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Characterization of Phage-Sensitive Mutants from a Phage-Insensitive Strain of *Streptococcus lactis*: Evidence for a Plasmid Determinant that Prevents Phage Adsorption†

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A phage-insensitive strain of *Streptococcus lactis*, designated ME2, was used as a prototype strain for the study of mechanisms and genetics of phage resistance in the lactic streptococci. Mutants sensitive to a *Streptococcus cremoris* phage, φ18, were isolated at a level of 17% from cultures of ME2 after sequential transfer at 30°C. Phage-sensitive mutants of ME2 were not fully permissive to φ18. The efficiency of plating of φ18 on the mutants was 5×10^{-7} as compared with $<10^{-9}$ for φ18 on ME2. Further characterization of the mutants showed that they efficiently adsorbed φ18 at levels of >99.8%, whereas ME2 adsorbed only 20 to 40% of φ18. These results suggest that increased phage susceptibility of the mutants may result from the loss of a mechanism that inhibits phage adsorption. Moreover, the high frequency of spontaneous mutation in ME2 indicates the involvement of an unstable genetic determinant in this phage defense mechanism. ME2 was shown to possess 13 plasmids ranging in size from 1.6 to 34 megadaltons. Of 40 mutants examined that had increased efficiencies of plating, all were missing a 30-megadalton plasmid, pME0030. These data suggest that pME0030 codes for a function that prevents phage adsorption. Further phenotypic characterization of the phage-sensitive mutants showed that some mutants were deficient in the ability to ferment lactose (Lac) and hydrolyze milk proteins (Prt). However, the Lac^+ and Prt^+ phenotype segregated independently of the phage-sensitivity phenotype. One phage-sensitive adsorption mutant, designated N1, was tested for susceptibility to 14 different phages. N1 showed increased capacity to adsorb 4 and to replicate 2 of these 14 phages, thereby indicating a phage resistance mechanism in ME2 that generalizes to phage interactions other than the specific φ18-ME2 phage-host interaction. These data provide evidence for a unique plasmid-linked phage defense mechanism in phage-insensitive strains of lactic streptococci.

The primary cause of slow acid production by lactic streptococci during dairy fermentations is lysis by bacteriophage (13). The occurrence of strains that demonstrate resistance to a wide variety of phage and maintain resistance during long-term commercial use is rare (15). Although a few phage-insensitive strains have been identified (3), the mechanisms for phage insensitivity in these strains are unknown. Investigation into the mechanisms of phage resistance could provide information that would aid in the selection, characterization, and ultimately the genetic construction of phage-resistant strains for commercial use.

Successful phage infection begins with phage adsorption to the host cell surface. Although many reports have been published on rates and levels of phage adsorption to the lactic streptococci (10, 25), little is known about the specific nature of their phage receptor sites. One notable exception is work done by Oram (24) that showed phage receptor activity for a *Streptococcus lactis* phage ml3 in a lipoprotein fraction of the cell membrane of strain ML3. Other phage receptors were located in the cell wall (25). Phage-resistant mutants isolated by challenge of phage-sensitive cultures with lytic phage have generally shown a decreased ability to adsorb the phage (11), presumably owing to chromosomal mutation that leads to cell surface, receptor site alterations. The involvement of unstable genetic elements, such as plasmid DNA, in coding for products that affect the adsorption

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efficiency of phage has not been reported in the lactic streptococci.

The intricate role that plasmid DNA plays in the genetics of the lactic streptococci has been unequivocally established (for reviews, see references 4, 9, and 18). Several metabolic traits important to the usefulness of the lactic streptococci in milk fermentation have been shown to be encoded by plasmids. However, phenotypes conferred by the majority of plasmid DNA species in the lactic streptococci remain unknown. The role that plasmid DNA may play in accelerating the adaptation of the lactic streptococci to the milk fermentation environment leads to the postulate that plasmid DNA elements may also be involved in coding for phage resistance traits in these organisms. The intense selective pressure resulting from lytic phage infection lends credence to this hypothesis. Earlier studies have shown that phage-sensitive mutants could be isolated at high frequency from cultures of lactic streptococci (2, 14, 27). In one case, the loss of a 10-megadalton (Mdal) plasmid was correlated with the loss of a phenotypic restriction and modification system, providing the first evidence for a plasmid-linked phage defense mechanism in the lactic streptococci (27). Plasmid linkage of phage resistance mechanisms has not been further reported.

The present study reports the examination of a phage-insensitive strain of *S. lactis* for instability of phage resistance. Phage-sensitive isolates demonstrated an increased ability to adsorb the challenging phage. All phage-sensitive mutants were missing a 30-Mdal plasmid, providing evidence that this plasmid genetically determines the ability of strain ME2 to prevent the adsorption of phage. The mechanism by which the plasmid-encoded product(s) interferes with phage adsorption remains unknown.

