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A novel N-linked flagellar glycan from *Methanococcus maripaludis*

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ABSTRACT

The archaea *Methanococcus maripaludis* strain Mm900 produces flagella that are glycosylated with an N-linked tetrasaccharide. Mass spectrometric analysis of flagellar tryptic peptides identified a number of tryptic glycopeptides carrying a glycan of mass 1036.4 Da, and fragmentation of the glycan oxonium ion indicated that the glycan was a tetrasaccharide. The glycan was purified, following extensive pronase digestion of flagellar filaments, by size-exclusion and anion-exchange chromatography. NMR spectroscopy revealed that the glycan had the following structure: Sug-4-β-ManNAc3NAmA6Thr-4-β-GlcNAc3NAcA-3-β-GalNAc-Asn where Sug is a novel monosaccharide unit, (5*S*)-2-acetamido-2,4-dideoxy-5-*O*-methyl-α-L-*erythro*-hexos-5-ulo-1,5-pyranose. This oligosaccharide has significant similarity to the oligosaccharide that was found previously in *Methanococcus voltae*.

1. Introduction

Flagellar proteins have been shown to be glycosylated in a diverse number of species of the domains Bacteria and Archaea.[1] In bacteria, glycosylation of flagellins is exclusively found to be O-linked, while in archaea the glycan is linked to the flagellin protein backbone via an N-linkage through a typical eukaryotic N-linked sequon (Asn-X-Ser/Thr). In archaea, the flagellar system is distinct from that of bacteria, and a body of evidence now indicates that the archaeal flagellar system much more closely resembles bacterial type IV pilus systems in both architecture and assembly.[2,3] Structural characterisation of flagellin glycans in archaea is thus far limited to only two species, *Methanococcus voltae* and *Halobacterium salinarum*,[4–6] while genetic investigation of the archaeal N-linked glycosylation process has been significantly advanced through mutagenesis and functional studies in *M. voltae*[7,8] and also in *Haloferax volcanii* where a pentasaccharide is N-linked to the S-layer protein.[9–13] The flagellin of *H. salinarum* is modified with N-linked sulfated oligosaccharides of glucose in (1→4)-linkage to hexuronic acids, while extensive structural characterisation of flagella from *M. voltae* revealed that the four flagellin structural proteins such as FlaA, FlaB1, FlaB2 and FlaB3 were all glycosylated with a trisaccharide composed of β-ManNAc3NAcA6Thr-4-β-GlcNAc3NAcA-3-β-GlcNAc-Asn. In addition, the same trisaccharide was identified on the respective S-layer proteins from these strains indicating a common N-linked glycosylation pathway for both processes. Studies have recently shown that *M. voltae* can also glycosylate both the flagellin and S layer with a tetrasaccharide composed of the afore-mentioned trisaccharide carrying an additional residue of either 220 or 262 Da mass.[14] A biological role for these glycans is yet to be determined although in *M. voltae* and *Methanococcus maripaludis*, flagellins that are nonglycosylated or have only truncated glycans attached result in either nonflagellated cells or flagellated cells that are less motile than the wildtype.[7,15] In addition, it has been recently demonstrated that a mutant of *M. maripaludis* that contains a deletion of an acetyltransferase gene (MMP0350) required for the biosynthesis of a glycan sugar was not only nonflagellated but also defective in attaching pili to the cell surface.[15]

In this work, we have elucidated the full structure of the flagellar glycan from *M. maripaludis* and presented the first example of a naturally occurring diglycoside of an aldulose as a component of the tetrasaccharide. In addition, the linkage of this novel tetrasaccharide to Asn is shown to occur via a β-GalNAc residue, which is in contrast to the related flagellar glycan from *M. voltae* that had been previously shown to occur via a β-GlcNAc residue.

