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Research Article

Conjugal Transfer and Stability of the Plasmids Determining Exopolysaccharide Production in *Lactococcus lactis* Strains

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Abstract: A 27.2 kb plasmid in *Lactococcus lactis* subsp. *lactis* ELL23, a 32.7 kb plasmid in *L. lactis* subsp. cremoris ELC5 and a 31.1 kb plasmid in *L. lactis* subsp. *cremoris* ELC19 were found to determine exopolysaccharide production (Eps^+) and lactose fermentation (Lac^+) abilities at the end of conjugal transfer studies. Conjugal transfer frequencies (per donor cell) and stability rates of Lac^+/Eps^+ plasmids were determined as $1.0 \times 10^{-2} - 2.8 \times 10^{-3}$ and 80-96%, respectively, after conjugal matings between the aggregative (Agg^+) wild type strains and Agg^+ recipient strain *L. lactis* subsp. *lactis* LLT40. On the other hand, conjugal transfer frequencies and stability rates of Lac^+/Eps^+ plasmids occurred as $1.5 \times 10^{-3} - 4.5 \times 10^{-4}$ and 65-80%, respectively, after conjugal matings between the same donor strains and non-aggregative (Agg^-) recipient strain *L. lactis* subsp. *lactis* ELL42-6.

Key Words: Exopolysaccharide, conjugation, plasmid, *Lactococcus lactis*

Lactococcus lactis Suşlarında Ekzopolisakkarit Üretimini Determine Eden Plazmidlerin Konjugal Aktarımı ve Stabilitesi

Özet: Konjugal aktarım çalışmaları sonucu, *Lactococcus lactis* subsp. *lactis* ELL23 suşunda 27.2 kb, *L. lactis* subsp. *cremoris* ELC5 suşunda 32.7 kb ve *L. lactis* subsp. *cremoris* ELC19 suşunda 31.1 kb büyüklükte plazmidlerin ekzopolisakkarit üretimi (Eps^+) ve laktoz fermentasyonu (Lac^+) özelliğinden sorumlu olduğu belirlendi. Agregasyon morfolojisine sahip (Agg^+) doğal tip verici suşlar ile, Agg^+ alıcı suş *L. lactis* subsp. *lactis* LLT40 kullanılarak gerçekleştirilen eşleştirmelerden sonra; Lac^+/Eps^+ plazmidlerin konjugal aktarım sıklığı, verici hücre başına, $1.0 \times 10^{-2} - 2.8 \times 10^{-3}$, konjugantlardaki stabilite oranları ise % 80-96 arasında saptandı. Diğer yandan, aynı verici suşlar ile agregasyon fenotipi içermeyen (Agg^-) alıcı suş *L. lactis* subsp. *lactis* ELL42-6 arasındaki eşleşmelerde; Lac^+/Eps^+ plazmidlerin konjugal aktarım sıklığı $1.5 \times 10^{-3} - 4.5 \times 10^{-4}$, stabiliteleri ise % 65-88 oranlarında gerçekleşti.

Anahtar Sözcükler: Ekzopolisakkarit, konjugasyon, plazmid, *Lactococcus lactis*

Introduction

Lactococci (*Lactococcus lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* biovar *diacetylactis*) play a major role in the production of fermented milk, sour cream and cheese (1). The type and character of the starter organism are the 2 most important factors determining the overall quality of the final product. The essential criteria for starter strain selection include acidification, aroma, flavor, stability and texture (2,3).

Many *L. lactis* strains used for manufacturing fermented milk products can produce exopolysaccharides, which are excreted into the milk during fermentation, prevent syneresis and ensure a proper texture and body of the end product (3-5). Although exopolysaccharide producing *L. lactis* strains could be used to produce smooth dairy foods with high viscosity without any need for thickeners or stabilizers, those strains have not been used extensively in commercial production because the phenotype is easily lost (6). Various reports describe the involvement of specific plasmids (>20 kb) in exopolysaccharide production in *L. lactis* strains (6-9). Currently, great effort is put into the identification and stabilization of exopolysaccharide production in *L. lactis* strains to achieve industrial starter cultures (10).

In this study, we identified 27.2, 31.1 and 32.7 kb plasmids that encode exopolysaccharide production (Eps^+)

and lactose fermentation (Lac^+) in *L. lactis* subsp. *lactis* ELL23, *L. lactis* subsp. *cremoris* ELC19 and *L. lactis* subsp. *cremoris* ELC5, respectively. The high frequencies of conjugal transfers and stabilities of Lac^+/Eps^+ plasmids in *L. lactis* were also examined.

