Breastfeeding is one of the main factors guiding the composition of the infant gut microbiota in the first months of life. This process is shaped in part by the high amounts of human milk oligosaccharides that serve as a carbon source for saccharolytic bacteria such as *Bifidobacterium* species. Infant-borne bifidobacteria have developed various molecular strategies for utilizing these oligosaccharides as a carbon source. We hypothesized that these species also interact with *N*-glycans found in host glycoproteins that are structurally similar to free oligosaccharides in human milk. Endo-β-/*N*-acetylglucosaminidases were identified in certain isolates of *Bifidobacterium longum* subsp. *longum*, *B. longum* subsp. *infantis*, and *Bifidobacterium breve*, and their presence correlated with the ability of these strains to deglycosylate glycoproteins. An endoglycosidase from *B. infantis* ATCC 15697, EndoBI-1, was active toward all major types of *N*-linked glycans found in glycosylated proteins. Its activity was not affected by core fucosylation or extensive fucosylation, antenna number, or sialylation, releasing several *N*-glycans from human lactoferrin and immunoglobulins A and G. Extensive *N*-deglycosylation of whole breast milk was also observed after coincubation with this enzyme. Mutation of the active site of EndoBI-1 did not abolish binding to *N*-glycosylated proteins, and this mutant specifically recognized Man₃GlcNAc₂(α1–6Fuc), the core structure of human *N*-glycans. EndoBI-1 is constitutively expressed in *B. infantis*, and incubation of the bacterium with human or bovine lactoferrin led to the induction of genes associated to import and consumption of human milk oligosaccharides, suggesting linked regulatory mechanisms among these glycans. This work reveals an unprecedented interaction of bifidobacteria with host *N*-glycans and describes a novel endoglycosidase with broad specificity on diverse *N*-glycan types, potentially a useful tool for glycoproteomics studies. *Molecular & Cellular Proteomics* 11: 10.1074/mcp.M112.018119, 775–785, 2012.

Breast milk is an intriguing and complex fluid that supports the growth, development and protection of the newborn. Its composition includes essential nutrients such as lactose, fatty acids, and proteins (1), as well as a constellation of bioactive compounds critical for the protection and correct development of the infant in the first months of life (2). Breastfeeding is one of the main factors in the establishment of the intestinal microbiota in infants (3). The presence of certain species of *Bifidobacterium* is a major feature of breast-fed infants (4), and the dominance of these microorganisms is thought to be associated with beneficial health effects (5, 6). This enrichment has been explained by the ability of bifidobacteria to degrade and utilize human milk oligosaccharides (HMOs)¹ as a carbon source (7).

Proteins represent an important fraction of breast milk. A great variability exists among different proteins types and concentrations across different mothers and stages of lactation (8). Milk proteins are readily utilized by the infant (9) and are also critical in the protection of the newborn. For example, human lactoferrin (hLF) is one of the most abundant proteins in human milk, and hLF and its derived peptides display broad antimicrobial and anti-inflammatory effects, among several other biological activities (10, 11).

Virtually all secreted proteins in eukaryotes, including those in human milk, are glycosylated (12). Although some milk caseins are O-linked glycosylated, lactoferrin and immunoglobulins contain *N*-linked glycans (12, 13). Asparagine-linked glycosylation is one of the most common post-translational modifications of eukaryotic proteins (14). In general, the role

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¹ The abbreviations used are: HMO, human milk oligosaccharide; GlcNAc, *N*-acetylglucosamine; Man, mannose; Fuc, fucose; GH, glycosyl hydrolase; hLF, human lactoferrin; bLF, bovine lactoferrin; IgA, immunoglobulin A; IgG, immunoglobulin G; RNaseB, ribonuclease B; MRS, de Mann-Rogose-Sharp; ZMB-1, Zhang-Mills-Block-1; DHB, 2,5-dihydroxy/benzoic acid; PNGase F, peptide*N*-glycosidase F.
of N-linked glycosylation in folding, secretion, and resistance to proteolysis is understood for several proteins (15, 16), and several examples have exemplified the crucial role of N-glycans in protein function, such as bacterial recognition (17), intracellular signaling (18), and antigen binding and presentation (19).

Interestingly, certain microorganisms, mostly pathogens, have acquired the ability to cleave N-glycans from glycoproteins. This trait is associated with the use of these oligosaccharides as a carbon source (20) or altering the biological function of certain glycoproteins such as immunoglobulins (21). Bacterial endo-β-N-acetylglucosaminidases (EC 3.2.1.96; endoglycosidases) are widespread enzymes that cleave the N,N'-diacetyl chitobiose moiety characteristic of the pentasaccharide Man₃GlcNAc₂ found in all N-glycans (22). These enzymes belong to glycosyl hydrolase families GH18 or GH85. Prominent examples of GH18 enzymes are EndoH from Streptomyces plicatus (23), EndoE from Enterococcus faecalis (24), and EndoS from Streptococcus pyogenes (25). EndoD from Streptococcus pneumoniae (26) is a member of GH85. Their substrate specificities are usually limited to either high mannose or complex N-glycans and some require additional exoglycosidases for glycan release. EndoH is commonly used for glycoprotein deglycosylation and determination of the type of N-linked glycosylation; however, EndoH substrate limitations hamper its use in comprehensive glycoproteomic analysis.

