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Formation of the ether lipids archaetidylglycerol and archaetidylethanolamine in Escherichia coli

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In archaea, the membrane phospholipids consist of isoprenoid hydrocarbon chains that are ether-linked to a sn-glycerol 1-phosphate backbone. This unique structure is believed to be vital for the adaptation of these micro-organisms to extreme environments, but it also reflects an evolutionary marker that distinguishes archaea from bacteria and eukaryotes. CDP–archaeol is the central precursor for polar head group attachment. We examined various bacterial enzymes involved in the attachment of L-serine and glycerol as polar head groups for their promiscuity in recognizing CDP–archaeol as a substrate. Using a combination of mutated bacterial and archael enzymes, archaetidylethanolamine (AE) and archaetidylglycerol (AG) could be produced in vitro using nine purified enzymes while starting from simple building blocks. The ether lipid pathway constituted by a set of archael and bacterial enzymes was introduced into Escherichia coli, which resulted in the biosynthesis of AE and AG. This is a further step in the reprogramming of E. coli for ether lipid biosynthesis.

Key words: archaea, ether lipid biosynthesis, liquid chromatography–mass spectrometry (LC–MS), membrane proteins.

INTRODUCTION

The cytoplasmic membrane is an essential constituent of cells. It forms a barrier that separates the cytosol from the external milieu. In conjunction with energy-transducing complexes and transporter proteins, the phospholipid bilayer allows homeostasis of the intracellular concentration of nutrients and other metabolites within the cell [1]. The lipid composition of the cytoplasmic membrane differs between organisms and is one of the elements that distinguish archaea from bacteria and eukarya. This marked diversity of the lipid composition between archaea and all other organisms is also termed "the lipid divide" that may find its origin in the early stages of the evolution of life [2–5].

Archaeal lipids are composed of highly branched isoprenoid chains ether-linked to a glycerol 1-phosphate (G1P) backbone, compared with fatty acid chains ester-linked to the glycerol 3-phosphate (G3P) backbone as typically found in bacteria and eukarya. Besides these main characteristics, archaeal membranes display a further diversity in their lipid composition consisting of different modifications of the two major structures: sn-2,3-dihytyanylglycerol diether, called archaeol, and sn-2,3-dihytyanylglycerol tetraether lipid, known as caldarchaeol [4,6,7]. The biosynthetic pathway leading to the formation of archaene lipids has been studied in some detail [8–13], and most of the enzymes involved in the biosynthesis have been identified and characterized. However, the entire pathway is not completely understood, nor is it clear how caldarchaeol is formed. The isoprenoid building blocks isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) are synthesized via the mevalonate pathway in archaea [12] and are combined through sequential condensation reactions catalysed by geranylgeranyl diphosphate (GGPP) synthase and farnesylgeranyl geranyl diphosphate (FGPP) synthase, depending on the length of the isoprenoid chain product [14,15]. G1P in archaea and G3P in bacteria and eukarya are formed by similar reactions although the enzymes involved, i.e. glycerol-1-phosphate dehydrogenase (G1PDH) [16,17] and glycerol-3-phosphate dehydrogenase (G3PDH), are not evolutionarily related and belong to different protein families [8]. The elongated isoprenoid chain and G1P are subsequently linked together through ether linkages by two prenyltransferases. A cytosolic protein geranylgeranylgeranyl diphosphate (GGGPP) synthase (GGGPS) selectively attaches the isoprenoid chain to the G1P [18,19] leading to the first ether bond formation. Next, the di-O-geranylgeranylgeranyl diphosphate (DGGGPS) [20–22] catalyses the second ether bond formation linking another isoprenoid chain to the lipid precursor yielding

INTRODUCTION

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DGGGP. The following step concerns the activation of DGGGP via a CTP-transferring reaction by CDP–archaeol synthase (CarS) that was recently discovered [22]. CDP–archaeol is an important intermediate for the successive steps in lipid biosynthesis where the CDP group is replaced by a polar head group. Serine, ethanolamine, glycerol and myo-inositol are common polar head groups found throughout the three domains of life. The enzymes involved in the initial replacement of CMP from a CDP–alcohol with different types of polar head groups share mostly a common mode of action among eukarya, bacteria and archaea (Figure 1). A different mechanism to produce phosphatidylethanolamine (PE) has been described for eukarya that involves a direct replacement of the CDP group with ethanolamine [23,24].

