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Functional Expression of SAV3818, a Putative TetR-Family Transcriptional Regulatory Gene from *Streptomyces avermitilis*, Stimulates Antibiotic Production in *Streptomyces* Species

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Avermectin and its analogs are major commercial antiparasitic agents in the fields of animal health, agriculture, and human infections. Previously, comparative transcriptome analysis between the low-producer *S. avermitilis* ATCC31267 and the high-producer *S. avermitilis* ATCC31780 using a *S. avermitilis* whole genome chip revealed that 50 genes were overexpressed at least two-fold higher in *S. avermitilis* ATCC31780. To verify the biological significance of some of the transcriptomics-guided targets, five putative regulatory genes were individually cloned under the strong-and-constitutive promoter of the *Streptomyces* expression vector pSE34, followed by the transformation into the low-producer *S. avermitilis* ATCC31267. Among the putative genes tested, three regulatory genes including SAV213, SAV3818, and SAV4023 exhibited stimulatory effects on avermectin production in *S. avermitilis* ATCC31267. Moreover, overexpression of SAV3818 also stimulated actinorhodin production in both *S. coelicolor* M145 and *S. lividans* TK21, implying that the SAV3818, a putative TetR-family transcriptional regulator, could be a global upregulator acting in antibiotic production in *Streptomyces* species.

Keywords: SAV3818, avermectin, TetR-family regulator, *Streptomyces avermitilis*

Species of the genus *Streptomyces* are of major pharmaceutical interest because they synthesize a variety of bioactive secondary metabolites [5]. Among the thousands of *Streptomyces* metabolites, only a few have been reported to possess anthelmintic activity [6]. Among all the metabolites containing anthelmintic activity is an avermectin, which is typically produced by *S. avermitilis* and considered as one of the most important drugs in the market because of its superior commercial potency [4]. Avermectins, which belong to a family of macrocyclic lactones, have eight major components with structural differences at C5, C22–C23, and C26 [1, 3, 18]. Avermectin and the related compounds such as milbemycin and nemadectin are potent anthelmintic compounds currently used commercially in animal healthcare and agriculture [5, 9]. Owing to its excellent anthelmintic activities against a variety of nematode and arthropod parasites with a low level of side effects on the host organism, many molecular biological studies have concentrated on the elucidation of its biosynthesis and regulation. However, the molecular processes regulating the events leading to differentiation and simultaneous antibiotic biosynthesis are still poorly understood. Despite of the recent completion of the *S. avermitilis* genome sequencing and the discovery of numerous useful insights into *Streptomyces* genetics, most of the genetic factors regulating avermectin production remain unknown [11, 12, 19]. Moreover, the attempts to improve the productivity of this important anthelmintic veterinary medicine were largely pursued with little or no consideration of the genetic modifications inherent to the mutant strain in which avermectin overproduction occurs [10 16, 21]. Lately, omics-guided technologies including DNA microarray systems have been applied in the identification of multiple mutations present in industrial strains at the molecular genetic level [13, 14, 17]. In recent studies, we showed that comparative transcriptome analysis between the low-producer *S. avermitilis* ATCC31267 and the high-producer *S. avermitilis* ATCC31780 *via* a *S. avermitilis* whole genome chip revealed that 50 *S. avermitilis* genes were overexpressed at least 2-fold higher in *S. avermitilis* ATCC31780 [13]. Here, this short communication reports the functional expression of some of the putative regulatory genes related to avermectin overproduction identified *via S. avermitilis* microarray systems in *S. avermitilis*, *S. lividans*, and *S. coelicolor*, highlighting

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Table 1. List of putative regulatory genes isolated from *S. avermitilis* ATCC31267.

