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ORIGINAL PAPER

Molecular analysis of the glutamate decarboxylase locus in *Streptococcus thermophilus* ST110

G. A. Somkuti · J. A. Renye Jr. · D. H. Steinberg

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Abstract γ-aminobutyric acid (GABA) is generated from glutamate by the action of glutamic acid decarboxylase (GAD) and characterized by hypotensive, diuretic, and tranquilizing effects in humans and animals. The production of GABA by lactic acid starter bacteria would enhance the functionality of fermented dairy foods including cheeses and yogurt. The survey of 42 strains of the yogurt starter culture *Streptococcus thermophilus* by PCR techniques indicated the presence of a glutamate decarboxylase gene (*gadB*) in 16 strains. DNA sequencing data indicated that the GAD/GABA antiporter locus (*gadB/gadC*) in GAD^+ *S. thermophilus* strains is flanked by transposase elements (5′ and 3′) and positioned between the *luxS* (5′) and the HD-superfamily hydrolase genes (3′). The PCR amplification product of a ca. 2-kb genomic fragment that included the *gadB* and its putative promoter region was inserted into a shuttle vector, which was used to transform *Escherichia coli* DH5α. Subsequently, the recombinant plasmid pMEU5a-1/*gadB* (7.24 kb) was electrotransformed into the GAD-negative strain *S. thermophilus* ST128. The ST128 transformants carrying the plasmid-encoded *gadB* produced functional GAD enzyme as evidenced by the conversion of glutamate to GABA at a rate similar to strains with the *gadB/gadC* operon located on the chromosome. The results demonstrated the potential to impart to non-GABA-producing strains of *S. thermophilus* and other lactic acid bacteria the GAD^+ phenotype that improves their appeal in possible applications in the development of health-promoting functional foods.

Keywords *Streptococcus thermophilus* · GABA · Glutamic acid decarboxylase

Introduction

Gamma-aminobutyric acid (GABA) is a non-protein amino acid that is widely distributed in nature from microorganisms to plants and animals [30]. In addition to its role as a major inhibitory neurotransmitter in animals, GABA also has hypotensive, diuretic, and tranquilizer effects [11], promotes secretion of insulin by the pancreas [1], and may alleviate diabetic conditions [6]. Recent research has shown GABA to improve the level of plasma growth hormone and the rate of protein synthesis in the brain [29], and possibly contribute to the inhibition of certain lung adenocarcinomas [21]. In microbes, GABA has been reported to increase acid tolerance and ATP production [7, 21].

Since GABA has the potential for application as a bioactive ingredient in foods and pharmaceuticals, the development of functional foods containing GABA has attracted attention. Glutamic acid decarboxylases (GAD, EC 4.1.1.15) catalyze the irreversible α-decarboxylation of glutamic acid to GABA. GAD can be produced by many groups of microbes including lactic acid bacteria (LAB) [14]. Several species of LAB are "generally regarded as safe" (GRAS), and have been widely used in the production of fermented foods where, through the production of lactic acid, vitamins, flavor, and aroma compounds, bacteriocins, exopolysaccharides, and enzymes, they contribute to improved shelf

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G. A. Somkuti (✉) · J. A. Renye Jr. · D. H. Steinberg
Dairy and Functional Foods Research Unit, Eastern Regional Research Center, US Department of Agriculture (USDA-ARS), 600 E. Mermaid Lane, Wyndmoor, PA 19038, USA
e-mail: george.somkuti@ars.usda.gov

life, safety, texture, and sensory profile of finished products. The presence of GAD has been demonstrated in several major groups of LAB used in dairy fermentations, including lactococci [17], lactobacilli [9, 12, 13, 17], and *S. thermophilus* [31, 32]. Since the biomass of these harmless and food-grade microbes is ingested routinely by humans as natural food components, they are excellent candidates for evaluation as bacterial cell factories for GABA production.

