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Production of squalene by squalene synthases and their truncated mutants in *Escherichia coli*

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Squalene is a precursor of thousands of bioactive triterpenoids and also has industrial value as a lubricant, health-promoting agent, and/or drop-in biofuel. To establish an efficient *Escherichia coli*-based system for squalene production, we tested two different squalene synthases and their mutants in combination with precursor pathways. By co-expressing a chimeric mevalonate pathway with human or *Thermosynechococcus* squalene synthase, *E. coli* accumulated squalene up to 230 mg/L or 55 mg/g-DCW in flask culture. We also determined that a significant truncation of squalene synthase at the C-terminus retains partial cellular activity. The squalene-producing strain described herein represents a convenient platform for gene discovery and the construction of the pathway toward natural and non-natural hopanoids/steroids.

[**Key words:** Squalene synthase; Mevalonate pathway; Triterpenoid; Carotenoid; Bacterial production]

Squalene (2,6,10,15,19,23-hexamethyltetracosa-2,6,10,14,18,22-hexaene) is an acyclic triterpenoid that is a universal precursor for thousands of bioactive compounds, such as steroids and hopanoids. Squalene itself is also of value; for example, it is extensively used in the cosmetic industry as a moisturizer and an emollient due to its moderate antioxidant properties (1). Squalene has a variety of health-promoting functions, including tumor-suppressing (2–6), antibacterial/antifungal (7), immunity-boosting (8), and cholesterol-lowering (9) effects. Squalene has recently attracted attention as a feasible source of biofuels (10).

The search for hyperproducers of squalene has identified various promising microorganisms. *Pseudozyma* sp. JCC 207 accumulates squalene to approximately 7% of its dry cell weight (DCW) (11). *Aurantiochytrium* sp. 18W-13a and Yonez5-1 accumulate as much as 20% and 32%, respectively, of their DCW as squalene (12–14). These natural-born squalene hyperproducers are believed to have unique, strong metabolic networks suitable for squalene hyperproduction. However, genetic engineering of these organisms remains a daunting challenge, if not impossible. Thus, it is difficult to feed the squalene produced by these organisms into the triterpenoid pathways.

Alternatively, rapid-growing microorganisms such as *Saccharomyces cerevisiae* and *Escherichia coli* can be reengineered for the hyperproduction of squalene. The exceptional engineerability (fast growth, high transformation efficiency, and richness of genetic, proteomic, and metabolomic information) of these organisms has enabled their fast-track improvement to decent and robust producers of isoprenoid compounds (15–18), sometimes to levels as high as 25 g/L (amorpha-4,11-diene) (19) or 60 g/L (isoprene) (20). *S. cerevisiae* is the gold standard host organism for gene discovery and reconstruction of triterpenoid pathways due to its intrinsic capacity to synthesize triterpenoid (ergosterol). However, newly introduced triterpenoid pathways must compete with the ergosterol pathway for squalene and could cross-react with the ergosterol pathways. To increase the target products, sterol-reduced strains of *S. cerevisiae* have been used (21,22). The endogenous ergosterol pathway is essential to yeast and therefore cannot be completely eliminated (23). This residual ergosterol pathway (14 unique steps in total) could complicate the functional elucidation of new enzymes and compromise the yield and specificity of introduced pathways. Thus, a host system that entirely lacks triterpenoid pathways is also attractive. *E. coli* does not produce endogenous triterpenoids, and thus biosynthesized squalene should be stably accumulated without being consumed/converted into other unwanted compounds. However, only one study has reported a systematic effort to develop a hyperproducing strain of *E. coli* (24).

Squalene is biosynthesized via the head-to-head condensation of two molecules of farnesyl diphosphate, which is catalyzed by the single enzyme squalene synthase (SQS) (Fig. 1). Farnesyl diphosphates are available in *E. coli*, and thus, the production of squalene in *E. coli* is achieved simply by expressing SQS. SQSs are typically slow, membrane-bound enzymes with $k_{cat} \sim 1/s$. As demonstrated for bisabolene synthesis (25), selecting an appropriate enzyme can have a substantial effect on the production level. The identification and removal of unnecessary domains can also

