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Flagellin and Outer Surface Proteins from *Borrelia burgdorferi* Are Not Glycosylated▽

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We investigated the presence of glycoproteins in *Borrelia burgdorferi*. We did not find any evidence for glycosylation of the major outer membrane proteins OspA and OspB or the structural flagellar proteins FlaB and FlaA. We suggest that glycoproteins present on the surface of *B. burgdorferi* may be tightly bound culture medium glycoproteins.

Lyme disease is a tick-borne disease caused by the spirochete *Borrelia burgdorferi* and is a chronic disease characterized by skin, joint, heart, and neurological sequelae. Mammalian receptors which bind Lyme disease spirochetes, as well as bacterial ligands which promote cell interactions, are believed to be critical to the infectious process (6). Sambri and colleagues (15) reported on the glycosylation of two outer membrane (OM) proteins from *B. burgdorferi*, OspA and OspB, which stained following periodate oxidation and were sensitive to enzymatic deglycosylation by peptide *N*-glycosidase F (PNGase F; EC 3.5.1.52; New England Biolabs), which specifically cleaves between the innermost GlcNAc and Asn residues. These outer surface proteins were shown to be lipoproteins (3) which play a role in colonization and survival in the tick (12, 14) and which are implicated in later stages of human disease (1). Evidence for glycosylation has been presented for another borrelial protein, FlaA, which is associated with periplasmic flagella (PF), and posttranslational modification was also suggested for the structural protein of flagella, FlaB (5, 8). This protein was shown to be stained following periodate oxidation and by the digoxigenin (DIG)-labeled lectins *Sambucus nigra* agglutinin and *Galanthus nivalis* agglutinin. The protein was also sensitive

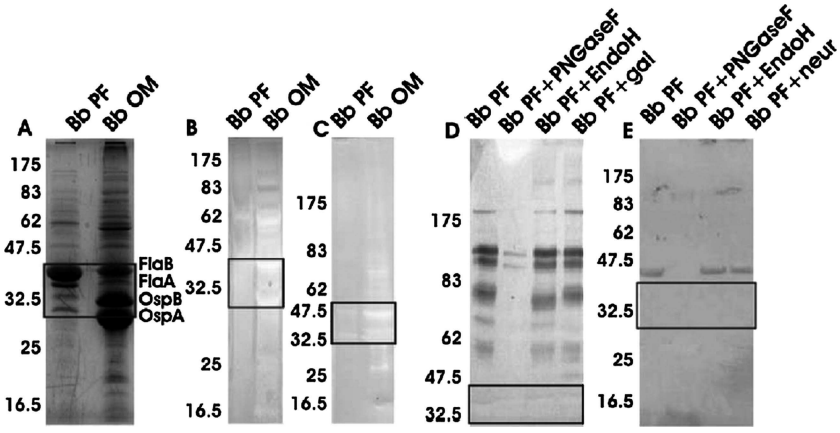

FIG. 1. Detection of glycoproteins in *Borrelia burgdorferi* PF and OM fractions. Crude PF and OM preparations were obtained by modification of the method described by Ge et al. (8). The OM fraction was obtained following Triton X-100 extraction of cells. After removal of bacterial cells by centrifugation, the OM fraction was collected from the supernatant by ultracentrifugation. PF were obtained by shearing of the Triton X-100-extracted cell pellets following their resuspension in PBS and vortexing in the presence of 1-mm-diameter glass beads. Crude PF preparations were obtained from the supernatant fraction by ultracentrifugation. (A) Coomassie brilliant blue-stained SDS-PAGE gel; (B) DIG glycan detection; (C) *G. nivalis* agglutinin lectin reactivity; (D) *S. nigra* agglutinin lectin reactivity; (E) *M. amurensis* agglutinin lectin reactivity. Numbers at the left are molecular masses (in kilodaltons). Bb, *B. burgdorferi*; EndoH, endoglycosidase H; gal, β-galactosidase; neur, neuraminidase.

