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# A General Method for Plasmid Isolation in Lactobacilli

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**Abstract.** A simple procedure for rapid isolation and detection of plasmid DNA from *Lactobacillus* species is described. Using an alkaline-detergent lysis method, plasmid DNA was released and characterized from cells treated with either mutanolysin or lysozyme for 1 h at 0°C. Treatment of cells with either enzyme at 37°C for 1 h was detrimental to plasmid isolation and characterization in some *Lactobacillus* species. The procedure was effective with small volumes of cells and allowed rapid characterization of plasmid DNA in *Lactobacillus plantarum, Lactobacillus acidophilus, Lactobacillus helveticus,* and *Lactobacillus bulgaricus* strains.

Recent progress in the genetics of lactic acid bacteria, particularly the group N streptococci, has established the critical involvement of plasmid DNA in numerous activities related to fermentative performance [2, 5, 10]. Unfortunately, plasmid genetics of the lactobacilli have not kept pace with the advances in molecular genetics, and little information is presently available on plasmid determinants or genetic transfer mechanisms for a bacterial genus critical to many industrial food fermentations. Undermining progress in *Lactobacillus* genetics has been the difficulty in achieving cell lysis with lysozyme [4, 18] and developing reliable procedures for plasmid DNA isolation. Although plasmid DNA has been reported in *Lactobacillus acidophilus* [13, 21], *Lactobacillus reuteri* [21], *Lactobacillus casei* [3], *Lactobacillus fermentum* [7], and *Lactobacillus helveticus* [20, 21], detection has been limited to a select number of strains within each species [13, 21]. Considering the ubiquity of plasmid DNA throughout Gram-positive and Gram-negative bacteria, the inability to demonstrate plasmid DNA throughout *Lactobacillus* species could reflect the ineffectiveness of current plasmid isolation procedures for this genus.

The reported sensitivity of Gram-positive bacteria to *N*-acetylmuramidase from *Streptomyces globisporus*, mutanolysin [14, 17, 22], suggests that DNA yields from lactobacilli could be enhanced upon employing this enzyme in plasmid isolation procedures. This report describes a general method for plasmid isolation in *Lactobacillus* species effective using either lysozyme or mutanolysin to achieve cell lysis. Although mutanolysin was more effective, plasmid detection in the lactobacilli was dependent more on the temperature conditions established for enzyme activity than on the specific enzyme used.

## Materials and Methods

**Strains and culture conditions.** All *Lactobacillus* strains were propagated in MRS broth (Difco Laboratories, Detroit, MI) at 37°C and maintained as described previously [11]. *Escherichia coli* V517 was propagated in nutrient broth (BBL, Cockeysville, MD) at 37°C. Growth conditions and methods for plasmid isolation from *E. coli* V517 were described previously [12, 15]. Strains used in this study, and their sources, are given in Table 1.

**Cell lysis.** Log phase cells from 200-ml MRS broth cultures (0.1% inoculum, 37°C) were harvested by centrifugation when the optical density (OD) at 650 nm reached 1.0. Pelleted cells were washed once in 20 ml of TES buffer [16] and resuspended in 2 ml of 25% (wt/vol) sucrose in 50 m*M* Tris-HCl, pH 7.5. Two 925-μl aliquots were removed and placed in an ice bath. Mutanolysin (Miles Laboratories, Elkhart, IN) or lysozyme (Sigma Chemical Company, St. Louis, MO) was added to one aliquot to a final concentration of 75 μg/ml (75 μl of a 1-mg/ml suspension in sterile distilled $H_2O$). Sterile distilled $H_2O$ (75 μl) was added to the second aliquot and both aliquots placed at 37°C. Samples containing 50 μl were removed from each aliquot at 0, 30, and 60 min and added to cuvettes containing 2 ml of 50 m*M* Tris-HCl, pH 7.0. After mixing, the $OD_{650}$ was determined. The percent relative lysis at each sampling period was calculated from the $OD_{650}$ of control samples (no enzyme) and $OD_{650}$ of enzyme-treated samples. The $OD_{650}$ of control aliquots approximated 0.5 and 0.6 and remained stable throughout the 60 min incubation.

