

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

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Abstract A \(\beta\)-galactosidase gene of \(Bifidobacterium\) adolescentis Int57 (INT57) was cloned using the shotgun method. The sequence of the $\beta\mbox{-galactosidase}$ 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.

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-

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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 EcoRI. 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 Na₂CO₃. 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 EcoRI and ligated into the EcoRI site of pBR322, and finally transformed into E. coli DH5\alpha. 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 EcoRI DNA restriction enzyme had taken place. The analysis showed that the inserted DNA fragment consisted of two EcoRI 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 retransformed 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

5' EcoR I **GAATTCCGCTTCCGACGTGGAGTTTGCGGCAACCGCCGAATATCATGCCTGGCTCAAGCA** 60 GGGCCGTCAGCTTCCGGCGTTCATGTATTCCAAGAAGGAACGTCAGCAGCTCGGCATCGA AGCCTGATTTCATTCAGTTTCAGTTTCCGGCTTCTGTATACGAAAAGACGTCTTTTCGAT 180 240 TGGACTTGCGGTAATTGTCGTCAAAAGATAAATTTGTTAGGGAAGCTAACAAAAAACA ORF I (beta-gal) 360 $\underline{\textbf{GGAG}} \\ \textbf{TGGATatgactca} \\ \textbf{acgtagagcctatcgttggcccgtcccgttgtctggacagcag}$ MTQRRAYRWPVPLSGQQ gaacgcatctggtacggaggtgactacaacccggaccagtggcccgaggaagtgtgggat ERIWYGGDYNPDQWPEEVWD $\tt gatgacgtccgtotgatgaagaaggcgggcgtgaacctcgtgtccgtcggcatcttctca$ D D V R L M K K A G V N L V S V G I F S tgggogaagatogagaogagogaaggcgtgtaogatttcgactggotogacogcatcatcAKIETSEGVYDFDWLDRII $\tt gacaagetgggogaggocggcgtcgccgtggatttggcgtccgccaccgcttcgccgccg$ D K L G E A G V A V D L A S A T A S P P 660 ${\tt atgtggotoacccaggogcatoccgaggtgctgttggaaggactaccgcggcgacgttgtgc}$ M W L T Q A H P E V L W K D Y R G D V C cagcctggcgcacgccagcattggaggcccacgagcccggtgttccgcgagtacgcgctg Q P G A R Q H W R P T S P V F R E Y A L ${\tt aagctgtgccgtgccgatgcccgagcattacaagggcaatccgtacgtggtcgcgtggcat}$ K L C R A M A E H Y K G N P Y V V A W H gtgagcaacgagtacggctgccacaaccgtttcgactattccgaggacgccgaacatgcg V S N E Y G C H N R F D Y S E D A E H A $\verb|tccagcagtggtgcgaggaacgctacggcaccatcgacgcggtgaacgacgcgtggggg|$ FQQWCEERYGTIDAVNDAW ${\tt accgcgttctgggcacagcgcatgaacgacttcagtgagatcgtgcctccgcgcttcatc}$ TAFWAQRMNDFSEIVPPRFI ggcgacggcaacttcatgaacccgggcaagctgctcgacttcaagcggttcagctcggac 1020 G D G N F M N P G K L L D F K R F S S D $\tt gcgttgaaggcgttotacatcgacgagcgcgacacgottgcgagatcacgccggatotgc \ 1080$ ALKAFYI AERDTLARS RRIC $\verb|cotgaccacgaacttatggtgtccgcgtccggttcggtgttggactacgacgattgggc 1140|\\$ PDHELMVSASGSVLDYDDWG gacgaggtcgatttcgtgtcgaacgaccattacttcattccgggcgaggcccacttggac 1200 DEVDFVS N D H Y F I P G E A H L D gagotogogttotoogogagootggtogacggcatogcacgcaaggatocgtggttootg 1260 ELAFSASLVDGIARKDPWFL atggagcattccaccagcgcggtcaactggcgtgagatcaactaccgcaaggagcccggc 1320 M E H S T S A V N W R E I N Y R K E P G cagctggtgogogactctttggctcatgtggccatgggtgcggacgcggtgtgctacttc 1380 Q L V R D S L A H V A M G A D A V C Y F cagtggaggcagtccaaggcaggcgggagaagttccattccgcgatggtgccccacgcg 1440 Q W R Q S K A G A E K F H S A M V P H A ggcgeggettcggcgtgttggggggtgtgggcgcgetttgaacaaactg 1500 G E D S A V F R D V C E L G A D L N K L tccgacgagggcattctgggatcgggctcgcgaagtcccgtgtggocgtggtattcgaca 1560 DEGILGSRLAKSRVAVVFD ${\tt tacgagtccgaatgggccaccgaacacaccaccaccacgccacgcaccacgcacctgcatcacgtg} \ \ 1620$