2. Results and discussion

2.1. Mass spectrometry analysis of flagellar proteins

Attempts to determine the intact mass of respective flagellin monomers by mass spectrometry (MS) were unsuccessful, presumably due to poor ionisation of the intact proteins from the filaments. This had previously been observed for flagellins from *M. voltae*.[4] MS analysis was carried out on the tryptic digest of

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flagella purified from *M. maripaludis*. Tryptic peptides were analysed by nano-LC–MS/MS, and most of the resulting MS/MS spectra could be assigned to tryptic peptides from one of the three flagellar proteins expressed by *M. maripaludis*, FlaB1, 2 and 3 (Fig. 1). However, a number of spectra could not be easily identified and appeared to be derived from glycopeptides containing the same unusual glycan (See Supplementary Table S1). In some instances, the amino acid sequence of the glycopeptides could be determined from their fragment-ion patterns (Fig. 2). A simple subtraction of the peptide mass from that of the glycopeptide determined the mass of the glycan to be 1036.4 Da. Furthermore, each glycopeptide contains one or more N-linked sequons, suggesting that the glycan is N-linked as was found previously for *M. voltae* flagellar modifications.[4] Evidence for the N-linkage can be seen in the MS/MS spectrum of the tryptic glycopeptide, $T^{146-166}$ derived from FlaB1 protein (Fig. 2), where the y-fragment ions that contains the asparagine from the N-linked sequon (y_{11} and greater) are modified with a HexNAc residue (+203 Da).

Initially, the composition and sequence order of the glycan were difficult to determine as the terminal monosaccharide residue was very labile and did not produce a distinctive oxonium ion in the low-mass region of the glycopeptide MS/MS spectra. In addition, ions that arise from the sequential loss of sugar residues from the precursor ion (neutral loss) or from a charge-reduced precursor ion were not in high abundance in these spectra. Nevertheless, the general structure and composition of the glycan were elucidated due to a distinctive group of oxonium ions that were found in all glycopeptide MS/MS spectra, including the intact glycan ion at *m/z* 1037.4. These ions are identified in Figure 2.

Using mass spectrometry, we determined that the glycan is a tetramer consisting of a HexNAc residue (203 Da) linked to the asparagine residue on the peptide, followed by a di-*N*-acetyl hexuronic acid (258 Da), a 358 Da residue and capped with a 217 Da residue at its non-reducing end. The 358 and 217 Da sugars were not observed before and were considered novel. Nevertheless, this glycan resembles that found on *M. voltae* flagellin, which is N-linked to the peptide through a GlcNAc residue, while the second sugar is di-*N*-acetyl-D-glucuronic acid.[4] Unlike the *M. voltae* flagellin proteins where every N-linked sequon from each flagellin protein was found to carry a glycan, in *M. maripaludis*, N^{26} of the first N-linked sequon of FlaB1 and FlaB2 was not glycosylated, suggesting that these sites are not accessible in *M. maripaludis* for attachment of a glycan. A total of nine sites of N-linked glycosylation were identified in FlaB1, FlaB2 and FlaB3 (Fig. 1, Supplementary Table 1). The mass of each glycopeptide was correlated to a single tetrasaccharide moiety at each N-linked site.

2.2. Purification of flagellar glycan

The flagellar glycan was purified following extensive pronase digestion of purified flagella using size-exclusion and anion-exchange chromatography. Following desalting, the column fractions were monitored by MS to identify glycan-containing samples. These were subsequently lyophilised and examined by NMR spectroscopy.

2.3. NMR structural analysis of pronase-digested glycopeptide

Standard homo- and heteronuclear NMR experiments were used to characterise the structure of the flagellar glycan. The structure is presented in Figure 3, and chemical shifts for each sugar residue and derivative are presented in Table 1. Spectra assignment revealed four monosaccharide spin systems. Residues C and D were identified on the basis of chemical shifts and coupling constants as β-GalNAc and β-GlcNAc3NAcA. Residue B had a β-manno configuration as was determined from coupling constants and an NOE between H-1 and H-3, and H-5. It had amino groups at C-2 and C-3 (C-2 at 53.4 and C-3 at 57.1 ppm). ^{13}C chemical shifts for this monosaccharide were close to those of the same sugar in *Pseudomonas aeruginosa* O3a,c polysaccharide, where β-D-ManNAc3NAmA is substituted by β-D-FucNAc.[16] The amino group at C-3 of 2,3-diaminomannuronic acid was substituted by an amidine residue, as followed from the observation of the HMBC correlation between proton B3 and C-1 of the amidine group, which agreed with the characteristic low field shift of the C-3 signal for amidine substitution (compared with acetylation). Mass spectral data confirmed that the amidine unit was attached to residue B. The linkage between residue B and threonine was determined from HMBC data as described previously.[4] Threonine was differentiated from *allo*-threonine based on the position of its C-4 signal (20.6 ppm,

