

Low-pH Adaptation and the Acid Tolerance Response of *Bifidobacterium longum* Biotype longum[∇]

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Bifidobacteria are one of the main microbial inhabitants of the human colon. Usually administered in fermented dairy products as beneficial microorganisms, they have to overcome the acidic pH found in the stomach during the gastrointestinal transit to be able to colonize the lower parts of the intestine. The mechanisms underlying acid response and adaptation in *Bifidobacterium longum* biotype longum NCIMB 8809 and its acid-pH-resistant mutant *B. longum* biotype longum 8809dpH were studied. Comparison of protein maps, and protein identification by matrix-assisted laser desorption ionization–time of flight mass spectrometry analysis, allowed us to identify nine different proteins whose production largely changed in the mutant strain. Furthermore, the production of 47 proteins was modulated by pH in one or both strains. These included general stress response chaperones and proteins involved in transcription and translation as well as in carbohydrate and nitrogen metabolism, among others. Significant differences in the levels of metabolic end products and in the redox status of the cells were also detected between the wild-type strain and its acid-pH-resistant mutant in response to, or as a result of, adaptation to acid. Remarkably, the results of this work indicated that adaptation and response to low pH in *B. longum* biotype longum involve changes in the glycolytic flux and in the ability to regulate the internal pH. These changes were accompanied by a higher content of ammonium in the cytoplasm, likely coming from amino acid deamination, and a decrease of the bile salt hydrolase activity.

Bifidobacteria are anaerobic, gram-positive, nonmotile, and nonsporulating irregular or branched fermentative rod-shaped bacteria in which the acid susceptibility is dependent on the strain (31). As with other intestinal bacteria, they have been shown to provide protection against gastrointestinal disorders, but it has also been proposed that they exercise other health-promoting effects, like the inhibition of pathogenic microorganisms, antimutagenic and anticarcinogenic activities, prevention of diarrhea, immune modulation, and reduction of serum cholesterol levels (42, 48). Because of these properties, several species belonging to the genus *Bifidobacterium* are considered probiotics, and they are largely applied in dairy products (28).

Acid tolerance in bifidobacteria is of particular importance, as this property is closely related to their use in human nutrition. In general, it can be considered that bifidobacteria have a weak acid tolerance with the exception of *Bifidobacterium animalis* (32), which can survive acidic pH better than the other species and which is usually detected as the sole viable bifidobacterium species in fermented milks (21). In addition, it has been shown that the effect of pH in the stomach and

duodenum promotes changes in the microbiota, excluding the growth of some species like bifidobacteria or *Escherichia coli*, while *Lactobacillus* or some yeasts are favored (35). Bifidobacteria are consumed in fermented milks, where they are added at the beginning of the fermentation process together with the starter culture which acidifies milk by lactose fermentation, reaching a pH usually lower than 4.6 (46). After ingestion, and in order to get to their ecological niche, i.e., the large intestine, bacteria must survive passage through the low-pH environment of the stomach. Then, bifidobacteria have to cope with acid stress from their storage, through their distribution and to their delivery, which usually results in their loss of viability and therefore in a reduction of the potential probiotic effects (7, 35).

It is known that exposure of bacteria to stress factors (heat, bile salts, or acid pH) can provide protection against further hostile environmental conditions (5, 17, 34). For instance, the acid tolerance response has been evidenced in *Bifidobacterium longum* and *B. animalis* (32, 41) and in other gram-positive bacteria such as *Listeria monocytogenes* (13), *Enterococcus faecalis* (16), or *Lactococcus lactis* (37). Mechanisms underlying acid tolerance used by gram-positive bacteria include not only proton pumping via the F₁F₀-ATPase but also changes in the cell membrane and regulatory mechanisms, alterations in different metabolic pathways, and amino acid decarboxylation (12).

With the purpose of understanding how bifidobacteria can

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adapt to acid stress, we used as a model microorganism *B. longum* biotype longum NCIMB 8809, a potential probiotic strain (36), and *B. longum* biotype longum 8809dpH, a mutant derived thereof, which we isolated as acid pH resistant. The physiological properties and proteomes of the two strains were compared.

MATERIALS AND METHODS

Strains and culture conditions. The bacterial strains used in this study were *B. longum* biotype longum NCIMB 8809 (wild-type [WT] strain) (NCIMB, Aberdeen, Scotland, United Kingdom), which was isolated from nursing stools, and its acid-pH-resistant mutant *B. longum* biotype longum 8809dpH. Strains were routinely grown in MRS (BD Diagnostic Systems, Sparks, MD, unless indicated otherwise) supplemented with 0.05% L-cysteine (Sigma, St. Louis, MO) (MRSC), at 37°C and under anaerobic conditions (Bactron anaerobic/environmental chamber; Sheldon Manufacturing Inc., Cornelius, OR) in an atmosphere of 5% CO₂, 5% H₂, 90% N₂. For the isolation of acid-resistant mutants, cells from an overnight culture of *B. longum* biotype longum NCIMB 8809, previously subcultured in standard conditions, were washed in phosphate-buffered saline and used to inoculate (1%) fresh MRSC (Scharlau, Barcelona, Spain) adjusted at different pH values (5.0, 4.0, 2.0, and 1.25) with 1 N HCl. These cultures were incubated at 37°C for 16 h, and then aliquots were plated on MRSC at neutral pH and incubated at 37°C for 3 to 4 days to recover possible acid-resistant strains. The acquisition of a stable acid resistance phenotype in the mutants was determined by testing their ability to survive in simulated gastric conditions (3 g/liter pepsin, adjusted at pH 2.0 with HCl; Sigma) after daily cultivation in MRSC for 2 weeks as previously described (10).

For the proteomic and physiological experiments, one colony isolated on MRSC agar was grown overnight. Aliquots of 2.5 ml of those precultures were washed twice in MRSC adjusted to pH 7.0 or pH 4.8, used to inoculate 250 ml of fresh MRSC adjusted to pH 7.0 or pH 4.8, and incubated anaerobically until mid-exponential phase (optical density at 600 nm [OD₆₀₀], 0.5).

Molecular identification of *Bifidobacterium* mutant strain. Chromosomal DNA from *Bifidobacterium* isolates was extracted according to the method of Wilson et al. (51), which was slightly modified by including an incubation step with lysozyme (50 mg/ml) at 37°C for 30 min prior to the extraction procedure. Then, DNA of *B. longum* was isolated using a GenElute bacterial genomic DNA kit (Sigma) and used as a template for PCR amplifications. Primers (Bif164, 5'-CATCCGGCATTACCACCC, and Bif662, 5'-CCACCGTTACACGGGAA) specific for the 16S rRNA gene sequence (22, 29) were used to amplify a partial sequence of the 16S gene (19). For the amplification of the gene coding for *O*-acetylhomoserine (thiol)-lyase and its 500-bp upstream region, the forward primer 5'-CTCCAATCAATCAGACGGATGTAC (CysDF1) and the reverse primer 5'-CCCAATATGGAGAGGCATTATGAACG (CysDR2) were used. For the amplification of the 3.8-kb sequence containing the gene of the methionine synthase and the upstream region, we used the forward primer 5'-CGATGAGCGTCAACCTGCTGGACG (MetEF2) and the reverse primer 5'-GCTCGGGTTTATGTAAGGAAATCAGC (MetER). PCR fragments were purified with the GenElute PCR cleanup kit (Sigma) and sequenced in the Servicio de Secuenciación de ADN (SSAD, CIB, Cantoblanco, Madrid, Spain).

