Title

Construction of an artificial biosynthetic pathway for hyperextended archaeal membrane lipids in the bacterium Escherichia coli

Authors

Ryo Yoshida and Hisashi Hemmi*

Affiliations

Department of Applied Biosciences, Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, Aichi 460-8601

*Corresponding author

Hisashi Hemmi

E-mail: hhemmi@agr.nagoya-u.ac.jp

© The Author(s) 2020. Published by Oxford University Press. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted reuse, distribution, and reproduction in any medium, provided the original work is properly cited.
Abstract

Archaea produce unique membrane lipids, which possess two fully saturated isoprenoid chains linked to the glycerol moiety via ether bonds. The isoprenoid chain length of archaean membrane lipids is believed to be important for some archaee to thrive in extreme environments because the hyperthermophilic archaean *Aeropyrum pernix* and some halophilic archaee synthesize extended C25,C25-archaean diether-type membrane lipids, which have isoprenoid chains that are longer than those of typical C20,C20-diether lipids. Natural archaean diether lipids possessing longer C30 or C35 isoprenoid chains, however, have yet to be isolated. In the present study, we attempted to synthesize such hyperextended archaean membrane lipids. We investigated the substrate preference of the enzyme *sn*-2,3-(digeranylflavesyl)glycerol-1-phosphate synthase from *Aeropyrum pernix*, which catalyzes the transfer of the second C25 isoprenoid chain to the glycerol moiety in the biosynthetic pathway of C25,C25-archaean membrane lipids. The enzyme was shown to accept *sn*-3-hexaprenylglycerol-1-phosphate, which has a C30 isoprenoid chain, as a prenyl acceptor substrate to synthesize *sn*-2-geranylflavesyl-3-hexaprenylglycerol-1-phosphate, a supposed precursor for hyperextended C25,C30-archaean membrane lipids. Furthermore, we constructed an artificial biosynthetic pathway by introducing 4 archaean genes and 1 gene from *Bacillus subtilis* in the cells of *Escherichia coli*, which enabled the *E. coli* strain to...
produce hyperextended C25,C30-archaeal membrane lipids, which have never been reported so far.
1. Introduction

The structure of archaeal membrane lipids is one of the most striking features that distinguishes the organisms of the domain Archaea from those of the domains Bacteria and Eucarya (1, 2). Archaeal membrane lipids with two fully saturated C20 isoprenoid chains that are linked to a glycerol moiety via ether bonds are the major components of membrane in some groups of archaе such as halophilic archaе or exist as minor components in many archaе. These types of archaеal membrane lipids are designated as “diether lipids” because they possess two ether bonds. The production of these lipids is considered an important adaptation mechanism for archaе to thrive in harsh environments such as high temperature and extremely high or low pH, because a cell membrane composed of these lipids maintains a low leakage of ions and small molecules over a wide range of temperatures (3, 4). Interestingly, various archaеal membrane lipids are produced depending on the species of archaе and their growth conditions (5–7). Many thermophilic archaе mainly synthesize tetraether lipids, which are believed to be formed by the dimerization of diether lipids. Some reports have shown that the ratio of tetraether lipids in the cells of thermophilic archaе is increased along with an elevation of the growth temperature, suggesting that these lipids help archaе adapt to more extreme environments (8–10). On the other hand, for some archaе that do not produce tetraether
lipids, the length of hydrophobic chains in diether lipids is considered an important feature that allows adaptation to extreme environments. “Extended” C25,C25-archaeal membrane lipids, which have hydrophobic isoprenoid chains that are longer than those of typical C20,C20-diether lipids, are produced in several extremophilic archaea such as the hyperthermophilic archaeon *Aeropyrum pernix* and some halophilic archaea of a few genera, and also in a mesophilic methanogen *Methanomassiliicoccus luminyensis* (11–13). The C25,C25-diether lipids supposedly form a thicker cell membrane that functions as a more impenetrable barrier for ions and small molecules. In fact, the liposomes formed by the C25,C25-archaeal membrane lipids isolated from *A. pernix* are known to maintain low leakage even at 100°C (14). Presumably, archaeal diether lipids with hydrophobic chains that are longer than those of C25,C25-archaeal membrane lipids would form cell membranes that are more stable. Although such “hyperextended” archaeal lipids are yet to be discovered in nature, the physical properties are of great interest.

The biosynthetic pathway that yields the hydrocarbon core structure of C25,C25-archaeal membrane lipids has already been identified from *A. pernix* (15) (Figure 1A). First, geranylfarnesyl pyrophosphate (GFPP) is synthesized via GFPP synthase from a molecule of dimethylallyl pyrophosphate (DMAPP) and 4 molecules of isopentenyl pyrophosphate (IPP) (16). Next, sn-3-(geranylfarnesyl)glycerol-1-phosphate (GFGP)
synthase catalyzes the transfer of a geranylfnanesyl group from GFPP to \( sn \)-glyceol-1-phosphate (G1P), which is produced by G1P dehydrogenase (G1PDH) from dihydroxyacetone phosphate, to synthesize GFGP. Then, \( sn \)-2,3-(digeranylfnanesyl)glycerol-1-phosphate (DGFGP) synthase transfers a geranylfnanesyl group from GFPP to the glycerol moiety of GFGP to form DGFGP. Finally, 10 double bonds in the isoprenoid chains of DGFGP are fully reduced by the action of geranylfnanesyl reductase. Therefore, the production of hyperextended diether archaeal lipids, which have isoprenoid chain(s) longer than C25, might be achieved by modification of the biosynthetic pathway for C25,C25-archaeal membrane lipids. Interestingly, \textit{Bacillus subtilis} is known to have a GFGP synthase homolog called PcrB. The enzyme transfers a heptaprenyl (C35) group from heptaprenyl pyrophosphate (HepPP) to G1P to form \( sn \)-3-heptaprenylglycerol-1-phosphate (HepGP), the physiological function of which remains unclear (17–19) (Figure 1B). We speculated that if DGFGP synthase could accept prenynl donor and acceptor substrates with longer chains, such as HepPP and HepGP, respectively, utilization of PcrB and suitable prenynl pyrophosphate synthases could enable the synthesis of hyperextended archaeal membrane lipids. A membrane intrinsic prenynltransferase, UbiA, which is involved in ubiquinone biosynthesis, catalyzes the transfer of a C30-50 prenynl chain from prenynl pyrophosphate to \( p \)-hydroxybenzoate. Structural analysis of the \textit{A. pernix} UbiA
homolog (20) has demonstrated the existence of a “lateral portal” that faces the hydrophobic region of a cell membrane, and allows the enzyme to accept a long prenyl pyrophosphate as a prenyl donor substrate, while the UbiA homolog from *Archaeoglobus fulgidus* (21) has a large internal cavity that could likely accommodate prenyl pyrophosphate. Because *A. pernix* DGFGP synthase belongs to the UbiA superfamily and has a sequence that approximates that of the *A. pernix* UbiA homolog (30% identity), we expected this enzyme to accept longer prenyl donor substrates such as HepPP.

