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A Single Mutation in the Gene Responsible for the Mucoid Phenotype of *Bifidobacterium animalis* subsp. *lactis* Confers Surface and Functional Characteristics

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Exopolysaccharides (EPS) are extracellular carbohydrate polymers synthesized by a large variety of bacteria. Their physiological functions have been extensively studied, but many of their roles have not yet been elucidated. We have sequenced the genomes of two isogenic strains of *Bifidobacterium animalis* subsp. *lactis* that differ in their EPS-producing phenotype. The original strain displays a nonmucoid appearance, and the mutant derived thereof has acquired a mucoid phenotype. The sequence analysis of their genomes revealed a nonsynonymous mutation in the gene Balat_1410, putatively involved in the elongation of the EPS chain. By comparing a strain from which this gene had been deleted with strains containing the wild-type and mutated genes, we were able to show that each strain displays different cell surface characteristics. The mucoid EPS synthesized by the strain harboring the mutation in Balat_1410 provided higher resistance to gastrointestinal conditions and increased the capability for adhesion to human enterocytes. In addition, the cytokine profiles of human peripheral blood mononuclear cells and *ex vivo* colon tissues suggest that the mucoid strain could have higher anti-inflammatory activity. Our findings provide relevant data on the function of Balat_1410 and reveal that the mucoid phenotype is able to alter some of the most relevant functional properties of the cells.

The genus *Bifidobacterium* includes commensal microorganisms commonly found in the human gut. Some strains, belonging mainly to the species *Bifidobacterium longum*, *Bifidobacterium breve*, *Bifidobacterium bifidum*, and *Bifidobacterium animalis*, have been used as probiotics, since there is scientific evidence that links the administration of these bacteria with specific health benefits (1, 2). In particular, *B. animalis* subsp. *lactis* has a robust phenotype that allows growth at high numbers in commercial applications under nonanaerobic conditions. Furthermore, strains of this subspecies survive the gastric passage and reach the intestinal tract in a metabolically active state, being also the most common representatives of bifidobacteria in functional food products (3). Because they are more resistant to harsh environmental conditions, strains of *B. animalis* subsp. *lactis* have been studied more than those of other members of the genus *Bifidobacterium*, and clinical studies demonstrated their health-promoting attributes in some gastrointestinal disorders and allergic processes (4–6).

Exopolysaccharides (EPS) are extracellular layers of carbohydrates typically found in microorganisms (7). EPS production has been involved in a variety of physiological processes that have implications for the producer microorganism and the host (8, 9). Specifically, in intestinal bacteria some of the most relevant roles attributed to EPS are the favoring of intestinal colonization and survival (10–12) and immunomodulation (11, 13, 14).

A common set of functional modules, including the synthesis of activated sugar precursors, cytoplasmic assembly of the EPS unit, export of the EPS repeating unit, and polymerization/chain length determination of the final EPS skeleton, seem to be necessary for EPS production (15–17). These functions are encoded in specific gene clusters (EPS clusters) that are present in the genomes of the large majority of *Bifidobacterium* species; the genetic content of these clusters is highly variable (18). Remarkably, bacteria can have different surface phenotypes, depending on the presence of mutations in the EPS genes (19). In bifidobacteria, the particular contribution of specific genes has not yet been determined.

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TABLE 1 Strains, plasmids, and oligonucleotide primers used in this study

Strain, plasmid, or oligonucleotide	Description or sequence (5′ to 3′)	Source or reference
Strains		
B. animalis subsp. *lactis* A1dOx	Ox gall-resistant derivative, plasmid free, EPS^+	IPLA collection (20)
B. animalis subsp. *lactis* IPLA-R1	Mutant of the strain A1dOx, plasmid free, EPS^+, mucoid phenotype	IPLA collection (20)
B. animalis subsp. *lactis* $DSM10140^T$	Culture collection, plasmid free, EPS^+	DSMZ collection
E. coli TG1	*supE thi-1* Δ(*lac-proAB*) Δ(*mcrB-hsdSM*)*5* (r_K^- m_K^-) F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	Lucigen
E. coli DH11S	*mcrA* Δ(*mrr-hsdRMS-mcrBC*) Δ(*lac-proAB*) Δ(*rec1398*) *deoR rpsL srl-thi*-F′ *proAB*$^+$ *lacI*^{q}ZΔM15	Invitrogen
B. lactis DSM10140-ΔBalat_1410	DSM10140 lacking the gene Balat_1410	This work
B. lactis DSM10140-ΔBalat_1410-pAM1 (ΔBalat_1410 strain)	DSM10140-ΔBalat_1410 complemented with pAM1	This work
B. lactis DSM10140-ΔBalat_1410-pAM1-Balat_1410 (Balat_1410 strain)	DSM10140-ΔBalat_1410 complemented with pAM1 + Balat_1410	This work
B. lactis DSM10140-ΔBalat_1410-pAM1-Balat_1410^{S89L} (Balat_1410^{S89L} strain)	DSM10140-ΔBalat_1410 complemented with pAM1 + Balat_1410^{S89L}	This work
Plasmids		
pAM1	*E. coli-Bifidobacterium* shuttle cloning vector; Amp^r Em^r[a]	(23)
pAM1-Balat_1410	Gene Balat_1410 from DSM10140 in pAM1, Amp^r Em^r	This work
pAM1-Balat_1410^{S89L}	Gene Balat_1410^{S89L} from IPLA-R1 in pAM1, Amp^r Em^r	This work
pJL74	*E. coli-Bifidobacterium* shuttle cloning vector; Amp^r Sp^r; integrative, nonreplicative in *Bifidobacterium*	(35)
pJL-dst-Balat_1410	Downstream (2.7 kb) region of the gene Balat_1410 in pJL74; Amp^r Sp^r	This work
pJL-dst/upst-Balat_1410	Upstream (3 kb) and downstream (2.7 kb) region of the gene Balat_1410 in pJL74; Amp^r Sp^r	This work
Oligonucleotides		
Balat_1410-UPST-F	tatataGGGCCCGTCACCTCGTCACCATGAGC[b]	This work
Balat_1410-UPST-R	tatataAAGCTTTAACATAGCACCGCGTGCC	This work
Balat_1410-DST-F	tatataGAATTCGGCACCGCTCAAGTCAGAAC	This work
Balat_1410-DST-R	tatataGTCGACCCACGAGAGCACACGAAGAC	This work
Integration1-F	CGTATGAGCGGAATCTGCAG	This work
Integration1-R	CACGGTACCATTTCTTGCTG	This work
Integration2-F	GCAGTGGCTGAATCTTCTCC	This work
Integration2-R	CTCGCACCTGATCTGCCTTA	This work
Spec-F	GGAGAAGATTCAGCCACTGC	This work
Spec-R	TTAGTCGTCGTATCTGAACC	This work
Balat_1410KO-F	GCTGGTCTCTTGCTGAATCT	This work
Balat_1410KO-R	CTCCTCACAGAGAGTGTTCT	This work
Balat_1410+pr-F	GCCGACTCTAGAACATGACAGCACCTCGCTTCGTTG	This work
Balat_1410+pr-R	GCCGACTCTAGATCATTACGATTTACGATTTTTTCTATTTTTTTCAGC	This work

