

The pathway-specific regulator AveR from *Streptomyces avermitilis* positively regulates avermectin production while it negatively affects oligomycin biosynthesis

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Abstract The function of the regulatory protein AveR in *Streptomyces avermitilis* was examined. An *aveR* deletion mutant abolished avermectin production and produced more oligomycin, and its phenotype was complemented by a single copy of the *aveR* gene. Removal of the C-terminal HTH domain of AveR abolished avermectin biosynthesis, indicating the importance of HTH domain for AveR function. Promoter titration and promoter probe assays suggested that the transcription of *aveA1*, encoding polypeptide AVES1 of avermectin PKS, was activated by AveR. Chromatin immunoprecipitation (ChIP) assay showed that the predicted promoter regions of both the *ave* cluster and the *olm* cluster were target sites of AveR, and the DNA-binding activity of AveR was dependent on its HTH domain. RT-PCR analysis revealed that the transcriptions of *ave* structural genes were dependent on AveR, but that of *olm* structural genes and putative pathway-specific regulatory genes increased in the *aveR* mutants. Consistent with these observations, overexpression of *aveR* successfully increased avermectin production. These results indicated that *aveR* encodes a pathway-specific activator essential for avermectin biosynthesis and it also negatively affects oligomycin biosynthesis.

Keywords *Streptomyces avermitilis* · *aveR* gene · Pathway-specific activator · Avermectin biosynthesis · Oligomycin biosynthesis

Introduction

Streptomyces are Gram-positive, filamentous soil bacteria with complex life cycle and ability to produce a wide variety of secondary metabolites, including many commercially important antibiotics. The production of secondary metabolites involves the expression of clustered biosynthetic genes that are activated in a growth phase-dependent manner. This expression pattern is often controlled by pathway-specific regulatory genes (Bibb 2005). Analysis of these regulatory genes is crucial for understanding the mechanism of regulation and for construction of overproducing strains.

Avermectins, a series of potent anthelmintic and insecticidal macrolide antibiotics (A1a, A1b, A2a, A2b, B1a, B1b, B2a, and B2b) produced by *Streptomyces avermitilis*, are used widely in the medical, veterinary, and agricultural fields (Burg et al. 1979; Ikeda and Omura 1997). *S. avermitilis* also produces small amount of a separate family of macrolide antibiotics called oligomycins, which are strongly toxic compounds (Pinna et al. 1967; Omura et al. 2001). Although the *S. avermitilis* genome has been sequenced (Omura et al. 2001; Ikeda et al. 2003) and the avermectin biosynthetic pathway has been elucidated (Ikeda and Omura 1997; Ikeda et al. 1999, 2001), most of the regulatory factors and mechanisms in avermectin production remain unknown. Genes coding for regulatory factors involved in avermectin biosynthesis include *aveR* (Ikeda et al. 1993, 1999; Kitani et al. 2009), *aveR1/aveR2* (Stutzman-Engwall and Price 2001), *orfX* (Hwang et al. 2003), *afsK-av* (Rajkarnikar et al. 2006), and *aveI* (Chen et al. 2008). *aveR*, located at the far

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left of the avermectin biosynthetic gene cluster (Fig. 1), encodes a putative pathway-specific regulator. *aveR* mutants generated by Tn4560 mutagenesis did not produce avermectins and failed to convert any intermediates after aglycon formation (Ikeda et al. 1993). Sequence analysis revealed that the product of *aveR* was a LAL family regulator (large ATP-binding regulators of the LuxR family) (Wilson et al. 2001; Kitani et al. 2009), which contains an N-terminal NTP binding domain and a C-terminal LuxR-like helix-turn-helix DNA-binding domain. The role of potential regulatory protein AveR regarding the onset of the avermectin is recently described to be positive by gene disruption and feeding experiments, while a higher amount of *aveR* resulted in complete loss of avermectin (Kitani et al. 2009). In this paper, evidence for AveR as a pathway-specific activator for avermectin biosynthesis as well as a negative regulator for oligomycin biosynthesis is given, and in contrast to the findings of Kitani et al. (2009) overexpression of *aveR* enhanced avermectin production in wild-type strain *S. avermitilis* ATCC31267.

Materials and methods

Strains, plasmids, and growth conditions

Streptomyces avermitilis ATCC31267 (wild-type strain) was grown at 28°C and used as a host strain for gene propagation and gene disruption. Solid YMS medium (Ikeda et al. 1988) and liquid YEME (Kieser et al. 2000) medium with 25% sucrose were used for sporulation and growth of mycelia for the purposes of extracting DNA and preparing protoplasts, respectively. Seed medium and fermentation medium (Chen et al. 2007) were used for avermectin production. Soluble fermentation medium II (5% soluble starch, 1.2% yeast extract, 0.05% K₂HPO₄·3H₂O, 0.05% MgSO₄·7H₂O, 0.4% KCl, 0.0005% CoCl₂·6H₂O) was used to cultivate mycelia for growth, ChIP, and RT-PCR analysis. RM14 (MacNeil and Klapko 1987) was used for regeneration of protoplasts and for selection of transformants. *E. coli* DH5α and *E. coli* BL21 (DE3) (Novagen, Shanghai, China) were used as cloning host and expression host, respectively. *E. coli* ET12567 (*dam dcm hsdS*) (MacNeil and Klapko 1987) was used to propagate non-methylated DNA for transformation in *S. avermitilis*. *E. coli* strains were grown at 37°C in Luria–Bertani (LB) medium, and transformed as

described by Sambrook et al. (1989). *S. lividans* TK54 was grown at 30°C on YMS agar and used as a host strain for promoter probe assay. Antibiotics used in this study were described previously (Zhao et al. 2007).

Plasmid pIJ2925 (Janssen and Bibb 1993) was used for routine cloning and subcloning experiments. pKC1139 (Bierman et al. 1992) was used to construct gene disruption mutants via homologous recombination. pSET152 (Bierman et al. 1992) was used to introduce a single copy of *aveR* into *S. avermitilis*. pIJ963 (Kieser et al. 2000) was used to provide hygromycin resistance gene (*hyg*) for constructing gene replacement vector. *Streptomyces* high copy number (50–100) vector pIJ486 (Kieser et al. 2000) containing reporter gene *neo* was used to construct plasmids for promoter titration and promoter probe assay. Vector pIJ4090 (Kieser et al. 2000) was used to provide the *Streptomyces* strong constitutive promoter *ermE***p*. pET-28a (+) (Novagen), a cloning/expression vector for conferring N-terminal His6 tag on expressed proteins, was used for gene overexpression in *E. coli*. TA vectors pMD18-T (TaKaRa, Dalian, China) and pGEM[®]-T Easy (Promega, Beijing, China) were used for cloning PCR products.

