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Glyco-engineering in *Archaea*: differential N-glycosylation of the S-layer glycoprotein in a transformed *Haloferax volcanii* strain

Doron Calo,[1] Ziqiang Guan[2] and Jerry Eichler[1]*

[1]*Department of Life Sciences, Ben Gurion University, Beersheva 84105, Israel.*

[2]*Department of Biochemistry, Duke University Medical Center, Durham, NC 27710, USA.*

Summary

Archaeal glycoproteins present a variety of N-linked glycans not seen elsewhere. The ability to harness the agents responsible for this unparalleled diversity offers the possibility of generating glycoproteins bearing tailored glycans, optimized for specific functions. With a well-defined N-glycosylation pathway and available genetic tools, the haloarchaeon *Haloferax volcanii* represents a suitable platform for such glyco-engineering efforts. In *Hfx. volcanii*, the S-layer glycoprotein is modified by an N-linked pentasaccharide. In the following, S-layer glycoprotein N-glycosylation was considered in cells in which AglD, the dolichol phosphate mannose synthase involved in addition of the final residue of the pentasaccharide, was replaced by a haloarchaeal homologue of AglJ, the enzyme involved in addition of the first residue of the N-linked pentasaccharide. In the engineering strain, the S-layer glycoprotein is modified by a novel N-linked glycan not found on this reporter from the parent strain. Moreover, deletion of AglD alone and introduction of the AglJ homologue from *Halobacterium salinarum*, OE2528R, into the deletion strain resulted in increased biosynthesis of the novel 894 Da glycan concomitant with reduced biogenesis of the pentasaccharide normally N-linked to the S-layer glycoprotein. These findings justify efforts designed to transform *Hfx. volcanii* into a glyco-engineering 'workshop'.

Introduction

It is now clear that *Archaea*, like *Eukarya* and *Bacteria*, are capable of N-glycosylation, the covalent addition of glycan moieties to select asparagine (Asn) residues of target proteins. Indeed, archaeal glycoproteins reveal diversity in the composition of their N-linked glycan moieties not seen elsewhere (for review, see Eichler and Adams, 2005; Calo *et al*., 2010a). Combining those agents responsible for generating the broad diversity seen in N-linked glycan composition together with the stability of archaeal proteins in the face of physically challenging surroundings (Danson and Hough, 1998) could lead to the creation of glycoproteins designed to function optimally under specific environmental conditions. Such efforts are of biotechnological significance in light of reports assigning archaeal glycosylation roles in enhancing protein solubility in hypersaline conditions (Mengele and Sumper, 1992), in offering protection to proteins exposed to highly acidic surroundings (Zahringer *et al*., 2000) and in contributing to thermoarchaeal protein stabilization (Albers *et al*., 2004). Specifically, these and other desired traits could be enhanced upon introducing non-native glycans into proteins of interest.

The development of a suitable archaeal host for such glyco-engineering efforts requires an understanding of N-glycosylation in that organism. In *Haloferax volcanii*, a halophilic archaeon first isolated from the Dead Sea (Mullakhanbhai and Larsen, 1975), *agl* (*a*rchaeal *gly*cosylation) genes encoding proteins involved in the assembly and attachment of a pentasaccharide to select Asn residues of the surface (S)-layer glycoprotein, a reporter of N-glycosylation in this species (Sumper *et al*., 1990), have been described (Abu-Qarn and Eichler, 2006; Abu-Qarn *et al*., 2007; 2008; Yurist-Doutsch *et al*., 2008; 2010; Kaminski *et al*., 2010; Magidovich *et al*., 2010). *Haloferax volcanii* AglJ, AglG, AglI, AglE and AglD are glycosyltransferases involved in the assembly the N-linked pentasaccharide, with AglJ, AglG, AglI and AglE, respectively, catalysing the addition of the first four pentasaccharide residues to a common dolichol phosphate carrier, and AglD adding the fifth and final pentasaccharide residue, mannose, to a distinct dolichol phosphate molecule (Abu-Qarn *et al*., 2007; 2008; Yurist-Doutsch *et al*., 2008; Guan *et al*., 2010; Kaminski *et al*., 2010). AglB is the oligosaccharyltransferase, ultimately responsible for glycan-based modification of the *Hfx. volcanii* S-layer glycoprotein (Abu-Qarn *et al*., 2007). In addition, N-glycosylation roles have been

Received 19 December, 2010; accepted 6 January, 2011. *For correspondence. E-mail jeichler@bgu.ac.il; Tel. (+972) 8646 1343; Fax (+972) 8647 9175.

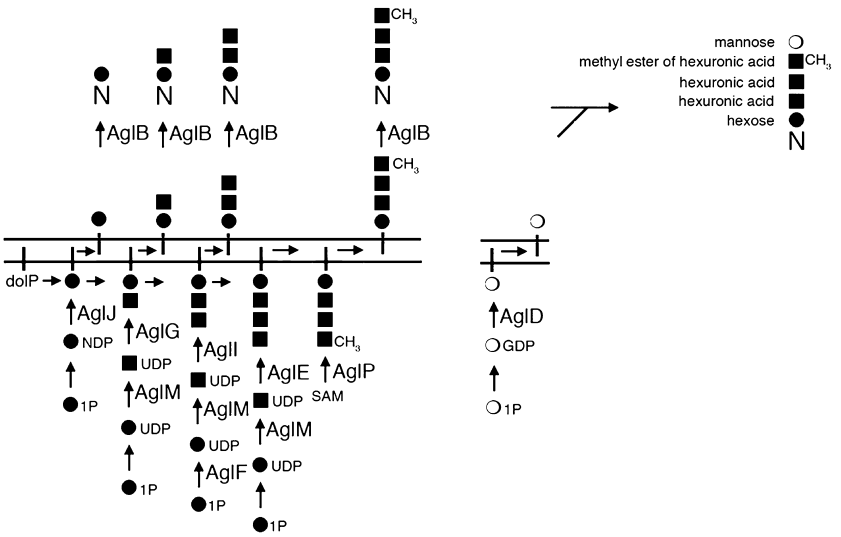

Fig. 1. Schematic depiction of current understanding of the *Hfx. volcanii* N-glycosylation pathway. The *Hfx. volcanii* S-layer glycoprotein is modified at select Asn residues by a pentasaccharide comprising a hexose, two hexuronic acids, a methyl ester of hexuronic acid and a terminal mannose residue. Refer to the text for details. 1P, 1-phosphate; dolP, dolichol phosphate; NDP, nucleoside diphosphate; SAM, *S*-adenosyl-L-methionine.

