Supporting Information

Kitani et al. 10.1073/pnas.1113908108

SI Materials and Methods

Construction of *aco* Mutant. A segment upstream of *aco* was amplified by the primer pair aco-up-Fw/aco-up-Re, and a segment downstream of *aco* was amplified by the primer pair aco-dw-Fw/aco-dw-Re. A cosmid clone CL_236_H08 (http://avermitilis.ls.kitasato-u.ac.jp/) was used as a template in above PCR. The two resultant segments were digested with *Hin*dIII and *Eco*RI and cloned together into *Eco*RI-cut pKU450 (1). The resultant plasmid was cleaved by *Hin*dIII and ligated with a kanamycin-resistant gene amplified by the primer pair aphII-H-Fw/aphII-H-Re using pKU474 (2) as a template to yield pLT115. An *Eco*RI fragment, recovered from pLT115, was inserted into a unique *Eco*RI site of pKU250 (3), thereby yielding pLT116. pLT116 was reintroduced into *E. coli dcm Δ(srl-recA)*::Tn*10* (pUB307::Tn*7*), and resultant transformants were conjugated with spores of *S. avermitilis* KA320 (isogenic strain of MA-4648, ATCC 31267, NRRL 8165, NCBIM 12804, DSM 46492, and K139 that lacks SAP2 plasmid). Exconjugants were selected by thiostreptone-resistance and were spread onto YMS medium (4) for sporulation. Desired *aco* mutants by allelic replacement of homologous regions between chromosome and recombinant plasmid were selected by thiostreptone-sensitive and neomycin-resistant phenotype. The genotype of mutant designated as *S. avermitilis* Δ*aco* strain was confirmed by Southern blot hybridization. For genetic complementation, the *aco* gene with its upstream region was amplified by the primer pair aco-com-Fw/aco-com-Re, and then cloned into a pENTR vector (Invitrogen) to generate an entry clone. The entry clone was used with pLT113 (2) in an LR reaction (LR Clonase Enzyme Mix; Invitrogen) to yield pLT117. By intergenic conjugation and integration, pLT117 was introduced into the *aco* mutant. The correct integration in the exconjugants was confirmed by PCR.

Construction of *avaA* Mutant. A 6.8-kb *Kpn*I fragment (position: 2,764,749–2,771,504 nt) of a cosmid clone CL_236_E08 (http://avermitilis.ls.kitasato-u.ac.jp/) was subcloned into the *Kpn*I site of pIJ2925 (5). The plasmid pIJ2925::6.8-kb-*Kpn*I was digested with *Xho*I. The linear fragment was digested partially with *Bam*HI, and the largest segment (containing 2,764,749–2,767,117 nt and 2,768,975–2,771,504 nt) was recovered. The purified segment was ligated with 2.0-kb *Xho*I/*Bam*HI *aad*(*3″*)-Ω from pHP45-Ω (6). The ligation products were introduced into *E. coli* DH5α, and desired clones were selected by resistance to ampicillin (50 μg/mL), streptomycin (50 μg/mL), and spectinomycin (100 μg/mL). The resultant plasmid was digested with *Bgl*II, and the largest 8.2-kb segment was ligated with *Bam*HI-digested pKU250. The desired plasmid pKU250::*ΔavaA*-cassette was isolated as ampicillin-, streptomycin-, and spectinomycin-resistant transformants. pKU250::*ΔavaA*-cassette was reintroduced into *E. coli dcm Δ(srl-recA)*::Tn*10* (pUB307), and the resultant transformants were conjugated with spores of the *S. avermitilis* wild-type strain. The exconjugants were selected by thiostreptone resistance (20 μg/mL). After the spores cultivated onto YMS medium were harvested, desired *avaA* mutants were selected by thiostreptone-sensitive and streptomycin/spectinomycin resistance. The region deleted *avaA* (2,767,118–2,767,655 nt) was confirmed by PCR and Southern blot hybridization.

Chirality Determination of a Hydroxy Group at C-10 Position of Avenolide. Natural avenolide (**1**), synthetic (4*S*,10*R*)-**1**, and (4*S*,10*S*)-**1** samples were dissolved in ethanol (~50 μg/mL), and a portion of each sample (5 μL) was subjected to chiral HPLC. Separation conditions are as follows: DAICEL CHIRALPAK IA (4.6 mm × 250 mm); ethanol was used as a mobile phase at flow rate 0.3 mL/min and detection at 210 nm.

