

Substrate Promiscuity: AglB, the Archaeal Oligosaccharyltransferase, Can Process a Variety of Lipid-Linked Glycans

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Across evolution, N-glycosylation involves oligosaccharyltransferases that transfer lipid-linked glycans to selected Asn residues of target proteins. While these enzymes catalyze similar reactions in each domain, differences exist in terms of the chemical composition, length and degree of phosphorylation of the lipid glycan carrier, the sugar linking the glycan to the lipid carrier, and the composition and structure of the transferred glycan. To gain insight into how oligosaccharyltransferases cope with such substrate diversity, the present study analyzed the archaeal oligosaccharyltransferase AglB from four haloarchaeal species. Accordingly, it was shown that despite processing distinct lipid-linked glycans in their native hosts, AglB from *Haloarcula marismortui*, *Halobacterium salinarum*, and *Haloferax mediterranei* could readily replace their counterpart from *Haloferax volcanii* when introduced into *Hfx. volcanii* cells deleted of *aglB*. As the four enzymes show significant sequence and apparently structural homology, it appears that the functional similarity of the four AglB proteins reflects the relaxed substrate specificity of these enzymes. Such demonstration of AglB substrate promiscuity is important not only for better understanding of N-glycosylation in *Archaea* and elsewhere but also for efforts aimed at transforming *Hfx. volcanii* into a glycoengineering platform.

N-glycosylation is a posttranslational modification that occurs in all three domains of life. In each case, a core or fully assembled glycan is assembled on a phosphorylated polyprenol lipid carrier, namely, dolichol in *Eukarya* and *Archaea* and undecaprenol in *Bacteria*, and transferred to select Asn residues of a target protein by the actions of an oligosaccharyltransferase (OST) (1–4). In higher *Eukarya*, the OST is a multisubunit complex, with the Stt3 (staurosporine- and temperature-sensitive) protein serving as the catalytic subunit (5, 6). In *Bacteria* and *Archaea*, the OST comprises a single subunit, namely, the Stt3 homologues PglB and AglB, respectively (7, 8).

While members of all three domains perform N-glycosylation, the diversity presented by the N-linked glycans added to target proteins varies greatly across evolution. In particular, the N-linked glycans that decorate archaeal glycoproteins offer a degree of architectural and compositional variability not seen in either their bacterial or eukaryal counterparts (4, 9). Archaeal N-glycosylation also presents diversity at the level of the glycan-charged lipid carrier, with both dolichol phosphate (DoIP) and dolichol pyrophosphate (DoIPP) reportedly serving this role (10–15). Moreover, archaeal N-glycosylation relies on several different sugars to provide the link between the oligosaccharide and either the lipid carrier or target protein Asn residues (4). As such, the archaeal OST must cope with a degree of substrate variety not encountered by either its eukaryal or bacterial homologues. Finally, while the presence of a sequon (the motif Asn-X-Ser/Thr, where X is any residue but proline) is required for N-glycosylation, not every sequon in an archaeal (or indeed, eukaryal or bacterial) glycoprotein is modified (16, 17). Thus, AglB must also decide whether or not a given sequon Asn residue in a target protein is processed.

The various options that AglB must consider are further demonstrated when N-glycosylation in the haloarchaea *Haloferax volcanii*, *Haloarcula marismortui*, and *Halobacterium salinarum* is considered. Despite the fact that phylogenetic analysis has shown the similarities of AglB in these organisms (18, 19), the enzyme

processes different substrates in each case. In *Hfx. volcanii*, AglB transfers a DoIP-bound tetrasaccharide comprising a hexose, two hexuronic acids, and a methyl ester of hexuronic acid to target Asn residues (15, 20, 21). In *Har. marismortui*, AglB processes DoIP charged with pentasaccharide corresponding to a similar, if not identical, tetrasaccharide, capped with a terminal mannose (22). In *Hbt. salinarum*, AglB seemingly encounters a more complex scenario. Here, the S-layer glycoprotein is modified by both a glucose-linked sulfated glycan assembled on DoIP and an N-acetylglucosamine-linked repeating sulfated pentasaccharide assembled on DoIPP (10–14). In addition, glycosylation of the Asn-2 position was shown to occur even upon replacement of the native Ser-4 residue of the sequon by Val, Leu, or Asn, suggesting that AglB in this species also recognizes Asn residues that are not part of a sequon (23).

Presently, it is unclear whether such versatility on the part of AglB in these closely related organisms represents species-specific traits that have yet to be identified or whether haloarchaeal AglB is instead a promiscuous enzyme able to work with a variety of glycan-charged lipid substrates. With this in mind, AglB from *Hfx. volcanii*, *Har. marismortui*, *Hbt. salinarum*, and *Haloferax mediterranei* were functionally compared in the present study.

MATERIALS AND METHODS

Strains and growth conditions. *Hfx. volcanii* WR536 (H53) parent strain (24) cells were grown in medium containing 3.5 M NaCl, 0.16 M MgSO₄,

Received 23 September 2013 Accepted 31 October 2013

Published ahead of print 8 November 2013

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AEM.03191-13>.

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doi:10.1128/AEM.03191-13

1 mM MnCl₂, 5 mM KCl, 3 mM CaCl₂, 0.3% (wt/vol) yeast extract, 0.5% (wt/vol) tryptone, 50 mM Tris-HCl, pH 7.2 (25). *Hfx. volcanii* Δ aglB strain cells (16) were grown in similar medium containing 0.5% (wt/vol) Casamino Acids instead of yeast extract and tryptone. *Har. marismortui* cells were grown in medium containing 3.6 M NaCl, 0.39 M MgSO₄, 1.5 mM MnCl₂, 3 mM CaCl₂, 1% (wt/vol) yeast extract (26). *Hbt. salinarum* cells were grown in medium containing 3.4 M NaCl, 0.01 M MgSO₄, 26 mM KCl, 10 mM trisodium citrate, 0.3% (wt/vol) yeast extract, 0.5% (wt/vol) tryptone, and trace amounts of metals (ZnSO₄, MnSO₄, FeSO₄, and CuSO₄) dissolved in 0.1 N HCl (27). *Hfx. mediterranei* cells were grown in medium containing 3.3 M NaCl, 0.2 M MgSO₄, 6.7 mM KCl, 6 mM CaCl₂, 0.17 M MgCl₂, 5.6 mM NaBr, 0.5% (wt/vol) yeast extract, pH 7.2 (28). All cells were grown at 42°C.

Plasmid construction. To introduce *Clostridium thermocellum* cellulose-binding domain (CBD)-tagged *Hfx. volcanii*, *Hbt. salinarum*, or *Har. marismortui* AglB into *Hfx. volcanii* Δ aglB cells, plasmid pWL-CBD-AglD (29) was first linearized using NdeI and KpnI, releasing the *aglD* sequence. PCR amplification then was performed using a forward primer spanning the 3' end of the CBD coding sequence and the 5' end of the *aglB* sequence of interest and a reverse primer spanning the 3' end of the *aglB* sequence of interest and the sequence downstream of the KpnI site, together with genomic DNA from the species of interest as the template. The sequences of the primer pairs employed (Hvfor and Hvrev [*Hfx. volcanii* *aglB*], Hsfor and Hsrev [*Hbt. salinarum* *aglB*], or Hmafor and Hmarev [*Har. marismortui* *aglB*]) are listed in Table S1 in the supplemental material, as are all other primers employed in this study. The amplified PCR fragment and the linearized plasmid were joined using an In-Fusion multiple fragment cloning kit (Clontech) according to the manufacturer's instructions to yield the desired construct. To introduce CBD-tagged *Hfx. mediterranei* AglB into *Hfx. volcanii* Δ aglB cells, the *aglB* sequence was PCR amplified from *Hfx. mediterranei* genomic DNA using the primer pair Hmefor and Hmever (*Hfx. mediterranei* *aglB*), designed to introduce NdeI and KpnI sites at the 5' and 3' ends of the amplified sequence, respectively. The amplified fragment was then ligated into plasmid pWL-CBD-AglD pretreated with NdeI and KpnI so as to remove the *aglD* insert.

