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Role of Extracellular Transaldolase from *Bifidobacterium bifidum* in Mucin Adhesion and Aggregation

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The ability of bifidobacteria to establish in the intestine of mammals is among the main factors considered to be important for achieving probiotic effects. The role of surface molecules from *Bifidobacterium bifidum* taxon in mucin adhesion capability and the aggregation phenotype of this bacterial species was analyzed. Adhesion to the human intestinal cell line HT29 was determined for a collection of 12 *B. bifidum* strains. In four of them—*B. bifidum* LMG13195, DSM20456, DSM20239, and A8—the involvement of surface-exposed macromolecules in the aggregation phenomenon was determined. The aggregation of *B. bifidum* A8 and DSM20456 was abolished after treatment with proteinase K, this effect being more pronounced for the strain A8. Furthermore, a mucin binding assay of *B. bifidum* A8 surface proteins showed a high adhesive capability for its transaldolase (Tal). The localization of this enzyme on the surface of *B. bifidum* A8 was unequivocally demonstrated by immunogold electron microscopy experiments. The gene encoding Tal from *B. bifidum* A8 was expressed in *Lactococcus lactis*, and the protein was purified to homogeneity. The pure protein was able to restore the autoaggregation phenotype of proteinase K-treated *B. bifidum* A8 cells. A recombinant *L. lactis* strain, engineered to secrete Tal, displayed a mucin- binding level more than three times higher than the strain not producing the transaldolase. These findings suggest that Tal, when exposed on the cell surface of *B. bifidum*, could act as an important colonization factor favoring its establishment in the gut.

Bifidobacteria are members of the intestinal microbiota of humans, and some strains are able to exert health-promoting effects, thus being considered to have some probiotic features (2, 21). They are especially relevant in infants, constituting one of the predominant populations in their gut microbiota (48). Because of their beneficial effects, the functional characteristics of specific strains are being extensively studied with the aim of selecting those more suitable for different target populations, such as children, the elderly, or people suffering from immune disorders (3, 25, 31). In this regard, it is generally believed that a *Bifidobacterium* strain intended for use as a probiotic needs to survive gastrointestinal transit and colonize, at least transiently, the gut (45).

Intestinal colonization factors have extensively been studied in pathogenic bacteria, as well as in gut commensals and probiotics (42, 50). While in enteric pathogens they are undesirable traits and their presence constitutes an important safety issue, probiotic bacteria (i.e., *Lactobacillus* and *Bifidobacterium* strains) use them to persist in the intestine, enabling these microorganisms to accomplish their effects. In fact, some of these characteristics, such as adherence to mucus and/or human epithelial cells and cell lines, are recommended by the Food and Agriculture Organization of the United Nations–World Health Organization (FAO/WHO) as *in vitro* tests to screen potential probiotics (2).

Adhesion to the intestinal epithelium has been extensively studied in *Lactobacillus* and, to a lesser extent, in *Bifidobacterium*. Specialized intestinal epithelial cells secrete mucin, a complex glycoprotein mixture that is the main component of mucus and protects the mucosal surface limiting bacterial entry (32). However, some *Lactobacillus* display on their surface adhesins able to mediate the attachment to the mucus layer (4). This process is mainly mediated by proteins, although saccharide moieties and lipoteichoic acids have also been involved (50, 53). The proteins playing a role in the mucus adhesion phenotype of lactobacilli are mainly secreted and surface-associated proteins, either anchored to the membrane through a lipid moiety or embedded in the cell wall (14, 40, 52, 53). More recently, the potential contribution of the so-called moonlighting proteins, those able to switch between unrelated functions depending on the cell location, has also been described, i.e., elongation factor Tu and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (15, 19, 41). Regarding bifidobacteria, the involvement of surface proteins in the interaction with human plasminogen or enterocytes has been reported in *Bifidobacterium animalis* subsp. *lactis* and *Bifidobacterium bifidum*, respectively. Under certain circumstances, these proteins could play a role in facilitating the colonization of the human gut through degradation of the extracellular matrix of cells or by facilitating a close contact with the epithelium (5, 6, 7, 17, 42). Also, a pilus-type IV mediated host colonization and persistence mechanism has recently been demonstrated in *Bifidobacterium breve* (30).

