Interspecific Protoplast Fusion between *Streptococcus cremoris* and *Streptococcus lactis*

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Interspecific recombinants have been produced between *Streptococcus cremoris* H-61 and *S. lactis* J-1 by polyethylene glycol-induced protoplast fusion. All of the fusants obtained showed mixed physiological properties of the two parents, and possessed plasmids derived from both parents at random. Physiological properties of primary colonies were stably maintained among the progenies after the single-colony isolation procedure. Similarly, in most of the fusants the plasmid profiles of the primary colonies were stably maintained, but one lost 2 out of the 7 plasmid bands. However, there was no indication that plasmids from either one of the parents were preferentially lost. These results showed that interspecific genetic transfer occurred on chromosomal and plasmid DNA on the protoplast fusion and that the fusants obtained were not heterokaryons, but true recombinants.

The economic value of lactic streptococci, which include *Streptococcus cremoris*, *S. lactis* and *S. lactis* subsp. *diacetylactis*, is well recognized, and efforts to improve strains of this genus have been made by using random mutagenesis. With the progress of molecular genetics in lactic streptococci, useful genetic transfer systems, such as transformation,¹⁾ transduction²⁾ and conjugation,^{3,4)} have been demonstrated. Whilst these developments are of potential value, a fourth method of a gene transfer system, protoplast fusion, appears to have broad utility for effective genetic recombination. The fusion process has been shown to generate large numbers of random recombinants. Furthermore, because the fusion is neither strain- nor species-specific, this method of gene transfer may be advantageous for strain improvement.

Techniques for the production and regeneration of protoplasts in lactic streptococci have been established,⁵⁾ and recently intraspecific genetic recombination by protoplast fusion was reported.^{6,7)} In this communication, we report the formation of interspecific hybrids between *S. cremoris* and *S. lactis* by polyethylene glycol-induced fusion of their proto-

plasts.

MATERIALS AND METHODS

Bacterial strains. *S. cremoris* H-61 and *S. lactis* J were maintained in our stock culture collection. *S. lactis* J-1 was a spontaneous Lac⁻ mutant derived from *S. lactis* J and was resistant to 1 mg/ml streptomycin. These strains were maintained by biweekly transfer in basal-glucose or basal-lactose broth.⁸⁾

Protoplast fusion. Protoplasts of the strains, *S. cremoris* H-61 and *S. lactis* J-1, were prepared by treatment with lysozyme and α -amylase, and treated with polyethylene glycol (PEG) 6000 as described previously.⁷⁾ The PEG-treated were diluted by adding four ml of Tris-HCl-magnesium-sucrose buffer,⁷⁾ and then 0.1 ml aliquots were plated on regeneration medium.⁷⁾ After 5 days' incubation at 30°C, the plates were replicated with sterile velvets onto selective medium⁷⁾ containing 500 μ g streptomycin per ml. The plates were incubated at 30°C for 2 days and Lac⁺ Stm^r primary colonies were isolated. Then the physiological properties and plasmid profiles of the colonies were checked.

Physiological characteristics. Growth in basal-glucose broth in the presence of 4% sodium chloride or at 40°C was observed as visible turbidity. Arginine hydrolysis was investigated with Nessler's reagent to detect ammonia by the method of Niven *et al.*⁹⁾ Test organisms were inoculated into basal broth containing 0.04% bromocresol purple (BCP) and maltose as a sole carbon source, and

then incubated at 30°C for 24 hr. Acid production from maltose was confirmed from the color of the indicator dye.

Plasmid analysis. Plasmid DNA from the strains of lactic streptococci was prepared after lysis by the method of LeBlanc and Lee.¹⁰⁾ *S. faecalis* plasmids pAM α , pAM β and pAM γ , and *Bacillus subtilis* pTP5 were isolated for use as mobility references by the methods of LeBlanc and Lee¹⁰⁾ and Gryczan *et al.*,¹¹⁾ respectively. Agarose gel electrophoresis was carried out as described by Meyers *et al.*¹²⁾ in a horizontal gel at 30 V for 16 hr. 20 μ l plasmid DNA and 10 μ l tracking dye per sample were electrophoresed in a 0.5 or 0.7% agarose gel. The gels were stained for 60 min in 0.5 μ g of ethidium bromide per ml and then photographed under a long-wave ultraviolet light.

Stability of fusants. To examine the stability of some phenotypes in the fusants obtained, the organisms were streaked on BCP-lactose indicator medium.¹³⁾ A typical Lac⁺ colony was transferred to a tube of basal-lactose broth and grown at a 30°C for 16 hr. Then the culture was streaked over the surface of indicator medium for the isolation of Lac⁺ colonies. All plates were incubated at 30°C for 2 days. The procedure was repeated three times. 9 to 10 typical Lac⁺ colonies were isolated from each fusant, and then their properties were checked.

