Avenolide, a *Streptomyces* hormone controlling antibiotic production in *Streptomyces avermitilis*

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Gram-positive bacteria of the genus *Streptomyces* are industrially important microorganisms, producing >70% of commercially important antibiotics. The production of these compounds is often regulated by low-molecular-weight bacterial hormones called autoregulators. Although 60% of *Streptomyces* strains may use γ-butyrolactone–type molecules as autoregulators and some use furan-type molecules, little is known about the signaling molecules used to regulate antibiotic production in many other members of this genus. Here, we purified a signaling molecule (avenolide) from *Streptomyces avermitilis*—the producer of the important anthelmintic agent avermectin with annual world sales of $850 million—and determined its structure, including stereochemistry, by spectroscopic analysis and chemical synthesis as (4*S*,10*R*)-10-hydroxy-10-methyl-9-oxo-dodec-2-en-1,4-olide, a class of *Streptomyces* autoregulator. Avenolide is essential for eliciting avermectin production and is effective at nanomolar concentrations with a minimum effective concentration of 4 nM. The *aco* gene of *S. avermitilis,* which encodes an acyl-CoA oxidase, is required for avenolide biosynthesis, and homologs are also present in *Streptomyces fradiae*, *Streptomyces ghanaensis*, and *Streptomyces griseoauranticus*, suggesting that butenolide-type autoregulators may represent a widespread and another class of *Streptomyces* autoregulator involved in regulating antibiotic production.

structure elucidation | actinomycetes | intercellular communications | microbial hormones

Bacteria commonly use small extracellular signaling molecules to control complex physiological traits such as biofilm formation, pathogenicity, and antibiotic production (1, 2). Autoregulators are signaling molecules that are able to trigger antibiotic production in the genus *Streptomyces*, Gram-positive bacteria that have the ability to produce a plethora of antibiotics with important medical and agricultural applications that are encoded by biosynthetic gene clusters. One well-studied family of autoregulators consists of the γ-butyrolactones, which are active at nanomolar concentrations and elicit antibiotic production by modulating the DNA binding activity of cognate receptor proteins; hence, they have been referred to as bacterial hormones (3). The most characterized γ-butyrolactone autoregulator is A-factor (compound **2**) (Fig. 1*A*), discovered as the first example in the early 1960s, which triggers both streptomycin production and sporulation in *Streptomyces griseus* (4, 5). Fourteen γ-butyrolactone autoregulators have since been identified in several *Streptomyces* species (3, 6). At least 60% of *Streptomyces* species appear to synthesize this type of signaling molecule (7), whereas signaling molecules produced by many other species still remain to be unraveled, largely because of extremely low concentrations in the culture medium. Recently, a furan-type autoregulator, methylenomycin furan (MMF), was shown to act as an additional class of *Streptomyces* autoregulator, although the minimum concentration required for inducing antibiotic production was not reported (8). Unlike γ-butyrolactone–type autoregulators, furan

autoregulators are produced by enzymes encoded by plasmid-borne genes, and their distribution across the genus *Streptomyces* remains unclear.

Streptomyces avermitilis is used for the industrial production of the important anthelmintic agent avermectin. A semisynthetic derivative of avermectin, ivermectin (22,23-dihydroavermectin B1), has been used as an agricultural pesticide and antiparastic agent since 1985. Ivermectin plays a major role in animal health and is also being used in humans to eradicate onchocerciasis, one of the neglected tropical diseases in Africa (9, 10), and strongyloidiasis in Asia (11). However, little was known about the extracellular signaling molecule that might regulate avermectin production. Here we report the isolation and structural elucidation of another type of signaling molecule termed "avenolide" for antibiotic production in the industrial microorganism *S. avermitilis*.

