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Identification and Characterization of the Unique *N*-Linked Glycan Common to the Flagellins and S-layer Glycoprotein of *Methanococcus voltae**

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The flagellum of *Methanococcus voltae* is composed of four structural flagellin proteins FlaA, FlaB1, FlaB2, and FlaB3. These proteins possess a total of 15 potential *N*-linked sequons (N*X*(S/T)) and show a mass shift on an SDS-polyacrylamide gel indicating significant post-translational modification. We describe here the structural characterization of the flagellin glycan from *M. voltae* using mass spectrometry to examine the proteolytic digests of the flagellin proteins in combination with NMR analysis of the purified glycan using a sensitive, cryogenically cooled probe. Nano-liquid chromatography-tandem mass spectrometry analysis of the proteolytic digests of the flagellin proteins revealed that they are post-translationally modified with a novel *N*-linked trisaccharide of mass 779 Da that is composed of three sugar residues with masses of 318, 258, and 203 Da, respectively. In every instance the glycan is attached to the peptide through the asparagine residue of a typical *N*-linked sequon. The glycan modification has been observed on 14 of the 15 sequon sites present on the four flagellin structural proteins. The novel glycan structure elucidated by NMR analysis was shown to be a trisaccharide composed of β-Man*p*NAcA6Thr-(1–4)-β-Glc-*p*NAc3NAcA-(1–3)-β-Glc*p*NAc linked to Asn. In addition, the same trisaccharide was identified on a tryptic peptide of the S-layer protein from this organism implicating a common *N*-linked glycosylation pathway.

Glycosylation of prokaryotic proteins is now well accepted, and examples of *N*- and *O*-glycosylation and of attachment of glycosylphosphatidylinositol anchors can now be found in the literature (1, 2). However, in contrast to eukaryotic glycosylation systems, prokaryotic systems display considerable diversity in the structure of the respective glycans and the proximal monosaccharide linkage. As a consequence there is considerable interest in determining the structural and genetic basis of glycan production among these diverse prokaryotic systems.

The archaeal flagellum is a unique motility structure that is distinct from the well characterized bacterial flagellum (3). In contrast to bacterial flagellar assembly where newly synthesized flagellin is incorporated at the distal tip of the filament, it is believed that mature archaeal flagellin is incorporated at the base of the filament. In recent studies, the assembly of archaeal flagellum has been shown to more closely resemble a second bacterial motility system, the type IV pilus, where the structural protein pilin is synthesized with an unusual signal peptide and a hydrophobic N terminus. In Archaea, signal peptidases have been shown to cleave a signal peptide of the preflagellin proteins to produce mature flagellin, which is then incorporated into the filament (4). Flagellated archaeal species have one to five flagellin genes organized into a *fla* locus (3). The marine archaeon *Methanococcus voltae* has four flagellin structural genes organized in two transcriptional units: one unit contains *flaA*, whereas the second unit contains *flaB1*, *flaB2*, and *flaB3* along with other co-transcribed accessory genes (3, 5). Early work demonstrated that FlaB1 and FlaB2 are the major species of the extended filament, whereas FlaB3 is localized proximal to the cell surface and is a major component of the curved hook region. The minor component FlaA is believed to be distributed throughout the filament (6).

Glycosylation of archaeal flagellin using glycoprotein-specific stains such as thymol sulfuric acid or periodic acid-Schiff is commonly reported (7, 8), although a detailed structural analysis of the post-translational modification present is limited to a single organism, *Halobacterium salinarum* (formerly *Halobacterium halobium*) (9). The flagellin of this organism has been shown to be glycosylated with *N*-linked sulfated oligosaccharides composed of glucose in 1,4 linkage to hexuronic acids (10, 11). This post-translational modification is also found on purified S-layer protein from this organism. This is in marked contrast to recent studies of bacterial flagellin glycosylation in *Campylobacter*, *Helicobacter*, *Pseudomonas*, and *Listeria* where each unique glycan was shown to be *O*-linked to the respective flagellin proteins (12–15).

Although the *M. voltae* flagellins did not react with glycoprotein-specific stains, these proteins possess a total of 15 potential *N*-linked sequons (N*X*(S/T)) and show a mass shift on an SDS-polyacrylamide gel suggesting significant post-translational modification. In this study we provide a detailed structural characterization of the unique glycan found on all of the *M. voltae* flagellin proteins and demonstrate that the S-layer protein is also post-translationally modified with the same novel trisaccharide.

EXPERIMENTAL PROCEDURES

Microbial Strains and Growth Conditions—M. voltae cells were grown in Balch medium III under a head space gas mixture of 80% H_2, 20% CO_2 at 37 °C as described previously (8).

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[S] The on-line version of this article (available at http://www.jbc.org) contains Supplemental Table 1.

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Flagellin and S-layer Purification—Crude flagellar filament preparations were isolated by shearing followed by banding in a KBr gradient (8). For isolation of flagella containing some attached basal structure (whole intact flagella), cells were extracted with the non-ionic detergent OP-10 without prior flagella shearing as described by Bardy *et al.* (6). S-layer protein was released from *M. voltae* membranes by heat treatment at 60 °C for 1 h in 50 mM HEPES buffer as described previously (16).

Preflagellin Peptidase Reaction—A non-glycosylated form of FlaB2 was produced in *Escherichia coli* as described previously (17). This form has the signal peptide of FlaB2 still attached. To remove this, *E. coli* membranes containing FlaB2 were used as substrate in an *in vitro* preflagellin peptidase assay using *M. voltae* membranes as a source of the preflagellin peptidase. Both the unprocessed and processed forms of FlaB2 are detected in a Western blot using anti-flagellin antisera (17).

Enzymatic Digestion of Flagellin and S-layer Protein—Flagellin or S-layer protein extract (50–200 μg) was digested with either trypsin (Promega, Madison, WI) or Glu-C (Roche Applied Science) at a ratio of 40:1 (protein:enzyme, v/v) in 100 mM ammonium bicarbonate, pH 8.5, at 37 °C overnight. Double digestion was achieved by overnight incubation of the tryptic digest with Glu-C at the same ratio (40:1) at 37 °C.

Nano-liquid Chromatography-Electrospray Tandem Mass Spectrometry Analysis—Protein digests were analyzed by nano-LC[1]-MS/MS using a Q-TOF 2 hybrid quadrupole time-of-flight mass spectrometer coupled to a CapLC capillary HPLC system (Waters, Milford, MA). The digests (0.5–1 μg) were separated on a 75-μm-inner diameter × 150-mm Inertsil ODS 3, 5-μm nano-HPLC column (Dionex/LC Packings, Sunnyvale, CA) using the following gradient conditions: 5–60% acetonitrile, 0.2% formic acid in 30 min; 60–90% in 5 min. The mass spectrometer was set to automatically acquire MS/MS spectra on doubly, triply, and quadruply charged ions. All MS/MS spectra were examined manually for the presence of unusual modifications.

