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Archaeal phospholipids: Structural properties and biosynthesis☆

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ABSTRACT

Phospholipids are major components of the cellular membranes present in all living organisms. They typically form a lipid bilayer that embroiders the cell or cellular organelles, constitute a barrier for ions and small solutes and form a matrix that supports the function of membrane proteins. The chemical composition of the membrane phospholipids present in the two prokaryotic domains Archaea and Bacteria are vastly different. Archaeal lipids are composed of highly-methylated isoprenoid chains that are ether-linked to a glycerol-1-phosphate backbone while bacterial phospholipids consist of straight fatty acids bound by ester bonds to the enantiomeric glycerol-3-phosphate backbone. The chemical structure of the archaeal lipids and their compositional diversity ensures the required stability at extreme environmental conditions as many archaea thrive at such conditions including high or low temperature, high salinity and extreme acidic or alkaline pH values. However, not all archaea are extremophiles, and the presence of ether-linked phospholipids is a phylogenetic marker that distinguishes Archaea from other life forms. During the past decade, our understanding of the biosynthesis of archaeal lipids has progressed resulting in the characterization of the main biosynthetic steps of the pathway including the reconstitution of lipid biosynthesis in vitro. Here we describe the chemical and physical properties of archaeal lipids and membranes derived thereof, summarize the existing knowledge about the enzymology of the archaeal lipid biosynthetic pathway and discuss evolutionary theories associated with the "*Lipid Divide*" that resulted in the differentiation of bacterial and archaeal organisms. This article is part of a Special Issue entitled: Bacterial Lipids edited by Russell E. Bishop.

1. Introduction

Phospholipids are major constituents of all living cells. They are involved in a wide variety of cellular processes, most notably their properties allow the formation of a phospholipid bilayer which forms a barrier to separate the inside and outside of the cell as well as a matrix to support the diverse functions of membrane proteins. Lipids play an important role in protein translocation, DNA replication and cell division, signal transduction, transport and many other cellular mechanisms [1,2]. Phospholipids consist of hydrophobic hydrocarbon chains, which are compacted together through non-covalent bonds and hydrophobic effects. These interactions determine a specific lipid arrangement in the membrane bilayer such that the hydrocarbon chain is oriented to the interior of the membrane and the different polar groups, which are attached to the lipid tails linked to glycerophosphate backbone, face the outer surface of the membrane that is in contact with the water phase. These properties ensure a low membrane permeability for cellular constituents such as nutrients and ions, while the organization into a lipid bilayer provides the perfect matrix in which the membrane-integral proteins are embedded [3]. The membrane lipid composition represents a taxonomic signature that distinguishes the different kingdoms of life. Bacterial and eukaryotic lipids have a different chemical structure compared to the phospholipids of Archaea. Archaeal phospholipids are composed of highly methylated isoprenoid chains that are ether-linked to a glycerophosphate backbone, glycerol-1-phosphate (G1P). This differs from the straight fatty acids bound by ester bonds to the enantiomeric form of the glycerophosphate backbone, glycerol-3-phosphate as found in bacteria and eukarya. Although ether-linked phospholipids can also be found in some bacteria and in eukarya, they are usually not the major component of the membrane. It is believed that the chemical lipid structure of the ether lipids contributes to the survival in extreme environments in which many archaeal organisms thrive [4,5]. Hyperthermophile archaea, for instance, that grow at temperatures above 85 °C and up to 121 °C contain so-called membrane spanning tetraether lipids with one or more cyclic structures in their isoprenoid side chains which confer an even greater stability to membranes allowing these organisms to survive at such high

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temperature [6,7]. The unique composition and structure of the archaeal lipids are also reflected in the biosynthetic pathway. In the past decades, many studies have characterized the main enzymatic steps leading to the biosynthesis of ether lipids revealing common mechanisms in lipid biosynthesis but also steps that are unique to archaea [8]. Nevertheless, still some critical steps in biosynthesis are unresolved nor is it known how archaeal phospholipid biosynthesis is regulated. The availability of a multitude of archaeal genome sequences has provided a greater insight in the genetic basis of biosynthesis resulting in the identification of missing enzymatic steps. This information provided the basis for further evolutionary considerations on hypotheses on how bacteria and archaea may have evolved from a common ancestor, a process that is also referred to as the lipid divide.

This review focuses on the structural aspects of archaeal lipids, their biosynthesis and physical properties, and also discusses recent evolutionary insights on the lipid divide.

2. Structural diversity of archaeal phospholipids

Bacterial membrane lipids consist of two fatty acid residues (FARs) that are ester-linked to hydroxyls of a glycerol moiety derived from glycerol 3-phosphate [3] (Fig. 1). Usually the hydrocarbon chain of these molecules comprises an even number of carbon atoms and chains contain one or more double bonds between carbon atoms, typically in a *cis* configuration. In bacteria, 16- or 18-carbon fatty acids with one unsaturation per single chain are the most commonly found in phospholipid species. Archaeal membrane lipids instead have a rather different chemical structure; they are recognizable as lipids from their structure but produced in a different way. The hydrocarbon chain is characterized by a repetition of a five-carbon unit with a methyl group at every fourth carbon of a saturated isoprene unit which leads to the formation of isoprenoid chains with different lengths [9,10]. These branched chains, using with a carbon number of 20 are then linked via ether bonds to glycerol 1-phosphate, which is the enantiomeric form of the bacterial counterpart (Fig. 1). Besides the general structure outlined above, an enormous variety of archaeal lipids are found in nature that differ in phytanyl chain length, composition and configuration as well as various modifications at the polar head groups. These lipids are all based on two main lipid cores, a C_{20} *sn*-2,3-dyacilglycerol diether lipid termed archaeol or a C_{40} glycerol-dialkylglycerol tetraether lipid, commonly known as caldarchaeol. Further, archaeol based lipids with an aberrant isoprenoid chain length (C_{25}) [9,11], a condensation in a macrocyclic glycerol diether [12] or the presence of a tetritol diether [13] are examples of modifications that are often found in the ether lipids of halophilic archaea and in some extreme (hyper)thermophilic archaea (Fig. 2). An even higher degree of variation is found among the tetraether structures. Unlike the diether lipids, these types of lipids are composed by two isoprenoid chains with a 40 carbon number linked to two identical or different polar groups. These lipids span the entire thickness of the membrane, and they form a monolayer rather than a bilayer (Fig. 3). The first tetraether characterized is caldarchaeol which corresponds to an antiparallel arrangement of the two diether molecules connected through their isoprenoid chains [14]. The parallel isomer of the caldarchaeol was later found in some *Sulfolobus* and *Thermoplasma* species and it is termed isocaldarchaeol [15]. A covalent bond between two carbon atoms in the isoprenoid chain leads to a particular tetraether structure found in hyperthermophilic organisms, termed the H-shaped caldarchaeol [15]. A species specific structure of *Sulfolobales* is named nonitol, and is characterized by C_{40} diphytanyl chains linked to a C_6 polyol at both ends. When the polyol groups have an isomeric orientation, the nonitol structure is referred to as calditol [16,17]. An additional feature found among tetraethers of hyperthermophile archaea is the presence of cyclopentane rings in the hydrophobic chain region. The number of rings vary depending on the growth temperature, reaching a maximum of 8 rings per molecule at the highest growth temperatures possible which is around 121 °C. The presence of cyclohexane rings as found in the lipids of some *Sulfolobus* species defined a new set of tetraether structures named crenarchaeol [11,18] (Fig. 3). Additional diversity in the archaeal tetraether structures occurs with hydrocarbon chain lengths such as C_{30}, C_{31} or C_{35} [19]. Finally, unsaturated diether lipids are found in some psychrophilic (or cryophilic) archaea [20] that grow at very low temperatures (−20 to 10 °C).

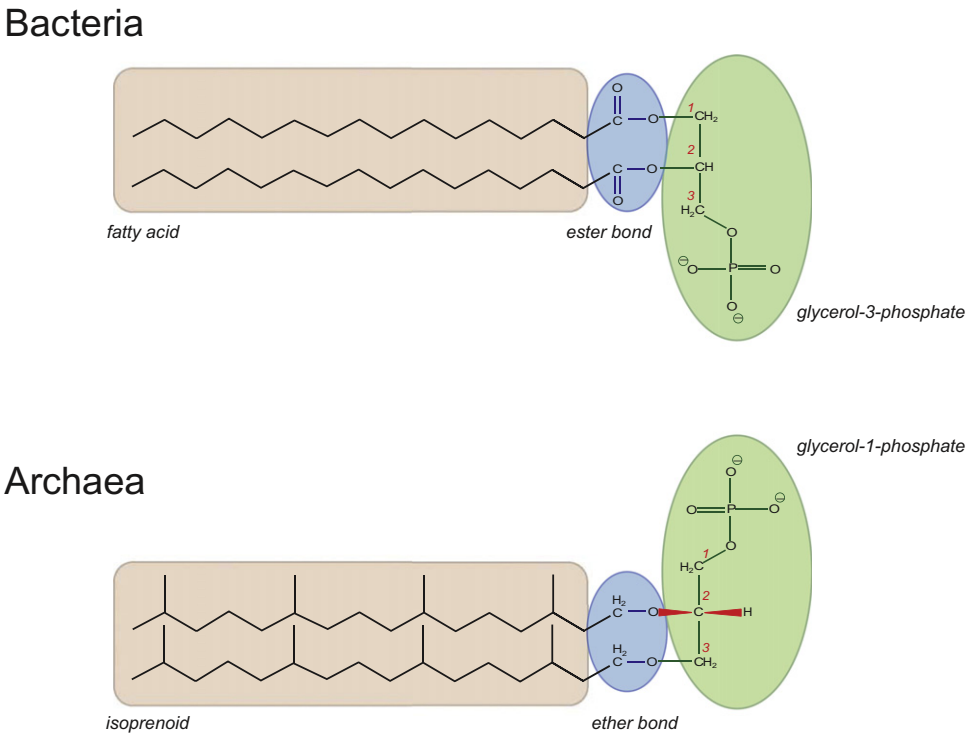

Fig. 1. Bacterial and archaeal lipid structure. Schematic representations of the bacterial and archaeal lipids which highlight the three main differences: hydrocarbon chain (pink), linkages (blue) and glycerophosphate backbone (green).