Results

Decreased Levels of Avermectin in *aco* Mutant Are Restored by Addition of Organic Solvent Extracts of Wild-Type Culture Broth. Genes involved in autoregulator biosynthesis and that encode the autoregulator receptor protein are frequently clustered at the same locus in various *Streptomyces* species (12). *sav3702-3706* of *S. avermitilis*, which is located far away from the avermectin gene cluster (*sav935-953*), consists of three genes, *avaR1* (*sav3705*), *avaR2* (*sav3702*), and *avaR3* (*sav3703*), that encode homologs of γ-butyrolactone–autoregulator receptor proteins and two genes, *aco* (*sav3706*) and *cyp17* (*sav3704*), that encode an acyl-CoA oxidase and a cytochrome P450 hydroxylase (CYP154B2), respectively (Fig. 2*A*). Loss of the *S. fradiae aco* and *cyp17* homologs abolished production of the antibiotic tylosin together with an unidentified compound that interacts with the autoregulator receptor (13). To determine whether *S. avermitilis aco* is important in avermectin production, a knockout mutant was constructed (*SI Materials and Methods*) and evaluated for avermectin production. The level of avermectin production in the *aco* mutant was markedly reduced to ~6% of wild-type levels at 138 h of cultivation (Fig. 2*B*). The introduction of an intact copy of *aco* with its upstream region into the *aco* mutant restored avermectin production to the level of the wild-type strain (Fig. 2*B*). These results confirmed that *aco* is indeed involved in avermectin production, although *aco* has no assigned functions in the context of avermectin biosynthesis itself. Thus, impaired pathways of signal transduction for the avermectin production could explain why the remarkable re-
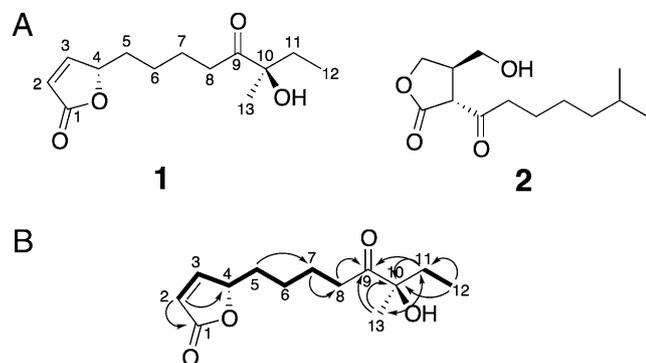
Fig. 1. Chemical structures of natural avenolide (**1**) and A-factor (**2**) as a representative γ-butyrolactone autoregulator (*A*). COSY (bold lines) and HMBC (arrows) correlations of avenolide (**1**) (*B*). Double arrow indicates cross peak in HMBC of **1**.

duction was observed in the *aco* mutant. To test this hypothesis, we added organic solvent extracts from the culture broth into the *aco* mutant (Fig. 2*C*). The defect in the avermectin production was restored completely by the addition of an ethyl acetate (AcOEt) extract of the wild-type culture broth. Interestingly, an AcOEt extract of the *aco* mutant was unable to induce avermectin production, which suggests that signaling molecule(s) eliciting avermectin production is present in the wild-type strain, but not in the *aco* mutant. Our subsequent hypothesis was that the signaling
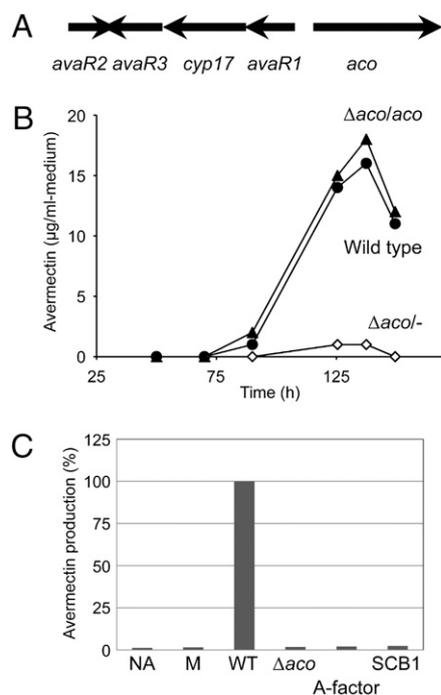

Fig. 2. Avermectin production in the *aco* mutant. (*A*) Organization of the *avaR* genes and the avenolide biosynthetic genes in *S. avermitilis*. (*B*) Production profiles of avermectin in the *S. avermitilis* wild-type strain and *aco* mutant. Filled circles, open diamonds, and filled triangles indicate time courses of avermectin production in the wild-type strain, the *aco* mutant (Δ*aco/−*), and *aco*-complemented *aco* mutant (Δ*aco/aco*), respectively. (*C*) Restoration of avermectin production in the *aco* mutant with the AcOEt extracts from *S. avermitilis* strains and γ-butyrolactone autoregulators. NA, no addition; M, AcOEt extract from production medium. The AcOEt extract or synthetic γ-butyrolactone autoregulator (A-factor and SCB-1, 246 nM) were added into the culture of the *aco* mutant at 48 h of cultivation, and the amounts of avermectin were measured in the culture broth after 144 h of cultivation.

molecule might belong to the family of γ-butyrolactone autoregulators. To test this hypothesis, we added representative γ-butyrolactone autoregulators (A-factor, IM-2, virginiae butanolides, or SCB1) (3) into the culture of the *aco* mutant, resulting in no recovery of avermectin production. All of these results implied that a low-molecular-weight (low-MW) factor distinct from known γ-butyrolactone autoregulators was likely to be involved in avermectin biosynthesis.