HPLC Purification of Tryptic Peptides—Fractionation of the flagellin tryptic digests was carried out using an Agilent 1100 series HPLC system equipped with a diode array detector (Agilent Technologies, Palo Alto, CA). The digests (25–40 μg) were separated on a 4.6 × 250-mm Jupiter C_{18} reverse phase column (Phenomenex, Torrance, CA) using the following gradient conditions: 5–40% acetonitrile, 0.2% formic acid (25 min); 40–95% (4 min); hold at 95% (1 min). The diode array detector was set at 254 nm to monitor the separation. Fractions were collected every minute starting at 4 min. The fractions were immediately evaporated to dryness on a SpeedVac preconcentrator; redissolved in 20 μl of acetonitrile, 5% acetic acid; and analyzed by MALDI-TOF MS using a Voyager-DE STR (Perseptive/Applied Biosystems, Foster City, CA). Dihydroxybenzoic acid was used as the MALDI matrix.

ESI-MS/MS and Front-end Dissociation MS/MS Analysis—HPLC fractions containing potential glycopeptides were analyzed by nano-ESI-MS/MS. Approximately 3 μl of each fraction were loaded into a nanoelectrospray emitter (Waters) and analyzed using the Q-TOF 2. Selected precursor ions were initially examined by MS/MS (50–80-eV collision energies, laboratory frame of reference), which confirmed the presence or absence of a glycan modification. More extensive sequencing analysis often required the use of front-end collision-induced dissociation to fragment the ions as they entered the mass spectrometer. This was achieved by increasing the orifice voltage from 30 to 90 V. Selected fragment ions were then analyzed by MS/MS as described above. This method was used to determine the site of glycan linkage and to generate fragment ion spectra of the glycan residue oxonium ions. Accurate mass measurement of the glycan oxonium ions in selected glycopeptide MS/MS spectra was achieved by using a number of the neighboring peptide fragment ions as internal mass standards. Plausible elemental formulas were then generated for each glycan greatly aiding the process of identifying the unusual glycan moieties.

β-Elimination of Tryptic Peptides—To further confirm the nature of the glycan linkage, β-elimination was performed on a number of the glycopeptide-containing HPLC fractions (18). Approximately 5 μl of the selected fractions were dried, dissolved in 100 μl of 25% ammonium hydroxide (aqueous), and allowed to stand at room temperature for 6–18 h. The samples were then evaporated to dryness; redissolved in 10 μl of 5% acetonitrile, 5% acetic acid; desalted using C_{18} Ziptips (Millipore, Billerica, MA); and analyzed by MALDI-TOF MS in the manner described above. Flagellin from *Campylobacter jejuni* 81–176 was used as a positive control. This protein is extensively modified with *O*-linked glycans (12).

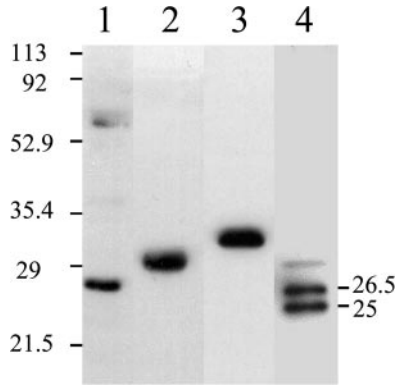

FIG. 1. **SDS-PAGE/Western blot analysis of *M. voltae* flagellin proteins.** *Lanes 1–3* are membrane preparations of *M. voltae* obtained by osmotic lysis, and *lane 4* is an analysis of a preflagellin peptidase assay using membranes from *E. coli* expressing recombinant *M. voltae* FlaB2 flagellin and *M. voltae* membranes as enzyme source. *Lane 1* was developed with FlaA-specific antiserum, *lane 2* and *lane 4* were developed with anti-FlaB2 antiserum, and *lane 3* was developed with FlaB3 antiserum. *Lane 4*, FlaB2 flagellin produced in *E. coli* with no glycosylation but with the 12 amino acid signal peptide present (*upper band*, 26.5 kDa) or removed during the assay (*lower band*, 25 kDa).

Glycan Purification—Approximately 620 μg of flagellin protein containing an estimated 90 μg of glycan were digested with Pronase in 0.1 M Tris-HCl, 2 mM $CaCl_2$, pH 8.0 (50:1 flagellin to Pronase). After incubation for 24 h at 37 °C a second aliquot of Pronase was added (same ratio), and the digestion was allowed to continue for another 24 h. The final digest was applied in turn to a Biogel P4 and to a P2 size exclusion column (Bio-Rad) in the manner described previously (5). The eluate from each size exclusion column was collected in 2-ml fractions concentrated to 20 μl and analyzed by nano-ESI-MS/MS as described above. Glycan-containing fractions were then analyzed by NMR.

NMR Analysis of Purified Glycan—NMR spectra were acquired at 20 °C on Varian INOVA spectrometers operating at 500 and 600 MHz (Varian Associates Inc., Palo Alto, CA). The purified glycan was lyophilized and redissolved in either D_2O or, for experiments where exchangeable proton resonances were observed, 90% H_2O. The sample volume was ~150 μl. The measured pH in D_2O (pD) uncorrected for D_2O was 3. Standard two-dimensional homonuclear COSY, TOCSY, and NOESY spectra and selective 1D-^{1}H TOCSY and NOESY spectra were acquired for the assignment of proton resonances within the glycan and were performed as described previously (19, 20). Proton assignments were then used to identify cross-peaks in heteronuclear ^{1}H-^{13}C heteronuclear single quantum coherence and HMBC spectra required for complete structure determination. ^{1}H and ^{13}C chemical shifts were referenced with respect to the methyl group of an internal acetone standard, appearing at 2.225 and 31.1 ppm, respectively. All spectral data were acquired using standard sequences provided with VNMR 6.1 software (Varian Associates Inc.) and were processed with the software package TOPSPIN (Bruker Biospin, Billerica, MA).

RESULTS

Intact Mass Analysis of M. voltae Flagellin—The apparent molecular masses of the *M. voltae* flagellins as determined by Western blotting with flagellin-specific antisera after SDS-PAGE are: FlaA, 27.5 kDa; FlaB1, 31 or 33 kDa; FlaB2, 31 or 33 kDa; and FlaB3, 34.5 kDa (Fig. 1). The antiserum raised against FlaB2 also reacts with FlaB1, and the two proteins have masses of 31 and 33 kDa. This is higher than the molecular masses deduced from the corresponding genes: FlaA, 23,905 Da; FlaB1, 22,513 Da; FlaB2, 22,799 Da; and FlaB3, 25,523 Da. When FlaB2 is overexpressed in *E. coli*, a protein of ~26.5 kDa is detected by FlaB2 antiserum, corresponding to the FlaB2 protein with the signal peptide of 12 amino acids still attached. This signal peptide is not present on the mature

[1] The abbreviations used are: LC, liquid chromatography; COSY, correlated spectroscopy; ESI, electrospray ionization; feCID, front end collision-induced dissociation; GlcNAc, 2-acetamido-2-deoxyglucose; GlcNAc3NAcA, 2,3-diacetamido-2,3-dideoxyglucuronic acid; HexNAc, *N*-acetylhexosamine; HMBC, heteronuclear multiple bond coherence; ManNAcA, 2-acetamido-2-deoxymannuronic acid; MS, mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; HPLC, high pressure liquid chromatography; MS/MS, tandem MS.

flagellin protein isolated from intact flagella filaments of *M. voltae*. This signal peptide can be removed from the *E. coli*-produced FlaB2 in an *in vitro* preflagellin peptidase assay as shown in Fig. 1, *lane 4* (17). This processed FlaB2 produced in *E. coli* has an apparent molecular mass of 25 kDa, which represents a difference in molecular mass from the mature FlaB2 flagellin protein isolated from the methanogen of about 6–8 kDa. This is suggestive of significant post-translational modification of the protein occurring in *M. voltae.* Earlier attempts to stain the *M. voltae* flagellins with glycoprotein stains such as the periodic acid Schiff reagent were unsuccessful (21). Attempts to determine the intact mass of the respective flagellins by mass spectrometry (MALDI-TOF MS and ESI-MS) have so far failed.