FlaB1

ASGIGTLIVF	IAMVLVAAVA	ASVLI**NTS**GF	LQQKASTTGK
ESTEQVASGL	LINGITGSVG	TSDVKLLAIY	LAPNAGSSAI
DLAQTKVMLD	YNGKSVVLGY	GGNQDMSSG**N**	**SS**VFS**NDT**GA
TATTFQVNIL	QDYDDSAVDN	AVINKGDAVA	LIVDV**NAS**FA
GEIPERTAIS	GKVQPEFGAP	GVISFTTPAS	YTTTLVELQ

FlaB2

ASGIGTLIVF	IAMVLVAAVA	ASVLI**NTS**GF	LQQKASTTGK
DSTEQVASGL	QIMGISGYQA	GTANA**NIT**KL	AIYITPNAGS
AAIDMNQVVL	TLSDGTTKTV	TKYDTTAYT**N**	**LT**AGGDLY**NT**
TTV**NWS**KLAD	TTEFGIVEIQ	DADLSFTSSA	PVINKGDIVA
IIVSGVSFDT	RMEISGTVQP	EFGAPGVISF	TTPSTFTEKV
VSLQ			

FlaB3

AVGIGTLIIF	IAMVLVAAIA	ASVIINTAGK	LQHKASTVGE
ESTEQVASGI	QVLKVIGHAD	TKTTIDKLAV	MVAPNVGGEI
DLSTTILTLG	TGDAKYSLVY	DSTQHNADVK	DDGSDSIFDE
TEWGSGSKYG	LIVLQDSD**NS**	**T**AEATHPTIN	YGDKVYITVT
MDV**NST**SKVS	GEVIPDYGAS	GIVEFRAPSV	FTETVVTLQ

Figure 1. Assignment map of flagellin proteins from *M. maripaludis*. The predicted amino acid sequence for the mature flagellin proteins, FlaB1, FlaB2 and FlaB3, is presented, and the N-linked sequons are highlighted. Peptides and glycopeptides identified by nanoLC–MS/MS of the flagellar tryptic digest are underlined. Note that all N-linked sites are occupied with the exception of N^{26} in FlaB1 and FlaB2. Only the unmodified peptide, T^{1-34}, was observed by nanoLC–MS/MS for these two flagellin proteins.

