APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1986, p. 1264–1271
0099-2240/86/061264-08$02.00/0

Vol. 51, No. 6

Conjugal Transfer of Bacteriophage Resistance Determinants on pTR2030 into *Streptococcus cremoris* Strains†

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Received 12 December 1985/Accepted 12 March 1986

Agar surface conjugal matings were used to introduce heat-sensitive phage resistance (Hsp^+) determinants carried on the conjugal plasmid pTR2030 into *Streptococcus cremoris* KH, HP, 924, and TDM1. Lactose-fermenting (Lac^+) transconjugants were selected from matings of Lac^- variants of *S. cremoris* KH, HP, 924, and TDM1 with *Streptococcus lactis* ME2 or a high-frequency donor, *S. lactis* T-EK1 (pTR1040, Lac^+; pTR2030, Hsp^+). For all of the *S. cremoris* strains examined, select Lac^+ transconjugants were completely resistant to plaquing by their homologous lytic phages. In all cases the plaquing efficiencies were less than 10^{-9}. Acquisition of a 30-megadalton plasmid (pTR2030) in the *S. cremoris* phage-resistant transconjugants was demonstrated by direct plasmid analysis, by hybridization with ^{32}P-labeled probes, or by conjugal transfer of pTR2030 out of the phage-resistant transconjugants into a plasmid-cured recipient, *S. lactis* LM2302. Acid production, coagulation ability, and proteolytic activity of phage-resistant transconjugants in milk were comparable to those of their phage-sensitive parents. Further, *S. cremoris* phage-resistant transconjugants were not attacked by phage in starter culture activity tests, which included a 40°C incubation period. The results demonstrated that phage resistance determinants on pTR2030 could be conjugally transferred to a variety of *S. cremoris* strains and confer resistance to phage under conditions encountered during cheese manufacture. Phage-resistant transconjugants of *S. cremoris* M43 and HP were also constructed without the use of antibiotic markers to select conjugal recipients from mating mixtures.

Conjugal transfer of phage defense mechanisms to lactic streptococci offers a powerful genetic approach to developmental programs targeted to the improvement of dairy starter cultures. Several phage defense mechanisms have now been identified in group N streptococci and correlated with plasmid DNA. These include restriction and modification activities (2, 23), prevention of phage adsorption (4, 24), and other undefined mechanisms that completely eliminate the plaquing ability of phage (17, 27) or reduce the burst size of infecting phage without inhibition of plaquing efficiency (12). Conjugation has been used successfully to identify the plasmids involved by transfer of plasmid-linked defense mechanisms to lactic streptococci, rendering them resistant to phage (2, 12, 17, 27). However, these investigations have employed few lactic streptococcal strains as conjugal recipients, and the transconjugants exhibited phage resistance in varied degrees. McKay and Baldwin (17) demonstrated conjugal transfer of a 40-megadalton (MDa) plasmid (pNP40) from *Streptococcus lactis* subsp. *diacetylactis* DRC3 to *S. lactis* LM0230. The transconjugants carrying pNP40 demonstrated complete resistance to c2 phage at 21 and 32°C, but restriction did not occur after incubation at 37°C. Consecutive transfers of the transconjugant at 37°C resulted in 100% loss of phage resistance due to pNP40 instability. Klaenhammer and Sanozky (12) found that when a 30-MDa plasmid, pTR2030, was transferred from *S. lactis* ME2 into *S. lactis* LM0230, the burst size and plaque size of infecting phage were reduced without a significant reduction in the efficiency of plaquing. However, normal plaque and burst size were restored when the transconjugants were propagated and challenged with phage at 40°C. High-temperature incubation was also shown to rapidly destabilize pTR2030 in *S. lactis* LM0230 transconjugants.

