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Genome Mining Reveals Two Novel Bacterial Sesquiterpene Cyclases: (−)-Germacradien-4-ol and (−)-*epi*-α-Bisabolol Synthases from *Streptomyces citricolor*

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Terpenoids are the largest family of natural products, with over 24000 known compounds.[1] They have various biological functions, and act as flavorings, pigments, antibiotics, hormones, and antitumor agents. All terpenoids are derived from the C_5 precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). The successive condensation of IPP and DMAPP gives rise to linear isoprenyl diphosphate precursors of various chain lengths, such as geranyl diphosphate (C_{10}), farnesyl diphosphate (FPP, C_{15}), and geranylgeranyl diphosphate (C_{20}). These linear substrates are generally cyclized by terpenoid cyclases to produce the parent skeletons of monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), and other homologues. Terpenoid cyclases are therefore one of the key enzymes in pathways leading to the diverse range of terpenoids.

To date, most terpenoids have been isolated from plants and fungi, and only a few from bacteria. A large number of eukaryotic terpenoid cyclases have been reported,[2,3] although there are only a few reports covering bacterial terpenoid cyclases. Most of the reported bacterial terpenoid cyclases are sesquiterpene cyclases (SCs), including those involved in the synthesis of geosmin, pentalenene, *epi*-isozizaene, avermitilol, germacrene A, 8a-*epi*-α-selinene, (−)-δ-cadinene, and (+)-T-muurolol.[4–14] However, recent genome-sequencing projects have revealed a large number of potential bacterial SC genes. Terpenoids, therefore, seem to be significantly more widely distributed in nature than has been previously appreciated. This prompted us to characterize several previously undescribed bacterial sesquiterpene cyclase homologue genes found in genome sequences that have been released to public databases. Recently, we discovered four novel bacterial terpene synthases: the 1,8-cineole synthase CnsA[15] and linalool/nerolidol synthase LnsA (unpublished data) from *Streptomyces clavuligerus*, and the (+)-caryolan-1-ol synthase GcoA[16] and (+)-epicubenol synthase GecA (unpublished data) from *Streptomyces griseus*. These findings strongly suggest that a broad variety of terpene synthases are produced by diverse *Streptomyces* species. In this study, we turned our attention to a *Streptomyces* genome sequence that has not yet been released. We found two SC homologue genes in the draft genome sequence of *Streptomyces citricolor* NBRC 13005 and demonstrated that they are sesquiterpene cyclases responsible for the synthesis of (−)-germacradien-4-ol and (−)-*epi*-α-bisabolol.

S. citricolor NBRC 13005 is known to produce the antibiotic aristeromycin.[17] Draft genome sequencing of *S. citricolor* revealed at least two putative single-domain SC homologues, SC1 and SC2 (these sequences have been deposited with the DNA Data Bank of Japan with accession numbers AB621338 and AB621339, respectively). SC1 (316 amino acids) and SC2 (313 amino acids) show 32 and 29% amino acid sequence identity, respectively, with the pentalenene synthase from *Streptomyces* sp. UC5319. Both the aspartate-rich (DDXXD/E) and NSE/DTE (N/DDXXS/TXX(K/R)E) motifs, which are responsible for the formation of the Mg^{2+} diphosphate–enzyme complex as well as the ionization of the substrate,[2] are conserved in SC1 (**D**^{80}**D**QF**D** and **N**^{218}DVR**S**FAQ**E**) and SC2 (**D**^{84}**D**RF**D** and **N**^{225}DLC**S**LQR**E**).

