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Comparative Transcriptome Analysis for Avermectin Overproduction *via* *Streptomyces avermitilis* Microarray System

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Abstract Avermectin and its analogs are major commercial antiparasitic agents in the fields of animal health, agriculture, and human infections. To increase our understanding about the genetic mechanism underlying avermectin overproduction, comparative transcriptomes were analyzed between the low producer *S. avermitilis* ATCC31267 and the high producer *S. avermitilis* ATCC31780 *via* a *S. avermitilis* whole genome chip. The comparative transcriptome analysis revealed that fifty *S. avermitilis* genes were expressed at least two-fold higher in *S. avermitilis* ATCC31780. In particular, all the avermectin biosynthetic genes, including polyketide synthase (PKS) genes and an avermectin pathway-specific regulatory gene, were less expressed in the low producer *S. avermitilis* ATCC31267. The present results imply that avermectin overproduction in *S. avermitilis* ATCC31780 could be attributed to the previously unidentified fifty genes reported here and increased transcription levels of avermectin PKS genes.

Keywords: *Streptomyces*, avermectin, transcriptomics, microarray

Streptomyces is widely known for its ability to produce a variety of commercially valuable enzymes and secondary metabolites [5, 16, 17]. Avermectins, produced by *Streptomyces avermitilis*, are a series of 16-membered pentacyclic macrolactone type I polyketides attached to a disaccharide of methylated deoxysugar L-oleandrose polyketides, exhibiting excellent anthelmintic activity against a variety of nematode and arthropod parasites with a low level of side effects on the host organism [18]. Eight major avermectin compounds produced by *S. avermitilis* have structural differences at C5, C22-C23, and C26 [1, 2, 13, Fig. 1]. The complete biosynthetic mechanism for avermectin has been elucidated through several experiments, including precursor labeling, blocked mutants analyses, intermediates characterization, and *in vitro* studies of avermectin biosynthetic enzymes [8, 10, 14]. The nucleotide sequence of the avermectin biosynthetic gene cluster and the complete genome of *S. avermitilis* have now been analyzed [7, 9]. However, the precise biosynthetic sequence and many details of its biosynthesis have yet to be elucidated. In particular, the genetic factors regulating avermectin biosynthesis remain mostly unknown except for a putative pathway-specific regulatory gene, *aveR* [9]. Like the biosynthesis of other secondary metabolites in *Streptomyces* species, the biosynthesis of avermectin is believed to be tightly regulated at complicated genetic levels, thereby leading to very low avermectin productivity in wild-type *S. avermitilis* ATCC31267 cultures [18]. Although researchers have attempted to improve the productivity of this important anthelmintic veterinary medicine, most strain improvement strategies were largely pursued with little or no consideration of the genetic modifications inherent to the mutant strain in which avermectin overproduction occurs [6, 11, 18]. Recently, 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. Erythromycin-producing *Saccharopolyspora erythraea* and tylosin-producing *S. fradiae* were successfully analyzed *via* the use of sequenced *S. coelicolor* DNA microarray systems, the first such work using industrial actinomycetes [12]. Here, we report the identification of previously unidentified genes related to avermectin overproduction, *via S. avermitilis* microarray systems, as well as further characterization of avermectin PKS gene expressions, *via* reverse transcriptase (RT)-PCR.

The avermectin overproducer *S. avermitilis* ATCC31780 and the low producer *S. avermitilis* ATCC31267, purchased from the American Type Cell Collection, were grown in shake flask cultures in NDYE media [15]. In order to produce avermectins, 10% (v/v) *S. avermitilis* stock solution was inoculated into 25 ml of a culture medium in a 250-ml

