

# N-Glycosylation of *Haloferax volcanii* Flagellins Requires Known Agl Proteins and Is Essential for Biosynthesis of Stable Flagella

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N-glycosylation, a posttranslational modification required for the accurate folding and stability of many proteins, has been observed in organisms of all domains of life. Although the haloarchaeal S-layer glycoprotein was the first prokaryotic glycoprotein identified, little is known about the glycosylation of other haloarchaeal proteins. We demonstrate here that the glycosylation of *Haloferax volcanii* flagellins requires archaeal glycosylation (Agl) components involved in S-layer glycosylation and that the deletion of any *Hfx. volcanii* *agl* gene impairs its swimming motility to various extents. A comparison of proteins in CsCl density gradient centrifugation fractions from supernatants of wild-type *Hfx. volcanii* and deletion mutants lacking the oligosaccharyltransferase AglB suggests that when the Agl glycosylation pathway is disrupted, cells lack stable flagella, which purification studies indicate consist of a major flagellin, FlgA1, and a minor flagellin, FlgA2. Mass spectrometric analyses of FlgA1 confirm that its three predicted N-glycosylation sites are modified with covalently linked pentasaccharides having the same mass as that modifying its S-layer glycoprotein. Finally, the replacement of any of three predicted N-glycosylated asparagines of FlgA1 renders cells nonmotile, providing direct evidence for the first time that the N-glycosylation of archaeal flagellins is critical for motility. These results provide insight into the role that glycosylation plays in the assembly and function of *Hfx. volcanii* flagella and demonstrate that *Hfx. volcanii* flagellins are excellent reporter proteins for the study of haloarchaeal glycosylation processes.

Despite their relatively recent discovery, prokaryotic glycoproteins have already been implicated in a wide range of functions, including pathogenesis, cell protection, motility, transport, and signaling (8, 18, 19, 25, 32, 34–36, 40). As in eukaryotes, examples of both O-linked and N-linked protein glycosylation have been identified in archaea and bacteria (30, 37). However, while researchers have studied the mechanisms of protein glycosylation in bacteria extensively, with a primary focus on O-linked glycosylation, prevalent in bacteria, much less is known about the glycosylation process in archaea. However, this paradigm is now rapidly changing with the elucidation of various archaeal N-glycosylation systems (11, 19, 29).

The surface (S)-layer glycoprotein of the haloarchaeon *Halobacterium salinarum* was the first noneukaryotic N-glycosylated protein to be reported (28), and subsequent efforts revealed that *Hbt. salinarum* flagellins are similarly modified (44). However, details of the molecular aspects of archaeal glycosylation did not surface until years later, with the publication of N-glycosylation studies of methanogenic archaea. Those studies revealed a set of archaeal glycosylation (*agl*) genes encoding enzymes that catalyze the assembly and attachment of N-linked glycans to target proteins (for reviews, see references 11 and 19). AglB, the most conserved of the *agl*-encoded proteins, is an oligosaccharyltransferase (OST), an enzyme that covalently links a fully assembled oligosaccharide to the appropriate asparagine residue of a target protein in the final step of glycan synthesis. Apart from the AglB homologs, little to no homology exists among the Agl components. This lack of homology is easily understood given the extremely diverse nature of the glycans found on glycoproteins produced by very closely related archaeal species. For instance, while the S-layer glycoprotein and flagella of *Methanococcus voltae* are found to be adorned with a trisaccharide [ $\beta$ -ManpNAcA6Thr-(1–4)- $\beta$ -GlcNac3NAcA-(1–3)- $\beta$ -GlcNac-Asn, added sequentially by AglH, AglK/C, and AglA, respectively] (43), the closely related meth-

anogen *Methanococcus maripaludis* has flagellins that are decorated with a tetrasaccharide moiety [Sug-4- $\beta$ -ManpNAcA6Thr-(1–4)- $\beta$ -GlcNac3NAcA-(1–3)- $\beta$ -GlcNac-Asn, added sequentially by an undefined glycosyltransferase, AglO, AglA, and AglL, respectively] (22). Furthermore, a rudimentary analysis of the S-layer glycoprotein of *Hbt. salinarum* revealed its modification by the addition of two vastly different N-linked polysaccharide moieties, along with an O-linked moiety, suggesting that multiple systems of glycosylation can sometimes exist even within the same organism (27).

In the model haloarchaeon *Hfx. volcanii*, 11 Agl components involved in the assembly and attachment of an N-linked pentasaccharide to the *Hfx. volcanii* S-layer glycoprotein have been identified thus far (11). Of these, only the haloarchaeal oligosaccharyltransferase AglB shows significant homology to nonhaloarchaeal Agl components. The characterization of the other Agl proteins has also shed some light on the basic structures of the individual sugars within the *Hfx. volcanii* pentasaccharide, although the identities of the first four sugars in this glycan chain have not been directly identified (3, 4, 21, 26, 46, 47). Like the glycosyltransferases found in other archaeal species, all of these Agl enzymes function through an indirect method of synthesis, adding sugar residues sequentially to a transient lipid carrier (dolichol monophosphate in archaea rather than dolichol diphosphate in eu-

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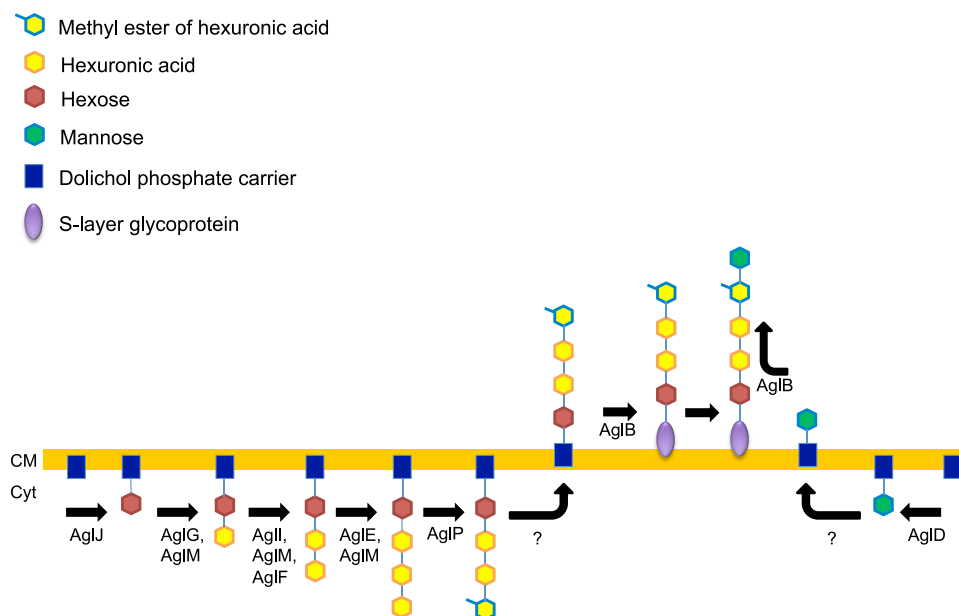
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**FIG 1** *Hfx. volcanii* S-layer glycoprotein N-glycosylation model. The glycosylation mechanism in *Hfx. volcanii* can be described as two distinct processes. The initial process involves the synthesis and transfer of the first four sugar residues in the pentasaccharide. AglJ is the glycosyltransferase responsible for the addition of the hexose at position 1 in the pentasaccharide (21). The addition of the second sugar, a hexuronic acid, at position 2 is mediated by the glycosyltransferase AglG (46). Another hexuronic acid is added at position 3 with the help of AglI. AglM appears to be a UDP-glucose dehydrogenase involved in the conversion of UDP-glucose to UDP-glucuronic acid during the syntheses of the three hexuronic acids at positions 2, 3, and 4. AglF is a glucose-1-phosphate uridylyltransferase that acts in conjunction with AglM to assemble the hexuronic acid at position 3 but plays no role in the addition of hexuronic acid at position 2 or 4 (47). AglE adds another hexuronic acid to position 4 with the help of AglM (2). This hexuronic acid is modified by a methyltransferase, AglP, to a methyl ester (26). This tetrasaccharide serves as the substrate for an unknown flippase that flips the glycan and its dolichol monophosphate carrier from the cytoplasmic leaflet of the cell membrane to the extracellular leaflet. The tetrasaccharide is transferred to its final destination on the S-layer glycoprotein by AglB. A dolichol phosphate mannose synthase, AglD, transfers the mannose, which it synthesizes to a dolichol monophosphate carrier that is separate from the one that holds the tetrasaccharide. Like the tetrasaccharide, this mannose is flipped across the cell membrane by an unknown flippase and then added directly to the tetrasaccharide moiety that had already been attached to the S-layer glycoprotein via the first process described above. AglB is also believed to be involved in the transfer of the final mannose (17). Cyt, cytoplasm. CM, cytoplasmic membrane.

karyotes) before the transfer of the completed glycan by AglB to its final protein target. For a more detailed look at this process, refer to Fig. 1.

