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Expression of β-Galactosidase Gene of *Lactococcus lactis* ssp. *lactis* ATCC 7962 in *Lactococcus lactis* ssp. *lactis* MG1363

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Abstract

A 4.4 kb DNA fragment encompassing *lacA* (galactoside acetyltransferase) and *lacZ* (β-galactosidase) genes from *Lactococcus lactis* ssp. *lactis* ATCC 7962 (*L. lactis* 7962) was introduced into a Lac⁻ strain, *Lactococcus lactis* ssp. *lactis* MG1363 (*L. lactis* MG1363) by using a lactococcal expression vector, pMG36e and expression level of *lacZ* was examined. Growth rates and β-galactosidase (β-gal) activities of MG1363 cells carrying recombinant plasmid, pMLZ3, on M17 broth containing different carbon sources (1%, w/v) were examined. Contrary to the expectations, MG1363 [pMLZ3] grown on lactose showed the lowest enzyme activity (17 units) and cells grown on galactose had the highest β-gal activity (41 units). Cells grown on glucose had intermediate activity (33 units). These activities are about one tenth of the values observed in *L. lactis* 7962 where *lacZ* is present as a single-copy gene in the chromosome. When the cellular concentrations of *lacZ* transcript were examined using slot blot hybridization, it was found that MG1363 [pMLZ3] produced sufficient amounts of transcript. These results indicate that either proteolytic degradation of β-gal or other regulatory mechanisms prevent the translation or accumulation of β-gal in *L. lactis* MG1363 cells. In regard to regulation, the presence of the *ccpA* gene in *L. lactis* MG1363 was confirmed by Southern blot.

Key words: *Lactococcus lactis*, β-galactosidase, expression vector, *ccpA*

INTRODUCTION

β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2. 1.23) hydrolyzes lactose into its constituting monosaccharides, glucose and galactose and is distributed in the intestines of mammals, plant seeds, and various microorganisms (1). β-gal and its structural gene, *lacZ*, from bacteria have been extensively studied. In particular, the *E. coli lacZ* gene is widely used as a reporter gene for various gene regulation studies and is also used for constructing chimeric genes with other genes (2-4). β-gal is a very important enzyme for many lactic acid bacteria (LAB), especially for LAB used for cheese and yogurt production (5). β-gal is important for the industrial utilization of cheese-whey, a waste product of the cheese industry and contains a lot of lactose. β-gal-treated whey can be used as a substrate for ethanol production by yeasts (6). β-gal is also used for the production of lactose-free dairy products for lactose- intolerant individuals (6). Many studies have been conducted on the β-gal from various LAB and these include the distribution of β-gal activities among LAB strains (7), the role of β-gal in the utilization of lactose (5) and β-gal gene cloning (8-10). LAB metabolize lactose via two different systems: a phosphoenolpyruvate lactose phosphotransferase (PEP-PTS) system with phospho-β-gal (p-β-gal) and a lactose permease system with β-gal (11). β-gal digests lactose into glucose and galactose, and the galactose moiety is then metabolized by the Leloir pathway enzymes (12). p-β-gal digests lactose-6-p into glucose and galactose-6-p and the galactose-6-p is then further metabolized into triose phosphates by the enzymes of the D- tagatose-6-p pathway (11). In most *Lactococcus lactis* strains, the predominant enzyme for lactose utilization 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 (11). Unlike other lactococci, *L. lactis* 7962 uses β-gal as the major lactose utilizing enzyme and in this respect, *L. lactis* 7962 is an atypical strain (13). The complete *gal/lac* operon of *L. lactis* 7962 has been previously cloned and the nucleotide sequences of *lacZ* determined (9). *L. lactis* MG1363 is a Lac⁻, plamid-free derivative from *L. lactis* NCDO 712 (14). Because of the Lac⁻ phenotype, *L. lactis* MG1363 did not grow fast on milk or other lactose-based media. We tried to investigate the effect of *lacZ* gene (from 7962) expression on the growth of MG1363. Here, we report the introduction of *lacA-lacZ* genes together with *lacA* promoter into *L. lactis* MG1363 and the degree of expression of *lacZ* in MG1363.

