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Mucin Degradation by *Bifidobacterium* Strains Isolated from the Human Intestinal Microbiota▽†

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The presence of the genes *engBF* (*endo*-α-*N*-acetylgalactosaminidase) and *afcA* (1,2-α-L-fucosidase) was detected in several intestinal *Bifidobacterium* isolates. Two strains of *Bifidobacterium bifidum* contained both genes, and they were able to degrade high-molecular weight porcine mucin in vitro. The expression of both genes was highly induced in the presence of mucin.

The human intestine is covered with a protective mucus layer, which plays an important role in the mucosal barrier system and is crucial for preventing adhesion and binding by many pathogens, toxins, and other damaging agents present in the intestinal lumen (5, 13). The mucus mainly consists of water (ca. 95%) and glycoproteins (1 to 10%), as well as electrolytes, antibodies, and nucleic acids (13). Furthermore, this mucus has been reported to serve as a source of nutrients for bacterial growth (4). Thus, bacteria that are able to survive, multiply, and colonize within the mucus layer display an adaptative advantage to persist in the gastrointestinal tract. Nevertheless, the interactions of the gut microbiota, especially probiotic bacteria, with intestinal mucus are poorly understood.

Bifidobacterium species are common inhabitants of the gastrointestinal tract, and they have received special attention because of their health-promoting effects in humans. The long history of safe use of these bacteria in the functional food industry remains the best proof of their safety, as the risk of infection is assumed to be very low (18). Adherence to mucus is one of the main in vitro tests for the study of probiotic strains (2), and some bifidobacteria have been found to be highly adhesive (8); however, mucin degradation has been considered an undesirable characteristic of probiotics (21), since it is believed that it could favor alteration of the intestinal mucosal barrier. In this context, the aim of this work was to evaluate the presence of genes potentially involved in mucus degradation and the ability of several *Bifidobacterium* strains to grow in the presence of mucus as the only carbon source and to degrade it.

Two bifidobacterial exocellular glycosidases potentially acting on sugar chains of mucin glycoproteins have been described (9). They have been functionally characterized by using synthetic substrates, but their involvement in intestinal mucin degradation has never been studied before. The product of the *engBF* gene of *Bifidobacterium longum* JCM1217 is an *endo*-α-*N*-acetylgalactosaminidase (E.C. 3.2.1.97). This enzyme catalyzes the hydrolysis of the *O*-glycosidic α-linkages between galactosyl β-1-3 *N*-acetylgalactosamine and a serine or threonine residue in mucin-type glycoproteins (6, 9). On the other hand, 1,2-α-L-fucosidases (E.C. 3.2.1.63) release terminal α-linked L-fucose from oligosaccharides of glycoconjugates, including mucin glycoproteins. A member of this last family of enzymes is present in *Bifidobacterium bifidum* JCM1254 (10, 14) and is encoded by the gene *afcA*. Both proteins are about 1,960 amino acids in length and contain a signal peptide and a membrane anchor at the N and C termini, respectively. Thus, they are predicted to be displayed on the bacterial surface, thereby enabling the catalytic domains to gain access to extracellular substrates.

