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Cloning and Expression of the UDP-Galactose-4-Epimerase Gene (*galE*) Constituting the *gal/lac* Operon of *Lactococcus lactis* ssp. *lactis* ATCC7962

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Abstract The gene (*galE*) encoding UDP-galactose-4-epimerase, operative in the galactose metabolic pathway, was cloned together with the β-galactosidase gene (*lacZ*) from *Lactococcus lactis* ssp. *lactis* ATCC7962 (*L. lactis* 7962). *galE* was found to have a length of 981 bps and encoded a protein with a molecular mass of 36,209 Da. The deduced amino acid sequence showed a homology with GalE proteins from several other microorganisms. A Northern analysis demonstrated that *galE* was constitutively expressed by its own promoter. When galactose or lactose was added into medium, the *galE* transcription was induced by several upstream promoters. The structure of the *gal/lac* operon of *L. lactis* 7962 was partially characterized and the gene order around *galE* was *galT-lacA-lacZ-galE-orfX*.

Key words: *Lactococcus lactis* ssp. *lactis* ATCC7962, *galE*, UDP-galactose-4-epimerase, *gal/lac* operon

In lactococci, galactose is transported into the cell via two different pathways: a phosphoenolpyruvate (PEP)-dependent galactose phosphotransferase system (PTS) and a galactose permease system [22]. In PEP: galactose PTS system, galactose enters into the cell as a phosphorylated sugar, galactose-6-phosphate, which is further metabolized by the enzymes in the tagatose-6-phosphate pathway [19]. In contrast, galactose permease transports galactose as a free sugar and the sugar is sequentially converted to α-galactose, galactose-1-phosphate, and glucose-1-phosphate by the enzymes in the Leloir pathway: mutarotase (GalM), galactokinase (GalK), and galactose-1-phosphate uridylyltransferase (GalT), respectively. UDP-galactose-4-epimerase (GalE) catalyzes a reversible conversion between UDP-glucose and UDP-galactose. *Lactococcus lactis* ssp. *lactis* ATCC7962 (*L. lactis* 7962) has been found to include the enzymatic activities of both pathways [5]. We previously cloned a β-galactosidase gene (*lacZ*) from *L. lactis* 7962 chromosome as a 10-kb *Pst*I fragment (pCKL11, Fig. 1) [6] and determined the nucleotide sequences of *lacZ* and flanking regions [12]. During close examination of the nucleotide sequence, we found the presence of other genes: *lacA* (galactoside acetyltransferase gene) [9], *galT*, and *galE* in the vicinity of *lacZ* [11]. In this paper, we show that *galE* along with other genes constitutes a *gal/lac* operon and its transcription is regulated by multiple promoters.

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Materials and Methods

Bacterial Strains and Vectors

L. lactis 7962 was obtained from the American Type Culture Collection. *E. coli* MC1061 was used as the host for the subcloning and construction of the deletion derivatives, and *E. coli* JM109 as the host to express the *galE*. pBluescript II KS (-), pUC18, and pUC19 were used to subclone the fragments of pCKL11 containing the 10-kb *Pst*I-fragment.

Molecular Cloning and Determination of Nucleotide Sequence

Plasmid DNA from *E. coli* was isolated using a method of Birnboim and Daly [4]. Standard molecular cloning and transformation experiments were performed as previously described [20]. An Erase-a-base system (Promega) was used to generate nested sets of deletion mutants according to the manufacturer's instruction. A Cy5™ AutoRead™ Sequencing Kit (Pharmacia Biotech.) was used to determine

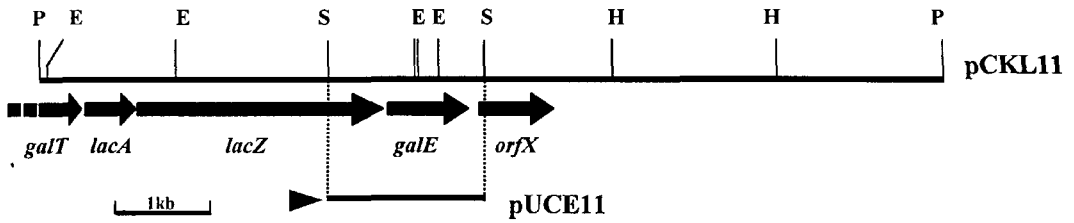

Fig. 1. The organization of genes in the *gal/lac* operon of *L. lactis* 7962.
Relevant restriction sites used for the cloning works are indicated, and the size and orientation of the genes are illustrated with black arrows. The 1,988-bp *Sac*I fragment was subcloned into pUC19 and then designated pUCE11. The arrowhead indicates the direction of the transcription from the *lac* promoter. E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sac*I; *galT*, galactose-1-phosphate uridylyltransferase gene; *lacA*, galactoside acetyltransferase gene; *lacZ*, β-galactosidase gene; *galE*, UDP-galactose-4-epimerase; *orfX*, unknown ORF.

the nucleotide sequence. The nucleotide sequence was analyzed with DNASIS and CLUSTAL V software packages. A deduced amino acid homology search was performed by BLAST on the Genbank database. The Genbank accession number of the nucleotide sequence is U60828.

