The autoregulator receptor homologue AvaR3 plays a regulatory role in antibiotic production, mycelial aggregation and colony development of *Streptomyces avermitilis*

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The γ-butyrolactone autoregulator receptor has been shown to control secondary metabolism and/or morphological differentiation across many *Streptomyces* species. *Streptomyces avermitilis* produces an important anthelmintic agent (avermectin) and two further polyketide antibiotics, filipin and oligomycin. Genomic analysis of *S. avermitilis* revealed that this micro-organism has the clustered putative autoregulator receptor genes distant from the antibiotic biosynthetic gene clusters. Here, we describe the characterization of *avaR3*, one of the clustered receptor genes, which encodes a protein containing an extra stretch of amino acid residues that has not been found in the family of autoregulator receptors. Disruption of *avaR3* resulted in markedly decreased production of avermectins, with delayed expression of avermectin biosynthetic genes, suggesting that AvaR3 positively controls the avermectin biosynthetic genes. Moreover, the disruption caused increased production of filipin without any changes in the transcriptional profile of the filipin biosynthetic genes, suggesting that filipin production is indirectly controlled by AvaR3. The *avaR3* disruptant displayed fragmented growth in liquid culture and conditional morphological defects on solid medium. These findings demonstrated that AvaR3 acts as a global regulator that controls antibiotic production and cell morphology.

INTRODUCTION

The genus *Streptomyces* consists of Gram-positive, soil-dwelling bacteria characterized by a complex developmental life cycle and the ability to produce a variety of secondary metabolites, including antibiotics with important applications in human and veterinary medicine, and agriculture. The initiation of secondary metabolism typically coincides with the post-exponential phase in liquid-grown culture and with the onset of aerial mycelium formation on solid culture. One of the well-known regulatory systems for secondary metabolism is the signalling system with the γ-butyrolactone autoregulator as a small diffusible signalling molecule (Bibb, 2005; Takano, 2006). In this system, binding of the autoregulator to its cognate receptor causes the receptor to lose DNA-binding activity toward autoregulatory elements (AREs), which are frequently located in the promoter regions of target genes (Folcher *et al.*, 2001), and in turn activates transcription of the target genes, which results in the coordinated expression of regulatory and biosynthetic genes involved in secondary metabolism. The autoregulator signalling system is involved not only in the onset of biosynthesis of secondary metabolites, but also occasionally in the onset of morphological differentiation (Folcher *et al.*, 2001; Healy *et al.*, 2009; Horinouchi, 2007).

To date, many genes encoding autoregulator receptors and their homologues have been reported to control antibiotic production, and the following three groups are recognised based on their gene loci and properties in the regulatory system. First, an autoregulator receptor gene, often accompanied by genes encoding a homologue of the autoregulator receptor, is found within an antibiotic...
biosynthetic gene cluster, and both of them operate at several regulation layers of biosynthesis of the cognate antibiotic. This type of regulation is regarded as a pathway-specific system for antibiotic production. In *Streptomyces virginiae*, BarA, a γ-butyrolactone autoregulator receptor, directly controls the transcription of barB, which encodes a receptor homologue (Kinoshita et al., 1997; Nakano et al., 2000). Multiple regulatory genes under the control of BarA and BarB coordinate the expression of virginiamycin biosynthetic genes, which lie in the flanking regions of the barA and barB genes (Matsumo et al., 2004; Pulsawat et al., 2007, 2009). Second, a gene encoding an autoregulator receptor in the gene cluster for antibiotic biosynthesis simultaneously controls production of both the corresponding antibiotic and another antibiotic whose biosynthetic genes are distal to the locus of the receptor gene. Representative regulators of this type are ScbR (SCO6265) and ScbR2 (SCO6286) of *Streptomyces coelicolor* A3(2), which regulate the expression of a cryptic type I polyketide biosynthetic gene cluster (SCO6269–SCO6288) by repressing a pathway-specific regulatory gene and indirectly stimulate production of actinorhodin and undecylprodigiosin (Gottelt et al., 2010; Takano et al., 2005). Third, an autoregulator receptor gene has no adjacent biosynthetic gene clusters but regulates the expression of antibiotic biosynthetic genes that are located apart from the locus of the receptor gene, as exemplified by ArpA of *Streptomyces griseus*. ArpA modulates self-resistance and biosynthesis of streptomycin and other metabolites in concert with aerial mycelium formation by controlling the pleiotropic transcriptional regulator AdpA in the A-factor regulatory cascade (Horinouchi, 2007).

