J. Microbiol. Biotechnol. (2005), **15**(4), 800–808

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Characterization of the α-Galactosidase Gene from *Leuconostoc mesenteroides* SY1

KIM, JONG HWAN, JAE-YONG PARK, SEON-JU JEONG, JIYEON CHUN, JONG HOON LEE[3], DAE KYUN CHUNG[2], AND JEONG HWAN KIM[1]*

Division of Applied Life Science, Graduate School, and [1]Institute of Agriculture & Life Science, Gyeongsang National University, Jinju 660-701, Korea
[2]School of Biotechnology and Institute of Life Science and Resources, Kyung Hee University, Suwon 449-701, Korea
[3]Department of Food Science and Biotechnology, Kyonggi University, Suwon 442-760, Korea

Received: November 18, 2004
Accepted: February 26, 2005

Abstract *Leuconostoc mesenteroides* SY1, an isolate from kimchi, was able to ferment α-galactosides, such as melibiose and raffinose. α-Galactosidase (α-Gal) activity was higher in cells grown on melibiose and raffinose than cells grown on galactose, sucrose, and fructose. α-Gal activity was not detected in cells grown on glucose, indicating the operation of carbon catabolite repression (CCR). A 6 kb DNA fragment was PCR amplified using a primer set based on the nucleotide sequence of a putative α-galactosidase gene (*aga*) from *L. mesenteroides* ATCC 8293. Nucleotide sequencing of the 6 kb fragment confirmed the presence of *aga* and other genes involved in the galactosides utilization, and the gene order was *galR* (transcriptional regulator)-*aga*-*galK* (galactokinase)-*galT* (galactose-1-phosphate uridylyltransferase). Northern blotting experiment showed that *aga*, *galK*, and *galT* constituted the same operon, that the transcription was induced by galactosides, such as melibiose and raffinose, whereas *galR* was independently transcribed as a monocistronic gene, and that the level of transcription was fairly constant. The *aga* was overexpressed in *E. coli* BL21(DE3) using pET26b(+) vector, and α-Gal was accumulated in *E. coli* as an inclusion body.

Key words: *Leuconostoc mesenteroides*, α-galactosidase gene, overexpression, catabolite repression

α-Galactosidase (α-D-galactoside-galactohydrolase, EC.3.2.1.22) catalyzes the hydrolysis of 1,6-linked α-galactose residues from oligosaccharides and polymeric galactomannans [15]. α-Galactosidases (α-Gals) are known to occur widely among microorganisms, plants, and animals, and some of them have been purified and characterized [5]. In the sugar beet industry, α-Gals have been used to increase the sucrose yield by eliminating raffinose, which prevents the crystallization of beet sugar [24]. Raffinose and stachyose in soybeans are known to cause flatulence, one of the major drawbacks of soy products, which must be overcome if more wide consumption of soy products is desired. Flatulence is caused because humans are deficient in pancreatic α-Gal, and α-galactosides are degraded in the large intestine by anaerobic bacteria, such as *Clostridium* spp. and *Bacteroides* spp., yielding considerable amount of CH_4, CO_2, and H_2. The accumulation of flatulent rectal gas provokes gastrointestinal distress, such as abdominal pain, nausea, and diarrhea [20]. α-Gal has the potential to alleviate these symptoms by removing α-galactosides, when added to soymilk [6]. Several microorganisms are known to produce α-Gals [1]. Among the LAB (lactic acid bacteria), *Lactobacillus plantarum*, *L. fermentum*, *L. brevis*, and *Lactococcus raffinolactis* used in vegetable fermentations can hydrolyze α-galactosides to digestible carbohydrates. Recently, α-Gal-encoding genes from several LAB were cloned and characterized [2, 4]; however, not much is known about the modes of gene regulation. In lactobacilli, α-Gals have been characterized at the biochemical and physiological levels [8, 18]. However, no data are so far available regarding the molecular characterization of the α-Gal gene from *Leuconostoc mesenteroides*.

In this work, we characterized the α-Gal gene (*aga*) and neighboring genes involved in galactose degradation from *L. mesenteroides* SY1, a strain isolated from kimchi. This study provides the first genetic analysis of the *aga* gene from *L. mesenteroides*.

*Corresponding author
Phone: 82-55-751-5481; Fax: 82-55-753-4630;
E-mail: jeonghkm@gsnu.ac.kr

Table 1. Bacterial strains and plasmids used in this study.

Bacterial strain and plasmid	Relevant characteristic	Reference
Strain		
Escherichia coli		
DH5α	φ80d*lacZ*ΔM15Δ*(lacZYA-orgF)*U169*recAlendA1, hsdR17*(r_k^-, m_k^+)*phoA supE44λ-thi-1, gyrA96 relA1*	Invitrogen Life Technologies
BL21(DE3)	hsdS gal(*λcIts857 indl Sam7 nin5 lacUV5-T7 gene 1)*	Novagen
Leuconostoc mesenteroides SY1	Isolated from kimchi	This study
Plasmid		
pUC19	Cloning vector for DNA sequencing, Ap^r	NEB
pET26b(+)	Overexpression vector for α-galactosidase gene, km^r	Novagen
pAGA6	pUC19 plus *galR, aga, galK* and *galT* of *L. mesenteroides* SY1	This study
pAGA2.2	pET26b(+) plus *aga* of *L. mesenteroides* SY1	This study

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

The bacterial strains and plasmids used in this work are summarized in Table 1. *Leuconostoc mesenteroides* SY1 was isolated from regular cabbage kimchi purchased at a local supermarket. Biochemical properties and 16S rDNA sequencing data were used to identify SY1 [12]. SY1 was grown at 30°C in MRS broth (Difco Lab., Detroit, U.S.A.) supplemented with glucose, galactose, melibiose, fructose, sucrose, or raffinose at a 1% (w/v) concentration or on MRS plate solidified with 1.5% agar. *E. coli* was grown at 37°C in LB broth (Sigma-Aldrich, U.S.A.). Carbohydrate fermentation was tested in bromocresol purple medium (MRS broth fortified with 40 mg of bromocresol purple/l). Sugars were filter-sterilized and added to autoclaved media at the final concentration of 1%. When required, antibiotics (Sigma-Aldrich, U.S.A.) were added as follows: for *E. coli*, 50 μg of ampicillin per ml and 60 μg of kanamycin per ml.

