J. Microbiol. Biotechnol. (2001), **11**(1), 106–111

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Molecular Cloning and Characterization of the β-Galactosidase Gene from *Bifidobacterium adolescentis* Int57

PARK, MYEONG-SOO[1], HYEON-JIN YOON[2], SEONG LYUL RHIM[3], AND GEUN EOG JI[4]*

[1]*Research Center for Agriculture and Life Sciences, Seoul National University, Suwon 441-744, Korea*
[2]*Department of Food Science and Technology, Seoul National University, Suwon 441-744, Korea*
[3]*Department of Genetic Engineering, Hallym University, Kangwon-Do, Chuncheon 200-702, Korea*
[4]*Department of Food Science and Nutrition, Seoul National University, Seoul 151-742, Korea*

Received: September 26, 2000
Accepted: December 8, 2000

Abstract A β-galactosidase gene of *Bifidobacterium adolescentis* Int57 (INT57) was cloned using the shotgun method. The sequence of the β-galactosidase gene existing in the sequenced 3,260-bp fragment showed higher than 40% homology with other bacterial β-galactosidase genes. The expression in *Escherichia coli* suggested that the β-galactosidase might have a monomeric, dimeric, or tetrameric protein structure. This is probably the first peer-reviewed sequence analysis of the β-galactosidase gene of the genus *Bifidobacterium.*

Key words: β-Galactosidase gene, sequence, expression, *Bifidobacterium adolescentis* Int57

Bifidobacteria is a Gram-positive, strict anaerobe of pleomorphic, coryneform rods. This bacterial genus was shown to be a predominant component of the human intestinal flora, especially in breast-fed infants [4, 16]. Bifidobacteria are also well known for their important roles in the proper balance of normal intestinal flora and beneficial effects for the health of human beings. This may be as a consequence of an altered intestinal pH, specifically through the release of acetic and lactic acids. It is known that bifidobacteria produce several enzymes that hydrolyze non-digestible oligosaccharides, including in the group of prebiotics, which cannot be digested in the upper part of the gastrointestinal tract. A few of the genes encoding the hydrolysis enzymes were cloned from bifidobacteria and sequenced [11, 17, 22]. β-Galactosidase is one of the hydrolysis enzymes of bifidobacteria and is also produced in eukaryotes and several other bacteria. Recently, Van Laere *et al.* [9] characterized the β-galactosidase of bifidobacteria which is engaged in the digestion of transgalactooligosaccharides (TOS). Several studies have been published concerning the isolation and characterization of plasmids from bifidobacteria [3, 12, 20], and vector construction using *Bifidobacterium* plasmid [2, 18, 19]. The classical antibiotics selection marker genes such as kanamycin, ampicillin, and tetracycline cannot be used for the transformation of bifidobacteria for the application in food industries. In view of these properties, the bifidobacterial β-galactosidase gene may be a good candidate for the development of the vector system. In this report, the molecular cloning and the expression of the β-galactosidase gene from *B. adolescentis* Int57 in *E. coli* are discussed.

*Corresponding author
Phone: 82-2-880-8749; Fax: 82-2-880-6282;
E-mail: geji@bifido.com

Materials and Methods

Bacterial Strains, Media, and Plasmids

E. coli DH5α was used for transformation, and cultured at 37°C in Luria broth with vigorous shaking. Ampicillin at the concentration of 50 μg/ml and 40 μl of X-gal (20 mg/ml in dimethylformamide) were used for the selection of transformed bacteria. *B. adolescentis* Int57 (INT57) was used for both gene cloning and enzyme characterization. The bacteria strain was grown in Brain Heart Infusion (BHI) broth (Difco, Detroit, U.S.A.) supplemented with 0.05% (final concentration) cysteine HCl at 37°C. The plasmid vectors pBR322 and pUC19 were used for shotgun cloning of the β-galactosidase gene and sequencing of the gene, respectively.

Chemicals and Enzymes

All restriction and modifying enzymes were purchased from Promega (Wisconsin, U.S.A.). X-gal (5-bromo-4-chloro-3-indonyl-β-D-galactoside), PNPG (*p*-nitrophenyl-β-D-

galactopyranoside) and agarose were from Sigma Chemical Co. (St. Louis, U.S.A.).

