Studies on the Protoplast Fusion of *Lactobacillus casei*

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*Lactobacillus casei*의 세포융합에 관한 연구

백영진 · 유 민 · 김영기 · 배형석 · 김현욱*

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The best conditions for the protoplast fusion of *Lactobacillus casei* have been searched for in this study. Antibiotic resistance was used as the selective marker for enumerating and selecting the recombinants. Antibiotic resistant mutants were isolated after treating cells with N-methyl-N'-nitro-N'-nitrosoguanidine. High frequency fusion of protoplasts of *L. casei* strains were obtained in the presence of 40% (wt/vol) polyethylene glycol 4,000 after 1 min at 30°C at around neutral pH. Spontaneous mutations of drug-resistance of *L. casei* were two or three orders lower than the recombination frequency. Recombination frequencies were about 10^4 per parent cells employed.

Lactobacillus casei, the normal inhabitant of the animal intestinal cavity, is understood to play the important roles for the human health[13] and has been used for supplementation to the daily diets in the form of liquid yogurt mostly in Japan, Korea, and in some oriental countries.

With the progress of molecular genetic of lactic acid bacteria, useful genetic transfer systems, such as transduction[8] conjugation[1,9], and transformation[6] have been demonstrated. Protoplast fusion is one of the promising methods of gene transfer in both genetic studies and improvement of starter strains for the use in dairy industries. Futhermore, because the fusion is neither strain nor species-specific, this method has broader use for the genetic recombination. Recently genetic recombinations through protoplast fusion induced by PEG have been reported in *L. casei* (Y.J. Baek, Ph. D. thesis, The Seoul National University, Seoul, Korea, 1985), as efficient as with lactic streptococci.[2,11,12] *L. casei* is slow in lactose fermentation and in proteolysis of the milk proteins, and has many bacteriophages known to attack the

cells. These properties make the dairy industries suffer with many difficulties in economy and processes. Accordingly the better strains of *L. casei* free of these problems have been searched for in many ways and became the reasonable hope in recent years.

In this paper, we describe the attempt to develop the protoplast fusion system as one of the practical gene-mixing tool for the strain development of *L. casei*. For the development of the protoplast fusion of *L. casei* strains, the factors affecting the optimization of genetic recombination by protoplast fusion have been studied.

Materials and Methods

Bacterial strains and media

The bacteria used throughout this study are listed in Table 1. The strains were maintained in IL stab stock culture and transferred to reconstituted 10% (wt/vol) skimmilk before use. Culture were grown in TCM media[15] con-

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) Methicillin resistant (DP^R) Hostacillin resistant (HC^R)	NTG-induced mutant of *L. casei* 3012

*NTG : N-methyl-N'-nitro-N'-nitrosoguanidine

taining 0.5% glycine for cell crops. Protoplast formation buffer (PFB) consisted of 20mM potassium phosphate (pH 6.8), 6mM CaCl$_2$, 6mM MgCl$_2$, and 1M sucrose as a stabilizer. Regeneration medium (RM) was TCM with 0.2% glucose, 0.8M sucrose and agar (hard medium 1.3%, soft medium 0.6%). Three ml of sterilized soft medium contained with the final concentrations of 6mM MgCl$_2$, 6mM CaCl$_2$ and 0.3ml of horse serum (GIBCO LAB) were stirred gently before pouring onto the hard regeneration medium plate. TCM agar medium containing the appropriate concentration of antibiotics was used as selective medium (SM). The final concentration of antibiotics in selective medium was 25µg per ml for streptomycin (SM; Han Dok Pharmaceutical Co. LTD), 0.5µg per ml for lincomycin (LM, Korea-Upjon Co.), 0.5 I.U. per ml for hostacillin (HC, Handok Pharmaceutical Co. LTD), and 5µg per ml for methicillin (DP, Dae Han Pharmaceutical Co. LTD).

