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Identification of the First Bacterial Monoterpene Cyclase, a 1,8-Cineole Synthase, that Catalyzes the Direct Conversion of Geranyl Diphosphate

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Terpenoids are the largest family of natural products, with over 24000 known examples. They have various biological functions, and act as flavorings, pigments, antibiotics, hormones and antitumor agents. Most terpenoids have been isolated from plants and fungi, and only a few have been isolated from bacterial species to date. 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 (GPP, 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. Therefore, terpenoid cyclases are the first key enzymes in pathways leading to the diverse range of terpenoids. A large number of terpenoid cyclases have been reported from eukaryotes, and these have been extensively studied.[1,2] In contrast, at present only a small number of reports deal with bacterial terpenoid cyclases. Most of the bacterial terpenoid cyclases reported are sesquiterpene cyclases (SCs) and only one bacterial monoterpene cyclase, 2-methylisoborneol synthase, has been reported.[3–5] 2-Methylisoborneol is a representative bacterial monoterpenoid and has an earthy odor. 2-Methylisoborneol synthase, which shows a very low amino acid sequence similarity to bacterial SCs, catalyzes the conversion of a unique substrate, 2-methyl GPP. No bacterial monoterpene cyclase that efficiently catalyzes the direct conversion of GPP to a monoterpene has been reported to date, and the 1,8-cineole synthase reported here is the first example of such a bacterial monoterpene cyclase.

SCs catalyze the cyclization of FPP (C_{15}) to generate more than 300 hydrocarbon skeletons. Bacterial SCs show no significant amino acid sequence identity with eukaryotic SCs. However, two characteristic metal ion binding motifs are universally conserved among SCs: the aspartate-rich motif (DDXXD/E) and the NSE/DTE motif (N/DDXXS/TXX(K/R)E). These motifs are responsible for Mg^{2+}–diphosphate–enzyme complex formation, as well as the ionization of the substrate.[1] In addition, crystal structures of bacterial, fungal and plant SCs have revealed a common α-helical fold similar to that first observed for an avian FPP synthase.[1] Bacterial and eukaryotic SCs, therefore, appear to have the same enzymology, despite insignificant overall sequence identity. To date, bacterial SCs have only been found in *Streptomyces* and *Nostoc*. Germacradienol/geosmin synthase from three *Streptomyces* species and *Nostoc punctiforme* PCC 73102 have been characterized.[6–9] Geosmin has an earthy odor and is particularly prevalent in actinomycetes, cyanobacteria and myxobacteria. Germacradienol/geosmin synthase is atypical in that it consists of two terpene cyclase domains. Typical single-domain SCs characterized from *Streptomyces* and *Nostoc* include those involved in the synthesis of pentalenene, *epi*-isozizaene, avermitilol, germacrene A and 8a-*epi*-α-selinene.[10–15]

Very recently, (−)-δ-cadinene and (+)-T-muurolol synthases have been identified in *Streptomyces clavuligerus* ATCC 27064.[16] We found more than 80 uncharacterized single-domain SC homologue genes in a variety of bacterial genomes using a BLAST P search with the pentalenene synthase from *Streptomyces* sp. UC5319 as a query. Many of these homologues show approximately 30% amino acid sequence identity to known SCs; this indicates that these homologues might catalyze different reactions to generate a variety of terpenoids. This result prompted us to characterize several previously undescribed bacterial SC homologues. In a series of our studies, we characterized several SC homologues from *S. clavuligerus* ATCC 27064. Characteristically, *S. clavuligerus* ATCC 27064, a producer of the β-lactamase inhibitor clavulanic acid, contains more than ten single-domain SC homologues.[17] The SSCG_00536 gene product (330 amino acids) shows 34% amino acid sequence identity with the pentalenene synthase of *Streptomyces* sp. UC5319. We named this gene *cnsA* (1,8-cineole synthase). Both the aspartate-rich motif (**DD**HF**D**, from Asp81 to Asp85) and the NSE/DTE motif (**N**DVL**S**LEK**E**, from Asn220 to Glu228) are conserved in CnsA.