Despite the strides made toward a comprehensive understanding of the Agl-mediated glycosylation of the S-layer glycoprotein in *Hfx. volcanii*, this cell wall glycoprotein is large and lacks a testable function, hence limiting the analyses that can be performed. Studies of methanogens have shown that the biosynthesis of their flagella requires the modification of flagellar subunits by the addition of sugar moieties and have indicated that archaeal flagella are a good system with which to study the effects that glycosylation can have on the structure and function of a protein (41). Our preliminary studies have also strongly suggested that the subunits of *Hfx. volcanii* flagella, the flagellins FlgA1 and FlgA2, are glycosylated, as each of these proteins contains predicted glycosylation sites, and deletion mutants lacking AglB are nonmotile (39).

In this study, we investigated the effects of various *agl* gene deletions on the glycosylation of *Hfx. volcanii* flagella. Our results indicate that the same Agl components are involved in the biosynthesis of the pentasaccharide that decorates both the S-layer and the flagella of *Hfx. volcanii* and that *Hfx. volcanii* swimming motility requires that its flagellins be decorated at the targeted sites by a polysaccharide containing at least a subset of the sugars found in the wild-type glycan chain, as has also been observed for the methanogens. We have also shown that a replacement of the asparagine

in any of three predicted N-linked glycosylation sites with a glutamine prevents FlgA1 expressed in *trans* from complementing a chromosomal *flgA1* gene deletion. By expressing these replacement mutants in a strain where all other glycoproteins are properly glycosylated, we were able to demonstrate definitively that the swimming motility and flagellar stability defects observed for *agl* deletion mutants are not due solely to a defective glycosylation of proteins other than the flagellins.

## MATERIALS AND METHODS

**Reagents.** All enzymes used for standard molecular biology procedures were purchased from New England BioLabs, except for iProof High-Fidelity DNA polymerase, which was purchased from Bio-Rad. The ECL Plus Western blotting system detection and horseradish peroxidase-linked sheep anti-mouse antibodies were purchased from Amersham Biosciences. The polyvinylidene difluoride membrane, MF membrane filters (0.025  $\mu\text{m}$ ), and Ultracel-3K membrane were purchased from Millipore. DNA and plasmid purification kits and anti-His antibodies were purchased from Qiagen. NuPAGE gels, buffers, reagents, and the Pro-Q Emerald 300 glycoprotein stain kit were purchased from Invitrogen. Difco agar and Bacto yeast extract were purchased from Becton, Dickinson, and Company. Peptone was purchased from Oxoid. 5-Fluoroorotic acid (5-FOA) was purchased from Toronto Research Biochemicals. All other chemicals and reagents were purchased from either Fisher or Sigma.

**Strains and growth conditions.** The plasmids and strains used in this study are listed in Table 1. *Hfx. volcanii* strains were grown at 45°C in liquid or on solid complex (MGM) (13) or defined (CA) (13) medium.

TABLE 1 Strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Reference or source
<b>Plasmids</b>		
pTA131	Amp <sup>r</sup> ; pBluescript II with BamHI-XbaI fragment from pGB70 harboring <i>pfdx-pyrE2</i>	7
pRV1- <i>ptna</i>	Amp <sup>r</sup> Nov <sup>r</sup> ; inducible <i>ptna</i> promoter	24
pTA963	Amp <sup>r</sup> , <i>pyrE2</i> and <i>hdrB</i> markers, inducible <i>ptna</i> promoter	5
pUC19	Amp <sup>r</sup>	45
pMT7	pRV1- <i>ptna</i> carrying <i>flgA1</i> -His	This study
pMT8	pRV1- <i>ptna</i> carrying <i>flgA2</i> -His	This study
pMT9	pTA963 carrying <i>flgA1</i> -His	This study
pJY1	pTA131 carrying chromosomal flanking regions of <i>flgA1</i>	This study
pST1	pTA963 carrying <i>flgA1</i> -His with an N70Q mutation	This study
pST2	pTA963 carrying <i>flgA1</i> -His with an N115Q mutation	This study
pST3	pTA963 carrying <i>flgA1</i> -His with an N172Q mutation	This study
pST4	pTA963 carrying <i>flgA1</i> -His with N70Q, N115Q and N172Q mutations	This study
<b>Strains</b>		
DH5α	<i>E. coli</i> F <sup>-</sup> φ80d <i>lacZ</i> ΔM15 ( <i>lacZYA-argF</i> )U169 <i>recA1 endA1 hsdR17</i> (r <sub>K</sub> <sup>-</sup> m <sub>K</sub> <sup>-</sup> ) <i>phoA supE44 thi-1 gyrA96 relA1</i>	Invitrogen
DL739	<i>E. coli</i> MC4100 <i>recA dam-13::Tn9</i>	10
H53	<i>Hfx. volcanii</i> Δ <i>pyrE2</i> Δ <i>trp</i>	7
MT2	H53 Δ <i>flgA1</i> Δ <i>flgA2</i>	39
MT13	H53 containing pTA963	This study
MT14	H53 Δ <i>flgA1</i>	This study
MT15	MT14 containing pTA963	This study
MT16	MT14 containing pMT9	This study
Δ <i>aglB</i>	H53 Δ <i>aglB</i>	1
Δ <i>aglJ</i>	H53 Δ <i>aglJ</i>	33
Δ <i>aglG</i>	H53 Δ <i>aglG</i>	46
Δ <i>aglM</i>	H53 Δ <i>aglM</i>	47
Δ <i>aglF</i>	H53 Δ <i>aglF</i>	46
Δ <i>aglI</i>	H53 Δ <i>aglI</i>	46
Δ <i>aglE</i>	H53 Δ <i>aglE</i>	3
Δ <i>aglP</i>	H53 Δ <i>aglP</i>	26
Δ <i>aglD</i>	H53 Δ <i>aglD</i>	1
JY1	H53 Δ <i>aglB</i> containing pMT7	This study
JY2	H53 Δ <i>aglJ</i> containing pMT7	This study
JY3	H53 Δ <i>aglG</i> containing pMT7	This study
JY4	H53 Δ <i>aglM</i> containing pMT7	This study
JY5	H53 Δ <i>aglF</i> containing pMT7	This study
JY6	H53 Δ <i>aglI</i> containing pMT7	This study
JY7	H53 Δ <i>aglE</i> containing pMT7	This study
JY8	H53 Δ <i>aglP</i> containing pMT7	This study
JY9	H53 Δ <i>aglD</i> containing pMT7	This study
JY10	H53 Δ <i>aglB</i> containing pMT8	This study
JY11	H53 Δ <i>aglJ</i> containing pMT8	This study
JY12	H53 Δ <i>aglG</i> containing pMT8	This study
JY13	H53 Δ <i>aglM</i> containing pMT8	This study
JY14	H53 Δ <i>aglF</i> containing pMT8	This study
JY15	H53 Δ <i>aglI</i> containing pMT8	This study
JY16	H53 Δ <i>aglE</i> containing pMT8	This study
JY17	H53 Δ <i>aglP</i> containing pMT8	This study
JY18	H53 Δ <i>aglD</i> containing pMT8	This study
ST1	MT14 containing pST1	This study
ST2	MT14 containing pST2	This study
ST3	MT14 containing pST3	This study
ST4	MT14 containing pST4	This study

Plates contained 1.5% agar, unless mentioned otherwise. To ensure equal concentrations of agar in all plates, the agar was completely dissolved prior to autoclaving, and the autoclaved medium was stirred before plates were poured. *Hfx. volcanii* strain H53 (7) was grown in CA medium supplemented with tryptophan and uracil (50-μg/ml final concentration) or in MGM medium without any supplements. For the selection of the

Δ*flgA1* (Hvo\_1210) deletion mutant (see below), 5-FOA was added at a final concentration of 150 μg/ml in CA medium, and uracil was added at one-fifth its normal concentration, i.e., a 10-μg/ml final concentration, during 5-FOA selection. *Hfx. volcanii* strain H53 and the Δ*flgA1* and Δ*agl* mutants transformed with pRV1-*ptna* (24) were grown in medium supplemented with novobiocin (2-μg/ml final concentration in plates and

1- $\mu$ g/ml final concentration in liquid). To induce expression from plasmid pRV1-*ptna*, tryptophan was added to the medium (100- $\mu$ g/ml final concentration) approximately 1 h before the cells were harvested. The same strains transformed with pTA963 were grown in CA medium supplemented only with tryptophan. *Escherichia coli* strains were grown at 37°C in NZCYM medium supplemented with ampicillin (200  $\mu$ g/ml), when necessary (9).

**Generation of a chromosomal *flgA1* deletion.** Chromosomal deletions were generated by using a homologous recombination (pop-in pop-out) method previously described by Allers and Ngo (6). Plasmid constructs for use in the pop-in pop-out knockout process were generated by using overlap PCR as described previously by Tripepi et al. (39) (primers used are listed in Table S1 in the supplemental material). To confirm the chromosomal replacement event at the proper location on the chromosome, colonies derived from these techniques were screened by PCR (primers used are listed in Table S1 in the supplemental material). The identities of the PCR products were verified by sequencing using the same primers. The *flgA1* deletion mutant generated in strain H53 was designated MT14.

