CHAPTER IV

RESULTS AND DISCUSSION

4.1. Preliminary screening of glutamate decarboxylase, amylase, and proteinase producing isolates. Totally 116 strains of LAB isolated from various sources were investigated for GABA production, starch and/or casein hydrolyzing abilities. 23 isolates with abilities of interest were selected for further characterized. The list of screened isolates is showed in Table 4.1.General characteristics of selected isolates are shown in Table 4.2.

Table 4.1. LAB isolates: their abilities and source of isolate

Strain	Cell[a]	GABA production	Amylase by starch plate	Proteinase by casein plate	Source (local name)
L13	R	+++	-	+	Japanese pickle (Senmaizukae)
LSF 8-12	R	++	-	++	Fermented fish (Pla-som)
LSF 8-13	R	++	-	++	Fermented fish (Pla-som)
P 46-1	R	+	-	+	Pickle (Pak-sian-dorng)
FS 73-1	R	+	-	-	Fermented fish (Pla-ra), Ubonrajthanii
SR 11-2	R	+	-	-	Soy sauce
SEA 62-2	R	+	-	-	Pine apple
SEA 85-1	R	+	-	-	Sugar cane, Ubonrajthanii
SEA 104-1	R	+	-	-	Rambutan
SEA 138-1	R	+	-	-	Cantaloupe
SG 1-1	C	w	-	-	Grape, Nakornpratom
SG 1-2	C	w	-	-	Grape, Nakornpratom
SG 1-3	C	w	-	-	Grape, Nakornpratom
SB 2-3	R	-	+++	-	Raw Strach, Songkla
U 3-1	R	-	++	-	Fermented starch
N 2-1A	C	-	+	-	Fermented starch
N 12-9	C	-	+	-	Rice (Khao-chae), Nakornrajsrima
FP 15-1	C	-	w	-	Fermented tea leaves (Miang), Chaingmai
933	C	-	w	-	Fermented beef sausage (Mum)
378-1	C	-	w	-	Fermented starch
SCR 1P	R	-	-	++	Red frog crab
SCC 1	C	-	-	++	Red frog crab
SMC 1	C	-	-	+++	Dog feces

+, Positive (number of + corresponded to level of activity); −, negative; w: weakly positive.

[a] Cell morphology: R, rod; C, cocci.

Table 4.2. Characteristics of the selected GABA, starch hydrolyzing and/or casein hydrolyzing LAB isolates

Strain	Hydrolysis of			Growth						Acid production from																	
	Arginine	Esculin	Starch	15/45 °C	pH 4.5	pH 5.0	pH 9.6	6.5 % NaCl	8.0 % NaCl	Amygdalin	Arabinose	Galactose	Gluconate	Glycerol	Lactose	Maltose	Manitol	Melibiose	Melezitose	Salicin	Sorbitol	Sucrose	Trehalose	Xylose	Lactic acid isomer(s)	G+C content (mol%)
L13	+	-	-	+/-	+	+	-	+	+	-	-	-	w	-	-	-	-	-	-	-	-	-	-	+	DL	46.0
LSF 8-12	+	ND	-	+/+	+	+	-	+	+	-	+	w	-	-	-	+	-	-	-	-	-	-	-	+	DL	ND
LSF 8-13	+	ND	-	+/+	+	+	-	+	+	-	+	+	-	-	-	+	-	-	-	-	-	-	-	+	DL	ND
FS 73-1	+	+	-	+/+	+	+	+	+	+	ND	+	+	ND	ND	+	+	ND	+	ND	ND	ND	+	ND	+	DL	ND
SR 11-2	-	-	-	+/-	+	+	-	+	+	-	+		-	-	w	-	-	-	-	-	w	w	+	-	L	ND
SEA 62-2	-	-	-	+/-	+	+	-	+	-	+	+	+	-	w	+	+	-	+	+	+	+	+	+	-	DL	ND
SB2-3	-	+	+	+/-	+	+	-	-	-	+	-	+	-	+	+	+	+	-	-	+	+	+	+	-	DL	ND
U3-1	-	+	+	+/-	+	+	-	+	-	+	-	+	-	+	+	+	+	-	-	+	+	+	+	-	DL	ND
N2-1A	-	+	+	+/+	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	L	35.3
N12-9	+	+	+	+/+	-	+	+	+	-	+	w	w	w	-	-	w	-	-	-	w	-	-	+	-	L	45.2
FP 15-1	-	w	w	+/+	-	+	+	-	-	-	-	-	-	-	-	+	+	-	-	+	-	+	+	-	L	37.8
933	+	-	-	+/+	-	+	+	+	w	+	-	+	+	+	+	-	-	-	-	+	-	+	-	-	L	37.9
378-1	-	w	w	+/+	-	-	+	-	-	-	-	-	-	-	-	w	-	-	-	-	-	+	+	-	L	ND
SCR 1P	-	-	-	+/+	+	+	-	+	+	+	+	+	+	-	-	+	-	+	+	+	-	+	+	-	DL	ND
SCC 1	+	-	-	+/+	-	+	+	+	+	w	-	w	w	w	-	w	w	-	w	+	-	-	-	-	L	ND
SMC 1	+	-	-	+/+	+	+	+	+	-	w	-	+	+	w	-	+	w	-	w	+	w	w	-	-	L	ND

+, Positive; −, negative; w: weakly positive; ND, not determine.

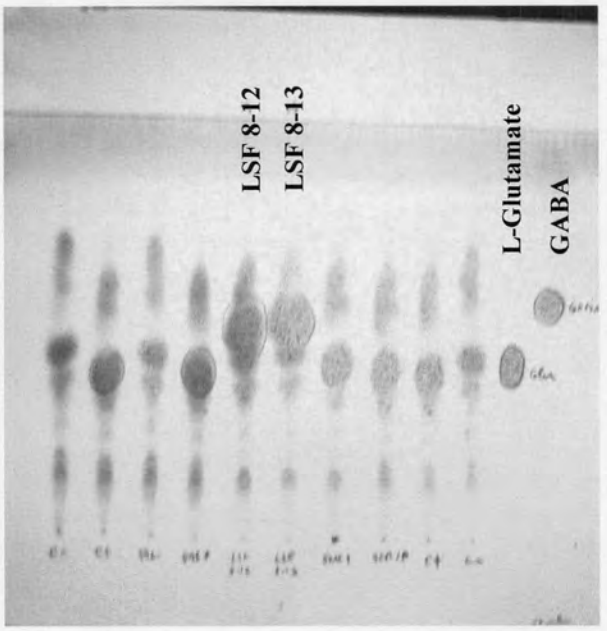


Fig. 4.1. Screening of GABA producing strains by TLC

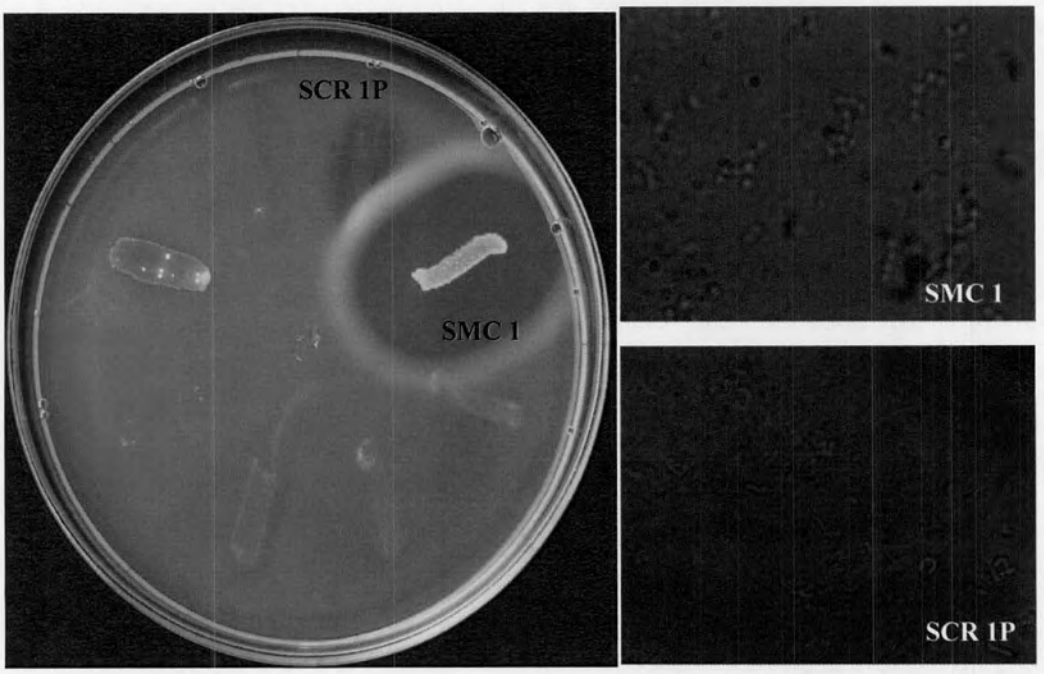


Fig. 4.2. Screening of proteinase producing strain by casein agar plate

TLC plate analysis for GABA producing LAB were done by spotting culture brorth of isolates then GABA detected by ninhydrin solution (Ueno et al., 1997). Strain LSF 8-12 and LSF 8-13 shown GAD activity (Fig. 4.1). Casein hydrolyzing isolates SMC 1 and SCR 1P photos and activity on casein plate are shown (Fig.4.2).

4.2. Identification of the isolated strains

4.2.1 Glutamate decarboxylase producing strains

4.2.1.1 The novel high GABA producing isolate of *Lactobacillus* sp. nov. L13 from Japanese pickle

The strain L13 was isolated from *Senmaizuke,* a Japanese traditional pickle after cultivation on GYP agar plate at 30℃ for 5 days. Single colonies were grown anaerobically in GYP liquid medium containing 5% sodium glutamate at 30℃ for 48 h, then the culture supernatant of each isolate was analyzed for production of GABA by thin layer chromatography (Ueno *et al.*, 2007). The cells were Gram-positive, catalase-negative, non-motile, nonspore- forming short rods (1.0-5.0 x 0.5-0.8 μm, Fig. 4.3). Colonies on GYP agar appear white and circular with a smooth surface and edges (1.0-2.0 mm in diameter after 2 days of growth). Strain L13 growth at 15°C but not at 45 °C. The strain L13 was able to grow up to 8.0 % NaCl; the specific growth rate at 5.0% NaCl is 50% (100% without NaCl). Facultative anaerobic and produced DL-lactic acid heterofermentatively from glucose (5.7% L-lactate and 0.7 % D-lactate). HPLC analysis revealed that it also produced 3% ethanol as other metabolite. L13 produced acid from D-xylose, D-ribose, D-galactose, D-fructose, gluconate, 2-keto gluconate, and *N*-acetylglucosamine. L13 was negative for acid production from glycerol, erythritol, arabinose, L-xylose, adonitol, β-methyl-D-xyloside, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, sorbitol, alpha-methyl-D-mannoside, alpha-methyl-D-glucoside, D-amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fructose, L-fructose, arabitol, and 5-keto gluconate. Ammonia is not produced from arginine. Peptidoglycan structure was A4a L-Lys–D-Asp type and the DNA G + C content is 46 mol%.

The 16S rRNA gene sequence (1,443 bp) of the strain L13 was determined and indicated that the strain showed 98% identity to *Lactobacilllus parabrevis* and *Lactobacillus hammesii.* In a neighbour-joining dendrogram (Fig. 4.4 a) based on 16S rRNA gene sequences obtained from this study and from the GenBank database, the strain L13 clearly belonged to the genus *Lactobacillus*, and were positioned very close to *Lactobacillus parabrevis*, which was isolated from wheat and deposited in the ATCC as *L. brevis* (Spiller, 1987), and recently it was reclassified as *L. parebrevis* sp.

nov. (ATCC 53295^T; Vancanneyt *et al.*,2006). *rpoA* gene (527 bp) sequences were determined and analyzed (Fig. 4.4 b). The position of the strain L13 clearly separated from *Lactobacillus parabrevis*. DNA sequence similarity matrixes of 16S rDNA and *rpoA* gene were shown in appendixIII. *rpoA* gene shown high discrimination power that of 16S rDNA. that DNA–DNA hybridization analysis was performed including the most closely related strain based on 16S rRNA gene sequence analysis. DNA–DNA relatedness values of L13 to *Lactobacillus parabrevis* sp. nov. ATCC 53295^T was below 16%. This value is below the threshold of 70% suggested for species delineation (Stackebrandt & Goebel, 1994), indicating that strain L13 represents a separate genomic species.

Based on physiological, biochemical analyses, DNA G + C content and DNA–DNA hybridization of the genomic DNA as shown in Table 4.3 indicated that the strain L13 represent a novel species of the genus *Lactobacillus*.

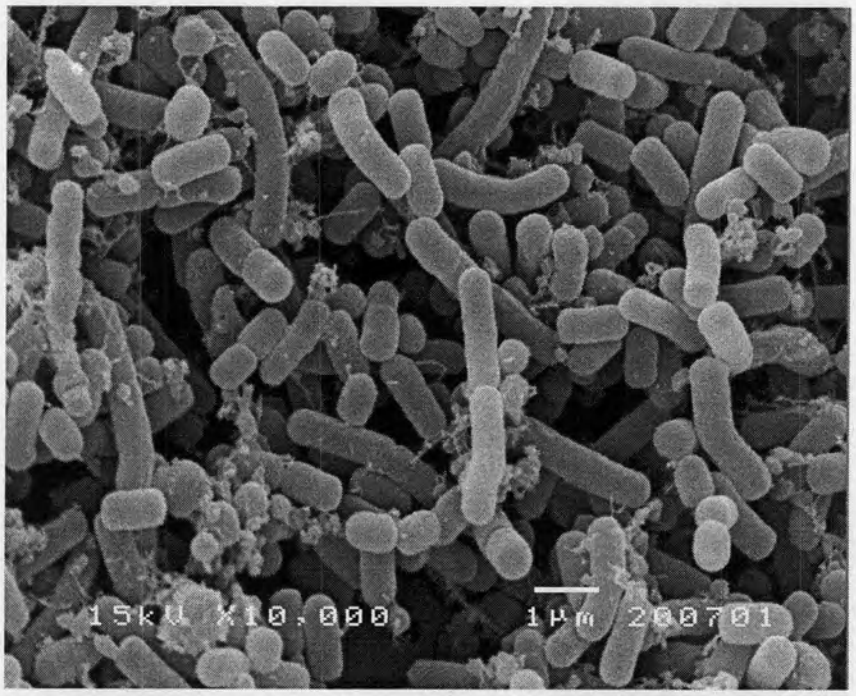


Fig. 4.3. Scanning electron micrograph of L13 grown on GYP agar plate at 30 °C . Bar, 1μm

Table 4.3. Differential characteristics of strain L13 and closely related *Lactobacillus* species. Strains: 1, L13^T; 2, *L. parabrevis* ATCC 53295^T (Data from Vancanneyt *et al.*, 2006); 3, *L. hammesii* CIP 108387^T (Data from Valcheva *et al.*, 2005).

Characteristics	**1**	**2**	**3**
NH_3 from arginine	−	+	−
Growth at 15/45°C	+/-	+/-	+/-
Acid production from:			
L-Arabinose	−	+	+
D-Xylose	+	+	+
Methyl beta-xyloside	−	+	+
Mannose	−	−	+
Mannitol	−	−	+
N-acetylglucosamine	w	+	+
Aesuculin	−	−	+
Cellobiose	−	−	+
Melibiose	−	−	−
Trehalose	−	−	+
Raffinose	−	−	−
Gluconate	w	+	ND
2-keto gluconate	w	−	ND
D-arabitol	−	+	ND
D-glucose	w	+	+
D-fructose	w	+	+
Maltose	−	+	+
Ribose	+	+	ND
Peptidoglycan type	L-Lys-D-Asp	L-Lys-D-Asp	L-Lys-D-Asp
G+C content (mol %)	46	49	52.6

+, Positive; −, negative; w: weakly positive; ND, not detemined; L-Lys-D-Asp, L-lysine-D-aspartic acid

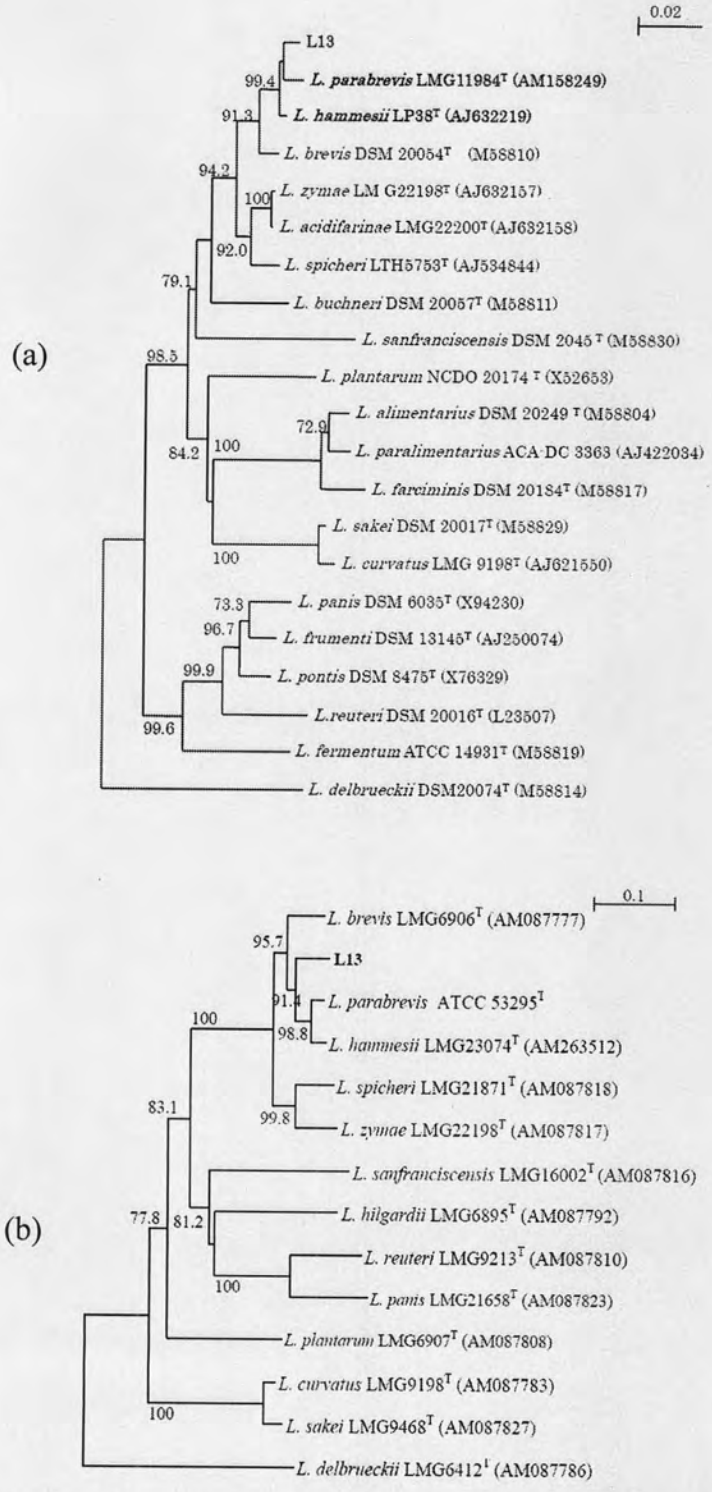


Fig. 4.4. Phylogenetic tree derived from sequence analysis of 16S rRNA gene (a) and *rpoA* gene (b) shown the position of *Lactobacillus sp. nov.* L13 among selected lactobacilli. The tree was generated by the neighbor-joining method. Bootstrap values based on 1000 replications are given at nodes. Bar (a) 2 substitutions and (b) 2 substitutions per 100 nucleotide positions.

4.2.1.2 GABA producing isolates *L. brevis* LSF 8-13, *L. plantarum* SR 11-2, SEA 62-2, and FS 73-1

The selected GABA producing isolates *L. brevis* LSF 8-13, *L. plantarum* SR11-2, SEA 62-2, and FS 73-1 were characterized as previously shown in Table 4.1. 16S rRNA genes were amplified and analyzed as shown in Fig. 4.5.

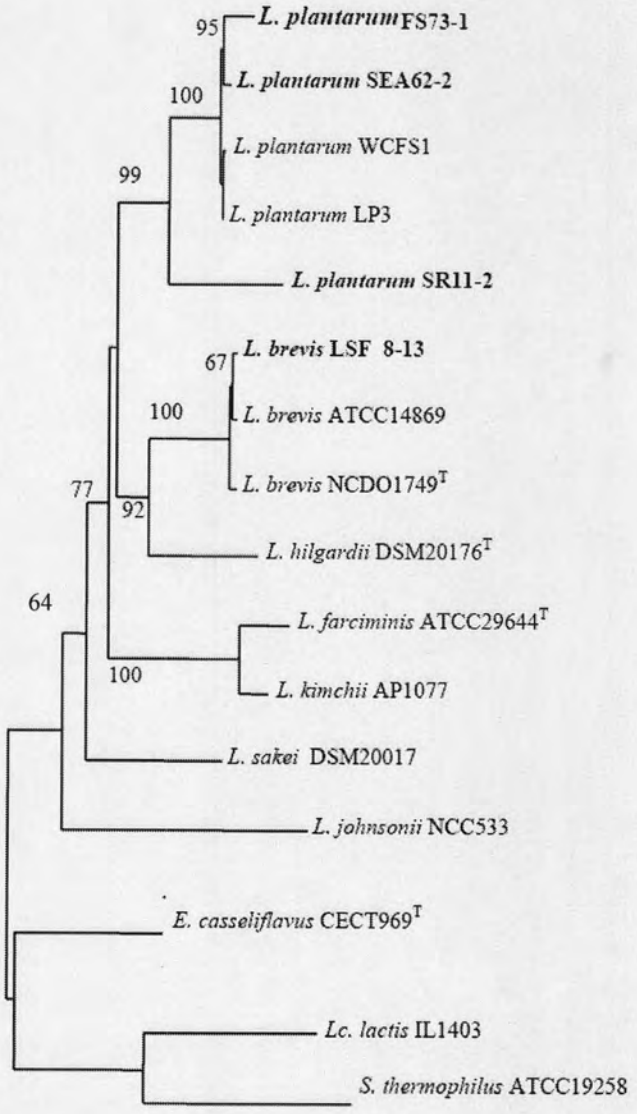


Fig. 4.5. Phylogenetic tree derived from 16S rRNA gene sequence analysis showing the position of selected GABA producing isolates *L. brevis* LSF 8-13, *L. plantarum* SR 11-2, SEA 62-2, and FS 73-1 among selected lactobacilli. The tree was generated by the neighbor-joining method. Bootstrap values based on 1000 replications are given at nodes. Bar, 2 substitutions per 100 nucleotide positions.