Sensitivity of *B. longum* biotype longum NCIMB 8809 and its acid-pH-resistant mutant *B. longum* biotype longum 8809dpH to artificial gastrointestinal conditions. The ability of *B. longum* to survive in in vitro conditions simulating the passage through the stomach and intestine was evaluated. For this purpose, cell suspensions of each strain (10⁸ to 10⁹ cells/ml) were incubated in sterile saline solution (0.5% [wt/vol] NaCl), containing 3 g/liter pepsin (Sigma) and adjusted at pH 2.0 with HCl, at 37°C for 120 min. Aliquots were withdrawn at different times (0, 90, and 120 min) to determine plate counts on MRSC agar. After exposure to simulated gastric conditions, cells were collected by centrifugation (8,000 × g, 10 min) and washed in sterile saline solution. Then, cell pellets were suspended in saline solution containing pancreatin (1 g/liter; Sigma) and bile salts (0.5% [wt/vol] oxgall; Sigma) and incubated at 37°C for 120 min. Aliquots were also withdrawn at different incubation times (0, 90, and 120 min) to determine plate counts.

A more detailed study of the ability of the WT and acid-resistant strains to survive in MRSC adjusted at a range of acid pH values (2.5, 3.5, 4.0, and 5.0) with HCl was also carried out. Cell suspensions were incubated at 37°C, and samples were withdrawn at different times (0 and 90 min) to determine the plate counts and viability with the LIVE/DEAD BacLight Bacterial Viability stain kit (Invitrogen Co., Carlsbad, CA), according to the manufacturer's instructions. Data

acquisition was performed using an EPICS-XL-MCL flow cytometer (Coulter Corporation, Miami, FL).

The different abilities of the WT and mutant strains to grow in the presence of different concentrations of bile salts (0.5, 1.0, 2.0, and 3.0% [wt/vol]) were also determined (10). The assayed concentrations of bile included those present in physiological conditions (from 0.3 to 2.0%) (34). Briefly, cells from overnight cultures were harvested by centrifugation (6,000 × g, 4°C, 10 min), washed with phosphate-buffered saline, and diluted in fresh medium supplemented with the respective concentrations of bile salts to reach a final OD₆₅₅ of 0.1. Bacterial growth was monitored by measuring the OD₆₅₅ in a Microplate Reader Model 550 (Bio-Rad, Hercules, CA). Growth curves were analyzed with a modified Gompertz model (52).

2D electrophoresis, statistical analysis, and protein identification. Cell extracts were obtained and two-dimensional (2D) electrophoresis was basically performed as described previously (44). Briefly, 350 µg of protein from bifidobacterial extracts was loaded into strips with a pH range of 4 to 7 and focused for 60,000 V · h, and the second dimension was carried out in a 12.5% polyacrylamide-sodium dodecyl sulfate gel. This pH range covers 71.5% of the theoretical proteins of *B. longum* NCC2705 (45). Proteic spots were visualized with Bio-Safe Coomassie blue staining. Spot detection and volume quantitation were carried out with ImageMaster 2D Elite (version 3.10; Amersham Biosciences). At least three independent analyses for each growth condition were performed. An effect of acidic pH on the expression of proteins was considered if the mean normalized spot volume varied at least 1.5-fold and was confirmed by analysis of variance at the significance level of $P < 0.05$ using pH as a factor with two categories, pH 7.0 and pH 4.8. Proteins were identified using the MOWSE (Molecular Weight Search) score (39).

Estimation of the redox balance, the intracellular pH, and the bile salt hydrolase (BSH) activity. The fluorescence properties of buffered cell suspensions (OD₆₀₀ of 0.6) in 50 mM Tris-HCl buffer, pH 7.0, were monitored in an Eclipse fluorescence spectrophotometer (Varian, Inc., Palo Alto, CA). The intensity values corresponding to NADH were calculated from the 413-nm emission at $\lambda_{ex} = 316$ nm, whereas for flavin adenine dinucleotide (FAD) the intensity values were calculated from the 436-nm emission at $\lambda_{ex} = 380$ nm, according to the work of Ammor et al. (1). The redox ratio was deduced from the NADH- and FAD-related signals using the equation $redox_{ratio} = FAD_{intensity} / (FAD_{intensity} + NADH_{intensity})$ (25).

Measurements of intracellular pH values of buffered cell suspensions, grown at pH 7.0 or 4.8, at two different external pHs (7.0 and 4.8) were recorded for the WT and the acid-resistant mutant as previously described (43), and the quantitative determination of the BSH activity was performed according to the method of Noriega et al. (33).

Intracellular amino acid and NH₃ determination. For the extraction of amino acids and ammonia, 2 ml of *B. longum* cultures at an OD₆₀₀ of 0.5 was harvested by centrifugation, washed twice, resuspended in the same volume of 50 mM Tris-HCl adjusted to pH 7, and boiled for 15 min. Cell debris was discarded by centrifugation (13,000 × g, 15 min, 4°C). The supernatants were evaporated in a vacuum concentrator (Eppendorf AG, Hamburg, Germany), resuspended in 20 µl of 0.1 N HCl, and derivatized with dabsyl chloride (Sigma) in the presence of 0.15 mM sodium bicarbonate for 15 min at 70°C, as previously described (26). Ten microliters of the derivatized samples was separated in a Novapak C₁₈ column (Waters Corporation, Milford, CA) at a constant temperature of 50°C, and the amino acid and NH₃ derivatives were detected with a UV detector at a wavelength of 436 nm.

High-pressure liquid chromatography and gas chromatography analysis. For glucose and acetic, lactic, and formic acid determination, studies were performed with buffered cell suspensions. Ten milliliters of cultures at an OD₆₀₀ of 0.6 was collected by centrifugation (10,000 × g for 15 min). The bacterial pellet was washed twice with 50 mM Tris-HCl buffer, pH 7.0, and resuspended in 10 ml of 50 mM Tris-HCl buffer, pH 5.6, containing 25 mM glucose. The suspension was incubated with constant mild stirring for 4 h at 37°C. Cells were removed from the suspension by centrifugation, and the supernatant was analyzed by high-pressure liquid chromatography according to the method of Sánchez et al. (44) to quantify acid levels. Results presented are the means of at least three separate experiments. The ethanol in supernatants of resting cells was determined by dynamic headspace extraction and gas chromatography-mass spectrometry analysis as previously described by Ruas-Madiedo et al. (40).

