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Effect of Temperature and Carbon Source on the Expression of β-Galactosidase Gene of *Lactococcus lactis* ssp. *lactis* ATCC 7962

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Abstract The effects of growth temperature and a carbon source on the expression of β-galactosidase gene of *Lactococcus lactis* ssp. *lactis* ATCC 7962 (*L. lactis* 7962) were investigated. At 25°C, *L. lactis* 7962 had a higher β-galactosidase activity than cells grown at 30°C or 37°C, although cells grew most quickly at 37°C. The highest β-galactosidase activity was observed in cells grown in M17 with lactose (1%) followed by cells grown in a galactose (1%) medium. *L. lactis* 7962 exhibited the minimum β-galactosidase activity in glucose media, indicating catabolite repression. When the cellular levels of β-galactosidase mRNA were examined using slot blot hybridization, no significant differences were observed between cells grown at 25°C and cells at 30°C or 37°C in the same media. This suggests that the quantity of β-galactosidase mRNA may not be the reason for the higher β-galactosidase activities of *L. lactis* 7962 at 25°C. The level of *ccpA* (Catabolite Control Protein) transcript remained almost constant during the exponential growth phase irrespective of a carbon sourse.

Key words: *Lactococcus lactis* ssp. *lactis* ATCC 7962, β-galactosidase, growth temperature, *ccpA*, catabolite repression

β-Galactosidase (β-gal) is a very important enzyme for many lactic acid bacteria (LAB), especially for LAB used in dairy fermentations [16]. Many studies have focused on the distribution of β-gal activities among LAB strains [4], the role of β-gal in the utilization of lactose [16] and β-gal structural gene cloning [5, 9, 15]. LAB metabolize lactose via two different systems: a phosphoenolpyruvate lactose phosphotransferase (PEP-PTS) system with phospho-β-gal (p-β-gal) [8] and a lactose permease system with β-gal [4]. β-gal digests lactose into glucose and galactose, and the galactose moiety is then metabolized by the Leloir pathway [8]. p-β-gal digests lactose-6-phosphate into glucose and galactose-6-phosphate and the galactose-6-phosphate is then further metabolized into triose phosphates by the enzymes of the D-tagatose-6-phosphate (tagatose-6-p) pathway [4]. In most *L. lactis* strains the predominant enzyme is p-β-gal, and the p-β-gal gene and other genes involved in lactose transport and the tagatose-6-p pathway have been thoroughly characterized [18]. Unlike other lactococci, *L. lactis* 7962 includes β-gal as its major lactose utilizing enzyme. In this respect, *L. lactis* 7962 is an atypical strain and has been the subject of speculations and studies on the role of *lac* operon genes [3]. The complete *gal/lac* operon genes of *L. lactis* 7962 have been previously cloned and the nucleotide sequences of the β-gal gene (*lacZ*) determined [9]. Accordingly, this report analyzes the expression of β-gal in *L. lactis* 7962 under different growth conditions such as carbon sources and growth temperatures. CcpA is known as a negative regulator for a group of genes under catabolite repression [10]. CcpA binds to *cre* (catabolite responsive element) sequences, thus preventing RNA polymerase from binding to the promoter sequences [17]. Conserved *cre* sequences were found in the 5' regulatory region of *orf1*, the first gene of the *gal/lac* operon of *L. lactis* 7962 [8]. The concentrations of *ccpA* mRNA in *L. lactis* 7962 cells were examined during growth in media containing either glucose, lactose, or galactose in the context of *lacZ* expression.

Materials and Methods

Bacterial Cultures and Media

L. lactis 7962 (our lab stock) was grown at 30°C without shaking in M17 broth (Difco Lab, Detroit,

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U.S.A.) containing 1% level of either glucose, galactose, or lactose.