4.2.2 Amylase producing strains

4.2.2.1 The novel isolate of *Enterococcus camelliae* sp. nov. FP15-1^T, isolated from fermented tea leaves in Thailand

The FP15-1 was isolated from *Miang*, a fermented tea leaves. Cells were Gram-positive, non-motile, non-spore-forming cocci which arranged in pairs and in chains. Cells were spherical or ovoid, 0.5-1 μm in diameter. Colonies on GYP agar plate were circular, raise or low convex with entire margins and nonpigmented. The strain did not hydrolyze arginine, shown slightly hemolysis on horse blood agar and unable to grow in litmus milk medium. Growth occurred at pH 5.0 to 9.6 and at 15°C to 45°C, and in 2 to 6% NaCl. The strain was negative for catalase, hydrolysis of gelatin, reduction of nitrate, production of gas from glucose, facultatively anaerobic and produced glucose fermentatively. Acid was produced aerobically from D-glucose, D-fructose, D-Cellobiose, esculin, D-mannose, and D-ribose but failed to produced acid from D-amygdalin, L-arabinose, D-galactose, gluconate, glycerol, lactose, D-melibiose, D-melezitose, D-sorbitol, D-xylose, α-methyglucoside, raffinose, L-rhamnose, and inulin. Riboflavin, niacin and calcium-pantothenate were required for growth. DMK-7 was the major menaquinone. Straight-chain fatty acid of $C_{18:1}$ was a dominant composition. The DNA G+C content of type strain is 37.8 mol%.

The almost complete 16S rDNA gene sequence (1490 bp) of FP 15-1^T had the highest similarity to 16S rRNA gene sequence of enterococci. Pairwise sequence alignments indicated that the closest relatives of strain FP 15-1^T were *Enterococcus italicus* (99.2%), *Enterococcus saccharolyticus* (98.3%), *Enterococcus sulfureus* (98.1%) and *Enterococcus casseliflavus* (97.0%). Lower sequence similarities (<97%) were found with other described species of the genus *Enterococcus.* The 16S rRNA gene and *rpoA* gene based phylogenetic tree of strain FP 15-1^T with other enterococci are shown in Fig. 4.6 and 4.7, respectively. Both trees clearly revealed distinct position of the representative strain FP 15-1^T from the nearest neighbor *E. italicus.* The *rpoA* gene sequence of the strain FP15-1^T showed 93.8, 88.3 and 88.1% similar to *E. italicus*, *E. sulfureus* and *E. saccharolyticus*, respectively. Therefore, *rpoA* gene confirmed that the strain FP 15-1 is a separate species with more discriminatory than 16S rRNA. DNA sequence similarity matrixes of 16S rDNA and *rpoA* gene were shown in appendixIII. Hybridization level of 21.6% was found between the type strain of *E. italicus* and the strain FP 15-1^T; this clearly indicated and confirmed that the strain FP15-1^T should be proposed as a novel species of the genus

Enterococcus. The characteristics differentiating the novel species from its other closest enterococci are shown in Table 4.4

Therefore, this strain represent a novel species of the genus *Enterococcus,* for which the name *Enterococcus camelliae* sp. nov. (*ca. mel. li'. ae* N. L. gen. n. *camelliae* of *Camellia*, fermented tea (*Camellia sinensis*) leaves, a source of the strain isolated) is proposed. The type strain is FP 15-1 ^{T} (KCTC 13133^{T}=NBRC 101868^{T} =NRIC 0105^{T} =TISTR 932^{T} =PCU 277 ^{T}).

Table 4.4. Differential characteristics of FP 15-1^{T} and related *Enterococcus* species. Species: 1, FP15-1^{T}; 2, *E. italicus*; 3, *E. saccharolyticus* DSM 20726^{T}; 4, *E. sulfureus* DSM 6905^{T}. Data for 2, 3, and 4 were obtained from Fortina *et al.* (2004). +, Positive; d, variable; –, negative; w, weak; nd, no data.

Characteristics	**1**	**2**	**3**	**4**
Growth in 6.5% NaCl	–	–	+	+
Growth at 10 °C	–	w	+	+
Acid Production from:				
Galactose	–	+	nd	+[a]
Lactose	–	+	nd	+[a]
L-Arabinose	–	–	–	–
Melezitose	–	–	+	+
Melibiose	–	–	+	+
Raffinose	–	–	+	+
Ribose	–	–	+	+
Sorbitol	–	d	+	–
DNA G + C content (mol%)	37.8	39.9-41.1	37.2	38.4

[a] data were taken from Manero & Blanch.(1999) based on biochemical characteristics of *E. sulfureus* ATCC 49903^{T}

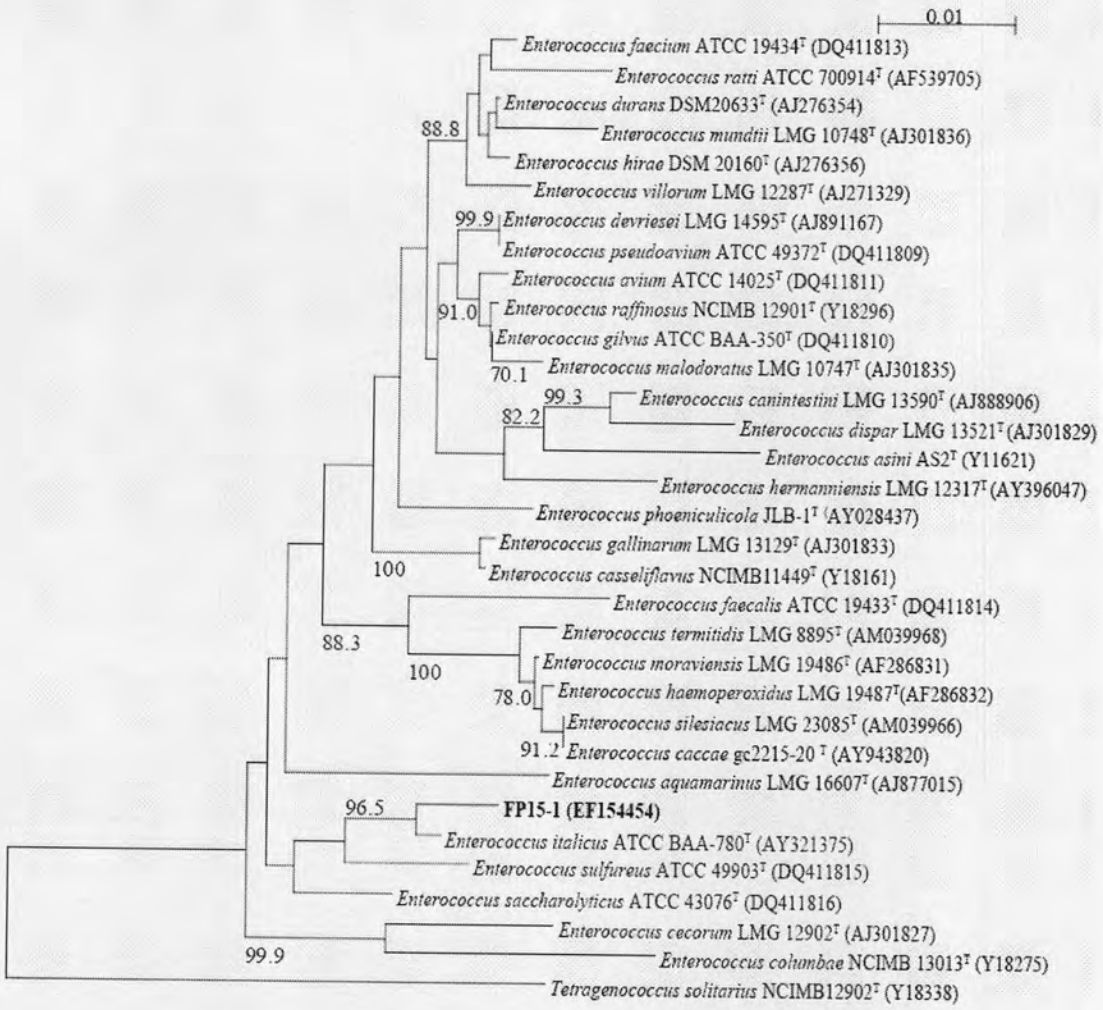


Fig. 4.6. Phylogenetic tree based on 16S rRNA gene sequences, showing the relationships between strain FP15-1^T and related bacterial species. The branching pattern was generated by the neighbor-joining method. Based on 1000 replications, bootstrap percentages less than 50 % are shown. Bar, 1 substitutions per 100 nucleotide positions.

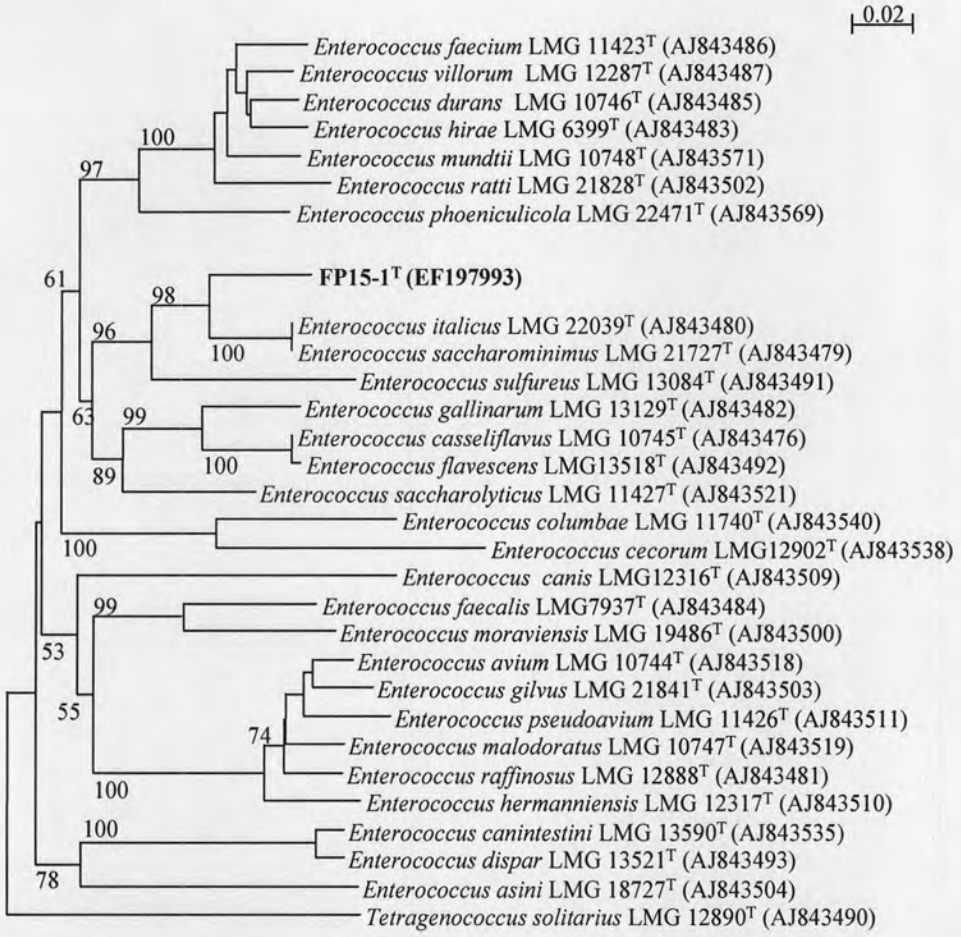


Fig. 4.7. Phylogenetic tree based on *rpoA* gene sequences, showing the relationships between strain FP15-1^T and related bacterial species. The branching pattern was generated by the neighbor-joining method. Based on 1000 replications, bootstrap percentages less than 50 % are shown. Bar, 2 substitutions per 100 nucleotide positions.

4.2.2.1 Amylase producing isolates

The selected starch hydrolyzing strains were the rods, SB2-3 and U3-1 and the coccal, N12-9 and N2-1A. The isolates were characterized as previously shown in Table 4.1 and 4.2. On the basis of the phenotypic characteristics and DNA-DNA similarity studied, the strains SB3-2 and U3-1 belonged to genus *Lactobacillus,* and the strains N12-9 and N2-1A were included in genus *Enterococcus*. Strains SB2-3 and U3-1 were identified as *Lactobacillus plantarum.* The 16S rDNA sequencing and phylogenetic analysis studied revealed that the strain N12-9 was *E. gallinarum* and N2-1A was *Enterococcus* sp. nov. On the acid production, SB2-3 and U3-1 produced high amount of acid in medium containing 5-7% starch while N2-1A did in the 2-7% starch after 5 days incubation. SB2-3 produced high acid at pH 5 after incubated for 3-5 days while U3-1 did after incubated for 3-4 days and N2-1A did at pH 6.8 for 5 days. In addition, 1% yeast extract was suitable for SB2-3 and U3-1 and N2-1A while 0.5% peptone was for SB2-3 and 1% peptone for U3-1 and N2-1A, in their acid production. DNA-DNA hybridization results of the isolates with the closely related type strains were shown in Table 4.5. 16S rRNA genes of N2-1A and N12-9 were amplified and analyzed as showed in Fig. 4.8.

Table 4.5. DNA-DNA similarity of starch hydrolyzing strains

		% Similarity with labeled strains		
Species	**Strain**		**NRIC 1067T**	**NRIC 1069T**
	SB2-3		**102.3**	**35.9**
	U3-1		**101.3**	**27.0**
***L. plantarum* NRIC 1067T**			**100.0**	**24.1**
***L. pentosus* NRIC 1069T**			**25.0**	**100.0**
		NRCI 1145T	**TISTR 379T**	**TISTR 943T**
	N2-1A	**18.3**	**10.5**	**17.5**
	N12-9	**19.6**	**9.7**	**11.2**
***E. faecium* NRIC 1145T**		**100.0**	**ND**	**14.6**
***E. faecalis* TISTR 379T**		**ND**	**100.0**	**ND**
***E. hirae* TISTR 943T**		**15.0**	**ND**	**100.0**

TISTR, Thailand Institute of Scientific and Tecnological Research, Bangkok, Thailand

NRIC, NODAI Research Institute Culture Collection, Tokyo University of Agriculture, Tokyo, Japan

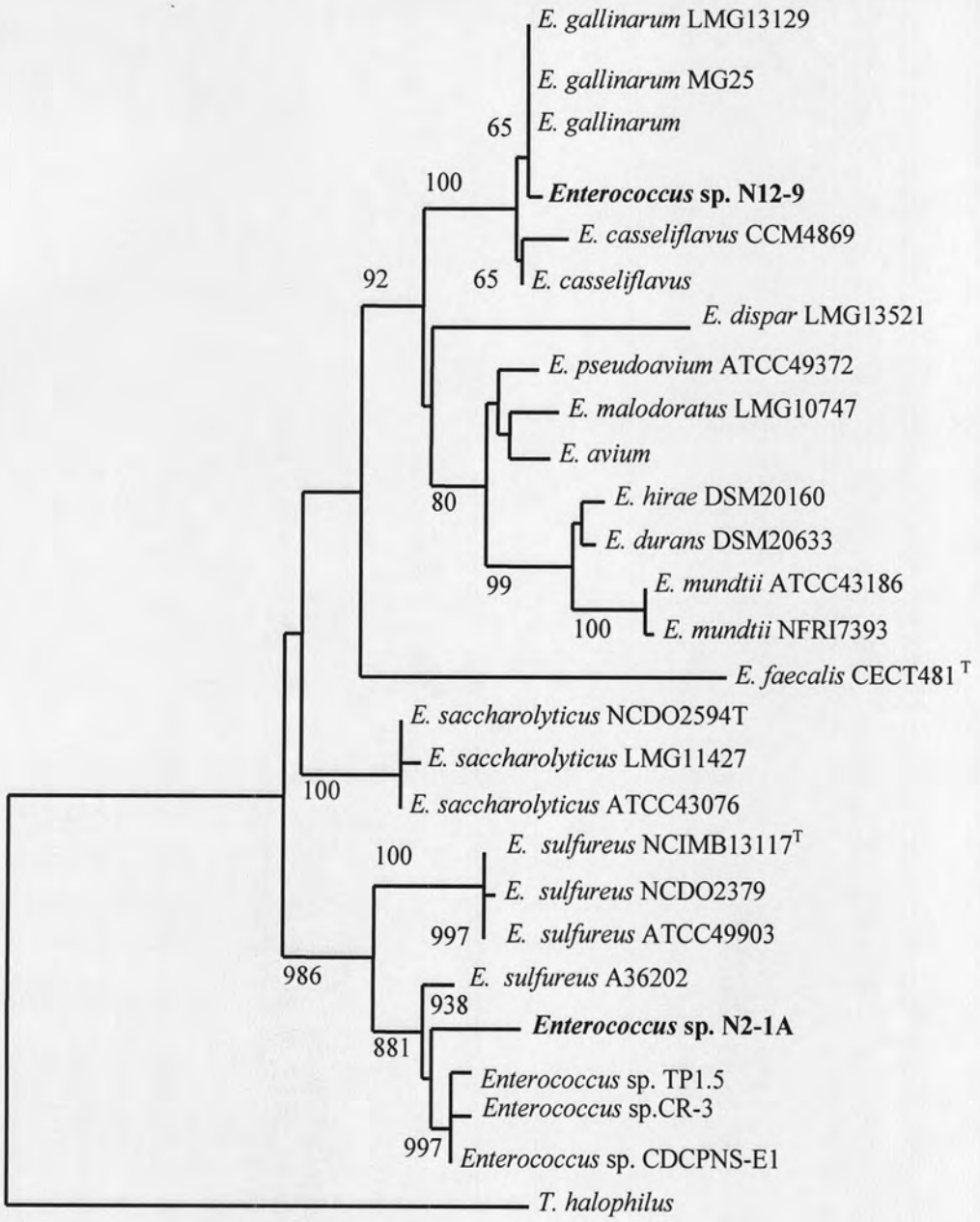


Fig. 4.8. Phylogenetic tree derived from 16S rRNA gene sequence analysis showing the position of selected amylase producing isolates N2-1A and N12-9 among selected enterococci. The tree was generated by the neighbor-joining method. Bootstrap values based on 1000 replications are given at nodes.

4.2.3 Proteinase producing strains

The selected proteinase producing isolates were selected by ability of hydrolyzing of skim milk on agar plate. The isolates of SCR 1P and SMC 1 were rod and cocci respectively. Characteristics are previously shown in Table 4.1 and 4.2. 16S rRNA genes of the isolates were amplified and analyzed as shown in Fig. 4.9. The position of the strain SCR 1P was closely related to *Lactobacillus sakei* and the strain SMC 1 was closely related to *Enterococcus faecalis*

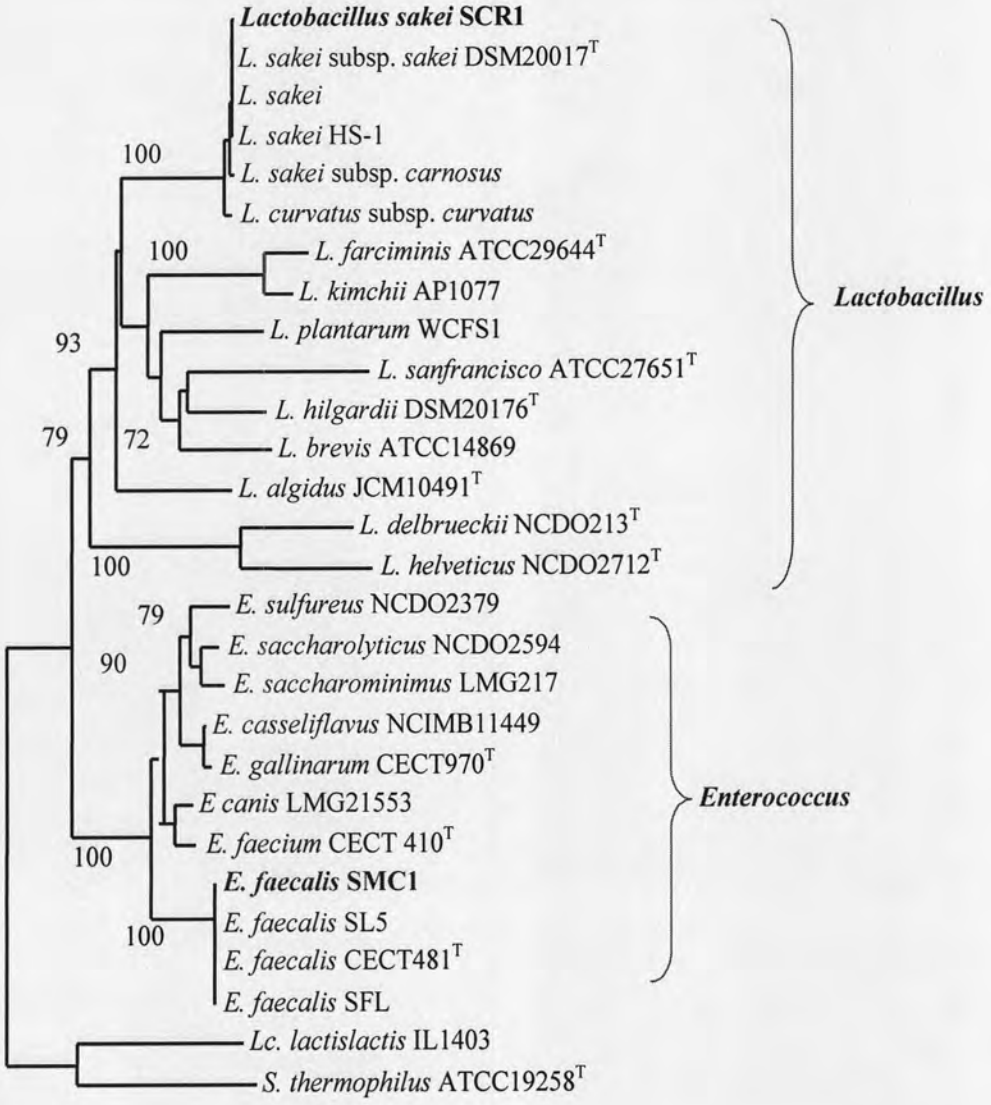


Fig. 4.9. Phylogenetic tree derived from 16S rRNA gene sequence analysis showing the position of selected amylase producing isolates SCR 1P and SMC 1 among selected lactobacilli and enterococci. The tree was generated by the neighbor-joining method. Bootstrap values based on 1000 replications are given at nodes.

