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AglR is required for addition of the final mannose residue of the N-linked glycan decorating the *Haloferax volcanii* S-layer glycoprotein

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Abstract

Background—Recent studies of *Haloferax volcanii* have begun to elucidate the steps of N-glycosylation in Archaea, where this universal post-translational modification remains poorly described. In *Hfx. volcanii*, a series of Agl proteins catalyzes the assembly and attachment of a N-linked pentasaccharide to the S-layer glycoprotein. Although roles have been assigned to the majority of Agl proteins, others await description. In the following, the contribution of AglR to N-glycosylation was addressed.

Methods—A combination of bioinformatics, gene deletion, mass spectrometry and metabolic radiolabeling served to show a role for AglR in archaeal N-glycosylation at both the dolichol phosphate and reporter glycoprotein levels.

Results—The modified behavior of the S-layer glycoprotein isolated from cells lacking AglR points to an involvement of this protein in N-glycosylation. In cells lacking AglR, glycan-charged dolichol phosphate, including mannose-charged dolichol phosphate, accumulates. At the same time, the S-layer glycoprotein does not incorporate mannose, the final subunit of the N-linked pentasaccharide decorating this protein. AglR is a homologue of Wzx proteins, annotated as flippases responsible for delivering lipid-linked O-antigen precursor oligosaccharides across the bacterial plasma membrane during lipopolysaccharide biogenesis.

Conclusions—The effects resulting from *aglR* deletion are consistent with AglR interacting with dolichol phosphate-mannose, possibly acting as a dolichol phosphate-mannose flippase or contributing to such activity.

General significance—Little is known of how lipid-linked oligosaccharides are translocated across membrane during N-glycosylation. The possibility of *Hfx. volcanii* AglR mediating or contributing to flippase activity could help address this situation.

Keywords

Archaea; Dolichylphosphate-mannose; *Haloferax volcanii*; N-glycosylation; S-layer glycoprotein

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1. Introduction

Members of all three domains of life, *i.e.* Eukarya, Bacteria and Archaea, perform N-glycosylation, namely the covalent attachment of glycans to select Asn residues of target proteins. Whereas the pathways of this post-translational modification in Eukarya and Bacteria are relatively well defined, much regarding the archaeal version of this universal protein-processing event remains unknown [1-4]. In recent years, however, studies on the halophilic archaeon, *Haloferax volcanii*, have offered significant insight into N-glycosylation in Archaea.

In *Hfx. volcanii*, select Asn residues of the S-layer glycoprotein, a reporter of N-glycosylation in this species, are decorated with a pentasaccharide comprising a hexose, two hexuronic acids, a methyl ester of hexuronic acid and a mannose [5-7]. A series of Agl (*a*rchaeal *g*lycosylation) proteins assembles these sugar residues eventually comprising the N-linked pentasaccharide onto dolichol phosphate (DolP) carriers [7]. Specifically, AglJ, AglG, AglI, and AglE sequentially add the first four pentasaccharide residues onto a common DolP, while AglD adds the final pentasaccharide residue, mannose, to a distinct DolP [5,8-10]. The DolP-bound tetrasaccharide (and its precursors) is transferred to select S-layer glycoprotein Asn residues by the archaeal oligosaccharyltransferase, AglB [5]. Mannose, the final pentasaccharide residue, is subsequently transferred from it DolP carrier to the N-linked tetrasaccharide [7,11]. Other Agl proteins, such as AglF, AglM and AglP, serve sugar-processing roles important for N-glycosylation.

Despite these advances in delineating the *Hfx. volcanii* N-glycosylation pathway, enzymes catalyzing several steps of the process remain to be identified. For instance, it is not clear how mannose finds its way from DolP to the N-linked tetrasaccharide decorating the S-layer glycoprotein. In *Hfx. volcanii*, genes encoding the Agl proteins involved in N-glycosylation (apart from *aglD*) are found within a single cluster [12]. While many of the products of the *agl* gene clusters have been demonstrated as participating in N-glycosylation, in other cases, evidence for such involvement has yet to be provided. For instance, while *aglR* is co-transcribed with *aglE* [12], the requirement for AglR in N-glycosylation has yet to be addressed. In the following, an analysis of the effects of *aglR* deletion points to AglR serving a role in DolP-mannose processing, possibly mediating or contributing to *Hfx. volcanii* DolP-mannose flippase activity.

2. Materials and methods

2.1. Strains and growth conditions

The *Hfx. volcanii* parent H53 strain and the isogenic strains deleted of *aglR* were grown in medium containing 3.4 M NaCl, 0.15 M $MgSO_4$, 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 [13]. The *Hfx. volcanii* strains deleted of *aglB* and *aglD* were previously described [14].

2.2. Deletion of aglR

Deletion of *aglR* was performed as previously described [14,15]. To amplify approximately 500 bp-long regions flanking the coding sequence of *aglR*, the aglR-5′upfor (gggctcgagGGAGTTCATCAATATGGTCCG; genomic sequence in capital letters) and aglR-5′uprev (cccaagcttTCGTGTTATTACCGCACCACGGA) primers, directed against the upstream flanking region, and the aglR-3′downfor (ggggatccGGTATGACTAGGTCGCAAGTA) and aglR-3′downrev (ccctctagaATGCTCTCTTTCATTTGCATATT) primers, directed against the downstream flanking region, were employed. *Xho*I and *Hind*III sites were introduced using the aglR-5′

upfor and aglR-5′uprev primers, respectively, while *Bam*HI and *Xba*I sites were introduced using the aglR-3′downfor and aglR-3′downrev primers, respectively.