In addition, autoaggregation was shown to play a role in favoring persistence and survival of intestinal bacteria in the human gastrointestinal tract (12). The aggregation phenotype of some probiotic bacteria can also promote coaggregation with pathogens, thus contributing to the removal of the pathogen in the oral or intestinal environment and decreasing its potential virulence (23, 44). Several aggregation factors have been described in *Lacto-*

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid[a]	Relevant origin, phenotype, and/or genotype[b]	Source or reference
Strains		
B. bifidum		
LMG13195	Intestine of infant	
DSM20239	Breast-fed infant	
DSM20456^T	Breast-fed infant	26
DSM20082	Intestine of adult	
DSM20215	Intestine of adult	
A8	Dairy product	46
D119	Human feces	46
L22	Human feces	46
JCM7002	Human feces	
JCM7003	Human feces	
KCTC3357	Human feces	
KCTC5082	Human feces	
L. lactis		
NZ9000	*L. lactis* MG1363 derivative carrying *pepN*::*nisRK*	11
NZ9700	Nisin-producing strain	22
NZ9000-HTal	Cm^r; NZ9000 derivative carrying pNZ8048-HTal	This study
NZ9000-SecTal	Cm^r; NZ9000 derivative carrying pNZ8110-SecTal	This study
Plasmids		
pNZ8048	Cm^r; inducible expression vector, *nisA* promoter	11
pNZ8110	Cm^r; pNZ8048 derivative, Usp45 signal peptide	11, 49
pNZ8048-HTal	Cm^r; pNZ8048 derivative, carrying His tag *tal*	This study
pNZ8110-SecTal	Cm^r; pNZ8110 derivative, carrying *tal*	This study

[a] LMG, Belgian Coordinated Collections of Microorganisms; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; JCM, Japan Collection of Microorganisms; KCTC, Korean Collection for Type Cultures.
[b] Cm^r, chloramphenicol resistance.

bacillus, and for some of them a relation between the aggregation phenotype and the persistence of the microorganisms in the gut has been reported (14, 51, 54). *B. bifidum* has a strong autoaggregation phenotype under *in vitro* conditions, although the molecular basis behind this phenomenon has not yet been elucidated (8). Remarkably, some authors have demonstrated a strong association between mucin adhesion and autoaggregation; cells with high adhesion capability possess a strong aggregation phenotype (14, 27).

In this context, the aim of the present study was to evaluate novel *B. bifidum* surface proteins able to contribute to its intestinal colonization capability.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains and plasmids used throughout the present study are listed in Table 1. *B. bifidum* strains were grown in MRS broth (Difco/BD Diagnostic Systems, Sparks, MD) supplemented with 0.25% (wt/vol) L-cysteine (Sigma Chemical Co., St. Louis, MO) (MRSc) and were incubated at 37°C in an anaerobic chamber (Whitley MG500 anaerobic workstation; DW Scientific, Shipley, United Kingdom) in an atmosphere of 10% CO_2, 10% H_2, and 80% N_2. *Lactococcus lactis* strains were grown in M17 broth (Oxoid, Ltd., Hampshire, United Kingdom) containing 0.5% (wt/vol) glucose (GM17) at 32°C. Chloramphenicol (5 mg liter^{-1}) was added to the medium for the selection of recombinant *L. lactis* strains containing pNZ plasmids. For the expression of the *B. bifidum* transaldolase gene (*tal*) in the strains *L. lactis* NZ9000-HTal and NZ9000-SecTal, 0.001% (vol/vol) of the supernatant from an overnight culture of the nisin-producing strain *L. lactis* NZ9700 (containing ~10 ng of nisin A ml^{-1}) was added to the cultures at an optical density at 600 nm (OD_{600}) of 0.2, and the inductions were performed until the cultures reached an OD_{600} of ~0.8.

Characterization of *B. bifidum* strains. (i) Adhesion of *B. bifidum* strains to HT29 cells. The cell line was maintained in McCoy broth (Sigma) supplemented with 3 mM L-glutamine, 10% (vol/vol) heat-inactivated fetal bovine serum, and a mixture of antibiotics (50 mg of penicillin, 50 mg of streptomycin, 50 mg of gentamicin, and 1.25 mg of amphotericin B liter^{-1}). Medium and supplements were purchased from Sigma. Incubations took place at 37°C in 5% CO_2 in an SL water jacketed CO_2 incubator (Sheldon Mfg., Inc., Cornelius, OR). The culture medium was changed every 2 days, and the cell lines were trypsinized with 0.25% trypsin-EDTA solution (Sigma) according to standard procedures (10). For adhesion experiments, 10^5 cells ml^{-1} were seeded in 24-well plates, incubated to confluence, and used at a mean age of 13 ± 1 days old.

Twelve *B. bifidum* strains and the reference strain *B. animalis* subsp. *lactis* BB12, grown in 10 ml of MRSc for 24 h, were harvested by centrifugation, washed twice with Dulbecco phosphate-buffered saline (PBS) buffer (Sigma), and resuspended in McCoy medium without antibiotics at a concentration of ~10^8 CFU ml^{-1}. The bacterial suspensions were added in a ratio of epithelial cells to bacteria of 1:10. 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). After the incubation period, the wells were gently washed three times with Dulbecco PBS buffer to remove the nonattached bacteria. Trypsin-EDTA solution was added to release the adhered cells, and bacterial counts were carried out in agar-MRSc to determine the number of adhered bacteria. The results were expressed as the percentage of bacteria adhered with respect to the amount of bacteria added to the monolayer (CFU of adhered bacteria × 100/CFU of added bacteria). Experiments were repeated at least twice, using two independent bacterial cultures and two different HT29 plates. In each plate, the samples were also tested in duplicate.

