The extracellular wall-bound beta-*N*-acetylglucosaminidase from *Lactobacillus casei* is involved in the metabolism of the human milk oligosaccharide lacto-*N*-triose

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Running title: Lacto-*N*-triose utilization by *Lactobacillus casei*

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ABSTRACT

Human milk oligosaccharides (HMOs) are considered to play a key role in establishing and maintaining the infant gut microbiota. Lacto-*N*-triose forms part of both type 1 and type 2 HMOs and also of the glycan moieties of glycoproteins. Upstream of the previously characterized gene cluster involved in lacto-*N*-biose and galacto-*N*-biose metabolism from *Lactobacillus casei* BL23, there are two genes, *bnaG* and *manA*, encoding for a β-*N*-acetylglucosaminidase precursor and a mannose-6P isomerase, respectively. In this work, we have shown that *L. casei* is able to grow in the presence of lacto-*N*-triose as a carbon source. Inactivation of *bnaG* abolished the growth of *L. casei* on this oligosaccharide, demonstrating that BnaG is involved in its metabolism. Interestingly, whole cells of *bnaG* mutant are totally devoid of β-*N*-acetylglucosaminidase activity, suggesting that BnaG is an extracellular wall-attached enzyme. The purified BnaG enzyme in addition to hydrolyze lacto-*N*-triose into *N*-acetylglucosamine and lactose, also catalyzed the hydrolysis of 3'-*N*-acetylglucosaminyl-mannose and 3'-*N*-acetylgalactosaminyl-galactose. *L. casei* can be cultured in the presence of 3'-*N*-acetylglucosaminyl-mannose as carbon source but curiously, the *bnaG* mutant strain was not impaired in its utilization. These results indicated that the assimilation of 3'-*N*-acetylglucosaminyl-mannose was independent of BnaG. Enzyme activity and growth analysis with a *manA* knockout mutant showed that ManA is involved in the utilization of the mannose moiety of 3'-*N*-acetylglucosaminyl-mannose. This is the first report describing the physiological role of a β-*N*-acetylglucosaminidase in lactobacilli and it supports the metabolic adaptation of *L. casei* to the *N*-acetylglucosaminide-rich gut niche.

INTRODUCTION

Glycans in human milk are present as free oligosaccharides or conjugated bound to proteins and lipids (1, 2), and they have been proposed to directly influence the composition of the infant gut microbiota (3, 4). Furthermore, the free human milk oligosaccharides (HMOs), the third largest solid component in milk, act as prebiotics to promote colonization by beneficial bacterial species (5, 6). HMOs contain a lactose moiety (Galβ1-4Glc) at their reducing end, which is elongated by β1,3-linked lacto-*N*-biose units (Galβ1-3GlcNAc) to give the type 1 HMOs, including lacto-*N*-tetraose (Galβ1-3GlcNAcβ1-3Galβ1-4Glc), or by β1,3/6-linked *N*-acetyllactosamine units (Galβ1-4GlcNAc) to give the type 2 HMOs, such as lacto-*N*-neotetraose (Galβ1-4GlcNAcβ1-3Galβ1-4Glc). Further elongation of these core structures is made by the addition of fucose and sialic acid residues (1). Both types of HMOs contain a lacto-*N*-triose unit (GlcNAcβ1-3Galβ1-4Glc), highlighting the importance of this trisaccharide in the total pool of HMOs. In addition, lacto-*N*-triose and other *N*-acetylhexosaminyl-oligosaccharides also form part of the structure of glycans conjugated to proteins and lipids present in human milk. The carbohydrate moieties of these molecules would also have a prebiotic role, and besides the monosaccharides described above for HMOs they also contain *N*-acetylgalactosamine (GalNAc) and mannose (2).

Species belonging to the genera of *Bifidobacterium* and *Lactobacillus* are found as part of the gastrointestinal microbiota of infants (7-9). Specific *Bifidobacterium* strains are efficient consumers of HMOs (10), which is in agreement with a large number of conserved genes with the predicted capacity to hydrolyze those complex glycan structures (11). This is the case of genes encoding enzymes from the glycoside hydrolase family 20 (GH20) that comprises lacto-*N*-biosidases (EC 3.2.1.140) and β-*N*-acetylhexosaminidases (EC 3.2.1.52). They are endoglycosidases as the lacto-*N*-biosidase from *Bifidobacterium bifidum*, that degrades lacto-*N*-tetraose into lacto-*N*-

biose and lactose (12), or exoglycosidases with specificity for β(1-2), β(1-3), β(1-4) and/or β(1-6) glycosidic linkages (13-15). Unlike *Bifidobacterium* species, data about the capacity of *Lactobacillus* species to metabolize human milk and mucosa-associated glycans are limited. *Lactobacillus casei* species is commonly isolated from faeces of breast-fed infants (7, 8). In this work we used *L. casei* strain BL23 as a laboratory model because it is easy to manipulate genetically. We have previously characterized three α-L-fucosidases from this strain (16) and demonstrated that in *L. casei* species the utilization of fucosyl-α-1,3-*N*-acetylglucosamine, an abundant structural disaccharide present in human milk glycans, is dependent of the specific α-L-fucosidase AlfB (17). Recently, we have identified in *L. casei* BL23 the unique metabolic pathway for both lacto-*N*-biose and galacto-*N*-biose (Galβ1-3GalNAc), the latter is a core structure in mucin glycans. These disaccharides are transported and phosphorylated by the phosphoenolpyruvate:sugar phosphotransferase system (PTS), and then they are hydrolyzed into galactose-6P and the corresponding *N*-acetylhexosamine by the intracellular GH35 phospho-β-galactosidase GnbG (18). A gene encoding a GH20 β-*N*-acetylglucosaminidase from *L. casei* ATCC 27092 has also been cloned and the purified enzyme has been biochemically characterized (19), but neither natural substrates were tested nor was its physiological role determined for this enzyme. In this work we demonstrated that *L. casei* BL23 is able to grow in the presence of lacto-*N*-triose. The chromosomal inactivation of the *bnaG* gene, encoding an extracellular β-*N*-acetylglucosaminidase, prevented the growth on this carbohydrate, revealing for the first time the physiological role of a β-*N*-acetylglucosaminidase from *Lactobacillus*. Additionally, we showed that 3'-*N*-acetylglucosaminyl-mannose is also a fermentable carbon source for *L. casei* and that a mutant for *manA*, encoding a mannose-6P isomerase, is involved in its metabolism.

