Identification of a gene from *Streptomyces rimosus* M527 negatively affecting antibiotic biosynthesis and morphological differentiation

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Research

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Abstract

Background: The polyene macrolide rimocidin, produced by *Streptomyces rimosus* M527, was found to be highly effective against a broad range of fungal plant pathogens. Current understanding of the regulatory mechanism of rimocidin biosynthesis and morphological differentiation in *S. rimosus* M527 is limited. NsdA is considered as a negative regulator involved in morphological differentiation and biosynthesis of secondary metabolites in some *Streptomyces*.

Results: In this study, *nsdA_{sr}* was cloned from *S. rimosus* M527. The role of *nsdA_{sr}* in rimocidin biosynthesis and morphological differentiation was investigated by gene deletion, complementation, and over-expression. A Δ *nsdA_{sr}* mutant was obtained using CRISPR/Cas9. The mutant produced more rimocidin (46%) and generated more spores than the wild-type strain. Over-expression of *nsdA_{sr}* led to a decrease in rimocidin production and impairment of sporulation. Quantitative reverse transcription-PCR (qRT-PCR) analysis revealed that transcription of *rim* genes responsible for rimocidin biosynthesis was up-regulated in the Δ *nsdA_{sr}* mutant but down-regulated in the *nsdA_{sr}* over-expression strain. Similar effects have been described for *Streptomyces coelicolor* M145 and the industrial toyocamycin-producing strain *Streptomyces diastatochromogenes* 1628.

Conclusion: *NsdA_{sr}* is identified as a negative regulator of sporulation and antibiotic biosynthesis as well as the transcription of biosynthetic genes both in its host *S. rimosus* M527 and in model strain *S. coelicolor* and industrial producer strain *S. diastatochromogenes* 1628. This work will provide further information for understanding regulatory mechanisms controlling rimocidin biosynthesis in *S. rimosus* M527.

Background

Streptomyces are Gram-positive bacteria with complex morphological differentiation and secondary metabolism. They are well-known to produce a wide variety of valuable natural products with diverse biological activities [1-3]. In *Streptomyces*, the processes of morphological differentiation and antibiotic biosynthesis are tightly controlled via multiple levels of regulation that includes cluster-situated, pleiotropic, and global regulators that respond to numerous physiological and environmental conditions [4-6]. Among the many regulatory genes, a gene negatively affecting *Streptomyces* differentiation (*nsdA*) was first found in model strain *Streptomyces coelicolor* A3(2) [7]. The disruption of *nsdA* in *S. coelicolor* resulted in accelerated sporulation and increased production of three antibiotics, actinorhodin (Act), methylenomycin, and a calcium-dependent antibiotic. Subsequent studies report that *nsdA* negatively affects antibiotic biosynthesis and morphological differentiation in *Streptomyces*. For example, in *Streptomyces bingchengensis* and *Streptomyces lydicus* A02, disruption of *nsdA* caused increased production of secondary metabolites (milbemycin A4, nanchangmycin, and natamycin, along with greater production of pigment and spores than their wild-type strain [8, 9].

The tetraene rimocidin is a glycosylated polyketide [10]. Like other macrolides, rimocidin is synthesized in *Streptomyces* sp. by so-called type I modular polyketide synthases [11]. Because of its broad range of biological activity against pathogenic fungi, rimocidin possessed some potential as a fungicide for controlling plant diseases and became an attractive target for research [12-14]. *Streptomyces rimosus* M527 (China Center for Type Culture Collection (CCTCC) M2013270), which was originally isolated in 2011 from soil, is also known as rimocidin producer [15]. In 2019, a gene transfer system for the strain was developed [16] and the genome of the strain was sequenced (GenBank accession No. GCA_004196335.1). Recently rimocidin production in the strain could be enhanced by ribosome engineering [17]. Our knowledge about regulatory mechanisms controlling rimocidin biosynthesis and about morphological differentiation lagged far behind our knowledge about the biosynthesis of rimocidin [11, 18, 19]. Elucidation and manipulation of regulatory networks have recently been shown to be an efficient strategy for further improving production of various industrial compounds [20, 21]. For example, Luo et al. [22] identified a transcriptional regulator PhaR in *Streptomyces roseosporus* L30. Deletion of *phaR* led to an increased expression of the gene cluster resulting in a 6.14-fold increased daptomycin production.

In the genome sequence of *S. rimosus* M527 *nsdA_{sr}* (GenBank accession No. MN395042) was identified which encodes a protein with high similarity to NsdA from *S. coelicolor*. This study aimed to explore the role of *nsdA_{sr}* in morphological differentiation and rimocidin biosynthesis in *S. rimosus* M527. Construction of a mutant of *S. rimosus* M527 with a *nsdA_{sr}* deletion was accomplished using the CRISPR/Cas9 system. NsdA_{sr} was identified as a negative regulator of rimocidin biosynthesis by repressing transcription of structural genes. Furthermore, heterologous expression of *nsdA_{sr}* in *Streptomyces diastatochromogenes* 1628 and *S. coelicolor* M145 also affected morphological differentiation and inhibited natural product biosynthesis.

