

Streptococcus cremoris M12R Transconjugants Carrying the Conjugal Plasmid pTR2030 Are Insensitive to Attack by Lytic Bacteriophages†

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Conjugal transfer of lactose-fermenting ability (Lac⁺), nisin resistance (Nis^r), and phage resistance (Hsp⁺) was demonstrated in matings between *Streptococcus lactis* ME2 (donor) and *Streptococcus cremoris* M43a (recipient), a derivative of M12R. Transconjugants were detected by transfer of Lac⁺ and were found to exhibit Nis^r and harbor a 40-megadalton plasmid (pTR1040). Fifty-six percent of Lac⁺ transconjugants were resistant to the *S. cremoris* M12R lytic phage. Efficiency of plaquing for phage m12r · M12 on a phage-resistant transconjugant, T2r-M43a, was $<4.3 \times 10^{-10}$. Five additional phages which were virulent for *S. cremoris* M12R and isolated from industrial sources failed to plaque on *S. cremoris* T2r-M43a. Mating experiments with T2r-M43a revealed that phage resistance was accompanied by high-frequency conjugation ability (Tra⁺) and the appearance of both pTR1040 and pTR2030 encoding Lac⁺ Nis^r and Tra⁺ Hsp⁺, respectively, in transconjugants of *S. lactis* LM2302. Phage-sensitive Lac⁺ transconjugants of *S. cremoris* M43a (T2s-M43a) showed no conjugal ability. These observations confirmed that pTR2030 was present and responsible for the phage resistance and conjugal ability exhibited by the *S. cremoris* transconjugant T2r-M43a. Unlike the *S. lactis* LM2302 transconjugant carrying pTR2030, resistance of T2r-M43a to phage was not affected at high temperatures (35 to 40°C) or destabilized in repeated transfers through a starter culture activity test. These results demonstrated that phage resistance conferred by pTR2030 in the *S. cremoris* transconjugant was effective against industrially significant phages under fermentation conditions normally encountered during cheese manufacture.

The majority of lactic streptococci used as starter cultures in dairy fermentations are highly susceptible to attack by lytic bacteriophage. Consequently, the nonaseptic nature of the milk fermentation and the ubiquity of phages in the dairy environment result in recurrent starter culture failures due to bacteriophage attack (5). In recent years, mechanisms of phage resistance carried by select lactic streptococci have been detected and, in some cases, found to be encoded by plasmid DNA elements. Specific examples include restriction and modification systems (2, 19), suppression of phage adsorption (3, 20), and other undetermined, yet powerful, mechanisms of phage resistance (13, 21). Identification of phage resistance mechanisms encoded by plasmid DNA elements provides exciting opportunities to isolate and disseminate the responsible genetic determinants to phage-sensitive lactic streptococci. Conjugation was used successfully by McKay and Baldwin (13) to transfer a plasmid (pNP40) encoding phage and nisin resistance from *Streptococcus lactis* subsp. *diacetylactis* DRC3 to a plasmid-cured derivative of *S. lactis* C2, LM0230. Virulent phage showed no ability to plaque on the resistant *S. lactis* LM0230 transconjugants when challenged at 32°C. Chopin et al. (2) conjugally transferred restriction and modification activities encoded on a 28-megadalton (MDa) plasmid (pIL6) from *S. lactis* IL594 to a plasmid-cured derivative of IL594 deficient in restriction and modification activities. Recent studies in our laboratory have demonstrated that conjugal matings

between *S. lactis* ME2 and LM0230 can also yield phage-resistant transconjugants (8). Resistance was manifested by a reduction in the burst size and plaque size of infecting phage without affecting the efficiency of plaquing (EOP), which remained at 1.0. Genetic determinants for phage resistance in the transconjugants were associated with a 30-MDa plasmid (pTR2030) which also conferred conjugal transfer ability at high frequency (Tra⁺).

These studies indicate that conjugal transfer of phage resistance determinants to phage-sensitive lactic streptococci will be a forthcoming strategy for starter culture development. However, in the studies of McKay and Baldwin (13) and Klaenhammer and Sanozky (8), the phage resistance acquired was inoperative when the transconjugants were propagated at elevated temperatures (37 to 40°C). Since these temperatures are routinely encountered in cheesemaking (10), the phage defense mechanisms identified in the *S. lactis* LM0230 transconjugants (8, 13) would not effectively protect starter cultures from phage attack throughout the fermentation process. However, it was not determined whether the heat-sensitive phage resistance (Hsp⁺) carried by pTR2030 would express similar levels of resistance and retain its sensitivity to heat when introduced into other lactic streptococcal strains susceptible to different phages. The objective of this investigation was to introduce the conjugal plasmid pTR2030 into a strain of *Streptococcus cremoris* and examine (i) phage resistance in the *S. cremoris* transconjugants and (ii) stability of the transconjugants to phage challenge in starter culture activity tests conducted with 40°C incubations. After matings with *S. lactis* ME2, *S. cremoris* M12R transconjugants harboring pTR2030 were

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TABLE 1. Bacterial strains, plasmids, and phages

