Establishment of an Efficient Fermentation System of Gamma-Aminobutyric Acid by a Lactic Acid Bacterium, *Enterococcus avium* G-15, Isolated from Carrot Leaves

Takayoshi TAMURA, Masafumi NODA, Moeko OZAKI, Masafumi MARUYAMA, Yasuyuki MATOBA, Takanori KUMAGAI, and Masanori SUGIYAMA*

Department of Molecular Microbiology and Biotechnology, Graduate School of Biomedical Sciences, Hiroshima University; 1–2–3 Kasumi, Minami-ku, Hiroshima 734–8551, Japan.
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In the present study, we successfully isolated a carrot leaf-derived lactic acid bacterium that produces gamma-aminobutyric acid (GABA) from monosodium L-glutamate (L-MSG) at a hyper conversion rate. The GABA-producing bacterium, identified as *Enterococcus* (*E.*) *avium* G-15, produced 115.7±6.4 g/l GABA at a conversion rate of 86.0±5.0% from the added L-MSG under the optimum culture condition by a continuous L-MSG feeding method using a jar-fermentor, suggesting that the bacterium displays a great potential ability for the commercial-level fermentation production of GABA. Using the reverse transcription polymerase chain reaction (RT-PCR) method, we analyzed the expression of genes for the GABA transporter and glutamate decarboxylase, designated *gadT* and *gadG*, respectively, which were cloned from the *E. avium* G-15 chromosome. Both genes were expressed even without the added L-MSG, but their expression was enhanced by the addition of L-MSG.

Key words plant-derived lactic acid bacteria; *Enterococcus avium*; gamma-aminobutyric acid

Lactic acid bacteria (LAB) are a heterogeneous group of Gram-positive facultative anaerobic microorganisms that produce large amounts of lactic acid. LAB, which are seen in milk and the human intestine, are used for the manufacture of yogurt and cheese. They are referred to as animal-derived LAB. On the other hand, LAB exist on the surface of fruits, vegetables, and flowers. These LAB, designated plant-derived LAB, have higher adaptation characteristics for withstanding harsh conditions like plant stems and leaf surfaces. The bacteria are useful to make traditional fermented foods, such as pickles, soy sauce, 'miso,' and 'kimchi.' Thus, LAB are roughly classified into two groups according to differences in their growth environment. The characteristics of plant-derived LAB should be distinguishable from those of animal-derived LAB. Recently, LAB are receiving attention for their probiotic potential for humans.

Gamma-aminobutyric acid (GABA), a four-carbon non-protein amino acid, is produced by animals and plants. Being an analog of glutamic acid, GABA has been reported to possess several physiological functions such as neurotransmission,[1—3] tranquilization,[4—6] antihypertension,[7] prevention of diabetic condition,[8,9] and diuretic effects.[10] Now, several functional foods supplemented with GABA are produced to satisfy the market demand.

GABA is biosynthesized by microorganisms such as *Lactobacillus* (*Lb.*) *paracasei*,[6] *Lb. brevis*,[11—13] and *Streptococcus salivarius*[14] *via* decarboxylation of glutamic acid by glutamate decarboxylase (EC 4.1.1.15) which is a pyridoxal phosphate-dependent enzyme. Due to the commercial demand of GABA, the screening of bacteria producing a large amount of the amino acid[15,16] and the improvement of the efficient production system[17—19] are being investigated actively. The production of GABA by a batch fermentation system has been reported using various microorganisms, but the productivity has remained on a low level, and the manufacturing costs continue to be high.

In the present study, we successfully isolated carrot leaf-derived LAB, which produce GABA from monosodium L-glutamate (L-MSG) at a hyper conversion rate. The GABA producer, which is designated G-15, was identified as *Enterococcus* (*E.*) *avium*. The optimum culture conditions for a high production of GABA were found to involve a continuous L-MSG feeding method using a jar-fermentor with *E. avium* G-15, suggesting that *E. avium* G-15 displays a great potential ability for the commercial-level fermentation production of GABA. A functional analysis of genes for GABA hyper-production was also conducted.

