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Inactivation of the Glutamate Decarboxylase Gene in *Lactococcus lactis* subsp. *cremoris*

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***Lactococcus lactis* subsp. *lactis* strains show glutamate decarboxylase activity, whereas *L. lactis* subsp. *cremoris* strains do not. The *gadB* gene encoding glutamate decarboxylase was detected in the *L. lactis* subsp. *cremoris* genome but was poorly expressed. Sequence analysis showed that the gene is inactivated by the frameshift mutation and encoded in a nonfunctional protein.**

Lactococcal strains are essential to cheese manufacture and in the early stages of ripening. The species *Lactococcus lactis* is subdivided into *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* on the basis of physiological properties. *L. lactis* strains can be classed as two phylogenetic groups by Southern hybridization (2) and by DNA sequence analyses (8, 11). It has been proposed that the subspecies diagnoses be redefined to reflect these natural relationships (2). However, the classification of subspecies based on phenotypes is of primary importance in the dairy industry. Phenotypes to distinguish two subspecies of *L. lactis* have been reported (1, 5), and classification is based on the following criteria: growth in 4% NaCl, pH 9.2, at 40°C; the ability to hydrolyze arginine; and sensitivity to lithium chloride. These criteria, however, have not yet been elucidated at the molecular level. Recently, a novel criterion for distinguishing *L. lactis* subsp. *lactis* from *L. lactis* subsp. *cremoris* was reported; glutamate decarboxylase (GAD; EC 4.1.1.15) activity was observed in *L. lactis* subsp. *lactis* and not in *L. lactis* subsp. *cremoris* (6).

GAD catalyzes the irreversible decarboxylation of glutamate to γ-aminobutyric acid (GABA). GAD constitutes a glutamate-dependent acid resistance mechanism with a glutamate-GABA antiporter (10). The introduction of a defect in the *gadC* and *gadB* genes, which encode the glutamate-GABA antiporter and GAD, has been shown to reduce acid resistance in *L. lactis* (9). Based on the sequence analysis, it has been suggested that *L. lactis gadCB* forms an operon present in one copy in the chromosome (7, 9). Here we studied the sequence of *gadB* genes in *L. lactis* subsp. *lactis* and in *L. lactis* subsp. *cremoris*.

L. lactis subsp. *lactis* biovar diacetylactis ATCC 13675, *L. lactis* subsp. *lactis* ATCC 19435, and *L. lactis* subsp. *cremoris* ATCC 19257 were obtained from the American Type Culture Collection (Manassas, Va.). Other strains tested were from laboratory collections and had been isolated from dairy products or cheese starters (6). The bacteria were maintained in sterile litmus milk and subcultured once a week. TYG medium consisted of 0.5% tryptone (Difco), 0.5% yeast extract (Difco), and 1.0% glucose, the pH of which was adjusted to 7.0 with 1 M NaOH. Actively growing cultures were obtained by transferring 1% inoculum to TYG or modified M17 (9) and incubating at 30°C for 16 h.

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Genomic DNA from *L. lactis* was isolated by the method described previously (7). Total RNA was isolated as follows: actively growing culture (0.5 ml) was transferred to 50 ml of TYG medium or M17 supplemented with 50 mM L-glutamate and 0.3 M NaCl. Cells were harvested at late log phase by centrifugation at 1,800 $\times$ *g* for 20 min and resuspended with 100 μl of 50 mM Tris-Cl (pH 7.4) containing 25% (wt/vol) sucrose, 3 mM $MgCl_2$, and 0.1 mg of lysozyme per ml (12). The suspension was incubated at 4°C for 10 min. Total RNA was extracted from the suspension with 1.5 ml of ISOGEN (Nippongene, Toyama, Japan) according to the manufacturer's instructions. The RNA fractions were treated with DNase I (3.5 U) in 50 μl of 100 mM sodium acetate buffer (pH 5.0) containing 5 mM $MnCl_2$ at 37°C for 10 min to remove any contaminating DNA. RNA was extracted with phenol-chloroform, precipitated with 2-propanol, and stored at −80°C until use.