MATERIALS AND METHODS

Bacterial strains, culture conditions and plasmids. The strains and plasmids used in this study are listed in Table 1. The *Lactobacillus casei* strains were routinely grown at 37ºC under static conditions on MRS medium (Difco). *Escherichia coli*, which was used as host in cloning experiments, was grown in Luria-Bertani medium at 37ºC (Oxoid). The corresponding solid media were prepared by adding 1.8 % agar. *E. coli* DH10B transformants were selected with ampicillin (100 μg/ml) and *E. coli* BE50 with ampicillin (100 μg/ml) and kanamycin (25 μg/ml). *L. casei* transformants were selected with erythromycin (5 μg/ml).

Vector pRV300 (20) was used for cloning experiments with *E. coli* and for insertional inactivation of genes in *L. casei*. Vector pQE80 (Qiagen) was used for protein overproduction. *E. coli* and *L. casei* strains were transformed by electroporation with a Gene Pulser apparatus (Bio-Rad Laboratories) as recommended by the manufacturer (*E. coli*) or as described earlier (*L. casei*) (21).

Culture of *L. casei* strains with oligosaccharides. The *L. casei* strains were grown overnight at 37ºC under static conditions on sugar-free MRS fermentation medium (18). Overnight cultures were diluted to an OD_{550} of 0.05 in 100 μl of MRS medium containing 2 mM of lacto-*N*-neotetraose, lacto-*N*-triose, 3'-*N*-acetylglucosaminyl-mannose, 3'-*N*-acetylgalactosaminyl-galactose, lactose, *N*-acetylglucosamine or mannose. The first four oligosaccharides were obtained from Carbosynth (Compton, Berkshire, UK). Bacterial growth was monitored for 24 h by spectrophotometric measurements every 30 min at 550 nm in 96-well plates at 37ºC without shaking in a POLARstar Omega microplate reader (BMG Labtech, Offenburg, Germany). At least three independent biological replicates for each growth curve were obtained. Results were expressed as means ± standard deviations. The growth rates

(μ) were calculated by using the Gompertz model (Graph Pad Software, San Diego, CA, USA).

DNA manipulation and sequencing. Total DNA was isolated from *L. casei* BL23 as described before (21). Recombinant DNA techniques were performed by following standard procedures (22). All PCR reactions were performed with the Expand High Fidelity PCR System (Roche). DNA sequencing was carried out by the Central Service of Research Support of the University of Valencia (Spain). M13 universal and reverse primers or custom primers hybridizing within the appropriate DNA fragments were used for sequencing. Sequence analyses were carried out with DNAMAN 4.03 for Windows (Lynnon BioSoft) and sequence similarities were analyzed with the BLAST program (23).

Construction of recombinant strains. DNA fragments containing part of *bnaG* and *manA* were obtained by PCR using *L. casei* BL23 chromosomal DNA and the oligonucleotides pairs: BnaGFow (5'-AGCTGCTGCTGATAACACC)/BnaGRev (5'-TCGGCTGGGCGAACTAAG) and ManAFow (5'-TTCATAGTGTCACGCAAGG)/ManA Rev (5'-TGAACGTCATGGTGCCATC), respectively. The PCR products were cloned into pRV300 digested with *Eco*RV. The resulting plasmids pRVbnaG and pRVmanA were cleaved at the unique *Sph*I and *Xag*I restriction sites present in the *bnaG* and *manA* coding regions, respectively. The two digested plasmids were then treated with the Klenow fragment of DNA polymerase I, ligated and transformed. Different constructs were selected in which a frameshift was introduced at the *Sph*I and *Xag*I sites in *bnaG* (pRVbnaG) and *manA* (pRVmanA), respectively. *L. casei* was transformed with each of these plasmids and one erythromycin-resistant clone of each gene carrying the plasmid integrated by single cross-over was grown in MRS without erythromycin for about 200 generations. Cells were plated on MRS and replica plated on MRS plus erythromycin. Antibiotic-sensitive clones were isolated and, among them,

146 one for each gene was selected (BL380 and BL381 strains) in which a second
147 recombination event led to the excision of the plasmid leaving a mutated *bnaG* and
148 *manA* copy, respectively, as was confirmed by sequence analysis of appropriate PCR
149 products.
150
151 **Oligosaccharide and monosaccharide analysis.** To determine the carbohydrates
152 present in the supernatants from the *L. casei* cultures, the cells were removed by
153 centrifugation and the cultures were analyzed by high-performance liquid
154 chromatography (HPLC) using a Jasco PU2080Plus system (*L. casei* cultures on lacto-
155 *N*-neotetraose, lacto-*N*-triose, 3'-*N*-acetylgalactosaminyl-galactose or lactose) or an
156 ICS3000 chromatographic system (Dionex) (*L. casei* cultures on 3'-*N*-
157 acetylglucosaminyl-mannose or mannose). The PU2080Plus system was coupled to a
158 refractive index detector (Jasco RI-2031 Plus) using a Rezex RCM-Monosaccharide
159 column (Phenomenex). The column was kept at 80ºC and the samples were eluted in
160 isocratic mode using water as the mobile phase at a flow rate of 0.6 ml/min. For the
161 Dionex system, with pulsed amperometric detection, a CarboPac PA100 column was
162 used. A combined gradient of 100 to 300 mM NaOH and 0 to 300 mM acetic acid was
163 used (100 mM NaOH during 2 min; 100 to 300 mM NaOH during 3 min; 300 mM NaOH
164 and 0 to 300 mM acetic acid during 15 min) at a flow rate of 1 ml/min. Oligo- and
165 monosaccharides were confirmed by comparison of their retention times with those of
166 standards (glucose, galactose, GlcNAc, GalNAc, mannose, fructose and the di, tri and
167 tetrasaccharides listed in Table 3).
168
169 **BnaG enzyme activity in whole cells and supernatants of *L. casei* cultures.** The
170 *L. casei* strains were grown overnight at 37ºC under static conditions on 10 ml of MRS
171 fermentation medium containing 0.5 % glucose or 0.5 % ribose. When the cultures
172 reached an OD_{550} of 1.5 (glucose) or 1.2 (ribose), cells were collected by centrifugation
173 at 8000 x *g* for 10 min, and the supernatants were subject to ultracentrifugation at