This report presents the results of a survey of *Streptococcus thermophilus* strains for GAD production, some of the architectural features of the GAD locus in the bacteriocin-producing *S. thermophilus* strain ST110, and the successful intra-species transfer of *gadB* among *S. thermophilus* strains by recombinant DNA techniques.

Materials and methods

Bacteria and culture conditions

The strains of the yogurt and cheese starter culture *Streptococcus thermophilus* used in the study were from an in-house collection. The cultures were grown in tryptone-yeast extract-lactose (TYL) medium adjusted to pH 6.5 before sterilization by autoclaving. Incubation of cultures (5 ml per tube) was at 37 °C for 16 h [24]. For enzyme assays, 200 ml of TYL was inoculated with a 16-h culture (0.1 %, v/v) with bacterial growth monitored at 660 nm and calibrated against cell dry weight measurements, using *S. thermophilus* NRRL-B59384 as the reference (Agricultural Research Service Culture Collection, NCAUR-USDA, Peoria, IL, http://nrrl.ncaur.usda.gov).

Survey of cultures for the presence of *gadB*

Polymerase chain reaction (PCR) was carried out with 1 µl fresh overnight growth of each strain as the template to screen for the GAD gene (*gadB*) using a protocol described previously [19]. First, the presence of the highly conserved 390-bp internal sequence common to microbial *gadB* genes was established with synthetic primers P1 (5′-ggtacatctacaa ttggttcttctga-3′ forward) and P2 (5′-aaaccaccagaagcagcRtc NacRtg-3′ reverse). This was followed by screening with primers P3 (5′-atgaatgagaagctattcagagagat-3′ forward) and P4 (5′-ttaatgatggaagccactgcggatg-3′ reverse) for the entire 1,380-bp ORF*gadB* gene [16]. PCR products were analyzed by gel electrophoresis in 1 % agarose in TAE buffer (0.04 M Tris, 0.02 M acetic acid, 0.001 M EDTA, pH 8.0) [19].

Sequence analysis of PCR products

PCR products from selected *S. thermophilus* strains that were obtained with primers P3 and P4 were purified with the QIAquick PCR purification kit (Qiagen Inc., Valencia, CA, USA). Nucleotide sequencing of each putative ORF-*gad*B was carried out in an ABI PRISM 3730 DNA analyzer (Perkin-Elmer, Wellesley, MA) with ABI PRISM Big Dye terminator cycle sequencing reagent. Nucleotide sequences were analyzed with Sequencer 4.2 (Gene Codes Corp., Ann Arbor, MI).

By using the primer walking technique and pairs of nested primers based on confirmed sequence of the putative ORF*gadB*, DNA analysis was extended to include the upstream and downstream polynucleotide stretches flanking *gadB* in both strands of DNA. The putative location of *gadB* on the *S. thermophilus* ST110 genome was determined by similarity searches between the 5,456-bp polynucleotide assembled from PCR products and sequences in GenBank databases (NCBI, http://www.ncbi.nlm.nih.gov), using the BLAST program [2].

Production of GABA by GAD in *S. thermophilus* ST110

GAD activity was tested by growing a culture of *S. thermophilus* ST110 in TYL at 37 °C overnight and adjusting to $OD_{660} = 1.0$, which corresponded to 375 µg/ml dry weight of biomass. The biomass recovered from 200 ml of medium by centrifugation (10,000 × *g*) were resuspended in 10 ml of 40 % ethanol for 30 min at 30 °C to induce permeabilization of cells, which is a convenient method to measure the activity of internalized enzymes [25]. After washing twice with 20 ml of 75 mM phosphate buffer (pH 4.5, POB), the permeabilized biomass was resuspended in 1 ml of POB and used as the source of GAD.

