Arch Microbiol (2000) 174 : 74–80
Digital Object Identifier (DOI) 10.1007/s002030000178

ORIGINAL PAPER

**Maddalena Rossi · Luigi Altomare
Antonio Gonzàlez Vara y Rodriguez · Patrizia Brigidi
Diego Matteuzzi**

Nucleotide sequence, expression and transcriptional analysis of the *Bifidobacterium longum* MB 219 *lacZ* gene

Received: 29 November 1999 / Revised: 5 May 2000 / Accepted: 8 May 2000 / Published online: 21 June 2000

Abstract The gene encoding β-galactosidase was isolated by functional complementation of *Escherichia coli* from *Bifidobacterium longum* MB219, which exhibited the highest activity among ten *Bifidobacterium* strains tested of the species *B. longum*, *B. breve*, *B. adolescentis*, *B. indicum*, *B. animalis* and *B. cuniculi*. The nucleotide sequence of the 5.0-kb fragment conferring the positive β-galactosidase phenotype to *E. coli* revealed the presence of a *lacZ*-type gene encoding a 1023-amino-acid protein that was preceded by a ribosome binding site. A sequence showing 72% identity with the proline tRNA of *Bacillus subtilis* and a gene probably encoding the DNA-3-methyladenine glycosydase I were located downstream from the *lacZ* gene, after a gap of 30–50 unsequenced base pairs. By primer-extension analysis, the transcription start site of the *lacZ* gene was mapped 65 nt upstream from the start codon, and it enabled identification of the –10 region of the putative promoter. The nucleotide sequence of *lacZ* and its deduced amino acid sequence were compared with those of β-galactosidase genes and enzymes from other microorganisms. High similarity was demonstrated between the *B. longum* β-galactosidase and its counterparts in *Lactobacillus delbruckii* subsp. *bulgaricus*, *Streptococcus salivarius* subsp. *thermophilus*, *E. coli*, *Clostridium acetobutylicum*, *Leuconostoc lactis*, *Klebsiella pneumoniae* and *Kluyveromyces marxianus* var. *lactis*, all belonging to the LacZ family. The *B. longum* MB219 *lacZ* gene was cloned in *Bifidobacterium* and its expression was observed in strains with otherwise low levels of endogenous activity. The expression increased by factors of 1.5–50 and enabled those strains that do not grow on lactose to use this sugar as sole carbon source.

M. Rossi (✉) · L. Altomare · A. Gonzàlez Vara y Rodriguez
P. Brigidi · D. Matteuzzi
Department of Pharmaceutical Sciences, University of Bologna,
Via Belmeloro 6, 40126, Bologna, Italy
e-mail: e056mr01@area.ba.cnr.it,
Fax: +39-51-2099734

Key words *Bifidobacterium longum* · LacZ · Expression · Transcriptional analysis · Nucleotide sequence

Introduction

Bifidobacteria are major constituents of the human intestinal microflora and interest in their therapeutic effects is increasing. Recently, several kinds of dairy and pharmaceutical products containing living bifidobacteria have been proposed as dietary adjuncts. Indications are that therapeutic use of orally administered bifidobacteria offers probiotic effects such as anti-tumorigenic activity (Reddy and Riverson 1993), immunological enhancement (Lee et al. 1993), maintenance of a proper balance of the normal intestinal microflora (Ibrahim and Bezkorovainy 1993), reduction of serum cholesterol levels, improvement of lactose intolerance and synthesis of B-complex vitamins (Tannok 1999).

The possibility of genetically manipulating bifidobacteria for metabolic activity is very promising, although not many genetic tools are available. Few *Bifidobacterium* genes have been characterized and analyzed for their structure, organization, expression and regulation (Minowa et al. 1990; Nunoura et al. 1996). Recently, *Escherichia coli*/*Bifidobacterium* shuttle vectors have been constructed (Rossi et al. 1996, 1998), electroporation protocols have been developed (Argnani et al. 1996; Rossi et al. 1997b), and heterologous genes have been cloned, but not expressed (Rossi et al. 1998). Characterization of new genes can increase the knowledge of bifidobacteria genetics, leading to the expression of heterologous genes and to the modification of the wild-type strain phenotypes.

β-Galactosidase, present in most lactic acid bacteria and bifidobacteria, hydrolyzes lactose to the monosaccharides glucose and galactose. This reaction is interesting for several reasons. First, lactose is digested with difficulty by a large part of the world population, especially by Asians; moreover, it has a low solubility in water, which

leads to the problems of concentrating the whey and preparing certain food items. Additionally, lactose has a relatively low level of sweetness. These problems can be overcome to a large extent by hydrolyzing lactose to its monosaccharides which are sweeter, more soluble, and more digestible than lactose (Smart et al. 1985).