Isolation of antibiotic resistant mutant

N-methyl-N'-nitro-N'-nitrosoguanidine (NTG) was used to obtain antibiotic resistant strains from *L. casei* YIT 9018 and *L. casei* 3012. Antibiotic mutation was induced by a NTG mutagenesis procedure similiar to that described by Miller.[10] The treated cells are plated on TCM medium containing antibiotic substances and bromocresol purple. The colonies after 3 days of incubation were streaked on TCM agar plates containing higher concentration of antibiotic substances than the minimal inhibitory concentration (MIC).

Protoplast formation

Cells were transferred two or three times in TCM broth

before use. The cells were cultured overnight at 37°C in 40 ml TCM broth containing 0.5% glycine. At the middle or late logarithmic growth phase (about 0.8-0.9 absorbance at 650nm), the cells were harvested by centrifugation, washed in 20mM potassium phosphate buffer, and suspended in 10-12ml of PFB. Mutanolysin (Dainippon Pharmaceutical Co. LTD., Japan) dissolved in 20mM potassium phosphate buffer was added to the cell suspension at the final concentration of 10µg per ml for *L. casei* C-M and 8µg per ml for *L. casei* 3-M. The cellmutanolysin mixture was incubated at 37°C for 10 to 20 min with gentle agitation in a shaking water bath. Protoplasts were searched for under the phase contrast microscope at 1600X.

Protoplast fusion and regeneration

The protoplast fusion was induced by using the method.[4] Protoplasts of two strains (*L. casei* C-M and *L. casei* 3-M) have been used throughout these experiments. Equal volumes (0.5ml; ca, 2-3X 10^9/ml protoplasts) of protoplast suspension of two antibiotic resistant strains were mixed and sedimented by Eppendorf centrifuge (15,000 rpm for 5 min at 4°C). The pelleted protoplasts were resuspended gently by an inoculating loop in 0.1 ml of PFB. Nine tenth ml of prewarmed PEG (Sigma Cheminical Co.) solution (30°C) was added and mixed gently. The protoplasts resuspended in 0.9 ml of PFB was used as control. At this stage, aggregation of protoplasts was observed. After incubation at 30°C for one minute, the PEG-treated protoplasts were immediately diluted in PFB. One tenth ml of appropriately diluted protoplast suspension was plated on the RM containing bromocresol purple (0.004%), and incubated at 30°C for 3-5 days.

Table 2. Minimum inhibitory concentration of 4 antibiotics for parent and mutants on TCM hard medium.

(unit : µg/ml)

The bacteria / antibiotics	Parent strains		Mutants	
	L. casei YIT9018	*L. casei* 3012	*L. casei* C-M	*L. casei* 3-M
Streptomycin (SM)	40	25	4,000	25
Lincomycin (LM)	0.3	0.8	0.5	1.0
Methicillin (DP)	5	20	5	40
*Hostacillin (HC)	0.5	2.0	0.5	4.0

*Hostacillin : I. U. /ml

Regenerated colonies were randomly transferred onto the selective medium by sterile toothpicks. After 3 days of incubation at 37°C, the colonies appeared on the selective medium were isolated. Then the antibiotic resistance of the colonies were checked.

Recombination frequency was calculated as follows;

Recombination frequency (%) = colony forming units (CFU) on selective media/Initial cell number of parent cells

Transmission electron microscopy

The specimen (protoplasts and fused cells) were prepared by the procedure described previously.[7] The ultra thin sections were mounted on copper grids, stained with uranyl acetate followed by Reynolds lead citrate. The prepared sections were observed and photographed by Hitach transmission electron microscope system (Model H-50, Japan).