Influence of Organic Solvent Extracts on DNA Binding Activity of AvaR1. The deduced gene product of *avaR1*, divergently situated from *aco*, showed the highest sequence similarity to other *Streptomyces* autoregulator receptors such as ArpA, BarA, and ScbR (14). These autoregulator receptors typically bind to autoregulator responsive element (ARE) sequences that are frequently found in the promoter region of the direct target genes (15). We found a 26-bp ARE-like sequence in the 128 bp upstream of the *aco* gene, suggesting that AvaR1 may bind directly to the *aco* upstream region. To confirm whether AvaR1 has DNA binding activity toward the *aco* upstream region, we performed a gel-shift assay as shown in Fig. 3*A*. The recombinant AvaR1 (rAvaR1) protein showed clear binding to the upstream region of *aco*, which suggests that AvaR1 recognizes the ARE-like sequence upstream of *aco*. To precisely identify the location and sequence of the AvaR1 binding site, DNase I footprinting analysis was carried out, which revealed that AvaR1 protected the region of 30-bp sequences, situated 126 bp upstream from the translational start codon of *aco* (Fig. 3*B*) and designated as *aco*-ARE. These results demonstrated that DNA binding activity of AvaR1 is sequence-specific.

To determine how such specific DNA binding activity is influenced or inhibited, an AcOEt extract of culture broth was assayed for the ability to prevent rAvaR binding to the *aco*-ARE sequence. AcOEt extract of the wild-type culture clearly inhibited AvaR1 from forming the AvaR1–*aco*-ARE complex (Fig. 3*A*), leading to 560 inhibitory units/mL medium (1 inhibitory unit is defined as the activity of releasing half of DNA-bound rAvaR1 from the *aco*-ARE sequence) (Fig. 3*C*). In contrast, AcOEt extract of the *aco* mutant showed almost no activity (<4% of the wild-type level), whereas the *aco*-complemented strain showed a similar level of inhibitory activity to the wild-type strain, implying that the factor inhibiting the DNA binding activity of AvaR1 seems to be synthesized by enzymatic function of Aco and that the factor is responsible for inducing avermectin production as a signaling molecule like γ-butyrolactone autoregulators. γ-Butyrolactone autoregulators are known to be resistant to proteases, heat, and acid treatments but sensitive to alkaline treatment that breaks an ester bond of the lactone ring (16, 17). Interestingly, the AvaR1 ligand was resistant to alkaline treatment (pH 11, 10 min) as well as proteases, heat, and acid treatments (Fig. S1). It therefore appears that the AvaR1 ligand is structurally different from γ-butyrolactone autoregulators, which implies that the AvaR1 ligand belongs to another class of signaling molecules controlling antibiotic production.