assigned to AglF, AglM and AglP. AglF is a glucose-1-phosphate uridyltransferase (Yurist-Doutsch *et al*., 2010), AglM is a UDP-glucose dehydrogenase (Yurist-Doutsch *et al*., 2010) and AglP is a methyltransferase (Magidovich *et al*., 2010). Figure 1 offers a schematic depiction of current understanding of the *Hfx. volcanii* N-glycosylation pathway.

With a relatively well-delineated N-glycosylation pathway and appropriate tools for genetic manipulation available (Allers and Mevarech, 2005), efforts to exploit *Hfx. volcanii* for the design of non-native N-linked glycans have begun. In initial glyco-engineering efforts, the ability of AglD homologues from *Halobacterium salinarum*, *Haloarcula marismortui* and *Haloquadratum walsbyi* to restore missing function to *Hfx. volcanii* Δ*aglD* cells confirmed the ability of introduced non-native glycosyltransferases to participate in the *Hfx. volcanii* N-glycosylation pathway (Calo *et al*., 2010b). In the present study, we have considered the effects of introducing a haloarchaeal homologue of AglJ, namely that predicted dolichol phosphate hexose synthase involved in adding the first residue of the N-linked pentasaccharide decorating the S-layer glycoproteins (Kaminski *et al*., 2010), into cells lacking *Hfx. volcanii* AglD, namely the mannosyl dolichol phosphate synthase involved in adding the final pentasaccharide residue (Guan *et al*., 2010). We report that the S-layer glycoprotein in *Hfx. volcanii* Δ*aglD* cells transformed to express *Hbt. salinarum* OE2528R presents an N-linked glycan profile distinct from that seen in the parent strain. These results offer further support to efforts aimed at developing *Hfx. volcanii* into a 'glyco-factory' capable of generating differentially glycosylated proteins.

Results

The introduction of Hbt. salinarum *OE2528R into* Hfx. volcanii *cells lacking AglD does not replace missing AglD function but affects N-glycosylation*

Earlier efforts demonstrated the ability of haloarchaeal homologues of *Hfx. volcanii* AglD to restore that activity lost in *Hfx. volcanii* Δ*aglD* cells (Calo *et al*., 2010b). To now assess the impact of transforming *Hfx. volcanii* Δ*aglD* cells to express a homologue of AglJ, assigned as the other dolichol phosphate hexose synthase involved in N-glycosylation in this species (Kaminski *et al*., 2010), on S-layer glycoprotein N-glycosylation, *Hbt. salinarum* OE2528R (61% identical, 74% similar to *Hfx. volcanii* AglJ) was introduced into the deletion strain. Expression of the haloarchaeal AglJ homologue in *Hfx. volcanii* cells deleted of *aglD* was confirmed by immunoblot via detection of the *Clostridium thermocellum* cellulose-binding domain (CBD) fused to *Hbt. salinarum* OE2528R at the gene level, using anti-CBD antibodies. In the transformed strain, a band corresponding to the combined molecular weight of the plasmid-encoded AglJ homologue and the CBD tag was detected (approximately 50 kDa) (Fig. 2A). In a control sample, prepared from Δ*aglD* cells transformed with plasmid-encoded CBD-AglD, an approximately 85 kDa band was observed, corresponding to the molecular weight of the fusion protein.

To investigate whether *Hbt. salinarum* OE2528R expressed in *Hfx. volcanii* Δ*aglD* cells could functionally replace absent AglD, the transformed strain was grown in the presence of [2-^{3}H] mannose, given the identification of mannose as the final residue of the N-linked pentasaccharide decorating the S-layer glycoprotein in the parent strain (Guan *et al*., 2010). In these experiments, the

