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Current Opinion in Structural Biology

Not just for Eukarya anymore: protein glycosylation in Bacteria and Archaea

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Of the many post-translational modifications proteins can undergo, glycosylation is the most prevalent and the most diverse. Today, it is clear that both *N*-glycosylation and *O*-glycosylation, once believed to be restricted to eukaryotes, also transpire in Bacteria and Archaea. Indeed, prokaryotic glycoproteins rely on a wider variety of monosaccharide constituents than do those of eukaryotes. In recent years, substantial progress in describing the enzymes involved in bacterial and archaeal glycosylation pathways has been made. It is becoming clear that enhanced knowledge of bacterial glycosylation enzymes may be of therapeutic value, while the demonstrated ability to introduce bacterial glycosylation genes into *Escherichia coli* represents a major step forward in glyco-engineering. A better understanding of archaeal protein glycosylation provides insight into this post-translational modification across evolution as well as protein processing under extreme conditions. Here, we discuss new structural and biosynthetic findings related to prokaryotic protein glycosylation, until recently a neglected topic.

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Current Opinion in Structural Biology 2008, **18**:544–550

This review comes from a themed issue on
Carbohydrates and glycoconjugates
Edited by Daan van Aalten and Natalie Strynadka

Available online 26th August 2008

0959-440X/$ – see front matter

DOI 10.1016/j.sbi.2008.06.010

Introduction

Long believed to be restricted to Eukarya, it is now clear that both Bacteria and Archaea are capable of glycosylation (for reviews, see [1,2,3•]). In fact, glycosylation in prokaryotes appears to lead to a greater diversity of glycan compositions and structures than that found in eukaryotic cells. Currently, heightened attention is focused on protein glycosylation in Bacteria, primarily because of the increasing frequency with which this post-translational modification is seen in pathogenic species [4•]. Accordingly, research conducted in several laboratories over the past decade has served to decipher the *N*-glycosylation pathway of the intestinal pathogen *Campylobacter jejuni*, making it the first bacterium for which such a complete pathway has been described [3•]. In addition, *O*-glycosylation has been shown to occur in several other bacterial pathogens, such as *Neisseria gonorhoeae* and *Helicobacter pylori* [4•], with glycosylation-defective mutants displaying attenuated virulence-associated properties. As such, pathways of protein glycosylation in these pathogens, especially those responsible for the biosynthesis of rare glycoprotein sugars not present in humans, may offer novel therapeutic targets. Moreover, the demonstration that the *C. jejuni pgl* gene cluster, responsible for *N*-glycosylation in this organism, can be expressed in *Escherichia coli*, and the fact that the *C. jejuni* oligosaccharyltransferase (OST) is of relaxed specificity, raise the hope that protein *N*-glycosylation will eventually be added to the menu of biological and biotechnological processes performed by this workhorse of molecular biology [5••,6••].

While significant strides are being made in understanding bacterial glycosylation, with, for instance, some 40 S-layer glycoprotein structures having been fully or partially elucidated (for a recent example, see [7]), much less is known of the steps involved in this post-translational modification in Archaea. This is despite the fact that the first noneukaryal *N*-glycosylated protein was discovered over three decades ago in the haloarchaea *Halobacterium salinarum* [8] and that *N*-glycosylated proteins are more prevalent in Archaea than in Bacteria [1,2]. Nonetheless, studies emerging from several groups have started to make inroads into identifying and characterizing components involved in the archaeal version of *N*-glycosylation. By contrast, virtually nothing is known of the archaeal version of *O*-glycosylation.

