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Extrachromosomal Elements in Group N Streptococci[1]

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The deoxyribonucleic acid (DNA) of *Streptococcus lactis* C2, *S. cremoris* B_1, and *S. diacetilactis* 18-16 was labeled by growing cells in Trypticase soy broth containing ^{3}H-labeled thymine. The cells were gently lysed with lysozyme, ethylenediaminetetraacetic acid, and sodium lauryl sulfate. The chromosomal DNA was separated from plasmid DNA by precipitation with 1.0 M sodium chloride. The existence of covalently closed circular DNA in the three organisms was shown by cesium chloride-ethidium bromide equilibrium density gradient centrifugation of the cleared lysate material. In an attempt to correlate the loss of lactose metabolism with the loss of plasmid DNA, lactose-negative mutants of these organisms were examined for the presence of extrachromosomal particles. Covalently closed circular DNA was detected in the lactose-negative mutants of *S. lactis* C2 and *S. diacetilactis* 18-16. In *S. cremoris* B_1, however, no covalently closed circular DNA was observed by using cesium chloride-ethidium bromide gradients. Electron micrographs of the satellite band material from *S. lactis* C2 and its lactose-negative mutant confirmed the presence of plasmid DNA. Three distinct plasmids having approximate molecular weights of 1.3×10^6, 2.1×10^6, and 5.1×10^6 were observed in both organisms.

The existence of extrachromosomal genetic determinants (plasmids) is well documented for members of the *Enterobacteriaceae*, *Staphylococcus aureus*, and species of *Pseudomonas* and *Bacillus* (3, 4, 10, 12). Plasmids are characteristically small, covalently closed, circular duplex deoxyribonucleic acid (DNA) molecules which may confer upon the host known genetic functions. Some of the metabolic functions known to be plasmid directed include the ability to conjugate, production of colicins, resistance to antibiotics, and resistance to inorganic ions (20). Recently, a plasmid isolated from *Bacillus pumilus* has been shown to be involved in sporulation (17). To our knowledge, the only reported evidence of plasmid DNA in streptococci was reported recently by Dunny et al. (6) in *Streptococcus mutans*.

The instability of lactose metabolism in strains of *S. lactis*, *S. cremoris*, and *S. diacetilactis* (13, 19, 21, 22, 26) prompted speculation that lactic streptococci may be carrying an extrachromosomal element that is responsible for the ability of cells to ferment lactose (19). Since plasmids or extrachromosomal particles have not previously been described in group N streptococci, it was of interest to obtain direct evidence for their occurrence in this group of microorganisms. In addition, the possibility that plasmid DNA may be responsible for the ability of cells to ferment lactose was examined.

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MATERIALS AND METHODS

Organisms. The strains used in this investigation were *S. lactis* C2, *S. cremoris* B_1, and *S. diacetilactis* 18-16, all obtained from our stock culture collection. The propagation and maintenance of these cultures were described previously (19). The lactose-negative mutants of these strains were isolated by treatment with acriflavine (19). These mutants were grown and maintained in lactic broth (7).

Labeling and extraction of DNA. The procedure used for isolation of plasmid DNA was similar to that described by Guerry et al. (9). The DNA of these organisms was labeled by growing the cells in 30 ml of Trypticase soy broth (pH 7.0; BBL) containing 250 μg of deoxyadenosine per ml (1) and 0.3 ml of [^{3}H]thymine (1 mCi/ml, 18 Ci/mmol, New England Nuclear Corp.) for 8 h at 32 C. Cells were harvested by centrifugation at 5,000 × *g*, washed with 0.03 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 8.0) containing 0.005 M ethylenediaminetetraacetic acid (EDTA) and 0.05 M NaCl (TES buffer), and resuspended in 1.0 ml of 25% sucrose in 0.05 M Tris at pH 8.0. Lysis of cells was accomplished by adding 0.2 ml of lysozyme (5 mg/ml in 0.25 M Tris, pH 8.0) and 0.4 ml of 0.25 M EDTA (pH 8.0) to the cell suspension followed by incubation at 37 C for 1.5 h. To facilitate lysis, 0.4 ml of 5% sodium lauryl