General Cloning Techniques and Sequence Analysis

The genomic DNA of INT57 was isolated by using the method of Demuyter *et al.* [6] and digested with *Eco*RI. The fragments were inserted in the same restriction site in the pBR322, which were then transformed in *E. coli* DH5α. The transformants were selected on agar solid plates containing X-gal, IPTG, and ampicillin. The positive blue colonies were selected and used for plasmid isolation. Plasmid DNA preparation from *E. coli*, restriction enzyme digestion, ligation, and transformation of *E. coli* were carried out according to the procedure described by Sambrook *et al.* [23]. Unidirectional deletion mutants for the DNA sequence were constructed with the Kilo-Sequencing Deletion Kit (TAKARA Shuzo Co., Shiga, Japan), and the nucleotides were determined using the BigDye terminator and ABI377 system (PE Applied Biosystems, CA, U.S.A.). The DNA and amino acid sequence data analyses were performed using the DNASIS and PROSIS programs (HITACHI Software Engineering Co., Japan), respectively. The homology search was done by using the World Wide Web server for BLAST search maintained at the National Center for Biotechnology and the programs BLASTN, BLASTP, BLASTX, and TBLASTN [1]. Multiple sequence alignment of related amino acid sequences were performed using the CLUSTAL V program [8].

Enzyme and Protein Assay

The β-galactosidase activities of INT57 and transformants were measured according to the method of Park *et al.* [21]. The strains were grown in BHI and LB media supplemented with 0.5% of glucose, galactose, and lactose. Aliquots of 1 ml were taken from the cell culture at various time periods. The harvested cells were washed twice with 1 ml of 0.1 M phosphate buffer (pH 6.0) and resuspended in 392 μl of the same buffer. Then, 8 μl of acetone-butanol (9:1) and 4 mM of PNPG (final concentration) was added. After vortexing and incubation at 45°C, the enzyme reaction was stopped by adding 600 μl of 0.5 M Na_2CO_3. The absorbance was measured at 400 nm. One unit of activity was defined as the amount of enzyme liberating one μmole of PNP per min according to the standard curve. Specific activity was defined as units per mg of protein. The protein concentration was measured by the method of Lowry *et al.* [13].

Activity Staining on Acrylamide Gel and SDS-PAGE

We have newly devised a proven method for the detection of β-Galactosidase activity by using X-Gal instead of PNPG or ONPG (*o*-nitrophenyl-β-D-galactopyranoside). The crude cell extract was prepared by sonication and electrophoresed on a non-denaturing polyacrylamide gel electrophoresis system according to Davis [5]. β-Galactosidase activity was then detected by incubating the gel in X-Gal solution (80 mg of X-Gal in 1 ml of 0.1 M phosphate buffer, pH 6.0) at 45°C until the blue color developed. For the molecular weight estimation of β-galactosidase, the active band was cut out from the native PAGE gel, electroeluted, and SDS-PAGE performed with the molecular weight size marker as described by Laemmli [10].

Detection of the β-Galactosidase Gene in INT57 by Southern Analysis

The 10-mg genomic DNA of INT57 was digested with *Eco*RI and electrophoretically separated in a 0.8% agarose gel. The genomic DNA was transferred to the hybridization paper of Hybond (Amersham) after denaturing, followed by neutralization. The 1.4 kb of DNA fragment was obtained by *Eco*RI digestion of the cloned pUBIGS. The fragment was digoxigenin-labeled using a hexanucleotide primer and Klenow enzyme as described by the manufacturer (DIG labeling and detection kits, Roche Biochemical) and used as the specific probe in the Southern analysis. Hybridization was performed overnight at 68°C in the blocking solution containing 5× SSC, 0.1% sarcosyl, and 0.02% SDS. After the hybridization process, the blotted paper was washed twice in 2× SSC, 0.1% SDS at room temperature and twice for 15 min in 0.5× SSC, 0.1% SDS at 68°C.

