Review

Lipid sugar carriers at the extremes: The phosphodolichols Archaea use in N-glycosylation

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Abstract

N-glycosylation, a post-translational modification whereby glycans are covalently linked to select Asn residues of target proteins, occurs in all three domains of life. Across evolution, the N-linked glycans are initially assembled on phosphorylated cytoplasmically-oriented polyprenoids, with polyproren (mainly C\textsubscript{35} undecaprenol) fulfilling this role in Bacteria and dolichol assuming this function in Eukarya and Archaea. The eukaryal and archaeal versions of dolichol can, however, be distinguished on the basis of their length, degree of saturation and by other traits. As is true for many facets of their biology, Archaea, best known in their capacity as extremophiles, present unique approaches for synthesizing phosphodolichols. At the same time, general insight into the assembly and processing of glycan-bearing phosphodolichols has come from studies of the archaeal enzymes responsible. In this review, these and other aspects of archaeal phosphodolichol biology are addressed.

1. Introduction

Life on Earth can be divided into three domains, namely Eukarya, Bacteria and Archaea [1]. The Archaea, first reported in 1977 [2], were initially thought of as extremophiles, given their abilities to thrive in some of the harshest environments on the planet, including those characterized by extremes of temperature, pH or salinity. However, it has since become clear that Archaea are major residents of so-called ‘normal niches’, such as ocean waters, soil and even intestinal flora [3–5]. In terms of their biology, Archaea can be considered a mosaic, presenting traits shared by Eukarya and/or Bacteria, together with elements that distinguish this form of life, such as the unique lipids comprising the archaeal membrane [6].

As in Eukarya and Bacteria, the archaeal cell is also surrounded by a phospholipid-based membrane. Yet, whereas eukaryal and bacterial phospholipids essentially comprise fatty acid side chains linked to a 1,2-sn-glycerol-3-phosphate backbone via ester bonds, their archaeal counterparts instead correspond to isoprenoid hydrocarbon side chains linked to a 2,3-sn-glycerol-1-phosphate backbone via ether bonds [6]. While such lipids are organized into the bilayer structure that delineates most archaeal cells, some Archaea are instead surrounded by membranes based on a lipid monolayer comprising tetraether lipids in which both ends of the isoprenoid side chains are ether-bound to glycerol phosphate backbones [7]. It is thought that the unusual physicochemical properties of their membrane lipids contribute to the abilities of Archaea to withstand the physical challenges presented by the hostile surroundings that they often inhabit, although how such distinctive phospholipid composition would benefit non-extremophilic archaea remains unclear [8–10]. For more information on archaeal phospholipids, the reader is directed to reviews on the topic [6,7,11–13].

In addition to isoprenoid-based phospholipids and glycolipids, the archaeal plasma membrane also includes isoprenoid-based non-bilayer-forming lipids. These include carotenoids, such as C\textsubscript{40} bacteriortubearins and C\textsubscript{60} carotenes [14], retinals, such as those associated with bacteriorhodopsin and other sensory and transport rhodopsins [15], and phosphodolichols, lipids involved in protein N-glycosylation [16,17]. In the following, current knowledge on the phosphodolichols that participate in archaeal N-glycosylation is reviewed.

2. The lipid glycan carriers of N-glycosylation across evolution

Long held to be an exclusively eukaryal post-translational modification, it is now clear that both Bacteria and Archaea can also perform N-glycosylation, i.e., the covalent attachment of mono- to polysaccharides to select Asn residues of target proteins [18]. Archaea, however, display a degree of diversity in all aspects of N-glycosylation that is unparalleled in either Eukarya or Bacteria [19]. Such diversity is evident at the level of the lipid carrier upon which N-linked glycans are assembled.
At present, the process of N-glycosylation is best understood in higher Eukarya [20,21]. Here, the sugar α-N-acetylgalactosamine (GlcNAc)-1-phosphate, donated by UDP-GlcNAc, is first added to dolichol phosphate (DolP) to yield dolichyl pyrophosphate (DolIPP)-bound GlcNAc-1-phosphate. Six additional nucleotide-activated sugars are next added to yield heptasaccharide-charged DolIPP. This moiety is then flipped across the endoplasmic reticulum (ER) membrane to face the ER lumen by an ATP-independent flipase that remains to be described. At this point, seven more sugars, each delivered from a distinct DolP carrier charged on the cytoplasmic face of the ER membrane and flipped to face the ER lumen, are added to yield a 14-member branched oligosaccharide that is ultimately transferred to select Asn residues of a nascent polypeptide entering the ER lumen by the multimeric oligosaccharyltransferase, with Stt3 serving as the catalytic subunit of the complex.

In Bacteria, where N-glycosylation is apparently restricted to only certain groups, the process involved has been best described in the pathogen *Campylobacter jejuni* [22,23]. Here, some 60 different proteins are modified by a heptasaccharide assembled by a pathway reminiscent of its eukaryal counterpart. In *C. jejuni*, N-glycosylation begins with the generation of di-N-acetylglucosamine-charged undecaprenyl pyrophosphate (UndIPP) via the fusion of a UDP-charged version of the sugar with undecaprenyl phosphate on the cytoplasmic face of the plasma membrane. Following glycan extension through the addition of six more sugars, the UndIPP-linked heptasaccharide is flipped across the membrane in a reaction involving the ABC transporter PglK. Once on the outer surface of the plasma membrane, the glycan is delivered to select Asn residues of target proteins by PglB, the bacterial oligosaccharyltransferase.