Construction of *aveR* deletion mutants

Using genomic DNA of ATCC31267 as template, a 0.5-kb fragment upstream of the *aveR* start codon was amplified by PCR with primers 93 (CTGAAGCTTACGGCATCA CGCTCACTGC) and 94 (CTGAGATCTCACGAGTGGG ATG CGCAGT) containing engineered sites *Hind*III and *Bg*III, and a 0.5-kb fragment downstream of the *aveR* stop codon was amplified with primers 95 (AGTCTGCAG CGTCTTCCGCCTCACGACATG) and 96 (ACTGAATTC ACACCCTGTTCGAGAAGTCGAG) containing *Pst*I and *Eco*RI. The two PCR fragments were digested with *Hind*III/*Bg*III and *Pst*I/*Eco*RI, respectively. The 1.7-kb hygromycin resistance gene (*hyg*) was excised from pIJ963 by *Pst*I/*Bg*III digestion. These three fragments were simultaneously ligated into *Hind*III/*Eco*RI-digested pKC1139 to generate pDIaveR (Fig. 2a).

Transformation of pDIaveR and selection of double-crossover recombination strains were performed as described previously (Zhao et al. 2007). The *aveR*-deleted mutants were confirmed by PCR analysis using primers 117 (GGTCACCGTGATCGTCACG), hhz (CCATCCCAGCT CGGCAAG), 118 (CTGGTGGCCGTTCACTACG) and

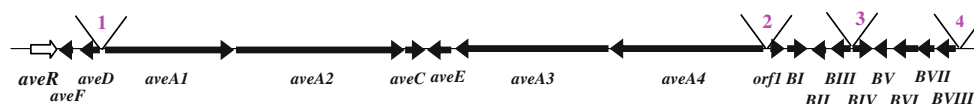
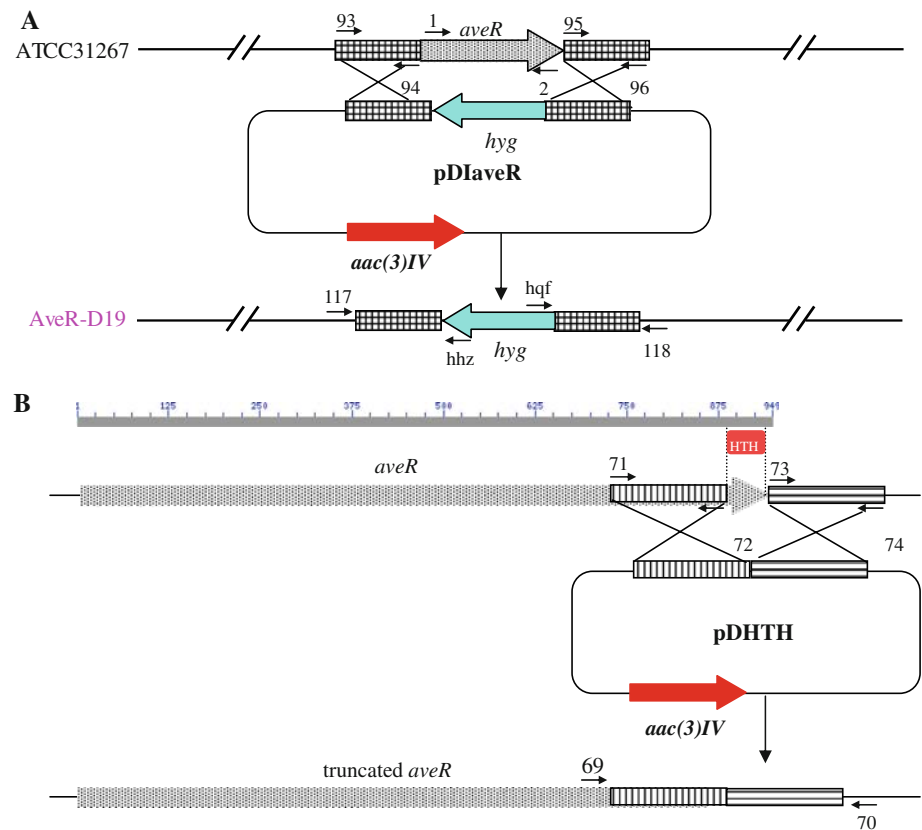


Fig. 1 Organization of the gene cluster for avermectin biosynthesis. Direction of transcription and relative sizes of ORFs are indicated (Ikeda et al. 1999); BI–BVIII: *aveBI*–*aveBVIII*. Numbers indicate putative promoter regions identified in the present study

Fig. 2 Schematic representation of the strategy used for deletion of the entire *aveR* gene (a), and its 3'-terminus (b), respectively. Long broad arrows indicate genes and their directions. Short small arrows indicate positions of primers used for cloning exchange regions and confirming gene deletions, as described in "Materials and methods". Double-crossover recombination led to replacement of *aveR* with hygromycin resistance gene *hyg*, and in-frame deletion of the 3'-terminus of *aveR*, which encodes the C-terminal HTH domain of AveR. The *aveR* deletion mutant was named as AveR-D19. The fusion in the truncated *aveR* used the original stop codon for *aveR*



hqf (CGGGATCGCCAATCTCTAC) (Fig. 2a). Primers 117 and 118 flank the exchange regions, while primers hhz and hqf are specific for *hyg*. When primer pairs 117/hhz and 118/hqf, which are specific for replacement of *aveR* with *hyg*, were used for PCR analysis of putative *aveR* deletion mutants, a 0.64-kb band and a 0.95-kb band appeared, respectively, whereas such bands were not detected when genomic DNA of wild-type strain ATCC31267 was used as the template. In contrast, when primers 1 (AACC ATATGCAGGGAGTTTCCTGTC) and 2 (GCGGAATT CATGTCGTGAGGCGGAAG) located within the deletion region of *aveR* were used, only the wild-type strain produced 2.9-kb PCR fragment as predicted (data not shown). These results indicate that *aveR* deletion mutants (*S. avermitilis* AveR-D19) were obtained in which the *aveR* gene was completely replaced with *hyg* by double-crossover recombination.

Complementation of *aveR* deletion mutants

A 3.1-kb DNA fragment carrying the promoter and coding region of *aveR* was amplified with primers 1* (ACGG ATGTCTCCAGGAAGG) and 2* (GGTCAGTTGGCCT GGTCCG) by PCR using genomic DNA of ATCC31267 as the template, and was then cloned into pGEM[®]-T Easy vector to produce pCZ10. The sequence of *aveR* in pCZ10

was verified by nucleotide sequencing. The 3.1-kb *SphI/SpeI* fragment of *aveR* was excised from pCZ10 and inserted into *SphI/XbaI*-digested pIJ2925 to produce pCZ11. The 3.1-kb *BglIII* fragment of *aveR* from pCZ11 was cloned into the *BamHI* site of pSET152 to give complementation plasmid pCZ13, which was integrated into the chromosome of *S. avermitilis* after transformation. The 3.1-kb *EcoRI/HindIII* fragment containing *aveR* from pCZ11 was cloned into the corresponding sites of pKC1139 to produce pCZ12 (Fig. S1A), which was used to introduce multi-copies of *aveR* into *S. avermitilis*.

