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N-glycosylation in Archaea – Expanding the process, components and roles of a universal post-translational modification

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ABSTRACT

While performed by all three domains of life, N-glycosylation in Archaea is less well described than are the parallel eukaryal and bacterial processes. Still, what is known of the archaeal version of this universal post-translational modification reveals numerous seemingly domain-specific traits. Specifically, the biosynthesis of archaeal N-linked glycans relies on distinct pathway steps and components, rare sugars and sugar modifications, as well as unique lipid carriers upon which N-linked glycans are assembled. At the same time, Archaea possess the apparently unique ability to simultaneously modify their glycoproteins with very different N-linked glycans. In addition to these biochemical aspects of archaeal N-glycosylation, such post-translational modification has been found to serve a wide range of roles possibly unique to Archaea, including allowing these microorganisms to not only cope with the harsh physical conditions of the niches they can inhabit but also providing the ability to adapt to transient changes in such environments.

1. A brief history of archaeal N-glycosylation

In 1938, Neuberger published the first conclusive description of a protein containing a covalently linked sugar-containing moiety [1]. In the decades that followed, protein glycosylation, and in particular, N-glycosylation, in which a glycan is attached to selected asparagine residues in a protein, was accepted to be a post-translational modification restricted to eukaryotes. This dogma was upended in 1976, when the surface (S)-layer glycoprotein from the halophile *Halobacterium salinarum* provided the first example of N-glycosylation in the prokaryotic world [2]. Today, it is clear that N-glycosylation is a protein-processing event that takes place across evolution.

At the time of providing the first example of non-eukaryotic N-glycosylation, *Hbt. salinarum* were still considered bacteria. However, with the 1977 re-alignment of the tree of life to account for three distinct branches, namely, Eukarya, Bacteria and Archaea [3], *Hbt. salinarum* were assigned to the newly described archaeal domain [4]. Originally defined as a distinct form of life on the basis of their unique 16S ribosomal RNA secondary structure [3], a variety of molecular traits that distinguished Archaea from Eukarya or Bacteria later emerged. These included membranes comprising ether-based phospholipids presenting a novel stereospecificity organized into monolayer or bilayer structures, and the use of biosynthetic pathways and metabolic strategies not seen elsewhere [5-9]. Moreover, despite being originally defined as extremophiles able to thrive in some of the most physically challenging environments on the planet, it subsequently became apparent that Archaea are also major constituents of so-called 'normal' biological niches [10, 11]. Most recently, the three-branched tree of life proposed by Woese [3, 12] has been challenged by evidence supporting the Eukarya as having emerged from within the Archaea [13].

Soon after the discovery of N-glycosylation in *Hbt. salinarum*, biochemical approaches were taken to define the composition of the N-linked glycan and gain insight into the pathway responsible for its biosynthesis. Although limited by the tools of the time, these efforts, nonetheless, revealed aspects of the process not seen elsewhere. For instance, a single protein, the S-layer glycoprotein, was shown to be modified by two very different N-linked glycans, with one being assembled on a dolichol pyrophosphate carrier and using an *N*-acetylhexosamine as the linking sugar, properties reminiscent of the eukaryal N-glycosylation pathway [14,15]. The second N-linked glycan, subsequently shown to modify other *Hbt. salinarum* glycoproteins [16], was the first shown to be assembled on a dolichol phosphate carrier and use a hexose as the linking sugar [17]. Moreover, this latter N-linked glycan was shown to be methylated at the lipid-linked stage but not when protein-bound, a phenomenon yet to be seen elsewhere [18]. Furthermore, this second polysaccharide represents the only known

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example of an N-linked glycan containing iduronic acid [19,20]. Not long after these intriguing findings were described, reports on archaeal N-glycosylation became far less frequent.

The dawn of the genome age brought renewed interest into archaeal N-glycosylation, as into other aspects of archaeal biology. With the development of genetic and other relevant experimental tools, the first components of archaeal N-glycosylation pathways were identified in the methanogen *Methanococcus voltae* and the halophile *Haloferax volcanii* [21,22]. In time, N-glycosylation pathways were subsequently fully or partially delineated in other archaea, including *Methanococcus maripaludis, Sulfolobus acidocaldarius, Haloarcula hispanica* and *Hbt. salinarum* [23-26]. Fig. 1 presents schematic depictions of two such pathways. In gaining insight into N-glycosylation pathways in these species, additional novel aspects of the process, some seemingly unique to Archaea, were revealed. For instance, in *Hfx. volcanii*, the N-linked pentasaccharide decorating glycoproteins in this species was shown to be assembled from a dolichol phosphate-bound tetrasaccharide precursor, which is transferred to target protein asparagine residues before the fifth sugar is transferred to the protein-bound tetrasaccharide from its own dolichol phosphate carrier [27]. To the best of our knowledge, this represents the sole example of an N-linked glycan being modified by additional lipid-linked sugars. At the same time, another haloarchaeon from the Dead Sea, *Haloarcula marismortui*, decorates its glycoproteins with what seems to be the same or at least a very similar pentasaccharide, yet here, the entire glycan is assembled on a single dolichol phosphate carrier [28]. In *M. maripaludis*, dolichol phosphate was shown to be charged with N-acetylglucosamine in the alpha conformation, in contrast to the beta conformation presented by linking sugars added to lipid carriers in Eukarya and Bacteria [29]. This difference points to a fundamental difference in the mechanism of action of archaeal dolichol phosphate glycosyltransferases that catalyze the addition of a linking sugar to the dolichol phosphate carrier upon which N-linked glycans are