Nucleotide sequence accession numbers. The genes coding for MetE and CysD, and their upstream regions, were sequenced in both strains, NCIMB 8809 and 8809dpH, and the sequences were submitted under accession numbers EF453722 and EF453723.

TABLE 1. Viability of *B. longum* biotype longum NCIMB 8809 and *B. longum* biotype longum 8809dpH after exposure to different acid pH values in MRSC and effect of bile concentration on growth rates^a

pH	% Viability (SYTO9) ^b		Culturable bacteria (log CFU/ml) ^c		Bile (%)	Relative growth rate (% μ) ^d	
	NCIMB 8809	8809dpH	NCIMB 8809	8809dpH		NCIMB 8809	8809dpH
6.4	94.87 ± 6.62	97.39 ± 1.15	8.08 ± 0.66	8.78 ± 0.54	0.0	100.00	100.00
5.0	68.16 ± 4.30	96.84 ^e ± 0.86	2.67 ± 1.20	8.60 ^e ± 0.90	0.5	45.07 ± 8.20	93.00 ^e ± 5.00
4.0	32.89 ± 1.74	94.06 ^e ± 1.27	1.52 ± 0.70	8.64 ^e ± 0.50	1.0	23.88 ± 7.56	81.20 ^e ± 3.45
3.5	1.79 ± 0.50	77.58 ^e ± 1.80	0.00 ± 0.50	6.39 ^e ± 0.33	2.0	3.94 ± 1.05	72.27 ^e ± 5.00
2.5	0.00 ± 0.10	44.05 ^e ± 1.70	0.00 ± 0.00	5.56 ^e ± 0.65	3.0	0.70 ± 1.00	65.64 ^e ± 4.56

^a Values, except for percent bile, are expressed as means ± standard deviations of at least three independent experiments.

^b Viability was determined by flow cytometry with the LIVE/DEAD kit after 90 min of exposure to acidified MRSC.

^c CFU were counted on MRSC after 90 min of exposure to acidified MRSC.

^d Data are expressed as the percentages of the growth rate (μ ; h⁻¹) obtained in the absence of bile, which was given a value of 100%.

^e Significant difference between WT and mutant strains ($P < 0.05$).

RESULTS

Isolation and characterization of an acid-resistant mutant in *B. longum* biotype longum. In order to isolate acid-resistant mutants from *B. longum* biotype longum NCIMB 8809, cell suspensions of this strain were incubated at different pH values (1.25, 2.0, 4.0, and 5.0), and then plated on MRSC. Small colonies could be obtained only from cultures at pH 4.0 and 5.0, those of pH 4.0 being selected for further characterization and confirmation of their genus and species identity by specific PCR (22, 29). The 16S rRNA gene of one of these isolates, named *B. longum* biotype longum 8809dpH, was partially sequenced, showing 100% identity with its counterpart.

B. longum biotype longum 8809dpH showed significantly higher survival than the WT in simulated gastrointestinal conditions after 90 and 120 min of incubation. While the colony counts of the WT decreased more than 5 units after 90 min of incubation (from 8.85 ± 0.17 to 3.83 ± 0.28 log CFU/ml) and were undetectable (<3 log CFU/ml) after 120 min, the mutant did not show a significant decrease in survival after 120 min (from 8.98 ± 0.10 to 8.78 ± 0.30 log CFU/ml; values are

expressed as means ± standard deviations of at least triplicate experiments). The stability of the acid resistance phenotype of the mutant was also confirmed by the absence of significant variations ($P < 0.05$) in its ability to survive in gastric juices after daily cultivation for 2 weeks in nonacidified MRSC (data not shown).

The viabilities of WT and mutant strains after exposure to a wide range of pH values in MRSC as well as their abilities to grow in the presence of bile are shown in Table 1. The numbers of both culturable (plate counts in MRSC) and viable (LIVE/DEAD kit) cells of the mutant strain were significantly higher ($P < 0.05$) than those of the WT after exposure to a pH range from 2.5 to 5.0. The mutant also showed a significantly ($P < 0.05$) better ability than the WT to grow in the presence of all tested bile concentrations (0.5 to 3%).

Constitutive differences between WT and acid-pH-resistant mutant at neutral pH. The cytoplasmic protein pools were analyzed by means of 2D electrophoresis, and both WT and mutant strains were compared after growth at pH 7.0 (Fig. 1). Under these conditions, the production of nine proteins was

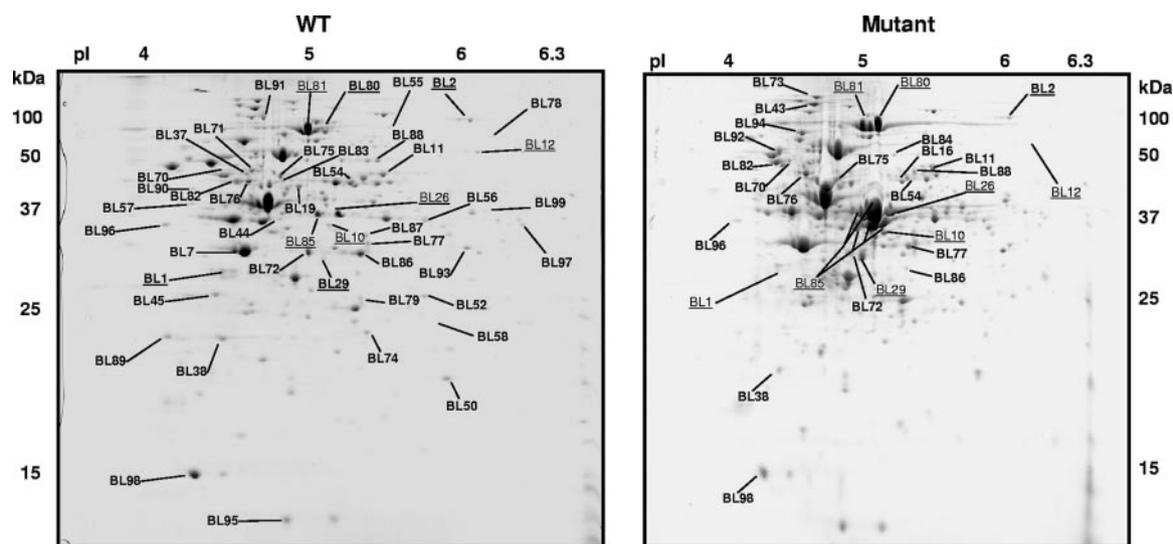


FIG. 1. Representative 2D gels containing cytosolic extracts from mid-exponential-phase cells of *B. longum* biotype longum NCIMB 8809 (left) and *B. longum* biotype longum 8809dpH (right) grown in MRSC initially adjusted to pH 7.0. Spots identified by peptide mass fingerprinting are labeled, and the corresponding identifications are listed in Tables 2 and 3. Spots that showed variations between the WT and the mutant are underlined, whereas those that modified their quantities at pH 4.8 are in bold.

