Supporting Information

Komatsu et al. 10.1073/pnas.0914833107

SI Materials and Methods

Construction of SUKA2 and SUKA3 Mutants. Two segments of *Bsp*HI-*Bgl*II (5,846–8,806 nt) from cosmid clone CL_090_H01, *Bam*HI (1,494,518–1,497,112 nt) from cosmid clone CL_214_C05, and the *Bam*HI segment of the kanamycin/neomycin resistance gene *aphII* from Tn*5* amplified by PCR were ligated with a large *Nco*I-*Bam*HI segment of pGM160*Δaac(3)I::oriT*. The correct recombinant plasmid from kanamycin-resistant transformants was isolated by confirmation of restriction digestion, and the resultant plasmid carrying *sav6*, *aphII*, and *sav1205* was introduced into *Streptomyces avermitilis ΔolmA* (Δ3,557,725–3,594,005 nt) by conjugation. After selection of neomycin- and thiostrepton-resistant exoconjungants generated by a single cross-over recombination event, they were propagated in antibiotic-free medium to excise the vector region by a second single cross-over recombination. The recombinant progeny were selected by their thiostrepton-sensitive and neomycin-resistance phenotype, and the desired deletion mutants were confirmed by clamped homogeneous electrical field (CHEF) electrophoresis. The *aphII* region in the large-deletion mutants was removed by a second double cross-over recombination event using two segments of *Bsp*HI–*Mlu*I (5,846–7,734 nt) from CL_090_H01 and *Mlu*I-*Bam*HI (1,494,893–1,497,112 nt) from CL_214_C05 on *Nco*I/*Bam*HI sites of pGM160*Δaac(3)I::oriT*. Exoconjugants resistant to thiostrepton were propagated onto antibiotic-free medium, and the desired deletion mutants were then selected by their thiostrepton- and neomycin-sensitive phenotype. Finally, the deletion in the progeny was reconfirmed by CHEF electrophoresis. The resultant mutant was named as SUKA2 with SUKA being the abbreviated name for Special Use of Kitasato Actinobacteria.

A *Xho*I-*Bsr*GI (77,213–81,746 nt) segment from cosmid clone CL_240_F01 was subcloned into the large *Sal*I-*Acc*65I segment of pIJ2925. The segment containing *hph* (hygromycin B resistance) with *loxP* sequence at the upstream end was amplified by PCR and introduced at the unique *Eco*RV site of the recombinant plasmid carrying a 4,533-bp *Xho*I-*Bsr*GI segment. The resultant plasmid was selected by ticarcillin and hygromycin B resistance, and the desired plasmid with the correct direction of *loxP* was confirmed by restriction digestion. The cassette containing *S. avermitilis* DNA and *loxP-hph* was digested with *Bgl*II, the large segment was introduced into the *Bam*HI site of pKU250, and the resultant plasmid was introduced into *S. avermitilis* wild type by conjugation. After selection of exoconjugants with thiostrepton, allelic replacement clones generated by double cross-over recombination events were selected by their thiostrepton-sensitive and hygromycin B-resistant phenotype. The changes in the chromosomal region were confirmed by PCR amplification. Another region around 1.59 Mb was also processed by similar procedures. An *Acc*65-*Eco*RI (1,591,859–1,598,761 nt) segment from CL_238_C05 was subcloned into the *Eco*RI site of pUC19 using an *Acc*65I-*Eco*RI synthetic linker. A *Mlu*I segment carrying *aphII-loxP* prepared by PCR amplification was introduced into a unique *Mlu*I site of pUC19::6,902-bp *Acc*65I-*Eco*RI. After selection by the ticarcillin- and kanamycin-resistance phenotype, the direction of the *loxP* sequence was confirmed by restriction digestion, and the cassette containing *S. avermitilis* DNA and *aphII-loxP* was recovered by digestion with *Eco*RI. The cassette was introduced into the *Eco*RI site of pKU250, and the resultant recombinant plasmid was introduced by conjugation into *S. avermitilis sav71::loxP-hph* constructed as described above. The desired recombinant progeny generated by double cross-over recombination events were selected by the thiostrepton-sensitive, neomycin- and hygromycin B-resistance phenotype, and the chromosomal regions were reconfirmed by PCR. The *cre*-expression plasmid, pKU471, was introduced into *S. avermitilis sav71::loxP-hph sav1286::aphII-loxP* by conjugation. To excise the ca. 1.51-Mb region between the two directed *loxP* sequences by Cre-*loxP* site-specific recombination, exoconjugants were grown on yeast extract-malts extract-soluble starch medium (YMS; ref.1) containing 2% xylose to induce the expression of *cre.* After sporulation, thiostrepton-, hygromycin B-, and neomycin-sensitive progenies were selected, the desired large-deletion mutant was confirmed by CHEF electrophoresis, and the correct progeny was defined as SUKA3.

