Characterization of Restriction-Modification Plasmids from *Lactococcus lactis* ssp. *cremoris* and Their Effects When Combined with pTR2030[1]

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ABSTRACT

Three different restriction-modification plasmids (pTRK12, pTRK30, pTRK317) were isolated from an industrial starter strain, *Lactococcus lactis* ssp. *cremoris* TDM1. A lactose-fermenting transconjugant, *Lactococcus lactis* ssp. *lactis* NCK40, was isolated from matings between *L. lactis* ssp. *cremoris* TDM1 and a plasmid-free recipient. The NCK40 transconjugant contained a 100-kb self-transmissible plasmid (pTRK11) encoding for restriction-modification and a 13.5-kb plasmid (pTRK10) encoding proteolytic activity. Following isolation of lactose-negative derivatives from NCK40, a 30.5-kb plasmid, pTRK12, was identified that encoded proteolytic and restriction-modification of the identical specificity as pTRK11. Restriction analyses and hybridization experiments indicated that pTRK12 contained sequences from pTRK11 and all of pTRK10. Cotransformation of total plasmid DNA from *L. lactis* ssp. *cremoris* TDM1 with vector pVS2 identified two other restriction-modification plasmids, pTRK30 (28.0 kb) and pTRK317 (15.5 kb). Efficiencies of plaquing for phage c2 on restriction-modification transconjugants and transformants was 10^{-2} to 10^{-4}. The specificity of restriction-modification activities conferred by each of the three plasmids was different. When the abortive infection plasmid pTR2030 was combined with pTRK30, both phage inhibition phenotypes were expressed. However, when pTR2030 was combined with pTRK12, the abortive infection phenotype was not fully expressed. Significant cell death occurred when abortive infection cells containing only pTR2030 were infected with phage. Combining the restriction-modification system of pTRK30 with pTR2030 significantly improved cell survival following phage infection. Operation of restriction-modification systems in conjunction with the abortive defense mechanism maximized cell survival. The data suggest that cell death is minimized when the lytic cycle is halted by restriction before abortive infection responses induce phage abortion and kill the cell.

(Key words: *Lactococcus lactis* ssp. *cremoris*, restriction-modification plasmids, plasmids, phage)

Abbreviation key: Ads = adsorption prevention, Cm = chloramphenicol (r = resistant), COI = center of infection, Em = erythromycin (r = resistant), EOP = efficiency of plaquing, Hsp = abortive infection (+), Lac = lactose-fermenting (+) or negative (–), Ori = origin of replication, R/M = restriction-modification (+) or not (–), Tc = tetracycline (r = resistant), Tra = self-transmissible (+) or not (–).

INTRODUCTION

Phage-resistant lactococci are important in maintaining consistent fermentations during the cheese making process. Restriction-modification (R/M) activities are widely dispersed in lactococci and contribute to the phage resistance exhibited by some strains (4, 6, 7, 16, 24, 33, 34, 35). Restriction and modification mech-

Received May 22, 1990.
Accepted September 24, 1990.
[1]Paper Number 12412 of the Journal Series of North Carolina Agricultural Research Service. The use of trade names in this publication does not imply endorsement by the North Carolina Agricultural Research Service of the products named nor criticism of similar ones mentioned.
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anisms have been linked to plasmids found in *Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *cremoris* strains. Individually, these systems can restrict the plaquing efficiency of phages to levels between 10^{-1} and 10^{-4} (6, 8, 11, 12, 13, 15, 27, 34, 40). When the specificity of R/M activities identified on plasmids is different (6, 11, 12, 13), combinations of two or more R/M systems can exhibit increased phage restriction [efficiency of plaquing (EOP) = 10^{-5} to 10^{-6}] (4, 6, 11, 17, 34, 40). Although R/M phenotypes for lactococcal strains are routinely described, physical and genetic characterization of the plasmids, which encode R/M systems, remains limited (12, 27).

Restriction-modification activities naturally complement other mechanisms of resistance such as abortive infection (**Hsp**) or prevention of adsorption (**Ads**) in naturally phage-insensitive strains (11, 12, 13, 27, 35). In some cases, R/M and Hsp are encoded on the same plasmid (10, 13, 27). The combined effects of Hsp and R/M encoded by pTR2030 represent a significant barrier to development of phage 31 (14, 38, 39). Considering this enhanced utility of combined R/M and Hsp mechanisms, construction of strains in which both mechanisms operate is desirable.

This study identified three plasmids from *L. lactis* ssp. *cremoris* TDM1 that encode R/M activities of different specificity. Physical and genetic characterization of two of the R/M plasmids localized the genetic determinants for origin of replication (**Ori**) and R/M: *ori* and *r/m*. The ability of these R/M plasmids to complement Hsp was evaluated.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Bacteriophage

The bacterial strains, plasmids, and bacteriophages used in this study are listed in Table 1. Lactose-fermenting (**Lac$^+$**) *L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris* strains and their bacteriophages were propagated at 30°C in M17 broth (41) or M17 + .5% glucose M17G for lactose-negative (Lac$^-$) derivatives. *Escherichia coli* strains were propagated at 37°C in LB broth with shaking (37). The following concentrations of antibiotics were used: spectinomycin at 300 μg/ml, rifampicin at 50 μg/ml, streptomycin at 1000 μg/ml, erythromycin (**Em**) at 2 μg/ml, chloramphenicol (**Cm**) at 4 μg/ml for *L. lactis*, Cm at 25 μg/ml, and tetracycline (**Tc**) at 10 μg/ml for *E. coli*. Lactose-negative variants were isolated on lactose indicator agar following transfer in M17G broth at 30°C. Plasmid curing was performed by repeated transfer in M17G broth containing 1.0 μg/ml ethidium bromide.

