Specific point mutations in *Lactobacillus casei* ATCC 27139 cause a phenotype switch from Lac− to Lac+

Yu-Kuo Tsai, Hung-Wen Chen, Ta-Chun Lo and Thy-Hou Lin

Lactose metabolism is a changeable phenotype in strains of *Lactobacillus casei*. In this study, we found that *L. casei* ATCC 27139 was unable to utilize lactose. However, when exposed to lactose as the sole carbon source, spontaneous Lac+ clones could be obtained. A gene cluster (lacTEGF–galKETRM) involved in the metabolism of lactose and galactose in *L. casei* ATCC 27139 (Lac−) and its Lac+ revertant (designated strain R1) was sequenced and characterized. We found that only one nucleotide, located in the lacTEGF promoter (lacTp), of the two lac–gal gene clusters was different. The protein sequence identity between the lac–gal gene cluster and those reported previously for some *L. casei* (Lac+) strains was high; namely, 96–100% identity was found and no premature stop codon was identified. A single point mutation located within the lacTp promoter region was also detected for each of the 41 other independently isolated Lac+ revertants of *L. casei* ATCC 27139. The revertants could be divided into six classes based on the positions of the point mutations detected. Primer extension experiments conducted on transcription from lacTp revealed that the lacTp promoter of these six classes of Lac+ revertants was functional, while that of *L. casei* ATCC 27139 was not. Northern blotting experiments further confirmed that the lacTEGF operon of strain R1 was induced by lactose but suppressed by glucose, whereas no blotting signal was ever detected for *L. casei* ATCC 27139. These results suggest that a single point mutation in the lacTp promoter was able to restore the transcription of a fully functional lacTEGF operon and cause a phenotype switch from Lac− to Lac+ for *L. casei* ATCC 27139.

INTRODUCTION

*Lactobacillus casei* is a facultative, heterofermentative member of the lactic acid bacteria (LAB) found in many food products, and has received considerable attention in recent years due to its claimed probiotic properties (health-promoting live culture). For example, *L. casei* DN-114001 (Turchet *et al.*, 2003) and *L. casei* Shirota (Ezendam & van Loveren, 2008) have been extensively studied and are widely available in functional foods. The bioconversion of lactose, which is the primary carbon and energy source in milk, into lactic acid is an essential process in industrial dairy fermentations and is carried out by LAB. The metabolic pathways for lactose utilization have been established in several LAB (de Vos & Vaughan, 1994) (Supplementary Fig. S1). Some strains of LAB have been found to have more than one system to transport lactose. For example, *L. casei* ATCC 393 has two lactose assimilation mechanisms, the chromosomal lactose-specific phosphoenolpyruvate-dependent phosphotransferase system (PTS) and a permease/β-galactosidase system encoded by plasmid pLZ15 (Chassy *et al.*, 1976; Flickinger *et al.*, 1986). When transported via the lactose-specific PTS (Lac-PTS), lactose is phosphorylated and then hydrolysed by phospho-β-galactosidase (P-β-Gal) to glucose and galactose 6-phosphate. The galactose 6-phosphate produced is then metabolized via the tagatose 6-phosphate pathway. The Lac-PTS operon lacTEGF of *L. casei* ATCC BL23 (Gosalbes *et al.*, 1997) encodes an antiterminator protein (LacT), the LacE and LacF elements of Lac-PTS, and a P-β-Gal (LacG). On the
other hand, if lactose is metabolized by the permease/β-
galactosidase system, it will be hydrolysed by β-galactosidase
to produce glucose and galactose. The galactose is then
metabolized via the Leloir pathway. The galactose genes of
the Leloir pathway are transcribed as the operon galKETRM
in L. casei 64H (Bettenbrock & Alpert, 1998).