**Plasmid isolation.** *Lactobacillus* cultures were propagated overnight (9–12 h) in 10 ml of MRS broth. A sample of 4 ml of culture

Table 1. Bacterial strains

| Organism | Source |
|---|---|
| *Escherichia coli* V517 | F. L. Macrina et al. [15] |
| *Lactobacillus acidophilus* C7 | NCSU[a] [13] |
| *Lactobacillus acidophilus* PA3 | NCSU [13] |
| *Lactobacillus acidophilus* M1 | Miles Laboratories, Elkhart, IN |
| *Lactobacillus acidophilus* 11088 | J. L. Johnson et al. [8] |
| *Lactobacillus bulgaricus* 10 | NCSU |
| *Lactobacillus helveticus* 261 | NCDO[b] |
| *Lactobacillus helveticus/jugurt* 103 | NCDO |
| *Lactobacillus lactis* 1438 | NCDO (also ATCC[c] 12315) |
| *Lactobacillus plantarum* 352 | NCDO |
| *Lactobacillus plantarum* YIT0068 | Yakult Institute for Microbiological Research, Tokyo, Japan |

[a] Culture Collection at North Carolina State University, Department of Food Science.
[b] National Collection of Dairy Organisms, University of Reading, Shinfield, England.
[c] American Type Culture Collection, Rockville, MD.

was placed in a sterile polypropylene culture tube (17 × 100 mm, Fisher Scientific, Raleigh, NC) and centrifuged at 6000 *g* for 10 min at 4°C. The pellet was resuspended in 10 ml of fresh MRS broth and the cell suspension incubated for 2 h at 37°C. Cells were then harvested by centrifugation and resuspended in 1 ml of cold (4°C) 25% (wt/vol) sucrose in 50 m*M* Tris-HCl, 5 m*M* $Na_2$ EDTA, pH 7.5. After holding the cell suspension for 10 min in an ice bath, 75 μl of mutanolysin or lysozyme (1 mg/ml in 50 m*M* Tris-HCl, 5 m*M* $Na_2$ EDTA, pH 7.5) was added and the sample held in an ice bath for 1 h. For some experiments, cell-enzyme samples were held at 37°C for 1 h. Cells were pelleted by centrifugation and the supernatant discarded. A lysis solution (500 μl) was added directly to the cell pellet and the pellet gently disrupted with a plastic disposable pipet. During the lysis procedure, the pH of the cell suspension, following addition of the lysis solution, should approximate pH 12.2. To achieve this pH, the volume of 10 *N* NaOH added to the lysis solution can be varied from 8 to 12 μl. The lysis solution was a modification of that reported by Kado and Liu [9] and was composed of 50 m*M* Tris, 5 m*M* $Na_2$ EDTA, 50 m*M* glucose, and 3% SDS. Immediately before use, 10 μl of 10 *N* NaOH was mixed with 1 ml of lysis solution. The sample was heated at 62°C for 1 h and then allowed to cool slowly to room temperature (approximately 15 min). A quantity of 50 μl 2 *M* Tris, pH 7.0, was added and mixed, followed by addition of 70 μl 5 *M* NaCl. The sample was transferred by disposable pipet to a 1.5-ml polypropylene Eppendorf centrifuge tube. The appearance of lysates at this step were often viscous, but remained turbid. Deproteinization of the sample was conducted with 500 μl of 3% NaCl-saturated phenol (redistilled, Bethesda Research Laboratories, MD). After addition of phenol, the tube was shaken vigorously for 3 s and held at room temperature for 5 min. To facilitate phase separation, 300 μl chloroform was added (repeat shaking for 3 s) prior to centrifugation for 5 min in an Eppendorf 5414 microfuge. After centrifugation, the aqueous phase above a white protein interface was removed, added to a second Eppendorf tube, and extracted once with 600 μl of chloroform:isoamyl alcohol (24:1, vol/vol). After 5 min at room temperature, the sample was again centrifuged for 5 min. After removal of the aqueous phase (500 μl), 1 ml of cold (−70°C) 95% ethanol was added and the sample held at −70°C for 1 h. DNA was pelleted by centrifugation for 10 min in an Eppendorf microfuge. Ethanol was removed by aspiration and the pellets dried under vacuum for 10 min. DNA pellets were dissolved in 20 μl TES buffer and analyzed by agarose gel electrophoresis [16].