Results

Cloning and identification of putative *nsdA_{sr}* from *S. rimosus* M527

Referencing the available genome sequence of *S. rimosus* M527 (GenBank accession No. GCA_004196335.1), a *nsdA* orthologue *nsdA_{sr}* gene from *S. rimosus* M527 was cloned. The nucleotide sequence was submitted to the GenBank database under the accession number MN395042. The 1476 bp *nsdA_{sr}* gene encodes a protein of 491 amino acids. A phylogenetic tree was constructed to show the relationship between NsdA_{sr} and known NsdA homologues in other *Streptomyces* species (Additional file 1: Figure S1). NsdA_{sr} (boldface) was very similar to known NsdA proteins submitted to National Center for Biotechnology Information (NCBI). Among them, NsdA_{sr} showed the highest similarity to NsdA from *S. griseoflavus* (KOG64483, 99.6% identical amino acids).

Deletion of *nsdA_{sr}* gene increases rimocidin production and acceleration of sporulation

To further determine the role of *nsdA_{sr}* in rimocidin biosynthesis and morphological differentiation, knock-out plasmid pWHU2653- Δ *nsdA_{sr}* was created (Figure 1A). The construction of the *nsdA_{sr}* disruption mutant M527- Δ *nsdA_{sr}* was achieved using the CRISPR/Cas9-CodA(sm) combined system. The deletion of *nsdA_{sr}* was validated by PCR with the corresponding verification primers P5/P8 and P9/P10. As shown in Figure 1B, a 1476 bp *nsdA_{sr}* gene could be amplified from M527 (lane 1), but not from M527- Δ *nsdA_{sr}* (lane 2). In addition, amplicons of 5.7 kb (lane 3) and 4.2 kb (lane 4) represented products of the wild-type strain M527 and the mutant strain M527- Δ *nsdA_{sr}*. These results indicate that the *nsdA_{sr}* gene was deleted in mutant M527- Δ *nsdA_{sr}*.

To investigate the effect of the *nsdA_{sr}* gene on rimocidin production, a shake-flask fermentation experiment was performed using mutant *S. rimosus* M527- Δ *nsdA_{sr}* and wild-type *S. rimosus* M527. Samples were periodically collected and analyzed to determine the concentration of rimocidin in the fermentation broth using HPLC (Additional file 2: Figure S2). After 96 h, the level of rimocidin produced by *S. rimosus* M527- Δ *nsdA_{sr}* had reached 318.5 mg/L, an increase of 46% compared to *S. rimosus* M527 (Figure 2), suggesting that *NsdA_{sr}* acts as a negative regulator in rimocidin biosynthesis.

To confirm that the deletion of the *nsdA_{sr}* gene was the sole reason for the increase in rimocidin production, the mutant M527- Δ *nsdA_{sr}* was complemented by introducing an integrative plasmid pSET152::*nsdA_{sr}* in which the *nsdA_{sr}* gene was driven by its own promoter, to generate the complemented strain M527- Δ *nsdA_{sr}*/pSET152::*nsdA_{sr}*. The complemented strain M527- Δ *nsdA_{sr}*/pSET152::*nsdA_{sr}* produced rimocidin at levels comparable with levels produced by the wild-type strain (Additional file 2: Figure S2 and Figure 2).

Moreover, to further investigate the effect of *nsdA_{sr}* on morphological differentiation, scanning electron microscopy (SEM) was employed to examine morphological characteristics of wild-type M527 and mutant M527- Δ *nsdA_{sr}* strains. More abundant sporulation in mutant M527- Δ *nsdA_{sr}* was observed (Figure 3). Moreover, no significant difference between *S. rimosus* M527 and the complemented strain M527- Δ *nsdA_{sr}*/pSET152::*nsdA_{sr}* was found (Figure 3). These results indicate that *NsdA_{sr}* negatively affected sporulation. In addition, the expression of the empty plasmid pSET152 in *S. rimosus* M527 had no effect on morphological differentiation or rimocidin production (data not shown).

Construction of recombinant strain *S. rimosus* M527-NA_{sr}

NsdA_{sr} was predicted to be a negative regulator, suggesting that an increased copy number of *nsdA_{sr}* might play a negative role in the morphological differentiation and further depress rimocidin production. The gene *nsdA_{sr}* was placed under the control of promoter *ermE*^{*} in plasmid pIB139 to create pIB139-*nsdA_{sr}* (Additional file 3: Figure S3). The plasmid pIB139-*nsdA_{sr}* was then introduced into *S. rimosus* M527 by intergeneric conjugation [16], to generate the recombinant strain *S. rimosus* M527-NA_{sr} resistant

to 300 µg/mL apramycin (Additional file 4: Figure S4). The integration of plasmid pIB139-*nsdA_{sr}* into the chromosome of *S. rimosus* M527 was verified by PCR (Additional file 5: Figure S5).

Over-expression of *nsdA_{sr}* gene negatively affects sporulation behavior and rimocidin production, and represses transcription of *rim* genes *S. rimosus* M527

Morphological characteristics of the wild-type *S. rimosus* M527 and the recombinant M527-NA_{sr} when cultivated on MS agar plates at 28°C were assessed. Both *S. rimosus* M527 and M527-NA_{sr} produced white aerial hyphae, however, a difference in sporulation was observed between both strains (Figure 3). The wild-type strain gave rise to abundant yellow spores, whereas the recombinant strain hardly sporulated.

