Applied and Environmental Microbiology

Improved Squalene Production via Modulation of the Methylerythritol 4-Phosphate Pathway and Heterologous Expression of Genes from *Streptomyces peucetius* ATCC 27952 in *Escherichia coli*

Gopal Prasad Ghimire, Hei Chan Lee and Jae Kyung Sohng
***Appl. Environ. Microbiol.* 2009, 75(22):7291. DOI: 10.1128/AEM.01402-09.**
Published Ahead of Print 18 September 2009.

Applied and Environmental Microbiology, Nov. 2009, p. 7291–7293 Vol. 75, No. 22
0099-2240/09/$12.00 doi:10.1128/AEM.01402-09

Improved Squalene Production via Modulation of the Methylerythritol 4-Phosphate Pathway and Heterologous Expression of Genes from *Streptomyces peucetius* ATCC 27952 in *Escherichia coli*▽†

Gopal Prasad Ghimire, Hei Chan Lee, and Jae Kyung Sohng*

Institute of Biomolecule Reconstruction (iBR), Department of Pharmaceutical Engineering, Sun Moon University, #100 Kalsan-ri, Tangjeong-myeon, Asansi, Chungnam 336-708, South Korea

Received 15 June 2009/Accepted 10 September 2009

Putative hopanoid genes from *Streptomyces peucetius* were introduced into *Escherichia coli* to improve the production of squalene, an industrially important compound. High expression of *hopA* and *hopB* (encoding squalene/phytoene synthases) together with *hopD* (encoding farnesyl diphosphate synthase) yielded 4.1 mg/liter of squalene. This level was elevated to 11.8 mg/liter when there was also increased expression of *dxs* and *idi*, *E. coli* genes encoding 1-deoxy-D-xylulose 5-phosphate synthase and isopentenyl diphosphate isomerase.

Squalene, an industrially important compound obtained primarily from the liver oil of deep-sea sharks and whales, is an important ingredient in skin cosmetics due to its photoprotective role (2, 7). The decreased cancer risk associated with high olive oil consumption could result from high squalene content (12, 16). Squalene has a chemopreventive effect on colon cancer (14). Moreover, squalene has wide applications in fine chemicals, magnetic tape, and low-temperature lubricants and as an additive in animal feed (1).

The use of shark liver oil is limited, due to the presence of environmental pollutants, such as polychlorinated biphenyls, heavy metals, and methylmercury residues, as well as an unpleasant fishy odor and taste (17, 19). Moreover, the presence of similar compounds, such as cholesterol, in the oils from marine animal liver can make squalene purification difficult. In addition, squalene production is limited by uncertain availability because of international concern for the protection of marine animals. Squalene has also been obtained from plant sources (4, 10, 11, 18), but very few methods can produce sufficient quantities at the desired purity level for pharmaceutical and industrial applications (6). The use of engineered microbial cell factories for the biosynthesis of squalene may be a suitable alternative to address these issues.

In the genome project for *Streptomyces peucetius* ATCC 27952, a cluster of genes which comprises five open reading frames, encoding hopanoid biosynthesis, has been detected and annotated. Even though these open reading frames share sequence homology with genes involved in hopanoid biosynthesis, no plausible hopanoid products have been isolated from *S. peucetius* in all laboratory cultures. Therefore, the hopanoid biosynthetic gene cluster of *S. peucetius* was considered "cryptic" in the present study. We were interested in activating the so-called "cryptic" hopanoid biosynthetic gene cluster of *S. peucetius* to produce pharmaceutically important compounds by using genetic engineering tools. Isoprenoid production in *Escherichia coli* has been extensively studied and reviewed (5, 8, 9, 15, 20, 21), but very few reports detail squalene formation in *E. coli* by the use of exogenous genes (13). In the present study, we introduced three cryptic genes (*hopABD*) from the hopanoid biosynthesis gene cluster from *S. peucetius* that catalyzed squalene production and also modulated the 2-C-methyl-D-erythritol 4-phosphate pathway in *E. coli* to enhance squalene production (Fig. 1).

