1 **Title**

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

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5 **Authors**

6 Akinori Katabami¹, Ling Li¹, Miki Iwasaki¹, Maiko Furubayashi¹, Kyoichi Saito¹
7 and Daisuke Umeno^{1,2*}

8
9 **Affiliations**

10 ¹Department of Applied Chemistry and Biotechnology, Chiba University, 1-33
11 Yayoi-cho, Inage, Chiba, 263-8522, Japan

12 ²Precursory Research for Embryonic Science and Technology (PRESTO), Japan
13 Science and Technology Agency (JST), 4-1-8 Honcho, Kawaguchi, Saitama,
14 332-0012, Japan.

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16 **Running Title**

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18
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* Corresponding author. E-mail: umeno@faculty.chiba-u.jp, Tel/Fax:
+81-43-290-3413

Abstract (<250 words)

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.

1 **Introduction :**

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

10 The search for hyperproducers of squalene has identified various promising
11 microorganisms. *Pseudozyma sp.* JCC 207 accumulates squalene to approximately
12 7% of its dry cell weight (DCW) (11). *Aurantiochytrium sp.* 18W-13a and Yonez5-1
13 accumulate as much as 20% and 32%, respectively, of their DCW as squalene
14 (12-14).

15 These natural-born squalene hyperproducers are believed to have unique, strong
16 metabolic networks suitable for squalene hyperproduction. However, genetic
17 engineering of these organisms remains a daunting challenge, if not impossible. Thus,
18 it is difficult to feed the squalene produced by these organisms into the triterpenoid
19 pathways.

20 Alternatively, rapid-growing microorganisms such as *Saccharomyces cerevisiae*
21 and *Escherichia coli* can be reengineered for the hyperproduction of squalene. The
22 exceptional *engineerability* (fast growth, high transformation efficiency, and richness
23 of genetic, proteomic, and metabolomic information) of these organisms has enabled
24 their fast-track improvement to decent and robust producers of terpenoid 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 $k_{cat} \sim 1/\text{sec}$. As demonstrated for bisabolene synthesis (25), selecting an appropriate enzyme can have a substantial effect on production. The identification and removal of unnecessary domains can also dramatically improve enzyme expression and solubility, as has been

1 demonstrated for SQSs from human (26), *Trypanosoma* (27), rat (28), and yeast (29),
2 as well as SQS activity (26).

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

10

Materials and Methods

Bacterial strains and reagents. *E. coli* XL10-Gold Kan^R (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 mg/mL and 30 mg/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'-TCTAGAAAGGAGGATTACAAA-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 *T. 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. (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 to enable 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 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 ($147,000 \text{ M}^{-1} \text{ cm}^{-1}$).

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 3,300 ×g for 30 min, and the hexane phase was collected. The hexane was then evaporated with a vacuum concentrator. The extracts 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 ref. (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 by the molar extinction coefficient (ε) of β-carotene (138,900 M⁻¹cm⁻¹ at 450 nm) and dividing by the ε value for dehydrosqualene (49,800 M⁻¹cm⁻¹ at 287 nm). To obtain the dry cell weight (DCW), cells harvested by centrifugation from the 45 mL culture and were freeze-dried. We obtained the following relationship between OD₆₀₀ and DCW per milliliter (g-DCW/mL): 1

- 1 $OD_{600} = 0.3 \text{ mg-DCW/mL}$. We found this equation was robustly applicable to all the
- 2 culture conditions we used throughout this work.

Results

Functional expression of squalene synthases

SQSs have been cloned from various species, and some have been biochemically and structurally characterized in detail. Based on the available biochemical parameters, we chose SQSs from human (hSQS; $k_{\text{cat}}/K_m^{\text{FPP}} = 0.51 \mu\text{M}^{-1} \text{s}^{-1}$) (26) and from *Thermosynechococcus elongatus* BP-1 (tSQS; $k_{\text{cat}}/K_m^{\text{FPP}} = 1.8 \mu\text{M}^{-1} \text{s}^{-1}$) (34). The membrane-binding domains of hSQS (30 residues of the N-terminus and 47 residues of the C-terminus) were truncated based on previous reports (26), while tSQS was expressed as the full-length sequence.

The SQS genes were sub-cloned into pUC18-derived plasmids, yielding pUC-*hsqs* and pUC-*tsqs*. The transformation of *E. coli* with these plasmids resulted in *E. coli* XL1-Blue capable of producing/accumulating squalene: hSQS-expressing cells produced 4.2 mg of squalene/L in 48 h, while tSQS-expressing cells produced 3.5 mg of squalene/L (**FIG. 2**). This production level is similar to that (4.1 mg/L) reported for *Streptomyces peucetius* SQS (encoded by *hopAB*) (24). We also tested the production capability of *Saccharomyces cerevisiae* SQS ($k_{\text{cat}}/K_m^{\text{FPP}} = 0.21 \mu\text{M}^{-1} \text{s}^{-1}$) (35) in *E. coli* (not shown). We observed only a modest difference among the three SQSs tested, indicating that the cellular activity of SQS is not the limiting factor for squalene production. Note that the k_{cat} values of SQSs ($0.4\text{--}3 \text{s}^{-1}$) are relatively high compared to other terpenoid synthases/cyclases ($0.005\text{--}5 \text{s}^{-1}$).

The effect of the overexpression of isopentenyl diphosphate synthase on squalene accumulation.