Nano-LC-MS/MS Analysis of Flagellin Peptide Digests—Initially the tryptic digest of the flagellin proteins was analyzed by nano-LC-MS/MS. Many of the MS/MS spectra could be readily assigned to unmodified flagellin tryptic peptides. However, quite a number of the spectra were derived from peptides modified with what appeared to be a 779-Da glycan composed of three residues with masses of 318, 258, and 203 Da, respectively (Fig. 2*A*). Moreover the neutral loss fragmentation patterns suggested that the glycan is linked to the peptides via the 203-Da residue (which is almost certainly a *N*-acetylhexosamine (HexNAc)) and that the 318-Da moiety is the terminal residue. This was confirmed by the presence of the strong oxonium ion at *m/z* 319.1 and the almost complete absence of the HexNAc oxonium ion at *m/z* 204.1. The slightly weaker oxonium ion at *m/z* 259.1 and the disaccharide ion at *m/z* 577.2 (Fig. 2*A*) suggest that the 258-Da residue is centrally located within the linear trisaccharide.

Although it was possible to infer the peptide sequence from the mass of the glycopeptide, the majority of the glycopeptide MS/MS spectra contained insufficient peptide fragment ions to confirm their identities. In these instances, feCID-MS/MS was performed on the deglycosylated peptide fragment ions to provide this evidence. For example, nano-ESI-feCID-MS/MS analysis of the deglycosylated fragment ion at *m/z* 890.4 observed in Fig. 2*A* confirmed the identity of this peptide as $T^{100-106}$ from FlaB2 flagellin (Fig. 2*C*).

A concerted effort was made to map the four flagellar proteins to identify as many sites of glycosylation as possible (Fig. 3). Nano-LC-MS and MS/MS analyses were performed on multiple tryptic digests, on Glu-C digests, and on tryptic/Glu-C double digests of the sheared flagellin isolates. This worked well for FlaB1 and FlaB2, the most abundant proteins in the flagellin. To improve the coverage of the less abundant proteins the same analyses were carried out on intact flagellin preparations that retained a partial anchoring structure. It has been demonstrated previously that the FlaB3 protein is enriched in these preparations (6). Improved coverage was obtained for FlaB1 (77%), FlaB2 (98%), and FlaB3 (71%). Two peptides for FlaA (13%) were also observed, including one glycopeptide (see Supplemental Table 1). These results are consistent with the fact that FlaA is the least abundant of the four flagellin components of the flagellar filament (6). In addition, FlaA may be resistant to enzymatic digestion as a consequence of its location within the flagellar filament.

Linkage Site Determination—Examination of the glycopeptide amino acid sequences suggested that the glycan may well be *N*-linked to asparagine residues in the classic eukaryotic *N*-linkage consensus sequon (Asn-Xaa-(Ser/Thr)). In fact, our data indicated that 14 of the 15 *N*-linked sequons may carry the glycan. The only site that remained uncharacterized was the first *N*-linked sequon (^{26}NTS^{28}) of the FlaA flagellin protein. However, no peptides, modified or otherwise, were detected

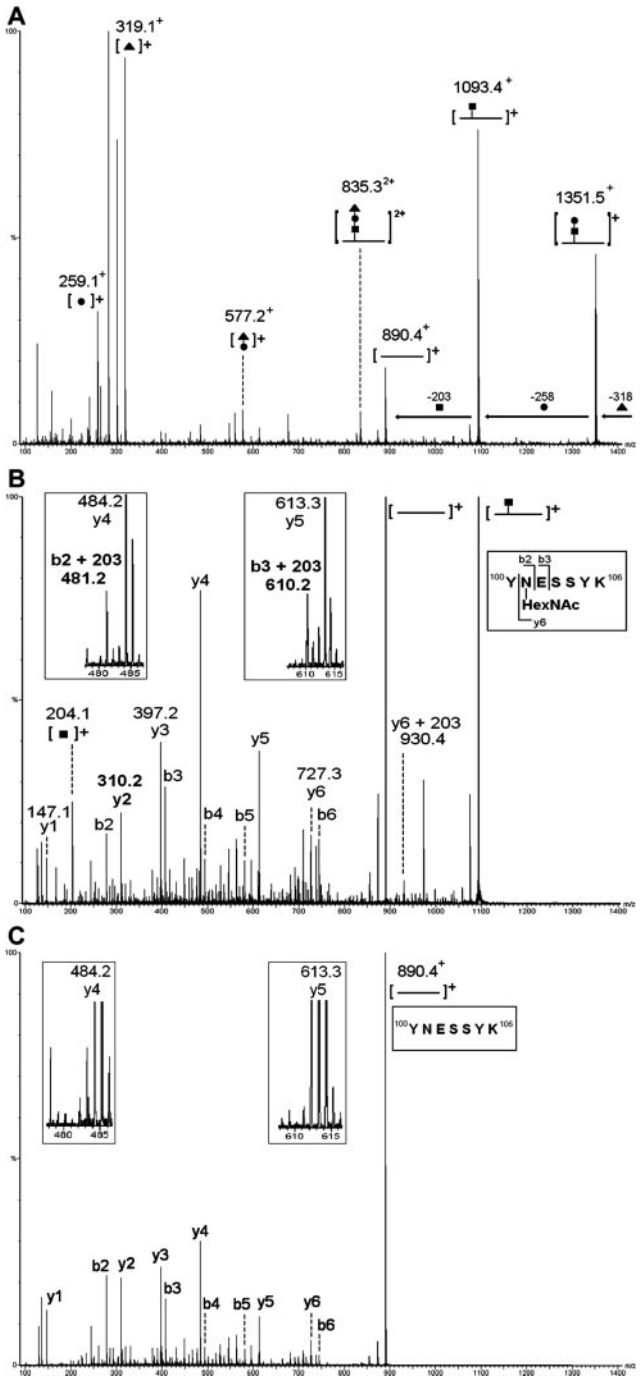

FIG. 2. **Mass spectrometry analysis of the modified tryptic peptide ^{100}YNESSYK^{106} from the protein FlaB2.** *A*, nano-LC-MS/MS spectrum of the doubly protonated $T^{100-106}$ glycopeptide ion 835.3^{2+}. The spectrum is dominated in the high *m/z* range by the sequential loss of the carbohydrate moieties (indicated in the spectrum by an *open arrow*) and in the low *m/z* range by the carbohydrate oxonium ions (*m/z* 319.1 and 259.1 plus their dehydration products). The presence of additional mass due to glycan components on respective oxonium ions is indicated by schematic depiction within the *brackets*. The *straight line* represents the tryptic peptide, and each additional *symbol* represents monosaccharide constituents of the glycan (■, *m/z* 204 (203 Da); ●, *m/z* 259 (258 Da); ▲, *m/z* 319 (318 Da)). *B*, second generation MS/MS spectrum of the fragment ion at *m/z* 1093.5 corresponding to the $T^{100-106}$ peptide plus the linking HexNAc. The glycopeptide was fragmented by front-end collision-induced dissociation (orifice voltage = 80 V) to produce this ion. The modified b2 and b3 fragment ions at *m/z* 481.2 and 610.2, respectively (*insets*), clearly indicate that the glycan is *N*-linked. *C*, second generation MS/MS spectrum of the fragment ion at *m/z* 890.4 corresponding to the unglycosylated $T^{100-106}$ peptide. *Insets* show the expanded spectrum of corresponding areas from Fig. 2*B* where the modified b2 and b3 fragment ions were observed from the glycosylated form of the peptide.