In Archaea, where the first example of non-eukaryal N-glycosylation was reported [24], the process remains the least well understood. In part, this is due to the fact that the vast majority of identified strains cannot be grown in the laboratory, and of the small number of cultivatable strains, genetic tools are only available for few. Furthermore, the N-linked glycans decorating archaeal glycoproteins demonstrate enormous variability in terms of composition and architecture, reflecting the variety of N-glycosylation processes employed in Archaea [19,25]. Still, what is known reveals that as in the other two domains, N-glycosylation in Archaea begins with the assembly of a glycan on one or more phosphorylated dolichol carriers. For instance, the first four sugars of the N-linked pentasaccharide decorating glycoproteins in the halophile (‘salt-loving’ organism) *Halofexer volcanii* (optimal growth in 1.5–2.5 M NaCl [26]) are assembled on a common DolP carrier, while the final sugar is delivered from a distinct DolP [27]. As such, DolP serves both as lipid glycan carrier and glycan donor in this organism. By contrast, the similar N-linked pentasaccharide attached to glycoproteins of a second halooarchaea also originating from the Dead Sea, *Halobacterium marismortui* (optimal growth in 3.5–4 M NaCl [28]), is completely assembled on a single DolP carrier [29]. In yet another halophilic archaee, *Halobacterium salinarum* (optimal growth in 3.9 M NaCl [30]), the surface (S)-layer glycoprotein, the building block of the S-layer surrounding the cell, is simultaneously modified by two distinct N-linked glycans, one initially assembled on DolP, the second on DolPP [31,32]. In the thermocadophile *Sulfolobus acidocaldarius* (optimal growth at 80 °C and pH 2 [33]), DolP is charged with the same hexasaccharide as N-linked to glycoproteins in this organism, as well as its derivatives, although DolP and hexose-charged DolP have also been detected [34,35]. In the related species *Sulfolobus solfataricus* (optimal growth at 87 °C and pH 3.5–5 [36]), DolP is charged with the first six sugars of the heptasaccharide N-linked to glycoproteins in this organism; the final sugar could thus be derived from hexose-charged DolP [37]. As such, it appears that N-glycosylation in Archaea relies on a variety of lipid-linked glycan processing strategies.

The dolichols and polyenolins that serve as lipid glycan carriers across evolution (Fig. 1; Table 1) are examples of polysaccharide oligosaccharides, a family of hydrophilic polymers containing linearly linked isoprene subunits, members of which are found in all organisms [38,39]. While both contain up to 25 isoprene subunits and present a hydroxyl group at the so-called α end, dolichols include a saturated α position isoprene subunit, whereas polyenolins, including C55, undecaprenol, do not. The dolichols used in eukaryal N-glycosylation can, however, be distinguished from their archaeal counterparts in that all of the dolichols shown to participate in archaeal N-glycosylation to date also present an additional saturated isoprene subunit at the ω position, namely that isoprene farthest from the α end of the molecule [27,29,32,34,35,37,40,41]. The significance of saturation of the ω position isoprene is not clear, since in vitro studies revealed how C55,60 DolP saturated solely at the α position could be glycan-charged using enzymes involved in N-glycosylation in the methanogen *Methanococcus voltae* (grown in the laboratory in a 80% H2/20% CO2 atmosphere [42]). Moreover, this glycan-charged lipid could serve as substrate for the archaeal oligosaccharyltransferase AqGb. At the same time, in the thermophile *Pyrococcus horikoshii* (optimal growth at 98 °C [43]), both undecaprenol phosphate and dolichol phosphate were reported to serve as substrates for DolP mannose synthase, the enzyme that charges DolP with mannose, raising questions as to the importance of ω position isoprene saturation in archaeal N-glycosylation [44].

### 3. The diversity of archaeal phosphodolichols

In addition to the saturated ω position isoprene subunit that differentiates archaeal dolichol from the eukaryal version of this lipid, the dolichols employed in archaeal N-glycosylation also present various species-specific characteristics. These differences are mostly manifested in terms of dolichol length, degree of phosphorylation and the extent of internal isoprene subunit saturation (Fig. 2).

To date, phosphorylated dolichols, many charged with the same glycans as N-linked to glycoproteins in the same species, have been detected in Archaea representing several phenotypes and belonging to different phyla. The first phosphorylated dolichols detected in Archaea were in *Hbt. salinarum*, soon after this halophile provided the first example of non-eukaryal N-glycosylation, namely the S-layer glycoprotein [24,45,46]. Here, C55,60 DolP is modified by a sulfated tetra/pentasaccharide N-linked to ten N-glycosylation sites of the protein, while C55,60 DolPP is modified by a distinct sulfated pentasaccharide that apparently assembles into a chain when linked to the Asn-2 position of the protein [31,32,46,47]. It is, however, not known whether the chain of repeating sulfated pentasaccharides is assembled on a single DolPP carrier (and if so, on which side of the membrane) and only then transferred to the protein, or alternatively, whether such polymerization results from the transfer of the subunit glycan to the protein from a series of glycan-charged DolPP carriers assembled on the cytoplasmic face of the membrane and then flipped to face the exterior, where N-glycosylation transpires [48].