To measure GABA production, 1 ml of permeabilized ST110 cell suspension was mixed with 1 ml of 100 mM sodium glutamate (MSG) prepared in POB (pH 4.5) and incubated at 37 °C for up to 120 min. The reaction was stopped by removing cells at 10,000 × *g* for 15 min at 4 °C. Samples of the cell-free supernatants (100–250 µl) were used to measure the amount of GABA produced by the GAD of strain ST110.

GABA concentrations were measured by a spectrophotometric assay [33] using commercial GABase (Sigma-Aldrich, St. Louis, MO, USA). The assay procedure is based on the coupled reaction of a transaminase (EC 2.6.1.19) that converts GABA in the presence of α-ketoglutarate to succinic semialdehyde that, in turn, is converted by a specific dehydrogenase (EC 1.2.1.16) to succinate with the stoichiometric reduction of $NADP^+$ at pH 8.6 at 25 °C. GAD activity was calculated as the amount of GABA (µM) produced by 1 mg of dry biomass of *S. thermophilus* ST110 in 60 min. All assays were carried out in triplicate.

Production of GABA in fermented milk by *S. thermophilus* ST110

Skim milk supplemented with 0, 25, 50, and 100 mM MSG was inoculated with 1 % (v/v) of an overnight culture of ST110 and incubated at 41 °C for 6 h to simulate conditions of yogurt production. Reaction mixtures were checked for pH, centrifuged at 10,000 rpm at 4 °C for 15 min, and supernatants were tested for GABA concentration using 250-µl samples.

Transfer of GAD activity to non-producing strains

The cloning strategy for the transfer of GAD activity to GAD$^-$ strains of *S. thermophilus* made use of the shuttle vector pMEU5a [23] and involved two approaches for the transport of *gad*B. In one, the pMEU5a vector was fitted with the chromosomal promoter ST2201 of *S. thermophilus* [26] and the insert included only the ORF*gadB*. In another approach, the insert was lengthened by 300-bp at the 5′ end of *gad*B to include the endogenous promoter of *gad*B present in *S. thermophilus* ST110. In both approaches, the inserts were fitted with recognition sites for the restriction endonucleases *Xba*I and *Xma*I, and amplified by PCR for insertion into the pMEU5a vector double-digested with *Xba*I and *Xma*I. These strategies are illustrated in Fig. 1.

Expression vectors were isolated from ampicillin-resistant (AmpR) transformants of *E. coli* DH5α and purified by CsCl/ethidium bromide ultracentrifugation [27]. The purified plasmids were used to electrotransform another thermophilic yogurt and cheese starter culture, the GAD$^-$ strain *S. thermophilus* ST128, following a protocol described previously [24]. Following 48-h incubation on TYL-agar with 15 µg/ml erythromycin, transformants (EmR) from each electroporation were picked into TYL and screened by PCR amplification for the presence of *gad*B.

GABA production by recombinant strains of *S. thermophilus* ST128

The expression of the GAD gene cluster with its endogenous promoter (pMEU5a/*gadB*-P1) or with the ST2201 chromosomal promoter (pMEU5a/*gadB*-P2) in *Streptococcus thermophilus* ST128 was compared with the level of GAD activity present in ST110/*gadB*-chr. Overnight cultures grown in TYL were adjusted to OD660 = 1.0, and the biomass from 200 ml of each culture was processed under standard conditions to determine GAD activity.

Results

Detection of *gadB* in *S. thermophilus* strains

Of the 42 strains of *S. thermophilus* tested, 20 yielded a 390-bp PCR product that corresponded to a conserved region of microbial *gadB* genes (Fig. 2a). However, only 16 of these strains yielded a 1,380-bp PCR product, which

