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Plasmids, Lactic Acid Production, and *N*-Acetyl-D-Glucosamine Fermentation in *Lactobacillus helveticus* subsp. *jugurti*

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Two lactobacillus strains, *Lactobacillus helveticus* subsp. *jugurti* S13-8 and *L. helveticus* subsp. *jugurti* S36-2, were examined for the presence of plasmids. Plasmids of 16.45, 13.03, and 11.83 kilobases (kb) were found in the first, low lactic acid-producing strain; their function is not presently known. A single plasmid species of 13.17 kb was found in the second, high lactic acid-producing strain. This plasmid was found to be associated with lactic acid production and *N*-acetyl-D-glucosamine fermentation.

Lactic acid bacteria are used in the dairy industry for their ability to produce lactic acid from lactose. Recently it was shown that the ability of *Streptococcus lactis* to ferment lactose is due to the presence of a plasmid (4, 11). It has been shown that plasmids and lactose metabolism in *Streptococcus cremoris* B_1 are correlated (1). Extrachromosomal elements of unknown function have been observed in strains of *Lactobacillus casei* isolated from humans (3, 14). Plasmids have been implied in the lactose metabolism of *L. casei* (8). In addition, strains of *L. helveticus* subsp. *jugurti* vary in their capacity to produce lactic acid (13). A study was therefore carried out to determine why some of these strains are capable of producing more lactic acid than others. Two strains of *L. helveticus* subsp. *jugurti*, a high acid producer and a low acid producer, were chosen initially for study as a model system. This paper presents the first evidence of plasmids in cheese starter cultures of *L. helveticus* subsp. *jugurti* and their involvement in *N*-acetyl-D-glucosamine fermentation and lactic acid production.

MATERIALS AND METHODS

Organisms. The strains used in this study were *L. helveticus* subsp. *jugurti* S36-2, a high acid producer, and *L. helveticus* subsp. *jugurti* S13-8, a low acid producer. These strains were obtained from the Institute of Agricultural Microbiology, University of Bologna, Bologna, Italy. They were originally isolated from Parmigiano-Reggiano cheese starters (12, 13).

Media and culturing conditions. The organisms were maintained by monthly transfer in a suspension of powdered skim milk (10% wt/vol)-yeast extract (0.1% wt/vol) in water and sterilized by autoclaving at 115°C for 30 min. Cultures were incubated until the milk coagulated and held between transfers at 5°C. Lactobacilli MRS broth was used to propagate the organisms in a liquid medium and was prepared as directed by the manufacturers (7). Agar (1.5%) was added to lactobacilli MRS broth to make plating media. Incubation for surface colonies was in an atmosphere of hydrogen and CO_2 within a GasPak anaerobic (BD & Co.) system. In all cases during this study a temperature of 42°C was used for incubations.

Characterization of lactobacilli. Parent and isolate strains were subjected to the API lactobacillus characterization system as directed by the manufacturers.

Treatment of lactobacilli with acriflavine. Cultures grown in lactobacilli MRS broth were transferred to fresh medium containing 3,6-diamino-10-methyl-acridinium chloride (acriflavine) (7 μg/ml) and incubated for 24 h. Cultures were then plated on lactobacilli MRS agar. After incubation, individual colonies were isolated and cultured in lactobacilli MRS broth. The above procedure was also carried out in the presence of 1% glucose.

Assay of lactic acid. Lactic acid produced by organisms after culturing in skim milk-yeast extract medium for 8 h was determined colorimetrically with ferric chloride reagent as described by Lunder (10). L(+) lactic acid was analyzed enzymatically by the method described (10). D-Lactic acid was estimated by difference.

Radioactive labeling of bacterial cultures. Overnight cultures in lactobacilli MRS broth were incubated at 1% into 1 liter of lactobacilli MRS broth containing [^{3}H]thymidine (2.5 μCi/ml), deoxyadenosine (250 μg/ml), and 0.02 M D-L threonine to facilitate cell lysis as described by Chassy (2). After overnight incubation, bacteria were harvested by centrifugation at 5,000 × *g*, washed twice, and suspended in 50 ml of 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 8.0) containing 0.005 M ethylenediaminetetraacetic acid and 0.05 M NaCl (TES buffer).

Preparation of bacterial lysates. To the cell

suspension, lysozyme was added to a concentration of 1 mg/ml and incubated for 3 h at 42°C. Sodium lauryl sulfate was added to a concentration of 1% plus Pronase (1 mg/ml), as suggested by Lee and Rieman (9), and incubation was continued for 3 h. This procedure resulted in highly viscous lysates although they were not always clear. Further lysis was aided by freezing to −20°C followed by rapid thawing. Before plasmid isolation on cesium chloride-ethidium bromide density gradients, lysates were sheared, alkaline denaturated, phenyl extracted, and ethanol precipitated as described by Currier and Nester (5). This precipitate was collected by centrifugation at 600 × *g* for 5 min and then dissolved with gentle swirling in a minimum of 0.1 M ethylenediaminetetraacetic acid (pH 8.0). The concentrated deoxyribonucleic acid (DNA) solution was dialyzed against TES buffer overnight.