4.3. Identification of Glutamate decarboxylase gene (*gadB*)

GABA-producing lactic acid bacteria isolates were examined for a glutamate decarboxylase encoding gene (*gad B*) using rapid PCR-based method. Based on search DNA data bank, five *gad B* genes of lactic acid bacteria were obtained. The PCR primers were designed from highly conserved regions of multiple alignments of the *gad B* nucleotide sequences (Fig. 4.29, including isolated genes) and presumed *gad B* encoding gene of *Lactobacillus plantarum* WCFS1 complete genome sequence. The annealing poison of *gadB* primers on L. plantarum *gadB* was shown on Fig. 4.10. Four representative strains of *L. brevis* LSF8-13 (isolated from fish fermented food, Pla-som), *L. farciminis* FS 73-1 (isolated from fish fermented food, Pla-ra), *L. plantarum* SCR 11-2 and SEA62-2 (isolated from soy sauce and pine apple, respectively) were found to have the expected 1.4 kb *gad B* gene (Fig.4.11) by using Fw &Rv GADP primers (Table 3.4). The amplified fragments were confirmed as *gad B* gene by nested PCR using inner primers of conserved regions (Fig. 4.12).

```
>L.plantarum WCSF1 gadB gi|28272563:31469-32878

Fw GADP ──────►
ATGGCAATGT TATACGGTAA ACACAATCAT GAAGCTGAAG AATACTTGGA ACCAGTCTTT GGTGCGCCTT CTGAACAACA

TGATCTTCCT AAGTATCGGT TACCAAAGCA TTCATTATCC CCTCGAGAAG CCGATCGCTT AGTTCGTGAT GAATTATTAG
                                                          Fw GAD1 ──────►
ATGAAGGCAA TTCACGACTG AACCTGGCAA CTTTTTGTCA GACCTATATG GAACCCGAAG CCGTTGAATT GATGAAGGAT

ACGCTGGCTA AGAATGCCAT CGACAAATCT GAGTACCCCC GCACGGCCGA GATTGAAAAT CGGTGTGTGA ACATTATTGC

CAATCTGTGG CACGCACCTG ATGACGAACA CTTTACGGGT ACCTCTACGA TTGGCTCCTC TGAAGCTTGT ATGTTAGGCG

GTTTAGCAAT GAAATTCGCC TGGCGTAAAC GCGCTCAAGC GGCAGGTTTA GATCTGAATG CCCATCGACC TAACCTCGTT
                  Fw GAD2 ──────►
ATTTCGGCTG GCTATCAAGT TTGCTGGGAA AAGTTTTGTG TCTACTGGGA CGTTGACATG CACGTGGTCC CAATGGATGA

GCAACACATG GCCCTTGACG TTAACCACGT CTTAGACTAC GTGGACGAAT ACACAATTGG TATCGTCGGT ATCATGGGCA

TCACTTATAC CGGTCAATAT GACGACCTAG CCGCACTCGA TAAGGTCGTT ACTCACTACA ATCATCAGCA TCCCAAATTA
                                                                    ◄────── Rv GAD3
CCAGTCTACA TTCACGTCGA CGCAGCGTCA GGTGGCTTCT ATACCCCATT TATTGAGCCG CAACTCATCT GGGACTTCCG

GTTGGCTAAC GTCGTTTCGA TCAACGCCTC CGGGCACAAG TACGGTTTAG TTTATCCCGG GGTCGGCTGG GTCGTTTGGC

GTGATCGTCA GTTTTTACCG CCAGAATTAG TCTTCAAAGT TAGTTATTTA GGTGGGGAGT TGCCGACAAT GGCGATCAAC

TTCTCACATA GTGCAGCCCA GCTCATTGGA CAATACTATA ATTTCATTCG CTTTGGTATG GACGGTTACC GCGAGATTCA

AACAAAGACT CACGATGTTG CCCGCTACCT GGCAGCCGCT CTGGATAAAG TTGGTGAGTT TAAGATGATC AATAACGGAC
                                                    ◄────── Rv GAD4
ACCAACTCCC CCTGATTTGT TACCAACTAG CCTCGCGCGA AGATCGTGAA TGGACCCTTT ATGATTTATC GGATCGCCTA

TTAATGAACG GTTGGCAAGT ACCAACGTAT CCTTTACCTG CTAATCTGGA ACAACAAGTC ATCCAACGAA TCGTCGTTCG

GGCTGACTTT GGCATGAATA TGGCCCACGA TTTCATGGAT GACCTGACCA AGGCTGTCCA TGACTTAAAC CACGCCCACA
                                  ◄────── Rv GADP
TTGTCTATCA TCATGACGCG GCACCTAAGA AATACGGATT CACACACTGA
```

Fig. 4.10. Map of full length GAD plantarum and inner primer of conserved region on *L.plantarum* WCFS1 predicted *gadB.*

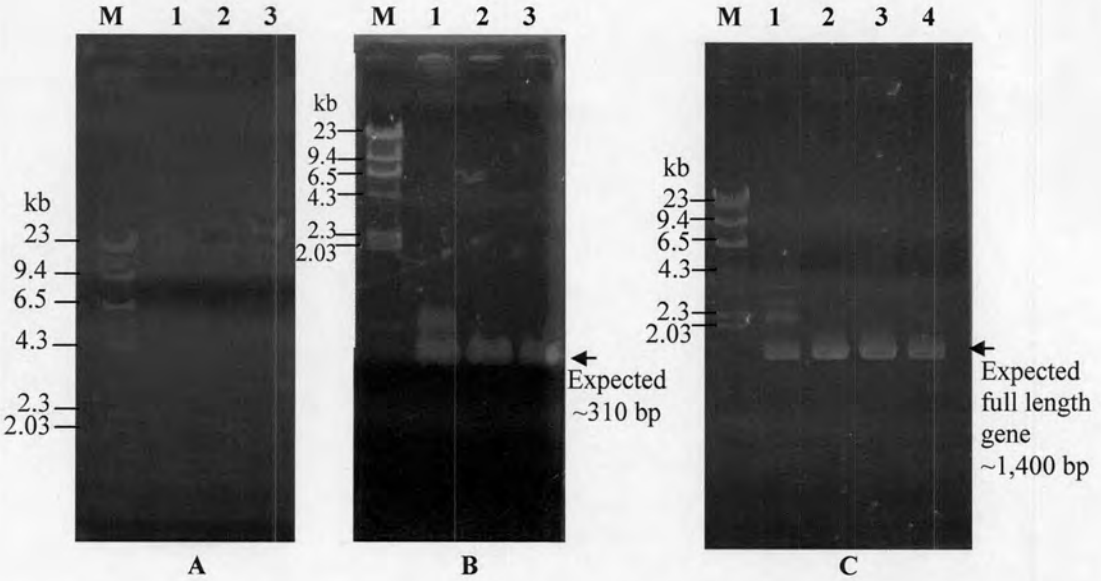


FIG. 4.11. PCR amplification of *gadB* gene from GABA producing isolated strains. A. Chromosomal DNA extracted using labiase, B. PCR products using GAD2 and GAD3primers (Annealing at 47°C) and C. PCR products using GAD plantarum CDS primers(Annealing at 43°C) Lanes: M, lamda/*Hin*dIII marker; 1, LSF8-13 (*L. brevis*); 2. FS73-1(*L. farciminis*) ; 3, SEA62-2 (*L. plantarum*); 4, SR11-2 (*L.* plantarum.)

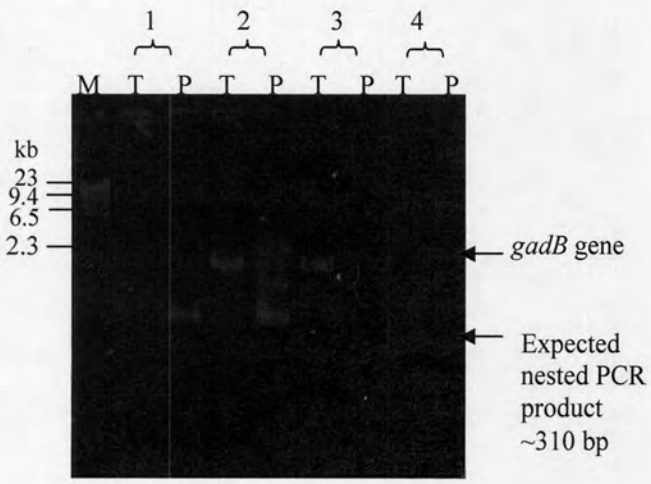


FIG. 4.12 . Confirmation of the full length amplified *gadB* of the representative strains by Nested PCR amplification using inner primers GAD2-3. Lanes: 1, LSF8-13; 2, FS73-1; 3, SEA62-2; 4, SR11-2; M, lamda DNA/*Hin*dIII marker; T, Full length amplified fragments used as template; and P, PCR product.

Interestingly, the high GABA producing strain *Lactobacillus sp.* L13 newly isolated from Japanese pickle was investigated and found to contain the different amplified PCR products pattern of *gadB* gene compared with the other isolates (Fig. 4.13). All of four representative GABA producing lactobacilli LSF8-13, FS7311, SEA62-2 and SR11-2 were found to have *gadB* gene (approximately 1.4 kb amplified fragment) proven by highly conserved regions within the gene (Fig. 4.12). In case of the high GABA producing strain LSF8-13 which was identified as *L. brevis* showed more than 4 amplified fragments using full length primers (Fig. 4.11, C, lane 1) even increasing annealing temperature. It may cause by repeat gene in genome. The expected band (~1.4 kb) were purified and confirmed as *gadB* encoding genes by nested PCR using the inner primes of conserved region.

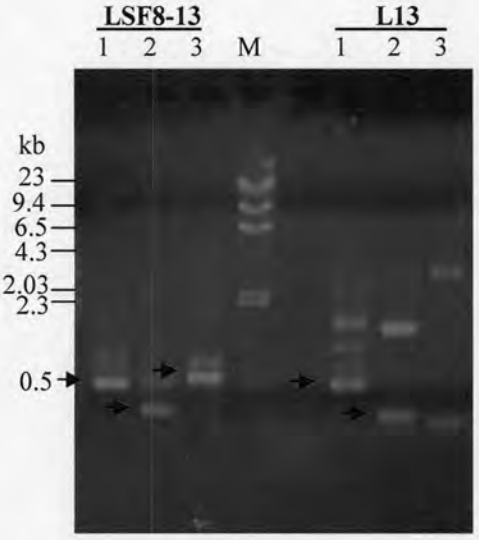


FIG. 4.13. PCR products of LSF8-13 and L13 genomic DNA amplified by inner primers of conserved regions; 1. GAD1-3; 2. GAD2-3; and 3. GAD1- 4. M: lamda DNA/*Hin*dIII marker. The expected PCR amplification products by GAD1-3, GAD2-3 and GAD1- 4 are approximate 615, 310 and 700 bp respectively. Arrowed bands are expected products.

Expected PCR product size using different inner primer sets Forward GAD1-Reverse GAD4 (GAD1-4), Forward GAD2-Reverse GAD4 (GAD2-4), Forward GAD1-Reverse GAD3 (GAD1-3) and Forward GAD2-Reverse GAD3 (GAD2-3) are 1010, 700, 620 and 310 bp, respectively. The 1.4 kb amplified product containing expected conserved region was cloned and sequenced. The nucleotide sequence

analysis of the full length amplified *Lactobacillus brevis* LSF8-13 *gadB* consisted of an open reading frame (ORF) of 1410 bases and the deduced amino acid of 469 residues showed 99 % identities to *L. plantarum* WCFS1 GAD and 49% to that of *L. brevis* IFO 12005 GAD and *L. brevis* ATCC367 GAD. The nucleotide and deduced amino acid sequences of the full length *Lactobacillus brevis* LSF8-13 *gadB* were shown in Fig. 4.14. PCR amplification of the high GABA producing *Lactobacillus* sp. L13 newly isolated form Japanese pickle using full length GAD *plantarum* primers and/or GAD *brevis* primers revealed non specific PCR products (data not shown). Using inner primers of conserved regions (GAD1-3 and GAD2- 3) showed expected bands while unexpected band was found by GAD1- 4 primers (Fig. 4.13).

The sequence analysis of the purified products of the 310 bp and 620 bp obtained from the GAD2-3 and GAD1-3 show conserved nucleotide sequence of *gadB* in *Lactobacillus* sp. L13. Phylogenetic analysis of the sequence of *gadB* genes (~620 bp) of representative isolates comparing with known *gadB* was shown in Fig. 4.14. The full length GAD *plantarum* CDS and *bre* GAD primers were used in combination of the different species: *bre* GAD forward – Rev GAD *plantarum* CDS and Forward GAD *plantarum* CDS – *bre* GAD Reverse were used to amplification of the full length *gadB* of *Lactobacillus* sp. L13. No amplification products were gain.

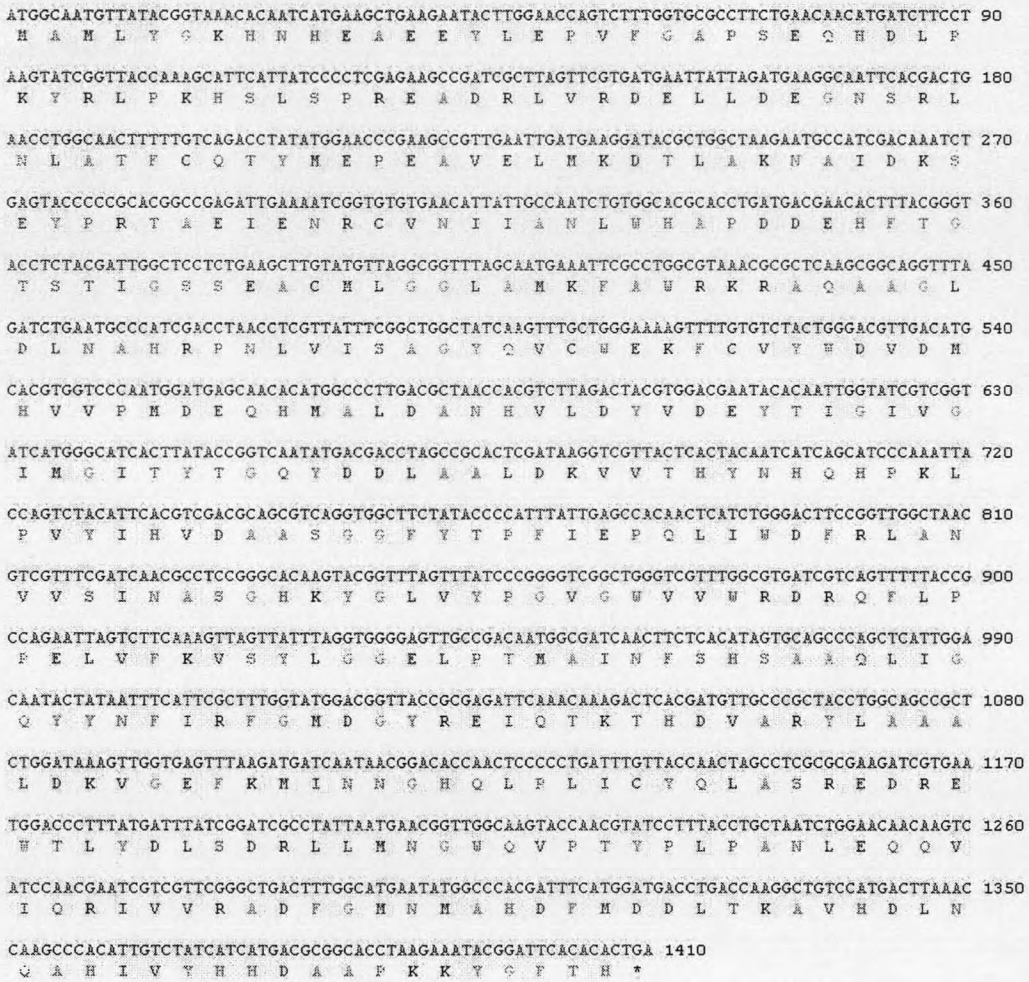


Fig. 4.14. The nucleotide and deduced amino acid sequences of the full length *gadB* of *Lactobacillus brevis* LSF8-13

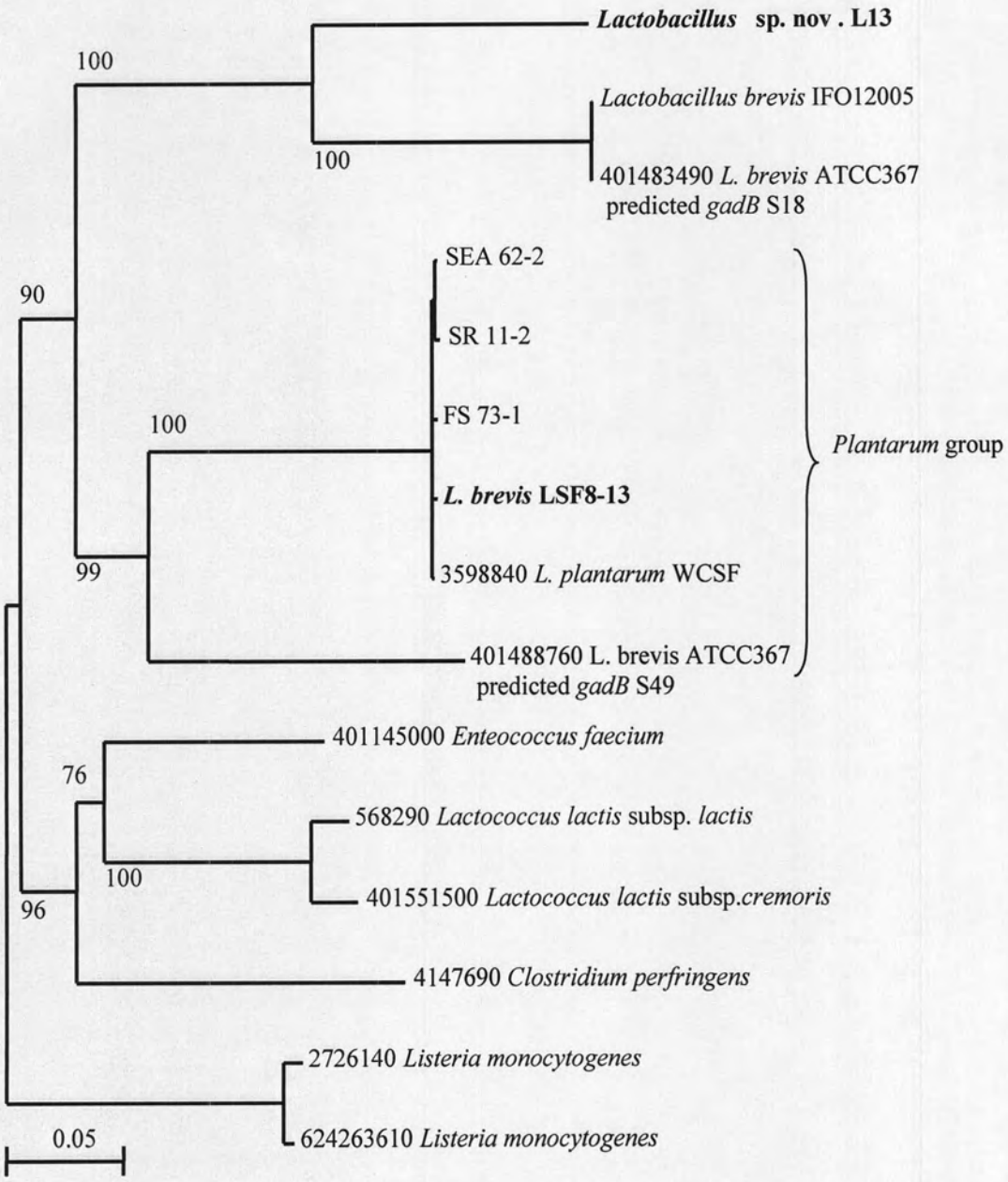


Fig. 4.15. Phylogenetic analysis of the conserved regions (~615 bp) of glutamate decarboxy lase genes (*gadB*) of representative isolates comparing with known *gad*B.

4.3.1 TAIL-PCR for identification of the novel glutamate decarboxylase gene from the novel isolate of *Lactobacillus* sp. L13

The inner primers of conserved regions in combination with the full length GAD *plantarum* CDS, *bre* GAD primers and inner primers of conserved regions (Table X) were investigated. Approximately 810 bp and 1,800 bp PCR product was obtained from *bre* GAD Fw and Reverse GAD3 (Fig. 4.16). The bands were purified, cloned into pGEM-T easy and sequenced. The sequence analysis of the ~810 bp band showed conserved among *gadB* .

