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Microscale Method for Rapid Isolation of Covalently Closed Circular Plasmid DNA from Group N Streptococci†

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A method for rapid purification of plasmid DNA from lactic streptococci, utilizing microliter quantities of reagents, was developed by combination of a short lysozyme-mutanolysin cell wall digestion with a modification of the *Escherichia coli* plasmid isolation procedure of McMaster et al. (Anal. Biochem. **109**:47–54, 1980). The preparations obtained were highly enriched for covalently closed circular DNA, and the method was applicable to plasmids of at least 40 megadaltons. Centrifugation in CsCl-ethidium bromide density gradients was not required.

Group N streptococci have for centuries played an essential role in the production of fermented dairy foods. Some of the most important metabolic properties of the lactic streptococci, such as the fermentation of lactose or the degradation of milk proteins, are often determined by plasmid-carried genes (5, 9). The study of plasmids has therefore become essential for the understanding of the genetics and physiology of group N streptococci, as well as being a valuable aid in strain identification (10). Most known strains of these bacteria carry at least two, and frequently more, plasmid species, which makes the interpretation of the band patterns obtained by agarose gel electrophoresis (26) considerably difficult if a preparation contains significant amounts of DNA which is in other than the covalently closed circular (CCC) state. This paper describes a simple procedure for rapid isolation of plasmid DNA from lactic streptococci, which yields preparations highly enriched for CCC DNA and utilizes micro quantities of reagents. The method was developed by combining previously available knowledge regarding the proper conditions for lysis of streptococcal cells (4, 10, 16, 20, 25, 27, 32) with a modification of an *Escherichia coli* plasmid isolation procedure (24).

MATERIALS AND METHODS

Bacterial strains. *Streptococcus cremoris* Wg2-2 was a gift from M. Teuber (Federal Dairy Research Center, Kiel, West Germany), and *Streptococcus lactis* MG1261 was kindly provided by M. J. Gasson (National Institute for Research in Dairying, Shinfield, Reading, United Kingdom). Other strains were from the culture collection maintained in this laboratory.

Chemicals. The sodium salt of triisopropylnaphthalenesulfonic acid (24) was from Eastman Kodak Co. Phenol (Mallinckrodt) was redistilled, washed three times with 0.5 M NaCl, and stored at 4°C after addition of 0.1% 8-hydroxyquinoline.

Enzymes. Lysozyme was dissolved daily (20 mg/ml) in sterile 25% sucrose solution. Mutanolysin (17, 27, 32) was dissolved at 5,000 U/ml in 0.1 M potassium phosphate buffer (pH 6.2) and stored at −25°C until used. RNase A was prepared as a 10-mg/ml solution in sterile water, heated at 70°C for 30 min, and stored at −25°C. All enzymes were from Sigma Chemical Co.

Culture growth. Streptococcal lysis medium was an M17 formulation (30) containing 0.5% yeast extract and 20 mM DL-threonine (4) (and 0.5% glucose instead of lactose when used with lactose-negative strains). A total of 16 μl of late-exponential-phase culture in M17 broth was used to inoculate 8 ml of streptococcal lysis medium, and the culture was incubated at 20°C (25°C for *S. cremoris* Wg2-2) for ca. 12 h (absorbance at 660 nm with a l-cm light path, 0.70; late-log phase).

Cell lysis and isolation of plasmid DNA. A Beckman model 11 microcentrifuge was used in all centrifugation steps after cell harvesting. The horizontal (vertical slides) rotor is here designated rotor 1, and the model 13.2 fixed-angle rotor is here designated rotor 2.

Cells were harvested by centrifugation at 4°C, suspended in 1.2 ml of sterile distilled water, transferred to 1.5-ml microcentrifuge tubes, and centrifuged in the cold at 2,390 × *g* for 5 min (rotor 1). The supernatant was discarded, and the tube was placed on ice. (The volume of the cell pellet at this point was ca. 30 μl.) Lysozyme solution (100 μl) was added, the cells were thoroughly resuspended by vortexing, and the preparation was returned to ice. After addition of 20 μl of mutanolysin solution and vortexing the cells, the tubes were transferred to a 37°C water bath and incubated for 3 min, and 210 μl of 0.079 M Tris-hydrochloride in 25% sucrose solution (pH 8.1) was added. The tubes were rapidly vortexed, incubated for another 3.5 min at 37°C (16), and chilled on ice for 2 min. With preparations still on ice, 60 μl of 10% triisopropylnaphthalenesulfonic acid in 250 mM Na_2 EDTA (pH 8.0) was added, and the samples were given four gentle inversions, quickly followed by the addition of 25 μl of 3.0 N NaOH and four more gentle inversions. Tubes were then transferred to a water bath at 30°C and incubated for 7 min while mixing by gentle inversions. The pH at this point was 12.0 to 12.2, and a clear solution was obtained.

