Isolation and Characterization of Plasmid DNA in *Lactobacillus acidophilus*

Kazuo KANATANI, Kazushi YOSHIDA, Takatsugu TAHARA,
Hirosumi MIURA, Masaru SAKAMOTO
and Masao OSHIMURA

Tamon Sake Brewing Co., Ltd., Research Laboratory,
1-13-11, Higashi-cho, Nishinomiya-shi,
Hyogo 662, Japan

Received January 28, 1991

Lactobacillus acidophilus strain TK8912 was found to carry six plasmids having molecular masses of 40, 17, 10.5, 5, 2, and 1.8 megadaltons (Mdal). An acriflavin-induced, Lac^- mutant had lost a 17-Mdal plasmid (pLA102) and phospho- β -galactosidase (P- β -gal) activity. Since strain TK1-2TL (Lac^+ transformant) restored P- β -gal activity, pLA102 is likely to encode a P- β -gal gene required for lactose metabolism in strain TK8912. It is also suggested that the gene for the tagatose 6-phosphate pathway, which is responsible for galactose metabolism, is encoded by the 40-Mdal plasmid pLA101.

Dairy lactic acid bacteria, including species of both the *lactococci* and *lactobacilli*, use lactose as their primary energy source. Two different metabolic pathways for lactose use have been reported in lactic acid bacteria (Fig. 1).¹⁾ One pathway requires phosphorylative transloca-

tion of lactose via the phosphoenolpyruvate-dependent phosphotransferase system (PTS).²⁻⁵⁾ The lactose 6-phosphate thus formed is hydrolyzed by phospho- β -galactosidase (P- β -gal),⁶⁾ giving D-glucose and D-galactose 6-phosphate.⁷⁾ The latter is then

Fig. 1. Lactose and Galactose Metabolic Schemes in Some Lactic Acid Bacteria.

converted to D-tagatose 6-phosphate (Tag-6P) and further metabolized to triose phosphates by the three enzymes of the Tag-6P pathway.⁸⁾ An alternative pathway involves the active transport of lactose by permease,^{9,10)} and further hydrolysis of lactose by β -galactosidase. The resulting D-galactose is converted to galactose 1-phosphate and further to glucose 6-phosphate by the Leloir pathway, and is metabolized *via* glycolysis together with the glucose moiety of lactose.

It has been suggested that some genes for lactose metabolism are associated with plasmid DNA in lactic acid bacteria,^{11–15)} since lactose metabolizing ability is unstable and readily lost by curing agents. For example, the gene for P- β -gal is associated with the plasmid DNA in *Lactobacillus casei*.^{16–18)}

We have isolated Lac[–] mutants that cannot metabolize lactose from *L. acidophilus* TK8912 grown on glucose medium. Therefore, this study was undertaken to ascertain whether the loss of lactose metabolism is correlated with the loss of a plasmid responsible for lactose metabolism.

Materials and Methods

Bacteria and culture conditions. The *L. acidophilus* TK8912 used in this study was obtained from our stock culture collection (Table I). MRS medium¹⁹⁾ was routinely used as the growth medium. Carbohydrates were added at a final concentration of 1% (wt/vol). The pH of all media was adjusted to 6.9. Cells used for assay of enzyme activities were grown for at least 10 generations on MRS broth supplemented with an appropriate carbohydrate.

Curing of plasmid. Mutants of *L. acidophilus* defective in lactose metabolism were isolated by treating with acriflavin.²⁰⁾ A 1% transfer from an overnight culture was made into MRS broth containing acriflavin concentration ranging from 0 to 20 μ g/ml. After 48 hr of incubation at 37°C, samples were transferred to fresh broth containing the same concentration of acriflavin, and incubated for 48 hr more. Dilutions of the culture were plated on bromocresolpurple (0.004%)–lactose indicator agar.²⁰⁾ Plates were incubated at 37°C for 48 hr, and lactose-defective mutants were purified from white colony isolates.