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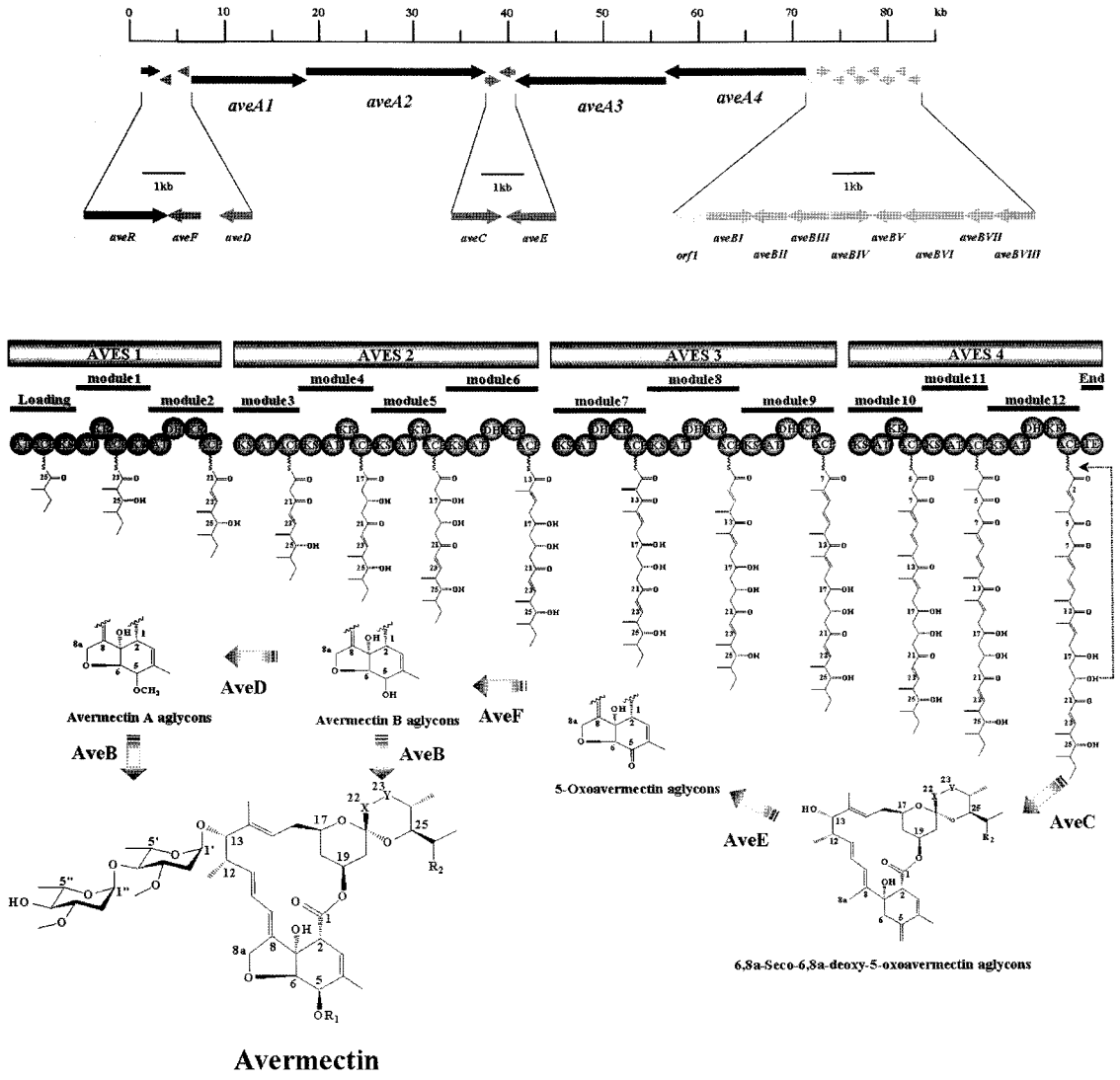

Fig. 1. Avermectin biosynthetic pathway and chemical structures.
Avermectins [R1, R2, X-Y]; A1a [CH_3, C_2H_5, CH=CH]; A1b [CH_3, CH_3, CH=CH]; A2a [CH_3, C_2H_5, CH_2-CH(OH)]; A2b [CH_3, CH_3, CH_2-CH(OH)]; B1a [H, C_2H_5, CH=CH]; B1b [H, CH_3, CH=CH]; B2a [H, C_2H_5, CH_2-CH(OH)]; B2b [H, CH_3, CH_2-CH(OH)].

