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Protein N-glycosylation in Archaea: defining *Haloferax volcanii* genes involved in S-layer glycoprotein glycosylation

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Summary

In this study, characterization of the N-glycosylation process in the haloarchaea *Haloferax volcanii* was undertaken. Initially, putative *Hfx. volcanii* homologues of genes involved in eukaryal or bacterial N-glycosylation were identified by bioinformatics. Reverse transcription polymerase chain reaction (RT-PCR) confirmed that the proposed N-glycosylation genes are transcribed, indicative of true proteins being encoded. Where families of related gene sequences were detected, differential transcription of family members under a variety of physiological and environmental conditions was shown. Gene deletions point to certain genes, like *alg11*, as being essential yet revealed that others, such as the two versions of *alg5*, are not. Deletion of *alg5-A* did, however, lead to slower growth and interfered with surface (S)-layer glycoprotein glycosylation, as detected by modified migration on SDS-PAGE and glycostaining approaches. As deletion of *stt3*, the only component of the oligosaccharide transferase complex detected in Archaea, did not affect cell viability, it appears that N-glycosylation is not essential in *Hfx. volcanii*. Deletion of *stt3* did, nonetheless, hinder both cell growth and S-layer glycoprotein glycosylation. Thus, with genes putatively involved in *Hfx. volcanii* protein glycosylation identified and the ability to address the roles played by the encoded polypeptides in modifying a reporter glycoprotein, the steps of the archaeal N-glycosylation pathway can be defined.

Introduction

Of the various post-translational modifications proteins can experience, glycosylation is probably the most prevalent. Protein glycosylation involves a wide range of polysaccharide moieties linked to either selected Asn (N-glycosylation) or Ser or Thr residues (O-glycosylation). In Eukarya, N-glycosylation begins when soluble activated sugars assemble into a heptasaccharide of defined composition on a dolichol pyrophosphate carrier embedded in the cytoplasmic leaflet of the ER membrane (Kornfeld and Kornfeld, 1985; Burda and Aebi, 1999; Spiro, 2002; Helenius and Aebi, 2004). The lipid-linked structure is then flipped across the membrane and, once oriented towards the ER lumen, the oligosaccharide moiety is further augmented by the sequential attachment of seven additional monosaccharides, transferred from other lumen-facing dolicholpyrophosphate carriers. The resulting branched polysaccharide is transferred by the Stt3 subunit of the multimeric oligosaccharide transferase (OST) onto the Asn residues of Asn–Xaa–Ser/Thr motifs found in nascent polypeptide chains co-translationally translocating into the ER lumen (Silberstein and Gilmore, 1996; Yan and Lennarz, 2005).

Long-thought to be an exclusively eukaryal trait, it is now clear that prokaryotes are also capable of performing N-glycosylation. With the identification of genes involved in this post-translational modification in *Campylobacter jejuni* (Szymanski *et al*., 1999; Linton *et al*., 2002) and the synthesis of N-glycosylated proteins by *Escherichia coli* transformed to express the *C. jejuni* gene products (Wacker *et al*., 2002), it has become apparent that Bacteria are also capable of N-glycosylation. In *C. jejuni*, the products of the *pgl* locus act to assemble a lipid-linked branched heptasaccharide that is subsequently flipped across the cytoplasmic membrane (Szymanski *et al*., 2003; Linton *et al*., 2005). The polysaccharide is then presumably transferred to selected Asn residues by PglB, a homologue of Stt3 (Feldman *et al*., 2005). Thus, while the proposed events that take place during bacterial N-glycosylation are highly reminiscent of the parallel process in Eukarya, differences exist, including the size and composition of the lipid-linked glycan moieties in each domain as well as the relative simplicity of the bacterial oligosaccharide transferase. Furthermore, the oligosaccharide entity is linked to Asn residues of the target protein through a *N*-acetylglucosamine (GlcNAc) residue in Eukarya while in Bacteria, the linking sugar is bacillosamine (2,4-diacetamido-2,4,6-trideoxyglucose) (Young *et al*., 2002). Finally, whereas the glycan moiety can be

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further modified following transfer to its polypeptide target in the eukaryal system (Varki, 1998), no such modulation has been observed in Bacteria.

In contrast to Bacteria, where N-glycosylation is still considered a rare event, numerous archaeal species contain Asn-modified glycoproteins. Indeed, the surface (S)-layer glycoprotein of the halophilic Archaea *Halobacterium salinarum* was the first prokaryotic glycoprotein to be described in detail (Mescher and Strominger, 1976a; Lechner and Sumper, 1987). Nonetheless, present understanding of protein N-glycosylation in Archaea is lacking. Much of what is known of archaeal N-glycosylation reveals similarities to the parallel eukaryal process. *Hbt. salinarum* and *Haloferax volcanii* have been shown to contain phospho- and/or pyrophosphodolichol linked to a variety of monosaccharides, including glucose, mannose and GlcNAc, as well as a tetrasaccharide moiety containing mannose, galactose and rhamnose (Mescher *et al*., 1976; Kuntz *et al*., 1997). In addition, archaeal homologues of selected eukaryal enzymes involved in N-glycosylation have been described. For example, a GlcNAc transferase in *Hbt. salinarum* membranes has been partially characterized (Mescher *et al*., 1976), dolichylphosphate mannose synthase was purified from *Thermoplasma acidophilum* and partially characterized (Zhu and Laine, 1996), and a putative dolichylphosphate glucose synthase was identified in *Hfx. volcanii* homogenates (Zhu *et al*., 1995). Antibiotics that block various aspects of the eukaryal N-glycosylation process have also been shown to interfere with protein glycosylation in Archaea (Wieland *et al*., 1980; Bayley *et al*., 1993; Zhu *et al*., 1995; Grogan, 1996). Moreover, studies on halophilic Archaea have also provided insight into the localization of protein glycosylation, pointing to the external surface of the plasma membrane (Lechner and Wieland, 1989), the topological homologue of the lumen-facing leaflet of the ER membrane, as the site of this processing event. Accordingly, membranes from *Sulfolobus acidocaldarius* contain an externally oriented pyrophosphatase activity possibly involved in the recycling of dolicholpyrophosphate oligosaccharide carriers (Meyer and Schafer, 1992).

Despite these similarities, numerous aspects of archaeal N-glycosylation find no parallel in Eukarya. While glycans are linked to Asn residues of eukaryal glycoproteins almost exclusively through GlcNAc, Archaea can use other linking sugars (Lechner and Wieland, 1989). The eukaryal glycosylation machinery modifies the Asn residue of the Asn–Xaa–Ser/Thr motif, where Xaa is any residue apart from Pro (Kornfeld and Kornfeld, 1985; Burda and Aebi, 1999; Spiro, 2002; Helenius and Aebi, 2004). In *Hbt. salinarum*, however, it was reported that exchanging the Ser residue of an Asn–Ala–Ser motif within the S-layer glycoprotein sequence with a Val, Leu or Asn did not prevent N-glycosylation (Zeitler *et al*., 1998). Together, these observations suggest that the archaeal oligosaccharide transferase works differently from its eukaryal counterpart or could point to the existence of a second oligosaccharide transferase that functions in a novel manner. In further distinction from the eukaryal N-glycosylation process, in which the lipid-linked oligosaccharide is not chemically modified (Varki, 1998), transient and permanent modification of the dolichol-linked glycan have been observed in *Hbt. salinarum* and *Methanothermus fervidus* respectively (Lechner *et al*., 1985; Karcher *et al*., 1993). Finally, the N-linked glycan moieties of archaeal glycoproteins are smaller yet often more varied in content than those found in eukaryal glycoproteins (Eichler and Adams, 2005).

Thus, like much of archaeal biology, the process of N-glycosylation in Archaea reveals aspects common to the eukaryal and bacterial systems yet also invokes traits unique to this life form. As a first step towards a systematic analysis of archaeal N-glycosylation, the following study has identified open reading frames (ORFs) putatively involved in N-glycosylation in the halophile *Hfx. volcanii* and addressed their expression profiles under various growth conditions. Using gene deletion techniques, the essential nature of several of these genes was considered as was their involvement in the glycosylation of a reporter glycoprotein, the S-layer glycoprotein.