Cloning of gene clusters for streptomycin, cephamycin C, and pladienolide biosyntheses. A cosmid clone, SGR4C6, containing the entire streptomycin biosynthetic gene cluster of *S. griseus* was digested with *Mse*I to isolate a 41.2-kb insert. The insert was subcloned into the *Nde*I site of pKU465cos by in vitro packaging (1) to generate pSM1. The chromosomal DNA of *S. clavuligerus* was sheared by repeated passing through a 23-gauge needle (500 times) to generate about 40-kb fragments. After purification by agarose-gel electrophoresis, the 40-kb fragments were treated with Bal31 for 15 s at 25 °C, and both ends of the fragments were filled by T4 DNA polymerase and T4 polynucleotide kinase in the presence of dNTPs and ATP. The blunt-ended DNA fragments were ligated with the large *Swa*I segment of pKU402 (1), and the ligated DNA was subjected to in vitro packaging. Clones carrying the entire gene cluster for cephamycin C were confirmed by detection of two regions, *blaC* and *pcbR,* by PCR amplification. One of these clones (1D4) was digested with *Ase*I to generate the 40-kb fragment that was subcloned into pKU465cos by in vitro packaging to generate pCEF2. Mycelia of *S. platensis* were embedded into 0.5% low-melting temperature agarose before overnight digestion with 2 mg/mL of lysozyme. The resulting protoplasts generated were lysed by the addition of 1% SDS and 1 mg/mL of proteinase K at 50 °C for 24 h. After removal of SDS and proteinase K by repeated washing with 50 mM EDTA (pH 8.0), DNA embedded in the agarose plug was digested with *Xba*I. From 70- to 100-kb fragments were purified by CHEF electrophoresis. The purified DNA fragments were ligated with a large *Nhe*I segment of pKU503, and *Escherichia coli* DH10B was transformed by electroporation with ligated DNA. The desired clone was selected by the insert size and amplification of two regions, *pldAI* and *pldR,* by PCR to generate pPLD30.

Construction of a cassette for expression of the ads gene. A synthetic *ads* gene (AB530988) encoding the amorpha-4,11-diene synthase of *Artemisia annua* (Q9AR04) was synthesized (OPERON Biotechnologies K.K.) according to *Streptomyces* codon usage, and restriction sites *Nde*I and *Xba*I were added at the 5′ and 3′ ends, respectively. A gene-encoding farnesyl diphosphate synthetase (*ptlB*; 3,753,107–3,754,120 nt) from the *S. avermitilis* neopentalenolactone biosynthetic gene cluster (2) was prepared by PCR with template DNA from *S. avermitilis* cosmid CL_216_D07 using primer pairs, forward: 5′-GCTCTAGACTCGACAGG*AGGA*GCGTC**ATG**ACCGTGACCCCGGAAAGTCTG-3′ (underlined characters indicate *Xba*I site, bold characters corresponded to the start codon of *ptlB*, and *boxed* characters are rRNA binding site) and reverse: 5′-CTCGAGAAGCTTACTAGT**TCA**CACCTCCCGGTCGACGACGAAGTGTCCGAG-3′ (underlined characters indicate *Hin*dIII site and bold characters corresponded to the stop codon of *ptlB*). The amplified segment was digested with *Xba*I and *Hin*dIII and joined with a large segment of pKU460::*rpsJp* (3). The resultant recombinant plasmid was digested with

*Nde*I and *Xba*I, and the digested DNA was ligated with a 1.66-kb *Nde*I/*Xba*I synthetic DNA to generate pKU460::*rpsJp-ads-ptlB*.