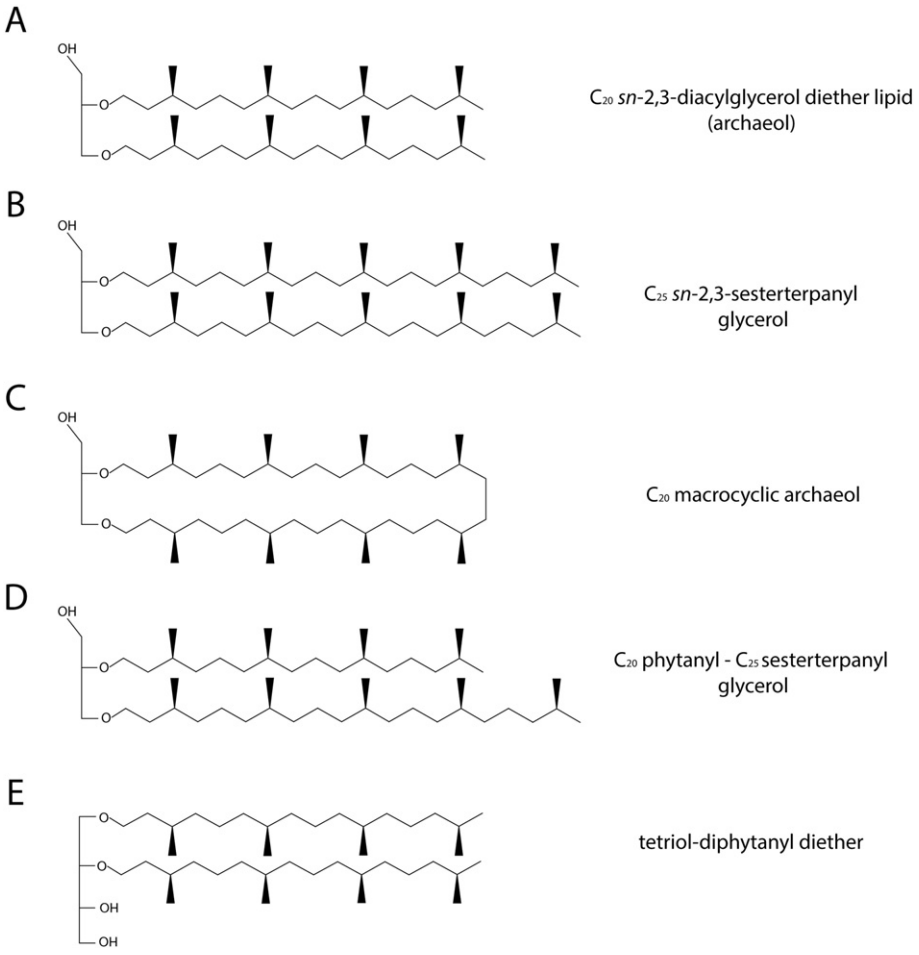

Fig. 2. Structures of archaeal diether lipids. (A) Basic chemical structure of diether lipids (archaeol). (B) Modification of the archaeol structure where the C_{20} isoprenoid chains are replaced by a C_{25} hydrocarbon chain. (C) Macrocyclic archaeol structure. (D) Further modification constituted by an asymmetric atom of carbon composition in the isoprenoid chains. (E) Tretriol-archaol diether lipid where the glycerol is replaced by a polyol molecule.

Further polar head group modifications are possible with multiple sugars [21] thereby increasing even more the diversity and variability of archaeal lipids. Evidently, the described archaeal lipid diversity can be seen as a unique taxonomic marker that adds to our understanding of the phylogenetic relationships between archaeal organisms.

3. Physiochemical properties of archaeal lipids

Biological membranes are typically composed of a bilayer of phospholipids that interacts with each other via non-covalent bonds such as Van der Waals and electrostatic interactions. One of the most important properties of a membrane is to ensure the proper lipid mobility and to maintain a constant fluidity by adapting the lipid composition in response to physical environments, e.g. temperature and pressure. Lipid bilayers can have a complex melting behavior and can undergo a transition from a crystal, or gel phase into a liquid state. At the growth temperature of a given organism, membranes are in a liquid-crystalline state in which a high degree of lipid movement ensures proper functioning of membrane proteins and maintenance of the barrier function to prevent passive diffusion of ions and other molecules [22]. The chemical structure of the lipids has a great impact on membrane fluidity such that by changing the lipid composition, organisms can control membrane fluidity in response to environmental factors. However, how organisms ensure the liquid-crystalline phase at different temperatures varies from species to species. Bacterial membranes composed of 16- or 18-carbon fatty acid residues (FARs), for instance, have a melting point over a narrow range of temperature (40 to 50 °C), while the introduction of one or more unsaturated bonds vastly lowers the melting point to temperatures even below 0 °C. Thus in order to keep the membrane in a liquid-crystalline phase at different temperatures, as most common mechanisms, organisms may change the lipid acyl chain length, the degree of saturation or the ratio of iso/anteiso branching [23–25]. In contrast, archaeal membranes typically do not undergo drastic changes in lipid composition in response to temperature variations due to the unique ability of their phytanyl chains to maintain a liquid-crystalline phase over a very wide range of growth temperatures (10 to 100 °C) [26]. The drastic modifications are seen only at the extremes of the temperature range, as will be outlined below. The different types of lipids found in archaeal organisms (some examples are listed in Table 1) support two important membrane properties required for life under extreme conditions, which are thermal stability and low ion/proton permeability.

3.1. Thermal stability

The unique stability of membranes in archaea can be attributed to the presence of branched methyl groups in the isoprenoid chain, tetraether structures, and macrocyclic rings which, together with an extensive network of hydrogen bonds between the polar head groups, determine a tight membrane packing [27]. The covalent linkage between the hydrocarbon chains of the tetraether lipids is a further major factor that contributes to the remarkable stability of archaeal membranes, but this particularly holds for extremely high growth temperatures and low pH. The monolayer configuration of tetraether lipids contributes to stability as it allows for a tighter lipid packing compared to diether lipids. This configuration prevents the melting of the inner and outer halves

