KOR. JOUR. MICROBIOL September, 1989, p. 181-187 Vol. 27, No. 3

Phospho-β-galactosidase gene located on plasmid in *Lactobacillus casei*

Moon, Kyung-Hee, Chung-Hee Park, Soon-Young Choi, You-Mee Lee, Tae-Han Kim*, Yung-Chil Hah and Kyung-Hee Min**

Department of Biology, Sookmyung Women's University, Seoul 140-742, Korea
** Central Laboratory, Il Dong Phar. Co. Ltd. and*
*** Department of Microbiology, Seoul National University*

플라스미드에 존재하는 *Lactobacillus casei* 의 phospho-β-galactosidases 유전자

문경희 · 박정희 · 최순영 · 이유미 · 김태한* · 하영칠 · 민경희**

숙명여자대학교 생물학과, 일동제약(주) 중앙연구실*
서울대학교 자연과학대학 미생물학과**

ABSTRACT : Plasmid DNA was isolated from *Lactobacillus casei* SW-M1(Lac^{+}strain). The curing frequencies of pPLac plasmid from *L. casei* SW-M1 showed 43% for acriflavin treatment and 53% for ethidium bromide treatment after 3 times transfer. On the charaterization of pPLac plasmid, it was found that the plasmid contained gene encoding phospho-β-galactosidase for lactose utilization. Lactose-PTS(phosphotransferase system) was involved in membrane transport system in Lac^{+} strain. Induction of phospho-β-galactosidase was specially effective by galactose, lower effect with lactose and glucose but not by IPTG(isopropyl-β-D-thiogalactoside). This result showed that induction of phospho-β-galactosidase by IPTG did not appeared. The catabolite repression of phospho-β-galactosidase synthesis by glucose was not found in *L. casei*.

KEY WORD ☐ Phospho-β-galactosidase gene, plasmid, *Lactobacillus casei*

Recent progress in the genetics of lactic acid bacteria has established the critical involvement of plasmid DNA in numerous activities related to fermentation (Chassy *et al.*, 1976 ; Davies & Gasson, 1981 ; Kempler & McKay 1981). However, plasmid genetics of the lactobacilli have not kept pace with the advances in molecular genetics, and little information is presently available on plasmid determinants or genetic transfer mechanisms for a bacterial genus and it is critical to many industrial food fermentations. Although plasmid DNA has been reported in *Lactobacillus acidophilus* (Klaengammer & Sutherland, 1980 ; Vescoro *et al.*, 1981), *Lactobacillus reuteri* (Vescoro *et al.*, 1981), *Lactobacillus casei* (Citti *et al.*, 1981), *L. fermentum*(Chassy *et at.*, 1978), and *Lactobacillus helveticus* (Vescoro *et al.*, 1981 ; Smiley *et al.*, 1978) detection has been limited to a selected number of strains (Klaenhammer & Sutherland, 1980 ; Vescoro *et al.*, 1981).

In lactic acid bacteria, lactose can enter the cell by two different pathways. *In Streptococcus thermophilus* (Tinson *et al.*, 1982) and most lactobacilli (Kandler, 1983) lactose is transported as such and then hydrolyzed by a β-galactosidase. In group N streptococci (Okamoto & Moriachi, 1979) and in some lactobacilli such as *L. casei* (Chassy & Tompson, 1983) the sugar is transported and phosphorylated by a phosphoenolpyruvate-dependent phosphotransferase system. The lactose phosphate is then split by a β-phosphogalactosidase. It has generally been observed that when the lactose is phosphorylated prior to its hydrolysis, a plasmid carries the genes coding for this part of metabolism (Hofer, 1977 ; Chassy *et al.*,1978).

In this paper, we describe the isolation and characterization of a *lactobacillus casei* SW-M1 strain which possesses a lactose-utilizing plasmid. The curing frequency, carbohydrate utilization, the exis-

tence of carbohydrate-phosphotransferase system, phospho-β-galactosidase induction, and the effect of catabolite repression of pPLac plasmid encoding gene for phospho-β-galactosidase were examined.

