NsdB, a TPR-like-domain-containing protein negatively affecting production of antibiotics in *Streptomyces coelicolor* A3(2)

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Abstract: Tetratricopeptide repeat (TPR) domains usually mediate protein-protein interactions. NsdA, one of the 70 proteins containing TPR-like domains in *Streptomyces coelicolor* A3(2), was previously found to negatively control sporulation and antibiotic production. Here we show that elimination of *SCO*7252, which encodes another of these proteins, also caused overproduction of two antibiotics, actinorhodin and CDA, but did not affect morphological differentiation. Disruption of *SCO*1593, encoding another of the family, had no obvious phenotypic effects. In surface-grown cultures, expression of *SCO*7252, which was named *nsdB*, was initiated at about 30 h, like that of *nsdA*. Analysis *in silico* of the 70 predicted TPR-like-containing proteins of *S. coelicolor* showed that 32 of them contained only TPR-like domains, and 25 of the remainder contained additional DNA-binding domains, implying that they might control gene expression directly.

Keywords: *nsdA*; *nsdB*; TPR-like
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1 INTRODUCTION

The tetratricopeptide repeat (TPR) is a degenerate 34-amino-acid sequence identified in a wide variety of proteins in all the three kingdoms of organisms[1,2]. A TPR motif contains two antiparallel α-helices, and tandem arrays of TPR motifs generate a right-handed helical structure with an amphipathic channel which might interact with a target protein. The basic function of TPR domains is to mediate protein-protein interactions, and some TPR-containing proteins are associated with multiprotein complexes. TPR-containing proteins are involved in diverse cellular functions such as cell cycle control, transcription and splicing, protein transport and protein folding. The TPR-like helical (InterPro accession number, IPR011990) includes TPR and several other types of α/α-repeats families: i.e. the pentatricopeptide repeat (PPR; IPR002885), SelI-like repeat (SLR; IPR006597) and HAT repeat (IPR003107) which are distantly homologous to TPRs[3,4].

Proteins that contain TPR-like motifs are common in streptomycetes, which are Gram-positive mycelial bacteria that can produce many secondary metabolites and undergo complex morphological differentiation. Among the many genes found to have a role in regulating these processes, some encode proteins with TPR-like motifs. In *Streptomyces coelicolor*, they include: ActII-4, RedD and CdaR, the transcriptional activators of gene clusters for production of the blue-pigmented polyketide actinorhodin (Act), red-pigmented prodigiosins (Red) and calcium-dependent antibiotic (CDA), respectively[5,6,7,8]. AfsR, a global positively regulator of production of at least three antibiotics[9]; LipR, the transcriptional activator of an extracellular lipase gene *lipA*[10]; and TcrA (SCO5433), an AfsR-like protein that negatively controls secondary metabolism under defined conditions[11]. Another example is NrsA (also named ORF1590 and ORF1422), a protein negatively controlling sporulation in *S. griseus*, which has an orthologue (SCO4114) in *S. coelicolor*[12]. Recently, we have shown that another TPR-like-containing protein, NsdA (SCO5582), negatively controls sporulation and antibiotic production in *S. coelicolor*[13].

In this study we have studied two other NsdA-like TPR-like-containing proteins (SCO7252 and SCO1593) in *S. coelicolor*. One of them, *SCO*7252, was found to have a role in negatively controlling Act and CDA production. So we named *SCO*7252 *nsdB* (a gene negatively affecting *Streptomyces* physiological differentiation), following the name of *nsdA* (a gene negatively affecting *Streptomyces* differentiation)[13]. We also systematically analyze the do-

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main organization of all the predicted TPR-like-containing proteins in *S. coelicolor* and discuss the role of TPR-like motifs in the secondary metabolism and morphological differentiation of *Streptomyces*.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Reagents and kits: DNA restriction and modifying enzymes were purchased from Takara Biotechnology (Dalian, China). Taq DNA polymerase was purchased from BioStar, Canada. DNA gel extraction kit for purifying DNA fragments from agarose gels was purchased from Axygen Scientific, Inc. Dig DNA Labeling and Detecting Kit was purchased from Roche.