Isolation of plasmid DNA. A 2% transfer from an overnight culture was made into 5 ml of MRS broth containing 1% glucose. Cells were grown for 3 hr at 37°C, pelleted, and suspended in 400 μ l of 6.7% sucrose (in 50 mM Tris, 5 mM EDTA, pH 8.0). To this suspension, mutanolysin (Seikagaku Kogyo, Tokyo, Japan; *N*-acetylmuramidase SG) and lysozyme (Wako Pure Chem. Ind., Osaka, Japan; from egg white) were added (final concentrations 25 and 500 μ g/ml, respectively). Cells were incubated for 30 min at 37°C, and then lysed by addition of a solution containing EDTA (0.25 M in 50 mM Tris, pH 8.0) and 20% (wt/vol) sodium dodecyl sulfate (in 50 mM Tris, 20 mM EDTA, pH 8.0). Plasmid DNA was extracted from the lysate by the method of Anderson and McKay.²¹⁾

Agarose gel (0.7%) electrophoresis was done in TAE buffer.²²⁾

Transformation. Transformation by electroporation was done by the method of Chassy *et al.*²³⁾ After the cells were used for a single pulse of electroporation (25 μ F at 5000 V/cm), serial dilutions of the samples were plated on bromocresolpurple lactose and galactose indicator plates. After incubation at 37°C, Lac⁺ and Gal⁺ transformants were purified from yellow colonies.

Enzyme assays. Phosphoenolpyruvate-dependent PTS activities were assayed in permeabilized cells (toluene-acetone (1:9)) which were prepared by the method of LeBlanc *et al.*²⁴⁾ with modification. The modification

Table I. STRAINS OF *Lactobacillus acidophilus*

Strain	Phenotype	Plasmids (Mdal)	Derivation
TK8912	Lac ⁺ Gal ⁺	40, 17, 10.5, 5, 2, 1.8 ^a	Wild type
TK1-1	Lac [–] Gal ⁺	40, 10.5, 5, 2, 1.8	From TK8912 after curing with acriflavin (This study)
TK1-2	Lac [–] Gal [–]	10.5, 5, 2, 1.8	From TK8912 after curing with acriflavin (This study)
TK1-2TL	Lac ⁺ Gal [–]	17, 10.5, 5, 2, 1.8	Transformant of TK1-2 with pLA102 (This study)
TK1-2TG	Lac [–] Gal ⁺	40, 10.5, 5, 2, 1.8	Transformant of TK1-2 with pLA101 (This study)

^a The molecular mass of each plasmid harbored by *L. acidophilus* TK8912 was measured by the method of Meyers *et al.*,²⁹⁾ using four plasmids (28.0, 6.6, 6.0 and 1.4 Mdal) from *L. plantarum* ATCC 8014^{30,31)} as markers.

involved the use of 20 μ l of a permeabilized cell (220 μ g of dry cell weight) suspension and carbohydrate substrate added to a final concentration of either 10 mM glucose, 5 mM lactose, or 40 mM galactose.⁹⁾ β -galactosidase and phospho- β -galactosidase activities were assayed in a 500- μ l reaction mixture containing 50 mM sodium-potassium phosphate buffer (pH 7.2), either 1 mM *o*-nitrophenyl- β -D-galactopyranoside (ONPG) or 1 mM ONPG-6-phosphate (ONPG-6P), and 50 μ l of a permeabilized cell suspension. The reaction mixtures were incubated for 30 min at 37°C and the reaction was stopped by adding 1 ml of 5% Na₂CO₃. After a brief centrifugation to remove the cells, the absorbance at 420 nm was measured.