Construction of cassettes for expression of regulatory genes. The regulatory gene *strR* was amplified by PCR with template DNA from *S. griseus* cosmid SGR4C6 using the primer pair, forward: 5′-GGCTCGAGCATATGGAGCATATTTCAGGGAACAGC-3′ (underlined characters indicate *Nde*I site and bold characters correspond to the start codon of *strR*) and reverse: 5′-AACTCGAGATTAATGTGATGCAGTGGTTCACGTGCT-3′ (underlined characters indicate *Ase*I site). The amplified DNA was digested with *Nde*I and *Ase*I and ligated with *Nde*I-cut pKU493*hph* containing *aveRp*, *ermEp*, or *rpsJp*. After transformants were isolated on Luira-Bertani agar medium containing hygromycin B (200 μg/mL), the desired clones with correct direction of *strR* were confirmed by restriction digestion. Because *pldR* was relatively large in size, a 4.75-kb *Mfe*I/*Ase*I fragment containing *pldR* of pPLD30 was subcloned into the large *Eco*RI/*Ase*I fragment of pKU493*aad*. Each promoter, *aveRp* (1,131,568–1,132,041 nt in *S. avermitilis*), *ermEp* (831,544–831,820 nt in *Saccharopolyspora erythraea* NRRL 2338), or *rpsJp* (5,986,588–5,987,134 nt in *S. avermitilis*), was introduced using the λ *red*-mediated recombination system described previously (2). The regulatory gene *ccaR* was amplified by PCR with template DNA from *S. clavuligerus* cosmid 1D4 using primer pair, forward: 5′-AAGGGACCCATATGAACACCTGGAATGATGTGACG-3′ (underlined characters indicate *Nde*I site and bold characters correspond to the start codon of *ccaR*) and reverse: 5′-GTGAGATCTAGAACGTTTCTTCAGCGTTGGTTCAGG–3′ (underlined characters indicate *Xba*I site). Amplified DNA digested with *Nde*I and *Xba*I was ligated with large *Nde*I-*Xba*I fragments of pKU493*hph* containing *ermEp* or *rpsJp*.

Both intact *bdpA* and *adpA* genes with promoters were cloned by PCR with template DNA from *S. avermitilis* cosmid CL_237_A06 and *S. griseus* cosmid SGR1A7 using primer pairs, forward for *bdpA*: 5′-CTCGAGGAATTCCCACACGGCGGGAAATACGC-3′ (underlined characters indicate *Eco*RI site) and reverse for *bdpA*: 5′-CTCGAGAAGCTTGATCCACACCATGCGATCGTTCAT-3′ (underlined characters indicate *Hin*dIII site), and forward for *adpA*: 5′-CTCGAGGAATTCCCTTGGGTTCAGGGATTTCGTGAC-3′ (underlined characters indicate *Eco*RI site) and reverse for *adpA*: 5′-CTCGAGAAGCTTGGTCATCTCGCAGTCGATCCACAC-3′ (underlined characters indicate *Hin*dIII site), respectively. After each segment amplified was digested with *Eco*RI and *Hin*dIII, segments digested were ligated with the large *Eco*RI/*Hin*dIII fragment of pKU493*hph*.