DNA Techniques

Routine DNA manipulations were carried out according to the standard procedures [19]. Restriction enzymes, alkaline phosphatase, and T4 DNA ligase (TaKaRa, Japan) were used according to the instructions provided. Transformation of *E. coli* was performed as described by Dower *et al.* [7]. Plasmid DNA from *E. coli* was isolated according to the standard procedure [19]. Total DNA from *Leuconostoc msenteroides* SY1 was prepared as described by Jhon and Lee [10].

Cloning and Sequencing of *aga* from *L. mesenteroides* SY1

For the cloning of the *aga* gene from *L. mesenteroides* SY1, a PCR approach was employed. GeneAmp PCR system 2400 (Perkin-Elmer Biosystems, Foster City, U.S.A.) was used and 1 μg of genomic DNA from SY1 was used as a template. Two oligonuclelotide primers (a-gal-F1: 5'-ATGAATTCCTCTGTCAGTTTCTGACGAAACTCG-3' and a-gal-R1:5'-ATGAATTCTCAAACCAGCTTGGCCT-GCTTC-3') were synthesized (Bionix, Korea) based on the sequence of a putative *aga* gene from *L. mesenteroides* ATCC 8293 (ZP_00063083). PCR was performed in a total 50 μl: 1 μg of template DNA; 10 pmol of each primer; dNTPs, 0.25 mM each; 2 U of Ex *Taq* (Takara, Japan) DNA polymerase; and 1× Ex-*Taq* DNA polymerase buffer. PCR amplification was carried out under the following condition: prePCR, predenaturation for 5 min at 94°C, followed by 2 cycles (denaturation 94°C, 45 s; annealing 58°C, 40 s; extension 72°C, 2 min); PCR, 25 cycles (denaturation 94°C, 45 s; annealing 64°C, 40 s; extension 72°C, 2 min); and a final extension for 7 min at 72°C. PCR products were analyzed on a 0.8% agarose gel. The amplified fragment was recovered using a PCR purification kit (Nucleogen. Korea), digested with *Eco*RI, and the resulting fragments were extracted from agarose gel (0.8%) using E.Z.N.A.™ Gel Extraction Kit (Omega Bio-tek, Doraville, U.S.A.). Relevant DNA fragment was cloned into pUC19, and recombinants were selected by blue-white screening [17]. DNA sequence of the entire cloned fragment was determined using the primer-walking method.

Preparation of Cell Extracts and α-Gal Activity Measurement

L. mesenteroides SY1 was grown in MRS with a carbon source (1%) until the OD_{600} reached around 1.0. Cells were recovered by centrifugation (5,000 ×*g*, 15 min at 4°C) and resuspended with 1 ml of PBS (phosphate buffered saline, pH 7.0). Cells were sonicated for 30 s followed by cooling on ice for 30 s, and this cycle of sonication-cooling was repeated four times. Disrupted cells were centrifuged and the supernatant was obtained as cell extract. α-Gal activity was measured by the method of Church *et al.* [3] using McIlvaine buffer. The method is based on the measurement of the absorbance at 400 nm caused by p-nitrophenol (PNP), which is released by the action of the enzyme from its specific substrate, p-nitrophenyl-α-galactopyranoside

(PNPG). The reaction mixture consisted of 10 mM PNPG, 50 μl; 100 mM McIlvaine buffer (pH 5.8) [16], 50 μl; cell extract, 100 μl. The reaction mixture, 200 μl, was incubated at 45°C for 15 min, and the reaction was stopped by the addition of sodium carbonate (3 ml, 0.25 M). The protein concentration was determined using the RC/DC proteins assay kit (BioRad, U.S.A.) and BSA as the standard protein.

One unit of enzyme (U) was defined as the amount of enzyme that released 1.0 μmol of PNP from the substrate PNPG per min under the assay conditions. The results were expressed as U/mg protein. For α-Gal activity measurements, *E. coli* cells were cultivated in M9 minimal media [19] until the OD_{600} reached about 1.0. Cell extracts preparation and enzyme assay were done as described above.

SDS-PAGE

L. mesenteroides SY1 was cultivated overnight in MRS with an appropriate carbon source at 30°C. Cells from 10 ml culture were recovered by centrifugation (5,000 ×*g*, 20 min at 4°C) and resuspended in 1 ml of PBS (phosphate buffered saline, pH 7.0). Cell extract for SDS-PAGE was prepared as described above. SDS-PAGE of the protein extracts was done according to the method of Laemmli [13] using a Mini ProteanIII system (Bio-Rad, U.S.A.). Proteins were stained with Coomassie brilliant blue R-250. The molecular mass of denatured α-Gal was estimated by comparison with standard proteins (Precision Plus, Bio-Rad, U.S.A.).