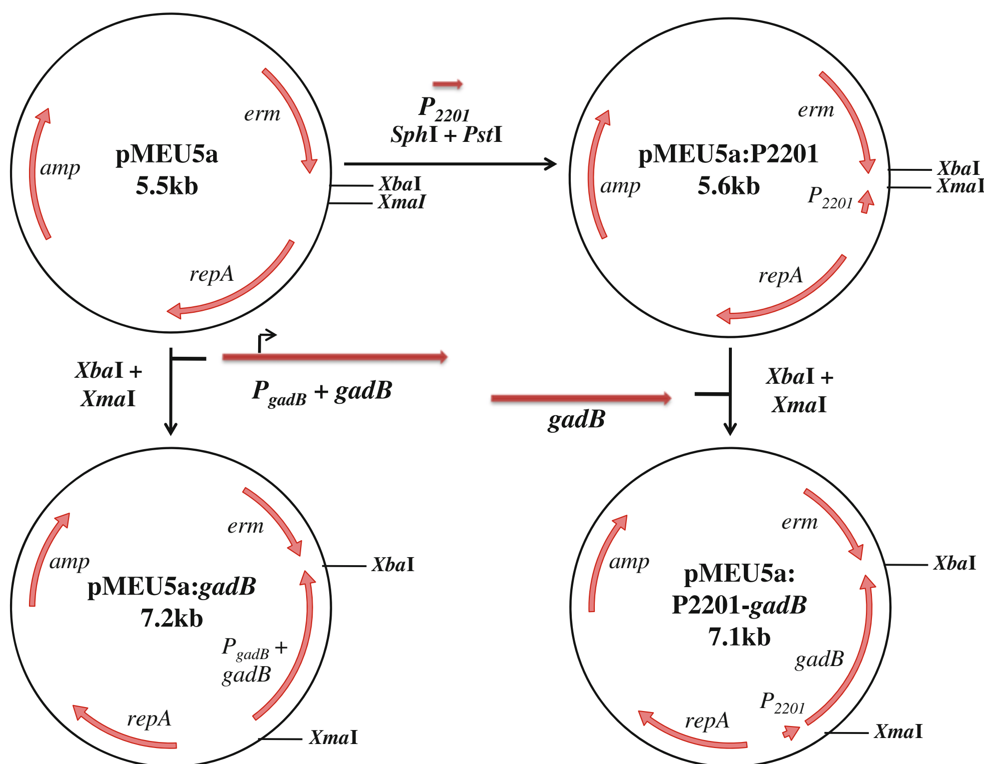

Fig. 1 Construction of vectors for the transport of *gadB* of *Streptococcus thermophilus* ST110