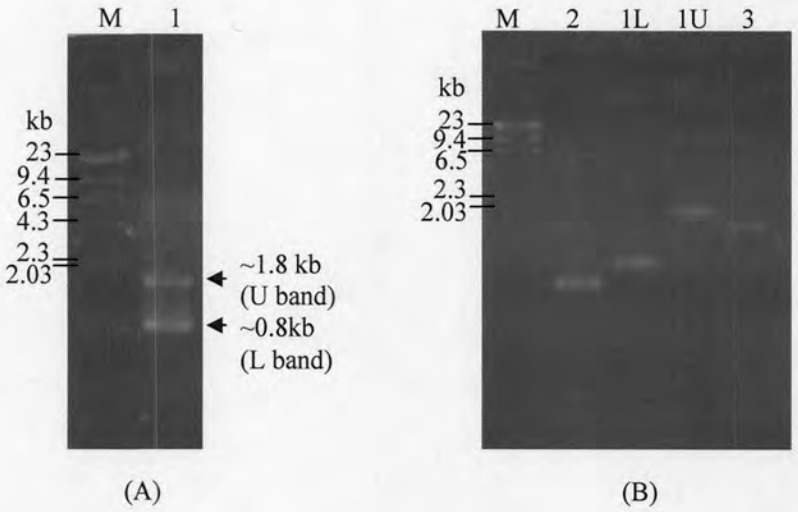
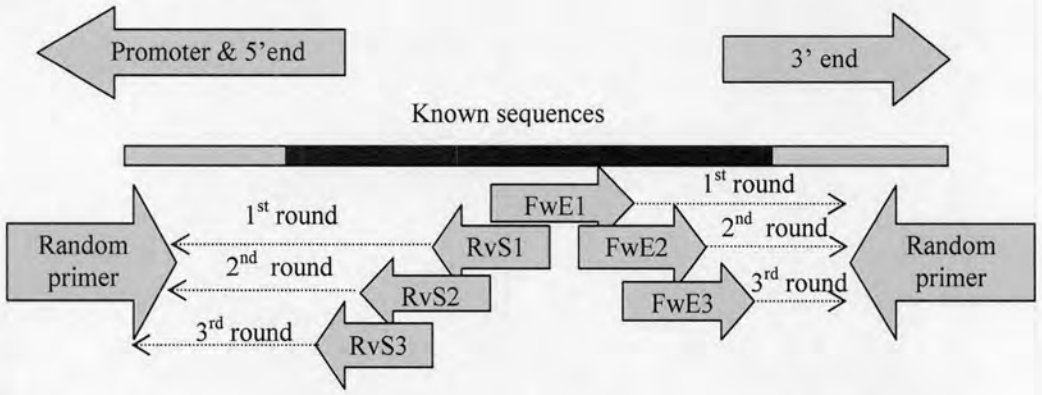


Fig. 4.16. Amplification products of *Lactobacillus* sp.nov.L13 using species combination primers of *bre* GAD Fw and Reverse GAD3 (lane 1A). M: lamda DNA/*Hin*dIII marker. The arrowed bands were purified as showed in (B) lane 1L (~0.8 kb) and 1U (~1.8 kb). Lane 2: purified band of L13 GAD1-3 which will be DIG-labeled and used as probe for hybridization, Lane 3: purified band of full length *gadB* of LSF8-13.

The specific PCR primers for TAIL-PCR were designed from the 810 bp known sequence to get the 3'end and 5' end including the promoter and ribosome binding site. TAIL-PCR were performed using different random primers in combination with specific primers. The strategy to get the whole gene by using TAIL-PCR method was shown in Scheme. 4.1. PCR primers used for TAIL-PCR methods were showed in Table. 3.6. Purified band of L13 GAD1-3 (Fig. 4.16.(B) land 2) was labeled and used as probe for detection of TAIL-PCR products and copy number of *gadB* gene in L13 genome.



Scheme 4.1. The schematic outline to obtain the whole *gadB* from *Lactobacillus* sp. L13 by using TAIL-PCR method

The 3' end region was successfully amplified by the random primer AD3 and Nini256 in combination with specific forward primers (FwE1, FwE2, FwE2N, FwE3, and FwE3N) subsequently. The expected amplified products decreased in size gradually. The amplified bands were detected by hybridization with the 610 bp probe of L13 GAD1-3 then the detected bands (Fig. 22) were cloned, sequenced and searched for overlapping region with the known sequences. L13_917 and L13_1151 primers (Table. 3.6) were designed from the revealed sequence and used as sequencing and specific primers in combination with the random primers walking at 3' end to obtain and confirm stop codon of the *gadB* gene of *Lactobacillus* sp. L13

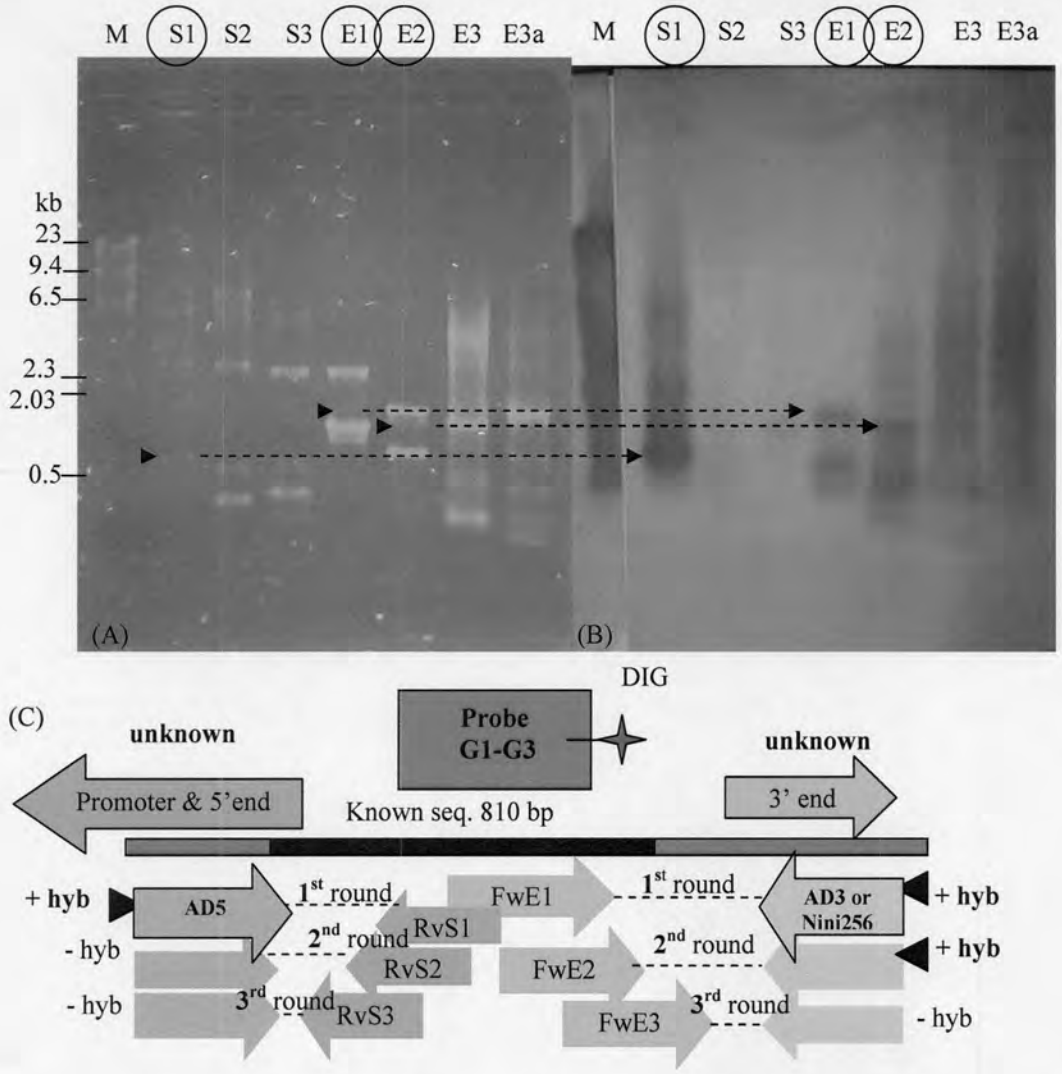


Fig. 4.17. TAIL-PCR amplification products (A) and results of hybridization (B) the arrowed bands showed positives signal of the hybridized bands of TAIL-PCR products of RvS1, E1 and E2. The scheme (C) shows strategy of the detection method. The RvS2, RvE3 and FwE3N products are out of regions to be hybridized by the probe G1-G3.

PCR amplification of full length gadB gene from *Lactobacillus* sp. nov L13 were investigated by reverse primers of L13_Rv1413 (Table 3.6) which was designed from 3' end revealed result of TAIL-PCR in combination with *bre* GAD Fw primer (Table 3.4). The PCR products was a single band with expected size of approximately 1.4 kb (Fig. 4.18) confirmed specificity of the primer used then the band were purified, cloned and sequenced to confirm the 3' end region of the *gadB*. However, the amplified gene may contains mismatch bases at the start codon caused by the different species of *bre* GAD Fw primer. The 5' end of the gene should be identified and confirmed.

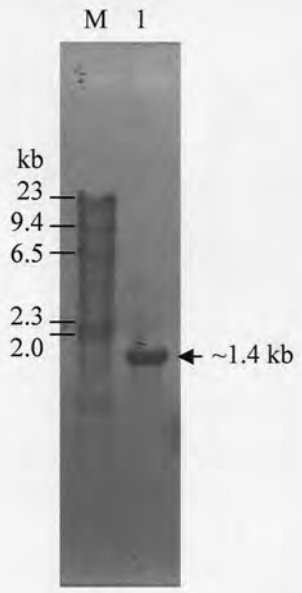


Fig. 4.18. Specific amplified PCR product of *Lactobacillus* sp. nov L13 *gadB* using *bre* GAD Fw and L13_Rv1413 primers

The 5' end region was obtained by the random primer AD5 in combination with specific forward primers (RvS1, RvS2 and RvS3 respectively). The RvL13_155 primer (Table 3.6) was designed from the revealed sequence and used as sequencing and a specific primer in combination with the random primer AD5 walking at 5' end to obtain and confirm start codon and promoter regions of the *gadB* gene of *Lactobacillus* sp. L13. The amplified bands were hybridized with 230 bp DIG-label probe of *bre* GAD Fw- RvS2, cloned, sequenced and searched for overlapping region with the known sequences.

The start codon was found to be ATG. The predicted promoter and ribosome binding site were found by promoter prediction programs, PPP (Prokaryote Promoter Prediction, http://bioinformatics.biol.rug.nl/websoftware/ppp/)and BPROM (Bacterial sigma70 promoter recognition program http://www.softberry.com). Sequences data were aligned. The full length *Lactobacillus* sp. L13 *gadB* consisted of an open reading frame (ORF) of 1437 bases and encoded a protein of 478 amino acid residues.

The deduced amino acid sequenced was searched for the conserved domain compared with others GAD in the conserved domain database. The deduced amino acid sequence shown conserved domain of pyridoxal-5'- phosphate decarboxylase and glutamate decarboxylase at the middle of the gene (Fig. 4.19). The nucleotide and

deduced amino acid sequences of the full length *Lactobacillus* sp. L13 *gadB* including predicted promoter regions were shown in Fig. 4.20.

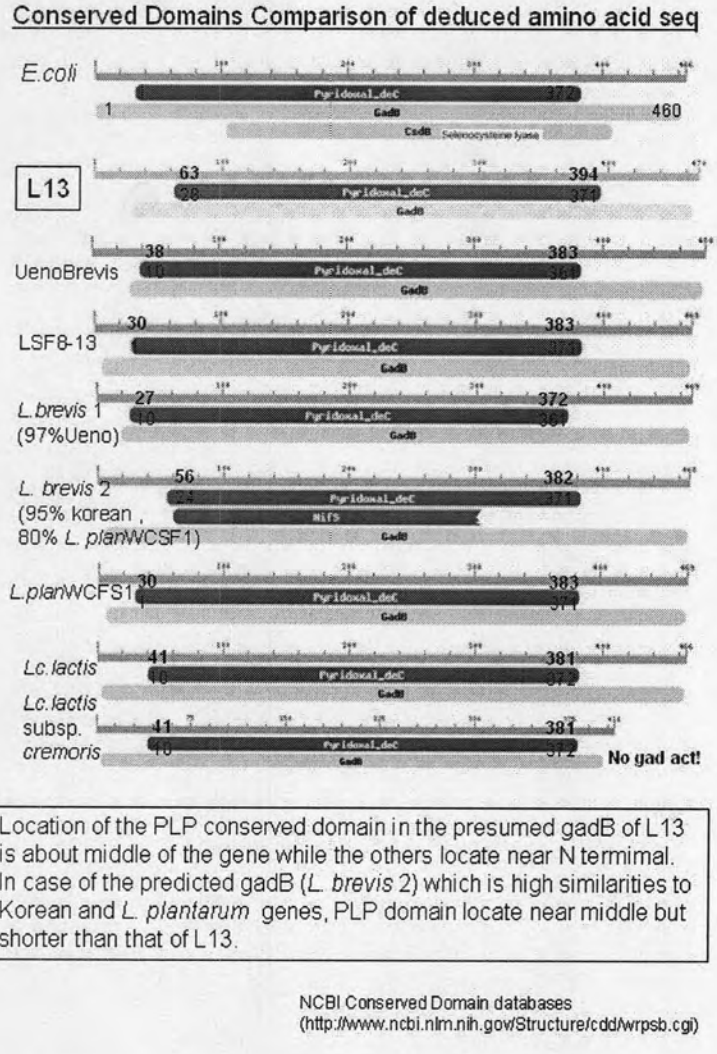


Fig. 4.19. Comparison of conserved domain comparison among glutamate decarboxylases from different species.

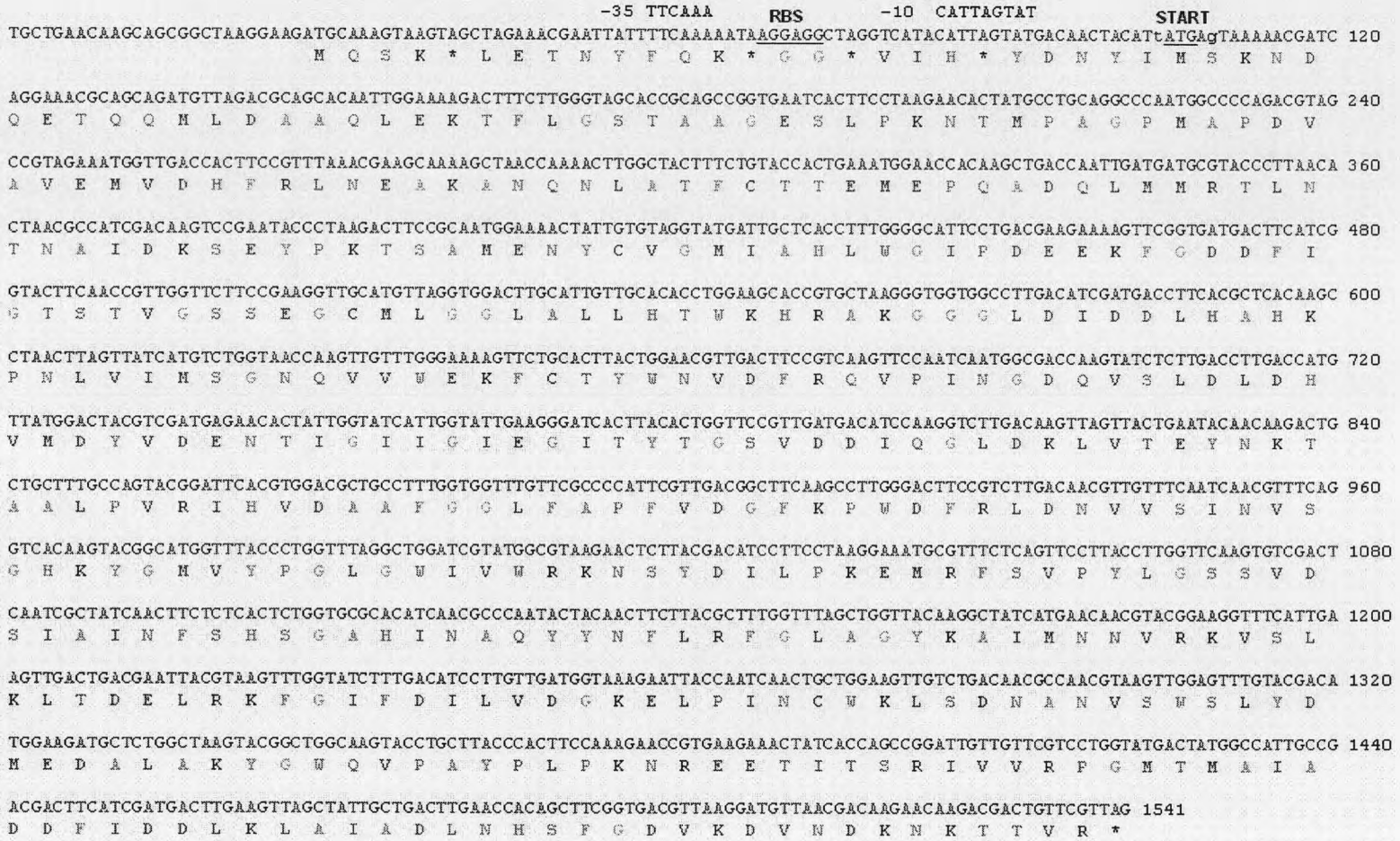


Fig. 4.20. The nucleotide and deduced amino acid sequences of the full length *Lactobacillus* sp. L13

The 1437 bp full length amplified fragment of *Lactobacillus* sp. L13 *gadB* was DIG-labeled and used as a hybridization probe for detection of *gadB* on the genome. *Lactobacillus* sp. L13. Genomic DNA was digested with restriction enzymes and subjected to Southern blotting (Fig. 4.21). A single band was detected with *Bam* HI, *Eco*R I, *Hind* III, and *Pst* I digested. Suggested, there is only one glutamate decarboxylase gene in the genome of *Lactobacillus* sp. L13.

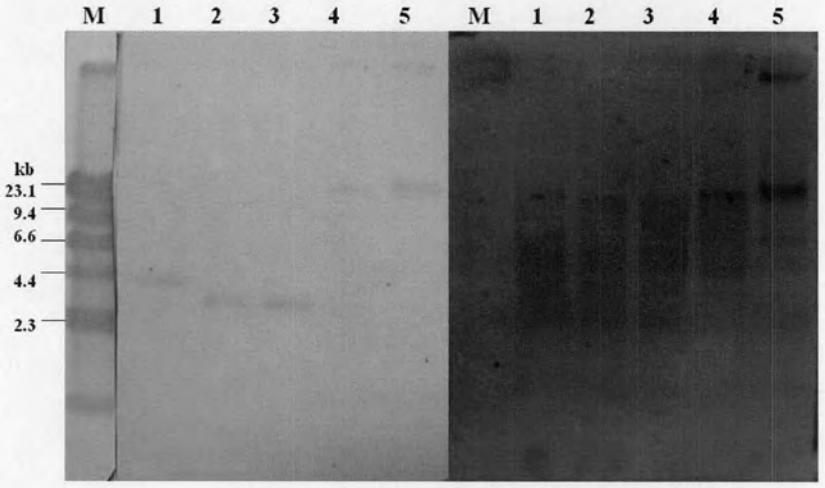


Fig. 4.21. Southern blot analysis of *Lactobacillus* sp. L13. The DNA was digested with *Bam* HI (lane 1), *Eco*R I (lane 2), *Hind*III (lane 3), *Pst* I (lane 4) and Uncut DNA (lane 5). The blot was probed a digoxigenin-labeled 1.4 kb *Lactobacillus* sp. L13 *gadB* amplified fragment.

4.3.2 Restriction analysis of the identified *gadB* of *Lactobacillus* sp. nov. L13 and *L. brevis* LSF 8-13

The revealed sequences of the *gadB* were restriction analysis using Bioedit program version 1.83. The restriction analysis of gadB from *Lactobacillus* sp. nov. L13 and *L. brevis* LSF 8-3 by 6 bases-cutter enzymes were list in Table 4.6. and 4.7, respectively. Enzymes that do not cut the genes will be selected for further experiments of subcloning and construction of expression clone. Enzymes that cut the gene will be used for restriction analysis of the recombinant clones.

Table 4.6. Restriction site of 6 bases-cutter enzymes for *gadB* of *Lactobacillus* sp. nov. L13

Enzyme	Recognition	frequency	Positions
AccI	GT'mk_AC	1	972
AclI	AA'CG_TT	3	558, 834, 849
BsaAI	yAC'GTr	2	760, 1115
BsaJI	C'CnnG_G	4	697, 812, 880, 958
BsiWI	C'GTAC_G	1	1078
BspHI	T'CATG_A	1	1068
BsrI	ACTG_Gn'	3	557, 682, 746
BsrFI	r'CCGG_y	1	80
Cac8I	GCn'nGC	3	111, 115, 1244
ClaI	AT'CG_AT	2	474, 1347
DraI	TTT'AAA	1	165
EaeI	y'GGCC_r	1	1326
FspI	TGC'GCA	1	1008
HincII	GTy'rAC	6	151, 562, 799, 973, 1101, 1408
HpaI	GTT'AAC	1	1408
Hpy8I	GTn'nAC	9	151, 419, 562, 763, 799, 877, 973 1101, 1408
Hpy188III	TC'nn_GA	6	17, 344, 602, 704, 827, 1069
MfeI	C'AATT_G	2	47, 233
MscI	TGG'CCA	1	1328
NlaIV	GGn'nCC	3	126, 219, 682
NspI	r_CATG'y	1	412
PmlI	CAC'GTG	1	760
PstI	C_TGCA'G	1	115
SalI	G'TCGA_C	1	971
SfcI	C'TryA_G	1	111
SnaBI	TAC'GTA	1	1115
StyI	C'CwwG_G	3	697, 812, 958

Table 4.7. Restriction site of 6 bases-cutter enzymes for *gadB* of *L. brevis* LSF 8-13

Enzyme	Recognition	frequency	Positions
AccI	GT'mk_AC	3	522, 726, 738
Acc65I	G'GTAC_C	1	359
AgeI	A'CCGG_T	1	650
ApoI	r'AATT_y	1	413
AseI	AT'TA_AT	1	1202
AvaI	C'yCGr_G	2	123, 857
BanI	G'GyrC_C	2	359, 1381
BclI	T'GATC_A	1	1107
BglII	A'GATC_T	1	451
Bme1580I	G_kGCm'C	1	837
BmgBI	CAC'GTC	2	589, 736
BsaAI	yAC'GTr	2	544, 601
BsaJI	C'CnnG_G	3	857, 858, 1329
BsaWI	w'CCGG_w	2	650, 798
BseYI	C'CCAG_C	3	504, 867, 978
BsiEI	CG_ry'CG	2	136, 288
Bsp1286I	G_dGCh'C	1	837
BspHI	T'CATG_A	2	28, 1372
BsrI	ACTG_Gn'	3	53, 530, 722
BsrBI	CCG'CTC	1	1079
BsrFI	r'CCGG_y	1	650
BstYI	r'GATC_y	1	451
Cac8I	GCn'nGC	4	246, 333, 490, 1063
EaeI	y'GGCC_r	1	285
EagI	C'GGCC_G	1	285
HincII	GTy'rAC	2	535, 739
HindIII	A'AGCT_T	1	384
Hpy8I	GTn'nAC	8	20, 310, 428, 523, 535, 604, 727 739
Hpy188III	TC'nn_GA	9	29, 125, 146, 174, 1052, 1082 1166, 1247, 1373
KpnI	G GTAC'C	1	363
MfeI	C'AATT_G	1	615
NlaIV	GGn'nCC	7	51, 213, 361, 376, 549, 1175, 1383
NspI	r_CATG'y	1	541
PmlI	CAC'GTG	1	544
PvuI	CG_AT'CG	1	136
SalI	G'TCGA_C	1	737
SmaI	CCC'GGG	1	859
SmlI	C'TyrA_G	2	123, 435
StyI	C'CwwG_G	1	1329
XhoI	C'TCGA_G	1	123
XmaI	C'CCGG_G	1	857

Bgl II cannot be used for construction of expression clone of *gadB* of *L. brevis* LSF 8-13 since this enzyme cut the gene so *Sph* I and *Bam* HI were selected to be used as adapter restriction size for subcloning.

