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Properties of the Fusants of *Lactobacillus acidophilus* 88 and *Lactobacillus casei* subsp. *casei* KCTC 1121

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Protoplast fusion between *L. casei* KCTC 1121 and *L. acidophilus* 88 was attempted to obtain improved strains. The fusants produced a bacteriocin against indicator strains, making a smaller inhibition zone compared to that of *L. acidophilus* 88. After culturing for 2 months on selective medium, the selected fusants were still stable without segregation. Fusants showed higher lipase activity compared to those of the two parent strains. Fusant No. 4, 11, and 15 exhibited excellent lactic acid productivity. Fusant No. 4 and 15 exhibited improved proteolysis ability compared to the two parent strains. Whereas *L. casei* possessed both β-galactosidase and phospho-β-galactosidase activities, and *L. acidophilus* 88 had only β-galactosidase activity, the fusants had both the intermediate enzyme activities. Cell size of the fusants was greater than that of the parents.

Lactobacillus among lactic acid bacteria (LAB) produces lactic acid using lactose, and provides flavor to fermented food and helps in the ripening of dairy products by producing protease and lipase. *Lactobacillus* is one of the most important microorganisms in dairy production. Therefore, the development and improvement of *Lactobacillus* is necessary for the improvement of fermentation processes.

For strain improvement of yield and functionality, various techniques such as transformation (17), conjugation (5), transduction (19), protoplast fusion (13), and electrofusion (6) were used. Protoplast fusion along with gene cloning is widely used for the development of industrial microorganisms because it produces a new strain regardless of the intrinsic characteristics of microorganisms.

LAB are widely used in the dairy industry to produce yogurt including drinking yogurt and other beverages and also, as medical aids and feed additives due to their beneficial, physiological activity such as Ca^{++} ion absorption (24), prevention of abnormal flora formation and the protection of mucous tissue in the intestine (7). Recent studies showed that LAB helps in the reduction of blood cholesterol (23) and the increase of immune activity as an adjuvant. Especially, the enhancement of immune activity by LAB is due to protection from pathogenic bacteria and anticancer activities (16) through the mechanism of interferences on induction, mitogen activations and cellular immunity activation.

Lactobacillus casei is a normal fermentative lactic acid bacterium and is a mesophilic starter microorganism used for the production of liquid yogurt and cheese. It shows high lactose assimilation activity and high productivity of lactic acid (4). *Lactobacillus acidophilus* used as a thermophilic starter for acidophilus milk production. It prevents the intestinal disease caused by pathogenic bacteria (25), and inhibits the enzyme production of cancer-causing bacteria (8). *L. acidophilus* is the main source of bacteriocin (2), and it is widely used for food preservation. Bacteriocin is a protective bactericidal agent produced by various microorganisms and kills other microorganisms by degrading cell membrane, inhibiting rRNA transcription as well as synthesis of peptidoglican, lipid, and DNA (1, 9). Studies on the application, characterization and production of bacteriocin from *L. acidophilus* are currently being carried out and it has a great potential in the fermentation industry for the application and production of bacteriocin.

This study focuses on the isolation of fused *Lactobacillus* between *L. casei* and *L. acidophilus* by the methods previously described (11, 12) and the production of a stable fused protoplast. After physiological characterization of the fused *Lactobacillus* those carrying the highest genetic stability and desired properties were selected.

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MATERIALS AND METHODS

Microorganism and Medium

The microorganisms used in this study are *Lactobacillus casei* subsp. *casei* KCTC 1121 and *Lactobacillus acidophilus* 88 (20). From these original strains, the antibiotic resistant strains were isolated and used for protoplast fusion. Physiological evaluations were carried out to identify the fused strains which had genetic stability and high growth rate. MRS medium (3) was used for complete medium and MRS medium containing 2 mg/ml streptomycin and 600 μg/ml kanamycin for isolation of fused cells.

Screening of Bacteriocin Production

Screening of bacteriocin-producing original and fused strains was carried out by the Staskawicz's method with slight modification (28). After growing *Lactobacillus* sp. overnight in MRS broth at 37°C, 5 μl of precultured broth were spotted onto 1.5% hard agar plates and cultured for 6 h. Then, 200 μl of the cultured indicator microorganism in 4 ml of 0.8% top agar was overlaid on the surface of hard agar and cultured overnight at 37°C in the anaerobic gas-pak system. The bacteriocin production activity was detected by the formation of clear zone. *Lactobacillus helveticus* CNRZ 1096 was used as an indicator strain.