In the present study, we attempted to construct artificial biosynthetic pathways for hyperextended archael diether lipids with hydrophobic chains that would be longer than those of the C25,C25-archael membrane lipids. Contrary to our expectations based on the characteristics of the UbiA-superfamily prenyltransferases, however, *in vitro* study of the substrate preferences of *A. pernix* DGFGP synthase revealed that this enzyme does not accept longer (>C25) prenyl donor substrates. The enzyme, however, could accept a longer prenyl acceptor substrate, *sn*-3-hexaprenylglycerol-1-phosphate (HexGP), which allowed the synthesis of a supposed precursor for hyperextended C25,C30-archael membrane lipids. We constructed a plasmid containing 4 archael genes and the *B. subtilis* PcrB gene, which was sufficient to produce the precursor in *E. coli* cells. An *E. coli* strain harboring the plasmid produced unsaturated C25,C30-archael membrane lipids with diacylglycerol- and
phosphatidylglycerol-like structures.
2. Material and Methods

2.1 Materials

Precoated reversed-phase thin-layer chromatography (TLC) plates, silica gel RP-18 F254S, were purchased from Merck Millipore, Germany. [1-14C]IPP (55 Ci/mol) was purchased from American Radiolabeled Chemicals, Inc., USA. Non-labeled IPP and DMAPP were donated by Dr. Chikara Ohto, Toyota Motor Co., Japan.

2.2 General procedures

Restriction enzyme digestions, transformations, and other standard molecular biological techniques were carried out as described by Sambrook et al (22).

2.3 Cultivation of microorganisms and extraction of their genomes

*B. subtilis* and *Saccharolobus solfataricus* (former *Sulfolobus solfataricus*) were provided by the RIKEN BRC through the Natural Bio-Resource Project of the MEXT, Japan.

*B. subtilis* was cultured in a LB medium at 37°C. *S. solfataricus* was cultured in an ATCC1304 *S. solfataricus* medium at 70 °C. The genomic DNAs of *B. subtilis* and *S. solfataricus* were extracted from cells using a DNA extraction kit, Geno Plus™ Mini.
2.4 Recombinant expression and purification of *B. subtilis* PcrB and archaeal enzymes

All plasmids used in this study are listed in Table 1. PCR reactions were performed using KOD plus NEO DNA polymerase (TOYOBO, Japan) and the primers shown in Table 2. The *BSU06600* gene encoding *B. subtilis* PcrB was amplified using the genomic DNA of *B. subtilis* as a template. The amplified gene was digested by restriction enzymes *NdeI* and *BamHI*, and was then ligated into pET15b and digested by the same restriction enzymes to construct pET15b-BsPcrB. *E. coli* BL21 (DE3) transformed by the 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, 1.0 mM IPTG was then added for induction. After an additional 24 h of incubation, the cells were harvested and disrupted by sonication in a HisTrap binding buffer that contained 20 mM potassium phosphate, pH 7.4, 0.5 M NaCl, and 10 mM imidazole. The homogenate was centrifuged at 4,000 g for 30 min, and the supernatant was recovered as a crude extract. The supernatant fraction was loaded into a HisTrap crude FF column (GE Healthcare, USA), which had been equilibrated with HisTrap binding buffer. The column was washed with HisTrap wash buffer containing 20 mM potassium phosphate, pH 7.4, 0.5 M NaCl, and 60 mM imidazole. 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 synthesis of radiolabeled substrates. The level of purification was confirmed by SDS-PAGE.

For expression and purification of *S. solfataricus* hexaprenyl pyrophosphate (HexPP) synthase and *A. fulgidus* HepPP synthase, the *E. coli* BL21(DE3) strain was transformed using pET-PTH, which includes the gene that encodes *S. solfataricus* HexPP synthase (23) and pET3a-AF1551 that encodes *A. fulgidus* HepPP synthase (24) via cultivation 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 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS)-NaOH buffer, pH 7.0. The homogenate was centrifuged at 4,000 g for 30 min, and each supernatant was recovered as a crude extract. The crude extracts were heated at 55 °C for 30 min, and the denatured proteins were removed by centrifugation at 4,000 g for 30 min. The supernatant fractions were used for the synthesis of radiolabeled substrates.

Purified *Sulfolobus acidocaldarius* geranylgeranyl pyrophosphate (GGPP) synthase, *A. pernix* GFPP synthase, GFGP synthase, and DGFGP synthase were prepared as described elsewhere (15, 25, 26).
2.5 Synthesis of a radiolabeled substrate for radio-TLC assay

