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OSMAN ÇATALOLUK

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The Development of a Modified Method for Isolating Plasmids from Exopolysaccharide Producing Lactobacillus Species Using Conventional Plasmid Isolation Methods

Osman ÇATALOLUK
Department of Medical Biology & Genetics, Faculty of Medicine, Gaziantep University, 27310, Kampus, Gaziantep – TURKEY
E-mail: ocatal@gantep.edu.tr

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Abstract: Lactic acid bacteria (LAB) are a key element in the dairy industry. The gene products encoded by the plasmids of LAB carry out fermentation. To date, many successful plasmid isolation studies have been conducted with *Escherichia coli, Staphilococcus, Streptococcus, Salmonella, Listeria* and many strains of LAB. However, since LAB comprise a conglomerate of seven different genera, each with its own physiological and morphological characteristics, and produce a large amount of exopolysaccharide coat, it is extremely difficult to obtain plasmids by conventional methods. A field isolate of lactobacillus known as *Lactobacillus salivarius* M7 was utilized to obtain plasmids by all known techniques. A modified technique was optimized from the methodologies of Klaenhammer (1983) and Burger et al. (1994). The plasmids obtained with this technique were easily separated into a cleaner and clearer upper phase. There was no need to perform cesium chloride centrifugation, and RNA remnants were totally eliminated. Plasmids obtained with this technique can safely be used in further molecular biological techniques, including sequencing and cloning.

Key Words: Plasmid, Plasmid isolation, *Lactobacillus salivarius* M7

Geleneksel Plasmid İsolasyon Metodları Kullanılarak Exopolisakkarid Üreten Laktobasillus Bakterilerinden Plasmid Elde Edilmesi ile İlgili Yöntem Geliştirilmesi

Özet: Laktik asit bakterileri (LAB) süt ve süt ürünleri endüstrisinin vazgeçilmez elemanıdır. Mayalanma bu bakterilerin sahip oldukları plasmidlerin gen ürünleri tarafından sağlanır. *E.coli, Staphilococcus, Streptococcus, Salmonella, Listeria* ve bir çok laktik asit bakteri türünden başarılı plasmid isolasyon çalışmaları yapılmıştır. Fakat LAB farklı yedi generadan oluşan bir grup olduğundan, her bir tür, alt tür ve bireyin kendine has özellikleri vardır. özellikle laktobasil gurubu bakterilerin yoğun egzopolisakkarid üretmelerinden dolayı plasmid elde etmek oldukça zordur. Bu amaçla sahadan izole edilen bir laktobasil türü olan *Lactobacillus salivarius* M7'den plazmid izolasyonu yapılması için şu ana kadar plasmid izolasyonunda kullanılan bütün teknikler denenmiştir. Klaenhammer (1983) ile Burger ve ark. (1994)'nın teknikleri modifiye edilerek ortak bir yöntem geliştirilmiştir. Bu teknikle elde edilen plazmidlerin üst fazda daha kolay ayrıldıkları ve sezyum kloridle yapılan ultra santrifüje gerek kalmadığı ve RNA kalıntılarından arındığı anlaşılmıştır. Bu teknikle elde edilen plasmidlerin dizi analizleri ve klonlama gibi daha ileri moleküler genetik çalışmalarda da rahatlıkla kullanılabildiği görülmüştür.

Anahtar Sözcükler: Plazmid, Plazmid isolasyonu, *Lactobacillus salivarius* M7

Introduction

The plasmids of both Gram-negative and Gram-positive bacteria are involved in metabolic activity, membrane protein production, the establishment of progressive infection and resistance. Many successful attempts have been made to isolate and sequence the genes of plasmids from many different species, including *Escherichia coli*, *Staphylococcus, Streptococcus, Salmonella, Listeria* and lactic acid bacteria (LAB) spp. The first plasmid isolation was carried out from LAB (1). Extra chromosomal elements were detected in group-N streptococci by dye-buoyant density centrifugation. Laters SDS was utilized together with lysosyme for spheroplast formation (2). According to this methodology, the incubation of cells in the presence of DL-threonine, followed by the incubation of the cells in the presence of lysozyme for approximately 30 min, would be enough for a complete lysis. However, it was shown that incubating the cells for more than 20 min in the presence of lysozyme would lead to the loss of plasmids of 30 M-dal and larger (3). Plasmids larger than 30 M-dal from were isolated from *Escherichia, Salmonella, Erwinia* and

Pseudomonas simply by increasing the temperature and the pH of the lysis solution (4). The same method was utilized for lactic streptococci (5). Soon, a general method for plasmid isolation from lactobacilli was developed (6). However, since a new enzyme, mutanolysin, was utilized together with lysozyme, plasmids larger than 70 M-dal could not be obtained. Finally a new method for plasmid isolation from lactobacilli was introduced (7). In this study we examined all these methodologies in order to optimize a means of isolating the plasmids that *Lactobacilus salivarius* M7 harbors.

