Chapter 4

Engineering of *Escherichia coli* with a hybrid heterochiral membrane

Manuscript in preparation

Antonella Caforio¹,##, Melvin Siliakus²,##, Marten Exterkate¹, Samta Jain¹,⁴, Varsha R. Jumde³, Ruben L.H. Adringa³, Servé W. M. Kengen, John van der Oost², Adriaan J. Minnaard³ and Arnold J.M. Driessen¹*

¹Department of Molecular Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, 9747 AG Groningen, The Netherlands; The Zernike Institute for Advanced Materials, University of Groningen, 9747 AG Groningen, The Netherlands

²Department of Microbiology, Wageningen University, Dreijenplein 10, 6703 HB Wageningen, The Netherlands

³Stratingh Institute for Chemistry, University of Groningen, 9747 AG Groningen, The Netherlands

⁴Present address: Department of Medicine, Section of Infectious Diseases, Boston University School of Medicine, 02118 Boston, Massachusetts, United States of America

Both authors equally contributed to the work
Abstract

The last universal common ancestor (LUCA) is the most recent organism from which all organisms now living on Earth have a common descent. The membrane composition of LUCA represents an unresolved aspect in the differentiation of Bacteria (and Eukarya) and Archaea. The driving force behind this segregation has often been attributed to the chemical instability of a mixed membrane composed of a racemic mixture of glycerol-1P ether and glycerol-3P ester based lipids. However, such mixed membranes have never been reproduced in living cells. Here, we present for the first time a stable hybrid heterochiral membrane through lipid engineering of the bacterium *Escherichia coli*. By using a combination of metabolic engineering to boost isoprenoid biosynthesis and heterologous expression of the archaeal ether lipid biosynthetic pathway genes, an *E. coli* strain was obtained with up to 32% of archaeal lipids in the lipidome with the expected chirality. This resulted in viable cells but with altered cell growth, morphology and robustness towards environmental stress. The hybrid heterochiral membrane bacterial strain sheds new light on the lipid divide and opens novel possibilities for bio-industrial applications.
**Introduction**

A widely accepted hypothesis on the separation of the three domains of life Archaea, Bacteria and Eukarya is the existence of a common living ancestor, known as LUCA (last universal common ancestor) or cenancestor, from which the archaea and bacteria have diverged. Based on genomic analysis, predictions have been made about the organization of the transcriptional and translational machinery present in LUCA during the early stages of evolution [1]. The cell membrane of LUCA has attracted particular attention. Although the acellular theory states that the ancestor cell was characterized by the absence of a membrane [87] or surrounded by mineral membranes [88], most theories claim the presence of a cenancestor with a defined cellular membrane as a consequence of the need of compartmentation and self replication [158]. The existence of a phospholipid based membrane in the ancestor cell is further supported by phylogenetic studies that revealed a high conservation of the mevalonate pathway for the synthesis of the isoprenoid building blocks in archaea, eukarya and some bacterial species [5,9]. Also the presence of conserved membrane proteins such as the ATPase [159], redox proteins for respiration [160] and proteins involved in secretion like Sec and YidC [161] suggests that LUCA was embroidered by a phospholipid based cellular membrane. However, the chemical identity of the membrane lipids of LUCA remains an unresolved question considering the remarkable differences between archaeal and bacterial lipids and the hereinto "lipid divide" that differentiates these two domains of life. Archaeal lipids are composed of isoprenoid chains, connected via ether linkages to a glycerol-1-phosphate (G1P) backbone while in bacteria and eukarya, phospholipids are based on straight-chain fatty acid esters linked to the enantiomeric glycerol-3-phosphate (G3P) backbone. Therefore, the stereochemical configuration of the lipids represents a crucial aspect to take into consideration during the divide between archaea and bacteria.

Assuming that the pre-cell was characterized by non-stereoselective enzymes [89] and abiotic catalysis, a racemic mixture of both G1P and G3P must have been present. Thus, the downstream enzymes of the lipid biosynthetic pathway should have had the capability of recognizing both
substrates leading to the formation of a heterochiral membrane. However, the existence of ancestral pre-cells with heterochiral membranes is assumed to be an unstable situation and expected to evolve towards a more stable homochiral membrane upon the occurrence of stereoselective enzymes and the differentiation between archaea and bacteria [87,89]. In contrast, in vitro experiments using liposomes composed of a mixture of archaeal and bacterial lipids showed a higher stability of mixed liposomes than those composed of only archaeal [90] or bacterial lipids in term of the temperature dependent permeability [91]. Although some bacteria are known to produce small quantities of ether lipids as well [162], so far no consistent evidence of the coexistence of substantial amounts of two phospholipids with opposite chirality has been observed in the membrane of any living cell. Few studies attempted to reproduce an in vivo heterochiral mixed membrane by introducing the partial [21,53] or almost entire [163] ether lipid biosynthetic pathway into the bacterium *Escherichia coli*, but the levels of ether lipids produced were minor and less than 1% compared to the endogenous *E. coli* lipid content.

Here we report the engineering of a hybrid heterochiral membrane in a viable bacterial cell. Via the upregulation of the synthesis of the isoprenoid building blocks and the co-expression of the archaeal lipids biosynthetic pathway genes, archaeal lipids with the G1P configuration were produced in *E. coli*, replacing nearly the complete endogenous pool of phosphatidylglycerol for archaetidylglycerol. In addition, we uncover substrate promiscuity of key enzymes of the archaeal lipid biosynthetic pathway, which supports the existence of a common ancestor with a heterochiral membrane from which archaea and bacteria diverged.

**Results**

**Lipid biosynthesis engineering in *E. coli***

Almost all enzymes involved in the biosynthesis of diether lipids in Archaea have been identified and characterized [2,6,21,41,127,163] (Figure S1). The first metabolic step is the isoprenoid building blocks biosynthesis. The two isoprenoid units, isopentenyl-diphosphate (IPP) and
dimethylallyl-diphosphate (DMAPP), are widespread in nature and used in many other biosynthetic processes such as carotenoids, steroids or quinones. Their condensation by the enzyme geranylgeranyl diphosphate (GGPP) synthase leads to an isoprenoid chain of twenty carbon atoms [19,44]. The glycerophosphate backbone (G1P) is synthetized in Archaea by the glycerol-1-phosphate dehydrogenase (G1PDH) [28,29]. Even though bacteria have a different glycerophosphate configuration (G3P) conferred by the evolutionary unrelated enzyme glycerol-3-phosphate dehydrogenase (G3PDH) [2], G1PDH is also found in some bacteria [31]. GGPP and G1P are linked via two ether bonds catalyzed by two different archaeal enzymes: geranylgeranylglycerol phosphate (GGGP) synthase [35,46] and digeranylgeranylglycerol phosphate (DGGGP) synthase [38,41]. CDP-archaeol formation from DGGGP involves the recently discovered CarS enzyme [41]. Next, the replacement of the CDP moiety present in CDP-archaeol with a polar head group such as glycerol-3-phosphate or L-serine [163] leads to the formation of archaetidylglycerol (AG) and archaetidylethanolamine (AE), respectively. In Archaea, the isoprenoid chains are further saturated, but the exact mechanism of this process has not yet been fully resolved.

Thus, in order to reproduce a hybrid heterochiral membrane in the bacterial host *E. coli* strain JM109DE3, a composite pathway was developed that consists of both bacterial and archaeal enzymes (Table S1) in order to yield the unsaturated archaetidylglycerol (AG) and archaetidylethanolamine (AE) which are counterparts of the bacterial phosphatidylglycerol (PG) and phosphatidylethanolamine (PE), respectively. In previous work, this resulted in less than 1% of unsaturated AG and AE of the total lipidome [163]. To achieve much higher amounts of ether lipids, the endogenous MEP-DOXP pathway, responsible for IPP and DMAPP synthesis in *E. coli* was upregulated. Two synthetic operons composed of only the native *IDI* gene and *IDI*, *IspD*, *IspX* and *DSX* genes were integrated at the ‘ori’ macrodomain of the *E. coli* chromosome [164,165] (Figure S2 A). The two obtained strains (listed in Table 1) containing the single *IDI* gene (*E. coli* IDI+) and the entire operon *IDI*, *IspD*, *IspX* and *DSX* (*E. coli* MEP/DOXP+), respectively. These were tested for inducible MEP-DOXP upregulation using a reporter that converts IPP and
DMAPP to the red carotenoid lycopene [166]. The upregulation with 10 µM of Isopropil-β-D-1-tiogalattopiranoside (IPTG), resulted in a 2.5 and 5.3-fold increase in lycopene production by the engineered strains, respectively (Figure S2 B). The successful increase of isoprenoid building block production represented the starting point for archaeal ether lipids production. Using a system of two compatible vectors (Figure S2 A), up to six ether lipid genes were introduced into the E. coli IDI+ and E. coli MEP/DOXP+ strains leading to E. coli IDI+EL+ and E. coli MEP/DOXP+EL+ strains, respectively. This concerned the crtE gene from Pantoea ananatis [166,167] encoding a GGPP synthetase, araM from Bacillus subtilis [31] specifying the G1P dehydrogenase, MmarC7_1004 [35,46] and MmarC7_RS04845 [38,39] from Methanococcus maripaludis encoding the GGGP and DGGGP synthases, respectively, AF1749 (CarS) from Archaeoglobus fulgidus [41] encoding the CDP-archaeol synthase and pssA from Bacillus subtilis encoding a phosphatidylserine synthase (PssA) [47,163] (Table S1). For the attachment of glycerol as polar head group and the conversion of L-serine into ethanolamine, the endogenous enzymes Psd, PgsA and PgpA of E. coli were exploited due their ability to recognize the archaeol derivatives [163] (Figure S1).