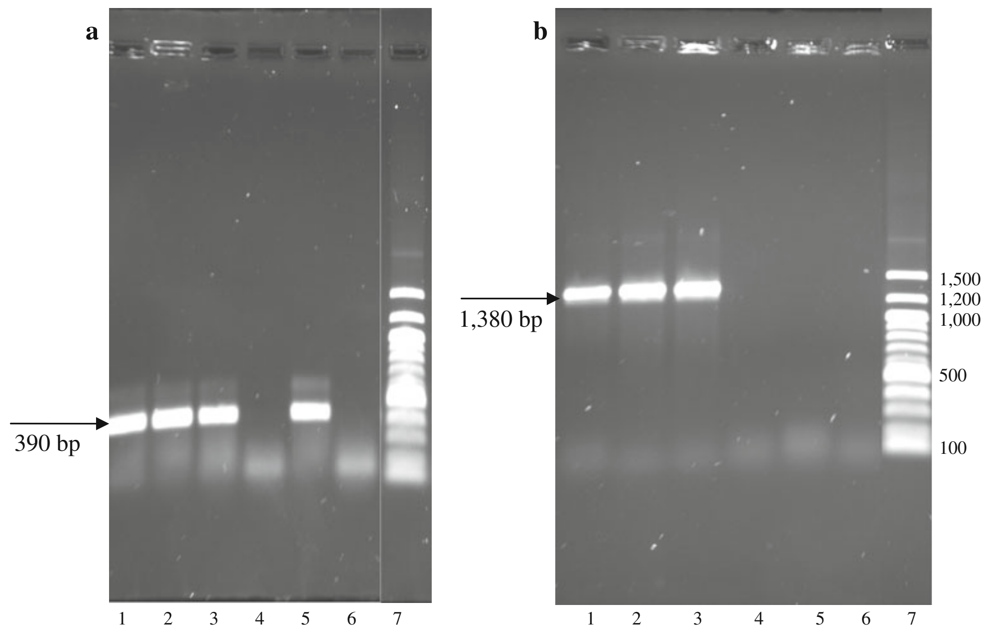

Fig. 2 PCR products obtained with primers for the internal conserved region (**a**) and the full ORF*gadB* (**b**) in six different *Streptococcus thermophilus* strains (*lanes 1–6*); MW marker, kb (*lane 7*)

theoretically corresponded to the structural *gadB* gene encoding the 459-amino-acid glutamic acid decarboxylase (GAD) enzyme (Fig. 2b). Thus, in approximately 50 % of *S. thermophilus* cultures tested the *gadB* was either absent or present in a truncated form.

Position of the *gadB* locus in the *S. thermophilus* ST110 genome

The characterization of the GAD locus and defining its approximate location on the genome were carried out in *S. thermophilus* ST110, which is a strain noted for the production of a bacteriocin with potential for application as a food preservative [5]. This involved the extension of DNA sequencing to the upstream and downstream regions flanking *gadB* in ST110 by the primer walking strategy, which yielded a ca. 5,800-bp polynucleotide fragment. Various parts of the entire sequence of this polynucleotide including *gadB* and its flanking regions were used in similar searches by BLAST analysis of databank sequences. The BLAST analysis of the translational product of the *gadB* locus in strain ST110 (NH_2-MNEKLFREIME…) showed near identity with the *gadB* of *S. thermophilus* strain ND03 (accession number CP002340.1, NH_2-MDEKLFREIME…) with the exception of Asn replacing Asp at the N-terminus of GAD. Further comparison with the complete published genomic sequences of other *S. thermophilus* strains such as LMG-18311 (accession number NC_006448), LMD-9 (accession number NC_008532), CNRZ-1066 (accession number NC_006449), and JIM-8232 (accession number FR875178) revealed that these strains are apparently devoid of the GAD locus or lack even a truncated form of *gadB*. Downstream of *gadB* in *S. thermophilus* ST110, the ORF for a glutamate/GABA antiporter gene (*gadC*) was identified (Fig. 3), which was also reported in strains Y2 [16] and ND03 [28] and is assumed to contribute to the acid-resistant phenotype described for bacterial species expressing GAD [4].

Sequence analysis revealed that in the *S. thermophilus* strain ST110, the *gad*B/*gadC* complex is flanked by transposase elements Tn1216 (5′ and 3′) and Tn1546, and positioned downstream from the *luxS* and upstream from the HD superfamily hydrolase genes (Fig. 3). Alignment of sequence data showed similar features in the GAD^+ strain *S. thermophilus* NDO3 in which the *gad*B/*gadC* complex is downstream from the *luxS* [28]. However, in strain NDO3, an additional ca. 7-kb polynucleotide stretch including transposase (Tn981) and transcriptional regulatory (rggB) elements is present upstream from the HD superfamily hydrolase gene. In other known genomic sequences of

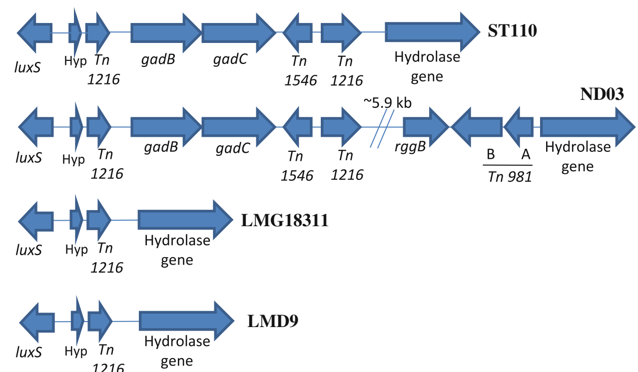

Fig. 3 Putative location of the *gadB/gadC* complex on the chromosome of *S. thermophilus* ST110

S. thermophilus strains lacking the *gadB/C* complex (LMD-9, LMG-18311, CNRZ-1066), only a single copy of Tn1216 is positioned between the *luxS* (5′) and HD superfamily hydrolase (3′) genes (Fig. 3).