The various plasmids were introduced into *Hfx. volcanii* Δ aglB cells essentially as described previously (30). To confirm the introduction of DNA encoding the different CBD-tagged haloarchaeal AglB proteins into *Hfx. volcanii* Δ aglB cells, PCR amplifications were performed using primer pairs comprising a forward primer directed against a region within the CBD-encoding sequence and a reverse primer directed against a region within the *aglB* sequence in question (i.e., Hvcdb and Hvaglb [*CBD-Hfx. volcanii* *aglB*], Hscdb and Hsaglb [*CBD-Hbt. salinarum* *aglB*], Hmacdb and Hmaaglb [*CBD-Har. marismortui* *aglB*], or Hmecdb and Hmeaglb [*CBD-Hfx. mediterranei* *aglB*], respectively).

Topology and homology modeling. The topology of the four haloarchaeal AglB proteins was predicted by the TMHMM (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>), PredictProtein (<http://www.predictprotein.org>), SOSUI (<http://bp.nuap.nagoya-u.ac.jp/sosui/>), TopPred (<http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html>), and HMMTOP (<http://www.enzim.hu/hmmtop/>) servers. To predict the structure of each haloarchaeal AglB sequence, homology modeling was performed using Phyre2 software (<http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index>). The template chosen by the software was the structure of the C-terminal domain of *Archaeoglobus fulgidus* AglB (PDB code 3VGP), which was determined to be the highest-scoring model for each of the haloarchaeal AglB proteins. The HHpred program (<http://toolkit.tuebingen.mpg.de/hhpred>) also identified the same template as being the highest-scoring solved structural homologue available. Once homology models were generated using the default settings of the Phyre2 software, these were compared by superimposing each model onto that generated for *Hfx. volcanii* AglB using the UCSF-Chimera program (<https://www.rbvi.ucsf.edu/chimera/index.html>), also using the default settings.

MS. Liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI MS/MS) analysis of total lipid extracts from *Hbt.*

salinarum and *Hfx. mediterranei* was performed as described previously (15), and LC-ESI MS/MS analysis of the *Hfx. volcanii* S-layer glycoprotein also was performed as described previously (31).

Cellulose-based protein purification and immunoblotting. Cellulose-based capture of CBD-tagged AglB proteins was performed as previously described (32). Immunoblotting of the purified protein pool was performed using polyclonal antibodies raised against the *C. thermocellum* cellulose-binding domain (1:10,000; obtained from Ed Bayer, Weizmann Institute of Science) (32). Antibody binding was detected using goat anti-rabbit horseradish peroxidase (HRP)-conjugated antibodies (1:4000; Bio-Rad, Hercules, CA) and an ECL enhanced chemiluminescence kit (Amersham, Buckingham, United Kingdom).

[2-³H]mannose radiolabeling. [2-³H]mannose radiolabeling was performed as described previously (22). *Δ*aglB cells transformed to express the various haloarchaeal CBD-tagged AglB proteins were grown to mid-exponential phase and incubated with 8 μ l [2-³H]mannose (23.8 mCi/mmol; PerkinElmer, Boston, MA) in a volume of 200 μ l for 17 h. The samples were then precipitated with 15% (wt/vol) trichloroacetic acid and examined by SDS-PAGE. The S-layer glycoprotein was identified by Coomassie staining and by fluorography upon exposure to film. To quantitate the level of radioactive mannose incorporation, densitometry was performed using NIH ImageJ software.

RESULTS

Haloarchaea contain a variety of glycan-charged phosphodolichols. To compare AglB from the four haloarchaeal species addressed in this study, a description of the glycan-charged phosphodolichol content of a total lipid extract from each organism, as provided by LC-ESI MS, is first required. Accordingly, since LC-ESI MS analysis of glycan-charged DolP from *Hfx. volcanii* and *Har. marismortui* has been previously reported (15, 22), the present study considered this lipid population in *Hbt. salinarum* and *Hfx. mediterranei*. When the phosphodolichol-bound glycan population of *Hbt. salinarum* was examined, DolP modified by a hexose and a hexuronic acid was observed (Fig. 1A). The same analysis also identified a hexose-charged DolP species that likely corresponds to a precursor of the DolP-charged disaccharide (not shown). LC-ESI MS analysis also identified C₅₅- and C₆₀-DolPP bearing a tetrasaccharide (Fig. 1B) comprising an *N*-acetylhexosamine linked to a hexuronic acid containing methylated hexuronic acid and sulfated *N*-acetylhexosamine branches (Fig. 1C). In addition, it was confirmed that both the α - and the ω -position isoprenes are saturated in *Hbt. salinarum* DolP (Fig. 1D) and DolPP (not shown). LC-ESI MS analysis of an *Hfx. mediterranei* total lipid extract revealed the presence of C₅₀- and C₅₅-DolP modified by two *N*-acetylhexuronic acids (Fig. 2). Here too, the α - and the ω -position DolP isoprenes are saturated (not shown). Thus, these results, together with earlier findings (15, 22), demonstrate that *Hfx. volcanii*, *Har. marismortui*, *Hbt. salinarum*, and *Hfx. mediterranei* AglB each contain distinct glycan-charged lipid carriers.

The four haloarchaeal versions of AglB share structural similarities. To better understand how the four haloarchaeal OSTs would process such different lipid-linked oligosaccharides, the topologies, sequences, and structures of the four proteins were compared with the hope of uncovering species-specific traits. The various topology prediction servers consulted all agreed that the haloarchaeal AglB proteins are transmembrane proteins, containing between 10 and 16 transmembrane domains each. The majority of the servers also predicted the intracellular orientation of the N terminus and the extracellular orientation of the soluble C-terminal catalytic domain in each case. Alignment of the four haloarchaeal AglB sequences revealed that the *Har. marismortui*, *Hbt.*