These studies have reported genetic and phenotypic responses at higher growth temperatures that would limit the practical usefulness of phage-resistant transconjugants carrying pTR2030 or pNP40 in the dairy industry. Numerous cases have been documented describing the effects of high-temperature incubation on phage proliferation in group N streptococci (9). In contrast, conjugal transfer of pTR2030 from *S. lactis* ME2 into *Streptococcus cremoris* M43a resulted in transconjugants that were not susceptible to bacteriophages isolated from commercial cheese plants (efficiency of plaquing, $<10^{-9}$) and were unaffected by challenge with phage at 36°C after heat shock at 40°C or during starter culture activity tests (27). Therefore, in a *S. cremoris* host, protection by pTR2030-induced phage resistance was not undermined by the high temperatures that are encountered in cheesemaking. Considering that *S. cremoris* species do not grow at 40°C, phage may not develop at these temperatures even if thermosensitive mechanisms of phage resistance are present (27). Therefore, *S. cremoris* strains may be the preferred hosts for genetic efforts to construct phage-resistant starter cultures. In this regard, however, the *S. cremoris* phage-resistant transconjugant T2r-M43a described previously (27) failed to demonstrate acceptable levels of acid production or proteolytic activity in milk.

The objectives of this study were to conjugally transfer pTR2030-induced phage resistance to additional *S. cremoris* strains and examine the transconjugants for fermentative properties and resistance to phages which attack these different strains. The successful recovery of transconjugants that were phage resistant (Hsp^+) and which demonstrated acceptable fermentative activities prompted development of

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† Paper no. 10234 of the Journal Series of the North Carolina Agricultural Research Service, Raleigh, NC 27695-7624.

TABLE 1. Bacterial strains, plasmids, and phages

Strain	Phenotype[a]	Homologous phage	Description	Relevant plasmids	Reference or source
S. lactis ME2	Lac$^+$ Hsp$^+$		Conjugal donor of Lac$^+$ and Hsp$^+$	pTR1040, pTR2030	(12)
S. lactis T-EK1	Lac$^+$ Hsp$^+$ Str^s	c2	Conjugal donor of Lac$^+$ and Hsp$^+$	pTR1040, pTR2030	This study
S. lactis T-RS1	Lac$^+$ Hsp$^+$ Str^r	c2	Conjugal donor of Lac$^+$ and Hsp$^+$	pTR1040, pTR2030	(12)
S. lactis T-RS1a	Lac$^-$ Hsp$^+$ Str^r	c2	Lac$^-$ Hsp$^+$ variant of T-RS1	pTR2030	(12)
S. lactis LM2302	Lac$^-$ Ery^r Str^r	c2	Plasmid free	None	(29)
S. lactis LM0230	Lac$^-$ Str^r	c2	Plasmid free	None	(29)
S. lactis C2	Lac$^+$ Prt$^+$	c2	Fast acid producer		(20)
E. coli V517			Source of reference plasmids		(15)
E. coli K-12 sublines (J5, J53, C600)			Source of reference plasmids		(23)
S. cremoris KH	Lac$^+$ Str^s	kh	Parent strain		(24)
S. cremoris KHA	Lac$^-$ Str^s	kh	Derivative of KH		This study
S. cremoris KHA2	Lac$^-$ Str^r	kh	Derivative of KHA		This study
S. cremoris T-KH1	Lac$^+$ Hsp$^+$ Str^r	kh	Transconjugant from mating between T-EK1 and KHA2	pTR1040, pTR2030	This study
S. cremoris HP	Lac$^+$ Str^s	hp	Parent strain		(24)
S. cremoris HPA	Lac$^-$ Str^s	hp	Derivative of HP		This study
S. cremoris HPA4	Lac$^-$ Str^r	hp	Derivative of HPA		This study
S. cremoris T-HP14	Lac$^+$ Hsp$^+$ Str^r	hp	Transconjugant from mating between T-EK1 and HPA4	pTR1040, pTR2030	This study
S. cremoris T-HPS1	Lac$^+$ Hsp$^+$ Str^s	hp	Transconjugant from mating between T-EK1 and HPA	pTR1040, pTR2030	This study
S. cremoris T-HPS2	Lac$^+$ Hsp$^+$ Str^s	hp	Transconjugant from mating between T-EK1 and HPA	pTR1040, pTR2030	This study
S. cremoris 924	Lac$^+$ Str^s	924	Parent strain		(24)
S. cremoris 924EB	Lac$^-$ Str^s	924	Derivative of 924		This study
S. cremoris 924EB1	Lac$^-$ Str^r	924	Derivative of 924EB		This study
S. cremoris T-9249	Lac$^+$ Hsp$^+$ Str^r	924	Transconjugant from mating between T-EK1 and 924EB1	pTR1040, pTR2030	This study
S. cremoris TDM1	Lac$^+$ Str^s	18	Parent strain		(24)
S. cremoris TDM1R3	Lac$^-$ Str^r	18	Derivative of TDM1		This study
S. cremoris TM-TDM1R3	Lac$^+$ Hsp$^+$ Str^r	18	Transconjugant from mating between ME2 and TDM1R3	pTR1040, pTR2030	This study
S. cremoris M43	Lac$^-$ Str^s	m12r.M12	Derivative of M12R		(27)
S. cremoris M43a	Lac$^-$ Str^r	m12r.M12	Derivative of M43		(27)
S. cremoris T-M432	Lac$^+$ Hsp$^+$ Str^s	m12r.M12	Transconjugant from mating between T-EK1 and M43	pTR1040, pTR2030	This study
S. cremoris T-M434	Lac$^+$ Hsp$^+$ Str^s	m12r.M12	Transconjugant from mating between T-EK1 and M43	pTR1040, pTR2030	This study