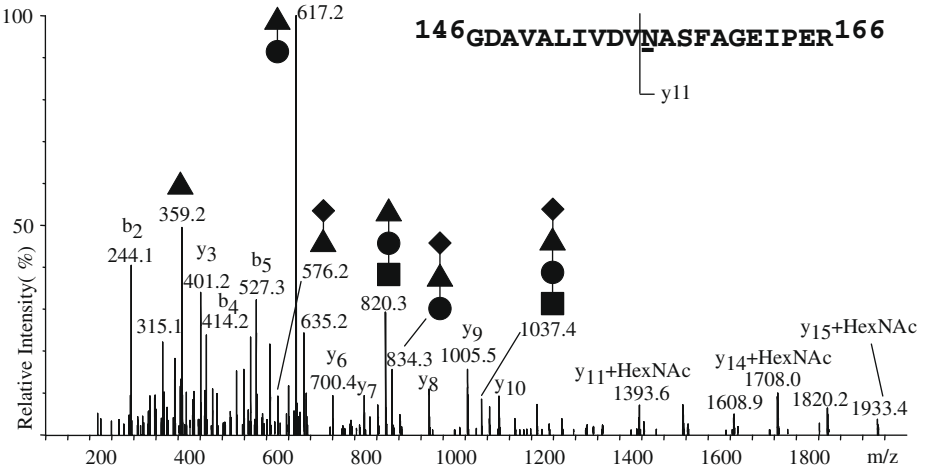

Figure 2. NanoLC–MS/MS analysis of the tryptic glycopeptide, $T^{146-166}$. MS/MS spectrum of the triply protonated glycopeptide ion at *m/z* 1060.5. The amino acid sequence of this peptide is presented in the inset, and the site of the N-linkage is underlined. The b- and y-fragment ions arising from the fragmentation of the peptide bonds are indicated in the spectrum. Note that the y-fragment ions containing the N^{156} residue (y_{11} and greater) are all heavier than that predicted by extra 203 Da, indicating that the glycan is N-linked through a HexNAc residue. The major carbohydrate oxonium ions are identified in this spectrum using symbols to indicate the sugar residues present (■ 203 Da; ● 258 Da; ▲ 358 Da and ♦ 217 Da). The intact glycan oxonium ion is observed at *m/z* 1037.4 and loss of the 203 Da (■) and 217 Da (♦) sugars yields the ions at *m/z* 834.3 and 820.3, respectively. The intense oxonium ion at *m/z* 617.2 corresponds to the 258 Da (●) and 358 Da (▲) sugar residues. The weaker ion at *m/z* 576.2 corresponds to the 358 Da (▲) and 217 Da sugars (♦). The only single residue oxonium ion observed originates from the 358 Da sugar (▲, *m/z* 359).

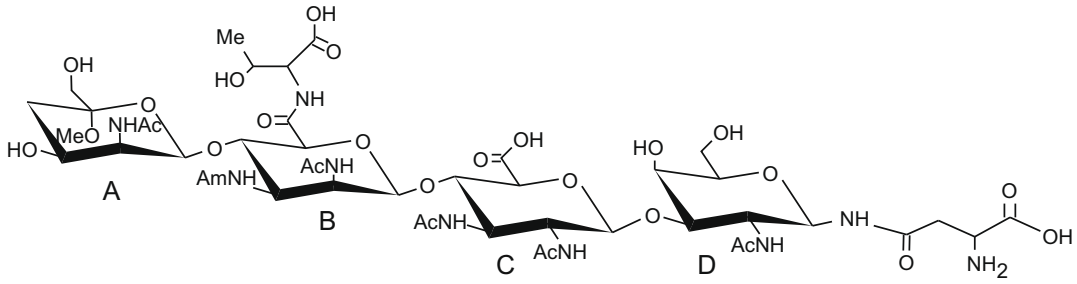

Figure 3. Structure of *M. maripaludis* flagellar glycan.

Table 1
NMR chemical shifts of flagellar glycan[a]

Unit		1	2	3	4	5/Me	6a/b
β-GalNAc **D**	H	5.03	4.06	3.84	4.19	3.73	3.73
	C	79.6	50.6	81.5	68.9	77.6	60.4
β-GlcNAc3NAcA **C**	H	4.68	3.87	3.95	3.78	3.81	
	C	103.5	54.5	54.5	79.2	77.7	176.4
β-ManNAc3NAcA **B**	H	4.74	4.45	4.13	3.87	4.18	
	C	100.1	51.6	57.1	74.7	77.5	176.9
A	H	4.78	4.28	4.07	1.43/2.00		3.38/3.69
		$J_{1,2}$ 1.8	$J_{2,3}$ 4	$J_{3,4ax}$ 12	$J_{3,4eq}$ 5	$J_{4ax,4eq}$ 13	$J_{6a,6b}$ 11.8
	C	97.0	53.4	65.6	34.7	101.2	62.4
A OMe	H					3.26	
	C					50.0	
Thr	H		4.18	4.27	1.24		
	C	176.9	61.4	70.0	20.6		
Asn	H		3.97	2.91/2.91			
	C	174.3	52.1	36.0			

[a] Amidine signal (C-1, H-2/C-2): 168.0, 2.26/19.9; *N*-acetates (not assigned to particular locations): 176.1, 1.96/23.3; 176.1, 1.96/23.3; 175.9, 2.00.23.3; 177.3, 2.07/23.3; 177.5, 2.14/23.3 ppm.

expected for *allo*-threonine is 17.3 ppm). The absolute configuration of the threonine residue has not been determined.

