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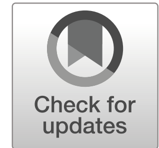
APPLIED GENETICS AND MOLECULAR BIOTECHNOLOGY



AveI, an AtrA homolog of *Streptomyces avermitilis*, controls avermectin and oligomycin production, melanogenesis, and morphological differentiation

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Abstract
Streptomyces avermitilis is well known as the producer of anthelmintic agent avermectins, which are widely used in agriculture, veterinary medicine, and human medicine. *aveI* encodes a TetR-family regulator, which is the homolog of AtrA. It was reported that deletion of *aveI* caused enhanced avermectin production. In this study, we investigated the regulatory function of the AveI in *S. avermitilis*. By binding to the 15-nt palindromic sequence in the promoter regions, AveI directly regulates at least 35 genes. AveI represses avermectin production by directly regulating the transcription of the cluster-situated regulator gene *aveR* and structural genes *aveA1*, *aveA3*, and *aveD*. AveI represses oligomycin production by repressing the CSR gene *olmRII* and structural genes *olmC*. AveI activates melanin biosynthesis by activating the expression of *melC1C2* operon. AveI activates morphological differentiation by activating the expression of *ssgR* and *ssgD* genes, repressing the expression of *wblI* gene. Besides, AveI regulates many genes involved in primary metabolism, including substrates transport, the metabolism of amino acids, lipids, and carbohydrates. Therefore, AveI functions as a global regulator in *S. avermitilis*, controls not only secondary metabolism and morphological differentiation, but also primary metabolism.

Keywords AveI · *Streptomyces avermitilis* · Avermectin production · Secondary metabolism · Morphological differentiation

Introduction

The genus *Streptomyces* is the known producer of more than 70% antibiotics used in medicine, veterinary practice, and agriculture. Genome sequencing has revealed that some *Streptomyces* strains harbor tens of putative gene clusters responsible for secondary metabolites production (Choudoir et al. 2018; Craney et al. 2013). Individual antibiotic is usually biosynthesized by a large gene cluster, including cluster-situated regulator (CSR) encoding gene or genes. Various pleiotropic regulators sense developmental state, nutrient availability, diverse stresses, and then transmit the signals to the CSR genes to regulate antibiotic biosynthesis (Liu et al. 2013a; van Wezel and McDowall 2011). Elucidation of the antibiotic regulatory networks will provide strategies for increasing antibiotic productivity and "awakening" cryptic antibiotic production.

S. avermitilis is well known as the producer of 16-membered macrolide avermectins. Avermectins and its derivative, ivermectin, have potent anthelmintic activities and are widely used in human and veterinary medicine, and agriculture (Ikeda and Omura 1997). In addition to avermectins, *S. avermitilis* also produces 26-membered macrolide oligomycin and polyene macrolide filipin (Ikeda et al. 2014; Xu et al. 2010). *aveR* is the only CSR gene of avermectin biosynthetic gene cluster, activating transcription of *ave* structural genes (Guo et al. 2010; Kitani et al. 2009). Similar to other CSR genes, the expression of *aveR* is also under complex regulatory networks. Several regulators have been shown to directly regulate the expression of *aveR*. The response regulator PhoP of two-component PhoR–PhoP system, which

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controls phosphate metabolism, represses *aveR* transcription by binding to a PHO box located downstream of *aveR* transcriptional start site (Yang et al. 2015). A pseudo γ-butyrolactone receptor homolog AvaR2 (Zhu et al. 2016) and a butenolide-type autoregulator receptor homolog AvaR1 (Zhu et al. 2017) both repress avermectin production by binding to the promoter region of *aveR*. GlnR, the master regulator of nitrogen metabolism, directly stimulates avermectin production through *aveR* (He et al. 2016). Redox-sensing regulator Rex directly represses avermectin production by binding to the ROP in the *aveR* promoter region (Liu et al. 2017). SAV742, an AraC-family regulator, inhibits avermectin production through direct control of *ave* structural genes (*aveA1-aveD*, *aveA4-orf1*, and *aveF*), rather than the CSR gene (Sun et al. 2016). Some regulators have been demonstrated to control avermectin production indirectly, including SAV3818 (Duong et al. 2009), SAV7471 (Liu et al. 2013b), SAV576 and SAV577 (Guo et al. 2013, 2014), and AveT (Liu et al. 2015).

AveI (SAV4110) is a homolog of AtrA (actinorhodin-associated transcriptional regulator) from *S. coelicolor* A3(2) (Chen et al. 2008; Uguru et al. 2005). Deletion of *aveI* in *S. avermitilis* led to increased avermectin B1a production, which could be complemented by either *aveI* or its homologous gene *atrA-c* from *S. coelicolor* (Chen et al. 2008). Comparative transcriptomic analyses between *aveI* deletion mutant and WT showed that AveI affected expression of avermectin, oligomycin, and filipin biosynthetic gene clusters. The genes involved in precursor biosynthesis for antibiotics were upregulated, while genes involved in protein synthesis and fatty acid metabolism were downregulated in *aveI* mutant (Chen et al. 2009). These results suggested that AveI may function as a global regulator controlling not only secondary metabolism, but also primary metabolism. However, so far, the direct gene targets of AveI remain to be identified.

The TetR-family regulator AtrA is highly conserved in Streptomycetes. AtrA was first characterized as a transcription activator in *S. coelicolor* A3(2), stimulating the transcription of *act*II-ORF4, the pathway-specific activator gene of actinorhodin biosynthetic gene cluster (Uguru et al. 2005). AtrA-c also directly activates the expression of *nagE2* and *ssgR*, which encode the major permease for N-acetylglucosamine and the activator for cell division-related gene *ssgA* (Kim et al. 2015; Nothaft et al. 2010). AtrA-gr of *S. griseus* binds to an inverted repeat between two AdpA-binding sites upstream promoter of *strR*, the transcriptional activator gene for streptomycin production. AtrA-gr only has a conditionally positive effect on streptomycin biosynthesis, probably through stimulating the AdpA-dependent transcriptional activation of *strR* (Hirano et al. 2008). AtrA-p in *S. pristinaespiralis* positively regulates pristinamycin production by directly stimulating the transcription of two CSR genes of pristinamycin gene cluster, *spbR*, and *papR5* (Wang et al. 2015). AtrA-r in *S. roseosporus* positively controls daptomycin production by binding directly to the promoter region of *dptE* (Mao et al. 2015). *S. globisporus* AtrA-gl stimulates lidamycin production by binding to the promoter regions of lidamycin CSR genes, *sgcR1* and *sgcR2*. The DNA-binding activity is inhibited by interaction with heptaene, a biosynthetic intermediate of lidamycin (Li et al. 2015).

Though AtrA has a common role in regulating antibiotic production in Streptomycetes, the mechanism how AveI (AtrA homolog) modulates avermectin production is still unknown. Besides, unlike the positive control of antibiotic production by AtrA in most cases, which is quite unusual for a TetR-family regulator (usually functioning as a repressor), AveI has a negative regulatory role in avermectin production. In this study, we provide evidences that AveI serves as a global regulator in *S. avermitilis*. AveI negatively regulates avermectin and oligomycin production by directly binding to the promoter regions of CSR and structural genes, positively regulates melanogenesis and morphological differentiation, and controls a variety of genes involved in primary metabolism.

Materials and methods

Strains and growth conditions

The *S. avermitilis* strains used in the present study included ATCC31267 (wild-type strain), DaveI (an *aveI* deletion mutant of WT), CaveI (a complementation strain of *aveI* deletion by *aveI*), and CAU69 (an avermectin high-producer). *S. avermitilis* strains were grown at 28 °C on YMS agar for sporulation, in modified liquid YEME medium (25% sucrose) for growth of mycelia for protoplast preparation, and on RM14 medium for regeneration of protoplasts (Macneil and Klapko 1987). Seed medium and fermentation medium I were used for avermectin production and fermentation medium II was used for growth analysis as described previously (Jiang et al. 2011). *E. coli* JM109 and Rosetta (DE3) were used for plasmid construction and protein expression, respectively. *E. coli* strains were cultured at 37 °C in LB medium.

