N-glycosylation in *Haloferax volcanii*: adjusting the sweetness

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Long believed to be restricted to Eukarya, it is now known that all three domains of life perform N-glycosylation, the covalent attachment of glycans to select target protein asparagine residues. Still, it is only in the last decade that pathways of N-glycosylation in Archaea have been delineated. In the haloharocaeon *Haloferax volcanii*, a series of Agl (archaeal glycosylation) proteins is responsible for the addition of an N-linked pentasaccharide to modified proteins, including the surface S-layer glycoprotein, the sole component of the surface layer surrounding the cell. The S-layer glycoprotein N-linked glycosylation profile changes, however, as a function of surrounding salinity. Upon growth at different salt concentrations, the S-layer glycoprotein is either decorated by the N-linked pentasaccharide introduced above or by both this pentasaccharide as well as a tetrasaccharide of distinct composition. Recent efforts have identified Agl5–Agl15 as components of a second *Hfx. volcanii* N-glycosylation pathway responsible for generating the tetrasaccharide attached to S-layer glycoprotein when growth occurs in 1.75 M but not 3.4 M NaCl-containing medium.

**Keywords:** Archaea, *Haloferax volcanii*, N-glycosylation, post-translational modification, protein glycosylation, S-layer glycoprotein

To cope with the challenges associated with life in a hypersaline environment, halophilic Archaea like *Haloferax volcanii* rely on a variety of strategies manifested at the molecular level. For instance, haloarchaeal proteins present more acidic residues and fewer basic residues than do their non-halophilic homologs (Lanyi, 1974; Fukushi et al., 2003). While this approach allows haloarchaeal proteins to fold and function properly in the presence of molar concentrations of salt, modified amino acid composition does not allow such proteins to adapt to fluctuations in their surroundings. Instead, post-translational modifications offer proteins a route through which to respond to changing conditions in a transient manner. In the case of the *Hfx. volcanii* S-layer glycoprotein, the sole component of the protein shell surrounding the cell (Sumper et al., 1993), changes in environmental salinity are reflected in a modified N-glycosylation profile (Guan et al., 2012).

The *Hfx. volcanii* S-layer glycoprotein contains seven putative sites of N-glycosylation (Sumper et al., 1993), at least two of which are modified by a pentasaccharide resembling a hexose, two hexuronic acids, a methyl ester of hexuronic acid, and a mannose (Abu-Qarn et al., 2007; Guan et al., 2010; Magidovich et al., 2010). Genetic and biochemical approaches have served to identify a series of Agl (archaeal glycosylation) proteins responsible for the assembly and attachment of this N-linked glycan. AglJ, AglG, AglI, and AlgE are glycosyltransferases that sequentially add the first four sugars of the N-linked pentasaccharide responsible for the assembly and attachment of the N-linked pentasaccharide to a common dolichol phosphate carrier (Abu-Qarn et al., 2007; Guan et al., 2010; Calo et al., 2011; Cohen-Rosenzweig et al., 2012; Kaminski et al., 2012). In addition, other Agl proteins serve various sugar-processing or other roles that contribute to pentasaccharide assembly, such as AglJ, a glucose-1-phosphate uridyltransferase, AglM, a UDP-glucose dehydrogenase, AglP, a methyltransferase, and AglQ, an isomerase (Yuriot-Douitsch et al., 2008; Magidovich et al., 2010; Yuriot-Douitsch et al., 2010; Arbiv et al., 2013). The most recent version of the Agl pathway is presented in Figure 1.

When first described, *Hfx. volcanii* was reported to grow at NaCl concentrations ranging from 1 M to 4 M (Mullahshabbi and Larsen, 1975). In deciphering the *Hfx. volcanii* N-linked glycosylation pathway responsible for the assembly and attachment of the N-linked pentasaccharide decorating S-layer glycoprotein Asn-13 and Asn-83 delineated above, cells were grown in medium containing 3.4 M NaCl. However, when the S-layer glycoprotein was considered in cells grown in medium containing only 1.75 M NaCl, a different N-glycosylation profile was observed. When grown at the lower salinity, S-layer glycoprotein Asn-13 and Asn-83 were still modified by the pentasaccharide described above, although to a lesser extent than when the same cells were grown in 3.4 M NaCl-containing medium. What was more striking was that Asn-498, a position not modified when growth occurs at the higher salinity, was decorated by a novel “low salt” tetrasaccharide comprising a sulfated...
hexose, two hexoses and a rhamnose when cells were raised at the lower salinity (Guan et al., 2012). Moreover, the same tetrasaccharide was detected on dolichol phosphate in cells raised in 1.75 M NaCl-containing medium. Indeed, dolichol phosphate bearing the low salt tetrasaccharide had been previously reported when *Hfx. volcanii* cells were grown in medium containing only 1.25 M NaCl (Kuntz et al., 1997). Thus, both dolichol phosphate and the S-layer glycoprotein present bound glycans that differ as a function of growth medium salinity. Furthermore, medium salinity also dictated whether N-glycosylation sites in the S-layer glycoprotein were processed and to what extent. The finding that the *Hfx. volcanii* S-layer glycoprotein can be simultaneously modified by two very different N-linked glycans had also been reported to be true in a second haloarchaeon, namely *Halobacterium salinarum* (for a review, see Lechner and Wieland, 1989). However, unlike the situation in *Hbt. salinarum*, where relatively little is known of the pathway(s) recruited for N-glycosylation, work in the last decade has provided considerable insight into this post-translational modification in *Hfx. volcanii*, including the recently solved pathway of low salt tetrasaccharide assembly.