Residue A was identified as 2-acetamido-2,4-dideoxyhexose (two H-4 protons, ^{13}C NMR C-2 signal at 34.7 ppm, C-4 signal at 53.4 ppm). No H-5 proton was present. HMBC correlations from H-4 and H-6 identified the C-5 resonance at 101.2 ppm. HMBC and NOE correlations showed that an OMe group was attached to C-5 (Figs. 4 and 5). Thus, this monosaccharide was the 5-methyl-

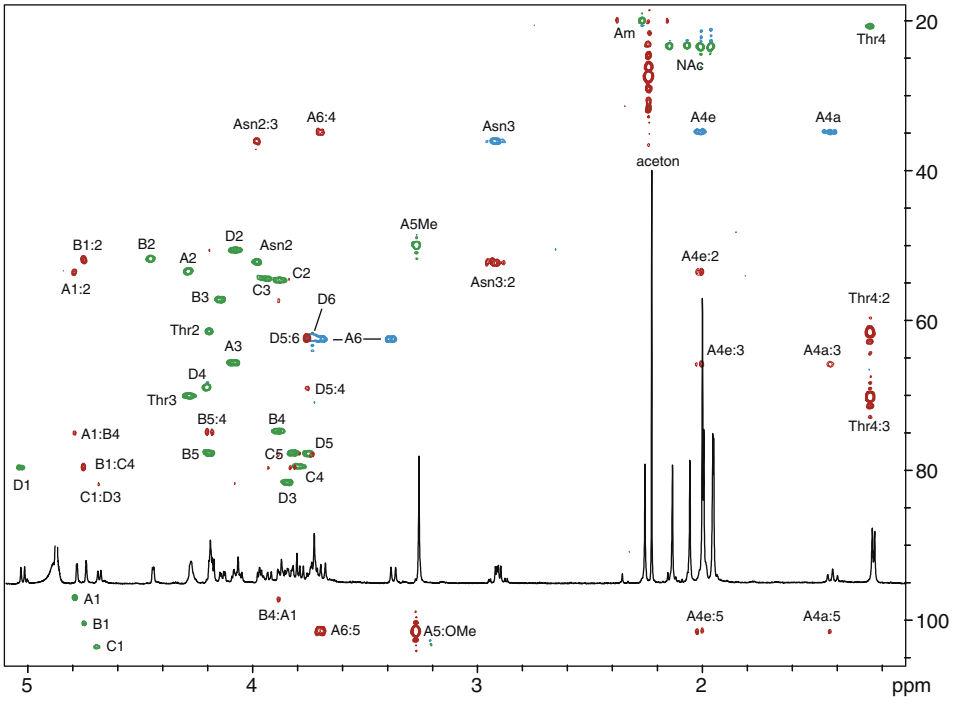

Figure 4. Overlap of ^{1}H, HSQC (green, cyan) and HMBC (red) spectra of the *M. maripaludis* flagellar oligosaccharide.