Gene deletion and complementation

To produce an *aveI* null mutant, two DNA fragments flanking *aveI* gene were amplified by PCR using ATCC31267 genomic DNA as template. A 521-bp fragment upstream (position − 480 to + 41 from start codon) and a 563-bp fragment downstream (position − 42 to + 521 from stop codon) of *aveI* were amplified with primers aveI-up-1/aveI-up-2 and aveI-dw-1/aveI-dw-2, respectively (Table S1). The amplified fragments were recovered, digested by *Eco*RI/*Xba*I and *Xba*I/*Hin*dIII, respectively, and were cloned into pKC1139 to generate the

aveI deletion vector pKCDaveI. The resulting plasmid was introduced into *S. avermitilis* ATCC31267 protoplasts. Double-crossover mutants were selected as described previously (Yang et al. 2015). The mutants were confirmed by PCR using primers listed in Table S1. The gene-deleted strain was termed as DaveI. To complement DaveI, a DNA fragment (1606 bp) carrying *aveI* gene with its promoter was amplified by PCR using primers aveI-C-1 and aveI-C-2 (Table S1). The fragment was recovered, digested by *Eco*RI/*Bam*HI, and then ligated to the integrative vector pSET152 to produce vector pSET-aveI, which was introduced into DaveI to obtain complemented strain.

Fermentation and HPLC analysis of avermectin and oligomycin production

Fermentation conditions and HPLC analysis of avermectin and oligomycin production were performed as described previously (Luo et al. 2014).

RNA extraction and qRT-PCR analysis

Mycelia of *S. avermitilis* from fermentation medium I or YMS agar were collected, frozen in liquid nitrogen, and ground to a fine powder. RNA was extracted using TRIzol reagent (Tiangen, Beijing, China) following the manufacturer's instructions, and was treated with DNase I (TaKaRa, Shiga, Japan) to remove chromosomal DNA contamination. RNA samples (2 μg each) were reverse transcribed by M-MLV (RNase H^-; TaKaRa), and qRT-PCR analysis was performed using FastStart Universal SYBR Green Master (ROX) by an ABI 7900HT Sequence Detection System with primer pairs listed in Table S1. PCR included a 10 min preincubation at 95 °C, followed by 40 cycles of denaturation at 95 °C for 10 s, and annealing and extension at 60 °C for 30 s. 16S *rRNA* was used as the internal control. The relative expression level was calculated using the comparative Ct method. Gene expression was determined in triplicate.

Overexpression and purification of His_6-AveI

The 859 bp coding region of *aveI* was amplified using primers aveI-E-1 and aveI-E-2, digested by *Eco*RI/*Bam*HI, and then cloned into pET28a (+) to produce pET-aveI. After confirmation by DNA sequencing, pET-aveI was introduced into *E. coli* Rosetta (DE3) for overexpression of AveI with a His_6 tag at N terminus. *E. coli* Rosetta (DE3) harboring pET-aveI was grown at 37 °C in LB with 50 μg ml^{-1} kanamycin to an OD_{600} of 0.4–0.6, induced by 0.1 mM IPTG, and incubated for 5 h at 28 °C. The cells were harvested, washed, resuspended in lysis buffer (20 mM Tris base, 500 mM NaCl, 5 mM imidazole, 5% glycerol, pH 7.9), and disrupted by sonication on ice. After centrifugation, His_6-AveI was purified from the supernatant using Ni^{2+}-NTA resin (Qiagen, Germany) according to the manufacturer's protocol.

Electrophoretic mobility gel shift assays (EMSAs)

EMSAs were carried out using a DIG Gel Shift Kit (2nd Generation, Roche) according to the manufacturer's instructions. DNA probes used for EMSA were amplified by PCR with the primers listed in Table S1 and labeled with Digoxigenin-11-ddUTP using recombinant terminal transferase. The binding mixture (20 μl) contained 0.3 nM DIG-labeled DNA probe, varying quantities of His_6-AveI, and 1 μg of poly[d(I–C)]. After incubation at 25 °C for 30 min, protein-bound and free probes were separated by electrophoresis on 5.0% native polyacrylamide gels with 0.5 × TBE buffer as running buffer. Then, DNA probes were electroblotted onto a positively charged nylon membrane, and the signals were detected by chemiluminescence and recorded on X-ray film.

DNase I footprinting assays

For DNase I footprinting assays, DNA fragments were amplified by PCR with FAM-labeled primers (Table S1) and purified from the agarose gel. 400 ng FAM-labeled probes and varying quantities of His_6-AveI were incubated at 25 °C for 30 min in a 25 μl volume. 0.017 U DNase I was added to the mixture. After incubation at 37 °C for 40 s, the digestion was stopped with 10 μl of 0.2 M EDTA (pH 8.0). DNA samples were extracted and subjected to capillary electrophoresis. Electrophoregrams were analyzed using GeneMarker software v2.2.0.

Results

AveI represses avermectin and oligomycin production, and stimulates melanogenesis and morphological differentiation

Chen et al. (2008) have reported that deletion of *aveI* enhanced avermectin production in *S. avermitilis*. To fully assess the function of AveI in secondary metabolism, morphological differentiation, and primary metabolism, we constructed the *aveI* deletion mutant (DaveI) in *S. avermitilis* wild-type strain by homologous recombination. Deletion of *aveI* did not affect growth in fermentation media (Fig. S1). Compared to the WT strain, avermectin and oligomycin productions of DaveI were ~ 3.1-fold and 1.47-fold of those in WT, and the productions were restored to the WT levels when an *aveI* gene was introduced into DaveI (Fig. 1a). When the strains were grown on RM14 medium, which favor melanin production, DaveI grew slower and produced less melanin than WT, while melanin

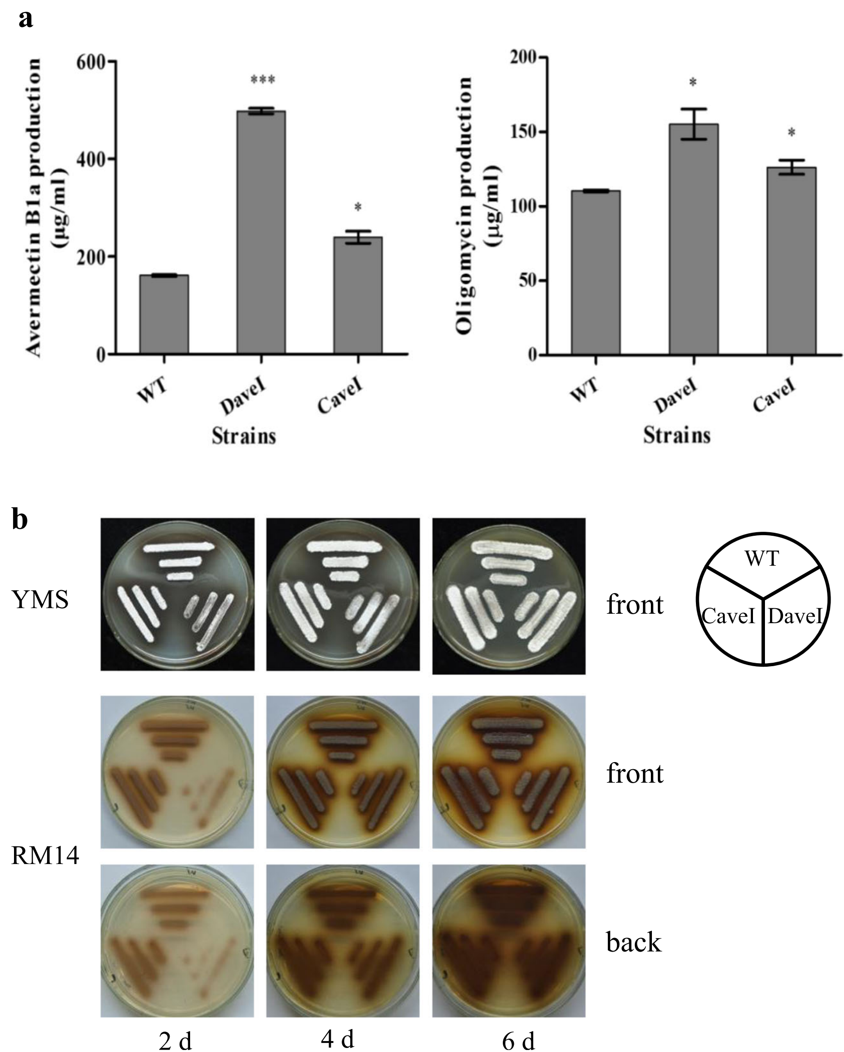

Fig. 1 Effects of *aveI* deletion on avermectin, oligomycin, and melanin production, and morphology. **a** Avermectin and oligomycin production in WT, DaveI, and CaveI. Values are mean ± SD of three replicate flasks cultured in FM-I. *P* values were determined by Student's *t* test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. **b** Phenotype of *aveI* deletion mutant. The indicated strains were grown on YMS and RM14 media for 2, 4, or 6 days. DaveI, *aveI* deletion mutant. CaveI, complemented strain of DaveI

production was restored to WT levels in the complemented strain of DaveI (Fig. 1b). When grown on YMS media, DaveI mutant displayed delays of aerial hyphae formation and sporulation in comparison with WT and complemented strain (Fig. 1b). These findings indicated that AveI had a negative regulatory role in both avermectin and oligomycin production, but a positive regulatory role in melanogenesis and morphological differentiation.