The niche that infant gut-associated bifidobacteria colonize is characterized by high amounts of milk oligosaccharides, as well as proteins or peptides arriving from breast milk or from the developing infant gut. Although some bifidobacteria apparently can use mucin O-linked oligosaccharides as a carbon source (27, 28), whether these microorganisms can interact with N-glycosylated proteins has yet to be addressed. In this work, we explored the ability of infant gut isolates of bifidobacteria to release N-glycans from host glycoproteins, and we also describe some of the properties of a bifidobacterial endoglycosidase that releases N-glycans from diverse host glycoproteins.

**EXPERIMENTAL PROCEDURES**

**Bacteria and Media—** The Bifidobacterium strains used in this study (supplemental Table S1) were obtained from the Japanese Collection of Microorganisms (Riken Biosource Center, Japan), the American Type Culture Collection (Manassas, VA), and the University of California Davis Viticulture and Enology Culture Collection (Davis, CA). For routine experiments, bifidobacteria were grown on de Mann-Rogrose-Sharp (MRS) broth supplemented with 0.05% (w/v) L-cysteine (Sigma-Aldrich). Chemically defined Zhang-Mills-Block-1 (ZMB-1) medium (29) was used for evaluation of bacterial growth on glycoproteins or transcriptional analyses. The cells were anaerobically grown in a vinyl chamber (Coy Laboratory Products, Grass Lake, MI) at 37 °C for 24 h, in an atmosphere consisting of 5% carbon dioxide, 5% hydrogen, and 90% nitrogen.

**Chemicals—** Cyanogen bromide-activated Sepharose 4B beads, ribonuclease B from bovine pancreas (RNaseB), immunoglobulin G from human serum (IgG), immunoglobulin A from human colostrum (30), lactoferrin from human milk (hLF), lactoferrin from bovine milk (bLF), and 2,5-dihydroxybenzoic acid (DHB) were all obtained from Sigma-Aldrich. Graphitized carbon cartridges were purchased from Grace Davison Discovery Sciences (Deerfield, IL). All of the chemicals used were either of analytical grade or better. Claristear yeast mannan protein was a gift from DSM Food Specialties ( Parsippany, NJ).

**Incubations and Growth of Bifidobacteria on Glycoproteins—** Bifidobacterial isolates were grown on 2 ml of MRS with no carbon source (mMRS), supplemented with 2% lactose to mid-late exponential phase. Two hundred µl of culture were centrifuged for 1 min at 12,000 × g and resuspended in 200 µl of mMRS supplemented with 5 mg/ml of RNaseB. Incubations were run for 18 h, and supernatants were recovered after centrifugation 1 min at 12,000 × g. A 1:10 dilution of each supernatant was denatured in glycoprotein denaturing buffer (0.5% SDS and 40 mM DTT), analyzed on 4–15% precast SDS-PAGE gels (Bio-Rad), and stained with Bio-safe Coomassie Blue (Bio-Rad). Growth of specific bacteria was also analyzed on 96-well plates containing 200 µl of ZMB-1 medium supplemented with 10 mg/ml of hLF or bLF or 50 mg/ml of Claristear yeast mannan protein. The cultures were inoculated at 2% and grown for 72 h in a PowerWave microplate reader (BioTek Instruments, Inc., Winoosky, VT) under anaerobic conditions at 37 °C. Growth was monitored using the Gen5 1.10 software (BioTek). The cultures were grown in triplicate, and controls with no glycoprotein and no bacteria were run and subtracted from the A₆₀₀ values.