Bifidobacterium is a promising source for the production of this enzyme because it is a food-approved organism. Therefore, the cloning of this gene may be of commercial value, in addition to its potential use as a selection marker in food-grade cloning vectors. Characterization of the *B. longum* gene encoding β-galactosidase would also increase our understanding of transcription signals in this organism, thereby facilitating the construction of expression vectors. Thus, the aims of the current study were to screen the β-galactosidase activity of several *Bifidobacterium* strains, to isolate and to characterize the gene of the strain with the highest level of activity, and then to clone and express this gene in other bifidobacteria. Finally, characterization of the transcription signals that regulate the β-galactosidase gene could provide a significant advance in the expression of foreign genes in species of this genus.

Materials and methods

Bacterial strains and growth conditions

Escherichia coli ER1458 [(FΔ*lacU169 lon-100 araD139 rpsL* (*Str*^{r}) *supF mcrA zjj202::Tn10* (*Tet*^{r}) *hsdR2* ($r_k^-m_k^+$) *mcrB1 serB28* (New England Biolabs)] was used for general cloning purposes and was grown at 37 °C in Luria broth (LB) or minimal medium (Sambrook et al. 1989), supplemented with essential nutrients as indicated by the auxotrophic markers. Ampicillin was utilized to select *E. coli* transformants at 100 μg ml^{-1}. Isopropyl-β-D-thiogalactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-thiogalactopyranoside (X-gal) were utilized at concentrations of 50 μg ml^{-1} and 40 μg ml^{-1}, respectively.

The *Bifidobacterium* strains *B. longum* MB219 and MB260, *B. bifidum* MB254, *B. breve* MB226, *B. animalis* MB209, *B. indicum* MB101, *B. cuniculi* MB279, MB280 and MB281 (collection of the Institute of Agricultural Microbiology, University of Bologna, Italy), and *B. adolescentis* INT-57 (Nam et al. 1995) were grown anaerobically at 37 °C in MRS medium (Difco) containing 0.05% cysteine, or in Iwata medium (Iwata and Morishita 1989) containing glucose 1% (w/v) (IMG) or lactose 1% (w/v) (IML) as sole carbon source. Chloramphenicol was added to the appropriate media at the following concentrations: for *B. longum* MB260, *B. bifidum* MB254, *B. breve* MB226, *B. animalis* MB209, *B. indicum* MB101, *B. cuniculi* MB280 and MB281 5 μg ml^{-1}; for *B. cuniculi* MB279 15 μg ml^{-1}.

Plasmids

The *E. coli* cloning vector pGM3 was used to clone the gene of *B. longum* MB219 encoding β-galactosidase and was constructed by deletion of the promoters T7 and SP6 from the plasmid pSP73 (Promega). pSP73 was digested with *Xho*I and *Hpa*I and blunt-ended with T4 DNA polymerase, and the largest fragment (2413 bp) was self-ligated, resulting in pSP1, and transferred to *E. coli* ER1458 by transformation. The same strategy was used to construct pGM3 from pSP1 by digestion with *Bgl*II and *Nde*I, blunt-ending with T4 DNA polymerase and self-ligation of the 2320-bp fragment.

The *E. coli*/*Bifidobacterium* shuttle vector pDG7 (Matteuzzi et al. 1990) was utilized to clone the gene encoding β-galactosidase into *Bifidobacterium*.

The plasmids pKK232–8 (Promega) and pBR322 (Pharmacia) were used as cloning vectors to determine the sequence of the fragment carrying the gene encoding β-galactosidase.

DNA isolation, manipulation and transformation

A 20-ml culture was grown overnight in MRS broth supplemented with cysteine (0.05%) to isolate chromosomal DNA from *B. longum* MB219. The cells were harvested by centrifugation and washed with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6). The pellet was resuspended in 1 ml of TE containing 15 mg lysozyme ml^{-1}, then incubated at 37 °C for 3 h. The cells were lysed by the addition of 3 ml lysis buffer (100 mM Tris-HCl, 400 mM NaCl, 2 mM EDTA, pH 8.2), 220 μl SDS (10%, w/v) and 150 μl proteinase K (stock solution 20 mg ml^{-1} in H_2O). The suspension was incubated for 2 h at 60 °C. NaCl-saturated solution (1 ml) was added, and the sample was inverted gently a few times. The pellet was collected by centrifugation at 5000×*g* at room temperature, and the clear solution was transferred to a fresh tube. The DNA was precipitated with 2.5 volumes of cold ethanol. The chromosomal DNA was spooled out with a glass rod, washed with ethanol 70% (w/v), dried, and then dissolved in 300 μl TE.

DNA manipulations were carried out under the conditions specified by the manufacturer or according to standard procedures as described by Sambrook et al. (1989). DNA fragments from agarose gel were purified using the QiaexII Kit (Qiagen). *Bifidobacterium* plasmid extraction and electroporation were performed according to Rossi et al. (1997b).