Strain	Phenotype ^a	Homologous phage	Description	Reference
<i>S. lactis</i> ME2	Lac ⁺ Nis ^r Hsp ⁺		Conjugal donor of pTR1040 (Lac ⁺ Nis ^r) and pTR2030 (Hsp ⁺ Tra ⁺)	20
<i>S. cremoris</i> M12R	Lac ⁺ Prt ⁺ Nis ^s	m12r-M12	Wild type	This study
<i>S. cremoris</i> M12	Lac ⁺ Prt ⁺ Nis ^s	m12r-M12	Single-colony isolate of M12R	This study
<i>S. cremoris</i> M43	Lac ⁺ Prt ⁻ Nis ^s	m12r-M12	Single-colony isolate of M12R	This study
<i>S. cremoris</i> M43a	Lac ⁻ Prt ⁻ Nis ^s Str ^r	m12r-M12	Derivative of M43	This study
<i>S. lactis</i> 11454	nisin producer			ATCC ^b
<i>S. lactis</i> LM0230	Lac ⁻ Nis ^s Hsp ⁻ Str ^r	c2	Plasmid-cured strain of <i>S. lactis</i> C2	15
<i>S. lactis</i> LM2302	Lac ⁻ Nis ^s Hsp ⁻ Str ^r Ery ^r	c2	Plasmid-cured strain of <i>S. lactis</i> C2	24
<i>S. cremoris</i> T2r-M43a	Lac ⁺ Nis ^r Prt ⁻ Tra ⁺ resistant to m12r-M12		Transconjugant from mating between ME2 × M43a, harboring pTR1040 and pTR2030	This study
<i>S. cremoris</i> T2s-M43a	Lac ⁺ Nis ^r Prt ⁻ Tra ⁻ sensitive to m12r-M12	m12r-M12	Transconjugant from mating between ME2 × M43a, harboring pTR1040	This study
<i>S. lactis</i> T-LS1	Lac ⁺ Hsp ⁺	c2	Transconjugant from mating between T2r-M43a × LM2302, harboring pTR1040 and pTR2030	This study
<i>S. lactis</i> T-LS2	Lac ⁺ Hsp ⁻	c2	Transconjugant from mating between T2r-M43a × LM2302, harboring pTR1040	This study
<i>S. lactis</i> T-RS17	Lac ⁺ Nis ^r Hsp ⁺	c2	Transconjugant from mating between ME2 × LM0230, harboring pTR1040, pTR2030, pTR3025, and pTR4002	8
<i>E. coli</i> V517			Source of reference plasmids; 32, 5.2, 3.5, 3.0, 2.2, 1.7, 1.5, and 1.2 MDa	11

^a Phenotype abbreviations: Lac⁺, lactose fermenting; Lac⁻, lactose negative; Prt⁺, proteinase positive; Prt⁻, proteinase negative; Nis^r, nisin resistant; Nis^s, nisin susceptible; Hsp⁺, heat-sensitive phage resistance; Hsp⁻, phage susceptible; Str^r, streptomycin resistant (1,000 µg/ml); Ery^r, erythromycin resistant (15 µg/ml).

^b ATCC, American Type Culture Collection.

resistant to phage attack in plaque assays and starter culture activity tests.

MATERIALS AND METHODS

Bacteria, phages, and culture conditions. Bacterial strains and phages used in this study are shown in Table 1. Cultures were maintained as frozen stocks at -20°C in the appropriate growth medium plus 10% glycerol. Group N streptococci and their phages were propagated in M17 broth at 30°C as described previously (23). Lactose (0.5%) and glucose (0.5%) were used as carbohydrate supplements for lactose-fermenting (Lac⁺) and non-lactose-fermenting (Lac⁻) cultures, respectively. *Escherichia coli* strains were propagated in brain heart infusion broth (BBL Microbiology Systems Cockeysville, Md.) at 37°C.

Isolation of Lac⁻ Str^r variants of *S. cremoris* M12R. For use as conjugation recipients, Lac⁻ variants of *S. cremoris* M12R were isolated on lactose-indicator agar (15) after 24 h of growth in M17-glucose broth containing 2 µg of ethidium bromide per ml. A Lac⁻ variant of M43 was purified by single-colony isolation on lactose-indicator agar and propagated through broth, and 0.1 ml was spread over the surface of M17-glucose agar plates containing 250, 500, and 1,000 µg of streptomycin sulfate (Sigma Chemical Co., St. Louis, Mo.) per ml. After 24 to 48 h at 30°C, single colonies appearing on plates with the highest concentration of streptomycin were picked and streaked onto M17-glucose agar containing 1,000 µg of streptomycin per ml. One streptomycin-resistant (Str^r) variant was selected in this manner and designated M43a. The identity of the Lac⁻ Str^r variant was confirmed by plasmid composition and susceptibility to the *S. cremoris* M12R lytic phage (m12r · M12). *S. cremoris* M43a was maintained in M17-glucose broth containing 1,000

µg of streptomycin per ml but was propagated once in the absence of streptomycin before use in conjugation experiments.

Conjugation. Conjugal matings were performed by using agar-surface matings as described by McKay et al. (14). Transconjugants were scored by selection of Lac⁺ colonies on lactose-indicator agar (15) containing either 1,000 µg of streptomycin per ml for *S. cremoris* M43a recipients or 1,000 µg of streptomycin per ml plus 15 µg of erythromycin per ml for *S. lactis* LM2302 recipients. Lac⁺ transconjugants resulting from matings between *S. lactis* ME2 and the *S. cremoris* M43a recipient were purified and confirmed as *S. cremoris* M43a derivatives by growth in Reddy broth (17) and plasmid analysis.