Bacteriophage Resistance and Proteolytic Activity Assays

Bacteriophage titers, EOP, and assays for centers of infection and cell death were conducted as described previously (38, 39). A multiplicity of infection of 10 was used in cell death experiments with phage p2. Proteolytic activity of Lac$^+$ and Lac$^-$ cultures was determined in 11% reconstituted skim milk or 11% reconstituted skim milk + 1% glucose, respectively, using a 18-h incubation period at 22°C as described previously (38).

Isolation and Physical Characterization of Plasmids

Small- and large-scale methods for isolation and detection of plasmids from lactococci were performed as described previously by Anderson and McKay (1). Small-scale plasmid isolations of *E. coli* were performed using the standard alkaline lysis procedure of Birnboim and Doly (3). Large-scale isolation of *E. coli* was conducted as described by Rodriguez and Tait (31). Agarose gel electrophoresis of plasmid DNA, digestion of DNA with restriction endonucleases, and isolation of DNA fragments by electroelution was conducted as described by Maniatis et al. (25).

Gene Transfer Experiments

Conjugal matings and selection of Lac$^+$ transconjugants were conducted as described by McKay et al. (26). Protoplast transformation was conducted as described previously (22, 23) with modifications (14). Vector pVS2 (Em^r Cm^r) (42) was used in cotransformation experiments in a vector:plasmid DNA (wt/wt) ratio of 1:50. The erythromycin-resistant transformants were screened for phage resistance (R/M) by cross streaking against dilutions of phage c2 swabbed on M17G agar plates. All transfor-

TABLE 1. Bacteria, plasmids, and phages.

Bacteria, plasmids, and phage	Relevant characteristics[1]	Source
Bacteria		
Lactococcus lactis ssp. *cremoris* TDM1	Lac^+ industrial starter culture isolate	(38)
Lactococcus lactis ssp. *lactis* LM0230	Lac^- R^-/M^-, plasmid-free transformation recipient	(43)
LM2301	*str*-1 Lac^- R^-/M^-, plasmid-free recipient	(43)
LM2302	*str*-1 *ery*-2, Lac^- R^-/M^-, plasmid-free recipient	(26)
LM2345	*spc*-4 *rif*-5, Lac^- R^-/M^-, plasmid-free recipient	(2)
T-EK1	Lac^+ Hsp^+, pTR1040 pTR2030, conjugal donor for pTR2030	(38)
NCK40	*str*-1 *ery*-2, Lac^+ R^+/M^+ Prt^+, pTRK11 pTRK10	TDM1 × LM2302 transconjugant
NCK41	*str*-1 *ery*-2, Lac^- R^+/M^+ Prt^+, pTRK12	Lac^- derivative of NCK40
NCK42	*str*-1 *ery*-2, Lac^- R^- Prt^+, pTRK10	Lac^- derivative of NCK40
NCK43	*str*-1 *ery*-2, Lac^+ R^- Tra^+, pTRK14	TDM1 × LM2302 transconjugant
NCK44	*spc*-4 *rif*-5, Lac^+ R^+/M^+ Tra^+, pTRK11	NCK40 × LM2345 transconjugant
NCK71	Em^r R^+/M^+ Prt^+, pTRK12 pVS2	Em^r transformant of LM0230
NCK72	Em^r R^+/M^+, pTRK30 pVS2	Em^r transformant of LM0230
NCK85	Em^r Lac^+ Hsp^+ R^+/M^+, pTR1040 pTR2030 pTRK30 pVS2	T-EK1 × NCK72 transconjugant
NCK86	Em^r Lac^+ Hsp^+ R^+/M^+, pTR1040 pTR2030 pTRK12 pVS2	T-EK1 × NCK71 transconjugant
NCK300	Em^r, Ori^+, pSA34-pTRK10 (7.0-kb *Hin*dIII fragment)	Em^r transformant of LM0230
NCK301	Em^r R^+/M^+, pSA34-pTRK12 (*Nru*I clone, pTRK301)	Em^r transformant of LM0230
NCK304	Em^r R^-/M^-, pSA34-pTRK12 (*Xba*I clone, pTRK304)	Em^r transformant of LM0230
NCK317	Em^r R^+/M^+, pTRK317 pTRK10 pVS2	Em^r cotransformant of LM0230 with plasmid DNA from TDM1
Escherichia coli HB101	pSA34 transformation recipient	(5)
MSA34	HB101 (pSA34), source of pSA34	(36)
Bacteriophage		
c2	Prolate phage for LM0230, LM2302, LM2345, and their derivatives	(19)
p2	Small isometric phage for LM0230, LM2302, LM2345, and their derivatives	(12)
p2.NCK72	Phage p2 propagated on NCK72	This study
Plasmids		
pTR1040	Lac^+ Nis^r Tra^-, 71.7 kb	(19)
pTR2030	Hsp^+ Tra^+, 46.2 kb	(19)
pTRK10	Prt^+, 13.5 kb	This study
pTRK11	Lac^+ Tra^+ R^+/M^+, 100 kb	This study
pTRK12	R^+/M^+ Prt^+, 30.5 kb	This study
pTRK14	Lac^+ Tra^+ R^-, 95 kb	This study
pTRK30	R^+/M^+, 28.0	This study
pTRK301	pSA34-pTRK12 *Nru*I clone, 36.5 kb	This study
pTRK304	pSA34-pTRK12 *Xba*I clone, 36.5 kb	This study
pTRK317	R^+/M^+, 17 kb	This study
pTRK320	pSA34-pTRK10 Ori^+ clone, 14.2 kb	This study
pVS2	Cm^r Em^r, 5.1 kb	(42)
pSA3	Cm^r Tc^r Ori^+, *E. coli*; Em^r Ori^+, *L. lactis*, 10.2 kb	(9)
pSA34	Cm^r Tc^r Ori^+, *E. coli*; Em^r Ori^-, *L. lactis*, 6.0 kb	(36)

[1] Lac^+ = Lactose-fermenting; Hsp^+, phage resistance (abortive infection); R^+/M^+, restriction and modification; R^-, nonrestrictive host (the corresponding modification activity was not evaluated in R^- cells); Prt^+, proteolytic activity; Tra^+, self-transmissible; Ori^+, origin of replication; *str*-1, streptomycin resistance (1000 μg/ml); *ery*-2, Em^r, erythromycin resistance (2 μg/ml); Cm^r, chloramphenicol resistance (4 μg/ml); *spc*-4, spectinomycin resistance (300 μg/ml); *rif*-5, rifampicin resistance (50 μg/ml), Tc^r, tetracycline resistance (10 μg/ml).