For DNA hybridization, *Sal*I partially restricted chromosomal DNA from *B. longum* MB219 was size-fractionated on a 0.8% agarose gel and transferred onto a nylon membrane (Hybond N, Amersham Pharmacia Biotech) by vacuum-supported diffusion using the Vacuum Blotter system from Bio-Rad. A PCR-generated DNA fragment, spanning the entire *lacZ* gene sequence (BETA-F: 5′-CGATCTCAAGCAGAGCCTTGAC-3′, nt 281–302, and BETA-R: 5′-GGAATCATCGCCGCCAAC-3′, nt 3137–3120) was labeled with digoxigenin-dUTP and used as a probe. Blotting, labeling, hybridization, washing and detection were performed using the non-radioactive DIG DNA labeling and detection kit.

Fragment sizes were determined by comparing mobilities with those of the standard reference DNA Molecular-Weight Marker II, DIG-labeled (Boehringer Mannheim).

Cloning of the β-galactosidase gene

Chromosomal DNA of *B. longum* MB219 was digested with *Sal*I and size-fractionated on 0.7% agarose gels. Fragments from 2 to 10 kb were isolated and ligated to *Sal*I-linearized and dephosphorylated pGM3 vector. Ligation mixture was used to transform *E. coli* ER1458, and β-galactosidase-proficient transformants were selected on LB plates supplemented with X-gal and IPTG. Appropriate subclones were made by using the *E. coli*/*Bifidobacterium* shuttle vector pDG7 (Matteuzzi et al. 1990).

RNA purification.

Total RNA from *B. longum* MB219 was isolated from an exponential phase culture grown in MRS broth supplemented with cysteine (0.05%), using the Cell RiboLyser and the RNA Extraction Kit Blue (Hybaid), according to the protocols specified by the manufacturer.

Primer-extension experiments

The primers used for the primer-extension experiments were Pem_1 (5′-AGTCCGGTTCACCTCGAACACCGT-3′, nt 224–

201) and Pem_2 (5′-CGTACCACTTGTGGCTGGAAT-3′, nt 252–232). For the primer-extension experiments, 10 μg of vacuum-dried total RNA were dissolved in 4.8 μl H_2O. After the addition of 1 μl of IRD800-marked primer (2 pmol), 2 μl of buffer (50 mM Tris-HCl pH 7.9, 1.25 M KCl) and 1 μl of RNase inhibitor (10 U μl^{-1}, Gibco-BRL), the mixture was denatured at 80 °C for 5 min and hybridized at 32 °C for 3 h. For synthesizing the cDNA strand, 10 μl of reverse trancriptase buffer (250 mM Tris-HCl, pH 8.3, 125 mM KCl, 15 mM $MgCl_2$), 5 μl of 0.1 M dithiothreitol, 2.5 μl of dNTP mix (10 mM), and 22.5 μl H_2O were mixed. This solution was added to the hybridization mixture and, after the addition of 1 μl of reverse transcriptase (200 U μl^{-1}), the reaction was carried out at 37 °C for 1 h.

The primer-extension product was ethanol-precipitated, dried under vacuum, then dissolved in 2.5 μl of H_2O plus 2 μl of loading buffer (10 mM EDTA, 0.1% xylene cyanole, 0.1% bromophenol blue). Aliquots (1.5 μl) of the sample were loaded onto a 6% (w/v) polyacrylamide sequencing gel. For exact location of the transcriptional start site, sequencing reactions using the plasmid pQU1 and the same oligonucleotides used for the primer extensions were co-electrophoresed.

DNA sequence analysis

The nucleotide sequence was determined on both DNA strands using the DNA Sequencing Kit dRhodamine Terminator Cycle Sequencing ABI PRISM and the automatic sequencer ABI PRISM 310 Genetic Analyzer (PE Applied Biosystem).

The nucleotide sequences of the 3.3-kb *Sal*I–*Eco*RV fragment, harboring the β-galactosidase gene of *B. longum* MB219 and the 1.7-kb *Eco*RV–*Sal*I fragment located downstream from this gene, were determined using as a template the plasmids pQU1 and p58M, respectively, and synthetic oligonucleotides. Modified nucleotides were used to solve the sequence downstream from the gene encoding β-galactosidase and to join the contigs. Every nucleotide was determined on average 2.7 times, at least once on each strand.

The sequence data obtained were compiled and analyzed, where not differently specified, on a UNIX computer using the Genetic Computer Group Programs Package (Madison, Wis.).