*Streptomyces avermitilis* produces a family of polyketide macrocyclic lactones known as avermectins, which have potent antiparasitic and broad-spectrum activities against nematodes and arthropod parasites (Burg et al., 1979), together with two other families of polyketide antibiotics, filipins and oligomycins. The biosynthetic pathways of the three types of antibiotic have been predicted and partially elucidated (Ikeda et al., 1999; Omura et al., 2001), while genetic information on the regulatory mechanism of antibiotic production has so far been limited. With respect to avermectin production, we recently reported that AveR, which is encoded by a gene situated on the left-hand extremity of the avermectin biosynthetic gene cluster, is a positive LAL-family regulator for controlling avermectin biosynthetic genes (Kitani et al., 2009). Further understanding of the mechanisms by which avermectins and other antibiotics are regulated is necessary to achieve a high-yield production of antibiotics as well as to arrest the production of unwanted secondary metabolites. In this study, we characterized avaR3, which encodes a γ-butyrolactone autoregulator receptor homologue, and demonstrated that AvaR3 functions as a global regulator for avermectin production and cell morphology both in liquid culture and on solid medium.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** *S. avermitilis KA320* (isogenic to MA-4680) was obtained from the culture collection of the Kitasato Institute. *Escherichia coli* DH5α was used for general DNA manipulation, and *E. coli* F′ dcm Δ(srl-recA)306::Tn10 carrying pUB307-aph::Tn7 was used for *E. coli* Streptomyces conjugation. *E. coli* strains were grown in 2× yeast extract-trypptone (YT) medium, with appropriate antibiotics when necessary. pKU451, pKU474 (Supplementary Fig. S1) and pKU250 (Komatsu et al., 2010) were used to construct a vector for gene disruption. For complementation, pENTR (Invitrogen) was used for DNA cloning, and pSET152 (Bierman et al., 1992) was used to introduce DNA into *Streptomyces*. For DNA manipulation in *Streptomyces*, standard procedures were performed according to Kieser et al. (2000). The *Streptomyces* strains were grown at 28°C on YMS medium (Ikeda et al., 1987) supplemented with 10 mM MgCl₂ and 10 mM CaCl₂ (YMS-MC medium) for sporulation. ISP medium (Becton Dickinson) was used for intergenic conjugation from *E. coli* into *Streptomyces*. Spores (7.0×10⁸ c.f.u.) of *S. avermitilis* strains were inoculated into 70 ml APM medium (Kitani et al., 2009) in a 500 ml baffled flask, and mycelia were harvested after 48 h of cultivation. The mycelia were washed, resuspended in fresh APM medium and stored at −80°C until use as a seed culture. All the primers are listed in Supplementary Table S1.

**Construction of the avaR3 deletion mutant.** A segment upstream of *avaR3* (nucleotides 4582958–4584926) was amplified by the primer pair avaR3-up-Fw-avaR3-up-Re, while a segment downstream of *avaR3* (nucleotides 4580176–4582218) was amplified by the primer pair avaR3-dw-Fw-avaR3-dw-Re. The two resultant segments were digested with HindIII and SpeI, and inserted together into the HindIII site of pKU451, resulting in pLT341. The fidelity of the amplified region was confirmed by DNA sequencing. A 1.7 kb DNA fragment containing a kanamycin-resistant gene marker (amplified by the primer pair aph-Fw/aph-Re using pKU474 as template) was digested by SpeI and cloned into the SpeI site of pLT341 to yield pLT342. A 5.7 kb HindIII fragment, recovered from pLT342, was transferred into pKU250 at the HindIII site, thereby yielding the *avaR3*-disruption plasmid pLT343. The resultant plasmid was introduced by intergenic conjugation into *S. avermitilis* KA320, and the wild-type gene was replaced with the disrupted allele by homologous recombination. The genotype of candidates for the *avaR3* deletion was confirmed by PCR.

