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Biosynthetic machinery for C_{25},C_{25}-diether archaeal lipids from the hyperthermophilic archaeon *Aeropyrum pernix*

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ABSTRACT

Archaea that thrive in harsh environments usually produce membrane lipids with specific structures such as bipolar tetraether lipids. Only a few genera of archaea, which are hyperthermophiles or halophiles, are known to utilize diether lipids with extended, C_{25} isoprenoid hydrocarbon chains. In the present study, we identify two prenyltransferases and a prenyl reductase responsible for the biosynthesis of C_{25},C_{25}-diether lipids in the hyperthermophilic archaeon *Aeropyrum pernix*. These enzymes are more specific to C_{25} isoprenoid chains than to C_{20} chains, which are used for the biosynthesis of ordinary C_{20},C_{20}-diether archaeal lipids. The recombinant expression of these enzymes with two known archaeal enzymes allows the production of C_{25},C_{25}-diether archaeal lipids in the cells of *Escherichia coli*.

1. Introduction

Archaeal membrane lipids are easily identified by unique structures that typically involve two linear, fully saturated C_{20} isoprenoid (phytanyl) chains linked to a glycerol moiety via ether bonding [1–4]. They form a membrane that maintains low levels of ion and small-molecule leakage across a wide range of temperatures [5], which supposedly allows archaea to thrive in harsh environments such as acidic hot springs and basic salt lakes. Some archaea, mostly thermophiles but also mesophiles such as some methanogens, produce specific tetraether lipids that are believed to form by dimerization of the typical diether lipids depicted above. These dipolar (or two-headed) tetraether lipids, which have C_{40} isoprenoid (biphytanyl) chains that sometimes contain 5- or 6-membered internal ring structures, produce a strong effect to reduce leakage through the membranes containing them [6], suggesting they help archaea adapt to extreme conditions. Another type of specific lipid produced by extremophilic archaea, such as the hyperthermophilic archaea *Aeropyrum pernix* and the halophilic archaea of a few genera, includes “extended” diether lipids that have C_{25} isoprenoid chains, which are longer than the usual C_{20} chains. The C_{25},C_{25}-diether lipids isolated from *A. pernix* are known to form liposomes that are characterized by low leakage even at temperatures approaching 100 °C [7].

The biosynthetic pathways of ordinary C_{20},C_{20}-diether archaeal lipids are already known, and almost all the enzymes involved in these pathways have been identified from several examples of thermophilic or mesophilic archaea (Fig. 1) [1,3]. The precursor for the glycerol moiety of the lipids, *sn*-glycerol 1-phosphate (G1P), is formed by G1P dehydrogenase from dihydroxyacetone phosphate. Two C_{20} isoprenoid chains with double bonds are transferred from geranylgeranyl pyrophosphate (GGPP), which is biosynthesized from dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP), to G1P via the actions of two prenyltransferases: geranylgeranylglyceryl phosphate (GGGP) synthase and digeranylgeranylglyceryl phosphate (DGGGP) synthase. All double bonds in the isoprenoid chains are then reduced by geranylgeranyl reductase (GGR). We have succeeded in reconstructing this pathway in *E. coli* cells by introducing the genes of these enzymes from a mesophilic methanogenic archaeon, *Methanosarcina acetivorans* [8]. These cells produce a C_{20},C_{20}-diether lipid, diphytanylglyceryl phosphoglycerol (or archaetidylglycerol), along with its

Abbreviations: DGFGP, digeranylfarnesylglyceryl phosphate; DGGGP, digeranylgeranylglyceryl phosphate; DGFGP-glycerol, digeranylfarnesylglyceryl phosphoglycerol; DMAPP, dimethylallyl pyrophosphate; EIC, Extracted ion chromatogram; G1P, *sn*-glycerol 1-phosphate; GFGP, geranylfarnesylglyceryl phosphate; GGGP, geranylgeranylglyceryl phosphate; GFPP, geranylfarnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; GGR, geranylgeranyl reductase; IPP, isopentenyl pyrophosphate.