baffle flask containing small glass beads, followed by constant shaking at 200 rpm at 30°C. Both the supernatant and cell samples were harvested during a 7-day culture period, followed by HPLC analysis for avermectin titers and optical density measurements for cell growth [6, 11]. In order to determine whether differences existed between the low producer and the over producer at the level of the transcriptomes, total RNA was isolated at the stationary stage (132 h after inoculation). It was determined that *S. avermitilis* ATCC31780 produced approximately 1.8-fold higher amounts of avermectins than *S. avermitilis* ATCC31267 (data not shown). A *S. avermitilis* whole genome microarray chip containing 12,000 oligonucleotides was commercially manufactured based on the publicly available complete genome sequence information (CombiMatrix Co., Seattle, U.S.A.). Total RNA was isolated from each *S. avermitilis* strain by using TriZol-reagent (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's instructions, and purified using a RNeasy mini kit (Qiagen, Valencia, CA, U.S.A.). Fluorescently labeled probes for an oligo microarray analysis were prepared using an Amino allyl MessageAmp aRNA kit (Ambion Inc., Texas, U.S.A.). The microarrays were assembled with hybridization caps and rehydrated with RNase-free water at 65°C for 10 min. After rehydration, a blocking solution (6× SSPE, 20 mM EDTA, 0.05% Tween-20, 5× Denhardt's Solution, 0.05% SDS, 100 ng/μl Sonicated Salmon Sperm DNA) was added and the arrays were incubated at the hybridization temperature (45°C) for 30 min. The cRNA mixtures were fragmented in 1× fragmentation solution (40 mM Tris-Acetate, pH 8.1, 100 mM KOAc, 30 mM MgOAc) at 95°C for 20 min. The fragmented cRNA sample was added to a hybridization solution (6×

SSPE, 0.05% Tween-20, 20 mM EDTA, 25% DI Formamide, 0.05% SDS, 100 ng/μl Sonicated Salmon Sperm DNA) and denatured for 3 min at 95°C. The blocking solution was removed from the hybridization chamber and the hybridization solution was applied to the arrays. Hybridization was carried out in a Fisher Scientific Isotemp hybridization incubator for 18 h at 45°C under gentle rotation. Following hybridization, arrays were washed at hybridization temperature for 5 min with 6× SSPE, 0.05% Tween-20 prewarmed to 45°C. Washings continued with 3× SSPE, 0.05% Tween-

Table 1. List of *S. avermitilis* genes with 2-fold increase in the overproducer *S. avermitilis* ATCC31780.

Gene no.	GenBank no.	Proposed function	Ratio (31780/31267)
SAV128	NP_821302	Two-component system response regulator	2.16
SAV213	NP_821387	RNA polymerase ECF-subfamily sigma factor	2.40
SAV424	NP_821599	RNA polymerase ECF-subfamily sigma factor	2.27
SAV573	NP_821748	Hypothetical protein	2.04
SAV594	NP_821769	Gas vesicle synthesis protein	2.09
SAV696	NP_821871	Hypothetical protein	2.26
SAV724	NP_821899	Dehydrogenase	2.17
SAV856	NP_822031	Thioesterase	2.17
SAV1235	NP_822410	Magnesium- or manganese-dependent protein phosphatase	2.16
SAV1405	NP_822580	Amidohydrolase	2.03
SAV1610	NP_822786	Ferredoxin	2.55
SAV1686	NP_822862	Oxidoreductase	2.04
SAV1904	NP_823080	Hypothetical protein	2.02
SAV2090	NP_823266	Xylitol oxidase	2.05
SAV2377	NP_823553	Cytochrome P450	3.63
SAV2441	NP_823617	Hypothetical protein	2.10
SAV2472	NP_823648	ppGpp synthetase/hydrolase	2.14
SAV2486	NP_823662	Glutamate transporter permease	2.00
SAV2895	NP_824071	Modular polyketide synthase	2.11
SAV2899	NP_824075	Modular polyketide synthase	2.17
SAV2929	NP_824105	Hypothetical protein	2.04
SAV2985	NP_824161	Methyltransferase	2.61
SAV3445	NP_824622	Phosphoribosylglycinamide formyltransferase	2.15
SAV3536	NP_824713	Cytochrome P450	2.57
SAV3746	NP_824923	Hypothetical protein	2.34
SAV3793	NP_824970	Hypothetical protein	2.83
SAV3818	NP_824995	TetR-family transcriptional regulator	2.00
SAV3829	NP_825006	Enoyl-CoA hydratase	2.02
SAV4023	NP_825200	GntR-family transcriptional regulator	6.37
SAV4053	NP_825230	ABC transporter ATP-binding protein	2.05
SAV4498	NP_825675	Hypothetical protein	2.30
SAV4507	NP_825684	Peptidase	2.25
SAV4536	NP_825713	Two-component system sensor kinase	2.03
SAV4618	NP_825795	rRNA or tRNA methyltransferase	2.39
SAV4688	NP_825865	L-Aspartate oxidase	2.07
SAV5017	NP_826194	Cholesterol oxidase	2.04
SAV5146	NP_826323	ABC transporter ATP-binding protein	2.37
SAV5215	NP_826392	Hypothetical protein	2.01
SAV5316	NP_826493	D-Ribose ABC transporter permease protein	2.56
SAV5346	NP_826523	Hypothetical protein	3.92
SAV5590	NP_826767	Hypothetical protein	3.29
SAV5782	NP_826959	Aldose 1-epimerase	2.68
SAV5833	NP_827010	Secreted protein	2.46
SAV6183	NP_827359	Hypothetical protein	2.03
SAV6469	NP_827645	Hypothetical protein	2.35
SAV6785	NP_827961	RNA polymerase ECF-subfamily sigma factor	2.25
SAV6892	NP_828068	Hydrolase	2.08
SAV7442	NP_828618	Endo-1,3-beta-glucanase	2.15
SAV7494	NP_828670	Hypothetical protein	2.08