100000 x *g* for 60 min to remove cell debris. The cell pellets washed twice with phosphate buffered saline (PBS) and resuspended in this buffer to an OD_{550} of 2. The BnaG enzyme activity was determined at 37ºC with 2.5 mM 4-nitrophenyl (NP)-*N*-acetyl-β-D-glucosaminide (*p*NPGlcNAc) in 96-well plates. The 4-nitrophenol released was measured by following continuous changes in absorbance at 404 nm using a POLARstar Omega microplate reader (BMG Labtech, Offenburg, Germany). Reaction mixtures (100 μl) containing the substrate in 100 mM sodium phosphate buffer pH 6.5, were initiated by adding 75 μl of cell suspension or culture supernatant.

Expression and purification of His-tagged BnaG and ManA. The coding regions of *bnaG* and *manA* were amplified by PCR using chromosomal DNA from *L. casei* BL23 as template and the primers pairs: 2860BamHIFW (5'-TTTTGGATC CGCTGATAACACCTTGAAAAGCG)/2860HindIIIRV (5'-TTTTAAGCTTTTACCGGTCT GGATTCACAATGG) and 2870BamHIFW (5'-TTTTGGATCCTTGACAGAACCATTATT TTTAAAACC)/2870HindIIIRV (5'-TTTTAAGCTTTTATGCGTCTACCCCTGGAGTAG), respectively, with added restriction sites to the 5´- and 3´-ends (underlined). The PCR fragments were cleaved with the corresponding restriction enzymes and cloned into pQE80 digested with the same enzymes. The resulting plasmids pQEbnaG and pQEmanA were used to transform *E. coli* BE50, and the correct sequence of the inserts was confirmed by DNA sequencing. One clone of each, PE168 (pQEbnaG) and PE169 (pQEmanA), was grown in 0.5 l Luria-Bertani medium with appropriate antibiotics at 20ºC under agitation. When the cultures reach an OD_{550} of 1.0, IPTG (1 mM) was added and incubation was continued for 5 h. Cells were harvested by centrifugation and resuspended in lysis buffer (Tris·HCl 50 mM pH 7.5, NaCl 100 mM, Na_2SO_4 50 mM, dithiothreitol 0.5 mM and phenylmethysulfonyl fluoride 1mM). The recombinant proteins were purified as described previously (18). Fractions containing the proteins of interest were analysed by SDS-PAGE gels, pooled, dialysed against

Tris·HCl 100 mM, pH 7.5, containing 20% glycerol and kept frozen at –80ºC. Protein concentrations were determined by spectrophotometric measurements at 280 nm in a NanoDrop Instrument (Thermo Scientific, Wilmington, USA).

His-tagged BnaG enzyme activity. The activity of the purified His-tagged BnaG enzyme was assayed at 37ºC with different 2/4-NP-sugars (Table 2) at 5 mM in 96-well plates. The 2/4-nitrophenol released was measured by following continuous changes in absorbance at 404 nm using a POLARstar Omega microplate reader (BMG Labtech, Offenburg, Germany). Reaction mixtures (100 μl) containing the substrate in 100 mM Tris-HCl buffer, pH7.0, were initiated by adding 1 μg of enzyme. The optimal pH was determined with 5 mM *p*NPGlcNAc using 100 mM phosphate-citrate buffer, (pH 3.0 to 7.0) and 100 mM glycine-NaOH buffer (pH 7.5 to 9.5). The optimal temperature reaction was analysed in a range from 20 to 60ºC at the optimal pH. Kinetic studies were performed in 100 mM Tris-HCl buffer, pH 7.0, at 37ºC with 4 *p*NPGlcNAc ranging from 0.5 to 7.5 mM.

In order to determine the ability of BnaG to hydrolyse natural oligosaccharides, several substrates were tested (Table 2). The reactions (100 μl) were performed at 37ºC for 16 h using 4mM substrate in 100 mM Tris-HCl buffer, pH7.0. The reaction mixtures were analysed by HPLC using the Jasco PU2080Plus system as described above.

His-tagged ManA enzyme activity. The activity of the purified His-tagged ManA enzyme was assayed as previously described (24) with some modifications. A typical reaction mixture (100 μl) contained 50 mM Tris-HCl buffer, pH 8.0, 5 mM $MgCl_2$, 1 mM $NADP^+$, 0.5 U of phosphoglucose isomerase, 1.0 U of glucose-6P dehydrogenase and 3 mM mannose-6P. The reactions were initiated by adding 2 μg of enzyme. The activity was assayed at 37ºC in 96-well plates and the NADPH formation was monitored by

following continuous changes in absorbance at 340 nm using a POLARstar Omega microplate reader (BMG Labtech, Offenburg, Germany). Kinetic studies were performed with mannose-6 P concentrations ranging from 1.0 to 10 mM.

Nucleotide sequence accession numbers. The sequences of the truncated genes were deposited in GenBank under accession numbers KT954009 and KT954010.

RESULTS AND DISCUSSION

***L. casei* β-*N*-acetylglucosaminidase BnaG is an extracellular wall-attached protein.** Analysis of the genome surroundings of the *gnb* operon, which is involved in the utilization of lacto-*N*-biose and galacto-*N*-biose in *L. casei* BL23 (18), revealed the presence of two genes, one named here as *bnaG* (LCABL_02860) and *manA* (LCABL_02870) that are annotated as a putative β-*N*-acetylglucosaminidase precursor and a putative mannose-6P isomerase, respectively (Fig. 1A). The deduced amino acid sequence of *bnaG* showed a conserved protein domain GH20_DspB_LnbB-like (cd06564), ranging from amino acids 38 to 377 (25). Sequence analysis using SignalP-4.0 (http://www.cbs.dtu.dk) additionally showed that BnaG displayed an N-terminal signal peptide for secretion (amino acids 1 to 32), and a predicted C-terminal sortase-dependent cell wall-anchoring domain (amino acids 533 to 569) was already described (26). In order to determine if the *bnaG* gene was responsible of the β-N-acetylglucosaminidase activity in whole cells of *L. casei* BL23 (26), a mutant in *bnaG* was constructed (strain BL380). The results showed that in the wild-type the activity was higher in ribose than in glucose grown cells (Table 2), confirming previous studies that showed that the β-N-acetylglucosaminidase activity in *L. casei* was subjected to catabolic repression by glucose (27). β-N-acetylglucosaminidase activity was also found in the supernatants of BL23 strain (Table 2), suggesting that part of the enzyme is liberated as a free enzyme to the culture medium. Interestingly, whole cells and supernatants of the BL380 mutant are totally devoid of activity (Table 2), indicating that *bnaG* gene is responsible of the β-N-acetylglucosaminidase activity present in *L. casei*.

