APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1978, p. 944-952
0099-2240/78/0036-0944$02.00/0

Vol. 36, No. 6
Printed in U.S.A.

Isolation and Characterization of Plasmid DNA in *Streptococcus cremoris*†

L. D. LARSEN AND L. L. McKAY*

Department of Food Science and Nutrition, University of Minnesota, St. Paul, Minnesota 55108

Received for publication 11 September 1978

Nine industrially important strains of *Streptococcus cremoris* (HP, AM_2, ML_1, WC, C_3, R_1, E_8, KH, and Wg_2) were shown to possess a diversity of plasmid molecules. Molecular weights of plasmids were determined from their relative mobilities after agarose gel electrophoresis and via electron microscopy. To illustrate the varied plasmid sizes, strain HP contained plasmids of 26, 18, 8.5, 3.3, and 2 megadaltons (Mdal); strain ML_1 contained plasmids of 29, 18, 9, 4, 2.2, and 1.8 Mdal; and strain AM_2 had plasmids of 42, 27, 16, and 8.4 Mdal. The numbers of plasmids observed in the other strains were 6, 5, 5, 7, 5, and 4 for C_3, E_8, KH, R_1, WC, and Wg_2, respectively. A spontaneous proteinase-negative (Prt^-) mutant of HP was missing the 8.5-Mdal plasmid, which suggests that in this strain proteinase activity could be linked to this particular plasmid. A lactose-negative (Lac^-) Prt^- mutant of ML_1 lacked the 2.2-Mdal plasmid. Under the conditions employed, antibiotic sensitivity and heavy-metal susceptibility did not correlate with the missing plasmid in Prt^- HP or in the Lac^- Prt^- ML_1. Curing experiments with AM_2, using acridine dyes and elevated temperatures, did not yield Lac^- variants. AM_2 was also cultured at high dilution rates in a chemostat for 168 h by using a buffered milk or lactic broth at 18 or 32°C with no selection of Lac^- derivatives. The inability to obtain Lac^- variants under conditions known to facilitate plasmid elimination suggests that lactose metabolism is not plasmid-mediated in AM_2.

Cords et al. (2) made the first physical demonstration of plasmid DNA in the group N streptococci. Subsequent investigations have centered on *Streptococcus lactis* because of the availability in strain C2 of a transducing system (23, 26) enabling limited genetic analysis. Four to six different sizes of plasmids have been reported in each of the four strains examined (5). In *S. lactis* C2, determinants for lactose metabolism and proteinase activity have been linked to plasmid DNA by curing experiments and transductional analysis (19). Resistance or sensitivity to specific metal ions (6) and production of the antibiotic nisin (11, 15) have also been correlated with plasmid DNA in certain strains.

S. cremoris B_1 was shown by Anderson and McKay (1) to contain only two plasmids, and one was linked to lactose fermentation. Sinha (30) recently reported a satellite peak of covalently closed circular (CCC) DNA in a cesium chloride-ethidium bromide (CsCl-EB) density gradient of cleared lysate material from *S. cremoris* ML_1. Several investigators (20, 28) have also speculated that the appearance of slow acid-producing variants of *S. cremoris*, resulting from deficiencies in the cell wall-associated proteinase, might be linked to the loss of plasmid DNA.

Since few studies of plasmids in *S. cremoris* have been made, we screened several strains of *S. cremoris* for plasmid DNA to determine whether they would resemble *S. cremoris* B_1 or whether they would contain a multiplicity of plasmids as do strains of *S. lactis*. In the present investigation, the sizes of the plasmid DNA molecules isolated from nine strains of *S. cremoris* were characterized by using electron microscopy (EM) and/or agarose gel electrophoresis. In addition, the plasmid profiles of several parent strains and selected metabolic-deficient mutants are reported.

MATERIALS AND METHODS

Microorganisms and plasmids. *S. cremoris* HP and its spontaneous Prt^- derivative (designated EX1 in our laboratory) were received from F. A. Exterkate (Netherlands Institute for Dairy Research, Ede, The Netherlands). *S. cremoris* strains AM_2, C_3, ML_1, WC, R_1, E_8, Wg_2, and KH were from the bacterial stock collection maintained in our laboratory. The strain designations and relevant phenotypes are shown in Table 1. *Escherichia coli* K-12 sublines J5, J53, and

† Scientific Journal Series paper no. 10396, Minnesota Agricultural Experiment Station, St. Paul, MN 55108.

C600, with resident plasmids RP4, Sa, and RSF1010, respectively, were provided by J. H. Crosa (University of Washington, Seattle). These previously characterized (3, 10, 12) plasmids served as mobility reference standards during agarose gel electrophoresis.

Media and culture conditions. *E. coli* was maintained on nutrient agar slants and grown in brain heart infusion broth at 37°C for the isolation of plasmid DNA. *S. cremoris* strains were maintained with biweekly transfer in Elliker broth (7) or M17 broth (32) and stored at 4°C. Lac⁻ mutants were maintained in glucose broth (7).

For isolation of plasmid DNA from *S. cremoris*, the strains were grown in Trypticase soy broth, Elliker broth, or a revised (14) Elliker broth, termed LB, which contains 2.4 g of D,L-threonine per liter. As an alternative to LB, cells were grown in steamed (1 h at 100°C), nonfat dry milk (11% wt/vol) containing 2.4 g of D,L-threonine per liter. The cells from the latter were recovered after growth by raising the pH to 6.9 with 6 N NaOH, adding 0.4% (wt/vol) sodium citrate, mixing thoroughly, and then holding on ice for 30 min before centrifuging to pellet the cells (31).