sulfate was added and the tubes were left at 25 C until lysis was complete. A 0.4-ml amount of 5.0 M NaCl (final concentration of 1.0 M) was added, and the tubes were stored overnight at 4 C. The tubes were then warmed to room temperature and centrifuged at 17,000 × *g* for 30 min, and the cleared supernatant was stored at 4 C until further testing.

Preparation of CsCl-ethidium bromide gradients. The cleared lysate material was subjected to CsCl-ethidium bromide equilibrium density gradient centrifugation. Gradients were prepared by the addition of 1 to 2 ml of cleared lysate to 8.5 ml of CsCl solution (20 g/14 ml of TES buffer) and 0.58 ml of ethidium bromide (5 mg/ml) (8). This mixture was mixed thoroughly, and the refractive index was adjusted to 1.3925 ± 0.0001 by the addition of TES buffer or CsCl. The above solution was placed in polyallomar centrifuge tubes and centrifuged for 60 h at 37,000 rpm in a number 40 fixed-angle rotor of a Beckman L3-50 centrifuge. After centrifugation, samples were fractionated by puncturing the bottom of the tubes with a needle and collecting about 60 10-drop fractions. These fractions were precipitated by the addition of 0.5 ml of cold 10% trichloroacetic acid to each tube of collected material. One drop of herring sperm DNA solution (5 μg/ml) was added as carrier. The precipitated material was collected on membrane filters (0.45 μm, HA; Millipore Corp.) that were presoaked in cold 5% trichloroacetic acid plus 5 μg of thymine/ml, washed twice with cold 5% trichloroacetic acid, and the filters were dried in an oven at 60 C. The filters were then placed in scintillation vials containing 10 ml of scintillation fluor of the following composition: 2,5-dinitrophenyloxazole, 6.0 g; 1-4-bis-(5-phenyloxazolyl)benzene, 0.3 g; and toluene, 1 liter. The samples were counted in a Beckman liquid scintillation spectrometer.

Electron microscopy of plasmid DNA. Fractions from the CsCl-ethidium bromide density gradient centrifugation that contained the fast-sedimenting species of DNA (covalently closed circles) were pooled and dialyzed against TES buffer to remove the majority of CsCl and ethidium bromide. After removal, the DNA from this band was prepared for electron microscopy by a modification of the Kleinschmidt technique (5).

RESULTS AND DISCUSSION

Although extrachromosomal particles have been isolated and characterized from a number of bacterial species, their presence in group N streptococci has not been reported. Knittel et al. (15) demonstrated the presence of a population of DNA molecules with a lower average guanosine plus cytosine content in some, but not all, lactic streptococci. These authors suggested that the satellite band of DNA represented the presence of an episome. Their assumption was based on the presence of a pronounced shoulder on a curve of DNA melting-point determinations. More recently, Pearce and Skipper (43rd Annu. Rep. N. Zealand Dairy Res. Inst., p. 36–37, 1971) have considered the spontaneous loss of proteinase production in lactic streptococci. By using procedures similar to those of Knittel et al. (15), these authors attempted to obtain physical evidence for this conclusion by comparing the DNA isolated from the proteinase-positive and -negative organisms. Their evidence neither confirms nor rules out the presence of plasmid DNA. McKay et al. (19) proposed that lactic streptococci could be carrying a genetic element that is responsible for the ability of cells to ferment lactose. The loss of this element could cause the cell to become lactose negative. No direct evidence for the existence of plasmid DNA was presented. In this communication, we describe the isolation of plasmid DNA from the group N streptococci.

