Improved production of antifungal angucycline Sch47554 by manipulating three regulatory genes in *Streptomyces* sp. SCC-2136

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Running title: Three regulatory genes in Sch47554 biosynthesis

Highlights:

- Three regulatory genes in the biosyntheiss of Sch47554 were functionally characterized.
- The production of antifungal natural product Sch47554 was significatnly improved by manipulating the three regulatory genes in *Streptomyces* sp. SCC-2136.

Abstract

Sch47554 and Sch47555 are two angucyclines with antifungal activities against various yeasts and dermatophytes from *Streptomyces* sp. SCC-2136. The *sch* gene cluster contains several putative regulatory genes. Both *schA4* and *schA21* were predicted as the TetR family transcriptional regulators, while *schA16* shared significant similarity to the AraC family transcriptional regulators. Although Sch47554 is the major product of *Streptomyces* sp. SCC-2136, its titer is only 6.72 mg/L. This work aimed to increase the production of this promising antifungal compound by investigating and manipulating the regulatory genes in the Sch47554 biosynthetic pathway. Disruption of *schA4* and *schA16* led to a significant increase in the production of Sch47554, whereas the titer was dramatically decreased when *schA21* was disrupted. Overexpression of these genes gave opposite results. The highest titer of Sch47554 was achieved in *Streptomyces* sp. SCC-2136/Δ*schA4* (27.94 mg/L), which is significantly higher than the wild type. Our results indicate that SchA4 and SchA16 are repressors, while SchA21 acts as an activator. This work thus provides an initial understanding of functional roles of regulatory elements in the biosynthesis of Sch47554. Several efficient producing strains of Sch47554 were constructed by disrupting or overexpressing particular regulatory genes, which can be further engineered for industrial production of this medicinally important molecule.

Keywords: Regulatory protein; antifungal agents; *Streptomyces*; Sch47554; angucyclines

Abbreviations: SARP, *Streptomyces* antibiotic regulatory protein; *E. coli*, *Escherichia coli*; ORFs, open reading frames; HPLC, High Performance Liquid Chromatography; LC-MS, Liquid Chromatography-Mass Spectrometry; NMR, Nuclear Magnetic Resonance; PCR, Polymerase Chain Reaction; *sch*, biosynthetic gene cluster for Sch47554 and Sch47555; HTH, helix-turn-helix; PKS, Polyketide Synthase; KO, Knockout; OE, Overexpression.

1. INTRODUCTION

Gram-positive, filamentous, soil-inhabiting bacteria of *Streptomyces* belong to the phylum of Actinobacteria and have been recognized as one of the richest sources of biologically active secondary metabolites. They have a complex life cycle of morphological differentiation, which begins with spore germination in order to form branched vegetative hyphae [1]. The subsequent differentiation results in the formation of sporogenic aerial hyphae, followed by the chains of unigenomic spores [2]. During the initiation of morphological differentiation, most of the *Streptomyces* produce an extraordinary diversity of bioactive secondary metabolites, including a wide variety of pharmaceutically important natural products with various biological activities such as antimicrobial, anticancer, antifungal, antiviral, and anti-cholesterol properties [3-6]. The morphological differentiation and secondary metabolite production are tightly controlled by a complex network of regulators that sense and respond to plenty of physiological and environmental stimuli, including environmental stress, nutrition depletion, growth rate and small signaling molecules such as γ-butyrolactone and ppGpp [7-10]. The regulators function at several hierarchical levels, starting from the highest level of regulation (also known as global or pleiotropic regulators), which involves pleiotropic genes that play significant roles in morphological differentiation as well as secondary metabolite production. The lowest level of regulation utilizes pathway-specific regulatory genes (also known as cluster-situated regulators) that are located in the biosynthetic gene clusters and acts as a master switch for the biosynthesis of corresponding secondary metabolites [7, 10-12].

Pathway-specific regulatory genes can either activate or repress the expression of certain genes in the gene clusters. Biosynthetic gene clusters might have different numbers of positive pathway-specific regulatory genes. For instance, there is one in the actinorhodin biosynthetic gene cluster [13], whereas the daunorubicin gene cluster has three [14]. Some gene clusters, such as that for tylosin biosynthesis [15], contain both activators and repressors

in the same gene cluster. Bacterial pathway-specific regulators are classified into approximately 50 families based on the sequence alignment, structural and functional criteria [11]. Among the known regulators in *Streptomyces*, the LuxR family transcriptional factors and *Streptomyces* antibiotic regulatory proteins (SARPs) are mainly activators as in the examples of GdmRI, GdmRII, ActII-orf4 and RedD [13, 16]. The TetR family transcriptional regulators often function as repressor as in the examples of TdrK, AlpZ and SAV576 [17-19]. However, some of the TetR family regulators were characterized as activators, such as GdmRIII, SlgR1 and SAV3818 [20-23]. Similar to the TetR family regulators, the AraC family transcriptional regulators can also function as either activator or repressor [24-26]. Knowledge of the function of regulatory proteins at molecular level is of great interest. It would potentially enhance the yield of secondary metabolites through either inactivation of repressors or overexpression of activators and a combinatorial approach of both inactivation and overexpression.

Streptomyces sp. SCC-2136 (ATCC 55186) produces Sch47554 and Sch47555 that exhibit antifungal activity against various yeasts (*Candida albicans*, *C. tropicalis* and *C. stellatoidea*) and dermatophytes (*Trichophyton mentagrophytes*, *T. rubrum*, *T. tonsurans* and *Microsporum canis*) [27]. In addition to reported antifungal activities, Sch47554 and Sch47555 (Figure 1) might potentially possess antitumor and antimicrobial activities, and enzyme inhibitory and agonistic activities due to their structural similarity to other angucycline metabolites [28]. The diverse and promising biological activities of angucyclines excite the attentions of researcher to study the biosynthesis of these pharmaceutically important natural products as well as to enhance the production of these compounds. The initial sequence analysis of biosynthetic gene cluster (*sch*) for Sch47554 and Sch47555 revealed a total of 55 open reading frames (ORFs) in a region of 77.5 kb (NCBI accession number: AJ628018). In our previous work, we proposed the biosynthetic pathway in which

minimal PKS enzymes (SchP6, SchP7, and SchP8) led to the production of SEK15 from acetyl-CoA and nine units of malonyl-CoA. The addition of ketoreductase (SchP5), aromatase (SchP4) and cyclase (SchP9) to the mimimal PKS produced UWM6, which was subsequently converted to rabelomycin by an oxygenase (SchP10). Upon another oxygenation step by SchP3, aglycone backbone was synthesized and further modified by three glycosyltransferases, which resulted in the biosynthesis of Sch47554 and Sch47555 [29, 30]. Additionally, based on the sequence similarity analysis, the *sch* gene cluster contains several ORFs that may produce genes with a potential regulatory function. Two putative regulatory genes, *schA4* and *schA21*, were annotated as the TetR family transcriptional regulators, while *schA16* shared significant similarity to the AraC family transcriptional regulators. In addition to well-known families of regulatory genes, Basnet et al. predicted the putative functions of *schA25* and *schA27* as repressor-response regulator and transcriptional factor, respectively [29].