By combining gene deletions with mass spectrometric analysis of glycan-charged dolichol phosphate and S-layer glycoprotein-derived peptides, it was demonstrated that the Agl proteins responsible for assembly of the N-linked pentasaccharide are not involved in the biosynthesis of the low salt tetrasaccharide (Kaminski et al., 2013a). As such, efforts were directed at identifying genes encoding proteins comprising a second N-glycosylation pathway. Delineating components of the pathway responsible for generating the low salt tetrasaccharide initially relied on previous work showing that all of the *Hfx. volcanii* genes involved in the assembly of the N-linked pentasaccharide decorating S-layer glycoprotein Asn-13 and Asn-83, with the exception of aglD, are found in a single cluster spanning *HVO_1517* (aglF) to *HVO_1531* (aglM; Yurist-Doutsch and Eichler, 2009; Yurist-Doutsch et al., 2010). As such, the *Hfx. volcanii* genome sequence (Hartman et al., 2010) was scanned for clustered open reading frames (ORFs) annotated as serving some glycosylation-related roles. Those ORFs spanning the region from *HVO_1504* to *HVO_2061* represent one such cluster. The involvement of the products of *HVO_2046* to *HVO_2061* in the biogenesis of the low salt tetrasaccharide was subsequently confirmed in a series of experiments involving gene deletions combined with mass spectrometry-based examination of dolichol phosphate and the S-layer glycoprotein. Given
Agl15, the intact low salt tetrasaccharide is assembled on dolichol Asn-13 and Asn-83, a process that also occurs in low salt conditions (Guan et al., 2012). Furthermore, because cells lacking Agl8 and Agl9 contribute to the addition of the next sugar, define the actions of Agl5, Agl6, and Agl7, as well as their order of action. While the enzyme responsible for adding the second sugar of the low salt tetrasaccharide, a hexose, to sulfated hexose-ribonucleotide can only be supposed. Likewise, the reason why Agl15 serves as a flipase, mediating the translocation of low salt tetrasaccharide-charged dolichol phosphate (and likely dolichol phosphate bearing a hexose) to S-layer glycoprotein, for that matter) Hfx. volcanii S-layer glycoprotein Asn-498 position when the cells are grown in low salt (1.75 M NaCl) containing medium. See the text for details. In the figure, dolichol phosphate is in purple, hexasose in yellow and rhamnose is in pink. The bottom half of the figure corresponds to the cell interior.

Presently, the reason why the Hfx. volcanii S-layer glycoprotein (and the Hbt. salinarum S-layer glycoprotein, for that matter) can be modified by two distinct N-linked glycans as a function of environmental salinity can only be supposed. Likewise, the reason why Agl15 serves as a flipase, mediating the translocation of low salt tetrasaccharide-charged dolichol phosphate (and likely dolichol phosphate bearing a hexose) to S-layer glycoprotein, for that matter) Hfx. volcanii S-layer glycoprotein Asn-498 position when the cells are grown in low salt (1.75 M NaCl) containing medium. See the text for details. In the figure, dolichol phosphate is in purple, hexasose in yellow and rhamnose is in pink. The bottom half of the figure corresponds to the cell interior.

Although Hfx. volcanii seemingly relies on two different pathways for the assembly of the two N-linked glycans decorating the S-layer glycoprotein, only one oligosaccharyltransferase, namely the enzyme responsible for the transfer of the lipid-linked glycan to a target protein, has been identified in this organism. In Hfx. volcanii, AglB is the only homolog of the euarkyl oligosaccharyltransferase catalytic subunit, SM3, or its bacterial counterpart, PglB (Magidovich and Eichler, 2009; Kaminski et al., 2013a). As such, the absence of AglB prevented the glycosylation of S-layer glycoprotein Asn-13 and Asn-83 by the pentasaccharide normally attached at these positions (Abu-Qarn et al., 2007). On the other hand, aglB deletion had no effect on the appearance of the low salt tetrasaccharide added to the Asn-498 position (Kaminski et al., 2013a). Thus, a currently unidentified and novel oligosaccharyltransferase seems to be involved in the delivery of the low salt tetrasaccharide (and its precursors) from dolichol phosphate to S-layer glycoprotein Agl15. The same may be the case in Hbt. salinarum, where one of the two N-linked glycans decorating the S-layer glycoprotein in this species is transferred from a dolichol phosphate carrier while the second glycan is delivered from a dolichol pyrophosphate carrier (for review, see Lechner and Wieland, 1989; Cohen-Rosenzweig et al., 2013).

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AUTHOR CONTRIBUTIONS
All authors made substantial contributions to the acquisition, analysis, and interpretation of data described in this report. All authors critically reviewed the report and approved the final version. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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