The Journal of Antibiotics (2012) 65, 551–558

www.nature.com/ja

ORIGINAL ARTICLE

Identification of the SGR6065 gene product as a sesquiterpene cyclase involved in (+)-epicubenol biosynthesis in *Streptomyces griseus*

Chiaki Nakano, Takeaki Tezuka, Sueharu Horinouchi* and Yasuo Ohnishi

Recent bacterial genome sequencing projects have shown the presence of many putative sesquiterpene cyclase (SC) genes, especially in the Gram-positive, filamentous bacterial genus *Streptomyces*. We describe here the characterization of a SC gene (SGR6065, named *gecA*) from *Streptomyces griseus*. Overexpression of *gecA* in *Streptomyces lividans* produced a sesquiterpene, which was isolated and determined to be (+)-epicubenol using spectroscopic analyses. The N-terminal histidine-tagged GecA protein was produced in *Escherichia coli*. Incubation of the recombinant GecA protein with farnesyl diphosphate (FPP) yielded (+)-epicubenol as the major product. The K_m value for FPP and the k_{cat} value for (+)-epicubenol formation were calculated to be 254 ± 7.1 nM and $0.026 \pm 0.001\ s^{-1}$, respectively. The k_{cat}/K_m value ($0.10\ s^{-1}\ \mu M^{-1}$) was broadly comparable to those reported for known bacterial SCs. (+)-Epicubenol was detected in the crude cell lysate of wild-type *S. griseus*, but not in a *gecA*-knockout mutant, indicating that GecA is a genuine (+)-epicubenol synthase. Although (+)-epicubenol synthases have been previously purified and characterized from the liverwort *Heteroscyphus planus* and *Streptomyces* sp. LL-B7, no (+)-epicubenol synthase gene has been cloned to date. The *gecA* gene is thus the first example of an (+)-epicubenol synthase-encoding gene. (+)-Epicubenol production was not controlled by the microbial hormone A-factor that induces morphological differentiation and production of several secondary metabolites in *S. griseus*.
The Journal of Antibiotics (2012) **65,** 551–558; doi:10.1038/ja.2012.68; published online 8 August 2012

Keywords: (+)-epicubenol; genome mining; natural product; sesquiterpene cyclase; *Streptomyces griseus*

INTRODUCTION

Terpenoids are one of the major classes of natural products and have various biological roles, including as flavorings, pigments, antibiotics and hormones.[1] They are biosynthesized from the C_5 precursors isopentenyl diphosphate and dimethylallyl diphosphate. In the biosynthesis of terpenoids, terpenoid cyclases are key enzymes in all pathways leading to the diverse range of terpenoids. Terpenoid cyclases catalyze the cyclization of linear isoprenyl diphosphate, which is produced by the successive condensation of isopentenyl diphosphate and dimethylallyl diphosphate, to produce the parent skeletons of terpenoids. In contrast to the large number of terpenoid compounds produced by plants and fungi, bacteria produce only a small number of terpenoids. However, recent genome sequencing projects have revealed many potential bacterial terpenoid cyclase genes, especially sesquiterpene cyclase (SC) genes in *Streptomyces*. SCs catalyze the cyclization of farnesyl diphosphate (FPP; C_{15}) into any of 300 known hydrocarbon skeletons. Using a genome mining approach, several bacterial SC homologs have been identified from *Streptomyces* and *Nostoc* species. These proteins are involved in the synthesis of germacradienol/geosmin,[2–5] pentalenene,[6] *epi*-isozizaene,[7,8] avermitilol,[9] germacrene A,[10] 8a-*epi*-α-selinene,[10] (−)-δ-cadinene[11] and (+)-T-muurolol.[11] Germacradienol/geosmin synthase is a representative bacterial SC, but is atypical in that it consists of two terpene cyclase domains. Others are typical single-domain SCs. Recently, we reported the identification and characterization of five bacterial single-domain SC homologs: the (+)-caryolan-1-ol synthase GcoA[12] in *Streptomyces griseus*, the 1,8-cineole synthase CnsA[13] and linalool/nerolidol synthase LnsA[14] in *Streptomyces clavuligerus*, and the (−)-germacradien-4-ol synthase SC1[15] and (−)-*epi*-α-bisabolol synthase SC2[15] in *Streptomyces citricolor*. CnsA is the first bacterial monoterpene synthase known to catalyze the direct conversion of geranyl diphosphate,[13] whereas LnsA is the first bacterial acyclic terpene synthase that shows significant amino-acid sequence similarity to bacterial SCs.[14] These studies and the presence of a large number of terpene cyclase homologs in bacteria indicate that terpenoids are likely to be significantly more widely distributed in nature than previously appreciated.[16]

Department of Biotechnology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo, Japan
*Deceased on 12th July 2009.
Correspondence: Professor Y Ohnishi, Department of Biotechnology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan.
E-mail: ayasuo@mail.ecc.u-tokyo.ac.jp
Received 20 April 2012; revised 14 July 2012; accepted 18 July 2012; published online 8 August 2012

The bicyclic sesquiterpene alcohol (+)-epicubenol has been isolated previously not only from plants,[17,18] but also from three *Streptomyces* species.[19] The enzymatic conversion of FPP to (+)-epicubenol has been previously performed using a purified enzyme, as well as a crude extract, prepared from the liverwort *Heteroscyphus planus*[20,21] and *Streptomyces* sp. LL-B7.[22–25] Cane *et al.* have presented extensive experimental evidence for a cyclization mechanism of the (+)-epicubenol synthase from *Streptomyces* sp. LL-B7 (Figure 1). The authors demonstrated that the reaction is initiated by isomerization of FPP to (*3R*)-nerolidyl diphosphate, which then undergoes conversion to the intermediate (*Z, E*)-germacradienyl cation, **2**. After a 1,3-hydride shift and a second cyclization, the *cis*-fused muurolyl cation, **3**, is generated, and then a 1,2-hydride shift occurs to give the bicyclic cation, **4**. Finally, this cation is quenched by the addition of a water molecule to produce (+)-epicubenol.[22–25] Nabeta *et al.*[20] demonstrated the same reaction mechanism using cell-free extracts of cultured cells of *H. planus*. To date, however, no gene encoding an (+)-epicubenol synthase has been cloned from any organism.

In 2008, we reported the complete genome sequence of *S. griseus* IFO13350.[26] We have long studied the A-factor regulatory cascade that leads to secondary metabolism and morphological differentiation in this famous streptomycin producer.[27] A-factor (2-isocapryloyl-3*R*-hydroxymethyl-γ-butyrolactone) is produced by the action of AfsA, which is the key enzyme for A-factor biosynthesis, and is gradually accumulates in a growth-dependent manner.[28] When the A-factor concentration reaches a critical level, A-factor binds the A-factor-specific receptor, which has bound and repressed the promoter of *adpA*, and dissociates A-factor-specific receptor from the promoter, resulting in induction of *adpA* transcription.[29] The AraC/XylS family transcriptional regulator AdpA then activates a number of genes required for morphological differentiation and secondary metabolism, forming an AdpA regulon.[30,31] We identified two single-domain SC homologs (SGR2079 and SGR6065) in the complete genome of *S. griseus*. Last year, we demonstrated that SGR2079 (named GcoA) is the first SC known to synthesize (+)-caryolan-1-ol, and that the production of this secondary metabolite is under the control of A-factor.[12] AdpA appeared to directly activate transcription of *gcoA*. In contrast, DNA microarray analysis predicted that transcription of the SGR6065 gene is independent of AdpA.[26] SGR6065 (358 amino acids) shows 28% amino-acid sequence identity with the pentalenene synthase from *Streptomyces* sp. UC5319,[32] and has both the aspartate-rich motif (**DD**QL**D**, from Asp81 to Asp85) and the NSE/DTE motif (**N**DVY**S**FEK**E**, from Asn226 to Glu234), which are responsible for the Mg^{2+}-diphosphate–enzyme complex formation, as well as the ionization of the substrate.[33] Here we describe the characterization of the function of SGR6065 *in vivo* and *in vitro*. We reveal that SGR6065 is an (+)-epicubenol synthase, and have named the SGR6065 gene '*gecA*' (*S. griseus* epicubenol synthase). (+)-Epicubenol was detected in the crude cell lysate of wild-type *S. griseus* and an *adpA*-deleted mutant, but not in a *gecA*-knockout mutant, indicating that GecA is a genuine (+)-epicubenol synthase and that (+)-epicubenol production is not controlled by A-factor in *S. griseus*.

