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Identification and Functional Characterization of an *afsR* Homolog Regulatory Gene from *Streptomyces venezuelae* ATCC 15439

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Sequencing analysis of a 5-kb DNA fragment from *Streptomyces venezuelae* ATCC 15439 revealed the presence of one 3.1-kb open reading frame (ORF), designated as *afsR-sv*. The deduced product of *afsR-sv* (1,056 aa) was found to have high homology with the global regulatory protein AfsR. Homology-based analysis showed that *afsR-sv* represents a transcriptional activator belonging to the *Streptomyces* antibiotic regulatory protein (SARP) family that includes an N-terminal SARP domain containing a bacterial transcriptional activation domain (BTAD), an NB-ARC domain, and a C-terminal tetratricopeptide repeat domain. Gene expression analysis by reverse transcriptase PCR (RT-PCR) demonstrated the activation of transcription of genes belonging to pikromycin production, when *afsR-sv* was overexpressed in *S. venezuelae.* Heterologous expression of the *afsR-sv* in different *Streptomyces* strains resulted in increased production of the respective antibiotics, suggesting that *afsR-sv* is a positive regulator of antibiotics biosynthesis.

Keywords: *afsR-sv*, regulatory gene, reverse transcriptase PCR, SARP family, *Streptomyces venezuelae*

Streptomyces venezuelae ATCC 15439 is a producer of several macrolide antibiotics that include the 12-membered polyketides methymycin, neomethymycin [16, 18], and novamethymycin, and the 14-membered polyketides narbomycin, pikromycin [15, 16], neopikromycin, and novapikromycin [14]. Usually, genes for the production of individual secondary metabolites are arranged in clusters, and most but not all of these clusters contain pathway-specific regulatory genes whose expression frequently depends on genes that are required for the production of several secondary metabolites. The production is activated when the transcription of the pathway-specific regulators reach a threshold in the cells in response to specific environmental and physiological signals [29].

Interestingly, a number of regulators exert pleiotropic effects in *Streptomyces*. AfsR is one of these regulators that acts as a transcriptional factor in both the regulation of secondary metabolism in *Streptomyces coelicolor* A3(2) and morphological differentiation in *Streptomyces griseus*. Moreover, AfsR is a protein belonging to the *Streptomyces* antibiotic regulatory protein (SARP) family, which consists of three major functional domains: an N-terminal SARP domain, a central ATPase domain, and a C-terminal tetratricopeptide repeat (TPR) domain. An example of SARPs includes ActII-ORF4, which functions in actinorhodin production in *Streptomyces coelicolor* A3(2) [1]. Transcriptional activation by SARPs is expected to occur *via* an interesting and novel mechanism. As a transcriptional activator, AfsR is phosphorylated on its threonine residues by a protein serine/threonine kinase, AfsK, which enhances its DNA-binding activity and causes it to bind the promoter elements, including −35, of *afsS*, thus resulting in activation of *afsS* transcription. AfsS then activates transcription of ActII-ORF4, a pathway-specific transcriptional activator in the actinorhodin biosynthetic gene cluster, in an unknown way.

Subsequent studies have shown that the AfsK/AfsR system is widely distributed in *Streptomyces* and influences secondary metabolism and morphogenesis [7, 19]. It has been reported that *afsR* overexpression led to the overproduction of the pigmented antibiotics actinorhodin and undecylprodigiosin and A-factor in *S. lividans*. Moreover, the *afsR* deletion mutant could not produce a detectable amount of actinorhodin [12]. In this study, we report the identification and functional characterization of an *afsR* homolog global regulatory gene, designated as *afsR-sv*, from *S. venezuelae* ATCC 15439 and present evidence that the *afsR-sv* gene is involved in the regulation of pikromycin biosynthesis. Furthermore, we also observed that *afsR-sv* influences the production of doxorubicin in *S. peucetius* and actinorhodin in *S. lividans* TK24.