Table 2. Effect of mutanolysin or lysozyme on the percent relative lysis[a] of *Lactobacillus* species

| Treatment | Time (min) at 37°C | *Lactobacillus* species | | | | | |
|---|---|---|---|---|---|---|---|
| | | *L. acidophilus* | | *L. plantarum* | | *L. helveticus* | |
| | | PA3 | C7 | YIT | 352 | 261 | 103 |
| Mutanolysin (75 μg/ml) | 30 | 98.4 | 98.2 | 79.7 | 91.4 | 96.9 | 92.3 |
| | 60 | 99.9 | 99.9 | 92.2 | 99.9 | 99.9 | 99.9 |
| Lysozyme (75 μg/ml) | 30 | 50.0 | 51.4 | 0.6 | 0.8 | 44.8 | 38.5 |
| | 60 | 76.6 | 69.1 | 15.6 | 14.5 | 36.7 | 53.8 |

[a] Percent relative lysis: calculated as percent loss in optical density at 650 nm.

Electrophoresis was conducted on 0.7% agarose gels (Seakem ME agarose, FMC Corp., Rockland, MD) in Tris-borate buffer, pH 8.2 [16]. Samples of 5–20 μl were mixed with an equal volume of agarose beads [19], loaded onto gels and subjected to electrophoresis at 100 V for 3–5 h. Gels were stained and photographed as described previously. Agarose beads were prepared by adding 20 mg agarose and 1 mg bromophenol blue to 10 ml of 10 m*M* Tris-HCl, 20 m*M* $Na_2$ EDTA, 10% glycerol, pH 8.0. The mixture was autoclaved, cooled to room temperature, and then passed repeatedly (3×) through a tuberculin syringe. Agarose beads were stored at 4°C.

## Results

**Lysis by mutanolysin or lysozyme.** Lysis of three *Lactobacillus* species was examined after treatment with either mutanolysin or lysozyme (Table 2). In all six strains examined, mutanolysin acted quickly and was more effective than lysozyme in generating osmotically fragile cells. *Lactobacillus plantarum* species were particularly insensitive to lysozyme. These data are consistent with previous reports noting the resistance of lactobacilli to lysozyme [4, 18] and sensitivity of Gram-positive bacteria to mutanolysin [14, 17, 22].

**Plasmid detection.** Noting the effectiveness of mutanolysin on generation of osmotically fragile lactobacilli, *L. acidophilus* PA3 and C7 were examined for plasmid DNA by a cleared lysate procedure for lactobacilli [13] modified by substitution of 75 μg/ml mutanolysin for lysozyme. Figure 1 shows that *L.*

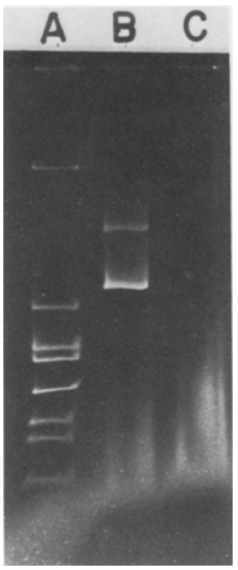


Fig. 1. Electrophoresis of plasmid DNA from *Lactobacillus acidophilus* PA3 (B) and C7 (C). DNA was isolated as described previously [13] using mutanolysin for 1 h at 37°C, prior to lysis with sodium dodecyl sulfate. Track A contains mobility plasmids from *Escherichia coli* V517.