**Endoglycosidase Sequence Determination—** Protein coding sequences belonging to GH18 found in the genomes of B. infantis ATCC 15697 (Bion_2468), B. infantis 157F (BLIF_1310), and E. faecalis OGI1RF (EndoEv) were aligned using MUSCLE. Conserved regions were selected and converted to DNA to design degenerate primers (supplemental Table S2). A similar approach was used with sequences encoding GH85 enzymes, found in the published genome sequences of Bifidobacterium longum DJO10A (BLD_0197), B. longum NCC2703 (BL1335), and Bifidobacterium breve UCC2003. Genomic DNA was prepared from overnight cultures on MRS for each strain used in this study using the DNeasy blood and tissue kit (Qiagen). Fifty-µl PCRs contained 1 unit of Phusion DNA polymerase (Finzymes, Vantaa, Finland), 1 ng of DNA, 0.2 mM of dNTPs, and 2.5 µM of each degenerate primer (supplemental Table S2) and were run in a PTC200 Thermo Cycler (MJ Research, Ramsey, MN). The PCR program included an initial denaturation at 98 °C for 30 s, 30 cycles of denaturation at 98 °C 10 s, annealing at 55 °C for 30 s, extension at 72 °C 1 min, and a final extension at 72 °C for 7 min. PCR products were purified using the Qiaquick PCR product purification kit (Qiagen), and sequenced at the University of California Davis DNA sequencing facility. Sequences encoding GH18 enzymes were analyzed using BioEdit 7.1.3 and later expanded and fully determined using the DNA Walking SpeedUp Premix Kit (Seegene, Rockville, MD) and the TSP142 primers listed in supplemental Table S2. GH85-encoding gene sequences were directly determined using primers GH85cF and GH85cR.

**Bioinformatic Analyses—** The Integrated Microbial Genomes (31) database was used to find GH18 and GH85 protein sequences in Bifidobacterium genomes and to determine genetic landscapes for GH18-type and GH85-type genes found in the genomes of B. infantis ATCC 15697, B. infantis 157F, and B. longum DJO10A. Multiple sequence alignments were performed using MUSCLE, using the maximum likelihood algorithm in MEGA version 5.0.

**Gene Cloning and Expression—** Genomic DNA from B. infantis ATCC 15697, B. infantis SC142, and B. longum DJO10A was amplified using the cloning primers indicated in supplemental Table S2, targeting GH18 or GH85 sequences. Signal peptides and transmembrane domains were not amplified to facilitate protein expression and
purification from Escherichia coli. Gene amplification by PCR, cloning, protein expression, and purification were performed as described (32). Induction was performed with 0.5 mM isopropl β-D-thiogalactoside at 28 °C (for EndoB1-1, EndoB1-2 and EndoB1-1mut) or with 1 mM isopropl β-D-thiogalactopyranoside at 37 °C (for EndoBB). The proteins were concentrated using Amicon Ultra 30-kDa 4-ml columns, and buffer was exchanged for sodium saline citrate 1× using Bio-Gel P-30 in SSC buffer columns (Bio-Rad).

**Glycoprotein Digestion by Bifidobacterial Endoglycosidases—Optimal enzymatic conditions for endoglycosidases EndoB1-1, EndoB1-2, and EndoB1 were determined by incubation with RNaseB. The reactions were performed in a 10 μl volume and included 4 μg of RNaseB, 1 μg of each enzyme, and 4 μl of 0.2 mM Na2HPO4 with pH values between 5.0 and 7.0 at 37 °C. The reactions were run for 1 h, stopped with 1 μl Na2CO3, treated with the denaturing buffer described above, and loaded into 4–15% precast polyacrylamide SDS gels. The optimal temperature for each reaction was determined at each optimal pH, and reactions were performed at 4, 30, 37, 45, 55, and 65 °C for 1 h. Heat resistance was evaluated by incubating each glycosidase at 95 °C for 1, 5, and 30 min, and enzyme reactions were then carried out under optimal conditions. Digestions of hLF and bLF (Sigma) were performed under optimal conditions using 4 μg of each glycoprotein and incubated for 18 h with 1 μg of each endoglycosidase, or 1 μl of glycerol-free peptide:N-glycosidase F (PNGase F); 500 units/μl; New England Biolabs, Ipswich, MA). Finally 20 μl of a fresh breast milk sample (kindly provided by a lactation study directed by Jennifer Smilowitz at University of California Davis) was incubated for 18 h at 37 °C with 10 μg of EndoB1-1, 10 μg of EndoB1-1 D184N, or 1 μl of PNGase F in 20 mM Na2HPO4, pH 5.0. Lactoferrin and human milk digestions were evaluated in 7.5% precast SDS-PAGE gels under denaturing conditions. All of the experiments were performed at least in duplicate.

**EndoB1-1 Immobilization to Sepharose Beads—To eliminate unwanted interferences and contamination from the enzyme solution, EndoB1-1 was immobilized to Sepharose beads activated with cyanogen bromide. This also allowed for multiple usage of the enzyme on different samples over a few weeks. Cyanogen bromide-activated Sepharose beads of 40–165-μm diameter were covalently coupled to EndoB1-1 via the well established coupling chemistry (33). The actual immobilization of EndoB1-1 to the Sepharose beads was achieved using a slightly modified version of the protocol reported earlier (34, 35). In this study 150 mg of the lyophilized Sepharose 4B beads were coupled to 300 μl of EndoB1-1 prior to the glycoprotein digestion.

