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Activation of Cryptic *hop* Genes from *Streptomyces peucetius* ATCC 27952 Involved in Hopanoid Biosynthesis S

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Genes encoding enzymes with sequence similarity to hopanoids biosynthetic enzymes of other organisms were cloned from the hopanoid (*hop*) gene cluster of *Streptomyces peucetius* ATCC 27952 and transformed into *Streptomyces venezuelae* YJ028. The cloned fragments contained four genes, all transcribed in one direction. These genes encode polypeptides that resemble polyprenyl diphosphate synthase (*hopD*), squalene-phytoene synthases (*hopAB*), and squalene-hopene cyclase (*hopE*). These enzymes are sufficient for the formation of the pentacyclic triterpenoid lipid, hopene. The formation of hopene was verified by gas chromatography/mass spectrometry.

Keywords: Cryptic genes, genome, heterologous expression, hopanoid biosynthesis, *S. peucetius*

Terpenoids represent one of the major classes of natural products, with more than 40,000 different chemical structures described to date, and have been isolated from plant, animal, and microbial species [21, 24]. Hopanoids comprise a class of pentacyclic triterpenoid lipids found in a wide range of gram-positive and -negative bacteria, but not in archaea [23]. Hopene is synthesized from the universal precursor isopentenyl diphosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP), also called "isoprene units," (Fig. 1), which are formed by two different metabolic routes [9, 14, 20, 25]. Hopanoids have a structure similar to that of sterols [4]. They are cyclized from squalene [1, 7], whereas sterols are synthesized from squalene epoxide [4, 24]. Hopanoids are involved in regulating membrane fluidity and stability, comparable to sterols in the membranes of eukaryotes, but the structural variants of hopanoids indicate that they may have some other interesting functions [11, 12].

Genome sequencing projects have revealed that *Streptomyces* bacteria have the genetic potential to produce large numbers of natural products that can be observed under routine laboratory conditions. Cryptic genes for the synthesis of secondary metabolites have been studied in actinomycetes [5, 6, 10, 15, 16]. A cluster of genes for hopanoid biosynthesis has been detected and annotated in *S. peucetius*. This cluster contains a pair of putative squalene/phytoene synthases (*hopAB*), a squalene/phytoene dehydrogenase (*hopC*), a polyprenyl diphosphate synthase (*hopD*), and a squalene-hopene cyclase (*hopE*) in the same orientation (Fig. 2A). We isolated lipids from the wild-type strain of *S. peucetius* under laboratory culture conditions, but no hopanoid was detected. It suggests that hopanoid biosynthesis is tightly regulated in *S. peucetius*, allowing their presence to escape detection.

To date, squalene-hopene cyclase (SHC) is the only enzyme involved in the biosynthesis of hopanoid lipids that has been characterized at the genetic level [19]. Previously, we cloned and heterologously expressed squalene-hopene cyclase (HopE, previously named "Spterp25", GenBank Accession No. ACA52082) in *E. coli* BL21 (DE3)

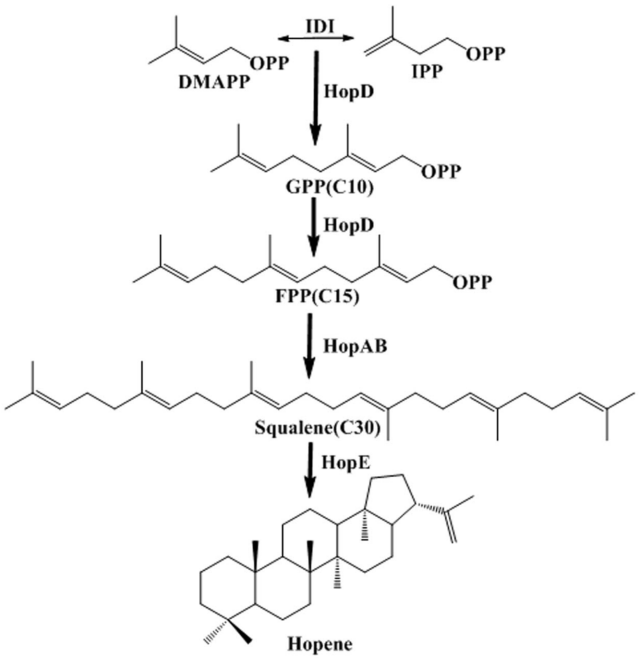

Fig. 1. Biosynthetic pathway for the biosynthesis of hopene from isopentanyl diphosphate (IPP) and its isomer, dimethylallyl diphosphate (DMAPP).