RNA Isolation, Northern Hybridization, and Slot Blot

Total RNA was isolated from *L. mesenteroides* SY1 cells grown in MRS broth (Difco Lab., Detroit, U.S.A) containing 1% glucose, melibiose, and raffinose using FastRNA Pro Blue kit (Q.BIO, U.S.A.). RNA (20 μg) was separated on an agarose-formaldehyde gel [19]. Nucleic acids were transferred to a positively charged nylon membrane (Amersham Biosciences, U.S.A.) and fixed by UV exposure. PCR fragments were labeled with ^{32}P using the RediprimeII DNA labeling system (Amersham Biosciences, U.S.A.) and used as probes. Prehybridization (1 h) and hybridization (overnight) were performed in ULTRAhyb™ (Ambion, U.S.A.) at 50°C, and two post-hybridization washes were done at the same temperature in 2× SSC (1× SSC; 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% sodium dodecyl sulfate prior to film exposure (Hyperfilm™ MP, U.S.A.). Stripping of the membrane was done by immersing the membrane in 0.1% boiling sodium dodecyl sulfate solution. The membrane was successively hybridized with different probes. Slot blot was performed using a slot-blot system (PR648, Amersham Pharmacia Biotech Inc., U.S.A.) according to the protocol provided by the manufacturer. Internal fragment of *aga* (800 bp) was obtained by PCR (agaF-1: 5'-TGCGTGAACGTCAAATGGAGC-3' and agaR-1: 5'-GTCAAACGATCCGCCAAATCATA-3') and used as a probe.

Overexpression of the *aga* Gene in *E. coli*

For overexpression of *aga* in *E. coli*, the *aga* gene was amplified by PCR and subcloned into a pET26b(+) (Novagen, Madison, U.S.A.) [14]. Two oligonucleotide primers containing a unique *Nde*I site (agaF: 5'-AGTAATTCCATATGGCTAATGCAAACATTTCTT-3') and *Xho*I site (agaR: 5'-ATACTCGAGTTATTCAGCTTGCCATTTGA-3') were used for the amplification. Amplified fragment was digested with *Nde*I and *Xho*I and ligated with pET26b(+). The resulting recombinant plasmid, pAGA2.2, was introduced into *E. coli* BL21(DE3) (Novagen, Madison, U.S.A.). BL21(DE3) cells harboring pAGA2.2 were grown overnight at 37°C, and inoculated into fresh LB medium (1%, v/v) and cultured at 37°C. When the OD_{600} of the culture reached 0.8, isopropyl-thiogalactoside (IPTG) was added to the final concentration of 1 mM to induce *aga*, and the culture was further incubated for 3 h at 30°C. Protein extract was prepared essentially according to the protocol provided by the manufacturer (Novagen, Madison, U.S.A.).

Nucleotide Sequence Accession Number

The DNA sequences described in this study are available in the GenBank database under the accession number AY753204.

Results and Discussion

Effect of Different Carbon Source on the Growth and α-Gal Activity of *L. mesenteroides* SY1

Growth and α-Gal activity of *L. mesenteroides* SY1 on the different carbon source are shown in Fig. 1. *L. mesenteroides* SY1 could grow on glucose, fructose, sucrose, melibiose, and raffinose, but could not grow on galactose (Fig. 1A). α-Gal activity was higher in melibiose and raffinose-grown cells than galactose, sucrose, and fructose-grown cells, and the activity was not detected in glucose-grown cells, indicating the operation of carbon catabolite repression (Fig. 1B). The highest enzyme activity (98 U/mg) was observed when raffinose was used as a carbon source. The enzyme activity increased during the exponential growth phase and reached a peak after 18 h of incubation (Fig. 1B), roughly coinciding with the increase in cell numbers (Fig. 1A). Virtually no activity was detected in *L. mesenteroides* cells cultivated in the medium containing glucose (Fig. 1B). Other carbohydrates assayed showed different patterns. In the case of melibiose, the enzyme production increased gradually during the first 12 h of incubation, no variations being observed up to 9 h. However, α-Gal activity was not detected in cells grown on galactose. The activity of α-Gal was 66-fold higher in raffinose and 62-fold higher in melibiose, when compared with the value obtained with galactose. These results are in good agreement with those reported by Wong [23] and Zeilinger *et al.* [25], who

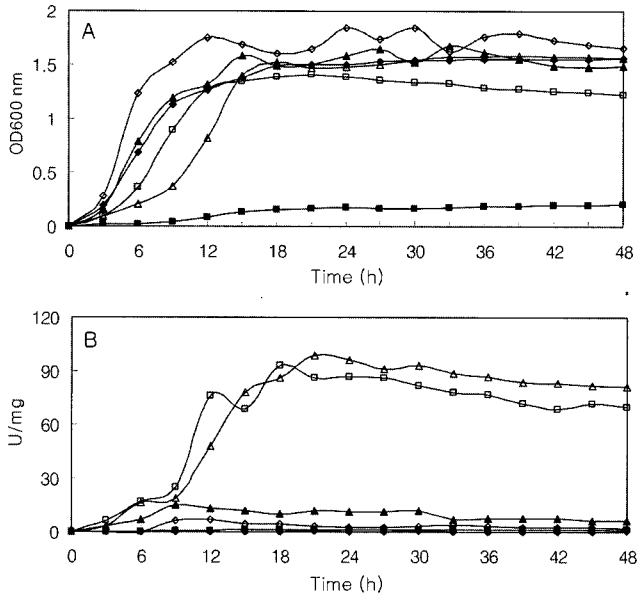

Fig. 1. Growth and α-Gal activity of *L. mesenteroides* SY1.
(A) Growth curve of *L. mesenteroides* SY1 in MRS media containing different carbon sources. (B) α-Gal activity of *L. mesenteroides* SY1 in MRS media containing different carbon sources. ◆, glucose; ■, galactose; ▲, fructose; ◇, sucrose; □, melibiose; △, raffinose.

found induction of α-Gal from *Azotobacter vinelandii* and *Trichoderma reesei*, respectively, by free galactose and oligosaccharides having terminal α-1,6-galactoside bonds. A similar phenomenon was observed in *Saccharomyces cerevisiae* by Tsuyumu and Adams [21]. No β-galactosidase and β-glucosidase activities were detected from *L. mesenteroides* SY1 (data not shown).