Construction of disruption mutants of bdpA, adpA, and rsbV. To construct deletions of *bdpA* in *S. avermitilis*, two segments were prepared by PCR amplification with template DNA from *S. avermitilis* cosmid CL_237_A06 using the primer pair of the upstream region, forward: 5′-CTCGAGAAGCTTCCGTCACTCCGTCGATCACGTC-3′ (underlined characters indicate *Hin*dIII site) and reverse: 5′-CTCGAGACTAGTCTCATACTGCTAAGCCCCCCTCGG-3′′ (underlined characters indicate *Spe*I site) and the primer pair of the downstream region, forward: 5′-CTCGAGACTAGTTAGGACTGCCGTGACAGCAGGGCG-3′ (underlined characters indicate *Spe*I site) and reverse: 5′-CTCGAGGAATTCACCCAGGCGATCTCCGCGACCTC-3′ (underlined characters indicate *Eco*RI site), respectively. Cloning vector pRED and resistance marker *loxP-aphII-loxP* were also amplified by PCR using the primer pair of pRED vector, forward: 5′-CTCGAGAAGCTTCCATTCATCCGCTTATTATC-3′ (underlined characters indicate *Hin*dIII site) and reverse: 5′-CTCGAGGAATTCTGCCAGGAAGATACTTAACAG-3′ (underlined characters indicate *Eco*RI site) and the primer pair for the resistance marker, forward: 5′-CTCGAGACTAGTCTTCATGAGCTCAGCCAATC-3′ (underlined characters indicate *Spe*I site) and reverse: 5′-CTCGAGACTAGTTGCCGTATTTGCAGTACCAG-3′ (underlined characters indicate *Spe*I site), respectively. After amplification of above segments, the upstream segment digested with *Hin*dIII and *Spe*I, the downstream segment digested with *Spe*I and *Eco*RI, the vector segment digested with *Eco*RI and *Hin*dIII, and the resistance-marker segment digested with *Spe*I were ligated together in a molar ration of 1:1:1:1. *E. coli* DH5α was transformed with the ligated products, the transformants were selected by chloramphenicol (30 μg/mL) and kanamycin (50 μg/mL), and the desired clones were confirmed by restriction digestion. The vector region of the disruption cassette was replaced by pKU257 using the homologous regions *cat* and p15A in both vectors using the λ *red*-mediated recombination system (4). The pKU257 plasmid carrying the disruption cassette was introduced into SUKA5 by conjugation, and the desired disruptants obtained by allelic replacement were selected by using the thiostrepton-sensitive and neomycin-resistance markers. The deletion region was confirmed by Southern hybridization and PCR amplification, and then, the resistance marker in the disruptant was removed by introducing *cre*-expression vector pKU471. Similar procedures were used for disruption of *adpA* in *S. griseus*. Two segments were prepared by PCR with template DNA from *S. griseus* cosmid SGR1A7 using a primer pair for the upstream region, forward: 5′-CTCGAGGAATTCGCCGGACGACGACCTGCTGGA-3′ (underlined characters indicate *Eco*RI site) and reverse: 5′-CTCGAGACTAGTCTCATGACGCTAAGCCCCCCTCGG-3′ (underlined characters indicate *Spe*I site) and a primer pair for the downstream region, forward: 5′-CTCGAGACTAGTTAGGGTGGGGCGCATGAACGACCG-3′ (underlined characters indicate *Spe*I site) and reverse: 5′-CTCGAGGAATTCGGACGTGGCCCTGATCGGCTTCGAC-3′ (underlined characters indicate *Eco*RI site), respectively. pRED was also amplified by PCR using the primer pair, forward: 5′-CTCGAGGAATTCCCATTCATCCGCTTATTATC-3′ (underlined characters indicate *Eco*RI site) and reverse (the same as the above pRED reverse primer). Four amplified segments, the upstream and downstream segments digested with *Eco*RI and *Spe*I, the pRED segment digested with *Eco*RI, and the above resistance marker segment digested with *Spe*I, were ligated together in the same 1:1:1:1 molar ratio. The vector region of the disruption cassette was also replaced by pKU257 using the same procedures described above. The pKU257 plasmid carrying the disruption cassette was introduced into *E. coli* GM2929 *hsdS::*Tn*10* to prepare unmethylated DNA, and *S. griseus* protoplasts were transformed by the derived unmethylated DNA preparation. The desired disruptants that had been generated by allelic replacement were selected by thiostreptone sensitivity and neomycin resistance, and the deletion region was confirmed by Southern hybridization. The *rsbV*-disruptant mutants of *S. avermitilis* were prepared by the same procedures as those used for the *bdpA*-disruptant mutants. The following primer pairs were used for amplification of upstream and downstream regions: forward for upstream: 5′-CTCGAGAAGCTTAGGAACGTGTCCAGCGGGTCGTC-3′ (underlined characters indicate *Hin*dIII site) and reverse for upstream region: 5′-CTCGACTAGTCCACGGATCCTCCAGCACCTTGC-3′ (underlined characters indicate *Spe*I site); forward for downstream region: 5′-CTCGAGACTAGTCTGACCCCGCCTCGGGCCCCTGT-3′ (underlined characters indicate *Spe*I site) and reverse for downstream region: 5′-CTCGAGGAATTCCCCCGAGGCCGAAGCCCTCGAGCA-3′ (underlined characters indicate *Eco*RI site).

1. Omura S, et al. (2001) Genome sequence of an industrial microorganism *Streptomyces avermitilis*: Deducing the ability of producing secondary metabolites. *Proc Natl Acad Sci USA* 98:12215–12220.

2. Jiang J, et al. (2009) Genome mining in *Streptomyces avermitilis*: A biochemical Baeyer-Villiger reaction and discovery of a new branch of the pentalenolactone family tree. *Biochemistry* 48:6431–6440.

3. Komatsu M, Tsuda M, Omura S, Oikawa H, Ikeda H (2008) Identification and functional analysis of genes controlling biosynthesis of 2-methylisoborneol. *Proc Natl Acad Sci USA* 105:7422–7427.

4. Tetzlaff CN, et al. (2006) A gene cluster for biosynthesis of the sesquiterpenoid antibiotic pentalenolactone in *Streptomyces avermitilis*.. *Biochemistry* 45:6179–6186.

