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Distinct glycan-charged phosphodolichol carriers are required for the assembly of the pentasaccharide N-linked to the *Haloferax volcanii* S-layer glycoprotein

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Abstract

In Archaea, dolichol phosphates have been implicated as glycan carriers in the N-glycosylation pathway, much like their eukaryal counterparts. To clarify this relation, highly sensitive liquid chromatography/mass spectrometry was employed to detect and characterize glycan-charged phosphodolichols in the haloarchaeon *Haloferax volcanii*. It is reported that *Hfx. volcanii* contains a series of C_{55} and C_{60} dolichol phosphates presenting saturated isoprene subunits at the α and ω positions and sequentially modified with the first, second, third and methylated fourth sugar subunits comprising the first four subunits of the pentasaccharide N-linked to the S-layer glycoprotein, a reporter of N-glycosylation. Moreover, when this glycan-charged phosphodolichol pool was examined in cells deleted of *agl* genes encoding glycosyltransferases participating in N-glycosylation and previously assigned roles in adding pentasaccharide residues one-four, the composition of the lipid-linked glycans was perturbed in the identical manner as was S-layer glycoprotein N-glycosylation in these mutants. By contrast, the fifth sugar of the pentasaccharide, identified as mannose in this study, is added to a distinct dolichol phosphate carrier. This represents the first evidence that in Archaea, as in Eukarya, the oligosaccharides N-linked to glycoproteins are sequentially assembled from glycans originating from distinct phosphodolichol carriers.

Keywords

Archaea; dolichol phosphate; *Haloferax volcanii*; N-glycosylation; S-layer glycoprotein

INTRODUCTION

N-glycosylation is a post-translational modification performed by all three domains of life, namely Eukarya, Bacteria and Archaea (Helenius and Aebi, 2004; Eichler and Adams, 2005; Szymanski and Wren, 2005; Weerapana and Imperiali, 2006). Presently, the pathway of N-glycosylation is best understood in higher Eukarya, where the oligosaccharide covalently linked to select Asn residues of a target protein is first assembled from seven soluble nucleoside-activated sugars sequentially added to a dolichol pyrophosphate carrier on the cytoplasmic face of the endoplasmic reticulum (ER) membrane. The charged lipid carrier is then flipped to face the ER lumen, at which point seven additional sugars, derived from

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individually charged and flipped phosphodolichol carriers, are added. Once assembled, the 14-meric oligosaccharide is transferred to the protein target (Burda and Aebi, 1999; Helenius and Aebi, 2004).

In contrast to the detailed delineation of the eukaryal N-glycosylation pathway, much less is known of this post-translational modification in Archaea. As in the ER membrane, glycan-charged phosphodolichol species have been detected in the archaeal plasma membrane. Moreover, evidence exists assigning glycan-charged phosphodolichols roles in archaeal N-glycosylation, in analogy to the function these lipids serve in the parallel eukaryal pathway (Burda and Aebi, 1999; Helenius and Aebi, 2004). For example, the identical methylated hexasaccharide moiety as attached to the *Methanothermus fervidus* surface (S)-layer glycoprotein is found on a dolichol pyrophosphate carrier in this species (Hartmann and Konig, 1989; Kärcher *et al.*, 1993). Likewise, the sulfated polysaccharide moiety N-linked to the *Halobacterium salinarum* S-layer glycoprotein and flagellin was detected on dolichol phosphate intermediates, while C_{60} dolichol phosphate species bearing glucose, mannose and N-acetylglucosamine units have also been observed in this organism (Mescher *et al.*, 1976; Lechner *et al.*, 1985; Sumper, 1987; Wieland *et al.*, 1980; Wieland *et al.*, 1985). Pulse-chase radiolabeling of *Hbt. salinarum* cells revealed the gradual transfer of the radiolabel from a lipid precursor to the S-layer glycoprotein (Wieland *et al.*, 1980), while the incorporation of radiolabeled glucose into *Haloferax volcanii* glycoproteins was shown to proceed through a glucose-containing phosphopolyisoprenol intermediate (Zhu *et al.*, 1995). Indeed, *Hfx. volcanii* membranes were reported to contain C_{55} and C_{60} dolichol phosphate charged with an α-linked mannosyl-(β1-4)-galactosyl group, and, to lesser extents, with a sulfated or phosphorylated dihexose and with a tetrasaccharide (comprising the hexoses, mannose and galactose, and the deoxyhexose, rhamnose), as well as with monosaccharides at radiochemical levels (Kuntz *et al.*, 1997). Not all of these phosphodolichol-charged glycans have, however, been detected on *Hfx. volcanii* glycoproteins (Abu-Qarn *et al.*, 2007; Magidovich *et al.*, 2010; Mengele and Sumper, 1992; Sumper *et al.*, 1990).

Thus, while glycan-charged phosphodolichols are implicated in archaeal N-glycosylation, numerous questions remain unanswered. Are those glycans found on dolichol phosphate carriers in Archaea, presumably destined to decorate target proteins, assembled from soluble, activated monosaccharides, from monosaccharides transferred from individual dolichol phosphate carriers or from both, as in Eukarya? If so, what is the relative contribution of each monosaccharide population in generating oligosaccharide-charged phosphodolichols? Is the protein-targeted oligosaccharide fully pre-assembled on a single phosphodolichol carrier in the archaeal cytoplasm or does assembly of the phosphodolichol-charged oligosaccharide involve steps that transpire on both sides of the membrane? Finally, one can also ask whether assembly of the N-linked glycan includes the addition of sugar subunits to a glycan already transferred from its phosphodolichol carrier to the protein target. Based on advances in describing the archaeal pathway of N-glycosylation made in the last five years (for review, see Yurist-Doutsch *et al.*, 2008a; Calo *et al.*, 2010), it may now be possible to address these and related questions.

