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Flagellin from *Listeria monocytogenes* Is Glycosylated with β-O-Linked *N*-Acetylglucosamine

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Glycan staining of purified flagellin from *Listeria monocytogenes* serotypes 1/2a, 1/2b, 1/2c, and 4b suggested that the flagellin protein from this organism is glycosylated. Mass spectrometry analysis demonstrated that the flagellin protein of *L. monocytogenes* is posttranslationally modified with O-linked *N*-acetylglucosamine (GlcNAc) at up to six sites/monomer. The sites of glycosylation are all located in the central, surface-exposed region of the protein monomer. Immunoblotting with a monoclonal antibody specific for β-O-linked GlcNAc confirmed that the linkage was in the β configuration, this residue being a posttranslational modification commonly observed in eukaryote nuclear and cytoplasmic proteins.

Listeria monocytogenes is a gram-positive bacterium responsible for listeriosis, a severe food-borne infection associated with the consumption of contaminated foods. Clinically, symptoms of listeriosis include meningitis, septicemia, spontaneous abortion, perinatal infections, and gastroenteritis (25). *Listeria* species are found throughout the food-processing environment (9, 17, 28, 36) and can survive adverse conditions such as high salt levels and both pH (32) and temperature (48) extremes. However, the organism does not survive thermal processing; contamination of food generally results from contact of a processed food with a spoiled surface prior to packaging (27) or through the use of contaminated ingredients in minimally processed foods. Postprocessing contamination remains an important issue, since *L. monocytogenes* can proliferate within a contaminated food product at refrigeration temperatures (27, 48).

In *L. monocytogenes*, motility is a thermoregulated phenotype (34). At temperatures of <30°C, the organism is flagellated with four to six peritrichous flagella. However, motility is reduced as growth temperatures increase to above 30°C. At 37°C, transcription of *flaA* appears to be shut down (34), although minor quantities of flagellin can be detected immunologically. Motility has been suggested to be down-regulated through the activity of PrfA, the transcriptional activator of the virulence genes (26).

The serotyping of *L. monocytogenes* strains is based on agglutination reactions utilizing both somatic (O) and flagellar (H) antigens (42). The flagellar H antigen correlates with the recognized pulse field fingerprinting-determined genomic divisions where the 1/2a and 1/2c strains fall within division I, and 1/2b and 4b fall within division II (6). The identification of H-antigen type is based on differences in the agglutination profile by a series of four cross-adsorbed polyvalent antisera (A, AB, C, and D) and allows the classification of the isolate into one of three groups having shared H antigens (AB, ABC, and BD) (42). For example, strains falling within the 4b and 1/2b serotypes agglutinate with H antisera A, AB, and C, whereas 1/2a serotypes agglutinate with only the A and AB antisera. However, the structural basis underlying the H serotype specificity remains unknown.

Flagella from a wide variety of bacteria are important as both colonization and pathogenicity factors. Flagella in *L. monocytogenes* are not believed to be an important virulence determinant for human disease (41, 49). However, there is considerable interest in the mechanisms by which this organism colonizes the surfaces found throughout the food processing environment (4, 7, 19, 23, 31, 47). Previous research has demonstrated that the organism can attach to the common surfaces used throughout the food processing environment (24, 43) and may also form biofilms (7). The molecular mechanisms governing both attachment and biofilm formation have yet to be studied in detail. However, the flagella of *L. monocytogenes* have been shown to be involved in the attachment of the organism at temperatures below 30°C to both stainless steel (47) and plant tissues (13).

Previous studies have indicated that *Listeria* flagellin undergoes some form of posttranslational modification (11, 33). Peel et al. (33) found that the flagella could be separated into multiple bands following extended sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and that these bands display antigenic heterogeneity. In contrast, Dons et al. (11) determined that only a single flagellin gene was present within the genome and noted a discrepancy between the predicted and actual molecular weights of the *L. monocytogenes* flagellin. The flagellins from a number of gram-negative (5, 14, 15, 21) and gram-positive (1, 20) bacteria have previously been identified as glycosylated. Structural studies on

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both *Campylobacter* and *Helicobacter* flagella have shown that the flagellin structural proteins are posttranslationally modified at multiple sites with a novel sialic acid-like sugar, Pse5Ac7Ac, and related derivatives, via an O-linked glycosylation process (40, 45). *Pseudomonas* flagellin is modified with a heterogeneous glycan composed of up to 11 monosaccharide units linked through a rhamnose residue (39). The sites of glycosylation on these flagellins are restricted to the central surface-exposed region of the assembled monomer, although the mechanistic basis of this process is currently unknown.