**Motility assay.** The motility assay was performed on motility plates (0.3% agar in MGM or CA medium). Agar media were supplemented with tryptophan, uracil, and/or novobiocin, depending on the strain being assayed. A toothpick was used to stab inoculate the agar, and halo sizes around the inoculation site were measured.

**Construction of expression vectors encoding proteins with C-terminal His tags.** Gene fusions that encode flagellins containing a C-terminal His tag were created; the inducible tryptophanase promoter (*tna*) (24) drove the expression of these gene fusions. The genes encoding FlgA1-His and FlgA2-His were cloned in a manner similar to that used for FlgA1/2-His, which was described previously by Tripepi et al. (39). Primers used specifically for the construction of pMT7 and pMT8 (pRV1-*ptna* expressing C-terminally His-tagged FlgA1 and FlgA2, respectively) are listed in Table S1 in the supplemental material.

pMT7 and pMT8 were isolated from *E. coli* DH5 $\alpha$  and transformed into *E. coli* DL739 (Table 1). Using the standard polyethylene glycol (PEG) (13), nonmethylated plasmid DNA isolated from *E. coli* DL739 was used to transform *Hfx. volcanii* strain H53.

**Site-directed mutagenesis of the glycosylation sites of FlgA1.** To generate point mutations in *flgA1*, encoding the FlgA1 protein, the QuikChange site-directed mutagenesis protocol (Stratagene) was used with modifications that are described below. The fragment encoding FlgA1-His was excised from pMT7 and cloned into pUC19 (45). The plasmid was then amplified by using three sets of oligonucleotide primers, each containing a point mutation that resulted in the replacement of an asparagine with a glutamine at positions N70Q, N115Q, and N172Q, respectively (see Table S1 in the supplemental material). We also generated a plasmid containing all three mutations using a series of PCRs on the mutated templates. PCR amplification of the pUC19 insert containing the sequence encoding the FlgA1-His fragment was performed by using Phusion high-fidelity DNA polymerase, which has a non-strand-displacing action and incorporates the mutagenic primers, resulting in a nicked circular strand. The methylated nonmutated parental DNA template was digested by using the restriction enzyme DpnI, upon which the remaining mutated DNA was transformed into *E. coli* DH5 $\alpha$ . Plasmid DNA was then isolated from transformed cells and sequenced to test for the presence of the point mutations. The mutated fragments were then excised from pUC19, cloned into pTA963 (5), and transformed into *E. coli* DH5 $\alpha$  strains. The plasmids were isolated from the DH5 $\alpha$  strains, transformed into *E. coli* DL739 strains, and finally isolated from strain DL739 and transformed into *Hfx. volcanii* H53.

**Growth curves.** Growth curves were generated by using a Biotek Power Wave X2 microplate spectrophotometer. *Hfx. volcanii* wild-type strain H53 and the *agl* deletion strains were incubated in MGM medium with continuous shaking at 45°C. Approximately 6  $\mu$ l of each culture (adjusted slightly for optical density [OD] differences) was transferred

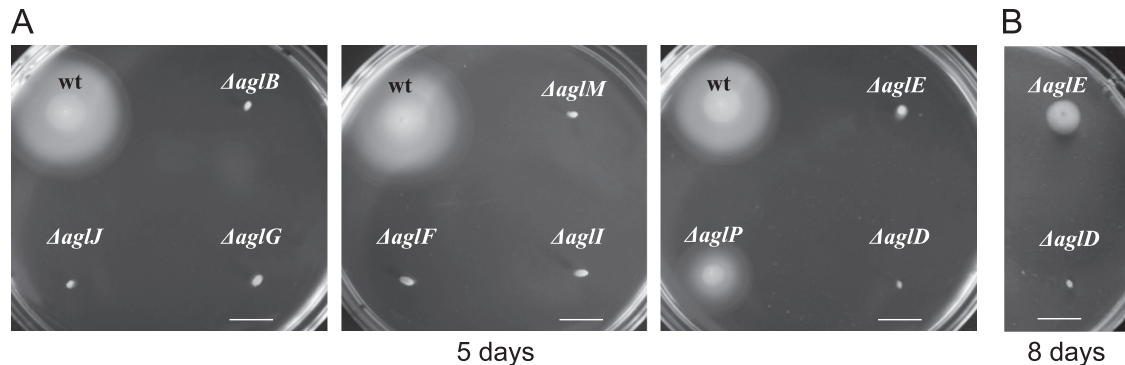
into 194  $\mu$ l of fresh medium and grown to the stationary phase, and OD recordings were taken every 30 min. The growth curves represent an average of cell densities determined in four independent experiments. Each experiment was performed on 10 different strains with eight replicates each, for a final total of 32 replicates per strain.

**Protein extraction, LDS-PAGE, and Western blotting.** Liquid cultures were grown until the mid-log phase (OD at 600 nm [OD<sub>600</sub>] of ~0.5). Subsequently, cells were collected by centrifugation at 4,300  $\times$  g for 10 min at 4°C. Cell pellets were resuspended and lysed in 1% NuPAGE lithium dodecyl sulfate (LDS) supplemented with 50 mM dithiothreitol (DTT) at -20°C. The supernatants of relevant strains were recentrifuged at 4,300  $\times$  g for 10 min to remove cellular contamination, secreted proteins were precipitated with cold trichloroacetic acid (TCA) (10%, vol/vol), and pellets were washed twice with cold acetone (80%, vol/vol) and then resuspended in 1% LDS buffer supplemented with 50 mM DTT at -20°C. The electrophoresis of the protein samples was performed with Bis-Tris NuPAGE gels (Invitrogen) under denaturing conditions by using morpholinepropanesulfonic acid (MOPS) at pH 7.7. Proteins were transferred from the gel onto a polyvinylidene difluoride membrane by using a Bio-Rad Transblot-SD semidry transfer cell at 15 V for 30 min. Western blots of whole-cell lysates of *Hfx. volcanii* strains expressing His-tagged constructs were probed with an anti-His antibody at a dilution of 1:1,000, followed by a secondary anti-mouse antibody at a dilution of 1:10,000. Antibody-labeled protein bands were identified by using the Amersham ECL Plus Western blotting detection system.

**Isolation and purification of flagella.** The isolation of flagella was performed as described previously by Fedorov et al. (14), with modifications described below. To select for motile cells, colonies from a solid-agar plate were stab inoculated twice onto motility plates and then inoculated into 5 ml MGM liquid medium. Two liters of MGM medium were inoculated with this 5-ml culture, and the cultures were harvested at an OD<sub>600</sub> of approximately 0.2 by centrifugation at 8,700 rpm (JA-10 rotor; Beckman) for 30 min. The supernatant was centrifuged again (8,700 rpm for 30 min) and incubated at room temperature with 4% (wt/vol) PEG 6000 for 1 h. The PEG-precipitated proteins were then centrifuged at 16,000 rpm (JLA-16.250 rotor; Beckman) for 50 min at 4°C, and the flagella were purified by cesium chloride (CsCl) density gradient centrifugation (overnight centrifugation at 50,000 rpm) (VTI-65.1 rotor; Beckman). CsCl was dissolved in a 3 M NaCl saline solution to a final density of 1.37 g/cm. For analysis by LDS-polyacrylamide gel electrophoresis (PAGE) and Coomassie brilliant blue staining, CsCl gradient fractions were dialyzed against water. Glycosylation was tested by using the Pro-Q Emerald 300 glycoprotein gel and blot stain kit (Invitrogen).

**Mass spectrometry analysis.** The chosen protein bands were manually excised from the gels and subjected to in-gel digestion with either trypsin or the endoproteinase GluC or double digestion with both enzymes. Tryptic peptide extracts were analyzed by using a Thermo LCQ Deca XP Plus tandem mass spectrometer coupled to an LC Packings Ultimate Nano liquid chromatography system controlled by Thermo Xcalibur 2.0 software. Nano-liquid chromatography-tandem mass spectrometry (LC-MS/MS), autosampling, and chromatography were performed essentially as described previously (31). SEQUEST searches against the *Hfx. volcanii* protein database (NCBI) were accomplished by using Thermo Bioworks 3.3 software, and results were filtered by using standard values for  $X_{\text{corr}}$  (cross-correlation score) and  $\Delta\text{CN}$  (delta-correlation score) for the preliminary assignment (31). Three missed cleavages were allowed. Following automatic assignment, results were manually validated to assess whether the theoretical y- and b-ion profiles bore a high degree of similarity to the experimental MS/MS spectrum. Those that lacked pairs of corresponding y or b ions were assigned as false positives. Modified peptides were manually inspected.

**Electron microscopy.** Fifteen-microliter samples of CsCl gradient fractions were applied onto copper grids coated with carbon-Formvar. The grids were left for 30 min at room temperature, washed with water, blotted with a filter paper, and stained with 2% uranyl acetate for 1 min.