In *Hfx. volcanii*, a series of Agl (*a*rchaeal *gl*ycosylation) proteins has been shown to participate in the assembly and attachment of a pentasaccharide decorating select Asn residues of the S-layer glycoprotein, a reporter of N-glycoslation in this species (Abu-Qarn and Eichler, 2006; Abu-Qarn *et al.*, 2007; Abu-Qarn *et al.*, 2008; Magidovich *et al.*, 2010; Yurist-Doutsch *et al.*, 2008b; Yurist-Doutsch *et al.*, 2010; Kaminski *et al.*, 2010). The involvement of each of these proteins in the N-glycosylation process was demonstrated by examining the N-linked glycan profile of the S-layer glycoprotein isolated from *Hfx. volcanii* strains deleted of each of these *agl* genes, relative to the parent strain. As such, AglJ, AglG, AglI, AglE and AglD were shown to participate in the introduction of the five

sugar subunits comprising the S-layer glycoprotein-bound pentasaccharide (Abu-Qarn *et al.*, 2007; Abu-Qarn *et al.*, 2008; Yurist-Doutsch *et al.*, 2008b; Kaminski *et al.*, 2010) while AglB was shown to be the oligosaccharyltransferase, responsible for delivery of the glycan to the S-layer glycoprotein (Abu-Qarn and Eichler, 2006; Abu-Qarn *et al.*, 2007).

Now, to address the involvement of glycan-charged phosphodolichols in the biosynthesis of the *Hfx. volcanii* N-linked pentasaccharide, liquid chromatography/mass spectrometry (LC/MS) was employed to define the sugar profiles of phosphodolichol-linked glycan carriers in a *Hfx. volcanii* parent strain as well as from cells deleted of different *agl* genes. It is reported that *Hfx. volcanii* contains a series of dolichol phosphate molecules sequentially modified with the one, two, three, and four sugar subunits corresponding to the first four subunits of the pentasaccharide found on the Slayer glycoprotein. The fifth pentasaccharide subunit, mannose, is, by contrast, derived from its own phosphodolichol carrier. These findings thus not only provide the first direct evidence for the sequential assembly of an oligosaccharide on a dolichol carrier prior to the addition of that glycan to an archaeal protein but also that the assembly of N-linked oligosaccharides in Archaea involves glycans originating from distinct dolichol carriers, as occurs in Eukarya.

RESULTS

Hfx. volcanii contains a population of C_{55} and C_{60} dolichol phosphate molecules

In the present study, a total *Hfx. volcanii* lipid extract was subjected to normal phase liquid chromatography coupled with mass spectrometry. Figure 1A shows the total ion chromatogram of the NPLC-ESI/MS in the negative ion mode. The mass spectrum averaged from those acquired during the retention time of 20–21 min (Fig 1B) shows prominent ion peaks of *m/z* 849.695 (this and all reported values are for the monoisotopic ion peaks, unless otherwise stated) and *m/z* 917.757, corresponding to the $[M\text{-}H]^-$ ions of the C_{55} and C_{60} dolichol phosphates with two saturated isoprene units, respectively. These measured ion masses are in agreement with the calculated values of *m/z* 849.690 for the $[M\text{-}H]^-$ ion of the C_{55} dolichol phosphate and *m/z* 917.752 for the $[M\text{-}H]^-$ ion of the C_{60} dolichol phosphate. In addition, a very minor peak corresponding to C_{50} dolichol phosphate (*m/z* 781.671) was observed. MS/MS was performed on the $[M\text{-}H]^-$ ion at *m/z* 917.7 of C_{60} dolichol phosphate (Fig 1C); the obtained fragmentation pattern is consistent with the chemical structure previously described (Kuntz *et al.*, 1997), with the saturated isoprene units at both the α and the ω positions. The same saturation pattern also held true for C_{55} dolichol phosphate (not shown). This is in contrast to the C_{55} undecaprenol involved in N-glycosylation in Bacteria, where theα position is unsaturated and the longer dolichols involved in eukaryal N-glycosylation (C_{70}–C_{110}), where theα position is saturated (Jones *et al.*, 2009). The presence of two saturated isoprene units in archaeal dolichol phosphate is quite remarkable, considering that Bacteria contain only unsaturated polyprenol phosphate, while in eukaryal dolichols, only the α-isoprenes are saturated (Burda and Aebi, 1999). Recently, the long-sought reductase for converting polyprenol to dolichol in eukaryotic cells has been identified (Cantagrel *et al.*, 2010). At present, no archaeal polyprenol reductase has been described.

