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ORIGINAL RESEARCH PAPER

Heterologous biosynthesis of triterpenoid ambrein in engineered *Escherichia coli*

Di Ke · Qinggele Caiyin · Fanglong Zhao · Ting Liu · Wenyu Lu

Abstract

Objectives   To genetically engineer *Escherichia coli* for the heterologous biosynthesis of triterpenoid, ambrein, the main bioactive component of ambergris, by constituting a novel squalene-derived ambrein biosynthetic pathway in *E. coli*.

Results   The *ScERG9* gene encoding the squalene synthase (SS) was integrated into the *E. coli* genome to generate a squalene-producing strain that supplied the central precursor squalene for the formation of cyclic triterpenoids. The mutated squalene–hopene synthase (D377C SHC) and the tetraprenyl-β-curcumene cyclase (BmeTC) were co-expressed with SS to construct a novel ambrein biosynthetic pathway in *E. coli*. Ambrein was produced at 2.6 mg l^{-1}.

Conclusions   An *E. coli* chassis for ambrein production was constructed by combining the squalene synthesis module with the downstream cyclization module.

Keywords   Ambergris · Ambrein · *Escherichia coli* · Heterologous biosynthesis · Squalene · Triterpenoid

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Introduction

The tricyclic triterpenoid, ambrein ($C_{30}H_{52}O$), is the major component of ambergris which is secreted from sperm whale intestines (Ohloff et al. 1977). Ambergris is one of the most valued animal perfumes and fragrances (Kovatcheva et al. 2004). Oxidative degradation of ambrein is responsible for the scent of ambergris (Tanimoto and Oritani 1997). Furthermore, ambergris is widely used in Eastern folklore medicine to treat common colds (Taha et al. 1998) and migraine, and is also an aphrodisiac (Shen et al. 2007). Ambrein possesses antinociceptive (Taha 1992), anti-inflammatory (Shen et al. 2007), and aphrodisiac properties (Taha et al. 1995). Ambergris is either obtained via whaling or is washed ashore (Taha 1989). However, natural sources of ambergris are increasingly limited due to the banning of commercial whaling by many countries. Thus, metabolic engineering of

microorganisms has become a favorable alternative for ambrein production.

Although the endogenous ambrein pathway in sperm whales remains to be fully elucidated, Ueda et al. (2013) have developed a two-step enzymatic method for ambrein synthesis using the common triterpenoid precursor, squalene. The mutated squalene–hopene cyclase (D377C SHC) from *Alicyclobacillus acidocaldarius* catalyzes cyclization of squalene into the monocyclic 3-deoxyachilleol A (Sato and Hoshino 1999). The tetraprenyl-β-curcumene cyclase (BmeTC) from *Bacillus megaterium* is a multifunctional terpenoid cyclase that converts squalene into the bicyclic 8α-hydroxypolypoda-13,17,21-triene (Sato et al. 2011) and converts 3-deoxyachilleol A into the tricyclic ambrein (Ueda et al. 2013). However, heterologous production of ambrein in microbial systems has not been reported. Here, we selected *Escherichia coli*, as the host to realize the biosynthesis of ambrein (see Fig. 1).

The native methylerythritol-phosphate (MEP) pathway in *E. coli* can provide building blocks, including farnesyl pyrophosphate (FPP), for terpenoid production (Ajikumar et al. 2010). The first step in triterpenoid synthesis is condensation of two 15-carbon FPP molecules into 30-carbon intermediate squalene, which is catalyzed by squalene synthase (SS; Katabami et al. 2015). In the past decade, squalene production has been achieved by expressing SS from human (Katabami et al. 2015) or yeast (Li

et al. 2016) in *E. coli*. Here, we co-expressed SS, D377C SHC, and BmeTC in engineered *E. coli* to achieve ambrein production and established a novel ambrein biosynthetic pathway.