Results

Haloferax volcanii *contains genes responsible for the glycan-charging of lipids used in protein N-glycosylation*

Towards deciphering the molecular pathway of N-glycosylation responsible for modification of archaeal proteins, a partially completed *Hfx. volcanii* genome sequence (http://www.tigr.org; September 2005) was scanned for the presence of sequence homologues to genes implicated in various steps of eukaryal or bacterial N-glycosylation (Fig. 1). Using various human, mouse, fungal or bacterial gene sequences as probes in BLAST searches, putative *Hfx. volcanii* homologues, deemed as those sequences with an *e*-value of $<10e^{-5}$ and a local alignment score of 100, were identified, often in multiple copies (Table 1). Such an approach revealed that *Hfx. volcanii* apparently contains two genes homologous to human *alg5* and four homologues to mouse *dpm1*, respectively encoding proteins responsible for loading activated glucose and mannose residues onto dolichol carriers in the ER membrane (Burda and Aebi, 1999). In addition to these *alg5*- and *dpm1*-like sequences, single apparent homologues of fungal *alg2* and *alg11*, the products of which serve to add mannose residues to the GlcNAc-charged dolichol structure in the cytoplasmic leaflet of the ER membrane (Kornfeld and Kornfeld, 1985;

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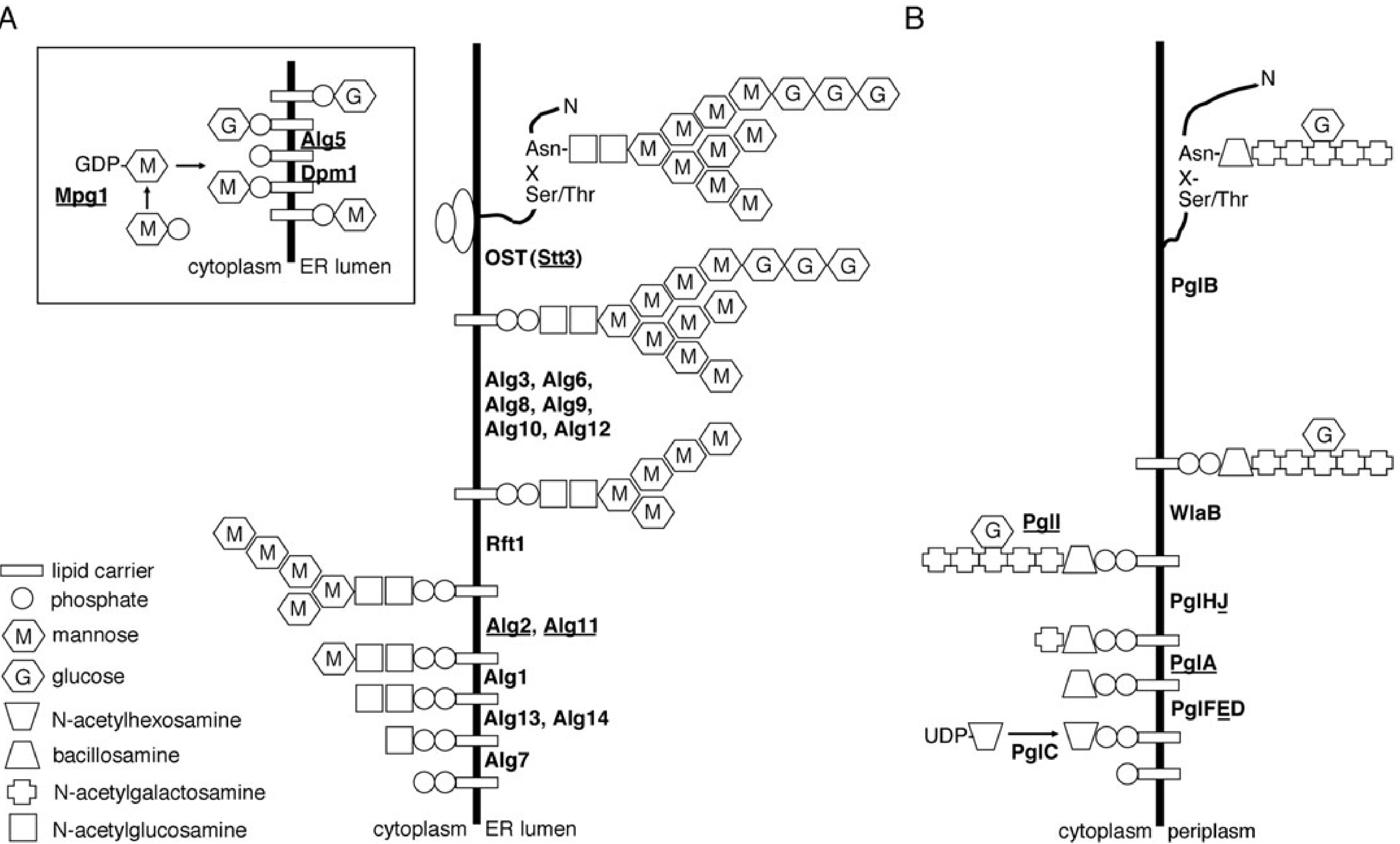

Fig. 1. N-glycosylation pathways in Eukarya and Bacteria. Schematic depictions of the steps and enzymes involved in protein N-glycosylation in (A) yeast and (B) *C. jejuni*. Those genes for which *Hfx. volcanii* homologues have been identified are underlined. Selected enzymes involved in activating sugars and loading activated sugars onto lipid carriers that are then flipped across the membrane before transfer to the polysaccharide-bearing dolichol carrier are shown in the inset of (A).

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Table 1. *Haloferax volcanii* contains ORFs homologous to eukaryal and bacterial genes involved in N-glycosylation.

Homologues to eukaryal genes		Homologues to *C. jejuni* genes	
Detected in *Hfx. volcanii*	Not detected in this study	Detected in *Hfx. volcanii*	Not detected in this study
alg2	*alg1*	*pglA*	*pglB*
alg5	*alg3*	*pglE*	*pglC*
alg11	*alg6*	*pglI*	*pglD*
dpm1	*alg7*	*pglJ*	*pglF*
mpg1	*alg8*		*pglG*
stt3	*alg9*		*pglH*
	alg10		*wlaB*
	alg12		
	alg13		
	alg14		
	rft1		

Burda and Aebi, 1999), were detected in *Hfx. volcanii*. Examination of the *Hfx. volcanii* genome also reveals the presence of sequences homologous to *C. jejuni* glycosyltransferases thought to be involved in N-glycosylation (Linton *et al*., 2005). These include apparent homologues to *pglA* and *pglJ*, the products of which are responsible for addition of *N*-acetylgalactosamine (GalNAc) residues to the growing undecaprenolpyrophosphate-linked polysaccharide structure, as well as to *pglI*, the product of which adds a glucose branch to this structure. Unexpectedly, *Hfx. volcanii* is also predicted to encode a homologue of *pglE*, the product of which is involved in the transformation of a *N*-acetylhexosamine subunit into bacillosamine, the linking sugar of the Asn-bound glycan moiety in *C. jejuni*. However, unlike the operon-like organization of those genes implicated in *C. jejuni* N-glycosylation (Wacker *et al*., 2002), the detected *Hfx. volcanii* genes putatively involved in N-glycosylation can either be found in a single major cluster or distributed throughout the genome.

Using the same stringent selection criteria, our sequence-based searches of the *Hfx. volcanii* genome failed to detect homologues of several other genes involved in eukaryal or bacterial N-glycosylation. Genes encoding Alg7, Alg13 or Alg14, involved in attaching GlcNAc residues to the dolichol carrier in the ER membrane (Kornfeld and Kornfeld, 1985), were not detected in the present search of *Hfx. volcanii*. Similarly, *Hfx. volcanii* homologues of *alg1*, involved in adding mannose subunits to the GlcNAc-bearing, ER-localized dolichol pyrophosphate entity (Kornfeld and Kornfeld, 1985; Burda and Aebi, 1999), were not identified. Homologues to genes encoding Alg3, Alg6, Alg8, Alg9, Alg10 or Alg12, proteins involved in the addition of mannose and glucose residues to the dolichol-bound oligosaccharide following its reorientation into the ER lumen of eukaryal cells (Kornfeld and Kornfeld, 1985), were not seen either. The *Hfx. volcanii* genome apparently does not contain homologues of the *pglC*, *pglD*, *pglF*, *pglG* or *pglH* genes, the products of which are implicated in assembly of the N-linked glycan moiety found on *C. jejuni* glycoproteins (Linton *et al*., 2005). Finally, the present search of the *Hfx. volcanii* genome also failed to detect homologues to yeast *rft1* or *C. jejuni wlaB*, the products of which are implicated in reorientation of the lipid-linked oligosaccharide across the ER and plasma membranes respectively (Helenius *et al*., 2002; Wacker *et al*., 2002).