MATERIALS AND METHODS

Culture Medium and Conditions De Man-Rogosa-Sharpe (MRS) broth (Merck KGaA, Darmstadt, Germany), M17 broth (Merck KGaA), brain heart infusion (BHI) broth (Difco, Franklin Lakes, NJ, U.S.A.), and GY (60 g glucose, 10 g yeast extract, 5 g tryptone, 2 g sodium acetate, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.01 g $MnSO_4 \cdot 5H_2O$, 0.01 g NaCl, 0.01 g $FeSO_4 \cdot 7H_2O$, and 0.5 g Tween 80 per liter, pH 6.8) medium were used for the LAB culture. A GY medium supplemented with pyridoxal phosphate (Sigma, St. Louis, MO, U.S.A.) of 0.1 g/l, known as a GYP medium, was mainly used to evaluate GABA productivity. We also prepared a GYP2 medium, in which the variety and contents of nitrogen sources and mineral ions were modified, to increase the productivity of GABA. The GYP2 medium consisted of 20 g polypeptone N, 12 g yeast extract (Difco), 5 g sodium acetate, 2 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.01 g $MnSO_4 \cdot 5H_2O$, 1 g Tween 80, 0.1 g pyridoxal phosphate, and 60 g glucose per liter, pH 6.8. When necessary, 1.5% (w/v) agar and the given concentration of L-MSG were added to each medium. All strains were stored at −80 °C until use by suspending cells into each medium containing 16% (v/v) glycerol.

Culture Conditions for the High Production of GABA

* To whom correspondence should be addressed. e-mail: sugi@hiroshima-u.ac.jp

For the seed culture, a portion (150 μl) of a freeze-stock culture of G-15 was inoculated into a GYP medium (50 ml) containing the given concentration of L-MSG in a 250-ml Erlenmeyer flask and incubated without agitation at 37 °C for 24 h. For the flask-scale test, the above-mentioned seed culture broth was inoculated at 2% (v/v) into the same 50-ml GYP medium in a 250-ml Erlenmeyer flask and incubated without agitation at 37 °C for 2—5 d. For the jar-fermentor scale test, seed culture broth prepared at the flask scale was inoculated at 2% (v/v) into a 1.6—2.0 l GYP medium in a 3 l jar-fermentor and incubated with agitation at 37 °C under N_2 gas aeration for 2—5 d. To maintain the given pH condition, the pH of the culture broth was automatically adjusted by NaOH or H_2SO_4. Bacterial growth was monitored by measuring the optical density ($OD_{610\,nm}$) in a spectrophotometer (Shimadzu UV-2400).

Isolation and Identification of GABA-Producing LAB from Plant Sources As an LAB-isolation source, a piece of vegetable leaf or a stick sample (about 1 cm^2), which was harvested from soil directly, was suspended into MRS broth supplemented with 0.5% (w/v) L-MSG and incubated anaerobically at 30 °C or 37 °C for 2—3 d. An aliquot of the culture, in which the GABA producing activity was confirmed, was plated onto MRS broth supplemented with 1.5% (w/v) agar and incubated at the given temperature for 2—3 d. Each colony generated on the plate was re-spread onto a fresh MRS agar medium to purify the colony. Gram-staining and organic acid- and catalase-production tests of the purified colony were carried out prior to the taxonomical identification of LAB species by 16S ribosomal DNA (rDNA).

To identify the GABA-producing LAB strains, the entire 16S rDNA sequence of each strain was determined as described previously[20—22] and compared with that of typical LAB obtained from the DNA Data Bank of Japan (DDBJ) website (http://www.ddbj.nig.ac.jp/Welcome-e.html). LAB species were identified by analysis of the sequence alignment using the ClustalW program (http://clustalw.ddbj.nig.ac.jp/top-e.html). Furthermore, the sugar fermentation profile on each strain was determined using a BD BBLCRYSTAL GP kit (Becton, Dickinson and Company, Franklin Lakes, NJ, U.S.A.).

DNA Preparation, Manipulation, and Sequencing Chromosomal DNA from LAB was isolated using DNA Zol Reagent (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's protocol. The nucleotide sequences were determined with the ABI PRIZM 310 genetic analyzer using the BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, U.S.A.) according to the manufacturer's protocol. The nucleotide sequence data was analyzed using ATGC and GENETYX software (GENETYX Corporation, Tokyo, Japan). The open reading frames (ORFs) were predicted using an ORF finder tool at NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The homology search was performed with the BLAST algorithm[23] utilizing the non-redundant database provided by NCBI.