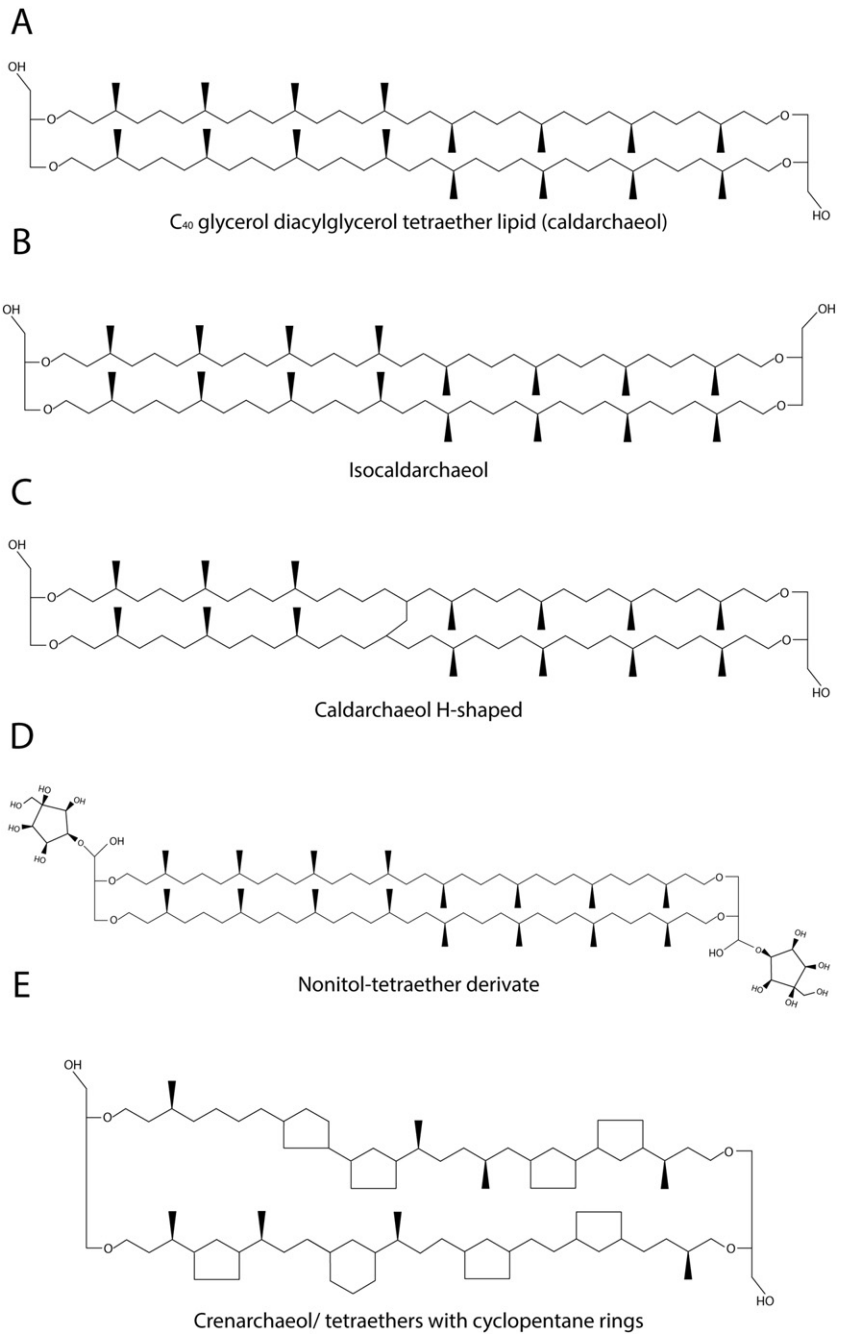

Fig. 3. Archaeal tetraether lipid structures. (A) Basic chemical structure of tetraether lipids (caldarchaeol). (B) Isomeric organization of the glycerol moiety which leads to the isocaldarchaeol formation. (C) H-shaped caldarchaeol structure with an intramolecular bond between two carbon atoms in the isoprenoid chain. (D) Nonitol-tetraether derivate in which the glycerol moiety is replaced by C_6 polyol molecules. (E) Creanarchaeol lipid structure with cyclopentane and cyclohexane rings typical of hyperthermophile organisms.

of the lipid bilayer at extremely high temperatures, thus maintaining an intact hydrophobic core thereby increasing stability. Further, the ether bond in the archaeal lipids are less prone to hydrolysis than the ester bond of bacterial lipids. The ether bond is resistant to lipid hydrolysis, an aspect that may contribute to stability at more extreme hot and acidic environments [28]. Also ether lipids are resistant to phospholipase treatment, while bacterial lipids are completely degraded by the successive action of different types of phospholipases. The membrane flexibility of tetraether lipid monolayers was compared to that of diether lipid bilayers through a molecular dynamics (MD) simulation. Herein, membranes composed of diether, acyclic tetraether and macrocyclic tetraether lipids were compared, confirming the importance of the monolayer structure in membrane stability [29]. In the simulation also the presence of cyclic rings was shown to increase membrane rigidity and resistance against external mechanical stresses. The presence of cyclic rings decreases the mobility of the hydrophobic region thereby affecting the packing and membrane organization [30]. Indeed, hyperthermophilic archaea increase the degree of cyclization upon an increase in growth temperature [9]. They add cyclopentane rings to the diphytanyl chain mounting up to a maximum of eight per chain. The MD simulations show that the presence of these cyclic structures reduces the rotational freedom of the chain thereby contributing to the maintenance of membrane fluidity and dynamics at elevated temperatures either in archaeal-like [31,32] and bacterial-like lipids [33] containing cyclopropane rings. Membranes composed of tetraether lipids with cyclopentane rings are more tightly packed and the lipid-lipid interaction are energetically more stable [34]. Tight membrane packing caused by the presence of cyclic rings results in a shortening

Table 1
Distribution of glycerol dialkyl glycerol tetraethers in Archaea.

Phylum	Species	pH	Temp (°C)	GDGTs/type	Ref.
Euryarchaeota	*Thermoplasma acidophilum*	1–2	30–60	95%/0–4 ring	[135]
	Picrophilus torridus	1.3	45–62	100%/0–8 ring	[136]
	Pyrococcus furiosus	7	85	69%/0 ring	[137]
	Archaeoglobus fulgidus	5.5–8	60–95	Major/0 ring	[138]
	Methanosarcina mazei	6–7	35	0%/0 ring	[9]
	Halobacteriales	6–7	0–40	0%/0 ring	[15]
Crenarchaeota	*Sulfolobus acidocaldarius*	2–3	70	100%/0–8 ring	[9]
	Caldivigra maquilingensis	4	83	100%/cyclopentane rings	[139]
	Ignisphaera aggregans	5.4–7	95	30%/0–4 ring, H-shaped	[140]
	Acidilobus sulfurireducensis	3–5	65–81	40%/0–7 ring	[141]
	Vulcaniseta distributa	3.5–5	65–89	100%/0 + cyclopentane rings	[142]
Thaumarchaeota	*Nitrosopumilis maritimus*	7	28	100%/0–4 ring, iso-, crenarchaeol	[143]
	Nitrososphaera viennensis	6.5	37	100%/0–4 ring, iso-, crenarchaeol	[144]

GDGT, glycerol dialkyl glycerol tetraethers.

of the distance between the phosphate moieties of neighboring polar head groups leading to more energetically favorable electrostatic interactions. The same MD simulation revealed that stability is also gained from the interactions between the polar head groups through hydrogen bonding. Fine tuning of the ratio of tetraethers and diethers with and without macrocyclic structures [35] is a further mechanism by which archaea can maintain membrane fluidity and stability over wide ranges of temperatures.

At lower temperatures, lipids will crystallize and the membranes may adopt a gel-like structure having a consequence of a drastic decrease in stability and an increased permeability. Psychrophilic organisms therefore increase the fraction of unsaturated isoprenoid chains in their lipids with decreasing temperature which is the main means to maintain membrane fluidity at low temperatures [36–38].

3.2. Ion permeability

The second fundamental property that membranes should fulfill is to function as a barrier against the free diffusion of ions and solutes into and out of the cell. This property is critical for cellular homeostasis such as to maintain a constant intracellular pH and ion strength, and a high electrochemical gradient of protons or sodium ions necessary to drive energy requiring processes. The archaeal lipids also influence the membrane permeability, in particular at high temperatures, salinity and extreme pH values. The presence of bulky branched methyl groups in the phytanyl chains reduces the degree of movement of these lipids and because of van der Waals interactions between the hydrocarbon chains, stable membrane packing is achieved [39]. As a consequence, membranes containing these lipids show a low permeability for small ions or solute molecules, a property that is also exploited in black lipid membranes where often phytanyl chain phospholipids are used in experimentation. Furthermore, several in vitro studies demonstrated that tetraether-based liposomes are equipped with a much lower ion-permeability than diether- or diester based liposomes. For instance, liposomes composed of a total lipid extract derived from *Thermoplasma acidophilum* [40] or *Sulfolobus acidocaldarius* [41,42], that both mostly contain tetraether lipids, are equipped with a very low permeability towards protons at high temperatures. The presence of cyclopentane rings further reduces the permeability [43–45]. Also, the presence of two or more sugar units as constituents of the polar head group contributes to the low proton permeability [46]. In this respect, there is a striking correlation between the effective proton permeability and membrane stability, factors that are both influenced by lipid packing. Obviously, this is in particular relevant for hyperthermophiles and hyperthermoacidophiles as the proton permeability increases with temperature, and at such high temperature becomes an issue causing higher maintenance energy. Proton tight membranes are also highly relevant for acidophiles that grow at extremely low pH (below pH 2.5) values. Thus, the chemical structure of archaeal lipids and derived modifications assures a proper balance between membrane stability and fluidity and ensures a low proton permeability even in extreme environments at which some archaea thrive.

3.3. Applications of archaeal lipids

The extreme high thermal stability and low permeability of liposomes composed of archaeal lipids has attracted the attention for biotechnological and biomedical applications. This in particular concerns liposome mediated drug encapsulation and targeting where formulation of advanced archaeal lipid-based liposomes can impact the efficiency of drug targeting. Classically, ester-bonded phospholipid liposomes have been exploited extensively as carrier systems to deliver drugs [47,48], but these liposomes are readily phagocytosed by macrophages and thus quickly cleared from the blood while also content retention can be an issue. Instead, archaeal-lipid based liposomes, named archaeosomes, appear more suitable for this purpose due to their intrinsic chemical properties and biocompatibility with human tissue [49]. They act as better adjuvants able to evoke a stronger immune response against cancer cells or in infectious diseases as compared to the ester-based phospholipid liposomes [50,51]. The enhanced adjuvant activity is due to their higher stability which allows them to fuse with immune cells [52], promoting a full humoral response via improved antigen delivery to the antigen processing compartment [53]. Archaeosomes are equipped with an exceptional stability and therefore they can even survive several autoclaving cycles at pH 4–10 without any apparent stability loss or content release [54]. This is in particular of interest to pharmaceutical applications since autoclaving represent a common sterilization method. Furthermore, since archaeosomes are also stable at very low pH, encapsulated drugs may even reach targets in the human gastro-intestinal tract pass through the stomach [55]. This makes archaeosomes attractive for potential biomedical applications when targets are difficult to reach, and at the same time allow for slow dose release over lengths of time [56]. Obviously, the flip-side of the high stability of the archaeosomes is that release is too slow, and therefore for effective release of the drugs smart solutions are needed. One of these avenues is the incorporation of switchable channels that may affect release under predetermined conditions.