SDS-PAGE of the Expressed UDP-Galactose-4-Epimerase Protein

Log-phase cells grown in LB broth were harvested, washed, and resuspended in a 100 mM Tris buffer (pH 7.5). The suspended cells were lysed by ultrasonication and the cell-free extracts were collected by centrifugation. The protein concentration of the cell-free extracts was determined by a Bio-Rad protein assay kit (Bio-Rad). SDS-PAGE was conducted using the method of Laemmli [13], with a 10% gel. Each lane contained 0.1 mg of protein.

RNA Isolation and Northern Analysis

L. lactis 7962 was grown in M17 broth (Difco Lab.) containing 0.5% (w/v) level of either galactose, lactose, or glucose to an OD_{600} of 0.5. The cells were harvested and washed with DEPC (diethylpyrocarbonate)-treated water. Following incubation in TE (pH 8.0) containing lysozyme (10 mg/ml) for 30 min, their RNAs were extracted using a RNeasy Midi Kit (Qiagen). The RNA was resuspended in DEPC-treated water and stored in liquid nitrogen. For a transcript analysis, equal quantities were fractionated using a 1% formaldehyde agarose gel. A half of the gel containing the RNA ladder (0.24 to 9.5 kb, Gibco BRL) was stained with ethidium bromide (0.5 μg/ml in H_2O). The transfer of the RNA to a nylon membrane (Hybond-N, Amersham), hybridization, and analysis were all carried out according to the standard molecular cloning methods [20]. The position of the DNA probe used in a Northern hybridization is shown in Fig. 5. The probe (831 bps) was made by PCR with primer pairs (galEF, ATACGTGGGA-AGTCACGCGGT; galER, ATCTGGGTCTCCTGCACGG) (Fig. 2). The PCR fragments were labeled with [α-^{32}P] dATP using the random primed DNA labeling kit (Roche Molecular Biochemicals, Germany) and purified with a spin column packed with a Sephadex G-50 (Sigma).

RESULTS AND DISCUSSION

Nucleotide and Amino Acid Sequences of the UDP-Galactose-4-Epimerase Gene (*galE*)

In the previous paper, we reported the cloning and characterization of *lacZ* from *L. lactis* 7962 [12]. Also, we showed that *lacZ* was a part of the *gal/lac* operon (Fig. 1) [11]. The *galE* gene was a member of this operon and designated based on homology with other genes. The *galE* nucleotide sequence is shown in Fig. 2. *galE* consists of 981 bps and encodes a calculated molecular mass of 36,209-Da. The putative ribosome-binding site (RBS, GGAGG at

