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Molecular Cloning of a β-D-Galactosidase Gene from *Lactococcus lactis* subsp. *lactis* 7962

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The β-galactosidase gene from *Lactococcus lactis* subsp. *lactis* ATCC 7962 was cloned and its enzymatic properties were characterized, with a view to assessing its potential use as a selection marker in the food-grade cloning vector. Chromosomal DNA from *L. lactis* subsp. *lactis* 7962 was cleaved with *Pst*I and ligated into pBR322 for transformation into *Escherichia coli* TG1. Transformants showing β-galactosidase activity possessed the pBR322 plasmid containing a 10 kilobase (kb) *Pst*I fragment and this plasmid was named pCKL11. The cloned β-galactosidase gene came from the chromosomal DNA of *L. lactis* subsp. *lactis* 7962 was confirmed by Southern hybridization. A restriction map of pCKL11 was constructed from the cleavage of both pCKL11 and the purified 10 kb insert fraqment. The optimum pH of the β-galactosidase determined with the *E. coli* harboring the pCKL11 was 7.0. The optimum temperature was 50°C, while the pI of the enzyme was 7.4. These values were the same as those of the enzyme from the parent strain.

Lactococcus lactis subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, and *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* are traditionally used in the manufacture of a variety of fermented dairy products. These strains are economically important due to their ability to produce large amounts of lactic acid and utilized extensively in the dairy fermentations as starter cultures. Due to the potential role of recombinant DNA technology in strain improvement, researchers have developed several plasmid cloning vectors for the dairy lactococci, and most of them use antibiotic resistance as selection markers (1). However, antibiotic selection is not suitable for the starter cultures utilized in dairy and food fermentations. Carbohydrates utilization genes are potential alternative selection markers. The cloned β-galactosidase gene is a suitable candidate as a plasmid selection marker for use with food-grade organisms.

The *E. coli* β-galactosidase (LacZ) has been sucessfully utilized as a selection marker in several cloning vectors, because of the availability of easy and sensitive detection method. Among the mesophilic dairy lactococci, *L. lactis* subsp. *lactis* ATCC 7962 is unusual in that it contains β-galactosidase activity (7, 9). Most of *Lactococcus lactis* subsp. *lactis* strains contain phospho-β-galactosidase (P-β-gal), but do not contains β-galactosidase (β-gal) activity (9). *L. lactis* subsp. *lactis* has been questioned in the past classification, because it contains P-β-gal like other *L. lactis* strains; but its cell extracts do not react with group N antiserum (3).

In this study, the β-galactosidase gene from *L lactis* subsp. *lactis* 7962 was cloned and its enzymatic properties were characterized. We believe this cloned gene is one of the most suitable candidates as a plasmid selection marker for its use with food-grade organisms, especially for the mesophilic dairy lactococci host. The cloning of β-gal from *L. lactis* subsp. *lactis* have commercial value, in addition to its potential use as a selection marker on a food-grade cloning vector.

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MATERIALS AND METHODS

Bacterial Strains, Plasmids and Media

The bacteria strains and plasmids used in this experiment are listed in Table 1. *Lactococcus lactis* subsp. *lactis* ATCC 7962 was obtained from the American Type Culture Collection (Rockville, Md). It was grown at 30°C in M17 medium (18) supplemented with 0.5% glucose (M17-G) or 0.5% lactose (M17-L). *E. coli* TG1 was used as a recipient in all transformation experiments, and all transformants were grown in LB medium (12) containing tetracycline (25 μg/ml) by incubation at 37°C.

DNA Manipulation

Total genomic DNA from *L. lactis* subsp. *lactis* was

Table 1. The bacterial strains, and plasmids used in this experiment.