Apart from the complete pentasaccharide, Hfx. volcanii contains dolichol phosphates charged with the same glycan series as found N-linked to the S-layer glycoprotein

Earlier studies revealed the *Hfx. volcanii* S-layer glycoprotein to be modified by a N-linked pentasaccharide comprising a hexose, two hexuronic acids, a methyl ester of hexuronic acid and a final hexose (Abu-Qarn *et al.*, 2007; Magadovich *et al.*, 2010). In addition, S-layer glycoprotein-derived peptides have also been shown to be modified by glycans comprising the first, the first two, the first three and the first four sugar subunits of the N-linked pentasaccharide (Abu-Qarn *et al.*, 2007; Abu-Qarn *et al.*, 2008; Magidovich *et al.*, 2010;

Yurist-Doutsch *et al.*, 2008b; Yurist-Doutsch *et al.*, 2010). To assess whether similar glycans also decorate *Hfx. volcanii* dolichol phosphate, phosphodolichol linked glycans were profiled by NPLC/MS in the total lipid extract of *Hfx. volcanii*. Fig 2 (left panels) reveals the presence of dolichol phosphate species modified by a glycan comprising one to four saccharides, while MS/MS analysis confirmed the nature of the sugars added to the C_{60} dolichol phosphate (Fig 2, right panels). Specifically, the fraction eluting during the retention time of 16–16.5 min contains C_{55} and C_{60} dolichol phosphate species modified by a hexose (peaks at *m/z* 1011.724 and 1079.797, respectively; Fig 2A). In addition, a major peak at the *m/z* 1055.714, corresponding to a previously described sulfoglycolipid (Sprott *et al.*, 2003), was also observed. The fraction eluting during the retention time of 20.8–21.3 min contains C_{55} and C_{60} dolichol phosphate species modified by a hexose and a hexuronic acid (peaks at *m/z* 1187.793 and 1255.858, respectively; Fig 2B), while the fraction eluting during the retention time of 26–27 min contains C_{55} and C_{60} dolichol phosphate species modified by a hexose and two hexuronic acids (peaks at *m/z* 1363.832 and 1431.895, respectively; Fig 2C). Finally, the fraction eluting during the retention time of 35.5–36 min contains C_{55} and C_{60} dolichol phosphate species modified by a hexose, two hexuronic acids and a methyl ester of hexuronic acid (their doubly charged ions $[M\text{-}2H]^{2-}$ are observed at *m/z* 766.43 and 810.46, respectively; Fig 2D). The structure of the C_{60} phosphodolichol-linked tetrasacchride was verified by MS/MS (Fig 2D, right panel). The most prominent ion at *m/z* 207 in the MS/MS spectrum derived from the $[M\text{-}2H]^{2-}$ ion at *m/z* 810.46 is derived from the terminal (i.e. the fourth) sugar. Its methanol-less (32 Da) ion, shown at *m/z* 175, provides additional evidence for this sugar subunit being a methyl ester of hexuronic acid.

Finally, although the S-layer glycoprotein is ultimately modified by a N-linked pentasaccharide, no pentasaccharide-modified dolichol phosphate species was detected. Instead, only dolichol phosphate species sequentially charged with the first four saccharides comprising the pentasaccharide N-linked to the *Hfx. volcanii* S-layer glycoprotein were observed.

Hfx. volcanii cells lacking components of the N-glycosylation machinery present dolichol phosphates void of or bearing truncated glycans

To assess whether the various glycan-charged phosphodolichols described in the previous section are involved in the N-glycosylation of the S-layer glycoprotein, the dolichol phosphate-derived species from *Hfx. volcanii* cells deleted of *aglG*, *aglI*, *aglE* and *aglD* were considered. Previous efforts implicated the products of these genes, predicted glycosyltransferases, in the respective addition of the second, third, fourth and fifth saccharides of the pentasaccharide decorating the S-layer glycoprotein, although direct biochemical proof for such activity has yet to be provided (Abu-Qarn *et al.*, 2007; Abu-Qarn *et al.*, 2008; Yurist-Doutsch *et al.*, 2008b).

When the total lipid extract from cells lacking AglG was analyzed by NP-LC/MS/MS as above, only hexose-modified C_{55} and C_{60} phosphodolichols were detected (Fig 3). Likewise, cells deleted of *aglI* or *aglE* presented dolichol phosphate species containing mono- and disaccharides and mono-, di- and trisaccharides, respectively. Consistent with these results, a recent study (Kaminski *et al.*, 2010) showed that in the absence of AglJ, involved in adding the first sugar subunit of the S-layer glycoprotein N-linked pentasaccharide, the level of a hexose-modified phosphodolichol species was significantly decreased, relative to what is seen in the parent strain.

When dolichol phosphate-derived species from cells lacking AglD, implicated in adding the fifth and final subunit of the S-layer glycoprotein N-linked pentasaccharide, were compared with those of the parent strain, a very different effect was seen than observed in cells lacking AglG, AglI or AglE. *Hfx. volcanii* membranes include three hexose-modified

phosphodolichol species retained at 14.77, 16.06 and 16.92 min. Of these, only the hexose-modified phosphodolichol with a retention time of 16.92 min was eliminated in the *aglD* deletion strain (Fig 4). This implies that AglD is a dolichol phosphate hexose synthase that catalyzes the addition of a hexose residue to the lipid carrier. To identify the monosaccharide apparently added to the dolichol phosphate carrier by AglD, a mannose-charged C_{55} phosphodolichol standard was examined as above. The mannose-modified phosphodolichol peak eluted at 16.99 min, just as was that peak missing from cells lacking AglD. Thus, the fifth and final pentasaccharide subunit, added to its own dolichol phosphate carrier and previously reported to be a hexose (Abu-Qarn *et al.*, 2007), is now identified as mannose.