Cloning and Nucleotide Sequence Analysis of the Glutamate Decarboxylase-Encoding Gene Cluster The restriction enzyme-digested chromosomal DNA was fractionated using 0.5% (w/v) agarose gel electrophoresis and transferred to a Hybond-N+ membrane (GE Healthcare, Little Chalfont, U.K.) using the standard protocol.[24] A probe to clone the glutamate decarboxylase gene was polymerase chain reaction (PCR)-amplified with a sense primer, 5′-GGATTATGTGGAYGAVTACACMATTGGTAT-3′, and an antisense primer, 5′-TGCTGCACTRTGDGAGAAGTTG-ATVGCCAT-3′. In this case, the chromosomal DNA of *E. avium* G-15 was used as a template. The primers were designed on the basis of the nucleotide sequences of glutamate decarboxylase genes from *Lb. plantarum* WCFS1 (AL935262.1), *Lb. brevis* OPK3 (DQ168031), and *E. faecium* D0 (AAAK03000022). PCR was conducted under the following conditions: after 5 min at 96 °C, 3 cycles of 30 s at 96 °C, 20 s at 50 °C, and 30 s at 72 °C, 3 cycles of 30 s at 96 °C, 20 s at 45 °C, and 30 s at 72 °C, and 3 cycles of 30 s at 96 °C, 20 s at 40 °C, and 30 s at 72 °C followed by 29 cycles of 30 s at 96 °C, 20 s at 35 °C, and 30 s at 72 °C, and, finally, a 7-min extension period at 72 °C. Labeling of the probe and detection were performed using an AlkPhos direct labeling and detection kit (GE Healthcare) according to the manufacturer's instructions. After determination of the appropriate single-restriction enzyme for inverse PCR,[25] *E. avium* G-15 chromosomal DNA was digested with several restriction enzymes. The purified DNA fragment was self-ligated using a DNA Ligation kit (Takara Bio Inc., Shiga, Japan). A portion of the ligated DNA mixture was used for inverse PCR as a template together with properly designed primer pairs. The nucleotide sequence of the PCR product was determined by the direct sequencing method.

RNA Extraction and Reverse Transcription (RT)-PCR Analysis Total RNA from *E. avium* G-15 was extracted as follows: the harvested cells (0.5—3 g) were disrupted by glass beads (ϕ=2 mm) with modified Kirby mix[26] [1% (w/v) sodium *N*-lauroylsarcosine, 6% (w/v) sodium 4-amino salicylate, 6% (w/v) phenol, and 0.1 M Tris–HCl, pH 8.0] containing 6% (v/v) 3-methyl-1-butanol. After phenol/chloroform extraction and 2-propanol precipitation of the resulting sample, the contaminated DNA was digested with DNase I. The purity and integrity of the RNA sample were checked by agarose gel electrophoresis. A portion of the total RNA was used to synthesize cDNA using the Prime Script RT-PCR kit (Takara Bio Inc., Shiga, Japan) and random oligomers consisting of 9-nucleotides according to the manufacturer's protocol. For the RT-PCR analysis, *atpA*, *gadG*, *gadT*, and *gadR* as targets were amplified using each cDNA sample as a template and the following gene-specific primers: 5′-CCGTTG-TACATGAATTCTTCACC-3′ and 5′-CGTCCAGTNGAA-GCAATGGC-3′ for *atpA*; 5′-TTAAAAGAGGATTCTTCA-CGAGAATGGA-3′ and 5′-TTAATGCGTAAATCCGTAA-GTTTTGATTTC-3′ for *gadG*; 5′-ATTTTAGCGATTGCC-TTGGATGCGAT-3′ and 5′-GGAACGAAAGAAATCAGC-AAAGCAAAA-3′ for *gadT*; and 5′-ATTGACGAGAATCA-TTGTTTGTCGATTG-3′ and 5′-CTCGAGCATACTGTAT-TCTTTA-3′ for *gadR*. PCR was done as follows: a cycle of 5 min at 96 °C followed by 35 cycles of 30 s at 96 °C, 30 s at 55 °C, and 30 s at 72 °C, and, finally, a 7-min extension period at 72 °C.

Mutagenicity and Acute Oral Toxicity Tests The mutagenicity test (*umu* test) of the *E. avium* G-15 culture broth was performed using a Umulac AT test kit (ProteinPurify Ltd., Maebashi, Japan) according to the manufacturer's protocol.

A test for acute oral toxicity was performed by the New

Drug Development Research Center, Inc. Fifteen 5-week-old Crl:CD (Sprague-Dawley (SD)) male rats, which were purchased from the Charles River Laboratories Japan, Inc. were housed individually in stainless-steel cages. After 1 week of acclimation, the rats were randomly divided into three groups with five members each: a high-dose group with G-15 cells (1.8×10^{12} colony forming unit (cfu)/kg), a low-dose group (0.9×10^{12} cfu/kg), and a sterile water-only group. Each cell suspension or water was administrated orally to each group of rats using a sterile probe once a day for 2 weeks. During the experiment, the body weight, activity, behavior, and general health status of the rats were recorded at 1-, 3-, 7-, 10-, and 14-d points after the first oral administration. After administration, the animals were euthanized with diethyl ether, and then histological observations of some organs were performed. The same experiment was also carried out with female rats.