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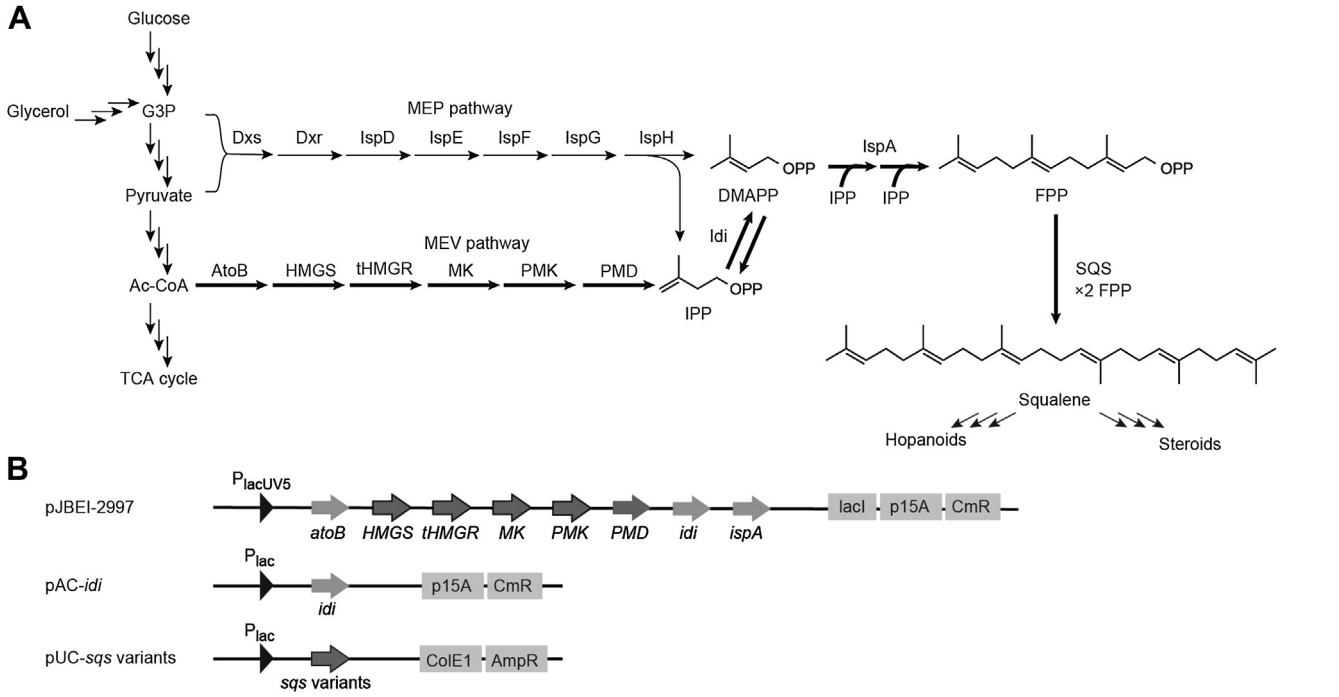

FIG. 1. Endogenous and synthetic pathways for squalene. (A) The MEP pathway is the endogenous isoprenoid pathway found in *E. coli*. Using farnesyl diphosphate as the starting material, squalene is produced by exogenously expressed squalene synthases (SQSs). The production of squalene can be enhanced by overexpressing the rate-limiting steps (Idi in this work) or/and adding an alternative supply route (MEV pathway). The enzyme steps overexpressed in this study are indicated in thick arrows. (B) The plasmid constructs used in this work are shown. pJBEI-2997 are derived from Peralta-Yahya et al. (25). The genes with black outline indicates that they are codon-optimized for *E. coli*.

dramatically improve enzyme expression, solubility, and activity, as has been demonstrated for SQSs from human (26), *Trypanosoma* (27), rat (28), and yeast (29).

In this work, we aimed to establish an efficient *E. coli* producer of squalene. We compared two different SQSs from different sources (bacteria and human). Truncation analysis revealed that significant portions of the C-terminal regions of these SQSs are not essential for their structure. By increasing the supply of precursor using synthetic mevalonate pathways previously constructed by Peralta-Yahya et al. (25), we achieved squalene production of up to 55 mg/g-DCW (230 mg/L) in flask culture.

MATERIALS AND METHODS

Bacterial strains and reagents *E. coli* XL10-Gold Kan (Stratagene, La Jolla, CA, USA) was used for DNA cloning, while XL1-Blue (Stratagene) was used for carotenoid or squalene production analysis. Carbenicillin (carb) and chloramphenicol (cm) were used at 50 μg/mL and 30 μg/mL, respectively. All reagents were purchased from Nacalai Tesque (Kyoto, Japan).