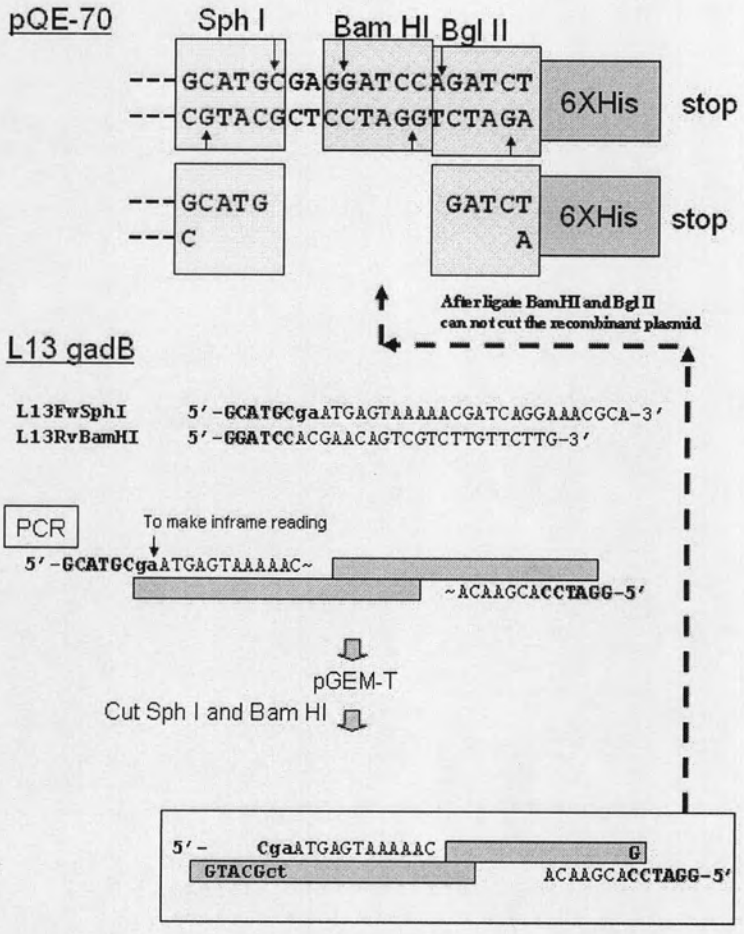
4.4. Construction of vector for expression of *gadB* from *Lactobacillus* sp. nov. L13 and *Lactobacillus brevis* LSF8-13

The *L. brevis* LSF8-13 and *Lactobacillus* sp. nov. L13 *gadB* open reading frame was re-amplified for restriction modification of 5' and 3' end adding of *Sph* I and *Bam* HI restriction site (Table.4.8, LSF8-13Fw*Sph*I & LSE8-13Rv*Bam*HI and L13Fw*Sph*I & L13Rv*Bam*HI). The synthetic primers with restriction site were also designed to control the inserted gene to be in frame by adding nucleotides and the stop codon of the genes were omitted. The modified *gadB* fragments were cloned into pGEMT-Easy (Promega). The recombinant clone of *Lactobacillus* sp. L13 and *gadB* *L. brevis* LSF8-13 with *Sph* I and *Bam* HI were subjected for restriction cut for preparation of insert for construction of expression plasmid.

Table 4.8. PCR primers used for cloning of *gadB*

Amplification	Primer name	Sequence (5'-3')	Tm (°C)
Full length LSF8-13 *gadB* with enzyme restriction site	LSF8-13 Fw*Sph*I	GCATGCGAATGGCAATGTTA	62.3
	LSF8-13 Rv*Bam*HI	GGATCCGTGTGTGAATCC	60.1
Full length L13 *gadB* with enzyme restriction site	L13 Fw*Sph*I	GCATGCGAATGAGTAAAAACGAT CAGGAAACGCA	72.5
	L13 Rv*Bam*HI	GGATCCACGAACAGTCGTCTTGT TCTTG	69.3
	aL13 Fw*Sph*I	GCTGCGCATGCGAATGAGTAAAA ACG	69.8
	aL13 Rv*Bam*HI	CGGGATCCACGAACAGTCGTCTT	68.7

The recombinant plasmids of *Lactobacillus* sp. L13 *L. brevis* LSF8-13 *gadB* in pGEM-T easy were subjected for sequencing analysis to confirmed the inserted genes then the clones will be cut with *Sph* I and *Bam* HI and subcloned into vector pQE70 double cut with *Sph* I and *Bam*HI, for expression under the control of the *lac* promoter and for purification of 6xHis-tagged recombinant GAD. The strategy to construct the expression clone is shown in scheme 4.2. The recombinant clone was checked for the size of recombinant plasmid and by restriction analysis (Fig.4.22) The recombinant expression plasmids which contained the inserted gene of interest were sequenced to confirm the gene and its reading frame and the histidine-tagged at the end of the genes



Scheme 4.2. The strategy to construct the expression plasmid of *gadB* from *Lactobacillus* sp. L13 in pQE-70 histidine-tagged expression vector.

The recombinant clones for expression of *Lactobacillus* sp. L13 *gadB* were first cut with *EcoR*I to avoid for contamination of pGEMT-Easy vector that the plasmid will be cut twice and two bands of 3 kb and 1.4 kb will be found (Fig.4.22) Recombinant clones the strain L13 and LSF 8-13 were analyzed by cutting with restriction enzymes (Fig. 4.23 and 4.24, respectively).

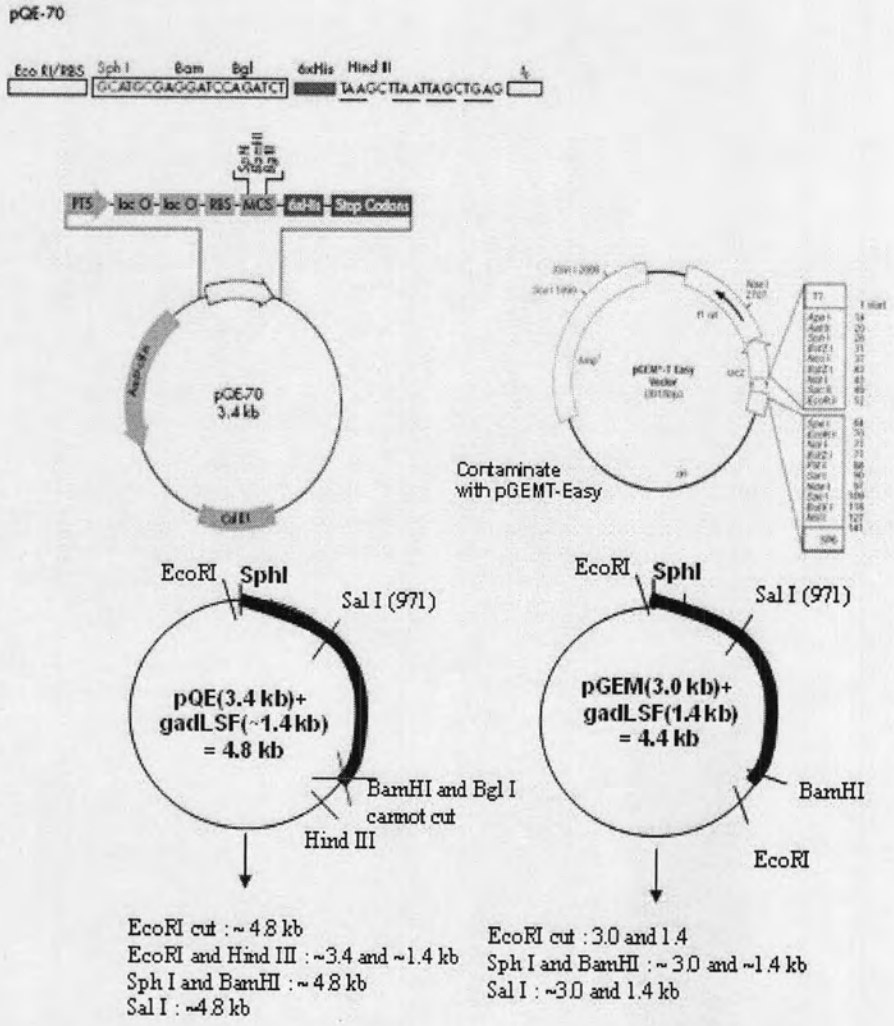


Fig. 4.22. Map of plasmid pQE-70 and pGEMT-Easy with inserted gene of L13 *gadB*.

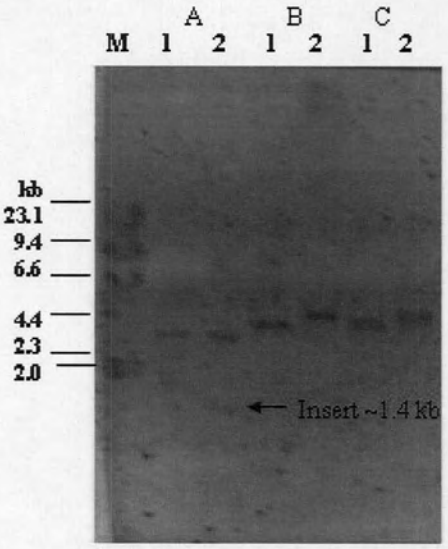


Fig. 4.23. Restriction analysis of the recombinant plasmids extracted from clone number 1 and 2. of the L13 strain. A, double cut with *EcoR* I and *Hind*III. B, double cut with *Sph* I and *Bgl* II. C, cut with *Sal* I .

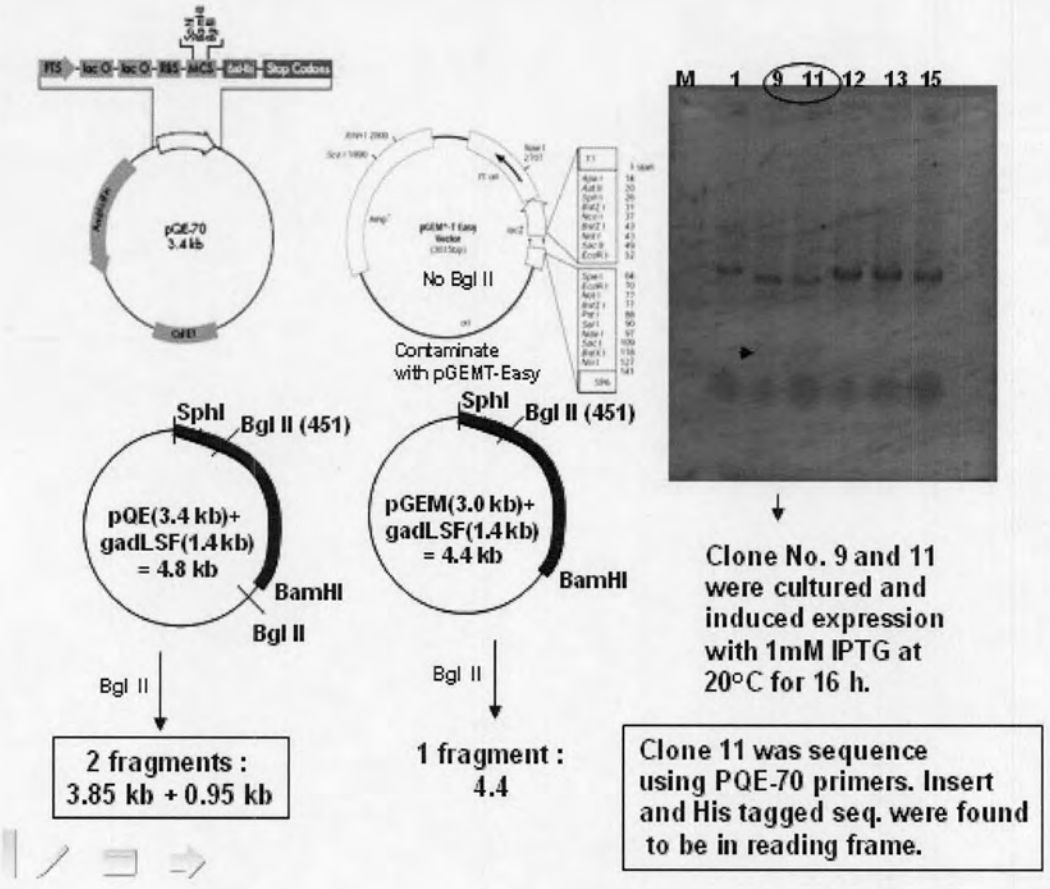


Fig. 4.24. Map of restriction analysis of the recombinant clones of the strain LSF8-13 *gadB* in pQE-70 and the result of restriction analysis of the selected clones.

Recombinant *gadB* clones of both LSF8-13 and L13 were sequenced to confirm the coding sequences and reading frame. The clone were sequenced using specific primer for the expression vector pQE70

4.5. Expression and purification of *gadB* from *Lactobacillus* sp. nov. L13 and *Lactobacillus brevis* LSF8-13

First of all, GABA producing activity of the isolated strains *Lactobacillus* sp. nov. L13 and *Lactobacillus brevis* LSF8-13 were compared by TLC (Ueno et al., 1997). The isolates were cultured in GYP medium supplemented with 1-5% glutamic acid of 5 days. The culture supernatants of each day were analyzed by TLC. The strain L13 showed high level of GABA productivity. 5% of glutamic acid in culture medium of strain L13 was completely converted to GABA within 48 h of incubation at 30°C while the strain LSF8-13 changed 1% glutamic acid to GABA by 2 days of incubation at the same condition. Next step, the two recombinant clones of LSF8-13, GABA producing activity from crude extract of clones were checked in comparison with the crude extract activity of the wild type *Lactobacillus* sp. L13, the extract of host *E.coli*, and purified fractions of the clones through HiTrap affinity column charge with Ni^{2+} ion as shown in Fig. 4.25. The Gad activity assay was performed as described previously in Chapter III. Ammonium sulfate was added to reaction mixture for testing of the effect of sulfate ion on Gad activity as the activity of *L. brevis* has been influenced by the ammonium sulfate in the reaction mixture (Ueno *et al.*, 1997). The ammonium sulfate had no significant effect on Gad activity of the crude extract of L13 (spot no. 2 & 3 in Fig 4.25). The clones of LSF8-13 had no activity of Gad comparing with the host *E. coli*.

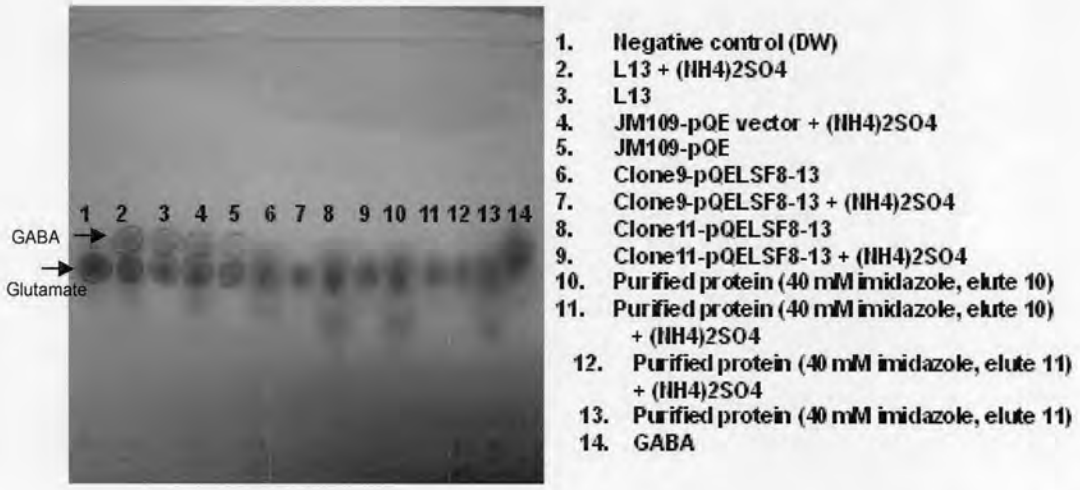


Fig. 4.25. TLC analysis for glutamate decarboxylase activity to catalyses L-glutamic acid to γ-aminobutyric acid (GABA)

After getting the expression of L13 and the complete sequence of the identified gene were confirmed, Gad activity of the recombinant clones of both L13 and LSF8-13 were again investigated of GABA productivity by modified decarboxylase test (LB agar supplemented with 0.05 % glucose, 100 μg/ml ampicillin,1% glutamic acid, 0.5% PLP and 0.001% bromothymol blue indicator). pH of medium was adjusted to 7.0 to get blue color. 0.01 M IPTG were added after sterile. After 1 day of incubation, the media turned yellow with gas production. The recombinant clone of L13 shown high activity in production of gas. The medium again turned blue (Fig. 4.26). Crude extract of the clones were checked for GABA productivity on TLC plate (Ueno *et al.*, 1997) in compared with crude extract of host *E. coli* as shown in Fig. 4.27. Requirement of PLP were investigated. The recombinant clone of L13 showed Gad acitivity depending on the present of PLP in reaction mixture clearly different from the background activity of the host *E.coli*. The Gad activity of recombinant clone of LSF8-13 was not detected.

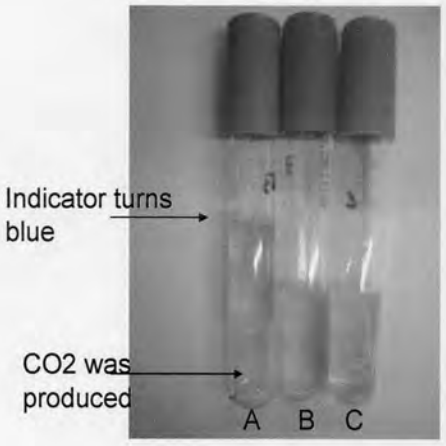


Fig. 4.26. Gad activity detection of the recombinant clones in modified decarboxylase medium containing 1% glutamic acid. A. clone L13, B. clone LSF 8-13, and C. host *E. coli* harboring pQE70 without inserted gene. The recombinant clone L13 *gadB* shown high activity in producing of CO_2 gas and the indicator turned back to blue color.

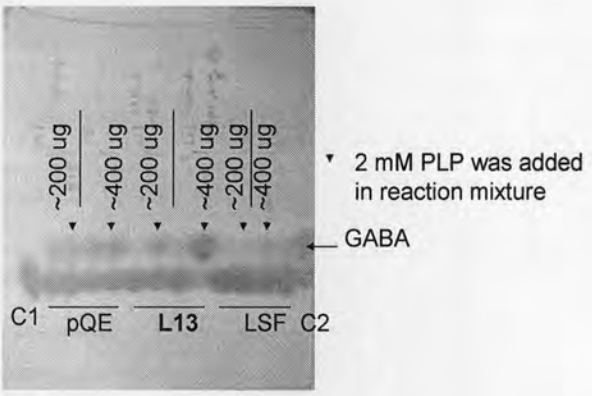


Fig. 4.27. The TLC plate analysis of GABA production by activities of the recombinant clones L13 and LSF 8-13 *gadB* comparing with the host E. coli containing pQE70 vector without inserted gene. C1 and C2 are glutamic acid and mixed glutamic acid-GABA used as standard control. The crude extract of the clones were mixed with the reaction with/without PLP. The crude protein concentrations were 200 and 400 μg, respectively. The clone L13 shown high GAD activity with PLP in reaction mixture clearly discriminated from the background of the host *E.coli.*

The clones were then induced with 0.01 mM IPTG at 25 ^{o}C for overnight (18 h). Cell pellet were collected by centrifugation and lysed by Multi-Beads shocker. The supernatant and cell debris were separated by centrifugation. The crude extract of induced, non-induced clones and host JM109 with an empty plasmid pQE70 were preliminary checked and compared the crude protein patterns on SDS-PAGE. Crude extract of the induced clone were found to contain an expressed protein band compared with that of the host cell (data not shown) then 6xHis-tagged recombinant *gadB* in the crude supernatant was loaded through HiTrap affinity column charge with Ni^{2+} ion. The eluted protein were collected and loaded onto SDS-PAGE. The SDS-PAGE analysis of the recombinant clone of strain L13 (pQE70-L13 *gadB*-histagged) in *E.coli* JM 109 (Fig. 4.28) showed the expressed protein in crude extract (lane C) and a single band of purified protein (lane E1 & E2) of approximately 59 kDa. Protein concentration of crude protein, flow through, wash, and elute fractions were quantified by Bradford method (Bradford, 1976). The Gad activity of the purified

enzyme (E1&E2) can be detected on TLC plate. The recombinant clone of strain LSF 8-13 was induced expression, purified and analyzed. The purified LSF 8-13 recombinant protein was also approximately ~56 kDa with no activity was detected (data not shown).