In conclusion, it is proposed that those enzymes previously assigned roles in adding the first four saccharide subunits of the pentasaccharide N-linked to the *Hfx. volcanii* S-layer glycoprotein sequentially attach their respective sugar substrates to a common dolichol phosphate carrier. By contrast, AglD, previously implicated in adding the fifth saccharide (now identified as mannose) to the N-linked glycan decorating the S-layer glycoprotein, adds its substrate to a distinct dolichol phosphate carrier.

DISCUSSION

It has been long known that Archaea contain glycan-bearing phosphodolichols and, like their eukaryal counterparts (Burda and Aebi, 1999), roles for these lipids in N-glycosylation were postulated (Mescher *et al.*, 1976; Mescher and Storminger, 1978; Lechner *et al.*, 1985a; Lechner *et al.*, 1985b; Sumper, 1987; Wieland *et al.*, 1980; Wieland *et al.*, 1985). Accordingly, dolichol phosphates charged with either the identical or slightly modified versions of the glycans decorating glycoproteins in *Hbt. salinarum* and *M. fervidus* were reported (cf. Lechner and Wieland, 1989). Since little or nothing is known of the N-glycosylation process in these species, much related to the involvement of dolichol phosphates in N-glycosylation in Archaea remained a matter of speculation. The recent identification of a series of *agl* genes involved in the N-glycosylation of the *Hfx. volcanii* S-layer glycoprotein (for review, see Yurist-Doutsch *et al.*, 2008a; Calo *et al.*, 2010), however, now makes it possible to address the precise role of dolichol phosphates as putative glycan carriers in the archaeal version of this post-translational modification.

Kuntz *et al.* (1997) first reported the presence of C_{55} and C_{60} dolichol phosphates saturated at both the α and the ω positions in *Hfx. volcanii*, including glycan-modified species. The *Hfx. volcanii* dolichol phosphate pool was reportedly modified by mannosyl-galactosyl groups, and to a lesser extent, by sulfated or phosphorylated dihexosyl moieties and by a tetrasaccharide that includes mannose, galactose and rhamnose, sugars not detected as components of the N-linked glycans reported to decorate the S-layer glycoprotein at the time (Sumper *et al.*, 1990; Sumper and Mengele, 1992). However, given the revision of the originally reported composition of the glycan N-linked to the *Hfx. volcanii* S-layer glycoprotein from a string of linear glucose residues (as well as a glucose-, idose- and galactose-containing polysaccharide) (Sumper *et al.*, 1990; Sumper and Mengele, 1992) to a pentasaccharide comprising two hexoses, two hexuronic acids and a methyl ester of hexuronic acid (Abu-Qarn *et al.*, 2007; Magidovich *et al.*, 2010), the present study revisited the composition of glycans decorating dolichol phosphates in *Hfx. volcanii* in an attempt to link these glycan-charged lipids to the N-glycosylation process.

In recent work from our group (Kaminski *et al.*, 2010), AglJ was shown to add the first hexose subunit of the N-linked S-layer glycoprotein pentasaccharide to a dolichol phosphate carrier. In the present report, it was revealed that the next three subunits of the pentasaccharide are sequentially added to that AglJ-generated monosaccharide-charged

carrier, through the respective actions of AglG, AglI and AglE. Since no hexuronic acid-charged phosphodolichol was detected, it seems that pentasaccharide subunits two and three are added from soluble, activated species. Likewise, the methyl ester of hexuronic acid found at position four of the N-linked pentasaccharide is added to the existing trisaccharide-charged phosphodolichol from a soluble methylated hexuronic acid species, since neither a dolichol phosphate modified with only a methyl ester of hexuronic acid nor a tetrasaccharide-charged phosphodolichol bearing a hexuronic acid at position four was detected. These observations, moreover, offer support to the earlier assignment of the nucleoside-hexose dehydrogenase, AglM, shown to catalyze the *in vitro* conversion of UDP-glucose to UDP-glucuronic acid and likely involved in the biogenesis of pentasaccharide subunits two, three and four, and of AglP, the SAM-dependent methyltransferase responsible for modifying the fourth subunit of the pentasaccharide subunit, as being soluble enzymes (Magidovich *et al.*, 2010; Yurist-Doutsch *et al.*, 2010).

In contrast to the sequential assembly of the first four pentasaccharide subunits onto a common dolichol phosphate, the fifth subunit of the pentasaccharide, mannose, was detected on its own distinct lipid carrier. The finding that AglD, involved in the addition of the fifth pentasaccharide subunit, acts in a manner seemingly independent of the other Agl proteins involved in generating the oligosaccharide decorating the *Hfx. volcanii* S-layer glycoprotein is not unexpected, given that *aglD* is the only gene not found in the *agl* gene island present in the *Hfx. volcanii* genome (Yurist-Doutsch and Eichler, 2009). Thus, the observation that the same sugar subunits are found on both dolichol phosphate carriers and the S-layer glycoprotein, four of which are sequentially added to the lipid carrier in the same order as found on the modified protein, together with the fact that deletion of *agl* genes compromised dolichol phosphate glycosylation in a manner reminiscent of the effects of the same gene deletions on *Hfx. volcanii* S-layer glycoprotein N-glycosylation (Abu-Qarn *et al.*, 2007; Abu-Qarn *et al.*, 2008; Yurist-Doutsch *et al.*, 2008b), directly links dolichol phosphates, acting as mono- and oligosaccharide carriers, to the archaeal N-glycosylation process (Fig 5).