gatooggacgtggtcggcgtgcgcgtggaggaattoggtgaaggttotcgtaccotcato 1920 D P D V V G V R V E E F G E G S R T L I V G E V D R H V F A V V G G G E R T A S ggcaagcoagatcatcgtgtogtagaagctggcgtcacccgaatcggctgcgatgtacac 2040 G K P D H R V V E A G V T R I G C D V H gccgatgcttttoagotttttggccgcctcgtaatagtcgtcccacgtgcgaatcttgga 2100 A D A F Q L F G R L V I V V P R A N L G ggcatcgatgtotacctgotcgaacacgotgtogttgtagaagaacgccattggaccgga 2160 G I D V Y L L E H A V V V E E R H W T G atccatgggaaggoogtacacgcggccgttcagotgcaccgaagcccacgtaccgggcgt 2220 I H G K A V H A A V Q L H R S P R T G R gtagaaatogccgtagccttccgtacgcccggtgatcgccaccagctggccgcttaccgc 2280 V E I A V A F R T P G D R H Q L A A Y R gtactggcgcaacgcatagtattccaattgcaccacgtcgggaatgccgtaaccgtcttg 2340 V L A Q R I V F Q L H H V G N A V T V L aatggcgttgttgagattgtcgtaaccgcttgtgttcttccaaataacgtggatgtcggg 2400 NGVVEIVVTACVLPNNVDVG attttccttttcgaaaccggcaatgacttgtttcatgctcggctcccacgaccatacggt 2460 I F L F E T G N D L F H A R L P R P Y G gatttcagttcgggaatcgacgttggctccgcatgcagccgcactggccgccatgatggc 2520 D F S S G I D V G S A C S R T G R H D G cgcacagcagatggcgatgagtcgtttcacoagtttoaagccgattccttccgtttgacg 2580 RTADGDESFHOFQADSFRLT ttttcgtacgaaaacgtacgtagacatacgcattcgagtgtacgtcagtcgcccgaacaa 2640 F S Y E N V R R H T H S S V R Q S P E Q aggcttgctatcttttgaTTTCTTCATTTCAGCCACGCAATCCGAATGCGCAATCCACAA 2700 RLAIF * CAATAGGGCGTCGGCCAATCAACGATTGGTTGACTTGCCGACACCCATAATGTTGCGATT 2760 ATTTGCTTGCTGTCCGCTTACTGCTTAGTGACGATCACGCCGTTCGGTTTAATCGAGGCC 2820 GTTTCGCCGTCCACGTCCGCGAACGAGGAGACCACGACTTCGCCTTCCACCGGAACCCGG 2880 ACCGTCTCATGCGTGCGGTTGAACGAGAACACGAACCTCGAACCGTCCGCGCCCTCACGT 2940 TCGACGTGCAGCACTCGGCCGTCGTTTCCGCCCGCTTCGGCCATGCCGAGCGAATCAAGA 3000 ATTTCCGGCAGGCTCAACGCGATGCCGTCCCGCCCGAGGCGCGCCCCACGTACACGCTG 3060 CGACCTTCGCCGAACGTGTGCGCCACGATCGCCGGAACGCCGTCCATACCCGTCCACGGA 3120 TCGTCCTTGAACGTCGCCAGCACGGTGGCCGACTCGTCGACCGAGCCGATCACATCGGCG 3180 ATGTCATGCGCAACAGCGCCGTTCGACAGATCGAGGTGATCCGGCACGCCCGTGAAATCG 3240 3260 TTGCCCATAGGCATGAATTC 3 EcoR I