Materials and Methods

Microorganisms

Bacterial strains used in this study are listed in Table 1. Lactose positive (Lac^+) strains were propagated in M17 broth (11) with 0.5% lactose at 30 °C. Lactose negative (Lac^-) strains were propagated at 30 °C in M17 broth with 0.5% glucose substituted for lactose.

Bacterial stocks were prepared as previously described (11) and stored in 20% glycerol at -40 °C.

Genetic Manipulations and Conjugation

A streptomycin resistant (Str^r, 150 µg/ml) and kanamycin resistant (Km^r, 50 µg/ml) derivative of strain *L. lactis* subsp. *lactis* ELL42-6 was isolated by successive selection for spontaneous mutation for resistance, first to Str, then to Km (12).

For conjugation experiments, filter matings were conducted as described by McKay et al. (13). Donor and recipient cell solutions (1.5 ml each) were mixed and collected on a sterile membrane filter (pore size 0.45 µm; Sartorious, Germany). Filters were transferred to M17 agar plates and incubated at 30 °C for 24 h. After incubation, filters were removed to sterile flasks and rinsed with 1.0 ml of 0.85% NaCl to obtain a cell suspension. Cells were plated on lactose indicator agar supplemented with Str (150 µg/ml) and Km (50 µg/ml) to select for Lac^+, Str^r and Km^r recombinants, and were tested for exopolysaccharide production as described by Dierksen et al. (3).

Plasmid Analysis

The lysis procedure of Anderson and McKay (14) was used to isolate plasmid DNA from lactococcal strains. Purification of plasmids in cesium chloride-ethidium bromide density gradients and analysis on agarose gels were performed as described previously (15). Plasmid sizes were estimated on agarose gels by comparing their relative mobility to commercial cccDNA markers (supercoiled DNA ladder, Lot48H1462; Sigma Chem. Co., USA).

Plasmid Stability Test

The stability of the plasmids in conjugants was investigated by growing the culture for 75 generations

Table 1. Bacterial strains used in this study.

Strain	Phenotype	Comments/Source
L. lactis subsp. *lactis*		
ELL23	Lac^+, Eps^+,Agg^+,Str^s, Km^s	Donor, this study
ELL42-6	Lac^-, Eps^-, Agg^-, Str^r, Km^r	Recipient, this study
LLT40	Lac^-, Eps^-, Agg^+, Str^r, Km^r	Recipient, P. Şanlıbaba*
L. lactis subsp. *cremoris*		
ELC5	Lac^+, Eps^+, Agg^+, Str^s, Km^s	Donor, this study
ELC19	Lac^+, Eps^+, Agg^+, Str^s, Km^s	Donor, this study
Conjugants		
ELL23XELL42-6	Lac^+, Eps^+, Agg^+, Str^r, Km^r	This study
ELL23XLLT40	Lac^+, Eps^+, Agg^+, Str^r, Km^r	This study
ELC5XELL42-6	Lac^+, Eps^+, Agg^+, Str^r, Km^r	This study
ELC5XLLT40	Lac^+, Eps^+, Agg^+, Str^r, Km^r	This study
ELC19XELL42-6	Lac^+, Eps^+, Agg^+, Str^r, Km^r	This study
ELC19XLLT40	Lac^+, Eps^+, Agg^+, Str^r, Km^r	This study

Lac^+/Lac^-: ferments/does not ferment lactose; Str^r/Str^s: resistant/sensitive to streptomycin (150 µg/ml); Km^r/Km^s: resistant/sensitive to kanamycin (50 µg/ml); Eps^+/Eps^-: produces/does not produce exopolysaccharide; Agg^+/Agg^-: aggregation/non-aggregation morphology; * : Department of Food Engineering, Faculty of Agriculture, University of Ankara.

(transfer of overnight cultures 8 times) in a 10% reconstitute skim milk at 30 °C, after which individual colonies were tested for lactose fermentation ability and exopolysaccharide production (16).