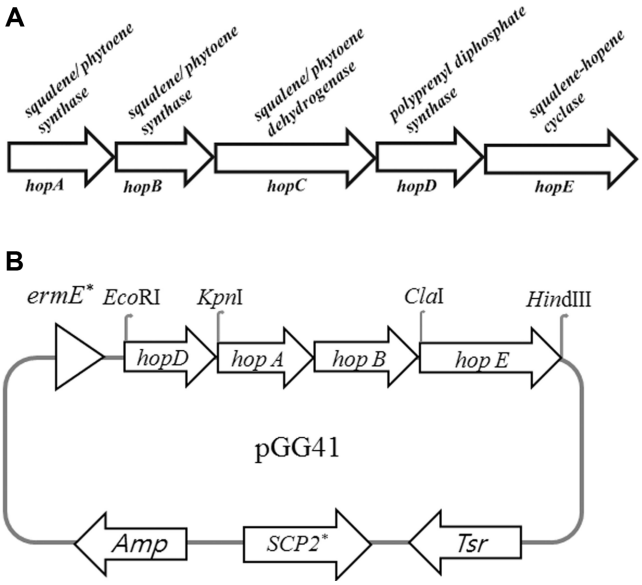

Fig. 2. (**A**) Hopanoid biosynthetic gene cluster of *S. peucetius*, and (**B**) construction of expression recombinant pGG41 from *S. peucetius* cloned into pIBR25.

pLysS and performed *in vitro* enzyme assays to characterize HopE functionally as the first step in an examination of hopanoid biosynthesis. This enzyme catalyzed the complex cyclization of squalene to the pentacarbocyclic hopene and represents the key reaction in the biosynthesis of hopanoids [7]. Furthermore, the expression of squalene/phytoene synthases (HopA, GenBank Accession No. FJ529811; or HopB, GenBank Accession No. FJ529812) independently produced minimal amounts of squalene by *E. coli*. Overexpression of polyprenyl diphosphate synthase (HopD, GenBank Accession No. FJ529814) along with HopA and HopB increased squalene levels, the precursor of hopene, by *E. coli* [8]. These results confirmed the activation of cryptic genes in *E. coli* when they are genetically engineered. Therefore, in the present study, we were interested to activate *S. peucetius*'s so-called "orphan" hopanoid biosynthetic gene cluster in *Streptomyces*.

To activate the hopanoid biosynthesis genes in the heterologous host, PCR primers (Table 1) targeting the *hop* genes were designed based on the *hop* gene cluster sequences of our previously published data [7, 8]. Three PCR fragments were first subcloned into the pGEM-T-easy vector and the inserts were verified by sequencing. They were subsequently further subcloned in pGEM7-Zf(+) vector. The *Eco*RI-*Hin*dIII fragment containing four genes obtained from the pGEM7-Zf(+) vector was cloned into *Eco*RI-*Hin*dIII-digested pIBR25 to construct pGG41 (Fig. 2B). The insertion of target genes was verified by restriction digestion. The protoplast was prepared as described in the protocol of Kieser *et al*. [13]. Transformation of pGG41 in *S.*

Table 1. List of PCR primers used in this study.

Primers	Oligonucleotide sequences (5′-3′)	Restriction site
HopD F	ATAGAATTCAGGAGCCGGCTCGCGCCGCCC	*Eco*RI
HopD R	TGAGGTACCCAGGGGAGGTAACGATGGCGA	*Kpn*I
HopA/B F	ATAGGTACCAGGAGCTCCGAGAGTGCTTTG	*Kpn*I
HopA/B R	TAAATCGATGCGCACCCGTCCGGGGGATCA	*Cla*I
HopE F	AGCATCGATAGGAGCAACGAAGGGGAGAAC	*Cla*I
HopE R	TGGAAGCTTTCTAGACCCGGGAGTCCCTCATCA	*Hin*dIII