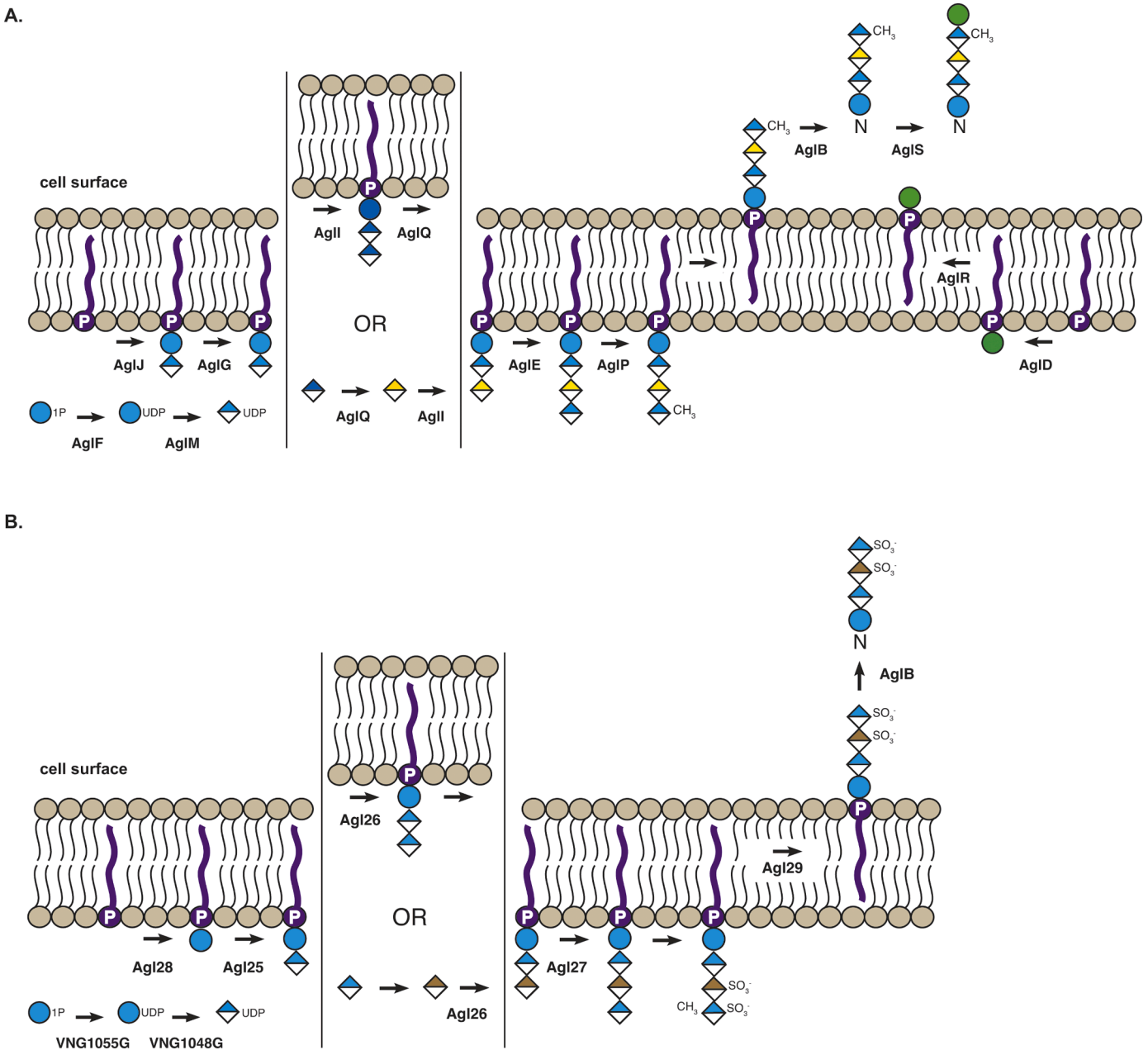

Fig. 1. Schematic depictions of two archaeal N-glycosylation pathways. A. *Hfx. volcanii*. B. *Hbt. salinarum*. Legend: blue circle – glucose; green circle – mannose; half-blue diamond – glucuronic acid; half-yellow diamond – galacturonic acid; half-brown diamond – iduronic acid; purple lipid – dolichol phosphate.

assembled from that of their eukaryal and bacterial counterparts, despite structural similarities [30]. Finally, structural comparisons of oligosaccharyltransferases across the three domains revealed a catalytic motif apparently unique to a sub-set of the archaeal protein, AglB [31].