Materials and methods

Strains and medium

Escherichia coli MEP43 was derived from strain ZF43 (Li et al. 2015) by deleting the β-carotene synthesis module. The MEP43 strain was used as the parent for establishing the ambrein-producing strain. *E. coli* DH5α was utilized to amplify the plasmids. All the strains and plasmids used or constructed in this study are summarized in Supplementary Table 1. *E. coli* strains were cultivated at 30 or 37 °C in lysogeny broth supplemented with 100 mg ampicillin l^{-1} and 34 mg chloramphenicol l^{-1} as required. *E. coli* strains were also cultivated at 30 °C in liquid terrific broth (TB) supplemented with 20 g glycerol l^{-1}. Ampicillin (100 mg l^{-1}) was added as required.

Genome editing and plasmid construction for gene expression in *E. coli*

The *ScERG9* gene (Li et al. 2016) is the gene encoding the SS from *Saccharomyces cerevisiae*, the gene was PCR-amplified with the primers tERG9F and tERG9R

Fig. 1 Schematic of the ambrein biosynthetic pathway in *Escherichia coli*. *FPP* farnesyl pyrophosphate, *SS* squalene synthase, *D377C SHC* mutated squalene–hopene cyclase, *BmeTC* tetraprenyl-β-curcumene cyclase
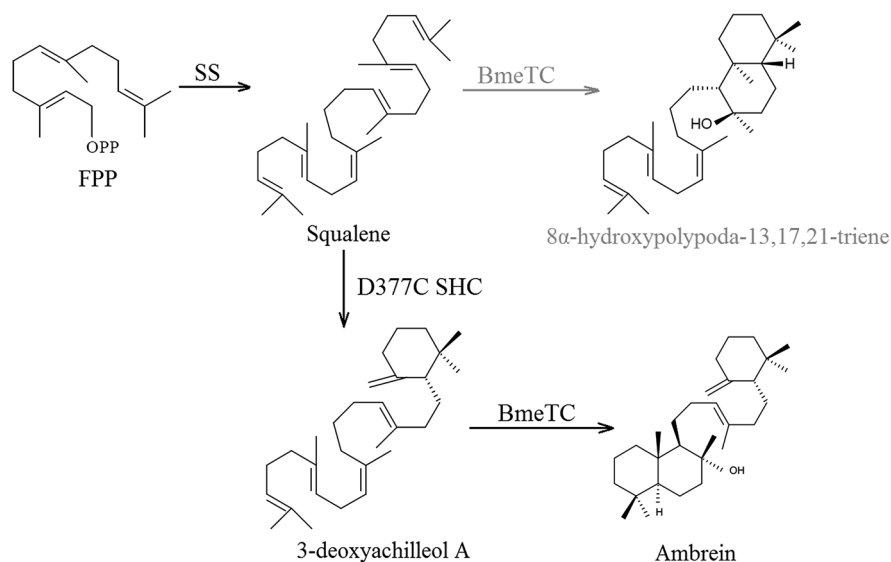
using the pSQ1 plasmid (Li et al. 2016) as template. The *ScERG9* operon, which is controlled by a strong promoter (J23119), was integrated into the *E. coli* genome at the *lacZ* locus through CRISPR-Cas9 genome editing (Li et al. 2015) to generate strain LK01. The 0.5 kb-homologous-arms, *lacZ*-up/*lacZ*-down, were amplified with the primers lacZuF, lacZuR, lacZdF, and lacZdR using the genomic DNA of *E. coli* MEP43 as template. The *ScERG9* operon, *lacZ*-up, and *lacZ*-down were assembled to be the donor DNA via fusion PCR. The gRNA targeting the *lacZ* locus was expressed in pGRB-*lacZ*, which was constructed according to the methods of Li et al. (2015) using the primers lacZE1D and lacZE1U. The donor DNA and pGRB-*lacZ* were introduced into *E. coli* MEP43 to integrate *ScERG9* into the genome, following the procedures described by Li et al. (2015).