Nisin resistance. Nisin resistance of *S. cremoris* recipients and transconjugants was determined subjectively by using culture supernatants from the nisin-producing strain, *S. lactis* 11454. Supernatant from an overnight culture of *S. lactis* 11454 in M17-lactose broth was filter sterilized (Acrodisc, 0.45 µm; Gelman Sciences Inc., Ann Arbor, Mich.) and diluted 10-fold in fresh M17 broth. Nisin activity was confirmed by spotting 5 µl of the preparation onto cell lawns of known nisin-resistant (*S. lactis* ME2) and nisin-susceptible (*S. lactis* LM0230) indicators (8, 13). Test culture lawns were then spotted with 5 µl of active nisin and examined for zones of inhibition after overnight incubation at 30°C. Cell lawns were prepared exactly as described for bacteriophage plaque assays (23).

Bacteriophage assays. The extent of phage activity on parents, recipients, and transconjugants was determined in M17 broth and by plaque assays on M17 agar as described by Terzaghi and Sandine (23). Levels of phage adsorption and EOP were determined as described previously (18, 19).

Phages virulent to *S. cremoris* M12R isolated from six

TABLE 2. Conjugal transfer of Lac⁺, Nis^r, and Hsp⁺ to *S. cremoris* M43a

Mating pair ^a		Lac ⁺ Str ^r recombinants ^b			
Donor	Recipient	No./ml	Frequency/donor	% Nis ^r	% Hsp ⁺
<i>S. lactis</i> ME2	<i>S. cremoris</i> M43a	19,010	1.1×10^{-4}	100 ^c	57 ^d
<i>S. lactis</i> ME2	Cell-free filtrate from M43a	0	$<5.9 \times 10^{-9}$		
Cell-free filtrate from ME2	<i>S. cremoris</i> M43a	2	NA	NA	NA
ME2 plus DNAase ^e	<i>S. cremoris</i> M43a	15,000 ^f	8.8×10^{-5}	ND	ND
<i>S. lactis</i> ME2		0	$<5.9 \times 10^{-9}$		
	<i>S. cremoris</i> M43a	0	$<5.9 \times 10^{-9}$		

^a Donor cells were 1.7×10^8 CFU/ml.

^b Recombinants were selected on lactose indicator agar containing 1,000 µg of streptomycin per ml. NA, Not applicable; spontaneous Str^r mutants of donor. ND, Not determined.

^c Ten recombinants were examined for resistance to nisin.

^d Fifty recombinants were examined for resistance to m12r-M12 phage in broth lysis experiments.

^e Mating performed in the presence of 100 µg of DNAase I per ml.

^f Estimated numbers.

cheese plants were examined for host ranges by spotting 5 µl of purified phage suspension in M17 broth on lawns of bacterial cells (23). Thirty different lactic streptococcal strains were tested with each phage.

Starter culture activity test. The starter culture activity test was a modification of that described by Heap and Lawrence (4). Cultures were prepared by overnight incubation at 30°C in 11% reconstituted skim milk (steamed for 1 h) or in 11% reconstituted skim milk containing 0.25% Casamino Acids (Difco Laboratories, Detroit, Mich.) for growth of proteinase-negative strains. The phage suspension (200 µl, $\sim 10^8$ PFU/ml) was mixed with 200 µl of milk culture and incubated at room temperature for 10 min to allow adsorption of phage. Reconstituted skim milk (9.6 ml) containing 40 µg of bromocresol purple (Sigma) per ml was added, and the tubes were sequentially incubated for 3 h at 30°C, 3 h and 10 min at 40°C, and 2 h and 30 min at 30°C. After the incubation, milk samples were examined for pH and phage titer. Consecutive starter culture activity tests were conducted with m12r · M12 phage to determine whether virulent phage could be generated which was active against the *S. cremoris* T2r-M43a transconjugant. Repeated cycles of the activity test were conducted as above except that 100 µl of m12r · M12 phage suspension ($\sim 10^8$ PFU/ml; prepared in M17 broth) and 100 µl of whey collected from the previous activity test were mixed with 200 µl of cells before milk inoculation.

Plasmid isolation. Plasmid DNA was isolated from lactic streptococcal strains by the method of Klaenhammer (6) with the modifications described by Sanders and Klaenhammer (20). For large-scale isolation of plasmid DNA, the method of Anderson and McKay (1) was used with the following modifications. Cell suspensions were not vortexed after lysis of cells. NaOH (3.0 N) was added to the lysate until a pH of 11.8 to 12.3 was achieved (approximately 2.0 ml). After the addition of phenol, 55 ml of chloroform was added to each bottle, and centrifugation was performed at $10,400 \times g$ for 30 min. After a second extraction with chloroform-isoamyl alcohol, the bottles were centrifuged at $10,400 \times g$ for 10 min to facilitate phase separation. Plasmid DNA was precipitated with 2 volumes of 95% ethanol by overnight incubation at -20°C . Plasmid DNA was purified through cesium chloride-ethidium bromide density gradients as described by Klaenhammer et al. (7). To isolate plasmid DNA from *E. coli* strains, a cleared lysate procedure (7) was used followed by purification through cesium chloride-ethidium bromide density gradients.

Electrophoresis was conducted on 0.65% horizontal

agarose gels (SeaKem ME agarose; FMC Corp., Rockland, Maine) in Tris-acetate buffer (40 mM Tris, 12 mM sodium acetate, 1 mM Na₂EDTA; pH was adjusted to 7.8 with glacial acetic acid). Samples of 5 to 20 µl were mixed with agarose beads (22) equal to one-half the sample volume before loading onto the gels. Gels were electrophoresed at 50 V for 4 to 5 h or 75 V for 1.75 to 2 h.