**Glycan Release by EndoB1-1—The model glycoproteins used in this study were RNaseB, bLF, hLF, IgA, and IgG. Each glycoprotein was individually digested with bead-immobilized EndoB1-1 while thoroughly rinsing the beads after each digestion to eliminate cross-contamination. Glycoproteins were prepared in 0.2 mM Na2HPO4, pH 5.0, at 1 mg/ml in a final volume of 300 μl in 1.5-ml tubes. The digestion mixture including the beads was incubated at 37 °C overnight with gentle agitation. The resultant digestion mixture was then carefully drawn out following centrifugation. Purification of the resultant glycans was then achieved via solid phase extraction (36) using C18 and graphitized carbon cartridges as earlier described by our group (37). In this study, a clean mixture of the resultant glycans was then eluted with 9 ml of 0.05% trifluoroacetic acid in 40% ACN in water (v/v) followed by vacuum drying using a SpeedVac prior to MS analysis.

**Instrumentation—Glycans purified by SPE were completely dried in a SpeedVac, reconstituted in 50 μl of deionized water, and directly analyzed by mass spectrometry. Glycan stock solutions (0.75 μl) were individually spotted on a stainless steel MALDI target with each spot mixed with an equal volume of DHB matrix solution made up of 0.05 mg/ml DHB in 50% ACN:50% water. The glycan-DHB spots were then allowed to dry prior to the actual MS analyses. In this study, an IonSpec HiRes MALDI FT-ICR mass spectrometer (Lake Forest, CA) equipped with an external ion source based on a third harmonic Nd:YAG laser (355 nm) and a 7.0 Tesla actively shielded superconducting magnet, served as the platform for all the experiments described herein. Glycans were analyzed with the MALDI FT-ICR MS instrument in both the positive and negative ion modes. Once released by the EndoB1-1 enzyme and detected via MS analysis, glycan assignment was achieved by a combination of accurate mass measurement and the use of an in-house tool (oligosaccharide calculator). The oligosaccharide calculator takes into account the various combinations of monosaccharides that match up in mass to the observed glycan mass within a predetermined error window using maltoligo- saccharides (38). This information is integrated with biological knowledge of N-linked glycosylation, such as connectivity and position of each monosaccharide. The cartoons of N-glycans represent putative structures.

**Site-directed Mutagenesis—A plasmid containing the EndoB1-1 coding sequence (described above) was resynthesized with mutagenic primers AmpR and 2468mutT (supplemental Table S2) using the Change-IT multiple mutation site-directed mutagenesis kit (USB Corporation, Santa Clara CA) and following the manufacturer’s instructions. Mutated plasmids were cloned into Top10 competent cells (Invitrogen) and, after the proper mutation was verified, were transformed into BL21 competent cells. EndoB1-1 D184N was purified as described in the previous section, with induction carried on with 0.5 mM isopropl β-D-thiogalactopyranoside at 28 °C for 6 h. Glycan Array Analysis—Purified EndoB1-1 D184N (100 μl/ml, 200 μl) was analyzed for glycan binding to the Mammalian Printed Array version 5.0 (provided by the Consortium for Functional Glycomics). Protocols are available at http://www.functionalglycomics.org. Detection was performed using an anti-His-FITC antibody (Invitrogen).

**B. infantis Gene Expression—**B. infantis cells were grown on ZMB-1 medium with 2% lactose or glucose as describe above. Six ml of an exponential culture (A600 0.8–1) were centrifuged for 1 min at 12,000 × g and immediately resuspended in 5 ml of prewarmed ZMB-1 supplemented with either hLF or bLF (5 mg/ml). The cultures were rapidly returned to anaerobic conditions, and 1 ml of each culture was taken anaerobically every hour. One ml of the original culture grown on lactose or glucose (t = 0) and hourly time points of incubations with bLF or hLF (t = 1–3 h) were centrifuged at 12,000 × g for 1 min, and the pellet was resuspended in 1 ml of RNAlater (Ambion, Austin, TX). The experiment was done in duplicate. Cell suspensions were stored overnight at 4 °C and then at 80 °C until use. RNA extraction, quality check, and cDNA conversion were performed as previously described (39). Relative quantification for genes listed in supplemental Table S2 was performed in a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) and using the Fast Sybr Green Master Mix (Applied Biosystems). The reaction conditions were as recommended by the manufacturer using 0.5 μM of each primer. Primers for quantitative PCR were designed using the NCBI primer design tool, checking for specificity across the B. infantis ATCC 15697 genome (supplemental Table S2).