**FIG 2** Deletion of any of the known *Hfx. volcanii* *agl* genes interferes with swimming motility. (A) *Hfx. volcanii* H53 (wild type [wt]) and  $\Delta$ *agl* strains were stab inoculated into 0.3% agar in complex medium (MGM) and incubated at 45°C for 5 days. (B) For the *Hfx. volcanii*  $\Delta$ *aglE* strain, a motility halo is clearly visible after 8 days of incubation. A plate lacking the wild-type stab was used for this longer incubation, as the wild-type motility halo would have migrated across much of the plate at this time (horizontal bar, 1 cm). All inoculations were repeated at least three times for the verification of the phenotype.

Grids were then analyzed by using a Philips Tecnai 12 instrument operating at 120 kV and a Gatan US1000 2K-by-2K (2,024- by 2,024-pixel) camera.

## RESULTS

***Hfx. volcanii* motility is compromised in  $\Delta$ *agl* strains.** We previously showed that an *Hfx. volcanii* strain lacking the oligosaccharyltransferase AglB is nonmotile (39). To determine whether the eight other Agl components known to be involved in the glycosylation of the *Hfx. volcanii* S-layer glycoprotein (11) (Fig. 1) are also required for *Hfx. volcanii* swimming motility, we assayed the motilities of  $\Delta$ *aglJ*,  $\Delta$ *aglG*,  $\Delta$ *aglM*,  $\Delta$ *aglF*,  $\Delta$ *aglI*,  $\Delta$ *aglE*,  $\Delta$ *aglP*, and  $\Delta$ *aglD* strains. As shown previously, when H53 (the parent strain) is stab inoculated, robust halos form on MGM–0.3% agar motility plates after 5 days of incubation at 45°C, while  $\Delta$ *aglB* cells are nonmotile, with growth being restricted to a small dense region at the stab site (Fig. 2A). We determined that all eight additional *agl* mutant strains examined in this study exhibited defective or limited motility. Specifically, motility halos were not observed for stab-inoculated  $\Delta$ *aglJ*,  $\Delta$ *aglG*,  $\Delta$ *aglM*,  $\Delta$ *aglF*, or  $\Delta$ *aglI* strains, even after 10 days of incubation, indicating that the glycosylation of the flagellins and/or other glycoproteins required for flagellum biosynthesis with moieties containing only one or two sugars is not sufficient to produce functional flagella (Fig. 2A). Conversely, although the *Hfx. volcanii*  $\Delta$ *aglE* strain exhibited little motility after 5 days of incubation (Fig. 2A), a notable halo was observed after an additional 3 days (i.e., 8 days total) (Fig. 2B), suggesting that the addition of moieties containing the first three sugars of the pentasaccharide to the flagellins and/or flagellar biosynthesis proteins is sufficient for the assembly of functional flagella. Even more striking,  $\Delta$ *aglP* cells generated motility halos with diameters that were at least half of those of H53 halos. As the methylation of the fourth sugar by the methyltransferase AglP is essential to the addition of the fifth and final sugar to the polysaccharide moiety (see below) (26), it appears that this mannose is not required for *Hfx. volcanii* motility. However, the  $\Delta$ *aglD* strain, in which the fourth sugar of the moiety modifying the flagellins is presumably methylated, is surprisingly nonmotile (Fig. 2A). Perhaps, altering the conformation of the structure associated with this tetrasaccharide, via the methylation of the fourth sugar, inhibits the formation of functional flagella.

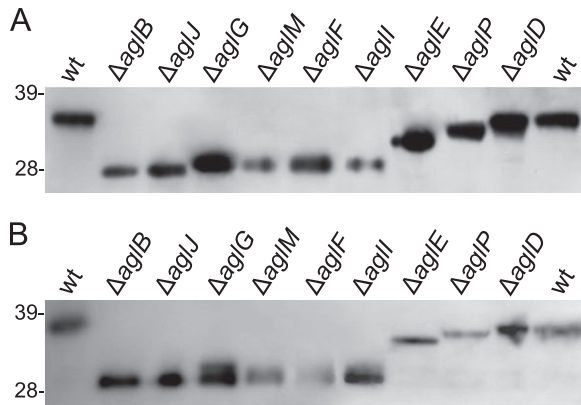
All deletion strains remained viable through 10 days of incu-

bation on motility plates, as determined by streaking them onto new agar plates, ruling out cell death as the cause of the motility defects, and none of the *agl* deletion mutants displayed an obvious growth defect when streaked onto solid medium (see Fig. S1A in the supplemental material). However, since the motility assays were carried out with semisolid media, we also compared the growth rates of the *agl* deletion strains and the H53 parent strain in liquid media. Interestingly, while all nonmotile *agl* mutants, except for  $\Delta$ *aglD*, have doubling times in MGM liquid medium that are similar to that of H53, the  $\Delta$ *aglE* and  $\Delta$ *aglP* strains exhibited growth defects (see Fig. S1B in the supplemental material).

**Flagellins expressed in most  $\Delta$ *agl* strains migrate faster than those expressed in H53 under PAGE.** To obtain evidence for the correlation between *Hfx. volcanii* swimming motility and flagellin glycosylation, we investigated the glycosylation status of flagellins expressed in the various  $\Delta$ *agl* strains based on their migration rates under LDS-PAGE. Although the expected molecular masses of His-tagged FlgA1 (FlgA1-His) and FlgA2 (FlgA2-His) are approximately 25 kDa and 24 kDa, respectively, the LDS-PAGE migration of these proteins corresponded to apparent masses of 36 kDa and 38 kDa, respectively (39) (Fig. 3). These flagellins are composed of a high percentage of negatively charged amino acids compared to their nonhalophilic counterparts, perhaps partially explaining the slower-than-expected migration of these proteins (15, 23). However, the unusually large shift in the expected migration rates of these two proteins is also consistent with glycosylation at their potential N-glycosylation sites (39).

Indeed, Western blot analysis revealed that FlgA1-His and FlgA2-His isolated from an *Hfx. volcanii*  $\Delta$ *aglB* mutant migrated significantly faster than the same proteins expressed in H53 (FlgA1-His and FlgA2-His isolated from the  $\Delta$ *aglB* strain migrate at close to 28 kDa and 30 kDa, respectively) (Fig. 3).

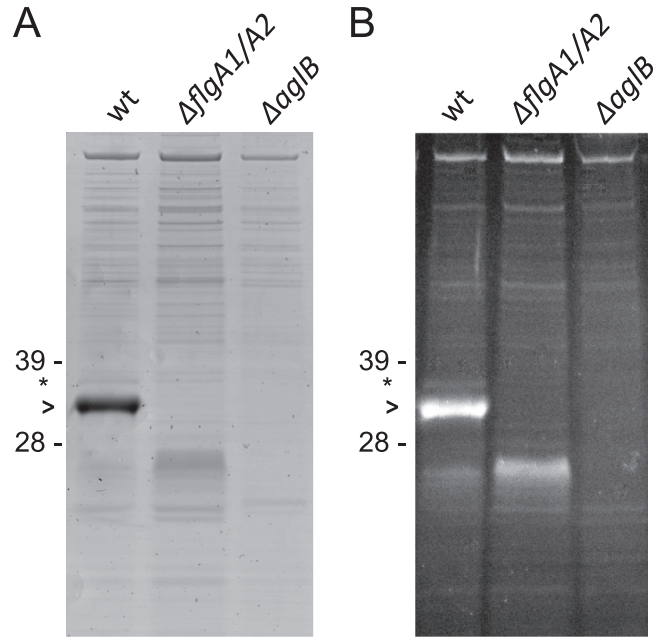
We also determined the migration rates of FlgA1-His isolated from other  $\Delta$ *agl* strains. Western blot analysis of proteins isolated from these strains following separation by LDS-PAGE showed that the FlgA1-His expressed in all but the  $\Delta$ *aglD* mutant strain migrates faster than when expressed in the wild-type strain. For example, FlgA1 expressed in a strain that lacks AglJ, which catalyzes the addition of the first sugar of the pentasaccharide, migrates at the same rate as that of the flagellin expressed in the  $\Delta$ *aglB* strain, suggesting that the lack of the first sugar prevents the addi-



**FIG 3** Flagellins expressed in *Hfx. volcanii*  $\Delta agl$  strains other than the  $\Delta aglD$  strain migrate faster than when expressed in the wild type. Western blot analysis was performed on protein extracts from *Hfx. volcanii* H53 (wild type) or  $\Delta agl$  strains grown to the mid-log phase in MGM medium and expressing either FlgA1-His (A) or FlgA2-His (B), encoded by genes expressed under the regulation of a *trp*-inducible promoter. His-tagged proteins were detected by using anti-His antibodies. Comparable amounts of protein were loaded into each lane of panel A as well as panel B. There was twice as much sample loaded for expression in panel B as in panel A, since FlgA1-His appears to be more abundant than FlgA2-His in the cell extracts. The migration of molecular mass standards is indicated on the left (in kDa).

tion of the others, as was observed for the glycosylation of the S-layer glycoprotein (Fig. 3A) (21). The migration rate of FlgA1 does not appear to increase linearly as the number of sugars added increases (Fig. 3A). For example, a small decrease in the LDS-PAGE migration rate of FlgA1-His expressed in the  $\Delta aglG$ ,  $\Delta aglM$ ,  $\Delta aglF$ , and  $\Delta aglI$  strains was observed compared to the migration of the same protein isolated from the  $\Delta aglJ$  and  $\Delta aglB$  strains. Conversely, FlgA1-His expressed in either the *Hfx. volcanii*  $\Delta aglE$  or  $\Delta aglP$  strain migrated significantly more slowly than the same protein isolated from the  $\Delta aglG$ ,  $\Delta aglM$ ,  $\Delta aglF$ , and  $\Delta aglI$  strains. This, as well as the lack of a size shift of flagellins expressed in the  $aglD$  deletion mutant, may be due to factors other than size, such as the pI of the protein. It should be noted that the anti-His antibody detected two protein bands, migrating as a doublet, in extracts isolated from several *agl* strains expressing either FlgA1-His or FlgA2-His in *trans*, suggesting that the flagellins may undergo a second type of modification in addition to N-glycosylation.