As part of a continuing effort to further understand the role of flagellin glycosylation, we undertook this study to determine if the flagellin of *L. monocytogenes* is indeed glycosylated and to identify the sites of glycosylation and structural nature of the glycosyl moiety.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *L. monocytogenes* strains CLIP23485, 2568, 568, and 394 (serotypes 4b, 1/2c, 1/2a, and 1/2b, respectively) were obtained from the Listeria Reference Service collection in the Bureau of Microbial Hazards. Cultures of *L. monocytogenes* were grown on tryptic soy yeast extract agar (Difco, Detroit, Mich.) containing 0.5% (wt/vol) yeast extract and were incubated at 37°C for 24 h. Both O- and H-antigen types for each strain were confirmed by agglutination testing according to the manufacturer's instructions (Denken Seiken Co., Ltd., Tokyo, Japan).

For production of flagellar filaments, strains were first inoculated into tryptic soy broth-yeast extract motility medium (0.5% agar) and incubated at 21°C for 72 h. Cells from motility medium were used to inoculate 2.0 liters of brain heart infusion (BHI) broth and incubated without agitation at 21°C for 72 h.

Isolation of genomic DNA. Genomic DNA from each of the four serotypes of *L. monocytogenes* was isolated as previously described (20).

Isolation of flagellar filaments. Flagellar filaments were isolated by shearing whole cells with a Waring blender. Briefly, cells grown for 72 h in BHI broth were recovered by centrifugation (8,000 × *g*, 20 min) and resuspended into 50 ml of Tris-buffered saline (TBS) (0.02 M Tris-HCl and 0.15 M NaCl [pH 7.0]), and the cells were subjected to three 30-s shearing cycles at maximum output. Whole cells were removed by centrifugation (8,000 × *g*, 30 min), and the supernatant was retained. Flagellar filaments were recovered from the supernatant by ultracentrifugation (100,000 × *g*, 30 min). The resulting pellets were resuspended in TBS containing 1% Triton X-100, and the filaments were recovered from the detergent wash by ultracentrifugation and were washed twice in TBS. Large-scale flagellar preparations for glycosyl characterization were prepared as described above from *L. monocytogenes* 1174. However, cultures were grown in 8 liters of BHI broth at room temperature for a 72-h period.

Cloning and sequencing of flagellin genes. Flagellin genes were amplified from each respective strain by PCR with the following primers based on the N- and C-terminal sequences of the full-length cloned flagellin sequences previously reported (11). Primer F1 (5′-ATGAAAGTAAATACTAATATCATTAGCTTG-3′) corresponded to the N-terminal amino acid sequence MKVNTNIISL, and primer R1 (5′-GCTGTTAATTAATTGAGTTAACAT-3′) corresponded to the C-terminal flagellin sequence MLTQLINS*. The resulting PCR-amplified products were analyzed by gel electrophoresis, and the PCR products were recovered by using glass milk (Bio 101, La Jolla, Calif.) and cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, Calif.). Each PCR-amplified flagellin gene was sequenced with forward and reverse M13 universal primers. An Applied Biosystems 373 DNA sequencer and Taq cycle sequencing kits with terminator chemistry were used to sequence the plasmid DNA. Custom primers were synthesized on an Applied Biosystems 394 DNA/RNA synthesizer.

Protein electrophoresis and glycoprotein staining. SDS-PAGE was carried out with 12% polyacrylamide gels. Proteins were visualized by Coomassie blue R-250 staining. Confirmation for flagellin glycosylation was carried out by using a DIG glycan detection kit (Roche Diagnostics, Laval, Quebec, Canada) according to the manufacturer's instructions.