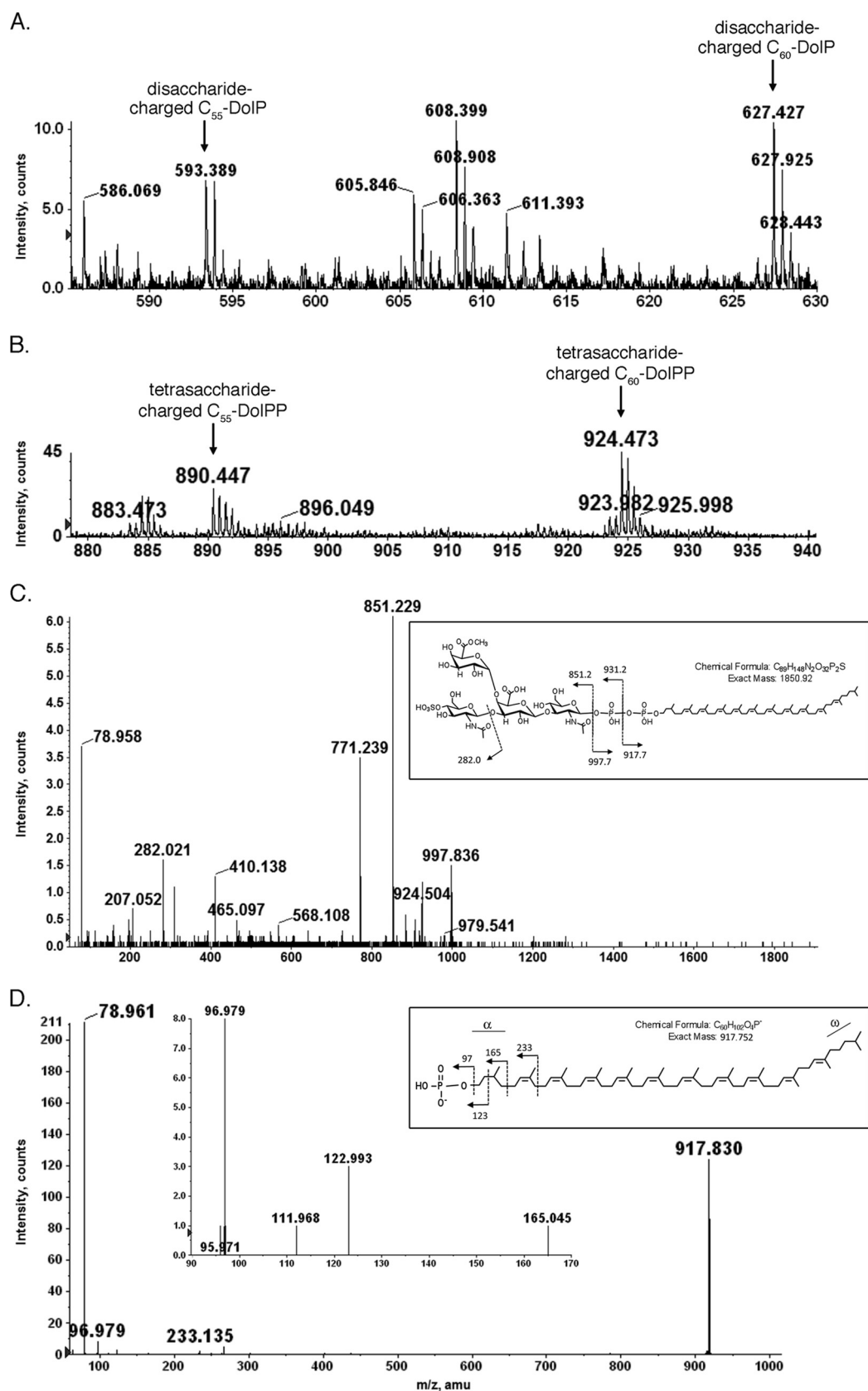


FIG 1 Glycan-charged phosphorylated dolichol species in *Hbt. salinarum*. LC-ESI MS analysis of a total *Hbt. salinarum* lipid extract reveals the presence of C_{55} and C_{60} DolP modified by a disaccharide comprising a hexose and a hexuronic acid (A) and C_{55} and C_{60} DolPP modified by a tetrasaccharide comprising a *N*-acetylhexosamine, a hexuronic acid, a methylated hexuronic acid, and a sulfated *N*-acetylhexosamine (B). In panels A and B, the glycan-charged DolP and DolPP species, respectively, are presented as doubly charged $[M-2H]^{2-}$ ions. (C) MS/MS profile of tetrasaccharide-modified C_{60} DolPP identified in panel B. The inset shows the fragmentation pattern. (D) MS/MS profile of *Hbt. salinarum* C_{60} DolP reveals that both the α - and ω -position isoprene subunits are saturated. The inner profile shows an expanded view of fragments with m/z values between 90 and 170. The boxed inset presents the fragmentation pattern.