MATERIALS AND METHODS

Strains and culture conditions

All *Lactobacillus strains* were propagated in MRS broth(Difco Laboratories, Detroit, MI) at 37℃ and maintained as described previously (Klaenhammer *et al.*, 1984).

MRS(Man Rogosa), TCM(Tomochika medium), BCP(Bromocresol purple) media were used (Bae *et al.*, 1985 ; Klaenhammer & Sutherland, 1984 ; McKay *et al.*, 1972).

Plasmid isolation

The plasmid isolation method was a modification of procedure developed by Maniatis (1982). Log phase cells from 300ml of TCM broth cultures (2% inoculum, 37℃) were harvested by centrifugation when the optical density(OD) at 600nm reached 1.0. Pelleted cells were washed once with 22.5ml of 20mM potassium phosphate buffer(pH 6.8) and resuspended with 22.5ml of the same buffer containing 1M sucrose, 0.6M $MgCl_2$ and 0.6M $CaCl_2$ and placed in an ice bath.

Table 1. *Bacterial strains and plasmid.*

Stranis and plasmid	Relevant genotype or phenotype	Source and reference
Lactobacillus casei SW-M1	Chromosomal *Lac'* ; contains lactose utilization plasmid pPLac	This work
SW-M2	*Lac⁻*	Cured strain of SW-M1,
Plasmid pPLac	*Lac'* ; 60kb(39Md)	This work

Lysozyme (Sigma Chemical Co.) was added to a final concentration of 20-40μg/ml(904 μl of 10mg/ml of Tris-HCl, pH 8.0) (Klaenhammer & Kleeman, 1984 ; Maniatis *et al.*, 1982). The samples were also added with SDS to be cleared lysate. The following procedures are the same as method of Maniatis (1982).

For the small scale isolation of plasmid, N-acetyl muramidase(Sigma Chemical Co.) instead of lysozyme was used to a final concentration of 30 μg/ml in 10mM Tris-HCl, pH 8.0.

Curing experiment

An inoculum of overnight culture of strain *L. casei* SW-Ml was added into 5ml of MRS broth contained curing agent at an initial OD at 600nm of 0.02. The cultures with acriflavin or ethidium bromide were then incubated for 24hrs at 37℃ without shaking (lst times transfer), diluted, and plated onto lactose-BCP agar plates. The same method was repeated to 3 times transfer.

Carbohydrate utilization

MRS broth cultures contained lg lactose, glucose, galactose were incubated for overnight at 37℃ without shaking, centrifugated, and the supernatant was obtained. Acid production by carbohydrate utilization was measured with pH meter (Bae *et al.*, 1985).

Cell-free extract

The cells were washed and resuspended in 100mM sodium phosphate buffer(pH 6.8). The cell suspension was shaken at 37℃ for lh prior to consuming the metabolic materials and then stirred for 5 min in a vortex mixer after addition of toluene at a final concentration of 10 μl/ml.

Determination of β-galactosidase and phospho-β-galactosidase activity

The assay of β-galactosidase were performed as preveously described for *Streptococcus lactis* 7962 (Citti *et al.*, 1965). The assay of phospho-β-galactosidase was applied by the method of McKay *et al.*,(1970).

Sugar-phosphotransferase activity

In vivo phosphotransferase activity was assayed in toluene-treated cells by indirect method of Hamilton *et al.*,(1978). The sugar-dependent release of pyruvate from PEP was assayed with lactic dehydrogenase and NADH.

RESULTS AND DISCUSSION

Growth of *Lactobacillus casei* SW-Ml

For the determination of optimal culture period for plasmid DNA isolation, the cells were incubated

in TCM medium containing 0.5% glycine at 37℃. As shown in Figure 1, the cell growth was increased very slowly until 6 hours, thereafter the cells were rapidly grown to 16 hours. Among the several intervals of culture time, plasmid DNA was efficiently isolated from 15 hours culture. Therefore, the plasmid isolation was carried out at the same condition described above.

pPLac plasmid isolation

Lactobacillus casei SW-Ml(Lac⁺ strain) was used for plasmid isolation. Figure 2 shows that *L. casei* SW-Ml((Lac⁺ strain) contained plasmid DNA.

