Identification of a Gene Negatively Affecting Antibiotic Production and Morphological Differentiation in *Streptomyces coelicolor* A3(2)

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SC7A1 is a cosmid with an insert of chromosomal DNA from *Streptomyces coelicolor* A3(2). Its insertion into the chromosome of *S. coelicolor* strains caused a duplication of a segment of ca. 40 kb and delayed actinorhodin antibiotic production and sporulation, implying that SC7A1 carried a gene negatively affecting these processes. The subcloning of SC7A1 insert DNA resulted in the identification of the open reading frame SCO5582 as *nsdA*, a gene negatively affecting *Streptomyces* differentiation. The disruption of chromosomal *nsdA* caused the overproduction of spores and of three of four known *S. coelicolor* antibiotics of quite different chemical types. In at least one case (that of actinorhodin), this was correlated with premature expression of a pathway-specific regulatory gene (*actII-orf4*), implying that *nsdA* in the wild-type strain indirectly repressed the expression of the actinorhodin biosynthesis cluster. *nsdA* expression was up-regulated upon aerial mycelium initiation and was strongest in the aerial mycelium. *NsdA* has DUF921, a *Streptomyces* protein domain of unknown function and a conserved SXR site. A site-directed mutation (S458A) in this site in NsdA abolished its function. Blast searching showed that NsdA homologues are present in some *Streptomyces* genomes. Outside of streptomycetes, NsdA-like proteins have been found in several actinomycetes. The disruption of the *nsdA*-like gene *SCO4114* had no obvious phenotypic effects on *S. coelicolor*. The *nsdA* orthologue *SAV2652* in *S. avermitilis* could complement the *S. coelicolor* *nsdA*-null mutant phenotype.

Soil-dwelling actinobacteria of the genus *Streptomyces* are mycelial sporulating organisms that are the major natural source of antibiotics. The genetically well-studied *Streptomyces coelicolor* A3(2) produces at least four antibiotics, blue-pigmented polyketide actinorhodin (Act), red-pigmented prodigiosins (Red), a lipopeptide calcium-dependent antibiotic (CDA), and the SCP1-plasmid-encoded cyclopentanone antibiotic methylenomycin (Mmy). The triggering of physiological differentiation (secondary metabolism) in mycelial sporulating organisms that are the major natural source of antibiotics. The genetically well-studied *Streptomyces coelicolor* A3(2) produces at least four antibiotics, blue-pigmented polyketide actinorhodin (Act), red-pigmented prodigiosins (Red), a lipopeptide calcium-dependent antibiotic (CDA), and the SCP1-plasmid-encoded cyclopentanone antibiotic methylenomycin (Mmy). The triggering of physiological differentiation (secondary metabolism) in *Streptomyces* concurs with the initiation of morphological differentiation, and both processes are under rigorous genetic modulation via a hierarchical regulatory network, integrating various physiological and environmental signals (4, 10).

Pathway-specific regulatory genes, such as *actII-orf4*, *redD*, *edaR*, and *mmrR*, are at the bottom of the regulatory network, each controlling one antibiotic biosynthetic pathway (4). Global regulators, such as *bldA* (16), *bldB* (14), *bldD* (15), and *bldG* (5), perform the highest-level regulation and affect both morphological and physiological differentiation (9, 10). At intermediate levels in the regulatory cascades, many regulatory genes, such as *afsB* (22), *abaA* (17), *absB* (7), *afsK-afsR* (21, 56), and *bldG* (5), are at the bottom of the regulatory network, each controlling one antibiotic biosynthetic pathway (4).

Recent microarray data have indicated a cross-regulation among disparate antibiotic biosynthetic pathways and even some back regulation from cluster-situated regulators to a “higher level” pleiotropic regulatory gene (23).

