ORIGINAL ARTICLE

Role of *nsdA* in negative regulation of antibiotic production and morphological differentiation in *Streptomyces bingchengensis*

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To investigate the function of *nsdA* in *Streptomyces bingchengensis*, it was cloned and sequenced, which presented an 89.89% identity with that of *S. coelicolor*. The λ RED-mediated PCR-targeting technique was used to create *nsdA* replacement in the *S. bingchengensis* 226541 chromosome. The *nsdA* disruption mutant, BC29, was obtained, which produced more pigment and spores than did the ancestral strain. HPLC analysis revealed that the disruption of *nsdA* efficiently increased milbemycin A₄ production and nanchangmycin production by 1.5-fold and 9-fold, respectively. Complementation of the *nsdA* mutation restored the phenotype and antibiotic production. These results showed that *nsdA* negatively affected sporulation and antibiotic production in *S. bingchengensis*.

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Keywords: antibiotic production; *nsdA*; sporulation; *Streptomyces bingchengensis*

INTRODUCTION

Streptomyces have attracted great interest because of their well-known ability to produce a great variety of antibiotics and other secondary metabolites.¹ Secondary metabolism is a very complex regulatory network.² Pathway-specific regulatory genes, such as *actII-orf4*, *redD*, *cdaR* and *mmvR*, are at the bottom of the regulatory network, each controlling one antibiotic biosynthetic pathway.³ Global regulators, such as *bldA*,⁴ *bldB*,⁵ *bldD*⁶ and *bldG*,⁷ perform the highest level regulation and affect both morphological and physiological differentiation.^{8,9} At intermediate levels in the regulatory cascades, many regulatory genes, such as *afsB*,¹⁰ *abaA*,¹¹ *absB*,¹² *afsK-afsR*^{13,14} and *tcrA*,¹⁵ and two-component systems, such as *afsQ1-afsQ2*,¹⁶ *absA1-absA2*,^{17,18} *cutS-cutR*¹⁹ and *phoR-phoP*,²⁰ have been identified, which regulate the synthesis of two or more antibiotics. *absA1-absA2*, *cutS-cutR*, *phoR-phoP*, *tcrA* and some pathway-specific repressors regulate antibiotic production in a negative manner, as mutations in these genes resulted in the overproduction of the corresponding antibiotic(s). Global regulators may also play a negative role, as in the case of the A-factor receptor protein, ArpA, in *Streptomyces griseus*. When A-factor is absent, ArpA binds to the *adpA* promoter and represses the expression of *AdpA*, which is an activator of a regulon that consists of operons involved in mycelial differentiation and antibiotic production. The *arpA*-null mutants produced more streptomycin and formed aerial hyphae earlier than the wild-type strain did.^{21–23} Recent microarray data have indicated a cross-regulation among disparate antibiotic biosynthetic pathways and even some backregulation from

cluster-situated regulators to a “higher level” pleiotropic regulatory gene.²⁴ These studies showed that regulating antibiotic biosynthesis and mycelial differentiation is important for understanding the factors affecting antibiotic yield.

Among the many regulatory genes, *nsdA* was able to negatively control sporulation and antibiotic production, which was first found in *Streptomyces coelicolor* A3(2) in 2006.²⁵ Subsequent studies showed that *nsdA* is widely existent and conserved in *Streptomyces*, such as *S. hygroscopicus* 10~22, *S. species* FR-008, *S. lividans* ZX64, *S. aureofaciens* 211, *S. albus* JA3453, *S. hygroscopicus* 5008, *S. avermitilis* NRRL8165 and *S. coelicolor* M145.²⁶ The disruption of chromosomal *nsdA* resulted in the overproduction of spores and three of four known *S. coelicolor* antibiotics of different chemical types.²⁶ It suggested that *nsdA* might have some general effects on antibiotic production. However, the function of *nsdA* was less investigated.