\[^{14}\text{C}]\text{GFPP} \text{was prepared from DMAPP and }^{[14}\text{C}]\text{IPP} \text{(American Radiolabeled Chemicals, Inc., USA) using }A. \text{ pernix} \text{GFPP synthase as described in our previous report (26). For the synthesis of }^{[14}\text{C}]\text{HexPP and }^{[14}\text{C}]\text{HepPP, we first prepared }^{[14}\text{C}]\text{GGPP using }S. \text{ acidocaldarius} \text{GGPP synthase from DMAPP and }^{[14}\text{C}]\text{IPP as described in our previous study (25). The resultant 1-butanol solution containing }\sim 56 \text{ pmol }^{[14}\text{C}]\text{GGPP} \text{(corresponding to }20,000 \text{ dpm) then was dried under a stream of }\text{N}_2 \text{ gas. To dissolve the residue, we added }200 \mu\text{L of }0.1 \text{ M MOPS-NaOH buffer, pH 7.0, containing }0.2 \text{ mmol }\text{MgCl}_2, 0.1\% \text{Triton-X, }0.2 \text{ nmol IPP, and a suitable amount of either }S. \text{ solfataricus} \text{ HexPP synthase or }A. \text{ fulgidus} \text{ HepPP synthase. The mixtures were incubated at }55^\circ\text{C for }60 \text{ min. The products were extracted with 1-butanol saturated with water and used as substrates for the reaction of }A. \text{ pernix} \text{ DGFGP synthase. A radiolabeled prenyl acceptor substrate for }A. \text{ pernix} \text{ DGFGP synthase, }^{[14}\text{C}]\text{GFPG}, \text{was prepared using }A. \text{ pernix} \text{ GFGP synthase as described in our previous report (15). For the syntheses of }^{[14}\text{C}]\text{sn-3-hexaprenylglycerol-1-phosphate (HexGP) and }^{[14}\text{C}]\text{sn-3-heptaprenylglycerol-1-phosphate (HepGP), a 1-butanol solution containing }\sim 28 \text{ pmol of }^{[14}\text{C}]\text{HexPP or }^{[14}\text{C}]\text{HepPP} \text{(both corresponding to }10,000 \text{ dpm) was dried under a stream of }\text{N}_2 \text{ gas. Then, }200 \mu\text{L of }0.1 \text{ M MOPS-NaOH buffer, pH 7.0, containing }0.2 \text{ mmol}
MgCl₂, 10 nmol α-glycerophosphate (racemic mixture), and a suitable amount of the purified *B. subtilis* PcrB was added to dissolve the residue. These mixtures were incubated at 37°C for 150 min, and the products were extracted with 1-butanol saturated with water and used as substrates for the reaction of *A. pernix* DGFGP synthase. The concentrations of the radiolabeled substrates were determined by measuring radioactivity with an LSC-7200 liquid scintillation counter (ALOKA, Japan). To confirm the formation of the expected radiolabeled substrates, each substrate was treated with potato acid phosphatase (SigmaAldrich, USA) according to a method established by Fujii et al. (27), and their hydrolysates were extracted with *n*-pentane to be analyzed by reversed-phase TLC using a precoated plate, silica gel RP-18 F₂₅₄S (Merck Millipore, Germany) developed with acetone/H₂O (19:1). The distribution of radioactivity on the TLC plate was visualized using a Typhoon FLA9000 multifunctional scanner (GE Healthcare, USA).

2.6 Radio-TLC assay for DGFGP synthase

To know if *A. pernix* DGFGP synthase accepts C30 or C35 prenyl donor and acceptor substrates, a prenyl donor substrate, ~10 pmol [¹⁴C]GFPP or ~14 pmol [¹⁴C]HexPP or ~14 pmol [¹⁴C]HepPP, and a prenyl acceptor substrate, ~10 pmol [¹⁴C]GFGP
or ~14 pmol $[^{14}\text{C}]$HexGP or ~14 pmol $[^{14}\text{C}]$HepGP, each corresponding to 5,000 dpm, were mixed and dried under a stream of N$_2$ gas. Then 200 µL of 0.1 M MOPS-NaOH buffer, pH 7.0, containing 0.2 mmol MgCl$_2$, 0.3 nmol 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate, and a suitable amount of purified $A$. pernix DGFGP synthase was added to dissolve the residue. These mixtures were incubated at 60 °C for 30 min, and the products were extracted with 1-butanol saturated with water. The products were analyzed by reversed-phase TLC following phosphatase treatment as described above.

### 2.7 Construction of a biosynthetic pathway of hyperextended archaeal membrane lipids in $E$. coli

For the construction of a plasmid containing 5 genes sufficient for the biosynthesis of C25,C30-archaeal membrane lipids in $E$. coli, the $BSU6600$ gene encoding $B$. subtilis PcrB was amplified using pET15b-BsPcrB as a template, KOD plus NEO DNA polymerase, and the primers shown in Table 2. The amplified gene and $EcoRI$-digested pBAD-MA3686, which contains the gene of G1PDH from $\text{Methanosarcina acetivorans}$ (28), were introduced into the $E$. coli ME9783 strain (National BioResource Project, Japan), which enables $\text{in vivo}$ $E$. coli cloning based on homologous recombination (29). The plasmid in which the $BSU6600$
gene was inserted was extracted from an *E. coli* clone and was designated pBAD-C30ALB2. In a similar manner, a *SSO2345* gene encoding *S. solfataricus* HexPP synthase was amplified using the genomic DNA of *S. solfataricus* as a template and was inserted into *EcoRI*-digested pBAD-C30ALB2 to construct pBAD-C30ALB3. Next, a DNA fragment containing *APE0159* and *APE1764* genes encoding *A. pernix* DGFGP synthase and GFPP synthase, respectively, was amplified from pBAD-C25ALB4, which had been constructed in our previous report for the biosynthesis of C25,C25-archaeal membrane lipids in *E. coli* (15). The DNA fragment was inserted into *EcoRI*-digested pBAD-C30ALB3 to construct pBAD-C30ALB5.

An *E. coli* TOP10 strain transformed by pBAD-C30ALB5 or an empty plasmid, pBAD-18 (30), was cultivated at 37°C for 24 hours in 1 L of LB medium supplemented with 100 mg/L ampicillin and 0.02% L-arabinose. After cultivation, cells were harvested and then dissolved with 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 1,000 g for 15 min. The supernatant was recovered and dried under a N2 stream. 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 under a N2 stream. The
residue was dissolved with 1.0 mL of methanol/2-propanol (1:1) per 1 g of wet cells.

The lipid extracts were analyzed by LC-ESI-MS using 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 (28). 10 µL of each lipid extract was injected into a COSMOSIL 5C18-AR-II packed column (2.0 x 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 was 100% of eluent A for 0-20 min, a linear gradient with 0-80% of eluent B for 20-50 min, and 100% of eluent B for 50-70 min.