20 for 5 min at room temperature (RT), followed by 0.5× SSPE, 0.05% Tween-20 for 5 min at RT, and 2× PBS, 0.1% Tween-20 for 5 min at RT. Final washing steps were performed at room temperature for 5 min each in 2× PBS, 0.1% Tween-20, followed by two rounds of 2× PBS with no detergent. The microarrays were imaged using a GenePix 4000B Array Scanner (Axon Instruments, Union City, CA, U.S.A.). Imaging was performed while the array was wet with 2× PBS under a LifterSlip glass coverslip (Erie Scientific, Portsmouth, NH, U.S.A.). Scanned images were analyzed with GenePix 6.0 software (Axon Instruments, Union City, CA, U.S.A.) to obtain gene expression ratios.

The comparative microarray data revealed that fifty *S. avermitilis* genes showed at least a 2-fold increase of their transcription levels in *S. avermitilis* ATCC31780 (Table 1). Among the identified genes was the SAV4023 (GenBank No. NP825200) gene encoding a GntR-family transcriptional regulator with an approximately 6.3-fold increase of its transcription in *S. avermitilis* ATCC31780. Recently, the SCO7168, a similar GntR-family transcriptional regulator found in *S. coelicolor*, was proved to be required for both morphogenesis and antibiotic production through controlling an ABC transporter transcription in response to a carbon source [3]. This implies that avermectin overproduction could be attributed to complex interactions of SAV4023, the detailed mechanism of which should be further elucidated. The comparative microarray data also revealed that all the avermectin biosynthetic genes including PKS genes were less expressed in the low producer *S. avermitilis* ATCC31267 than in the overproducer *S. avermitilis* ATCC31780 (Table 2). In particular, the expression of *aveR*, an avermectin pathway-specific regulatory gene, was approximately 80% less expressed in the low producer *S. avermitilis* ATCC31267 (Table 2).