The Escherichia coli membrane is composed of 70–80% PE, 20–25% phosphatidylglycerol (PG) and 5% or less cardiolipin (diphosphatidylglycerol) as the major phospholipid components [1,25,26]. PG represents the main anionic phospholipid and is important for various cellular processes such as the initiation of DNA synthesis [1,27–29] and protein translocation [30–32], whereas PE is the main zwitter ionic lipid. In order to re-programme E. coli for the synthesis of archaeal ether lipids, it will be essential to produce the two archaeal lipids archaetidylethanolamine (AE) and archaetidylglycerol (AG) to accommodate at least the polar head group composition. For polar head group modification, the branch point in bacteria lies with CDP–diacylglycerol (CDP–DAG), produced by CDP–DAG synthase encoded by the \textit{cdsA} gene [33]. CDP–DAG acts as a substrate for two sets of enzymes. For the formation of PE, the CDP group of CDP–DAG is replaced with L-serine by phosphatidylserine (PS) synthase (Pss) leading to the production of PS. Next, PS is decarboxylated by PS decarboxylase (Psd) converting it into PE. In the other biosynthetic pathway the CDP group of CDP–DAG is initially replaced by a G3P moiety by PG synthase (Pgs) leading to the formation of phosphatidylglycerol phosphate (PGP). The enzyme phosphatidylglycerol phosphatase (Pgp) removes the phosphate resulting in the formation of PG [1,25,26,34]. In archaea, the formation of the corresponding archaeal lipids, AE and AG, seem to take place via very similar mechanisms (Figure 1). The two biosynthetic branches diverge from the CDP–archaeol towards the formation of AE or AG. The former is produced by the sequential action of two enzymes, archaetidylserine (AS) synthase (Ass) [35] and AS decarboxylase (Asd) which replace the CDP group with L-serine whereupon a decarboxylation reaction yields the AE. Likewise, AG synthesis involves the enzyme AG phosphate (AGP) synthase (Ags) which attaches a G3P to CDP–archaeol forming AGP which is subsequently dephosphorylated into AG by the action of AGP [36,37].
The bacterial and archaean synthase enzymes involved in polar head group attachment all contain a well conserved domain, identified as D-G-(X)g-(X)g in the PROSITE database that classifies these proteins as members of the CDP–alcohol phosphatidyltransferase family (Supplementary Figure S1) [36,38]. Previous bioinformatics analysis [36,37,39] revealed a wide distribution of these enzymes in bacteria and archaea, suggesting the existence of an ancestral enzyme in the last universal common ancestor (LUCA) [38] able to produce both archaea- and bacteria-like lipids. Therefore, this could indicate a universal common ancestor (LUCA) [38] able to produce both suggesting the existence of an ancestral enzyme in the last.

**Table 1 Expression vectors used in the present study**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRSF-Duet-1</td>
<td>Expression vector (KanR), T7 promoter</td>
<td>Novagen</td>
</tr>
<tr>
<td>pET-Duet-1</td>
<td>Expression vector (AmpR), T7 promoter</td>
<td>Novagen</td>
</tr>
<tr>
<td>pCDF-Duet-1</td>
<td>Expression vector (Sp3R), T7 promoter</td>
<td>Novagen</td>
</tr>
<tr>
<td>pACYC-Duet-1</td>
<td>Expression vector (CmR), T7 promoter</td>
<td>Novagen</td>
</tr>
<tr>
<td>pSJ112</td>
<td>Synthetic gene encoding codon-optimized DGGGPS from A. fulgidus with a N-terminal His-tag cloned into pRSF-Duet vector using primers 41 and 42</td>
<td>Jain et al. [23]</td>
</tr>
<tr>
<td>pSJ135</td>
<td>PCR product of ispA gene with N-terminal His-tag from E. coli K12 genomic DNA containing a double mutation Y79H and S140T. PCR product of idi gene with a His-tag at the N-terminus from E. coli K12 genomic DNA. Both genes were cloned into pCDF-Duet vector using primers 62, 63, 24 and 57.</td>
<td>Present study</td>
</tr>
<tr>
<td>pSJ138</td>
<td>Synthetic gene encoding codon-optimized DGGGPS from M. maripaludis with a N-terminal His-tag, PCR product of araM with C-terminus His-tag from B. subtilis genomic DNA. Both genes were cloned into pET-Duet vector using primers 70, 71, 11 and 12.</td>
<td>Present study</td>
</tr>
<tr>
<td>pSJ140</td>
<td>Synthetic gene encoding codon-optimized DGGGPS from A. fulgidus with a N-terminal His-tag and redesigned ribosome-binding site AGGACGTAAACAT cloned into pET-Duet vector using primers 32, 20, 84 and 86.</td>
<td>Present study</td>
</tr>
<tr>
<td>pSJ148</td>
<td>PCR product of cdsA with N-terminal His-tag from E. coli genomic DNA cloned into pACYC-Duet vector using primers 103 and 106.</td>
<td>Present study</td>
</tr>
<tr>
<td>pAC004</td>
<td>PCR product of pss with C-terminal His-tag from B. subtilis genomic DNA cloned into pACYC-Duet vector using primers 89 and 90.</td>
<td>Present study</td>
</tr>
<tr>
<td>pAC008</td>
<td>PCR product of pss with C-terminal His-tag from E. coli genomic DNA cloned into pACYC-Duet vector using primers 533 and 534.</td>
<td>Present study</td>
</tr>
<tr>
<td>pAC011</td>
<td>PCR product of pss with C-terminal His-tag from E. coli genomic DNA cloned into pET-Duet vector using primers 542 and 543.</td>
<td>Present study</td>
</tr>
<tr>
<td>pAC015</td>
<td>PCR product of pgsA with C-terminal His-tag from E. coli genomic DNA cloned into pRSF-Duet vector using primers 551 and 552.</td>
<td>Present study</td>
</tr>
<tr>
<td>pAC017</td>
<td>PCR product of pgpA with C-terminal His-tag from E. coli genomic DNA cloned into pET-Duet vector using primers 562 and 563.</td>
<td>Present study</td>
</tr>
</tbody>
</table>

**MATERIALS AND METHODS**

**Bacterial strain and cloning procedures**

_E. coli_ and _Bacillus subtilis_ genomic DNA were used as template for the amplification of genes encoding the bacterial enzymes. _E. coli_ DH5α (Invitrogen) was used for cloning. The primers and the plasmids used in the present study are listed in Tables 1 and 2. _E. coli_ BL21 (DE3) or Lemo21 (DE3) [40] was used as a protein overexpression host strain and grown under aerobic conditions at 37°C in LB medium supplemented with the required antibiotics, kanamycin (50 μg/ml), chloramphenicol (34 μg/ml), streptomycin (50 μg/ml) and ampicillin (50 μg/ml) in conjunction with 0.2% glucose added when necessary.