MATERIALS AND METHODS

Bacterial strains and phages. Lactic streptococcal strains and phages listed in Table 1 were propagated in M17 broth (30) or in M17 broth with glucose substituted for lactose (M17-glc broth) as previously described (26). Phage preparations were titrated at 30°C by the method of Terzaghi and Sandine (30). *Escherichia coli* K-12 sublines J5, J53, and C600, harboring plasmids RP4 (34 Mdal), Sa (23 Mdal), and RSF1010 (5.5 Mdal), respectively, have been previously described for use as electrophoretic mobility standards (27). *E. coli* V517, harboring plasmids of 35.8, 4.8, 3.7, 3.4, 2.6, 2.0, 1.8, and 1.4 Mdal (17), was obtained from F. L. Macrina (Virginia Commonwealth University, Richmond, Va.).

Isolation of phage-sensitive mutants. Before use, *S. lactis* ME2 was purified through single-colony isolation on M17 agar. The plasmid content of the purified isolate was identical to the profile of the parental culture, ME2. For plasmid-curing studies, ME2 was propagated in M17-glc broth at 30 or 42°C or in M17-glc broth containing 5 μg of acriflavin per ml at 30°C. Cultures were transferred (2%) after 24 h of incubation and were plated on M17-glc agar after 0, 5, and 10 transfers. After incubation (24 h at 30°C), single colonies were picked into 2 ml of M17-glc broth, incubated 8 to 10 h at 30°C, and tested for phage sensitivity by plaque assay with a high-titer phage φ18 preparation.

Phenotypic characterization of parent and mutant strains. Activity test. The activity test (7) was em-

TABLE 1. Bacterial strains and phages

Species	Strain	Homologous phage	Source[a]
Streptococcus lactis	ME2		Miles Laboratories
	SK2	φsk2	L. L. McKay, U. Minn.
	C2	φc2	L. L. McKay, U. Minn.
	ML3	φml3	L. L. McKay, U. Minn.
	M18		L. L. McKay, U. Minn.
Streptococcus cremoris	TDM1	φ18	Miles Laboratories
	KH		L. L. McKay, U. Minn.
	924		L. L. McKay, U. Minn.
	HP		L. L. McKay, U. Minn.
	799	φ799	E. I. Garvie, NCDO
	TR	φtr	E. I. Garvie, NCDO
	B1		L. L. McKay, U. Minn.
	205	φT189/205	W. E. Sandine, OSU
Streptococcus diacetylactis	18-16	φ18-16	L. L. McKay, U. Minn.
Streptococcus spp. strain	TD40	φ40	Miles Laboratories
	TD29	φ29	Miles Laboratories
	TD32	φ32	Miles Laboratories
	TD23	φ23	Miles Laboratories
	TD31	φ31	Miles Laboratories
	TD39	φ39	Miles Laboratories
	TD43	φ43	Miles Laboratories

[a] Abbreviations: U. Minn., University of Minnesota, St. Paul; NCDO, National Collection of Dairy Organisms, Shinfield, United Kingdom; OSU, Oregon State University, Corvallis.

ployed with the modifications described previously (26).

Spot test. Cultures tested for sensitivity to phage composites were cultivated at 30°C for 4 to 6 h to the late log phase of growth. Samples of culture (0.3 ml) were added to tubes (18 by 40 mm) containing 1 drop of 1 M $CaCl_2$. Tempered (46°C) M17 overlay agar (2.5 ml) was added, and the tubes were mixed gently and poured onto M17 agar plates. After solidification, 20 μl of assay phage was spotted onto the lawn. Plates were incubated upright at 30°C overnight and examined for zones of lysis.

Carbohydrate fermentation and hydrolysis of milk proteins. For rapid screening of the lactose-fermenting and proteolytic abilities of strain ME2 mutants, mutants were grown overnight in M17-glc broth and were inoculated (1%) into steamed (60 min) 10% reconstituted nonfat dry milk with 40 mg of bromocresol purple per liter as a pH indicator. Milk was either used with no supplement or was supplemented with 1% glucose, 0.25% acid casein hydrolysate (vitamin free), or both glucose and casein hydrolysate. Cultures were grown 12 h at 30°C and were examined for milk coagulation and acid production. To differentiate between growth and acid production from lactose, cultures were grown in a defined medium described by Harvey and Collins (6) as modified by Moustafa and Collins (22), except that both sodium acetate and sodium thioglycolate were omitted. Lactose (final concentration, 0.5%) was added as a concentrated solution filtered through a 0.22-μm filter (Gelman Instrument Co., Ann Arbor, Mich.). Cells for inoculation were centrifuged 7,700 × *g*, washed, and suspended in 0.31 mM KH_2PO_4 (pH 7.2). Cuvettes containing the defined medium were inoculated (2%). Growth was followed spectrophotometrically at 650 nm.

