CHAPTER III

EXPERIMENTAL

3.1. Preliminary screening of glutamate decarboxylase, amylase and proteinase producing strain

Lactic acid bacteria (LAB) were isolated from fermented foods, fruits, animals, etc. in Thailand by enrichment of the samples in MRS broth (Difco, de Man et al., 1960) pH ~ 6.2-6.4 for lactobacilli group and pH ~7.2 for enterococci group. Incubated for 2-3 days at room temperature, the enriched cultures were streaked on MRS agar supplemented with 0.2% $CaCO_3$. Clear zone forming colonies with catalase negative were selected and re-purified by streaking on MRS agar medium for 2-3 times to assure purity. The purified isolates were screened for the activities of interest, characterized describes as followed, and preserved in 10% sterile skimmed milk and/or 15% glycerol at -20°C for short term storage. The characterized strains were lyophilized and kept at 4°C for long term storage.

GABA-producing LAB were screened by their ability to produce γ-aminobutyric acid (GABA) from glutamate in GYP medium containing 0.5-1% L-monosodium glutamate at 30 ° C for 1-4 days then the cultured supernatant of each isolate was analyzed for production of GABA by thin layer chromatography (Ueno *et al.*, 2007). Amylase producing LAB were screened by their ability to hydrolyze soluble starch in MRS agar plate without glucose. Proteinase producing LAB were screened by their ability to make clear zone on MRS agar plate supplemented with 1% skim milk or on buffered caseinate agar (Martley et. al., 1970).

3.2. Bacterial strains and growth conditions

The high GABA producing strain *Lactobacillus* sp. nov. L13, from "Senmaizukae" a traditional Japanese pickle in Kyoto area was isolated by Assistant Professor Kazumi Hiraga at Kyoto Institute of Technology, Kyoyo, Japan. The strain was subjected to be studied of glutamate decarboxylase (*gadB*). The strain was grown in GYP liquid medium containing 5% sodium glutamate at 30°C without aeration.

The type strains of validly described species: *Lactobacillus parabrevis* sp. nov. ATCC 53295^T and *Lactobacillus hammesii* sp. nov. CIP 108387^T were obtained to compare by DNA-DNA hybridization with the isolate of *Lactobacillus* sp. nov. L13; *E. italicus* KCTC 5373^T was obtained to be compared with the isolate of

Enterococcus camilliae sp. nov. FP15-1^T. *Lactobacillus plantarum* NRIC 1067^T and *Lactobacillus pentosus* NRIC 1069^T were obtained to be compared with the isolates of SB2-3 and U3-1 which belonged to *Lactobacillus plantarum*. *Enterococcus faecium* NRIC 1145^T, *Enterococcus faecalis* TISTR 379^T and *Enterococcus hirae* TISTR 943^T were obtained to be compared with the isolates of N2-1A and N12-9 which was included in genus *Enterococcus*.

Host *E. coli* DH5α (strain with high-efficiency cloning, enables α-complementation), JM109 (endonuclease (*endA*) deficient, greatly improving the quality of miniprep DNA, recombination (*recA*) deficient, improving insert stability, and *lacI*q*ZΔM15* gene on the F′ episome, allowing blue-white screening for recombinant plasmids) and derivatives were grown in Luria-Bertani medium (Sambrook & Russell 2001) with aeration. To select for *E. coli* strain carrying recombinant plasmid pGEM-T easy, pQE70 and/or its derivatives, ampicillin (Sigma, St. Louis, MO) was added to media to a final concentration of 100 μg ml^{-1}. Host *E. coli* and recombinant clones were maintained as frozen stocks at -20 and -80°C in liquid media containing 15% glycerol. Culture media used in this study are in Appendix I.

3.3. Identification of isolates

3.3.1 Morphological and cultural characteristics

Cell form, cell size, cell arrangement and Gram stain were determined under the microscope. Colonies appearance on MRS agar was observed. Motility was detected by the appearance of stab cultures in soft agar.

3.3.2 Physiological and biochemical characteristics

Catalase test A loop full of bacterial culture was smeared on a microscopic slide then 3% of H_2O_2 was dropped over. Gas bubble indicated a positive catalase reaction

O/F test, gas and acid production from glucose The bacteria were grown in MRS broth containing durham tube and incubated at 30°C for 1-2 days. The gas production was indicated by the air bubble trapped inside durham tube. Changing in purple color of bromocresol purple to yellow indicated the acid production. Oxidation and fermentation test was examined in soft agar (Whittenbury, 1963).