The *S. bingchengensis* used in this study was isolated from soil samples collected from Harbin, China. As a novel strain, *S. bingchengensis* produces at least two kinds of antibiotics, the polyether nanchangmycin and macrolide compound milbemycins, A₃ and A₄, (Figure 1). In addition, it also generates new milbemycins, β_{13} , β_{14} , α_{28} , α_{29} α_{30} , and two new seco-milbemycins.^{27–29} The mixture of milbemycins A₃ and A₄ was marketed as an acaricide for the control of mites in 1990.³⁰ Subsequently, in animal health fields, 5-oxime derivatives of milbemycins, A₃ and A₄, were found to be highly effective as anthelmintics and were marketed. However, nanchangmycin is an antibiotic that affects cation transport, and is effective in

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Figure 1 The structures of milbemycins, A3 and A4, and nanchangmycin.

treating coccidiosis in chickens.³¹ The identification of new genes that regulate antibiotic biosynthesis and mycelial differentiation is important for understanding the factors affecting antibiotic yield. In this study, we report the identification of a new gene, *nsdA*, negatively affecting both processes. In light of our results, we propose a novel view of improving the output of milbemycins and nanchangmycin in *S. bingchengensis*.

MATERIALS AND METHODS

Microorganisms, cloning vectors and cultivation

For routine subcloning, *Escherichia coli* DH5 α was cultivated and transformed according to the method of Sambrook *et al.*³² ET12567 was used to propagate unmethylated DNA for introduction into *Streptomyces bingchengensis*_226541 by transformation or conjugation. *E. coli* BW25113/pIJ790 (gifts from Professor Andrew Hesketh) was the host for λ RED-mediated PCR-targeting mutagenesis. pIJ773 was used as the template for the amplification of a disruption cassette containing the apramycin resistance gene, *aac(3)IV*, and the RK2 origin of transfer (*oriT*), flanked by recognition sites for FLP recombinase.³³ *E. coli*–*Streptomyces* shuttle plasmid, pHZ1358 (provided by Dr Yinghua Zheng), was used to construct the *nsdA* gene-replacement vector. pIJ8600, which integrated into the *S. bingchengensis*_226541 chromosome by site-specific recombination at the bacteriophage Φ C31 attachment site, *attB*,^{34,35} was used to introduce single copies of genes into the *S. bingchengensis*_226541 chromosome.

S. bingchengensis was isolated from soil samples collected in Harbin, China. *S. bingchengensis* has been stored at the China General Microbiology Culture Collection Center (accession no.: CGMCC1734), and the 16S rDNA sequence was determined (accession no.: DQ449953 in National Center for Biological Information). Several *Streptomyces* media were used. MS agar³⁶ was used to make spore suspensions and for plating-out conjugations with *E. coli* ET12567 containing the RP4 derivative, pUZ8002.³⁷ R2YE was used for protoplast transformation.³⁶ Yeast extract–malt extract was a liquid medium.³⁶ All *Streptomyces* cultivations were carried out at 30 °C.

Genetic procedures

Standard genetic procedures with *E. coli* and *in vitro* DNA manipulations were carried out as described by Sambrook *et al.*³² Recombinant DNA manipulations in the *Streptomyces* species and isolation of genomic DNA were performed as described by Kieser *et al.*³⁶ DNA restriction and modifying enzymes were used as recommended by the manufacturers (BRL, Carlsbad, CA, USA and Takara Biotechnology Ltd Company, Dalian, China). DNA fragments were purified from agarose gels with the GeneClean kit II (BIO101, Beijing, China).

DNA sequencing and analysis

Primers were designed by using the data obtained from the *S. coelicolor* and *S. avermitilis* genome sequences.²⁵ Total DNA was isolated from *S. bingchen-*

