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Studies on the Genetic Recombination by Intraspecific Fusion of *Lactobacillus casei* Protoplast

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*Lactobacillus casei*의 同種間 細胞融合에 依한 遺伝子 再組合에 関한 研究

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After intraspecific fusion of *Lactobacillus casei* protoplasts, the recombinants have been studied for their lactose utilization, protease activity and phage resistance. *L. casei* C-M phenotypes constituted 46% of the fused cells when tested against phages, and *L. casei* 3-M phenotypes 42% of the fused cells, and 12% of the recombinants developed the resistance to both parent types of phages. The acid production and proteolytic activity of recombinants evidenced the similar trends. There was no difference in *Hind* III digests of plasmid DNA between parent cells and recombinants, but the recombinant cells were found to possess only one type of plasmid, either of *L. casei* C-M or of *L. casei* 3-M.

Protoplast fusion became one of the gene mixing tools in recent times. Protoplast fusion of *Lactobacillus casei* cells was successful[3] and the optimum conditions of the protoplast formation as well as the protoplast regenerations have been reported.[2] The first genetic analysis of the fused protoplast has been made on the *Bacillus* species.[8, 16] An extensive genetic analysis was done with the hybrid protoplast of *Streptomyces coelicolor*.[7] Recombination of chromosomal genes (maltose and streptomycin-resistance) and transfer of plasmid genes (lactose and erythromycin-resistance) were investigated with the fused cells of *Streptococcus lactis* 712.[6] Okamoto et al.[15] reported that the interspecific hybrids between *S. cremoris* and *S. lactis* possessed the plasmids of the two parent strains at random.

As an attempt to study the genetics of the protoplast fusion, we investigated the physiological characteristics and the plasmid profiles of the parent strains as well as the intraspecific recombinants of *Lactobacillus casei*.

Materials and Methods

Bacteria, phages and media

The bacteria used throughout this study are listed in Table 1. The cultures were maintained in ILS and grown in reconstituted skimmilk (10% wt/vol) before use. The culture for cell crops was grown in TCM media[17] containing 0.5% glycine. The phage J-1 and SGT were obtained from the Japan Yakult Central Research Institute. The phage ϕ 3M-1 and ϕ 3M-2 were isolated from the drainages of Korea Yakult plant. The newly isolated phages were purified by three suc-

Key words; Physiological characteristics, Genetic analysis

Table 1. The list of *L. casei* strains.

The bacteria	Genotype or Phenotype	Source
Lactobacillus casei, YIT 9018	wild type	Korea Yakult Institute
Lactobacillus casei, C-M	Streptomycin resistant (SM^R)	*NTG-induced mutant of *L. casei* YIT 9018
Lactobacillus casei, 3012	wild type	Institüt für Milchforschung, Kiel, West Germany
Lactobacillus casei, 3-M	Lincomycin resistant (LM^R)	NTG-induced mutant of *L. casei* 3012
	Methicillin resistant (DP^R)	
	Hostacillin resistant (HC^R)	
Lactobacillus casei, Recombinant 101, 102, 207	*L. casei* C-M type	
Lactobacillus casei Recombinant 303, 309, 310	*L. casei* 3-M type	
Lactobacillus casei Recombinant 104	Plasmid-cured	

*NTG : N-methyl-N′-nitro-N-nitrosoguanidine.

cessive single plaque isolation. The phages were stored as lysate at 4°C, and the phage lysates were employed for the study.

Titratable acidity

Nine grams of the culture were weighed into a tared 100 ml beaker. Equal amount of carbon dioxide-free distilled water was added to the sample and mixed thoroughly. Three to four drops of phenolphthalein indicator solution were added and it was titrated with 0.1 N sodium hydroxide to the first definite (30 sec) color change from white to pink. Acidity was expressed as the total amount of titratable lactic acid (1 ml of 0.1 N NaOH = 0.009g of lactic acid). Acidity(%) = ml of 0.1 N NaOH x 0.009 x 100 / weight of sample.