SAV Gene number in *S. avermitilis*	Proposed function	Homologous SCO gene number in *S. coelicolor*	Amino acid identities to each other
SAV213	RNA polymerase ECF-subfamily sigma factor	NP[a]	NP
SAV424	RNA polymerase ECF-subfamily sigma factor	SCO0159	73%
SAV3818	TetR-family transcriptional regulator	SCO4421	87%
SAV4023	GntR-family transcriptional regulator	SCO4188	75%
SAV6785	RNA polymerase ECF-subfamily sigma factor	SCO1564	76%

[a]NP, homologous gene is not present in *S. coelicolor*.

the biological significance of these putative target genes involved in antibiotic production in *Streptomyces* species.

Two strains of *S. avermitilis*, the wild-type strain ATCC 31267 and the avermectin high-producing mutant strain ATCC31780, were purchased from the American Type Culture Collection. *S. lividans* TK21 and *S. coelicolor* M145 were cultured on R2YE agar plate [15]. *E. coli* DH5α and the *Streptomyces* expression vector pSE34 [15], were used for the cloning and expression experiments according to standard molecular biology procedures. Based on the publicly available genome sequence information of *S. avermitilis* (http://avermitilis.ls.kitasato-u.ac.jp./), each of the five target genes (Table 1) was individually amplified using a polymerase chain reaction (PCR) (BioRad, U.S.A.) with the XbaI-containing forward primer and the HindIII-containing reverse primer. The PCR primer pairs (5'-3') were as follows: SAV213 (tctagaaacgacagggtgatccatga, aagctttcgggcttgttgaggatcca), SAV424 (tctagagggccggtgtgaaggtcg, aagcttcaggtcactggatgccgg), SAV6785 (tctagagttggagtgacagtcggc, aagcttcgccagttgtccctcac), SAV3818 (tctagacgatcacccctcggtaag, aagcttcttcgtgctgcttcgtac), SAV4023 (tctagagacgtctcataaccacct, aagcttctgtgacagcgcctagtt). The PCR conditions used for the high G+C DNA amplification program were as follows: denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 68°C for 2 min [15]. All of the five PCR-amplified target genes include the putative upstream ribosome binding site, start codon, and stop codon sequences. Each of the five PCR-amplified target genes was then cloned into the pGEM T-easy vector (Takara, Japan), which was followed by complete sequence verification. The target genes were then subcloned into the *Streptomyces* expression vector pSE34 [15]. Each of the five plasmids was introduced into *S. avermitilis* ATCC31267 using the polyethylene glycol (PEG)-mediated protoplast transformation method, followed by the thiostrepton (*tsr*) selection method [15]. The level of avermectin production was determined by culturing each