Mass spectrometry. The *L. monocytogenes* flagellins were analyzed by nanoelectrospray mass spectrometry (nESMS) on a Waters Q-TOF Ultima mass spectrometer (Manchester, United Kingdom) to determine the molecular mass of the intact glycoprotein. The flagellin protein sample was dialyzed in H_2O (0.2% formic acid) by using a Centricon YM-10 membrane filter (Millipore) with a molecular mass cutoff of 10 kDa to remove salts. This solution was infused into the mass spectrometer at a flow rate of 0.5 μl/min. For the identification of glycosylation sites, the flagellins were digested with trypsin (Promega, Madison, Wis.) and analyzed by using CapLC (Waters, Milford, Mass.) coupled to a nESMS interface. Peptide separation was achieved by using a linear gradient of 10 to 40% acetonitrile (0.2% formic acid) for 50 min and 80% acetonitrile (0.2% formic acid) from 50.1 min to 60 min on a homemade Jupiter C18 column (10 cm by 150 μm in diameter, 5-μm particle size) (Phenomenex, Torrance, Calif.). The flow rate was set to 600 nanoliters/min. All tandem MS spectra were acquired by using data-dependent experiments with Ar as a collision gas. Second-generation product ion spectra were obtained by increasing the RF Lens1 on the Q-TOF Ultima instrument from 50 to 125 V to form fragment ions in the orifice-skimmer region, while the desired precursor ion was selected by the quadrupole. The precise identification of the glycosylation site was achieved by using β elimination with ammonium hydroxide to leave a modified Ser/Thr residue that could be located by tandem mass spectrometry (35). The tryptic digest was subjected to alkaline hydrolysis with 1 ml of NH_4OH for 18 h at 50°C, dried, redissolved in water, and analyzed by liquid chromatography (LC)-nESMS. Tryptic peptides showing the characteristic mass shift following β elimination were subjected to tandem MS analyses to locate the position of the modified residue.

Monosaccharide analysis of flagellin. Flagellin (2 mg) was hydrolyzed with 2 N trifluoroacetic acid at 120°C for 2 h, and the sample was dried under a stream of nitrogen and then dissolved in 0.4 ml of water and treated with sodium borohydride ($NaBH_4$) overnight at room temperature. The $NaBH_4$ was destroyed with acetic acid and dried under a stream of nitrogen. Residual $NaBH_4$ was evaporated with subsequent additions of methanol. Acetylation was performed with 0.4 ml of acetic anhydride at 100°C for 1 h with residual sodium acetate as the catalyst. Acetic anhydride was evaporated under nitrogen, and the derived alditol acetate was extracted into $CHCl_3$ and examined by gas-LC-MS (GLC-MS) on a Varian Saturn II mass spectrometer. The GLC-MS was equipped with a 30-m DB-17 capillary column (the temperature was raised from 180 to 260°C at 3.5°C/min), and MS was performed in the electron impact mode. The elution profile from the GLC-MS was compared to sugar standards.

Western blotting. Flagellin protein was resolved by SDS-PAGE and transferred to nitrocellulose membrane. Blots were incubated for 3 h at room temperature with purified mouse monoclonal antibody specific to β-O-linked GlcNAc at a dilution of 1:1000 (Covance Research Products, Princeton, N.J.). Following washing with phosphate-buffered saline-Tween (0.02%), blots were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG and IgM at a dilution of 1:1000, washed three times in phosphate-buffered saline-Tween (0.05%), and developed by using a Bio-Rad AP conjugate substrate kit in accordance with the manufacturer's instructions.

Nucleotide sequence accession numbers. The *L. monocytogenes* flagellin gene sequences described here have been deposited in GenBank as AY660548, AY660549, and AY660550.

RESULTS

Genetic analysis of flagellin genes from various serotypes. The *flaA* structural gene from strains, 568, 394, 2568, and CLIP23485, belonging to serotypes 1/2a, 1/2b, 1/2c, and 4b, respectively, were amplified from genomic DNA by PCR with the flagellin-specific primers F1 and R1. The resulting amplicons were electrophoresed to confirm the correct sizes, purified from the gel, cloned, and sequenced to determine the amino acid sequences of the translated proteins. The predicted amino acid sequence and mass of each respective flagellin were determined from the DNA sequence data. As had been previously reported, a very high degree of conservation was found among the predicted flagellin DNA sequences from the four serotypes examined when compared to *L. monocytogenes* EGD (12), which translated into only a single amino acid substitution for each flagellin protein (Fig. 1). The predicted amino acid sequences of flagellin from strain 394 (serotype 1/2b) and CLIP23485 (serotype 4b) were identical, as would be expected; the strains share a common lineage and both fall within the division II (6). Furthermore, the identical substitution occurs in two additional serotype 4b full-length flagellin sequences from strains F2365 and H7858 (EAL07249 and AAT03507,