The mutated squalene–hopene cyclase gene (*D377C SHC*) was synthesized with codon usage optimization. The tetraprenyl-β-curcumene cyclase gene (*BmeTC*) was amplified from the genomic DNA of *B. megaterium* (CGMCC 1.10466). The sequences for *D377C SHC* and *BmeTC* are shown in Supplementary Table 2. The *D377C SHC* gene fragment was amplified with the primers CASHC-SacIF/CASHC-BamHIR and cloned into p5C between the *Sac*I and *Bam*HI restriction sites to generate the pLs plasmid. The *BmeTC* gene fragment was amplified with the primers BmeTC-KpnIF/BmeTC-KpnIR and cloned into p5C at the *Kpn*I restriction site to generate pLb. The *D377C SHC-BmeTC* co-expression cassette was constructed via fusion PCR, in which the individual *D377C SHC* and *BmeTC* fragments were amplified with the primers CASHC-EcoRIF/SHCBR and BmeTCSF/CBmeTC-KpnIR, respectively. Then, the *D377C SHC-BmeTC* cassette was cloned into p5C between the *Eco*RI and *Kpn*I restriction sites to yield pLsb.

The oligonucleotide primers are listed in Supplementary Table 3.

Shake-flask cultures and metabolite extraction

Shake-flask cultures were performed in triplicate. *E. coli* recombinants were initially inoculated into 4 ml LB containing 100 mg ampicillin l^{-1} as required, and subsequently cultivated overnight at 30 °C and 220 rpm. The culture (0.3 ml) was then transferred into a 250 ml shake-flask containing 30 ml TB medium, and then incubated at 30 °C and 220 rpm. Upon reaching an OD_{600} of 0.6, gene expression was induced by adding 0.5 mM IPTG to the broth.

After 48 h of fermentation, metabolites were extracted using *n*-hexane following the method of Li et al. (2016). Samples were collected and then analyzed by GC–MS/HPLC.

Analytical methods

Putative squalene and ambrein products were identified using a GC–MS system which was the same as Li et al. (2016). Compound separation was achieved under the same operational conditions reported by Ueda et al. (2013). Squalene standard was purchased from Sigma-Aldrich. A gray ambergris sample (3.2 g; HuaZhuang Pha, China) was dissolved in *n*-hexane. The soluble fraction containing ambrein was analyzed by GC–MS and the total ion mass spectra were compared with the data from the NIST Database (Supplementary Fig. 1). The soluble fraction of ambergris in *n*-hexane was used directly as the ambrein standard for metabolite analysis.

Squalene produced by the recombinant strains was quantitatively analyzed by HPLC (Li et al. 2016), whereas ambrein was quantitatively analyzed by GC-FID. The ambrein yields were expressed as mean ± SD (bars) of three independent measurements (see Fig. 3). A GC system equipped with a Rxi-1HT capillary column (30 m × 0.32 mm × 0.25 μm) was used for analysis. Compound separation was achieved following the above mentioned heating process employed for GC–MS analysis. Epifriedelanol ($C_{30}H_{52}O$) was added to the samples as the internal standard to quantify ambrein yield (Supplementary Fig. 2). The relative response factor (RRF) was set to 0.99 (calculations are described in detail as a Supplementary method).

Results

Construction of ambrein biosynthetic pathway in *E. coli*

Following the presumptive ambrein synthetic pathway (Fig. 1), three reactions sequentially catalyzed by SS, D377C SHC, and BmeTC were incorporated in *E. coli*

to achieve ambrein production. Firstly, the *ScERG9* operon under the control of the constitutive J23119 promoter was assembled into the *E. coli* MEP43 genome to generate the transformed LK01 strain. Shake-flask fermentation of LK01 led to intracellular accumulation of squalene at 18.4 mg l^{-1}. Afterwards, the recombinant plasmids pLs, pLb, and pLsb were introduced into LK01 strains to generate the strains LKs, LKb, and LKsb, respectively. The LKsb strain, which harbors the genes for the complete ambrein biosynthetic pathway, was cultured to evaluate ambrein production, LKs and LKb were taken as controls.

Identification of ambrein produced in *E. coli*

The strains LK01, LKs, LKb, and LKsb were cultivated according to the methods described above, and the corresponding intracellular metabolites were analyzed by GC–MS. Based on the relative retention times (Rt) and mass spectral comparisons with an external ambrein standard, the peak at Rt 19.1 min (Fig. 2a) corresponded to the desired ambrein product. Thus, strain LKsb produces ambrein in detectable levels. Ambrein production was not detected in strain LKs or LKb, but possible 3-deoxy-achilleol A and 8α-hydroxypolypoda-13,17,21-triene were produced in LKs and LKb, respectively (Fig. 2a). Thus, heterologous biosynthesis of ambrein was successfully achieved via the co-expression of SS, D377C SHC, and BmeTC in *E. coli*.