Recently, saturation of the α and ω position isoprenes of *Hbt. salinarum* C56,60 DolP and C55,60 DolPP was demonstrated. In these studies, DolP was detected bearing a disaccharide precursor of the tetra/pentasaccharide, while DolPP was shown to be modified by the first four sugars of the repeating pentasaccharide; DolPP bearing repeats of this glycan was not observed [32]. However, the first demonstration of dolichol saturation at both ends of the lipid was reported in the case C55,60 DolP bearing a tetrasaccharide in *Hfx. volcanii* [40]. Much later, the tetrasaccharide attached to this DolP was shown to modify at least one Asn of the S-layer glycoprotein when the cells were grown in 1.7 M NaCl [49], considered as low salinity for this halophile [26]. Indeed, the most convincing evidence for the involvement of DolP in archaeal N-glycosylation has come from studies on *Hfx. volcanii*, where the deletion of genes encoding components of the N-glycosylation pathway yielded the same effects on the glycans linked to both DolP and to a target protein, the S-layer glycoprotein [27]. Subsequently, C55,60 DolP saturated at both the α and ω position isoprenes was observed in other haloarchaea, namely *Har. marismortui*, *Hfx. volcanii* and *Pyrococcus furiosus*, with the nature of α,ω isoprene saturation in archaeal N-glycosylation.
Fig. 1. Lipid glycan carriers used across evolution. Examples of lipid glycan carriers from Eukarya (human C95 DolPP (trans, cis 16 isoprenes) charged with the tetradecasaccharide GlcNAc2-Man9-Glc3 where Glc is glucose, GlcNAc is N-acetylglucosamine and Man is mannose), Bacteria (C. jejuni C50 UndPP (trans, cis 8 isoprenes) charged with the heptasaccharide GalNAc-α1,4-GalNAc-α1,2-GalNAc-α1,3-dinAcBac where diNacBac is 2,4-diacetamido-2,3,6-trideoxy-D-glucopyranose, GalNAc is N-acetylgalactosamine and Glc is glucose) and Archaea (Hfx. volcanii C60 DolP (trans, cis 8 isoprenes, predicted) charged with methyl-O-4-GlcA-β1,4-GalA-α1,4-GlcA-β1,4-glucose where GalA is galacturonic acid and GlcA is glucuronic acid). In each lipid, the arrow depicts the α position isoprene while the arrowhead depicts the ω position isoprene. In the eukaryal and archaeal lipids, the α position isoprene is saturated, whereas the ω position isoprene is saturated only in the archaeal lipid.

Table 1
A comparison of lipid glycan carriers used in N-glycosylation across evolution.

<table>
<thead>
<tr>
<th>Eukarya</th>
<th>Bacteria</th>
<th>Archaea</th>
</tr>
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<tbody>
<tr>
<td>Lipid glycan carrier</td>
<td>DolP</td>
<td>UndPP</td>
</tr>
<tr>
<td>Length</td>
<td>C70–C105</td>
<td>C50–C60</td>
</tr>
<tr>
<td>Positions of saturated isoprenes</td>
<td>α-position isoprene</td>
<td>α- and ω-position isoprenes; variable number of internal position isoprenes</td>
</tr>
<tr>
<td>Location</td>
<td>ER membrane</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>Topology of sugar addition</td>
<td>Sugars added on both sides of the membrane</td>
<td>Sugars added only on the cytosolic side of the membrane</td>
</tr>
<tr>
<td>Number of lipid glycan carriers used per N-glycosylation event</td>
<td>Multiple</td>
<td>Single</td>
</tr>
<tr>
<td>Chemical modification of lipid-linked glycan</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Recycling after glycan transfer to target protein</td>
<td>DolPP is dephosphorylated to DolP, flipped to face the ER lumen and recharged to yield DolPP-glycan</td>
<td>UndPP is dephosphorylated to UndP, flipped to face the cytoplasm and recharged to yield UndPP-glycan</td>
</tr>
</tbody>
</table>
where the lipid was modified by the same pentasaccharide as found on the S-layer glycoprotein in this species [29], and *Haloflexix mediterranei*, where the lipid was modified by a disaccharide [32]. Presently, nothing else is known about N-glycosylation in *Hfx. mediterranei*.

Haloarchaea, such as those considered above, belong to the Euryarchaeota, one of the better-studied archaeal phyla [1,50]. The same phylum also includes the methanogens, namely Archaea able to reduce carbon dioxide with hydrogen to generate methane [51]. To date, phosphorylated dolichols have only been characterized in two species. In *Methanococcus maripaludis* (grown in the laboratory in a 80% H2/20% CO2 atmosphere [52]), a lipid extract was shown to contain C55 DolP saturated at two non-specified positions and modified by a trisaccharide that corresponds to a precursor of the tetrasaccharide N-linked to glycoproteins in this organism [53,54]. Moreover, the enzyme thought to add the initial sugar to the lipid carrier in *M. maripaludis* was shown to be more active when presented with C55,60 DolP rather than with longer C85–105 DolP, as found in eukaryotes [54]. In contrast, older studies conducted on *Methanothermus fervidus* (optimal growth at 83 °C [55]), where the S-layer glycoprotein is modified by an N-linked hexasaccharide, detected sugar-modified C55 DolPP [56].