AveI regulates avermectin production by directly repressing *aveR* and *ave* structural genes

Avermectin was overproduced by deletion of *aveI* gene. To test whether AveI affects avermectin production through regulating *ave* genes, we carried out qRT-PCR analysis using RNAs isolated from DaveI and WT cultured in FM-1 for 2 days (exponential phase) and 6 days (stationary phase). The transcriptional levels of avermectin biosynthetic CSR gene *aveR* and structural genes *aveA1*, *aveA3*, *aveA4*, *aveD*, and *aveF* were increased in DaveI at both time points, particularly on 6 day (Fig. 2a). The augmented expression of *ave* genes is consistent with the avermectin overproduction in DaveI. The findings suggest that AveI represses the expression of *ave* genes. As TetR-family regulators are usually autoregulated, it was tested whether AveI regulates its own expression, we also examined the expression of *aveI* using the same RNAs. The transcriptional level of *aveI* was increased in DaveI at both 2 days and 6 days (Fig. 2a), indicating that *aveI* is negatively autoregulated.

An N-terminal His_6-tagged AveI protein was overexpressed in *E. coli* Rosetta (DE3) and purified for EMSAs (Fig. S2). The promoter regions of *aveI*, *act*II-ORF4, *aveR*, *aveA3*, *aveA4*, and *aveF* and the intergenic region of *aveA1-aveD* were DIG labeled for EMSAs. His_6-AveI bound to the promoter region of *aveI*, and the shifted bands could be abolished by addition of 500-fold excess of unlabeled specific competitor DNA but not by unlabeled nonspecific DNA, indicating that the binding was specific (Fig. 2b). AveI is negatively autoregulated by binding to its promoter region. His_6-AveI could bind specifically to the promoter

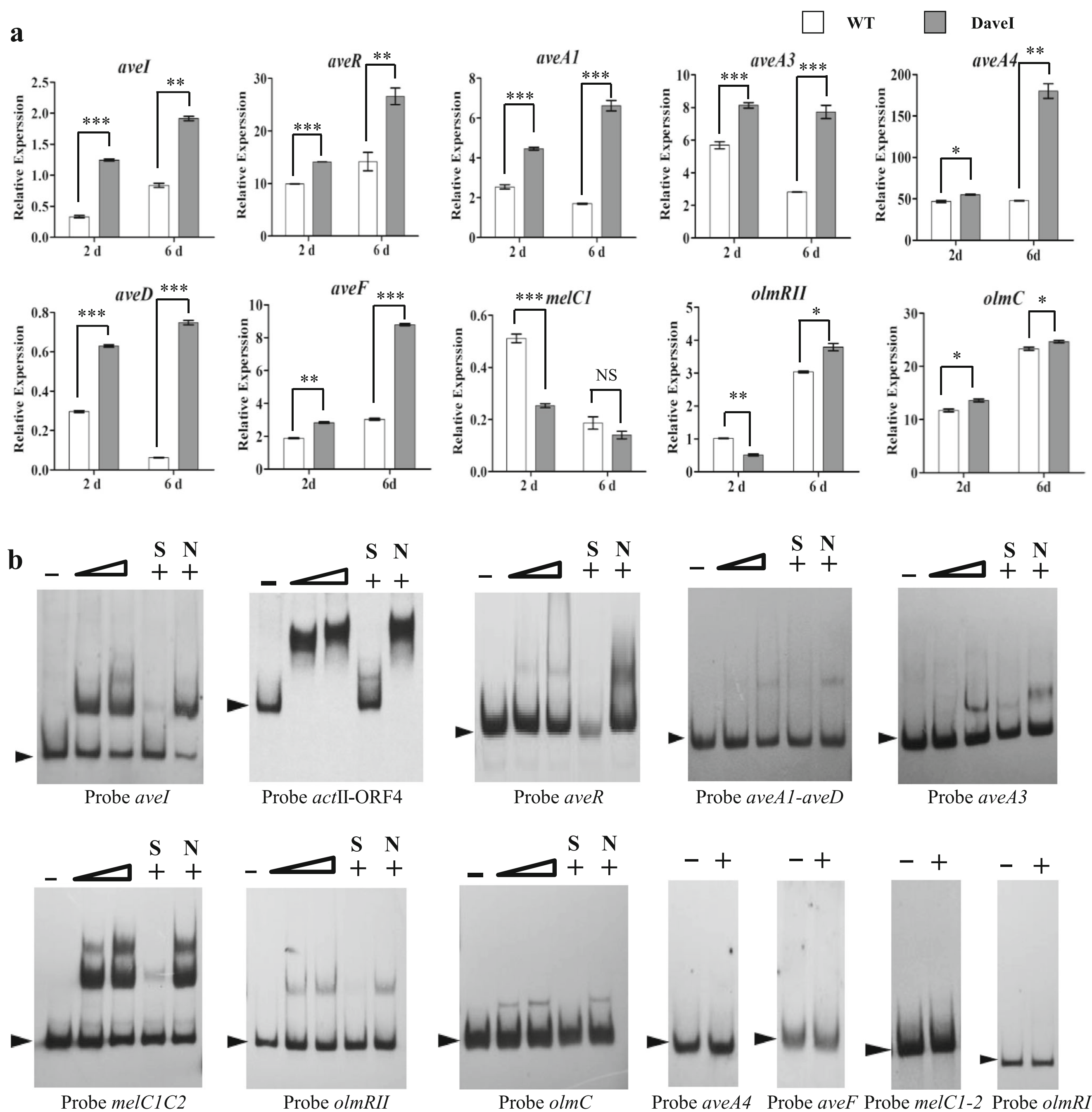

Fig. 2 AveI represses avermectin and oligomycin production, and activates melanogenesis. **a** qRT-PCR analysis of *ave*, *olm*, and *melC* genes in DaveI and WT. RNAs were prepared from cells cultured in FM-I for 2 and 6 days. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS, not significant. **b** Binding of His_6-AveI to the promoter regions of the above genes by EMSA. Concentrations of His_6-AveI for probes: 0.15 and 0.3 μM. Competition assays were carried out using 500-fold excess of unlabeled specific (S) and non-specific (N) DNAs. Arrow: free probe

region of *act*II-ORF4 in *S. coelicolor*, implying that AveI and AtrA have similar binding site. His_6-AveI bound specifically to the promoter regions of *aveR* and *aveA3*, and the intergenic region of *aveA1-aveD*, but not to the promoter regions of other *ave* genes (Fig. 2b). Therefore, AveI represses avermectin production by directly regulating the transcription of the CSR gene *aveR* and structural genes *aveA1*, *aveA3*, and *aveD*. The improved expression of *aveA4* and *aveF* was probably caused by the enhanced expression of *aveR*.

AveI directly represses *olm* genes and activates expression of *melC1C2* gene

EMSAs were also used to determine whether AveI directly regulates oligomycin biosynthesis and melanogenesis. His_6-

AveI bound specifically to the promoter regions of oligomycin biosynthetic CSR gene *olmRII* and structural gene *olmC* (Fig. 2b), but not to the promoter regions of CSR gene *olmRI* and other structural genes. The transcriptional levels of *olmRII* and *olmC* were increased in DaveI, in accordance with the increased oligomycin production in DaveI, indicating the negative role of AveI in regulating oligomycin production (Fig. 2a). *S. avermitilis* contains two melanin biosynthetic operons: *melC1C2* and *melC1-2C2-2*. His_6-AveI bound specifically to the promoter region of *melC1C2* operon, but not to that of *melC1-2C2-2* (Fig. 2b). The expression of *melC1* was greatly reduced in DaveI (Fig. 2a), which was consistent with the decreased melanin production in DaveI. Thus, AveI positively controls melanogenesis through directly activating the expression of *melC1C2*.

