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# Cloning and Functional Characterization of the Germacradienol Synthase (*spterp13*) from *Streptomyces peucetius* ATCC 27952

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**Sequence analysis of the metabolically rich genome of *Streptomyces peucetius* ATCC 27952 revealed a 2,199 bp sesquiterpene alcohol (germacradienol) synthase-encoding gene from the germacradienol synthase/terpene cyclase gene cluster. The gene was named *spterp13*, and its putative function is as a germacradienol synthase/terpene cyclase. The amino acid sequence of Spterp13 shows 66% identity with SAV2163 (GeoA) from *S. avermitilis* MA-4680 and 65% identity with SCO6073 from *S. coelicolor* A3(2), which produces germacradienol/geosmin. The full-length recombinant protein was heterologously expressed as a his-tagged fusion protein in *Escherichia coli*, purified, and shown to catalyze the $Mg^{2+}$-dependent conversion of farnesyl diphosphate to the germacradienol, which was verified by gas chromatography/mass spectrometry.**

**Keywords:** Geosmin, germacradienol, heterologous expression, sesquiterpene cyclase, *Streptomyces peucetius*.

Terpenoid or isoprenoid metabolites represent one of the largest and most diverse groups of natural products that have physiological and commercial importance, which include antibiotics, hormones, antitumor agents, flavor and odor constituents, and pigments [12, 24, 25]. Over 20,000 terpenoids have been reported, and most of them have been isolated from plants or fungi, and very few of them have been isolated from prokaryotes. Terpene synthases (also known as terpene cyclases) are responsible for the formation of over 20,000 different products that stem from linear isoprenoid precursors [14]. Although *Streptomyces* are a rich source of secondary metabolites of all structural classes, no more than a dozen terpenoid metabolites have been obtained from them [2, 3]. Only a few of the thousands of useful natural products produced by *Streptomyces* are cyclic terpenoids [19]. Many streptomycetes produce earthy or musty odors [23].

The characteristic odor of moist soil is due to the presence of two terpene derivatives, 2-methylisoborneol and geosmin [5, 7]. Geosmin is a well-known odiferous metabolite that is produced by a wide variety of microorganisms, including the genera actinomycetes, myxobacteria, cyanobacteria, fungi, and by higher plants such as liverworts and red beets [20]. Geosmin has an exceptionally low detection threshold (on the order of 10 to 100 parts per trillion), and is associated with the characteristic odor of freshly turned earth and with the unpleasant off-flavors in water, wine, fish, and other foodstuffs [6, 15]. Farnesyl diphosphate is the immediate precursor of cyclic sesquiterpenes [8]. There are several biosynthetic pathways of isoprenoid synthesis in microorganisms, one or more of which may lead to the production of germacradienol and geosmin [4, 11] (Fig. 1).

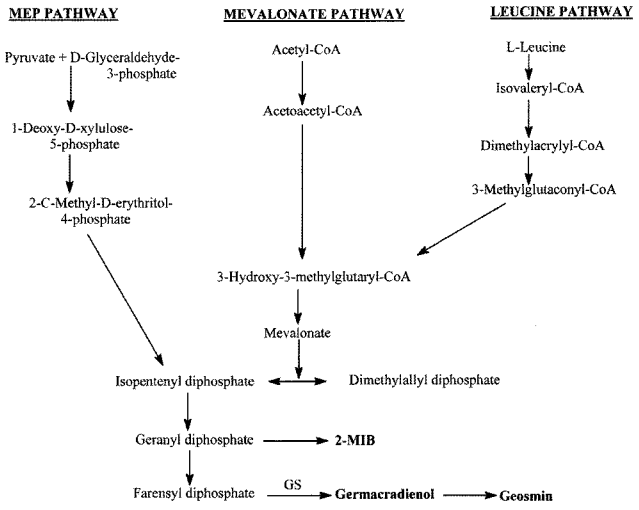


**Fig. 1.** Simplified biosynthetic scheme for the formation of 2-methylisoborneol (MIB) and geosmin in streptomycetes and myxobacteria.