2.8 Availability of materials and resources

Any unique materials and resources presented in the manuscript may be available from the authors upon reasonable request and through a materials transfer agreement.
3. Results

3.1 Synthesis of Radio-labeled substrates for DGFGPS assay

For the *in vitro* assay of DGFGP synthase, we prepared \[^{14}C\]GFPP, \[^{14}C\]HexPP, and \[^{14}C\]HepPP as candidates for prenyl donor substrates, and \[^{14}C\]GFGP, \[^{14}C\]HexGP, and \[^{14}C\]HepGP as possible prenyl acceptor substrates. GFPP was synthesized using *A. pernix* GFPP synthase from non-labeled DMAPP and \[^{14}C\]IPP. HexPP and HepPP were synthesized using *S. solfataricus* HexPP synthase, and *A. fulgidus* HepPP synthase, respectively, from \[^{14}C\]GGPP and non-labeled IPP. The formation of the substrates was confirmed by radio-TLC analysis following phosphatase treatment. As shown in Figure 2, the radioactive spots that corresponded to C25, C30 or C35 prenyl alcohol emerged, confirming that radiolabeled GFPP, HexPP and HepPP were synthesized as major products. In the analysis of \[^{14}C\]HepPP, a faint spot corresponding to geranylgeraniol, which arose from unreacted \[^{14}C\]GGPP, was observed. The amount of unreacted GGPP estimated from the density of the spot was, however, much lower than that of HepPP, suggesting that most of the GGPP was converted to HepPP. To synthesize the prenyl acceptor substrates, *A. pernix* GFGP synthase and its homolog prenyltransferase PcrB from *B. subtilis* were expressed in *E. coli* and purified. \[^{14}C\]GFGP was successfully synthesized using GFGP synthase. To synthesize \[^{14}C\]HexGP and \[^{14}C\]HepGP, we reacted \[^{14}C\]HexPP and \[^{14}C\]HepPP, respectively, with PcrB in the
presence of an excess amount of G1P. Hydrophobic products and unreacted substrates extracted from the reaction mixture were analyzed by reversed-phase TLC following phosphatase treatment. As shown in Figure 2, new spots with values for $R_f$ of 0.59 and 0.53 were considered to emerge from alcohols from HexGP and HepGP, respectively, because of the disappearance of the spots of the alcoholic forms of HexPP and HepPP that had values for $R_f$ of 0.53 and 0.47, respectively, and because a similar level of increase in the value of $R_f$ was also observed between the alcohols from GFPP ($R_f$ 0.59) and GFGP ($R_f$ 0.65). These results suggested that HexPP and HepPP were almost completely consumed to synthesize HexGP and HepGP, respectively.

### 3.2 In vitro assay of *A. pernix* DGFGP synthase

To test the capability of DGFGP synthase to accept substrates longer than the original C25 substrates GFPP and GFGP, recombinant DGFGP synthase was prepared by purification via heat treatment and affinity column chromatography. First, DGFGP synthase was reacted with the original prenyl acceptor substrate $[^{14}\text{C}]$GFGP and various prenyl donors: $[^{14}\text{C}]$GFPP, $[^{14}\text{C}]$HexPP, and $[^{14}\text{C}]$HepPP. Radio-TLC analysis of the reaction products extracted with 1-butanol was performed following phosphatase treatment. As shown in Figure 3A, the spot of DGFGP-derived alcohol with an $R_f$ of 0.32 emerged when
GFPP and GFGP were used, confirming the occurrence of the original DGFGP synthase reaction. As HexPP was used instead of GFPP, a new faint spot with an \( R_f \) of 0.28 emerged. Because the difference in \( R_f \) between this spot and that corresponding to alcohol from HexPP was comparable to that observed in the analysis of DGFGP and GFPP, this result suggested the formation of \( sn\)-2-hexaprenyl-3-(geranylfarnesyl)glycerol-1-phosphate, a precursor for C30,C25-archaeal membrane lipids, by prenyltransfer reaction from HexPP to GFGP. However, the amount of the reaction product estimated from the density of the spot was much lower than that of DGFGP. When HepPP was used as the prenyl donor substrate, two new spots were detected. The faint spot with an \( R_f \) of 0.38 that is indicated by the asterisk in Figure 3A, likely corresponded to the alcoholic form of \( sn\)-2-geranylgeranyl-3-(geranylfarnesyl)glycerol-1-phosphate synthesized from GGPP, which had been mixed in the solution of HepPP as shown in Figure 2, and GFGP. The other very faint spot with an \( R_f \) of 0.25 was considered to arise from the alcoholic form of \( sn\)-2-heptaprenyl-3-(geranylfarnesyl)glycerol-1-phosphate, suggesting that the heptaprenyl group was transformed to GFGP to form the precursor of C35,C25-archaeal membrane lipids, while the amount of the product was almost negligible compared with that of the original reaction.

Next, DGFGP synthase was reacted with \([^{14}\text{C}]\text{HexGP}\), as the prenyl acceptor
substrate, and each of the donor substrates. When HexGP and GFPP were used for the reaction, a new dense spot with an \( R_f \) of 0.28 arose, suggesting the formation of \( sn-2 \)-geranylfarnesyl-3-hexaprenylglycerol-1-phosphate, a precursor of C25,C30-archaeal membrane lipids (Figure 3B). The density of the spot demonstrated that the amount of the reaction product was comparable to that of DGFGP from the original reaction with GFPP and GFGP. When either HexPP or HepPP was used instead of GFPP, no new spot was detected. These results demonstrate that DGFGP synthase is capable of accepting HexGP as the effective prenyl acceptor only when the donor substrate is GFPP.

Finally, DGFGP synthase was reacted with \([^{14}\text{C}]\)HepGP and each of the prenyl donor substrates. When GFPP was used for the reaction, a faint spot with an \( R_f \) of 0.40 was observed (Figure 3C, shown by an asterisk). The spot did not correspond to the alcoholic form of the expected product from the reaction between GFPP and HepGP, but possibly did correspond to that of the \( sn-2 \)-geranylfarnesyl-3-(geranylgeranyl)glycerol-1-phosphate that was synthesized from GFPP and GGGP, which had been mixed to a small extent into the solution of HepGP (Figure 2). With HexPP and HepPP, no new spot was observed. These results suggested that DGFGP synthase is incapable of accepting HepGP as the prenyl acceptor.