Bacterial strains, plasmids, media, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1, and the oligonucleotides used in this study are summarized in Table 2. *S. peucetius* was cultured in R2YE liquid medium at 28°C for 5 days on a rotary shaker (230 × *g*) to isolate genomic DNA. *E. coli* strains were grown in LB medium at 37°C or plated onto agar plates for subcloning and DNA manipulation and supplemented with ampicillin (100 μg/ml), kanamycin (100 μg/ml), and spectinomycin (35 μg/ml) (all purchased from Sigma, St. Louis, MO) wherever required for plasmid maintenance. *E. coli* strains were grown in 2× YT medium (Difco Bacto tryptone, Difco Bacto yeast extract, Nacl, and 5 N NaOH) at 20°C for squalene production on a rotary shaker (220 × *g*), and the cells were induced with 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), with 0.5% (wt/vol) glycerol used as a carbon source plus 0.5% (wt/vol) $MgSO_4$-$7H_2O$.

In vivo heterologous expression of *hopA*, *hopB*, and *hopD*. To characterize the putative genes encoding squalene synthases, *hopA* and *hopB*, and the putative gene encoding farnesyl diphosphate synthase, *hopD*, and their involvement in squalene biosynthesis, the recombinants pHOP-A and pHOP-B were separately transformed into *E. coli* BL21(DE3). The transformants were grown in 2× YT medium at 20°C for 48 h after 0.1 mM IPTG induction with 0.5% (wt/vol) glycerol as a carbon source, and lipids were isolated. The process of squalene extraction and analytical methods (thin-layer chromatography [TLC], high-pressure liquid chromatography [HPLC], and nuclear magnetic resonance [NMR]) are given in the supplemental material. The formation of squalene was detected by TLC

* Corresponding author. Mailing address: Institute of Biomolecule Reconstruction (iBR), Department of Pharmaceutical Engineering, Sun Moon University, #100 Kalsan-ri, Tangjeong-myeon, Asansi, Chungnam 336-708, South Korea. Phone: 82-41-530-2246. Fax: 82-41-544-2919. E-mail: sohng@sunmoon.ac.kr.

† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 18 September 2009.

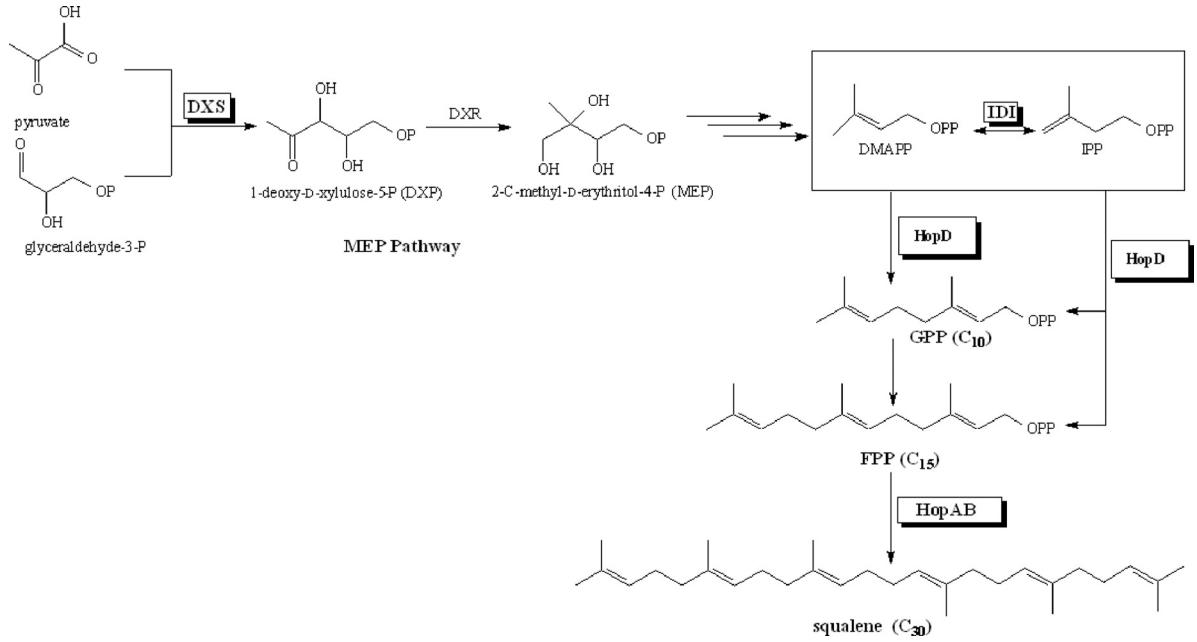

FIG. 1. Schematic representation of the engineered squalene biosynthetic pathway in *E. coli* BL21(DE3). DXS and IDI were overexpressed for the modulation of the MEP pathway to increase and balance the IPP pool, HopD (farnesyl diphosphate synthase) was overexpressed to increase FPP, and HopA and HopB (HopAB) (squalene synthases) were overexpressed to overproduce squalene. The recombinant enzymes overexpressed in *E. coli* are boxed.