Isolation and Structure Elucidation of AvaR1-Interactive Ligand. To isolate the AvaR1 ligand, we prepared 2,000 L of wild-type culture broth, which was adsorbed on the synthetic adsorbent HP-20. Active compounds were recovered as a 100% methanol eluate. Half of the methanol–eluate concentrate, showing specific activity of 1.6×10^3 units/mg residue to dissociate the rAvaR1–DNA complex, was extracted with chloroform and chromatographed on a silica gel column, followed by further purification by HPLC on a preparative C_{18} reverse-phase column. The purified compound (1.2 mg, colorless oil) possessed a specific activity of 1.0×10^7 units per mg, indicating 6.3×10^3-fold purification with a yield of 67%. No other fractions showed any DNA dissociation activity, implying that the compound purified is the sole avermectin-eliciting factor.
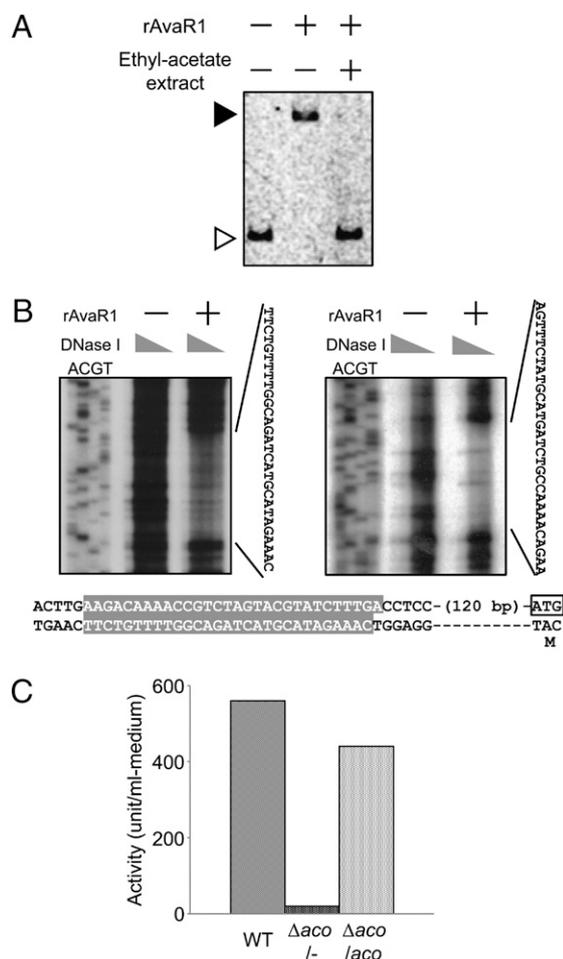
Fig. 3. Influence of AcOEt extract on DNA binding activity of rAvaR1 and avermectin production. (*A*) Effect of *S. avermitilis* extract on DNA binding activity of rAvaR1. The AcOEt extract prepared from the wild-type culture broth after 70 h of cultivation was used in the gel-shift assay. (*B*) Determination of rAvaR1 binding site in the upstream region of *aco* by DNase I footprinting analysis. (*Upper*) The upstream region of *aco* was labeled in the antisense (*Left*) and sense (*Right*) strands. ^{32}P-labeled fragment was incubated with DNase I (40–5 μg/mL) in the absence (−) or presence (+) of purified rAvaR1 (0.6 μg). The sequences protected by the presence of rAvaR1 are shown to the right of each image. *Lower* shows nucleotide sequence of the upstream region of *aco*. The rAvaR1 binding site is highlighted by a gray box. The putative start codon of *aco* gene is shown in an open box. (*C*) Avenolide activity in the *aco* mutant. Avenolide activity at 48 h of cultivation was detected by using the gel-shift assay. One unit of avenolide activity was defined as described in *Materials and Methods*.

The structure of the active compound (**1**) was assigned as a 2(5*H*)-furanone derivative on the basis of spectroscopic evidence (olefinic proton signals at δ7.45 and δ6.12 and quaternary carbon at δ173.0). The high-resolution fast atom bombardment mass spectrometry (HR-FAB-MS; positive mode) showed a molecular ion peak at *m/z* 263.1261 [M+Na]$^+$, corresponding to a molecular formula of $C_{13}H_{20}O_4$. The proton signals at δ5.05 indicated a characteristic methine signal of butenolides from marine actinomycetes (18, 19). The ^{1}H-^{1}H-COSY spectrum of **1** showed the connections from C-2 to C-8 and between C-11 and C-12. Furthermore, the connection from C-8 to C-11 via a ketone (C-9, δ212.2) and quaternary carbon with a hydroxyl and a methyl group (C10, δ79.9) was demonstrated on the basis of heteronuclear multiple bond correlation (HMBC) spectrum of **1** (Fig. 1*B*). From these data, we elucidated the planner structure of **1** as 10-hydroxy-10-methyl-9-oxo-dodec-2-en-1,4-olide (Fig. 1*A*), which

has not been isolated from any natural sources. The absolute configuration at the C-4 position was determined as 4*S* by comparison with a circular dichroism (CD) spectrum of stereochemically known butenolide (20). To further establish the absolute configuration of **1**, we synthesized two candidates (4*S*,10*R* and 4*S*,10*S* forms of **1**) (21) and purified by chiral chromatography, together with the 4*R*,10*R* form of **1** (Fig. 4). The identical elution time of the natural compound with the 4*S*,10*R* form on chiral HPLC (Fig. S2) together with the highest activity of the 4*S*,10*R* form (Fig. 4) confirmed that the natural compound has the absolute configuration of 4*S*,10*R*. The 4*S*,10*S* isomer possessed half the activity of the 4*S*,10*R* isomer, whereas the 4*R*,10*R*-isomer and 10-deoxy form (21) showed much reduced activity, suggesting that the 4*S*-absolute configuration and the presence of a hydroxyl group at C-10 are important for the binding to or signal perception by AvaR1.