MATERIALS AND METHODS

General analytical methods

NMR spectra were recorded in C_6D_6 on a JNM-A500 spectrometer (JEOL, Tokyo, Japan). Chemical shifts in ^{1}H- and ^{13}C-NMR spectra (p.p.m.) were relative to 7.15 and 128.0 p.p.m. for the solvent peak of C_6D_6 solution. The coupling constant *J* was given in Hz. GC-MS analysis was performed using a JEOL JMS-Q1000 GC K9 instrument under electronic impact at 70 eV with an Intercap 5MS/Sil capillary column (15 m × 0.25 mm ID, 0.25 μm film thickness, GL science, Tokyo, Japan), the oven temperature being elevated from 60 (3-min hold) to 260 °C (20 °C min^{-1}). The specific rotation value was measured with a JASCO DIP-1000 digital polarimeter (Jasco, Tokyo, Japan) at room temperature.

Bacterial strains, plasmids, media and chemicals

Escherichia coli strains JM109 and BL21(DE3), plasmids pUC19 and pColdI, restriction enzymes and other DNA-modifying enzymes used for DNA

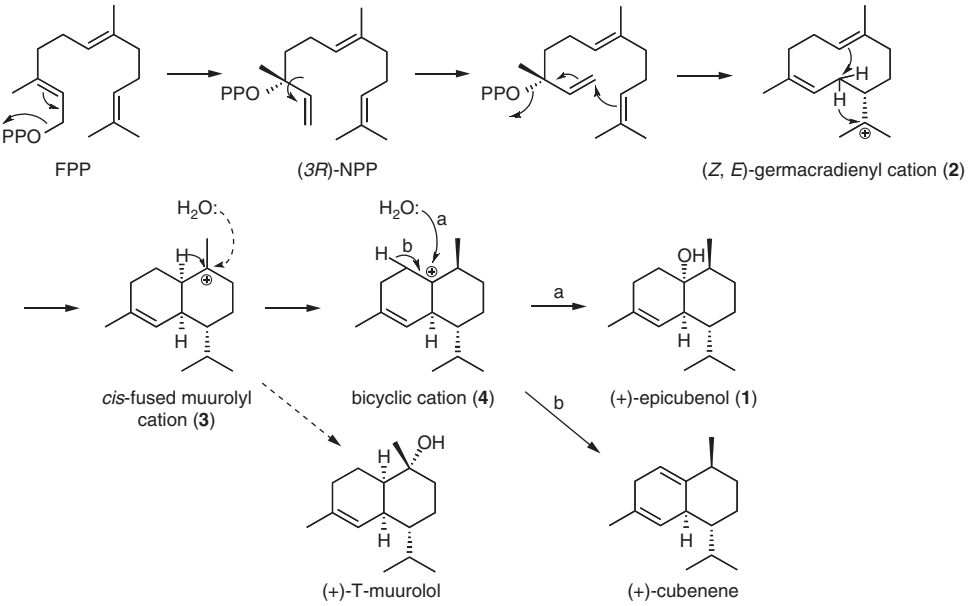

Figure 1 Reactions proposed to be catalyzed by (+)-epicubenol synthase. (+)-Epicubenol synthase first catalyzes the isomerization of FPP to (*3R*)-nerolidyl diphosphate and its subsequent ionization-initiated cyclization to yield (*Z, E*)-germacradienyl cation (**2**). It then catalyzes a 1,3-hydride shift and a second cyclization to give the *cis*-fused muurolyl cation (**3**). A 1,2-hydride shift then occurs to form the bicyclic cation (**4**), **4** is then quenched by the addition of a water molecule to produce (+)-epicubenol (path a). (+)-Cubenene is produced by deprotonation of **4** (path b). The structure of (+)-T-muurolol, which is produced by the addition of a water molecule to the *cis*-fused muurolyl cation (**3**), is also shown.

manipulations were purchased from Takara Bio (Otsu, Japan). *S. griseus* IFO13350 was obtained from the Institute of Fermentation, Osaka, Japan (IFO). *S. lividans* TK21 was obtained from David A. Hopwood (John Innes Centre, Norwich, UK). Plasmid pHSA81, used for heterologous expression of *gecA* in *S. lividans*, was obtained from Michihiko Kobayashi (University of Tsukuba, Ibaraki, Japan). *S. lividans* and *S. griseus* were cultured in yeast extract–malt extract (YEME) medium (glucose, 1%; sucrose, 34%; yeast extract, 0.3%; malt extract, 0.3%; bacto peptone, 0.5%; $MgCl_2 \cdot 6H_2O$, 0.1%; pH 7.0) and YMPD medium,[26] respectively. FPP was purchased from Sigma-Aldrich (St Louis, MO, USA). Authentic (−)-germacradien-4-ol was produced by the *in vitro* reaction of recombinant SC1 with FPP.[15]

Construction of plasmids

Chromosomal DNA of *S. griseus* was used as a template for the PCR amplification of *gecA* with primer I 5′-CCGAATTC*CAT**ATG***GACAGC GAACTGCCGGACATCTAC-3′ (*Eco*RI site underlined, *Nde*I site italicized and the start codon of *gecA* shown in boldface) and primer II 5′-CCGAAG CTT*GGATCC*TCAGCGGGCCCGGGGAGCCTCG-3′ (*Hin*dIII site underlined and *Bam*HI site italicized). The PCR product was digested with *Eco*RI and *Hin*dIII, and cloned between the *Eco*RI and *Hin*dIII sites of pUC19. The DNA fragments amplified by PCR were sequenced to confirm that no PCR error was introduced. The resultant plasmid pUC19-*gecA* was digested with *Nde*I and *Bam*HI, and cloned between the *Nde*I and *Bam*HI sites of pColdI and pHSA81, resulting in pColdI-*gecA* and pHSA81-*gecA*, respectively.

Analysis of terpenoids produced by recombinant *S. lividans* strains

S. lividans harboring pHSA81-*gecA* (or an empty vector pHSA81) was cultivated at 30 °C for 72 h in YEME medium (100 ml) supplemented with 5 μg ml^{-1} thiostrepton. Cells were harvested by centrifugation and resuspended in 50 mM Tris–HCl (pH 8.0, 20 ml), then disrupted by sonication. The low-molecular-weight molecules were then extracted with ethyl acetate from the crude cell lysate. The organic layer was dried with Na_2SO_4, and evaporated to dryness. The residue was dissolved in ethyl acetate (5 ml) for GC-MS analysis.