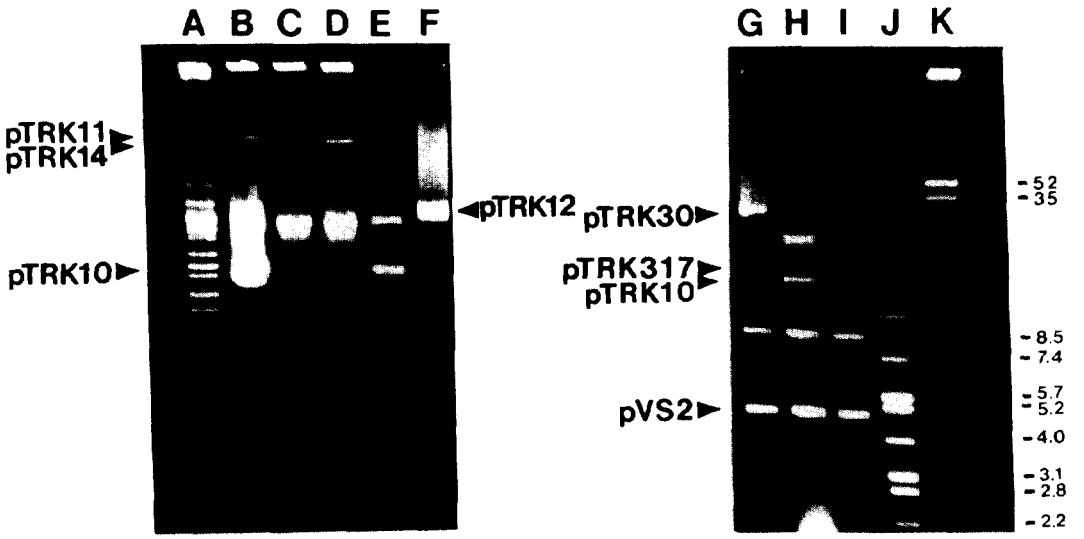

Figure 1. Plasmid DNA from *Lactococcus lactis* ssp. *cremoris* TDM1, lane A; NCK40 (pTRK11, pTRK10) lane B; NCK44 (pTRK11) lane C; NCK43 (pTRK14), lane D; NCK42 (pTRK10), lane E; NCK41 (pTRK12), lane F; NCK72 (pTRK30, pVS2) lane G; NCK317 (pTRK317, pTRK10, pVS2), lane H; pVS2, lane I; and molecular weight standards: 52.3, 7.4, 5.7, 5.2, 4.0, 3.1, 2.8, 2.2 kb, lane J; and 52, 35, 8.5 kb, lane K.

mants and transconjugants were examined for plasmid profile and phenotype. Linkages were assayed by correlation of phenotype with plasmid acquisition or loss. Transformation of *E. coli* competent cells was conducted as described by Maniatis et al. (25).

Molecular Cloning

General procedures for DNA manipulations and cloning were as described by Maniatis et al. (25). The Ori search vector pSA34 (36), a deletion derivative of the *E. coli* and *L. lactis* ssp. *lactis* shuttle vector pSA3 (9), was used in all cloning experiments. Vector pSA34 was Em^r Ori^- for *L. lactis*, Cm^r Tc^r Ori^+ for *E. coli*, and carried unique cloning sites for *Nru*I, *Sal*I, *Sph*I, *Bam*HI, *Eco*RV, *Hin*dIII, and *Xba*I within the Tc gene. Cloning of the origin of replication (*ori*) from pTRK10 was conducted in *E. coli* HB101 using pSA34 and recombinants evaluated for replication in *L. lactis* ssp. *lactis* LM0230. To localize the R^+/M^+ and Ori^+ determinants on pTRK12 and pTRK30, partial digestion fragments of pTRK12 or pTRK30 were generated using diluted restriction endonucleases and ligated to pSA34 completely digested with the appropriate enzyme. Ligation mixes were transformed directly into *L. lactis* ssp. *lactis* LM0230. The Em^r Ori^+ transformants were isolated and examined for pSA34-pTRK12 or pSA34-pTRK30 recombinants.

RESULTS

Identification of Restriction-Modification Plasmids

Total plasmid DNA from *L. cremoris* TDM1 was cotransformed with vector pVS2 (Cm^r Em^r) into *L. lactis* ssp. *lactis* LM0230 using protoplast transformation. Two types of phage-resistant transformants represented by *L. lactis* ssp. *lactis* NCK72 and NCK317 were isolated. *Lactococcus lactis* ssp. *lactis* NCK72 contained a 28.0-kb plasmid (pTRK30) and pVS2 (5.1-kb, Cm^r Em^r) (Figure 1, lane G). Phage c2 plaqued at an efficiency of 1.0×10^{-4} on NCK72 (Table 2). *Lactococcus lactis* ssp. *lactis* NCK317 contained 3 plasmids, pTRK317 (15.5 kb), pTRK10 (13.5 kb), and pVS2 (Cm^r Em^r) (Figure 1, lane H). Phage c2 plaqued at an EOP of 10^{-2} on NCK317 (Table 2). Host-controlled phage replication was demonstrated in both NCK72 and NCK317, indicating that pTRK30 and pTRK317 encoded R/M activities (Table 2). Derivatives of NCK317, which were cured of pTRK317 but retained pTRK10 and pVS2,

TABLE 2. Host-controlled phage replications of phage c2 on *Lactococcus lactis* ssp. *lactis* derivatives harboring different restriction-modification systems.