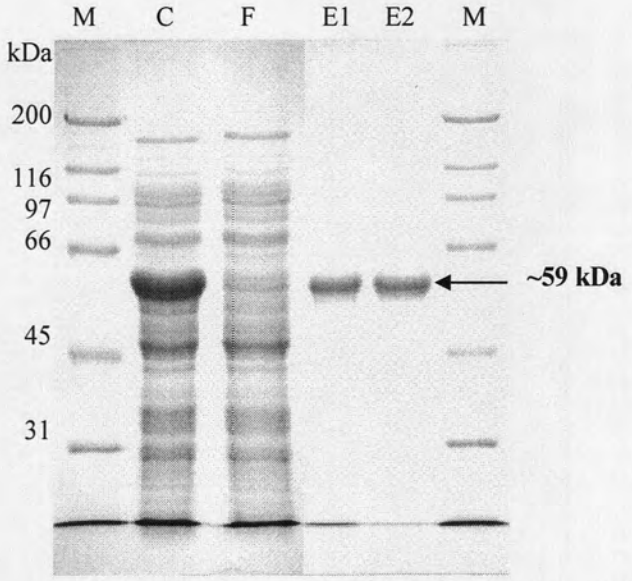


Fig. 4.28. SDS-PAGE analysis (12 % w/v gel) of the purified GAD of *Lactobacillus* sp. nov. L13 induced expression in E.coli JM109. Lane M. broad range SDS-PAGE standards (Biorad) with their size listed in kilodalton (kDa), C. crude extract of JM109 harboring plasmid pQE-70-L13 *gadB* , F. HiTrap-Ni column flow through of , E. purified protein GAD eluted by 100 mM imidazole. The calibration curve of protein standards was in appendix III.

4.6. Comparative analysis of the isolated *gadB*

Identity and similarity (%) of DNA sequence (1326 bp) and amino acid sequence (449 residues) of L13 with others are shown in Table 4.9. The multiple alignment of *gadB* available in database including our data were performed both gene and deduced amino acid. Alignments of nucleotide sequence and deduced amino acid sequence of L13 with others are shown in Fig. 4.29 and 4.30, respectively.

Table 4.9. Percent identity and similarity of DNA sequence and amino acid sequence of L13 with others related genes.

GAD	Ref.	% identity of DNA	% identity of amino acid	% similarity of amino acid
Lactobacillus sp. L13	This study	100	100	100
L. brevis IFO12005 (Ueno *et al*)	Unpublished	72.3	81.9	88.7
L. brevis ATCC367 S18	Makarova *et al.*, 2006	72.4	82.2	88.9
L. plantarum WCFS1	Kleerebezem, *et al.*, 2003	57.9	51.5	66.7
L. brevis LSF8-13	This study	57.7	51.3	62.2
L. brevis ATCC367 S49	Makarova *et al.*, 2006	56.6	50.6	56.2
L. brevis OPK-3	Park, K. and Oh, S. 2007	56.5	50.4	65.5
Lactococcus lactis ssp. *lactis*	Nomura *et al.*, 1999	57.8	52.0	69.1
Escherichia coli K12 *gadB*	Smith *et al*, 1992	49.5	36.9	56.9
Escherichia coli K12 *gadA*	Smith *et al*, 1992	49.2	37.1	56.9

Fig. 4.29. Alignment of glutamate decarboxylase gene (*gadB*)

```
                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                          10         20         30         40         50         60         70         80
L13gadB          ---ATGAGTA AAAACGATCA GGAAACGCAG CAGATG-TTA GACGCAGCAC AATTGGAAAA GACTTTCTTG GGTAGCACCG
L.brevisIFO      ATGATGAATA AAAACGATCA GGAAACACAG CAGATGATTA ATAAT-GTGG ATTTAGAAAA AACGTTTTTA GGCAGTGTCG
L.brevisS18      ---------- ---------- ---------- ---ATGATTA ATAAT-GTGG ATTTAGAAAA AACGTTTTTA GGCAGTGTCG
L.plantarum      ----ATGGCA ATGTTATACG GTAAACACAA -TCATGAA-- GCTGAAGAAT A-CTTGGAA- -CCAGTCTTT GGTGCGCCTT
L.bre LSF8-13    ----ATGGCA ATGTTATACG GTAAACACAA -TCATGAA-- GCTGAAGAAT A-CTTGGAA- -CCAGTCTTT GGTGCGCCTT
L.brevisS49      ----ATGGCT ATGTTGTATG GAAAACACAC -GCATGAA-- ACAGATGAGA C-GCTCAAA- -CCAATCTTC GGGGCCAGCG
L.brevisOPK      ---------- -------ATG GAAAACACAC -GCATGAA-- ACAGATGAGA C-GCTCAAA- -CCAATCTTC GGGGCCAGCG
Lc.lactis lac    ---------- ATGTTATACG GAAAAGAAAA -TCGCGAT-- GAAGCAGAGT T-CTTGGAA- -CCAATTTTT GGTTCAGA-A
Lc.lactis cre    ---------- ATGTTATACG GAAAAGAAAA -TCGCGAT-- GAAGCAGAGT T-CTTGGAA- -CCAATTTTT GGTTCAGA-A
EcoligadB        ---------- -----ATGGA TAAGAAGCAA GTAACGGATT TAAGGTCGGA A-CTACTCGA TTCACGTTTT GGTGCGA--A
EcoligadA        ---------- -----ATGGA CCAGAAGCTG TTAACGGATT TCCGCTCAGA A-CTACTCGA TTCACGTTTT GGCGCAA--A
Consensus                                          *                                 *    **  **

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                          90        100        110        120        130        140        150        160
L13gadB          CAGCCGGTGA ATCACTTC-- CTAAGAACAC TATGCCTGCA GGCCCAATGG CCCCAGAC-- ----GTAGCC GTAGAAATGG
L.brevisIFO      AAGCCGGGCA ATCCTTAC-C C-ACCTATAC ATTACCAGAT GATCCCATGG CACCGGAT-- ----GTTGCC GCTCAATTGG
L.brevisS18      AAGCCGGGCA ATCCTTAC-C C-ACCAATAC ATTACCAGAT GATCCCATGG CACCGGAT-- ----GTTGCC GCTCAATTGG
L.plantarum      CTGAAC-AAC ATGATCTT-C CTAAGTATCG GTTACCAAAG CATTCATTAT CCCCTCGA-- ----GAAGCC GATCGCTTAG
L.bre LSF8-13    CTGAA-CAAC ATGATCTT-C CTAAGTATCG GTTACCAAAG CATTCATTAT CCCCTCGA-- ----GAAGCC GATCGCTTAG
L.brevisS49      CTGAAC-GCC ACGACCTC-C CCAAATATAA ATTGGCAAAG CACGCGCTCG AGCCCCGT-- ----GAAGCC GATCGATTGG
L.brevisOPK      CTGAACTGCC ACGACCTC-C CCAAATATAA ATTGGCAAAG CACGCGCTCG AGCCCCGTCC TCGAGAAGCC GATCGCTTAG
Lc.lactis lac    AGTGA-ACAA GTGGATTTGC CTAAATATAA ATTAGCTCAA CAATCAATTG AACCTCGA-- ----GTGGCC TATCAGTTAG
Lc.lactis cre    AGTGA-ACAA GTGGATTTGC CTAAATATAA ATTAGCTCAA CAATCAATTG AACCTCGA-- ----GTGGCC TATCAGTTAG
EcoligadB        GTCTATTTCC ACTATCGCA- GAATCAAAAC GTTTTCCGCT GCACGAAATG CGCGACGA-- ---TGTCGCA TTCCAGATTA
EcoligadA        GGCCATTTCT ACTATCGCG- GAGTCAAAAC GATTTCCGCT GCACGAAATG CGCGATGA-- ---TGTCGCA TTTCAGATTA
Consensus                                    *      *  *                  *           *  **         *
```

Fig. 4.29. Alignment of glutamate decarboxylase gene (*gadB*), **(continued)**

```
                    170        180        190        200        210        220        230        240
L13gadB         TTGACCACTT CCGTTTAAAC GAAGCAAAAG CTAACCAAAA CTTGGCTACT TTCTGTACCA CTGAAATGGA ACCACAAGCT
L.brevisIFO     TGGAACACTA TCGTTTAAAT GAAGCCAAGG CTAATCAAAA CCTAGCGACC TTCTGTACCA CGCAAATGGA ACCACAAGCC
L.brevisS18     TGGAACACTA TCGTTTAAAT GAAGCCAAGG CTAATCAAAA CCTAGCGACC TTCTGTACCA CGCAAATGGA ACCACAAGCC
L.plantarum     TTCGTGATGA ATTATTAGAT GAAGGCAATT CACGACTGAA CCTGGCAACT TTTTGTCAGA CCTATATGGA ACCCGAAGCC
L.bre LSF8-13   TTCGTGATGA ATTATTAGAT GAAGGCAATT CACGACTGAA CCTGGCAACT TTTTGTCAGA CCTATATGGA ACCCGAAGCC
L.brevisS49     TTCGCGATCA ACTATTGGAT GAAGGAAACT CGCGGCTGAA TCTCGCCACG TTCTGTCAGA CTTACATGGA ACCGGAAGCG
L.brevisOPK     TTCGCGATCA ACTATTGGAT GAAGGAAACT CGCGGCTGAA TCTCGCCACG TTCTGTCAGA CTTACATGGA ACCGGAAGCG
Lc.lactis lac   TTCAAGATGA AATGTTAGAT GAAGGGAACG CTCGTTTAAA TTTAGCCACA TTCTGTCAAA CTTATATGGA ACCTGAAGCA
Lc.lactis cre   TTCAAGATGA AATGTTAGAT GAAGGAAATG CTCGTTTAAA TTTAGCCACA TTCTGTCAAA CTTATATGGA ACCTGAAGCA
EcoligadB       TCAATGACGA ATTATATCTT GATGGCAACG CTCGTCAGAA CCTGGCCACT TTCTGCCAGA CCTGGGACGA CGAAAATGTC
EcoligadA       TCAATGATGA ATTATATCTT GATGGCAACG CTCGTCAGAA CCTGGCCACT TTCTGCCAGA CCTGGGACGA CGAAAACGTC
Consensus       *     *        *      ** *  **   *       **   * ** **  ** **    * *       **      * *

                    250        260        270        280        290        300        310        320
L13gadB         GACCAATTGA TGATGCGTAC CCTTAACACT AACGCCATCG ACAAGTCCGA ATACCCTAAG ACTTCCGCAA TGGAAAACTA
L.brevisIFO     GATGAATTAA TGAAGAACGC GTTGAATACC AATGCGATTG ATAAATCGGA ATACCCTAAG ACCGCGGCAA TGGAAAATTA
L.brevisS18     GATGAATTAA TGAAGAACGC GTTGAATACC AATGCGATTG ATAAATCGGA ATACCCTAAG ACCGCGGCAA TGGAAAATTA
L.plantarum     GTTGAATTGA TGAAGGATAC GCTGGCTAAG AATGCCATCG ACAAATCTGA GTACCCCCGC ACGGCCGAGA TTGAAAATCG
L.bre LSF8-13   GTTGAATTGA TGAAGGATAC GCTGGCTAAG AATGCCATCG ACAAATCTGA GTACCCCCGC ACGGCCGAGA TTGAAAATCG
L.brevisS49     GTTGAACTCA TGAAAGATAC ACTGGAGAAA AACGCCATCG ATAAATCCGA GTATCCTCGG ACCGCTGAAA TTGAAAATCG
L.brevisOPK     GTTGAACTCA TGAAAGATAC ACTGGAGAAA AACGCCATCG ATAAATCCGA GTATCCTCGG ACCGCTGAAA TTGAAAATCG
Lc.lactis lac   GTCAAACTAA TGAGTCAAAC CTTGGAAAAA AATGCAATTG ATAAATCGGA ATATCCAAGA ACAACTGAAA TTGAGAACCG
Lc.lactis cre   GTCAAACTAA TGAGTCAGAC CTTGGAAAAA AATGCGATTG ATAAATCGGA ATATCCAAGA ACAACTGAAA TTGAAAACCG
EcoligadB       CACAAATTGA TGGATTTATC CATTAACAAA AACTGGATCG ACAAAGAAGA ATATCCGCAA TCCGCAGCCA TCGACCTGCG
EcoligadA       CATAAATTGA TGGATTTGTC GATCAATAAA AACTGGATCG ACAAAGAAGA ATATCCGCAA TCCGCAGCCA TCGACCTGCG
Consensus          ** * * **       *   *    *   **    ** * * * **    **  ** **      *  * *  * * **
```

Fig. 4.29. Alignment of glutamate decarboxylase gene (*gadB*), **(continued)**

```
                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         330        340        350        360        370        380        390        400
L13gadB          TTGTGTAGGT ATGATTGCTC ACCTTTGGGG CATTCCTGAC GAAGAAAAGT TCGGTGATGA CTTCATCGGT ACTTCAACCG
L.brevisIFO      CTGTGTCAGC ATGATTGCTC ACCTATGGGG AATTCCTGAC AATGAAAAGA TTTACGATGA TTTCATTGGG ACCTCAACTG
L.brevisS18      CTGTGTCAGC ATGATTGCTC ACCTATGGGG AATTCCTGAC AATGAAAAGA TTTACGATGA TTTCATTGGG ACCTCAACTG
L.plantarum      GTGTGTGAAC ATTATTGCCA ATCTGTGGCA CGCACCTGAT GACGAACA-- ---------- CTTTACGGGT ACCTCTACGA
L.bre LSF8-13    GTGTGTGAAC ATTATTGCCA ATCTGTGGCA CGCACCTGAT GACGAACAC- ---------- -TTTACGGGT ACCTCTACGA
L.brevisS49      TTGCGTTAAT ATCATTGCCA ACCTCTGGCA TGCTCCAGAA GCTGAGTC-- ---------- GTTCACTGGC ACCTCGACGA
L.brevisOPK      TTGCGTTAAT ATCATTGCCA ACCTCTGGCA TGCTCCAGAA GCTGAGTC-- ---------- GTTCACTGGC ACCTCGACGA
Lc.lactis lac    TTGCGTCAAC ATGATCGCTG ACCTTTGGAA TGCGAGTGAA AAAGAAAA-- ---------- ATTTATGGGG ACTTCAACGA
Lc.lactis cre    TTGCGTCAAC ATGATCGCTG ACCTTTGGAA TGCGAGTGAA AAAGAAAA-- ---------- ATTTATGGGG ACTTCAACAA
EcoligadB        TTGCGTAAAT ATGGTTGCCG ATCTGTGGCA TGCG-CCTGC GCCGAAAAA- ----TGGTCA GGCCGTTGGC ACCAACACCA
EcoligadA        TTGCGTAAAT ATGGTTGCCG ATCTGTGGCA TGCG-CCTGC GCCGAAAAA- ----TGGTCA GGCCGTTGGC ACCAACACCA
Consensus         ** **     **  * **   * ** ***                 **                          **  **    **

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         410        420        430        440        450        460        470        480
L13gadB          TTGGTTCTTC CGAAGGTTGC ATGTTAGGTG GACTTGCATT GTTGCACACC TGGAAGCACC GTGCTAAGGG TGGTGGCCTT
L.brevisIFO      TAGGTTCTTC TGAAGGATGT ATGTTAGGCG GCTTGGCGCT ACTACATAGT TGGAAGCACC GGGCCAAGGC AGCTGGTTTT
L.brevisS18      TAGGTTCTTC TGAAGGATGT ATGTTAGGCG GCTTGGCGCT ACTACATAGT TGGAAGCACC GGGCCAAGGC AGCTGGTTTT
L.plantarum      TTGGCTCCTC TGAAGCTTGT ATGTTAGGCG GTTTAGCAAT GAAATTCGCC TGGCGTAAAC GCGCTCAAGC GGCAGGTTTA
L.bre LSF8-13    TTGGCTCCTC TGAAGCTTGT ATGTTAGGCG GTTTAGCAAT GAAATTCGCC TGGCGTAAAC GCGCTCAAGC GGCAGGTTTA
L.brevisS49      TTGGTTCCTC CGAGGCCTGC ATGCTGGCCG GTTTGGCGAT GAAGTTTGCT TGGCGTAAGC GCGCCAAAGC GAACGGTCTT
L.brevisOPK      TTGGTTCCTC CGAGGCCTGC ATGCTGGCCG GTTTGGCGAT GAAGTTTGCT TGGCGTAAGC GCGCCAAAGC GAACGGTCTT
Lc.lactis lac    TTGGTTCTTC AGAAGCTTGT ATGCTTGGTG GAATGGCCAT GAAGTTTTCT TGGCGCAAGC GAGCAGAAAA ATTGGGATTA
Lc.lactis cre    TTGGTTCTTC AGAAGCTTGT ATGCTTGGTG GAATGGCCAT GAAGTTTTCT TGGCGCAAGC GAGCAGAAAA ATTAGGCCTA
EcoligadB        TTGGTTCTTC CGAGGCCTGT ATGCTCGGCG GGATGGCGAT GAAATGGCGT TGGCGCAAGC GTATGGAAGC TGCAGG----
EcoligadA        TTGGTTCTTC CGAGGCCTGT ATGCTCGGCG GGATGGCGAT GAAATGGCGT TGGCGCAAGC GTATGGAAGC TGCAGG----
Consensus        * ** ** **  ** *  **  *** * *  * *  * **  *           ***    * * *    *       **
```

Fig. 4.29. Alignment of glutamate decarboxylase gene (*gadB*), **(continued)**

```
                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         490        500        510        520        530        540        550        560
L13gadB          GACATCGATG ACCTTCACGC TCACAAGCCT AACTTAGTTA TCATGTCTGG TAACCAAGTT GTTTGGGAAA AGTTCTGCAC
L.brevisIFO      GATATTGAAG ACCTGCATAG CCACAAGCCC AACTTGGTCA TCATGTCAGG TTACCAAGTT GTTTGGGAAA AGTTCTGTAC
L.brevisS18      GATATTGAAG ACCTGCATAG CCACAAGCCC AACTTGGTCA TCATGTCAGG TTACCAAGTT GTTTGGGAAA AGTTCTGTAC
L.plantarum      GATCTGAATG -----CCCAT ----CGACCT AACCTCGTTA TTTCGGCTGG CTATCAAGTT TGCTGGGAAA AGTTTTGTGT
L.bre LSF8-13    GATCTGAATG -----CCCAT ----CGACCT AACCTCGTTA TTTCGGCTGG CTATCAAGTT TGCTGGGAAA AGTTTTGTGT
L.brevisS49      GACTTAACTG -----CCCAT ----CAACCT AATATTGTCA TCTCAGCCGG TTATCAAGTT TGTTGGGAAA AATTCTGTGT
L.brevisOPK      GACTTAACTG -----CCCAT ----CAACCT AATATTGTCA TCTCAGCCGG TTATCAAGTT TGTTGGGAAA AATTCTGTGT
Lc.lactis lac    GATATTAATG -----CGAAA ----AAGCCA AACTTAGTTA TTTCATCTGG TTATCAAGTT TGCTGGGAAA AATTCTGTAT
Lc.lactis cre    GATATTAATG -----CGAAA ----AAGCCA AACTTAGTCA TTTCCTCTGG TTATCAAGTT TGCTGGGAAA AATTCTGTGT
EcoligadB        CAAACCAACG -------GAT ----AAACCA AACCTGGT-G TGCGGTCCGG TA--CAAATC TGCTGGCATA AATTCGCCCG
EcoligadA        CAAACCAACG -------GAT ----AAACCA AACCTGGT-G TGCGGTCCGG TA--CAAATC TGCTGGCATA AATTCGCCCG
Consensus         *       *                  **  **  * **   *     * **     *** *     *** * * * **

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         570        580        590        600        610        620        630        640
L13gadB          TTACTGGAAC GTTGACTTCC GTCAAGTTCC AATCAATGGC GACCAAGTAT CTCTTGACCT TGACCATGTT ATGGACTACG
L.brevisIFO      CTATTGGAAT GTCGAGATGC GCCAAGTGCC AATTAATGGT GACCAAGTTT CCTTAGATAT GGATCATGTG ATGGATTATG
L.brevisS18      CTATTGGAAT GTCGAGATGC GCCAAGTGCC AATTAATGGT GACCAAGTTT CCTTAGATAT GGATCATGTG ATGGATTATG
L.plantarum      CTACTGGGAC GTTGACATGC ACGTGGTCCC AATGGATGAG CAACACATGG CCCTTGACGT TAACCACGTC TTAGACTACG
L.bre LSF8-13    CTACTGGGAC GTTGACATGC ACGTGGTCCC AATGGATGAG CAACACATGG CCCTTGACGC TAACCACGTC TTAGACTACG
L.brevisS49      CTATTGGGAC ATCGACATGC ATGTCGTTCC CATGGACGAT GACCACATGT CCTTGAATGT CGATCACGTG TTAGATTACG
L.brevisOPK      CTATTGGGAC ATCGACATGC ATGTCGTTCC CATGGACGAT GACCACATGT CCTTGAATGT CGATCACGTG TTAGATTACG
Lc.lactis lac    TTATTGGGAT ATTGAAATGC GAGAAGTGCC AATGGATAAA GAACATATGT CAATCAATTT GGACAAGGTG ATGGATTATG
Lc.lactis cre    TTATTGGGAT ATTGAAATGC GAGAAGTACC AATGGATAGA GAACATATGT CAATCAATTT GGATGAAGTG ATGGATTATG
EcoligadB        CTACTGGGAT GTGGAGCTGC GTGAGATCCC TATGCGCCCC GGTCAGTTGT TTATGGACCC GAAACGCATG ATTGAAGCCT
EcoligadA        CTACTGGGAT GTGGAGCTGC GTGAGATCCC TATGCGCCCC GGTCAGTTGT TTATGGACCC GAAACGCATG ATTGAAGCCT
Consensus         ** *** *   * **  * *       * **  **          **  *     *  *     *    *   * **
```