Only the Stt3 subunit of the eukaryal oligosaccharide transferase complex is detected in Hfx. volcanii

The transfer of the completed oligosaccharide structure from its dolichol carrier to selected Asn residues of nascent eukaryal polypeptides in the ER lumen is mediated by the OST complex (Silberstein and Gilmore, 1996; Yan and Lennarz, 2005). Whereas the roles played by most of the components of this multimer are presently unknown, the yeast Stt3 subunit and its homologues in other eukaryotes are thought to be responsible for the actual transfer (Zufferey *et al*., 1995; Nilsson *et al*., 2003). In *C. jejuni*, oligosaccharide transferase activity is fulfilled by a single polypeptide, the Stt3 homologue, PglB (Feldman *et al*., 2005). Sequence alignment of Stt3 homologues from various eukaryal and archaeal sources as well as *C. jejuni* PglB reveals a five-residue motif (WWDXG) implicated in the transferase activity of the proteins (Wacker *et al*., 2002; Yan and Lennarz, 2002). In *Hfx. volcanii*, only a single sequence containing this motif was observed. Topology prediction software (http://www.expasy.org) describes *Hfx. volcanii* Stt3 as a multispanning membrane protein with the C-terminal half of the protein, including the WWDYG motif (amino acid residues 638–642), exposed to the cell exterior. Sequence comparisons suggest, furthermore, that the *Hfx. volcanii* protein is more similar to its eukaryal than its *C. jejuni* counterpart. No homologues of other subunits of the eukaryal OST were detected in *Hfx. volcanii* nor indeed in any other Archaea examined thus far, although *Archaeo-*

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globus fulgidus reportedly encodes for two Stt3 proteins (Burda and Aebi, 1999).

ORFs putatively involved in Hfx. volcanii *N-glycosylation also exist in other haloarchaea*

Having identified ORFs putatively involved in N-glycosylation in the *Hfx. volcanii* genome, the presence of homologous sequences was next considered in those halophilic archaea for which complete genome sequence information is available, i.e. *Halobacterium* sp. NRC-1 (Ng *et al*., 2000), *Haloarcula marismortui* (Baliga *et al*., 2004) and *Natronomonas pharaonis* (Falb *et al*., 2005). As presented in Table 2, homologues were detected for the majority of *Hfx. volcanii* sequences identified. Of those sequences that share homology with eukaryal genes, current annotation of the completed haloarchaeal genomes also predicts functions related to protein N-glycosylation in many cases. For instance, the different *dpm1* homologues detected in *Hfx. volcanii* are annotated as a galactosyltransferase, a glycosyltransferase, a dolichol-phosphate-glucose synthetase or a dolichyl-phosphate-β-D-mannosyltransferase in the completed haloarchaeal genomes. Similarly, the *Hfx. volcanii* sequences homologous to eukaryal *alg5* are annotated as glycosyltransferases or dolichol-phosphate-glucose-transferases in the other haloarchaea. In other cases, those sequences thought to participate in *Hfx. volcanii* N-glycosylation are annotated as encoding for enzymes involved in other saccharide-based processes. For example, some homologues of *mpg1* in *Hfx. volcanii* are predicted to be glucose 1-phosphate thymidylyltransferases in other genomes.

The current annotations of the *Halobacterium* sp. NRC-1, *Har. marismortui* and *Nmn. pharaonis* genomes were also analysed to determine whether any genes had been identified and annotated as being involved in N-glycosylation in these genomes, yet overlooked in the current search of the *Hfx. volcanii* genome. Accordingly, *Har. marismortui* was shown to contain an addition annotated *dpm1* homologue not identified in our bioinformatics-based searches. A homologous sequence, termed *Hdpm1*, was subsequently shown to also exist in *Hfx. volcanii*. In contrast, no such homologue was detected in either *Halobacterium* sp. NRC-1 or *Nmn. pharaonis*.

When the predicted *Hfx. volcanii* homologues of genes encoding components involved in bacterial N-glycosylation were used to search the completed haloarchaeal genomes, the annotations assigned to the obtained sequences predict functions other than those pertaining to N-glycosylation. Moreover, for one sequence for which a homologue of a *C. jejuni* sequence was detected in *Hfx. volcanii*, i.e. *pglE*, no homologues were noted in *Halobacterium* sp. NRC-1, *Har. marismortui* or *Nmn. pharaonis*.

Table 2. Distribution and current annotation of putative N-glycosylation genes in haloarchaea.

Gene[a]	*Hfx. volcanii*	*Halobacterium* sp. NRC-1		*Har. marismortui*		*Nmn. pharaonis*	
	No.	No.	Annotated functions	No.	Annotated functions	No.	Annotated functions
Eukaryal genes							
Sugar nucleotide transferases							
mpg1	5	4	Glucose 1-P thymidylyltransferase, dTDP-glucose pyrophosphorylase	4	LPS glycosyltransferase, glucose 1-P thymidylyltransferase	4	Sugar nucleotidyltransferase, glucose-1-P thymidylyltransferase
Glycosyltransferases							
alg2	1	1	LPS glycosyltransferase	1	Glycosyltransferase	1	Hexosyltransferase
alg5	2	2	Dolichol-P-glucose-transferase	2	Dolichol-P-glucose-transferase	2	Glycosyltransferase
alg11	1	1	Hypothetical protein	1	LPS glycosyltransferase	1	Hexosyltransferase
dpm1	4	2	Galactosyltransferase, glycosyltransferase	4	Glycosyltransferase, dolichol-P-glucose synthetase	2	Glycosyltransferase, dolichyl-P-β-D-mannosyltransferase
Hdpm1[b]	1	–		1	Dolichyl-P-β-D-mannosyltransferase	–	
Oligosaccharyltransferase							
stt3	1	1	Oligosaccharyltransferase	1	Oligosaccharyltransferase	1	Oligosaccharyltransferase
Bacterial genes							
pglA	2	1	Hypothetical protein	1	LPS glycosyltransferase	1	Glucose 1-P thymidylyltransferase
pglE	1	–		–		–	
pglI	1	1	Hypothetical protein	1	Hypothetical protein	1	Glucosyltransferase
pglJ	1	1	LPS biosynthesis (*rfbU2*)	1	LPS biosynthesis	1	Hexosyltransferase

a. Sequences of eukaryal and bacterial genes shown or proposed to be involved in protein N-glycosylation were used as templates in BLAST searches of the *Hfx. volcanii*, *Halobacterium* sp. NRC-1, *Har. marismortui* and *Nmn. pharaonis* genomes.
b. Annotated *Har. marismortui* ORF used as template in BLAST search of the *Hfx. volcanii*, *Halobacterium* sp. NRC-1 and *Nmn. pharaonis* genomes.

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Table 3. *Haloferax volcanii* ORFs for which RNA is transcribed.

Homologues to eukaryal genes					Homologues to *C. jejuni* genes	
Gene	Log phase	Stationary phase	Low salt	Heat shock	Gene	Log phase
alg5-A	+	–	–	+	*pglA-A*	+
alg5-B	+	–	+	+	*pglA-B*	+
alg11	+	nd	nd	+	*pglE*	+
					pglI	+
dpm1-A	+	+	+	+	*pglJ*	+
dpm1-B	+	–	+	+		
dpm1-C	+	–	+	+		
dpm1-D	+	+	+	+		
Hdpm1[a]	–	–	–	+		
mpg1-A	+	+	+	+		
mpg1-B	+	+	+	+		
mpg1-C	+	+	+	+		
mpg1-D	+	–	+	+		
mpg1-E	+	–	–	+		
stt3	+	–	+	+		

a. Homologue of an annotated *Har. marismortui dpm1* gene.
nd, not determined.

Finally, examination of haloarchaeal genomes also revealed the presence of a single annotated oligosaccharide transferase, homologous to *Hfx. volcanii stt3*.

Haloferax volcanii *ORFs putatively involved in N-glycosylation are differentially expressed*

Having used bioinformatics approaches to identify various ORFs putatively involved in N-glycosylation in *Hfx. volcanii*, efforts were next directed at confirming that these sequences correspond to true genes. As transcription of mRNA offers strong support that a given ORF encodes an expressed protein, a series of reverse transcription polymerase chain reaction (RT-PCR) experiments was performed. In these experiments, the RNA content of *Hfx. volcanii* cells was isolated, with any extraneous DNA co-captured digested with DNase. The isolated RNA was then employed as a template in the synthesis of single-stranded cDNA, using reverse transcriptase. This cDNA served, in turn, as the template in PCR reactions that also included primers directed to the particular gene sequence being addressed. To control for the appearance of PCR products due to contaminating DNA that survived the DNase treatment, PCR was performed on RNA samples not exposed to reverse transcriptase. Finally, the obtained PCR products were subjected to sequence analysis to confirm their identities.