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 negatively affecting both processes.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and growth conditions.** *Streptomyces coelicolor* A3(2) strains (Table 1) were manipulated as described previously (27). For routine subcloning, *Escherichia coli* K-12 strains DH5α (43) and ET12567 (dam dcm hsdS) (34) were grown and transformed according to the method of Sambrook et al. (43). ET12567 was used to propagate unmethylated DNA for introduction into *S. coelicolor* by transformation or conjugation. Bacillus *mycoides* Flagge ATCC 6462 was purchased from the China Center for Type Culture Collection. *E. coli* BW25113/pIJ790 was the host for *Amp*--mediated PCR-targeted mutagenesis (20). pJ773 or pJ778 was used as the template for the amplification of a disruption cassette containing the apramycin resistance gene *aac(3)IV* or streptomycin/spectinomycin resistance gene *aadA* and the RK2 or-
This page contains a detailed description of molecular biology experiments involving Streptomyces coelicolor. The text explains the construction of a gene replacement vector, the use of PCR to introduce mutations, and the transformation of Streptomyces spp. with the gene of interest. The page also includes tables listing the characteristics of plasmids constructed in the study and the strains used.

**Table 1. S. coelicolor strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Features</th>
<th>Source</th>
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<tbody>
<tr>
<td>M145</td>
<td>Wild type</td>
<td>27</td>
</tr>
<tr>
<td>M600</td>
<td>Wild type</td>
<td>27</td>
</tr>
<tr>
<td>J1501</td>
<td>hisA1 uraA1 strA1 Pgl− SCP1− SCP2−</td>
<td>27</td>
</tr>
<tr>
<td>J1506</td>
<td>J1501/SCP1+</td>
<td>C. J. Bruton and K. F. Chater</td>
</tr>
<tr>
<td>YX2</td>
<td>M145 ΔnsdA (870 bp)::aac(3)IV</td>
<td>This study</td>
</tr>
<tr>
<td>LW9</td>
<td>M145 Δnsd::aac(3)IV</td>
<td>This study</td>
</tr>
<tr>
<td>LW6</td>
<td>M145 ΔSCO4141::aadA</td>
<td>This study</td>
</tr>
<tr>
<td>LW4</td>
<td>M600 ΔSCO4141::aadA</td>
<td>This study</td>
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**Table 2. Plasmids constructed in this study and their characteristics**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Characteristic(s)</th>
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</thead>
<tbody>
<tr>
<td>pHZ2701</td>
<td>33-kb EcoRI fragment of SCO4114 inserted in pHJL401</td>
</tr>
<tr>
<td>pHZ2702</td>
<td>12-kb EcoRI fragment of SCO4114 inserted in pHJL401</td>
</tr>
<tr>
<td>pHZ2703</td>
<td>18-kb BglII fragment of SCO7A1 inserted in pHJL401</td>
</tr>
<tr>
<td>pHZ2704</td>
<td>8-kb BglII fragment of SCO7A1 inserted in pHJL401</td>
</tr>
<tr>
<td>pHZ2705</td>
<td>6-kb BglII fragment of SCO7A1 inserted in pHJL401</td>
</tr>
<tr>
<td>pHZ2706</td>
<td>3-kb Xhol fragment removed from pHZ2703</td>
</tr>
<tr>
<td>pHZ2707</td>
<td>3-kb and 8-kb Xhol fragments removed from pHZ2703</td>
</tr>
<tr>
<td>pHZ2712</td>