In the present work, the presence of *engBF* and *afcA* was tested in 22 strains of *Bifidobacterium* (Table 1) by using the primers Fuc-F (5′-TTCAACGAGGAGACGCTGTGGACCGG) and Fuc-R (5′-GCCAGTAGTTCATCTGGAGGTTCAC-3′) and the primers Nac-F (5′-CGTCAACTGGCAGGATGGCGCAATC-3′) and Nac-R (5′-CACCTTGAAGTGCTGGATGAACTTAG-3′), designed to amplify internal fragments of 1,072 and 943 bp from the sequences of *afcA* (AY303700) and *engBF* (AY836679), respectively. The annealing temperature for both amplifications was 45°C. The amplified DNA fragments are located in the conserved sequence coding for the catalytic domain of the enzymes. None of the genes were detected in the *Bifidobacterium animalis*, *Bifidobacterium pseudocatenulatum*, and *Bifidobacterium breve* strains tested. This could be due to the lack of similar genes in the genomes of these microorganisms or to a relatively low identity at the DNA level between these three species and the species *B. longum* and *B. bifidum*, from which the *afcA* and *engBF* genes were initially sequenced. The gene *engBF* was present in all of the strains of *B. longum* (14 isolates) and *B. bifidum* (2 isolates), but only the *B. bifidum* strains contained *afcA* and *engBF* (Table 1). All of the PCR products were sequenced, and the predicted protein sequences were subjected to BLASTp analysis (20) and compared with homologous sequences by using as a query the sequences of *B. bifidum* D119 and L22 (Fig. 1). Both AfcA and EngBF internal fragments displayed

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TABLE 1. Microorganisms used in this study and amplification by PCR of the genes *afcA* and *engBF*

Strain	Origin	PCR product[a]	
		afcA	*engBF*
B. animalis IPLA4549[b]	Culture collection	−	−
B. bifidum D119[b]	Human feces	+	+
B. bifidum L22[b]	Human feces	+	+
B. longum C51	Human feces	−	+
B. longum C61[b]	Human intestinal mucosa	−	+
B. longum C72	Human feces	−	+
B. longum D12[b]	Human feces	−	+
B. longum H64	Human feces	−	+
B. longum H92	Human feces	−	+
B. longum L23	Human feces	−	+
B. longum L43	Human feces	−	+
B. longum L44	Human feces	−	+
B. longum L45	Human feces	−	+
B. longum M14	Human intestinal mucosa	−	+
B. longum M25[b]	Human feces	−	+
B. longum M44	Human feces	−	+
B. longum NCIMB8809[b]	Culture collection[c]	−	+
B. pseudocatenulatum E114[b]	Human feces	−	−
B. pseudocatenulatum E514	Human feces	−	−
B. pseudocatenulatum M115	Human intestinal mucosa	−	−
B. pseudocatenulatum M63[b]	Human feces	−	−
B. breve NCIMB8807[b]	Culture collection[c]	−	−

[a] Positive amplification (+) indicates that the sequence of the PCR product is a homolog of that held in the NCBI database (accession numbers AY303700 and AY836679).
[b] Strain selected for mucin-degrading assays.
[c] NCIMB, National Collection of Industrial, Marine, and Food Bacteria.

the highest homology scores with several proteins from the normal gut microbiota (*Ruminococcus* sp., *Clostridium perfringens*, *Bacteroides* sp., and *Enterococcus faecalis*; identities of 37 to 74% for EngBF and of 28 to 39% for AfcA), indicating the ubiquity of these mucin-degrading enzymes among the bacteria populating the intestine.

For growth experiments, a selection of *Bifidobacterium* strains was used, taking into account the species and the presence or absence of glycosidase genes (Table 1). Cells were grown in a defined medium (BM [see the supplemental material]) to evaluate their ability to use mucin as a carbon source. Fresh stabilized fecal samples (1) obtained from a healthy adult donor who had not received antibiotics for the previous 6 months were used as the positive control. Bacterial cultures grown overnight in 10 ml MRSC medium under standard conditions (16), and stabilized fecal samples were washed with the same volume of sterile 50 mM pH 7.0 phosphate buffer and resuspended in 2 ml of sterile Ringer solution (0.25 strength; Oxoid). This suspension was used to inoculate at 2% (vol/vol) 10 ml of BM with or without mucin (3 g/liter mucin from porcine stomach type III [Sigma]), and samples were withdrawn after 24 and 48 h of incubation. Differences in growth were evident among the two strains of *B. bifidum* (D119 and L22), *B. breve* NCIMB8807, and *B. longum* NCIMB8809, all of them displaying the highest growth in the presence of mucin after 48 h (Fig. 2). None of the *B. animalis* and *B. pseudocatenulatum* strains lacking both glycosidase genes or the three *B. longum* human isolates harboring the *engBF* gene reached differences in optical density at 600 nm (OD_{600}) of greater than 0.5. In relation to this, it has previously been shown that *B. bifidum* was the only species among 29 *Bifidobacterium* species tested that was able to ferment porcine gastric mucin (3). Also, an analysis of 18 different intestinal bacterial species showed