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MATERIALS AND METHODS

Bacterial cultures and media

Bacterial strains and plasmids used for this work are described in Table 1. *L. lactis* 7962 and *L. lactis* MG1363 were grown at 30°C without shaking in M17 broth (Difco Lab, Detroit, USA) containing 1% (w/v) level of either glucose, galactose, or lactose. *L. lactis* cells harboring pMG36e or its derivatives were cultured in M17 broth with erythromycin (5 μg/ml). *E. coli* DH5α cells and recombinant *E. coli* cells were cultured in LB containing appropriate antibiotics [ampicillin (Ap), 50 μg/ml; erythromycin (Em), 200 μg/ml; tetracycline (Tc), 10 μg/ml] at 37°C.

β-gal assay

The β-gal activities of *L. lactis* cells were measured according to the Miller method (15). *L. lactis* MG1363 cells were grown at 30°C in M17 broth and 1 ml aliquots were taken at various time points. The cells were resuspended in 1 ml of a Z buffer (Na_2HPO_4 7 H_2O 1.61 g, NaH_2PO_4 H_2O 0.55 g, KCl 0.075 g, $MgSO_4$ 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_3Citrate $2H_2O$ 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.

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

t: time in min taken for color development, A_{600}: absorbance value of culture at 600 nm

DNA manipulations

L. lactis chromosomal DNA was isolated as described by Jeong et al. (16), 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 (17) and plasmid prep from *L. lactis* was done by using O'Sullivan and Klaenhammer method (18). Competent cell preparation and electroporation of *E. coli* were done according to the method of Dower et al. (19). Electroporation of *L. lactis* strains was done according to the method of Holo and Nes (20). A southern blot experiment was carried out according to the published methods (21) and probes were radioactively labelled using a random prime labelling kit (Amersham Pharmacia Biotech., RPN1600Y, USA) and α-P^{32}-dATP.

RNA isolation

Total RNA from *L. lactis* was prepared by the following method. *L. lactis* cells were grown in 100 ml of M17 broth containing a 1% level of either glucose, galactose or lactose. After 8 h of cultivation at 30°C, a cell pellet was 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, USA). Cell extracts were obtained by centrifugation and 20 μl of Proteinase K (20 mg/ml) was added. The cell extract was incubated for 1 hr at 55°C followed by phenol/chloroform extractions and ethanol precipitation. The pellet obtained by ethanol precipitation was 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 unit/μl, Promega, Madison, USA) was added to remove any contaminating DNA. After 1 hr of digestion at 37°C, RNA was recovered after 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.

Table 1. Bacterial strains and plasmids used for this work

Bacterial strain or plasmid	Description	Reference
E. coli		
DH5α	Φ80*dlacZ*△M15, *recA1*, *endA1*, *gyrA96*, *thi*-1, *hsdR17* (r_K^-, m_K^+), *supE44*, *relA1*, *deoR*, △ (*lacZYA-argF*)U169	
Lactococcus lactis subsp. *lactis*		
MG 1363	plasmid-free derivative of NCDO 712, Lac^-	14
Lactococcus lactis subsp. *lactis*		
ATCC 7962		13
plasmids		
pCKL11	pBR322 containing an 11 kb *Pst*I fragment which encompasses *lac* operon genes of *L. lactis* 7962	9
pMG36e	Em^r, 3.6 kb; expression vector carrying origin of a cryptic plasmid, pWV01	22
pMLZ3	pMG36e containing a 4.4 kb *lacA-lacZ* gene in *Eco*RI site	This study
pKF-gal	pKF18 containing a 3 kb *Pst*I-*Sal*I fragment containg *lacZ*	24

Slot blot hybridization

Slot blot hybridization was performed by using a slot blot apparatus (Hoefer PR600, USA). Twenty μg of the RNA samples were loaded onto each slot according to the procedure provided by the manufacturer. A hybond N^- nylon membrane (Amersham Pharmacia Biotech., RPN203B, USA) was used and the RNA was immobilized onto the membrane by UV-crosslinking (BioRad, GS linker II, USA). Prehybridization and hybridization were performed at 43°C in a hybridization chamber (Hybaid, shake'n'stack, UK) 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.