Deletion of the 3'-terminal portion of *aveR*

A 0.5-kb DNA fragment upstream of the coding region for C-terminal HTH domain of AveR was amplified by PCR with primers 71 (ATCAAGCTTCCGCGACTTCCTCAC CG, *HindIII*) and 72 (ACTGAGCTCGGACTCGCTCAGC AG, *SacI*). A 0.5-kb DNA fragment downstream of the HTH coding region was also amplified with primers 73 (ACTGAGCTCCGACACCTCGCGGACCAG, *SacI*) and 74 (GCTGAATTCTGTTCAGGATCAATGTGCG, *EcoRI*). These two PCR fragments were then digested with *HindIII/SacI* and *SacI/EcoRI*, respectively, and were simultaneously ligated into *HindIII/EcoRI*-digested pKC1139 to generate pDHHTH (Fig. 2b). The deletion has been controlled by

DNA sequencing, which confirmed the desired in-frame deletion of 51 codons and the usage of the original *aveR* stop codon.

pDHTH was transformed into ATCC31267, and the expected deletion mutants were selected using the same strategy as for selection of *aveR* deletion mutants and confirmed by PCR analysis. When primers 69 (GACGCCAAGGAGTGC GC) and 70 (ACACCCTGTTCG CAGAAGT CGAG) flanking the exchange regions were used, the mutant strains generated a 1.35-kb PCR product (Fig. 2b), whereas the wild-type strain generated a 1.5-kb PCR product (data not shown). These results indicate that an internal 150-bp fragment of *aveR*, which encodes the C-terminal HTH domain, was deleted in these mutants.

Construction of plasmids for promoter titration and promoter probe assay as well as for overproduction of AveR

A 327-bp DNA fragment containing the putative *aveAI* promoter was amplified by PCR with primers 6 (ATCATGGTCGGGAACCTCC) and 7 (TCACCGCTAGGCAATGCTC), and was cloned into pMD18-T to produce pJL1311. The 327-bp fragment was cut from pJL1311 with *Bam*HI/*Hind*III and inserted into *Bam*HI/*Hind*III-digested pIJ486 to generate pJL62, in which the 327-bp *aveAI* promoter was located upstream of the reporter gene *neo*. The recombinant plasmid pJL62 was transformed into protoplasts of ATCC31267 for promoter titration assay, and was also used in promoter probe assay to detect activity of *aveAI* promoter.

In order to enhance expression of AveR in *Streptomyces*, the plasmid pJL66 was constructed. There is a rare TTA codon 1390–1392 nucleotides downstream of the start codon of *aveR*. To change the TTA leucine codon to a CTG leucine codon, site-directed mutagenesis was performed in vitro by PCR. Two primer pairs were designed: primers 22 (TCGGCGGCCGCTTCTACCAG, *Not*I) and 23 (GACGAGCAGAGGGACGAGCAGGGCCGTCTGATGGCCGGTC); and primers 24 (GACCGGCCATCAGACGGCCCTGCTCGTCCCTCTGCTCGTC) and 25 (GTGTGCGGCCGCTCGCTG, *Not*I). Primer 23 and primer 24 were complementary to opposite strands of the TTA codon region of *aveR*, and contained the desired mutation as indicated by shading above. PCR products amplified with primers 22/23 and 24/25, respectively, were mixed and then used as template for PCR with primers 22/25 again. The resulting 0.9-kb PCR product was cloned into pMD18-T to give pCTG. The inserted 0.9-kb DNA fragment in pCTG was sequenced to confirm that it contained the designed TTA → CTG mutation without other mutation. The 0.9-kb *Not*I fragment from pCTG was subsequently ligated with *Not*I-digested pCZ11 to give pCZ11*, which contained mutated

aveR (called *aveR**). The *aveR** fragment was excised from pCZ11* with *Bg*III/*Hind*III, and cloned into the corresponding sites of pIJ4090 to generate pJL63. pJL63 was digested with *Bg*III, and the resulting 3.2-kb fragment containing strong constitutive promoter *ermE***p* and *aveR** was ligated with *Bam*HI-digested pSET152 to give AveR-high expression plasmid pJL66 (Fig. S1B), in which *aveR* gene was controlled by strong promoter *ermE***p* and contained TTA → CTG mutation.

Overexpression and purification of the recombinant AveR

A DNA fragment encoding the 394 carboxy-terminal amino acids of AveR was obtained by PCR with primers 68 (CATATGACAGCGGCCTTGTGGGC, *Nde*I) and 2 (containing *Eco*RI site). The PCR fragment digested with *Nde*I/*Eco*RI was inserted into the corresponding sites in pET-28a (+) to generate pET-AveRc (Fig. S1C), which was further confirmed by DNA sequencing, and then introduced into *E. coli* BL21 (DE3) for protein overexpression.

After induction by IPTG, the recombinant protein His6-AveRc was expressed as inclusion body in *E. coli*. Cells containing His6-AveRc were harvested, washed twice with PBS buffer, and resuspended in 5 ml PBS buffer. The cell suspension was treated with lysozyme on ice, and then disrupted by sonication. After centrifugation, the supernatant was removed, and the pellet was washed three times with 4 M urea containing 1% Triton X-100. 8 M urea was added to solubilize inclusion bodies in the pellet, and the solution was centrifuged. The supernatant was dialyzed against a stepwise decrease in urea concentration solution (4 M, 2 M, 1 M urea and PBS buffer); each dialysis was performed over a period of 24 h. The purified protein was diluted with PBS buffer to 1 mg ml⁻¹ for use in antibody induction.

Preparation of antibodies against AveR protein

The purified recombinant protein His6-AveRc was mixed with Freund's complete adjuvant and injected into a rabbit. After 2 weeks, the antigen was injected into the same rabbit with Freund's incomplete adjuvant. Further booster immunizations were given at 2-week intervals. The rabbit was bled 2 weeks after each boost, and serum was prepared. Each serum was stored at 4°C, and its potency checked by ELISA. After several booster immunizations, the immune serum reached a high potency and was used as a source of anti-AveR antibodies for subsequent Western blotting and CHIP assay.