4. Archaeal ether lipid biosynthesis pathway

During the last decades, most of the biosynthetic steps of the archaeal phospholipid biosynthesis pathway have been elucidated now providing a detailed insight in how ether lipids are synthesized (Fig. 4). Individual biosynthetic steps have been analyzed biochemically and recently, also the entire pathway has been reconstituted with purified protein components. Since the number of sequenced archaeal genomes have vastly increased during the last few years, advanced bioinformatics now helps to elucidate the remaining unresolved biosynthetic steps. In

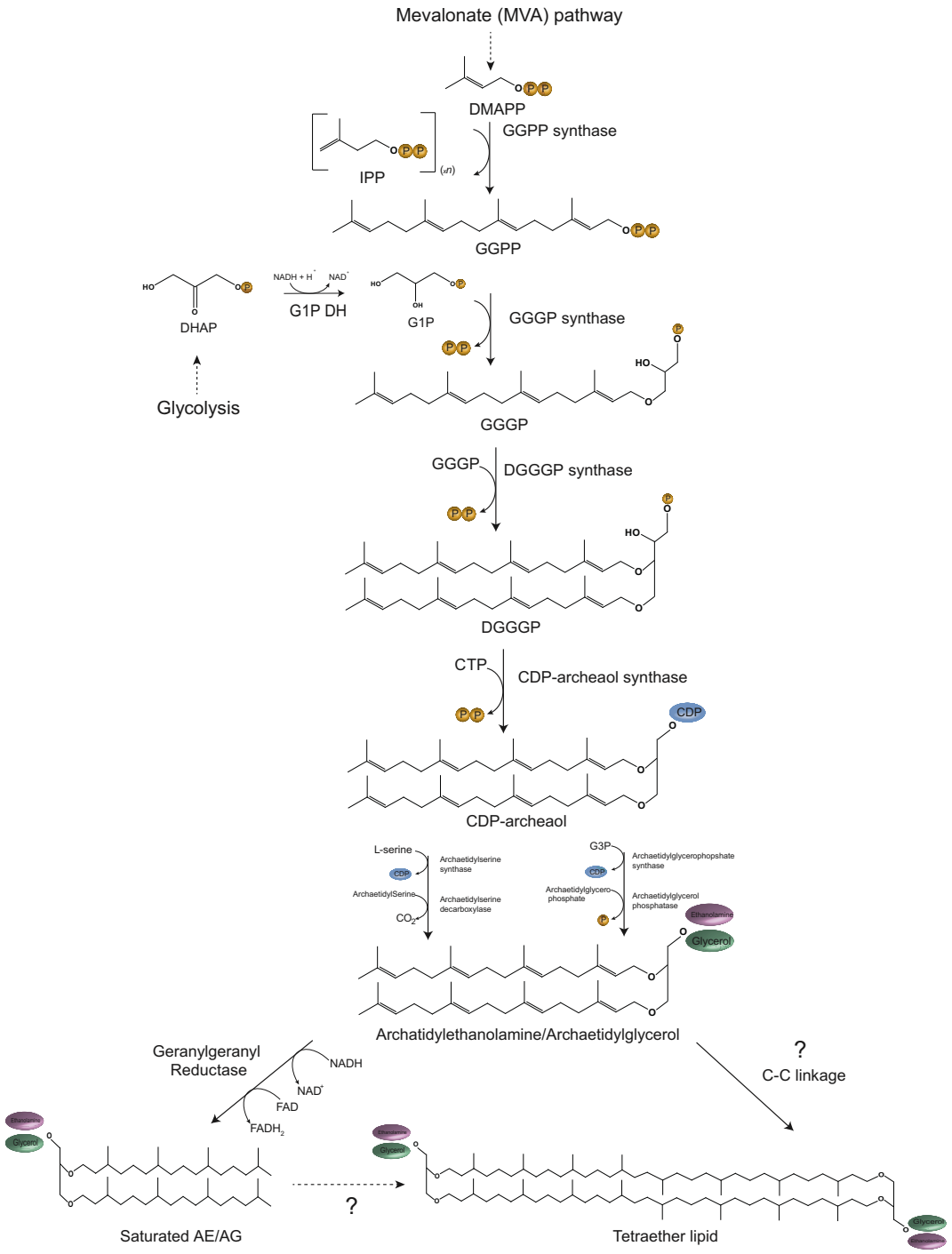

Fig. 4. Schematic representation of the archaeal lipid biosynthetic pathway. The main biosynthetic steps that lead to the synthesis of final archaeal lipids from the simple building blocks are depicted in the scheme. Ethanolamine and glycerol are the two polar head groups whose attachment mechanism has been described. The mechanism for the tetraether formation is unknown and could proceed via a head-to-head condensation of saturated AE/AG or exploit the presence of the double bonds in a different mechanism. Isopentenyl pyrophosphate (IPP), dimethylallyl pyrophosphate (DMAPP), geranylgeranyl diphosphate (GGPP), dihydroxyacetone phosphate (DHAP), glycerol-1-phosphate (G1P), geranylgeranylglyceryl phosphate (GGGP), digeranylgeranylglyceryl phosphate (DGGGP).

the following sections, the main biosynthetic steps that lead to the formation of diether phospholipids from simple isoprenoid building blocks are described in detail.

4.1. Synthesis of isoprenoid building blocks

The hydrocarbon chain of the archaeal lipids consists of a serial repetition of isoprene units that are synthesized from isopentenyl pyrophosphate (IPP) and the isomeric form dimethylallyl pyrophosphate (DMAPP). These two simple units are not only used for phospholipid biosynthesis, but are also building blocks of a great variety of molecules such as carotenoids, steroids, and ubiquinones. Although these building blocks are universally used to synthesize isoprenoids, the actual biosynthetic pathway differs between the various domains of life. Two main pathways have been identified, the mevalonate (MVA) pathway and the MVA-independent pathway known as 2-C-methyl-D-erythitol 4-phosphate/1-deoxy-D-xylulose-5-phosphate (MEP-DOXP) pathway [57–59]. The MVA pathway leads to the formation of IPP and DMAPP

via an initial condensation of acetyl-CoA molecules to form acetoacetyl-CoA which is further condensed to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) (Fig. 5). The latter is then reduced to form a mevalonate intermediate, which undergoes phosphorylation and decarboxylation reactions up to the formation of IPP and DMAPP [60,61]. The MVA pathway was initially believed to be the universal pathway responsible for isoprenoid precursor synthesis. However, the MEP-DOXP pathway was identified at a later stage and found to be present in bacteria, algae and plants. Herein, the IPP is synthetized from glyceraldehyde-3-phosphate and pyruvate through the action of seven enzymatic steps [62]. Archaea and non-photosynthetic eukaryotes depend for isoprenoid precursor biosynthesis exclusively on the MVA pathway [63,64]