The confirmed involvement of archaeal dolichol phosphate sugar carriers in *Hfx. volcanii* N-glycosylation provides novel insight into the mechanism of this post-translational modification. Earlier work had shown that Agl proteins involved in adding sugars found on the pentasaccharide N-linked to the *Hfx. volcanii* S-layer glycoprotein are membrane proteins oriented towards the cytoplasm, pointing to dolichol phosphate sugar charging as occurring within the confines of the cell (Plavner and Eichler, 2008). Hence, as no pentasaccharide-charged phosphodolichol species could be detected, it is possible that transfer of pentasaccharide subunit five occurs directly onto the S-layer glycoprotein-linked tetrasaccharide. Indeed, tetrasaccharide-modified S-layer glycoprotein-derived peptides have been observed (Abu-Qarn *et al.*, 2007; Magidovich *et al.*, 2010). Alternatively, transfer of the fifth sugar subunit from its own dolichol phosphate carrier to the lipid-linked tetrasaccharide and subsequent transfer to the S-layer glycoprotein may occur too rapidly to be detected here. This explanation for our inability to detect a *Hfx. volcanii* pentasaccharide-modified phosphodolichol species is unlikely, since such an entity was readily observed upon examination of the dolichol phosphate pool of another halophilic archaea originating from the Dead Sea, namely *Haloarcula marismortui* (Z.G. et al., in preparation). To determine whether the fifth and final subunit of the pentasaccharide is added to the tetrasaccharide-charged phosphodolichol to yield a potentially short-lived pentasaccharide-charged lipid carrier or directly to the tetrasaccharide-modified S-layer glycoprotein, additional biochemical studies are required. It is, however, clear that methylation of the fourth pentasaccharide subunit is important for addition of pentsaccharide subunit five, since no N-linked pentasaccharide is detected in the Δ*aglP* mutant, where methylation of the fourth pentasaccharide subunit fails to occur (Magidovich *et al.*, 2010). On the other hand,

the actions of AglP are not essential for modification of the S-layer glycoprotein by the tetrasaccharide formed in the absence of this methyltransferase.

In conclusion, despite considerable progress made in understanding archaeal N-glycosylation in recent years (Yurist-Doutsch *et al.*, 2008a; Calo *et al.*, 2010), many questions still remain unanswered. For instance, why does N-glycosylation in some Archaea, such as *Hfx. volcanii*, rely on dolichol phosphate while other species rely on dolichol pyrophosphate or both, as in the case of *Hbt. salinarum* (31)? What is/are the flippase(s) involved in N-glycosylation in *Hfx. volcanii*? Finally, do the glycan-modified phosphodolichol species originally reported by Kuntz *et al.* (1997) participate in any *Hfx. volcanii* post-translation modification? Continued examination of the *Hfx. volcanii* N-glycosylation pathway will likely provide answers to these and other outstanding questions.

EXPERIMENTAL PROCEDURES

Strains and growth conditions

The *Hfx. volcanii* parent strain WR536 (H53) and the same strain deleted of *aglG*, *aglI*, *aglE* or *aglD* were grown in complete medium containing 3.4 M NaCl, 0.15 M $MgSO_4 \bullet 7H_20$, 1 mM $MnCl_2$, 4 mM KCl, 3 mM $CaCl_2$, 0.3 % (w/v) yeast extract, 0.5 % (w/v) tryptone, 50 mM Tris-HCl, pH 7.2, at 40°C (Mevarech and Werczberger, 1985). The preparation of *Hfx. volcanii* strains deleted of *aglG*, *aglI*, *aglE* and *aglD* was previously reported (Abu-Qarn and Eichler, 2006; Abu-Qarn *et al.*, 2008; Yurist-Doutsch *et al.*, 2008).

Isolation of the Hfx. volcanii lipid fraction

The total lipid contents of the *Hfx. volcanii* parent strain and of *Hfx. volcanii* Δ*aglG*, Δ*aglI*, Δ*aglE* and Δ*aglD* cells were extracted as follows. Cells were harvested (8,000 g, 30 min, 4°C) and frozen at −20°C until extraction was performed. At that point, the pelleted cells (15 g) were thawed, resuspended in 20 mL double-distilled water (DDW) and DNase (1.7 μg/ml; Sigma, St. Louis, MO) and stirred overnight at room temperature. Methanol and chloroform were added to the cell extract to yield a methanol:chloroform:cell extract ratio of 2:1:0.8. After stirring for 24 h at room temperature, the mixture was centrifuged (1,075 g, 30 min, 4°C). The clarified supernatants were collected, combined and filtered through glass wool. Chloroform and DDW were added to the filtrate to yield a chloroform:DDW:filtrate ratio of 1:1:3.8, in a separating funnel. After separation, the lower clear organic phase, containing the total lipid extract, was collected into a round bottom flask and evaporated in a rotary evaporator at 35°C. For analysis of the dolichol phosphate-derived species, the total lipid extracts were subjected to normal phase LC/MS analysis without pre-fractionation.