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Materials and Methods

Bacterial Strains and Growth Conditions

Escherichia coli XL1-Blue MRF' (Stratagene, U.S.A.) was used to prepare recombinant plasmids and to construct the cosmid library [21]. Luria-Bertani (LB) medium was used for *E. coli* propagation. For transformation of *Streptomyces*, plasmid DNA was isolated after amplification in *E. coli* ET12567 (John Innes Centre, U.K.). All *Streptomyces* strains were grown at 28°C in R2YE media [9] in order to prepare the protoplasts and isolate plasmid DNA. Recombinant plasmids were transformed into protoplasts of *S. peucetius* ATCC 27952, *S. lividans* TK24, and *S. venezuelae* ATCC 15439 according to the standard protocol [9]. Thiostrepton (12.5 μg/ml for *S. peucetius* and 50 μg/ml for *S. lividans* TK24) and apramycin (500 μg/ml for *S. venezuelae*) were used to select recombinant strains.

SCM liquid medium (0.01% calcium chloride, 0.01% ferrous sulfate, 1.05% MOPS, 1.5% soluble starch, 2% soytone, and 0.15% yeast extract) was used as production media for pikromycin from *S. venezuelae* and its recombinant strains [14]. NDYE medium (11.2% maltose, 0.7% yeast extract, 21.4% $NaNO_3$, 1.15% K_2HPO_4, 23.8% HEPES, 0.6% $MgSO_4 \cdot 6H_2O$, 4% NaOH), supplemented with 1 ml of inorganic solution (1 mg of ferrous sulfate, 1 mg of magnesium chloride, and 1 mg of zinc sulfate per 1,000 ml distilled water), was used for the production of doxorubicin from *S. peucetius* and its recombinant strains [8]. YEME medium (1% glucose, 0.3% malt extract, 0.5% peptone, 34% sucrose, 0.3% yeast extract, and 10 mM magnesium chloride) was used as production media for actinorhodin from *S. lividans* TK24 and its recombinant strains. Reverse transcriptase polymerase chain reaction (RT-PCR) was carried out using APM medium (6% glucose, 2% malt extract, 1.5% MOPS, 0.2% sodium chloride, and 0.8% yeast extract) to grow multiple cultures, following the time course, of *S. venezuelae* and *S. venezuelae*/pASV152.

Construction of Genomic Library and Screening

Genomic DNA was isolated from *S. venezuelae* by lysozyme treatment and phenol-chloroform extraction according to the method described by Kieser *et al.* [9]. A cosmid library of *S. venezuelae* was constructed by partial Sau3AI digestion of chromosomal DNA, and ligation into the XbaI-BamHI sites of SuperCosI. Packaging was performed with Gigapack III XL (Stratagene, U.S.A.) and transduced into *E. coli* XL1-Blue MRF'. Common primers, CPF and CPR (Table 1), were designed, where S may be replaced by G or C, based on the conserved residues from *afsR-sp* (*S. peucetius*), *afsR-g* (*S. griseus*) and *afsR* (*S. coelicolor*). The 600-bp DNA fragment was amplified from *S. venezuelae* using those primers. The PCR products were ligated into the pGEM-T easy vector, sequenced, and used as probes for library screening. Southern blot analysis was performed on Hybond-N nylon membranes (Amersham, Braunschweig, Germany) with digoxigenin-labeled probes by using the DIG high prime DNA labeling and detection starter kit (Boehringer, Mannheim, Germany).

DNA Sequencing and Computer-Assisted Sequence Analysis

Restriction fragments of approximately 300 to 5,000 bp were obtained from the cosmid and subcloned into the pGEMT-Easy vector (Promega, U.S.A.). Sequencing was performed according to the dideoxynucleotide chain termination method on an automatic sequencer. The DNASIS software package (version 2.1, 1995; Hitachi Software Engineering, San Bruno, CA, U.S.A.) and BLAST (NCBI) were used for sequence analysis and homology search in the GenBank database, respectively.

Table 1. Primers used in this study.