**Complementation of the avaR3 mutant.** A Gateway Reading Frame Cassette C1.1 (Invitrogen) was cloned into the EcoRV site of pSET152 to yield pLT113 as a destination vector. A 1.2 kb DNA fragment (nucleotides 4581968–4583157) containing the entire *avaR3* gene and the *avaR3* upstream region was PCR-amplified by the primer pair *avaR3*-comp-Fw-avaR3-comp-Re, and then cloned into a pENTR vector according to the manufacturer's instructions to generate an entry clone. The entry clone was used with pLT113 in an LR reaction (LR Clonase Enzyme Mix, Invitrogen), resulting in pLT362. By intergenic conjugation, pLT362 was introduced into the *S. avermitilis* strain. The correct integration in the exconjugants was confirmed by PCR.

**Analysis of antibiotic production.** The seed culture was inoculated into 70 ml APM medium in a 500 ml baffled flask at a final OD₆₀₀ of 0.3, and cultivated at 28°C. The culture broth was sampled periodically, and mixed with an equal volume of methanol. After centrifugation, the supernatant was analysed by HPLC system as described previously (Kitani et al., 2009). The amounts of four avermectin components, A1a, A2a, B1a and B2a, were quantified using an authentic sample of avermectins. For filipin III production, the supernatant was separated and quantified by reverse-phase HPLC.
Transcriptional analysis by RT-PCR. Total RNA was prepared from mycelium grown in APM medium by using an RNeasy Mini kit (Qiagen), and treated with DNase I (Takara Bio). The cDNA was synthesized using SuperScript III RNase H− Reverse Transcriptase (Invitrogen) and Random Primers (Invitrogen) according to the manufacturer’s instructions. The PCR amplification was performed by using GoTaq Green Master Mix (Promega KK) under the following conditions: 95 °C for 10 min, followed by 40 cycles consisting of 15 s at 95 °C for denaturation, and 1 min at 65 °C for annealing and extension. A final dissociation stage was run to generate a melting curve and consequently verify the specificity of the amplification products. Gene expression was measured in triplicate and normalized to the mRNA level of the rpoD gene (sav2444) using the relative standard curve method.

Determination of transcriptional start points. A GeneRacer kit (Invitrogen) was used with total RNA extracted from 48 h mycelium for rapid amplification of 5′ cDNA ends (RACE) according to the manufacturer’s instructions without dephosphorylation of RNA. The cDNA was synthesized using SuperScript III RNase H− Reverse Transcriptase and Random Primers. The first round of RACE-PCR was performed with a gene-specific reverse primer and the GeneRacer 5′ Primer using GoTaq Green Master Mix. The second round of RACE-PCR was carried out with a gene-specific nested primer and the GeneRacer 5′ Nested Primer using the initial PCR product as template. PCR products were cloned into a pCR4-TOPO vector (Invitrogen) for sequencing.

RESULTS

Effects of avaR3 disruption on antibiotic production in S. avermitilis

A homology search with a FASTA program against the S. avermitilis genome demonstrated that there are three homologues of autoregulator receptor genes (sav3702, sav3703 and sav3705). The deduced gene product of sav3705, designated avaR1 (S. avermitilis autoregulator receptor gene 1), showed the highest sequence similarity (39–48 % identity) to γ-butyrolactone autoregulator receptors, such as ArpA, BarA and ScbR. Notably, two homologue genes (sav3702 and sav3703), whose products showed moderate similarity (25–34 % identity) to the autoregulator receptors but much higher similarity (36–48 % identity) to receptor homologues (BarB and ScbR2), were found to be present in the adjacent region of the avaR1 gene (Fig. 1a). Thus, these genes were designated avaR2 and avaR3, respectively. Sequence alignment with known autoregulator receptors (Fig. 1b) revealed that the predicted products of the three avaR genes had two features: a highly conserved helix–turn–helix (HTH) DNA-binding domain at the N terminus (Pfam00440), and a conserved tryptophan residue at the centre of proteins, which is proposed to be an important amino acid residue for the function of the autoregulator receptor in responding to the binding of the γ-butyrolactone autoregulator (Natsume et al., 2004). These findings indicated that genes encoding homologues of autoregulator receptors are clustered at the same locus in S. avermitilis (nucleotides 4,581,428–4,585,275), suggesting that they are probably involved in the regulation of secondary metabolism and/or morphological differentiation.