Cesium chloride-ethidium bromide density gradient centrifugation. The above DNA solution adjusted to 9.0 ml with TES buffer was added to 9.0 g of cesium chloride; to this solution was added 0.675 ml of ethidium bromide solution (10 mg/ml in distilled water). The solution was centrifuged to equilibrium in cellulose nitrate tubes (50 to 70 h at 40,000 rpm [International ultracentrifuge model B-60 fixed-angle rotor-type 269]). Gradients were fractionated from the top by a density gradient fractionator. Approximately 50 fractions were collected. The refractive index (n_D^{25}) was determined for several fractions with a Zeiss Abbe refractometer.

The radioactivity of fractions was assayed after placing 5 μl directly into scintillation vials with water (1 ml) and Aquasol Universal LSC cocktail scintillation fluid (15 ml) by counting in a Searle liquid scintillation spectrometer.

Electron microscopy of plasmid DNA. Fractions containing covalently closed circular (CCC) DNA were pooled, and ethidium bromide was removed by four extractions with isopropyl alcohol saturated with 6 M NaCl and finally dialyzed against 0.001 M ethylenediaminetetraacetic acid–0.01 M Tris-hydrochloride buffer (pH 8.2) to remove the CsCl. After removal, the CCC DNA was prepared for electron microscopy by a modification of the Kleinschmidt technique (6). Electron microscope grids were prepared by using preparations that had been stored at 4°C for about 1 month to allow single-strand nicking. An A.E.I. EM6M electron microscope was used to visualize the DNA. Photographs were taken and projections were traced on paper. Contour lengths were measured with a curvimeter. The sizes of plasmids were determined in kilobase (kb) pairs by comparing plasmid contour lengths with those of a simian virus 40 (SV40) viral DNA of 5.16 kb introduced into samples at the time of grid preparation. This technique eliminated error due to any stretching that might have occurred in preparation of grids.

Materials. Bacterial culture media were obtained from Difco Laboratories, Detroit Mich.; 3,6-diamino-10-methyl-acridinium chloride (acriflavine) was obtained from Fluka AG. Buchs, Switzerland; deoxyadenosine was obtained from Koch-Light Laboratories, Colnbrook, England; [^{3}H]thymidine was obtained from The Radiochemical Centre, Amersham, England; β-nicotinamide adenine dinucleotide came from Sigma Chemical Co., St. Louis, Mo.; L-lactate dehydrogenase came from Boehringer Mannheim Corp., Mannheim, West Germany; lysozyme was obtained from Serva, Heidelberg, Germany; Pronase came from Calbiochem, La Jolla, Calif.; GasPak Anaerobic system was obtained from BBL, Division of Becton Dickinson & Co., Rutherford, N.J.; API lactobacillus identification system was from API Systems S.A., Versciex, France; and the density gradient fractionator was obtained from Instrument Specialities Co., Lincoln, Neb. SV40 viral DNA was the gift of P. K. Wellauer, Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland, All other chemicals, unless otherwise stated, were from E. Merck, Darmstadt, West Germany.

RESULTS AND DISCUSSION

After acriflavine treatment of strain S13-8, no colonies that differed in their response to any of the 50 API diagnostic tests could be isolated. However, after acriflavine treatment of strain S36-2, approximately 60% of colonies isolated were found to have lost their ability to ferment *N*-acetyl-D-glucosamine, whereas less than 1% of colonies of this type were isolated from control cultures. No *lac* isolates of strain S36-2 could be induced by this treatment even when treatment and isolation were made in the presence of glucose. Isolates of strain S36-2 incapable of fermenting *N*-acetyl-D-glucosamine were tested for their ability to produce lactic acid in skim milk medium. All isolates tested were found to have reduced lactic acid-producing ability and to a level similar to that of the poor acid-producing strain S13-8 (Table 1). Further acriflavine treatment of these isolates was unsuccessful in obtaining isolates with further reduction in lactic acid-producing ability. Neither could isolates be obtained after acriflavine treatment of strain S13-8 that had further reduced acid-producing ability. Strains of *L. helveticus* subsp. *jugurti* produce both L- and D-lactic acid. However, the proportion of these two forms remained similar for reduced acid-producing strains as for the parent strains (Table 1). Consequently, reduction of lactic acid production was not due to an inability to produce one of the isomers.