As summarized in Table 3, RNA for most, but not all, *Hfx. volcanii* sequences implicated in N-glycosylation was transcribed in cells grown to mid-logarithmic phase ($OD_{550} = 1.0$) in complete medium. RNA was detected for four of the five *Hfx. volcanii dpm1* homologues (Fig. 2A); no mRNA was transcribed for *Hdpm1*, that sequence annotated as *dpm1* in the *Har. marismortui* genome but not identified in our initial search of *Hfx. volcanii* (see above). Hence, given that a member of the *dpm1* gene family was not expressed under the growth conditions considered, a series of experiments was performed to determine whether transcription of any of the *dpm1* sequences could be developmentally or environmentally regulated. As such, *Hfx. volcanii* cells were grown to stationary phase, in hyposaline conditions or at elevated

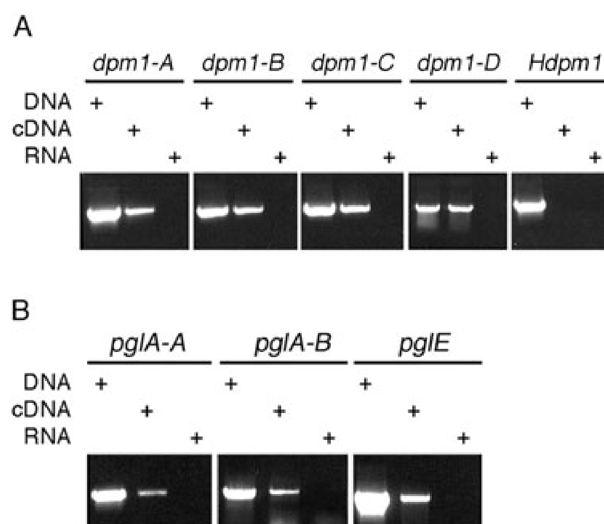

Fig. 2. RT-PCR reveals that *Hfx. volcanii* homologues of eukaryal and bacterial genes involved in N-glycosylation are transcribed. RNA from *Hfx. volcanii* cells grown in complete medium to mid-exponential phase was isolated and used to prepare cDNA. The cDNA then served as template in PCR amplifications using primers against *Hfx. volcanii* (A) *dpm1-A*, *dpm1- B*, *dpm1-C*, *dpm1-D* or *Hdpm1*; (B) *pglA-A*, *pglA-B* or *pglE*. In each case, PCR amplifications were also performed using total DNA as a positive control and total RNA as a negative control.

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temperatures. In each case, RT-PCR was performed using primers directed against the different *dpm1* sequences. In *Hfx. volcanii* cells grown to stationary phase ($OD_{550} = 3.5$), only mRNA encoding for *dpm1-A* and *dpm1-D* was transcribed. In contrast, mRNA for *dpm1-B* and *dpm1-C*, transcribed in cells grown to mid-logarithmic phase, was not detected. As observed with cells grown to mid-logarithmic phase, no mRNA encoding *Hdpm1* was detected in cells grown to stationary phase. When the cells were grown in low-salt medium containing 1.75 M NaCl (i.e. half the concentration in the complete medium), the same pattern of *dpm1* gene transcription as obtained in cells grown to logarithmic phase was observed. To address gene expression upon heat shock, *Hfx. volcanii* cultures were transferred to a 60°C environment for 45 min, after which time RT-PCR was performed. Such exposure to this elevated temperature resulted in the transcription of all *dpm1* genes, including *Hdpm1*.

Differential RNA transcription was also observed when RT-PCR was performed on *Hfx. volcanii* cells grown under various conditions using primers directed at members of the *mpg1* and *alg5* families. In cells grown to logarithmic phase or subjected to heat shock, all five *mpg1* genes were transcribed. Cells grown to stationary phase only transcribed *mpg1-A*, *mpg1-B* and *mpg1-C* while at low-salt concentrations, all except *mpg1-E* were transcribed. In the case of *alg5-A* and *alg5-B*, both genes were transcribed in cells grown to logarithmic phase, yet neither was transcribed in cells grown to stationary phase. In cells grown in low-salt conditions, *alg5-B* but not *alg5-A* was transcribed, while both were transcribed in cells exposed to elevated temperatures.

To address the transcription of *Hfx. volcanii stt3*, RNA was once again isolated from cells at different developmental stages (i.e. logarithmic or stationary phases) or from cells exposed to various environmental conditions (i.e. low salt or heat shock) and used to generate cDNA that was subsequently employed in PCR amplifications. As summarized in Table 3, the *Hfx. volcanii stt3* gene was transcribed under the various environmental conditions tested, suggesting the central role of the encoded protein in the growing cell (see below). No *stt3* transcription was detected in stationary-phase cells.

Finally, of the *Hfx. volcanii* sequences identified as homologues to *C. jejuni* genes implicated in N-glycosylation, RNA was transcribed for both *pglA*-like sequences as well as for the *pglE*, *pglI* and *pglJ* homologues (Fig. 2B).

In Hfx. volcanii*, deletion of* alg11 *is lethal*

Employing a recently developed protocol for gene deletion in *Hfx. volcanii*, the essential nature of several genes implicated in N-glycosylation was considered. In this approach, DNA encoding the upstream and downstream flanking 400–500 nucleotides of the gene to be deleted, separated by the *Hfx. volcanii* tryptophan synthase-encoding *trpA* gene, is inserted into the *pyrE*-containing vector, plasmid pTA131 (Allers *et al*., 2004), where *pyrE* encodes for orotate phosphoribosyl transferase, an enzyme involved in uracil biosynthesis. The plasmid is then integrated into the genome of the uracil and tryptophan auxotrophic *Hfx. volcanii* strain WR536 (Allers *et al*., 2004) by plating onto casamino acids lacking uracil and tryptophan. To replace the native gene of interest with the plasmid-containing genetic material, i.e. the flanking regions of that gene separated by the *trpA* sequence, the transformed cells are grown without tryptophan but in the presence of uracil and 5-fluoroorotic acid (5-FOA), a toxic uracil analogue. As the transformed cells become resistant to 5-FOA only in the absence of *pyrE*, the integrated *pyrE*-containing plasmid is expelled, along with the native gene being addressed, which is now replaced in the genome by *trpA*.

To test the essential nature of *alg11*, a plasmid for replacement of the gene with the *Hfx. volcanii trpA* sequence was devised (Table 4). A series of PCR amplifications was then undertaken to follow the integration of the plasmid-encoded *trpA* as well as the expulsion of *alg11* from transformed cells. When genomic DNA extracted from cells transformed with the plasmid served as the template in a PCR amplification together with primers raised against the flanking regions of *alg11*, plasmid integration was revealed (Fig. 3, lane 2). When tryptophan was deleted from the growth medium of cells incorporating the plasmid encoding replacement of *alg11* with *trpA* at the same time as uracil and 5-FOA were added, i.e. conditions under which the native *alg11* gene is expelled, no cell growth was detected (lane 3). If, however, the plasmid-integrating cells were transferred to medium containing tryptophan, uracil and 5-FOA, the integrated plasmid was expelled from the genome, leaving the native *alg11* locus intact and thus ensuring cell survival (lane 4).

Deletion of alg5-A *hinders* Hfx. volcanii *cell growth and S-layer glycoprotein glycosylation*

In contrast to the lethal effects of *alg11* deletion, the Alg5-A- and Alg5-B-encoding genes could be readily replaced by the plasmid-encoded *Hfx. volcanii trpA* gene upon transfer of the transformed cells to tryptophan-free, uracil- and 5-FOA-containing growth medium (Fig. 4A, lane 3 of each panel). To confirm that the native Alg5-A- or Alg5-B-encoding genes had indeed been replaced in cells of the deletion strains, RT-PCR was performed using primers directed against either *alg5-A* or *alg5-B*

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Table 4. Primers used in this study.