Proteolytic activity

Proteolytic activity was determined by the procedure of Hull[9] using the Folin-Ciocalteau reagent. The pregrown cultures (0.5% v/v) in reconstituted skimmilk (10%, w/v) were inoculated into 100 ml fresh reconstituted skimmilk in 200 ml Elrenmyer flask. The inoculated skimmilk was incubated for 5 days at 37°C and the proteolytic activity of the culture was examined.

Protoplast fusion

Protoplasts of the parent strains (*L. casei* C-M and *L. casei* 3-M) were prepared by treating the cells with mutanolysin, and fused in the presence of polyethylene glycol (PEG) 4000 as described previously.[3] The PEG-treated protoplasts were immediately diluted in protoplast formation buffer.[3] One tenth ml of appropriately diluted protoplast suspension was plated on the regeneration medium[3] containing bromocresol purple (0.004%), and incubated at 30°C for 3-5 days. Regenerated colonies were randomly transferred onto the selective medium[3] by using sterile toothpicks. After 3 days of incubation at 37°C, the colonies appeared on the selective medium were picked for further tests. The physiological properties, plasmid profiles, and bacteriophage susceptibilities of the selected cultures were checked subsequently to search for the fused cells.

Bacteriophage susceptibility

The susceptibility of the bacteria to the phages was tested by the method of Murata.[13] The experimental host bacteria were cultured overnight in TCM broth at 37°C. The overnight-grown cells were transferred again into fresh MRT broth, and incubated for about 4 hrs at 37°C to reach the cell density of about $2\text{-}4 \times 10^8$ cells/ml. Two and half ml of the soft agar containing 0.2 ml of the actively growing cells was poured onto the hard agar plate spreaded with 0.1 ml of phage solution. The plates were incubated overnight at 37°C and checked for the plaque formation.

Plasmid analysis

Plasmid DNA of *Lactobacillus* strains was prepared after lysis (using mutanolysin) by the method of Bae et al.[1, 10] Agarose gel electrophoresis of plasmid DNA was carried out as described previously[12] on a horizontal gel at 100V for 3 hrs, and 20 μl plasmid DNA containing 5 μl tracking dye per

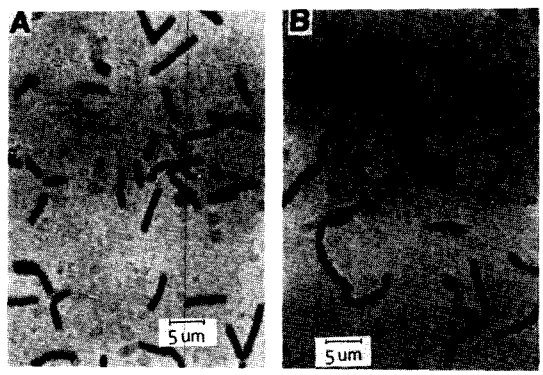

Fig. 1. Phase contrast micrographs of *L. casei* C-M and *L. casei* 3-M.
(A) *L. casei* C-M, (B) *L. casei* 3-M.

sample was run on a 0.6% agarose gel (Sigma Chemical Co., Type II). The gel was stained for 30-60 min in ethidium bromide solution (0.5 μg/ml), photographed by Polaroid camera (Polaroid MP-4 Land Camera, Polaroid Corp., USA) under UV illumination.

Plasmid analysis by restriction enzymes

Digestions of plasmids by restriction enzymes (The New England Biolabs, Mass. USA) were carried out in the appropriate assay buffer in sterile microcentrifuge tubes (Total volume was 1.5ml). The digestion mixtures were analyzed on a 1% agarose gel prepared as described previously.[11] The electrophoresis was run for 4 hrs at 80V. After electrophoresis, the gels were stained for 30 min in ethidium bromide solution (0.5 μg per ml). The stained gels were photographed by Polaroid camera under UV illumination. *Hind* III-digested *lambda* DNA and *Bst* E II-digested *lambda* DNA were used as molecular weight references.[14]