Total DNAs from *L. lactis* were digested, separated on 1.0% agarose, and transferred to Hybond-N (Amersham). The Southern blots were hybridized with the 2.4-kb *Xba*I-*Eco*RI fragment of *L. lactis* 01-7 *gadCB* (7). Hybridization was performed at 68°C. The filter was washed twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% sodium dodecyl sulfate at room temperature and then twice in 0.1× SSC–0.1% sodium dodecyl sulfate at 68°C.

The *gadCB* fragments were amplified by PCR, using the total *L. lactis* DNA or the first-strand cDNA as the template. The PCR primers were designed from the published sequence of *L. lactis gadCB* (7). The sense primer was 5′-GTTTTGTT GTGACTGCTATCTTGCCA-3′ (nucleotides 704 to 729), and the antisense primer was 5′-TTTTTGGGAAGTGGATAAG CAGGCA-3′ (nucleotides 2136 to 2112). The length of the expected amplified fragment was 1,433 bp. Fifty microliters of each PCR mixture contained 200 ng of DNA, 20 pmol of each of the primers, reagent mix, and AmpliTaq Gold DNA polymerase (Perkin-Elmer, Foster City, Calif.). PCR amplification was conducted with a GeneAmp PCR System 2400 (Perkin-Elmer). The following 35 or 45 cycles of amplification were performed: denaturation for 30 s at 94°C, annealing for 30 s at 55°C, and extension for 90 s at 72°C. Amplified double-stranded DNA was purified by electrophoresis on a 1.5% agarose gel for direct sequencing. Both strands of purified DNA were sequenced with a *Taq* Dye Terminator cycle sequencing kit and a model 373A DNA sequencer (both from Applied Biosystems, Foster City, Calif.). From the nucleotide sequence, the amino acid sequence was deduced. The amplification of *ldh* was carried out as described previously (3).

Total RNA (200 ng) was added to RTG reverse transcrip-

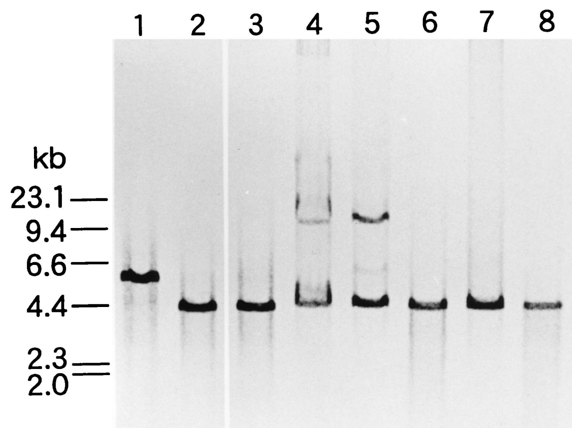

FIG. 1. Southern blot analysis of *L. lactis gadB*. The DNA was digested with *Eco*RI. The blot was probed with a digoxigenin-labeled 2.4-kb *Xba*I-*Eco*RI fragment (7). Lanes: 1, *L. lactis* subsp. *lactis* biovar diacetylactis 01-7; 2, *L. lactis* subsp. *lactis* biovar diacetylactis ATCC 13675; 3, *L. lactis* subsp. *lactis* ATCC 19435; 4, *L. lactis* subsp. *lactis* 712; 5, *L. lactis* subsp. *lactis* SlN; 6, *L. lactis* subsp. *cremoris* ATCC 19257; 7, *L. lactis* subsp. *cremoris* H-61; 8, *L. lactis* subsp. *cremoris* HP.