<td>870-bp BamHI fragment removed from pHZ2706</td>
</tr>
<tr>
<td>pHZ2714</td>
<td>3.5-kb BamHI fragment of pHZ2703 inserted in pHJL401</td>
</tr>
<tr>
<td>pHZ2719</td>
<td>4.1-kb BclI fragment of pHZ2706 inserted in pHZ199</td>
</tr>
<tr>
<td>pHZ2718</td>
<td>870-bp nsdA internal BamHI fragment of pHZ2717 replaced by 1.7-kb BamHI fragment from pHP451a (IV)</td>
</tr>
<tr>
<td>pHZ2731</td>
<td>2.9-kb Stul-Bell fragment of pHZ2706 inserted in pHJL401</td>
</tr>
<tr>
<td>pHZ2733</td>
<td>1.3-kb SacI fragment of pHZ2731 inserted in pHZ2705</td>
</tr>
<tr>
<td>pHZ2735</td>
<td>Carrying nsdA S458A mutation; 1-kb SacI fragment of pHZ2731 replaced by same fragment of pHZ2733* with TCC→GCC mutation</td>
</tr>
<tr>
<td>pHL123</td>
<td>nsdA gene in SCO4114::aadA carried by an oriT+::aadA cassette from pHJ773</td>
</tr>
<tr>
<td>pHL127</td>
<td>1.7-kb SacI-PstI fragment containing aac(3)IV from pSET152 replaced by 1.3-kb SacI-PstI fragment containing nsdA from pHZ778</td>
</tr>
<tr>
<td>pHL128</td>
<td>2.9-kb Stul-Bell fragment of pHZ2706 inserted in pHL127</td>
</tr>
<tr>
<td>pHL129</td>
<td>2.9-kb Stul-Bell fragment of pHZ2706 inserted in pHL127 carrying a TCC→GCC mutation in nsdA S458 site</td>
</tr>
<tr>
<td>pHL156</td>
<td>544-bp PCR fragment of nsdA promoter region inserted between the KpnI-BamHI sites of pHJL401</td>
</tr>
<tr>
<td>pHL17</td>
<td>BglII-EcoRI region of pHJL401 replaced by an oriT+::aadA cassette from pHJ773</td>
</tr>
<tr>
<td>pHL133</td>
<td>931-bp fragment of promoterless xylE amplified from pSET151 inserted between the BglII-BamHI sites of pHJL17</td>
</tr>
<tr>
<td>pHL134</td>
<td>1.3-kb nsdA::oriT fragment inserted into the EcoRI site of pHL133</td>
</tr>
<tr>
<td>pHL134</td>
<td>544-bp fragment of nsdA promoter region inserted into the BglII site of pHL134</td>
</tr>
<tr>
<td>pHL561</td>
<td>2.2-kb fragment (containing SAV2652 and 637-bp upstream region) of S. avermitilis inserted in pHL127</td>
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</tbody>
</table>
SCD72SS was conjugated into M145 and M600. Five spectinomycin-streptomycin-resistant and kanamycin-sensitive strains (named LW6) were obtained from M145, and six (named LW4) were obtained from M600. The S. coelicolor chromosome was amplified with primers actII-4a (5′-CACTTATTGGGACGTCTCAT-3′) and the reverse primer actII-4b (5′-CACCGTGAATTTCACTGTG-3′). The 743-bp PCR product contained a large part of the structural gene actII-orf4, encoding an amino acid sequence 4 to 250 of ActII-orf4. The actII-orf4 probe was used to detect the corresponding gene in DNA labeling and detection kit (Roche). A digoxigenin-labeled synthetic oligonucleotide PI6865 (5′-CGGCCTTCCAGCCACCCAG-3′) was used as a probe for the detection of 16S rRNA. Twenty micrograms of total RNA was loaded for each sample and fractionated on a 1% formaldehyde-MOPS (morpholinepropanesulfonic acid) agarose gel, transferred to a HybondN membrane, hybridized with digoxigenin-labeled probe at 53°C in hybridization buffer containing 50% formamide.