Results and Discussion

As shown in Fig. 1, *L. casei* C-M usually forms single or paired cells, while *L. casei* 3-M forms short chains. Since *L. casei* is used for the manufacture of liquid yogurts in Korea as well as in some other Asian countries, its proteolytic activity, the rapidity of lactose fermentation, and the good flavor production are considered to be important. When we examined these properties of *L. casei* C-M and *L. casei* 3-M, *L. casei* C-M was found to be less active in proteolysis and less rapid in lactose fermentation than *L. casei* 3-M, as shown in Fig. 2 and Fig. 3. *L. casei* C-M required lysine and threonine for growth while *L. casei* 3-M required none of the amino acids. Therefore, it is reasonable that we attempt to introduce these genes of *L. casei* 3-M into *L. casei* C-M, which could ferment lactose more rapidly, degrade the milk protein faster and for the growth, while keeping the flavor production and the resistance to the organic acids. The antibiotic resistance of two mutants (*L. casei* C-M and *L. casei* 3-M) was found to be very stable with no detectable loss of resistance even after prolonged storage and repeated subculturing for about one year. *L. casei* YIT 9018 and *L. casei* C-M grew and produced acid in milk media as well as *L. casei* 3012 and *L. casei* 3-M did.

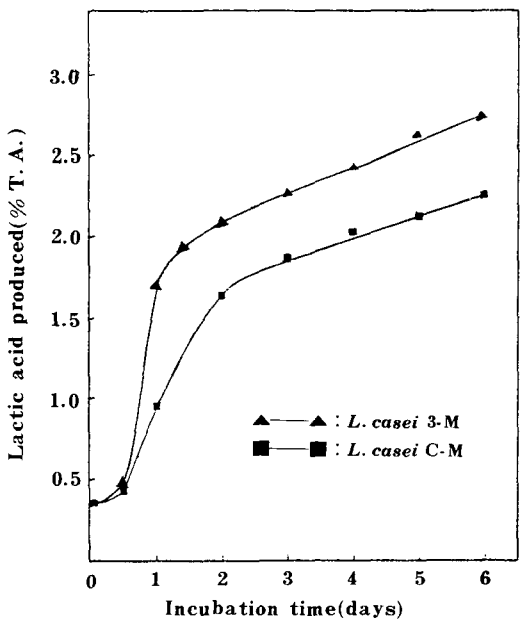

Fig. 2. Acid production of *L. casei* C-M and *L. casei* 3-M in 16% reconstituted skimmilk added 3% glucose at 37℃.

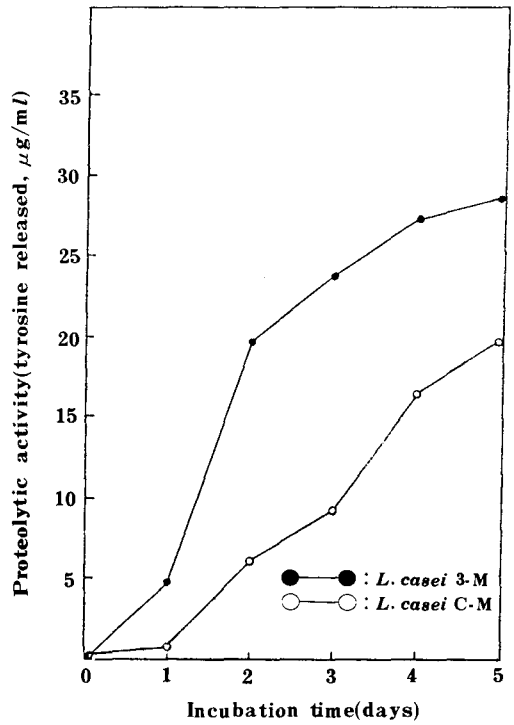

Fig. 3. Proteolytic activity of *L. casei* C-M and *L. casei* 3-M in 10% reconstituted skimmilk at 37℃.