Induction of Avermectin Production by AvaR1-Interactive Ligand Avenolide. To confirm that compound **1** was the missing factor in the *aco* mutant and is required to elicit avermectin production, synthetic 4*S*,10*R*-**1** was added to the *aco* mutant. Avermectin production was restored to wild-type levels by the addition of 4*S*,10*R*-**1**, mimicking the addition of wild-type AcOEt extract to the *aco* mutant (Fig. 5*A*). Thus, the compound was named "avenolide" (for *S. avermitilis* bute<u>nolide</u>).

Finally, to determine whether the identified avenolide elicits avermectin production at nanomolar concentrations, we tested it on the *S. avermitilis aco* mutant at final concentrations ranging from 2 to 15 nM (Fig. 5*B*). Avenolide (**1**) triggered avermectin production at a minimum effective concentration of 4 nM, together with saturation at 50–150 nM, resulting in avermectin production up to 1.8-fold of that of the wild-type strain (wild-type strain, 15 μg/mL; *aco* mutant with 150 nM avenolide, 27 μg/mL). Thus, avenolide (**1**) is a hormone-like signaling molecule effective at nanomolar concentrations in eliciting avermectin production.

Discussion

Intercellular communication by low-MW signaling molecules has been reported in a variety of microorganisms, including Gram-negative bacteria (such as *Vibrio fischeri*) (22) and Gram-positive bacteria (such as *Bacillus subtilis* and *Streptomyces* species) (23). The discovery of signaling molecules involved in the regulation of secondary metabolism, typically in antibiotic production, has played a key role in developing our understanding of the molecular mechanisms of microbial intercellular communication and opens many possibilities for the manipulation of antibiotic production. We have shown here that avenolide is an essential signaling molecule for avermectin production in *S. avermitilis* effective at nanomolar concentrations and is therefore another class of *Streptomyces* autoregulators distinct from γ-butyrolactone and furan autoregulators. Four nanomolar avenolide is sufficient to induce avermectin production in the *aco* mutant. This result is well in line with the concentrations and signaling activities of other microbial hormones, which range from micromolar (homoserine lactones) (24) and submicromolar (0.05 μM PI factor) (25) to nanomolar (AI-2 and γ-butyrolactone autoregulators) (26). The activity of avenolide (**1**) is clearly dependent on the butenolide structure; 4*R*-**1** had only 4% of the activity of 4*S*-**1**. SCB1, a typical butanolide that possessed the highest binding activity toward rAvaR1 of all of the γ-butyrolactones tested (Fig. S3), had negligible elicitor activity compared with the avenolide analogs (Fig. 4). Hence, the addition of the γ-butyrolactone autoregulator did not influence avermectin production. The presence of the hydroxyl group at C-10 is also crucial for recognition of avenolide by its receptor protein AvaR1, although its configuration appears less important.

Elucidation of avenolide (**1**) as a signaling molecule now allows us to explore the distribution of this chemical signal in strepto-

Compound		Relative Activity (%)
(4S,10R)-1		100
(4S,10S)-1		50
(4R,10R)-1		4.2
(4S)-10-deoxy-1		1.2
SCB1		0.0038

Fig. 4. Comparison of dissociation activity against the rAvaR1–DNA complex among avenolide and synthetic avenolide analogs.

mycetes. Our previous findings based on screening of autoregulator producers suggested that ~60% of *Streptomyces* species have the ability to produce a γ-butyrolactone–type autoregulator (7), but little was known about molecules used as the signaling molecules for antibiotic production by other species. The furantype autoregulator (typically MMF) is one such example. However, the sparse occurrence of homologs of the MMF biosynthetic