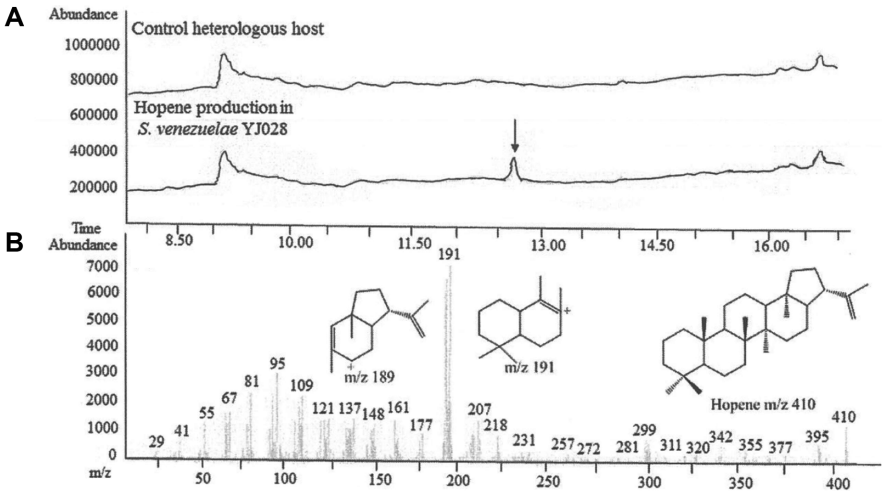

Fig. 3. GC-MS analysis of the isolated product (hopene).
(**A**) GLC traces containing hopene (shown by arrow at retention time 12.7 min) along with the control heterologous host strain that does not produce hopene. (**B**) GLC-MS fractionation analysis of hopene. The mass peak at *m/z* 410 and base peak at *m/z* 191 are indicative of hopene.

venezuelae YJ028 was confirmed by plasmid isolation, followed by PCR and restriction digestion.

Finally, to produce and detect the hopanoid, *S. venezuelae* YJ028 and *S. venezuelae* YJ028/pGG41 were inoculated into R2YE as seed, supplemented with required antibiotic, and transferred into R2YE medium. After incubation, each culture broth was harvested by centrifugation (6,000 rpm, 15 min). The supernatant was discarded and the pellet was washed twice with water. The cell pellet was frozen overnight at −20°C. The lipids were extracted using the method described by Bligh and Dyer [3] with slight modification. The extracted product was dissolved in chloroform and analyzed by GC/MS. The pentacyclic product hopene (retention time: 12.7 min. Mass peak *m/z* = 410, base peak *m/z* = 191) was detected only from the transformant, indicating the functionality of the cloned genes in *Streptomyces* (Figs. 3A and 3B).

The results presented herein contribute to the understanding regarding the cryptic hopanoid biosynthesis genes involved in the synthesis of hopanoid. Successful transfer of these genes produced hopene in a heterologous host. Taken together, the understanding of the cryptic genes involved in the hopanoids biosynthetic pathway are interesting from a purely cell biological and genetic perspective, and furthermore there is an additional motivation provided by evolutionary biology. The role of the hopanoids as membrane reinforcers has been supported by experiments [2, 17, 18, 22], but numerous issues concerning the haponoids are still unresolved: structural, biosynthetic, and functional studies are currently being pursued.

Acknowledgments

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References

1. Anding C, Rohmer M, Qurisson G. 1976. Nonspecific biosynthesis of hopane triterpenes in a cell-free system from *Acetobacter rancens*. *J. Am. Chem. Soc*. **98:** 1274-1275.
2. Bisseret P, Wolff G, Albrecht AM, Tanaka T, Nakatani Y, Ourisson G. 1983. A direct study of the cohesion of lecithin bilayers: the effect of hopanoids and α,ω- dihydroxycarotenoids. *Biochem. Biophys. Res. Commun*. **110:** 320-324.
3. Bligh EG, Dyer WJ. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol*. **37:** 911-917.
4. Corey EJ, Russey WE, Ortiz de Montellano PR. 1966. 2,3-Oxidosqualene, an intermediate in the biological synthesis of sterols from squalene. *J. Am. Chem. Soc*. **88:** 4750-4751.
5. Ghimire GP, Oh TJ, Liou K, Sohng JK. 2008. Identification of a cryptic type III polyketide synthase (1,3,6,8-tetrahydroxynaphthalene synthase) from *Streptomyces peucetius* ATCC 27952. *Mol. Cells* **26:** 362-367.
6. Ghimire GP, Oh TJ, Lee HC, Kim BG, Sohng JK. 2008. Cloning and functional characterization of germacradienol synthase (*spterp13*) from *Streptomyces peucetius* ATCC 27952. *J. Microbiol. Biotechnol*. **18:** 1216-1220.
7. Ghimire GP, Oh TJ, Lee HC, Sohng JK. 2009. Squalene-hopene cyclase (Spterp25) from *Streptomyces peucetius*: sequence analysis, expression and functional characterization. *Biotechnol. Lett*. **31:** 565-569.