To examine in vivo terpenoid production by CnsA, the *cnsA* gene was heterologously expressed in a conventional *Streptomyces* host, *Streptomyces lividans*. The recombinant *S. lividans* strain harboring *cnsA* (or the empty vector pHSA81 as a negative control) was grown at 30 °C for 72 h. Then, low molecular weight molecules were extracted with ethyl acetate from the culture broth. When the sample was analyzed by GC-MS, a single major product was detected (Figure 1 A). We had assumed that the product should be a sesquiterpene because CnsA was an SC homologue. Contrary to our expectation, however, the MS spectrum of the product had *m/z* 154, which corresponded to a monoterpene alcohol $C_{10}H_{18}O$ (Figure 1 B). Comparison of the MS spectrum of the product with a GC-MS database (National Institute of Standards and Technology Libraries, NIST08) indicated that it was the bicyclic monoterpene, 1,8-cineole. The MS spectra of representative monoterpene alcohols, including 1,8-cineole, 1,4-cineole, isoborneol, borneol, α-terpinol, and fenchol, which were derived from the database,

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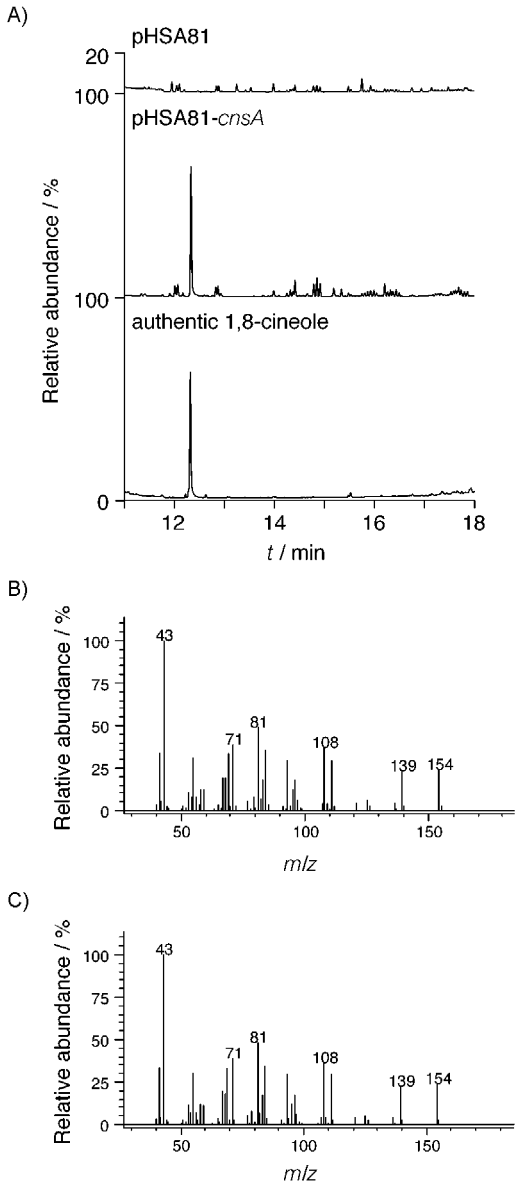
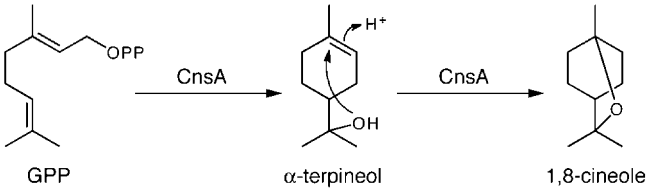


Figure 1. GC-MS analysis of extracts from the culture broth of recombinant *S. lividans* strains. A) GC-MS chromatograms of *S. lividans* strains harboring empty vector pHSA81 and pHSA81–*cnsA*, and authentic 1,8-cineole. B) MS spectrum of the product generated by *S. lividans* strain harboring pHSA81–*cnsA*. C) MS spectrum of authentic 1,8-cineole.

are shown in Figure S1 in the Supporting Information. They are easily distinguishable by fragment ion peak compositions. Finally, the structure of the product was confirmed to be 1,8-cineole by comparison with an authentic sample by using GC-MS (Figure 1, see Scheme 1 for structure). There was a complete agreement between the product and authentic 1,8-cineole not only on retention time but also on fragment ion peak composition.