With the recent advances in sequencing and recombinant DNA technologies, rational strain improvement through metabolic engineering and synthetic biology has become a powerful tool to increase the titers of natural products for industrial purposes. Some of widely used metabolic engineering techniques for yield improvement are to increase the precursor supply, delete or tune the competing pathways or unwanted by-products, heterologous expression of the entire biosynthetic pathway, overexpression of rate-limiting enzymes and manipulation of regulatory genes [31, 32]. Typically, the best yields are achieved through a combination of several approaches. Moreover, manipulation of regulatory genes through overexpression of activator genes and/or deletion of repressor genes has been successfully performed in order for the improved yield of natural products [33]. For instance, overexpression of SARP from the mithramycin biosynthetic gene cluster in *Streptomyces argillaceus* led to a 16-fold increase in the mithramycin titer [34]. In addition, the inactivation

of GntR family transcriptional repressor increased the yield of platensimycin and platencin by 100-fold compared to the wild type strain [35]. The aim of the present study was to identify the functional roles of the TetR and AraC family transcriptional regulators in the *sch* gene cluster in *Streptomyces* sp. SCC-2136 for the improved production of the major antifungal compound Sch47554. The results presented in this study indicate that SchA4 and SchA16 are repressors, while SchA21 is an activator based on the inactivation and overexpression of corresponding regulatory genes. Manipulation of these regulatory genes significantly increased the production of Sch47554. This study paves the way for further engineering of this producing strain for enhanced production of these medicinally important angucyclines.

2. MATERIALS AND METHODS

2.1. Plasmids, strains, media and growth conditions

Plasmids and bacterial strains used in this study are listed in Table 1. *Streptomyces* sp. SCC-2136 was used as the parent strain for gene disruption and overexpression of regulatory proteins. *Streptomyces* sp. SCC-2136 was grown in YM broth (4 g/L yeast extract, 10 g/L malt extract, 4 g/L glucose) for 5 days for genomic DNA extraction. *Streptomyces* sp. SCC-2136 and its derivative strains were grown at 28 °C for 10 days on YM agar plates (4 g/L yeast extract, 10 g/L malt extract, 4 g/L glucose, and 20 g/L agar) for product analysis. MS (20 g/L mannitol, 20 g/L soy flour, 20 g/L agar and 10 mM $MgCl_2$) and ISP4 (DIFCO^{TM}) media were used for conjugation and single crossover homologous recombination, respectively. *Escherichia coli* XL1-Blue was used as the cloning host for plasmid construction and amplification. *E. coli* ET12567 was used for the introduction of gene knockout and overexpression plasmids into *Streptomyces* sp. SCC-2136 through intergenic conjugation. *E. coli* strains were routinely grown in LB medium at 37 °C. Apramycin (50 μg/mL), kanamycin (50 μg/mL), chloramphenicol (25 μg/mL), ampicillin (50 μg/mL) and nalidixic acid (25 μg/mL) were supplemented when appropriate.

Standard molecular biology protocols were performed as previously described [36]. Genomic DNA samples of *Streptomyces* sp. SCC-2136 and its derivative strains were extracted with a ZR Fungal/Bacterial DNA Miniprep Kit (Irvine, CA, USA). Plasmid DNA extraction from *E. coli* cells was performed using the Thermo Scientific GeneJET Plasmid Miniprep Kit (Thermo Scientific). PCR reactions were performed with an Arktik™ Thermal Cycler (Thermo Scientific) using Phusion DNA polymerase (Thermo Scientific). Oligonucleotide primers were ordered from Sigma-Aldrich and dissolved in TE buffer to the concentration of 100 ng/mL. Restriction enzymes used in this study were purchased from New England BioLabs.

2.2. Plasmid construction for targeted gene disruption and overexpression of regulatory genes

Targeted gene disruption plasmids were constructed based on the temperature-sensitive vector pKC1139 [37]. Firstly, we PCR-amplified a 456-bp (from A 77 to C 532), 538-bp (from G 125 to A 662), and 508-bp (from A 77 to A 584) fragment of *schA4*, *schA16* and *schA21*, respectively, from the genomic DNA of *Streptomyces* sp. SCC-2136 using the primers listed in Table 2. These PCR products were ligated into the pJET1.2 cloning vector to yield pRY15, pRY16 and pRY17 (Table 1), respectively. The plasmids were sent out for DNA sequencing using the Sanger method (Eton Bioscience). Subsequently, the partial DNA fragments of *schA4*, *schA16*, and *schA21* genes were excised from pRY15, pRY16 and pRY17 using XbaI and HindIII, and then ligated into pKC1139 between the same sites to yield pRY19, pRY20 and pRY21 (Table 1), respectively. These plasmids were subsequently used for targeted gene disruption of *schA4, schA16* and *schA21* in *Streptomyces* sp. SCC-2136 through intergenic conjugation using *E. coli* ET12567. Positive exconjugants for *Streptomyces* sp. SCC-2136/Δ*schA4*, Δ*schA16* and Δ*schS10* mutants were transferred to ISP4 plates supplemented with apramycin and incubated at 37 °C for about 10 days to allow the plasmids to integrate

into the genome and yield the single-crossover mutants. The positive mutants were shuttled back and forth to YM and ISP4 plates supplemented with apramycin for two generations to guarantee the positive mutants. Positive exconjugants after genome integration were subjected to product analysis and PCR confirmation of gene knockouts using primers listed in Table 2. The details of intergenic conjugation protocols were described in literature [38].

For overexpression of regulatory genes, an integrative expression plasmid for *Streptomyces*, pSET152, was used [39]. We firstly amplified *schA4*, *schA16* and *schA21* genes by PCR from the genomic DNA of *Streptomyces* sp. SCC-2136 using the primers listed in Table 2. These PCR products were inserted into pJET1.2 cloning vector to yield pOKF7, pOKF8 and pOKF9 (Table 1), respectively. After sequencing, each regulatory gene was excised from pOKF7, pOKF8 and pOKF9 with XbaI and subsequently ligated into pSET52 using the same site, yielding pOKF10, pOKF11 and pOKF12 (Table 1), respectively. The direction of the genes was double-checked using the NdeI restriction site, which was designed in the primers for direction check. These plasmids were transformed into *Streptomyces* sp. SCC-2136 through intergenic conjugation using *E. coli* ET12567. Positive exconjugants were streaked on YM plates supplemented with apramycin for product analysis.

2.3. Extraction, purification and product analysis

Streptomyces sp. SCC-2136 and engineered strains were grown for 10 days at 28 ºC on YM agar plates supplemented with apramycin. The plates were chopped and extracted with a mixture of solvents consisting of 89% ethyl acetate, 10% methanol, and 1% acetic acid (v/v). The resulting extracts were dried *in vacuo*, and the residues were redissolved in methanol for HPLC analysis. We purified the major compound Sch47554 by HPLC with an Agilent Eclipse XDB-C18 column (5 μm, 250 mm × 4.6 mm) to prepare a standard curve for the product analysis of engineered strains. This purified compound was characterized by LC-MS and NMR (Figures. S1 and S2). Low-resolution and high-resolution ESI-MS spectra were

obtained on Agilent 6130 and 6210 LC-MS, respectively. Proton NMR was recorded on a Bruker AvanceIII HD Ascend-500 NMR instrument.