Fig. 5. Effect of avenolide on avermectin (AVM) production. Synthetic (4S,10R)-**1** was added into the culture of the *aco* mutant at 48 h of cultivation. (*A*) HPLC chromatogram of AVM production in the *aco* mutant by addition of synthetic (4S,10R)-**1**. Synthetic (4S,10R)-**1** was added at a final concentration of 15 nM. Peaks eluted at 4.5, 5.2, 6.2, 8.7, 9.5, and 12.3 min are AVM derivatives, such as AVM B2a, AVM A2b, AVM A2a, AVM B1a, AVM A1b, and AVM A1a, respectively. A peak eluted at 7.1 min is oligomycin A (OLM). (*B*) AVM production with increasing concentrations of synthetic (4S,10R)-**1**. Error bars represent SDs from triplicate experiments.

genes (only one example among sequenced *Streptomyces* genomes) suggests that the furans might be a minor group of *Streptomyces* autoregulator. In contrast, gene arrangements similar to the *aco/cyp17/avaR* cluster are found frequently in the genomes of *Streptomyces* strains (Fig. 6). Aco presumably introduces a double bond into the C-2–C-3 positions of avenolide (**1**). Our preliminary data demonstrated that Cyp17, encoding the cytochrome P450 hydroxylase CYP105B2, is also involved in the production of avenolide, generating avenolide (**1**) from (4S)-10-deoxy-**1**. In contrast, *avaA* (*sav2269*; 1.8 Mb away from the *aco/cyp17/avaR* cluster and 1.6 Mb away in the opposite side from the avermectin cluster), which encodes a homolog of well-studied AfsA family protein required for the formation of phosphorylated butenolides for γ-butyrolactone autoregulator (27) and MMF (8), does not participate in avenolide (**1**) production (Fig. S4). These results support our hypothesis that other streptomycetes may produce avenolide-type autoregulators and that they are acting as a general class of signaling molecule in regulating antibiotic production in *Streptomyces*. It will be interesting to determine the mechanism by which this unique class of signaling molecules initiates antibiotic production. The identification of avenolide as a unique *Streptomyces* autoregulator should allow the construction of high-level producers of antibiotics, including the important therapeutic agent
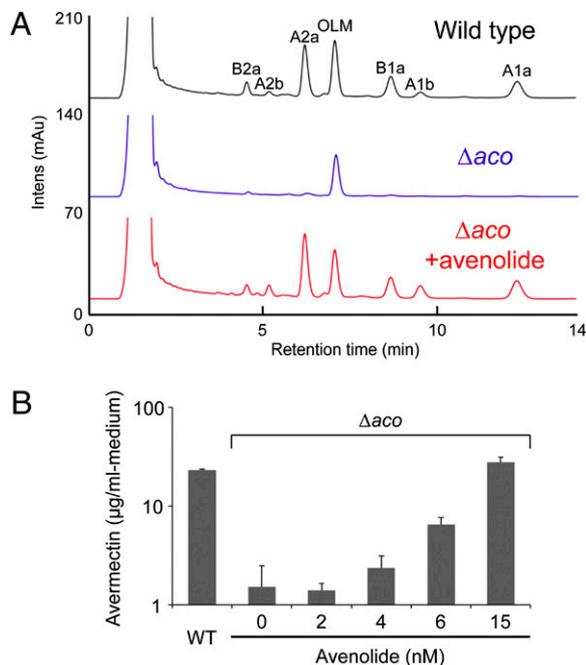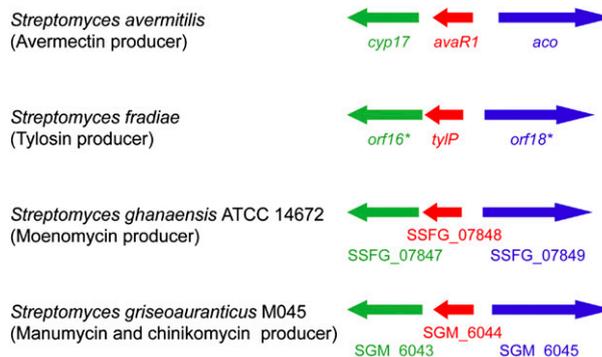

Fig. 6. The biosynthetic gene clusters of the avenolide family of autoregulators. Genes are color-coded by the predicted function of their products: *avaR1* homologs (red), *aco* homologs (blue), and *cyp17* homologs (green).

avermectin, as well as facilitate genetic understanding of regulatory mechanism for microbial antibiotic production. The result could also lead to the discovery of clinically useful natural compounds by activating the numerous silent natural product gene clusters prevalent in actinomycete genomes.