Results

To determine whether the involvement of the exopolysaccharide production in *L. lactis* strains is related to self-transmissible plasmids, conjugal mating experiments were performed between exopolysaccharide producing (Eps^+) donor stains and exopolysaccharide non-producing (Eps^-) recipient strains. Conjugants were selected as Lac^+, Str^r and Km^r on lactose indicator agar plates and were tested for exopolysaccharide production. Plasmid profiles showed that all Eps^+, Lac^+, Str^r and Km^r conjugant strains contained only one plasmid with different molecular sizes (Figures 1, 2 and 3). These results indicated that the exopolysaccharide production and lactose fermentation ability are associated with the 27.2 kb, 31.1 kb and 32.7 kb plasmids in *L. lactis* subsp. *lactis* ELL23, *L. lactis* subsp. *cremoris* ELC19 and *L. lactis*

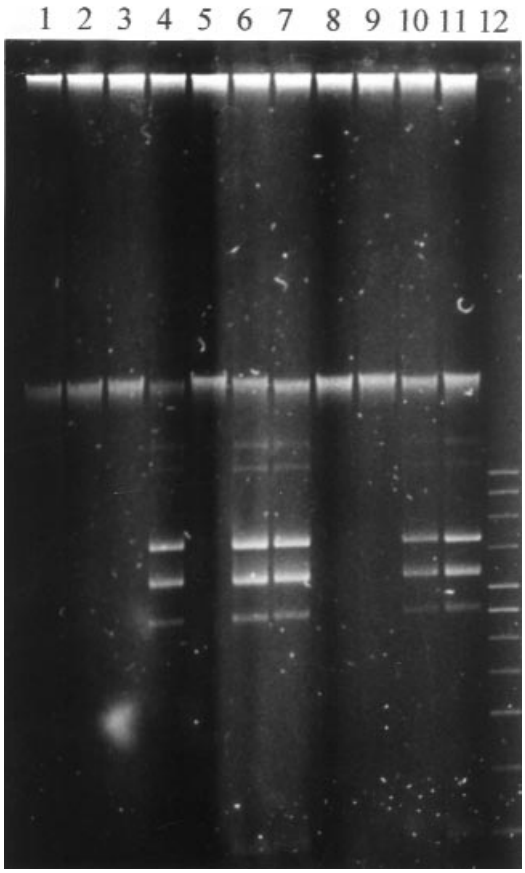

Figure 1. Plasmid profiles of the donor strain *L. lactis* subsp. *lactis* ELL23 and the conjugants obtained from ELL23XELL42-6 and ELL23XLLT40 matings.
Lanes 1, 2 and 3 (Conjugants from ELL23XELL42-6 matings, Lac^+/Eps^+): 27.2 kb; lanes 4, 6, 7, 10 and 11 (ELL23, wild type strain, Lac^+/Eps^+); 27.2, 19.3, 17.0, 10.9, 8.9 and 7.4 kb; lanes 5, 8 and 9 (conjugants from ELL23XLLT40 matings, Lac^+/Eps^+): 27.2 kb; lane 12 (ccc plasmid DNA marker): 16.2, 14.1, 12.2, 10.2, 8.0, 7.2, 6.0, 5.0, 4.0, 2.9 and 2.1 kb.

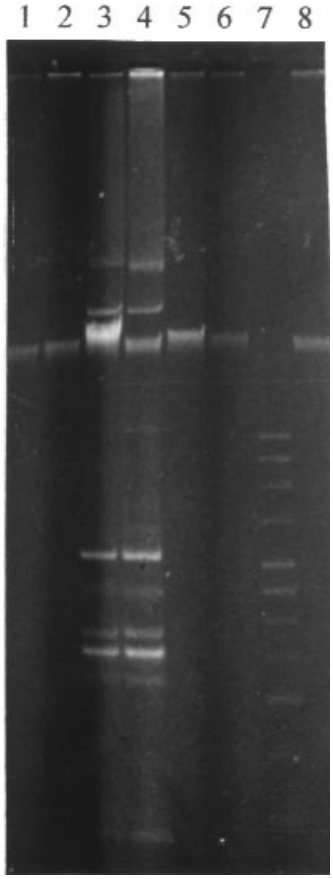

Figure 2. Plasmid profiles of the donor strain *L. lactis* subsp. *cremoris* ELC19 and the conjugants obtained from ELC19XELL42-6 and ELC19XLLT40 matings.
Lanes 1 and 2 (conjugants from ELC19XELL42-6, Lac^+/Eps^+): 31.1 kb; lanes 3 and 4 (ELC19, wild type strain, Lac^+/Eps^+): 47.6, 39.2, 31.1, 17.1, 9.8, 8.6, 7.1, 5.4, 5.2, 4.5 and 2.0 kb; lanes 5, 6 and 8 (conjugants from ELC19XLLT40, Lac^+/Eps^+): 31.1 kb; lane 7 (ccc plasmid DNA marker): 16.2, 14.1, 12.2, 10.2, 8.0, 7.2, 6.0, 5.0, 4.0, 2.9 and 2.1 kb.