E. coli synthesizes isoprenoid precursors using the non-mevalonate pathway that proceeds from glyceraldehyde 3-phosphate (G3P) and pyruvate (**FIG. 1**). In the 7 steps of the pathway, over-expression of 1-deoxyxylulose-5-phosphate synthase (Dxs) (36, 37), 1-deoxy-D-xylulose 5-phosphate reductoisomerase (Dxr) (36), and isoprenyl diphosphate isomerase (Idi) (38) were reported to improve the titer of the pathway, increasing the production level of various isoprenoid compounds. This was also the case for squalene: the production level of squalene in *E. coli* was elevated threefold, reaching 11.8 mg/L, by combined over-expression of *idi* and *dxs* (24). The over-expression of *idi* has been the most popular strategy to increase the cellular production of isoprenoid compounds: Kajiwara *et al.* tested the effect of over-expression of *idi* from various sources and observed 4.5- and 2.5-fold increases in lycopene and beta-carotene production. The molar ratio of IPP/DMAPP produced by endogenous IspH is biased toward IPP (5:1 to 6:1) (39-42), and this ratio shift to 3:7 by the activity of Idi (40). We sub-cloned *E. coli* *idi* under the control of the lac promoter on a pACYC-based plasmid. Co-transformation of the resultant plasmid pAC-*idi* with pUC-*hsqs* or pUC-*tsqs* resulted in squalene production of 12.9 mg/L and 13.5 mg/L, respectively (**FIG. 2**). The fold improvement (3- to 4-fold) achieved by co-expression with *idi* is similar to that reported for carotenoids (38).

The effect of adding mevalonate pathway on squalene accumulation.

Another strategy to increase the isoprenoid supply is to introduce the mevalonate pathway, a six-step pathway starting from acetyl-CoA (43). Many groups have re-constructed mevalonate pathways from various sources, and the introduction of these pathways has been reported to be highly effective in improving terpenoid

production (25, 44-60). Interestingly, the effect of adding the MEV pathway on triterpenoid production has never been reported in *E. coli*.

In this study, we adopted the chimeric mevalonate pathway created by Peralta-Yahya *et al.* (25). Five genes, a truncated mutant of HMG-CoA reductase (*tHMGR*), HMG-CoA synthase (*HMGS*), mevalonate kinase (*MK*), phosphomevalonate kinase (*PMK*), and mevalonate diphosphate decarboxylase (*PMD*), from *S. cerevisiae* and three endogenous genes, acetyl-CoA acetyltransferase (*atoB*), isoprenyl diphosphate isomerase (*idi*), and farnesyl diphosphate synthase (*ispA*), were integrated into a single operon in which all genes were codon-optimized for expression in *E. coli*. The resultant plasmid, pJBEI-2997, was used to co-transform XL1-Blue with pUC-*hsqs* or *tsqs* to evaluate the ability of the chimeric mevalonate pathways to increase squalene formation. The effect was profound: the average yield of squalene was 230 mg/L (54 mg/g-DCW) for XL1-Blue harboring pUC-*hsqs* and pJBEI-2997, an approximately 55-fold increase compared to XL1-Blue harboring pUC-*hsqs* alone (**Fig. 2**). Similarly, an approximately 40-fold improvement in squalene production was observed for *E. coli* expressing tSQS (150 mg/mL, 55 mg/g-DCW) (**Fig. 2**). It should be noted that these values are for shaking flask without any effort in fermentation engineering. Still, these levels of production are 13- to 20-fold of the highest reported value (11.8 mg/L) (24) for *E. coli*, and are comparable with the highest value for (191.9 mg/L) (61) reported for *S. cerevisiae*. On the yield per dry cell weight-basis, the values we obtained (54 mg/g-DCW for hSQS-expressing *E. coli* and 55 mg/g-DCW for tSQS-expressing *E. coli*) were about three fold of the highest values reported for engineered *S. cerevisiae* (18.5

mg/g-DCW) (62) and were about one fifth of the natural squalene hyperproducer *Aurantiochytrium Yonez5-1* (317 mg/g-DCW) (14).

The effect of temperature on squalene accumulation by the hyperproducing strain.

Because five components (tHMGR, HMGS, MK, PMK, PMD) of the chimeric mevalonate pathway used here originated from yeast, we sought to determine whether the optimal temperature for these enzymes differed from 37°C, which was initially adopted as part of the culture conditions (**FIG. 3**). We compared the performance of the squalene hyperproducing strain at 30°C and 37°C. XL1-Blue was transformed with JBEI-2997 and pUC-*hsqs*, and the resultant strain was cultured at 30°C or 37°C (**FIG. 3**) to compare cell growth (OD) and productivity (accumulated squalene, mg/L). In both cultures, the level of squalene increased over time, but the time-profile of squalene accumulation was different. The culture at 37°C rapidly began accumulating squalene, while the culture at 30°C began accumulating squalene approximately 24 h after inoculation, despite similar increases in cellular density at the two temperatures. The maximum production of squalene was 180 mg/L and 100 mg/L at 37°C and 30°C, respectively.

Evaluation of truncated SQS mutants.

With the aim of identifying a reading frame with higher cellular activity in *E. coli* and better defining the minimal set of amino acids required for SQS catalytic activity, we constructed and evaluated the cellular activity of a series of truncated hSQS and tSQS mutants.