Acid production of the parent strains and the recombinants were examined during 4 days of incubation in 10% (w/v) reconstituted skimmilk. The recombinants could be classified into 4 groups depending on their acid production and illustrated in Fig. 4. The first group constituted the

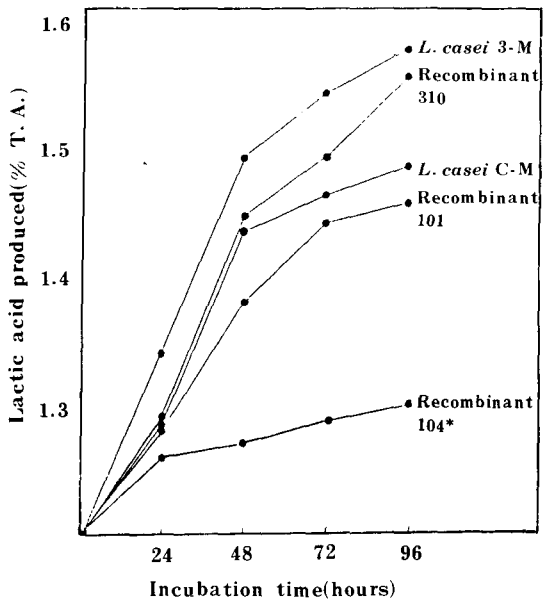

Fig. 4. Lactic acid production of parent strains and several recombinants in 10% reconstituted skimmilk.

*Recombinant 104 : Plasmid cured cell.

recombinants which produce acid lower than that by *L. casei* C-M, the second group, which produce similar to that by *L. casei* C-M, the third group, which produce about half of that by two parent strains, and the fourth group, which produce similar amount to that by *L. casei* 3-M.

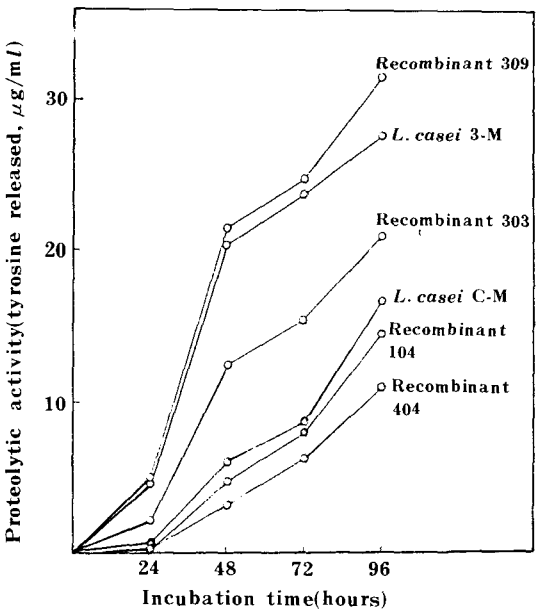

Fig. 5. Proteolytic activity of parent strains and recombinants in 10% reconstituted skimmilk

Table 2. The bacteriophage sensitivity of parent strains and recombinants to *Lactobacillus casei* phages.

Phages / Bacterial strains	*L.casei* C-M phage		*L.casei* 3-M phage		Occurrence of recombinants (%)
	J-1	SGT	ϕ3M-1	ϕ3M-2	
Parent. *L.casei* C-M	+	+	−	−	
L.casei 3-M	−	−	+	+	
Recom- C-M type	+	+	−	−	46.3
binants. 3-M type	−	−	+	+	31.8
3M-A	−	−	+	−	7.3
3M-B	−	−	−	+	2.4
Mixed type of C-M & 3-M	−	−	−	−	12.2

+ : lysis of bacterial strain
− : no lysis

Depending upon the proteolytic activity, the recombinants could be divided into 5 groups, and only one out of 41 recombinants evidenced the improved proteolysis compared to that of *L. casei* 3-M. Fourteen percent of the recombinants exhibited decreased proteolytic activity as compared to the parents.

The proteolytic activity of recombinants appeared to give the similar phenotypic variations as that of acid production of recombinants, as shown in Fig. 5. Eight of 16 recombinants had the mixed characteristics of the two parent strains in lactic acid production and proteolytic activity. There seems to

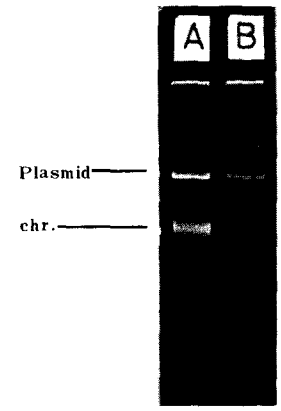

Fig. 6. Agarose gel electrophoretogram of plasmid DNA of *L. casei* C-M (A) and *L. casei* 3-M (B).