(R_f value of ~0.9) (see Fig. S1 in the supplemental material), gas chromatography/mass spectrometry (retention time of 43.5 min) (see Fig. S2 in the supplemental material), and HPLC (retention time of ~25.4 min) (Fig. 2, lines II and III) and compared with authentic squalene. Thus, *hopA* and *hopB* genes encode squalene synthases. Transformation of recombinant pHOP-D in *E. coli* along with *hopA* or *hopB* enhanced squalene production (data not shown), but along with *hopAB*, it markedly increased squalene production (4.1 mg/liter), indicating that the *hopD* gene might encode the FPP synthase (Fig. 2, line IV).

Effect of amplification of the *E. coli dxs* and *idi* genes to enhance squalene production. Squalene is synthesized in *E. coli* by the expression of the *hopA* or *hopB* gene, and its production is further increased by incorporation of the *hopD* gene, as mentioned above. 1-Deoxy-D-xylulose 5-phosphate synthase (DXS) and isopentenyl diphosphate isomerase (IDI) are two enzymes involved in isoprenoid biosynthesis in the methylerythritol phosphate (MEP) pathway that can limit isoprenoid production yields in *E. coli* (3). To further improve squalene production, we amplified *idi* and *dxs* genes from the genomic DNA of *E. coli* and generated plasmid pIDXS and transformed pHOP-AB and pHOP-D plasmids into BL21(DE3) to overexpress *hopA*, *hopB*, *hopD*, *idi*, and *dxs* genes. The transformant was grown in 2× YT medium as described above, and the lipid was isolated. Squalene production increased considerably (11.8 mg/liter), as verified by HPLC (Fig. 2, line V) and TLC.

Purification of squalene and structural elucidation. We purified squalene by the use of a simple preparative TLC as shown

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source
Strains		
Streptomyces peucetius ATCC 27952	Wild-type strain, doxorubicin producer	ATCC
Escherichia coli		
XL1-Blue MRF′	Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F′ *proAB lacI*^{q}*Z*ΔM15 Tn*10* (Tet^r)]	Stratagene
BL21(DE3)	B strain carrying F^- *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3)	Invitrogen
Plasmids		
pGEM-T Easy vector	*E. coli* general cloning vector; Amp^r	Promega
pET-28a(+)	*E. coli* expression vector; Kan^r	Novagen
pETDuet-1	*E. coli* expression duet vector; Amp^r	Novagen
pCDFDuet-1	*E. coli* expression duet vector; Sm^r	Novagen
pHOP-A	pETDuet-1 carrying PCR product of *hopA* from *S. peucetius*	This study
pHOP-B	pETDuet-1 carrying PCR product of *hopB* from *S. peucetius*	This study
pHOP-AB	pETDuet-1 carrying PCR products of *hopA* and hopB from *S. peucetius*	This study
pHOP-D	pET-28a carrying PCR product of *hopD* from *S. peucetius*	This study
pIDXS	pCDFDuet-1 carrying PCR products of *idi* and *dxs* from *E. coli*	This study

TABLE 2. Primers used in this study

Primer name	Sequence (5′→3′)[a]	Restriction site
Sp-21:F	AAG**GAATTC**GTGGCGCGACGACCTCATG	EcoRI
Sp-21:R	GAT**AAGCTT**CCTTCCATCGTCCGGCTCA	HindIII
Sp-22:F	GCG**CATATG**AGCCGGACGATGGAG	NdeI
Sp-22:R	ATA**CTCGAG**CACCCGTCCGGGGGATCA	XhoI
Sp-24:F	ATA**GAATTC**CGGCTCGCGCCGCCCATG	EcoRI
Sp-24:R	GCG**AAGCTT**GGGCGGTAACGATGGCGA	HindIII
Duet idi:F	AGT**CCATGG**TTATGCAAACGGAACACG	NcoI
Duet idi:R	GCC**GGATCC**AATGTCGGGGTTTTTTTA	BamHI
Duet dxs:F	CCC**CATATG**AGTTTTGATATTGCCAA	NdeI
Duet dxs:R	AAC**AGATCT**GGAGTGGAGTAGGGATTA	BglII

[a] The restriction sites are indicated by boldface letters.

in the supplemental material. Several fractions rich in squalene, detected by preparative TLC with the Sigma standard, were scraped from the plates. The lipid was collected and dissolved in chloroform-methanol (1:1 [vol/vol]), and combined supernatants were brought to dryness by using a rotary evaporator to yield a colorless liquid. The purified squalene was dissolved in $CDCl_3$ and subjected to NMR. The structure of purified squalene was verified by NMR spectra (see Fig. S3 in the supplemental material). The NMR spectra were consistent with the literature (6).

