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Evidence for Plasmid Linkage of Restriction and Modification in *Streptococcus cremoris* KH†

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Restriction and modification have been demonstrated in *Streptococcus cremoris* KH cells when infected by *Streptococcus lactis* C2 phage (designated c2) at an efficiency of plating of 2×10^{-7}. The growth of c2 phage through KH cells produces modified progeny phage capable of unrestricted growth on KH cells. The ability of single-colony isolates of *S. cremoris* KH cultures to restrict and modify c2 phage was found to be variable. From 2 to 6.5% of colonies isolated were partially deficient in restrictive capacity, permitting a greater plaquing ability by c2 phage of 1.8 to 2.9 log cycles. No completely restrictionless mutants were isolated from 1,000 colonies examined. Mutants were shown to be deficient in both restriction and modification capabilities of the same specificity. The frequent occurrence of a genotypic change that resulted in the loss of both restriction and modification capacities indicated the involvement of plasmid deoxyribonucleic acid in genetically determining this specific restriction and modification system. *S. cremoris* KH was found to harbor 11 plasmid molecules, with molecular weights ($\times 10^6$) estimated to be 50, 41, 24, 18, 10, 7.4, 3.3, 3.0, 2.8, 2.5, and 1.5. Of the 27 mutants examined, 25 were missing the 10-megadalton plasmid. This consistent plasmid difference among the majority of mutants isolated supports the involvement of this plasmid in restriction and modification. Plasmid linkage of restriction and modification systems provides a genetic mechanism for the rapid development of phage-sensitive starter cultures due to the inherent instability of extrachromosomal elements.

Streptococcus cremoris strains that are used industrially for milk fermentation are often selected for phage resistance before commercial use. However, lysis by lytic phage still frequently occurs, resulting in incomplete fermentation (12). Successful phage infection of selected starter strains is thought to occur through the appearance of previously undetected lytic bacteriophage from environmental contamination (13), phage mutation (8), lysogenic starter strains (16), host-controlled modification of heterospecific phage (25), or the accumulation of phage-sensitive variants within starter culture populations (14).

Few researchers have studied the presence of phage-sensitive variants within lactic streptococcal cultures, since most studies have been directed toward the isolation of industrially useful, phage-resistant variants (15; R. K. Thunell and W. E. Sandine, Abstr. Annu. Meet. Am. Dairy Sci. Assoc., 1980, abstr. IB3, p. 37). Sinha (27) reported that during selection for acriflavine resistance in *S. cremoris* ML1, mutants which demonstrated sensitivity to phage to which the parental culture was resistant were readily isolated. This phenotype did not appear to be due to changes in phage adsorption or to the operation of a host-controlled restriction and modification system. Limsowtin et al. (14) found members of single strains of lactic streptococci successively transferred in milk or broth to be composed of several types of isolates with variable phage sensitivity. In one strain, the variability was attributed to the loss of restriction and modification systems of different specificities. This indicated that variability of restrictive capacity among members of a single strain may occur and subsequently contribute to the phage sensitivity of starter cultures.

Several unstable traits inherent to the lactic streptococci such as lactose (19), casein (18), and citrate (9) metabolism are genetically determined by plasmid deoxyribonucleic acid (DNA). In these studies, metabolically deficient mutants were isolated at levels far greater than expected through simple chromosomal mutation (21). Analogously, the frequent isolation of mutants

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deficient in restrictive capacity would indicate the possible involvement of plasmids in genetically determining this trait. However, the genetic control of restriction and modification in the lactic streptococci has not been previously studied.

Studies on plasmid characterization in the lactic streptococci have been aided by the presence of genetic transfer systems in *Streptococcus lactis* and *Streptococcus diacetylactis* strains (5, 20, 22). In contrast, although *S. cremoris* strains harbor a wide variety of plasmid molecules (11), plasmid characterization studies in this species have been less conclusive. Larsen and McKay (11) linked the 8.5-megadalton (Mdal) plasmid to proteolytic capacity in *S. cremoris* HP. Studies with *S. cremoris* B1 linked three phosphoenolpyruvate-phosphotransferase lactose-specific enzymes to the 36-Mdal plasmid (1). However, according to Sherman's classifications (26), B1 is an atypical *S. cremoris* strain, in that it hydrolyzes arginine and grows at 40°C (unpublished data). Lactose-negative variants were isolated from *S. cremoris* ML1, C3, and WC, but plasmid linkage of lactose metabolism was not established (11). Therefore, the function of plasmids harbored by *S. cremoris* strains is largely unknown.