Plasmid curing

Further studies were conducted with *L. casei* SW-Ml to determine the presence of gene encoding for lactose metabolism on the plasmid DNA. The curing experiment of plasmid DNA from the host cell was carried out under various conditions. *L. casei* SW-Ml was treated with acriflavin or ethidium bromide various conditions in order to determine optimal concentrations(Table 2,3). Lac-strains were isolated by the method of BCP(bromocresol purple) plate contained lactose. It was found that the curing frequencies of plasmid DNA from *L. casei* SW-Ml showed 43% for acriflavin and 53% for ethidium bromide after 3 times transfer. *L. casei* SW-Ml and its derivative SW-M2 were used for the determination of plasmid on agarose gel electrophoresis. As shown in Figure 2, it suggests that the plasmid in Lac⁺ strain contained gene encoding lactose utilization.

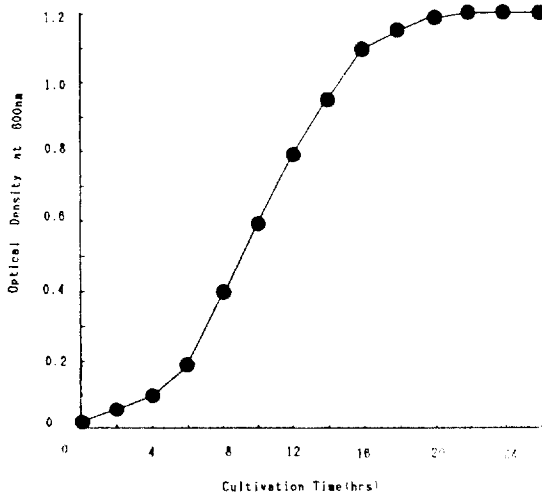

Fig. 1. *Growth curve of Lactobacillus casei SW-W1 on Tomochika medium(TCM).*

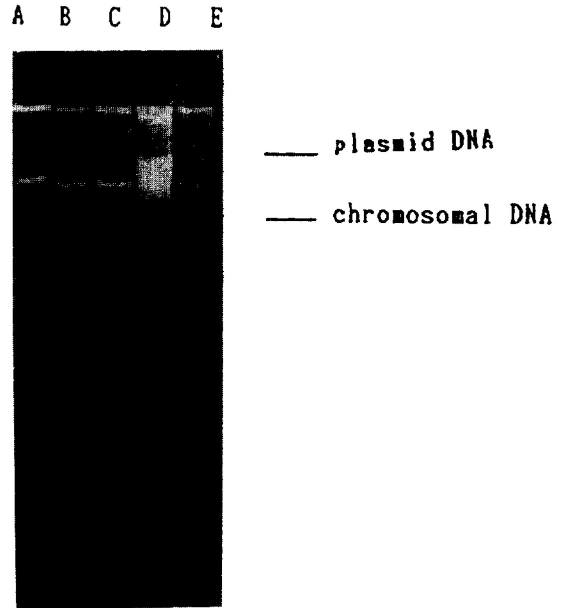

Fig. 2. *Plasmid DNA detection from L. casei Lac⁺ and Lac⁻ strain.*
The Lac⁺ strain of *L. casei* was treated with N-acetylmuramidase. 30μg/ml(A). The same strain was treated with lysozyme, 20μg/ml(B) and 40μg/ml(C), and Lac⁻ strain with 20μg/ml of lysozyme (D) and 30μg/ml of N-acetylmuramidase(E).

Characterization of pPLac plasmid

Test of carbohydrates utilization

From the result of Table 4, glucose and galactose were used for energy sources in Lac⁺ and Lac⁻ strains, but Lac⁻ strains did not used lactose. The results were tested for the acid production by the carbohydrate utilization.