In addition to halophiles and methanogens, the Euryarchaeota also include thermophilic archaean where phosphodolichols involved in N-glycosylation have been characterized, namely *Pyrococcus furiosus* and *Archaeoglobus fulgidus*. In *P. furiosus* (optimal growth at 100 °C [57]), C40,45,70 DolP modified by glycans containing up to the first six sugars found in the N-linked heptasaccharide that decorates glycoproteins in this organism was described [37,41]. As such, *P. furiosus* DolP is the longest phosphodolichol involved in archaeal N-glycosylation known to date. More striking, however, was the finding that in addition to being saturated at the α and ω position isoprenes, *P. furiosus* DolP is also saturated at up to four more internal isoprene positions, with these additional sites of saturation being closer to the ω than the α end of the molecule. In *A. fulgidus* (optimal growth at 83 °C [58]), C55,60 DolP displays saturation of not only the α and ω position isoprenes but also of up to four additional internal isoprene positions [37]. Moreover, *A. fulgidus* DolP is apparently modified by two distinct heptasaccharides [37]. The different lipid-linked glycans could reflect the fact that *A. fulgidus* encodes multiple versions of the oligosaccharyltransferase AglB [59], responsible for the transfer of the glycan moiety from the lipid carrier to select Asn residues of modified proteins (see below), raising the possibility that different versions of the enzyme process distinct glycan substrates.

In addition to the phosphodolichols involved in N-glycosylation studied in representatives of the Euryarchaeota, these lipids have also been considered in three members of a second well-studied archaeal phylum, the Crenarchaeota [1,50]. *S. acidocaldarius* is the only crenarchaeote for which insight into the N-glycosylation process is available. Here, C40,45,50 DolP, in some cases charged with one of three different hexoses, and C45 DolPP modified by the hexasaccharide added

**Fig. 2.** Phosphodolichols used in archaeal N-glycosylation. Archaeal N-glycosylation relies on phosphodolichols that differ in terms of length, degree of phosphorylation and the extent of internal isoprene subunit saturation. Shown are major phosphodolichol species used (or present) in N-glycosylation in different Archaea.
to target proteins in N-glycosylation, as well as by glycans comprising the first four of five sugars of this structure, were detected [34,35]. In S. acidocaldarius DolP and DolIPP, up to six isoprenes are saturated, including those at the α and ω positions. In the related thermocarbo-
dilopho S. solfataricus, αC6 and C4α DolIPP modified by the first six sugars of the N-linked heptasaccharide assembled in this species [60] and saturated at the α and ω isoprene positions, as well as at up to four additional internal position isoprenes, were identified [37]. Moreover, it has been suggested that the two phosphodolichol glycan carriers used in S. solfataricus N-glycosylation differ not only in length (C8α and C6α) but also in terms of stereochemistry [37]. At the same time, Pyrobac-
ulm calidifontis (optimal growth at 90–95 °C [61]) was reported to contain C9α,55 DolIPP modified by a decasaccharide also shown to be N-
linked to a glycopeptide generated by proteolytic digestion of a membrane fraction prepared from this organism [37]. Similarly to the phosphodolichols in other crenarchaeotes considered to date, P. calidifontis DolIPP is saturated at the α position isoprene, up to five internal position isoprenes and at the ω position isoprene [37].

Despite the limited number of archaeal species in which phospho-
dolichols either shown or likely to be involved in N-glycosylation have been characterized, general considerations on the use of these lipids may have already been revealed. With the exception of M. fervidus, where older technologies were employed, it would appear that N-
glycosylation in the Euryarchaeota relies on DolP as glycan lipid carrier, with the N-linked glycan either being assembled on a single DolP carrier or alternatively, being assembled on the modified protein from precursors delivered from at least two DolP carriers. In contrast, Crenarchaeota rely on DolIPP as lipid glycan carrier, with DolIPP possibly serving as the carrier of individual sugars either delivered to DolIPP-linked precursors of N-linked glycans or to glycans already transferred from DolIPP carriers to target protein Asn residues. As such, it would appear that euryarchaeotes rely on a more bacterial-like N-glycosyla-
tion process, whereas crenarchaeotes rely on a process reminiscent of that employed in Eukarya. Indeed, phylogenetic analyses of known Archaea position the Crenarchaeota proximal to the ancestor of modern-day eukaryotes [62,63].

In addition, the fact that DolP and DolIPP from both thermophilic euryarchaeotes and crenarchaeotes include saturated isoprenes at internal positions points to dolichol isoprene reduction as being important for survival at high temperatures. Such enhanced saturation, relative to what is seen in mesophilic archaea, would reduce the movement of phosphodolichols in the membranes of thermophilic archaea, a property that could be important for the enzyme-mediated processing of these glycan carriers. At the same time, dolichol length does not seem to be sensitive to temperature, since both the shortest (C10) and longest (C90) archaeal phosphodolichols known to date have been detected in thermophiles [37,41].

4. Phosphodolichol biosynthesis in Archaea

Phosphodolichol synthesis occurs in two stages [38,39]. In the first stage, the two building blocks of isoprenoid biosynthesis, isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP), are generated from acetyl CoA. This is followed by the second stage of dolichol biosynthesis in which a series of condensation reactions between DMAPP and several IPP moieties occurs. The resulting polymer is then further processed to yield the phosphorylated dolichols used in archaeal N-glycosylation.