Unlike typical domain structures of the γ-butyrolactone autoregulator receptor, the predicted AvaR3 protein has an extra stretch of 75 aa residues between the HTH DNA-binding domain and the conserved tryptophan residue (Fig. 1b). Such an extra stretch is very rare and only known in AlpW (stretch of 24 aa residues) of Streptomyces ambofaciens among the autoregulator receptors (Bunet et al., 2011). A search of the current database demonstrated that there is no protein that contains the stretch possessed by AvaR3. The nucleotide sequence encoding the 75 aa stretch had a relatively low G+C content at the third letter of the codon, i.e. 73 % versus an average of 92 % in Streptomyces genes (Ishikawa & Hotta, 1999). The avaR3 gene was constitutively expressed throughout cultivation (Fig. 1c), suggesting that AvaR3 may have a physiological function. In order to investigate the in vivo role of avaR3 in S. avermitilis, an avaR3 deletion (ΔavaR3) mutant was constructed by allelic replacement. With respect to antibiotic production, avermectin production of the wild-type strain was readily detectable after 72 h of cultivation and increased until 120 h of cultivation, whereas the ΔavaR3 mutant produced trace amounts of avermectins (less than 1 % of the amount produced by the wild-type strain) (Fig. 2a). In contrast, the ΔavaR3 mutant showed a sevenfold increase in production of filipin III at 72 h of cultivation in comparison with that of the wild-type strain (Fig. 2b). With respect to production of oligomycin A, there was no difference between the wild-type strain and the ΔavaR3 mutant (data not shown). Complementation of the ΔavaR3 mutant, with the genome-integrative plasmid pLT362 containing the entire avaR3 gene and its upstream region, restored the production of avermectins and filipin III to the level of the wild-type strain (Fig. 2a, b). These results clearly indicated that AvaR3 functions as a positive regulator of avermectin production and has a negative effect on filipin production.
Influence of AvaR3 on the transcription of avermectin biosynthetic genes

To elucidate the function of AvaR3 in transcriptional regulation of antibiotic biosynthetic genes, the pattern of gene expression was examined by RT-PCR analysis (Fig. 3). All of the investigated avermectin biosynthetic genes were found to be transcribed in the wild-type strain at 48 h of cultivation, and avermectin production began several hours after the 48 h cultivation time point. However, in the ΔavaR3 mutant, the expression at 48 h of five biosynthetic genes, aveA1, aveA4, aveBI, aveBIII and aveBVIII, still remained at low levels similar to those observed at 24 h of cultivation, while the other three genes, aveD, aveBIV and aveG, were transcribed normally. At 72 h of cultivation, the transcriptional levels of these five genes were elevated. The avaR3-complemented exconjugant showed the same temporal expression pattern of the avermectin biosynthetic genes as the wild-type strain, suggesting that AvaR3 upregulates the expression of a part of the ave cluster. AveR positively controls the expression of both polyketide biosynthetic and post-polyketide modification genes in avermectin biosynthesis (Kitani et al., 2009). Reduced amounts of aveR transcripts were also detected throughout the cultivation of the ΔavaR3 mutant, in contrast to the results with the wild-type strain. This result indicated that AvaR3 is necessary to expedite the transcription of aveR, implying that the remarkable decrease of avermectin production is due to a lower and delayed transcription of the avermectin biosynthetic genes regulated by aveR. Surprisingly, major filipin biosynthetic genes (pte), including the putative pathway-specific regulatory genes pteF and pteR, were transcribed in the ΔavaR3 mutant in a manner similar to that of the wild-type strain (Supplementary Fig.