β-Galactosidase assays

β-Galactosidase standard assays using permeabilized cells of bifidobacteria were performed with *o*-nitrophenyl-β-D-galactopyranoside (ONPG) at 37 °C in Z buffer (100 mM sodium phosphate pH 7.0, 10 mM KCl, 1 mM $MgSO_4$, 50 mM mercaptoethanol). Cells from a stationary-phase culture (16–18 h) were harvested by centrifugation at 6000×*g* for 10 min at 0 °C and resuspended in Z buffer. The optical density was adjusted to an OD_{600} of 1.4, which corresponded to approximately 109 cells ml^{-1} and yielded approximately 260 μg protein ml^{-1}. Cells were permeabilized by mixing 0.5 ml of suspension at the appropriate dilution with 0.5 ml of buffer Z containing Triton X-100 (5%, w/v) and incubating the sample at 37 °C for 5 min; 0.2 ml of ONPG (4 mg ml^{-1}) was added and incubated for a further 30 min. The reaction was stopped by adding 0.5 ml of 1 M Na_2CO_3. The sample was centrifuged at 13,000×*g* for 5 min, and the *o*-nitrophenol released into the supernatant was determined spectrophotometrically at 420 nm. One unit of β-galactosidase was defined as the amount of enzyme required to release 1 μmol of *o*-nitrophenol per min under the conditions described above. The specific activity was defined in terms of units (mg protein)$^{-1}$.

β-Galactosidase in *E. coli* was assayed according to the procedure described by Miller (1972) and was expressed in Miller units.

Each experiment was repeated three to four times with similar results. The activity values reported represent the mean of these independent experiments.

Biochemicals, reagents and primers

All chemicals, unless otherwise specified, were provided by Sigma or Merck. The enzymes and the reagents used for molecular biological procedures were from Pharmacia, Boehringer Mannheim or MBI Fermentas. All primers were from M-Medical Genenco (Florence, Italy).

Results and discussion

β-Galactosidase activities of *Bifidobacterium* spp.

β-Galactosidase activity was detected in permeabilized cells of ten strains of bifidobacteria belonging to the species *B. longum*, *B. breve*, *B. adolescentis*, *B. indicum*, *B. animalis* and *B. cuniculi*. Under the assay conditions used, the strains exhibited the activities presented in Table 1. The human intestinal strains *B. longum* MB219 and

Table 1 Growth of different wild-type and recombinant strains of *Bifidobacterium*. Cells were grown in Iwata media containing glucose or lactose and specific activity of β-D-galactosidase is expressed as units (mg protein)$^{-1}$. +++:Optimal growth, +poor growth, – no growth

Strain	Glucose		Lactose	
	Growth	β-Galactosidase activity	Growth	β-Galactosidase activity
B. longum MB 219	+++	112.3	+++	173.8
B. longum MB 260	+++	63.8	+++	79.2
B. bifidum MB 254	+++	64.2	+++	162.7
B. breve MB 226	+++	55.3	+++	117.7
B. adolescentis INT-57	+++	11.9	+++	43.0
B. indicum C 410	+++	4.2	–	
B. animalis MB 209	+++	21.9	+++	85.2
B. animalis MB 209+pDG7	+++	20.7	+++	90.7
B. animalis MB 209+pDM9	+++	107.3	+++	155.6
B. cuniculi MB 279	+++	1.9	–	
B. cuniculi MB 279+pDG7	+++	1.9	–	
B. cuniculi MB 279+pDM9	+++	94.2	+++	103.6
B. cuniculi MB 280	+++	1.6	–	
B. cuniculi MB 280+pDG7	+++	1.5	–	
B. cuniculi MB 280+pDM9	+++	57.7	+++	50.8
B. cuniculi MB 281	+++	2.3	+	4.6

MB260, *B. bifidum* MB254 and *B. breve* MB226 had the highest activities. These strains were able to use lactose as carbon source.

B. adolescentis INT-57 and *B. animalis* MB209 had lower levels of β-galactosidase activity, but could still grow using lactose as sole carbon source. *B. cuniculi* MB279, MB280 and MB281, isolated from rabbit feces, and *B. indicum* C410, isolated from bee intestine, were not able to grow on lactose and had very low β-galactosidase activity.

The activities in human strains and in *B. animalis* were slightly higher when grown on medium containing lactose than when grown on glucose; nevertheless this enzyme seems to be constitutive in these bifidobacteria. It is noteworthy that the strains with low β-galactosidase activity were the ones isolated from herbivorous animals (*B. cuniculi*) or from insects (*B. indicum*), both of which do not utilize milk.

Cloning and expression of the β-galactosidase gene in *E. coli*

In order to isolate the β-galactosidase genetic determinants of *B. longum* MB219, *E. coli* ER1458, which lacks the entire *lac* operon, was complemented with shotgun clones carrying fragments from chromosomal DNA of *B. longum* MB219 in pGM3. Seven colonies, out of approximately 30,000 transformants, showed a β-galactosidase-positive phenotype when tested on LB agar supplemented with X-gal and IPTG. The clone carrying the smallest insert contained a 5.0-kb fragment of DNA partially digested with *Sal*I, and the recombinant plasmid was designated pS3.

A restriction map of the insert in pS3 was generated, and fragments were subcloned into *E. coli* to determine the smallest fragment carrying the gene encoding β-galactosidase (Fig. 1). The fragments *Eco*RV (3.4 kb), *Bam*HI (3.4 kb) and *Pvu*II (3.0 kb) were directly cloned or blunt-end cloned in the unique *Bam*HI site of the high-copy number *E. coli*/*Bifidobacterium* shuttle vector pDG7.