We recently reported that the dehydrosqualene desaturase CrtN from *Staphylococcus aureus* converts squalene into yellow carotenoid pigments (63), permitting the colorimetric detection of squalene produced in the cell. We created several truncated mutants of hSQS and tSQS and co-transformed *E. coli* with the plasmid for the expression of each of the mutants and pAC-*crtN-idi* to detect the possible formation of carotenoid pigments (FIG. 4). The pigmentation level of cells expressing hSQS₃₁₋₃₅₁ was nearly identical to that of cells expressing hSQS₃₁₋₃₇₀, indicating that the last helix is disposable for hSQS activity. Indeed, HPLC-based product analysis of the *E. coli* culture expressing this mutant alone revealed that squalene was accumulated with somewhat low yield but without observable formation of dehydrosqualene (FIG. 5). By contrast, carotenoid pigment did not accumulate when the truncated mutants hSQS₃₁₋₃₁₅, hSQS₃₁₋₃₁₁, and hSQS₃₁₋₃₀₅ were co-expressed with pAC-*crtN-idi*. These mutants lack the part of JK-loop, which is proposed to directly interact with the NADPH-binding region (64). The loss of SQS function was confirmed by HPLC analysis of extracts of *E. coli* expressing these mutants (FIG. 5).

We also created truncated mutants of tSQS. Like hSQS, the C-terminal truncation at amino acid 351 of tSQS resulted in active SQS, with a pigmentation score approximately 70% of that observed for full-length tSQS (FIG. 4). Cellular accumulation of squalene by *E. coli* expressing tSQS₁₋₃₅₁ was approximately half of that observed for the non-truncated version of tSQS, without formation of detectable level of dehydrosqualene (FIG. 5). In contrast to hSQS, the mutants tSQS₁₋₃₁₉, tSQS₁₋₃₁₃, tSQS₁₋₃₀₇ (corresponding to the C-terminal truncation mutants hSQS₃₁₋₃₁₅, hSQS₃₁₋₃₁₁, hSQS₃₁₋₃₀₅, respectively) produced lower but non-negligible

pigmentation compared to non-truncated tSQS (25-30% of non-truncated tSQS) (FIG. 4).

Interestingly, these mutants had no SQS activity, as confirmed by extract analysis of *E. coli* expressing these mutants. However, these mutants retained a certain level of dehydrosqualene synthesis, which can be attributed to the absence of the JK-loop, one of the most important NADPH-interacting regions (FIG. S1 and S2). In contrast to the hSQS mutants, these results indicate that tSQS can fold without the JK-loop and downstream residues, which comprise a significant portion of the protein structure. In addition, our results also demonstrate that the truncated tSQS mutants are still capable of catalyzing head-to-head condensation of two farnesyl diphosphates. However, the loss of the NADPH-binding domain (JK-loop and neighboring regions) resulted in a switching of product specificity toward non-reductive compounds such as dehydrosqualene. In addition to dehydrosqualene, we observed significantly increasing of the **peak 3** (at 4 mins in FIG. 6) in products of truncated tSQSs (tSQS₁₋₃₁₉, tSQS₁₋₃₁₃, and tSQS₁₋₃₀₇). We have not yet been able to determine the chemical structure of this compound. Based on the higher polarity (early elution time) of this molecule, we assume that this compound is a hydroxylated molecule such as presqualene alcohol, hydroxysqualene or hydroxybotryococcene (65). At present, we do not know what it is and how it forms. It is possible that the SQS mutants were so slow in the second half reaction, thereby allowing the endogenous pyrophosphate enzymes to intercept the presqualene pyrophosphate to form the presqualene alcohol (66). Or, the truncated mutants cannot perfectly shield the water molecules from the reaction pocket, leading to the quenching of the carbocation intermediate with the water molecule.

Discussion

The removal of membrane-anchoring and organelle-targeted domains often improves the expression of genes from higher eukaryotes. SQSs are integrally associated with the membrane and are resistant to solubilization. Various studies have attempted to prepare soluble SQS using deoxycholate (67, 68) or non-ionic detergents (69-71). To minimize complications due to the membrane-bound nature of SQS, attempts have been made to generate an enzyme that is more amenable to study. Because the C-terminal region of SQS is highly hydrophobic and serves as a membrane anchor (72, 73), genetic truncation has enabled the preparation of soluble SQSs. The truncation of the hydrophobic region retained the activity of yeast enzyme (29, 35). The N-terminal domains of SQS are not hydrophobic, but enzymatic truncation of rat SQS to produce a soluble, active enzyme revealed that 33 amino acids can be removed from the N-terminus of the enzyme without loss of activity (28). Accordingly, Thompson *et al.* removed the first 30 residues from hSQS and observed no apparent decrease in activity (26). Moreover, the elimination of 30 amino acids from the N terminus and 47 C-terminal amino acids resulted in a doubling of apparent activity. The doubly truncated mutant hSQS₃₁₋₃₇₀ can be readily overexpressed in *E. coli* and purified to homogeneity and has become the standard for *in vitro* analysis studies of this enzyme (64, 74, 75). However, hSQS₃₁₋₃₇₀ retains a C-terminal helix that is partially missing in bacterial SQSs, and the homologous dihydrosqualene synthases (CrtMs) do not contain this helix (**FIG. S1**). In this work, we found that the helix-M in hSQS is removable without losing its activity. Further truncation resulted in the loss of cellular activity of hSQS, while tSQS showed surprising tolerance to the removal of its C-terminal residues. Truncation up

1 to 52 aa, removing three additional helices and a part of the JK-loop known to be
2 involved in NADPH binding, resulted in the complete loss of activity as squalene
3 synthase, but showed detectable activity as dehydrosqualene synthase, together with
4 the activity yielding novel, probably hydroxylated condensation products. We do not
5 know how this compound forms, but it is apparent that the truncated tSQS mutant
6 retains the folding capability as well as its catalytic activity. It is intriguing to what
7 extent could this enzyme **be** truncated further without completely losing the activity,
8 and whether this mutant could fully-recover its activity as squalene/
9 dehydrosqualene synthase.

