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Intra- and Extracellular β-Galactosidases from *Bifidobacterium bifidum* and *B. infantis*: Molecular Cloning, Heterologous Expression, and Comparative Characterization

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Three β-galactosidase genes from *Bifidobacterium bifidum* DSM20215 and one β-galactosidase gene from *Bifidobacterium infantis* DSM20088 were isolated and characterized. The three *B. bifidum* β-galactosidases exhibited a low degree of amino acid sequence similarity to each other and to previously published β-galactosidases classified as family 2 glycosyl hydrolases. Likewise, the *B. infantis* β-galactosidase was distantly related to enzymes classified as family 42 glycosyl hydrolases. One of the enzymes from *B. bifidum*, termed BIF3, is most probably an extracellular enzyme, since it contained a signal sequence which was cleaved off during heterologous expression of the enzyme in *Escherichia coli*. Other exceptional features of the BIF3 β-galactosidase were (i) the monomeric structure of the active enzyme, comprising 1,752 amino acid residues (188 kDa) and (ii) the molecular organization into an N-terminal β-galactosidase domain and a C-terminal galactose binding domain. The other two *B. bifidum* β-galactosidases and the enzyme from *B. infantis* were multimeric, intracellular enzymes with molecular masses similar to typical family 2 and family 42 glycosyl hydrolases, respectively. Despite the differences in size, molecular composition, and amino acid sequence, all four β-galactosidases were highly specific for hydrolysis of β-D-galactosidic linkages, and all four enzymes were able to transgalactosylate with lactose as a substrate.

Since they were first discovered by Tissier (33), the bifidobacteria have been investigated extensively by several scientists (e.g., references 23 and 27). In recent years, bifidobacteria have attracted particular attention due to their promising health-promoting properties, for example, reduction of harmful bacteria and toxic compounds in the intestine, prevention of dental caries, reduction of total cholesterol and lipid in serum, and relief of constipation (2, 5, 10, 17, 36, 41). Therefore, live probiotic bifidobacteria, which may improve the microbial balance of the human gastrointestinal tract, have been used to supplement dairy products for many years. Another approach to increase the number of beneficial bacteria in the human intestine is to selectively stimulate their growth by supplementing food with ingredients which can only be metabolized by such bacteria. Certain oligosaccharides, the so-called prebiotics, have been shown to exert this growth-stimulating effect on probiotic bacteria, including bifidobacteria.

So far, most of the probiotic bacteria and the prebiotic oligosaccharides have been used in combination with dairy products, and since these products often contain large amounts of lactose, much attention has been focused on the enzyme β-galactosidase (EC 3.2.1.23), which is involved in the bacterial metabolism of lactose. In addition to normal hydrolysis of the β-D-galactoside linkage in lactose, some β-D-galactosidase enzymes may catalyze the formation of galactooligosaccharides through transfer of one or more D-galactosyl units onto the D-galactose moiety of lactose. This transgalactosylation reaction (12) has been shown to be a characteristic of β-galactosidase enzymes from a great variety of bacterial and fungal species (7, 19, 21, 40).

Galactooligosaccharides produced from lactose by transgalactosylation specifically stimulate growth of bifidobacteria (39), and recently Van Laere et al. (37) have described a novel β-galactosidase from *Bifidobacterium adolescentis* that preferentially hydrolyzes galactooligosaccharides. Therefore, it is generally accepted that a structural and catalytic characterization of the β-galactosidase enzymes of probiotic bacteria is of central importance for an understanding of their health-promoting effects.

Lactose hydrolysis and transgalactosylation properties of the enzyme have been studied in several probiotic bacteria including bifidobacteria (6, 7, 25, 26, 30, 35, 37), but so far only one DNA sequence of a bifidobacterial β-galactosidase gene has been published (*Bifidobacterium longum*; EMBL accession no. AJ242596) (24), and another sequence has been deposited in a database (*Bifidobacterium breve*; EMBL accession no. E05040). In this paper, we describe the molecular cloning, sequencing and characterization of three different β-galactosidase enzymes from *Bifidobacterium bifidum* (DSM20215) and one enzyme from *Bifidobacterium infantis* (DSM20088).