[a] Amp^r, Em^r, and Sp^r, resistance to ampicillin, erythromycin, and spectinomycin, respectively.
[b] Restriction enzyme sites are underlined.

In previous studies, we have shown that *B. animalis* subsp. *lactis* strain IPLA-R1 can produce an EPS that generates a mucoid (or ropy) phenotype. IPLA-R1 was spontaneously obtained, after several consecutive cultures, from the strain A1dOx, which was derived from the yogurt isolate A1 after adaptation to increasing concentrations of ox gall (20). In the present work, we aimed to further study our models of EPS-producing bifidobacteria (and their polymers), and we wanted to address whether the mucoid phenotype could be connected with a specific genetic background. For that purpose, we have sequenced the genomes of mucoid and nonmucoid strains, and we investigated more deeply the genetic basis and the functional characteristics of this phenotype.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. The bifidobacterial strains were routinely cultivated in MRSC broth (MRS Difco [BD Biosciences, San Diego, CA] containing 0.05% L-cysteine-HCl [Sigma Chemical Co., St. Louis, MO]) at 37°C under anaerobic conditions (80% N_2, 10% CO_2, 10% H_2) in an MG500 chamber (Don Whitley Scientific, West Yorkshire, United Kingdom). *Escherichia coli* strains were grown in Luria-Bertani (LB) broth at 37°C under stirring conditions (200 rpm).

Frozen stocks (stored with 20% glycerol at −80°C) were plated on the surface of agar-MRSC or agar-LB plates, and a single colony per strain was used to inoculate 10 ml broth. After overnight incubation, this culture was used to inoculate (1% vol/vol) fresh broth. Spectinomycin (100 μg ml^{-1}), ampicillin (100 μg ml^{-1}), and erythromycin (2.5 μg ml^{-1}) were added when necessary.

Chromosomal and plasmid DNA isolation and analyses. Chromosomal DNA from bifidobacteria was isolated using a GenElute bacterial genomic DNA kit (Sigma-Aldrich, Dorset, United Kingdom), according to the manufacturer's recommendations. The first lysis step was modified with the addition of lysozyme (10 mg ml^{-1}) (Merck, Darmstadt, Germany) and mutanolysin (5 U) (Sigma-Aldrich) and additional incubation at 37°C for 1 h. Plasmid DNA was isolated using a commercial GenElute

plasmid miniprep kit (Sigma-Aldrich) and a Qiagen plasmid midi kit (Qiagen, Hilden, Germany), according to the manufacturer's recommendations. For Gram-positive strains, the first lysis step was modified as indicated above.

Chromosomal DNA and plasmids were checked by electrophoresis in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA [pH 8]) on 0.8 to 1% agarose gels and then visualized with ethidium bromide staining (0.5 μg ml^{-1}). The DNA concentration was measured in a Gen5 Take3 module (BioTek, VT, USA).

DNA manipulations and molecular techniques. The PCR products were purified using a QIAquick gel extraction kit (Qiagen). Purified plasmids and amplicons were sequenced at Macrogen, Inc. (Seoul, South Korea). The Platinum *Taq* DNA polymerase high fidelity was from Invitrogen (Life Technologies, Guilford, CT). The restriction endonucleases were supplied by Roche Diagnostics (Barcelona, Spain), and T4 DNA ligase was obtained from Sigma-Aldrich. All reagents were used according to the manufacturer's instructions.

Genome sequencing and comparison of genomes. Total DNA of the two *Bifidobacterium animalis* subsp. *lactis* strains (A1dOx and IPLA-R1) was extracted using a GenElute bacterial genomic DNA kit (Sigma-Aldrich), following the instructions provided by the manufacturer with a modification of the lysis step, as indicated in "Chromosomal and plasmid DNA isolation and analyses." Sequencing was performed using an Illumina HiSeq 2000 sequencer at Macrogen, Inc. Totals of 3,465,257,480 (A1dOx) and 3,476,287,488 (IPLA-R1) bases were sequenced, generated by 34,309,480 (A1dOx) and 34,418,688 (IPLA-R1) paired-end reads of 101-bp average length; this resulted in a theoretical genome sequence coverage of approximately 17-fold. More than 97% of the paired-end reads were successfully mapped to the *B. animalis* subsp. *lactis* strain DSM10140 complete genome sequence (RefSeq accession no. CP001606) using Maq software (21) with the maq.pl script and the following parameters: maq.pl easyrun -d query-genome-paired -D 2500 -E 0 -p DSM10140.fa paired-end-file_1.fastq paired-end-file_2.fastq. Single nucleotide polymorphisms (SNPs) were then identified and filtered using the Maq option SNPfilter -q 40 -w 5 -N 2 -d 3 -D 256 -n 20 -Q 40, as recommended for bacterial genomes (21).

Furthermore, the draft genomes of the two strains were *de novo* assembled using Velvet sequence assembler 1.2.05 software (22), followed by contig N_{50} value optimization with VelvetOptimiser 2.2.0 software (Victorian Bioinformatics Consortium, Monash University). These programs were run at the Cluster de Modelización Científica, at Oviedo University (http://cms.uniovi.es). The final assembly results were sets of 315 (A1dOx) and 400 (IPLA-R1) unoriented contigs, with total lengths of 1,905,359 bp (A1dOx) and 1,886,812 bp (IPLA-R1).