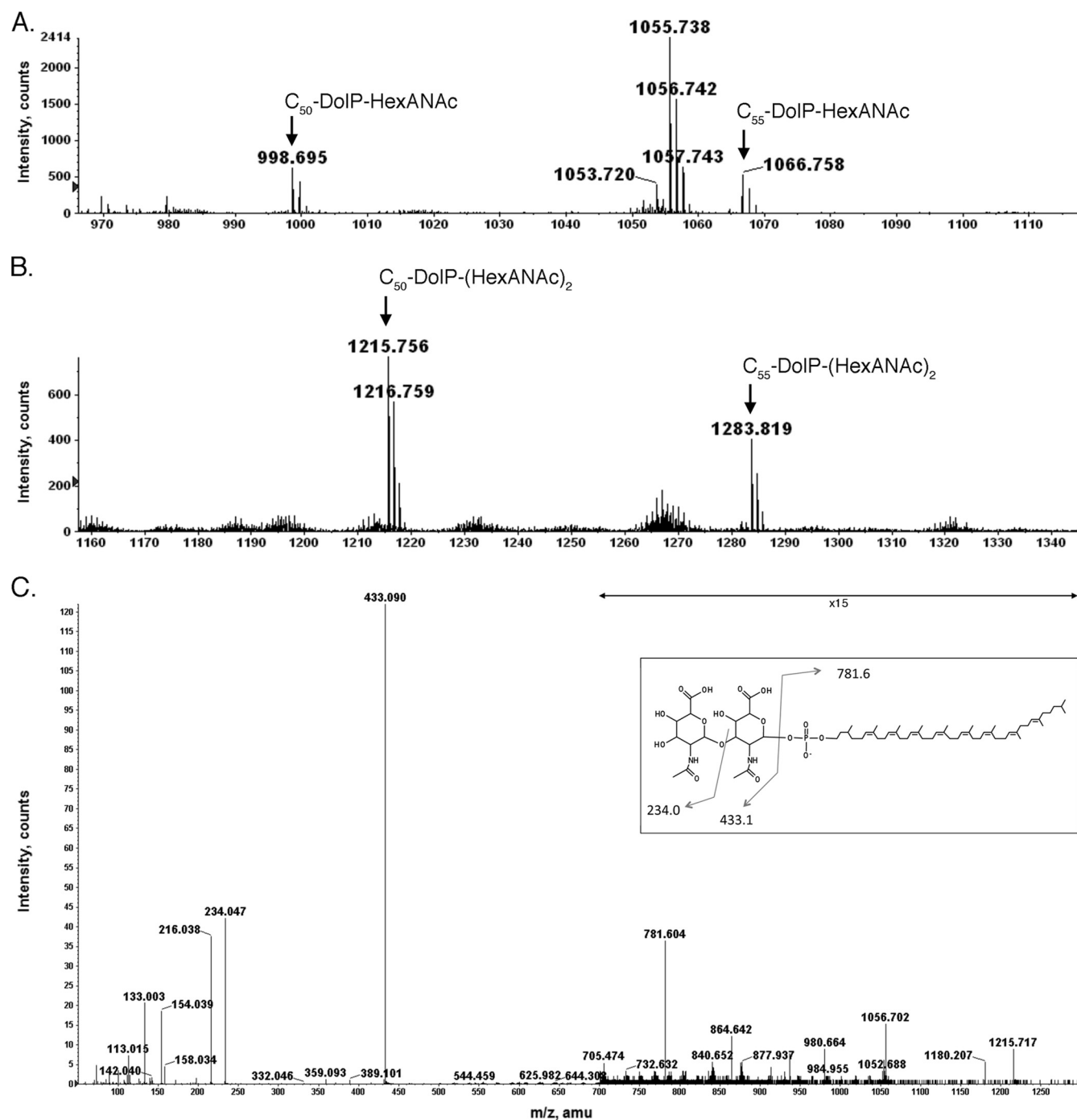


FIG 2 Glycan-charged Dol species in *Hfx. mediterranei*. LC-ESI MS analysis of a total *Hfx. mediterranei* lipid extract reveals the presence of C_{50} and C_{55} DolP modified by one (A) and two (B) *N*-acetylhexuronic acid subunits. The identity of the major peak at m/z 1055.734 is not known. (C) MS/MS profile of di-*N*-acetylhexuronic acid-modified C_{50} DolP identified in panel B. The inset shows the fragmentation pattern. The arrows indicating $\times 15$ reflect magnification of the ion peaks in the corresponding region of the m/z values on the graph.

salinarum, and *Hfx. mediterranei* proteins share 43, 47, and 72% identity, respectively, with *Hfx. volcanii* AglB. The similarities of the four AglB proteins is even more striking when one aligns those motifs implicated in the catalytic activity of the enzyme (Fig. 3A) (7, 33, 34). The DXD motif, found in the first extracellular loop, is essential and thought to bind dolichol (pyro)phosphate via a divalent cation, most commonly Mn^{2+} or Mg^{2+} (35). While the first

Asp is not strictly conserved in all OST proteins, the Asp at the third position is absolutely conserved in all Stt3/PglB/AglB proteins. The four AglB sequences addressed here each contain this motif, assuming the identical GND sequence. The WWDYG motif, important for OST function (6, 7, 36), is also identical in the four haloarchaeal sequences and is assigned to the soluble C-terminal domain in each case. Some differences are, however, de-

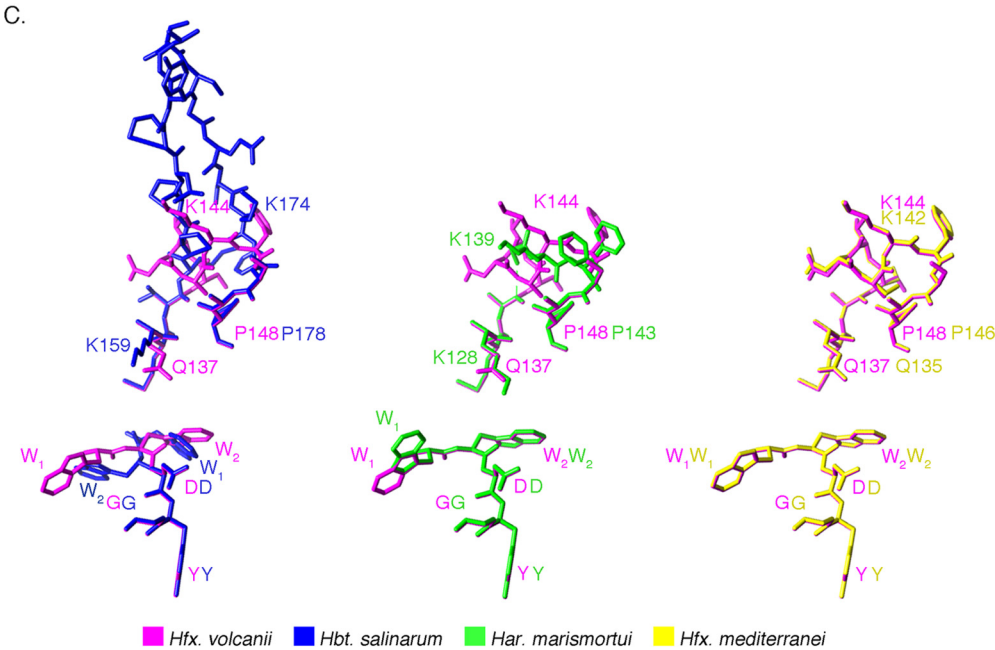
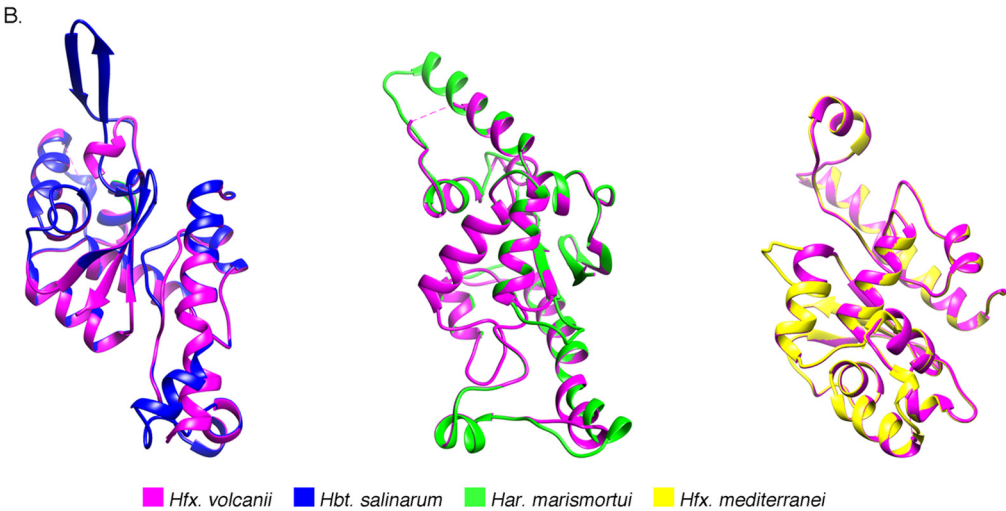
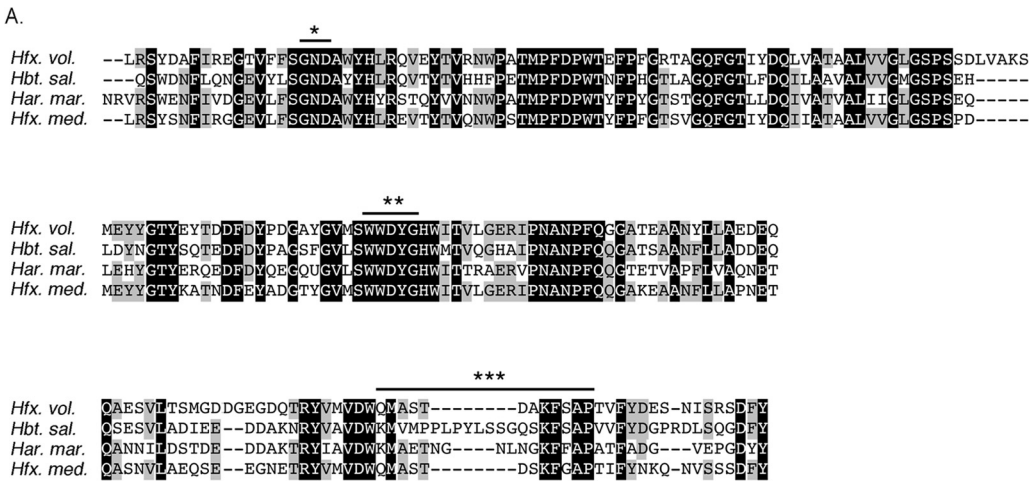