To confirm deletion of *aglR* at the DNA level, PCR amplification was performed using forward primers directed against either an internal region of *algR* (aglR-for; ATGAACGAAAGTGACGACATTTCC) or *trpA* (cccgaattcTTATGTGCGTTCCGGATGCG) together with a reverse primer against a region downstream of *aglR* (aglR-5′downrev), respectively yielding primer pairs a and b, or using primers aglR-for and aglR-rev (TCAACCAAGACTTTCAGATAGCAAC), designed to amplify a section of the *aglR* coding region (primer pair c). Reverse-transcription (RT)-PCR was performed as described previously [14], using primer pair c to test for *algR* transcription, so as to confirm *aglR* deletion at the RNA level.

2.3. Mass spectrometry

The total lipid contents of the *Hfx. volcanii* parent and Δ*aglR* cells were extracted and subjected to liquid chromatography-electrospray ionization mass spectrometry (LC-ESI/MS) and tandem mass spectrometry (MS/MS) analysis as reported [10]. Cells were harvested (8000 *g*, 30 min, 4 °C), resuspended in double-distilled water (DDW) (1.33 ml DDW/g cells) 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 (1075 *g*, 30 min, 4 °C). The supernatant fractions 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-bottomed flask and evaporated in a rotary evaporator at 35 °C. For analysis of the dolichol phosphate pool, the total lipid extracts were subjected to normal phase LC/MS analysis without pre-fractionation.

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-column splitter diverted ~10% of the LC flow to the ESI source of the Q-Star XL mass spectrometer, with MS settings as follows: ion spray voltage=−4500 V, curtain gas=20 psi, ion source gas 1=20 psi, de-clustering potential=−55 V and focusing potential=−150 V. Nitrogen was used as the collision gas for MS/MS experiments. Data acquisition and analysis were performed using the instrument's Analyst QS software.

LC-ESI/MS/MS analysis of S-layer glycoprotein tryptic fragments was performed as previously described [16]. The protein contents of *Hfx. volcanii* cells were separated on 7.5% polyacrylamide gels and stained with Coomassie R-250 (Fluka, St. Louis MO). For in-gel digestion of the S-layer glycoprotein, the relevant bands (identified *via* the unique SDS-PAGE migration and staining pattern of the protein) were excised, destained in 400 μl of 50% (vol/vol) acetonitrile (Sigma, St Louis, MO) in 40 mM NH_4HCO_3, pH 8.4, dehydrated

with 100% acetonitrile, and dried using a SpeedVac drying apparatus. The S-layer glycoprotein was reduced with 10 mM dithiothreitol (Sigma) in 40 mM NH_4HCO_3 at 56 °C for 60 min and then alkylated for 45 min at room temperature with 55 mM iodoacetamide in 40 mM NH_4HCO_3. The gel pieces were washed with 40 mM NH_4HCO_3 for 15 min, dehydrated with 100% acetonitrile, and SpeedVac dried. The gel slices were rehydrated with 12.5 ng/μl of mass spectrometry (MS)-grade Trypsin Gold (Promega, Madison, WI) in 40 mM NH_4HCO_3. The protease-generated peptides were extracted with 0.1% (v/v) formic acid in 20 mM NH_4HCO_3, followed by sonication for 20 min at room temperature, dehydration with 50% (v/v) acetonitrile, and additional sonication. After three rounds of extraction, the gel pieces were dehydrated with 100% acetonitrile, dried completely with a SpeedVac, resuspended in 5% (v/v) acetonitrile containing 1% formic acid (v/v) and infused into the mass spectrometer using static nanospray Econotips (New Objective, Woburn, MA). The protein digests were separated on-line by nano-flow reverse-phase liquid chromatography (LC) by loading onto a 150-mm by 75-μm (internal diameter) by 365-μm (external diameter) Jupifer prepacked fused silica 5-μm C_{18} 300 Å reverse-phase column (Thermo Fisher Scientific, Bremen, Germany). The sample was eluted into the LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific) using a 60-min linear gradient of 0.1% formic acid (v/v) in acetonitrile/0.1% formic acid (1:19, by volume) to 0.1% formic acid in acetonitrile/0.1% formic acid (4:1, by volume) at a flow rate of 300 nl/min.

2.4. [2-^{3}H]-mannose radiolabeling

[2-^{3}H]-mannose radiolabeling was performed according to [17]. Cells of the parent and Δ*aglR* strains grown to mid-exponential phase were incubated with 6 μl [2-^{3}H]-mannose (23.8 mCi/mmol; PerkinElmer, Boston MA) in a volume of 100 μl. Sixty min later, the samples were precipitated with 15% (w/v) trichloroacetic acid and separated by SDS-PAGE. The S-layer glycoprotein was identified by Coomassie-staining and fluorography and exposure to film.