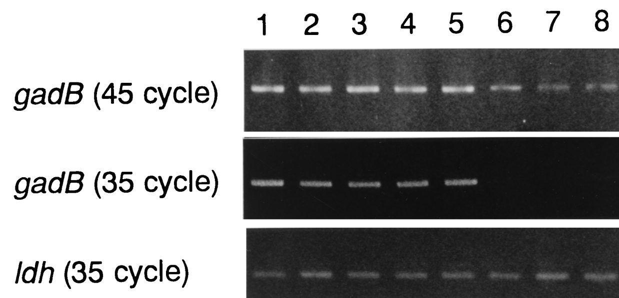

FIG. 2. RT-PCR analysis for *gadB* mRNA. Lanes: 1, *L. lactis* subsp. *lactis* biovar diacetylactis 01-7; 2, *L. lactis* subsp. *lactis* biovar diacetylactis ATCC 13675; 3, *L. lactis* subsp. *lactis* ATCC 19435; 4, *L. lactis* subsp. *lactis* 712; 5, *L. lactis* subsp. *lactis* SlN; 6, *L. lactis* subsp. *cremoris* ATCC 19257; 7, *L. lactis* subsp. *cremoris* H-61; 8, *L. lactis* subsp. *cremoris* HP.

tion (RT)-PCR reagent with the d(N)$_6$ random hexamer (Amersham Pharmacia Biotech, Piscataway, N.J.). The first-strand synthesis reaction was carried out at 42°C for 30 min. The mixture was heated at 95°C for 5 min, and then the *gadB*-specific primer was added. The *gadB* amplification was performed as described above.

In order to study the genetic basis of non-GABA productivity, three *L. lactis* subsp. *cremoris* strains were compared with six *L. lactis* subsp. *lactis* strains by Southern hybridization. A fragment of *L. lactis* 01-7 *gadCB* was used as a probe. Positive hybridizing bands were observed with all strains (Fig. 1). Restriction fragment length polymorphism was observed among the strains examined. PCR amplification of six *L. lactis* subsp. *lactis* and three *L. lactis* subsp. *cremoris* strains was performed. The 1.4-kb fragments were amplified from each of the samples (data not shown). The sizes of the amplified products corresponded to the sizes predicted from the published *gadCB* sequence (7), and no nonspecific amplification was observed. We believe that the pair of primers designed was useful for the amplification of *L. lactis gadB*. The results indicated that the *gadCB* genes are also present in *L. lactis* subsp. *cremoris* and that they are not grossly rearranged by insertions or deletions of large fragments.

To detect a *gadB* transcription, RT-PCR analysis was performed with the successful primer pair by using the first-strand cDNA as a template. The 1.4-kb positive signals were observed with both *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* when amplification was performed with 45 cycles (Fig. 2). The signals of *L. lactis* subsp. *cremoris* could not be detected with 35 cycles of amplification, while that of *L. lactis* subsp. *lactis* was clearly observed. The amplifications of *ldh* were observed at equivalent intensities, indicating that the RNAs from *L. lactis* subsp. *cremoris* were not degraded. *L. lactis gadB* was transcribed to mRNA not only in *L. lactis* subsp. *lactis* but also in *L. lactis* subsp. *cremoris*, although the amount of the latter was slight. When the cells were cultured in the medium without glutamate and NaCl, no amplification was observed with any of the strains investigated (data not shown).

The *gadB* gene of *L. lactis* subsp. *cremoris* ATCC 19257 was sequenced and compared with that of *L. lactis* subsp. *lactis* (7). The DNA sequence from ATCC 19257 revealed close homology to *L. lactis* subsp. *lactis* 01-7 (95.4%). However, a one-base deletion of adenine and a one-base insertion of thymine were detected within the coding region, resulting in frameshift mutations. The regions around these two mutations were subsequently sequenced in other *L. lactis* subsp. *cremoris* strains to confirm that the mutations are common. The adenine deletion was conserved in six of seven strains (the exception was H-61), and the thymine insertion was detected in all seven strains (Fig. 3). The resulting frameshift from these mutations created stop codons and was thought to affect GadB of *L. lactis* subsp. *cremoris*. Among the adenine-deleted strains and in strain H-61, the GadB proteins were truncated to 102 and 405 amino acids, respectively, while the counterpart of *L. lactis* subsp. *lactis* typically contains 466 amino acids. Given that the coding region of strain H-61 *gadB* was not sequenced completely, the product might be further truncated by additional mutations within the unsequenced region.