Phage[1]	Efficiency of plaquing on strains				
	LM2302	NCK44 (pTRK11)	NCK41 (pTRK12)	NCK72 (pTRK30)	NCK317 (pTRK317)
c2.LM2302	1.0	5.5×10^{-3}	8.0×10^{-3}	1.0×10^{-4}	1.2×10^{-2}
c2.NCK44	.9	1.0	ND[2]	ND	ND
c2.NCK44.LM2302	1.0	4.7×10^{-3}	ND	ND	ND
c2.NCK41	1.0	1.0	1.0	1.2×10^{-2}	ND
c2.NCK41.LM2302	1.0	7.5×10^{-3}	1.1×10^{-2}	3.7×10^{-4}	ND
c2.NCK72	1.0	ND	3.2×10^{-2}	1.0	ND
c2.NCK72.LM2302	.9	ND	4.0×10^{-2}	1.3×10^{-4}	ND
c2.NCK317	.9	ND	1.1×10^{-2}	2.0×10^{-4}	1.0
c2.NCK317.LM2302	1.0	ND	2.3×10^{-3}	7.8×10^{-4}	1.5×10^{-2}

[1]Suffixes following phage designation indicates the hosts through which the phage was propagated.

[2]Not determined.

did not exhibit R^+/M^+. Similarly, derivatives of NCK72 cured of pTRK30 lacked restriction activity (R^-).

Conjugal matings between *L. lactis* ssp. *cremoris* TDM1 and plasmid-free recipient *L. lactis* ssp. *lactis* LM2302 yielded Lac^+ transconjugants at a frequency of 1.9×10^{-5}. Phage c2 exhibited an EOP of 1.3 and 4.1×10^{-3} on the lactose-fermenting (Lac^+) transconjugants *L. lactis* ssp. *lactis* NCK43 and *L. lactis* ssp. *lactis* NCK40, respectively. *Lactococcus lactis* ssp. *lactis* NCK43 (Lac^+ R^-) harbored a single plasmid, pTRK14 (Figure 1, lane D; approximately 95 kb) that encoded Lac^+ but not R/M. *Lactococcus lactis* ssp. *lactis* NCK40 [Lac^+ proteolytic (Prt^+) R^+/M^+] harbored two plasmids of ca. 100 kb (pTRK11) and 13.5 kb (pTRK10) (Figure 1, lane B). A Lac^- R^- Prt^+ derivative of NCK40 lost pTRK11 but retained pTRK10 (Figure 1, lane E). This provided correlative evidence that pTRK10 encoded Prt^+. To confirm that pTRK11 encoded Lac^+ and R^+/M^+, second round conjugal matings were conducted between *L. lactis* ssp. *lactis* NCK40 and a plasmid-free recipient *L. lactis* ssp. *lactis* LM2345. One Lac^+ transconjugant, *L. lactis* ssp. *lactis* NCK44, contained only pTRK11 (Figure 1, lane C) and demonstrated R^+/M^+ (Table 2). Restriction of phage c2 by NCK44 was at the same level and specificity as NCK40 (data not shown). Loss of Lac^+ and R^+/M^+ phenotypes from NCK44 was correlated with curing of pTRK11. Both pTRK11 and pTRK14 were self-transmissible plasmids (Tra^+) and transferred concomitantly with Lac^+ in second round matings (data not shown).

Identification of a Recombinant Plasmid, pTRK12, Encoding Restriction-Modification and Proteolytic Activities

Upon consecutive transfer of *L. lactis* ssp. *lactis* NCK40 (pTRK11, Lac^+ R^+/M^+ Tra^+; pTRK10, Prt^+) in M17G broth, two types of Lac^- variants were isolated. *Lactococcus lactis* ssp. *lactis* NCK42, a derivative of NCK40, contained only pTRK10 (Figure 1, lane E) and was Prt^+ but did not restrict phage c2. The second derivative, *L. lactis* ssp. *lactis* NCK41, retained R^+/M^+ and Prt^+ and harbored a single 30.5-kb plasmid, designated pTRK12 (Figure 1, lane F). The specificity and level of R/M encoded by pTRK12 in NCK41 was identical to that of pTRK11 in NCK44 (Table 2). *Lactococcus lactis* ssp. *lactis* NCK41 was transferred twice in M17G with 1.0 μg/ml ethidium bromide and examined for R^- derivatives. The R^- derivatives were isolated at a frequency of 6.1% and had lost pTRK12. Plasmid pTRK12 (R^+/M^+) was cotransformed with vector pVS2 (Cm^r Em^r) into a plasmid-free strain. One representative transformant, *L. lactis* ssp. *lactis* NCK71, contained pTRK12 and pVS2 (Cm^r Em^r) and exhibited Prt^+ and R^+/M^+ at the same level and specificity as that of *L. lactis* ssp. *lactis* NCK41 (pTRK12).