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                10        20        30        40        50        60
  1 TGAAGTTAGT TTTAAATTAA CGTGATGATA CAAATTTTCA AGAATTTGAT ATAATATAGA
                          lacZ stop -35                    -10
 61 GAAAATGGAG GTTCTTATGA CGGTTTTAGT TCTTGGTGGA GCAGGATACG TGGGAAGTCA
          RBS      galE start                    galEF------------
121 CGCGGTAGAT ATGCTTCTTA AACGTGGTTA CGATGTGGCA GTGGTGGATA ATTTAGTGAC
    ---->
181 AGGCCACCGT GAATCTGTTC CAGCAAATGT ACGCTTTTAC GAGGGTGATG TGCGTGATCA
241 TGCTTTCTTA GCTAGTGTTT TTGAAAAAGA AAATATTGAA GGAATCATGC ATTTTTGTGC
301 TTACTCTTTA GTGGGTGAGT CAATGCAAAA ACCTTTAATG TATTTTAATA ATAATGTTGG
361 TGGTGCTCAA GTAATTCTAG AAACGATGGA AGAATTCGGA GTAAAACATA TTGTTTTCTC
421 AAGTACAGCA GCTACATTTG GAATTCCAGA AAAAAGTCCA ATTTCAGAAA AAACACCTCA
481 AAAACCAATT AATCCTTATG GTGAAAGCAA ATTAATCATG GAAAAAATGA TGAAATGGCA
541 ATCGCAAGCA ACTGATATGA CTTATGTCGC TTTACGTTAT TTCAATGTTG CAGGGGCTAA
601 GGATGATGGT TCAATTGGTG AAGCTCATAA AAATGAAACA CATTTGATTC CAATTATTTT
661 GCAAACAGCA CTTGGCCAAA GGGAATTCAT TACTATTTAT GGTGATGACT ATCACACTCC
721 TGATGGAACT TGTATTCGTG ATTATATTGA TATGGAAGAT TTGATTGAAG CACACATCAA
781 AGCACTAGAG TATCTTAAAG CGGGCGGTCA GTCTGACCAA TTTAATCTCG GTTCAAGTAA
841 AGGCTATTCA AATCTTGAAG TACTTGAAAC AGCACCAAAA GTAACAGGTA AAGAAATTCC
901 AAGCCAAATG GGTGAACGCC GTGCAGGAGA CCCAGATGAA CTAGTTGCTG ATTCAACAAA
                    <------------ galER
961 AGCTGGTGAA ATTTTGGGTT GGAAAGCACA AAATGATTTA GAGCACATTA TTACGAACGC
1021 TTGGAAATGG CACCAAAGTC ATCCAAAAGG TTACTAGTTA ATTATCTAAA ATTCGAAAAA
                          galE stop                          >>>>
1081 AAAAAGCTCA AATTTTTGAG CTTTTTTTGT ATGTAATTGT CATGAAAATG TAATGGTAAT
     >>>>>>>>> >> <<<<<<<<<<<<<<<
1141 TGTGATAATT ATTAATAAAA AAATTGATAT AATGAAGTGG ATGAAAAAAA GACAGTTAAG
                                              orfX start
1201 AAGAAATAAA
```

Fig. 2. Nucleotide sequence of *galE*.
The stop and start codons of *galE* and those of contiguous genes (*lacZ* and *orfX*) are underlined. Putative -35 and -10 promoter sequences and ribosome-binding site (RBS) are indicated in bold face. Inverted repeats putatively capable of forming a stem-loop structure are indicated by arrowheads under sequences. Primer pairs (galEF and galER) used to amplify the 831-bp probe for a Northern hybridization are indicated as arrows under the corresponding sequences.

Table 1. The % amino acid identities between pairs of UDP-galactose-4-epimerase from different microorganisms.

Strains[a]	Lclac	Stli	Sthe	Ecoli	Rhime[b]	Klulc[c]	Ref.
Lclac	100						This work
Stli	50.2	100					[1]
Sthe	60.4	57.7	100				[18]
Ecoli	39.3	42.9	38.9	100			[14]
Rhime	43.6	45.8	44.8	10.9	100		[7]
Klulc	39.6	31.7	40.4	47.3	38.4	100	[23]

Pair alignments between homologous proteins were made with the CLUSTAL V program.

[a]Lclac, *Lactococcus lactis* 7962; Stli, *Streptomyces lividans*; Sthe, *Streptococcus thermophilus*; Ecoli, *Escherichia coli*; Rhime, *Rhizobium meliloti*; Klulc, *Kluyveromyces lactis.*

[b]*exoB* of *Rhizobium meliloti* was aligned.

[c]COOH terminal fragment of *gal10* was aligned.