The GAD gene has been observed to exist in the *L. lactis* subsp. *cremoris* genome and to transcribe to mRNA. The transcription was not induced by acid and chloride stress, which do induce the expression of *gadCB* in *L. lactis* (9). The transcription was therefore insufficient. Because of the frameshift resulting from a one-base insertion or deletion within the coding region, the translated protein was not functional. Given that a 55-residue segment around the active-center lysine of GAD is highly conserved (4), the segment is considered important to the expression of enzymatic activity. An adenine deletion, which creates a stop codon, was observed in six of seven GAD-negative strains. Protein synthesis was terminated by the stop codon, and the truncated protein was considered to show no activity because the active-center lysine was not synthesized. Although the remaining strain, H-61, was also GAD negative (6), the adenine deletion was not detected in it. In H-61 GadB, the active-center lysine could be translated but the C-terminal region was not synthesized as a result of the thymine insertion. As a result of the deformation, GadB in strain H-61 could not be folded exactly and thus could not fulfill its function. For expression of GAD activity, it is necessary that the conserved segment exist not only around the active center but also in the sequence of the C-terminal region. Given that the thymine insertion was observed in all *L. lactis* subsp. *cremoris* strains, such a mutation would be more remarkable than the adenine deletion.

This study indicated that *gadB* of *L. lactis* subsp. *cremoris* was present but was poorly expressed and encoded in a nonfunctional protein. To our knowledge, this is the first study to

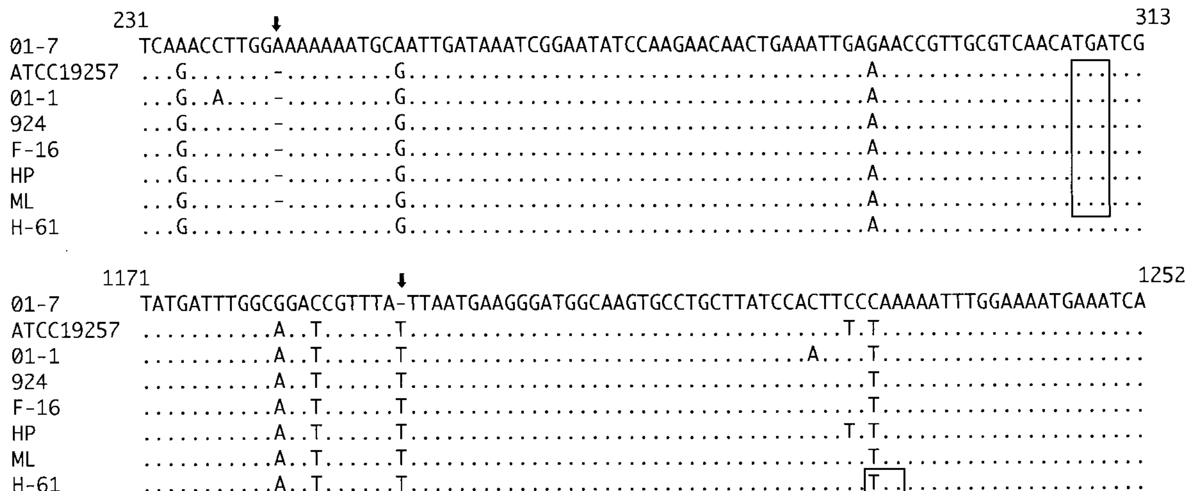

FIG. 3. Comparison of the nucleotide sequences of *gadB* from *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*. The first line is the sequence from *L. lactis* subsp. *lactis* strain 01-7 (7). The second and the following lines are from *L. lactis* subsp. *cremoris*. The insertion and the deletion sites are indicated with arrows. The stop codons created by the frameshift are boxed. Numbers are counted from the translational start point of GadB of strain 01-7.

establish the nature of lesions affecting the criteria that distinguish *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* at the molecular level.

Nucleotide sequence accession numbers. The DDBJ accession numbers for the sequences reported in this paper are AB033218 to AB033230.

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