[a] Lac, lactose-fermenting ability; Prt, proteinase activity; Hsp$^+$, phage resistance; Hsp$^-$, phage sensitivity; Str^r, streptomycin resistance (1,000 μg/ml); Ery^r, erythromycin resistance (15 μg/ml).

a new strategy to recover phage-resistant transconjugants in the absence of antibiotic selection.

MATERIALS AND METHODS

Bacterial strains and bacteriophages. The bacterial strains and bacteriophages used in this study are listed in Table 1. Lactose-fermenting (Lac$^+$) *S. lactis* and *S. cremoris* strains and their bacteriophages were propagated in M17 broth at 30°C (28). Lac$^-$ strains were propagated in M17-glucose broth (23) at 30°C. *Escherichia coli* strains were propagated at 37°C in brain heart infusion broth (BBL Microbiology Systems, Cockeysville, Md.). All stock cultures were stored at −30°C in growth medium containing 10% (vol/vol) glycerol. Frozen stock cultures were thawed and transferred twice before used in experiments. Bacteriophage titers and efficiency of plaquing were determined as described previously (22, 28).

Isolation of Lac$^-$ derivatives. Lac$^-$ variants of *S. cremoris* KH, HP, 924, and TDM1 were isolated after repeated transfers in M17-glucose broth containing either 2 μg ethidium bromide (Sigma Chemical Co., St. Louis, Mo.) per ml or 3.5 μg of acriflavin hydrochloride (Sigma) per ml. From the Lac$^-$ derivatives, mutants resistant to 1 mg of streptomycin per ml (Str^r) were isolated as described previously (27) and used as recipients in conjugation experiments. Lac$^-$ Hsp$^+$, Lac$^+$ Hsp$^-$, and Lac$^-$ Hsp$^-$ derivatives of *S. cremoris* T-KH1 and T-HP14 (Lac$^+$ Hsp$^+$) were isolated as described above and used to evaluate the effects of pTR1040 and pTR2030 on the proteolytic activities of the strains in milk.