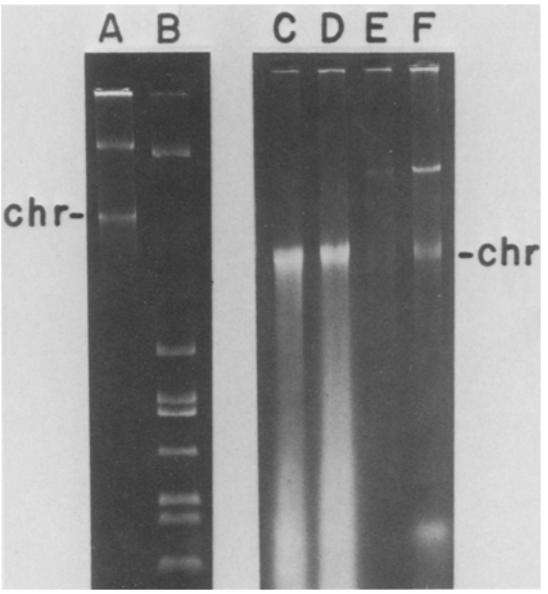


Fig. 2. Electrophoresis of plasmid DNA from *Lactobacillus acidophilus* C7. Conditions of plasmid isolation were as described in *Materials and Methods* using: mutanolysin, 0°C, 1 h (A and F); lysozyme, 37°C, 1 h (C); mutanolysin, 37°C, 1 h (D); and lysozyme, 0°C, 1 h (E). Track B contains mobility plasmids from *Escherichia coli* V517. "Chr" indicates contaminating chromosomal fragments (also indicated on Figs. 4 and 5).

*acidophilus* PA3 (Tract B) contained two plasmid DNA species corresponding to the 13.7 and 6.3 megadalton molecules previously reported [13]. Alternatively, *L. acidophilus* C7 (Tract C), reported devoid of plasmid DNA using lysozyme [13], again showed no detectable plasmid DNA when mutanolysin was employed. Therefore, despite excellent cell lysis conditions, plasmid DNA could not be extracted from C7 using mutanolysin in the cleared lysate procedure described previously [13]. However, *L. acidophilus* C7 does contain a single 38 megadalton plasmid detectable when mutanolysin incubation was conducted at 0°C for 1 h rather than at 37°C for 1 h (Fig. 2A). The effect of incubation temperature, during lysozyme or mutanolysin digestion, on plasmid yields of *L. acidophilus* C7 is shown in Fig. 2 (C–F). Using the procedure described herein, incubation at 37°C for 1 h with mutanolysin or lysozyme resulted in a failure to detect the 38 megadalton plasmid. Alternatively, incubation at 0°C for 1 h with either enzyme resulted in a detectable plasmid band. These data demonstrate that extended 37°C incubation during cell wall digestion is detrimental to isolation of the large plasmid in *L. acidophilus* C7. Although lysozyme was not as effective as mutanolysin in preparing osmotically fragile cells in C7, plasmid DNA was readily isolated from cells digested with 75 μg/ml lysozyme at 0°C for 1 h.

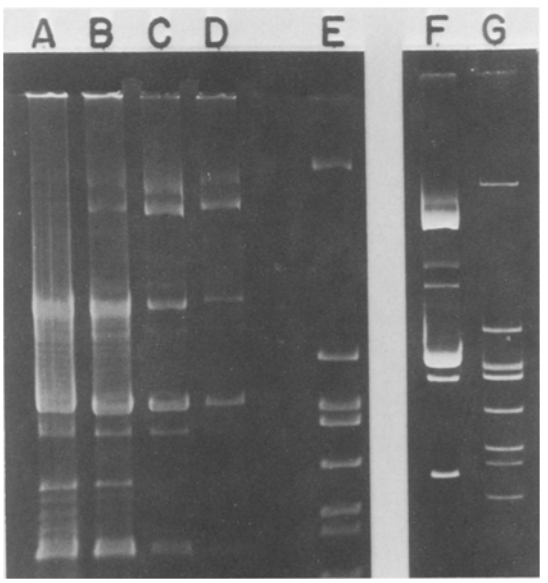


Fig. 3. Electrophoresis of plasmid DNA from *Lactobacillus helveticus* 261. Conditions of plasmid isolation were as described in *Materials and Methods* using: lysozyme, 37°C, 1 h (A); mutanolysin, 37°C, 1 h (B); mutanolysin, 0°C, 1 h (C); lysozyme, 0°C, 1 h (D). Track F contains CsCl–EB purified plasmid DNA. Tracks E and G contain mobility plasmids from *Escherichia coli* V517.