Substrate specificity and enzymatic characteristics of *L. casei* β-*N*-acetylglucosaminidase BnaG. The His-tagged β-*N*-acetylglucosaminidase BnaG from *L. casei* without the N-terminal signal peptide and the C-terminal cell wall-

anchoring domain was expressed in *E. coli*. The purified protein showed a molecular weight of 55 kDa, in agreement with the calculated mass of the 6x(His)-tagged protein (55.250 Da; Fig. 2). The recombinant BnaG hydrolyzed *p*NPGlcNAc and 2-NP-β-D-galactopyranoside, however the latter was poorly hydrolyzed (Table 3). The catalytic properties of BnaG using *p*NPGlcNAc as substrate were determined (Table 3). Although the deduced amino acid sequences of BnaG and the *N*-acetylglucosaminidase from *L. casei* ATCC 27092 (19) showed high homology (91.7 % identity), the *Km*, optimum pH and temperature were different. The *Km* (6.4 μM) of the enzyme from the ATCC 27092 strain showed that the affinity for the substrate *p*NPGlcNAc was about 350-fold higher than that of BnaG. The modifications of the sequence described above for the purified BnaG could account for those differences between both β-*N*-acetylglucosaminidases. Indeed, the enzyme from the ATCC 27092 strain showed a lower molecular weight (39 kDa) (19) than that determined for BnaG. Regarding natural oligosaccharides, BnaG is able to degrade lacto-*N*-triose into GlcNAc and lactose. However, it does not hydrolyze lacto-*N*-tetraose or lacto-*N*-neotetraose, which contain the same GlcNAcβ1,3-glycosidic linkage, indicating that BnaG showed exoglycosidase but not endoglycosidase activity. BnaG degrades also the disaccharides 3'-*N*-Acetylglucosaminyl-mannose, 3'-*N*-Acetylgalactosaminyl-galactose, galacto-*N*-biose and lacto-*N*-biose, although the last two were cleaved very inefficiently (Table 3). These results indicated that the β-*N*-acetylglucosaminidase BnaG from *L. casei* showed specificity for β1,3-glycosidic linkages, and within these *N*-Acetylhexosaminyl-β1,3-linked sugars are better substrates than their corresponding hexoses.

***L. casei* ferments lacto-*N*-triose and hydrolyzes it by using the β-*N*-acetyl-glucosaminidase BnaG**. In order to determine the capacity of *L. casei* BL23 to metabolize lacto-*N*-triose, 3'-*N*-acetylglucosaminyl-mannose and 3'-*N*-

288 acetylgalactosaminyl-galactose, which were substrates *in vitro* for the β-*N*-acetyl-
289 glucosaminidase BnaG, the growth profile in MRS basal medium supplemented with 2
290 mM of those oligosaccharides was determined independently. *L. casei* BL23 was able
291 to grow in the presence of lacto-*N*-triose and 3'-*N*-Acetylglucosaminyl-mannose,
292 respectively, as fermentable carbon sources (Fig. 3A). However, the presence of 3'-*N*-
293 acetylgalactosaminyl-galactose resulted in a diminished growth, compared with the
294 residual growth of *L. casei* in non-supplemented MRS basal medium (Fig. 3A). The
295 growth pattern of *L. casei* in the presence of lactose as a positive control is also shown
296 (Fig. 3A). To determine if the β-*N*-acetyl-glucosaminidase BnaG was involved in the
297 utilization of lacto-*N*-triose and 3'-*N*-acetylglucosaminyl-mannose in *L. casei* BL23, the
298 mutant strain BL380 (*bnaG*) was cultured in the presence of those oligosaccharides,
299 respectively, as carbon sources. Interestingly, this mutant exhibited a diminished
300 growth with lacto-*N*-triose which was comparable to that of the negative control (non-
301 supplemented MRS basal medium), indicating that BnaG is necessary for the utilization
302 of this oligosaccharide (Fig. 3B). Sugar content analysis of the culture supernatants
303 detected lacto-*N*-triose in the supernatants from BL380 (*bnaG*), while it was completely
304 consumed by the wild-type BL23 strain. In the wild type strain, the resulting products
305 (GlcNAc and lactose) from the lacto-*N*-triose hydrolysis by BnaG were also exhausted.
306 The above results suggested that BnaG is an extracellular enzyme, and then it will
307 hydrolyze lacto-*N*-triose outside the cells into GlcNAc and lactose. GlcNAc would be
308 then transported inside the cells by a yet unknown PTS and/or a PTS-independent
309 permease (28). The lactose moiety would be internalized by the already characterized
310 PTS^{Lac} into the cells as lactose-6P, and this phosphorylated sugar will be degraded by
311 the phospho-β-galactosidase LacG into galactose-6P and glucose (29) (Fig. 1B).
312 Although 3'-*N*-Acetylglucosaminyl-mannose is an *in vitro* substrate for BnaG, the
313 results showed that the growth rate (0.120 h^{-1}) of BL380 (*bnaG*) is similar to the rate of
314 the wild-type (0.113 h^{-1}) (Fig. 3B), suggesting that its metabolism is independent of that

glycosidase. Sugar content analysis of the culture supernatants did not detect 3'-*N*-Acetylglucosaminyl-mannose either in BL23 or BL380 culture supernatants.

