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Rapid PCR-Based Method Which Can Determine Both Phenotype and Genotype of *Lactococcus lactis* Subspecies

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A highly efficient, rapid, and reliable PCR-based method for distinguishing *Lactococcus lactis* subspecies (*L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*) is described. Primers complementary to positions in the glutamate decarboxylase gene have been constructed. PCR analysis with extracted DNA or with cells of different *L. lactis* strains resulted in specific fragments. The length polymorphism of the PCR fragments allowed a clear distinction of the *L. lactis* subspecies. The amplified fragment length polymorphism with the primers and the restriction fragment length polymorphism of the amplified products agreed perfectly with the identification based on genotypic and phenotypic analyses, respectively. Isolates from cheese starters were investigated by this method, and amplified fragments of genetic variants were found to be approximately 40 bp shorter than the typical *L. lactis* subsp. *cremoris* fragments.

Lactococcal strains are essential to milk fermentation, especially in the cheese-making process, providing optimal conditions for curd formation and for the development of texture and flavor. It is important to the dairy industry to identify new strains of *Lactococcus lactis* for cheese manufacture. Dairy lactococcal strains are subdivided into *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* based on a few phenotypic tests: growth in 4% NaCl, pH 9.2, at 40°C; the ability to hydrolyze arginine (7); and sensitivity to lithium chloride (1).

Recently, a novel criterion for distinguishing *L. lactis* subsp. *lactis* from *L. lactis* subsp. *cremoris* has been reported: glutamate decarboxylase (GAD; EC 4.1.1.15) activity, which has been observed in *L. lactis* subsp. *lactis* and not in *L. lactis* subsp. *cremoris* (8). 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 (12). The *gadB* gene encoding *L. lactis* subsp. *cremoris* GAD was apparently inactivated by a frameshift mutation resulting from an adenine deletion or a thymine insertion and encoded a nonfunctional protein (10).

It has been discovered in recent years that *L. lactis* strains can be divided into two phylogenetic groups based on genotypic analysis (6, 11, 13), and it has been proposed that the subspecies diagnoses be redefined to reflect these natural relationships (6). The new taxonomic system requires the transfer of several strains across subspecies lines, from *L. lactis* subsp. *lactis* to *L. lactis* subsp. *cremoris* and vice versa.

As classification according to phenotypic criteria is complicated and requires skill, simple, fast, and reliable molecular methods have been developed (3, 5). Classification based on these methods, however, coincides with genotypic identification and does not always correlate with phenotypic characterization. The classification of *L. lactis* subspecies based on phenotypic characteristics is of primary importance in the dairy industry, as phenotypes directly reveal the abilities required in milk fermentation. It has been reported that PCR analysis with oligonucleotide primers designed for the *rrnB-rrnC* region correlates with phenotypic characterization (2).

In this paper, we describe useful PCR primers for distinguishing *L. lactis* subspecies. Genotypic and phenotypic characteristics of *L. lactis* can be determined by one PCR and subsequent nuclease digestion. Results of amplified fragment length polymorphism analysis with these primers agreed with the genotypic identification, and the restriction fragment length polymorphism (RFLP) of the amplified products concurred with the phenotypic identification.

In addition, 37 isolates of *L. lactis* were examined by the PCR-based method and by phenotypic differentiation, with strains being differentiated to the subspecies level. Four of 20 isolates of *L. lactis* subsp. *cremoris* were found to have shorter fragments than the other *L. lactis* subsp. *cremoris* isolates. The amplified fragments of these strains were sequenced.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *L. lactis* subsp. *lactis* strains ATCC 9936 and ATCC 19435, *L. lactis* subsp. *lactis* biovar diacetylactis ATCC 13675, *L. lactis* subsp. *cremoris* ATCC 19257, *Leuconostoc mesenteroides* ATCC 8293, and *Lactobacillus casei* ATCC 393 were obtained from the American Type Culture Collection (Manassas, Va.). *Enterococcus faecalis* IFO 12964 was obtained from the Institute for Fermentation, Osaka (Osaka, Japan). *Streptococcus thermophilus* 9Y was from laboratory collections. Other *L. lactis* strains and their growth conditions have been described previously (8). Phenotypic characteristics were determined as described previously (8). Genotypic characterization of *L. lactis* was performed by PCR analysis with primers PALA-4 and PALA-14, as described by Garde et al. (5). Actively growing cultures were obtained by transferring a 1% inoculum to tryptone-yeast extract-glucose (10), M17 broth (14) containing 0.5% glucose (GM17), or MRS broth (4) and then incubating the cultures at 30°C for 16 h.

