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A Plasmid of *Lactococcus lactis* subsp. *lactis* ML8 Linked with Lactose Metabolism and Extracellular Proteinase

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Three distinct plasmids, with approximate molecular weights of 1, 4.5, and 33 megadaltons, were found in *Lactococcus lactis* subsp. *lactis* (*L. lactis*) ML8. Slow acid-producing mutants of *L. lactis* ML8, isolated by plasmid curing with acriflavine treatment, lacked the 33-megadalton plasmids. The plasmid-cured mutant showed lactose-negative (Lac$^-$) characteristics and the alteration of extracellular proteinase pattern. The possible involvement of extracellular proteinase with the 33-megadalton plasmid is highlighted in this research.

Lactococcus lactis subsp. *lactis* and *cremoris* (*L. lactis* and *L. cremoris*) have been used as starters in the manufacture of cheese and other fermented dairy products. The lactococci are all nutritionally fastidious, so their proteolytic systems are important both as a means of making protein and peptide nitrogen available for growth and as an agent of the ripening process which gives the foods their characteristic rheological and organoleptic properties. However, the proteolytic activity is not a stable property. Several workers (3, 21) have shown that certain pure cultures of *L. lactis* or *L. cremoris* that rapidly coagulate milk can be divided into fast- and slow-coagulating strains. The coagulation of milk by the slow strains is delayed compared with that of the fast-strains. Westhoff *et al.* (21) examined spontaneous slow variants of *L. lactis* no. 3 and attempted to identify the proteinase that was lost during conversion from fast-strain to slow-variants. The proteinase isolated from the membrane fraction of disrupted cells of the variants was similar to that of the parent strain. In a subsequent publication (22), they examined the possible involvement of the intracellular proteinase in the slowness of the variant.

Including proteinase activity, several important metabolic traits are unstable in lactococci. Throughout the research on plasmids in lactococci, the existence of a wide complement of plasmids and some characteristics related with the plasmids were elucidated (14). Properties that have been linked to plasmids include metabolism of nutrients such as lactose (1, 6, 10, 15, 18, 19), protein (6, 10, 15, 18), sucrose, glucose, mannose, xylose, and galactose, resistance to bacteriophages including bacteriophage adsorption, restriction-modification systems, production of antibiotics such as diplococcin, nisin, and kanamycin, resistance to nisin, and resistance to inorganic ions (14).

In the present study, we selected the 33-megadalton (Mdal) plasmid-cured mutants of *Lactococcus lactis* subsp. *lactis* ML8 by acriflavine treatment and examined the alteration of extracellular proteinase (ECP) to elucidate the involvement of the plasmid with extracellular proteinase which can be a factor influencing milk coagulation.

MATERIALS AND METHODS

Bacterial Strains and Media

L. lactis ML8 was obtained from the stock culture of the Department of Food Science and Technology, Seoul National University and *L. lactis* ML3 was kindly supplied by L. L. Mckay, Department of Food Science and Nutrition, University of Minnesota. The cultures were maintained by biweekly transfer in 10% nonfat dry milk (NFDM) at 30°C until milk coagulation and stored at -20°C. Lactose broth (Difco) was used to monitor the growth of organisms in the liquid media. Plasmid-cured mutants were maintained in MRS broth (Difco) at 30°C. For isolation of plasmid DNA, MRS broth supplemented with 20 mM DL-threonine was used (13). Casein medium containing casein solubilized in 0.1 N sodium hydroxide, yeast extract, glucose at the concentration of 1%, 0.2%, and 1% in 0.1 M phosphate buffer (pH 7) was used for

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ECP preparation. The pH was adjusted with 0.1 M HCl to 7.

Plasmid Curing and Selection of Mutants

Curing of plasmids was achieved by adding 6 μg of acriflavine per ml to the 1% inoculum of the culture in lactose broth and reacting for 3 h (9, 17). After 3 h-incubation, the culture was diluted with water and plated on milk agar plate containing 10% NFDM, 1.5% agar, and 0.004% bromocresol purple (BCP) for selection of slow acid-producing mutants (6). On this plate, the color of the medium changes to yellow as the pH of the medium decreases.

Preparation and Electrophoresis of Plasmid DNA

Extraction and detection of plasmid DNA was performed according to the method of Anderson and Mckay (2).