Construction of nsdA promoter-egfp and nsdA promoter-xylE fusion plasmids. To construct an nsdA promoter-egfp fusion plasmid, a 544-bp fragment from -18 to -561 upstream of the nsdA GTG start codon was amplified with primers psdAp1 (5′-ACGGGGTACCCATATGCCTCGAG-3′) and orf1590-2 (5′-GTCCGCGAGACCGATCTCG-3′), where underlined letters indicate engineered restriction sites and boldface letters indicate the start codon of nsdA. The fragment was then digested by KpnI and BamHI and ligated into pBluescript KS (46), generating pHLI16. Then the BglII-EcoRI fragment of pHLI16 was ligated between the BglII and EcoRI sites of pBluescript KS, generating pHLI17, which contained the nsdA promoter region fused to egfp, and the apramycin-resistant gene aux(3)IV.

To construct an nsdA promoter-xylE fusion plasmid, a 931-bp fragment of the xylE coding region and 11 bp upstream of its ATG start codon was amplified with primers xylE40230 (5′-TGAATTCCTGGCGAGCGGCAT-3′) and xylE-3′ (5′-GCGTTCGGCGCGGACCTTCTTG-3′), where the desired restriction sites and boldface letters indicate the start codon of xylE. The fragment was then digested by BglII and ligated between the BglII and BamHI sites of pHLI17, generating pHLI133. A 1.3-kb nsdA-xylE fragment was amplified from JPHZ2735, containing the desired mutation. The mutagenesis PCR was carried out following the manufacturer’s instructions. Primers psdAr1 (5′-GCCTTACGGCCGGCAG-3′) and psdAr2 (5′-GCCTTACGGCCGGCAG-3′) were used to detect nsdA. Primers phrBrt1 (5′-GGCCACCCGAAAGCGGTCGC-3′) and phrBrt2 (5′-GGCCGGGAGCGGTCGC-3′) were used to detect hrdB (the RNA polymerase principal sigma factor gene as an internal control). RT-PCR conditions were as follows: 42°C for 1 h, 95°C for 2 min, 25 cycles of 95°C for 30 s, 55°C for 30 s, and 68°C for 20 s.

Site-directed mutagenesis. The site-directed mutagenesis of nsdA was carried out following the instruction manual for the Stratagene QuikChange site-directed mutagenesis kit. First a 1.3-kb SacI fragment, encoding the last 312-amino-acid sequence (including S458) and the downstream intergenic region, was cloned into pBluescript KS to generate pHZ2733. pHZ2733 was then used as the PCR template for site-directed mutagenesis. Two synthetic oligonucleotides, W40230 (5′-GGTCCGAGGGCGAAGCGGTCAACACC-3′), where the desired mutation was underlined) and W40231 (5′-GGGCTTCGAGGCGGACCTTCGTTG-3′), were designed complementary to opposite strands of the S458-encoding region of nsdA and containing the desired mutation. The mutation PCR was carried out with Pfu DNA polymerase. Dimethyl sulfoxide was added to the reaction mixture to a final concentration of 5% (vol/vol). After temperature cycling, the PCR product was treated with DpnI to digest the methylated parental DNA template and to select for mutation-containing synthesized DNA. The mixture was then sequenced to transform E. coli DH5α. Plasmid DNA from two transformants was sequenced to show that the inserted DNA fragments contained the desired mutation and there was no other mutation. Then the 1.3-kb SacI fragment was excised from the mutated plasmid pHZ2733 and used to replace the same fragment in pHZ2735 to generate pHZ2735. The 2-kb fragment from 479 bp upstream to 40 bp downstream of nsdA in pHZ2735 was sequenced to confirm the designed TCCGCC mutation and that there was no other mutation.

RESULTS

Identification of nsdA, a gene negatively affecting differentiation. In the course of trying to clone a gene that had been mapped genetically to the 5′ o’clock region of the linkage map, thirty cosmids containing insert DNA from the corresponding region of the chromosome of S. coelicolor A3(2) (strain M145) were separately introduced into strain J1501 by protoplast transformation. Since they contained no Streptomyces replication origin, the cosmids could be maintained only via homologous recombinational integration into the chromosome. We found it interesting that all SC7A1 transformants on R2YE medium showed delayed sporulation and formed blue pigment poorly and late compared with the parent J1501. The transformation of a different A3(2) derivative, M145, by SC7A1 showed similar results (Fig. 1), implying that one or more genes carried by this cosmid negatively affect sporulation and pigmentation.

SC7A1 carries a 46-kb insert with 41 annotated genes (http://www.sanger.ac.uk/Projects/S_coelicolor) (3). To identify the possible negative gene(s), restriction fragments of SC7A1 were subcloned in pBluescript KS and then used to transform M145 (Fig. 1). Streptomyces cosmid SC7A1 and some subclones carrying nsdA on pHJL401, a medium-copy-number SCP2*-based vector, delay actinorhodin production of M145. The cultures were grown on R2YE for 5 days at 30°C. The back of the plate is shown.
2). Only subclones with orf26 (i.e., SCO5582) caused the pigment delay phenotype on R2YE medium (Fig. 1). In the S. coelicolor chromosome, SCO5582 is followed by five genes in the same transcriptional direction (Fig. 2). Whether they are cotranscribed with SCO5582 has not been documented. Since pHZ2721, containing only one entire open reading frame, i.e., SCO5582, was sufficient to delay pigmentation and sporulation (Fig. 1), extra copies of the downstream genes were not required for the negative function. We therefore designated SCO5582 as nsdA, meaning a gene negatively affecting Streptomyces differentiation.