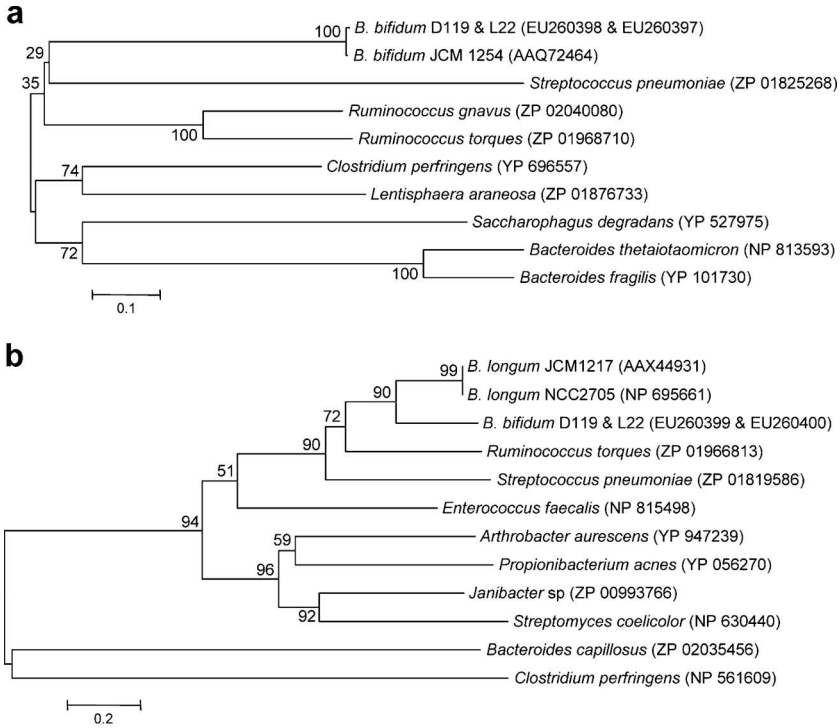

FIG. 1. Phylogenetic relationship analysis of the internal amino acid sequences of AfcA (Y665 to V950) (a) and EngBF (A614 to I903) (b). Database accession numbers are in parentheses. Trees were constructed with the software MEGA4 (www.megasoftware.net), with the matrix of pair distances between sequences by using the neighbor-joining cluster algorithm. The numbers at the branches are bootstrap values (confidence limits). The bar scale refers to the number of amino acid substitutions per site.