**Fig. 1.** Organization of the avaR genes in *S. avermitilis* at nucleotides 4 581 428 to 4 585 275 (a), sequence alignment of the N-terminal regions of AvaR proteins with those of γ-butyrolactone autoregulator receptors (b), and transcriptional analysis of the avaR3 gene in the wild-type strain (c). (a) Bent arrows indicate transcriptional units of the avaR genes. (b) The amino acid sequence alignment was created using CLUSTAL W. The numbers indicate the amino acid positions within each sequence. The secondary structure elements of CprB are shown above its sequence. The filled triangle indicates an important residue for conformational changes of the autoregulator receptor upon binding of the γ-butyrolactone autoregulator, as proposed by Natsume et al. (2004). Black and grey boxes in the alignment indicate positions at which the same amino acid is found in at least seven and five of the eight sequences, respectively. CprB (BA238748) and ScbR (CA07628) from *S. coelicolor* A3(2); AvaR1 (BAC71417), AvaR2 (BAC71414) and AvaR3 (BAC71415) from *S. avermitilis*; ArpA (BAA36282) from *S. gliseus*; BarA (BA06981) from *S. virgineus*; Ty1P (AAD40801) from *Streptomyces fradiae*. (c) Total RNA was prepared from mycelium at the indicated cultivation times. PCR with 25 cycles of amplification was used for transcripts of the avaR3 and rpoD genes. The rpoD gene was used as a control.
S2), in spite of the overproduction of filipin in the ΔavaR3 mutant. This observation suggested that AvaR3 has a negative effect on the production of filipin without transcriptional control of the pte gene cluster.

**Antibiotic production of the ΔavaR3 mutant on solid medium**

To investigate the contribution of avaR3 to the regulation of antibiotic production on solid medium, we assessed the production of avermectin, filipin and oligomycin on APM agar medium and YMS-MC medium (Table 1). In the cultivation on APM agar medium, the wild-type strain produced 74 μg avermectins (ml medium)$^{-1}$, whereas the ΔavaR3 mutant showed a 79% reduction (16 μg ml$^{-1}$) in avermectin production. Meanwhile, filipin production of the ΔavaR3 mutant increased to a level 3.3-fold greater than that of the wild-type strain. No differences in oligomycin production were detected between the wild-type strain and the ΔavaR3 mutant (data not shown). These phenotypic changes attributable to the avaR3 mutation were partially restored by introduction of the intact avaR3 gene. Similarly, cultivation of the ΔavaR3 mutant on YMS-MC medium resulted in a 69% decrease of avermectin production and a 1.7-fold increase of filipin production with no change in oligomycin production. Under these medium conditions, the changes in avermectin and filipin production were restored to the wild-type level by the introduction of the intact avaR3 gene. These phenomena on solid medium were similar to the antibiotic production in liquid cultivation, indicating that AvaR3 is also involved in the production of avermectins and filipin III on solid medium.

**avaR3 mutation affects cell morphology on solid culture and in liquid culture**

Some γ-butyrolactone autoregulator receptors control both morphological differentiation and antibiotic production (Folcher et al., 2001; Healy et al., 2009; Horinouchi, 2007). To clarify whether AvaR3 is involved in the morphological control of *S. avermitilis*, we carefully examined morphological characteristics of the ΔavaR3 mutant on a variety of solid media. On YMS-MC medium, where the ΔavaR3

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**Fig. 2.** Production profiles of avermectin (a) and filipin (b) in the wild-type strain (■), ΔavaR3 mutant (○) and avaR3-complemented exconjugant (▲) in liquid culture. Error bars, SD from triplicate cultures.

**Fig. 3.** Gene expression analysis by RT-PCR of avermectin biosynthetic genes in the wild-type strain (WT), ΔavaR3 mutant (ΔavaR3) and avaR3-complemented exconjugant (ΔavaR3/avaR3). Total RNA was isolated from mycelia harvested at 24, 48 and 72 h of cultivation. Avermectin production started between 48 and 72 h of cultivation. In the PCR, the following numbers of amplification cycles were used: 27 cycles for the aveR and aveBIII transcripts, 25 cycles for the rpoD transcript, and 30 cycles for the transcripts of the other genes.
mutant showed less avermectin production and more filipin production, no differences in morphology were detected between the wild-type strain and the ΔavaR3 mutant. On APM agar medium, however, the ΔavaR3 mutant showed a clear defect in aerial mycelium formation at the time point at which the wild-type strain produced an abundant lawn of aerial mycelium, although the ΔavaR3 mutant grew normally until the stage of substrate mycelium (Supplementary Fig. S3), suggesting that the process of aerial mycelium formation is most likely impaired in the ΔavaR3 mutant. To determine the effect of avaR3 disruption on development more quantitatively, the degree of sporulation was investigated by counting a number of spores. Fig. 4(a) demonstrates that the number of spores in the ΔavaR3 mutant was 5.8-fold lower than that of the wild-type strain, indicating the clear involvement of AvaR3 in morphological development. Complementation by intact avaR3 restored sporulation, but only partially (35% of the wild-type strain). We suggested that integration at the attB site might have an adverse effect. In fact, integration of an empty vector alone (ΔavaR3/pSET152; Fig. 4a) impaired sporulation severely. A similar adverse effect due to attB site integration was also observed on avermectin production (Table 1) when the mutant strain was cultured on APM agar medium alone, suggesting that avaR3 and the attB site adjacent gene(s) may have a similar level of importance in antibiotic production and morphological development, when grown on APM agar medium.

