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A comparison of methods for isolating large plasmid DNA from lactococci

Porównanie metod izolacji dużych plazmidów z bakterii z rodzaju *Lactococcus*

SUMMARY

Lactococcus lactis harbor large plasmids encoding industrially important traits. For many molecular protocols it is vital to achieve large quantity of pure plasmid DNA. However, little information is available on how different extraction methods compare, especially regarding large extrachromosomal DNA. As a step towards understanding the process of isolation of plasmid DNA, plasmid extraction with six different methods was performed in this study accompanied by an analysis of the main extraction phases. Also the influence of the used reagents on yield and DNA purity was indicated. The aim of this study was to evaluate the methods for extraction of large plasmid DNA from lactococcal cells in terms of DNA quality and purity.

STRESZCZENIE

Bakterie mlekowe należące do gatunku *Lactococcus lactis* posiadają duże plazmidy kodujące wiele cech ważnych dla przemysłu spożywczego. Możliwość wydajnej izolacji pozachromosomalnych elementów genetycznych jest pierwszym, bardzo ważnym etapem dalszych manipulacji materiałem genetycznym bakterii mlekowych w technologii ekspresji genów. Obecnie brak w literaturze dostępnych informacji na temat wpływu różnych metod ekstrakcji na wydajność izolacji dużych, niskokopijnych plazmidów. W niniejszej pracy porównano sześć różnych protokołów ekstrakcji plazmidowego DNA, szczegółowo analizując poszczególne etapy izolacji wyjaśniono wpływ zastosowanych odczynników na jakość i ilość uzyskanego plazmidowego DNA. Celem przeprowadzonych

badań było wskazanie najbardziej efektywnej metodyki izolacji dużych, niskokopijnych plazmidów z bakterii mlekowych, która prowadziłaby do uzyskania wysokiej jakości DNA.

Key words: *Lactococcus lactis,* large plasmids, DNA isolation

INTRODUCTION

Lactococcus lactis is a fermentative lactic acid bacterium (LAB) used in food fermentation. LAB are used by the dairy industry not only as starter microorganisms to manufacture fermented products, but also for the preservation of food. For these reasons, LAB have attracted intensive research interest in recent years: in particular, much effort has been devoted to the genetic characterization of *L. lactis*, which resulted in cloning and characterization of many industrially relevant traits (Hill and Ross, 1998). *Lactococcus* spp. is known to harbor plasmids, with most strains carrying 4 to 7 different plasmids ranging in size from 3 to 80 kb (Dougherty et al., 1998). Many industrially significant traits are plasmid-encoded, including resistance to phages or antibiotics, lactose catabolism, and production of proteolytic enzymes, bacteriocins or exopolysaccharides (von Wright and Sibakov, 1998; Mäyrä-Mäkinen and Bigret, 1998). Many plasmids carrying industrially valuable traits have been recently described (Mills et al., 2006). Some of these are large extrachromosomal elements, e.g. the 60 kb plasmid pMRC01 found in *L. lactis* subsp. *lactis* DPC3147 encoding for conjugative functions, bacteriophage resistance, and lacticin production (Ryan et al., 1996). Van Kranenburg (2000) has sequenced an *eps* (exopolysaccharide) gene cluster located in *Lactococcus lactis* on large (42,180 kb) pNZ4000 encoding for exopolysaccharide biosynthesis plasmid. A food-grade host/ vector system has been constructed (Cotter et al., 2003), which can be effectively used for cloning and overexpression of industrially important traits, e.g., high phage resistance in the *L. lactis* strains (Boucher et al., 2001). This system provides valuable tools for the genetic enhancement of starter cultures.

Plasmids also provide a genetic tool for differentiation among lactococcal species and strains. The two of the commonly used methods are plasmid profiling and RAPD fingerprinting of plasmid DNA (de la Plaza et al., 2006; Mainville et al., 2006).

For the above reasons, isolation of pure and high quality plasmid DNA is an important element of many protocols and preparations. However, although there are reports of different methods of plasmid DNA extraction, there is only scarce literature comparing the isolation procedures especially with respect to large extrachromosomal elements.

