Towards Glycoengineering in Archaea: Replacement of *Haloferax volcanii* AglD with Homologous Glycosyltransferases from Other Halophilic Archaea

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Like eukarya and bacteria, archaea also perform N-glycosylation. However, the N-linked glycans of archaeal glycoproteins present a variety not seen elsewhere. Archaea accordingly rely on N-glycosylation pathways likely involving a broad range of species-specific enzymes. To harness the enormous applied potential of such diversity for the generation of glycoproteins bearing tailored N-linked glycans, the development of an appropriate archaeal glycoengineering platform is required. With a sequenced genome, a relatively well-defined N-glycosylation pathway, and molecular tools for gene manipulation, the haloarchaeon *Haloferax volcanii* (*Hfx. volcanii*) represents a promising candidate. Accordingly, cells lacking AglD, a glycosyltransferase involved in adding the final hexose of a pentasaccharide N-linked to the surface (S)-layer glycoprotein, were transformed to express AglD homologues from other haloarchaea. The introduction of nonnative versions of AglD led to the appearance of an S-layer glycoprotein similar to the protein from the native strain. Indeed, mass spectrometry confirmed that AglD and its homologues introduce the final hexose to the N-linked S-layer glycoprotein pentasaccharide. Heterologously expressed haloarchaeal AglD homologues contributed to N-glycosylation in *Hfx. volcanii* despite an apparent lack of AglD function in those haloarchaea from where the introduced homologues came. For example, although functional in *Hfx. volcanii*, no transcription of the Halobacterium salinarum aglD homologue, OE1482, was detected in cells of the native host grown under various conditions. Thus, at least one AglD homologue works more readily in *Hfx. volcanii* than in the native host. These results warrant the continued assessment of *Hfx. volcanii* as a glycosylation “workshop.”

The discovery of a protein N-glycosylation system in the bacterium *Campylobacter jejuni* (30, 35) and the functional transfer of the corresponding gene locus into *Escherichia coli* (33) have raised the possibility of using the latter, a workhorse of molecular biology, for the heterogenous expression of glycosylated proteins (16, 18, 28, 34). Such efforts have gained further impetus with the introduction of the pilin glycosylation systems from *Pseudomonas aeruginosa* and *Neisseria meningitidis* into *E. coli*, resulting in customized protein O-glycosylation (14–16).

The prospect of creating an archaeal system capable of generating glycoproteins bearing tailored glycan moieties is also enticing, particularly if such a system could exploit the broad diversity in composition of Asn-linked oligosaccharides decorating archaeal glycoproteins (see reference 12). Archaeal proteins, moreover, often possess the added benefit of being able to withstand physical conditions that would normally lead to protein denaturation and aggregation, properties possibly augmented by glycosylation. Unfortunately, progress in creating archaeal “glyco-factories” had been hampered by the limited information available on the workings of the archaeal N-glycosylation pathway (see reference 19). Over the last few years, however, genes encoding many of the enzymes involved in N-glycosylation in three different archaeological species, i.e., *Haloferax volcanii* (*Hfx. volcanii*), *Methanococcus voltae*, and *Methanococcus maripaludis*, have been identified (for a review, see references 4, 10, and 37).

In *Hfx. volcanii*, products of the *agl* genes are responsible for adding a pentasaccharide to select Asn residues of the surface (S)-layer glycoprotein, a protein that serves as a reporter of N-glycosylation in this species. Specifically, AglJ, AglG, AglI, AglE, and AglD are predicted glycosyltransferases (2, 3, 38). AglM, a UDP-glucose dehydrogenase, and AglF, a glucose-1-phosphate uridylyltransferase, are involved in the biosynthesis of pentasaccharide hexuronic acids (39). AglP is a methyltransferase that participates in the generation of the methyl ester of hexuronic acid found at position four of the N-linked pentasaccharide decorating the S-layer glycoprotein (21). Finally, AglB serves as the oligosaccharyltransferase in *Hfx. volcanii* (2). Examination of the *Hfx. volcanii* genome reveals that all of the genes encoding these proteins, with the exception of *aglD*, are grouped into a single cluster (36).