Contrary to our expectations, the results from \textit{in vitro} enzyme assay showed that
DGFGP synthase has a relatively specific substrate preference, particularly for its donor substrate. The fact that the enzyme effectively catalyzes the reaction between GFPP and HexGP, however, suggests the possibility that an artificial synthetic pathway of hyperextended C25,C30-archaeal membrane lipids could yet be constructed.

3.3 Construction of an artificial biosynthetic pathway of hyperextended archaeal membrane lipids in *E. coli*

To construct the artificial biosynthetic route for C25,C30-archaeal membrane lipids in *E. coli* cells, we introduced the genes of *A. pernix* DGFGP synthase and GFPP synthase, *S. solfataricus* HexPP synthase, *B. subtilis* PcrB, and *M. acetivorans* G1PDH into the same plasmid to construct pBAD-C30ALB5 (Figure 4). Lipids extracted from the *E. coli* strain harboring the plasmid were analyzed via LC-ESI-MS. As shown in Figure 5A, a positive ion peak with an *m/z* of 864.1, which was not observed in the analysis of the negative control sample, was eluted at ~16 min of retention time. This suggests that a diacylglycerol-like C25,C30-archaeal membrane lipid (C25,C30-OH), i.e., *sn*-2-geranylfarnesyl-3-hexaprenylglycerol, actually was produced because the *m/z* value corresponded to [C25,C30-OH + Na]⁺. To confirm the production of C25,C30-OH, we performed LC-ESI-MS/MS analysis for this ion. Detected major fragment ions with *m/z* values of 521.6 and
453.5 could be reasonably explained via the conceivable fragmentation of C25,C30-OH, as shown in Figure 5B. In addition, a positive ion peak with an $m/z$ of 1,040.0 was eluted with the same retention time, which suggested that actually a phosphatidylglycerol-like C25,C30-archaeal membrane lipid (C25,C30-PG), i.e., $sn$-2-geranylfarnesyl-3-hexaprenylglycerol-1-phosphoglycerol was produced because this $m/z$ value corresponded to [C25,C30-PG + 2Na]$^+$. The identical elution time of the two ion peaks suggests that C25,30-OH might arise from the decomposition of C25,30-PG through ionization.
4. Discussion

In the present study, an *in vitro* assay of *A. pernix* DGFGP synthase was performed in an effort to construct artificial biosynthetic pathways for hyperextended archaeal membrane lipids. Although *A. pernix* DGFGP synthase could not accept longer prenyl donors such as HexPP and HepPP as proper substrates, the enzyme efficiently accepted the C30 prenyl acceptor substrate HexGP when GFPP was the donor substrate. This enzyme belongs to the UbiA superfamily, and therefore is considered to have a structure similar to UbiA, which is a bacterial prenyltransferase involved in ubiquinone biosynthesis (31). Structural analysis has suggested that the *A. pernix* UbiA homolog, which is presumably responsible for the biosynthesis of *A. pernix*-specific demethylmenaquinones or methionaquinones with a fully/partially-saturated C30 prenyl side-chain, can accommodate a long prenyl donor substrate because it has a lateral portal through which the long prenyl chain protrudes into the hydrophobic region of a cell membrane (20). Unlike UbiA that accepts a small aromatic prenyl acceptor substrate, however, DGFGP synthase utilizes two large substrates, both of which contain a prenyl group. It is unclear whether both of the substrates are accommodated in the active pocket of the enzyme, or if one or two prenyl groups protrude into the cell membrane. Considering the results from the *in vitro* assay that demonstrated the stricter preference of the enzyme for prenyl
donors over prenyl acceptors, the prenyl chain of the prenyl acceptor substrate possibly extends through the lateral portal. Given the fact that HepGP was not accepted even when the donor was GFPP, however, the substrate recognition mechanism is probably more complicated.

Furthermore, we succeeded in constructing an artificial biosynthetic pathway for hyperextended C25,C30-archaeal membrane lipids in *E. coli* cells. Some of the produced lipids had a phosphoglycerol head group. This modification of the polar head group was also observed when other types of archaeal membrane lipids were produced in *E. coli* (15, 28, 32–35). Because such modification occurs at the cell membrane, at least part of the produced C25,C30-archaeal membrane lipids are considered to be included in the cell membrane of *E. coli*. It should be noted that double bonds remain in the produced C25,C30-archaeal membrane lipids. Archaeal lipids possessing unsaturated isoprenoid chains are reportedly quite rare in archaea, except for a hyperthermophilic methanogen, *Methanopyrus kandleri*, and a few examples of psychrophilic archaea (36–38). In a future study, we will attempt to saturate the lipids. To date, *E. coli* strains have been constructed with the ability to produce various archaeal membrane lipids that possess C20,C20-, hydroxylated C20,C20-, or C25,C25-core structures (15, 28, 32–34, 39). Caforio et al. recently reported the construction of an *E. coli* strain that produced *sn*-2,3-
(digeranylgeranyl)glycerol-based lipids as main components of the cell membrane, constituting up to 20-30% of whole lipids (35). Intriguingly, the strain had a slightly more robust cell membrane, which demonstrated that the addition of archaeal lipids could affect the properties of the bacterial cell membrane. The present study is the first to construct an *E. coli* strain by synthesizing hyperextended archaeal membrane lipids. Boosting the production of this lipid will elucidate the effect of producing hyperextended lipids on *E. coli* cells. Moreover, by comparing the phenotype of the *E. coli* strain producing C25,C30-archaeal membrane lipids with those of strains producing C20,C20- or C25,C25-lipids, we should gain a better understanding of how the chain-lengths of diether archaeal membrane lipids confer a tolerance of harsh environments and why such lipids have not been discovered in nature.
Supplementary Data available at SYNBIO online.