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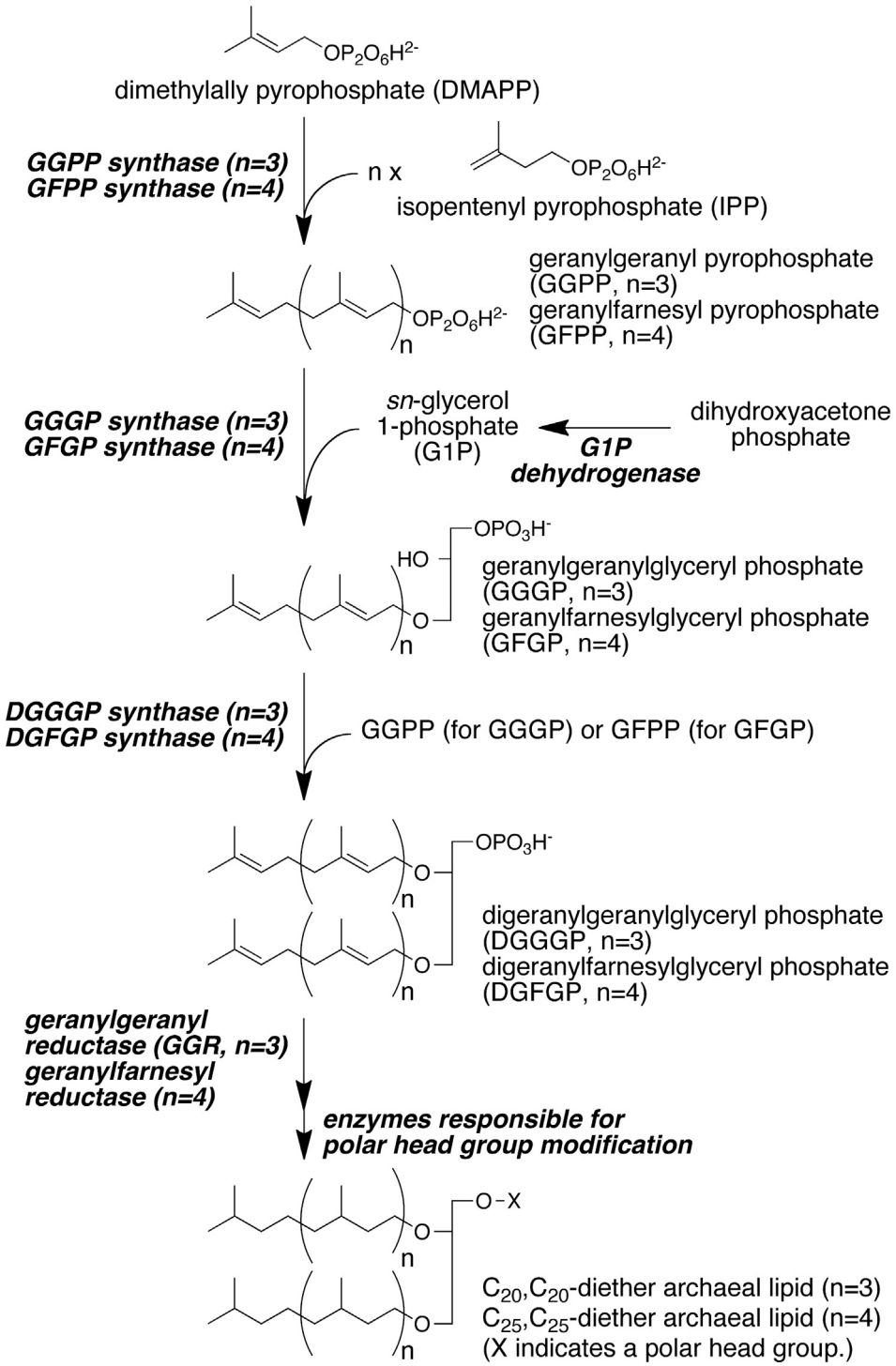

Fig. 1. The biosynthetic pathways of C_{20},C_{20}- and C_{25},C_{25}-diether archaeal lipids.

unsaturated precursors. Additionally, some archaeal enzymes that catalyze the modification of the polar head groups of membrane lipids have also been discovered. In contrast, only geranylfarnesyl pyrophosphate (GFPP, or farnesylgeranyl pyrophosphate) synthase has been identified as the enzyme responsible for the biosynthesis of extremophile-specific extended diether lipids [9,10].

In this study, we identified two new prenyltransferases and one prenyl reductase from *A. pernix*, which are responsible for the biosynthesis of C_{25},C_{25}-diether lipids (Fig. 1). The specificities of these enzymes toward biosynthetic precursors with C_{25} isoprenoid chains are in sharp contrast with those of the enzymes involved in C_{20},C_{20}-diether lipid biosynthesis, and this enabled us to synthesize a C_{25},C_{25}-diether lipid, disesterterpanylglyceryl phosphoglycerol (or C_{25},C_{25}-archaetidylglycerol), in *E. coli* cells by introducing five archaeal genes.