Lactose metabolism is a changeable phenotype in strains of
L. casei (Christensen et al., 2004). In this study, we found that
L. casei ATCC 27139 was a lactose-negative strain. However, spontaneous mutants with a Lac + phenotype
were obtained when ATCC 27139 was kept on a lactose-
containing medium. In order to characterize the Lac −
phenotype of L. casei ATCC 27139, the lacTEGF and
galKETRM operons of L. casei ATCC 27139 were sequenced. Unlike those of L. casei 64H and ATCC 334
strains (Bettenbrock & Alpert, 1998; Makarova et al., 2006), the two operons were found to be linked together
and organized as a lacTEGF–galKETRM gene cluster, as
reported previously for Lactobacillus rhamnosus TCELL-1
(Tsai & Lin, 2006) and L. casei BL23 (GenBank accession
no. FM177140). The protein sequence identity between the
lac–gal gene cluster and those reported previously for some L. casei (Lac +) strains (Gosalbes et al., 1997; Bettenbrock &
Alpert, 1998) was high, 96–100% identity was found and
no premature stop codon was identified. Sequence
comparisons of the 42 independently isolated Lac +
revertants of ATCC 27139 revealed that single point
mutations had occurred in the lacTEGF promoter (lacTp)
region of every isolate. These revertants could be divided
into six classes based on their point mutations. The lacT
promoter activity of these six classes of Lac + revertants was
detected by using primer extension experiments, whereas
that of L. casei ATCC 27139 was undetectable. The effect of a single point mutation on the lacTp promoter on the
transcription of the lacTEGF operon was further studied by
using Northern blotting experiments. We found that the
lacTEGF operon of the Lac + revertant (strain R1) of L.
casei ATCC 27139 was fully transcribed and was induced by
lactose but suppressed by glucose. However, no blotting
signal was detected for L. casei ATCC 27139. These results
suggest that the inability of L. casei ATCC 27139 to grow
on lactose could be caused by naturally occurring
mutations in the lacTp promoter.

METHODS

Bacterial strains and growth conditions. The bacterial strains
used in this study are described in Table 1. L. casei strain R1 was
identified at the species level by determining its 16S rRNA sequence
(1428 bp), which was found to be the same as that of L. casei ATCC
27139 (GenBank accession no. EU670679) and L. casei neotype strain
ATCC 334 (D86517). L. casei strains were cultured at 37 °C in Mann–
Rogosa–Sharpe (MRS; Difco) broth or Lactobacillus-carrying medium
(LCM) supplemented with 0.5 or 1% filter-sterilized carbohydrates
(Effthymiou & Hansen, 1962). Ribose (non-inducing, non-repressing
sugar), lactose (inducer) and glucose (repressor) were used as
carbohydrates as previously described for studying the regulation of
the lacTEGF operon in L. casei (Alpert & Siebers, 1997; Gosalbes et al.,
1997, 1999, 2002; Monedero et al., 1997). For preparing the agar
plates, 1.5% agar (Amresco) was added to the medium. The growth
of cells was monitored by determining the OD600 using an Amersham
GeneQuant pro spectrophotometer.

Accumulation of Lac + colonies and viability assay. L. casei ATCC
27139 (Lac +) was grown in liquid LCM supplemented with 1% lactose at 37 °C overnight to late-exponential growth phase. This
culture was diluted 10−4-fold in 20 ml fresh LCM supplemented with
1% glucose. Then it was incubated at 37 °C until the late-exponential
growth phase (about 23–25 h), at which point the viable cell number
was estimated to be ~3.5 × 109 cells ml−1. The cells were harvested by
centrifugation at 8000 g for 5 min at room temperature and then
washed twice before being resuspended in 7 ml sterile physiological
saline (0.9% sodium chloride). Aliquots of 0.1 ml resuspension
containing 1 × 106 cells were spread on LCM agar plates supplemented
with 1% lactose and 0.004% chlorophenol red. The fermentation
of lactose would acidify the growth medium and produce a colour
change in the agar plates from purple to yellow. The cells were plated
in quintuplicate and incubated at 37 °C, and this experiment was
repeated at least three times. The emergence of new revertants was
recorded daily throughout 7 days.

On each day, the number of viable Lac + cells on LCM agar plates
supplemented with 1% lactose and 0.004% chlorophenol red was
counted by taking agar plugs (avoiding Lac + colonies) from one of a
set of five plates. Bacteria on 25 mm2 agar plugs were vortexed with
1 ml sterile physiological saline and the cell suspensions were
gradually diluted before being spread on MRS plates to determine
the presence of viable cells. Viable bacterial counts were determined

Table 1. Bacterial strains used in this study

<table>
<thead>
<tr>
<th>L. casei strain*</th>
<th>Relevant characteristic</th>
<th>Type of mutation</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 27139</td>
<td>Wild-type Lac− strain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R1–26</td>
<td>Lac + class I revertant</td>
<td>Transition</td>
<td></td>
</tr>
<tr>
<td>R27–36</td>
<td>Lac + class II revertant</td>
<td>Insertion</td>
<td></td>
</tr>
<tr>
<td>R37–38</td>
<td>Lac + class III revertant</td>
<td>Insertion</td>
<td></td>
</tr>
<tr>
<td>R39–40</td>
<td>Lac + class IV revertant</td>
<td>Transition</td>
<td></td>
</tr>
<tr>
<td>R41</td>
<td>Lac + class V revertant</td>
<td>Deletion</td>
<td></td>
</tr>
<tr>
<td>R42</td>
<td>Lac + class VI revertant</td>
<td>Transversion</td>
<td></td>
</tr>
</tbody>
</table>

†BCRC, Bioresources Collection and Research Center, Hsin-Chu, Taiwan, ROC.
daily and were normalized by the size of Petri dish (8.5 cm diameter) (Yang et al., 2001).