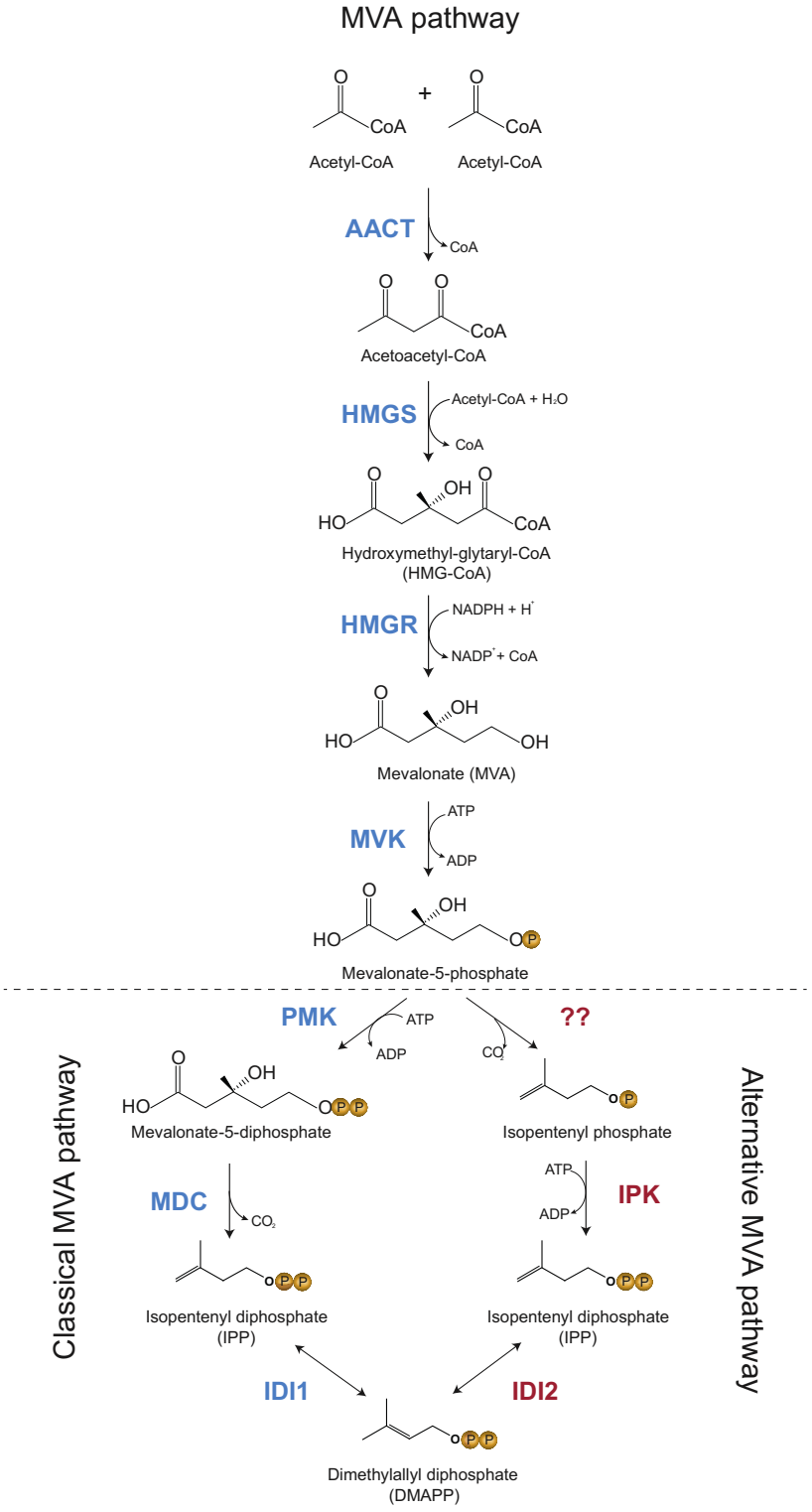

Fig. 5. Schematic representation of the synthesis of isoprenoid building blocks. The MVA pathway for the synthesis of IPP and DMAPP is indicated in the scheme. The alternative MVA pathway with the three specific archaeal enzymes is highlighted in red, whereas the enzymes involved in the classical MVA pathway are colored in blue. Acetoacetyl-CoA thiolase (AACT), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), mevalonate kinase (MVK), phosphomevalonate kinase (PMK), mevalonate-5-decarboxylase (MDC), isopentenyl phosphate kinase (IPK) and isopentenyl diphosphate isomerase (IDI).

and they do not contain the MEP-DOXP pathway enzymes. Interestingly, the MVA pathway in archaea differs from that in eukaryotes in the final steps of the isoprenoid unit synthesis [65,59,66]. The three enzymes of the classical MVA pathway, phosphomevalonate kinase (PMK), diphosphomevalonate decarboxylase (MDC) and isopentenyl diphosphate isomerase (IDI), do not share any sequence homology with the corresponding archaeal enzymes that define this alternative MVA pathway (Fig. 5), with the exception of Sulfolobales species which have the classical MVA pathway [67]. In particular the enzyme involved in the phosphorylation of mevalonate to isopentenyl phosphate via a decarboxylation reaction has still not been identified in archaea. However, a decarboxylase enzyme that converts MVA to isopentenyl phosphate was identified in the specific bacterial phylum Chloroflexi, along with a isopentenyl phosphate kinase (IPK) [68]. IPK which is involved in the synthesis of IPP, is instead highly conserved in archaea, and was described for the first time by Grochowski et al. in 2006 [69]. Furthermore, the last step of the alternative MVA pathway is catalyzed by an archaeal specific isomerase, named IDI2, which does not share any sequence homology with the IDI1 enzyme of the classical MVA pathway even though these enzymes catalyze the same biochemical reaction [59]. Thus, the distribution of the different isoprenoid pathways in the three domains of life shows a clear organismal correlation, since the MEP-DOXP pathway is typical for bacteria, while the classical MVA pathway is found in eukaryotes and the alternative MVA pathway is characteristic for archaea.

The two isoprenoid building blocks IPP and DMAPP subsequently undergo sequential condensation reactions that exploit the reactivity of the allylic diphosphate of IPP finally leading to the formation of polyisoprenoid chains that differ in length with the following main products: geranyl (C_{10}), farnesyl (C_{15}), geranylgeranyl (C_{20}), and farnesylgeranyl (C_{25}) isoprenoid chains. However, also longer isoprenoid chains may also be formed. The condensation reaction is catalyzed by enzymes of the prenyl transferase superfamily [70,71]. Generally archaeal lipids contain the geranylgeranyl or farnesylgeranyl isoprenoid chain, and the synthesis of these chains is catalyzed by two enzymes: geranylgeranyl diphosphate (GGPP) and farnesylgeranyl diphosphate (FGPP) synthase, respectively [72–74]. Structural and functional studies have demonstrated that the volume of the hydrophobic pocket of the substrate binding site is a determining factor in the final length of the isoprenoid chain. The substrate binding site is highly conserved among members of this protein family. The size of the aforementioned hydrophobic pocket can be enlarged by site-directed mutagenesis resulting in the formation of longer isoprenoid chains [75,76].

4.2. Glycerophosphate backbone synthesis

The glycerophosphate backbone of the archaeal ether lipids is derived from glycerol-1-phosphate (G1P). G1P in archaea and G3P in bacteria and eukaryotes result from the reduction of dihydroxyacetone phosphate (DHAP) using NADH (or NADPH) and divalent ions as cofactors [77]. This reaction is catalyzed by G1P dehydrogenase (G1PDH) and G3P dehydrogenase (G3PDH), respectively. Even though these two enzymes catalyze a similar enzymatic step (Fig. 4), they belong to vastly different protein superfamilies that do not share any sequence or structural homology [8,78]. The key enzymatic difference lies in the opposite orientation of the binding of the nicotinamide ring of the cofactor. The archaeal G1P dehydrogenase transfers the *pro*-R-hydrogen of glycerol to NADH in contrast to the bacterial and eukaryotic G3P dehydrogenases where the transfer of *pro*-S-hydrogen occurs [79] thereby resulting in a discrete stereospecificity [80]. G1PDH enzymes have been purified and characterized from many archaeal sources [81–83], but the enzymes are not unique to archaea per se as they are also found in some Gram positive and negative bacteria. For instance, the *araM* gene of *Bacillus subtilis* encodes a G1PDH that is needed for the production of an ether-like lipid whose actual function is still unknown [84].

4.3. Formation of ether bond linkages

The condensation of the isoprenoid chains to the glycerophosphate backbone is carried out by two different enzymes. The first ether linkage that brings together G1P and GGPP is catalyzed by a cytoplasmic protein geranylgeranylglyceryl diphosphate (GGGP) synthase (Fig. 4). In the proposed mechanism, the enzyme is involved in the transfer of a GGPP unit to the C3 oxygen atom of glycerol-1-phosphate which acts as an acceptor for the electrophilic charged carbon atom of the isoprenoid chain [85]. GGGPS enzymes can be classified in two main phylogenetic groups that differ in their selectivity towards isoprenoid chains of different lengths. Both archaeal and bacterial GGGPS belong to group I, that comprises dimeric enzymes while in group II both dimeric and hexameric enzymes are found [86]. Crystal structures of GGGPS enzymes from the two different groups revealed some striking differences in the organization of the hydrophobic pocket that accommodates the isoprenoid chain. The difference of group I and II GGGPS enzymes in specificity towards polyprenyl diphosphates with a defined chain length can be attributed to a set of amino acid residues that localize to the binding groove. The hydrophobic binding pocket of GGGPS enzymes from group II is characterized by "limiter residues", making them specific to accommodate only shorter polyprenyl chains [86,87].

The second ether bond between GGGP and a second GGPP molecule is catalyzed by the membrane associated enzyme di-*O*-geranylgeranylglyceryl diphosphate (DGGGP) synthase (Fig. 4). This protein belongs to a wide protein superfamily of ubiquinone-biosynthetic (UbiA) prenyltransferases, whose members are involved in diverse functions such as the synthesis of heme, chlorophyll and ubiquinones of the respiratory system. The DGGGP synthase is conserved in archaea even though a quite high sequence divergence was found in the archaea phylum Thaumarcheota, most likely due to the abundant presence of cyclohexane rings in the lipid structure [88]. DGGGP synthase activity was for the first time observed in the membrane fraction of the archaeon *Methanothermobacter marburgensis* [89]. The corresponding gene was later identified in *Sulfolobus solfataricus* and the corresponding enzyme was purified and shown to be highly specific towards GGPP and GGGP [90]. In another study, DGGGPS from *Archaeoglobus fulgidus* was expressed at high level in *E. coli*. By employing a coupled reaction with the GGGPS enzyme, GGPP could be successfully coupled to G1P to yield DGGGP in vitro [91] as well as in vivo by coexpression of the ether lipid enzymes in *E. coli* [92,93].

