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SarA influences the sporulation and secondary metabolism in *Streptomyces coelicolor* M145

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The filamentous bacteria *Streptomyces* exhibit a complex life cycle involving morphological differentiation and secondary metabolism. A putative membrane protein gene *sarA* (*sco4069*), sporulation and antibiotic production related gene A, was partially characterized in *Streptomyces coelicolor* M145. The gene product had no characterized functional domains and was highly conserved in *Streptomyces*. Compared with the wild-type M145, the *sarA* mutant accelerated sporulation and dramatically decreased the production of actinorhodin and undecylprodigiosin. Reverse transcription-polymerase chain reaction analysis showed that SarA influenced antibiotic production by controlling the abundance of *actII-orf4* and *redZ* messenger RNA.

Keywords *Streptomyces coelicolor*; sporulation; antibiotic production; *sarA*

The life cycle of streptomycetes is remarkably intriguing for a prokaryote, as it encompasses a series of structurally differentiated states and physiological changes [1]. Colonies germinate from spores and continue to grow by forming a mat of branched hyphae called substrate mycelium. In response to some signals, including A factor, ppGpp, SapB, SapT and chaplins, the substrate hyphae cease and aerial hyphae begin to form [2–5]. These aerial hyphae then undergo synchronous septation leading to the formation of unigenomic spores [6]. Coinciding with the onset of aerial mycelium formation is the production of secondary metabolites, which have many important commercial medical applications, such as antibacterial, antitumor and immunosuppression activities [7].

Sporulation of *Streptomyces coelicolor* (*S. coelicolor*), a well-studied model for the actinomycetes genus, is probably affected by metabolite, morphological, homeostatic and stress-related checkpoints. Sigma factors and the regulators encoded by the *whi* and *bld* genes are known to be implicated [8]. Secondary metabolism is typically affected by the nature and levels of the carbon and nitrogen source as well as by the availability of phosphate and small signaling molecules, such as ppGpp and r-butyrolactone [9]. It has also been shown that certain regulators are involved in the pleiotropic control of antibiotic production including AbsA1/A2, AfsR/K, PhoR/P and regulators encoded by *bld* genes [10–14]. Although there has been limited understanding of the regulatory mechanism involved in the production of actinorhodin (Act) and undecylprodigiosin (Red) in *S. coelicolor*, it has been established that these antibiotics are regulated directly by the pathway-specific transcriptional regulators ActII-ORF4, RedD and RedZ [11,15–19].

In this study we characterized a new putative membrane protein, SarA (SCO4069), which negatively regulates sporulation in *S. coelicolor* M145. The sporulation and antibiotic production related gene A (*sarA*) mutant decreased the production of Act and Red by influencing the pathway-specific activators ActII-ORF4 and RedZ at

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the mRNA level.

Materials and Methods

Bacterial strains, plasmids, and growth conditions

The bacterial strains, plasmids and primers used in this study are listed in **Table 1**. *Escherichia coli* (*E. coli*) DH5α [20] was used for plasmid propagation. Mannitol Soya flour medium (MS) [21] agar was used to generate spores and for selection of *Streptomycete* exoconjugants. YBP medium agar (2 g yeast extract, 2 g beef extract, 4 g Bacto-peptone, 1 g $MgSO_4$, 5 g NaCl, 15 g agar and 10 g glucose combined with 1 l water) was used to screen for phenotypes. Yeast extract-malt extract medium (YEME) [21] was used to cultivate mycelia to prepare genomic DNA and supplemented minimal medium solid (SMMS) liquid medium [21] was used to prepare RNA. The conjugation of *E. coli* ET12567/pUZ8002 with *Streptomycetes* was performed as described [21]. Antibiotics were added, whenever necessary, at following final concentrations: 50 μg/ml ampicillin, 33 μg/ml chloramphenicol, 30 μg/ml kanamycin and 25 μg/ml thiostrepton.