Conjugation. Conjugal matings were conducted on the surface of milk-glucose agar plates as described by McKay et al. (18). Lac$^+$ transconjugants were detected on lactose indicator agar (19) containing either 1 mg of streptomycin per ml for selection of *S. cremoris* conjugal recipients KHA2, HPA4, 924EB1, TDM1R3, and M43a; or 1 mg of streptomycin per ml plus 15 μg of erythromycin per ml when *S. lactis* LM2302 (Lac$^-$ Str^r Ery^r) was used as a recipient. Lac$^+$ Str^r transconjugants resulting from matings between *S. lactis* donors (T-EK1 or ME2) and *S. cremoris* recipients were

purified by single-colony isolation on lactose indicator agar with 1,000 μg of streptomycin per ml and confirmed as *S. cremoris* derivatives by growth on Reddy differential agar (21) and plasmid analysis. Transformation (13) and transduction were ruled out as mechanisms of gene transfer by conducting experiments with DNase and culture filtrates as described previously (12).

To isolate a high-frequency donor for use in conjugation experiments, agar matings were conducted between *S. lactis* T-RS1 (Lac^+ Hsp^+ Str^r) and a derivative of *S. lactis* LM0230 that was susceptible to streptomycin. Fifty suspect Lac^+ transconjugants were purified on lactose indicator agar, screened for streptomycin susceptibility, and examined for small plaques when challenged with c2 phage (Hsp^+). One Lac^+ Hsp^+ Str^s transconjugant was isolated, found to carry pTR1040 and pTR2030, and designated *S. lactis* T-EK1.

A procedure was developed to detect phage-resistant transconjugants in matings between *S. lactis* T-EK1 and *S. cremoris* recipients without use of antibiotics to select recombinants. Conjugation was conducted as described by McKay et al. (18), with the following modifications. Cells were recovered from mating plates with 2 ml of M17 broth per plate. Serial dilutions between 10^{-1} and 10^{-4} were made in 10% M17 broth. Samples (500 μl) from each cell dilution (10^0 to 10^{-4}) were added to a mixture of 500 μl of c2 phage (10^9 PFU/ml) and 100 μl of 1 M $CaCl_2$. The tubes were incubated for 15 min at room temperature to allow phage adsorption. Phage c2 was used in the cell mixtures to reduce levels of Lac^+ donor cells (*S. lactis* T-EK1) that might interfere with detection of Lac^+ *S. cremoris* transconjugants. To detect transconjugants, 200 μl of each cell-phage mixture was spread plated on each of five Reddy differential agar plates (21) and incubated for 48 h at 30°C. On this medium, Lac^+ *S. lactis* colonies and Lac^- *S. cremoris* colonies are white, whereas Lac^+ *S. cremoris* colonies are yellow. Suspect *S. cremoris* Lac^+ transconjugants (yellow colonies) were purified on Reddy agar, and their identity was confirmed by plasmid analysis before evaluation of phage resistance.

Plasmid analysis. Rapid methods for isolation and detection of plasmids from lactic streptococci were performed as described previously by Klaenhammer (10) with the modifications described by Sanders and Klaenhammer (25). Large-scale plasmid isolations were performed by the method of Anderson and McKay (1), with the modifications described by Steenson and Klaenhammer (27). Plasmid DNA from large-scale preparations was purified through cesium chloride-ethidium bromide density gradients (11). A cleared lysate procedure (11) and cesium chloride-ethidium bromide gradients were used for purification of plasmids from *E. coli* V517 and K-12 sublines for use as molecular weight standards.

TABLE 2. Conjugal transfer of lactose metabolism and phage resistance into *S. cremoris*

Mating pair		Lac^+ Str^r Transconjugants	
Donor (Lac^+ Str^s)	Recipient (Lac^- Str^r)	Lac^+ frequency/donor	% Phage resistant[a]
S. lactis T-EK1	*S. cremoris* KHA2	4.5×10^{-6}	25
S. lactis T-EK1	*S. cremoris* HPA4	4.0×10^{-4}	20
S. lactis T-EK1	*S. cremoris* 924EB1	1.1×10^{-4}	28
S. lactis T-EK1	*S. cremoris* TDM1R3	6.9×10^{-5}	0
S. lactis ME2	*S. cremoris* TDM1R3	5.5×10^{-5}	62
S. lactis T-EK1	*S. cremoris* M43a	3.3×10^{-2}	57
S. lactis T-EK1	*S. lactis* LM2302	1.2×10^{-2}	ND[b]

[a] Single-colony isolates of 60 Lac^+ transconjugants were examined for resistance to homologous phage by a standard plaque assay.
[b] ND, Not determined.