Results and Discussion

Search for the marker genes

Through extensive search for usable marker genes of *L. casei* YIT 9018 and *L. casei* 3012, antibiotic resistances have been selected as the selective traits to ascertain the identity of each strains and the fused cells. NTG has been used to get the desired antibiotic resistant mutants from *L. casei* YIT 9018
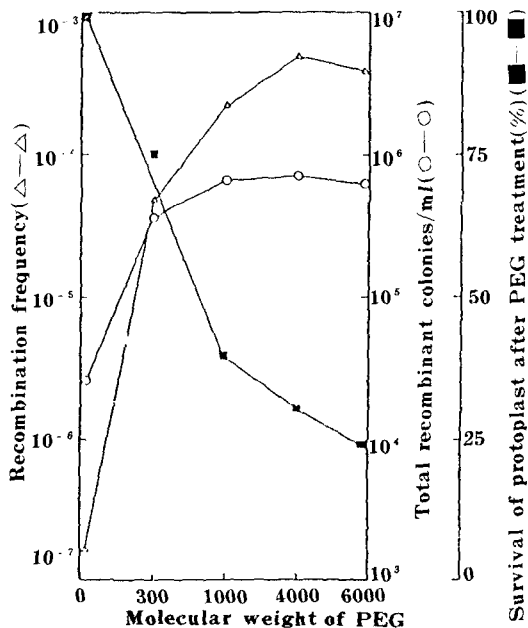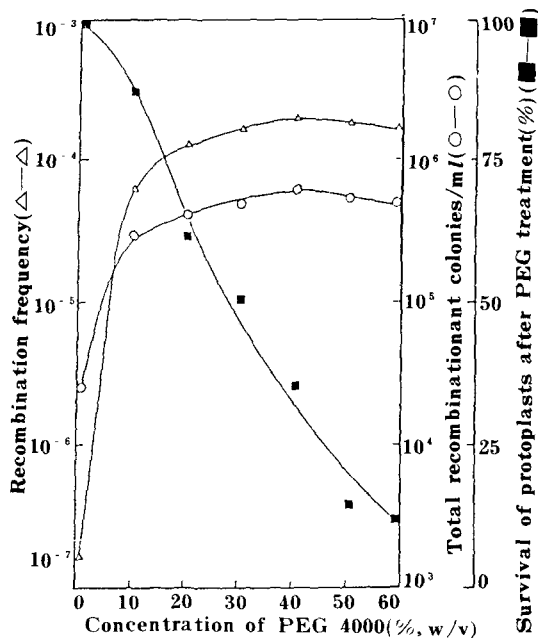
Fig. 2. Effect of the concentrations of PEG 4000 on recombination frequency and survival of protoplasts after PEG treatment.

and *L. casei* 3012. Antibiotic resistance of mutants was amplified by subculturing on TCM agar containing higher concentration of antibiotics than MIC.

As illustrated in Table 2, *L. casei* C-M possessed much greater resistance to streptomycin (4,000 μg/ml) than *L. casei* 3-M (25 μg/ml) while *L. casei* 3-M evidenced greater resistances to lincomycin 1.0 μg/ml), methicillin (40 μg/ml) and hostacillin (4.0 I.U./ml) than *L. casei* C-M. This different resistance of four antibiotics allowed us design a selective antibiotic medium system which could select each mutants and their hybrid cells.

The antibiotic resistance of two mutants was found to be very stable with no detectable loss of resistance, even after prolonged maintenance and repeated subculturing for about one year. *L. casei* YIT 9018 and *L. casei* C-M performed almost identical growth and acid production in milk media as *L. casei* 3012 and *L. casei* 3-M. Eventually the selective medium, which could exclude *L. casei* C-M as well as *L. casei* 3-M but select for the hybrid cells of two mutant strains, is formulated, containing 25 μg/ml of streptomycin, 0.5 μg/ml of lincomycin, 5 μg/ml of methicillin, and 0.5 I.U./ml of hostacillin in TCM medium. The antibiotic resistance genes were not appeared to be on plasmid DNA, because plasmid-free of *L. casei* possessed the identical antibiotic resistance. Therefore the traits used as genetic markers may be situated

Fig. 1. Effects of molecular weight of PEG, on recombination frequency and survival of protoplasts after PEG treatment.