Analytical Condition of GABA, L-MSG, and Lactic Acid with HPLC The concentrations of L-MSG and GABA were determined with the *o*-phthaldealdehyde (OPA) derivatization method using a CAPCELL PAK HPLC column (4.6×250 mm) with fluorometric detection (excitation and emission wavelengths of 340 and 490, respectively). HPLC was carried out at a flow rate of 1.0 ml/min and ambient column temperature. When a solution consisting of 5 mM 1-octane sulfonate sodium salt and a 100 mM sodium acetate buffer (pH 4.7) was used as mobile phase, the retention time of L-MSG and GABA was 2.4 and 8.0 min, respectively. The concentration of lactic acid was also determined by HPLC using Shodex Rspak KC-G (6.0×50 mm) and Shodex Rspak KC-811 (8.0×300 mm) columns as the pre-column and main column, respectively. The column was used at 60 °C and at a flow rate of 0.8 ml/min using 3 mM perchloric acid.

RESULTS

Isolation and Identification of GABA-Producing Microorganisms To obtain GABA-producing LAB, 22 vegetable samples were employed. A piece of each plant material, such as a leaf and stick from vegetables, was suspended in an MRS medium and incubated at 30 °C or 37 °C. After the 3 d incubation, the sample was transferred to an MRS medium supplemented with 0.5% (w/v) L-MSG at 30 °C or 37 °C. We observed that the sample with a carrot leaf, designated No. 15, contained GABA. Therefore, an aliquot of this sample was inoculated into each MRS-, BHI-, and M17-agar medium supplemented with 1% (w/v) $CaCO_3$ and cultured at 37 °C for 3 d under anaerobic conditions. Colonies carrying a clear circle formed by the resolution of $CaCO_3$ were selected and inoculated into MRS-, BHI-, and M17 media without agar and then incubated for a few days under the above conditions. We obtained GABA-producing LAB from the carrot leaf. These bacteria, designated G-15, were cocci. We confirmed that the GABA productivity of the G-15 strain was higher than those of *Lb. hilgardii* NBRC15886 and *Lb. brevis* 823A as GABA-producing bacteria. A preliminary experiment demonstrated that the G-15 strain converts most of the extracellulary added L-MSG to GABA.

The G-15 strain, which is a Gram-positive bacterium, was a catalase-negative facultative anaerobe. Since the bacterium produced lactic acid and acetic acid by utilizing glucose, the G-15 strain was suggested to be a lactic acid bacterium that takes hetero-type fermentation. The sugar utilization test suggested that the bacterium is *E. avium*. The entire 16S rDNA sequence analysis with the DDBJ database confirmed that this strain is identical to *E. avium* (DDBJ Accession No.: AB548684). We named the strain *E. avium* G-15.

Safety Evaluation of G-15 as a Fermentation Material for Food Manufacture The *umu*-test confirmed that the *E. avium* G-15 culture supernatant does not give mutagenesis. In addition, an acute toxicity test by oral administration of the concentrated culture broth demonstrated that significant activity changes and intake-related illness or death were not observed. Histological analysis did not reveal any symptoms of inflammation or obvious differences in the appearance of some organs in animals.

Effects of the Cultivation Condition on the GABA Productivity We first evaluated the GABA productivity on *E. avium* G-15 at a flask scale. When 10 and 50 g L-MSG was added to a GYP medium, the GABA content in each 24 h-culture broth was 3.5 and 2.7 g/l, respectively. The result shows that the productivity is not dependent on the concentration of added L-MSG. When a GYP2 medium containing higher nitrogen sources than the GYP medium was used, the cell growth was stimulated. In addition, L-MSG (50 g/l) added to the culture broth was completely consumed during 24 h cultivation, and the GABA production was 28.9 g/l. By using GYP2 medium, the GABA productivity was increased by 10.7 times.

We also examined the effect of added L-MSG on the GABA productivity at the jar-fermentor scale. First, when the GYP medium supplemented with 50 g L-MSG was used without a pH control, the cell growth reached a steady state after 10 h culture, and the GABA productivity was 5.2 g/l at 48 h culture (Table 1). When the culture medium was controlled at pH 5, the GABA content in the 48 h culture broth was 10.1 g/l at 48 h. However, the cell growth was almost the same level as that without the pH control. When the medium

Table 1. The Effect of pH Control Level on the Cell Growth and the GABA Productivity by *E. avium* G-15 at Jar-Fermentor Scale Using GYP and GYP2 Medium

Medium	pH control	pH			Cell growth (A_{610})			GABA (g/l)	
		10 h	24 h	48 h	10 h	24 h	48 h	24 h	48 h
GYP medium	non	4.2	4.0	3.9	3.5	5.0	5.0	3.5	5.2
	5.0	5.0	4.9	5.1	3.5	5.5	6.5	5.5	10.1
	6.5	6.4	6.4	6.5	5.0	7.0	8.0	0.2	2.4
GYP2 medium	5.0	5.0	4.9	4.9	5.0	15	15	21.5	25.7
	6.5	6.5	6.6	6.5	6.5	18	26	11.7	12.0

Table 2. The Effect of the Initial Concentration and Feeding of L-MSG on the GABA Productivity by *E. avium* G-15 at Flask Scale Using GYP2 Medium