Sequence Analysis of the *aga* Locus from *L. mesenteroides* SY1

A 6 kb DNA fragment containing *aga* and other genes involved in α-galactosides utilization was cloned into pUC19 (pAGA6), and the whole fragment was sequenced. The determined G+C content was 38.7%. Sequence analysis of the 6 kb DNA fragment revealed the presence of four open reading frames (ORFs) (Fig. 2A), and sequences corresponding to the beginning and end of each gene are shown in Fig. 2B. Putative functions were attributed to each protein product based on the predicted motifs in the peptide chains, and also from amino acid similarity with proteins of known function (Table 2). Each of the four ORFs had 99% homology with the corresponding annotated gene from the genome of *L. mesenteroides* ATCC 8293 (AABH02000011), and was named accordingly. *L. mesenteroides* ATCC 8293 was isolated

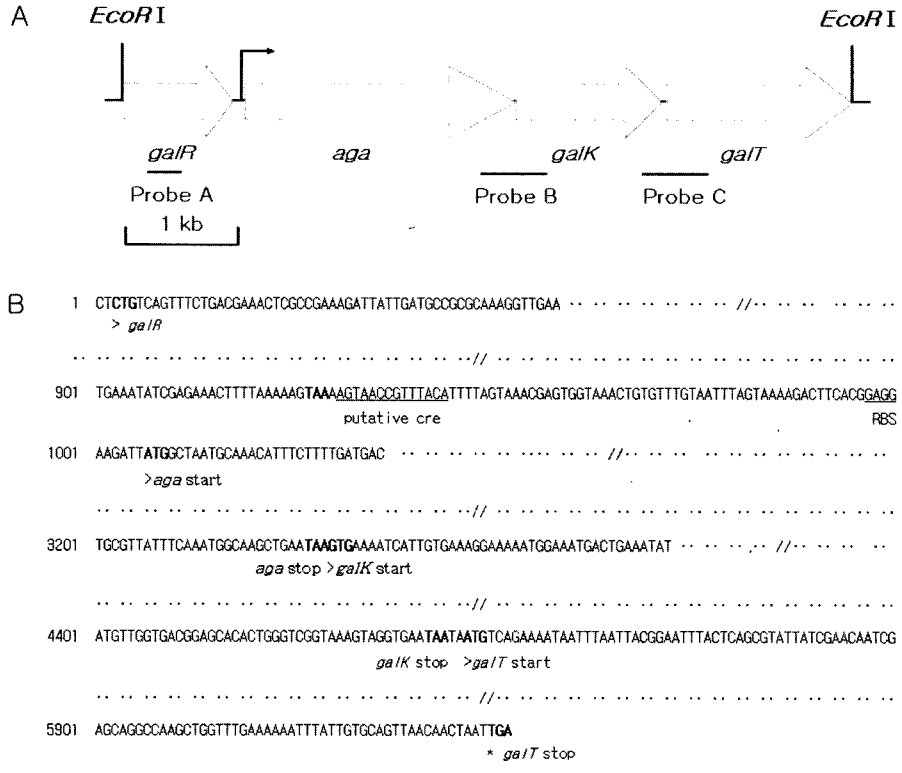

Fig. 2. Characterization of the *aga* locus of *L. mesenteroides* SY1.
(A) Four ORFs were identified, and putative functions were assigned to corresponding protein products according to the conserved motifs and similarity with proteins of known functions (Table 1). Brackets located under the figure identify transcripts, with dotted lines indicating the putative limits of transcription. Positions of restriction sites used for cloning or sequencing are indicated above the figure. (B) Sequence of parts of the 6 kb fragment. The start codon and stop codon of each gene are marked in bold. The putative *cre* site and ribosome binding site (SD) of *aga* are underlined.

Table 2. ORFs identified from the 6 kb insert in pAGA6.

ORF	Size	Mol. mass (kDa)	Proposed function	Characteristics
GalR (*galR*)	308 aa	34.8	Transcriptional regulator	GalR (*galR*) family; up to 26% identity over the entire protein sequence with orthologues found in various Gram-positive organisms, such as *L. raffinolactis*
Aga (*aga*)	740 aa	84.3	α-Galactosidase	Up to 66% identity over the entire protein sequence with orthologues found in various Gram-positive organisms, such as *Oenococcus oeni* PSU-1
GalK (*galK*)	404 aa	44.6	Galactokinase	Up to 54% identity over the entire protein sequence with orthologues found in various Gram-positive organisms, such as *Lactococcus lactis* subsp. *lactis* Il1403
GalT (*galT*)	501 aa	57.0	Galactose-1-phosphate uridylyltransferase	Up to 53% identity over the entire protein sequence with orthologues found in various Gram-positive organisms, such as *Enterococcus faecium*

from fermented olive product, therefore, *L. mesenteroides* SY1 seems likely to share the same metabolic capacities, including galactosides utilization, with *L. mesenteroides* ATCC 8293. The first ORF might encode a protein of 308 amino acids (aa), and the protein GalR has 26% overall homology to other regulatory proteins belonging to the GalR/LacI family. The cloned 6 kb fragment contained only the structural gene encoding GalR (*galR*) and did not contain any promoter sequence, which might be present in the upstream region. The second ORF can encode a protein of 740 aa and the expected 84.3 kDa protein has 66% homology with known α-Gals from LAB. Therefore, the gene product corresponds to α-Gal. The third ORF can encode a protein of 404 aa (MW, 44.6 kDa) and has up to 54% homology with galactokinases (*galK* gene product) from various Gram (+) organisms. The last ORF might encode a protein of 501 aa (MW, 57 kDa), which shows up to 53% homology with galactose-1-phosphate uridylyltransferase (*galT* gene