Fla A

1 ATGVGTLIVF IAMVLVAAVA ASVLI**NTS**GF LQQKASSTGT ESTEQVSTGL KMFQTSGKLN
61 EPIIDRLTIY VTPSPGSKPV DLKNTKLLMN RWTFQPPPVS YSSTYFENNN KQIFDVTGSK
121 AWNNGAILPE YNFGVIVIQD DDGSCTAESP VIGKGDMAVI TI**NCT**NLDLA PRTRLNGYLQ
181 SEIGFKTQFT YILPNAYDKT EDVVILQ

Fla B1

1 ASGIGTLIVF IAMVLVAAVA ASVLI**NTS**GF LQQKASTTGK ESTEQVASGL QVTGVTGV**NS**
61 **T**DSE**NIT**HIV AYITPKAGSS AIDLSQAKLF VTYNGVSAVL KA**NES**AIDGT SGTPDVLSAT
121 LLS**NTS**ATEY QVVSLQDFDG SVAKNQVINK GDLVAIRIST GQVFSSTKGI PTRAHVSGKL
181 QPEFGAPGII EFTTPATFTT KVVELQ

Fla B2

1 ASGIGTLIVF IAMVLVAAVA ASVLI**NTS**GF LQQKASTTGK ESTEQVASGL QISQVMGMH**N**
61 **NS**NI**NKT**AIY ISPNAGSSAI DLSQAVIMLS DGSNKRVYKY **NES**SYKDLTN GGDIFDNANV
121 EWIKATATKF GIVVIQDADE SCTAANPVIN KGDLVAITL**N** **TT**SFSTTPRT SITGTVQPEF
181 GAPGIISFTT PATYL**NDS**KV VQLQ

Fla B3

1 AVGIGTLIIF IALVLVAAVA ASVIINTAGK LQHKAAVVGQ ETTQQVASGL QVVKITGHSV
61 DQYNLDKIAI LVSPNIGDEI DLATTVVTFS TDDRKMSLLY DSS**NAS**NGGK VRLSTA**NGT**S
121 DIFKYDDVYA IGAWPFEDPT YGQSDQDPNE KKFGIVVLQD MDNSVSGEHP TVNYGDKVLL
181 AINIGNIVGE NIGNRIRIQG EVVPEFGAPG IIDFTTPPVY ANRVVALQ

S-layer Protein

1 VEKIGDVEGF KVIDNGEPTA DIVVGSTAAA ADVVSAANVA AKVGSMMFKE GEAASGSAKL
61 TVKASAESDD ANLKSLLT**NG** **T**NDFTELDAG KEAFVVAAAD SDYSDALI**NA** **T**TGFANIADN
121 VLYDQAKLAA AVSLGDLSTL SVVKDIDPSD WYADKNKAAD VATKDYYDQD GDAVEMLMAT
181 VASNDDGKSL TVDEDGVLYA SIAYDDDNED FQRATQVLKE GNRLPFLGEE YALVKLDTDD
241 DIVYLGKEVF DGVLKEGDTY NIGDGYELKV VAILKSGDEY KISLQLMKDG KVVAEKFDKV
301 SATSALKMIY TPGNIGIVVN EAWENVGQDY GYGSTLITKD VIALELGEEY IPDWEVVTIE
361 KDTTTDNTKD SKMTLSDDKI TKDNTYGIGL QYVGDEEDNF KSGKAIKIAK YAELELDDED
421 KEDTKLNLFF SMDETKEATL AAGQKVTVLN SDITLSEVMA DAKAPVAFKA PLAVLDTEVS
481 LDAANKKLIL VGGPVANALT KELADAGKIE MTVESPATLA VVAGAANGND VLVVAGGDRA
541 ATAEAANALI EML

FIG. 3. **Assignment map of flagellins and S-layer protein from *M. voltae*.** The predicted amino acid sequence of each mature flagellin structural protein and S-layer protein is presented (NCBI accession numbers A41316, B41316, C41316, D41316, and AAA93515). Peptides identified by nano-LC-MS/MS analysis of the trypsin and/or Glu-C digestions are *underlined*. The 17 existing *N*-linked sequons are indicated in *boldface*. Each characterized peptide containing an *N*-linked sequon was bearing a 779-Da glycan modification.

from this region of FlaA. To determine the exact site of glycan attachment we performed nano-ESI-feCID-MS/MS on the fragment ion of the $T^{100-106}$ glycopeptide bearing only the 203-Da monosaccharide (Fig. 2*B*). The obtained spectrum contained ions that were attributed to b_2 and b_3 peptide fragments bearing the extra 203-Da mass (Fig. 2*B*, *insets*). These two ions were not present in the deglycosylated fragment ion *m/z* 890.4 as shown in Fig. 2*C* (*insets*). In addition, the only modified ion of the y series clearly visible on the spectrum is the y6 + 203 ion at *m/z* 930.4, which includes the asparagine residue. This pattern of ions could only occur if the monosaccharide is *N*-linked to the asparagine residue of the *N*-linked sequon ^{101}NES^{103}. No ions corresponding to *O*-linkage on either of the serines present in this peptide were observed. To confirm this finding we performed β-elimination of selected glycopeptide-containing fractions. Although the reaction successfully removed the *O*-linked glycans from the positive control (*C. jejuni* flagellin), no modification of the *M. voltae* flagellin glycopeptides was observed (data not shown). This confirms that the glycan linkage on these flagellin proteins is not *O*-linked. This *N*-linkage through an Asn residue was confirmed for an S-layer peptide (see below).

MS/MS Analysis of the Glycan Moiety—Accurate mass measurements were performed on the glycan oxonium ions in the MS/MS spectra illustrated in Fig. 2 to determine possible empirical formulas for the unknown glycans. The known masses of peptide fragment ions were used as internal standards in a manner similar to that described previously (12). The accurate masses and the top ranked, plausible elemental compositions are presented in Table I. The measured masses were within ±0.002 Da of the actual masses of the sugar residues as identified by NMR. The accurate mass and elemental formula for the *N*-linked sugar clearly indicated that this is a HexNAc, whereas that for the 258-Da sugar suggested that this is a di-*N*-acetyl hexuronic acid.

TABLE I
Accurate masses of the glycan oxonium ions and most plausible empirical formula

Shown are the accurate monoisotopic masses of the glycan oxonium ions in Fig. 2 calculated using the "lock mass" function of Masslynx software (Waters). The fragment ions y1 (*m/z* 147.1134), y2 (*m/z* 310.1767), b2 (*m/z* 278.1141), and b3 (*m/z* 407.1567) from the peptide ^{100}YNESSYK^{106} were used as reference. Hydrogen mass = 1.0078 Da.