To examine in vivo terpenoid production by SC1 and SC2, their genes were heterologously expressed in a conventional *S. lividans* host. The recombinant *S. lividans* strains harboring the SC1 and SC2 genes were grown at 30 °C for 72 h. The low-molecular-weight molecules were extracted with ethyl acetate from the mycelia, and the extracts were analyzed by GC-MS for the production of sesquiterpenes. A single major product, **1**, was detected in extracts of the recombinant *S. lividans* strain harboring the SC1 gene (Figure 1 A). The mass spectrum of product **1** had peaks at *m/z* 222 and 207; corresponding to the molecular ion of sesquiterpene alcohol ($[M(C_{15}H_{26}O)]^+$) and its fragment ion ($[M-CH_3]^+$), respectively (Figure 1 B). On the other hand, a single major product **2** was detected from extracts of the recombinant *S. lividans* strain harboring the SC2 gene (Figure 1 A). The mass spectrum of product **2** had peaks at *m/z* 204 and 207; corresponding to the fragment ions of sesquiterpene alcohol, $[M-H_2O]^+$ (dehydrated form) and $[M-CH_3]^+$ (demethylated form), respectively. (Figure 1 B). For structural determination, products **1** and **2** were purified by SiO_2 column chromatography to yield 9.8 mg of **1** and 14.0 mg of **2** (from 4 L culture each). NMR and specific-rotation analyses of **1** and **2** revealed their structures; **1** was the sesquiterpene alcohol (−)-germacradien-4-ol (Figure S1 in the Supporting Information, see Scheme 1 A for structure)[18,19] and **2** was the sesquiterpene alcohol (−)-*epi*-α-bisabolol (Figure S2, see Scheme 1 B for structure).[20] The ^{1}H NMR spectra and specific

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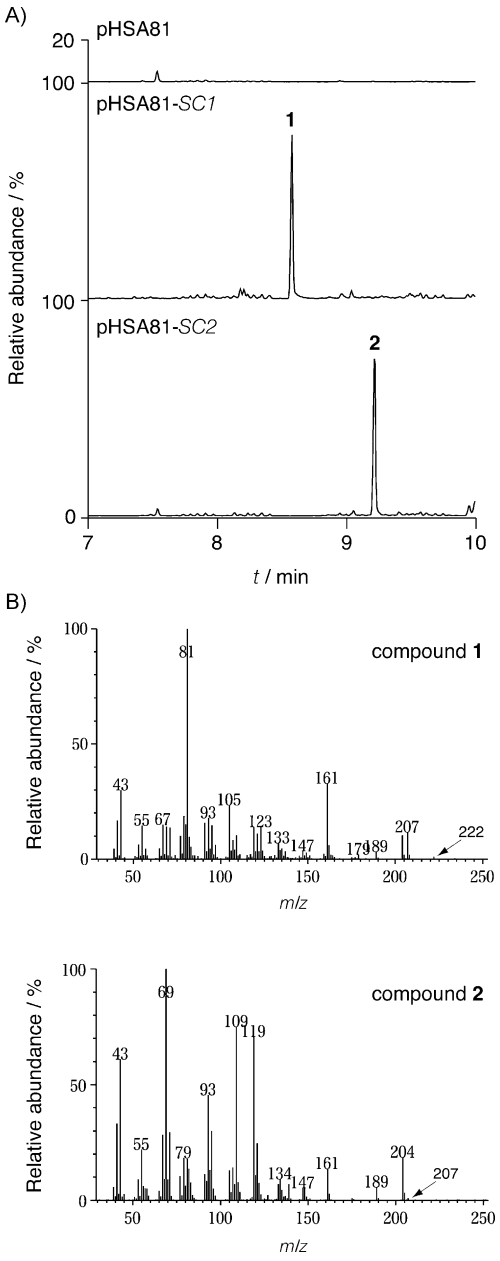
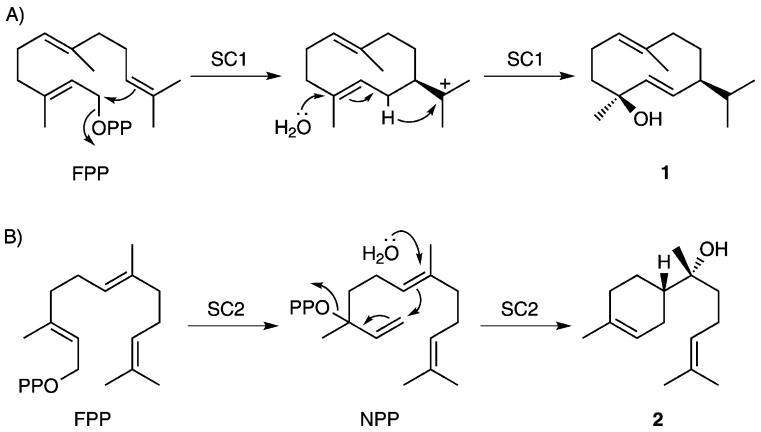