Isolation of plasmid DNA. DNA from cells grown 10 h at 32°C in Trypticase soy broth or Elliker broth was isolated by following the previously described lysozyme (0.6 mg/ml, 1.5 h, 37°C)-sodium dodecyl sulfate-salt procedure (2). DNA from cells grown 5 to 7 h in LB was isolated by using a shortened lysozyme (2 mg/ml, 8 to 10 min, 37°C)-SDS-salt protocol (14). These crude lysates were centrifuged at 22,000 × *g* for 30 min to sediment most of the chromosomal DNA and membranes. The supernatant (cleared lysate) containing the supercoiled DNA was further purified by the dye-buoyant density procedure of Radloff et al. (29). CsCl-EB gradients contained 2 to 3 ml of cleared lysate, 4.5 ml of TES buffer [50 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 30 mM tris(hydroxymethyl)aminomethane (Tris)], pH 8.0, 7 g of CsCl, and 250 μg of EB per ml and were adjusted with TES buffer to obtain a refractive index of 1.3885 to 1.3914. Gradients were formed in Beckman Type 40 or 50 Ti fixed-angle rotors at 37,000 to 41,000 rpm at 15°C for 48 to 62 h.

To identify the satellite band of CCC plasmid DNA in the CsCl-EB density gradients, the cells were grown in the presence of 100 μCi of [^{3}H]thymine per ml and 250 μg of deoxyadenosine per ml. The fractionation of the gradient and the counting procedures were previously described (2). The fractions containing the satellite band of plasmid DNA were then identified and pooled. For nonlabeled preparations, the satellite band was detected by using long-wave UV illumination and was removed from the tube with a needle and syringe (14).

EM and agarose gel electrophoresis of plasmid DNA. To obtain open circular (OC) DNA molecules necessary for contour length measurements, the CCC plasmid DNA was held at 4°C for 10 days. Plasmid DNA was then prepared for EM observation by the Kleinschmidt technique as previously described (2, 4). The lengths of molecules having well-defined contours were determined as measured with string overlays or a Hewlett Packard 9107A digitizer. The molecular weight was then calculated by using the equivalence 1.0 μm = 2.07 × 10^6 daltons (16). The agarose gel electrophoresis procedure and isolation of the mobility reference plasmids from *E. coli* were previously described (25). A 10- to 30-μl amount of DNA suspension plus 10 μl of sodium dodecyl sulfate-glycerol-bromophenol blue were applied to a 0.65% agarose (SeaKem, Marine Colloids, Inc.) vertical slab gel (13.8 by 17.2 by 0.3 cm) and subjected to electrophoresis at 40 mA constant current for 3 h.

TABLE 1. *Strains of S. cremoris utilized in this investigation*

Strain designation	Description	Relevant phenotype
AM_2	Parent	Lac⁺ Prt⁺
C_3	Parent	Lac⁺ Prt⁺
LL03	38°C induced mutant of C_3	Lac⁻ Prt⁻
HP	Parent	Lac⁺ Prt⁺
EX1	Spontaneous Prt⁻[a]	Lac⁺ Prt⁻
ML_1	Parent	Lac⁺ Prt⁺
LL01	38°C induced mutant of ML_1	Lac⁻ Prt⁻
WC	Parent	Lac⁺ Prt⁺
LL04	38°C induced mutant of WC	Lac⁻ Prt⁻
R_1	Parent	Lac⁺ Prt⁺
E_8	Parent	Lac⁺ Prt⁺
KH	Parent	Lac⁺ Prt⁺
Wg_2	Parent	Lac⁺ Prt⁺

[a] See Exterkate (8).

Antibiotic resistance and metal ion sensitivity. To test for antibiotic resistance, antibiotic sensitivity disks (Difco Laboratories) were used (6). Antibiotic sensitivity was also examined by incorporating graded concentrations of antibiotic into Elliker agar and preparing replicate inocula with a Steers device. Metal ion sensitivity was evaluated by using blank disks saturated with a predetermined quantity of heavy-metal salts (6).

RESULTS

Plasmid content of several *S. cremoris* strains as determined by EM. Early observations by Anderson and McKay (1) of plasmid DNA in *S. cremoris* B_1 and the now well-established existence of plasmids in strains of *S. lactis* (6, 15, 20) created interest in the content and possible role(s) of plasmids in other strains of *S. cremoris*. Initial observations using *S. cremoris* HP, C_3, and ML_1 revealed that these strains incorporated less [^{3}H]thymine and were less amenable to lysis than were the strains of *S. lactis* (data not shown). Extensive EM observations of the pooled fractions in the satellite regions from a CsCl-EB density gradient revealed few plasmid molecules. The small quantity of DNA observed could have resulted from poor incorporation of label, inefficient lysis be-

cause *S. cremoris* is less sensitive to lysozyme than *S. lactis* (17), insufficient cell numbers, degradative nuclease activity, or a combination of these factors. Larger volumes of cells were then prepared to possibly increase the quantity of plasmid DNA recovered. *S. cremoris* HP was grown in 450 ml of Elliker broth for 10 h, and the cells were lysed by using the same ratio of reagents to culture volume as used in a 30-ml preparation (2). CsCl-EB gradients of the cleared lysate from the larger volume revealed two visible bands of DNA under UV illumination. A plasmid band was also visible from 250 ml of culture, but not from 150- or 30-ml preparations. By using the larger culture volumes, enough DNA was isolated from HP, EX1, AM_2, ML_1, and LL01 to permit construction of plasmid histograms of each strain via EM.