Plasmid DNA has been found to code for restriction and modification systems in many bacteria, including *Escherichia coli* (2), *Pseudomonas* species (7), and *Salmonella typhimurium* (29). To determine whether plasmids play a role in coding for restriction and modification in *S. cremoris*, single-colony isolates of *S. cremoris* KH were examined for variability of restrictive capacity. Mutants partially deficient in both restriction and modification activities were readily isolated and lacked the 10-Mdal plasmid. Plasmid linkage of a restriction and modification system in *S. cremoris* provides evidence for a genetic mechanism operating in the development of phage-sensitive starter cultures.

MATERIALS AND METHODS

Bacterial strains, phages, and plasmids. *S. lactis* C2, *S. cremoris* KH, and their homospecific phages, c2 and kh, respectively, were obtained from L. L. McKay (University of Minnesota, St. Paul). *E. coli* K-12 sublines J5, J53, and C600, with resident plasmids RP4 (34 Mdal), Sa (23 Mdal), and RSF1010 (5.5 Mdal), respectively (23, 24), were provided by J. H. Crosa (University of Washington, Seattle). *E. coli* V517, harboring 35.8-, 4.8-, 3.7-, 3.4-, 2.6-, 2.0-, 1.8-, and 1.4-Mdal plasmids (17), was obtained from F. L. Macrina (Virginia Commonwealth University, Richmond). *E. coli* 711, with resident 62-Mdal plasmid R1*drd*19 (23), was provided by P. Totten (University of Washington, Seattle). The plasmids harbored by *E. coli* strains were used as mobility reference standards during agarose gel electrophoresis.

Media and culture conditions. Lactic streptococci and their phages were propagated in M17 broth as previously described (25). For isolation of plasmid DNA, the lactic streptococcal strains were grown for 6 h at 30°C in 40 ml of Elliker broth (4) with 20 mM DL-threonine (Sigma Chemical Co., St. Louis, Mo.). *E. coli* strains were propagated for plasmid isolation at 37°C for 6 h in 40 ml of brain heart infusion broth.

Isolation of restriction-deficient mutants. Cultures of *S. cremoris* KH were grown in M17 broth (28), containing glucose instead of lactose (M17-glc broth), for 18 to 24 h at 30 or 37°C with designated concentrations of acriflavine hydrochloride (AF; Sigma Chemical Co.). Mutants were also isolated from cultures of KH grown through a starter activity test (6) modified as described previously (25). After growth under the specified conditions, cultures were sonified with a model 5110 Branson Sonifier (Branson Instruments, Inc., Stanford, Conn.) for 15 s at 3 A to break chains. Cultures were then plated on M17-glc agar and were incubated for 36 to 48 h at 30°C. Individual colonies were picked with sterile toothpicks, placed into 2 ml of M17-glc broth, and grown for 10 to 12 h at 30°C. The phage sensitivity of these isolates was determined by plaque assay as described previously (25, 28). Assay plates were incubated overnight and examined for an increased level of plaque-forming ability by c2 phage in the mutants as compared with c2 phage on KH cells. Mutants were then titrated with serially diluted c2 phage to determine the exact efficiency of plating (EOP = phage titer on restrictive host ÷ phage titer on nonrestrictive host).

Determination of modification capacity of restriction mutants. The modification capacity of 23 restriction mutants was determined by using phage isolated from single plaque isolates of c2 phage titrated on each mutant. Phage lysates were prepared for experimental use by adding the single-plaque isolate and 1 drop of 1 M $CaCl_2 \cdot 7H_2O$ to 10 ml of M17-glc broth inoculated with 0.25 ml of an overnight culture of mutant cells. After 10 h at 30°C, cultures were centrifuged to remove cellular debris and were filter sterilized through a 0.45-μm filter (Acrodisc, Gelman Instrument Co., Ann Arbor, Mich.). Phage were then serially diluted and titrated on 6-h cultures of C2, KH, and the mutant from which they were isolated.

Isolation, purification, and electrophoresis of plasmid DNA. Cellular lysis was achieved by the lysozyme-sodium dodecyl sulfate-salt procedure as described previously (10), except lysozyme digestion was increased to 15 min to enhance lysis. Cleared lysates were subjected to cesium chloride-ethidium bromide (CsCl-EB) density gradient centrifugation to purify covalently closed circular plasmid DNA (10). The isolation of *E. coli* plasmid DNA for use as mobility reference standards followed the procedure described by Meyers et al. (23). For additional clarity, plasmid mobility standards were also purified through CsCl-EB density gradients.