Chromatin immunoprecipitation (CHIP) assay

Streptomyces avermitilis cultures grown in fermentation medium II for 40 h were cross-linked by addition of

formaldehyde (final concentration 1%) for 30 min at room temperature. 125 mM glycine (final concentration) was added to stop the reaction, and incubation was continued for 5 min with gentle shaking. Mycelia were collected, washed twice with cold PBS buffer, and stored in 300 mg (wet weight) aliquots at -80°C until use. 300 mg frozen mycelia was ground into a fine powder in liquid nitrogen, resuspended in 3 ml immunoprecipitation (IP) buffer (Grainger et al. 2004) containing 1 mM PMSF, and subjected to sonication, which broke down the DNA to 500–1,000 bp fragments. After centrifugation (10 min, 12,000 rpm, 4°C), the supernatant was removed and used as input sample (positive control) in immunoprecipitation experiments.

An 800- μl input sample was pre-cleared with 50 μl 50% protein G agarose/salmon sperm (SS) DNA (Upstate, catalog no. 16-201) for 60 min at 4°C with gentle rotation. Agarose was spun down, supernatant was transferred to a new tube, and 80 μl anti-AveR antibodies was added. After incubation overnight with gentle rotation, 70 μl 50% protein G agarose/SS DNA was added, and incubation continued 4 h at 4°C . An immunoprecipitation experiment without antibody was run as negative control. The protein G agarose beads were washed as described by Grainger et al. (2004). To elute immune complexes, the beads were incubated in 300 μl freshly made elution buffer (1% SDS, 0.1 M NaHCO_3) at room temperature for 15 min with rotation. A total of 12 μl 5 M NaCl was added to the eluate or to 300 μl positive-control sample, and cross-links were reversed by incubation at 65°C for 5 h. The positive-control sample was subsequently treated in the same manner as the experimental sample. Residual protein was degraded by addition of 200 $\mu\text{g ml}^{-1}$ Proteinase K at 45°C for 1 h, followed by phenol/chloroform extraction. The DNA was precipitated with ethanol and resuspended in 30 μl TE, whereas the positive-control sample was resuspended in 100 μl TE. To determine the identity of the immunoprecipitated DNA, PCR using primer sets shown in supplementary Table S1 was performed on 2 μl immunoprecipitated DNA or control DNA for 25–30 cycles.

Western blotting and RT-PCR analysis

After grown in fermentation medium II for 40 h, mycelia of *S. avermitilis* were collected, flash-frozen in liquid nitrogen, and ground into a fine powder. For Western blotting analysis, cells were suspended in ice-cold extraction buffer containing protease inhibitors (50 mM HEPES, pH 7.4, 137 mM NaCl, 10% glycerol, 1 mM PMSF, 1 $\mu\text{g ml}^{-1}$ leupeptin, 1 $\mu\text{g ml}^{-1}$ pepstatin A). Cell debris was removed by centrifugation, and the supernatant was used as total protein extract. 100 μg total protein of each sample was separated in 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis, after which the proteins were transferred

onto PVDF membrane. Polyclonal antiserum raised against AveR was used at a dilution of 1:1,500. Western blots were developed using an ECL detection system (Amersham, Beijing, China).

For RNA preparation, total RNA was isolated from the ground mycelial paste using Trizol reagent (Invitrogen, Shanghai, China) according to the manufacturer's instructions, and the sample was treated with DNase I (Promega) to remove the contaminating chromosomal DNA. RT-PCR was conducted with TaKaRa One Step RNA PCR Kit. As a negative control, the DNase-treated RNA sample was used as a template for PCR, to confirm that the amplified products were not derived from chromosomal DNA. For semiquantitative analysis, samples were taken at four-cycle intervals between cycles 24 and 35 to compare non-saturated PCR product formation. Data were verified in three independent experiments. *hrdB* gene, which encodes the major sigma factor in *Streptomyces*, was used as positive control for RT-PCR assay. For oligonucleotides, see supplementary Table S2.

Fermentation and HPLC analysis of products

Fermentation of *S. avermitilis* ATCC31267 and its mutants was performed as described previously (Chen et al. 2007). Avermectins and oligomycins in fermentation culture were identified by HPLC analysis as described by Chen et al. (2007).

