APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1989, p. 2702–2709
0099-2240/89/102702-08$02.00/0

Vol. 55, No. 10

Localization of Separate Genetic Loci for Reduced Sensitivity towards Small Isometric-Headed Bacteriophage sk1 and Prolate-Headed Bacteriophage c2 on pGBK17 from *Lactococcus lactis* subsp. *lactis* KR2†

LARRY L. McKAY,* MICHAEL J. BOHANON,‡ KAYLA M. POLZIN, PATRICIA L. RULE,§ AND KATHLEEN A. BALDWIN

Department of Food Science and Nutrition, University of Minnesota, 1334 Eckles Avenue, St. Paul, Minnesota 55108

Received 10 March 1989/Accepted 22 July 1989

The mechanism of reduced sensitivity to the small isometric-headed bacteriophage sk1 encoded on a 19-kilobase (kb) *Hpa*II fragment subcloned from pKR223 of *Lactococcus lactis* subsp. *lactis* KR2 was examined. The reduced sensitivity to phage sk1 was due to a modest restriction/modification (R/M) system that was not active against prolate-headed phage c2. The genetic loci for the R/M system against sk1 and the abortive phage infection (Abi) mechanism effective against phage c2 were then localized by restriction mapping, subcloning, and deletion analysis. The restriction gene was localized to a region of a 2.7-kb *Eco*RV fragment and included an *Eco*RI site within that fragment. The modification gene was found to be physically separable from the restriction gene and was present on a 1.75-kb *Bst*EII-*Xba*I fragment. The genetic locus for the Abi phenotype against phage c2 was localized to a region containing a 1.3-kb *Eco*RI fragment. Attempts to clone the c2 Abi mechanism independent of the sk1 R/M system were unsuccessful, suggesting that expression of the *abi* genes required sequences upstream of the modification gene. Some pGBK17 (vector pGB301 plus a 19-kb *Hpa*II insert fragment) transformants exhibited the R/M system against phage sk1 but lost the Abi mechanism against phage c2. These transformants contained a 1.2- to 1.3-kb insertion in the Abi region. The data identified genetic loci on a cloned 19-kb *Hpa*II fragment responsible for restriction activity and for modification activity against a small isometric-headed phage and for Abi activity against prolate-headed phage c2. A putative insertion element was also found to inactivate the *abi* gene(s).

Bacteriophage infection of lactococci used in milk fermentation processes is of major concern to the dairy fermentation industry (6). The genetic determinants for resistance to these bacteriophages are often plasmid encoded (for recent reviews, see references 6, 17, and 30). Recent studies have involved cloning and subcloning of these phage resistance genes in an attempt to localize and characterize them for subsequent strain improvement by recombinant DNA techniques (30).

In a previous communication, a 36-kilobase (kb) plasmid (pKR223) from *Lactococcus lactis* subsp. *lactis* KR2 (previously designated *Streptococcus lactis* subsp. *diacetylactis*) was shown to encode a modest 20 to 90% reduction in efficiency of plating (EOP) of both small isometric- and prolate-headed bacteriophages when transferred to the plasmid-free derivative of *L. lactis* subsp. *lactis* C2, LM0230. The gene(s) responsible for this reduced phage sensitivity was localized on a 19-kb *Hpa*II restriction fragment of pKR223 which was inserted into the *Hpa*II site on streptococcal cloning vector pGB301. The resulting plasmid was designated pGBK17. With prolate-headed phage c2, the reduced sensitivity exhibited by pGBK17 included reduced burst size (46 PFU compared with 94 PFU for LM0230) and production of fewer infective centers (<10%) than with infections of LM0230. Thus, in the presence of pGBK17, phage c2 infection was reduced and the burst size of those phage that did infect was also smaller. The c2 plaques formed on pGBK17 were translucent, smaller, and more difficult to visualize than the clear plaques formed on LM0230. It was also concluded that pGBK17 did not encode a restriction/modification (R/M) system active against phage c2 and that the reduced phage sensitivity was due to an abortive phage infection (Abi$^+$), as described for some other lactic streptococcal host systems (2, 18). In contrast, the mechanism of reduced phage sensitivity exhibited by pGBK17 on the small isometric-headed phage sk1 was not examined other than to note that it appeared to be different from that of the prolate-headed phage and that clear rather than translucent plaques were formed.

The current study focused on locating the genetic loci on the 19-kb *Hpa*II fragment of pKR223 which encode reduced sensitivity toward prolate-headed and small isometric-headed phages. By subcloning and deletion analysis, more than one genetic locus was shown to be involved in expressing the reduced phage sensitivity.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *L. lactis* strains used in this study included LM0230, a plasmid-free derivative of *L. lactis* C2 (9); JK301, an LM0230 transformant harboring pGB301 (19); and GBK17, an LM0230 transformant containing a 19-kb fragment from pKR223 cloned into the *Hpa*II site of pGB301 (21). This plasmid containing the insert was designated pGBK17. The *Escherichia coli* strains used were HB101 (8) and DH5α (13). Before use, the *L. lactis* strains were grown in M17 broth (33) containing 0.5% glucose

* Corresponding author.
† Paper 17016 of the contribution series of the Minnesota Agricultural Experiment Station.
‡ Present address: The Upjohn Co., Kalamazoo, MI 49007.
§ Present address: Vitek Systems, Inc., Hazelwood, MO 63166.

(M17-G), and *E. coli* was grown in LB broth (22) with shaking at 37°C. *L. lactis* strains containing pGB301 (3) or pSA3 (7) were grown in the presence of erythromycin (5 μg/ml). *E. coli* strains containing pSA3 were grown in the presence of tetracycline (12.5 μg/ml) or chloramphenicol (100 μg/ml).

Plasmid DNA isolation, restriction mapping, and agarose gel electrophoresis. *L. lactis* plasmid DNA was isolated by the method of Anderson and McKay (1). *E. coli* plasmid DNA was isolated by the alkaline lysis procedure (22) or the rapid boiling method (15). Plasmid DNA was purified by cesium chloride-ethidium bromide density gradient centrifugation and desalted as described previously (21). Restriction digests were conducted as described by Maniatis et al. (22). Agarose gel electrophoresis was performed through 0.6 to 1.5% agarose gels in Tris-acetate buffer (pH 8.0; 22) at 4 V/cm, followed by staining in ethidium bromide (0.5 μg/ml).