In liquid culture, the avaR3 mutation showed a 1.7- to 1.9-fold increase in the OD_{600} value after 48 to 72 h cultivation, respectively (data not shown), although the dry cell weight of the ΔavaR3 mutant was lower than that of the wild-type strain after 72 h of cultivation (Fig. 4b). Micrographs (Fig. 4c) revealed that the ΔavaR3 mutant possessed more fragmented mycelia, with a remarkable decrease in the mean diameter of the pellet (73 ± 6 μm), while the wild-type strain produced typical large pellets with a mean diameter of 154 ± 14 μm, indicating that the increased OD_{600} value of the ΔavaR3 mutant would have resulted from the increased dispersion due to the decreased pellet size during cultivation, and that AvaR3 may play an important role in mycelial aggregation in liquid culture.

Transcriptional start points of the avaR genes

ARE sequences, to which γ-butyrolactone autoregulator receptor proteins typically bind, are frequently found in the promoter region of their own genes as well as those of the direct target genes (Folcher et al., 2001). To analyse the promoter region of avaR3, 5’-RACE analysis was carried out (Supplementary Fig. S4), which revealed that the transcriptional start point (tsp) of avaR3 was A, situated 25 nt upstream from the translational start codon of avaR3 (Fig. 5a). In front of the tsp, a possible −10 region was identified, which was similar to the consensus −10 region of streptomycetes Esr70-like promoters, although no typical −35 region was detected (Kang et al., 1997; Strohl, 1992). As shown in Fig. 5a (a), an ARE-like sequence was found at −36 to −11 nt from the tsp of avaR3, and designated avaR3-ARE.

Similarly, avaR1-tsp was located at G, 102 nt upstream of the translational start codon, and avaR2-tsp at T, 43 nt upstream of the translational start codon (Fig. Sb, c and Supplementary Fig S4), and both possessed probable promoters, resembling the consensus −10 region of streptomycetes Esr70-like promoters (avaR1), or −10 and −35 regions of the σ^{bdh}_{bdh}-type promoters of S. coelicolor A3(2) (avaR2). As shown in Fig. 5(b, c), ARE-like sequences were identified in the upstream regions of these two genes as avaR1-ARE, which was situated at −93 to −68 nt from the avaR1-tsp, and as avaR2-ARE, which covers both the avaR2-tsp and the putative −10 region.

Regarding the gene organization, avaR3 runs in the same direction as the upstream avaR1 and cyp17 (sav3704) (Fig. 1a), which encodes a cytochrome P450 hydroxylase, CYP154B2 (Lamb et al., 2003). Because no plausible transcriptional terminator exists in the 3′ regions of cyp17 and avaR1, avaR3 may form a tricistronic operon with cyp17 and avaR1. RT-PCR analysis using RNA from the 48 h mycelia (Supplementary Fig. S5) detected transcripts containing the intergenic regions of cyp17–avaR3 and

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**Table 1. Antibiotic production of the S. avermitilis wild-type strain, a ΔavaR3 mutant and an avaR3-complemented exconjugant on solid media**