Aerial mycelium formation was delayed, but more spores were eventually produced in nsdA-null mutants. An nsdA mutant, YX2, was constructed by replacing an nsdA internal 870-bp BamHI fragment with the apramycin resistance gene aac(3)IV via homologous recombination. The gene replacement was confirmed by Southern hybridization (data not shown).

On different solid media, YX2 differed from M145 in the rate and extent of sporulation (Fig. 3). On MS and R2YE media, the mutant began to grow aerial mycelium about 1 to 2 days later and sporulated 2 to 3 days later, but both aerial mycelium amounts and eventual sporulation levels appeared to be greater than those of the parent strain. When we plated out diluted spores harvested from colonies on MS medium grown for 10 days and counted the CFU, YX2 spore suspensions reproducibly formed about twice as many CFU/colony as did M145. On SMMS medium, M145 lawns sporulated poorly after 13 days' growth but YX2 sporulated well and produced nearly twenty times more spores than did M145 (data not shown). In liquid culture YEME, in which S. coelicolor does not sporulate, the biomass was not changed by the gene disruption (data not shown). Complementation of the altered developmental time course was obtained by introducing pHZ2731 into YX2 (data not shown). To confirm that the phenotype was not attributable to any residual part of nsdA in YX2, the whole nsdA gene was replaced by an oriT+aac(3)IV cassette by using PCR targeting. Seven mutant strains, LW9-2 to LW9-8, were obtained easily and were confirmed by PCR (see Materials and Methods). These strains had phenotypes very similar to that of YX2 and could be fully complemented by pHL128, a plasmid containing an nsdA gene and a specti-
nomycin-/streptomycin-resistant gene *aadA* (Fig. 3). This convincingly confirmed the *nsdA* mutant phenotype.

**nsdA** disruption elevated biosynthesis of three antibiotics.

When cultured on SMMSS or MS solid medium, *nsdA* mutants YX2 and LW9 produced more blue pigment (the polyketide antibiotic actinorhodin or Act) than did M145 (Fig. 4A and B). On R2YE, they became pigmented a few hours to 2 days sooner than did M145, although all strains produced large amounts of Act upon longer incubation (5 to 6 days, data not shown). The change of Act production and timing was reversed to the wild-type level by the introduction of *aadA* back into YX2 or LW9 (Fig. 4), excluding potential polar effects on adjacent genes as an explanation and suggesting that *nsdA* in the wild-type strain represses Act production.

In addition to Act, the production of the lipopeptide antibiotic CDA was elevated by *nsdA* disruption, as indicated by the inhibition of a CDA-sensitive *Bacillus mycoides* strain, and the overproduction phenotype was complemented by reintroducing *nsdA* (Fig. 5A). In view of the fact that the Act and CDA biosynthetic pathways are quite unlike each other, this result suggested that *nsdA* might be having some general effect on secondary metabolism. We therefore further investigated this possibility by introducing the biosynthetic pathway for another kind of antibiotic, the epoxycyclopentanone methyl-

**FIG. 4.** *nsdA* disruption results in the overproduction of actinorhodin on (A) SMMSS (6-day culture) and (B) MS media (4-day culture). The backs of the plates are shown. YX2 and LW9, *nsdA* disruption mutants. M145, parent strain. pHZ2731 carried *nsdA* inserted into pIJ8600. pHLL127 is a pSET152-derived plasmid with *aac(3)IV* replaced by *aadA*. pHLL128 carried *nsdA* inserted into pHLL127. pHLL129 carried the *nsdA* with the S458A mutation inserted in pHLL127. The *nsdA*-null mutation phenotype was complemented by pHLL128 but not by pHLL129.

emomycin (Mmy), into an *nsdA* mutant. This was carried out by the conjugal introduction of the plasmid SCP1, which carries the *mmy* genes (and which had been previously eliminated at an early stage of the M145 pedigree). Compared to that of M145/SCP1, YX2/SCP1 gave a larger inhibition zone on the indicator *S. coelicolor* J1501, which is Mmy sensitive because it lacks SCP1, and the Mmy overproduction phenotype was complemented by reintroducing *nsdA* (Fig. 5B). This conclusion was not affected by the slight complication that, for reasons that have yet to be investigated, the control strain YX2/SCP1/pIJ8600 gave a larger inhibition zone than did YX2/SCP1.