The size and distribution of plasmids observed in Prt^+ HP and EX1, a Prt^- derivative of HP, are shown in Fig. 1. According to the data, *S. cremoris* HP contains at least five plasmids having measurements of 0.87, 1.59, and 3.91, 9.03, and 14.3 μm. These values correspond to molecular weights of 1.8×10^6, 3.3×10^6, 8.1×10^6, 18.7×10^6, and 29.7×10^6. EX1, however, was missing the 8.1-Mdal plasmid, suggesting that it may be linked to proteinase activity in HP. Figure 2 shows the plasmid distribution seen for AM_2. This strain appears to possess plasmids

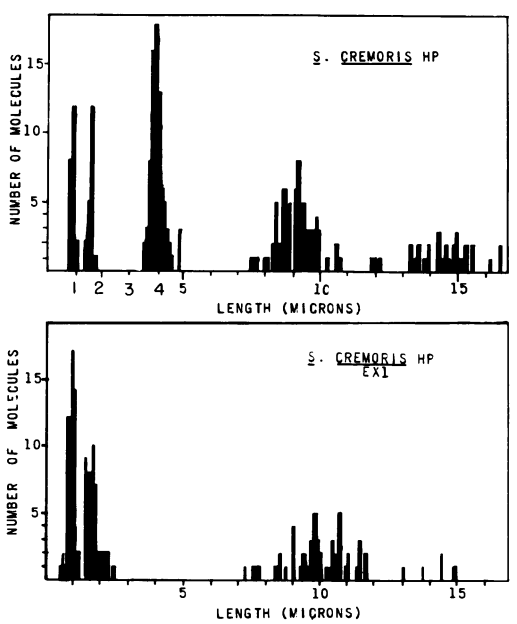

FIG. 1. *Distribution of contour lengths of circular molecules of DNA observed in S. cremoris HP and its spontaneous Prt^- mutant EX1; 1 μm = 2.07×10^6 daltons.*

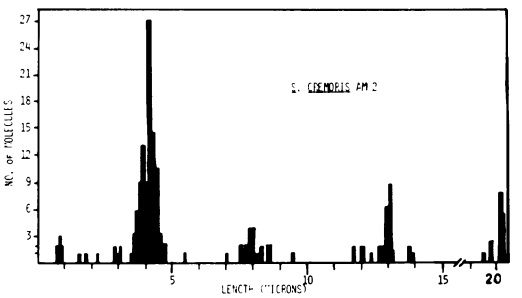

FIG. 2. *Distribution of contour lengths of circular molecules of DNA observed in S. cremoris AM_2.*

having molecular weights of 8.4×10^6, 15.9×10^6, 26.9×10^6, and 41.7×10^6 Mdal. Histograms of ML_1 and LL01, a Lac^- Prt^- derivative of ML_1 (see below) were also prepared, and each strain appeared to contain plasmids of 1.8, 3.9, 8.1, 18.2, and 29.4 Mdal (histograms not presented). Typical electron micrographs of the OC forms of the plasmids obtained from HP and AM_2 are presented in Fig. 3.

Isolation of mutants. Since three strains of *S. cremoris* (HP, AM_2, and ML_1) were shown to possess a diversity of plasmid molecules, it was of interest to determine whether any of these plasmids were linked to lactose metabolism and proteinase activity as was previously described for plasmids from *S. lactis* (20, 21). Growth at near-maximal temperature has been shown to increase the proportion of Lac^- Prt^- variants in *S. lactis* (22). In attempting to isolate similar variants from *S. cremoris*, purified single-colony isolates of strains C_3, HP, ML_1, and WC were inoculated into Elliker broth at 38°C for 18 h and then screened for the appearance of Lac^- derivatives on bromocresol purple lactose indicator plates (12). Following one transfer at 38°C, 0.5 and 7% of the colonies from strains ML_1 and WC, respectively, were Lac^-. Eight successive transfers at 38°C were necessary to obtain Lac^- variants from *S. cremoris* C_3. While screening for Lac^- mutants of strain HP, we observed numerous small, white colonies, indicating no lactose fermentation at 32°C in 48 h on bromocresol purple indicator plates. Extended incubation caused a gradual change of the indicator to yellow, indicating delayed lactose fermentation. These same colonies, however, definitely produced acid from lactose on bromocresol purple indicator plates at 21°C. Although McDonald (18) was able to isolate Lac^- variants of *S. cremoris* HP at 18°C, we did not find any Lac^- derivatives from this strain.

Strain AM_2 would not grow at 38°C and was, therefore, serially transferred at 35°C. We were unable to observe any Lac^- variants through 21

consecutive transfers at 35°C. The apparent stability of lactose metabolism in AM_2 was further examined by using chemical agents known to increase the frequency of plasmid loss (13, 27). AM_2 was serially transferred in Elliker broth or M17 containing acriflavin (4×10^{-6} M), EB (1×10^{-6} M), or acridine orange (2×10^{-6} M) at 32 or 35°C. No Lac^- variants were obtained. Continuous culture has also been used to produce a dramatic loss of lactose fermentation in *S. lactis* C2 (19). AM_2 was cultured in Elliker broth in a chemostat (24) for 144 h at 32°C. The temperature was then raised to 34 to 35°C for 72 h. Periodic plating on M17 agar and bromocresol