The metabolism of lacto-*N*-triose resulting from the catabolism of lacto-*N*-neotetraose has been previously reported in *B. bifidum* (13). This tetra-saccharide was sequentially hydrolyzed from its non-reducing terminus by two extracellular exo-enzymes, with β-galactosidase and β-*N*-acetylglucosaminidase activity, respectively (13). *L. casei* is able to grow in the presence of lacto-*N*-triose, whereas it was not able to ferment lacto-*N*-neotetraose (data not shown), suggesting that this strain lacks a β-galactosidase activity able to convert the tetra-saccharide into galactose and lacto-*N*-triose, at least under our experimental conditions. Analysis of the available genome sequences of lactobacilli (http://www.ncbi.nlm.nih.gov/genomes) showed that the *bnaG* gene was only present in the *L. casei* and *Lactobacillus paracasei* species. Notably, it was not conserved in the phylogenetically related *Lactobacillus rhamnosus* species. The lack of *bnaG* gene in these latter species correlated with the inability of two strains of *L. rhamnosus* (strains ATCC 9595 and ATCC 53103 [GG]) to metabolize lacto-*N*-triose. However, *L. rhamnosus* strain HN001 was able to grow using lacto-*N*-triose as carbon source (G. N. Bidart, J. Rodríguez-Díaz and M.J. Yebra, unpublished results), suggesting that in addition to BnaG, lactobacilli have other enzymes involved in the catabolism of this tri-saccharide.

ManA is required for mannose and 3'-*N*-Acetylglucosaminyl-mannose metabolism in *L. casei*. As shown above 3'-*N*-Acetylglucosaminyl-mannose was a carbon source for *L. casei* BL23, and a mutant deficient in the general component enzyme I of the PTS (28) was unable to ferment that disaccharide, which remained non-hydrolyzed in the culture supernatant (data not shown). These results suggest that 3'-*N*-Acetylglucosaminyl-mannose is transported and phosphorylated by the PTS and it might be hydrolyzed inside the cells by a yet unknown phospho-β-glycosidase (Fig.

342 1B). The PTS phosphorylates the incoming disaccharides at the non-reducing end (18,
343 29), therefore the intracellular products generated from 3'-*N*-Acetylglucosaminyl-
344 mannose would probably be GlcNAc-P and mannose. We analyzed if the *manA* gene
345 was involved in the metabolism of that disaccharide. The deduced amino acid
346 sequence of *manA* showed a 53 % and 48 % identity with the characterized type I
347 mannose-6P isomerase (EC.5.3.1.8) from *Streptococcus mutans* (30) and *Bacillus*
348 *subtilis* (31), respectively. The *manA* gene from *L. casei* BL23 codes for a 323 amino
349 acids protein, without a signal peptide or a cell wall-anchoring motif, and it was
350 expressed in *E. coli*. The purified His-tagged protein showed a molecular weight of 37
351 kDa, in agreement with the calculated mass of the 6x(His)-tagged protein (36.815 Da;
352 Fig. 2). The ManA activity was analyzed by following the conversion of mannose-6P
353 into fructose-6P and it exhibited a Vmax of 1.11 μmol/mg protein/min and a Km of 2.78
354 mM. This and the results presented below also suggested that the mannose resulting
355 from the hydrolysis of 3'-*N*-Acetylglucosaminyl-mannose would be phosphorylated by a
356 mannose kinase at the C-6 position. Mannose-6P will be converted into the glycolytic
357 intermediary fructose-6P by ManA (Fig. 1B).

358 To determine if the mannose-6P isomerase ManA was involved in the utilization of
359 3'-*N*-Acetylglucosaminyl-mannose in *L. casei* BL23, a mutant in *manA* was constructed
360 (strain BL381). Interestingly, this mutant showed a growth rate (0.064 h^{-1}) lower than
361 the wild-type strain BL23 (0.113 h^{-1}) with that disaccharide as carbon source in the
362 culture medium (Fig. 4), indicating that *manA* is involved in the utilization of 3'-*N*-
363 Acetylglucosaminyl-mannose. However, mannose was not detected in the culture
364 supernatants from BL381, which is in agreement with the fact that the maximum cell
365 density reached by this strain was similar to that of the wild-type BL23. The growth of
366 strain BL381 (*manA*) with mannose as carbon source was lower (0.026 h^{-1}) to that of
367 the negative control (non-supplemented MRS basal medium) (0.038 h^{-1}) (Fig. 4B),
368 indicating that ManA is necessary for the utilization of mannose in *L. casei*. Curiously,
369 after 24 h the culture reached an OD similar to that of the control, which indicates that

370 BL381 growth is recovering. These results and the growth profile of BL381 strain in the
371 presence of 3'-*N*-Acetylglucosaminyl-mannose suggested that additional genes
372 involved in the metabolism of the mannose might be expressed. Using the deduced
373 amino acid sequence of ManA in BLAST searches against the *L. casei* BL23 genome
374 (32), another mannose-6P isomerase was found (LCABL_06290). It showed a 47 %
375 homology (30 % identity) with the ManA characterized here. Hence, the catabolism of
376 3'-*N*-Acetylglucosaminyl-mannose in the *manA* mutant might depend on this additional
377 mannose-6P isomerase able to utilize the mannose-6P generated.

378 *Lactobacillus casei* species is associated with the infant and adult human
379 gastrointestinal tract and some strains are considered beneficial for the host (7, 8, 33).
380 The establishment of this species in the gut and its interaction with the host will rely on
381 its nutritional capacities. The extracellular localization of the β-*N*-acetylglucosaminidase
382 study here and its activity on human milk and mucosa-linked glycans probably
383 constitute a molecular strategy of these bacteria to survive in the gut.