Polyacrylamide gel electrophoresis and silver staining. An 8% polyacrylamide gel was prepared as described by Maniatis et al. (22), and electrophoresis was conducted at 20 mA (16 V/cm) in a cooled vertical gel apparatus (SE600; Hoefer Scientific Instruments, San Francisco, Calif.) using Tris-borate buffer until the bromophenol blue dye was within 2 cm of the bottom of the gel (about 1.5 h). DNA size markers consisted of lambda DNA digested with *Bst*EII and DNA marker V (*Hae*III digestion of pBR322 DNA; Boehringer Mannheim Biochemicals, Indianapolis, Ind.). The DNA was visualized by silver staining with a silver stain kit (Bio-Rad Laboratories, Richmond, Calif.) as suggested by the manufacturer.

Subcloning fragments of pGBK17. Two methods of subcloning were used. To form deletion derivatives pMN1 through pMN9 (see Fig. 1), pGBK17 was digested with the enzyme necessary to remove the fragment(s) to be deleted, the mixture was self-ligated, and the DNA was transformed into *L. lactis* LM0230. Em^r transformants were selected, and deletion derivatives were identified by restriction analysis of the plasmid DNA. Subclones pMN10 through pMN12 (see Fig. 1) were formed by isolating the desired fragment of pGBK17 from ultrapure low-melting-point agarose and removing the agarose by the method of Maniatis et al. (22), except that the buffer used to dilute the agarose consisted of 20 mM Tris hydrochloride (pH 8.0), 1 mM EDTA, and 250 mM NaCl and the first extraction was made with 3% NaCl-saturated phenol. The purified fragment was then ligated to vector pSA3 and transformed into *E. coli* DH5α, and the desired clones were identified by restriction analysis of Cm^r Tc^s transformants. Plasmid DNA was then isolated from these clones and used to transform *L. lactis* LM0230. Transformants were selected on M17-G containing erythromycin (5 μg/ml).

All ligations were conducted as described by Maniatis et al. (22), with a vector-insert ratio of 1:4 and T4 DNA ligase. *L. lactis* transformation was conducted as described by Froseth et al. (11), with 1 μg of DNA. *E. coli* transformation was performed as described by Hanahan (13), with 500 ng of DNA.

Bacteriophage assays. *L. lactis* transformants were screened for sensitivity to small isometric-headed phage sk1 (14) and to prolate-headed phage c2 (16) as previously described, with plaque assays (10). The presence of an R/M system (R^+/M^+) was evaluated as described by Kruger and Bickle (20) and by Pearce (27). Bacteriophages sk1 and c2 were first purified from single plaques and propagated on the R^-/M^- homologous host *L. lactis* LM0230 (14) to obtain high-titer lysates. EOP on test strains was determined as described previously (10). A single plaque from the cell lawns of the test strain was then picked and propagated on the test strain in phage broth (33) to obtain high-titer lysates. The EOP of this lysate on various hosts was used to assess R/M activity in the original test strain. Loss of a modification was determined by allowing the phage to form plaques on the desired host after propagating it through R^-/M^- *L. lactis* LM0230. Modified phages were designated by suffixes indicating the last host for propagation, for example, sk1·LM0230·GBK17.

RESULTS

Restriction mapping of the 19-kb *Hpa*II insert. To facilitate deletion and subcloning of fragments that may affect reduced phage sensitivity, a map of the 19-kb *Hpa*II fragment of pKR223 was constructed (Fig. 1). Instead of 17 kb, as originally reported (21), the size of the *Hpa*II insert appeared to be 19 kb.

Operation of an R/M system in GBK17 active against phage sk1 but not phage c2. To determine whether the 19-kb *Hpa*II fragment in pGBK17 encoded an R/M system active against phage sk1, the phage was propagated on *L. lactis* LM0230 after a single plaque was picked from this host. Phage sk1 propagated on LM0230 showed a modest EOP of 0.05 when allowed to form plaques on *L. lactis* GBK17 (Table 1). Phage picked from the plaques formed on GBK17 was not restricted by GBK17 or JK301, and EOPs of 1.0 and 1.1, respectively, were obtained. However, after propagation of sk1·GBK17 on JK301, the phage again showed a low EOP of 0.02 on GBK17. These results suggest that an R/M system is involved in retarding phage sk1 development on GBK17.

The results obtained with strains JK301 and GBK17 and phage c2 (Table 2) initially suggested that the sk1 R/M system in GBK17 was also active against phage c2. However, this result was complicated by the fact that GBK17 is R^+/M^+ Abi^+. To circumvent this problem, R^+/M^+ Abi^- strain MN1 (Fig. 1) was used to assess restriction activity against phage c2. The results (Table 2) indicated that phage c2 was not restricted by MN1. Furthermore, phage propagated through MN1 still exhibited the reduced EOP on GBK17. Phage picked from MN1 plaques, propagated through R^-/M^- JK301, and then allowed to form plaques on MN1 also was not restricted (EOP, 1.0). These results imply that phage c2 is not restricted by the sk1 R/M system and that the reduced EOP on R^+/M^+ Abi^+ GBK17 observed for phage c2 is due to the Abi^+ phenotype.

Location of separate genetic loci for reduced sensitivity to phages c2 and sk1 on the 19-kb *Hpa*II fragment. *Eco*RI cleaved pGBK17 into detectable fragments of 19.8, 6.3, 1.5, and 1.3 kb, the largest of which contained the vector pGB301. Deletion derivatives were made by digesting pGBK17 with *Eco*RI, religating it, and transforming it into *L. lactis* LM0230. Em^r transformants were then screened for sensitivity to phages c2 and sk1. All isolates except one (MN1) were sensitive to both phages at levels observed for hosts lacking the 19-kb *Hpa*II insert. MN1, however, retained restrictive activity toward phage sk1 (Table 1) but lost the reduced sensitivity to phage c2. The c2 plaques formed on GBK17 were translucent and pinpoint to 0.5 mm in diameter, in contrast to the clear plaques formed on JK301 and MN1, which were 0.5 to 3 mm in diameter (Fig. 2).