TABLE 2. Proteins whose production was affected by the acquisition of acid tolerance in the strain *B. longum* biotype longum 8809dpH

COG ^a	Spot no. ^b	Putative function ^c	Gene	Accession no.	Molecular mass ^d	pI ^e	Cov. ^f	MOWSE ^g	Prod. ^h
Carbohydrate transport and metabolism	BL81	Xylulose-5-phosphate/fructose-6-phosphate phosphoketolase	<i>xfp</i>	BL0959	92.5	5.0	57	3.59E + 09	Down
	BL26	Glyceraldehyde-3-phosphate dehydrogenase C	<i>gap</i>	BL1363	37.7	5.2	18	2.55E + 07	Up
	BL29	UDP-glucose 4-epimerase	<i>galE1</i>	BL1644	37.3	5.1	34	1.02E + 05	Up
Energy production and conversion	BL2	Aldehyde-alcohol dehydrogenase 2	<i>adh2</i>	BL1575	98.9	5.9	16	4.92E + 09	Down
Amino acid transport and metabolism	BL80	Methionine synthase	<i>metE</i>	BL0798	85.4	5.2	47	4.32E + 25	Up
	BL10	Cystathionine gamma-synthase	<i>metB</i>	BL1155	41.9	5.3	77	1.08E + 11	Up
	BL12	Probable ATP binding protein of ABC transporter for peptides	<i>dppD</i>	BL1390	73.2	5.9	64	1.12E + 15	Down
	BL85	<i>O</i> -Acetylhomoserine (thiol)-lyase	<i>cysD</i>	BL0933	47.6	5.1	48	1.77E + 07	Up
Cell envelope biogenesis, outer membrane	BL1	BSH	<i>bsh</i>	BL0796	35.1	4.7	60	4.59E + 11	Down

^a COG, cluster of orthologous genes.

^b Spot numbers refer to the proteins labeled in Fig. 1.

^c Putative functions were established according to the cluster of orthologous genes for *B. longum* NCC2705.

^d Theoretical molecular mass expressed in kDa.

^e Theoretical isoelectric point expressed in pH.

^f Percentage of sequence coverage.

^g Molecular weight search score.

^h Prod., production. Up-regulation (up) or down-regulation (down) of matching proteins between *B. longum* biotype longum NCIMB 8809 and *B. longum* biotype longum 8809dpH.

clearly affected in the mutant, five and four of them being significantly over- and underproduced, respectively, compared with the WT (Table 2).

Interestingly, the main differences between strains were identified as the methionine synthase (MetE, spot BL80); the *O*-acetylhomoserine (thiol)-lyase (CysD, spot BL85); and, to a lesser extent, the cystathionine gamma-synthase (MetB, spot BL10). To determine if the changes in production of MetE and CysD were related to mutations of their structural gene, the genes coding for these proteins and their upstream regions were sequenced in both strains. No changes were detected between *B. longum* biotype longum NCIMB 8809 and the mutant DNA sequences (accession numbers EF453722 and EF453723), suggesting a pleiotropic effect of a mutation located somewhere else.

In addition, the mutant showed changes in the isoform distribution of two important proteins of the carbohydrate catabolism, i.e., xylulose-5-phosphate/fructose-6-phosphate phosphoketolase (Xfp) and glyceraldehyde-3-phosphate dehydrogenase C (Gap). Two other enzymes involved in carbohydrate metabolism were affected: UDP-glucose 4-epimerase (GalE1) was overproduced, and the aldehyde-alcohol dehydrogenase 2 (Adh2) was underproduced. Surprisingly, BSH was underproduced in the acid-pH-resistant mutant.

Different response mechanisms are induced at low pH in the WT and mutant strains. When the WT strain was grown at pH 4.8 and its growth was compared to that at neutral pH, 41 spots were detected to be differentially produced, 18 of which were overproduced and 23 underproduced. When the mutant strain was cultured at pH 4.8, it showed variations in the production levels of 20 spots, which were smaller in number than the WT strain, 17 and 3 of them being over- and underproduced, respectively (Fig. 1; Table 3). In a general view, up-regulated

spots are predominant in the mutant, whereas down-regulated ones are more abundant in the WT strain. On the other hand, enzymes involved in carbohydrate metabolism, energy production and conversion, and amino acid metabolism groups are generally overproduced, and down-regulated spots are spread among the other functional groups.

Influence of low-pH adaptation on energy recycling under pH challenge. The production of several enzymes involved in different steps of glycolysis was found to be modulated by acid pH in both strains (Fig. 2). Alpha-1,4-glucosidase was overproduced in both strains. Furthermore, the WT strain also showed an increase in the production of phosphoglucomutase and UDP-glucose 4-epimerase. All these enzymes are related to the utilization of complex carbohydrate sources, and they fuel the bifid shunt. Interestingly, transaldolase was highly underproduced after growth at acidic pH in the WT (ratio, -29.19) whereas the mutant increased its amount by a factor of 5.56. Theoretically, this should favor the formation of glyceraldehyde 3-phosphate from fructose 6-phosphate faster in the mutant than in the WT strain. In accordance, a decrease of the glucose consumption was observed for the mutant strain when grown at pH 4.8, together with a moderate increase of the total carbon balance of the bifid shunt (Table 4). However, in the WT strain no noticeable changes in these two features were detected.

Impact of low pH on the BSH of *B. longum* biotype longum. BSH was constitutively down-regulated in the mutant, as well as in the WT, as a response to acidic pH (Tables 2 and 3). Furthermore, cytoplasmic BSH activity followed the same pattern, correlating with the enzyme levels. Indeed, at pH 7.0 the BSH activity is constitutively diminished in cell extracts of the mutant (2.73 ± 0.41 U/mg protein) compared with the WT (10.55 ± 2.79 U/mg protein) and decreases in the WT at pH

TABLE 3. Spot identification by mass spectrometry against the theoretical peptide database of *B. longum* NCC2705 for the strains *B. longum* biotype longum NCIMB 8809 and *B. longum* biotype longum 8809dpH in MRSC adjusted initially to pH 4.8 or 7^a

