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.

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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

Abbreviations: HTH, helix–turn–helix; qRT-PCR, quantitative RT-PCR; RACE, rapid amplification of 5' cDNA ends.

Six supplementary figures and a supplementary table are available with the online version of this paper.

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 coordinately regulate the expression of virginiamycin biosynthetic genes, which lie in the flanking regions of the *barA* and *barB* genes (Matsuno *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* DH5 α 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 $\times$ yeast extract-tryptone (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 4 (Becton Dickinson) was used for intergenic conjugation from *E. coli* into *Streptomyces*. Spores (7.0×10^8 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 *Hind*III and *Spe*I, and inserted together into the *Hind*III 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 *Spe*I, and cloned into the *Spe*I site of pLT341 to yield pLT342. A 5.7 kb *Hind*III fragment, recovered from pLT342, was transferred into pKU250 at the *Hind*III 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 C.1 (Invitrogen) was cloned into the *Eco*RV 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 *avaR3* deletion mutant. 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 an 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.

(Mightysil RP-18GP column, 4.6 × 250 mm, Kanto Chemical) using CH₃CN/MeOH/H₂O (30:20:50 by volume) as solvent and detection at 336 nm. To measure antibiotic production on solid medium, spores (1.0×10^5 c.f.u.) were inoculated on 2.5 ml YMS-MC medium and APM agar medium (APM medium containing 1.5 % agar), and incubated at 28 °C for 8 and 10 days, respectively. The culture was diced and extracted with methanol (5 ml). The supernatant was collected by centrifugation and analysed by the HPLC system described above. The HPLC analysis of antibiotic production in liquid culture and on solid medium was performed at least twice independently.

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: 97 °C for 3 min, followed by discrete cycles (as described in the legend to Fig. 3) at 97 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min. The absence of DNA contamination was confirmed by RT-PCR without reverse transcriptase.

Quantitative RT-PCR (qRT-PCR) was performed using an Applied Biosystems 7300 Real-Time PCR system and SYBR Green PCR Master Mix (Applied Biosystems) according to the supplier's recommendations. The reaction parameters were as follows: 95 °C for 10 min, followed by 40 cycles consisting of 15 s at 97 °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 ambifaciens* 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 (Δ *avaR3*) 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 Δ *avaR3* mutant produced trace amounts of avermectins (less than 1 % of the amount produced by the wild-type strain) (Fig. 2a). In contrast, the Δ *avaR3* 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 Δ *avaR3* mutant (data not shown). Complementation of the Δ *avaR3* 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.

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 *ava3* 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 (BAA28748) and ScbR (CAA07628) from *S. coelicolor* A3(2); AvaR1 (BAC71417), AvaR2 (BAC71414) and AvaR3 (BAC71415) from *S. avermitilis*; ArpA (BAA36282) from *S. griseus*; BarA (BAA06981) from *S. virginiae*; TylP (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 *ava3* and *rpoD* genes. The *rpoD* gene was used as a control.

Influence of AvaR3 on the transcription of avermectin biosynthetic genes

To elucidate the function of *ava3* 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 Δ *ava3* mutant, the expression at 48 h of five biosynthetic genes, *aveA1*, *aveA4*, *aveB1*, *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 *ava3*-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 Δ *ava3* 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 Δ *ava3* mutant in a manner similar to that of the wild-type strain (Supplementary Fig.

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

S2), in spite of the overproduction of filipin in the Δ *avaR3* 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 Δ *avaR3* 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 μ g avermectins (ml medium) $^{-1}$, whereas the Δ *avaR3* mutant showed a 79% reduction (16 μ g ml $^{-1}$) in avermectin production. Meanwhile, filipin production of the Δ *avaR3* 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 Δ *avaR3* mutant (data not shown). These phenotypic changes attributable to the *avaR3*

Fig. 3. Gene expression analysis by RT-PCR of avermectin biosynthetic genes in the wild-type strain (WT), Δ *avaR3* mutant (Δ *avaR3*) and *avaR3*-complemented exconjugant (Δ *avaR3/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 *aveBVIII* transcripts, 25 cycles for the *rpoD* transcript, and 30 cycles for the transcripts of the other genes.

mutation were partially restored by introduction of the intact *avaR3* gene. Similarly, cultivation of the Δ *avaR3* 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 Δ *avaR3* mutant on a variety of solid media. On YMS-MC medium, where the Δ *avaR3*