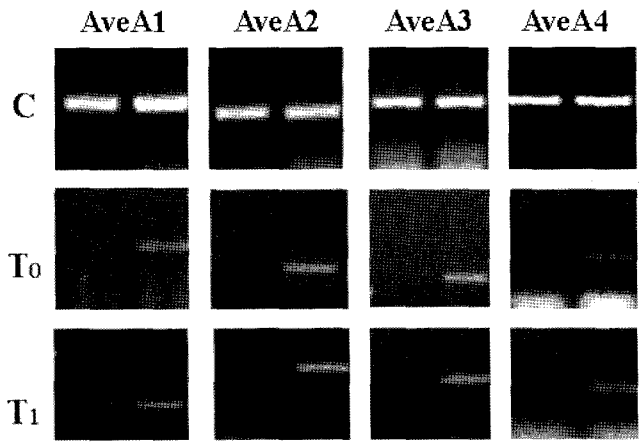

Fig. 2. Gene expression analysis of the avermectin PKS genes by RT-PCR.
First and second lanes of each panel were from *S. avermitilis* ATCC31267 and *S. avermitilis* ATCC31780, respectively. Panel C, PCR with total DNA; panel T0, RT-PCR with total RNA isolated at exponential growth stage (36 h); panel T1, RT-PCR with total RNA isolated at stationary growth stage (132 h); Each primer pair (20 mer) was designed to generate PCR product of approximately 150 to 250 bp. RT-PCR primer sequences (5'-3') were as follows: aveA1 (AAACCGCCTGGGAAACCATC, CGCAGTTCGACACCCATGAC), aveA2 (GCGACTGGTCAAGTCAGCGA, GTACTGCGCGCCTCCTCAGT), aveA3 (CACCTCGTACACCCGCTGTG, GTCCATAGGCCTGCGGAATG), aveA4 (CGAGGACCCTGGTGTTCGAC, ACAATGGCAATCGGCTCGTC).

Since the efficient expression of avermectin PKS genes is believed to be the critical first step for avermectin biosynthesis and these genes serve as putative targets of the AveR regulator, a comparative RT-PCR analysis for four avermectin PKS genes between two *S. avermitilis* strains was performed with *ExTaq* polymerase (TaKaRa, Japan) with 5 ng of total RNA as a template. Conditions for the first strand cDNA synthesis were 50°C for 60 min followed

Table 2. List of avermectin biosynthetic genes and their comparative expression levels.

Gene no.	GenBank no.	Avermectin biosynthetic gene (proposed function)	Ratio (31780/31267)
SAV935	NP_822110	*aveR* (pathway-specific positive regulator)	1.80
SAV936	NP_822111	*aveF* (C5-ketoreductase)	1.05
SAV937	NP_822112	*aveD* (C5-*O*-methyltransferase)	1.21
SAV938	NP_822113	*aveA1* (polyketide synthase)	1.30
SAV939	NP_822114	*aveA2* (polyketide synthase)	1.35
SAV940	NP_822115	*aveC* (CHC-B2:CHC-B1 ratio)	1.65
SAV941	NP_822116	*aveE* (cytochrome P450 hydroxylase)	1.12
SAV942	NP_822117	*aveA3* (polyketide synthase)	1.82
SAV943	NP_822118	*aveA4* (polyketide synthase)	1.06
SAV945	NP_822120	*aveBI* (glycosyl transferase)	1.12
SAV946	NP_822121	*aveBII* (dTDP-glucose 4,6-dehydratase)	1.30
SAV947	NP_822122	*aveBIII* (glucose-1-phosphate thymidyltransferase)	1.28
SAV948	NP_822123	*aveBIV* (dTDP-4-keto-6-deoxy-L-hexose 4-reductase)	1.59
SAV949	NP_822124	*aveBV* (dTDP-4-keto-6-deoxyhexose 3,5-epimerase)	1.21
SAV950	NP_822125	*aveBVI* (dTDP-4-keto-6-deoxy-L-hexose 2,3-dehydratase)	1.68
SAV951	NP_822126	*aveBVII* (dTDP-6-deoxy-L-hexose 3-*O*-methyltransferase)	1.14
SAV952	NP_822127	*aveBVIII* (dTDP-4-keto-6-deoxy-L-hexose 2,3-reductase)	1.28

by 96°C for 2 min, and the amplification conditions were 30 cycles of 96°C for 15 s, 62°C for 30 s, and 72°C for 1 min. As expected, four avermectin PKS bands were considerably less efficiently amplified in the low producer *S. avermitilis* ATCC31267 (Fig. 2), suggesting that high expression of *aveR* is necessary to stimulate the avermectin PKS genes. In conclusion, a comparative transcriptome analysis using a *S. avermitilis* whole-genome microarray and comparative RT-PCR for avermectin PKS gene expression revealed that avermectin overproduction in *S. avermitilis* ATCC31780 could be attributed to the previously unidentified fifty genes reported here and efficient transcription levels of avermectin PKS genes.

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