**Expressions and purification of ether lipid enzymes**

The bacterial proteins _B. subtilis_ PssA and _E. coli_ PsA were expressed in _E. coli_ BL21 strain and induced with 1 mM IPTG. _E. coli_ PssA was induced with 0.5 mM IPTG in the same overexpression strain _E. coli_ BL21, whereas _E. coli_ PgsA and PgpA were expressed in _E. coli_ Lemo strain and induced with 0.4 mM IPTG and 0.5 mM L-rhamnose. After 2.5 h of induction, the cytoplasmic and membrane fractions were separated as described in a previous study [23]. The inner membrane vesicles (IMVs) of _E. coli_ expressing the membrane proteins (_B. subtilis_ PssA and _E. coli_ Psd, PgsA and PgpA) were isolated as previously described [41]. The _E. coli_ IMVs harbouring the _B. subtilis_ PssA and _E. coli_ Psd were resuspended in buffer A (50 mM Tris/HCl, pH 7.5, 300 mM NaCl and 10% glycerol) and 0.5 mg/ml IMVs were solubilized in 2% DDM (n-dodecyl-β-D-maltopyranoside) detergent for 1 h at 4°C. A centrifugation (173,400 g) step for 30 min at 4°C removed the insolubilized materials and the supernatant was incubated with Ni-NTA (Ni2+-nitritodiacetic acid) beads (Sigma) for 1 h at 4°C. The Ni-NTA beads were washed 10 times with 40 column volumes (CV) of buffer B (50 mM Tris/HCl, pH 7.5, 300 mM NaCl, 10% glycerol and 0.2% DDM) supplemented with 20 mM imidazole, and the proteins were eluted three times with 0.5 CV of buffer B supplemented with 250 mM imidazole. The _E. coli_ IMVs (1 mg/ml) containing the _E. coli_ PgsA and PgpA were...
resuspended in buffer C (50 mM Tris/HCl, pH 7.5, 150 mM NaCl and 10% glycerol). The solubilization steps were performed as above. The E. coli PgsA-bound beads were washed five times with 40 CV of buffer D (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 10% glycerol and 0.2% DDM) supplemented with 10 mM imidazole and eluted three times with 0.5 CV of buffer D supplemented with 250 mM imidazole. The Ni-NTA beads containing E. coli PgpA were washed 10 times with 40 CV of buffer D supplemented with 10 mM imidazole and the protein was eluted with 0.5 CV of buffer D supplemented with 300 mM imidazole.

For the purification of the soluble protein E. coli PssA, the cytoplasmic fraction was incubated with Ni-NTA beads in buffer C overnight at 4°C. The beads were washed three times with 40 CV of buffer C supplemented with 10 mM imidazole, once with 40 CV of buffer C supplemented with 60 mM imidazole and eluted with 2 CV of buffer C supplemented with 300 mM imidazole.

The purity of the eluted proteins were assessed on SDS/PAGE (12% gel) stained with Coomassie Brilliant Blue and the protein concentration was determined by measuring the absorbance at 280 nm. The other cytosolic proteins (E. coli isopentenyl diphosphate isomerase (Idi), E. coli mutant farnesyl diphosphate synthase (IspA), B. subtilis glycerol-1-phosphate dehydrogenase (AraM) and Methanococcus maripaludis DGGPS, A. fulgidus CarS and E. coli CDP-diglyceride synthase (CdsA)) used in the present study were expressed and purified as described previously [22].

### In vitro archaeal lipids production

**In vitro** reactions were performed in 100 μl of assay buffer containing a final concentration of 50 mM Tris/HCl, pH 7.5, 10 mM MgCl₂, 52.5 mM NaCl, 87.5 mM imidazole, 0.07% DDM and 3.5% glycerol. Where specified, 100 μM synthetic DGGPS, 100 μM PA (phosphatidic acid) (C₄,₆), 2 mM CTP, 10 mM G3P, 10 mM L-serine, 20 mM EDTA and the indicated amount of purified enzymes were added to the reaction mixture. For the in vitro reconstitution of AE and AG, 100 μl of reaction volume was used containing the following assay buffer: 50 mM Tris/HCl, pH 7.5, 10 mM MgCl₂, 15 mM NaCl, 25 mM imidazole, 0.02% DDM, 1% glycerol, 0.5 mM DTT, 0.1 mM farnesyl pyrophosphate (FPP), 0.1 mM IPP, 2 mM nicotinamide adenine dinucleotide (NADH) and 2 mM dihydroxyacetone phosphate (DHAP). Reactions were incubated at 37°C for 1 h, as described previously [22] and the products were extracted twice with 0.3 ml of n-butanol. Extracted lipids were evaporated under a stream of nitrogen gas and resuspended in 50 μl of methanol for LC–MS analysis.