In addition to gaining insight into the pathways involved in archaeal N-glycosylation, progress was also made in defining the lipid carriers upon which N-linked glycans are assembled. Such efforts not only showed archaea-specific lipid chemistry, namely, saturation of dolichol phosphate at both the alpha and omega positions (as opposed to only the alpha position seen in eukaryal dolichol phosphate) but also revealed that phylogenetic considerations were involved in relying on dolichol or dolichol pyrophosphate as the lipid glycan carrier [32-34]. At the same time, phylogenetic approaches addressing the distribution of the oligosaccharyltransferase AglB revealed the almost universal nature of N-glycosylation in Archaea, while distinguishing between versions of AglB found in certain archaeal phyla as being more similar to its bacterial homologue PglB and in others to the eukaryal AglB counterpart Stt3 [35]. These findings, moreover, offer support for the proposed evolutionary proximity of Eukarya and the TACK and Asgard archaea [36].

In summary, these observations argue that despite the fact that little is known of N-glycosylation in the vast majority of archaeal species, what has been learned from even the limited number of examples studied to date nonetheless reveals that domain-specific traits appear at all levels of the archaeal N-glycosylation process.

2. Unique sugars found in archaeal N-linked glycans – selected examples

Although the pathways used to assemble the N-linked glycans decorating archaeal glycoproteins are largely limited to those handful of species for which genetic manipulation is currently possible, far more progress has been realized in describing the sugar composition and architecture of archaeal N-linked glycans, with a few dozen such structures now described. These efforts have revealed a degree of diversity not seen in either eukaryal or bacterial N-glycosylation [37-39]. Moreover, it is becoming increasingly clear that archaeal N-linked glycans can include sugars not used elsewhere in N-glycosylation or sugars modified by unusual chemical groups or at positions not seen before. In this section, selected examples of these phenomena are described.

As noted above, the N-linked tetrasaccharide decorating *Hbt. salinarum* glycoproteins includes iduronic acid [19,20], a common sugar component of eukaryal glycosaminoglycans, like heparin, an anti-coagulant, or heparin sulfate, found in the extracellular matrix of animal tissues [40-42]. In bacteria, iduronic acid has also been found as a component of cell wall polysaccharides, albeit in only few species [43-47]. Not only does the *Hbt. salinarum* N-linked tetrasaccharide represent the only known example of iduronic acid in a protein-bound glycan, this iduronic acid is sulfated at the O-3 position, a modification never reported before [20]. While the iduronic acids of glycosaminoglycans can also be sulfated, this is only seen at the O-2 position [41,42].

The N-linked tetrasaccharide decorating *M. maripaludis* glycoproteins includes a novel monosaccharide ((5S)−2-acetamido-2,4-dideoxy-5-O-methyl-α-L-*erythro*-hexos-5-ulo-1,5-pyranose) as the terminal sugar, corresponding to the first example of a diglycoside of an aldulose [48]. The same glycan also includes a 2,3-diamino-mannuronic acid acylated at the N-3 position with an acetamidino group and modified with an acetyl group at the N-2 position, as occurs in the lipopolysaccharides of some Gram-negative bacteria [49-51], as well as a threonine linked to the C-6 carbonyl group. This sugar, found at the third position of the tetrasaccharide, represents the first example of a mannuronic acid being modified in this manner [39]. The related methanogen *M. voltae* decorates its glycoproteins with a similar N-linked trisaccharide that ends with a 2-acetamido-2-deoxy-β-mannuronic acid, with the carbonyl group at C-6 forming an amide bond with the amino group of threonine [52].

The N-linked trisaccharide decorating the S-layer glycoproteins of another methanogen, *Methanoculleus marisnigri*, provided the first known example of an N-linked glycan that includes a sugar modified by a glyceric acid [53]. Specifically, the second sugar of this N-linked glycan corresponds to 2,3-diaminoglucuronic acid N-acylated with acetic acid and glyceric acid residues, as well as being amidated at the C-6 position. Moreover, as described below, the *M. marisnigri* S-layer glycoprotein is simultaneously modified by a related, yet distinct N-linked disaccharide.