3. Results

3.1. AglR contributes to Hfx. volcanii N-glycosylation

Transcription of a given open reading frame offers support for the assignment of that sequence as corresponding to a true protein-encoding gene. Accordingly, previous RT-PCR efforts had revealed the transcription of *aglR* [12]. Moreover, the co-transcription of *aglR* and *aglE* implies that AglR serves a role in *Hfx. volcanii* N-glycosylation. Now, as a first step in directly determining whether AglR participates in N-glycosylation, *Hfx. volcanii* cells deleted of the encoding gene were generated according to the so-called ‘pop-in/pop-out’ technique developed for this organism [15]. In this approach, the gene of interest (in this case, *aglR*) is replaced in the genome by *trpA*, encoding tryptophan synthase, in a *Hfx. volcanii* strain auxotrophic for tryptophan. To confirm replacement of *aglR* by *trpA* at the DNA level, PCR was performed using primers directed at either *aglR* (primer pairs a and c) or *trpA* (primer pair b) (Fig. 1A). Whereas *aglR* was solely detected in the parent strain, only the deletion strain contained *trpA*. To confirm deletion of *aglR* at the RNA level, RT-PCR was performed. Whereas a PCR product corresponding to *aglR* was generated when genomic DNA or cDNA prepared from parent strain cells served as template, no such products were generated when the same templates generated from Δ*aglR* cells were used (not shown).

In earlier studies on *Hfx. volcanii* N-glycosylation, efforts focused on the processing of the S-layer glycoprotein, a reporter of this post-translational modification in this species (for review, see [4]). The effects of *aglR* deletion on the S-layer glycoprotein were thus considered by addressing the SDS-PAGE migration of this reporter in cells lacking AglR. In

Δ*aglR* cells, the S-layer glycoprotein migrated faster than did the same protein from the parent strain (Fig. 1B). Such enhanced migration was also noted with cells lacking the oligosaccharyltransferase, AglB, and was previously shown to occur upon deletion of other *Hfx. volcanii* N-glycosylation pathway components [9,10,14,18]. Although the unusual migration of the S-layer glycoprotein in SDS-PAGE (due to the reduced ability of the protein to bind SDS because of the highly acidic nature of the protein [19]) does not allow any specific conclusions as to the precise role of AglR, these results nonetheless reveal that AglR contributes to N-glycosylation of the *Hfx. volcanii* S-layer glycoprotein.

3.2. In Hfx. volcanii cells lacking AglR, glycan-charged DolP accumulates

Earlier findings reported that the first four residues of the pentasaccharide ultimately N-linked to the S-layer glycoprotein are sequentially added to a common DolP carrier, whereas the final pentasaccharide residue (*i.e.* mannose) is derived from a distinct monosaccharide-charged DolP [7]. Thus, toward more precisely defining the role of AglR, the glycan-charged DolP pool of Δ*aglR* cells was examined. Normal phase LC-ESI/MS [10] of glycan-charged C_{55} and C_{60} DolP in parent and Δ*aglR* strain cells revealed that the level of tetrasaccharide-charged C_{55} DolP (*m/z* 776.449) was increased some 20-fold in Δ*aglR* cells, as compared to the parent strain, while tetrasaccharide-charged C_{60} DolP (*m/z* 810.481) levels showed a close to 13-fold increase (Fig. 2A; all peaks correspond to doubly-charged $[M–2H]^{2-}$ ion species, unless otherwise noted). A similar phenomenon was observed when the levels of the trisaccharide-charged lipid carriers were considered in parent strain and mutant cells. In this case, the levels of trisaccharide-charged C_{55} and C_{60} DolP (*m/z* 681.419 and 731.644, respectively) were increased 9- and 6-fold in Δ*aglR* cells, respectively (Fig. 2B). These increases are evident when one considers the unchanged levels of the singly-charged, non-DolP-related peaks at *m/z* 805.685 and 731.644, relative to tetra- and trisaccharide-charged C_{55} and C_{60} DolP in each strain. At the same time, no changes in the levels of the major *Hfx. volcanii* sulphated glycolipid, 6-HSO_3-D-Man*p*-α1,2-D-Glc*p*α1,1-[*sn*-2,3-di-*O*-phytanylglycerol] (S-GL-1), present in the same sample injection as the glycan-charged DolP populations considered above were seen in the deletion strain cells ($[M–H]^-$ ion peak at *m/z* 1055.755 [20]), relative to the parent strain cells ($[M–H]^-$ ion peak at *m/z* 1055.737) (Fig. 2C). In the case of disaccharide-charged C_{55} and C_{60} DolP, comparable levels were observed in the mutant and parent strain cells (not shown).

When levels of monosaccharide-modified DolP species were considered, effects of *aglR* deletion were also observed. *Hfx. volcanii* contains several different monosaccharide-modified DolP species. The major species, generated through the actions of AglJ, serves as the hexose-charged DolP core onto which sugar residues two through four of the pentasaccharide ultimately N-linked to the S-layer glycoprotein are added [10]. A second, AglD-generated mannose-modified DolP species serves as the donor of the final N-linked pentasaccharide residue [7]. The predicted glycosyltransferase, HVO_1613, modifies a third hexose-bearing DolP [10], although the contribution of this species to N-glycosylation is unclear. Analysis of normal phase LC-extracted ion chromatograms (EIC) derived from the various hexose-modified DolP $[M–H]^-$ ions detected at *m/z* 1079.814 from parent and Δ*aglR* strain cells revealed the AglD-generated DolP-Man species (retained at 17.06 min in the parent strain) to be increased some 2.5-fold in cells lacking AglR (retained at 16.57 min in the Δ*aglR* strain) (Fig. 2D). In contrast, *aglR* deletion had no significant effect on the levels of the AglJ-generated species, with only 16% less of this species being detected in that strain lacking AglR (retained at 15.81 min) than in the parent strain (retained at 16.30 min). Hence, in the absence of AglR, both tetrasaccharide- and mannose-linked dolichol phosphate accumulate.