To evaluate the effect of *nsdA_{sr}* gene on rimocidin production, recombinant strain *S. rimosus* M527-NA_{sr} and control strain *S. rimosus* M527 were cultured in shake-flask fermentation. As shown in Figure 2, a large decrease in rimocidin production was observed for the recombinant strain M527-NA_{sr}. After 96 h, the amount of rimocidin produced by M527-NA_{sr} reached the highest level of 91.1 mg/L, a 58.2% decrease in the yield of rimocidin produced by the *S. rimosus* M527 (218.2 mg/L). These results indicate that *nsdA_{sr}* negatively affects on *S. rimosus* M527 morphological differentiation and rimocidin biosynthesis. Accordingly, the expression of empty plasmid pIB139 in *S. rimosus* M527 had no effect on morphological differentiation and rimocidin production (data not shown).

The partial sequence of the *rim* gene cluster involved in rimocidin biosynthesis in *S. rimosus* M527 was cloned and published (GenBank accession No: MK300953). The putative functions of 10 *rim* genes (*rimA* to *rimK*) located in the cluster have been analyzed, and these genes are anticipated to be responsible for rimocidin biosynthesis [11, 17]. To test whether NsdA_{sr} regulates rimocidin biosynthesis through affecting transcription of *rim* genes, we performed qRT-PCR analysis using total RNA of *S. rimosus* M527, mutant M527-Δ*nsdA_{sr}*, complemented strain M527-Δ*nsdA_{sr}*/pSET152::*nsdA_{sr}* and *S. rimosus* M527-NA_{sr}, after 48 and 84 h of fermentation. As compared with wild-type, transcriptional levels of *rim* genes were obviously increased in M527-Δ*nsdA_{sr}*, and the increase was diminished in the complemented strain. Transcriptional levels of *rim* genes were repressed in strain *S. rimosus* M527-NA_{sr} (Figure 4). NsdA_{sr} thus impairs gene expression at the transcriptional level for all *rim* genes and further decreases rimocidin production in over-expressing recombinant strains.

Heterologous expression of the *nsdA_{sr}* gene negatively affects sporulation behavior and antibiotic production of *S. coelicolor* M145 and *S. diastatochromogenes* 1628

To determine whether the *nsdA_{sr}* has similar negative effects on *S. coelicolor* M145 and *S. diastatochromogenes* 1628, the plasmid pIB139-*nsdA_{sr}* was also introduced into *S. coelicolor* M145 and *S. diastatochromogenes* 1628 by intergeneric conjugation, to generate the recombinant strains M145-NA_{sr} and 1628-NA_{sr}, both resistant to 50 µg/mL apramycin (data not shown). The integration of plasmid

pIB139-*nsdA_{sr}* into the chromosome of *S. coelicolor* M145 (Additional file 6: Figure S6) and *S. diastatochromogenes* 1628 (Additional file 7: Figure S7) was verified by PCR, respectively. When grown on Gauze's No. 1 medium for 6 days, blue pigment corresponding to Act, which diffuses into the agar, can be detected earlier and is increased in wild-type *S. coelicolor* M145 comparing with the recombinant strain M145-NA_{sr} (Figure 5A). Sporulation behaviors of *S. coelicolor* M145-NA_{sr} was also delayed because of the introduction of the *nsdA_{sr}* gene. Similarly, heterologous expression of the *nsdA_{sr}* gene had negative effects on sporulation and toyocamycin(TM) production of *S. diastatochromogenes* 1628. Nearly no difference between control strain *S. diastatochromogenes* 1628 and *S. diastatochromogenes* 1628-NA_{sr} in terms of cell growth was observed (Figure 5B). However, after 24 h of cultivation, the sporulation of *S. diastatochromogenes* 1628-NA_{sr} was obviously impaired. TM production for *S. diastatochromogenes* 1628-NA_{sr} was greatly reduced (Figure 5C). After 84 h, TM production by 1628-NA_{sr} was reduced by 52%.

Additionally, the effect of *nsdA_{sr}* on the transcription of the respective genes involved in biosynthesis of Act and TM was investigated. In *S. coelicolor*, Act biosynthesis is dependent on the transcriptional activation of the Act biosynthesis cluster by the ActII-orf4 protein, and increased expression of *actII-orf4* results in the overproduction of Act [23-26]. Total RNA from *S. coelicolor* M145 and M145-NA_{sr} was isolated after 48 and 84 h of cultivation. Semi-quantitative RT-PCR analysis results indicated that gene *actII-orf4* was down-regulated in *S. coelicolor* M145-NA_{sr} (Figure 6A), indicating that heterologous expression of *nsdA_{sr}* weakens transcription of *actII-orf4*. As an internal control, the expression of 16S rDNA was comparable in the two strains.

As expected, semi-quantitative RT-PCR analysis also indicated that heterologous expression of *nsdA_{sr}* had similar negative effects on the transcription of five *toy* genes(*toyA*, *toyB*, *toyE*, *toyF*, and *toyG*). Compared to the wild-type strain, the transcriptional levels of all *toy* genes were greatly decreased in 1628-NA_{sr} (Figure 6B).