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519 **TABLE 1**. Strains and plasmids used in this study[a]

Strain or plasmid	Relevant genotype or properties	Source or reference[a]
Strains		
Lactobacillus casei		
BL23	Wild type	CECT 5275
BL126	BL23 *ptsI*	(28)
BL380	BL23 *bnaG* (frameshift at *Sph*I site)	This work
BL381	BL23 *manA* (frameshift at *Xag*I site)	This work
Escherichia coli		
DH10B	F^- *endA1 recA1 galE15 galK16 nupG rpsL* Δl*acX74* Φ80l*acZ*ΔM15 *araD139*	Invitrogen
BE50	BL21(DE3) containing pREPGroES/GroEL	(34)
PE168	BE50 containing pQEbnaG	This work
PE169	BE50 containing pQEmanA	This work
Plasmids		
pRV300	Suicide vector carrying Erm^R from pAMβ1	(20)
pRVbnaG	pRV300 with a frameshift at *Sph*I site in *bnaG*	This work
pRVmanA	pRV300 with a frameshift at *Xag*I site in *manA*	This work
pQE80	*E. coli* expression vector; Amp^R	Qiagen
pQE bnaG	pQE80 containing *bnaG*-coding region	This work
pQEmanA	pQE80 containing *manA*-coding region	This work

520 [a]CECT, Colección Española de Cultivos Tipo; Erm^R, erythromycin resistance; Amp^R,
521 ampicillin resistant.

TABLE 2. *N*-acetylglucosaminidase activity in whole cells and supernatants of *L. casei* cultured in MRS fermentation medium with glucose or ribose[a]

	N-acetylglucosaminidase activity[b] (nmol/min/OD)	
Strains	Glucose	Ribose
Whole cells		
BL23 (WT)	0.223 ± 0.015	2.962 ± 0.011
BL380 (*bnaG*)	0.0 ± 0.013	0.0 ± 0.015
Supernatants		
BL23 (WT)	0.247 ± 0.003	0.934 ± 0.075
BL380 (*bnaG*)	0.0 ± 0.0	0.0 ± 0.0

[a]*N*-acetylglucosaminidase activity was determined with 4-nitrophenyl-β-D-glucosaminide as the substrate.

[b]Each value represents the mean of four different measurements ± standard deviation.

530 **TABLE 3**. Activity and characterization of enzyme BnaG

Substrate[a] (Structure)	**Activity[b]**
4-NP-*N*-acetyl-β-D-glucosaminide	+
2-NP-β-D-galactopyranoside	+/-
4-NP-β-D-glucopyranoside	-
2-NP-1-thio-β-D-galactopyranoside	-
4-NP-β-D-glucuronide,	-
4-NP-α-D-glucopyranoside	-
4-NP-α-D- galactopyranoside	-
4-NP-α-L- fucopyranoside	-
Lacto-*N*-triose (GlcNAcβ1-3Galβ1-4Glc)	+
3'-*N*-Acetylglucosaminyl-Man (GlcNAcβ1-3Man)	+
3'-*N*-Acetylgalactosaminyl-Gal (GalNAcβ1-3Gal)	+
Galacto-*N*-biose (Galβ1-3GalNAc)	+/-
Lacto-*N*-biose (Galβ1-3GlcNAc)	+/-
Lacto-*N*-tetraose (Galβ1-3GlcNAcβ1-3Galβ1-4Glc)	-
Lacto-*N*-neotetraose (Galβ1-4GlcNAcβ1-3Galβ1-4Glc)	-
6'-Galactopyranosyl-GlcNAc (Galβ1-6GlcNAc)	-
4'-Mannopyranosyl-GlcNAc (Manβ1-4GlcNAc)	-
4'-Galactofuranosyl-GlcNAc (Galβ1-4GlcNAc)	-
Lactose (Galβ1-4Glc)	-
N-acetyl-lactosamine (Galβ1-4GlcNAc)	-
Lactulose (Galβ1-4Fru)	-
Maltose (Glcα1-4Glc)	-
Maltotriose (Glcα1-4Glcα1-4Glc)	-
Characterization[c]	
V_{max} (μmol (mg protein/min)	33.3
K_m (mM)	2.3
Optimal pH	6.5
Optimal temperature (ºC)	53

531 [a]Carbohydrates used as substrates. NP, nitrophenyl; Glc, glucose; Gal,
532 Galactose; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine;
533 Man, mannose; Fru, fructose.
534 [b]+, substrate is totally hydrolyzed after 16 h reaction in the conditions described
535 in the "Materials and methods" section; +/-, substrate is partially hydrolyzed
536 after 16 h reaction in the conditions described in the "Materials and methods"
537 section; -, no activity detected.
538 [c]The enzyme activity was determined with 4-nitrophenyl-β-D-glucosaminide as
539 the substrate.

FIGURE LEGENDS

FIG. 1. (A) Schematic presentation of the 11.2 kbp *Lactobacillus casei* BL23 DNA fragment containing the β-*N*-acetylglucosaminidase precursor and a mannose-6P isomerase in the surroundings of the galacto-*N*-biose (*gnb*) operon (18). The first (*gnbR*) and last (*gnbA*) genes of this operon are represented. (B) Schematic presentation of proposed pathways for Lacto-*N*-triose and 3'-*N*-Acetylglucosaminyl-mannose metabolism in *L. casei* BL23. PTS: phosphoenolpyruvate: phosphotransferase system; GlcNAc: *N*-acetylglucosamine; Gal: galactose; Man: mannose; LacG: phospho-β-galactosidase; NagA: *N*-acetylglucosamine-6P deacetylase; NagB: glucosamine-6P deaminase

FIG. 2. Coomasie brilliant blue stained 10% SDS-PAGE showing the His-tagged proteins β-*N*-acetylglucosaminidase BnaG and mannose-6P isomerase ManA. P indicates protein standards and the numbers on the right are the molecular weights.

FIG. 3. (A) Growth curves of *Lactobacillus casei* wild type strain BL23 on MRS basal medium without carbon source (black), with lacto-*N*-triose (red), 3'-*N*-Acetylglucosaminyl-mannose (blue), 3'-*N*-Acetylgalactosaminyl-galactose (dark cyan) or lactose (green). (B) Growth curves of *L. casei* mutant strain BL380 (*bnaG*) on MRS basal medium without carbon source (black), with lacto-*N*-triose (red), 3'-*N*-Acetylglucosaminyl-mannose (blue) or lactose (green). Data presented are mean values based on at least three replicates. Error bars indicate standard deviations.

FIG. 4. Growth curves of *Lactobacillus casei* wild type strain BL23 (A) and *L. casei* mutant strain BL381 (*manA*) (B) on MRS basal medium without carbon source (black),

566 with 3'-*N*-Acetylglucosaminyl-mannose (blue) or mannose (pink). Data presented are
567 mean values based on at least three replicates. Error bars indicate standard deviations.