The *nsdA* mutation did not affect the production of the red-pigmented prodigines, in liquid or on solid media. We also found that extracellular amylase activity was not influenced by the *nsdA* gene replacement (data not shown). Thus, although the mutant phenotype was strikingly pleiotropic, it did not affect all aspects of secondary metabolism or stationary-phase biology.

**Actinorhodin overproduction in the *nsdA* mutant was associated with the up-regulation of the pathway-specific regulator *actII-orf4*.** The expression of many antibiotic biosynthesis clusters is regulated by pathway-specific regulators, most of which are transcriptional activators. In *S. coelicolor*, Act biosynthesis has been shown to depend on the transcriptional activation of the Act biosynthesis cluster by ActII-orf4 protein, and increased expression of ActII-orf4 results in the overproduction of Act (4). The transcription of *actII-orf4* in the *nsdA* mutant YX2 was therefore analyzed by Northern blotting. Total RNA was isolated from different developmental stages of M145 and YX2 grown on SMMSS medium. As shown in Fig. 6, the transcription of *actII-orf4* increased markedly and was comparatively early in YX2, suggesting that the negative effect of *nsdA* on Act production involved the repression of the transcription of the pathway-specific activator *actII-orf4*, either directly or indirectly.

*nsdA* expression was developmentally regulated and was strongest in the aerial mycelium. The developmental effects of *nsdA* led us to investigate its expression in relation to colony differentiation. A 544-bp fragment from −18 to −561 upstream of the *nsdA* GTG start codon was amplified by PCR and fused to a promoterless *egfp* gene. The resulting plasmid pHL117 was conjugated into M145. Fluorescence signals were not detected in newly grown germ tubes and substrate mycelium but were relatively high in aerial mycelium and spore chains (Fig. 7A). The signal became weaker in spore chains.

**FIG. 6.** Northern blot analysis indicating an increase of *actII-orf4* mRNA in the *nsdA* mutant strain. Total RNA was isolated from solid SMMSS cultures grown for 28, 40, 52, 64, and 76 h. The same blot was stripped and hybridized with probe for 16S rRNA.

**FIG. 5.** *nsdA* gene disruption results in the overproduction of CDA (A) and methylenomycin (B). Compared to that of M145, YX2 culture plugs generated a larger inhibition zone with the CDA-sensitive *Bacillus mycoides* indicator strain. Similarly, compared to that of M145/SCP1, YX2/SCP1 gave a larger inhibition zone on the Mmy-sensitive indicator *S. coelicolor* J1501. The CDA and methylenomycin overproduction phenotype was complemented by pHZ2731 but not by pIJ8600.

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**nsdA** disruption elevated biosynthesis of three antibiotics. When cultured on SMMSS or MS solid medium, *nsdA* mutants YX2 and LW9 produced more blue pigment (the polyketide antibiotic actinorhodin or Act) than did M145 (Fig. 4A and B). On R2YE, they became pigmented a few hours to 2 days sooner than did M145, although all strains produced large amounts of Act upon longer incubation (5 to 6 days, data not shown). The change of Act production and timing was reversed to the wild-type level by the introduction of *aadA* back into YX2 or LW9 (Fig. 4), excluding potential polar effects on adjacent genes as an explanation and suggesting that *nsdA* in the wild-type strain represses Act production.

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after prolonged incubation. Thus, nsdA expression is strong only in aerial mycelium.