AveI directly activates the expression of *ssgR* and *ssgD* genes and represses *wblI* gene

DaveI displayed delayed morphogenesis compared with WT. To identify the direct targets of AveI involved in morphological differentiation, we performed EMSAs using His_6-AveI and the promoter regions of *bldC*, *ftsZ*, *ssgD*, *ssgR*, *ssgY*, *wblB*, and *wblI*. His_6-AveI bound specifically to the promoter regions of *ssgD*, *ssgR*, and *wblI*, but not to the other promoters (Fig. 3a). qRT-PCR analysis showed that the transcriptional levels of *ssgD* and *ssgR* were decreased, while the transcriptional level of *wblI* was increased in DaveI (Fig. 3b), indicating that AveI affects morphological differentiation through directly activating *ssgR* and *ssgD* genes and repressing *wblI* gene. The positive control of *ssgR* by AveI is same as the findings that AtrA activates the transcription of *ssgR*, which in turn activates *ssgA* transcription in *S. coelicolor* (Kim et al. 2015).

Prediction and verification of the AveI regulon

To fully understand the regulatory role of AveI in *S. avermitilis*, we used the palindromic consensus sequence (5′-GGAAT-n5-ATTCC-3′) of ArtA (Wang et al. 2015) to scan the *S. avermitilis* genome, through the PREDetector web-based application (http://predetector.hedera22.com/login) to search for putative AveI target genes. The genome-wide search revealed 440 putative AveI target genes with score > 6.5 (Table S2). About half of the target genes (203) are unknown or unclassified genes, the others are involved in regulatory function (58), transport (44), amino acids and related molecules metabolism (28), carbohydrate metabolism (17), protein synthesis, folding, and modification (13), DNA synthesis, repair, recombination, modification, and packaging (9), secondary metabolism (9), fatty acid and lipid metabolism (7), differentiation (4), and other metabolisms. Nine of them (*aveA1*, *aveD*, *aveI*, *melC1*, *olmC*, *olmRII*, *ssgD*, *ssgR*, and *wblI*) have been confirmed to be directly regulated by AveI.

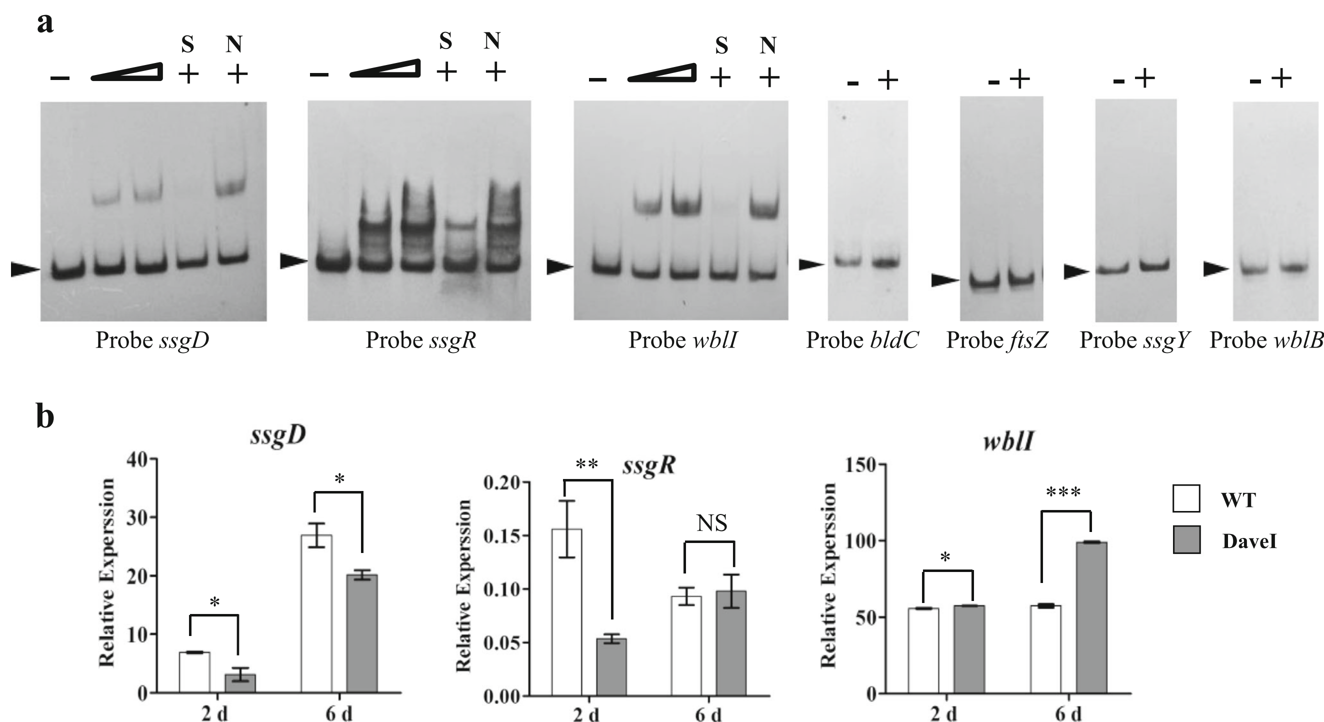

Fig. 3 The regulatory role of AveI in morphology. **a** Binding of His_6-AveI to the promoter regions of genes involved in morphology by EMSA. EMSA conditions as described for Fig. 2. **b** qRT-PCR analysis of the related genes in DaveI and WT. RNAs were prepared from cells grown on YMS for 2 and 6 days. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS, not significant