**Funding**

This work was supported in part by JSPS KAKENHI Grants Number 18K19170, 19H04651, and 20H02899, for H.H.; by grants-in-aid from the Institute for fermentation, Osaka, the Noda Institute for Scientific Research, and the Nagase Scientific Technology Foundation, for H.H.; and, by JSPS KAKENHI Grant Number 19J21282 for R.Y.

Conflict of interest statement. None declared.
References

1. Koga Y, Morii H. (2007) Biosynthesis of Ether-Type Polar Lipids in Archaea and Evolutionary Considerations. *Microbiol. Mol. Biol. Rev.* 71, 97–120.

2. Caforio A, Driessen AJM. (2017) Archaeal phospholipids: Structural properties and biosynthesis. *Biochim. Biophys. Acta. Mol. Cell. Biol. Lipids* 1862, 1325–1339.

3. Yamauchi K, Doi K, Kinoshita M, Kii F, Fukuda H. (1992) Archaeobacterial lipid models: highly salt-tolerant membranes from 1,2-diphytanylglycerol-3-phosphocholine. *Biochim. Biophys. Acta. Biomembr* 1110, 171–177.

4. Van de Vossenberg JLCM, Driessen AJM, Konings WN. (1998) The essence of being extremophilic: The role of the unique archaeal membrane lipids. *Extremophiles* 2, 163–170.

5. Koga Y, Morii H. (2005) Recent Advances in Structural Research on Ether Lipids from Archaea Including Comparative and Physiological Aspects. *Biosci. Biotechnol. Biochem.* 69, 2019–2034.

6. Ulrih NP, Gmajner D, Raspor P. (2009) Structural and physicochemical properties of polar lipids from thermophilic archaea. *Appl. Microbiol. Biotechnol.* 84, 249–60.

7. Uda I, Sugai A, Itoh YH, Itoh T. (2001) Variation in molecular species of polar lipids from *Thermoplasma acidophilum* depends on growth temperature. *Lipids* 36, 103–105.

8. Shimada H, Nemoto N, Shida Y, Oshima T, Yamagishi A. (2008) Effects of pH and Temperature on the Composition of Polar Lipids in *Thermoplasma acidophilum* HO-62. *J. Bacteriol.* 190, 5404–5411.

9. Lai D, Springstead JR, Monbouquette HG. (2008) Effect of growth temperature on ether lipid biochemistry in *Archaeoglobus fulgidus*. *Extremophiles* 12, 271–278.

10. Matsuno Y, Sugai A, Higashibata H, Fukuda W, Ueda K, Uda I, Sato I, Itoh T, Imanaka T, Fujiwara S. (2009) Effect of Growth Temperature and Growth Phase on the Lipid Composition of the Archaeal Membrane from *Thermococcus kodakaraensis*. *Biosci. Biotechnol. Biochem.* 73, 104–108.

11. Morii H, Yagi H, Akutsu H, Nomura N, Sako Y, Koga Y. (1999) A novel phosphoglycolipid archaetidyl(glucosyl)inositol with two sesterterpanyl chains from the aerobic hyperthermophilic archaean *Aeropyrum pernix* K1. *Biochim. Biophys. Acta. Mol. Cell. Biol. Lipids* 1436, 426–436.

12. de Rosa M, Gambacorta A, Nicolaus B, Grant WD. (1983) A C25,C25 Diether Core Lipid from Archaeobacterial Haloalkaliphiles. *Microbiology* 129, 2333–2337.

13. Becker KW, Elling FJ, Yoshinaga MY, Sollinger A, Urih T, Hinrichs K-U. (2016) Unusual Butane- and Pentanetriol-Based Tetraether Lipids in *Methanomassiliicoccus luminyensis*, a Representative of the Seventh Order of Methanogens. *Appl. Environ. Microbiol.* 82, 4505–4516.

14. Shimada H, Yamagishi A. (2011) Stability of Heterochiral Hybrid Membrane Made of Bacterial

https://mc.manuscriptcentral.com/synbio
sn'-G3P Lipids and Archaeal sn'-G1P Lipids. *Biochemistry* 50, 4114–4120.

15. Yoshida R, Yoshimura T, Hemmi H. (2018) Biosynthetic machinery for C25,C25-diether archaeal lipids from the hyperthermophilic archaeon *Aeropyrum pernix*. *Biochem. Biophys. Res. Commun.* 497, 87–92.

16. Tachibana A, Yano Y, Otani S, Nomura N, Sako Y, Taniguchi M. (2000) Novel prenyltransferase gene encoding farnesylgeranyl diphosphate synthase from a hyperthermophilic archaeon, *Aeropyrum pernix*. *Eur. J. Biochem.* 267, 321–328.

17. Guldan H, Matysik P-M, Bocola M, Sterner R, Babinger P. (2011) Functional Assignment of an Enzyme that Catalyzes the Synthesis of an Archaea-Type Ether Lipid in Bacteria. *Angew. Chemie. Int. Ed.* 50, 8188–8191.

18. Ren F, Feng X, Ko T-P, Huang C-H, Hu Y, Chan H-C, Liu Y-L, Wang K, Chen C-C, Pang X, He M, Li Y, Oldfield E, Guo R-T. (2013) Insights into TIM-Barrel Prenyl Transferase Mechanisms: Crystal Structures of PcrB from Bacillus subtilis and *Staphylococcus aureus*. *ChemBioChem* 14, 195–199.

19. Peterhoff D, Beer B, Rajendran C, Kumpula E-P, Kapetanious E, Guldan H, Wierenga RK, Sterner R, Babinger P. (2014) A comprehensive analysis of the geranylgeranylglyceryl phosphate synthase enzyme family identifies novel members and reveals mechanisms of substrate specificity and quaternary structure organization. *Mol. Microbiol.* 92, 885–899.

20. Cheng W, Li W. (2014) Structural Insights into Ubiquinone Biosynthesis in Membranes. *Science* 343, 878–881.

21. Huang H, Levin EJ, Liu S, Bai Y, Lockless SW, Zhou M. (2014) Structure of a Membrane-Embedded Prenyltransferase Homologous to UBIAD1. *PLoS Biol.* 12:1–11.