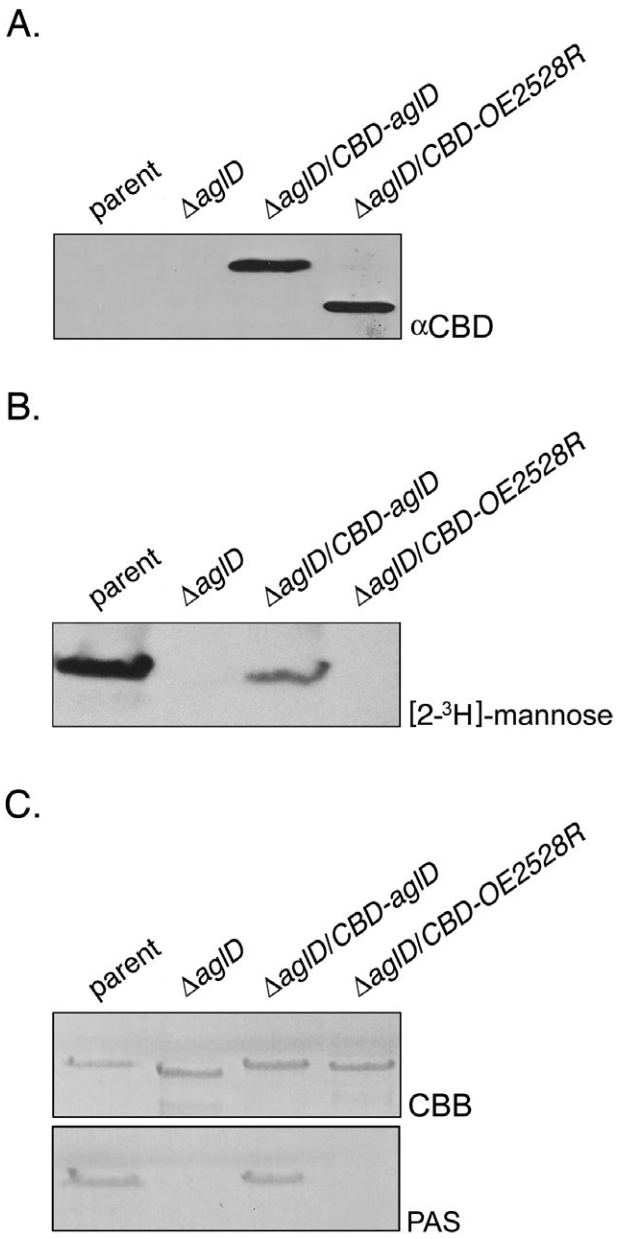

Fig. 2. N-glycosylation is affected upon expression of *Hbt. salinarum* OE2528R in *Hfx. volcanii* Δ*aglD* cells. Cells of the *Hfx. volcanii* parent strain, the Δ*aglD* strain, Δ*aglD* cells transformed to express CBD-AglD and Δ*aglD* cells transformed to express CBD-OE2528R were (A) probed with antibodies to *C. thermocellum* CBD (α-CBD) by immunoblot, (B) grown in the presence of [2-^{3}H]-mannose and processed for SDS-PAGE and fluorography or (C) subjected to SDS-PAGE and stained by Coomassie blue (CBB; upper panel) or PAS glycostain (lower panel). In (A), the positions of CBD-AglD and CBD-OE2528R are shown in the corresponding lanes, while in (B) and (C), only the S-layer glycoprotein is shown.

extent of [2-^{3}H] mannose incorporation into the N-linked glycan was determined by SDS-PAGE and fluorography. Such analysis revealed the incorporation of radiolabel into the S-layer glycoprotein of cells of the parent strain and of Δ*aglD* cells transformed to express CBD-tagged AglD but not in cells lacking AglD (Fig. 2B), confirming the presence of mannose in the N-linked pentasaccharide decorating the S-layer glycoprotein and the importance of AglD for addition of this residue, as well as the fact that the presence of the CBD moiety does not interfere with enzyme function. In the case of *Hfx. volcanii* Δ*aglD* cells transformed to express CBD-tagged *Hbt. salinarum* OE2528R, no [2-^{3}H] mannose-derived radioactivity was associated with the S-layer glycoprotein, reflecting that mannose is not an element of any glycans decorating the S-layer glycoprotein in this strain.

In cells lacking AglD, the S-layer glycoprotein migrates faster in SDS-PAGE than in the parent strain and can no longer be stained by PAS glycostain (Kaminski and Eichler, 2010). Previous studies revealed that both changes are reversed upon complementation of the mutant cells with a plasmid-encoded CBD-tagged version of AglD, as well as with CBD-tagged versions of the AglD homologues, *Hbt. salinarum* OE1482, *Har. marismortui* rrnAC1873 and *Hqr. walsbyi* HQ1489 (Calo *et al*., 2010b). However, in the case of *Hfx. volcanii* Δ*aglD* cells transformed to express CBD-tagged *Hbt. salinarum* OE2528R, only partial restoration of S-layer glycoprotein behaviour was observed. Specifically, although S-layer glycoprotein SDS-PAGE migration in the transformed strain was returned to that position seen for this protein from the parent strain (Fig. 2C, top panel), PAS glycostaining of the S-layer glycoprotein was not restored (Fig. 2C, bottom panel). In a control experiment, where *Hfx. volcanii* Δ*aglD* cells transformed to express CBD-tagged *Hbt. salinarum* OE2546F, a homologue of *Hfx. volcanii* AglI (51% identity, 68% similarity), neither SDS-PAGE migration nor PAS glycostaining of the S-layer glycoprotein was restored to what is seen in the native strain (not shown). As such, the effect on S-layer glycoprotein behaviour seen with the *Hfx. volcanii* Δ*aglD* strain transformed to express the AglJ homologue, *Hbt. salinarum* OE2528R, is unlikely an artefact of transformation but rather could reflect the presence of a modified version of the N-linked glycan decorating the S-layer glycoprotein, distinct from what is seen in cells of the parent or Δ*aglD* strains.

A novel N-linked glycan decorates the S-layer glycoprotein in Hfx. volcanii ΔaglD *cells expressing* Hbt. salinarum *OE2528R*

The pentasaccharide covalently linked to select Asn residues of the *Hfx. volcanii* S-layer glycoprotein comprises a hexose, two hexuronic acids, a methyl ester of a hexuronic acid and a mannose (Abu-Qarn *et al*., 2007; Guan *et al*., 2010; Magidovich *et al*., 2010). To determine whether *Hfx. volcanii* Δ*aglD* cells transformed to express the AglJ homologue, *Hbt. salinarum* OE2528R, modified the composition of this pentasaccharide or present a