Figure 1. A) GC-MS chromatograms of extracts from a crude cell lysate of *S. lividans* strains harboring empty vector pHSA81, pHSA81-*SC1*, and pHSA81-*SC2*. B) Mass spectra of compounds **1** and **2**.

rotation values of **1** and **2** corresponded well with the reported data. (−)-Germacradien-4-ol (**1**) and (−)-*epi*-α-bisabolol (**2**) have been isolated previously from plants,[18–21] but not from bacteria.

To allow in vitro analysis, SC1 and SC2 were produced as N- and C-terminally His-tagged proteins, respectively, in *Escherichia coli* and purified by nickel–nitrilotriacetic acid (Ni-NTA) affinity chromatography (Figure S3). In vitro incubation of the recombinant SC1 and SC2 proteins with FPP produced **1** and **2**, respectively, as the main products (Figure 2). The yields of **1** and **2** were estimated to be >85 and >90%, respectively, of the total sesquiterpene products produced, as calculated from their peak area in GC-MS chromatograms. Plant SCs involved in the biosynthesis of germacradien-4-ol and α-bisabolol are known,[22–24] although the relative and absolute configurations of the products have not been determined. However, these plant germacradien-4-ol and α-bisabolol synthases produce several by-product sesquiterpenes, at least in vitro. *Santalum album* L and *Pinus sylvestris* germacradien-4-ol synthases produced 38.9 and 41.5% (of total sesquiterpenes), respectively, germacradien-4-ol.[22,23] The production of α-bisabolol as a percentage of the total sesquiterpenes produced by *Sspi*BS from *Santalum spicatum* is 53.6 and 57.1% in the presence of Mg^{2+} and Mn^{2+}, respectively.[24] Thus, the product specificities of the bacterial germacradien-4-ol and α-bisabolol synthases are significantly higher than those of corresponding plant enzymes. It should be noted that SC1 and SC2 show no significant amino acid sequence identity with plant germacradien-4-ol and α-bisabolol synthases, respectively. It is usual that bacterial SCs show no significant sequence identity with corresponding eukaryotic SCs, although two characteristic metal ion binding motifs (aspartate-rich and NSE/DTE motifs) are universally conserved among SCs. We postulate that SC1 catalyzes the formation of **1** via the (*E*,*E*)-germacradienyl cation, while SC2 catalyzes the conversion of FPP to nerolidyl diphosphate (NPP) followed by an ionization-initiated cyclization (Scheme 1).

SC1 was optimally active at a temperature of 55 °C and had relatively high activity over a broad pH range (7.0–10.0; Figure S4B and A). The reaction catalyzed by SC1 did not proceed in the absence of $MgCl_2$, and a concentration of at least 2.5 mM $MgCl_2$ was required for maximum activity (data not shown). SC1 activity was examined in the presence of other divalent metal ions (2.5 mM). No activity was observed for Fe^{2+}, Zn^{2+}, or Cu^{2+}, but activities of 12, 6, and 6% (relative to the activity observed in the presence of Mg^{2+}) were achieved in the presence of Co^{2+}, Mn^{2+}, and Ni^{2+}, respectively. SC2 showed a temperature optimum at 35 °C and a pH optimum at 8.0 to 8.6 (Figure S4D and C). The reaction catalyzed by SC2 also did not proceed in the absence of $MgCl_2$, and a concentration of at least 15 mM $MgCl_2$ was required for maximum activi-



Scheme 1. Proposed reactions catalyzed by SC1 and SC2. A) SC1 converts FPP to (−)-germacradien-4-ol (**1**) via the (*E*,*E*)-germacradienyl cation. B) SC2 converts FPP to (−)-*epi*-α-bisabolol (**2**) via NPP.