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EGD (1/2a)        MKVNTNIISLKTQEYLRKNNEGMTQAQERLASGKRINSSLDDAAGLAVVTRMNVKSTGLD
394 (1/2b)        MKVNTNIISLKTQEYLRKNNEGMTQAQERLASGKRINSSLDDAAGLAVVTRMNVKSTGLD
CLIP 23485(4b)    MKVNTNIISLKTQEYLRKNNEGMTQAQERLASGKRINSSLDDAAGLAVVTRMNVKSTGLD
568 (1/2a)        MKVNTNIISLKTQEYLRKNNEGMTQAQERLASGKRINSSLDDAAGLAVVTRMNVKSTGLD
2568(1/2c)        MKVNTNIISLKTQEYLRKNNEGMTQAQERLASGKRINSSLDDAAGLAVVTRMNVKSTGLD

EGD (1/2a)        AASKNSSMGIDLLQTADSALSSMSSILQRMRQLAVQSSNGSFSDEDRKQYTAEFGSLIKE
394 (1/2b)        AASKNSSMGIDLLQTADSALSSMSSILQRMRQLAVQSSNGSFSDEDRKQYTAEFGSLIKE
CLIP 23485(4b)    AASKNSSMGIDLLQTADSALSSMSSILQRMRQLAVQSSNGSFSDEDRKQYTAEFGSLIKE
568 (1/2a)        AASKNSSMGIDLLQTADSALSSMSSILQRMRQLAVQSSNGSFSDEDRKQYTAEFGSLIKE
2568(1/2c)        AASKNSSMGIDLLQTADSALSSMSSILQRMRQLAVQSSNGSFSDEDRKQYTAEFGSLIKE

EGD (1/2a)        LDHVADTTNYNNIKLLDQTATGAATQVSIQASDKANDLINIDLFNAKGLSAGTITLGSGS
394 (1/2b)        LDHVADTTNYNNIKLLDQTATGAATQVSIQASDKANDLINIDLFNAKGLSAGTITLGSGS
CLIP 23485(4b)    LDHVADTTNYNNIKLLDQTATGAATQVSIQASDKANDLINIDLFNAKGLSAGTITLGSGS
568 (1/2a)        LDHVADTTNYNNIKLLDQTATGAATQVSIQASDKANDLINIDLFNAKGLSAGTITLGSGS
2568(1/2c)        LDHVADTTNYNNIKLLDQTAIGAATQVSIQASDKANDLINIDLFNAKGLSAGTITLGSGS
                                      *

EGD (1/2a)        TVAGYSALSVADADSSQQATEAIDELINNISNGRALLGAGMSRLSYNVSNVNNQSIATKA
394 (1/2b)        TVAGYSALSVADADSSQEATEAIDELINNISNGRALLGAGMSRLSYNVSNVNNQSIATKA
CLIP 23485(4b)    TVAGYSALSVADADSSQEATEAIDELINNISNGRALLGAGMSRLSYNVSNVNNQSIATKA
568 (1/2a)        TVAGYSALSVADADSSQQATEAIDELINNISNGRALLGAGMSRLSYNVSNVNNQSIATKA
2568(1/2c)        TVAGYSALSVADADSSQQATEAIDELINNISNGRALLGAGMSRLSYNVSNVNNQSIATKA
                                  *

EGD (1/2a)        SASSIEDADMAAEMSEMTKYKILTQTSISMLSQANQTPQMLTQLINS
394 (1/2b)        SASSIEDADMAAEMSEMTKYKILTQTSISMLSQANQTPQMLTQLINS
CLIP 23485(4b)    SASSIEDADMAAEMSEMTKYKILTQTSISMLSQANQTPQMLTQLINS
568 (1/2a)        SASSIEDADMAAEMSEMTKYKIPTQTSISMLSQANQTPQMLTQLINS
2568(1/2c)        SASSIEDADMAAEMSEMTKYKILTQTSISMLSQANQTPQMLTQLINS
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FIG. 1. Comparison of the amino acid sequences of *L. monocytogenes* flagellins. Amino acid residues are written in single-letter code, and variable amino acids are underlined, in boldface, and marked with asterisks.