Pimers	Nucleotide sequences (5'–3')
CPF	GCSGGSATCGGSGGSGTSGGSAAGACSA
CPR	CTCGTCSGCSAGCTTSGCSGCSAGSACSGA
afsR-svF	AAAGAATTCGTACGGGCAGGGGGAGCT
afsR-svR	ATAAAGCTTGAACGCCCAGGCCGGGAC
KS-RTF	CGAACGGTTTCGCGGAGCAGACTCATG
KS-RTR	GAAGTCGTGGTGCACCACGGTCACATG
pikD-RTF	CATCTCAGGGCCGTTCTTGACGCATCC
pikD-RTR	CGTAGTAGTGGGCGAGTAACTGGCGTAC
16SF	CCTTCGGGTTGTAAACCTCTTTCAGCA
16SR	CAACACCTAGTTCCCAACGTTTACGGC

S may be replaced by either G or C.

The amino acid sequence of AfsR-sv was aligned with its homologs using the ClustalX program, and the conserved domain was analyzed using the Pfam 22.0 (Pfam Consortium) [26] and NCBI database.

DNA Sequence Accession Numbers

The nucleotide sequence of *afsR-sv* reported in this paper has been deposited in the NCBI nucleotide sequence database under Accession No. EF612792.

Construction of *afsR-sv* Expression Plasmids

Two oligonucleotides, afsR-svF and afsR-svR (Table 1), were synthesized with EcoRI and HindIII sites, respectively. They were then used to amplify the *afsR-sv* from the genomic DNA of *S. venezuelae* under the following PCR conditions: a denaturation at 97°C for 7 min, 30 cycles of denaturation at 97°C for 1 min, annealing at 62°C for 10 min, and polymerization at 47°C for 1 min. DNA amplification was performed in a total volume of 20 μl with 4 μl of PCR premix solution from Genechem Co., Korea. The PCR product (3,171 bp) obtained was purified and cloned into the EcoRI and HindIII sites of pIBR25 [24] under the control of the *ermE** promoter in order to generate the expression recombinant plasmid pASV25. Similarly, the purified PCR product was subcloned into pGEM-7Z (+) and then cloned into the XbaI and BamHI sites of the integration vector pSET152 in order to generate the integration recombinant plasmid pASV152.

Comparison of Growth Rate

To study the growth rate, *S. venezuelae, S. venezuelae*/pSET152, and *S. venezuelae*/pASV152 were grown in SCM media at 28°C after 36 h of incubation in R2YE seed media. The cell pellets were collected at intervals of 12 h by centrifuging 50 ml of culture broth of each strain at 6,000 ×*g*. Cell pellets were washed with distilled water and dried at 72°C in a vacuum oven to constant weight. Dried cell pellets were obtained for growth rate analysis.

Analysis of Antibiotic Production

The plasmids pASV152 and pSET152 were integrated into the genomic DNA of *S. venezuelae* to generate *S. venezuelae*/pASV152 and *S. venezuelae*/pSET152, respectively. The expression plasmids pIBR25 and pASV25 were transformed into *S. peucetius* generating *S. peucetius*/pIBR25 and *S. peucetius*/pASV25, respectively. Similarly, pIBR25 and pASV25 were also transformed into *S. lividans* TK24 to generate *S. lividans* TK24/pIBR25 and *S. lividans* TK24/pASV25,

respectively. These transformations were done by following the PEG-mediated protoplast transformation method [9].

S. venezuelae, *S. venezuelae*/pSET152, and *S. venezuelae*/pASV152 were grown in 50 ml of liquid SCM medium for 60 h at 28°C. The culture broth of each strain was centrifuged for 15 min at 6,000 ×*g* to remove cell pellets. The supernatants were extracted with 2 volumes of ethyl acetate, and the extract was dried under reduced pressure using a rotary evaporator and reconstituted by 1.5 ml of methanol. A 15-µl aliquot of the extract obtained was then analyzed on high-performance liquid chromatography (HPLC) using a reverse-phase C18 column (Mytstil RP-18, 4.6×250 mm×5 µm) with 80% acetonitrile in 5 mM ammonium acetate buffer containing 0.05% acetic acid, adjusted to pH 8 with ammonium hydroxide. Detection was carried out with a UV absorbance detector, monitoring peaks at 220 nm for 66 min at a flow rate of 1 ml/min. The major peak corresponding to pikromycin was confirmed by liquid chromatography/mass spectrometry (LC/MS) analysis.