To confirm that the plasmid in MN1 was responsible for the reduced sensitivity to phage sk1, pMN1 was both cured from MN1 and transformed into LM0230. Cured derivatives gained full sensitivity to phage sk1, and transformants that

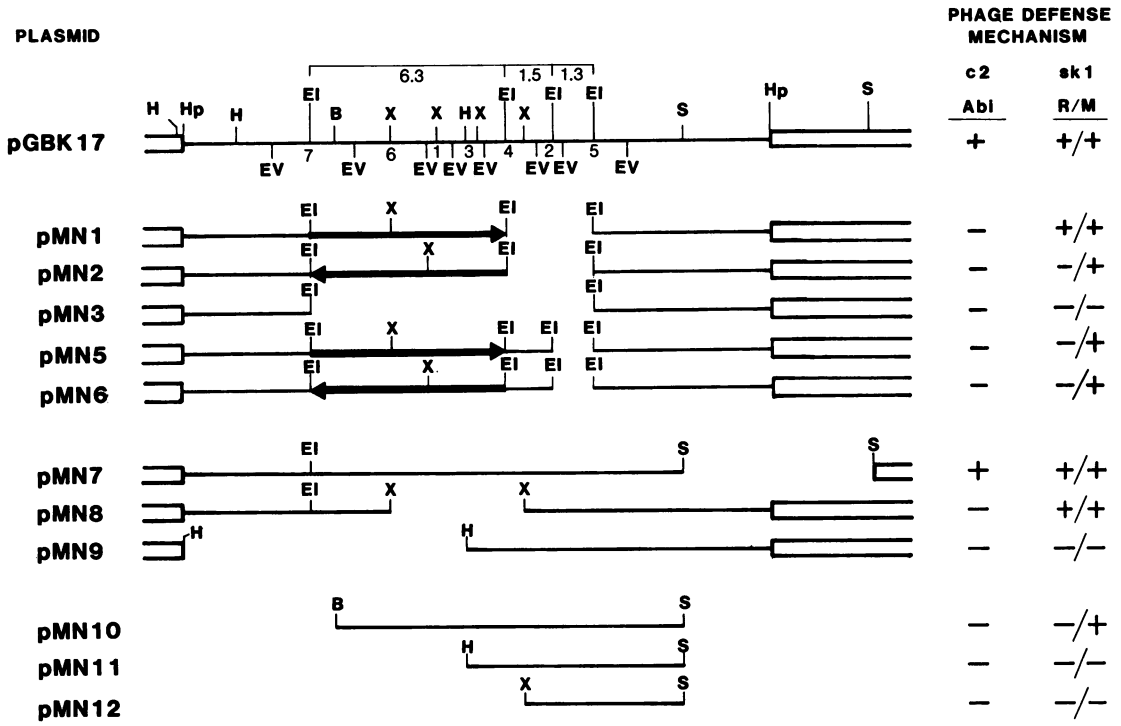

FIG. 1. Restriction maps of pGBK17 (numbers above the line, sizes in kilobases; numbers below the line, restriction fragment designations), the 19-kb *Hpa*II fragment of pKR223 cloned into streptococcal vector pGB301, and various deletion derivatives and subclones of pGBK17. Mapping of the restriction enzyme cleavage sites, except for *Eco*RV, was performed by using single, double, and sometimes triple digestions. *Eco*RV sites were mapped by using various deletion derivatives and subclones of pGBK17. The ordering of *Eco*RV fragments 1 and 6 was accomplished by restriction analysis. The bar represents pGB301 DNA, and the thin line represents the 19-kb *Hpa*II fragment cloned from pKR223. The arrow represents the orientation of the 6.3-kb *Eco*RI fragment. Designations of restriction enzymes: B, *Bst*EII; EI, *Eco*RI; EV, *Eco*RV; H, *Hae*III; Hp, *Hpa*II; S, *Sph*I; X, *Xba*I. Phage defense mechanism refers to Abi against prolate-headed phage c2 and R/M activity against the small isometric-headed phage sk1.

acquired the plasmid gained resistance to the phage (data not shown). To correlate particular restriction fragments with phage resistance phenotypes, plasmid DNAs from transformants MN1(pMN1), MN2(pMN2), MN3(pMN3), MN5(pMN5), and MN6(pMN6) were isolated and cleaved with various restriction enzymes to determine the *Eco*RI fragments present and their orientations (Fig. 1). As expected, all contained the 19.8-kb *Eco*RI fragment encompassing the vector pGB301. Plasmid pMN1 lacked the 1.5- and 1.3-kb *Eco*RI fragments but possessed the 6.3-kb *Eco*RI fragment in the same orientation as in pGBK17. Plasmid pMN2 also lacked the two small *Eco*RI fragments and contained the 6.3-kb *Eco*RI fragment in the orientation opposite to that of pGBK17. The plasmid from MN3 lacked the 6.3-, 1.5-, and 1.3-kb *Eco*RI fragments. pMN5 contained the 6.3- and 1.5-kb fragments in the parental position and orientation. The plasmid from MN6 resembled pMN2, except for the presence of the 1.5-kb fragment in pMN6.

The loci responsible for phage sk1 and c2 resistances were further localized by developing additional deletion derivatives. An *Xba*I deletion of pGBK17 (Fig. 1, pMN8) resulted only in loss of resistance to phage c2. The restrictive activity

TABLE 1. Host-controlled R/M activity of phage sk1 by pGBK17 and several derivatives of this plasmid

Phage	Relative[a] EOP on:					
	JK301 (pGB301)	GBK17 (pGBK17)	MN1 (pMN1)	MN2 (pMN2)	MN3 (pMN3)	MN10 (pMN10)
sk1	1.1	0.05	0.14	1.4	1.2	1.3
sk1·GBK17	1.1	1.0				
sk1·GBK17·JK301	1.0	0.02				
sk1·MN1	1.0	0.95	1.0			
sk1·MN1·JK301	1.0	0.02	0.10			
sk1·MN2	1.0	0.77		1.0		
sk1·MN2·JK301	1.0	0.01		0.75		
sk1·MN3	0.88	0.07			1.0	
sk1·MN3·JK301	1.0	0.02			0.95	
sk1·MN10	1.2	0.85				1.0
sk1·MN10·JK301	1.0	0.02				0.97

[a] Phage sk1 was initially propagated on *L. lactis* LM0230 (EOP, 1.0).

TABLE 2. Absence of host-controlled R/M activity toward phage c2 by pGBK17

Phage	Relative[a] EOP on:		
	JK301 (pGB301)	GBK17 (pGBK17)	MN1 (pMN1)
c2	1.0	0.1	0.82
c2·MN1	1.1	0.2	1.0
c2·MN1·JK301	1.0	0.15	1.0

[a] Phage c2 was initially propagated on *L. lactis* JK301.

against phage sk1 was retained. Deletion of the 6.0-kb *Sph*I fragment from pGBK17, as in MN7, did not affect the phage resistance mechanisms exhibited against phage c2 or sk1 but did eliminate the Cm^r phenotype (Fig. 1). Deletion of the *Hae*III region (Fig. 1, pMN9) resulted in full sensitivity to both phage types.