L-MSG (g/l)		GABA (g/l)			Glutamate		
Initial	Feeding	24 h	48 h	120 h	24 h	48 h	120 h
50		26.3	27.0		2.2	3.3	
100		20.4	49.8	55.1	45.1	3.9	2
150		3.4	10.7	26.0	100.3	88.9	79.8
100	60			84.8			2.1

pH was maintained at 6.5, the cell growth and lactic acid content were higher than they were at pH 5, but the GABA productivity was suppressed. When the GYP2 medium supplemented with 50 g L-MSG/l was used, the amount of GABA contained in the 48 h culture broth was 25.7 and 12.0 g/l under the pH 5- and 6.5-controlled conditions, respectively.

We investigated the effect of the initial concentration of L-MSG added to the GYP2 medium on the GABA productivity at a flask scale (Table 2). When 50 g L-MSG/l was added, almost all L-MSG was converted to GABA, and the content reached 26.3 g/l after the 24 h culture. When 100 g L-MSG/l was added, although the GABA productivity decreased at the 24 h culture, it reached 49.8 g/l after the 48 h culture. When 150 g L-MSG/l was added, the conversion of L-MSG to GABA was obviously reduced to 3.4 g/l at the 24 h culture. Even after 120 h, the GABA content was a low level of 26.0 g/l, suggesting that the GABA productivity by *E. avium* G-15 is inhibited when a high concentration of L-MSG is present in the culture medium. We then examined whether GABA production was increased by feeding of L-MSG. After *E. avium* G-15 was cultured for 24 h in a GYP2 medium containing 100 g L-MSG/l, 60 g L-MSG/l was added to the culture. As a result, the GABA content in the 120 h culture broth increased to 84.8 g/l.

Next, we expected an increment in the GABA productivity by a fed-batch culture at a jar-fermentor scale using a GYP2 medium containing 50 g L-MSG/l at an initial concentration. After inoculation, 50 g L-MSG was added to the GYP2 medium (1.6 l) in the jar-fermentor at cultivation time 22, 30, 46, 52 h (a sum of 200 g L-MSG/l). As shown in Fig. 1, when the pH of the culture broth was kept at 5.0, the GABA productivity reached 66.0±2.4 g/l, and the conversion ratio of L-MSG to GABA was 78.1±3.5%. However, at the 46 h culture, the GABA production rate decreased, and L-MSG without conversion to GABA remained in the medium (more than 20 g/l). This suggests that the metabolic pathway for GABA production might be repressed by the L-MSG lodged in the medium. Therefore, we tried to establish a feeding method efficient for GABA production from L-MSG at a high conversion rate. When 200 g/l L-MSG in total was continuously added to the culture broth at a cultivation time between 22 h and 52 h, which is at a speed of 10.7 g/h, the survival L-glutamate was lower than 10 g/l (data not shown), and GABA productivity in the 70 h culture reached 77.1±6.4 g/l (Fig. 1A). The conversion ratio of GABA from L-glutamate with the continuous feeding culture was higher (86.0±5.0%) than that with the fed-batch culture (78.1±3.5%).

The effect of aeration on GABA production by a fed-batch culture at the jar-fermentor scale was examined. Since *E. avium* G-15 is a Gram-positive, facultative anaerobic cocci occurring in pairs or in tetrads, it can grow in an aerobic environment, but it is necessary to culture it under a condition without vigorous agitation. We examined the effect of aeration on GABA production by a fed-batch culture at the jar-fermentor scale using a GYP2 medium containing 40 g L-MSG/l at an initial concentration. When air (160 ml/min) was introduced into the medium, the GABA productivity after 48 h cultivation was the maximum of 4.0 g/l, suggesting that oxygen suppresses the production of GABA, as shown in Fig. 2B. When the G-15 strain was cultured under aeration with nitrogen gas, the growth reached a stationary stage at 24 h cultivation after a lag stage for 10 h and the following exponential stage of growth (Fig. 2A). However, the growth was inhibited by oxygen (air). In addition, after G-15 was grown for 10 h under aeration, the culture was conducted under nitrogen gas. As a result, the GABA productivity was almost the same as that for cultivation under the successive