TABLE 1 Quality of homology model generation and superimposition

Structure/model and strain analyzed	Confidence ^a	% identity	No. matched residue pairs	RMSD (Å) ^b
<i>A. fulgidus</i> AglB structure (PDB code 3VGP)				
<i>Hfx. volcanii</i>	100	31		
<i>Hbt. salinarum</i>	100	30		
<i>Har. marismortui</i>	100	32		
<i>Hfx. mediterranei</i>	100	34		
<i>Hfx. volcanii</i> AglB model				
<i>Hbt. salinarum</i> AglB			150	0.26
<i>Har. marismortui</i> AglB			156	0.27
<i>Hfx. mediterranei</i> AglB			167	0.13

^a As described in reference 37.^b RMSD, root mean square deviation.

tected in the DK motifs of the proteins. The DK motif contributes to the formation of the sequon +2 Ser/Thr recognition pocket that guides the side chain carboxamide group of the target Asn residue into the catalytic site, together with the invariable WWD part of the WWDYG motif (34, 36). Although the DK motif is not identical in the four haloarchaeal proteins, with insertions of differing lengths appearing in *Hbt. salinarum* and *Har. marismortui* AglB, consensus of the DK motif is conserved in the four proteins, except for the initial Glu residue. This residue is replaced by Gln in the *Hfx. volcanii* and *Hfx. mediterranei* proteins and is changed to a Lys in *Hbt. salinarum* and *Har. marismortui* AglB.

To compare AglB from the four haloarchaea at the structural level, homology models of the C-terminal soluble domain of each protein were generated. The models obtained for the different AglB proteins are highly similar, with the highest correlation being observed between the models for the two *Haloferax* protein domains (Fig. 3B and Table 1). At the same time, insertions within the DK motif of the *Hbt. salinarum* and *Har. marismortui* proteins led to the most apparent structural differences from the *Hfx. volcanii* AglB domain. A closeup view of the WWDYG and DK motifs (Fig. 3C) further highlighted the strong similarity of AglB in the two *Haloferax* strains, with these motifs predicted to share identical structures. Less correlation was observed between these regions of the *Hfx. volcanii* and *Har. marismortui* proteins. The lowest degree of correlation was observed between the *Hfx. volcanii* and *Hbt. salinarum* models. Still, despite these differences, the four haloarchaeal AglB proteins are predicted to share considerable structural similarity.

***Hfx. volcanii* AglB can be functionally replaced by its haloarchaeal homologues.** Given their sequence and structural similarities, it was next considered whether the ability of the four haloarchaeal AglB proteins to process such different lipid-linked glycans instead reflects relaxed stringency of these enzymes in terms of substrate specificity. Accordingly, the ability of plasmid-

carried *Har. marismortui*, *Hbt. salinarum*, and *Hfx. mediterranei* aglB to complement an *Hfx. volcanii* ΔaglB strain was tested. In each case, a plasmid encoding the nonnative version of AglB bearing an N-terminal *C. thermocellum* CBD tag was introduced into *Hfx. volcanii* ΔaglB cells, and N-glycosylation of the *Hfx. volcanii* S-layer glycoprotein was considered by LC-ESI MS.

Initial efforts verified the introduction of plasmids encoding CBD-tagged AglB from *Hfx. volcanii* and other haloarchaea into *Hfx. volcanii* ΔaglB cells. After having confirmed that aglB had indeed been replaced in the deletion strain by the tryptophan synthase-encoding *trpA* gene (Fig. 4A, first two images from the left), the introduction of a plasmid encoding CBD-tagged AglB from *Hfx. volcanii*, *Hbt. salinarum*, *Har. marismortui*, or *Hfx. mediterranei* into the deletion strain was confirmed by PCR using the appropriate primers (Fig. 4A, third through sixth images from the left). Expression of the plasmid-encoded proteins was then verified by cellulose-based chromatography followed by immunoblotting of any captured proteins using anti-CBD antibodies. Such analysis revealed that CBD-tagged AglB from all four haloarchaea was expressed in the transformed *Hfx. volcanii* ΔaglB cells, although the tagged enzyme from *Hfx. mediterranei* was far better expressed than were the other versions of the protein (Fig. 4B). Indeed, the extract from these cells had to be diluted 100-fold to obtain an antibody-stained band comparable in intensity to those obtained from the same cells transformed to express CBD-tagged AglB from *Hfx. volcanii*, *Hbt. salinarum*, or *Har. marismortui*.

N-glycosylation of the S-layer glycoprotein in the transformed *Hfx. volcanii* ΔaglB cells was considered next. In these studies, LC-ESI MS was employed to examine whether a trypsin-generated S-layer glycoprotein fragment containing Asn-13 from the transformed cells was modified as in the parent strain, where this residue was previously shown to be decorated by a pentasaccharide comprising a hexose, two hexuronic acids, a methyl ester of hexuronic acid, and a mannose (15, 20, 21). LC-ESI MS confirmed that no pentasaccharide-modified Asn-13-containing peptide was present in the deletion strain cells (Fig. 5A), despite the fact that the nonmodified peptide could be readily detected (Fig. 5B). The ability of CBD-tagged *Hfx. volcanii* AglB to restore OST activity to the ΔaglB strain was confirmed, as reflected by MS/MS detection of an m/z 1224.47 $[M + 2H]^{2+}$ ion peak, corresponding to the pentasaccharide-modified S-layer glycoprotein Asn-13-containing fragment (31). Such analysis also revealed the presence of $[M + 2H]^{2+}$ peaks at m/z 1143, 1048, 960, and 872, corresponding to the same peptide modified by the tetra-, tri-, di-, and monosaccharide precursors of the N-linked pentasaccharide (Fig. 5C). This finding demonstrates that the presence of the fused CBD moiety did not interfere with AglB activity.

When the same S-layer glycoprotein-derived peptide from *Hfx. volcanii* ΔaglB cells transformed to express CBD-tagged *Hbt. salinarum*, *Har. marismortui*, or *Hfx. mediterranei* AglB was similarly examined, the same fragmentation pattern was observed as

FIG 3 Comparison of *Hfx. volcanii* (*Hfx. vol.*), *Hbt. salinarum* (*Hbt. sal.*), *Har. marismortui* (*Har. mar.*), and *Hfx. mediterranei* (*Hfx. med.*) AglB sequences and predicted structures. (A) Multiple-sequence alignment of the regions containing the putative XXD, WWDYG, and DK motifs important for AglB function, as performed by ClustalW. Residues common to all four sequences are on a black background, while residues common to three sequences are on a gray background. The three motifs are marked with a bar and asterisks and span the following residues: *Hfx. volcanii* (37 to 119, 614 to 675, and 676 to 727), *Hbt. salinarum* (44 to 120, 640 to 761, and 702 to 760), *Har. marismortui* (40 to 119, 540 to 601, and 602 to 653), and *Hfx. mediterranei* (41 to 118, 618 to 679, and 680 to 729). (B) Homology model of the soluble C-terminal domain of AglB from *Hfx. volcanii* (magenta) with the models of the same domains of *Hbt. salinarum* (blue), *Har. marismortui* (green), and *Hfx. mediterranei* (yellow) AglB superimposed. (C) Closeup view of the superimposed active sites formed by the WWDYG and DK motifs of the AglB proteins, as described for panel B. Selected residues are highlighted for comparison.