Discussion

The polyene macrolide rimocidin shows strong antifungal activity and can be used to treat plant fungal diseases. *S. rimosus* M527 is a rimocidin producer, but its rimocidin production is very limited. Accordingly, significant focus has been placed on developing genetic transformation methods and expression systems [16] or ribosome engineering technology combined with new fermentation conditions to improve rimocidin production [17]. The long-term goal of our research is to better understand regulatory mechanisms involved in rimocidin biosynthesis. Such understanding will allow rational modification of regulatory networks to achieve up- or down-expression of specific gene(s) to control the desired metabolic flow. This will eventually lead to an increased rimocidin production and more potent and useful rimocidin analogues. To date, a lack of knowledge of the regulation of rimocidin biosynthesis has prevented titer enhancement of rimocidin production by rational strategies.

The *nsdA* gene, which is found and conserved in some *Streptomyces*, negatively affects morphological differentiation and secondary metabolite production [7-9]. Recently, by genome mining of *S. rimosus* M527, the *nsdA* homologue *nsdA_{sr}* was found, which encodes a 491-amino-acid protein that shares up to 75% amino acids with other NsdA proteins. An obvious question is whether *nsdA_{sr}* has similar effects on sporulation behavior and rimocidin production.

An efficient gene disruption technology in *S. rimosus* M527 will make it possible to determine gene function through characterization of deletion mutant phenotypes. And it will certainly contribute to a better understanding of the relationship between regulators and rimocidin biosynthesis. Unfortunately, biotechnological methods available in *Streptomyces* are limited. Typically, for gene disruption in *Streptomyces*, single crossover integration of a suicide plasmid can be employed, resulting in disruption of the gene of interest with a selectable marker [27]. However, the limited number of selectable markers limits the reusability of this approach. Alternatively, clean genomic deletions can be made *via* double-crossover integration. However, this multistep process is often time-consuming and laborious [27-29]. Auspiciously, the appearance of clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) protein systems provides an ideal solution to these problems. Compared with traditional methods, the application of CRISPR/Cas9 has several advantages, including higher efficiency and ease of operation [30]. The establishment of CRISPR/Cas9-based genetic manipulation approaches in *Streptomyces* opened a new era for genome engineering of this type of microorganism [31]. A few CRISPR/Cas9 systems for genome editing have been developed and applied in various *Streptomyces* species, such as *Streptomyces coelicolor* [32] and *Streptomyces lividans* [33]. By co-expression with customized single guide RNAs (sgRNAs), *Streptococcus pyogenes* Cas9 nuclease can mediate a double-strand break in the sgRNA binding region; efficient double crossover can be achieved when the homologous templates are provided in the same plasmid. Among them, pWHU2653, based on engineered CRISPR/Cas9 combined with the counter selection system CodA(sm), has been developed and was successfully used to rapidly and effectively delete the Act polyketide chain length factor gene *actI-ORF2* [34]. In this study, in order to clarify the relationship of *nsdA_{sr}* to morphological differentiation and rimocidin biosynthesis in *S. rimosus* M527, pWHU2653 was adopted for the construction of a *nsdA_{sr}* deletion mutant using CRISPR/Cas9 system. Results show that gene deletion of *nsdA_{sr}* results in an increase in rimocidin production. Complementation of *nsdA_{sr}* reduced rimocidin production to levels comparable with production in the wild-type strain. qRT-PCR analysis revealed that *rim* genes were up-regulated in the *nsdA_{sr}* mutant and down-regulated in the *nsdA_{sr}* over-expression strain. In addition, deletion and over-expression of *nsdA_{sr}* led to acceleration and delaying of sporulation, respectively. The qRT-PCR result demonstrated that the decreased rimocidin production caused by the introduction of *nsdA_{sr}* was attributed to the inhibition of transcription of rimocidin biosynthetic genes. These results support the conclusion that NsdA_{sr} plays a negative role in morphological differentiation and rimocidin production in *S. rimosus* M527.

Whether the negative effect of NsdA_{sr} is universal to other *Streptomyces* is still unknown. In this study, the deduced amino acid sequence of NsdA_{sr} showed 77.6% similarity to NsdA_{sc} from model strain *S.*

coelicolor. The disruption of the *nsdA_{sc}* gene resulted in the overproduction of Act in *S. coelicolor*. Thus, the heterologous expression of *nsdA_{sr}* in *S. coelicolor* was considered and performed. As expected, NsdA_{sr} had negative effects on sporulation and Act biosynthesis as well as on transcription of the pathway-specific regulator, *actII-orf4*. This result confirmed that NsdA_{sr} is not only a homologue of NsdA_{sc} but also has similar effects. Subsequently, by heterologous expression of *nsdA_{sr}*, similar negative effects on morphological differentiation and antibiotic production were observed in *S. diastatochromogenes* 1628, which is considered a significant industrial producer of the nucleoside antibiotic, TM [35, 36].

Conclusions

In conclusion, *nsdA_{sr}* cloned in this study is identified as a negative regulator of sporulation and antibiotic biosynthesis as well as the transcription of biosynthetic genes both in its host *S. rimosus* M527 and in model strain *S. coelicolor* and industrial producer strain *S. diastatochromogenes* 1628. However, the exact molecular mechanism of rimocidin biosynthesis and other metabolic pathways affected by NsdA_{sr} remains unknown. This point could be elucidated by RNA-seq or -omics strategies using the *nsdA_{sr}* deletion mutant and wild-type strains. Further studies are needed to elucidate other key genes or metabolic pathways involved in rimocidin biosynthesis.