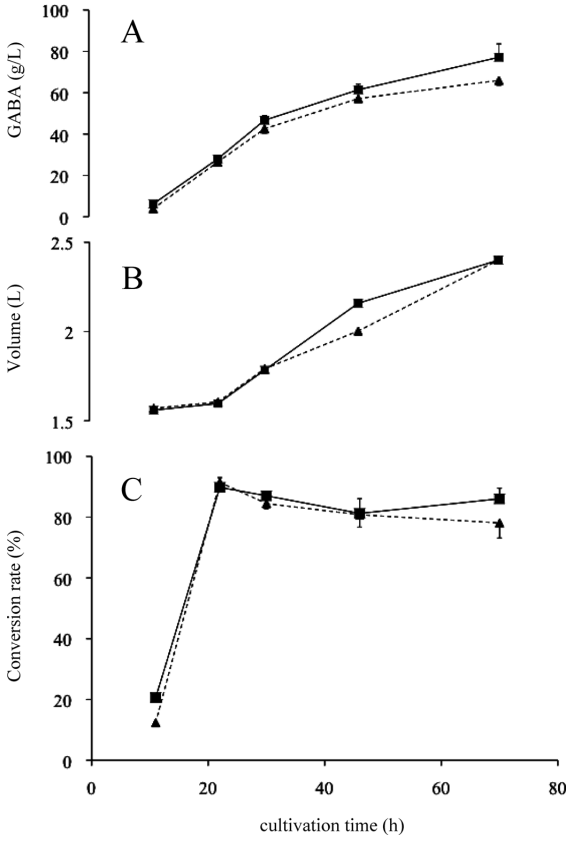


Fig. 1. Effect of the Feeding Manner of L-MSG on the GABA Productivity by *E. avium* G-15 at a Jar-Fermentor Scale Using a GYP2 Medium

L-MSG was added at a cultivation time 22, 30, 46, and 52 h (closed triangles with a broken line) or continuously fed between 22 h and 52 h (closed squares with a solid line). (A) GABA productivity. (B) Change of the total volume increased by the addition of L-MSG. (C) Conversion rate from glutamate to GABA (mol%).

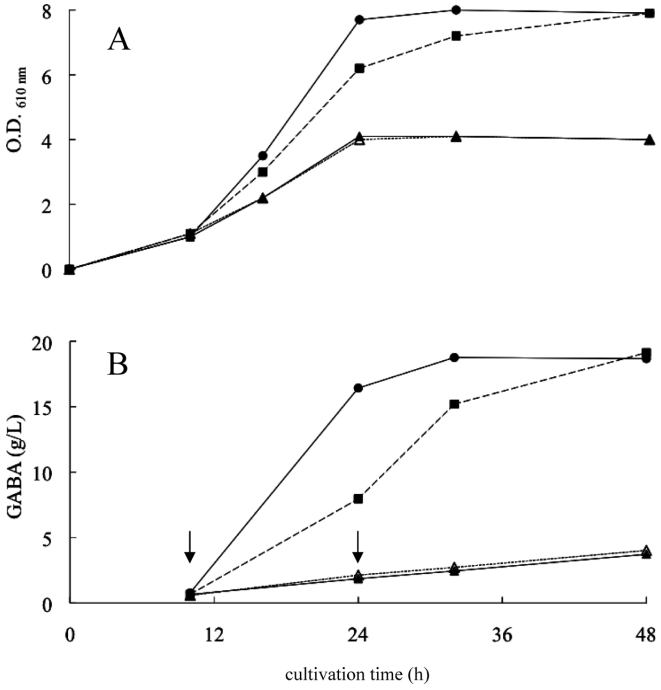
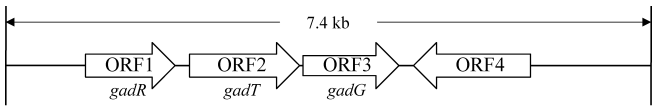


Fig. 2. Aeration Effect on Growth (A) and GABA Productivity (B) of *E. avium* G-15

Symbols: under aerobic conditions (open triangles with a dotted line); under anaerobic conditions (closed circles with a solid line); change to nitrogen gas at 10 h (closed squares with a broken line) and 24 h (closed triangles with a solid line). The arrows indicate the point of aeration gas exchange.



ORF	aa	Best BLAST match	Identity (%)
ORF1	232	*E. faecalis* V583 transcriptional regulator	32
ORF2	503	*Lc. lactis* subsp. *lactis* IL1403 Glu-GABA antiporter	88
ORF3	466	*Lc. lactis* subsp. *lactis* IL1403 glutamate decarboxylase	90
ORF4	503	*E. faecium* D0 hypothetical protein	48

Fig. 3. Gene Organization of the Glutamate Decarboxylase Loci and Similarities of ORFs in the *gad* Cluster

nitrogen gas. However, the start for GABA production was somewhat late (Fig. 2B). On the other hand, cultivation under nitrogen gas was also conducted after G-15 was grown for 24 h under aeration, and it was observed that GABA productivity was the same as that for cultivation with aeration alone (Fig. 2B). These results suggest that the ability to convert L-MSG to GABA in *E. avium* G-15 is acquired only during the lag-to-exponential stage of growth but not during the stationary stage.