COG ^b	Spot no. ^c	Putative function ^d	Gene	Accession no.	Cov. ^e	MOWSE ^f	WT ratio ^g	Mutant ratio ^h
Carbohydrate transport and metabolism	BL70	Probable alpha-1,4-glucosidase	<i>aglA</i>	BL0529	50	3.49E + 13	11.90	1.60
	BL71	Phosphoglucomutase	<i>pgm</i>	BL1630	60	8.17E + 12	1.67	— ⁱ
	BL16	Pyruvate kinase	<i>pyk</i>	BL0988	55	2.64E + 10	—	2.18
	BL7	Transketolase	<i>ikt</i>	BL0716	25	1.08E + 10	2.09	—
	BL29	UDP-glucose 4-epimerase	<i>galE1</i>	BL1644	34	1.02E + 05	2.13	—
	BL72	Transaldolase	<i>tal</i>	BL0715	65	1.00E + 08	-29.19	5.56
	BL73	Beta-galactosidase	<i>lacZ</i>	BL0978	39	2.14 + E16	—	2.14
	BL55	Sugar kinase		BL1341	30	1.52E + 07	-14.94	—
	BL74	Ribulose-phosphate 3-epimerase	<i>rpe</i>	BL0753	36	1.06E + 03	-1.68	—
Energy production and conversion	BL75	ATP synthase alpha chain	<i>atpA</i>	BL0359	65	6.70E + 16	1.68	5.09
	BL76	ATP synthase beta chain	<i>atpD</i>	BL0357	78	3.83E + 17	1.75	3.72
	BL77	Lactate dehydrogenase	<i>ldh2</i>	BL1308	77	3.44E + 10	3.50	1.93
	BL2	Aldehyde-alcohol dehydrogenase 2	<i>adh2</i>	BL1575	16	4.92E + 09	-17.42	-3.61
	BL78	Phosphoenolpyruvate carboxylase	<i>ppc</i>	BL0604	45	4.85E + 16	-1.83	—
	BL79	Succinyl coenzyme A synthetase alpha chain	<i>sucD</i>	BL0733	50	3.74E + 10	-4.29	—
Amino acid transport and metabolism	BL80	Methionine synthase	<i>metE</i>	BL0798	19	1.45E + 15	1.87	—
	BL37	Xaa-Pro aminopeptidase I	<i>pepP</i>	BL1350	67	5.05E + 16	1.79	—
	BL82	Glutamine synthetase 1	<i>ghnA1</i>	BL1076	58	3.04E + 12	1.74	4.30
	BL83	Acetylornithine aminotransferase	<i>argD</i>	BL1061	71	2.97E + 09	2.73	—
	BL44	Probable BCAA aminotransferase	<i>ilvE</i>	BL0852	13	2.09E + 07	1.51	—
	BL84	Dihydroxy acid dehydratase	<i>ilvD</i>	BL1788	48	1.14E + 11	—	2.02
	BL57	Peptidase M20 homolog		BL1072	48	1.28E + 10	-2.79	—
	BL86	Ketol-acid reductoisomerase	<i>ilvC2</i>	BL0531	50	2.96E + 09	20.41	7.17
Nucleotide transport and metabolism	BL87	GMP reductase		BL1500	72	4.17E + 10	2.50	—
	BL11	Bifunctional purine protein PurH	<i>purH</i>	BL0735	19	3.89E + 13	-1.54	-1.68
	BL88	IMP dehydrogenase	<i>guaB</i>	BL1722	76	1.38E + 17	-2.03	2.18
	BL89	Probable MTA/SAH nucleosidase	<i>pfs</i>	BL0318	74	4.69E + 06	-285.4	—
Coenzyme metabolism	BL90	Probable bifunctional folate synthesis	<i>sulD</i>	BL1685	20	2.48E + 04	-7.53	—
Translation, ribosomal structure, and biogenesis	BL91	Polyribonucleotide nucleotidyltransferase	<i>pnpA</i>	BL1546	58	5.08E + 20	4.21	—
	BL92	30S ribosomal protein S1	<i>rpsA</i>	BL0992	74	2.05E + 13	—	2.09
	BL19	Glutamyl-tRNA synthetase	<i>gluX</i>	BL0469	66	1.27E + 18	1.61	—
	BL93	Tryptophanyl-tRNA synthetase	<i>trpS</i>	BL0600	23	3.86E + 03	1.76	—
	BL94	Elongation factor G	<i>fusA</i>	BL1098	65	1.39E + 19	—	2.29
	BL38	Probable 50S ribosomal protein L25	<i>rplY</i>	BL0853	10	3.23E + 07	-1.82	1.53
	BL50	Ribosome recycling factor	<i>frr</i>	BL1506	15	1.51E + 04	-2.59	—
	BL95	30S ribosomal protein S6	<i>rpsF</i>	BL0416	82	3.70E + 04	-8.80	—
	BL58	Hypothetical RNA methyltransferase		BL1394	19	3.29E + 03	-5.92	—
Transcription	BL56	N utilization substance homolog	<i>nusA</i>	BL1615	57	4.42E + 08	-1.82	—
	BL96	Probable transcription antitermination protein	<i>nusG</i>	BL1288	41	2.97E + 07	-2.15	2.49
Posttranslational modification, protein turnover, and chaperones	BL97	Chaperone protein	<i>dnaJ</i>	BL0719	56	1.11E + 10	-3.01	—
	BL98	GroES	<i>groES</i>	BL1558	68	4.18E + 02	-2.29	2.02
Cell envelope biogenesis, outer membrane	BL1	BSH	<i>bsh</i>	BL0796	60	4.59E + 11	-12.88	—
DNA replication, recombination, and repair	BL99	RecA protein	<i>recA</i>	BL1415	14	6.29E + 02	-2.97	—
	BL54	Putative ATPase involved in DNA repair		BL0016	53	3.65E + 07	1.73	5.87
	BL43	DNA-directed RNA polymerase beta chain	<i>rpoB</i>	BL1205	47	7.89E + 22	—	-1.87

Continued on following page

TABLE 3—Continued

COG ^b	Spot no. ^c	Putative function ^d	Gene	Accession no.	Cov. ^e	MOWSE ^f	WT ratio ^g	Mutant ratio ^h
Signal transduction mechanisms	BL45	Response regulator of two-component system		BL0005	56	5.21E + 05	-3.88	—
General function prediction only	BL52	Probable ATP binding protein of ABC transporter		BL0870	71	3.11E + 08	-3.88	—

^a Only spots which were over- or underproduced by a factor of greater than 1.5 are included.
^b COG, cluster of orthologous genes.
^c Spot numbers refer to the proteins labeled in Fig. 1.
^d Putative functions were assigned according to the cluster of orthologous genes for *B. longum* NCC2705.
^e Percentage of sequence coverage.
^f Molecular weight search score.
^g Ratio of WT strain value for growth at pH 4.8 to WT strain value for growth at pH 7 for each spot.
^h Ratio of mutant strain value for growth at pH 4.8 to mutant strain value for growth at pH 7 for each spot.
ⁱ —, not significant.

4.8 (2.75 ± 1.72 U/mg protein) but not in the mutant (3.11 ± 1.04 U/mg protein).