### In vivo archaeal lipids synthesis

Engineered E. coli strains were aerobically grown at 37°C in 200 ml of LB medium supplemented with required antibiotics [kanamycin (25 μg/ml), chloramphenicol (17 μg/ml), streptomycin (25 μg/ml) and ampicillin (25 μg/ml), 0.2% of glucose and 1 mM NiCl₂]. The cells were induced with 0.25 mM IPTG and, after 3.5 h, the total membrane fractions were isolated as previously described [23]. The internal standard Eicosane (20 μM) was added to the total membrane fractions (8 mg/ml) and lipids were extracted by means of the Bligh and Dyer method [42]. The chloroform-extractable lipid fraction was washed with the aqueous phase of a blank Bligh and Dyer extraction solution and evaporated under a stream of nitrogen gas. The evaporated samples were resuspended with 0.3 ml 1:2 chloroform/methanol, evaporated under a stream of nitrogen and finally resuspended in 100 μl of methanol for LC–MS analysis.
LC–MS analysis

The sample from in vitro reactions was analysed using an Accela1250 HPLC system coupled with an ESI–MS Orbitrap Exactive (Thermo Fisher Scientific). A volume of 5 μl of each sample was used for the analysis. The LC–MS method parameters used in the present study were the same as described previously [22]. The capillary and the tube lens voltage were set to −75 V and −190 V respectively. For the samples from in vivo experiments, a sample volume of 5 μl was injected into a Shim-pack XR-ODS/C8/phenyl column with dimension 3.0 mm × 75 mm (Shimadzu) operating at 55°C with a flow rate of 400 μl/min. Mobile phase A [10 mM ammonium formate with 0.1% formic acid in water/acetoneitrile (40:60, v/v)] and mobile phase B [10 mM ammonium formate with 0.1% formic acid in acetonitrile/propan-2-ol (10:90, v/v)] were used as follows: initial conditions started with 40 % B, a linear gradient was started in 2 min from 40 % to 43 % B, gradient from 43 % to 50 % B in 0.1 min, 54 % B isocratic for the next 9.9 min, linear gradient from 54 % to 70 % B in 0.1 min, 99 % B isocratic for the following 5.9 min, gradient from 99 % to 40 % B in 0.1 min and 40 % B isocratic for the last 1.9 min [43]. The MS settings used for this analysis were the same as described above. Thermo XCalibur processing software was used for the data analysis and the Genesis algorithm for automated peak detection and integration was applied to this analysis.

RESULTS

Archaetidylethanolamine formation by bacterial PgsA and PgpA enzymes

PG synthesis in E. coli proceeds via two enzymatic steps. The first reaction is catalysed by phosphatidylethanolamine synthase. E. coli contains two individual genes that encode this enzyme activity [44,45]. The pgsA gene exclusively functions in PG formation [46], whereas the pgsB gene is also involved in the synthesis of the lipid A core of lipopolysaccharide (LPS) [47]. The resulting PGP is dephosphorylated to PG. In E. coli, three main PGs are found, encoded by the ppga, ppgB and ppgC genes [48]. PgpB exhibits a narrow substrate specificity dephosphorylating only PGP [49], whereas PgpB also shows phosphatase activity towards DAG pyrophosphate [50], PA and lysophosphatidic acid [51,52]. Bioinformatics analysis [36,39] revealed the occurrence of Pgs homologues in archaea belonging to the CDP–alcohol phosphatidyltransferase family [36,39] revealed the occurrence of Pgs homologues in archaea [53,54] and archaea [55]. The Pgs-II enzymes contain a highly conserved domain present in CDPA–alcohol phosphatidylintransferases [55]. Previously, Ass activity was identified in the membrane fraction of Methanothermobacter thermautotrophicus [56]. Secondary structure analysis of the archaeal members of the CDPA–alcohol phosphatidylintransferase family indicates the presence of eight conserved transmembrane domains (TMDs) comparable to the corresponding bacterial protein family (Figure 3A). Interestingly, the PssA sequence of B. subtilis is substantially smaller than the other members of this family (only 177 amino acid instead of 451 amino acids of the E. coli Pss-I), but it shows the core of five TMDs (Figure 3B) but lacking two C-terminal TMDs. A membrane fraction of B. subtilis incubated with the different archaea-like substrates showed AS formation, whereas such activity could not be demonstrated with E. coli membranes [56] suggesting a more narrow substrate specificity of the E. coli Pss-I and Pgs-II enzymes. Therefore, the pssA gene of B. subtilis was cloned and overexpressed in E. coli BL21 strain under the control of the T7 promoter. The protein was solubilized from the membrane with DDM and purified by Ni-NTA affinity chromatography (Figure 3C). Coupled in vitro reactions were performed using DGGGP as initial substrate along with the A. fulgidus CarS and CTP. Products were extracted with n-butanol and analysed by LC–MS (Figure 3F). In the presence of the B. subtilis PssA, AS (m/z = 802.53 [M-H]− formation was observed in the presence of L-serine and Mg2+ (Figure 3D, lane 3). PssA showed no activity towards DGGGP indicating the strict requirement of a CDPA-activated intermediate for the reaction (Figure 3D, lane 4). Moreover, no AS was formed in presence of EDTA (Figure 3D, lane 5) which chelates divalent cations, consistent with a requirement of Mg2+ for enzymatic activity [57]. As a control, formation of PS by B. subtilis PssA was demonstrated in the presence of PA (C18:1), CTP and the E. coli CdsA (Figure 3D, lane 6). To examine the activity of the E. coli PssA towards CDPA–archaeol, the enzyme was also overexpressed and purified (Figure 3C). In the presence of CDPA–archaeol as substrate, no AS formation was observed demonstrating that the E. coli PssA (the Pss-I enzyme) indeed does not recognize the archaeal precursor (Figure 3E, lane 1). The enzyme, however, converted CDPA–DAG reaction, the conversion of AGP into AG (m/z = 789.55 [M-H]−) was observed when also the E. coli PgpA was included in the reaction (Figure 2B, lane 4). The E. coli PgsA was unable to use archaeal DGGGP as substrate (Figure 2B, lane 5). As a control, the activities of PgsA and PgpA were also tested towards the bacterial substrate CDPA–DAG. The latter was produced by incubating the purified E. coli CdsA protein with its substrates PA (C18:1) and CTP (Figure 2C, lane 2). The formation of PGP (m/z = 853.50 [M-H]−) was detected only in the presence of the E. coli PgsA and G3P (Figure 2C, lane 3), which was further converted into the final product PG (m/z = 773.54 [M-H]−) upon the addition of the E. coli PgpA (Figure 2C, lane 4). These results demonstrate that the E. coli PgsA and PgpA recognize and convert the archaea substrate CDPA–archaeol and AGP respectively, forming the archaeal polar lipid AG.
Figure 2  In vitro demonstration of AG biosynthesis involving the E. coli PgsA and PgpA