3.3. In cells lacking AglR, the final mannose residue of the N-linked glycan decorating the S-layer glycoprotein is not added

To define further the contribution of AglR to N-glycosylation, parent and Δ*aglR* strain S-layer glycoprotein-derived tryptic peptides, including the N-terminal ^{1}ERGNLDADSESFNK^{14} fragment that contains the glycosylated Asn-13 residue [5], were analyzed by LC-ESI/MS/MS [16]. As presented in Fig. 3 (left panels), the Asn-13-containing S-layer glycoprotein-derived peptide generated from the parent strain is modified by a pentasaccharide (doubly-charged $[M–2H]^{2–}$ ion peaks observed at *m/z* 1224.98, top left panel) comprising a hexose, two hexuronic acids, a methyl ester of hexuronic acid and a terminal mannose residue [5,6], as well as by precursor tetra- (*m/z* 1143.95), tri- (*m/z* 1048.42), di- (*m/z* 960.41) and monosaccharides (*m/z* 872.38) (second through bottom left panels, respectively; in each case, the doubly-charged $[M–2H]^{2–}$ ion peaks are shown). In cells lacking AglR, however, no pentasaccharide-modified Asn-13-containing peptide is observed (Fig. 3, top right panel). Yet, as observed in the parent strain, versions of the same peptide modified by precursor tetra- (*m/z* 1143.95), tri- (*m/z* 1048.42), di- (*m/z* 960.41) and monosaccharides (*m/z* 872.39) were detected (Fig. 3, second to fifth panels on the right; in each case, the doubly-charged $[M–2H]^{2–}$ ion peaks are shown).

To confirm both the earlier determination of mannose as the final residue of the N-linked pentasaccharide [7,11] and the importance of AglR for its addition, parent, Δ*aglD* and Δ*aglR* strain cells were incubated with 2-[^{3}H]-mannose, and the incorporation of this radiolabeled sugar into the S-layer glycoprotein was addressed. While parent strain cells readily incorporated 2-[^{3}H]-mannose, no such labeling was seen in cells deleted of *aglD*, encoding the glycosyltransferase responsible for charging DolP with this final pentasaccharide residue [5] (Fig. 4, top panel, center and left lanes, respectively). Likewise, when Δ*aglR* cells were challenged with 2-[^{3}H]-mannose, no radiolabel was incorporated into the S-layer glycoprotein (Fig. 4, top panel, right lane). Densitometric quantification of the Coomassie-stained S-layer glycoprotein bands (Fig. 4, bottom panel) confirmed that comparable levels of S-layer glycoprotein from each strain had been loaded onto the gel (parent: 97.48±12.1, Δ*aglR*: 87.14±12.1, Δ*aglD*: 91.44±6.6, (in each case, n=3); values in arbitrary units).

3.4. AglR is a homologue of Wzx, a predicted lipid-linked oligosaccharide flippase

Toward more precisely defining the role of AglR in *Hfx. volcanii* N-glycosylation, a bioinformatics approach was taken. The topology prediction servers, HMMTOP (http://www.enzim.hu/hmmtop/), SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/), TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/.), TopPred (http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html) and TMpred (http://www.ch.embnet.org/software/TMPRED_form.html), all agreed that the 476 amino acid residue-containing AglR is a multi-membrane-spanning protein, containing 11 or 12 trans-membrane domains. An InterProScan search (http://www.ebi.ac.uk/Tools/InterProScan/) recognized a Polysacc_synt (PF01943) domain in AglR in the region between amino acids 155–204. The Polysacc_synt domain is seen in protein involved in polysaccharide biogenesis and is found in RfbX (COG2244), also known as WzxB, a hydrophobic protein containing 12 potential trans-membrane domains [21,22]. The Wzx proteins are annotated as flippases responsible for delivering lipid-linked O-antigen precursor oligosaccharides across the bacterial plasma membrane in an ATP-independent manner during lipopolysaccharide biogenesis [21,23,24]. In the case of AglR, the various topology prediction algorithms assign the Polysacc_synt domain to a segment of the protein spanning the fifth and sixth trans-membrane domains and partially facing the cell exterior.

To further examine whether AglR shares resemblance to Wzx proteins, several bacterial Wzx sequences were used as query in BLAST searches against the *Hfx. volcanii* genome. Using the *Escherichia coli* O32 Wzx sequence (ACD37057.1) as query, AglR was identified as a homologue, with an e value of 2e – 9. When this search was repeated using the *E. coli* O59 Wzx (AAV74382.1) or the *E. coli* O177 Wzx sequences (AAY728255.1) as query, AglR was again identified as a homologue, with e values of 4e – 5 and 1e – 5, respectively.

4. Discussion

Based on the physico-chemical properties of phospholipids, cellular membranes present a hydrophobic barrier to the transfer of hydrophilic molecules. Many biological processes, however, require that this barrier be overcome. To achieve this, membranes contain a wide array of substrate-translocating proteins, including flippases, a class of proteins dedicated to the delivery of lipid species across membranes [25]. In N-glycosylation, flippases catalyze the transfer of lipid-linked oligosaccharides (LLOs) from that side of the membrane where they are charged to the opposing membrane face, where the LLO glycan cargo is delivered to other lipid-bound glycans or to target proteins. Presently, relatively little is known of the flippases that catalyze the translocation of LLOs involved in N-glycosylation across membranes or their mechanisms of action. This is particularly true in Archaea, where research efforts are hampered, in part, by a lack of appropriate molecular tools.