It was therefore decided to determine whether plasmids were present in strains S13-8 and S36-2 and isolates of S36-2 with reduced acid production. Satellite CCC DNA peaks were observed with both CsCl-ethidium bromide density gradients prepared from strains S13-8 and S36-2 (Fig. 1). No satellite peaks were found in any of the S36-2 reduced acid-producing isolates (data not shown).

After electron microscopic observation of these satellite peaks, three species of plasmid were found in strain S13-8, corresponding to 16.45, 13.03, and 11.83 kb (Fig. 2–4). Strain S36-

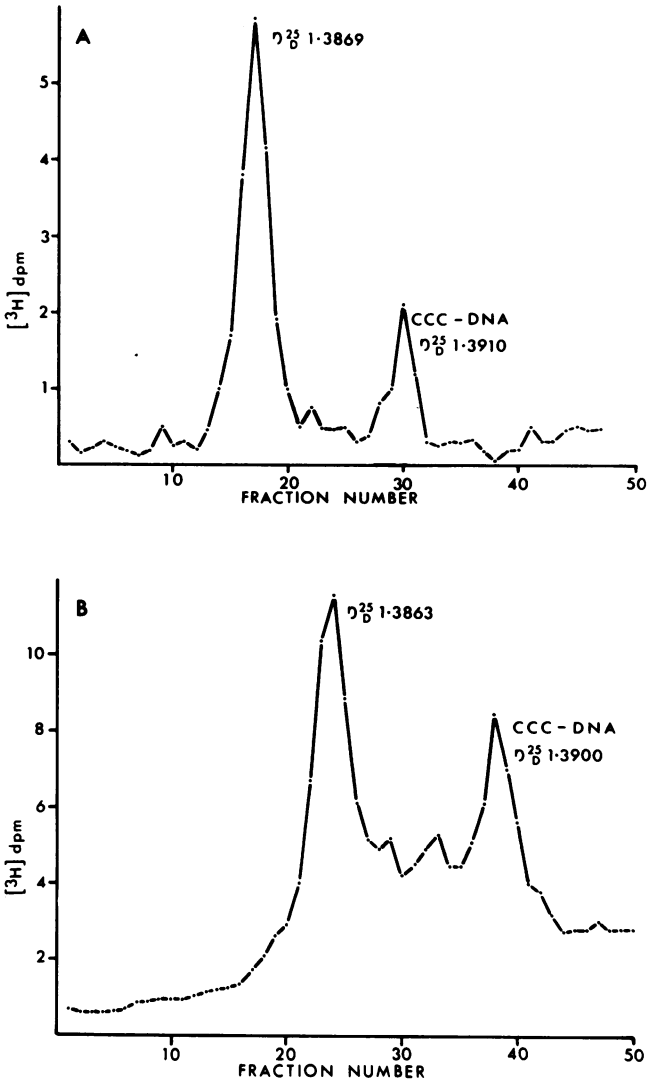

FIG. 1. *Elution profiles of CsCl-ethidium bromide density gradients of DNA from cleared lysate material of L. helveticus subsp. jugurti S36-2 (A) and L. helveticus subsp. jugurti S13-8 (B). Because gradients were fractionated from the top, the fractions from the bottom of tubes are those in the right hand portions of the figure.*

TABLE 1. *Lactic acid production in strains of L. helveticus subsp. jugurti*

Strain	% Lactic acid in skim milk	Relative proportion of L-lactic acid/D-lactic acid
S13-8	1.5	57/43
S36-2	2.4	52/48
S36-2 isolate 1	1.5	54/46
S36-2 isolate 2	1.6	53/47
S36-2 isolate 3	1.35	55/45
S36-2 isolate 4	1.5	54/46

2 was found to contain only one species of plasmid corresponding to 13.17 kb (Fig. 5). No plasmids were observed in CsCl-ethidium bromide-density gradient fractions prepared from the S36-2 isolate cultures, even from fractions where peaks would be expected to occur. Therefore, loss of *N*-acetyl-D-glucosamine fermenting ability and reduced lactic acid-producing ability can be correlated with loss of the 13.17-kb plasmid in strain S36-2.