(A) Coomassie Blue-stained SDS/PAGE gels showing the Ni-NTA-purified proteins PgsA (21 kDa) and PgpA (20.5 kDa) from E. coli. In vitro reactions using (B) DGGGP or (C) PA (C18:1) and the purified proteins as specified, to test the substrate specificity of the E. coli PgsA and PgpA. Total ion counts from LC–MS data were normalized using DDM as internal standard. The data are the averages for three experiments ± S.E.M. (D) Schematic representation of the in vitro reactions. The purified enzymes used in the experiments are highlighted in blue and the mass spectra from the LC–MS runs of the corresponding products are depicted in the red boxes: DGGGP (m/z = 715.51 [M-H]−), CDP-ol (m/z = 1020.54 [M-H]−), AGP (m/z = 869.51 [M-H]−) and AG (m/z = 789.55 [M-H]−) in (B), and PA (C18:1) (m/z = 699.50 [M-H]−), CDP–DAG (m/z = 1004.54 [M-H]−), PGP (m/z = 853.50 [M-H]−) and PG (m/z = 773.54 [M-H]−) in (C).

into PS (m/z = 786.53 [M-H]−) in a coupled enzyme assay using PA as substrate (Figure 3E, lane 4).

In archaea and bacteria, AE and PE are produced by a decarboxylation reaction of L-serine. In E. coli, the Psd is encoded by the psd gene that specifies a membrane-associated pro-enzyme which undergoes an autocatalytic internal cleavage [58] leading to two subunits, the α-subunit containing a pyruvoyl prosthetic group and a β-subunit [59,60]. Previous bioinformatics analysis [36] identified an archaeal hypothetical protein as a potential Asd showing sequence similarity to the bacterial Psd (Supplementary Figure S3). Moreover, a similar operon conservation of the two genes pss/psd, typical of several bacterial species, was found in some archaea [36]. However, the archaeal Asd has not been biochemically characterized. Because of the general mechanism of the decarboxylation reaction [23], the possibility exists that the endogenous Psd of E. coli is able to recognize AS. Therefore, the E. coli psd gene was overexpressed in E. coli BL21 strain under the control of the T7 promoter. Membranes bearing overexpressed levels of Psd were solubilized with DDM and the enzyme was purified by Ni-NTA affinity chromatography (Figure 4A). The α-subunit showed a slower migration on SDS/PAGE, as expected on the basis of its predicted molecular mass. This is probably due
Figure 3  In vitro AS synthesis by the B. subtilis PssA

(A) Averaged hydrophathy profile of the bacterial Pss proteins (blue line) and archaeal ones (red line) is aligned highlighting their conservation in the two kingdoms. The bacterial hydrophobicity profile is based on a multiple sequence alignment of 260 sequences sharing between 40% and 80% sequence identity. Likewise, the archaeal hydrophobicity plot is based on a multiple sequence alignment of 38 archaeal sequences having a sequence identity between 30% and 70%. The membrane topology prediction is depicted above the plot. (B) Hydrophathy profile alignment of B. subtilis PssA (purple line) and the averaged hydrophathy profile of the bacterial Pss protein family (blue line). The conserved TMDs are indicated by roman numbers. (C) Coomassie Blue-stained SDS/PAGE gels showing the Ni-NTA-purified proteins B. subtilis PssA (21 kDa) and E. coli PssA (53.6 kDa). Specificity of the bacterial B. subtilis PssA (D) and E. coli PssA (E) towards DGGGP and PA (C18:1) as assessed by means of an in vitro assay using the purified enzymes. Total ion counts from LC–MS data were normalized using DDM as internal standard. The data are the averages for three experiments ± S.E.M. (F) Schematic representation of the performed in vitro reactions. The mass spectra from the LC–MS runs of the two products AS (m/z = 802.54 [M-H]⁻) and PS (m/z = 786.53 [M-H]⁻).
to the presence of a covalently attached pyruvyl prosthetic group that may affect the folding state in SDS/PAGE [59]. The enzymatic activity of the E. coli Psd was tested by in vitro coupled reactions as described above and product formation was detected by LC–MS (Figure 4C). Conversion of AS to AE (m/z = 758.55 [M-H]−) by the E. coli Psd was observed in the presence of Mg2+, L-serine, B. subtilis PssA, A. fulgidus CarS, CTP and DGGGP (Figure 4B, lane 4).