Materials and Methods

This study was carried out with the strains of *L. salivarius* M7 and *L. plantarum* (data not included) obtained from human and mouse dental plaque at the Department of Medical Biology and Genetics, Gaziantep University, Turkey and at the Department of Microbiology, TNO Institute, The Netherlands. Cultures were subculturred at bi-weekly intervals in de Man Rogosa Sharpe (MRS) broth and stored at 4 ºC. Strain designation was as previously described (8).

One liter of MRS broth was prepared, inoculated with freshly prepared 1% (v/v) *L. salivarius* M7 culture, and grown at 37 ºC to log phase (8-12 h) at OD_{600}= 0.6. Cells were harvested by centrifugation (3000 x g, 5 min, 4 ºC). The cells were washed in 100 ml STE buffer (0.1 M NaCl, 10 mM Tris HCl, 1 mM EDTA, pH 6.0) twice and resuspended in 120 ml of 20% w/v sucrose in 50 mM Tris HCl - 1mM EDTA, pH 8.0. This solution was divided equally into 12 tubes. The tubes were then separated into four groups. The pH levels of the groups were adjusted to 5.3, 6.0, 6.5 and 7.0, respectively. To the first tubes of all four groups were added 10 mg/ml of lysozyme and 40 U of mutanolysin. To the second tubes of the groups were added 6 mg/ml of lysozyme and 60 U of mutanolysin. To the third tubes were added 4 mg/ml of lysozyme and 100 U mutanolysin. To the last tubes of the four groups were added 1.5 mg/ml of lysozyme and 150 U of mutanolysin. The first tubes of all groups were incubated at 0 ºC, the second tubes were incubated at 25 ºC and the last tubes were incubated at 37 ºC. The cells in each of the four groups were incubated for 7 to 15 min. After incubation, 1000 ml of phenol:chloroform was added to each tube and mixed for 5 min on a rotary shaker. The tubes were placed on ice for 5 min and then centrifuged at 13,000 x g for 5 min. The upper phase was collected in a fresh Eppendorf tube and treated with absolute alcohol and 3M NaAc. The plasmids were precipitated by centrifugation at 14,000 x g for 15 min, washed with 70% alcohol and dried in a vacuum chamber at 1000 Bar for 5 min. The plasmids were resuspended in 50 ml of TE buffer and stored for further use. A sample of 5 ml was withdrawn from each tube and electrophoresed on 0.7% agarose gel (Sigma) in 1XTAE buffer with EtBr (1 mg/ml) for 1 h at 100 V. The plasmids were visualized on UV.

Results

The methods of Chassy (1976) (Figure 1), Klaenhammer (1978) (Figure 2), Sullivan (1993) (Figure 3) and Burger (1994) were used to obtain plasmids from exopolysaccharide, producing *L. salivarius* M7. However, none of these methods produced consistent results. Therefore, these methodologies were employed to derive and modify a new method. The results of the experiments carried out with the four groups of samples were as follows; with group 1 (0 ºC and pH 6.5, in 10 mg/ml of lysozyme and 40 U of mutanolysin), chromosomal DNA was easily removed with the cell debris, leaving a clear lysate. In groups 2 and 3, at the same temperature and pH, (6 mg/ml of lysozyme and 60 U of mutanolysin and 4 mg/ml of lysozyme and 100 U of mutanolysin, respectively), similar results were obtained. However, the

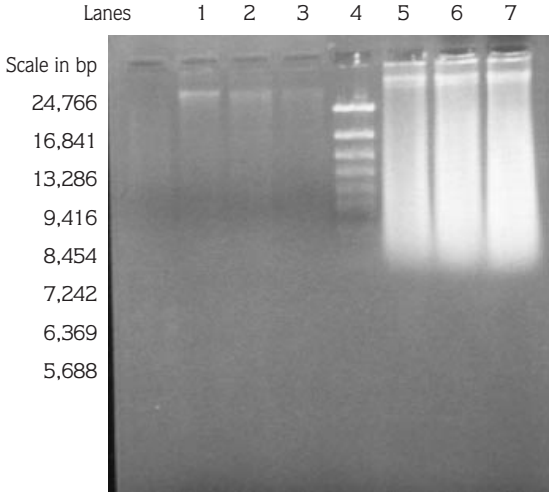

Figure 1. The result of plasmid isolation by the Chassy method (1976) at 37 ºC with an incubation time of 20 min Lanes: 1, 2 and 3, chromosomal DNA; 5, 6 and 7, plasmids; 4, marker DNA, *Escherichia coli* V517 (Reference 14).