Knockout mutant generation. The plasmid pJL74 (Table 1) was used as the starting material for the construction of the plasmids to achieve the knockout (KO) in *B. animalis* subsp. *lactis* DSM10140, with deletion of the gene Balat_1410. The plasmid was constructed by cloning in two steps the flanking regions of the gene Balat_1410 in the plasmid pJL74. The downstream region (2.7 kb) and upstream region (3 kb) were amplified by PCRs using the specific primers Balat_1410-DST-F/R and Balat_1410-UPST-F/R, respectively, and the chromosomal DNA of the strain DSM10140 was used as a template. The 2.7-kb PCR product of the downstream region was purified, digested with EcoRI and SalI, and purified before cloning into pJL74, previously digested with the same enzymes and purified from agarose gel. Ligation was performed overnight at 16°C. The ligation mixture was purified and transformed into *E. coli* Tg1 competent cells by heat shock (42°C, 2 min) to obtain the intermediate construction pJL-dst-Balat_1410 that was verified by the use of restriction enzymes. Similarly, the upstream region (3 kb) was inserted into the ApaI and HindIII sites of pJL-dst-Balat_1410 and transformed into *E. coli* Tg1 competent cells to obtain the final plasmid construction pJL-dst/upst-Balat_1410.

Then, pJL-dst/upst-Balat_1410 was electrotransformed in *B. animalis* subsp. *lactis* DSM10140 by electroporation. The colonies grown after 72 h in MRSC with spectinomycin (100 μg ml^{-1}) were checked for plasmid integration (single crossover) in the chromosome by PCRs using the specific primers Integration1-F/R, Spec-F/R, and Integration2-F/R. The bacterial cells with the integrated plasmid were grown in 10 ml MRSC broth without antibiotics, and two subcultures per day were grown during 5 days. Then, bacterial suspensions were plated into agar-MRSC plates without antibiotics for 48 h. Afterwards, each colony was grown on agar-MRSC plates with and without spectinomycin to select the non-antibiotic-resistant colonies due to the looseness of the plasmid. These colonies were checked by PCRs using the specific primers Balat_1410KO-F/R to analyze the deletion of the Balat_1410 gene in the loop out of the plasmid. The strain lacking the gene Balat_1410 was named DSM10140-ΔBalat_1410.

Complementation: plasmid construction and transformation. The pAM1 plasmid was used as the starting material to achieve the plasmids required for the complementation of the gene Balat_1410 in *B. animalis* subsp. *lactis* strain DSM10140-ΔBalat_1410. The gene Balat_1410 and its promoter were amplified by PCRs using the specific primers Balat_1410+pr-F/R and the chromosomal DNA of the strains DSM10140 and IPLA-R1 as a template. The only difference between the two amplicons was the nonsynonymous mutation in the gene Balat_1410 at position 266. The 2.5-kb PCR products were purified, digested with XbaI, and purified before being cloned into pAM1, previously digested with the same enzyme, and purified. Ligation was performed overnight at 16°C. Then, the ligation mixture was purified and electrotransformed into *E. coli* strain DH11S electrocompetent cells to obtain the final constructions pAM-Balat_1410 (Balat_1410 from DSM10140) and pAM-Balat_1410^{S89L} (mutated Balat_1410 from IPLA-R1). The two inserts within the two plasmids were sequenced to ensure that only the right DNA fragments had been introduced.

Both plasmids extracted from *E. coli* were transferred into *B. animalis* subsp. *lactis* DSM10140-ΔBalat_1410. The electroporation of the bifidobacteria was done with electrocompetent cells by electrotransformation using a Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, CA), according to the manufacturer's instructions. Electrocompetent *B. animalis* subsp. *lactis* cells were prepared by optimizing previously reported methods (23). In short, fresh MRSC broth (200 ml) supplemented with 0.5 M sucrose was inoculated (1% vol/vol) with an overnight culture and incubated at 37°C for 6 to 7 h until the optical density at 600 nm (OD_{600}) was 0.3 to 0.5. Then, cells were chilled on ice for 10 min, washed twice with ice-cold sucrose-citrate buffer (0.5 M sucrose, 1 mM citrate buffer [pH 6]), and resuspended in 1 ml of the same buffer. Cells were chilled on ice for 30 min. Electrotransformation was performed at 25 μF, 200 Ω, and 2 kV. Cells were immediately diluted in 1 ml MRSC broth and incubated at 37°C under anaerobic conditions for 4 h and subsequently plated on agar-MRSC plates with erythromycin (2.5 μg ml^{-1}). After 48 to 72 h, colonies were inoculated in 10 ml MRSC broth with erythromycin, incubated for 24 to 48 h at 37°C under anaerobic conditions, and stored at −80°C.

The stabilities of the original plasmid pAM1 and derivatives (pAM-Balat_1410 and pAM-Balat_1410^{S89L}) were assayed by growing the *B. animalis* subsp. *lactis* transformed strains in MRSC broth without antibiotics for 50 generations. Then, the antibiotic resistance levels of the bacterial suspensions were monitored by plating them on agar-MRSC plates with and without antibiotics (2.5 μg ml^{-1} erythromycin) and incubation for 48 h. Finally, plasmids were monitored by plasmid extraction from antibiotic-resistant colonies as described before.

Purification and analysis of EPS by size exclusion chromatography-multiangle laser light scattering. The biomass of each bifidobacterial strain, collected from the surface of at least 100 agar-MRSC plates grown under standard conditions, was used to purify the initial crude EPS by means of ethanol precipitation and dialysis (24). A further purification step was carried out using sequential DNase and pronase treatments, followed by trichloroacetic acid (TCA) precipitation, dialysis (molecular mass cutoff of 12 to 14 kDa), and freeze-drying (25).

Purified EPS were analyzed by size exclusion chromatography (SEC)

coupled with multiangle laser light scattering (MALLS) detection (25). Briefly, EPS (5 mg ml^{-1}) were dissolved in 0.1 M $NaNO_3$ (mobile phase) and 50 μl (volume injection) was separated at 40°C with a flow rate of 0.45 ml min^{-1} in two TSKgel columns ($G3000PW_{XL}$ plus $G5000PW_{XL}$), protected with a TSKgel guard column (Sigma-Aldrich). The chromatograph (Waters, Milford, MA) had an Alliance 2690 module injector, a photodiode array (PDA) 996 detector (checked at 280 nm for protein detection), a 410 refraction index (RI) detector, and Empower software. Each EPS fraction was quantified (in micrograms) with the RI detector using the calibration equations determined with dextran standards (Sigma-Aldrich) of different sizes (26). In addition, a third static MALLS detector (Dawn Heleos II; Wyatt Europe GmbH, Dembach, Gemany) was coupled in series to analyze the weight-averaged molar mass (M_w) distribution of the EPS fractions using the software Astra 3.5 (Wyatt Europe GmbH).