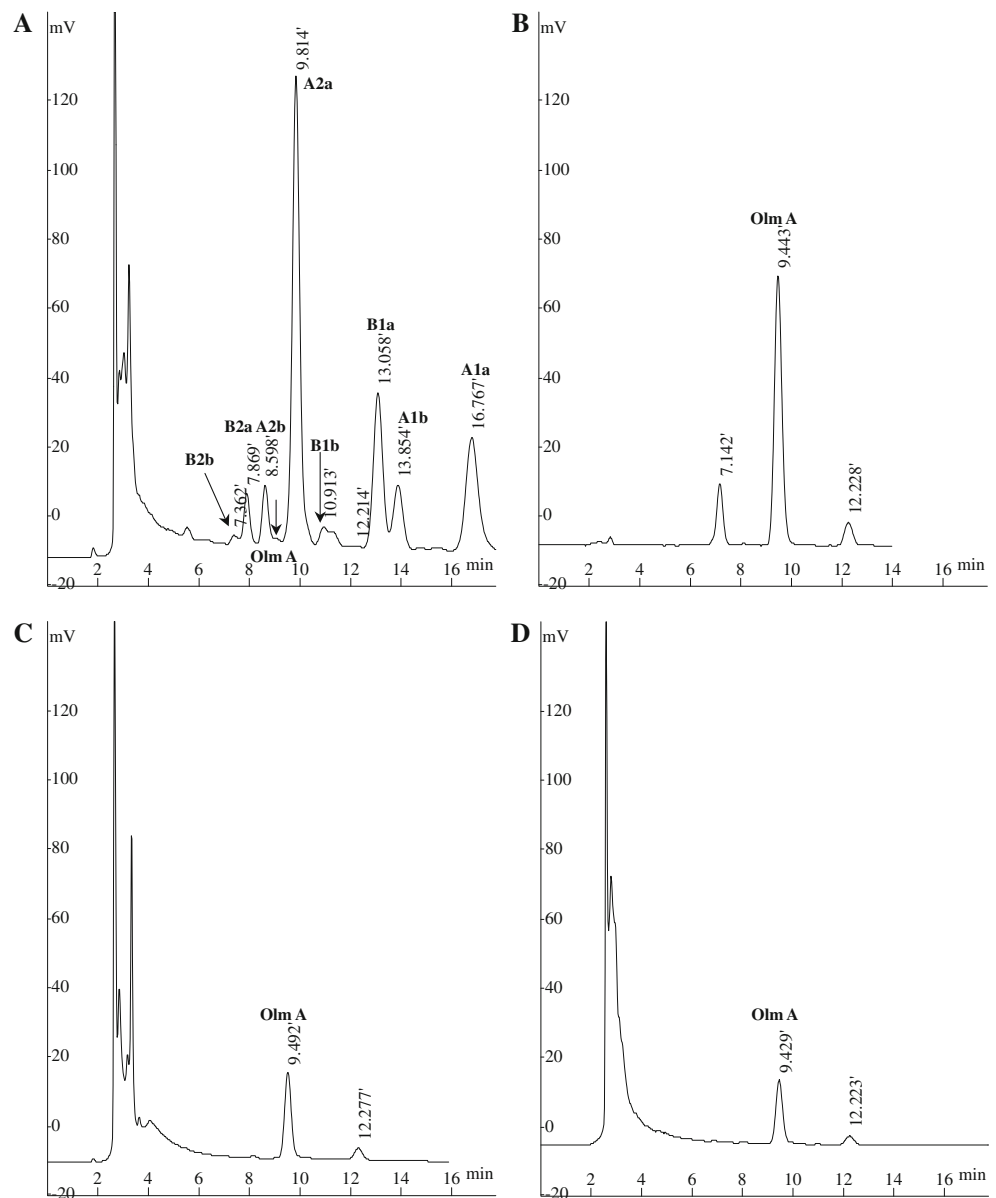
Results

Deletion of *aveR* abolished avermectin biosynthesis

The entire *ave* gene cluster contains 18 ORFs spanning a distance of 82-kb (Ikeda et al. 1999) (Fig. 1). The *aveR* gene for the putative regulator AveR contains 2,850 nucleotides and encodes a protein of 949 amino acids (predicted molecular mass, 101 kDa) including an N-terminal NTP-binding domain (a.a. 17–233), and a C-terminal HTH DNA-binding domain (a.a. 892–941) belonging to the LuxR family (Wilson et al. 2001). To study its function, an *aveR* deletion strain was constructed (Fig. 2a) and designated as *S. avermitilis* AveR-D19. AveR-D19 displayed normal growth on YMS solid medium (data not shown). HPLC analysis revealed no avermectin peaks in fermentation culture of AveR-D19 (Fig. 3c). In contrast, wild-type strain ATCC31267 produced all eight avermectin components (Fig. 3a).

To confirm that deletion of *aveR* was the sole reason for loss of avermectin production, a 3.1-kb DNA fragment containing *aveR* and its promoter region was reintroduced into AveR-D19 through the pSET152-based complementation

Fig. 3 HPLC analysis of fermentation products from **a** wild-type strain ATCC31267; **c** *aveR* deletion mutant AveR-D19; **d** *aveR* truncation mutant AveR-DHTH. **b** Standards for oligomycins. *A1a*, *A1b*, *A2a*, *A2b*, *B1a*, *B1b*, *B2a*, *B2b* eight components of avermectin; *OlmA* oligomycin A



plasmid pCZ13. The complementation strain restored avermectin production, but at a level of ca. 50% of wild-type production (data not shown). This result suggests that *aveR* is required for avermectin biosynthesis, and that *aveR* function may depend on position in the *S. avermitilis* chromosome.

The HTH domain of AveR is essential for avermectin productivity

As in other members of the LAL family, an HTH domain was found in the C-terminal region of AveR. To evaluate the function of this domain, a 150-bp internal fragment in the 3'-terminal portion of *aveR* encoding the HTH domain was deleted (Fig. 2b). The *aveR* truncation mutant was

designated as AveR-DHTH. Growth of AveR-DHTH was similar to that of wild-type strain. HPLC analysis showed that no avermectin was produced by AveR-DHTH (Fig. 3d). Thus, the C-terminal HTH domain is essential for AveR function, and is probably responsible for DNA binding.

In vivo titration of AveR with *aveA1* promoter

Generally, pathway-specific regulators directly affect expression of linked structural genes via binding to their promoter regions. *aveA1*, which encodes the polyketide synthase AVES1 required for avermectin aglycon biosynthesis, might be the target of AveR. To test this, a multi-copy promoter titration plasmid pJL62 carrying the *aveA1*

promoter region was constructed and transformed into ATCC31267. The avermectin production was reduced significantly in the resulting transformants (data not shown). A plausible explanation of this finding is that titration of AveR with the 327-bp *aveA1* promoter region at high copy number affected interaction of AveR with the corresponding chromosomal region, and that AveR is a transcriptional activator of *aveA1*.

Analysis of AveR activity as transcriptional activator by promoter probing

The promoter probe plasmid pIJ62, in which the reporter *neo* gene is under control of the *aveA1* promoter, was transformed into *S. avermitilis* ATCC31267 and *aveR* deletion mutant AveR-D19. The resulting transformants were cultured on YMS agar containing 5 $\mu\text{g ml}^{-1}$ kanamycin. No growth of these transformants was observed after 10 days incubation, possibly because the *aveA1* promoter in pIJ62 was very weak due to limited number of AveR regulator in cells. Therefore, the AveR-high expression plasmid pJL66 was constructed and co-transformed with pIJ62 into *S. lividans* TK54 instead. The co-transformants TK54 (pIJ62 + pJL66) grew well even on YMS agar containing 150 $\mu\text{g ml}^{-1}$ kanamycin, whereas the control transformants TK54 (pJL62 + pSET152) did not grow on YMS agar containing 50 $\mu\text{g ml}^{-1}$ kanamycin (Fig. 4b, c), indicating that *aveA1* promoter is inactive without AveR. Both TK54 (pIJ62 + pJL66) and TK54 (pJL62 + pSET152) grew on thiostrepton-containing YMS plates (Fig. 4a) because pJL62 contains thiostrepton-resistant gene *tsr*. The same growth patterns were obtained for ATCC31267 (pIJ62 + pJL66) and ATCC31267 (pJL62 + pSET152) grown on YMS agar containing corresponding antibiotics (data not shown). Thus, promoter probe assay confirmed that AveR positively regulates the activity of *aveA1* promoter.

Identification of AveR target promoters by ChIP assay

To screen the DNA targets of AveR in *ave* cluster, we established a ChIP assay using *S. avermitilis* strains treated with formaldehyde to cross-link AveR to its DNA targets.

For the wild-type strain, avermectins were detected by HPLC after 36-h growth in fermentation medium II, suggesting that AveR may interact with its targets and thereby inducing avermectin production prior to this time point. We therefore cross-linked *S. avermitilis* cells with formaldehyde after 40-h growth in fermentation medium II, extracted and fragmented the cross-linked DNA by sonication, and performed immunoprecipitation with anti-AveR antibodies to select DNA fragments attached to AveR, using a sample without antibody as a negative control. PCR assays were conducted using primers that flank the predicted promoter regions. Based on sequence analysis, the *ave* gene cluster contains six major putative transcriptional units: *aveD-aveF*, *aveA1-aveA2-aveC*, *aveA4-aveA3-aveE*, *orf1-aveBI*, *aveBIII-aveBII*, and *aveBVIII-aveBVII-aveBVI-aveBV*. Four promoter regions were therefore chosen at intergenic regions between divergently transcribed genes in the *ave* gene cluster: 327-bp *aveD-aveA1* intergenic region; 260-bp *aveA4-orf1* intergenic region; 126-bp *aveBIII-aveBIV* intergenic region; and 238-bp region immediately upstream of *aveBVIII* (Fig. 1). Primer pairs used for the four promoter regions are shown in Table S1. PCR products with correct size were obtained with these primers when immunoprecipitated DNA or positive control DNA from wild-type strain was used as template (Fig. 5a). In contrast, these primers amplified DNA fragments only from positive control DNA, not from anti-AveR-immunoprecipitated DNA of mutant strain AveR-DHTH (Fig. 5b). No correct PCR bands were obtained using all the primer pairs when negative control DNA without antibody was used as template (Fig. 5). These results demonstrate that the four promoter regions were selectively enriched as a result of ChIP using anti-AveR antibodies, and that AveR binds specifically to these regions with its C-terminal HTH domain.