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

Y E S E W A T E H T A T P T Q H V H H V

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% identity, GenBank accession number: g114936, [7]), *Caldicellalosiruptor* sp. 14B, (38%, g4160520), and *Thermotoga maritima* (34%, g4980811).

	MANDALIA DE LINE DE LA CONTROL
INT57	MTGRRAYRWPVPLSGGGERIWYGGDYNPDGWPEEVWDDDVRLMKKAGVNLVSVGIFSWAKIETSEGVYDFDWLDRIIDKLGEAGVAVDLASATASPPM
В.	MNVLSSICYGGDYNPEQWPE-EIWYEDAKLMQKAGVNLVSLGIFSWSKIEPSDGVFDFEWLDKVIDILYDHGVYINLGTATATTPA
gi 4160520	MGKIKLKKFLHGGDYNPDGWTEDVWEKDIEFMKYYNVNAVSMPIFSWAGLGPNEDEFTFEWLDKIINKLYSNGIHVILATPTASOPA
gi 4980811	MVNPKLPVIWYGGDYYPEGWDEETFERDIRMFKEAGINVVTIGVFSWSLIQPDENTYDFSFFEKVMDRLYKEGIYVCLATPTSAPPH
gi 551691	MISSKLPKMFYGGDYNPEGWDH-ETHLEDLRMFKLAGIDIATINVFSWALIOPDEVTYNFEGLDQLINSLYESGVYICLATSTAAHPA
gi 1945714	MMSKLEKTHVTKAKFMLHGGDYNPDGWLDRPDILADDIKLMKLSHTNTFSVGIFAWSALEPEEGVYGFEWLDDIFERIHSIGGRVILATPSGARPA
	* * * * * * * * * * * * * * * * * * * *
INT57	WLTQAHPEVLWKDYRGDVCQPGARQHWRPTSPVFREYALKLCRAMAEHYKGNPYVVAWHVSNEYGCHNRFDYSEDAEHAFQQWCEERYG-TIDAVNDAWG
В.	WFVKKYPDSLPIDESGVILSFGSRQHYCPNHPQLITHIKRLVRAIAERYKNHPALKMWHVNNEYACHVSKCFCENCAVAFRKWLKERYK-TIDELNERWG
gi 4160520	CSLKSIPDVLPVDIHGRORKHGARONYCPNSPNFKNAARRIVEEMAKRYKDHPAVIMWHISNEY——GPYCYCENCAKAFROWLKDRYK-TLDELNKRWN
gi 4980811	WITCKYPEILFTDVNGVKREKGGRONFCPNSEKYRYFARNIAEKLAEHFKDHPALVLWHVNNEYLNYCYCDICRGKFONWLKEKYG-TLDELNRRWN
• :	WMAKRYPDVLRVDADGRKRKFGGRHNSCPNSPTYRKYSEKIADKLAERYKDHPAVLVWHISNEYGGDCYCDNCEKAFRVYLKERYG-TLDQLNKAWN
gi 551691 gi 1945714	WLSQTYPEVLRVNASRVKQLHGGRHNHCLTSKVYREKTRHINRLLAERYGHHPALLMWHISNEYGGDCHCDLCGHAFREWLKSKYDNSLKTLNHAWW
g1 1945/14	* * * * * * * * * * * * * * * * * * *
INT57	TAFWAQRMNDFSEIVPPRFIGDGNYMN-PGKLLDFKRFSSDALKAFYIAERDTLARSRRICPDHELMVSASGSVLDYDDWGDEVDF
В.	TNFWGGRYNHWDEINPPRKAPTFINPSGE-LDYYRFMNDSILKLFLTEKEILREVTPDIPVSTNFMGSFKPLNYFGWAGHVDI