Increased IPP and DMAPP production dramatically stimulated the synthesis of unsaturated AG (Figure S2 C). A higher amount of this lipid was observed in the E. coli MEP/DOXP+EL+ strain, harboring the entire MEP-DOXP operon compared to the E. coli IDI+EL+ strain containing only the IDI gene and the control strain (Figure S2 C). A second operon, containing the crtE, araM and MA3969 genes, was further integrated into the bacterial chromosome, but this did not improve ether lipids production compared to plasmid based expression (data not shown). Despite the presence of the B. subtilis pssA gene for the synthesis of unsaturated AE, only a very low amount of this lipid was detected. The use of alternative Ribosome Binding Sites (RBS) or the addition of L-serine to the growth medium [168] did not improve AE synthesis (data not shown). As previously shown [169], overexpression of the B. subtilis PssA impairs cell growth likely because of elevated levels of the non-bilayer lipid PE which is lethal to the cells [148]. Thus, the study further focuses on the increased AG levels.
Ether lipid production optimization

The obtained engineered E. coli strain (E. coli MEP/DOXP*EL*) was further optimized in terms of growth and induction to achieve the highest amounts of ether lipids possible. Herein, ether lipid synthesis was analyzed in a defined minimal medium (OPT1), optimized for increased isoprenoid production [170]. The total lipid analysis revealed a 2.1-fold increased AG production when the strain was grown in OPT1 medium compared to growth in rich LB medium (Figure S3 A). Further optimization was performed by inducing the engineered E. coli strain with 100 μM of IPTG at different growth phases. Induction at the beginning of growth (OD$_{600}$ = 0.0) yielded the highest amounts of AG compared to induction at the early (OD$_{600}$ = 0.3) and mid-exponential (OD$_{600}$ = 0.6) growth phase (Figure S3 B). Next, different IPTG concentrations were used to induce AG production. The distribution of the main bacterial phospholipids PE, PG, and cardiolipin (CL) and the archaeal lipid AG was compared among the different strains using LC-MS [163] (Figure S3 C), TLC (Figure 1A) and lipid quantitation via phosphorous determination. Lower amounts of inducer resulted in a higher AG lipid production. From the quantitative TLC analysis, the PG content decreased from 52% in the wild type to less than 5% in the strain induced with 10 μM IPTG (Figure 1B), while the AG content increased up to 32%. Higher amounts of IPTG (50-100 μM) resulted in less AG production and higher amounts of PG. With the decrease in PG content, we noted triacylglyceride accumulated in the cells consistent with their decreased demand because of the increased levels of AG production (Figure 1A). These data demonstrate that essentially the entire PG pool can be replaced by AG, resulting in a hybrid membrane.

Ether lipid chirality and archaeal enzyme substrate promiscuity

The G1P configuration is the most striking features that distinguishes archaeal and bacterial phospholipids. However, to ascertain that the correct archaeal lipid chirality was present, a strain was constructed that lacks the G1PDH due to an araM gene deletion (E. coli MEP/DOXP*EL* AraM). Surprisingly, in the absence of G1PDH, still high levels of AG were detected, although lower as compared to the strain bearing a functional araM (Fig. S3 C). This finding raised the question if E. coli can produce G1P
Figure 1 | Thin Layer Chromatography (TLC) based quantitation of in vivo archaeal lipids synthesis. (A) TLC of lipids extracts from wild type E. coli, heterochiral mixed membrane E. coli induced with different IPTG concentrations and the E. coli strain harboring the entire ether lipid pathway but lacking the araM gene. (B) Relative quantitation of the spots detected in the TLC. Each lipid species was calculated as percentage of the total amount of lipid phosphorous detected in each lane.
or whether the ether lipid pathway contains non stereoselective enzymes. The first ether lipid enzyme involved in recognition of G1P is GGGPS, which links the long isoprenoid chain GGPP to the glycerophosphate backbone of G1P. This enzyme is considered to be highly stereoselective, as GGGPS of *Thermoplasma acidophilum* exhibited only a low enzymatic activity with G3P [46] and as confirmed by the resolved G1P and GGPP binding sites in the protein structures of GGGPS from *A. fulgidus* [36] and *Methanothermobacter thermoautotrophicus* [35]. However, analysis of the activity of GGGPS from *M. maripaludis* in vitro using GGPP as substrate revealed a remarkable non selectivity towards G1P and G3P (Figure 2A, lane 2 and 3). Also, purified G3P acyltransferase from *E. coli* (PlsB), involved in the attachment of the glycerophosphate backbone to the fatty acid chain [94,171], was tested for its specificity towards G3P and G1P. To this end an in vitro system was used to synthetize acyl-CoA by condensation of oleic acid and CoA by the *E. coli* FadD enzyme [172,173] (Figure 2B, lane 1) which is subsequently converted by PlsB into lysophosphatidic acid (LPA). LPA production was observed only in presence of G3P (Fig. 2B, lane 2) and no product was detected with G1P (Figure 2B, lane 3) demonstrating a very high stereoselectivity of PlsB. Kinetic analysis of the GGGPS enzyme with G1P and G3P showed 9 times higher preference of the enzyme towards G1P (Kₘ = 5.8 ± 1.6 µM) as compared to G3P (Kₘ = 46.7 ± 6 µM) (Figure 2C and D). The weaker chiral specificity of GGGPS could potentially account for AG formation in the absence of G1PDH. Therefore, to conclusively establish the configuration of the diether lipids in the engineered *E. coli* strains, both enantiomers of AG were prepared chemically and compared with the AG produced in *E. coli*. In short, saponification of the total lipid extract allowed the subsequent purification of the ether lipids by chromatography on silica. Chemical synthesis of AG with G1P (Figure 2E, panel II), and AG with G3P (Figure 2E, panel I) configuration was carried out according to our previous work [41]. All three samples were converted into their corresponding Mosher’s ester and analyzed by ¹H- and ¹⁹H-NMR [174]. Readily distinguished diastereotopic shifts in the ¹H-NMR (Figure 2E) showed unambiguously that the AG produced by the engineered strains both with (Figure 2E, panel IV) and without (Figure 2E, panel V) G1PDH have the archeal G1P...
Figure 2 | Chirality of the ether lipid biosynthesis in E. coli and stereoselectivity of the archaeal GGGPS. Specificity of archaeal M. maripaludis GGGPS (A) and the bacterial E. coli PlsB (B) enzymes towards G1P and G3P. Kinetic analysis of M. maripaludis GGGPS using different concentration of G1P (C) or G3P (D). Total ion counts are normalized using DDM as internal standard. Results are the averages of two experiments ± S.E.M. (E) NMR spectra of Mosher’s ester derivatized AG. Synthetic AG with G3P configuration (I), synthetic AG with G1P configuration (II), a mixture of both (III), AG from the E. coli strain expressing the whole ether lipid biosynthetic pathway (IV) and from the E. coli strain harboring the AraM gene deletion (V). The red boxes highlight the diagnostic signals.
configuration. This confirms a high selectivity of the ether lipid enzymes for G1P \textit{in vivo}, but also indicates that \textit{E. coli} harbors an endogenous mechanism of G1P production.

**Growth and cell morphology**

The replacement of the endogenous PG pool for AG impacted bacterial growth (Figure 3A). The \textit{E. coli} MEP/DOXP-EL+ strain showed a long lag phase of \textasciitilde16 hours before growth commenced with a growth rate similar to the parental strain. Both the non-induced and induced (10 µM) cells showed a similar growth behavior likely because of leakage of the promoter used to express the archaeal lipid enzymes as evidenced by the presence of AG in non-induced cells (Figure S3 C). With increased IPTG concentration (up to 100 µM), the lag phase shortened to \textasciitilde8 hours but growth proceeded with a slower rate. Ether lipid biosynthesis caused an elongation of the cell length (Figure 3B, panel II) as evidenced by Scanning Electron Microscopy (SEM). The observed phenotype affects the majority of the cells with cell lengths ranging between 2 and 12 µm, compared to 500 nm of control \textit{E. coli} cells. In particular, at higher IPTG levels, the engineered cells exhibited lobular appendages which extrude from the cell surface, ranging between 100 and 500 nm in diameter. These bulges occur at the cell poles or on the side. It appears that cell division takes place at these appendages sites, leaving at time a scar on the mother cell and the formation of small daughter cells (Figure 3B, panel III). At higher inducer levels, the phenomenon is more frequent and filamentous extrusions are formed connecting cells and cellular aggregates. Released extrusions could readily be isolated from the supernatant after high speed centrifugation of a cell culture. Total lipid analysis on the isolated appendages revealed a mixture of archaeal and bacterial lipids similar to the lipid content of the mother cells, (Figure S3 D) excluding the hypothesis that these structures are the result of lipid segregation. Also, the SDS-PAGE protein profile of cells and the isolated appendages was similar (data not shown). We hypothesize that these extrusions are formed as a result of high level lipid production.

To further examine the aberrant division mechanism, \textit{E. coli} cells were stained with the dyes FM4-64 and DAPI that stain lipids and DNA,
Figure 3 | Growth and cell morphology analysis of the heterochiral mixed membrane strains. (A) Growth of the *E. coli* MEP/DOXP-EL+ strain with all the ether lipids enzymes (not induced (---), induced with 10 μM (—), and induced with 100 μM (——) of IPTG) compared with two negative control strains (*E. coli* JM109DE3 wild type (—) and *E. coli* MEP/DOXP+ strain with the integrated MEP-DOXP operon (——)). The data are the averages of three biological replicates ± S.E.M. (B) Scanning Electron Microscopy (SEM) of wild type *E. coli*, the heterochiral mixed membrane strain induced at a later (0.3 OD$_{600}$) and earlier (0.03 OD$_{600}$) growth phase using 100 μM of IPTG. (I) Field of cells. (II) Altered cell shape and length. (III) Aberrant cell division and formation of bulges and shreds. (C) Effect of mixed heterochiral membranes on *E. coli* cells detected by double staining with FM4-64 and DAPI. (I-II) Lipid staining showing elongated and thinner cells in the engineered strain compared to the control. (III) Presence of membrane associated spots in the engineered strain. (V) Double staining with FM4-64 and DAPI showing the presence of appendages surrounded by a lipid layer and the presence of DNA. (IV-VI) Presence of irregular division sites in engineered cells compared to the symmetrical division septum present in the wild type cells.
respectively. The FM4-64 staining confirmed the presence of elongated and thinner cells in the engineered strain compared to wild type cells (Figure 3C, I-II). Furthermore, the lipid staining also signified the presence of intense membrane associated spots in the induced strain (Figure 3C, III) that possibly correspond to accumulation of anionic lipids in highly induced cells. Interestingly, the appendages contain genetic material as evidenced by the DAPI staining (Figure 3C, V). Finally, the double staining revealed the presence of irregular division sites in the elongated cells (Figure 3C, VI) compared to the typical mid-cell septum present in growing wild type cells (Figure 3C, IV). These data suggest that a high level of induction of archaeal lipid biosynthesis result in aberrant cell division.