Plasmid stability

Stability of pMLZ3 in *L. lactis* MG1363 was examined. Actively growing MG1363 [pMLZ3] cells in M17 broth containing Em (5 μg/ml) were used to inoculate fresh M17 broth (inoculum size, 1%) without Em and the culture was incubated for 24 hr at 30°C. Grown cells again were used to inoculate fresh medium without Em. Continued subculturing in fresh M17 broth at daily intervals was repeated for up to 9 days. At 3, 6 and 9 days, aliquots of culture were taken and serially diluted with 1/10 M17 broth. 0.1 ml of serially diluted samples was spread onto M17 plates with and without Em, and incubated at 30°C for 48 hrs. The percentage of cells still harboring pMLZ3 was calculated as follows.

$$\text{Percentage of cells keeping pMLZ3} = \frac{\text{number of cells on M17 Em plate}}{\text{number of cells on M17 plate}} \times 100$$

RESULTS AND DISCUSSION

Introduction of *lacZ* gene from *L. lactis* 7962 into *L. lactis* MG1363

A 4.4 kb *Eco*RI fragment containing *lacA-lacZ* genes of *L. lactis* 7962 with upstream *lacA* promoter sequences was obtained from pCKL11 (9). An 11 kb *Pst*I fragment containing *gal/lac* operon genes was isolated from pCKL11 first and cut with *Eco*RI under partial digestion conditions (Fig. 1). The 4.4 kb fragment was isolated and purified from an agarose gel (0.8%) by using a gel extraction kit (Omega Biotek, Doraville, GA USA), and ligated with pMG36e (22). pMG36e is a lactococcal expression vector based on a 2.2 kb broad-host-range plasmid, pWV01, of *L. lactis* ssp. *cremoris* Wg2 and carries an erythromycin (Em) resistance marker. The 8.0 kb recombinant plasmid was obtained and named as pMLZ3. Fig.1 shows the construction procedures for pMLZ3 and the positions of *lacA* and *lacZ* genes on the 4.4 kb fragment with *lacA* promoter sequences. pMLZ3 was introduced into *L. lactis* MG1363 competent cells by electroporation and transformants which formed colonies on a M17 plate containing Em (5 μg/ml) were obtained. Plasmid DNA from a transformant was prepared and checked by agarose gel electrophoresis after

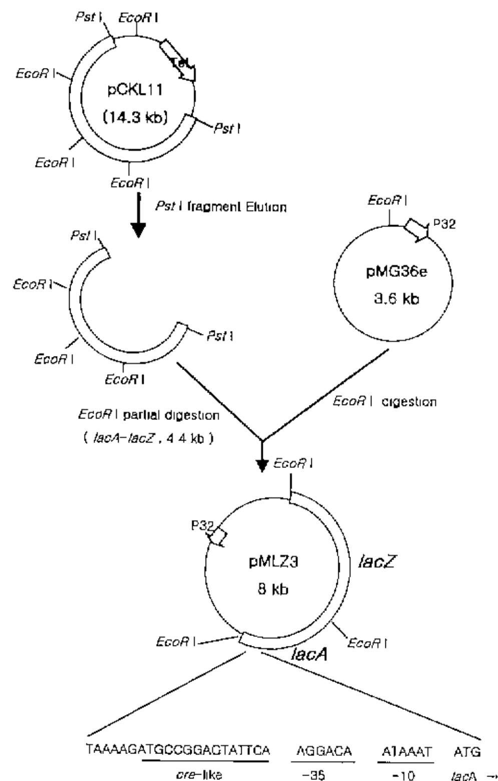

Fig. 1. Procedures for constructing pMLZ3. pMLZ3 is based on pMG36e and a 4.4 kb fragement containing *lacA-lacZ* genes from *L. lactis* 7962 is isolated from pCKL11. Upstream of *lacA*, lactococcal promoter sequences (-35, -10) are located. A *cre*-like sequence is also found. Transcription of *lacZ* depends on *lacA* promoter and the order of transcription is *lacA-lacZ*.

restriction digestion with *Eco*RI (Fig. 2). The transformation frequency of MG1363 for pMLZ3 was estimated to be around 3×10^3 CFUs/μg DNA.