10 Squalene is a universal precursor for thousands of triterpenoids. Because *E.*
11 *coli* does not have triterpenoid pathways, the squalene produced in *E. coli*
12 accumulates without being metabolized. In addition, the lack of endogenous
13 pathways guarantees the *intactness* of the introduced triterpenoid pathways. Thus,
14 researchers can freely explore novel genes of interest and/or novel combinations of
15 triterpenoid genes, with minimal concern for competition for the precursor
16 (squalene) (21, 22) and/or cross-reactions of introduced pathways with pre-existing
17 (endogenous) enzymes that would complicate identification and purification. At
18 present, oxidosqualene synthesis has not been established in *E. coli* because it
19 requires the functional expression of association factors (NADPH-cytochrome C
20 reductase, squalene carrier protein (76-79) and likely others). Once the expression of
21 these factors is achieved, the squalene-producing *E. coli* strain would serve as an
22 ideal platform for the characterization, combinatorial expression, and efficient
23 production of triterpenoids, including sterols and plant-derived triterpenoids.

References

1. **Kohno, Y., Egawa, Y., Itoh, S., Nagaoka, S., Takahashi, M. and Mukai, K.:** Kinetic study of quenching reaction of singlet oxygen and scavenging reaction of free radical by squalene in n-butanol, *Biochim. Biophys. Acta*, **1256**, 52-56 (1995).
2. **Smith, T. J., Yang, G. Y., Seril, D. N., Liao, J. and Kim, S.:** Inhibition of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced lung tumorigenesis by dietary olive oil and squalene, *Carcinogenesis*, **19**, 703-706 (1998).
3. **Rao, C. V., Newmark, H. L. and Reddy, B. S.:** Chemopreventive effect of squalene on colon cancer, *Carcinogenesis*, **19**, 287-290 (1998).
4. **Newmark, H. L.:** Squalene, olive oil, and cancer risk: a review and hypothesis, *Cancer Epidemiol. Biomarkers Prev.*, **6**, 1101-1103 (1997).
5. **Budiyanto, A., Ahmed, N. U., Wu, A., Bito, T., Nikaido, O., Osawa, T., Ueda, M. and Ichihashi, M.:** Protective effect of topically applied olive oil against photocarcinogenesis following UVB exposure of mice, *Carcinogenesis*, **21**, 2085-2090 (2000).
6. **Aioi, A., Shimizu, T. and Kuriyama, K.:** Effect of squalene on superoxide anion generation induced by a skin irritant, lauroylsarcosine., *Int. J. Pharm.*, **113**, 159-164 (1995).
7. **Smith, T. J.:** Squalene: potential chemopreventive agent, *Expert Opin. Investig. Drugs*, **9**, 1841-1848 (2000).
8. **Kelly, G. S.:** Squalene and its potential clinical uses, *Altern. Med. Rev.*, **4**, 29-36 (1999).
9. **Miettinen, T. A. and Vanhanen, H.:** Serum concentration and metabolism of cholesterol during rapeseed oil and squalene feeding, *Am. J. Clin. Nutr.*, **59**, 356-363 (1994).
10. **Rude, M. A. and Schirmer, A.:** New microbial fuels: a biotech perspective, *Curr. Opin. Microbiol.*, **12**, 274-281 (2009).
11. **Chang, M. H., Kim, H. J., Jahng, K. Y. and Hong, S. C.:** The isolation and characterization of *Pseudozyma* sp. JCC 207, a novel producer of squalene, *Appl. Microbiol.*

Biotechnol., **78**, 963-972 (2008).

12. **Kaya, K., Nakazawa, A., Matsuura, H., Honda, D., Inouye, I. and Watanabe, M. M.:**

Thraustochytrid *Aurantiochytrium* sp. 18W-13a accumulates high amounts of squalene,

Biosci. Biotechnol. Biochem., **75**, 2246-2248 (2011).

13. **Nakazawa, A., Matsuura, H., Kose, R., Kato, S., Honda, D., Inouye, I., Kaya, K. and**

Watanabe, M. M.: Optimization of culture conditions of the thraustochytrid

Aurantiochytrium sp. strain 18W-13a for squalene production, Bioresour. Technol., **109**,

287-291 (2012).

14. **Nakazawa, A., Kokubun, Y., Matsuura, H., Yonezawa, N., Kose, R., Yoshida, M.,**

Tanabe, Y., Kusuda, E., Thang, D. V., Ueda, M. and other 4 authors: TLC screening of

thraustochytrid strains for squalene production, J. Appl. Phycol., **26**, 29-41 (2014).