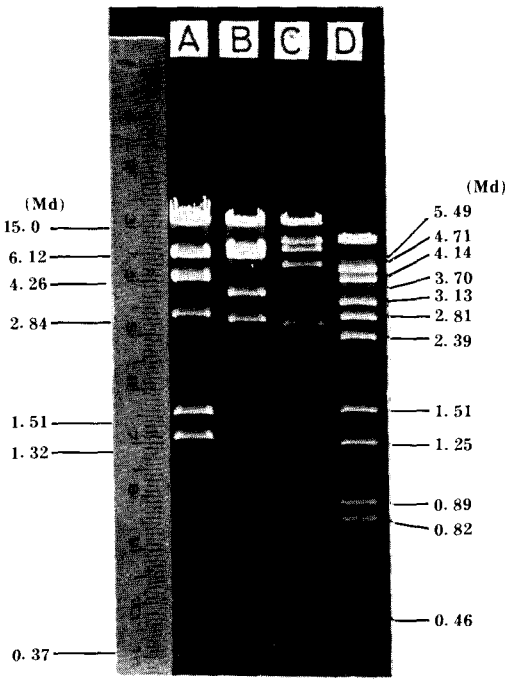

Fig. 7. Agrose gel electrophoretogram of *Pst* 1-digest of plasmid DNA of *L. casei* C-M and *L. casei* 3-M.

A) *Hind* III digest of *lambda* DNA
B) *L. casei* C-M plasmid
C) *L. casei* 3-M plasmid
D) *Bst* E II digest of *lambda* DNA

be no close relationship between lactose fermentation and proteolytic activity of these *L. casei* cells, which must be clarified through further genetic studies of *L. casei*.

Lactobacillus phages are uaually known to have a very narrow host specificity.[4, 5] *L. casei* C-M did not reveal any alteration in the sensitivity to phage J-1 as mentioned previously. This narrow host range of *Lactobacillus casei* phages could hopefully be used to identify the recombination-associated changes of the parent strains and recombinants.

As evidenced in Table 2, 19 out of 41 recombinants (46.3%) were lysed by the phages J-1 and SGT, 13 out of 41 recombinants (31.8%) were lysed by the phages ϕ3M-1 and ϕ3M-2. Although 4 out of 41 recombinants (9.7%) were lysed only either by the phages ϕ3M-1 or ϕ3M-2, but 5 out of 41 recombinants (12.2%) were not lysed by any of these phages mentioned above.

The plasmids appeared on agarose gel electrophoretogram of the parent strains (*L. casei* C-M and *L. casei* 3-M) are shown in Fig. 6. As evidenced in Fig. 6, each parent strain possessed only one large plasmid. As shown in Fig. 7, the plasmid digests of the two plasmids from the parent strains were shown to give quite different patterns. The plasmid of *L. casei* C-M was a approximately 39 Mdal, while that of *L. casei* 3-M was a 40 Mdal when measured using the fragments of *Hind* III-digested *lambda* DNA and *Bst* E II-digested *lambda* DNA as references.[14]

The plasmids of the parent strains and recombinants of *L. casei* cells were isolated and examined by agarose gel electrophoresis as shown in Fig. 8. The studied recombinant evidenced a single plasmid band as the parent strain, which suggests that recombinant possesses a single plasmid similar to that of parent strains. Antibiotic resistance genes were not found to be on plasmid DNA, because plasmid free derivatives of *L. casei* have the intact antibiotic resistance as shown in Fig. 8, which suggests that the resistance genes may be on chromosomal DNA.