22. J. Sambrook, EF. Fritsch, T. Maniatis. Molecular Cloning: A Laboratory Manual, second ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

23. Hemmi H, Ikejiri S, Yamashita S, Nishino T. (2002) Novel Medium-Chain Prenyl Diphosphate Synthase from the Thermoacidophilic Archaeon *Sulfolobus solfataricus*. 184, 615–620.

24. Hemmi H, Takahashi Y, Shibuya K, Nakayama T, Nishino T. (2005) Menaquinone-Specific Prenyl Reductase from the Hyperthermophilic Archaeon *Archaeoglobus fulgidus*. *J. Bacteriol.* 187, 1937–1944.

25. Hemmi H, Shibuya K, Takahashi Y, Nakayama T, Nishino T. (2004) (S)-2,3-Di-geranylgeranylglyceryl Phosphate Synthase from the Thermoacidophilic Archaeon *Sulfolobus solfataricus*. *J. Biol. Chem.* 279, 50197–50203.

26. Mori T, Ogawa T, Yoshimura T, Hemmi H. (2013) Substrate specificity of undecaprenyl diphosphate synthase from the hyperthermophilic archaeon *Aeropyrum pernix*. *Biochem. Biophys. Res. Commun.* 436, 230–234.
27. Hiroshi F, Tanetoshi K, Kyozo O. (1982) Efficient enzymatic hydrolysis of polyprenyl pyrophosphates. *Biochim. Biophys. Acta. - Lipids Lipid Metab.* 712, 716–718.

28. Yokoi T, Isobe K, Yoshimura T, Hemmi H. (2012) Archaeal phospholipid biosynthetic pathway reconstructed in *Escherichia coli*. *Archaea* 2012.

29. Nozaki S, Niki H. (2019) Exonuclease III (XthA) Enforces In Vivo DNA Cloning of *Escherichia coli* To Create Cohesive Ends. *J. Bacteriol.* 201, e00660-18.

30. Guzman L-M, Belin D, Cartee RT, J. M, Beckwith J. (2000) Tight Regulation, Modulation, and High-Level Expression by Vectors containing the Arabinose Pbad promoter. *J. Bacteriol.* 177, 4121–4130.

31. Li W. (2016) Bringing Bioactive Compounds into Membranes: The UbiA Superfamily of Intramembrane Aromatic Prenyltransferases. *Trends Biochem. Sci.* 41, 356–370.

32. Isobe K, Ogawa T, Hirose K, Yokoi T, Yoshimura T, Hemmi H. (2014) Geranylgeranyl Reductase and Ferredoxin from *Methanosarcina acetivorans* Are Required for the Synthesis of Fully Reduced Archaeal Membrane Lipid in *Escherichia coli* Cells. *J. Bacteriol.* 196, 417–423.

33. Caforio A, Jain S, Fodran P, Siliakus M, Minnaard AJ, van der Oost J, Driessen AJM. (2015) Formation of the ether lipids archaetidylglycerol and archaetidylethanolamine in *Escherichia coli*. *Biochem. J.* 470, 343–355.

34. Mori T, Isobe K, Ogawa T, Yoshimura T, Hemmi H. (2015) A phytoene desaturase homolog gene from the methanogenic archaeon *Methanosarcina acetivorans* is responsible for hydroxyarchaeol biosynthesis. *Biochem. Biophys. Res. Commun.* 466, 186–191.

35. Caforio A, Siliakus MF, Exterkate M, Jain S, Jumde VR, Andringa RLH, Kengen SWM, Minnaard AJ, Driessen AJM, van der Oost J. (2018) Converting *Escherichia coli* into an archaeabacterium with a hybrid heterochiral membrane. *Proc. Natl. Acad. Sci.* 115, 3704–3709.

36. Hafenbradl D, Keller M, Thiericke R, Stetter KO. (1993) A Novel Unsaturated Archaeal Ether Core Lipid from the Hyperthermophile *Methanopyrus kandleri*. *Syst. Appl. Microbiol.* 16, 165–169.

37. Koga Y, Nakano M. (2008) A dendrogram of archaea based on lipid component parts composition and its relationship to rRNA phylogeny. *Syst. Appl. Microbiol.* 31, 169–182.

38. Gibson JAE, Miller MR, Davies NW, Neill GP, Nichols DS, Volkman JK. (2005) Unsaturated diether lipids in the psychrotrophic archaeon *Haloarcula marismortui*. *Syst. Appl. Microbiol.* 28, 19–26.

39. Lai D, Lluncor B, Schröder I, Gunsalus RP, Liao JC, Monbouquette HG. (2009) Reconstruction of the archaeal isoprenoid ether lipid biosynthesis pathway in *Escherichia coli* through digeranylgeranylglycerol phosphate. *Metab. Eng.* 11, 184–191.
Table 1. Plasmids used in the study

| Plasmid          | Characteristics                                                                 | Reference     |
|------------------|---------------------------------------------------------------------------------|---------------|
| pET-15b          | Amp<sup>a</sup>, pBR322<sub>origin</sub>, P<sub>T7</sub>, His-tag              | Novagen       |
| pET-HisGGPS      | Amp<sup>a</sup>, pBR322<sub>origin</sub>, P<sub>T7</sub>, His-tag, Saci0092    | (25)          |
| pET-15b-gfps     | Amp<sup>a</sup>, pBR322<sub>origin</sub>, P<sub>T7</sub>, His-tag, APE1764     | (26)          |
| pET-PTH          | Amp<sup>a</sup>, pBR322<sub>origin</sub>, P<sub>T7</sub>, SS02345              | (23)          |
| pET3a-AF1551     | Amp<sup>a</sup>, pBR322<sub>origin</sub>, P<sub>T7</sub>, AF1551               | (24)          |
| pET48b-APE0621   | Kan<sup>a</sup>, pBR322<sub>origin</sub>, P<sub>T7</sub>, Trx-tag, His-tag, APE0621 | (15)          |
| pET15b-APE0159   | Amp<sup>a</sup>, pBR322<sub>origin</sub>, P<sub>T7</sub>, His-tag, APE0159     | (15)          |
| pET15-BsPerB     | Amp<sup>a</sup>, pBR322<sub>origin</sub>, P<sub>T7</sub>, His-tag, BSU06600   | This study    |
| pBAD18           | Amp<sup>a</sup>, pBR322<sub>origin</sub>, P<sub>ARA</sub>                     | (30)          |
| pBAD-MA3686      | Amp<sup>a</sup>, pBR322<sub>origin</sub>, P<sub>ARA</sub>, MA3686              | (28)          |
| pBAD-C25ALB4     | Amp<sup>a</sup>, pBR322<sub>origin</sub>, P<sub>ARA</sub>, APE0159, APE1764, APE0621, MA3686 | (15)          |
| pBAD-C30ALB2     | Amp<sup>a</sup>, pBR322<sub>origin</sub>, P<sub>ARA</sub>, BSU06600, MA3686 | This study    |
| pBAD-C30ALB3     | Amp<sup>a</sup>, pBR322<sub>origin</sub>, P<sub>ARA</sub>, SS02345, BSU06600, MA3686 | This study    |
| pBAD-C30ALB5     | Amp<sup>a</sup>, pBR322<sub>origin</sub>, P<sub>ARA</sub>, APE0159, APE1764, SS02345, BSU06600, MA3686 | This study    |