S. peucetius, *S. peucetius*/pIBR25, and *S. peucetius*/pASV25 were grown in 50 ml of liquid NDYE medium for 84 h at 28°C. 50 ml of culture broth of each strain was centrifuged for 15 min at 6,000 ×*g* to remove cell pellets. The supernatant was extracted with 2 volumes of $CHCl_3$:CH_3OH. The extract was dried under reduced pressure using a rotary evaporator and reconstituted with 1.5 ml of methanol. A 15-µl aliquot of the extract obtained was analyzed by HPLC using a reverse-phase C18 column with 100% methanol (solvent B) and distilled water (solvent A, pH 2.34) for 71 min, with a flow rate of 1 ml/min. Detection was carried out using a UV absorbance detector, monitoring peaks at 254 nm. Authentic doxorubicin was used as a reference.

S. lividans TK24, *S. lividans* TK24/pIBR25, and *S. lividans* TK24/pASV25 were cultured in 50 ml of liquid YEME medium for 9 days at 28°C. After centrifugation for 15 min at 6,000 ×*g* to remove cell pellets, the pH of the supernatants was adjusted at 2.0 with 1 N HCl and extracted with chloroform. The amount of the blue-colored antibiotic actinorhodin was determined by measuring the optical density of the extracts at 633 nm using a UV spectrophotometer (Shimadzu, Japan) [3]. In every case, *Streptomyces* strains transformed with only vector were used as a control, and the productions were averaged from four separate cultivations and extractions.

RNA Sample Preparation and RT-PCR Analysis

For total RNA isolation, *S venezuelae* and *S venezuelae*/pASV152 were cultured in APM medium. 5-ml aliquots of each culture at about 48 to 72 h were suspended in RNA protect Bacteria Reagent (Qiagen) for 5 min. An RNeasy Mini kit (Qiagen) was used for RNA isolation according to the instructions supplied by the manufacturer. Contaminant DNA in the sample was eliminated by using RNase-free DNase (Qiagen) and verified by PCR analysis with the RNA as the template. The total RNA concentration and purity were determined by measuring the optical density at 260 and 280 nm in a spectrophotometer.

RT-PCR was performed with a QuantiTech SYBER Green RT-PCR Kit (Qiagen). Primers used for RT-PCR analysis are listed in Table 1. An equal amount of RNA (1.5 µg) was used for RT-PCR in every case. The reaction conditions were as follows: first-strand cDNA synthesis at 50°C for 30 min; initial denaturation at 95°C for 15 min; and 45 cycles of 1 min at 94°C, 1 min at 63°C, and elongation at 72°C for 2 min. RT-PCR products were electrophoresed on 1% agarose gel and visualized using ethidium bromide staining. Negative controls were carried out with *Taq* DNA polymerase without reverse transcripts in order to confirm that the amplified products were not derived from chromosomal DNA that could have contaminated the RNA preparations. The 16S rRNA gene from *S. venezuelae* was used as a positive internal control. For further confirmation, the results obtained were crosschecked by running RT-PCR with decreased PCR cycles (*i.e.*, 30 cycles).