Robustness of cells harboring a heterochiral mixed membrane

Archaeal ether lipids have been associated with extremophilicity and robustness, even though not all Archaea are extremophiles. Therefore, the survival of the strains with a heterochiral mixed membrane upon a heat and cold shock was tested. Three different engineered E. coli strains (JM109DE3, MEP/DOXP+ and MEP/DOXP+EL+) were exposed to elevated temperatures for two minutes and recovered for one hour at 37 °C. The non-induced and induced (10 μM IPTG) E. coli MEP/DOXP+EL+ strain, showed an overall higher survival and ability to survive exposure to 55 °C and 58 °C compared to the two control strains JM109DE3 and E. coli MEP/DOXP+ that do not survive when exposed to temperatures above 50 °C (Figure 4A). Cells were also exposed to freezing at -80 °C. The cells containing an induced heterochiral mixed membrane were remarkable more tolerant to this treatment than the control strains (Figure 4B) as evidenced by the higher CFU count but only when cells were induced with 10 μM IPTG. Finally, the tolerance to the organic solvent butanol was tested by exposing the strains for two minutes to different concentrations. A higher resistance of the non-induced and induced (10 μM IPTG) E. coli MEP/DOXP+EL+ strain harboring the entire ether lipid biosynthetic pathway was observed compared to the controls (Figure 4C). This was most notable when the cells were treated with 2% of butanol. Taken together these data demonstrate that the presence of archaeal lipids in the
bacterial membrane renders the engineered cells more resistant to different types of environmental stress.

**Figure 4** Robustness of *E. coli* with a heterochiral mixed membrane. The *E. coli* strain with all ether lipids enzymes MEP/DOXP*EL* (not induced – yellow; and induced with 10 μM IPTG - red) was compared with the wild type strain JM109DE3 (blue) and the strain harboring the integrated MEP-DOXP operon MEP/DOXP⁺ (green) for survival against exposure to different environmental stresses. (A) Heat shock, (B) Freezing at -80 °C, and (C) Butanol tolerance. The data were normalized against the CFU of untreated samples. The results are the averages of four biological replicates ± S.E.M.

**Discussion**

The “Lipid Divide” represents a critical event during the differentiation of the two domain of life Bacteria and Archaea, both originating from the last universal common ancestor (LUCA). According to the discordant hypothesis, the instability of a heterochiral mixed membrane in the common ancestor triggered the segregation of archaea and bacteria towards a more stable homochiral membrane [4,91]. While it is inherently difficult to test such a hypothesis *in vivo*, as the conditions of early evolution would need to be replicated, *in vitro* data using pure lipid liposomes failed to demonstrate the assumed instability. Also, so far no biological evidence has been reported for instable mixed heterochiral membranes in a living cell. Here we reproduced a viable bacterial cell with a heterochiral mixed membrane composed of bacterial and archaeal lipids through the introduction of the archaeal ether lipid biosynthetic pathway into *E. coli*. Such a heterochiral mixed membrane may be a biological
model for the coexistence of these two lipid species which might have characterized the membrane of the common cenancestor.

We have previously reported the introduction of a fully functional ether lipid pathway into the bacterium *E. coli* and the synthesis of the two archaean lipids AG and AE [163]. However, the level of the ether lipids was very low compared to the bacterial lipidome (less than 1%) as also encountered in other studies [40,53]. In the present work, a higher level of isoprenoid units (IPP and DMAPP) was accomplished by a combination of the chromosomal integration of an inducible MEP-DOXP pathway [175] and the use of a statistically optimized medium [170]. Further strain optimization yielded an engineered bacterial strain in which the nearly complete PG pool is replaced by the archaean AG. Importantly, the remarkable decrease of the PG content in favor of a high amount of newly synthetized AG demonstrates the functional integration of the ether lipid biosynthetic pathway in these cells.

A critical element of the introduced pathway and the generation of a mixed heterochiral membrane is the validation of the proper stereochanical configuration of the introduced ether lipids. The configuration of the glycerophosphate backbone represents one of the most distinctive differences between bacterial and archaean lipids. With no exception, bacterial membranes are characterized by G3P-based lipids while archaia have G1P-based lipids [91]. The enzymes involved in the synthesis of G3P and G1P, G3PDH and G1PDH respectively, do not share any sequence and functional homology being members of evolutionary different protein families [2]. Moreover, since there is no mechanism known in *E. coli* for the production of G1P, the engineered *E. coli* strain lacking the introduced G1PDH, should not produce archaean lipids. However, the araM gene was found to be redundant which raises questions on the stereoselectivity of the archaean enzymes. Biochemical analysis using purified GGGPS from *M. maripaludis* suggest a preference for G1P over G3P, exhibiting a nine times higher affinity for G1P than G3P, and in the presence of saturating amounts of G3P, high levels of GGGP could be detected. In contrast, the analogous enzyme PlsB from *E. coli* produces LPA only in presence of G3P exhibiting a high stereoselectivity. Despite this lower stereoselectivity, even in the absence of the G1PDH AraM, the *in vivo*
synthesized archaeal lipids were derived from the G1P configuration. Thus, our data indicate that there must be a mechanism of G1P formation in *E. coli*. A possible mechanism of G1P formation is the phosphorylation of glycerol by glycerolkinase and the reductive phosphorylation of dihydroxyacetone by glycerol phosphate dehydrogenase. Although these enzymes are known to generate G3P, for none the chiral specificity has been examined in detail. Taken together, the data demonstrate that the lipid biosynthesis engineering resulted in the formation of a heterochiral mixed membrane in *E. coli*. The high stereoselectivity of the bacterial enzyme PlsB compared to the weak stereoselectivity of the archaeal enzyme GGGPS that carries out an analogous reaction, raises the possibility that the primordial insurgence of archaeal organisms was followed by the differentiation into bacteria. In this way the appearance of higher stereoselective enzymes as PlsB could have triggered the differentiation of bacterial organisms from the ancient cells, which further evolved in archaeal cell, keeping the primordial ability to survive in extreme environments and acquiring a specific membrane lipid composition. We never detected the formation of a possible archaeal counterpart of cardiolipin, di-archaetidylglycerol which suggests that the bacterial cardiolipin synthetase does not recognize the different chirality of the archaeal lipid.

A major question is if such heterochiral mixed membrane affects the cell characteristics. The engineered bacterial strains show a long lag phase of approximately 16 hours before growth commenced at growth rates comparable to the wild type. Genome sequencing of the adapted strain did not reveal any apparent mutation (data not shown) as expected for such short adjustment period. The restoration of growth could result from a metabolic adaptation of the bacterial strain and/or a tailoring of the expression of heterologous enzymes for the viable production of the archaeal ether lipids. On the other hand, strong induction of the archaeal lipid pathway causes severe cell stress as growth slows down and the cell morphology changes. Whereas the majority of engineered cells show elongated and thinner cells compared to the wild type strain, high induction also causes the formation of lobular appendages that are eventually released from the cells. Lipid analysis on these isolated
extrusions revealed the presence of a mixture of archaeal and bacterial lipids much akin the mother cell excluding the hypothesis of immiscibility and segregation of the archaeal lipids with the endogenous lipids as possible cause of this phenomenon. The extrusions also contain genetic material and likely originate from non-symmetrical cell division caused by the high level of archaeal lipid biosynthesis. The suggestion that archaeal lipids interfere with cell division is consistent with an important role of lipids in this process [176,177]. As the introduced ether lipid biosynthetic pathway is not fully integrated in the cellular and phospholipid homeostasis, we speculate that the shredding as seen under conditions of high induction is the result of high level overproduction of lipids that does not keep pace with other processes of cellular growth resulting in the formation of irregular division sites thereby clearing the cells from excess lipids.

Importantly, under conditions of moderate induction that lead to the nearly complete replacement of PG with AG, the archaeal lipids did not confer any toxicity to the bacterial cell. Archaea are well known to be able to survive under extreme conditions such as high temperatures [3], thus one may expect that the presence of archaeal lipids into a bacterial cell membrane could partially confer this ability. Indeed, a higher tolerance to heat treatment compared to control strains was observed. It should be stressed that the archaeal lipids are unsaturated and possibly, saturation will further enhance the survival to heat stress. Cells were also found to be more tolerant to freezing at -80 °C, a feature that can be attributed to the presence of the high concentration of unsaturated archaeal lipids which confers increased membrane fluidity needed to survive extreme cold temperatures [178,179]. Finally, the cells with the engineered membranes exhibited a higher tolerance against the organic solvent butanol. Although the acquired features of robustness are subtle, they are significant and demonstrate that bacteria gain properties by the presence of archaeal lipids rather than being detrimental to the cell’s physiology.

The work described in the present study represents a unique approach to address a possible coexistence of archaeal and bacterial phospholipids as a heterochiral mixed membrane in a living bacterial cell. Despite the fact that the bacterial integral membrane proteins have evolved to function in
an ester-bond based phospholipid membrane, the near to complete replacement of one of the key lipid species of *E. coli*, phosphatidylglycerol for its archaeal counterpart, resulted in viable cells showing growth rates indistinguishable from that the parental strain. Our findings contrast the hypothesis of the instability of such membranes. The strategy described here may be applied to microorganism of industrial relevance to render them more robust with a higher tolerance to toxic products, organic solvents or byproducts without loss of productivity in bio-industrial processes. Further, it will be of interest to exploit the *E. coli* strains with archaeal phospholipids for the functional overproduction of archaeal membrane proteins.

**Materials and Methods**

**Operon integration and cloning procedures**

*E. coli* MG1655 genomic DNA was used as template for the amplification of the *IDI*, *IspDF* and *DXS* genes encoding for the MEP-DOXP operon. The primers and the plasmids used for the integration of the operon into *E. coli* are listed in Table 2 and 3. The three genes were cloned into the same plasmid vector, which was used as a template for the integration of the *lox71-kanR-lox66* selection marker cassette. The selection marker cassette along with the MEP-DOXP operon or the single *IDI* gene was amplified by PCR in order to get a DNA fragment for the integration into *E. coli* JM109DE3 competent cells via electroporation. *E. coli* cells containing the integrated operon were transformed with a plasmid expressing the Cre recombinase to remove the selection marker. The obtained *E. coli* strains (Table 1) containing the integrated *IDI* gene and the MEP-DOXP operon were used as basic strains for the following strain engineering. The primers and plasmids used for expressing the ether lipids genes in the engineered *E. coli* strains are listed in Table 2 and 3.