	Forward primers	Reverse primers
Homologues to eukaryal genes		
alg5-A	ATGGGTCGCCCGACAGAACG	TCACTTCGAGTCAGCGAGTTCGTG
alg5-B	ATGTCGCAGTCTGTCGGGGTG	TCAGTCATCCGCGACCTCC
alg11	ATGCGGGTCGTCTGCGAGGACGGTT	TCAGTTCGCGTCCGACTCGGCGGCGTCCG
dpm1-A	ATGAGCACGAGATCCCAATCAG	TTATTCCCCAAGAGAGCCCAACAG
dpm1-B	ATGACTTCGACTCTACCGTTTG	TCATTGTTCCTCCGATCTTAG
dpm1-C	ATGCCCACCCCCGATGCCGTC	TCACTCCAGTTCTTCGATTC
dpm1-D	ATGACCGAGTACACCTTCGACGACC	TCAGAGTCGGAGCCGTTCTTCC
Hdpm1	ATGTATCGCGGTAACGCCGTGG	CTAACCCCCGATTACCTCGC
mpg1-A	ATGAAAGGCGTACTTCTCTC	TTAGAGTTTCAGTTGGGAGT
mpg1-B	ATGCAAGCTGTTGTCCTCGC	CTACTCGGTCGCCTGTGTC
mpg1-C	ATGAAGGCAGTCGTCCTTGCC	TTACTGCCCGTTCGTCAAC
mpg1-D	ATGCAGACAGTCATTCTCG	TCACTCGTCTCTCCCGTTCG
mpg1-E	ATGAACGGCCGTCTCACCG	TCAGTGAGTCACCTCCACACGC
stt3-I(1-1632)	ATGAGTGACGAGCAAACAAAGTATTCG	CCCATACGAGATGTCGTCGAAGCG
stt3-II(1472-3039)	GGGCAGCCATCATCACGTCGGCAG	CGGCAGGTCGAGTTCCTGTTCGTTC
Homologues to bacterial genes		
pglA-A	ATGAAGCCCAAGCAAACACTC	CTACGCCGCCTCGGCGTACAT
pglA-B	ATGTCTAGTGACTACTCGAAC	CTACCTTTGTTTAGTGAGTTG
pglE	ATGAGTGCAGACCACATCTC	TCACCTCGCCACCTCCGCGTAGT
pglI	ATGGCTGATTCTCCGTTTCC	TCAGCGGGTGTTCCCGCGAACG
pglJ	GACCGCCTCGCCGATACCG	GTCACCGATGACCTCACTCGCC
Deletion primers		
alg5-A 5′ up	gggctcgagGAAGTCGCCGTCGAGTATGG	cccaagcttCGTCGTATGGCCGCTATCC
alg5-A-3′ down	gggggatccCGTGCGCAGTCGGCTCAGT	ccctctagaCGAACGAACCTGCCCCGAC
alg5-B 5′ up	gggctcgagCGCGTCGGTCGATGTCGAG	cccaagcttGCACGACCATTCACTCAATGGG
alg5-B 3′ down	gggggatccTCGGCTCTCGGCGCTCGTCT	ccctctagaGACGTAGTTCAGGAGCATTGAC
alg11 5′ up	gggctcgagGCGCAGACTCAGCAGTCGTAG	cccaagcttTCGCCGCCCGCGAGGACCGC
alg11 3′ down	gggggatccCGGAGTCGCGGAGTCGGCAC	ccctctagaGGCGAATCACTAAGTGCTCG
stt3 5′ up	gggctcgagGGTTTGCGAGCGACCCAGTCG	cccaagcttTGTGACCAACAACCGCCAAG
stt3 3′ down	gggggatccCACGAGCCGAGACGGCGACGA	ccctctagaGCGCGTCGCCGTGCTCGGAC
trpA	gggaagcttCGTGGATAAAACCCCTCGTTG	

Genomic DNA sequences are in capitals.

(Fig. 4B, top and bottom respectively). In each case, control experiments, performed using genomic DNA from untreated cells as PCR template, yielded a single band, corresponding to either *alg5-A* (top, lane 1) or *alg5-B* (bottom, lane 1). Bands respectively corresponding to *alg5-A* or *alg5-B* were also detected when cDNA prepared from the *alg5-B* deletion strain was used as PCR template in a reaction including primers directed against *alg5-A* (top right, lane 4) or using cDNA prepared from the *alg5-A* deletion strain in a reaction including primers directed against *alg5-B* (bottom left, lane 4). In contrast, no such bands appeared when amplification was performed using cDNA obtained from the *alg5-A* deletion strain as the PCR template together with primers directed against *alg5-A* (top left, lane 4) or using cDNA obtained from the *alg5-B* deletion strain together with primers directed against *alg5-B* (bottom right, lane 4). Similarly, no bands were detected in any reactions when RNA served as PCR template (lane 3 of each panel). Thus, the absence of *alg5-A* and *alg5-B* from the respective deletion strain was confirmed at both the DNA and RNA levels.

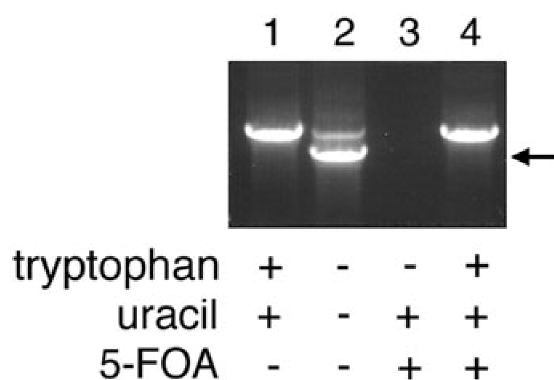

Fig. 3. Gene deletion identifies *Hfx. volcanii alg11* as an essential gene. PCR amplification was performed using primers directed against stretches within the flanking regions of *alg11* and as template, DNA isolated from background strain cells (lane 1), plasmid-incorporating cells (lane 2), cells from which the plasmid and native *alg11* (lane 3) or the plasmid and *trpA* gene (lane 4) were expelled. The arrow on the left depicts the position of the plasmid-encoded material used to replace the native gene.

Having shown that neither *alg5-A* nor *alg5-B* is essential for *Hfx. volcanii* survival, the effects on cell growth resulting from deletion of these genes was addressed. As shown in Fig. 5A, *Hfx. volcanii* cells lacking either *alg5-A* or *alg5-B* grew considerably slower in complete medium than did the background WR536 strain, with the *alg5-A*-

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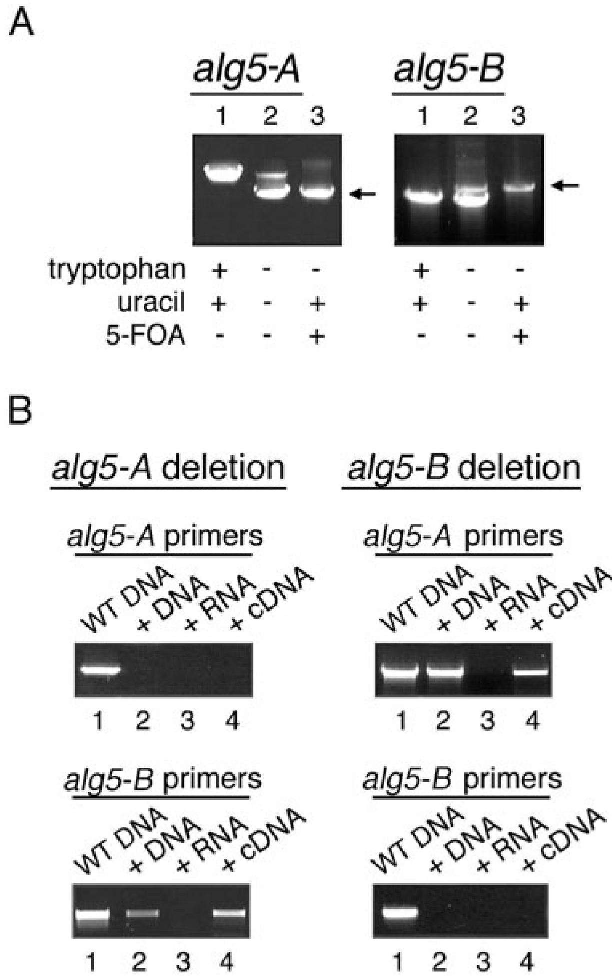

Fig. 4. Gene deletion shows that neither *alg5-A* nor *alg5-B* is essential for *Hfx. volcanii* survival.
A. PCR amplification was performed using primers against the flanking regions of either *alg5-A* (left) or *alg5-B* (right) and as template, DNA isolated from background strain cells (lane 1), from plasmid-incorporating cells (lane 2), or from cells in which the plasmid and native *alg5-A* or *alg5-B* had been expelled (lane 3).
B. RT-PCR was performed using primers against *alg5-A* (top) or *alg5-B* (bottom). As template, DNA (lane 2), RNA (lane 3) or cDNA (lane 4) from cells lacking either *alg5-A* (left) or *alg5-B* (right) were used. In each panel, DNA from background strain cells served as template in control PCR amplifications (lane 1).

lacking strain growing somewhat slower than the *alg5-B*-deleted cells (top and bottom respectively).