Results and Discussion

Screening of Cosmid Library and Sequence Analysis of the *afsR* Homolog Gene

A cosmid library of *S. venezuelae* was constructed in Super-Cos 1, and screening was carried out by using 600-bp PCR fragments as probes as described in the Materials and Methods section. Sequencing analysis of the cosmid revealed an open reading frame (ORF) that constitutes 3,168 nucleotides, where ATG and TGA were assigned as putative start and stop codons, respectively. The start codon was preceded by the putative ribosome binding site (GGGGGAG). Homology searches for the ORF showed a high degree of homology to *afsR* encoding global regulatory genes from *S. coelicolor* and other *Streptomyces*. This *afsR* homolog gene of *S. venezuelae* was designated as *afsR-sv*. In fact, AfsR-sv encodes 1,056 amino acids with ATP binding sites (A-type: ^{407}GIGGV**GKT**^{414}; and B-type: ^{485}LVLL**D**^{489}) and a catalytic domain (^{440}AEPET^{465}). AfsR-sv exhibited 76% identity with AfsR-av from *S. avermitilis* MA-4680, 74% with AfsR-sp from *S. peucetius*, and 72% identity with AfsR from *S. coelicolor* A3(2). Conserved domain analysis of AfsR-sv from NCBI and Pfam 22.0 revealed the presence of major functional domains of the *Streptomyces* antibiotic regulatory protein (SARP) family of transcriptional activators, including an N-terminal SARP domain, a bacterial transcriptional activation domain (BTAD), an NB-ARC domain, and a C-terminal tetratricopeptide repeat (TPR) domain (Fig. 1). In addition to these major functional domains, AfsR-sv possesses two additional domains: DnrI, a DNA binding transcriptional activator of the SARP family [23], and MalT, an ATP-dependent transcriptional activator of the LuxR family [20, 22]. Owing to the presence of major functional domains that are characteristics of the SARP

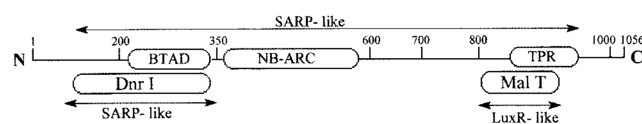

Fig. 1. Domain analysis of AfsR- sv. AfsR-sv possesses the major functional domains of the *Streptomyces* antibiotic regulatory protein (SARP) family.

N-terminal: SARP domain, a bacterial transcriptional activation domain (BTAD), an NB-ARC domain, and a DNA binding transcriptional activator of the SARP family (DnrI). C-terminal: tetratricopeptide repeat (TPR) domain and ATP-dependent transcriptional activator of the LuxR family (MalT).

family, *afsR-sv* could be classified as a member of the SARP family of transcriptional activators and considered to function as a global regulator of secondary metabolites in *S. venezuelae*.

afsR-sv Overexpression Increases the Production of Pikromycin

S. venezuelae/pSET152 and *S. venezuelae*/pASV152 were generated as described in the Materials and Methods section. There were no morphological differences between *S. venezuelae*/pASV152 and the wild-type strain when grown in R2YE and SCM media (data not shown). However, *S. venezuelae*/pASV152 showed a higher growth rate when compared with the wild-type strain (Fig. 2). HPLC and LC/MS analyses of the extracts isolated from 50-ml culture broths of *S. venezuelae*, *S. venezuelae*/pSET152, and *S. venezuelae*/pASV152 after 60 h incubation at 28°C revealed that *S. venezuelae*/pASV152 produced more pikromycin in SCM liquid medium as compared with that of the *S. venezuelae* and *S. venezuelae*/pSET152. *S. venezuelae*/pASV152 exhibited an approximately 4.85-fold increase in pikromycin production when compared with wild-type *S. venezuelae*. Similarly, analysis of the production of pikromycin after various culture periods (12 to 96 h) revealed that the rate of pikromycin production by *S. venezuelae*/pASV152 at 60 to 96 h was higher than that of the wild-type strain (Fig. 3A). These results clearly demonstrated that overexpression of *afsR-sv* influenced the production of pikromycin in *S. venezuelae* during the stationary phase.

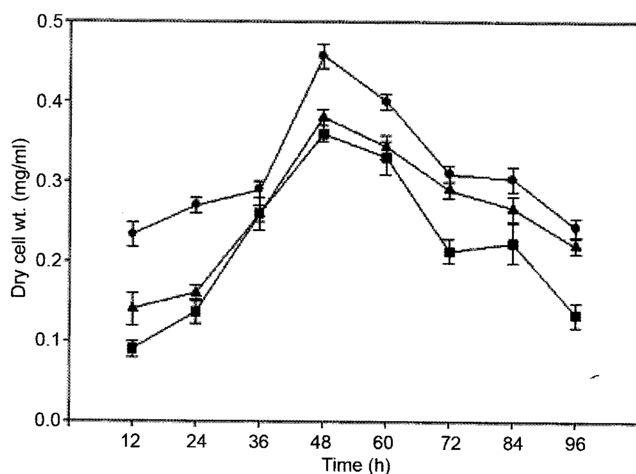

Fig. 2. Comparison of the growth rate between *S. venezuelae* and its transformants (*S. venezuelae*/pSET152 and *S. venezuelae*/pASV152).