β-gal activities of *L. lactis* MG1363 cells harboring pMLZ3

Growth and β-gal activities of *L. lactis* MG1363 [pMLZ3] in M17 broth were measured. As shown in Fig. 3, MG1363 [pMG36e] (- control) grown on lactose showed the poorest degree of growth (OD_{600} = 0.73 at 6 h). This was expected considering the Lac^- phenotype of the MG1363 strain. *L. lactis* cells grew quickly on glucose and entered into the stationary phase in 6 hr whereas it took longer to reach the same absorbance on galactose. The growth rate of 7962 was higher than that of MG1363 in all three carbon sources tested. β-gal activities of MG1363 [pMG36e] on glucose, lactose, or galactose were in the range of 1-3 units (Fig. 4). β-gal activities of MG1363 [pMLZ3] were at least 10-fold higher (19~41 units) than those of MG1363[pMG36e] but the degree of growth was not significantly different (OD_{600} values; 0.77 versus 0.73 at 6 h cultivation on lactose, see Fig. 3B). The results indicate that introduction of the *lacZ* gene into MG1363 does not complement the Lac^- phenotype of MG1363. MG1363 cells on glucose grew quickly and reached the maximum β-

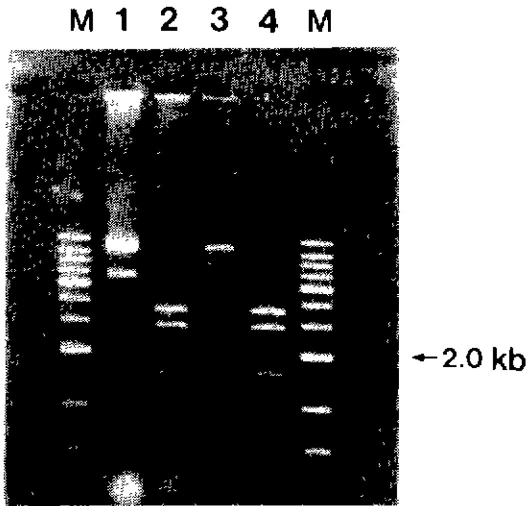

Fig. 2. Restriction digestion of pMLZ3 with *Eco*RI. M, 1kb DNA ladder (GeneRuler™ MBI Fermentas, Vilnius, Lithuania); 1, pMLZ3 from *E. coli* DH5α ; 2, pMLZ3 from *E. coli* DH5α (*Eco*RI digested); 3, pMLZ3 from *L. lactis* MG1363; 4, pMLZ3 from *L. lactis* MG1363 (*Eco*RI digested). When *Eco*RI-digested, pMLZ3 produces 3 fragments and the largest, 3.6 kb, fragment corresponds to pMG36e. A *Eco*RI site is located 5' region of *lacZ* (470 bp downstream from ATG). 2.8 kb fragment contains most of *lacZ* and the smallest 1.6 kb fragment contains *lacA* promoter, *lacA*. and 5' part of *lacZ*.