Table 1 Putative AveI target genes tested by EMSA

#	Accession number	Gene	Function	Sequence	ATG distance	Score	EMSA
Regulatory function							
1	*SAV577*		TetR-family transcriptional regulator	GGAACGAACAATTCC	− 361	11.6	+
2	*SAV2270*	*avaL1*	Gamma-butyrolactone-dependent transcriptional regulator	GGAAGGTTGGATTCC	− 46	10.4	+
3	*SAV2901*	*olmRII*	LuxR-family transcriptional regulator of *olm* gene cluster	AGAATCTGGGATTGC	− 90	7	+
4	*SAV3216*	*wblI*	Putative WhiB-family transcriptional regulator; putative role in cell cycle control	GGAACCGCACCCTCC	− 131	6.6	+
5	*SAV4042*	*glnR*	Putative DNA-binding response regulator	GGAACCAGACGTTAT	− 105	7.3	−
6	*SAV4110*	*aveI*	Putative TetR-family transcriptional regulator	GGGAGACGTCATTAC	− 28	6.6	+
7	*SAV4268*	*ssgR*	Putative IclR-family transcriptional regulator	GGAACCTGGCGTTCC	− 326	9.3	+
8	*SAV4997*	*wblB*	Putative WhiB-family transcriptional regulator; putative role in cell cycle control	GAAATCTGCCATTAC	− 3	7.3	−
Secondary metabolism							
9	*SAV936*	*aveF*	C-5 ketoreductase	GTGATGAGCGATTCC	− 58	9.6	−
10	*SAV937*	*aveD*	C5-O-methyltransferase	GGAACGGCACAGCCC	− 148	6.6	+
11	*SAV938*	*aveA1*	Type I polyketide synthase AVES 1	GGAACGGCACAGCCC	− 203	6.6	+
12	*SAV1136*	*melC1*	Tyrosinase co-factor protein	GGAACTTCCCCCTCC	− 185	6.4	+
13	*SAV2269*	*avaA*	Putative gamma-butyrolactone biosynthesis protein	GGAAGGTTGGATTCC	− 160	10.4	+
14	*SAV2376*	*pks9-4*	Putative 3-oxoacyl-ACP synthase I	GGAATCGTCGATGCC	− 95	8.4	+
15	*SAV2903*	*olmC*	Thioesterase	CGAATTAGGGGTTCC	− 219	7.7	+
16	*SAV5361*	*melC1-2*	Putative tyrosinase co-factor protein	AGAATCCCTCATTCG	− 327	7.5	−
17	*SAV6633*	*nrps5*	Putative non-ribosomal peptide synthetase	GGAGGCTCTGATTCC	− 176	8.8	+
	SAV6633	*nrps5*	Putative non-ribosomal peptide synthetase	GGGATGTTCCGGTCC	− 77	6.5	+
Metabolism of amino acids and related molecules							
18	*SAV2710*	*leuA1*	Putative 2-isopropylmalate synthase	GGAATGACCGGTTCC	− 50	11.8	+
19	*SAV2717*	*ilvE*	Putative branched-chain amino acid amino-transferase	GCATTCCCTCATTCC	− 95	6.9	−
20	*SAV3115*	*pepD1*	Putative serine protease	AGAATCGTCGATTCC	− 376	11.1	+
21	*SAV4376*	*bkdF*	Branched-chain alpha keto acid dehydrogenase E1 alpha subunit	GGAGTGGACTCTTCC	− 379	7.2	−
22	*SAV4858*	*fahA*	Putative fumarylacetoacetase	GGAGGGTTCAATTCC	− 118	7.8	+
23	*SAV4963*	*glmS1*	Putative L-glutamine-D-fructose-6-phosphate amidotransferase	AGAATTTCCGATTTC	− 65	7.3	−
24	*SAV5600*		Putative peptidase M4	GGAACCCCGGAGTCC	− 86	8.1	+
25	*SAV5601*	*leuA2*	Putative 2-isopropylmalate synthase	GGAAAGTGGCATTGC	− 303	8.1	+
26	*SAV6025*	*pepA*	Putative leucyl aminopeptidase (cytosol aminopeptidase)	GGAATGGCTGATTCA	− 324	9.6	+
	SAV6025	*pepA*	Putative leucyl aminopeptidase (cytosol aminopeptidase)	GGTTTCGCTCATTCC	− 191	6.7	+
27	*SAV6046*		Putative aminotransferase	GGAGTCGGGCATTCC	− 156	9.2	+
28	*SAV6399*	*aspC2*	Putative aspartate aminotransferase	GGAACCCACAGTTCC	− 202	9.3	+
29	*SAV6634*		Putative peptidase	GGAGGCTCTGATTCC	− 375	8.8	+
Carbohydrate metabolism							
30	*SAV803*	*pgmA*	Putative phosphoglucomutase	GGAAGGTCGGCATCC	− 246	6.6	−
31	*SAV1662*	*galE6*	Putative UDP-glucose 4-epimerase	GGAACCTGGCATTCC	− 65	10.7	+
32	*SAV1671*		Putative oxidoreductase	GGAACAGGTGGTCCC	− 284	7.7	+
33	*SAV3287*	*pckA*	Putative phosphoenolpyruvate carboxykinase	GTAAGTTACGGTTCC	− 338	7.9	−
34	*SAV3533*	*eno*	Putative phosphopyruvate hydratase	GGAACAAAAGAGCCC	− 170	6.7	−
35	*SAV3566*	*ppc*	Putative phosphoenolpyruvate carboxylase	GTAATCGGCCATCCC	− 268	8.9	+
36	*SAV3979*	*gpmA1*	Putative phosphoglycerate mutase	GTGATCACACGTTAC	− 142	6.6	−

Table 1 (continued)

#	Accession number	Gene	Function	Sequence	ATG distance	Score	EMSA
37	*SAV5126*	*maeB2*	Putative NAD-dependent malic enzyme	GTAACAAACGATTCC	− 296	9.6	−
38	*SAV5800*	*aceE1*	Putative pyruvate dehydrogenase E1 component	GTAATCGAGACGTCC	− 120	6.5	−
39	*SAV6627*	*idnO*	Putative gluconate 5-dehydrogenase	CGGATGGCTCATTCC	− 3	7.3	−
Metabolism of lipids and fatty acid							
40	*SAV1665*	*echA6*	Putative enoyl-CoA hydratase/isomerase	GAAATCAGCGATTAT	− 177	6.6	−
41	*SAV1681*	*fadA4*	Putative acetyl-CoA acetyltransferase	GGGAGGGAGAGTTCC	− 150	6.8	−
42	*SAV1682*	*mcr*	Putative fatty acid-CoA racemase	GGAGTGGGACATTCC	− 145	9.5	+
43	*SAV3567*	*fadS3*	Putative fatty acid desaturase	GTAATCGGCCATCCC	− 45	8.9	+
Energy metabolism							
44	*SAV6047*	*ctaC*	Putative cytochrome c oxidase subunit II (complex IV)	GGAGTCGGGCATTCC	− 187	9.2	+
Transport and binding proteins							
45	*SAV4072*	*pstS*	Phosphate ABC transporter substrate-binding protein	GGACGCAACCGTTCC	− 213	7.8	−
46	*SAV6224*	*livK3*	Putative branched-chain amino acid ABC transporter substrate-binding protein	AGAACGCCCTGTTCC	− 269	7	+
47	*SAV6400*	*gltI2*	Putative ABC transporter substrate-binding protein	GGAACCCACAGTTCC	− 32	9.3	+
48	*SAV6419*	*pitH2*	Putative low-affinity inorganic phosphate transporter	GGAATTCCGGCCTCC	− 168	7.1	+
49	*SAV7416*	*rhaT*	Putative ribose import ATP-binding protein RbsA 2	GGAATGAGTCATTTC	− 166	10	−
Cell division and differentiation							
50	*SAV570*	*ssgY*	Putative SsgA homolog	GGGCTCGGTGATTCC	− 259	9	−
51	*SAV1687*	*ssgD*	Putative cell division protein	GGATTGGCTCATTCC	− 82	10.9	+
Protein synthesis, folding, and modification							
52	*SAV4991*	*groES1*	Putative GroES	GGAACTTCCCGCTCC	− 263	6.7	+
53	*SAV7237*	*dnaK2*	Chaperone protein dnaK2	GGACCCGTGCATTCC	− 175	8.4	−

To test whether AveI binds to the promoter regions of other putative target genes, we selected 42 genes with predicted gene function associated with regulation function, secondary metabolism, carbon metabolism, energy metabolism, and protein folding for EMSAs (Table 1).

EMSA results showed that His_6-AveI bound specifically to the promoter regions of 25 putative AveI target genes (*aspC2*, *avaA*, *avaL1*, *ctaC*, *fadS3*, *fahA*, *galE6*, *gltI2 groES1*, *ilvk3*, *leuA1*, *leuA2*, *maeB1*, *mcr*, *nrpS5*, *pepA*, *pepD1*, *pitH2*, *pks9-4*, *ppc*, *SAV577*, *SAV1671*, *SAV5600*, *SAV6046*, and *SAV6634*) (Fig. 4), but not to the promoter regions of *aceE1*, *bkdF*, *cdh*, *dnaK2*, *echA6*, *eno*, *fadA4*, *glmS1*, *glnR*, *gpmA1*, *idnO*, *ilvE*, *maeB2*, *pckA*, *pstS*, and *rhaT*. qRT-PCR analysis demonstrated that the transcriptional levels of 14 genes (*avaA*, *avaL1*, *ctaC*, *fadS3*, *galE6*, *groES1*, *maeB1*, *mcr*, *pepA*, *pitH2*, *ppc*, *SAV577*, *SAV6046*, and *SAV6634*) from 25 newly identified AveI target genes were decreased in DaveI (Fig. 5), indicating the positive control of AveI on these genes. The transcriptional levels of *aspC2*, *fahA*, *gltI2*, *leuA1*, *leuA2*, *ilvK3*, *nrpS5*, *pepD1*, *pks9-4*, *SAV1671*, and *SAV5600* were increased in DaveI (Fig. 5), suggesting the negative regulatory role of AveI on these genes. The findings indicate that AveI plays a pleiotropic role in primary metabolism, secondary metabolism, and morphological differentiation in *S. avermitilis* and acts as dual role of a repressor and an activator.