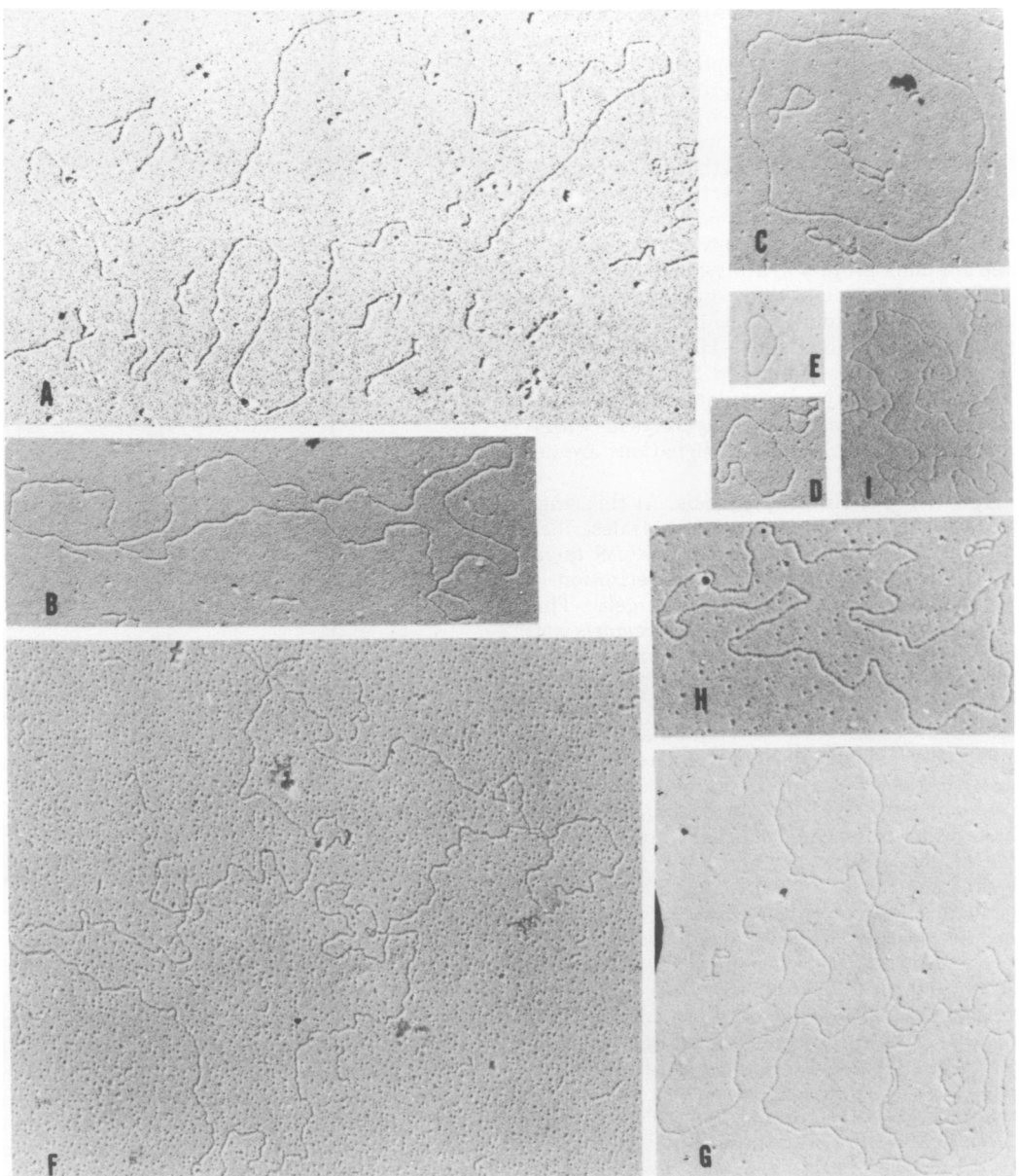

FIG. 3. *Electron micrographs of the open circular forms of plasmids observed in S. cremoris HP. (A) 30 Mdal; (B) 19 Mdal; (C) 8 Mdal; (D) 3.3 Mdal; (E) 1.8 Mdal, and S. cremoris* AM_2*; (F) 41 Mdal; (G) 27 Mdal; (H) 16 Mdal; (I) 8 Mdal. Original magnification ca. ×9,870. Electron micrographs were prepared and molecular weights determined as described in the text.*

purple lactose agar indicated that the cell population maintained a level of about 4.2×10^7 colony-forming units per ml, but no Lac^- variants were detected. McDonald (18) reported that, under chemostat conditions, the pH and temperature markedly influenced the rate of selection of Lac^- variants from HP and that Lac^- derivatives of HP could be obtained at 18°C but not at 32°C. Strain AM_2 was, therefore, grown at 18°C in M17 and in 2% nonfat dry milk containing 19.0 g of β-glycerophosphate per liter. During 144 h of chemostat growth in each medium, no Lac^- mutants were obtained.

The Lac^- mutants which were isolated from C_3, ML_1, and WC were further characterized with respect to proteinase activity. They were found to be $Lac^- Prt^-$ on the basis of their ability to coagulate milk supplemented with both glucose and milk protein hydrolysate within 24 h at 21°C (19), but not in milk supplemented only with glucose or milk protein hydrolysate. The spontaneous Prt^- variant of HP obtained from Exterkate (8) was confirmed to be $Lac^+ Prt^-$ based on its ability to coagulate only milk supplemented with milk protein hydrolysate within 24 h at 21°C. The mutant designations are described in Table 1.

Agarose gel electrophoresis. At this stage in the study, Klaenhammer et al. (14) described an improved method of lysing strains of *S. lactis* for the isolation and rapid characterization of plasmid DNA by using agarose gels. This method was also applicable to *S. cremoris* and therefore eliminated the time-consuming nature and expense of characterizing plasmid content via EM. All strains of *S. cremoris* previously examined by EM were analyzed on agarose gels to validate previous plasmid characterization.