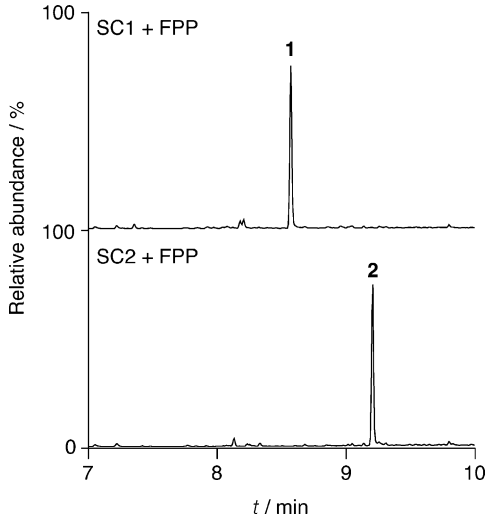


Figure 2. In vitro reactions of recombinant SC1 and SC2 proteins. GC-MS profiles of products generated by SC1 and SC2 from FPP.

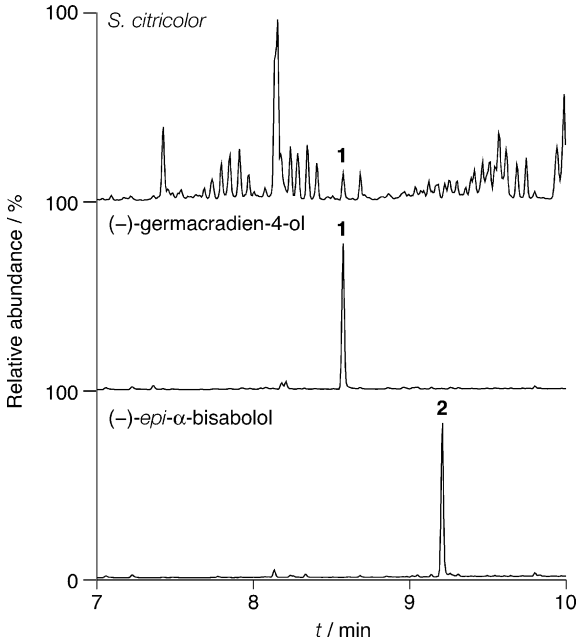


Figure 3. GC-MS chromatograms of the extracts of *S. citricolor*, and (−)-germacradien-4-ol (**1**) and (−)-*epi*-α-bisabolol (**2**).

ty (data not shown). SC2 activity was examined in the presence of other divalent metal ions (15 mM). No activity was observed for Fe^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+}, or Cu^{2+}, but 16% activity (relative to the activity observed in the presence of Mg^{2+}) was achieved in the presence of Mn^{2+}. The K_m and k_{cat} values of SC1 and SC2 were measured at 35 °C and pH 8.0 (50 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES)) in the presence of 2.5 and 15 mM, respectively, of $MgCl_2$. The K_m and k_{cat} values of SC1 were found to be 115 ± 3.5 nM and 0.198 ± 0.004 s^{-1}, respectively, and those of SC2 were 334 ± 19.9 nM and 0.123 ± 0.002 s^{-1}. The k_{cat}/K_m values (1.72 $s^{-1}\,\mu M^{-1}$ for SC1 and 0.37 $s^{-1}\,\mu M^{-1}$ for SC2) were broadly comparable to those reported previously for known bacterial SCs.[8,10,12,13]

Finally, we analyzed an extract of wild-type *S. citricolor* mycelia by GC-MS for the production of **1** and **2**. The production of **1** by *S. citricolor* was confirmed; however, **2** was not observed (Figure 3). (−)-*epi*-α-bisabolol (**2**) may be converted into another compound in *S. citricolor* by a putative P450 mono-oxygenase whose gene is apparently co-transcribed and co-translated with the SC2 gene. It is also possible that the SC2 gene is not expressed in *S. citricolor* under the standard laboratory culture conditions employed.