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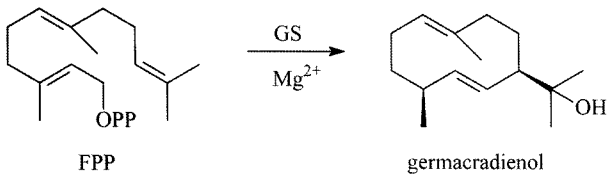


**Fig. 2.** Cyclization of farnesyl diphosphate to germacradienol.

We recently determined the genome sequence of the 8.7 Mb genome of *Streptomyces peucetius* ATCC 27952, which produces clinically important anthracycline chemotherapeutic agents of the polyketide class of antibiotics, daunorubicin and doxorubicin [21]. From the genome mining of *S. peucetius*, we found one open reading frame of 2,199 bp, named *spterp13*, which encodes a putative protein of 732 aa with significant similarity to *S. avermitilis* MA-4680 (SAV2163, GeoA) and *S. coelicolor* A3(2) (SCO6073) [1]. We expect that Spterp13 acts as a germacradienol/geosmin synthase because sequence alignment of the deduced amino acids of the Spterp13 protein with the known germacradienol/geosmin synthases provides strong bootstrap support for heterologous expression. To investigate the function of *spterp13*, the *spterp13* gene was cloned and heterologously expressed in *Escherichia coli*, and an *in vitro* enzyme assay was carried out. Our results demonstrate that Spterp13 is a key enzyme for the cyclization of farnesyl diphosphate (FPP) to germacradienol (Fig. 2).

## MATERIALS AND METHODS

### Bacterial Strains, Media, and Culture Conditions

*S. peucetius* ATCC 27952 was grown in 50 ml of R2YE medium at 28°C in a shaking incubator for 5 days. *E. coli* XL1-Blue MRF' and *E. coli* BL21 (DE3) (Stratagene, La Jolla, CA, U.S.A.) were used as hosts for plasmid preparation and expression, respectively [18, 22]. pET32a(+) (Novagen, U.S.A.) was used as an expression vector [17]. *E. coli* strains were grown in Luria Bertani (LB) medium, both in liquid culture and agar plates, with appropriate antibiotics for selection. Synthetic oligonucleotides were synthesized by GenoTech (Daejeon, Korea). A polymerase chain reaction (PCR) kit and enzymes were purchased from Takara (Japan). All chemicals were purchased from Sigma unless otherwise stated. Database searches were performed by using the BLAST, FASTA, and CLUSTALW programs.

### DNA Manipulation

Genomic DNA was isolated from *S. peucetius* using the Kirby mix procedure [13]. The *spterp13* (2.2 kb) was obtained using the primers TS-f (5'-CAT GAA TTC CGA AAG GCC CTG GCG CAC-3') and TS-r (5'-ATT AAG CTT ATC CCG GTC GGG CAT GCC-3'). PCR was carried out in a thermal cycler (Takara, Japan) under the following conditions: 94°C for 7 min, followed by 30 cycles of 94°C for 1 min, 62°C for 1 min, 72°C for 1 min, and finally 72°C for 7 min. The amplification mixture was prepared in a total volume of 20 μl containing 5 μl of PCR premix [Buffer (10 mM Tris-HCl, pH 9.0, 1.5 mM $MgCl_2$, and 40 mM KCl), 1 mM dNTP, and *Taq* DNA polymerase] with dimethyl sulfoxide (DMSO).

All of the PCR products were extracted, purified, and ligated into the pGEM-T easy vector (Promega, U.S.A.). The ligated mixture was transformed into *E. coli* XL1-Blue MRF' by heat pulse transformation. Positive clones containing the recombinant plasmid DNA were isolated and sequenced prior to cloning into expression vectors to verify that no mutation had been introduced during PCR amplification. A fragment containing EcoRI-HindIII was recovered from one such clone and then subcloned into the pET32a(+) vector (Novagen, U.S.A.) for construction of pGGGS. The purified pGGGS was then transformed into *E. coli* BL21 (DE3) for heterologous expression.

### Expression and Purification of Sptrep13

*E. coli* BL21 (DE3) harboring pGGGS was grown in 3 ml of LB culture medium. For the expression of Spterp13, *E. coli*-pGGGS was transferred from an overnight culture to 400 ml of fresh LB containing ampicillin (100 μg/ml) at 37°C and 250 rpm. At an $OD_{600}$ of 0.6, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.7 mM, and the incubation was continued at 37°C for 8 h. The induced culture was harvested, and the cell pellet was resuspended in 40 ml of lysis buffer [50 mM Tris-HCl, 0.5 mM EDTA, 10% glycerol (v/v), pH 8.2]. Lysozyme (1 mg/ml) and β-mercaptoethanol were added to the cell suspension, which was then incubated at 32°C, with shaking at 100 rpm for 15 min, followed by the addition of $MgCl_2$ (5 mM) and Triton X-100 (0.1%) after 10 min. All subsequent procedures were carried out at 4°C. The soluble fraction was collected by centrifugation at 10,000 rpm for 15 min and purified by $Co^{2+}$-affinity chromatography (TALON Purification Kit, Clontech, Takara Bio Company, U.S.A.) according to the instructions supplied by the manufacturer. The purified fractions were desalted by dialysis and concentrated by Centricon with Ultracel YM-10. The purity of the recombinant protein was evaluated by 12% SDS-PAGE, as described by Laemmli [16].