The importance of this work is that it provides further information for understanding regulatory mechanisms controlling rimocidin biosynthesis in *S. rimosus* M527. In addition, the successful application of a CRISPR/Cas9 system based on pWHU2653 in this study will provide a basis for efficient genome manipulation for the overproduction of rimocidin.

Materials And Methods

Materials

Q5 High-Fidelity Master Mix with GC-buffer was purchased from NEB. Restriction endonucleases, Miniprep, and Gel Extraction kits were purchased from TaKaRa Biotechnology Co. Ltd. 5-Fluorocytosine (5FC) was purchased from Aladdin. Oligonucleotide primer synthesis and DNA sequencing of PCR products were performed by Shanghai Sunny Biotechnology Co. Ltd. China.

Strains, plasmids, and primers

The strains and plasmids used in this study are listed in Table 1. Strains were used as follows: Rimocidin producer *S. rimosus* M527 has been deposited in the China Center for Type Culture Collection (CCTCC: M2013270). Act producer *S. coelicolor* M145 was provided by Prof. Andreas Bechthold (University of Freiburg, Freiburg, Germany). TM producer *S. diastatochromogenes* 1628 has been deposited in the China General Microbiological Culture Collection Center (CGMCC No. 2060) [37]. *Escherichia coli* JM109 was used as a general host for gene cloning and plasmid construction. Methylation-deficient strain, *E. coli*

ET12567/pUZ8002, was used as the donor for plasmid transfer to *Streptomyces* by intergeneric conjugation.

Table 1 Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strain		
<i>E. coli</i> JM109	General cloning host	Our lab
<i>E. coli</i> ET12567/pUZ8002	<i>Cm^r</i> , <i>Km^r</i> , donor strain for conjugation	Our lab
<i>S. rimosus</i> M527	Rimocidin producer	CCTCC 2013270
<i>S. diastatochromogenes</i> 1628	Toyocamycin producer	CGMCC 2060
<i>S. coelicolor</i> M145	Actinorhodin producer	Prof. Andreas
M527- Δ <i>nsdA_{sr}</i>	<i>nsdA_{sr}</i> gene deletion mutant, derived from M527 strain	This work
M527- Δ <i>nsdA_{sr}</i> /pSET152:: <i>nsdA_{sr}</i>	<i>nsdA_{sr}</i> complemented strain, mutant M527- Δ <i>nsdA_{sr}</i> with integrative plasmid pSET152:: <i>nsdA_{sr}</i>	This work
M527-NA _{sr}	M527 with integrative vector pIB139- <i>nsdA_{sr}</i>	This work
M145-NA _{sr}	M145 with integrative vector pIB139- <i>nsdA_{sr}</i>	This work
1628-NA _{sr}	1628 with integrative vector pIB139- <i>nsdA_{sr}</i>	This work
Plasmids		
pSET152	Integrative plasmid, <i>apr^r</i> , <i>oriT_{RK2}</i> , ϕ C31 <i>int/attP</i>	Our lab
pIB139	Derivative of integrative plasmid pSET152, harboring a <i>Perme[*]</i> promoter, <i>apr^r</i> , <i>oriT_{RK2}</i> , ϕ C31 <i>int/attP</i>	Our lab
pWHU2653	Scas9, sgRNA cloning cassette, <i>codA(sm)</i> , <i>apr^r</i> , <i>ori(coEI)</i>	[34]
pWHU2653- Δ <i>nsdA_{sr}</i>	Derived from pWHU2653, for deletion of <i>nsdA_{sr}</i> containing up- and down-stream homologous arms of <i>nsdA_{sr}</i>	This work
pSET152:: <i>nsdA_{sr}</i>	Derived from pSET152, harboring <i>nsdA_{sr}</i> driven by its own promoter	This work
pIB139- <i>nsdA_{sr}</i>	<i>nsdA_{sr}</i> gene under the control of promoter <i>Perme[*]</i> in pIB139	This work

Plasmid pWHU2653 [34], a gift from Prof. Sun YH, was used for disruption of the *nsdA_{sr}* gene using the CRISPR/Cas9 system. The primers (restriction sites are underlined) used in this study are listed in Table 2.

Table 2 Primers used in this study

Primers	Description	Source or reference
P1	5'-CACCACCACCACTGAGCTAGCTTCAGACGTGTCTA-3'	This work
P2	5'-TCCGTGTCCGGCGTCGACCTGCTGGATCCT-3'	This work
P3	5'-AGGTCGACGCCGGACACGGAGTTTTAGAGC-3'	This work
P4	5'-GTCGACTAGAGGATCCCCGGGTATCTAGAAA-3'	This work
P5	5'-CGTCGACCTGCAGGCATGCAAGCTTCGCGTCGTCCACCAGCACCTCG-3'	This work
P6	5'-AATCGGAATGGGGGCGTTCCACAGCACTCCCACAGACCCCGG-3'	This work
P7	5'-GGTCTGTGGGAGTGCTGTGGAACGCCCCCATTCCGATTGC-3'	This work
P8	5'-GAGTGCTTGCGGCAGCGTGAAGCTTGGCATGGTCGGCCTTACGGACAG-3'	This work
P9	5'-ACCGCATATGGTGGGCGGCAGTGGCGGCAC-3' (<i>NdeI</i>)	This work
P10	5'-ACCGTCTAGATTATCAGACGGCCTCCGCGCCGG-3' (<i>XbaI</i>)	This work
P11	5'-ACCGCATATGCGGCAGCCGGACCGAGCAGT-3' (<i>NdeI</i>)	This work