Analysis of the deletion derivatives and their corresponding c2 and sk1 phage resistance phenotypes suggested that a genetic locus responsible for resistance to phage c2 is located in the region of the 1.5- and 1.3-kb *Eco*RI fragments, since loss of one (MN5) or both (MN1) of these fragments coincided with loss of c2 resistance (Fig. 1). Regarding the locus that determines the restriction activity against phage sk1, restriction activity against phage sk1 was functional if the 6.3-kb *Eco*RI fragment was in the parental orientation, as in pMN1, but not if the fragment was inverted, as in pMN2 and pMN6 (Fig. 1). The exception, pMN5, is discussed below. Moreover, restriction activity was lost upon deletion of the 6.3-kb *Eco*RI fragment, as in pMN3, or the *Hae*III fragment, as in pMN9, but was maintained in *Xba*I deletion derivative pMN8 (Fig. 1). Since all transformants resistant to phage sk1 have the *Eco*RI site within *Eco*RV fragment 7 intact, while this site is disrupted in all sk1-sensitive transformants, the locus for restriction activity against phage sk1 must lie in the region of the *Eco*RI site within fragment 7 of the *Eco*RV digest (Fig. 1).

Based on the above-described information and the restriction map presented in Fig. 1, attempts to clone the region believed to be responsible for Abi^+ in response to phage c2 were initiated. MN10(pMN10), MN11(pMN11), and MN12(pMN12) are transformants containing the *Bst*EII-*Sph*I, *Hae*III-*Sph*I, and *Xba*I-*Sph*I restriction fragments of pGBK17, respectively (Fig. 1). However, all three transformants exhibited full sensitivity to phage c2.

Analysis of the R^- phenotype in MN5. Although the *Eco*RI site of *Eco*RV fragment 7 appeared to be intact in pMN5, MN5 was R^-. Several restriction digestions were conducted to search for a previously undetected difference among pMN5, pMN1, and pGBK17 in the region of the restriction (*r*) gene. Restriction digestions with *Eco*RI showed no detectable difference among these three clones, even when electrophoresed through a high-percentage (1.5%) agarose gel or when large amounts of DNA were used to detect previously undetected small fragments (data not shown). However, an *Eco*RV digest appeared to indicate a slight increase in the size of *Eco*RV fragment 7 in pMN5 (data not shown). To verify and further localize this difference, the three plasmids were subjected to double digestion with *Eco*RV and *Bst*EII. The results are shown in Fig. 3. *Bst*EII cuts *Eco*RV fragment 7 into 1.65- and 1.0-kb fragments (Fig. 3, lanes 1 and 2). Although the 1.0-kb fragment was present in all three plasmids, the 1.65-kb fragment was missing in pMN5 (lane 4). Instead, an additional 1.7-kb fragment was present. Therefore, *Eco*RV fragment 7 of pMN5 appeared to contain approximately 50 base pairs (bp) of additional DNA located within the *Eco*RV-*Bst*EII subfragment containing the *Eco*RI site (Fig. 1). The most likely explanation for the source of the additional DNA was that the original clone, pGBK17, contained an undetected *Eco*RI fragment of approximately 50 bp which was ligated into the *Eco*RI site of *Eco*RV fragment 7 during formation of the deletion derivatives. To verify this, the plasmids were digested with *Eco*RI and electrophoresed on a polyacrylamide gel. pGBK17 contained a previously undetected 64-bp fragment which was also present in pMN5 but was missing from pMN1 (Fig. 4). Therefore, the R^- phenotype of pMN5 appeared to be due to the presence of a 64-bp *Eco*RI fragment ligated to the *Eco*RI site in *Eco*RV fragment 7 of pMN5. This finding further localizes the *r* gene to the region of the *Eco*RI site of *Eco*RV fragment 7.

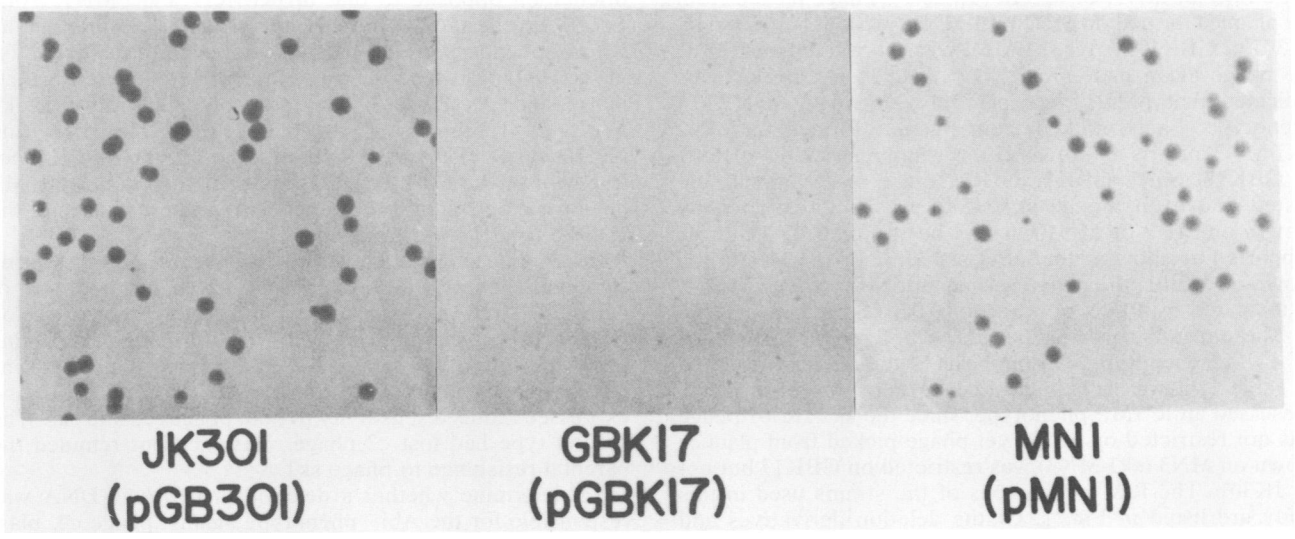

FIG. 2. Plaque morphology of phage c2 on *L. lactis* JK301, an LM0230 transformant harboring the streptococcal vector pGB301; on GBK17, an LM0230 transformant containing a 19-kb fragment from pKR223 cloned into pGB301; and on MN1, a deletion derivative of pGBK17 lacking the 1.5- and 1.3-kb *Eco*RI fragments of the 19-kb insert. Phage assays were conducted at 32°C.