S. venezuelae, *S. venezuelae*/pSET152 and *S. venezuelae*/pASV152, were grown in SCM media at 28°C after 36 h of incubation in R2YE seed media. The cell pellets were collected at intervals of 12 h by centrifuging 50 ml of culture broth of each strain at 6,000 ×*g*. Cell pellets were washed with distilled water and dried at 72°C in a vacuum oven to constant weight. Dried cell pellets were obtained for growth rate analysis. Maximum growth was observed at approximately 48 h in SCM medium. Square, *S. venezuelae*; triangle, *S. venezuelae*/pSET152; and circle, *S. venezuelae*/pASV152.

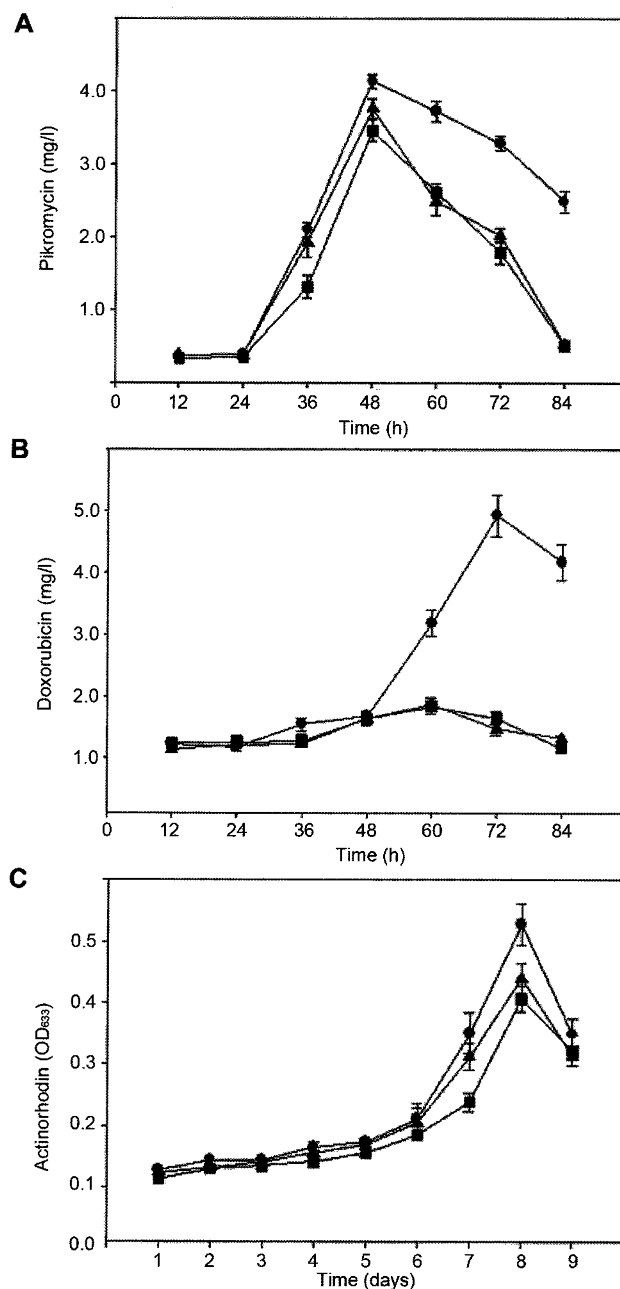

Fig. 3. Comparison of influence of *afsR-sv* on the production of pikromycin, doxorubicin and, actinorhodin.