*ensis*_226541,³⁶ and a DNA fragment containing a coding region for the *nsdA* was amplified with *nsdA* R (5'-CCTCGGCGAAGATGTCCTCC-3') and *nsdA* L (5'-TCTCCGTCGAGGACCTGGGC-3') primers. The amplified fragment was ligated into a pMD18-T vector to obtain the recombinant plasmid, pBC106. Then, *nsdA* was subcloned into the *Bgl*II site of an *E. coli*–*Streptomyces* shuttle plasmid, pHZ1358, to obtain pBC118, with *nsdA* 2L (5'-TCGAGATCTGTACGCGGGGATC-3', wherein underlined letters indicate restriction sites) and *nsdA* 2R (5'-GCATGCCTGCAGATCTACGATC-3'). Thereafter, pBC118 was used as a template for the following PCR mutagenesis experiments. Subclone sequencing was performed by Takara Biotechnology Ltd Company. DNA and deduced protein sequences were analyzed using publicly available programs at the following websites: <http://www.ncbi.nlm.nih.gov/blast/>, <http://watson.nih.go.jp/~jun/cgi-bin/frameplot-3.0b.pl> and <http://motif.genome.jp/>.

nsdA replacement and verification

The *nsdA* gene replacement vector was constructed by λ RED-mediated PCR-targeting mutagenesis.^{33,38} Two PCR primers, BC-*nsdA*-F and BC-*nsdA*-R, corresponding to the regions upstream and downstream of the *nsdA* coding region (underlined), respectively, were used to amplify *aac(3)IV*+*oriT* from pIJ773.

BC-*nsdA*-F: 5'-CCGCCGCGGCTTCCTCGGCACCTCGCTCGCGCTCTCCGATTCGGGGATCCGTCGACC-3'

BC-*nsdA*-R: 5'-GCGAAGGTGTCCTCGGCCATCCGCACGGCCCGCTTGCACTGTAGGCTGGAGCTGCTTC-3'

The amplified fragment was used to replace the *nsdA* in pBC118. The resulting plasmid, named pBC929, was verified by PCR and restriction analysis, and then passed through the non-methylating *E. coli* ET12567/pUZ8002 before reintroduction into *S. bingchengensis*_226541 by conjugation. The gene mutant was verified by PCR analysis.

In the complementation experiments, the intact *nsdA* fragments were amplified by the primers, P3 (5'-GAAGATCTCCTCGTTACCTCCATCAC-3', where underlined letters indicate restriction sites) and P4 (5'-GAAGATCTTCTGCACGCTGTCCGCCT-3'), and then inserted into the *Bam*HI site of pIJ8600 to give pJC3. The resulting vectors were introduced into the *nsdA* disruption mutant.

Fermentation and analysis of antibiotic production

The seed for preculture was spores. The medium for sporulation contained sucrose 4.0 g, yeast extract 2.0 g, malt extract 5.0 g and skimmed milk powder 1.0 g in 1 l water. The pH was adjusted to 7.0 with 1 M NaOH, 20 g of agar was added and this mixture was sterilized at 121 °C for 30 min. The spore suspension was prepared from agar plates incubated at 28 °C for 7–8 days.

A spore suspension of the culture of *S. bingchengensis*, 1.0 ml, was transferred to a 250-ml Erlenmeyer flask that contained 25 ml of the seed medium comprising sucrose 0.25 g, polypeptone 0.1 g and K₂HPO₄ 1.25 mg. The

inoculated flasks were incubated at 28 °C for 42 h on a rotary shaker at 250 r.p.m. Then, 8.0 ml of the culture was transferred into 1-l Erlenmeyer flasks containing 100 ml of the fermentation medium consisting of sucrose 8.0%, soybean powder 1.0%, yeast extract 0.2%, meat extract 0.1%, CaCO₃ 0.3%, K₂HPO₄ 0.03%, MgSO₄·7H₂O 0.1% and FeSO₄·7H₂O 0.005%. The medium pH was 7.2 before sterilization. Fermentation was carried out at 28 °C for 8 days on a rotary shaker at 250 r.p.m.^{27,28,29}