The classic route for generating IPP and DMAPP is known as the mevalonate pathway [64]. Here, three acetyl CoA molecules are condensed and reduced using NADPH to yield mevalonate via the sequential actions of acetacetyl-CoA thiolase, 3-hydroxy-3-methylglutaryl (HMG-CoA) synthase and HMG-CoA reductase. Mevalonate is subsequently phosphorylated by mevalonate-5-kinase and phosphome-
valonate kinase to yield mevalonate-5-phosphate and mevalonate pyrophosphate, respectively. The latter compound is processed by mevalonate pyrophosphate decarboxylase to generate IPP, which is thenomerized by IPP isomerase to yield DMAPP (Fig. 3).

Genomic analyses revealed the presence of the first four enzymes of the classic mevalonate pathway in Archaea, namely acetacetyl-CoA thiolase, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase, HMG-CoA reductase and mevalonate-5-kinase [65,66]. Subse-
quent efforts not only confirmed the functionality of archaeal versions of these enzymes but also revealed unique properties in several instances. For example, Hfx. volcanii HMG-CoA reductase is regulated at the transcription level by environmental salinity, pointing to a link between the cell surroundings and the biosynthesis of isoprenoids, including phosphodolichols [67]. Moreover, Hfx. volcanii HMG-CoA reductase is the first such enzyme that is not sensitive to feedback inhibition by its substrate, acetacetyl-CoA [68]. Likewise, known feedback inhibitors of mevalonate-5-kinases do not inhibit the Metha-
osarcina maezii enzyme, making it the sole member of a third class of mevalonate kinases defined on the basis of inhibition profile [69].

At the same time, genomic scanning failed to detect archaeal homologues of mevalonate pyrophosphate decarboxylase in most species [65,66,70], suggesting the existence of an alternate route for the conversion of mevalonate-5-phosphate to IPP. Initial support for this hypothesis came with the identification of an isopentenyl phos-
phate kinase in Methanocaldococcus jannaschii (optimal growth at 85 °C in a 80% H2/20% CO2 atmosphere; first isolated from a hydrothermal vent on the Pacific Ocean floor [71]), namely an enzyme that synthesizes IPP via the phosphorylation of isopentenyl phosphate [72]. Genomic analyses revealed that other Archaea also contain the gene encoding this protein, with the activity of recombinant isopente-
neryl phosphate kinase from Methanothermobacter thermoautotrophicus (optimal growth at 65–70 °C in a 80% H2/20% CO2 atmosphere [73]) and Thermoplasma acidophilum (optimal growth at 59 °C and pH 1–2 [74]) having been demonstrated [75]. Subsequent efforts uncovered the existence of both phosphomevalonate decarboxylase, responsible for converting mevalonate-5-phosphate into isopentenyl phosphate, and isopentenyl phosphate kinase in Hfx. volcanii, thereby confirmed the existence of an alternate route for IPP biosynthesis in Archaea (Fig. 3) [76]. Nonetheless, there are archaeal species that rely on the classic mevalonate pathway despite also encoding components of the alternate pathway, such as S. solfataricus [77]. Here too, Archaea have introduced unusual variations not seen elsewhere. For instance, while the meva-
lonate pyrophosphate decarboxylase in S. solfataricus exists as a dimer, like its eukaryal and bacterial counterparts, the archaeal enzyme is unique in that it does not require allosteric regulation to maintain activity in this thermoadilopho [78]. Recently, evidence for the existence of yet another pathway for IPP biosynthesis in Archaea has been presented. In T. acidophilum, mevalonate-3-kinase converts mevalonate into mevalo-
nate-3-phosphate, which is then converted into mevalonate-3,5-bispho-
phate by mevalonate-3-phosphate-5-kinase [79,80]. Mevalonate-3-
kinase was subsequently described in Picrophilus torridus (optimal growth at 60 °C and pH 1.8–2 [81], with sequence analysis suggesting the enzyme to also exist in other members of the Thermoplasmatales, the order that includes these two species [82]. Presently, however, the mevalonate-3,5-bisphosphate decarboxylase that would be required for the appearance of IPP by this novel route has yet to be identified (Fig. 3). Finally, the IPP isomerases used by Archaea to convert IPP into DMAPP are, for the most part, orthologous of a bacterial, rather than the eukaryal version of this enzyme [83]. This, despite the fact that many Bacteria do not use the MVA pathway but rather rely on a distinct pathway for IPP biosynthesis termed the MEP/DOPX pathway, as do plants [84,85]. Members of the archaeal order Haloarchaeales, however, encode both versions of the isomerase [66].

To generate phosphodolichols from the five-carbon precursors IPP and DMAPP, there is a need for elongation steps in which DMAPP and multiple IPP moieties polymerize to yield an isoprenoid chain, dephos-
phorylation steps to yield a polyprenol, saturation steps to reduce the isoprene subunit at the α end of the dolichol molecule (and, in Archaea,
the isoprene subunit at the ω position, and in some cases, at more internal positions) and finally, phosphorylation steps. While the details of many of these reactions are relatively well described in Eukarya (and in undecaprenol synthesis in Bacteria, where saturation steps are not required), much less is known about the route(s) taken in Archaea.

Polyprenol biosynthesis begins with DMAPP serving as the acceptor for an IPP molecule to generate C10 geranyl pyrophosphate [38,39]. This reaction, as well as the addition of a second IPP to generate C15 farnesyl pyrophosphate, involves trans-condensations catalyzed by isoprenyl pyrophosphate synthases, also termed prenyltransferases, members of an enzyme family responsible for generating polyprenol chains of increasing length [86]. Following the condensation of IPP moieties in trans, additional IPP units are added in cis by a cis-prenyltransferase to generate a trans/cis-polyprenol pyrophosphate.