The two recombinant plasmids containing the 3.4-kb *Eco*RV fragment that differed with respect to the orientation of the insert (pDM9 and pDM9R) conferred β-galactosidase activity to *E. coli* ER1458. *E. coli* ER1458 (pDM9) and (pDM9R) were able to grow on minimal medium with lactose as sole carbon source. In accordance with these observations, permeabilized cells of *E. coli* ER1458 (pDM9) and (pDM9R) exhibited high β-galactosidase activity (both about 2850 Miller units). The expression of β-galactosidase gene from a *Bifidobacterium* strain in *E. coli* was expected since β-galactosidase genes of *B. adolescentis* (Rossi et al. 1997a) and *B. infantis* (Hung and Lee 1998) have been expressed in *E. coli*. Therefore, the amount of enzyme synthesized was not affected by the orientation of the inserted fragment, showing that vector-located sequences did not affect expression of the gene. These results demonstrated that the original β-galactosidase gene promoter was present on the cloned

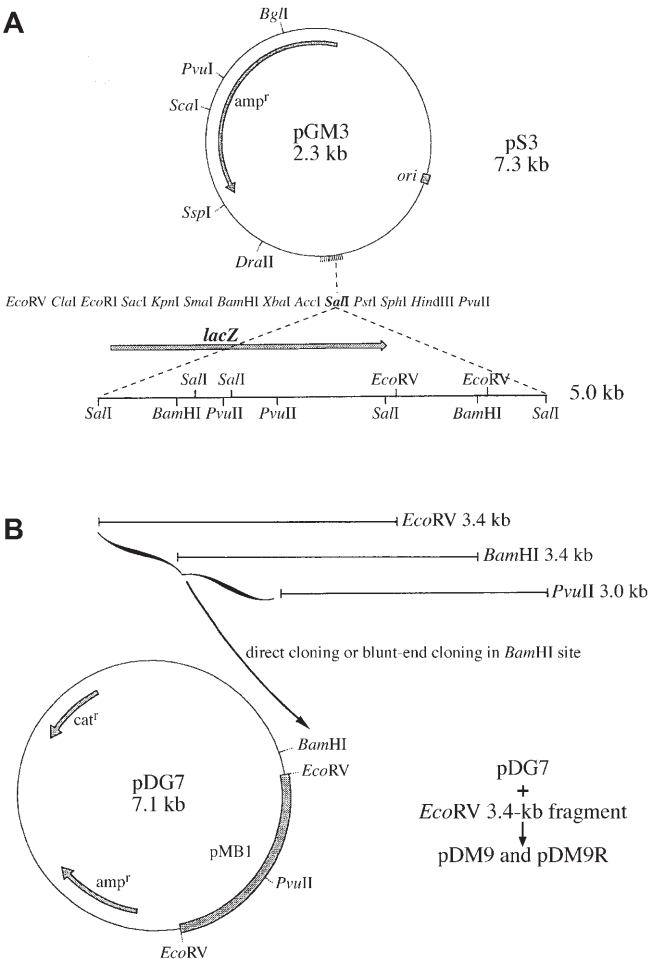

Fig. 1 **A** Cloning of a 5.0-kb *Sal*I fragment from a partial *Sal*I digest of genomic DNA of *Bifidobacterium longum* MB219 into the *Sal*I site of the vector plasmid pGM3. **B** Subcloning of portions of the 5.1-kb *Eco*RVI–*Pvu*III fragment of plasmid pS3 into the *E. coli*/*Bifidobacterium* shuttle vector pDG7

DNA fragment and that the *B. longum* transcription and translation signals were recognized by *E. coli*. As expected, β-galactosidase activity in *E. coli* was not induced by growth in the presence of lactose or IPTG.

Nucleotide sequence analysis

The organization of the *B. longum* MB219 gene encoding β-galactosidase was analyzed by sequencing the 3.4-kb *Eco*RV fragment of pS3 that conferred the β-galactosidase-positive phenotype. To determine the sequence, this fragment was cloned into the blunt-ended *Sal*I site of pKK232–8, yielding the structurally stable plasmid pQU1. The nucleotide sequence determined was 3317 bp long. However, it was not possible to complete the sequence of the region downstream from the gene encoding β-galactosidase (named *lacZ* gene according to the similarities further presented) and to reach the *Eco*RV restriction site using primers that hybridize to the vector or to the *lacZ* gene.