To test mutants rapidly for fermentation of several carbohydrates, a microtiter plate procedure was employed. The basal identification medium consisted of Proteose Peptone no. 3, 20 g/liter; yeast extract, 10 g/liter; 1 M $MgSO_4$, 2 ml/liter; Na_2PO_4, 4 g/liter; pH 6.5. Sugar solutions were prepared 4% (wt/vol) in distilled water, filtered through a 0.45-μm filter, and stored at −20°C in 1-ml portions. Cultures to be tested were streaked onto M17-glc agar and incubated at 30°C for 48 h, and surface growth was added to double-strength identification medium to equal a no. 5 McFarland standard. To the wells of a 96-well microplate, 50-μl sugar soutions and 50-μl culture suspensions were added. Plates were covered with a fitted lid and were incubated at 30°C for 24 h. Controls included sugar and uninoculated double-strength identification broth; sterile distilled water and double-strength identification broth; and sterile distilled water and culture suspension. After incubation, 50 μl of indicator solution (0.2 g of bromocresol purple and 3.7 ml of 0.01 M NaOH in 46.3 ml of distilled water) was added to each well, and results were scored as positive or negative based on color change of the indicator. The proteolytic activity of ME2 mutants on milk proteins were determined by an *o*-phthaldialdehyde assay (1).

Phage adsorption. Phage adsorption was conducted as described previously (26).

Isolation of plasmid DNA. Two procedures were used for the purification of plasmid DNA.

(i) To isolate plasmid DNA from lactic streptococci and from strains harboring mobility reference plasmids, the procedure of Klaenhammer et al. (12) was used, except that cleared lysates were treated with 100 to 150 μl of 3 N NaOH to decrease open circular forms and chromosomal DNA. After 10 min at room temperature, lysates were neutralized with 750 μl of 2 M Tris-hydrochloride, pH 7.0, before extraction with chloroform-isoamyl alcohol (24:1). Samples intended for purification through cesium chloride-ethidium bromide density centrifugation were not denatured with alkali before gradient formation.

(ii) The alternative procedure used for the isolation of plasmid DNA from strain ME2 and its mutants was derived from a procedure designed for isolation of plasmid DNA from the lactobacilli (T. R. Klaenhammer, in press). This procedure was useful for rapid screening of mutants since plasmid DNA samples were ready for electrophoretic analysis in only 7 h. To 9 ml of M17 or M17-glc broth, a 1-ml inoculum of an overnight culture of ME2 or its mutants was added. Cultures were grown at 30°C for 75 min, pelleted by centrifugation, and suspended in 1 ml of 25% (wt/vol) sucrose in 50 mM Tris-hydrochloride–5 mM EDTA, pH 7.5. Cells were chilled on ice for 5 min. Lysozyme (50 μl, freshly prepared at 10 mg/ml in 250 mM Tris, pH 8.0) was added, and cells were held on ice for 60 min. Cells were pelleted by centrifugation, and the supernatant fluid was discarded. Lysis solution (500 μl; 50 mM Tris–5 mM EDTA–50 mM glucose–3% sodium lauryl sulfate, mixed with 7.5 μl of 10 N NaOH per ml immediately before use) was added directly to the cell pellet. The cell pellet was gently mixed with the lysing solution by using a plastic pipette, and the suspension was heated at 62°C for 1 h. After cooling to room temperature, the lysate was transferred to a 1.5-ml microcentrifuge tube, and 10 μl of 0.25 M EDTA and 50 μl of 2 M Tris-hydrochloride (pH 7.0) were added. After gentle mixing, 65 μl of 5 M NaCl was added. Redistilled phenol (Bethesda Research Laboratories, Gaithersburg, Md.) was saturated with 3% NaCl, and 500 μl was added to the cell lysate. Preparations were mixed vigorously and incubated at room temperature for 5 min. This was followed by the addition of 300 μl of chloroform-isoamyl alcohol (24:1) with gentle mixing. Preparations were centrifuged in an Eppendorf 5414 microcentrifuge (Brinkmann Instruments, Inc., Westbury, N.Y.) for 5 min. The aqueous phase was removed and transferred to a clean centrifuge tube. A second extraction was performed with 500 μl of chloroform-isoamyl alcohol (24:1) followed by centrifugation for 5 min. The aqueous phase was removed (up to 500 μl) and added to a fresh tube, and the DNA was precipitated with 1 ml of cold (−20°C) 95% ethanol at −70°C for 1 h. The plasmid DNA was pelleted by centrifugation for 10 min in the Eppendorf microcentrifuge. Ethanol was aspirated off the white pellet, and the pellet was vacuum dried for 10 min. Pellets were suspended in 25 μl TES buffer (30 mM Tris, 5 mM EDTA, 50 mM NaCl, pH 8.0). Pellets dissolved immediately, and purified plasmid was subjected to agarose gel electrophoresis.