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2. Materials and methods

2.1. General procedures

Restriction enzyme digestions, transformations, and other standard molecular biological techniques were carried out, as described by Sambrook et al. [11].

2.2. Cultivation of Aeropyrum pernix

A. pernix was provided by the RIKEN BRC through the Natural Bio-Resource Project of the MEXT, Japan, and was cultured in a JXT medium supplemented with 4 mM $Na_2S_2O_3 \cdot 5H_2O$ at 85 °C.

2.3. Recombinant expression and purification of archaeal enzymes

The genomic DNA of *A. pernix* was extracted from cells using a DNA extraction kit, Geno Plus™ Mini (VIOGENE, USA). Each of the *A. pernix* genes, *APE_0621* and *APE_0159*, hypothetically encoding geranylfarnesylglyceryl phosphate (GFGP) synthase and digeranylfarnesylglyceryl phosphate (DGFGP) synthase, respectively, was amplified using the primers shown in Table 1, the genome of *A. pernix* as a template, and KOD plus DNA polymerase (TOYOBO, Japan). Only the *APE_0621* gene was re-amplified via nested PCR, for which the forward primer was changed, using the first amplified fragment as a template. The amplified genes were digested by restriction enzymes (*Eco*RI and *Not*I for *APE_0621*, and *Nde*I and *Bam*HI for *APE_0159*) and then ligated into either a pET48b(+) or a pET15b vector cut with the same restriction enzymes to construct pET48b-APE_0621 and pET15b-APE_0159, respectively.

E. coli BL21(DE3) transformed with each plasmid was cultivated at 37 °C in 1 L LB medium supplemented with 100 mg/L ampicillin. When the culture reached an optical density of 0.5, then 1.0 mM IPTG was added for induction. After an additional 24 h of incubation, the cells were harvested and disrupted by sonication in HisTrap binding buffer that contained 20 mM potassium phosphate, pH 7.4, 0.5 M NaCl, and 10 mM imidazole, prepared following the manufacturer's instructions. The homogenate was centrifuged at 4000 g for 30 min, and the supernatant was recovered as a crude extract. The crude extract was heated at 60 °C for 30 min, and the denatured proteins were removed by centrifugation at 6000 g for 30 min. Note that in the case of the purification of APE_0159, CHAPS at a final concentration of 0.2% was added to the crude extract to solubilize the recombinant protein prior to heat treatment. The supernatant fraction was loaded into a HisTrap crude FF column (GE Healthcare, USA), which had been equilibrated with the HisTrap binding buffer. The column was washed with HisTrap wash buffer containing 20 mM potassium phosphate, pH 7.4, 0.5 M NaCl, and either 80 or 40 mM imidazole for the purification of APE_0621 and APE_0159, respectively. Then, the recombinant proteins were eluted with a HisTrap elution buffer containing 20 mM potassium phosphate, pH 7.4, 0.5 M NaCl, and 500 mM imidazole, to be used for characterization. The level of purification was determined by SDS-PAGE.

Purified *A. pernix* GFPP synthase, *Sulfolobus solfataricus* GGGP synthase, and *S. solfataricus* DGGGP synthase were prepared as described elsewhere [12,13].

2.4. Synthesis of radiolabeled substrates for radio-TLC assay

Radiolabeled substrates for enzyme assay, [^{14}C]GFPP, [^{14}C]GGPP, and [^{14}C]GGGP, were prepared as described elsewhere using *A. pernix* GFPP synthase, *Sulfolobus acidocaldarius* GGPP synthase, and both *S. acidocaldarius* GGPP synthase and *S. solfararicus* GGGP synthase, respectively [12,13]. The only exceptions were that DMAPP was used as the allylic substrate for each reaction and that NaCl was not added when the reaction products were extracted with 1-butanol. For the synthesis of [^{14}C]GFGP, a reaction mixture containing, in a final volume of 200 μL, 1.0 nmol [1-^{14}C]IPP (2.04 GBq/mmol, American Radiolabeled Chemicals, Inc., USA), 1.0 nmol DMAPP, 0.5 μmol $MgCl_2$, 10 nmol α-glycerophosphate (racemic mixture), 10 μmol sodium phosphate buffer, pH 7.2, and suitable amounts of *A. pernix* GFPP synthase and purified APE_0621, was incubated at 60 °C for 30 min. The product of the reaction was extracted with 1-butanol saturated with water, and used as [^{14}C]GFGP. The concentrations of the radiolabeled substrates were determined by measuring radioactivity with an LSC-5100 liquid scintillation counter (ALOKA, Japan).