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.91 (d, J = 7.8 Hz, 1H), 7.64 (d, J = 7.8 Hz, 1H), 6.94 (d, J = 9.8 Hz, 1H), 6.85 (dd, J = 10.2, 3.5 Hz, 1H), 6.71 (dd, J = 10.2, 3.5 Hz, 1H), 6.43 (d, J = 9.8 Hz, 1H), 6.13 (d, J = 10.2 Hz, 1H), 6.10 (d, J = 10.2 Hz, 1H), 5.61 (d, J = 3.4 Hz, 1H), 5.35 (d, J = 3.5 Hz, 1H), 4.83 (d, J = 10.0 Hz, 1H), 4.76 (q, J = 6.7 Hz, 1H), 4.62 (q, J = 6.8 Hz, 1H), 3.58 (dd, J = 9.1, 6.1 Hz, 1H), 3.43 (ddd, J = 14.5, 10.1, 4.8 Hz, 2H), 3.25 (dd, J = 13.4, 3.0 Hz, 1H), 2.56 (d, J = 13.3 Hz, 1H), 2.49 (dd, J = 15.6, 2.9 Hz, 1H), 2.40 – 2.31 (m, 2H), 2.30 – 2.24 (m, 1H), 1.90 – 1.85 (m, 1H), 1.83 (d, J = 15.5 Hz, 1H), 1.49 (s, 3H), 1.46 (d, J = 6.7 Hz, 3H), 1.41 (d, J = 6.8 Hz, 3H), 1.39 (d, J = 6.1 Hz, 3H).

The titers of Sch47554 for all strains were calculated using the standard curve prepared with the purified compound. Three replicates were analyzed for production formation for all strains. Depending on the production level, the crude extracts of all strains were diluted with different ratios in order to be in the range of the standard curve and then analyzed by HPLC.

3. RESULTS AND DISCUSSION

Actinomycetes have possessed a huge potential to produce novel, pharmaceutically useful secondary metabolites with a wide variety of biological activities [40]. Particularly, streptomycetes have been recognized as one of the richest sources of biologically active secondary metabolites [41]. However, the production titers of those important compounds are often low, and sometimes it is too low to detect the products with current analytical techniques [42, 43]. To expand Nature's chemical repertoire and to increase the titers of known natural products, researchers have come up with various solutions: heterologous expression, co-cultivation, fermentation engineering and strain engineering [7]. In particular, strain engineering can be conducted though both metabolic engineering of rate-limiting and/or

competing pathways, and engineering regulatory networks of secondary metabolites in native strains in which secondary metabolite biosynthesis is tightly regulated by global and/or pathway-specific regulatory proteins [40, 42, 44-47]. In this study, we investigated the roles of three regulatory genes in the *sch* biosynthetic gene cluster with the purpose of enhancing the production of Sch47554 in *Streptomyces* sp. SCC-2136.

3.1. Sequence analysis of three putative regulatory proteins in the *sch* biosynthetic gene cluster

BLAST analysis revealed that SchA4 and SchA21 are putative TetR family transcriptional factors, while SchA16 is a putative AraC family transcriptional factor. SchA4 and SchA21 exhibit high sequence homology to LanK (72% and 76%, respectively) and SimR (80% and 63%, respectively), both of which are regulatory proteins in the biosynthetic gene clusters of landomycin A and simocyclinone [48-50]. SchA16 is highly similar to some of the characterized AraC family transcriptional factors such as AdpA (78%), RapG (72%), NanR4 (75%) and SAV742 (78%) [24-26, 51]. We also performed a multiple amino acid sequence alignment for those regulatory genes against some of the characterized regulatory proteins from various *Streptomyces* strains in order to identify the conserved domains (Figure 2). The overall conserved structure of the TetR family transcriptional factors consists of nine α helices, and the DNA-binding domain is composed of helices 1 to 3 [52]. Both SchA4 and SchA21 consist of conserved DNA-binding domains similar to other reference TetR family transcriptional factors (Figures 2A and 2B). On the other hand, the AraC family transcriptional factors (Figure 2C) possess a C-terminal conserved helix-turn-helix (HTH) DNA-binding domain with the consensus of 15 amino acid residues (**A**xxxxx**S**xxx**L**xxx**F**xxxx**G**xxxxxxxxxxx**R**xxx**A**xxx**L**xxxxxxxxx**I**/**V**x**I**/**V** xxxx**G**(**F**/**K**)xxxxxx**F**xxx**F**(**R**/**K**)xxxx**G**x**P**, where x is any amino acid) [53]. SchA16 has high similarity to this consensus sequence. All in all, BLAST and Clustal Omega multiple

sequence alignments for these regulatory proteins indicated that SchA4 and SchA21 belong to the TetR family, while SchA16 is a member of the AraC family transcriptional factors.

3.2. Characterization of SchA4 as a repressor and enhanced production of Sch47554 by disrupting *schA4*

Based on the multiple amino acid sequence alignment and analysis of the conserved domains, SchA4 was predicted to be a TetR family transcriptional factor. To characterize the role of *schA4*, we first disrupted this gene on the genome of *Streptomyces* sp. SCC-2136. A single crossover approach was used (Figure 3). Apramycin resistant exconjugants were obtained from intergenic conjugation between *Streptomyces* sp. SCC-2136 and *E. coli* ET12567/pRY19. The correct mutant was verified by PCR using vector- (RV-M and M13-47) and genome-specific primers (Figure 3). The 1.50 kb and 1.55 kb PCR products were amplified from the genome of *Streptomyces* sp. SCC-2136/Δ*schA4*, whereas the wild type genome did not yield these fragments (Figure S3A). This indicated that the *schA4* gene was successfully disrupted.

Upon confirmation of gene inactivation, the positive exconjugants were grown on YM plates supplemented with apramycin for product analysis. As shown in Figure 4A, *Streptomyces* sp. SCC-2136/Δ*schA4* showed the same product profile as the wild type. The major peak was isolated and characterized as Sch47554 by a comparison of its MS and NMR data (Figures S1 and S2) with those reported. However, the titer of Sch47554 in *Streptomyces* sp. SCC-2136/Δ*schA4* reached 27.94 mg/L, which is significantly higher than the wild type (6.72 mg/L) (Figure 4B). This result suggests that SchA4 acts as a repressor, similar to most regulatory proteins in the TetR family [17, 18, 54-56]. For instance, SAV576 negatively controls the biosynthesis of avermectin in *Streptomyces avermetilis* [18]. Similarly, AlpZ is another TetR family transcriptional factor with a repressor role in the biosynthesis of angucyclinone antibiotic alpomycin in *Streptomyces ambofaciens* [17].

To ensure that the above result was not due to a polar effect caused by insertion of the disruption plasmid into the gene cluster (Figure 3), we next overexpressed *schA4* in *Streptomyces* sp. SCC-2136. Overexpression approach has been used to investigate the function of regulatory proteins. For instance, Yoo et al. overexpressed three regulatory genes (*rapY*, *rapR* and *rapS*) using pSET152 integrative expression plasmid to understand the function of regulatory elements in rapamycin biosynthesis [54]. Kuščer et al. also exploited a pSET152-based integrative expression system to overexpress regulatory proteins in rapamycin-producing wild type strain, which led to an increase in the titer of rapamycin [24]. Thus, we used the pSET152 integrative expression system to overexpress the corresponding regulatory genes. First, the *schA4* gene was ligated into pSET152 to yield pOKF10, which was subsequently introduced into *Streptomyces* sp. SCC-2136 through intergenic conjugation. The resulting strain was grown on YM plates supplemented with apramycin for product analysis. As shown in Figure 5A, *Streptomyces* sp. SCC-2136/OE-*schA4* produced the same metabolites as the wild type. The titer of Sch47554 in this strain was determined to be 2.42 mg/L (Figure 5B), which is much lower than the wild type. This result further supports that SchA4 is a repressor for the biosynthetic pathway of Sch47554. Enhanced expression of SchA4 would have down-regulated the pathway, resulting in a lower production titer of the target compound.