respectively) (30) and the full-length *Listeria innocua* flagellin sequence (12) (Q92DW3), all of which are available in the NCBI database. The published flagellin protein sequences for serotype 1/2a strains EGD and F6854 (12, 30) differed by a single residue (position number 263, Leu to Pro) from strain 568, which is also a serotype 1/2a strain. Strain 2568 (serotype 1/2c) differed from all of these strains by a single residue (position number 141, Thr to Ile) within the central core region of the flagellin protein. Serotype 1/2a and 1/2c strains fall within the division I lineage (6).

Glycan staining of *Listeria* flagellin. Purified flagellin from the various *L. monocytogenes* strains (Fig. 2A) were analyzed following SDS-PAGE by using a DIG glycan kit. Each purified flagellin monomer reacted strongly in this assay, confirming the presence of carbohydrate molecules which can be oxidized by mild periodate treatment (Fig. 2B) and which are covalently linked to flagellin protein.

Structural analysis of *L. monocytogenes* flagellin. To determine the degree of glycosylation and to identify the sites of modification on the flagellin monomer, mass spectrometry was employed for detailed structural analysis. An electrospray mass spectrometry analysis of purified flagellin from *L. monocytogenes* CLIP23485 showed four well-defined components at 31,050, 31,254, 31,457, and 31,660 Da, corresponding to the molecular mass of the monomeric FlaA protein (30,444 Da) with additional modifications accounting for 606, 810, 1,013 and 1,215 Da (Fig. 3). The predominant flagellin species had a molecular mass of 31,254 Da.

Analysis of the tryptic digest identified the tryptic peptide T_{135}-T_{154}, to be modified with one to two *N*-acetylhexosamine (HexNAc) residues, and T_{168}-T_{214}, to be modified with two to three 203-Da residues (data not shown). A total of 90% se-

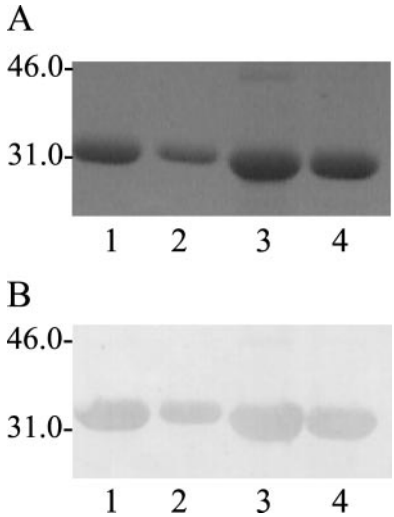

FIG. 2. Glycan staining of *Listeria* flagellin. A. Coomassie blue stain. B. DIG glycan stain. Lanes 1, CLIP23485 (serotype 4b); lanes 2, 2568 (serotype 1/2c); lanes 3, 568 (serotype 1/2a); lanes 4, 394 (serotype 1/2b). Molecular masses (in kDa) are shown on the left.

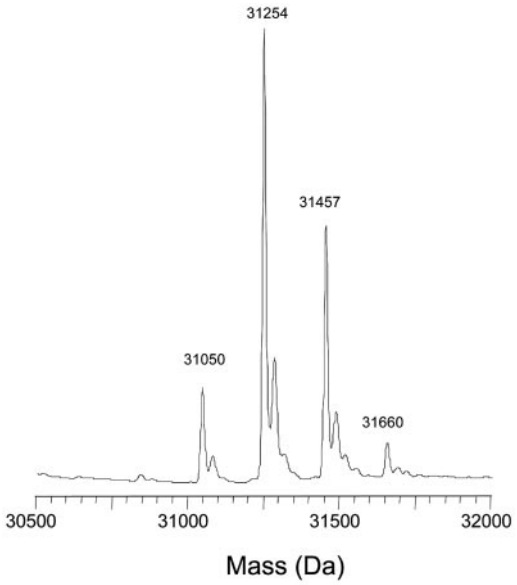

FIG. 3. Intact mass analysis of CLIP23485 flagellin. The reconstructed molecular mass profile of CLIP23485 flagellin (theoretical mass, 30,444 Da) shows peaks at 31,050, 31,253, 31,456, and 31,495 Da, corresponding to the attachment of three, four, five, and six HexNAc residues, respectively. No signal corresponding to unmodified flagellin was observed.