Fermentation of carbohydrates Basal medium supplemented with various kinds of carbohydrates were investigated: D-glucose, D-fructose, D-manose, D-

cellobiose, esculin, D-mannose, moltose, mannitol, sucrose, salicin, glycerol, erythriol, D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, adonitol, D-galactose, rhamnose, inositol, sorbitol, D-amygdalin, lactose, α-methyl-D-glucoside, inulin, D-melibiose, D-melezitose, D-sorbitol, D-xylose, raffinose, D-trehalose, and gluconate, to give a final concentration of 1% carbohydrates and a pH indicator, phenol red, which makes the medium red (pH 7.0). Incubated for 3 days at 30°C. Changing in red color of phenol red to yellow indicated the acid production. Volume of 0.1 N NaOH used for titration of the culture broth to make the yellow color indicator turns red indicates the level of acid production activity and range of sugars utilized by the test strain.

The API 50 CH strip (bioMerieux) multi test system was also used for the study of the carbohydrate metabolism of isolated strains. It consists of 50 microtubes each containing an anaerobic zone (the tube portion), for the study of fermentation and an aerobic zone (the cupule portion), for the study of oxidation or assimilation. Carbohydrate substrates were listed in Table 3.1 Fermentation was detected by changing of indicator from purple to yellow after incubating the culture at 30°C for 2 days.

Table 3.1. List of carbohydrates substrate contains in API 50 CH strip

COMPOSITION OF THE STRIP

STRIP 0-9 tube / substrate	STRIP 10-19 tube / substrate	STRIP 20-29 tube / substrate	STRIP 30-39 tube / substrate	STRIP 40-49 tube / substrate
0 CONTROL	10 GALactose	20 α-Methyl-D-Mannoside	30 MELibiose	40 D TURanose
1 GLYcerol	11 GLUcose	21 α-Methyl-D-Glucoside	31 Sucrose	41 D LYXose
2 ERYthritol	12 FRUctose	22 N-Acetyl-Glucosamine	32 TREhalose	42 D TAGatose
3 D ARAbinose	13 MaNnosE	23 AMYgdalin	33 INUlin	43 D FUCose
4 L ARAbinose	14 SorBosE	24 ARButin	34 MeLeZitose	44 L FUCose
5 RIBose	15 RHAmnose	25 ESCulin	35 RAFfinose	45 D ARabitoL
6 D XYLose	16 DULcitol	26 SALicin	36 Starch	46 L ARabitoL
7 L XYLose	17 INOsitol	27 CELlobiose	37 GLYcoGen	47 GlucoNaTe
8 ADOnitol	18 MANnitol	28 MALtose	38 XyLiTol	48 2-Keto-Gluconate
9 β Methyl-D-Xyloside	19 SORbitol	29 LACtose	39 GENtiobiose	49 5-Keto-Gluconate

Analysis of lactic acid isomers Lactic acid in culture broth was detected by enzymatically (Okada *et al.*, 1978).

Arginine and esculin hydrolysis The differential ability of enterococci to produce the enzyme capable of hydrolyzing arginine was tested with media that contained glucose, arginine, yeast extract, tryptone and potassium phosphate. The hydrolysis of arginine by the enzyme arginine dihydrolase resulted in the formation of

ammonia which was detected by 2-3 drops of Nessler's Reagent after 2 days incubation, the broth media turned orange for positive reaction. No color change indicted negative. Strain grown on agar-based medium which included 0.1% esculin and 0.05% ferric chloride; a brown/black coloration indicates a positive test of esculin hydrolysis.

Starch hydrolysis Amylase activity was detected by clear zone on modified MRS agar without glucose supplemented with soluble starch or by added of iodine solution. Iodine reacted with starch produced a dark brown or blue/black color. Hydrolyzed starch indicated by a clear zone around the bacterial growth.

Protein hydrolysis Proteinase activity was detected by clear zone on MRS agar supplemented with 1% skimmed milk.

Nitrate reduction test The bacterial cell were grown in Nitrate broth and incubated at 30°C for 2 days. Nitrate reductase was examined by dropped sulfanilic acid and α-naphthylamine into culture broth. The positive results were indicated by the appearance of pale pink to deep red in broth.

Cell wall composition analysis Cells were hydrolyzed with 6N HCl at 100 °C for 18 h. then cooling down and dropped on TLC plate No. 5577. The solvent system of pyridine: methanol: water: 6N HCl (10:80:26:4) was used. 0.2% of ninhydrin solution was sprayed on the developed TLC plate. (Komagata & Suzuki, 1987)

Cellular fatty acids analysis Methyl esters of fatty acids were prepared as described by Ikemoto *et al.* (1978) and cellular fatty acid compositions were analyzed by gas-liquid chromatography system, a model GC-14 A (Shimadzu Corp., Kyoto, Japan) equipped with a CBP1 (OV-1) type capillary column 25 m by 0.25 mm inside diameter at 180-220°C and a flame ionized detector. Gas-liquid chromatograms were calculated by Chromatopac C-R 4 A data-processor (Shimadzu Corp.).