Primers P1-P4 were used for amplification of sgRNA. Primers P5 and P6 were used for amplification of up-stream homologous arms of *nsdA_{sr}*. Primers P7 and P8 were used for amplification of down-stream homologous arms of *nsdA_{sr}*. Primers P9 and P10 were used for amplification of *nsdA_{sr}*. *NdeI* and *XbaI* restriction enzyme sites are underlined. Primers P11 and P10 were used for amplification of a 1776-bp DNA fragment containing the coding region of *nsdA_{sr}* and its 300-bp upstream promoter region.

Media and culture conditions

E. coli strains were cultured using liquid or solid LB medium containing appropriate antibiotics at 37 °C. Antibiotics were used in the following concentrations: apramycin (100 µg/mL), chloramphenicol (25 µg/mL), ampicillin (100 µg/mL), and kanamycin (50 µg/mL). To generate spores, *Streptomyces* cells were sprayed on MS medium [17] and incubated for 5-6 days at 28°C. Collected spores were washed with water and preserved in water/glycerol (1:1, v/v) at -80°C.

2CMC solid medium [16] was used for conjugation. CP liquid medium [38] was used as seed medium. MS, YMG [39], and Gauze's No. 1 medium were used for morphological observation. Gauze's No. 1 medium composes of 20 g of starch, 1 g of KNO₃, 0.5 g of KH₂PO₄, 0.5 g of MgSO₄, 0.5 g of NaCl, 0.01 g of FeSO₄, and 20 g of agar per liter.

S. rimosus M527 and its derivatives were incubated using the method of Zhao et al. [17]. *S. diastatochromogenes* 1628 and its derivative were incubated using the method of Xu et al. [35]. *S. coelicolor* M145 and its derivative were incubated using the method of Zhao et al. [38].

Construction of the $\Delta nsdA_{sr}$ mutant and its complementation

Plasmid pWHU2653, a delivery vector containing the sgRNA cloning cassette and counterselection marker CodA(sm), was developed for genome editing in *Streptomyces* using the CRISPR/Cas9 system [34, 40]. sgRNA consists of an exchangeable 20 nt guide sequence that matches the target DNA and an invariant scaffold that binds to Cas9 in pWHU2653. In this study, a protospacer adjacent motif (PAM) for sgRNA and a 20 nt target guide sequence AGGTCGACGCCGACACGGA of the *nsdA_{sr}* gene extended by PAM were selected according to the guide design tool (<https://zlab.bio/guide-design-resources>). To construct the double-enzyme digestion sgRNA cloning cassette, PCR was used to generate the sgRNA with the target sequence using pWHU2653 as the template. Primers P1/P2 were used to amplify the upstream fragment, whereas another pair of primers P3/P4 were used to amplify the downstream fragment. The two fragments, each end flanked by a 20 bp homology sequence from pWHU2653, were spliced together by overlap extension PCR, yielding a 0.3 kb sgRNA fragment in which expression of sgRNA is under control of the constitutive promoter ermE*. The 0.3 kb sgRNA cloning cassette was inserted into pWHU2653 between *NheI*/*XbaI* using an infusion cloning kit, generating plasmid pWHU2653-sgRNA. Subsequently, the 2.1 kb upstream homologous arm (UHA) and 2.1 kb downstream homologous arm (DHA) of the *nsdA_{sr}* start codon were amplified by primer pairs P5/P6 and P7/P8 from genomic DNA of *S. rimosus* M527, respectively. The resulting DNA fragments, UHA and DHA, were ligated into the *HindIII* site of pWHU2653-sgRNA using Gibson assembly methods as described by Gibson et al. [41], yielding plasmid pWHU2653- $\Delta nsdA_{sr}$ for gene knock out.

The constructed pWHU2653- $\Delta nsdA_{sr}$ was introduced into the wild-type strain *S. rimosus* M527 by intergeneric conjugation as described by Song et al. [16]. Single apramycin-resistant exconjugants were patched on 2CMC agar, containing apramycin and nalidixic acid (300 and 100 µg/mL, respectively), and grown at 28 °C for four or five generations. Genomic DNA was extracted from mycelium grown on the plate and amplified by PCR using primers P9/P10 to verify the deletion of *nsdA_{sr}*. To obtain plasmid-free progeny, single exconjugants were picked and streaked on 2CMC agar containing 800 µg/mL 5FC and grown in the dark at 28 °C for 3 or 4 days. The 5FC^R colonies were then replicated to 2CMC with and without apramycin to confirm plasmid loss. The $\Delta nsdA_{sr}$ mutants were named *S. rimosus* M527- $\Delta nsdA_{sr}$.