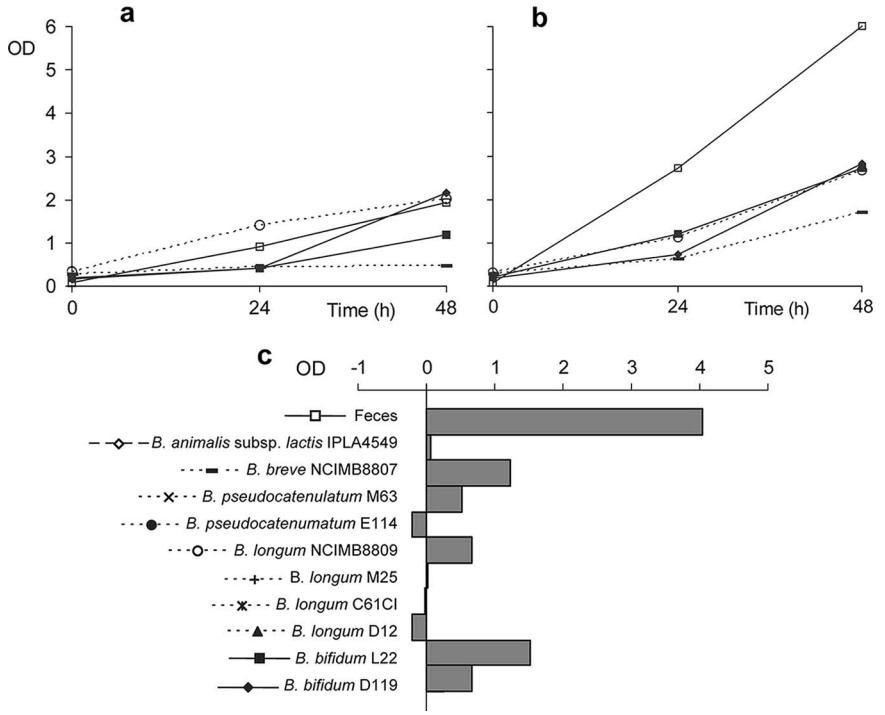

FIG. 2. OD_{600}s of cultures of *Bifidobacterium* strains reaching values higher than 0.5 and a fecal sample growing in BM in absence (a) or presence (b) of mucin. The difference in OD_{600} between the presence and absence of mucin for 10 *Bifidobacterium* strains is depicted for cultures at 48 h (c). Results are the average of three independent cultures, and the coefficients of variation ranged between 1 and 35%.

that only *Ruminococcus torques* and *B. bifidum* were able to partially ferment mucin (19). It is noteworthy that in a recent study using human fecal samples as inocula to colonize porcine gastric mucin, the sequences most commonly recovered from mucin were from *B. bifidum* and *Ruminococcus*, suggesting the competence of these bacteria to colonize this specific substrate (12).

Mucin degradation was analyzed by gel permeation chromatography. Samples obtained after 48 h of incubation from BM containing mucin were centrifuged, and supernatants were collected to check polymer degradation. Samples were isocratically separated at 0.450 ml/min by using 0.1 M $NaNO_3$ in two columns of TSK-Gel $G3000PW_{XL}$ and $G5000PW_{XL}$ (Supelco) placed in series as previously described (17). Several concentrations of dextran standards (Fluka) with different molecular masses (5×10^3, 5×10^4, 8×10^4, 2.7×10^5, 6.7×10^5, 1.4×10^6, and 4.9×10^6 Da) were run for molecular mass calibration and quantification. In BM containing mucin, a high-molecular-mass peak ($\sim 5 \times 10^5$ Da) and several small peaks ($< 1 \times 10^3$ Da) were apparent in the chromatograms (Fig. 3a), whereas after 48 h of incubation with stabilized fecal samples, the high-molecular-mass peak completely disappeared and changes in the other peaks were also detected. Thus, we considered the decrease in the highest-molecular-mass peak to be an indicator of mucin degradation. Accordingly, most of the strains tested were unable to degrade mucin (Fig. 3b). However, the two *B. bifidum* strains were able to degrade more than 80% of the high-molecular-mass mucin glycoproteins after 48 h of incubation. Furthermore, *B. longum* NCIMB8809 and *B. breve* NCIMB8807 displayed an intermediate level of degradation compared with the other *Bifidobacterium* strains, in spite of the fact that none of the genes were present in the *B. breve* strain. Thus, probably other genes, not considered in the present work, or genes with a similar function but with low homology to those used in the present study could also be involved in the degradation of mucin by *B. breve* NCIMB8807.