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Fifteen different bifidobacterial strains were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany, and analyzed for their ability to synthesize galactooligosaccharides. Two strains, *B. bifidum* DSM20215 and *B. infantis* DSM20088, which were able to synthesize oligosaccharides, were selected for this study. The strains were grown anaerobically using TPY medium

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(28) at 37°C with BBL GasPak Anaerobic systems (Becton Dickinson and Co., Cockeysville, Md.). DNA cloning was performed using the *Escherichia coli* strains (i) MT102, a derivative of MC1000 (*hsdR*-K12) (4), (ii) XL-1–5, an F^- derivative of XL1-Blue (3), and (iii) ER1458 (22). The *E. coli* strains were grown in Luria-Bertani (LB) medium (18) supplemented with 100 μg of ampicillin/ml and 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal)/ml when appropriate. The plasmid pBluescript KS(−) (Stratagene Cloning Systems, La Jolla, Calif.) was the vector used for DNA fragment cloning.

Chemicals and enzymes. Chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.). Restriction enzymes and other enzymes used for DNA manipulation were from New England Biolabs, Inc. (Beverly, Mass.) and were used according to the instructions of the manufacturer.

Isolation of β-galactosidase genes. Chromosomal DNA was prepared from a cell pellet harvested from 500 ml of TPY culture. The cells were resuspended in 4.4 ml of lysis solution (20 mM Tris-HCl, 20 mM $MgCl_2$, 20% glucose, 5 mg of lysozyme/ml, and 350 U of mutanolysin/ml [pH 6.5]) and incubated at 37°C for 60 min. Cells were lysed by the addition of 12 ml of TEN buffer (100 mM Tris-HCl, 1 mM EDTA, and 100 mM NaCl [pH 7.5]), 1.6 ml of 10% sodium dodecyl sulfate (SDS), 1.6 ml of 0.5 M EDTA (pH 7), and 0.3 ml of proteinase K (20 mg/ml), followed by incubation at 37°C for 60 min. Five milliliters of phenol and 5 ml of chloroform were added, and the extraction was repeated until the water phase could easily be separated from the interphase. The genomic DNA was precipitated with isopropanol, resuspended in 10 mM Tris-HCl–1 mM EDTA (pH 8.0), and treated with RNase. The genomic DNA was then digested with restriction enzymes, ligated into pBluescript KS(−), digested with the same enzymes, and treated with alkaline phosphatase. Digestion of *B. bifidum* genomic DNA was performed using *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, *Sac*I, *Kpn*I, *Apa*I, and *Sal*I, whereas *B. infantis* DNA was digested with *Kpn*I. Ligation mixtures were used to transform *E. coli* MT102, and β-galactosidase-producing clones were identified as blue colonies on X-Gal-containing plates.

Preparation of cell lysates. *E. coli* cells harboring the recombinant β-galactosidase genes were lysed with a French pressure cell. Harvested cells from a 750-ml culture of *E. coli* ER1458 (optical density at 450 nm = 1) were washed with 50 ml of 50 mM sodium phosphate–10 mM $MgCl_2$ (pH 6.8) and then resuspended in 7 ml of the same buffer. The French pressure cell was operated at 196 MPa. Alternatively, the cells were resuspended in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, and 50 mM β-mercaptoethanol [pH 7.0]), mixed with glass beads (33% [vol/vol], 212 to 300 μm; Sigma), and lysed in an ultrasonic bath by incubation twice for 5 min. Cell debris was removed by centrifugation, and the supernatant was used directly for enzyme activity measurements and enzyme characterization.