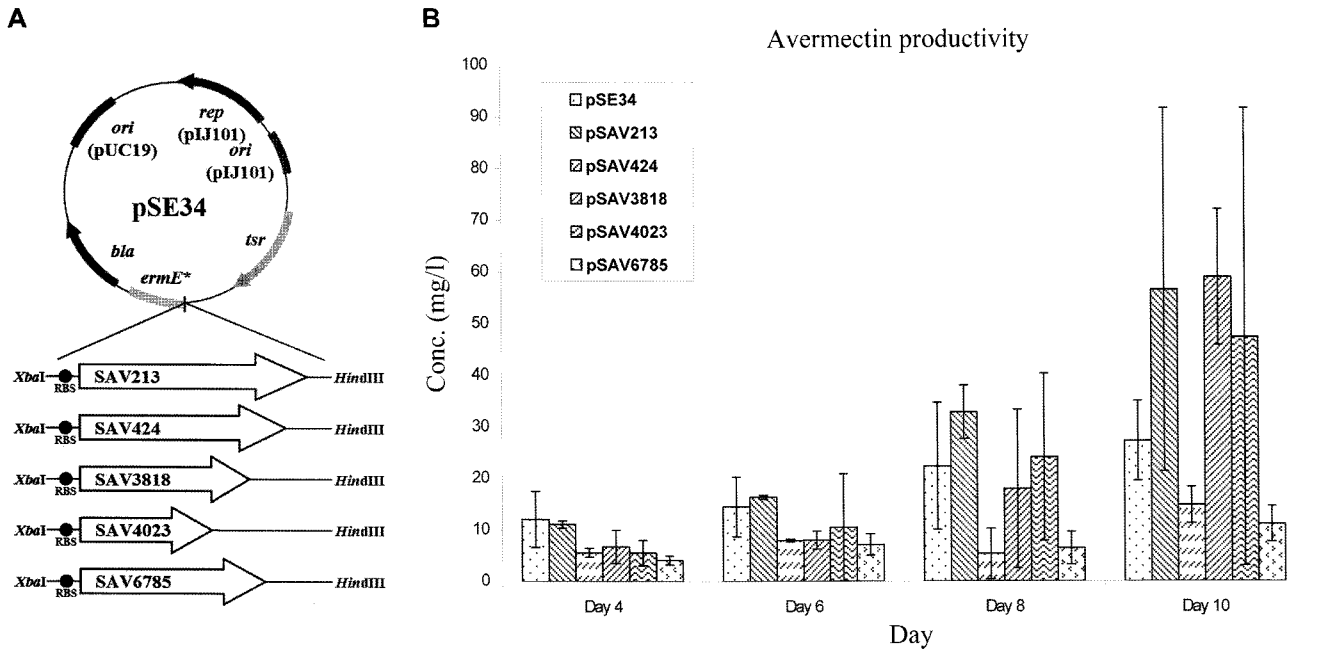

Fig. 1. A. Schematic maps of pSAV213, pSAV424, pSAV3818, pSAV4023, and pSAV6785. **B**. Avermectin compounds concentration (average of triplicates) of the *S. avermitilis* ATCC31267 transformants analyzed by quantitative HPLC assay. *P*-values in t-test with the day 10 samples of SAV213, SAV424, SAV3818, SAV4023, and SAV6785 were 0.12, 0.06, 0.02, 0.48, and 0.03, respectively.

of the *S. avermitilis* transformants containing the plasmids in 50 ml of aNDYE medium containing 50 μg/ml thiostrepton with constant shaking at 200 rpm at 30°C for 10 days, followed by quantitative HPLC analysis [10, 20]. The level of sporulation and actinorhodin production was visualized by culturing each of the *S. lividans* and *S. coelicolor* transformants containing the plasmids on a R2YE plate containing 50 μg/ml thiostrepton at 30°C for 6 days.

In our recent studies, we have shown that the comparative transcriptome analysis between the low-producer *S. avermitilis* ATCC31267 and the high-producer *S. avermitilis* ATCC31780 *via* a *S. avermitilis* whole genome chip revealed that 50 *S. avermitilis* genes were expressed at least 2-fold higher at the transcription level in the high producer [13]. Among the identified genes were putative regulatory genes encoding RNA polymerase ECF-subfamily sigma factors (SAV213, SAV424, and SAV6785), a GntR-family transcriptional regulator (SAV4023), and a TetR-family transcriptional regulator (SAV3818) (Table 1). To further verify the biological significance of these putative regulatory genes presumably involved in avermectin overproduction, each of the five genes was PCR-amplified and cloned into the *Streptomyces* expression vector pSE34 (Fig. 1A). The level of avermectin productivity of each culture was quantitatively measured and compared by HPLC analysis. As shown in Fig. 1B, *S. avermitilis* ATCC31267 transformants containing the SAV424 or SAV6785 gene failed to show any significant increase in avermectin productivity compared with the *S. avermitilis* ATCC31267, implying that the overexpression of these genes is not directly related to avermectin overproduction. Although culture-to-culture variations of the cells containing both SAV213 and SAV4023 were significantly high, the three regulatory genes (SAV3818, SAV213, and SAV4023,) exhibited some stimulatory effects on avermectin production in *S. avermitilis* ATCC31267 (Fig. 1B). These results suggest that the overexpression of these putative regulatory genes, especially SAV3818, may play an important role in the stimulatory network of avermectin production in *S. avermitilis* ATCC31267.