Agarose gel electrophoresis was carried out, as described by Meyers et al. (23), in a vertical slab gel electrophoresis cell at 30 mA (constant current) and ~95 V for 2.5 h. From 10 to 20 μl of CsCl-EB density gradient plasmid-purified DNA and 5 μl of tracking

dye per sample were electrophoresed in a 0.6 or 0.8% agarose (Seakem, Marine Colloids, Inc., Rockland, Maine) gel (0.3 by 16 by 14 cm). The gel was stained for 20 min in 0.4 μg of EB per ml and was photographed under short-wave ultraviolet light (Ultraviolet Light Products, San Gabriel, Calif.).

RESULTS

Isolation and properties of restriction-deficient mutants. Data for the isolation of restriction-deficient mutants under specific growth conditions are shown in Table 1. From 2 to 6.5% of single-colony isolates of *S. cremoris* KH showed an increased sensitivity to c2 phage, with the highest frequency occurring from broth cultures grown at 37°C containing 1.0 μg of AF per ml. The range in EOP among the 50 mutants examined for sensitivity to c2 phage varied from 1.3×10^{-4} to 1.0×10^{-5}. Since the EOP of c2 phage grown through the parental culture, KH, was 2.1×10^{-7}, c2 phage replication on the mutant was increased by 1.8 to 2.9 log cycles. Comparative levels of restriction mutants were isolated from all culture treatment conditions, including growth at 30°C, 37°C, and 37°C with AF and through the starter activity test (6). Therefore, cultures of *S. cremoris* KH contained high levels of variants altered in restrictive capacity, irrespective of the growth conditions.

The testing of over 1,000 isolates of KH did not reveal any completely restrictionless mutants (designated $r^- m^-$). Since the original EOP of c2 phage on *S. cremoris* KH was very low, 2.1×10^{-7}, it seemed probable that more than one restriction and modification system operated in this strain. However, it was not known whether the multiple restriction and modification functions were coded by unstable plasmids or by chromosomal genes. The frequent occurrence of $r^- m^-$ mutants would indicate unstable plasmid involvement in all restriction and modification capacities present. In an attempt to isolate $r^- m^-$ mutants, two restriction-deficient mutants, designated C45 and N86, were grown at 30°C with and without AF, and isolates were screened for a further decrease in restrictive capacity. Of 548 isolates of N86 and 520 isolates of C45, no variants with enhanced sensitivity to c2 phage were detected. Therefore, although partially phage-sensitive variants were readily isolated from the KH culture, fully permissive KH isolates were not found in over 2,000 examined. This indicates the presence of only one unstable restriction and modification system in this strain.

TABLE 1. *Instability of restrictive capacity in S. cremoris KH*

Incubation condition[a]		No. of colonies isolated	No. of restriction-deficient isolates	%
Temp (°C)	AF concn (μg/ml)			
30	0	100	4	4.0
37	0	104	4	3.8
37	1.0	107	7	6.5
37	2.0	135	5	3.7
SAT[b]		100	2	2.0

[a] Cultures were plated after one transfer under indicated conditions.

[b] SAT, Starter activity test (6, 25).

During screening for restriction mutants, variants of KH were found that displayed increased resistance to c2 phage. From 13 to 54% of variants isolated showed greater resistance than did the parental culture to c2 phage. Levels of phage replication in these resistant isolates were below detection with the titer of c2 phage used. In some cases very few plaques, less than five, were produced on plates with undiluted phage. This represents an EOP of less than or equal to 10^{-8}. Resistant mutants were more frequently isolated from cultures grown in AF at 37°C. Whereas 13% of single-colony isolates of a KH culture grown at 30°C demonstrated reduced plaquing efficiency, 41 and 54% of these resistant variants were isolated from cultures grown at 37°C with 0.5 and 1.0 μg of AF per ml, respectively. Representative mutants completely insensitive to c2 phage were tested for sensitivity to their homospecific phage, kh, and were found to be sensitive to this phage at the parental level. This result indicates that the resistance to c2 phage was not a pleiotropic effect generalizing to other KH lytic phage. Plasmid analysis of four phage-resistant isolates did not reveal any consistent plasmid difference. The mechanism of this phage resistance was not investigated further.