Protoplast Fusion Using Polyethylene Glycol

The procedure for protoplast fusion was carried out as shown in Fig. 1. Equal amounts of two protoplast suspensions were mixed and centrifuged. After resuspending the protoplast pellet with 0.1 ml of protoplast-forming buffer, 0.9 ml of polyethylene glycol (PEG) was added. In order to stop the fusion, 2 ml of protoplast fusion buffer (PFB) was added and centrifuged. The pellet was resuspended with 1 ml of PFB. After proper dilution, the fused protoplasts were spread on agar plates containing 2 mg/ml streptomycin and 600 μg/ml kanamycin and agar plate without antibiotics and cultured for 3 to 5 days.

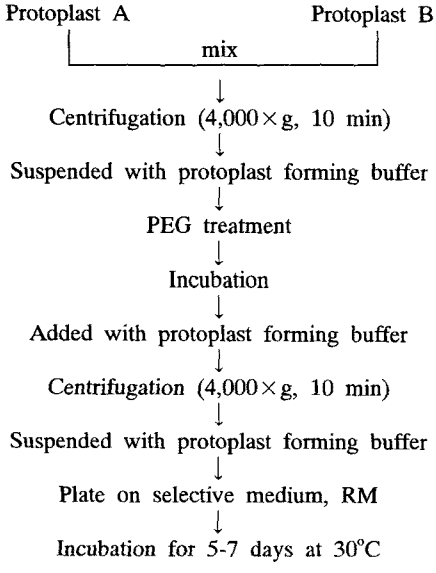

Fig. 1. Procedure of protoplast fusion between *Lactobacillus casei* subsp. *casei* KCTC 1121 and *Lactobacillus acidophilus* 88.

Selection of Stable Fusants (26)

Successive culture of the fusants every weeks was carried out for the selection of a stable strain without segregation. After 10 successive cultures, the stable fusants were isolated and spread on the complete medium. The colonies on the plate were transferred to complete and selective medium by the replication method. Genetic stability was determined by the ratio of colonies grown on the selective medium to the complete medium. Fifteen stable fusants were isolated and used for further studies.

Acid Productivity

Acid productivity from *Lactobacillus* was measured according to the method of Kaneko *et al.* (14). Cells were inoculated into 10% reconstituted skim milk and cultured at 37°C. The pH at each hour was measured for acid productivity.

β-Galactosidase Activity

β-Galactosidase activity was determined by the methods of Okamoto *et al.* (21) and Mckay *et al.* (19) with modifications. After centrifugation the cell pellet was washed twice with 50 mM sodium phosphate buffer (pH 7.0) and resuspended with the same buffer. Acetone and toluene (9 : 1, v/v) was added at a concentration of 10 μl/ml and the cell suspension was vortexed for 5 minutes. The toluenized cell (0.1 ml) was mixed with 0.2 ml of 12 mM *o*-nitrophenyl-D-galactopyranoside (ONPG) in 50 mM sodium phosphate buffer (pH 7.0) and reacted at 37°C. The reaction was stopped by adding 2 ml of 0.5 M Na_2CO_3 and left for 10 minutes at room temperature. Absorbance at 420 nm was measured and the protein content of 1 ml of toluenized cell recorded. Specific activity was defined as the amount of *o*-nitrophenol in nanomole released by 1 mg of β-galactosidase by 1 minute of reaction.

Activity of Phospho-β-galactosidase

After centrifugation the cell pellet was washed twice with 50 mM sodium phosphate buffer (pH 7.0) and resuspended with the same buffer. Toluene was added at a concentration of 10 μl/ml and the cell suspension was vortexed for 5 minutes. The toluenized cell (0.5 ml) was mixed with 2 ml of 50 mM sodium phosphate buffer (pH 7.0) containing 3 mM *o*-nitrophenyl-D-galactopyranoside-6-phosphate (ONPG-6-P) and left at 37°C for 10 minutes. The reaction was terminated by adding 2 ml of 0.5 M Na_2CO_3. After 10 minutes the absorbance at 420 nm was

measured. The specific activity was measured by the same method as used for β-galactosidase.