2.1.2 Oligonucleotides used in this study were synthesized by AuGCT (Beijng, China) and are listed in table 1.

Table 1 Oligonucleotides used in this study

Name of Oligonucleotides	Sequence (from 5′→3′)
P5H1-40cd1	CTACTGTCAACTCAGCCGAGAACCTGGGGGCTTGTAGTGATTCCGGGGATCCGTCGACC
P5H1-40cd2	GCCCGGGTGACACCCTAGGTCCGCGAGCGCGGCGCGCCTATGTAGGCTGGAGCTGCTTC
SC5H1	CGGAATTCGGACGAACGAGCGGTATGCC
SC5H1B	CGGGATCCATGCTCTTCGCTCCCGTCGT
P5H1-40cv2	CTATGGATCCGCCAGCGGGACAGGACGGAC
P5H1-40chb	CTATGGATCCACCAGGAGCACGGAGCGTC
PI35-15cd1	CACTACGCTGTCGGCACCGATTCACCGGGGGCACCCATGATTCCGGGGATCCGTCGACC
PI35-15cd2	GGGCGCAGGGGCGGGACGGACGGGACGGACAGGACGCCTA TGTAGGCTGGAGCTGCTTC
p40crt1	GGCCATGGGCCACCCCCAGCTG
p40crt2	CCGTCGTGTGGCCGTTTCCCCG
phrdBrt1	GCGCACCCGAAAGAGCGTCGCAG
phrdBrt2	CGGCGGGAGCGGTCGCCTTCC

2.1.3 Bacterial strains, plasmids and growth conditions: *Streptomyces coelicolor* strains (Table 2) were manipulated as described previously[14]. *Escherichia coli* K-12 strains DH5α[15] and ET12567 (*dam dcm hsdS*)[16] were grown on Luria-Bertani (LB) or LB-Agar media[15] at 37°C. ET12567/pUZ8002[17] was used to propagate unmethylated DNA for introduction into *S. coelicolor* by conjugation. *Bacillus mycoides* Flugge ATCC6462, purchased from the China Center for Type Culture Collection (CCTCC), was used as a bioassay indicator of CDA activity. *E. coli* BW25113/pIJ790 was the host for λRED-mediated PCR-targeted mutagenesis[18]. pIJ778 was used as the template for amplification of a disruption cassette containing the streptomycin/spectinomycin resistance gene *aadA*, and the RK2 origin of transfer (*oriT*), flanked by recognition sites for FLP recombinase[18]. Cosmids SC5H1 and SCI35[19], carrying *nsdB* and *SCO*1593 respectively, were used to construct gene replacement plasmids. pSET152[20], which integrates into the *S. coelicolor* chromosome by site-specific recombination at the bacteriophage ϕC31 attachment site, i.e. *attB*[21], was used to construct complementary plasmids. Plasmids constructed in this study are listed in Table 2.

Table 2 Streptomyces strains and plasmids used or constructed in this study

Strains or plasmids	Features	Source
S. coelicolor M145	Wild type	[14]
S. coelicolor ZL1	M145 Δ *SCO*1593 ::*aadA*	This study
S. coelicolor ZL2	M145 Δ*nsdB* ::*aadA*	This study
SC5H1	Cosmid carrying *nsdB*	[19]
SCI35	Cosmid carrying *SCO*1593	[19]
pSET152	Replicates in *E. coli*, integrates into the chromosome of *S. coelicolor*	[21]
pHL405	SC5H1 Δ*nsdB* ::*aadA*	This study
pHL407	SCI35 Δ*SCO*1593 ::*aadA*	This study
pHL422	A 2.3 kb fragment of *nsdB* inserted in pSET152	This study

Several different *Streptomyces* media were used. MS agar[14] was used to make spore suspensions, plating out conjugations with *E. coli* ET12567/pUZ8002[17], extracting total RNA for RT-PCR analysis and phenotype observation. Minimal medium (MM) and R5[14] were also used in phenotype observation. Difco nutrient agar (DNA) medium was used for CDA analysis[14]. All *Streptomyces* cultivation was at 30℃.