Thermophilic archaea also present N-linked glycans bearing unusual sugars or sugars presenting unusual modifications. For instance, the N-linked glycan attached to membrane proteins of *Thermoplasma acidophilum* [54] includes a particular heptose isomer thus far only seen in the lipopolysaccharides of a limited number of bacteria [55-58]. At the same time, the 6-deoxy-6-C-sulfo-D-galactose (6-C-sulfo-D-fucose) found in the same N-linked glycan has not been previously described and is only one of two known monosaccharides presenting a direct C-S bond [39]. The glycan attached to selected asparagine residues of membrane proteins in *Thermococcus kodakarensis* comprises five sugars and a *myo*-inositol phosphate [59]. This represents the first example of inositol as a component of an N-linked glycan. Finally, *Sulfolobus acidocaldarius* contains an N-linked glycan that includes 6-deoxy-6-sulfoglucose (6-sulfoquinovose), found in glycolipids in plants [60,61] and elsewhere [62,63]. Uniquely, 6-sulfoquinovose is beta-linked in the archaeal glycan; elsewhere, linkage of this sugar relies on the alphaconformation.

Several of the different N-linked glycans discussed in this section are portrayed in Fig. 2.

3. In archaea, multiple N-glycans can simultaneously modify the same protein

Not only do archaeal N-linked glycans contain sugars not previously described or never having been previously reported as being part of such structures elsewhere, it has also been shown that numerous archaeal glycoproteins can be simultaneously modified by clearly distinct N-linked glycans. Such a phenomenon has not been seen, to the best of our knowledge, in either of the other two domains of life.

The addition of N-glycans of very different composition to the same protein was first shown in the case of the *Hbt. salinarum* S-layer glycoprotein. This glycoprotein is modified at ten of twelve putative N-glycosylation sites by a tetrasaccharide assembled on a dolichol phosphate carrier, with glucose serving as the linking sugar [17,64]. At an eleventh N-glycosylation site, asparagine-2, the same protein is reportedly modified by ten-fifteen repeats of a distinct pentasaccharide linked to the protein via a *N*-acetylhexosamine and assembled on a dolichol pyrophosphate carrier [14,15,64]. Whereas the majority of players involved in the biogenesis of the former N-linked glycan have been described [65-67], virtually nothing is known of how the latter is assembled.

In the case of the *Hfx. volcanii* S-layer glycoprotein, three different N-linked glycans have been reported to simultaneously modify the protein [68-70], with distinct biosynthetic pathways having been delineated for two of these glycans [68,71,72]. At least two of these N-linked glycans seem to be differentially added as a function of the surrounding salinity (see below). In *M. marisnigri*, the S-layer glycoprotein is simultaneously modified by an N-linked trisaccharide and an N-linked disaccharide, with both glycans seemingly originating from a common early precursor [53]. Finally, whereas archaellins from *Sulfolobus shibatae* migrate as a single diffuse glycoprotein band in SDS-PAGE, two bands of similar molecular mass and identical N-terminal sequence can be distinguished, suggesting the existence of two variants of the protein presenting different glycosylation profiles [73]. This, however, remains to be conclusively shown.

