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Simple and Rapid Method for Isolating Large Plasmid DNA from Lactic Streptococci†

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A procedure for the rapid isolation of plasmid DNA larger than 30 megadaltons from lactic streptococci is described. This protocol can be used on a preparative scale to isolate sufficient quantities of plasmid DNA required for restriction analysis, cloning, or transformation experiments. A scaled-down protocol is very useful for rapidly screening the plasmid content of streptococcal strains. With this methodology, previously undetected large plasmids were observed.

A significant problem that exists with current methods for isolating large plasmids from dairy streptococci is that the yield of large plasmids is very low. This observation was initially reported in studies linking the ability to metabolize lactose to a 30-megadalton (Mdal) plasmid in *Streptococcus lactis* C2 (11, 12) and to a 36-Mdal plasmid in *Streptococcus cremoris* B_1 (1). Plasmid DNA was prepared as a cleared lysate by the method of Cords et al. (3), purified by cesium chloride-ethidium bromide density gradient centrifugation, and examined by electron microscopy. Very few of the 30- or 36-Mdal plasmids were observed. The scarcity of these plasmids was attributed to a combination of low plasmid copy number and inefficient cellular lysis (1, 12). Subsequently, Klaenhammer et al. (8) developed cellular growth and lysis conditions which permitted the direct detection of these plasmids by agarose gel electrophoresis. Recently, Chopin and Langella (2) isolated plasmid DNA from 60 strains of group N streptococci with the method described by Gasson and Davies (6), and reported that plasmids larger than 30 to 45 Mdal could not be consistently isolated by this method. To isolate and characterize 60-Mdal plasmids from transconjugants of *S. lactis* ML3, Walsh and McKay (15) modified a procedure developed by Hansen and Olsen (7).

Although methodology has been developed which permits the reliable detection of plasmids larger than 30 Mdal, the yield of such plasmid DNA is still low. For example, plasmid DNA from 80 ml of cells was required to visualize the 60-Mdal plasmid from *S. lactis* PW1 on an agarose gel (15). Clearly, the limited availability of large plasmid DNAs has severely limited their physical characterization by techniques such as restriction mapping and heteroduplex analysis. To remove this limitation, we have developed a simple, rapid method for efficiently isolating large plasmids from lactic streptococci.

The method described below is a composite method. Cellular lysis is accomplished by a modification of the method described by Klaenhammer et al. (8), and the resulting lysate is enriched for large plasmid DNA by a modification of the method of Currier and Nester (4). This method is suitable for both small-scale screening and preparative purification of large plasmids from lactic streptococci.

MATERIALS AND METHODS

Bacterial strains. *S. lactis* strains used in this study were obtained from the stock culture collection maintained in our laboratory. Cultures were maintained in M-17 broth (14) containing either lactose or glucose at 0.5%; the cultures were transferred at biweekly intervals.

Growth of cells and DNA preparation. Strains from which plasmid DNA was to be extracted were grown overnight in the appropriate M-17 broth at 32°C. The resulting culture provided a 2% inoculum for a modified Elliker broth medium designated lysis broth (8). Strains were propagated for 4 h at 32°C and were harvested by centrifugation. Pelleted cells could be used immediately for plasmid DNA preparation or frozen at −70°C for use the following day.

Plasmid purification protocol. The protocol for purifying large plasmid DNA is presented in Table 1. Column 1 details the steps involved in this method, and columns 2 and 3 define the volumes of reagents used and other details for either screening or preparative plasmid purification protocols. The screening protocol was designed to be performed in a 1.5-ml Eppendorf centrifuge tube. All reagents were mixed immediately after addition by vortexing at low speed for 1 s, with the exception of the 3.0 N NaOH and the 2.0 M Tris-hydrochloride, pH 7.0. These reagents were mixed by inversion. Centrifugations were per-

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TABLE 1. Lysis protocol for lactic streptococci

Step	Details of following protocol:	
	Screening (1.5–10 ml)[a]	Preparative (600 ml)[a]
Resuspend pelleted cells in 6.7% sucrose–50 mM Tris–1 mM EDTA, pH 8.0	379 μl	30 ml
Warm to 37°C		
Add lysozyme (10 mg/ml in 25 mM Tris, pH 8.0)	96.5 μl	7.5 ml
Incubate for 5 min at 37°C		
Add 0.25 M EDTA–50 mM Tris, pH 8.0	48.2 μl	3.75 ml
Add sodium dodecyl sulfate (20% [wt/vol] in 50 mM Tris–20 mM EDTA, pH 8.0)	27.6 μl	2.25 ml
Mix immediately		
Incubate for 5 to 10 min at 37°C to complete lysis		
Vortex at highest setting for 30 s in an appropriate tube	1.5-ml Eppendorf	15 ml per tube (25 by 150 mm)
Add fresh 3.0 N NaOH	27.6 μl	2.40 ml
Mix gently by intermittent inversion or swirling for 10 min	Inversion	Swirl in 250-ml centrifuge bottle
Add 2.0 M Tris-hydrochloride, pH 7.0	49.6 μl	3.90 ml
Continue gentle mixing for 3 min		
Add 5.0 M NaCl	71.7 μl	5.7 ml
Add phenol saturated with 3% NaCl; mix thoroughly	700 μl	55.8 ml
Centrifuge	5 min	5,000 rpm in GSA rotor, 10 min
Remove upper phase and extract with chloroform-isoamyl alcohol (24:1)	700 μl	55.8 ml
Remove upper phase, precipitate with 1 vol of isopropanol		
Incubate at 0°C	>30 min	>60 min
Centrifuge	5 min	8,000 rpm in GSA rotor, 20 min
Remove excess isopropanol and resuspend in 10 mM Tris–1 mM EDTA, pH 7.5	20 μl	1,200 μl
Examine 5 to 10 μl by agarose gel electrophoresis		

[a] The culture volume used in each protocol is indicated in parentheses.

formed at room temperature in an Eppendorf centrifuge.