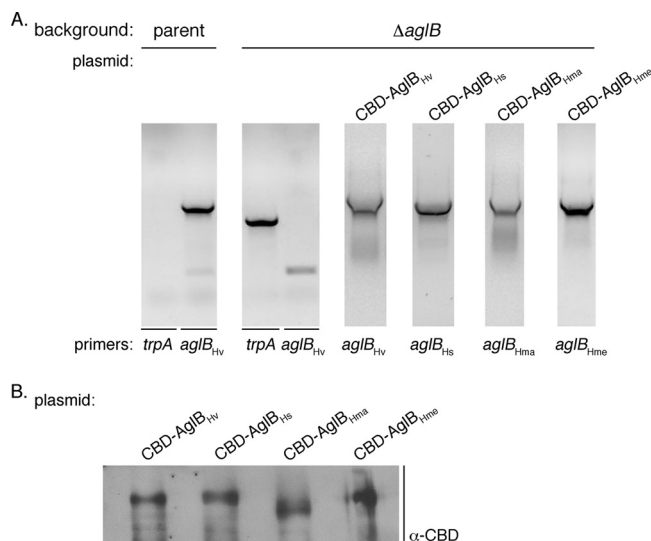


FIG 4 Transformation of *Hfx. volcanii* Δ aglB cells with plasmids encoding CBD-tagged versions of haloarchaeal AglB. (A) A series of PCR amplifications were performed using DNA templates from the *Hfx. volcanii* parent strain (right-most panel) or Δ aglB cells (five other panels) and appropriate primers to confirm the introduction of plasmids of interest into the deletion strain cells. The primers used are listed in Table S1 in the supplemental material. In the right-most panel, genomic DNA from *Hfx. volcanii* Δ trpA cells served as the template in PCR amplifications using primers to trpA (left lane) or aglB (right lane). In the second panel, to confirm replacement of aglB by trpA in the deletion strain, DNA from *Hfx. volcanii* Δ aglB cells served as the template in PCR amplifications using primers to trpA (left lane) or aglB (right lane). Using a forward primer to a region with the CBD-encoding sequence and reverse primers to sequences within *Hfx. volcanii* (Hv; third panel), *Hbt. salinarum* (Hs; fourth panel), *Har. marismortui* (Hma; fifth panel), or *Hfx. mediterranei* (Hme; sixth panel) aglB, together with DNA isolated from *Hfx. volcanii* Δ aglB cells transformed with plasmids carrying CBD-tagged *Hfx. volcanii*, *Hbt. salinarum*, *Har. marismortui*, or *Hfx. mediterranei* aglB, introduction of the non-native DNA into the deletion strain was confirmed. (B) Immunoblotting of total cell extracts (100 μ l) confirms the expression of CBD-tagged *Hfx. volcanii* AglB (131 kDa), *Hbt. salinarum* AglB (132 kDa), *Har. marismortui* AglB (125 kDa), and *Hfx. mediterranei* AglB (130 kDa) in *Hfx. volcanii* Δ aglB cells. Note that the *Hfx. mediterranei* extract is diluted 100-fold.

that in deletion strains transformed to express CBD-tagged *Hfx. volcanii* AglB (Fig. 5D to F). As such, *Hbt. salinarum*, *Har. marismortui*, and *Hfx. mediterranei* AglB can functionally replace their *Hfx. volcanii* counterpart both in terms of the substrate processed and the Asn target modified (Table 2). These observations demonstrate that haloarchaeal AglB proteins display substrate promiscuity.

To assess the relative efficiencies of the different haloarchaeal AglB proteins introduced into *Hfx. volcanii* Δ aglB cells in catalyzing S-layer glycoprotein N-glycosylation, [2-³H]mannose radio-labeling was performed. Since the incorporation of [2-³H]mannose requires prior AglB-mediated transfer of the first four pentasaccharide subunits from DolP to select S-layer glycoprotein Asn residues (15), assessing the extent of S-layer glycoprotein radiolabeling by [2-³H]mannose reflects the relative efficiencies of the different haloarchaeal AglB proteins. Accordingly, equivalent amounts of protein extract from *Hfx. volcanii* Δ aglB cells transformed to express the various CBD-tagged AglB proteins were separated by SDS-PAGE. Coomassie staining revealed that comparable amounts of S-layer glycoprotein were present in the different samples (Fig. 6, upper). At the same time, all incorporated

the radioactive sugar into the N-linked glycan decorating the S-layer glycoprotein, albeit to different extents (Fig. 6, lower). Relative to the efficiency of [2-³H]mannose incorporation presented by cells of the deletion strain expressing CBD-tagged *Hfx. volcanii* AglB (taken as 100%), CBD-tagged *Hbt. salinarum*, *Hfx. mediterranei*, and *Har. marismortui* AglB-expressing *Hfx. volcanii* Δ aglB cells incorporated [2-³H]mannose into the S-layer glycoprotein 86, 70, and 32% as effectively, respectively. If, however, one considers that the *Hfx. mediterranei* enzyme is expressed some 100-fold more than are any of the other versions of AglB introduced into the *Hfx. volcanii* Δ aglB cells, then the efficiency of *Hfx. mediterranei* AglB in the transformed cells would be less than 1%, assuming that the entire *Hfx. mediterranei* AglB population is active in the nonnative host.

DISCUSSION

In all three domains of life, N-linked glycans are initially assembled on phosphorylated polyisoprenoid lipids (38). From here, the glycan is transferred to select target protein Asn residues by the actions of an OST (2–4). In doing so, the archaeal OST, AglB, encounters substrate diversity not seen by either its bacterial or eukaryal counterparts both at the level of the glycan moiety, where unparalleled architectural and compositional variability is encountered (4, 9), and at the level of the lipid carrier, where differences in carrier length, extent of isoprene subunit saturation, degree of phosphorylation, and linking sugar identity arise. In this study, the ability of *Hbt. salinarum*, *Har. marismortui*, and *Hfx. mediterranei* AglB to replace their *Hfx. volcanii* counterpart was shown, despite the fact that each OST processes different substrates in the native host.

The confirmation that DolP- and DolPP-bound glycans are detected in *Hbt. salinarum* (Fig. 1), as well as the ability of *Hbt. salinarum* AglB to replace its *Hfx. volcanii* counterpart (Fig. 5 and 6 and Table 2), are among the more intriguing findings of this study. Presently, it is not clear whether the single AglB encoded by *Hbt. salinarum* is responsible for processing both the DolP- and DolPP-based glycan carriers. If not, then it remains to be determined which lipid-linked glycan is processed by AglB, and more importantly, how the other lipid-linked glycan is delivered to target Asn residues in this haloarchaea. If *Hbt. salinarum* AglB only processes the DolP-bound tetrasaccharide comprising a glucose and three glucuronic acids sulfated at nonspecified positions, then the ability of *Hbt. salinarum* AglB to replace the *Hfx. volcanii* protein is reasonable, given that in *Hfx. volcanii*, AglB delivers a similar tetrasaccharide that includes a hexose as the linking sugar to target Asn residues (15). If, however, *Hbt. salinarum* AglB normally processes the DolPP-linked glycan in the native host, then the ability of this AglB to functionally replace its *Hfx. volcanii* counterpart points to an enzyme that shows promiscuity both in terms of the phosphorylated state of the dolichol carrier and at the level of the linking sugar.