While the data presented above suggest that FlgA1 and FlgA2 contain the same pentasaccharide as the S-layer glycoprotein and that the components involved in the addition of the pentasaccharide to S-layer glycosylation are also required for flagellin glycosylation, it is possible that FlgA1 and FlgA2 contain additional sugars that are not added to the S-layer glycoprotein. Therefore, we set out to identify a protocol that would allow us to purify *Hfx. volcanii* flagella for mass spectrometry analyses. Two-liter cultures of a wild-type strain and a strain lacking the coregulated genes encoding the two *Hfx. volcanii* flagellins, *flgA1* and *flgA2*, were each spun down, and the supernatants, upon PEG precipitation, were separated by using cesium chloride (CsCl) gradient centrifugation. Samples of seven CsCl gradient fractions were collected, separated by gel electrophoresis, and stained with Coomassie brilliant blue. Fraction 4 of the wild-type sample contained a prominent protein band migrating at an apparent molecular mass of approximately 35 kDa that was not observed in any of the frac-



**FIG 4** *AglB* is required for the biosynthesis of stable flagella. CsCl-purified samples of strain H53 (wild type) and the  $\Delta flgA1 \Delta flgA2$  and  $\Delta aglB$  mutant strains stained with Coomassie brilliant blue (A) and Pro-Q Emerald glycoprotein stain (B) show the presence of glycosylated protein bands corresponding to molecular masses of FlgA1 (35 kDa) and FlgA2 (37 kDa) in the wild-type fraction, which were absent in both the  $\Delta flgA1 \Delta flgA2$  and  $\Delta aglB$  mutant strains. The migration of molecular mass standards is indicated on the left (in kDa). >, FlgA1; \*, FlgA2.

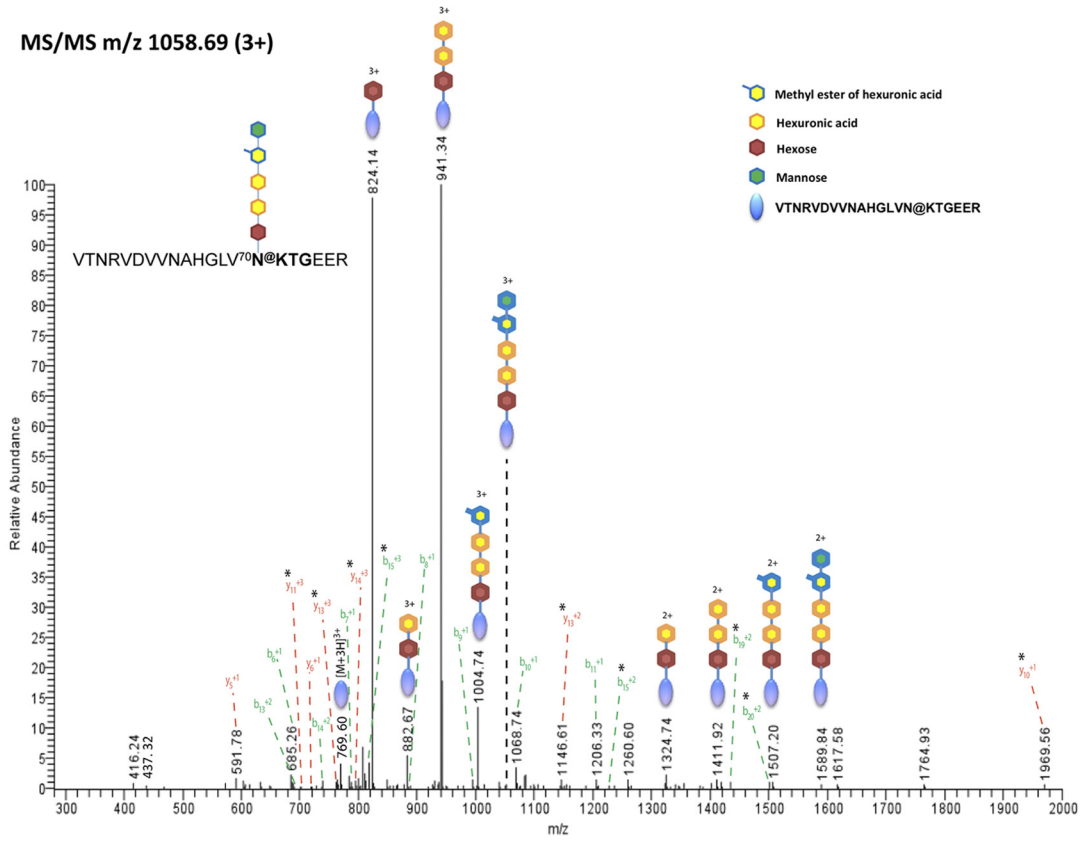
tions generated from the  $\Delta flgA1 \Delta flgA2$  strain (Fig. 4A). Mass spectrometry confirmed that this 35-kDa band, which was also stained with the Pro-Q Emerald 300 glycoprotein gel and blot stain kit (Fig. 4B), contained mostly FlgA1 (see Fig. S2 and Table S2 in the supplemental material). In the same CsCl fractions, an additional faint band was observed to migrate at a rate consistent with a protein having a molecular mass of 37 kDa, the same molecular mass as that of FlgA2. Mass spectrometry confirmed that this band contained FlgA2, and in agreement with Coomassie blue staining, a comparison of the number of MS/MS spectra of the 35-kDa and 37-kDa bands strongly supports that the *Hfx. volcanii* flagellum is composed of a major flagellin, FlgA1, as well as a minor flagellin, FlgA2.

MS/MS of FlgA1 glycoprotein-derived proteolytic peptides revealed the presence of three peptides containing the predicted glycosylation sites modified by the pentasaccharide moiety ( $m/z$  1,058.69 [3+], 1,230.27 [2+], and 1,435.56 [2+]), as identified for the S-layer glycoprotein (Fig. 5A to C). These data not only confirm that the same five sugars modify the flagellins and S-layer glycoprotein but also indicate that no additional sugars absent from the S layer are added to the flagellins. The identified glycosylation sites are consistent with three predicted N-glycosylation sites in the FlgA1 protein sequence (NetNGlyc 1.0) (Fig. 5D). The peptides containing three additional sequons were likely too large (or too small in the double-digested samples) to be detected by LC-MS/MS.

**Glycosylation appears to be required for biosynthesis of stable flagella.** Nonglycosylated flagellins appear to be stable in a  $\Delta aglB$  strain, based on the Western blot analyses presented above