Adhesion to HT29 cells. The adhesion ability of *B. animalis* subsp. *lactis* DSM10140 and derivative strains was studied with the epithelial intestinal cell line HT29, derived from human colon adenocarcinoma. The cell line maintenance, culture conditions, and reagents were described elsewhere (27). For the adhesion experiments, 10^5 cells ml^{-1} were seeded in 48-well plates and incubated to confluence (monolayer of approximately 10^7 cells ml^{-1}), at about 13 days. Bacterial suspensions from 18-h cultures were harvested by centrifugation, washed with phosphate-buffered saline (PBS), and resuspended in McCoy's medium (10^8 cell ml^{-1}) without antibiotics. Then, 250 μl of each bacterial suspension was added (2.5×10^7 bacteria), in triplicate, to the HT29 monolayer wells (bacteria/HT29 cell ratio of 10:1), and three independent plates were assayed. The plates were incubated for 1 h at 37°C in 5% CO_2 in a Heracell 240 incubator (Thermo Electron LDD GmbH, Langenselbold, Germany). Then, 250 μl of each well was eliminated (containing the nonadhered bacteria). The HT29 monolayer was broken down with the addition of 150 μl (μg ml^{-1}) of trypsin (Sigma-Aldrich) and 15 min of incubation at 37°C in a 5% CO_2 incubator. Finally, 150 μl of McCoy's medium was added to recovered HT29 cells, and the bacteria adhered. The number of bacteria initially added (bacterial suspensions) and adhered to the HT29 cells was determined by counts on agar-MRSC plates. The percentage of adhesion of each strain was calculated as follows: CFU bacteria adhered · 100/CFU bacteria added.

PBMC stimulation and cytokine analyses. The immunomodulation capability of *B. animalis* subsp. *lactis* DSM10140 and derivative strains was determined using an *in vitro* model based on the cocultivation of human peripheral blood mononuclear cells (PBMCs) with the UV-irradiated strains in 48-well plates. The PBMCs were obtained as described by López et al. (27). The UV-irradiated bacterial suspensions were prepared as described by López et al. (28). The UV-irradiated bacterial suspensions in PBS were centrifuged and resuspended in RPMI 1640 (Sigma-Aldrich) (10^7 cells ml^{-1}), and 250 μl was added to each well (2.5×10^6 bacteria added) (bacteria/PBMC ratio of 5:1). Concanavalin A (Sigma-Aldrich) was used as a positive control (final concentration of 10 μg ml^{-1}) for PBMC stimulation. Each factor was tested in duplicate per plate, and 8 independent plates (1 per donor) were used. Plates were incubated for 4 days at 37°C in a 5% CO_2 incubator. Then, 500 μl of each well was collected to analyze the cytokine profile as described previously by López et al. (27). Transforming growth factor-beta (TGF-β) levels were determined using a human TGF-β1 Quantikine enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Abingdon, United Kingdom), following the manufacturer's instructions.

Chemical simulation of the gastrointestinal transit. The survival capability of *B. animalis* subsp. *lactis* DSM10140 and derivative strains in a chemically simulated gastrointestinal transit was analyzed using an *in vitro* model modified from Sánchez et al. (29). In short, overnight cultures were centrifuged, washed with PBS, and resuspended in 0.85% NaCl (10^9 cells ml^{-1}). These bacterial suspensions were used to inoculate 1% (vol/vol) of a simulated gastric juice (GJ) (125 mM NaCl, 7 mM KCl, 45 mM $NaHCO_3$, 3 g $liter^{-1}$ pepsin [Sigma-Aldrich], adjusted with HCl to pH 3) and incubated at 37°C in aerobic conditions for 1 h. Then, bacterial suspensions were centrifuged, resuspended in simulated duodenal juice (DJ) (1% ox gall bile salts [Sigma-Aldrich], 0.1% pancreatin [Sigma-Aldrich], adjusted with NaOH to pH 8), and incubated at 37°C in anaerobic conditions for 2 h. Bacterial survival was quantified by counts on agar-MRSC plates after 1 h of incubation in GJ and after 1 and 2 h of incubation in DJ. The percentage of survival of each strain was calculated as follows: log CFU bacteria recovered · 100/log CFU bacteria inoculated.

Transmission electron microscopy (TEM). Cells from a fresh overnight culture were recovered and encapsulated in gelatin and then were fixed in a PBS solution containing 2% glutaraldehyde and 4% paraformaldehyde during 3 h at room temperature. Subsequently, cells were treated with 1% osmium tetroxide containing 0.8% potassium ferricyanide for 60 min at 4°C. Samples were dehydrated through a graded acetone series, embedded in Epon 812 resin, and polymerized for 48 h at 60°C. Ultrathin sections were obtained on a Leica EM UC6 ultramicrotome and mounted on Formvar carbon-coated grids. Sections were stained with uranyl acetate and lead citrate and examined in a JEOL 1011 transmission electron microscope at 100 kV with an ES1000W Erlangshen charge-coupled device (CCD) camera.

Cryo-scanning electron microscopy (cryo-SEM). Strains were grown on the surface of agar-MRSC plates as described previously. An isolated colony attached to the specimen holder of a CT-1000C cryo-transfer system (Oxford Instruments, Oxford, United Kingdom) interfaced with a JEOL JSM-5410 scanning electron microscope was picked up and frozen in N_2 slush at −210°C. The sample was then fractured and transferred from the cryostage to the microscope sample stage, where the condensed surface water was sublimated by controlled warming to −90°C. Afterwards, the sample was transferred again to the cryostage in order to coat it with gold by sputtering. Finally, the sample was transferred to the microscope sample stage to be viewed at an accelerating voltage of 15 keV and at ×5,000 magnification.