Fig. 4.29. Alignment of glutamate decarboxylase gene (*gadB*), **(continued)**

```
                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         650        660        670        680        690        700        710        720
L13gadB          TCGATGAGAA CACTATTGGT ATCATTGGTA TTGAAGGGAT CACTTACACT GGTTCCGTTG ATGACATCCA AGGTCTTG-A
L.brevisIFO      TTGATGAAAA TACGATTGGG ATTATCGGAA TTGAGGGCAT TACGTACACA GGCTCCGTTG ATGATATTCA AACGCTAG-A
L.brevisS18      TTGATGAAAA TACGATTGGG ATTATCGGAA TTGAGGGCAT TACGTACACA GGCTCCGTTG ATGATATTCA AACGCTAG-A
L.plantarum      TGGACGAATA CACAATTGGT ATCGTCGGTA TCATGGGCAT CACTTATACC GGTCAATATG ACGACCTAGC CGCACTCG-A
L.bre LSF8-13    TGGACGAATA CACAATTGGT ATCGTCGGTA TCATGGGCAT CACTTATACC GGTCAATATG ACGACCTAGC CGCACTCG-A
L.brevisS49      TGGATGACTA CACCATTGGT ATCGTTGGCA TTATGGGCAT CACTTATACT GGACAATACG ACGATTTAGC CCGATTAG-A
L.brevisOPK      TGGATGACTA CACCATTGGT ATCGTTGGCA TTATGGGCAT CACTTATACT GGACAATACG ACGATTTAGC CCGATTAG-A
Lc.lactis lac    TTGATGAGTA CACGATTGGT GTAGTTGGTA TTATGGGGAT TACTTATACT GGTCGTTATG ATGATATCAA AGCTTTGG-A
Lc.lactis cre    TTGATGAATA TACGATTGGG GTAGTTGGAA TTATGGAGAT TACTTATACT GGTCGTTATG ATGATATCAA AGCTTTGG-A
EcoligadB        GTGACGAAAA CACCATCGGC GTGGTGCCGA CTTTCGGCGT GACCTACACT GGTAACTATG A-GTTCCCAC AACCGCTGCA
EcoligadA        GTGACGAAAA CACCATCGGC GTGGTGCCGA CTTTCGGCGT GACCTACACC GGTAACTATG A-GTTCCCAC AACCGCTGCA
Consensus          ** **  *  ** ** **   * *   *      *   *  ** ** **  **       * * *                * *

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         730        740        750        760        770        780        790        800
L13gadB          CAAGTTAGTT ACTGAATACA ACAAGACTG- -CTGCTTTGC C-AGTACGGA TTCACGTGGA CGCTGCCTTT GGTGGTTTGT
L.brevisIFO      TAACCTCGTG ACCGAATATA ATAAGACCG- -CGACGATGC C-GGTACGGA TTCACGTTGA TGCTGCCTTT GGTGGCCTGT
L.brevisS18      TAACCTCGTG ACCGAATATA ATAAGACCG- -CGACGATGC C-GGTACGGA TTCACGTTGA TGCTGCCTTT GGTGGCCTGT
L.plantarum      TAAGGTCGTT ACTCACTACA ATCATCAGCA TCCCAAATTA CCAGTCTACA TTCACGTCGA CGCAGCGTCA GGTGGCTTCT
L.bre LSF8-13    TAAGGTCGTT ACTCACTACA ATCATCAGCA TCCCAAATTA CCAGTCTACA TTCACGTCGA CGCAGCGTCA GGTGGCTTCT
L.brevisS49      TGCCGTTGTA GAGCGGTACA ATCGGACGA- --CTAAGTTC CCGGTATATA TCCATGTCGA TGCCGCTTCC GGCGGATTTT
L.brevisOPK      TGCCGTTGTA GAGCGGTACA ATCGGACGA- --CTAAGTTC CCGGTATATA TCCATGTCGA TGCCGCTTCC GGCGGATTTT
Lc.lactis lac    TAATTTAATT GAAGAATATA AT-AAACAG- -ACAGACTAT AAAGTTTATA TTCACGTAGA TGCTGCTTCA GGAGGACTTT
Lc.lactis cre    TAATTTGATT GAAGAATATA AT-AAACAG- -ACAGACTAT AAAGTTTATA TTCACGTAGA TGCTGCTTCA GGAGGACTTT
EcoligadB        CGATGCGCTG GATAAATTCC AGGCCGATA- --CCGGTATC GACATCGACA TGCACATCGA CGCTGCCAGC GGTGGCTTCC
EcoligadA        CGATGCGCTG GATAAATTCC AGGCCGACA- --CCGGTATC GACATCGACA TGCACATCGA CGCTGCCAGC GGTGGCTTCC
Consensus              *       *    *                      *    * * **  * **  ** **     ** **  *
```

Fig. 4.29. Alignment of glutamate decarboxylase gene (*gadB*), **(continued)**

```
                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         810        820        830        840        850        860        870        880
L13gadB          TCGCCCCATT CGTTGACGGC TTCAAGCCT- -TGGGACTTC CGTCTTGACA ACGTTGTTTC AATCAACGTT TCAGGTCACA
L.brevisIFO      TCGCGCCGTT CGTCGATGGC TTTAACCCG- -TGGGACTTC CGGTTGAAGA ACGTGGTTTC CATTAACGTT TCGGGCCATA
L.brevisS18      TCGCGCCGTT CGTCGATGGC TTTAACCCG- -TGGGACTTC CGGTTGAAGA ACGTGGTTTC CATTAACGTT TCGGGCCATA
L.plantarum      ATACCCCATT TATTGA--GC CGCAACTCAT CTGGGACTTC CGGTTGGCTA ACGTCGTTTC GATCAACGCC TCCGGGCACA
L.bre LSF8-13    ATACCCCATT TATTGA--GC CACAACTCAT CTGGGACTTC CGGTTGGCTA ACGTCGTTTC GATCAACGCC TCCGGGCACA
L.brevisS49      ACACGCCGTT TATTGA--AC CCGAGCTCAA GTGGGACTTC CGTTTAAACA ACGTGATTTC CATCAATGCC TCCGGCCACA
L.brevisOPK      ACACGCCGTT TATTGA--AC CCGAGCTCAA GTGGGACTTC CGTTTAAACA ACGTGATTTC CATCAATGCC TCCGGCCACA
Lc.lactis lac    ATGCTCCCTT TGTTGA--GC CAGAACTTGA GTGGGATTTC CGTTTGAAAA ATGTCATTTC AATCAATACC TCAGGCCATA
Lc.lactis cre    ATGCTCCTTT TGTTGA--GC CAGAACTTGA GTGGGATTTC CGTTTGAAAA ATGTCATTTC AATCAATACT TCAGGACATA
EcoligadB        TGGCACCGTT CGTCGC--CC CGGATATCGT CTGGGACTTC CGCCTGCCGC GTGTGAAATC GATCAGTGCT TCAGGCCATA
EcoligadA        TGGCACCGTT CGTCGC--CC CGGATATCGT CTGGGACTTC CGCCTGCCGC GTGTGAAATC GATCAGTGCT TCAGGCCATA
Consensus           * ** **   * *    *    *        ***** *** **  *        **    **  ** *      ** ** ** *

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         890        900        910        920        930        940        950        960
L13gadB          AGTACGGCAT GGTTTACCCT GGTTTAGGCT GGATCGTATG GCGTAAGAAC TCTTACGACA TCCTTCCTAA GGAAATGCGT
L.brevisIFO      AGTACGGGAT GGTTTACCCT GGGTTGGGGT GGATTGTTTG GCGGCACGAC ACGGCTGATA TTTTACCCGC AGAAATGCGA
L.brevisS18      AGTACGGGAT GGTTTACCCT GGGTTGGGGT GGATTGTTTG GCGGCACAAC ACGGCTGATA TTTTACCCGC AGAAATGCGA
L.plantarum      AGTACGGTTT AGTTTATCCC GGGGTCGGCT GGGTCGTTTG GCGTGATCG- TC----AGTT TTT-ACC-GC CAGAATTAGT
L.bre LSF8-13    AGTACGGTTT AGTTTATCCC GGGGTCGGCT GGGTCGTTTG GCGTGATCG- TC----AGTT -TTTACC-GC CAGAATTAGT
L.brevisS49      AATATGGCTT GGTTTATCCC GGAGTCGGCT GGGTAATCTG GCGTGACCAA C-----AGTA TCT-ACC-AA AAGAGCTGGT
L.brevisOPK      AATATGGCTT GGTTTATCCC GGAGTCGGCT GGGTAATCTG GCGTGACCAA C-----AGTA TCT-ACC-AA AAGAGCTGGT
Lc.lactis lac    AATATGGTTT AGTTTATCCT GGTGTAGGTT GGGTTTTGTG GCGTGACAAA A-----AATA TTT-ACC-AG AAGAATTAAT
Lc.lactis cre    AATATGGTTT AGTATATCCT GGTGTAGGTT GGGTCTTGTG GCGTGACAAA A-----AATA TTT-ACC-TG AAGAGTTAAT
EcoligadB        AATTCGGTCT GGCTCCGCTG GGCTGCGGCT GGGTTATCTG GCGTGACGAA G-----AAGC GCT-GCC-GC AGGAACTGGT
EcoligadA        AATTCGGTCT GGCTCCGCTG GGCTGCGGCT GGGTTATCTG GCGTGACGAA G-----AAGC GCT-GCC-GC AGGAACTGGT
Consensus        * *  **  *  *     *   **    ** * ** *  * ** ***  *                    **       *
```

Fig. 4.29. Alignment of glutamate decarboxylase gene (*gadB*), **(continued)**

```
                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                            970        980        990       1000       1010       1020       1030       1040
L13gadB             TTCTCAGTTC CT--TACCTT GGTTC--AAG TGTCGACTCA ATCGCTATCA ACTTCTCTCA CTCTGGTGCG CACATCAACG
L.brevisIFO         TTCCAAGTGC CA--TATCTA GGTAA--GAC CGTTGATTCA ATCGCCATTA ACTTCTCACA CAGTGGTGCC CATATCAGTG
L.brevisS18         TTCCAAGTGC CA--TATCTA GGTAA--GAC CGTTGATTCA ATCGCCATTA ACTTCTCACA CAGTGGTGCC CATATCAGTG
L.plantarum         CTTCAAAGTT -AGTTATTTA GGTGGGGAGT TG--CCGACA ATGGCGATCA ACTTCTCACA TAGTGCAGCC CAGCTCATTG
L.bre LSF8-13       CTTCAAAGTT -AGTTATTTA GGTGGGGAGT TG--CCGACA ATGGCGATCA ACTTCTCACA TAGTGCAGCC CAGCTCATTG
L.brevisS49         CTTTAAGGTC -AGCTACTTG GGTGGTGAAC TA--CCTACG ATGGCCATCA ACTTCTCCCA CAGTGCCTCC CAATTAATCG
L.brevisOPK         CTTTAAGGTC -AGCTACTTG GGTGGTGAAC TA--CCTACG ATGGCCATCA ACTTCTCCCA CAGTGCCTCC CAATTAATCG
Lc.lactis lac       TTTTAAAGTA -AGTTATCTT GGAGGAGAAC TA--CCAACG ATGGCCATTA ATTTTTCTCA TAGTGCCTCT CAATTAATTG
Lc.lactis cre       TTTTAAAGTA -AGTTATCTT GGAGGAGAAT TA--CCAACA ATGGCGATTA ATTTTTCTCA CAGTGCTTCT CAATTAATCG
EcoligadB           GTTCAACGTT -GACTACCTG GGTGGTCAAA TT--GGTACT TTTGCCATCA ACTTCTCCCG CCCGGCGGGT CAGGTAATTG
EcoligadA           GTTCAACGTT -GACTACCTG GGTGGTCAAA TT--GGTACT TTTGCCATCA ACTTCTCCCG CCCGGCGGGT CAGGTAATTG
Consensus            *   *         **  *  **                  *   * ** ** * * ** ** *      *      **  * *  *

                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                            1050       1060       1070       1080       1090       1100       1110       1120
L13gadB             CCCAATACTA CAACTTCTTA CGCTTTGGTT TAGCTGGTTA -CAAGGCTAT CATGAACAAC GTACGGAAGG TTTCATTGAA
L.brevisIFO         CGCAATACTA CAATTTCATT CGATTTGGAT TATCAGGTTA -CAAGACGAT CATGCAAAAT GTTCGGAAGG TGTCATTGAA
L.brevisS18         CGCAATACTA CAATTTCATT CGATTTGGAT TATCAGGTTA -CAAGACGAT CATGCAAAAT GTTCGGAAGG TGTCATTGAA
L.plantarum         GACAATACTA TAATTTCATT CGCTTTGGTA TGGACGGTTA CCGCGAGATT CAAACAAAGA CTCACGATGT TGCCC-GCTA
L.bre LSF8-13       GACAATACTA TAATTTCATT CGCTTTGGTA TGGACGGTTA CCGCGAGATT CAAACAAAGA CTCACGATGT TGCCC-GCTA
L.brevisS49         GTCAGTATTA CAACTTTATT CGCTTTGGTT TTGATGGCTA TCGTGAAATT CAAGAAAAAA CTCACGACGT TGCCC-GCTA
L.brevisOPK         GTCAGTATTA CAACTTTATT CGCTTTGGTT TTGATGGCTA TCGTGAAATT CAAGAAAAAA CTCACGACGT TGCCC-GCTA
Lc.lactis lac       GTCAATATTA TAATTTTGTA CGTTATGGAT TTGATGGATA TAAAGCTATT CATGAGAGAA CACATAAAGT AGCCA-TGTT
Lc.lactis cre       GTCAATACTA TAATTTTGTA CGTTATGGAT TTGATGGATA TAAAGCTATT CATGAGAGAA CGCATAAAGT AGCCA-TGTA
EcoligadB           CACAGTACTA TGAATTCCTG CGCCTCGGTC GTGAAGGCTA TACCAAAGTA CAGAACGCCT CTTACCAGGT TGCCG-CTTA
EcoligadA           CACAGTACTA TGAATTCCTG CGCCTCGGTC GTGAAGGCTA TACCAAAGTA CAGAACGCCT CTTACCAGGT TGCCG-CTTA
Consensus             ** ** **   * **  *  **    **       ** **            **              * *    *
```

Fig. 4.29. Alignment of glutamate decarboxylase gene (*gadB*), **(continued)**

```
                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         1130       1140       1150       1160       1170       1180       1190       1200
L13gadB          GTTGACTGAC GAATTACGTA AGTTTGGTAT CTTTGACATC CTTG---TTG AT-----GGT AAAGAAT-TA CCAATCAACT
L.brevisIFO      GCTGACGGCA GCTCTGAAAA CGTATGGGAT TTTCGATATT TTAG---TTG AT-----GGG TCACAGC-TA CCAATTAACT
L.brevisS18      GCTGACGGCA GCTCTGAAAA CGTATGGGAT TTTCGATATT TTAG---TTG AT-----GGG TCACAGC-TA CCAATTAACT
L.plantarum      CCTGGCAGCC GCTCTGGATA AAGTTGGTGA GTTTAAGATG ATCA---ATA AC-----GGA CACCAAC-TC CCCCTGATTT
L.bre LSF8-13    CCTGGCAGCC GCTCTGGATA AAGTTGGTGA GTTTAAGATG ATCA---ATA AC-----GGA CACCAAC-TC CCCCTGATTT
L.brevisS49      TCTCGCGAAA TCGCTCACTA AATTAGGGGG CTTTTCCCTC ATTA---ATG AC-----GGC CACGAGT-TA CCGCTGATCT
L.brevisOPK      TCTCGCGAAA TCGCTCACTA AATTAGGGGG CTTTTCCCTC ATTA---ATG AC-----GGC CACGAGT-TA CCGCTGATCT
Lc.lactis lac    TTTAGCAAAA GAAATTGAAA AAACTGGAAT GTTTGAAATT ATGA---ACG AT-----GGG TCACAAT-TG CCAATTGTCT
Lc.lactis cre    TTTAGCAGAA GAAATTGAAA AAACAGGAAT GTTTGAGATT ATGA---ACG AT-----GGG TCACAAT-TG CCAATTGTCT
EcoligadB        TCTGGCGGAT GAAATCGCCA AACTGGGGCC GTATGAGTTC ATCTGTACGG GTCGCCCGGA CGAAGGCATC CCGGCGGTTT
EcoligadA        TCTGGCGGAT GAAATCGCCA AACTGGGGCC GTATGAGTTC ATCTGTACGG GTCGCCCGGA CGAAGGCATC CCGGCGGTTT
Consensus          *  *         *    *      **     *      *   *                 **          *  **       *

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         1210       1220       1230       1240       1250       1260       1270       1280
L13gadB          GCTGGAAGTT GTCTGACAAC GCCAACGTAA GTTGGAGTTT GTACGACATG GAAGATGCTC TGGC-TAAGT ACGGCTGGCA
L.brevisIFO      GTTGGAAACT AGCGGACGAT GCGCCGGTTG GTTGGACGTT GTATGATTTG GAGTCCGAGC TGGC-TAAGT ATGGTTGGCA
L.brevisS18      GTTGGAAACT AGCGGACGAT GCGCCGGTTG GTTGGACGTT GTATGATTTG GAGTCCGAGC TGGC-TAAGT ATGGTTGGCA
L.plantarum      GTTACCAACT AGCCTCGCGC GAAGATCGTG AATGGACCCT TTATGATTTA TCGGATCGCC TATT-AATGA ACGGTTGGCA
L.bre LSF8-13    GTTACCAACT AGCCTCGCGC GAAGATCGTG AATGGACCCT TTATGATTTA TCGGATCGCC TATT-AATGA ACGGTTGGCA
L.brevisS49      GTTATGAACT CACTGCCGAT TCTGATCGCG AATGGACCCT CTACGATTTA TCCGATCGGT TATT-AATGA AGGGCTGGCA
L.brevisOPK      GTTATGAACT CACTGCCGAT TCTGATCGCG AATGGACCCT CTACGATTTA TCCGATCGGT TATT-AATGA AGGGCTGGCA
Lc.lactis lac    GCTATAAATT AAAAGAAGAT TCAAATCGAG GTTGGAATCT TTATGATTTG GCGGACCGTT TATT-AATGA AGGGATGGCA
Lc.lactis cre    GCTACAAATT AAAAGAAAAT TCAAATCTTG GTTGGAATCT TTATGATTTG GCAGATCGTT TATTTAATGA AGGGATGGCA
EcoligadB        GCTTCAAACT GAAAGATGGT GAAGATCCGG GATACACCCT GTATGACCTC TCTGAACGTC TGCG-TCTGC GCGGCTGGCA
EcoligadA        GCTTCAAACT GAAAGATGGT GAAGATCCGG GATACACCCT GTACGACCTC TCTGAACGTC TGCG-TCTGC GCGGCTGGCA
Consensus        * *   *  *                        *  *   *  ** **  *            *      *    ** *****
```

Fig. 4.29. Alignment of glutamate decarboxylase gene (*gadB*), **(continued)**

```
                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         1290       1300       1310       1320       1330       1340       1350       1360
L13gadB          AGTACCTGCT TAC-CCACTT CCAAAGAACC GTGAAGAAAC TATCACCAGC CGGATTGTTG TTCGTCCTGG TATGACTATG
L.brevisIFO      AGTCCCAGCT TAC-CCGCTG CCAAAGAATC GCGACGATGT GACAATTAGC CGGATCGTGG TACGCCCATC CATGACCATG
L.brevisS18      AGTTCCGGCA TAT-CCACTG CCAAAGAATC GCGACGATGT GACAATTAGC CGGATCGTGG TACGCCCATC CATGACCATG
L.plantarum      AGTACCAACG TAT-CCTTTA CCTGCTAATC TGGAACAACA AGTCATCCAA CGAATCGTCG TTCGGGCTGA CTTTGGCATG
L.bre LSF8-13    AGTACCAACG TAT-CCTTTA CCTGCTAATC TGGAACAACA AGTCATCCAA CGAATCGTCG TTCGGGCTGA CTTTGGCATG
L.brevisS49      GGTTCCCACC TAT-CCCTTA CCAAAAAACA TGACGGACCG CGTTATTCAA CGGATCGTGG TTCGGGCTGA CTTTGGTATG
L.brevisOPK      GGTTCCCACC TAT-CCCTTA CCAAAAAACA TGACGGACCG CGTTATTCAA CGGATCGTGG TTCGGGCTGA CTTTGGTATG
Lc.lactis lac    AGTGCCTGCT TAT-CCACTT CCCAAAAATT TGGAAAATGA AATCATTCAA CGTTTAGTGA TTCGAGCAGA TTTTGGGATG
Lc.lactis cre    AGTGCCTGCT TAT-CCACTT CCTAA----- ---------- ---------- ---------- ---------- ----------
EcoligadB        GGTTCCGGCC TTCACTCTCG GCGGTGAAGC CACC-GACAT CGTGGTGATG CGCATTATGT GTCGTCGCGG CTTCGAAATG
EcoligadA        GGTTCCGGCC TTCACTCTCG GCGGTGAAGC CACC-GACAT CGTGGTGATG CGCATTATGT GTCGTCGCGG CTTCGAAATG
Consensus         ** **  *  *   *       *

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         1370       1380       1390       1400       1410       1420       1430       1440
L13gadB          GCCATTGCCG ACGACTTCAT CGATGACTTG AAGTTAGCTA TTGCTGACTT GAACCACAGC TTCGGTGACG TTAAGGATGT
L.brevisIFO      ACGATTGCCG ATGATTTCTT GGATGATTTG AAATTAGCAA TTGATGGATT AAATCACACA TTTGGCGTGA CGACCACCGT
L.brevisS18      ACGATTGCCG ATGATTTCTT GGATGATTTG AAATTAGCAA TTGATGGATT AAATCACACA TTTGGCGTGA CGACCACCGT
L.plantarum      AATATGGCCC ACGATTTCAT GGATGACCTG ACCAAGGCTG TCCATGACTT AAACCACGCC CACATTGT-- CTATCATCAT
L.bre LSF8-13    AATATGGCCC ACGATTTCAT GGATGACCTG ACCAAGGCTG TCCATGACTT AAACCAAGCC CACATTGT-- CTATCATCAT
L.brevisS49      AGTATGGCCC ACGACTTTAT TGATGATCTA ACCCAAGCCA TTCACGATCT CGACCAAGCA CACATCGT-- TTTCCATAGT
L.brevisOPK      AGTATGGCCC ACGACTTTAT TGATGATCTA ACCCAAGCCA TTCACGATCT CGACCAAGCA CACATCGT-- TTTCCATAGT
Lc.lactis lac    AATATGGCAT TTAACTATGT TCAAGATATG CAAGAAGCAA TTGAGGCTTT AAATAAGGCT CATATTCT-- ATATCATGA-
Lc.lactis cre    ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
EcoligadB        GACTTTGCTG AACTGTTGCT GGAAGACTAC AAAGCCTCC- ---CTGAAAT ATCTCAGC-- --GATCAC-- CCGAAACTGC
EcoligadA        GACTTTGCTG AACTGTTGCT GGAAGACTAC AAAGCCTCC- ---CTGAAAT ATCTCAGC-- --GATCAC-- CCGAAACTGC
Consensus
```