Effect of cultivation temperature on ambrein production

Effective control of cultivation temperature can help increase the ambrein yield in the recombinant strain LKsb. Temperatures ranging from 25 to 37 °C were tested to determine the optimum temperature for ambrein production. As shown in Fig. 3, 30 and 34 °C were optimal for ambrein biosynthesis in LKsb, corresponding to yields of ∼ 2.5 mg l^{-1} which were 1.4- and 2.5-times higher than the yields obtained at 25 °C (1.8 mg l^{-1}) and 37 °C (1 mg l^{-1}), respectively.

Discussion

The triterpenoid ambrein, a crucial bioactive component of ambergris, is a valuable ingredient of perfumes and a potential medical feedstock. In this study, we constructed a novel ambrein biosynthetic pathway in genetically-engineered *E. coli* and provided an ambrein-producing system. To achieve this goal, an *E. coli* chassis (LK01) was constructed to supply precursor squalene, a key intermediate of the triterpenoid biosynthesis. The complete ambrein biosynthetic pathway was established by co-expression of D377C SHC and BmeTC with SS in the *E. coli* strain LKsb. Ambrein was produced at a yield of 2.55 mg l^{-1} in LKsb at the optimum temperature (Fig. 3). Thus, an alternative ambrein production platform was created in *E. coli*. To our knowledge, our study is the first to report ambrein production in recombinant microorganisms.

The established LKsb chassis can also be further engineered to improve ambrein yield by increasing the supply of precursors. For instance, introduction of the heterologous mevalonate pathway (Zhang et al. 2014) and modification of central metabolic modules (Jing et al. 2013) are two generally employed methods to enhance terpenoid productions in *E. coli*. The LKsb strain also generated an undesired by-product (peak 3 in Fig. 2a), which was likely caused by nonspecific catalysis of BmeTC (Fig. 1), so further engineering of BmeTC to improve its catalytic specificity might be useful for improving ambrein production.

To date, only a few triterpenoids have been developed for biosynthesis in engineered *E. coli*. The favorable formation of squalene and 2,3-oxidosqualene (Li et al. 2016) highlights the potential of *E. coli* as a host for triterpenoid production, and the present study also expanded the list of microbially derived triterpenoids. Our strategy used for constructing the ambrein synthetic pathway can also be employed to some other potential hosts such as *S. cerevisiae*. Thus, our findings provide new possibilities to achieve industrial-scale production of ambrein.