The effect of absence of *alg5-A* on the biogenesis of the S-layer glycoprotein, a well-characterized reporter of *Hfx. volcanii* protein glycosylation (Sumper *et al*., 1990; Mengele and Sumper, 1992), was next considered. Equivalent amounts of the WR536 background strain or cells deleted of *alg5-A* were subjected by SDS-PAGE and the separated proteins were probed by Coomassie blue or periodic acid-Schiff reagent staining (Fig. 5B). Although Coomassie staining revealed that both strains contained similar amounts of S-layer glycoprotein, differences in apparent molecular weight were apparent, with the S-layer glycoprotein from the deletion strain migrating faster than the same protein in the background strain (top). When the S-layer glycoprotein from each cell type was glycostained by periodic acid-Schiff reagent, only the reporter from the background strain was labelled (middle).

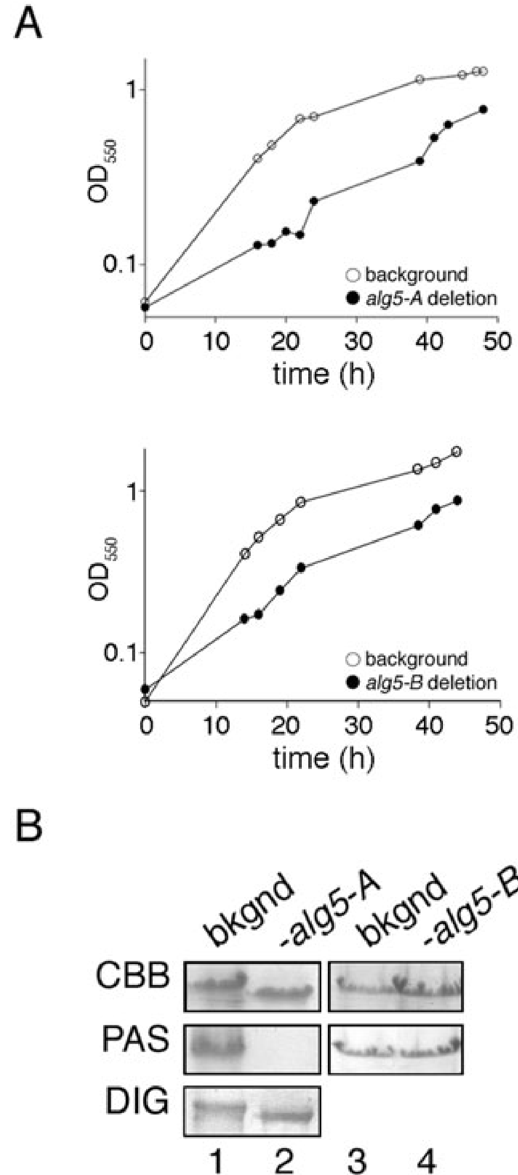

Fig. 5. The effects on *Hfx. volcanii* cell growth and protein glycosylation of *alg5-A* or *alg5-B* deletion.
A. *Haloferax volcanii* WR356 cells containing or lacking *alg5-A* (top) or *alg5B* (bottom) were grown in complete media. Growth was measured at an optical density at 550 nm (OD_{500}). Shown are values obtained from one of two repeats.
B. Equivalent aliquots of *Hfx. volcanii* WR356 cells (bkgnd) (lanes 1 and 3) and *Hfx. volcanii* WR356 cells lacking either *alg5-A* (lane 2) or *alg5-B* (lane 4) were separated by 7.5% SDS-PAGE and either Coomassie blue-stained (CBB; top) or glycan-stained with periodic acid-Schiff reagent (PAS; middle) or a DIG-based reagent (DIG; bottom).

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When, however, a more sensitive digoxigenin (DIG)-based glycan staining technique was employed, the S-layer glycoprotein in both the background and deletion strains was labelled (bottom). Such DIG-based staining moreover confirmed the differences in apparent molecular weight between the S-layer glycoproteins in the background and the deletion strains noted upon Commassie staining (compare top and bottom).

In contrast, despite the detrimental effect of its absence on cell growth (Fig. 5A, bottom), deletion of *alg5-B* had no apparent effect on *Hfx. volcanii* S-layer glycoprotein processing, as judged by the comparable migration of the protein in both the background and deletion strains in SDS-PAGE, as well as by the comparable periodic acid-Schiff reagent glycostaining of the protein in each cell type (Fig. 5B, top and middle, compare lanes 3 and 4). Thus, it seems that the absence of *alg5-A* but not of *alg5-B* interferes with *Hfx. volcanii* S-layer glycoprotein glycosylation.

Deletion of stt3 *does not affect cell viability but hinders cell growth and S-layer glycoprotein glycosylation*

The essential nature of *stt3*, encoding the only member of the eukaryal OST detected in *Hfx. volcanii*, was next considered. As with *alg11*, *alg5-A* and *alg5-B* above, Stt3-encoding DNA was replaced with the *Hfx. volcanii trpA* sequence in the deletion strain. Once again, PCR amplification was performed to follow genome integration of the introduced plasmid as well as the subsequent expulsion of the plasmid together with *stt3*. However, given the difficulty in amplifying the extremely long (3039 nucleotides) *stt3* sequence, the fate of the gene was followed by dual PCR amplifications using forward primers raised against internal sequences of either *stt3* or *trpA* and a reverse primer directed against a sequence within the flanking region downstream to *stt3*. Using these primers, plasmid integration was confirmed (Fig. 6A, lanes 2a and 2b). When the transformed cells were transferred to conditions in which both the integrated plasmid and the native *stt3* gene were expelled, only the primer pair directed against an internal sequence of *trpA* and the flanking region downstream to *stt3* yielded a PCR amplification product (lane 3b). No PCR product was obtained using the second primer pair directed against an internal sequence of *stt3* and the flanking region downstream to *stt3* (lane 3a), pointing to replacement of *stt3* by *trpA*. Deletion of *stt3* was further confirmed when PCR amplification was performed using DNA from the deletion strain as template and primers directed against the *stt3* coding region (Fig. 6B).

The absence of *stt3* was next ascertained at the RNA level by RT-PCR. No amplification product was detected in reactions employing either genomic DNA or cDNA obtained from *stt3*-deleted cells as template, together with primers directed against the Stt3-encoding gene (Fig. 6C, top, lanes 2 and 4 respectively). Similarly, no such bands were seen either when RNA served as template (lane 3). A PCR product of the expected size was only obtained when DNA from background cells served as template (lane 1). In contrast, when the same experiment was repeated using primers directed against *sec11b*, an essential gene encoding a type I signal peptidase in *Hfx. volcanii* (Fine *et al*., 2006), the expected PCR product was obtained using either total DNA or cDNA (Fig. 6C, bottom, lanes 2 and 4 respectively) or DNA from background cells as template (lane 1).

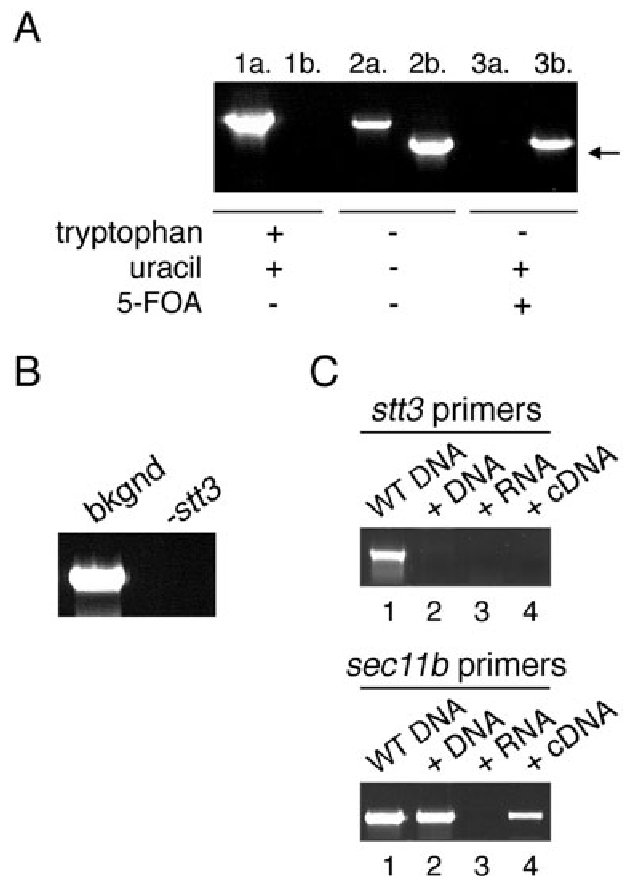

Fig. 6. *Haloferax volcanii stt3* is not an essential gene.
A. PCR amplification was performed using a forward primer against either an internal *stt3* sequence (lanes 1a, 2a and 3a) or an internal *trpA* sequence (lanes 1b, 2b and 3b) and a reverse primer directed against a sequence within the downstream flanking region of *stt3*. As template, DNA isolated from background strain cells (lanes 1a and 1b), from plasmid-incorporating cells (lanes 2a and 2b), or from cells in which the plasmid and native *stt3* had been expelled (lane 3a and 3b).
B. PCR amplification was performed using primers against the *stt3* coding sequence together with genomic DNA isolated from the background strain (bkgnd) or the *stt3* deletion strain (–*stt3*) as template.
C. RT-PCR was performed using primers against *stt3* (top) or *sec11b* (bottom). As template, DNA (lane 2), RNA (lane 3) or cDNA (lane 4) from cells lacking *stt3* was used. In each panel, DNA from background strain cells served as template in control PCR amplifications (lane 1).

