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Expression of the Galactose Mutarotase Gene from *Lactococcus lactis* ssp. *lactis* ATCC7962 in *Escherichia coli*

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Abstract The structure of *gal/lac* operon of *Lactococcus lactis* ssp. *lactis* ATCC7962 was partially characterized and the gene (*galM*) encoding galactose mutarotase was cloned together with the order: *galA-galM-galK-galT*. The *galM* was found to be 1,027 bp in length and encoded the protein of 37,609 Da calculated molecular mass. The deduced amino acid sequence showed a homology with GalM proteins from several other microorganisms. Thus, the *galM* gene was expressed in *Escherichia coli* and the product was identified as a 38 kDa protein which corresponded to the size estimated from DNA sequence. Mutarotase activity of the IPTG induced recombinant was 2.7 times increased against that of the host strain.

Key words: *Lactococcus lactis* ssp. *lactis* ATCC7962, *galM*, galactose mutarotase

Lactococcus lactis strains are known to have an ability to use galactose via either the Leloir pathway or the tagatose-6-phosphate pathway [1, 15]. For the utilization of galactose via the Leloir pathway, a highly specific galactose permease system with low affinity for lactose has been described. Up to now, *gal* genes have been characterized in *L. lactis* ssp. *lactis* ATCC7962 (*L. lactis* 7962) and *L. lactis* ssp. *cremoris* MG1363 [5].

Unlike other lactococci, *L. lactis* 7962 has a higher affinity for galactose than lactose, and utilizes lactose by hydrolyzing it with β-galactosidase [3, 6, 7, 13]. In these respects, the strain has been instrumental in the studies of the genes of galactose operon. Recently, the whole genes consisting of the complete *gal* operon have been characterized. To elucidate the whole *gal* operon structure, a 10-kb *Pst*I fragment and 7-kb *Hpa*I fragment containing β-galactosidase gene (*lacZ*) were cloned by our group [9] and Vaughan *et al.* [16], and the nucleotide sequences of the fragments were determined. Both of the works elucidate only the partial structure of operon. Our DNA sequence analysis of the cloned 10-kb fragment revealed the gene cluster of galactose and lactose utilization with the gene order, *galT-lacA-lacZ-galE* [11]. The gene cluster formed *gal/lac* operon that contains *lacA* and *lacZ* genes in the *gal* operon. Since the 10-kb fragment contained a partial sequence of 3' end of *galT*, another 7.4-kb *Eco*RI fragment corresponding to the upstream region of *galT* was cloned by chromosome walking. DNA sequence analysis revealed that the 7.4-kb fragment contained the rest of the *gal/lac* operon genes [10]. The whole *gal/lac* operon genes of *L. lactis* 7962 were determined as follow: *galA-galM-galK-galT-lacA-lacZ-galE* [J. M. Lee *et al.* 2000. *Abstr. Annu. Meet. Kor. Soc. Appl. Microbiol.*, Seoul, Korea, p. 326].

An investigation of regulation and function of genes was made in regards to *gal/lac* operon. In this report, we studied the function of *galM* gene through the expression in *E. coli* and the determination of galactose mutarotase activity.

Galactose mutarotase (GalM) catalyzes the interconversion of α- and β-anomer of aldose that is currently an identified member of the *gal* operon of *E. coli* and a participant in the Leloir pathway [2]. The presence of GalM was reported to be essential for an efficient utilization in *E. coli* as the cleavage of this β-galactoside by β-galactosidase gives glucose and β-D-galactose, whereas α-D-galactose is identified to be the only substrate for galactokinase.

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MATERIALS AND METHODS

Bacterial Strains and Vectors

L. lactis 7962 was obtained from ATCC (American Type Culture Collection). *Escherichia coli* JM109 was used as the host for cloning and expression. pT7Blue(R) (Novagen, U.S.A.) and pUC19 were used to clone the fragment of amplified *galM* gene by PCR (Polymerase Chain Reaction).

Amino Acid Sequence Alignments

A deduced amino acid homology search was performed by BLAST on the GenBank database and the amino acid sequence alignments were analyzed with CLUSTAL W software.