3.3. Identification of SchA16 as an AraC-family repressor and enhanced production of Sch47554 by disrupting *schA16*

The multiple amino acid sequence alignment predicted that SchA16 is an AraC family transcriptional factor. Similar to other characterized AraC family regulators, SchA16 has 15 amino acid consensuses for the conserved DNA-binding domain. To understand its role in the biosynthesis of Sch47554, we used a similar approach to disrupt this gene. The correct conjugant was selected by apramycin resistance and verified using PCR. Two fragments, 1.50

297 kb and 1.40 kb, respectively, were amplified from the genome of *Streptomyces* sp. SCC-
298 2136/Δ*schA16* using genome- and vector-specific primers, while the wild type gave no PCR
299 products using the same primers (Figure S3B). Production of Sch47554 by this mutant was
300 confirmed by HPLC (Figure 4A) and the titer of this compound was determined to be 17.43
301 mg/L (Figure 4B). This represents a nearly 200% increase compared to the wild type strain,
302 suggesting that SchA16 plays a role of repressor in the biosynthesis of Sch47554.

303 We then amplified the *schA16* gene and ligated it into pSET152 to yield the
304 overexpression plasmid pOKF11. This plasmid was introduced into *Streptomyces* sp. SCC-
305 2136 to overexpress SchA16. Although this engineered strain produced Sch47554 (Figure
306 5A), the titer of this compound was only 1.78 mg/L (Figure 5B), which is much lower than
307 the wild type. This result was consistent with that from the disruption of *schA16*. In general,
308 the AraC family regulators are considered as activators with some exceptions [11]. For
309 instance, NanR4 and RapG act as activator in the biosynthesis of nanchangmycin and
310 rapamycin, respectively [24, 26]. However, SAV742 is a repressor in the biosynthesis of
311 avermectin in *Streptomyces avermitilis* and its deletion increased the yield of avermectin [25],
312 same as what we observed in the disruption of *schA16*. Similar to the TetR family regulators,
313 the AraC family regulators could exhibit both positive and negative roles in the secondary
314 metabolite production. Our results support that SchA16 is a repressor in the biosynthetic
315 pathway of Sch47554 and disruption of this gene can be an effective approach for improving
316 the production of angucyclines in this strain.

317 **3.4. Characterization of SchA21 as a TetR family activator and enhanced**
318 **production of Sch47554 by overexpressing *schA21***

319 BLAST analysis showed that SchA21 also belongs to the TetR family of transcriptional
320 factors. To under its role in the biosynthesis of Sch47554, we also disrupted this gene through
321 the single crossover homologous recombination approach shown in Figure 3. The genome of

322 the resulting mutant was extracted and subjected to PCR verification. Using the genome- and
323 vector-specific primers, we were able to amplify the 1.40 and 1.25 kb fragments from the
324 genome of *Streptomyces* sp. SCC-2136/Δ*schA21*, but none from the wild type (Figure S3C),
325 confirming that this strain is the correct mutant. Product analysis showed that this mutant did
326 produce the same products as the wild type (Figure 4A), but Sch47554 was only produced at a
327 very low level (0.39 mg/L, Figure 4B), indicating that disruption of SchA21 has significantly
328 repressed the biosynthetic pathway of Sch47554. This gene was then ligated into pSET152 to
329 yield the overexpression plasmid pOKF12. Overexpression of this gene in *Streptomyces* sp.
330 SCC-2136 led to significantly enhanced production of Sch47554, with a titer of 26.23 mg/L,
331 as shown in Figures 5A and 5B. The disruption and overexpression strains for SchA4 and
332 SchA21 led to the opposite results even though both regulatory proteins are in the same
333 family. The titer of Sch47554 increased significantly in the knockout strain for SchA4 by
334 approximately 4-fold, whereas it was dramatically decreased when *schA21* was disrupted
335 (Figure 4B). The overexpression strains for these TetR family transcriptional factors produced
336 consistent results with the gene-disrupted strains. Therefore, it can be concluded that SchA21
337 serves as an activator of the Sch47554 biosynthetic pathway. Although most known TetR
338 family transcriptional factors are repressors, there are some TetR family transcriptional
339 regulators that act as activators in the biosynthesis of secondary metabolites. As an example,
340 SAV3818 cloned from high avermectin-producing *S. avermitilis* ATCC 31780 was
341 overexpressed in the low avermectin-producing *S. avermitilis* ATCC 31267, showing a
342 stimulatory effect in the low avermectin-producing strain. In addition, the overexpression of
343 SAV3818 stimulated actinorhodin production in both *S. coelicolor* M145 and *S. lividans*
344 TK21, implying that this TetR-family transcriptional regulator might be a global up-regulator
345 acting in antibiotic production in *Streptomyces* species [21]. Another example is GdmRIII,
346 which not only plays a positive regulatory role in the biosynthesis of geldanamycin, but also

plays a negative role in elaiophylin biosynthesis in *Streptomyces autolyticus* CGMCC 0516 [23]. We successfully increased the titer of Sch47554 through the overexpression of TetR family activator, SchA21.

4. CONCLUSIONS

In conclusion, the roles of three putative regulatory genes in the *sch* biosynthetic gene cluster were investigated for enhanced production of a pharmaceutically important angucycline. Gene disruption of *schA4* and *schA16* led to a significant increase in the production of angucyclines. In contrast, disruption of *schA21* dramatically decreased the yield of Sch47554. The overexpression strains produced opposite results. Therefore, SchA4 and SchA16 were identified as repressors, while SchA21 acts as an activator. Our results demonstrated that both disruption of pathway repressors and overexpression of activators are effective ways to improve the production of target compounds in the producing strains. This work provides an initial understanding of regulatory elements in the biosynthesis of Sch47554, which will facilitate further engineering of this producing strain for efficient production of these medicinally important angucyclines.

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Table 1. Bacterial strains and plasmids used in this study

Strains	**Description**	**Source**
E. coli XL1-Blue	*rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 *sup*E44 *rel*A1 *lac*	Agilent Technologies
E. coli ET12567 (pUZ8002)	F- dam-13::Tn9 dcm-6 hsdM hsdR zjj-202::Tn10 recF143 galK2 galT22 ara-14 lacY1 xyl-5 leuB6 thi-1 tonA31 rpsL136 hisG4 tsx-78 mtl-1 glnV44	[30]
Streptomyces sp. SCC-2136/WT	*Streptomyces sp*. SCC-2136 wild type strain	ATTC 55186
Streptomyces sp. SCC-2136/Δ*schA4*	*Streptomyces sp.* SCC-2136 *schA4* knockout strain	This study
Streptomyces sp. SCC-2136/Δ*schA16*	*Streptomyces sp.* SCC-2136 *schA16* knockout strain	This study
Streptomyces sp. SCC-2136/Δ*schA21*	*Streptomyces sp.* SCC-2136 *schA21* knockout strain	This study
Streptomyces sp. SCC-2136/OE-SchA4	*Streptomyces sp.* SCC-2136 *schA4* overexpression strain	This study
Streptomyces sp. SCC-2136/OE-SchA16	*Streptomyces sp.* SCC-2136 *schA16* overexpression strain	This study
Streptomyces sp. SCC-2136/OE-SchA21	*Streptomyces sp.* SCC-2136 *schA21* overexpression strain	This study
Plasmids	**Description**	**Source**
pJET1.2	Cloning vector	Thermo Fisher
pKC1139	*E.coli-Streptomyces* shuttle plasmid contains a *Streptomyces* temperature-sensitive origin of replication	[37]
pSET152	ϕC31 int + attP integrative expression plasmid	[39]