Extraction and assay for Gal-1P and Gal-6P. Cultures were grown at 37°C in MRS broth containing 20 mM galactose. When the concentration reached 14 to 16 mM (Gal⁺ strain; 3 hr, Gal⁻ strain; 12 hr), cells were collected on membrane filters (pore diameter, 0.45 μ m), and filters were added to 0.15 volume of 5% *n*-butanol and incubated at 37°C for 10 min. The galactose-1-phosphate (Gal-1P) and galactose-6-phosphate (Gal-6P) in the supernatant were assayed by the method of Thomas *et al.*²⁵⁾

Results

Isolation and growth of Lac⁻ mutants

When strain TK8912 cells of *L. acidophilus* were treated with a plasmid-curing agent, acriflavin, lactose deficient mutants (Lac⁻) were readily isolated. The treatment with 15 μ g of acriflavin per ml was found to be most effective (Table II). The second treatment with acriflavin and the combination of other curing agents, such as mitomycin C (data not shown)

Table II. EFFECTS OF ACRIFLAVIN ON LOSS OF LACTOSE METABOLISM OF *L. acidophilus* TK8912

Acriflavin concentration (μ g/ml)	Percent of Lac ⁻ mutants	
	1st	2nd
0	0	0
5	0	0.3
10	0.7	3.1
15	4.1	19.8
20	3.5	15.7

A 1% transfer from an overnight culture was made into MRS broth containing acriflavin. After 48 hr of incubation at 37°C, samples were transferred to fresh broth containing acriflavin of the same concentration. After the 1st or 2nd incubation, dilutions of the culture were plated on bromocresolpurple (0.004%)–lactose indicator agar.²⁰⁾

significantly increased the frequency of Lac⁻ mutants, as was the case reported in *L. casei*.²⁶⁾ These results suggest that some plasmids may be responsible for lactose metabolism in strain TK8912.

Previous reports^{8,24,27,28)} indicated that genes for both lactose and galactose metabolism were encoded by identical plasmids in *Lactococcus lactis*. Thus, curing of such plasmids resulted in the Lac⁻ Gal⁻ phenotype. Therefore, galactose metabolizing activity was also measured in the Lac⁻ mutants. In our experiment, after curing treatment with acriflavin (15 μ g/ml, 2nd), 43% (59 of 138) of Lac⁻ mutants had a Gal⁻ phenotype. This indicates that the genes for lactose and galactose metabolisms are independently encoded by different plasmids.

Plasmid analysis

Plasmid profiles of the strain TK8912 (Lac⁺ Gal⁺), TK1-1 (Lac⁻ Gal⁺), and TK1-2 (Lac⁻ Gal⁻) were analyzed in Fig. 2. The parental strain had six plasmids of molecular masses of 40×10^6 , 17×10^6 , 10.5×10^6 , 5×10^6 , 2×10^6 ,

Fig. 2. Agarose Gel (0.7%) Electrophoresis Patterns of Plasmid DNA Isolated from *L. acidophilus* TK8912 and Its Mutants.

(A) TK8912, (B) TK1-1, and (C) TK1-2. (D) Molecular mass markers of plasmids obtained from *L. plantarum* ATCC8014^{30,31)} (bands identified top to bottom; 28.0, 6.6, 6.0, and 1.4 Mdal). "chr" denotes position of chromosomal DNA.

and 1.8×10^6 . In contrast, the lactose-deficient mutant TK1-1 ($\text{Lac}^- \text{Gal}^+$) lost the 17-Mdal plasmid pLA102, and TK1-2 ($\text{Lac}^- \text{Gal}^-$) further lost the 40-Mdal plasmid pLA101 (Fig. 2). In the isolation of Lac^- mutants from strain TK8912, over 99% of the Lac^- mutants lost pLA102, and all of the Gal^- mutants lost pLA101, suggesting that key enzymes for lactose and galactose metabolisms are encoded by the genes on plasmids pLA102 and pLA101, respectively.

Transformation and analysis of transformants

To show that the plasmids are responsible for lactose and galactose metabolism, strain TK1-2 ($\text{Lac}^- \text{Gal}^-$) was transformed by the plasmids pLA102 and pLA101 by electroporation. Although the efficiency of transformation was low, nine Lac^+ strains and four Gal^+ strains per μg of DNA were transformed. In the absence of pLA102 or pLA101, no Lac^+ or Gal^+ transformants were obtained under the same conditions. Plasmid profiles of transformants indicate the plasmid pLA102 (lane C) and pLA101 (lane D) were re-introduced into the strain TK1-2TL and TK1-2TG, respectively (Fig. 3).