Table 3. Effect of pH of PEG 4000 solution on recombination frequency.

pH	Regenerated cells (CFU/ml)	Recombinants (CFU/ml)			Total recombinants (CFU/ml)	Recombination frequency (CFU/ml)
		phenotype SM^R+HC^R	phenotype SM^R+LM^R	phenotype SM^R+DP^R		
4	1.1×10^6	$<10^4$	1.1×10^4	1.1×10^4	2.2×10^4	1.8×10^{-5}
5	1.1×10^6	$<10^4$	1.1×10^4	3.3×10^4	4.4×10^4	3.7×10^{-5}
6	1.8×10^6	5.4×10^4	7.1×10^4	3.5×10^4	1.6×10^5	1.3×10^{-4}
7	1.9×10^6	1.1×10^5	3.8×10^4	5.7×10^4	2.1×10^5	1.8×10^{-4}
8	1.8×10^6	1.8×10^4	1.8×10^4	3.6×10^4	7.2×10^4	6.0×10^{-5}
9	4.1×10^5	$<10^3$	$<10^3$	$<10^3$	$<10^4$	$<10^{-5}$

The number of parent cells was 1.2×10^9/ml (The number of *L. casei* C-M and *L. casei* 3-M strains was 1.0×10^9/ml, and 2.0×10^8/ml, respectively).

on chromosomal DNA. The spontaneous mutation was less than 10^7 cells per ml for *L. casei* C-M on the selective media containing the appropriate concentration of antibiotics.

Fusion of *L. casei* protoplasts

When protoplasts of *L. casei* C-M and of *L. casei* 3-M were mixed in equal number in 1M sucrose salt solutions and the hybrid cells were studied on the selective media after grown on the regeneration medium, and the recombination frequency was found to be 4.6×10^{-7} hybrid cells. The spontaneous mutation was less than 10^{-7} cells per ml for *L. casei* C-M and less than 10-8 cells per ml for *L. casei* 3-M on the selective media containing the proper concentration of antibitics. Spontaneous mutations of drug-resistance were two or three orders lower than the recombination frequency, which could conveniently exclude the chances of the confusions. However, when polyethylene glycol (PEG) was added to the fusion mixture of protoplasts, the fusion frequencies have been greatly enhanced as previously reported by several authors.[3,14]

It is reasonable to screen for the best PEG of the right molecular weight because PEG has wide variety of different molecular size. When four kinds of PEG between 300 and 6000 of molecular weight have been tested for their efficiencies of enhancing hybrid cell formations, PEG of MW 4000 was found to give the highest recombination frequency, 4.0 $\times$ 10^{-4} hybrid cells/ml, which is almost a thousand times higher frequency than that without PEG, as shown in Fig. 1. PEG 6000 also gave a good recombination but it was inconvenient to use because of its high viscosity. The optimal molecular weight range of PEG was different with different species of bacteria.[5]

The optimum concentration of PEG 4000 for the best cell fusion has been found to be 40%. As the concentration of PEG 4000 increases, the survival of the cells was greatly damaged although the fusion frequencies was not decreased dramatically. When 40% of PEG 4000 was employed, it gave 33% of cell survival and 3.0 $\times$ 10^{-4} CFU of hybrid cells per ml, as shown in Fing. 2.

**Table 4. Effect of reaction time with PEG 4000 on recombination frequency.**

Reaction time (min)	Regenerated cells (CFU/ml)	Recombinants (CFU/ml)			Total recombinants (CFU/ml)	Recombination frequency (CFU/ml)	Survival after PEG treatment (%)
		phenotype SM^R+HC^R	phenotype SM^R+LM^R	phenotype SM^R+DP^R			
0.5	5.3×10^6	1.6×10^5	1.6×10^5	2.1×10^5	5.3×10^5	2.9×10^{-4}	100
1	5.3×10^6	3.2×10^5	1.6×10^5	5.0×10^4	5.3×10^5	2.9×10^{-4}	100
5	4.7×10^6	2.3×10^5	5.0×10^4	1.4×10^5	4.2×10^5	2.3×10^{-4}	89
10	3.9×10^6	2.3×10^5	4.0×10^4	1.6×10^5	4.3×10^5	2.4×10^{-4}	74
20	3.5×10^6	1.4×10^5	1.1×10^5	3.0×10^4	2.8×10^5	1.6×10^{-4}	66
30	3.5×10^6	1.1×10^5	1.4×10^5	1.4×10^5	3.9×10^5	2.2×10^{-4}	66

The number of parent cells was 1.8×10^9 per ml (The number of *L. casei* C-M and *L. casei* 3-M strins was 1.5×10^9/ml and 2.7×10^8/ml, respectively).