(ii) Aggregation phenotype of selected *B. bifidum* strains. The *B. bifidum* strains A8, DSM20456, DSM20239, and LMG13195 were grown in 50 ml of MRSc for 18 h under standard conditions. Cultures were harvested by centrifugation, washed with PBS buffer (Oxoid), and resuspended in PBS buffer until an OD_{600} of 1 was reached. Aliquots of 10 ml were transferred to five tubes, harvested by centrifugation, and resuspended under the following conditions: condition 1, PBS buffer with 1 mg of proteinase K (EC 3.4.21.64; Molecular Biochemicals, Lewes, United Kingdom) ml^{-1}; condition 2, PBS buffer with 1 mg of lipase (EC 3.1.1.3; Sigma) ml^{-1}; condition 3, PBS buffer (control); condition 4, acetate buffer (pH 4.5) containing 5 mg of sodium metaperiodate (Merck, Darmstadt, Germany) ml^{-1}; and condition 5, acetate buffer (pH 4.5) (control for the metaperiodate treatment). All bacterial suspensions were incubated for 30 min at 37°C. After incubation, the bacterial suspensions were washed with acetate buffer (pH 3.8) and resuspended in 5 ml of the same buffer. Then, each tube was incubated in a bath at 37°C, measuring the OD_{600} at different times (0, 60, 120, and 180 min). Aggregation percentage was calculated as follows: [(OD_{600} of the control bacterial suspension/OD_{600} of the bacterial suspension after treatment)] × 100. The influence of the different treatments on cell viability was checked by plate counting before and after the treatments. Experiments were carried out at least in triplicate.

For the determination of the influence of surface proteins or pure transaldolase (see below) on the aggregation phenotype, the strain *B. bifidum* A8 was grown in 100 ml of MRSc for 18 h and subjected to a proteinase K treatment under the conditions described above. Several aliquots of the proteinase-treated suspensions in 5 ml of acetate buffer (pH 3.8) were incubated in a bath at 37°C. After 1 h of incubation, surface proteins extracted after Na_2CO_3 treatment (10 μg), pure transaldolase

from *B. bifidum* A8 (1 or 10 μg), bovine serum albumin (BSA; 1 or 10 μg), or pure histidine-tagged arabinofuranosidase (1 or 10 μg) (28) were added, and the OD_{600} was measured every hour for 4 h.

Functional characterization of surface proteins from *B. bifidum* A8. (i) Extraction of surface proteins by carbonate wash or sodium hydroxide treatment. Cultures (50 ml) of *B. bifidum* A8, grown in MRSc for 18 h, were harvested by centrifugation and washed with acetate buffer (pH 3.8), and the pellets were subjected to two different treatments to extract proteins noncovalently bound to the cell surface. Cells from 50 ml of culture were resuspended in 5 ml of 100 mM Na_2CO_3 buffer, and cell suspensions were incubated for 1 h on ice and harvested by centrifugation (Na_2CO_3 fraction) (35). Another washed cell pellet from 50 ml of culture was resuspended in 5 ml of 0.01 M NaOH, and an incubation at 37°C for 30 min under gentle agitation was performed (NaOH fraction) (36). The protease inhibitors EDTA and phenylmethylsulfonyl fluoride were added to all of the solutions at final concentrations of 5 and 1 mM, respectively. After these treatments, the supernatants were collected and filtrated through 0.45-μm-pore-size filters. The protein concentrations were determined by using a BCA protein assay kit (Pierce, Rockford, IL). Protein (40 μg) was precipitated using a methanol-chloroform protocol (55), solubilized in Laemmli buffer, and separated by SDS-PAGE. The protein profiles were visualized by standard Coomassie staining, and the proteins were identified by tandem mass spectrometry in a matrix-assisted laser desorption ionization–time of flight apparatus (Servicio de Proteómica, CNIC, Madrid, Spain).