Enzyme activities

The activities of phosphoenolpyruvate-dependent PTS on lactose (Lac), galactose (Gal), and glucose (Glu), as well as P- β -gal and

Fig. 3. Agarose Gel (0.7%) Electrophoresis Patterns of Plasmid DNA Isolated from Strains Used in the Transformation Experiment.

(A) *L. acidophilus* TK8912 (top to bottom; 40, 17, 10.5, 5, 2, and 1.8 Mdal), (B) TK1-2 (missing the 40- and 17-Mdal plasmids) is a $\text{Lac}^- \text{Gal}^-$ mutant of strain TK8912 used as the recipient, (C) TK1-2TL, Lac^+ transformant of strain TK1-2, and (D) TK1-2TG, Gal^+ transformant of strain TK1-2. "chr" denotes position of chromosomal DNA.

Table III. SUGAR-PTS, P- β -GALACTOSIDASE (P- β -gal), AND β -GALACTOSIDASE (β -gal) ACTIVITIES IN PERMEABILIZED CELLS OF *L. acidophilus* TK8912 AND ITS MUTANTS

Strain	Energy source for growth	Specific activity ^a			P- β -gal ^b	β -gal ^b
		Lac-PTS	Gal-PTS	Glu-PTS		
TK8912	Lactose	3.8	7.6	9.1	602	157
	Galactose	3.0	9.9	7.6	579	92
	Glucose	0	4.2	5.4	0	23
TK1-1	Galactose	2.8	8.3	7.8	0	81
	Glucose	0	5.2	5.6	0	29
TK1-2	Glucose	0	5.2	6.7	0	21
TK1-2TL ^c	Lactose	4.1	7.9	8.7	597	151
	Glucose	0	3.8	5.9	0	20
TK1-2TG ^d	Galactose	3.0	8.8	8.0	0	84
	Glucose	0	5.7	5.7	0	22

^a Specific activity are expressed as nanomoles of NADH oxidized per minute per milligram of dry cell weight.

^b Activities expressed as nanomoles of *o*-nitrophenyl- β -D-galactopyranoside (ONPG) (β -gal) or ONPG-6P (P- β -gal) hydrolyzed per minute per milligram of dry cell weight.

^c Lac^+ transformant.

^d Gal^+ transformant.

β -gal were assayed in strain TK8912 and its mutants (Table III). P- β -gal activity was fully induced by galactose or lactose in the parent strain, but the activity was not induced in strain TK1-1 and TK1-2, using any carbon sources. It is also noted that Lac-PTS activity was normally retained in strain TK1-1. More interestingly, strain TK1-2TL restored P- β -gal activity. Thus, taking the plasmid profiles (Figs. 2 and 3) into consideration together, it is conceivable that a P- β -gal gene resides on plasmid pLA102. A major contribution of β -gal to lactose metabolism seems to be very unlikely, since strain TK1-1 cells still retained a significant activity of this enzyme.

The level of Gal-PTS activity in strain TK1-2 (Lac⁻ Gal⁻) was nearly the same with that in strain TK1-1 (Lac⁻ Gal⁺), thus the defect of a certain enzyme of the Tag-6P pathway, which is responsible for phosphorylated galactose metabolism, may explain the loss of galactose metabolic activity in this mutant.

Intracellular concentrations of Gal-1P and Gal-6P in cells growing on galactose

Gal-1P is a key intermediate of the Leloir pathway, while Gal-6P is a key intermediate of the Tag-6P pathway (Fig. 1). Therefore, to distinguish the route responsible for galactose metabolism, intracellular Gal-1P and Gal-6P

were measured (Table IV). The fact that no Gal-1P was detected in any strain and that the intracellular Gal-6P was elevated 2-fold in Gal⁻ mutants, strongly suggest that the Tag-6P pathway is responsible for galactose metabolism in these strains.