Assays for β-galactosidase activity. Hydrolysis of *o*-nitrophenyl (ONP)-β-D-galactopyranoside at 37°C and pH 7.0 followed by measurement of absorbance at 420 nm was used for determination of β-galactosidase activity (18). Assays were performed with Z buffer (for β-galactosidases BIF1, BIF2, and INF1) or Z buffer containing 0.5% Triton X-100 (for BIF3), and the reactions were stopped by the addition of 1 M Na_2CO_3. Transgalactosylation assays were performed with 0.4 M lactose, 50 mM Na citrate, and 100 mM Na_2HPO_4 (pH 6.0), and the 50-μl reaction volumes were incubated for approximately 20 h at 40°C. A 5-min incubation at 95°C was used to stop the enzyme reaction. The reaction mixtures were analyzed by thin-layer chromatography on Silica Gel 60 plates (Merck) in a solvent containing butanol, 2-propanol, and water (3:12:4). Samples of 1 μl of diluted sample (1:1 dilution in water) were subjected to three runs. After being dried, the sugars were visualized by spraying with an orcinol reagent, followed by incubation at 100°C for 5 to 10 min.

Molecular mass determination. The native molecular mass of β-galactosidases expressed in *E. coli* was determined by analytical gel filtration on a Superdex 200 HR 10/30 column (Pharmacia) followed by β-galactosidase assay of collected fractions. The molecular mass markers used for calibration of the column were thyroglobulin (669 kDa), ferritin (440 kDa), human immunoglobulin G (160 kDa), transferrin (81 kDa), and ovalbumin (43 kDa). The molecular mass of the β-galactosidase subunits was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (15). Samples were reduced with dithiothreitol and loaded onto 7.5 or 10% minigels which were stained with Coomassie brilliant blue or silver stained.

Enzyme purification. The BIF2 β-galactosidase was purified from *E. coli* extract by anion-exchange chromatography on a 5-ml HiTrap Q column (Pharmacia) at pH 7.5, followed by gel filtration on a Sephacryl S-200 HR column (Pharmacia, 1.6 by 60 cm). The INF1 β-galactosidase was purified from *E. coli* extract by gel filtration on a Superdex 200 HR 10/30 column (Pharmacia) followed by anion-exchange chromatography at pH 7.5 on a Mini-Q column (Pharmacia). The BIF3 β-galactosidase was purified from *E. coli* extract by gel filtration on a Superdex 200 HR 10/30 column, run on a SDS–7.5% polyacrylamide gel as described above, transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon P; Millipore), and stained with Coomassie brilliant blue. Digestion of BIF3 polypeptide bound to PVDF with endoproteinase Lys-C and extraction of the resultant proteolytic peptides were performed as described (8). The peptide fragments were purified by reversed-phase chromatography on a SMART system (Pharmacia) equipped with a μRPC C2/C18 SC2.1/10 column (Pharmacia) using a gradient of 0 to 80% acetonitrile in 0.1% trifluoroacetic acid. Peptide sequencing was performed as described below. The expression level of BIF1 was too low to permit purification. Therefore, crude cell extract containing the BIF1 enzyme was used to determine the molecular weight by gel filtration.

N-terminal amino acid sequence analysis. Enzyme samples were run on SDS-polyacrylamide gels as above, transferred to PVDF membrane (Problott; PE Biosystems), stained with Coomassie brilliant blue, and analyzed by Edman degradation with a protein microsequencer (Procise; PE Biosystems).

DNA sequence analysis. DNA sequencing was carried out using Cy-5-labeled primers. Vector specific primers, T3 and T7, were used in the first sequencing reaction mixture, followed by reactions with sequence-specific primers. The reaction mixtures were run with an ALF Express sequencer (Pharmacia). Databases were searched for homologous proteins with the BLAST facility (1). Comparison of amino acid sequences was performed as a BestFit analysis with the Wisconsin software package, version 10.0 (Genetics Computer Group, Madison, Wis.). The gap creation and gap extension penalty parameters for the BestFit analysis were 8 and 2, respectively. Alignment of β-galactosidase protein sequences was performed with the ClustalX program (34). The aligned sequences were subsequently imported into the PAUP 4.0b4 program (32), where phylogenetic trees were generated using a neighbor-joining algorithm.

Nucleotide sequence accession numbers. The four β-galactosidase sequences were deposited in the EMBL nucleotide sequence database with the accession numbers AJ272131 (BIF1), AJ224434 (BIF2), AJ224435 (BIF3), and AJ224436 (INF1).