**Selection and identification of spontaneous mutations.** Independent cultures were grown and plated as described above. Only one revertant colony was picked from each culture every day. The DNA fragment containing the lacT promoter region (nucleotides 1–1586) was amplified by PCR using primers Lac393.5F and Lac393.1644R (Table 2) for the selected revertants. The mutations in the promoter region that are responsible for lactose metabolism were identified by sequencing and by comparison with the nucleotide sequence of the wild-type strain. The specific class of revertants of *L. casei* ATCC 27139 (Lac−) was designated for each strain in which a distinct mutation in the DNA sequence was identified.

**Stability of the revertants.** To examine whether the selected Lac− revertants were stable or not, representative clones were picked with a toothpick and seeded in liquid LCM supplemented with 1% glucose without lactose. The fully grown cultures were diluted (1 in 1000) in fresh LCM supplemented with 1% glucose and incubated repeatedly. After 12 rounds of repeat incubation, the bacteria had doubled approximately 120 times in the absence of lactose. The resulting bacterial populations were diluted 1.6 × 10−2-fold by using sterile physiological saline to maintain the viable cell count at ~2500 cells ml−1. Aliquots of 0.2 ml resuspended bacteria containing about 500 cells were spread on LCM agar plates (15 cm diameter) supplemented with 1% lactose and 0.004% chlorphenol red. The cells were plated in triplicate and bacterial growth was examined after 48 h incubation at 37°C.

**Carbohydrate fermentation.** The ability of *L. casei* ATCC 27139 (Lac−) and its Lac− revertant strains to ferment 49 carbohydrates was studied by using the API 50 CH kit (bioMérieux). Strains were grown in LCM supplemented with 1% glucose at 37°C overnight to the late-exponential growth phase, and 1 ml culture was harvested by centrifugation at 10,000 × g for 1 min. The cell pellets were washed twice with 1 ml sterile physiological saline and then suspended and added to API CHL medium according to the manufacturer’s protocol. The diluted cultures were loaded onto the API 50 CH test strips and the capsules were covered with mineral oil. The fermented strips were examined and recorded after being incubated at 37°C for 24 and 48 h.

**Sequencing of the lac–gal gene cluster from *L. casei* ATCC 27139.** Total DNA was extracted by phenol/chloroform and ethanol precipitation from the lysozyme-treated lactobacilli by using SDS (Alander et al., 1999). Based on the nucleotide sequence of the lacTEGF operon of *L. casei* BL23 (Gosalbes et al., 1997) (GenBank accession no. Z80834), and the galKETRM operon of *L. casei* 64H (Bettenbrock & Alpert, 1998) (AF005933) and *L. casei* ATCC 334 (Makarova et al., 2006) (CP000423), several sets of oligonucleotides were designed and used for PCR amplification of the corresponding *L. casei* ATCC 27139 (Lac−) genes. The length of the entire lac–gal gene cluster of *L. casei* ATCC 27139 (Lac−) obtained was 12,009 nt. The total sequence was determined by four overlapping PCR fragments using the following PCR primers: Lac393.5F–Lac393.4339R (nucleotides 1–4281), Lac393.4085F–Gal46H.2002R (nucleotides 4027–7041), Gal64H.1824F–Gal64H.5454R (nucleotides 6863–10,493) and Gal334.7656F–Gal334.9486R (nucleotides 10,257–12,009) (Table 2). The PCR products were separated by agarose gel electrophoresis and excised from the gel using a gel extraction kit (GeneMark).

**Nucleotide sequencing and sequence analysis.** The DNA sequences were determined by the DNA sequencing service of Mission Biotech, Taiwan. All the PCR products were sequenced using the dye-deoxynucleotide chain-termination method with an ABI Prism Big Dye terminator kit (Applied Biosystems) on an ABI Prism 3100 DNA sequencer (Applied Biosystems). The PCR products sequenced were amplified using the high fidelity TaKaRa Ex Taq DNA polymerase (TaKaRa Shuzo). All the sequence analyses and protein homology searches were conducted using the NCBI database (http://www.ncbi.nlm.nih.gov/). The isoelectric point (pI) and molecular mass of each gene product were calculated using the Expyase website (http://ttw.expasy.org/tools/protparam.html). The free energy of formation was calculated through the Vienna RNA secondary structure prediction website (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) using published parameters (Mathews et al., 1999).