**Table 1.** *E. coli* strains used in this study.

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Genome integration</th>
<th>Plasmids</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM109DE3</td>
<td>none</td>
<td>pETduet and pRSF-duet</td>
</tr>
</tbody>
</table>

146
Table 2. Cloning and expression vectors used in this study.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGFPuv</td>
<td>Cloning vector expressing <em>Aequorea Victoria</em> GFP</td>
<td>Clontech</td>
</tr>
<tr>
<td>pCR2.1 TOPO</td>
<td>Cloning vector with <em>lox71-kanR-lox66</em> gene cassette</td>
<td>ThermoFisher</td>
</tr>
<tr>
<td>pKD46</td>
<td>Cre-recombinase expressing vector</td>
<td>[164]</td>
</tr>
<tr>
<td>pRSF-Duet-1</td>
<td>Cloning and expression vector (Kan&lt;sup&gt;R&lt;/sup&gt;), T7 promoter</td>
<td>Novagen</td>
</tr>
<tr>
<td>pPET-Duet-1</td>
<td>Cloning and expression vector (Amp&lt;sup&gt;R&lt;/sup&gt;), T7 promoter</td>
<td>Novagen</td>
</tr>
<tr>
<td>pACYC-Duet-1</td>
<td>Cloning and expression vector (CM&lt;sup&gt;R&lt;/sup&gt;), T7 promoter</td>
<td>Novagen</td>
</tr>
<tr>
<td>pMS003</td>
<td><em>IDI</em> gene from <em>E. coli</em> MG1655 cloned into pGFPuv vector using primers BG3606 and BG3599</td>
<td>This study</td>
</tr>
<tr>
<td>pMS008</td>
<td><em>IspDF</em> genes from <em>E. coli</em> MG1655 cloned into pMS003 vector using primers BG3600 and BG3601</td>
<td>This study</td>
</tr>
<tr>
<td>pMS011</td>
<td><em>DXS</em> gene from <em>E. coli</em> MG1655 cloned into pMS008 vector using primers BG3602 and BG3603</td>
<td>This study</td>
</tr>
<tr>
<td>pMS051</td>
<td><em>lox71-kanR-lox66</em> gene cassette from pCR2.1 TOPO vector cloned into pMS003 vector using primers BG4429 and BG4430</td>
<td>This study</td>
</tr>
<tr>
<td>pMS053</td>
<td><em>lox71-kanR-lox66</em> gene cassette from pCR2.1 TOPO vector cloned into pMS011 vector using primers BG4429 and BG4430</td>
<td>This study</td>
</tr>
<tr>
<td>pMS016</td>
<td><em>crtE</em> gene from <em>Pantotea ananatis</em> cloned into the pACYC-Duet vector using primers BG3899 and BG3900</td>
<td>This study</td>
</tr>
<tr>
<td>pMS017</td>
<td><em>crtB</em> and <em>crtI</em> genes from <em>Pantotea ananatis</em> cloned into the pMS016 vector using primers BG3901 and BG3902</td>
<td>This study</td>
</tr>
<tr>
<td>pSJ130</td>
<td><em>araM</em> gene from <em>Bacillus subtilis</em> cloned into pET-duet vector using primers 70 and 71</td>
<td>[41]</td>
</tr>
<tr>
<td>pMS148</td>
<td><em>crtE</em> gene from <em>Pantotea ananatis</em> digested with EcoRI and cloned into the pSJ130 vector</td>
<td>This study</td>
</tr>
<tr>
<td>pMS148Δ</td>
<td>pMS148 vector containing a deleted version of the <em>araM</em> gene using the EcoRV and BmgBI</td>
<td>This study</td>
</tr>
<tr>
<td>pSP001</td>
<td>Codon-optimized GGGPS and DGGGPS genes from <em>M. maripaludis</em> cloned into pRSF-duet vector using the primers 11, 12, 39 and 40</td>
<td>[41]</td>
</tr>
<tr>
<td>pAC027</td>
<td>Codon optimized <em>carS</em> gene from <em>A. fulgidus</em> cloned into pSP001 vector using primers 583 and 584</td>
<td>This study</td>
</tr>
<tr>
<td>pAC029</td>
<td><em>pssA</em> gene from <em>B. subtilis</em> cloned into the pAC027 vector using the primers using primers 585 and 586</td>
<td>This study</td>
</tr>
</tbody>
</table>
Hybrid Heterochiral Membrane

**pSJ103** Codon optimized GGPS and DGGPS genes from *M. maripaludis* cloned into pRSF-duet vector using the primers 11 and 12

**pME001** *fadD* gene from *E. coli* MG1655 cloned into pRSF-Duet-1 vector using the primers PrME001 and PrME002

**pME002** *plsB* gene from *E. coli* MG1655 cloned into pet28b vector using the primers PrME003 and PrME004

### Table 3. Oligonucleotide primers used in the present study.

<table>
<thead>
<tr>
<th>Primers name</th>
<th>Primer sequence 5’ → 3’</th>
<th>Restriction enzyme site</th>
</tr>
</thead>
<tbody>
<tr>
<td>BG3606</td>
<td>GCCATGCCATGCAAACGGAACAG</td>
<td>SphI</td>
</tr>
<tr>
<td>BG3599</td>
<td>GGCTGAGTATATACTATGGCA</td>
<td>PstI</td>
</tr>
<tr>
<td>BG3600</td>
<td>GCCTGAGAGGAACTAATGGGAAC</td>
<td>XbaI</td>
</tr>
<tr>
<td>BG3601</td>
<td>GCTCTAGATTTTTTGGAGTCTTT</td>
<td>XbaI</td>
</tr>
<tr>
<td>BG3602</td>
<td>GCTCTAGAGGAGATATCTGAGT</td>
<td>XbaI</td>
</tr>
<tr>
<td>BG3603</td>
<td>GCGGTAGTTATGCGAGGCCC</td>
<td>Kpnl</td>
</tr>
<tr>
<td>BG4429</td>
<td>GACCGTACGTTCTTTTACTCCTTA</td>
<td>BsiWI</td>
</tr>
<tr>
<td>BG4430</td>
<td>GACGCTTAAGCTATCCTTCCTCTGA</td>
<td>AflII</td>
</tr>
<tr>
<td>BG3899</td>
<td>GAACGAATTCAAGCCGAGAATCGTC</td>
<td>EcoRI</td>
</tr>
<tr>
<td>BG3900</td>
<td>GAATCTAGAGCGCGAGACGATTT</td>
<td>AflII</td>
</tr>
<tr>
<td>BG3901</td>
<td>GCTAGATCTGAGTAAACAACATAGG</td>
<td>BglII</td>
</tr>
<tr>
<td>BG3902</td>
<td>CTTCATCGAGAAAGCAGATGGCGA</td>
<td>XhoI</td>
</tr>
<tr>
<td>70</td>
<td>GCCCGATATCAGTCAACCAGG</td>
<td>NdeI</td>
</tr>
<tr>
<td>71</td>
<td>GCCCGATGCTAGATGATGGTGAGG</td>
<td>XhoI</td>
</tr>
<tr>
<td>11</td>
<td>GCCGGAATTCAATGCAATGCCAACC</td>
<td>EcoRI</td>
</tr>
<tr>
<td>12</td>
<td>GCCGAAAGCTTTTACTTTTGGACG</td>
<td>HindIII</td>
</tr>
<tr>
<td>39</td>
<td>TCTTATATTCCTCTCTTAACTAATA</td>
<td>blunt</td>
</tr>
<tr>
<td>40</td>
<td>CATATTGAGCCGCACTCAGCAATC</td>
<td>blunt</td>
</tr>
<tr>
<td>583</td>
<td>CGGGCAATGATGCGGAGTCTGAGT</td>
<td>NcoI</td>
</tr>
<tr>
<td>584</td>
<td>CGGGGAATTTAGCTGGATGATGCTG</td>
<td>BamHI</td>
</tr>
<tr>
<td>585</td>
<td>CGGGCCGCCGCATTAGAATCTACAT</td>
<td>NotI</td>
</tr>
<tr>
<td>586</td>
<td>CGGGCTTAAGTATGATGCTGGAGG</td>
<td>AflII</td>
</tr>
<tr>
<td>PrME001</td>
<td>TACTAATGAGATATCAGTCTGAGA</td>
<td>EcoRI</td>
</tr>
<tr>
<td>PrME002</td>
<td>AGCTATCACGGCGGCTATTGCTGGTG</td>
<td>NotI</td>
</tr>
<tr>
<td>PrME003</td>
<td>CATCTGCATGAGCGGCGGCTGGAG</td>
<td>NcoI</td>
</tr>
<tr>
<td>PrME004</td>
<td>CTTCATGATTCTGAGCGGCTCTG</td>
<td>XhoI</td>
</tr>
</tbody>
</table>

### Bacterial strains and growth conditions

Engineered *E. coli* strains were grown under aerobic conditions at 37 °C in 200 ml of LB medium supplemented with the antibiotics ampicillin (100 μg/ml) and kanamycin (50 μg/ml). OPT1 medium [170] was prepared by autoclaving a solution based on glycerol 1% (v/v), KH₂PO₄ 2.4% (w/v), (NH₄)₂HPO₄ 0.4% (w/v), citric acid 0.17% (w/v) and by adding sterile 1
mM NiCl₂, 0.12 mM MgSO₄ and 1x MEM vitamin solution (Sigma-Aldrich). When not specified the cells were induced with 0.1 mM IPTG for 3 hours or overnight.

**Lycopene quantification**
Lycopene quantification was done as described by Yoon *et al.* [166] An aliquot of a growing culture (10 ml) was centrifuged at 4,700 xg for 10 minutes. The obtained pellet was washed with 1 ml of milli-Q water and further centrifuged at 12,000 xg for 5 minutes. The residual cell pellet was suspended in 500 μl of acetone and incubate 30 minutes at 55 °C to promote the lycopene extraction and then centrifuged for 20 minutes at 16,000 xg at 4 °C as previously described [166]. The extraction was repeated twice and the obtained lycopene extracts was additionally centrifuged at 16,000 xg for 2 minutes to remove possible impurities. Samples of 250 μl were then diluted with 750 μl of acetone and the amount of extracted lycopene was determined by measuring the absorbance at 472 nm. The lycopene concentration was calculated by means of a calibration curve and normalized to the dry cell weight (DCW).