Large-scale preparation and isolation of sesquiterpene

S. lividans harboring pHSA81-*gecA* was cultivated at 30 °C for 96 h in YEME medium (4 × 1 l) supplemented with 5 μg ml^{-1} thiostrepton. Cells were harvested by centrifugation and resuspended in 50 mM Tris-HCl (pH 8.0, 500 ml), then disrupted by sonication. Low-molecular-weight molecules were then extracted with ethyl acetate from the crude cell lysate. The organic layer was dried with Na_2SO_4 and evaporated to dryness. The crude materials were dissolved in a small amount of *n*-hexane. Sesquiterpene produced was purified by SiO_2 column chromatography with *n*-hexane:ethyl acetate (100:0 to 100:5), yielding 9.2 mg of **1**. The structure of **1** was determined by NMR using ^{1}H, ^{13}C, DEPT, ^{1}H-^{1}H COSY, NOESY, HMQC and HMBC experiments, and specific rotation analysis (Supplementary Figure S1).

Production and purification of the recombinant GecA protein

To produce N-terminal His_6-tagged fusion proteins of GecA, *E. coli* BL21(DE3) strain harboring pColdI-*gecA* was grown at 37 °C in Luria-Bertani medium (200 ml) supplemented with 50 μg ml^{-1} ampicillin. When the OD_{600} had reached 0.4, the culture was kept at 15 °C for 30 min. isopropylthiogalactoside (final 10 μM) was added and cultivation was continued for 24 h at 15 °C. Cells were harvested by centrifugation and resuspended in buffer A (50 mM Tris-HCl, 150 mM NaCl, 5 mM imidazole and 10% glycerol, pH 8.0, 40 ml). After sonication, cell debris was removed by centrifugation at 10 000 *g* for 20 min. Recombinant His-tagged protein was purified over a nickel-nitrilotriacetic acid superflow (Qiagen, Hilden, Germany) according to the manufacturer's instructions, with the exception that 10% glycerol was added to all buffers. The purified GecA protein was dialyzed against buffer B (10 mM Tris–HCl and 10% glycerol, pH 8.0). The purity of the recombinant proteins was checked by SDS–polyacrylamide gel electrophoresis. The protein concentration was measured with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

In vitro enzyme assay

For the analysis of sesquiterpenes, the reaction mixture consisted of 50 mM Tris–HCl (pH 8.0), 5 mM $MgCl_2$, 5 mM mercaptoethanol, 4.6 μM FPP and 0.5 μM GecA in a total volume of 2.5 ml. The reaction mixture, overlaid with *n*-hexane (1 ml), was incubated at 30°C for 4 h. The reaction was terminated by the addition of 0.5 M EDTA (pH 8.0, 200 μl) followed by immediate vortexing for 30 s. The product(s) was extracted with *n*-hexane and subjected to GC-MS analysis. For determination of the optimum conditions for the GecA reaction, an *in vitro* assay was performed as follows. The standard reaction mixture contained 50 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) (pH 8.0), 1 mM $MgCl_2$, 5 mM mercaptoethanol, 2.3 μM FPP and 29 nM GecA in a total volume of 2.5 ml. The reaction mixture, overlaid with *n*-hexane (1 ml), was incubated at 35 °C for 20 min. The data were obtained from three independent experiments.

Kinetic analysis

The reaction mixture (2.5 ml) contained 50 mM HEPES (pH 8.0), 1 mM $MgCl_2$, 5 mM mercaptoethanol, FPP (0.09 to 4.6 μM) and 29 nM GecA. Each reaction mixture was incubated at 35 °C for 3 min. Each reaction was terminated by the addition of 0.5 M EDTA (pH 8.0, 200 μl) followed by immediate vortexing for 30 s. The product was extracted with *n*-hexane and subjected to GC-MS analysis. We confirmed that the product formation was linear throughout this period. Steady-state parameters were determined by fitting the curve to $v = V_{max}[S]/(K_m + [S])$, where V_{max} is the maximum reaction rate, v is the initial velocity of formation of (+)-epicubenol by GecA and S is the concentration of FPP. The data were obtained from three independent experiments.

Gene disruption of *gecA* in *S. griseus*

A 1.63-kbp DNA fragment, which contained a 1.48-kbp region upstream from the *gecA* start codon and a 150-bp region downstream from the start codon, was amplified by PCR using primer III, (5′-GCC*AAGCTT*ATCCCGAAG-GACTCCTTGTAGAAG-3′ (*Hin*dIII site italicized) and primer IV, 5′-CGC*A AGCTT*ATCGGCTTGTTCGAATCGGTTCCTC-3′ (*Hin*dIII site italicized). Chromosomal DNA of *S. griseus* was used as a template for the PCR amplification. The PCR product was digested with *Hin*dIII and cloned into the *Hin*dIII site of pUC19, resulting in pUC-Δ*gecA*N. Similarly, a 1.48-kbp DNA fragment, which contained a 627-bp region upstream from the *gecA* stop codon and a 850-bp region downstream from the stop codon, was amplified by PCR using primer V, 5′-TGG*GGTACC*GTCACCTACCTCACGACCGCCAC GACCTG-3′ (*Kpn*I site italicized) and primer VI, 5′-GCG*GAATTC*TTACG GGCTGCGGCACGCGGTGGTCGTCGAGTC-3′ (*Eco*RI site italicized). The PCR product was digested with *Kpn*I and *Eco*RI and cloned between the *Kpn*I and *Eco*RI sites of pUC19, resulting in pUC-Δ*gecA*C. The kanamycin resistance gene (*aphII*) from Tn*5*[34] was inserted between the *Hin*dIII and *Kpn*I sites of the pUC19-Δ*gecA*C, resulting in pUC19-Δ*gecA*CK. The *Hin*dIII fragment excised from the pUC19-Δ*gecA*N was cloned in the *Hin*dIII site of pUC19-Δ*gecA*CK, resulting in pUC19-Δ*gecA*, in which the middle of *gecA* sequence (encoding Phe-51 to Leu-150) was replaced by the *aphII* sequence. pUC19-Δ*gecA* was linearized with *Dra*I and introduced by protoplast transformation into *S. griseus* IFO13350. Kanamycin-resistant transformants were selected, and the gene replacement, as a result of double crossover, was confirmed by Southern hybridization.

Examination of terpenoid production in *S. griseus*

The wild-type strain *S. griseus,* mutant Δ*gecA*::*aphII* and mutant Δ*adpA* were grown in YMPD medium (100 ml) at 30 °C for 72 h. Cells were harvested by centrifugation and resuspended in 50 mM Tris–HCl (pH 8.0, 20 ml), then disrupted by sonication. Low-molecular-weight molecules were then extracted with ethyl acetate from the crude cell lysate. The organic layer was dried with Na_2SO_4 and then analyzed by GC-MS.

S1 nuclease mapping

Total RNA was isolated with Isogen (Nippon Gene, Tokyo, Japan) from cells grown at 30 °C in YMPD liquid medium, as described previously.[35] S1 nuclease mapping was performed by a method described by Bibb *et al.*[36] and Kelemen *et al.*[37] Hybridization probes were prepared by PCR with a pair of

^{32}P-labeled and unlabeled primers. *hrdB* encoding a principal sigma factor of RNA polymerase was used to check the purity and amount of RNA used, as described previously.[29] Primers used in S1 nuclease mapping are listed in Supplementary Table 1.

