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NOTE

Expression of the Galactokinase Gene (*galK*) from *Lactococcus lactis* ssp. *lactis* ATCC7962 in *Escherichia coli*

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The whole *gal/lac* operon genes of *Lactococcus lactis* ssp. *lactis* 7962 were reported as follows: *galA-galM-galK-galT-lacA-lacZ-galE*. The *galK* gene encoding a galactokinase involved in one of the Leloir pathways for galactose metabolism was found to be 1,197 bp in length and encodes a protein of 43,822 Da calculated molecular mass. The deduced amino acid sequence showed over 50% homology with GalK proteins from several other lactic acid bacteria. The *galK* gene was expressed in *E. coli* and the product was identified as a 43 kDa protein which corresponds to the estimated size from the DNA sequence. The galactokinase activity of recombinant *E. coli* was about 8 times greater against that of the host strain and more than 3 times higher than the induced *L. lactis* 7962.

Key words: Lactococcus lactis ssp. *lactis* ATCC7962, *galK*, galactokinase

Lactic acid bacteria play a vital role in many commercial milk fermentations; their primary role is to ferment lactose to lactic acid. The end products of these fermentations confer necessary protection against spoilage, contribute to the generation of the desired flavors, and add to the texture of the final products. During their growth in milk, lactose is used as a primary energy source. Lactose metabolism in lactic acid bacteria is initiated by either a lactose permease system or a phosphoenolpyruvate lactose phosphotransferase system (PEP : PTS) (van Rooijen *et al.*, 1991). In the lactose permease system, lactose is hydrolyzed by the enzyme β-galactosidase (β-gal) into galactose and glucose, which are utilized in the Leloir and Embden-Meyerhof-Parnas (EMP) pathways, respectively (Poolman *et al.*, 1989). In the PEP : PTS, lactose is hydrolyzed by the enzyme phospho-β-galactosidase (p-β-gal) into galactose-6-phosphate and glucose, which are then utilized in the tagatose-6-phosphate and EMP pathways, respectively. In most *Lactococcus lactis* strains, p-β-gal is a predominant enzyme for lactose utilization. Other genes involved in lactose transport and the tagatose-6-phosphate pathway have been characterized (van Rooijen *et al.*, 1991). In view of lactose metabolism, *Lactococcus lactis* ssp. *lactis* ATCC7962 (*L. lactis* 7962) has a higher β-gal activity than p-β-gal activity (Crow and Thomas, 1984) and has a higher affinity for galactose than lactose (Kashket and Wilson, 1972). In these respects, the strain has been the subject of studies on the galactoside transport system and the genes of sugar utilization.

The whole *gal/lac* operon genes of *L. lactis* 7962 were determined as follows: *galA-galM-galK-galT-lacA-lacZ-galE* (U60828) (Lee *et al.*, 2000). Before the complete illumination of *gal/lac* operon of *L. lactis* 7962, a galactose gene cluster which has the gene order of *galA-galM-galK-galT-galE*, encoding a galactose permease, galactose mutarotase, galactokinase, galactose-1-phosphate uridyltransferase, and UDP-glucose-4 epimerase, respectively, has been reported for *L. lactis* ssp. *cremoris* MG1363 (Grossiord *et al.*, 1998). These gene products have over 85% identities to those from *L. lactis* 7962. The sequence homologies and gene order support the acquisition of *lac* genes between *gal* operon.