As shown in Fig. 9, no differences were found between plasmid digests of the parental strains and that of recombinants. Recombinant 102 and 207 (Wells D and E) were found to possess the plasmid of *L. casei* C-M, while recombinants 303 and 310 had the plasmid of *L. casei* 3-M based upon the restriction enzyme digest patterns of the plasmids. Microscopic observation and bacteriophage susceptibility of

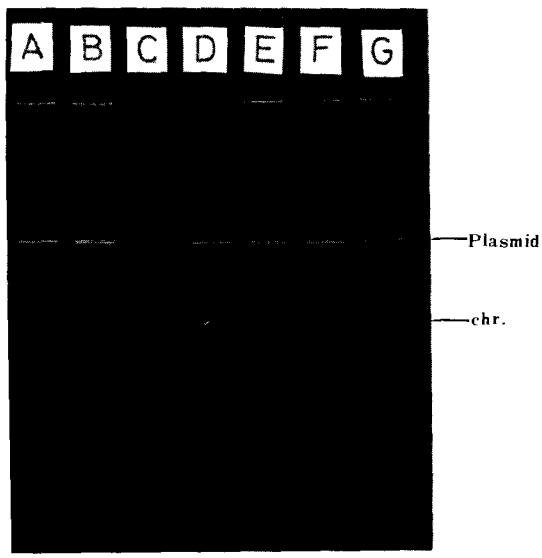

Fig. 8. Agarose gel electrophoretogram of plasmid DNA of the parent cells and the recombinants.

A) *L. casei* C-M, B) *L. casei* 3-M
C) Recombinant 104 (plasmid-cured cell)
D) Recombinant 102, E) Recombinant 207
F) Recombinant 303, G) Recombinant 310

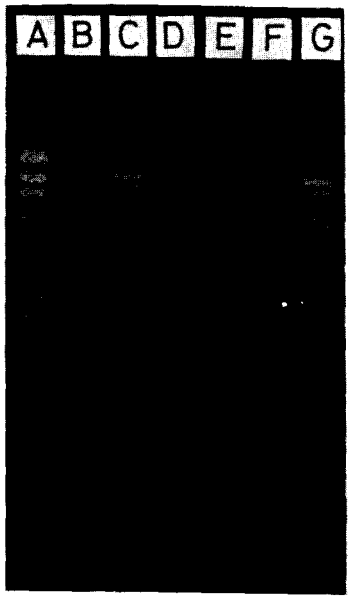

Fig. 9. Agarose gel electrophoretogram of *Hind* Ⅲ-digest of plasmid DNA of the parent cells and the recombinants.

A) *Hind* Ⅲ digest of *lambda* DNA
B) *L. casei* C-M, C) *L. casei* 3-M
D) Recombinant 102, E) Recombinant 207
F) Recombinant 303, G) Recombinant 310

the recombinant cells revealed that strain 101 and strain 104 appeared to be *L. casei* C-M type while strain 303, strain 309 and strain 404 appeared to be *L. casei* 3-M type in morphology.

These results suggest that the recombination of intraspecific protoplast fusion of *L. casei* appears to allow the extensive genetic exchange of the chromosomal DNA of the parent cells. More studies are needed to elucidate the gene recombination mechanisms during the protoplast fusion.

要 約

Lactobacillus casei 母細胞와 同種間 融合細胞間의 乳酸生成, 蛋白質 分解力, Bacteriophage 抵抗性 等의 生理的 特性과 表現型을 比較分析하였다. 두 母細胞사이에서 形成된 融合細胞는 母細胞를 侵入하는 bacteriophage에 対하여 抵抗性을 試驗한 結果 *L. casei* C-M表現型은 融合細胞 中 46%를 차지하였고, *L. casei* 3-M表現型은 42%였고, 12%의 融合細胞는 두 母細胞 bacteriophage에 抵抗性을 所有하였다. 融合細胞의 乳酸生成力과 蛋白質 分解力도 類似한 出現率을 나타내었다. Plasmid DNA 의 *Hind* Ⅲ分解物은 母細胞와 融合細胞間에 차이가 없었으나, 融合細胞들은 *L. casei* C-M 또는 *L. casei* 3-M중 어느 한 母細胞의 Plasmid만을 所有하고 있었다.

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