Figure 1

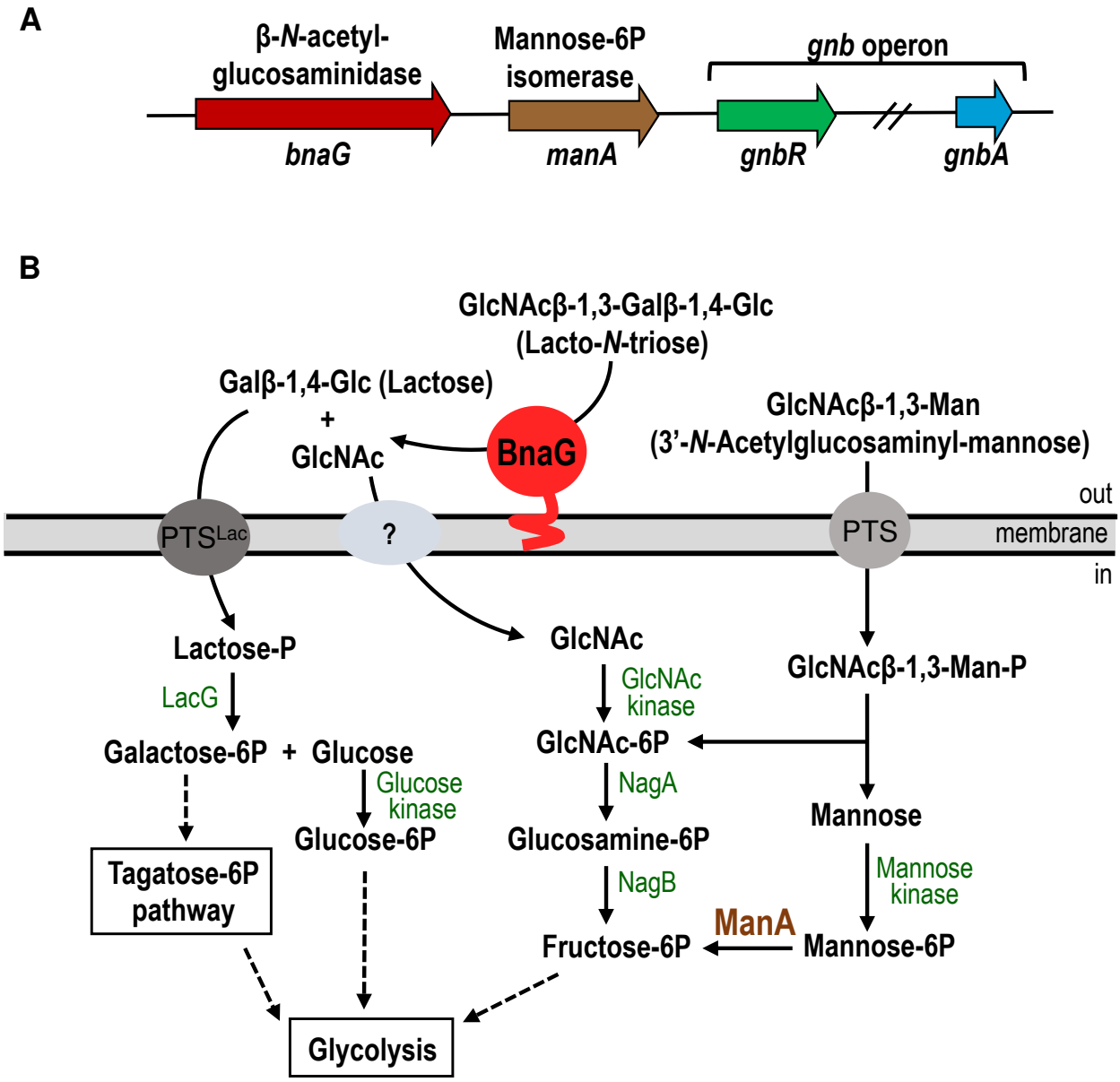

A
β-N-acetyl-glucosaminidase
Mannose-6P isomerase
gnb operon
bnaG
manA
gnbR
gnbA
B
GlcNAcβ-1,3-Galβ-1,4-Glc
(Lacto-N-triose)
Galβ-1,4-Glc (Lactose)
+
GlcNAc
BnaG
GlcNAcβ-1,3-Man
(3'-N-Acetylglucosaminyl-mannose)
out
membrane
in
PTSLac
?
PTS
Lactose-P
LacG
Galactose-6P + Glucose
Glucose kinase
Glucose-6P
Tagatose-6P pathway
GlcNAc
GlcNAc kinase
GlcNAc-6P
NagA
Glucosamine-6P
NagB
Fructose-6P
ManA
GlcNAcβ-1,3-Man-P
Mannose
Mannose kinase
Mannose-6P
Glycolysis