<table>
<thead>
<tr>
<th>Strain</th>
<th>APM agar</th>
<th>YMS-MC</th>
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<tbody>
<tr>
<td></td>
<td>Avermectins</td>
<td>Filipin III</td>
</tr>
<tr>
<td>Wild-type</td>
<td>74.0 ± 3.0</td>
<td>3.2 ± 0.7</td>
</tr>
<tr>
<td>ΔavaR3</td>
<td>15.8 ± 1.0</td>
<td>10.4 ± 3.2</td>
</tr>
<tr>
<td>ΔavaR3/pSET152</td>
<td>1.8 ± 0.6</td>
<td>10.9 ± 0.8</td>
</tr>
<tr>
<td>ΔavaR3/avaR3</td>
<td>31.2 ± 0.7</td>
<td>8.0 ± 0.5</td>
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</tbody>
</table>

*Data shown are mean±sd from triplicate samples.
avaR1–cyp17, and the tricistronic transcript. This finding, together with the fact that 5’-RACE analysis did not yield any distinct bands of the cyp17 transcript, led us to conclude that avaR3 is transcribed as a monocistronic mRNA and a tricistronic avaR1–cyp17–avaR3 mRNA. The presence of ARE in all three avaR genes suggested that AvaR3 might transcriptionally control the expression of itself, avaR1 and avaR2 via binding to AREs.

Transcriptional control by AvaR3 of the avaR genes

To examine whether AvaR3 regulates the transcription of the avaR genes, qRT-PCR analysis was performed as shown in Fig. 6. The mRNA level of avaR3 increased significantly in the ΔavaR3 mutant compared with that in the wild-type strain (2.8- to 4.8-fold), indicating that an autoregulatory circuit is present in avaR3 transcription. Increased mRNA levels of avaR1 and avaR2 in the ΔavaR3 mutant led us to conclude that AvaR3 also negatively regulates the transcription of both avaR1 and avaR2. These results indicated that all the avaR genes are under the control of AvaR3. avaR1 disruption did not give any of the phenotypic changes observed in the ΔavaR3 mutant (Supplementary Fig. S6), indicating that AvaR3 controls antibiotic production as well as cell morphology independently of AvaR1. Further work will be needed to find out whether AvaR2 participates in AvaR3-dependent phenomena.

DISCUSSION

The γ-butyrolactone autoregulator receptor and its homologues are distributed widely among streptomycetes, and control antibiotic production and/or morphological
differentiation. In this study, we have characterized AvaR3, an autoregulator receptor homologue in *S. avermitilis*, and have shown that it is responsible for the production of avermectin and filipin, and for the control of cell morphology, both in liquid medium and on solid medium. These findings led to the idea that AvaR3 is an activator-type global regulator for secondary metabolism and morphogenesis, except for filipin production (see Discussion below). In *S. griseus*, ArpA stimulates the onset of secondary metabolism and morphological differentiation in response to A-factor through transcriptional regulation of AdpA, a central transcriptional activator. Furthermore, a few AdpA homo-

![Fig. 5. Nucleotide sequences of the upstream regions of avaR3 (a), avaR1 (b) and avaR2 (c). The solid triangle indicates the transcriptional start point. The probable −35 and −10 regions are shown by dashed lines, and the putative ribosome-binding sites (RBSs) are shown in boxes. The putative AvaR3-binding sequences are indicated by solid lines.](image)

![Fig. 6. Gene expression analysis of the avaR3, avaR1 and avaR2 genes by qRT-PCR. WT, wild-type strain; ΔavaR3, ΔavaR3 mutant; ΔavaR3/avaR3, avaR3-complemented exconjugant. Total RNA was extracted from mycelia harvested at the indicated cultivation times. Fold-change is relative to the expression of each gene in the wild-type strain at 24 h of cultivation. The avaR3 transcript corresponding to +1 to +58 nt from the transcriptional start point of avaR3 was quantified. ND, The mRNA level of avaR3 was not determined in the avaR3-complemented strain, because the amplified regions upstream of avaR3 are present at two loci. Error bars, SD from triplicate experiments.](image)
logues in other _Streptomyces_ strains have been shown to be pleiotropic regulators for secondary metabolism and morphological differentiation (López-García _et al._, 2010; Pan _et al._, 2009). However, the transcription of _bdpA_ (sav5261), a plausible _adpA_ orthologue of _S. avermitilis_, is not influenced by the _avaR3_ disruption (data not shown). Thus, this finding indicates that _AvaR3_ has a _bdpA_-independent pathway for controlling avermectin production and cell morphology, suggesting that _S. avermitilis_ has a novel regulatory system via autoregulator receptors that globally regulates two representative features of streptomycetes.