Carbohydrate	Oxonium ion	Residue mass	Elemental composition
	m/z	*Da*	
N-Linked	204.087	203.079	$C_8H_{13}NO_5$
Second	259.092	258.084	$C_{10}H_{14}N_2O_6$
Distal	319.112	318.104	$C_{12}H_{18}N_2O_8$

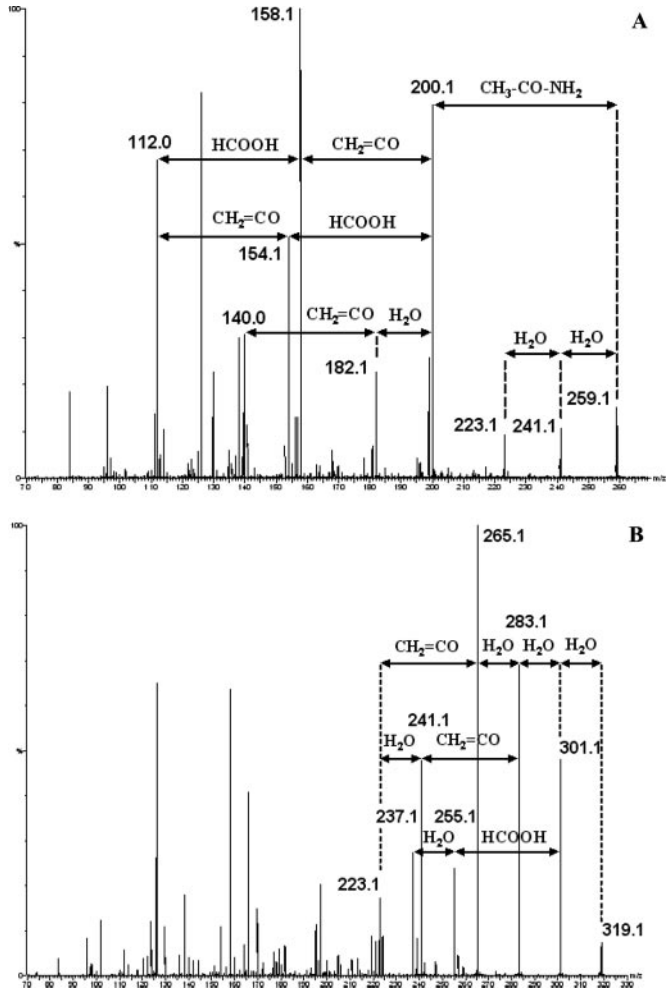

FIG. 4. **Analysis of the unusual carbohydrates obtained by nano-ESI-feCID-MS/MS.** MS/MS spectra of the oxonium ion at *m/z* 259.1 (*A*) and *m/z* 319.1 (*B*), respectively. The orifice voltage was raised to 60 V to fragment the glycopeptide ions as they entered the mass spectrometer. The collision activation energy was 20 V. Plausible neutral losses of molecules such as formic acid and water are indicated.

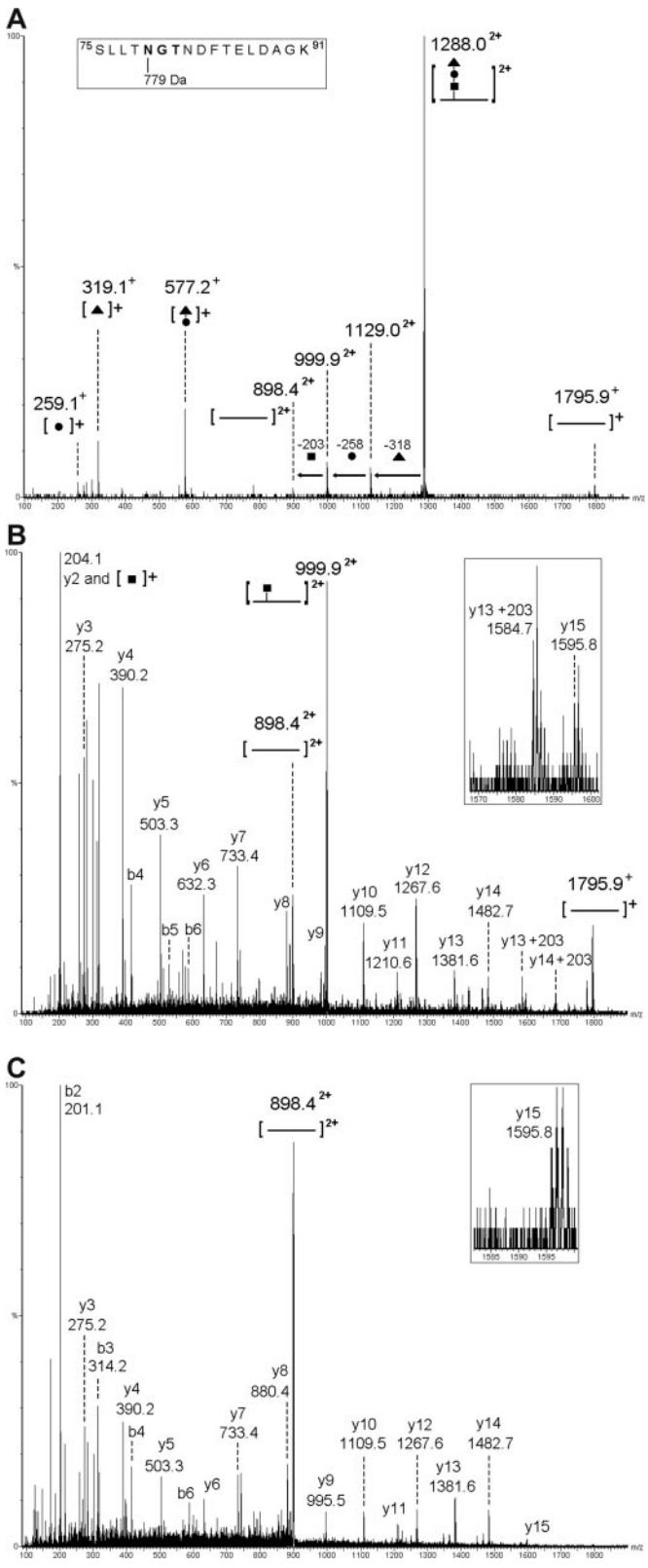

FIG. 5. **Mass spectrometry analysis of the modified tryptic peptide ^{75}SLLTNGTNDFTELDAGK^{91} from *M. voltae* S-layer protein.** *A*, nano-LC-MS/MS spectrum of the doubly protonated $T^{75–91}$ glycopeptide ion 1288.0^{2+}. This spectrum shares common features with the spectrum displayed in Fig. 2*A*: in the high *m/z* range, there is the sequential loss of the carbohydrate moieties (indicated in the spectrum by *open arrows*), and the carbohydrate oxonium ions could be seen in the low *m/z* range. The *straight line* between *brackets* represents the tryptic peptide, and each additional *symbol* represents monosaccharide constituents of the glycan (■, *m/z* 204 (203 Da); ●, *m/z* 259 (258 Da); ▲, *m/z* 319 (318 Da)). *B*, second generation MS/MS spectrum of the doubly protonated fragment ion at *m/z* 999.9^{2+} corresponding to the $T^{75–91}$ peptide plus the linking HexNAc. The glycopeptide was fragmented by front-end collision-induced dissociation (orifice voltage = 90 V) to produce this ion. The presence of the modified y13 fragment ion at *m/z* 1584.7 (*inset*) along with the absence of any shorter modified y ions clearly indicates that the glycan is *N*-linked. *C*, second generation MS/MS spectrum of the doubly protonated fragment ion at *m/z* 898.4^{2+} corresponding to the unglycosylated $T^{75–91}$ peptide. Front-end collision-induced dissociation (orifice voltage = 90 V) produced this ion. Fragmentation ions from y and b series allowed the definitive identification of the peptide as ^{75}SLLTNGTNDFTELDAGK^{91} from *M. voltae* S-layer protein. The *inset* shows the expanded spectrum of the corresponding area from Fig. 5*B* where the HexNAc-modified y13 fragment ion was observed from the glycosylated form of the peptide.