RESULTS AND DISCUSSION

Cloning of the β-Galactosidase Gene (β-*Gal*) from *B. adolescentis* Int57

For the cloning of β-galactosidase from INT57, genomic DNA was isolated and partially digested with *Eco*RI and ligated into the *Eco*RI site of pBR322, and finally transformed into *E. coli* DH5α. One out of approximately 10,000 transformants showed the blue color on the X-β-Gal plate. From the transformant, a plasmid was isolated and analyzed on the gel electrophoresis after the digestion with *Eco*RI DNA restriction enzyme had taken place. The analysis showed that the inserted DNA fragment consisted of two *Eco*RI fragments of about 1.4 and 1.9 kb. The plasmid was named as pBIG. For the confirmation of the β-galactosidase activity, the plasmid pBIG was re-transformed in *E. coli*, in which all the transformants showed the blue color on the X-β-Gal and ampicillin solid plates. In contrast, the *E. coli* with or without pBR322 showed no color formation (data not shown).

Sequence Analysis of the β-Galactosidase Gene

For the DNA sequence of the insert, the fragment was restricted to two fragments by *Eco*RI digestion, which were subcloned into pUC19. The resulted plasmids pUBIGL and pUBIGS containing 1.9 kb and 1.4 kb, respectively, were

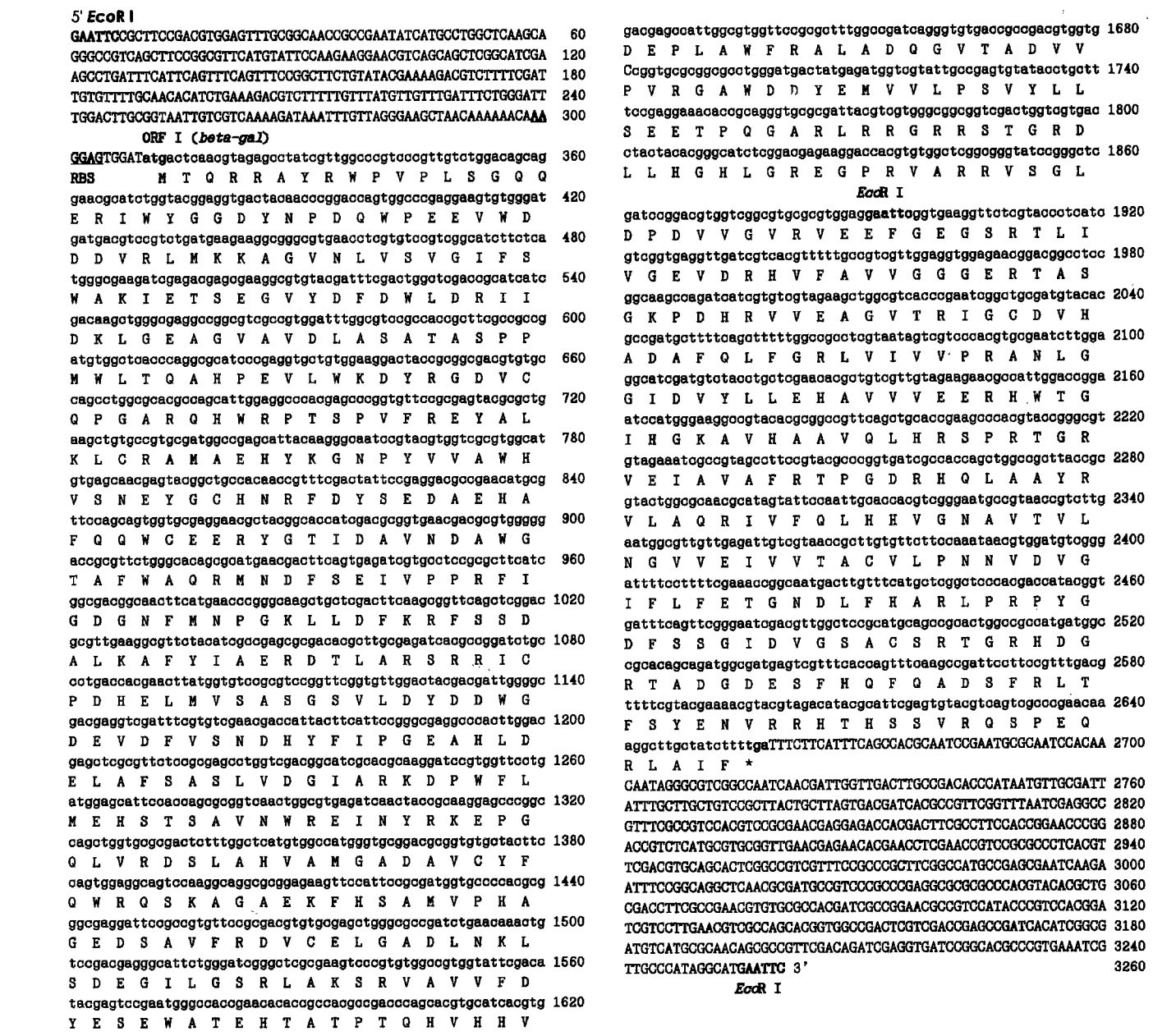

Fig. 1. Nucleotide sequence of *Eco*RI fragments and deduced amino acid sequence of the β-galactosidase gene (from 310 to 2,658 bp) of *Bifidobacterium adolescentis* Int57.