Determination of the precise AveI-binding sequence

To identify the precise AveI-binding site, we selected the promoter regions of *mcr* and *galE6* which have high affinity with His_6-AveI for DNase I footprinting analysis. DNase I footprinting assays were performed on 5′-end fluorescein-labeled probes of *mcr* (264 bp) and *galE6* (296 bp) in the presence of His_6-AveI. A 26-nt protected region (5′-ACGT CGGTGGAATGTCCCACTCCGGT-3′) was found in the *mcr* promoter region, extending from − 67 to − 42 relative to the predicted transcriptional start site of *mcr* (Fig. 6a), containing a 15-nt palindromic sequence (5′-GGAA TGTCCCACTCC-′), similar to the consensus sequence of ArtA. Combined with the qRT-PCR results that AveI

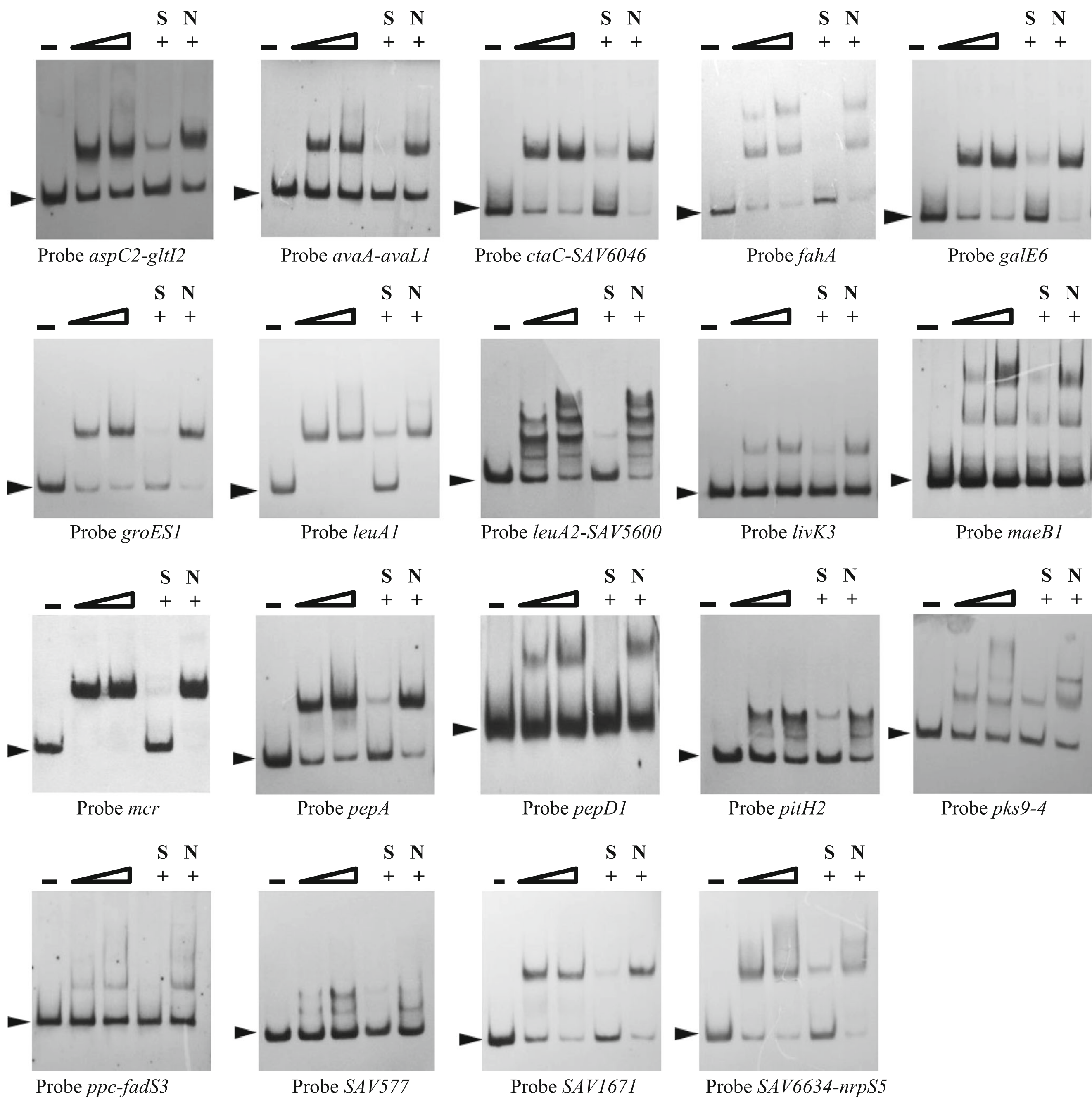

Fig. 4 Confirmation of putative AveI target genes by EMSA. EMSA conditions as described in Fig. 2

positively regulates *mcr* expression, AveI may help to recruit RNA polymerase to the promoter of *mcr* through binding to the site adjacent to the promoter and activate its transcription. A 15-nt palindromic ArtA consensus sequence (5′-GGAA CCTGGCATTCC-3′) was also found in the 31-nt AveI-protected region (5′-CACACCATCCCGGAACCTGG CATTCCAGGCA-3′) in the *galE6* promoter region (Fig. 6b). To confirm that the 15-nt palindromic sequence is the recognition sequence of AveI, we introduced mutation to the palindromic sequence in the *mcr* promoter region (probe A) to produce probe AM (Fig. 6c). EMSAs showed that His$_6$-AveI bound to probe A, but not to probe AM (Fig. 6c); therefore, the palindromic sequence (5′-GGAAT-n5-ATTCC-3′) is the AveI-binding site. The findings indicate that AveI has very similar binding sites as AtrA, suggesting the conserved regulatory role of AtrA homolog in *Streptomyces*.

Deletion of *aveI* increases avermectin production in an avermectin high-producer

Deletion of *aveI* could enhance avermectin production in *S. avermitilis* WT strain. We also tested the possibility of

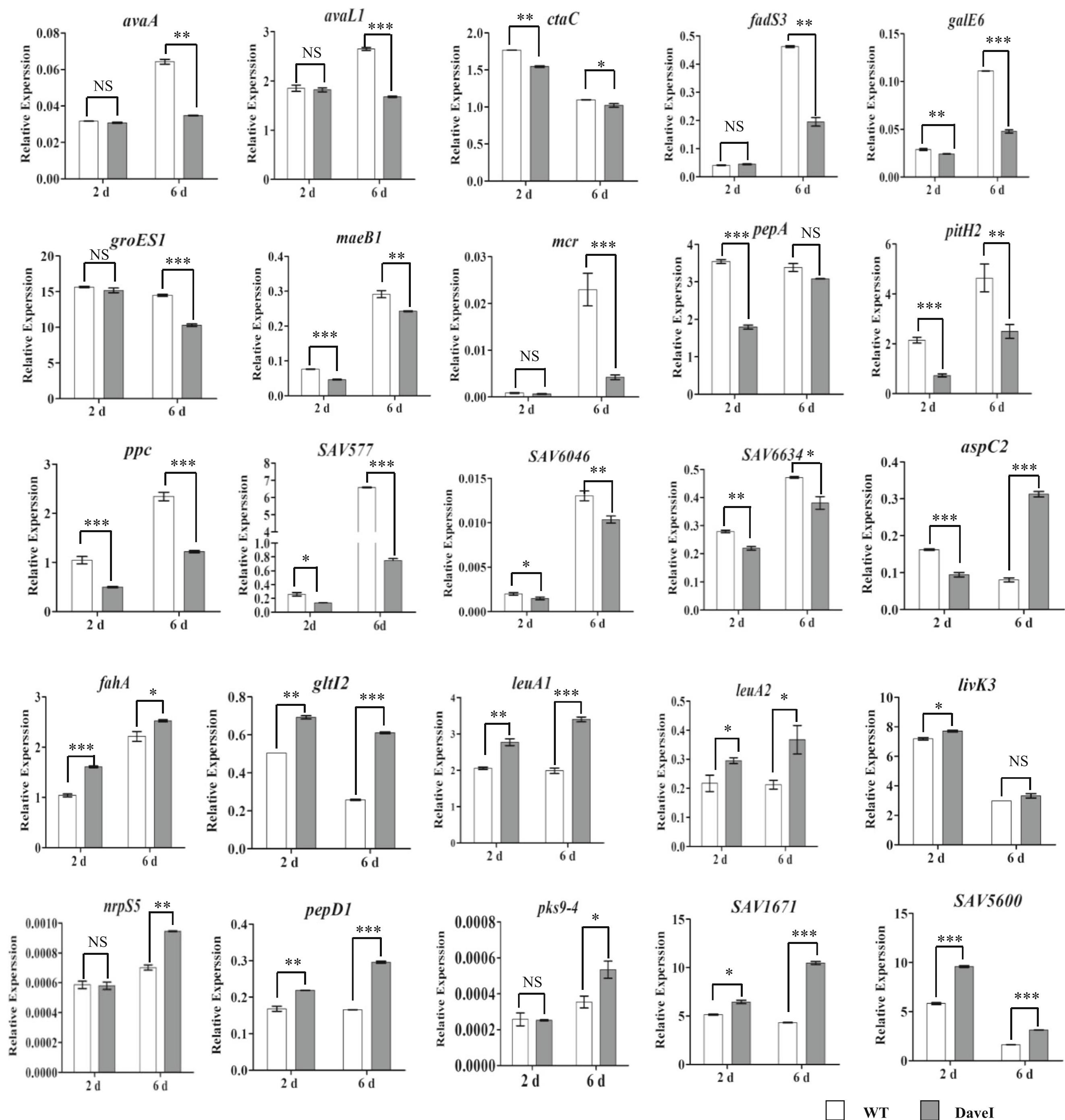

Fig. 5 qRT-PCR analysis of putative AveI target genes in DaveI and WT. RNAs were the same ones used in Fig. 2. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS, not significant

further improving avermectin production in an avermectin high-producer by *aveI* deletion. Compared to the avermectin B1a production (5470 μg/mL) of the parental strain CAU69, the production level of CAU69/DaveI was increased by ~ 14.3% and reached 6252 μg/mL (Fig. 7), indicating that deletion of *aveI* may provide an effective strategy to improve avermectin production in the avermectin high-producing strains.