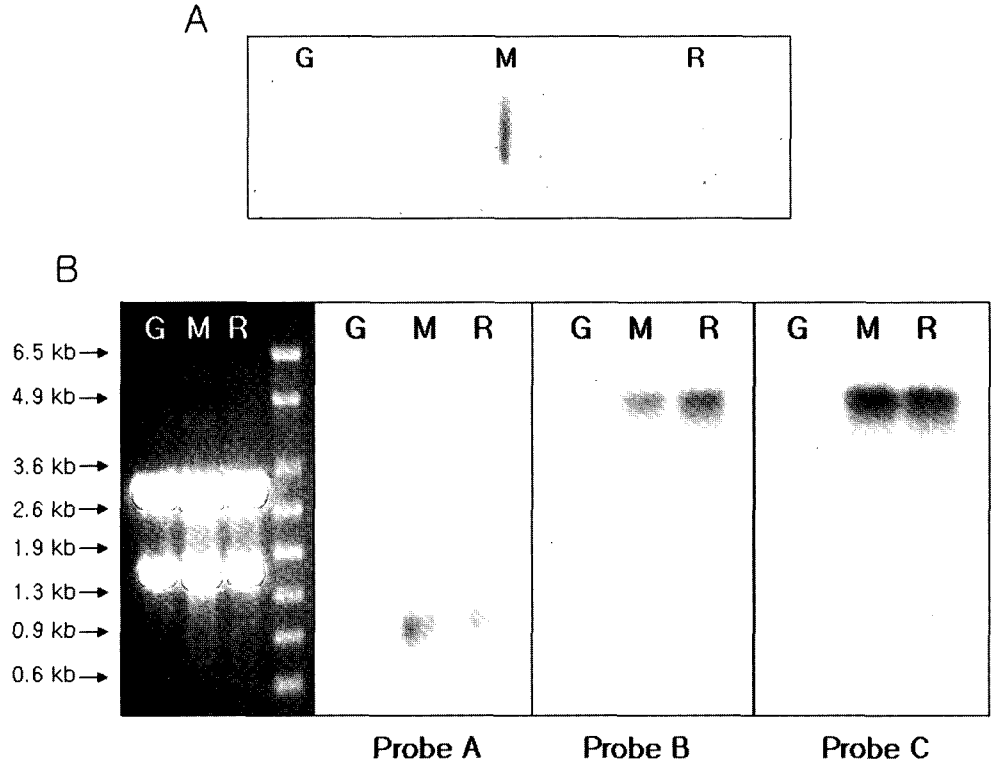

Fig. 3. Northern blot and slot blot of *L. mesenteroides* SY1 total RNA.
(A) Slot-blot hybridization of total RNA extracted from *L. mesenteroides* SY1 cells grown on different carbon sources (G, glucose; M, melibiose; R, raffinose). (B) Northern blot analysis of the *aga* locus. Total RNA was isolated from *L. mesenteroides* SY1 cells cultivated in the presence of glucose (G), melibiose (M), and raffinose (R). Ethidium bromide stained agarose gel (left) and the autoradiogram (right). ^{32}P-labeled probes were used to detect various transcripts; *galR* (probe A), *aga-galK* (probe B), and *galK-galT* (probe C).

product). The putative promoter sequence and a *cre* (catabolite-responsive element) were located upstream of the *aga* gene (Fig. 2). Two possible -35 promoter sequences were located at 900–905 nc (nucleotide) (TTGAAA) and 939–944 nc (TTTACA), and each deviated from the consensus -35 sequence (TTGACA) in one base. Putative -10 sequences located 16 or 17 nc downstream were much deviated from the consensus sequence. A region containing inverted repeats, which are able to form a stem-loop structure and thus act as an intrinsic transcriptional terminator, was found downsteam of *galR*. A *cre* was located upstream of *aga* and its sequence (AGTAACCGTTTACA) overlapped with a possible promoter sequence (TTTACA at 939–944 nc). When 1 μg of purified CcpA from *L. mesenteroides* SY1 was added to DNA fragment containing the *cre* of *aga*, CcpA bound to the DNA, causing gel mobility shift, whereas BSA did not (data not shown), indicating that *aga* expression is transcriptionally regulated through CcpA binding at *cre*. Slot-blot result also supports that transcription of *aga* was repressed, when glucose was present in the MRS (Difco Lab., Detroit, U.S.A.) medium (Fig. 3). The RBS (ribosome binding site) for *aga* (GGAGG) is located six nc upstream of the start codon. There is no space between the end of *aga* (TAA, 3227–3229 nc in Fig. 2) and the start of *galK* (GTG, 3230–3232 nc), and only a 2-bp space between the end of *galK* (TAA, 4442–4444 nc) and the start of *galT* (ATG, 4447–4449 nc). This close location of three genes is a strong indication that *aga*, *galK*, and *galT* belong to the same transcriptional unit; that is, they are part of the same operon. This was confirmed by Northern bolt analyses (see below).