The objective of the present work was to evaluate six different methods of extraction of large plasmid DNA from lactococcal cells in terms of DNA quality and purity.

MATERIAL AND METHODS

Bacterial strains and growth conditions

Two LAB strains were used in this study, *Lactococcus lactis* ssp. *cremoris* AC1 which was purchased from the DSMZ collection (Deutsche Sammlung von Mikroorganizm und Zellkulturen GmbH) and *L. lactis ssp. lactis* IBB 500 kindly provided by Prof. Bardowski, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw. Cultures were maintained in M-17 broth and transferred to a fresh medium at 24-h intervals.

The lactococcal strains were grown to the late log phase (8 h at 30°C) in 10 ml of M-17 broth and harvested by centrifugation or used as inoculum for the lysis broth (Elliker broth medium).

Large plasmid DNA extraction methods

Six different plasmid DNA extraction methods, hereafter referred to as methods 1–6 were evaluated using 10 ml of lactococcal cultures at the late log growth phase which corresponded to an OD_{600} value of 2.1 to 2.3. Precipitated plasmid DNA was resuspended in 20 µl TE buffer. The main steps of plasmid DNA isolation are described in Tab. 1.

Evaluation of plasmid DNA concentration and purity

UV Spectrophotometer

The quality of the plasmid DNA extracted by the different methods was assessed with the Smart Spec ™ Plus (Bio-Rad) spectrophotometer, using TE buffer as a reference. The ratio of absorbance at 260 nm and 280 nm was determined in order to assess the purity (protein contamination) and concentration of the plasmid DNA sample.

Agarose gel electrophoresis

5 µl of each sample was analyzed in 0.6% agarose gel containing ethidium bromide (0.5 µg/ml). Electrophoresis was performed in a Tris acetate buffer containing 40 mmol/L Tris, 20 mmol/L acetic acid, and 2 mmol/L EDTA, pH 8.0, at 100 V (3.6 V/cm). The gels were visualized by UV illumination. Plasmid DNA bands were assessed using Gel Doc 2000 software.

RESULTS AND DISCUSSION

Previous problems with isolation of plasmids from *Lactococcus lactis* have been caused by inefficient cell lysis and large amounts of resulting protein in the cell preparation (Duan et al., 1999). To overcome these problems, usually log-phase cultures have been used for plasmid DNA isolation. In some methods, the typical growth medium for *Lactococcus lactis* was replaced with lysis broth (Klaenhammer et al., 1978; Anderson and McKay, 1983; Saguir and Manca de Nadra, 2004). Duan et al. (1999) proposed the introduction of the acetone step into the protocol which facilitated complete lysis of the cells. For large plasmids, gentle lysis is best accomplished by suspending the bacteria in an isoosmotic solution of sucrose and treating them with lysozyme and EDTA, which removes much of the cell wall (Sambrook and Russel, 2001). In all the examined protocols, the cells are digested with lysozyme and the resulting protoplasts are lysed by adding an anionic detergent such as SDS. In the method by Klaenhamer et al. (1978), a positive effect of DEP (diethylpyrocarbonate) on large lactococcal plasmid isolation has been observed, which could be attributed to the inactivation of an intracellular nuclease bond to the plasmid at cell lysis. Moreover, to minimize the activity of endogenous nuclease, lysozyme digestion could be decreased from 20 to 7 min. Our results show that the most effective plasmid isolation is achieved with protocols by Saguir (2004) and Klaenhamer (1978). In both methods lysis broth is used, which induces formation of spheroplasts already during the culture and allows to shorten the time of incubation of cells with lysozyme (Figs. 1 and 2). The lowest yield of plasmid DNA was obtained after isolation with GenJET™ Plasmid Mini-

prep Kit (Fermentas). This may have been caused by incomplete lysis of bacterial cell or inefficient elution of plasmid DNA from the silica membrane.