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FlaA BB_0668

Residues mapped 230/344 (**66.8%**) Predicted MW 38816.14

*MVYMKRKAKSILFFLLSTVLFA***QETDGLAEGSKRAEPGELVLDFAELARDPSSTRLD***LTNYVDYV***YSGASGIVKPED**

MVVDLGINN**WSVLL***TPSARLQAYV***KNSVVAPAVVKSESKRYAGDTILGVRVLFPSYSQSSAMIMPPFKIPFYSG**

ESGNQFLGK*GLIDNIKTMKEIKVSVYSLGYEIDLEVLFEDMNGMEYAYSMGTLKF***KGWADLIWSNPNYIP**N**ISSRIIK**

DDVPNYPLASSKM*RFKAFRVS***KSHSSKEQNFIFYVKD***LRVLYD***KLSVSIDSDIDSESVFKVYETSGTESLRKL***KAH**

***ETFKRVLKLRE*KISMPEGSFQNFVEKIESEKPEESSPKN**

FlaB BB_0147

Residues mapped 286/336 (**85.1%**) Predicted MW 35764.66

MIINHN**TSAI**N**ASRNNGINAA**N**LSKT***QEKLSSGYRIN*RASDDAAGMGVSGK***INAQIRGLSQASR***N***TS*KAINFIQ**

TTEGNLNEVEKV*LV*RMKELAVQSG**N**GTYSDADRGSIQIEIEQLTDEINRIADQAQYNQMHMLS**N**KSASQNV**

RTAEELGMQPAKINTPASLSGSQASWTLRVHVGANQDEA*IAVNI*YAANVANLFSGEGAQTAQAAPVQE***GVQQ**

E*GAQQPAPATAPSQGGVNSPV**NVT**TTVDA**N**TSLAKIENAIRMISDQ*RANLGAFQNRLESIK**N**STEYAIENLKA**

SYAQIKDATMTDEVVAATTNSILTQSAMAMIAQANQVPQYVLSLLR

OspA (lp54) BB_A15

Residues mapped 203/273 (**74.3%**) Predicted MW 29367.41

*MKKYLLGIGLILALIA***CKQ**N**VSSLDEKNSVSVDLPGEMKVLVSKEKNKDGKYDLIATVDKLELKGTSDKN**N**GSG**

VLEGVKA*DKS***KVKLTISDDLGQTTLEVFKE***DGKTLVSKKVTSKD***KSSTEEKFNEKGEVSEKIITRADGTRLEYTGIK**

SDGSGKAKEVLK*GYVLEGTLTAEKTTLVVKEGTVTLSK**N**IS*KSGEVSVEL**N**DTDSSAATKKTAAWNSGTSTLTIT**

VNSK*KTKDLVFTKENTITVQQYDS**N**GTKLEGSAVEITKLDEIKNALK***

OspB (lp54) BB_A16

Residues mapped 216/296 (**72.9%**) Predicted MW 31714.86

*MRLLIGFALALALIG***CAQKGAESIGSQ*KENDLNLEDSSKKS**HQNA**KQDLPAVTEDSVSLFNGNKIF***VSKEK***N***SSGK**

YDL*RATIDQVELKGTSDKN**N**GSGTLEGSKPDKSKV*KLTVSADLNTVTLEAFDASNQKI***SSKVT*KKQGSITEETL**

KA*NKLDSKKLT*RS**N**GTTLEYSQITDAD**N**ATKAVE***TLKNSI*KLEGSLVGGKTT***VEI*KEGTVTLKRE***IEKDGKV*KVFL**

NDTAGSNKKT***G*KWEDSTSTLTISADSKKTKDLVFLTDGTITVQQYNTAGTSLEGSASEIK**N**LSELKN***ALK***

FIG. 2. Assignment maps of flagellins and outer surface proteins. The primary amino acid sequences of FlaA, FlaB, OspA, and OspB from *B. burgdorferi* B31 are shown. Boldface letters indicate peptides identified by MS. Italicized letters indicate unidentified peptides. Sites of potential N-linked glycosylation are indicated by increased font size. Boxed sequences indicate predicted signal sequences of FlaA, OspA, and OspB. MW, molecular weight.

to enzymatic deglycosylation by PNGase F, suggesting the N-linked attachment of glycan (8). Finally, Coleman and Benach (7) demonstrated that the immunodominant 41-kDa antigen recognized by sera of Lyme disease patients is the FlaB protein. Interestingly, the mass observed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels of 41,000 Da is considerably larger than the predicted mass of FlaB (35,742 Da), which may be due to either anomalous migration on SDS-PAGE gels (17) or posttranslational modification. The identification of flagellin structural proteins as glycoproteins has also been described for other spirochete species (2, 11, 21), although the precise structures of these glycans and mode of attachment remain to be determined.