### Germacradienol Synthase Assay

The enzyme assay was performed at 30°C in 1 ml of buffer [50 mM Tris-HCl, pH 8.2, containing 10% glycerol (v/v), 10 mM $MgCl_2$, 0.2 mM β-mercaptoethanol] and 800 nM FPP with 100 ng of purified protein. The mixture was immediately overlaid with 1.5 ml of HPLC-grade pentane for 3 h. The reaction mixtures were extracted twice with 2 ml of pentane/dichloromethane (5:1), and the combined organic layers were dried over $MgSO_4$ (anhydrous), concentrated, filtered, and subjected to gas chromatography/mass spectrometry (GC/MS) analysis.

### GC/MS Analysis

A 5-μl portion of the concentrated extract was analyzed by GC/MS (Shimadzu GC-17A, 70 eV, EI, positive-ion mode; 30 m×0.25 mm bond capillary column, 5% phenylmethylsilicon), using a temperature program of 50 to 280°C, and a temperature gradient of 20°C per min). The incubation yielded two products: germacradienol (r.t. 8.314 min, $[M^+]$ $m/z$=222, base peak $m/z$=82) and a sesquiterpene hydrocarbon (r.t. 8.322 min, $[M^+]$ $m/z$=204, base peak $m/z$=107).

### Nucleotide Sequence Accession Number

The sequence of *spterp13* reported here has been deposited in the GenBank under the accession number ABY50951.

## Results and Discussion

### Homology-based Structure and Function of Spterp13

The Spterp13 encodes a protein of 732 aa with a molecular mass of 80.63 kDa. The overall G+C content of the gene is 70%, which is characteristic of the genes in *Streptomyces*. Spterp13 shows high sequence similarity with the terpene cyclase genes in the database (data not shown). For example, it has the closest homology with SAV2163 (GeoA) (GenBank Accession No. NP_823339) of *S. avermitilis* MA-4680, having a 66% identity according to BLAST. It also exhibits 65% identity with SCO6073 (GenBank Accession No. NP_630182) from *S. coelicolor* A3(2), 60% identity with terpene synthase (GenBank Accession No. YP_483306) from *Frankia* sp. CcI3, and 59% identity from *Saccharopolyspora erythraea* NNRL 2338.

Several bacterial and fungal terpene synthases have been characterized to date and share many features such as a conserved aspartate-rich **DD**HFLE motif with a high proportion of aromatic amino acids and a downstream **NSE** triad **N**DLF**S**YQR**E**, the conserved $Mg^{2+}$-binding domains of 330 to 400 aa in length, which correspond to a subunit $M_r$ of 35 to 45 kDa [10]. The 732 aa Spterp13 of *S. peucetius* is unusual in the sense that it is about twice the size of a typical terpene cyclase. Notably, both the N- and C-terminal halves of the Spterp13 show significant sequence similarity to the germacradienol/geosmin cyclases, SAV2163 (725 aa) of *S. avermitilis* MA-4680 and SCO6073 (726 aa) of *S. coelicolor* A3(2). Like SAV2163 and SCO6073, Spterp13 shows variation of an aspartate-rich domain, with a **DD**HFLE motif in the N-terminal half and an unusual **DD**YYP motif in the C-terminal half. Both halves also have **NSE** motifs: **N**DLF**S**YQR**E** and **N**DLF**S**YQK**E** with the repetition of an **NSE** motif, **N**DLLT**S**RVQQF**E**, in the N-terminal half. The organization of protein domains and conserved $Mg^{2+}$-binding motifs in Spterp13, SAV2163, and SCO6073 are also similar (Fig. 3). The active-site residues

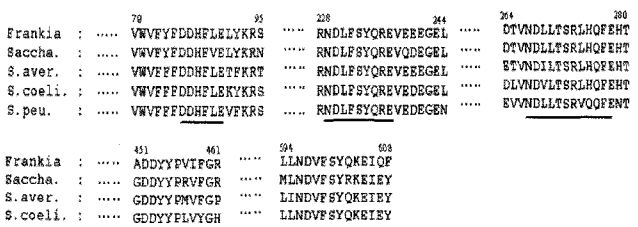


**Fig. 3.** Sequence alignment of Spterp13 with the other known proteins.

Frankia (GenBank Accession No. YP_483306) from *Frankia* sp. CcI3; Saccha. (GenBank Accession No. YP_001105388) from *Saccharopolyspora erythraea* NNRL 2338; S.aver. (GenBank Accession No. NP_823339) from *S. avermitilis* MA-4680; S.coeli. (GenBank Accession No. NP_630182) from *S. coelicolor* A3(2); and S.peu. (GenBank Accession No. ABY50951) from *S. peucetius* ATCC 27952. Only conserved regions are shown. Catalytic motifs and $Mg^{2+}$-binding domains are underlined.