4.4. CDP-archaeol synthesis

To prepare DGGGP for the attachment of a polar head group, an intermediate step is required that leads to the synthesis of a CDP-activated precursor also known as CDP-archaeol (Fig. 4). CDP-archaeol synthase activity was first detected in the membrane fraction of *Methanothermobacter thermoautotrophicus* [94] but it has taken more than a decade before the gene encoding for the CDP-archaeol synthase (named CarS) was identified. In bacteria an analogous biosynthetic step takes place where phosphatidic acid is activated to CDP-diacylglycerol by the CDP-diacylglycerol synthetase (CdsA). The activity of CarS is strictly Mg^{2+} dependent, and specific for the substrate DGGGP and the cofactor CTP [91]. Recently, the structure of CdsA from *Thermotoga maritima* was solved at 3.4 Å resolution [95], that catalyzes an analogous reaction as CarS but using phosphatidic acid as substrate. The structure shows that the enzyme is arranged as a dimer where each monomer is composed of nine transmembrane helices. The C-terminal domain exhibits the highest degree of conservation, as it bears the CTP binding site and interacts with divalent ions (Fig. 6A). Each of the monomers contains a Mg^{2+} ion that acts as an activator of the phosphate group on the lipid substrate promoting an attack on the α-phosphate of CTP (Fig. 6B) causing the transfer of CMP to phosphatidic acid, which most likely is positioned in a hydrophobic groove on the surface.

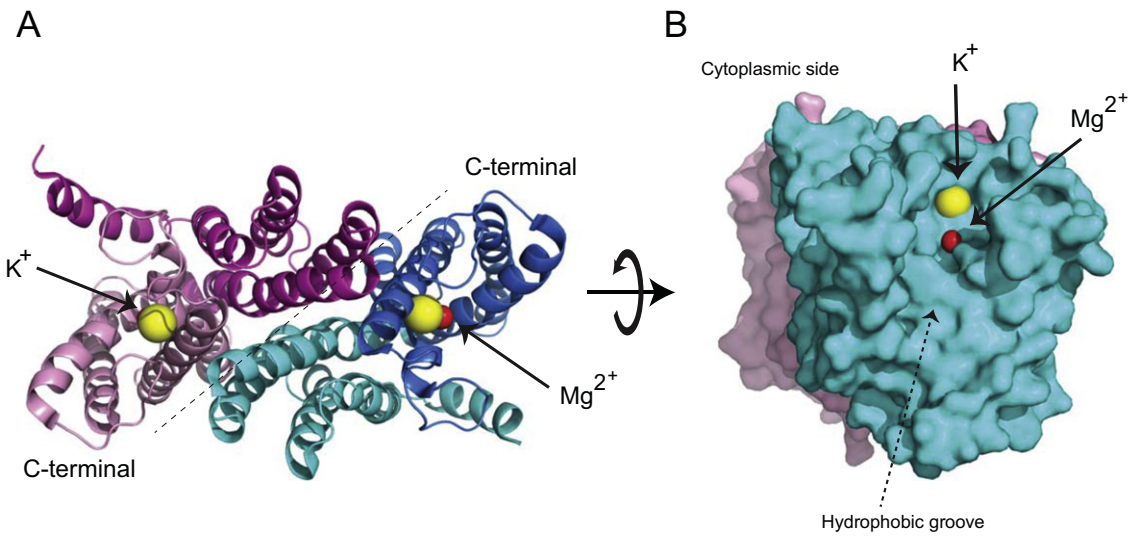

Fig. 6. Structure of CDP-diacylglycerol synthase (CdsA). (A) Crystal structure of the CDP-diacylglycerol synthetase of the bacterium *Thermotoga maritima* at 3.4 Å resolution (PDB: 4Q2E) [134] shows a dimeric organization. The two units are colored in magenta and light blue. The CTD domain is highlighted in each unit (pink and dark blue respectively) which interacts with the ions. The divalent ion Mg^{2+} is indicate in red and the monovalent ion K^{+} is highlighted in yellow. The dashed line represents the dimer interface. (B) Representation of the enzyme surface showing a hydrophobic groove as a putative substrate binding site and the CTP-binding pocket where the two ions reside.

Subsequently, the pyrophosphate is released in a process that is promoted by K^{+} which is also needed for efficient catalysis. CarS likely functions in a similar manner, but with CDP-archaeol formation no K^{+} requirement was observed. The identification of CarS allowed the complete reconstitution of the archaeal lipid biosynthetic pathway in vitro from the simple isoprenoid building blocks IPP and DMAPP up to CDP-archaeol formation utilizing a set of purified (membrane) proteins [91].

4.5. Attachment of polar head groups

Serine, ethanolamine, glycerol and *myo*-inositol are the most common polar head groups which replace the cytidine monophosphate (CMP) moiety of CDP-archaeol in archaea or CDP-diacylglycerol in bacteria and eukarya (Fig. 4). The enzymes that catalyze the corresponding transfer reactions are highly conserved in archaea and bacteria and they belong to same protein superfamily of CDP-alcohol phosphatidyltransferase [78,96–98]. In addition, there is a high degree of functional homology as for instance shown for the archaetidylserine (AS) synthase versus the phosphatidylserine (PS) synthase and the archaetidylglycerol (AG) synthase versus phosphatidylglycerol (PG) synthase. AS synthases catalyze the formation of AS from CDP-archeaol and L-serine and the enzymes are homologous to a subclass of bacterial PS synthases. The latter proteins are divided in two subclasses, where subclass I includes enzymes of Gram-negative bacteria, such as *E. coli* while subclass II, mostly concerns enzymes of Gram-positive bacteria, yeast and archaea. However, a recent study identified the presence of PssA of subclass II also in some Gram-negative bacterial species [99]. Initial studies using cell free extracts of *Methanothermobacter thermoautotropicus*, *B. subtilis*, and *E. coli* showed that both the AS synthase from *M. thermoautotropicus* and PS synthase of *B. subtilis* accept both CDP-archaeol and CDP-diacylglycerol as substrates. On the other hand, the *E. coli* PS synthase is specific only for CDP-diacylglycerol [100]. Likewise, in vitro studies utilizing the *B. subtilis* PS synthase demonstrated the high substrate promiscuity of this enzyme [93]. Further conversion into AE and PE involves the decarboxylation of the L-serine residue by an AS and PS decarboxylase, respectively. The archaeal and bacterial decarboxylases are highly homologous and the reactions proceed via the same mechanism [93]. These enzymes are not specific and accept either PS or AS as substrates.

Also the enzymes involved in the attachment of the glycerol group on the CDP-archaeol exhibit a high substrate promiscuity with respect to CDP-archaeol and CDP-diacylglycerol. This follows from observations that CDP-archaeol is readily converted into AG phosphate by the purified *E. coli* PG synthase in the presence of G3P, and then converted into AG by the *E. coli* PG phosphatase A [93]. Recently, the crystal structure of an archaeal member of CDP-alcohol phosphotransferase was solved at 2 Å resolution [101,102], revealing a dimeric form with two subunits of six transmembrane helices with a distinct N-terminal domain each. Although the mechanism of lipid binding was not resolved, the active site is probably close to the membrane and accessible from the cytosol consistent with the enzymatic property to bind a soluble and a membrane-bound substrate (Fig. 7A). The binding site contains a Ca^{2+} ion that probably forms a contact site with the CDP of the lipid intermediate during catalysis (Fig. 7B).

The replacement of the CDP group with *myo*-inositol is catalyzed by the archaetidylinositol (AI) phosphate synthase resulting in the formation of AI phosphate, which is further dephosphorylated to AI [103]. In bacteria a similar reaction takes place catalyzed by a phosphatidylinositol (PI) synthase. Likewise for the aforementioned AS/PS and AG/PG synthases, these enzymes belong to the same protein superfamily and show a broad substrate specificity [104].