In addition to *Hfx. volcanii*, the complete genome sequences of other halophilic archaea are available (at http://archaea.ucsc.edu/, http://www.halolex.mpg.de/public/, and http://edwards.sdsu.edu/halophiles/#aboutlink). Analysis of the genomes of *Halobacterium* sp. NRC-1 (24), *Halobacterium salinarum* (*Hbt. salinarum*) (26), *Haloarcula marismortui* (*Hma. marismortui*) (5), *Haloquadratum walsbyi* (*Hqr. walsbyi*) (7, 9), and *Natronomonas pharaonis* (13) reveals the presence of homologues to...
TABLE 1. Primers used in this study

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<th>Primer</th>
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<tr>
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<tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
</tbody>
</table>

*a Introduced restriction sites are underlined.

many Hfx. volcanii agl gene sequences, and, as in Hfx. volcanii, these homologous sequences, too, are often clustered into gene islands (20, 36).

While the involvement of haloarchaeal homologues of Hfx. volcanii Agl proteins in N-glycosylation in the native hosts remains to be shown, the ability to delete Hfx. volcanii of genes of interest and subsequently exploit appropriate vectors to complement the deletion allows for testing of the impact of introducing various haloarchaeal agl gene homologues into Hfx. volcanii lacking a given N-glycosylation gene. Accordingly, Hfx. volcanii cells with a deletion of aglD, involved in adding the final hexose to the pentasaccharide N-linked to the Hfx. volcanii S-layer glycoprotein (2), were transformed to express the AglD homologue from Hbt. salinarum, Hbt. OE1482, were generated, respectively. The sequences of the primer pair Hqtfor and Hqtrev and primer pair Hbfor and Hbrev, together with previously (1) using the primers listed in Table 1.

The protein contents of HbtcOE1482, were separated on 7.5% polyacrylamide gels (22) as were aglD/H11032 rrnAC1873, genomic DNA was isolated from Hma. marismortui, and 3 ends of the amplified sequence, and 3 were PCR amplified using primers Hmafor and Hmarev, designed to

RESULTS

Homologues of Hfx. volcanii AglD are found in other haloarchaea. Hfx. volcanii AglD is a component of the N-glycosylation apparatus of this species, involved in adding the final sugar subunit of the pentasaccharide decorating select Asn residues of the S-layer glycoprotein (2). Homology-based searches revealed that homologues of AglD are encoded by the genomes of Hma. marismortui (rrnAC1873; 67% identity), Hgr. walsbyi (1489A; 64% identity), and Hbt. salinarum (OE1482; 62% identity). Hfx. volcanii AglD (2), Hma. marismortui rrnAC1873, Hgr. walsbyi 1489A, and Hbt. salinarum OE1482 are all predicted to contain a soluble N-terminal domain of comparable size, followed by six C-terminal membrane-spanning domains (Fig. 1A). The sequences differ more in the C-terminal domain, that region of glycosyltransferases responsible for acceptor recognition and generally showing higher variability (8), than in the N-terminal region. All three AglD homologues, however, contain aspartic acid residues at positions equivalent to Hfx. volcanii D110, D112, and D201, residues predicted to form the DXD motif involved in donor binding and the catalytic base of the Hfx. volcanii enzyme, respectively (17).