Analysis of quinone system Quinone system was extracted from freeze-dried cells and purified as described by Collins *et al.* (1977; 1979). The purified quinones were analyzed by high-performance liquid chromatography (Tomaoka *et al.*, 1983).

3.3.3 Nucleic acid based identification of isolates

3.3.3.1 Isolation and purification of nucleic acids

Genomic DNA were isolated and purified by conventional chloroform/isoamyl alcohol (24:1) extraction followed by ethanol precipitation as describe by Saito & Miura (1963) or using the lytic enzyme, labiase, followed by SDS (Niwa *et al.*, 2005).

3.3.3.2 Identification based on sequences of nucleotides

DNA base composition and DNA reassociation

DNA base composition was performed as described by Tamaoka & Komagata, 1984. DNA-DNA hybridization was performed by colorimetric hybridization method at 40 ° C for 16 h as reported by Ezaki *et al.* (1989)

PCR amplification of the 16S rDNA and sequence analysis

The complete 16S rRNA gene was amplified by PCR using primers 8-27f and 1492r and sequenced according to Lane (1991). DNA sequence was determined with an ABI PRISM Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems) using ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The primers used for sequencing raction were listed in Table 3.2. The positions of sequencing primers on 16S rRNA were shown in Fig. 3.1.

Table 3.2. 16S rRNA sequencing primers (Lane, 1991).

Name	Sequence (5' > 3')	Length (bases)
8-27f	AGAGTTTGATC(A/C)TGGCTCAG	20
342r	CTGCTGC(C/G)(C/T)CCCGTAG	16
357f	CTCCTACGGGAGGCAGCAG	19
519r	G(A/T)ATTACCGCGGC(G/T)GCTG	18
530f	GTGCCAGC(A/C)GCCGCGG	16
1100r	GGGTTGCGCTCGTTG	15
1114f	GCAACGAGCGCAACCC	16
1392r	ACGGGCGGTGTGT(A/G)C	15
1406f	TG(C/T)ACACACCTCCCGT	16
1492r	TACGG(C/T)TACCTTGTTACGACTT	22
1525r	AAGGAGGTG(A/T)TCCA(A/G)CC	17

Amplification reactions were typically performed using *Taq* DNA polymerase (TaKaRa^{TM}). PCR reactions were composed of 37.5 µl sterile MilliQ water, 50 µl PCR buffer, 5.0 µl dNTPs (2 mM each), 0.5 µl forward primer (50 mM), 0.5 µl reverse primer (50 mM), 0.5 µl Taq DNA Polymerase (1 U μl^{-1}) and 1.0 µl template DNA (0.2 µg µl-1). The PCR conditions for amplification of 16S rDNA from purified

genomic DNA of LAB isolates normally included 95°C for 5 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min, followed by a single cycle of 72°C for 10 min. The amplified product(s) were checked by 0.8% agarose gel electrophoresis. DNA band(s) were stain by EtBr or SYBR green. The expected 1500 bp band of 16S rDNA was purified by freeze-thaw method or using purification kit (Qiagen)