**DNA amplification procedure.** Each of the 50 μl PCR mixtures contained 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 200 μM dTTP, 5 μl 10× reaction buffer, 0.5 μM primers, 2.5 U Ex Taq DNA polymerase (TaKaRa Shuzo) or 2 U Dynazyme (Finnzymes Oy), and 1 μl bacterial DNA solution (200–400 ng of DNA prepared as described above). All the amplification reactions were performed on a Gene Amp PCR System 2400 (Perkin-Elmer). Unless otherwise specified, the reactions were conducted using the following temperature-time profiles: an initial denaturation step at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 1–2 min. An additional extension of 5–10 min at 72°C was added to the final cycle. The length of the extension step was varied according to the length of DNA amplified.

**RNA isolation and Northern blot analysis.** *L. casei* strains were grown overnight to the late-exponential growth phase at 37°C in LCM supplemented with 0.5% glucose, 0.5% ribose, 0.5% ribose plus 0.5% lactose, 0.5% glucose plus 0.5% lactose, or 0.5% lactose. The overnight cultures were diluted with 30 ml of the same medium to OD600 of 0.05, and then incubated further at 37°C for 5–10 h until an OD600 of 0.8–1 was measured. The bacteria were harvested by centrifugation and resuspended in 0.5 ml SET buffer (0.45% sucrose, 8 mM EDTA, 15 mM Tris/HCl, pH 8.0). After being incubated with 30 μg lysozyme ml−1 at room temperature for 10 min, the bacterial protoplasts were harvested by centrifugation at 12,000 g for 2 min and the total RNA was extracted using the method of van Rooijen & de Vos (1990). The RNA size markers (0.5–10 kb) were obtained from Invitrogen. The RNA was fractionated using the method of van Rooijen & de Vos (1990). The size of the RNA from this agarose gel and blotted onto a positively charged nylon membrane (Hybond XL, GE Healthcare) according to the method of Sambrook & Russell (2001). The DNA probe (830 bp) against the lacTEGF operon was prepared by PCR with primers pro.3088F and pro.3917R (Table 2). The DNA probes were labelled by random priming with [α-32P]dATP (Izotop). The hybridization procedure was performed using the standard method described in the literature (Sambrook & Russell, 2001).

**Table 2.** Oligonucleotide primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lac393.1644R</td>
<td>CGTTCCGAGAATACAGA</td>
</tr>
<tr>
<td>Lac393.5F</td>
<td>TGTCGTAGGACCAAGT</td>
</tr>
<tr>
<td>Lac393.4339R</td>
<td>AATCCGGCATGATGATGC</td>
</tr>
<tr>
<td>Lac393.4085F</td>
<td>ACACCGCAGGAAATAGAG</td>
</tr>
<tr>
<td>Gal64H.2002R</td>
<td>CCAAGTGTGACCCGAA</td>
</tr>
<tr>
<td>Gal64H.1824F</td>
<td>CTGCGTGATGAGAAAG</td>
</tr>
<tr>
<td>Gal64H.5454R</td>
<td>CATGTTGGTGCAAGCCTC</td>
</tr>
<tr>
<td>Gal334.7656F</td>
<td>AGGGGTCTCATCATGTT</td>
</tr>
<tr>
<td>Gal334.9486R</td>
<td>CACTGCGCCAAATAAGAAC</td>
</tr>
<tr>
<td>pro.3088F</td>
<td>GCTGTTGCAACAACATGG</td>
</tr>
<tr>
<td>pro.3917R</td>
<td>AAGCGGTCCTGCAACTCG</td>
</tr>
<tr>
<td>LacT.fam</td>
<td>CGCCTAAATTAATAGTCACAATCC</td>
</tr>
</tbody>
</table>

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Primer extension experiment. To determine the exact 5′ end of each transcript, a non-radioactive primer extension (NAPE) analysis was performed (Yamada et al., 1998). The technique employs high temperature to minimize the complication resulting from the formation of secondary structures. The primer extension experiments were performed at 50 °C using the SuperScript III reverse transcriptase (Invitrogen) and primer LacT.fam (Table 2) according to the manufacturer’s instructions. The primer used in the NAPE experiment carried a fluorescent dye (6-Fam) covalently linked at its 5′ end (MDBio). Sample analyses were performed by the nucleic acid analysis service at Mission Biotech, Taiwan. These first-strand cDNAs were separated by an ABI 3730 capillary electrophoresis sequencer and the corresponding fluorescence intensity was quantified with the GeneMapper V3.7 software (Applied Biosystems).