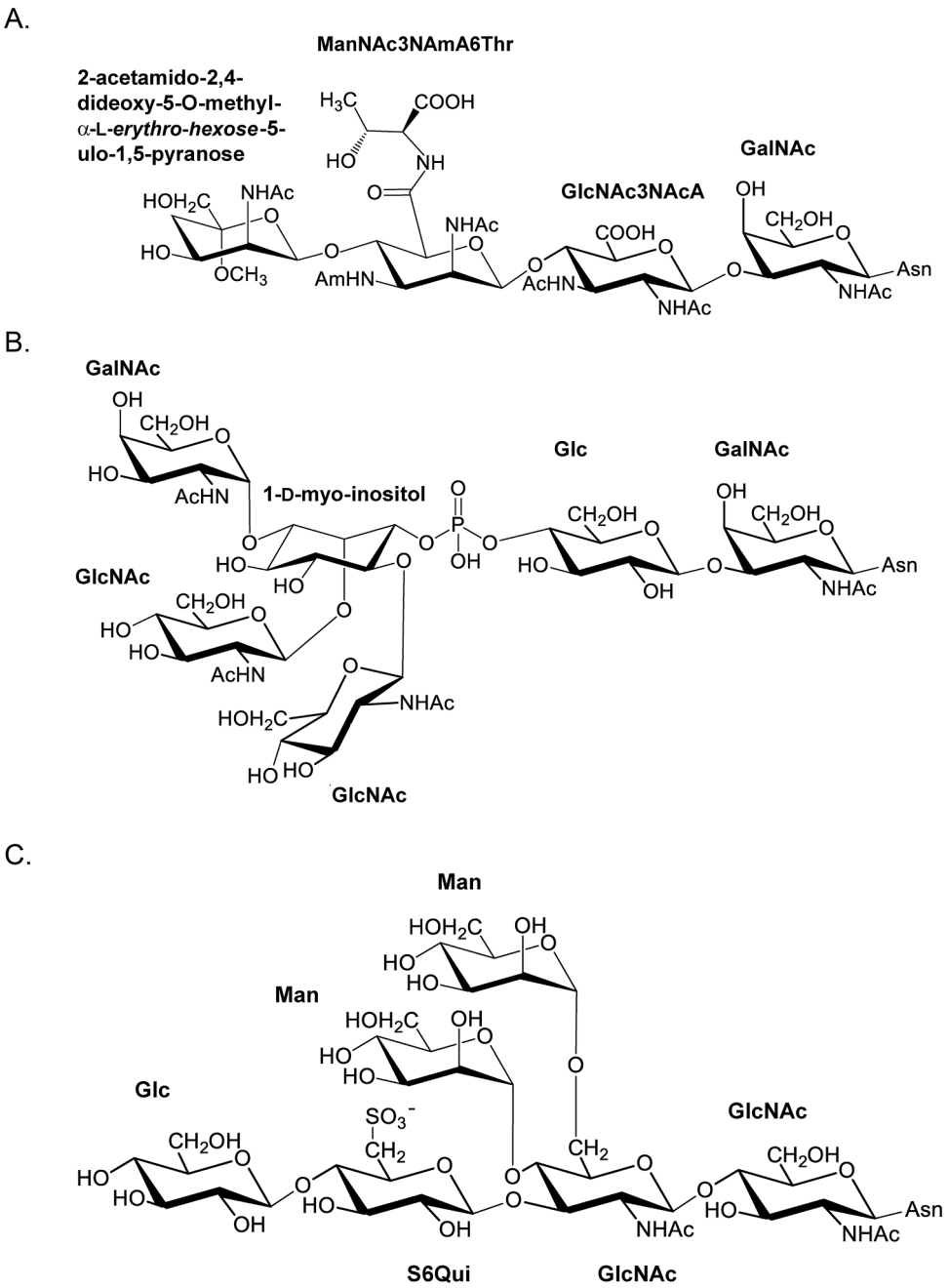

Fig. 2. Selected archaeal N-linked glycans. A. *M. maripaludis*. B. *T. acidophilum*. C. *S. acidocaldarius*. Abbreviations: Asn – asparagine; Glc – glucose; GalNAc – N-acetylgalactosamine; GlcNAc – N-acetylglucosamine; GlcNAc3NAcA - 2,3-diacetamido-2,3-dideoxy-glucuronic acid; Man – mannose; ManNAc3NamA6Thr - 3-acetamidino derivative of 2,3-diamino-2,3-dideoxymannuronic acid amidated with a threonine amino group; S6Qui - 6-deoxy-6-sulfoglucose.

4. N-glycosylation in archaea serves roles not (or not yet) seen elsewhere

Archaeal N-glycosylation has not only demonstrated its uniqueness in terms of the sugars recruited, the pathway steps involved and the simultaneous presence of very different N-linked glycans on the same protein, but also in terms of the roles it serves. Specifically, studies have revealed how archaeal N-glycosylation is responsible for behavior either unique to this domain of life, or which may be also shared by eukaryotes and/or bacteria but has yet to reported in these domains.

In addition to the S-layer glycoprotein, archaellins, the building blocks of the archaeal swimming device, the archaellum [73], are also common reporters of N-glycosylation in Archaea and were among the first archaeal proteins shown to be so-modified [16]. Moreover, the importance of archaellin N-glycosylation for cell motility, has been long known. In numerous species, it was reported that in response to gene deletions leading to the loss of N-glycosylation or to the assembly of highly truncated N-linked glycans, no archaella were detected and cell motility was lost ([74] and references therein). Accordingly, it was concluded that archaellin N-glycosylation was necessary for their proper assembly into archaella. More recently, however, an additional role for archaellin N-glycosylation was described. Using cryo-EM, structures of archaella from *Hbt. salinarum* parent strain cells and from cells deleted of genes encoding glycosyltransferases responsible for adding the last two sugars of the N-linked tetrasaccharide decorating archaellins were obtained [75]. Although earlier efforts had revealed how progressively shortening the N-linked tetrasaccharide to a trisaccharide or a disaccharide resulted in a loss of motility, with the degree of lost motility