Strain or Plasmid	Description	Reference
Strain		
Lactococcus lactis subsp. *lactis* 7962	wild type (starter culture)	(2)
E. coli TG1	*supE hsdΔ5 thi Δ(lac-proAB)* F'[*traD36 proAB*$^+$, *lacI*^{q} *lacZ*ΔM15]	(12)
Plasmid		
pBR322	Ap^r, Tet^r, cloning vector	
pCKl11	Tet^r, pBR322 containing the β-galactosidase gene from *L. lactis* subsp. *lactis* 7962	This work
pUC19	Ap^r, containing the amino-terminal fragment of β-galactosidase for α-complementation, and multicloning site. blue and white color selection cloning vector	(20)

prepared according to the method of Steen *et al.* (17). Plasmid DNA from *E. coli* was isolated by the alkaline lysis method (12). Elution of DNA fragments from agarose gels was carried out using a Gene Clean kit (Bio 101, Inc., La Lolla, CA). Restriction enzymes, DNA ligase, and alkaline phosphatase were purchased from Boehringer Mannheim Co. (Indianapolis, IN) and used under the conditions recommended by the manufacturer. Southern hybridization was performed as described by Southern (16). Labelling of the probe DNA with [α-^{32}P] dATP (3,000 *Ci*/mmol; Amersharm) was carried out by using a random-primed labeling kit (Boehringer Mannheim). Transformation of the *E. coli* strains with plasmid DNA was performed as described by Sambrook *et al.* (12). Transformants harboring the β-gal gene were detected on LB medium containing tetracycline (25 μg/ml), 5-bromo-4-chloro-3-indoly-β-D-galactoside (X-gal, 40 μg/ml), and 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

β-Galactosidase Assay

E. coli TG1 harboring pCKL11 was grown in LB broth containing 2% lactose (w/v) for 20-24 h at 37°C. *L. lactis* subsp. *lactis* 7962 was grown in M17 broth containing 2% lactose (w/v) for 20-24 h at 30°C. Two hundred ml of cells were harvested by centrifugation, washed twice with cold (4°C) deionized water, suspended in 20 ml of 0.01 M potassium phosphate buffer (pH 7.0), and ultrasonicated for 3 min. After the centrifugation at 12,000 rpm in a Sorvall SS-34 rotor at 4°C for 30 min, the supernatant (crude cell extract) was transferred into new tubes, and used for further characterization. To determine the β-gal activity in crude cell extracts, the method of Miller (8) was used. A 1 ml of crude cell extract and 1 ml of Z-buffer (8) or 0.2 ml of crude cell extract and 1 ml of Z-buffer were added according to the enzyme activity. Each tube was mixed well, and placed in a 37°C water bath for 5 min. A 0.4 ml of 10 mM *o*-nitrophenyl-β-D-galactopyranoside (ONPG) was added, and the mixture shaken. When the yellow color was developed sufficiently, 1 ml of 1 M Na_2CO_3 was added to terminate the enzyme-substrate reaction. The release of *o*-nitrophenol (ONP) was measured colorimetrically at 420 nm and the amount of ONP released determined.

Determination of Optimal Enzyme Assay Conditions

To determine the optimal temperature for the β-galactosidase, the activity of the enzyme was measured at different incubation temperature from 20 to 60°C. To determine the optimal pH, the enzyme reaction mixture was incubated at pH 5.0-9.0 with universal buffer (10) instead of Z-buffer.

Isoelectric Focusing

Isoelectric focusing was performed with a pH 3-9 Phast gel (Phamacia). After electrophoresis, the gel was incubated in the reaction mixture containing Z-buffer, β-naphthyl-D-galactopyranoside (50 μg/ml in Z-buffer; stock solution of 10 mg/ml in dimethyl formamide) and fast garnet GBC (1 mg/ml in Z buffer). The active β-gal band appeared as a red band within 30 min.