Gene expression analysis of the *ave* cluster by RT-PCR

To screen the structural genes controlled by *aveR*, transcripts from the *ave* cluster were analyzed by one-step RT-PCR applied to total RNA obtained from *S. avermitilis* wild-type strain after the onset of avermectin production,

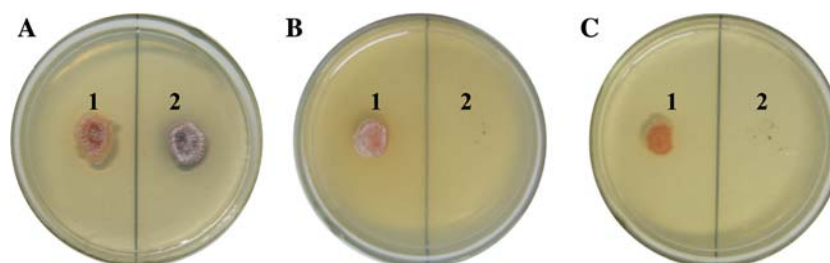


Fig. 4 Growth of *S. lividans* TK54 transformed with promoter probe plasmid pIJ62. Strains were grown for 7 days on YMS agar containing thiostrepton (a 50 $\mu\text{g ml}^{-1}$) or kanamycin (b 50 $\mu\text{g ml}^{-1}$,

c 150 $\mu\text{g ml}^{-1}$). 1 *S. lividans* TK54 transformed with pJL62 and pJL66; 2 *S. lividans* TK54 transformed with pJL62 and pSET152

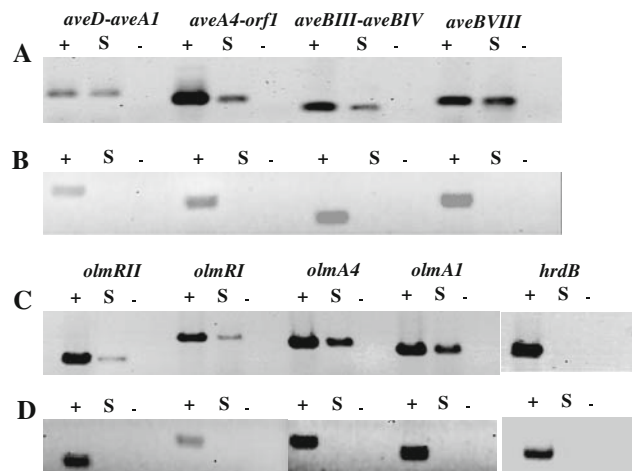


Fig. 5 ChIP analysis of AveR binding to *ave* promoter regions (**a, b**) and *olm* promoter regions (**c, d**) in vivo. Anti-AveR antibodies were used to immunoprecipitate AveR/DNA complexes from cells (**a, c** wild-type strain; **b** AveR-DHTH; **d** AveR-D19) treated with formaldehyde. PCR was performed with primers flanking putative promoter regions (Table S1). DNA used for PCR was total DNA prior to immunoprecipitation (positive control; lanes “+”), immunoprecipitated DNA (experimental sample; lanes “S”), and negative control DNA without antibody (lanes “-”). *hrdB* promoter region was used as a control

and concurrently from *aveR* deletion mutant AveR-D19. Most transcripts were visualized using 25 cycles of PCR, but some required 30 cycles. In negative controls, containing DNA polymerase but lacking reverse transcriptase, no PCR product was detected with any of the primers when fewer than 35 PCR cycles were used. RT-PCR showed that all structural genes of the *ave* gene cluster in wild-type strain were transcribed (Fig. 6). Corresponding transcripts were not observed for mutant strain AveR-D19. This gene expression pattern shows that AveR is required for the transcription of all avermectin biosynthetic genes.

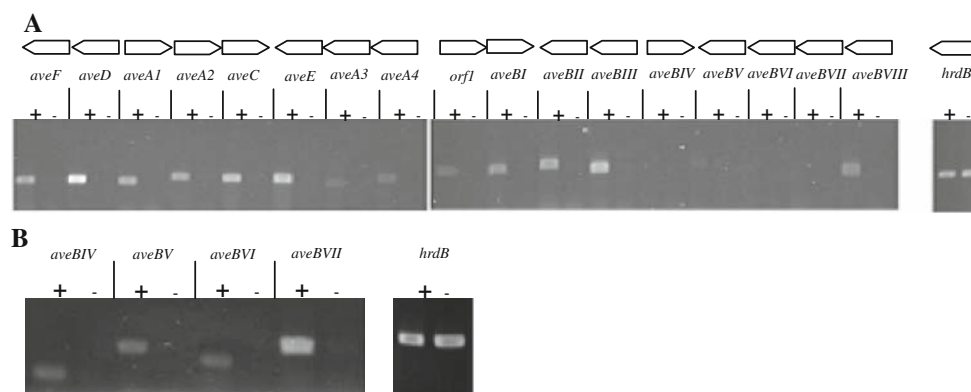


Fig. 6 RT-PCR analysis of the *ave* cluster. Analysis was carried out in *S. avermitilis* wild-type strain (+) and AveR-D19 (-). In general, 25 cycles of PCR were used (**a**); in case this generated no apparent