RESULTS

Accumulation of Lac+ revertants

The number of Lac+ colonies appearing after each day of incubation was scored as described in Methods. Fig. 1(a, b, c) present the single experimental results obtained on different days, while Fig. 1(d) shows the averaged results obtained from 12 independent experiments. These results show that most of the Lac+ revertant colonies appeared within 2–3 days and only a few Lac+ revertant colonies appeared between days 4 and 7. On LCM agar plates supplemented with 1% lactose and 0.004% chlorophenol red, L. casei ATCC 27139 was able to double in population during the first day and then remained in stationary phase for 2–7 days. The viability of the strain decreased significantly after this period (data not shown).

Stability of the revertant and carbohydrate fermentation

To analyse whether the Lac+ revertants were stable mutants or not, 12 different isolates (including all classes of Lac+ revertants found in this study) were picked and tested for the maintenance of the Lac+ phenotype in the

Fig. 1. Accumulation of Lac+ revertants and viability of L. casei ATCC 27139 (Lac−) after being spread on LCM agar plates supplemented with 1% lactose and 0.004% chlorophenol red. The Lac+ colony counts (○) representing the average number of revertants (from five individual plates) obtained from three independent experiments (a, b and c) on each day were plotted against the plating time. Error bars represent the standard deviation of five different plates counted. (d) The Lac+ colony counts (□) were averaged from 12 independent experiments. Error bars represent the standard deviation computed from 12 experiments. Agar plugs were removed daily from the plates and the number of viable cells (●) was determined as described in Methods.
absence of selective pressure. The colonies were able to retain the Lac⁺ phenotype in all the cases studied, indicating that they were stable mutants.

The ability of *L. casei* ATCC 27139 (Lac⁻) and its 42 Lac⁺ revertant strains (including all the Lac⁺ revertants found in this study) to ferment 49 carbohydrates was studied by using an API 50 CH kit (bioMérieux). According to these results (Supplementary Table S1), Lac⁻ revertants differ from *L. casei* ATCC 27139 (Lac⁻) only by their ability to ferment lactose, while the other fermentation patterns obtained were the same. This implies that the effect of the genetic switch is strictly limited to a specific metabolic pathway.

**Sequence analysis of the lac–gal gene cluster**

In order to characterize the mutations responsible for the metabolism switch for lactose, the lac–gal gene cluster of *L. casei* ATCC 27139 (Lac⁻) was sequenced and found to be organized as lacTEGF–galKETRM. As shown in Fig. 2(a) and Table 3, there were nine ORFs on the gene cluster identified. The same potential RBS, start codon and stop codon for each putative gene on the lacTEGF and galKETRM operons could also be found on well-known *L. casei* (Lac⁺) strains such as BL23, 64H and ATCC 334 (Gosalbes et al., 1997; Bettenbrock & Alpert, 1998; Makarova et al., 2006) (Supplementary Tables S2 and
Table 3. Characterization of ORFs identified in *L. casei* ATCC 27139 (*Lac−*)