**Fluorescence Assays—**Binding of EndoB1-1 D184N to different glycoproteins was determined after overnight coating in microtiter 96-well plates of 20 μmoles of RNaseB, bLF, hLF, or BSA in PBS buffer at room temperature. The experiment was performed in triplicate. The wells were washed with PBS three times and blocked after incubation with 3% BSA at room temperature for 1 h. Twenty μmol of EndoB1-1 D184N or BSA were added to the wells and incubated for 2 h at 37 °C in PBS buffer adjusted to pH 5.0. The wells were washed three times with 0.05% PBS-Tween 20 and incubated for 1 h with a 1:500 dilution of FITC–anti-His (C-terminal) antibody (Invitrogen). After four washes with PBS-Tween, fluorescence was monitored in a
Synergy2 Microplate reader (Biotek), at 485/530-nm emission/excitation. In another set of experiments, breast milk samples incubated overnight with EndoBI-1, EndoBI-1 D184N, or PNGase F as performed above were coated overnight in a microtiter 96-well plate at room temperature. After washing three times with PBS buffer, the wells were incubated with a 1:500 dilution of 5 mg/ml of fluorescein-labeled concavalin A (Vector Laboratories, Inc., Burlingame, CA) for 1 h at 37°C. The wells were washed four times with 0.05% PBS-Tween 20, and fluorescence was read as described above. The experiment with three replicates was repeated twice. Statistical analysis of the data was carried out by one-way analysis of variance, and the means were separated by Tukey’s honest significant differences test using the SPSS software package version 10.0.6. (SPSS Inc., Chicago, IL).

RESULTS

Infant Gut Isolates of Bifidobacteria Display Endo-N-acetylglucosaminidase Activity—Bovine ribonuclease B (RNaseB) is a 17-kDa model glycoprotein that contains one glycosylation site composed of high mannose N-linked glycans. Cleavage by endoglucosidases results in a molecule of 14 kDa (40). Overnight incubations of a panel of 76 bifidobacterial isolates (supplemental Table S1) with RNaseB suggested that endoglucosidase activity is present in only certain isolates. Incubation of B. infantis ATCC 15697 with 5 mg/ml of RNaseB led to a gradual deglycosylation of this glycoprotein over time (Fig. 1A). In general, other B. infantis strains degraded RNaseB weakly (Fig. 1C). Only a few B. longum subsp. longum (B. longum) isolates and none of the B. bifidum strains examined displayed this phenotype (Fig. 1B). Interestingly, certain isolates of B. breve such as KA179 and JCM7019 completely deglycosylated RNaseB (Fig. 1D).

Distribution of Endo-N-acetylgalactosaminidase Gene Sequences in Bifidobacteria—Based on known endo-N-acetylgalactosaminidase sequences found in bifidobacteria, degenerate primers were used to search for GH18 or GH85 sequences in the strains used in this study. Certain isolates were found to contain a gene encoding either a GH18 or GH85 enzyme. All strains containing one of these sequences cleaved RNaseB in vitro, and conversely strains lacking these genes did not show endoglucosidase activity (supplemental Table S1). This suggested a correlation between the presence of a GH18 or GH85 enzyme and the observed RNaseB deglycosylation phenotype.

Fig. 1. Endoglucosidase activity found in Bifidobacterium isolates. A, deglycosylation of RNaseB by B. infantis ATCC 15697 over time. B–D, overnight incubation with RNaseB was evaluated for other isolates of B. longum (B), B. infantis (C), or B. breve (D). E, phylogenetic representation of predicted endoglucosidase protein sequences found in bifidobacterial isolates. C: control reactions.

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A phylogenetic tree (Fig. 1E) classified these protein sequences in three types. One group was exclusively found in *B. infantis* strains including the type strain ATCC 15697 (termed GH18a) and distantly related to EndoE from *E. faecalis*. Another set of sequences found in strains of *B. infantis*, *B. breve*, and *B. longum* shared 60% amino acid identity with GH18a, and it was termed GH18b. Sequences belonging to GH85 were almost exclusively found in fecal *B. breve* isolates. Multiple alignments revealed a high degree of conservation of the proposed active site for each glycosidase family (supplemental Fig. S1) (24, 41). The genomic landscape for these genes supported their association with carbohydrate metabolism. Blon_2468 in *B. infantis* ATCC 15697 is located in a gene cluster that contains a phosphotransferase system specific for *N*-acetylglucosamine (supplemental Fig. S2). BLIF_1310 in *B. infantis* 157F (GH18b) and BLD_0197 in *B. longum* DJO10A (GH85) are located near ABC transporters predicted to import oligosaccharides and two or three putative α-mannosidases (supplemental Fig. S2).