2.2 DNA manipulations

Plasmid and genomic DNA isolation was carried out according to standard protocol[14,15]. DNA restriction and modifying enzymes were used as recommended by

the manufacturer. Southern hybridization was carried out with the Roche Dig DNA Labeling and Detecting Kit.

2.3 *SCO7252* gene replacement and mutant complementation

A *SCO7252* (*nsdB*) gene replacement vector was constructed by λRED-mediated PCR-targeted mutagenesis[18]. Two PCR primers, P5H1-40cd1 and P5H1-40cd2, corresponding to the regions immediately upstream and downstream of the *nsdB* coding region (underlined), were used to amplify *aadA* + *oriT* from pIJ778. In order to replace the entire *nsdB* from its GTG start codon to its TAG stop codon, the amplified DNA was introduced into the cosmid SC5H1 using λRED-mediated recombination[18]. The resulting *nsdB* gene replacement plasmid pHL405 was passed through the non-methylating *E. coli* ET12567/pUZ8002 before reintroduction into *S. coelicolor* M145 by conjugation. Spectinomycin/streptomycin resistant and kanamycin sensitive strains, presumptive double crossover replacement mutants, were obtained.

The *nsdB* mutation in ZL2 was verified by Southern hybridization carried out with the Roche Dig DNA Labeling and Detecting Kit. Total DNA of M145 and three candidate *nsdB* replacement strains was digested by Bcl Ⅰ. The probe was produced by amplifying a 993 bp fragment from SC5H1 using primers SC5H1 and SC5H1B, and then labeled using random hexanucleotide primers and Dig-dUTP. A representative confirmed mutant was designated ZL2.

To construct a plasmid for complementation of ZL2, we first amplified a 2.3 kb fragment from SC5H1 using primers P5H1-40cv2 and P5H1-40chb. The amplified DNA was digested by BamH Ⅰ (in bold) and then inserted into the BamH Ⅰ site of pSET152 to give pHL422.

2.4 Construction of *SCO1593* gene replacement mutants

Two PCR primers, PI35-15cd1 and PI35-15cd2 were used to amplify *aadA* + *oriT* from pIJ778. In order to replace the entire *nsdB* from its ATG start codon to its TAG stop codon, the amplified DNA was introduced into the cosmid SCI35 using λRED-mediated recombination[18]. The *SCO1593* gene replacement in the resulting cosmid pHL407 was conjugated into *S. coelicolor* M145. Four spectinomycin/streptomycin resistant and kanamycin sensitive strains were confirmed as double crossover replacement mutants by PCR analysis.

2.5 Bioassay of CDA

About 6×10^6 spores of *Streptomyces* strains were plated on MS medium and grown for 4 days. Agar plugs of these strains were placed onto plates of DNA medium with 0 or 10mM $CaCl_2$. Soft agar seeded with 10 μL of an over-night cultured indicator strain *Bacillus mycoides* Flugge ATCC6462 was overlaid around the plugs. Inhibition zones were observed after placing the plates for 10 hours at 4°C and overnight at 30℃.

2.6 RT-PCR

M145 RNA was isolated from *S. coelicolor* cultures grown on MS media covered with cellophane, using the method described by Kieser *et al*. (2000), and then treated with DNase I (RNase Free). 1.5 μg total RNA was used to synthesize the first strand cDNA by reverse transcriptase XL (AMV) following the manufacturer's instructions. Taq DNA polymerase was used to amplify cDNA. Primers p40crt1 and p40crt2 were used to detect *nsdB* mRNA. Primers phrdBrt1 and phrdBrt2 were used to detect mRNA of *hrdB* (the RNA polymerase principal sigma factor gene as an internal control). RT-PCR conditions were as follows: 42℃ for 1 hour, 95℃ for 2 min, 30 cycles of 95℃ for 30 s, 53℃ for 30 s and 68℃ for 20 s.

2.7 Domain organization analysis

The domain organization analysis of all the predicted TPR-like-containing proteins in *S. coelicolor* was conducted by searching the InterPro database (http://www.ebi.ac.uk/interpro/)[22], a database of protein families, domains and functional sites.