Restriction enzyme analysis. Restriction endonucleases were obtained from Bethesda Research Laboratories, Inc., Gaithersburg, Md. Restriction digestions were performed by the method of Maniatis et al. (12). Separation of DNA restriction fragments was accomplished on 0.8% agarose gels. Fragments generated by *Hind*III and *Eco*RI digestions of bacteriophage Lambda DNA (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) served as molecular weight standards.

RESULTS

Conjugation. Matings on milk-glucose agar plates between the donor, *S. lactis* ME2 (Lac⁺ Nis^r Hsp⁺ Str^s), and the recipient, *S. cremoris* M43a (Lac⁻ Nis^s Hsp⁻ Str^r), resulted in the transfer of Lac⁺ in a conjugation-like process (Table 2). Control experiments ruled out transduction and transformation as possible mechanisms of genetic transfer. Whereas *S. cremoris* M43a was susceptible to nisin, all Lac⁺ transconjugants examined exhibited nisin resistance. These observations were consistent with linkage of Lac⁺ and Nis^r to a single 40-MDa plasmid, pTR1040, in *S. lactis* ME2 and concurrent transfer of these phenotypes to *S. lactis* LM0230 (8).

Characterization of phage-resistant transconjugants. *S. cremoris* Lac⁺ transconjugants were further examined for resistance to virulent phage m12r · M12. In experiments conducted in broth, 57% of the M43a Lac⁺ transconjugants failed to lyse when challenged with phage (Table 2). Typical results comparing the effect of phage on *S. cremoris* M43a and a Lac⁺, phage-resistant transconjugant, T2r-M43a, are shown in Fig. 1. Further characterization revealed that m12r · M12 phage failed to produce plaques on *S. cremoris* T2r-M43a in plaque assays conducted at 30°C with high-titer phage preparations (Table 3). The EOP on T2r-M43a was less than 4.3×10^{-10} . When plaque assays were conducted at 36°C, or when the cells were heat shocked at 40°C for 1 h before plaque assays, T2r-M43a retained its resistance to m12r · M12 phage, displaying no plaque formation (data not shown). In contrast, m12r · M12 phage was fully active on *S. cremoris* M43a and on a Lac⁺ Nis^r transconjugant which was phage susceptible (T2s-M43a). Resistance by *S. cremoris*

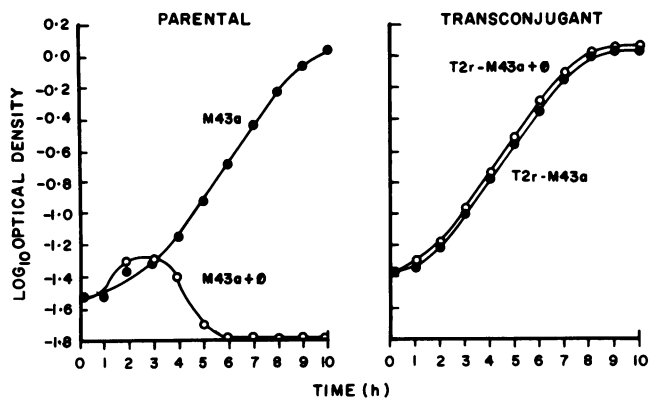


FIG. 1. Growth of *S. cremoris* M43a and the Lac⁺ transconjugant T2r-M43a in the presence (○; 10⁷ PFU/ml) and absence (●) of m12r · M12 phage. Optical density was determined at 590 nm.

T2r-M43a to phage was not due to reduced adsorption of the phage. Both the phage-susceptible strain M43a and its phage-resistant transconjugant, T2r-M43a, showed adsorption levels of 98 to 99% for phage m12r · M12 (Table 3).

Plasmid analysis. The plasmid compositions of the conjugation recipient *S. cremoris* M43a, four phage-resistant Lac⁺ transconjugants, including T2r-M43a, and five phage-sensitive Lac⁺ transconjugants are shown in Fig. 2. The acquisition of pTR1040 was apparent in all Lac⁺ transconjugants. Acquisition of pTR2030 by phage-resistant transconjugants could not be observed due to a resident plasmid of 30 MDa in M43a. When the plasmid profiles of phage-resistant transconjugants were initially analyzed, it was noted that there were alterations in the compositions of small plasmids (<10 MDa) which could not be attributed directly to acquisition of plasmid DNA from ME2. Apparently, recombination events led to the appearance of different plasmids in this size range. Whether these events were triggered by conjugation with ME2 is not known. However, it seemed possible that the acquired phage resistance in the transconjugants was due to this phenomenon. To address this possibility, five phage-susceptible Lac⁺ transconjugants were examined for plasmid DNA profiles, which were compared with the plasmid profiles of four phage-resistant transconjugants (Fig. 2). The phage-susceptible transconjugants also demonstrated alterations among the plasmids in this size range, suggesting that the recombination events transpired independently of the acquisition of phage resistance.