An nsdA promoter-xyle fusion plasmid was also constructed. xyle encodes a catechol 2,3-dioxygenase (C230) which converts colorless catechol to the yellow compound 2-hydroxy muconic semialdehyde and its activity can be quantitatively measured (25). Two strains, M145/pHL134 (a control strain with promoterless xylE) and M145/pHL134-nsdAp, were grown on SMMS medium covered with cellophane for 28 to 76 h. C23O activities were obviously detected in only M145/pHL134-nsdAp at 40, 52, 64, and 76 h. Phl134, M145/pHL134; nsdA, M145/pHL134-nsdAp. Error bars indicate standard deviations. (C) RT-PCR. M145 was grown on SMMS medium for 18 to 76 h. It began to form aerial mycelium (Am) and sporulate (Sp) at about 28 and 64 h, respectively. C23O activities were measured quantitatively (25). Two strains, M145/pHL134 (a control strain with promoterless xylE) and M145/pHL134-nsdAp, were grown on SMMS medium covered with cellophane for 28 to 76 h. C23O activities were obviously detected in only M145/pHL134-nsdAp at 40, 52, 64, and 76 h. Phl134, M145/pHL134; nsdA, M145/pHL134-nsdAp. Error bars indicate standard deviations. (C) RT-PCR. M145 was grown on SMMS medium for 18 to 76 h. It began to form aerial mycelium (Am) and sporulate (Sp) at about 28 and 64 h, respectively. nsdA was expressed at about 28 h. hrdb, as a control, was expressed throughout growth.

A conserved serine at position 458 in NsdA is required for its function. Bioinformatic analysis of NsdA using the Prosite database search tool Proscan (12) identified “SXR” at positions 458 to 460 as a putative protein kinase C phosphorylation site (28). This site is highly conserved in NsdA homologues (Fig. 8). To test whether this conserved motif is required for the negative function of NsdA, the serine residue was mutated to alanine to generate an NsdA (S458A) mutant. The nsdA(S458A) mutation in plasmid pHL129 was then introduced into the nsdA mutants to test for complementation (Fig. 4B). YX2/pHL129 and LW9/pHL129 still overproduced Act, just as YX2, LW9, and the vector controls YX2/pHL127 and LW9/pHL127 did, whereas the wild-type nsdA gene (YX2/pHL128 and LW9/pHL128) restored Act production to the low level typical of the wild type. This indicated that nsdA(S458A) had lost its negative function and that the hydroxyl group in the conserved serine residue is required for this function.

NsdA belongs to a protein family that also includes a Streptomyces sporation-associated regulator. nsdA encodes a 500-amino-acid protein that shares 28% sequence identity with the Streptomyces griseus sporation-associated protein P56 (2) and 31% identity (end to end) with the P56 orthologue SCO4114 of S. coelicolor. The S. coelicolor genome encodes three other proteins showing over 30% sequence identity with NsdA: SCO7252 (41%), SCO2192 (33%), and SCO4399 (32%). In the sequenced Streptomyces avermitilis genome (24), there is an orthologue with sequence identity over 80% to each of these NsdA-like proteins and NsdA itself. Streptomyces chartreusis, producing a potent antitumor agent chartreusin, contains an NsdA homologue ChaR2 (27% identity), whose gene is inside the chartreusin gene cluster (49). Blast searching indicated that besides Streptomyces, several other actinomycetes (i.e., cellulolytic Thermobifida fusca, GenBank accession number NC_007333; symbiotic nitrogen-fixing Frankia sp. strain NC_007777 and NZ_AAIH00000000; and the causative agent of nocardiosis, Nocardia farcinica, NC_006361) contain NsdA homologues. NsdA contains a conserved domain, DUF921, that is defined as a Streptomyces protein domain of unknown function. Proteins containing the DUF921 domain include several putative regulatory proteins from S. coelicolor and S. griseus (35). A search in the Superfamily server also revealed that NsdA, P56, and other homologous proteins in S. coelicolor and S. avermitilis have tetratricopeptide repeat (TRP)-like repeats (19) which mediate protein-protein interactions (13).

The S. griseus or1590, which encodes P56 (see above), was discovered as a suppressor of the morphological deficiencies of certain bld mutants of S. griseus (2, 29) and renamed to nsaA due to its negative role on sporulation (37). The nsaA counterpart in S. coelicolor is SCO4114 (91% sequence identity at the amino acid level). When present at a high copy number, SCO4114 suppressed the morphological deficiency of bld mutants of S. griseus (38), implying it as well plays a role in sporulation.