α-Gal Activity in *E. coli*

The α-Gal activity of *E. coli* DH5α cells harboring pAGA6 was measured (Table 3). The activity of cells harboring pAGA6 was higher than control cells with pUC19. When grown on raffinose, the former had 43.3 U/mg protein, whereas the latter had only 1.6 U/mg protein, but the activity was lower than *L. mesenteroides* cells. This result was unexpected, because the *aga* gene was present in *E. coli* as multiple copies. The *aga* promoter under this construction might not be efficient in *E. coli*. The degree of induction (activity on raffinose/activity on glucose) in cells with pAGA6 was lower than *E. coli* control. *E. coli* DH5α has its own α-Gal activity, but its contribution to the overall activity of cells with pAGA6 on raffinose is small. Probably, the higher induction ratio (7.3 fold) of control is caused by the basal level of α-Gal activity on glucose.

Table 3. α-Gal activity of *E. coli* DH5α with pAGA6.

	Activity (U/mg)	
	Glucose	Raffinose
pUC19	0.22	1.6
pAGA6	8.25	43.3

Slot-Blot Analysis

To quantify transcriptional activity from the *aga* gene, slot-blot hybridization experiments were performed. Total RNAs were prepared from *L. mesenteroides* SY1 cells grown on different carbon sources, and 20 μg was applied to a nylon membrane and used for hybridization with a probe. As shown in Fig. 3, *L. mesenteroides* SY1 produced *aga* transcript at the highest concentration when grown on melibiose, followed by raffinose. The least amount of transcript was synthesized in cells grown on glucose. The result confirmed again that *aga* transcription was induced when cells were exposed to α-1,6-

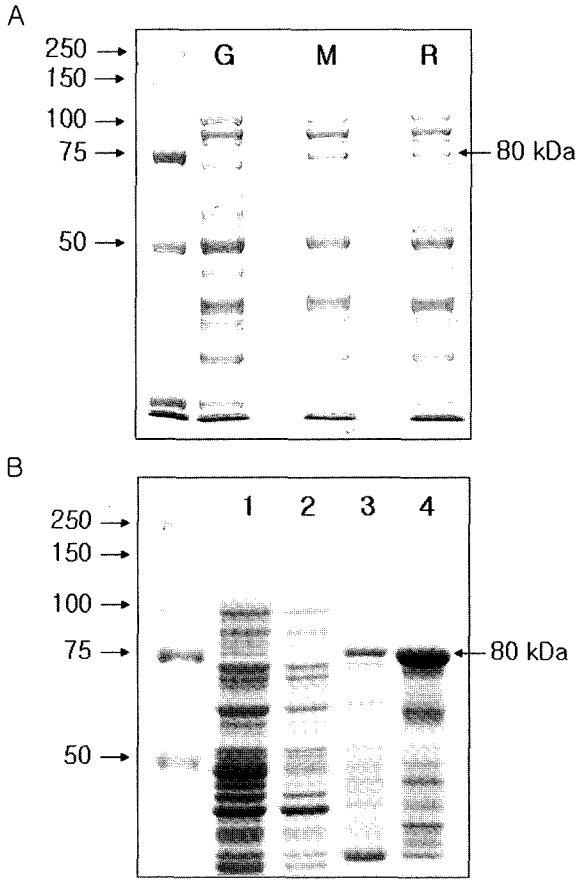

Fig. 4. SDS-PAGE analysis of α-Gals produced by *L. mesenteroides* SY1 (A) and *E. coli* BL21(DE3) (B).
A. *L. mesenteroides* SY1 was grown in MRS media containing different carbon sources (G, glucose; M, melibiose; R, raffinose). B. Total cellular protein extracts were prepared from *E. coli* BL21 (DE3) harboring pAGA2.2 (pET26b+*aga*) expressing the *L. mesenteroides aga* gene. 1, soluble fraction (no IPTG induction); 2, soluble fraction (IPTG induction, 1 mM); 3, insoluble fraction (no IPTG induction); 4, insoluble fraction (IPTG induction, 1 mM). Soluble fraction is the supernatant obtained after centrifugation of disrupted cells (by sonication), and the insoluble fraction is the pellet.

galactosides, such as melibiose and raffinose, and repressed by glucose.

Transcriptional Analysis of the *aga* Gene from *L. mesenteroides* SY1

To determine the number and size of transcripts from *aga* and neighboring genes, Northern blot analyses were performed with RNA samples prepared from *L. mesenteroides* SY1 cells grown on different carbon sources (Fig. 3B). The regulator gene, *galR*, was expressed as a monocistronic transcript of 1 kb in size (Fig. 3B), and the size agreed well with the size expected from the sequence data. *galR* transcription seemingly starts by a promoter located upstream of *galR* and terminates at the stem-loop structure in the *galR-aga* intergenic region. When probe B (spanning *aga* and *galK*) and probe C (spanning *galK* and *galT*) were used for hybridization, a single 5 kb transcript was detected, indicating that *aga*, *galK*, and *galT* genes were transcribed together as a single transcript. The expected size of *aga-galKT* transcript is 5 kb if transcription starts at the proposed promoter upstream of *aga* and ends at the terminator downstream of *galT*. The transcription of this operon was stimulated by galactosides such as melibiose and raffinose (Fig. 3A), and repressed by glucose (Fig. 3A), again confirming the operation of CCR.

Protein Analysis

When total cellular proteins from *L. mesenteroides* SY1 were examined by SDS-PAGE, a 80 kDa α-Gal band (Fig. 4A, indicated by an arrow on the right side) was observed from melibiose and raffinose grown cells, but was not detected in cells grown on glucose. *αga* from *L. mesenteroides* SY1 was overexpressed in *E. coli*. *E. coli* BL21 (DE3) harboring pAGA2.2 (pET26b(+) with *aga*) was grown up to the middle of the exponential growth phase, and then IPTG was added at the concentration of 1 mM. As shown in Fig. 4B, α-Gal was overproduced in *E. coli*. However, the enzyme was recovered only from insoluble fraction, indicating that α-Gal accumulated inside cell forming an inclusion body. The size on the SDS gel, 80 kDa, agreed well with the size of α-Gal produced in the original host, *L. mesenteroides* SY1. Currently, finding an efficient method for solubilizing α-Gal from inclusion body is under investigation. Overproduction of α-Gal in *E. coli* is necessary and desirable if an industrial process for removing α-galactosides from soymilk is to be developed. In this respect, *aga* overexpression in *E. coli* using the pET expression system might be useful as long as α-Gal inclusion body is easily dissociated and generates active enzyme.