High-frequency conjugation. Expression of phage resistance by T2r-M43a suggested acquisition of pTR2030 or phage resistance determinants carried by pTR2030. Since pTR2030 also carries Tra⁺ determinants (8), T2r-M43a was examined for high-frequency conjugation ability by mating with *S. lactis* LM2302, a Lac⁻ Hsp⁻ Str^r Ery^r plasmidless

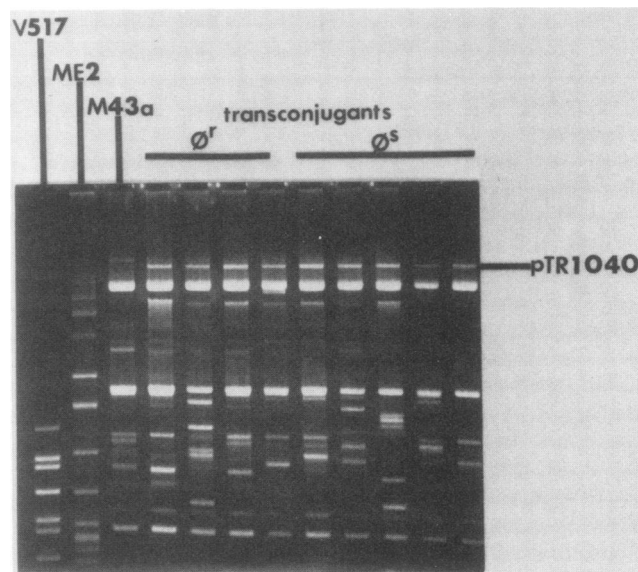


FIG. 2. Plasmid profiles of Lac⁺ transconjugants resulting from *S. lactis* ME2 × *S. cremoris* M43a conjugal matings. V517, *E. coli* plasmid mobility standards. φ^r, phage-resistant Lac⁺ transconjugants; φ^s, phage-susceptible Lac⁺ transconjugants. pTR1040 was not visible in the *S. lactis* ME2 profile (see reference 8).

derivative of *S. lactis* C2. Lac⁺ was transferred at a frequency of 2.6×10^{-2} per donor cell (Table 4). Of 30 transconjugants examined from this cross, 67% displayed the Hsp⁺ phenotype when challenged with c2 phage as described by Klaenhammer and Sanozky (8). When the parent, M43 (Lac⁺), or a Lac⁺ phage-susceptible transconjugant, T2s-M43a, was mated with LM2302, no transconjugants were generated (Table 4). Examination of Lac⁺ Hsp⁺ LM2302 transconjugants revealed that two plasmids of 40 and 30 MDa were transferred from T2r-M43a to LM2302 (Fig. 3). Correlation of Lac⁺ to the 40-MDa plasmid and Hsp⁺ to the 30-MDa plasmid was demonstrated in curing studies with *S. lactis* T-LS1 (data not shown). These results demonstrated that only phage-resistant transconjugants were capable of high-frequency conjugal transfer of Lac⁺, although the 40-MDa Lac⁺ plasmid was harbored by all transconjugants. A 30-MDa plasmid was transferred from T2r-M43a which conferred the Hsp⁺ phenotype to LM2302 transconjugants. These data suggest that pTR2030 was present and responsible for phage resistance in T2r-M43a. However, the level of expression of phage resistance in T2r-M43a was much greater than in LM2302 transconjugants carrying the 30-MDa plasmid.

Restriction endonuclease comparison of plasmids. After matings between *S. cremoris* T2r-M43a and LM2302, the

TABLE 3. Responses of transconjugant T2r-M43a and parent M43a to challenge with m12r-M12

Strain	Phage titer (PFU/ml)	EOP	Phage adsorption		
			Initial titer (PFU/ml)	Residual titer (PFU/ml)	% Adsorption
<i>S. cremoris</i> M43a	2.3×10^{10}	1.0	1.3×10^7	7.8×10^4	99.4
<i>S. cremoris</i> T2r-M43a ^a	<10	$<4.3 \times 10^{-10}$	1.3×10^7	2.6×10^5	98.0
<i>S. cremoris</i> T2s-M43a ^b	2.5×10^{10}	1.1	ND ^c	ND	ND

^a Phage-resistant Lac⁺ transconjugant resulting from mating of ME2 and M43a.

^b Phage-susceptible Lac⁺ transconjugant resulting from mating of ME2 and M43a.

^c ND, Not determined.

TABLE 4. High-frequency conjugation of Lac⁺ and phage resistance from *S. cremoris* Lac⁺ transconjugants

Mating pair donor ^a	Lac ⁺ Str ^r Ery ^r recombinants ^b		
	No./ml	Frequency/donor	% Phage resistant ^c
<i>S. cremoris</i> T2r-M43a ^d	1.7 × 10 ⁶	2.6 × 10 ⁻²	67 ^e
<i>S. cremoris</i> T2s-M43a ^f	0	<5.2 × 10 ⁻⁸	0
<i>S. cremoris</i> M43 ^g	0	<6.8 × 10 ⁻⁹	0

^a Recipients for all strains were *S. lactis* LM2302.

^b Recombinants were selected on lactose indicator agar containing 1,000 µg of streptomycin per ml and 15 µg of erythromycin per ml.

^c Hsp⁺ recombinants were detected in plaque assays.

^d Donor population was 6.3 × 10⁷ CFU/ml.

^e Thirty recombinants were examined.

^f Donor population was 1.9 × 10⁷ CFU/ml.

^g Donor population was 1.5 × 10⁸ CFU/ml.