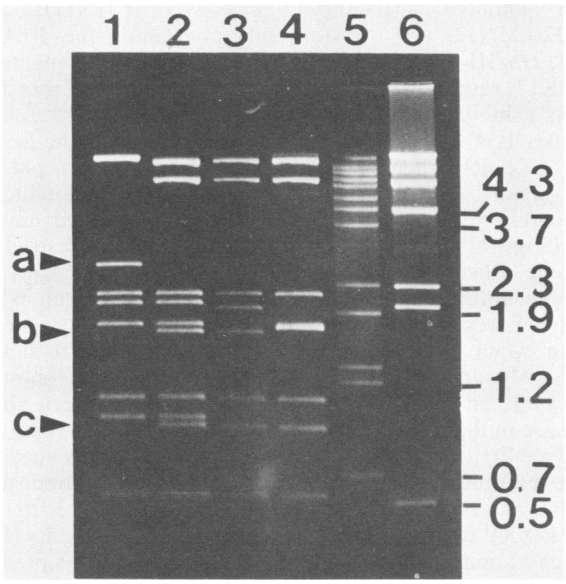

FIG. 3. Restriction digests of pGBK17 and deletion derivatives pMN1 and pMN5 showing that pMN5 differs within *Eco*RV fragment 7. DNAs were digested with *Eco*RV or *Eco*RV and *Bst*EII and electrophoresed in a 1.5% agarose gel. Arrowhead a indicates the 2.7-kb *Eco*RV fragment 7. Arrowheads b and c indicate the 1.65- and 0.95-kb fragments, respectively, produced by *Bst*EII digestion of *Eco*RV fragment 7. Lanes: 1, pGBK17 digested with *Eco*RV; 2, pGBK17 digested with *Eco*RV and *Bst*EII; 3, pMN1 digested with *Eco*RV and *Bst*EII; 4, pMN5 digested with *Eco*RV and *Bst*EII; 5, lambda DNA digested with *Bst*EII; 6, lambda DNA digested with *Hin*dIII. The numbers on the right indicate molecular sizes in kilobases.

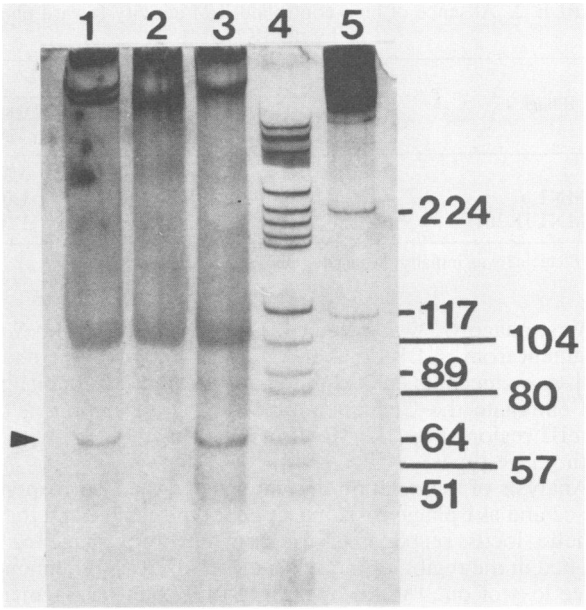

FIG. 4. Polyacrylamide gel showing that pGBK17 and pMN5 contain a previously undetected 64-bp *Eco*RI fragment not present in pMN1. Plasmid DNAs were digested with *Eco*RI, electrophoresed in an 8% polyacrylamide gel, and silver stained as described in Materials and Methods. The 64-bp fragment is indicated by the arrowhead. Lanes: 1, pGBK17; 2, pMN1; 3, pMN5; 4, DNA marker V; 5, lambda DNA digested with *Bst*EII. The numbers on the right indicate molecular sizes in base pairs.

Identification of modification activity independent of restriction activity. By propagating phage sk1 on various hosts and allowing it to form plaques on R^-/M^- JK301 and R^+/M^+ GBK17, it was possible to assess whether the phage had been modified during growth through the restrictive host. When phage sk1 was propagated through JK301 (sk1·JK301), the EOP was reduced on GBK17 and MN1 but not on MN2, MN3, or MN10 (Table 1). Phage picked from the plaques formed on MN1 (sk1·MN1) was not restricted by JK301 or GBK17. After sk1·MN1 was propagated on JK301, the phage again had a low EOP of 0.02 on GBK17. This indicated that pMN1, like pGBK17, encoded an R^+/M^+ phenotype. Phage picked from plaques formed on MN2 (sk1·MN2) or MN10 (sk1·MN10) was not restricted by JK301 or GBK17. Since GBK17 is R^+, these observations suggested that even though sk1·JK301 was not restricted by growth on MN2 or MN10, it had been modified. This was supported by showing that after sk1·MN2 or sk1·MN10 was grown on JK301, the phage was again restricted on GBK17, as indicated by EOPs of 0.01 and 0.02, respectively (Table 1). These results revealed that pMN2 and pMN10 possessed an R^-/M^+ phenotype. On the basis of the results presented in Table 1, it was concluded that pMN3, present in MN3, possessed an R^-/M^- phenotype, since the sk1·JK301 phage was not restricted on MN3, yet phage picked from plaques grown on MN3 (sk1·MN3) was restricted on GBK17 but not on JK301. The R/M phenotypes of the strains used in this study are listed in Fig. 1. Of the deletion derivatives and subclones examined, MN1, MN2, MN5, MN6, MN7, MN8, and MN10 were able to modify phage sk1, but MN1, MN7, and MN8 were the only hosts also able to restrict the phage. Hosts MN3, MN9, MN11, and MN12 were unable to restrict or modify phage sk1.

Location of the genetic locus for modification activity. On the basis of the R/M phenotypes of the GBK17 derivatives and the restriction maps of the altered pGBK17 plasmids in the various hosts (Fig. 1), the genetic region responsible for modifying phage sk1 was localized. MN1 was R^+/M^+ and had the 6.3-kb *Eco*RI fragment on pMN1 oriented as in the parental plasmid, pGBK17, which was also R^+/M^+ (Fig. 1). MN2 was R^-/M^+ and had the 6.3-kb fragment in the orientation opposite to that of pGBK17 and pMN1. Total *Eco*RI deletion of pGBK17, as in MN3, resulted in an R^-/M^- phenotype. These results suggested that the modifying gene(s) is located on the 6.3-kb *Eco*RI fragment. Furthermore, since MN8 was R^+/M^+ and contained only a 2.5-kb *Eco*RI-*Xba*I segment of the 6.3-kb *Eco*RI fragment and MN10 was R^-/M^+ and shared only the 1.75-kb *Bst*EII-*Xba*I fragment of the 6.3-kb *Eco*RI fragment with MN8, the M^+ phenotype appeared to be dependent on the presence of the 1.75-kb *Bst*EII-*Xba*I fragment (Fig. 1).

Insertional inactivation of Abi against phage c2. During subcloning of pGBK17 restriction fragments and use of pGBK17 DNA as a positive control for transformation efficiency, it was noticed that transformants containing pGBK17 were of two phage sensitivity types. One type was phenotypically identical to the parental strain GBK17 and expressed reduced sensitivity to both phages c2 and sk1. The second type had lost c2 phage resistance but retained the parental resistance to phage sk1.