<sup>a</sup>The fully annotated sequences of the plasmids used in this study are provided as Supplementary Data online.

https://mc.manuscriptcentral.com/synbio
Table 2. Primers used in the study

| Gene       | Primer sequence                                                                 | Constructed plasmid |
|------------|---------------------------------------------------------------------------------|---------------------|
| BSU06600   | forward: 5’-CAGCTCATATGTACGATGTAACGGAGTGG-3’                                  | pET15b-BsPcrB       |
|            | reverse: 5’-ACTGTGGATCCTTTAATCTGGCTCTCGGCTACAGCC-3’                           |                     |
| BSU06600   | forward: 5’-TTTTTTTGGGCTAGCAGATTAAGATATTATATGTACGATGTAACGGAGTGG-3’            | pBAD-C30ALB2        |
|            | reverse: 5’-TTTTATTTTGGGCTAGCAGATTAAGATATTATATGTACGATGTAACGGAGTGG-3’          |                     |
| SSO2345    | forward: 5’-TTTTTTTGGGCTAGCAGATTAAGATATTATATGTACGATGTAACGGAGTGG-3’            | pBAD-C30ALB3        |
|            | reverse: 5’-TTTTTTTGGGCTAGCAGATTAAGATATTATATGTACGATGTAACGGAGTGG-3’            |                     |
| APE0159    | forward: 5’-TTTTTTTGGGCTAGCAGATTAAGATATTATATGTACGATGTAACGGAGTGG-3’            | pBAD-C30ALB5        |
| and        | reverse: 5’-TTTTTTTGGGCTAGCAGATTAAGATATTATATGTACGATGTAACGGAGTGG-3’            |                     |
| APE1764    | reverse: 5’-TTTTTTTGGGCTAGCAGATTAAGATATTATATGTACGATGTAACGGAGTGG-3’            |                     |
Figure 1. Part of the biosynthetic pathway of extended C25,C25-archaeal membrane lipids in *A. pernix* (A) and the reaction catalyzed by PcrB from *B. subtilis* (B).
Figure 2. The radio-TLC analysis of enzymatically synthesized prenyl donor and acceptor substrates. Synthesized prenyl donors and acceptors for the DGFGP synthase reaction, which were extracted from reaction mixtures with 1-butanol, were hydrolyzed with acid phosphatase and then analyzed by reversed-phase TLC after pentane extraction. Radiolabeled GFPP was synthesized from non-labeled DMAPP and \([^{14}C]IPP\). Radiolabeled HexPP and HepPP were synthesized from \([^{14}C]GGPP\) and non-labeled IPP.

Radiolabeled GFGP, HexGP, and HepGP were synthesized from G1P and corresponding prenyl pyrophosphate, i.e., \([^{14}C]GFPP\), \([^{14}C]HexPP\), or \([^{14}C]HepPP\), respectively. ori., origin; s.f., solvent front.

96x108mm (300 x 300 DPI)
Figure 3. The radio-TLC analysis of the products of DGFGP synthase from various prenyl donor and acceptor substrates. The butanol-extracted products were hydrolyzed with acid phosphatase and analyzed by reversed-phase TLC after pentane extraction. Asterisks indicate the products from unexpected reactions as explained in the main text. s.f., solvent front; ori., origin; DGFGPS, *A. pernix* DGFGP synthase. (A) Analysis of the products from $[^{14}C]$GFPP and various prenyl donor substrates, i.e., $[^{14}C]$GFPP, $[^{14}C]$HexPP, and $[^{14}C]$HepPP. C30,C25 and C35,C25 indicate sn-2-hexaprenyl-3-(geranylfarnesyl)glycerol-1-phosphate and sn-2-heptaprenyl-3-(geranylfarnesyl)glycerol-1-phosphate, respectively. (B) Analysis of the products from $[^{14}C]$HexGP and each prenyl donor substrate. C25,C30 indicates sn-2-geranylfarnesyl-3-hexaprenylglycerol-1-phosphate. (C) Analysis of products from $[^{14}C]$HepGP and each prenyl donor substrate.
Figure 4. The artificial biosynthetic pathway of hyperextended C25,C30-archaeal membrane lipids constructed in this study.

167x135mm (300 x 300 DPI)
Figure 5. LC-ESI-MS analysis of lipids extracted from *E. coli* strains. (A) Analysis of the lipid samples extracted from *E. coli* harboring the empty vector pBAD18 (left panels), as the negative control, and pBAD-C30ALB5 (right panels). UV chromatogram at 210 nm is shown in the top panel. The extracted ion chromatogram of m/z 864.1 corresponding to [C25,C30-OH + Na]⁺ is shown in the middle panel. The extracted ion chromatogram of m/z 1040.0 corresponding to [C25,C30-PG + 2Na]⁺ is shown in the bottom panel. (B) MS/MS analysis of the ion with m/z of 864.1 in the lipid sample extracted from *E. coli* harboring pBAD-C30ALB5. Inset: Predicted fragmentation patterns corresponding to the major fragment ions are depicted.