quence coverage was obtained. A second-generation fragment ion spectrum of fragment ion (*m/z* 204) resulted in a fragmentation pattern consistent with that of a HexNAc residue (data not shown). Together, these results suggest that *L. monocytogenes* flagellin is modified with a variable number of HexNAc residues, ranging from three to six ($3 \times 203 = 609$ Da; $4 \times 203 = 812$ Da; $5 \times 203 = 1{,}015$ Da; $6 \times 203 = 1{,}218$ Da).

To assign precisely the location of the glycosylation sites on flagellin, the flagellin tryptic digest was then subjected to base-catalyzed hydrolysis in the presence of NH_4OH. Upon alkaline hydrolysis, O-linked Ser and Thr residues yield modified amino acids with neutral masses of 86 and 100 Da that can be identified by the corresponding mass shift in the product ion spectrum of the β-eliminated products as shown in Fig. 4 for tryptic peptide T_{135}-T_{154}. Using this approach, it was possible to map five sites of attachment on *L. monocytogenes* CLIP23485 flagellin (Fig. 5). Threonine residues T_{141}, T_{145}, T_{173}, and T_{175} and serine residue S_{181} were identified as the sites of O-linked GlcNAc (O-GlcNAc) attachment.

Conservation of glycosylation sites among *Listeria* serotypes. Intact mass analysis of flagellin from strains 386, 568, and 2568 confirmed that the protein from these strains was also glycosylated with GlcNAc. Interestingly, in strain 2568, T_{141} is replaced with an isoleucine, one of the glycosylation sites identified by alkaline hydrolysis for CLIP23485 flagellin. As a result of this amino acid substitution, the intact mass analysis of this strain showed a different molecular mass profile. The intact glycoprotein comprising six HexNAc residues was no longer observed. Rather, the mass spectrum showed peaks corresponding to the glycoprotein with only 3, 4, or 5 HexNAc residues attached (data not shown).

Chemical analysis of *Listeria* flagellin. The monosaccharide (alditol acetate) GC-MS analysis of hydrolyzed flagellin protein from strain CLIP23485 identified GlcNAc as the N-acetylated amino sugar, revealing the singly charged *m/z* 204 fragment ion in the tryptic peptide analysis. The presence of this monosaccharide was confirmed by using a GlcNAc standard as described in Materials and Methods (data not shown).

Specificity of O-linked *N*-acetylglucosamine. A monoclonal antibody which recognizes O-GlcNAc in a β-O-glycosidic linkage to both serine and threonine was utilized in immunoblotting experiments to determine the nature of the linkage found on *Listeria* flagellin (8). As can be seen in Fig. 6, *L. monocytogenes* CLIP23485 flagellin had a positive reaction with this antibody, indicating that the GlcNAc residue is attached to the protein backbone through a β-O-glycosidic linkage. Negative controls (*Campylobacter* flagellin, which is modified with O-linked Pse5Ac7Ac, and *Pseudomonas* flagellin, which is modified with a complex glycan) were not recognized by this antibody. Flagellin protein from strains 568, 394, and 2568 were