pRY15	Partial fragment of *schA4* gene in pJET1.2	This study
pRY16	Partial fragment of *schA16* gene in pJET1.2	This study
pRY17	Partial fragment of *schA21* gene in pJET1.2	This study
pRY19	Partial fragment of *schA4* gene (from pRY15) in pKC1139	This study
pRY20	Partial fragment of *schA16* gene (from pRY16) in pKC1139	This study
pRY21	Partial fragment of *schA21* gene (from pRY17) in pKC1139	This study
pOKF7	*schA4* gene in pJET1.2	This study
pOKF8	*schA16* gene in pJET1.2	This study
pOKF9	*schA21* gene in pJET1.2	This study
pOKF10	*schA4* gene (from pOKF8) in pSET152	This study
pOKF11	*schA16* gene (from pOKF9) in pSET152	This study
pOKF12	*schA21* gene (from pOKF10) in pSET152	This study

476 **Table 2.** Oligonucleotides used in this study

Primers	Oligonucleotides
schA4-SCKO-F-HindIII	aaAAGCTTacgtcgccacgcacgagttc
schA4-SCKO-R-XbaI	aaTCTAGAggaagaagcagaacgagctgat
schA16-SCKO-F-HindIII	aaAAGCTTggcacgcgaacgaggcgatc
schA16-SCKO-R-XbaI	aaTCTAGAtcggcgtacgcgctgacgct
schA21-SCKO-F-HindIII	aaAAGCTTaacgcctcaaccgccccgca
schA21-SCKO-R-XbaI	aaTCTAGAttcgcgctggaccagccctg
OE-schA4-F-XbaI-NdeI	aaTCTAGAgggagccCATATGtcagtccgccgagcggtcc
OE-schA4-R-XbaI	aaTCTAGAatgaccagcgtcgaagaaccgg
OE-schA16-F-XbaI-NdeI	aaTCTAGAggaggagccCATATGgcggacataagcaccca
OE-schA16-R-XbaI	aaTCTAGAtcagccctgaacgccgccgg
OE-schA21-F-XbaI-NdeI	aaTCTAGAgggagccCATATGtcagtccgccgagcggtcc
OE-schA21-R-XbaI	aaTCTAGAatggccactcggagaaaagaggagaagc
schA4-RY19-check-F	aaatgacgtagccgacgacg
schA4-RY19-check-R	aagtgacatcggtgttgtgc
schA16-RY20-check-F	aacagacggtgtggtcgaag
schA16-RY20-check-R	aaagaccacgaccgactctg
schA21-RY21-check-F	aagtgagccagtcgcagtgc
schA21-RY21-check-R	aacagtcgatcgcgttcttcag

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Figure Legends

Figure 1. Structures of Sch47554 (**1**) and Sch47555 (**2**).

Figure 2. Multiple amino acid sequence alignment for SchA4 (A), SchA21 (B), and SchA16 (C) with respect to the characterized TetR- and AraC-family transcriptional factors, respectively. SchA4 and SchA21 consist of the conserved DNA-binding domain of the TetR regulators, while SchA16 has the C-terminal conserved HTH DNA-binding domain. Asterisk (*) indicates positions which have a single, fully conserved residue. Colon (:) indicates conservation between groups of strongly similar properties. Period (.) indicates conservation between groups of weekly similar properties. GenBank accession numbers are: WP_010981587 (SAV_151), WP_052413891 (TcmR), WP_011028825 (SCO3201), WP_108908224 (TdrK), CAH10095 (SchA4), CAA60451 (RapY), CCC55902 (ClmR1), AAD13556 (LanK), WP_030240800 (Au1R), WP_053125906 (AlpW), WP_053125912 (AlpZ), Q9ZN78 (ArpA), WP_010982014 (SAV_576), AAK06798 (SimR), CAL99365 (SACE_0012), NP_733542 (SCO1712), WP_004193135 (TetR), BAG20734 (SgAtrA), EFE75762 (SrAtrA), CBA11576 (SlgR1), AFU52889 (GouR), CAH10121 (SchA21), WP_069862081 (GdmRIII), WP_009951472 (SACE_7301), EDY64415 (SSDG_03033), WP_006124065 (DepR1), WP_003968987 (AdpA), AAP42877 (NanR4), CAH10107 (SchA16), CAA60472 (RapG), BAC68452 (SAV_742).

Figure 3. Illustration of the single crossover recombination strategy for disruption for *schA4* in *Streptomyces* sp. SCC-2136.

Figure 4. Effects of disruption of *schA4*, *schA16* and *schA21* on the production of **1**. (A) HPLC traces for the extracts of wild type *Streptomyces* sp. SCC-2136 and mutant strains for *schA4*, *schA16* and *schA21*, respectively. The peak at the wavelength of 420 nm (in the dashed rectangle) is the antifungal compound Sch47554 (**1**). (B) A comparison of the titers of **1** in wild type *Streptomyces* sp. SCC-2138 with those in the mutants with disrupted *schA4*,

schA16 or *schA21*. 55186-WT: *Streptomyces* sp. SCC-2138 wild type strain, SchA4-KO: *Streptomyces* sp. SCC-2136/Δ*schA4*, SchA16-KO: *Streptomyces* sp. SCC-2136/Δ*schA16*, and SchA21-KO: *Streptomyces* sp. SCC-2136/Δ*schA21*. The average titers are shown on the top of the bars.

Figure 5. Effects of overexpression of *schA4*, *schA16* and *schA21* on the production of **1**. (A) HPLC traces for the extracts of wild type *Streptomyces* sp. SCC-2136 and overexpression strains for *schA4*, *schA16* and *schA21*, respectively. (B) A comparison of the titers of **1** in wild type *Streptomyces* sp. SCC-2136 with those in the engineered strains overexpressing *schA4*, *schA16* or *schA21*. 55186-WT: wild type strain; SchA4-OE: *Streptomyces* sp. SCC-2136-SchA4 overexpression strain; SchA16-OE: *Streptomyces* sp. SCC-2136-SchA16 overexpression strain; SchA21-OE: *Streptomyces* sp. SCC-2136-SchA21 overexpression strain. The average titers are shown on the top of the bars.