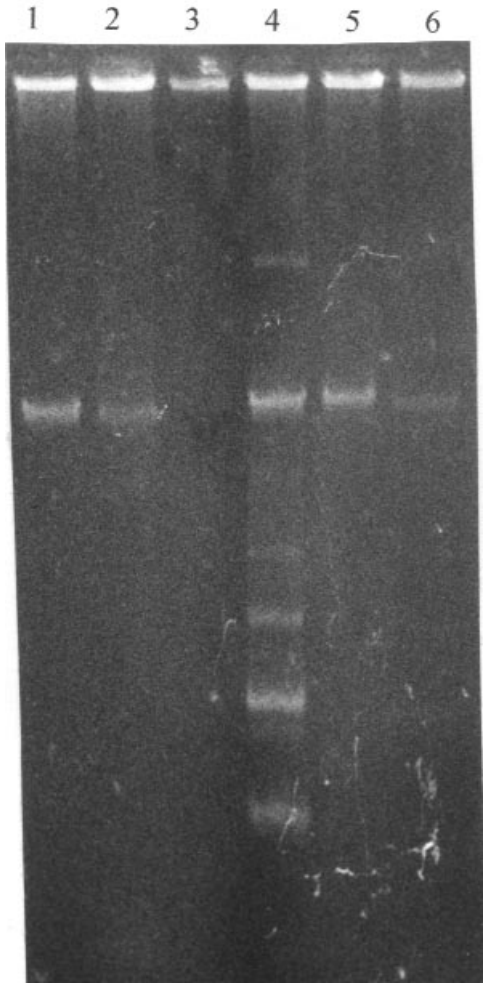

Figure 3. Plasmid profiles of *L. lactis* subsp. *cremoris* ELC5, *L. lactis* subsp. *lactis* ELL42-6 and, conjugants obtained from ELC5XELL42-6 and ELC5XLLT40 matings.
Lanes 1 and 2 (conjugants from ELC5XELL42-6 matings, Lac^+/Eps^+): 32.7 kb; lane 3 (ELL42-6, Lac^-/Eps^-): plasmid free mutant strain; lanes 4 (ELC5, wild type strain, Lac^+/Eps^+): 44.3, 32.7, 19.6, 10.4, 9.1 and 4.0 kb; lanes 5 and 6 (conjugants from ELC5XLLT40 matings, Lac^+/Eps^+): 32.7 kb.

subsp. *lactis* ELC5, respectively.

In this study, aggregative (Agg^+) wild type donor strains (*L. lactis* subsp. *lactis* ELL23, *L. lactis* subsp. *cremoris* ELC19 and *L. lactis* subsp. ELC5) and Agg^+ (*L. lactis* subsp. *lactis* LLT40) and non-aggregative (Agg^-, *L. lactis* subsp. *cremoris* ELL42-6) recipient strains were used to investigate the nature of the process by which conjugal transfer of lactose/exopolysaccharide plasmids occurred. Conjugal transfer frequencies for 27.2 kb, 31.1 kb and 32.7 kb plasmids, obtained from Agg^+ donor and Agg^- recipient matings, were determined at the rates of 4.5×10^{-4}, 2.8×10^{-4} and 1.5×10^{-3}, respectively. On the other hand, conjugal transfer frequencies were increased to 2.0×10^{-2} for 27.2 kb plasmid, 2.5×10^{-2} for 31.1 kb plasmid and 1.0×10^{-2} for 32.7 kb plasmid when Agg^+ recipient strain was used as donor with the Agg^+ wild type donor strains (Table 2).

Stabilities of lactose/exopolysaccharide plasmids in the wild type strains and the conjugants were investigated by growing these strains in 10% reconstitute skim milk at 30 °C for 75 generations. After this growing period, 100 colonies for each strain were tested for lactose fermentation ability/exopolysaccharide production and plasmid contents. Stability tests showed that there were no significant differences in the stability rates of lactose/exopolysaccharide plasmids of Agg^+ wild type donor strains and their conjugants obtained by using Agg^- recipient strain *L. lactis* subsp. *lactis* ELL42-6. In contrast stability rates increased to 96% for 27.2 kb plasmid, 90% for 31.1 kb plasmid and 94% for 32.7 kb plasmid in conjugant strains obtained from Agg^+ wild type donors and Agg^+ recipient (*L. lactis* subsp. *lactis* LLT40) matings, while the stability rates were 72% for 27.2 kb plasmid, 70% for 31.1 kb plasmid and 75% for 32.7 kb plasmid in Agg^+ wild type strains *L. lactis* subsp. *lactis* ELL23, *L. lactis* subsp. *cremoris* ELC19 and *L. lactis* subsp. *cremoris* ELC5, respectively (Table 3).