Impact of low pH on the physiology of *B. longum* biotype longum. Proteomic analysis showed several changes in the levels of enzymes of pyruvate catabolism (Tables 2 and 3). The production of aldehyde-alcohol dehydrogenase 2 was diminished after growth at pH 4.8 by a factor of 17.42 in *B. longum* biotype longum NCIMB 8809 and by a factor of 3.61 in the mutant, while lactate dehydrogenase (Ldh2) in the same conditions was up-regulated in both strains. No changes in the production of pyruvate formate-lyase (Pfl) or acetate kinase (AckA) were detected. In bifidobacteria, pyruvate is mainly transformed into lactic and acetic acids, giving a theoretical final acetic/lactic ratio of 3:2. Pyruvate can also theoretically be

transformed to formate by pyruvate formate-lyase. Our proteomic data indicate that the exposure to low pH in *B. longum* (WT and mutant) predominantly directed the reduction of pyruvate to lactate by Ldh2. These changes may have some consequences for the various end products generated from pyruvate and may thus also affect the regeneration of NAD⁺ and the NADH/NAD⁺ ratio. Therefore, we measured the different end products, lactic, formic, and acetic acids as well as ethanol (Table 4), and the fluctuations of intracellular pools of NADH and FAD⁺ (Fig. 3), whose fluorescence could be monitored under in vivo conditions (1). We did not detect a strong modification of the acetic/lactic acid ratio and of the ethanol production. However, we observed an increase in formate production in both strains after exposure to low pH. The WT

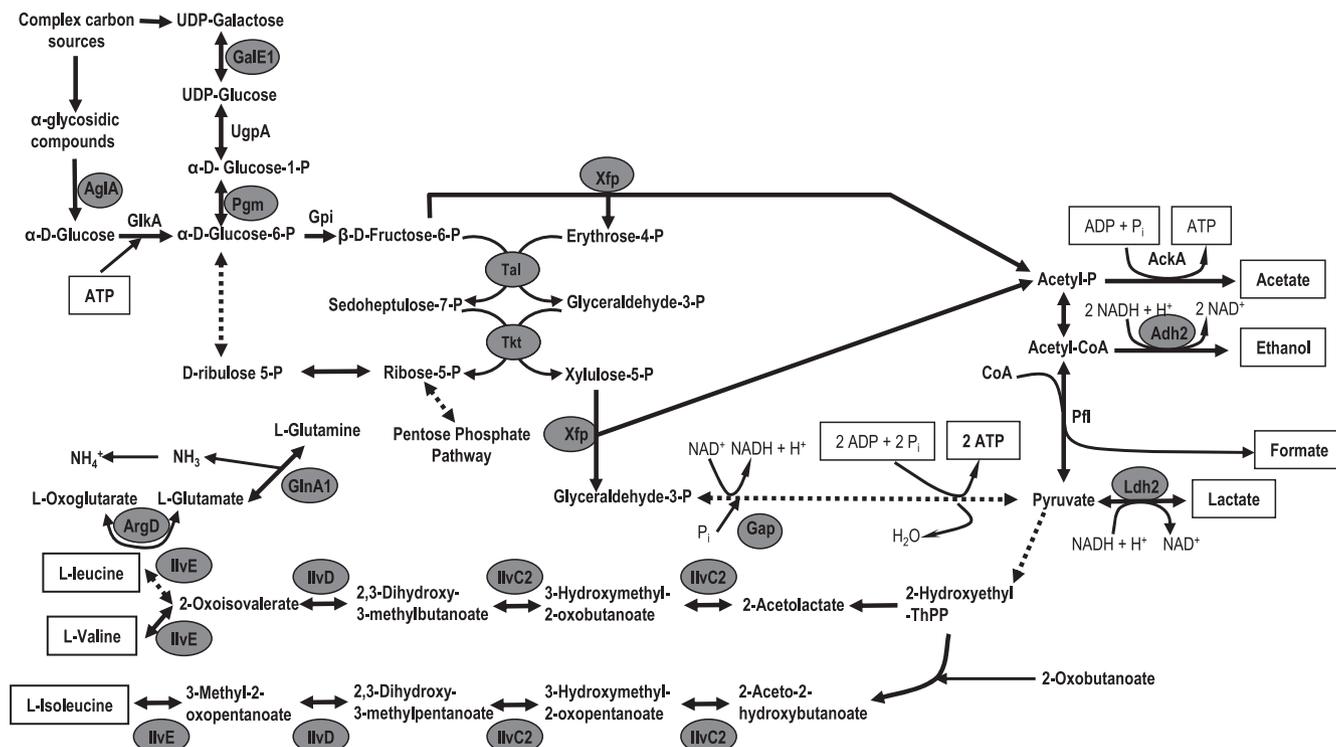


FIG. 2. Schematic representation of the carbon catabolic pathway, the bifid shunt, and the BCAA metabolism. Enzymes whose production was modified after adaptation or exposure to acid pH in *B. longum* are marked with a gray circle.

TABLE 4. Glucose consumed; ethanol and lactic, formic, and acetic acid production; acetic/lactic acid ratio; and carbon balance of buffered resting cells of *B. longum* biotype longum NCIMB 8809 and *B. longum* biotype longum 8809dpH grown in MRSC adjusted to pH 7.0 or pH 4.8^a

Strain and condition	Glucose consumed (mM)	Organic acid formation (mM)			Ethanol formation (mM)	Acetic/lactic ratio	Carbon balance
		Lactic acid	Formic acid	Acetic acid			
NCIMB 8809							
pH 7	6.235 ± 0.281	3.936 ± 0.270	0.000 ± 0.000	9.190 ± 0.692	0.204 ± 0.037	2.334 ± 0.041	0.811 ± 0.026
pH 4.8	6.461 ± 0.527	4.168 ± 0.235	0.459 ± 0.022	9.646 ± 0.519	0.205 ± 0.056	2.314 ± 0.022	0.838 ± 0.042
8809dpH							
pH 7	6.470 ± 0.337	3.190 ± 0.251	0.214 ± 0.010	7.808 ± 0.499	0.233 ± 0.012	2.459 ± 0.246	0.659 ± 0.012
pH 4.8	3.621 ± 1.096	2.694 ± 0.391	0.503 ± 0.073	5.424 ± 0.493	0.240 ± 0.064	2.025 ± 0.106	0.792 ± 0.048

^a Values are expressed as means ± standard deviations of at least three independent experiments.