Mutagenesis of *S. coelicolor* M145 and gene complementation

Insertional mutagenesis of M145 was conducted by *in vivo* transposition with plasmid pDZY101, a derivative transponson from IS204 which was first identified in *Nocardia asteroids* YP21 [22], through conjugation from *E. coli* ET12567/pUZ8002 to *S. coelicolor* M145. The exconjugants were selected by growth on MS media flooded with 30 μg/ml kanamycin. The pDZY101 carrying the replication region of pUC serial plasmids is capable of causing highly efficient random and stable mutagenesis with a single copy number in *S. coelicolor* M145. The chromosomal locations of the pDZY101 insertions were determined by sequencing the insertion plasmid flanking DNA through plasmid rescue.

sarA and its upstream DNA fragment was amplified by PCR using primer sets of Oxj138/139 (**Table 1**). It was then inserted into the *Sac*I/*Hind*III-digested pFDZ16, a *Steptomycete*/*E. coli* shuttle single integrate vector carrying genes encoding thiostrepton, kanamycin and ampicillin resistance, to give rise to plasmid pFDZ16-sarA for genetic complementation of *sarA* mutant K66. The plasmid was conjugated into the K66 from the donor *E. coli* ET12567/pUZ8002. The thiostrepton-resistant *Streptomyces* exoconjugant was designated as K66-sarA.

Quantification of antibiotics and assay of growth curves

Act and Red were assayed as previously described [21]. The bacteria grew in 30 ml SMMS liquid medium and was filtered to separate the supernatant from the pellet. For Act, KOH was added to the supernatant to a 1 M final concentration, and was then assayed at an optical density of 640 nm. For Red, the mycelia pellet was dried under vacuum conditions and extracted with 10 ml methanol (adjusted to pH 2) overnight at room temperature and the optical density was measured at 530 nm. Measurements were always taken from triplicate cultures. Growth curves of the prototype, the mutant K66 and the revertant strain K66-sarA were determined as described by Kieser *et al* [21]. Cultivation was performed by using 25-ml test tubes each containing 3 ml of YBP liquid medium with the inoculation of 2×10^7 spores per ml and incubated on a reciprocal shaker (200 rpm) at 30 ºC. Cultures were taken at each time point and weight.

Reverse transcription-polymerase chain reaction analysis

Methods for RNA isolation were performed according to the manual of Bacterial RNA Kit (Omega, Norcross GA, USA). Reverse transcription (RT) was performed according to the manual of High fidelity RNA PCR kit (TaKaRa, Otsu Shiga, Japan). The primers used for RT-PCR are shown in **Table 1**. PCR conditions were 94 ºC for 30 s, 60 ºC for 30 s and 72 ºC for 30 s in a total of 26 cycles. For *redD*, there were 32 cycles. Controls were performed using the RNA from the parent strain M145 or K66 without RT, and the results were negative.

Results

Identification of *sarA* in *S. coelicolor* M145

We used an *in vivo* transposition system to generate a collection of mutants with abnormalities in aerial mycelium differentiation and secondary metabolite production by conjugation plasmid pDZY101 from *E. coli* ET12567/pUZ8002 to *S. coelicolor* M145. Insertion mutant K66 showed accelerated sporulation and decreased antibiotic production. By sequencing the DNA flanking the pDZY101 insertion in K66, we identified a gene, *sarA* (*sco4069*), that was disrupted in K66 [**Fig. 1(A)**]. The *sarA* gene in *S. coelicolor* encodes a 664 amino acid protein with a calculated molecular mass of 69,158 Da without any characterized functional motif except for the trans-membrane domain. The proteins SAV4148 in *Streptomyces*