RESULTS

Isolation of β-galactosidase genes from *B. bifidum* DSM20215. Genes encoding β-galactosidase from *B. bifidum* DSM20215 were cloned by shotgun cloning. Chromosomal DNA was isolated, cut with restriction enzymes, and inserted into cloning vectors as described in Materials and Methods. Ligation mixtures were transformed into β-galactosidase-deficient *E. coli* cells, and β-galactosidase-producing transformants were identified on X-Gal indicator plates. Ligation mixtures with *Pst*I-restricted *B. bifidum* DSM20215 chromosomal DNA gave rise to five positive blue clones out of approximately 1,500 screened transformants, and mixtures with *Kpn*I-restricted DNA resulted in one positive clone out of approximately 600 transformants. Restriction enzyme analysis indicated that four of the five *Pst*I clones were identical, whereas the fifth *Pst*I clone was different from the four identical clones. The *Pst*I clones were denoted pBIF1 and pBIF3, respectively, and the single *Kpn*I clone was denoted pBIF2. The positions of the β-galactosidase genes on the cloned fragments were examined by subcloning the inserts of the plasmids pBIF1, pBIF2, and pBIF3, respectively, as described below.

Plasmid pBIF1 contained a 7.6-kb insert. Deletion of 3 kb from one end of the fragment to a *Bam*HI site (Fig. 1) and 1.8 kb from the other end to an *Eco*RI site totally eliminated β-galactosidase activity measured on X-Gal indicator plates. Another plasmid construct, in which a 1-kb *Pst*I-to-*Kpn*I fragment was deleted, showed increased β-galactosidase activity, indicating that the *Kpn*I site was close to the structural β-galactosidase gene. Therefore, this deletion mutant was chosen as an anchor during DNA sequencing by so-called primer walking (Fig. 1). The resulting 3.5-kb DNA sequence contained an open reading frame with a coding capacity of 1,020

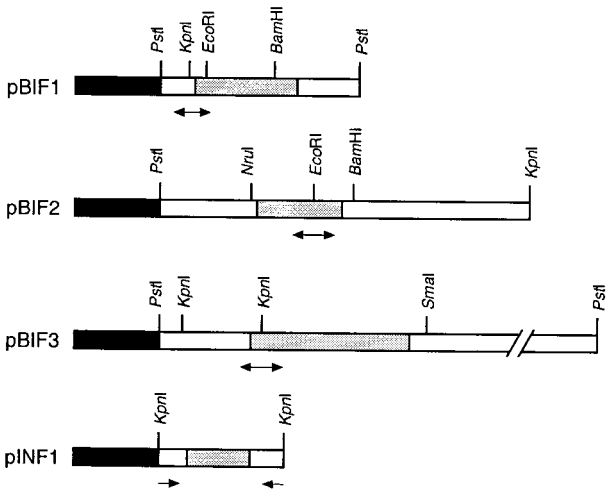


FIG. 1. Map of plasmids pBIF1, pBIF2, pBIF3, and pINF1. Black boxes indicate the cloning vector pBluescript KS(−), grey boxes show the position of β-galactosidase genes, and white boxes symbolize cloned sequences outside the β-galactosidase genes. Restriction enzyme sites used for mapping the β-galactosidase genes are shown above the maps, and the sites used as anchors in DNA sequencing by primer walking are indicated by arrows below the maps.

amino acid codons corresponding to a molecular mass of approximately 112 kDa (EMBL accession number AJ272131).

Plasmid pBIF2 was similarly subcloned in order to map the position of the β-galactosidase gene. β-Galactosidase activity was mapped to a 4.3-kb *Nru*I-to-*Bam*HI fragment in the middle of the original 13.6-kb *Kpn*I fragment. Further subcloning of a *Nru*I-to-*Eco*RI fragment resulted in transformants without β-galactosidase activity, indicating that the *Eco*RI site was located in the β-galactosidase gene (Fig. 1). Therefore, DNA sequencing of the β-galactosidase gene on plasmid pBIF2 was initiated at the *Eco*RI site and completed by primer walking. The resulting 3,700-bp DNA sequence contained an open reading frame of 1,044 amino acid codons corresponding to a polypeptide with a molecular mass of 117 kDa (EMBL accession number AJ224434).