2.5. Radio-TLC assay of prenyltransferases from A. pernix

For the assay of the prenyltransferase activity of APE_0621, ^{14}C-labeled prenyl diphosphate solved in 1-butanol, ~10 pmol [^{14}C]GFPP or ~14 pmol [^{14}C]GGPP (both corresponding to 5000 dpm), was dried with N_2. Then, a 200 μL solution containing 0.5 μmol $MgCl_2$, 10 nmol α-glycerophosphate (racemic mixture), 10 μmol sodium phosphate buffer, pH 7.2, and suitable amounts of the enzyme was added to dissolve the residue. Purified *S. solfataricus* GGGPS was also used instead of APE_0621 to compare substrate specificities.

Table 1
PCR primers used for plasmid construction.

Genes	Primer sequences	Plasmids constructed
APE_0621	1st forward: 5′-AACCTTGTGAGCGACGCCTACTTCG-3′ 2nd forward: 5′-CGATGGAATTCTATGGCGGTGAAGAGGAGGAGGC-3′ reverse: 5′-CATGTGCGGCCGCGAAGGCTAGGCGCTCTTGAAGGCC-3′	pET48b-APE_0621
APE_0159	forward: 5′-GATGACATATGAAGGCTGCTATCGAGATAACTAGGC-3′ reverse: 5′-ACTGCGGATCCTTAAATCCCGAGGGTACCCAGG-3′	pET15b-APE_0159
APE_0621	forward: 5′-TTTTTTTGGGCTAGCGAATTCAGGAGAATATAAATGGCG-GTGAAGAGGAGG-3′ reverse: 5′-TTTTTATTTGAGCTCTAATTACTAGGCGCTCTTGAAGGC-C-3′	pBAD-C_{25}ALB2
APE_1764	forward: 5′-TTTTTTTGGGCTAGCGAATTCAAGGAGTAATATATGAAG-TGGGATAGACTGTTTG-3′ reverse: 5′-CATTTATATTCTCCTGAATTACTACTTCTCCCTCTCCACA-ATATAGTCTAGAAG-3′	pBAD-C_{25}ALB3
APE_0159	forward: 5′-TTTTTTTGGGCTAGCGAATTCAAGAAGATATAAATGAAG-GCTGCTATCGAGATAACTAGG-3′ reverse: 5′-CATATATTACTCCTTGAATTATTAAATCCCGAGGGTACC-CAG-3′	pBAD-C_{25}ALB4
APE_1952	forward: 5′-TTGGGCTAGCGAATTCAGGAGAATATAAATGGCTGTGG-AGTATAAGTATGATGTCG-3′ reverse: 5′-ATATCTTCTTGAATTATTACCAATCGATGCCGAGACG-3′	pBAD-C_{25}ALB5
saci_0986	forward: 5′-TTGGGCTAGCGAATTCAGGAGAATATAAATGAAGGAAC-TTAAATATGACG-3′ reverse: 5′-ATATCTTCTTGAATTACTAACTTAAACTTTTGTTAAACTC-TG-3′	pBAD-C_{25}ALB4-SaGGR

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To assay the prenyltransferase activity of APE_0159, ~10 pmol [^{14}C]GFPP or ~14 pmol [^{14}C]GGPP was used as a donor substrate and mixed with ~10 pmol [^{14}C]GFGP or ~14 pmol [^{14}C]GGGP that served as the acceptor substrate. The radiolabeled substrates solved in 1-butanol were dried with N_2, and then a 200 μL solution containing 1.0 μmol $MgCl_2$, 3.2 μmol CHAPS, 20 μmol 3-(*N*-morpholino) propanesulfonic acid (MOPS)-NaOH buffer, pH 7.0, and suitable amounts of the enzyme was added to dissolve the residue. Purified *S. solfataricus* DGGGP synthase was also used instead of APE_0159 to compare substrate specificities.

These mixtures were incubated at 60 °C for 30 min, and the products were extracted with 1-butanol saturated with water. Then they were treated with acid phosphatase according to a method established by Fujii et al. [14], and their hydrolysates were extracted with *n*-pentane and analyzed by reverse-phase TLC using a pre-coated plate RP-18 (Merck Millipore, Germany) developed with acetone/H_2O (19:1). The distribution of radioactivity was detected using an FLA7000 multifunctional scanner (GE Healthcare).