RESULTS

Identification of a terpenoid produced by the *gecA* overexpression strain

To analyze *in vivo* function of GecA, a *gecA*-overexpressing *S. lividans* strain was constructed. Low-molecular-weight molecules were extracted from the recombinant strain (or the negative control strain containing the empty vector pHSA81) using ethyl acetate. When the sample was analyzed by GC-MS, major product **1** was detected (Figure 2a). The mass spectrum of product **1** had peaks at *m/z* 204 and 207, corresponding to fragment ions of dehydrated ($C_{15}H_{26}O-H_2O$) and demethylated ($C_{15}H_{26}O-CH_3$) forms, respectively, of a sesquiterpene alcohol (Figure 2b). Product **1** was purified by SiO_2 column chromatography, yielding 9.2 mg of **1** from the 4-l culture. The ^{1}H NMR spectrum displayed signals arising from one fully substituted methyl group (δ 1.54 [3H, s]), three doublet methyl groups (δ 0.78, 0.83 and 1.10 [each 3H, d, $J=7.0$ Hz]) and one olefinic proton (δ 5.39 [1H, bs]), while 12 remaining protons appeared in the δ 0.98–2.04 region. The ^{13}C NMR spectrum showed 15 carbon resonances; one C–H olefinic carbon (δ 122.5) and one fully substituted olefinic carbon (δ 133.9). By two-dimensional NMR spectroscopic analyses, including COSY, HMQC, HMBC and NOESY experiments (Supplementary Figure S1), we determined the structure and relative stereochemistry of **1** the bicyclic sesquiterpene alcohol, epicubenol. The NMR spectra and signal assignments of **1** agreed completely with those previously reported for (+)-epicubenol.[22] Finally, the absolute configuration of **1** was determined as (+) by comparing the specific rotation value of **1** ($[\alpha]_D$ +111.6) with that of (+)-epicubenol from *H. planus* ($[\alpha]_D$ +106.5).[20]

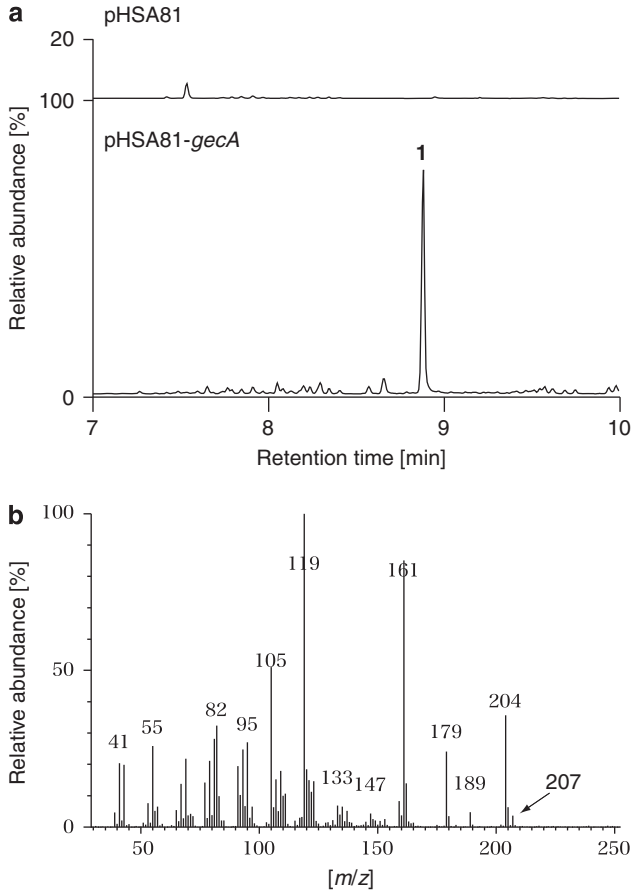

Figure 2 GC-MS analysis of extracts from crude cell lysate of recombinant *S. lividans* strains. (**a**) GC-MS chromatograms of extracts from crude cell lysate of *S. lividans* strains harboring empty vector pHSA81 and pHSA81-*gecA*. (**b**) Mass spectrum of **1**.

In vitro analysis of the GecA reaction

GecA was produced as an N-terminal His-tagged protein (MNHKVH$_6$IEGRH-GecA) in *E. coli*. The recombinant GecA was purified by nickel-nitrilotriacetic acid affinity chromatography to give a single major protein band of 43 kDa on SDS–PAGE (Supplementary Figure S2). Incubation of the recombinant GecA protein with FPP yielded (+)-epicubenol (**1**) as a major product, as determined by GC-MS analysis (Figure 3a). In addition to **1**, at least three minor products were detected in the *in vitro* GecA reaction (Figure 3a). A minor product (at 8.6 mm), **5**, was identified as germacradien-4-ol by comparison of the retention time and mass fragmentation pattern with those of the authentic sample (Figure 3a and b). Minor products **6** (at 8.3 mm) and **7** (at 7.8 mm) were predicted to be cubenene (see Figure 1 for structure) and β-gurjunene (see Figure 3c for structure), respectively, by comparison of the mass spectrum with a GC-MS database (National Institute of Standards and Technology Libraries, NIST08) (Supplementary Figure S3). Although the mechanism of the reaction catalyzed by the bacterial (+)-epicubenol synthase from *Streptomyces* sp. LL-B7 has previously been characterized, no kinetic analysis of the enzyme was reported. We therefore analyzed the kinetics of GecA. First, the optimum pH and temperature of the GecA reaction were examined. The optimum pH for (+)-epicubenol synthesis at 35 °C was found to be 8.0 (Figure 4a). The optimum temperature for (+)-epicubenol synthesis at pH 8.0 was found to be 35 °C (Figure 4b). The K_m value for FPP and the k_{cat} value for (+)-epicubenol formation under these optimized conditions (pH 8.0 and 35 °C), in the presence of 1 mM $MgCl_2$ (see below), were measured as 254 ± 7.1 nM and 0.026 ± 0.001 s^{-1}, respectively (Figure 4c). The k_{cat}/K_m value (0.10 s^{-1} μM^{-1}) was broadly comparable with those reported for known bacterial SCs.[7,9,10] GecA requires divalent metal ions for its activity. Mg^{2+} was the most effective among the divalent metal ions examined, and more than 1 mM $MgCl_2$ was required for maximum activity (data not shown). In the presence of Mn^{2+} (1 mM $MnCl_2$), GecA showed only 12% activity, relative to the activity in the presence of Mg^{2+}. In contrast, no activity was observed for Fe^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+} or Cu^{2+} (each at 1 mM as chloride salt).

Production of (+)-epicubenol by wild-type *S. griseus* and an *adpA*-deleted mutant but not by a *gecA*-knockout mutant

To examine the production of (+)-epicubenol in *S. griseus*, we analyzed the extracts of the crude cell lysate of the wild-type and *gecA*-disrupted strains by GC-MS (Figure 5). Because a fragment ion peak with *m/z* 119 was detected for (+)-epicubenol (**1**) (Figure 2b),[20] production of **1** was assessed by monitoring the fragment ion peak with *m/z* 119 in the GC-MS analysis (Figure 5). A peak at 8.9 min was detected in the selected ion chromatogram of the wild-type sample, but not in the Δ*gecA*::*aphII* sample. This peak was shown to come from (+)-epicubenol by comparison with the authentic sample (Figure 5). From this result, we concluded that *gecA* is not a cryptic gene and is responsible for (+)-epicubenol production in *S. griseus*.