In the present study, it was shown that in *Hfx. volcanii* cells lacking AglR, there is an accumulation of DolP-Man and an absence of the final mannose residue from the N-linked glycan decorating the S-layer glycoprotein. Such observations are consistent with AglR acting as a DolP-Man flippase or contributing to DolP-Man flippase-related activity. Indeed, the homology of AglR to Wzx, a bacterial protein thought to translocate lipid-linked O-antigen precursor oligosaccharides across the plasma membrane [21-23], supports such a role for the *Hfx. volcanii* protein. Although it remains to be demonstrated that the accumulated DolP-Man seen in Δ*aglR* is restricted to the cytosolic face of the membrane, mannose is added to the protein-bound tetrasaccharide on the external face of the membrane in the current working model of *Hfx. volcanii* N-glycosylation [11]. Indeed, the N-glycosylation of cell-impermeant peptides by a related haloarchaeal species, *Halobacterium salinarum*, points to the oligosaccharyltransferase, AglB as acting on the external surface of the cell [26]. At the same time, the accumulation of tetra- and trisaccharide-charged DolP species in the Δ*aglR* strain points to AglR as serving alternate roles. For instance, AglR could serve a more general flippase-related function, such as regulating access to a flippase or otherwise modulating flippase function. On the other hand, AglR could be required for the utilization but not the flipping of DolP-Man, as proposed for the eukaryal Lec35 protein [27]. It is also conceivable that AglR is responsible or otherwise involved in the transfer of the mannose residue from DolP-Man to the apparently target protein-bound tetrasaccharide. This possibility is, however, unlikely, as AglR does not contain any glycosyltransferase domains, such as those found in the *Hfx. volcanii* glycosyltransferases, AglJ, AglG, AglI, AglE and AglD. Moreover, unlike these glycosyltransferases, which present major soluble domains, AglR is predicted as being largely buried within the membrane bilayer, spanning the membrane 11–12 times. Finally, one could envisage AglR being involved in the membrane organization of other Agl proteins involved in *Hfx. volcanii* N-glycosylation.

If, however, AglR is indeed a DolP-Man flippase, then the fact that monosaccharide-modified S-layer glycoprotein was detected in Δ*aglR* cells suggests that AglR is able to distinguish between DolP species bearing mannose and other hexoses, in turn pointing to the involvement of multiple flippases in *Hfx. volcanii* N-glycosylation. A requirement for multiple flippases is also thought to be the case in eukaryal N-glycosylation [28,29]. Recently, the activity of a eukaryal DolP-Man flippase was assayed *in vitro* [29]. When the

same assay was attempted with *Hfx. volcanii*, namely assessing the ability of carboxy-2,2,6,6-tetramethylpiperidine 1-oxyl NO(+) to label externally exposed mannose subunits, no labeling was detected, possibly to due to low levels of DolP-Man or effects related to the hypersaline conditions required by *Hfx. volcanii* (not shown). Moreover, since the eukaryal DolP-Man flippase remains to be identified, sequence comparison with *Hfx. volcanii* AglR is not yet possible. Likewise, a BLAST search of the eukaryal protein database failed to identify any AglR homologue. This may be related to the unique composition of DolP in *Hfx. volcanii*, relative to its eukaryal counterpart [7,30].

At present, little is also known of LLO flippases in the other domains of life, namely Eukarya and Bacteria. Based on genetic studies, Rft1 was originally proposed as mediating the delivery of mannose$_5$-N-acetylglucosamine$_2$-charged dolichol pyrophosphate (DolPP-M5) across the ER membrane in an ATP-independent manner [31,32]. Subsequent biochemical analysis of DolPP-M5 translocation across ER-derived or proteoliposome membranes, however, revealed that Rft1 is not central to such activity [28,32,33]. As such, the agent responsible for DolPP-M5 flipping has yet to be defined and validated by genetic criteria. Indeed, it has been suggested that in the ER, Rft1 controls access to, rather than itself being, a flippase [28,34]. In the bacterium, *Campylobacter jejuni*, PglK has been given the role of the ATP-dependent flippase of the N-glycosylation pathway, serving to translocate undecaprenol pyrophosphate-linked heptasaccharide across the plasma membrane [35,36]. Nonetheless, some N-glycosylation persists in a *pglK* mutant, thus calling this assignment into question [37].

Defining the precise function of AglR will require *in vitro* reconstitution of its activity. As the list of experimental tools available for working with Archaea, in general, and *Hfx. volcanii*, in particular, continues to grow, such experiments may soon be possible.

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Abbreviations

ABC	ATP-binding cassette
CBB	Coomassie brilliant blue
DDW	double-distilled water
DolP	dolichol phosphate
DolPP-Man5	$mannose_5$-N-$acetylglucosamine_2$-charged dolichol pyrophosphate
EIC	extracted ion chromatograms
ER	endoplasmic reticulum
LC-ESI/MS	liquid chromatography-electrospray ionization mass spectrometry
LLO	lipid-linked oligosaccharide
Man	mannose
MS/MS	tandem mass spectrometry
RT-PCR	reverse transcriptase-PCR