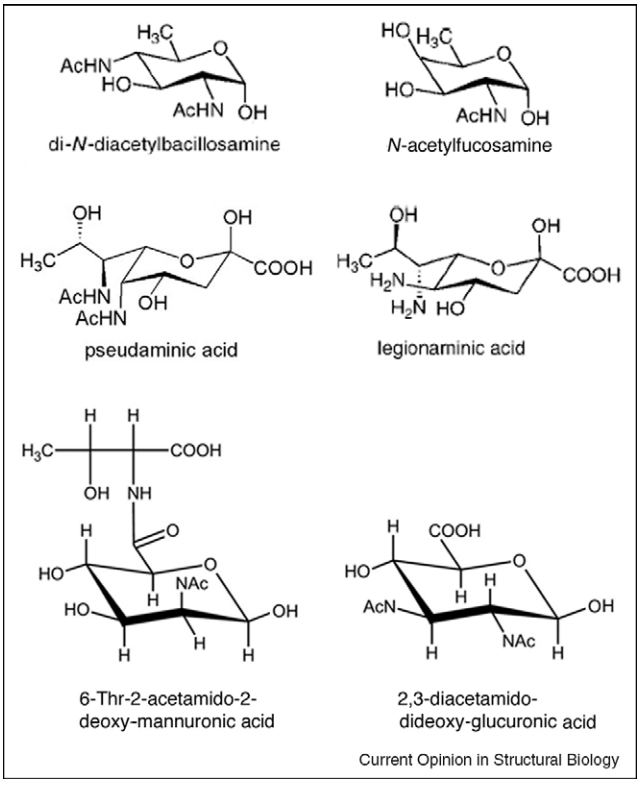
Bacterial protein glycosylation

Bacteria are capable of forming both protein-attached *N*-glycans and *O*-glycans. Such glycans can include rare monosaccharides, often serving as carbohydrate–peptide linking groups (Figure 1), in addition to more common sugars present in eukaryotic glycoproteins, such as glucose, galactose, *N*-acetylgalactosamine, and xylose.

N-Glycosylation — the *Campylobacter* Pgl proteins

Following the first isolation of an Asn-attached heptasacccharide from *C. jejuni* and its structural characterization [9••] (Table 1), the pathway responsible for its biosynthesis has been delineated. The *pgl* gene cluster

Figure 1



Rare monosaccharide constituents of archaeal and bacterial glycoproteins: di-*N*-acetylbacillosamine, *N*-acetylfucosamine, pseudaminic acid, legionaminic acid, 6-Thr-2-acetamido-2-deoxy-β-mannuronic acid, and 2,3-diacetamido-2,3-dideoxy-β-glucuronic acid.

encodes, among others, five putative glycosyltransferases (i.e. PglA, PglC, PglH, PglI, and PglJ) involved in the assembly of the heptasaccharide on a lipid carrier and PglB, responsible for transfer, en bloc, of the glycan from the carrier to protein (Figure 2) in a pathway similar to that employed in eukaryotes [10,11]. The same gene cluster also encodes PglF, a UDP-*N*-acetylglucosamine C-6 dehydratase [12], PglE, a C-4 aminotransferase [13,14], and PglD, an *N*-acetyltransferase [15] (Figure 3), responsible for transforming UDP-GlcNAc into di-*N*-acetylbacillosamine, the carbohydrate–peptide linking monosaccharide, as postulated over 40 years ago [16•]. The *in vitro* biosynthesis of the complete lipid-linked *C. jejuni* heptasaccharide from UDP derivatives of galactose and *N*-acetylglucosamine by coupling the required enzymes, followed by the transfer of the glycan to a target protein, is a particularly impressive achievement [12,15]. Structural insight into the reactions of bacterial *N*-glycosylation is now beginning to emerge with the recent solution of the crystal structure of PglD, solved in complex with acetyl-CoA as cosubstrate [17].

The central enzyme of the Pgl system is the OST, PglB. This 82 kDa integral membrane protein shares significant primary sequence similarity with STT3, an essential component of the nine-member OST complex of yeast and crucial for the proper functioning of all eukaryotic cells [5••]. In the case of *N*-glycosylation by the *C. jejuni* OST, the eukaryotic sequon, N-X-S/T is N-terminally extended to D/E-Z-N-X-S/T, where Z and X can be any amino acid except proline [18]. Although DQNAT is the optimal bacterial acceptor sequence [19], not all sequons are glycosylated, as elsewhere.

O-Glycosylation — a target for therapeutics?