Fig. 2 Identification of ambrein produced by LKsb. **a** GC profiles of (*A*) squalene standard, metabolite extracts from the recombinant *Escherichia coli* strains (*B*) LK01, (*C*) LKs, harboring the pLKs plasmid, (*D*) LKb, harboring the pLKb plasmid, (*E*) LKsb, harboring the pLKsb plasmid and (*F*) ambrein standard, **b** mass spectrum of squalene in sample LK01, **c** mass spectrum of squalene standard, **d** mass spectrum of ambrein in sample LKsb, and **e** mass spectrum of ambrein standard. (*1*) squalene, (*2*) possible 3-deoxyachilleol A, (*3*) possible 8α-hydroxypolypoda-13,17,21-triene, and (*4*) ambrein (mass spectra of *2* and *3* not shown)
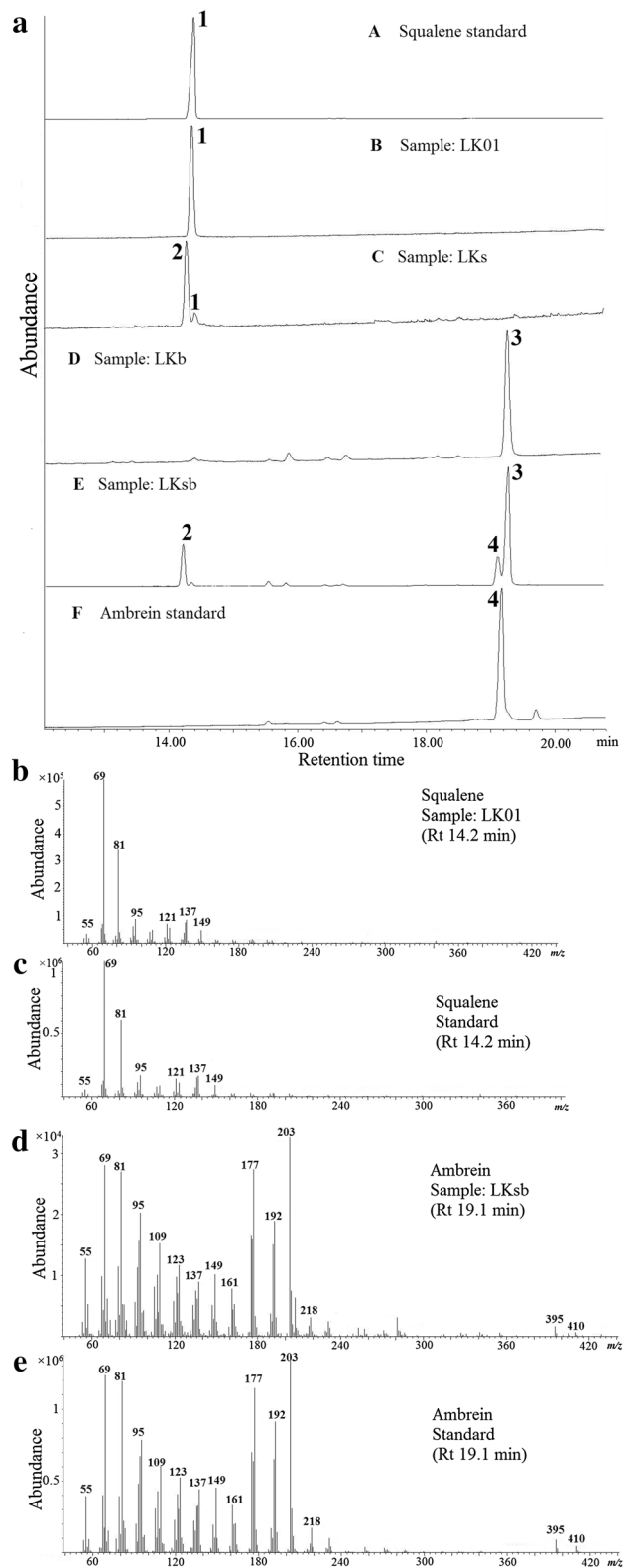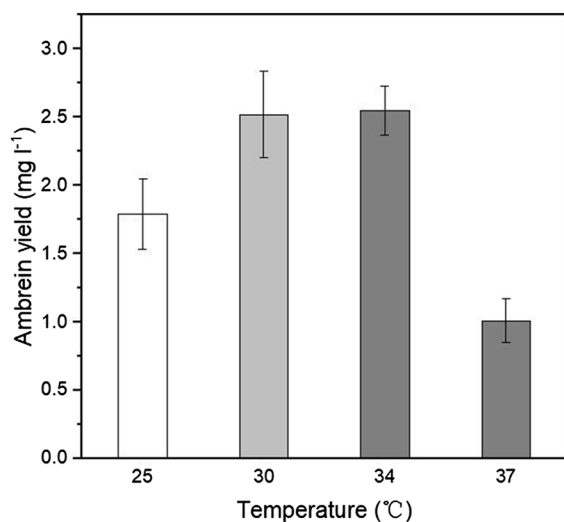
Fig. 3 Ambrein yields in LKsb at different cultivation temperatures

Supporting information Supplementary method—effective prediction of relative response factor RRF for epifriedelanol/ambrein in GC-FID analysis via a carbon number-based approach.

Supplementary Table 1—strains and plasmids used.

Supplementary Table 2—DNA sequences of exogenous genes used for the construction of the ambrein biosynthetic pathway in *Escherichia coli*.

Supplementary Table 3—oligonucleotide primers used in this study.

Supplementary Fig. 1—identification of the authentic ambergris containing ambrein via GC–MS.

Supplementary Fig. 2—quantitative analysis of ambrein production via GC-FID.

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