Three *Eco*RI sites are indicated at each corresponding site. The presumptive ribosome-binding site preceding the start codon is indicated. The nucleotide sequence is available in the NCBI and GenBank database under the accession number of AF213175.

used to determine the full sequences of inserted DNA fragments. Nested deletion sets of the two plasmids were made and fully sequenced for both strands as described in Materials and Methods. It was found that the inserted DNA fragment was identified to be composed of 3,260 bp and registered in GenBank under the accession number of AF213175. An ORF of 2,349 nucleotides was detected in this fragment and suggested to encode a protein of 87.9 kDa that was composed of 782 amino acids (Fig. 1). In the 7-bp region upstream of a translation start site at 310 bp, there was a presumptive ribosome binding site (AAGGAG). The amino acid sequences deduced from the ORF (Fig. 2.) showed high homologies in comparison with β-galactosidase from various microorganisms such as *B. breve* YIT 4010 (72% identity, patent number: JP 1993146296-A/1), *Bacillus stearothermophillus* (40% identitiy, GenBank accession number: g114936, [7]), *Caldicellalosiruptor* sp. 14B, (38%, g4160520), and *Thermotoga maritima* (34%, g4980811).

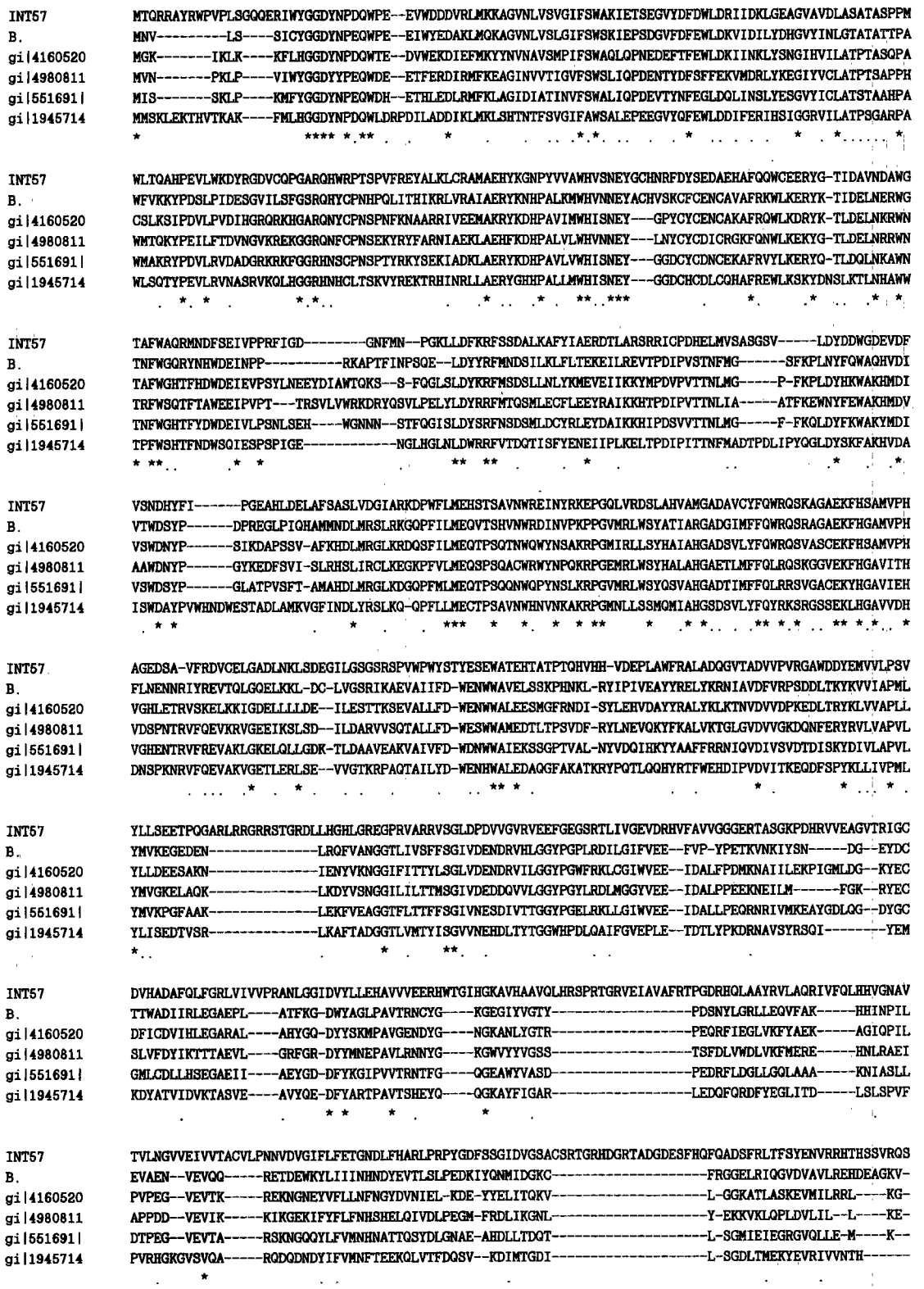

Fig. 2. Multiple sequence alignment of various β-galactosidases from *B. adolescentis* Int57 (INT57), *Bacillus stearothermophilus* (B), *Caldicellalosiruptor* sp. 14B (gil4160520), *Thermotoga maritima* (gil4980811), *Bacillus circulans* (gil5516911), and *Bacillus subtilis* (gil1945714).

The consensus line includes amino acid residues conserved in all the aligned sequences and marked as*. The gaps among those β-galactosidases are indicated as—, and the amino acid residues are indicated as belong to the same group.

From the sequence analysis, it was also found that the G+C content was 59.1%. The overall G+C contents of the genus *Bifidobacterium* is usually 55.0% to 67.0%, which is higher than that of most other bacteria. The G+C contents of the β-Gal I gene from *B. breve* YIT4010 (patent number: JP 1993146296-A/1), the LDH gene from *B. longum* aM101-2 [15], and the β-glucosidase gene from *B. breve* clb [17] were 63.6, 59.0, and 65.1%, respectively. For the detection of the β-galactosidase gene in INT57, Southern analysis was performed as described in Material and Methods. In this analysis, it was observed that the probe of the β-galactosidase gene was specifically hybridized with the genomic DNA fragment at the position of 1.4 kb of genomic DNA digested with *Eco*RI. The result indicates that the cloned β-galactosidase gene is located on the chromosomal DNA of INT57.