Physiological properties, 4% sodium chloride tolerance and ammonia from arginine, were examined. To examine the stability of lactose metabolism, a typical Lac⁺ clone was chosen from 9 or 10 isolates and grown under specified experimental conditions. The frequency of spontaneous Lac⁻ reversion from Lac⁺ was determined under the cultural conditions of growth in lactic broth.¹⁴⁾ The test organisms were grown in lactic broth at 30°C in the presence of 10 μ g/ml acridine orange or at elevated temperature (38°C). The cultures were diluted with saline and plated over the surface of indicator medium. After 48 hr incubation at 30°C, colonies were counted and the frequency of segregation from Lac⁺ to Lac⁻ was determined.

Plasmid profiles of all the isolates were determined electrophoretically by the method described above, and their stability was checked.

RESULTS

Formation and characterization of interspecific fusants

After PEG treatment of a mixture of protoplasts of *S. lactis* J-1 and *S. cremoris* H-61, Lac⁺ Stm^r clones were obtained at the frequency of about 10⁻⁵ per regenerated *S. lactis* J-1 protoplast. This value was lower than that reported for intraspecific fusion in *S. lactis*.⁷⁾ Five primary Lac⁺ Stm^r colonies, A-1, A-2, A-3, A-4 and A-5, were isolated and their characteristics were investigated.

Although there is no remarkable difference between *S. cremoris* and *S. lactis* with respect to morphology, *S. lactis* mostly forms pairs or short chains whereas *S. cremoris* forms long chains. Microscopic observation of the interspecific hybrids revealed that A-1, A-3 and A-4 form pairs or short chains, and A-2 and A-5 form long chains, suggesting that the former are the *S. lactis* type and the latter the *S. cremoris* type in morphology (Table I).

From a taxonomical point of view, *S. lactis* produces ammonia from arginine and grows at 40°C or in broth containing 4% sodium chloride or with maltose as a sole carbon source, but *S. cremoris* gives negative reactions in these physiological tests. As shown in Table I, all of the fusants could not grow at 40°C or in broth containing maltose. Therefore, these properties were the same as those of *S. cremoris*. On the other hand, the property of ammonia production from arginine was the same as that of *S. lactis*. A-1, A-3 and A-4

TABLE I. PHYSIOLOGICAL PROPERTIES OF *S. cremoris* H-61, *S. lactis* J-1 AND THEIR FUSANTS

Properties	Strain						
	H-61	A-1	A-2	A-3	A-4	A-5	J-1
Maltose fermentation	—	—	—	—	—	—	+
Arginine hydrolysis	—	+	+	+	+	+	+
Growth in the presence of 4% NaCl	—	+	—	+	+	—	+
Growth at 40°C	—	—	—	—	—	—	+
Morphology*	C	L	C	L	L	C	L

* C, *S. cremoris* type; L, *S. lactis* type.

FIG. 1. Agarose Gel Electrophoresis of Plasmid DNA from *S. cremoris* H-61 (well F), *S. lactis* (well G) and Their Fusants.

Well F shows (top to bottom) the banding of plasmids having molecular weights of 11.4, 7.7, 2.6, 2.1 and 1.8 Mdal. Well G shows the banding at the 9.5 and 5.0 Mdal positions. Wells A, B, C, D and E represent A-5, A-4, A-3, A-2 and A-1, respectively.

could grow in broth containing 4% sodium chloride, but A-2 and A-5 could not. These results showed that the properties of the five clones obtained were distinctly different from those of the parental strains and that they were interspecific recombinants between *S. cremoris* H-61 and *S. lactis* J-1.

Group N streptococci possess a diversity of plasmid molecules, some of which encode industrially important traits, such as lactose metabolism,¹⁵⁻¹⁷⁾ and protease¹⁵⁾ and citrate utilization,¹⁸⁾ and the plasmid profile is inherent to a specified strain. The plasmid profiles on agarose gel electrophoresis of *S. cremoris* H-61 and *S. lactis* J-1 are shown in Fig. 1. *S. cremoris* H-61 possessed 5 plasmid species of 11.4, 7.7, 2.6, 2.1 and 1.8 megadaltons (Mdal), and *S. lactis* J-1 possessed 9.5 and 5.0 Mdal plasmids. It was evident that

TABLE II. STABILITY OF PHYSIOLOGICAL PROPERTIES OF FUSANTS

Strains	Physiological properties			
	Arginine hydrolysis		4% NaCl tolerance	
	Primary*	Isolates**	Primary*	Isolates**
H-61	—		—	
A-1	+	10/10	+	10/10
A-2	+	10/10	—	10/10
A-3	+	9/9	+	9/9
A-4	+	10/10	+	10/10
A-5	+	9/9	—	9/9
J-1	+		+	

* Primary colony obtained after protoplast fusion.