Treatment with curing agents in various concentrations was performed with the addition of 2% inoculum(initial O.D. at 600nm was 0.02) of *L. casei* SW-M1 culture to 5ml MRS broth in cap tube and the cells were incubated for 1 day at 37℃. This transfer experiment was repeated 3 times for 3 days.

The presence of phospho-β-galactosidase gene in pPLac plasmid

It was found that pPLac plasmid in Lac⁺ strains had contained lactose utilization as described above. The experiment was conducted that the lactose utilization was originated from β-galactosidase or phospho-β-galactosidase. The result of this experiment was shown in Table 5. As shown in Table 5, Lac⁺ strains and Lac⁻ strains did not showed β-

galactosidase activity. Therefore, it was assumed that plasmid in Lac$^-$ strains contained gene for phospho- β-galactosidase

Evidence of carbohydrate-phosphotransferase system (PTS)

When carbohydrates were transported through the cell membrane by phosphotransferase system.

Table 2. *Elimination of plasmid from L. casei Lac$^+$ strain by acriflavin.*

Acriflavin conc. (μg/ml)	Number of transfer	Incubation (hr)	Cells/ml (X 10^9)	Frequency (%)
0	1	24	15	〈
	2	48	16	〈
	3	72	15	〈
2	1	24	15	〈
	2	48	17	〈
	3	72	28	〈
4	1	24	5.9	〈
	2	48	5.0	〈
	3	72	3.1	10
6	1	24	5.2	〈
	2	48	4.7	〈
	3	72	3.2	8
8	1	24	3.4	〈
	2	48	2.5	11
	3	72	1.6	43

Table 3. *Elimination of plasmid from L. casei Lac$^+$ strain by ethidium bromide.*

EtBr conc. (μg/ml)	Number of transfer	Incubation (hr)	Cells/ml (X10^8)	Frequency (%)
0	1	24	150	〈
	2	48	160	〈
	3	72	150	〈
3	1	24	73	〈
	2	48	59	〈
	3	72	32	7
7	1	24	38	〈
	2	48	26	5
	3	72	14	31
11	1	24	9.5	21
	2	48	6.6	47
	3	72	4.6	53

Table 4. *Carbohydrate utilization by Lac$^+$ and Lac$^-$ strains.*

Strain	Lac$^+$strain			Lac$^-$ strains		
	Culture time(h)					
Carbohydrates	0	24	48	0	24	48
Lactose	* 6.44	4.23	4.28	6.59	6.61	6.57
Glucose	6.30	4.26	4.34	6.40	4.26	4.33
Galactose	6.28	4.39	4.34	6.27	4.44	4.30

*Acid production (pH)

they are accepted phosphate of phosphoenol pyruvate (PEP) to be sugar-phosphate and PEP into pyruvate (Chassy and Thompson, 1983). The pyruvate was converted into lactic acid by lactate dehydrogenase with coupling of NADH to NAD as cofactor. By the above principle, it was found that carbohydrate-PTS reaction was measured indirectly by the NADH oxidation.

Therefore, this experiment was included to determine relative activity of lactose-PTS, galactose-PTS, and glucose-PTS (Table 6). From the above results, it was confirmed that lactose-PTS was the highest activity in galactose-PTS and glucose-PTS. This result is agreed with the result of Chassy and Thompson (1983) that membrane transport was involved in lactose-PTS, galactose-PTS, and glucose-PTS in *L. casei* strain.

Table 5. *Enzyme activity of β-galactosidase and phospho-β-galactosidase in cell-free extract of L. casei SW-M1 and cured strain.*

Strains	*Sp. activity of β-galactosidase	Sp. activity of P-β-galactosidase
L. Casei SW-W1	0.40	16.70
Cured strain SW-M2	0.40	0.70

* Expressed as nanomoles of 0-nitrophenol released per minute per milligram of protein.