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

Strain	Antibiotic production ($\mu\text{g ml}^{-1}$)*			
	APM agar		YMS-MC	
	Avermectins	Filipin III	Avermectins	Filipin III
Wild-type	74.0 $\pm$ 3.0	3.2 $\pm$ 0.7	64.6 $\pm$ 2.2	6.1 $\pm$ 0.3
Δ <i>avaR3</i>	15.8 $\pm$ 1.0	10.4 $\pm$ 3.2	19.8 $\pm$ 1.9	10.3 $\pm$ 0.5
Δ <i>avaR3</i> /pSET152	1.8 $\pm$ 0.6	10.9 $\pm$ 0.8	23.8 $\pm$ 1.0	10.7 $\pm$ 0.5
Δ <i>avaR3</i> / <i>avaR3</i>	31.2 $\pm$ 0.7	8.0 $\pm$ 0.5	83.1 $\pm$ 6.0	5.5 $\pm$ 0.4

*Data shown are mean $\pm$ SD from triplicate samples.

mutant showed less avermectin production and more filipin production, no differences in morphology were detected between the wild-type strain and the Δ *avaR3* mutant. On APM agar medium, however, the Δ *avaR3* 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 Δ *avaR3* 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 Δ *avaR3* 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 Δ *avaR3* 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 suspected that integration at the *attB* site might have an adverse effect. In fact, integration of an empty vector alone (Δ *avaR3*/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 OD₆₀₀ value after 48 to 72 h cultivation, respectively (data not shown), although the dry cell weight of the Δ *avaR3* mutant was lower than that of the wild-type strain after 72 h of cultivation (Fig. 4b). Micrographs (Fig. 4c) revealed that the Δ *avaR3* mutant possessed more fragmented mycelia, with a remarkable decrease in the mean diameter of the pellet (73 $\pm$ 6 μm), while the wild-type strain produced typical large pellets with a mean diameter of 154 $\pm$ 14 μm , indicating that the increased OD₆₀₀ value of the Δ *avaR3* 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 E σ ⁷⁰-like promoters, although no typical -35 region was detected (Kang *et al.*, 1997; Strohl, 1992). As shown in Fig. 5(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. 5b, c and Supplementary Fig. S4), and both possessed probable promoters, resembling the consensus -10 region of streptomycetes E σ ⁷⁰-like promoters (*avaR1*), or -10 and -35 regions of the σ^{HrdD} -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

Fig. 4. Effect of *avaR3* deletion on cell morphology on agar medium and in liquid medium. All experiments were conducted at least twice, and the results were highly reproducible. WT, wild-type strain; ΔavaR3 , ΔavaR3 mutant; $\Delta\text{avaR3/pSET152}$, ΔavaR3 carrying pSET152; $\Delta\text{avaR3/avaR3}$, *avaR3*-complemented exconjugant. Error bars, SD from triplicate cultures (a, b). (a) Spore numbers on agar medium. The spores (1.0×10^7 c.f.u.) of each strain were plated on 25 ml APM agar medium, followed by incubation for 10 days at 28 °C. (b) Growth curve in liquid culture measured by dry cell weight: wild-type strain ($\blacksquare$), ΔavaR3 mutant ($\circ$) and *avaR3*-complemented exconjugant ($\blacktriangle$). (c) Phase-contrast micrographs of the mycelia in liquid medium. Cultures were grown in APM medium for 72 h. The micrographs were taken with an Olympus BX50 microscope and an Olympus DP70 digital camera. Bars, 100 μm . The diameter was measured from 30 independent pellets, and calculated as mean and SD.

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

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.

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. 6. Gene expression analysis of the *avaR3*, *avaR1* and *avaR2* genes by qRT-PCR. WT, wild-type strain; Δ *avaR3*, Δ *avaR3* mutant; Δ *avaR3/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.

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 Δ *avaR3* 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 Δ *avaR3* 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 CslA, which is involved in polysaccharide synthesis at the outer surface of hyphae (de Jong *et al.*, 2009). The enhanced expression of *ssgA* leads to mycelial fragmentation in *S. coelicolor* A3(2) and *S. griseus* (Kawamoto *et al.*, 1997; van Wezel *et al.*, 2000), whereas the *cslA* 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 CslA 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 Δ *avaR3* 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 CslA-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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