Agarose gel electrophoretic migration of the *S. cremoris* HP CsCl-EB-purified plasmid DNA showed the presence of plasmids having molecular weights of 25×10^6, 17.7×10^6, 9×10^6, 3.4×10^6, and 1.9×10^6 (Fig. 4A). These values correlate closely with the previously identified plasmids in this strain obtained by EM. EX1, the spontaneous Prt^- variant of HP, was missing the 9-Mdal plasmid (Fig. 4B), thus supporting the EM data. However, when plasmids from *S. cremoris* ML_1 were examined by agarose gels, the 1.8-Mdal plasmid (calculated from EM) was resolved into two bands having molecular weights of 1.7×10^6 and 2.2×10^6 (Fig. 4E). LL01, the $Lac^- Prt^-$ variant of ML_1, was missing the 2.2-Mdal plasmid (Fig. 4D). The plasmids observed in ML_1 were 20, 10, 4, 2.2, and 1.7 Mdal. The 29.4-Mdal plasmid of ML_1 observed by EM was not visible in these gels. However, when these strains were examined by using ethanol-precipitated cleared lysates, the 29.4-Mdal plasmid was more easily observed (Fig. 5A and B, showing LL01 and ML_1, respectively). The top band in both wells has the mobility of a 28-Mdal plasmid, which was not apparent on gels from CsCl-EB density gradients. The second band is the 20-Mdal plasmid. The diffuse third band contains chromosomal fragments which are present in ethanol-precipitated cleared lysates. The 10-Mdal species which is observed in gels from CsCl-EB density gradients was partially obscured in this gel by the chromosomal band. The fourth band of wells A and B is a 4-Mdal

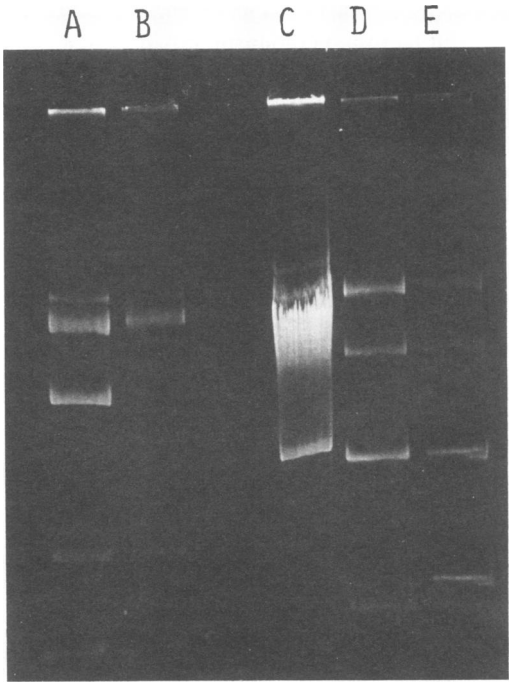

FIG. 4. *Agarose gel electrophoretic patterns of CsCl-EB purified plasmids obtained from S. cremoris HP, EX1, LL01, and ML_1. Cells were grown in LB for 5 h, treated with 2 mg of lysozyme per ml at 37°C for 8 min, and lysed with 1% sodium dodecyl sulfate. The cleared lysates were centrifuged to equilibrium in CsCl-EB gradients, and the satellite band was removed and analyzed by agarose gel electrophoresis. (A) S. cremoris HP showing (top to bottom) the 25-, 17.7-, 9.0-, 3.4- and 1.9-Mdal plasmids; (B) EX1 showing plasmid pattern similar to HP but missing the 9.0-Mdal plasmid; (C) E. coli mobility reference plasmids RP4 (34 Mdal), Sa (23 Mdal), and RSF1010 (5.5 Mdal) prepared from ethanol-precipitated cleared lysates; (D) LL01 showing the 20-, 10-, 4-, and 1.7-Mdal plasmids; (E) S. cremoris ML_1 plasmids of 20, 10, 4, 2.2, and 1.7 Mdal. The large diffuse band in well C is due to chromosomal fragments which are present in ethanol-precipitated cleared lysates.*

plasmid. The fifth band of well A and the fifth and sixth bands of well B are OC forms of the 1.7- and 2.2-Mdal plasmids. The characteristic migration of the CCC form of the 1.7-Mdal plasmid is seen in band 6 of well A and band 8 of well B. The 2.2-Mdal plasmid is only present in ML_1 (band 7 of well B). Well C in Fig. 5 contains the *E. coli* mobility markers.

S. cremoris WC and a Lac^- Prt^- variant of this strain (LL04) showed no difference in plasmid content (Fig. 5D and E). Each strain appeared to contain plasmid sizes of 21 and 18 (not distinctly separated in this gel) and of 5, 3, and 2 Mdal.

The number of plasmid species observed in *S. cremoris* AM_2 by gel electrophoresis did not correspond with the earlier EM data. Only two plasmids, of 27 and 16 Mdal, were detected in the gels, whereas four plasmids, of 41.7, 26.9, 15.9, and 8.4 Mdal, were seen by EM. Since the growth medium and time of incubation of the Cords procedure (2) was employed in EM determinations, in contrast to the Klaenhammer method (14) of using LB and 4 to 6 h of cell growth for preparation of gel data, *S. cremoris* AM_2 was reexamined by gels, using growth conditions previously described by Cords et al. (2). Plasmids from the cleared lysates of AM_2 grown under the latter conditions are shown in Fig. 6C. A 26-Mdal plasmid is observed as the top band. The broad band contains chromosomal material as well as the partially obscured 16.4- and 9.5-Mdal plasmids. However, when cleared lysates from cells propagated in milk were examined, it was found that the 42-Mdal plasmid was visible by gel electrophoresis. The top three bands in well A of Fig. 6 show the 42, 27, and 16.4-Mdal plasmids. The bright band contains chromosomal material and obscures the smallest plasmid, which here appears to have the mobility of an 11-Mdal plasmid.