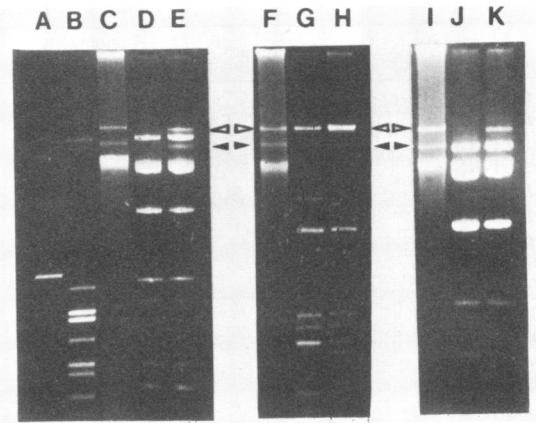

FIG. 1. Agarose gel electrophoresis of plasmid DNA from donor, recipient, and transconjugants. Lanes: A, *E. coli* J-C 34-, 23-, and 5.5-MDa molecular mass reference plasmids; B, *E. coli* V517 35.8-, 4.8-, 3.7-, 3.4-, 2.6-, 2.0-, 1.8-, and 1.4-MDa molecular mass reference plasmids; C, F, and I, *S. lactis* T-EK1 (conjugal donor); D, *S. cremoris* 924EB1 (recipient); E, *S. cremoris* T-9249 (transconjugant); G, *S. cremoris* KHA2 (recipient); H, *S. cremoris* T-KH1 (transconjugant); J, *S. cremoris* HPA4 (recipient); K, *S. cremoris* T-HP14 (transconjugant). Arrows designate the positions of pTR1040 (▷) and pTR2030 (▶).

Electrophoresis was conducted on 0.75% agarose horizontal gels (Seakem ME agarose; FMC Corp., Rockland, Md.) in Tris-acetate buffer (40 mM Tris, 12 mM sodium acetate, 1 mM disodium EDTA, pH 7.8). Plasmid preparations of 5 to 15 μl were mixed with 5 to 10 μl of agarose beads before loading into gels. Gels were electrophoresed at 6 V/cm for 2.5 h.

Southern transfer of plasmid DNA to nitrocellulose filters (Schleicher & Schuell Co., Keene, N.H.), and hybridization reactions were carried out as described previously (7, 26). ^{32}P-probes of pTR2030 were prepared by the random primer method (7, 16) or nick translation (Amersham Corp., Arlington Heights, Ill.) according to the manufacturer's specifications. Labeled probes of pTR2030 were prepared from *S. lactis* T-RS1a DNA which had been purified once through cesium chloride-ethidium bromide gradients.

Acid production, coagulation ability, and proteolytic activity. Reconstituted skim milk (11% RSM) and 11% RSM–1% glucose were steamed for 1 h and cooled to 22°C before inoculation with Lac^+ and Lac^- cultures, respectively. Cultures were propagated at 22°C overnight, and 0.4 ml was inoculated into 40 ml of 11% RSM or 11% RSM–1% glucose. After 18 to 22 h of incubation at 22°C, the cultures were examined for pH, coagulation, and proteolytic activity. Proteolysis was determined by the *o*-phthaldialdehyde spectrophotometric assay (3). Initial values for pH and free amino acids were determined from samples withdrawn immediately after inoculation of the milk.

Starter culture activity tests. The Heap and Lawrence activity test (6) was conducted in 11% RSM as described previously (24). Acid development and phage propagation were evaluated over a time-temperature incubation profile of 100 min at 30°C, 190 min at 40°C, and 100 min at 30°C. Cultures were propagated for 8 h at 30°C in 11% RSM before inoculation for the activity test.