Phylogenetic analysis of AglD homologues from both complete and incomplete haloarchaeal genomes (as listed at http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi?; accessed August 2009) reveals the evolutionary proximity of the various haloarchaeal versions of AglD, which cluster in a group distinct from Hfx. volcanii AglE, AglG, AglI, and AglJ, other glycosyl-
FIG. 1. Haloarchaeal homologues of \textit{Hfs. volcanii} AglD. (A) The amino acid sequences of \textit{Hfs. volcanii} AglD, \textit{Hma. marismortui} rrrnAC1873, \textit{Hqr. walsbyi} 1489A, and \textit{Hbt. salinarum} OE1482 were aligned using Tcoffee (http://tcoffee.vital-it.ch/cgi-bin/Tcoffee/tcoffee_cgi/index.cgi?stage1=1&daction=TCOFFEE::Regular). Identical residues at a given position in at least three sequences are highlighted against a black background.
Expression of AglD or its haloarchaeal homologues in ΔaglD Hfx. volcanii cells restores S-layer glycoprotein traits lost in the deletion strain. To test whether haloarchaeal AglD homologues are able to fulfill the role assumed by Hfx. volcanii AglD, Hfx. volcanii ΔaglD cells were transformed with plasmids encoding Hfx. volcanii AglD, Hma. marismortui rrnAC1873, Hqr. walsbyi 1489A, or Hbt. salinarum OE1482, with each protein bearing a N-terminal CBD tag. Immunoblotting using anti-CBD antibodies revealed that the different proteins were expressed at comparable levels in the Hfx. volcanii host (Fig. 2, upper panels). Next, experiments were undertaken to exploit the earlier observation that the behavior of the Hfx. volcanii S-layer glycoprotein is modified upon deletion of aglD. Specifically, upon deletion of aglD, the S-layer glycoprotein migrates faster in SDS-PAGE gels than does the same protein from cells encoding AglD. Furthermore, the S-layer glycoprotein in the deletion strain can no longer be labeled by the PAS glycocon. When cells lacking AglD are transformed to express a plasmid-encoded version of the protein, however, these effects on S-layer glycoprotein behavior are reversed (17). As shown in Fig. 2 (middle and lower panels), the same holds true when Hfx. volcanii cells lacking AglD were transformed to express Hma. marismortui rrnAC1873, Hqr. walsbyi 1489A, or Hbt. salinarum OE1482.

To further address whether the S-layer glycoprotein from Hfx. volcanii ΔaglD cells complemented to express Hfx. volcanii AglD, Hma. marismortui rrnAC1873, Hqr. walsbyi 1489A, or Hbt. salinarum OE1482 behaves similarly in each case, the susceptibility of the Hfx. volcanii S layer to added proteinase K was considered in each population of transformed cells. The S layer of Hfx. volcanii corresponds to a protein shell surrounding the cell and is thought to comprise a single component, namely, the S-layer glycoprotein (29). Moreover, the S-layer glycoprotein can be readily identified by SDS-PAGE because it is the dominant protein on the gel and because its migration as an approximately 180-kDa species clearly separates it from the bulk of Hfx. volcanii proteins. As presented in Fig. 3, the S layer of cells of the Hfx. volcanii parent strain was seemingly unaffected by a 3-h incubation with proteinase K, as reflected in the relatively constant level of the Coomassie-stained S-layer glycoprotein. In contrast, the S-layer glycoprotein was readily digested when cells with a deletion of aglD were similarly exposed to the protease. When, however, the deletion strain was transformed to express tagged versions of the absent Hfx. volcanii protein, of Hma. marismortui rrnAC1873, of Hqr. walsbyi 1489A, or of Hbt. salinarum OE1482, protease resistance of the S layer was restored to that level noted with the parent strain. In addition, Hfx. volcanii ΔaglD cells transformed to express CBD-tagged versions of Hfx. volcanii AglD, Hma. marismortui rrnAC1873, Hqr. walsbyi 1489A, and Hbt. salinarum OE1482 all presented similar growth profiles (data not shown). Finally, in cells of the deletion strain expressing plasmid-encoded AglD or any of its homologues, the extent of S-layer glycoprotein release into the growth medium was comparable (data not shown).

Together, these results reveal that AglD and each of its haloarchaeal homologues are functionally equivalent in terms of their ability to modify both S-layer glycoprotein processing and the ability of this reporter of N-glycosylation to assemble into a multimeric complex, which is reflected in S-layer stability.