Plasmids For the construction of pUC-*hsqs*, the sequence corresponding to residues 31-370 of human squalene synthase (*hsqs*) were amplified using pHSS16 (26) as a template. The PCR primers were designed so that the PCR product contained an *Xba*I site, ribosome binding sequence, and a seven base spacer (5'-TCTAGAAGGAGGATTACAAA-3') upstream of the reading frame of *hsqs* and a stop codon (TAA) followed by an *Xho*I site flanking downstream of the *sqs* gene. The resultant PCR product was inserted into the *Xba*I/*Xho*I sites of pUC18m (30). *E. coli* codon-optimized *Thermosynechococcus elongatus* BP-1 *sqs* (*tsqs*) was purchased from DNA 2.0 Inc. (Menlo Park, CA, USA). This gene was inserted into the *Xba*I/*Xho*I sites of pUC-*hsqs*, thereby replacing the *hsqs* gene to yield pUC-*tsqs*. The expression plasmids for the truncated variants were constructed by PCR amplifying the corresponding portions (residues 31-351, 31-315, 31-311, 31-309 for hSQS and 1-351, 1-319, 1-313, 1-307 for tSQS), followed by cloning into the *Xba*I/*Xho*I sites of pUC-*hsqs*. pJBEI-2997 (25) was purchased from Addgene Inc. (Plasmid 35151) (Cambridge, MA, USA). For the construction of pAC-*idi* and pAC-*crtN*-*idi*, Plac-*idi* was PCR amplified from pUC-*idi* (in which the *E. coli* amplified *idi* gene is inserted into the *Xba*I/*Xho*I sites of pUC18m) and ligated into the *Sal*I site of pACmod (31) and pAC-*crtN* (32), respectively.

Pigment analysis Plasmids were used to transform XL1-Blue cells, and the transformants were plated onto LB (carb/cm) agar plates for colony formation. Colonies were picked and inoculated into 2 mL of LB (carb/cm) medium in a 48-deep-well plate and cultured at 37°C, 1000 rpm, for 16 h. An aliquot (50 μL) of these pre-cultures was transferred to 2 mL of Terrific Broth (TB) (carb/cm) in a 48-deep-well plate and cultured at 30°C or 37°C, 1000 rpm, for 48 h. The cells were harvested, washed with 2 mL of saline, and centrifuged to obtain cell pellets; the supernatants were discarded. After vortexing, 1 mL of acetone was added to each of the cell pellets, followed immediately by vortexing for 3 min to extract the carotenoids and centrifugation. The absorbance spectra (350–650 nm at 5-nm intervals) of the acetone extracts were analyzed using a SpectraMax Plus384 Absorbance Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). The pigmentation level of each culture was determined from the lambda max (470 nm) of the resulting extract, using the molar adsorption coefficient of 4,4'-diaponeurosporene (147,000 M^{-1} cm^{-1}).

HPLC analysis of squalene and dehydrosqualene Plasmids were used to transform XL1-Blue cells, and the transformants were plated onto LB (carb/cm) agar plates to form colonies. The colonies were picked and inoculated into 2 mL of LB (carb/cm) medium in a 48-deep-well plate and cultured at 37°C, 1000 rpm, for 16 h. An aliquot of these pre-cultures was transferred to 10 mL of TB (carb/cm) in a CELLSTAR CELLreactor 50 mL Filter Tube (Greiner Bio-One, Frickenhausen, Germany) and cultured at 30 or 37°C and 200 rpm for 96 h in maximum. Cells were harvested, washed with 10 mL of saline, and centrifuged to obtain cell pellets; the supernatants were discarded. After brief vortexing, carotenoids were extracted by the addition of 10 mL of acetone, followed by vortexing for 5 min. One milliliter of hexane and 35 mL of 1% (w/v) NaCl were added, the samples were centrifuged at 3300 ×*g* for 30 min, and the hexane phase was collected. The hexane was then evaporated with a vacuum concentrator. The non-bolatile residues were then dissolved in 400 μL of (4:6) methanol/tetrahydrofuran for separation. A 25-μL aliquot of the final extract was analyzed using an HPLC system (Shimadzu Prominence, Shimadzu Corporation, Kyoto, Japan) equipped with a photodiode array detector and a Spherisorb ODS 2 column (250 × 4.6 mm, 5 μm particles; Waters, Milford, MA, USA). The mobile phase was acetonitrile/tetrahydrofuran/methanol (58:7:35 v/v, 2 mL min^{-1}). Squalene was identified and quantified by peak area using a calibration curve generated from known amounts of squalene (Nacalai Tesque). Dehydrosqualene was identified by the comparison of chromatogram and absorbance spectra with dehydrosqualene prepared by *in vitro* reaction using purified his-tagged CrtM and FPP (purchased from Sigma–Aldrich, St. Louis, MO, USA), based on the protocol reported by Ku et al. (33). Dehydrosqualene was quantified by peak area using a calibration curve generated from known amounts of β-carotene (quantified by absorbance) and then multiplying the result value by the molar extinction coefficient (ε) of β-carotene (138,900 M^{-1} cm^{-1} at 450 nm) and dividing by the ε value for