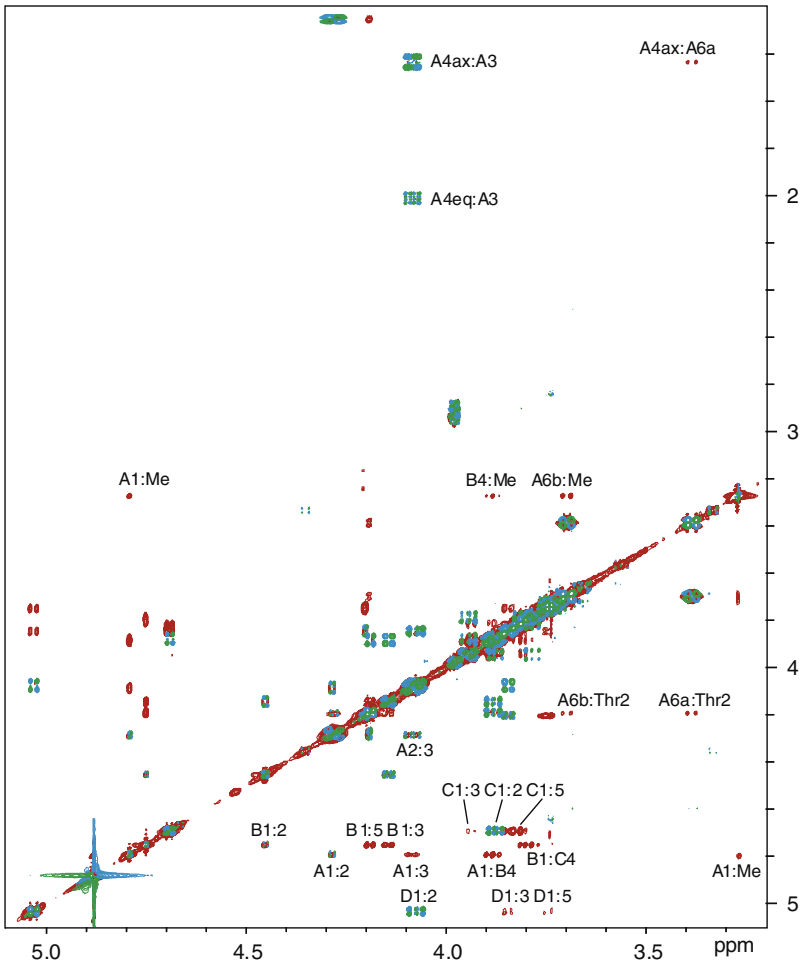

Figure 5. Overlap of COSY (green, cyan) and NOESY (red) spectra of the *M. maripaludis* flagellar oligosaccharide.

glycoside of a hexos-5-ulose. Vicinal coupling constants (Table 1) between ring protons corresponded to the α-*erythro* configuration of the C-1–C-3 fragment. Strong NOE correlations between protons A1:A3, A1:Me, A6b:Me, and A4ax:A6a indicated that the OMe group occupied the axial position in the $^4C_1$ conformation, and the whole configuration of this 2-acetamido-2,4-dideoxy-5-*O*-methyl-hexos-5-ulopyranose was isomorphic to the 4-deoxy-β-D-*manno* (D-configuration is discussed below).

The monosaccharide sequence as shown in Figure 3 followed from the NOE between protons A1:B4, B1:C4,5, C1:D3, A6a,b:Thr2; B4:Me and HMBC (proton:carbon, A1:B4, B1:C4, C1:D3, D1:Asn C-1) data.

The absolute configuration of the D-GalNAc was determined by GC–MS of acetylated 2-butyl glycosides.[17] The D-configuration of the residues B and C was deduced on the basis of glycosylation effects in the ^{13}C NMR spectra.[18] Thus, for the residue of β-GlcNAc3NAcA, the position of its C-1 signal (103.5 ppm) was closer to that expected for the same absolute configuration as D-GalNAc (104.7 ppm) than for different configurations (99.6 ppm). The C-3 and C-4 signals of GalNAc (81.5 and 68.9 ppm) were also closer to the DD combination (expected value 81.2 and 69.2 ppm) than to the LD combination (expected value 79.2 and 66.3 ppm). For the β-ManNAc3NAcA, its C-1 signal (100.1 ppm) is closer to the expected value for the DD-pair (100.5 ppm) than to that of LD-pair (101.4 ppm); C-3 signal of the GlcNAc3NAcA also fits slightly better for DD-pair. The same method was used for the determination of the configuration of residue A: A comparison of the position of the C-4 signal of ManNAc3NAmA6Thr (74.7 ppm) with that of the ManNAc3NAmA substituted with α-D-FucNAc (71.4 ppm) and β-D-FucNAc (75.1 ppm) from different *P. aeruginosa* polysaccharides[16] led to the conclusion that the monosaccharide A was (5*S*)-2-acetamido-2,4-dideoxy-5-*O*-methyl-α-L-*erythro*-hexos-5-ulo-1,5-pyranose, as shown in Figure 3.

3. Discussion

NMR spectroscopy and mass spectrometry have been utilised to deduce the structure of the flagellar glycan from *M. maripaludis*. In comparison to the structure elucidated for the flagellar glycan from the related organism *M. voltae*,[4,14] the glycan presented on the flagellins of this organism appears to be novel in structure. *M voltae* has been shown to glycosylate its flagellin with either the trisaccharide β-ManNAc3NAcA6Thr-4-β-GlcNAc3NAcA-3-β-GlcNAc-Asn or this trisaccharide carrying an additional mass of either 220 or 262 Da.[14] In contrast, we demonstrate in this study that the flagellar glycan from *M. maripaludis* is composed of a novel tetrasaccharide that is attached in an N-linkage via β-GalNAc. The significance of this linkage to the flagellin proteins via β-GalNAc rather than β-GlcNAc is not known, but may be a reflection of the specificity of the respective glycosyltransferases from each strain. In *M. voltae* AglH (Mv1751), it has been shown via complementation in yeast to function as the β-GlcNAc transferase.[8]