Now found within the membrane, the polyprenol pyrophosphate is next thought to undergo two rounds of dephosphorylation to yield polyisoprenol [87,88]. In Eukarya, saturation of the α position isoprene subunit is proposed to occur at this stage, yielding dolichol; in Bacteria, no such saturation occurs. Finally, dolichol is phosphorylated by dolichol kinase to generate DolP [87,89]. In Bacteria, an undecaprenol phosphokinase that catalyzes a similar reaction has been described [38,90]. Whereas there is considerable understanding of the steps taken to synthesize IPP and DMAPP in Archaea, insight into how Archaea assemble phosphodolichol from these building blocks remains only partial. Nonetheless, what is currently known has revealed unexpected aspects of each of the latter steps of phosphodolichol assembly, namely elongation, dephosphorylation, saturation and phosphorylation.

The sequential addition of IPP subunits to a DMAPP acceptor is
catalyzed by distinct prenyltransferases that catalyze condensation reactions as a function of substrate length [86]. To generate dolichol, the so-called short-chain prenyltransferases are required [91]. In most Archaea, the only members of this group are C_{20} geranylgeranyl pyrophosphate synthases [92,93]. These, however, can assemble trans-polyprenyl pyrophosphates of different lengths, as exemplified by the bi-functional enzyme from *M. thermoturricus* that is able to synthesize both farnesyl pyrophosphate and geranylgeranyl pyrophosphate [94]. Similar bi-functional activity was reported for geranylgeranyl pyrophosphate synthases from *S. acidocaldarius* and *Thermococcus kodakarensis* (optimal growth at 95 °C [95]) [96,97]. Based on such observations, it has been proposed that archaeal geranylgeranyl pyrophosphate synthases represent the ancestor of the farnesyl pyrophosphate synthases and geranylgeranyl pyrophosphate synthases presently found in Eukarya and Bacteria [98,99]. This hypothesis has, however, been questioned by others [100]. Finally, in *S. acidocaldarius*, genes encoding IPP isomerase (saci0091), geranyl pyrophosphate synthase (saci0092) and AglH, the glycosyltransferase responsible for adding the first sugar of the N-linked glycan to DolIPP (saci0093), lay adjacent to each other in the genome, with saci0092 and saci0093 being found on the same polycistronic mRNA [101,102]. This gene arrangement offers support for close interplay between phosphodolichol biosynthesis and N-glycosylation in this thermoacidophile.

In general, the substrate (or primer) of cis-prenyltransferases in Eukarya and Bacteria is C_{15} trans-farnesyl pyrophosphate [38,39,91], although evidence for the broader selectivity of these enzymes in plants has been offered [103,104]. In Archaea, both C_{15} trans-farnesyl pyrophosphate and C_{20} trans-geranylgeranyl pyrophosphate serve as cis-prenyltransferase substrates [92]. In *S. acidocaldarius*, apparently only trans-geranylgeranyl pyrophosphate serves this role [92]. The recent report of one of the three cis-prenyltransferases in *Methanosarcina acetivorans* being able to catalyze both head-to-tail and head-to-tail condensation reactions [105] could reflect yet another unique aspect of archaeal phosphodolichol biosynthesis. In the hyperthermophile *Aquopyrum pernix* (optimal growth at 90–95 °C [106]), the cis-prenyltransferase is unusual in that the substrate is C_{25} trans-geranyl-farnesyl pyrophosphate [107]. This could indicate the use of a unique lipid glycan carrier in this species. Accordingly, it is of note that when 168 archaeal genomes were considered for the presence of aglB, encoding the archaeal oligosaccharide transferase responsible for the transfer of glycans from phosphodolichol carriers to target protein Asn residues, *A. pernix* was only one of two species where no such gene was detected, raising the question of whether N-glycosylation occurs in this organism [108]. However, if N-glycosylation indeed occurs in *A. pernix*, the process may rely on an oligosaccharide transferase distinct from AglB. Lastly, it has been reported that in mammalian cells, cis-prenyltransferases require interaction with the NogoB receptor for stabilization of the enzyme [109]. In plants, where cis-prenyltransferases exist as enzyme families, it was shown that select family members that contribute to dolichol biogenesis also interact with a NogoB receptor homologue [103,110]. Although phylogenetic analysis has raised the possibility of such a protein in Archaea [105], evidence for the involvement of a NogoB receptor homologue in archaeal cis-prenyltransferase activity has yet to be presented.

Once the polyprenyl pyrophosphates destined to become phosphodolichols have been assembled, it is believed that they must next undergo dephosphorylation, saturation and phosphorylation [111], although conclusive evidence that this is indeed the order of events in Archaea remains wanting. With this in mind, a scheme of the possible steps taken during the latter phase of archaeal DolP biosynthesis, using *S. acidocaldarius* as a model system [34], is presented in Fig. 4. Indeed, it should be noted that alternate pathways leading to the appearance of DolP exist in other forms of life [112], and, therefore, possibly in Archaea as well. Nonetheless, in this review it is assumed that in Archaea, polyprenyl pyrophosphates are subjected to dephosphorylation, saturation and then phosphorylation to generate DoLP.