**Thin Layer Chromatography**
An aliquot (3 μl) of lipid extracts from the different *E. coli* strains was spotted on Silica Gel 60 (Merck) plates. A solvent mixture of chloroform, methanol and water (50:10:1) was used as mobile phase for the separation of the different lipid species which were detected by molybdenum blue [180]. A solvent system chart from Avanti ([http://avantilipids.com/tech-support/analytical-procedures/tlc-solvent-systems/](http://avantilipids.com/tech-support/analytical-procedures/tlc-solvent-systems/)) was used as reference for the lipid identification. The spots were relatively quantified using ImageJ software.

**Expression and purification of GGGPS, FadD and PIsB enzymes**
The archaeal protein GGGPS from *M. maripaludis* was expressed and purified as previously described [41]. The bacterial FadD and PIsB proteins from *E. coli* were overexpressed in *E. coli* BL21 induced with 1 mM IPTG as will be detailed elsewhere. After 2 hrs of induction, the cells were harvested (8,754 xg) and washed with buffer A containing 50 mM Tris-HCl.
Hybrid Heterochiral Membrane

pH 8.0, 100 mM KCl and 20% glycerol. After re-suspension, the cells were supplemented with 0.5 mg/ml of DNase and a complete EDTA free protease inhibitor tablet (Roche). The suspension was subjected to cell disruption at 13,000 psi and the cell lysate was centrifuged for 15 minutes at low spin (12,000 xg) to remove unbroken cells. The purification of the cytoplasmic protein FadD was performed by separation of the cytosolic fraction from membranes by a centrifugation step at 43,667 xg for 15 min. The supernatant was incubated with Ni-NTA beads (SigmaAldrich) in buffer A for 60 min at 4 °C. The beads were washed 5 times with 20 column volumes (CV) of buffer A supplemented with 10 mM imidazole and eluted 2 times with 2 CV of buffer A supplemented with 300 mM imidazole. The membrane protein P1sB was purified by a high-speed centrifugation at (235,000 xg) for 1 hour to isolate the membrane fraction. Total membrane (pellet) were suspended in buffer A and solubilized at 4 °C for 1 hour in 2% of n-dodecyl-β-D-maltopyranoside (DDM) detergent. Insolubilized materials were removed by centrifugation (15,800 xg) for 10 minutes and the supernatant was incubated with Ni-NTA beads for 90 min at 4 °C. The beads were washed 5 times with 40 CV of buffer B (0.05 % DDM, 50 mM Tris pH 8.0, 100 mM KCl, 20% glycerol) supplemented with 10 mM imidazole and eluted 3 times with 0.5 CV of buffer B supplemented with 300 mM imidazole. The purity of the proteins was checked by 15% SDS-PAGE, stained with Coomassie Brilliant Blue. Absorbance was measured at 280 nm to determine the concentration of purified protein.

**In vitro enzyme reactions**

*In vitro* reactions were performed in 100 μl of buffer containing a final concentration of 50 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 60 mM NaCl, 100 mM Imidazole, 0.08% DDM and 4% glycerol. Where specified, 100 μM GGPP, 10 mM G3P, 10 mM G1P and the indicated amount of purified enzymes were added to the reaction mixture. The reactions were incubated at 37 °C for 1 hour. Kinetic assays were performed using the same reaction mixture but the reactions were incubated at 37 °C for 2 hours. The coupled FadD-P1sB *in vitro* assay was performed in a 100 μl reaction volume containing: 50 mM Tris-HCl pH 8, 10 mM MgCl₂, 100 mM
KCl, 20% glycerol, 0.05% DDM, 2mM DTT, 2.67 mM lipids (DOPC: DOPG:DOPE, 1:1:1). Where specified, 300 μM oleic acid, 40 μM CoA, 1 mM ATP, 0.5 μM purified FadD, 1 μM purified PlsB, 10 mM G3P and 10 mM G1P were added to the reaction mixture. Reactions were incubated at 37 °C for 4 hours. The products were extracted two times 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 the LC-MS analysis.

**Lipid analysis**

*E. coli* strains induced for the archaeal lipids synthesis were grown as described above. The total membrane fractions were isolated and the total lipid content was extracted according to the Bligh and Dyer method [163]. Samples were then resuspended in 100 μl of methanol for LC-MS analysis, or total lipid quantitation by a colorimetric assay, based on the formation of a complex between phospholipids and ammonium ferrothiocyanate [181]. Samples (20 μl) were evaporated under a nitrogen stream and resuspended in 500 μl of chloroform; 250 μl of ferrothiocyanate reagent was then added to the chloroform layer, mixed for 1 min and allowed to phase separate for 5 minutes. The lower red phase was collected and the absorbance at 490 nm was measured and calibrated against standards. The obtained values were also used to normalized the LC-MS ion counts for amounts of individual lipids.

**LC-MS analysis**

The lipid extracts and the samples from *in vitro* reactions were analyzed using an Accela1250 high-performance liquid chromatography system coupled with an electrospray ionization mass spectrometry (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 this study to analyzed both type of samples were the same as described previously [163].

**Analysis of the configuration of the ether lipids**

All chemical reactions were carried out under a nitrogen atmosphere using oven-dried glassware and using standard Schlenk techniques. Reaction
temperature refers to the temperature of the oil bath. All reagents and
catalysts were purchased from Sigma-Aldrich, Acros, J&K Scientific and TCI
Europe and used without further purification unless otherwise mentioned,
any purification of reagents was performed following the methods
described by Armarego et al. [182]. TLC analysis was performed on Merck
silica gel 60/Kieselguhr F254, 0.25 mm. Compounds were visualized using
either Seebach’s reagent (a mixture of phosphomolybdic acid (25 g),
cerium (IV) sulfate (7.5 g), \( \text{H}_2\text{O} \) (500 mL) and \( \text{H}_2\text{SO}_4 \) (25 mL)), 2,4-DNP
stain (2,4-dinitrophenylhydrazine (12 g), conc. sulfuric acid (60 ml), water
(80 ml), ethanol (200 ml)) or elemental iodine. Flash chromatography was
performed using SiliCycle silica gel type SiliaFlash P60 (230 – 400 mesh)
as obtained from Screening Devices. GC-MS measurements were
performed with an HP 6890 series gas chromatography system equipped
with an HP1 or HP5 column (Agilent Technologies, Palo Alto, CA), and
equipped with an HP 5973 mass sensitive detector. High resolution mass
spectra (HRMS) were recorded on a Thermo Scientific LTQ Orbitrap XL
(ESI+, ESI- and APCI). \(^1\text{H}, ^{13}\text{C}-\) and \(^{19}\text{F}-\)NMR spectra were recorded on a
Varian AMX400 (400, 101 and 376 MHz, respectively) using CDCl\(_3\) as
solvent unless stated otherwise. Chemical shift values are reported in ppm
with the solvent resonance as the internal standard (CDCl\(_3\): \( \delta \) 7.26 for \(^1\text{H}, \delta \)
77.16 for \(^{13}\text{C}\)). Data are reported as follows: chemical shifts (\( \delta \)), multiplicity
(s = singlet, d = doublet, dd = double doublet, ddd = double double doublet,
td = triple doublet, t = triplet, q = quartet, b = broad, m = multiplet),
coupling constants \( J \) (Hz), and integration. Enantiomeric excesses were
determined by chiral HPLC analysis using a Shimadzu LC- 10ADVP HPLC
instrument equipped with a Shimadzu SPD-M10AVP diode-array detector.
Optical rotations were measured on a Schmidt+Haensch polarimeter
(Polartronic MH8) with a 10 cm cell (c given in g/mL) at ambient
temperature (±20 °C).

**Scanning Electron Microscopy and Bright Field Microscopy**
For the scanning electron microscopy analysis 150 µl of cell suspension
was immobilized on poly-L-lysine coated cover slips (Corning art. 354085)
for 1 hour. 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer pH 7.2
was added to the glass at room temperature for 1 hour. The sample was
rinsed three times in the same buffer and fixed for 1 hour in 1% OsO4 (w/v) in the same buffer. Two washes with water were performed, followed by a dehydration in a graded ethanol series (10, 30, 50, 70, 90, 100%) and dried with carbon dioxide (Leica EM CPD 300). The glasses were attached on a sample holder by carbon adhesive tabs (EMS Washington USA), sputter coated with tungsten (Leica EM SCD 500) and analyzed and digitally imaged with a field emission scanning electron microscope (FEI Magellan 400). Sample preparation, imaging and measurements were performed by the Wageningen Electron Microscopy Centre (WEMC) facility.

The bright field microscopy was performed on cells grown until exponential phase. Aliquots of 1 ml were centrifuged at max speed for 30 s on the top bench centrifuge. The obtained pellet was resuspended in 100 μl of Phosphate Buffered Saline (PBS, 58 mM Na$_2$HPO$_4$, 17 mM NaH$_2$PO$_4$ and 68 mM NaCl pH 7.3). The FM4-64 and DAPI dyes were added to the solution at the final concentration of 0.8 μM and 36 nM respectively [183]. The solution was incubated at room temperature for 10 minutes and centrifuged at max speed for 30 s. The stained cell pellet was suspended in 40 μl of PBS and spotted on agarose pad (1% w/v in PBS). Cells were imaged using a Nikon Ti-E-microscope (Nikon Instruments) equipped with a Hamamatsu Orca Flash 4.0 camera. The image analysis was performed by the software ImageJ [184].

**Robustness tests**

The engineered *E. coli* strains and the controls strains were grown and induced as described above. A dilution into fresh medium was performed to reach the OD$_{600} = 1.0$ and the obtained culture was diluted again for a dilution factor of 1000x in order to have approximately 10$^5$- $10^6$ cells/ml.

For the freezing survival, the cells aliquots of 20 μl were frozen in liquid nitrogen and kept at -80 °C for 4 days. An untreated cell sample was plated in a 100x dilution to be used as reference. Heat shock treatment was performed by exposing the strains to different temperatures (37 °C, 42 °C, 46 °C, 50 °C, 55 °C and 58 °C) for 2 minutes. The cells were then recovered by adding 980 μl of LB medium and incubated at 37 °C for 1 hour and plated for CFU counts. Butanol tolerance was tested by incubating cells in
Hybrid Heterochiral Membrane

LB supplemented with different butanol concentration (0%, 0.5%, 1%, 1.5%, 2%, and 2.5%) for 2 minutes and recovering the treated cells at 37°C for 1 hour. After all the treatments 100 μl of cells were plated on LB agar plate supplemented with the proper antibiotics (Amicillin 100 μg/ml and kanamycin 50 μg/ml) and incubated at 37°C overnight. The colony counting was performed using a developed plugin for the software ImageJ.