gi 4160520	TAFWGHTFHDWDEIEVPSYLNEEYDIAWTOKS-S-FGGLSLDYKRFMSDSLLNLYKMEVEIIKKYMPDVPVTTNLMGP-FKPLDYHKWAKHMDI
gi 4980811	TRFWSQTFTAWEEIPVPTTRSYLVWRKDRYQSVLPELYLDYRRFMTQSMLECFLEEYRAIKKHTPDIPVTTNLIAATFKEWNYFEWAKHMDV
gi 551691	TNFWGHTFYDWDEIVLPSNLSEHWGNNN-STFGGISLDYSRFNSDSMLDCYRLEYDAIKKHIPDSVVTTNLMGF-FKGLDYFKWAKYMDI
gi 1945714	TPFW9HTFNDWSQIESP9PIGENGLHGLNLDWRRFVTDGTISFYENEIIPLKELTPDIPITTNFMADTPDLIPYGGLDYSKFAKHVDA
91/1040/11	** * * * * * * * * *
INT57	VSNDHYFIPGEAHLDELAFSASLVDGIARKDPWFLMEHSTSAVNWREINYRKEPGOLVRDSLAHVAMGADAVCYFOWRQSKAGAEKFHSAMVPH
В.	VTWDSYPDPREGLPIOHAMMNDLMRSLRKGOPFILMEQVTSHVNWRDINVPKPPGVMRLWSYATIARGADGIMFFGWRQSRAGAEKFHGAMVPH
gi 4160520	VSWDNYPSIKDAPSSV-AFKHDLMRGLKRDQSFILMEQTPSQTNWQWYNSAKRPGMIRLLSYHAIAHGADSVLYFQWRQSVASCEKFHSAMVPH
gi 4980811	AAWDNYPGYKEDFSVI-SLRHSLIRCLKEGKPFVLMEQSPSQACWRWYNPQKRPGEMRLWSYHALAHGAETLMFFQLRQSKGGVEKFHGAVITH
gi 551691	VSWDSYPGLATPVSFT-AMAHDLMRGLKDGOPFMLMEGTPSQQNWQPYNSLKRPGVMRLWSYQSVAHGADTIMFFQLRRSVGACEKYHGAVIEH
gi 1945714	I SWDAYPYWHNDWESTADLAMKYGF INDLYRSLKQ-OPFILMECTPSAVNWHNYNKAKRPGMNLLSSMOMIAHGSDSVLYFQYRKSRGSSEKLHGAVVDH
8111240114	* * * * * * * * * * * * * * * * * * * *
INT57	AGEDSA-VFRDYCELGADLNKLSDEGI LGSGSRSPVWPWYSTYESEWATEHTATPTOHVHH-VDEPLAWFRALADGGVTADVVPVRGAWDDYEMUVLPSV
В.	FINENNRIYREVTOLGGELKKI-DC-LVGSRIKAEVAIIFD-WENWWAVELSSKPHNKL-RYIPIVEAYYRELYKRNIAVDFVRPSDDLTKYKVVIAPML
gi 4160520	VGHI.ETRVSKELKKIGDELLLLDEILESTTKSEVALLFD-WENWWALEESMGFRNDI-SYLEHVDAYYRALYKLKTNVDVVDPKEDLTRYKLVVAPLL
gi 4980811	VDSPNTRVFQEVKRVGEEIKSLSDILDARVVSQTALLFD-WESWWAMEDTLTPSVDF-RYLNEVQKYFKALVKTGLGVDVVGKDONFERYRVLVAPVL
gi 551691	VGHENTRYFREYAKI.GKELQLI.GDK-TLDAAVEAKVAIVFD-WDNWWAIEKSSGFTVAL-NYVDQIHKYYAAFFRRNIQVDIVSVDTDISKYDIVLAPVL
gi 1945714	DNSPKNRVFGEVAKVGETLERLSEVVGTKRPAGTAILYD-WENHWALEDAGGFAKATKRYPGTLGGHYRTFWEHDIPVDVITKEGDFSPYKLLIVPML
0 -1	
INT57	YLLSEETPOGARLRRGRRSTGRDLLHGHLGREGPRVARRVSGLDPDVVGVRVEEFGEGSRTLIVGEVDRHVFAVVGGGERTASGKPDHRVVEAGVTRIGC