**Enzymatic Properties of Bifidobacterial Endo-N-acetylglucosaminidases**—Based on the sequence alignments obtained (Fig. 1E), a representative gene of each glycosyl hydrolase type was cloned, expressed, and purified in *E. coli* (supplemental Fig. S3). The recombinant endo-β-N-acetylglucosaminidases from *B. infantis* ATCC 15697 (EndoBI-1; Blon_2468), *B. infantis* SC142 (EndoBI-2), and *B. longum* DJO10A (EndoBB; BLD_0197), all exhibited maximum glycolytic activity at pH 5.0, and their optimal temperatures ranged from 30 to 45 °C (supplemental Fig. S4). Interestingly, the enzymatic activity of EndoBI-1 and EndoBI-2 was not significantly impaired by incubation at 95 °C for 1 or 5 min, implying that they are heat-resistant enzymes (Fig. 2A). This property was not observed for EndoBB. Other properties of these enzymes are listed in Table I.

hLF contains core fucosylated complex *N*-glycans, predominantly at two glycosylation sites (42). BLF represents a minor fraction of bovine milk, and it contains mainly oligomannose *N*-linked glycans at five glycosylation sites (35). Overnight incubations of BLF and hLF with the three recombinant *Bifidobacterium* endoglycosidases indicated that EndoBI-1 and EndoBI-2 were able to cleave BLF and also hLF, as observed by discrete changes in molecular weight on SDS-PAGE gels (Fig. 2B). EndoBB did not display glycolytic activity against BLF or hLF (Fig. 2B).

**EndoBI-1 Cleaves the Chitobiose Core of High Mannose and Complex N-glycans**—To better determine the enzymatic properties of EndoBI-1, the enzyme was immobilized using Sepharose beads and incubated with several glycoproteins with varying glycosylation types. Glycans released were analyzed using MALDI FT-ICR mass spectrometry. Similarly to related endoglycosidases, EndoBI-1 acted on the chitobiose core of *N*-glycans, probably leaving a GlcNAc residue (and an α1–6 fucose in core fucosylated glycans) attached to the
glycosylated asparagine residue. Activity on bLF was detected, and predominantly oligomannose N-glycans were released, with a minor amount of complex/hybrid glycans (Fig. 3A) (43), consistent with the general N-glycosylation of the protein. N-Glycans released from RNaseB contained between five and nine mannose residues (supplemental Fig. S5) (40). EndoBl-1 was also shown to deglycosylate glycoproteins such as hLF, IgA, and IgG (Fig. 3, B and C, and supplemental Fig. S5). These proteins are characterized by complex core fucosylated glycans and are essentially resistant to several commercial endoglycosidases under native conditions. Glycans released from hLF and IgA were bi- and triantennary and contained up to two sialic acid residues and up to three fucoses attached to the lactosamine chains. The profile of the glycans released by EndoBl-1 was similar to PNGase F (10, 42, 44). IgG deglycosylation by EndoBl-1 revealed biantennary complex N-glycans with lesser fucosylation and sialylation compared to IgA, and similar to those observed after PNGase F cleavage (supplemental Fig. S5) (45, 46).

A Mutant of EndoBl-1 Binds the Core of N-linked Glycans—The conserved active site of GH18 enzymes includes the motif DXE where both Asp and Glu are crucial for activity (47). Asp184 in EndoBl-1 was replaced by site-directed mutagenesis to Asn184 (EndoBl-1 D184N). The mutated enzyme specifically bound to the core of N-glycans, Man3GlcNAc2, across 600 glycans in a mammalian glycan array (Fig. 4A). Interestingly, EndoBl-1 D184N also showed significant binding to the α1–6-fucosylated pentasaccharide, characteristic of human N-linked glycoproteins. When equimolar amounts of RNaseB, bLF, and hLF were coated to microwell plates, EndoBl-1 D184N showed a significant binding to these proteins compared with a nonglycosylated control (Fig. 4B).

EndoBl-1 Has Glycosidase Activity on Human Milk Glycoproteins—Breast milk is a complex fluid, characterized by diverse amounts of N-linked, O-linked, and nonglycosylated proteins. Overnight incubation of a fresh human milk sample with EndoBl-1 or PNGase F produced a shift in the molecular weight of mainly one protein, probably lactoferrin as deduced from its molecular weight (Fig. 5A). No change was observed when a breast milk sample was incubated with EndoBl-1 D184N. In a parallel experiment, the total amount of N-linked glycans, estimated as the amount of α-mannose detected by the lectin concavalin A conjugated to FITC, was determined in digested milk samples. EndoBl-1 and PNGase F, but not EndoBl-1 D184N, significantly decreased the amount of α-mannose in breast milk (Fig. 5B), suggesting an extensive removal of N-linked glycans.

Growth of Bifidobacteria on N-glycosylated Proteins—Vigorous growth of bifidobacteria was observed when 5% of yeast mannoprotein, heavily N-glycosylated cell wall proteins purified from Saccharomyces cerevisiae, were used as the sole carbon source (supplemental Fig. S6). A higher A600 was obtained under these conditions for B. breve SC139 and B. breve KA179, strains that showed high endoglycosidase
Bifidobacterial Endoglycosidases Active on Complex N-glycans

Human milk contains a constellation of factors that shape the microbial content of the infant gastrointestinal tract (49). They include a range of bioactive glycans (either as free HMOs or as conjugates bound to proteins or lipids) that simultaneously enrich beneficial commensals within, and deflect pathogens from, the intestine. The mechanism by which free HMOs enrich infant-borne bifidobacteria has been characterized at the molecular level (50, 51); however, relatively little work has explored the impact of human milk glycoproteins on the developing infant gut microbiota. A prebiotic character has been previously suggested for N-linked human milk lactoferrin (52, 53), as well as O-linked substrates such as bovine glycomacropeptide (54), a peptide derived from human milk.