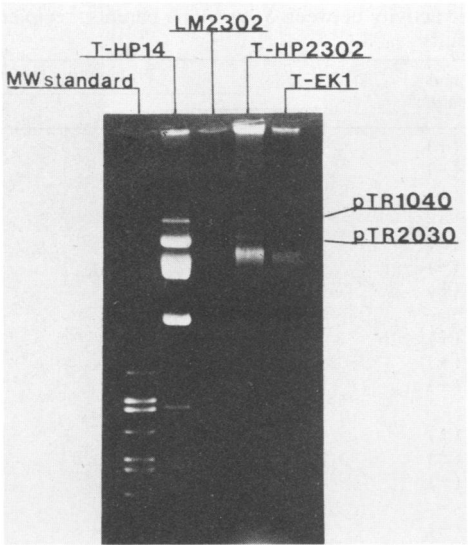

FIG. 2. Agarose gel electrophoresis of plasmid DNA from *S. cremoris* T-HP14 (donor), *S. lactis* LM2302 (recipient), and the Lac$^+$ Hsp$^+$ transconjugant, T-HP2302. The positions of pTR1040 and pTR2030 are indicated. Molecular mass reference plasmids from *E. coli* V517 are as in Fig. 1.

RESULTS

Conjugal transfer of Lac$^+$ and Hsp$^+$. Agar surface matings were conducted between *S. lactis* T-EK1 (Lac$^+$ Hsp$^+$ Str^s) and *S. cremoris* KHA2, HPA4, 924EB1, and TDM1R3 (Lac$^-$ Str^r) in attempts to transfer lactose-fermenting ability (Lac$^+$) and phage resistance (Hsp$^+$). Lac$^+$ was transferred into *S. cremoris* recipients at frequencies ranging from 10^{-4} to 10^{-6} recombinants per donor cell (Table 2). The frequencies for transfer of Lac$^+$ were substantially lower than those detected for *S. lactis* T-EK1 matings with *S. lactis* LM2302 or *S. cremoris* M43a (10^{-2} recombinants per donor cell). Control experiments eliminated the possibility of transduction or transformation modes of genetic transfer and established a requirement for cell-to-cell contact typical of a conjugationlike process (data not shown).

Lac$^+$ transconjugants from *S. cremoris* KHA2, HPA4, 924EB1, and TDM1R3 were examined for resistance to each strain's homologous lytic phage by plaque assays with high-titer phage preparations. Between 20 and 28% of the Lac$^+$ transconjugants from *S. cremoris* KHA2, HPA4, and 924EB1 were resistant to the recipient's homologous phage (Table 2). However in *S. cremoris* TDM1R3, none of 60 Lac$^+$ transconjugants was resistant to phage 18. Consequently, transfer of Lac$^+$ and phage resistance to TDM1R3 was attempted with *S. lactis* ME2 as a donor. From these crosses, Lac$^+$ transconjugants that demonstrated resistance to phage 18 were successfully recovered (Table 2).

Plasmid analysis. Plasmid analyses were conducted to determine whether a 30-MDa plasmid was present and correlated with the phage resistance exhibited by select Lac$^+$ transconjugants. Figure 1 compares the plasmid profiles of the Lac$^+$, phage-resistant *S. cremoris* transconjugants to their respective conjugal recipients and the donor *S. lactis* T-EK1. Clear acquisition of 30- and 40-MDa plasmids, corresponding to pTR2030 (Hsp$^+$) and pTR1040 (Lac$^+$), respectively, by the conjugal recipient *S. cremoris* 924EB1 was observed (Fig. 1, lanes D and E). Similarly, the plasmid profiles of transconjugant *S. cremoris* T-KH1 clearly showed pTR2030 (Fig. 1, lane H). However, upon examination of the plasmid profile of *S. cremoris* T-HP14, the presence of pTR2030 could not be detected due to a resident 30-MDa plasmid in *S. cremoris* HPA4 (Fig. 1, lanes J and K). To establish that pTR2030 was present in the phage-resistant transconjugant, matings were conducted with *S. lactis* LM2302 to attempt to transfer pTR1040 and pTR2030 out of T-HP14. In Lac$^+$ transconjugants of *S. lactis* LM2302 both 40- and 30-MDa plasmids were detected (Fig. 2). Transconjugants carrying the 30-MDa plasmid exhibited small plaques upon challenge with c2 phage. With Hsp$^+$ demonstrated, these data provided evidence that the 30-MDa plasmid transferred from *S. cremoris* T-HP14 to LM2302 was, in fact, pTR2030. The same analysis conducted for transconjugants T-KH1, T-9249, and TM-TDM1R3 provided similar evidence that pTR2030 was present and did not suffer significant physical or phenotypic alterations after residence in the *S. cremoris* transconjugants (data not shown). Further evidence for the presence of pTR2030 in T-KH1 and T-HP14 was provided by the accompanying study, where pTR2030 probes hybridized strongly at the 30-MDa position in plasmid profiles of T-KH1 and T-HP14 (8).