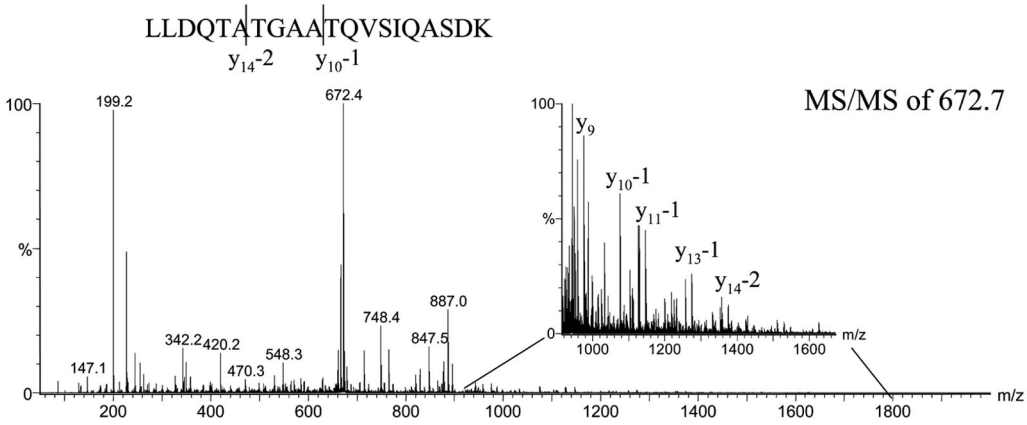

FIG. 4. Identification of glycosylation site by alkaline hydrolysis and tandem mass spectrometry. The product ion spectrum (*m/z* 672.7) from T_{135}-T_{154} following β elimination provided a series of y-type fragment ions, confirming the expected amino acid sequence. Shifts of 1 and 2 *m/z* units were observed for y11 and y14 fragment ions, consistent with modified Thr_{141} and Thr_{145}, confirming the locations of the HexNAc residues. MS/MS, tandem MS.

MKVNTNIISLKTQEYLR**KNNEGMTQAQER**LASGK**RINSSLDDAAGLAVVTRM**
NVKSTGLDAASK**NSSMGIDLLQTADSALSSMSSILQR**MR**QLAVQSSNGSFSDE**
DRKQYTAEFGSLIKELDHVADTTNYNNIKLLDQTATGAATQVSIQASDK**AND**
LINIDLFNAKGLSAGTITLGSGSTVAGYSALSVADADSSQEATEAIDELINNISN
GALLGAGMSRLSYNVSNVNNQSIATKASASSIEDADMAAEMSEMTKYKILTQ
TSISM LSQANQTPQMLTQLINS

FIG. 5. Assignment map of CLIP23485 flagellin. The primary amino acid sequence of FlaA from CLIP23485 is shown. Boldface letters indicate peptides identified by mass spectroscopy, and underlined peptides were shown to be glycosylated. Boxed residues are where carbohydrate is attached. Peptides not identified are indicated in lightface. The sequence coverage was 90%.

also positive by immunoblotting with this antibody (data not shown).

DISCUSSION

Our results demonstrate that *L. monocytogenes* flagellin is uniquely modified with β-*N*-acetylglucosamine in O linkage at up to six sites on each protein monomer. The precise sites of linkage for five of these residues were determined. These findings confirm previous studies suggesting that the flagellins of *Listeria* are posttranslationally modified (11, 33). Furthermore, results from ESMS analysis of flagellin from three additional flagellar H groups demonstrated that the modification is conserved regardless of the serotype. Although indirect evidence for glycosylation of flagellin from *Azospirillum brasilense* (29), *Aeromonas caviae* (14), *Spirochaeta aurantia* (3), *Clostridium tyrobutyricum* (2), *Butyrivibrio fibrisolvens* (20), and *Caulobacter crescentus* (21) has been provided in the literature, the only detailed structural characterization of the glycan moiety found on flagellins reported to date are those for *Pseudomonas aeruginosa* (39), *Campylobacter jejuni* (45), and *Helicobacter pylori* (40). This work represents the first structural characterization of flagellar glycosylation from a gram-positive organism. Unlike the novel glycans found on the flagella of these gram-negative organisms, the glycosylation of *Listeria* flagella is restricted to up to six sites, each containing a single O-linked GlcNAc residue.

Posttranslational modification of proteins by O-GlcNAc is a well-characterized monosaccharide modification of a number of eukaryotic nuclear and cytoplasmic proteins (46, 50). In comparison, no equivalent modification has been identified to date in prokaryotic systems. We present here the first description of a β-O-GlcNAc posttranslational modification on a prokaryotic protein (44). Unlike the glycosylation of *Campylobacter* and *Helicobacter* flagellin with pseudaminic acid, the addition of GlcNAc to *Listeria* flagellin would not require a novel glycan biosynthetic pathway as GlcNAc is a common biosynthetic precursor of numerous biochemical pathways. However, in eukaryotic systems, an important component of the O-GlcNAc modification is the O-GlcNAc transferase enzyme. An enzyme of equivalent function would be a prerequisite for the glycosylation of *L. monocytogenes* flagellin. However, a search of the *L. monocytogenes* EGD genome revealed no obvious candidate gene based on homology to known eukaryotic O-GlcNAc transferases. We are now pursuing the identification of this enzyme, a novel prokaryotic protein responsible for the O-linked glycosylation of *Listeria* flagellin.