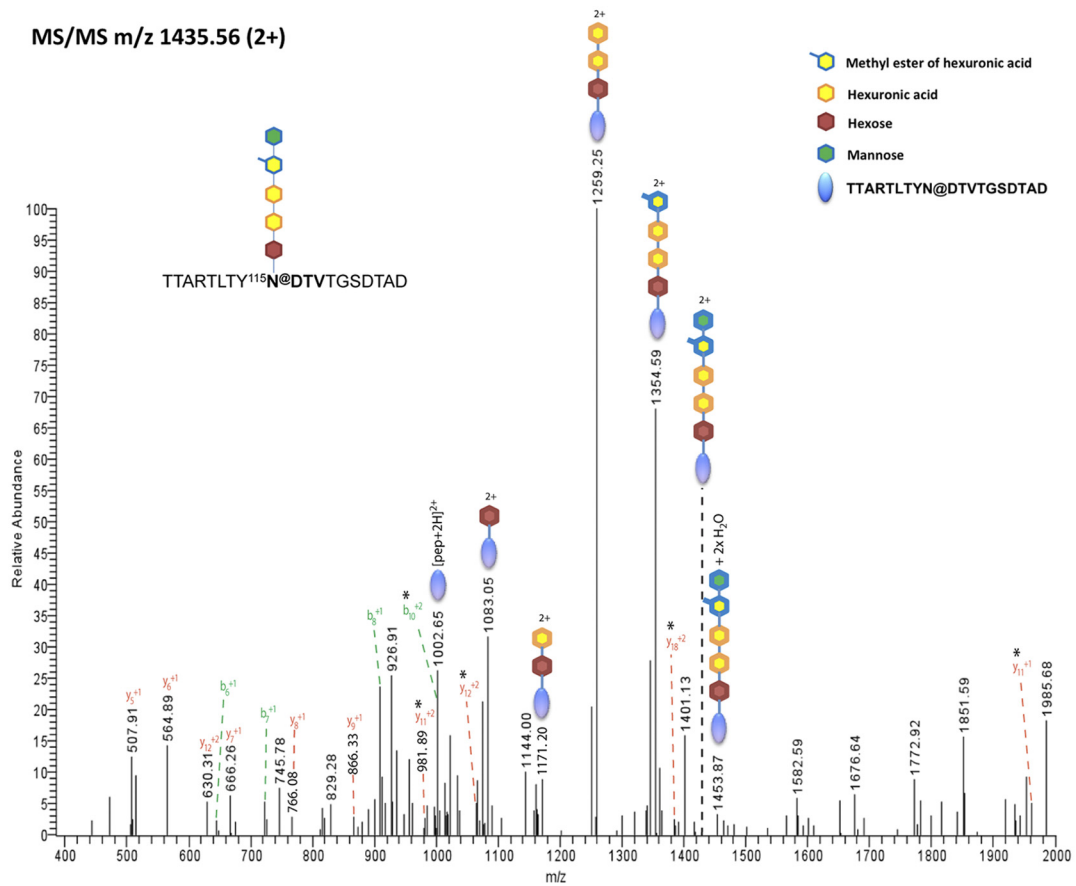
**A**

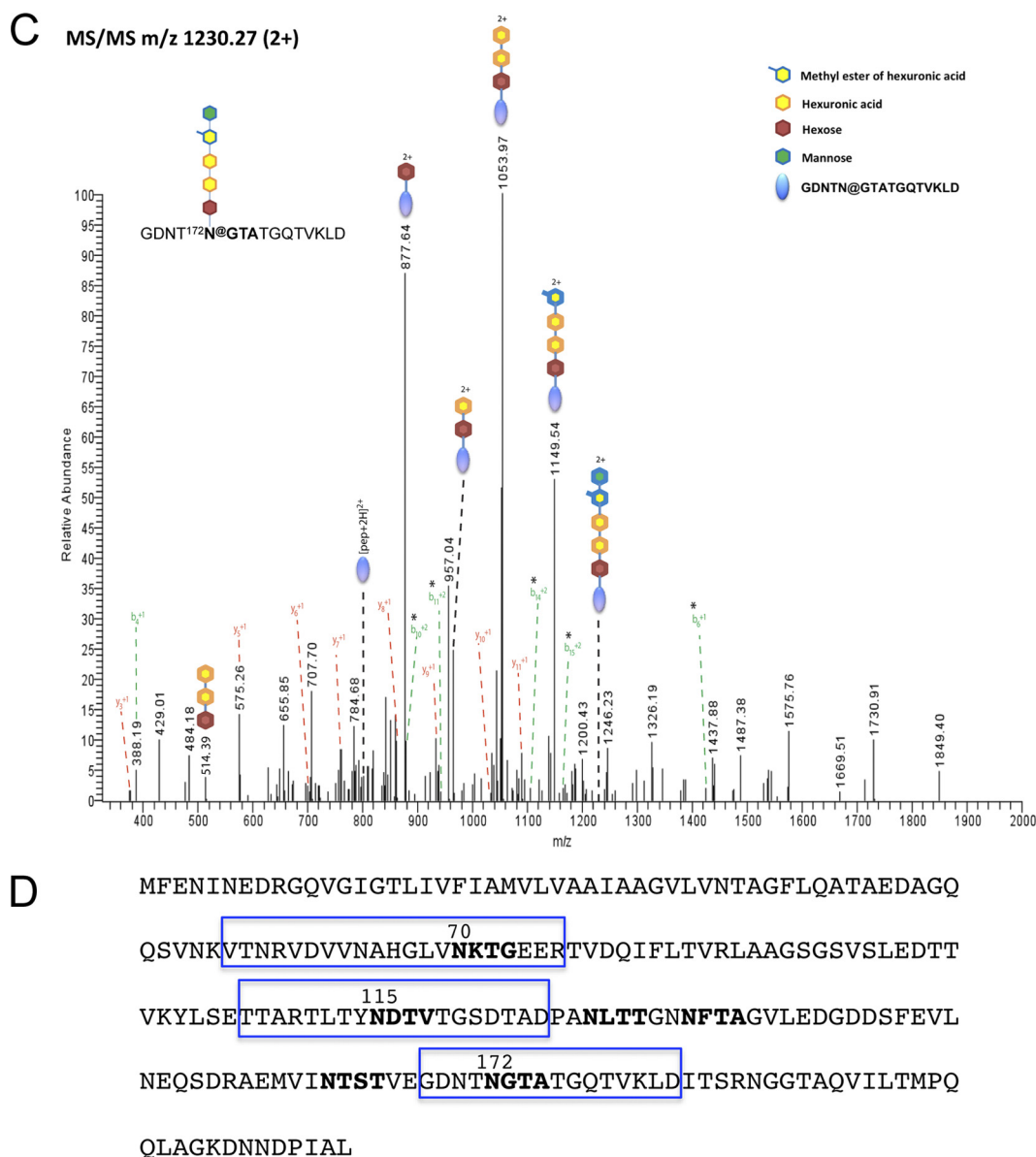
**MS/MS m/z 1058.69 (3+)**



**B**

**MS/MS m/z 1435.56 (2+)**



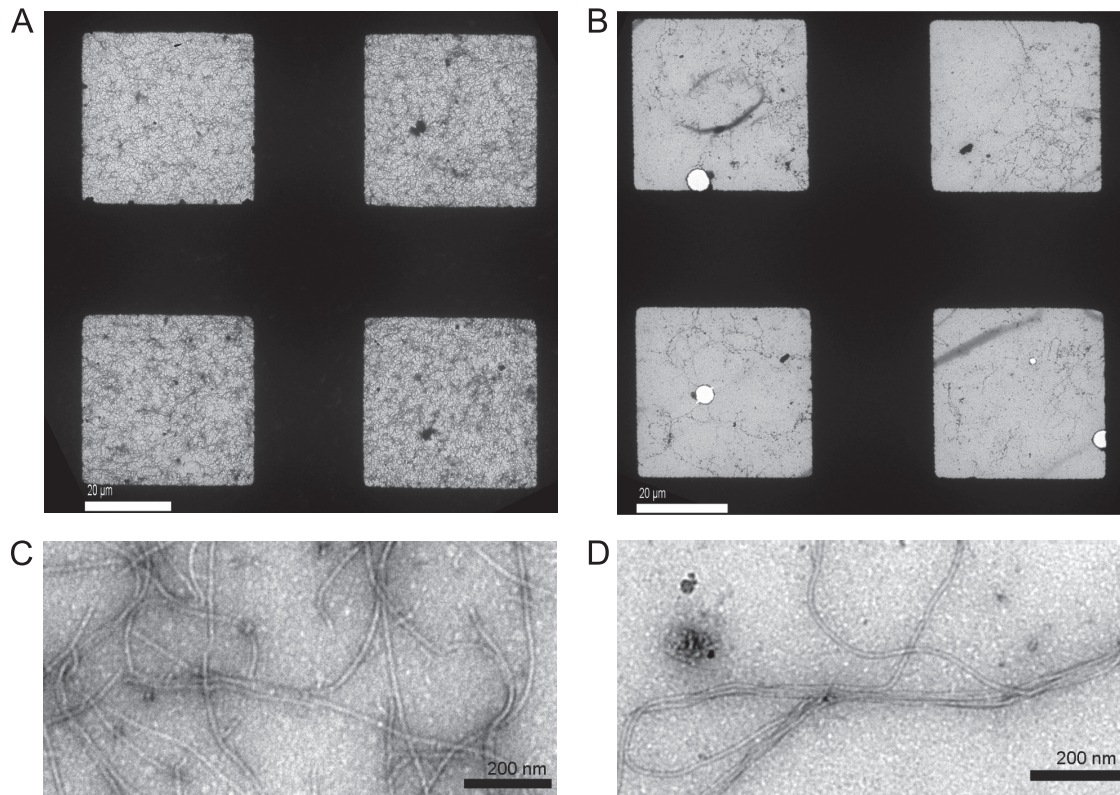


**FIG 5** Three predicted FlgA1 N-glycosylation sites are modified with the same pentasaccharides as its S-layer glycoprotein. Three distinct ion trap MS/MS spectra show all observed fragment ions that arose from backbone cleavage and fragmentation of the glycan moiety of intact peptides. The  $y$ - and  $b$ -ion fragments indicated by asterisks allowed the localization of the modification at the Asn residues in each peptide. (A) MS/MS of the precursor ion of  $m/z$  1,058.69 (3+) corresponding to the glycopeptide VTNRVDVVNAHGLVN@KTGEER (@ represents the glycosylated Asn residue). (B) MS/MS of the precursor ion of  $m/z$  1,230.27 (2+) corresponding to the glycopeptide GDNTN@GTATGQTVKLD. (C) MS/MS of the precursor ion of  $m/z$  1,435.56 (2+) corresponding to the glycopeptide TTARTLTYN@DTVTGSDTAD. Peptides containing the glycan (a pentasaccharide) are indicated at the top left of each panel. The identities of the glycan components are presented with color coding in the inset at the top right of panel A (the complete pentasaccharide is mannose-MethHexUA-HexUA-HexUA-hexose-Asn, where HexUA is hexuronic acid and MethHexUA is the methyl ester of hexuronic acid). (D) Amino acid sequences of *Hfx. volcanii* FlgA1 with Asn-Xaa-Ser/Thr sequons are shown in boldface type. The three sequons predicted by NetNGlyc 1.0. were identified as being modified by MS/MS. Peptides containing sugar modifications shown in panels A, B, and C are boxed.