Preparation of ECP

L. lactis ML8 and its mutants were grown in casein medium for ECP isolation. Casein media were inoculated with 1% seed culture, and incubated at 30°C for 12 h with the wild-type strain and for 14 h with the mutant strains. By then both strains reached stationary phase. After incubation, the culture broth was centrifuged at 10,000×g for 25 min, and the supernatant was used as crude ECP solution (4, 5, 20).

DEAE-Sephadex Chromatography

To the crude ECP solution, ammonium sulfate was added to 70% saturation after elimination of the precipitate with 25% saturation. The precipitate was dissolved in a small amount of 0.03 M phosphate buffer (pH 6.5) followed by dialysis against the same buffer overnight. The dialyzate was applied on a DEAE-Sephadex A-50 column (2.5×20 cm, Pharmacia) previously equilibrated with the same buffer. The column was washed with 150 ml of the same buffer and eluted by linear NaCl gradient from 0 to 1.2 M in 0.03 M phosphate buffer (pH 6.5) at a flow rate of 20 ml/h. Protein was determined by measuring absorbance at 280 nm.

Determination of Acid Production and Proteinase Activity

Acid production was determined by measuring pH in cultures of 1% seed culture inoculation.

Casein solubilized with 0.1 N NaOH and diluted with equal volume of 0.2 M phosphate buffer (pH 7.0) to give a final concentration of 1.0% of casein (final pH was adjusted to 7.0 with 0.1 N HCl) was used as the substrate solution for proteinase activity. For proteinase assay, an appropriate amount of enzyme solution was mixed with 3 ml of the substrate solution and incubated at 37°C for 1 h. The reaction was terminated by adding the same volume of 12% trichloroacetic acid (TCA) solution. The mixture was filtered (Whatman No. 42) and the amount of TCA soluble N in the filtrate was determined according to the method of Lowry *et al.* (16) and of Hull (12). One unit of proteinase activity was defined as the enzyme amount that produced 1.0 μg of TCA soluble tyrosine in 1 h.

RESULTS AND DISCUSSION

Selection and Characterization of the Plasmid-cured Mutants of *L. lactis* ML8

Several strains of *L. lactis* were shown to have a plasmid involved in lactose utilization and proteinase activity (6, 10, 15, 18). In order to screen proteinase-negative strains of *L. lactis* ML8, we tried plasmid curing. After 3 h-incubation in acriflavine containing lactose broth, the cell suspension of about 100 colony forming units (CFU) was plated onto milk agar plates. Slow acid-producing mutants can be screened by the size of yellow zone which is made as the result of pH decrease. As the pH of the medium decreases, the color of bromocresol purple (BCP) is changed from purple to yellow. Slow acid-producing mutants made small yellow zones compared with the wild strain. The mutants selected from the milk agar were grown in MRS broth for several generations and transferred to fresh milk agar to confirm whether the property was fixed. Finally, four mutants were selected and named as strain PD31, strain PD32, strain PD34, and strain PD43.

To elucidate the characteristics of the four mutants involved in slow acid production, we tested the growth and acid production of the mutants during growth (Fig. 1). Panel A shows the growth of both mutants and the wild-type strain in lactose broth which contained lactose as a carbon source. The wild-type strain reached a maximum population in 10 h while all the mutants ceased exponential growth after 6 h and the cell concentration (A_{580}) was about 1/4 of the wild-type strain indicating that lactose can not be utilized as a carbon source by the mutants. This result implies that the selected mutant strains PD31, PD32, PD34, and PD43, lost their lactose utilizing ability. Acid production of the mutants during growth was tested in milk broth supplemented with 2% glucose to reduce the decrease of growth resulting from their loss of lactose utilizing ability (Panel B of Fig. 1). The wild-type strain showed normal acid production until 16 h incubation but all four mutants stopped acid production after 12 h incubation and the pH of the broth remained around 5.2. This result suggests that the mutants may utilize the supplemented glucose for growth but glucose supplement was not sufficient for their growth to match that of the wild-type strain. From this result, the deficiency of available nitrogen nutrients or the proteolytic system to produce them is postulated as being the reason for the mutants not growing like the wild-type strain. Acid production and the growth of mutants were restored to normal levels by supplementing the milk broth with 2% glucose and 1% casein hydrolyzate (Panel C of Fig. 1). These results made it evident that the mutant strains PD31, PD32, PD