Table 5. Effect of temperature of the fusion mixture of cells and PEG 4000 on recombination frequency.

Temperature (℃)	Regenerated cells (CFU/ml)	Recombinants (CFU/ml)			Total recombinants (CFU/ml)	Recombination frequency (CFU/ml)
		phenotype $SM^R + HC^R$	phenotype $SM^R + LM^R$	phenotype $SM^R + DP^R$		
20	5.8×10^6	5.0×10^4	1.2×10^5	1.8×10^5	3.5×10^5	1.9×10^{-4}
30	4.7×10^6	2.8×10^5	1.9×10^5	2.4×10^5	7.1×10^5	3.9×10^{-4}
37	4.5×10^6	4.5×10^4	4.5×10^4	9.0×10^4	1.8×10^5	1.0×10^{-4}
40	2.0×10^2	$<10^2$	$<10^2$	$<10^2$	$<10^3$	$<10^{-6}$
50	0	0	0	0	0	0

The number of parent cells was 1.8×10^9 per ml (The number of *L. casei* C-M and *L. casei* 3-M strains was 1.0×10^9/ml, and 8.0×10^8/ml, respectively).
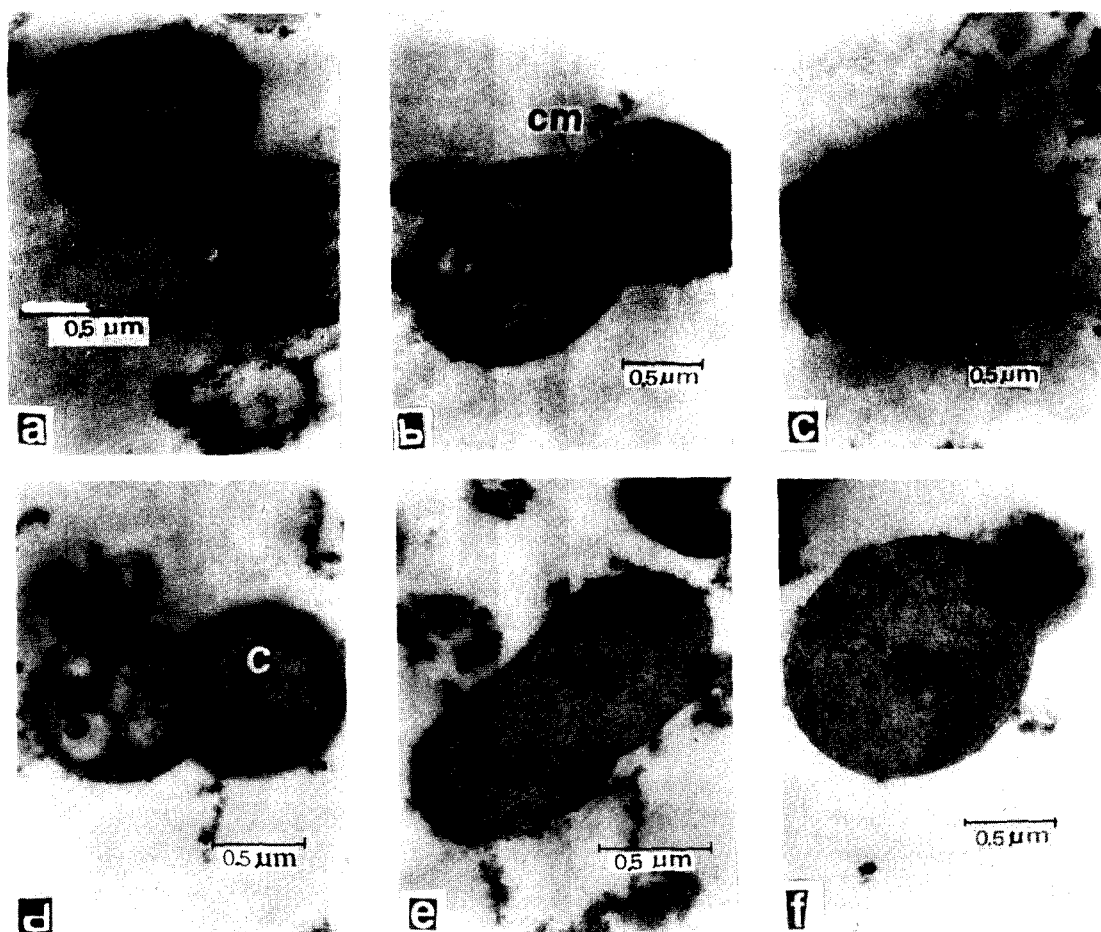
Fig. 3. Transmission electron micrographs of thin section of fusing. *L. casei* protoplasts.