For complementation of *nsdA_{sr}* in *S. rimosus* M527- Δ *nsdA_{sr}*, a 1776 bp DNA fragment containing the coding region of *nsdA_{sr}* and its 300 bp upstream promoter region was amplified by PCR using P11 and P10 as primers. The DNA fragment was inserted into the *Nde*I and *Xba*I sites of pSET152 to obtain pSET152::*nsdA_{sr}*. Introduction of pSET152::*nsdA_{sr}* and the empty vector pSET152 as a control into mutant M527- Δ *nsdA_{sr}* by conjugation resulted in the complemented strain *S. rimosus* M527- Δ *nsdA_{sr}*/pSET152::*nsdA_{sr}* and the control strain *S. rimosus* M527- Δ *nsdA_{sr}*/pSET152, respectively.

Over-expression/Heterologous expression of *nsdA_{sr}* in *S. rimosus* M527/*S. coelicolor* M145 and *S. diastatochromogenes* 1628

All recombinant DNA techniques were performed as described by Sambrook and Russell [42]. Plasmid pIB139 [43, 44] is a shuttle vector that replicates in *E. coli* and integrates site-specifically into *Streptomyces* chromosomes. Using *S. rimosus* M527 genomic DNA as a template, a 1476 bp *nsdA_{sr}* open reading frame (ORF) was amplified by PCR using primers P9 and P10 (Table 1). The PCR product was then digested with *Nde*I and *Xba*I and inserted into the corresponding sites of pIB139, yielding plasmid pIB139-*nsdA_{sr}*. Sequencing of the inserted gene fragment confirmed that the gene did not contain any mutations.

Subsequently, the introduction of the constructed pIB139-*nsdA_{sr}* into *S. rimosus* M527, *S. coelicolor* M145, and *S. diastatochromogenes* 1628 was conducted by intergeneric conjugation to yield recombinant strains *S. rimosus* M527-NA_{sr}, *S. coelicolor* M145-NA_{sr} and *S. diastatochromogenes* 1628-NA_{sr}, respectively. Recombinant strains were confirmed using apramycin resistance and PCR.

Morphological observation

To evaluate morphological differentiation, wild-type strain *S. rimosus* M527, the *nsdA_{sr}*-disrupted mutant M527- Δ *nsdA_{sr}*, the complemented strain M527- Δ *nsdA_{sr}*/pSET152::*nsdA_{sr}* and the recombinant strain M527-NA_{sr} were streaked on solid MS medium; the wild-type *S. diastatochromogenes* 1628 and recombinant strain 1628-NA_{sr} were streaked on solid YMG medium; and the wild-type *S. coelicolor* M145 and recombinant strain M145-NA_{sr} were streaked on Gauze's No. 1 medium. The morphology of all strains was observed after incubation for 6-7 days at 28°C. Morphological characteristics of the mycelia surface were examined under SEM (JSM-5410LV, JEOL, Tokyo, Japan).

Analysis of gene transcriptional levels by qRT-PCR

Extraction of RNA and analysis of transcriptional levels of *rim* genes were performed as described previously [17, 36]. Extraction of RNA, design of primers, and analysis of the transcription of *toy* genes were performed as described by Xu et al. [35]. Extraction of RNA, design of primers, and analysis of transcription of *actII-orf4* gene were performed as described by Zhao et al. [38].

Fermentation of antibiotic

Production of rimocidin by *S. rimosus* M527 was achieved using the method of Zhao et al. [17]. Production of TM by *S. diastatochromogenes* 1628 was achieved using the method of Ma et al. [36].

Analysis of antibiotic

Rimocidin was analyzed using high-performance liquid chromatography (HPLC) (Varian, USA) method described previously [17]. HPLC analysis of TM used the method of Ma et al. [36]. Gauze's No. 1 medium was used to identify the production of Act on agar media by directly evaluating the density of the blue color characteristic of this antibiotic.

Statistical analysis

All experiments were performed at least three times, and results were expressed as mean $\pm$ standard deviations (SD). Statistical analysis was performed with Student's *t*-test.

Abbreviations

qRT-PCR: quantitative RT-PCR; TM: toyocamycin; Act: actinorhodin; SEM: scanning electron microscopy; sgRNA: single guide RNA; 5FC: 5-Fluorocytosine; CCTCC: China Center for Type Culture Collection; CGMCC: China General Microbiological Culture Collection Center; PAM: protospacer adjacent motif; UHA: upstream homologous arm; DHA: downstream homologous arm; ORF: open reading frame; HPLC: high-performance liquid chromatography; SD: standard deviations. CRISPR/Cas: clustered regularly interspaced short palindromic repeats (CRISPR) /CRISPR-associated

Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and materials: All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Competing interests: The authors declare that they have no competing interests.

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Additional File Information

Additional file 1: Figure S1. Phylogenetic analysis of NsdA_{sr} using NCBI BLASTP.

Additional file 2: Figure S2. HPLC-analysis to measure the influence of NsdA_{sr} on rimocidin production.

Additional file 3: Figure S3. Map of constructed plasmid pIB139-*nsdA*_{sr}.

Additional file 4: Figure S4. Phenotypic verification of recombinant strains *S. rimosus* M527-NA_{sr}.

Additional file 5: Figure S5. PCR analysis of apramycin (*ap^r*) gene from recombinant strains *S. rimosus* M527- NA_{sr}.

Additional file 6: Figure S6. PCR analysis of apramycin (*ap^r*) gene from recombinant strains *S. coelicolor* M145- NA_{sr}.