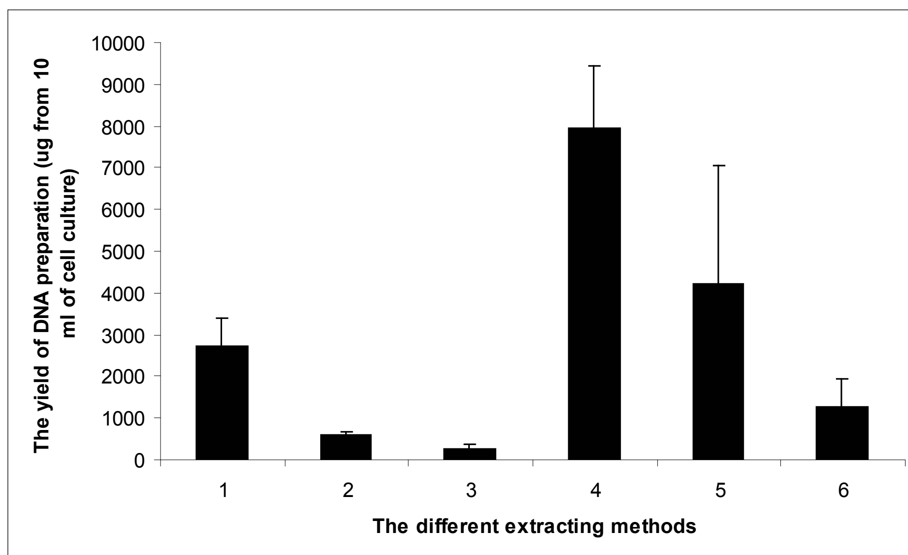

Fig. 1. The yield of plasmid DNA from *Lactococcus lactis* subsp. *cremoris* using six different extraction methods

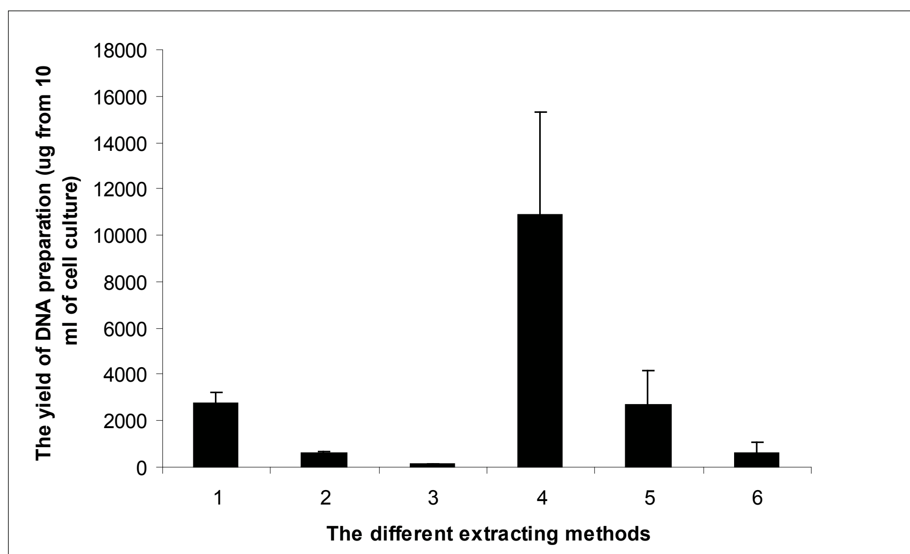

Fig. 2. The yield of plasmid DNA from *Lactococcus lactis* IBB500 using six different extraction methods