Human colonic mucosa organ culture *ex vivo*. Macroscopically normal colonic tissue from a distal region of resected intestine was obtained from 5 patients after adenocarcinoma surgery. The organ culture procedure was modified from Carol et al. (30). In short, colonic tissue was washed twice with sterile phosphate buffer. Then, mucosal samples between 25 and 35 mg were separated from the colonic tissue and placed, with the epithelial surface uppermost, on culture filter plates (15-mm-diameter wells with 500-μm bottom mesh, Netwell culture systems; Costar, Cambridge, MA) suspended over wells containing 1.5 ml of RPMI 1640 (Lonza BioWhittaker, Basel, Switzerland) supplemented with glutamine (2 mM) and bicarbonate (100 mM) and previously incubated in an atmosphere of 5% CO_2 for 30 min. Mucosal tissues were stimulated by the addition of phorbol 12-myristate 13-acetate (PMA) (10 ng ml^{-1}) (Sigma-Aldrich) and ionomycin (100 ng ml^{-1}) (Sigma-Aldrich) to the RPMI 1640. Then, 100 μl of bifidobacterial suspensions (10^{10} CFU ml^{-1}) in RPMI 1640 was added on top of the tissue and cocultivated for 6 h at 37°C in an atmosphere of 5% CO_2. Tissue controls without bacteria and without bacteria and stimulation were tested in each experiment. Three technical replicates for each experimental condition were used per donor.

After 6 h of incubation, tissues were collected in 400 μl of RNA*later* (Life Technologies) for RNA extraction, in 4% paraformaldehyde (Sigma-Aldrich) for immunohistochemistry assay, and in 1 ml phosphate buffer for lactate dehydrogenase (LDH) measurement. Supernatants were collected and stored at −80°C until cytokine analyses. Levels of the cytokines interleukin 10 (IL-10) and tumor necrosis factor-alpha (TNF-α) were quantified using ELISA kits (human IL-10 DuoSet ELISA kit [R&D Systems] and human TNF-α ELISA Ready-SET-Go [eBioscience, Hatfield, United Kingdom], respectively). Bifidobacterial counts from tissue and supernatants were obtained. Some of the mucosal tissue samples were directly frozen at −80°C without incubation as reference samples.

Tissue viability was assessed by measuring the LDH in the supernatant and tissue after 6 h of incubation, according to Llopis et al. (31). Briefly, tissue samples were homogenized in Tris-HCl (100 mmol l^{-1}, pH 7.4), and LDH activity was analyzed by the spectrophotometric method (pyru-

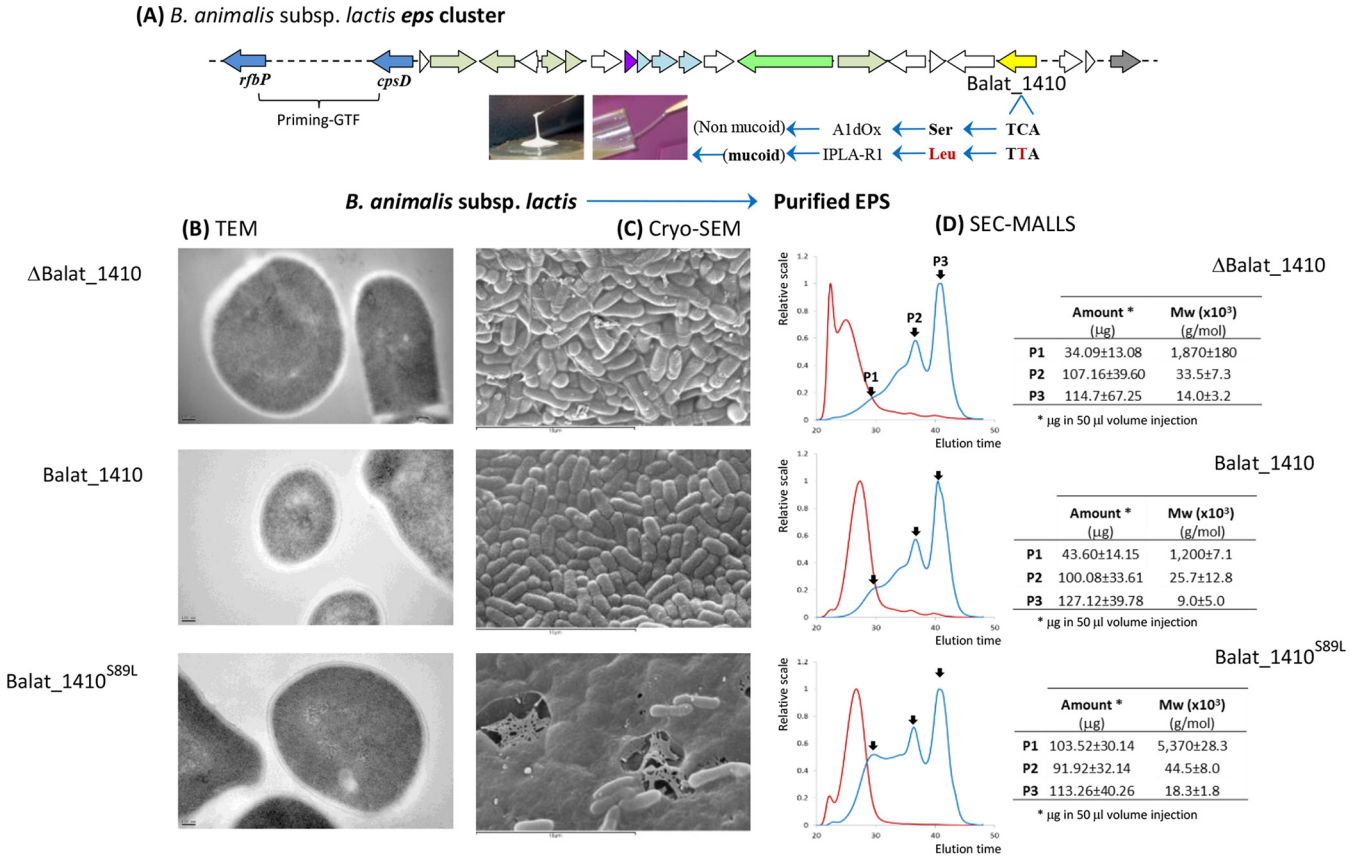

FIG 1 (A) Physical map of the *eps* cluster sequenced for the strains *Bifidobacterium animalis* subsp. *lactis* A1dOx and IPLA-R1 (32) showing a single nucleotide change in Balat_1410. (B and C) TEM (B) and cryo-SEM (C) analyses of the isogenic strains ΔBalat_1410 (top), Balat_1410 (middle), and Balat_1410^{S89L} (bottom). Bars, 100 nm (B) and 10 μm (C). (D) SEC-MALLS analysis of EPS purified from the three isogenic strains. The blue line represents the refraction index detection, and the red line represents the MALLS detection (angle of 90°). GTF, glucosyltransferase; P, peak; Mw (weight-averaged) molar mass.

vate-lactate) recommended by the Scandinavian Society of Enzymes. The percentage of tissue viability was calculated as follows: supernatant LDH (units liter^{-1})/tissue (units liter^{-1}) LDH · 100.