Bacterial *O*-glycosylation also makes use of several unusual sugars. The pili of *Neisseria meningitides* [20] and *Neisseria gonorrhoeae* [21] contain serine O-linked glycans, the first of which was reported 13 years ago as Gal-β1,3-Gal-α1,3-2,4-diacetamido-2,4,6-trideoxyhexose (DATDH) [20]. Despite the passage of time, the stereochemistry of

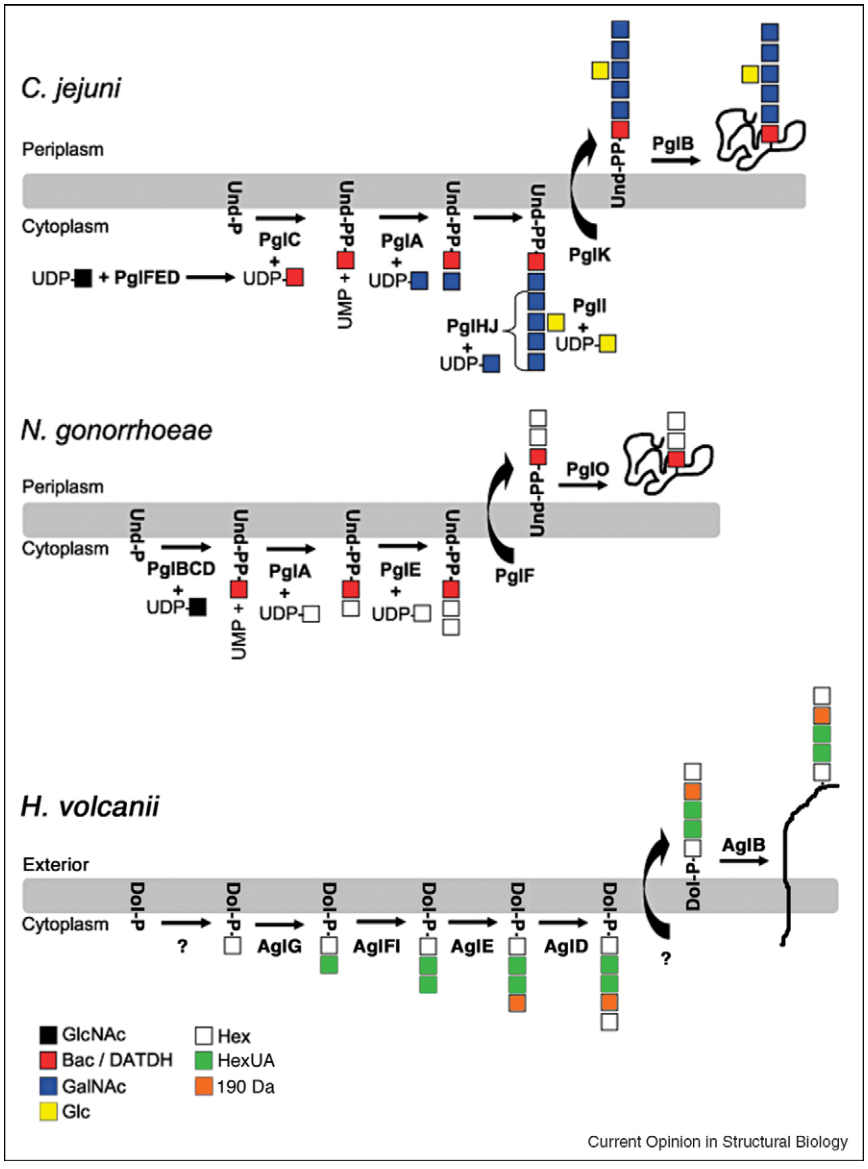
Table 1

Select protein-linked glycans in Bacteria and Archaea

Organism	Glycan structure	Reference
Bacteria		
N-linked		
Campylobacter jejuni	GalNAc-α1,4-GalNAc-α1,4-(Glc-β1,3)-GalNAc-α1,4-GalNAc-α1,4-GalNAc-α1,3-**$BacAc_2$**-Asn	[9••]
O-linked		
Neisseria gonorrhoeae	Gal-β1,4-Gal-α1,3-**DATH**-Ser	[21]
Pseudomonas aeruginosa 1244	5N(3-OH)But7NFmPse-α2,4-Xyl-β1-3**FucNAc**-β-Ser	[24]
Archaea		
N-linked		
Halobacterium salinarum	[GalNAc-3-1-(3fGal)GalA-4-1-(6GalA3OCH_3)GlcNAc-4-]$_{(10–15)}$-1-**GalNAc**-Asn	[46]
	(OSO_3)GlcA-[β1,4-GlcA(OSO_3)]$_2$-β1,4-**Glc**-Asn	[37]
Methanococcus voltae	ManNAcA6Thr-β1,4-GlcNAc3NAcA-β1,3-**GlcNAc**-Asn	[36]