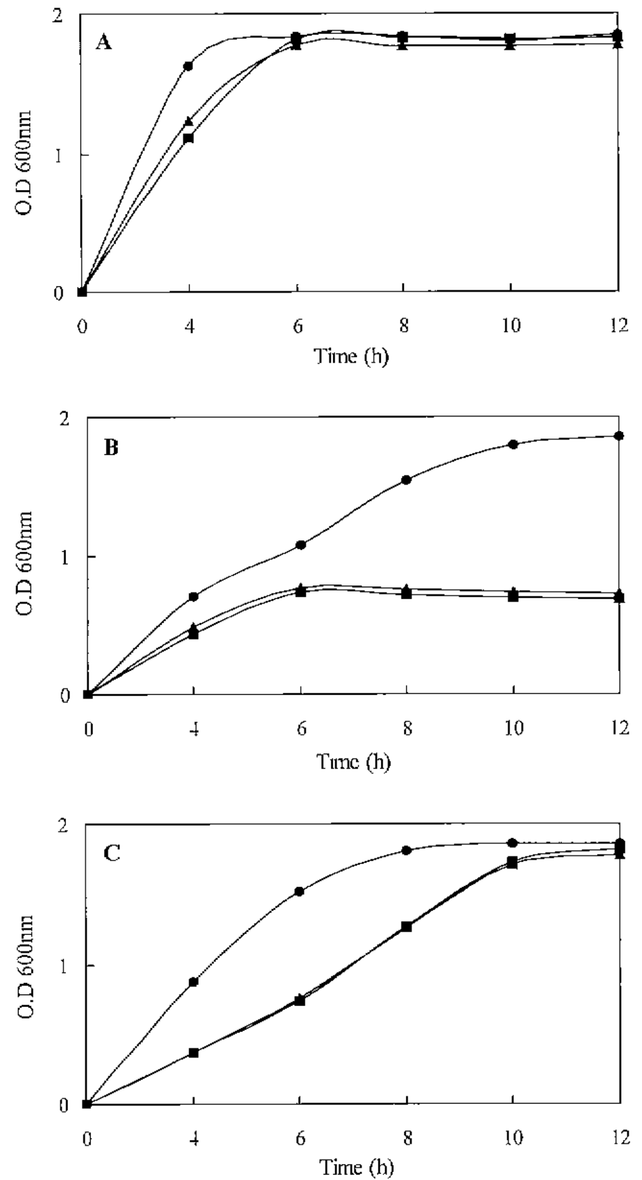

Fig. 3. Growth of *L. lactis* cells on M17 broth containing different carbon source. 1% level of either glucose, galactose, or lactose was included into M17. Fresh M17 broth was inoculated with 1% overnight culture grown in the same media and cultivated at 30°C. A, glucose; B, lactose; C, galactose. ■, MG1363[pMG36e], control; ▲, MG1363[pMLZ3]; ●, 7962 strain.

gal activities in 6 hr whereas cells on galactose reached the maximum level in 10 hr. An interesting observation was that β-gal activities of cells grown on galactose was the highest (41 units at 10 h) followed by cells grown on glucose (33 units at 10 h) and cells grown on lactose had the lowest activity (17 units at 10 h). This is contrary to the results observed in 7962 cells (23). In 7962, maximum activity of β-gal was observed in cells grown on lactose (325 units at 4 h) and cells grown on galactose had 263 units of activity at the same time point. Cells grown on glucose had very low activity (3.6 units), indicating the operation of catabolite repression in 7962 cells. Thus, expression of *lacZ* in MG1363 was quite different from that in the original host, 7962. Although the copy number of *lacZ* in MG1363 is at least 10 times higher than that in 7962 (single-copy), β-gal activity of MG1363[pMLZ3] is about one tenth of that in the 7962 strain. Thus, the efficiency of *lacZ* expression is very low in MG1363 although the reasons are not obvious. The lack of an efficient lactose transport system in MG1363 might be one reason for poor growth of MG1363[pMLZ3] on lactose. But the observed lower β-gal activities of MG1363[pMLZ3] is difficult to explain since cell extracts and ONPG were used for β-gal assays. Unstability of β-gal in MG1363 might be the reason (see below). The apparent lack of catabolite repression for *lacZ* in MG1363 could be due to either the absence of regulatory molecules such as CcpA or the lack of binding sites for regulatory molecules. In this respect, the presence of *ccpA* in MG1363 was investigated and confirmed through this study (see below).

Level of β-gal mRNA

In order to know whether the low β-gal activities of MG1363[pMLZ3] are due to low concentrations of β-gal mRNA, slot blot hybridization was performed. A 3 kb *Pst*I-*Sal*I fragment containing a promoterless whole *lacZ* gene from 7962 was isolated from pKF-gal (24) and used as a probe and the results are shown in Fig. 5. On the contrary to the β-gal activity measurements, MG1363[pMLZ3] grown on lactose showed the highest concentration of *lacZ* transcript and cells grown on glucose showed the lowest. The same results were obtained with RNA preps from 7962. These results indicate that the low β-gal activities observed in MG1363[pMLZ3] cells grown on lactose or galactose were not caused by poor transcription