Expression and Characterizaion of the β-Galactosidase

For the analysis of the expression and characterization of the enzyme, an activity staining with X-gal solution was performed as described in Materials and Methods (Fig. 3A). The activity staining test after native PAGE with INT57 cell free extract clearly showed the active protein bands (lanes 1-3) corresponding to 170 kDa (II), 350 kDa band (I), and a faint 88 kDa band (III). Similar band patterns were obtained in the analysis with *E. coli* (pBIG) cell free extract (lanes 4 and 5). The intensely stained band corresponding to 170 kDa was cut out from the gel (lanes 4 and 5 in Fig. 2A), and the protein was electroeluted. The protein was analyzed on SDS-PAGE (Fig. 3B) indicating a single band of molecular weight 88 kDa that was identical to that of the protein deduced from the DNA sequence analysis. These results suggested that the cloned ORF expressed the β-galactosidase, which may have a complex protein structure of a monomer, dimer, or tetramer. The major form of the β-galactosidase is a dimer (II in Fig. 3A), and the high molecular mass bands (I in Fig. 3A) are a tetramer, whereas a faintly observable band (III in Fig. 3A) is a monomer. Activity bands were also observed on the native PAGE analyzed with the supernatant of *E. coli* (pBIG) in the culture (lanes 6 and 7 in Fig. 3A). The strong expression of the 170 kDa band suggested that the dimeric form may be identified as a major secreted enzyme. In contrast, the tetrameric or monomeric form is only present in cell extract of *E. coli* (pBIG) and INT57. This result suggests that the dimeric form might be the secretable form. *E. coli*, in general, is limited in its ability to secrete proteins into the culture medium. However, we have observed that the high enzyme activity (about 200 units) of β-galactosidase was present in the broth medium after the 16-h culture of *E. coli* (BIG) (Fig. 4), most likely caused by the secretion system of the β-galactosidase. Such a high extracellular enzyme activity was also reported in an expression system of the pathogenic hemolysin, which was secreted across the membrane [14]. The secretable characteristics of this enzyme may be very useful for the expression and purification of the heterologous protein in *E. coli*. In this experiment, we have observed that the activity of the bifidobacterial β-galactosidase may be regulated by carbon sources (data not shown). In fact, the regulation should be studied more thoroughly in the near future.