Overexpression of *aveR* leads to overproduction of avermectin

As a general rule, overexpression of a pathway-specific transcriptional activator is associated with increased production of the corresponding antibiotic. To test this possibility, we introduced the AveR-high expression vector pJL66, in which *aveR* gene was controlled by *ermE***p* and contained TTA → CTG mutation, into wild-type strain, and analyzed its effects on cell growth and avermectin productivity in soluble fermentation medium II. Enhanced expression of *aveR* resulted in increased avermectin production, but growth of the transformant was similar to that of wild-type strain (Fig. 7a, b). Overexpression of *aveR* was confirmed by both Western blotting and semiquantitative RT-PCR analysis, comparing the wild-type strain with its transformant ATCC31267/pJL66 and *aveR* deletion mutant AveR-D19 (Fig. 7c). In addition, multi-copy plasmid pCZ12 (based on pKC1139) and integrative plasmid pCZ13 (based on pSET152) containing *aveR* with its own promoter were transformed into wild-type strain, respectively. The resulting transformants and their host strain ATCC31267 as well as vector control strains were separately cultured in fermentation medium for 10 days. HPLC analysis of fermentation products showed that transformant containing only pSET152 produced nearly the same amount of avermectin as host strain, while introduction of empty pKC1139 caused a small reduced avermectin production. In contrast, transformation of plasmid pCZ12 and pCZ13 containing *aveR* led to enhanced avermectin production (Fig. 7d), indicating that the increased production was due to the overexpression of *aveR* gene. These results further demonstrated the role of *aveR* as an important pathway-specific activator gene for avermectin biosynthesis.

product, analysis was repeated at 30 cycles in attempt to detect low level of transcripts (**b**)

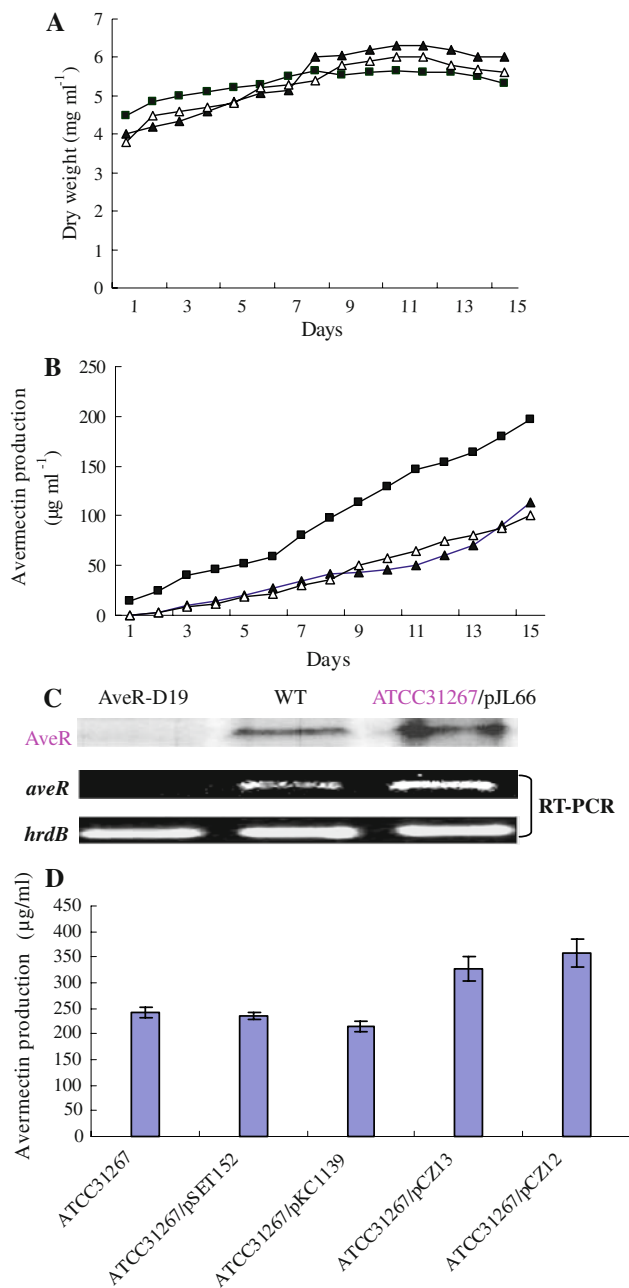


Fig. 7 Effect of enhanced expression of *aveR* on growth (a) and avermectin production (b) of *S. avermitilis* grown in soluble fermentation medium II. Filled triangles wild-type strain ATCC31267; open triangles transformant of wild-type strain containing control plasmid pSET152 (ATCC31267/pSET152); filled squares transformant of wild-type strain containing AveR-high expression vector pJL66 (ATCC31267/pJL66). c Western blotting and semiquantitative RT-PCR analysis of *aveR* in wild-type strain (WT), AveR-D19 and ATCC31267/pJL66. The same batch of cells was used for both Western blotting and RT-PCR analysis. d Comparison of avermectin production in various *S. avermitilis* strains grown in fermentation medium. Fermentation medium is usually used for avermectin production; however, it contains insoluble yeast meal. So, we used soluble fermentation medium II in a and b for analysis of cell growth as well as avermectin production in the same batch of cells. Avermectin production is lower in soluble fermentation medium II than that in fermentation medium

Deletion of *aveR* enhanced oligomycin biosynthesis

The *aveR* mutants lost the ability to produce avermectins, but produced oligomycins at a higher level than wild-type strain (Fig. 3c, d). In complementation experiments, DNA fragment containing *aveR* and its promoter region restored avermectin production as well as reduced oligomycin production to the level of wild-type strain in the mutant AveR-D19 (data not shown). To test whether *aveR* affects the transcription of oligomycin biosynthetic genes, semiquantitative RT-PCR analyses were performed to detect the expression of two selected polyketide synthase (PKS) genes (*olmA1* and *olmA4*) and two putative pathway-specific regulatory genes (*olmRI* and *olmRII*) in *olm* cluster. As shown in Fig. 8, all the detected *olm* genes were upregulated in the *aveR* mutants. In ChIP assays, the predicted promoter regions of the *olm* genes were amplified from both positive control DNA and anti-AveR-immunoprecipitated DNA (Fig. 5c). Since AveR, *OlmRI* and *OlmRII* are all members of the LAL family, antibodies raised against one might cross-react with the others. To investigate whether AveR antibodies immunoprecipitated *OlmRI* and/or *OlmRII*, the ChIP experiment was repeated in *aveR* null mutant AveR-D19 and showed that the *olm* promoter DNA was no longer immunoprecipitated (Fig. 5d). These data indicate that AveR has negative effects on oligomycin biosynthesis, and represses transcription of *olm* genes.

Discussion

We describe here a detailed functional analysis of the *aveR* gene, and demonstrate that *aveR* encodes a pathway-specific positive regulator required for transcription of all the avermectin biosynthetic genes by direct interaction with *ave* promoters. We also showed that *aveR* exerts a negative role on oligomycin production, but does not affect the growth of *S. avermitilis*. A very recent paper of Kitani et al. (2009) performed a number of similar experiments. As described in the following section only some results could be confirmed here.