Discussion

The ability to transfer genetic material via conjugation is widespread among lactococci. Conjugative transfer of various industrially important traits includes carbohydrate utilization, proteinase activity, bacteriophage resistance, and bacteriocin and exopolysaccharide production (17-20). Conjugation offers a powerful genetic approach for identifying and developing plasmid-linked characters in lactococcal starter strains (21). Due to the importance of plasmids in dairy fermentation processes, attempts to elucidate the functional properties of cryptic plasmids in food-fermenting microorganisms need to continue. The present study aimed to meet this particular need.

Our results and the data presented in the literature (22-24) showed similarities with the exopolysaccharide specific *eps* genes encoded on conjugally transferable lactose plasmids in *L. lactis* strains. Studies on a variety of conjugative lactose plasmids in lactococci have resulted in

Table 2. Conjugal transfer of lactose fermentation/exopolysaccharide production abilities in *L. lactis* strains.

Donor Strain	Recipient Strain	Conjugant Phenotype/ Plasmid Content (kb)	Transfer Frequency (Per Donor Cell)
L. lactis subsp. *lactis*	*L. lactis* subsp.*lactis*		
ELL23	ELL42-6	Lac^+, Eps^+, Agg^+, Str^r, Km^r/27.2	4.5×10^{-4}
ELL23	LLT40	Lac^+, Eps^+, Agg^+, Str^r, Km^r/27.2	2.0×10^{-2}
L. lactis subsp. *cremoris*			
ELC5	ELL42-6	Lac^+, Eps^+, Agg^+, Str^r, Km^r/32.7	1.5×10^{-3}
ELC5	LLT40	Lac^+, Eps^+, Agg^+, Str^r, Km^r/32.7	1.0×10^{-2}
ELC19	ELL42-6	Lac^+, Eps^+, Agg^+, Str^r, Km^r/31.1	2.8×10^{-3}
ELC19	LLT40	Lac^+, Eps^+, Agg^+, Str^r, Km^r/31.1	2.5×10^{-2}

Lac^+/Lac^-: ferments/does not ferment lactose; Str^r/Str^s: resistant/sensitive to streptomycin (150 µg/ml); Km^r/Km^s: resistant/sensitive to kanamycin (50 µg/ml); Eps^+/Eps^-: produces/does not produce exopolysaccharide; Agg^+/Agg^-: aggregation/non-aggregation morphology; kb: kilobase.

Table 3. Stabilities of lactose/exopolysaccharide specific plasmids in wild type *L. lactis* strains and their conjugants.

Strain	Lac^+/Eps^+ Plasmids (kb)	Stability (%)
L. lactis subsp. *lactis*		
ELL23	27.2	72
L. lactis subsp. *cremoris*		
ELC5	32.7	80
ELC19	31.1	65
Conjugants		
ELL23XELL42-6	27.2	70
ELL23XLLT40	27.2	96
ELC5XELL42-6	32.7	80
ELC5XLLT40	32.7	94
ELC19XELL42-6	31.1	66
ELC19XLL40	31.1	90

Lac^+: ferments lactose; Eps^+: produces exopolysaccharide; kb: kilobase.

a model in which the conjugation is considered as a 2-part process. The first component involves bringing the donor and recipient cells together to form the mating pair, and the second step involves enzymatic transfer of a single strand of conjugative plasmid into the recipient cell (20,25-27). Although the frequencies of lactose plasmids transfer were in general low, high frequency conjugal transfer of lactose genes was achieved by donor strains that exhibited aggregation morphology (28,29). The results of this study indicate that high frequency conjugal transfer of lactose/exopolysaccharide specific plasmids was associated with aggregation morphology, not only of donor strains, but also of recipient strains. An important requirement for the application of recombinant DNA technologies concerns the development of efficient and natural DNA transfer systems. From this point of view, our data are significant in starter strain improvement studies.

The success of commercial production of plasmid-encoded gene products and/or utilization of such gene products through live bacterial cultures in fermented foods mainly depends on the stability of plasmids (30). Thus, Agg^+ recipient strain *L. lactis* subsp. *lactis* LLT40, which regulated lactose/exopolysaccharide specific plasmids by high copy number, seems to be important for genetic manipulation in lactococci.

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