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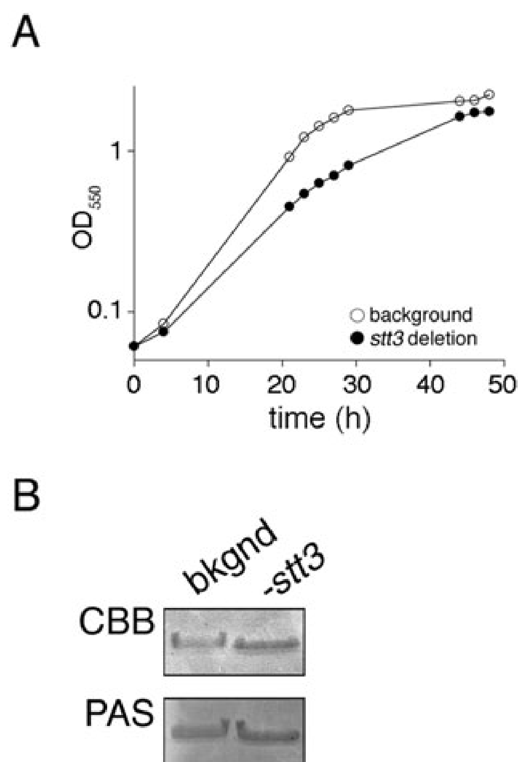

Fig. 7. Deletion of *Hfx. volcanii stt3* affects cell growth and the apparent molecular weight of the S-layer glycoprotein.
A. *Haloferax volcanii* WR356 cells containing or lacking *stt3* were grown in complete medium. Growth was measured at an optical density at 550 nm (OD_{500}). Shown are values obtained from one of three repeats.
B. Equivalent aliquots of *Hfx. volcanii* WR356 cells (bkgnd) and *Hfx. volcanii* WR356 cells lacking *stt3* (–*stt3*) were separated by 7.5% SDS-PAGE and either Coomassie blue-stained (CBB; top) or glycan-stained with periodic acid-Schiff reagent (PAS; bottom).

When the effect of *stt3* deletion on *Hfx. volcanii* cell growth was considered, it could be seen that the deletion strain grew slower than the background cells (Fig. 7A). Next, the effect of *stt3* deletion on S-layer glycoprotein glycosylation was addressed. Comparable amounts of the background and *stt3* deletion strains were examined by SDS-PAGE and probed by Coomassie blue or periodic acid-Schiff reagent staining (Fig. 7B). Coomassie staining revealed that the S-layer glycoprotein in the deletion strain migrated slightly faster than the S-layer glycoprotein in the background strain, reflecting a lower apparent molecular weight. When glycosylation of the S-layer glycoprotein was considered in the *stt3*-lacking cells, glycostaining comparable to that achieved in the background strain was observed.

Discussion

As completed genome sequences begin to accumulate, attention is being increasingly focused on describing living organisms in terms of their protein content. Much of this protein-based investigation addresses post-translational modification, a major source of proteomic complexity. Of the various processing events a newly synthesized polypeptide chain can experience, glycosylation is among the most prevalent. Although it has been predicted that glycan moieties are added to half of all eukaryal proteins (Apweiler *et al.*, 1999), such post-translational modification is not restricted to Eukarya. S-layer glycoproteins from halophilic archaea were the first reported examples of prokaryal glycoproteins (Mescher and Strominger, 1976b; Lechner and Sumper, 1987). Since, archaeal flagellins, transport proteins, secreted enzymes as well as other polypeptides have been shown to contain both N- and O-linked polysaccharides (Eichler and Adams, 2005). More recently, genes reportedly involved in protein glycosylation as well as bona fide O- and N-glycoproteins have also been observed in several bacterial strains (Szymanski and Wren, 2005). Nonetheless, glycoprotein research in prokaryotes remains in its infancy, with only a limited number of basic steps involved in prokaryotic glycosylation pathways described.

If, as proposed (Burda and Aebi, 1999; Helenius and Aebi, 2004), the complicated process responsible for protein N-glycosylation in Eukarya originated from a simpler archaeal system, then many of the core steps and central players involved in eukaryal protein N-glycosylation should also be present in Archaea. Accordingly, when the genome sequence of *Hfx. volcanii* was probed for the presence of genes involved in eukaryal N-glycosylation, putative homologues of several sequences were detected, including *alg11*, two versions of *alg5* and multiple copies of *mpg1* and *dpm1*. The profile of genes observed is in accord with previous analysis of the sugar content of *Hfx. volcanii* glycoproteins, where glucose, galactose and mannose were detected (Zhu *et al.*, 1995). However, given the relatively stringent definition of homology used in this study, it remains possible that many of the genes not identified in the present search of *Hfx. volcanii* indeed exist in this species, although less well conserved evolutionarily than the identified sequences. Moreover, it should be stressed that the assignment of the different sequences detected as glucosyltransferases, mannosyltransferases or other glycosyltransferases is based on the use of a limited number of bioinformatics tools. Given the challenge in correctly assigning function to glycosyltransferases based on sequence information alone (Coutinho *et al.*, 2003), it is well possible that the annotations made here will have to be modified following experimentation. As such, further study is required before it will be possible to directly compare the N-glycosylation pathways in the three domains of life.

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While the existence of *Hfx. volcanii* homologues to eukaryal genes involved in N-glycosylation suggests a common origin for the process in both domains of life, distinctions between the archaeal and eukaryal N-glycosylation processes are evident. The apparent absence of *Hfx. volcanii* genes encoding Alg3, Alg6, Alg8, Alg9, Alg10 or Alg12, proteins involved in modifying the lipid-bound oligosaccharide in Eukarya following its reorientation into the ER lumen (Kornfeld and Kornfeld, 1985), argues that the *Hfx. volcanii* N-glycosylation process assembles the complete polysaccharide chain within the confines of the cytoplasm. Such assembly of the lipid-linked oligosaccharide in Archaea is reminiscent of what transpires in the parallel bacterial system, as exemplified by *C. jejuni* (Szymanski *et al*., 2003). Accordingly, *Hfx. volcanii* encode homologues of genes involved in *C. jejuni* N-glycosylation such as *pglA*, *pglI* and *pglJ*. On the other hand, the presence of genes in *Hfx. volcanii* responsible for linking activated sugars to phosphodolichol carriers, together with detection of phosphodolichol-linked saccharides in Archaea (cf. Eichler and Adams, 2005), raises the possibility that the sugar subunits of lipid-linked oligosaccharide are transferred from dolichol-bound precursors in the cytosol, rather than being derived from soluble activated monosaccharides as takes place in the cytoplasm of *C. jejuni* (Szymanski *et al*., 2003) or during assembly of the cytoplasmically oriented, dolichol-bound heptasaccharide entity in Eukarya (Kornfeld and Kornfeld, 1985; Burda and Aebi, 1999; Spiro, 2002; Helenius and Aebi, 2004). Dolichol-linked monosaccharides are used for continuing modification of the lipid-linked oligosaccharide in Eukarya only following flipping of the structure across the ER membrane.