Concluding remarks. We heterologously expressed three cryptic genes (*hopABD*) from the hopanoid biosynthesis gene cluster of *S. peucetius* that catalyzed squalene production and also overexpressed the *idi* and *dxs* genes involved in providing additional isoprenoid precursors in the MEP pathway in *E. coli* to improve squalene production. We succeeded in obtaining 11.8 mg/liter of pure squalene under optimized conditions in the present study by shaking flask cultures. We believe that the use of genetically engineered *E. coli* could be an excellent alternative for producing commercial squalene.

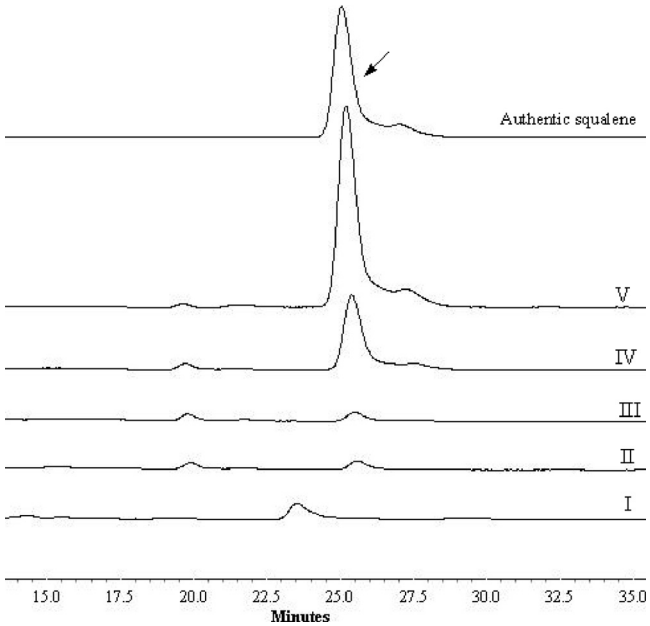

FIG. 2. Partial HPLC profiles of extracts from *E. coli* cells compared with that of authentic squalene as detected by UV absorption at 214 nm. *E. coli* BL21(DE3) harboring empty vectors pETDuet-1, pCDFDuet-1, and pET-28a(+) as a control (I); BL21(DE3) harboring the overexpressed *hopA* gene (II); BL21(DE3) harboring the overexpressed *hopB* gene (III); BL21(DE3) harboring the overexpressed *hopABD* genes (IV); and BL21(DE3) harboring the overexpressed *hopABD*, *idi*, and *dxs* genes (V).

Nucleotide sequence accession numbers. The nucleotide sequences reported in this paper have been deposited into the GenBank database under the accession numbers FJ529811 (*hopA*), FJ529812 (*hopB*), and FJ529814 (*hopD*).

This study was supported by the 21C Frontier Microbial Genomics and Application Center Program, the Ministry of Science and Technology (grant 11-2008-14-007-00), Republic of Korea.