**In vitro reconstitution of archaetidylethanolamine and archaetidylglycerol formation**

Previously, we have described the in vitro reconstitution of CDP-archaeol formation starting from the precursors IPP and DMAPP/FPP [22] using two bacterial enzymes and three archaeal enzymes that were overexpressed and purified from E. coli upon codon optimization. Due to the wider substrate specificity described above, the extension of this pathway for the reconstitution of the formation of AE and AG requires four additional bacterial enzymes described in the previous section.

The ether lipid biosynthetic pathway reconstitution [22] starts from the two isoprenoid building blocks IPP and FPP which undergo several cycles of condensation leading to isoprenoid chains with the required C20 length [61]. GGPP (m/z = 449.19 [M-H]−) formation was detected by LC–MS with an E. coli IspA [62] mutant as described previously [22] (Figure 5A, lane 2). Despite the unique feature of archaeal GIP dehydrogenases to synthesize the G1P, some bacterial enzymes are also able to perform this reaction [63]. The enzyme AraM (G1PDH) from B. subtilis [64] was used that produces the required glycerophosphate backbone which can be attached to the GGPP via the first ether bond leading to the synthesis of GGPP (m/z = 443.26 [M-H]−). M. maripaludis GGGPS (MmarC7_1004) [65] was used to catalyse the latter reaction which, in combination with IspA and G1PDH and the substrates IPP, FPP, DHAP and NADH, leads to the conversion of GGPP into GGGP (Figure 5A, lane 3). GGGP production (m/z = 715.51 [M-H]−) was observed in a subsequent reaction with the A. fulgidus GGGPS (AF0404) [20] (Figure 5A, lane 4).

Next, DGGGP was converted into CDP-archaeol (m/z = 1020.54 [M-H]−) by the A. fulgidus CarS (AF1740) in the presence of CTP [22] (Figure 5A, lane 5). CDP-archaeol is the precursor for the formation of AS (m/z = 802.51 [M-H]−) in the presence of the B. subtilis PssA and L-serine (Figure 5A, lane 6), which was further converted into AE (m/z = 758.54 [M-H]−) (Figure 5A, lane 7) by the E. coli Psd. In another reaction, the CDP-archaeol was converted into ACP (m/z = 869.51 [M-H]−) by the E. coli PgpA in the presence of G3P (Figure 5A, lane 8). ACP was then converted into AG (m/z = 789.54 [M-H]−) by the addition of the E. coli PgpA to the in vitro reactions (Figure 5A, lane 9). Taken together, the reactions described in the present study employing purified enzymes represent the in vitro reconstitution of the entire archaeal lipid pathway using a set of archaeal and bacterial enzymes.

**Archaetidylglycerol and archaetidylethanolamine formation in E. coli**

To reconstruct the entire archaeal ether lipid biosynthetic pathway into E. coli, a system of four compatible expression vectors was used to co-express seven ether lipid genes into E. coli. In this system, the vectors containing one or two genes each, as listed in Table 1, allowed the simultaneous expression of three archaeal enzymes (M. maripaludis GGGPS, A. fulgidus DGGGPS and A. fulgidus CarS) and four bacterial enzymes (E. coli Idi, E. coli mutant IspA, B. subtilis AraM and B. subtilis PssA). All genes were expressed under the control of the T7 promoter and induced with 0.25 mM IPTG for 3.5 h. Total lipids were extracted from the E. coli membrane fraction using the Bligh and Dyer method [42] and analysed by LC–MS. Four different engineered E. coli strains were compared, containing a different combination of archaeal lipid enzymes: (1) control harbouring only the empty vectors; (2) five ether lipid enzymes (E. coli Idi, E. coli mutant IspA, E. coli mutant IsfA, B. subtilis AraM, M. maripaludis GGGPS and A. fulgidus DGGGPS); (3) six ether lipid enzymes (E. coli Idi, E. coli mutant IspA, B. subtilis AraM, M. maripaludis GGGPS, A. fulgidus DGGGPS and A. fulgidus CarS); and (4) seven ether lipid enzymes (E. coli Idi, E. coli mutant IspA, B. subtilis AraM,
In vitro reconstitution of the archaeal ether lipid pathway

In vitro reactions were performed using a combination of purified enzymes as specified and the substrates IPP, FPP, DHAP and NADH in presence of Mg$^{2+}$ and 0.2 % DDM. Each product was detected by LC–MS: FPP (m/z = 381 [M-H]−), GGPP (m/z = 449.19 [M-H]−), GGGP (m/z = 443.26 [M-H]−), DGGGP (m/z = 715.51 [M-H]−), CDP–archaeol (m/z = 1020.54 [M-H]−), AS (m/z = 802.51 [M-H]−), AE (m/z = 758.54 [M-H]−), AGP (m/z = 869.51 [M-H]−) and AG (m/z = 789.54 [M-H]−). The total ion counts were normalized using DDM as internal standard. Results are averages for two experiments ± S.E.M.