** Number of isolates which exhibit the same properties as the primary colony/number of isolates obtained after the single-colony isolation procedure.

the plasmid profiles of the two parental strains were quite distinct. All of the fusants possessed 11.4 and 9.5 Mdal plasmids. The remaining plasmids, 1.8, 2.1, 2.6, 5.0 and 7.7 Mdal, were present in varying permutations across the five strains. These results showed that the fusants possessed plasmids of the two parents at random, and the tendency that the profiles were inclined toward either one of the parents was not observed. In addition to the plasmids mentioned above, strain A-1 possessed a 10.0 Mdal plasmid. A band corresponding to a similar molecular size was also detected in *S. cremoris* H-61, but it was too faint to be identified as a distinct plasmid. At present, therefore, we are not certain whether the 10 Mdal plasmid of A-1 originates from H-61 or not.

Genetic stability of fusants

To determine whether the interspecific hybrids were heterokaryons, single-colony isolation was performed for each of the hybrids three times, and then the physiological properties were checked. As shown in Table II, all of the isolates exhibited the same phenotypes as those of the primary colonies obtained after protoplast fusion.

Since many industrially important functions are encoded on plasmid DNA in lactic strep-

FIG. 2. Agarose Gel Electrophoresis of Plasmid DNA from Primary Colonies and Isolates of A-2 (A) and A-5 (B).

The bright diffuse bands at the bottom of the gels represent residual RNA. Electrophoresis in experiment (B) was conducted at 100 V for 4 hr.

tococci, it is important to elucidate whether the plasmids in a primary colony are stably maintained among the progeny. Examples of the plasmid pattern on agarose gel electrophoresis are shown in Fig. 2. It is evident that all plasmids in strain A-2, which contained all of the plasmids of both parents, are maintained among the progeny after the three single-colony isolation procedure. Similar results were obtained for strains A-1, A-3 and A-4 (data not shown). On the other hand, as to strain A-5, all of the isolates lost the 7.7 Mdal and 5.0 Mdal plasmids (Fig. 2B). This revealed that in some cases plasmid loss occurred among the progeny. However, it is likely that this loss occurred nonselectively and at random, because the 7.7 Mdal plasmid was derived from *S. cremoris* H-61 and the 5.0 Mdal plasmid from *S. lactis* J-1.

It is well known that the ability to ferment lactose is unstable and associated with plasmid DNA in many strains of group N streptococci.¹⁵⁻¹⁷ Considering the strain improvement of these organisms, it is important to pay attention to the activity and stability of lactose metabolism of the genetic recombinants obtained. The lactose-fermenting ability of *S. lactis* J, which was the Lac⁺ parent of *S. lactis* J-1, was unstable. 12.3% of the population consisted of Lac⁻ variants after 16 hr growth

TABLE III. STABILITY OF LACTOSE METABOLISM IN THE PARENTS AND THEIR FUSANTS

Strains	Frequency of Lac ⁻ (%)		
	Spontaneous	Curing conditions	
		Acridine orange*	Elevated temperature**
H-61	0	0	0
A-1	7.6	63.9	14.6
A-2	0	2.8	2.4
A-3	3.3	2.2	5.3
A-4	11.9	48.8	10.2
A-5	0	4.5	4.7
J	12.3	92.6	98.2

* The cells were incubated in the presence of 10 µg of acridine orange per ml.

** The cells were incubated at 38°C.

in lactic broth. Moreover, when *S. lactis* J was incubated at 38°C or in the presence of acridine orange (10 µg/ml), Lac⁻ variants appeared at the frequency of 98.2% and 92.6%, respectively. On the contrary, lactose metabolism of *S. cremoris* was very stable. Curing experiments, using acridine orange and at elevated temperature, did not yield Lac⁻ variants. The stability of the Lac⁺ property in five typical Lac⁺ interspecific fusants is shown in Table III.

DISCUSSION

Protoplast fusion should play an important role in strain improvement of industrially important organisms, for which the use of the general method of a genetic transfer system is limited. Intraspecific fusion of *S. lactis* has already been reported by several authors.^{6,7} This paper describes the formation of interspecific recombinants between *S. cremoris* and *S. lactis* by PEG-induced fusion of their protoplasts.