In the usual pattern of antibiotic regulation, a gene encoding a γ-butyrolactone autoregulator receptor resides inside or adjacent to an antibiotic biosynthetic gene cluster and exerts a negative effect on antibiotic production by binding of the receptor protein to the promoter region of the in-cluster pathway-specific regulatory gene (Bunet _et al._, 2008; Cundliffe, 2008). It was thus of interest that _AvaR3_ regulates the production of avermectin, whose biosynthetic gene cluster is 3.4 Mb away from the _avaR3_ locus. Moreover, although _AvaR3_ facilitates the expression of _aveR_, a pathway-specific regulatory gene for avermectin biosynthesis, no plausible ARE sequence is located in the region upstream of the _aveR_ gene, suggesting that _AvaR3_ is likely to control the transcription of _aveR_ without direct binding to the promoter.

In spite of the increased expression of avermectin biosynthetic genes at the late growth stage, the _ΔavaR3_ mutant still showed little production of avermectin and increased filipin production with no change of transcription of filipin biosynthetic genes. Because the biosynthetic pathways of both avermectin and filipin require malonyl-CoA and methylmalonyl-CoA as a common extender unit of the polyketide backbone (Ikeda _et al._, 1999; Omura _et al._, 2001), it is most likely that the delayed expression of the _ave_ genes, attributed to the delayed and weakened transcription of _aveR_ as the result of _avaR3_ disruption, would lead to the preferential use of precursors in filipin production at the middle growth stage, resulting in increased filipin production and a lack of the necessary precursors for avermectin production at the late growth stage. Regarding oligomycin production, although oligomycin biosynthesis shares common precursors (malonyl-CoA and methylmalonyl-CoA) with avermectin and filipin biosynthesis (Wei _et al._, 2006), because oligomycin production already reached a plateau before the onset of avermectin production in the wild-type strain (Supplementary Fig. S6), the surplus precursors resulting from the delayed expression of _ave_ genes in the _ΔavaR3_ mutant had no influence on oligomycin production. Thus, _AvaR3_ appears to act as a transcriptional activator for avermectin production by indirectly controlling the expression of _aveR_.

To date, the molecular mechanisms underlying mycelial aggregation in liquid cultivation remain poorly understood. Recently, two factors have been reported to be involved in the process of mycelial aggregation: SsgA, which is an activator of sporulation-specific cell division (Traag & van Wezel, 2008), and CsA, which is involved in polysaccharide synthesis at the outer surface of hyphae (de Jong _et al._, 2009). The enhanced expression of _sga_ leads to mycelial fragmentation in _S. coelicolor A3(2)_ and _S. griseus_ (Kawamoto _et al._, 1997; van Wezel _et al._, 2000), whereas the _csA_ mutation reduces the degree of mycelial aggregation in _S. coelicolor A3(2)_(Xu _et al._, 2008). We have demonstrated in this study that the _avaR3_ mutation results in fragmented growth in liquid culture. Putative genes encoding the SsgA and CsA orthologues are present in the genome of _S. avermitilis_ as SAV_4267_ and SAV_5219_, respectively. The transcriptional profile of these two genes in the _ΔavaR3_ mutant was nearly identical to that of the wild-type strain (data not shown), suggesting that _AvaR3_ regulates mycelial aggregation independently of an SsgA- and CsA-dependent pathway. To the best of our knowledge, this is the first report to note that an autoregulator receptor homologue participates in both mycelial clump formation in liquid culture and aerial mycelium formation on solid medium.

The completed _Streptomyces_ genome sequences have demonstrated that the putative receptor genes are frequently far from the antibiotic biosynthetic gene clusters, although the majority of reports on autoregulator receptors have so far concentrated on their pathway-specific functioning. Thus, it is expected that further understanding of the regulatory mechanism of _AvaR3_ will lead not only to higher yields in antibiotic production but also to the ability to awaken silent gene clusters for the discovery of novel natural compounds.

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