RNA Isolation

Total RNA from *L. lactis* 7962 was prepared by the following method. *L. lactis* 7962 was grown in 500 ml of M17 broth containing 1% level of either glucose, galactose, or lactose. At specific time points, 100 ml aliquots of culture were taken and cell pellets were obtained by centrifugation at 10,000×g for 10 min. The cells were resuspended in 2 ml of a lysis buffer (30 mM Tris HCl, pH 8.0, 100 mM NaCl, 5 mM EDTA, 1% SDS) and disrupted by sonication (Bandelin Sonopuls, HD60). Cell extracts were obtained by centrifugation and 20 µl of Proteinase K (20 mg/ml) was added. The cell extracts were incubated for 1 h at 55°C followed by phenol/chloroform extractions and ethanol precipitation. The pellets obtained by ethanol precipitation were resuspended in 95 µl of a DNase buffer (20 mM Tris HCl, pH 8.0, 10 mM $MgCl_2$) and 5 µl of RQ1 RNase-free DNase (1 U/µl, Promega, Madison, U.S.A.) was added to remove any contaminating DNA. After 1 h of digestion at 37°C, the RNA was recovered using phenol/chloroform extractions followed by ethanol precipitation. The RNA was dissolved in an appropriate volume of TE buffer (10 mM Tris HCl, pH 8.0, 1 mM EDTA) and the concentration was calculated by measuring the absorbance at 260 and 280 nm.

DNA Manipulations

L. lactis 7962 chromosomal DNA was isolated as described by Jeong *et al.* [7], except that M17 broth was used instead of MRS broth. Plasmid DNA from *E. coli* was isolated using the alkaline lysis method of Birnboim and Doly [2]. The 3 kb β-gal gene (*lacZ*) of *L. lactis* 7962 was obtained by PCR using pCKL11 as template DNA [9]. The forward primer (5'-GATGA**GTCGAC**CTGAAAGGAAT-3', *Acc*I site, bold and underlined) and reverse primer (5'-ACCGT-**CTGCAG**AACCTGGATTTTCTCTATA-3', *Pst*I site, bold and underlined) were used. A 30-cycle repeated protocol consisting of 45 s of strand denaturation (94°C), 2 min of primer annealing (50°C), and 2 min of primer extension (72°C) was used to amplify the DNA. The amplified DNA was digested with *Acc*I and *Pst*I, isolated from agarose gel using a gel extraction kit (Omega Biotek, Doraville, U.S.A.), and radioactively labelled using a random prime labelling kit (Amersham, RPN1600Y) and α-^{32}P-dATP. A 0.8 kb DNA fragment corresponding to the internal region of catabolite control protein gene (*ccpA*) was obtained by PCR using a primer pair based on known *ccpA* sequences of other Gram positive bacteria [13, 17]. The forward primer was 29-mer (5'-GA(C,T)TATCGG-CC(A,G)AATGC(A,T,C)GT(G,T)GC(G,A,T)CGTGG-3') and the reverse primer was 31-mer (5'-CGCAT(A,T,C) GC(A,T)AC(C,T)GC(A,T)CC(A,T)A(A,T)ATCATA(A,C) A(G,A,T)(C,T)G-3'). The PCR conditions were the same as described the above. The amplified fragment was cloned into a pGEM-T-easy vector (Promega, Madison, U.S.A.) and the identity of the clone was confirmed by nuclotide sequencing (unpublished results). The nuclotide sequencing was completed using Sanger's dideoxy method [14].

Slot Blot Hybridization

Slot blot hybridizations were performed for the RNA preparations using the slot blot apparatus (Hoefer PR600). Ten or 20 µg of RNA samples were loaded onto slots according to the procedure provided by the manufacturer. A hybond N^+ nylon membrane (Amersham, RPN203B) was used and the RNA was immobilized onto the membrane using UV-crosslinking (BioRad, GS linker II). Prehybridization and hybridization were performed at 43°C in a hybridization chamber (Hybaid) according to the procedure provided by the membrane manufacturer. The composition of the pre- and hybridization buffer was as follows: 50% formamide, 0.12 M Na_2HPO_4, pH 7.2, 0.25 M NaCl, 7% SDS, 1 mM EDTA.

β-Galactosidase Assay

The β-gal activities of *L. lactis* 7962 were measured according to the Miller method [12]. *L. lactis* 7962 was grown at 30°C in M17 broth and 1 ml aliquots were taken at various points in time. The cells were resuspended in 1 ml of a Z buffer ($Na_2HPO_4 \cdot 7\ H_2O$ 1.61 g, $NaH_2PO_4 \cdot H_2O$ 0.55 g, KCl 0.075 g, $MgSO_4 \cdot 7\ H_2O$ 0.0246 g, β-mercaptoethanol 0.27 ml, ddH_2O 98 ml, pH 7.0, 100 ml final volume) disrupted by sonication (30 s×5 times on ice), and left standing for 5 min at 28°C. Then, 200 µl of an ONPG solution (4 mg/ml, dissolved in an A buffer: K_2HPO_4 1.05 g, KH_2PO_4 0.45 g, $(NH_4)_2SO_4$ 0.1 g, Na_3 Citrate $2H_2O$ 0.05 g, 100 ml volume) was added and incubated at 28°C until a yellow color appeared. The reaction was stopped by adding 500 µl of 1 M Na_2CO_3. After centrifugation at 9,000×g for 15 min, a supernatant was obtained and its absorbance at 420 and 550 nm was measured. The β-gal units were calculated according to the following equation.