Haloarchaeal AglD homologues add a hexose to the final position of the S-layer glycoprotein N-linked pentasaccharide. Based on sequence homology considerations and the results of mass spectrometry studies, Hfx. volcanii AglD was designated as the glycosyltransferase responsible for adding the terminal hexose to the N-linked pentasaccharide decorating the S-layer glycoprotein (2). To determine whether AglD homologues from Hma. marismortui, Hqr. walsbyi, and Hbt. salinarum also catalyze the addition of a terminal hexose to the glycans N-linked to the Hfx. volcanii S-layer glycoprotein, mass spectrometry was performed on an Asn-13-containing glycopeptide derived from the S-layer glycoprotein (2) isolated from Hfx. volcanii cells deleted of aglD and subsequently transformed to express CBD-tagged versions of AglD, Hma. marismortui rrnAC1873, Hqr. walsbyi 1489A, or Hbt. salinarum OE1482. As reflected in Fig. 4 and summarized in Table 2, in each case, the peptide was shown to be decorated with a pentasaccharide comprising a hexose (predicted m/z, 1,743.62; measured m/z, 1,742.64 to 1,743.06), two hexuronic acids (predicted m/z, 1,919.62 and 2,095.62, respectively; measured m/z, 1,918.89 to 1,919.89 and 2,094.64 to 2,095.09, respectively), a methyl ester of hexuronic acid (predicted m/z, 2,285.62; measured m/z, 2,284.79 to 2,285.44), and a final hexose subunit (predicted m/z, 2,448; measured m/z, 2,447.94 to 2,448.72). In addition, the same peptide bearing the presumably precursor tetra-, tri-, di-, and monosaccharides was also detected. The detection of a pentasaccharide-modified peptide in ΔaglD cells transformed to express CBD-AglD (Fig. 4) confirms both that complementation of the deletion strain restores AglD function and that the added CBD moiety does not compromise enzyme activity. Furthermore, the fact that identical mass spectrometry profiles were obtained for the Asn-13-containing S-layer glycopeptide derived peptides from each of the strains engineered to express an AglD homologue (Fig. 4B to D) confirms that, like the native protein, each of the other haloarchaeal versions of AglD

Asterisks indicate the positions of the catalytically important Asp110, Asp112, and Asp201 residues in Hfx. volcanii AglD (17). (B) Phylogenetic analysis of Hfx. volcanii AglD and its haloarchaeal homologues. A phylogenetic tree showing the relation of available AglD homologues from haloarchaeal species was generated by the maximum parsimony method with the MEGA package (31). Bootstrap values are indicated at branch nodes and correspond to confidence levels obtained upon 500 trial repeats. The sequences used to construct the phylogenetic tree are Hfx. volcanii AglD, AgIE, AgIG, AgII, and AgI (Hov AglD, AgIE, AgIG, AgII, and AgI, respectively), as well as AglD homologues from Hbt. salinarum (Hsal OE1482R), Hma. marismortui (Hmar rrnAC1873), Hqr. walsbyi (Hwal HQ1489A), Halococcus mukohatae (Hmuk ZP_03873045.1), Holarhabdus utahensis (Huta ZP_03871024.1), Halogethecum borinquense (Hbor ZP_03997945.1), and Natronomonas pharaonis (Npha NP2244A). The sequences of haloarchaeal AglD homologues listed in panel A were identified in a BLAST search performed at http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi? (accessed July 2009) using the Hfx. volcanii AglD sequence as bait.
Hma. marismortui

Hfx. volcanii strain lacking adds a final hexose to the N-linked glycan decorating the Hfx. volcanii S-layer glycoprotein. Finally, the results reveal that the introduction of nonnative versions of AglD does not interfere with assembly of the pentasaccharide or the composition of its first four sugar residues.