3 RESULTS

3.1 Production of two antibiotics was elevated in nsdB null mutants

nsdB encodes a protein of 502 amino acids that is 41% identical to NsdA (500 amino acids) over 96% of the total length of NsdB. To analyse the function of *nsdB*, we disrupted it using PCR-targeted mutagenesis[18]. Three spectinomycin/streptomycin resistant and kanamycin sensitive strains (Fig. 1-A) were obtained from M145. The double crossover replacements were confirmed by Southern hybridization (Fig. 1-B).

When cultured on MS medium, the mutants began to produce blue pigment actinorhodin at about 1.5 days, about one day earlier than M145, and went on to produce more actinorhodin than M145 after culture for 3 days (Fig. 1-C). The mutants also produced more CDA than

M145 as indicated by inhibition of a CDA-sensitive *Bacillus mycoides* strain (Fig. 1-D). When cultured on defined medium MM, the extent of actinorhodin overproduction was less marked, though still perceptible. On the complex high osmolarity medium R5, production of prodiginines and actinorhodin were found to be unaffected by *nsdB* disruption. Aerial mycelium formation and sporulation were not affected by *nsdB* disruption on any of the three media tested. All the phenotypic changes of a representative mutant, ZL2, were complemented by introducing pHL422, an integrative plasmid carrying *nsdB*, excluding potential polar effects on adjacent genes or additional mutation in ZL2 as alternative explanations.

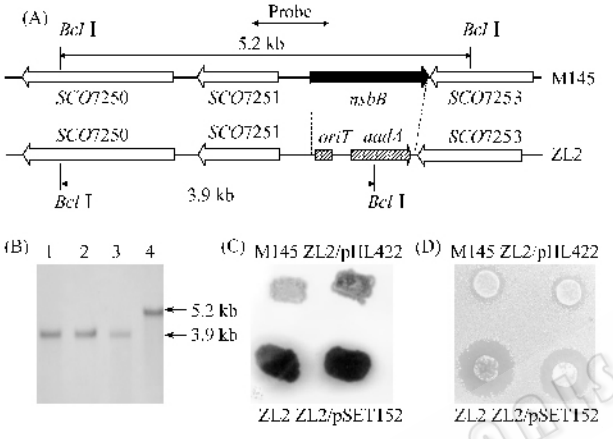

Fig.1 Disruption of *nsdB*. (A) Construction of *nsdB* gene replacement mutants (ZL2) from *S. coelicolor* M145. Coding regions are indicated by wide arrows. The probe used for Southern blot hybridization is shown by lines with two end arrows. *Bcl* I sites are also indicated in the map. (B) Southern blot confirmation of three *nsdB* gene replacement mutants (lanes 1 – 3), compared with the wild type strain M145 (lane 4). (C, D). *nsdB* gene disruption results in overproduction of actinorhodin (3-day culture) (C) and CDA (D) on MS medium. M145, parent strain; ZL2, *nsdB* gene replacement mutant; pHL422, complementing plasmid carrying *nsdB* inserted into pSET152. ZL2 produce more actinorhodin and CDA, and the null mutation phenotype was complemented by reintroducing *nsdB* in pHL422.

3.2 Expression of *nsdB* is developmentally regulated

To test the expression profile of *nsdB*, M145 was grown on MS medium for 18 to 68 hours. It began to form aerial mycelium at about 30 h, and sporulated at about 54h. *nsdB* expression was studied by RT-PCR. Fig. 2 shows that *nsdB* was expressed at about 30 hours. *hrdB*, as a control, was expressed throughout growth. The expression profile of *nsdB* was consistent with microarray data for M145 growing on the surface of R5 medium[23].

We had previously studied the expression of *nsdA*[24]. Interestingly, *nsdA* and *nsdB* have very similar expression profiles.

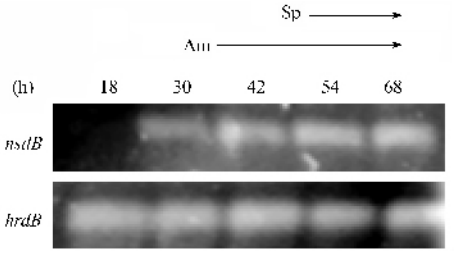

Fig.2 RT-PCR of *nsdB* and *hrdB* in M145. M145 was grown on MS media for 18 to 68 hours. It began to form aerial mycelium (Am) and sporulate (Sp) at about 30 h and 54 h respectively. *nsdB* was expressed at about 30 hours. *hrdB*, as a control, was expressed throughout growth.