Table 1 Strains, plasmids and primers used in this study

Strain, plasmid and primer	Description	Reference or source
Strains		
S. coelicolor		
M145	Prototroph SCP1$^-$ SCP2$^-$	[18]
K66	*sarA* gene knocked out M145 strain, *sarA*$^-$, *Kan*^{R}	This study
K66-sarA	K66 carrying plasmid pFDZ16-sarA	This study
K66-pFDZ16	K66 carrying plasmid pFDZ16	This study
E. coli		
DH5α	F$^-$ *rec*A *lac*ZΔM15	[16]
ET12567	*dam dcm hsd*S	[17]
Plasmids		
pFDZ16	Integration shuttle-vector of *E. coli* and *Streptomyces*. *kan*^{R}, *Thio*^{R}, *Amp*^{R}	This study
pFDZ16-sarA	Derivative plasmid from pFDZ16, containing the *sarA* gene and its promoter. *kan*^{R}, *Amp*^{R}, *Thio*^{R}	This study
Primers		
Oxj138	5′-ATGAGCTCGCCTCGCACAACTACCCC-3′	–
Oxj139	5′-ATAAGCTTTGCTCCCGACTCCCAACG-3′	–
Oxj201	5′-CCGGAGCCAGCCAAAGATC-3′	–
Oxj202	5′-GGAGGGCGTTGAGGACGTT-3′	–
Oxj203	5′-TGCTGACCAAGCCCGAGAA-3′	–
Oxj204	5′-CGGTGTACGTGGGACCTGAC-3′	–
Oxj205	5′-TGGTGCTGCTGCTCCTCAG-3′	–
Oxj206	5′-ATCCAGTCCCGCGTCCAA-3′	–
Oxj237	5′-CTCTGTCATGGCGCTCATTGA-3′	–
Oxj238	5′-TTCGCTGCGACGCTCTTT-3′	–

Amp^R, ampilcillin-resistant; *E. coli*, *Escherichia coli; kan*^{R}, kanamycin-resistant; *sarA*, sporulation and antibiotic production related gene A; *S. coelicolor*, *Streptomyces coelicolor*; *Thio*^{R}, thiostrepton-resistant.

avermitilis MA-4680, SGR3860 in *Streptomyces griseus* NBRC 13350 and SCAB47711 in *Streptomyces scabies* 87.22 have, respectively, a 77%, 68% and 66% similarity to the SarA protein [**Fig. 1(B)**]. BLAST results revealed that members of this type of protein are highly conserved and have only been identified in *Streptomcyes* thus far. Genes located immediately upstream and downstream of *sarA* are *purD* (or *sco4068*), *sco4070* and *purC* (or *sco4071*) in M145. Homologs of these genes are arranged in the same order in *Streptomyces avermitilis*, *Streptomyces griseus* and *Streptomyces scabies*. Because *purD*- and *purC*-encoded proteins participate in the biosynthesis of *de novo* purine nucleotide, we wondered if SarA also participated in this metabolic pathway. By testing the growth of *sarA* mutant on minimal medium agar, we found that the mutation of this gene does not cause auxotrophy and the mutant strain could grow well on this medium without any growth factor (data not shown). This result indicated that SarA was not essential for the purine nucleotide biosynthesis.

SarA influences the morphogenesis and secondary metabolism in a divergent way

The morphological phenotype of the *sarA* mutant was firstly screened on YBP medium (**Fig. 2**). The results showed that the *sarA* mutant sporulated earlier and better than the M145 strain, while the production of Act and Red dramatically decreased to a level that was hardly visible from the bottom of the plates. We also screened the phenotype on YBP with 1% mannitol instead of glucose and the results were the same (data not shown). To investigate whether the phenotype of antibiotic production in liquid medium is the same as that on solid medium, we tested the antibiotic production in SMMS liquid medium; the experiments showed that *sarA* mutant's production