30-MDa plasmid appearing in Hsp⁺ transconjugants of LM2302 was digested with *Eco*RI and *Hae*III and compared to restriction enzyme digests of pTR2030. Figure 4 shows that the fragment patterns generated were identical. These data demonstrate that the 30-MDa plasmid conjugally transferred from T2r-M43a to LM2302 was, in fact, pTR2030. Therefore, this plasmid had not incurred detectable genetic alterations during its introduction into, residence in, or transfer out of *S. cremoris* T2r-M43a. Similarly, *Eco*RI and *Hind*III digests of pTR1040 and the 40-MDa Lac⁺ Nis^r

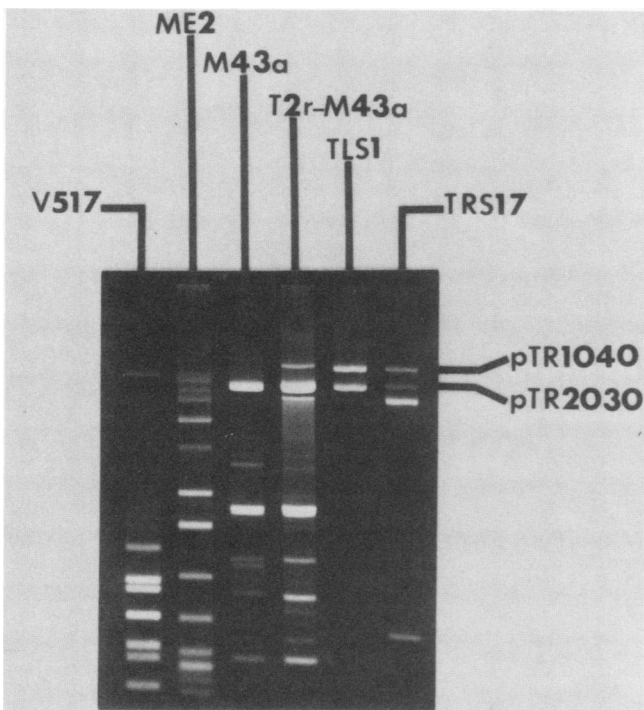


FIG. 3. Appearance of 40- and 30-MDa plasmids in *S. cremoris* T2r-M43a × *S. lactis* LM2302 transconjugants. T2r-M43a is a phage-resistant Lac⁺ transconjugant resulting from the *S. lactis* ME2 × *S. cremoris* M43a conjugal mating. TLS1 is a Lac⁺ Hsp⁺ transconjugant resulting from the T2r-M43a × LM2302 conjugal mating. TRS17 is a Lac⁺ Hsp⁺ transconjugant resulting from the ME2 × *S. lactis* LM0230 conjugal mating. V517, *E. coli* plasmid mobility standards. pTR1040 was not visible in the *S. lactis* ME2 profile (see reference 8).

plasmid transferred from T2r-M43a to LM2302 were identical (Fig. 4).

Starter culture activity tests. A starter culture activity test was performed to compare the abilities of phage-resistant and phage-susceptible transconjugants from *S. lactis* LM2302 and *S. cremoris* M43a to withstand challenge by virulent phage under simulated cheesemaking conditions (Table 5). Both the *S. lactis* LM2302 transconjugants T-LS1 and T-LS2 failed to produce acid during activity tests conducted in the presence of c2 phage. Although a small reduction in the final c2 phage population was observed for T-LS1 over T-LS2 at the end of the activity test, the presence of pTR2030 and the Hsp⁺ character expressed by T-LS1 did not protect this strain from phage-induced inhibition of acid development. Similarly, both *S. cremoris* M43 and the Lac⁺, phage-susceptible transconjugant T2s-M43a were susceptible to their virulent phage m12r · M12, and, therefore, acid development was inhibited in the presence of phage. In contrast, phage m12r · M12 failed to replicate on *S. cremoris* T2r-M43a and did not inhibit acid development during the starter culture activity test. Virulent phage also failed to develop toward *S. cremoris* T2r-M43a when this transconjugant was subjected to five successive cycles of the starter culture activity test in the combined presence of phage m12r · M12 and whey resulting from the previous cycles of the test.

Resistance of T2r-M43a to different *S. cremoris* M12R lytic phages. Examination of purified phages isolated from six different cheese plants revealed that they had slightly different host ranges (Table 6). These phages were used to challenge T2r-M43a in plaque assays (Table 7). All resulted in high titers when plaqued on the homologous host, *S. cremoris* M12. However, none of these phages produced plaques on *S. cremoris* T2r-M43a. These data demonstrate

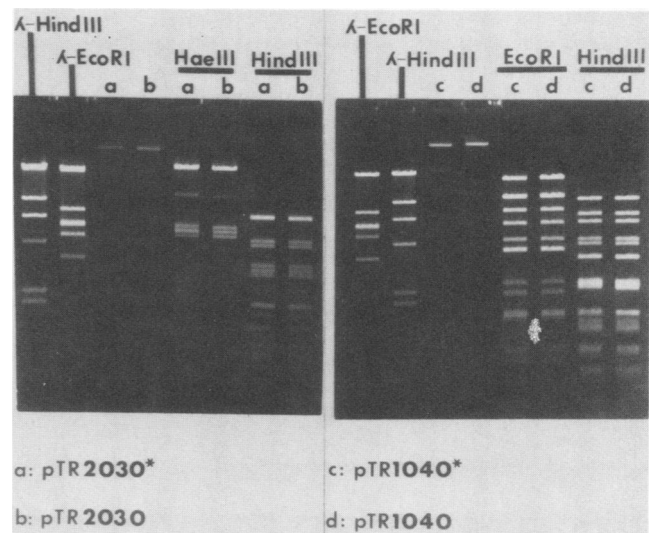


FIG. 4. Comparisons of restriction enzyme digest patterns of the 30- and 40-MDa plasmids transferred from T2r-M43a to LM2302 with pTR2030 and pTR1040, respectively. The 30-MDa plasmid (pTR2030*) and pTR2030 were digested with *Hae*III and *Hind*III. The 40-MDa plasmid (pTR1040*) and pTR1040 were digested with *Eco*RI and *Hind*III. Lambda DNA digested with *Eco*RI (fragment sizes of 14, 4.9, 3.8, 3.7, 3.2, and 2.3 MDa) and *Hind*III (fragment sizes of 15.3, 6.2, 4.3, 2.9, 1.5, and 1.3 MDa) served as mobility standards.