$$\text{β-gal unit} = 1,000 \times \frac{A_{420} - (1.75 \times A_{550})}{t \times A_{600}}$$

t: time in min taken for color development

RESULTS AND DISCUSSION

β-Galactosidase Activities of *L. lactis* 7962 during Growth at Different Temperatures

The effects of temperature on the growth and β-gal activities of *L. lactis* 7962 were examined and the results are shown in Figs. 1 and 2. In Fig. 1, the *L. lactis* 7962 grew most quickly at 37°C in a glucose medium. Initially, it was thought that *L. lactis* 7962 would not grow quickly

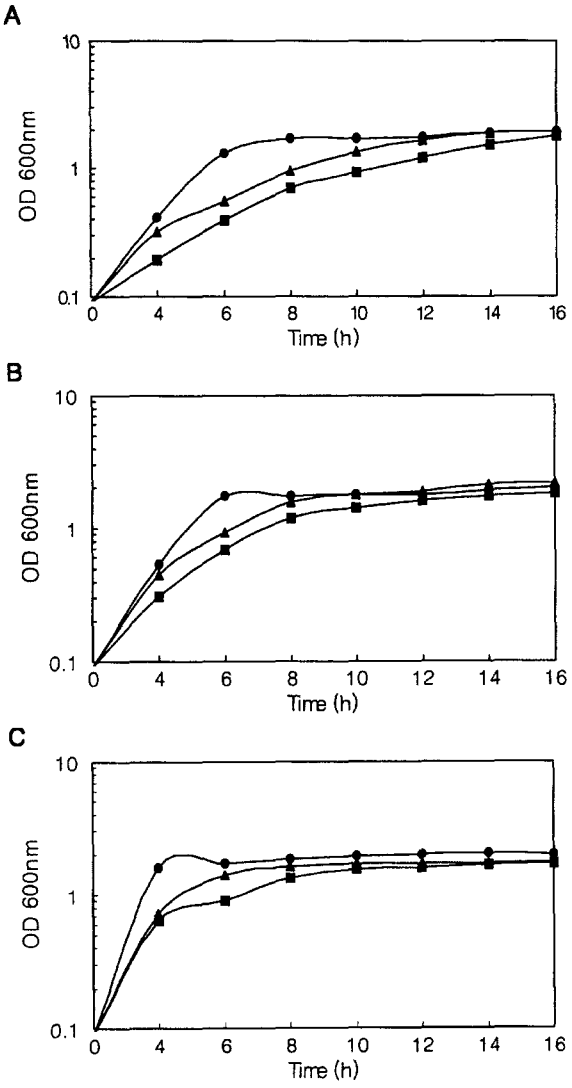

Fig. 1. Growth curves of *L. lactis* 7962 in M17 media at different temperatures.
A: 25°C; B: 30°C; C: 37°C. *L. lactis* 7962 was grown in M17 broth with lactose (1%, ■), galactose (1%, ▲), or glucose (1%, ●).