novel N-linked glycan, the S-layer glycoprotein from the transformed cells was digested with trypsin and analysed by liquid chromatography-electrospray ionization/mass spectrometry/mass spectrometry (LC-ESI/MS/MS). In agreement with earlier studies (Calo *et al*., 2010b), such analysis of a sample prepared from cells of the parent strain revealed the existence of a doubly charged $[M+2H]^{2+}$ ion peak at *m/z* 1224.97, corresponding to the N-terminal ^{1}ERGNLDADSESFNK^{14} fragment, with its Asn-13 residue modified by the pentasaccharide (Sumper *et al*., 1990; Abu-Qarn *et al*., 2007) (Fig. 3A). No such peak was detected in *Hfx. volcanii* Δ*aglD* cells transformed to express *Hbt. salinarum* OE2528R (Fig. 3D). However, both parent strain cells (Fig. 3B) and Δ*aglD* cells transformed to express *Hbt. salinarum* OE2528R (Fig. 3E) present a doubly charged $[M+2H]^{2+}$ ion peak at *m/z* 1143.44, corresponding to the Asn-13-containing glycopeptide modified by the first four subunits of the pentasaccharide attached at this position (Sumper *et al*., 1990; Abu-Qarn *et al*., 2007; Guan *et al*., 2010). The correct assembly of the first four residues of the N-linked pentasaccharide at the level of the protein target [and, by extension, at the level of the dolichol phosphate carrier on which the tetrasaccharide is assembled (Guan *et al*., 2010)] verifies that native AglJ function is not compromised upon introduction of *Hbt. salinarum* OE2528R, an AglJ homologue, into the Δ*aglD* cells. Still, since the level of the *m/z* 1143.44 peak shown is normalized to that level seen in the parent strain, it is clear that less of the tetrasaccharide-charged glycopeptide is found in the transformed strain. In contrast, the absence of AglD had no effect on the level of this peak (not shown). As such, it appears that the decrease in the level of the *m/z* 1143.44 peak represents a *Hbt. salinarum* OE2528R-related effect on the processing of the pentasaccharide N-linked to the S-layer glycoprotein. More striking, however, is the observation that *Hfx. volcanii* Δ*aglD* cells transformed to express *Hbt. salinarum* OE2528R present a doubly charged $[M+2H]^{2+}$ ion peak at *m/z* 1238.51 not seen in the parent strain (Fig. 3C and F).

To determine whether the doubly charged $[M+2H]^{2+}$ ion peak at *m/z* 1238.51 corresponds to a S-layer glycoprotein-derived glycopeptide, tandem mass spectrometry was performed. Fragmentation of the *m/z* 1238.51 species resulted in doubly charged product ion peaks at *m/z* 1150.74, 1062.78 and 981.67, corresponding to the neutral loss of one hexuronic acid (i.e. a loss of 176 Da), two hexuronic acids (i.e. a loss of 352 Da), and two hexuronic acids and a hexose (i.e. a loss of 514 Da) respectively (Fig. 4A). Examination of the LC-ESI/MS profile indeed revealed that as was the case for the doubly charged $[M+2H]^{2+}$ ion peak at *m/z* 1238.51, the engineered strain presents a doubly charged $[M+2H]^{2+}$ ion peak at *m/z* 1150.49 not seen in the parent strain (Fig. 4B, top panels). A doubly charged $[M+2H]^{2+}$ ion peak at *m/z* 1062.48 is also seen in the transformed strain. This peak is also noted in the parent stain, albeit to a much lower extent (Fig. 4B, bottom panels). No doubly charged $[M+2H]^{2+}$ ion peak at *m/z* at 981.46 was detected in either strain. These results thus point to the S-layer glycoprotein-derived Asn-13-containing tryptic fragment (1580.7 Da) from *Hfx. volcanii* Δ*aglD* cells transformed to express *Hbt. salinarum* OE2528R as being modified by a 894 Da glycan comprising an N-linking 380 Da moiety of unknown structure, a hexose and two hexuronic acid residues (Fig. 4A, inset).

The novel N-linked 894 Da glycan is present in ΔaglD *cells but is substantially augmented in the presence of* Hbt. salinarum *OE2528R*

The detection of a doubly charged $[M+2H]^{2+}$ ion peak at *m/z* 1062.48 in cells of the parent strain implies that these cells, to some extent, modify the S-layer glycoprotein with the precursor of the N-linked 894 Da glycan containing the 380 Da moiety of unknown character and a hexose. Since the complete 894 Da glycan was not detected, it was speculated that the presence of AglD somehow interferes with the biosynthesis of the complete glycan in the parent strain. To test this hypothesis, the levels of the doubly charged $[M+2H]2^{+}$ ion peak at *m/z* 1062.78, 1150.74 and 1238.51 in cells of the parent and Δ*aglD* strains were normalized to the levels of these peaks detected in Δ*aglD* cells transformed to express *Hbt. salinarum* OE2528R.

Comparison of the level of the doubly charged $[M+2H]^{2+}$ ion peak at *m/z* 1062.78 in the three *Hfx. volcanii* strains revealed a slight increase in the Δ*aglD* strain (Fig. 5A, middle panel), relative to what is seen in the parent strain (top panel), but still only close to half of that amount seen in Δ*aglD* cells transformed to express *Hbt. salinarum* OE2528R (bottom panel). On the other hand, the Δ*aglD* strain contained just close to 50% of the doubly charged $[M+2H]^{2+}$ ion peak at *m/z* 1150.74 seen in Δ*aglD* cells transformed to express *Hbt. salinarum* OE2528R (Fig. 5B, middle and bottom panels); only a negligible amount of this peak was detected in the parent strain (bottom panel). Finally, 10-fold more of the doubly charged $[M+2H]^{2+}$ ion peak at *m/z* 1238.51 was detected in Δ*aglD* cells (Fig. 5C, middle panel) than was present in the parent strain (top panel), while Δ*aglD* cells transformed to express *Hbt. salinarum* OE2528R (bottom panel) in turn presented 10-fold more of this peak that did cells of the deletion strain. It thus appears that whereas the presence of AglD prevents the assembly of the complete 894 Da glycan N-linked to the S-layer glycoprotein, the replacement of AglD by *Hbt. salinarum* OE2528R substantially augments such assembly.