Table 6. *Evidence of carbohydrate-PTS synthesis in L. casei SW-M1.*

Substrates	Relative activity(%)
Lactose	100
Galactose	85
Glucose	28

Table 7. *Induction of phospho-β-galactosidase in L. casei SW-M1 by various substrates and gratuitous inducer IPTG.*

Growth substrates	*Specific Activity of P-β-galactosidase (μmole/min/mg protein)×10^{-2}
Lactose	1.590
Galactose	3,017
Glucose	1.518
Maltose	336
Sucrose	275
IPTG	382
Lactose+Glucose	451
Maltose+IPTG	434

* Specific activities were expressed as μmole 0-nitrophenol released (mg protein)$^{-1}$ min^{-1}.

Induction of phospho-β-galactosidase

Carbohydrates effect on the induction of phospho-β-galactosidase was employed in this experiment. As shown in Table 7, the induction of phospho-β-galactosidase by galactose was the highest level compared with lactose and glucose. However, other carbohydrates, maltose, sucrose, and IPTG were lower effect on the enzyme induction. The induction of phospho-β-galactosidase was not involved in IPTG. Therefore, it was shown that the induction of this enzyme is different from the induction of β-galactosidase synthesis in lac operon.

The effect of catabolite repression

As compared with catabolite repression of β-galactosidase synthesis, glucose effect of phospho-β-galactosidase was examined in this Lac$^+$ strains. On the observation of growth curve of cell growth on the medium containing 0.2% glucose and lactose, it was found that diauxic growth did not appeared in *L. casei* SW-Ml as shown in Figure 3. Another experiment was conducted for the repression of phospho-β-galactosidase by glucose addition to the

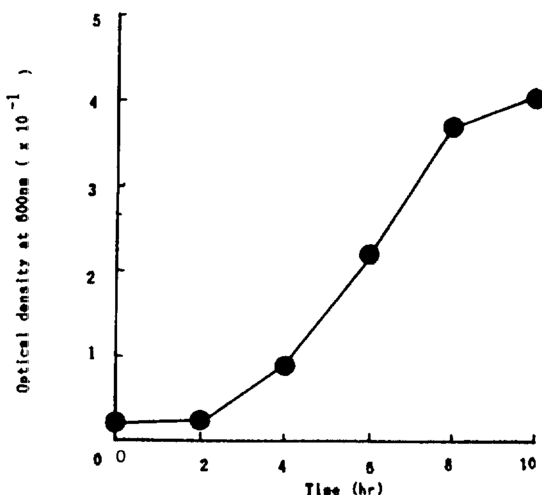

Fig. 3. *Growth of L. casei SW-M1 in MRS broth containing 0.2% glucose and 0.2% lactose.*

culture medium. Catabolite repression enzyme synthesis also tested using cAMP addition to the culture medium. The result showed in Figure 4, indicates that the catabolite repression of phospho-β-galactosidase synthesis by glucose was not found.

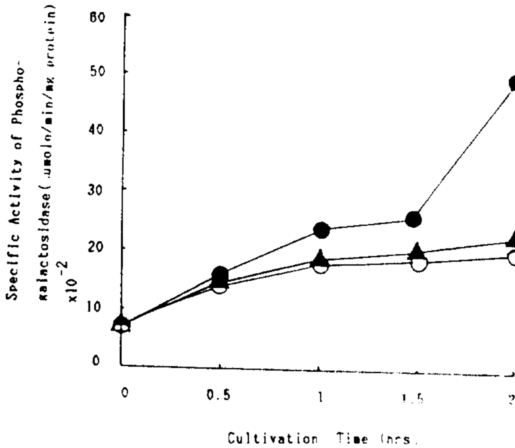

Fig. 4. *Effect of glucose on the activity of phospho-β-galactosidase in L. casei SW-M1.*
Cells were grown in 0.5% maltose broth for 6hrs., washed two times, and treated with toluene (10μl/ml). The cells transferred to three tubes containing 0.2% lactose(●), 0.2% lactose and 0.2% glucose (○), and 0.2% lactose plus 0.2% glucose plus 2mM cAMP(▲).