67 bp) is located upstream of the translational initiation site (ATG at 77 bp) by 6 bps. A putative - 10 promoter sequence (TATAAT) was found upstream of RBS and, upstream of the - 10 region by 1 bp, a TG doublet found in many

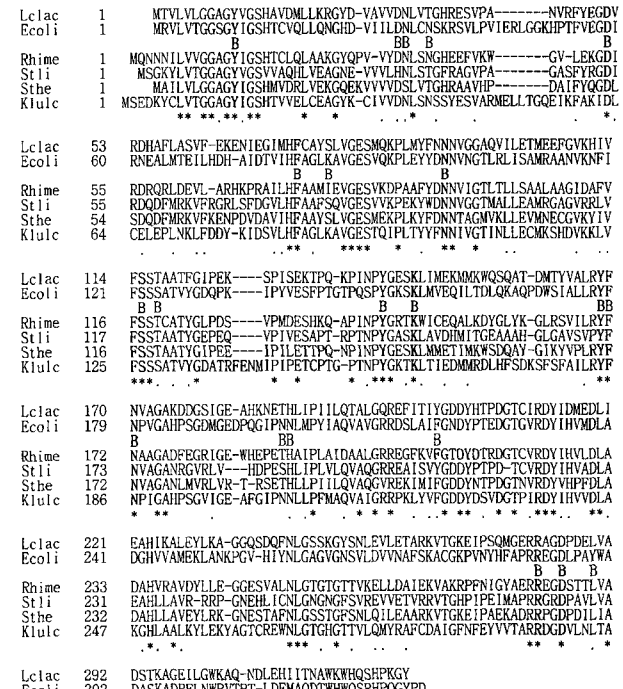

Fig. 3. Computer alignment of the deduced amino acid sequence of GalE with homologous proteins.

Identical (*) and conserved (•) residues in the sequences are indicated. Lclac, Ecoli, Rhime, Stli, Sthe, and Klulc denote the enzyme from *Lactococcus lactis* 7962, *Escherichia coli* [14], *Rhizobium meliloti* [7], *Streptomyces lividans* [1], *Streptococcus thermophilus* [18], and *Kluyveromyces lactis* [23], respectively. B indicates the residues forming the binding pocket for the coenzyme and substrate analogue. The identical residues are shown in bold.

lactococcal genes was present. A stem-loop structure followed the *galE* which could function as a transcriptional terminator. The % amino acid identities of GalE (Table 1) showed a homology with several other GalEs from other microorganisms. Interestingly, the GalE of *L. lactis* 7962 also showed a homology with ExoB in the exopolysaccharide and lipopolysaccharide biosynthesis pathway of *Rhizobium meliloti* [7]. This homology may be related to the fact that *L. lactis* 7962 has galactose in its cell wall [21]. The molecular structure of GalE from *E. coli* was previously determined by X-ray crystallography and revealed the amino acids within 3.5 Å of the atoms of the coenzyme and the substrate analogue binding pocket [3]. In a multiple deduced amino acid sequence alignment of the *E. coli* enzyme with five homologous enzymes, the residues forming the binding pocket for the coenzyme and the substrate analogue in *E. coli* epimerase (indicated as B in the Fig. 3) were well conserved in *L. lactis* 7962, and 7 among 21 residues deviated (Fig. 3). Mukherji and Bhaduri [16] reported the presence of an essential arginine residue at the substrate-binding region of the UDP-galactose-4-epimerase of *Saccharomyces fragilis.* The supposed substrate-binding arginine residue (Arg-292) of the *E. coli* epimerase was well conserved in all homologous enzymes (Fig. 3).

Identification of the UDP-Galactose-4-Epimerase Protein

The 1,988-bp *Sac*I fragment of pCKL11 encompassing the *galE* gene from *L. lactis* 7962 was inserted downstream from the *lacZ* promoter of pUC19 (Fig. 1) and transformed into *E. coli* JM109. The constructed plasmid was designated as pUCE11. When the proteins in the cell-free extract of *E. coli* JM109 containing pUCE11 were separated by SDS-

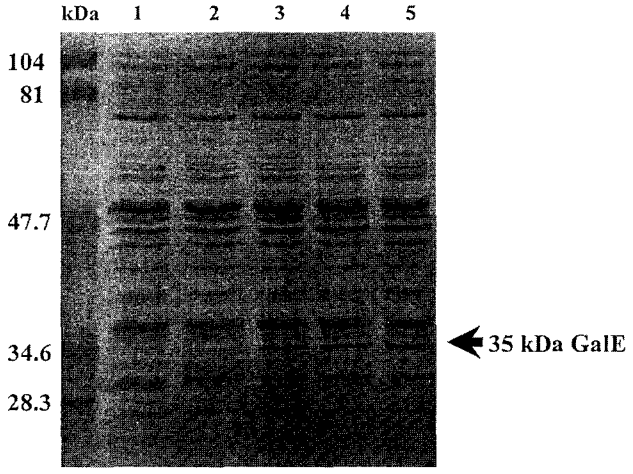

Fig. 4. SDS-PAGE of the protein extract from *E. coli* JM109 harboring pUCE11.

kDa, molecular weight marker, numbers on the left-side are the sizes of molecular weight in kDa. Lane 1, *E. coli* JM109; Lane 2, *E. coli* JM109 (pUC19); Lane 3, *E. coli* JM109 (pUCE11) no induction with IPTG; Lane 4, *E. coli* JM109 (pUCE11) induced by 1 mM IPTG; Lane 5, *E. coli* JM109 (pUCE11) induced by 2 mM IPTG.