PCR and restriction endonuclease digestion. Preparation of genomic DNA has been described previously (10). The sense primer (gadB21) was 5′-CGTTA TGGATTTGATGGATATAAAGC-3′, located within the *gadB* gene, and the antisense primer (GAD7) was 5′-ACTCTTCTTAAGAACAAGTTTAACAGC-3′, which is located downstream from the gene. Each 50 μl of PCR mixture contained 200 ng of genomic DNA, 20 pmol of each primer, reagent mixture, and

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TABLE 1. Sources of the *L. lactis gadB* gene sequences

Strain	Accession no.	Reference or source
L. lactis subsp. *lactis* biovar diacetylactis NIAI 01-7	AB010789	9
L. lactis subsp. *lactis* ATCC 19435	AB067750	This study
L. lactis subsp. *cremoris*		
MG1363	AF005098	12
ATCC 19257	AB033218	10
NIAI 01-1	AB033220	10
NIAI 53-2	AB067751	This study
924	AB033222	10
F-16	AB033224	10
HP	AB033226	10
ML	AB033228	10
H-61	AB033230	10

Ampli *Taq* gold DNA polymerase (Perkin-Elmer, Foster City, Calif.). PCR amplification was conducted with a GeneAmp PCR System 2400 (Perkin-Elmer). The PCR conditions were as follows: denaturation at 94°C for 9 min, followed by 45 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 60 s, with an additional extension of 7 min at 72°C after the last cycle. The amplified fragments were digested with *Ase*I restriction endonuclease (Toyobo, Tokyo, Japan) according to a supplier's instructions. The fragments were run on a 4% NuSieve GTG agarose gel (BioWhittaker Molecular Applications, Rockland, Maine) and were stained with ethidium bromide.

DNA sequence analysis. Amplified double-stranded DNA was purified by electrophoresis on a 4% NuSieve GTG agarose gel for direct sequencing. Both strands of purified DNA were sequenced with a DYEnamic ET terminator cycle sequencing kit (Amersham Pharmacia Biotech, Piscataway, N.J.) and a 373A DNA sequencer (Applied Biosystems, Foster City, Calif.). The DNA sequences used in this study are listed in Table 1. The 16S rRNA gene was amplified and sequenced as described previously (15).

Nucleotide sequence accession numbers. The sequences of the *gadB* genes of *L. lactis* subsp. *lactis* ATCC 19435 and *L. lactis* subsp. *cremoris* NIAI 53-2 have been assigned accession numbers AB067750 and AB067751, respectively.

RESULTS

The genotypes and phenotypes of *L. lactis* strains used in this study are summarized in Table 2. *L. lactis* strain MG1363 is classified as *L. lactis* subsp. *cremoris* based on its genotypic traits, although it shows the phenotypic abilities of *L. lactis* subsp. *lactis*. Strain MG1363 also exhibits GAD activity. Strain IL1403 showed the ability to produce GABA like the typical *L. lactis* subsp. *lactis* although it could not hydrolyze arginine.