Gene Organization around the Glutamate Decarboxylase-Encoding Gene, Designated *gad* To characterize glutamate decarboxylase in *E. avium* G-15 as a high GABA producer, we cloned a DNA fragment containing a gene encoding glutamate decarboxylase, designated *gad*, from the chromosomal DNA. Using a *gad* probe, which was designed on the basis of the nucleotide sequence of some known *gad* genes, we successfully obtained a 7.4-kb DNA fragment (DDBJ Accession No.: AB548685). Figure 3 shows a gene

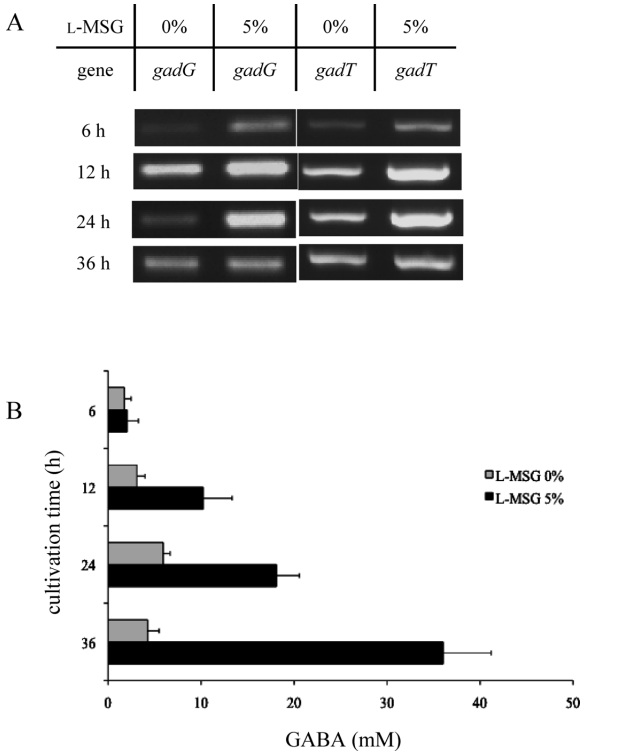


Fig. 4. Effect of L-MSG on GABA Production

RT-PCR analysis of *gadG* and *gadT* genes (A) and GABA production (B) with or without added L-MSG under anaerobic conditions.

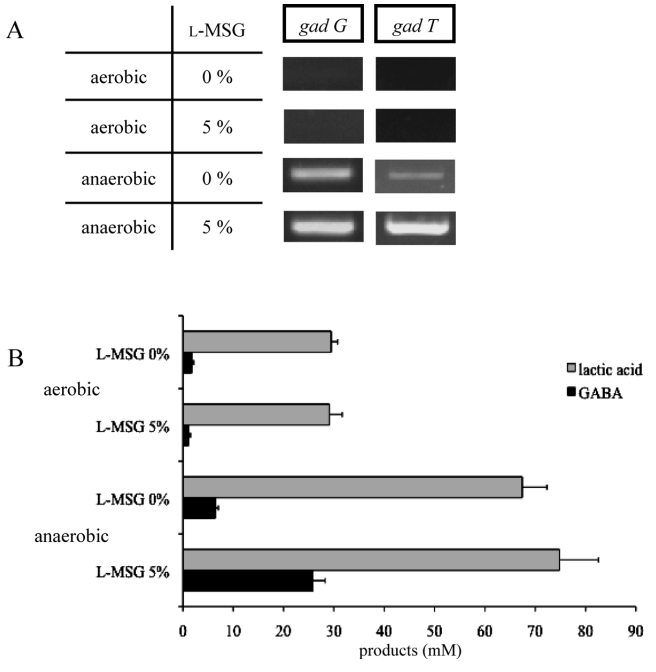


Fig. 5. Effect of Cultivation Conditions on Lactic Acid and GABA Production

RT-PCR analysis (A) and lactic acid and GABA production (B) with or without added L-MSG at 24 h, cultivation.

organization that consists of 4 open reading frames (ORFs), which are designated ORF1 to ORF4. ORF1, which is similar to a transcriptional regulator, was designated *gadR*. ORF2 and ORF3, which are similar to a GABA transporter and glutamate decarboxylase (EC 4.1.1.15), were designated *gadT*

and *gadG*, respectively. The sequences TTCCCC (-35 region) and AATAAT (-10 region), which are putative promoter sequences, are present upstream of *gadT*. The AAGAAG sequence, which is a putative ribosome-binding site, is also present 8 bp upstream of the *gadT* initiation codon. Although a terminator sequence is absent downstream of *gadT*, a hairpin structure as a putative terminator is observed downstream of *gadG*, suggesting that *gadT* may be co-transcribed with *gadG* by forming an operon.