(ii) Mucin binding experiments. Adhesion to mucin was performed as described previously (41). In brief mucin (2 mg, type III; Sigma) was used to coat the wells of F96 Maxisorp Immunoplates (Nunc, Roskilde, Denmark) for 1 h at 37°C and overnight at 4°C. The wells were then blocked with 1% (wt/vol) BSA (Sigma) in PBS for 1 h at 37°C and washed twice with PBS, and 100 μg of *B. bifidum* A8 surface proteins, from both NaOH and Na_2CO_3 fractions, was added to each well, followed by incubation at 37°C for 1 h. Unbound proteins were removed by several washes with PBS and, subsequently, 60 μl of 1% (wt/vol) SDS was added to each well, followed by incubation at 37°C for 1 h to extract the bound protein. Proteins that were able to adhere to the mucin layer were analyzed by SDS-PAGE, visualized by silver staining, and identified by a mass spectrometry analysis (Servicio de Proteómica, CNIC). At least two different surface protein extracts (biological replicates from two independent cultures) from each fraction (NaOH-extracted proteins and Na_2CO_3-extracted proteins) were used for mucus-binding assays, and several technical replicates with each extract were performed per plate.

(iii) Cloning of the transaldolase gene, protein purification, and Western blotting. The transaldolase gene (*tal*) was amplified from the genomic DNA of *B. bifidum* A8 by PCR using high-fidelity Platinum *Pfx* DNA polymerase (Invitrogen, Barcelona, Spain) and the primers TalF (5′-TCCGATTCATGACTGAAGCAACTCAGCGCACG-3′; the NcoI site is underlined) and TalHisR (5′-TCGGCTTCTAGATCAATGGTGATGGTGATGGTGGGCGTTCACGCGGTCGATG-3′; the XbaI site is underlined, including the codons for the synthesis of a His_6 tag). The PCR product was purified with a QIAquick PCR purification KIT (Qiagen, Ltd., Strasse, Germany), digested with RcaI and XbaI (RcaI yields compatible ends with NcoI), and ligated with the NcoI/XbaI-digested pNZ8048 vector, yielding pNZ8048-HTal. A similar protocol was used to clone *tal* into pNZ8110, which contains the coding sequence for the Usp45 signal peptide (49) and allows the secretion of the expressed protein outside the cell. In this case, the gene was amplified by using the primers SecTalF (5′-CCGAATGCCGGCATGACTGAAGCAACTCAGCG-3′) and SecTalR (5′-CCGAATGCCGGCTCAGGCGTTCACGCGGTCGA-3′), both containing an NaeI site (underlined). NaeI-digested pNZ8110 was ligated with the NaeI-digested PCR product, and the fragment orientation was checked by restriction analysis, yielding pNZ8110-SecTal. Plasmids were electroporated into *L. lactis* NZ9000 according to procedures described elsewhere (28), yielding the recombinant strains NZ9000-HTal and NZ9000-SecTal. The absence of PCR-introduced mutations was verified in the two plasmid constructs by sequencing both DNA strands on an ABI Prism sequencer (Applied Biosystems). Expression and purification of the histidine-tagged protein from NZ9000-HTal protein extracts was performed according to the method of Margolles and de los Reyes-Gavilán (28). After purification, the pure protein was dialyzed against PBS (Oxoid) and stored at −80°C.

For Western blot analysis, rabbit polyclonal IgG obtained against purified Tal was used for the primary immunoreaction. The polyclonal antibodies were obtained in the Laboratory of Biotechnological and Biomedical assays of the University of Oviedo (Oviedo, Asturias, Spain). Previous experiments showed a good specificity of the serum, and a single band with the proper molecular weight was detected by confronting the serum to total protein extracts of *B. bifidum* A8, using standard Western blot procedures. In brief, for Western blot analysis of the different *L. lactis* protein extracts (supernatant, cytoplasmic, and surface-associated proteins), ~30 μg of protein was run on SDS-PAGE gels, the samples were then transferred to polyvinylidene difluoride membranes (Amersham Biosciences, Buckinghamshire, United Kingdom), and immunoreactions were performed as previously described (39). The blots were developed with 4-chloro-1-naphthol.

(iv) Mucin binding experiments of recombinant *L. lactis*. *L. lactis* NZ9000 carrying the plasmid pNZ8110 (control; empty plasmid) or pNZ8110-SecTal were grown in 10 ml of GM17 at 32°C to an OD_{600} 0.2. The expression of the gene was induced as indicated above, and the cells were harvested by centrifugation and resuspended in PBS to OD_{600} of 1. Next, 100-μl portions of lactococci suspensions were added to each well of mucin-coated F96 Maxisorp Immunoplates, followed by incubation at 37°C for 1 h. After the wells were washed twice to remove nonattached bacteria, the well content was resuspended in 100 μl of PBS, and lactococcus counts were determined in GM17 agar. The results are expressed as the percentage of bacteria adhered with respect to the amount of bacteria added (% of CFU bacteria adhered/CFU of bacteria added). Experiments were carried out using two biological replicates, each tested in duplicate in two mucin-coated wells.