To analyze milbemycin A₄ and nanchangmycin titer, the samples were collected from fermentation flasks and processed for milbemycin extraction by mixing one volume of the whole culture with five volumes of ethanol and sonicated at room temperature for 30 min. The samples were centrifuged at 3250 g for 10 min and the supernatant was analyzed by HPLC using a 5-μl particle size NOVA-PAK (Waters, Milford, MA, USA) column (3.9×150 mm) eluted at a flow rate of 1.5 ml min⁻¹ with a 15-min linear gradient from 0 to 90% (V/V) of phase B. Phase A was MeCN-H₂O-MeOH (350:50:100, V/V/V) and phase B was MeOH. Chromatography was performed with a SHIMADZU LC-2010CHT HPLC system (Shimadzu Corporation, Kyoto, Japan) and detection was at 242 nm. As standard for antibiotic titer determination, a sample of milbemycin A₄ (100 μg ml⁻¹) and nanchangmycin (200 μg ml⁻¹) was used. The peak positions for milbemycin A₄ and nanchangmycin were determined compared with those for the standard compounds.

RESULTS

DNA sequencing and analysis

The nucleotide sequence of the 1.5-kb DNA fragment was amplified by PCR. Sequence analysis showed that the 1485-bp DNA fragment encoded a 494-amino-acid protein (GenBank EU779992), which shared 89.89% identity in the nucleotide sequence and 97.82% similarity at the amino-acid level compared with those of *S. coelicolor* (Table 1).

Replacement of *nsdA* gene

To understand the function of *nsdA*, the gene replacement vector, pBC929, was constructed using λRED-mediated PCR-targeting mutagenesis,³³ and then conjugated into *S. bingchengensis*_226541. The mutant strains were selected using apramycin and thiostrepton. Seven apramycin-resistant and thiostrepton-resistant (Am^RTsr^R) strains (named BC19) were obtained from conjugation plates, indicating the single cross-over events. Three apramycin-resistant and thiostrepton-sensitive (Am^RTsr^S) strains (named BC29) were obtained from conjugation plates, implying the loss of the plasmid by a second homologous recombination. Gene replacement events were confirmed by PCR using the *nsdA*2L-*nsdA*2R primers with genomic DNA from *S. bingchengensis*_226541 and gene disruptants. The expected 1.5-kb *nsdA* fragment was amplified from the *S. bingchengensis*_226541 genome by PCR. The PCR products from the double-cross-over recombination mutant, BC29, were larger than those from the ancestral strain by about 0.5 kb in size. However, the single-cross-

over recombination mutant, BC19, has both 1.5- and 2-kb gene fragments. These results showed that the *nsdA* was replaced precisely by an *oriT*+*aac*(3) IV in the homologous double-cross-over recombination mutant.

Phenotype of *nsdA* disruption mutant

The *nsdA* disruption mutant, BC29, was different from the ancestral strain in the rate and extent of sporulation. When cultured on an MS medium, the mutant BC29 began to produce a yellow pigment at 36 h, which was about 24 h earlier than that by *S. bingchengensis*_226541. The *nsdA* disruption mutant, BC29, gave rise to abundant gray spores grown for 72 h, whereas the ancestral strain produced white aerial hyphae at this time. Both pigment amounts and eventual sporulation levels in the mutant BC29 seemed to be greater than those of the ancestral strain (Figure 2). When we plated out diluted spores harvested from colonies on the MS medium grown for 10 days and counted the colony, BC29 spore suspensions reproducibly formed about twice as many colonies as did *S. bingchengensis*_226541.

All the phenotypic changes of the *nsdA* disruption mutant, BC29, were complemented by introducing pJC3, excluding potential polar effects on adjacent genes or an additional mutation in BC29 as alternative explanations. These results suggested that *nsdA* played a negative role in *S. bingchengensis*_226541 morphological differentiation.

Enhanced production of two antibiotics in *nsdA* disruption mutant

HPLC assays on the *nsdA* disruption mutant, BC29, revealed that disruption of *nsdA* remarkably increased antibiotic production. Milbemycin A₄ production increased from 670 to 1005 μg ml⁻¹, whereas nanchangmycin production improved from 230 to 2070 μg ml⁻¹. The yield of milbemycin A₄ and nanchangmycin increased by 1.5- and 9-fold, respectively (Figure 3). The change of milbemycin and nanchangmycin production was reversed to the ancestral level by the introduction of *nsdA* back into BC29 (data not shown), excluding potential polar effects on adjacent genes as an explanation. It inferred that *nsdA* had repressed the production of milbemycins and nanchangmycin by *S. bingchengensis*_226541.