Further databank searches revealed significant homology of the ST110 *gadB* with the known sequences reported in other bacteria of the LAB group, including *Lactococcus lactis* ssp. *lactis* (accession number NP-267446, 50 %), *Lactobacillus reuteri* (accession number EDX42043, 50 %), *Lb. plantarum* (accession number NP-786643, 48 %), and *Lb. brevis* (accession number AAZ95185, 47 %).

Production of GABA by GAD$^+$ *S. thermophilus* ST110

Under the experimental conditions used, TYL-grown cells showed the average production of 13.7 μM of GABA per mg of dry ST110 biomass in 1 h.

In milk fermented with ST110, the quantity of GABA produced was proportional to the concentration of exogenous glutamate (MSG) added to the milk. In the control, only a trace amount of GABA was detectable, which was most likely due to the limited amount of free glutamate available during milk fermentation (see Table 1).

Transformation of non-producing ST strains to GAD$^+$ phenotype

The construction of pMEU5a-based vectors harboring the *gadB* of *S. thermophilus* ST110 was carried out in *Escherichia coli* DH5α with the selection of transformants based on ampicillin resistance (AmpR). The correctness of vector construction was confirmed by PCR amplification of the *gadB* insert in pMEU5s:*gadB* (gadB-P1, ca. 1.7 kb), and also the ST2210 promoter-*gad*B fusion product in pMEU5a:ST2201-*gad*B (gadB-P2, ca. 1.6 kb).

Following the recovery and purification of the pMEU5a/*gadB* fusion products from *E. coli* DH5α, they were electrotransformed into the GAD$^-$ *S. thermophilus* ST128. PCR analysis confirmed that nine of the 12 *S. thermophilus* ST128 clones transformed with pMEU5a/*gadB*-P1 tested positive for the 1.7-kb fragment corresponding to the full-length *gadB* with its endogenous promoter. The 12 additional transformants obtained with the pMEU5a/*gadB*-P2 tested positive for the 1.6-kb DNA fragment containing the ST2210 promoter-*gad*B fusion product.

The amount of GABA produced by the ethanol-treated biomass of the recombinant strains was ca. 12 % higher than that obtained with the parent strain ST110 that has *gadB* located on the chromosome. This was probably due to the presence of multiple copies of the *gadB*-bearing vectors in the transformants resulting in an elevated level of GAD activity (Table 2).

Since the GABA concentrations attained with the two types of recombinant strains were similar, it was concluded that the expression of *gadB* was equally well controlled by its native promoter (P1) or an unrelated chromosomal promoter ST2201 (P2) derived from the GAD$^-$ strain ST128. It was also observed that cells collected earlier in the growth phase (i.e., $OD_{660} = 0.7–0.8$) had ca. 10 % higher level of GAD activity than overnight cells.

Discussion

The results of this study demonstrated that ca. 38 % of *S. thermophilus* strains surveyed apparently had the entire *gadB* (1,380 bp), classifying them as putative producers of GAD. The translational product of *gadB* in strain ST110 corresponded to a 459-amino-acid protein that was identical to the GAD of strain Y2 [32] but differed from the GAD of strain ND03 in which aspartic acid replaced asparagine at the N-terminus [28]. The sequence of GAD in ST110 also shared some degree of homology with the GAD found in other food-grade LAB species, such as lactococci and lactobacilli. Additionally, sequence analysis of apparently full-length PCR products showed evidence of frameshift mutation in several strains of *S. thermophilus*, resulting in truncated and inactive forms of GAD (data not shown).