To allow in vitro analysis, CnsA was produced as the N-terminally His-tagged protein in *Escherichia coli* and purified by nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography to give a single major protein band of 40 kDa on SDS-PAGE (Figure S2 in the Supporting Information). Incubation of the recombinant CnsA protein with GPP resulted in the production of 1,8-cineole (Figure 2). No product was detected when CnsA was incubated with FPP (Figure 2). From these results, we concluded that CnsA is a monoterpene cyclase capable of producing 1,8-cineole. 1,8-Cineole, which is a component of essential oils from *Eucalyptus polybractea* and many other plants, is used for pharmaceutical preparation, flavoring and cosmetics.[18] Several 1,8-cineole synthase genes have been cloned from different plants,[2] but none has been previously identified from bacteria. Moreover, 1,8-cineole has not been previously found in bacteria. We postulate that 1,8-cineole is produced via α-terpineol by CnsA (Scheme 1), as has been previously elucidated for the synthesis of 1,8-cineole in plants.[19] The K_M and k_{cat} values for GPP were calculated to be 170(±3.3) nM and 0.079(±0.002) s^{-1}, respectively. The k_{cat}/K_M value (0.46 $s^{-1}\,\mu M^{-1}$) of the bacterial 1,8-cineole synthase (CnsA) is broadly comparable with those reported for known bacterial SCs[10,12,14,15] but significantly higher than those measured (0.005, 0.003 and 0.0008 $s^{-1}\,\mu M^{-1}$) for plant 1,8-cineole synthases from *Arabidopsis thaliana*, *Nicotiana suaveolens* and *Salvia fruticosa*, respectively.[20–22]



Scheme 1. Proposed reactions catalyzed by CnsA. CnsA catalyzes the formation of 1,8-cineole by protonation of α-terpineol.

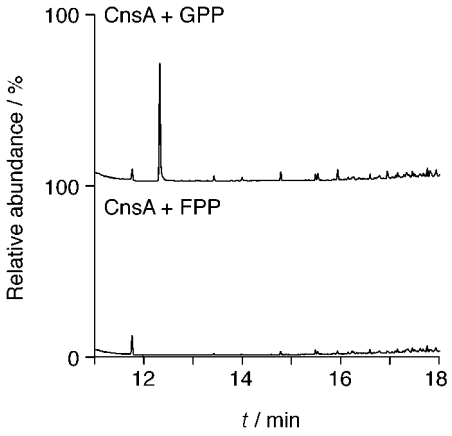


Figure 2. Functional analyses of the recombinant CnsA protein, in vitro. GC-MS profiles of products generated by CnsA with GPP or FPP.

Finally, we analyzed an extract of culture broth of the wild-type *S. clavuligerus* strain by GC-MS for the production of 1,8-cineole. However, the production of 1,8-cineole by *S. clavuligerus* was not observed. It seems likely that the *cnsA* gene was not expressed in *S. clavuligerus* under the standard laboratory culture conditions employed. It is also possible, however, that

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1,8-cineole is converted into an as yet unidentified product by *S. clavuligerus*.

In conclusion, we have identified the SSCG_00536 gene product (CnsA) as a 1,8-cineole synthase. Importantly, CnsA, which shows significant homology to bacterial SCs, is the first bacterial monoterpene cyclase to be identified that can efficiently catalyze the direct conversion of GPP. This suggests that bacterial SC homologues can include monoterpene cyclases, similar to plant SC homologues. Thus, several monoterpenes found in bacteria[23] could be synthesized by SC homologues. Recent genome sequencing projects have revealed a large number of potential bacterial terpenoid cyclase genes, most of which encode SC homologues. The identification of CnsA as a monoterpene cyclase allows us to speculate that the diversity of bacterial SC homologues means a diversity of not only sesquiterpenes but also monoterpenes in bacteria. Therefore, terpenoids are significantly more widely distributed in nature than has been previously appreciated, and are widespread among not only eukaryotes, but also bacteria.

Experimental Section

Bacterial strains, plasmids, media and chemicals: *E. coli* strains JM109 and BL21(DE3), plasmid pUC19, restriction enzymes and other DNA-modifying enzymes were purchased from Takara Biochemicals (Shiga, Japan). Plasmid pET16b was purchased from Novagen (Darmstadt, Germany). *S. clavuligerus* ATCC 27064 was obtained from the American Type Culture Collection. *S. lividans* TK21 was obtained from David A. Hopwood (John Innes Centre, Norwich, UK). Plasmid pHSA81, used for heterologous expression of *cnsA* in *S. lividans*, was obtained from Michihiko Kobayashi (University of Tsukuba, Ibaraki, Japan). *S. lividans* was cultured in yeast extract–malt extract (YEME) medium (1% glucose, 34% sucrose, 0.3% yeast extract, 0.3% malt extract, 0.5% bactopeptone, 0.1% $MgCl_2 \cdot 6H_2O$, pH 7.0). *S. clavuligerus* was cultured in soy medium (1.5% soybean flour, 0.47% soluble starch, 0.01% KH_2PO_4, 0.02% $FeSO_4 \cdot 7H_2O$, pH 6.8). GPP and FPP were purchased from Sigma. 1,8-Cineole was purchased from Wako (Osaka, Japan).