2.6. Production of C_{25},C_{25}-diether lipids in *E. coli*

Plasmids for the expression of multiple archaeal genes were constructed using an In-Fusion Advantage PCR cloning kit (Takara, Japan), which is similar to our previous construction of plasmids that allow the production of C_{20},C_{20}-diether lipids in *E. coli* [8,15]. The *APE_0621* gene was amplified using the primers shown in Table 1, and the amplified DNA fragment was inserted into *Eco*RI-digested pBAD-MA3686 [15], which contains the gene of G1P dehydrogenase from *M. acetivorans*, to construct pBAD-C_{25}ALB2. In a similar manner, the amplified fragment of *APE_1764*, which encodes *A. pernix* GFPP synthase, was inserted into *Eco*RI-digested pBAD-C_{25}ALB2 to construct pBAD-C_{25}ALB3. Then the *APE_0159* fragment was inserted into *Eco*RI-digested pBAD-C_{25}ALB3 to construct pBAD-C_{25}ALB4, which contains 4 archaeal genes that are rated sufficient for the biosynthesis of DGFGP. Finally, the *APE_1952* gene, which encodes a hypothetical prenyl reductase, was amplified from *A. pernix* genomic DNA, and the amplified fragment was inserted into *Eco*RI-digested pBAD-C_{25}ALB4 to construct pBAD-C_{25}ALB5. In a similar manner, an amplified fragment containing a gene of GGR from *S. acidocaridarius* (*saci_0986*) [16] was inserted into pBAD-C_{25}ALB4 to construct pBAD-C_{25}ALB4-SaGGR.

E. coli TOP10 transformed with pBAD-C_{25}ALB4, pBAD-C_{25}ALB5, or pBAD-C_{25}ALB4-SaGGR was statically cultivated at 37 °C in 1 L LB medium supplemented with 100 mg/L ampicillin and 0.02% L-arabinose. After cultivation, cells were harvested and then dissolved using 10 mL of 1-butanol/75 mM ammonium water/ethanol (4:5:11) per 1 g of wet cells. The mixture was heated to 70 °C and shaken vigorously for 1 min. After cooling to room temperature, the mixture was centrifuged at 1000 g for 15 min. The supernatant was recovered and dried with N_2. The residue was dissolved with 3.6 mL of 1-butanol/methanol/0.5 M acetate buffer, pH 4.6, (3:10:5) per 1 g of wet cells. Lipids in the mixture were extracted twice with 3 mL *n*-pentane and dried with N_2. The residue was dissolved with 1.0 mL of methanol/2-propanol (1:1) per 1 g of wet cells.

LC-ESI-MS analysis was performed with an Esquire 3000 ion trap system (Bruker Daltonics, USA) equipped with an Agilent 1100 Series HPLC system (Agilent Technologies, USA). The parameters for MS were the same as those described in our previous report [15]. Ten μL of each lipid sample was injected into a COSMOSIL 5C_{18}-AR-II packed column (2.0 × 150 mm, Nacalai, Japan) and eluted at a flow rate of 0.2 mL/min with eluent A: methanol/10 mg L^{-1} sodium acetate (9:1) and eluent B: 2-propanol. The procedure used 100% A for 0-20 min in a linear gradient with 0–80% B for 20-50 min, and then 100% of B for 50-70 min.