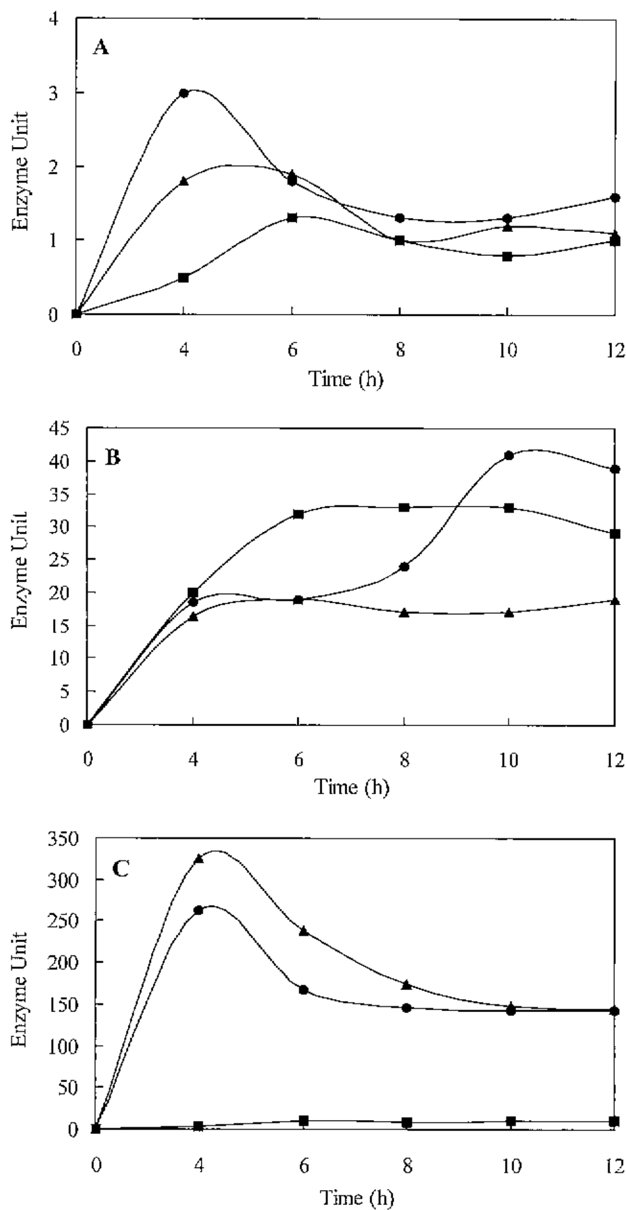

Fig. 4. β-gal activities of *L. lactis* cells. Growth conditions were same as described in Fig. 3. A, MG1363[pMG36e], control; B, MG1363[pMLZ3]; C, 7962 strain. ■, glucose; ▲, lactose; ●, galactose.

of *lacZ* in MG1363. As shown in Fig 5, RNA prep from MG1363[pMLZ3] cells grown on lactose showed the strongest hybridization signal. When compared with the hybridization signals of RNA preps from 7962, it can be concluded that sufficient amounts of *lacZ* transcripts are synthesized in MG1363 [pMLZ3]. The hybridization signal of RNA prep from cells grown on glucose was the weakest, indicating catabolite repression still works in MG1363 despite of the apparent lack of catabolite repression in terms of β-gal activities. We do not know the exact reasons why *lacZ* transcripts in MG1363 [pMLZ3] are not efficiently translated into active enzyme. Proteolytic degradation of β-gal in MG1363 might be one reason.

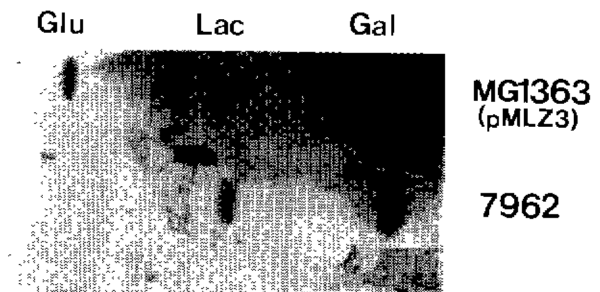

Fig. 5. Slot blot for detecting β-gal mRNA in *L. lactis* cells. Twenty μg of RNA was applied to each slot. In the first row, RNA from *L. lactis* MG1363 [pMLZ3] was applied and in the second row, RNA from *L. lactis* 7962 was applied. Glu, Lac, and Gal represent RNA from glucose-, lactose-, and galactose-grown cells, respectively.

Alternatively, translation of *lacZ* or accumulation of β-gal might be repressed in the MG1363 strain where carbohydrate metabolism including lactose utilization are regulated intricately by various mechanisms closely linked with PEP: PTS systems and non-PTS systems (25). Obviously, more studies are needed to answer this question.