To examine whether N-linked glycoproteins serve as potential growth substrates, endoglycosidase activity was tested across a large panel of bifidobacterial isolates obtained from infant feces. Only certain isolates of B. infantis (40%), B. longum (21%), and B. breve (36%) exhibited this activity, with the latter species showing the highest glycolytic activity on RNaseB. Genes encoding endo-β-N-acetylglucosaminidases belonging to GH18a, GH18b, or GH85 were found only on RNaseB. Genes encoding endo-β-N-acetylglucosaminidases were significantly induced by hLF and to a lesser extent bLF (Fig. 6B). In general the highest induction was observed after 1 h of incubation. These genes included Blon_2344, Blon_2347, Blon_0883, and Blon_2177, solute-binding proteins that bind different classes of HMO associated with ABC transporters, as well as Blon_2335 and Blon_2336, two key fucosidases in the B. infantis genome (32, 39).

**DISCUSSION**

Impact of hLF and bLF on B. infantis Gene Expression—The molecular response of B. infantis ATCC 15697 to bLF and hLF was tested by incubating the microorganism with 5 mg/ml of bLF or hLF in a resting cell assay. This revealed an increased expression of Blon_2468 (EndoBI-1) by comparison with cells grown on lactose (supplemental Fig. S7). Incubations with bLF or hLF resulted in higher expression of other genes adjacent to Blon_2468 including Blon_2470 and Blon_2471, encoding part of a phosphotransferase system specific for GlcNAc (Fig. 6A). A similar trend was observed for Blon_0177 and Blon_0178, genes also associated with phosphotransferase systems in B. infantis. Other genes induced by these glycoproteins were Blon_0881 and to a lesser extent Blon_0882, key enzymes that participate in metabolism of GlcNAc and sialic acid. Putative genes in B. infantis associated to mannose metabolism (Blon_2380, solute binding protein for manno-oligosaccharides, and Blon_0868 and Blon_0869, α-mannosidases) were not affected by the presence of bLF or hLF. Conversely, several genes associated with the import and consumption of HMO in B. infantis were significantly induced by hLF and to a lesser extent bLF (Fig. 6B). In general the highest induction was observed after 1 h of incubation. These genes included Blon_2344, Blon_2347, Blon_0883, and Blon_2177, solute-binding proteins that bind different classes of HMO associated with ABC transporters, as well as Blon_2335 and Blon_2336, two key fucosidases in the B. infantis genome (32, 39).
cytophaga canimorsus (20). These bacteria can extensively grow on different glycoproteins as a carbon source, and as in the case of EndoS from S. pyogenes, IgG-specific deglycosylation severely impairs its recognition by immune effectors, increasing bacterial survival in blood (25).

It is possible that bifidobacterial endoglycosidases could also modulate the activity of host glycoproteins. An increasing amount of evidence suggests a crucial role for N-glycans in the function of several host proteins (19, 50, 51). For example, recognition of Gram-positive bacteria by IgA is dependent on its glycosylation (17), and intracellular signaling and NF-κB activation of the toll-like receptor 3 (18) is modulated by N-glycans. C-type lectins, galectins, and sialic-acid-binding Ig-like lectins are immune and cell response mediators that specifically recognize different epitopes in N-glycans (22). Although lactoferrin N-linked glycosylation is variable during lactation (12), studies about the impact of glycosylation of this protein with regard to its resistance to proteolysis and iron binding (52–55) are contradictory. If certain Bifidobacterium isolates have the ability to remove N-glycans from lactoferrin, destabilization of the protein could favor to the production of antimicrobial peptides such as lactoferricin B or lactoferrampin. Interestingly, some studies have suggested that lactoferrin has a bifidogenic effect (56, 57). Although in the present work specific enzymes in bifidobacteria have been determined to deglycosylate human lactoferrin, more studies are needed to address more closely the impact of lactoferrin or its derived peptides on these microorganisms.