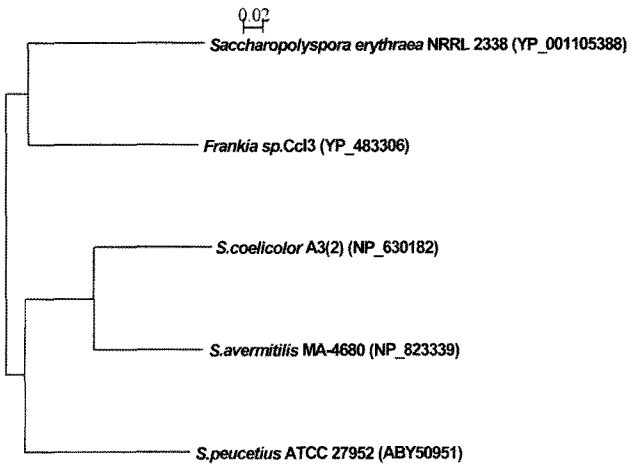


**Fig. 4.** Phylogenetic tree of Spterp13 with other known proteins.

YP_001105388, *Saccharopolyspora erythraea* NNRL 2338; YP_483306, *Frankia* sp. CcI3; NP_630182, *S. coelicolor* A3(2); NP_823339, *S. avermitilis* MA-4680; and ABY50951, *S. peucetius* ATCC 27952. The phylogenetic tree was drawn using the CLUSTALW and TREEVIEW programs.

are marked by germacradienol synthase taking the references of SAV2163 (GeoA) from *S. avermitilis* MA-4680 and SCO6073 from *S. coelicolor* A3(2) [1, 10]. Therefore, based on aa alignment and conserved domains, we suggest that Spterp13 can serve as a germacradienol synthase, similar to SCO6073 and SAV2163.

Comparison of the phylogenetic tree for Spterp13 and various terpene cyclases showed a distinct relationship between Spterp13 and other germacradienol synthase/geosmin synthase/terpene cyclase (Fig. 4). The SAV2163 and SCO6073 proteins, which were already characterized as germacradienol/geosmin synthases, occupied one group, whereas the other terpene cyclases were separated into another group.

### PCR Amplification, Overexpression, and Purification of Recombinant Spterp13

The 2,199 bp DNA fragment was amplified from the total DNA of *S. peucetius* by PCR. The amplified DNA was initially cloned into the pGEM-T easy vector. The fragment digested with EcoRI and HindIII was ligated with the pET32a(+) expression vector to prepare pGGGS. *E. coli* was transformed with pGGGS in order to express *spterp13* as the 6×his-tagged fusion protein. The expression of *E. coli* BL21 (DE3)-pGGGS was optimized in order to obtain a soluble protein. The induction with IPTG (0.7 mM) at 37°C for 8 h led to the excessive formation of soluble proteins. The soluble fraction was purified, desalted, and concentrated as described in Materials and Methods. The molecular mass of the recombinant Spterp13 was approximately 98 kDa, which is consistent with the predicted molecular mass of Spterp13 plus his-tag determined by SDS-PAGE analysis (Fig. 5).

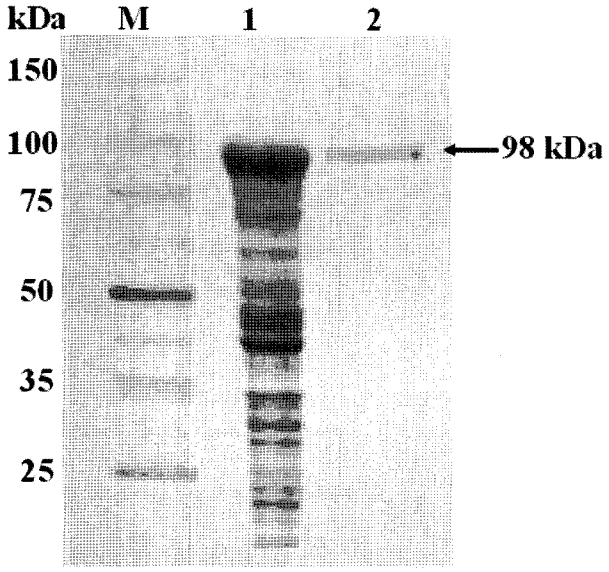


**Fig. 5.** SDS-PAGE analysis of recombinant overexpressed Spterp13 visualized by Coomassie staining.
Lane M, molecular mass markers; lane 1, crude extract of *E. coli* BL21 (DE3) carrying pGGGS; and lane 2, concentrated elution fractions containing purified Spterp13.