Linking sugars are shown in bold. Abbreviations used: $BacA_2$, di-*N*-acetylbacillosamine; DATH, 2,4-diacetamido-2,4,6-trideoxyhexose; FmPse, formyl-pseudaminic acid; FucNAc, *N*-acetylfucosamine; Gal, galactose; GalA, galacturonic acid; GalNAc, *N*-acetylgalactosamine; Glc, glucose; GlcA, glucuronic acid; GlcNAc, *N*-acetylglucosamine; GlcNAc3NAcA, 2,3-diacetamido-2,3-dideoxy-glucuronic acid; ManNAcA, *N*-acetylmannuronic acid; Xyl, xylose.

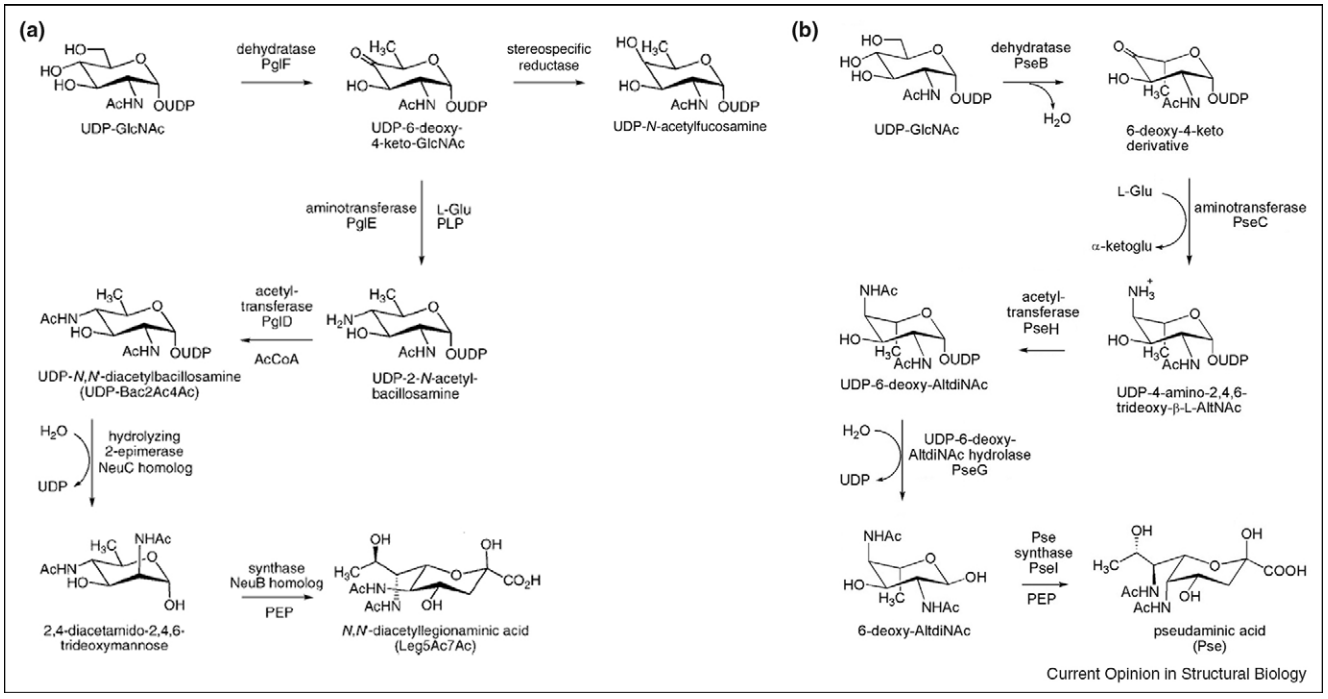
Figure 2



Models of N-linked and O-linked glycosylation in Bacteria and Archaea. Upper panel: *N*-glycosylation in *Campylobacter jejuni*. Middle panel: *O*-glycosylation of *Neisseria gonorrhoeae* pilin. Lower panel: *N*-glycosylation of the *Haloferax volcanii* S-layer glycoprotein. Bac, di-*N*-acetylbacillosamine; DATDH, Gal-β3-Gal-α3-2,4-diacetamido-2,4,6-trideoxyhexose; Dol-P, dolichyl phosphate; GalNAc, *N*-acetylgalactosamine; Glc, glucose; GlcNAc, *N*-acetylglucosamine; Hex, hexose; HexUA, hexuronic acid; UMP, uridine monophosphate; Und-PP, undecaprenyl-pyrophosphate; 190 Da, uncharacterized 190 Da saccharide. The top two panels are reproduced, with permission, from [34••].