Materials and Methods

Analysis of Avermectin Production. The derivative strains of *S. avermitilis* KA320 were cultivated in production medium (28). The culture broth was withdrawn and mixed with an equal volume of methanol. After centrifugation, the supernatant was analyzed by an HPLC system as described (29). The amounts of four avermectin components, A1a, A2a, B1a, and B2a, were quantified by using an authentic sample of avermectins.

Preparation of rAvaR1. An *avaR1* gene amplified by the primer pair avaR1-Fw/avaR1-Re was digested by *Nco*I and *Bam*HI and then inserted into the NcoI–BamHI sites of pET-3d (Merck), resulting in pLT118. *Escherichia coli* BL21(DE3) pLysS harboring pLT118 was inoculated into LB medium and the cultivation was continued at 37 °C until the OD_{600} reached 0.4. Isopropyl β-D-thiogalactopyranoside was then added to a final concentration of 0.5 mM, and the cultivation was continued for a further 4 h. After the collected cells were washed with 0.9% NaCl solution, they were resuspended in buffer A [20 mM triethanolamine-HCl (pH 7.0) containing 20% vol/vol glycerol, 1 mM 1,4-DTT, and 0.1 mM (*p*-amidinophenyl)methanesulfonyl fluoride] containing 50 mM KCl. After sonication and centrifugation, the supernatant was absorbed on a DEAE-Sephacel column preequilibrated with buffer A containing 50 mM KCl, and a fraction showing SCB1 binding activity was eluted with buffer A containing 150 mM KCl. The SCB1 binding assay was performed as described (30). The autoregulator binding activity of rAvaR1 was measured by observing the difference between the binding of the [3H]-labeled autoregulator (86 nM [3H]SCB1 [1.73 TBq/mmol]) in the absence and presence of 1,450-fold excess nonlabeled SCB1. The radioactivity was quantified by an LS-6000 liquid scintillation counter (Beckman Coulter). After the fraction was concentrated by ultrafiltration (UP-20; Advantec Toyo Kaisha), the concentrate was applied to a gel filtration column (Superrose 12 PC 3.2/30; GE Healthcare Bio-Sciences) and eluted with buffer A containing 200 mM KCl. Protein concentration was measured with Bio-Rad protein assay kit using BSA as a standard. Purity of rAvaR1 was analyzed by SDS/PAGE.

Avenolide Assays. Two bioassays were used to detect avenolide activity: a DNA dissociation activity on the rAvaR1–DNA complex in the gel-shift experiment and an avermectin-inducing activity with *S. avermitilis aco* mutant. Synthetic avenolide and the chiral analogs were dissolved in methanol and added at the specified concentrations for these bioassays. One unit of avenolide activity in the gel-shift experiment was defined as the potency for releasing half of DNA-bound rAvaR1 from the specific target DNA, which was measured by image analysis on Typhoon 9210 Variable Mode Imager.

Gel-Shift Assay. An upstream region of *aco* gene was amplified by PCR with the primer pair aco-gel-Fw/aco-gel-Re (Table S1). The amplified fragment was digested by *Bam*HI and then cloned into the *Bam*HI site of pUC19 (Takara Bio), resulting in pLT119. The DNA probe was labeled by PCR using pLT119 containing an *aco*-upstream region as a template with FITC-labeled M13-47 primer and RV primer. After FITC-labeled probe (7.5 ng) and rAvaR1 (0.2 μg) were mixed and incubated at 25 °C for 10 min, the reaction mixture was separated at 4 °C by electrophoresis. The gel-shift condition to detect DNA retardation was followed as described (31). To investigate effect of an ethyl-acetate extract as described below on DNA binding activity of rAvaR1, 2 μL of the extract was added into the reaction mixture, and the mixture was incubated for a further 5 min. For preparation of an ethyl-acetate extract, the culture supernatant, adjusted under pH 5.0 with acetate, was extracted with ethyl acetate. The ethyl-acetate broth was evaporated and dissolved in 20% ethanol.

DNase I Footprinting. DNase I footprinting was performed as described (32). DNA fragment was prepared by PCR using pLT119 as a template and the [32P]-labeled aco-gel-Fw or aco-gel-Re primers. Each [32P]-labeled primer was end-labeled with T4 polynucleotide kinase and ATP.