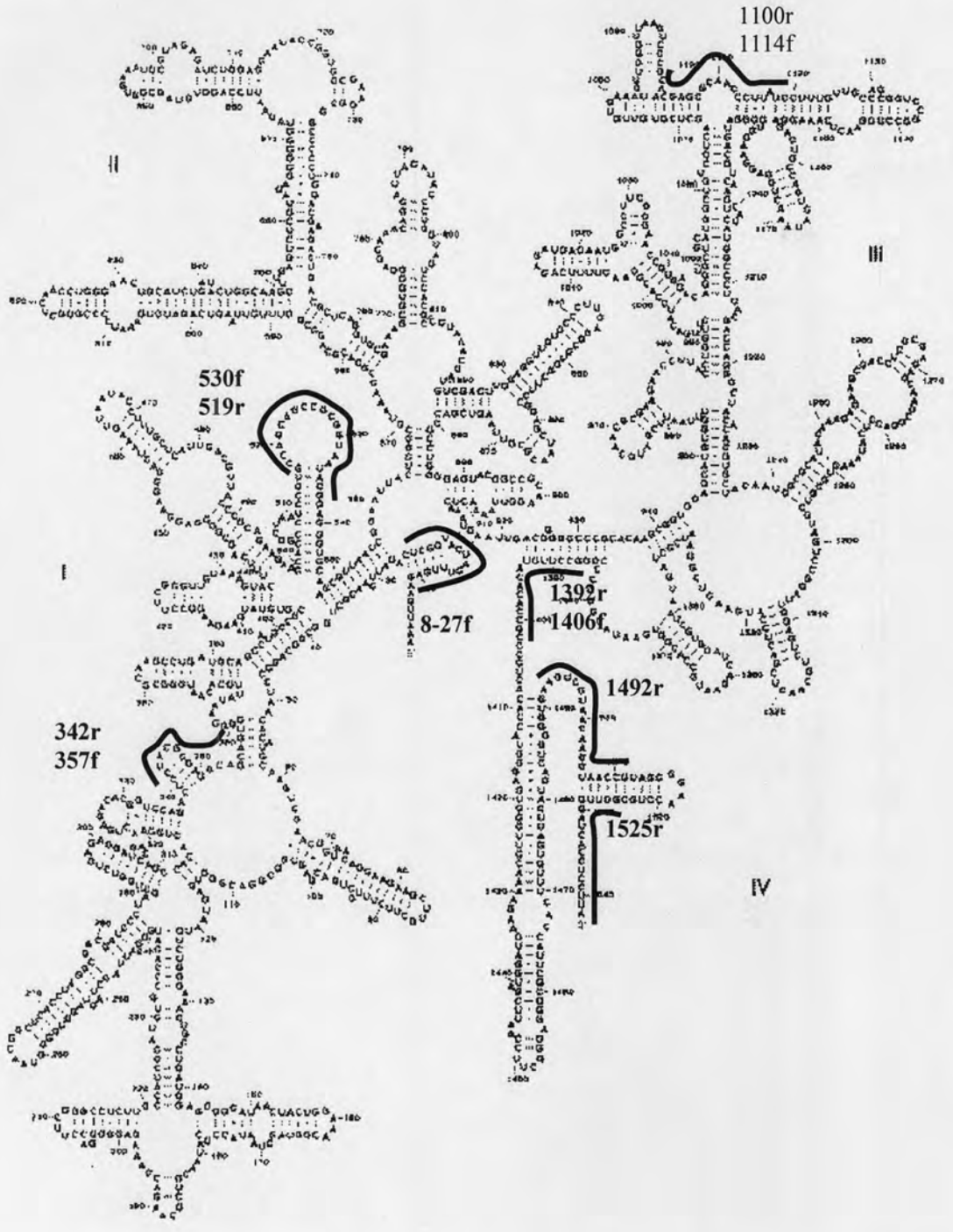

Fig. 3.1. The positions of sequencing primers on 16S rRNA.

Multi locus sequence analysis (MLSA)

The sequences of the primers used for amplification and sequencing of *phe*S and *rpo*A genes (Naser et al., 2005) are listed in Table 3.3. The primer combinations rpoA-21-F/rpoA-23-R, pheS-21-F/pheS-22-R and pheS-21-F/pheS-23-R were used for amplification of the target genes of isolated strains.

Table 3.3. Amplification and sequencing primers for MLSA. (Naser et al., 2005)

Primer name	Sequence (5' > 3')	Position
pheS-21-F	CAYCCNGCHCGYGAYATGC	557
pheS-22-R	CCWARVCCRAARGCAAARCC	1031
pheS-23-R	GGRTGRACCATVCCNGCHCC	968
rpoA-21-F	ATGATYGARTTTGAAAAACC	1
rpoA-23-R	ACHGTRTTRATDCCDGCRCG	802

PCR reactions were composed of 37.5 µl sterile MilliQ water, 50 µl PCR buffer, 5.0 µl dNTPs (2 mM each), 0.5 µl forward primer (50 mM), 0.5 µl reverse primer (50 mM), 0.5 µl Taq DNA Polymerase (1 U μl^{-1}) and 1.0 µl template DNA (0.2 µg µl-1). PCR was consisted of (1) 5 min at 95 °C, (2) 3 cycles of 1 min at 95 °C +2 min 15 s at 46 °C+1 min 15 s at 72 °C, (3) 30 cycles of 35 s at 95 uC+1 min 15 s at 46 °C+1 min 15 s at 72°C and (4) a final 7 min at 72 °C. In a few cases, an annealing temperature of 42 °C was used for the amplification of *rpo*A.