In conclusion, two single-domain SC homologues from *Streptomyces citricolor* have been characterized. In vivo and in vitro analyses showed that SC1 and SC2 are (−)-germacradien-4-ol and (−)-*epi*-α-bisabolol synthases, respectively. These SCs are the first enzymes of their kind to be identified in bacteria. Significantly, both SC1 and SC2 have high product specificity compared with the specificity observed in the corresponding plant enzymes. Crystal-structural analysis of several terpenoid cyclases suggests that active site pockets with product-like contours are responsible for the high product specificity.[25] It therefore seems likely that the active-site pockets of SC1 and SC2 have contours that are more product-like than those of plant germacradien-4-ol and α-bisabolol synthases. Crystal structures of SC1 and SC2 would be invaluable in elucidating the catalytic residues involved in the enzymes' respective reactions. Our study strongly supports the idea that genome mining is a useful approach in revealing the terpenoid diversity in bacteria. Analysis of the bacterial SC homologues found by future genome sequencing efforts will undoubtedly lead to the discovery of novel bacterial SCs.

Experimental Section

General analytical methods: NMR spectra were recorded in $CDCl_3$ on a JNM-A500 spectrometer (JEOL, Tokyo, Japan), the chemical shifts being given relative to the solvent peaks $\delta_H = 7.26$ and $\delta_C = 77.0$ ppm as internal references for ^1H and ^{13}C NMR spectra, respectively. GC-MS analysis was performed on a JEOL JMS-Q1000 GC K9 instrument equipped with an Intercap 5MS/Sil capillary column (15 m × 0.25 mm i.d., 0.25 μm film thickness, GL science, Tokyo, Japan) by using the electron-impact mode operated at 70 eV. A temperature gradient of 60 (3 min hold) to 260 °C (20 °C min^{-1}) was used. The specific rotation value was measured with a JASCO DIP-1000 digital polarimeter at room temperature.

Bacterial strains, plasmids, media, and chemicals: *E. coli* strains JM109 and BL21(DE3), plasmid pUC19 and restriction enzymes, and other DNA-modifying enzymes used for DNA manipulations were purchased from Takara Bio (Otsu, Japan). Plasmids pET16b and pET26b were purchased from Novagen (Darmstadt, Germany). *S. citricolor* NBRC 13005 was obtained from the NITE Biological Resource Center (Kisarazu, Japan). *S. lividans* TK21 was obtained from David A. Hopwood (John Innes Centre, Norwich, UK). Plasmid pHSA81 was obtained from Michihiko Kobayashi (University of Tsukuba, Ibaraki, Japan). *S. lividans* was cultured in YEME medium (pH 7.0), which consisted of glucose (1%), sucrose (34%), yeast extract (0.3%), malt extract (0.3%), Bacto-peptone (0.5%), and $MgCl_2 \cdot 6H_2O$ (0.1%). *S. citricolor* was cultured in medium A (pH 7.0), which consisted of glucose (2%), soluble starch (3%), soy bean flour (1%), corn steep liquor (1%), peptone (0.5%), and $CaCO_3$ (0.5%). FPP was purchased from Sigma.