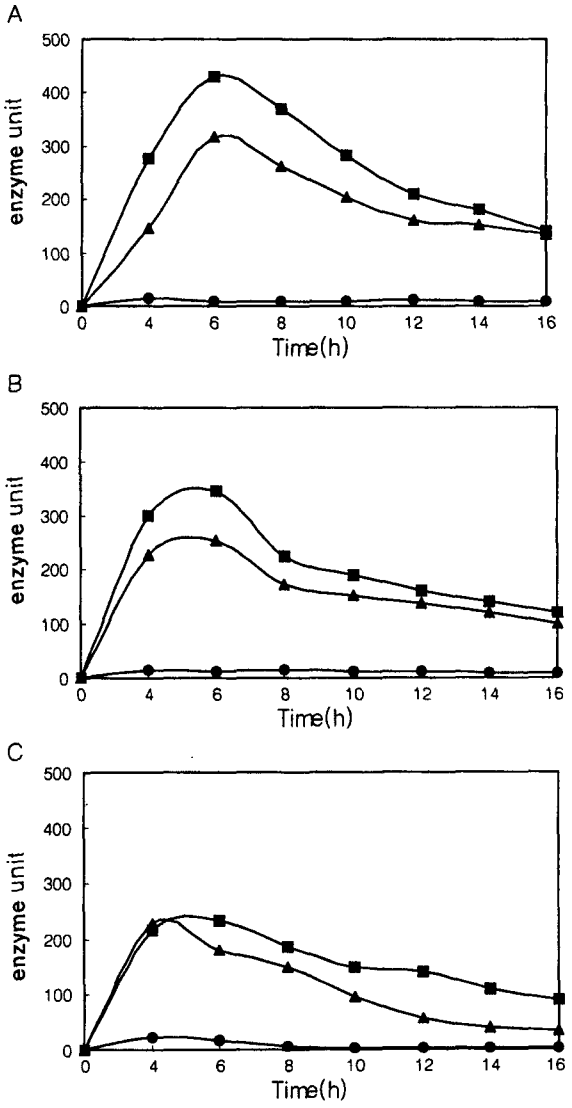

Fig. 2. Changes of β-gal activities of *L. lactis* 7962 during growth at different temperatures.
A: 25°C; B: 30°C; C: 37°C. *L. lactis* 7962 was grown in M17 broth with lactose (1%, ■), galactose (1%, ▲), or glucose (1%, ●).

at 37°C since *L. lactis* strains are known as mesophiles and their usual growth temperature is 30°C. At 37°C in glucose media, *L. lactis* 7962 entered into a stationary phase between 4 and 6 h after incubation started. However, in M17 broth with lactose, it took 16 h to reach the same optical density and in galactose media, it took 10 h. At 30 and 25°C (in glucose media), the *L. lactis* 7962 cells entered into a stationary phase around 6 and 8 h, respectively, indicating that the growth rate was proportional to temperature. *L. lactis* 7962 grew slower in M17 broth with lactose than in M17 with galactose or glucose, confirming the poor growth of *L. lactis* 7962 in a lactose medium. The rate-limiting step in the growth of *L. lactis* 7962 in a lactose medium is either the breakdown of lactose into galactose and glucose by β-gal or the transport of lactose into the cells. Alternatively, the low level of p-β-gal activity might be the culprit for the slow growth, as mentioned by Vaughan *et al.* [19]. *L. lactis* 7962 cells exhibited the highest β-gal activity (431 units) at 25°C in M17 broth with lactose (1%). The highest activity was observed at the 6 h point and the cells grown in galactose (1%) had 317 units at the same point in time. Absorbance values of the culture (600 nm) at that time were 0.39 and 0.55, respectively, corresponding to the mid-log phase of growth. Repeated experiments produced similar results although the maximum values fluctuated within the range of experimental error. In glucose media, β-gal expression was repressed and the activity remained at base level at all temperatures tested. At 25°C, the highest enzyme activity

in the presence of glucose was 14 units, indicating that glucose repressed β-gal more than 30 fold. At 30°C, the β-gal activity of *L. lactis* 7962 in M17 with lactose reached 345 units after 6 h of incubation. At the same time, the cells in M17 with galactose had 254 units of activity. *L. lactis* 7962 cells at 37°C showed the lowest β-gal activities although they grew quicker than cells at lower temperatures. The highest β-gal activities (234 units, after 6 h) were detected in cells grown in a lactose medium where the cells entered into a stationary phase around 12 h. Lorca and de Valdez [11] reported that *Lactobacillus acidophilus* CRL639 cells grown at 25°C in MRS (Merck) broth had higher β-gal activities than cells grown at 37°C.