TABLE 5. Acid production by Lac⁺ transconjugants during starter culture activity tests in the presence of virulent bacteriophage

Phage added, strain	Description	Phage titer (PFU/ml)		Final pH ^a
		Initial	Final	
c2				
<i>S. lactis</i> TLS1	Lac ⁺ Hsp ⁺ transconjugant of T2r-M43a × LM2302	— ^b	—	5.45
		1.4 × 10 ⁶	1.3 × 10 ⁹	6.45
<i>S. lactis</i> TLS2	Lac ⁺ Hsp ⁻ transconjugant of T2r-M43a × LM2302	—	—	5.35
		1.4 × 10 ⁶	5.9 × 10 ⁹	6.45
m12r · M12				
<i>S. cremoris</i> M43	Lac ⁺ Nis ^s Hsp ⁻ parent of M43a	—	—	5.85
		4.1 × 10 ⁶	5.2 × 10 ⁹	6.50
<i>S. cremoris</i> T2r-M43a	Lac ⁺ Nis ^f phage-resistant transconjugant of ME2 × M43a	—	—	5.95
		4.1 × 10 ⁶	2.7 × 10 ³	5.95
<i>S. cremoris</i> T2s-M43a	Lac ⁺ Nis ^f phage-susceptible transconjugant of ME2 × M43a	—	—	5.95
		4.1 × 10 ⁶	5.6 × 10 ⁹	6.50

^a Initial pH of milk, 6.6.^b —, Not applicable; no phage added.

that T2r-M43a displayed a high level of resistance to a group of phages isolated in the field which were capable of attacking the parent strain, *S. cremoris* M12R.

DISCUSSION

Correlation of phage resistance mechanisms with plasmid DNA in lactic streptococci (2, 3, 13, 19, 20) encourages attempts to transfer these mechanisms to phage-susceptible starter cultures by using plasmid-transfer techniques such as conjugation (14), transduction (16), and transformation (9). Chopin et al. (2), McKay and Baldwin (13), and Klaenhammer and Sanozky (8) have used conjugal transfer mechanisms to introduce phage resistance determinants into strains of *S. lactis*.

In this study, lactose-fermenting ability was conjugally transferred from *S. lactis* ME2 to *S. cremoris* M43a. The Lac⁺ transconjugants acquired a 40-MDa plasmid identical to pTR1040 and exhibited resistance to nisin. These data further confirmed the association of Lac⁺ and Nis^f with pTR1040 in *S. lactis* ME2 (8), and demonstrated that conjugal transfer to *S. cremoris* M43a occurred in the absence of

detectable changes in either phenotypic or genetic characteristics of this plasmid. Examination of the *S. cremoris* Lac⁺ transconjugants revealed that approximately 50% of the recombinants had become resistant to the virulent phage, m12r · M12. Although pTR2030 could not be observed directly in the transconjugants due to a resident 30-MDa plasmid in M43a, two lines of evidence suggested that the phage resistance exhibited by the Lac⁺ transconjugant T2r-M43a was imposed by the presence of pTR2030. First, the Lac⁺ phage-resistant transconjugant (T2r-M43a) demonstrated high-frequency conjugation ability, indicating the presence of Tra⁺ determinants associated with pTR2030; the Lac⁺ phage-sensitive transconjugant (T2s-M43a) failed to promote conjugal transfer. Secondly, the high-frequency conjugal matings between T2r-M43a and *S. lactis* LM2302 resulted in a Lac⁺ Hsp⁺ transconjugant (*S. lactis* T-LS1) which carried a 30-MDa plasmid encoding Hsp⁺ and Tra⁺. Comparison of restriction enzyme digests between pTR2030 and the 30-MDa plasmid appearing in T-LS1 confirmed its identity as pTR2030. We conclude from these data that the presence of pTR2030 in *S. cremoris* T2r-M43a was responsible for the phage resistance and high-frequency transfer ability exhibited by this transconjugant.

In the *S. cremoris* phage-resistant transconjugant, T2r-M43a, phage m12r · M12 failed to exhibit plaquing ability (EOP < 4.3 × 10⁻¹⁰). T2r-M43a continued to adsorb phage

TABLE 6. *S. cremoris* M12R phage host ranges^a

Strain	Phage reaction					
	m12r · M12	05	da	br	ot	sgl
M12	+	+	+	+	+	+
HP	+	+	+	+	+	+
ML3	+ ^b	+ ^c	—	—	—	—
C2	+ ^b	+ ^c	—	—	—	—
K375-1R1	—	+ ^c	—	—	—	—
205	—	+	—	—	—	—
M20CL1	+ ^b	+ ^d	—	+ ^d	—	+ ^b
TDM1	+ ^b	+	+	+	+	+
others ^f	—	—	—	—	—	—

^a Phages were first propagated through M12R variant M12. Phage suspensions were then spotted (5 μl) onto indicator lawns. Reactions: +, positive reaction (clear zones 7 to 14 mm in diameter); —, negative reaction (no zone).