Organization of the *aga* and Neighboring Genes in LAB

Genes involved in the utilization of α-galactosides such as raffinose and melibiose are found in some LAB [11]. The organization of those genes from five different LAB is shown in Fig. 5. *Lactococcus raffinolactis* has the same genetic organization as *L. mesenteroides* SY1. *Leuconostoc lactis* also has the same *aga-galKT* gene order, but the regulator gene *galR* is missing upstream of *aga*. Other LAB have more complex structure; for example, *Lactobacillus plantarum* has two copies of *galR* (*galR1* and *galR2*), which are separated by genes involved in galactose and lactose utilization. Upstream of *melA* (encoding α-Gal), a putative galactoside transporter (RafP) gene is located, and immediately downstream of *melA*, the *lacM* and *LacL* genes, each encoding a subunit of heterodimeric β-galactosidase, are present in the opposite direction [20]. *melA* and *rafP* are monocistronic genes. *galKET* genes are located further downstream in the same direction as *melA* (see Fig. 5). In

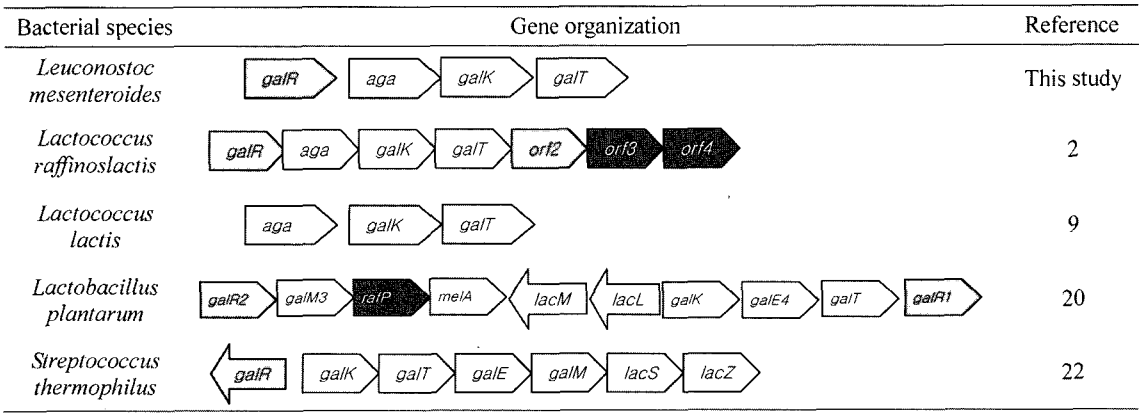

Fig. 5. Organization of α-Gal genes in LAB.

White, carbohydrate degradation enzymes; black, sugar transporters; gray, transcriptional regulators. Genes and their protein products: *galR*, transcriptional regulator; *aga* and *melA*, α-galactosidase; *galK*, galactokinase; *galT*, galactose-1-phosphate uridylyltransferase; *galM*, mutarotase; *lacS*, lactose transporter; *lacZ*, β-galactosidase.

St. thermophilus, *galR* is divergently transcribed from *galK*, and *galKTE* forms an operon. Downstream of the *gal* operon, a monocistronic *galM* (encoding galactose mutarotase), and *lac* operon genes, *lacS* and *lacZ*, are located in the same direction with the *gal* operon.

Most *Lactococcus lactis* strains used in the dairy industry do not utilize melibiose and raffinose, and they were isolated from raw milk or fermented milk products. On the other hand, *L. mesenteroides* SY1, isolated from kimchi, is able to utilize these sugars. *L. mesenteroides* ATCC 8293, isolated from olive, has the same ability. Therefore, the presence of α-galactosides-utilizing genes in *L. mesenteroides* strains reflects the likely adaptation of these organisms to natural environments, such as plant materials where α-galactosides are easily encountered. During kimchi preparation, various plant materials including Chinese cabbage are included, and the plant stuffs might be the source of α-galactosides. As far as the authors are aware, this is the first report on the genetic analysis of the α-galactosidase gene from *L. mesenteroides* strain. In this organism, α-galactosides metabolism is driven by an operon that contains three genes, *aga*, *galK*, and *galT*. The expression of these genes is under the CCR, and also probably controlled by a repressor gene, *galR*, located upstream of the operon.

Acknowledgments

This work was supported by a research grant from the KOSEF (Korea Science and Engineering Foundation) grant # R01-2003-000-10124-0. Jong-Hwan Kim and Jae-Yong Park were supported by Brain Korea 21 Project from the Ministry of Education, Korea. The authors are grateful for the financial supports.