Protease and Lipase Activity

Protease activity of fusants of *Lactobacillus* was measured by Lin *et al.*'s method (18). Lipase Activity of fusants was measured by Kwon and Rhee's method (15).

Thermal and Acidic Tolerance

The thermal and acid tolerance of the original strains and fusants were determined. The precultured broth (100 μl, 0.5%) was inoculated and cultured at 44, 46, 48 and 50°C to determine cell growth. Acid tolerance was determined by culturing cells at pH 3.0-5.0.

Observation of Cell by Optical Microscope

Cell suspensions and protoplasts were observed by optical microscope (Nikon, Microflex HFX-II) after fixation by flame and crystal violet followed by washing and drying.

Transmission Electron Microscopy

The transmission electron micrographs of the original strains and protoplasts were taken by Epon embedding and uranyl acetate and lead citrate dying using TEM (JEOL 100S Electron Microscope, Japan).

Estimation of Cell Volume

The cell volumes of both the original and protoplast strains were measured by Sipiczki and Ferenczy's method (27). The long and short axis were measured under a microscope and cell volume was calculated using the equation shown below.

$$\text{Cell volume } (V) = \frac{4}{3}\pi \times \frac{a}{2} \times \left(\frac{b}{2}\right)^2$$

(a, length of cells; b, width of cells)

RESULTS AND DISCUSSION

Protoplast Fusion by PEG and Selection of Stable Fusants

The fusants were prepared with the protoplasts of streptomycin resistant *L. casei* and kanamycin resistant *L. acidophilus*. The fusants were isolated on the selection plate containing 2 mg/ml streptomycin and 600 μg/ml kanamycin. Then, fusants were bred through ten successive cultures with 2 week intervals to obtain stable fusants. The stable fusants were named *Lactobacillus* sp. F1-F15 and used for the further experiments.

Bacteriocin and Acid Productivities of Fusants

The bacteriocin activity of fusants against *L. helveticus* CNRZ 1096 was determined. The fusants showed considerable bacteriocin activity as shown in Fig. 2. However, it was lower than that of *L. acidophilus* 88, the parent strain.

Acid productivity of fusants increased than the parent strains, especially, for fusants F11 and F15 (Fig. 3).

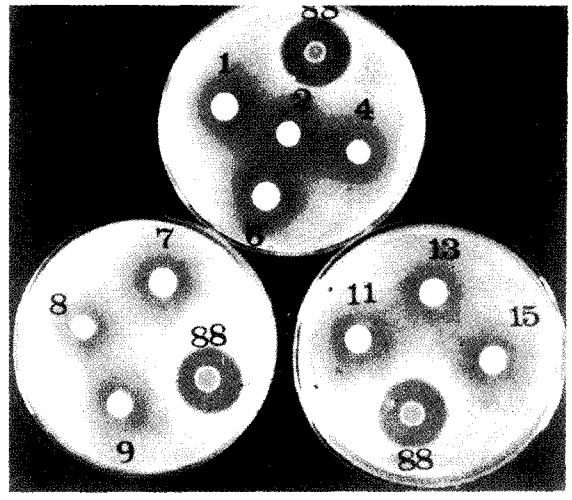

Fig. 2. Antagonistic activities of *Lactobacillus acidophilus* 88 and fusants overlaid with *Lactobacillus helveticus* CNRZ 1096.