Liquid Chromatography/Mass Spectrometry (LC/MS) and Tandem Mass Spectrometry (MS/MS)

Normal phase LC-ESI/MS of lipids was performed using an Agilent 1200 Quaternary LC system coupled to a QSTAR XL quadrupole time-of-flight tandem mass spectrometer (Applied Biosystems, Foster City, CA). An Ascentis Si HPLC column (5 μm, 25 cm × 2.1 mm) was used. Mobile phase A consisted of chloroform/methanol/aqueous ammonium hydroxide (800:195:5, v/v/v). Mobile phase B consisted of chloroform/methanol/water/aqueous ammonium hydroxide (600:340:50:5, v/v/v/v). Mobile phase C consisted of chloroform/methanol/water/aqueous ammonium hydroxide (450:450:95:5, v/v/v/v). The elution program consisted of the following: 100% mobile phase A was held isocratically for 2 min and then linearly increased to 100% mobile phase B over 14 min and held at 100% B for 11 min. The LC gradient was then changed to 100% mobile phase C over 3 min and held at 100% C for 3 min, and finally returned to 100% A over 0.5 min and held at 100% A for 5 min. The total LC flow rate was 300 μl/min. The post-columnsplitter diverted ~10% of the

LC flow to the ESI source of the Q-Star XL mass spectrometer, with MS settings as follows: IS = −4500 V, CUR = 20 psi, GS1 = 20 psi, DP = −55 V, and FP = −150 V. For MS/MS, collision-induced dissociation (CID) was performed with collision energy ranging from 40 V to 70 V (laboratory frame of energy) and with nitrogen as the collision gas. Data acquisition and analysis were performed using the instrument's Analyst QS software.

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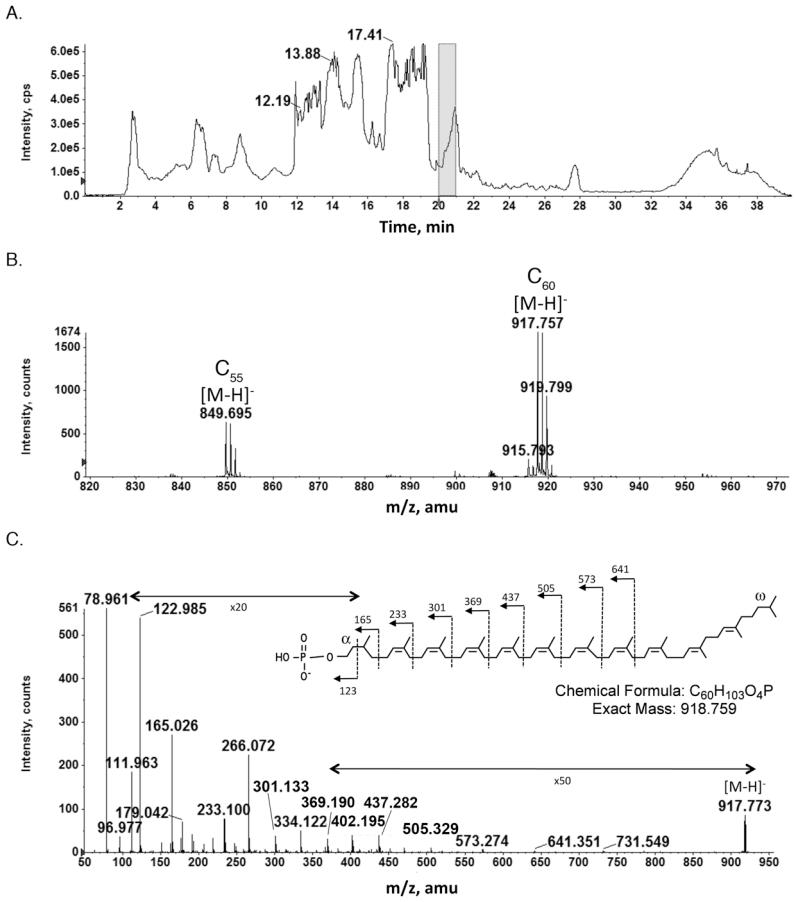

Fig 1.

Normal phase LC/MS identification of dolichol phosphate from the total lipid extract of *Hfx. volcanii*. A. Total ion chromatogram of the NP-LC/MS analysis in the negative ion mode. B. The $[M-H]^-$ ions of C_{55} and C_{60} dolichol phosphate detected at *m/z* 849.695 and 917.757, indicated by C_{55} and C_{60}, respectively. The mass spectrum shown is averaged from spectra acquired during the 20–21 min window, indicated by the shaded area in A. C. MS/MS of the $[M-H]^-$ ion of C_{60} dolichol phosphate. The inset shows the predicted chemical structure of dolichol phosphate (according to Kuntz *et al.*, 1997) and the MS/MS fragmentation scheme.