RESULTS

Identification of a β-Galactosidase Positive Clone

Chromosomal DNA was extracted from *L. lactis* subsp. *lactis* 7962 and cleaved with *Pst*I, and was ligated into the *Pst*I site of pBR322. These ligation mixtures were used to transform *E. coli* TG1. Three transformants out of 315 colonies were blue on LB medium supplemented with tetracycline, X-gal and IPTG. Each plasmid profile from these three transformants showed exactly the same pattern. The transformant harboring the recombinant plasmid was Tc^r, Ap^s, indicating that its plasmid contains a DNA fragment in the *Pst*I site. Recombinant plasmid was digested with *Pst*I and a 10 kb DNA fragment of the insert was observed. When this recombinant plasmid was retransformed into *E. coli* TG1,

the transformant showed blue on LB medium containing tetracycline, X-gal and IPTG. Only when this 10 kb *Pst*I fragment was present in pBR322, β-gal activity could be detected in *E. coli* TG1. We designated this recombinant plasmid as a pCKL11. Since *E. coli* TG1, with or without pBR322, forms white colonies on LB medium containing X-gal, this result indicated that the β-gal gene had been cloned into pBR322 on a 10 kb fragment.

β-Gal activities from crude cell extract of *L. lactis* subsp. *lactis* 7962 and *E. coli* TG1 harboring pCKL11 were measured respectively. β-Gal specific activity (mg ONP/mg protein/min) from transformed *E. coli* TG1 containing pCKL11 was 148.5 which value was thirty times higher than that of *L. lactis* subsp. *lactis* 7962.

Restriction Mapping of pCKL11

To construct a restriction map of pCKL11, it was tested for restriction with *Eco*RI, *Hin*dIII, *Pst*I, *Bam*HI, *Sal*I, *Sma*I, *Kpn*I, *Bst*EII, *Bgl*II, *Bgl*I, *Cla*I and *Xba*I. The 10 kb *Pst*I insert fragment had one *Xba*I site, two *Bgl*I sites, three *Eco*RI sites, and four *Hin*dIII sites, but no sites for the other enzymes used (Fig. 1).

Southern Analysis of Transformant Containing pCKL11

To confirm that the cloned β-gal gene came from the chromosomal DNA of *L. lactis* subsp. *lactis*, Southern hybridization was carried out (Fig. 2). DNAs were digested with endonuclease *Pst*I and the 10 kb insert fragment was used as a probe DNA. The digestion of chromosomal DNA of *L. lactis* subsp. *lactis* 7962 and pCKL11 are shown in Fig. 2, lane 2 and 3, respectively. Two strong and clean bands, both about 10 kb, were observed. However, no bands were observed with pBR 322 and pUC19 plasmid DNAs digested with *Pst*I (Fig 2B, lane 4 and 5, respectively).

Optimum pH and Temperature

The optimum pH and temperature of the β-gal from *E. coli* transformants and that of *L. lactis* subsp. *lactis* 7962 was observed to be pH 7.0 and 50°C, respectively (Fig. 3, 4).

The effect of pH and temperature on the β-gal ac-

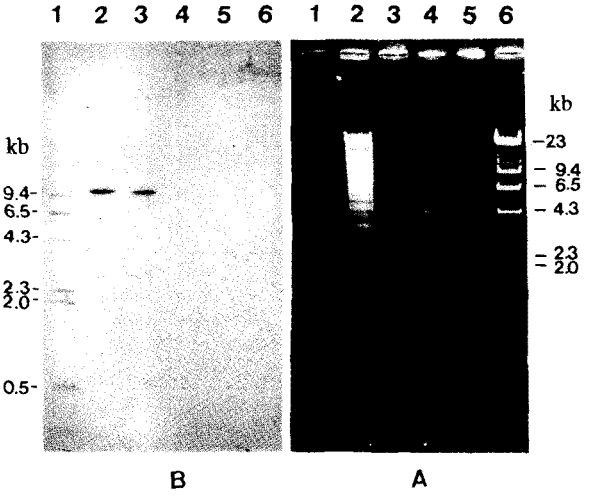

Fig. 2. Southern blot analysis of the genomic DNA of *L. lactis* subsp. *lactis* 7962.