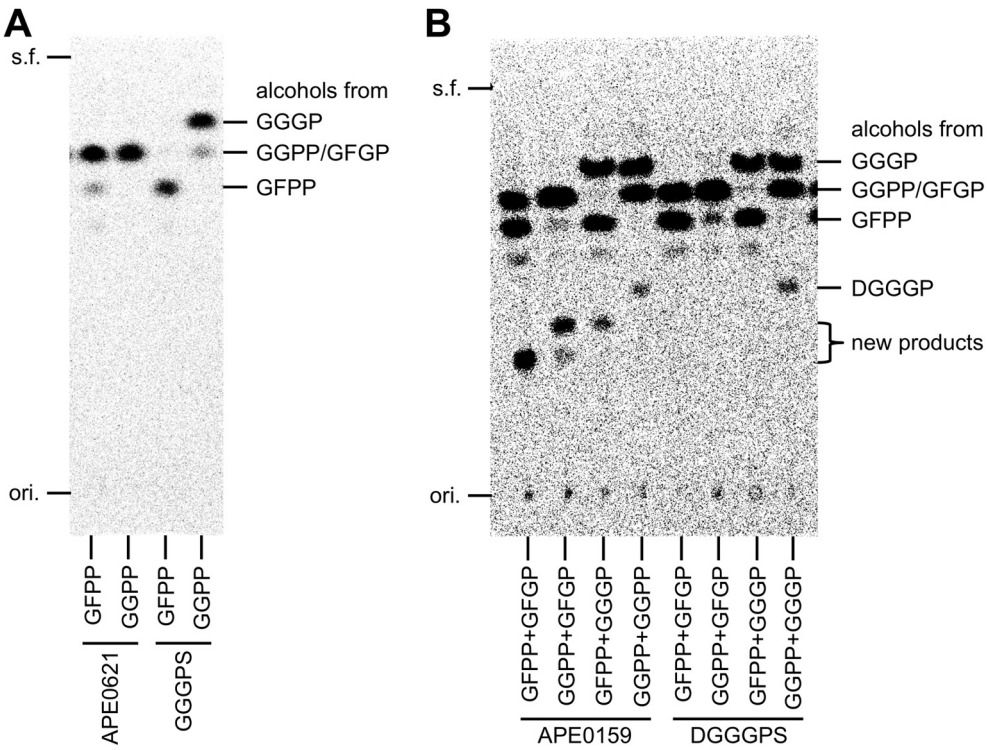

Fig. 2. Radio-TLC analysis of the products of archaeal prenyltransferases. (A) Analysis of the products from the reaction of [^{14}C]GFPP or [^{14}C]GGPP against G1P catalyzed by APE_0621 or *S. solfataricus* GGGP synthase. The products extracted with 1-butanol were hydrolyzed with acid phosphatase and analyzed by reversed-phase TLC after pentane extraction. (B) Analysis of the hydrolyzed products from the reaction between the prenyl donor substrate, [^{14}C]GFPP or [^{14}C]GGPP, and the acceptor substrate, [^{14}C]GFGP or [^{14}C]GGGP, catalyzed by APE_0159 or *S. solfataricus* DGGGP synthase. s.f., solvent front; ori., origin.

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3. Results and discussion

3.1. Identification of GFGP synthase and DGFGP synthase from A. pernix

The genes of the sole homologs of GGGP synthase and DGGGP synthase, APE_0621 and APE_0159, respectively, were cloned from *A. pernix*, and the recombinant expression of each homolog was performed in *E. coli*. The recombinant proteins were purified via heat treatment and affinity column chromatography. First, the GGGP synthase homolog was reacted with [^{14}C]GFPP and G1P. Radio-TLC analysis of the reaction product was performed following phosphatase treatment. As shown in Fig. 2A, a new spot with a R_f of 0.77 emerged along with a fading of the spot from the alcohol from GFPP, suggesting almost complete conversion of GFPP into GFGP. In contrast, the prenyltransferase reaction was not observed when [^{14}C]GFPP was changed to a comparable amount of [^{14}C]GGPP. Therefore, the *A. pernix* enzyme is thought to be GFGP synthase. Contrary to *A. pernix* GFGP synthase, GGGP synthase from *S. solfataricus* showed a strict preference toward GGPP and would not accept GFPP.

Next, the DGGGP synthase homolog from *A. pernix* was reacted with [^{14}C]GFPP and [^{14}C]GFGP. A new radioactive spot with a R_f value of 0.33 emerged in the TLC analysis of hydrolyzed products, suggesting the formation of DGFGP. When [^{14}C]GGPP was used instead of [^{14}C]GFPP, the amount of the reaction product estimated from the density of the radioactive spot (R_f of 0.42) was slightly lower but still comparable to that from the reaction with [^{14}C]GFPP. In contrast, the product from the reaction with [^{14}C]GGGP was a much lower amount, regardless of whether [^{14}C]GFPP or [^{14}C]GGPP was used as counter substrate. These results indicate that the chain length of the prenyl acceptor substrate is important for the enzyme, while that of the donor substrate does not significantly affect the activity. Given the fact that the best substrates are GFPP and GFGP,

