Note

Intraspecific Protoplast Fusion of *Lactobacillus plantarum*

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

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

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Lactobacillus plantarum is one of several lactic-acid bacteria widely used in the manufacture of many fermented food products.¹⁾ Although a few attempts involving a conjugal gene transfer system have been reported for this organism,^{2–4)} its practical improvement has depended almost completely on classical mutagenesis and selection; protoplast fusion and transformation, which have been applied successfully in other species of microorganisms,^{5–9)} have not been demonstrated yet. Therefore, it seems extremely important to establish genetic techniques applicable to this commercially important organism.

For this purpose, we recently established¹⁰⁾ the optimal conditions for protoplast formation and the subsequent regeneration of intact cells of *L. plantarum*. In this communication, we describe the construction of genetically altered *L. plantarum* strains by means of intraspecific protoplast fusion between mutants carrying different genetic markers.

L. plantarum IFO 3074 (obtained from the Institute for Fermentation, Osaka, Japan) was mutagenized¹¹⁾ with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG, 100 µg/ml) in 30 mM Tris–HCl buffer, pH 7.5 (Tris buffer)¹⁰⁾ at 37°C for 30 min or by UV irradiation (UVP Inc., San Gabriel, U.S.A.; type UVGL-58) for 3 min at a distance of 40 cm, mutants TS 63-11, TE 64-21 and TE 64-215 (see Table I) being obtained. Protoplast formation of the mutant cells was performed according to the procedure previously described.¹⁰⁾ Protoplasts were collected by centrifugation at 3000 × *g* as a precipitate and then suspended in SM-Tris buffer (Tris buffer, pH 7.5, containing 10% sucrose and 2.5 mM MgCl₂). A protoplast suspension (approximately 3.0 × 10⁸ CFU/ml) of each strain was routinely prepared and two protoplast suspensions thus obtained were mixed. Then, the protoplasts were immediately sedimented by centrifugation at 3000 × *g* and resuspended in 1 ml of a polyethylene glycol (PEG) solution [SM-Tris buffer, pH 7.5, containing 50% (w/v) PEG (MW 6000)]. The resultant suspension was incubated at 30°C for 2 min with gentle mixing, for protoplast fusion, and then centrifuged

Table I. *L. plantarum* MUTANT STRAINS USED

Strain	Phenotype	Reversion frequency
<i>L. plantarum</i>		
TS 63	Sm ^r	4.2 × 10 ^{−8}
TS 63-11	Sm ^r Ile [−]	3.4 × 10 ^{−8 a}
TE 64	Em ^r	3.9 × 10 ^{−8}
TE 64-21	Em ^r Leu [−]	2.7 × 10 ^{−8 a}
TE 64-215	Em ^r Leu [−] Phe [−]	2.5 × 10 ^{−8 b}

Abbreviations: Ile[−], isoleucine auxotrophy; Leu[−], leucine auxotrophy; Phe[−], phenylalanine auxotrophy.

Drug resistant mutants, TS 63 and TE 64, were derived from *L. plantarum* IFO 3074 by NTG treatment. Through second NTG treatment or UV irradiation, auxotrophic mutants were obtained. The reversion frequencies were determined after protoplasts of these mutants had been treated with PEG.

^a The reversion frequency of the second marker.

^b The reversion frequency of the third marker.

at 3000 × *g*. The precipitate was suspended in 10 ml of SM-Tris buffer. One milliliter of this suspension was plated on the regeneration medium (RM)¹⁰⁾ supplemented with 100 µg/ml streptomycin, 50 µg/ml erythromycin and auxotrophic amino acids. After 5 to 7 days incubation at 37°C, colonies formed were counted and replicated upon specified selection media (the basal medium¹¹⁾ supplemented with the nutrients required by each strain and 1.5% agar). Colonies formed on these plates were counted after 2 to 3 days incubation at 37°C.

When the protoplast fusion experiment were carried out with a streptomycin resistant (Sm^r, TS 63-11) mutant and erythromycin resistant (Em^r, TE 64-21 and TE 64-215) mutants, the frequencies of Sm^rEm^r colonies developing on RM supplemented with the two drugs and the three auxotrophic amino acids ranged from 4.7 to 6.3 × 10^{−7} of the plated protoplasts. These values were evidently higher than that (1 to 3 × 10^{−8}) reported for *L. fermentum*⁵⁾. When PEG was omitted or each protoplast preparation was plated separately, no Sm^rEm^r colonies were formed under our experimental conditions. Also, reversion to prototrophy as to drug resistant or nutrient markers occurred at the frequency of 10^{−8} of the plated protoplasts (Table I), which was one order lower than the fusion rates mentioned above. Thus, the results strongly suggest that Sm^rEm^r colonies arose on the fusion of protoplasts carrying different genetic markers and not as a consequence of spontaneous mutation.

To determine whether or not recombinants were actually constructed, the nutrient markers of Sm^rEm^r colonies were examined. As indicated in Table II, 14 out of 28 isolates (TS 63-11 and TE 64-21 as parents) or 10 out of 30 (TS 63-11 and TE 64-215) showed a mixed phenotype, a mixture of those of their parents. It was concluded from

Table II. RECOMBINANT FORMATION THROUGH PROTOPLAST FUSION

	Sm	Em	Ile	Leu	Phe	Number of clones obtained
	Parental					
TS 63-11	r	s	—	+	+	
TE 64-21	s	r	+	—	+	
	Fusant					
	r	r	—	+	+	6
	r	r	+	—	+	18
	r	r	+	+	+	12
	r	r	—	—	+	2
	Parental					
TS 63-11	r	s	—	+	+	
TE 64-215	s	r	+	—	—	
	Fusant					
	r	r	—	+	+	7
	r	r	+	—	—	13
	r	r	+	+	+	5
	r	r	—	—	—	2
	r	r	+	—	+	1
	r	r	+	+	—	2

Sm⁺Em⁺ colonies that developed on RM supplemented with the two drugs and the auxotrophic amino acids were replicated upon specified selection media supplemented with the nutrients required by each strain. After 2 to 3 days incubation at 37°C, colonies were counted.

these results that Sm⁺Em⁺ colonies obtained on protoplast fusion were true recombinants. This property of the Sm⁺Em⁺ phenotype was stably maintained for at least 5 transfers on the growth medium containing no drugs.

Protoplast fusion has been applied to *Streptococcus* spp.¹²⁻¹⁴⁾ and *Bacillus* spp.¹⁵⁻²⁰⁾ as a very useful technique for genetic recombination, nevertheless, this technique has not yet been extensively applied for the strain improvement of *Lactobacilli*. We demonstrated here the usefulness of protoplast fusion for the recombination of *L. plantarum* mutants carrying different auxotrophic and drug resistant markers. This technique seems to be applicable not only for the breeding a certain desired strain, but also for genetic analysis of the organism. At present, detailed studies on the fusants are in progress in our laboratory.

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