S. cremoris and *S. lactis* could be physiologically differentiated from a taxonomical point of view, i.e. maltose fermentation, growth in the presence of 4% sodium chloride and so on. It has been reported that *S. lactis* subsp. *diacetylactis* may exhibit variability with re-

spect to arginine hydrolysis, and that this trait may be an unstable property in some strains.²⁰⁾ In *S. lactis* J-1, the arginine-hydrolyzing ability was not encoded on plasmid DNA, because a plasmid free derivative of *S. lactis* J-1 produced ammonia from arginine (data not shown). So physiological traits used as genetic markers may be encoded on chromosomal DNA. Due to the lack of chromosomal maps in lactic streptococci, the number of crossovers required for recombinants, which exhibit specified phenotypes, is not clear. However, as shown in Table I, none of the clones obtained showed only one of parental phenotypes, all showed mixed physiological phenotypes of both parents. This showed that the five clones obtained in these experiments were interspecific recombinants between *S. cremoris* H-61 and *S. lactis* J-1, and that wide range genetic exchange occurred on chromosomal DNA. Furthermore, the results of plasmid analysis by agarose gel electrophoresis showed that strains A-2 and A-4 possessed all of the plasmids of both parents, and similarly A-1, A-3 and A-5 possessed not all but several plasmids derived from both parents; *S. cremoris* H-61 and *S. lactis* J-1 harbor 5 and 2 distinct plasmid species, respectively (Fig. 1). These results also supported the conclusion that the five clones obtained were interspecific fusants.

Lactic streptococci possess a diversity of plasmid molecules and the functions of most of these plasmids are still unknown. However, some genetic evidence has been obtained for specified plasmid linkage to important metabolic traits, such as lactose fermentation^{15~17)} and citrate utilization.¹⁸⁾ Considering that plasmids of both parents simultaneously existed in fusants and that wide range genetic recombination occurred on chromosomal DNA, protoplast fusion should play an important role in strain improvement.

Protoplast fusion was expected to produce di- or multi-nucleated products in a primary state. It was reported that with *Streptomyces antibioticus* marked segregation to the original genotypes was observed in many of the prototrophic colonies derived on fusion.²¹⁾ In

contrast, Schaeffer *et al.*²²⁾ showed that in *Bacillus subtilis* segregation and recombination occurred at an early step, and all of the colonies which appeared on regeneration medium were haploid recombinants. As for streptococcal fusants obtained in these experiments, the genetic stability as to physiological characteristics after the single-colony isolation procedure is especially noteworthy, suggesting the formation of true recombinants. Besides these chromosomal events, four of the five fusants stably maintained the plasmid profiles of the primary colonies even after single-colony isolation. On the other hand, strain A-5 lost the 7.7 Mdal and 5.0 Mdal plasmids. Since the former is derived from *S. cremoris* H-61 and the latter from *S. lactis* J-1, it is not likely that plasmids of either one of the parents only disappeared. However, considering that in lactic streptococci industrially important functions are encoded on plasmid DNA, the phenomenon of plasmid loss may be an obstacle for strain improvement.

Larsen and McKay²³⁾ reported that lactose metabolism of *S. cremoris* was generally a stable property, and in *S. cremoris* AM2 few Lac⁻ variants could be found even when the cells were treated in a variety of ways known to cause a high frequency of Lac⁻ variants in *S. lactis*. Similar results were obtained in this study. *S. cremoris* H-61 was treated by using common curing conditions but no Lac⁻ mutants were obtained. On the contrary, the proportion of Lac⁻ mutants in *S. lactis* J was extremely increased by the curing treatment (Table III). From the results of overall physiological characteristics, fusants could be classified into two parental types, that is, A-2 and A-5 were the *S. cremoris* type, and A-1, A-3 and A-4 the *S. lactis* type (Table I). In agreement with these results, the lactose-fermenting ability of strains A-1 and A-4 was as unstable as that of *S. lactis* J-1, especially Lac⁻ mutants increased in the population on treatment with acridine orange (Table III). Although strain A-3 seemed to be the *S. lactis* type, its lactose metabolism showed more stable characteristics than that of *S. lactis* J even under the curing

conditions. It is likely that the lactose-fermenting ability of this fusant was stabilized by the influence of the character of *S. cremoris* H-61.

It has been well documented that lactose metabolism in *S. lactis* and *S. lactis* subsp. *diacetylactis* is mediated by plasmid DNA.¹⁵⁻¹⁷⁾ In contrast, not enough genetic evidence has been obtained to elucidate the stability of lactose metabolism in *S. cremoris*.²³⁾ Fujita *et al.*¹⁹⁾ recently isolated Lac⁻ mutants of *S. cremoris* H-61, which simultaneously lost the 61 and 31 Mdal plasmids. This suggested that the lactose metabolism in *S. cremoris* H-61 was similarly encoded on plasmid DNA as in the case of *S. lactis*,^{16,17)} whereas its stability was distinctly different from in *S. lactis*. It is necessary to elucidate the mechanisms of stable inheritance of plasmids for strain improvement in lactic streptococci.

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