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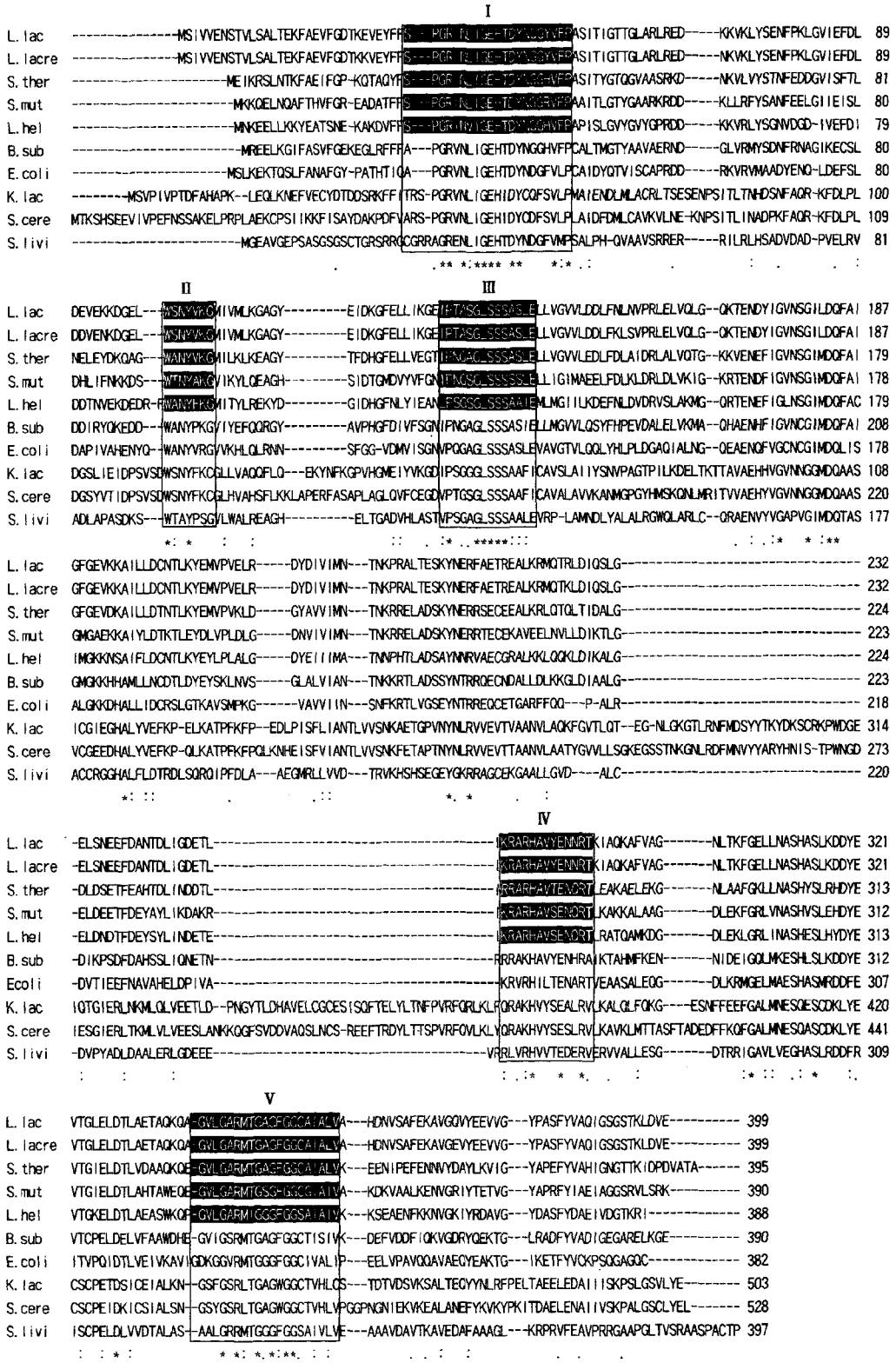

Fig. 1. Amino acid sequence alignment of galactokinases. L. lac, L. lacre, S. ther, S. mut, L. hel, B. sub, E. coli, K. lac, S. cere, and S. livi denote the enzyme from *L. lactis* 7962, *L. lactis* ssp. *cremoris* MG1363, *Strptococcus thermophilus*, *Streptococcus mutans*, *Lactobacillus helveticus*, *Bacillus subtilis*, *E. coli*, *Saccharomyces cerevisiae*, *Kluyveromyces lactis*, and *Streptomyces lividans*, respectively. Identical (*) and functionally related (●) amino acids are indicated. Gaps were introduced to maximize identity. Regions of significant homology are shown in boxes, labeled I to V. The highly conserved sequences between lactic acid bacteria are shaded.

The rate-limiting enzyme galactokinase (GalK) is involved in one of the Leloir pathways for galactose metabolism and converts α-galactose to galactose-1-phosphate. It can be used as a catabolic pathway for the degradation of galactose as an energy source and carbon source. In this report, we illuminated the *galK* gene from *L. lactis* 7962 and its expression in *E. coli*.

Amino acid sequence homology

The deduced amino acid sequence of GalK from *L. lactis* 7962 showed significant homologies with galactokinases from several other microorganisms (Fig. 1). The sequence identities are 92% with *L. lactis* ssp. *cremoris* MG1363 (Q9S6S2), 55% with *Streptococcus thermophilus* (AAD49612), 54% with *Streptococcus mutans* (P96993), 51% with *Lactobacillus helveticus* (Q00052), 43% with *Bacillus subtilis* (P39574), and 33% with *Escherichia coli* (P06976). Even though the sequences for *Saccharomyces cerevisiae* (NP_009576) and *Kluyveromyces lactis* (AAA35256) were longer than those of bacterial origin, as manifested by several gaps in sequence alignment, the five homologous regions are conserved between yeasts and bacteria and highly conserved between lactic acid bacteria. These regions were thought to be important for enzyme function and substrate binding. In particular, the region V contains a sequence motif, GXGXXG(X)nK, found within the ATP binding sites of numerous protein kinases (Debouck *et al.*, 1985; Mollet and Pilloud, 1991). As the result of active domain analysis by Inter Pro Scan program in ExPASy Molecular Biology Server (Http://www.expasy. ch), regions I and III are the galactokinase signature (GRINLIGEHTDY) and putative ATP binding domain (IPTASGLSSSAS), respectively.