(Fig. 3). Therefore, it is possible that the swimming motility defect caused by the lack of flagellin glycosylation is due to the disruption of flagellum assembly or, alternatively, an inhibited function of assembled flagella. Hence, we used the CsCl density gradient fractionation protocol to determine whether assembled flagella can be isolated from a  $\Delta aglB$  strain. Gel electrophoresis was employed to separate proteins isolated from CsCl density gradient fraction 4 of the  $\Delta aglB$  culture supernatant, followed by Coomassie brilliant blue staining of the gel. This stain did not detect either a protein

migrating at a rate corresponding to FlgA1 or a faster-migrating band that might contain nonglycosylated flagellins, consistent with the absence of stable flagella in the  $\Delta aglB$  strain (Fig. 4A). Consistent with it containing purified flagella rather than flagellin subunits released into the supernatant, CsCl density gradient fraction 4 was enriched for filaments having diameters of 12 to 14 nm, as determined by electron microscopy (Fig. 6A). This fraction must also contain type IV pilus-like structures that are not flagella, as electron microscopy showed that, albeit to a significantly lesser



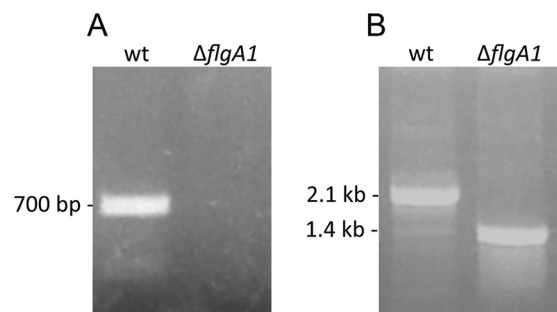


**FIG 6** *Hfx. volcanii* contains AglB-independent type IV pilus-like structures. Shown are transmission electron microscopy images of *Hfx. volcanii* strain H53 (wild type) and  $\Delta aglB$  CsCl fraction 4 stained with uranyl acetate. (A and B) While the abundance of observed surface filaments (diameter of between 12 and 14 nm) was highest in the wild-type fraction (A), a significant number of filaments of similar diameters were identified in CsCl fraction 4 of the  $\Delta aglB$  strain (B). (C and D) Magnification of filaments observed for the wild-type (C) and  $\Delta aglB$  (D) strains.

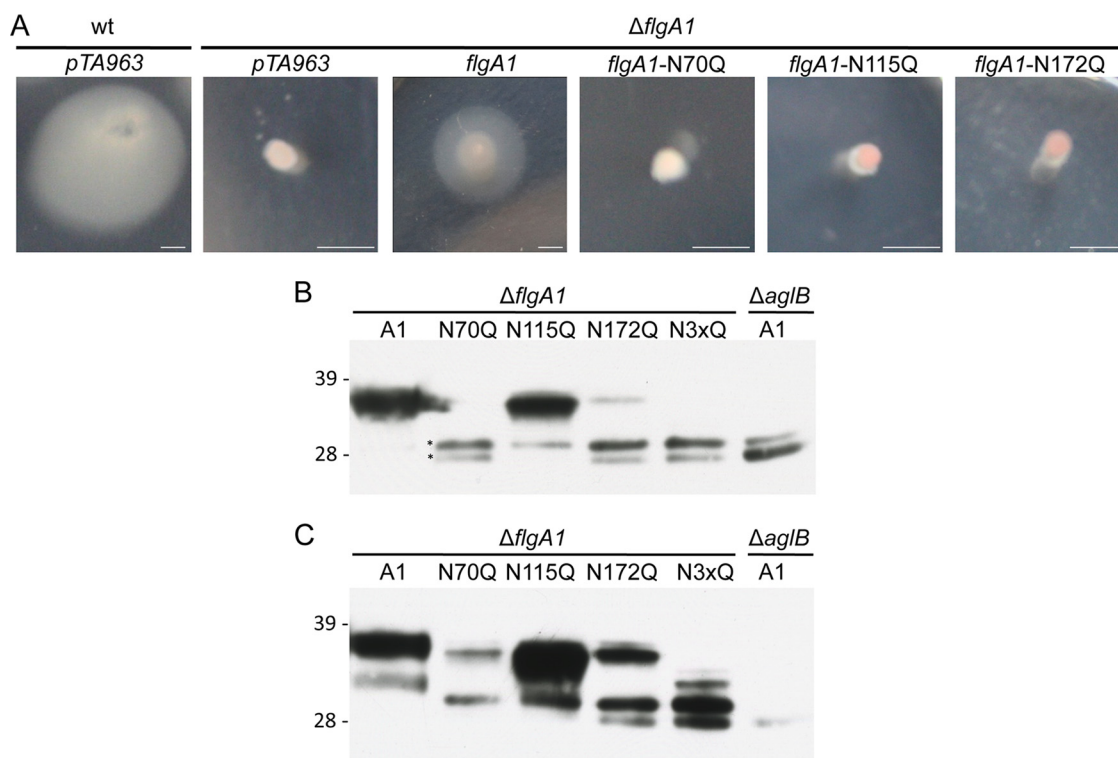
extent, a  $\Delta aglB$  strain produces filamentous structures (Fig. 6B). Consistent with these results, mass spectrometric analyses showed that CsCl density gradient fractions 4 for the wild-type and  $\Delta aglB$  strains all contain peptides of proteins predicted to be pilins, while flagellins were identified only in the wild-type fraction (38; data not shown). Taken together, the Coomassie brilliant blue staining, mass spectrometry, and electron microscopy data presented above suggest that glycosylation is necessary for flagellins to form a stable flagellum in *Hfx. volcanii*.

**Predicted FlgA1 glycosylation sites are critical for flagellum function.** The mass spectrometry data presented above strongly support the modification of at least three NetNGlyc-predicted peptides by the pentasaccharide moiety (Fig. 5; see also Fig. S2 in the supplemental material). Here we analyzed the importance of the glycosylation of each of these three glycosylation sites. While we previously showed that the deletion of FlgA1 and FlgA2 rendered cells nonmotile, in this study, we showed that the deletion of FlgA1 alone can render cells nonmotile (Fig. 7 and 8A). The motility of this mutant can be rescued, albeit not to wild-type levels, by the expression of *flgA1*-His from a plasmid under the control of the inducible *ptna* promoter (Fig. 8A). We took advantage of this observation to test the importance of each of the N-glycosylation modifications of FlgA1 by expressing mutant proteins in which one of the three identified FlgA1 glycosylation sites was replaced with a glutamine in an *Hfx. volcanii*  $\Delta flgA1$  strain. Unlike wild-type FlgA1-His, the replacement mutant constructs did not rescue the swimming motility of an *Hfx. volcanii*  $\Delta flgA1$  strain when ex-

pressed in *trans* (Fig. 8A). Western blot analyses of the cell fractions for cells expressing the His-tagged flagellin constructs revealed that the mutant constructs were expressed. However, the intensities of the protein bands detected for FlgA1N70Q-His and FlgA1N172Q-His were considerably lower than the intensities of bands containing the FlgA1-His or FlgA1N115Q-His proteins (Fig. 8B). In fact, only protein bands migrating at the same rate as that of FlgA1-His expressed in the  $\Delta aglB$  strain were detected in protein extracts isolated from the  $\Delta flgA1$  strain expressing FlgA1N70Q-His. Possibly, the glycosylation of this site in FlgA1



**FIG 7** Confirmation of the deletion of the *flgA1* locus in *Hfx. volcanii*. PCRs were performed using primers (see Table S1 in the supplemental material) against the *flgA1* gene (A) and flanking regions approximately 700 nucleotides upstream and downstream of the *flgA1* locus (B).