<table>
<thead>
<tr>
<th>Product of ORF</th>
<th>Length (aa)</th>
<th>pI</th>
<th>Molecular mass (kDa)</th>
<th>Potential RBS/N terminus/stop codon*</th>
<th>Proposed function</th>
<th>Homologous protein from <em>L. casei</em> (<em>Lac+</em>) strains†</th>
</tr>
</thead>
<tbody>
<tr>
<td>LacT</td>
<td>292</td>
<td>6.15</td>
<td>33.9</td>
<td>gcactGGAGGTatgaacattg/ MPKIAQIPNN/taa</td>
<td>Antiterminator protein</td>
<td>Identity (%) 100  Similarity (%) 100</td>
</tr>
<tr>
<td>LacE</td>
<td>577</td>
<td>5.69</td>
<td>62.4</td>
<td>gcctcaGAGGaaagaactc/ MNNVDFKLKPG/aag</td>
<td>Enzyme IBC lactose PTS</td>
<td>99 99</td>
</tr>
<tr>
<td>LacG</td>
<td>474</td>
<td>5.09</td>
<td>54.0</td>
<td>ttaacGAGGTatgtaacga/ MSQQLPQDFV/taa</td>
<td>P-β-Gal</td>
<td>99 100</td>
</tr>
<tr>
<td>LacF</td>
<td>112</td>
<td>4.63</td>
<td>12.5</td>
<td>aagggtgaacGAGGigaaatg/ MMATKEEISMS/taa</td>
<td>Enzyme IIa lactose PTS</td>
<td>100 100</td>
</tr>
<tr>
<td>GalK</td>
<td>388</td>
<td>4.91</td>
<td>42.5</td>
<td>ttcattGAGGTatgaga/ MNSTDVTKG7/taa</td>
<td>Galactokinase</td>
<td>96 97</td>
</tr>
<tr>
<td>GalE</td>
<td>331</td>
<td>5.69</td>
<td>36.3</td>
<td>aaaaagGAGGatattatag/ MTHAVLGGAG/aag</td>
<td>UDP-galactose 4-epimerase</td>
<td>100 100</td>
</tr>
<tr>
<td>GalT</td>
<td>486</td>
<td>6.06</td>
<td>54.1</td>
<td>agaaggattGGTGAGCgggaatg/ MTAISNHVGIT/tag</td>
<td>Galactose-1-phosphate uridylyltransferase</td>
<td>99 99</td>
</tr>
<tr>
<td>GalR</td>
<td>331</td>
<td>7.84</td>
<td>36.4</td>
<td>tcaatggaaacGGAGAaaagatg/ MTTITDIAKA/tag</td>
<td>Transcriptional regulator</td>
<td>99 99</td>
</tr>
<tr>
<td>GalM</td>
<td>336</td>
<td>5.12</td>
<td>37.1</td>
<td>tcggttataaGAGGagacaggtg/ MDVSVPEYQQ/tag</td>
<td>Aldose 1-epimerase (mutarotase)</td>
<td>98‡ 100‡</td>
</tr>
</tbody>
</table>

*Upper-case type indicates potential RBS; bold type indicates start codons.†LacTEGF was compared with GenBank accession number Z80834. GalKETRM was compared with GenBank accession number AF005933.‡Note that homology values computed are based on the partial sequence of galM in *L. casei* 64H (*Lac+*).*

S3). The protein sequence identity between the *lac–gal* gene cluster and those of the well-known *L. casei* (*Lac+*) strains was high and was around 96–100% (Table 3, Supplementary Table S4). A cre (catabolite responsive element)-like element was detected in the upstream region of the *lacT* gene, which was followed by a putative promoter (*lacTp*), a highly conserved ribonucleic anti-terminator sequence (Houman *et al.*, 1990; Aymerich & Steinmetz, 1992; le Coq *et al.*, 1995; Schnetz *et al.*, 1996), and a terminator structure (Fig. 2b). We found that, except for the promoter region, the sequences of these aforementioned regulatory regions were exactly the same as those reported for the *Lac+* strains *L. casei* BL23, 64H and ATCC 334 (Alpert & Siebers, 1997; Gosalbes *et al.*, 1997; Makarova *et al.*, 2006) (Supplementary Fig. S2). Three putative rho-independent terminators (T1, T2 and T3) were identified, and each of these putative terminators was found to be able to form a stem–loop-like secondary structure with corresponding free energies of formation estimated to be −26.6, −13.2 and −15.3 kcal mol⁻¹ (−111.3, −55.2 and −64.0 kJ mol⁻¹), respectively (Fig. 2c). These show that the major genetic components required to metabolize lactose in *L. casei* ATCC 27139 (*Lac−*) are as intact as those in *L. casei* (*Lac+) strains. Therefore, the genetic lesions responsible for lactose metabolism might be present in *L. casei* ATCC 27139 (*Lac−*).

Spectra of spontaneous mutations

To study how lactose was fermented in the *Lac+* revertants, 42 *Lac+* revertants of *L. casei* ATCC 27139 were independently isolated. These *Lac+* revertants were divided into six classes based on the positions of the point mutations detected (Fig. 3, Table 1). The *lacTEGF–galKETRM* gene cluster of *L. casei* strain R1 (a class I revertant) was PCR-amplified and sequenced. The DNA sequence was compared with that of the parent stain *L. casei* ATCC 27139 (*Lac−*). We found that these two *lac–gal* gene cluster sequences differed by only one nucleotide, which was identified to be in the *lacTp* promoter region (Fig. 3). We obtained the same result when comparing the partial sequence of the *lac–gal* gene cluster (nucleotides 1–6989) of yet another revertant strain, R27 (a class II revertant), with that of the parent strain *L. casei* ATCC 27139 (*Lac−*). We isolated 40 more *Lac+* revertants, and the corresponding *lacTp* promoter sequences (nucleotides 1–155) were PCR-amplified and sequenced. The DNA sequence analysis conducted for each of these 40 promoters also revealed that there was a point mutation present in the *lacTp* promoter region (Fig. 3).
Transcriptional analyses of the lacTp promoters