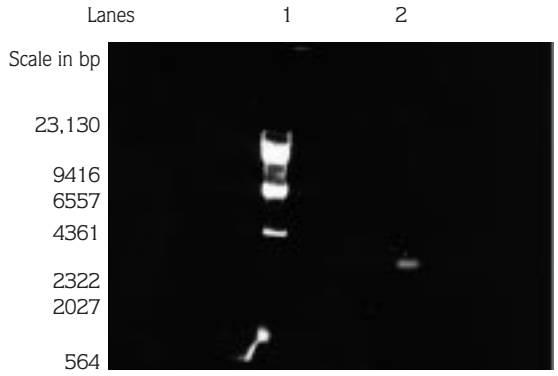

Figure 2. A plasmid obtained by Klaenhammer's methodology (1983) from *Lactobacillus salivarius* M7 at 0 ºC Lanes: 1, marker DNA cut by *Hin*d III; 2, a plasmid DNA of 2.7 kb.

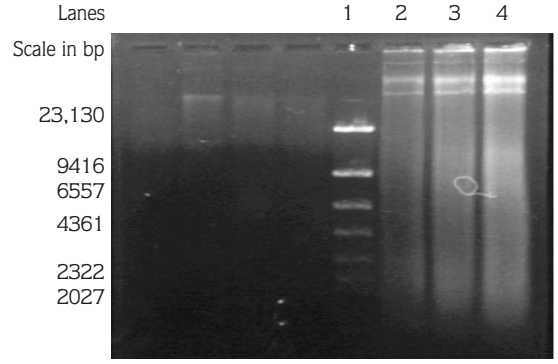

Figure 3. The plasmids obtained by the methodology of Sullivan (1993) from *Lactobacillus salivarius* M7. Lanes: 1, marker pEMBL-8; 2, 3 and 4, the plasmids.

amount of plasmid obtained with groups 2 and 3 was less than that obtained with group 1. With group 4, (0 ºC and pH 7.0, 1.5 mg/ml of lysozyme and 150 U of mutanolysin) a cloudy lysate was produced and no phase separation was observed. The plasmid yield was poor after phenol:chloroform extraction. With groups 3 and 1 (4 mg/ml of lysozyme, 100 U of mutanolysine and 10 mg/ml of lysozyme, 40 U mutanolysin, respectively), no efficient phase separation and plasmid yield were obtained. In group 1, (0 ºC, pH 8.0, 4mg/ml of lysozyme and 100 U of mutanolysin), even though there was still a cloudy lysate and no phase separation was observed, plasmid isolation was achieved after phenol:chloroform extraction. At 37 ºC, starting from pH 6.5, there was a gradual increase in the ability to separate phases, to obtain clear lysate and to increase the concentration of plasmids obtained. The clearest lysate was obtained at pH 8.0, yet phase separation was still incomplete and the interphase region was still cloudy, and during pipetting the interphase would start moving upwards. The yield was also less than that of group 1. At 25 ºC, with different pHs, the results obtained were very similar to those obtained at 0 ºC (Figure 4). However, the best result was obtained at 8.0 pH with 6 mg/ml of lysozyme and 60 U of mutanolysin. The plasmid yield was optimum with these parameters. The incubation time for all tubes was 7 min. In tubes with incomplete lysis the time was extended by 3-5 min. With the separate 12 tubes, the same parameters were tested with an extended incubation time of 30 min as control. Even though a very clear lysate was obtained with each tube, the author failed to obtain any plasmids. A sample of plasmid was purified with the cesium chloride ethidium bromide (CsCl-EB) method, although RNA remnants were still a problem with this technique, whereas the modified method did not leave such remnants (Figure 5).

Discussion

Since LAB have very particular nutritional requirements, they not only need amino acids, peptides, nucleic acid derivatives, vitamins, salt and fatty acid esters, but also fermentable carbohydrates. Evidence suggesting a link between lactose metabolism and plasmid DNA within LAB was based on the spontaneous loss of bacteria observed during growth (2). Incomplete

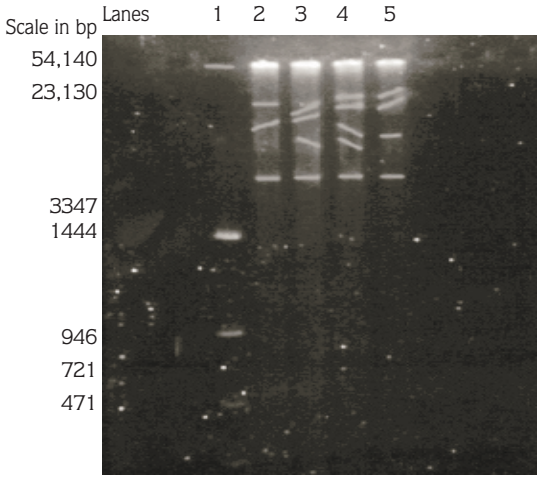

Figure 4. The plasmids obtained by the modified methods of Klaenhammer (1983) and Burger (1994) from *Lactobacillus salivarius* M7. Lanes: 1, marker DNA pEMBL-8; 2, 3, 4 and 5, plasmid DNAs after RNAse treatment.