534 Fig. 1

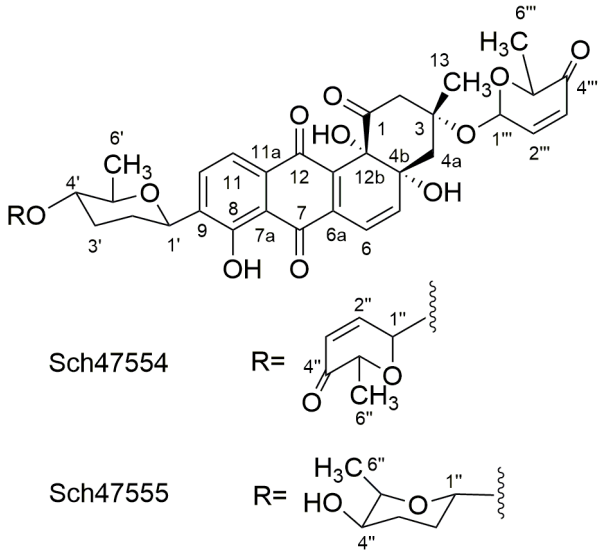

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Fig. 2

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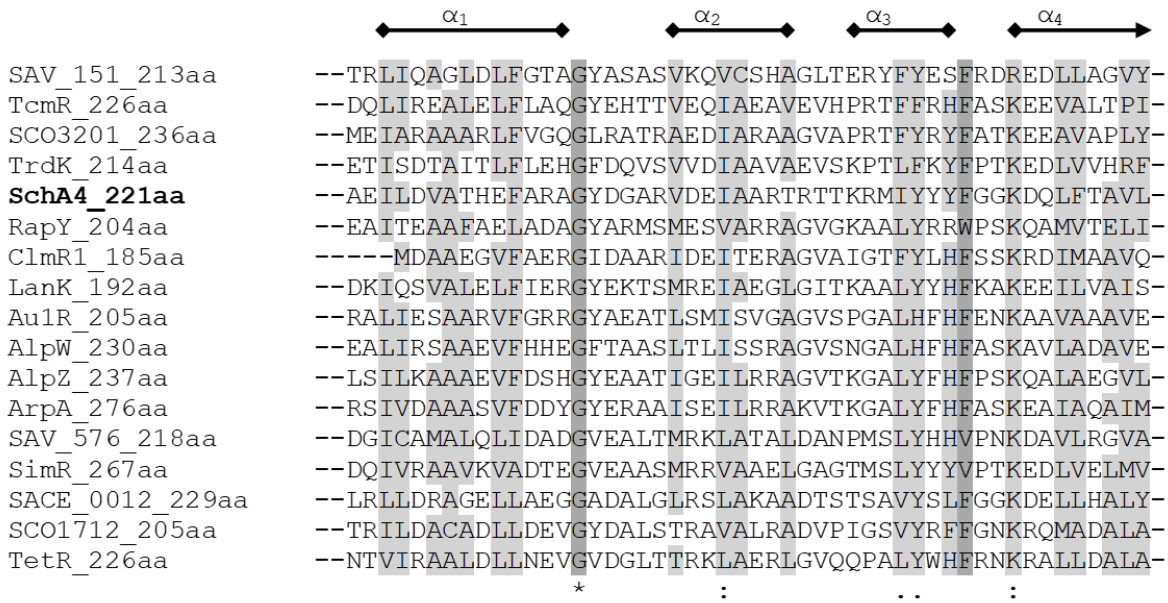
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α2
α3
α4
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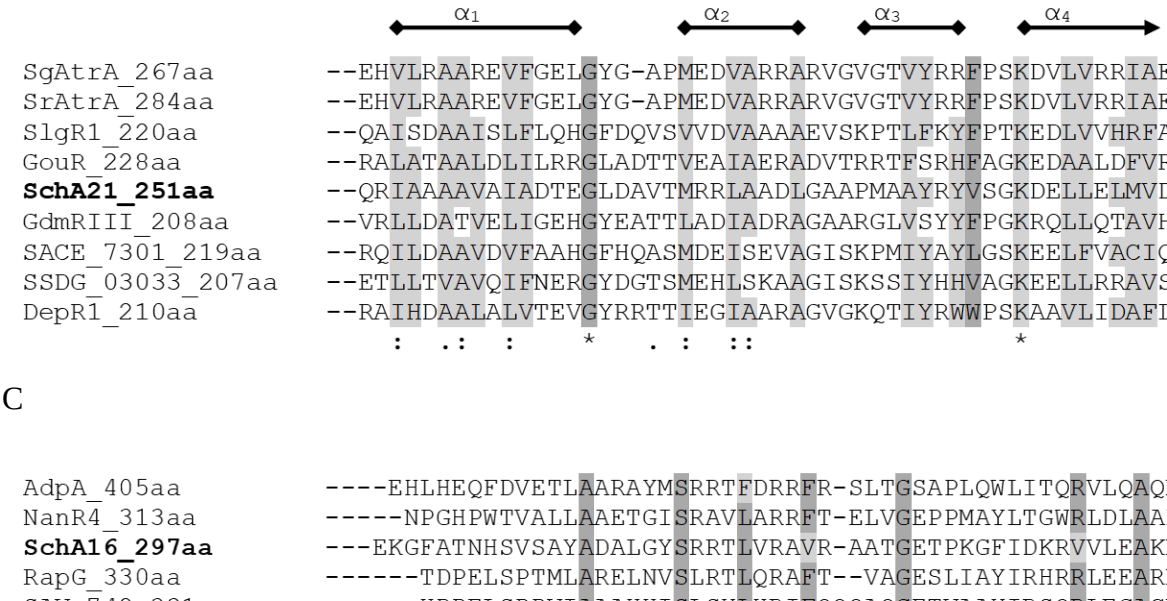
TcmR_226aa --DQLIREALELFLAQGYEHTTVEQIAEAVEVHPRTFFRHFASKEEVALTPI--
SCO3201_236aa --MEIARAAARLFVGQGLRATRAEDIARAAGVAPRTFYRYFATKEEAVAPLY--
TrdK_214aa --ETISDTAITLFLEHGFDQVSVVDIAAVAEVSKPTLFKYFPTKEDLVVHRF--
SchA4_221aa --AEILDVATHEFARAGYDGARVDEIAARTRTTKRMIYYYFGGKDQLFTAVL--
RapY_204aa --EAITEAAFAELADAGYARMSMESVARRAGVGKAALYRRWPSKQAMVTELI--
ClmR1_185aa -----MDAAEGVFAERGIDAARIDEITERAGVAIGTFYLHFSSKRDIMAAVQ--
LanK_192aa --DKIQSVALELFIERGYEKTSMREIAEGLGITKAALYYHFKAKEEILVAIS--
Au1R_205aa --RALIESAARVFGRRGYAEATLSMISVGAGVSPGALHFHFENKAAVAAAVE--
AlpW_230aa --EALIRSAAEVFHHEGFTAASLTLISSRAGVSNGALHFHFASKAVLADAVE--
AlpZ_237aa --LSILKAAAEVFDSHGYEAATIGEILRRAGVTKGALYFHFPSKQALAEGVL--
ArpA_276aa --RSIVDAAASVFDDYGYERAAISEILRRAKVTKGALYFHFASKEAIAQAIM--
SAV_576_218aa --DGICAMALQLIDADGVEALTMRKLATALDANPMSLYHHVPNKDAVLRGVA--
SimR_267aa --DQIVRAAVKVADTEGVEAASMRRVAAELGAGTMSLYYYVPTKEDLVELMV--
SACE_0012_229aa --LRLLDRAGELLAEGGADALGLRSLAKAADTSTSAVYSLFGGKDELLHALY--
SCO1712_205aa --TRILDACADLLDEVGYDALSTRAVALRADVPIGSVYRFFGNKRQMADALA--
TetR_226aa --NTVIRAALDLLNEVGVDGLTTRKLAERLGVQQPALYWHFRNKRALLDALA--