A series of primer extension analyses were conducted to study the transcription of lacTp promoters of L. casei ATCC 27139 (Lac−) plus the corresponding six classes of Lac+ revertants (strains R1, R27, R37, R39, R41 and R42). Primer LacT.fam (Fig. 2b, Table 2) was used in this experiment on the total RNA extracted from these strains grown in LCM supplemented with 0.5 % ribose plus 0.5 % lactose. An apparent signal corresponding to an oligonucleotide of 45 nt was detected for each of these six Lac+ revertants and the transcription site was mapped at a G residue (Fig. 2b). The distance between the −10 regions and the transcriptional start sites determined was 6 nt for strains R1, R27, R37, R39 and R42, and 5 nt for strain R41, all of which fall within the range of 6.9±2.7 bp reported for some lactobacilli (McCracken et al., 2000). Moreover, the detected transcriptional start site coincided with that of L. casei 64H (Lac+) (Alpert & Siebers, 1997). However, no such signal was detected for L. casei ATCC 27139 (Lac−) grown in LCM supplemented with 0.5 % glucose, 0.5 % ribose, 0.5 % ribose plus 0.5 % lactose, or 0.5 % glucose plus 0.5 % lactose (Fig. 4a). However, a strong signal at ~5.0 kb was found for strain R1 grown in LCM supplemented with 0.5 % ribose plus 0.5 % lactose or 0.5 % lactose (Fig. 4b). The size of this transcript coincided with the prediction that transcription started at the transcriptional start site of lacTEGF and terminated at the putative rho-independent terminator T2 (Fig. 2).

DISCUSSION

Stationary-phase (or adaptive) mutations are spontaneous mutations which occur in non-dividing or very slowly dividing microbial populations subjected to non-lethal selective conditions. Conversely, growth-dependent mutations occur in dividing cells. The phenomenon of stationary-phase mutations is conventionally observed by spreading a population of bacteria or yeast onto a nutrient-limited medium lacking some carbohydrates or amino acids (Cairns & Foster, 1991; Steele & Jinks-Robertson, 1992; Bull et al., 2000; Yang et al., 2001, 2006; Sung & Yasbin, 2002; Ross et al., 2006). The first revertant colonies to appear are assumed to reflect mutant cells that arose during growth, and colonies that continue to appear for periods of several days may result from stationary-phase mutations. In most of these cases of stationary-phase mutations, the number of revertants will accumulate in a
linear manner or with an upward inflection with time. Here, we have shown that most of the Lac+ colonies appeared within 2–3 days (Fig. 1). This indicates that most of the Lac− revertants of L. casei ATCC 27139 (Lac−) were caused by growth-dependent mutations.

Our sequencing results show that L. casei ATCC 27139 (Lac−) possesses the full complement of genes necessary for lactose metabolism, despite exhibiting a Lac− phenotype. After characterizing the promoter sequences of 42 isolated Lac+ revertants of L. casei ATCC 27139 (Lac−), we detected a point mutation in the lacTp promoter region of each of these isolates. These Lac+ revertants were divided into six classes based on the detected mutation. For class I revertants, a C-to-T substitution in the −10 box region caused a change of the original Lac− promoter sequence from TTTACA-N16-TACAAC to TTTACA-N16-TACAAT, and the lacTp promoter sequence was found to be identical to that of the functional lacTp promoter of L. casei 64H (Alpert & Siebers, 1997). In class II and III revertants, an insertion of one base had occurred in the region between the −35 and the −10 boxes of the lacTp promoter. This gave a promoter sequence of TTTACA-N17-TACAAC. Interestingly, the lacTp promoter sequence of the class III revertants was exactly the same as that of the functional lacTp promoter described for L. casei BL23 (Gosalbes et al., 1997). This reflects the fact that maximum promoter activity and open complex formation by RNA polymerase usually happen with promoters in which the length of spacer between the −35 and −10 boxes is 17 bp (Berman & Landy, 1979; Ackerson & Gralla, 1983; Mandecki & Reznikoff, 1982; Stefano & Gralla, 1982; Aoyama et al., 1983; Mandecki et al., 1985; Chatwin & Summers, 2001). For class IV, V and VI revertants, a point mutation was found in the −10 (TATAAC), −10 (TAAACT) and −35 (TTGACA) boxes of the lacTp promoter, respectively. These latter promoter sequences were found to have greater homology with the consensus promoter sequence (−35: TTgaca and −10: TAtaAT; T ≥ 75%, T 60–74%, t 40–59%) reported for some lactobacilli (McCracken et al., 2000). Therefore, these point mutations can be regarded as promoter-up mutations, which will enhance the level of transcription of the lacTEGF operon and allow the metabolism of lactose.