Amplification of *galM* Gene from *L. lactis* 7962 by PCR

The *galM* gene of *L. lactis* 7962 was obtained by PCR using pCKL27 which contained the 7.4-kb fragment as a template DNA (Fig. 1). The forward primer (5'-GTTGCA-GAATTCGAAGAACG-3') and the reverse primer (5'-TCTGTTAATGCTGATAATAC-3') were used. A 30-cycle repeated protocol consisting of 1 min of strand denaturation (94°C), 1 min of primer annealing (48°C), and 2 min of primer extension (72°C) was used to amplify the *galM* gene. The 1.2-kb amplified DNA was cloned into pT7Blue(R) vector and then transferred to the pUC19 vector.

SDS-PAGE of the Expressed Mutarotase Protein

Log-phase cells grown in LB broth were harvested, washed, and resuspended in 100 mM Tris · Cl 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 the Bio-Rad protein assay kit (Bio-Rad, U.S.A.). SDS-PAGE was conducted by the method of Laemmli [8], with 10% gel. Each lane contained 0.1 mg of protein.

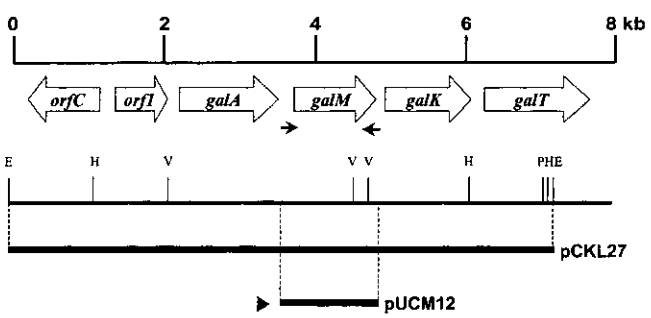

Fig. 1. The organization of the *gal/lac* operon genes in *L. lactis* 7962.

The extent and direction of each gene is illustrated with an 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: E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; V, *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 *galM* amplication by PCR.

Mutarotase Assay

Mutarotase was assayed as described by Gatz *et al.* [4] with some modification. In the presence of added β-D-glucose dehydrogenase and NAD, the conversion of α-D-glucose to β-D-glucose resulted in the oxidation of the latter to D-glucono-δ-lactone and the equimolar formation of NADH. Thus, log-phase cells grown in LB broth were harvested, washed, and resuspended in a 100 mM Tris · Cl buffer (pH 7.5). The suspended cells were lysed by ultrasonication and the cell-free extracts were collected by centrifugation. The supernatant was incubated at 45°C for 5 min to inactivate the NADH oxidase. The reaction mixture of the mutarotase assay contained 3 mM NAD, 30 U of glucose dehydrogenase, and 100 mM Tris · Cl buffer (pH 7.5); 850 μl of this mixture was pipetted into cuvette. After the mutarotase-containing solution was added to the sample cuvette and incubated at 25°C for 1 min, the absorption at 340 nm was followed until it remained constant. Then, 50 μl of a freshly prepared α-D-glucose solution (15 mg/25 ml, giving 166 μM in the reaction mixture) was added to the sample. The increase in absorption was recorded for 10 min.

RESULTS AND DISCUSSION

Nucleotide Sequence of the Upstream Genes of *gal/lac* Operon

The size of the insert in pCKL27, 7.4 kb, was large enough to contain all the upstream genes of the *gal/lac* operon not yet characterized. The nucleotide sequence of *gal/lac* operon was deposited on the Genbank database as the accession number, U60828. Through the sequence analyses, three ORFs and one partial ORF corresponding to the 5' part of *galT* were discovered. These ORFs were designated on the basis of their homology to the known genes in the data bank: *galA-galM-galK-galT* (Fig. 1).

Amino Acid Sequence Homology

The deduced amino acid sequence of GalM from *L. lactis* 7962 showed significant homologies with mutarotase (aldose 1-epimerase) of *Acinetobacter calcoaceticus* [4] and GalM from *L. lactis* ssp. *cremoris* MG1363 [5], *Streptococcus thermophilus* [12] and *E. coli* [2]. UDP galactose 4-epimerase homologous protein from *Pachysolen tannophilus* [14] also has significant homology.