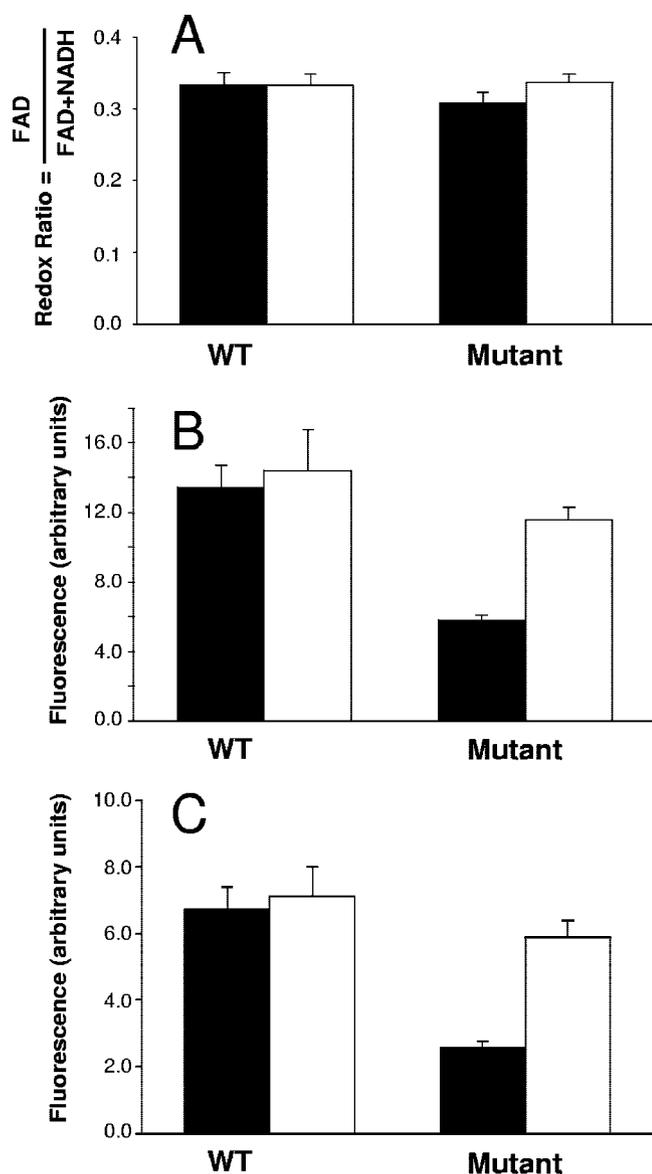


FIG. 3. Redox ratios (A), NADH-associated fluorescence (B), and FAD-associated fluorescence (C) of *B. longum* biotype longum NCIMB 8809 and *B. longum* biotype longum 8809dpH grown at neutral pH (solid bars) or acid pH (white bars). Vertical lines on the bars represent standard deviations.

strain did not produce any detectable formic acid at pH 7.0, whereas the mutant produced 0.214 ± 0.01 mM. At low pH the two strains produced similar amounts of formate (Table 4). As shown in Fig. 3, the growth of the WT and the mutant at pH 4.8 did not influence the global redox ratio ($FAD/[FAD + NADH]$), although in the case of the mutant strain the quantities of both FAD^+ and NADH increased significantly. This was correlated with the preservation of the relationship between acetic and lactic acid production (Table 4).

Several enzymes involved in amino acid metabolism are overproduced at low pH in the WT, some are also induced in the mutant, and one is up-regulated by low pH only in the mutant (Table 3). Among those, we observed *IlvC2*, *IlvD*, and *IlvE*, which are involved in the biosynthesis pathway of branched-chain amino acids (BCAA), and the glutamine synthetase *GlnA1*. As seen in Fig. 2, pyruvate can be driven to the formation of BCAA. Deamination of BCAA has been postulated as a mechanism of maintaining the internal pH of the cells (27). We have thus measured internal pH as well as the BCAA and NH_4^+ concentrations in cytoplasmic extracts of the WT and the mutant strains grown at neutral and acidic pHs (Fig. 4). A higher concentration of valine in *B. longum* biotype longum NCIMB 8809, together with a sharp increase of the ammonium content in both strains, was detected after growth at pH 4.8. This increase was less pronounced in the mutant. Moreover, acetylornithine aminotransferase (*ArgD*; overproduced in the WT) catalyzes a reaction that could serve as a way of recycling the 2-oxoglutarate formed by the BCAA aminotransferase as well as feeding the glutamine synthesis. Remarkably, the overproduction of glutamyl-tRNA synthetase detected in the WT under acidic conditions could also be related to this fact.

Finally, measurements of intracellular pH (pH_{in}) values at two different external pHs (pH_{out}) were recorded for the WT and the mutant strains (Fig. 4). As expected, the higher the pH_{out} , the higher the pH_{in} that was observed. Remarkably, when cells were grown at pH 7.0 and they were put in contact with a pH_{out} of 4.8, the WT was not able to maintain the pH_{in} above 6 whereas the mutant maintained it above 6.5. No significant differences between WT and mutant strains were observed when cells were grown at pH 4.8 (data not shown). Agreeing with this, we found the F_1F_0 -ATPase subunits *AtpA* and *AtpD* in higher amounts under acidic conditions in both

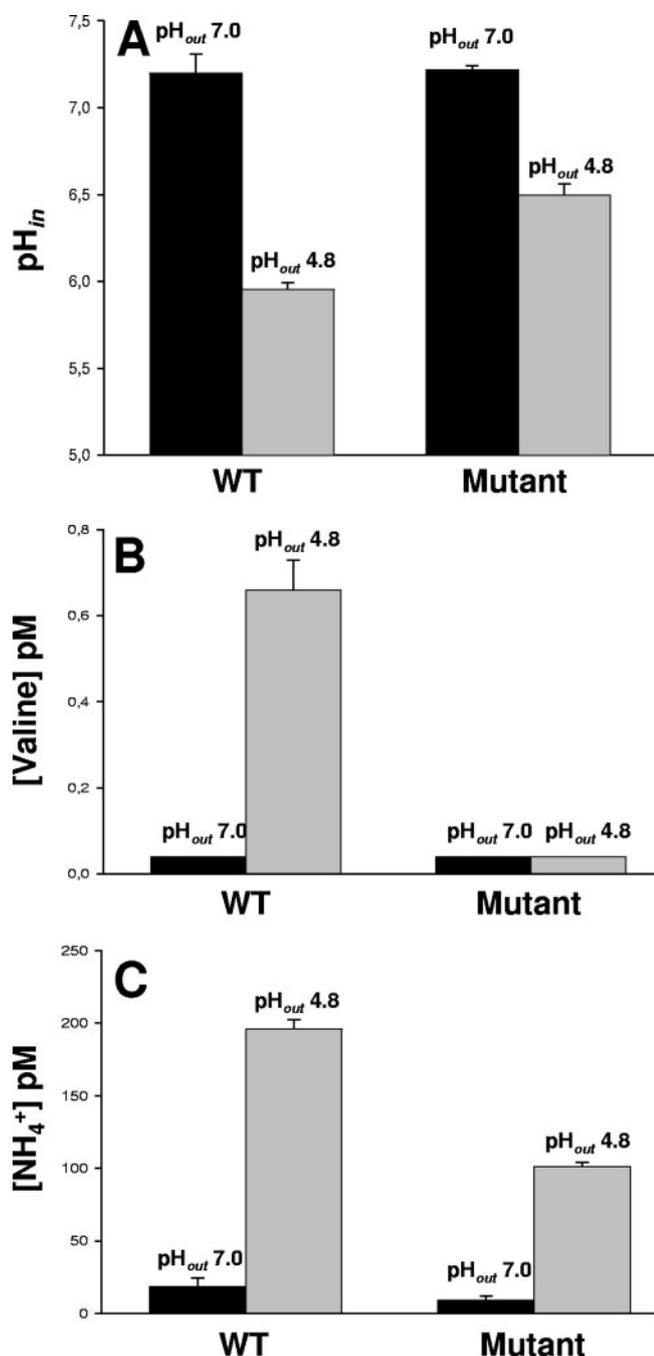


FIG. 4. (A) Intracellular pH of *B. longum* cells (grown in MRSC at pH 7.0) after suspension in buffer at pH 7.0 or pH 4.8. (B and C) Valine concentration (B) and NH₄⁺ concentration (C) measured in cell extracts of *B. longum* biotype longum NCIMB 8809 and *B. longum* biotype longum 8809dpH grown at neutral pH (black bars) or acid pH (grey bars).

strains, although the increase was much more pronounced in the mutant strain (Table 3).