Electrophoresis was conducted in a vertical electrophoresis unit (model 2001, LKB Instruments, Inc., Gaithersburg, Md.) with a running buffer consisting of 89 mM Tris, 2.5 mM EDTA, and 8.9 mM boric acid. Samples were loaded with an equal volume of agarose beads as a tracking dye (28). After electrophoresis for 3 to 3.5 h at 7.5 V/cm (constant voltage), gels were

TABLE 2. Sensitivity of several lactic streptococcal strains to a phage composite containing 63 different phages

Strain	Developed pH through SAT[a]		Spot test[b]
	No phage	Phage	
ME2	4.85	4.90	−
924	5.85	5.90	−
TR	5.45	5.65	−
TDM1	5.45	6.55	+
SK2	5.80	6.50	+
KH	5.40	6.10	+
C2	5.00	6.50	+
HP	5.65	6.60	+
799	6.15	6.10	+
ML3	5.05	6.50	+
B1	5.30	6.50	+
M18	5.70	6.10	+
205	6.00	6.50	+

[a] SAT, starter activity test; see text.
[b] +, Zone of lysis; −, no detectable lysis.

stained with ethidium bromide (0.3 μl/liter) and photographed.

RESULTS

Isolation of phage-sensitive variants of ME2. Thirteen strains of *S. lactis* and *Streptococcus cremoris* were examined for their sensitivity to a phage composite containing 63 different phages. All strains except ME2, 924, and TR showed sensitivity to phage as determined by a spot test and inhibition of acid production through one cycle of the starter activity test (Table 2). These results suggest a higher order of phage resistance in ME2, 924, and TR than in other lactic streptococcal strains. ME2 was found to develop sensitivity to one phage in the composite, φ18, after repeated trials through the starter activity test. However, when φ18 was assayed at high titer on ME2, no plaque-forming ability was demonstrated (efficiency of plating, $<1.4 \times 10^{-9}$). With φ18 as a test phage that demonstrated the potential for lytic development on strain ME2, the phage insensitivity of ME2 was further studied.

The genetic instability of a phage defense mechanism in ME2 was examined by testing for the occurrence of phage-sensitive mutants in cultures of ME2. Single-colony isolates of ME2 were subcultured under conditions which facilitate plasmid curing in the lactic streptococci (21). ME2 was transferred in M17-glc broth at 30 and 42°C and in M17-glc broth with 5 μg of acriflavin per ml at 30°C. Colonies isolated after 0, 5, and 10 transfers were tested for sensitivity to φ18. The results (Table 3) indicate that phage-sensitive variants in the ME2 cultures were readily generated upon subculture under all treatment conditions. All mutants isolated showed similar sensitivity to φ18, with an average efficiency of plating of 5.8×10^{-7}. This represents an increase of at least 2 log cycles over the efficiency of plating of φ18 on ME2. However, the ability of φ18 to plaque on ME2 mutants was still limited, indicating additional defense mechanisms in the mutants. The data in Table 3 show that nonselective growth conditions yielded the highest level of phage-sensitive mutants. After 10 transfers in M17-glc broth at 30°C, 17% of the population showed sensitivity to φ18. These results indicate a high degree of instability of phage resistance in this strain and suggest the involvement of an unstable genetic element.

To investigate factors potentially involved in the phage sensitivity of the ME2 mutants, the ability of the mutants to adsorb φ18 was determined. The results (Table 4) indicate that ME2 adsorbed φ18 poorly, at an efficiency of 20 to 40%, but a representative mutant, N1, adsorbed φ18 very efficiently. Of 20 phage-sensitive mutants tested, all were able to adsorb approximately 99.7% of φ18. Comparisons of the residual titers after adsorption of φ18 to ME2 versus the mutants consistently showed a difference of 2 to 2.5 log cycles. These data suggest that a

TABLE 3. Generation of phage-sensitive mutants in *S. lactis* ME2 cultures repeatedly transferred in M17-glc broth with or without acriflavin at 30 or 42°C

Temp (°C)	Medium	Phage-sensitive variants after no. of transfers:					
		0		5		10	
		No.[a]	%	No.	%	No.	%
30	M17-glc	0/100	0	13/100	13.0	17/99	17.2
	M17-glc + 5 μg of acriflavin per ml	0/100	0	3/100	3.0	0/98	0
42	M17-glc	0/100	0	1/88	1.1	NT[b]	NT

[a] Number of phage-sensitive variants/number of isolates tested.
[b] NT, Not tested.