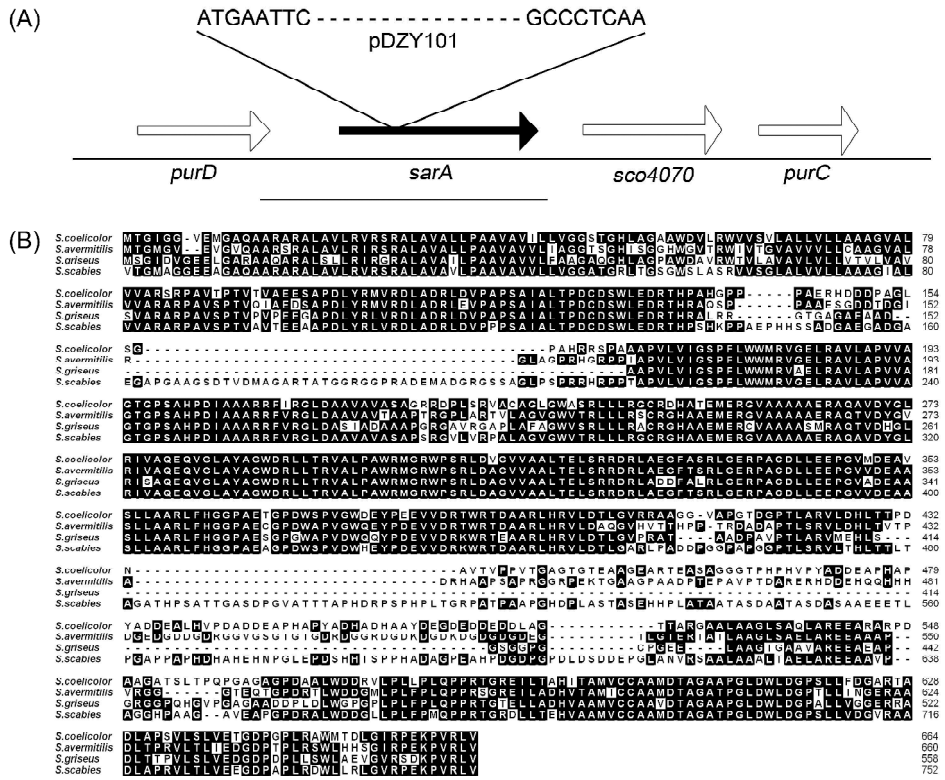

Fig. 1 SarA is highly homologous in *Streptomyces* (*S.*) (A) The location of *sarA* (*sco4069*) in *S. coelicolor* genome. The insertion of the pDZY101 plasmid in the K66 mutant is indicated above *sarA*. The bold line below *sarA* represents the DNA fragment used for genetic complementation in the K66 mutant. (B) Multiple amino acid sequence alignment of SarA from *S. coelicolor* with its homologous proteins, SAV4148 from *S. avermitilis*, SGR3860 from *S. griseus* and SCAB47711 from *S. scabies* (unfinished genome sequence data produced by the *S. scabies* Sequencing Group at the Sanger Institute (Cambridge Hinxton, UK) and can be obtained from http://www.sanger.ac.uk/Projects/S_scabies) using BioEdit 7.0.0 (Indiana University, Indiana Bloomington, USA).