15. **Keasling, J. D.:** Synthetic biology and the development of tools for metabolic engineering,

Metab. Eng., **14**, 189-195 (2012).

16. **Misawa, N.:** Pathway engineering for functional isoprenoids, Curr. Opin. Biotechnol., **22**,

627-633 (2011).

17. **Peralta-Yahya, P. P., Zhang, F., del Cardayre, S. B. and Keasling, J. D.:** Microbial

engineering for the production of advanced biofuels, Nature, **488**, 320-328 (2012).

18. **Wriessnegger, T. and Pichler, H.:** Yeast metabolic engineering--targeting sterol metabolism

and terpenoid formation, Prog. Lipid Res., **52**, 277-293 (2013).

19. **Tsuruta, H., Paddon, C. J., Eng, D., Lenihan, J. R., Horning, T., Anthony, L. C.,**

Regentin, R., Keasling, J. D., Renninger, N. S. and Newman, J. D.: High-level

production of amorpha-4,11-diene, a precursor of the antimalarial agent artemisinin, in

Escherichia coli, PLoS One, **4**, e4489 (2009).

20. **Cervin, M., Whited, G. M., Chotani, G. K., Valle, F., Fiorese, C., Sanford, K. J.,**

McAuliffe, J. C., Ferher, F. J., Puhala, A. S., Miasnikov, A. and Aldor, I. S.:

Compositions and methods for producing isoprene. Internatiol Publication Number
WO2009/076676 A2 (2009).

21. **Moses, T., Pollier, J., Almagro, L., Buyst, D., Van Montagu, M., Pedreno, M. A.,
Martins, J. C., Thevelein, J. M. and Goossens, A.:** Combinatorial biosynthesis of
sapogenins and saponins in *Saccharomyces cerevisiae* using a C-16alpha hydroxylase from
Bupleurum falcatum, Proc. Natl. Acad. Sci. USA, **111**, 1634-1639 (2014).

22. **Kirby, J., Romanini, D. W., Paradise, E. M. and Keasling, J. D.:** Engineering triterpene
production in *Saccharomyces cerevisiae*-beta-amyrin synthase from *Artemisia annua*, FEBS
J., **275**, 1852-1859 (2008).

23. **Lees, N. D., Skaggs, B., Kirsch, D. R. and Bard, M.:** Cloning of the late genes in the
ergosterol biosynthetic pathway of *Saccharomyces cerevisiae*--a review, Lipids, **30**, 221-226
(1995).

24. **Ghimire, G. P., Lee, H. C. and Sohng, J. K.:** 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 (2009).

25. **Peralta-Yahya, P. P., Ouellet, M., Chan, R., Mukhopadhyay, A., Keasling, J. D. and Lee,
T. S.:** Identification and microbial production of a terpene-based advanced biofuel, Nat.
Commun., **2**, 483 (2011).

26. **Thompson, J. F., Danley, D. E., Mazzalupo, S., Milos, P. M., Lira, M. E. and Harwood,
H. J., Jr.:** Truncation of human squalene synthase yields active, crystallizable protein, Arch.
Biochem. Biophys., **350**, 283-290 (1998).

27. **Urbina, J. A., Concepcion, J. L., Rangel, S., Visbal, G. and Lira, R.:** Squalene synthase
as a chemotherapeutic target in *Trypanosoma cruzi* and *Leishmania mexicana*, Mol.
Biochem. Parasitol., **125**, 35-45 (2002).

- 1 28. **Shechter, I., Klinger, E., Rucker, M. L., Engstrom, R. G., Spirito, J. A., Islam, M. A.,**
2 **Boettcher, B. R. and Weinstein, D. B.:** Solubilization, purification, and characterization of
3 a truncated form of rat hepatic squalene synthetase, *J. Biol. Chem.*, **267**, 8628-8635 (1992).
- 4 29. **Zhang, D., Jennings, S. M., Robinson, G. W. and Poulter, C. D.:** Yeast squalene synthase:
5 expression, purification, and characterization of soluble recombinant enzyme, *Arch.*
6 *Biochem. Biophys.*, **304**, 133-143 (1993).
- 7 30. **Umeno, D., Tobias, A. V. and Arnold, F. H.:** Evolution of the C₃₀ carotenoid synthase CrtM
8 for function in a C₄₀ pathway, *J. Bacteriol.*, **184**, 6690-6699 (2002).
- 9 31. **Schmidt-Dannert, C., Umeno, D. and Arnold, F. H.:** Molecular breeding of carotenoid
10 biosynthetic pathways, *Nat. Biotechnol.*, **18**, 750-753 (2000).
- 11 32. **Umeno, D. and Arnold, F. H.:** Evolution of a pathway to novel long-chain carotenoids, *J.*
12 *Bacteriol.*, **186**, 1531-1536 (2004).
- 13 33. **Ku, B., Jeong, J. C., Mijts, B. N., Schmidt-Dannert, C. and Dordick, J. S.:** Preparation,
14 characterization, and optimization of an *in vitro* C₃₀ carotenoid pathway, *Appl. Environ.*
15 *Microbiol.*, **71**, 6578-6583 (2005).
- 16 34. **Lee, S. and Poulter, C. D.:** Cloning, solubilization, and characterization of squalene
17 synthase from *Thermosynechococcus elongatus* BP-1, *J. Bacteriol.*, **190**, 3808-3816 (2008).
- 18 35. **LoGrasso, P. V., Soltis, D. A. and Boettcher, B. R.:** Overexpression, purification, and
19 kinetic characterization of a carboxyl-terminal-truncated yeast squalene synthetase, *Arch.*
20 *Biochem. Biophys.*, **307**, 193-199 (1993).
- 21 36. **Albrecht, M., Misawa, N. and Sandmann, G.:** Metabolic engineering of the terpenoid
22 biosynthetic pathway of *Escherichia coli* for production of the carotenoids β -carotene and
23 zeaxanthin, *Biotechnol. Lett.*, **21**, 791-795 (1999).
- 24 37. **Matthews, P. D. and Wurtzel, E. T.:** Metabolic engineering of carotenoid accumulation in
25 *Escherichia coli* by modulation of the isoprenoid precursor pool with expression of