To determine whether a deletion in pGBK17 DNA was responsible for the Abi^- phenotype against phage c2, plasmid pMN14 from a c2-sensitive transformant was restricted with *Eco*RI. The restriction pattern of pMN14 appeared to be identical to that of pGBK17 (data not shown). However,

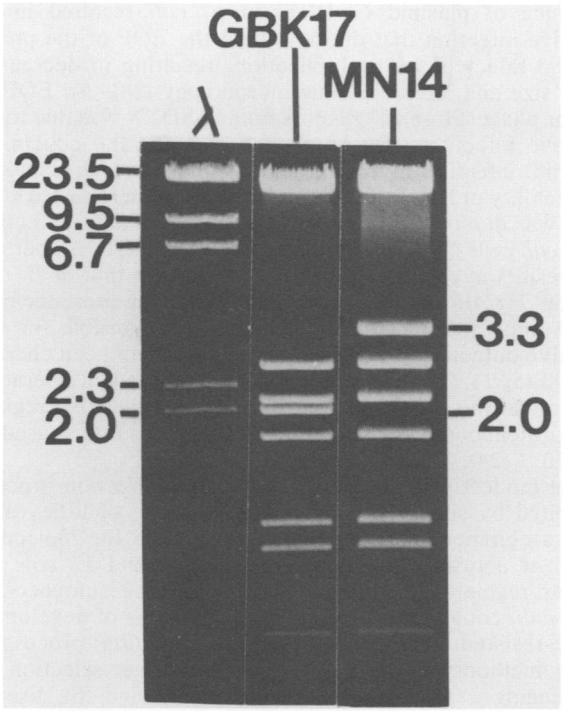

FIG. 5. *Eco*RV digest of pGBK17 (c2 phage resistant) and pMN14 (c2 phage sensitive) indicating a possible insertion into the 2.0-kb *Eco*RV fragment of pGBK17. Electrophoresis was in a 0.6% agarose gel. The sizes of the missing and new fragments in pMN14 are indicated in kilobases. Lanes: 1, lambda DNA digested with *Hin*dIII; 2, pGBK17; 3, pMN14.

restriction of pMN14 with *Eco*RV, which cuts the 19-kb *Hpa*II insert of pGBK17 into seven fragments (Fig. 1), revealed a new 3.3-kb *Eco*RV fragment and loss of the 1.9-kb fragment (*Eco*RV fragment 5 of pGBK17) (Fig. 5).

To determine the basis for the apparent increase in size of *Eco*RV fragment 5, additional restriction digestions of pGBK17 and pMN14 were performed. Since no difference between the *Eco*RI digests of the two plasmids was observed, it was suspected that the insertion had occurred in the portion of the 19.8-kb *Eco*RI fragment that overlaps *Eco*RV fragment 5 (Fig. 1). A 1.3-kb insertion into such a large fragment may not have been detectable by the agarose gel electrophoresis conditions used. To confirm this, an *Eco*RI-*Hpa*II double digestion was performed. *Hpa*II cleaved the 19.8-kb *Eco*RI fragment into the 9.8-kb *Hpa*II vector fragment and 5.8- and 4.2-kb *Hpa*II-*Eco*RI insert fragments, all allowing detection of a 1.3-kb insertion. The result of this digestion was that pMN14 lost the 5.8-kb *Hpa*II-*Eco*RI fragment and contained a new fragment of 7.0 kb (data not shown). Therefore, the DNA insertion in pGBK17 was concluded to be 1.2 to 1.4 kb and to have occurred in the 1.1-kb *Eco*RI-*Eco*RV fragment common to *Eco*RV fragment 5 and the 5.8-kb *Hpa*II-*Eco*RI fragment (Fig. 1).

To further characterize the insertion, additional restriction digestions of both plasmids were performed. These digestions revealed that pMN14 contained an additional 0.7-kb *Xba*I fragment and an additional 0.7-kb *Hae*III fragment, both mapping within the 1.1-kb *Eco*RI-*Eco*RV fragment (Fig. 6). Therefore, conversion of pGBK17 from reduced sensitivity to full sensitivity to phage c2 appeared to be the result of insertion of a DNA sequence containing two *Xba*I and two *Hae*III sites into the 1.1-kb *Eco*RI-*Eco*RV fragment. These restrictions also confirmed that the size of the insert was 1.2 to 1.3 kb.

DISCUSSION

The 19-kb *Hpa*II fragment subcloned from pKR223 in *L. lactis* KR2 codes for genes responsible for an R/M system effective against the small isometric-headed phage sk1 (this study) and for an Abi^+ phenotype effective against prolate-headed phage c2 (21). Genetic loci for the R^+/M^+ and Abi^+ phenotypes were localized by restriction mapping, subcloning, and deletion analysis. Both systems exhibited only a modest reduction in EOP of about 0.1.

R/M systems encoded by plasmid DNA are widely distributed among lactococci (for a review, see reference 17). Some of these have exhibited modest restriction activity like that of pGBK17. For example, Teuber (34) correlated the presence of a plasmid in *L. lactis* with restriction of a phage with an EOP of 0.1, and Chopin et al. (4) described an R/M phenotype on a plasmid from *L. lactis* IL594 that exhibited an EOP of 0.08. Higgins et al. (14) also demonstrated EOPs of 0.05 to 0.4 for some Lac^+ transconjugants containing cointegrate plasmids coding for R/M activity. It is unknown whether the modest R/M activity exhibited by pGBK17 is due to deficiency of its expression in LM0230 or whether more efficient R/M activity would be expressed if the plasmid were introduced into other strains. It was also of interest that R/M activity against phage sk1 but not against phage c2 was observed. As suggested by Klaenhammer (17), this difference in sensitivity could be related to the differences in