적 요

Lactobacillus casei SW-M1으로부터 lactose 이용 pPLac plasmid를 분리하였다. 이 plasmid 에 lactose이용 유전자가 존재하는지를 확인하기 위하여 plasmid curing을 실시한 결과, acriflavin 8μg/ml과 11 μg/ml EtBr를 처리한 후, 3차 접종 배양의 경우에 curing 빈도가 가장 높았다. Lac$^-$와 plasmid 가 cured 된 Lac$^-$strain의 당 이용능을 조사한 결과, glucose와 galactose이용능은 불변이나, lactose이용능만이 Lac$^-$strain에서 감소하였다. pPLac plasmid 의 lactose 분해능은 β-galactosidase 에 의한 것이 아니고, phospho-β-galactosidase에 의한 것으로 확인되었다. Lac$^+$strain의 carbohydrate가 막투과시 PTS과 관련이 있는가를 조사한 결과 lactose-PTS가 가장 활성이 높았으며, 그 다음이 galactose-PTS, glucose-PTS로 나타났다. 그러므로 lactose는 lactose-PTS(lactose-phosphotransferase system)에 의하여 glucose와 galactose-6-phosphate로 분해됨을 알 수 있었다. Phospho-β-galactosidase의 induction 실험에서는 galactose가 가장높은 induction효과를 보여 주었으며, lactose와 glucose는 높은 수준의 induction 을 나타내었으며, IPTG는 induction 효과가 없었다. Glucose와 lactose배지에서 *L. casie*는 diauxic growth나 phospho-β-galactosidase합성을 조사한 결과, catabolite repression을 받지 않는 것으로 나타났다.

참고문헌

1. Anderson, D. G., and L. L. McKay.1977. Plasmid, loss of lactose metabolism, and appearance of partial and full lactose fermenting revertants in *Streptococcus cremoris* Bl. *J. Bacteriol.* **129** : 367-377.
2. **Bae, H. S., Y. J. Baek, Y. K. Kim, M. Yoo and M. Y. Park.** 1985. Rapid and simple method for isolating plasmid DNA from lactic acid bacteria. *Kor. J. Appl. Microbiol. Bioeng.*, **13** : 289-296.
3. **Chassy, B. M.** 1976. A gentle method for the lysis of oral streptococci. *Biochem. Biophys. Res. Commum.***68** : 603-608.
4. **Chassy, B. M., E. M. Gibson, and A. Giuffrida.** 1976. Evidence for extrachromosomal elements in *Lactobacillus. J. Bacteriol.* **127** : 1576-1578.
5. **Chassy, B. M., E. M. Gibson, and A. Giuffrida.** 1978. Evidence for plasmid associated lactose metabolism in *Lactobacillus casei* subsp. *casei. Curr. Microbiol.* **1** : 141-144.
6. **Chassy, B. M., and A. Giuffrida.** 1980. Method for lysis of Gram positive, asporogenous bacteria with lysozyme. *Appl. Environ. Microbiol.* **39** : 153-158.
7. **Chassy, B. M., and J. Thompson.** 1983. Regulaton of lactose-phosphoenolpyruvate-dependent phosphotransferase system and β-D-phosphogalactoside galactohydrolase activities in *Lactobacillus casei. J. Bacteriol.* **154** : 1195-1203.
8. **Citti, J. E., W. E. Sandine, and P. R. Elliker.** 1965. β-Galactosidase of *Streptococcus lactis. J. Bacteriol.* **89** : 937-942.
9. **Davies, F. L., and M. J. Gasson.** 1981. Reviews of the progress of dairy science. Jour. Dairy Res. **48** : 368-376.
10. **Hamilton, I. R. and G. C. Y. Lo.** 1978. Co-induction of β-galactosidase in *Klebsiella. J. Bacteriol.* **138** : 691-698.
11. **Hofer, E. 1977. Involvement of plasmids in lactose metabolism in** *Lactobacillus casei* suggested by genetic experiments. *FEMS Miciobiol. Lett.* **1** : 169-170.