4.6. Double bond saturation and isoprenoid chain modifications

The isoprenoid chains of fully matured archaeal lipids are usually completely saturated. Hydrogenation of the double bonds is catalyzed by geranylgeranyl reductase (GGR) enzymes (Fig. 4). These enzymes belong to the family of GGR proteins that also includes members from bacteria [105] and plants [106] that are mainly involved in photosynthesis and carotenoid production [107]. Among the archaea, several GGR homologues are found with a highly conserved FAD binding site but lower conservation of the substrate binding domain including the conserved sequence motif YxWxFPx7-8GxG that is important for orienting the isoprenoid chain in a FAD parallel position to enable double bond reduction [108]. Several GGR enzymes from different archaeal sources have been investigated and characterized. Reductase activity was initially observed in a cell-free extract of the hyperthermophilic archaeon *Thermoplasma acidophilum* and attributed to a membrane associated protein [109]. The crystal structures of the GGR enzymes of *T. acidophilum* [108] and *Sulfolobus acidocaldarius* [110] were solved as FAD bound complexes. The proposed hydrogenation mechanism requires the presence of NADH or another reducing agent that transfers

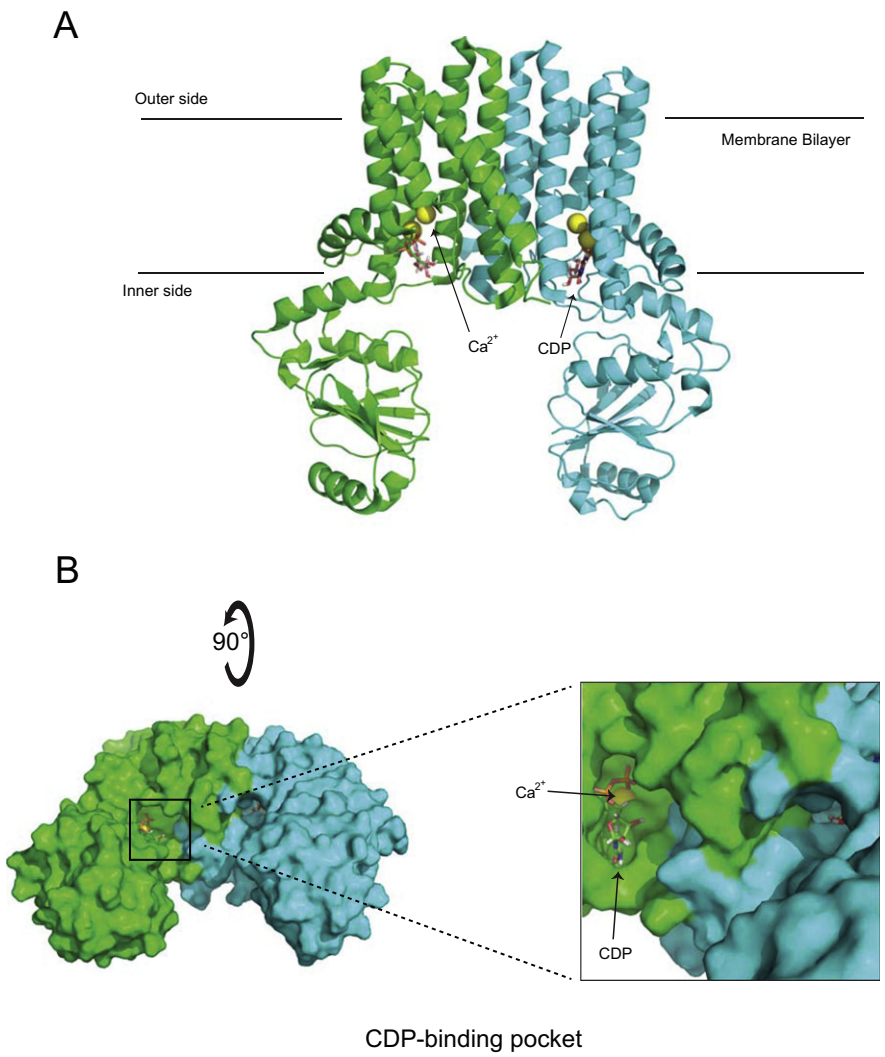

Fig. 7. Structure of CDP-alcohol phosphotransferase. (A) Crystal structure of CDP-alcohol phosphotransferase from *Archaeoglobus fulgidus* at 2.0 Å resolution (PDB: 4O6N) [101,102]. The two protomers of the dimer are shown in green and light blue. The active site is located close to the membrane but with a direct access to the cytoplasmic side according to the hydrophobic nature of the substrate and the hydrophilic properties of the head groups. The divalent ion Ca^{2+} is indicated by yellow spheres and the bound CDP is highlighted in a stick representation. (B) Representation of the enzyme surface showing the CDP-binding pocket with bound Ca^{2+}.

its electrons to the FAD cofactor whereupon the isoprenoid double bonds are reduced. The GGR exhibits a substrate preference in the order of DGGGP > GGGP ≫ GGPP with only 10% of activity towards GGPP compared to DGGGP [111]. The GGR from the mesophilic archaeon *Methanoscarcina acetivorans* was co-expressed in *E. coli* together with four genes of the archaeal ether lipid biosynthetic pathway and this allowed the formation of saturated DGGGP in vivo [112]. The reductase activity was enhanced when the ferredoxin gene, localized upstream of the GGR gene in *M. acetivorans*, was co-expressed but overall the degree of saturation was very low leaving most of the archaeal lipid unsaturated. This casts some doubts at what exact biosynthetic step saturation occurs and whether there are other, so far undiscovered enzymes that carry out this reaction more efficiently. Since CarS is active with unsaturated GGGP, saturation may occur after CDP-archaeol formation or even following polar head group attachment [8].

As mentioned above, hyperthermophiles adapt to high temperatures by the incorporation of cyclic structures in their lipid hydrophobic core. However, the exact mechanism and the specific enzymes involved have not yet been identified and characterized. A recent study, however, suggested an alternative pathway for ether lipid production, in which the synthesis of cyclic structures may take place in the early stages of the pathway, along with isoprenoid chain elongation [88]. The authors postulated this hypothesis on the basis of the amino acid sequence differences of the IPP synthase, GGGP synthase and DGGGP synthase enzymes which could give a great functional plasticity. Furthermore, the absence of genes homologous to the DGGGP synthase in Thaumarcheota may support this hypothesis, since the presence of a higher divergence DGGGP synthase could reflect the ability of this enzyme to accommodate bulky cyclohexane rings in the isoprenoid chain during synthesis. However, there is no biochemical evidence confirming this hypothesis.

4.7. Unusual C—C bond formation during tetraether lipid synthesis

The tetraether core is the most striking characteristic of membrane lipids in hyperthermophilic archaea. The biochemical reaction that leads to tetraether lipid formation is essentially unknown although several mechanisms have been proposed. One mechanism assumes that tetraether lipids arise from the tail-to-tail condensation of two diether lipids, but so far no direct evidence has been provided for such an unusual C—C bond formation reaction. Pulse-chase experiment using [^{14}C]-labeled melavonate added to *Thermoplasma acidophilum* cells indicate that there is a rapid incorporation of the radioactivity in the archaeol core and subsequently into the caldarchaeol core lipid, which would be consistent with a tail-to-tail condensation of the two C_{20} diethers leading to a C_{40} tetraether [113]. Terbinafine is a squalene

epoxidase inhibitor, and when added to *T. acidophilum* cells, fully saturated diether lipids like AG accumulate and tetraether lipid formation is blocked [114,115]. The authors argued that tetraether formation may take place once the polar head group is attached and the double bonds are saturated, but the experiment per se does not reveal the mechanism as the observed lipid end products may be formed via the regular biosynthetic route rather than being precursors that accumulate because of the inhibition of tetraether lipid formation. In another set of experiments, deuterated labeled diether precursors bearing saturated or unsaturated isoprenoid chains [116] were fed to cells, and the results suggested that unsaturation of the isoprenoid chains is important for tetraether lipid formation. This indeed supports the hypothesis that these lipids are formed by a tail-to-tail condensation of unsaturated diether lipids (Fig. 4). Recently, an alternative mechanism was proposed that involves a role of phytoene synthase in generating very long prenyl chains with a length of C40–44 that subsequently are condensed to G1P at both ends by versatile GGGPS and DGGGPS enzymes [88]. Although, this hypothesis cannot be excluded at this stage, the authors did not provide a convincing chemical mechanism on how such condensation reactions might occur. In analogy to the formation of diether lipids, this reaction would require that the long prenyl chains are modified with a pyrophosphate at both ends whereas the binding sites of GGGPS and DGGGPS should be able to recognize both orientations of the prenyl chain which seems enzymatically an unlikely event. Current experimental evidence points at a tail-to-tail condensation of two unsaturated diether lipids but the enzymatic mechanism remains to be elucidated.

5. Evolution of membrane lipid biosynthesis

How bacteria and archaea evolved and differentiated is one of the unsolved questions in evolution. The similarity between these two life forms in terms of genetic code and metabolism suggests the existence of a last universal common ancestor (LUCA) from which all domains of life have diverged. By means of large numbers of sequenced genomes, a common set of genes has been defined that must have been present in LUCA, also known as the cenancestor [117–119]. These mostly relate to enzymes involved in DNA replication and protein translation [120], but most other processes are not conserved and there is considerable variation between species. One of these processes is membrane lipid biosynthesis. As discussed in the previous sections, the membrane lipids of archaea and bacteria are distinct but the overall biosynthetic pathways follow similar schemes and likely evolved in parallel. The unique features of archaeal lipids are the presence of isoprenoid chains, the use of G1P as lipid backbone and the formation of ether bonds. Whereas isoprenoid biosynthesis is also observed in bacteria, the G1P dehydrogenase, the GGGPS and the DGGGPS are unique to archaea and these enzymes bear no homology to the bacterial G3P dehydrogenase, PlsB or PlsX/Y (depending on the bacterial species) [121] and PlsC that catalyze analogous steps. The archaeal and bacterial lipid biosynthesis pathways share steps of CDP-intermediate activation and polar head group attachment and the enzymes involved in those processes are more promiscuous with respect to substrate specificity and even share sequence homology [93,97].