In contrast to *Hbt. salinarum*, nothing is presently known of N-glycosylation or of N-glycoproteins in *Hfx. mediterranei*, although the complete genome of this species was recently reported (39). As such, it can only be assumed at this point that the di-N-acetylhexuronic group found attached to DolP in a total *Hfx. mediterranei* lipid extract is delivered to the S-layer glycoprotein previously reported as being glycosylated (40). If this assumption is valid, then the demonstration that *Hfx. mediterranei* AglB can replace its *Hfx. volcanii* counterpart seems to reflect the ability of

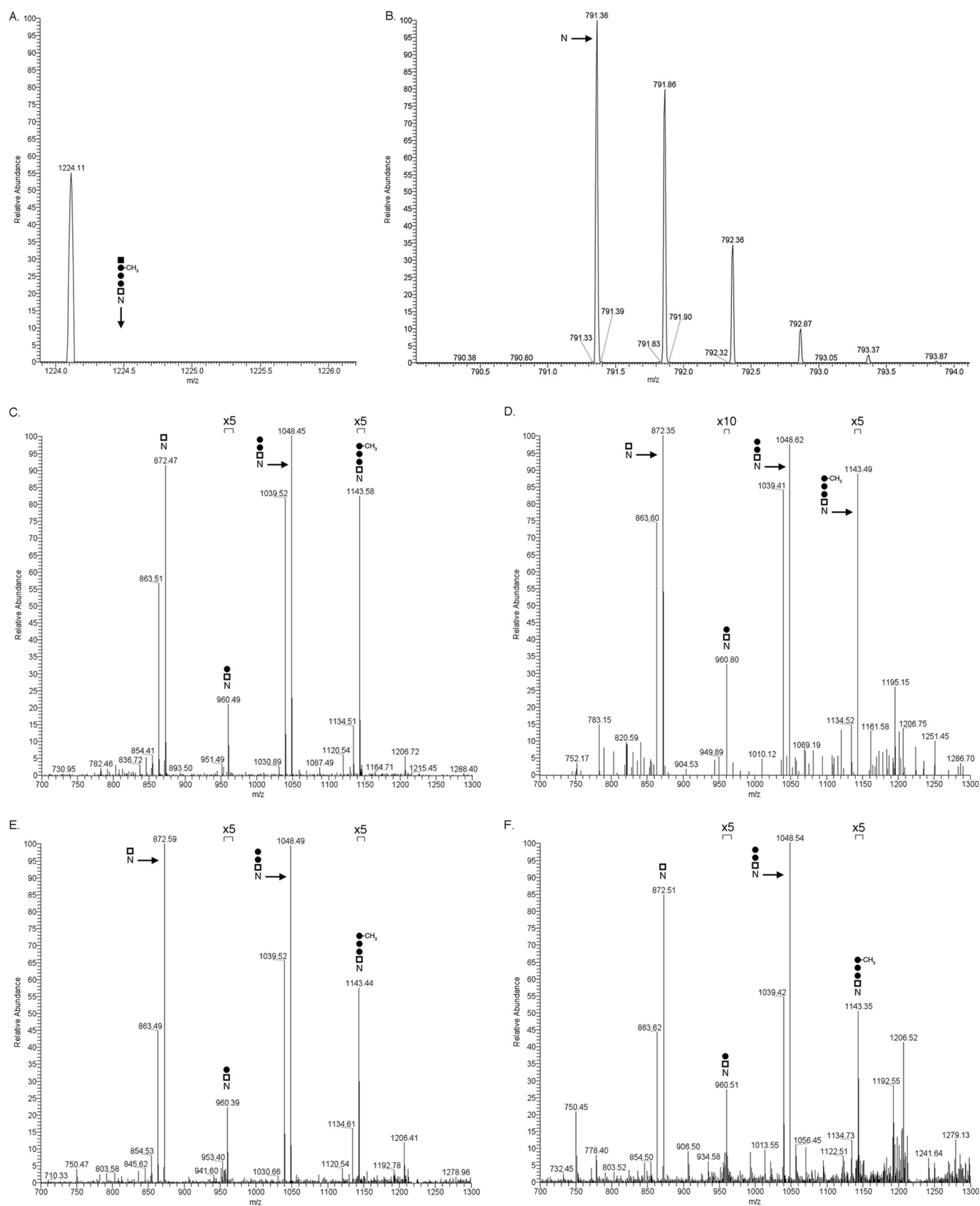


FIG 5 LC-ESI MS analysis of an S-layer glycoprotein Asn-13-containing peptide from *Hfx. volcanii* $\Delta aglB$ cells transformed to express CBD-tagged versions of haloarchaeal AglB. (A) The arrow indicates the expected position of the pentasaccharide-modified peak that is not detected in *Hfx. volcanii* $\Delta aglB$ cells. (B) The indicated monoisotopic $[M + 2H]^{2+}$ peak at m/z 791.36 corresponds to the nonglycosylated Asn-13-containing peptide detected in *Hfx. volcanii* $\Delta aglB$ cells. (C to F) MS/MS analysis of a pentasaccharide-charged peptide (m/z 1224.48) from *Hfx. volcanii* $\Delta aglB$ cells transformed to express CBD-tagged *Hfx. volcanii* (C), *Hbt. salinarum* (D), *Har. marismortui* (E), and *Hfx. mediterranei* AglB (F) identified by LC-ESI MS reveals the presence of mono- (m/z 872), di- (m/z 960), tri- (m/z 1048), and tetrasaccharide-charged (m/z 1143) fragments. The regions indicated by $\times 5$ and $\times 10$ reflect magnification of the ion peaks in the corresponding region of the m/z values on the graph. Symbols: N, Asn-13-containing peptide; open square, hexose; full circle, hexuronic acid; full square, mannose.