deoxyxylulose phosphate synthase, Appl. Microbiol. Biotechnol., **53**, 396-400 (2000).

38. **Kajiwara, S., Fraser, P. D., Kondo, K. and Misawa, N.:** Expression of an exogenous isopentenyl diphosphate isomerase gene enhances isoprenoid biosynthesis in *Escherichia coli*, Biochem. J., **324**, 421-426 (1997).

39. **Rohdich, F., Hecht, S., Gartner, K., Adam, P., Krieger, C., Amslinger, S., Arigoni, D., Bacher, A. and Eisenreich, W.:** Studies on the nonmevalonate terpene biosynthetic pathway: metabolic role of IspH (LytB) protein, Proc. Natl. Acad. Sci. USA, **99**, 1158-1163 (2002).

40. **Rohdich, F., Zepeck, F., Adam, P., Hecht, S., Kaiser, J., Laupitz, R., Grawert, T., Amslinger, S., Eisenreich, W., Bacher, A. and Arigoni, D.:** The deoxyxylulose phosphate pathway of isoprenoid biosynthesis: studies on the mechanisms of the reactions catalyzed by IspG and IspH protein, Proc. Natl. Acad. Sci. USA, **100**, 1586-1591 (2003).

41. **Adam, P., Hecht, S., Eisenreich, W., Kaiser, J., Grawert, T., Arigoni, D., Bacher, A. and Rohdich, F.:** Biosynthesis of terpenes: studies on 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase, Proc. Natl. Acad. Sci. USA, **99**, 12108-12113 (2002).

42. **Hintz, M., Reichenberg, A., Altincicek, B., Bahr, U., Gschwind, R. M., Kollas, A. K., Beck, E., Wiesner, J., Eberl, M. and Jomaa, H.:** Identification of (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate as a major activator for human gammadelta T cells in *Escherichia coli*, FEBS Lett., **509**, 317-322 (2001).

43. **Kuzuyama, T.:** Mevalonate and nonmevalonate pathways for the biosynthesis of isoprene units, Biosci. Biotechnol. Biochem., **66**, 1619-1627 (2002).

44. **Harada, H. and Misawa, N.:** Novel approach in the biosynthesis of functional carotenoids in *Escherichia coli*, Methods Mol. Biol., **892**, 133-141 (2012).

45. **Campos, N., Rodriguez-Concepcion, M., Sauret-Gueto, S., Gallego, F., Lois, L. M. and Boronat, A.:** *Escherichia coli* engineered to synthesize isopentenyl diphosphate and

1 dimethylallyl diphosphate from mevalonate: a novel system for the genetic analysis of the
2 2-C-methyl-d-erythritol 4-phosphate pathway for isoprenoid biosynthesis, *Biochem. J.*, **353**,
3 59-67 (2001).

4 46. **Martin, V. J., Pitera, D. J., Withers, S. T., Newman, J. D. and Keasling, J. D.:**
5 Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids, *Nat.*
6 *Biotechnol.*, **21**, 796-802 (2003).

7 47. **Vadali, R. V., Fu, Y., Bennett, G. N. and San, K. Y.:** Enhanced lycopene productivity by
8 manipulation of carbon flow to isopentenyl diphosphate in *Escherichia coli*, *Biotechnol.*
9 *Prog.*, **21**, 1558-1561 (2005).

10 48. **Yoon, S. H., Lee, Y. M., Kim, J. E., Lee, S. H., Lee, J. H., Kim, J. Y., Jung, K. H., Shin,**
11 **Y. C., Keasling, J. D. and Kim, S. W.:** Enhanced lycopene production in *Escherichia coli*
12 engineered to synthesize isopentenyl diphosphate and dimethylallyl diphosphate from
13 mevalonate, *Biotechnol. Bioeng.*, **94**, 1025-1032 (2006).

14 49. **Asadollahi, M. A., Maury, J., Schalk, M., Clark, A. and Nielsen, J.:** Enhancement of
15 farnesyl diphosphate pool as direct precursor of sesquiterpenes through metabolic
16 engineering of the mevalonate pathway in *Saccharomyces cerevisiae*, *Biotechnol. Bioeng.*,
17 **106**, 86-96 (2010).

18 50. **Morrone, D., Lowry, L., Determan, M. K., Hershey, D. M., Xu, M. and Peters, R. J.:**
19 Increasing diterpene yield with a modular metabolic engineering system in *E. coli*:
20 comparison of MEV and MEP isoprenoid precursor pathway engineering, *Appl. Microbiol.*
21 *Biotechnol.*, **85**, 1893-1906 (2010).