(v) Electron microscopy and immunogold labeling. To visualize the cell surface-associated Tal of *B. bifidum* A8, the conditions described previously were used (6). *B. bifidum* A8 was grown in 10 ml of MRSc for 18 h, harvested by centrifugation, washed twice with PBS, and rotated in PBS–1% BSA for 30 min. Cells were harvested by centrifugation, resuspended in 1 ml of rabbit polyclonal anti-Tal IgG (1:100 in PBS–1% BSA), and incubated for 1 h. After incubation the cells were washed three times with PBS, resuspended in PBS–1% BSA, and incubated for 30 min at room temperature. The bacteria were then resuspended in 1 ml of anti-rabbit IgG coupled to 12-nm gold particles (Jackson Immunoresearch Europe, Ltd., Suffolk, United Kingdom) (1:40 in PBS–1% BSA) and incubated for 45 min. The bacteria were then collected, washed twice in PBS–1% BSA, and fixed in PBS containing 4% glutaraldehyde and 2% paraformaldehyde for 3 h. Labeled samples were stored at 4°C until microscopy visualization, which was carried out at the Servicio de Microscopía CNB-CSIC (Cantoblanco, Madrid, Spain). As a negative control, the same treatment was performed in the absence of rabbit polyclonal anti-Tal IgG.

RESULTS AND DISCUSSION

Twelve strains of *B. bifidum* from different origins (infant feces, adult feces, and dairy products) were studied in order to determine phenotypic traits able to distinguish the different strains. First, their ability to adhere to HT29 cells (Table 2) was evaluated. This cell line has an enterocyte-like differentiation and is considered to be representative of surface or villus type cells also presenting mucus-producing Goblet cells. The adhesion of *B. bifidum* to HT29 ranged between 0.13 and 74.79% depending on the strain, while the reference strain *B. animalis* subsp. *lactis* BB12 displayed an adhesion of 1.03% ± 0.43%. In general, the majority of *B. bifidum* strains, compared to other bifidobacteria, showed good

TABLE 2 Adhesion of *B. bifidum* strains to HT29 cells

Strain	Adhesion to HT29 (%) ± SD[a]
A8	**6.90 ± 2.17**
D119	15.56 ± 2.03
DSM20082	20.33 ± 14.11
DSM20215	40.14 ± 10.74
DSM20239	**0.13 ± 0.03**
DSM20456	**3.25 ± 1.14**
JCM7002	1.84 ± 0.51
JCM7003	1.41 ± 0.49
KCTC3357	26.56 ± 2.79
KCTC5082	30.05 ± 11.73
L22	60.68 ± 28.22
LMG13195	**74.79 ± 15.07**

[a] Adherence is represented as the percentage of bacteria adhered with respect to the amount of bacteria added. Strains selected for further characterization are indicated in boldface.

adhesion properties to HT29 cells (10, 33). To our surprise, some strains, such as L22 and LMG13195, displayed extremely high adhesion capabilities (more than half of the cell population remained attached to the enterocyte layer after the adhesion assay). This could be related to the ability of some *B. bifidum* strains to degrade and metabolize mucin (34, 46, 47). The breakdown of the glycoprotein linkages within the mucin by some *B. bifidum* strains could facilitate access to the outer mucus layer and likely contributes to a tighter contact with the colonocyte.

Taking into account the different adhesion capabilities of our strains, four of them were selected for further studies: *B. bifidum* LMG13195 (high adhesion), *B. bifidum* DSM20239 (low adhesion), and *B. bifidum* A8 (intermediate adhesion), including the type strain *B. bifidum* DSM20456 for comparison purposes. Since the adhesion capability has been found to be linked to cell autoaggregation, the involvement of surface-exposed macromolecules in the aggregation phenomena of the four selected strains was studied first. Initial experiments led us to conclude that the four selected strains displayed a strong autoaggregation phenotype (data not shown), which is in accordance with previous results that detected high levels of autoaggregation in *B. bifidum* species (8). In this regard, the type of surface-exposed molecules may have a critical role in the aggregation of the bacteria (16). To clarify this point, the nature of the molecules involved in the aggregation of our strains was preliminarily characterized. Different treatments were carried out in order to determine the involvement of carbohydrate moieties (metaperiodate), proteins (proteinase K), and lipids (lipase). All experiments were performed in acetate buffer (pH 3.8) since the aggregation phenotype of *B. bifidum* is strictly dependent on the pH (8, 16). The treatment with metaperiodate was lethal for *B. bifidum*, and viable cells were not recovered after the treatment; for this reason, the role of carbohydrate moieties in aggregation could not be tested under our experimental conditions. The rest of the treatments did not affect the viability of the *B. bifidum* strains (data not shown). None of the treatments affected the autoaggregation ability of *B. bifidum* LMG13195 and DSM20239 (Fig. 1). However, the aggregation of *B. bifidum* A8 and DSM20456 was abolished after a treatment with proteinase K, the effect being more pronounced for strain A8, suggesting the involvement of surface-exposed proteins in the aggregation phenotype. In this regard, it is worth mentioning that previous studies suggested that proteins have a key role in the autoaggregating ability of *B. bifidum*, since protease-treated cells lost this characteristic phenotype (8).