We undertook this study to characterize the OspA, OspB, FlaA, and FlaB proteins from *B. burgdorferi* strain B31. Surprisingly, we were unable to demonstrate N-linked glycosylation by both indirect staining and detailed structural analysis. However, several other proteins from membrane preparations were stained as glycoproteins and were labeled by lectins. We provide preliminary evidence indicating that these proteins may be culture medium components which appear to bind tightly to the surfaces of Lyme borreliosis (LB) spirochetes.

Both crude PF and OMs were prepared from *B. burgdorferi* according to a modified method for isolation of periplasmic flagella using Triton X-100 (8) and analyzed by SDS-PAGE (Fig. 1A). The cells were washed three times with 10 mM phosphate-buffered saline (pH 7.2) and three times with 0.13 M phosphate buffer (pH 7.4) prior to isolation. The identification of OspA, OspB, FlaA, and FlaB from OM and PF preparations was made following gel band extraction, tryptic

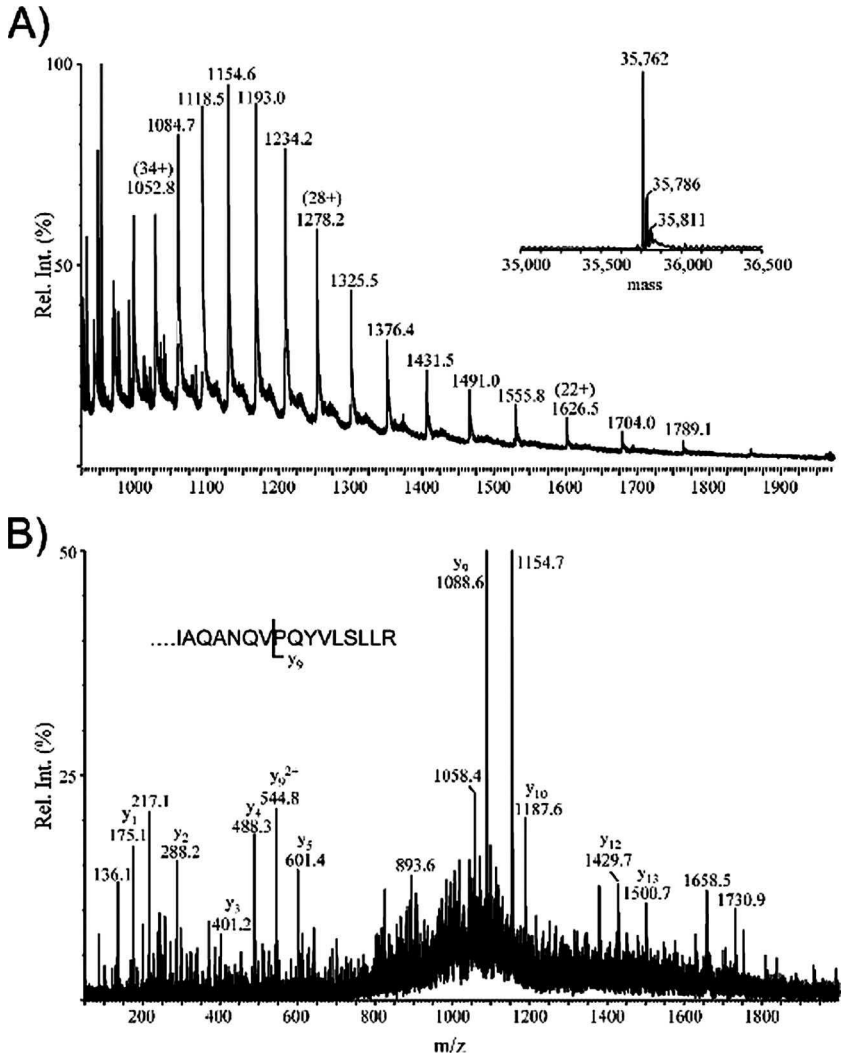

FIG. 3. Electrospray ionization (ESI)-MS analysis of FlaB flagellin protein from *B. burgdorferi*. (A) ESI-MS of the intact protein. The protein solution was infused into the ESI source of a Q-TOF2 mass spectrometer at a flow rate of 1 μl/min. The deconvoluted molecular mass profile of this protein is presented in the inset. (B) MS/MS analysis of the $(M + 31H)31^{+}$ ion at *m/z* 1,154.6. The collision offset was 30 V. The *y* fragment ions are indicated in the spectrum, and the corresponding portion of the C-terminal amino acid sequence from FlaB flagellin is presented in the inset. Rel. Int., relative intensity.