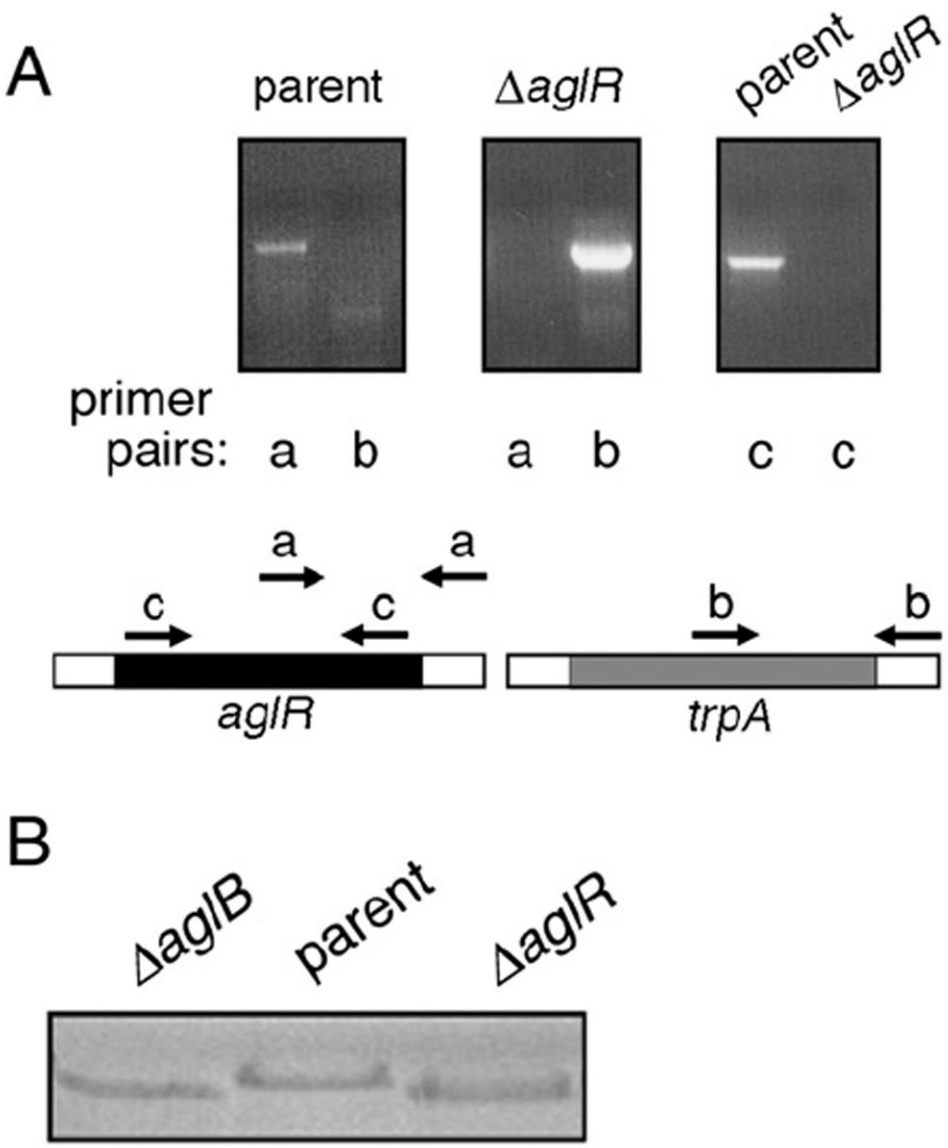

Fig. 1.

The absence of AglR affects *Hfx. volcanii* N-glycosylation. A. Left and middle panels: PCR amplification was performed using a forward primer directed to a sequence within the *aglR* coding region and a reverse primer directed at the *aglR* 3′ flanking region (primer pair a) or using a forward primer directed to a sequence at the start of the *trpA* sequence and the same reverse primer as above (primer pair b), together with genomic DNA from cells of the parent strain (parent; left panel) or from cells where *aglR* had been replaced with *trpA* (Δ*aglR*; middle panel), as template. Right panel: PCR amplification was performed using primer pair c directed against the *aglR* coding region, together with genomic DNA from cells of the parent strain (parent) or the *aglR*-deleted strain (Δ*aglR*) as template. The positions to which the various primer pairs bind are shown in the drawing below the panels. Note that *aglR* and *trpA* are surrounded by the same flanking regions. B. In the absence of AglR, the *Hfx. volcanii* S-layer glycoprotein migrates faster in SDS-PAGE than does the same protein from the parent strain. Similarly enhanced migration is seen for the S-layer glycoprotein from

cells lacking AglB, and hence incapable of performing N-glycosylation. Only that region of the Coomassie-stained gel containing the S-layer glycoprotein is shown.