A correlation seems to exist between mucin adhesion and autoaggregation in some *Lactobacillus* strains (14, 27). Thus, our next attempt was to characterize in depth the relationship between these two features in *B. bifidum*. In order to pursue this, *B. bifidum* A8 was used as a model strain, since the abolishment of its aggregation phenotype was very pronounced after protease treatment. The mucin adhesion assays of surface-associated proteins from *B. bifidum* A8 extracted with two different methods, NaOH and Na_2CO_3 treatments, led to the isolation of a protein that was collected, almost exclusively, from the mucin layer, suggesting a high binding affinity (Fig. 2). The protein was identified by mass spectrometry analysis as transaldolase (Tal, BBPR_1029) (46). This enzyme is involved in the pentose phosphate pathway and is responsible for the conversion of fructose-6-P and erythrose-4-P into sedoheptulose-7-P and glyceraldehyde-3-P, the latter entering directly into the glycolytic pathway (43). Although its cytoplasmic location is generally agreed, some clues point to the fact that in several *Bifidobacterium* species it could be released in the extracellular milieu. In a previous work from our group 14 proteins were identified in the extracellular proteome of *B. longum* NCIMB8809 (37), all of them displayed a predicted noncytoplasmic location (membrane, cell wall, or extracellular) excluding Tal, which lacks any apparent signal peptide. Furthermore, Tal was also detected in the extracellular proteome of the probiotic bacteria *B. animalis* subsp. *lactis* BB12 (13). In addition, in the present study we demonstrated the presence of Tal on the surface of *B. bifidum* A8 by visualizing the enzyme with immunogold particles (Fig. 3). In this regard, recent studies have indicated that many enzymes of carbon catabolism, either from prokaryotic or eukaryotic cells, are often cell wall associated and are able to perform "moonlighting" functions. For instance, in tumor cells the surface exposed α-enolase acts as a plasminogen receptor, mediating plasmin activation, extracellular matrix degradation, and supporting

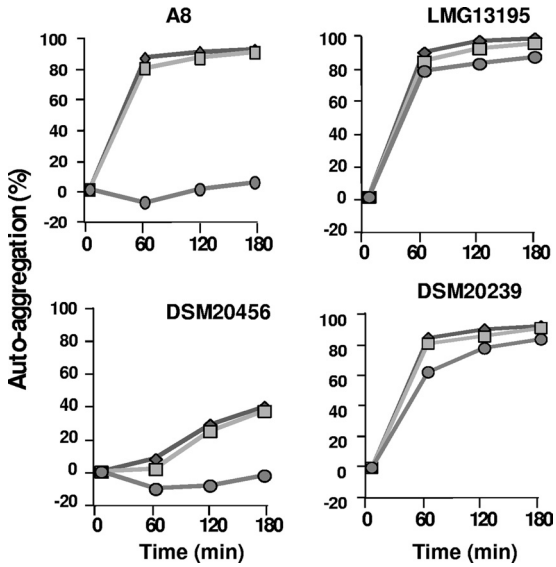

FIG 1 Representative plots displaying autoaggregation percentages of selected *B. bifidum* strains after proteinase K or lipase treatments. Symbols: diamonds, control (PBS); squares, lipase; circles, proteinase K.