Considering the aforementioned results, the two *B. bifidum* strains analyzed in this study seem to possess a higher capacity to degrade intestinal mucin. To establish a correlation between this capacity and the likely involvement of *afcA* and *engBF* in mucin degradation, the influence of mucin and/or glucose on the expression levels of the *afcA* and *engBF* genes in strain *B. bifidum* L22 was assessed. The bacterium was grown in BM in the presence of 20 g/liter glucose and/or 3 g/liter mucin to an OD_{600} of 1 ± 0.2 as described above. Specific primers were designed by using the sequences obtained from the genes *afcA* (forward, 5′-ACACCGCCGTCAAGAAAGC-3′; reverse, 5′-CGATCTTCACGCGGTCGTA-3′) and *engBF* (forward, 5′-GCTCCCAGGCGCAGAAC-3′; reverse, 5′-TGTTGAGGGCGACCTTCTTG-3′) of *B. bifidum* L22. Lysis, total RNA extraction, and quantitative PCR analysis were carried out as previously described (7), with an ABI Prism 7500 machine (Applied Biosystems). Both genes were strongly induced in the presence of mucin and mucin plus glucose but not in the presence of glucose alone (Fig. 4). This suggests that these two mucin-degrading glycosidases are transcriptionally regulated in *B. bifidum* by the presence of mucin.

In summary, we have shown for the first time that several intestinal bifidobacteria, especially two *B. bifidum* isolates, are able to degrade intestinal mucin in vitro to different