Recently, a galactose gene cluster was characterized from *L. lactis* ssp. *cremoris* MG1363 and the gene order reflects that of the biochemical reactions involved in degradation of galactose via the Leloir pathway, *galA-galM-galK-galT-galE*, in which *galM* is a galactose mutarotase gene [5].

The deduced amino acid sequences of GalA and GalK demonstrated over 50% similarities to sequences from

```
                                1                                  40
Lclc    ------------------------MEIKIRDFGLGSDLISLTNKAGVTISFTNLG---ARIVDWQKD
Lccre   ------------------------MEITTKDFGLGSSLISLTNKNDVTIAFTNLG---ARIVDWQKD
Accal   MKKLAILGVTVYSFAQLANAATLNVKSYGTTQNGQKVDLYTNSNNNGVSVSFISFGGVITQILTPDAQ
Ecoli   ----------------------MLN-ETPALAPDGQPYRLLTLRNNAGMVVTLMDWGATLLSARIPLSD
Strtr   ----------------------MKISCEIIGKVDSG-DVSKISMENNNGVVISTLTTGATLQEFLVPMET
Pacta   ------------------------DSESLTNYDRLNTVRTFNGKFEVSISNHG---ATIVAAKLN
                                                .     :  . ::   *
                                41                                 101
Lclc    GK--HLILGFDSAKEYLEKDAYP----GATVGPTAGRIKDGLVKISGKDYILNQNEGP-QTLHGGEES
Lccre   GK--HFILGFDSAQEYLEKDAYP----GATVGRTAGRIKDGLVDISGKTYHLNQNEAP-QTLHGGEDS
Accal   GKQNNIVLGFDDLKGYEVTDTKEGIHFGGLIGRYANRIGNAKFSLDGKTYNLEKNNGP-NSLHSGNPG
Ecoli   GSVREALLGCASPECYQDQAAFL----GASIGRYANRIANSRYTFDGETVTLSPSQGV-NQLHGGPEG
Strtr   GALKNIVLGFSDFEDYYKNNLCA----COSIGRVAGRIGKASYTHNMVLYSLPKNEGD-NCLHGGPKG
Pacta   GI--KLNLGFDNLKGYLREDNPF----FGATIGRVANRISKGDLLINGTHYQVGLNELHRTSLHGGTYG
        *  .  **  . : *                :*  *.**..   .    :  .:     **.*  .
                                102                                165
Lclc    IHTKLWTYEVTDLGAEVQVKFSLFSLVTNDGTNGYPGKIEMSVTHSFDQY---NKWKIHYEAISDKD-
Lccre   IHTKLWTYEINDLGDEVQVKF---SLVSNDGENGYPGKIEMSVTHSFDEE---NNWKIKYEAISDKD-
Accal   FDKRVWQVKPLVSKGETVKAS--LKLTSPNGDQGFPGKLDVEVIYSLSDQ---NEFKIEYKAKTDQP-
Ecoli   FDKRRWQ---IVNQNDRQVLF---ALSSDGDQGFPGNLGATVQYRLTDD---NRISITYRATVDKP-