Construction of plasmids: All PCR experiments were conducted by using the *S. clavuligerus* genomic DNA as a template. The absence of undesired alterations during PCR was confirmed by nucleotide sequencing. A 1.0 kb DNA fragment containing the *cnsA*-coding sequence was amplified by PCR with primer I, 5′-GGA GAATTC *CAT**ATG*** CCCGCC GGCCAC GAAGAG TTCGAC ATAC-3′ (EcoRI site underlined, NdeI site italicized and the start codon of *cnsA* in boldface) and primer II, 5′-CGG AAGCTT *GGATCC* TGGGGG CGGAGG GACGGG CGGGTC ACCAAG GGGTGG-3′ (HindIII site underlined and BamHI site italicized) and cloned between the EcoRI and HindIII sites of pUC19, resulting in pUC19–*cnsA*. The NdeI–HindIII fragment excised from pUC19–*cnsA* was cloned between the NdeI and HindIII sites of pHSA81, resulting in pHSA81–*cnsA*. The NdeI–BamHI fragment excised from pUC19–*cnsA* was cloned between the NdeI and BamHI sites of pET-16b, resulting in pET16b–*cnsA*.

Analysis of terpenoids produced by recombinant *S. lividans* strains: *S. lividans* strain harboring pHSA81–*cnsA* was inoculated into YEME medium (100 mL) containing thiostrepton (5 $\mu g\,mL^{-1}$) and grown at 30 °C for 72 h. Low molecular weight molecules were extracted with ethyl acetate (5 mL) from a portion (40 mL) of the culture broth of *S. lividans* strain harboring pHSA81–*cnsA*. The organic layer was dried with Na_2SO_4 and then analyzed by GC-MS.

Production and purification of the recombinant CnsA: For the production of N-terminally His_{10}-tagged CnsA, *E. coli* BL21(DE3) strain harboring pET16b–*cnsA* was inoculated into LB medium (200 mL) containing ampicillin (50 $\mu g\,mL^{-1}$) 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, 10% glycerol, pH 8.0, 40 mL), then disrupted by sonication. The crude cell lysate was prepared by removal of cell debris by centrifugation at 10000 *g* for 20 min. Recombinant protein with a His-tag was purified by using Ni-NTA superflow (Qiagen) according to the manufacturer's instructions, with the exception that glycerol (10%) was added to all buffers. The purified CnsA protein was dialyzed against buffer B (10 mM Tris-HCl, 10% glycerol, pH 8.0). The purity of the recombinant proteins was checked by SDS-PAGE. The protein concentration was measured with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

In vitro enzyme assay: The standard reaction mixture consisted of Tris-HCl (50 mM, pH 8.0), $MgCl_2$ (5 mM), mercaptoethanol (5 mM), GPP (5.5 μM) or FPP (4.6 μM), and CnsA (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, pH 8.0, 200 μL) followed by immediate vortexing for 30 s. The product was extracted with hexane (1 mL) and subjected to GC-MS analysis.

Kinetic analysis: The reaction mixture for CnsA consisted of HEPES (50 mM, pH 7.5), $MgCl_2$ (10 mM), mercaptoethanol (5 mM), GPP (0.05 to 2.7 μM) and CnsA (3.4 nM). The total volume of each reaction mixture was 5 mL. Each reaction mixture, overlaid with hexane (1 mL), was incubated at 30 °C for 3 min. Each reaction was terminated by the addition of EDTA (0.5 M; pH 8.0, 400 μL) followed by immediate vortexing for 30 s. The product was extracted with hexane (1 mL) and subjected to GC-MS analysis. We confirmed that product formation was linear throughout this period. Steady-state parameters were determined by fitting the curve to $v = V_{max}[S]/(K_M + [S])$. Data were obtained from three independent experiments.

Examination of terpenoid production in *S. clavuligerus*: *S. clavuligerus* was grown in soy medium (100 mL) at 30 °C for 72 h. Low molecular weight molecules were extracted with ethyl acetate (5 mL) from a portion (50 mL) of the culture broth. The organic layer was dried with Na_2SO_4 and then analyzed by GC-MS.

General analytical methods: GC-MS analysis was performed by using a GCMS-QP2010 instrument (SHIMADZU, Kyoto, Japan) equipped with an Intercap 5MS/Sil capillary column (30 m × 0.25 mm ID, 0.25 μm film thickness, GL Science) by using the EI mode operated at 70 eV. A temperature gradient of 40 (8 min hold) to 260 °C (20 $°C\,min^{-1}$) was used.

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