a) Protoplasts formation of *L. casei*. The cell wall was clearly left behind and protoplasts formed a spherical shape. b) Initial stage of protoplast fusion. Cell membrane (cm) between two protoplasts could be clearly observed. c) Cell membrane between two protoplasts could be hardly observed in these micrographs. d) Fusion was distinctly pro gressed and developed. "C" : chromosomal region.
e) The protoplasts seemed to reach the final stage of fusion. f) It seemed to be the final stage of fusion.

When pH of 40% PEG 4,000 solution was varied using several buffers from PH 4.0 to pH 9.0, the neutral pH (pH 7.0) gave the highest recombination frequency as shown in Table 3. If pH of the mixture was below pH 5 or above pH 8, the recombination frequency was decreased substantially.

As evidenced in Table 4, a short exposure of protoplasts to 40% PEG 4,000 for 0.5 to 1 min was enough for the efficient protoplast fusion. This suggested that effective fusion between individual protoplasts was probably achieved almost instantly after exposure of the protoplasts to PEG. Optimum fusion was found to take place within the first few minutes after mixing of the protoplast-PEG solution and there was no increase in recombination frequency when treated for longer than 10 min. It was also found that the survival of fused protoplast was significantly decreased by prolonging the PEG treatment for longer than 20 min.

When the protoplast fusion was proceeded at different temperature in 40% PEG 4,000 solutions, the best fusion was obtained at about 30°C. The fusion at 40°C yielded less than 10^{-6} colony forming units per ml. It was presumed that temperature may affect the stability of the protoplast membrane, and the optimum temperature of PEG treatment appears to be about 30°C, as evidenced in Table 5. High frequency fusion of protoplasts of *L. casei* strains were obtained in the presence of 40% (wt/vol) PEG 4,000 after 1 min at 30°C at around neutral pH. Under these conditions, the recombination frequency achieved was about three times better than that obtained by the classical crossing method. The recombination frequencies were about 10^{-4} per parent cells employed. This level of recombination frequency is similiar level to that of previous reports with lactic streptococci strains.[2,11] As shown in Fig. 3 the entire fusion steps of *L. casei* protoplasts were traced by the transmission electron microscopy of the proper fusion mixtures samples. Looking through the micrographs, every steps of protoplast fusion, like protoplast contact, actual fusion, membrane disappearance and cytoplasm fusion, could be confirmed visually.

요 약

L. casei 세포의 유전연구를 위한 도구로서 세포 융합 기술을 연구하였으며 융합세포(recombinant)를 선발하고 확인하기 위한 유전자 선발표지 인자로서 항생제 저항성이 이용되었고, 항생제 저항성 돌연 변이 균주는 nitrosoguanidine을 처리하여 분리하였 다. 선발 배지에서 항생제의 적절한 최종 농도는

streptomycin 25 μg/ml, hostacillin 0.5 I.U./ml, lincomycin 0.5 μg/ml 그리고 methicillin 5 μg/ml 로 확인되었다.

L. casei 균주에서 높은 세포융합은 PEG 분자량 4,000에서 40% 농도, 중성 부근의 pH, 30℃에서 약 1분간 처리하였을때 얻어졌다. 항생제 저항성 의 자연돌연변이주의 출현빈도는 세포융합 출현빈도 보다 $10^2 - 10^3$ 정도 낮은 수준으로 나타났다. 세포 융합 빈도는 모균에 대해 약 10^{-4} 비율로 나타났다.

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