3.3 *SCO1593* gene disruption did not affect development or antibiotic production

Another TPR-like-containing protein, *SCO*1593, has 446 amino acids and is 24% and 25% identical to NsdB and NsdA respectively over 70% of its length. To analyse the function of *SCO*1593, four *SCO*1593 null mutants were obtained. When cultured on MS, MM or R5 media, they were indistinguishable from the wild type strain M145.

4 DISCUSSION

TPR domains are present in a number of functionally unrelated proteins, and mediate a variety of different protein-protein interactions[1], so it is difficult to predict the function of a TPR-like-containing protein only by its TPR-like domain. However, many of these proteins have one or more additional domains, which may give clues to overall function. The InterPro database (a database of protein families, domains and functional sites, http://www.ebi.ac.uk/interpro/)[22] has predicted 70 proteins with TPR-like domains in *S. coelicolor*. The domain organization of these proteins is summarized in Fig. 3. About half [32 proteins: SCO0642, SCO1404, SCO1504, SCO1593, SCO1684, SCO1774, SCO1790, SCO1995, SCO2219, SCO2767, SCO2805, SCO2855, SCO2926, SCO3929, SCO4114, Q53959 (Internal ORF), SCO4399, SCO4548, SCO4632, SCO5205, SCO5220, SCO5433 (TcrA), SCO5582 (NsdA), SCO5675, SCO5740, SCO6168, SCO6492, SCO6696, SCO6947, SCO6994, SCO7252 (NsdB) and SCO7770] of the 70 proteins contain only one or several TPR-like domains without recognisable additional domains. NsdA, NsdB, TcrA, SCO1593 and the *S. griseus* NrsA homologue SCO4114[13,24] are in this group. However, only NsdA, NsdB and TcrA have been found to be "functional" by disruption analysis in *S. coelicolor*. 15 proteins [SCO0132, SCO0712 (LipR), SCO0877, SCO1331,