Characterization of phage-resistant transconjugants. To evaluate the level of phage resistance conferred by pTR2030 in each of the *S. cremoris* transconjugants, the efficiency of plaquing of homologous phage was compared among *S. cremoris* parents, recipients, and transconjugants (Table 3). Homologous lytic phage plaqued at high efficiencies on both the parent and recipient *S. cremoris* strains. In contrast, high-titer phage preparations did not form plaques on lawns of *S. cremoris* transconjugants T-KH1, T-HP14, T-9249, and TM-TDM1R3. Plaquing efficiencies were $<10^{-9}$ upon challenge of all four *S. cremoris* transconjugants with lytic phage. These data demonstrated that introduction of pTR2030 into different *S. cremoris* strains conferred a significant level of resistance to those phage which normally attack these strains.

Acid production, coagulation ability, and proteolytic activity. Acid production, coagulation ability, and proteolytic

TABLE 3. Efficiency of plaquing (EOP) of homologous phage on *S. cremoris* parents, Lac$^-$ Str^r variants, and transconjugants

S. cremoris strain	Description	Phage	PFU/ml	EOP
KH	Lac$^+$ parent	kh	5.6×10^9	1.0
KHA2	Lac$^-$ Str^r KH variant	kh	6.8×10^9	1.2
T-KH1	Lac$^+$ Hsp$^+$ Str^r transconjugant	kh	<10	$<1.8 \times 10^{-9}$
HP	Lac$^+$ parent	hp	6.7×10^9	1.0
HPA4	Lac$^-$ Str^r HP variant	hp	8.7×10^9	1.3
T-HP14	Lac$^+$ Hsp$^+$ Str^r transconjugant	hp	<10	$<1.5 \times 10^{-9}$
924	Lac$^+$ parent	924	1.9×10^9	1.0
924EB1	Lac$^-$ Str^r 924 variant	924	2.1×10^9	1.1
T-9249	Lac$^+$ Hsp$^+$ Str^r transconjugant	924	<10	$<5.3 \times 10^{-9}$
TDM1	Lac$^+$ parent	18	1.1×10^{10}	1.0
TDM1R3	Lac$^-$ Str^r TDM1 variant	18	1.1×10^{10}	1.0
TM-TDM1R3	Lac$^+$ Hsp$^+$ Str^r transconjugant	18	<10	$<9.1 \times 10^{-10}$

TABLE 4. Comparison of acid production, coagulation ability, and proteolytic activity between *S. cremoris* parents, recipients, and phage-resistant transconjugants