Supplemental Information
Supplementary information includes one table, three figures and supplemental experimental procedures.

Acknowledgments
This work was carried out within the research program of the biobased ecologically balanced sustainable industrial chemistry (BE-Basic). We thank Tiny Franssen Verheijen from the Wageningen Electron Microscopy Centre (WEMC) for providing the electron micrographs and Teunke van Rossum for providing the Lox-KanR-lox integration cassette. We also thank Anne-Bart Seinen for the graphic assistance and the development of a colony counting software and thank Anabela de Sousa Borges for the bright field microscopy assistance.

Authors contributions
A.C., M.S., A.D., S.K. and J.vd.O. conceived and designed the research. M.S. performed the operon integration, lycopene quantification, robustness tests and strain optimization. A.C. performed the total lipid analysis, the in vitro biochemical analysis of GGGPS, the robustness tests and the bright field microscope analysis. M.E. cloned the genes, purified the enzymes and performed the in vitro experiments for the PIsB activity assay. A.M. designed the synthesis of the synthetic standards for the total lipid stereochiral configuration analysis, which was performed by V. J. and R. H. The manuscript was written by the contributions of all the authors.
Competing financial interests
The authors declare that the research was conducted in absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplemental Information

Table S1 Combination of bacterial and enzymes used in this study for the \textit{in vivo} ether lipids production.

<table>
<thead>
<tr>
<th>Locus (gene)</th>
<th>Source</th>
<th>Protein expressed</th>
<th>Function</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>CrtE</td>
<td>\textit{P. ananatis}</td>
<td>GGPP synthesis</td>
<td>IPP+DMAPP → GGPP</td>
<td>[167]</td>
</tr>
<tr>
<td>BSU28760 (araM)</td>
<td>\textit{B. subtilis}</td>
<td>G1P dehydrogenase – His\textsubscript{6}</td>
<td>DHAP+NAD H→G1P</td>
<td>[31]</td>
</tr>
<tr>
<td>MmarC7_1004</td>
<td>\textit{M. maripaludis}</td>
<td>His\textsubscript{8}-GGGP synthase (codon optimized)</td>
<td>G1P+GGPP→ GGGP</td>
<td>[35]</td>
</tr>
<tr>
<td>MmarC7_RS04845</td>
<td>\textit{M. maripaludis}</td>
<td>His\textsubscript{8}-DGGGP synthase (codon optimized)</td>
<td>GGPP+GGGP → DGGGP</td>
<td>[21]</td>
</tr>
<tr>
<td>AF1740</td>
<td>\textit{A. fulgidus}</td>
<td>CarS-His\textsubscript{8} (codon optimized)</td>
<td>DGGGP+CTP → CDP---archaeol</td>
<td>[41]</td>
</tr>
<tr>
<td>pssA</td>
<td>\textit{B. subtilis}</td>
<td>PssA-His\textsubscript{8}</td>
<td>CDP---archaeol→ AS</td>
<td>[163]</td>
</tr>
<tr>
<td>psd</td>
<td>\textit{E. coli}</td>
<td>Psd-His\textsubscript{8}</td>
<td>AS→AE</td>
<td>[163]</td>
</tr>
<tr>
<td>pgsA</td>
<td>\textit{E. coli}</td>
<td>PgsA-His\textsubscript{8}</td>
<td>CDP---archaeol→ AGP</td>
<td>[163]</td>
</tr>
<tr>
<td>pgpA</td>
<td>\textit{E. coli}</td>
<td>PgpA---His\textsubscript{8}</td>
<td>AGP→AG</td>
<td>[163]</td>
</tr>
</tbody>
</table>
**Figure S1 related to Figure 1** Schematic representation of the biosynthetic pathway introduced into the bacterium *E. coli* for archaeal lipids synthesis. The bacterial MEP/DOXP pathway enzymes used to overproduce the isoprenoid building blocks IPP and DMAPP and the genes encoding the enzymes for the ether lipids synthesis are highlighted in blue. The scheme indicates all the biosynthetic steps introduced in the bacterium *E. coli* for the production of a heterochiral mixed membrane.
Figure S2 related to Figure 1 | E. coli metabolic engineering. (A) Schematic representation of the engineering of E. coli JM109DE3 showing the integration of the MEP-DOXP operon or the IDI gene into the chromosome and the three vectors harboring the ether lipids enzymes. (B) Effect of the chromosomal integration of the IDI gene and the IDI-IspDF-DXS operon on the synthesis of isoprenoid building blocks as monitored through the production of lycopene using 0 (white bars), 10 (grey bars) and 100 (black bars) μM IPTG for induction. (C) In vivo production of AG by engineered E. coli strains (JM109DE3, IDI-EL+ and MEP/DOXP·EL+) with improved IPP and DMAPP synthesis. Total ion count from LC-MS were normalized for the total amount of lipids present in each sample. The data are the averages of three biological replicates ± S.E.M. (AG: archaetidylglycerol)
Figure S3 related to Figure 1 | Optimization of archaeal lipid production in *E. coli*. Total lipid analysis of the *E. coli* MEP/DOXP−EL+ strain harboring the entire ether lipid biosynthetic pathway grown and induced under different conditions. (A) Comparison between rich LB medium and a defined minimal medium (OPT) optimized for the isoprenoid production. (B) Different growth phases and (C) IPTG concentrations including the JM109DE3 wild type strain, the engineered *E. coli* MEP/DOXP−EL+ strain and *E. coli* MEP/DOXP−EL+AraM− strain lacking the *araM* gene. Total ion counts are normalized for the total amount of lipids present in each sample. The data are the averages of three biological replicates ± S.E.M. (AG: archaeetylglycerol, CL: cardiolipin, PG: phosphatidylglycerol and PE: phosphatidylethanolamine). (D) Total lipid analysis of the heterochiral mixed membrane *E. coli* strain and the isolated bulges. The lobular appendages were separated from the bacterial cells by a centrifugation step at low speed (5403 xg) and high speed (235,000 xg). The obtained pellet was resuspend and the lipid analysis was performed and compared with the total lipidome of the isolated membranes from the same *E. coli* strain. The different total amount of lipids reflects the low starting material of the isolate bulges compared the total membranes. The total ion counts from LC-MS were normalized using eicosane as internal standard.
Chemical synthesis of the standard used for the phospholipid chirality analysis

Scheme 1:

Scheme 2:

Scheme 3:

Scheme 4:

2-(((3,4-dimethoxybenzyl)oxy)methyl)oxirane[185] (3). To a 100 mL 3-necked flask equipped with magnetic stirrer bar was added 25 mL of a 50% NaOH solution, epichlorohydrin (18.5 g, 15.6 mL, 0.2 mol) and
Bu₄NHSO₄ (1.5 mmol, 525 mg, 4 mol%). The resulting solution was cooled to 0 °C (ice/water-bath) after which neat 3,4-dimethoxybenzyl alcohol (37.5 mmol, 5.5 mL, 6.3 g) was added dropwise over 30 min while the solution was stirred vigorously. The resulting turbid mixture was allowed to warm up over a 5 h period, after which complete conversion was observed by TLC. The entire content of the flask was poured into 100 mL of ice water which was subsequently extracted with diethyl ether (3 x 50 mL). The combined organic layers were washed with brine (2 x 50 mL) dried over MgSO₄ and concentrated in vacuo. The resulting crude was further purified by column chromatography (1:3 EtOAc/pentane) to give 2-(((3,4-dimethoxybenzyl)oxy)methyl)oxirane as a pale yellow oil (94% yield, 7.9 g).

1H NMR (400 MHz, Chloroform-d) δ 6.94 – 6.78 (m, 3H), 4.52 (q, J = 11.6 Hz, 2H), 3.89 (s, 3H), 3.87 (s, 3H), 3.75 (dd, J = 11.5, 2.9 Hz, 1H), 3.41 (dd, J = 11.4, 5.9 Hz, 1H), 3.19 (td, J = 6.3, 3.2 Hz, 1H), 2.80 (t, J = 4.6 Hz, 1H), 2.61 (dd, J = 4.9, 2.7 Hz, 1H).

13C NMR (101 MHz, Chloroform-d) δ 149.0, 148.7, 130.4, 120.4, 111.1, 110.9, 73.2, 70.6, 55.9, 55.8, 50.8, 44.3.

3-((3,4-dimethoxybenzyl)oxy)propane-1,2-diol (4). Epoxide 3 (200 mg, 0.9 mmol) with 6 mL water was added to a 10 mL round-bottomed flask equipped with magnetic stirrer bar. To this mixture was added 0.2 mL of 10% aqueous sulfuric acid followed by stirring for 5 h at rt. The resulting acidic solution was neutralized with 1 M NaOH and extracted with ethyl acetate (3 x 5 mL). The combined organic layers were washed with brine (2 x 5 mL), dried over MgSO₄ and concentrated in vacuo which yielded the desired product as a colorless thick oil (98% yield, 210 mg).

1H NMR (400 MHz, Chloroform-d) δ 6.87 – 6.77 (m, 3H), 4.44 (s, 2H), 3.85 (s, 3H), 3.83 (s, 3H), 3.67 – 3.60 (m, 1H), 3.55 (dd, J = 11.5, 5.9 Hz), 3.52 – 3.42 (m, 2H), 3.03 (br s, 2H).
$^{13}$C NMR ($101$ MHz, Chloroform-$d$) δ 149.1, 148.8, 130.3, 120.5, 111.2, 111.0, 73.5, 71.5, 70.8, 64.1, 55.6, 55.9.

**(S)-3-((3,4-dimethoxybenzyl)oxy)propane-1,2-diol**[185] (5). A 25 mL flask equipped with a magnetic stirrer bar was charged with (S,S)-1 (70 mg, 0.005 equiv). The catalyst was exposed to 2-(((3,4-dimethoxybenzyl)oxy)methyl)oxirane (5 g, 22.3 mmol) and AcOH (25 μL, 0.2 equiv). The resulting red mixture was allowed to stir for 30 min in order to oxidize the catalyst. To the resulting brown mixture was added H$_2$O (220 μL, 0.55 equiv) and was stirred rt for 48 h. The final product was isolated as a brown oil by flash column chromatography (100% EtOAc) (45% yield, 2.2 g).