DNA sequencing and analysis

DNA sequencing was conducted with sequence-specific primers for each template of interest or experimental design. Sequence reactions were performed as mentioned previously in 3.3.3.2. The sequences was manipulated, manual edited, contig assembled, and analyzed using BioEdit version 7.0.1 (Hall, 1999). Sequence similarities to others sequence in the GenBank database were searched and analyzed by the Basic Local Alignment Search Tool (BLAST) link by BioEdit program or directly submitted to the NCBI webpage (http://www.ncbi.nlm.nih.gov/BLAST/).

16S rDNA, *rpo*A, and *phe*S sequences of the validly described type strains which related to isolated strains of interest were retrieved from the GenBank database. Multiple sequence alignment was done using CLUSTAL X version 1.83 (Thompson *et al.*, 1997). The phylogenetic trees were constructed based on the neighbor-joining methods (Saitou & Nei, 1987) with by Nj plot program (Perriere & Gouy, 1996). The confidence values of branches of the phylogenetic tree were determined using bootstrap analysis (Felsenstein, 1995) based on 1,000 resamplings.

3.4. Enzyme assay

Glutamate decarboxylase assay Enzyme solution (~50 µl) was mixed with 50 ul of 4M ammoniumsulfate or water (as a control) and incubated for 5 min at room temperature. 1.3 ml of substrate solution (9:1 volume of 20 mM Sodium glutamate in 50 mM Sodium acetate pH 4.6 and 2 mM pyridoxal-5'-phosphate) was added and incubate at 37 °C for 30 min. The reaction was stopped in boiling water for 5 min. Then the produced γ-aminobutyric acid (GABA) was analyzed by TLC (Kiesel gel F254, Meark). The solvent system of butanol: acetic acid: H_2O (3:2:1, v/v) was used (Ueno et al., 1997). One unit of enzyme activity was defined as the amount of enzyme that produced 1 µmol of GABA per min.

Amylase assay Enzyme solution (~100 µl) was mixed with 0.8 ml of 1.2% soluble starch in 0.1 M phosphate buffer, pH 6.0 and incubated for 10 min at room temperature. The reaction was terminated by adding 0.1 ml of 5M NaOH then the reducing sugar were determined by dinitrosalicylic acid reagent as described by Miller 1959. One unit of enzyme activity was defined as the amount of enzyme that permits the hydrolysis of 10 mg of starch in 30 min in the conditions of the assay.

Proteinase assay Enzyme solution (500 µl) was mixed with 1.3% casein in 50 mM Tris buffer, pH 7.5 and incubated for 30 min at 37°C. 0.44 M Trichloroacetic acid (TCA) was added, then centrifuged at 2,000 rpm for 10 min. 500 µl of supernatant was mixed with 2.5 ml of Na_2CO_3, 0.5 ml of 1:1 dilution of Folin phenol reagent was mixed and incubated for 20 min at 37°C. Measuring of an absorbance at 660 nm was performed and results were compared with the standard curve of tyrosine (Hiraga *et al.*, 2000). One unit of enzyme activity was defined as the amount of enzyme that hydrolyzes casein to produce color equivalent to 1.0 mmole (181 mg) of tyrosine per minute at pH 7.5 at 37°C

3.5 Glutamate decarboxylase, amylase and proteinase: gene analysis.

3.5.1 Comparative analysis of the genes

Glutamate decarboxylase, amylase and proteinase genes of LAB available on database were retrieved from GenBank database (http://www.ncbi.nlm.nih.gov/) and DOE genome sequence project (http://www.jgi.doe.gov/).

3.5.2 Multiple alignments of available *gad*B, amylase and proteinase gene on databases

Nucleotide sequences for glutamate decarboxylase and 16S rDNA of lactic acid bacteria were obtained from the NCBI (National Center for Biotechnology Information): (http://www.ncbi.nlm.nih.gov/) and The US Department of Energy Joint Genome Institute (http://www.jgi.doe.gov/). Multiple alignments were carried out using the Clustal X 1.83 program. Based on DNA seq. alignment of 3 predicted *gadB* genes from *Lactobacillus plantarum* WCFS1 (gi|28272563:31469-32878), and *Lactobacillus brevis* ATCC367 (2 predicted genes on S18 and S49. draft genome sequence gi|116098028:81489-82928 & gi|116098028:1806645-1808051) and 2 known *gadB* genes of *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* (BAA24585 & AB033218).

Two kinds of primer, inner primers of conserved region and full length GAD plantarum CDS primers from predicted *gadB* of *Lactobacillus plantarum* WCFS1 were designed listed in Table 3.4. In case of amylase and proteinase genes, the available known genes and predicted genes were analyzed as previous method used for *gadB*, primers for amylase and proteinase are listed in Table 3.5.