Nucleotide accession numbers. This whole-genome shotgun project has been deposited in the RAST server (http://rast.nmpdr.org/) under accession numbers 302911.15 (A1dOx strain) and 302911.16 (IPLA-R1 strain).

RESULTS AND DISCUSSION

A single nucleotide mutation in Balat_1410 is present in the mucoid EPS-producing *B. animalis* subsp. *lactis* strain IPLA-R1. *B. animalis* subsp. *lactis* IPLA-R1 displayed a mucoid phenotype compared with that of the strain A1dOx. The strain IPLA-R1 is a derivative of A1dOx, which, after consecutive generations, spontaneously acquired a mucoid phenotype (20). The sequencing of the EPS clusters (32) (Fig. 1A) in the wild-type and the mutant strains allowed the identification of a C to T transition in the gene Balat_1410 at position 266, which causes a codon change. As a consequence, a serine is substituted by a leucine in position 89 (S89L) of the protein. We sequenced the genomes of the two strains in search of other mutations with a potential role in the observed change of the phenotype. With the genome of the type strain as a template (total length of the *B. animalis* subsp. *lactis* DSM10140^{T} genome is 1,938,483 bp; RefSeq accession no. CP001606), the two draft genomes were compared, and the number of residues that matched in equivalent positions was 1,886,811 bp, corresponding to a theoretical alignment of 97.33% of the total genomes. No other mutations were found, suggesting that the mutated Balat_1410 gene could be involved in the acquisition of the mucoid phenotype in IPLA-R1. These results clearly point to a role of the gene Balat_1410 in shaping the macromolecular structure of the EPS and, as a consequence, in the surface characteristics of the cell. Mutations in the genes involved in the capsular polysaccharide of *Streptococcus pneumoniae* have previously been related to different colony morphology, i.e., rough, small, and mucoid (19). Remarkably, it has recently been shown that mutations in *epsC*, predicted to encode a tyrosine-protein kinase of an EPS gene cluster and involved in the regulation of capsular EPS biosynthesis in *Lactobacillus johnsonii*, cause increased levels of EPS and the appearance of a smooth colony variant (33). In relation to this, the Balat_1410 product is a hypothetical membrane-anchored protein with a predicted large soluble domain, theoretically involved in the chain length determination of the polymer (34) and harboring, in the mucoid strain, a serine to leucine substitution in the putative soluble part of the protein. The protein is homologous to Etk-like tyrosine kinases, suggesting that phosphorylation might regulate EPS synthesis under different environmental conditions in *B. animalis* subsp. *lactis*.

Generation of recombinant *B. animalis* subsp. *lactis* strains with different Balat_1410 backgrounds. In order to establish the genetic bases for the acquisition of a mucoid phenotype in *B.*

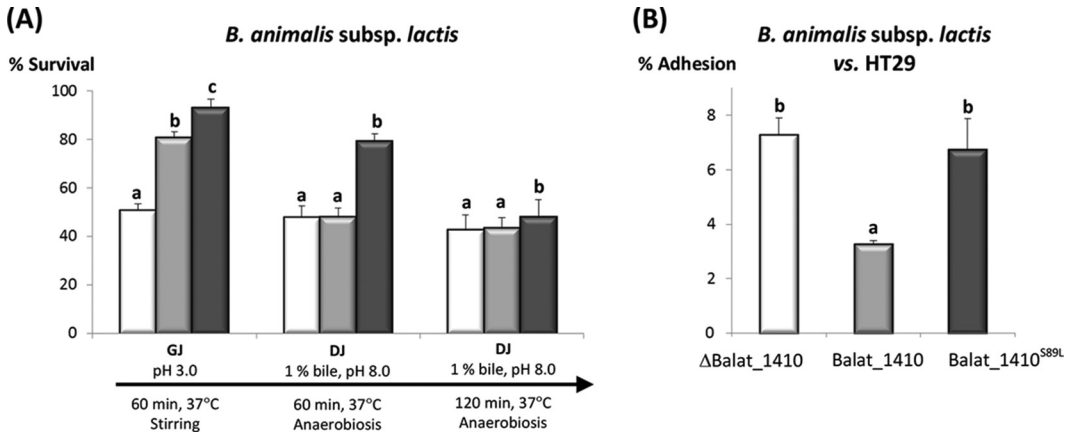

FIG 2 (A) Survival of the isogenic strains ΔBalat_1410 (white bar), Balat_1410 (light-gray bar), and Balat_1410^{S89L} (dark-gray bar) under sequential *in vitro*-simulated gastrointestinal transit. (B) Adhesion percentage of the three isogenic strains to the human colonocyte-like cell line HT29 (ratio 10:1, bacteria to HT29). Statistical analysis: one-way analysis of variance (ANOVA) and least significant difference mean comparison test; under each condition, strains that do not share a letter are significantly different ($P < 0.05$). GJ, gastric juice; DJ, duodenal juice.

animalis subsp. *lactis*, we constructed several isogenic recombinant strains. First, we removed Balat_1410 from the genome of the type strain *B. animalis* subsp. *lactis* DSM10140 (*B. lactis* DSM10140), using a knockout mutation system based on the integrative plasmid pJL74. This circular plasmid integrates into the *Bifidobacterium* genome by homologous recombination through a crossover event. Then, the integrated plasmid is lost through a second homologous recombination event, with the concomitant loss of the DNA sequence located between the two homologous fragments (35, 36). In our study, this resulted in *B. lactis* DSM10140-ΔBalat_1410 (lacking the gene Balat_1410). Subsequently, the *B. lactis* strain DSM10140-ΔBalat_1410 was complemented with the plasmid pAM1 (37), yielding the *B. lactis* DSM10140-ΔBalat_1410-pAM1 (ΔBalat_1410 strain), and with the plasmid pAM1, containing the gene Balat_1410 with its upstream region (pAM1-Balat_1410), yielding *B. lactis* DSM10140-ΔBalat_1410-pAM1-Balat_1410 (Balat_1410 strain). A similar strategy was performed using the mutated gene Balat_1410^{S89L}, yielding *B. lactis* DSM10140-ΔBalat_1410-pAM1-Balat_1410^{S89L} (Balat_1410^{S89L} strain) (for details, see Table 1; see also Fig. S1 in the supplemental material). Although a few works have reported gene deletion systems in *Bifidobacterium longum* (38, 39), to our knowledge, this work is the first report in scientific literature of a gene deletion system in *B. animalis* subsp. *lactis*, and it paves the way for new opportunities to study the functional characteristics of this commercially relevant bacteria.