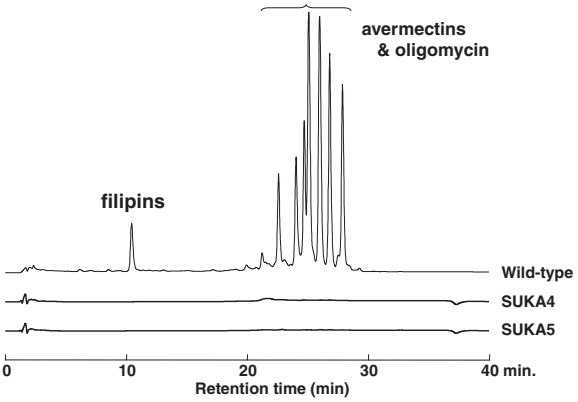

Fig. S1. HPLC profiles of EtOAc extracts from whole culture of *S. avermitilis* wild type and its large-deletion mutants, SUKA4 and SUKA5. The organic extract was subjected to octadodecylsilyl silica-HPLC (HyperSil 3μm; 4.6φ × 100 mm) developing with 30–80% acetonitrile in water at a flow rate of 0.6 mL/min (detection at 254 nm).

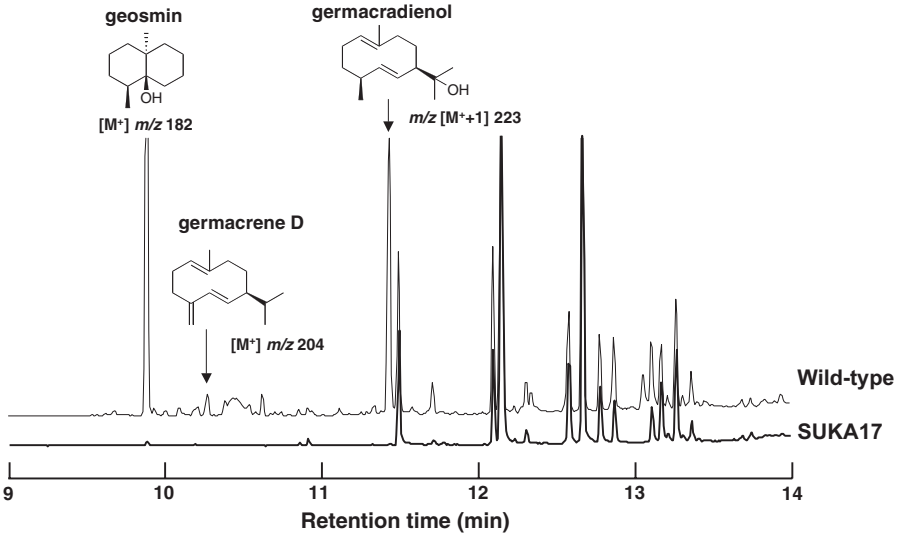

Fig. S2. GC-MS analysis of *n*-hexane extracts from *S. avermitilis* wild type and its large-deletion mutant SUKA17. A 5-μL portion of the extract was analyzed by GC-MS [Shimadzu GC-17A, 70 eV, electron impact, positive ion mode; 30 m × 0.25 mm neutral bond-5 capillary column (5% phenylmethylsilicon) using a temperature program of 50–280 °C and temperature gradient of 20 °C/min]. Three volatile sesquiterpenoids were identical with authentic samples prepared from *S. avermitilis*. The peaks eluted from 12.1 to 13.4 min were identical to short- and medium-chain fatty acids.

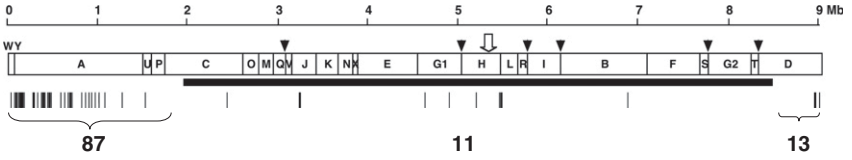

Fig. S3. Distribution of transposase genes on the *Ase*I physical map of the *S. avermitilis* linear chromosome. Horizontal closed bar indicates 6.50 Mb of the central core region. Vertical lines are transposase genes. Open arrow and filled triangles indicate replication origin *oriC* and ribosomal RNA operons *rrn*.