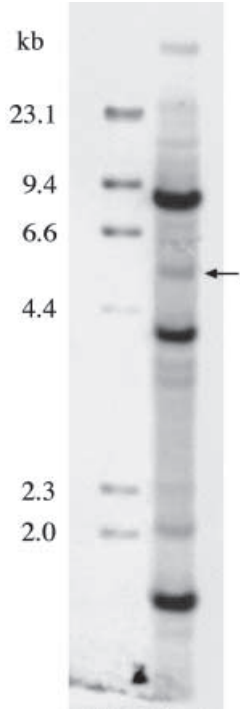

Fig. 2 Southern blot analysis of chromosomal DNA of *B. longum* MB219 partially digested with *Sal*I hybridized with the PCR-generated probe spanning the entire *B. longum* MB219 *lacZ* gene. *Right lane*, the 5.0-kb fragment is indicated by the *arrow*

In order to extend the sequence from *lacZ* to the *Eco*RV site and to determine the sequence of the 3′-flanking region, the 5.1-kb insert *Kpn*I-*Hin*dIII from pS3 was blunt-ended and cloned into pBR322 linearized with *Eco*RV, yielding the plasmid p58M. The sequence of the 1.7-kb region downstream from the *lacZ* gene was determined. The *Eco*RV restriction site located downstream from *lacZ* was reached, and the 40 bp upstream were determined using a primer hybridizing to the newly sequenced region; unfortunately, it was not possible to join the 3.4- and 1.7-kb fragments. It seemed that this region formed a secondary structure so strong that it could not be melted within the range of temperatures reached during the sequencing reaction. Nevertheless, it was possible to amplify a fragment of *B. longum* MB219 genomic DNA or plasmid p58M that included the gap using the primers GAP-F: 5′-GTTGGCGGCGATGATTCC-3′, nt 3120–3137 and GAP-R: 5′-AACGACGCTGGATACATTGC-3′, nt 3446–3427. The size of this product, compared with the size of sequenced regions flanking the gap, showed that the unsequenced portion is in the range of 30–50 bp.

After the molecular characterization of the 5.1-kb fragment, genomic DNA of *B. longum* MB219 partially digested with *Sal*I was analyzed by Southern blot hybridization using a PCR-generated probe spanning the entire *lacZ* gene (Fig. 2). The results confirmed that the 5.0-kb *Sal*I fragment inserted in pS3 does occur in the *B. longum* MB219 genome and was not rearranged during cloning. The entire sequence of the *Sal*I fragment of chromosomal DNA from *B. longum* MB219 is 4991 bp long. The EMBL/GenBank accession number for this nucleotide sequence is AJ242596.

The sequence revealed the existence of a single large ORF (*lacZ*) in the *Sal*I–*Eco*RV fragment of 3.4 kb, highly similar to other genes belonging to the *lacZ* family. The *lacZ* gene is 3072 bp long and encodes a protein of 1023 amino acids, with a calculated molecular mass of 114.5 kDa, starting with ATG (nt 147) and ending with codon TGA (nt 3218). The gene is preceded by a putative ribosome-binding site (AGGAG) located 3 bp upstream from the start codon. The gene has a G+C content of 65.1 mol%, higher than the G+C content of ca. 61 mol % for genomic *B. longum* DNA.

The 1.7-kb DNA fragment located downstream from the gap had a G+C content of 54 mol%, much lower than that of *B. longum*. Another ORF is found downstream from the *lacZ* gene in this fragment. This ORF starts with nucleotide 4019 (ATG codon) and stops at nucleotide 4573. The predicted gene product consists of 184 amino acids and showed 43% identity to the DNA-3-methyladenine glycosydase I from *P. fluorescens*.

A sequence (nt 3475–3554) with 72% identity to the tRNAPro of *B. subtilis* was found between this ORF and the *lacZ* gene.

Transcriptional analysis of the *lacZ* gene

In order to identify the transcriptional start site, primer-extension experiments were performed using primers Pem_1 and Pem_2 and approximately 10 μg of total RNA from *B. longum* MB219. With both primers, signals were obtained corresponding to the nucleotide adenine (nt 82) (Fig. 3). These results showed that transcription of the *B. longum* MB 219 *lacZ* gene starts 65 nt upstream from the presumed translational start.

A possible –10 consensus sequence (TTATCC, nt 68–73) can be found upstream from the *lacZ* transcriptional starting site. This motif is similar to the corresponding structures found for the promoters of the *B. longum ldh* gene (TATAGA, Minowa et al. 1985) and of the operon encoding the replicative functions of plasmid pMB1 of *B. longum* MB203 (TATTCG, Rossi et al. 1996). No sequence motif with similarity to the –35 sequences identified for the *ldh* gene (GTAGCAA) and for the pMB1 operon (GTAGCCA) promoters was found at the appropriate distance from the *lacZ* transcriptional starting site.

The promoter of the *B. longum* MB219 β-galactosidase gene is the first to be identified from a *Bifidobacterium* gene using the primer-extension technique. This result allows us to test the possibility of using the β-galactosidase promoter to express heterologous genes in bifidobacteria.