Attempts to express high level of full-length AveR protein for purification were unsuccessful in both *E. coli* and *S. avermitilis*. The *aveR* gene was cloned in a high-copy-number vector under control of a strong promoter: T7 promoter in *E. coli* to express His6-tagged AveR or *tipA* promoter in *S. avermitilis* to express native AveR. Both approaches yielded a very low level of the target protein, and MBP (maltose-binding protein) fusion protein system (BioLabs) in *E. coli* also failed. These results could not be explained by the presence of unusual codon TTA in the *aveR* gene, since the replacement of *aveR* gene in the

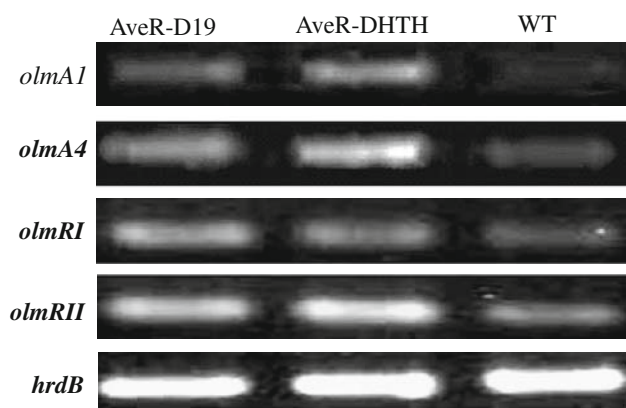


Fig. 8 Semiquantitative RT-PCR analyses of the *olmA1*, *olmA4*, *olmRI*, and *olmRII* transcript levels. Samples were collected from *S. avermitilis* wild-type strain, AveR-D19 and AveR-DHTH grown in fermentation medium II for 40 h

expression vector with mutated gene *aveR** containing TTA-to-CTG mutation did not lead to production of enough AveR protein for purification. Another possibility is that the large size of the AveR protein is a “bottleneck” in the translation process. We are not aware of any examples of intact LAL regulators in *Streptomyces* purified through overexpression. This makes it more difficult to understand the regulation mechanism of this novel regulatory protein family. A high level of truncated form of AveR containing the C-terminal HTH domain (AveRc), fused to a hexahistidine tag at its N-terminus, was therefore expressed as inclusion body, and was used for preparation of anti-AveR antibodies for ChIP assay. ChIP assay identified four AveR target promoter regions, all located within the *ave* cluster. The finding that AveR without HTH domain is unable to bind *ave* promoters demonstrated the DNA-binding activity of AveR HTH domain. The promoter regions (intergenic regions) identified in this study were predicted to contain divergent promoters. Sequence alignment of the promoter regions bound by AveR did not predict a consensus AveR-binding sequence; more experiments are needed to reveal the binding sites of AveR.

Overexpression studies with an extra copy of *aveR* containing TTA-to-CTG mutation, expressed under the control of strong constitutive promoter *ermE*_p* in wild-type *S. avermitilis* ATCC31267, demonstrated a positive effect of the *aveR* gene on avermectin production. This finding was further confirmed by the facts that multi-copies of *aveR* as well as an extra copy of *aveR* with its native promoter promoted avermectin production in ATCC31267. Our results are in contrast to the findings of Kitani et al. (2009), who reported that a higher amount of *aveR* resulted in complete loss of avermectin in wild-type strain KA320 and its spontaneous mutant K139. These authors disrupted *aveR* by gene replacement in *S. avermitilis*, and the resulting mutant lost avermectin productivity and was

unable to convert an avermectin intermediate to any avermectin derivatives. They concluded that AveR is a positive regulator for avermectin biosynthesis, but they postulated a maximum threshold concentration of *aveR* for the production of avermectin. The differing behavior of *aveR* may reflect differences in the parental strains used and the growth media adopted in the respective studies. They described KA320 as a wild-type strain isogenic to MA-4680 and since ATCC31267 is designated MA-4680, there should be no obvious difference in genetic background between strain KA320 and ATCC31267. However, they described that strain KA320 is phenotypically unstable and frequent reisolation is necessary; thus the spontaneous mutant K139 from KA320 is used as the major wild-type strain in their study, whereas the strain ATCC31267 was not so unstable during our experiments. The linear chromosome of *Streptomyces* easily undergoes large rearrangement, such as large deletions, circularization, arm replacement, and amplifications in terminal regions. We therefore analyzed the chromosomal structure of ATCC31267 by pulsed-field gel electrophoresis (PFGE) (Fig. S2) and showed that its *AseI* restriction patterns were consistent with the published data (<http://avermitilis.lskitasato-u.ac.jp/physicalmap>), suggesting no large rearrangement occurred in the chromosome of our strain. We sequenced the entire *aveR* gene and its promoter region of ATCC31267 again, but did not find any difference with the published sequence. As KA320 was so unstable, we are not sure if chromosomal rearrangement occurred in this strain and its mutant K139 used by Kitani et al. (2009). The discrepancies among these results still remain to be studied.

Like *aveR*, *olmRI* and *olmRII* are two putative pathway-specific regulatory genes belonging to the LAL family. Disruption of *olmRI* or *olmRII* resulted in loss of oligomycin production (our unpublished data), suggesting that *OlmRI* and *OlmRII* are positive regulators for oligomycin biosynthesis. The *aveR* mutants enhanced yield of oligomycin, and the transcript levels of *olm* structural and regulatory genes were consistent with the production of oligomycin, suggesting the possibility that the negative effect of *aveR* on oligomycin biosynthesis is mediated by regulatory gene *olmRI* and *olmRII*. However, ChIP results indicated that the AveR protein could bind putative *olmA1* and *olmA4* promoters as well as *olmRI* and *olmRII* promoters. Several tetratricopeptide repeats (TPRs), which are implicated in protein–protein interactions, were predicted in AveR, *OlmRI*, and *OlmRII*. It seems likely that the various LAL regulators present in the same bacterial cell may communicate with each other and form a complex regulatory network. Thus, AveR may repress *olm* genes by interacting with other proteins, such as *OlmRI* and *OlmRII*. Another possibility is that AveR affects the expression of *olm* genes through interacting directly with their promoters.

More experiments will be needed to clarify the regulatory mechanism of AveR on oligomycin biosynthesis. Furthermore, we still could not rule out the possibility that carbon flux contributes to the increased oligomycin production. Avermectin and oligomycin belong to type I polyketides, and both require acetate and propionate extender units for synthesis of polyketide backbone (Ikeda et al. 1999, 2001; Omura et al. 2001). Therefore, loss of avermectin production in *aveR* mutants might allow additional acetate and propionate to enter the biosynthetic pathway of oligomycin and stimulate oligomycin production.

aveR is the first pathway-specific regulatory gene characterized in *S. avermitilis*, and the present work also showed the pleiotropic effects of *aveR* on oligomycin biosynthesis. These results will contribute to elucidation of other pathway-specific regulatory factors and regulatory mechanisms of secondary metabolism in *S. avermitilis*. This knowledge will be useful in improving and modifying methods for production of secondary metabolites, including avermectin and oligomycin, by this industrially important bacterial strain.

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