digestion, and peptide assignment by mass spectroscopy (MS) analysis (Fig. 1A). Similar results were obtained for *Borrelia afzelii* and *Borrelia garinii* OM and PF preparations (data not shown). To determine if these proteins were glycosylated, we first used periodate oxidation (a Roche DIG glycan detection kit and a Pierce GelCode glycoprotein staining kit according to the manufacturers' instructions) for detection of glycoproteins in PF and OM samples. We did not observe any positive reaction with the proteins of interest (FlaA, FlaB, OspA, OspB) (Fig. 1B), although a number of higher-molecular-weight proteins appeared positive by this reaction. Using a Roche DIG glycan differentiation kit, which utilizes lectin binding, we observed no positive reaction of FlaA, FlaB, OspA, and OspB proteins with the lectins *G. nivalis* agglutinin (Fig. 1C), *S. nigra* agglutinin (Fig. 1D), and *Maackia amurensis* agglutinin (Fig. 1E) or with *Arachis hypogaea* (peanut) agglutinin and *Datura stramonium* agglutinin (data not shown). However, using the *Sambucus nigra* agglutinin lectin, we stained a number of high-molecular-mass protein bands (47,000 to 200,000 Da) in both OM and PF preparations (Fig. 1D). A single protein with a molecular mass of 45,000 Da in both PF and OM preparations was stained by the *M. amurensis* agglutinin lectin. All samples were subjected to enzymatic deglycosylation using PNGase F (Fig. 1D), endoglycosidase H (EC 3.2.1.96), β-galactosidase, and neuraminidase (all purchased from New England Biolabs) (data not shown), and we failed to observe any shift in the molecular weights of proteins OspA, OspB, FlaA, and FlaB. It should be noted that the sensitivity of this approach would detect changes only where the glycosylation process contributed a minimum of 5 to 10% of the protein mass. Again, several of the *S. nigra* agglutinin-reactive proteins with molecular masses higher than 47,000 Da were sensitive to enzymatic deglycosylation with PNGase F, as was the 45,000-Da *M. amurensis* agglutinin-reactive protein (Fig. 1D

and E). The specificity of staining reactions was verified in each case by using positive and negative controls as described by the manufacturer (data not shown).

Next, a concerted effort was made to map the OspA, OspB, FlaA, and FlaB proteins. Following excision of each protein band from an SDS-PAGE gel and proteolytic digestion, peptides were analyzed by MS. A number of classic eukaryotic N-linked sequon consensus sequences (*N*-X-S/T) (10) are present in the primary amino acid sequences as indicated in Fig. 2 (FlaA, two sites; FlaB, nine sites; OspA, five sites; and OspB, six sites). Of these, three are bacterial N-glycosylation sequons (D/E-X-*N*-X-S/T) (13) (one in FlaB and two in OspA). Nanovolume liquid chromatography–MS and MS/MS analysis performed by multiple tryptic digestion and GluC and tryptic/GluC double digestion was successful in assigning 79.5% of OspA peptides, 86.8% of OspB peptides, 92.8% of FlaB peptides, and 71.8% of FlaA peptides (Fig. 2). Peptides containing 20 of the 22 N-linked sequons from these proteins were identified and shown not to carry an N-linked glycan modification. Peptides containing the remaining two putative *N*-glycosylation sites (FlaB and OspB) were not identified. No evidence for peptides with masses that were anomalous to those of any of the four proteins was obtained, indicating that these proteins are not glycosylated with either N- or O-linked glycans.