DISCUSSION

Acid resistance has been proposed as a selection marker for potential probiotic bifidobacteria (8, 9, 38). To understand the mechanisms underlying this process, molecular techniques for

disrupting genes and controlling gene expression have been extensively used. However, the lack of efficient transformation systems and effective molecular tools for gene inactivation severely limits functional studies in *Bifidobacterium* species (50). In the present work we have undertaken this challenge by isolating an acid-resistant mutant of a strain of *B. longum* biotype longum.

To unravel the functions involved in acid response in *B. longum* and to search for those responsible for the acid-resistant phenotype of the mutant, we chose a proteomic approach. Three proteins involved in the synthesis of the sulfur amino acids methionine and cysteine, MetE, CysD, and MetB, were strongly overexpressed in the mutant, indicating the impact that the mutation(s) produced could have on sulfur metabolism in *B. longum* biotype longum 8809dpH.

It has been suggested that *B. longum* cysteine biosynthesis and sulfur assimilation may be accomplished by an atypical pathway involving homologs of cystathionine γ -synthase, cystathionine β -synthetase, and cystathionine γ -lyase, using succinylhomoserine and the H₂S or methanethiol produced by other colonic microbiota as substrates (45). However, we have to take into account that the mutant strain was obtained by natural selection, and the large number of proteins identified as being differentially produced between the WT and the mutant point to a mutation(s) in a global regulation system rather than in an individual gene. Therefore, the pleiotropically affected genes might partially mask some of the genes directly involved in acid resistance.

Remarkably, growth rates were higher for the mutant than for the WT in the presence of bile, which pointed to an association between the acquisition of resistance to acid pH and bile salt tolerance in *B. longum*, as postulated before for other *Bifidobacterium* strains (34, 41, 43). Related to this, this work shows that BSH was underproduced in the mutant and underproduced in the WT under acidic conditions. This enzyme is present in many bacterial species of the gastrointestinal tract and catalyzes the release of the amino acid moiety from the conjugated bile salt, rendering deconjugated bile acids. Its activity is one of the positive factors for probiotic selection (3), and its function has been extensively studied (6, 11, 23, 24, 47), but its physiological role in probiotic bacteria remains unclear. Deconjugated bile salts are more toxic than the conjugated ones for *Bifidobacterium* (18, 33), due to their higher detergent activity. Thus, the lower BSH activity detected as a result of the response of the WT and the adaptation of the mutant to low pH in *Bifidobacterium* could represent a way of counteracting bile toxicity. Interestingly, the BSH-encoding gene (BL0796) is located downstream from *metF* (BL0797) and *metE* (BL0798). As mentioned above, MetE is sensitive to low pH and regulated in the opposite way from BSH. We cannot thus yet exclude the possibility that those genes belong to a gene cluster that is pH regulated.

Two of the main changes in protein production were noted for proteins involved in nucleotide metabolism and in transcription/translation. Noticeably, 5'-methylthioadenosine/*S*-adenosylhomocysteine (MTA/SAH) nucleosidase displayed the highest down-regulation ratio at pH 4.8 (−285.4) in the WT, although such a change could not be detected in the mutant. MTA/SAH nucleosidase is a dual substrate-specific enzyme that plays a key role in regulating the cellular levels of the metabolites

MTA and SAH, products involved in bacterial intercellular communication in *Salmonella enterica* serovar Typhimurium 14028 and other gram-negative bacteria (4). In relation to transcriptional/translational processes, differences in behavior between the two strains were observed. Whereas as a response to low pH the strain NCIMB 8809 decreased the amount of the ribosomal protein homologs RplY, Frr, and RpsF, the mutant increased the production of FusA, RpsA, and RplY. The production of some ribosomal proteins has been shown to be dependent on several stress factors in gram-positive bacteria, such as osmotic shock, phosphate and amino acid starvation, and ethanol and heat treatment (2, 14, 15). Concerning chaperone proteins, GroES was overproduced in the mutant at low pH whereas the WT showed a diminution in the amount of both GroES and DnaJ. It has been shown that these proteins are up-regulated when the acid tolerance response is achieved, for example, in *Propionibacterium freudenreichii*, where the overproduction of GroES and GroEL corresponded with a late response to acid pH (20). Those findings agree with our own results as long as chaperone overproduction can be detected only in the late response to acid pH, represented in our model of study by the mutant strain.

On the other hand, the slightly more efficient carbon utilization by *B. longum* biotype longum 8809dpH under acidic conditions suggests that carbons coming from the carbohydrate metabolism may be more efficiently driven to acid formation. The subsequent ATP formation at the substrate level may be directed to a higher yield in the ATP production of the cell, which could feed the mechanisms of proton extrusion mediated by the F_1F_0 -ATPase. The maintenance of internal pH in bacteria lacking a respiratory chain, such as *Bifidobacterium*, is mainly due to this enzyme (30, 43, 49). The overproduction of the two intracellular subunits of the F_1F_0 -ATPase in the WT and mutant strains under acidic conditions strongly points to the involvement of this enzyme in the acid response of *B. longum*, which is in good agreement with the measurements of the intracellular pH. In this respect, it is worthwhile to remark that the higher NH_4^+ concentration detected in both strains at low pH may help to buffer the internal pH (27). Furthermore, the higher capacity of the mutant strain to overproduce AtpA and AtpD, together with the lower levels of ammonium and valine in its cytoplasm under low-pH conditions, with respect to the WT, is likely due to the acquisition of different mechanisms and mutations during the adaptation process.

In conclusion, acid adaptation and response to acid pH involve different mechanisms in *B. longum* biotype longum. We suggest that the response of this bacterium to acid stress involves a number of changes in the levels of different proteins that are jointly dedicated to reduce the impact of acidic conditions in the cell physiology, mainly by controlling the intracellular pH through different mechanisms. Some differences in how the WT or the mutant strain overcomes the acidic environment were evidenced, which could reflect the preadaptation of the mutant strain to the acidic challenge. As a response to low pH, the decrease of the levels of the enzyme BSH and its activity and the overproduction of enzymes related to the utilization of complex carbohydrates may indicate that a pH challenge triggers a response that might allow adaptation for further environmental stresses encountered in the gastrointestinal tract. This study establishes the basis for understanding

the low-pH response and adaptation in the probiotic bacterium *B. longum*.

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