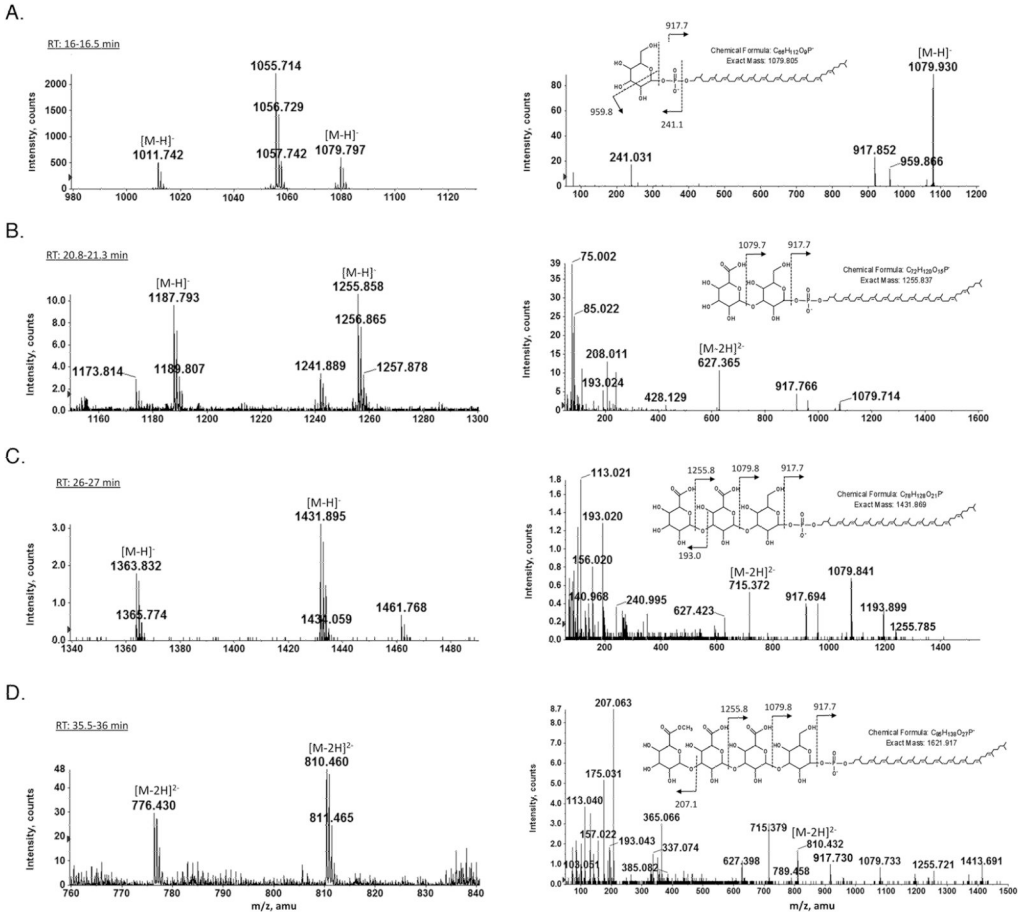

Fig 2.

Normal phase LC/MS/MS identification of mono-, di-, tri-, and tetrasacchride-charged dolichol phosphate from the total *Hfx. volcanii* lipid extract. The left panels show the $[M-H]^-$ ions of (A) hexose-modified, (B) hexuronic acid-hexose-modified and (C) dihexuronic-hexose-modified C_{55} and C_{60} phosphodolichol. The left panel of (D) shows the doubly charged $[M-2H]^{2-}$ ions of methyl ester of hexuronic acid-dihexuronic acid-hexose-modified C_{55} and C_{60} phosphodolichol, detected at *m/z* 766.43 and 810.46, respectively. RT refers to retention time. The right panels show the MS/MS spectra of the $[M-H]^-$ ion of (A) hexose-modified, (B) hexuronic acid-hexose-modified and (C) dihexuronic acid-hexose-modified C_{60} phosphodolichol. The right panel of (D) shows the MS/MS spectrum of doubly charged $[M-2H]^{2-}$ ions of methyl ester of hexuronic acid-dihexuronic acid-hexose-modified C_{60} phosphodolichol. The inset in each right panel shows the chemical structure of the glycan-charged C_{60} phosphodolichol and the MS/MS fragmentation scheme of the $[M-H]^-$ ion (or the $[M-2H]^{2-}$ ions in (D)). The arrows indicating ×20 and ×50 reflect magnification of the ion peaks in the corresponding region of the m/z values on the graph.

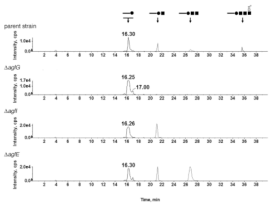

Fig 3.

LC/MS profiling of glycan-charged phosphodolichols in the parent and *agl* mutant strains. The presence or absence of mono-, di-, tri- and tetra-sacchride-modified phosphodolichols in each strain was revealed by generating extracted ion chromatograms. The $[M\text{-}H]^{-}$ ion at *m/z* 1079.8, the $[M\text{-}2H]^{2-}$ ion at *m/z* 627.4, the $[M\text{-}3H]^{3-}$ ion at *m/z* 476.6, and the $[M\text{-}3H]^{3-}$ ion at *m/z* 540.0 were selected for monitoring the mono-, di-, tri-, and tetracchride-modified phosphodolichols. Each of these 4 ions represents the highest-abundance charge state observed by ESI/MS of the individual glycan-modified C_{60} phosphodolichol species. Above each peak, schematic representation of the linked glycan is shown. The full circles correspond to hexose, the full squares correspond to hexuronic acid and the open square corresponds to the methyl ester of hexuronic acid. Note that the monosaccharide-modified phosphodolichol pool comprises several species.