Level of β-Galactosidase mRNA

In order to determine whether the β-gal activities of *L. lactis* 7962 at different temperatures represent the cellular β-gal mRNA levels, slot blot hybridization experiments were performed. Twenty μg of RNA was applied to each slot. The 3 kb *lacZ* gene was used as a probe and the results are shown in Fig. 3. As illustrated in Fig 3, there was no apparent difference in the *lacZ* mRNA level between cells grown at 25°C and cells grown at higher temperatures. The same results were produced with the RNA preparation obtained by a different method; RNAs prepared using the hot phenol method gave the same results. Thus, it was concluded that the quantity of *lacZ* mRNA was not affected much by growth temperature. Initially, it was suspected that low temperature-inducible promoter sequences might be present upstream of the *lacZ*, which were responsible for the higher β-gal induction at 25°C. However, the slot blotting results indicate that other mechanism(s) such as the degree of DNA supercoiling or conformational change of β-gal at different temperatures might be the reason for the lower enzyme activities at 37°C [6]. Alternatively, changes in the β-gal mRNA conformation could be the reason as observed with the *cIII* gene of bacteriophage lambda [1]. The lambda *cIII* gene product has a regulatory function in the lysis-lysogeny decision following infection. It was reported that at a lower temperature, the predominant form of the *cIII* message (structure B) allowed the efficient binding of mRNA to the 30 S ribosomal subunit, thereby permitting the sufficient translation of *cIII* mRNA [1]. However, at a higher temperature, the *cIII* mRNA took an alternative conformation (structure A) and in this structure, efficient translation was blocked, thus only a small amount of CIII product was generated. It is also recognized that the degree of expression of several other bacterial genes is determined by mRNA conformations which are affected by temperature [6]. Further studies are needed to elucidate the mechanism(s) responsible for the lower β-gal activity at 37°C. It will be interesting to find out whether the same mechanism(s) operates in both *L. lactis* 7962 and *Lactobacillus acidophilus* CRL639. For all the temperatures tested, few *lacZ* transcripts were detected for *L. lactis* 7962 cells grown in M17 broth with glucose (1%), indicating the preseuce of the catabolite repression in *L. lactis* 7962.

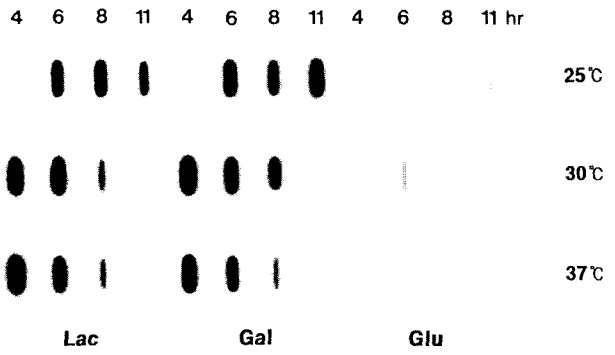

Fig. 3. Slot blot for detecting β-gal mRNA of *L. lactis* 7962.
Twenty μg of RNA was applied to each slot and the numbers on the top indicate incubation time and the numbers on the right side indicate incubation temperature. Lac, Gal, and Glu represent lactose, galactose, and glucose, respectively.

Level of *ccpA* Transcript

Changes in the level of the *ccpA* transcript during growth at 30°C were examined by slot blot hybridization and the autoradiogram is shown in Fig. 4. RNAs were prepared from cells grown in M17 broth with either galactose, lactose, or glucose (1%). Ten μg and 20 μg of RNA samples were applied to each slot and hybridized with a radioactively labelled 0.8 kb internal fragment of the *ccpA* gene. As shown in Fig. 4, the level of the *ccpA* transcript was almost constant during the first 8 h of incubation in the lactose or galactose media. Apparently, more *ccpA* transcripts were present in the cells grown in a lactose medium. *ccpA* transcripts were not detected after 11 h of incubation. This indicates that the transcription of the *ccpA* occurs mainly during the exponential growth phase. Interestingly, in cells grown in a glucose medium, the level of *ccpA* transcripts did not increase compared to that in cells in media with galactose or lactose. Also, in cells grown on a glucose

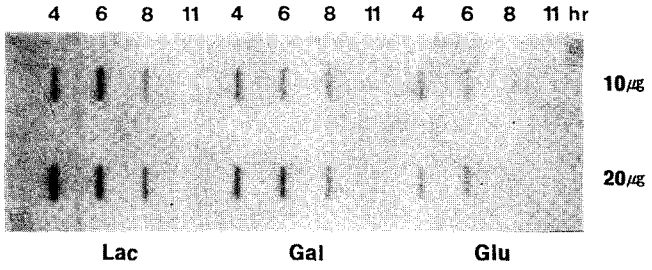

Fig. 4. Slot blot for detecting the *ccpA* homolog mRNA of *L. lactis* 7962.
Ten and 20 μg of RNA were applied to each slot. The numbers on the top indicate incubation time. Lac, Gal, and Glu represent lactose, galactose, and glucose, respectively.

medium, transcripts were not detected after 6 h of incubation. This may be due to the rapid growth of *L. lactis* 7962 in a glucose medium, thus a stationary phase is entered after 6 h.

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