Figure 5  In vitro reconstitution of the archaeal ether lipid pathway

In vitro reactions were performed using a combination of purified enzymes as specified and the substrates IPP, FPP, DHAP and NADH in presence of Mg$^{2+}$ and 0.2 % DDM. Each product was detected by LC–MS: FPP (m/z = 381 [M-H]−), GGPP (m/z = 449.19 [M-H]−), GGGP (m/z = 443.26 [M-H]−), DGGGP (m/z = 715.51 [M-H]−), CDP–archaeol (m/z = 1020.54 [M-H]−), AS (m/z = 802.51 [M-H]−), AE (m/z = 758.54 [M-H]−), AGP (m/z = 869.51 [M-H]−) and AG (m/z = 789.54 [M-H]−). The total ion counts were normalized using DDM as internal standard. Results are averages for two experiments ± S.E.M.

M. maripaludis GGGPS, A. fulgidus DGGGPS, A. fulgidus CarS and B. subtilis PssA). When the total phospholipid composition of E. coli was compared for the different strains, no major differences were observed for the four differently engineered E. coli strains (Figure 6A). However, a detailed analysis showed the presence of a peak corresponding to AE (unsaturated) in E. coli strain 4 (Figure 6B, lane 4) which contains all of the ether lipid genes required for synthesis compared with the other strains that lack the B. subtilis PssA (Figure 6B, lane 3) or both the A. fulgidus CarS and the B. subtilis PssA (Figure 6B, lane 2) or
Figure 6  Archaeal lipid production in E. coli

(A) Total lipid analysis of four different engineered E. coli strains containing a different combination of ether lipid enzymes. The lipids were extracted from the membrane fraction and analysed by LC–MS. The total ion counts of the several PE and PG species and of the archaeal lipids were normalized using Eicosane (m/z = 281.55 [M-H]−) as internal standard. The species are classified according to the number of carbon atoms and the number of unsaturated bonds in the acyl chains at the sn-1 and sn-2 positions. (B) Archaeal lipids production by different strains of E. coli upon the expression of the ether lipid biosynthetic genes. Results are the averages for three biological replicates ± S.E.M. (C) In vitro reactions using DGGGP and the purified E. coli CdsA, PgsA and PgpA proteins to determine the ability of CdsA to convert DGGGP into CDP–archaeol. Total ion counts from LC–MS data were normalized using DDM as internal standard. The data are the averages for three experiments ± S.E.M.
the control (Figure 6B, lane 1). Another archaea-specific product dierganlygeranylglyceryl phosphoglycerol (DGGGP-Gro) (m/z = 789.5 [M-H]−) was detected in the engineered E. coli strains, which is absent from the control strain (Figure 6B, lane 1). This compound was also detected in a previous study [66] and measured as the unsaturated form of AG. This lipid was observed only in the E. coli strains that contain the basic set of five ether lipid genes up to the genes required for the formation of DGGGP (Figure 6B, lane 2). However, an enhanced production of unsaturated AG was observed when the strain also contains the A. fulgidus CarS (Figure 6B, lane 3). Upon the introduction of the AS-producing B. subtilis PssA enzyme (strain 4), the amount of AG decreased and instead, AE was produced (Figure 6B, lane 4).

Formation of unsaturated AG has been attributed to an endogenous reaction in E. coli which directly attaches the glycerol moiety to DGGGP [66], as it was assumed that the E. coli PgsA and PgpA enzymes are not able to accept the archaeal intermediate as a substrate. As shown in the previous section, these two enzymes do recognize CDP–archaeol and convert it into AG. Therefore, the presence of unsaturated AG in the E. coli strain expressing the ether lipid genes is due to the low, but significant, activity of the endogenous CDP–DAG synthase towards DGGGP. To confirm this hypothesis, an in vitro reaction was performed where DGGGP was incubated in presence of the purified CDP–DAG synthase (E. coli CdsA) in the presence of CTP. Under those conditions, a significant fraction of the DGGGP was converted into CDP–archaeol (Figure 6C, lane 2) demonstrating that CdsA is active with the archaeal substrate. When the E. coli PgsA and PgpA and the substrate G3P were added to the reaction, the CDP–archaeol was converted into AG (Figure 6C, lane 3). Taken together, these data demonstrate that the low activity of the endogenous E. coli CdsA (in vitro less than 1% compared with CarS in our previous work [22]) towards DGGGP results in substantial AG production in vivo. It is concluded that with a limited set of genes the archaeal lipids AG and AE can be produced in E. coli.

**DISCUSSION**

For several decades, E. coli has been used for metabolic engineering such as the improvement of isoprenoid or carotenoid production [67–70]. Several attempts have been made to reconstruct the archaeal ether lipid biosynthetic pathway into E. coli. Gunsalus et al. [10] demonstrated production of DGGGP upon the overexpression of the endogenous E. coli Idi and expression of four enzymes (G1PDH, GGPPS, GGGPS and DGGGPS) from the hyperthermophilic archaeon A. fulgidus. Likewise, Yokoi et al. [66] employed ether lipid genes from the mesophilic archaeon Methanococccus maripaludis acetoacitrons to produce DGGGP and another compound that was identified as the PG-type derivative of DGGGP, named DGGGP-Gro. Isole et al. [71], in addition, expressed the M. acetivorans geranylgeranyl reductase for double bond reduction in conjunction with the four aforementioned archaeal enzymes [66]. They observed the in vivo production of archaeal lipids with a fully saturated isoprenoid chain only when the cofactor M. acetivorans ferredoxin was co-expressed.