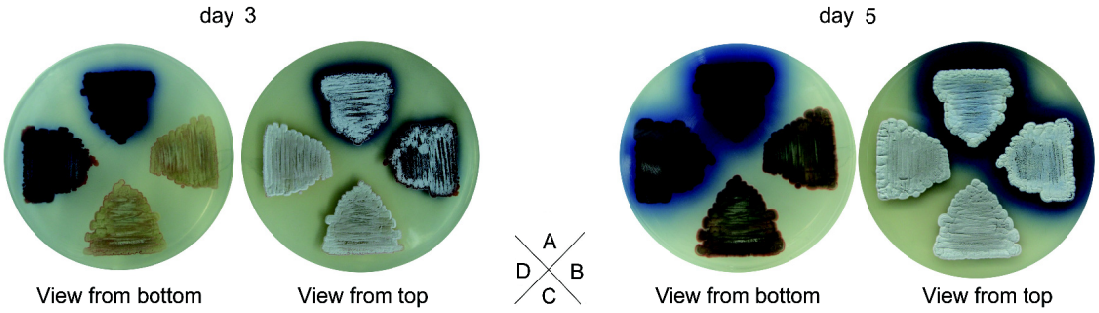

Fig. 2 Phenotypic display of antibiotic production and sporulation of *sarA* null mutant Colonies of the parent strain *Streptomyces coelicolor* M145 (A), the *sarA* null mutant K66 (B), the *sarA* mutant harboring the empty vector pFDZ16 [K66-pFDZ16] (C) and the complement strain K66-sarA (D) were grown on YBP medium containing 1% glucose for 3 d and 5 d. Left, view from below; right, view from on top; YBP medium, 2 g yeast extract, 2 g beef extract, 4 g Bacto-peptone, 1 g $MgSO_4$, 5 g NaCl, 15 g agar and 10 g glucose combined with 1 L water.

of Act and Red were lower when compared to M145's [**Fig. 3(A)**]. The phenotype was complemented by an integrative plasmid containing only *sarA*$^+$ with its 0.4 kb upstream probable promoter sequence. The growth curves of M145, *sarA* mutant and K66-sarA in liquid YBP medium were tested, and the results showed that there was no

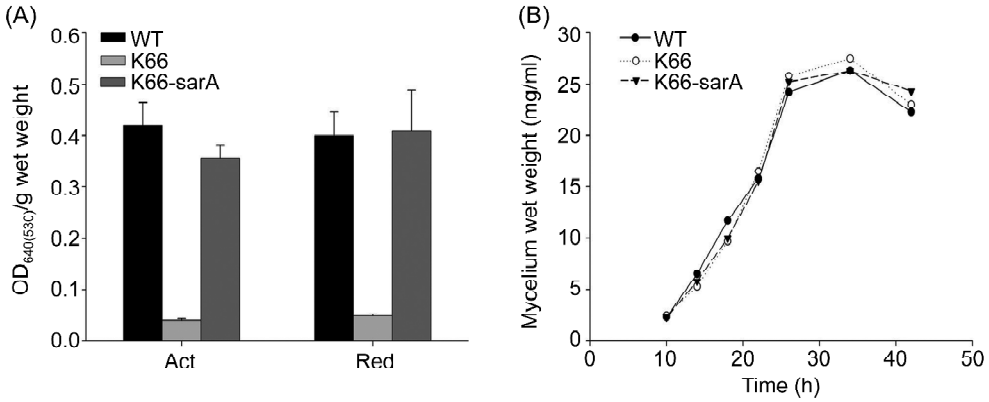

Fig. 3 Antibiotic production and growth curves of the *Streptomyces coelicolor* (*S. coelicolor*) M145 (WT), *sarA* null mutant K66 and the complemented strain K66-sarA (A) Actinorhodin (Act) and undecylprodigiosin (Red) production by *S. coelicolor* M145, K66 and K66-sarA. Incubation was carried out in supplemented minimal medium solid (SMMS) liquid medium at 30 ºC for 5 d. (B) Growth curves of M145, K66 and K66-sarA in YBP liquid medium with 1% glucose. OD, optical density; YBP medium, 2 g yeast extract, 2 g beef extract, 4 g Bacto-peptone, 1 g $MgSO_4$, 5 g NaCl, 15 g agar and 10 g glucose combined with 1 L water.

difference in their respective growth rates [**Fig. 3(B)**]. These data highlight the fact that SarA negatively regulates sporulation, though it has a positive influence on Act and Red production.