To support our hypothesis that a point mutation in the lacTp promoter was largely responsible for the production of the Lac+ phenotype of the revertants, the transcription of lacTp promoters of L. casei ATCC 27139 (Lac−) plus that of the corresponding six classes of Lac− revertants (strains R1, R27, R37, R39, R41 and R42) was studied by primer extension analyses. The same transcription start site was found in these six Lac+ revertants, whereas no primer extension product was detected in L. casei ATCC 27139 (Lac−). Furthermore, the transcript accumulation of the lacTEGF operon in L. casei ATCC 27139 (Lac−) and that of the corresponding Lac− revertant (strain R1) was studied by Northern blot analysis. A complete lacTEGF gene transcript in strain R1 was detected, similar to those observed by others for other L. casei (Lac+) strains (Alpert & Siebers, 1997). However, no blotting signal was detected for L. casei ATCC 27139 (Lac−). These results suggest that the lacTp promoter of L. casei ATCC 27139 (Lac−) could be silent or very weak. On the other hand, our Northern analysis showed that the transcription of the lacTEGF operon of strain R1 could be induced by lactose but suppressed by glucose. This in fact was consistent with the dual regulation mechanism reported for L. casei (Alpert & Siebers, 1997; Gosalbes et al., 1997, 1999, 2002; Monedero et al., 1997). The inducible, catabolite-repressed expression of the lacTEGF operon detected here suggests that L. casei ATCC 27139 (Lac−) originally might have been Lac+, but was converted to Lac−. The lactose metabolism in strain R1 or other Lac− revertants of L. casei ATCC 27139 (Lac−) could be also mediated through the tagatose 6-phosphate pathway, although the corresponding genes have not been defined experimentally. It is known that the pathway catabolizes galactose 6-phosphate generated from the metabolism of lactose transported via the Lac-PTS (lacTEGF operon).

Among lactobacilli, L. casei is known as a remarkably adaptive species that has been isolated from raw or fermented dairy products, fresh or fermented plant products, as well as the reproductive and intestinal tracts of humans and other animals (Kandler & Weiss, 1986). Earlier studies (Bringel & Hubert, 2003, 2004) have suggested that LAB evolve by progressively losing unnecessary genes upon adaptation to some specific habitats. DNA degeneration by spontaneous mutation may inactivate unnecessary genes during their adaptation to specific habitats in order to improve the growth or cell viability under the stressful or unusual conditions. Once LAB have adapted to some rich environments, they may lose the ability to synthesize many essential amino acids and vitamins. Most of these genetic lesions have been found to be located in genes rather than in the promoter regions (Delorme et al., 1993; Godon et al., 1993; Cavin et al., 1999; Nomura et al., 2000; Bringel & Hubert, 2003, 2004). A systematic attempt to isolate mutants that no longer require each of the essential amino acids has been undertaken for several lactobacilli (Morishita et al., 1974, 1981; Bringel & Hubert, 2003, 2004), including Enterococcus, Pediococcus and Lactococcus species (Deguchi & Morishita, 1992). Successful isolation of amino acid prototrophic revertants means that minor genetic lesions, such as point mutations, are postulated to be present in the parental strain, while failure to isolate mutants is often ascribed to the involvement of more extensive lesions (Morishita et al., 1981). The genetic lesions responsible for carbohydrate metabolism in LAB have also been studied by others (Erlandson et al., 2000; Vaughan et al., 2001; Lapierre et al., 2002). Vaughan et al. (2001) have isolated 10 Gal+ revertants from a galactose-negative strain, Streptococcus thermophilus CNZR 302, and found that they all resulted from point mutations occurring at three different positions in the galK promoter.
These authors proposed that poor expression of the gal genes in *S. thermophilus* strain CNRZ 302 was caused by some naturally occurring mutations in the galK promoter. Here, we observed that a point mutation in the lacTp promoter of *L. casei* ATCC 27139 (Lac<sup>−</sup>) could greatly affect the promoter activity and cause a phenotype switch from Lac<sup>−</sup> to Lac<sup>+</sup> in *L. casei* ATCC 27139. *L. casei* ATCC 27139 might have lost its ability to utilize lactose in a stressful environment that was no longer selective for lactose metabolism.

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**REFERENCES**


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