In the present study, we aimed to produce the two major archaea-like lipids AE and AG in E. coli as these lipids have the same polar head group as the major phospholipids PE and PG present in the E. coli membrane. However, this required a further understanding of the enzymatic steps required for polar head group attachment. Importantly, we have previously shown the production of CDP–archaeol in vitro by also including the CarS into the pathway that yielded DGGGP. Expression of CarS provides a means to substantially increase the production of the endogenously produced DGGGP-Gro that corresponds to AG (Figure 6B). During polar head group attachment, the CDP group from the CDP-activated precursor is replaced by a different polar head group. In bacteria and in archaea, the reactions involved in this process are very similar and mediated by the enzymes belonging to the CDP–alcohol phosphatidytransferase superfamily. Given the high sequence homology between archaeal and bacterial phosphatidytransferase [36,39], we investigated the substrate promiscuity of the enzymes PgsA and PgpA that are involved in PG formation and their ability to recognize CDP–archaeol. Using in vitro reactions, purified E. coli PgsA was able to produce AGP from CDP–archaeol which in turn was produced by the A. fulgidus CarS from DGGGP, CTP and G3P. By means of the E. coli phosphatase PgpA, the AGP was readily converted into AG. This demonstrates a high substrate promiscuity of these bacterial enzymes and alleviates the need to introduce archaeal enzymes into E. coli to perform these reactions. Indeed, when the ether lipid biosynthesis pathway up to the formation of CDP–archaeol is introduced into E. coli, AG formation is observed. However, CarS is not essential, as, even in its absence, some AG can be formed. The origin of this AG was previously unknown, but we now show that this is due to a low activity of the endogenous E. coli CdsA for DGGGP resulting in the formation of CDP–archaeol that is further converted by E. coli PgsA and PgpA into AG.

In contrast with AG formation, AE formation has not been observed previously in E. coli which must imply that the E. coli Psses are unable to recognize CDP–archaeol in order to produce the intermediate AS that by decarboxylation should be further converted into AE. Indeed, the purified E. coli PssA was inactive with this substrate. Structural analysis of the bacterial and archaeal members of CDP–alcohol phosphatidytransferase family indicated a high level of secondary structure conservation of these enzymes with eight transmembrane segments. The B. subtilis PssA is a truncated version of these enzymes, being significantly shorter but still containing the highly conserved protein core. Importantly, this enzyme was previously shown to be active with the archaea-like substrates [35]. Indeed, the purified B. subtilis PssA catalysed the production of AS from CDP–archaeol and L-serine. The next step, the decarboxylation of the serine moiety, seems less specific, as purified E. coli Psd mediated the decarboxylation of AS with the concomitant formation of AE. Thus, for the production of AE in E. coli cells, only the B. subtilis PssA needs to be introduced (Figure 6C).

Having established the exact sequence of reactions needed for the archaeal ether lipid biosynthetic pathway [22], including the polar head group attachment, the reconstitution of AE and AG formation in vitro could be realized. Using a combination of archaeal and bacterial enzymes and a breakdown in individual reactions, the synthesis of the unsaturated archaeal lipids AE and AG from the initial building blocks IPP, FPP and DHAP was achieved with nine purified enzymes. This defined the conditions needed for the reconstitution of AE and AG formation in vivo, using E. coli as a host. Since three endogenous E. coli enzymes (Psd, PgsA and PgpA) recognize the archaeal precursors, the in vivo reconstitution depends on the expression of seven ether lipid genes. This includes overexpression of the E. coli Idi to boost IPP formation and expression of a mutant IspA to generate GGPP; the B. subtilis AraM for GIP formation and B. subtilis PssA for AS formation, the key ether lipid biosynthetic proteins, the M. maripaludis GGGPS, the A. fulgidus DGGGPS and the A. fulgidus CarS. Although the conversion reactions appear efficient in vitro, the amounts of AE and AG produced in vivo are still low and, compared with the total E. coli lipid content, they are probably...
less than 1%. However, the expression of CarS elevated the levels 3-fold as compared to a previous report on AG formation [66].

The work described in the present study represents a unique strategy to synthesize archaeal ether lipids in bacteria. Although the levels are still low, it is important to realize that the production of AE and AG as reported in the present study is performed in the presence of a fully functional phospholipid biosynthetic pathway. High-level AE and AG production probably also requires the up-regulation of the entire pathway leading to IPP production. In addition, the pathway resulting in the reduction of the isoprenoid chains needs to be introduced to produce the saturated archaeatalyl compounds. Future studies should be directed towards a gradual down-regulation of the endogenous ester-bonded phospholipid biosynthetic pathway so that the endogenous lipids can be replaced by ether lipids. Such a bacterial strain could be used as an experimental model to examine the impact of the ‘lipid divide’ on the physiology and robustness of bacteria.

AUTHOR CONTRIBUTION
Antonella Caforio cloned the genes, purified the enzymes and performed the experiments. Melvin Silnik assisted in the cloning of genes and John van der Oost coordinated the pathway design. Adriaan Minnaard designed the DGGP synthesis, which was performed by Peter Fodran. The manuscript was written by the contributions of all authors.

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