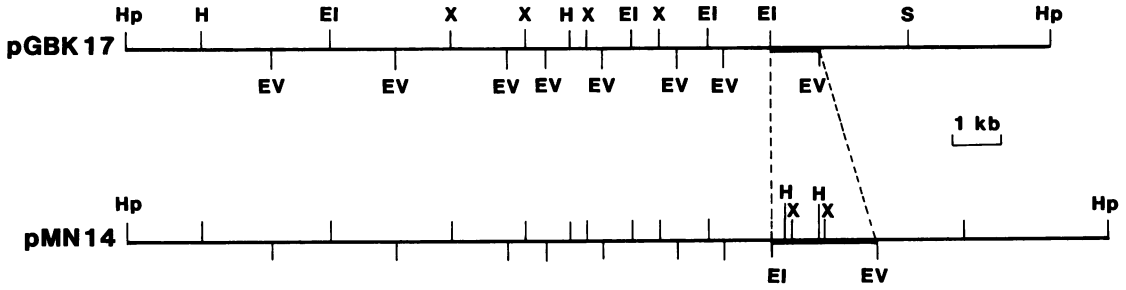

FIG. 6. Restriction maps of the *Hpa*II insert fragment from pGBK17, showing the location of the 1.1-kb *Eco*RI-*Eco*RV fragment (thick bar), and that from pMN14, showing the insertion of a DNA segment into this fragment (thick bar). The *Eco*RI-*Eco*RV fragment in pMN14 is 1.2 to 1.4 kb larger than the fragment from pGBK17 and contains two additional *Xba*I and *Hae*III sites. For abbreviations, see the legend to Fig. 1.

the species of infecting phage and to the length of the phage genome. The level of phage restriction has been shown to correlate with the estimated genome size of the infecting phage (17). Since prolate-headed phages have smaller genomes than small isometric-headed phages (17), the absence of R/M activity against phage c2 by pGBK17 could be explained by absence of appropriate target sites for the enzyme.

As indicated by Sanders (30), all curing and conjugation data on known lactococcal R/M plasmids support the linkage of both restriction and modification activities to the same plasmid. This is further supported by the cloning of a 19-kb *Hpa*II fragment from pKR223 which contained genes for an R/M system. In addition, it was found that modification activity could be expressed without restriction expression and that the genetic loci for modification activity appear to reside on a 1.75-kb *Bst*EII-*Xba*I fragment.

The EOP of phage c2 on GBK17 was determined to be 0.1 compared with that on JK301 (Table 2). One-step growth experiments of phage c2 on GBK17 and JK301 (21) revealed that the number of infective centers obtained at zero time on GBK17 was less than 10% of the number obtained on JK301, although both strains adsorbed more than 90% of the input phages. In addition, it was found that the burst sizes per infective center were 46 in GBK17 cells and 108 in JK301 cells. The latent period was about the same with both types of cells. These results indicated that phage c2 could produce progeny phage, to a limited extent, in only a small fraction of infected GBK17 cells. These results, coupled with the absence of an R/M system effective against phage c2 (this study; 21), are consistent with the operation of an abortive phage c2 infection process. The reason(s) for the absence of the Abi^+ phenotype in MN10, MN11, and MN12 is unknown. Since MN7 was Abi^+, it is possible that expression of *abi* genes requires sequences upstream of the modification gene. We also found that insertion of a putative insertion sequence resulted in loss of the Abi^+ phenotype against phage c2. The properties of this insertion sequence and its origin are being investigated.

Klaenhammer and Sanozky (18) were the first to demonstrate abortive infection in lactococci when they found that pTR2030 from *L. lactis* ME2, when conjugally transferred to *L. lactis* LM0230, limited the burst size and plaque size of phage c2 without affecting the EOP or level of adsorption. It was subsequently found that these transconjugants were unable to replicate small isometric-headed phages (16). In contrast to pTR2030-containing strains, pNP40 from *L. lactis* DRC3 completely inhibited the plaque-forming ability of phage c2 at 30°C, although the phage exhibited normal adsorption (23). The small isometric-headed phage sk1 exhibited a 10^{-4} reduction in EOP (unpublished data). It is unknown whether the latter resistance conferred by pNP40 was due to abortive infection.

It has become evident that plasmid-encoded abortive infection among lactococci is widespread (2, 6, 10, 12, 17, 18, 25). Cloning of DNA fragments that determine abortive phage infection in lactococci has also been initiated (17, 21, 31). As indicated by Sanders (30), one of the most significant observations about these abortive-infection phenotypes is that their total phage resistance is conferred in some host backgrounds. Others, however, like pTR2030 and pGBK17 when present in an *L. lactis* LM0230 background, conferred no reduction in EOP or a modest reduction of about 0.1 when phage c2 was used.

These results for abortive infection in lactococci are similar to those described for *E. coli*. For example, the presence of plasmid *Clo*DF13 in *E. coli* resulted in an abortive infection that did not affect the EOP of the phage but did affect phage multiplication, resulting in decreased burst size and altered plaque morphology (28). An EOP of 0.1 for phage T1 on *E. coli* containing pSD274 was due to an abortive infection (32). In *E. coli* carrying the colicin 1b plasmid, infection by phage BF23 is abortive and results in the inability of BF23 to form plaques (26). The reported 10^{-6} EOP was due to the appearance of mutant phage. Infection of *E. coli* cells carrying the F plasmid by phage T7 is abortive and results in an EOP of 0.1 compared with that of *E. coli* without F (24). The plaques that formed in the presence of F were pinpoint. The genetic loci on F responsible for the abortive outcome of T7 infection have already been characterized (5, 24, 29), and it is interesting that when operator-type mutations were isolated such that the negative regulatory protein could not function, the EOP of T7 decreased to 7×10^{-4} (24).

The modest plasmid-mediated abortive infection process exhibited by some lactococci appears to be of little value from a commercial standpoint. However, if the molecular bases of abortive infection in lactococci and *E. coli* are similar, regulatory mutants isolated from these lactococci, as in *E. coli*, could serve as an additional means of developing phage-resistant strains for milk fermentation processes. Other methods being examined include direct selection by challenging a sensitive host with lytic phage (6), use of conjugal transfer systems (17, 30), and use of recombinant DNA techniques (30).

ACKNOWLEDGMENTS

This research was supported in part by Kraft, Inc., Glenview, Ill., in part by the Minnesota-South Dakota Dairy Foods Research Center, and by Hatch and General Agricultural Research funds (project 18-62).