Plasmid pBIF3, containing an insert of approximately 20 kb, was further subcloned and the β-galactosidase activity of transformants harboring the deletion plasmids was determined. Since cleavage at one of the internal *Kpn*I sites in the 20-kb fragment abolished β-galactosidase activity, this site was chosen as an anchor for DNA sequencing (Fig. 1). The resulting 5.5-kb DNA sequence contained an open reading frame of 1,752 amino acid codons corresponding to a molecular mass of 188 kDa (EMBL accession number AJ224435).

Isolation of β-galactosidase genes from *B. infantis* DSM20088. Genes from *B. infantis* DSM20088 encoding β-galactosidase were isolated as described above for *B. bifidum*. Chromosomal DNA was restricted with *Kpn*I, inserted in a cloning vector, and transformed into β-galactosidase-deficient *E. coli* cells as described in Materials and Methods. Nine β-galactosidase producing clones out of approximately 5,000 transformants were isolated. DNA sequencing showed that all the clones were identical. One of the clones, pINF1, was selected for further analysis. DNA sequencing of a 4.3-kb *Kpn*I fragment by primer walking from the ends of the fragment revealed an open reading frame of 690 amino acid codons corresponding to a molecular mass of 77 kDa (EMBL accession number AJ224436).

Characterization of β-galactosidase genes from *B. bifidum* and *B. infantis*. A comparison of the open reading frames in plasmids pBIF1, pBIF2, pBIF3, and pINF1 showed only a minor degree of protein sequence similarity between the encoded β-galactosidases (Fig. 2). Especially, the pINF1 sequence seemed to be distantly related to the other three genes and to the *E. coli lacZ* gene, as indicated by the short stretches of homology that the BestFit analysis returned (data not shown). Despite the fact that all four genes are bifidobacterial β-galactosidase genes and that three of them were derived from the same strain, they showed surprisingly little resemblance to each other.

Database searches with full-length amino acid sequences as queries showed homology to other known β-galactosidase sequences. The highest scores of amino acid identity found by comparison to other β-galactosidase sequences deposited in databases were 36, 53, 35, and 50% for the pBIF1, pBIF2, pBIF3, and pINF1 reading frames, respectively. Subsequences around the catalytic domains—corresponding to the sequence around the glutamic acid residue at position 461 in the *E. coli lacZ* gene—were selected from 30 β-galactosidases previously deposited in databases and aligned with the four sequences from this work. The alignment shown in Fig. 2A was subsequently used to generate a phylogenetic tree by neighbor-joining analysis. The resulting tree (Fig. 2B) showed that the INF1 β-galactosidase from *B. infantis* was located in a group of enzymes previously designated as family 42 glycosyl hydrolases (shown as class I in Fig. 2) (http://afmb.cnrs-mrs.fr/~pedro/CAZY/db.html and reference 11) and that the three β-galactosidases, BIF1, BIF2, and BIF3, from *B. bifidum* belonged to the group of enzymes classified as family 2 glycosyl hydrolases (shown as class II in Fig. 2). However, a closer examination of the alignments showed that BIF3 was deeply rooted within the group of family 2 glycosyl hydrolases and that the enzymes BIF1 and BIF2 were only distantly related to other family 2 glycosyl hydrolases. A phylogenetic analysis using full-length sequences confirmed the results obtained with the catalytic domain subsequences (data not shown). As expected, the alignment analysis placed the four known 6-phospho-β-galactosidases (family 1) in the same subgroup (class III in Fig. 2).