To test whether these avermectin-stimulating genes are also functional in other *Streptomyces* species, the expression vectors containing these three genes (SAV213, SAV3818, and SAV4023) were also transformed into two most commonly used heterologous strains such as *S. coelicolor* M145 and *S. lividans* TK21, followed by the visual observation of each transformant grown on a R2YE plate containing 50 μg/ml thiostrepton at 30°C for 6 days. As shown in Fig. 2, both *S. lividans* and *S. coelicolor* transformants containing the SAV213 and SAV4023 genes showed neither significant phenotypic difference nor blue-pigment actinorhodin production compared with the control strains containing the pSE34 vector plasmid alone, even though overexpression of the SAV213 appeared to cause slight delay of sporulation in both strains. Interestingly, the overexpression of the SAV3818

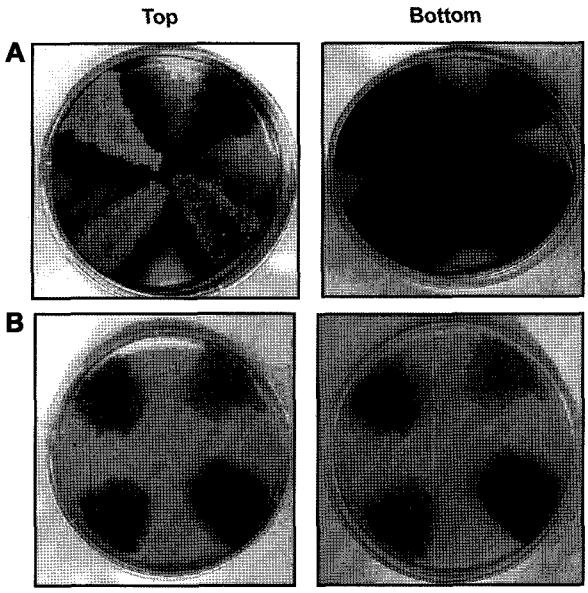

Fig. 2. Overexpression of target genes in *S. coelicolor* M145 (**A**) and *S. lividans* TK21 (**B**).
Top left of each plate, the transformant containing empty vector pSE34; top right, the transformant containing gene SAV213; bottom left, the transformant containing gene SAV4023; bottom right, the transformant containing gene SAV3818.

genes significantly stimulated the blue-pigment actinorhodin both in *S. lividans* and *S. coelicolor* (Fig. 2). Since a highly homologous gene of SAV3818 is also present in the *S. coelicolor* chromosome (SCO4421), it is very likely that the SAV3818 in *S. avermitilis* as well as SCO4421 in *S. coelicolor* could be both upregulators acting as putative TetR-family transcriptional regulators in antibiotic production in *Streptomyces* species. Unlike SAV3818, both SAV213 and SAV4023 might be limited to the *S. avermitilis* ATCC31267 strain as antibiotic stimulating genes, whose detailed mechanism remains to be further elucidated. *In silico* database search of the SAV3818 sequence exhibited that SAV3818 belongs to the members of the TetR/AcrR family in *E. coli*, where a TetR protein regulates an efflux pump involved in tetracycline resistance and a AcrR protein regulates a pump involved in multidrug resistance [2, 8]. Considering that TetR/AcrR family transcriptional regulators have been proved to modulate secondary metabolism *via* the expression of a putative four-component resistance-nodulation-cell-division efflux pump in Gram-negative enterobacterium *Serratia* sp. ATCC 39006 [7], SAV3818 or its homologous SCO4421 might also act as a transcriptional regulator involved in secondary metabolism of *Streptomyces* species.

In conclusion, the comparative transcriptomics followed by the functional expression can be an efficient approach to identify previously unknown avermectin-stimulating target genes identified from *S. avermitilis* ATCC31267. Potentially, further manipulation of this positive regulator such as SAV3818 may result in further improvements in the productivity

of pharmaceuticals produced by industrial *Streptomyces* strains, including those for which complete genome sequence information and knowledge of regulatory mechanisms at the molecular level are not currently available.

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