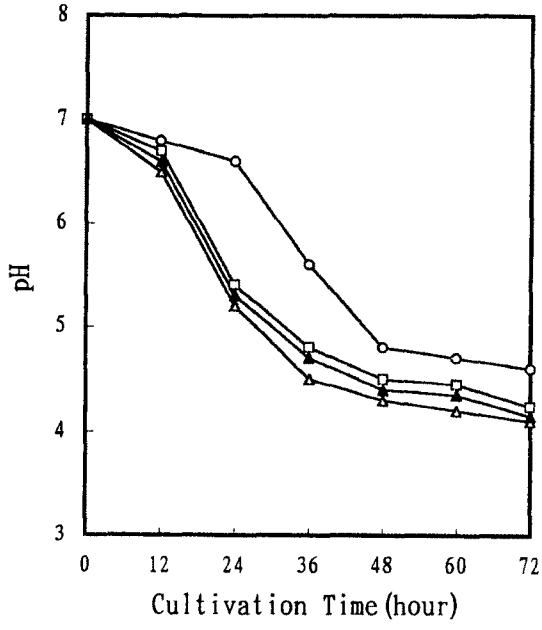

Fig. 3. Acid production during fermentation by test strains. —○—, *L. acidophilus*; —□—, *L. casei*; —△—, Fusant No. 11; --▲—, Fusant No. 15.

Activities of β-Galactosidase and Phospho-β-galactosidase

β-Galactosidase activity of each strains was measured by toluenized preparation of cells in the logarithmic phase of cell growth. β-Galactosidase activity of *L. acidophilus* 88 was high but that of *L. casei* was relatively low as shown in Table 1. This result agreed well with Premi *et al.*'s result (22) on the β-galactosidase activities of the two strains. Although most fusants showed

lower values of β-galactosidase activity than *L. acidophilus*, the parent strain, some fusants such as F4, F7, F8 and F12 showed higher activity than the original strain.

L. casei produces a specific enzyme, phospho-β-galactosidase and its activity was high as is shown in Table 1. However, *L. acidophilus* did not show phospho-β-galactosidase activity. Most of the fusants showed phospho-β-galactosidase activity except fusants F2, F8 and F13, indicating their genetic resemblance to the parent strain, *L. casei*. Fusants F5 and F15 showed relatively high phospho-β-galactosidase activity.

Jimeno *et al.* (10) reported that some of *L. casei* had phospho-β-galactosidase and some had β-galactosidase, and some strains had both enzyme activities. This experiment also confirmed that *L. casei* produced both enzymes and outfindings agreed with the results of Jimeno *et al.* (10). Most fusants also showed both enzyme activities.

Activities of Protease and Lipase

Protease activity of both parent and fusant strains was determined as shown in Table 2. The activity of *L. acidophilus* was higher than that of *L. casei*. Most fusants showed an intermediate value of activity but F2, F14 and F15 showed higher values compared to that of the parent strain, *L. acidophilus*.

Lipase activity of fusants showed intermediate values between that of *L. acidophilus* and *L. casei*. However, F 2, F6 and F10 showed higher activity than the original strains indicating the possibility of improved functionality by cell fusion (Table 2). As these two enzymes are key enzymes for the dairy industry, fusants with high levels of activities of these enzymes could be useful.

Thermal Stability of Cells

Lactobacillus can grow at temperatures over 40°C. Some can grow at near 50°C. As shown in Table 3, *L. casei* could grow at 48°C, while *L. acidophilus* grew slowly at 44°C. The fusants showed an intermediate range of temperature tolerance. Particularly F5 showed

Table 1. β-Galactosidase and phospho-β-galactosidase activity of parents and fusants.

Strains	β-Galactosidase Activity (nmol)*	Phospho-β-galactosidase Activity (nmol)*
L. casei	374	395
L. acidophilus	958	8
Fusant No. 1	418	279
Fusant No. 2	906	94
Fusant No. 3	521	243
Fusant No. 4	1009	69
Fusant No. 5	419	370
Fusant No. 6	823	75
Fusant No. 7	981	69
Fusant No. 8	1016	23
Fusant No. 9	356	578
Fusant No. 10	966	150
Fusant No. 11	743	342
Fusant No. 12	1105	83
Fusant No. 13	918	10
Fusant No. 14	502	98
Fusant No. 15	978	350

*Specific activity was expressed as nanomoles of *o*-nitrophenol liberated from ONPG per milligram of enzyme protein per minute.

Table 2. Protease and lipase activities of parents and fusants.