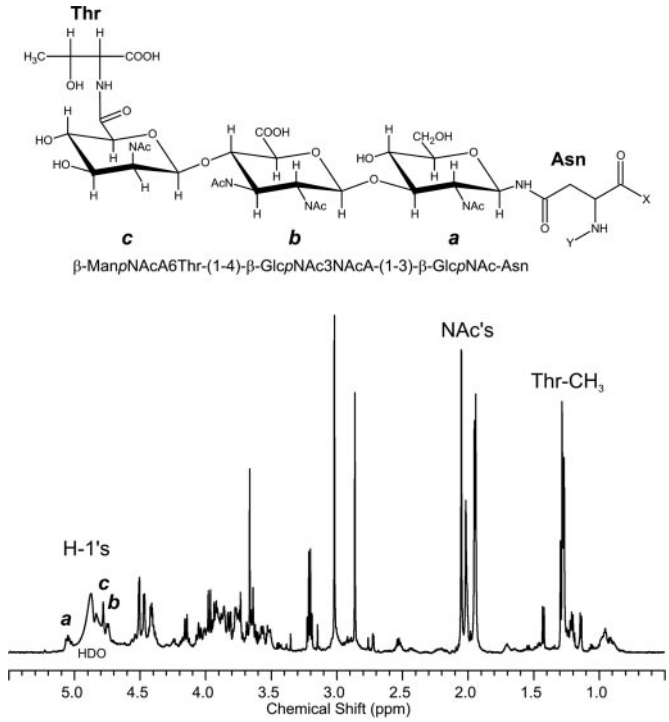

FIG. 6. **Structure and ^{1}H spectrum of the *N*-linked flagellin glycan.** Residue ***a*** is 2-acetamido-2-deoxy-β-glucose (β-GlcNAc) with C-1 *N*-linked to Asn, ***b*** is 2,3-diacetamido-2,3-dideoxy-β-glucuronic acid (β-GlcNAc3NAcA), and residue ***c*** is 2-acetamido-2-deoxy-β-mannuronic acid (β-ManNAcA) with the carbonyl group at C-6 forming an amide bond with the amino group of Thr. All sugars have the pyranose form and are drawn in the D-configuration. *X* and *Y* represent various amino acids to indicate heterogeneity in the peptide. The ^{1}H spectrum (*bottom*) was acquired with eight scans at 600 MHz using a cryogenically cooled probe. The H-1 anomeric, NAc, and Thr methyl resonances are labeled. The residual water resonance (*HDO*) was presaturated and digitally filtered.

The *m/z* 259 and 319 glycan oxonium ions were examined by feCID-MS/MS (Fig. 4). The fragmentation pattern for the *m/z* 259 ion was especially informative (Fig. 4*A*). The ion at *m/z* 200 (loss of 59 Da) likely comes from the loss of an *N*-acetyl group. The subsequent loss of a ketene (42 Da) from this ion yields the strong fragment ion at *m/z* 158.1 and suggests the presence of a second acetate group. Loss of formic acid (46 Da) forms the ion at *m/z* 112.0 and suggests the presence of a carboxylic acid moiety. This fragmentation pattern plus the putative elemental composition provided in Table I provided strong evidence indicating that this is a di-*N*-acetyl hexuronic acid. This was later confirmed by NMR analysis. The fragmentation pattern of *m/z* 319 oxonium ion presented in Fig. 4*B* also yielded important information about the terminal monosaccharide although not enough to suggest a possible structure. The MS/MS spectrum was dominated by the successive loss of three water molecules from the precursor ion and is indicative of the pres-

ence of at least three hydroxyl groups. As with the 258-Da carbohydrate, loss of a ketene group and loss of formic acid suggested that this carbohydrate contains one carboxyl group and one *N*-acetyl group.

S-layer Protein Glycosylation—In the MS/MS analysis of peptides from the intact flagella preparation we identified peptides from other *M. voltae* proteins, in particular the S-layer protein. There are two potential sites of *N*-linked glycosylation within the predicted sequence of this protein, and a tryptic peptide of increased mass due to an additional 779 Da was found during the nano-LC-MS/MS analysis of flagellin extracts. To confirm whether this protein was also glycosylated we digested a crude S-layer protein preparation and characterized a number of peptides by nano-LC-MS/MS (see Supplemental Table 1). We were able to obtain 59% coverage of the S-layer protein (Fig. 3) and we were also able to show that tryptic peptide $T^{75–91}$, which contains one of the *N*-linked sequons, is indeed *N*-glycosylated with the 779-Da glycan (Fig. 5).

NMR Structural Analysis of the Flagellin Glycan—Standard homo- and heteronuclear NMR techniques were used to completely characterize the structure of the novel *N*-linked flagellin glycan, which is presented in Fig. 6. The ^{1}H spectrum indicated the presence of three anomeric protons at 5.05, 4.77, and 4.74 ppm, respectively. Because anomeric protons often give rise to well resolved peaks, selective excitation of these resonances within TOCSY and NOESY experiments are effective methods to assign scalar-coupled protons within the glycan units, determine intraresidual coupling constants, and establish inter-residual glycan linkages (22, 23). TOCSY and NOESY spectra obtained through selective excitation of the three anomeric protons in the glycan are presented in Fig. 7. The vicinal coupling constants (8–10 Hz) and H-1 to H-3 and H-1 to H-5 intraresidual NOEs associated with the spectra from the anomeric protons at 5.05 (Fig. 7, residue ***a***) and 4.74 ppm (residue ***b***) are consistent with those of glucose residues in the β-pyranose configuration. The vicinal coupling constants of 1.3, 4.2, 9.9, and 9.8 Hz (±0.5 Hz) from H-1 to H-5, respectively, obtained from the TOCSY spectrum and the H-1 to H-3 and H-1 to H-5 NOEs observed in the NOESY spectrum acquired upon excitation of the anomeric resonance at 4.77 ppm (residue ***c***) are consistent with that of mannose in the β-pyranose configuration. The sharp doublet seen for proton resonances at position 5 in both residues ***b*** and ***c*** (Fig. 7) indicates the presence of a carbonyl group at position 6.

The two-dimensional homonuclear spectra of the glycan acquired in 90% H_2O revealed the presence of four NH moieties linked to the glycan units of the molecule. Through-bond connectivities observed in TOCSY and COSY spectra positioned an NH group at carbon 2 of each residue; the fourth NH moiety is linked to carbon 3 of residue ***b*** (Fig. 6). NOEs observed between each NH proton and a methyl group of acetate (located at ~2 ppm) confirmed that these substituents are *N*-acetyl groups. Assignment of the NH protons to *N*-acetyl groups was also established through the use of ^{1}H-^{13}C HMBC spectra acquired in both D_2O and 90% H_2O, which yielded observable connectivities from the methyl groups of acetate through to the NH and ring protons across the amide bond (Fig. 8). The position of the *N*-acetyl substituents was further confirmed upon observation of upfield carbon shifts of CH moieties at position 2 of all three residues and position 3 of residue ***b***.