Modification capacity of restriction mutants. Modified phage were isolated from crosses of c2 phage grown on each restriction-deficient mutant selected. Representative titers are shown in Table 2. Each modified phage was capable of high-level growth both on the mutant from which it was isolated and on C2 cells. However, plaquing ability on KH cells was reduced by 2.4 to 3.4 log cycles as compared with plaquing ability on the mutant. If restriction-deficient mutants retained the modification function, c2 phage replicated through them would be fully modified and immune to restrictive action by KH cells. Because this was not the case, restrictive action by KH cells against these phage indicated that both restriction and modification activities were lost simultaneously in the restriction-deficient mutants. Additionally, with tests plaquing several modified phage

TABLE 2. *Plaquing ability of c2 phage isolated after growth on restriction variants of S. cremoris KH*

Modified phage	Log PFU[a]/ml on host:			
	C2	KH	M41	Mutant[b]
mc2·E5[c]	10.2	6.28	9.52	9.51
mc2·C78	10.2	7.15	9.48	9.57
mc2·K50	10.1	6.11	9.15	9.23
mc2·N85	10.3	6.04	9.40	9.46
mc2·M41	10.6	7.00		9.64
mc2·KH	9.85	8.74	9.18	

[a] PFU, Plaque-forming units.

[b] Phage were plaqued on the mutant through which the phage was propagated.

[c] Phage are designated as mc2, followed by the mutant through which the phage was last propagated.

on a variety of mutant-host combinations, modified phage isolated from one mutant showed equal plaquing ability on all other mutants. Typical data (Table 2) reveal that four separate phage stocks, prepared by growth of c2 phage through one of the restriction-deficient mutants, E5, C78, K50, or N85, were fully replicative on a distinct mutant, M41. Therefore, all of these isolates possess the same replication pattern, indicating the loss of the same restriction and modification system in these mutants. A concomitant loss of both restriction and modification capabilities of the same specificity at a high frequency could be readily explained by the plasmid linkage of one specific restriction and modification system in KH.

Plasmid DNA content of *S. cremoris* KH and its restriction-deficient mutants. The likelihood of plasmid DNA involvement in genetically determining restriction and modification in KH led to an investigation of the plasmid content of KH and its restriction-deficient mutants. Larsen and McKay (11) reported the presence of five plasmid molecules in KH of 31, 19, 3.2, 2.7, and 2.2 Mdal. Our examination of KH plasmid DNA purified through CsCl-EB density gradients revealed 11 distinct plasmid molecules. The molecular weights were estimated by electrophoretic mobility through 0.6% agarose gel as compared with standard reference plasmids (Fig. 1). The plasmids isolated from KH with this method demonstrated molecular weights ($\times 10^6$) of 50, 41, 24, 18, 10, 7.4, 3.3, 3.0, 2.8, 2.5, and 1.5.

The 50-, 41-, and 24-Mdal plasmids consistently produced much fainter bands in gels, leaving the possibility that these bands were produced by open circular forms of other plasmids. However, their persistent appearance after CsCl-EB gradient purification supports their unique identity. The faint appearance of the bands may be due to a low plasmid copy number or to an inherent susceptibility to degradation during lysis (10). Separation of the 10- and 7.4-Mdal plasmids into two distinct bands was dependent on agarose concentration, with concentrations greater than 1% preventing resolution of these bands. In 1.2% agarose gels, the 1.5-Mdal plasmid sometimes appeared as three distinct bands. Similarly, but irrespective of agarose concentration, the 2.5-Mdal plasmid frequently separated into two bands. This phenomenon was also observed by Macrina et al. (17) with a 3.7-Mdal plasmid. These bands may represent different plasmid molecules of very similar size that are difficult to distinguish under the electrophoretic conditions used. Alternatively, since superhelical conformation can change the hydrodynamic properties of a specific plasmid molecule (30), these bands may represent different conformations of the same molecular weight plasmid.