TABLE 4. Adsorption of φ18 to *S. lactis* ME2 and N1 cells

Cells with φ18	Residual phage titer[a]	Adsorbed phage (%)[b]
ME2	2.9×10^6	40.8
N1	1.4×10^4	99.7
None (φ18 only)	4.9×10^6	0

[a] Cells and phage were mixed and allowed to adsorb at room temperature for 15 min. Mixtures were centrifuged, and phage remaining in the supernatants was titrated on TDM1 cells.

[b] Calculated as {[(titer of phage only) − (titer of cells plus phage)]/[titer of phage only]} × 100.

change in adsorption capacity was responsible for the phage sensitivity of the mutants.

Plasmid analysis of phage-sensitive variants. To determine the involvement of plasmid DNA in coding for the prevention of adsorption in strain ME2, ME2 and its phage-sensitive mutants were examined for plasmid DNA content. ME2 was found to harbor 13 distinct plasmid species of 34, 30, 20, 16, 7.5, 5.8, 3.6, 2.6, 2.0, 1.9, 1.7, and 1.6 Mdal (Fig. 1). The 1.9-Mdal band consistently separated into two bands under different electrophoresis conditions (data not shown). The plasmid profiles of eight phage-sensitive mutants isolated after repeated transfer in M17-glc broth at 30°C are shown in Fig. 2. The only plasmid missing from these phage-sensitive variants was the 30-Mdal plasmid, designated pME0030. These results provide strong correlative evidence that the ability of ME2 to resist phage adsorption is linked to pME0030.

Characterization of phage-sensitive mutants. Tests were conducted to determine whether the phage sensitivity phenotype in strain ME2 genetically segregated with other, more easily selectable, traits. Several mutants were screened for the ability to produce acid from sugars fermented by ME2. Lactose, galactose, maltose, salicin, sucrose, trehalose, and xylose were tested. None of the mutants missing only plasmid pME0030 showed any difference in the fermentation pattern of these sugars, suggesting independent genetic segregation of phage resistance and fermentation of these carbohydrates.

However, based on the ability to coagulate milk or milk supplemented with glucose, casein hydrolysate, or both, several phage-sensitive mutants were found to be slow acid producers (data not shown). The inability of two of these mutants to grow on lactose (Lac^-) is shown in Fig. 3. These data indicate that a deficiency in lactose-utilization was responsible, at least in part, for the slow acid production of these mutants in milk. In addition, Table 5 shows that hydrolysis of milk proteins was defective (Prt^-) for several mutants. Of 10 slow acid-producing, phage-sensitive mutants isolated, 8 were found to be Lac^- Prt^-, and 2 were Lac^- Prt^+. One isolate, P4, demonstrating wild-type phage resistance and harboring pME0030, was also Lac^- Prt^-. All slow acid-producing mutants were isolated after multiple transfer of ME2 in M17-glc with acriflavin or from growth at 42°C. Therefore, the level of mutants demonstrating multiple phenotypic changes was increased when selective curing conditions were used. The plasmid profiles of mutants isolated under selective curing conditions (Fig. 4) showed that all these mutants were missing several plasmids. However, no consistent pattern of plasmid loss was determined, and therefore, correlative evidence for the plasmid linkage of Lac and Prt to a specific plasmid species in ME2 was lacking. Multiple plasmid deficiencies and the phenotypic instability of Lac and Prt in these mutants strongly suggest the involvement of plasmid DNA in determining Lac and Prt phenotypes. Evidence for the plasmid linkage of Lac and Prt phenotypes in the lactic streptococci has been presented previously (18, 20). However, more

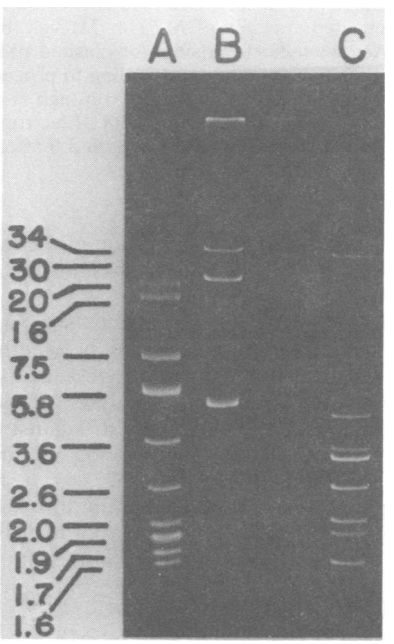

FIG. 1. (A) Plasmid DNA content of *S. lactis* ME2. (B) Electrophoretic mobility reference plasmids RP4 (34 Mdal), Sa (23 Mdal), and RSF1010 (5.5 Mdal). (C) Plasmids from *E. coli* V517 (35.8, 4.8, 3.7, 3.4, 2.6, 2.0, 1.8, and 1.4 Mdal). The 1.9-Mdal plasmid in ME2 separated into two bands under different electrophoretic conditions. Plasmid DNA was purified through cesium chloride-ethidium bromide gradient centrifugation and electrophoresed for 3.5 h at 7 V/cm in a 0.6% agarose gel.