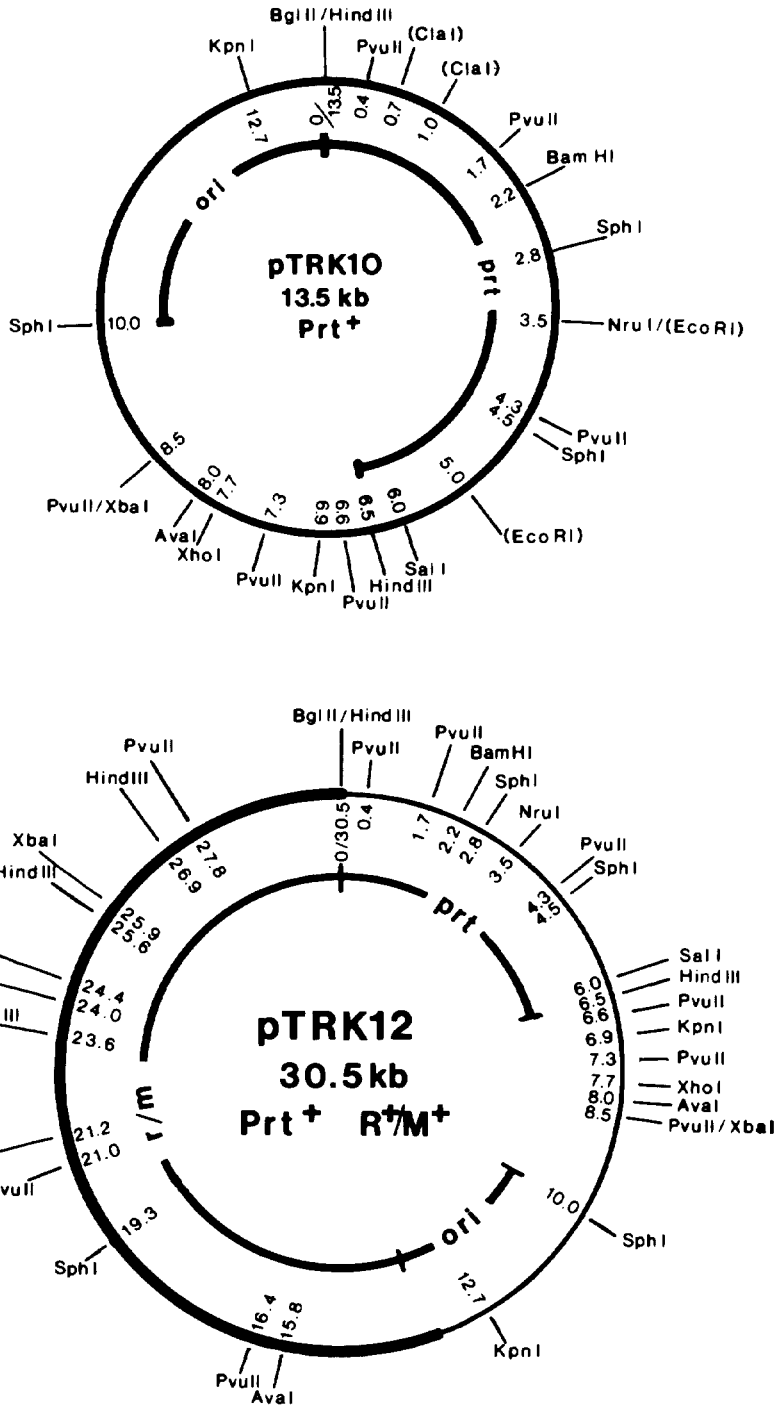

Figure 2. Circular restriction map of pTRK10 (Prt^+) and pTRK12 (R^+/M^+ Prt^+). The thick and thin lines denote regions contributed by pTRK11 (R^+/M^+) and pTRK10 (Prt^+ Ori^+), respectively. Prt^+ = Proteolytic, R^+/M^+ = restriction-modification, Ori^+ = origin of replication.

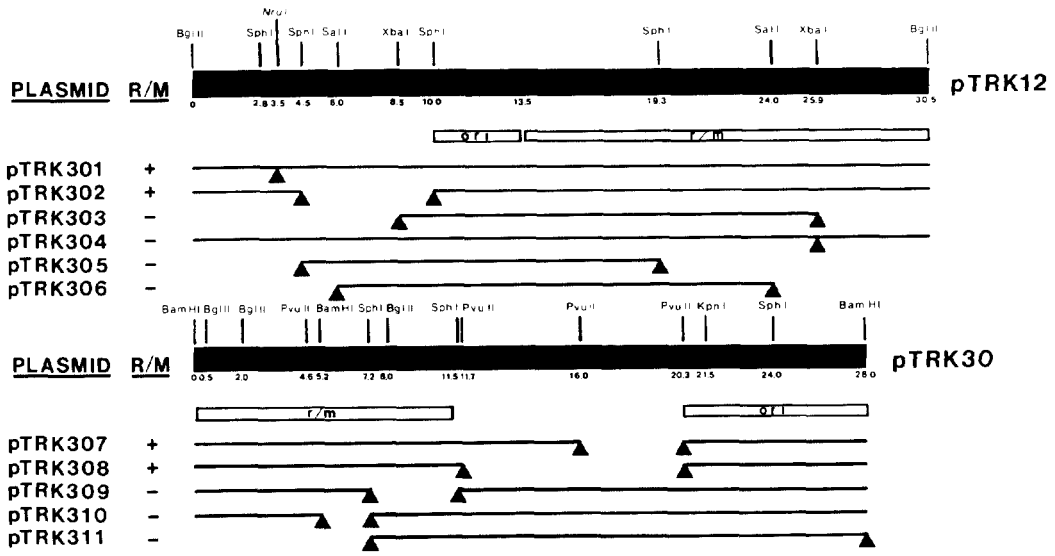

Figure 3. Localization of *ori* and *r/m* on pTRK12 and pTRK30 by analysis of pSA34-pTRK12 and pSA34-pTRK30 recombinants. Horizontal lines represent regions of pTRK12 or pTRK30. Solid triangles indicate the position of the pSA34 insertion.