LITERATURE CITED

1. **Anderson, D. G., and L. L. McKay.** 1983. A simple and rapid method for isolating large plasmid DNA from lactic streptococci. Appl. Environ. Microbiol. **46**:549–552.
2. **Baumgartner, A., M. Murphy, C. Daly, and G. F. Fitzgerald.** 1986. Conjugative co-transfer of lactose and bacteriophage resistance plasmids from *Streptococcus cremoris* UC653. FEMS Microbiol. Lett. **35**:233–237.
3. **Behnke, D., M. S. Gilmore, and J. J. Ferreti.** 1981. Plasmid pGB301, a new multiple resistance streptococcal cloning vehicle and its use in cloning of a gentamicin/kanamycin resistance determinant. Mol. Gen. Genet. **182**:414–421.
4. **Chopin, A., M.-C. Chopin, A. Moillo-Batt, and P. Langella.** 1984. Two plasmid-determined restriction and modification systems in *Streptococcus lactis*. Plasmid **11**:260–264.
5. **Cram, D., A. Ray, and R. Skurray.** 1984. Molecular analysis of F plasmid *pif* region specifying abortive infection of T7 phage. Mol. Gen. Genet. **197**:137–142.
6. **Daly, C., and G. Fitzgerald.** 1987. Mechanisms of bacteriophage insensitivity in the lactic streptococci, p. 259–268. *In* J. J. Ferretti and R. Curtiss III (ed.), Streptococcal genetics. American Society for Microbiology, Washington, D.C.
7. **Dao, M. L., and J. J. Ferretti.** 1985. *Streptococcus-Escherichia coli* shuttle vector pSA3 and its use in the cloning of streptococcal genes. Appl. Environ. Microbiol. **49**:115–119.
8. **Davis, R. W., D. Botstein, and J. R. Roth (ed.).** 1980. Advanced bacterial genetics, p. 140–141. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
9. **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.
10. **Froseth, B. R., S. K. Harlander, and L. L. McKay.** 1988. Plasmid mediated reduced phage sensitivity in *Streptococcus*

lactis KR5. J. Dairy Sci. **71**:275–284.

11. **Froseth, B. R., R. E. Herman, and L. L. McKay.** 1988. Cloning of nisin resistance determinant and replication origin on 7.6-kilobase *Eco*RI fragment of pNP40 from *Streptococcus lactis* subsp. *diacetylactis* DRC3. Appl. Environ. Microbiol. **54**:2136–2139.
12. **Gautier, M., and M.-C. Chopin.** 1987. Plasmid-determined systems for restriction and modification activity and abortive infection in *Streptococcus cremoris*. Appl. Environ. Microbiol. **53**:923–927.
13. **Hanahan, D.** 1985. Techniques for transformation of *E. coli*, p. 109–135. *In* D. M. Glover (ed.), DNA cloning: a practical approach. Vol. I. IRL Press, Oxford.
14. **Higgins, D. L., R. B. Sanozky-Dawes, and T. R. Klaenhammer.** 1988. Restriction and modification activities from *Streptococcus lactis* ME2 are encoded by a self-transmissible plasmid, pTN20, that forms cointegrates during mobilization of lactose-fermenting ability. J. Bacteriol. **170**:3435–3442.
15. **Holmes, D. S., and M. Quigley.** 1981. A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. **114**: 193–197.
16. **Jarvis, A. W., and T. R. Klaenhammer.** 1986. Bacteriophage resistance conferred on lactic streptococci by the conjugative plasmid pTR2030: effects on small isometric-, large isometric-, and prolate-headed phages. Appl. Environ. Microbiol. **51**:1272–1277.
17. **Klaenhammer, T. R.** 1987. Plasmid-directed mechanisms for bacteriophage defense in lactic streptococci. FEMS Microbiol. Rev. **46**:313–325.
18. **Klaenhammer, T. R., and R. B. Sanozky.** 1985. Conjugal transfer from *Streptococcus lactis* ME2 of plasmids encoding phage resistance, nisin resistance, and lactose-fermenting ability: evidence for abortive infection of virulent bacteriophage. J. Gen. Microbiol. **131**:1531–1541.
19. **Kondo, J. K., and L. L. McKay.** 1984. Plasmid transformation of *Streptococcus lactis*: optimization and use in molecular cloning. Appl. Environ. Microbiol. **48**:252–259.
20. **Kruger, D. H., and T. A. Bickle.** 1983. Bacteriophage survival: multiple mechanisms for avoiding the deoxyribonucleic acid restriction systems of their hosts. Microbiol. Rev. **47**:345–360.
21. **Laible, N. J., P. L. Rule, S. K. Harlander, and L. L. McKay.** 1987. Identification and cloning of plasmid deoxyribonucleic acid coding for abortive phage infection from *Streptococcus lactis* ssp. *diacetylactis* KR2. J. Dairy Sci. **70**:2211–2219.
22. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **McKay, L. L., and K. A. Baldwin.** 1984. Conjugative 40-megadalton plasmid in *Streptococcus lactis* subsp. *diacetylactis* DRC3 is associated with resistance to nisin and bacteriophage. Appl. Environ. Microbiol. **47**:68–74.
24. **Miller, J. F., and M. H. Malamy.** 1984. Regulation of the F-factor *pif* operon: *pifO*, a site required in *cis* for autoregulation, titrates the *pifC* product in *trans*. J. Bacteriol. **160**:192–198.
25. **Murphy, M. C., J. L. Steele, C. Daly, and L. L. McKay.** 1988. Concomitant conjugal transfer of reduced bacteriophage sensitivity mechanisms with lactose- and sucrose-fermenting ability in lactic streptococci. Appl. Environ. Microbiol. **54**:1951–1956.
26. **Nisioka, T., and H. Ozeki.** 1968. Early abortive lysis by phage BF23 in *Escherichia coli* K-12 carrying the colicin 1b factor. J. Virol. **2**:1249–1254.
27. **Pearce, L. E.** 1978. The effect of host-controlled modification on the replication rate of a lactic streptococcal bacteriophage. N. Z. J. Dairy Sci. Technol. **13**:166–171.
28. **Pol, H. V. D., E. Veltkamp, and H. J. J. Nykamp.** 1980. Genetic information of the bacteriocinogenic plasmid *Clo*DF13 involved in the inhibition of the multiplication of double stranded DNA phages. Mol. Gen. Genet. **178**:535–540.
29. **Rotman, G. S., R. Cooney, and M. H. Malamy.** 1983. Cloning of the *pif* region of the F sex factor and identification of a *pif* protein product. J. Bacteriol. **155**:254–264.
30. **Sanders, M. E.** 1988. Phage resistance in lactic acid bacteria. Biochimie **70**:411–422.
31. **Simon, D., and A. Chopin.** 1988. Construction of a vector plasmid family and its use for molecular cloning in *Streptococcus lactis*. Biochimie **70**:559–566.
32. **Taylor, D. E., and R. B. Grant.** 1978. Bacteriophage inhibition properties of H_2 plasmids, p. 214–216. *In* D. Schlessinger (ed.), Microbiology—1978. American Society for Microbiology, Washington, D.C.
33. **Terzaghi, B. E., and W. E. Sandine.** 1975. Improved medium for lactic streptococci and their bacteriophages. Appl. Microbiol. **29**:807–813.
34. **Teuber, M.** 1986. Construction of bacteriophage resistant dairy starter cultures, p. 539–547. *In* E. Magnien (ed.), Biomolecular engineering in the European Community: achievements of the research programme (1982–1986)—final report. Martinus Nijhoff, Dordrecht, The Netherlands.