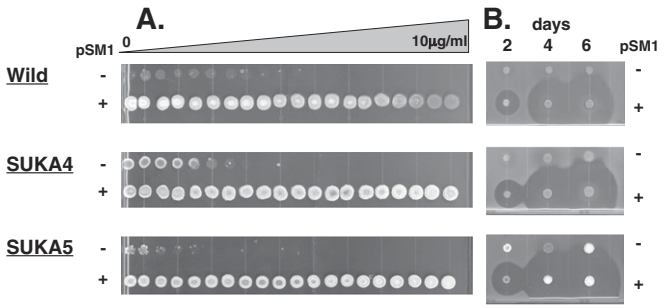

Fig. S4. Streptomycin susceptibility (*A*) and production (*B*) in *S. avermitilis* wild type, its large-deletion mutants, and their transformants that carry pSM1 encoding the gene cluster for streptomycin biosynthesis. To examine streptomycin susceptibility, diluted spore suspensions of all strains were spotted onto streptomycin-gradient (0–10 μg/mL of streptomycin) YMS agar, and the plate was incubated for 4 days at 30 °C. For streptomycin production, each strain was grown on YMS agar medium at 30 °C for 2, 4, and 6 days. After cultivation, *Esherichia coli* DH5α as an indicator organism was overlaid with soft agar (the same composition of YMS except that agar was added to 0.5%), and plates were incubated at 37 °C for 24 h.

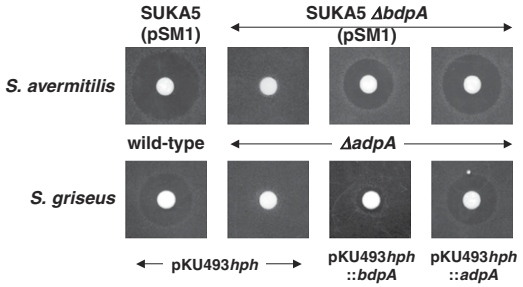

Fig. S5. Streptomycin productivity in the *bdpA* disruptant of *S. avermitilis* (*Upper*) and *adpA* disruptant of *S. griseus* (*Lower*) and the restoration of streptomycin production by introduction of *bdpA* (*sav5261*) and *adpA* (*sgr4742*). Each strain was grown in avermectin production synthetic medium at 28 °C for 5 days. A 50-μL sample of culture supernatant was spotted onto a paper disk (8 ϕmm), the paper disk was put onto a nutrient agar plate seeded with spores of *Bacillus subtilis* ATCC 6633, and the plate was incubated at 37 °C for 24 h.