Table 1. Compilation of methods used for the isolation of plasmid DNA

Method	Cell lysis	Deproteination purification	Plasmid DNA precipitation	Culture media	References
1	GTE buffer, pH 8.0, lysozyme, RNase A 2% alkaline SDS, acetone	phenol/chloroform	isopropanol 70% ethanol	M-17 broth	[7]
2	TES buffer, pH8.0, 0.1% SDS, lysozyme, 1% alkaline SDS	acetic buffer, pH 4.8	isopropanol LiCl	M-17 broth	[13]
3	GenJET™ Plasmid Miniprep Kit (Fermentas)				
4	buffer I Tris-EDTA 6.7% sucrose, pH 8.0 buffer II Tris-EDTA 20% SDS, pH 8.0, lysozyme buffer III Tris-EDTA, pH 8.0	phenol saturated with 3% NaCl	chloroform-isoamyl alcohol isopropanol	Elliker broth	[1]
5	TES buffer (pH 8.0) with lysozyme buffer EDTA-Tris/HCl pH 8.0, 1% alkaline SDS	potassium acetate	phenol-chloroform-isoamyl alcohol, chloroform, isopropanol	M-17 broth supplemented with 15% tomato juice	[15]
6	buffer I TES-NaCl buffer (pH 8.0) buffer II Tris-EDTA 25% sucrose, pH 8.0, lysozyme DEP, 1% alkaline SDS	NaCl	chloroform-isoamyl alcohol, isopropanol	Elliker broth	[9]

Table 2. Optical density ratios of plasmid DNA extracted using methods 1–6

Species	1	2	3	4	5	6
	$OD_{260/280}$	$OD_{260/280}$	$OD_{260/280}$	$OD_{260/280}$	$OD_{260/280}$	$OD_{260/280}$
Lactococcus lactis ssp. *cremoris*	1.878625 ±0.0170	1.86435 ±0.0227	1.62285 ±0.0391	1.906675 ±0.0772	1.822667 ±0.0074	1.874375 ±0.0313
Lactococcus lactis IBB500	1.9474 ±0.0417	1.83035 ±0.0300	1.465567 ±0.0955	1.880975 ±0.0602	1.724975 ±0.1513	1.8847 ±0.0399

The next important step of plasmid DNA isolation is purification from proteins and RNA contaminants. For this purpose phenol/chloroform precipitation are used (Duan et al.,1999) or phenol saturated with salt (Anderson and McKay, 1983). In other methods, salt solutions (potassium acetate, NaCl) are used, which protect DNA through strengthening of hydrogen bonds between complementary strands of DNA (Klaenhamer et al., 1978; Saguir and Manca de Nadra, 2004). The best results of DNA purification were obtained with (Palamarczyk et al., 2002) method in which contaminants were precipitated with isopropanol and LiCl (Tab. 2). Lithium chloride is a strong dehydrating reagent which lowers the solubility of RNA and strips proteins from the DNA. The contaminating high-molecular weight RNA and proteins can then be removed by low-speed centrifugation (Barlow et al., 1963).

A comparison of plasmid DNA yields and purity obtained using the different extraction methods indicates that some factors are crucial for efficient isolation of large plasmid DNA from gram positive bacteria. The optimal growth phase of bacterial culture is the late log phase, which for *Lactococcus lactis* was 8 h. The next important step is cells lysis. For lactococci, the best result was achieved when partial cell lysis was performed already during bacterium growth in a special kind of lysis medium (Elliker broth). For complete cell lysis, digestion with lysosyme must be performed, but an optimal effect is obtained when the time of incubation does not exceed 20 min. If one decides to prolong this step, an inhibitor of exogenous nuclease DEP should be added. The last important step is plasmid DNA precipitation. The preferred reagent is isopropanol, as a milder precipitation factor than ethanol. Addition of LiCl significantly improves the purity of the isolated DNA.

It should be pointed out that the results obtained from all the examined methods were consistent for the two strains *L. lactis* ssp. *cremoris* AC1 and *L. lactis* ssp. *lactis* IBB 500. On the basis of the analysis of the crucial steps in all the methods, a new isolation protocol may be proposed that would include the best described elements from methods 2, 5, and 6.

In conclusion, an improved large plasmid DNA extraction method should consist of the following steps: cell lysis using TES-NaCl buffer (pH 8.0), Tris-EDTA 25% sucrose, pH 8.0, lysozyme DEP, and 1% alkaline SDS, deproteination by potassium acetate, and DNA precipitation with isopropanol and LiCl. Preferred growth medium is lysis Elliker broth. The protocol proposed here yields clean plasmid DNA. The procedure should be applicable to other lactic acid bacteria species.

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