References

1. Annunziato, M. E., R. R. Mahoney, and R. E. Mudgett. 1986. Production of α-galactosidase from *Aspergillus oryzae* grown in solid state culture. *J. Food Sci.* **51:** 1370–1371.
2. Boucher, I., C. Vadeboncoeur, and S. Moineau. 2003. Characterization of genes involved in the metabolism of α-galactosides by *Lactococcus raffinolactis*. *Appl. Environ. Microbiol.* **69:** 4049–4056.
3. Church, F. C., S. P. Meyers, and V. R. Srinivasan. 1980. Isolation and characterization of alpha-galactosidase from *Pichia guilliermondii*, pp. 339–348. *In* L. A. Underkofler and M. L. Wulf (eds.), *Developments in Industrial Microbiology*, vol. 21. Lubrecht & Cramer, Arlington, VA, U.S.A.
4. Coombs, J. and J. E. Brenchley. 2001. Characterization of two new glycosyl hydrolases from the lactic acid bacterium *Carnobacterium piscicola* strain BA. *Appl. Environ. Microbiol.* **67:** 5094–5099.
5. Dey, P. M. and E. Del Campillo. 1984. Biochemistry of the multiple forms of glycosidases in plants. *Adv. Enzymol. Relat. Areas Mol. Biol.* **56:** 141–249.
6. Dey, P. M., S. Patel, and M. D. Brownleader. 1993. Induction of α-galactosidase in *Penicillium ochrochloron* by guar (*Cyamopsis tetragonoloba*) gum. *Biotechnol. Appl. Biochem.* **17:** 361–371.
7. Dower, W. J., J. F. Miller, and C. W. Ragsdale. 1988. High efficiency transformation of *E. coli* by high voltage electroporation. *Nucleic Acids Res.* **16:** 6127–6145.
8. Garro, M. S., G. F. de Valdez, G. Oliver, and G. S. de Giori. 1996. Influence of carbohydrates on the alpha-galactosidase activity of *Lactobacillus fermentum*. *Curr. Microbiol.* **33:** 302–305.
9. Grossiord, B., E. E. Vaughan, E. Luesink, and W. M. de Vos. 1998. Genetics of galactose utilization via the Leloir pathway in lactic acid bacteria. *Lait* **78:** 77–84.
10. Jhon, D. Y. and S. H. Lee. 2003. Complete DNA sequence and analysis of a cryptic plasmid isolated from *Lactobacillus bifermentans* in kimchi. *J. Microbiol. Biotechnol.* **13:** 1018–1020.
11. Kelly, W. J., G. P. Davey, and L. J. H. Ward. 1998. Conjugative transfer of raffinose metabolism in *Lactococcus lactis*. *FEMS Microbiol. Lett.* **167:** 145–149.
12. Kim, S. J., J. H. Kim, J. Y. Park, H. T. Kim, S. J. Jeong, Y. L. Ha, H. D. Yun, and J. H. Kim. 2004. Cold adaptation of *Lactobacillus paraplantarum* C7 isolated from kimchi. *J. Microbiol. Biotechnol.* **14:** 1071–1074.
13. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.
14. Lim, J. M., Y. H. Jang, H. R. Kim, Y. T. Kim, T. J. Choi, J. K. Kim, and S. W. Nam. 2004. Overexpression of arylsulfatase in *E. coli* and its application to desulfatation of agar. *J. Microbiol. Biotechnol.* **14:** 777–782.
15. Margolles-Clark, E., M. Tenkanen, E. Luonteri, and M. Penttila. 1996. Three α-galactosidase genes of *Trichoderma reesei* cloned by expression in yeast. *Eur. J. Biochem.* **240:** 104–111.
16. McIlvaine, T. C. 1921. A buffer solution for colorimetric comparison. *J. Biol. Chem.* **49:** 183–186.
17. Nam, S. J., J. Y. Park, J. K. Kim, Y. L. Ha, H. D. Yun, and J. H. Kim. 2004. Cloning of *pdh* genes encoding subunits of pyruvate dehydrogenase complex from *Lactobacillus reuteri* ATCC 55739. *J. Microbiol. Biotechnol.* **14:** 197–201.
18. Nigatu, A., S. Ahrné, and G. Molin. 2000. Temperature-dependent variation in API 50 CH fermentation profiles of *Lactobacillus* species. *Curr. Microbiol.* **41:** 21–26.
19. Sambrook, J. and D. W. Russell. 2001. *Molecular Cloning: A Laboratory Manual*, 3rd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., U.S.A.
20. Silvestroni, A., C. Connes, F. Sesma, G. Savoy de Giori, and J.-C. Piard. 2002. Characterization of the *melA* locus for α-galactosidase in *Lactobacillus plantarum*. *Appl. Environ. Microbiol.* **68:** 5464–5471.
21. Tsuyumu, S. and B. G. Adams. 1974. Dilution kinetic studies on yeast populations: *In vivo* aggregation of galactose

utilizing enzymes and positive regulator molecules. *Gene* **77:** 919–923.
22. Vaillancourt, K., S. Moineau, M. Frenette, C. Lessard, and C. Vadeboncoeur. 2002. Galactose and lactose genes from the galactose-positive bacterium *Streptococcus salivarius* and the phylogenetically related galactose-negative bacterium *Streptococcus thermophilus*: Organization, sequence, transcription, and activity of the *gal* gene products. *J. Bacteriol.* **184:** 785–793.
23. Wong, T. Y. 1990. Melibiose is hydrolyzed exocellularly by an inducible exo-α-galactosidase in *Azotobacter vinelandii*. *Appl. Environ. Microbiol.* **56:** 2271–2273.
24. Yamane, T. 1971. Decomposition of raffinose by α-galactosidase. An enzymatic reaction applied in the factory-process in Japanese beet sugar factories. *Sucr. Belge/Sugar Ind. Abstr.* **90:** 345–348.
25. Zeilinger, S., D. Kristufek, I. Arisan-Atac, R. Hodits, and C. Kubicek. 1993. Conditions of formation, purification, and characterization of an α-galactosidase of *Trichoderma reesei* RUT C-30. *Appl. Environ. Microbiol.* **59:** 1347–1353.