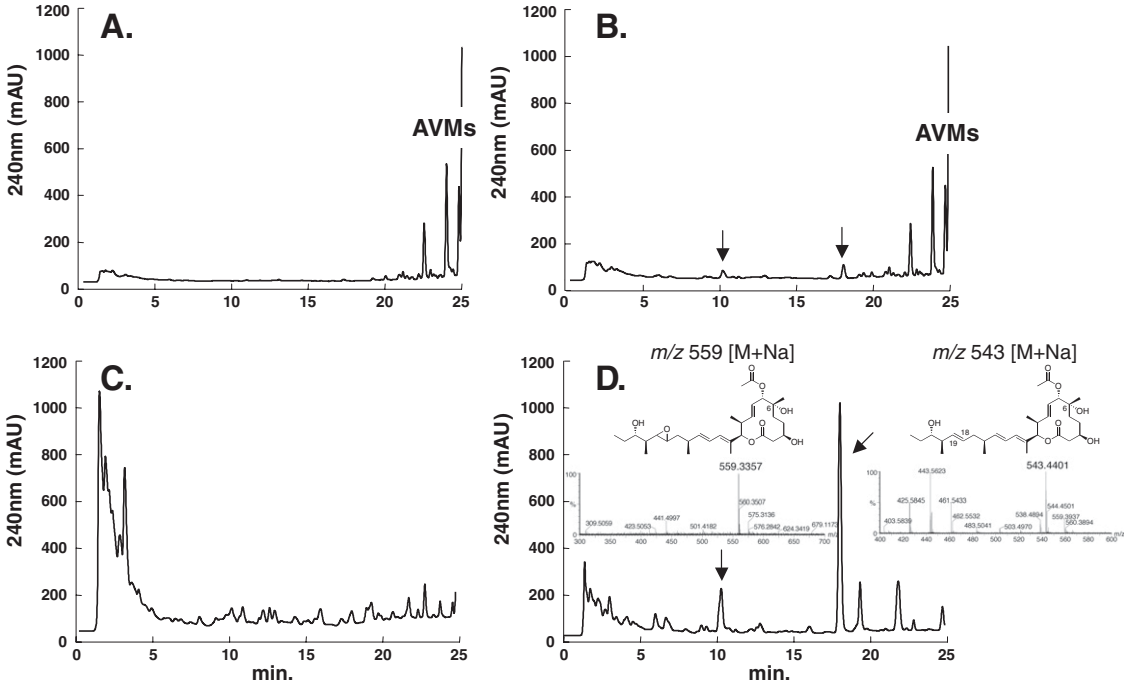

Fig. S6. HPLC-MS analysis of products from *S. avermitilis* wild type carrying pPLD30 (*A*) and wild type carrying pPLD30 and pKU493*aad*::*ermEp-pldR* (*B*). Each culture filtrate was extracted with EtOAc, and the organic layer was concentrated 10-fold under reduced pressure. The culture filtrate (5 μL) of SUKA5 carrying pPLD30 (*C*) and SUKA5 carrying pPLD30 and pKU493*aad*::*ermEp-pldR* (*D*) was directly analyzed by HPLC-MS. Analytical conditions for HPLC were as follows: octadodecylsilyl silica-HPLC (3 μm; 2.0ϕ × 100 mm), detection (240 nm), mobile phase (40–90% linear gradient of acetonitrile in water), flow rate (0.2 mL/min). Two peaks that eluted at 10.15 (*m/z* 559 [M+Na]) and 17.45 min (*m/z* 543 [M+Na]) were identical to authentic samples of pladienolide B and Δ18,19-pladienolide B, respectively, and the peaks from wild-type exoconjugants, which eluted after 22.5 min, were also identical to authentic samples of avermectins (AVMs) and oligomycin.

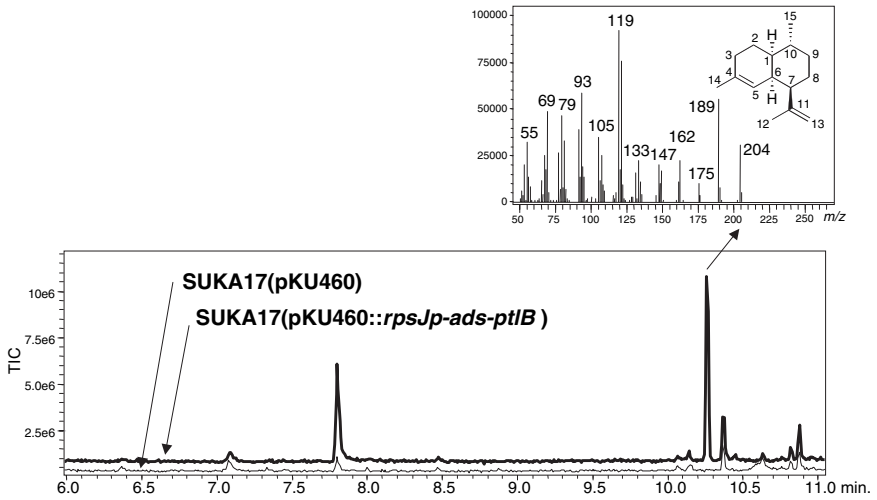

Fig. S7. GC-MS analysis of *n*-hexane extracts from *S. avermitilis* SUKA17 and its exoconjugant carrying the synthetic operon for amorpha-4,11-diene synthase (*ads*) and farnesyl diphosphate synthase (*ptlB*). A portion (1 μL) of the *n*-hexane extract of whole broth was directly analyzed by GC-MS (Shimadzu GC-17A, 70 eV, electron impact (EI), positive ion mode; 30 m × 0.25 mm neutral bond-5 capillary column; 5% phenylmethylsilicon using a temperature program of 50–280 °C and a temperature gradient of 20 °C/min). The compound that eluted at 10.25 min was purified by silica-gel chromatography (*n*-pentane/dichloromethane) of the *n*-hexane extract. NMR assignments and EI-MS fragmentation of the compound purified; ^{1}H-NMR δ (500MHz, $CDCl_3$) 5.06 5-H, 4.86 13-Hα, 4.64 13-Hβ, 2.55 6-H, 1.97 3-Hα, 1.93 7-H, 1.82 2-Hβ, 1.76 1-H, 1.74 12-H_3, 1.67 9-Hβ, 1.59 14-H_3, 1.56 2-Hα, 1.49 8-Hα, 1.39 10-H, 1.31 2-Hβ, 1.24 8-Hβ, 0.95 9-Hα 0.87 15-H_3, EI-MS: *m/z* (%) = 204 (33) $[M]^+$, 189 (60), 175 (11), 162 (24), 147 (22), 133 (24), 119 (100), 105 (39), 93 (64), 79 (50), 69 (53), 55 (35).