В.,	YMVKEGEDENLRQFVANGGTLIVSFFSGIVDENDRVHLGGYPGPLRDILGIFVEEFVP-YPETKVNKIYSNDG-EYDC
gi 4160520	YLLDEESAKNIENYVKNGGIFITTYLSGLVDENDRVILGGYPGWFRKLCGIWVEEIDALFPDMKNAIILEKPIGMLDGKYEC
gi 4980811	YMVGKELAQKLKDYVSNGGILILTTMSGIVDEDDQVVLGGYPGYLRDLMGGYVEE-IDALPPEEKNEILMFGK-RYEC
gi 551691	YMVKPGFAAKLEKFVEAGGTFLTTFFSGI VNESDI VTTGGYPGELRKLLGI WVEEI DALLPEGRNRI VMKEAYGDLGGDYGC
gi 1945714	YLISEDTVSRLKAFTADGGTLVMTYISGVVNEHDLTYTGGWHPDLQAIFGVEPLETDTLYPKDRNAVSYRSQIYEM
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INT57	DVHADAFQLFGRLVIVVPRANLGGIDVYLLEHAVVVEERHWTGIHGKAVHAAVQLHRSPRTGRVEIAVAFRTPGDRHQLAAYRVLAQRIVFQLHHVGNAV
В.	TTWADIIRLEGAEPLATFKG-DWYAGLPAVTRNCYGKGEGIYVGTYPDSNYLGRLLEQVFAKHHINPIL
gi 4160520	DFICDVIHLEGARALAHYGQ-DYYSKMPAVGENDYGNGKANLYGTRPEQRFIEGLVKFYAEKAGIQPIL
gi 4980811	SLVFDYIKTTTAEVLGRFGR-DYYMNEPAVLRNNYGKGWVYYVGSSTSFDLVWDLVKFMEREHNLRAEI
ICE4 CO4 I	
gi 551691	GMLCDLLHSEGAEIIAEYGD-DFYKGIPVVTRNTFGQGEAWYVASDPEDRFLDGLLGQLAAAKNIASLL
gi 1945714	GMLCDLLHSEGAEIIAEYGD-DFYKGIPVVTRNTFGQGEAWYYASDPEDRFLDGLLGGLAAAKNIASLL KDYATVIDVKTASVEAVYQE-DFYARTPAVTSHEYQQGKAYFIGARLEDQFQRDFYEGLITDLSLSPVF
-	
-	KDYATVIDVKTASVEAVYQE-DFYARTPAVTSHEYQQGKAYFIGARLEDQFQRDFYEGLITDLSLSPVF
gi 1945714 INT57	KDYATVIDVKTASVEAVYQE-DFYARTPAVTSHEYQQGKAYFIGARLEDQFQRDFYEGLITDLSLSPVF * * * * TVLNGVVEIVVTACVLPNNVDVGIFLFETGNDLFHARLPRPYGDFSSGIDVGSACSRTGRHDGRTADGDESFHQFQADSFRLTFSYENVRRHTHSSVRQS
gi 1945714 INT57 B.	KDYATVIDVKTASVEAVYQE-DFYARTPAVTSHEYQQGKAYFIGARLEDQFQRDFYEGLITDLSLSPVF * * * * TVLNGVVEIVVTACVLPNNVDVGIFLFETGNDLFHARLPRPYGDFSSGIDVGSACSRTGRHDGRTADGDESFHQFQADSFRLTFSYENVRRHTHSSVRQS EVAENVEVQQRETDEWKYLIIINHNDYEVTLSLPEDKIYQNMIDGKCFRGGELRIQGVDVAVLREHDEAGKV-
gi 1945714 INT57 B. gi 4160520	KDYATVIDVKTASVEAVYQE-DFYARTPAVTSHEYQQGKAYFIGARLEDQFQRDFYEGLITDLSLSPVF * * * * TVLNGVVEIVVTACVLPNNVDVGIFLFETGNDLFHARLPRPYGDFSSGIDVGSACSRTGRHDGRTADGDESFHQFQADSFRLTFSYENVRRHTHSSVRQS EVAENVEVQQRETDEWKYLIIINHNDYEVTLSLPEDKIYQNMIDGKCFRGGELRIQGVDVAVLREHDEAGKV- PVPEGVEVTK