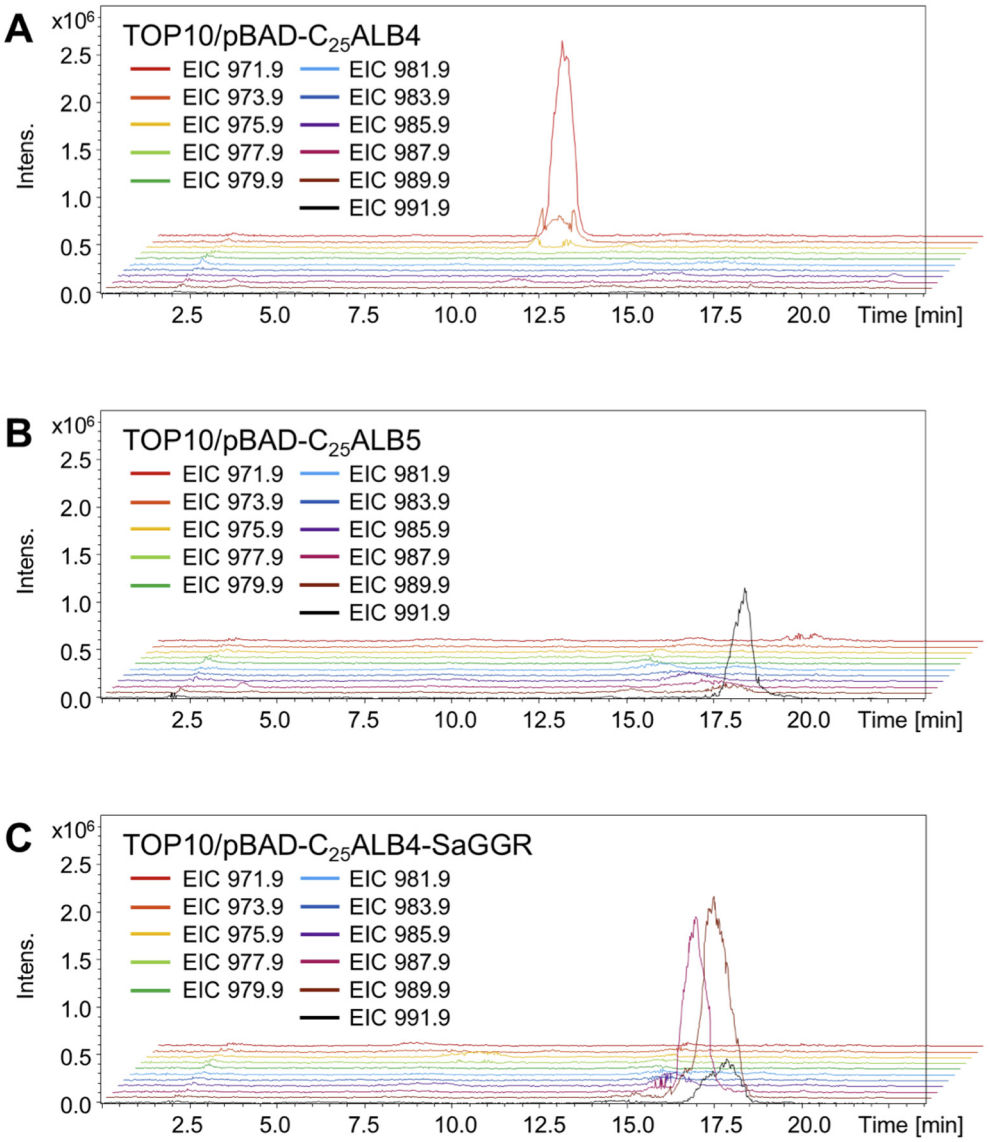

Fig. 3. LC-ESI-MS analysis of lipids extracted from *E. coli* cells. Shown are extracted ion chromatograms (EICs) from the analysis of the lipid samples from *E. coli* harboring pBAD-C_{25}ALB4 (A), pBAD-C_{25}ALB5 (B), or pBAD-C_{25}ALB4-SaGGR (C). Red, ion with *m/z* of 971.9 ± 1.0, which corresponds to [DGFGP-glycerol+2Na]$^+$; orange, *m/z* of 973.9 ± 1.0; yellow, *m/z* of 975.9 ± 1.0; pale green, *m/z* of 977.9 ± 1.0; green, *m/z* of 979.9 ± 1.0; cyan, *m/z* of 981.9 ± 1.0; dark blue, *m/z* of 983.9 ± 1.0; purple, *m/z* of 985.9 ± 1.0; magenta, *m/z* of 987.9 ± 1.0; brown, *m/z* of 989.9 ± 1.0; black, *m/z* of 991.9 ± 1.0, which is derived from the fully reduced product, disesterterpanylglyceryl phosphoglycerol. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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the *A. pernix* enzyme is thought to be DGFGP synthase. By contrast, *S. solfataricus* DGGGP synthase catalyzed the formation of DGGGP from GGPP and GGGP, but would not accept the substrates with a C_{25} chain.