SarA regulates the antibiotic production by controlling the abundance of the ActII-ORF4 and RedZ mRNA

The expression of antibiotic biosynthesis clusters is normally regulated by pathway-specific activators [17–19]. In *S. coelicolor*, Act and Red biosynthesis have been shown to depend on the transcriptional activation of the Act and Red biosynthesis clusters by ActII-ORF4, RedD and RedZ proteins respectively. RedD, the direct transcriptional activator for the biosynthesis Red cluster, is RedZ dependant [11,15]. Down-regulated expression of these proteins results in the decreased production of Act or Red. The transcription of *actII-orf4*, *redD* and *redZ* in the sarA mutant K66 were therefore analyzed by RT-PCR. Total RNA was isolated from two developmental stages of M145 and K66 grown on SMMS liquid medium cultured for 36 h and 80 h. As shown in **Fig. 4**, the transcription of *actII-orf4*, *redD* and *redZ* decreased markedly in the later stage in K66 compared to that in M145, suggesting that SarA regulated the Act and Red production by controlling the mRNA abundance of the ActII-ORF4 and RedZ.

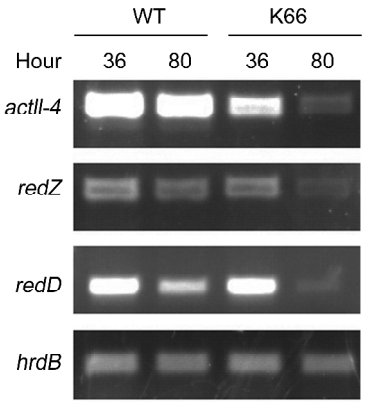

Fig. 4 Reverse transcription-polymerase chain reaction results of the wild-type M145 (WT) and K66 mutant The transcriptional levels of the pathway-specific activators were detected using primer pairs (**Table 1**). *actII-orf4*, oxj205/206; *redZ*, oxj203/204; *redD*, oxj201/202; *hrdB* (control), oxj237/238. Measurements were separately performed in duplicate, and the results were the same.

Discussion

In this study *sarA* (*sco4069*) in *S. coelicolor* was identified by gene disruption as a gene negatively affecting sporulation but positively influencing the production of Act and Red. SarA belongs to a putative membrane protein family that has so far been only found in *Streptomyces*. Levels of *actII-orf4*, *redZ* and *redD* mRNA decreased dramatically at a late time point in the *sarA* mutant, suggesting exerted either by the activated genes that were regulated by SarA over long time periods or by the effects on mRNA half-life. Though the disruption of *sarA* dramatically decreased the production of antibiotics in *S. coelicolor* M145, the sporulation of the strain was accelerated rather than delayed. The cause of this paradox

remains unknown. One possible explanation is that SarA exists as a membrane protein, senses the extracellular or intracellular signals, and balances the nutrients and energy between aerial mycelium morphogenesis and antibiotic production. Since the growth rate of *sarA* mutant in liquid culture and aerial mycelium formation on solid medium were not changed, and this mutant maintained the prototrophic phenotype, it seems that the effect of SarA on sporulation and antibiotics production of *S. coelicolor* M145 is not correlated with primary metabolism.

In conclusion, *sarA* encodes a putative membrane protein, which is a representative of a new family of *Streptomyces*-specific proteins. The presence of SarA and its homolog exclusively in *Streptomyces* could imply that this type of protein plays an important role in controlling the development of these streptomycetes.

References

1 Chater KF. Genetics of differentiation in *Streptomyces*. Annu Rev Microbiol 1993, 47: 685–713

2 Hesketh A, Chen WQ, Ryding J, Chang S, Bibb M. The global role of ppGpp synthesis in morphological differentiation and antibiotic production in *Streptomyces coelicolor* A3(2). Genome Biol 2007, 8: R161

3 Capstick DS, Willey JM, Buttner MJ, Elliot MA. SapB and the chaplins: connections between morphogenetic proteins in *Streptomyces coelicolor*. Mol Microbiol 2007, 64: 602–613

4 Willey JM, Willems A, Kodani S, Nodwell JR. Morphogenetic surfactants and their role in the formation of aerial hyphae in *Streptomyces coelicolor*. Mol Microbiol 2006, 59: 731–742