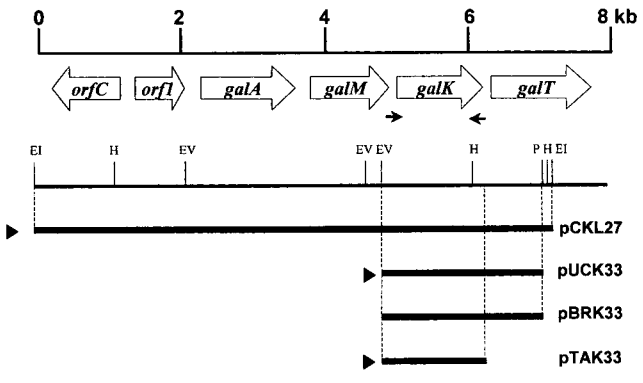

Fig. 2. The organization of the *gal/lac* operon genes in *L. lactis* 7962. The extent and direction of each gene is illustrated with open arrows (*galA*, galactose permease gene; *galM*, galactose mutarotase gene; *galK*, galactokinase gene; *galT*, galactose-1-phosphate uridyltransferase gene; *orfC* and *orf1*, unknown ORFs). Restriction sites are: EI, *Eco*RI; H, *Hin*dIII; P, *Pst*I; EV, *Eco*RV. The arrowhead indicates the direction of the transcription from the *lac* promoter while the arrows indicate the position and direction of primers used for *galK* amplification by PCR.

GalK gene complementation

For the functional analysis of expressed *galK* gene product, the *galK* gene and the flanking region of *L. lactis* 7962 were subcloned from pCKL27 (Lee *et al.*, 2000) which contains three ORFs and one partial ORF corresponding to *galA, galM, galK*, and *galT* genes of *gal/lac* operon (Fig. 2). Plasmid DNA pCKL27 was digested with *Eco*RV and *Pst*I and then ligated into plasmids pUC18 and pBR322. The *Eco*RI-*Pst*I digested 2.4-kb fragment of pCKL27 subcloned in pUC18 and pBR322 was designated pUCK33 and pBRK33, respectively. Sugar utilization of transformants was identified on MacConkey agar (Difco, USA) supplemented with various carbon sources. Formation of red colonies indicated the complementation of *galK* gene mutation. Neither of the plasmids complemented the growth or sugar utilization of *galK* mutant host (*E. coli* HB101) on MacConkey agar containing galactose as a sole carbon source (Table 1). Therefore, the 1.2-kb *galK* of *L. lactis* 7962 was amplified by PCR using pCKL27 as the template DNA in order to eliminate the effects from the flanked region of *galK* gene. The forward primer (5'-TAGAGGAGAGAAGAAATGTCTATAG-3') and reverse primer (5'-GAGTGCTTGAATTTGCTATCACTAA-3') were used. The amplified fragment was cloned into pT7Blue vector (Novagen, U.S.A) and named pTAK33. The plasmid containing the only *galK* gene (pTAK33) complemented the growth and sugar utilization of the host on MacConkey agar containing galactose as a sole carbon source.

Identification of the galK gene product

For the identification of the gene product, transformed 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 cell-free extracts were separated by SDS-PAGE with a 10% gel (Laemmli, 1970). The protein concentration of the cell-free extracts was determined by a Bio-Rad protein assay kit (Bio-Rad, USA) and each lane contained 0.1 mg of protein. When cell extracts of the transformants were separated, 43-kDa overexpressed protein bands were observed

Table 1. Complementation of *E. coli galK* mutant on MacConkey agar supplemented with various carbon sources[a]

E. coli strain	Carbon source added to MacConkey agar		
	Glucose	Lactose	Galactose
HB101	+	-	-
HB101 (pBR322)	+	-	-
HB101 (pUC18)	+	-	-
HB101 (pBRK33)	+	-	-
HB101 (pUCK33)	+	-	-
HB101 (pTAK33)	+	-	-

[a]Complementation of *E. coli galK* mutation was detected by the red colony formation (+) on MacConkey agar plates. The strains incapable of metabolizing each carbon source formed white colonies (–)