being proportional to the extent of glycan truncation [66,67], these more recent efforts revealed how such compromised archaellin N-glycosylation had no impact on archaellin structure or their packing into archaellum filaments. This apparent paradox was solved when it was shown that although the loss of the last or the last two tetrasaccharide sugars did not perturb archeallum architecture, it instead led to dramatic clustering of both isolated and cell-attached archaellum filaments (Fig. 3) [75]. The clustering-induced loss of motility in the two glycosyltransferase-lacking strains was attributed to an inability to separate archaellum filaments, a process needed for effective swimming [76]. In contrast, in the parent strain, where the complete N-linked tetrasaccharide decorates archaellins and where far less archaellum bundling was seen, the N-linked glycans that protrude from the surface of archaellin filaments prevented filament aggregation. The ability of the complete N-linked tetrasaccharide to prevent archaellum filament clustering could be due to electrostatic repulsion mediated by the highly negatively charged N-linked glycan that includes a glucuronic acid, a sulfated iduronic acid and a sulfated glucuronic acid, in addition to a linking glucose unit. Alternatively, the N-linked tetrasaccharides could introduce steric hinderance that would prevent any protein-protein interactions underlying archaellum filament bundling. A similar phenomenon of filament clustering due to compromised N-glycosylation of archaeal cell surface structures other than archaella was observed with *Hfx. volcanii* cells lacking the oligosaccharyltransferase and hence, unable to perform N-glycosylation. Here, the loss of pilin glycosylation by the mutant cells, with pilins corresponding to the building blocks of an additional assembly protruding from the cell surface, resulted in the aggregation of these assemblies, termed pili, and the formation of microcolonies [77]. Much as archaellum filaments must separate to elicit swimming [76], maintaining the separation of adhesive pili is necessary for surface adhesion instead of the microcolony formation that occurs with the clustering of pili from proximal cells. It is also of note that N-glycosylation was recently reported as being able to directly prevent the aggregation of eukaryal proteins [78].

In considering the roles served by archaeal N-glycosylation, one can also ask how archaea exploit the fact that even the relatively limited number of archaeal N-linked glycans that have been described thus far display so much more variety in terms of both content and structure than do their bacterial and eukaryal counterparts. As more is learnt of the functions assumed by N-glycosylation in Archaea, partial answers to this question are beginning to appear. Studies addressing interactions between archaeal cells of the same or different species have pointed to N-glycosylation as introducing specificity into such contacts. The importance of N-glycosylation to *Hfx. volcanii* mating, and hence the maintenance of a species barrier, was demonstrated when the efficiency of pairing was compared in parent strain cells and strains deleted of genes encoding central N-glycosylation pathway components. These studies found mating to be proportionally compromised when N-glycosylation (presumably of the S-layer glycoprotein) was absent in one or both partners [79]. Moreover, even among very closely phylogenetically related *Haloferax* species collected from sampling ponds in the same area, it was shown that although the major N-glycosylation gene cluster tended to be conserved, a second N-glycosylation gene cluster was highly variable. It was thus concluded that even genetically- and geographically-related strains can present distinct N-glycosylation profiles, such that no two isolates present an identical profile [80]. Likewise, differences in N-glycosylation profiles were also offered as the explanation for why cells of three closely related *Sulfolabales* species could readily mate with cells of the same species but not with members of the other two species [81]. The link between N-glycosylation and the maintenance of species barriers is also supported by the observation that even closely related *Hbt. salinarum* species present distinct N-glycosylation pathway gene clusters [82].

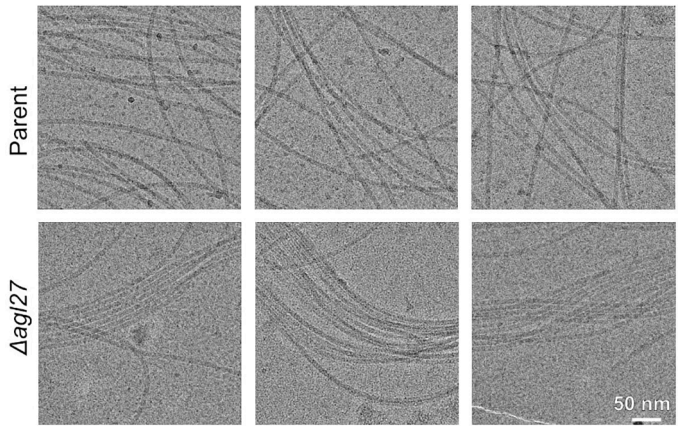

Fig. 3. Pertrubed *Hbt. salinarum* N-glycosylation leads to archaellum filament bundling. Representative cryo-EM micrographs of archaellum filament samples in the spent growth medium of parent strain cells (parent; top panels) and of a strain lacking Agl27, responsible for adding the fourth sugar of the N-linked tetrasaccharide decorating *Hbt. salinarum* archaellins (*Δagl27*; bottom panels). Taken from 75 with permission.