Additional file 7: Figure S7. PCR analysis of apramycin (*ap^r*) gene from recombinant strains *S. diastatochromogenes* 1628-NA_{sr}.

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Figures

Figure 1

Construction of mutant *S. rimosus* M527-ΔnsdAsr. Map of plasmid pWHU2653 -ΔnsdAsr. A. The sgRNA consists of the 20 nt target gene specific guide sequence of *S. rimosus* M527 (yellow) and the invariant scaffold RNA (green). Light orange parallelograms connect the identical UHA and DHA sequences on pWHU2653 and the *S. rimosus* M527 chromosome where homologous recombination can take place. B. PCR verification of the mutant *S. rimosus* M527-ΔnsdAsr. M: DL10000 DNA Marker. Lane 1, PCR products of nsdAsr gene were amplified by using the primers P9/P10 from wild-type strain *S. rimosus* M527; lane 2, PCR products of nsdAsr gene were amplified by using the primers P9/P10 from mutant M527-ΔnsdAsr; lane 3, PCR product amplified from the wild-type *S. rimosus* M527 by using the primers P5/P8; lane 4, PCR product amplified from the mutant *S. rimosus* M527-ΔnsdAsr by using the primers P5/P8.

Figure 2

Determination of effects of NsdAsr on rimocidin production. Rimocidin production of the wild-type strain *S. rimosus* M527 (●), *S. rimosus* M527- Δ nsdAsr (◆), *S. rimosus* M527- Δ nsdAsr/pSET152::nsdAsr (▲) and *S. rimosus* M527-NA_{sr} (■) in shake-flask cultures. All shake-flask fermentations were carried out in 250 mL flasks with a working volume of 40 mL at 200 rpm and 28°C. The medium was inoculated at 5% (v/v). The error bars were calculated from three different batches of fermentation.

A

B

S. rimosus M527

M527- Δ nsdA_{sr}

M527- Δ nsdA_{sr}/pSET152::nsdA_{sr}

M527-NA_{sr}

Figure 3

NsdAsr negatively affected on morphological differentiation. Morphological characteristics of the wild-type strain *S. rimosus* M527, *S. rimosus* M527- Δ nsdAsr, *S. rimosus* M527- Δ nsdAsr/pSET152::nsdAsr, and *S. rimosus* M527-NAAsr. A. Picture of an agar plate taken after 7 days of growth. B. Scanning electron micrographs SEM of four strains grown on MS medium for 7 days.

Figure 4

Summary of qRT-PCR analysis using rim genes (rimA~rimK) involved in rimocidin biosynthesis. The following strains were investigated: M527: *S. rimosus* M527; M527- Δ nsdAsr: *S. rimosus* M527- Δ nsdAsr; M527- Δ nsdAsr/pSET152::nsdAsr: *S. rimosus* M527- Δ nsdAsr/pSET 152::nsdAsr and M527-NAsr: *S. rimosus* M527-NAsr. SigBsr was used as an internal control. The cells were harvested from the fermentation broth after 48 and 84 h. Error bars were calculated by measuring the standard deviations of the data from three replicates of each sample. (*) indicates statistically significant results ($0.01 < P\text{-value} < 0.05$). (**) indicates highly statistically significant results ($P\text{-value} < 0.01$). rimA: type I polyketide synthase gene; rimB: type I polyketide synthase gene; rimC: tyrosine phosphatase gene; rimD: cholesterol oxidase gene; rimE: glycosyl transferase gene; rimF: aminotransferase gene; rimG: cytochrome P450 monooxygenase gene; rimH: ferredoxin gene; rimJ: crotonyl-CoA reductase gene; rimK: acetyltransferase gene.

Figure 5

Heterologous expression of *nsdAsr* in *S. coelicolor* M145 and *S. diastatochromogenes* 1628 and its effects. A. Visual observation of morphological differentiation and Act biosynthesis by *S. coelicolor* M145 and *S. coelicolor* M145-NA_{sr} grown on the Gauze's No.1 media. Spores (1×10^8) were streaked on Gauze's No.1 agar media and then incubated at 28 °C for 6 days. The reverse sides of the plates are shown to indicate the amount of Act produced. B. Detection of morphological differentiation of *S.*

diastatochromogenes 1628 and *S. diastatochromogenes* 1628-NA_{sr}. Spores (1×10⁸) were streaked on MS agar media and then incubated at 28 °C for 6 days. C. Detection of TM production of *S. diastatochromogenes* 1628 and *S. diastatochromogenes* 1628-NA_{sr} in shake-flask cultures. All shake-flask fermentations were carried out in 250 mL flasks with a working volume of 40 mL at 200 rpm and 28°C. The medium was inoculated at 5% (v/v). Error bars were calculated from three different batches of fermentation.

A

B

Figure 6

Summary of qRT-PCR analysis. A. Studies on the regulator actII-orf4 are shown. Here the following strains were investigated: M145: *S. coelicolor* M145; M145-NAsr: *S. coelicolor* M145-NAsr. B. Studies on toy genes (toyA~toyM) involved in TM biosynthesis are shown. Here the following strains were investigated: 1628: *S. diastatochromogenes* 1628; 1628-NAsr: *S. diastatochromogenes* 1628-NAsr. 16S rDNA was used as the positive internal control. Cells were harvested from fermentation broth at 48 and 84 h, respectively.

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