***B. animalis* subsp. *lactis* Balat_1410^{S89L} synthesizes a high-molecular-weight polymer and displays a different cell surface.** Transmission electron microscopy (TEM) showed that the strains containing Balat_1410, either the native or the mutated gene, produce an outer cell surface layer which is absent in the ΔBalat_1410 strain. On the other hand, cryo-scanning electron microscopy (SEM) clearly differentiated the mucoid strain (Balat_1410^{S89L}) from the other two strains, by revealing a thick and continuous veil covering the cell colony that was absent in the ΔBalat_1410 and Balat_1410 strains (Fig. 1B and C). Although the two microscopic techniques were not able to independently differentiate the three strains, their combination clearly revealed differences in the surface appearance of the three strains, most likely due to different configurations of production and attachment of the EPS. To corroborate this assumption, we performed surface EPS isolation from the three strains and the isolated fractions were analyzed by size exclusion chromatography coupled with a multiangle laser light scattering detector (SEC-MALLS). Three different peaks were consistently present in the chromatograms obtained for the three strains. The relative abundance of each peak was different, showing that the peak with the highest molecular weight (P1 in Fig. 1D) was the minority fraction in ΔBalat_1410 and Balat_1410 (accounting for <16% of the total amount of EPS), but its presence was increased up to one-third of the total EPS amount in the strain Balat_1410^{S89L}. A high-molecular-weight (HMW) polymer, rich in rhamnose, was also very abundant in the mucoid strain, *B. animalis* subsp. *lactis* IPLA-R1, which contains the Balat_1410^{S89L} gene (32). This finding is consistent with a modified polymer mass in the strain containing the mutated gene and suggests that Balat_1410 is involved in determining the chain length of the EPS in *B. animalis* subsp. *lactis*.

Acid and bile resistance, as well as adhesion to intestinal cells, is modified in the strain with the mucoid phenotype. The strain Balat_1410^{S89L} was able to survive the acid conditions better than the Balat_1410 strain, and both improved their viability compared with that of the strain lacking Balat_1410. A subsequent exposure to duodenal juice containing 1% bile extract showed that the strain containing the mutated gene behaved the best, as far as viability was concerned, independently of the time of exposure (Fig. 2A). These results suggest that the presence of the HMW EPS fraction (associated with the presence of mucoid EPS) on the cell surface could protect against gastrointestinal stress. In addition, the potential to persist in the human gut, as affected by the different surface properties, was approached by analyzing the adhesion capacity with human intestinal cells. For this purpose, we have used HT29 monolayers, which have the capability of synthesizing mucus that could have an influence on the adhesion process (40). The adhesion to HT29 cells was higher for the Balat_1410^{S89L} strain than for the Balat_1410 strain, but it was similar to the adhesion of the ΔBalat_1410 strain (Fig. 2B). Since the strain lacking Balat_1410 has previously been shown to display a nonorganized surface layer compared with those of the other two con-