REFERENCES

1. **Bhattacharjee, P., V. B. Shukla, R. S. Singhal, and P. R. Kulkarni.** 2001. Studies on fermentation production of squalene. World J. Microbiol. Biotechnol. **15:**811–816.
2. **Budin, J. T., W. M. Breene, and D. H. Putnam.** 1996. Some compositional properties of seeds and oils of eight *Amaranthus* species. J. Am. Oil Chem. Soc. **73:**475–481.
3. **Farmer, W. R., and J. C. Liao.** 2001. Precursor balancing for metabolic engineering of lycopene production in *Escherichia coli*. Biotechnol. Prog. **17:**57–61.
4. **Guil-Guerrero, J. L., F. Garcia-Maroto, P. Campra-Madrid, and F. Gomez-Mercado.** 2000. Occurrence and characterization of oils rich in γ-linolenic acid part II: fatty acids and squalene from Macaronesian *Echium* leaves. Phytochemistry **54:**525–529.
5. **Harada, H., F. Yu, S. Okamoto, T. Kuzuyama, R. Utsumi, and N. Misawa.** 2008. Efficient synthesis of functional isoprenoids from acetoacetate through metabolic pathway-engineered *Escherichia coli*. Appl. Microbiol. Biotechnol. **81:**915–925.
6. **He, H. P., Y. Cai, M. Sun, and H. Corke.** 2002. Extraction and purification of squalene from Amaranthus grain. J. Agric. Food Chem. **50:**368–372.
7. **Jahaniaval, F., Y. Kakuda, and M. F. Marcone.** 2000. Fatty acid and triacylglycerol compositions of seed oils of five *Amaranthus* accessions and their comparison to other oils. J. Am. Oil Chem. Soc. **77:**847–852.
8. **Kim, S. W., and J. D. Keasling.** 2001. Metabolic engineering of the nonmevalonate isopentenyl diphosphate synthesis pathway in *Escherichia coli* enhances lycopene production. Biotechnol. Bioeng. **72:**408–415.
9. **Kirby, J., and J. D. Keasling.** 2009. Biosynthesis of plant isoprenoids: perspectives for microbial engineering. Annu. Rev. Plant Biol. **60:**335–355.
10. **Lehmann, J. W.** 1996. Case history of grain amaranth as an alternative crop. Cereal Foods World **41:**399–411.
11. **Marcone, M. F.** 2000. First report of the characterization of the threatened plant species *Amaranthus pumilus* (Seabeach Amaranth). J. Agric. Food Chem. **48:**378–382.
12. **Newmark, H. L.** 1999. Squalene, olive oil, and cancer risk: review and hypothesis. Ann. N. Y. Acad. Sci. **889:**193–203.
13. **Perzl, M., I. G. Reipen, S. Schmitz, K. Poralla, H. Sahm, G. A. Sprenger, and E. L. Kannenberg.** 1998. Cloning of conserved genes from *Zymomonas mobilis* and *Bradyrhizobium japonicum* that function in the biosynthesis of hopanoid lipids. Biochim. Biophys. Acta **1393:**108–118.
14. **Rao, C. V., H. L. Newmark, and B. S. Reedy.** 1998. Chemopreventive effect of squalene on colon cancer. Carcinogenesis **19:**287–290.
15. **Rohmer, M., M. Knani, P. Simonin, B. Sutter, and H. Sahm.** 1993. Isoprenoid biosynthesis in bacteria: a novel pathway for the early steps leading to isopentenyl diphosphate. Biochem. J. **295:**517–524.
16. **Smith, T. J.** 2000. Squalene: potential chemopreventive agent. Exp. Opin. Investig. Drugs **9:**1841–1848.
17. **Storelli, M. M., E. Ceci, A. Storelli, and G. O. Marcotrigiano.** 2003. Polychlorinated biphenyl, heavy metal and methylmercury residues in hammerhead sharks: contaminant status and assessment. Mar. Pollut. Bull. **46:**1035–1039.
18. **Sun, H., D. Wiesenborn, K. Tostenson, J. Gillespie, and P. Rayas-Duarte.** 1997. Fractionation of squalene from amaranth seed oil. J. Am. Oil Chem. Soc. **74:**413–418.
19. **Turoczy, N. J., L. J. Laurenson, G. Allinson, M. Nishikawa, D. F. Lambert, C. Smith, J. P. Cottier, S. B. Irvine, and F. Stagnitti.** 2000. Observations on metal concentrations in three species of shark (*Deania calcea*, *Centroscymnus crepidater*, and *Centroscymnus owstoni*) from southeastern Australian waters. J. Agric. Food Chem. **48:**4357–4364.
20. **Vadali, R. V., Y. Fu, G. N. Bennett, and K. Y. San.** 2005. Enhanced lycopene productivity by manipulation of carbon flow to isopentenyl diphosphate in *Escherichia coli*. Biotechnol. Prog. **21:**1558–1561.
21. **Yoon, S.-H., J.-E. Kim, S.-H. Lee, H.-M. Park, M.-S. Choi, J.-Y. Kim, S.-H. Lee, Y.-C. Shin, and J. D. Keasling.** 2007. Engineering the lycopene synthetic pathway in *E. coli* by comparison of the carotenoid genes of *Pantoea agglomerans* and *Pantoea ananatis*. Appl. Microbiol. Biotechnol. **74:**131–139.