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

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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.

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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

Abbreviations: *cre*, catabolite responsive element; LAB, lactic acid bacteria; P-β-Gal, phospho-β-galactosidase; PTS, phosphoenolpyruvate-dependent phosphotransferase system.

The GenBank accession numbers for the sequences of the 16S rDNA and *lac*–*gal* gene cluster for *L. casei* ATCC 27139 are EU670679 and EU670680, respectively.

Two supplementary figures, showing the lactose and galactose metabolism pathways that have been identified in lactic acid bacteria and a comparison of promoter region and regulatory genetic elements of the *lacTEGF* operon in *L. casei* strains, and four supplementary tables, of API 50 CH fermentation profiles of *L. casei* ATCC 27139, potential RBSs and start codons, comparisons of the start and stop codons of the *lacTEGF* and *galKETRM* operons, and estimates of the homology of the *lac* and *gal* operons in *L. casei* ATCC 27139 (Lac[−]) and *L. casei* (Lac⁺) strains, are available with the online version of this paper.

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 rDNA 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 (Efthymiou & 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% glucose 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 $\sim 3.5 \times 10^9$ 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 1×10^9 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

<i>L. casei</i> strain*	Relevant characteristic	Type of mutation	Source
ATCC 27139	Wild-type Lac^- strain		BCRC†
R1–26	Lac^+ class I revertant	Transition	This study
R27–36	Lac^+ class II revertant	Insertion	This study
R37–38	Lac^+ class III revertant	Insertion	This study
R39–40	Lac^+ class IV revertant	Transition	This study
R41	Lac^+ class V revertant	Deletion	This study
R42	Lac^+ class VI revertant	Transversion	This study

*R1–26, revertant strains 1–26; R27–36, revertant strains 27–36; R37–38, revertant strains 37–38; R39–40, revertant strains 39–40.

†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 *lacTp* 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^{-6} -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 % chlorophenol 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 diluted in 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–Gal64H.2002R (nucleotides 4027–7041), Gal64H.1824F–Gal64H.5454R (nucleotides 6863–10 493) and Gal334.7656F–Gal334.9486R (nucleotides 10257–12009) (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 dideoxy 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 ExPASy website (<http://tw.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 30 s and extension at 72 °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 mg 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 on a 0.8 % formaldehyde–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

Primer	Sequence (5'–3')
Lac393.1644R	CGTTCGGAACATAAGCAA
Lac393.5F	TCGTCGATGAGCCAAAGT
Lac393.4339R	AATCCGCATCAGTATGTC
Lac393.4085F	ACACCGCAGGAAATGAAG
Gal64H.2002R	CTGCCGTAGATGAGAAGAC
Gal64H.1824F	CCAAGTGTTCAGCCAGGAA
Gal64H.5454R	CATGTTGTGCAAGCCATC
Gal334.7656F	AGCGGGTCATCATACTTG
Gal334.9486R	CAC TGCCCAAAATAGAAC
pro.3088F	GCTGGTCAACAACACTGG
pro.3917R	AAAGCGTCCTGCAACTCG
LacT.fam	CGCCTAATTAAATAGTCACAATCC

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

Fig. 2. (a) Organization of the *lac-gal* gene cluster determined for *L. casei* ATCC 27139 (Lac⁻). The open arrows indicate the localization and length of the genes (the directions of transcription are indicated). The positions of putative terminators (stem-loop structures) identified are also shown. The black line below the genes represents the DNA probe used in the Northern blot experiments. (b) Nucleotide sequence upstream of the *lacT* gene. Bold type indicates the potential -35 and -10 regions. The *cre*-like element, ribonucleic antiterminator (RAT) region, potential RBS and start codon are underlined. The transcriptional start site (bold type) determined by the primer extension experiments for *L. casei* strains R1, R27, R37, R39, R41 and R42 (Lac⁺) is indicated as +1. The inverted repeats capable of forming a stem-loop structure are indicated by arrowheads underneath the sequences. The oligonucleotide (LacT.fam) used in primer extension experiments is indicated by an arrow. (c) The three putative rho-independent terminators identified are depicted as stem-loop structures.