Fig. 4.29. Alignment of glutamate decarboxylase gene (*gadB*), **(continued)**

```
                 ....|....| ....|....| ....|....| ....|....|
                         1450       1460       1470       1480
L13gadB          TAACGACAAG AA-CAAGACG ACTGTTCGTT AG--------
L.brevisIFO      TGATCAAGAT AA-CAAGACC ACCGTTCG-- AAGT--TAA-
L.brevisS18      TGATCAAGAT AA-CAAGACC ACCGTTCG-- AAGT--TAA-
L.plantarum      GACGCGGCAC CT-AAGAAAT ACGGATTC-- ACACACTGA-
L.bre LSF8-13    GACGCGGCAC CT-AAGAAAT ACGGATTC-- ACACACTGA-
L.brevisS49      GATCCGCAAC CT-AAAAAAT ACGGGTTC-- ACGCACTAA-
L.brevisOPK      GATCCGCAAC CT-AAAAAAT ACGGGTTC-- ACGCACTAA-
Lc.lactis lac    AGAGCCTGAA AATAAAACAT ATGGATTT-- ACTCACTAA-
Lc.lactis cre    ---------- ---------- ---------- ----------
EcoligadB        AGGGTATTGC CC--AACAGA ACAGCTTTAA ACATACCTGA
EcoligadA        AGGGTATTGC CC--AGCAGA ACAGCTTTAA ACACACCTGA
Consensus
```

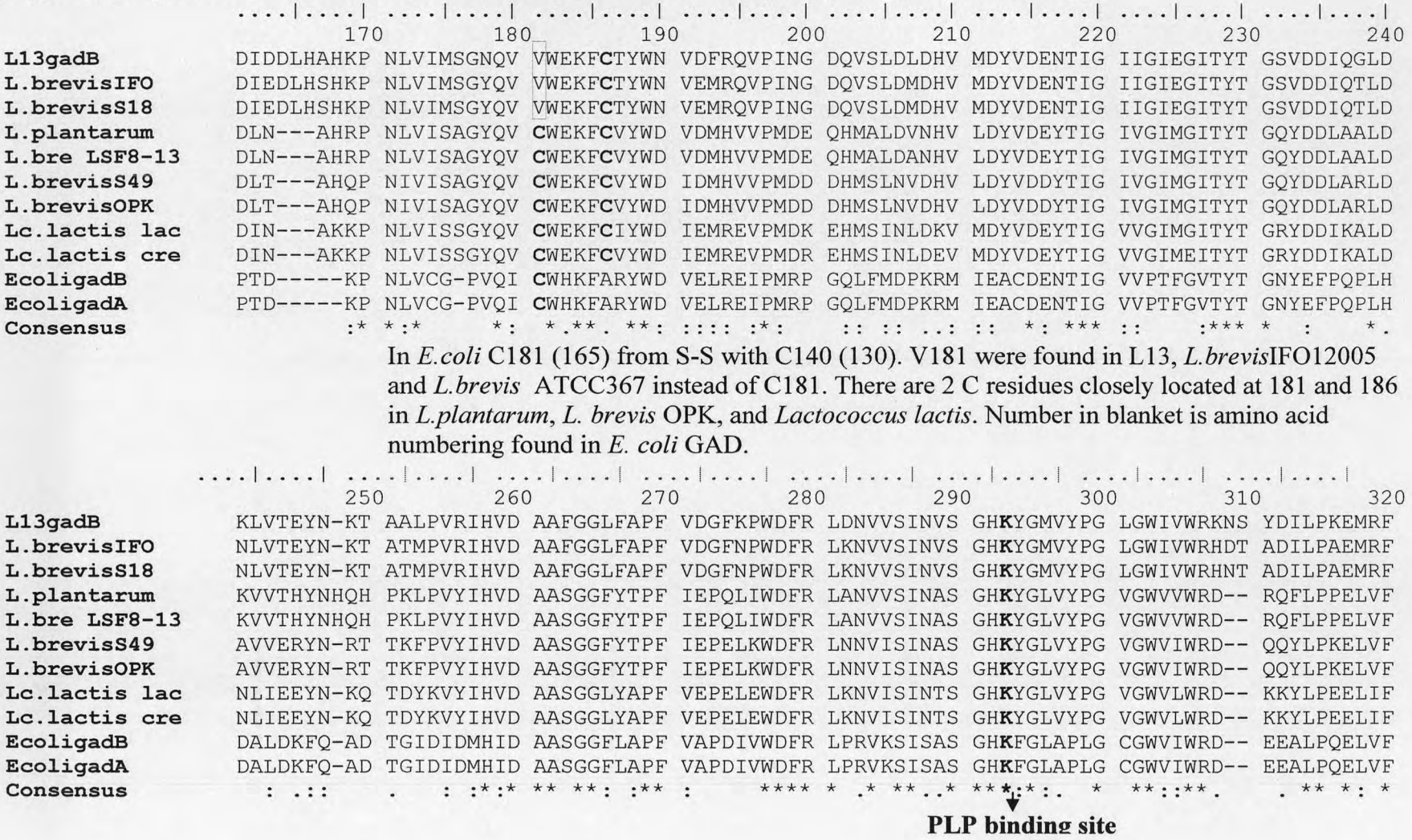
Fig. 4.30. Alignment of deduced amino acid from glutamate decarboxylase gene (*gadB*)

```
                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                          10         20         30         40         50         60         70         80
L13gadB          --MSKNDQET QQMLDAAQLE KTFLGSTAAG ESLPKNTMPA GPMAPD--VA VEMVDHFRLN EAKANQNLAT FCTTEMEPQA
L.brevisIFO      -MMNKNDQET QQMINNVDLE KTFLGSVEAG QSLPTYTLPD DPMAPD--VA AQLVEHYRLN EAKANQNLAT FCTTQMEPQA
L.brevisS18      ---------- --MINNVDLE KTFLGSVEAG QSLPTNTLPD DPMAPD--VA AQLVEHYRLN EAKANQNLAT FCTTQMEPQA
L.plantarum      MAMLYGKHN- HEAEEYLEP- --VFGAPSEQ HDLPKYRLPK HSLSPR--EA DRLVRDELLD EGNSRLNLAT FCQTYMEPEA
L.bre LSF8-13    MAMLYGKHN- HEAEEYLEP- --VFGAPSEQ HDLPKYRLPK HSLSPR--EA DRLVRDELLD EGNSRLNLAT FCQTYMEPEA
L.brevisS49      MAMLYGKHT- HETDETLKP- --IFGASAER HDLPKYKLAK HALEPR--EA DRLVRDQLLD EGNSRLNLAT FCQTYMEPEA
L.brevisOPK      MENTRMKQM- RRSNQSS--- ----GPALNC HDLPKYKLAK HALEPRPREA DRLVRDQLLD EGNSRLNLAT FCQTYMEPEA
Lc.lactis lac    --MLYGKEN- RDEAEFLEP- --IFGSESEQ VDLPKYKLAQ QSIEPR--VA YQLVQDEMLD EGNARLNLAT FCQTYMEPEA
Lc.lactis cre    --MLYGKEN- RDEAEFLEP- --IFGSESEQ VDLPKYKLAQ QSIEPR--VA YQLVQDEMLD EGNARLNLAT FCQTYMEPEA
EcoligadB        --MDKKQVTD LRS-ELLDS- --RFGAKSIS TIAESKRFPL HEMRDD--VA FQIINDELYL DGNARQNLAT FCQTWDDENV
EcoligadA        --MDQKLLTD FRS-ELLDS- --RFGAKAIS TIAESKRFPL HEMRDD--VA FQIINDELYL DGNARQNLAT FCQTWDDENV
Consensus                     :          *.         .  :.    :     *  .:: .     :.::. **** ** *  : :.

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                          90        100        110        120        130        140        150        160
L13gadB          DQLMMRTLNT NAIDKSEYPK TSAMENYCVG MIAHLWGIPD EEKFGDDFIG TSTVGSSEGC MLGGLALLHT WKHRAKGGGL
L.brevisIFO      DELMKNALNT NAIDKSEYPK TAAMENYCVS MIAHLWGIPD NEKIYDDFIG TSTVGSSEGC MLGGLALLHS WKHRAKAAGF
L.brevisS18      DELMKNALNT NAIDKSEYPK TAAMENYCVS MIAHLWGIPD NEKIYDDFIG TSTVGSSEGC MLGGLALLHS WKHRAKAAGF
L.plantarum      VELMKDTLAK NAIDKSEYPR TAEIENRCVN IIANLWHAPD DE----HFTG TSTIGSSEAC MLGGLAMKFA WRKRAQAAGL
L.bre LSF8-13    VELMKDTLAK NAIDKSEYPR TAEIENRCVN IIANLWHAPD DE----HFTG TSTIGSSEAC MLGGLAMKFA WRKRAQAAGL
L.brevisS49      VELMKDTLEK NAIDKSEYPR TAEIENRCVN IIANLWHAPE AE----SFTG TSTIGSSEAC MLAGLAMKFA WRKRAKANGL
L.brevisOPK      VELMKDTLEK NAIDKSEYPR TAEIENRCVN IIANLWHAPE AE----SFTG TSTIGSSEAC MLAGLAMKFA WRKRAKANGL
Lc.lactis lac    VKLMSQTLEK NAIDKSEYPR TTEIENRCVN MIADLWNASE KE----KFMG TSTIGSSEAC MLGGMAMKFS WRKRAEKLGL
Lc.lactis cre    VKLMSQTLEK NAIDKSEYPR TTEIENRCVN MIADLWNASE KE----KFMG TSTIGSSEAC MLGGMAMKFS WRKRAEKLGL
EcoligadB        HKLMDLSINK NWIDKEEYPQ SAAIDLRCVN MVADLWHAPA PKN--GQAVG TNTIGSSEAC MLGGMAMKWR WRKRMEAAGK
EcoligadA        HKLMDLSINK NWIDKEEYPQ SAAIDLRCVN MVADLWHAPA PKN--GQAVG TNTIGSSEAC MLGGMAMKWR WRKRMEAAGK
Consensus         :**  :: . * ***.***: :: ::  **. ::*.**  .   :       * *.*:****.* **.*:*:    *::* :  *
```

D94 is the important residue for substrate binding and loop taking up while C140 (130) from S-S with C181(165) in *E.coli* GAD. Number in blanket is amino acid numbering found in *E.coli* GAD.

Fig. 4.30. Alignment of deduced amino acid from glutamate decarboxylase gene (*gadB*) (continued)



In *E.coli* C181 (165) from S-S with C140 (130). V181 were found in L13, *L.brevis*IFO12005 and *L.brevis* ATCC367 instead of C181. There are 2 C residues closely located at 181 and 186 in *L.plantarum*, *L. brevis* OPK, and *Lactococcus lactis*. Number in blanket is amino acid numbering found in *E. coli* GAD.

Fig. 4.30. Alignment of deduced amino acid from glutamate decarboxylase gene (*gadB*) (continued)

```
                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         330        340        350        360        370        380        390        400
L13gadB          SVPYLGSSVD SIAINFSHSG AHINAQYYNF LRFGLAGYKA IMNNVRKVSL KLTDELRKFG IFDILVDG-- -KELPINCWK
L.brevisIFO      QVPYLGKTVD SIAINFSHSG AHISAQYYNF IRFGLSGYKT IMQNVRKVSL KLTAALKTYG IFDILVDG-- -SQLPINCWK
L.brevisS18      QVPYLGKTVD SIAINFSHSG AHISAQYYNF IRFGLSGYKT IMQNVRKVSL KLTAALKTYG IFDILVDG-- -SQLPINCWK
L.plantarum      KVSYLGGELP TMAINFSHSA AQLIGQYYNF IRFGMDGYRE IQTKTHDVAR YLAAALDKVG EFKMINNG-- -HQLPLICYQ
L.bre LSF8-13    KVSYLGGELP TMAINFSHSA AQLIGQYYNF IRFGMDGYRE IQTKTHDVAR YLAAALDKVG EFKMINNG-- -HQLPLICYQ
L.brevisS49      KVSYLGGELP TMAINFSHSA SQLIGQYYNF IRFGFDGYRE IQEKTHDVAR YLAKSLTKLG GFSLINDG-- -HELPLICYE
L.brevisOPK      KVSYLGGELP TMAINFSHSA SQLIGQYYNF IRFGFDGYRE IQEKTHDVAR YLAKSLTKLG GFSLINDG-- -HELPLICYE
Lc.lactis lac    KVSYLGGELP TMAINFSHSA SQLIGQYYNF VRYGFDGYKA IHERTHKVAM FLAKEIEKTG MFEIMNDG-- -SQLPIVCYK
Lc.lactis cre    KVSYLGGELP TMAINFSHSA SQLIGQYYNF VRYGFDGYKA IHERTHKVAM YLAEEIEKTG MFEIMNDG-- -SQLPIVCYK
EcoligadB        NVDYLGGQIG TFAINFSRPA GQVIAQYYEF LRLGREGYTK VQNASYQVAA YLADEIAKLG PYEFICTGRP DEGIPAVCFK
EcoligadA        NVDYLGGQIG TFAINFSRPA GQVIAQYYEF LRLGREGYTK VQNASYQVAA YLADEIAKLG PYEFICTGRP DEGIPAVCFK
Consensus        .* ***  :  ::*****:.. .:: .***:* :* *  **   :     .*:   *:  : . *  :.::  *      :*  *::

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         410        420        430        440        450        460        470        480
L13gadB          LSDNANVSWS LYDMEDALAK YGWQVPAYPL PKNREETITS RIVVRPGMTM AIADDFIDDL KLAIADLNHS FGDVKDVNDK
L.brevisIFO      LADDAPVGWT LYDLESELAK YGWQVPAYPL PKNRDDVTIS RIVVRPSMTM TIADDFLDDL KLAIDGLNHT FGVTTTVDQD
L.brevisS18      LADDAPVGWT LYDLESELAK YGWQVPAYPL PKNRDDVTIS RIVVRPSMTM TIADDFLDDL KLAIDGLNHT FGVTTTVDQD
L.plantarum      LASREDREWT LYDLSDRLLM NGWQVPTYPL PANLEQQVIQ RIVVRADFGM NMAHDFMDDL TKAVHDLNHA HIVYHHDAAP
L.bre LSF8-13    LASREDREWT LYDLSDRLLM NGWQVPTYPL PANLEQQVIQ RIVVRADFGM NMAHDFMDDL TKAVHDLNQA HIVYHHDAAP
L.brevisS49      LTADSDREWT LYDLSDRLLM KGWQVPTYPL PKNMTDRVIQ RIVVRADFGM SMAHDFIDDL TQAIHDLDQA HIVFHSDPQP
L.brevisOPK      LTADSDREWT LYDLSDRLLM KGWQVPTYPL PKNMTDRVIQ RIVVRADFGM SMAHDFIDDL TQAIHDLDQA HIVFHSDPQP
Lc.lactis lac    LKEDSNRGWN LYDLADRLLM KGWQVPAYPL PKNLENEIIQ RLVIRADFGM NMAFNYVQDM QEAIEALNKA HILYHEEPEN
Lc.lactis cre    LKENSNLGWN LYDLADRLFN EGMASACLST S--------- ---------- ---------- ------No GAD activity
EcoligadB        LKDGEDPGYT LYDLSERLRL RGWQVPAFTL GGEATDIVVM RIMCRRGFEM DFAELLLEDY KASLKYLS-D HPKLQGIAQQ
EcoligadA        LKDGEDPGYT LYDLSERLRL RGWQVPAFTL GGEATDIVVM RIMCRRGFEM DFAELLLEDY KASLKYLS-D HPKLQGIAQQ
Consensus        *       :. ***: . *    *   .  .
```

Fig. 4.30. Alignment of deduced amino acid from glutamate decarboxylase gene (*gadB*) (continued)

```
                    ....|..

L13gadB             NKTTVR-
L.brevisIFO         NKTTVRS
L.brevisS18         NKTTVRS
L.plantarum         KKYGFTH
L.bre LSF8-13       KKYGFTH
L.brevisS49         KKYGFTH
L.brevisOPK         KKYGFTH
Lc.lactis lac       KTYGFTH
Lc.lactis cre       -------
EcoligadB           NSFKHT-
EcoligadA           NSFKHT-
Consensus
```

4.7 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 submitted to 3D –JIGSAW server program (Bates *et al.* 2001) at http://www.bmm.icnet.uk/servers/3djigsaw/. The submitted amino acid were analyzed and changed to protein data bank (pdb) format. The pdb file were view by RasWin 2.7.3.1 molecular graphics visualization tool (http://www.rasmol.org). The 3D structures were compared and shown in fig 4.31.

Fig.4.31 3D structure of GAD proteins from the strain IFO12005, L13, and LSF8-13. The number of predicted H-bonds, helices, strands, and turn of each protein were shown under their structure.

The structures were rotated, compared, and marked for PLP-binding domains, predicted active sites and/or different residues. Variation sites were found at the N-terminal loop. PLP-binding lysine (K) residues were located at 288 and 289 in the strain L13 and IFO12005 respectively (Fig. 4.32). Deletion residues at 121 and 122 were deleted in the unexpressed GAD of the strain L13. Difference were found at these residues in the strain L13 and LSF8-13. Glycine (G) 121 was found in L13 while tyrosine (Y) 122 was found in IFO12005. Difference loops and residues were marked and shown in Fig 4.32 and 4.33.

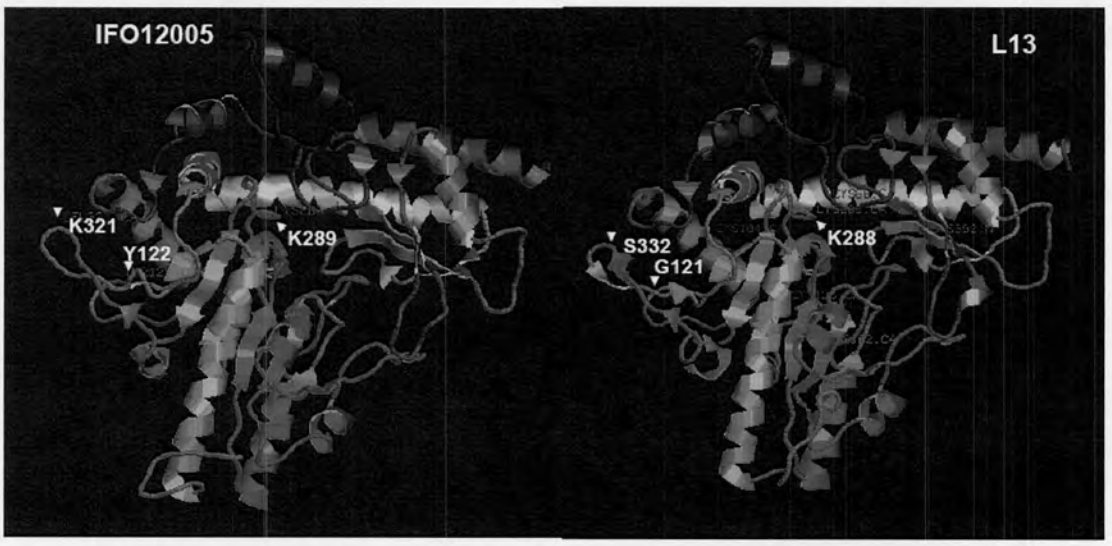


Fig 4.32 PLP-binding lysine (K) residues and different residues on loop structure of the strain L13 and IFO12005 which were deleted in unexpressed GAD of strain LSF8-13

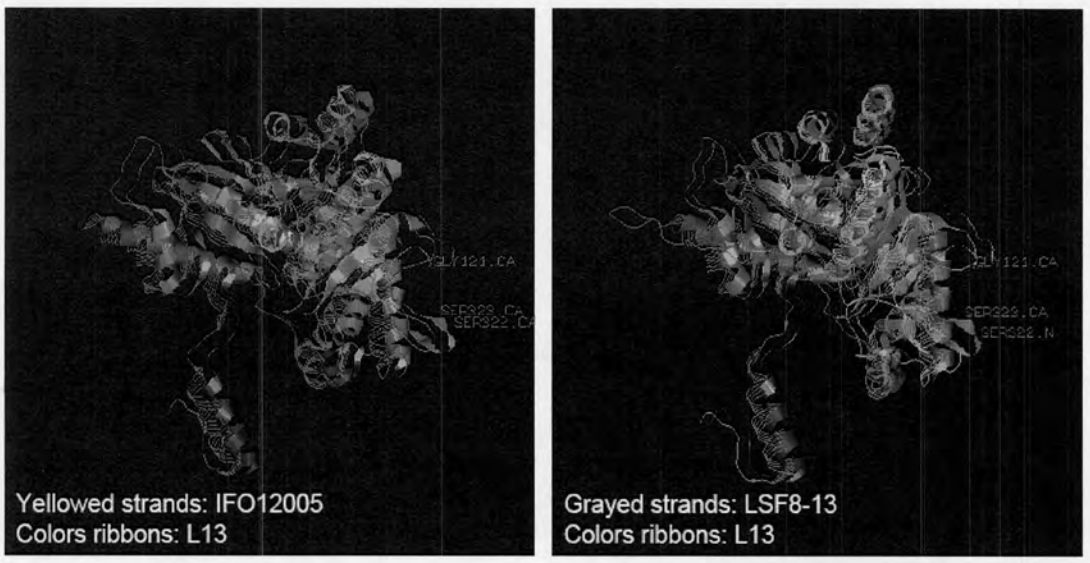


Fig 4.33 Superimposed structure of L13 compared with that of IFO12005 and LSF8-13 shown difference residues at the right side loops structure.

By comparison of the 3D models and alignment of deduced amino acids, the residues phenylalanine (F) 120 and glycine (G) 121 in the strain L13 seem to be important residues since this position was different in the lower GABA producing strain of IFO12005. Isoleucine (I)121 and tyrosine (Y) 122 were found in IFO12005. Interestingly, deletions of these residues were found in that of the unexpressed LSF8-13 GAD. These residues were predicted to from loop structure and found to locate near another loop which consisted of variable residues among compared GADs. Two serine (S) 322 and 323 were found in L13 while lysine (K) 321 and threonine (T) 322 were found in IFO12005, these residues were found to from loop closed to the previously mentioned loop. The residues were labeled as shown in Fig 4.32 and 4.33. These residues should be further studies by mutation to confirm their important properties in catalyze of glutamate to GABA.