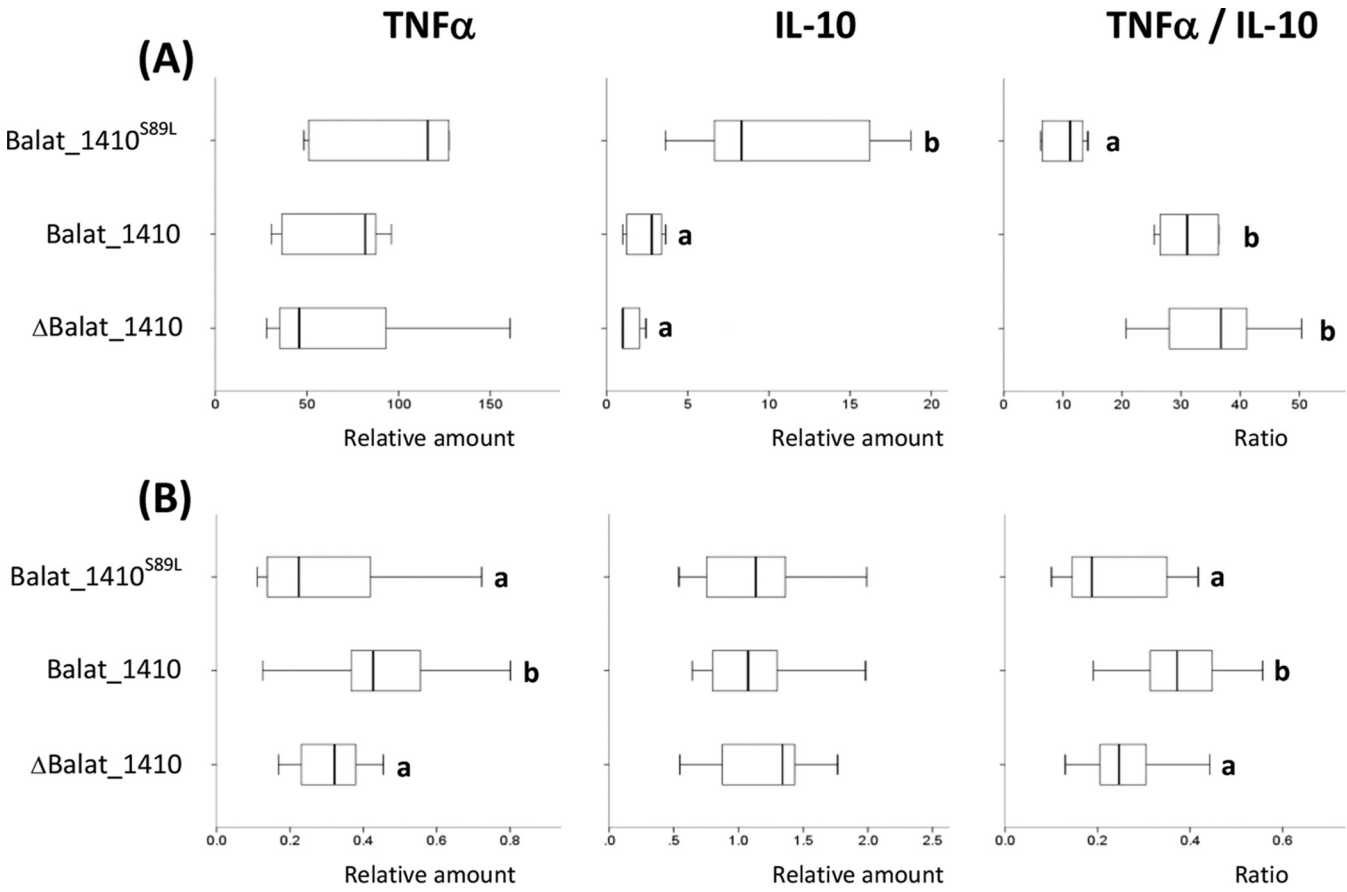

FIG 3 Relative amounts (concentration in the strain sample divided by the concentration in the control sample) of TNF-α (proinflammatory cytokine) and IL-10 (anti-inflammatory cytokine) measured in supernatants of human cells cocultivated with the isogenic strains *B. lactis* ΔBalat_1410, Balat_1410, and Balat_1410^{S89L}. (A) *In vitro* coculture with PBMCs from seven healthy donors for 4 days, at a ratio of 5:1 (UV-inactivated bacteria to PBMCs) (control samples in RPMI medium). (B) *Ex vivo* coculture with ascending colonic tissue (from the healthy tissue of five cancer patients) for 6 h (bifidobacteria added at about 1×10^9 CFU/sample) (control samples in RPMI medium). The ratio TNF-α/IL-10 was calculated to determine the potential anti-inflammatory profile of the strains under study. Box and whiskers show median, interquartile range (25% percentile and 75% percentile), and minimum and maximum values. Statistical analysis: nonparametric Mann-Whitney test; strains that do not share a letter are significantly different ($P < 0.05$).

structs, this particular result suggests that in the strains containing Balat_1410, cell surface macromolecules have lower accessibility to the extracellular milieu. Taken together, these data may indicate a favorable tendency of the cells with mucoid phenotypes to better survive and colonize the gut.

The role of EPS in the survival and colonization ability of gut bacteria has always been controversial. A polysaccharide utilization locus that is conserved among intestinal *Bacteroides*, one of the most abundant species in the human gut microbiota, mediates stable and resilient gut colonization, and deletion of the genes involved in polysaccharide biosynthesis results in colonization defects in mice (12). Our data suggest that EPS could have an influence on survival and adhesion. However, it has been shown that colonization and persistence characteristics of mice of two isogenic strains of *B. breve* UCC2003 producing two different extracellular polymers seem to be similar. Furthermore, the lack of EPS does produce a significantly lower persistence of the strain in mouse cecum and colon and does not prevent colonization by the gut pathogen *Citrobacter rodentium* (11). In contrast, a mutant of *L. johnsonii*, with a deleted EPS biosynthesis cluster and lacking the EPS outer layer, had an increased residence time in mice (10). We have to bear in mind the possibility that the lack of EPS in lactobacilli could make other molecules interact with the gut epithelium, increasing the residence time (41). These results suggest that the mechanisms governing residence time in the colon may not be the same for members of these two genera and highlight the importance of knowing the structural-functional features of each polymer in establishing ecological roles. Furthermore, the presence of pili in some species, such as *B. bifidum* and *Lactobacillus rhamnosus*, may also play a role in adhesion (42, 43). One way or another, our results clearly indicate the involvement of *B. animalis* subsp. *lactis* EPS in colonization/residence time/survival in processes that mimic the conditions of the gastrointestinal tract.

Presence of mucoid EPS triggers the production of anti-inflammatory signals in PBMCs and *ex vivo* human colonic tissues. The immunomodulatory capacity of the *B. animalis* subsp. *lactis* strains was tested using two experimental models: *in vitro* coculture with human peripheral blood mononuclear cells (PBMCs) and *ex vivo* coculture with ascending human colonic tissue (see Fig. S2 in the supplemental material). The strain with the mucoid phenotype exhibited a cytokine production pattern different from that of the nonmucoid strains (see Tables S1 and S2 in the supple-

mental material). The production of the anti-inflammatory cytokine IL-10 was significantly higher when the mucoid strain Balat_1410^{S89L} was cocultivated *in vitro* with human PBMCs (Fig. 3A), and significantly lower production of TNF-α was measured after cocultivation with colonic biopsy specimens (Fig. 3B) in the mucoid strain compared with that for the strain containing the wild-type Balat_1410 gene. A low TNF-α/IL-10 ratio has been used as an indicator of the anti-inflammatory capacity of some bacteria and antigens (28). Overall, the TNF-α/IL-10 ratio was lower for the Balat_1410^{S89L} strain than for the Balat_1410 and ΔBalat_1410 strains. This difference was, however, not statistically significant between the ΔBalat_1410 and Balat_1410^{S89L} strains using *ex vivo* viable colon tissues. Several immune system-stimulating capabilities were associated with different *Bifidobacterium* strains, some of which show proinflammatory profiles, whereas others display potential anti-inflammatory activity (28, 42, 44). However, in very few cases has the immunological effect of bifidobacteria been associated with the presence of EPS. In *B. breve* UCC2003, EPS-producing variants failed to elicit a strong immune response compared with that for nonproducing ones (11). In *Lactobacillus rhamnosus* GG, it has been suggested that EPS form a protective shield against host innate immune molecules in the intestine (13). In other commensal bacteria, such as *Bacteroides fragilis*, the extracellular polysaccharide A mediates a regulatory T cell (Treg) response that triggers production of IL-10 during commensal colonization (45). In relation to this, Hidalgo-Cantabrana and coworkers (46) highlighted the effect of the experimental models (*in vivo* versus *in vitro*) on their results, thus calling for caution when results obtained with different cell /animal models are compared.

In conclusion, most bifidobacteria contain EPS gene clusters in their genomes (18, 47), but the functional roles of the enzymatic machinery responsible for the synthesis of the EPS and of the polymers themselves are largely unknown. In our study, we have demonstrated that Balat_1410 is not essential for EPS production in *B. animalis* subsp. *lactis*. However, two variants of this gene confer different surface properties and phenotypic characteristics. The mucoid phenotype, most likely due to a larger abundance of an HMW EPS, is able to alter some of the most relevant intestinal colonization and persistence properties of the cells, as well as their immune system-stimulating capability. Further experiments are required to determine if these phenotypes confer an adaptive advantage to the mucoid strains and if the mucoid phenotype constitutes a positive selection criterion of *B. animalis* subsp. *lactis* strains intended to be used as probiotics.

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