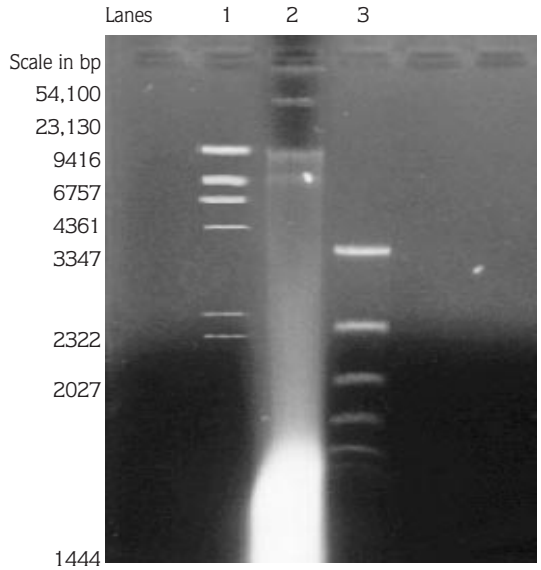

Figure 5. The result of the CsCl-Eb plasmid isolation method in *Lactobacillus salivarius* M7. Lanes: 1, marker, DNA cut with *Hin*d III; 2, plasmids of *Lactobacillus salivarius* M7 with the CsCl-EB method; 3, marker pEMBL-8.

fermentation or acidification during fermentation suggested that these plasmids may have a commercial value. However, the inability to isolate plasmid DNA molecules prevents further study of the nature of the plasmids of LAB, and adequate characterization of plasmid DNA in lactobacilli has been inhibited by inefficient and lengthy cellular lysis procedures. Lysis broth, containing DL-threonine, was found to be an effective culture medium for the growth of lactobacilli. Log-phase cells grown in lysis broth were efficiently lysed by SDS after both 7 min of digestion with 4 mg/ml of lysozyme and 100 U of mutanolysin at 37 ºC and 10 min of 6 mg/ml of lysozyme and 60 U of mutanolysin digestion at 0 ºC.

The lysozyme insensitivity of many bacteria recently investigated for plasmid DNA has necessitated longer periods of lysozyme digestion or the addition of the enzyme mutanolysin for spheroplast formation (2). However, increasing the lysozyme digestion period beyond 20 min at 37 ºC resulted in a loss of plasmid species. It was previously shown that long proteolytic or lysozyme treatment allows endogenous nucleases to become active (10). Thus, loss of plasmid may be due to the presence of intracellular nucleases, and not a direct result of lysozyme activity per se in an extended incubation period.

Conventional lysis and plasmid purification techniques have routinely been ineffective for lactobacilli. In a previous study, only one out of eight strains of lactobacilli contained plasmid DNA (11). The same authors were unable to detect plasmids in nine strains of *L. plantarum*. The difficulty in isolating plasmid DNA from lactobacilli may be associated with the lysozyme insensitivity of this genus (12), the length of time spent during the lysis process, the pH or the temperature of the environment in which lysis took place. All the previous methodologies except for the methods of Klaenhammer (1983) and Dick (1994) continued to employ a 37 ºC incubation for 1 h to facilitate cell wall digestion by lysozyme (3-7, 9, 10, 12, 13). It was shown that extended incubation at 37 ºC was unnecessary and, in fact, could be detrimental to plasmid isolation in some *lactobacillus* species. The employment of 0 ºC compensated for this effect and substantially reduced the time of incubation, to 7 min, by using an effective cell wall lytic enzyme, mutanolysin. The effects of three different temperatures, pHs and incubation times were also examined by this author. Extended incubation time at elevated temperatures was detrimental to plasmid yield. However, the best plasmid harvest was obtained at 25 ºC and pH 8.0. We observed no difference between the effects of lysozyme and mutanolysin. Moreover, the effect of lysozyme was better than that of mutanolysin. The amounts of mutanolysin greater than 60 U were the leading cause of plasmid loss, especially when plasmids larger than 50 kb were present. Thus the method described in this study was derived from the methodologies of Klaenhammer (1983) and Dick (1994). This method is suitable for use with either mutanolysin or lysozyme, or both. It is rapid, convenient and effective. Detergent lysis under alkaline conditions appeared very effective in the release of plasmid DNA from cells, but did not totally eliminate minor contaminating linear chromosomal DNA or nicked, open circular molecules. However, the plasmid patterns were comparable to CsCl-EB purified samples. In a previous study, this author employed plasmid DNA obtained with this modified technique in the sequencing and cloning of the gene encoding the salivaricin B activity of *L. salivarius* M7.

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