As with other glycosylated bacterial flagellins, the sites of modification are located in the central surface-exposed region of the protein (37). This region is responsible for the antigenic variability observed for the flagella from a variety of bacteria (18). While diversity in glycan structure has been shown to contribute to serospecificity of *Campylobacter* flagella (22), this is unlikely to be the case for *Listeria*. The flagellins from three different serotypes (1/2a, 1/2b, and 4b) displayed an identical pattern of glycosylation with β-*N*-acetylglucosamine, clearly demonstrating that this posttranslational modification is likely not a contributing factor in the serological differentiation of these *L. monocytogenes* strains, but likely represents a shared antigen common among all of the H serotypes (42).

Inhibition of the flagellar glycosylation process through site-directed mutagenesis in *Campylobacter* and *Helicobacter* led to loss of motility, while in *Pseudomonas*, flagellar assembly and motility were unaffected by the lack of flagellin glycosylation. The future identification and characterization of *Listeria* glycosylation genes will permit the determination of the role of the glycosylation process in flagellar assembly and motility of this organism.

Flagella have been shown to be important in the initial attachment of the organism to various surfaces at temperatures

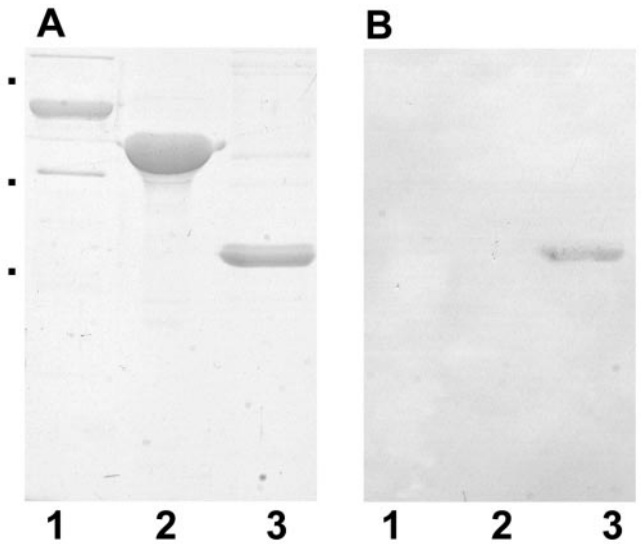

FIG. 6. Immunoreactivity of flagellin with β-O-GlcNAc antibody. A. SDS-PAGE of flagellin proteins stained with Coomassie blue. B. Western blot of flagellin proteins reacted with β-O-GlcNAc monoclonal antibody (1:1000). Lane 1, *C. jejuni* 81-176 flagellin; lane 2, *P. aeruginosa* PAO flagellin; lane 3, *L. monocytogenes* CLIP23485 flagellin. Molecular mass markers (■) are at 78,000, 45,700, and 32,500 Da.

of less than 30°C (13, 47). Flagellar glycosylation may have an important role in environmental interactions particularly within the food processing environment, where temperatures are generally maintained at much lower than ambient levels, which are optimal for the production of flagella. Previous reports (16, 38) described the growth of *Listeria* at lower temperatures as part of a consortium associated with bacterial species which form biofilms. It remains to be established if the presence of O-linked GlcNAc on the flagella filament acts to facilitate such interactions through lectin-like interactions.

Whether glycosylation is essential for flagellar assembly or in some way facilitates interactions of *Listeria* either with other cells or with environmental substrates is currently unknown. While flagellin expression has been shown to be down-regulated at 37°C (49) and may not be essential for pathogenesis in human infection, a recent study indicated that flagella of *L. monocytogenes* grown at 20°C played a role in Caco-2 cell invasion (10). The molecular basis of this process was not determined, but the potential of O-linked GlcNAc residues on the flagellin protein to facilitate such interactions cannot now be overlooked.

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