Discussion

Lactic acid bacteria are widely used in dairy manufactures to obtain lactic acid from milk. This important ability to ferment lactose is however unstable and is often lost spontaneously.^{12,32,33} The loss of lactose metabolizing ability is enhanced by elevating temperature,^{3,8,34} curing agents,^{15,20,35} or omitting lactose in the growth medium,¹⁶ and is usually associated with a loss of plasmids.

In this communication, we have isolated Lac⁻ mutants which cannot metabolize lactose, by treating *L. acidophilus* TK8912 with acriflavin (Table II). Loss of P- β -gal, the key enzyme for lactose metabolism, is associated with the loss of plasmid pLA102. Moreover, strain TK1-2TL which was reintroduced with pLA102, restored P- β -gal activity. From these results, plasmid pLA102 is extremely likely to encode a P- β -gal gene in *L. acidophilus* TK8912.

In lactic acid *lactococci*, it is known that genes for P- β -gal³⁶ and lactose-specific PTS^{3,24} are associated with the same plasmid. In contrast, the Lac-PTS activity of Lac⁻ mutants in *L. acidophilus* TK8912 is retained normally while the P- β -gal is totally depleted. Thus plasmid pLA102 appears to encode only the gene for P- β -gal in this strain.

It is known that galactose can be metabolized either by the Leloir pathway or by the tagatose 6-phosphate (Tag-6P) pathway in lactic acid bacteria (Fig. 1). In *L. acidophilus* TK8912, Tag-6P pathway is the route responsible for galactose metabolism, since growing cells on galactose contained Gal-6P substantially but no detectable Gal-1P (Table IV). These results, together with the plasmid profile of Gal⁺ transformant (TK1-2TG), suggest that the key enzyme of the Tag-6P pathway is encoded by

Table IV. INTRACELLULAR CONCENTRATION OF Gal-1P AND Gal-6P IN *L. acidophilus* TK8912 AND ITS MUTANTS GROWING ON GALACTOSE^a

Strain	Phenotype	Intracellular concentration (mM) ^b	
		Gal-1P	Gal-6P
TK8912	Lac ⁺ Gal ⁺	0.1 >	1.9 ± 0.2
TK1-1	Lac ⁻ Gal ⁺	0.1 >	2.1 ± 0.2
TK1-2	Lac ⁻ Gal ⁻	0.1 >	3.8 ± 0.3
TK1-2TL ^c	Lac ⁺ Gal ⁻	0.1 >	4.1 ± 0.4

^a Culture conditions and measurements of intracellular Gal-1P and Gal-6P concentrations are described in the text. At sampling, the dry weight of cells from each culture were approximately 11 mg.

^b The results are expressed as means ± standard deviations for three separate experiments.

^c Lac⁺ transformant.

the gene(s) on plasmid pLA101.

Acknowledgment. We are grateful to Dr. Keiji Sano, School of Pharmacy, Kobe-Gakuin University, for his warm encouragement throughout this study and reading the manuscript.