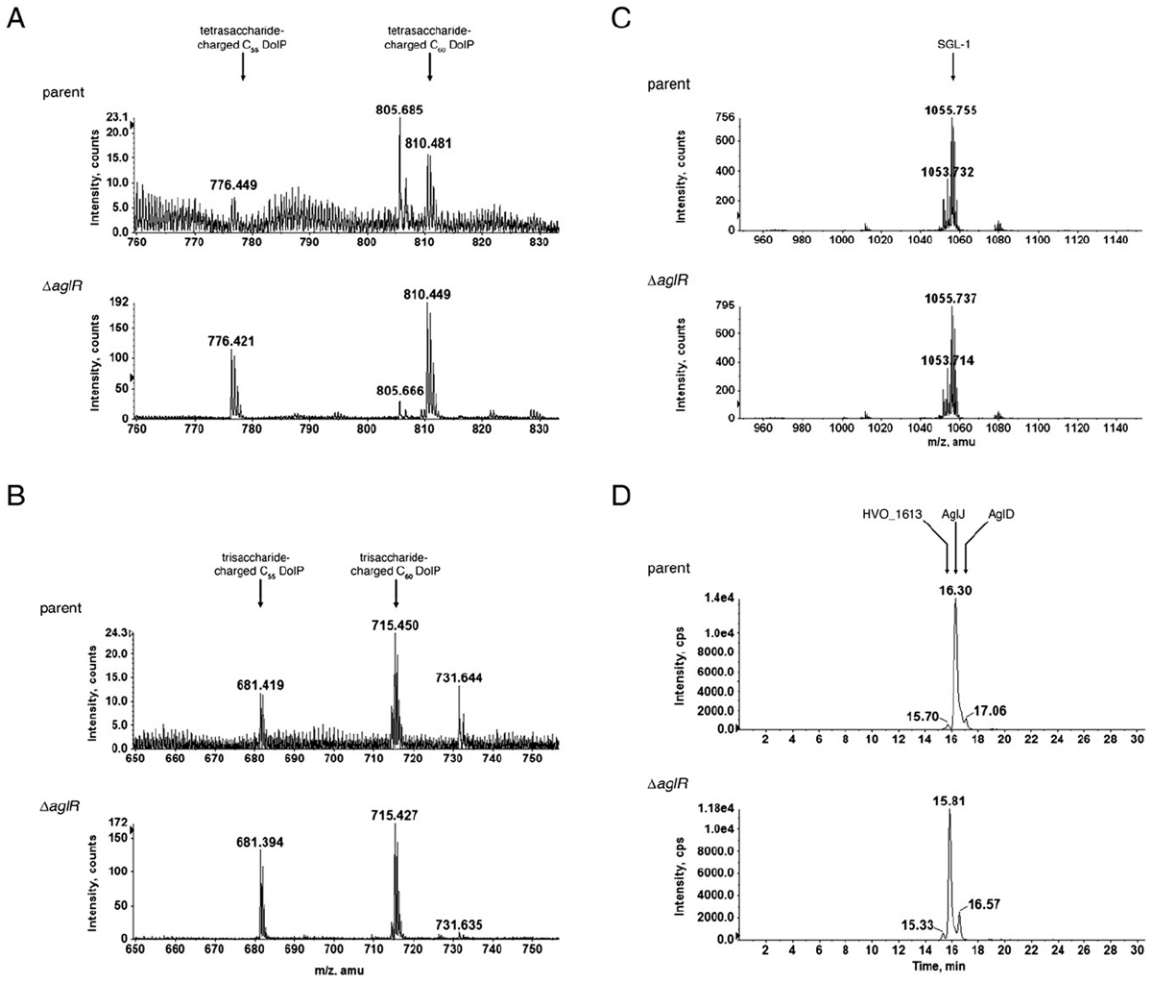

Fig. 2.

Glycan-charged DolP accumulates in Δ*aglR* cells. Normal phase LC/MS/MS analysis of (A) tetra- and (B) trisaccharide-charged DolP from the total lipid extract of cells of the *Hfx. volcanii* parent strain (upper panels) and of Δ*aglR* cells (lower panels) was performed. A. Doubly charged $[M–2H]^{2–}$ ions of methyl ester of hexuronic acid-dihexuronic acid-hexose-modified C_{55} and C_{60} DolP are shown. B. Doubly charged $[M–2H]^{2–}$ ions of dihexuronic acid-hexose-modified C_{55} and C_{60} DolP are shown. Non-DolP-related peaks at *m/z* 805.685 (A) and 731.644 (B) serve as internal controls for changes in glycan-charged DolP peaks as a function of *aglR* deletion. Note that different y-axis scales are used in the profiles of the parent and the Δ*aglR* cells. C. In both parent and the Δ*aglR* cells, identical levels of the sulfo-glycolipid, SGL-1 [20], are detected as $[M–H]^{–}$ ion peaks at *m/z* 1055.7. D. EICs of the hexose-charged DolP $[M–H]^{–}$ ion at *m/z* 1079.8 from parent (upper panel) and Δ*aglR* strain cells (lower panel) are shown. The enzymes responsible for generating the three monosaccharide-charging DolP species are indicated above each peak. In Δ*aglR* cells, the AglD-generated species accumulates.