Table 3.4. Primers used for amplification of full length and conserved regions of *gadB*

Amplification	Primer name	Sequence (5'-3')	Tm (°C)
Full length primers GAD *plantarum* CDS	Fw GADP	ATGGCAATGTTATACG	49.0
	Rv GADP	TCAGTGTGTGAATCC	46.1
Inner primers of conserved regions	Fw GAD1	ATGGAACCNSAAGC	43.9
	Fw GAD2	CAAGTTKKYTGGGAAAA	53.3
	Rv GAD3	AAMCGGAARTCCCA	51.8
	Rv GAD4	AAATCRTAVARVKTCCA	43.3
Full length primer GAD *brevis*	*bre* GAD Fw	ATGATGAATAAAAACGATCAG	54.3
	bre GAD Rv	TTAACTTCGAACGGTGG	56.6

Table 3.5. Primers used for amplification of amylase and proteinase.

Amplification	Primer name	Sequence (5'-3')	Tm (°C)
L. plantarum amylase with spHI and BamHI site	aAMYP_FwSphI	GCTGCGCATGCGAGTGAAAAAA AAG	68.7
	aAMYP_RvBamHI	CGGGATCCCGAACTGCTTGAT	67.0
Predicted *E. faecium* amylase	Fw-amyEf	ATGTATACAAAACTTTTACAAG	52.7
	Rv-amyEf	TTATTTTATTATTTTCAGCCACA	54.9
Predicted proteinase	EfaeciPepV_Fw	TATGCGATGAAGATCATT	53.1
	EfaeciPepV_Rv	TTATTTCGCCAAACGATA	54.1
	EfaecaSProt_Fw	ATGAAAAAGTTCTCCATAC	52.4
	EfaecaSProt_Rv	TTACGCTGCTGGCACA	61.6

3.5.3 PCR amplification of the genes of interest

Amplification reactions were performed using *Taq* DNA polymerase (TaKaRa^{TM}). PCR reactions were composed of 37.5 µl sterile MilliQ water, 50 µl PCR buffer, 5.0 µl dNTPs (2 mM each), 0.5 µl forward primer (50 mM), 0.5 µl reverse primer (50 mM), 0.5 µl Taq DNA Polymerase (1 U μl^{-1}) and 1.0 µl template DNA (0.2 µg µl-1). The PCR conditions for amplification of 16S rDNA from purified genomic DNA of LAB isolates normally included 95°C for 5 min, followed by 30 cycles of 94°C for 30 s, annealing temperature was depended on the Tm of the specific primer for 30 s, and 72°C for 2 min, followed by a single cycle of 72°C for 10 min. The amplified product(s) were checked by 0.8% agarose gel electrophoresis. DNA band(s) were stained by EtBr or SYBR green. The expected 1500 bp band of 16S rDNA was purified by freeze-thaw method or using purification kit (Qiagen)

3.6 Identification of glutamate decarboxylase gene from the high GABA producing *Lactobacillus* sp. nov L13 and *L. brevis* LSF 8-13

3.6.1 Isolation of *gadB* from *Lactobacillus* sp. nov. L13

Template DNA: Genomic DNA was extracted from the isolated strain as described previously on genomic DNA extraction section and used as template for TAIL-PCR amplifications.

Amplification of core fragment of *Lactobacillus* sp. L13 *gadB*: The forward primer for full length of *Lactobacillus brevis* IFO12005 (*bre* GAD Fw primer) and the inner primers of conserved regions of predicted *gadB* in *L. plantarum* genome (Reverse GAD3 primer) were used for amplification of the core fragment of *Lactobacillus* sp. L13 *gadB.* The amplified fragment was sequenced and confirmed to contain conserved domain among group of glutamate decarboxylase enzymes

available on databases. The sequence result was used as a core fragment of known sequence for designation of nested sequence specific primers for TAIL-PCR. The primers were listed in Table 3.4.

Oligonucleotide primers: Specific primers for each walking step in both 3' and 5' end were designed based on the known sequence and revealed walking sequence during experiments. The nested sequence specific primers are as follows: forward TAIL-PCR primers for walking on the 3' end region(FwE1, FwE2, FwE2N, FwE3, and FwE3N); reverse TAIL-PCR primers for walking on the 5' end region (RvS1, RvS2, RvS3, and RvL13-155). The arbitrary degenerate primers for use in combination with the specific primers are as follows: AD3, Nini256, and AD5. All primers used were listed in Table 3.6.