Using the RT-PCR method, we analyzed the expression of a *gad* gene cluster in *E. avium* G-15 cultured in a GYP medium (Fig. 4A). The expression of *gadT* and *gadG* in an anaerobic environment was observed even without the added L-MSG, whereas the expression was enhanced by the addition of L-MSG, suggesting that the expression of the *gad* gene cluster is induced by glutamate. In addition, although the expression of *gadT* and *gadG* was low during the 6 h cultivation, it reached a maximum at the 12—24 h cultivation time and then decreased thereafter. In addition, when 5% L-MSG was extracellularly added to the medium, the GABA production was remarkably increased with the culture time but scarcely increased without the added L-MSG (Fig. 4B). On the other hand, the expression of *gadT* and *gadG* under aerobic conditions was not observed regardless of 24 h cultivation with or without added L-MSG (Fig. 5A). The production of GABA and lactic acid in an anaerobic environment was also higher than that in an aerobic environment (Fig. 5B).

DISCUSSION

As GABA-producing microorganisms, many LAB strains classified into the genera *Lactococcus*, *Lactobacillus*, and *Streptococcus* have been previously isolated.[10—14,16] In the *Enterococcus* strains, *E. casseliflavus* has been only reported to be the GABA-producing strain.[15] In this study, we found that *E. avium* G-15 from carrot leaves has high potency for GABA production. *E. avium* does not display acute toxicity, and mutagenesis tests demonstrated that the bacterium does not display mutagenesis, intake-related illness, or death, suggesting that GABA produced by this bacterium is useful as a food material.

The bacterial growth rate was stimulated by increasing the concentration of nitrogen sources in GYP as a start medium. As described in the result section, by using a GYP2 medium, the GABA productivity was increased by 10.7 times. The increment of GABA productivity might be achieved not only by reinforcing the source of nutrition necessary for growth, but also by maintaining a pH level that is suitable for the conversion to GABA by a change in the medium composition. In this study, we found that the medium pH controlled at 5.0 is the best for GABA production. When the pH in the medium was controlled at 6.5, the cell growth was somewhat improved, and the lactate production was 2-fold higher. However, the GABA productivity decreased. From this observation, the GABA production by *E. avium* G-15 might be independent of the primary metabolism, such as growth and respiratory activity.

It is well known that GABA is produced from L-MSG in LAB by the catalytic activity of glutamate decarboxylase (EC 4.1.1.15).[27—30] Accordingly, to obtain higher amounts of GABA, the manner in which a large amount of L-MSG is fed into the culture medium of a GABA-producing microorganism is very important. On a fed-batch culture using a jar-fermentor, we observed that the GABA productivity is repressed when large amounts of L-MSG are present in the culture medium. To reduce the concentration of L-MSG in the medium as possible, we devised a method for the gradual feeding of L-MSG instead of a batch-culture method. Although *E. avium* G-15 produced 133.7 g GABA/l as a champion datum, the average productivity with a standard error was 115.7±6.4 g/l. When fed with 250 g L-MSG/l, *E. avium* G-15 produced GABA at a 86.0±5.0% conversion rate. In a previous study,[16] it was reported that *Lactobacillus* sp. L-13 produced 650 mM (67.0 g/l) GABA when cultured for 3 d. We demonstrated that *E. avium* G-15 produces 115.7±6.4 g/l GABA in the same cultivation time, suggesting that this strain is a hyper GABA-producing bacterium.

The repression mechanism of GABA production by large amounts of L-MSG in the culture medium is unclear. In the present study, we observed that; although the concentration of L-MSG was lower at the late than at the early stage of growth, the GABA productivity was repressed at the late stage (data not shown). If the feeding of L-MSG is strictly controlled during the middle and late stages of growth, it will be possible to obtain higher amounts of GABA.

On the other hand, we observed that *gadT* is co-expressed with *gadG*. As shown in Figs. 2 and 4, at the exponential stage of growth, *E. avium* G-15 starts to produce GABA and increases the production, and during this growth period, the expression of *gadT* and *gadG* also rapidly increases. However, after a stationary stage of growth is attained (24 h cultivation), both GABA productivity and the expression of the *gad* gene cluster decrease, revealing that the GABA production may be synchronized with growth. When the pH in the culture medium was raised to 6.5, the GABA productivity decreased in spite of cell growth stimulation (Table 1). The result may be due to the biological significance for the GABA production in the microorganism. It seems the GABA production is a mechanism that may keep intracellular pH neutral by consuming the proton, which was shown to increase in the acid stress.[31] This observation suggests that these bacteria may lack the ability to produce GABA under the neutral pH condition.

We confirmed biochemically that the pH optimal for the catalysis of glutamate decarboxylase of *E. avium* G-15 is 4.4, and this enzyme loses the catalytic activity completely at pH 6.5 (data not shown). *E. avium* G-15 is useful as a practical bacterium for GABA production. Further analysis is in progress to show how *E. avium* G-15 produces GABA at a high level.

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