To further evaluate the plasmid composition of *S. cremoris* strains, E_8, R_1, Wg_2, C_3, and KH were examined by using agarose gel electrophoresis. Since culture conditions apparently influenced the observation of certain plasmid bands, ethanol-precipitated cleared lysates from cells grown in LB and milk plus D,L-threonine were

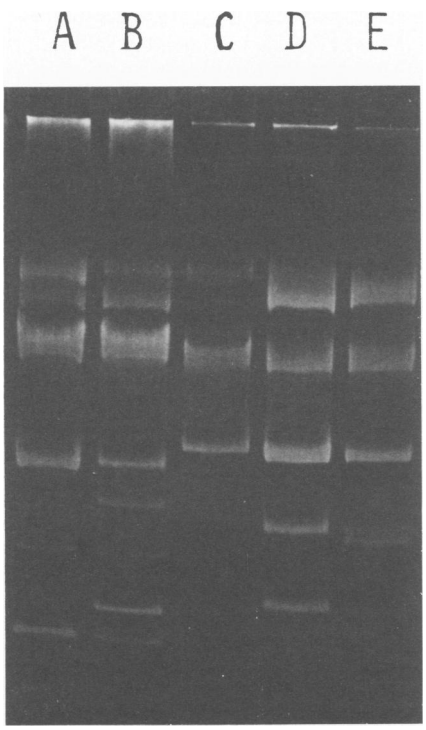

FIG. 5. *Agarose gel electrophoresis of ethanol-precipitated DNA from cleared lysates of LL01 (A), S. cremoris ML_1 (B), E. coli mobility reference plasmids RP4 and Sa which are not distinctly separated in this gel and RSF1010 (C), S. cremoris WC (D), and LL04 (E).*

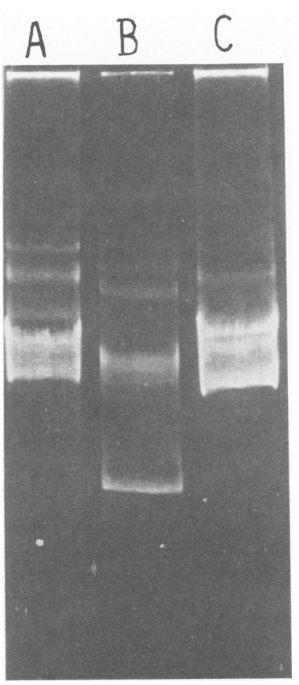

FIG. 6. *Agarose gel electrophoresis of ethanol-precipitated DNA from cleared lysates of S. cremoris AM_2. (A) AM_2 grown for 4.5 h in 11% nonfat dry milk containing D,L-threonine; (B) E. coli mobility reference plasmids RP4 (a light band), and the more distinct bands showing Sa, chromosomal fragments and RSF1010 (bottom band); (C) AM_2 grown for 10 h in Trypticase soy broth.*

each examined. CsCl-EB gradients from LB-grown cells were used to identify OC molecules and to remove chromosomal material. No difference in plasmid composition was observed between the LB- and milk-grown cells in these strains (Table 2).

Antibiotic and metal ion sensitivities. Antibiotic resistance has frequently been associated with plasmid DNA. Strains AM_2, HP EX1, C_3, LL03, ML_1, and LL01 were examined for resistance to the commonly employed antibiotics: erythromycin, clindamycin, chloromycetin, gentamicin, streptomycin, kanamycin, tetracycline, vancomycin, furadantin, gantrisin and sulfathiazole. No differences in antibiotic resistance between parents and mutants were observed. However, resistance which might be linked to one or more of the cryptic plasmids present was apparent in individual strains.

No obvious differences in inhibitory patterns between parent strains and their lactose and/or proteinase variants were observed with the following: NaF (0.1 M), $MgSO_4$ (0.1 M), $Na_2C_2O_7$ (4×10^{-3} M), $FeSO_4$ (2.0 M), MoO_3 (0.1 M), K_2TeO_3 (saturated), KI (0.1 M), Na_2SiO_3 (1.0 M), $VOSO_4$ (1.0 M), $Zn(NO_3)_2$ (0.1 M), $CdSO_4$ (10^{-4} M), $K(SbO)C_4H_6O_6$ (0.1 M), $Hg(NO_3)_2$ (10^{-2} M), $Pb(NO_3)_2$ (0.1 M), Na_2HAsO_4 (0.1 M), $NaAsO_2$ (0.1 M), K_2CrO_4 (10^{-2} M), and $CuSO_4$ (0.1 M).

TABLE 2. *Sizes of plasmids in S. cremoris strains as determined by EM and agarose gels*

Strain	No. of molecules measured	Length (μm)[a]	Molecular wt ($\times 10^6$) based on:	
			Contour length	Electrophoretic mobility
AM_2	187	4.07 ± 0.30	8.43	9.5
	15	7.7 ± 0.82	15.9	16.4
	10	13.0 ± 0.65	26.9	27
	6	20.1 ± 0.86	41.7	42
HP	77	0.87 ± 0.08	1.8	1.9
	69	1.59 ± 0.09	3.3	3.4
	77	3.91 ± 0.21	8.1	9
	137	9.03 ± 0.67	18.7	17.7
	36	14.34 ± 0.72	29.7	25
ML_1	154	0.89 ± 0.13	1.84	1.7 and 2.2[b]
	87	1.89 ± 0.20	3.89	4
	43	3.76 ± 0.27	8.1	10
	56	8.45 ± 0.77	18.2	20
	16	14.20 ± 0.82	29.4	28
C_3				2.0, 2.7, 15, 19, 27, and 31
E_8				2.7, 3.5, 9, 28, and 34
KH				2.2, 2.7, 3.2, 19, and 31
R_1				2.2, 7.7, 9, 20, 25, and 30
WC				2, 3, 5, 18, and 21
Wg_2				2, 3, 18, and 29

[a] Each value indicates mean ± standard deviation.