Although expressed in Hfx. volcanii, Hbt. salinarum OE1482 is not transcribed in the native host. While the various halooarchaeal homologues of AglD can functionally replace the absent Hfx. volcanii protein, the roles played by these proteins in their native hosts are not known. Accordingly, RT-PCR was first conducted to confirm that the Hma. marismortui rrnAC1873, Hqr. walsbyi 1489A, and Hbt. salinarum OE1482 gene sequences are indeed transcribed in their native hosts. Accordingly, RNA isolated from Hma. marismortui and Hbt. salinarum cells grown to mid-logarithmic phase in rich medium and from a standing culture of Hqr. walsbyi was used to generate cDNA. PCR was then performed using primers directed against the rrnAC1873, 1489A, and OE1482 gene sequences. Figure 5A (left panels) shows that only a transcript-derived PCR product for the Hma. marismortui aglD homologue was detected. For each species, RT-PCR of 16S rRNA was performed as a positive control (Fig. 5B, upper panel). The transcription of PCR product for the Figure 5A (right panels) shows that only a transcript-derived PCR product for the Hfx. volcanii aglD was readily transcribed in their native hosts as determined by RT-PCR (Fig. 5B, middle panel). As above, no transcript-derived PCR product was detected from a standing culture of Hqr. walsbyi grown in hyposaline medium (not shown), or from cells subjected to a 15- or 45-min heat shock (data not shown). In contrast, the neighboring OE1488 sequence was readily transcribed in Hbt. salinarum cells grown to mid-logarithmic phase in rich medium, as well as in cells grown to stationary phase (Fig. 5B, lower panel). In these experiments, as above, RT-PCR of 16S rRNA was performed as a positive control (Fig. 5B, upper panel). The transcription of Hbt. salinarum OE1482 in Hfx. volcanii but apparently not in the native host offers
encouragement to continue developing \textit{Hfx. volcanii} as a glycoengineering platform.

Finally, given the extremely low rate at which \textit{Hqr. walsbyi} grows in the laboratory, transcription of 1489A was not further pursued.

\section*{DISCUSSION}

The detection of the oligosaccharyltransferase-encoding \textit{aglB} gene in numerous species suggests that N-glycosylation is widespread in \textit{Archaea} (20). Moreover, the heterogeneity seen in the N-linked glycans of archaeal glycoproteins identified to date (approximately 12) implies that an enormous array of enzymes is involved in this posttranslational modification. With the ultimate aim of harnessing this variety for applied purposes, a suitable platform for hosting chimeric N-glycosylation pathways is needed. Offering the opportunity for simple heterologous expression of nonnative proteins, possessing a relatively well-defined N-glycosylation

TABLE 2. Summary of mass spectrometry results

<table>
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\(^a\) The composition of the glycan decorating each peak is indicated as follows: hexose; hexuronic acid; a methyl ester of hexuronic acid; N, the modified Asn-13 residue of the S-layer glycoprotein-derived glycopeptide (\textit{\textit{ERGLNDADSESFK}}\textit{\textit{N}}).

The detection of the oligosaccharyltransferase-encoding \textit{aglB} gene in numerous species suggests that N-glycosylation is widespread in \textit{Archaea} (20). Moreover, the heterogeneity seen in the N-linked glycans of archaeal glycoproteins identified to date (approximately 12) implies that an enormous array of enzymes is involved in this posttranslational modification. With the ultimate aim of harnessing this variety for applied purposes, a suitable platform for hosting chimeric N-glycosylation pathways is needed. Offering the opportunity for simple heterologous expression of nonnative proteins, possessing a relatively well-defined N-glycosylation transformed to express AglD or its haloarchaeal homologues. (A) \textit{Hfx. volcanii} \textit{aglD} cells transformed to express CBD-tagged AglD. (B) \textit{Hfx. volcanii} \textit{aglD} cells transformed to express CBD-tagged \textit{Hma. marismortui} \textit{rnnAC1873}. (C) \textit{Hfx. volcanii} \textit{aglD} cells transformed to express CBD-tagged \textit{Hqr. walsbyi} 1489A. (D) \textit{Hfx. volcanii} \textit{aglD} cells transformed to express CBD-tagged \textit{Hbt. salinarum} OE1482. The arrows indicate the positions of the pentasaccharide-modified peak in each strain. The composition of the glycan decorating each peak is indicated as follows: hexose; hexuronic acid; and a methyl ester of hexuronic acid. Only the modified Asn-13 residue (N) of the S-layer glycoprotein-derived glycopeptide (\textit{\textit{ERGLNDADSESFK}}\textit{\textit{N}}) is indicated. Also shown above each peak is the charge (\textit{z}) applied (+2 or +3).
to the S-layer glycoprotein. Still, the unequal incorporation of radiolabeled sugar subunits in the various transformed cell populations (D. Calo and J. Eichler, unpublished observations) could reflect differences in the actions of the different glycosyltransferases. It will be possible to confirm this hypothesis once ongoing efforts have successfully defined the precise composition of the S-layer glycoprotein N-linked pentasaccharide.