Archaeal protein N-glycosylation thus may not only present unique mechanistic properties, but also is able to adapt to conditions facing the cell. When the transcription profile of the *Hfx. volcanii* sequences implicated in N-glycosylation was addressed by RT-PCR, clear effects of the physiological status of the cell on mRNA expression were observed. For example, *Hfx. volcanii* cells in stationary phase no longer transcribe mRNA from almost any of the genes examined, in accord with the reduced protein synthesis that transpires at this stage (Zaigler *et al*., 2003; Ring and Eichler, 2004). Differential expression of several members of the proposed *dpm1* and *mpg1* gene families was observed not only as a function of growth phase, but also in response to environmental conditions. For instance, *Hdpm1* was only transcribed upon heat shock. Given that *Hdpm1* is found thus far only in *Hfx. volcanii* and *Har. marismortui*, it is conceivable that this gene is related to survival in the Dead Sea, from whence both species originate (Mullakhanbhai and Larsen, 1975; Oren *et al*., 1990).

The present report also revealed the involvement of at least two of the *Hfx. volcanii* genes addressed in this study, i.e. *stt3* and *alg5-A*, in the glycosylation of the S-layer glycoprotein. The glycosylation profile of the *Hfx. volcanii* S-layer glycoprotein has been extensively studied and was shown to comprise both N- and O-linked glycan structures (Sumper *et al*., 1990). Indeed, it has been proposed that the protein contains two distinct N-linked glycan structures (Mengele and Sumper, 1992). Whereas a glucose-galactose disaccharide is employed for hydrogen fluoride-sensitive O-glycosylation (Sumper *et al*., 1990), the seven proposed N-linked glycans are thought to contain either glucose alone or a mixture of glucose, galactose and idose (Sumper *et al*., 1990; Mengele and Sumper, 1992). Deletion of *stt3* resulted in enhanced migration of the S-layer glycoprotein, in agreement with earlier experiments on the *Hbt. salinarum* S-layer glycoprotein in which antibiotic treatment known to interfere with proper glycosylation led to a similar decrease in apparent molecular weight (Mescher and Strominger, 1976b). The ability of *Hfx. volcanii* to survive the deletion of *stt3* suggests that N-glycosylation is not an essential requirement in the species. Moreover, it would appear that the O-glycosylation experienced by the S-layer glycoprotein (Sumper *et al*., 1990) is responsible for glycan staining of the protein in the *stt3* deletion strain. The S-layer glycoprotein in the *alg5-A* deletion strain also migrated faster on SDS-PAGE than did the wild-type protein, again likely reflecting a decrease in apparent molecular weight due to reduced glycosylation. The failure of periodic acid-Schiff reagent to stain the S-layer glycoprotein in this strain further points to the detrimental effect of *alg5-A* deletion on glycosylation of the reporter. Glycosylation of the S-layer glycoprotein was not, however, totally blocked in the absence of *alg5-A* as the protein was labelled using a more sensitive glycan-staining technique. The failure of the periodic acid-Schiff reagent to stain the S-layer glycoprotein in the *alg5-A* deletion strain could thus reflect a role for Alg5-A, responsible for transferring activated glucose residues (Burda and Aebi, 1999), in both N- and O-glycosylation in *Hfx. volcanii*, where the S-layer glycoprotein contains N- and O-linked glycans that include glucose subunits (Sumper *et al*., 1990; Mengele and Sumper, 1992). The finding that deletion of *alg5-B* also resulted in slower cell growth but had no apparent effect on S-layer glycoprotein glycosylation suggests either that the activity absent in cells lacking this gene can be compensated for by another protein or that Alg5-B does not participate in S-layer glycoprotein glycosylation. Alternatively, it is possible that the methods of glycan analysis employed in the present study are unable to detect an effect on S-layer glycoprotein glycosylation in the *alg5-B* lacking cells. Finally, deletion of *alg11* resulted in a loss in viability, suggesting this gene as

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being essential. The essential nature of *alg11* but not of other genes implicated in N-glycosylation tested here could point to an additional role for this gene or to incorrect annotation on our part. The role of the product of this gene in *Hfx. volcanii* protein N-glycosylation thus awaits experimental verification.

With a set of genes putatively involved in N-glycosylation now identified in *Hfx. volcanii*, a proven technique for gene deletion (Bitan-Banin *et al*., 2003; Allers *et al*., 2004), a system for rapid purification of haloarchaeal proteins (Ortenberg and Mevarech, 2000; Irihimovitch and Eichler, 2003) and a well-characterized reporter protein in the form of the S-layer glycoprotein (Sumper *et al*., 1990; Mengele and Sumper, 1992; Konrad and Eichler, 2002), dissection of the archaeal N-glycosylation process can proceed at the molecular level.

Experimental procedures

Growth conditions

Haloferax volcanii WR536 was obtained from M. Mevarech (Tel Aviv University) and grown in complete medium containing 3.4 M NaCl, 0.15 M $MgSO_4 7H_2O$, 1 mM $MnCl_2$, 4 mM KCl, 3 mM $CaCl_2$, 0.3% (w/v) yeast extract, 0.5% (w/v) tryptone, 50 mM Tris-HCl, pH 7.2, at 40°C (Mevarech and Werczberger, 1985). In low-salt medium, only 1.75 M NaCl was added. In casamino acids medium, yeast extract and tryptone were replaced by casamino acids (Difco, Detroit, MI) at a final concentration of 0.5% (w/v). *E. coli* were grown in Luria–Bertani medium.

RT-PCR

To perform RT-PCR, specific forward and reverse oligonucleotide primers were designed for each *Hfx. volcanii* gene under consideration (Table 4). RNA isolation was carried out using an Easy-spin RNA extraction kit (Intron Biotechnology, Kyungki-Do, Korea), according to the manufacturer's instructions. RNA concentration was determined spectrophotometrically. After contaminating DNA was eliminated with a DNAFree kit (Ambion, Austin, TX), single-stranded cDNA was prepared for each sequence from the corresponding RNA (2 μg) using random hexamers (150 ng) in a SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). The single-stranded cDNA was then used as PCR template in a reaction containing the appropriate forward and reverse primer pairs. cDNA amplification was monitored by electrophoresis in 1% agarose gels. The sequences of the PCR products were determined to confirm their identity. In control experiments designed to exclude any contribution from contaminating DNA, PCR amplification was performed on total RNA prior to cDNA preparation.

Gene deletion

To test the essential natures of *Hfx. volcanii alg5-A*, *alg5-B*, *alg11* and *stt3* sequences, each gene was deleted as previously described (Allers *et al*., 2004). Briefly, strains in which one of these genes was replaced by *Hfx. volcanii trpA* were prepared using plasmids pIDT-Alg5A, pIDT-Alg5B, pIDT-Alg11 or pIDT-Stt3. To construct the plasmids, 400–500 bp fragments lying upstream from the first codon of the ORF containing the *Hfx. volcanii* gene of interest were PCR amplified using forward and reverse primers designed to introduce XhoI and HindIII restriction sites at the 5′- and 3′-ends of the fragments respectively. Fragments (400–500 bp) encoding the downstream flanking region of the ORF in question were PCR amplified using forward and reverse primers designed to introduce XbaI and BamHI restriction sites at the 5′- and 3′-ends of the fragments respectively. The amplified fragments were digested with XhoI and HindIII (upstream fragments) or XbaI and BamHI (downstream fragments) and purified by electrophoresis in 1% agarose gels and using a Nucleospin Extract kit (Macherey-Nagel, Duren, Germany). The appropriate upstream and downstream fragments were then ligated into plasmid pTA131 (Allers et al., 2004), predigested with the appropriate restriction enzymes. To generate plasmids pIDT-Alg5A, pIDT-Alg5B, pIDT-Alg11 or pIDT-Stt3, the *Hfx. volcanii trpA* gene was cloned by PCR from plasmid pTA132 (Allers et al., 2004), respectively, using forward and reverse plasmids trpAfor and trpArev. The cloned gene was inserted between the EcoRI and HindIII sites of the set of pTA131 plasmids containing the flanking regions. *Hfx. volcanii* WR536 was subsequently transformed with plasmid pIDT-Alg5A, pIDT-Alg5B, pIDT-Alg11 or pIDT-Stt3 and plated onto casamino acids medium. Transformants were screened for integration of the plasmid-derived genes at the corresponding locus by PCR analysis. Plasmids were excised by plating onto agar in casamino acid medium supplemented with 10 μg ml^{-1} uracil and 50 μg ml^{-1} 5-FOA.

Glycan detection

The glycan moiety of the S-layer glycoprotein was glycostained by periodic acid-Schiff reagent, as previously described (Dubray and Bezard, 1982) or by a DIG-based staining kit (Roche), employed according to the manufacturer's instructions.

Acknowledgements

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