Figure 1A shows that, when the cleared lysate material of *S. lactis* C2 was subjected to CsCl-ethidium bromide density gradient centrifugation, the presence of a peak at higher density than that of the chromosomal DNA was observed. The position of this peak is consistent with that predicted for covalently closed circular (CCC) DNA molecules (23). If plasmid is responsible for lactose metabolism in lactic streptococci, CCC DNA may be absent in the lactose-negative variant. Results shown in Fig. 1B suggest that CCC DNA is also present in the lactose-negative cells.

Since CCC DNA was detected in both lactose-positive and -negative cells, no simple relationship appeared to exist between the presence of this plasmid and lactose metabolism. Alternatively, more than one species of plasmid DNA could be present in a single cell. This means that the loss of one plasmid species would not be detected by CsCl-ethidium bromide gradient centrifugation, since all CCC DNA would band together. To obtain evidence for the existence of more than one plasmid, electron micrographs of the dense peak obtained for CsCl-ethidium bromide centrifuga-

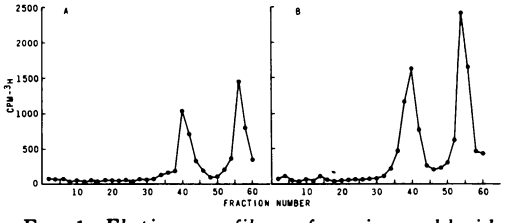

FIG. 1. *Elution profiles of cesium chloride-ethidium bromide gradients of DNA from cleared lysate material of S. lactis C2 (A) and its lactose-negative variant (B). The DNA was labeled with [^{3}H]thymine and extracted as described in Materials and Methods.*

tion of *S. lactis* C2 and its lactose-negative mutant were taken. Electron micrographs revealed the presence of at least one and possibly three distinct plasmid species in both organisms. Molecular weight estimates of the plasmid DNA were obtained by contour length measurements of the open circular DNA (16). Both strains appear to be carrying a small plasmid with a molecular weight of about 1.3×10^6 and another small plasmid with a molecular weight of about 2.1×10^6. A larger plasmid with a molecular weight of about 5.1×10^6 was also observed in both organisms. It is recognized that these observations were made on only a small number of molecules (nine, six, and four molecules, respectively), and therefore the estimates of molecular weight are only approximate. The open circular DNA species isolated from the lactose-negative mutant are illustrated in Fig. 2. The presence of the same plasmid species in both the lactose-negative mutant and the parent strain suggests against, but does not rule out, the plasmid DNA being directly responsible for lactose metabolism in *S. lactis* C2.

Since the existence of plasmid DNA was shown in *S. lactis* C2, it was of interest to determine its presence in other group N streptococci. Figures 3A and B show results of a CsCl-ethidium bromide centrifugation of cleared lysates from *S. diacetilactis* 18-16 and its lactose-negative derivative. The results indicate that both organisms contained CCC DNA as evidenced by the existence of a dense peak separate from the chromosomal DNA.

When *S. cremoris* B_1 was examined, the presence of CCC DNA was detected (Fig. 4A). The lactose-negative derivative of *S. cremoris* B_1 contained very little, if any, CCC DNA (Fig. 4B). The degree of [^{3}H]thymine labeling appeared to be approximately the same in both instances, since the same amount of chromosomal label was obtained. Unlike the other two streptococci examined, in this organism there appears to be a relationship between lactose metabolism and the presence of plasmid DNA. In the case of *S. lactis* C2, the examination of a larger number of molecules will be required before a possible relationship between lactose metabolism and plasmid DNA can be determined. In addition, electron micrographs of plasmid DNA from *S. diacetilactis* 18-16 and *S. cremoris* B_1 are needed to clarify any relationship which may exist.