Comparison of β-galactosidases

The amino acid sequence deduced from the gene encoding β-galactosidase of *B. longum* MB219 was compared with that of other β-galactosidases extracted from the Swiss-Prot protein database. The enzyme was found to be a member of the *E. coli* LacZ family, being similar in size and amino acid sequence to the β-galactosidases of *E. coli*

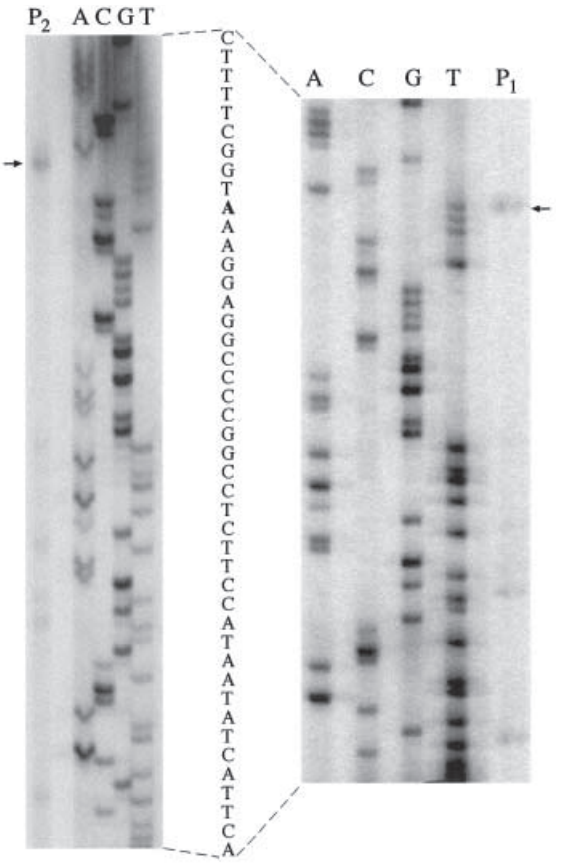

Fig. 3 Primer-extension analysis of the transcriptional starting site in front of the *B. longum* MB219 β-D-galactosidase gene. *Left* The product of extension of primer Pem_2 (*lane P2*) and total *B. longum* MB219 RNA and the products of the sequencing reaction using the same primer on plasmid pQU1. *Right* The product of extension of primer Pem_1 (*lane P1*) and total *B. longum* MB219 RNA and the products of the sequencing reaction using the same primer on plasmid pQU1. The relevant nucleotide sequence representing the coding strand is shown

(55.0% similarity and 33.1% identity), *Lactobacillus delbruckii* subsp. *bulgaricus*, *Streptococcus thermophilus*, *Clostridium acetobutylicum*, *Leuconostoc lactis*, *Klebsiella pneumoniae* and *Kluyveromyces marxianus* var. lactis. The amino acid sequence exhibited the highest sequence similarity with the corresponding enzyme from *L. delbruckii* subsp. *bulgaricus* (64.5% similarity and 46.2% identity). The alignment of *B. longum* MB219 and *E. coli* β-galactosidases showed that the proposed active site residue (Glu-461), identified by active-site-directed inhibitors (Herrchen and Legler 1984), and the tyrosine residue at site 503, involved in the reaction (Schmidt et al. 1989) of *E. coli* enzyme, are conserved in the *B. longum* enzyme (Glu-468 and Tyr-513).

Furthermore, it is remarkable that the amino acid sequence of a *Bifidobacterium breve* β-galactosidase, encoding a protein of 77.4 kDa (accession number E05040), shows no sequence similarity with the sequenceof enzymes of the LacZ family. This is in agreement with the experimental evidence that bifidobacteria possess several β-galactosidase isoenzymes (Roy et al. 1994).

Expression of the *lacZ* gene from pDM9 in *Bifidobacterium*

The expression of the *lacZ* gene of *B. longum* MB219 was under the control of its own promoter in pDM9, the recombinant plasmid based on the *E. coli*/*Bifidobacterium* shuttle vector pDG7. Plasmid DNA from *E. coli* ER1458 was isolated and used to transform bifidobacteria of different species: *B. longum* MB260, *B. bifidum* MB254, *B. breve* MB226, *B. animalis* MB209, *B. cuniculi* MB279 and MB280. Electroporation of *B. cuniculi* MB279 and MB280 with pDM9 yielded few Cm^R transformants. Furthermore, this result was often not reproducible.

The transformed clones of *B. longum* MB260, *B. bifidum* MB254 and *B. breve* MB226 were shown to harbor the expected 10.6-kb plasmid pDM9. The clones were analyzed for β-galactosidase activity. These hosts had high levels of endogenous β-galactosidase activity and slightly higher expression was detected in clones transformed with pDM9 than with the vector pDG7.