B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α2
α3
α4
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SrAtrA_284aa --EHVLRAAREVFGELGYG-APMEDVARRARVGVGTVYRRFPSKDVLVRRIAE-
SlgR1_220aa --QAISDAAISLFLQHGFDQVSVVDVAAAAEVSKPTLFKYFPTKEDLVVHRFA-
GouR_228aa --RALATAALDLILRRGLADTTVEAIAERADVTRRTFSRHFAGKEDAALDFVR-
SchA21_251aa --QRIAAAAVAIADTEGLDAVTMRRLAADLGAAPMAAYRYVSGKDELLELMVD-
GdmRIII_208aa --VRLLDATVELIGEHGYEATTLADIADRAGAARGLVSYYFPGKRQLLQTAVH-
SACE_7301_219aa --RQILDAAVDVFAAHGFHQASMDEISEVAGISKPMIYAYLGSKEELFVACIQ-
SSDG_03033_207aa --ETLLTVAVQIFNERGYDGTSMEHLSKAAGISKSSIYHHVAGKEELLRRAVS-
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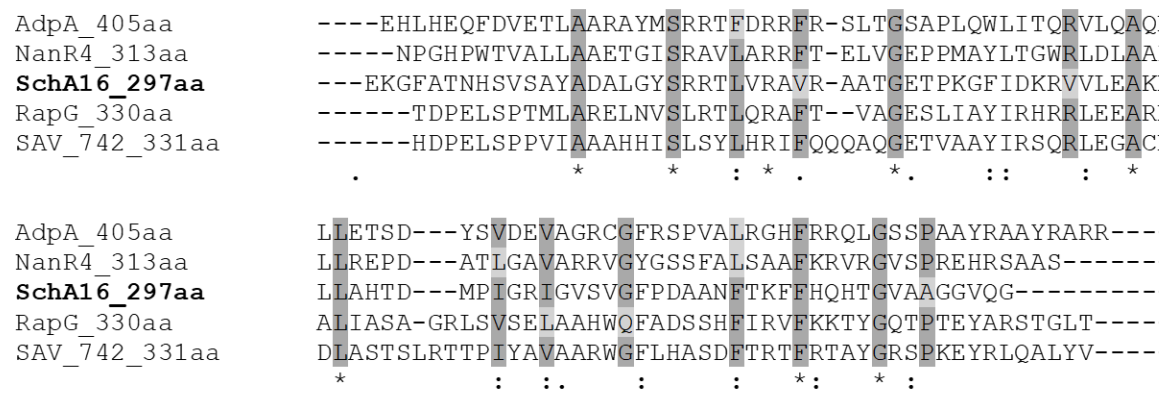
C


AdpA_405aa ----EHLHEQFDVETLAARAYMSRRTFDRRFR-SLTGSAPLQWLITQRVLQAQR
NanR4_313aa -----NPGHPWTVALLAAETGISRAVLARRFT-ELVGEPPMAYLTGWRLDLAAD
SchA16_297aa ---EKGFATNHSVSAYADALGYSRRTLVRAVR-AATGETPKGFIDKRVVLEAKR
RapG_330aa ------TDPELSPTMLARELNVSLRTLQRAFT--VAGESLIAYIRHRRLEEARR
SAV_742_331aa ------HDPELSPPVIAAAHHISLSYLHRIFQQQAQGETVAAYIRSQRLEGACR
AdpA_405aa LLETSD---YSVDEVAGRCGFRSPVALRGHFRRQLGSSPAAYRAAYRARR----
NanR4_313aa LLREPD---ATLGAVARRVGYGSSFALSAAFKRVRGVSPREHRSAAS-------
SchA16_297aa LLAHTD---MPIGRIGVSVGFPDAANFTKFFHQHTGVAAGGVQG----------
RapG_330aa ALIASA-GRLSVSELAAHWQFADSSHFIRVFKKTYGQTPTEYARSTGLT-----
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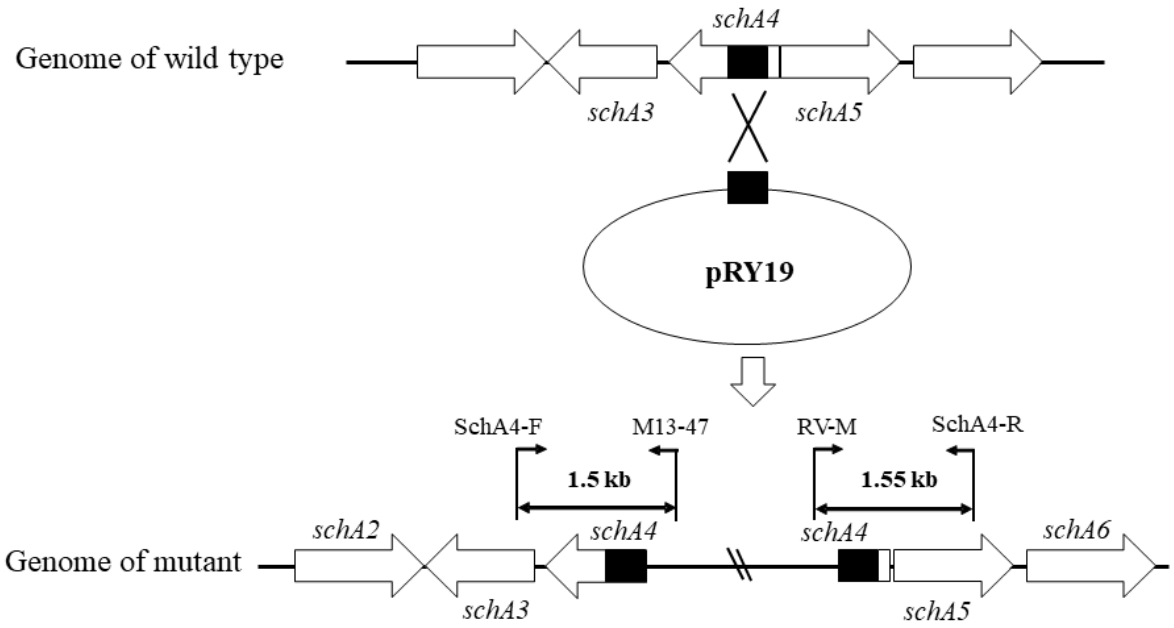
559 Fig. 3



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572 Fig. 4

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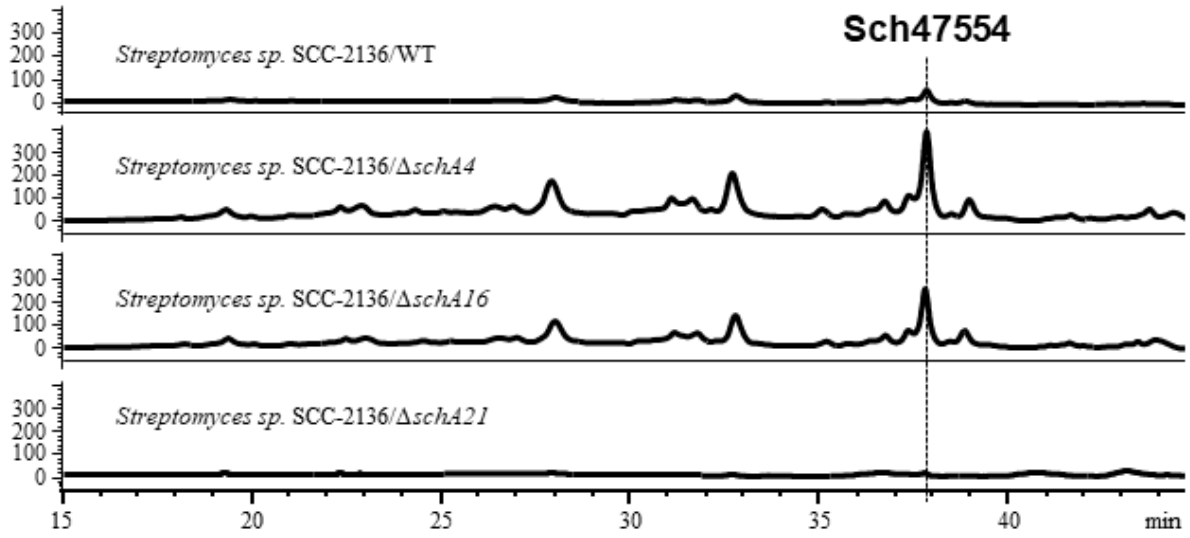