DATDH has yet to be unambiguously determined, though it is believed to be that of bacillosamine. *Neisseria* spp. type IV pilin has been shown to be glycosylated at Ser63 by a derivative of DATDH in which the position 4 acetamide is replaced by a glyceramide, CH_2OH–$CH_2OHCONH$ [22]. By contrast, pili of *Pseudomonas aeruginosa* contain O-linked pseudaminic acid (Pse) (Figure 1), an analog of sialic acid [23], as do flagellin proteins of other Gram-negative pathogens, such as *H. pylori* [24] and *Campylobacter coli* [25], the latter of which also contains another rare monosaccharide sharing the same stereochemistry as sialic acid, that is, legionaminic acid (Figure 1) [26]. In *P. aeruginosa* strain 1244, pilin proteins contain a glycan of unusual structure, namely a trisaccharide composed of hydroxybutyryl-formyl-pseudaminic acid α2,4-linked to xylose, in turn β1,4-linked to *N*-acetylfucosamine (Table 1) [22]. The latter sugar is attached to the hydroxyl of the pilin terminal Ser, the carboxyl of which must be unsubstituted [27]. Mention should also be made of the threonine-linked oligomers of α-arabinofuranosides found in type IV pilins of *P. aeruginosa* [28].

Figure 3



Pathways for the biosynthesis of **(a)** di-*N*-acetylbacillosamine, *N*-acetylfucosamine, and *N,N′*-diacetyllegionaminic acid [16•,25,32] and **(b)** pseudaminic acid [29].

Pilin and flagellin glycosylation is, in many cases, necessary for proper assembly of the corresponding filaments, for bacterial motility, for colonization, and hence, for virulence. As such, deciphering bacterial *O*-glycosylation pathways will open novel avenues for combating bacterial diseases. Till date, the pathways of Pse [13,29–31] and legionaminic acid [25,32] biosynthesis have been defined (Figure 3). Interestingly enough, the immediate precursor of legionaminic acid is di-*N*-acetylbacillosamine. In their active forms, both Pse and legionaminic acid are attached to CMP, just like *N*-acetylneuraminic acid (Figure 2). Remarkably, the conversion of UDP-GlcNAc to CMP-Pse was achieved in a single reaction combining the six enzymes involved in its biosynthesis [30]. This represents the first complete *in vitro* enzymatic synthesis of a sialic acid like sugar and sets the groundwork for future small-molecule inhibitor screening and design. Indeed, CMP-Pse was found to be a potent inhibitor of PseB, the first enzyme of the Pse pathway in *Campylobacter coli* and *H. pylori* [33]. This observation led to the conclusions that Pse levels are feedback-regulated within the bacterial cell and that PseB represents a key control point for Pse production, underscoring the importance of this enzyme as a therapeutic target.

The three-dimensional structure of another central component in Pse biosynthesis, that is, the aminotransferase PseC (HP0366), has been determined in complex with pyridoxal phosphate alone and in combination with the UDP-4-amino-4,6-dideoxy-L-AltNAc (*N*-acetylaltrosamine) intermediate [31]. This is the first time the structure of a nucleotide–sugar aminotransferase has been cocrystallized with its natural ligand, revealing the enzyme to form a homodimer, where each monomer contributes to the active site.