Physical and Genetic Characterization of pTRK12, pTRK30, and pTRK317

A [^{32}P]pTRK10 probe hybridized strongly to pTRK12 (data not shown). To evaluate the physical relatedness of pTRK10 (Prt$^+$, 13.5 kb) and pTRK12 (R$^+$/M$^+$ Prt$^+$, 30.5 kb), physical maps of the two plasmids were generated (Figure 2). Plasmid pTRK12 was composed of pTRK10 (Prt$^+$) plus an additional 17.0-kb region. This was consistent with retention of the pTRK10-derived Prt$^+$ phenotype in pTRK12 and the high copy number (Figure 1, lane F).

The genetic determinants for Prt$^+$ encoded on pTRK10 and pTRK12 were localized by comparison with known proteinase genes on pLP712, pSK11, and pWV05 (20). The *Cla*I, *Eco*RI, *Bam*HI, and *Sal*I digests were performed on a 6.5-kb *Hin*dIII fragment from pTRK10 (Prt$^+$) (Figure 2). The locations of restriction sites within this region were identical to those identified on the completely sequenced 6.5-kb *Hin*dIII fragment of pWV05 (20, 21).

Plasmids pTRK10 (Prt$^+$) and pTRK12 (R$^+$/M$^+$ Prt$^+$) appeared to be high copy number replicons (Figure 1, lanes B and F). To localize the pTRK10 *ori*, the Ori$^+$ search vector pSA34 (36) was used to clone the 7.0- and 6.5-kb *Hin*dIII fragments of pTRK10. The Cm^r Tc^r transformants were recovered in *E. coli* and pSA34-pTRK10 recombinants containing either the 7.0- or 6.5-kb *Hin*dIII fragments. When these plasmids were transformed into *L. lactis* ssp. *lactis* LM0230, only pSA34 clones containing the 7.0-kb *Hin*dIII fragment generated Em^r transformants and therefore carried the *ori*. To subclone the *ori*, pSA34 and pTRK10 (7.0-kb *Hin*dIII) recombinants were digested with *Hin*dIII and one enzyme (*Sph*I or *Eco*RV), which cleaved within the 7.0-kb *Hin*dIII fragment and a site within pSA34. These fragments were ligated, transformed into *L. lactis* ssp. *lactis* LM0230, and Em^r transformants selected. One transformant carried a pSA34-pTRK10 recombinant plasmid, pTRK320, which carried a 3.5-kb *Hin*dIII-*Sph*I fragment of pTRK10 (internal to the 7.0-kb *Hin*dIII fragment) and demonstrated the high copy characteristics of pTRK10 and pTRK12. Therefore, the pTRK10 *ori* was located between map positions 10.0 and 13.5 kb within the 3.5 kb *Hin*dIII-*Sph*I fragment (Figure 2). By comparison of the pTRK10 and pTRK12 maps, the pTRK12 *ori* was deduced to be located between the 10.0- and 13.5-kb map sites (Figure 3).

TABLE 3. Combined effects of plasmid encoded restriction-modification (R^+/M^+) and abortive infection (Hsp^+) systems.

Strain	Plasmid composition	EOP[1,2]	Plaque morphology (diameter)
T-EK1	pTR2030 (Hsp^+)	3.2×10^{-1}	Small (<2 mm)
NCK86	pTRK12 (R^+/M^+) pTR2030 (Hsp^+)	5.9×10^{-3}	Small and medium (1 to 2 mm)
NCK85	pTRK30 (R^+/M^+) pTR2030 (Hsp^+)	7.4×10^{-6}	Faint small (<2 mm)

[1]Efficiency of plaquing against phage c2.

[2]For EOP of strains carrying only pTRK12, pTRK11, or pTRK30, see Table 2; plaque morphologies were medium (1.5 mm).

Because pTRK12 retained R^+/M^+ with the same specificity as pTRK11 (100-kb, Lac^+ Tra^+ R^+/M^+) and pTRK11 sequences hybridized to pTRK12 (data not shown), the 17.0-kb region of pTRK12, distinct from pTRK10, was thought to encode genetic determinants for R^+/M^+. pTRK12 was partially digested with *Bam*HI, *Sph*I, *Nru*I, and *Xba*I, ligated to pSA34 cut with the appropriate enzyme, and transformed into *L. lactis* LM0230. The Em^r transformants were recovered and evaluated for R/M. All R^+/M^+ transformants restricted phage c2 at an EOP = 10^{-3}. To localize *r/m* genes, R^+/M^+ and R^- clones were compared by restriction mapping to define the regions cloned and relative position of pSA34 (Figure 3). The pTRK12 *r/m* genes were localized between map positions 13.5 and 30.5 kb. This region includes an *Xba*I (25.9 kb) site at which insertion of pSA34 via cloning into the entire pTRK12 replicon inactivated R^+/M^+. Phage c2 propagated through *L. lactis* NCK304 (R^-, pTRK304) did not yield modified phage, indicating that inactivation of both restriction and modification activities occurred upon disruption of the *Xba*I site in pTRK12.

The pTRK30 *r/m* genes were localized in a manner similar to that described for pTRK12. A restriction map of pTRK30 and approximate location of the R/M determinants are shown in Figure 3. The pTRK30 *r/m* genes were localized between the *Bam*HI (0 kb) and *Pvu*II (11.7 kb) sites. Interruption of sequences around the *Sph*I (7.2 kb) site inactivated R/M activity. The pTRK30 *ori* was localized within the 7.7 kb *Pvu*II (20.3 kb) and *Bam*HI (28.0 kb) fragment.

A physical map was constructed for pTRK317 (15.5 kb) (data not shown). This plasmid appeared unrelated to either pTRK12 or pTRK30 and was not characterized further.