TAIL-PCR procedure:

Round 1st. 50 ng of template DNA, 50 pmole of each specific primer (FwE1 or RvS1) and random primer (AD3, NiNi or AD5), 1x *Ex Taq* Buffer and 2.5 unit of *TaKaRa Ex Taq*™ HS polymerase (Takara Bio Inc.) were used in reaction mixture for the primary reaction. The conditions for amplification were as follows. Denaturation at 95 °C for 5 min, 5 cycles of 30 s at 94 °C, 30 s annealing at 55-60 °C (depending on an average Tm of the specific primer used minus 5 °C), and 3 min at 72 °C, 2 cycles of 30 s at 94 °C, 2 min at 25 °C, and then ramp to 72 °C in 3 min, 15 cycles of 30 s at 94 °C, 30 s at 55-60 °C , 3 min at 72 °C, 30 s at 94 °C, 30 s at 55-60 °C, 3 min at 72 °C, 30 s at 94 °C, 1 min at 44 °C, final extension at 72 °C for 5 min. Then immediately kept at 4 °C.

Round 2nd. The primary reaction product was diluted with sterilized distilled water to 1/50, and then the 2nd reaction was set up as previous with the next specific primers (FwE2, FwE2N or RvS2 etc.) in combination with the random primer used. PCR conditions were 95 °C for 9 min of denaturation, 15 cycles of 30 s at 94 °C, 1 min at 55-60 °C, 3 min at 72 °C, 30 s at 94 °C, 1 min at 64 °C, 3 min at 72 °C, 30 s at 94 °C, 1 min at 44 °C, 3 min at 72 °C, final extension at 72 °C for 5 min. Then immediately kept at 4 °C.

Round 3rd. The second reaction product was diluted with sterilized distilled water to 1/50, and then the 3rd reaction was set up as previous with the next specific primer (FwE3, FwE3N or RvS3 etc.) in combination with the random primer used. PCR conditions were performed as the 2nd round conditions.

Table 3.6. PCR primers used for TAIL-PCR methods

Amplification (ref.)	Primer name	Sequence (5'-3')	Tm (°C)
Arbitrary degenerate primers (Liu et al., 1995)	AD3	AGWGNAGWANCAWAGG	46.5-54.6
	AD5	NTCGASTWTSGWGTT	44.6-51.7
	Nini256	NGTCGASWGANANGAA	50.0-55.9
Forward TAIL-PCR primers for 3' end region (This study)	FwE1	AACCAAGTTGTTTGGGAAAAGTT	61.9
	FwE2	GGTTCCGTTGATGACATCCA	62.5
	FwE2N	TATGGACTACGTCGATGAGAACA CTA	64.7
	FwE3	AAGCCTTGGGACTTCCGGTT	66.5
	FwE3N	ATACAACAAGACTGCTGCTTT	60.5
	L13_971	GTGTCGACTCAATCGCTATC	60.6
	L13_1151	GTAAAGAATTACCAATCAACTG	55.4
	L13_ Rv1413	TTAAACATCCTTAACGTCACC	58.3
	L13_ Rv1542	CTAACGAACAGTCGTCTTG	58.2
Reverse TAIL-PCR primer For 5' end promoter region (This study)	RvS1	CAGAACTTTTCCCAAACAACT	59.2
	RvS2	TCAGCTTGTGGTTCCATTT	59.8
	RvS3	TTTCCTGATCGTTTTTATTCATCAT	59.5
	RvL13_ 155	TCAACCATTTCTACGGCTAC	59.8
	L13_ minus119	GCTAAGGAAGATGCAAAGTAA	58.3

All TAIL-PCR products were loaded on 0.7% agarose gel electrophoresis for analysis of specific amplified products with decreasing in fragment size gradually. The expected bands were purified and sequenced. The DNA sequences of TAIL-PCR products were searched for overlapping region with the known fragment and aligned subsequently together to get the whole gene. The open reading frame (ORF), predicted promoter region and ribosome biding site were analyzed by BioEdit program version 7.0.1 and PPP (Prokaryote Promoter Prediction, http://bioinformatics.biol.rug.nl/websoftware/ppp/) and BPROM (Bacterial sigma 70 promoter recognition program http://www.softberry.com) respectively.

3.6.2 Isolation of *gadB* from *Lactobacillus brevis* LSF8-13

Template DNA: Genomic DNA was extracted from the isolated strain as described previously on genomic DNA extraction section and used as PCR template for Rapid PCR-based method.