The seemingly species-specific nature of archaeal N-glycosylation is also thought to mediate the specificity of other interactions. For instance, in the *Sulfolabales*, exposure to ultraviolet light can lead to the appearance of pili that mediate cell aggregation, resulting in DNA exchange [83]. The species-specificity of such interactions was shown to be mediated by a defined region of the pilin UspA protein that interacts with the N-linked glycan decorating components of the S-layer from the same species [81]. Strain-specific N-glycosylation is also thought to play a role in the selectivity of viruses for targeted archaeal cells. In *Saccharolobus solfataricus*, the decreased cell surface glycosylation resulting from a perturbed S-layer resulted in a decreased degree of viral infection [84]. The link between N-glycosylation and virus selectivity could explain why the coat protein of the HRPV-1 virus and S-layer glycoprotein of the *Halorubrum* sp. PV6 host present the same N-linked glycan [85,86].

5. Changing N-glycosylation in response to environmental changes

N-glycosylation has been long held to afford extremophilic archaea with the ability to cope with the harsh environments they can inhabit. Specifically, N-glycosylation is thought endow extremophilic archaeal proteins the ability to remain folded, and thus functional, in the face of the physical challenges presented by such conditions [24,87–90]. However, the relation between N-glycosylation and surviving difficult surroundings may be more profound, given how several studies have demonstrated how archaea can modify their N-glycosylation profiles as a function of changing growth conditions. Altering the content and/or positions of N-linked glycans decorating their glycoproteins provides Archaea with a strategy to rapidly and reversibly respond to shifting environments.

In *Hfx. volcanii*, the N-glycosylation profile of the S-layer glycoprotein was shown to change as a function of salinity [68]. In 3.4 M NaCl-containing medium, four of seven putative sites of N-glycosylation were shown to present a pentasaccharide comprising a glucose, a glucuronic acid, a galacturonic acid, a methylated glucuronic acid and a mannose [69]. However, a considerably different glycosylation pattern was observed when *Hfx. volcanii* were grown in the presence of only 1.7 M NaCl. At this lower salinity, a distinct tetrasaccharide comprising a sulfated hexose, two hexoses and a rhamnose was bound to asparagine-498, a position not modified in cells raised at the higher salinity, while less of the pentasaccharide was detected [68]. Subsequent efforts considered N-glycosylation in medium containing an intermediate salt concentration (i.e., 2.7 M NaCl). In these conditions, both N-linked glycans were detected [91], raising the possibility that *Hfx. volcanii* gradually shift their N-glycosylation profile as the

surrounding salt level increases or decreases. It was further shown that the assembly of each N-linked glycan relies on a distinct N-glycosylation pathway, with each pathway also apparently involving a different oligosaccharyltransferase [68]. Specifically, the loss of AglB, the canonical archaeal oligosaccharyltransferase reminiscent of its eukaryal and bacterial counterparts, did not prevent addition of the 'low salt' tetrasaccharide to the S-layer glycoprotein, yet resulted in a loss of glycosylation by the N-linked pentasaccharide.

Methanolobus psychrophilus offer another example of how N-glycosylation changes as a function of the environment. Earlier efforts had shown that growing this psychrophilic ('cold-loving') methanogen in trimethylamine at 18 °C over a period of years provided this species with the ability to grow at 30 °C [92]. It was subsequently shown that the S-layer glycoprotein was N-glycosylated at asparagine-94 when *M. psychrophilus* were grown at 30 °C in the presence of elevated levels of trimethylamine and methanol but not at 18 °C [93]. Growth in medium containing 20 mM trimethylamine or 40 mM methanol also led to the N-glycosylation of S-layer glycoprotein asparagine-879, a position not modified when the trimethylamine and methanol concentrations were reduced to 10 mM and 20 mM, respectively. In contrast, other sites of S-layer glycoprotein N-glycosylation were unaffected by these shifts in growth conditions, pointing to the specific temperature- and substrate-related nature of N-glycosylation profile modulation.

M. maripaludis also revises the composition of the N-linked glycan decorating its glycoproteins in response to growth temperature changes. When grown at 34 °C, the major archaellin in this methanogen is N-glycosylated by the tetrasaccharide described above, comprising N-acetylglucosamine, di-N-acetylglucuronic acid, 2,3-diamino-2,3-dideoxymannuronic acid amidated with a threonine amino group and a novel terminal sugar, 2-acetamido-2,4-dideoxy-5-O-methyl-hexos-5-ulo-1,5-pyranose [48]. Yet, when grown at 40 °C, *M. maripaludis* fails to add the threonine on the third sugar and lacks the fourth and final sugar [94]. This microorganism can also add different versions of the N-linked glycan to different target proteins. While archaellins are modified by the above-described tetrasaccharide, pilins are modified by the same glycan containing an additional branched hexose [95]. It is still unclear how such protein-specific N-glycosylation is realized.