22 51. **Rico, J., Pardo, E. and Orejas, M.:** Enhanced production of a plant monoterpene by
23 overexpression of the 3-hydroxy-3-methylglutaryl coenzyme A reductase catalytic domain in
24 *Saccharomyces cerevisiae*, *Appl. Environ. Microbiol.*, **76**, 6449-6454 (2010).

25 52. **Redding-Johanson, A. M., Batth, T. S., Chan, R., Krupa, R., Szmidt, H. L., Adams, P.**

D., Keasling, J. D., Lee, T. S., Mukhopadhyay, A. and Petzold, C. J.: Targeted proteomics for metabolic pathway optimization: application to terpene production, *Metab. Eng.*, **13**, 194-203 (2011).

53. **Song, A. A., Abdullah, J. O., Abdullah, M. P., Shafee, N., Othman, R., Tan, E. F., Noor, N. M. and Raha, A. R.:** Overexpressing 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) in the lactococcal mevalonate pathway for heterologous plant sesquiterpene production, *PLoS One*, **7**, e52444 (2012).

54. **Zhou, Y. J., Gao, W., Rong, Q., Jin, G., Chu, H., Liu, W., Yang, W., Zhu, Z., Li, G., Zhu, G., Huang, L. and Zhao, Z. K.:** Modular pathway engineering of diterpenoid synthases and the mevalonic acid pathway for miltiradiene production, *J. Am. Chem. Soc.*, **134**, 3234-3241 (2012).

55. **Alonso-Gutierrez, J., Chan, R., Batth, T. S., Adams, P. D., Keasling, J. D., Petzold, C. J. and Lee, T. S.:** Metabolic engineering of *Escherichia coli* for limonene and perillyl alcohol production, *Metab. Eng.*, **19**, 33-41 (2013).

56. **Yang, J., Nie, Q., Ren, M., Feng, H., Jiang, X., Zheng, Y., Liu, M., Zhang, H. and Xian, M.:** Metabolic engineering of *Escherichia coli* for the biosynthesis of alpha-pinene, *Biotechnol. Biofuels*, **6**, 60 (2013).

57. **Zheng, Y., Liu, Q., Li, L., Qin, W., Yang, J., Zhang, H., Jiang, X., Cheng, T., Liu, W., Xu, X. and Xian, M.:** Metabolic engineering of *Escherichia coli* for high-specificity production of isoprenol and prenol as next generation of biofuels, *Biotechnol Biofuels*, **6**, 57 (2013).

58. **Bentley, F. K., Zurbruggen, A. and Melis, A.:** Heterologous expression of the mevalonic acid pathway in cyanobacteria enhances endogenous carbon partitioning to isoprene, *Mol Plant*, **7**, 71-86 (2014).

59. **Gupta, D., Summers, M. L. and Basu, C.:** Engineering an isoprenoid pathway in *Escherichia coli* for production of 2-methyl-3-buten-2-ol: a potential biofuel, *Mol.*

Biotechnol., **56**, 516-523 (2014).

60. **Yuan, J. and Ching, C. B.:** Combinatorial engineering of mevalonate pathway for improved amorpha-4,11-diene production in budding yeast, *Biotechnol. Bioeng.*, **111**, 608-617 (2014).

61. **Tokuhiro, K., Muramatsu, M., Ohto, C., Kawaguchi, T., Obata, S., Muramoto, N., Hirai, M., Takahashi, H., Kondo, A., Sakuradani, E. and Shimizu, S.:** Overproduction of geranylgeraniol by metabolically engineered *Saccharomyces cerevisiae*, *Appl. Environ. Microbiol.*, **75**, 5536-5543 (2009).

62. **Mantzouridou, F. and Tsimidou, M. Z.:** Observations on squalene accumulation in *Saccharomyces cerevisiae* due to the manipulation of HMG2 and ERG6, *FEMS Yeast Res.*, **10**, 699-707 (2010).

63. **Furubayashi, M., Li, L., Katabami, A., Saito, K. and Umeno, D.:** Construction of carotenoid biosynthetic pathways using squalene synthase, *FEBS Lett.*, **588**, 436-442 (2014).

64. **Liu, C. I., Jeng, W. Y., Chang, W. J., Shih, M. F., Ko, T. P. and Wang, A. H.:** Structural insights into the catalytic mechanism of human squalene synthase, *Acta Crystallogr. D Biol. Crystallogr.*, **70**, 231-241 (2014).

65. **Jarstfer, M. B., Zhang, D. L. and Poulter, C. D.:** Recombinant squalene synthase. Synthesis of non-head-to-tail isoprenoids in the absence of NADPH, *J. Am. Chem. Soc.*, **124**, 8834-8845 (2002).

66. **Edmond, J., Popják, G., Wong, S.-M. and Williams, V. P.:** Presqualene Alcohol: FURTHER EVIDENCE ON THE STRUCTURE OF A C₃₀ PRECURSOR OF SQUALENE, *J. Biol. Chem.*, **246**, 6254-6271 (1971).

67. **Agnew, W. S. and Popjak, G.:** Squalene synthetase. Solubilization from yeast microsomes of a phospholipid-requiring enzyme, *J. Biol. Chem.*, **253**, 4574-4583 (1978).