Neutralization was effected by the addition of 30 μl of 1.0 M sodium acetate (pH 5.0) followed by five gentle inversions. Phenol reagent (600 μl) was immediately added, and the samples were thoroughly mixed by two rounds of four gentle inversions. Addition of 150 μl of chloroform was rapidly followed by six gentle inversions, and the preparations were immediately centrifuged at 11,900 × *g* at room temperature for 12 min (rotor 2). A pipettor with large-bore tips (15) was used to collect 320 μl of the clear aqueous phase (top), carefully avoiding the white, disk-shaped interface which separated it from the yellow organic phase (bottom). Failure to avoid this interface resulted in contamination with

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chromosomal (chr) or open circular (OC) DNA (8, 24). The aqueous phase was transferred to a fresh 1.5-ml tube, 6 μl of RNase solution was added, and the preparations were mixed by inversion and incubated in a 37°C water bath for 20 min. A total of 35 μl of 5.0 M NaCl was added, followed by gentle mixing, the addition of 265 μl of 30% polyethylene glycol (molecular weight, 6,000) solution, and more gentle mixing. Samples were then incubated in a salt-ice bath at −11°C for 1.5 h. Centrifugation in the cold at 11,200 × *g* for 15 min (rotor 1) produced a pellet containing precipitated DNA and polyethylene glycol. The supernatant was discarded, 500 μl of 50 mM Tris-hydrochloride–5 mM Na_2 EDTA (pH 8.0) solution was added to the pellet, and five gentle inversions were given to the tubes. Dissolution of the pellet was completed with the addition of 900 μl of chloroform and gentle but very thorough mixing, which was continued for another 10 min. Samples were then chilled on ice for 10 min before centrifugation in the cold at 11,900 × *g* for 12 min (rotor 2). The preparations were returned to ice, and 300 μl of the clear aqueous phase (top) was collected. (Low temperature helped to prevent resolubilization of the white interface into the aqueous phase.) In a fresh tube, 600 μl of cold, 100% ethanol was added to the aqueous phase, and the tubes were inverted five times and incubated at −25°C for 1.5 h. The precipitated plasmid DNA was collected by centrifugation in the cold at 11,200 × *g* (rotor 1) for 20 min. The supernatant was discarded, and the tubes were thoroughly dried (inverted for 15 min in a laboratory hood) before addition of 24 μl of loading buffer (10% glycerol, 0.025% bromphenol blue, 50 mM Tris-hydrochloride, 50 mM NaCl, 10 mM Na_2 EDTA, pH 8.0 [11, 23]). The DNA was allowed to dissolve overnight at 4°C (7) and to warm up to room temperature before electrophoresis. A total of 6 μl was loaded into a 10-mm gel slot.

When sample purity was not important, e.g., when screening multiple isolates, the procedure needed to be carried only as far as the phenol extraction; plasmid DNA could be visualized by direct loading of ca. 8 μl of the dense, sucrose-containing aqueous phase into a 5-mm gel slot. The obvious advantages of this procedure were the ability to process more isolates in a shorter time and same-day electrophoresis. (The complete procedure was applied to the samples shown in Fig. 1.)

Agarose gel electrophoresis. Electrophoresis buffer was 40 mM Tris base–20 mM sodium acetate–2 mM EDTA, pH adjusted to 8.1 with glacial acetic acid (10). The final concentration of acetate in this system was ca. 38 mM. Horizontal electrophoresis in 0.70% agarose gels was carried out in submarine mode with buffer recirculation (14) for 4.0 h. A low-voltage gradient (3.0 V/cm) was employed to optimize the separation of large plasmids (11, 22, 23). After electrophoresis, the gel was stained with ethidium bromide (0.5 μg/ml) for 30 min, destained in water for 20 min, and photographed through an orange filter on a model C-63 Ultraviolet Products transilluminator with Polaroid type 47 film. Plasmid mobilities were measured directly on photographic prints. Plasmid molecular masses were estimated from a logarithmic plot of mass versus relative mobility (26, 31), constructed on the basis of *E. coli* V517 standards (21).

RESULTS AND DISCUSSION

The plasmid profiles of representative strains of lactic streptococci are shown in Fig. 1, lanes A to E. Information previously published on these or closely related strains, gathered by use of centrifugation in CsCl-ethidium bromide density gradients, electron microscopy, or agarose gel electrophoresis (1, 13, 16, 19, 28), is in good agreement with the data shown here. (*S. lactis* C2 carried in this laboratory is somewhat different from that held by McKay's group, even though both strains are derived from *S. lactis* NCDO 712; this was previously shown by Davies et al. [10].) It is evident that the present procedure is effective in reducing chr and OC DNA contamination and recovering plasmids in the 1- to 40-megadalton range. The amount of DNA loaded in each lane of Fig. 1 is equivalent to that obtained from 2.0 ml of culture. High enrichment with CCC DNA greatly facilitates the interpretation of plasmid profiles (10), especially for strains containing multiple plasmid species.