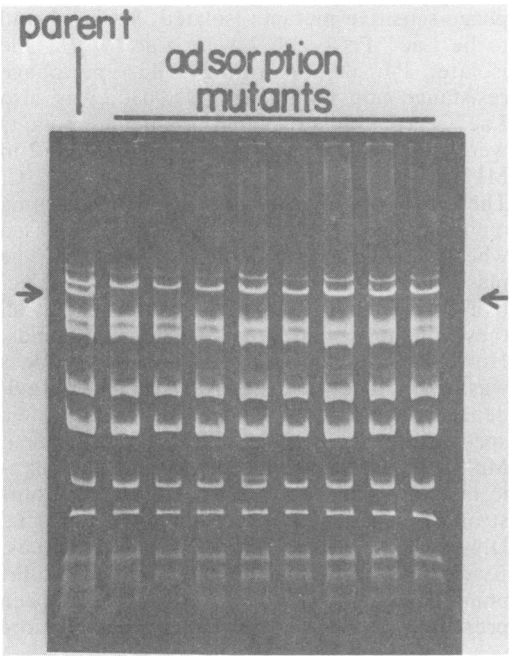

FIG. 2. Plasmid DNA content of mutants of ME2 which efficiently adsorb ϕ18. Mutants were isolated after repeated transfer of ME2 in M17-glc broth at 30°C. Arrows indicate position of plasmid pME0030. Plasmid DNA was isolated according to procedure (i) (see text) and was not purified through a cesium chloride-ethidium bromide gradient. Electrophoresis was conducted for 3.5 h at 7 V/cm in a 0.6% agarose gel.

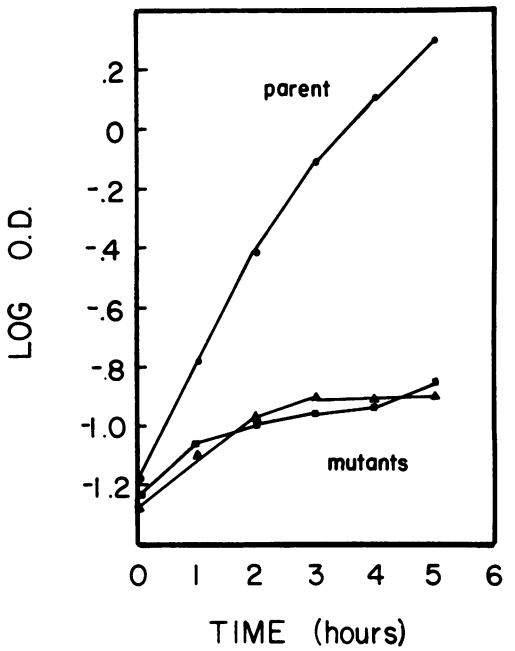

FIG. 3. Growth of *S. lactis* ME2 (●) and phage-sensitive mutants J5 (▲) and E3 (■) in defined medium containing 5 g of lactose per liter as the sole carbohydrate source.

extensive phenotypic description of the mutants is necessary to support the plasmid linkage of these traits in this strain.

Sensitivity of ME2 mutants to several lactic streptococcal phages. To determine whether the increased sensitivity of the phage-sensitive mutants to ϕ18 generalized to other phages, 13 additional phages were tested against strain ME2 and an efficiently adsorbing mutant, N1 (Table 6). Sensitivity to phage was tested by using phage adsorption, acid inhibition through a starter activity test, lysis by a spot test, lysis of broth cultures, and plaque-forming ability. The results show that ϕ18 was the only phage to react with N1 cells in all five tests. The parental culture, ME2, was unaffected by ϕ18. However, three other phages, ϕ23, ϕc2, and ϕml3, showed a significantly increased ability to adsorb to N1 as opposed to ME2, although none of these three phages showed replication ability. In contrast, ϕ29 and ϕ40 showed inhibition of N1 through the starter activity test and the spot test without showing any significant ability to adsorb to N1. The remaining phages tested demonstrated no detectable difference in their interactions between N1 and ME2 cells. These results indicate that the loss of plasmid pME0030 from ME2 leads to a mutant strain showing altered interactions with more than one phage (5 out of 13 tested). This change in sensitivity to groups of phage is not unprecedented (29) and suggests that the action of the gene products of pME0030 is not solely directed toward the specific interaction of ϕ18 with ME2 but has a more generalized effect.

DISCUSSION

Although many properties important to the industrial usefulness of the lactic streptococci

TABLE 5. Spectrophotometric assay of milk protein hydrolysis by *S. lactis* ME2 (Lac^+ Prt^+) and mutants (Lac^- Prt^-) J5, E3, E6, and D6

Strain	A_{340}[a]		
	0 h	23 h	Change
ME2	0.225	0.420	0.195
J5	0.248	0.268	0.020
E3	0.280	0.305	0.025
E6	0.275	0.295	0.020
D6	0.282	0.265	−0.017

[a] Increased absorbance at 340 nm (A_{340}) indicates the reaction of *o*-phthaldialdehyde and β-mercaptoethanol with α-amino groups released during the hydrolysis of milk proteins (1).