DISCUSSION

In this paper, *nsdA* in *S. bingchengensis*_226541 was identified, which encoded a 483-amino-acid protein that shared a 97.82% sequence identity with the NsdA of *S. coelicolor*. Blast searching indicated that there was an ortholog with sequence identity over 80% to each of these NsdA ortholog proteins in *Streptomyces*. NsdA contained a conserved domain, DUF921, which was defined as a *Streptomyces* protein domain of unknown function. Proteins containing the

Table 1 Comparison of *nsdA* homologous genes of different *Streptomyces*

	GenBank accession numbers	Length of gene/protein (bp/amino acid)	Similarity with <i>nsdA</i> of <i>S. bingchengensis</i> _226541	
			Amino acid	Nucleotide
<i>Streptomyces bingchengensis</i> _226541	EU779992	1450/494	100%	100%
<i>S. avermitilis</i> MA-4680	SAV2652	1476/491	83.93%	83.33%
<i>S. lividans</i> ZX64	DQ478681	1503/500	97.82%	89.77%
<i>S. qingfengmyceticus</i> A553	DQ478680	1503/500	75.00%	76.13%
<i>S. coelicolor</i> M145	EU779992	1450/483	97.82%	89.89%
<i>S. hygroscopicus</i> 5008	DQ478679	1527/508	86.11%	84.15%
<i>S. albus</i> JA3453	DQ478678	1473/490	81.94%	82.06%
<i>S. spicificus</i> FR-008	DQ478677	1485/494	71.23%	79.39%

Figure 2 (a–c) The effect of *nsdA* disruption on morphological differentiation grown for 72 h. (a) *S. bingchengensis*_226541: ancestral strain; (b) BC29: *nsdA* disruption mutant in which *nsdA* was replaced by an *oriT*+*aac(3)*/IV cassette; (c) BC29 complemented by pJC3 (a pIJ8600-derived plasmid containing *nsdA*).

Figure 3 HPLC analysis of milbemycin A₄ and nanchangmycin in (a) ancestral strain *Streptomyces bingchengensis*_226541 and (b) *nsdA* disruption mutant, BC29.

DUF921 domain included several putative regulatory proteins from *S. coelicolor* and *S. griseus*.³⁹ A search in the superfamily server also revealed that NsdA and other homologous proteins in *Streptomyces* had tetratricopeptide repeat-like repeats,⁴⁰ which mediated protein–protein interactions.⁴¹ We are currently using an *E. coli* two-hybrid system to seek NsdA-interacting proteins. The disruption of the *SCO4114* homologous gene, which had earlier been shown to prevent premature sporulation septation of *S. griseus*, had no obvious phenotypic effects on *S. bingchengensis* (data not shown). Possibly, there are differences in the manner deployed in the two species.

nsdA negatively affects both antibiotic biosynthesis and mycelial differentiation. The repressing effect was obvious even when the *nsdA* gene was disrupted. Gene disruption mutant, BC29, showed abundant gray spores in contrast to the white phenotype of the ancestral strain, and markedly increased the yield of milbemycin A₄ and nanchangmycin. In view of the fact that the structures of milbemycin A₄ and nanchangmycin are quite unlike each other, this result suggested that

nsdA might have some general effects on secondary metabolism. As the disruption of chromosomal *nsdA* resulted in higher productions of Act, CDA, Mmy and spores in *S. coelicolor*, *actII-orf4* mRNA was increased in an *nsdA* mutant, suggesting that the negative effect of *nsdA* on Act biosynthesis was exerted at the level of transcription of the pathway-specific activator gene.^{25,42} Although the genomic library of *S. bingchengensis* is under way, the new transcription factors, including pathway-specific regulators of milbemycin A₄ and nanchangmycin biosynthesis cluster, will be identified, which may be correlated with *nsdA*, suppressing antibiotic production. The elucidation of correlations between *nsdA* and its corresponding factors could be helpful in enhancing antibiotic yield.

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