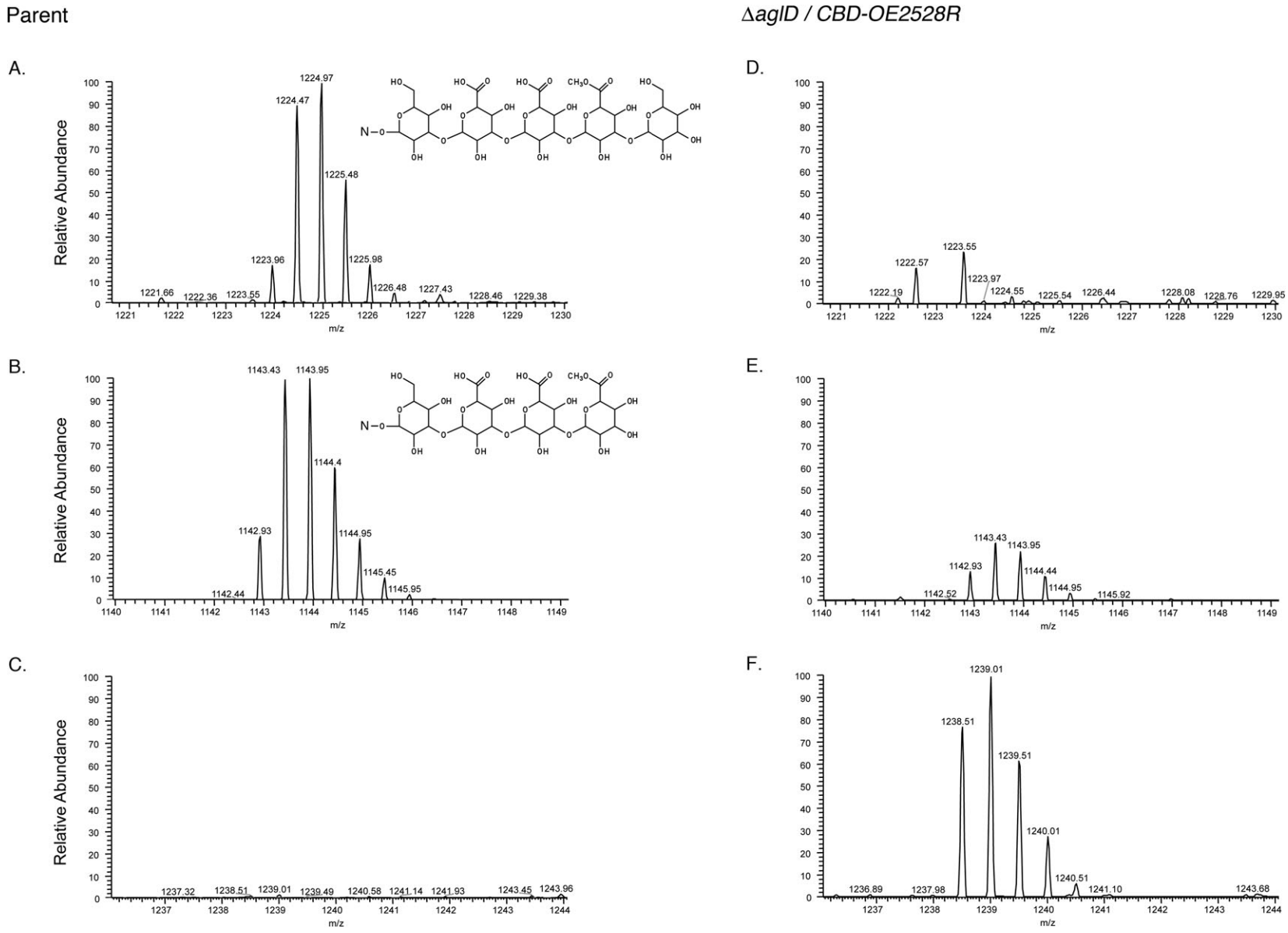

Fig. 3. The S-layer glycoprotein is differentially glycosylated in *Hfx. volcanii* parent strain cells and in Δ*aglD* cells transformed to express CBD-OE2528R. LC-ESI/MS analysis of the Asn-13-containing tryptic peptide derived from the S-layer glycoprotein from cells of the parent strain (A–C) or from Δ*aglD* cells transformed to express CBD-OE2528R (D–F). Shown are doubly charged $[M+2H]^{2+}$ ion peaks at *m/z* 1224.97, corresponding to the peptide modified with the N-linked pentasaccharide (A and D), at *m/z* 1143.95, corresponding to the peptide modified with the tetrasaccharide precursor of the N-linked pentasaccharide (B and E) and at *m/z* 1238.51, corresponding to the modified peptide seen only in the transformed strain (C and F). In each case, the higher level of the peak is taken as 100%, while the lower level of the peak in the other strain is expressed as the percentage thereof. The structures of the N-linked pentasaccharide and tetrasaccharide are shown in the insets of the profile showing that parent strain-derived peak modified by each glycan, with the N corresponding to the Asn-13 residue.