Presence of *ccpA* gene in *L. lactis* MG1363

CcpA (catabolite control protein) is a negative regulator for transcription of genes under catabolite repression including *gal* operon of *L. lactis* (26). CcpA represses transcription of genes by binding to a *cis*-acting *cre* (catabolite responsive element) site, located in the promoter region of genes. The presence of the *ccpA* gene among various gram-positive bacteria including bacilli, staphylococci, lactic acid bacteria, and some actinomycetes has been reported (27). Since the presence of *ccpA* was not previously reported for *L. lactis* MG1363 and the transcription of *lacZ* from *L. lactis* 7962 is most likely repressed by CcpA, the presence of the *ccpA* gene in *L. lactis* MG1363 chromosome was examined by Southern blot experiment. Complete *ccpA* gene from *L. lactis* 7962 was previously cloned by us (manuscript in preparation) and a 0.8 kb fragment corresponding to the internal region of the *ccpA* was obtained by PCR and used as a probe. The southern blot autoradiogram is shown in Fig. 6. As shown in Fig. 6, the presence of *ccpA* in *L. lactis* MG1363 was confirmed. A 10 kb *Eco*RI fragment from MG1363 hybridized with the probe whereas a 4.5 kb *Eco*RI fragment from 7962 was detected, indicating differences in restriction map between these two strains. *ccpA* of *L. lactis* MG1363 might affect the transcription of *lacZ* from 7962 introduced into MG1363 since a potential *cre* site (TGCCGGAGTATTCA, 7737-7750 nt in U60828) is located just upstream of *lacA* promoter sequences and transcription of *lacZ* depends on this promoter. In fact, RNA from MG1363 [pMLZ3] grown on glucose showed the weakest hybridization signal. This can be interpreted that transcription was repressed although β-gal activities of MG1363[pMLZ3] on glucose was higher than that on lactose as mentioned above. Cloning of *ccpA* from MG1363 and functional studies such

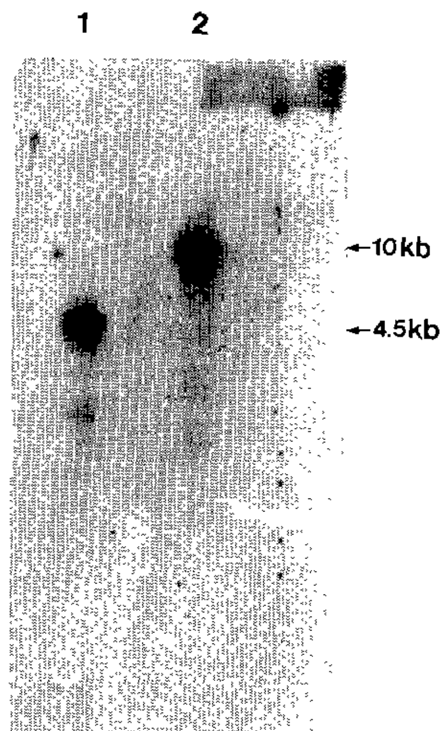

Fig. 6. *Southern blot for detecting the ccpA gene in L. lactis* MG1363. Chromosomal DNA (10 μg) was digested with *Eco*RI and DNA fragments were separated by agarose gel electrophoresis. 0.8 kb PCR fragment corresponding to the internal region of the *ccpA* gene from *L. lactis* 7962 was used as a probe. 1. *Eco*RI-digested *L. lactis* 7962 chromosomal DNA, 2. *Eco*RI-digested *L. lactis* MG1363 chromosomal DNA. Hybridizing signals were detected at 4.5 kb and 10 kb regions, respectively.

Table 2. Stability of pMLZ3 in *L. lactis* MG1363

Strain	Incubation time (days)			
	0	3	6	9
MG1363 [pMLZ3]	100%	72.9	46.6	24.3

as complementation of *ccpA*⁻ mutant with MG1363 CcpA are needed.

Stability of pMLZ3 in *L. lactis* MG1363

The results of stability tests for pMLZ3 in *L. lactis* MG1363 are summarized in Table 2. pMLZ3 was stable without antibiotic selection. In the absence of erythromycin, MG1363 lost pMLZ3 slowly, so after 9 days of daily subculturing in M17 media, 75.7% of MG1363 cells lost pMLZ3. The result indicates that pMG36e is reasonably stable in MG1363 and thus useful for the genetic engineering of the MG1363 strain where use of an antibiotic selection marker is not desirable.

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