The preparative protocol was usually performed in a 250-ml centrifuge bottle. All reagents were mixed by swirling. Shearing the lysate was performed by dispensing 13- to 15-ml portions of the lysate into screw-cap test tubes (25 by 150 mm). Each tube was vortexed at full speed for 30 s, and the sheared lysates were pooled before denaturation. Isopropanol precipitates could be stored at 0 or −20°C overnight.

Agarose gel electrophoresis. Agarose gel electrophoresis was performed in a Tris acetate buffer containing 40 mM Tris, 20 mM acetic acid, and 2 mM Na_2EDTA (pH 8.1). Gels contained 0.6% agarose, and electrophoresis was performed at 100 V (3.6 V/cm) for 5 h. Gels were stained with 0.5 μg of ethidium bromide per ml and photographed through a red filter on Polaroid type 107 film.

RESULTS AND DISCUSSION

The plasmid purification protocol described in Table 1 solves the problem of isolating substantial quantities of large plasmids from dairy streptococci. To demonstrate the effectiveness of this protocol, we isolated plasmid DNA from 10 ml of *S. lactis* PW1, NP2, ML3, and PW2. DNA equivalent to that present in 5.0 ml of cells was loaded onto the gel shown in Fig. 1. Covalently closed circular plasmids of 80 Mdal (NP2), 60 Mdal (PW1, ML3, and PW2), and 33 Mdal (ML3) are clearly identified above the chromosomal DNA band. It is significant that both the 80-Mdal plasmid in *S. lactis* NP2 and the 60-Mdal plasmid in *S. lactis* ML3 were not detected before the development of this method. It appears that the 80-Mdal plasmid is linked with bacteriocin production (13) and the 60-Mdal plasmid is a recombinant plasmid involved with lactose metabolism, cell aggregation, and conjugal transfer (15). Therefore, although previous phenotypic instability implied the presence of a plasmid, our protocol may be useful in actually identifying that presence (5, 9).

To estimate the minimum number of cells required to permit the detection of all observed plasmids, the DNA preparations were diluted and electrophoresed (Table 2). The different volumes of cells required to visualize respective plasmids probably reflect the relative copy num-

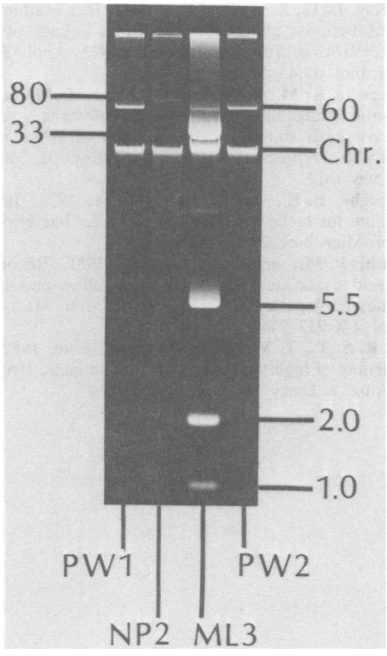

FIG. 1. Agarose gel electrophoresis of plasmid DNA isolated from *S. lactis* PW1, NP2, ML3, and PW2. Plasmid sizes are designated in Mdal. Chr., Chromosomal DNA band.

ber of the plasmids. For routine screening, a volume of DNA equivalent to that present in 2.5 ml of cells is loaded onto a gel.

The presence of the chromosomal DNA band may mask the presence of plasmid DNA during screening experiments. To detect whether plasmid DNA was present in the chromosomal band, we returned a stained gel to the electrophoresis apparatus, and electrophoresis was continued for 1 to 2 h in the presence of 0.5 μg of ethidium bromide per ml. At this concentration of ethidium bromide, the mobility of the putative comigrating plasmid is very low (10) compared with the mobility of the chromosomal DNA fragments. The resulting difference in mobilities of previously comigrating species permitted the identification of plasmids previously hidden by the chromosomal DNA band (data not shown).

Another asset of this plasmid purification protocol is its simplicity. Key developments in simplifying this protocol were (i) determining the quantities of 3.0 N NaOH and 2.0 M Tris-hydrochloride required for the alkaline denaturation-renaturation steps and (ii) developing alternate stirring regimens for use in the denaturation-renaturation steps. The former eliminated the need for time-consuming pH measurements; the latter permitted many samples to be manipulated at one time. The result is a very rapid method for the purification of plasmid DNA on both screening and preparative scales. With our method, plasmid DNA from 24 strains can be prepared and loaded onto a gel within a 4-h period.

TABLE 2. Minimum cell volumes for plasmid detection

Strain	Plasmid size (Mdal)	Minimum culture vol (ml) required to detect plasmid
S. lactis	60[a]	1.70
	33	0.10
	5.5	0.10
	2.0	0.10
	1.0	0.10
S. lactis PW1	60	0.25
S. lactis PW2	60	0.25
S. lactis NP2	(80)[a]	1.70

[a] Parentheses indicate previously undetected plasmid.

In conclusion, the methodology detailed above represents another contribution to the growing list of plasmid purification protocols adapted for use with lactic streptococci (8, 9, 15, 16). However, this methodology is distinct in that it permits the efficient isolation of quantities of large plasmid DNA required for physical characterization experiments. A scaled-down version of this methodology is useful in screening the plasmid content of many strains of lactic streptococci.

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