Further studies were conducted with *L. helveticus* 261 and *L. plantarum* 352 to determine the

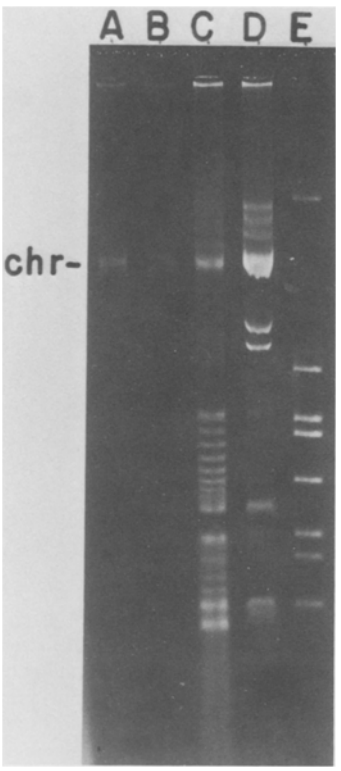


Fig. 4. Electrophoresis of plasmid DNA from *Lactobacillus plantarum* 352. Conditions of plasmid isolation were as described in *Materials and Methods* using: lysozyme, 37°C, 1 h (A); lysozyme, 0°C, 1 h, (B); mutanolysin, 37°C, 1 h (C); and mutanolysin, 0°C, 1 h (D). Track E contains mobility plasmids from *Escherichia coli* V517.

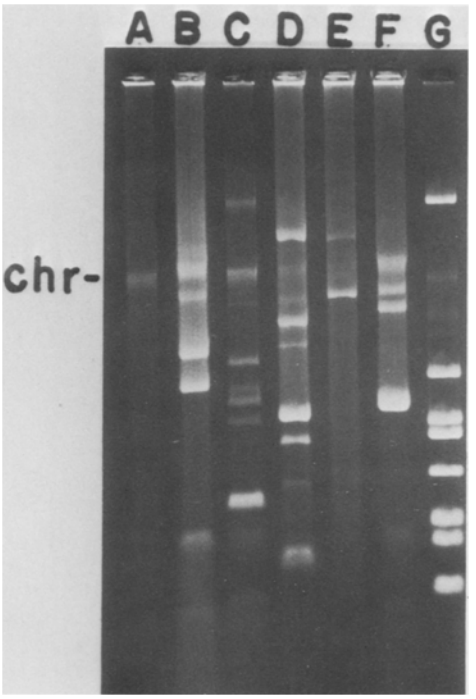


Fig. 5. Electrophoresis of plasmid DNA from *Lactobacillus lactis* 1438 (A); *Lactobacillus acidophilus* M1 (B); *Lactobacillus plantarum* YIT0068 (C); *Lactobacillus bulgaricus* 10 (D); *Lactobacillus helveticus* 103 (E); *Lactobacillus acidophilus* 11088 (F); and *Escherichia coli* V517 (G). Conditions of plasmid isolation in lactobacilli were as described in *Materials and Methods* using mutanolysin 0°C, 1 h.