Construction of plasmids: The *S. citricolor* genomic DNA was used as a template for PCR. The absence of undesired alterations during PCR was confirmed by nucleotide sequencing. A 1.0 kb DNA fragment containing the SC1 coding sequence was amplified by PCR with primer I, 5′-ACC GAATTC *CAT**ATG*** TCCGAC GACACC TCACTT GAGCTT C-3′ (EcoRI site underlined, NdeI site italicized, and the start codon of *SC1* shown in bold) and primer II, 5′-CGG *GGATCC* TCAGCC GTGGTG GTAGCG GCGGGT GTGGGA CTCCCA GTG-3′ (BamHI site italicized). The amplified DNA fragment was cloned between the EcoRI and BamHI sites of pUC19 to give pUC19-*SC1*. The NdeI–BamHI fragment excised from pUC19-*SC1* was cloned between the NdeI and BamHI sites of pHSA81 and pET16b to give pHSA81-*SC1* and pET16b-*SC1*, respectively.

A 1.0 kb DNA fragment containing the SC2 coding sequence was amplified by PCR with primer III, 5′-GGA GAATTC *CAT**ATG*** AACTCC CTCCTC CACGCC GCAGAG TTAG-3′ (EcoRI site underlined, NdeI site italicized, and the start codon of *SC2* shown in bold) and primer IV, 5′-TCG *AAGCTT* AAGGCC CGTGCG TGTCAT GGGTGG TAG-3′ (HindIII site italicized). The amplified DNA fragment was cloned between the EcoRI and HindIII sites of pUC19 to give pUC19-*SC2*. The NdeI–HindIII fragment excised from pUC19-*SC2* was cloned between the NdeI and HindIII sites of pHSA81 to give pHSA81-*SC2*.

A 1.0 kb DNA fragment containing the SC2 coding sequence was amplified by PCR with primers III and V, 5′-GCG *AAGCTT* TGGGTG GTAGCG GCGCGA CGTG-3′ (HindIII site italicized). The amplified DNA fragment was cloned between the EcoRI and HindIII sites of pUC19 to give pUC19-*SC2*-2. The NdeI–HindIII fragment excised from pUC19-*SC2*-2 was cloned between the NdeI and HindIII sites of pET26b to give pET26b-*SC2*.

Analysis of terpenoids produced by recombinant *S. lividans* strains: *S. lividans* strains harboring pHSA81, pHSA81-*SC1*, and pHSA81-*SC2* were inoculated into YEME medium (100 mL) containing thiostrepton (5 μg mL^{-1}) and grown at 30 °C for 72 h. Cells were harvested by centrifugation and resuspended in Tris·HCl (50 mM, 20 mL, pH 8.0), then disrupted by sonication. The low-molecular-weight molecules were then extracted with ethyl acetate (10 mL × 2) 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 sesquiterpenes: *S. lividans* strains harboring pHSA81-*SC1* and pHSA81-*SC2* were inoculated into YEME medium (total 4 L for each strain) containing thiostrepton (5 μg mL^{-1}) and grown at 30 °C for 96 h. Cells were harvested by centrifugation and resuspended in Tris·HCl (50 mM, pH 8.0, 500 mL), then disrupted by sonication. Low-molecular-weight molecules were then extracted with ethyl acetate (2× 100 mL) 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 hexane. Each sesquiterpene was purified by SiO_2 column chromatography with hexane/ethyl acetate (100:0 to 100:5) to give **1** (9.8 mg) and **2** (14.0 mg). The structure of each sesquiterpene was determined from NMR spectra by using ^{1}H,^{13}C, DEPT, ^{1}H,^{1}H COSY, NOESY, HMQC, and HMBC experiments, and specific rotation analysis.

Production and purification of the recombinant SC1 and SC2 proteins: For the production of N-terminally His_{10}-tagged SC1, *E. coli* BL21(DE3) harboring pET16b-*SC1* was inoculated into LB medium (200 mL) containing ampicillin (50 μg mL^{-1}) and grown at 26.5 °C for 24 h. For the production of C-terminally His_6-tagged SC2, *E. coli* BL21(DE3) harboring pET26b-*SC2* was inoculated into LB medium (400 mL) containing kanamycin (25 μg mL^{-1}) and IPTG (10 μM), and grown at 26.5 °C for 24 h. Cells were harvested by centrifugation and resuspended in buffer A (50 mM Tris·HCl, 150 mM NaCl, 5 mM imidazole and 10% glycerol, 40 mL, pH 8.0), then disrupted by sonication. The crude cell-free extract was prepared by removing cell debris by centrifugation at 10000 *g* for 20 min. The recombinant protein with a His tag was purified by using a Ni-NTA superflow (Qiagen) according to the manufacturer's instructions, with the exception that glycerol (10%) was added to all buffers. The purified SC1 and SC2 proteins were dialyzed against buffer B (10 mM Tris·HCl, 10% glycerol, pH 8.0) and buffer B containing NaCl (150 mM), respectively. The purity of the recombinant proteins was checked by SDS-PAGE (Figure S3). The protein concentration was measured with a NanoDrop 2000 spectrophotometer (Thermo Scientific).