Of 55 restriction-deficient mutants isolated, 27 were examined for plasmid DNA content. Of these, 25 were missing the 10-Mdal plasmid. Figure 2 compares the plasmid content of KH

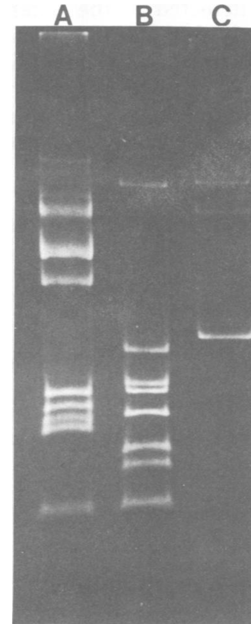

FIG. 1. *Agarose gel electrophoresis of CsCl-EB-purified plasmid DNA from S. cremoris KH (A). Molecular weights (×10⁶) of the 11 plasmids detected in S. cremoris KH were estimated as 50, 41, 24, 18, 10, 7.4, 3.3, 3.0, 2.8, 2.5, and 1.5, by electrophoretic mobility relative to reference plasmids on a 0.6% agarose gel. (B) Reference plasmids harbored by E. coli V517, with molecular weights (×10⁶) of 35.8, 4.8, 3.7, 3.4, 2.6, 2.0, 1.8, and 1.4 Mdal. (C) RP4 (34 Mdal), Sa (23 Mdal), and RSF1010 (5.5 Mdal).*

with that of a restriction-deficient mutant, M41. The 10-Mdal plasmid is missing in M41, but is present in the fully restrictive parent, KH. This is strongly supportive of the role of this plasmid in genetically determining this restriction and modification system. The mutants retaining the 10-Mdal plasmid demonstrated intermediate phenotype. The EOP of these mutants was only one log cycle greater than the EOP of KH. Their increased phage sensitivity, therefore, may be due to a mutation in a phage defense mechanism other than restriction and modification.

The restrictive capacity conferred by the 10-Mdal plasmid was also seen against the kh phage. kh phage, propagated through a restriction-deficient mutant, was fully replicative on the mutant from which it was isolated, but was restricted by the parental culture, KH, and by fully restrictive isolates retaining the 10-Mdal plasmid. Phage replication was reduced by 1.9 to 2.8 log cycles in restrictive hosts.

Further examination of Fig. 2 shows that M41 is also missing the 2.8-Mdal plasmid. This plasmid was consistently missing in all mutants examined. However, isolates missing this plasmid were readily detected in the parental culture, and loss of only this plasmid did not correlate with loss of restrictive activity. Additionally, the modification capacity of mutants missing only the 2.8-Mdal plasmid was equal to the parental level. Therefore, the 2.8-Mdal plasmid does not appear to be involved with this restriction and modification system.

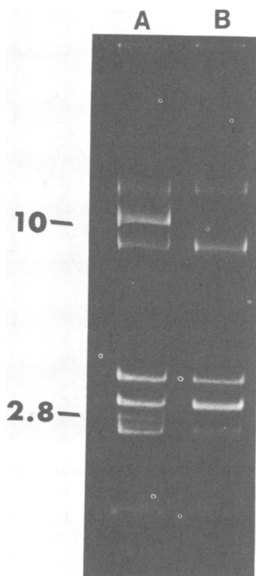

FIG. 2. *Electrophoresis of CsCl-EB-purified plasmid DNA from S. cremoris KH (A) and restriction-deficient mutant M41 (B) in 0.8% agarose gel. The 10- and 2.8-Mdal plasmids are indicated.*

DISCUSSION

Restriction-deficient mutants were readily isolated from cultures of KH treated through a variety of growth conditions. Neither direct nor stepwise isolation techniques resulted in isolation of an $r^- m^-$ variant. The limitations of the selection assay prevented large-scale screening from $r^- m^-$ mutants, but the data presented here indicate that completely restrictionless mutants do not occur at high frequency and, therefore, not all restrictive capacities within KH are unstable. The occurrence of only a reduced level of restrictive capacity in KH could be explained by the presence of more than one independently operating restriction and modification system. Multiple restriction and modification systems were recently reported in one lactic streptococcal strain (3). Additionally, the high parental level of restriction is consistent with the presence of multiple restriction and modification systems. Only one system, therefore, demonstrated genetic instability, and any remaining restriction and modification systems most likely are determined by the chromosome or other stable genetic elements.

The frequent occurrence of restriction-deficient mutants suggested the involvement of plasmid DNA in determining restrictive capacity. The evidence for plasmid-coded modification capacity came from examining the replication patterns of phage isolated after growth through a mutant. The restricted growth of these phage on KH cells indicated that growth of phage through a mutant did not result in full modification of the phage. Therefore, modification ability was concomitantly lost with restrictive capacity. In experiments with *E. coli* K-12 and B, which possess chromosomally linked restriction and modification systems, roughly equal numbers of $r^- m^+$ and $r^- m^-$ mutants were isolated (31). In this study, the absence of $r^- m^+$ mutants could be easily explained by the loss of a plasmid molecule coding for both restriction and modification activities.