Culture growth before lysis at 20°C for 12 h was employed because, at least for some plasmids, the amount of plasmid DNA relative to the total DNA in the cell increases at lower growth rates (6, 12, 18, 29). Under these conditions, use of a small inoculum (0.2%, more or less, depending on the strain) permitted combining the convenience of overnight incubation with the obtainment of cultures in late-exponential phase of growth, which are more amenable to lysis than are stationary-phase cells (16).

The quantity of cells used was important for the success of the procedure. An excess of cells frequently led to poor lysis, because of insufficient digestion of the cell wall by lysozyme and mutanolysin, in the short (16) incubation period. A scarcity of cells was also undesirable, because of the recovery of inadequate amounts of plasmid DNA. A 30-μl cell pellet, obtained after washing in a microcentrifuge tube (see above), was an appropriate quantity for lysis by the present method.

Lysozyme was added to washed cells in a 25% unbuffered

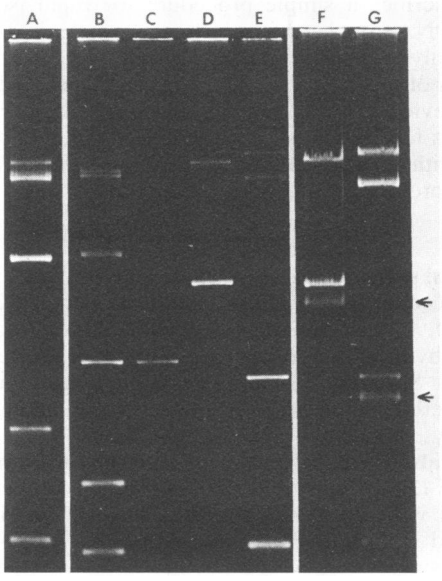

FIG. 1. Plasmid profiles of lactic streptococci. Apparent molecular masses (in megadaltons) are indicated in parentheses after the identity of each strain. Lane A, *S. cremoris* Wg2-2 (1.5, 3.4, 13, 32, 40), a derivative of *S. cremoris* Wg2 (described in reference 28). Lane B, *S. lactis* C2 (1.4, 2.3, 5.6, 13, 33, 35). Lane C, *S. lactis* MG1261 (5.6); both lanes B and C are derivatives of *S. lactis* NCDO 712 (described in references 10 and 13). Lane D, *S. cremoris* B1 (11, 40; described in references 1 and 16). Lane E, *S. lactis* C10 (1.5, 5.0, 32, 45; described in references 16 and 19). The next two lanes show artifact bands (arrows) produced in *S. cremoris* B1 (lane F) and in *S. lactis* C10 (lane G) by excessive denaturation in alkali (see text). Lanes F and G were run in a separate gel.

sucrose solution, with Tris-hydrochloride added later, according to the findings of Metcalf and Deibel (25), which were also explored by Davies et al. (10). Use of a small amount of mutanolysin (17, 27, 32) in the presence of a high concentration of lysozyme was extremely helpful in promoting lysis of streptococcal cells.

Lysis under highly alkaline conditions allows little time for the action of endogenous nucleases (3, 24) and eliminates the necessity of reducing the viscosity of the lysate through shearing (3, 8). Vortexing of lysed cells was eliminated to avoid contamination with chr DNA fragments.

Alkali denaturation followed by renaturation and phenol extraction in high salt (8, 24) were the most critical steps in the procedure in regard to the elimination of chr and OC DNA. It was important that neutralization be immediately followed by the addition of phenol. Presumably, only CCC plasmid molecules could renature properly in this short interval and thus escape precipitation caused by the addition of phenol.

Extreme care had to be employed with regard to the exposure time and pH during the alkali denaturation step. Excesses in either or both lead, upon neutralization, to the irreversible denaturation of CCC plasmid molecules into collapsed circles (14, 24). This results in artifacts that are sometimes difficult to detect, since only one plasmid species in a multiple-plasmid strain may be affected. Examples of such artifacts are shown in Fig. 1, lanes F and G. In these preparations, cell lysates were exposed to pH 12.4 for 12 min, the conditions originally employed by McMaster et al. (24). Use of a less severe denaturation treatment, as described here, permitted elimination of the artifact bands.

Phenol extraction was carried out in the presence of the 25% sucrose solution used to stabilize spheroplasts before lysis. The density of the phenolic phase was smaller than that of the aqueous phase. This would be very inconvenient when collecting the latter after centrifugation, because of heavy losses and contamination with phenol and with precipitated chr and OC DNA. This problem was solved by the addition of chloroform after the phenol extraction, followed by brief mixing and centrifugation. The density of the organic phase was sufficiently increased by the added chloroform, so that reversal of phases no longer occurred. Avoidance of the white interface produced after centrifugation was essential to minimize contamination with chr and OC DNA. Reagent volumes were increased by 50% relative to those used by McMaster et al. (24), so percent losses at interfaces could be diminished.

Recently, two new rapid screening procedures for isolation of plasmid DNA from lactic streptococci appeared (2, 33). The present method has an advantage over one of these procedures (2) in that contamination with chr DNA is virtually eliminated.

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