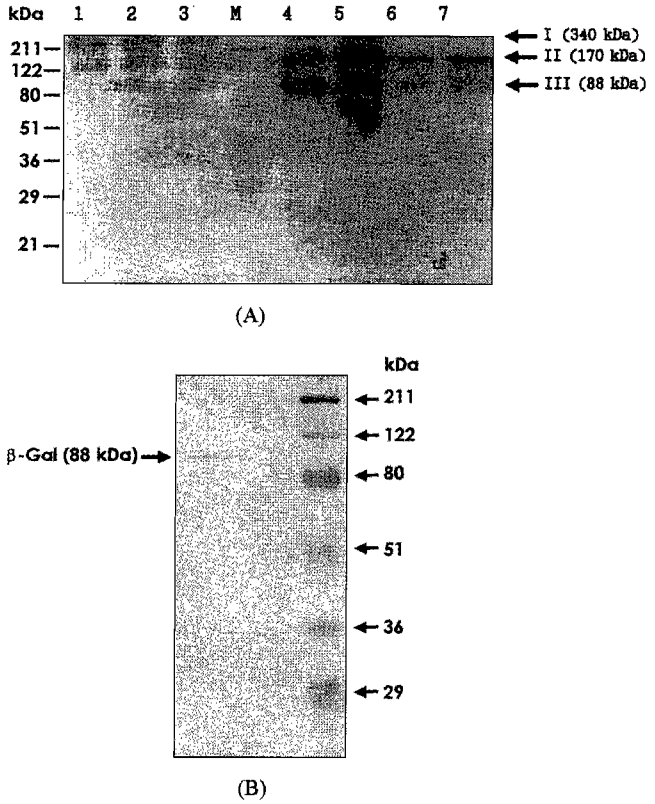

Fig. 3. Activity staining on native polyacrylamide gel.
(A) Lanes 1-3: culture broth of INT57; 4-5: cell extract of BIG; 6-7: supernatant of BIG; M: size marker (denatured and prestained marker, Bio-Rad, U.S.A.); sizes are indicated on the left. Putative multimeric bands are indicated as I, II, and III with molecular mass. (B) Denaturing SDS-PAGE analysis of the electroeluted β-galactosidase from BIG.

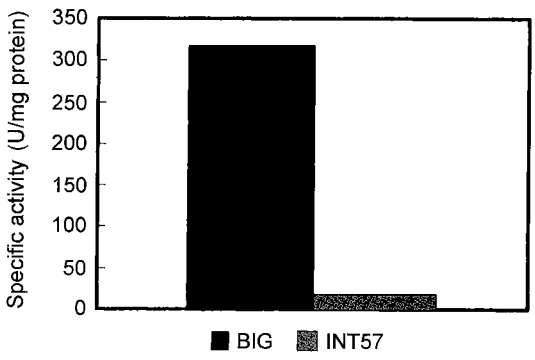

Fig. 4. Specific activities of β-galactosidase in *E. coli* (pBIG) and INT57. The cultured cells were disrupted by sonication.

Acknowledgments

This work was partially supported by the Ministry of Agriculture and Forestry (19980222) and the author are thankful for this.