Table 3. Characterization of ORFs identified in *L. casei* ATCC 27139 (Lac⁻)

Product of ORF	Length (aa)	pI	Molecular mass (kDa)	Potential RBS/N terminus/stop codon*	Proposed function	Homologous protein from <i>L. casei</i> (Lac ⁺) strains†	
						Identity (%)	Similarity (%)
LacT	292	6.15	33.9	gcactgGGAGGtgatgacaac t tg/ MPKIAQIFNN/taa	Antiterminator protein	100	100
LacE	577	5.69	62.4	gcgctcaGGAGGaaaagactc a tg/ MNKVFDKLP/tga	Enzyme IIBC lactose PTS	99	99
LacG	474	5.09	54.0	ttaacaGGAGGtattaagca a tg/ MSKQLPQDFV/taa	P-β-Gal	99	100
LacF	112	4.63	12.5	aaggattgaatcGGAGGgaaa a tg/ MMATKEEISM/taa	Enzyme IIA lactose PTS	100	100
GalK	388	4.91	42.5	ttcaattaGGAGGtattgacg g tg/ MNSTDVTKGF/taa	Galactokinase	96	97
GalE	331	5.69	36.3	aaaaaagGGAGAtattttatt a tg/ MTIAVLGGAG/tga	UDP-galactose 4-epimerase	100	100
GalT	486	6.06	54.1	agaaggatttGGTGAccggaa t tg/ MTAISNHVGT/tag	Galactose-1-phosphate uridylyltransferase	99	100
GalR	331	7.84	36.4	tcaatggaacGGAGAaaacg a tg/ MTTITDIAKA/tag	Transcriptional regulator	99	99
GalM	336	5.12	37.1	tcgtttataaaGGAGGacaag a tg/ MDVSVEPYGQ/tag	Aldose 1-epimerase (mutarotase)	98‡	100‡

*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 strain *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⁻). To examine whether the mutation detected in the *lacTp* promoter region could also occur in other Lac⁺ revertants of *L. casei* ATCC 27139 (Lac⁻) or not, we isolated 40 more Lac⁺ revertant strains, and the corresponding *lacTp* promoter sequences (nucleotides 1–1586) were amplified by PCR using primers Lac393.5F and Lac393.1644R (Table 2). A 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).

	-35	-10
ATCC 27139	ACGTTTACAT-TCTGTTTCAGAT-ATGGTACAACCTTG	
Class I (26)	-----T-----	
Class II (10)	-----A-----	
Class III (2)	-----T-----	
Class IV (2)	-----T-----	
Class V (1)	-----	
Class VI (1)	-----G-----	
BL23	-----T-----	
64H, ATCC 334	-----T-----	

Fig. 3. Comparison of the sequences of the *lacTp* promoter regions identified for *L. casei* ATCC 27139 (Lac⁻), *L. casei* ATCC 27139 (Lac⁺) revertants (six classes), *L. casei* BL23 (Lac⁺), *L. casei* 64H (Lac⁺) and *L. casei* ATCC 334 (Lac⁺). The -35 and -10 regions of the *lacTp* promoter are underlined. The number of isolates that correspond to each class is shown in parentheses.

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. These results suggest that the *lacTp* promoter of these six classes of Lac⁺ revertants was functional while that of *L. casei* ATCC 27139 was not.

To study the effect of a single point mutation on the *lacTp* promoter region on the transcription of the entire *lacTEGF* operon, a series of Northern hybridization experiments were performed (Fig. 4). A probe (Fig. 2a) was used to monitor the transcription of the *lacTEGF* operon in *L. casei*

ATCC 27139 (Lac⁻) and the corresponding Lac⁺ revertant (strain R1). No hybridization 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

Fig. 4. Northern blot analysis for RNA isolated from *L. casei* ATCC 27139 (Lac⁻) (a) and strain R1 (Lac⁺) (b) using an internal DNA fragment of the *lacTEGE* operon (see Fig. 2a) as the probe. Cells were grown in LCM supplemented with 0.5 % glucose (lane 1), 0.5 % ribose (lane 2), 0.5 % ribose plus 0.5 % lactose (lane 3), 0.5 % glucose plus 0.5 % lactose (lane 4), or 0.5 % lactose (lane 5).

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: TAaAT; 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* CNRZ 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⁻) could greatly affect the promoter activity and cause a phenotype switch from Lac⁻ to Lac⁺ 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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