Combinations of Restriction-Modification and Abortive Infection

To determine the phenotypic effect of combining R/M and Hsp systems in the same host background, pTR2030 (Hsp^+) was introduced into strains carrying pTRK12 (R^+/M^+) or pTRK30 (R^+/M^+) and evaluated for resistance against phage c2. The R^+/M^+ activities encoded by pTR2030 are not exhibited against phages c2 and p2. Therefore, the contribution of pTR2030 to phage resistance in this host background is limited to the effects of Hsp^+. Conjugation was used to transfer pTR2030 (Hsp^+ Tra^+) into *L. lactis* ssp. *lactis* NCK71 (pTRK12, R^+/M^+; pVS2, Em^r) or NCK72 (pTRK30, R^+/M^+; pVS2, Em^r). Transconjugants NCK86 (pTR2030, Hsp^+; pTRK12, R^+/M^+) and NCK85 (pTR2030, Hsp^+; pTRK30, R^+/M^+) were isolated and examined for resistance using phage c2 (Table 3). Phage c2 plaqued at a reduced efficiency on NCK86, but plaque sizes were larger and heterogeneous (1 to 2 mm in diameter) in comparison with Hsp^+ (<.2 mm, homogeneous). The presence of pTRK12 interfered with full expression of Hsp^+ by pTR2030. We examined whether or not specific pTRK12 phenotypes negatively affected Hsp^+. Aberrant plaque sizes for phage c2 were still apparent in a strain in which pTR2030 (Hsp^+) and the pSA34-pTRK12 (R^-) recombinant pTRK304 were combined. Heter-

TABLE 4. Efficiency of plaquing, center of infection formation, and percentage of cell death by phages p2 and p2.NCK72 on *Lactococcus lactis* ssp. *lactis* NCK85 (pTR2030, pTRK30).

Phage	Host	Plasmids	EOP[1]	COI[2]	% Cell death[3]
p2	T-EK1	pTR2030	$<10^{-9}$	2.1×10^{2}	78
p2	NCK72	pTRK30	10^{-4}	<10	<1[4]
p2	NCK85	pTR2030 pTRK30	$<10^{-9}$	<10	8
p2	LM2301	none	1.0	1.2×10^{4}	94
p2.NCK72[5]	NCK85	pTR2030 pTRK30	$<10^{-9}$	9.2×10^{4}	93
p2.NCK72	LM2301	none	1.0	1.4×10^{6}	95

[1]Efficiency of plaquing = (titer on heterologous host/titer on homologous host).

[2]Centers of infection.

[3]Percentage cell death = (cfu/ml – cfu/ml of phage infected cells)/(cfu/ml).

[4]No detectable cell death.

[5]Phage p2 propagated on NCK72 (pTRK30).

ogenous plaque sizes were not observed when pTR2030 (Hsp^+) was combined with pSA34 and pTRK10 (*Hin*dIII 7.0-kb fragment, *ori* clone), pSA3, or pTRK10. These data indicated that neither the pTRK12 *ori* nor *r/m* gene expression induced the aberrant plaque morphologies observed for strains carrying pTR2030 (Hsp^+) and pTRK12 (R^+/M^+).

When pTR2030 (Hsp^+) was combined with pTRK30 (R^+/M^+) in *L. lactis* ssp. *lactis* NCK85, an increase in resistance to phage c2 was observed (Table 3). The R^+/M^+ encoded by pTRK30 operated independently of Hsp^+. Plaques were faint and small, indicating that Hsp^+ was fully operational. The EOP was reduced one log cycle below hosts containing pTRK30 (R^+/M^+) alone (see Table 2). Phage c2 modified by replication on NCK85 (pTR2030, Hsp^+; pTRK30, R^+/M^+) plaqued at an EOP of 9.1×10^{-1} on NCK72 (pTRK30, R^+/M^+) and an EOP of 1.8×10^{-1} with faint, small plaques on NCK85. These data indicated that R/M activities exhibited in NCK85 for phage c2 were specific for pTRK30 and not pTR2030.

To examine further the combined effects of pTRK30 (R^+/M^+) and pTR2030 (Hsp^+), we assayed the EOP, center of infection formation (COI), and cell death of *L. lactis* NCK72 (pTRK30) infected with phage p2 and modified p2.NCK72 (Table 4). Neither phage p2 nor p2.NCK72 formed plaques on *L. lactis* ssp. *lactis* NCK85 (pTR2030, Hsp^+; pTRK30, R^+/M^+). Formation of COI were not detected on NCK85 cells infected with unmodified phage p2. However, COI assays did demonstrate that phages were released from modified p2.NCK72 infected NCK85. Because p2.NCK72 formed COI on NCK85 and p2 did not, we conclude that R^+/M^+ was responsible for preventing COI formation in NCK85. Approximately 93% of *L. lactis* ssp. *lactis* NCK85 (pTRK30, pTR2030) cells infected with p2.NCK72 died, but only 8% cell death was detected for NCK85 infected with phage p2 (Table 4). As with COI formation, pTRK30 (R^+/M^+) acted to minimize cell death in NCK85. Although pTR2030 alone lowers the EOP of phage p2 to $<10^{-9}$, cells aborting the infection still die at levels of 78% (Table 4). These data indicate that addition of pTRK30 (R^+/M^+) to an existing Hsp system encoded by pTR2030 improved host survival.

DISCUSSION

Three plasmid-encoded R/M systems from *L. lactis* ssp. *cremoris* TDM1 were isolated and characterized. Plasmids pTRK12 (30.5 kb), pTRK30 (28.0 kb), and pTRK317 (15.5 kb) were identified via conjugation or cotransformation strategies. Each plasmid encoded R/M activities of different specificity. Description of these R/M plasmids further established that multiple R/M systems exist in lactococci that can serve as a barrier to phage attack (4, 6, 11, 12, 15, 16, 17, 27, 34, 35).