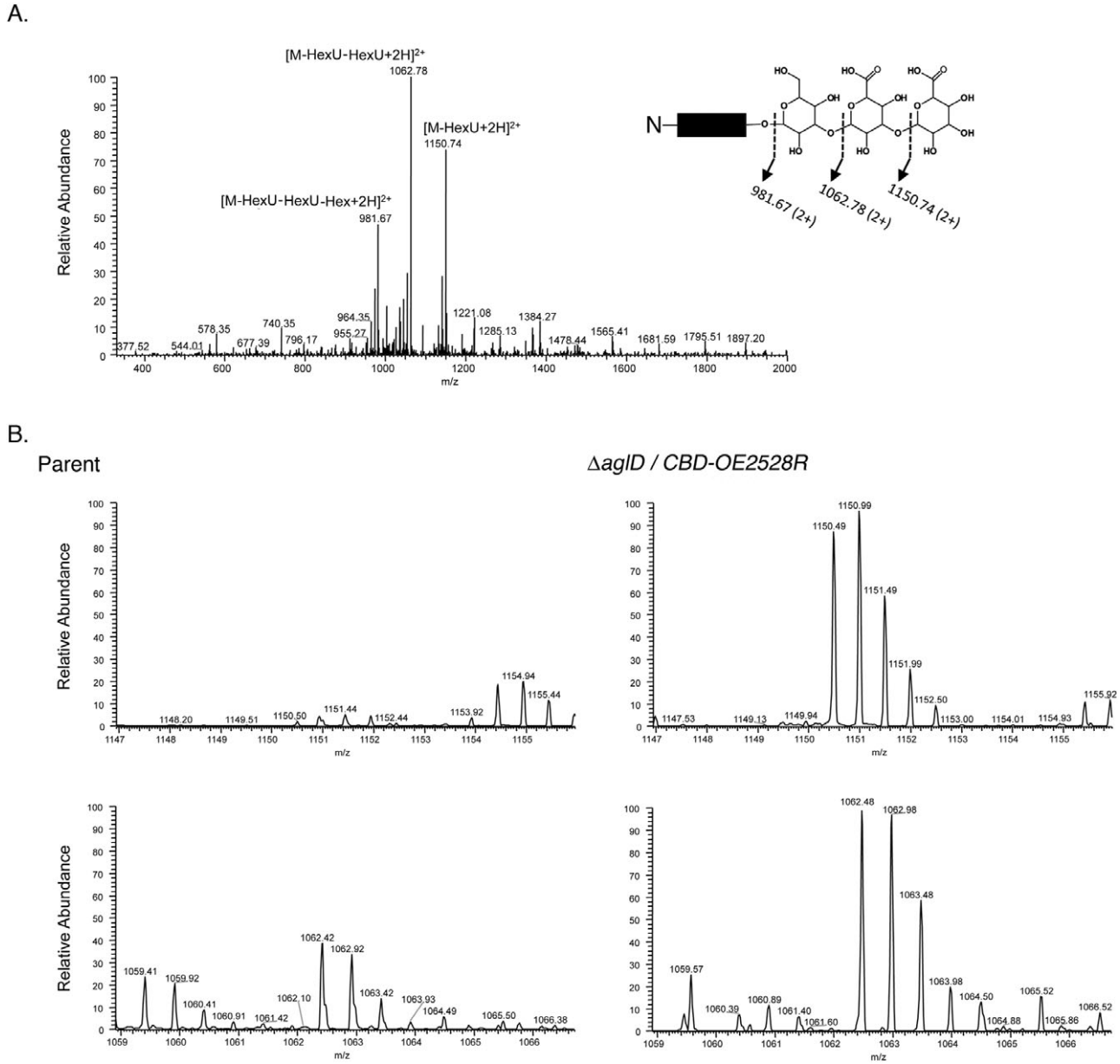

Fig. 4. The LC-ESI/MS peak seen at *m/z* 1238.5 represents the S-layer glycoprotein-derived Asn-13-containing tryptic peptide modified by a novel glycan.
A. Tandem MS (MS/MS) fragmentation pattern of the doubly charged $[M+2H]^{2+}$ ion peak at *m/z* 1238.5. Labelled are the doubly charged product ions resulting from the loss of one or two hexuronic acid (HexU) residues or two HexU residues and a single hexose (Hex) residue. The inset shows a schematic version of the fragmentation. The black box corresponds to the unknown 380 Da moiety of the glycan, while N corresponds to Asn-13.
B. LC-ESI/MS analysis of the S-layer glycoprotein-derived Asn-13-containing tryptic peptide from parent strain (left panels) or from Δ*aglD* cells transformed to express CBD-OE2528R (right panels) reveals peaks modified by precursors of the novel 894 Da glycan. The levels of the doubly charged $[M+2H]^{2+}$ ion peaks at *m/z* 1150.74 and 1062.78 (upper and lower panels respectively) in the parent strain are expressed as percentages of the levels of the same peaks in the engineered strain, taken as 100%.

Discussion

The ability to harness the various glycosyltransferases and other saccharide-processing enzymes responsible for generating the wide variability seen in the composition of N-linked glycans decorating archaeal glycoproteins (Eichler and Adams, 2005) carries enormous potential for the biosynthesis of glycoproteins bearing tailored glycans. With a relatively well-delineated N-glycosylation pathway (Calo *et al.*, 2010a) and the availability of genetic tools for manipulating this strain (Allers and Mevarech, 2005), *Hfx. volcanii* offers a suitable platform for hosting such