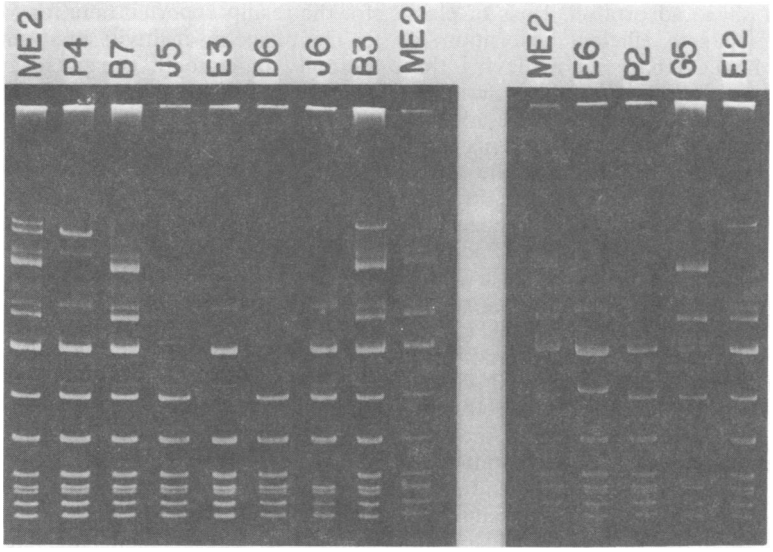

FIG. 4. Plasmid DNA content of slow acid-producing mutants of *S. lactis* ME2. Mutant designations are as indicated. All mutants were isolated after repeated transfer at 30°C in M17-glc broth containing 5 μg of acriflavin per ml, except G5, which was isolated after transfer at 42°C. Plasmids from the mutants and from ME2 in the first track were purified by procedure (ii) (see text) and contain contaminating open circular forms. Plasmids from ME2 in tracks 9 and 10 were purified through cesium chloride-ethidium bromide gradients and represent covalently closed circular forms. Electrophoresis was conducted for 3.0 h at 7 V/cm in a 0.65% agarose gel.

are genetically unstable, the most variable of these in practice is phage resistance. This is not surprising since the phage sensitivity of a strain is a function of two separately evolving genetic entities, the bacterium and the phage. Changes in the populations of phage due to host-controlled modification, new sources of phage, or genetic mutation of present phage, in addition to changes in genetic makeup of starter strains by mutation or by the loss of genetically unstable phage defense mechanisms, can lead to an overall change in the phage resistance patterns of a strain. Phage-resistant mutants which no longer adsorb phage can be readily isolated from cultures of cells lysed by lytic phage (2, 11). This type of phage resistance is most easily explained by chromosomal mutation leading to changes in phage receptor sites, and plasmid involvement is doubtful. In contrast, *S. lactis* ME2 appears to possess a plasmid-linked system that effectively

TABLE 6. Interaction of several lactic streptococcal phages with *S. lactis* ME2 and N1 cells

Phage	Adsorption (%)		SAT[a]		Spot test		Broth lysis		Plaque formation	
	ME2	N1	ME2	N1	ME2	N1	ME2	N1	ME2	N1
φ18	40	99.8	−	+	−	+	−	+	−	+
φ40	0	0	−	+	−	+	−	−	−	−
φ29	21	9	−	+	−	+	−	−	−	−
φ32	26	0	−	−	−	−	−	−	−	−
φ23	39	99.9	−	−	−	−	−	−	−	−
φc2	39	95	−	−	−	−	−	−	−	−
φ31	0	0	−	−	−	−	−	−	−	−
φsk2	5	0	−	−	−	−	−	−	−	−
φml3	0	91.4	−	−	+	+	−	−	−	−
φ18-16	18	14	−	−	−	−	−	−	−	−
φtr	0	20	−	−	−	−	−	−	−	−
φ799	0	0	−	−	−	−	−	−	−	−
φ39	0	0	−	−	−	−	−	−	−	−
φT189/205	0	0	−	−	−	−	−	−	−	−

[a] SAT, Starter activity test.

interferes with phage adsorption. Loss of plasmid pME0030 leads to efficient adsorption of φ18. The biochemical basis of achieving this effect is unknown. However, the genetic instability of the capacity to prevent phage adsorption provides evidence for a novel, plasmid-coded phage defense mechanism in the lactic streptococci.