Strains	Protease Activity (μg/ml)*	Lipase Activity (unit/ml)**
L. casei	5.4	0.17
L. acidophilus	8.7	0.32
Fusant No. 1	6.2	0.01
Fusant No. 2	8.9	1.70
Fusant No. 3	1.0	0.15
Fusant No. 4	9.6	0.34
Fusant No. 5	7.3	0.12
Fusant No. 6	5.2	1.26
Fusant No. 7	4.7	0
Fusant No. 8	7.5	0.23
Fusant No. 9	6.3	0.32
Fusant No. 10	3.5	0.43
Fusant No. 11	9.0	0.27
Fusant No. 12	0	0.24
Fusant No. 13	4.7	0.32
Fusant No. 14	6.4	0.23
Fusant No. 15	9.6	1.09

*Tyrosine content. **One unit was defined as the amount of enzyme that produced 1 μmole of free fatty acid per minute.

Table 3. Effect of temperature on the growth of parents and fusants.

Strans	Temperature (°C)			
	44	46	48	50
L. casei	+	+	+	−
L. acidophilus	(+)	−	−	−
Fusant No. 1	+	(+)	−	−
Fusant No. 2	+	(+)	−	−
Fusant No. 3	+	+	−	−
Fusant No. 4	+	(+)	−	−
Fusant No. 5	+	+	+	−
Fusant No. 6	+	−	−	−
Fusant No. 7	+	−	−	−
Fusant No. 8	+	−	−	−
Fusant No. 9	+	−	−	−
Fusant No. 10	+	−	−	−
Fusant No. 11	+	+	−	−
Fusant No. 12	+	+	−	−
Fusant No. 13	+	−	−	−
Fusant No. 14	+	+	−	−
Fusant No. 15	+	+	−	−

+, growth; (+), partial growth; −, no growth.

Table 4. Effect of pH on the growth of parents and fusants.

Strains	pH 3	pH 4	pH 5
L casei	(+)	+	+
L. acidophilus	−	−	+
Fusant No. 1	−	(+)	+
Fusant No. 2	(+)	+	+
Fusant No. 3	−	(+)	+
Fusant No. 4	−	(+)	+
Fusant No. 5	−	(+)	+
Fusant No. 6	−	(+)	+
Fusant No. 7	−	−	+
Fusant No. 8	−	(+)	+
Fusant No. 9	−	(+)	+
Fusant No. 10	−	(+)	+
Fusant No. 11	−	+	+
Fusant No. 12	−	(+)	+
Fusant No. 13	−	+	+
Fusant No. 14	−	(+)	+
Fusant No. 15	−	(+)	+

+, growth; (+), partial growth; −, no growth.

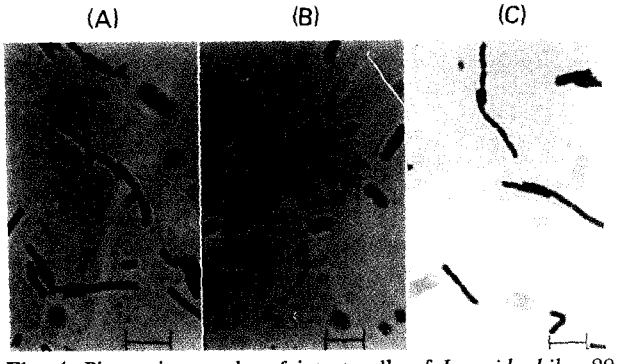

Fig. 4. Photomicrographs of intact cells of *L. acidophilus* 88 (A), protoplast of *L. acidophilus* 88 (B) and fusant No. 11 (C). 1,000×; indicator bar, 5 μm.

better growth than *L. casei*, the parent strain at 48°C.

Acid Stability of Cells

The acid sensitivity of fusants and parent cells was evaluated (Table 4). *L. casei* and F2 showed weak growth at pH 3.0. Most of fusants grew at pH 4.0, but not *L. acidophilus* 88.

Determination of Cell Volume

Fig. 4 shows the optical micrographs of *L. acidophilus* 88, the protoplast, and fusants. *L. acidophilus* was a typical rod-type bacillus, while the fusants were of an extended shapes.

The protoplasts of *L. acidophilus* and *L. casei*, and the fusion process were observed by TEM. Fig. 5A shows the contact point between two protoplasts prepared by PEG treatments. The initial fusion stage could be observed by the integration of two plasma membrane (Fig. 5B). The late stage of fusion to form a fused cell body is shown in Fig. 5C.