The *gad*B of *S. thermophilus* ST110 and its flanking regions were sequenced and its location on the ST110 chromosome was tentatively identified. The presence of transposase genes for Tn1216 and Tn1546 in the downstream and upstream sequences flanking the *gadB/gadC* operon implied that an ancestral strain of *S. thermophilus* may have originally acquired these ORFs by horizontal gene transfer.

Table 1 GABA production in skim milk fermented by *S. thermophilus* ST110

MSG (mM)	pH (6 h)	GABA (μM)
0	4.34	33
25	4.62	481
50	4.87	655
100	5.04	648

Table 2 GABA production by recombinant strains of *S. thermophilus* ST128

Strain	GABA (μM/mg dry biomass)
ST110 (control)	13.70
ST128	0
ST128/gadB-P1	15.49
ST128/gadB-P2	15.36

The order of *gadB* and *gadC* in ST110 was identical to that found in *S. thermophilus* strain Y2 [16] and strain ND03 [28] but opposite from that reported in *L. lactis* ssp. *lactis* [20] and *Lb. brevis* [8]. The extension of nucleotide sequencing past the 5′ and 3′ ends of the *gadB/gadC* complex, similarity searches in databanks, and comparison with known genomic sequences of several *S. thermophilus* strains (accession numbers CP002340, NC_006448, NC_008532, NC_006449, and FR875178) allowed the putative assignment of the GAD operon between the *luxS* (5′) and the HD superfamily hydrolase (3′) genes.

The $GABA^+$ phenotype was transferable by electro-transformation to other strains of *S. thermophilus* that lack GAD activity but may have other important attributes as starter cultures (e.g., production of flavor and aroma compounds). This was accomplished by using the conventional pMEU5a-based cloning vectors in which the expression of *gadB* was controlled by either its endogenous promoter or the unrelated chromosomal promoter ST2201 that had been previously used for gene transport [3, 19]. Although the cloning of *gadB* from *S. thermophilus* Y2 [16] and *Lb.* brevis OPK-3 [18] in *Escherichia coli* has been reported, this work demonstrates the applicability of the electroporation technique to the successful intra-species transfer of the GAD^+ phenotype to a non-GABA producing strain of the food-grade starter culture *S. thermophilus*. Although the *S. thermophilus* strains used in the study have not been evaluated for attributes (acid, flavor, and aroma compound production, compatibility with *Lb. delbrueckii* ssp. *bulgaricus* starter cultures) preferred by industry, ours is a convenient approach to impart a valuable phenotype to industrial strains that have been already selected for such properties. Further, to avoid reliance on antibiotic resistance genes in the selection of genetic transformants, work is continuing on the development of a total food-grade system for the transport of *gadB* into new LAB hosts.

The results of milk fermentation experiments indicated that the amount of free glutamate available in milk for GABA production is limited, and GABA could be detected only if the milk was supplemented with MSG, which is a drawback similar to that reported for other food systems [14]. Potentially, GABA-producing *S. thermophilus* strains could be used in co-culture with other LAB possessing high levels of protease and peptidase activities that may increase the free glutamate concentration in milk. A similar approach was successful in the development of a new type of milk product that was co-fermented with *Lactococcus lactis* ssp. *lactis* and *Lb. casei* [10].

On account of the recognized beneficial physiological effects of GABA and the increasing consumer interest in the health-promoting properties of foods, commercial demand for GABA-enriched foods is expected to rise. Therefore, the availability of natural food-grade GAD^+ strains of *S. thermophilus* and strains transformed to the GAD^+ phenotype may facilitate the development of this important microbe for a variety of applications as ingredients in dairy-based functional foods, and as microbial factories for industrial GABA production.

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