8. Ghimire GP, Lee HC, Sohng JK. 2009. Improved squalene production *via* modulation of the methylerythritol 4-phosphate pathway and heterologous expression of genes from *Streptomyces peucetius* ATCC 27952 in *Escherichia coli*. *Appl. Environ. Microbiol*. **75:** 7291-7293.
9. Goldstein JL, Brown MS. 1990. Regulation of the mevalonate pathway. *Nature* **343:** 425-430.
10. Jones GH, Hopwood DA. 1984. Activation of phenoxazinone synthase expression in *Streptomyces lividans* by cloned DNA sequences from *Streptomyces antibioticus*. *J. Biol. Chem*. **259:** 14158-14164.
11. Kannenberg EL, Perzl M, Muller P, Hartner T, Poralla K. 1996. Hopanoid lipids in *Bradyrhizobium* and other plant-associated bacteria and cloning of the *Bradyrhizobium japonicum* squalene-hopene cyclase. *Plant Soil* **186:** 107-112.
12. Kannenberg EL, Poralla K. 1999. Hopanoid biosynthesis and function in bacteria. *Naturwissenschaften* **86:** 168-176.
13. Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA. 2000. *Practical Streptomyces Genetics*. The John Innes Foundation, Norwich, England.
14. Meyer O, Grosdemanage-Billard C, Tritsch D, Rohmer M. 2003. Isoprenoid biosynthesis *via* the MEP pathway. Synthesis of (3R,4S)-3,4-dihydroxy-5-oxohexylphosphonic acid, an isosteric analogue of 1-deoxy-D-xylulose 5-phosphate, the substrate of the 1-deoxy-D-xylulose 5-phosphate reducto-isomerase. *Org. Biomol. Chem*. **1:** 4367-4372.
15. Palmu K, Ishida K, Mantsala P, Hertweck C, Mesta-Ketele M. 2007. Artificial reconstruction of two cryptic angucycline antibiotic biosynthetic pathways. *Chembiochem* **8:** 1577-1584.
16. Parker LL, Betts PW, Hall BG. 1988. Activation of a cryptic gene by excision of a DNA fragment. *J. Bacteriol*. **170:** 218-222.
17. Poralla K, Kannenberg E, Blume A. 1980. A glycolipid containing hopane isolated from *Bacillus acidocaldarius* has a cholesterol like function in membranes. *FEBS Lett.* **113:** 107-110.
18. Poralla K, Hartner T, Kannenberg E. 1984. Effect of temperature and pH on the hopanoid content of *Bacillus acidocaldarius*. *FEMS Microbiol. Lett*. **23:** 253-256.
19. Poralla K, Muth G, Harter T. 2000. Hopanoids are formed during transition from substrate to aerial hyphae in *Streptomyces coelicolor* A3 (2). *FEMS Microbiol. Lett*. **189:** 93-95.
20. Qurisson G, Albrecht P, Rohmer M. 1979. The hopanoids: paleochemistry and biochemistry of a group of natural products. *Pure Appl. Chem*. **51:** 709-729.
21. Roberts SC. 2007. Production and engineering of terpenoids in plant cell culture. *Nat. Chem. Biol*. **3:** 387-395.
22. Rohmer M, Bouvier-Nave P, Ourisson G. 1984. Distribution of hopaniods in prokaryotes. *J. Gen. Microbiol*. **130:** 1137-1150.
23. Sahm H, Rhomer M, Bringer-Meyer S, Sprenger GA, Welle R. 1993. Biochemistry and physiology of hopanoids in bacteria. *Adv. Microb. Physiol*. **35:** 247-273.
24. Van Tamelen EE, Willett JD, Clayton RB, Lord KE. 1966. Enzymatic conversion of squalene 2,3-oxide to lanosterol and cholesterol. *J. Am. Chem. Soc*. **88:** 4752-4754.
25. Withers ST, Keasling JD. 2007. Biosynthesis and engineering of isoprenoid small molecules. *Appl. Microbiol. Biotechnol*. **73:** 980-990.