Discussion

In this investigation, we demonstrated that AveI acts as a repressor regulating avermectin and oligomycin production, and also as an activator controlling melanogenesis and morphological differentiation (Fig. 8). At least 35 genes have been confirmed to be AveI targets by EMSAs, most of them are associated with primary metabolism. *pepA*, *pepD1*, *SAV5600*,

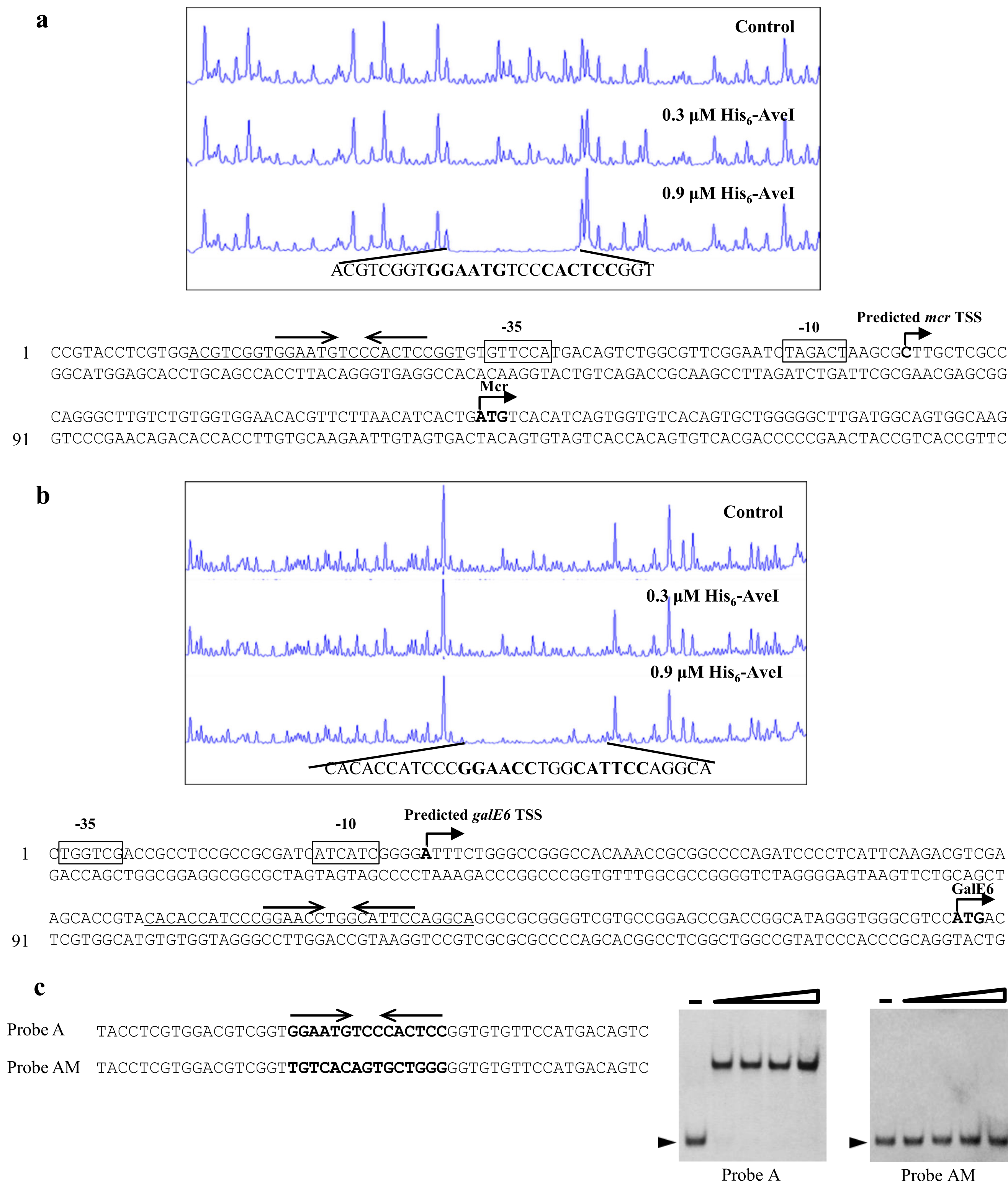

Fig. 6 Determination of AveI-binding sites. **a** and **b** DNase I footprinting assays of the promoter regions of *mcr* (**a**) and *galE6* (**b**) with His_6-AveI. Upper fluorograms: control reaction. Protection fluorograms were obtained with increasing amounts of His_6-AveI. Nucleotide sequences of *mcr* and *galE6* promoter regions are shown below fluorograms. Underlines: AveI-protected sequences. Arrows: palindromic sequences. Boxes: predicted − 35 and − 10 elements of promoters. Black bent arrows and boldface letters: translational start codon. **c** EMSAs of probe A of *mcr* promoter region and mutated probe AM to identify the AveI-binding site. Mutation was introduced into the palindromic sequence of protected region in probe A to produce mutated probe AM. Concentrations of His_6-AveI for probes: 0.075, 0.15, 0.225, and 0.3 µM

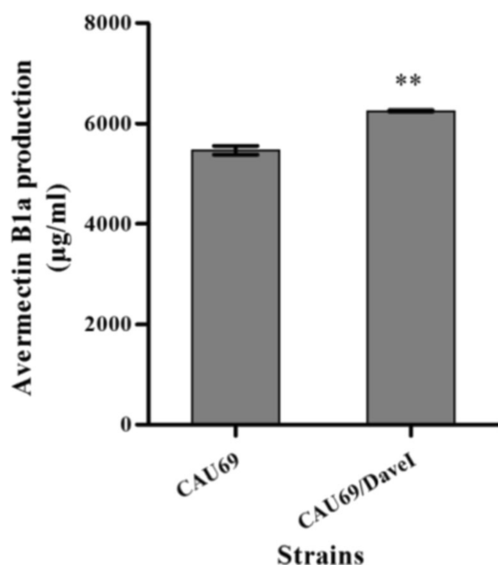

Fig. 7 Deletion of *aveI* enhanced avermectin production in an avermectin high-producing strain. Values are mean ± SD of three replicate flasks cultured in FM-I. CAU69, an avermectin high-producing strain. CAU69/DaveI, *aveI* deletion mutant of CAU69. $^{**}P < 0.01$

and *SAV6634* encode proteases or peptidases, which digest proteins or peptides to produce amino acids, and *aspC2*, *SAV6046*, *fahA*, *leuA1*, and *leuA2* are involved in the metabolism and biosynthesis of amino acids. *fadS3* and *mcr*, encoding a fatty acid desaturase and racemase, are associated with metabolism of lipids. *galE6* (encoding a UDP-glucose 4-epimerase), *maeB1* (encoding a malate dehydrogenase), *ppc* (encoding a phosphoenolpyruvate carboxylase), and *SAV1671* (encoding an oxidoreductase) are associated with carbohydrate metabolism. *ctaC* (encoding a cytochrome c oxidase subunit II) is involved in energy metabolism. *gltI2*, *ilvK3*, and *pitH2*, encoding putative ABC transporter substrate-binding proteins and a low-affinity inorganic phosphate transporter, are involved in substrates transport. These findings were consistent with the transcriptomics analysis results of the *aveI* mutant vs. WT that *aveI* deletion affects a variety of genes in both primary and secondary metabolic pathways (Chen et al. 2009). Therefore, AveI acts as a global regulator in *S. avermitilis*, controls not only secondary metabolism and morphological differentiation, but also primary metabolism. Although TetR-family regulators usually function as repressors, about half of the identified target genes are under the positive control of AveI. The mechanism of activation by AveI probably involves competition with repressors in the promoter regions of target genes or allowing activators to bind, as observed for AtrA-gr in *S. griseus* which activates streptomycin biosynthesis, probably through facilitating the AdpA-dependent transcriptional activation of *strR* (Hirano et al. 2008).