B. animalis MB209 had a low endogenous β-galactosidase activity, whereas *B. cuniculi* MB279 and MB280 had very low activities and could not grow on lactose as the sole carbon source. The successful introduction of plasmids pDM9 and pDG7 into these strains enabled the analysis of *lacZ* expression due to pDM9 (Table 1). *B. animalis* clones harboring pDM9 had a fivefold higher β-galactosidase activity when grown on IMG, and a 1.7-fold higher activity when grown on IML. In *B. cuniculi* MB279 and MB280 transformed with pDM9, the β-galactosidase activity was about 50-fold higher than in these hosts transformed with pDG7, and the clones transformed with pDM9 were able to grow on IML using lactose as the sole carbon source. Furthermore, whenever the plasmid pDM9 was isolated from these clones and introduced into *E. coli* ER1458, blue colonies appeared on plates containing X-gal.

This is the first report of gene expression in bifidobacteria, and it demonstrates the possibility of expressing a gene isolated from a *Bifidobacterium* strain in bifidobacteria of other species. This result is in agreement with the knowledge that this genus is phylogenetically not very deeply branched.

References

Argnani A, Leer RJ, van Luijk N, Pouwels PH (1996) A convenient and reproducible method to genetically transform bacteria of the genus *Bifidobacterium*. Microbiology 56:713-715

Herrchen M, Legler G (1984) Identification of an essential carboxylate group at the active site of the *lacZ* β-galactosidase from *Escherichia coli*. Eur J Biochem 138:527–531

Hung M, Lee BH (1998) Cloning and expression of β-galactosidase gene from *Bifidobacterium* infantis into *Escherichia coli*. Biotech Lett 7:659–662

Ibrahim SA, Bezkorovainy A (1993) Inhibition of *Escherichia coli* by Bifidobacteria. J Food Protein 56:713–715

Iwata M, Morishita T (1989) The presence of plasmids in *Bifidobacterium* breve. Lett Appl Microbiol 9:165–168

Lee J, Ametani A, Enomoto A, Sato Y, Motoshima H, Ike F, Kaminogawa S (1993) Screening for the immunopotentiating activity of food microorganism and enhancement of the immune response by *Bifidobacterium* adolescentis M101-4. Biosci Biotech Biochem 57:2127–2132

Matteuzzi D, Brigidi P, Rossi M, Di Gioia D (1990) Characterisation and molecular cloning of *Bifidobacterium longum* cryptic plasmid pMB1. Lett Appl Microbiol 11:220–223

Miller HJ (1972) Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Minowa T, Iwata S, Sakai H, Masaki H, Otha T (1990) Sequence and characteristics of the *Bifidobacterium longum* gene encoding L-lactate dehydrogenase and the primary structure of the enzyme: a new future of the allosteric site. Gene 85:161–168

Nam MJ, Heo TR, So JS (1995) Molecular cloning and expression in *Escherichia coli* of β-galactosidase gene of a *Bifidobacterium* sp. INT-57. Seventh European Congress in Biotechnology, February 19–23, 1995, Nice

Nunoura N, Ohdan K, Tanaka K, Tamaki H, Yano T, Inui M, Yukawa H, Yamamoto K, Kumagai H (1996) Cloning and nucleotide sequence of the β-D-glucosidase gene from *Bifidobacterium breve* clb, and expression of β-D-glucosidase activity in *Escherichia coli*. Biosci Biotech Biochem 60:2011–2018

Reddy BS, Rivenson A (1993) Inhibitory effect of *Bifidobacterium longum* on colon, mammary, and liver carcinogenesis induced by 2-amino-3-methylimidazo [4,5-f] quinoline, a food mutagen. Cancer Res 53:3914–3918

Rossi M, Brigidi P, Gonzalez Vara y Rodriguez A, Matteuzzi D (1996) Characterization of the plasmid pMB1 from *Bifidobacterium longum* and its use for shuttle vector construction. Res Microbiol 147:133–143

Rossi M, Altomare L, Brigidi P, So JJ (1997a) Expression of *Bifidobacterium* adolescentis β-galactosidase in *Bifidobacterium* spp. Eighth European Congress in Biotechnology, August 17–21, 1997, Budapest

Rossi M, Brigidi P, Matteuzzi D (1997b) An efficient transformation system for *Bifidobacterium* spp. Lett Appl Microbiol 24:33–36

Rossi M, Brigidi P, Matteuzzi D (1998) Improved cloning vectors for *Bifidobacterium* spp. Lett Appl Microbiol 26:101–104

Roy D, Berger JL, Reuter G (1994) Characterization of dairy-related *Bifidobacterium* spp. based on their β-galactosidase electrophoretic patterns. Int J Food Microbiol 23:55–70

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Schmidt BF, Adams RM, Redquadt C, Power S, Mainzer SE (1989) Expression and nucleotide sequence of the *Lactobacillus bulgaricus* β-galactosidase gene cloned in *Escherichia coli*. J Bacteriol 171:625–635

Smart JB, Crow VL, Thomas TD (1985) Lactose hydrolysis in milk and whey using β-galactosidase from *Streptococcus thermophilus*. N Z J Dairy Sci Technol 20:43–56

Tannock GW (ed) (1999) Probiotics: a critical review. Horizont Scientific, Norfolk, England