At first, the *gadB* sequences of *L. lactis* subsp. *cremoris* strains ATCC 19257 and MG1363 were compared with those of *L. lactis* subsp. *lactis* NIAI 01-7 and ATCC 19435 (Fig. 1). Two specific bases were observed only in the sequence of strain 01-7 (C^{2331} and T^{2382}). At the 3′ untranslated region of *L. lactis* subsp. *cremoris gadB* (including strain MG1363), three fragmentary deletions were observed: a 24-bp deletion upstream of the stem-loop structure of the transcription terminator and two 6-bp deletions downstream of the stem-loop. Further, an additional 1-bp deletion in GAD-negative strains such as *L. lactis* subsp. *cremoris* ATCC 19257 was observed (Fig. 1). These deletions were also commonly observed in another six strains of GAD-negative *L. lactis* subsp. *cremoris* NIAI 01-1, 924, F-16, H-61, HP, and ML (data not shown). The PCR primers were designed to amplify the sequences comprising both the thymine insertion within the coding region (10) and the deletions at the 3′ untranslated region. The positions of these primers on the sequence determined by Nomura et al. (9) were 1901 to 1926 and 2502 to 2476, respectively. The lengths of the expected amplified fragments were 602 bp for the *L. lactis* subsp. *lactis*-type strain and 564 bp for *L. lactis* subsp. *cremoris*.

TABLE 2. Phenotypic and genotypic characteristics of *L. lactis* strains

Strain	NH_3 from arginine	GABA from glutamate	Amplification by PCR of:	
			1,131 bp	~700 bp
L. lactis subsp. *lactis*				
ATCC 19435	+	+	+	+
NIAI 527	+	+	+	+
ATCC 9936	+	+	+	+
IL1403	−	+	+	+
L. lactis subsp. *lactis* biovar diacetylactis				
ATCC 13675	+	+	+	+
NIAI 01-7	+	+	+	+
DRC1	+	+	+	+
17 isolates[a]	+	+	ND[b]	ND
L. lactis subsp. *cremoris*				
MG1363	+	+	+	−
ATCC 19257	−	−	+	−
NIAI 01-1	−	−	+	−
924	−	−	+	−
F-16	−	−	+	−
HP	−	−	+	−
ML	−	−	+	−
H-61	−	−	+	−
20 isolates[a]	−	−	ND	ND

[a] Isolated in previous work (8).
[b] ND, not determined.

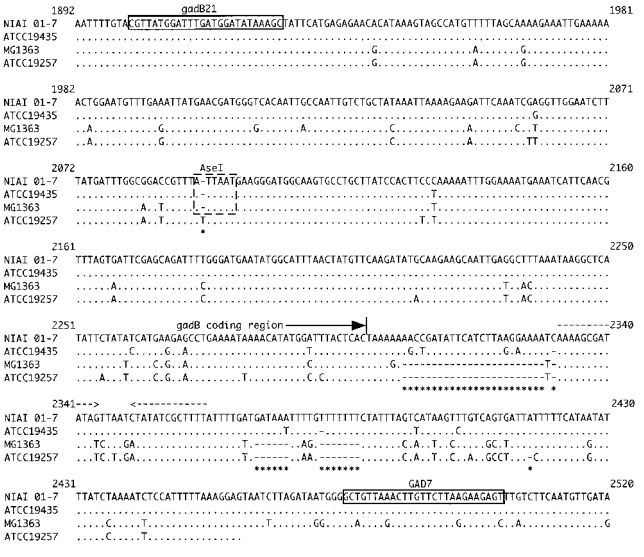

FIG. 1. Multiple alignment of *gadB* sequences from *L. lactis* subsp. *lactis* NIAI 01-7, *L. lactis* subsp. *lactis* ATCC 19435, *L. lactis* subsp. *cremoris* MG1363, and *L. lactis* subsp. *cremoris* ATCC 19257. PCR primers are boxed. Dashed box, *Ase*I restriction site; dashed arrows, inverted repeat, suggestive of a transcription terminator (not including the poly[T] stretch); asterisks, positions of insertions or deletions. The numbering is according to the sequence of *L. lactis* subsp. *lactis* NIAI 01-7 reported by Nomura et al. (9).