Figure 2

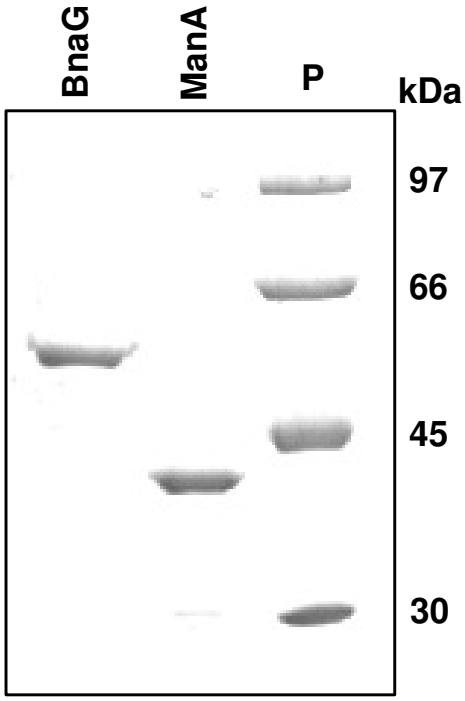


Figure 3

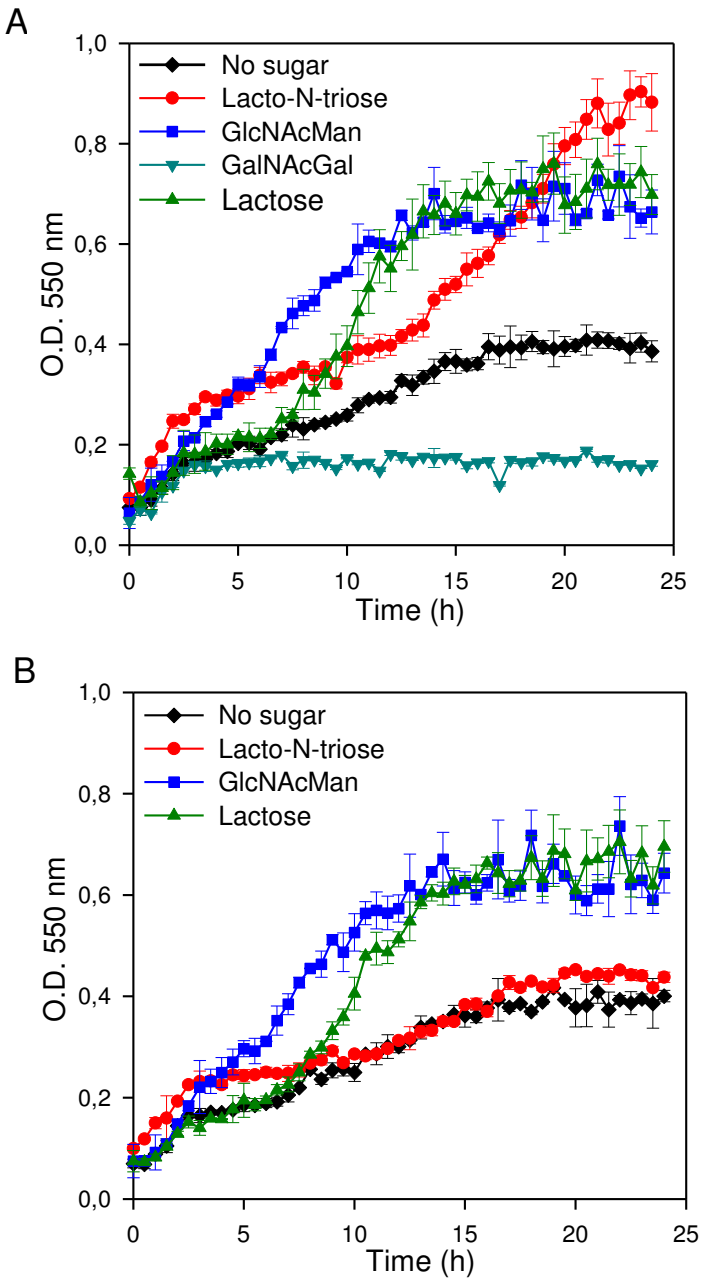
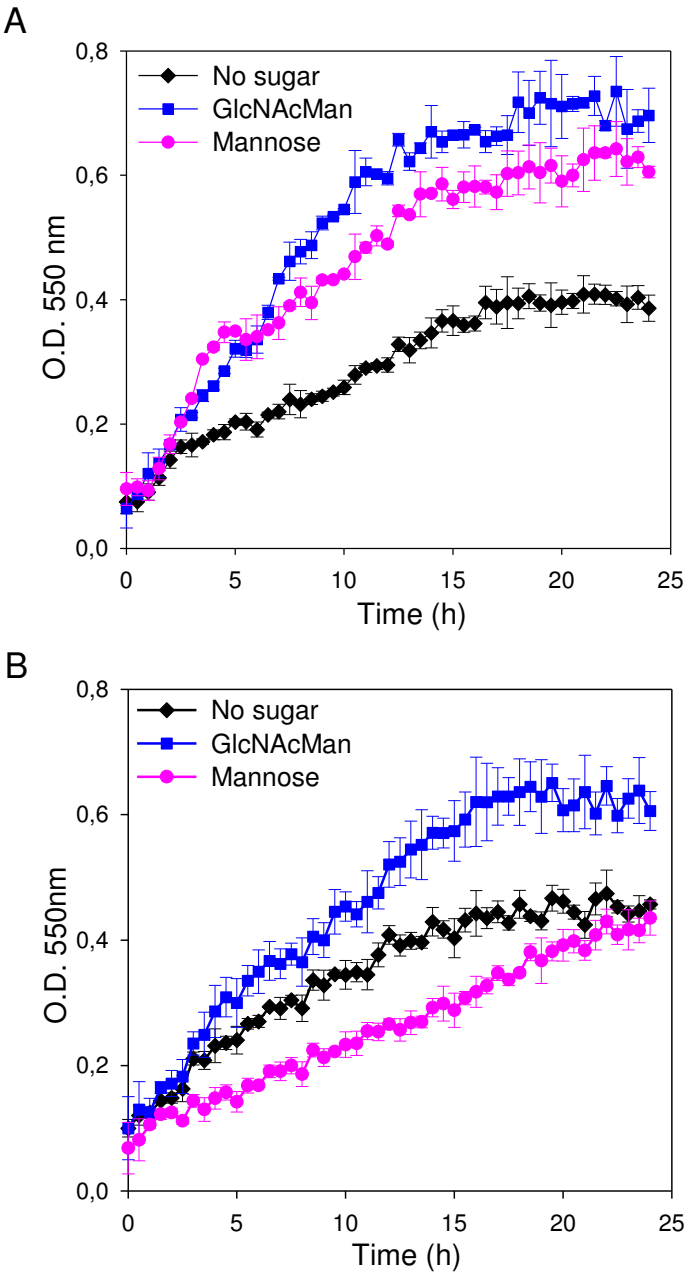

A
No sugar
Lacto-N-triose
GlcNAcMan
GalNAcGal
Lactose
O.D. 550 nm
Time (h)
B
No sugar
Lacto-N-triose
GlcNAcMan
Lactose
O.D. 550 nm
Time (h)

Figure 4


A
No sugar
GlcNAcMan
Mannose
O.D. 550 nm
Time (h)
0,0
0,2
0,4
0,6
0,8
0
5
10
15
20
25
B
No sugar
GlcNAcMan
Mannose
O.D. 550nm
Time (h)
0,0
0,2
0,4
0,6
0,8
0
5
10
15
20
25