To investigate this further, we constructed SCO4114-null
mutants LW6 and LW4 from M145 and M600, respectively, by replacing the entire open reading frame with the streptomycin/spectinomycin resistance cassette (nsdA) by using the PCR-targeting strategy (20). When cultured on solid MS, R2YE, and MM media, LW6 and LW4 were indistinguishable from the parent strains in sporulation and pigment production (data not shown), suggesting that SCO4114 is not essential for growth and does not negatively regulate differentiation in S. coelicolor, as had been proposed previously (37). Alternatively, any negative regulatory effects of SCO4114 may be masked by other effects of culture conditions or additional negative regulators.

The NsdA orthologue SAV2652 in S. avermitilis showed 84% amino acid identity with NsdA in S. coelicolor. To investigate the function of this gene, we amplified a fragment containing this gene from the total DNA of S. avermitilis and inserted it into pH127. The resulting plasmid pH561 was conjugated to LW9, the nsdA-null mutant strain. We observed that pH561 can complement the phenotype of LW9 to the wild-type level just like pH128 can (carrying nsdA from S. coelicolor). This indicated that SAV2652 in S. avermitilis may function like nsdA in S. coelicolor.

**DISCUSSION**

In this study, nsdA (SCO5582) in S. coelicolor was identified by gene dosage effects and gene disruption as a gene negatively affecting antibiotic production and sporulation. The repressing effect was obvious even when the supernumerary gene was introduced on plasmids that integrate at the prophage 4C31 attachment site (data not shown). Such insertions usually involve one or sometimes two tandem copies. This implies that the natural effective levels of NsdA must be very precisely controlled.

The disruption of nsdA resulted in higher productions of Act, CDA, Mmy, and spores. 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. A newly identified transcription factor AtmA (48), which activated actII-orf4, is a potential target of a NsdA-controlling pathway on Act. Possibly, NsdA causes a deficiency in the transcription of other cluster-situated genes, such as cadA (homologous to actII-orf4 and redD) in the case of CDA (42) and mmyB in the case of methylenomycin (40).

Despite the negative effects of extra copies of nsdA on antibiotic production and sporulation, aerial mycelium formation was delayed, rather than accelerated, in nsdA-null mutants. The cause of this paradox remains to be elucidated. Possible explanations might lie either in the redirection of nutrients and energy to antibiotic production at the expense of aerial growth or in the complexity of cross-regulation between secondary metabolism and morphological differentiation, as indicated by microarray experiments (23) and the complex phenotypes of mutants (32). Since the disruption of nsdA did not completely abolish aerial mycelium formation, it can be regarded as important but not essential for development.

Huang’s microarray data (23) had suggested that the expression of nsdA (SC7A1.26) was up-regulated from about 32 h after inoculating on the surface of R5 medium. Our results showed that its expression was also growth-phase dependent in SMMS medium: unexpectedly, it was expressed mainly in the aerial mycelium. Antibiotic production generally appears to be confined to the older substrate mycelium, as exemplified by redD, the pathway-specific regulator of RED, which was expressed in only ageing substrate mycelium (46). Thus, one role of nsdA may be to suppress antibiotic production in aerial mycelium.

NsdA belongs to a protein family containing a function-unknown domain, DUF921, which is so far found in only streptomycetes and several other actinomycetes. One member of this family, the S. griseus P56 protein, which is thought to be involved in the regulation of sporulation, contains a typical helix-turn-helix motif in its amino-terminal region (2). However, NsdA does not contain a helix-turn-helix DNA binding domain, leaving open the question of whether NsdA works via binding to its target gene. NsdA also has a TPR-like domain, which may mediate protein-protein interactions. Another TPR-containing protein TcrA related to secondary metabolism of S. coelicolor has recently been reported (33). We are currently using an E. coli two-hybrid system to seek NsdA-interacting proteins. The disruption of another member of this gene family, SCO4114, which had previously been shown to prevent premature sporulation septation of another streptomycetes organism (S. griseus), had no obvious phenotypic effects on S. coelicolor. Possibly, there are differences in the way that different members of this paralogous family are deployed in the two species. Other differences in the central regulation of their sporulation have been described previously (11). These may perhaps account for the comparatively rapid and synchronous sporulation of S. griseus and its ability to sporulate in submerged culture.

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**REFERENCES**