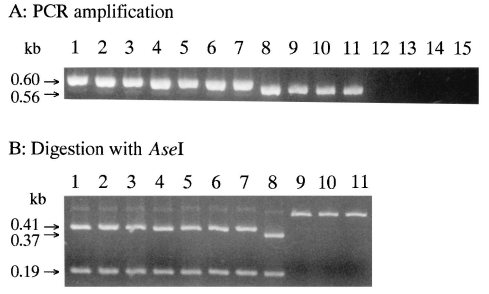

FIG. 2. PCR amplification and digestion with *Ase*I of *L. lactis gadB*. Conditions for PCR and electrophoresis are described in Materials and Methods. Lanes: 1, *L. lactis* subsp. *lactis* biovar diacetylactis ATCC 13675; 2, *L. lactis* subsp. *lactis* biovar diacetylactis NIAI 01-7; 3, *L. lactis* subsp. *lactis* biovar diacetylactis DRC1; 4, *L. lactis* subsp. *lactis* ATCC 19435; 5, *L. lactis* subsp. *lactis* NIAI 527; 6, *L. lactis* subsp. *lactis* ATCC 9936; 7, *L. lactis* subsp. *lactis* IL1403; 8, *L. lactis* subsp. *cremoris* MG1363; 9, *L. lactis* subsp. *cremoris* ATCC 19257; 10, *L. lactis* subsp. *cremoris* H-61; 11, *L. lactis* subsp. *cremoris* HP; 12, *S. thermophilus* 9Y; 13, *Leuconostoc mesenteroides* ATCC 8293; 14, *E. faecalis* IFO 12964; 15, *Lactobacillus casei* ATCC 393.

PCR amplification of seven *L. lactis* subsp. *lactis* strains and four *L. lactis* subsp. *cremoris* strains was performed. Specific DNA amplification was observed with all *L. lactis* strains tested, but not with other species such as *S. thermophilus* 9Y, *Leuconostoc mesenteroides* ATCC 8293, *E. faecalis* IFO 12964, and *Lactobacillus casei* ATCC 393 (Fig. 2A). The sizes of the amplified products were approximately 600 bp for *L. lactis* subsp. *lactis* and *L. lactis* subsp. *lactis* biovar diacetylactis (Fig. 2A, lanes 1 to 7) and approximately 560 bp for *L. lactis* subsp. *cremoris* (Fig. 2A, lanes 8 to 11). The product from strain MG1363, which is a GAD-positive *L. lactis* subsp. *cremoris* strain, revealed close to 560 bp that were like those of the GAD-negative strains, as predicted from the DNA sequence (12) (Fig. 2A, lane 8).

PCR fragments of *L. lactis* subsp. *lactis* were cut into portions of approximately 190 and 410 bp with endonuclease *Ase*I, while those of *L. lactis* subsp. *cremoris* were not (Fig. 2B). The PCR product of *L. lactis* subsp. *cremoris* MG1363 was also digested into two fragments as were those of *L. lactis* subsp. *lactis*, although the sizes of the resulting fragments were different (approximately 190 and 370 bp) (Fig. 2B, lane 8). The results of the PCR-RFLP analyses are summarized in Table 3. For each strain, digestion with *Ase*I appeared to correspond to the GAD phenotype.

Bacterial cells were subsequently investigated with PCR templates instead of extracted DNA. A bacterial culture grown in tryptone-yeast extract-glucose, GM17, or MRS medium was sequentially diluted with sterile deionized water, and a 1-μl portion was used as a template. In addition, a colony on the agar plate was picked up with a sterile toothpick and then suspended directly into a PCR mixture. Amplification was observed with a culture broth diluted from 10- to 10^4-fold but not with undiluted broth. The reaction was also observed with a cell from the colony. The PCR fragments resulting from both cell preparations were the same as those obtained with the extracted DNA (data not shown). These results indicate that vegetative cells can be substituted for genomic DNA as a template for PCR.