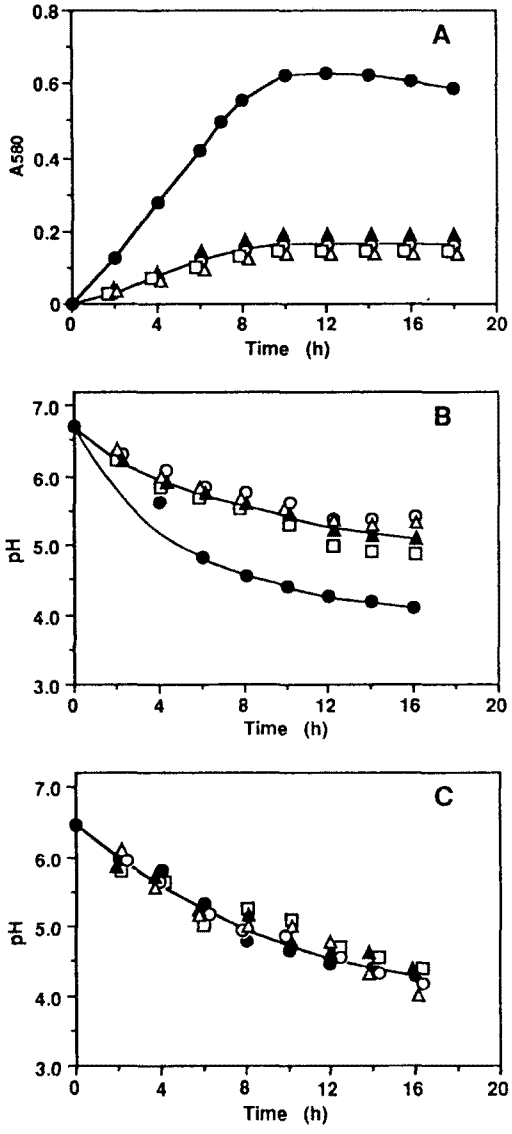

Fig. 1. Growth and acid production of *L. lactis* ML8 and its mutants.

Panel A, growth in lactose broth. Panel B, acid production in milk broth supplemented with 2% glucose. Panel C, acid production in milk broth supplemented with 2% glucose and 1% casein hydrolyzate. Symbols: ●, wild strain ML8; ○, strain PD31; ▲, strain PD32; △, strain PD34; □, strain PD43.

34, and PD43 lack the ability of lactose metabolism (Lac$^-$) and a certain proteolytic system.

Plasmid Profiles of Wild-type Strain and Mutants

To identify the cured plasmid by acriflavine treatment, we extracted plasmids from both wild-type strain and mutants. In *L. lactis* ML8, three plasmid species were observed corresponding to the sizes, 1.0, 4.5, and 33 Mdal. These were determined by observing relative mobility against the plasmid DNAs of *L. lactis* ML3 (Fig. 2). The molecular weight of plasmid DNAs in *L. lactis* ML3 had been determined by Anderson and Mckay (2) as 1.0, 2.0, 5.5, and 33 Mdal. The three plasmids of *L. lactis* ML8 always appeared in replicate electrophoresis, while the feeble plasmid band, shown in the 13 Mdal position, seemed to be a nicked circular form produced during the purification procedure.

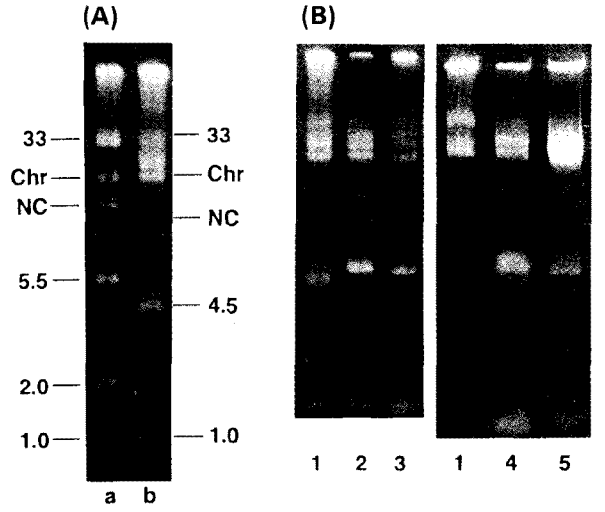

Fig. 2. Agarose gel electrophoresis of the plasmid DNAs of *L. lactis* ML8 and its plasmid-cured mutants.