Isolation of Avenolide (1). The vegetative culture of *S. avermitilis* KA320 grown in the medium containing 5 g of glucose, 15 g of soy flour, and 5 g of yeast extract per liter of tap water (adjusted to pH 7.2) at 30 °C for 2 d was inoculated to the fermentation medium containing 60 g of glucose, 2 g of NaCl, 0.5 g of K_2HPO_4, 0.1 g of $MgSO_4 \cdot 7H_2O$, 2 g of $(NH_4)_2SO_4$, 2 g of yeast extract, 0.05 g of $FeSO_4 \cdot 7H_2O$, 0.05 g of $MnSO_4 \cdot 4H_2O$, 0.05 g of $ZnSO_4 \cdot 7H_2O$, and 5 g of $CaCO_3$ per liter of tap water (adjusted to pH 7.0) and grown at 28 °C for 24 h. The level of avenolide in all purification steps was evaluated by using the gel-shift assay. About 2,000 L of the culture filtrate was mixed with 200 L of a synthetic adsorbent Diaion HP-20 (Mitusbishi Chemical Co. Ltd.). The adsorbent was washed with 2 volumes of water, and avenolide was successively eluted with 2 volumes of 100% methanol. After methanol eluate was concentrated to 1 L, half of the concentrate was extracted with chloroform (200 mL × 5). The organic layers were combined and concentrated under the reduced pressure. The brownish oily residue (2.2 g) was subjected to silica gel column chromatography (70~230 mesh, 30 × 150 mm in chloroform), the active fractions were eluted with chloroform/methanol (99:1), and the fractions were combined and concentrated to dryness (107.5 mg). The yellowish oily material was subjected to preparative HPLC (PEGASIL ODS; 20 × 250 mm, 5 μm, developing with 20% aqueous acetonitrile, flow rate 9.0 mL/min, and detection at 210 nm). Because avenolide was eluted at 45–60 min, the fractions were combined and concentrated to obtain as an oily material (4.7 mg). Final purification of the active fraction (4.2 mg) by preparative HPLC (PEGASIL ODS; 10 × 250 mm, 5 μm, developing with 15% aqueous acetonitrile, flow rate 4.0 mL/min, and detection at 210 nm) gave 1.2 mg of avenolide (**1**) eluted at 73.0 min. Fractions were assayed for avenolide activity as described above.

Physicochemical Properties of Avenolide (1). Avenolide (**1**): colorless oil; $[\alpha]_D^{23}$ +22.0° (c 0.01, methanol); HR-FAB-MS (positive mode) $C_{13}H_{20}O_4Na$ [M+Na]+ (obsd. 263.1261, calcd. 263.1259); UV (methanol) λ_{max} (log ε) 208.0 nm (4.13); CD (methanol) λ_{max} (Δε): 208 (+4.4) nm. [13C] NMR (125 MHz, δ ppm in CDCl3): 212.2 (C-9, s), 173.0 (C-1, s), 156.1 (C-3, d), 121.8 (C-2, d), 83.0 (C-4, d), 79.0 (C-10, s), 35.5 (C-8, t), 33.1 (C-5, t), 32.5 (C-11, t), 25.3 (C-13, q), 24.8 (C-6, t), 23.3 (C-7, t), 7.8 (C-12, q). [1H] NMR (500 MHz, δ ppm in CDCl3): 7.45 (1H, dd, *J* = 5.9, 1.4 Hz, H-3), 6.12 (1H, dd, *J* = 5.9, 2.1 Hz, H-2), 5.05 (1H, m, H-4), 2.57 (1H, dddd, *J* =14.5, 8.2, 6.4, 3.2 Hz, H-8), 2.48 (1H, dddd, J = 14.5, 7.8, 6.4, 3.7 Hz, H-8), 1.83 (1H, m, H-5), 1.72 (1H, m, H-5′), 1.72 (2H, quint., *J* = 7.3 Hz, H-11), 1.69 (2H, m, H-7), 1.46 (2H, m, H-6), 1.34 (3H, s, CH3-13), 0.80 (3H, t, *J* = 7.3 Hz, CH3-12).

Chemical and Analytical Methods. NMR ([1H], 500 MHz; [13C], 125 MHz) spectra were obtained on JEOL JNM-ECP 500 FT NMR system. Chemical shifts are reported on the δ scale, using tetramethylsilane as an internal reference. Molecular mass spectra were measured on a JEOL JMS-700 Mstation spectrometer. CD spectra and optical rotations were recorded on JASCO J-720 and JASCO DIP-1000 polarimeter, respectively.

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