TOCSY spectra of the trisaccharide revealed the presence of a spin system consistent with that of the amino acid threonine with signals proportional to proton resonances within the glycan units. In the HMBC spectra, couplings were observed between the NH and CHα moieties of threonine with the CH and carbonyl groups at positions 5 and 6 of

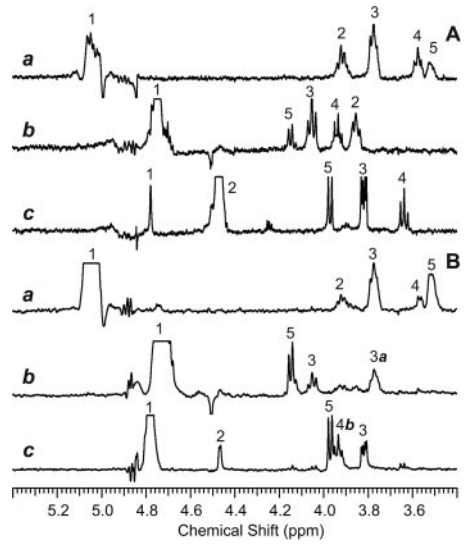

FIG. 7. **Selective excitation proton spectra of the *N*-linked flagellin glycan.** *A*, TOCSY spectra with a mixing time of 80 ms acquired upon selective excitation of the anomeric resonances of residues ***a***, ***b***, and ***c***. *Numbered peaks* correspond to the resonances within the glycan units coupled to the anomeric proton. *B*, NOESY spectra with a mixing time of 800 ms acquired upon selective excitation of the anomeric resonances of residues ***a***, ***b***, and ***c***. *Labeled peaks* correspond to both intraresidual NOEs within the glycan units and inter-residual NOEs across the glycosidic bond. *Residue* and *atom numbers* correspond to the glycan structure presented in Fig. 6.

residue ***c*** (Fig. 8), confirming that this amino acid is *N*-linked to residue ***c*** through an amide bond. Due to the small amount of purified material, optical experiments needed to determine the chiral configuration of threonine could not be performed. It is also worth noting that the small amount of glycan available for analysis by NMR presented difficulties with regard to sensitivity. Signal-to-noise improvement was achieved through the use of a cold probe in experiments run on the spectrometer operating at 600 MHz, enabling the acquisition of insensitive spectra (*e.g.* ^{1}H-^{13}C HMBC) within reasonable periods of time (overnight).

Proton assignments deduced from homonuclear spectra enabled the identification of cross-peaks in carbon-correlated proton spectra of the trisaccharide (Table II). The upfield carbon chemical shift near 79 ppm for the anomeric proton resonance at 5.05 ppm in ^{1}H-^{13}C heteronuclear single quantum coherence spectra established residue ***a*** as the proximal *N*-linked residue. In addition, this upfield carbon chemical shift clearly established the linkage of the glycan as *N*-linked and not *O*-linked. Comparison of the ^{13}C chemical shifts for residue ***a*** with those of β-Glc*p*NAc-Asn (24) indicated a glycosidation shift of 8 ppm for C-3***a***. Comparison of the ^{13}C chemical shifts of residue ***b*** with those of -4)-β-Glc*p*NAc3NAcA (25, 26) was consistent with a glycosidic link at C-4***b,*** although there were some discrepancies with the assignment for the C-4 and C-5 resonances of Hashimoto *et al.* (25). The ^{13}C chemical shifts for the C-2 to C-5 resonances of residue ***c*** were in accord with those of the monosaccharide β-Man*p*NAcA (27). There was a difference of 5 ppm for the C-6 resonance due to a substitution at C-6***c*** with Thr. The proton and ^{13}C chemical shifts for Thr and C-6 of residue ***c*** were similar to those found in GalA6Thr (28). Hence the general linkage pattern of the glycan was therefore determined to be ***c***-^{4}***b***-^{3}***a.*** This linkage pattern was confirmed through the observation of appropriate inter-residual couplings in ^{1}H-^{13}C HMBC spectra and inter-residual NOEs, H-1***b*** to H-3***a*** and H-1***c*** to H-4***b*** in the NOESY spectra (Fig. 7*B*). Hence the *N*-linked glycan structure was determined to be β-Man*p*NAcA6Thr-(1–4)-β-Glc*p*NAc3NAcA-(1–3)-β-Glc*p*NAc-

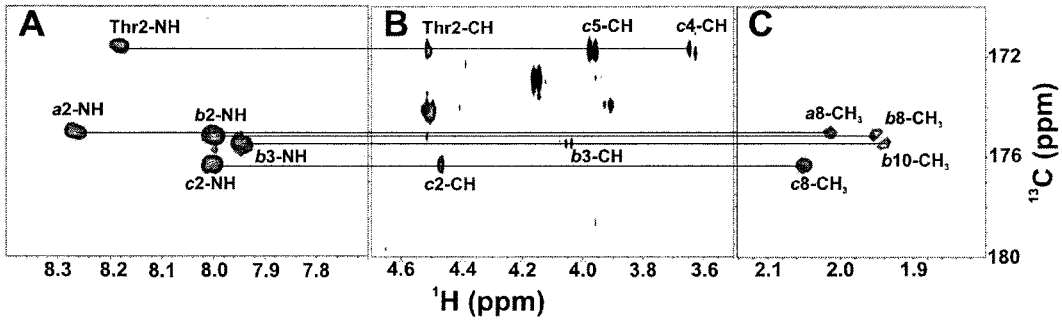

FIG. 8. **^{1}H-^{13}C HMBC spectrum of the *N*-linked flagellin glycan.** *Solid lines* are drawn to show the couplings across the amide bond in *N*-acetyl substituents, and the *dashed line* connects resonances across the amide bond formed by the amino group of threonine with the C-6 carbonyl moiety of residue ***c***. *A*, acquired in 90% H_2O, shows the exchange-labile amide protons. *B* and *C*, acquired in D_2O, show the glycan ring and *N*-acetyl methyl resonances, respectively. *Residue* and *atom numbers* correspond to the glycan structure presented in Fig. 6.

TABLE II
^{1}H and ^{13}C chemical shifts of the N-linked flagellin trisaccharide from M. voltae

All reported shifts are based on spectra acquired at 20 °C in D_2O, pD 3, with the exception of NH moieties whose shifts are based on spectra acquired in 90% H_2O. Spectra were referenced with respect to the methyl group of internal acetone appearing at 2.225 and 31.1 ppm in the ^{1}H and ^{13}C dimensions, respectively. Residue atom numbers and types are based on the glycan structure presented in Fig. 6.