Panel A shows the migration profile of the plasmid DNAs of *L. lactis* ML3 (lane a) and *L. lactis* ML8 (lane b). Panel B shows the migration profile of the plasmid DNAs from strains ML8 (lane 1), PD31 (lane 2), PD32 (lane 3), PD34 (lane 4), and PD43 (lane 5). Plasmid sizes are designated in Mdal. Chr, chromosomal DNA band; NC, nicked circular DNA band.

In case of the mutant strains, the lack of the 33-Mdal plasmid was easily detected. The lack of the 33-Mdal plasmid in the mutant strains brought us to the conclusion that the 33-Mdal plasmid in *L. lactis* ML8 is responsible for lactose metabolism and proteinase activity. These data coincide with the findings of Klaenhammer *et al.* (12), and Kuhl *et al.* (13) that the largest plasmid of 33-45 Mdal in the *L. lactis* C10, ML3, M18 strains are linked with lactose metabolism and proteinase activity.

DEAE-Sephadex Chromatography of Crude ECP

The crude ECPs of wild-type strain and mutant strain PD34 were separated with a DEAE-Sephadex A-50 column and the separation patterns of both strains were compared under the same conditions to evaluate the plasmid curing effect. The concentrated sample produced by salting out with ammonium sulfate was applied to the column after dialysis. In the case of the wild-type strain, enzyme activity was detected primarily in the two parts. Fraction I was eluted by the buffer containing 0-0.2 M NaCl and fraction II was eluted by the buffer containing 0.6-0.9 M NaCl (Fig. 3). The crude ECP of the mutant

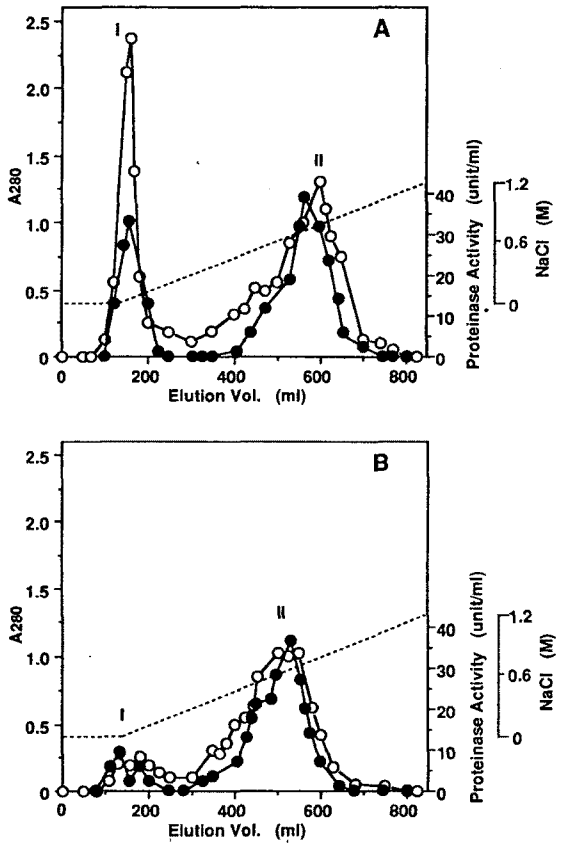

Fig. 3. DEAE-Sephadex column chromatographies of the ECPs from *L. lactis* ML8 (A) and its plasmid-cured mutant, strain PD34 (B).
The effluent from the column was collected and absorbance at 280 nm (○) and proteinase activity (●) were measured. The dotted line indicates NaCl concentration.

strain PD34 was also separated into the two parts which were eluted by the buffer containing 0-0.2 M NaCl and 0.6-0.9 M NaCl (Fig. 3). However, the proteinase activity and protein quantity of fraction I of the mutant was reduced by about three times, while those of fraction II maintained a relatively constant level similar to that of the wild-type strain. It is well known that the proteolytic system of *L. lactis* and *L. cremoris* is composed of several different proteolytic enzymes (7, 8, 11). This suggests that peak I and II are composed of several different proteolytic enzymes and one of the proteinases in peak I is encoded on the 33-Mdal plasmid in the wild-type strain. Though, some research has shown the identity of plasmids involved in proteinase activity and lactose utilization, the specific correlation between a plasmid and extracellular proteinase has not previously been reported. In this research, we suggest the involvement of the 33-Mdal plamid of *L. lactis* ML8 with extracellular proteinase.

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