The conversion of polyprenyl pyrophosphates to polyenol requires dephosphorylation. In Eukarya, the enzyme(s) responsible has (have) yet to be described, although polyenol pyrophosphate phosphatase and polyenol pyrophosphate activities have been observed in whole-cell homogenates [113]. In Bacteria, undecaprenol pyrophosphate is converted to undecaprenol phosphate by the actions of UppP [114]. The resulting mono-phosphorylated polymer serves as the bacterial lipid glycan carrier. In Archaea, it is assumed that dephosphorylation also occurs. Indeed, undecaprenol (and dolichol) has been observed in *M. acetivorans* [115]. Likewise, the detection of dolichol in *S. acidocaldarius* [34] could reflect the initial dephosphorylation of polyenol pyrophosphates, although here too, the enzyme(s) responsible remain(s) to be identified. Finally, while roles for polyenol and dolichol have been proposed in Bacteria and Eukarya, respectively [117], it is not known whether dolichol serves any biological function in Archaea.

In the presumed next step in phosphodolichol biosynthesis, saturation of the isoprene subunit found at the α position of polyenol is thought to occur. In Archaea, saturation of the isoprene subunit at the ω position would likely also occur at this point, as would the saturation of other isoprene subunits found at more internal positions in those in species where highly saturated phosphodolichols exist. A polyenol reductase responsible for saturation of the α position isoprene subunit of polyenol has been identified in Eukarya [116]. The enzyme, encoded by the SRD5A3 gene in humans, has no archaeal homologue. As such, it would appear that Archaea rely on a distinct enzyme for such activity. Furthermore, given that eukaryal cells lacking SRD5A3 can still generate some 30% of the dolichol found in normal cells [116], it would seem that at least one more eukaryal polyenol reductase must exist. One can speculate that Archaea, lacking a SRD5A3 gene, could instead contain homologues of this(these) additional polyenol reductase(s), considering the high number of saturated isoprenes in archaeological phosphodolichols. To date, archaeal proteins showing polyenol reductase activity have been described. *S. acidocaldarius* contains a geranylgeranyl reductase that is able to saturate three of the four double bonds in geranylgeranyl pyrophosphate, leaving the Δ^2 double bond intact and thereby yielding a substrate for prenyltransferases in a reaction that would result in the removal of the pyrophosphate group from the geranylgeranyl pyrophosphate substrate [117]. Recent crystallographic analysis of *S. acidocaldarius* geranylgeranyl reductase bound to geranylgeranyl pyrophosphate has provided insight into the catalytic mechanism of this enzyme [118]. In contrast to the geranylgeranyl reductase of *T. acidophilum*, which uses NADPH as electron donor [119], the *S. acidocaldarius* enzyme does not use either NADPH or NADH, and as such, the source of electrons used in the saturation reaction in this species is not known [117]. In the case of *M. acetivorans* geranylgeranyl reductase, ferrodoxin is thought to serve as electron donor [120]. In addition, reductases that saturate the ω position isoprene subunit of polyenol, all members of the geranylgeranyl reductase family, have also been identified. These enzymes, however, affect the saturation status of other lipids as well, raising the question of whether these reductases are truly components of the phosphodolichol biosynthesis pathway in Archaea [115,121]. Indeed, the observation that *S. acidocaldarius* contains versions of C_{45} DolP presenting saturation at the α position isoprene subunit, at the α and ω position isoprene subunits and at these two positions together as well as at one-four more internal positions, suggests that multiple reductases contribute to phosphodolichol biosynthesis here [34]. Finally, considering that two or more of the isoprene subunits comprising archaeal dolichols are saturated, it has been proposed that archaeal dolichols should be called archaeoprenoids, so as to distinguish them from their less saturated eukaryal counterparts [122].

In what is assumed to be the final step of DolP biosynthesis, dolichol is phosphorylated. In Eukarya, this reaction is catalyzed by a CTP-dependent dolichol kinase [123,124]. Homology-based searches of
archaeal genomes have identified homologues of eukaryal dolichol kinase in only a limited number of species, implying that dolichol phosphorylation in Archaea relies on a distinct enzyme. Once the lipid-linked glycan has been transferred by the oligosaccharyltransferase to a target protein, the lipid carrier can be recycled for subsequent rounds of glycan-charging. In Eukarya, for example, the DolPP that remains following N-glycosylation undergoes dephosphorylation to yield DolP, which is subsequently returned to the cytoplasmic leaflet of ER, where it is again recruited as a glycan carrier [125,126]. In Archaea, incubation of glycan-charged DolPP from *S. solfataricus* or *P. calidifontis* with a membrane fraction containing the oligosaccharyltransferase AglB and an peptide that contained an Asn residue suitable for glycosylation resulted in the appearance of DolP, suggesting that a dephosphorylation reaction, similar to that which occurs in Eukarya, also transpires in Archaea where DolPP serves as the lipid glycan carrier [37]. Indeed, externally oriented pyrophosphatases from *S. acidocaldarius* and *Sulfolobus tokodaii* (optimal growth at 80 °C and pH 2.5–3 [127]) have been described and proposed to participate in DolPP recycling [128–130].