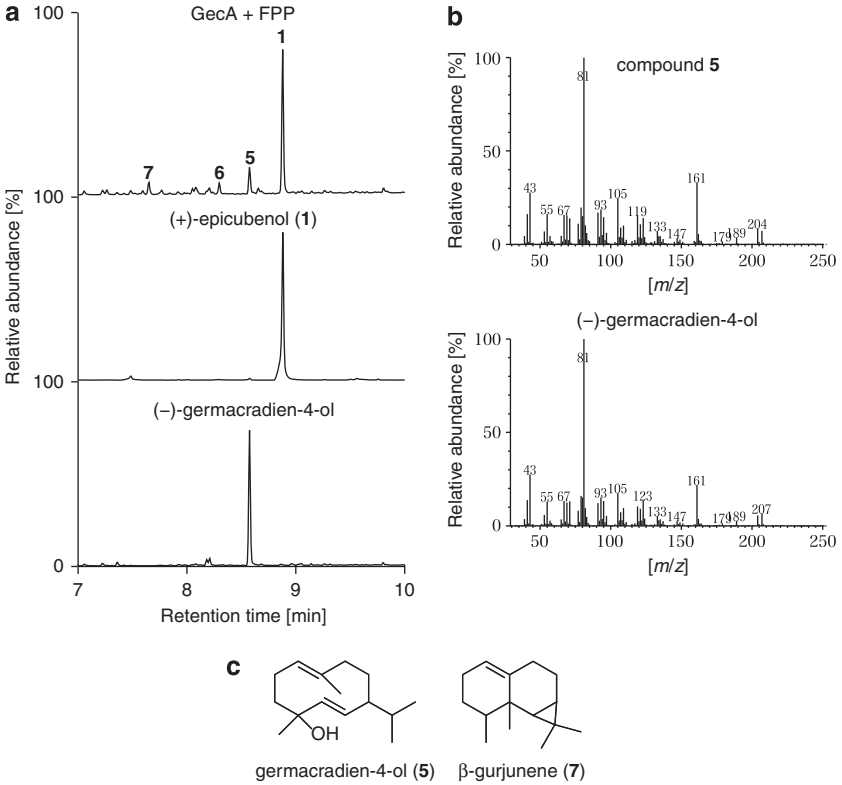

Figure 3 *In vitro* reaction of recombinant GecA protein. (**a**) GC-MS chromatograms of products generated by GecA with FPP, (+)-epicubenol and (−)-germacradien-4-ol. (**b**) Mass spectrum of **5** and (−)-germacradien-4-ol. (**c**) Structures of germacradiene-4-ol (**5**) and β-gurjunene (**7**).

Because *gecA* did not appear to be a direct target of AdpA,[31] a master regulator of secondary metabolism and morphological differentiation in *S. griseus*, we also analyzed the extracts of the crude cell lysate of an *adpA*-deleted (Δ*adpA*) mutant by GC-MS (Figure 5). As expected, the production of **1** by the Δ*adpA* mutant could be detected, confirming that (+)-epicubenol production is not controlled by A-factor in *S. griseus*. Interestingly, the Δ*adpA* mutant produced more (+)-epicubenol than the wild-type strain.

Transcription profiles of *gecA* and *gcoA* in the wild-type and *adpA*-deleted strains

As described above, the Δ*adpA* strain produced more (+)-epicubenol than the wild-type strain. In contrast, (+)-caryolan-1-ol, which is synthesized by another single-domain SC, GcoA, is produced in the wild-type *S. griseus* strain, but not in the Δ*adpA* mutant.[12] Therefore, we analyzed expression profiles of *gecA* and *gcoA* in the wild-type and Δ*adpA* strains by S1 nuclease mapping. RNAs were extracted from each strain grown for 12 (mid log phase), 21 (late log phase) and 32 h (stationary phase) in liquid YMPD. *gecA* was transcribed throughout growth; the transcription was slightly more active in mid log phase (Figure 6). No significant difference in the amount of *gecA* transcript was observed between the wild-type and Δ*adpA* strains (Figure 6), indicating that the observed difference in the productivity of (+)-epicubenol could not be ascribed to differences in the transcriptional level of *gecA*. Unexpectedly, transcription of *gcoA* was detected in the Δ*adpA* mutant strain, similar to the wild-type strain, almost constantly throughout growth (Figure 6). This result apparently contradicted the observation that the Δ*adpA* strain did not produce (+)-caryolan-1-ol.[12] Presence of a TTA codon in *gcoA* and regulation of the tRNA-UUA gene by AdpA could explain this apparent contradiction (see Discussion).

DISCUSSION

In this study, we demonstrated that GecA is a SC involved in (+)-epicubenol synthesis by *S. griseus*. (+)-Epicubenol synthases have been previously purified and characterized from the liverwort *H. planus* and *Streptomyces* sp. LL-B7. However, no (+)-epicubenol synthase gene has been cloned to date, and *gecA* is thus the first example of an (+)-epicubenol synthase-encoding gene. The identification of GecA as an (+)-epicubenol synthase enabled a phylogenic comparison of the (+)-epicubenol synthase to be made with other known SCs and SC homologs. Six close homologs of GecA were found in other *Streptomyces* species; *S. griseus* XylebKG-1 (SACT1_6398, 100% amino-acid identity with GecA), *Streptomyces roseosporus* NRRL 11379 (SrosN1_010100003716, 95%), *Streptomyces* sp. W007 (SPW_4380, 95%), *S. roseosporus* NRRL 15998 (SSGG_00557, 94%), *Streptomyces* sp. SirexAA-E (SACTE_0873, 84%) and *Streptomyces flavogriseus* ATCC 33331 (Sfla_5399, 81%). These GecA homologs are presumed to be (+)-epicubenol synthases. Very recently, Citron *et al.*[38] reported that *S. griseus* IFO13350 (NBRC13350), *S. roseosporus* NRRL15998 and *S. flavogriseus* ATCC33331 produce epicubenol. SSGG_00557 and Sfla_5399 therefore should be responsible for the production of epicubenol. Together with the observation that three *Streptomyces* species, *Streptomyces* sp. LL-B7, sp. LL-B5a and sp. LL-100-1 (Eren), produce (+)-epicubenol,[19] this sesquiterpene alcohol appears to be a relatively common secondary metabolite in the genus *Streptomyces*.

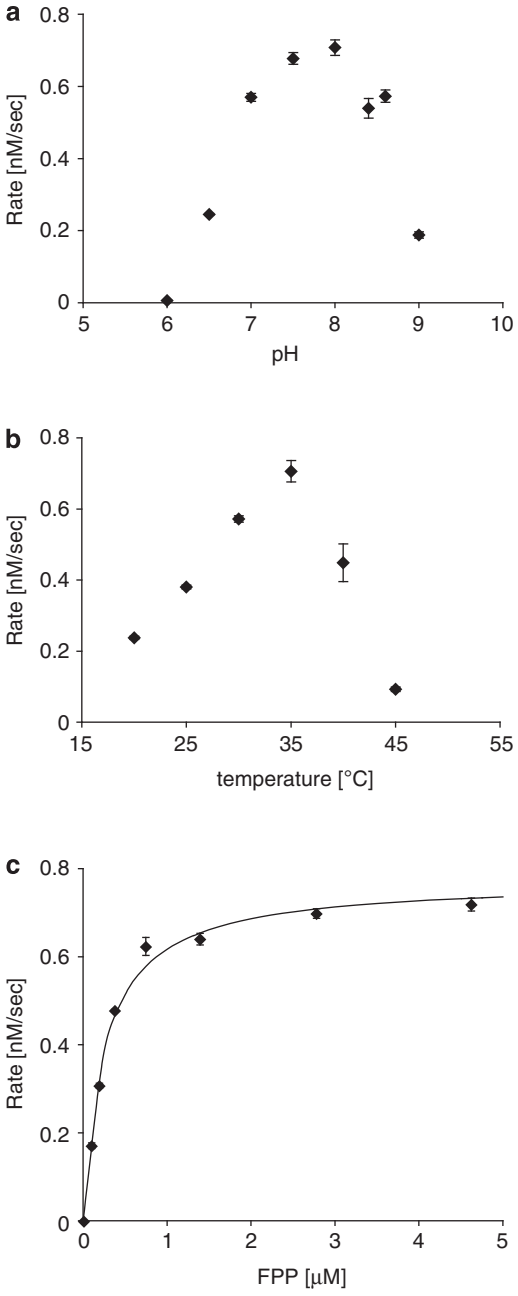

Figure 4 Analysis of the optimum pH and temperature for the GecA reaction and kinetic analysis of GecA. (**a**) Recombinant GecA was incubated with FPP at 35 °C at various pHs; 50 mM 4-morpholineethanesulfonic acid (MES) buffer (pH 6.0–6.5), 50 mM HEPES buffer (pH 7.0–8.4) and 50 mM *N*-cyclohexyl-2-aminoethanesulfonic acid (CHES) buffer (pH 8.6–9.0) were used. (**b**) Recombinant GecA was incubated with FPP in 50 mM HEPES buffer (pH 8.0) at various temperatures (20–45 °C). (**c**) The rate–FPP concentration profile for (+)-epicubenol synthesis from FPP are shown. The data were obtained from three independent experiments.