Phage propagated on restriction-deficient mutants replicate equally well on other mutants as on the propagating host. This demonstrates that all mutants are deficient in a restriction and modification system of the same specificity. Such a constancy of phenotype would be expected if the same genotypic change that resulted from the loss of a specific plasmid molecule occurred in all of the mutants. The modification pattern reported here is different from a report by Limsowtin et al. (14), working with the

lactic streptococcal strain 240 and three isolates of 240 that varied in restrictive capacity, B_1, G_1, and G_2. Although B was fully permissive to phage grown through G_1 or G_2, G_1 and G_2 were restrictive to phage grown through each other, indicating the loss of restriction and modification systems of different specificities.

Typical restriction-deficient mutants were isolated with an EOP from 1.8 to 2.9 log cycles greater than for KH. Also, the plaquing ability of phage propagated through a mutant was 2.4 to 3.4 log cycles fewer on KH than on the restriction mutant. This observation led to the conclusion that modification capacity was missing from the mutants. However, since the action of a restriction and modification system is independent of other restriction and modification systems harbored by that strain, it would be expected that any phage lacking modification would be restricted at the same rate by any cell harboring the same restriction specificities. A discrepancy in replication ability of c2 phage and mc2·mutant phage, both of which lack modification of the one unstable restriction and modification system, is seen in the data presented here. In some cases, the difference between mc2·mutant phage replication on KH and on the mutant (range of 2.4 to 3.4 log cycles) is greater than the difference between c2 phage replication on KH and on the mutant (range of 1.8 to 2.9 log cycles). Therefore, it appears that fewer partially modified phage (mc2·mutant) may escape restriction than may escape with the completely unmodified phage (c2). The significance of this observation is unclear.

The characteristics of the readily isolated restriction mutants, coupled with the consistent absence of a specific plasmid molecule in the restriction-deficient mutants, strongly support the linkage of restriction and modification to plasmid DNA. The presence of genetic transfer mechanisms in KH would enable further substantiation of the plasmid linkage of this trait. However, genetic transfer mechanisms have not been identified in *S. cremoris*, preventing this approach. It may be possible that the very presence of restriction and modification systems in the lactic streptococci hinders the elucidation of genetic transfer systems in these strains. Identification of restriction and modification could be essential in the construction of recipient strains. Consistent with this, in previous studies on conjugation (20) and transduction (22), the recipient strains used were derivatives of *S. lactis* C2, a strain that, according to available evidence, does not appear to confer any restriction specificities (25).

Culture conditions did not appear to drastically alter the frequency of mutant isolation. Although incubation at elevated temperatures and growth in AF have been reported to increase the levels of lactose-negative variants in lactic streptococcal populations (21), similar treatments of KH in this study did not significantly enhance the occurrence of restriction-deficient mutants. These mutants appeared to be inherent to the culture, and growth conditions that cause the conversion of a wild-type phenotype to a mutant phenotype or that favor the outgrowth of variants inherent to the population were not clarified. Larsen and McKay (11) found that *S. cremoris* strains are phenotypically stable to rigorous plasmid-curing techniques when isolates were screened for lactose fermentative ability. Since the strains harbor many plasmids, this may reflect either the stability of these strains to plasmid curing or the inability to detect plasmid curing due to the selection of chromosomally determined phenotypes. Our study indicates that perhaps *S. cremoris* strains are stable to current plasmid-curing techniques.

The ubiquity of lytic lactic streptococcal bacteriophage in a cheese-making environment dictates that the survival of *S. cremoris* strains is largely dependent on their ability to resist phage attack. *S. cremoris* strains have been almost exclusively isolated from bulk starter cultures that have been propagated for long periods of time (13), often exposed to a diversity of phage. Since plasmids aid in rapid adaptation, the plasmid linkage of restriction and modification may represent the adaptation of these microorganisms to growth in the presence of phage. The maintenance of this trait on a plasmid, however, provides a means to rapidly lose restrictive capacity during growth under nonselective conditions. Therefore, plasmid linkage of restriction and modification provides a genetic mechanism for the generation of phage-sensitive starters.

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