TABLE 3. PCR fragments and RFLP analyses for *gadB* gene in *L. lactis*

Strain	PCR fragment of:		Digestion with *Ase*I
	~600 bp	~560 bp	
L. lactis subsp. *lactis* biovar diacetylactis			
ATCC 13675	+	−	+
NIAI 01-7	+	−	+
DRC1	+	−	+
L. lactis subsp. *lactis*			
ATCC 19435	+	−	+
NIAI 527	+	−	+
ATCC 9936	+	−	+
IL1403	+	−	+
L. lactis subsp. *cremoris*			
MG1363	−	+	+
ATCC 19257	−	+	−
H-61	−	+	−
HP	−	+	−
S. thermophilus 9Y	−	−	ND[a]
Leuconostoc mesenteroides ATCC 8293	−	−	ND
E. faecalis IFO 12964	−	−	ND
Lactobacillus casei ATCC 393	−	−	ND

[a] ND, not determined.

Thirty-seven isolates from cheese starters were investigated by this method. Based on some phenotypic characteristics, 17 of the 37 strains were identified as *L. lactis* subsp. *lactis* and 20 were identified as *L. lactis* subsp. *cremoris* (8). Fragments with the expected lengths were amplified from all 17 strains of *L. lactis* subsp. *lactis* and from 16 of 20 strains of *L. lactis* subsp. *cremoris* (data not shown). The PCR fragments of the remaining *L. lactis* subsp. *cremoris* strains, 53-2, 53-4, 53-6, and 53-8, were slightly shorter than those of the typical *L. lactis* subsp. *cremoris* strains (Fig. 3). The apparent sizes of the fragments were estimated to be approximately 520 bp. The fragments of strains 53-2 and 53-6 were sequenced, and it was found that the sequences were identical to each other. The sequences were compared with those of common *L. lactis* subsp. *cremoris* strains (Fig. 4). A further 43-bp deletion from the end of the coding region to the transcription terminator was observed. Two deletions downstream of the stem-loop (both 6 bp) were conserved. The expected length of each amplified fragment was 521 bp, which was the size estimated from gel electrophoresis.

The 16S rRNA gene sequences of strains 53-2 and 53-6 were determined. The sizes of the amplified sequences were 300 bp. The two sequences were identical to each other and were found to have an identity to *L. lactis* subsp. *cremoris* (11). These results suggest that strains 53-2 and 53-6 should be classified as *L. lactis* subsp. *cremoris*, although *gadB* of these strains exhibited genetic polymorphism.

DISCUSSION

To improve starter cultures for the dairy industry, isolation and classification of new *L. lactis* strains from nature have been carried out. Dairy products are made by good use of pheno-

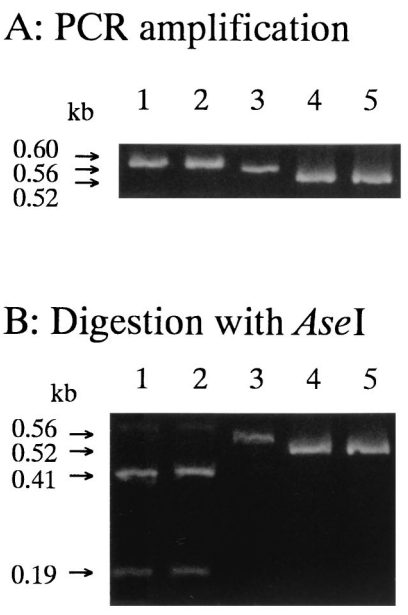

FIG. 3. PCR amplification and digestion with *Ase*I of the *gadB* gene in isolates from cheese starters. Conditions for PCR and electrophoresis are described in Materials and Methods. Lanes: 1, *L. lactis* subsp. *lactis* biovar diacetylactis NIAI 01-7; 2, *L. lactis* subsp. *lactis* ATCC 19435; 3, *L. lactis* subsp. *cremoris* ATCC 19257; 4, *L. lactis* subsp. *cremoris* 53-2; 5, *L. lactis* subsp. *cremoris* 53-6.