The β-galactosidase encoded by the pBIF3 sequence was found to be quite large compared to what is normally observed for *lacZ* group enzymes. Sequence analysis showed that the homology to known β-galactosidases was located in the N-terminal part of the reading frame, whereas no homology between the C-terminal half of the BIF3 enzyme and other β-galactosidases could be detected. A separate BLAST search with the C-terminal part revealed homology to enzymes known to contain a galactose binding domain, e.g., sialidase from *Micromonospora viridifaciens* (9), galactose oxidase from *Dactylium dendroides* (14) and sialidase from *Clostridium septicum* (EMBL accession no. X63266). As shown in Fig. 3, the amino acid residues known to bind galactose in sialidase and galactose oxidase are conserved in the BIF3 sequence, implying that the BIF3 β-galactosidase contains a galactose binding site.

Signal peptide prediction using the SignalP program described by Nielsen et al. (20) (http://www.cbs.dtu.dk/services/SignalP/) showed that the first 32 amino acid residues of the

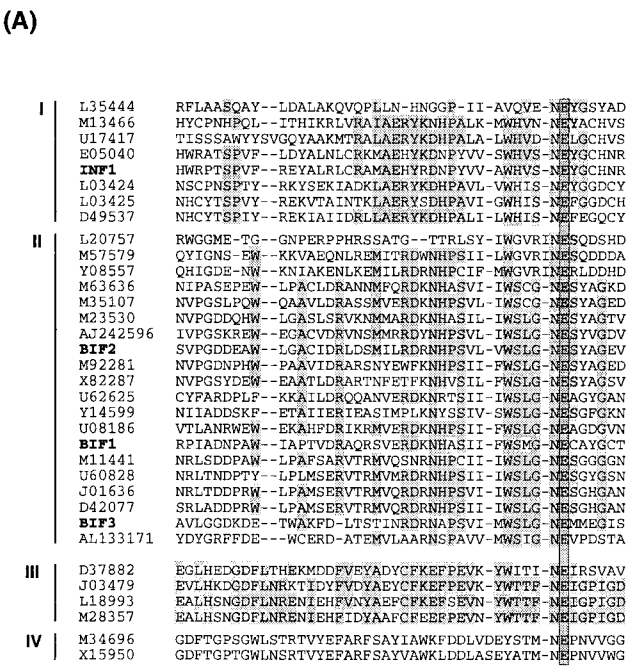
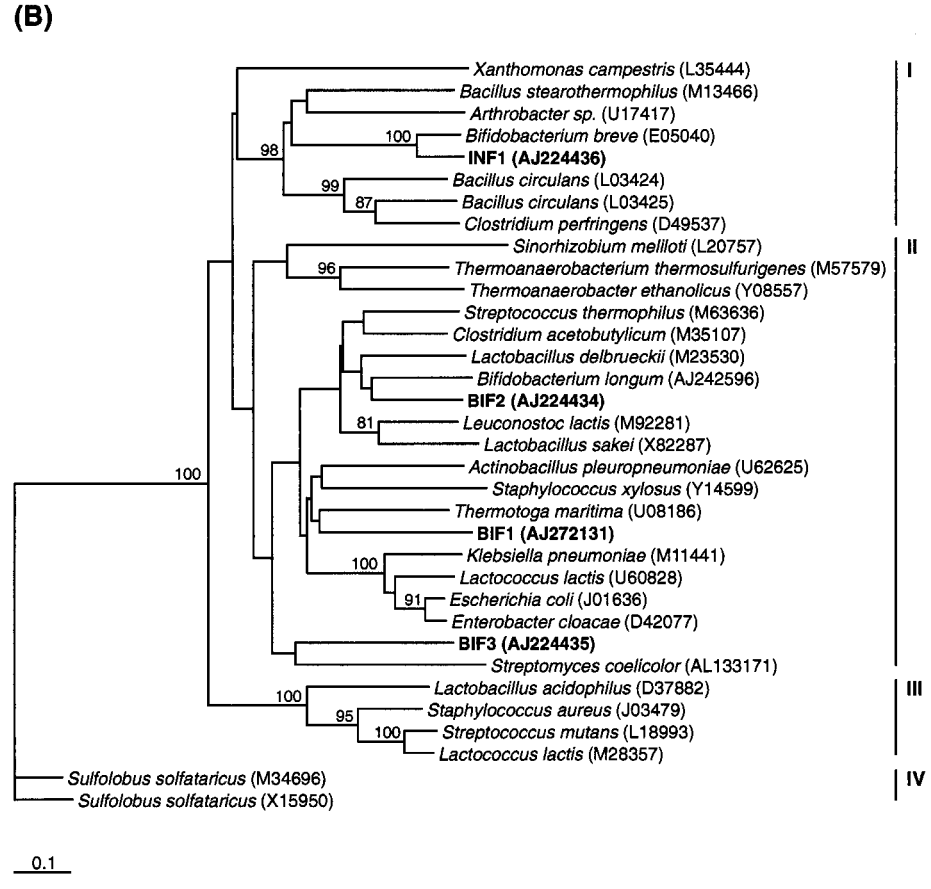