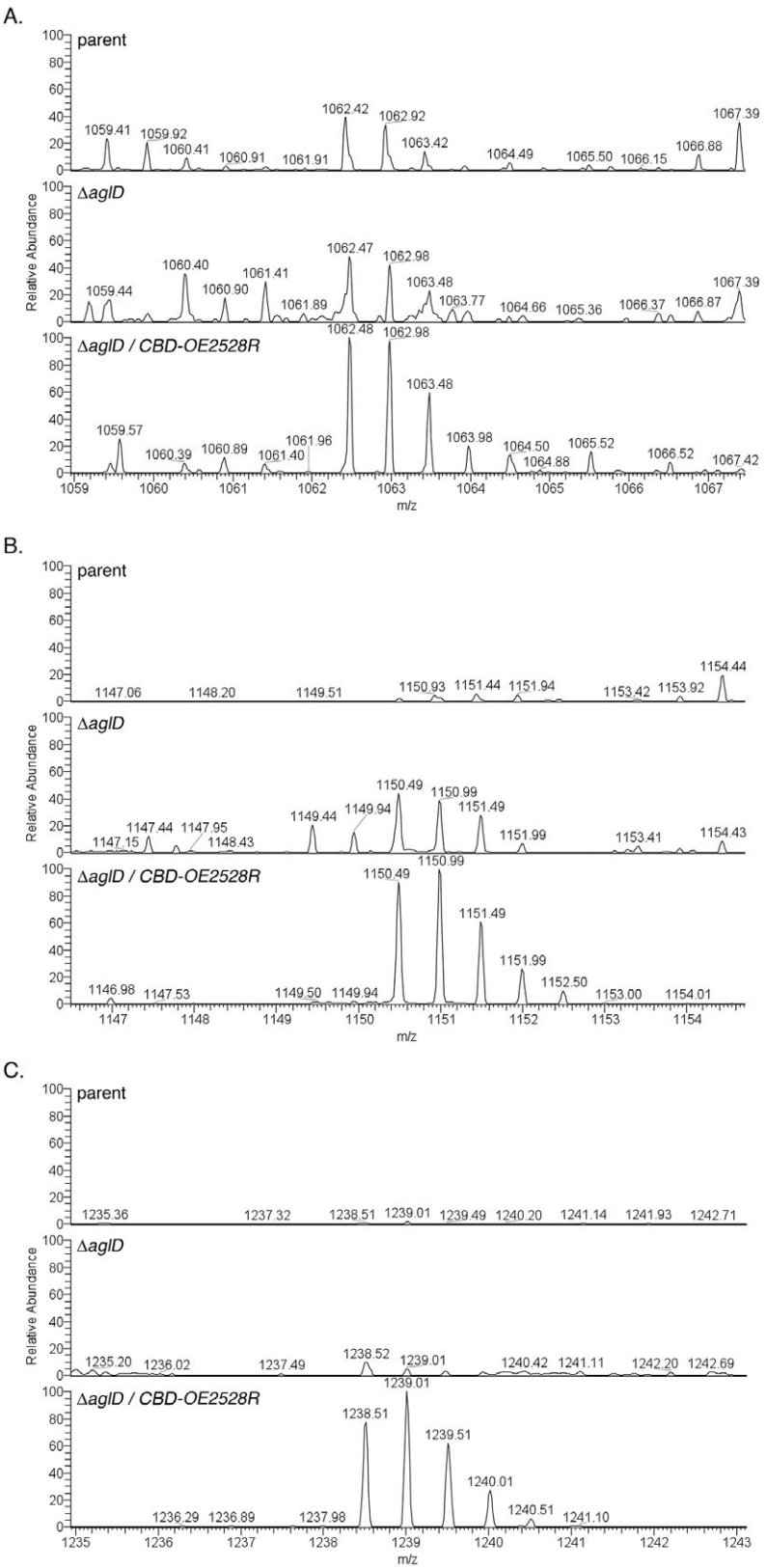

Fig. 5. Precursors of the novel glycan decorating the S-layer glycoprotein-derived Asn-13-containing fragment differentially accumulate in different *Hfx. volcanii* strains. The relative levels of the novel glycan or its precursors attached to the S-layer glycoprotein-derived Asn-13-containing fragment [doubly charged $[M+2H]^{2+}$ ion peaks at *m/z* 1062.78 (A), 1150.74 (B) and 1238.5 (C)] in cells of the parent strain (top panels), Δ*aglD* cells (middle panels) and Δ*aglD* cells transformed to express CBD-OE2528R (bottom panels) are shown. In each case, the level of the peak in the engineered strain is taken as 100%, with the levels of the peaks in the other two strains expressed as the percentage thereof.

chimeric glycosylation systems. Here, the effects of introducing a *Hbt. salinarum* homologue of *Hfx. volcanii* AglJ, OE2528R, into the *aglD* deletion strain further justify the suitability of *Hfx. volcanii* as a glyco-engineering 'workshop'.

The results show that introduced *Hbt. salinarum* OE2528R did not simply mediate the addition of a novel fifth residue to the glycan N-linked to the S-layer glycoprotein in place of the absent mannose residue normally found at this position. Instead, the presence of *Hbt. salinarum* OE2528R in the *aglD* deletion strain compromised the amount of N-linked tetrasaccharide corresponding to the first four residues of the pentasaccharide normally generated. This shows that while introduced *Hbt. salinarum* OE2528R has an effect on *Hfx. volcanii* N-glycosylation, it does not compete with AglJ, responsible for charging a dolichol phosphate with the first residue of the pentasaccharide (Kaminski *et al*., 2010). Rather, *Hfx. volcanii* Δ*aglD* cells transformed to express *Hbt. salinarum* OE2528R decorated the S-layer glycoprotein with a novel 894 Da N-linked glycan comprising two hexuronic acids, a hexose and a 380 Da component of unknown composition, with preliminary evidence suggesting this moiety to include an additional hexuronic acid residue. The presence of unidentified residues in N-linked glycans decorating archaeal glycoproteins has also been reported in *Methanococcus voltae*, where both the S-layer glycoprotein and flagellin are modified by an N-linked trisaccharide of defined composition that can include an additional, as yet unidentified, 220 or 262 Da residue (Chaban *et al*., 2009). The same glycan, like the N-linked glycan decorating glycoproteins in *Methanococcus maripaludis*, also includes several highly modified residues (Voisin *et al*., 2005; Kelly *et al*., 2009).