The high degree of similarity and organization of the lipid biosynthetic pathways and the widespread presence of isoprenoid biosynthesis genes in all the organisms, supports the hypothesis of an original common ancestor. Several hypotheses have been proposed to explain the remarkable 'lipid divide' that must have led to differentiation of LUCA into the archaea and bacteria. The so-called acellular theory [122] proposes the independent separation of archaeal and bacterial organisms from a non-cellular ancestor developed on pyrite surfaces. In the primordial stage, chemical reactions may have led to the synthesis of a racemic mixture of the two enantiomeric forms of glycerophosphate. The appearance of stereoselective enzymes then pushed the evolution of two independent lipid biosynthetic pathways that were at the basis of the differentiation of archaea and bacteria (Fig. 8A). Martin and Russel [123] proposed a related theory that assumes the existence of an early stage where a non-free living universal ancestor originated in a prebiotic chemical environment. Their ancestor model was characterized by iron monosulphide precipitates that may have acted as cell-like compartments that are connected to form a network from which primordial archaeal and bacterial forms have emerged to completely differentiate with the acquisition of the proper enantioselective enzymes (Fig. 8B). Other hypotheses assume that the cenancestor already harbored a lipid membrane before the lipid divide occurred. The high conservation of the mevalonate pathway involved in the synthesis of isoprene units in archaea, eukarya and some bacteria [63,67] and the omnipresence of conserved membrane proteins such as the conserved ATPase [124], SecYE and YidC [125] support the presence of a lipid bilayer in the universal ancestor cell. The Wächtershäuser theory [126] proposes an initial stage of chemical and acellular metabolism on pyrite surfaces from which an initial pre-cell evolved. The pre-cell stage was then characterized by a racemic mixture of chemically synthetized chiral lipids from which bacterial, archaeal and eukaryotic organisms diverged in a three stage process during evolution triggered by environmental pressure (Fig. 8C). The existence of such heterochiral membranes was considered to be chemically unstable and unfavorable conditions for cellular life. Thus, when stereoselective enzymes evolved with specificity towards the enantiomeric form of glycerophosphate, evolution towards a more stable homochiral membrane may have led to the segregation of bacteria and archaea whereupon the lipid biosynthetic pathways underwent further parallel evolution as suggested by Peretó et al. [127] (Fig. 8D). However, experiments in vitro performed with liposome composed of mixtures of archaeal and bacterial lipids [128,129] do not support the immiscibility theory and actually demonstrate that mixed liposomes are equipped with a higher thermal stability and lower proton permeability than liposomes composed of only archaeal or bacterial lipids. Nevertheless, the presence of a universal ancestor cell whose membrane was characterized by a racemic mixture of lipids is a likely possibility. Environmental pressure and the progressive evolution of an enzyme with increased stereoselectivity may have led to the lipid divide without the need to assume membrane instability, and this may eventually have driven the segregation of bacterial and archaeal cells.

6. Conclusion and perspectives

Recently, many mechanistic aspects of the archaeal lipid biosynthetic pathway have been resolved now resulting in a more complete insight in the enzymology of diether lipid biosynthesis. However, several prominent features still remain unresolved most notably the mechanism of tetraether lipid formation for which different hypothesis have been proposed (see Section 4.7). Also the mechanisms of the various usual isoprenoid chain modification as depicted in Figs. 2 and 3, as well as the mechanism by which cells regulate the isoprenoid chain composition in response to environmental conditions have barely been studied. Further, archaeal lipids are in particular resistant to degradation and for this reason, they can be detected as fossil remnants in sediments [130–132]. In this respect, also little is known about ether lipid turnover and degradation. These represent challenging questions for the future.

One further challenge is to replicate the presumed mixed heterochiral membrane of the cenancestor, i.e., a mixture of both ether and ester lipids. To this end, several attempts were made to introduce the ether lipid biosynthetic pathway into *Escherichia coli*. By the expression of four genes encoding a GGPP synthase, G1P dehydrogenase, GGGP synthase and DGGGP synthase from the hyperthermophile *Archaeoglobus fulgidus* [92], or mesophile *Methanosarcina acetivorans* [133], part of the ether lipid pathway could be reconstructed in *E. coli*. By the addition of the GGR and thioredoxin gene of *M. acetivorans* also partially saturated archaeal lipid could be generated [112]. Recently,

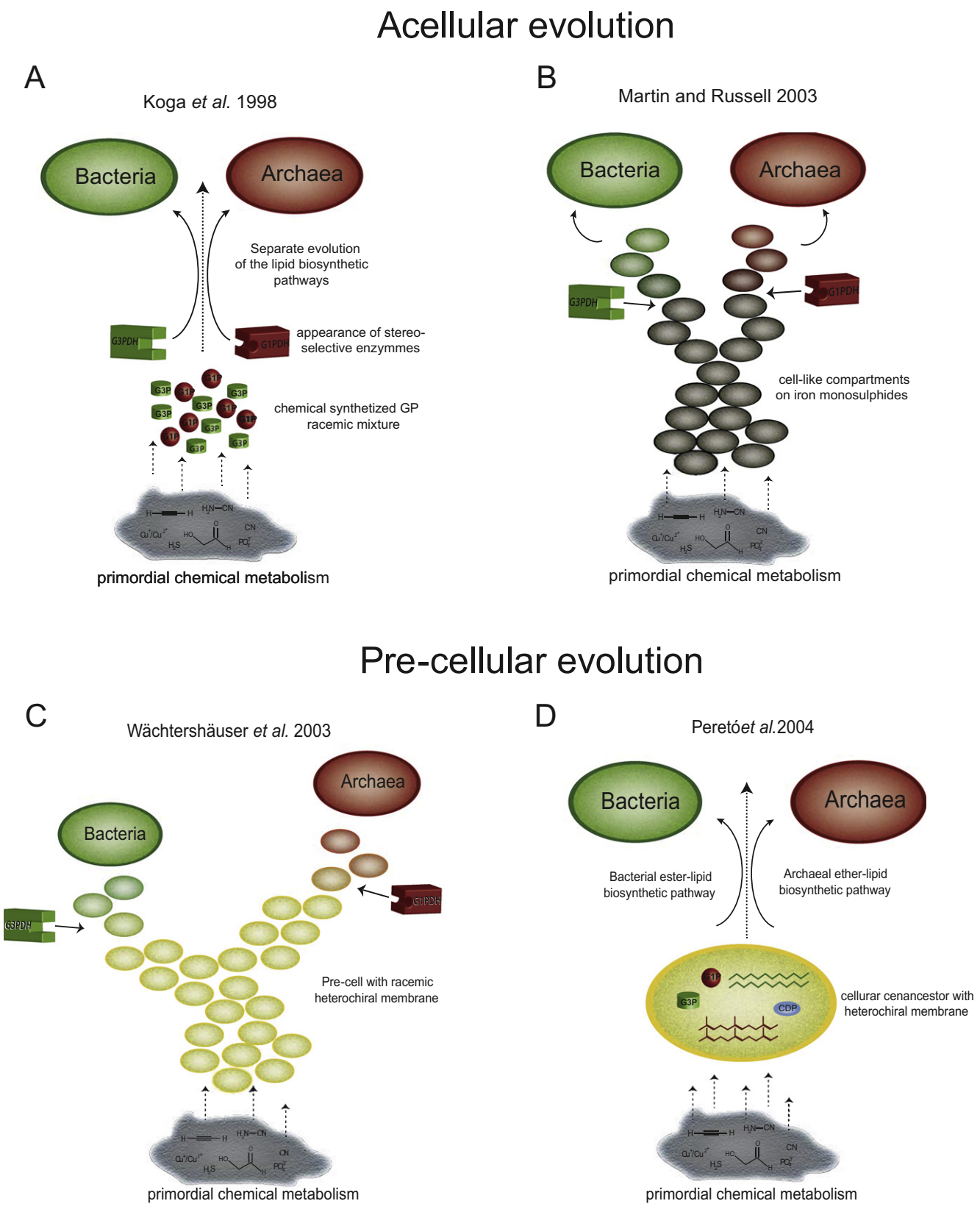

Fig. 8. Schematic representation of proposed evolutionary models for the differentiation of Archaea and Bacteria. The acellular evolution models included are the (A) Koga and (B) Martin and Russell theories. The pre-cellular evolution models shown are the (C) Wächtershäuser and (D) Peretó hypothesis. See text for further explanation. Abbreviation: G1P (glycerol-1-phosphate), G3P (glycerol-3-phosphate), G1PDH (glycerol-1-phosphate dehydrogenase), G3PDH (glycerol-3-phosphate dehydrogenase), CDP (cytidine diphosphate).

the complete ether lipid biosynthetic pathway was introduced in *E. coli* allowing the biosynthesis of the two unsaturated ether lipids AG and AE [93]. However, in all of these experiments, the amounts of ether lipids produced are very low and less than 1% of the total bacterial lipid fraction. Thus, further optimization and pathway refactoring is necessary to replicate a mixed heterochiral membrane in living cells. Such a

'synthetic' organism could serve various means. It may provide new insights in the evolutionary mechanisms underlying the differentiation of bacteria and archaea, but such an 'archaeabacterium', i.e., a synthetic cell with both bacterial and archaeal properties may also serve as a production host for archaeal membrane proteins as alternative to the mostly less genetically accessible archaea.

Transparency document

The Transparency document associated with this article can be found, in online version.

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