GH18 and GH85 endoglycosidases specifically cleave the N,N₁-diacyctylchitobiose core of N-linked glycans. Here we studied some of the enzymatic properties of EndoBI-1 and EndoBI-2, representatives of two clades of GH18 sequences found in bifidobacteria (Fig. 1E). Although their amino acid sequences were only 60% identical and their genetic landscapes were different (supplemental Fig. S2), their active sites were conserved, and both acted on bLF and hLF, containing oligomannose and complex N-glycans, respectively. Further description of the N-glycans released from EndoBI-1 by mass spectrometry indicated that it was also active on IgA and IgG. The enzyme did not recognize O-linked glycans or HMO (data not shown). In general the specificity of most known endoglycosidases is limited to high mannose glycans (for example EndoH (23)). EndoS from S. pyogenes acts solely on IgG (25), and the affinities of EndoEα from E. faecalis for proteins other

FIG. 5. EndoBI-1 activity in breast milk. A, SDS-PAGE gel of overnight incubation of human milk (lane 1, control) with EndoBI-1 (lane 2), EndoBI-1 D184N (lane 3), or PNGase F (lane 4). Protein identities were deduced from Ref. 12. B, amount of N-glycosylation (proportional to α-mannose) in samples from A. The error bars represent S.D. from triplicate experiments. The asterisks represent samples with p < 0.05 compared with control.

FIG. 6. Fold changes in gene expression for B. infantis ATCC 15697 genes during time coincubation with bLF or hLF, as indicated in the figure legend. Numbers in the x-axis represent B. infantis ATCC 15697 locus tags (Blon). The error bars represent S.D. from three biological replicates. A, genes associated to GlcNAc metabolism and located close to EndoBI-1. B, genes previously described to be associated or induced by HMO.
than RNaseB have not been further studied (24). Endoglycosidasises F1, F2, and F3 from *Elzabethkingia mircoila* show a preference for either high mannose or complex oligosaccharides (58). By comparison, several features suggested that EndoBI-1 might prove useful as a novel tool for diverse applications in proteomics and glycoproteomics research. First, EndoBI-1 is heat stable because incubation at 95 °C for 5 min did not severely impact its activity. Moreover, the glycolytic activity found on a varied range of target glycoproteins revealed that this enzyme cleaved the chitobiose core of high mannose N-glycans (RNaseB and bLF), and core α1–6-fucosylated or bi- or triantennary complex N-glycans with up to two sialic acid and up to three fucose residues decorating the lactosamine chains (Fig. 3 and supplemental Fig. S4). More precise kinetic studies are required for determining the impact of these modifications on enzyme activity. These analyses were done using native glycoproteins, and a much greater deglycosylation rate after denaturation is expected. Moreover, EndoBI-1 was active directly on human milk—a complex matrix of lipids, oligosaccharides, and proteins with disparate glycosylation types—successfully removing a significant proportion of the total amount of N-glycans (Fig. 5). Finally, EndoBI-1 will be useful in applications such as detection and characterization of glycosylated regions of proteins under non-denaturing conditions and also for improved determination of glycosylation sites of core and noncore fucosylated N-glycans, after chitobiose cleavage and trypsination.

Further evidence for the affinity of EndoBI-1 for N-linked glycans was determined in a parallel set of experiments. An active site mutant of the enzyme (EndoBI-1 D184N) lacked catalytic activity, but it retained the ability to bind to Man$_3$GlcNAc$_2$(α1–6Fuc) in a glycan array. Although this suggests that this mutation does not significantly alter the binding pocket of this enzyme, further structural studies are required to understand the mechanism of action of EndoBI-1 and the wide range of N-linked glycans that it recognizes.

We also partially characterized EndoBB (BLD_0197) from *B. longum* DJO10A, representative of GH85 sequences found in infant gut bifidobacteria. The activity of this enzyme was much more limited, cleaving RNaseB but not bLF or hLF. Bifidobacterial GH85 enzymes are distantly related to EndoD from *S. pneumoniae* (26). EndoD acts on complex core fucosylated N-glycans but only when lactosamine chains have been trimmed by exoglycosidasises (26). It is possible that *B. breve* GH85 endoglycosidasases collaborate with additional glycosyl hydrolases. The presence of α-mannosidasises and an ABC importer for oligosaccharides adjacent to these genes suggests that their function is related. It is also possible that these clusters are also active on mannoose-based oligosaccharides from plant origin.

Strains of *B. infantis* have been studied for their remarkable ability to use HMO as the sole carbon source (59). Genes induced by HMO in *B. infantis*, such as solute binding proteins and α-fucosidasises (32, 39), were also up-regulated by hLF and bLF (Fig. 6). These results suggested that, although not extensively using these glycoproteins as a carbon source (at least under the conditions tested in this study), *B. infantis* was still able to respond to these substrates in a similar fashion as to HMO.

In conclusion, in this work we described the interaction of infant gut-associated bifidobacteria with N-linked glycans found in host glycoproteins such as those found in breast milk, and we determined the discrete molecular determinants associated with this interaction. Finally, we have characterized the enzymatic properties of EndoBI-1 from *B. infantis*, which showed a remarkable activity on a wide range of host N-linked glycans.

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