Chiral HPLC analysis on a Lux® 5 μm Cellulose-3 column, $n$-heptane : $i$-PrOH = 90 : 10, 40 °C, flow = 1 mL/min, UV detection at 274 nm, $t_R$(major): 25.29 min, $t_R$(minor): 29.06 min, 97% ee

$^1$H NMR (400 MHz, Chloroform-$d$) δ 6.87 – 6.77 (m, 3H), 4.44 (s, 2H), 3.85 (s, 3H), 3.83 (s, 3H), 3.67 – 3.60 (m, 1H), 3.55 (dd, $J$ = 11.5, 5.9 Hz, 1H) 3.52 – 3.42 (m, 2H), 3.03 (br s, 2H).

$^{13}$C NMR ($101$ MHz, Chloroform-$d$) δ 149.1, 148.8, 130.3, 120.5, 111.2, 111.0, 73.5, 71.5, 70.8, 64.1, 55.6, 55.9.

[α]$^D_{20}$ = -2.4 (c = 0.1 g/mL, CHCl$_3$).

**(R)-3-((3,4-dimethoxybenzyl)oxy)propane-1,2-diol** Error! Bookmark not defined. (15). This compound was prepared with the same synthetic procedure that was used for **(S)-3-((3,4-dimethoxybenzyl)oxy)propane-1,2-diol**.
dimethoxybenzyl)oxy)propane-1,2-diol (5), using (R,R)-1 as catalyst (45% yield). Error! Bookmark not defined.

Chiral HPLC analysis on a Lux® 5 µm Cellulose-3 column, n-heptane : i-PrOH = 90 : 10, 40 °C, flow = 1 mL/min, UV detection at 274 nm, $t_R$(minor): 26.01 min, $t_R$(major): 29.21 min, 95% ee.

$^1$H NMR (400 MHz, Chloroform-$d$): Same as reported for compound 5

$^{13}$C NMR (101 MHz, Chloroform-$d$): Same as reported for compound 5

$[\alpha]^D_{20} = +2.4$ (c = 0.1 g/mL, CHCl$_3$).

The spectral data correspond to those previously reported[124]

ethyl (6$E$,10$E$)-7,11,15-trimethyl-3-oxohexadeca-6,10,14-trienoate Error! Bookmark not defined. (7). An oven dried Schlenk flask equipped with magnetic stirrer bar was charged with NaH (60% dispersion, 136 mg, 3.3 equiv). The mineral oil was removed by 3 successive washings with pentane. The remaining white solid was dried in vacuum, suspended in dry THF (2.5 mL) and cooled to 0 °C (ice/water-bath). To the resulting suspension, freshly distilled ethyl acetoacetate (400 mg, 3 equiv) was added dropwise over 15 min after which the solution turned light yellow. After stirring for an additional 15 min at 0 °C, a solution of n-BuLi in hexanes (1.6 M, 1.95 mL, 3 equiv) was added over 15 min. The resulting dark yellow solution was allowed to stir further for 15 min at 0 °C. Farnesyl bromide (6) (286 mg, 1 mmol) in 0.55 mL of dry THF was added dropwise over 10 min. The resulting orange suspension was quenched by the addition of HCl (1 M, 1.5 mL). The aqueous layer was separated and extracted with Et$_2$O (3 x 2 mL), the organic layers were combined, washed with brine, dried over MgSO$_4$ and concentrated in vacuo. The obtained crude oil was further purified by flash column chromatography after which the pure aceto-ester was obtained as a pale yellow oil (10% Et$_2$O in pentane) (yield 93%).
$^1$H NMR (400 MHz, Chloroform-\textit{d}) \ \delta \ 12.06 \ (s, \ 0.2H), \ 5.10 \ – \ 4.99 \ (m, \ 3H), \\
4.22 \ – \ 4.08 \ (m, \ 2H), \ 3.38 \ (d, \ 2H), \ 2.52 \ (t, \ J = 7.4 \ Hz, \ 2H), \ 2.24 \ (q, \ J = 7.4 \ Hz, \ 2H), \ 2.08 \ – \ 1.88 \ (m, \ 8H), \ 1.63 \ (s, \ 3H), \ 1.56 \ (m, \ 9H), \ 1.23 \ (t, \ J = 7.1 \ Hz, \ 3H).

The spectral data correspond to those previously reported\cite{125}.

\begin{center}
\includegraphics[width=0.8\textwidth]{figure}
\end{center}

ethyl \ (2\textit{Z},6\textit{E},10\textit{E})-3-[(diethoxyphosphoryl)oxy]-7,11,15-trimethylhexadeca-2,6,10,14-tetraenoate\textsuperscript{Error! Bookmark not defined.} (8). An oven dried Schlenk flask equipped with magnetic stirrer bar was charged with NaH (60\% dispersion, 46 mg, 1.15 mmol, 1.15 equiv). The mineral oil was removed by 3 washings with pentane. The remaining white solid was dried in vacuum and suspended in dry Et$_2$O (4.5 mL). The suspension was cooled to 0 °C (ice/water-bath) and a solution of ethyl (6\textit{E},10\textit{E})-7,11,15-trimethyl-3-oxohexadeca-6,10,14-trienoate (7) in dry Et$_2$O (1.5 mL) was added over 15 min. The resulting yellow homogeneous mixture was stirred for 15 min at 0 °C and for 15 min at rt. The solution was again cooled to 0 °C and neat diethylchlorophosphate was added over 5 min. The resulting mixture was stirred for 15 min at 0 °C after which the reaction was quenched by addition of saturated aqueous NH$_4$Cl solution (3 mL). The organic layer was separated and the aqueous layer was extracted with Et$_2$O (3 x 3 mL). The combined organic layers were washed with saturated aqueous NaHCO$_3$ (3 x 3 mL), brine (3 x 3 mL), dried over MgSO$_4$ and the solvent was removed \textit{in vacuo}. The resulting yellow oil (400 mg) was used without further purification in the successive step.

\begin{center}
\includegraphics[width=0.8\textwidth]{figure2}
\end{center}

ethyl \ (2\textit{E},6\textit{E},10\textit{E})-3,7,11,15-tetramethylhexadeca-2,6,10,14-tetraenoate\textsuperscript{Error! Bookmark not defined.} (9). An oven dried Schlenk flask
Hybrid Heterochiral Membrane

equipped with magnetic stirrer bar was charged with CuI (340 mg, 1.8 mmol, 1.8 equiv) and 1.1 mL Et₂O. The resulting suspension was treated with MeLi (1.6 M in Et₂O, 2.25 mL 3.6 equiv) upon which the mixture turned bright yellow. After complete addition of the MeLi the mixture was homogeneous and colorless. The mixture was cooled to -78 °C (cryostat, acetone-bath) and a solution of the phosphate 8 (400 mg) in dry Et₂O (1.4 mL) was added dropwise such that the phosphate solution was cooled by the cold wall of the Schlenk flask, during which the color of the reaction mixture turned to yellow. After complete addition the resulting bright orange solution was stirred at -78 °C for 1 h, after which the mixture was allowed to warm up to -45 °C and was stirred for an additional 2 h. The resulting dark red mixture was quenched by adding 130 μL of MeI and after stirring for 10 minutes the entire content of the Schlenk flask was carefully poured into a saturated aqueous solution of NH₄Cl (5 mL) and NH₄OH (25%, 6 mL) during which gas evolution was observed. The resulting mixture was stirred until it became homogeneous. The layers were separated, the aqueous layer was extracted with Et₂O (3 x 5 mL), the organic layers were combined and washed with an aqueous solution of NH₄OH (25%, 2 x 8 mL), brine (2 x 8 mL), dried and concentrated in vacuo. 290 mg of a yellow oil was obtained and used in the successive reaction without further purification.

\[
(2E,6E,10E)-3,7,11,15\text{-tetramethylhexadeca-2,6,10,14-tetraen-1-ol (10).}
\]

To an oven dried Schlenk flask under N₂ atmosphere was added ethyl (2E,6E,10E)-3,7,11,15-tetramethylhexadeca-2,6,10,14-tetraenoate (9), from the previous step in dry toluene (3 mL). The resulting solution was cooled to -78 °C (cryostat, acetone-bath) and a solution of DIBAL (1 M in CH₂Cl₂, 2.6 mL, 3 equiv) was added dropwise over 20 min. The resulting mixture was stirred at -78 °C for 1h after which full consumption was observed by TLC (30% Et₂O in pentane, I₂-stain, \( R_f \sim 0.3 \)). The reaction was quenched by dropwise addition of MeOH over 10 min (gas evolution observed) after which the mixture stirred until gas evolution ceased. The
solution was allowed to warm up to ambient temperature and was allowed to stir for 10 min. The resulting mixture was poured into a 1:1 saturated aqueous solution of NH₄Cl/1N HCl solution (20 mL) and was stirred until a clear separation of layers was observed. The aqueous layer was extracted with Et₂O (3 x 10 mL) and the combined organic layers were washed with water (2 x 10 mL), brine (3 x 10 mL), dried over MgSO₄ and concentrated in vacuo. The residual yellow oil was purified by flash column chromatography (30% Et₂O in pentane) which yielded 181 mg of a yellow oil (63% yield over 3 steps).

The spectral data correspond to those previously reported. The spectral data correspond to those previously reported.  