The observed mass of FlaB from SDS-PAGE gels had been shown to be approximately 5,000 Da higher than that predicted by the primary sequence. To confirm that we had not missed any sites of modification by peptide analysis, we next determined the intact mass of FlaB from the PF. Infusion of the PF protein sample into the mass spectrometer resolved a single major protein species with a mass of 35,760 ± 10 Da, which corresponds to the predicted mass of FlaB (35,742 Da). The mass discrepancy of 18 Da is most likely attributable to the oxidation of a methionine residue during sample preparation (Fig. 3). Signals corresponding to the FlaA, OspA, and OspB proteins were not found in the mass spectra, probably due to the low levels of ionization of these proteins.

It has been previously shown that the surface of *B. burgdorferi* is able to bind a number of lectins (9, 18, 20). DIG glycan detection and lectin labeling indicated that OM and PF preparations contained reactive proteins which did not correspond to FlaA, FlaB, OspA, or OspB. Immunogold labeling of *B. burgdorferi* cells grown in BSK-H medium demonstrated that the surfaces of spirochete cells were reactive to both *S. nigra* agglutinin lectin (Fig. 4A) and anti-rabbit serum (Fig. 4B). As complete BSK-H medium contains rabbit serum, we next investigated whether the source of these glycan-positive proteins was from the BSK-H medium. Examination of the medium alone by SDS-PAGE analysis and glycan-*S. nigra* agglutinin detection revealed that some of the OM-positive proteins corresponded to glycan-positive medium components (Fig. 4C). An immunoglobulin heavy-chain protein of approximately 83,000 Da has previously been shown to be coisolated from complete BSK-H medium (16) and may correspond to one of the *S. nigra* agglutinin-positive proteins identified in this study in both BSK-H medium and OM preparations (Fig. 4C).

In conclusion, FlaA, FlaB, OspA, and OspB proteins purified from LB spirochetes grown under standard laboratory conditions in BSK-H medium are not glycosylated. Obviously,

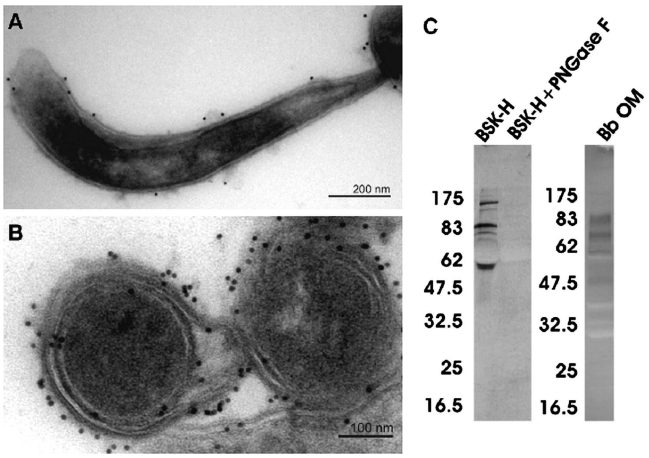

FIG. 4. Surface-associated binding of medium glycoproteins to *B. burgdorferi*. (A) Cryosection and *S. nigra* agglutinin lectin labeling of *B. burgdorferi* cells. (B) Cryosection of *B. burgdorferi* cells labeled with anti-rabbit serum. (C) *S. nigra* agglutinin lectin staining of diluted BSK medium and *B. burgdorferi* (Bb) OM. Numbers at the left are molecular mass markers (in kilodaltons).

this does not rule out the possibility that the organism may be able to glycosylate proteins while growing in vivo in an arthropod vector or mammalian host, and the recent development of dialysis membrane chamber implants in a rat peritoneum for the in vivo cultivation of LB spirochetes (4) may facilitate such studies. While earlier work indicated that the borrelial OspA, OspB, and FlaA proteins carry N-linked glycans, the extensive structural analysis performed in this study indicates that N-linked glycosylation did not occur. Moreover, bioinformatics analysis of six spirochete genomes revealed no evidence of a conserved STT3 (*pglB*) oligosaccharyltransferase, which is the enzyme required for the transfer of N-linked glycans from a lipid carrier to an asparagine residue of the protein (19). Still, we cannot rule out the possibility of the presence of a novel glycosyltransferase in the genome of *B. burgdorferi* and the possibility of its activation at different stages of the complex life cycle of LB spirochetes.

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