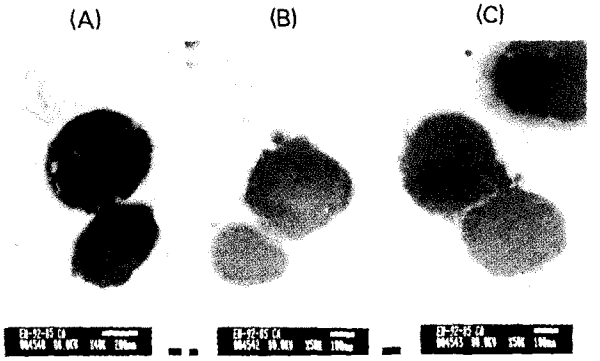

Fig. 5. Transmission electron micrographs of protoplast fusion between *L. casei* and *L. acidophilus* 88.
(A) contacting stage (40,000×), (B) initial stage (50,000×), (C) final stage (50,000×).

Table 5. Cell size and capacity of the parents and fusants.

Strains	Cell size		
	Length (μm)	Width (μm)	Volume (μm^3)
L. casei	0.7 − 2.8	0.3 − 0.5	0.03 − 0.37
L. acidophilus	1.1 − 3.5	0.3 − 0.5	0.05 − 0.46
Fusant No. 1	1.2 − 4.2	0.4 − 0.5	0.10 − 0.55
Fusant No. 2	1.0 − 2.8	0.3 − 0.7	0.05 − 0.72
Fusant No. 3	1.2 − 4.2	0.3 − 0.7	0.06 − 1.08
Fusant No. 4	1.2 − 4.2	0.4 − 0.5	0.10 − 0.55
Fusant No. 5	1.0 − 3.5	0.5 − 0.7	0.13 − 0.90
Fusant No. 6	2.8 − 4.2	0.4 − 0.7	0.23 − 1.08
Fusant No. 7	4.2 − 7.0	0.5 − 0.7	0.55 − 1.80
Fusant No. 8	2.8 − 4.2	0.4 − 0.5	0.37 − 1.08
Fusant No. 9	1.2 − 4.2	0.4 − 0.5	0.10 − 0.55
Fusant No. 10	2.8 − 4.2	0.5 − 0.7	0.37 − 1.08
Fusant No. 11	1.0 − 2.8	0.4 − 0.5	0.08 − 0.37
Fusant No. 12	1.2 − 4.2	0.5 − 0.7	0.16 − 1.08
Fusant No. 13	2.8 − 4.2	0.4 − 0.7	0.23 − 1.08
Fusant No. 14	4.2 − 7.0	0.5 − 0.7	0.55 − 1.80
Fusant No. 15	2.8 − 4.2	0.5 − 0.7	0.37 − 1.08

The cell volumes of fusants, *L. acidophilus* and *L. casei* were measured. The volume of *L. casei* was larger than that of *L. acidophilus* as shown in Table 5. The size of fusants was much larger than that of the parent strains.

From these experiments, several good strains with bacteriocin activity which is essential for the dairy fermentation industry, were prepared. Particularly strains F4, F11 and F15 strains showed superior characteristics to the parent strains (Table 6). Cell fusion provides a simple and quick method to improve cell properties for industrially important cells such as *Lactobacillus* strains which are hard to deal with using gene cloning techniques.

Table 6. Comparison of characteristics of the parent strains and fusants.

Test	*L. casei*	*L. acidophilus*	F4	F11	F15
β-Galactosidase activity[a]	374	958	1009	743	978
Phospho-β-galactosidase activity[a]	395	8	69	342	350
Protease activity[b]	5.4	8.7	9.6	9.0	9.6
Lipase activity[c]	0.17	0.32	0.34	0.27	1.09
Lactic acid production (pH)	4.24	4.6	4.15	4.1	4.15
Cell volume (μm^3)	0.03~0.37	0.05~0.46	0.10~0.55	0.08~0.37	0.37~1.08

[a]Specific activity was expressed as nanomoles of *o*-nitrophenol librated from *o*-nitrophenyl-D-galactopyranoside (ONPG) per milligram of enzyme protein per minute. [b]Tyrosine content. [c]One unit was defined as the amount of enzyme that produced 1 μmole of free fatty acid per minute.

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