Although the novel 894 Da N-linked glycan noted in Δ*aglD* cells transformed to express *Hbt. salinarum* OE2528R was not detected on the S-layer glycoprotein from cells of the *Hfx. volcanii* parent strain, the S-layer glycoprotein was modified by an early 542 Da precursor of this glycan in parent strain cells. More of this and more advanced precursors of the 894 Da N-linked glycan were shown to decorate the S-layer glycoprotein of cells deleted of *aglD*. Indeed, trace amounts of the complete glycan were detected in cells of the Δ*aglD* strain, albeit at levels some 20-fold less than seen in Δ*aglD* cells transformed to express *Hbt. salinarum* OE2528R. At the same time, the expression of *Hbt. salinarum* OE2528R in Δ*aglD* cells led to a decrease in the level of the tetrasaccharide-bearing precursor of the pentasaccharide decorating the S-layer glycoprotein in the parent and Δ*aglD* strains. These observations suggest that in the absence of AglD, the activities of other enzymes involved in N-glycosylation, namely those involved in the biogenesis of the 894 Da glycan, are encouraged. This effect is, moreover, enhanced when *Hbt. salinarum* OE2528R is introduced into the deletion strain. It would thus appear that the presence or absence of AglD affects the relative importance or two different N-glycosylation pathways. Introduced *Hbt. salinarum* OE2528R, seemingly catalysing a reaction performed to a much lesser extent by a native, as yet unidentified N-glycosylation enzyme, subsequently shifts this balance substantially. Recently, examination of the N-linked glycan decorating *M. maripaludis* pili distinguished it from that glycan N-linked to the S-layer glycoprotein and flagellin in the same species through the presence of an additional hexose branch, pointing to the ability of *Archaea* to generate different N-linked glycans in a single species (Ng *et al*., 2011). In the case of the *Hfx. volcanii* S-layer glycoprotein, the results presented here point to different N-linked glycans being added to the same protein as a function of the presence or absence of certain N-glycosylation pathway enzymes. The *Hbt. salinarum* S-layer glycoprotein is also modified by two distinct N-linked glycans, although nothing is known of the biosynthesis of these glycans (Lechner and Wieland, 1989).

Based upon analysis of the dolichol phosphates upon which N-linked glycans are assembled in *Hfx. volcanii* (Guan *et al*., 2010; Kaminski *et al*., 2010), the existence of different N-linked glycans in this species is not unexpected. Preliminary experiments observed differences in the profile of the glycan-charged dolichol phosphate pool in *Hfx. volcanii* cells including or lacking AglD (Z. Guan and J. Eichler, unpubl. obs.). Earlier analysis of the *Hfx. volcanii* dolichol phosphate pool reported the presence of species modified by glycans that included sulfated or phosphorylated hexoses and the deoxyhexose, rhamnose (Kuntz *et al*., 1997), although none of these glycans was shown to be N-linked to the S-layer glycoprotein, the major glycoprotein in this species (Sumper *et al*., 1990; Abu-Qarn and Eichler, 2006). It is conceivable that these dolichol phosphate-bound glycans contribute to the 380 Da component of the novel 894 Da N-linked glycan reported here.

In conclusion, the finding that a non-native homologue of a *Hfx. volcanii* N-glycosylation pathway glycosyltransferase can modify the glycosylation of a reporter glycoprotein in a novel manner offers encouragement for efforts to develop *Hfx. volcanii* into a glyco-engineering platform. At the same time, this study also makes it clear that much remains to be learned about N-glycosylation in *Hfx. volcanii*.

Experimental procedures

Strains and growth conditions

The *Hfx. volcanii* parent strain WR536 (H53) (obtained from M. Mevarech, Tel Aviv University), the same strain deleted of *aglD* and the deletion strain transformed to express *C.*

thermocellum cellulose-binding domain-tagged *Hbt. salinarum* OE2528R were grown in complete medium containing 3.4 M NaCl, 0.15 M $MgSO_4 \cdot 7H_2O$, 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).

The preparation of *Hfx. volcanii* strains deleted of *aglD* was previously reported (Abu-Qarn and Eichler, 2006), as was preparation of the *aglD* deletion strain transformed to express CBD-AglD (Calo *et al*., 2010b). *Haloferax volcanii* Δ*aglD* cells transformed to express CBD-tagged *Hbt. salinarum* OE2528R were generated by introducing plasmid pWL-CBD-OE2528R into the deletion strain. To generate plasmid pWL-CBD-OE2528R, genomic DNA was isolated from *Hbt. salinarum* R1 (obtained from D. Oesterhelt, Max Planck Institute) and *OE2528R* was PCR-amplified using primers OE2528for (CCCAGATCTATGAGCGAGGAGTACGAG) and OE2528rev (CCCGGTACCTCACTTGTTCAGGCGC), designed to introduce BglII and KpnI sites at the 5′ and 3′ ends of the amplified sequence respectively. The amplified fragment was then ligated into plasmid pWL-CBD-AglD (Plavner and Eichler, 2008), pre-treated with BglII and KpnI so as to remove the *aglD* insert.

LC-ESI/MS/MS analysis

LC-ESI/MS/MS analysis of the *Hfx. volcanii* S-layer glycoprotein was performed as previously described (Calo *et al*., 2010b).

[2-^{3}H]-mannose radiolabelling of the S-layer glycoprotein

For [2-^{3}H]-mannose radiolabelling of the S-layer glycoprotein, 100 μl aliquots of *Hfx. volcanii* cells were incubated with 6 μl of [2-^{3}H]-mannose (23.8 mCi mmol^{-1}; PerkinElmer, Boston, MA). One hour later, the protein content of the cells was precipitated with 15% (w/v) trichloroacetic acid and separated by SDS-PAGE. The incorporation of radiolabel was revealed following fluorography and exposure to film.

Other methods

Proteins bearing the CBD moiety were detected by immunoblot, using polyclonal antibodies raised against the *C. thermocellum* cellulose-binding domain (obtained from Ed Bayer, Weizmann Institute of Science; 1:10 000). Antibody binding was detected using goat anti-rabbit horseradish peroxidase (HRP)-conjugated antibodies (1:2500, Bio-Rad, Hercules, CA) and an ECL enhanced chemiluminescence kit (GE Healthcare, Piscataway, NJ). Periodic acid-Schiff reagent (PAS) glycoprotein staining was performed as described previously (Dubray and Bezard, 1982).

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