12. **Ishiwa, H., and S. Iwata.** 1980. Drug resistance plasmids in *Lactobacillus fermentum. J. Gen. Appl. Microbiol.* **26**: 71-74.
13. **Kandler, O.** 1983. Antonie van Lweeuwenhoek. J. *Microbiol.* **49**: 209-224.
14. **Kempler, G. M., and L. L. McKay.** 1981. Biochemistry and genetics of citrate utilization in *Streptococcus lactis* subsp. *diacetylatis. J. Dairy Sci.* **64**: 1527-1539.
15. **Klaenhammer, T. R., and S. M. Sutherland.** 1980. Detection of plasmid deoxyribonucleic acid in an isolate of *Lactobacillus acidophilus. Appl. Environ. Microbiol.* **39**: **671-674.**
16. **Klaenhammer, T. R., E. G. Kleeman.** 1984. Growth characteristics bile sensitivity, freeze damage in variants of *Lactobacillus acidophilus. Appl. Environ. Microbiol.* **41**: 1461-1467.
17. **Kleanhammer. T. R.** 1984. A general method for plasmid isolation in *Lactobacilli. Curr. Microbiol.* **10**: 23-28.
18. **Kornberg, H. L., and R. E. Reeves.** 1972. Inducible phosphoenolpyruvate-dependent hexose phosphotransfersae activities in *Escherichia coli. Biochem. J.* **128** : 1339-1344.
19. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193**: 269-275.
20. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: A Laboratory mannual, Cold Spring Harbor Laboratory. pp. 366.
21. **McKay, L. L., L. A. Walter, W. E. Sandine, and P. R. Elliker.** 1969. Involvement of phosphoenolpyruvate in lactose utilization by group N *strepcocci. J. Bacteriol.* **999**: 603-610.
22. **McKay. L. L., A. Miller III, W. E. Sandine, and P. R. Elliker.** 1970. Mechanisms of lactose utilization by lactic acid streptococci: enzymatic and genetic analyses. *J. Bacteriol.*, **102**: 804-809.
23. **McKay, L. L., K. A. Baldwin, E. A. Zottola.** 1972. Loss of lactose metabolism in lactic streptococci. *Appl. Mcrobiol.* **23**: 1090-1096.
24. **Meyers, J. A., D. Sanchez, L. P. Elwell, S. Falkow.** 1976. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. *J. Bacteriol.*, **127**: 1529-1537.
25. **Morse, M. L., K. L. Hill, J. B. Egan, and W. Hengstenberg.** 1968. Metabolism of lactose in *Staphylococcus aureus* and its genetic basis. *J. Bacteriol.* **95**: 2270-2274.
26. **Neujahr, H. Y., B. Borstad., and I. Logardt.** 1973. Factors affecting the resistance of *Lactobacillus fermentum* to lysozyme. *J. Bacteriol.*, **116**: 694-698.
27. **Okamoto, T., and T. Moriachi.** 1979. Distribution of β-galactosidase and β-phospho-galactosidase activity among lactic streptococci. *Agr. Biol. Chem.*, **43**: 2389-2390.
28. **Smiley, M. B., and V. Fryder.** 1978. Plasmids, lactic acid production and N-acetyl-D-glucosamine fermentation in *Lactobacillus helveticus* subsp. *jugurti. Appl. Environ. Microbiol.*,**35**: 777-781.
29. **Tinson, W., A. J. Hillier, and G. R. Jago.** 1982. Metobolism of *Streptococcus thermophilus.* 3. Influence on the level of bacterial metabolites cheddar cheese. *Aust. J. Dairy Technol.*, **37**: 17-21.
30. **Vescoro, M., V. Bottazzi, P. G. Satta., and F. Dellaglio.** 1981. Evidence of plasmid deoxyribonucleic acid in *Lactobacillus. Microbiologica.* **4**: 413-419.

(Received May 7, 1989)