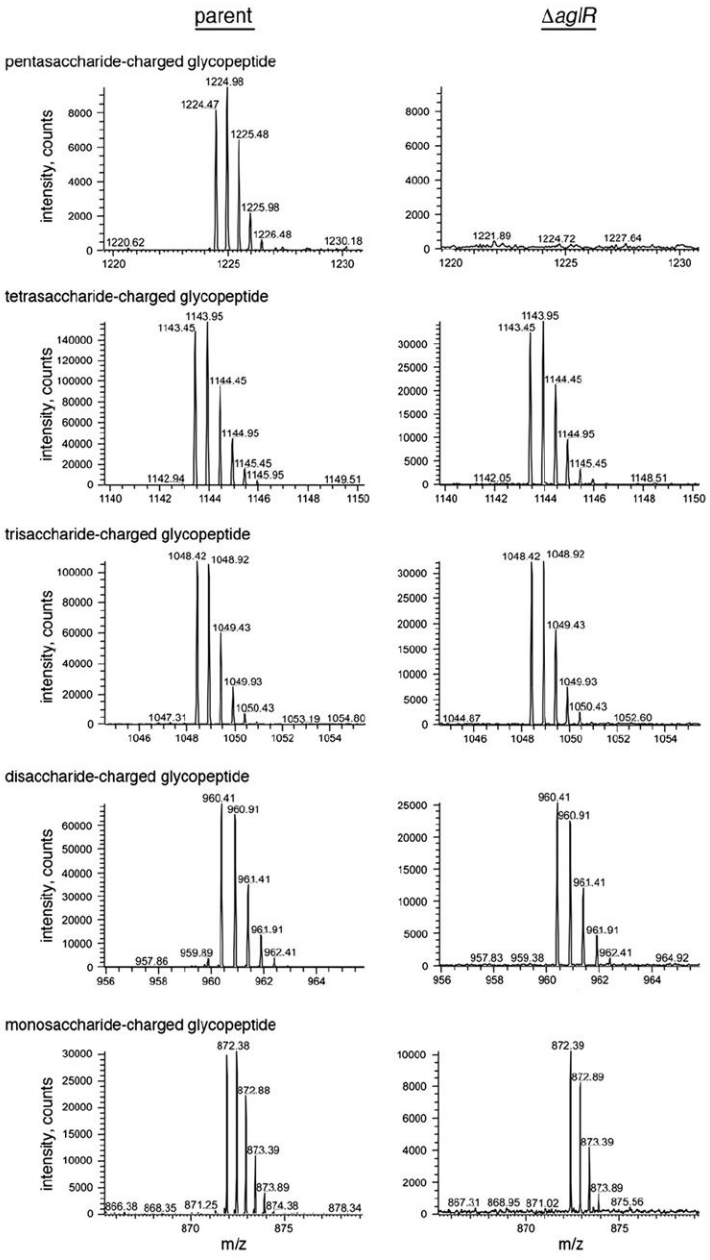

Fig. 3.

LC-ESI/MS/MS of an Asn-13-containing *Hfx. volcanii* S-layer glycoprotein-derived glycopeptide. The LC-ESI/MS/MS spectra of Asn-13-containing tryptic peptides derived from the S-layer glycoprotein from parent (left panels) or Δ*aglR* (right panels) strain cells are shown. The top to bottom panels show peaks corresponding to penta-, tetra-, tri-, di- and monosaccharide-modified peptides, as indicated. Above each peak, the doubly-charged $[M–2H]^{2–}$ ion species mass is indicated.

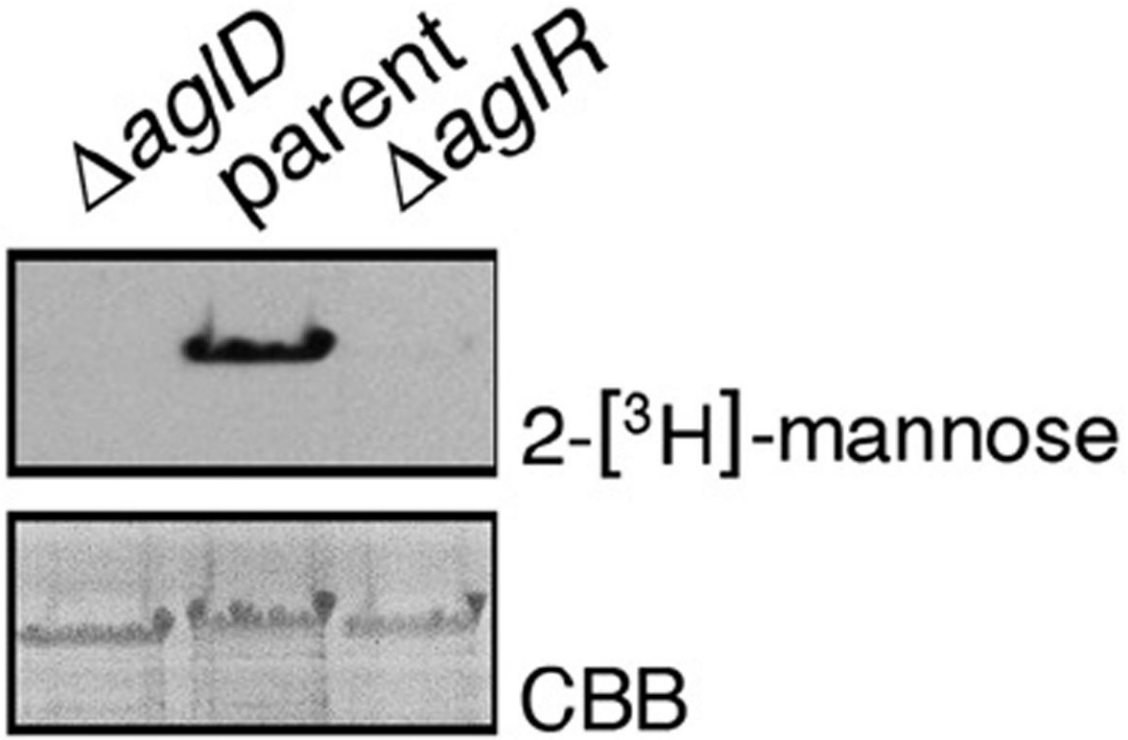

Fig. 4.

Δ*aglR* cells lack the final mannose residue of the S-layer glycoprotein N-linked glycan. The S-layer glycoprotein of 2-[^{3}H]-mannose-treated parent, Δ*aglD* and Δ*aglR* strain cells was separated by SDS-PAGE and examined by fluorography (top panel; 2-[^{3}H]-mannose) or Coomassie brilliant blue staining (bottom panel; CBB).