5 Kodani S, Lodato MA, Durrant MC, Picart F, Willey JM. SapT, a lanthionine-containing peptide involved in aerial hyphae formation in the *Streptomycetes*. Mol MicrobioL 2005, 58: 1368–1380

6 Ohnishi Y, Seo JW, Horinouchi S. Deprogrammed sporulation in *Streptomyces*. FEMS Microbiol Lett 2002, 216: 1–7

7 Challis GL, Hopwood DA. Synergy and contingency as driving forces for the evolution of multiple secondary metabolite production by *Streptomyces* species. Proc Natl Acad Sci USA 2003, 100: 14555–14561

8 Chater KF. Regulation of sporulation in *Streptomyces coelicolor* A3(2): a checkpoint multiplex? Current Opinion in Microbiology 2001, 4: 667–673

9 Bibb M. Regulation of secondary metabolism in streptomycetes. Curr Opin Microbiol 2005, 8: 208–215

10 Chater KF, Chandra G. The use of the rare UUA codon to define "expression space" for genes involved in secondary metabolism, development and environmental adaptation in *Streptomyces*. J Microbiol 2008, 46: 1–11

11 White J, Bibb M. bldA dependence of undecylprodigiosin production in *Streptomyces coelicolor* A3(2) involves a pathway-specific regulatory cascade. J Bacteriol 1997, 179: 627–633

12 Sola-Landa A, Moura RS, Martin JF. The two-component PhoR-PhoP system controls both primary metabolism and secondary metabolite biosynthesis in *Streptomyces lividans*. Proc Natl Acad Sci USA 2003, 100: 6133–6138

13 McKenzie N, Nodwell J. Phosphorylated AbsA2 negatively regulates antibiotic production in *Streptomyces coelicolor* through interactions with pathway-specific regulatory gene promoters. J Bacteriol 2007, 189: 5284–5292

14 Lee P, Umeyama T, Horinouchi S. afsS is a target of AfsR, a transcriptional factor with ATPase activity that globally controls secondary metabolism in *Streptomyces coelicolor* A3(2). Mol Microbiol 2002, 43: 1413–1430

15 Guthrie E, Flaxman C, White J, Hodgson D, Bibb M, Chater KF. A response-regulator-like activator of antibiotic synthesis from *Streptomyces coelicolor* A3(2) with an amino-terminal domain that lacks a phosphorylation pocket. Microbiology 1998, 144: 727–738

16 Aceti DJ, Champness WC. Transcriptional regulation of *Streptomyces* coelicolor pathway-specific antibiotic regulators by the absA and absB loci. J Bacteriol 1998, 180: 3100–3106

17 Bibb M. 1995 Colworth Prize Lecture. The regulation of antibiotic production in *Streptomyces coelicolor* A3(2). Microbiology 1996, 142: 1335–1344

18 Gramajo HC, Takano E, Bibb MJ. Stationary-phase production of the antibiotic actinorhodin in *Streptomyces coelicolor* A3(2) is transcriptionally regulated. Mol Microbiol 1993, 7: 837–845

19 Takano E, Gramajo HC, Strauch E, Andres N, White J, Bibb MJ. Transcriptional regulation of the redD transcriptional activator gene accounts for growth-phase-dependent production of the antibiotic undecylprodigiosin in *Streptomyces coelicolor* A3(2). Mol Microbiol 1992, 6: 2797–2804

20 Sambrook J, Russell DW. Molecular Cloning: A Laboratory Manual, 3rd edn. New York: Cold Spring Harbor Laboratory Press, 2001

21 Kieser T, Bibb MJ, Butter MJ, Chater KF, Hopwood DA. Practical *Streptomyces* Genetics. Norwich, England: The John Innes Foundation, 2000

22 Yao WS, Yang YL, Chiao JS. IS*204:* an insertion sequence from *Nocardia asteroides* (*mexicana*) YP21. Plasmid 1994, 32: 262–269