[b] The 1.84-Mdal plasmid, which plotted as a broad peak with results from electron micrographs, was resolved into two discrete plasmid species by gel electrophoresis.

DISCUSSION

As revealed by direct observation with EM and/or by relative mobilities using agarose gel electrophoresis, each strain of *S. cremoris* examined in this study was shown to possess a distinct set of plasmid molecules. Assuming a 10% variation in molecular weight between contour length measurements and relative mobility determinations (25), the plasmid constitution observed by agarose gel electrophoresis is consistent with EM observations. However, in *S. cremoris* AM_2, the conditions of growth appeared to influence the recovery of specific plasmids as observed in agarose gels. Under gel conditions, growth in milk was necessary to recover the 42-Mdal plasmid. Stationary-phase cells (10 h at 32°C) were needed to demonstrate the 8- to 9-Mdal plasmid in AM_2. In *S. cremoris* ML_1, the 29-Mdal plasmid was more easily observed by using gels prepared from ethanol-precipitated cleared lysates than from CsCl-EB-purified DNA samples. Similar observations were made for the plasmid DNA isolated from *S. lactis* C2 strain LM0231 (16; T. R. Klaenhammer, Ph.D. thesis, University of Minnesota, St. Paul, 1978).

The diversity of plasmid molecules observed in the *S. cremoris* strains raises the question of which, if any, are missing when the cell loses the ability to ferment lactose and/or to produce the proteinase system. Strains were treated in a variety of ways known to cause a high frequency of Lac^- variants in *S. lactis* (5, 18, 22). In contrast to *S. lactis,* however, few Lac^- variants could be found in the *S. cremoris* strains. *S. cremoris* AM_2 was treated extensively by using common curing conditions but again failed to elicit Lac^- variants. This observation suggests that lactose metabolism might be a stable characteristic in this strain. Whether this stability in AM_2 results from chromosomal linkage or from plasmid resistance to curing (24) is presently unknown.

It has been postulated that the loss of cell-bound proteinase might result from the loss of plasmid DNA in *S. cremoris* (8, 20, 28). Only in *S. cremoris* HP were Lac^+ Prt^- mutants readily isolated. Characterization of plasmids in EX1, a spontaneous Lac^+ Prt^- variant of HP, revealed

the absence of the 8- to 9-Mdal plasmid. This strain was also shown by Exterkate (8) to be deficient in two cell-wall associated proteolytic activities as well as in an intracellular proteolytic activity. Although not enzymatically characterized, Lac^+ Prt^- variants of HP isolated in this study were also missing the same plasmid (data not shown). The above results imply that the 8- to 9-Mdal plasmid observed in HP is linked to the proteinase system of this strain. Since Exterkate (8) observed that three of the five different proteolytic activities present in HP were missing in EX1, the results presented here suggest that all proteolytic activities in HP may not be plasmid-associated or at least not all are linked to the same plasmid.

Exterkate has also shown that the proteolytic enzyme system of *S. cremoris* ML_1 differs from other *S. cremoris* strains in that none of the five proteolytic activities are observed (9). Although LL01 was characterized as Lac^- Prt^-, normal coagulation of milk supplemented with glucose and milk protein hydrolysate was delayed beyond 48 h and sometimes indefinitely. Whether the absence of the 2.2-Mdal plasmid in LL01 is related to this phenotype is not known. It appears that a more generalized genetic defect in carbohydrate metabolism or an unidentified proteinase or peptidase system may be present in LL01. In any event, this phenotype did not resemble Lac^- Prt^- mutants previously described from *S. lactis* C2 (7, 21).

Sinha (30) has recently selected mutants of ML_1, in elevated concentrations of the curing agent acriflavine which show altered phage and temperature sensitivities. The nearly unchanged size of a satellite peak of DNA in a CsCl-EB gradient from his isolate led to his conclusion that plasmids were neither eliminated nor amplified. Our report of at least six distinct plasmids in ML_1 suggests that the loss or amplification of one plasmid species alone would not produce a discernible change in the nature of a satellite peak. It therefore remains to be determined whether the altered sensitivity to phage and temperature of the mutants isolated by Sinha are due to changes in the plasmid composition.

ACKNOWLEDGMENTS

This research was supported in part by Dairy Research Incorporated, Chicago, Ill.