typic properties of starter organisms. Therefore, it is necessary to know the phenotypes of isolates for application to the dairy industry. However, the traditional microbiological characterization is usually time-consuming and requires skill. The simple and reliable methods elucidating phenotypic characteristics are extremely useful in obtaining new strains. A nucleic acid-based approach can provide technical simplicity and accuracy for differentiation of strains. For *L. lactis* subspecies identification, Southern hybridization (11) and PCR (3, 5) have been developed. These methods, however, are based on genetics. Genotypic identification does not always correlate with phenotypes. We, therefore, investigated a PCR-RFLP technique that specifically differentiates *L. lactis* subspecies.

In this study, some fragmentary deletions were observed at the 3′ untranslated region of *L. lactis* subsp. *cremoris gadB*. Since the PCR primers were designed to amplify the deletion region, the lengths of the products from *L. lactis* subsp. *cremoris* were 37 bp shorter than those of *L. lactis* subsp. *lactis*. The amplified fragment length polymorphism concurrent with the genotypic classification.

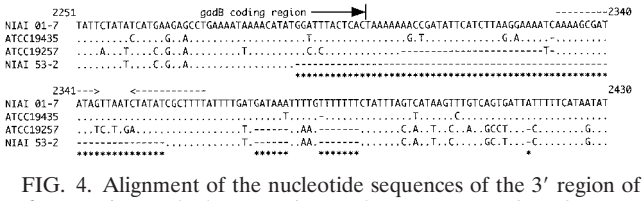

FIG. 4. Alignment of the nucleotide sequences of the 3′ region of *L. lactis gadB*. Dashed arrows, inverted repeat, suggestive of a transcription terminator (not including the poly[T] stretch); asterisks, positions of insertions or deletions. Base pair numbering is as in Fig. 1.

A common thymine insertion within the coding region of *L. lactis* subsp. *cremoris gadB* has been observed (10). The insertion site is included in the amplified region with primers gadB21 and GAD7. An *Ase*I site present in the amplified region of *L. lactis* subsp. *lactis* is absent in the counterpart of *L. lactis* subsp. *cremoris* due to this thymine insertion. Thus, the PCR products of *L. lactis* subsp. *lactis* can be cut with *Ase*I into two fragments, while those of *L. lactis* subsp. *cremoris* cannot. Digestion with *Ase*I appears to occur concurrently with GAD activity, making it a criterion for phenotypic classification (8).

Isolation of new strains is usually performed by picking a single colony grown on an agar plate. In this study, *L. lactis* could be detected by PCR using the cells of isolates as templates. After isolation from a plate, the isolates can be subsequently subjected to PCR without cultivation and DNA preparation.

The primers in this study identified all *L. lactis* strains tested and distinguished them to the subspecies level. The non-*L. lactis* strains showed no positive result. The assay is not affected by physiological cell parameters, and the identification procedure leads to reliable results in fast and easy steps. Unlike other available molecular techniques, this PCR assay can be used to determine genotypes and phenotypes of *L. lactis* subspecies.

Some mutations in the primer recognition sequence make it impossible to amplify a fragment. In this case, the fragment cannot be detected even if the unknown organism is *L. lactis*. Since there was a strain, 53-2, with a deletion greater than that found in a typical *L. lactis* subsp. *cremoris* strain, it is possible that there is a variant with a further deletion in the sequence complementary to the primers. It is also possible that the recognition sequence is replaced and becomes impossible to anneal with the primers.

The restriction enzyme site used for RFLP analysis is conserved in *L. lactis* subsp. *lactis* strains currently investigated. If there is a variant in which GAD is inactivated by mutations of other parts in *gadB* gene, even if the sequence of the restriction site is typical, the results of RFLP and phenotyping will not be in agreement.

This PCR-RFLP technique is useful for screening and grouping new lactococcal isolates. It is critical that the techniques used to differentiate between *L. lactis* subspecies allow for correlation between phenotypic and genotypic identification.

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