References

- 1) D. L. Bissett and R. L. Anderson, *J. Bacteriol.*, **117**, 318 (1974).
- 2) L. L. McKay, L. A. Walter, W. E. Sandine and P. R. Elliker, *J. Bacteriol.*, **99**, 603 (1969).
- 3) L. McKay, A. Miller III, W. E. Sandine and P. R. Elliker, *J. Bacteriol.*, **102**, 804 (1970).
- 4) P. W. Postma and S. Roseman, *Biochim. Biophys. Acta*, **457**, 213 (1976).
- 5) J. Thompson, *J. Bacteriol.*, **140**, 774 (1979).
- 6) K. G. Johnson and I. J. McDonald, *J. Bacteriol.*, **117**, 667 (1974).
- 7) J. Thompson, *J. Bacteriol.*, **144**, 683 (1980).
- 8) V. L. Crow, G. P. Davey, L. E. Pearce and T. D. Thomas, *J. Bacteriol.*, **153**, 76 (1983).
- 9) L. Premi, W. E. Sandine and P. R. Elliker, *Appl. Microbiol.*, **24**, 51 (1972).
- 10) E. R. Kashket and T. H. Wilson, *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 2866 (1973).
- 11) L. L. McKay, K. A. Baldwin and J. D. Efstathiou, *Appl. Environ. Microbiol.*, **32**, 45 (1976).
- 12) D. G. Anderson and L. L. McKay, *J. Bacteriol.*, **129**, 367 (1977).
- 13) T. R. Klaenhammer, L. L. McKay and K. A. Baldwin, *Appl. Environ. Microbiol.*, **35**, 592 (1978).
- 14) G. M. Kempler and L. L. McKay, *Appl. Environ. Microbiol.*, **37**, 1041 (1979).
- 15) S. A. Kuhl, L. D. Larsen and L. L. McKay, *Appl. Environ. Microbiol.*, **37**, 1193 (1979).
- 16) M. Shimizu-Kadota, *Appl. Environ. Microbiol.*, **53**, 2987 (1987).
- 17) M. Shimizu-Kadota, *Biochimie.*, **70**, 523 (1988).
- 18) L.-Ja Lee, J. B. Hansen, E. K. Jagusztyn-Krynicka and B. M. Chassy, *J. Bacteriol.*, **152**, 1138 (1982).
- 19) J. C. De Man, H. Rogosa and H. E. Sharpe, *J. Appl. Bacteriol.*, **23**, 130 (1960).
- 20) L. L. McKay, K. A. Baldwin and E. A. Zottola, *Appl. Microbiol.*, **23**, 1090 (1972).
- 21) D. G. Anderson and L. L. McKay, *Appl. Environ. Microbiol.*, **46**, 549 (1983).
- 22) J. Sambrook, E. F. Fritsch and T. Maniatis, "Molecular Cloning," Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989, p. 6.7.
- 23) B. M. Chassy and J. L. Flickinger, *FEMS Microbiol. Lett.*, **44**, 173 (1987).
- 24) D. J. LeBlanc, V. L. Crow, L. N. Lee and C. F. Garon, *J. Bacteriol.*, **137**, 878 (1979).
- 25) T. D. Thomas, K. W. Turner and V. L. Crow, *J. Bacteriol.*, **144**, 672 (1980).
- 26) B. M. Chassy, E. M. Gibson and A. Giuffrida, *Curr. Microbiol.*, **1**, 141 (1978).
- 27) G. M. Demko, S. J. B. Blanton and R. E. Benoit, *J. Bacteriol.*, **112**, 1335 (1972).
- 28) Y. H. Park and L. L. McKay, *J. Bacteriol.*, **149**, 420 (1982).
- 29) J. A. Meyers, D. Sanchez, L. P. Elwell and S. Falkow, *J. Bacteriol.*, **127**, 1529 (1976).
- 30) A. W. Shrago and W. J. Dobrogosz, *Appl. Environ. Microbiol.*, **54**, 824 (1988).
- 31) W. M. Cosby, I. A. Casas and W. J. Dobrogosz, *Appl. Environ. Microbiol.*, **54**, 2599 (1988).
- 32) J. D. Efstathiou and L. L. McKay, *J. Bacteriol.*, **130**, 257 (1977).
- 33) T. W. Wolfe and L. L. McKay, *J. Dairy Sci.*, **67**, 950 (1984).
- 34) J. D. Efstathiou and L. L. McKay, *Appl. Environ. Microbiol.*, **32**, 38 (1976).
- 35) L. L. McKay and K. A. Baldwin, *Appl. Environ. Microbiol.*, **36**, 360 (1978).
- 36) S. M. Maeda and M. J. Gasson, *J. Gen. Microbiol.*, **132**, 331 (1986).