Quite surprisingly, the bacterial pilin *O*-glycosylation and *N*-glycosylation pathways resemble each other (Figure 2). The *N. meningitides pgl* gene cluster, responsible for the O-linked glycosylation of pilin in this species, displays significant homology to the *pgl* cluster of *C. jejuni*. On the basis of the results of combined reverse genetics and mass spectrometry, a neisserial pilin glycosylation pathway paralleling that proposed for *C. jejuni N*-linked protein glycosylation has been proposed [34••]. Like the N-linked oligosaccharide of *C. jejuni*, the *O*-linked oligosaccharide of *N. gonorhoaea* is also preassembled through the sequential addition of nucleotide-activated monosaccharides onto a lipid carrier (Figure 2). This is extremely unusual, since all other *O*-glycans studied till date are assembled via sequential addition of their monosaccharide constituents directly onto a protein acceptor. Finally, as with the *C. jejuni N*-glycosylation pathway, the *O*-glycosylation systems of *P. aeruginosa* 1244 and *N. meningitidis* MC58 have been expressed in *E. coli* [35•]. In both cases, assembled oligosaccharides are transferred,

en bloc, from a lipid carrier by the action of the OSTs PilO (in *P. aeruginosa*) or PglL (in *N. meningitidis*). Although both PilO and PglL show relaxed glycan specificity, the former activity is restricted to short oligosaccharides. By contrast, PglL is able to transfer diverse oligosaccharides and polysaccharides. Such functional characterization supports the concept that despite their low sequence similarity, PilO and PglL belong to a new family of 'O-OTAses' that transfer oligosaccharides from lipid carriers to hydroxylated amino acids in proteins [35•]. To date, no such activity has been identified in eukaryotes.

Archaeal *N*-glycosylation

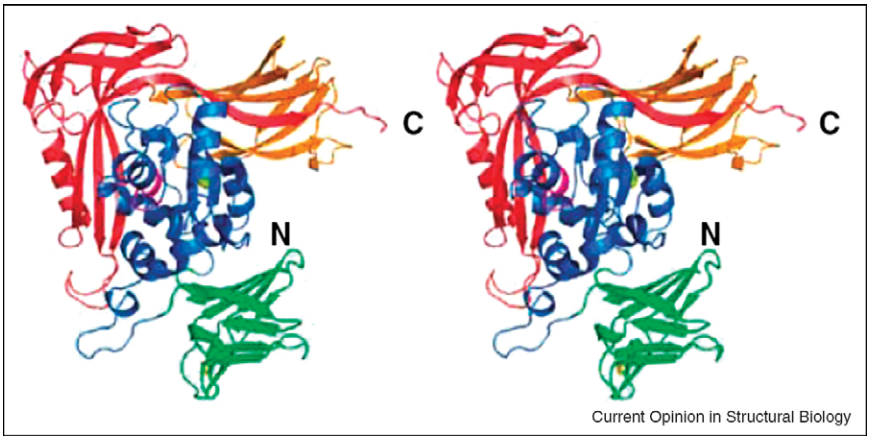
Post-translational modification in extreme conditions

In contrast to bacterial *N*-glycosylation, which is still considered a relatively rare event, *N*-glycosylation of archaeal proteins is more widespread. Although the sugar content of several archaeal glycoproteins has been known for several years, more detailed structural information on a limited number of glycans has recently become available. In the methanoarchaea *Methanococcocus voltae*, mass spectrometry, together with NMR analysis, has revealed the structure of the glycan entity decorating 15 of the 17 sequons distributed among the 4 flagellin proteins of this species (FlaA, FlaB1, FlaB2, and FlaB3), as well as the S-layer glycoprotein, as a novel trisaccharide, 6-*O*-threonyl-ManNAcβ1,4GlcNAc3NAcAβ1,3GlcNAc [36] (Table 1). The similar modification of both flagellins and the S-layer glycoprotein points to a common *N*-glycosylation mechanism in these cells. Mass spectrometry has also provided initial characterization of the pentasaccharide decorating at least two of the seven putative sequons of the S-layer glycoprotein in the haloarchaea *Haloferax volcanii*. Instead of the linear string of β1,4-linked glucose residues originally reported as decorating the protein [37], it now appears that the protein is modified by a pentasaccharide comprising two hexoses, two hexuronic acids, and a 190 Da species [38]. Recent *in vitro* studies using either isolated membranes or affinity-purified OST from the hyperthermophile *Pyrococcus furiosus* have examined the glycan moiety transferred to a fluorescently tagged, sequon-presenting hexapeptide [39,40••]. Mass spectrometry revealed the Asn-attached oligosaccharide to be composed of two *N*-acetylhexosamines, two hexoses, one hexuronic acid, and two pentoses, with GalNAc serving as the protein-linking sugar [40••].