In an earlier publication (19), it was suggested that the spontaneous loss of lactose metabolism may be due to the loss of a prophage that carried the lactose genes. It has been found that the lactose-negative mutants, when treated with ultraviolet light, undergo lysis with subsequent release of phage. This appears to rule out the possibility that the lactose genes in *S. lactis* C2 are carried on a phage. The presence of the very small plasmid species (1.3×10^6 daltons) in *S. lactis* C2 leads one to another interesting speculation as to the possible mechanism involved in the loss of lactose metabolism. It has been shown that some spontaneous mutations can be due to strong polar mutations that occur as a result of the insertion of a few

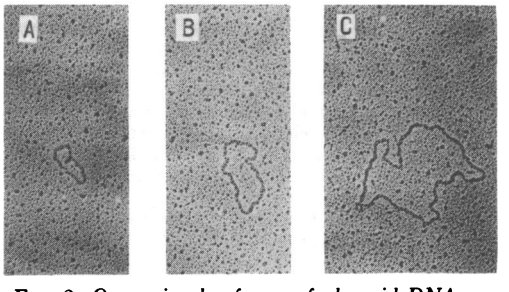

FIG. 2. *Open circular forms of plasmid DNA species isolated from the lactose-negative mutant of S. lactis C2. Magnification factor, 50,000. Molecular weight estimates: A, 1.3 × 10⁶; B, 2.1 × 10⁶; and C, 5.1 × 10⁶. Plasmids with approximately the same molecular weights were also observed in the parent strain.*

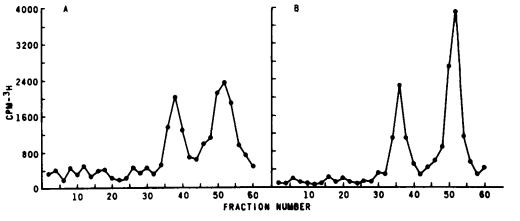

FIG. 3. *Elution profiles of cesium chloride-ethidium bromide gradients of DNA from cleared lysate material of S. diacetilactis 18-16 (A) and its lactose-negative variant (B). The DNA was labeled with [³H]thymine and extracted as described in Materials and Methods.*

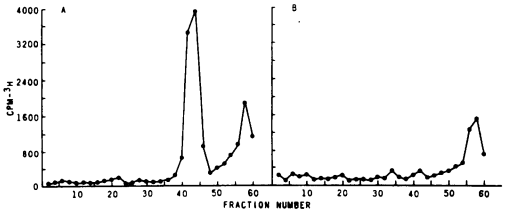

FIG. 4. *Elution profiles of cesium chloride-ethidium bromide gradients of DNA from cleared lysate material of S. cremoris B_1 (A) and its lactose-negative variant (B). The DNA was labeled with [³H]thymine and extracted as described in Materials and Methods.*

nucleotide pairs into the continuity of a gene in the *gal* (11, 14, 25) and the *lac* operon (18) of *Escherichia coli* K-12 and the *CIIOP* operon (2) of phage λ. In the case of the *gal* operon, Jordan et al. (14) showed that the molecular weight of the largest insertion was approximately 1.2×10^6. The origin of this inserted material is unknown. Saedler and Heib (24) suggested a few small plasmids exclusively carrying insertion sequences as the most likely source of insertion material. These authors were unable to detect small plasmids exclusively carrying insertion sequences in *E. coli* K-12. The insertions are made in multiple copies per cell (24); therefore, if the lactose-negative mutants were due to insertion of plasmid, the extrachromosomal species of the two strains would appear identical in CsCl-ethidium bromide gradients or by electron microscopy. The possibility that the spontaneous loss of lactose metabolism in lactic streptococci is due to insertion of plasmid DNA into the bacterial chromosome is presently under investigation.

The presence of extrachromosomal elements in group N streptococci has been established. The function of these elements is presently unknown. Further research is being conducted in an effort to establish their function, and a variety of *S. lactis* C2 mutants are being examined in this regard. In addition, other strains of *S. lactis*, *S. cremoris*, and *S. diacetilactis* are being examined for the presence of extrachromosomal particles.

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