FIG. 2. Amino acid sequence comparison of active-site regions in selected β-galactosidases. (A) Sequences corresponding to the region around the catalytic Glu_{461} in the *lacZ* enzyme of *E. coli* were aligned using the ClustalX program. The BIF1, BIF2, BIF3, and INF1 sequences were obtained in this study. The other sequences are identified by database accession numbers. The conserved glutamic acid residue (E) is shown in frames, and conserved residues within classes I, II, and III are shaded. (B) Neighbor-joining analysis of the alignment shown in Fig. 2A. The *Sulfolobus* sequences were used as an outgroup. Results from a bootstrap analysis ($n = 100$) are shown for the junctions with a value above 80.



BIF3 reading frame constituted a potential signal peptide. N-terminal protein sequencing of BIF3 β-galactosidase expressed in *E. coli* confirmed that the predicted signal peptide was indeed cleaved off when BIF3 was expressed in *E. coli* (see below). The three other β-galactosidase sequences showed no signs of a signal peptide, when analyzed similarly (data not shown).

The untranslated sequences (UTS) (20) upstream of the

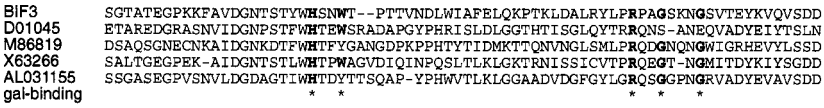


FIG. 3. Identification of a galactose binding domain in BIF3. The amino acid sequence of the BIF3 β-galactosidase (this study) was aligned to (i) galactose binding domains in sialidase from *M. viridifaciens* (accession no. D01045) and galactose oxidase from *D. dendroides* (accession no. M86819), (ii) sialidase from *C. septicum* (accession no. X63266), and (iii) a protein of unknown function from *Streptomyces coelicolor* (accession no. AL031155). Amino acid residues, which have been found by X-ray crystallography to interact with the bound galactose moiety in sialidase from *M. viridifaciens*, are marked with an asterisk below the sequences.

open reading frames of the β-galactosidase genes were examined for putative transcription and translation signals. UTS from other bifidobacterial genes were compared to the UTS from the four β-galactosidase genes described here, but no obvious transcription initiation signals were identified. However, when sequences immediately upstream of the translation initiation ATG codon were compared, potential base pairing to the 3′-end of *Bifidobacterium* 16S rRNA was evident (Fig. 4).

Heterologous expression of *B. bifidum* and *B. infantis* β-galactosidases in *E. coli*. The *Bifidobacterium* β-galactosidase genes on plasmids pBIF1, pBIF2, pBIF3, and pINF1 were expressed in *E. coli* under growth conditions which would normally repress expression from the inducible *E. coli lacZ* promoter located in the flanking region in the cloning vector. This observation indicated that endogenous, internal bifidobacterial sequences upstream of the β-galactosidase genes may serve as transcription initiation signals in *E. coli*. Similarly, initiation of translation may be facilitated by the putative *E. coli* ribosome binding sites (AGGA) (Fig. 4) which were observed immediately upstream of the open reading frame in all the β-galactosidase genes. The β-galactosidase activity of *E. coli* cells expressing BIF1, BIF2, BIF3, or INF1 was exclusively found in cell extracts (BIF1, BIF2, and INF1) or in cell extract and membrane fraction (BIF3), whereas no activity was found in the growth medium.