^b Turbid zone.^c Few indistinct plaques.^d Few distinct plaques.^e Turbid zone containing few indistinct plaques.^f Twenty-two additional strains were examined.TABLE 7. Plaque assays of virulent phages on *S. cremoris* M12 and the phage-resistant transconjugant *S. cremoris* T2r-M43a

Phage ^a	Titer (PFU/ml)		EOP on strain T2r-M43a ^c
	M12 ^b	T2r-M43a	
m12r · M12	2.8 × 10 ¹⁰	<10	<3.6 × 10 ⁻¹⁰
05	3.8 × 10 ¹⁰	<10	<2.7 × 10 ⁻¹⁰
da	4.6 × 10 ⁹	<10	<2.2 × 10 ⁻⁹
br	5.8 × 10 ⁹	<10	<1.7 × 10 ⁻⁹
ot	1.9 × 10 ¹⁰	<10	<5.3 × 10 ⁻¹⁰
sgl	3.2 × 10 ¹⁰	<10	<3.1 × 10 ⁻¹⁰

^a Propagated through *S. cremoris* M12.^b EOP was 1.0 for all phages on *S. cremoris* M12.

at a high efficiency, eliminating reduced phage adsorption as a contributing factor in the resistance mechanism. Unlike the complete resistance exhibited by *S. cremoris* T2r-M43a toward phage m12r · M12, *S. cremoris* LM0230 transconjugant T-RS1 (8) and *S. lactis* LM2302 transconjugant T-LS1 harboring pTR2030 demonstrated a reduction in burst size while allowing infection by phage c2 at unrestricted levels (EOP = 1.0). Therefore, in the apparent absence of genotypic differences in pTR2030, phenotypic expression of phage resistance in the *S. lactis* and *S. cremoris* transconjugants was remarkably different. These differences could be accounted for by different levels of host-induced expression of the phage-resistance determinants carried on pTR2030 or could simply reflect the difference in the phage-host combinations examined and the susceptibility of different phages to the resistance mechanism conferred by pTR2030. These possibilities are currently being investigated in our laboratory. In addition, it cannot be disregarded that minor or temporary genetic alterations in pTR2030 occurred during its introduction and residence in *S. cremoris* T2r-M43a, which were not detected in the restriction enzyme analysis and could account for the difference in phage resistance expressed.

The starter culture activity test described by Heap and Lawrence (4) establishes fermentation conditions encountered during cheesemaking under which the appearance and action of virulent bacteriophage against dairy starter cultures can be evaluated. When propagated through five cycles of the activity test in the presence of phage m12r · M12, *S. cremoris* T2r-M43a was not affected, whereas the phage-susceptible transconjugant T2s-M43a and T-LS1 were both susceptible to phage within the first cycle of the activity test. The susceptibility of T-LS1 to phage c2 in the test reflects both the heat lability of this phage resistance mechanism and the genetic instability of pTR2030 at 40°C (8). Similarly, McKay and Baldwin (13) showed that transconjugants of *S. lactis* LM0230 harboring the thermosensitive plasmid pNP40 became susceptible to phage when grown at 37°C and gradually lost activity during propagation through repeated cycles of the starter culture activity tests with periods of incubation at 37°C. Consequently, little protection from phage has been afforded *S. lactis* LM0230 transconjugants harboring pTR2030 or pNP40 (13) when propagated in starter culture activity tests. Phage resistance conferred by pTR2030 effectively protected the *S. cremoris* transconjugant T2r-M43a from the appearance or action of lytic phage in the activity test. *S. cremoris* M43a and its transconjugants do not grow at 40°C. Therefore, under conditions in which thermosensitive plasmids might be destabilized (8, 13) or heat-sensitive phage defense mechanisms might be inactivated (18, 21), *S. cremoris* T2r-M43a does not provide a replicating host for phage proliferation.

Although *S. cremoris* T2r-M43a was unaffected by phage m12r · M12 in starter culture activity tests, the acid-producing ability of the transconjugant in milk was poor. The conjugal recipient, M43a, was deficient in proteinase activity (unpublished data), and matings with *S. lactis* ME2 did not restore proteolytic activity to the transconjugants. Therefore, the phage-resistant transconjugants described here would be of little industrial significance. Efforts to introduce pTR2030 into Prt⁺ conjugal recipients of *S. cremoris* have been successful and will be presented in a subsequent report.

During this study, it was also of interest to determine whether *S. cremoris* T2r-M43a was susceptible to phages appearing in the field where its parent strain, *S. cremoris* M12R, was being used. Six phages with slight differences in

host range showed no virulent activity against the phage resistant transconjugant. It cannot be determined from these data whether the phages examined were different or simply the same phage with slightly altered host ranges. Nevertheless, from a practical standpoint, it is highly significant that virulent phages appearing in commercial fermentations against *S. cremoris* M12R were ineffective against the transconjugant T2r-M43a harboring pTR2030. The mechanism of phage resistance imposed by pTR2030, location of the responsible genetic determinants, and effectiveness against different lactic streptococcal phages are under investigation.

In conclusion, our study has shown that conjugal matings between *S. lactis* ME2 and a phage-susceptible strain of *S. cremoris* yield transconjugants which harbor pTR2030 and are resistant to virulent phage in plaque assays and starter culture activity tests. Strategies for the conjugal dissemination of pTR2030 among lactic streptococci, particularly *S. cremoris* strains, may prove useful in programs emphasizing genetic development of phage-resistant starter cultures for dairy fermentations.

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