In vitro enzyme assay: The standard reaction mixture consisted of Tris·HCl (50 mM, pH 8.0), $MgCl_2$ (5 mM), mercaptoethanol (5 mM), FPP (4.6 μM), and enzyme (0.5 μM) in a total volume of 2.5 mL. The reaction mixture, overlaid with hexane (1 mL), was incubated at 30 °C for 4 h. The reaction was terminated by the addition of EDTA (0.5 M, 200 μL, pH 8.0) followed by immediate vortexing for 30 s. The products were extracted with hexane (1 mL) and subjected to GC-MS analysis. To determine the optimum conditions for the SC1 and SC2 reactions, an in vitro assay was performed as follows. The standard SC1 reaction mixture consisted of HEPES (50 mM, pH 8.0), $MgCl_2$ (2.5 mM), mercaptoethanol (5 mM), FPP (2.3 μM), and SC1 (2.7 nM) in a total volume of 2.5 mL. The standard SC2 reaction mixture consisted of HEPES (50 mM, pH 8.0), $MgCl_2$ (15 mM), mercaptoethanol (5 mM), NaCl (150 mM), FPP (2.3 μM), and SC2 (8.5 nM) in a total volume of 2.5 mL. The reaction mixture, overlaid with hexane (1 mL), was incubated at 35 °C for 10 min. The data were obtained from three independent experiments.

Kinetic analysis: The reaction mixture for SC1 consisted of HEPES (50 mM, pH 8.0), $MgCl_2$ (2.5 mM), mercaptoethanol (5 mM), FPP (0.05 to 4.6 μM), and SC1 (2.7 nM). The reaction mixture for SC2 consisted of HEPES (50 mM, pH 8.0), $MgCl_2$ (15 mM), mercaptoethanol (5 mM), NaCl (150 mM), FPP (0.18 to 4.6 μM), and SC2 (8.5 nM). The total volume of each reaction mixture was 2.5 mL. Each reaction mixture was incubated at 35 °C for 3 min. Each reaction was terminated by the addition of EDTA (0.5 M, 200 μL, pH 8.0) followed by immediate vortexing for 30 s. The product was extracted with hexane (2 mL) 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 $v = V_{max}[S]/(K_m + [S])$. The data were obtained from three independent experiments.

Examination of terpenoid production in *S. citricolor*: The wild-type strain *S. citricolor* was grown in medium A (100 mL) at 30 °C for 96 h. Cells were harvested by centrifugation and resuspended in Tris·HCl (50 mM, 20 mL, pH 8.0), then disrupted by sonication. Low-molecular-weight molecules were extracted with ethyl acetate (5 mL) from the crude cell lysate. The organic layer was dried with Na_2SO_4 and then analyzed by GC-MS.

Abbreviations: DEPT: distortionless enhancement by polarization transfer, FPP: farnesyl diphosphate, HEPES: 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, IPTG: isopropyl-β-D-thiogalactopyranoside, Ni-NTA: nickel-nitrilotriacetic acid, NPP: nerolidyl diphosphate, SC: sesquiterpene cyclase, YEME: yeast extract–malt extract.

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Keywords: cyclization · genome mining · sesquiterpene cyclases · *Streptomyces citricolor* · terpenoids

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