Residue	Atom (type)	δ_H	δ_C
		ppm	*ppm*
GlcNAc (*a*)	1 (CH)	5.05	79.3
	2 (CH)	3.91	53.8
	3 (CH)	3.77	83.4
	4 (CH)	3.57	69.2
	5 (CH)	3.51	78.1
	6 (CH_2)	3.75, 3.87	61.4
	7 (C=O)		175.0
	8 (CH_3)	2.02	23.1
	1 (NH)	8.79	
	2 (NH)	8.26	
GlcNAc3NAcA (*b*)	1 (CH)	4.74	101.9
	2 (CH)	3.86	54.4
	3 (CH)	3.94	78.3
	4 (CH)	4.05	53.8
	5 (CH)	4.15	75.1
	6 (COO)		173.0
	7 (C=O)		175.1
	8 (CH_3)	1.96	23.1
	9 (C=O)		175.4
	10 (CH_3)	1.94	23.1
	2 (NH)	8.00	
	4 (NH)	7.94	
ManNAcA (*c*)	1 (CH)	4.77	100.6
	2 (CH)	4.47	53.9
	3 (CH)	3.82	72.5
	4 (CH)	3.64	69.6
	5 (CH)	3.97	77.0
	6 (CON)		171.7
	7 (C=O)		176.3
	8 (CH_3)	2.05	23.1
	2 (NH)	8.00	
Thr	1 (COO)		174.2
	2 (CH)	4.50	59.4
	3 (CH)	4.41	68.0
	4 (CH_3)	1.27	20.1
	2 (NH)	8.18	

Asn. The absolute configuration of the sugars could not be determined due to the low amount of material available. However, in previous structural studies of glycans containing these sugars, only the D-configuration has been found.

DISCUSSION

Our results demonstrate that the flagellin structural proteins FlaA, -B1, -B2, and -B3 and the S-layer protein of *M. voltae* are uniquely modified with a novel *N*-linked trisaccharide. These findings confirm previous indirect reports of glycosylation for both these important surface-associated proteins in *Methanococcus* (3). Although S-layer protein glycosylation has been extensively studied for a number of archaeal species (for reviews, see Refs. 2 and 29), prior to this work, detailed structural characterization of flagellin glycosylation was available for only a single species. The flagellin and S-layer proteins of *H. salinarum* were shown to be *N*-glycosylated with sulfated oligosaccharides (11, 30). In contrast, structural characterization of flagellar glycosylation for a number of bacterial species has demonstrated that the flagellar glycan is linked to the protein backbone through *O*-linkage to serine/threonine in all cases. In addition, the studies have revealed significant diversity in the composition of the glycans attached; *C. jejuni* and *Helicobacter pylori* flagellins are glycosylated with pseudaminic acid (Pse) and related derivatives (12, 13), *Listeria monocytogenes* flagellin is glycosylated with GlcNAc (15), and *Pseudomonas aeruginosa* type-a flagellin is glycosylated with a rhamnose-linked complex glycan (14). So it appears that although *O*-linkage is more common to bacterial flagellar glycosylation systems, *N*-linkage may be the mechanism of choice for glycosylation of archaeal flagellins. Although the significance of this is currently unknown it is important to recognize that the archaeal flagellum is quite distinct in composition and mechanism of assembly from bacterial flagellum.

The detection of *N*-linked glycans on the flagellins of *M. voltae* and earlier on *H. salinarum* has important implications for the assembly of archaeal flagella. The current model of *N*-linked glycosylation involves the assembly of the glycan on a lipid carrier on the cytoplasmic side of the cytoplasmic membrane followed by a flipping of the complete glycan to the periplasmic side of the membrane before its transfer to the target protein. This process would not be possible for bacterial flagellins that are transferred from the cytoplasm through the hollow growing flagella structure before incorporation at the distal tip of the filament (31). The bacterial flagellins are never exposed to the periplasmic side of the membrane, and this may explain why *O*-linked but not *N*-linked glycans are found on bacterial flagellins. The presence of *N*-linked glycans on archaeal flagellins would be consistent with a type IV pilin-like assembly at the base of the structure and where the subunits cross the membrane prior to incorporation. Evidence has been presented in the halophiles that the transfer of the glycan to the flagellins does in fact occur on the periplasmic side of the membrane (10, 32).

The identification of both S-layer and flagellin having the same *N*-linked glycosylation pattern in both *H. salinarum* and *M. voltae* points to a common glycosylation process for these proteins that cross the cytoplasmic membrane. Archaeal flagellins are believed to cross the cytoplasmic membrane, most likely via a Sec secretion pathway, and so would be exposed to a processing environment similar to that of the S-layer proteins

(33–35). Interestingly in *C. jejuni* an *N*-linked glycosylation pathway has been described that was shown to glycosylate a diverse group of cell surface and periplasmic proteins with a unique oligosaccharide (20, 36). It now remains to be established whether indeed *N*-linked glycosylation in Archaea and Bacteria is an important component of a particular protein secretion pathway.

Uronic acids are common components of many surface-associated glycoconjugates including a number of well characterized acidic bacterial capsular polysaccharides and lipopolysaccharides. The diacetamido-substituted uronic acid of 258 Da was previously identified as a component of the repeating unit of the *O*-specific polysaccharide of the phenol-extracted lipopolysaccharide of *Thiobacillus* sp. IFO-14570 (37). In addition the substitution of glucuronic and mannuronic acids with amino acids such as threonine, serine, and alanine has been reported previously (28, 38, 39), although the biological role of the unique terminal *N*-acetyl mannuronic acid described here bearing such a modification remains unknown. Of particular significance in the current study is the characterization of β-GlcNAc as the linkage sugar to asparagine for this novel glycan. This linkage is the same as that used in higher organisms for the attachment of a large number of complex and polymannose oligosaccharides, which have considerable biological significance (41–43), and so points to the potential of utilizing selected bacterial strains or recombinant enzymes from these strains for glycoengineering purposes as described recently (44). Preliminary characterization of a number of other prokaryotic glycoproteins (for a review, see Ref. 45) including *Thermoplasma acidophilum* (46) and *Streptococcus sanguis* (40) has indicated that a GlcNAc asparagine linkage may also be present, although structural confirmation for each protein is still required.

Structural characterization of glycoproteins is a time-consuming and challenging process especially in cases where only limited amounts of protein can be recovered from biological samples. In the current study, we have successfully completed the structural assignment of a unique complex trisaccharide using a combination of mass spectroscopy and NMR technology. MS was used to identify glycopeptides and linkage sites from each flagellin monomer. We successfully demonstrated that all but one of the 15 *N*-linked sequons from the four flagellin structural proteins were glycosylated with trisaccharide. The 15th site that resides on the FlaA protein was never characterized probably due to low abundance of the protein in the flagellar preparations and may indeed also be glycosylated. In addition, accurate mass measurements of each monosaccharide and secondary MS/MS fragmentation patterns provided preliminary structural information. NMR analysis of microgram quantities of purified glycan, using cold probe technology to improve sensitivity, provided the detailed linkage and stereochemical assignments for each monosaccharide. The data provided in this analysis point to the potential to obtain detailed structural information from nanomolar quantities of glycan from other biological samples where material is limited.

From a biological perspective very little is known on the role of respective *N*- and *O*-linked glycans found on the myriad of flagellins and S-layer proteins studied to date from both Archaea and Bacteria or the mechanistic basis of assembly. The structural characterization of the unique glycan components found in these organisms will facilitate the identification of glycan biosynthetic components and so lead to a comprehensive understanding of the similarities and differences of each process in prokaryotes when compared with eukaryotes.

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Identification and Characterization of the Unique *N*-Linked Glycan Common to the Flagellins and S-layer Glycoprotein of *Methanococcus voltae*

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