These results also could indicate that the lower level of lactic acid-producing ability is

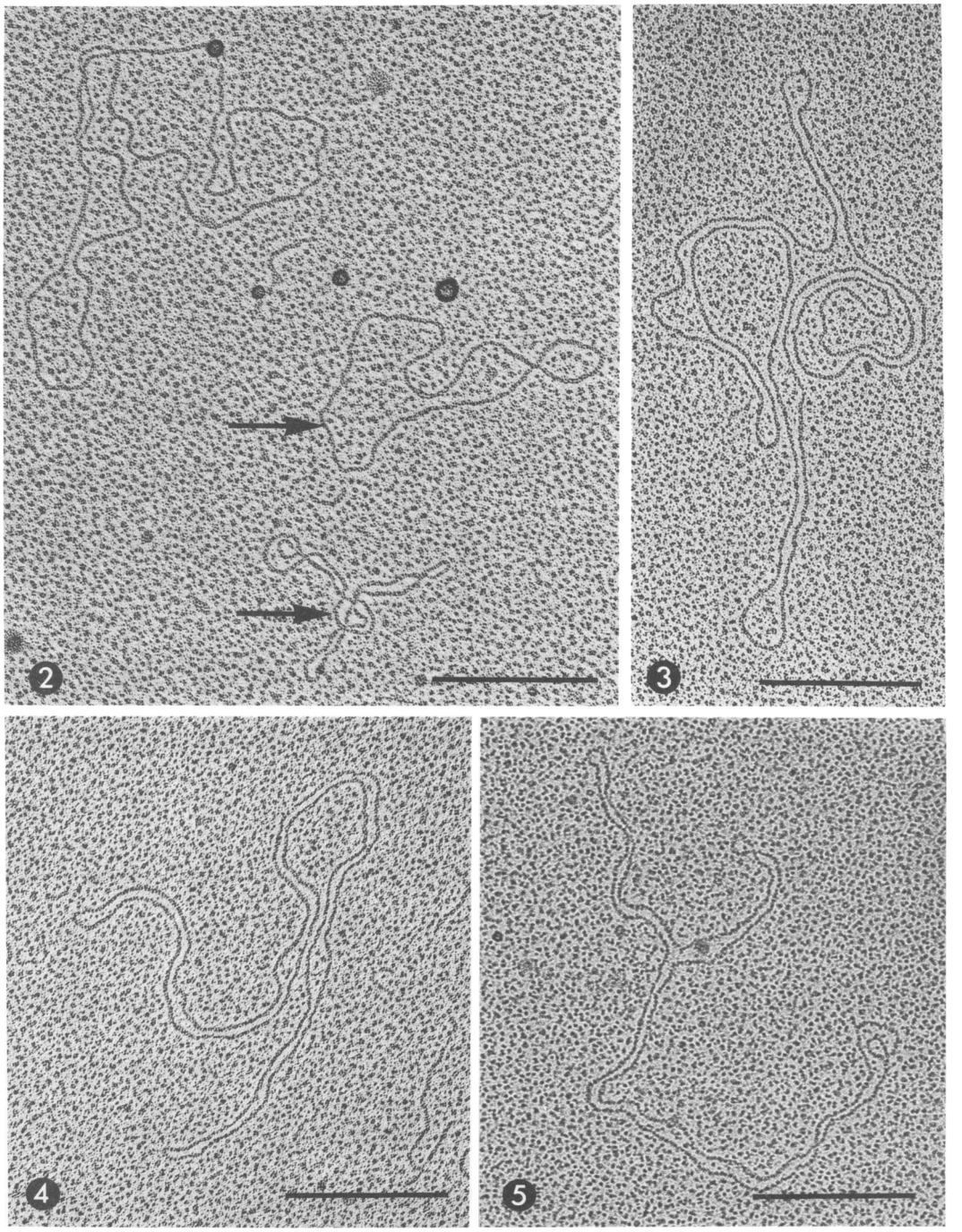

FIG. 2. *Electron micrograph of the 11.83-kb plasmid from strain S13-8, with SV40 virus DNA (arrows). Bar in each figure represents 1 kb (1,000 base pairs).*

FIG. 3. *Electron micrograph of the 16.45-kb plasmid from strain S13-8.*

FIG. 4. *Electron micrograph of the 13.03-kb plasmid from strain S13-8.*

FIG. 5. *Electron micrograph of the 13.17-kb plasmid from strain S36-2.*

under the control of chromosomally located genes in strain S36-2. Since acridine treatment of strain S13-8 did not produce isolates with further reduced acid-producing ability, it would appear that, in this strain also, its low acid-producing ability is under the control of chromosomally located genes. That there was no variation in the proportions of lactic acid (D or L) after curing strain S36-2 indicates that the 13.17-kb plasmid probably contains a positive effector of the chromosomal lactic acid determinant, which results in increased acid production. Research is in progress to develop a transformation system in these strains to determine whether stimulation of acid production can occur if the 13.17-kb plasmid is introduced to low acid-producing strains.

These results differ from those of Hofer (8) with *L. casei* since in our strains lactose metabolism appears to be only partially controlled by plasmid-located genes, whereas, in *L. casei,* lactose metabolism is completely lost after acriflavine treatment. Further study is in progress to determine the functions of the three plasmids in strain S13-8.

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