Strtr   MQVQNWNYYVTNLNDDYVETKF---IRRLYSSYDGFPGDVTVSISYRLNNN---NRLTILFEAFDVTES
Pacta   YNKRTFLGPIVKTNEKEKETTMEFVLIDLDGTEGYPGDVETKVIYTVRDTGVGGELGIEYEAKLLEES
        .  : :                  .. :*:**.:   : : .  :   ..   * :.*
                                166                                227
Lclc    ----TVFNPTGHVYFNLN--GDASESVENHGLRLAASRFVPLKDQTEIVRGDIVDIKNTDLDFRQEKQ
Lccre   ----TVFNPTGHVYFNLN--GDASKSIENHQLKLAASRFVPLKDQTEIVRGDIVDTKNTDLDFRQEKQ
Accal   ----TVVNLTNHSYFNLSGAGNNPYGVLDHVVQLNAGR-ILVTDQNSLPTGEIASVAGTPFDFRMPKA
Ecoli   ----CPVNMTNHVYFNLD--GEQSD-VRNHKLQILADE-YLPVDEGGIPHDGLKSVAGTSFDFRSAKI
Strtr   ----TVFNPTNHVYFNLS---DKQD-LSSHELQIYSDY-RLELDSELIPTGQKINVDETNYDFRKTTD
Pacta   GRDSTAVSLTNHSYWNIG---NQPS-IEGTHIKLVSNKHLESNPLDSTPTGKIVTS--TDLDSQNSAK
           .. *.* *:*:.  :   : .  ::: :.          .      * * :
                                228                                291
Lclc    LSNAFNSNMEQVQLVKGIDHPFLLDQLGLDKE-QARLTLDDTS---ISVFTDQPSIVIFTANFGDLGT
Lccre   LSKALESSMEQVQLVGGIDHPFLLDEQSLEKE-QARLSLDDLS---VSVYTDQPSIVIFTANFGDLGT
Accal   IVKDIRANNQQLAYGYGYDQTWVINQKSQGKLNLAAIVVDPKSKRTMQVLTTEPSVQMYTADHLLGNI
Ecoli   IASEFLADDDQRK-VKGYDHAFLLQAKGDGKK-VAAHVWSADEKLQLKVYTTAPALQFYSGNF-LGGT
Strtr   LLPRIEANN------G-FDDAFVVGGGTCDHVKEVAILHDKESGDGIEIFSNRNGLVIFTNDDIEDNI
Pacta   LGPDGPVFD----YCFVTKQQDKLDTRNDELR-VVATATHPKTRIAFTTLTTEPAFQFYTGDG--VDV
        :           .. :    .          .  :  .. ::: :   .
                                292                                342
Lclc    LYHEKKQVHHG---GITFECQVSPGSEQIPELG--DISLKAGEKYQATTIYSLHTK--
Lccre   VYHGKNQVHHG---GITFECQVSPGSQQIPELG--DISLKAGDEYQATTIYSLHTN--
Accal   VGANGVLYRQAD--ALALETQHFPDSPNQPTFP--STRLNPNQTYNSVTVFKFGVQK-
Ecoli   PSRGTEPYADWQ--GLALESEFLPDSPNHPEWPQPDCFLRPGEEYSSLTEYQFIAE--
Strtr   YFARDKGKMAKRREAIAMEAQTLPDAVNHKGFG--DIILDKGHSVNYEIGFQYFNSSR
Pacta   A--GVFTKRSG------FCLEASRYIYNPKWFI-----PLNKGEVYGSYTIYRFEN---
                                :  :    :      * ..     :
```