effects of incubation temperature and type of lytic enzyme on plasmid yields. Plasmid DNA from *L. helveticus* 261 was isolated under all treatment conditions. Either lysozyme or mutanolysin at 0°C for 1 h gave good plasmid DNA yields (Fig. 3C and D) with patterns comparable to cesium chloride–ethidium bromide purified plasmid DNA from *L. helveticus* 261 (Fig. 3F). Incubation at 37°C for 1 h with lysozyme or mutanolysin resulted in a "stair-step" pattern between plasmids of molecular weights ($\times 10^6$) ranging from 1 to 10 (Fig. 3A and B). It was also observed that the lysozyme sample incubated at 37°C for 1 h was missing the two large molecular weight plasmids. Similar results were obtained with *L. plantarum* 352 (Fig. 4). Mutanolysin at 0°C for 1 h resulted in good yields, whereas incubation at 37°C yielded a stair-step pattern and loss of large plasmid molecules. Use of lysozyme at 75 μg/ml was totally ineffective for plasmid isolation from *L. plantarum* 352. Failure to detect plasmid DNA could be directly attributed to the resistance of *L. plantarum* 352 to 75 μg/ml lysozyme (Table 2). However, increasing the lysozyme concentration to 750 μg/ml gave results identical to those with mutanolysin (data not shown). The stair-step bands isolated from *L. plantarum* 352 and *L. helveticus* 261 during enzyme incubation at 37°C for 1 h were carried with covalently closed circular (ccc) DNA through cesium chloride–ethidium bromide (CsCl–EB) purification (data not shown). Therefore, these molecules maintained their superhelical conformation and were not simply degradative products generated solely by nicking of ccc DNA. This banding pattern was strikingly similar to topoisomers of ccc DNA generated by nicking and religation of nicked molecules [6]. The specific cause and nature of these stair-step bands in the lactobacilli were not further investigated. However, the results demonstrated that extended 37°C incubation with mutanolysin or lysozyme altered the banding pattern of small plasmids and either eliminated large plasmids or reduced them to nondetectable levels.

**Plasmid distribution in *Lactobacillus* species.** Six strains of lactobacilli were examined for plasmid DNA using the mutanolysin (0°C, 1 h) procedure to determine the usefulness of this method for rapid

plasmid purification and identification in this genus. With the exception of *L. lactis* 1438, plasmid DNA was detected in all strains examined (Fig. 5).

## Discussion

Conventional lysis and plasmid purification techniques have routinely been ineffective for lactobacilli. In a previous study from our laboratory, only one of eight strains of *L. acidophilus* was shown to harbor plasmid DNA [13]. Similarly, Vescovo et al. [21] identified plasmid DNA in only 19% of *L. acidophilus*, 27% of *L. helveticus*, and 5% of *L. bulgaricus* strains examined. Furthermore, plasmid DNA was not detected in nine strains of *L. plantarum* examined. Difficulty in isolation of plasmid DNA from lactobacilli could be attributed directly to the lysozyme insensitivity of this genus [18]. Noting the level of resistance, investigators have reported methods to optimize lysozyme action [1, 4] and thus enhance cell lysis and plasmid DNA yields. However, these methods continue to employ a 37°C incubation for 1 h to facilitate cell wall digestion by lysozyme [4]. Data presented in this study demonstrated that extended incubation at 37°C is unnecessary and, in fact, may be detrimental to plasmid isolation in some *Lactobacillus* species. A similar reponse to 37°C incubation with lysozyme was previously reported for a 30 Mdal plasmid from *Streptococcus lactis* C2 [12]. The events reponsible for plasmid loss or alteration noted for the lactobacilli are unknown. However, it is apparent that failure to detect plasmid DNA in a number of *Lactobacillus* strains could be the direct result of the extended incubation times at 37°C typically used for plasmid isolation procedures. Compensation for this effect could employ 0°C, as was done in this study, or, alternatively, a substantial reduction of incubation time using an effective cell wall lytic enzyme, such as mutanolysin.

The method described in this study was suitable using either mutanolysin or lysozyme and was 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, plasmid patterns were consistent with CsCl–EB purified samples, and comparisons of plasmid content can easily be made without routine density gradient purification of DNA samples. The method was applicable to *L. acidophilus, L. bulgaricus, L. helveticus*, and *L. plantarum* species, and further, provides the first evidence for the presence of plasmid DNA in *L. plantarum*. The availability of a rapid and effective method for plasmid isolation in the lactobacilli will, hopefully, facilitate future developments on the plasmid genetics of this genus.

ACKNOWLEDGMENTS

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