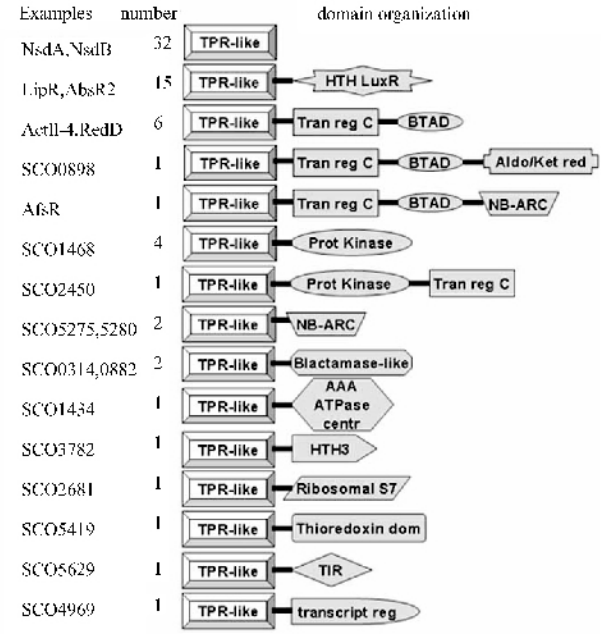

Fig. 3 Domain organization of the 70 TPR-like-containing proteins predicted from the genome sequence of *S. coelicolor* M145. Examples of proteins and the total number of proteins with the relevant domain organizations are shown on the left of the figure. Further details of the domains are as follows: TPR-like, IPR011990, Tetratricopeptide-like helical; HTH LuxR, IPR000792, DNA-binding helix-turn-helix (HTH) domain present in transcriptional regulators of the LuxR/FixJ family; Tran reg C, IPR001867, Transcriptional regulatory protein C-terminal which may play a role in DNA binding; BTAD, IPR005158, Bacterial transcriptional activator domain found in a small set of bacterial regulatory proteins that mainly occur in the streptomycetes and the closely related Mycobacteria[25]; Aldo/Ket red, IPR001395, Aldo/keto reductase family including a number of related monomeric NADPH-dependent oxidoreductases; NB-ARC, IPR002182, a signal motif shared by plant resistance gene products and regulators of cell death in animals; Prot Kinase, IPR000719, Protein kinase; Blactamase-like, IPR001279, Beta-lactamase-like; AAA ATPase centr, IPR003959, AAA ATPase central region; HTH3, IPR001387, DNA binding Helix-turn-helix domain type 3; Ribosomal S7, IPR000235, Ribosomal protein S7-like; Thioredoxin dom, IPR013766, Thioredoxin domain; TIR, IPR000157, the Toll/interleukin-1 receptor homologous region; transcript reg, IPR000944, Transcriptional regulator containing Rrf2 and some other proteins that may represent a family of probable transcriptional regulators.

SCO4263, SCO5065, SCO5506, SCO6193, SCO6993 (AbsR2), SCO7093, SCO7134, SCO7137, SCO7143, SCO7173, SCO7295] contain an additional HTH-LuxR DNA binding domain (IPR000792). Eight proteins [SCO2259, SCO3217 (CdaR), SCO4116, SCO5085 (ActII-4), SCO5877 (RedD), SCO6288, SCO0898, SCO4426 (AfsR)] contain a Tran Reg C DNA binding (IPR001867) and a BTAD (IPR005158) domain. Five proteins (SCO1468, SCO2666, SCO3102, SCO7240 and SCO2450) contain at least an additional Prot Kinase domain (IPR000719). Other domain organizations with only one or two examples are shown in Fig. 3.

It is noteworthy that, overall, about 36 % of the proteins contain an additional DNA binding domain. Among these, five have been found to regulate some aspect of secondary metabolism: AfsR and AbsR2 affect more than one pathway, while ActII-4, RedD and CdaR are the pathway-specific regulators for, respectively, actinorhodin, undecylprodigiosin and calcium-dependent antibiotic (CDA) biosynthesis. ActII-4 has been shown to recognize and bind specific regions in the actinorhodin biosynthetic gene cluster, thus activating the transcription of act genes[26]. However, NsdA, NsdB and TcrA were found to contain no additional DNA binding domains. How could these proteins be "functional" to the development of *Streptomyces*? We speculate that these proteins possibly contain undefined domains that play a role in DNA binding. Alternatively, their effects on secondary metabolism may be indirect. The TPR and NB-ARC domain of AfsR function in recruiting RNA polymerase[25], so NsdA, NsdB and TcrA might also interact with RNA polymerase or certain transcriptional regulators (for example, ActII-4, RedD *etc.*) to control antibiotic production or morphological differentiation.

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NsdB——天蓝色链霉菌 A3(2)中一个负调控抗生素产量蛋白的研究

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摘　要:TPR(tetratricopeptide repeat)是在很多蛋白中均被发现到的一个含有 34 个氨基酸的蛋白重复序列,其基本功能是参与蛋白间的相互作用。天蓝色链霉菌 A3(2)中有 70 个蛋白含有类 TPR 结构域,NsdA 是其中的一个,研究发现该蛋白对天蓝色链霉菌的产孢和产素都有负调控作用。本研究中发现基因 *SCO*7252 和 *SCO*1593 编码含 TPR 结构的蛋白,中断 *SCO*7252 基因后菌株放线紫红素和钙依赖抗生素产量均提高,但形态分化没有明显变化,基因 *SCO*1593 中断后菌株在产孢产素及形态等各方面均未受到影响。基因 *SCO*7252 被命名为 *nsdB*,RT-PCR 分析表明,该基因在生长 30h 时开始表达。通过生物信息分析表明,天蓝色链霉菌的 70 个含类 TPR 结构的蛋白中有 32 个仅含该结构域,有 25 个另外含有 DNA 结合区域,这些暗示着它们可能直接控制基因的表达。

关键词:nsdA;nsdB 类 TPR 结构域

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