1. Ueki M, et al. (2009) Nocardamin production by *Streptomyces avermitilis*. *Actinomycetologica* 23:34–39.
2. Miyamoto KT, Kitani S, Komatsu M, Ikeda H, Nihira T (2011) The autoregulator receptor homologue AvaR3 plays a regulatory role in antibiotic production, mycelial aggregation and colony development of *Streptomyces avermitilis*. *Microbiology* 157: 2266–2275.
3. Komatsu M, Uchiyama T, Ōmura S, Cane DE, Ikeda H (2010) Genome-minimized *Streptomyces* host for the heterologous expression of secondary metabolism. *Proc Natl Acad Sci USA* 107:2646–2651.
4. Ikeda H, Kotaki H, Ōmura S (1987) Genetic studies of avermectin biosynthesis in *Streptomyces avermitilis*. *J Bacteriol* 169:5615–5621.
5. Janssen GR, Bibb MJ (1993) Derivatives of pUC18 that have *Bgl*II sites flanking a modified multiple cloning site and that retain the ability to identify recombinant clones by visual screening of *Escherichia coli* colonies. *Gene* 124:133–134.
6. Prentki P, Krisch HM (1984) *In vitro* insertional mutagenesis with a selectable DNA fragment. *Gene* 29:303–313.

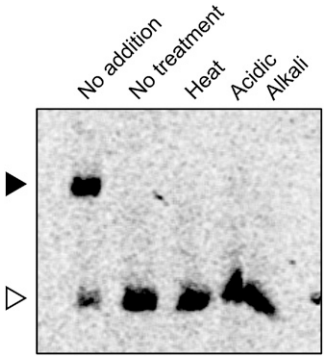

Fig. S1. AvaR1-interactive ligand properties. Gel-shift assay was carried out with the wild-type AcOEt extracts exposed to various treatments. Heat, 100 °C for 30 min; acidic, pH 1 for 30 min; alkali, pH 11 for 10 min. The treated extracts were adjusted to the initial pH (6.0), evaporated, and dissolved in 20% ethanol. A black arrowhead indicates the position of the rAvaR1–DNA complex, and a white arrowhead indicates the position of the probe DNA only.

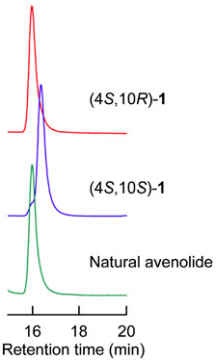

Fig. S2. HPLC chromatogram for assignment of the chirality of avenolide. Natural avenolide was eluted at 16.0 min, synthetic 10*R*-**1** was eluted at 16.0 min, and 10*S*-**1** was eluted at 16.3 min.

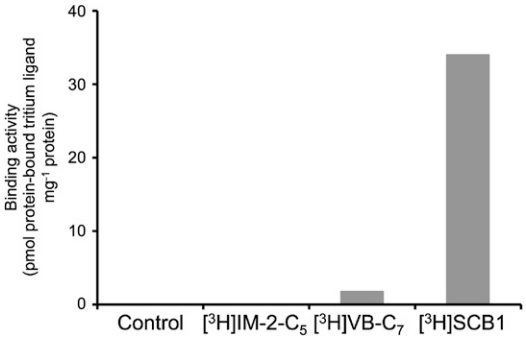

Fig. S3. Autoregulator binding activity of purified rAvaR1 with tritium-labeled γ-butyrolactone autoregulators. Cell-free extract from the *E. coli* cell harboring pET-3d was used as a control.

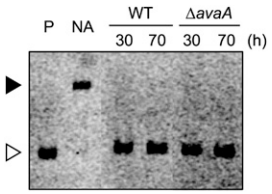

Fig. S4. Effect of an *avaA* mutation on the AvaR1-interactive ligand. Black and white arrowheads are used as described in Fig. S1. P, probe DNA; NA, no addition of the AvaR1-interactive ligand. AcOEt extract prepared from the culture broth at the indicated time was added into the reaction mixture.

Table S1. Oligonucleotide primers used in this study

Primer	Sequence (5-3′)*
For construction of *aco* mutant	
aco-up-Fw	CTCGAGAATTCTGGGCGAAGAGCATGTTGTCGTT
aco-up-Re	CTCGAGAAGCTTGAACAGGTTGTAGTGGATGCTCG
aco-dw-Fw	CTCGAGAAGCTTAACCTGGTGATCTGGGTGAAGG
aco-dw-Re	CTCGAGAATTCGACCACGCCGAGCGACATCTGAA
aphII-H-Fw	CTCGAGAAGCTTCAGTGAGTTCGAGCGACTCGAGA
aphII-H-Re	CTCGAGAAGCTTCTGGTACCGAGCGAACGCGTA
For construction of complemented Δ*aco* strain	
aco-com-Fw	CACCAATGGCTCGCTCCTGCCG
aco-com-Re	CGAACGGCACGAAGAGTGAGC
For expression of recombinant AvaR1 protein	
avaR1-Fw	CATGCCATGGCGCGTCAGGAACGAGCCATTCGG
avaR1-Re	CGCGGATCCGGACAGTCACTCCAACGTCCGTGC
For preparation of probe in gel-shift assay	
aco-gel-Fw	AAGGATCCAGAAGGGCTGAGGAAGGGCT
aco-gel-Re	AAGGATCCCGCCCTCTCACAAACCACTC

*Restriction sites are underlined.