A. Pikromycin production by *S. venezuelae* ATCC 15439 (square), *S. venezuelae*/pSET152 (triangle), and *S. venezuelae*/pASV152 (circle) at the indicated time intervals. The amount of pikromycin produced by *S. venezuelae*/pASV152 was approximately 4.85 times greater than that produced by the wild-type strain when pikromycin was extracted after 60 h of incubation from the respective strain. **B.** Doxorubicin production by *S. peucetius* ATCC 27952 (square), *S. peucetius*/pIBR25 (triangle), and *S. peucetius*/pASV25 (circle) at the indicated time intervals. The amount of doxorubicin produced by *S. peucetius*/pASV25 was increased by approximately 8.04-fold, when doxorubicin was extracted after 72 h of incubation from the respective strain. **C.** Actinorhodin production by *S. lividans* TK24 (square), *S. lividans* TK24/pIBR25 (triangle), and *S. lividans* TK24/pASV25 (circle) at the indicated time intervals. Actinorhodin production by *S. lividans* TK24/pASV25 was increased by approximately 1.5-fold, when actinorhodin was extracted after 8 days of incubation from the respective strain. Bars above the columns indicate standard errors.

Effect of *afsR-sv* on Antibiotic Production in Other *Streptomyces* Species

To study the influence of *afsR-sv* on the production of antibiotics other than pikromycin, the *afsR-sv* under the control of the *ermE** promoter was heterologously expressed in *S. peucetius* (doxorubicin producer) and *S. lividans* TK24 (actinorhodin producer). *S. peucetius, S. peucetius*/pIBR25, and *S. peucetius*/pASV25 were cultured in NDYE medium and doxorubicin was isolated as described in the Materials and Methods section. The amount of doxorubicin produced by *S. peucetius*/pASV25 was approximately 8.04 times greater than that produced by the wild-type strain (Fig. 3B). S. *lividans* TK24, *S. lividans* TK24/pIBR25, and *S. lividans* TK24/pASV25 were cultured in YEME medium, and actinorhodin was extracted as described in Materials and Methods. The amount of actinorhodin produced by *S. lividans* TK24/pASV25 was approximately 1.5 times greater than that produced by the wild-type strain (Fig. 3C). Similarly, analysis of doxorubicin and actinorhodin productions after various culture periods revealed that *S. peucetius*/pASV25 produced higher levels of doxorubicin around 72 h and *S. lividans* TK24/pASV25 produced higher levels of actinorhodin around 8 days when compared with the wild-type strains. These results clearly demonstrated that although the regulatory networking of *afsR-sv* in *Streptomyces* is not uniform, AfsR-sv acts as a positive regulator and increases the antibiotics production when heterologously expressed in different *Streptomyces*. Consistent with other AfsR, *afsR-sv* also exerts pleiotropic effects on the production of multiple secondary metabolites.

Transcriptional Analysis

To further confirm the regulatory effect of *afsR-sv* in the pikromycin biosynthesis, RT-PCR analysis was carried out to assess the expression levels of two representative genes: the ketosynthase (KS) gene and *pikD* (encoding the pathway-specific activator of the pikromycin gene cluster) [31, 32]. The results showed that the transcript levels of both KS and *pikD* were increased in the *afsR-sv* overexpressed strain (*S. venezuelae*/pASV152) when compared with *S. venezuelae*, and this finding is consistent with the increased production of pikromycin. Moreover, to assess whether the regulation of *afsR-sv* in the KS and *pikD* biosynthetic genes is growth phase-dependent, RT-PCR was performed using multiple cultures according to the time course described for *S. venezuelae* and *S. venezuelae*/pASV152 (Fig. 4). In both *S. venezuelae* and *S. venezuelae*/pASV152, the transcript levels of both genes increased at around 60 h and reached the maximal level at around 72 h, after which the transcript level was found to be decreased, suggesting that the regulation of *afsR-sv* in the pikromycin biosynthetic gene cluster was growth-phase- and time-dependent. However, the transcript levels of these genes in *S. venezuelae*/pASV152 were observed to be higher at earlier and later phases as compared