In addition to containing β-GalNAc and β-GlcNAc3NAcA, the *M. maripaludis* flagellar glycan also contains a 3-acetamidino derivative of 2,3-diamino-2,3-dideoxymannuronic acid amidated with a threonine amino group (β-ManNAc3NAmA6Thr), as well as a unique terminal sugar, 2-acetamido-2,4-dideoxy-5-*O*-methyl-hexos-5-ulo-1,5-pyranose. The presence of this sugar to our knowledge is the first example of a naturally occurring diglycoside of an aldulose. As the genome sequence of *M. maripaludis* is available, and genetic techniques exist that allow targeted studies of individual genes, it will now be possible to identify the biosynthetic enzymes responsible for the production of this novel structure.[19,20] The biological role of the tetrasaccharide, and more significantly the novel terminal sugar, is currently unknown but it is clearly of relevance in the emerging field of prokaryotic glycobiology. The structural elucidation of the glycan monosaccharides may now facilitate the identification of corresponding biosynthetic enzymes.

4. Experimental

4.1. Growth of *M. maripaludis* and extraction of flagellar filaments

M. maripaludis strain Mm900 is a Δ*hpt* mutant of *M. maripaludis* strain S2 (formerly LL) that was developed for markerless mutagenesis.[20] It was grown at 30 °C in Balch medium III under an atmosphere of CO_2–H_2 (20:80).[20] Crude flagellar filament preparations were obtained by detergent extraction of cell membranes followed by banding in a KBr gradient.[21]

4.2. MS analysis of flagellin tryptic peptides

For MS characterisation of flagellar glycan, flagellar filaments (~200 μg) were digested overnight with trypsin (Promega, Madison WI) at a ratio of 30:1 (protein:enzyme, v/v) in 50 mM ammonium bicarbonate at 37 °C. The protein digest was analysed by nano-LC–MS/MS using a QTOF Ultima hybrid quadrupole time-of-flight mass spectrometer coupled to a NanoAquity UPLC system. The digest was separated on a 100 μm × 100 mm, 1.7 μm BEH130 C_{18} column (Waters) using a standard gradient of acetonitrile and 0.2% formic acid (400 nL/min.). MS/MS spectra were acquired on doubly, triply and quadruply charged ions. The resulting MS/MS spectra were submitted to the Mascot® search engine (Matrix Science Ltd., London, UK) and searched against the NCBInr database. Unidentified spectra were then examined manually for the presence of post-translational modifications.

4.3. Purification of flagellar glycan

To obtain sufficient amounts of pure glycan for structural analysis, we digested 2.5 mg of flagellar filaments with pronase (50 μg) in 0.1 M Tris HCl, 2 mM $CaCl_2$, pH 8.0. After incubation at 37 °C for 24 h, a second aliquot of pronase was added (30 μg), and digestion was allowed to continue for 24 h. The lyophilised digest was resuspended in distilled H_2O and applied to a Bio-Gel P4 size-exclusion column. Fractions were collected and examined by infusion-MS/MS on a LTQ linear ion-trap mass spectrometer (ThermoFisher Scientific), and those containing glycan were pooled and applied to a Bio-Gel P2 column. The eluate was collected in 1-mL fractions, concentrated and again examined by MS to identify fractions containing glycan. Fractions were pooled and applied to an anion-exchange Hitrap Q column, and glycan was eluted with a linear gradient of NaCl (0–1 M, 1 h). Desalting was performed on Sephadex G15 prior to analysis by NMR spectroscopy.

4.4. NMR spectroscopic analysis

NMR experiments were carried out with a Varian INOVA 600 MHz (^1H) spectrometer with a Varian Z gradient probe at 25 °C in 3-mm tube with acetone internal reference (2.225 ppm for ^1H and 31.5 ppm for ^{13}C) using the standard pulse sequences DQCOSY, TOCSY (mixing time 120 ms), NOESY (mixing time 200 ms), HSQC and HMBC (100 ms long-range transfer delay).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2009.01.006.

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