Plasmid-coded interference with phage adsorption has been documented in other bacterial systems. Robillard et al. (N.-J. Robillard, T. M. Koehler, R. Murray, and C. B. Thorne, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, H54, p. 115) observed that curing *Bacillus anthracis* of a large plasmid resulted in increased sensitivity to *Bacillus cereus* phages due to increased adsorption capacity. However, investigation into the cause of altered adsorption was not conducted. Olsen et al. (23) conducted a detailed study on a phage, PRD1, that attacked certain pseudomonads and *Enterobacteriaceae*. The adsorbing ability of the phage was dependent on cell wall alterations coded by drug resistance plasmids of certain incompatibility groups. Similarly, pME0030 may block phage adsorption as a result of secondary structural changes that serve another purpose in the cell. However, in this study, no phenotype examined was found to cosegregate with phage-sensitivity genes. This may have resulted from not testing for the necessary trait. Alternatively, genes carried on plasmid pME0030 may be solely directed toward a phage defense mechanism.

Lysogenic conversion has been shown to result in surface structure alterations that lead to the loss of phage receptors. A model example is the lysogenization of *Salmonella anatum* by phage ε15 (15, 31), which results in a change in cellular O-antigen specificity, with a concomitant loss of receptors for ε15 and gain in receptors for phage ε34. Although ε15 is not known to exist extrachromosomally, conversions of surface structure may result in other systems from plasmid-residing lysogenic phage (5). This type of lysogenic conversion is thought to provide a mechanism for superinfection immunity. Lysogeny has been extensively documented in the lactic acid bacteria (8, 16, 19), and therefore lysogenic phage genes, defective or fully functional, could be considered to be an integral part of the lactic streptococcal genetic system. Therefore, the ability of strain ME2 to prevent phage adsorption could possibly be caused by lysogenic conversion owing to plasmid-linked prophage. Loss of the phage genes through plasmid curing would result in a change of adsorption specificity. The lysogenic nature of ME2 compared with its phage-sensitive mutants has not yet been studied. However, this type of lysogenic conversion is one possible explanation for the results reported here for ME2.

The phage insensitivity of strain ME2 is only partially understood. The efficiency of plating of the phage-sensitive mutants is very low, 5×10^{-7}. Therefore, this strain possesses mechanisms that prevent growth of the phage after adsorption. Continued study of efficiently adsorbing mutants of ME2 will, we hope, provide insight into other phage defense mechanisms in this strain. Our studies indicate, however, that the phage resistance of commercially phage-insensitive strains of the lactic streptococci is likely to result from the cumulative effects of several distinct systems.

The interactions of strain ME2 cells with several phages in addition to φ18 were affected by the loss of plasmid pME0030. The ME2 mutant N1 was able to adsorb φml3, φc2, and φ23 more efficiently than ME2 could and was able to replicate φ29 and φ40, whereas ME2 could not. These results indicate the involvement of pME0030 in affecting the sensitivity of ME2 to a variety of phages. A plasmid-encoded gene product(s) that interferes with the susceptibility of a lactic streptococcal strain to a variety of phages could be significant in the control of phage infection of starter cultures. However, insufficient data are available on the mechanism of action of this gene product(s) to speculate on its general usefulness against phage attack. The different types of interactions observed in this study (adsorption without replication, replication without efficient adsorption, efficient adsorption with limited phage production) may illustrate several facets of phage resistance remaining in N1. Prevention of the full-scale proliferation of phage on N1 may be caused by a general incompatibility with the host or may reflect specific phage defense mechanisms that remain in this strain. Why pME0030 should effect adsorption by some phages and proliferation of other phages is not known. Perhaps it exhibits more than one effect that can act independently, as with φ29, φ40, φml3, φc2, and φ23, or in combination, as with φ18.

The curing study conducted here reemphasizes the extreme lability of some plasmids harbored by the lactic streptococci. Transfer of a purified isolate only five times in M17-glc broth yielded 13% phage-sensitive variants. Repeated transfer under nonselective conditions generated mutants missing only one plasmid and deficient in only the selected phenotype. Increased cellular stress by using acriflavin or growth at elevated temperatures was more effective in curing multiple plasmids from strains and resulted in mutants lacking several properties, including phage resistance, lactose-fermenting ability, and proteolytic activity. Genetically heterogeneous cultures deficient in traits necessary for

successful commercial use could result quickly from the maintenance of cultures by repetitive subculturing. Cultures containing significant proportions of slow acid producing variants can maintain suitable milk-coagulating ability (11). However, cultures containing a significant proportion of phage-sensitive variants, although perhaps able to execute the fermentation, would have the undesirable property of providing a potential host for the release of lytic phage. Therefore, maintenance of starter cultures in a manner that would minimize the genetic variability resulting from plasmid loss is essential for consistent starter culture performance, especially in the light of evidence that indicates the plasmid linkage of phage resistance mechanisms in the lactic streptococci.

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