Amplification of full length *gadB* Based the presumed *gadB* gene of *Lactobacillus plantarum* WCFS1 complete genome sequence. PCR was performed using the full length GAD plantarum CDS primers (Forward GADP and Reverse GADP). The amplified fragment of expected size was confirmed for *gadB* conserved domain sequence by different inner primer sets GAD1-GAD4, GAD2-GAD4, GAD1-

GAD3 and GAD2-GAD3, respectively. The primer sequences were listed in Table 3.4. Annealing temperature used depending on average Tm of primer pair minus 5°C.

3.6.3 Southern blot hybridization analysis

Genomic DNA of *Lactobacillus* sp. L13 and *L. brevis* LSF8-13 were separately digested with restriction enzymes (*Bam*HI, *Eco*RI, *Hind* III and *Pst* I). The restriction digested of genomic DNA were fractionated by electrophoresis on 0.7% agarose gel, and transferred to Hybond-N nylon membranes (Amersham). The blots were hybridized with digoxigenin (DIG)-labeled PCR products corresponding to the whole *gadB* of *Lactobacillus* sp. L13 or *L. brevis* LSF8-13. The hybridized probes on blot were detected by chromogenic methods using NBT/BCIP (Roche Molecular Biochemicals)

3.7 Cloning and sequencing of the presumed *gad*B

The amplified *gad B* genes were purified by GeneCleanII Kit. The purified gene fragments were inserted into the pGEMT-easy kit (Promega) and cloned in to *E. coli* DH5α for sequencing purpose. Sequencing reaction were performed using ABI PRISM Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems) and ABI PRISM 310 Genetic Analyzer (Applied Biosystems) The sequence were assemble and analyzed using BioEdit program version 7.0.1.

3.8 Construction of expression vector for the isolated *gad*B

To prepare the expression clone of the both presumed *gadB*, 2 sets of primer with restriction site of *Sph*I and *Bam*HI at 5' and 3' end (L13FwSphI - L13RvBamHI and LSF8-13FwSphI- LSF8-13RvBamHI) were designed from the complete sequenced analyzed of the whole amplified genes from *Latobacillus* sp. L13 and *L. brevis* LSF8-13, respectively. The amplified PCR products with restriction sites were cloned into pGEMT-easy (Promega), and fully sequenced to confirm the absence of any mutations. Then both inserted genes, recovered after digestion with *Sph* I and *Bam*HI, were inserted in-frame into the expression vector pQE-70 by cutting with the appropriate restriction enzymes (*Sph* I and *Bam*HI or *Sph* I and *Bgl* II). Strategy of construction showed in scheme 4.2. The genes and the expression vector were ligated using DNA ligase of the pGEMT-easy ligation kit (Promega) at 4 °C for O/N. The ligation mixtures were transformed into the competent cell of *E. coli* JM109

(Wako).The expression clones were confirmed by restriction analysis and also sequenced to confirm the in-frame inserted genes.

3.9 Expression and purification of the presumed *gadB*

The transformed cells cultured of O/N were inoculated at 1:20 dilution in 100 ml of LB medium containing 100 μg/ml ampicillin, and cultivated further at 37 °C. At an approximately OD_{600} = 0.4, expression was induced by adding 1 mM isopropyl thio-β-D-galactoside and cultured at 20 °C for 16 h. *E.coli* cells were harvested by centrifugation at 3,000 rpm for 5 min and resuspended in 2 ml of lysis and binding buffer pH 7.0 containing 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cell suspensions were distrupted using Multi-beads shocker (Yasui Kikai, Osaka, Japan). To separate beads from the lysate, crude extract were centrifuged at low speed 3,000 rpm for 5 min. The soluble extracts were obtained by centrifugation at 13,000 g for 20 min. The supernatants were loaded onto the HiTrap chelating HP affinity column (Amersham pharmacia biotech) charged with Ni^{2+} ions and washed with 10 column volumes of binding buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 7.0, 10 mM imidazole), with 10 column volumes of binding buffer with increasing the concentration of imidazole to 20 mM. Recombinant (His) 6-tagged protein were eluted with 5 column volumes of 40 mM immidazole in binding buffer. The soluble extracts, flow through, wash, and elution fractions were loaded on SDS/PAGE (10%). Gels were stained with Coomassie Brillant Blue.

3.10 3D structure analysis

The deduced amino acid sequence of isolated *gadB* from *Lactobacillus* sp. L13 and *L. brevis* LSF8-13 and that of *Lactobacillus brevis* IFO12005 (Ueno *et al.* unpublished data) were compared. Protein 3D structure models (PDB file) were constructed from the deduced amino acid sequences by 3D –JIGSAW program (Bates *et al.* 2001). The 3D structure models were view by RasWin 2.7.3.1 molecular graphics visualization tool (http://www.rasmol.org)