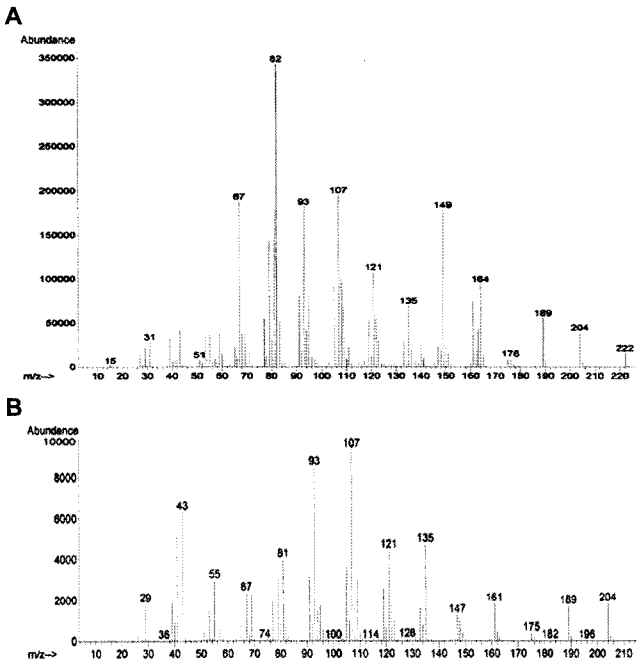


**Fig. 6.** GC/MS spectra of the generated products, germacradienol (**A**) and sesquiterpene hydrocarbon (**B**), by germacradienol synthase.

### Characterization of Recombinant Spterp13

The protein encoded by Spterp13 was overexpressed in *E. coli*. To confirm that the purified synthase was indeed generating germacradienol, the recombinant protein was incubated with FPP in the presence of $Mg^{2+}$ as described in the Materials and Methods section, and the resulting products were analyzed by GC/MS. The GC/MS gave two products: germacradienol (r.t. 8.314 min, $[M^+]$ *m/z*=222, base peak *m/z*=82) as a major product, and a detectable amount of sesquiterpene hydrocarbon of unassigned structure (r.t. 8.322 min, $[M^+]$ *m/z*=204, base peak *m/z*=107) (Figs. 6A and 6B). The structure of germacradienol was verified by comparison with the spectra of the corresponding reference compounds in the Automatic Mass Spectral Deconvolution and Identification System (AMDIS) program (Version 2.65) and also by comparing the GC/MS spectrum of germacradienol produced by *S. coelicolor* SCO6073 [9]. Since the same GC/MS was detected in the enzymatic conversion of FPP by SAV2163 from *S. avermitilis* MA-4680, we called it a sesquiterpene hydrocarbon by taking the reference of the Cane group [1].

We also tried to isolate compounds from the wild strain but no products that are biosynthetically related to germacradienol synthase were detected even though they were screened for secondary metabolite production. We assumed *spterp13* to be a "silent" gene under the laboratory condition administrated. The combined evidence from sequence analysis and enzymatic conversion conclusively established that the *spterp13* gene encodes a germacradienol synthase and that this enzyme catalyzes an essential step in the biosynthesis of geosmin. The N- and C-terminal domains of Spterp13 are similar to those of the SCO6073 and SAV2163 proteins. It has been reported that the *S. coelicolor* SCO6073 N-terminal domain catalyzes the $Mg^{2+}$-dependent cyclization of FPP to germacradienol/germacrene D, whereas the highly homologous C-terminal domain, previously thought to be catalytically silent, catalyzes the $Mg^{2+}$-dependent germacradienol/geosmin. Presently, the study in SCO6073 proved that the N- and C-terminal domains each harbor distinct and independently functioning active sites [10]. Although geosmin was not detected in the *in vitro* assay of the full-length recombinant Spterp13 protein, the formation of germacradienol seems to be a committed step in the formation of geosmin, the characteristic odoriferous degraded sesquiterpene of several *Streptomyces* species. Owing to its unusual size and repeated conserved motifs, further experiments are needed to establish the function of the individual halves between the N- and C-terminals of Spterp13.

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