Fig. 2. Alignment of the deduced amino acid sequence of *L. lactis* 7962 GalM (Lclc) with homologous proteins.
L. lactis ssp. *cremoris* MG1363 GalM (Lccre), mutarotase from *A. calcoaceticus* (Accal), GalM from *E. coli* (Ecoli) and *St. thermophilus* (Strtr), UDPgalactose 4-epimerase from *P. tannophilus* (Pacta). Identical (*) and functionally related (·) amino acids are indicated. Gaps were introduced to maximize identity. The numbers corresponded to the amino acid positions of the *L. lactis* 7962 GalM.

other lactic acid bacteria but the sequence of GalM exhibited 26% identity and 46% similarity with the GalM from *St. thermophilus*. The scores show little difference from those of *A. calcoaceticus*, *E. coli* and *P. tannophilus*. Exceptionally, *L. lactis* ssp. *cremoris* MG1363 have 85% identity and 96.5% similarity. Alignment of the deduced amino acid sequence of *L. lactis* 7962 GalM with homologous proteins is shown in Fig. 2.

Identification of the Galactose Mutarotase Protein

The 1.2-kb amplified fragment of pCKL27 encompassing the *galM* gene from *L. lactis* 7962 was cloned in pT7Blue(R) and then inserted downstream from the *lac* promoter of pUC19 vector (Fig. 1). The constructed plasmid was

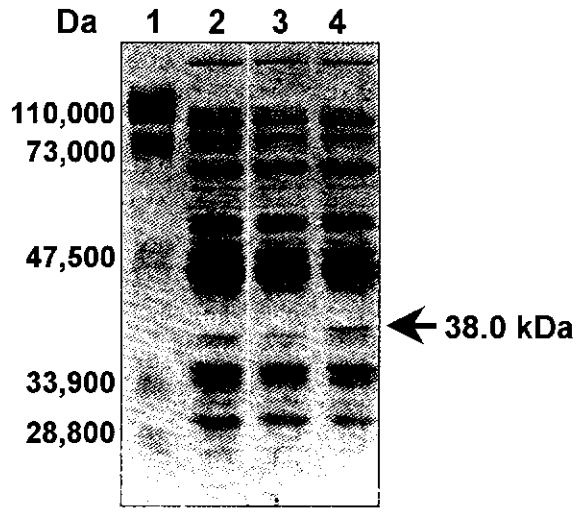

Fig. 3. SDS-PAGE of the protein extract from *E. coli* JM109 harboring pUCM12.
The sizes of molecular weight markers are shown in dalton (Da). Lane 1, molecular weight marker; Lane 2, *E. coli* JM109; Lane 3, *E. coli* JM109 (pUCM12); Lane 4, *E. coli* JM109 (pUCM12) induced by 1 mM IPTG.

designated as pUCM12. When the proteins in the cell-free extract of *E. coli* JM109 containing pUCM12 were separated by SDS-PAGE, a protein band of 38 kDa was observed (Fig. 3). The size agreed well with the predicted size of 37,609 Da that was calculated from the *galM*

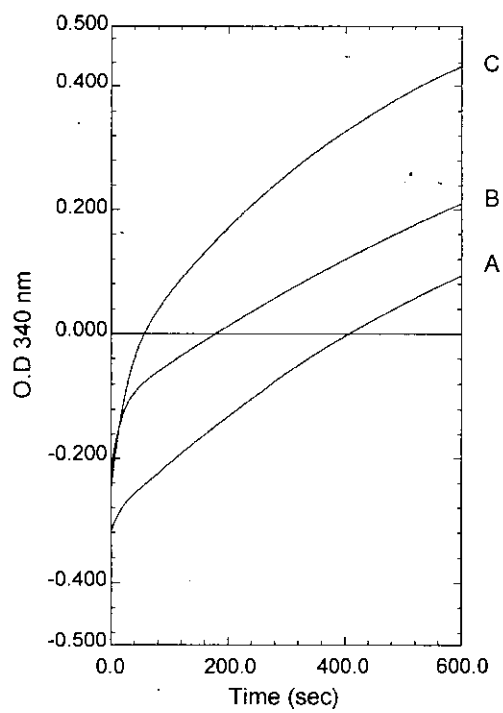

Fig. 4. Mutarotase activity of whole cell protein extract of transformant.
A, *E. coli* JM109; B, *E. coli* JM109 (pUCM12); C, *E. coli* JM109 (pUCM12) induced by 1 mM IPTG.

sequences. The band appeared when the cell was induced with IPTG, suggesting that the protein might be produced under the control of *lac* promoter of pUC19 vector.

Expression of *galM* in *E. coli*

Mutarotase activity of the recombinant *E. coli* strain JM109 containing pUCM12 was measured for 10 min by the increase in the absorption spectrum. The absorption spectrum is shown in Fig. 4. For quantitative evaluation, we compared the activities by the slopes of the spectra for 1 min at the initial stage. Mutarotase activity of the recombinant was about 1.7 times higher than that of the host strain which also contains its own mutarotase gene. The IPTG induced recombinant shows 2.7 times higher activity than that of the host. Even though we could not detect the significant increase of mutarotase activity by the recombinant strain owing to the background activity of the host, this information might reinforce the prediction of the gene identity through homology analysis.

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