PAGE, a protein band of 35 kDa was observed (Fig. 4). The size agreed well with the predicted size of 36,209-Da calculated from the *galE* sequence. The intensity of the band did not increase even when the cell was induced by IPTG, suggesting the operation of its own promoter in *E. coli*.

Transcriptional Analysis of *galE*

In *L. lactis* 7962, the activities of the enzymes in the Leloir pathway including GalE were induced with galactose and lactose [5]. To determine the nature of the induction of the *galE* and the transcriptional organization, total RNAs from cells grown on media with galactose, lactose, or glucose were analyzed by a Northern hybridization. Several large transcripts whose syntheses were induced during growth on galactose or lactose appeared (Fig. 5A). These transcripts, more intense in the galactose-grown cells than in the lactose-grown cells, were estimated to be 10.3, 8.6, 7.0, 6.2, and 4.9 kb in size. The syntheses of these transcripts, except 1.2 kb, were induced by galactose and lactose but repressed by glucose (Fig. 5A, lane 3), indicating the operation of carbon catabolite repression [10]. The 1.2-kb mRNA corresponded to the size of the *galE*, indicating that *galE* was constitutively transcribed from its own promoter and terminated by a stem-loop structure.

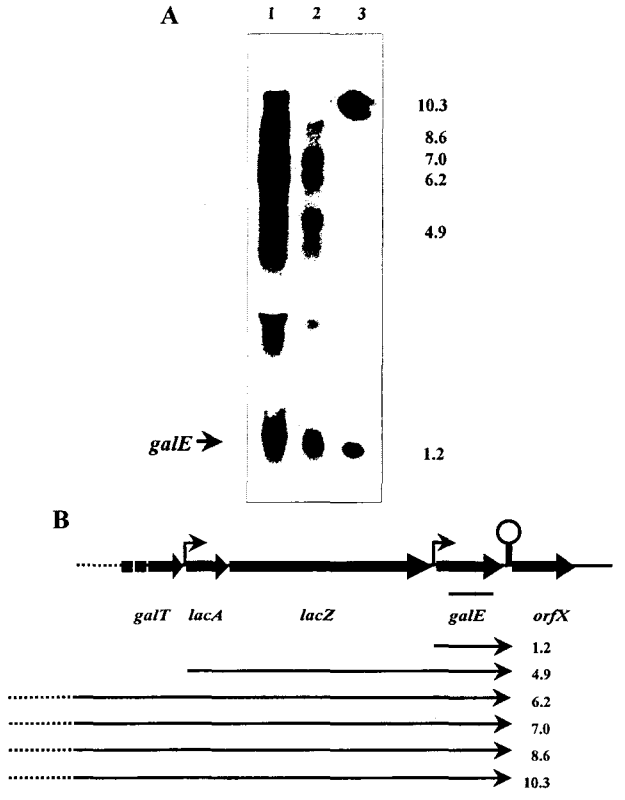

Fig. 5. Northern hybridization analysis (A) and illustrations of transcripts containing *galE* (B).
The total RNAs from *L. lactis* 7962 grown on a medium containing either 0.5 % (w/v) galactose (lane 1), lactose (lane 2), or glucose (lane 3) were electrophoresized. 831-bp *galE* probe obtained by PCR was used for hybridization. The estimated sizes of transcripts are indicated in the right-side (in kb). The transcripts are represented as lines with arrows and their estimated lengths (in kb) are indicated. The positions of potential promoters (black arrows) and the terminator (♀) are indicated. The location of probe used for the hybridization is underlined below *galE*.

gal/lac Operon of *L. lactis* 7962

In the *gal/lac* operon of *Streptococcus thermophilus* [17, 18], the *lacZ* encoding β-galactosidase forms an operon with upstream *lacS* (lactose permease) and the *lacSZ* operon follows the *gal* genes encoding the Leloir enzymes: galactokinase (GalK), galactose-1-phosphate uridylyltransferase (GalT), UDP-galactose-4-epimerase (GalE), and mutarotase (GalM). The *gal/lac* operon structure of *L. lactis* 7962 is unique, as compared with those of other lactic acid bacteria, such as *St. thermophilus* [17, 18] and *Lactobacillus bulgaricus* [15] in terms of the position of *galE*, which is separated from other *gal* genes. In many organisms, *galK*, *galT*, and *galE* are present in a single operon, constituting the Leloir pathway for galactose metabolism [1, 2, 24]. Alternatively, *galE* in *Erwinia stewartti* is not linked to *galK* and *galT*; rather, *galE* is linked to the genes encoding enzymes involved in the biosynthesis of extracellular polysaccharide [8]. A manuscript is in preparation which will describe the complete *gal/lac* operon of 7962 including the characterization and regulation of *galA*, *galM*, *galK*, and *galT* upstream genes.

Acknowledgments

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