The spectral data correspond to those previously reported.  

\[
(2E,6E,10E)-1\text{-chloro-3,7,11,15-tetramethylhexadeca-2,6,10,14-tetraene}
\]

\[
(2E,6E,10E)-1\text{-chloro-3,7,11,15-tetramethylhexadeca-2,6,10,14-tetraene}
\]

(2E,6E,10E)-1-chloro-3,7,11,15-tetramethylhexadeca-2,6,10,14-tetraene. N-chlorosuccinimide (145 mg, 1.1 mmol, 1.3 equiv.) was suspended in dry CH₂Cl₂ (2.5 mL) under N₂ atmosphere in a pre-dried Schlenk flask. The turbid mixture was cooled to -30 °C (acetone/liquid N₂ bath) after which dimethyl sulfide (90 μL, 1.25 mmol, 1.5 equiv.) was added dropwise. The reaction mixture was stirred for 10 min at -30 °C after which it was allowed to warm up to 0 °C for 10 min. The resulting solution was cooled to -40 °C and geranylgeraniol (10, 240 mg, 0.83 mmol) in dry CH₂Cl₂ (1 mL) was added dropwise over 15 min. The resulting suspension was allowed to warm up over 1 h to -20 °C after which the acetone/liquid N₂ bath was replaced by an ice/water bath and the resulting suspension was stirred for another h at 0 °C after which it was poured into pentane (20 mL). The pentane mixture was decanted from the white precipitate, the white crystals were washed with pentane (20 mL) and decanted again, this process was repeated three times. The pentane extracts were combined and evaporated obtaining geranylgeranyl chloride as a yellow oil quantitatively which was used immediately for the dialkylation of (S)-3-(((3,4-dimethoxybenzyl)oxy)propane-1,2-diol.
An oven dried Schlenk flask equipped with magnetic stirrer bar was charged with NaH (60% dispersion in mineral oil, 33 mg, 825 μmol, 2 equiv). The mineral oil was removed by three successive washings with pentane and then dried under vacuum. The resulting white solid was suspended in DMSO (0.6 mL) and stirred at 60 °C (oil bath) until a clear solution was observed (45 min). The pale yellow solution was allowed to cool down to rt after which (S)-3-((3,4-dimethoxybenzyl)oxy)propane-1,2-diol 5, (83 mg, 340 μmol) in dry DMSO (freshly distilled from CaH, 0.6 mL) was added in a dropwise manner. After the solution was stirred for 1.5 h at rt, the crude mixture of geranylgeranyl chloride 11, (250 mg, 2.2 equiv) in dry DMSO (0.2 mL) was added dropwise over 20 min. The resulting reaction mixture was stirred for 16 h after which it was poured into a saturated aqueous solution of NH₄Cl (5 mL). The aqueous layer was extracted with diethyl ether (3 x 5 mL). The combined organic layers were washed with brine, dried and concentrated in vacuo. The resulting crude was further purified by flash column chromatography (20 % diethyl ether in pentane) to afford the dialkylated product (31% yield over 2 steps, 41 mg).

HRMS-ESI+ (m/z): [M + Na]+ calculated for C₅₂H₈₂O₅Na, 809.6055; found, 809.6041.

The spectral data correspond to those previously reported.[186]
1-(3,4-dimethoxybenzyl)-2,3-bisgeranylgeranyl-sn-3-glycerol (16). This compound was prepared with the same synthetic procedure that was used for 1-(3,4-dimethoxybenzyl)-2,3-bisgeranylgeranyl-sn-glycerol, (12).

\(^1\)H NMR (400 MHz, Chloroform-\(d\)): Same as reported for 12

\(^{13}\)C NMR (101 MHz Chloroform-\(d\)): Same as reported for 12

2,3-bisgeranylgeranyl-sn-glycerol (13). 1-(3,4-dimethoxybenzyl)-2,3-bisgeranylgeranyl-sn-glycerol (12) (35 mg, 45 \(\mu\)mol) was dissolved in CH\(_2\)Cl\(_2\) (0.9 mL) to which water (0.1 mL) was added in order to form a biphasic system. The solution was cooled to 0 °C (ice/water bath) and DDQ (12.5 mg, 55 \(\mu\)mol, 1.2 equiv) was added in portions. The resulting green mixture was stirred for 2 h at 0 °C during which it turned light brown. The entire mixture was filtered over a small silica pad with CH\(_2\)Cl\(_2\) and purified by flash column chromatography (20% diethyl ether in pentane, I\(_2\) and 2,4-DNP stain) which afforded the desired product as a viscous yellow oil (70% yield, 20 mg).

HRMS-ESI+ (\(m/z\)): [M + Na]\(^+\) calculated for C\(_{43}\)H\(_{72}\)O\(_3\)Na, 659.5374; found, 659.5369.

The spectral data correspond to those previously reported. Error! Bookmark not defined.
2,3-bisgeranylgeranyl-sn3-glycerol (17). This compound was prepared with the same synthetic procedure that was used for 2,3-bisgeranylgeranyl-sn-glycerol 13.

$^1$H NMR (400 MHz, Chloroform-d): Same as reported for 13

HRMS-ESI+ (m/z): [M + Na]$^+$ calculated for C$_{43}$H$_{72}$O$_3$Na, 659.5374; found, 659.5371.

Saponification of ester-lipids from natural lipid extract mixture. 480 mg NaOH was added to a mixture of MeOH (0.6 mL) and CH$_2$Cl$_2$ (4.4 mL) and stirred until all NaOH was dissolved. To this resulting stirred solution was added the natural lipid extract (40 mg) from the modified E. coli cultures in CH$_2$Cl$_2$ (1 mL) such that the resulting mixture had a 1:9 MeOH/CH$_2$Cl$_2$ ratio and a 2 N NaOH concentration. The solution was allowed to stir for 80 h at rt during which it became turbid. The mixture was filtered over celite and the residue was washed with CH$_2$Cl$_2$. The filtrate was neutralized with 1 M aqueous HCl, transferred to a separatory funnel and separated. The aqueous layer was extracted with CH$_2$Cl$_2$ (3 x 5 mL) and the combined organic layers were washed with brine (2 x 5mL), dried over MgSO$_4$ and concentrated in vacuo. The obtained residue was further purified by column chromatography (20% Et$_2$O in pentane) in order to yield 7 mg of the natural di-ether glycerol lipid (20) as a yellow oil with some co-eluted impurities.

HRMS-ESI+ (m/z): [M + Na]$^+$ calculated for C$_{43}$H$_{72}$O$_3$Na, 659.5374; found, 659.5371.
Alcohol 13 (5 mg, 7.8 μmol) was dissolved in pyridine (0.2 mL) under N₂ atmosphere. (S)-(+)-MTPA-Cl (6 mg, 5 μL, 23.4 μmol, 3 equiv, Mosher’s chloride) was added dropwise and the reaction mixture was stirred for 2 h at rt before it was quenched with 0.2 mL saturated NaHCO₃ solution. A small quantity of water (1 mL) was added and the aqueous mixture was extracted with ethyl acetate (3 x 1 mL), the organic layers were combined, washed with brine (2 x 1 mL) dried over MgSO₄ and concentrated in vacuo. The residue was filtered over silica with pentane and the residue was washed with a large amount of pentane. The filtrate was concentrated in vacuo in order to yield a yellow crude mixture containing the desired Mosher’s ester (6 mg).

**1H NMR (599 MHz, Chloroform-d)** δ 7.57 – 7.50 (m, 2H), 7.42 – 7.37 (m, 3H), 5.42 – 5.23 (m, 1H), 5.16 – 5.05 (m, 6H), 4.69 (s, 1H), 4.51 (dd, J = 11.5, 3.5 Hz, 1H), 4.37 – 4.32 (m, 1H), 4.09 – 4.05 (m, 2H), 4.00 – 3.96 (m, 2H), 3.74 (m, 1H), 3.55 (s, 3H), 3.49 (m, J = 10 Hz, 1H), 3.42 (m, J = 10.0 Hz, 1H), 2.11 – 1.94 (m, 24H), 1.68 (s, 6H), 1.60 (s, 18H), 1.55 (s, 6H).

HRMS-ESI+ (m/z): [M + Na]⁺ calculated for C₅₃H₇₉F₃O₅Na, 875.5772; found, 875.5772.
Hybrid Heterochiral Membrane

2,3-bisgeranylgeranyl-sn3-glycerol (+)-Mosher’s ester derivative (18). This compound was prepared with the synthetic route, identical to that used for 2,3-bisgeranylgeranyl-sn-glycerol Mosher’s ester derivative (14).

1H NMR (599 MHz, Chloroform-d): δ 7.57 – 7.51 (m, 2H), 7.41 – 7.36 (m, 3H), 5.29 (m, 1H), 5.18 – 5.07 (m, 6H), 4.71 – 4.65 (m, 1H), 4.56 (dd, J = 11.5, 3.5 Hz, 1H), 4.37 – 4.32 (m, 1H), 4.07 (d, J = 6.5 Hz, 2H), 3.96 (d, J = 6.5 Hz, 2H), 3.75 – 3.71 (m, 1H), 3.56 (s, 3H), 3.49 (m, J = 10 Hz, 1H), 3.42 (m, J = 10 Hz, 1H), 2.12 – 1.94 (m, 24H), 1.64 (s, 6H), 1.59 (s, 18H), 1.56 (s, 6H).

HRMS-ESI+ (m/z): [M + Na]+ calculated for C_{53}H_{79}F_{3}O_{5}Na, 875.5772; found, 875.5773.

Naturally derived di-ether lipid (+)-Mosher’s ester derivative (19). This compound was prepared with the synthetic route, identical to that used for 2,3-bisgeranylgeranyl-sn-glycerol Mosher’s ester derivative (14).

1H NMR (599 MHz, Chloroform-d): Same as reported for 14

HRMS-ESI+ (m/z): [M + Na]+ calculated for C_{53}H_{79}F_{3}O_{5}Na, 875.5772; found, 875.5773.

Naturally derived di-ether lipid (+)-Mosher’s ester derivative from G1P deficient strain (20). This compound was prepared with the synthetic route, identical to that used for 2,3-bisgeranylgeranyl-sn-glycerol Mosher’s ester derivative (14).

1H NMR (599 MHz, Chloroform-d): Same as reported for 14

HRMS-ESI+ (m/z): [M + Na]+ calculated for C_{53}H_{79}F_{3}O_{5}Na, 875.5772; found, 875.5773.
Hybrid Heterochiral Membrane

(S)-enantiomer:
racemic mixture:
Hybrid Heterochiral Membrane

(R)-enantiomer:
Racemic mixture:
Hybrid Heterochiral Membrane

[Chemical structures and spectra images]

178
Chapter 4

Synthetic $sn$-1 Mosher’s ester lipid (14)

Synthetic $sn$-3 Mosher’s ester lipid (18)
Hybrid Heterochiral Membrane

Mixture of \( sn-1 \) and \( sn-3 \) Mosher's ester lipids
Naturally derived Mosher's ester lipid (19)

Naturally derived Mosher's ester lipid (G1P-deficient strain) (20)