Over the past two years, in parallel to efforts aimed at describing the structure of N-linked glycan structures, major strides have been made in identifying archaeal genes whose products are involved in the protein *N*-glycosylation process. In *H. volcanii* and *M. voltae*, several *agl* (*a*rchaeal *gl*ycosylation) genes have been cloned and their products functionally characterized via combined gene deletion and mass spectrometry approaches [41••,42••]. In *M. voltae*, AglA serves to add the terminal sugar subunit of the N-linked trisaccharide, namely a modified mannuronic acid with a covalently attached threonine residue (Table 1) [42••], while AglH, a homolog of the yeast Alg7 protein, is involved in addition of the Asn-bound linking sugar, GlcNAc [43]. In *H. volcanii*, AglD, AglE, AglF, AglG, and AglI participate in the addition of the second to fifth sugar subunits of the still incompletely characterized pentasaccharide linked to the S-layer glycoprotein (Figure 2) [38,44,45].

In both *M. voltae* and *H. volcanii*, *aglB* encodes the sole component of the archaeal OST. Indeed, the archaeal OST, comprising a single subunit homologous to the core STT3 subunit of the multimeric eukaryotic complex, offers a simplified model system for understanding the mechanism of the enzyme. Accordingly, Kohda and

Figure 4



The 3D structure of the soluble carboxyl terminal domain of *Pyrococcus furiosus* AglB/STT3. Stereo-view of the carboxyl terminal soluble domain of AglB/STT3 (residues 471–967). The core domain is shown in blue, the insertion domain is shown in green, and the peripheral domains are shown in red and orange. The WWDYG catalytic motif is shown in magenta, while the C638–C658 disulfide bond is shown as yellow sticks. The yellow sphere represents a bound metal ion. Reprinted by permission from Macmillan Publishers Ltd: [40••], copyright 2008.

colleagues recently solved the crystal structure of the *P. furiosus* STT3/AglB C-terminal domain to 2.7 Å, for the first time providing structural insight into OST activity [40••] (Figure 4). This soluble domain assumes a compact, globular structure that can be divided into four regions, based on tertiary folds. The central core domain, largely consisting of α-helices, includes the WWDYG motif implicated in the catalytic activity of the enzyme. The insert domain corresponds to a 10-stranded antiparallel β-barrel structure found in the central core domain and appears to be unique to *P. furiosus* and its close relatives. Two peripheral domains, composed mostly of β-strands, surround the central core. Multiple alignments of *P. furiosus* STT3/AglB and its homologs from Archaea, Bacteria, and Eukarya revealed the presence of a conserved DxxK motif. Examination of the 3D structure of *P. furiosus* Stt3/AglB reveals the Asp and Lys residues of this motif are found on the same side of a long helix, positioned in proximity to the catalytic WWDYG motif. It was suggested, therefore, that the DxxK sequence represents a new motif that also participates in the activity of the enzyme, probably interacting with the pyrophosphate moiety of the lipid carrier on which N-linked oligosaccharides are assembled and transported. Subsequent site-directed mutagenesis of these residues in the yeast enzyme has provided experimental support for this hypothesis.

Conclusions

Researchers are only now beginning to appreciate the variety of prokaryotes capable of protein glycosylation and the wide range of monosaccharides found in the glycan moieties of prokaryotic glycoproteins. At the same time, prokaryotic protein glycosylation pathways are being delineated. Continued investigation into the bacterial *N*-glycosylation and *O*-glycosylation processes will advance glyco-engineering efforts as well as the development of new antibacterial agents. A more comprehensive understanding of *N*-glycosylation and *O*-glycosylation in Archaea also carries enormous applied potential, given the possible links between glycosylation of archaeal proteins and their ability to withstand diverse physical challenges. Clearly, the future looks sweet for the field of bacterial and archaeal protein glycosylation.

Acknowledgements

The authors wish to thank Martin Young and Susan Logan for their insightful comments. The Eichler laboratory is supported by the Israel Science Foundation (grant 30/07), the US Air Force Office for Scientific Research (grant FA9550-07-10057), and the US Army Research Office (grant W911NF-07-1-0260).

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