6. N-glycosylation in archaea as a marker of evolution

When Archaea were first defined on the basis of 16S ribosomal RNA secondary structure, they were assigned to a distinct branch of the universal tree of life [3]. In time, it became clear that Archaea could be divided into the more bacteria-like Euryarcheota and the more eukarya-like Crenarchaeota [12]. As more archaeal genomes were sequenced, novel archaeal phyla were described. Today, Archaea can be divided into four major phylogenetic groups, namely, the DPANN, TACK and Asgard super-phyla and the phylum Euryarchaeota, all defined according to various molecular markers [96–99]. At the same time, phylogenetic evidence further argues that the Asgard archaea and Eukarya arose from a common ancestor, although the relation between the two groups of organisms still requires further investigation [13,100]. While archaeal N-glycosylation has been far less well investigated than have the parallel bacterial and eukaryal systems, evidence showing that aspects of the archaeal process differ along phylogenetic group lines is accumulating, in addition to demonstrating similarites between N-glycosylation in the TACK and Asgard archaea and Eukarya.

Across evolution, N-glycosylation begins with the assembly of a glycan from soluble nucleotide-activated sugars onto a cytoplasm-facing lipid carrier [101]. While phosphorylated unsaturated polyprenol serves as the lipid carrier upon which N-linked glycans are assembled in Bacteria, Archaea and Eukarya instead rely on phosphorylated isoprene-based dolichols for this purpose [33,34,102–104]. In Eukarya, N-linked glycans are assembled on dolichol pyrophosphate, with initial sugars added to the growing glycan originating as soluble precursors and subsequently added sugars being transferred to the growing dolichol pyrophosphate-bound glycan from their own dolichol phosphate carriers [105,106]. In contrast, Archaea use both dolichol phosphate and dolichol pyrophosphate as glycan lipid carrier, with the lipid-bound glycan being assembled from soluble precursors [33,34]. Specifically, dolichol phosphate serves as the glycan lipid carrier in Euryarchaeota [33,34], although, as noted above, *Hbt. salinarum* has been reported to use both dolichol phosphate and dolichol pyrophosphate for this purpose [15,107], and at least one example of augmentation of a protein-bound N-linked glycan by transfer of a dolichol phosphate-bound sugar has been reported [27]. At the same time, Crenarchaeota (part of the TACK super-phylum) employ dolichol pyrophosphate as lipid glycan carrier, like Eukarya [33,34,104]. Moreover, the crenarchaeotes *S. acidocaldarius* and *S. solfataricus* also contain hexose-charged dolichol phosphate which could serve as intermediates in assembly of the dolichol pyrophosphate-bound glycan, although this has yet to be demonstrated [33,108]. In *S. acidocaldarius* and *S. solfataricus* this glycan corresponds to a tri-branched hexasaccharide and a di-branched heptasaccharide, respectively, both based on a chitobiose (i.e. a pair of N-acetylglucosamines) core, as also found at the core of eukaryal N-linked glycans [109,110]. The crenarchaeote *Pyrobaculum calidifontis* presents the most eukaryal-like N-linked glycan yet reported in Archaea, namely, a di-branched glycan comprising nine mannoses and a core of two N-acetylated N-acetylglucosamines [111].

Distinction in aspects of N-glycosylation that reflect phylogenetic divisions of the Archaea can also be seen when one addresses the archaeal oligosaccharyltransferase AglB. Like the bacterial oligosaccharyltransferase PglB and the Stt3 catalytic subunit of the oligosaccharyltransferase complex found in higher eukaryotes, beginning with yeast, AglB also contains a WWDXG motif involved in the mechanism of action of this enzyme. Yet while various residues are found at the variable fourth position of this motif in Euryarchaeota, a tyrosine is found here in AglB from the TACK and Asgard Archaea, just as in eukaryal Stt3 [35]. Likewise, other sequence motifs seem to be common to AglB from the TACK and Asgard Archaea and to eukaryal Stt3, such as the DK motif [112,113] or the DNXTZNXS/T motif [114].

7. Conclusions

As our knowledge of Archaea continues to expand, numerous accepted concepts in various realms of biology, such as evolution, genetics, biochemistry, and cell biology, to name but a few, have been revised. In the specific case of N-glycosylation, despite the relatively little that is known of the archaeal version of this universal post-translational modification at present, it is, nonetheless, clear that members of this domain have developed unique N-glycosylation strategies, pathway steps and substrates. It is thus not unreasonable to expect that continued exploration of archaeal N-glycosylation will provide additional surprises.

CRediT authorship contribution statement

Zlata Vershinin: Writing – review & editing. **Marianna Zaretsky:** Writing – review & editing. **Jerry Eichler:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Jerry Eichler reports financial support was provided by Israel Science Foundation. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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