REFERENCES

1. Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* **215:** 403-410.
2. Argnani, A., R. J. Leer, N. van Luijk, and P. H. Pouwels. 1996. A convenient and reproducible method to genetically transform bacteria of the genus *Bifidobacterium*. *Microbiology* **142:** 109-114.
3. Bourget, N., J. M. Simonet, and B. Decaris. 1993. Analysis of the genome of five *Bifidobacterium breve* strains: Plasmid content, pulsed-field gel electrophoresis genome size estimation and *rrn* loci number. *FEMS Microbiol. Lett.* **110:** 11-20.
4. Bezkorovainy, A. and R. Miller-Catchpole. 1989. *Biochemistry and Physiology of Bifidobacteria*. CRC Press, Boca Raton, U.S.A.
5. Davis, B. J. 1964. Disc electrophoresis II method and application to human serum proteins. *Ann. NY Acad. Sci.* **121:** 404-427.
6. Demuyter, P., P. Leblond, B. Decaris, and J. M. Simonet. 1988. Characterization of two families of spontaneously amplifiable units of DNA in *Streptomyces ambofaciens*. *J. Gen. Microbiol.* **134:** 2001-2007.
7. Haruhisa, H., F. Tsuyoshi, N. Feiji, and O. Hirosuke. 1986. Structure of a β-galactosidase gene of *Bacillus stearothermophilus*. *J. Bacteriol.* **166:** 722-727.
8. Higgins, D. G. 1994. CLUSTAL V: Multiple alignment of DNA and protein sequences. *Methods Mol. Biol.* **25:** 307-318.
9. Van Laere, K. M., T. Abee, H. A. Schols, G. Beldman, and A. G. Voragen. 2000. Characterization of a novel β-galactosidase from *Bifidobacterium adolescentis* DSM 20083 active towards transgalactooligosaccharides. *Appl. Environ. Microbiol.* **66:** 1379-1384.
10. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680-685.
11. Leder, S., W. Hartmeier, and S. P. Marx. 1999. α-Galactosidase of *Bifidobacterium adolescentis* DSM 20083. *Current Microbiology* **38:** 101-106.
12. Lee, H. J., M. S. Park, K. H. Lee, and G. E. Ji. 2000. Characterization of plasmids from *Bifidobacterium* sp. *J. Microbiol. Biotechnol.* **10:** 753–758.
13. Lowry, O. H., N. J., Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265-271.
14. Mackman, N., K. Baker, L. Gray, R. Haigh, J. M. Nicaud, and I. B. Holland. 1987. Release of a chimeric protein into the medium from *Escherichia coli* using the C-terminal secretion signal of haemolysin. *EMBO J.* **6:** 2835-2841.
15. Minowa, T., S. Iwata, H. Sakai, H. Masaki, and T. Ohta. 1989. Sequence and characteristics of the *Bifidobacterium longum* gene encoding L-lactate dehydrogenase and the primary structure of the enzyme: A new feature of the allosteric site. *Gene* **85:** 161-168.
16. Mitsuoka T. 1984. The effect of nutrition on intestinal flora. *Nahrung* **28:** 619-625.
17. Nunoura, N., K. Ohdan, K. Tanaka, H. Tamaki, T. Yano, M. Inui, H. Yukawa, K. Yamamoto, and H. Kumagai. 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. Biotechnol. Biochem.* **60:** 2011-2018.
18. Park, M. S., D. W. Shin, K. H. Lee, amd G. E. Ji. 2000. Characterization of plasmid pKJ36 from *Bifidobacterium longum* and construction of an *E. coli-Bifidobacterium* shuttle vector. *J. Microbiol. Biotechnol.* **10:** 312-320.
19. Park, M. S., D. W. Shin, K. H. Lee, and G. E. Ji. 1999. Sequence analysis of plasmid pKJ50 from *Bifidobacterium longum*. *Microbiology* **145:** 585-592.
20. Park, M. S., K. H. Lee, and G. E. Ji. 1997. Isolation and characterization of two plasmids from *Bifidobacterium longum*. *Lett. Appl. Microbiol.* **25:** 5-7.
21. Park, H. K., D. H. Kang, S. H. Yoon, K. H. Lee, S. K. Lee, and G. E. Ji. 1992. Enzyme pattern of *Bifidobacterium* sp. Int57. *Korean J. Appl. Microbiol. Biotechnol.* **26:** 647-654.
22. Rossi, M., L. Altomare, A. Gonzalez Vara y Rodriguez, P. Brigidi, and D. Matteuzzi. 2000. Nucleotide sequence, expression and transcriptional analysis of the *Bifidobacterium longum* MB 219 *lacZ* gene. *Arch Microbiol.* **174:** 74-80.
23. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY, U.S.A.