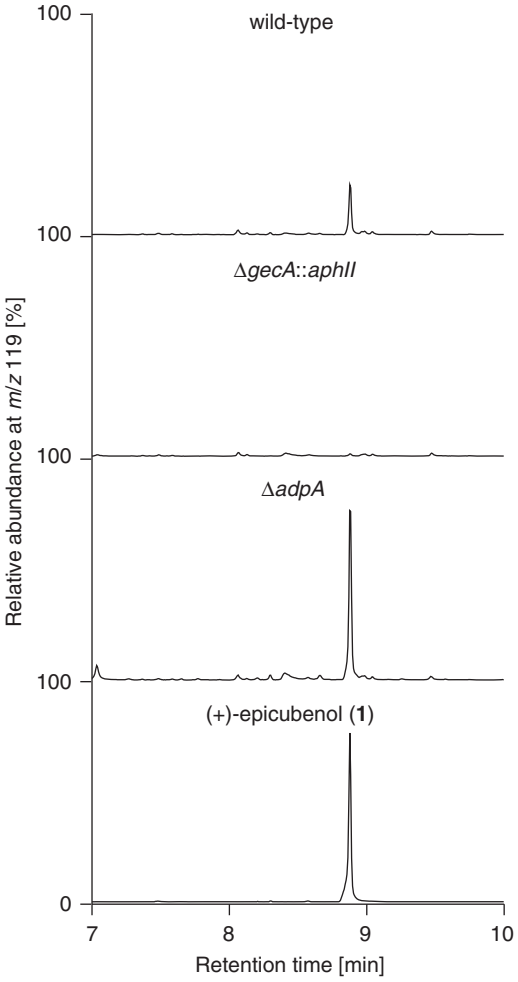

Figure 5 GC-MS analysis of terpenoids produced by *S. griseus*. GC-MS chromatograms of the extracts of wild-type *S. griseus* and mutants Δ*gecA*::*aphII* and Δ*adpA*, and (+)-epicubenol.

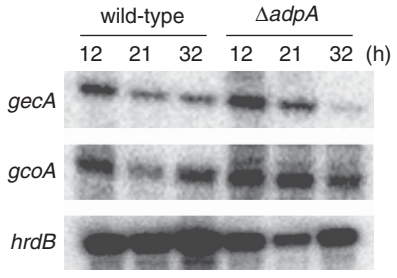

Figure 6 Time course of transcription of *gecA* and *gcoA*, as determined by low-resolution S1 nuclease mapping. RNA was prepared from the wild-type and Δ*adpA* strains grown at 30 °C in liquid YMPD for the times indicated above the panels. The *hrdB* gene encoding a principal sigma factor was used as an internal control.

GecA catalyzes the conversion of FPP into (+)-epicubenol, probably with the same reaction mechanism as has been previously elucidated for the (+)-epicubenol synthase from *Streptomyces* sp. LL-B7 (Figure 1). Three minor products, germacradien-4-ol and the putative products cubenene and β-gurjunene, were detected in the *in vitro* GecA reaction. Germacradien-4-ol and β-gurjunene are predicted to be generated from the (*E*, *E*)-germacradienyl cation formed by ionization-initiated cyclization of FPP (Supplementary Figure S4). Thus, we postulate that GecA produces small amount of (*E*, *E*)-germacradienyl cation, in addition to (*Z*, *E*)-germacradienyl cation (**2**), at least in the *in vitro* reaction. The multiproduct terpene synthase from *Medicago truncatula* generates both (*E*, *E*)

and (*Z*, *E*)-germacradienyl cations.[39] However, it is also possible that germacradien-4-ol is generated from a reaction intermediate, (*Z*, *E*)-germacradienyl cation (**2**), of the (+)-epicubenol synthesis (Figure 1).[40] Cubenene can also be generated from another reaction intermediate, bicyclic cation (**4**) (Figure 1). The SC involved in (+)-epicubenol biosynthesis in *H. planus* also produces (+)-cubenene; an *in vitro* enzyme reaction yielded both (+)-epicubenol and (+)-cubenene with a molar ratio of 1:3,[21] indicating that the major product is (+)-cubenene. In contrast, GecA produces only a small amount of cubenene (Figure 3a). In the synthesis of (+)-epicubenol, the bicyclic cation (**4**) is quenched by the addition of a water molecule ('path a' in Figure 1).[25] Cane *et al.*[25] speculated that the same water molecule might serve as the Lewis base in the deprotonation reaction ('path b' in Figure 1) that would lead to the formation of cubenene. In the reaction pocket of the *H. planus* (+)-cubenene/(+)-epicubenol synthase, deprotonation of the bicyclic cation (**4**) may occur much more easily compared with GecA. Moreover, it is interesting that the reactions catalyzed by (+)-T-muurolol synthase are partially identical to those catalyzed by GecA, although (+)-T-muurolol synthase from *S. clavuligerus* shows a low amino-acid sequence similarity (25% identity) to GecA. In (+)-T-muurolol synthesis, the *cis*-fused muurolyl cation (**3**) is produced, and this intermediate is quenched by the addition of a water molecule (Figure 1).[11]

We reported that transcription of *gcoA* was enhanced by exogenous A-factor in an A-factor-deficient mutant, as determined by DNA microarray analysis (fold change 4.1–5.5; $P<0.01$; 4–12 h after A-factor addition).[31] Our recent transcriptome analysis also showed that *gcoA* was downregulated in the absence of *adpA* (fold change 3.8; $P=0.003$).[41] In contrast, our previous transcriptome analysis showed that *gcoA* was slightly upregulated in the Δ*adpA* strain compared with the wild-type strain (fold change 1.3; $P=0.001$).[26] In the present study, we found that the transcription profile of *gcoA* was similar in the wild-type and Δ*adpA* strains, as determined by S1 nuclease mapping. This result indicated that AdpA should not necessarily be required for the transcription of *gcoA*. The lack of (+)-caryolan-1-ol production by the Δ*adpA* strain could be explained by the presence of a TTA codon (for Leu-6) in *gcoA*. The UUA leucine codon is very rare in *Streptomyces*, and *bldA* encodes the only tRNA species able to read the UUA codon efficiently. Recently, it was shown that AdpA bound to the upstream region of *bldA* and activated transcription of *bldA*.[42] Thus, the TTA-containing *gcoA* gene is presumably not translated efficiently in the Δ*adpA* mutant and GcoA is not produced in the Δ*adpA* mutant, which results in no production of (+)-caryolan-1-ol.