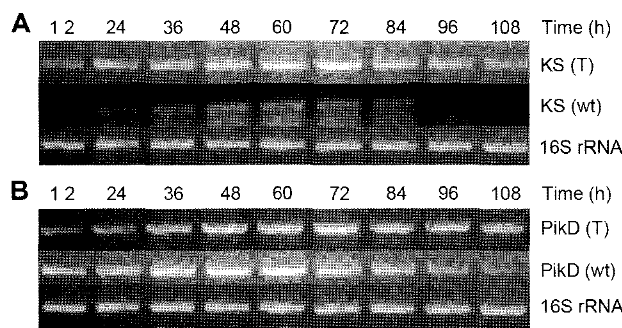

Fig. 4. Time-dependent transcriptional analysis of ketosynthase (**A**) and *pikD* (**B**) by RT-PCR in *S. venezuelae* and *S. venezuelae*/pASV152 strains.

The influence of *afsR-sv* on the transcription of ketosynthase and *pikD* was observed to be growth-phase-dependent. The transcription of both ketosynthase and *pikD* was found to be higher at around 48 to 72 h in *S. venezuelae*/pASV152 as compared with *S. venezuelae*. The experiments were repeated three times. 16S rRNA, internal control; KS, ketosynthase; wt, *S. venezuelae*; T, *S. venezuelae*/pASV152.

to those of *S. venezuelae*. Even when the RT-PCR was carried out with decreased PCR cycles, the transcript levels of both KS and *pikD* were found to be increased in the *afsR-sv* overexpressed strain than that in wild strain.

In this study, we reported the identification and characterization of an *afsR* homolog global regulatory gene, designated *afsR-sv*, from *S. venezuelae*. Sequencing and conserved domain analyses of *afsR-sv* identified an N-terminal SARP domain containing a bacterial transcriptional activation domain (BTAD), an NB-ARC domain, and a C-terminal tetratricopeptide repeat domain, which are the major functional domain of SARP family of thanscriptional activators. The global regulatory genes, even though not closely linked to the biosynthetic genes, keep control of antibiotic biosynthetic pathways. Moreover, these genes have been found to play a significant role in secondary metabolism and morphological differentiation in *Streptomyces*. Among them, the *afsR-sp* gene from *S. peucetius* was found to regulate the production of doxorubicin [17], and *afsR-g* from *S. griseus* was found to be involved in morphological differentiation [30]. In addition, the *afsR*2 gene from *S. lividans* was reported to highly stimulate two structurally unrelated antibiotics, actinorhodin and undecylprodigiosin, in both *S. lividans* and *S. coelicolor* [10, 11]. Consistent with these results, overexpression of *afsR-sv* in *S. venezuelae* increased pikromycin production by about 4.85-fold. Likewise, heterologous expression of *afsR-sv* resulted in increased production of actinorhodin by about 1.5-fold in *S. lividans* TK24 and doxorubicin by about 8.04-fold in *S. peucetius*. In this context we observed a much higher level of doxorubicin production in *S. peucetius* when compared with the levels of pikromycin and actinorhodin production by *S. venezuelae* and *S. lividans* TK24, respectively. These findings indicate that several factors other than the *afsR-sv* are engaged to affect the production of secondary metabolites in *Streptomyces*.

As in the case of *S. coelicolor* A3(2), where AfsR shows pleiotropic effects on secondary metabolism, the effect of *afsR-sv* is not also uniform in regulatory networking in *Streptomyces*.

RT-PCR assays demonstrated that *afsR-sv* functions as a positive regulator and is involved in the regulation of pikromycin biosynthesis in *S. venezuelae*. Overexpression of *afsR-sv* in *S. venezuelae* seemed to activate the transcription of ketosynthase and a pathway-specific regulator, both resulting in the overproduction of pikromycin. RT-PCR analysis also revealed that antibiotic production in *Streptomyces* species generally depends on the growth phase and involves the expression of physically clustered regulatory and biosynthetic genes. However, the mechanism of regulation by *afsR-sv* in *S. venezuelae* has yet to be elucidated. In conclusion, *afsR-sv* is a global regulatory gene in *S. venezuelae*, which belongs to the SARP family of transcriptional activators, regulates the pikromycin biosynthetic genes, and acts as a positive regulator of antibiotic production in *Streptomyces* strains.

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