All DNAs was digested with restriction enzyme indicated, seperated by 0.7% agarose gel electrophoresis (A), and was transferred into a nitrocellulose membrane (B). It was hybridized with the 10 kb *Pst*I fragment in pCKL11 which was labelled by nick translation with [α-^{32}P] dATP at 42°C for 15 h. lane 1, λ DNA/*Hin*dIII (^{32}P-labelled); lane 2, *L. lactis* subsp. *lactis* 7962 genomic DNA/*Pst*I; lane 3, pCKL11/*Pst*I; lane 4, pBR322/*Pst*I; lane 5, pUC19/*Pst*I; lane 6, λ DNA/*Hin*dIII.

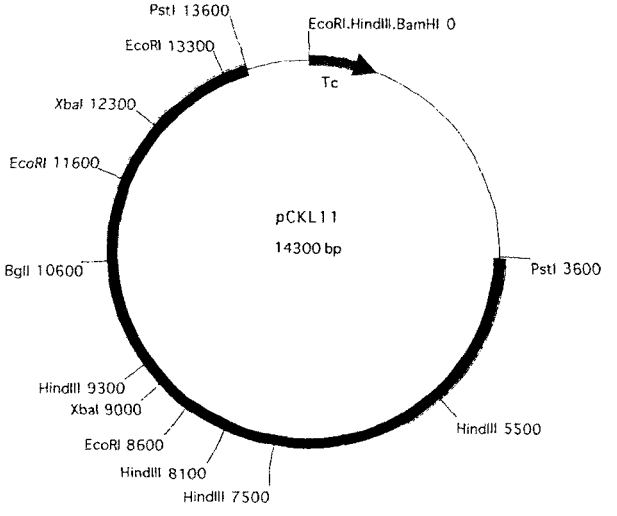

Fig. 1. Restriction enzyme map of pCKL11.

No sites were found in the insert for *Bam*HI, *Bgl*II, *Bst*EII, *Cla*I, *Kpn*I, *Sal*I, and *Sma*I.

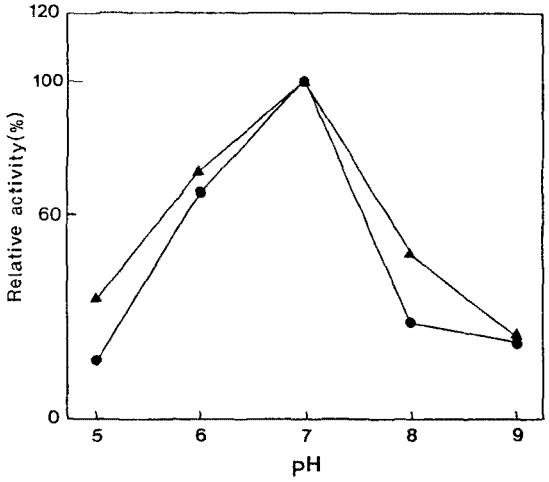

Fig. 3. Effect of pH on the β-galactosidase activity of pCKL 11/*E. coli* (▲) and *L. lactis* subsp. *lactis* 7962 (●).

The β-galactosidase activity was measured at pHs ranging from 5 to 9. The method of Miller (8) were used to determine the enzyme activities.

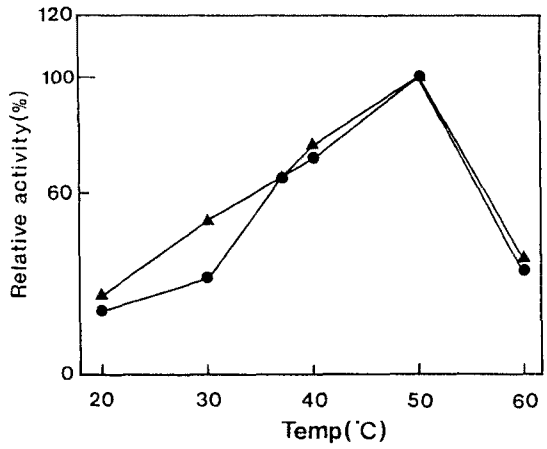

Fig. 4. Effect of temperature on the β-galactosidase activity of pCKL11/*E. coli* (▲) and *L. lactis* subsp. *lactis* 7962 (●). The β-galactosidase activity was measured at temperatures ranging from 20 to 60°C. Enzyme activities were determined by the method of Miller (8).