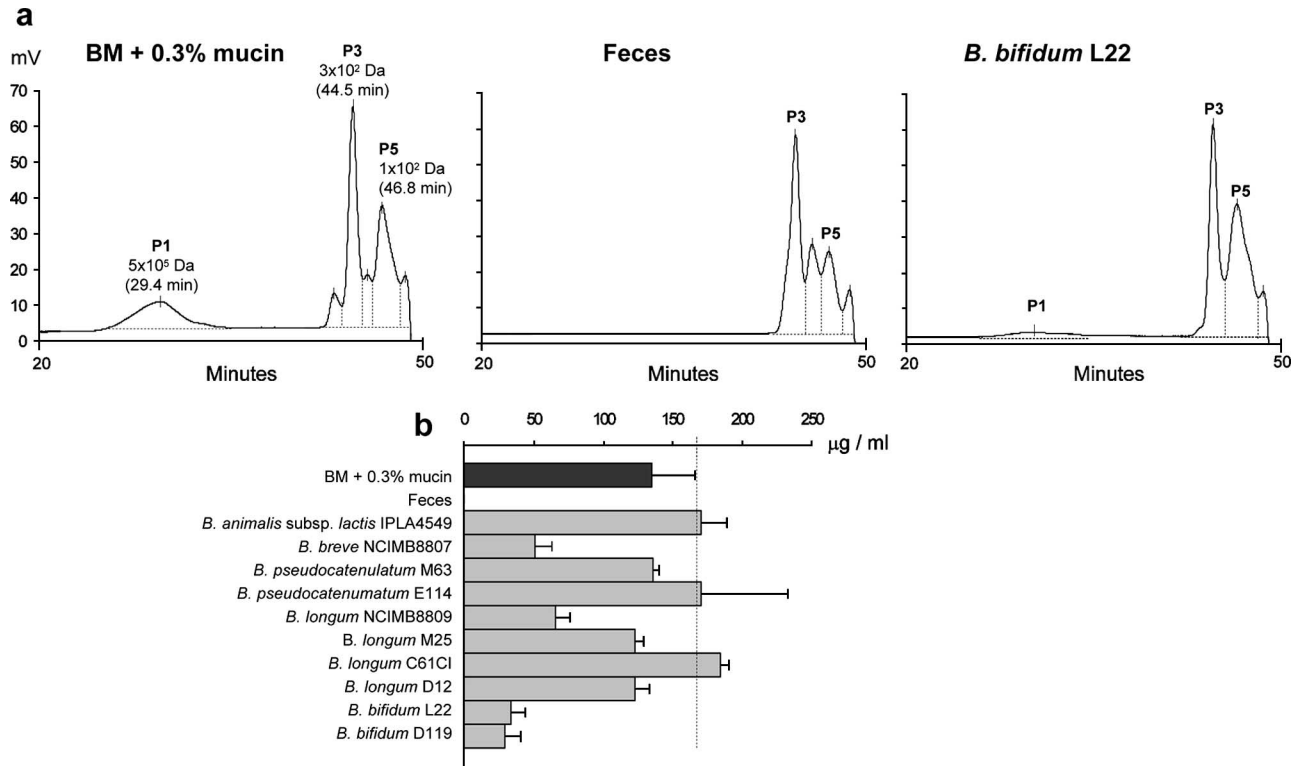

FIG. 3. Degradation of mucin by 10 *Bifidobacterium* strains after 48 h of growth in BM in the presence of mucin. (a) Chromatograms of BM containing 0.3% mucin, the supernatant of the positive mucin-degradation control (feces) after 48 h of incubation, and *B. bifidum* L22 culture supernatant after 48 h of incubation. (b) Amount of the highest ($\sim 5 \times 10^5$ Da) peak. The dotted line indicates the maximum value of mucin initially present in the culture medium. For sample peak quantification (μg/ml), the linear regression equations ($R^2 = 0.994 \pm 0.004$) for each standard were calculated from at least three replicated measurements of each concentration. A linear regression equation ($R^2 = 0.995$) was also calculated to determine the molecular masses (in daltons) of the sample peaks.

extents. This degradation capacity seems to have a correlation with the presence of two genes coding for extracellular glycosidases, *afcA* and *engBF*. Intraspecies differences were also detected in *B. longum*, since only strain NCIMB8809 was able to degrade mucin significantly; however, all *B. longum* strains have *engBF*, suggesting that the presence of this gene is not the only factor affecting the degradation capacity in question. In addition, *B. breve* NCIMB8807 is able to degrade mucin to some extent but it seems not to harbor these genes. The reactions catalyzed by the products of the *afcA* and *engBF* genes could represent the first step in the degradation of mucin sugars by *B. longum* and *B. bifidum*. The galactosyl β-1-3 *N*-acetylgalactosamine released extracellularly from mucin glycoconjugates by EngBF could be transported into the cell by a putative ABC transporter, entering the galacto-*N*-biose metabolic pathway recently described in *B. longum* (11, 15) and finally being metabolized through the glycolytic pathway or by amino sugar metabolism, thus serving as a carbon and energy source for bifidobacteria. Results shown here open an interesting debate on probiotic safety criteria regarding intestinal mucus degradation, which was until now considered a hazardous selection property.

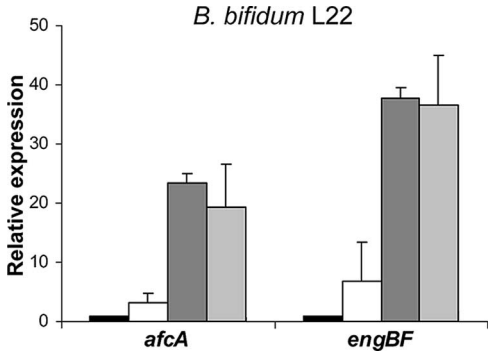

FIG. 4. Levels of *B. bifidum* L22 *afcA* and *engBF* gene expression in four culture media, i.e., BM (black), BM plus 2% glucose (white), BM plus 0.3% mucin (dark gray), and BM plus 2% glucose plus 0.3% mucin (light gray). The expression levels shown are relative to those obtained in the control culture (BM). Experiments were carried out in duplicate and analyzed in duplicate in two independent PCR runs.

Nucleotide sequence accession numbers. The partial nucleotide sequences of *afcA* obtained in this study are available in the GenBank database under accession numbers EU260397 and EU260398 for positive strains appearing in the same order given in Table 1 from top to bottom. The partial nucleotide sequences of *engBF* obtained in this study are available in the GenBank database under accession numbers EU260399 to EU260414 for positive strains appearing in the same order given in Table 1 from top to bottom.

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