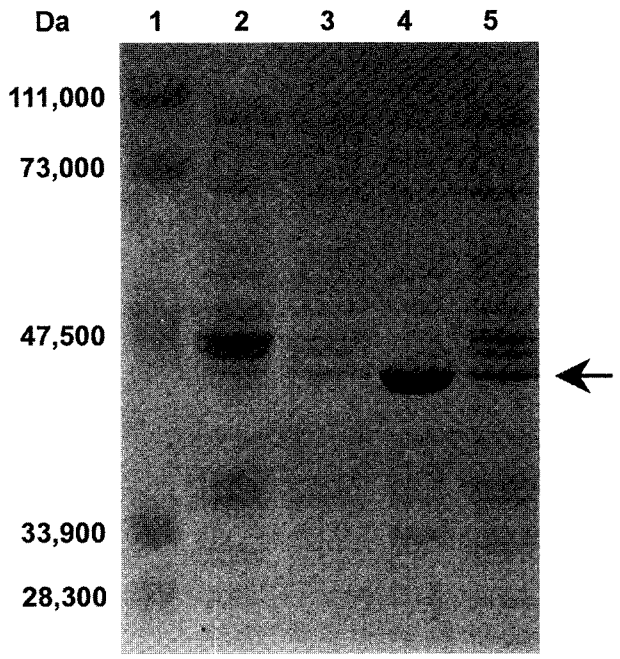

Fig. 3. SDS-PAGE of the expressed galactokinase protein in *E. coli*. Lane 1, molecular weight standard; lane 2, *E. coli* HB101; lane 3, *E. coli* HB101 (pBRK33); lane 4, *E. coli* HB101 (pUCK33); lane 5, *E. coli* HB101 (pTAK33).

Table 2. Mean (n=5) galactokinase activity measurements

E. coli strain[b]	Specific activity[a] (U/mg of protein)
HB101	7.8
HB101 (pBR322)	8.5
HB101 (pUC18)	4.0
HB101 (pBRK33)	20.7
HB101 (pUCK33)	22.8
HB101 (pTAK33)	62.4

[a]One unit of galactokinase activity was expressed as the 0.01 decrease of absorbance at 340 nm per minute per milligram of cell protein.
[b]*E. coli* was cultured in LB broth and 50 mg/ml ampicillin was added when needed.

Table 3. Galactokinase activity of *L. lactis* 7962

Carbon source	Specific activity[a] (U/mg of protein)
Glucose	8.3
Lactose	17.4
Galactose	17.9

[a]One unit of galactokinase activity was expressed as the 0.01 decrease of absorbance at 340 nm per minute per milligram of cell protein.
[b]*L. lactis* 7962 was cultured in M17 broth supplemented with 0.5% (w/v) each carbon source.

(Fig. 3). The size agreed well with the predicted size of 43,822 Da calculated from the nucleotide sequence of cloned *galK* gene.

Expression of the galK gene in E. coli

For the determination of galactokinase activity, log-phase cells were harvested, washed, and resuspended in buffer, pH 7.5 (100 mM Tris, 10 mM $MgCl_2$, 100 mM NaF, 1 mM dithiothreitol, 100 mM triethanolamine). The cell suspensions were cooled on ice, and lysed by ultrasonication. The lysates were cleared by centrifugation, and the supernatants were assayed for galactokinase activity using the NADH-ADP coupled enzyme assay of Thomas and Crow (Thomas and Crow, 1984) with modification. Assay mixtures contained supernatants, 2 mM ATP, 0.4 mM NADH, 2 mM phosphoenolpyruvate, 2 U/ml of pyruvate kinase, 5 U/ml of lactate dehydrogenase. The reaction was started by addition of 10 mM galactose and oxidation of NADH was measured by absorbance at 340 nm. Galactokinase activity of the recombinant *E. coli* strains containing pBRK33 or pUCK33 was about 2.5 times higher than that of the *galK* mutant strain, *E. coli* HB101. Even though GalK production was higher in pUCK33 containing the recombinant, the recombinant containing pTAK33 showed a higher activity (Table 2). This result might have a relationship with that of the complementation test on MacConkey agar and be attributed to the flanked region of *galK* gene. But the inconsistent results between the amount of expressed protein on SDS-PAGE and enzyme activity take us to the conclusion that the overexpressed protein band can be the expression of other genes or production of an inclusion body. The expression of galactokinase activity in pBRK33 containing the recombinant suggests the possible existence of a promoter on the upstream of *galK* gene and the galactokinase activities of the *L. lactis* 7962 grown in M17 broth (Difco, USA) supplemented with glucose, lactose, and galactose suggest the existence of catabolite repression by glucose (Table 3). Galactokinase activity of the recombinant *E. coli* strain HB101 (pTAK33) was about 8 times higher than that of strain HB101 and more than 3 times higher than the induced *L. lactis* 7962.

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