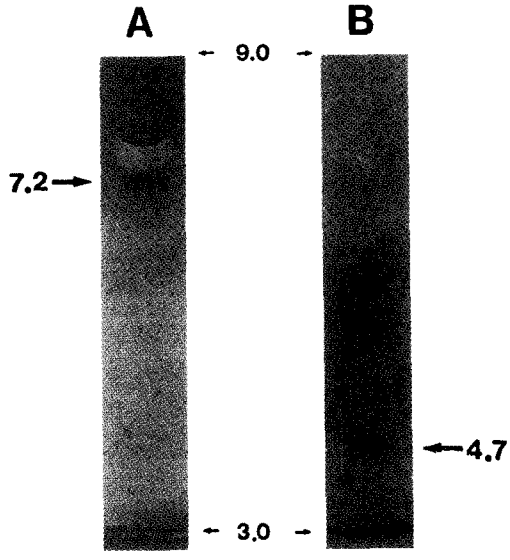

Fig. 5. Determination of isoelectric points by Phast system. A, β-galactosidase from *E. coli* TG1 containg pCKL11; B, β-galactosidase from *E. coli* TG1 containg pUC19. The activity of β-galactosidase was detected as a red band in electrophoresis gel system. β-naphthyl-D-galactopyranoside was used as substrate and fast garnet GBC was used as a dye coupler.

tivity of these two strains is almost the same (Fig. 3, 4).

pI Value

Isoelectric focusing of the cell extracts indicated that both the parent strain *L. lactis* subsp. *lactis* 7962 and the cloned β-gal proteins displayed similar isoelectric points. The isoelectric point of the *E. coli* TG1 harboring pCKL 11 was 7.2, whereas the *E. coli* TG1 harboring pUC19 was 4.7 (Fig. 5).

DISCUSSION

β-Gal is present in various plants, animals, and microorganisms (6, 19). The β-gal of *E. coli* has been studied extensively in regard to its mechnism of protein systhesis, genetic control, and enzyme induction. The use of lactose by lactic acid bacteria is important in the commercial production of various fermented food and dairy products, therefore the induction, purification, and properties of this lactococcal enzyme have been investigated (4, 5, 11, 14, 15).

Studies on the β-gal gene from *Streptococcus salivalius* subsp. *thermophilus* strains (7, 13) have been reported among thermophilic lactic acid bacteria This study is the first and only report about cloning and characterization of β-gal from the mesophilic dairy lactococci. The presence of β-gal in the mesophilic dairy lactococci is unusual. *Lactococcus lactis* subsp. *lactis* 7962 contains P-β-gal activity, but extraordinally it also contains high β-gal activity. The ratio of β-gal to P-β-gal activities are 16:1 from *L. lactis* subsp. *lactis* 7962 (9). A comparison was made (3) between 7962 and known strains of *L. lactis* subsp. *lactis* using physiological properties and the GC content in the DNA. *L. lactis* subsp. *lactis* 7962 was distinguished as an atypical strain of *L. lactis* subsp. *lactis*. Schoroeder *et al.* carried out Southern blot hybridization with ^{32}P-labelled *lacZ* from *S. salivalius* subsp. *thermophilus* A054 as a probe, to determine the homology of β-gal gene among the different species of *S. salivalius* subsp. *thermophilus*, and *L. lactis* subsp. *lactis* 7962 (13). According to their results, no hybridization signal was detected from *L. lactis* subsp. *lactis* 7962, even though *L. lactis* subsp. *lactis* 7962 produces both a β-gal and P-β-gal (13). It can be deduced β-gal gene from *L. lactis* subsp. *lactis* 7962 has different structural pattern from the others. The exact location and complete nucleotide sequence of β-gal gene in pCKL11 should be determined by further experiments. Thereafter, it will be possible to explain how different the sequence of β-gal gene from *L. lactis* subsp. *lactis* 7962 and that of other lactic acid bacteria.

This study has values for the production of β-gal by food microorganism and its potential to be used as a food-grade selection marker.

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