While Hma. marismortui rnrAC1873, Hqr. walsbyi 1489A, and Hbt. salinarum OE1482 all proved to be functional in Hfx. volcanii, the true role of these proteins in their native hosts remains to be defined. In the present study, it was shown that transcripts of Hma. marismortui rnrAC1873 could be detected in the native host. This could point to similarities between the glycan putatively decorating the 40 putative N-glycosylation sites of the Hma. marismortui S-layer glycoprotein, rnrAC0971, and its N-linked Hfx. volcanii counterpart. Specifically, as AgID is assigned as the glycosyltransferase catalyzing the addition of a hexose residue to the methyl ester of hexuronic acid found at position four of the glycan N-linked to the Hfx. volcanii S-layer glycoprotein (2, 21), the transcription of Hma. marismortui rnrAC1873 in the native host could reflect a similar linkage being found in the Hma. marismortui S-layer glycoprotein.

In contrast to Hma. marismortui rnrAC1873, no transcription of Hbt. salinarum OE1482 was detected in cells grown in any of several conditions tested, unlike the neighboring OE1488 or 16S rRNA sequences. Still, despite a failure to observe the transcription of OE1482 in cells grown to log-arithmic and stationary phases, under low-salt conditions or following heat shock in the present report, a PCR product-based DNA microarray study nonetheless reported a 1.15-fold increase in OE1482 transcription in cells growing under anaerobic and phototropic conditions, relative to aerobic conditions (32). Yet the basal level of the transcript was not reported, nor was OE1482 transcription confirmed by RT-PCR using specific primers. Moreover, OE1482 was not detected when the membrane proteomes of Hbt. salinarum cells grown under the same anaerobic and phototropic or aerobic conditions were addressed (6). How thus can the presence of a seemingly nontranscribed glycosyltransf erase-encoding gene in the Hbt. salinarum genome be explained? It is conceivable that OE1482 is expressed under a specific set of conditions not tested here. Alternatively, the OE1482 transcript may not be stable under the conditions tested. Regardless, the results presented here demonstrate the ability of Hfx. volcanii to express foreign enzymes and incorporate them into its N-glycosylation pathway, even though such enzymes are not central to this post-translational modification in the native host. Indeed, although Hbt. salinarum OE1482 expressed in Hfx. volcanii readily catalyzed the addition of a hexose residue to a methyl ester of a hexuronic acid (as Hfx. volcanii AgID is thought to do [2, 21]), the N-linked glycans decorating the Hbt. salinarum S-layer glycoprotein and flagellin do not include a hexose added to a hexuronic acid (19). Finally, as with Hbt. salinarum OE1482, transcription of Hqr. walsbyi 1489A in the native host was not detected either. In this case, however, it is premature to draw any conclusions, given the general lack of information on this species and its low growth rate under laboratory conditions. Thus, the possibil-
ity remains that this protein is expressed under different growth conditions than the single situation tested here.

The experiments described in this study represent the first steps in assessing the suitability of Hfx. volcanii as a platform for the synthesis of variable N-linked glycans relying on introduced N-glycosylation pathway components originating from other archaeal species. The results reveal that the nonnative glycosyltransferases considered here are functional in Hfx. volcanii, even though they are not constitutively recruited and/or possibly do not catalyze different reactions in their native host.

In the future, the ability of other haloarchaeal proteins to replace their Hfx. volcanii homologues will be tested, as will the functionality of other glycosylation-related enzymes found in haloarchaea but not in Hfx. volcanii.

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REFERENCES