LITERATURE CITED

1. **Anderson, D. A., and L. L. McKay.** 1977. Plasmids, loss of lactose metabolism, and appearance of partial and full lactose-fermenting revertants in *Streptococcus cremoris* B_1. J. Bacteriol. **129**:367-377.
2. **Cords, B. R., L. L. McKay, and P. Guerry.** Extrachromosomal elements in group N streptococci. J. Bacteriol. **117**:1149-1152.
3. **Crosa, J., L. K. Luttropp, and S. Falkow.** 1975. Nature of R-factor replication in the presence of chloramphenicol. Proc. Natl. Acad. Sci. U.S.A. **72**:654-658.
4. **Davis, R. W., M. Simon, and N. Davidson.** 1971. Electron microscope heteroduplex methods for mapping regions of base sequence homology in nucleic acids. Methods Enzymol. **21**:413-428.
5. **Efstathiou, J. D., and L. L. McKay.** 1976. Plasmids in *Streptococcus lactis*: evidence that lactose metabolism and proteinase activity are plasmid linked. Appl. Environ. Microbiol. **32**:38-44.
6. **Efstathiou, J. D., and L. L. McKay.** 1977. Inorganic salts resistance associated with a lactose-fermenting plasmid in *Streptococcus lactis*. J. Bacteriol. **130**: 257-265.
7. **Elliker, P. R., A. Anderson, and G. Hanneson.** 1956. An agar culture medium for lactic acid streptococci and lactobacilli. J. Dairy Sci. **39**:1611-1612.
8. **Exterkate, F. A.** 1976. The proteolytic system of a slow lactic-acid-producing variant of *Streptococcus cremoris* HP. Neth. Milk Dairy J. **30**:3-8.
9. **Exterkate, F. A.** 1976. Comparison of strains of *Streptococcus cremoris* for proteolytic activities associated with the cell wall. Neth. Milk Dairy J. **30**:95-105.
10. **Falkow, S., P. Guerry, R. W. Hedges, and N. Datta.** 1975. Polynucleotide sequence relationships among plasmids of the I compatibility complex. J. Gen. Microbiol. **85**:65-76.
11. **Fuchs, P. G., Zajdel Jolanta, and W. T. Dobrzanski.** 1975. Possible plasmid nature of the determinant for production of the antibiotic nisin in some strains of *Streptococcus lactis*. J. Gen. Microbiol. **88**:189-192.
12. **Heffron, F., C. Ruebens, and S. Falkow.** 1975. Transduction of a plasmid DNA sequence which mediates ampicillin resistance: molecular nature and specificity of insertion. Proc. Natl. Acad. Sci. U.S.A. **72**:3623-3627.
13. **Hirota, Y.** 1960. The effect of acridine dyes on mating type factors in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **46**:57-64.
14. **Klaenhammer, T. R., L. L. McKay, and K. A. Baldwin.** 1978. Improved lysis of group N streptococci for isolation and rapid characterization of plasmid DNA. Appl. Environ. Microbiol. **35**:592-600.
15. **Kozak, W., M. Rajchert-Trzpil, and W. T. Dobrzanski.** 1974. The effect of proflavin, ethidium bromide and an elevated temperature on the appearance of nisin-negative clones in nisin producing strains of *Streptococcus lactis*. J. Gen. Microbiol. **83**:295-302.
16. **Lang, D.** 1970. Molecular weights of coliphages and coliphage DNA. III. Contour length and molecular weight of DNA from bacteriophages T_4, T_5, and T_7 and from bovine papilloma virus. J. Mol. Biol. **54**:557-565.
17. **McDonald, I. J.** 1971. Filamentous forms of *Streptococcus cremoris* and *Streptococcus lactis*. Observations on susceptibility to lysis. Can. J. Microbiol. **17**:897-902.
18. **McDonald, I. J.** 1975. Occurrence of lactose-negative mutants in chemostat cultures of lactic streptococci. Can. J. Microbiol. **21**:245-251.
19. **McKay, L. L., and K. A. Baldwin.** 1974. Simultaneous loss of proteinase and lactose-utilizing enzyme activities in *Streptococcus lactis* and reversal of loss by transduction. Appl. Microbiol. **28**:342-346.
20. **McKay, L. L., and K. A. Baldwin.** 1976. Plasmid distribution and evidence for proteinase plasmid in *Streptococcus lactis* C2. Appl. Microbiol. **29**:546-548.
21. **McKay, L. L., K. A. Baldwin, and J. D. Efstathiou.** 1976. Transductional evidence for plasmid linkage of lactose metabolism in *Streptococcus lactis* C2. Appl. Environ. Microbiol. **32**:45-52.
22. **McKay, L. L., K. A. Baldwin, and E. A. Zottola.** 1972. Loss of lactose metabolism in lactic streptococci. Appl. Microbiol. **23**:1090-1096.
23. **McKay, L. L., B. R. Cords, and K. A. Baldwin.** 1973.

Transduction of lactose metabolism in *Streptococcus lactis* C2. J. Bacteriol. **115**:810–815.

24. **Macrina, F. L., and E. Balbinder.** 1972. Genetic characterization of a stable F'*lac* plasmid. J. Bacteriol. **112**: 503–512.
25. **Meyers, J. A., D. Sanchez, L. P. Elwell, and S. Falkow.** 1976. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol. **127**:1529–1537.
26. **Molskness, T. A., W. E. Sandine, and L. R. Brown.** 1974. Characterization of Lac^+ transductants of *Streptococcus lactis*. Appl. Microbiol. **28**:753–758.
27. **Novick, R. P.** 1969. Extrachromosomal inheritance in bacteria. Bacteriol. Rev. **35**:210–263.
28. **Pearce, L. E., N. A. Skipper, and B. D. W. Jarvis.** 1974. Proteinase activity in slow lactic-acid producing variants of *Streptococcus lactis*. Appl. Microbiol. **27**: 933–937.
29. **Radloff, R., W. Bauer, and J. Vinograd.** 1967. A dye buoyant density method for the detection and isolation of closed circular duplex DNA: the closed circular DNA in Hela cells. Proc. Natl. Acad. Sci. U.S.A. **57**: 1514–1520.
30. **Sinha, R. P.** 1977. Acriflavine-resistant mutant of *Streptococcus cremoris*. Antimicrob. Agents Chemother. **12**: 383–389.
31. **Stadhouders, J., L. A. Jansen, and G. Hup.** 1969. Preservation of starters and mass production of starter bacteria. Neth. Milk Dairy J. **23**:182–199.
32. **Terzaghi, B. E., and W. E. Sandine.** 1975. Improved medium for lactic streptococci and their phages. Appl. Microbiol. **29**:807–813.