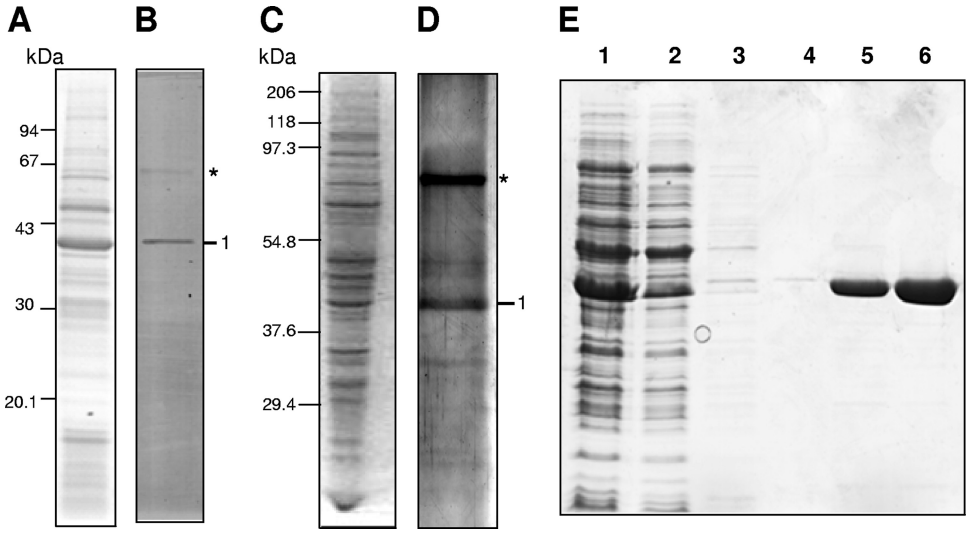

FIG 2 SDS-PAGE of *B. bifidum* A8 surface proteins extracted with NaOH (A) or with Na_2CO_3 (C) and proteins recovered from the mucus layer after the mucus binding assay with the surface proteins extracted with NaOH (B) or with Na_2CO_3 (D). Markers: 1, transaldolase; *, BSA. (E) SDS-PAGE of samples taken during transaldolase purification. Lanes: 1, cytoplasmic protein extract of *L. lactis* NZ9000-HTal; 2, flowthrough; 3 and 4, protein eluted from the Ni-NTA resin after washing with 10 mM imidazole (lane 3) and 30 mM imidazole (lane 4); 5, pre-elution step; 6, purified histidine-tagged Tal eluted from the Ni-NTA column with 150 mM imidazole.

anaerobic proliferation (9). The same role for α-enolase has been suggested in pathogenic bacteria, facilitating tissue invasion via extracellular matrix degradation (18). Also, surface-localized GAPDH acts as a mucin binding protein in several bacteria (1, 41). Interestingly, *B. longum* transaldolase is highly underproduced in acidic pH (38) and, theoretically, this should facilitate its passage through the stomach, preventing adhesion and directing it to the large gut, its natural habitat.

The data obtained in the present study point to a role of the surface-exposed transaldolase as a mucin binding protein in *B. bifidum*. To demonstrate this hypothesis, bifidobacterial *tal* was cloned, and the mucin adhesion ability of the recombinant bacterium was studied. The lack of proper molecular tools to construct isogenic mutants in *B. bifidum* led us to select *L. lactis* NZ9000 as a host, since this bacterium has been shown to be an adequate host to produce several *Bifidobacterium* proteins and, furthermore, it naturally lacks a transaldolase gene (24, 28, 29). A Western blot analysis confirmed that the protein was present in the three subcellular locations of the recombinant strain and absent in the control strain containing the empty plasmid pNZ8110 (Fig. 4A). Furthermore, the *L. lactis* strain expressing Tal displayed a mucin binding level more than three times higher than the nonproducing strain (Fig. 4B). Therefore, these experiments further support the role of Tal as an adhesin able to bind mucin. In relation to this, it has been suggested that not only proteinaceous factors play a role in the adhesion phenotype of *B. bifidum*, and the partial involvement of sugar moieties has also been indicated (16).

Finally, since mucin adhesion and autoaggregation are two characteristics often related in bacteria, the role of Tal in the autoaggregation phenotype of *B. bifidum* A8 was also checked. As seen in Fig. 5, proteinase K-treated cells, which have lost the intrinsic autoaggregation trait, restored this phenotype by adding a surface protein extract from the same bacterium or pure Tal, but not by adding histidine-tagged arabinofuranosidase (tested to rule out the possible effect of a histidine tag in the aggregation phenotype) or BSA. This indicates that Tal is a specific aggregation factor in *B. bifidum* A8 that can contribute, at least partially, to the strong autoaggregation phenotype found in some strains of this *Bifidobacterium* species.

In conclusion, to the best of our knowledge the findings reported in this article are the first to suggest an extracellular role for transaldolase in *Bifidobacterium*. Until now there was no informa-

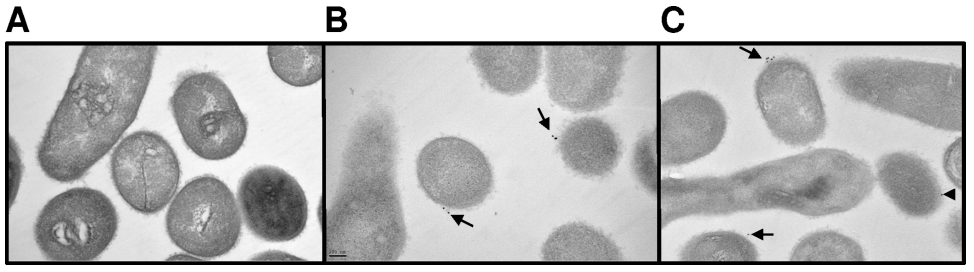

FIG 3 Visualization of extracellular transaldolase of *B. bifidum* A8 by immunogold electron microscopy. (A) Control, *B. bifidum* A8 marked only with antibody against rabbit IgG carrying gold particles of 12 nm. (B and C) *B. bifidum* A8 labeled first with polyclonal rabbit IgG antibody against transaldolase and second with antibody against rabbit IgG carrying gold particles of 12 nm. Bar, 100 nm.