68. **Shechter, I. and Bloch, K.:** Solubilization and purification of trans-farnesyl pyrophosphate-squalene synthetase, *J. Biol. Chem.*, **246**, 7690-7696 (1971).
69. **Kuswik-Rabiega, G. and Rilling, H. C.:** Squalene synthetase. Solubilization and partial purification of squalene synthetase, copurification of presqualene pyrophosphate and squalene synthetase activities, *J. Biol. Chem.*, **262**, 1505-1509 (1987).
70. **Sasiak, K. and Rilling, H. C.:** Purification to homogeneity and some properties of squalene synthetase, *Arch. Biochem. Biophys.*, **260**, 622-627 (1988).
71. **Kroon, P. A. and Threlfall, D. R.:** Squalene synthase from cell suspension cultures of *Tabernaemontana divaricata*, *Phytochemistry*, **45**, 1157-1163 (1997).
72. **Jennings, S. M., Tsay, Y. H., Fisch, T. M. and Robinson, G. W.:** Molecular cloning and characterization of the yeast gene for squalene synthetase, *Proc. Natl. Acad. Sci. USA*, **88**, 6038-6042 (1991).
73. **Robinson, G. W., Tsay, Y. H., Kienzle, B. K., Smith-Monroy, C. A. and Bishop, R. W.:** Conservation between human and fungal squalene synthetases: similarities in structure, function, and regulation, *Mol. Cell. Biol.*, **13**, 2706-2717 (1993).
74. **Pandit, J., Danley, D. E., Schulte, G. K., Mazzalupo, S., Pauly, T. A., Hayward, C. M., Hamanaka, E. S., Thompson, J. F. and Harwood, H. J., Jr.:** Crystal structure of human squalene synthase. A key enzyme in cholesterol biosynthesis, *J. Biol. Chem.*, **275**, 30610-30617 (2000).
75. **Liu, C. I., Jeng, W. Y., Chang, W. J., Ko, T. P. and Wang, A. H.:** Binding modes of zaragozic acid A to human squalene synthase and staphylococcal dehydrosqualene synthase, *J. Biol. Chem.*, **287**, 18750-18757 (2012).
76. **Ono, T. and Bloch, K.:** Solubilization and partial characterization of rat liver squalene epoxidase, *J. Biol. Chem.*, **250**, 1571-1579 (1975).
77. **Ritter, M. C. and Dempsey, M. E.:** Specificity and role in cholesterol biosynthesis of a

- 1 squalene and sterol carrier protein, J. Biol. Chem., **246**, 1536-1539 (1971).
- 2 78. **Tai, H. H. and Bloch, K.:** Squalene epoxidase of rat liver, J. Biol. Chem., **247**, 3767-3773
- 3 (1972).
- 4 79. **Yamamoto, S. and Bloch, K.:** Studies on squalene epoxidase of rat liver, J. Biol. Chem.,
- 5 **245**, 1670-1674 (1970).

Figure legends

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). (B) The plasmid constructs used in this work are shown.

FIG. 2 The effect of booster plasmids on squalene formation in *E. coli*. *E. coli* XL1-Blue harboring pUC-*hsqs* or pUC-*tsqs* and booster plasmid (pAC-*idi* or pJBEI-2997) were cultured in TB medium at 37°C for 48 h. The amount of squalene was analyzed by HPLC. The bars indicate the average of three samples, and the error bars indicate the standard deviation.

FIG. 3 The effect of temperature on the time-course of squalene accumulation. *E. coli* XL1-Blue were transformed with JBEI2997 and pUC-*hsqs* and subjected to time course analysis of (A) the level of squalene accumulated (mg) per culture volume (L) and (B) the change in culture cell density. The analysis was conducted in TB culture at 30°C (open circles) or 37°C (closed circles).

FIG. 4 Carotenoid production by *E. coli* co-expressing truncated SQS variants with the squalene desaturase CrtN. The SQSs carried on pUC18m vectors were expressed in *E. coli* XL1-Blue harboring pAC-*crtN-idi*, and the resultant transformants were cultured in TB medium at 37°C for 48 h. Carotenoid production was analyzed by extracting the carotenoid pigments with acetone and measuring the absorbance. The bars indicate the average of three samples, and the error bars indicate the standard deviation. Asterisk indicates non-detectable levels of the product.

FIG. 5 Squalene/dehydrosqualene production by *E. coli* expressing SQS variants. The SQSs carried on pUC18m vectors were expressed in *E. coli* XL1-Blue harboring pJBEI-2997, and the transformants were cultured in TB medium at 37°C for 48 h. The amounts of (A) squalene and (B) dehydrosqualene were analyzed by HPLC. The bars indicate the average of three samples, and the error bars indicate the standard deviation. Asterisk indicates non-detectable levels of the product.

FIG. 6 HPLC analysis of the products of *E. coli* expressing truncated SQSs by HPLC. The plasmids belonging to the pUC-*tsqs* series were introduced into *E. coli*, and the production of squalene and dehydrosqualene by the culture was analyzed by HPLC. The chromatogram drawn in black indicates the absorbance at 207 nm, while gray indicates absorbance at 287 nm. 1, squalene; 2, dehydrosqualene; 3, unknown product. The peaks with an asterisk (at 10.3 min) were identified as quinone, based on the characteristic maximum absorbance at 207 nm and 275 nm.

Figure1

FIGURES

Figure2

Figure3

Figure4

Figure5

Figure6