5. Phosphodolichol-processing enzymes that participate in archaeal N-glycosylation

In terms of both glycan composition and glycan architecture, archaeal N-glycosylation presents a degree of diversity not seen in either Eukarya or Bacteria [19]. Fittingly, what is known of archaeal N-glycosylation pathways reveals the involvement of numerous species-specific components [25]. In contrast, the oligosaccharyltransferase AglB is highly conserved across Archaea. Indeed, like PglB, the bacterial oligosaccharyltransferase, and Stt3, the catalytic subunit of the eukaryal oligosaccharyltransferase, AglB also contains the WWDXG and other motifs important for activity. AglB, moreover, presents a topology similar to those of PglB and Stt3 [131,132]. Solution of the crystal structures of the complete AglB protein from *A. fulgidus*, as well as of the catalytic domain from *P. furiosus* and *S. acidocaldarius* phosphodolichols; when the position of a double bond is only speculated in a *S. acidocaldarius* lipid in the figure, a dotted line appears). Dolichol would undergo phosphorylation to yield dolichol phosphate (DolP). Alternatively, polyisoprenol pyrophosphate could undergo reduction reactions, followed by removal of one phosphate group to yield DolP or both phosphate groups, followed by re-phosphorylation, again yielding DolP, as thought to occur in Eukarya. Abbreviations used: DolP, dolichol phosphate; DolPP, dolichol pyrophosphate; GGPP, geranylgeranyl pyrophosphate.

Fig. 4. Potential pathways for the biosynthesis of DolP in Archaea. The biogenesis of DolP in *S. acidocaldarius* offers an example of the potential pathways used for synthesizing phosphodolichols in Archaea. Initially (not shown), three isopentenyl pyrophosphates and dimethylallylpyrophosphate combine to form geranylgeranyl pyrophosphate (GGPP). Where internal phosphodolichol isoprenes are reduced, as in *S. acidocaldarius*, three of the four double bonds in GGPP may be reduced at this stage; the α position isoprene remains unsaturated. Elongation occurs when additional isoprene subunits (four-six in the case of *S. acidocaldarius*) are added to the α end of GGPP to generate polyisoprenyl pyrophosphate. The generation of dolichol requires the removal of the two phosphate groups, followed by reduction of the α position isoprene subunit (and possibly other subunits elsewhere, as in the case of *S. acidocaldarius* phosphodolichols; when the position of a double bond is only speculated in a *S. acidocaldarius* lipid in the figure, a dotted line appears). Dolichol would undergo phosphorylation to yield dolichol phosphate (DolP). Alternatively, polyisoprenol pyrophosphate could undergo reduction reactions, followed by removal of one phosphate group to yield DolP or both phosphate groups, followed by re-phosphorylation, again yielding DolP, as thought to occur in Eukarya. Abbreviations used: DolP, dolichol phosphate; DolPP, dolichol pyrophosphate; GGPP, geranylgeranyl pyrophosphate.
replaced the magnesium ion normally associated with the enzyme) [135]. This sulfate group interacted with His-81, His-162, Trp-215 and Arg-246, residues that are conserved in other AglB sequences. Site-directed mutagenesis showed the importance of the positive charges of His-81, His-162 and Arg-246. Based on these observations, it was assumed that the sulfate ion mimicked the phosphate group of the DolP that serves as the glycan lipid carrier in A. fulgidus N-glycosylation [37]. Accordingly, a docking model showed a good fit of the DolP dolichol chain within the hydrophobic groove on the protein surface leading to these residues. Once a crystal structure of AglB from an archaeal species that relies on DolPP as glycan lipid carrier becomes available, it will be of interest to see if the same architecture applies.

Flipases, catalyzing the translocation of glycan-charged and glycan-free phosphodolichols across the membrane [136], are also components of N-glycosylation pathways in intimate contact with DolP and/or DolPP. In Archaea, the only known protein proposed to act as a flipase or to play a flipase-assisting role is Hfx. volcanii AglR [137]. Upon deletion of the encoding gene, only the first four sugars of the N-linked pentasaccharide normally decorating glycoproteins in this species were protein-bound. It was previously shown that this tetrasaccharide is assembled on a single DolP carrier, while the fifth sugar (mannose) is derived from its own DolP carrier [27]. Moreover, in the aglR-deleted strain, mannoside-charged DolP accumulated. As such, it would appear that AglR is involved in translocating mannoside-charged DolP across the membrane. Accumulation of tetrasaccharide-charged DolP was, however, also noted in the absence of AglR, pointing to a more general flipase or flipase-related role for the protein. Further support for AglR acting as a flipase or playing a flipase-supporting role comes from sequence homology searches that revealed the similarity of AglR to Wzx proteins. In Bacteria, members of this group of proteins are thought to act as flipases responsible for translocating lipid-linked O-antigen precursor oligosaccharides across the membrane during lipopolysaccharide biosynthesis [138].

6. Conclusions and future prospects

Until recently, the study of Archaea had been hampered by the inability to cultivate the vast majority of identified strains in the laboratory, as well as the few cultivatable strains for which genetic tools were available. However, as recent advances have expanded the list of species amenable to molecular studies, it is likely that novel solutions employed by these unusual organisms to address a variety of biological challenges will be revealed. In terms of phosphodolichols, it is conceivable that not only will the enzymes catalyzing all of the steps of known pathways be identified but that additional archaeal-specific steps will be identified. In addition, other more specific mysteries may be solved. For instance, both DolP and DolPP serve as lipid glycan carriers in this post-translational modification, and it will likely continue to provide novel examples of Nature's creativity in the face of such challenges.

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