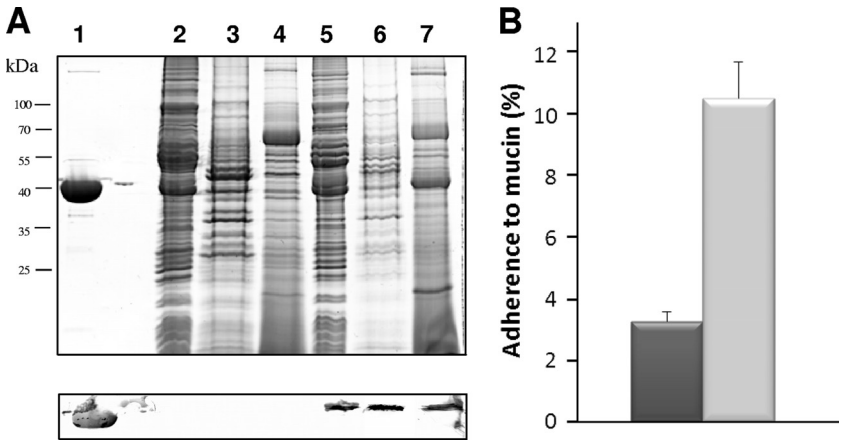

FIG 4 (A) SDS-PAGE (upper panel) and Western blot (lower panel) analyses of different protein fractions from *L. lactis* NZ9000-SecTal. Lanes (upper panel): 1, 10 μg of purified transaldolase; 2, 3, and 4, cytoplasmic, surface-associated, and secreted protein fractions of *L. lactis* NZ9000 containing pNZ8110, respectively; lanes 5, 6, and 7, cytoplasmic, surface-associated, and secreted protein fractions of *L. lactis* NZ9000 containing pNZ8110-SecTal, respectively. The lower panel shows Western blotting using of the same samples run in a twin gel. (B) Percentage of adhesion to mucin of *L. lactis* NZ9000 containing pNZ8110 (dark gray) and *L. lactis* NZ9000 containing pNZ8110-SecTal (light gray). The plots represent the means and standard deviations of four measurements.

tion about putative aggregation factors in bifidobacteria, and specific mucin binding proteins had not been described. We have demonstrated, *in vitro*, that the extracellular transaldolase from *B. bifidum* A8 can fulfill both functions. Bifidobacterial transaldolase is abundant in the infant gut, and its dominance in the feces of newborn infants might be related to the high abundance of bifidobacteria in this population group (20). Transaldolase, as has been demonstrated for other glycolytic enzymes, can be recruited on the cell surface, and it could be secreted via a nonclassical secretion mechanism or a thus-far-uncharacterized translocation pathway that remains to be elucidated. Our findings suggest that Tal, when exposed on the cell surface, could act as an important colonization factor favoring the establishment of *B. bifidum* in the intestinal tract.

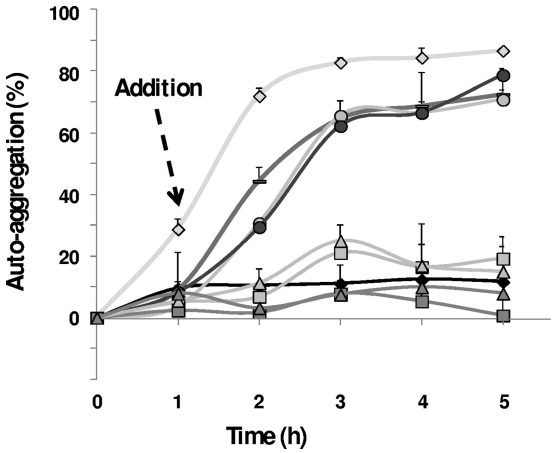

FIG 5 Autoaggregation ability of proteinase K-treated *B. bifidum* A8 in the presence of different proteins or surface protein extracts. Symbols: gray diamond, control (non-proteinase K-treated) strain; black diamond, proteinase K-treated strain; —, addition of 10 μg of A8 surface proteins; gray circle, addition of 1 μg of A8 transaldolase; black circle, addition of 10 μg of A8 transaldolase; light gray box, addition of 1 μg of BSA; dark gray box, addition of 10 μg of BSA; light gray triangle, addition of 1 μg of histidine-tagged arabinofuranosidase; dark gray triangle, addition of 10 μg of histidine-tagged arabinofuranosidase.

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