Similar to its homolog AtrA in other Streptomycetes, AveI regulates the biosynthesis of several secondary metabolites in

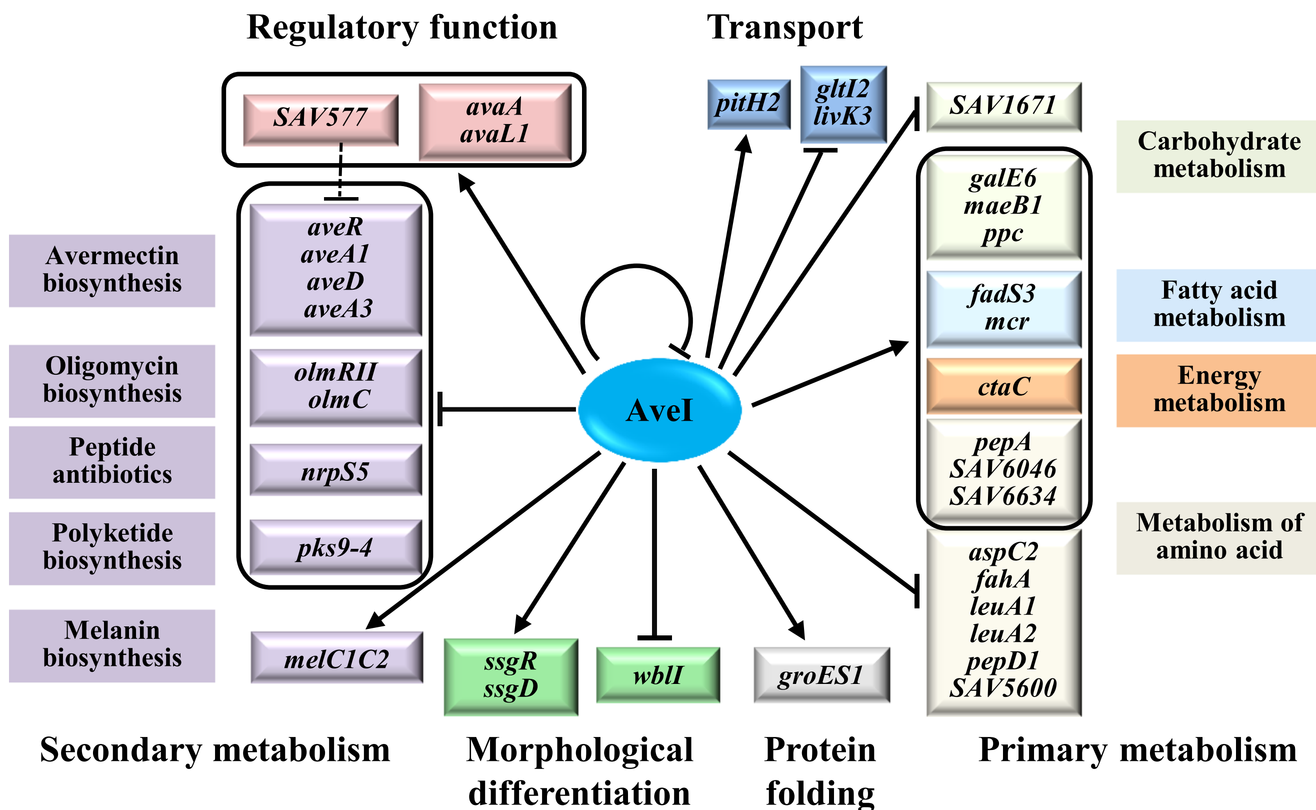

Fig. 8 Proposed model of AveI-mediated regulatory network in *S. avermitilis*. Solid lines, direct control. Dashed lines, indirect control. Arrows, activation. Bars, repression

S. avermitilis. AveI negatively regulates avermectin production by repressing the transcription of CSR gene *aveR* and structural genes *aveA1*, *aveA3*, and *aveD*. AveI also negatively regulates oligomycin production by repressing the CSR gene *olmRII* and the structural gene *olmC*. AveI positively regulates melanin biosynthesis by activating the expression of *melC1C2* operon. *nrpS5* (*SAV6633*, encoding a non-ribosomal peptide synthetase) and *pks9-4*(*SAV2376*, encoding putative 3-oxoacyl-ACP synthase I of PKS9) are also under the direct negative control of AveI. Besides, among the AveI target genes, *avaA* (*SAV2269*, encoding a γ-butyrolactone biosynthesis protein, homolog of AfsA) and *avaL1* (*SAV2270*, encoding a γ-butyrolactone-dependent transcriptional regulator) belongs to γ-butyrolactone regulatory system, which triggers antibiotic production and morphological differentiation in *Streptomyces*. *SAV577* (encoding a TetR-family transcriptional regulator) downregulates avermectin biosynthesis indirectly (Guo et al. 2014). Therefore, AveI may also regulate secondary metabolism through the cascaded regulation of other regulators. Genes associated with several primary metabolic pathways, such as fatty acid metabolism and protein synthesis, were found to be under positive control of AveI. Therefore, AveI is also possibly involved in directing the carbon flux from primary to secondary metabolism in *S. avermitilis*.

The SsgA-like proteins are a family of proteins that control cell division and sporulation in actinobacteria (Noens et al. 2005, 2007). In *S. coelicolor* A3(2), the transcription of *ssgA* is activated by the regulator SsgR (Traag et al. 2004). AtrA-c activates the transcription of *ssgR*, which in turn activates the transcription of *ssgA* (Kim et al. 2015). In *S. avermitilis*, *ssgR* is also under the positive control of AveI. Another *ssgA*-like gene, *ssgD*, is also under the positive control of AveI. WhiB-like proteins (Wbl) are small transcription factor-like proteins essential for sporulation in actinobacteria (Fowler-Goldsworthy et al. 2011; Molle et al. 2000). The expression of *wblI* is under the negative control of AveI. Therefore, AveI controls morphological differentiation by regulating the expression of *ssgR*, *ssgD*, and *wblI*. It is interesting to mention that some targets of AveI are conserved in *Streptomyces*. Besides *ssgR*, γ-butyrolactone receptor protein encoding gene *spbR* in *S. pristinaespiralis* and *avaA* in *S. avermitilis* are also the targets of AtrA homolog (Wang et al. 2015). SsgR and γ-butyrolactone receptor protein are involved in morphological differentiation of *Streptomyces*, suggesting that AtrA homologs may play a conserved role in development.

AtrA (the AveI homolog) has been intensively studied to regulate secondary metabolism in several *Streptomyces* strains (Chen et al. 2008; Hirano et al. 2008; Li et al. 2015; Mao et al. 2015; Uguru et al. 2005; Wang et al. 2015). AtrA-c and AtrA-gr activate actinorhodin and streptomycin biosynthesis through activating the expression of the CSR genes *actII-ORF4* in *S. coelicolor* and *strR* in *S. griseus*, respectively (Hirano et al. 2008; Uguru et al. 2005). AtrA-p activates pristinamycin production through activating the expression of two CSR genes *spbR* and *papR5* (Wang et al. 2015). *S. globisporus* AtrA activates lidamycin production by activating one of the CSR genes, *sgcR1* (Li et al. 2015). AtrA-r positively regulates daptomycin production via activating the structure gene *dptE* in *S. roseosporus* (Mao et al. 2015). AveI activates melanin biosynthesis by activating the expression of structure genes *melC1C2* operon. However, AveI represses avermectin production and oligomycin production through repressing the transcription of CSR genes *aveR* and *olmRII* and structural genes *aveA1*, *aveA3*, *aveD*, and *olmC* in *S. avermitilis*. Although the regulatory role of AtrA homolog in antibiotic biosynthesis is conserved in various *Streptomyces*, the molecular mechanism for each secondary metabolite is relatively diverse.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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