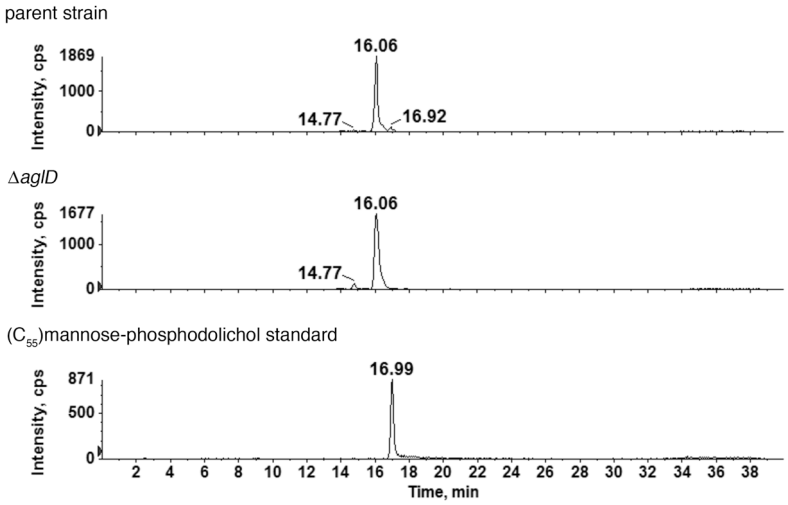

Fig 4.

The absence of AglD eliminates mannose-modified phosphodolichol. Normal phase LC extracted ion chromatograms (EIC) of the dolichylphosphate-hexose $[M-H]^-$ ion at *m/z* 1079.8 from the parent strain (upper panel) and the Δ*aglD* (middle panel) are shown. The peaks at different retention times reflect the existence of three different dolichylphosphate-hexose species (Kaminski *et al.*, 2010). The 16.92 min peak is eliminated in the mutant, as compared with the parent strain, suggesting AglD to be specific for the formation of the third monosaccharide-modified phosphodolichol species. The monosaccharide-modified phosphodolichol peak affected by the absence of AglD is retained at the position of a mannose-modified phosphodolichol standard (16.99 min; lower panel). The identities of the two other monosaccharide-modified phosphodolichols with retention times of 14.77 and 16.06 min, respectively, remain to be determined. While the results shown address C_{55} dolichol phosphate, similar results were obtained with C_{60} dolichol phosphate (not shown).

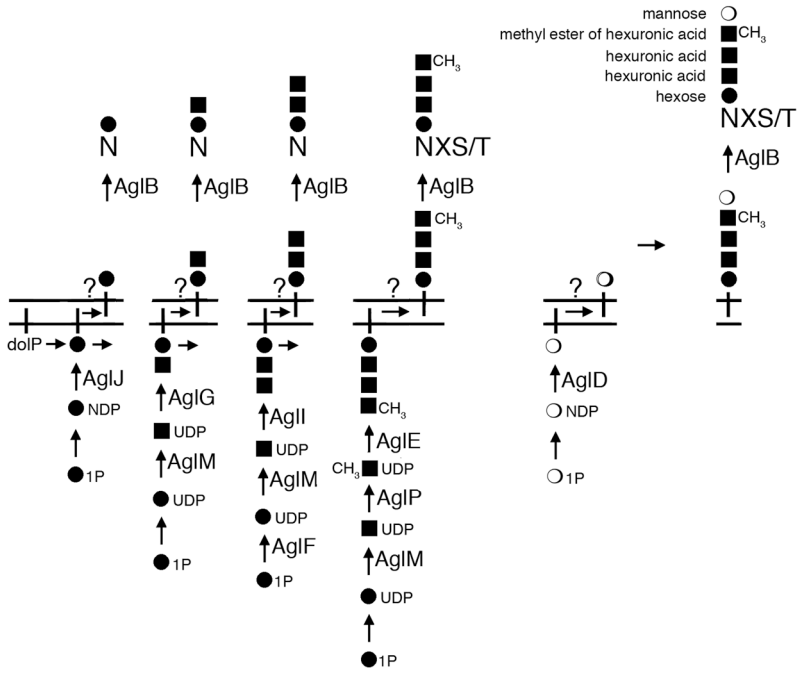

Fig 5.

The working model of the *Hfx. volcanii* N-glycosylation pathway. Select Asn residues of the *Hfx. volcanii* S-layer glycoprotein are modified by a pentasaccharide comprising a hexose, 2 hexuronic acids, a methyl ester of hexuronic acid and a terminal mannose residue. Based on the findings of the present study and earlier reports (16,17,23–25,33), a working model of the *Hfx. volcanii* N-glycosylation pathway is provided. AglJ, AglG, AglI, AglE and AglD are assigned roles in either modifying dolichol phosphates or adding sugars to dolichol phosphate-bound sugars. AglB serves as the oligosaccharyltransferase, while AglF, AglP and AglM serve various sugar processing roles. At present, the flippase(s) responsible for delivering the lipid-charged glycans across the plasma membrane remain to be defined and are indicated by question marks. dolP, dolichol phosphate; NDP, nucleoside diphosphate.