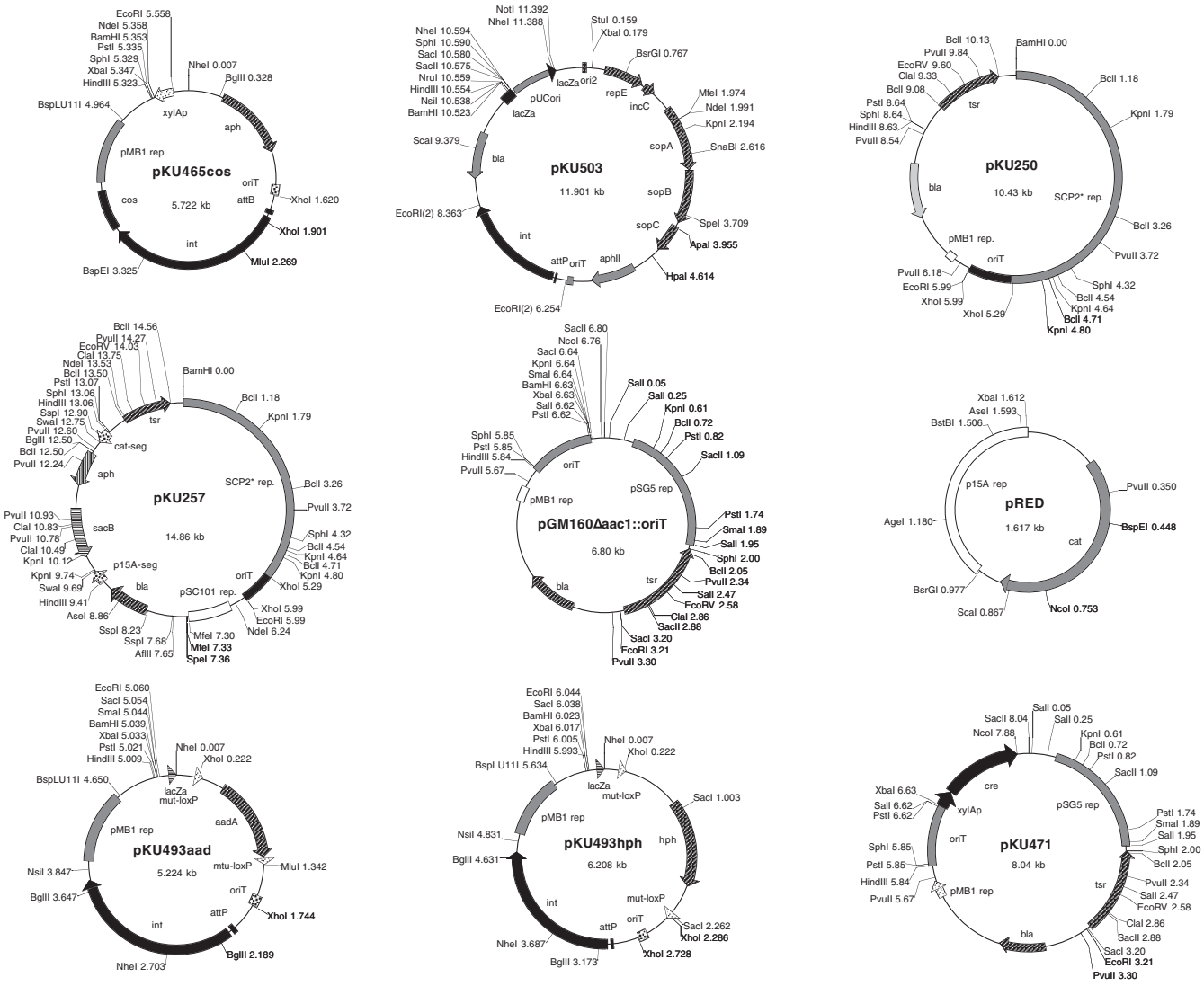

Fig. S8. Restriction maps of pKU465cos, pKU503, pKU250, pKU257, pGM160*Δaac(3)I::oriT,* pRED, pKU493*aad*, pKU493*hph*, and pKU471. Both *attP* and *int* genes in pKU465cos and pKU503 were derived from actinophage ϕC31, and *attP* and *int* genes in pKU493*aad* and pKU493*hph* were from actinophage ϕK38-1.