gi 1945714 INT57 B.	KDYATVIDVKTASVEAVYQE-DFYARTPAVTSHEYQQGKAYFIGARLEDQFQRDFYEGLITDLSLSPVF * * * * TVLNGVVEIVVTACVLPNNVDVGIFLFETGNDLFHARLPRPYGDFSSGIDVGSACSRTGRHDGRTADGDESFHQFQADSFRLTFSYENVRRHTHSSVRQS EVAENVEVQQRETDEWKYLIIINHNDYEVTLSLPEDKIYQNMIDGKGFRGGELRIQGVDVAVLREHDEAGKV- PVPEGVEVTKREKNGNEYVFLLNFNGYDVNIEL-KDE-YYELITQKVL-GGKATLASKEVMILRRL
gi 1945714 INT57 B. gi 4160520	KDYATVIDVKTASVEAVYGE-DFYARTPAVTSHEYQGKAYFIGARLEDGFQRDFYEGLITDLSLSPVF * * * * TVLNGVVEIVVTACVLPNNVDVGIFLFETGNDLFHARLPRPYGDFSSGIDVGSACSRTGRHDGRTADGDESFHGFQADSFRLTFSYENVRRHTHSSVRQS EVAENVEVQQ
gi 1945714 INT57 B. gi 4160520 gi 4980811	KDYATVIDVKTASVEAVYQE-DFYARTPAVTSHEYQQGKAYFIGARLEDQFQRDFYEGLITDLSLSPVF * * * * TVLNGVVEIVVTACVLPNNVDVGIFLFETGNDLFHARLPRPYGDFSSGIDVGSACSRTGRHDGRTADGDESFHQFQADSFRLTFSYENVRRHTHSSVRQS EVAENVEVQQRETDEWKYLIIINHNDYEVTLSLPEDKIYQNMIDGKGFRGGELRIQGVDVAVLREHDEAGKV- PVPEGVEVTKREKNGNEYVFLLNFNGYDVNIEL-KDE-YYELITQKVL-GGKATLASKEVMILRRL

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 EcoRI. 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

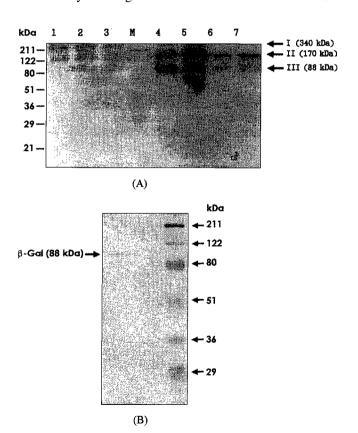


Fig. 3. Activity staining on native polyacrylamide gel. (A) Lanes 1-3: culture broth of INT57; 4-5: cell extract of BIG; 6-7: supernatant of BIG; M: size marker (denatured and prestained marker, Bio-Rad, U.S.A.); sizes are indicated on the left. Putative multimeric bands are indicated as I, II, and III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

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 βgalactosidase 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.

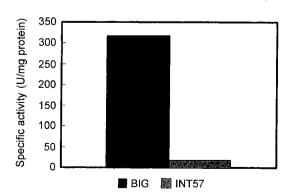


Fig. 4. Specific activities of β -galactosidase in *E. coli* (pBIG) and INT57. The cultured cells were disrupted by sonication.

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