Genome sequencing has revealed that *S. griseus* IFO13350 has more than 34 (putative) biosynthesis gene clusters (or genes) thought to be involved in secondary metabolite production.[26] However, the products of most of these clusters are yet to be identified. The secondary metabolites of *S. griseus* IFO13350 that have been identified to date are streptomycin,[43] grixazone,[44] 1,4,6,7,9,12-hexahydroxyperylene-3,10-quinone,[45] alkylquinones,[46] a carotenoid,[47] geosmin,[48] 2-methylisoborneol,[48] and (+)-caryolan-1-ol.[12] The production of streptomycin, grixazone, hexahydroxyperylene-3,10-quinone and (+)-caryolan-1-ol has been shown to be under the control of the A-factor regulatory cascade, either directly or indirectly. In contrast, the production of alkylquinones, geosmin, and 2-methylisoborneol does not appear to be controlled by A-factor. The production of the carotenoid is cryptic under normal culture conditions. In this study, we succeeded in identifying (+)-epicubenol as a secondary metabolite produced by *S. griseus* and confirmed that its production is not controlled by A-factor. In contrast, production of (+)-caryolan-1-ol is controlled by A-factor through the translational regulation of *gcoA* by the BldA tRNA, which is produced in an AdpA-dependent manner. Thus, *S. griseus* produces two sesquiterpene alcohols using single-domain SCs; (+)-caryolan-1-ol, whose production is A-factor dependent, and (+)-epicubenol, whose production is independent of A-factor. This finding provides new insight into the regulation of secondary metabolism by A-factor in *S. griseus*. It should be noted that neither the *gcoA* mutant nor the *gecA* mutant showed any phenotypic changes with respect to growth or morphological differentiation.

Very recently, the production of a great variety of volatile terpenoids from 35 strains, including 31 actinomycetes and four strains from other taxa, was reported.[38] This result, together with several genome mining studies for SC homologs by our group[12–15] and others,[2–11] indicates that terpenoids are significantly more widely distributed in nature than was previously appreciated, being widespread among not only eukaryotes, but also bacteria.

ACKNOWLEDGEMENTS

This work was supported in part by the Targeted Proteins Research Program (TPRP) of the Ministry of Education, Culture, Sports, Science and Technology of Japan, and a Funding Program for Next Generation World-Leading Researchers from the Bureau of Science, Technology, and Innovation Policy, Cabinet Office, Government of Japan.

1 Chappell, J. Biochemistry and molecular biology of the isoprenoid biosynthetic pathway in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **46,** 521–547 (1995).
2 Cane, D. E. & Watt, R. M. Expression and mechanistic analysis of a germacradienol synthase from *Streptomyces coelicolor* implicated in geosmin biosynthesis. *Proc. Natl Acad. Sci. USA* **100,** 1547–1551 (2003).
3 Cane, D. E., He, X., Kobayashi, S., Ōmura, S. & Ikeda, H. Geosmin biosynthesis in *Streptomyces avermitilis*. *J. Antibiot.* **59,** 471–479 (2006).
4 Ghimire, G. P., Oh, T. -J., Lee, H. C., Kim, B. -G. & Sohng, J. K. Cloning and functional characterization of the germacradienol synthase (*spterp13*) from *Streptomyces peucetius* ATCC 27952. *J. Microbiol. Biotechnol.* **18,** 1216–1220 (2008).
5 Giglio, S., Jiang, J., Saint, C. P., Cane, D. E. & Monis, P. T. Isolation and characterization of the gene associated with geosmin production in cyanobacteria. *Environ. Sci. Technol.* **42,** 8027–8032 (2008).
6 Tetzlaff, C. N., You, Z., Cane, D. E., Takamatsu, S., Ōmura, S. & Ikeda, H. A gene cluster for biosynthesis of the sesquiterpenoid antibiotic pentalenolactone in *Streptomyces avermitilis*. *Biochemistry* **45,** 6179–6186 (2006).
7 Lin, X., Hopson, R. & Cane, D. E. Genome mining in *Streptomyces coelicolor*: Molecular cloning and characterization of a new sesquiterpene synthase. *J. Am. Chem. Soc.* **128,** 6022–6023 (2006).
8 Takamatsu, S., Lin, X., Nara, A., Komatsu, M., Cane, D. E. & Ikeda, H. Characterization of a silent sesquiterpenoid biosynthetic pathway in *Streptomyces avermitilis* controlling *epi*-isozizaene albaflavenone biosynthesis and isolation of a new oxidized *epi*-isozizaene metabolite. *Microb. Biotechnol.* **4,** 184–191 (2011).
9 Chou, W. K. W., Fanizza, I., Uchiyama, T., Komatsu, M., Ikeda, H. & Cane, D. E. Genome mining in *Streptomyces avermitilis*: cloning and characterization of SAV_76, the synthase for a new sesquiterpene, avermitilol. *J. Am. Chem. Soc.* **132,** 8850–8851 (2010).
10 Agger, S. A., Lopez-Gallego, F., Hoye, T. R. & Schmidt-Dannert, C. Identification of sesquiterpene synthases from *Nostoc punctiforme* PCC 73102 and *Nostoc* sp. strain PCC 7120. *J. Bacteriol.* **190,** 6084–6096 (2008).
11 Hu, Y., Chou, W. K. W., Hopson, R. & Cane, D.E. Genome mining in *Streptomyces clavuligerus*: expression and biochemical characterization of two new cryptic sesquiterpene synthases. *Chem. Biol.* **18,** 32–37 (2011).
12 Nakano, C., Horinouchi, S. & Ohnishi, Y. Characterization of a novel sesquiterpene cyclase involved in (+)-caryolan-1-ol biosynthesis in *Streptomyces griseus*. *J. Biol. Chem.* **286,** 27980–27987 (2011).
13 Nakano, C., Kim, H. -K. & Ohnishi, Y. Identification of the first bacterial monoterpene cyclase, a 1,8-cineole synthase, that catalyzes the direct conversion of geranyl diphosphate. *Chembiochem* **12,** 1988–1991 (2011).
14 Nakano, C., Kim, H. -K. & Ohnishi, Y. Identification and characterization of the linalool/nerolidol synthase from *Streptomyces clavuligerus*. *Chembiochem* **12,** 2403–2407 (2011).
15 Nakano, C., Kudo, F., Eguchi, T. & Ohnishi, Y. Genome mining reveals two novel bacterial sesquiterpene cyclases: (−)-germacradien-4-ol and (−)-*epi*-α-bisabolol synthases from *Streptomyces citricolor*. *Chembiochem* **12,** 2271–2275 (2011).
16 Cane, D. E. & Ikeda, H. Exploration and mining of the bacterial terpenome. *Acc. Chem. Res.* **45,** 463–472 (2012).