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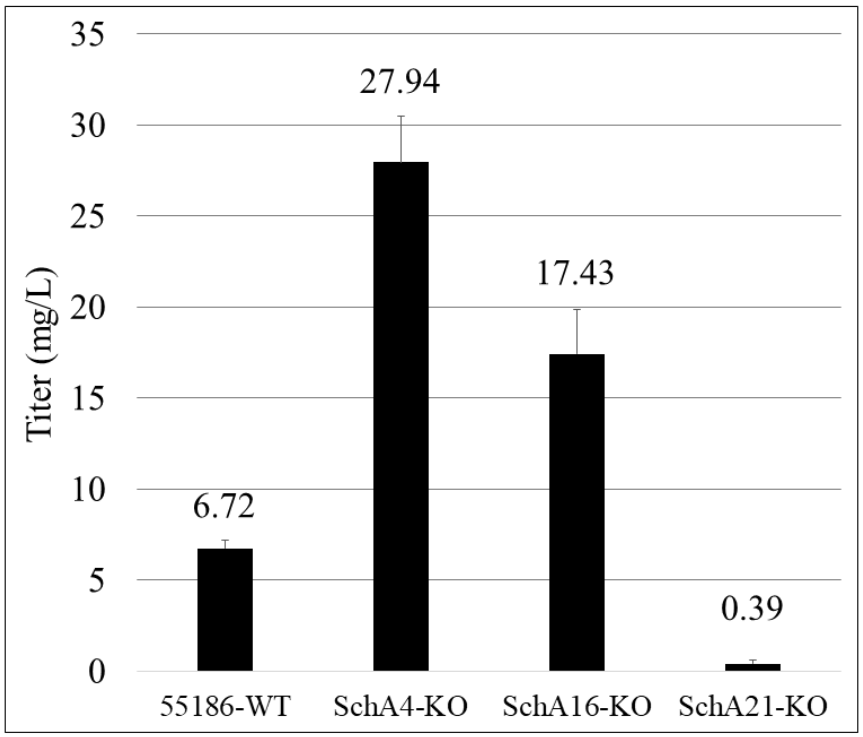
575 B



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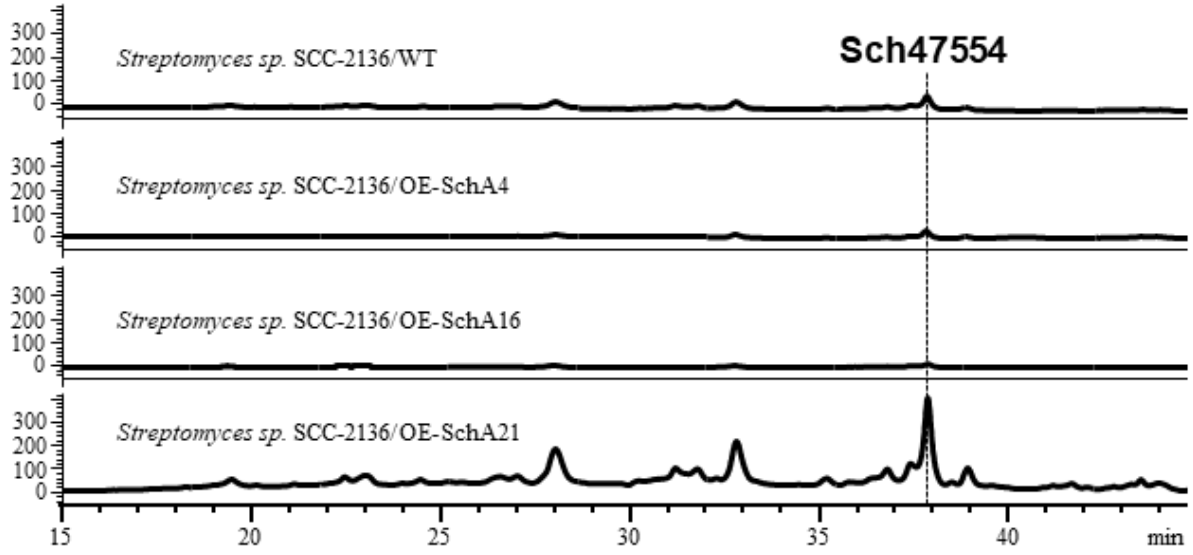
578 Fig. 5

579 A



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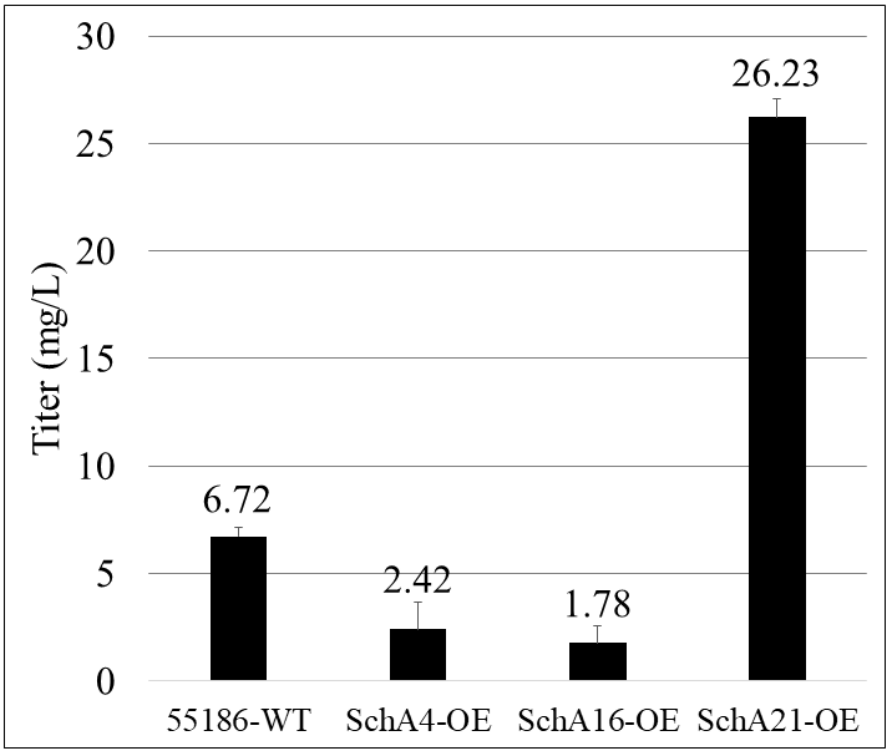
581 B



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