The native molecular masses of the recombinant β-galactosidases were determined by gel filtration, and the subunit sizes were determined by SDS-PAGE of the purified enzymes and/or calculated from the DNA sequence (Table 1). The native molecular mass of the BIF1 β-galactosidase was 620 kDa, and the size of the open reading frame corresponded to a subunit molecular mass of 112 kDa (Table 1). Taken together, these data suggest that BIF1 is a hexameric β-galactosidase.

Recombinant BIF2 β-galactosidase produced in *E. coli* exhibited a native molecular mass of approximately 236 kDa and a subunit size of approximately 130 kDa. Since the length of the open reading frame in the DNA sequence corresponded to 117 kDa, the BIF2 β-galactosidase is probably a dimeric enzyme. The BIF2 β-galactosidase was purified from *E. coli* and subjected to N-terminal sequence analysis. The N-terminal amino acid sequence (MNTTDDQRKN) confirmed that the predicted open reading frame of the DNA sequence was actually translated.

The BIF3 β-galactosidase produced in *E. coli* showed a native molecular mass of approximately 180 kDa when sonication with glass beads was used for cell lysis. Homogenization with a French press, however, led to a molecular mass of 360 kDa for the active enzyme. In both cases, the subunit size determined by SDS-PAGE was approximately 182 kDa. Since both extraction procedures resulted in an enzymatically active BIF3 enzyme, the BIF3 β-galactosidase is apparently active as a dimeric molecule and also—contrary to almost any other β-galactosidase—as a monomeric molecule. Further experiments are required, however, to determine whether the enzyme exists as a monomer or a dimer in vivo. The N-terminal amino acid sequence of BIF3 β-galactosidase produced in *E. coli* was VEDATRSDSTTQMS. This sequence corresponded to the sequence V_{33}-E_{34}-D_{35}-A_{36} found in the N-terminal part of the BIF3 open reading frame, implying that the first 32 amino acids of the BIF3 β-galactosidase constitute a signal peptide which is cleaved off posttranslationally. The processing site observed between amino acid residues Ala_{32} and Val_{33} is identical to the one predicted by the SignalP computer program (20). The exceptionally large open reading frame of BIF3 was further verified by amino acid sequence analysis of internal peptide fragments derived from the recombinant enzyme. BIF3 β-galactosidase was purified to homogeneity and digested with endoproteinase Lys-C. The peptide fragments were separated by high-pressure liquid chromatography, and selected peptide peaks were then analyzed with a protein sequencer. Six peptide sequences, spanning a wide range of the BIF3 amino acid sequence, were identified (F_{73}-Q_{82}, M_{384}-H_{393}, W_{433}-N_{442}, I_{906}-S_{915}, T_{1317}-Q_{1326}, and V_{1418}-T_{1425}). All the peptide sequences completely matched the amino acid sequence deduced from the DNA sequence, thus confirming that the large BIF3 reading frame was indeed translated.

Recombinant INF1 β-galactosidase expressed in *E. coli* showed a native molecular mass of approximately 140 kDa, and SDS-PAGE of the purified enzyme indicated a subunit molecular mass of 73 kDa, which is in agreement with the subunit size of 77 kDa predicted from the DNA sequence. Therefore, we conclude that the INF1 β-galactosidase is prob-

TABLE 1. Structural properties of recombinant *B. bifidum* and *B. infantis* β-galactosidases produced in *E. coli*

Enzyme	Molecular mass[a] determined by:			Proposed molecular structure	Signal peptide
	Gel filtration	SDS-PAGE	DNA sequence analysis		
BIF1	620	ND	112	Hexamer	−
BIF2	236	130	117	Dimer	−
BIF3	180 and 360	182	188	Monomer and dimer	+
INF1	140	73	77	Dimer	−

[a] Molecular mass values are in kilodaltons. ND, not determined.

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