17 Connolly, J. D., Phillips, W. R. & Huneck, S. (+)-*Ent*-epicubenol from the liverwort *Scapania undulata*. *Phytochemistry* **21,** 233–234 (1982).
18 Nabeta, K., Katayama, K., Nakagawara, S. & Katoh, K. Sesquiterpenes of cadinane type from cultured cells of the liverwort, *Heteroscyphus planus*. *Phytochemistry* **32,** 117–122 (1993).
19 Gerber, N. N. Sesquiterpenoids from actinomycetes: cadin-4-ene-1-ol. *Phytochemistry* **10,** 185–189 (1971).
20 Nabeta, K., Kigure, K., Fujita, M., Nagoya, T., Ishikawa, T., Okuyama, H. & Takasawa, T. Biosynthesis of (+)-cubenene and (+)-epicubenol by cell-free extracts of cultured cells of *Heteroscyphus planus* and cyclization of [^{2}H]farnesyl diphosphates. *J. Chem. Soc. Perkin Trans. 1,* 1935–1939 (1995).
21 Nabeta, K., Fujita, M., Komuro, K., Katayama, K. & Takasawa, T. *In vitro* biosynthesis of cadinanes by cell-free extracts of cultured cells of *Heteroscyphus planus*. *J. Chem. Soc. Perkin Trans. 1,* 2065–2070 (1997).
22 Cane, D. E., Tandon, M. & Prabhakaran, P. C. Epicubenol synthase and the enzymatic cyclization of farnesyl diphosphate. *J. Am. Chem. Soc.* **115,** 8103–8106 (1993).
23 Cane, D. E. & Tandon, M. Biosynthesis of (+)-epicubenol. *Tetrahedron lett.* **35,** 5355–5358 (1994).
24 Cane, D. E. & Tandon, M. Epicubenol synthase and the stereochemistry of the enzymatic cyclization of farnesyl and nerolidyl diphosphate. *J. Am. Chem. Soc.* **117,** 5602–5603 (1995).
25 Cane, D. E. & Ke, N. Epicubenol synthase. Origin of the oxygen atom of a bacterial sesquiterpene alcohol. *Bioorg. Med. Chem. Lett.* **10,** 105–107 (2000).
26 Ohnishi, Y., Ishikawa, J., Hara, H., Suzuki, H., Ikenoya, M., Ikeda, H., Yamashita, A., Hattori, M. & Horinouchi, S. Genome sequence of the streptomycin-producing microorganism *Streptomyces griseus* IFO 13350. *J. Bacteriol.* **190,** 4050–4060 (2008).
27 Horinouchi, S. & Beppu, T. Hormonal control by A-factor of morphological development and secondary metabolism in *Streptomyces*. *Proc. Jpn. Acad. Ser. B* **83,** 277–295 (2007).
28 Kato, J., Funa, N., Watanabe, H., Ohnishi, Y. & Horinouchi, S. Biosynthesis of γ-butyrolactone autoregulators that switch on secondary metabolism and morphological development in *Streptomyces*. *Proc. Natl Acad. Sci. USA* **104,** 2378–2383 (2007).
29 Ohnishi, Y., Kameyama, S., Onaka, H. & Horinouchi, S. The A-factor regulatory cascade leading to streptomycin biosynthesis in *Streptomyces griseus*: identification of a target gene of the A-factor receptor. *Mol. Microbiol.* **34,** 102–111 (1999).
30 Ohnishi, Y., Yamazaki, H., Kato, J., Tomono, A. & Horinouchi, S. AdpA, a central transcriptional regulator in the A-factor regulatory cascade that leads to morphological development and secondary metabolism in *Streptomyces griseus*. *Biosci. Biotechnol. Biochem.* **69,** 431–439 (2005).
31 Hara, H., Ohnishi, Y. & Horinouchi, S. DNA microarray analysis of global gene regulation by A-factor in *Streptomyces griseus*. *Microbiology* **155,** 2197–2210 (2009).
32 Cane, D. E., Sohng, J. -K., Lamberson, C. R., Rudnicki, S. M., Wu, Z., Lloyd, M. D., Oliver, J. S. & Hubbard, B. R. Pentalenene synthase. purification, molecular cloning, sequencing, and high-level expression in *Escherichia coli* of a terpenoid cyclase from *Streptomyces* UC5319. *Biochemistry* **33,** 5846–5857 (1994).
33 Christianson, D. W. Structural biology and chemistry of the terpenoid cyclases. *Chem. Rev.* **106,** 3412–3442 (2006).
34 Beck, E., Ludwig, G., Auerswald, E. A., Reiss, B. & Schaller, H. Nucleotide sequence and extract localization of the neomycin phosphotransferase gene from transposon Tn5. *Gene* **19,** 327–336 (1982).
35 Tezuka, T., Hara, H., Ohnishi, Y. & Horinouchi, S. Identification and gene disruption of small noncoding RNAs in *Streptomyces griseus*. *J. Bacteriol.* **191,** 4896–4904 (2009).
36 Bibb, M. J., Janssen, G. R. & Ward, J. M. Cloning and analysis of the promoter region of the erythromycin resistance gene (*ermE*) of *Streptomyces erythraeus*. *Gene* **41,** 357–368 (1986).
37 Kelemen, G. H., Brian, P., Flärdh, K., Chamberlin, L., Chater, K. F. & Buttner, M. J. Developmental regulation of transcription of *whiE*, a locus specifying the polyketide spore pigment in *Streptomyces coelicolor* A3(2). *J. Bacteriol.* **180,** 2515–2521 (1998).
38 Citron, C. A., Gleitzmann, J., Laurenzano, G., Pukall, R. & Dickschat, J. S. Terpenoids are widespread in actinomycetes: a correlation of secondary metabolism and genome data. *Chembiochem* **13,** 202–214 (2012).
39 Garms, S., Köllner, T. G. & Boland, W. A multiproduct terpene synthase from *Medicago truncatula* generates cadalane sesquiterpenes via two different mechanisms. *J. Org. Chem.* **75,** 5590–5600 (2010).
40 Yoshikuni, Y., Martin, V. J. J., Ferrin, T. E. & Keasling, J. D. Engineering cotton (+)-δ-cadinene synthase to an altered function: germacrene D-4-ol synthase. *Chem. Biol.* **13,** 91–98 (2006).
41 Higo, A., Hara, H., Horinouchi, S. & Ohnishi, Y. Genome-wide distribution of AdpA, a global regulator for secondary metabolism and morphological differentiation in *Streptomyces*, revealed the extent and complexity of the AdpA regulatory network. *DNA Res.* **19,** 259–274 (2012).
42 Higo, A., Horinouchi, S. & Ohnishi, Y. Strict regulation of morphological differentiation and secondary metabolism by a positive feedback loop between two global regulators AdpA and BldA in *Streptomyces griseus*. *Mol. Microbiol.* **81,** 1607–1622 (2011).
43 Tomono, A., Tsai, Y., Yamazaki, H., Ohnishi, Y. & Horinouchi, S. Transcriptional control by A-factor of *strR*, the pathway-specific transcriptional activator for streptomycin biosynthesis in *Streptomyces griseus*. *J. Bacteriol.* **187,** 5595–5604 (2005).
44 Higashi, T., Iwasaki, Y., Ohnishi, Y. & Horinouchi, S. A-factor and phosphate depletion signals are transmitted to the grixazone biosynthesis genes via the pathway-specific transcriptional activator GriR. *J. Bacteriol.* **189,** 3515–3524 (2007).
45 Funa, N., Funabashi, M., Ohnishi, Y. & Horinouchi, S. Biosynthesis of hexahydroxyperylenequinone melanin via oxidative aryl coupling by cytochrome P-450 in *Streptomyces griseus*. *J. Bacteriol.* **187,** 8149–8155 (2005).
46 Funabashi, M., Funa, N. & Horinouchi, S. Phenolic lipids synthesized by type III polyketide synthase confer penicillin resistance on *Streptomyces griseus*. *J. Biol. Chem.* **283,** 13983–13991 (2008).
47 Lee, H. -S., Ohnishi, Y. & Horinouchi, S. A σ^B-like factor responsible for carotenoid biosynthesis in *Streptomyces griseus*. *J. Mol. Microbiol. Biotechnol.* **3,** 95–101 (2001).
48 Komatsu, M., Tsuda, M., Ōmura, S., Oikawa, H. & Ikeda, H. Identification and functional analysis of genes controlling biosynthesis of 2-methylisoborneol. *Proc. Natl Acad. Sci. USA* **105,** 7422–7427 (2008).

Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)