Physical characterization of plasmid pTRK12 (R^+/M^+, Prt^+) revealed that this plas-

mid resulted from in vivo recombination events. Plasmid pTRK12 (R^+/M^+, Prt^+) was isolated only after destabilization of Lac^+ from transconjugants carrying pTRK11 (Lac^+ Tra^+ R^+/M^+) and pTRK10 (Prt^+). The identical specificity of pTRK11 and pTRK12 R/M activities and relation of pTRK10 and pTRK12 sequences suggest that pTRK12 was generated from recombination between pTRK11 and pTRK10. It is possible that pTRK12 is a resolution product of pTRK11 although unlikely because pTRK11 is Prt^-. Hybridization experiments indicated that *r/m* sequences on pTRK12 did not exist on a 30.5 kb plasmid in the parental strain, *L. lactis* ssp. *cremoris* TDM1 (unpublished data). This further suggested that pTRK11 (Lac^+ Tra^+ R^+/M^+) may have originally formed via recombination between a conjugal Lac^+ plasmid and a R^+/M^+ plasmid in TDM1, which was not pTRK12. Higgins et al. (12) reported a similar event where a conjugal Lac^+ R^+/M^+ plasmid was formed due to cointegration between plasmid pTN20 (Tra^+ R^+/M^+) and pTR1040 (Lac^+) in *L. lactis* ssp. *lactis* N1. Further investigation is needed to elucidate the mechanism by which *r/m* sequences in TDM1 recombined with a conjugal Lac^+ plasmid and the events responsible for formation of the unique Prt^+ R^+/M^+ plasmid, pTRK12.

Concurrent operation of Hsp and R/M mechanisms in lactococci represents a significant barrier to phage development (13, 18, 27, 39). Multiple forms of resistance within a single host provide a distinct advantage over operation of individual Hsp or R/M mechanisms in isolation. Enhanced levels of resistance can be achieved if different stages in the phage lytic cycle are targeted. It is therefore of interest to study cooperative resistance mechanisms. In this study, we examined phage resistance due to the combined effect of pTR2030-encoded Hsp and R/M encoded by newly identified plasmids, pTRK12 or pTRK30. Although the plasmids were stably maintained within each host, the combined effects of Hsp and R/M depended on the individual plasmids involved. When R^+/M^+ encoded by pTRK12 was combined with pTR2030 (Hsp^+), phage restriction was apparent, but the typical plaque size reduction indicative of Hsp^+ was not exhibited. Atypical and unstable resistance characteristics were observed for some TDM1 transconjugants carrying pTR2030 (38; W. D. Sing, M.S. Thesis, North Carolina State University), suggesting that interference occurred between pTR2030 and resident R^+/M^+ plasmids. This case represented an attempt to combine R/M and Hsp that did not yield full cooperativity between resistance phenotypes. In this study, expression of pTRK12 encoded R/M activities or *ori* did not interfere with Hsp. Therefore, genetic efforts to recombine *r/m* with *hsp* determinants on the same replicon and achieve full expression of both phenotypes should be feasible. Sequences exclusive of the pTRK12 *ori* and *r/m* genes may cause interference with full expression of Hsp. These regions may bind regulatory proteins essential for full expression of Hsp^+ or directly encode Hsp^+ repressors as described by Miller and Malamy (28) and Miller et al. (29) for plasmids in the *E. coli* (*pif* gene)-T7 system.

When pTRK30 (R^+/M^+) was combined with pTR2030 (Hsp^+) in *L. lactis* ssp. *lactis* LM0230, R/M and Hsp mechanisms operated cooperatively to give reduced size plaques (Hsp) with a concurrent reduction in EOP (R/M) when challenged with phage c2. Cooperation between Hsp and R/M inhibited phage c2 plaque formation to an extent where plaques were indiscernible. Strains carrying pTR2030 (Hsp^+) and pTRK30 (R^+/M^+) completely inhibited plaque formation by small isometric phage p2. Although pTR2030 (Hsp^+) alone eliminated plaque formation by phage p2 (14), the combination of pTRK30 and pTR2030 represented a greater degree of resistance as evidenced by reductions in COI assays and prevention of cell death. From the parameters evaluated, inhibition of phage development resembled the combined action of Hsp and R/M encoded by pTR2030 against ϕ31 (39). However, in this study, an additional level of phage inhibition was achieved by combining R/M (pTRK30) and Hsp (pTR2030) on separate plasmids. The cooperative effects of pTRK30 (R^+/M^+) and pTR2030 (Hsp^+) in the same strain created the strongest inhibition of phage p2 demonstrated thus far in our laboratory.

Compatible expression of R/M and Hsp to achieve increased levels of phage resistance further supported that strains can be constructed with cooperative defenses encoded on separate plasmids. Therefore, the potential exists to use such plasmids in forthcoming strategies to improve the activity and longevity of dairy starter cultures. Gene transfer techniques such as con-

jugation and transformation provided a useful strategy to introduce two different resistance mechanisms into one strain. However, obtaining multiple levels of phage resistance may be more difficult than simply combining the appropriate plasmids. In certain cases, plasmid selection was critical to obtaining complementation between phage resistance mechanisms. This would infer that construction strategies utilizing separate plasmid components may be equally reliant on plasmid selection and definition of specific genes and resistance mechanisms involved. The natural tendency for lactococci to carry several phage resistance mechanisms and the obvious benefits of multiple defenses may necessitate recombination events involving *r/m* sequences to facilitate compatible existence and expression.

ACKNOWLEDGMENTS

This study was supported in part by Miles Inc., Biotechnology Products Division, Elkhart, IN. We thank Mary Ellen Sanders for providing pSA34 and helpful discussions on cloning strategies for R/M plasmids in lactococci. We would like to thank C. Hill and D. A. Romero for their comments on this manuscript.

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