TABLE 2 Restoration of AglB function upon introduction of haloarchaeal AglB into *Hfx. volcanii* Δ aglB cells

CBD-tagged protein expressed by transformed <i>Hfx. volcanii</i> Δ aglB	N-glycosylation of <i>Hfx. volcanii</i> S-layer glycoprotein Asn-13	Composition of Asn-13-linked glycan ^a
<i>Hfx. volcanii</i> AglB	Yes	Hex-(HexA) ₂ -HexA(methyl ester)-Hex
<i>Hbt. salinarum</i> AglB	Yes	Hex-(HexA) ₂ -HexA(methyl ester)-Hex
<i>Har. marismortui</i> AglB	Yes	Hex-(HexA) ₂ -HexA(methyl ester)-Hex
<i>Hfx. mediterranei</i> AglB	Yes	Hex-(HexA) ₂ -HexA(methyl ester)-Hex

^a Abbreviations: Hex, hexose; HexA, hexuronic acid; HexA(methyl ester), methyl ester of hexuronic acid.

these extremely similar enzymes (72% identity) to process very different substrates, with hexose and *N*-acetylhexuronic acid acting as the linking sugar in the *Hfx. volcanii* and *Hfx. mediterranei* DolP-linked glycans, respectively. Moreover, the assumed ability of *Hfx. mediterranei* AglB to process a glycan attached to C₅₀- and C₅₅-DolP in the native host and C₅₅- and C₆₀-DolP when introduced into *Hfx. volcanii* points to an additional level of AglB substrate promiscuity, namely, DolP length. Still, it should be stressed that *Hfx. mediterranei* AglB was present at levels some 100-fold higher than that of any other version of AglB introduced into the *Hfx. volcanii* Δ aglB cells, yet this level of the *Hfx. mediterranei* enzyme was only 30% as functionally effective as the native *Hfx. volcanii* enzyme, as reflected by [2-³H]mannose incorporation. As such, and assuming the entire population of introduced *Hfx. mediterranei* AglB to be active, the ability of *Hfx. mediterranei* AglB to replace its *Hfx. volcanii* counterpart is limited. This in turn argues that despite their sequence and predicted structural similarities, the two *Haloferax* enzymes are designed to process very different substrates. Differentiation based on substrate apparently is not related to the different environments the two species inhabit, since identical glycosylation-related gene arrangements are seen in *Hfx. denitrificans*, isolated from a saltern in California, and *Hfx. volcanii*, originally from the Dead Sea, as well as in *Hfx. mediterranei*, first found in a saltern near Alicante, Spain, and *Hfx. mucosum*, originating from Shark Bay, Australia (19), apparently reflecting identical pathways of protein glycosylation in each pair of *Haloferax* species despite their distinct geographic origins.

Of the four haloarchaeal species considered in this study, detailed information on the N-glycosylation process is only available in the case of *Hfx. volcanii*. Here, the Agl (archaeal glycosylation) pathway is responsible for the assembly and attachment of a pentasaccharide N-linked to at least two proteins (4). In *Har. marismortui*, the S-layer glycoprotein is decorated by the same or a very

similar pentasaccharide, although different routes for assembling this glycan are employed by the two species (22). As such, the ability of *Har. marismortui* AglB to replace its *Hfx. volcanii* counterpart is not unexpected, despite the fact that sequence alignment and phylogenetic analysis show AglB from *Hfx. volcanii* and *Har. marismortui* to be the least similar of the four haloarchaeal AglB sequences considered in this study (19).

In addition to exposing the promiscuous nature of haloarchaeal AglB, this study also served to further our understanding of N-glycosylation in this group of *Archaea*. While no genes or proteins involved in N-glycosylation in *Hbt. salinarum* have been identified experimentally, structural and biochemical studies performed some 30 years ago have provided insight into the mechanism of such posttranslational modification in this organism (14). These previous efforts had implicated DolPP, apparently charged with a repeating sulfated pentasaccharide, as a glycan carrier in *Hbt. salinarum*, based on the ability of bacitracin, an antibiotic that interferes with the recycling of polyprenol pyrophosphates, to interfere with N-glycosylation in this species (11, 41). As such, the detection of DolPP bearing a glycan similar to that attached to the Asn-2 position of the *Hbt. salinarum* S-layer glycoprotein offers the first direct evidence for DolPP contributing to N-glycosylation in this organism (or indeed, any archaeon). Still, the LC-ESI MS-based findings reported here describe a glycan of slightly different composition than those previously reported. Unlike the previously described DolPP-bound pentasaccharide comprising sulfated *N*-acetylglucosamine linked to a galacturonic acid subunit containing galactofuranose and *N*-acetylglucosamine-methylated galacturonic acid branches, with the last four sugar subunits being sulfated at undetermined positions (14), the LC-ESI MS/MS analysis performed here instead revealed C₅₅- and C₆₀-DolPP as bearing a tetrasaccharide comprising an *N*-acetylhexosamine linked to a hexuronic acid containing methylated hexuronic acid and sulfated *N*-acetylhexosamine branches (Fig. 1C, inset). In addition, the earlier efforts had reported that select Asn residues of the S-layer glycoprotein and the archaeellin (or the archaeal flagellin [42]) are modified by a tetrasaccharide comprising a glucose and three glucuronic acids sulfated at nonspecified positions and derived from a C₆₀-DolP carrier (15). Moreover, it was previously shown that the DolP-linked glycan is sulfated, implying that such glycan modification occurs at the lipid-linked rather than at the protein-linked stage (12, 43). In the present study, C₅₅- and C₆₀-DolP modified by hexose and by hexose-hexuronic acid, likely precursors of the DolP-bound tetrasaccharide, were detected. The fact that the hexose detected at position one and the hexuronic acid detected at position two of the DolP-bound disaccharide were not methylated or sulfated, respectively, points to these chemical modifications occurring on the lipid-bound sugar rather

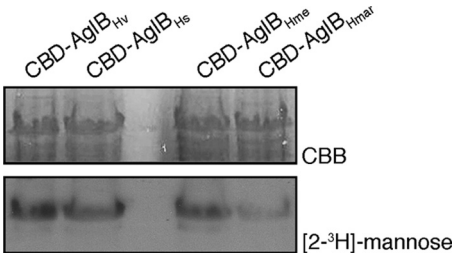


FIG 6 Haloarchaeal AglB proteins replace *Hfx. volcanii* AglB with differing efficiencies. The extent to which *Hfx. volcanii* Δ aglB cells transformed to express CBD-tagged *Hfx. volcanii*, *Hbt. salinarum*, *Hfx. mediterranei*, and *Har. marismortui* AglB incorporate [2-³H]mannose into the N-glycan decorating the S-layer glycoprotein was compared, as revealed by SDS-PAGE and Coomassie brilliant blue staining (CBB; upper) or fluorography ([2-³H]mannose; lower). Shown is a representative of two repeats of the experiment.

than involving the attachment of a methylated hexose to DolP or a sulfated sugar to hexose-charged DolP.

In conclusion, by revealing the ability of AglB from *Hbt. salinarum*, *Har. marismortui*, or *Hfx. mediterranei* to replace their *Hfx. volcanii* counterpart, the present study has demonstrated the relaxed stringency of the enzyme (at least from these haloarchaeal species) with respect to the size and composition of the lipid-charged glycan it processes, as well as to the linking sugar and the length of the dolichol-based glycan carrier substrate. The ability of AglB to process nonnative substrates is, however, not without limit. For instance, *in vitro* studies demonstrated that *A. fulgidus* AglB could not process a lipid-linked oligosaccharide substrate from *Pyrococcus furiosus* or *vice versa*, reflecting specificity of the enzyme in each case (34). Defining the limits of AglB promiscuity may find practical application in on-going efforts aimed at exploiting *Hfx. volcanii* as a platform for glycoengineering (44, 45).

ACKNOWLEDGMENTS

J.E. is supported by the Israel Science Foundation (grant 8/11) and the U.S. Army Research Office (W911NF-11-1-520). The mass spectrometry facility in the Department of Biochemistry of the Duke University Medical Center and Z.G. are supported by LIPID MAPS Large Scale Collaborative Grant number GM-069338 from the NIH.

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