**FIG 8** Predicted N-glycosylation sites are crucial for *Hfx. volcanii* motility. (A) *Hfx. volcanii* H53 (wild type) or the  $\Delta flgA1$  strain transformed with expression vector pTA963 or pTA963 expressing either FlgA1-His or FlgA1-His constructs in which a predicted N-glycosylation site asparagine (N) is replaced with a glutamine (Q) under the control of an inducible *trp* promoter. Strains stab inoculated into complex medium (MGM) with 0.3% agar were incubated at 45°C for 5 days. Motility halos indicate the presence of functional flagella. All stabs and incubations were repeated at least three times for the verification of the phenotype. Horizontal bars, 0.5 cm. (B and C) Western blot analysis of cell extracts (B) and TCA-precipitated supernatant fractions (C) from the *Hfx. volcanii*  $\Delta flgA1$  strain transformed with pTA963 expressing either FlgA1-His (wild type) or FlgA1N-Q-His constructs and the *Hfx. volcanii*  $\Delta aglB$  strain transformed with pTA963 expressing FlgA1-His. His-tagged proteins were detected by using anti-His antibodies. Comparable amounts of protein were loaded into each lane. There was twice as much protein loaded for samples in panel C as for samples in panel B, to show the mutated flagellins in the supernatant fractions. While slower-migrating bands (\*) were detected in all N-Q-His samples, only proteins from the wild type and the N115Q mutant consistently contained a band that migrated at the expected molecular mass in the cell fraction. The migration of molecular mass standards is indicated on the left (in kDa).

affects the modification of the other targeted sites in the same protein. Interestingly, while FlgA1-His can barely be detected in the supernatant fraction of the  $\Delta aglB$  strain, consistent with the CsCl density gradient centrifugation results, all replacement mutants expressed in the  $\Delta flgA1$  strain are clearly present in the supernatant fractions. In future structure-function studies, we will determine whether these nonfunctional flagellin replacement mutants can form stable flagella.

Finally, while the FlgA1 replacement mutants each migrate slightly faster than wild-type flagellin, which is consistent with the lack of a pentasaccharide, FlgA1 lacking all three glycosylation sites migrates significantly faster than the wild-type proteins does. However, even FlgA1 lacking all three predicted glycosylation sites migrates significantly more slowly than FlgA1 produced by the  $\Delta aglB$  strain, suggesting that, consistent with the additional three predicted sequons (Fig. 5), additional N-glycosylation occurs in these strains (Fig. 8).

## DISCUSSION

Studies of the N-glycosylation of S-layer glycoproteins in two methanogens, a *Sulfolobus* strain, and two haloarchaeal species have clearly demonstrated that the details of the glycosylation process vary significantly across archaeal species, even among closely

related organisms (11, 19, 29). Moreover, there can be important differences in how various sites within the same protein are glycosylated (28). The glycosylation of certain sites within the protein can even depend upon the specific environmental conditions under which the organism is grown, as was demonstrated recently for *Hfx. volcanii* (16). However, although glycosylation may stabilize the S layer, the lack of a well-defined functional assay makes assessing the importance of the pentasaccharide moieties, or any derivatives, that decorate the S-layer glycoprotein difficult. Further complicating matters, the S-layer glycoprotein is also O-glycosylated, making functional analyses even more complex. These limitations can be avoided by studying the effects of the disruption of the glycosylation of archaeal flagellum subunits, as was demonstrated previously for the methanogens *M. maripaludis* and *M. voltae*, since they are relatively small, do not appear to be O-glycosylated, and have a function that can be easily assayed (19).

Using CsCl density gradient centrifugation, we have developed a method to purify flagella, allowing us to demonstrate that FlgA1 is the major *Hfx. volcanii* flagellin and that FlgA2 is a minor flagellin. In addition to providing an important insight into the biosynthesis of *Hfx. volcanii* flagella, this has allowed us to accumulate enough *Hfx. volcanii* FlgA1 to complete a thorough biochemical

analysis of its glycosylation. Taking advantage of this, we developed several lines of evidence indicating that *Hfx. volcanii* flagellins are glycosylated and that this glycosylation is required for the formation of a functional flagellum.

Using *agl* deletion mutants known to inhibit S-layer glycoprotein glycosylation in *Hfx. volcanii*, we determined that flagellins expressed in all mutants but one ( $\Delta aglD$ ) migrate faster than flagellins expressed in the wild type, consistent with flagellum subunits lacking sugars having lower molecular weights. Moreover, these data provide direct evidence that flagellins are glycosylated through a process that employs a set of components similar to that involved in the glycosylation of the S-layer glycoprotein. While these mobility shifts are reminiscent of size shifts observed for *Methanococcus* S-layer glycoproteins and flagellins in *agl* mutant strains, it is interesting that the *Hfx. volcanii* S-layer glycoprotein mobility shifts do not correlate with the number of glycans added (4, 21). It should be noted that in the *aglG*, *aglI*, *aglF*, and *aglM* deletion strains, AglB may transfer fewer polysaccharide chains to some targeted substrates. Such a substrate specificity may explain why, in these particular *agl* deletion strains, AglB might not attach a polysaccharide at each N-linked site predicted for FlgA1, which may account for the higher-than-expected electrophoretic mobility of FlgA1 proteins in these mutants than that of FlgA1 made in strains lacking AglP and AglE (Fig. 3).

Mass spectrometry analyses not only confirmed the modification of the three asparagines predicted to be glycosylated by NetNGlyc but also revealed that FlgA1 is modified by the same sugar moieties as the S-layer glycoprotein. This is consistent with observations of methanogens, where, although the sugars added to the structures in each species are distinct, within an organism the cell wall and flagellar subunits do appear to be modified by the same saccharides (12). Similarly, as was shown previously for *M. maripaludis* motility, it appears that, at the very least, a minimal-length glycan attached to the flagellins is required for functional flagella and that cells lacking AglB cannot produce stable flagella (20, 41). Finally, our preliminary data suggesting the presence of pili in the  $\Delta aglB$  strain are also consistent with data from an analysis of an *M. maripaludis* strain lacking the acetyltransferase required for the proper assembly of flagella and pili, which revealed that although flagella were absent, pili were found in the supernatant fractions of cultures of this mutant strain (42). Studies are under way to determine whether the filaments enriched for in CsCl density gradient fraction 4 of the  $\Delta aglB$  strain are indeed pili, as suggested by mass spectrometric analyses, which identified peptides of a predicted type IV pilin-like protein in fraction 4 for the  $\Delta aglB$  culture supernatant. Thus far, the failure to raise antibodies against *Hfx. volcanii* flagellins, possibly due to the extensive glycosylation of these proteins, has prevented us from distinguishing flagella from non-flagellum type IV pilus-like structures and hence from excluding the possibility that a subset of these surface filaments are nonglycosylated flagella. Comparing and contrasting pilus and flagellum biosynthesis in *Hfx. volcanii* may yield some interesting insights, especially considering that glycosylation is apparently not required for the assembly of at least a subset of pili.

While the data described above clearly showed that, as in other archaeal systems studied, the subunits of the S-layer glycoprotein and flagella are glycosylated in a similar manner, it has not been determined whether the lack of glycosylation directly or indirectly affects flagellum motility and biosynthesis. A lack of glycosylation

could interfere with flagella anchoring to the cell surface, possibly due to an absence of N-glycosylation of the S-layer glycoprotein, hence resulting in the degradation of the protein in the supernatant. Having identified three glycosylation sites in the major flagellin by mass spectrometry and having demonstrated that FlgA1 expressed from a plasmid can complement an *flgA1* deletion strain, we showed that the specific amino acid residues targeted for glycosylation in the flagellins are absolutely required for the flagellum to function properly. We demonstrated this by showing that the expression of site-directed *flgA1* mutants lacking one of the three identified glycosylation sites of FlgA1 does not rescue swimming motility. Moreover, while the N-glycosylation of each of these three predicted glycosylation sites is critical to motility, Western blot analysis of proteins isolated from cells and supernatant fractions of cells expressing the His-tagged replacement mutant flagellin constructs indicated that glycosylation differentially affects the stability of FlgA1, depending upon which of the glycosylation sites in the flagellin are modified by the addition of a polysaccharide moiety. Why this is so is one of many questions that can be addressed now that we have purified the glycosylated form of FlgA1, the major flagellin, and generated an *flgA1* deletion mutant in which altered FlgA1 constructs can be expressed in *trans*. In future experiments, combined characterizations of *flg* and *agl* mutants using electron microscopy, high-resolution mass spectrometry, and functional assays will allow us to clarify the role that glycosylation plays in the proper assembly and function of *Hfx. volcanii* cell surface structures.

We can now begin to determine the importance of specific sugars to the structure and function of the *Hfx. volcanii* flagellum and the significance of the modifications of specific sugars, such as why the fourth sugar must be methylated prior to the addition of the mannose cap in the flagellar pentasaccharide and why, when the fifth sugar is absent, the methylation of the fourth sugar renders the cells nonmotile. Interestingly, the *agl* deletion mutants in which swimming motility was least affected are those that exhibit growth defects, suggesting that the various steps in the glycosylation process affect the functions of *Hfx. volcanii* glycoproteins to various degrees. Whether this is due to differences in the way different protein complexes form or because specific protein functions are differentially affected is unknown.

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