The substrate preferences of the prenyltransferases from *A. pernix*, GFGP synthase and DGFGP synthase, demonstrated that they are responsible for the biosynthesis of C_{25},C_{25}-diether lipids in the archaeon, as well as previously identified GFPP synthase [10]. These results suggest that other enzymes catalyzing the downstream reactions of the biosynthesis of C_{25},C_{25}-diether lipids in *A. pernix*, such as the reductase for geranylfarnesyl groups, might also be specific toward intermediates with C_{25} isoprenoid chains.

3.2. Production of C_{25},C_{25}-diether lipids in *E. coli* and identification of geranylfarnesyl reductase from *A. pernix*

To identify the prenyl reductase responsible for C_{25},C_{25}-diether lipid biosynthesis from *A. pernix*, we constructed an *E. coli* strain that can produce DGFGP-based polar lipids. With a protocol similar to that used for the construction of a strain that holds *M. acerivorans* genes and thus produces digeranylgeranylglyceryl phosphoglycerol [15], we introduced the genes of *M. acetivorans* G1P dehydrogenase, *A. pernix* GFPP synthase, *A. pernix* GFGP synthase, and *A. pernix* DGFGP synthase in the same plasmid to construct pBAD-C_{25}ALB4. As shown in Fig. 3A, a positive ion with an *m/z* of 971.9 was eluted at ~12 min in the LC-ESI-MS analysis of the lipid extracted from *E. coli* cells harboring the plasmid, which suggested the formation of digeranylfarnesylglyceryl phosphoglycerol (DGFGP-glycerol) because the *m/z* value corresponded to $[\text{DGFGP-glycerol}+2\text{Na}]^+$. The glycerol modification of DGFGP is likely catalyzed by the endogenous enzymes of *E. coli*. Then, we added the gene of a GGR homolog of *A. pernix*, APE_1952, into the pBAD-C_{25}ALB4 to construct pBAD-C_{25}ALB5. We expected that the GGR homolog would be the prenyl reductase responsible for lipid biosynthesis because it has a homology toward known archaeal lipid-biosynthetic GGRs that is higher than that of other homologs such as APE_0759 and APE_1622. The ion peak derived from DGFGP glycerol was diminished in the analysis of lipid extracted from *E. coli* transformed with pBAD-C_{25}ALB5, whereas an ion with an *m/z* value larger by 20 units was eluted at ~18 min (Fig. 3B). This suggests the formation of disesterterpanylglyceryl phosphoglycerol, which is synthesized via the reduction of all 10 double bonds in DGFGP-glycerol. In contrast, when the gene of *S. acidocaldarius* GGR was introduced into pBAD-C_{25}ALB4 to construct pBAD-C_{25}ALB4-SaGGR, the lipid from the *E. coli* harboring the plasmid contained a smaller ratio of the fully reduced lipid, and the major components of DGFGP-glycerol-derived lipids were those that retained one or two double bonds (Fig. 3C). This suggests that the full reduction of C_{25} geranylfarnesyl groups is difficult for *S. acidocaldarius* GGR, which is supposedly optimized for C_{20} geranylgeranyl groups. By contrast, the partially reduced products were nearly absent in *E. coli* harboring pBAD-C_{25}ALB5, which suggests that the prenyl reductase from *A. pernix* is dedicated to reduction of geranylfarnesyl groups. Therefore, we propose that the enzyme is geranylfarnesyl reductase.

The present work identified three enzymes involved in C_{25},C_{25}-diether lipid biosynthesis in *A. pernix* and succeeded in constructing *E. coli* strains that produced a fully reduced C_{25},C_{25}-diether lipid, disesterterpanylglyceryl phosphoglycerol. The hydrophobic moiety structure of the lipid is the same as that of membrane lipids that are produced in *A. pernix*, although the major lipids in the archaeon are disesterterpanylglyceryl phosphoinositol and disesterterpanylglyceryl phospho(glucosyl)inositol [17]. However, this report describes the recombinant production of an extremophile-specific lipid in bacterial cells. It seems quite intriguing to speculate as to whether the production of this unique extended lipid will have any physiological effect on the bacterial hosts.

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Transparency document

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