Strain[a]	Description	pH and coagulation[b]	Proteolytic activity	
			A_{340}[c]	Phenotype[d]
S. cremoris KH	Parent	4.6 (+)	+0.19	+
S. cremoris KHA2	Lac$^-$ Str^r	5.2 (−)	+0.05	−
S. cremoris T-KH1	Lac$^+$ Hsp$^+$ Str^r	4.5 (+)	+0.18	+
S. cremoris HP	Parent	4.5 (+)	+0.18	+
S. cremoris HPA4	Lac$^-$ Str^r	5.0 (−)	+0.08	−
S. cremoris T-HP14	Lac$^+$ Hsp$^+$ Str^r	4.7 (+)	+0.19	+
S. cremoris 924	Parent	4.6 (+)	+0.20	+
S. cremoris 924 EB1	Lac$^-$ Str^r	4.5 (+)	+0.19	+
S. cremoris T-9249	Lac$^+$ Hsp$^+$ Str^r	4.6 (+)	+0.20	+
S. cremoris TDM1	Parent	4.5 (+)	+0.22	+
S. cremoris TDM1R3	Lac$^-$ Str^r	4.9 (±)	+0.14	+
S. cremoris TM-TDM1R3	Lac$^+$ Hsp$^+$ Str^r	4.7 (+)	+0.21	+
S. lactis C2	Lac$^+$ Prt$^+$	4.7 (+)	+0.17	+
S. lactis LM0230	Lac$^-$ Prt$^-$ Str^r	6.2 (−)	−0.02	−
Uninoculated		6.5 (−)	−0.04	

[a] Lac$^+$ parent and transconjugant strains were propagated in 11% RSM, and Lac$^-$ recipients were propagated in 11% RSM–1% glucose medium.
[b] Initial pH was 6.5. pH and coagulation were determined after incubation for 18 h at 22°C. +, Full coagulation; ±, partial coagulation; −, no coagulation.
[c] Change in adsorbance at 340 nm as measured by an *o*-phthaldialdehyde spectrophotometric assay (3) after 22 h at 22°C.
[d] Proteolytic activity: +, proteolytic; −, proteolytic deficient.

activity of phage-resistant transconjugants were compared with those of parents and recipients to determine whether the introduction of pTR2030 affected fermentative activity (Table 4). Transconjugants carrying pTR2030 showed no significant alteration in their ability to produce acid or coagulate milk when compared with parental strains after 18 h at 22°C in milk. The proteolytic activity of the transconjugants was also comparable to that of parental strains after 22 h at 22°C in milk (Table 4). Each transconjugant culture exhibited comparable levels of proteolytic activity and acid production to the reference fast-acid producing strain, *S. lactis* C2.

Closer examination of the proteolytic activity data indicated that *S. cremoris* transconjugants T-KH1 and T-HP14 carrying pTR1040 and pTR2030 exhibited higher proteolytic activity than their corresponding proteolysis-deficient recipients, *S. cremoris* KHA2 and HPA4. This suggested that either pTR1040 or pTR2030 could carry a proteolytic determinant. To investigate this possibility, a comparison was made between *S. cremoris* T-KH1 and T-HP14 and each of their Lac$^-$ Hsp$^+$, Lac$^+$ Hsp$^-$, and Lac$^-$ Hsp$^-$ derivatives (Table 5). Although Lac$^-$ Hsp$^+$, Lac$^+$ Hsp$^-$, and Lac$^-$ Hsp$^-$ derivatives were isolated from *S. cremoris* T-KH1, only Lac$^-$ Hsp$^+$ derivatives could be isolated from *S. cremoris* T-HP14 due to stability of the Hsp$^+$ phenotype in this host. Therefore, *S. cremoris* HPA4 (Lac$^-$ Hsp$^-$) and a phage-susceptible transconjugant, *S. cremoris* T-HP1 (Lac$^+$ Hsp$^-$), were used to represent the two remaining phenotypes. In our comparison, both the Lac$^+$ Hsp$^+$ transconjugant *S. cremoris* T-KH1 and Lac$^+$ Hsp$^-$ derivatives demon-

TABLE 5. Comparison of proteolytic activity between *S. cremoris* T-KH1 and T-HP14 derivatives

S. cremoris strain[a]	Description	Plasmid composition	Proteolytic activity[b]
KH	Parent		0.24
T-KH1	Lac$^+$ Hsp$^+$ transconjugant of T-EK1 × KHA2	pTR1040, pTR2030	0.23
T-KH1-25	Lac$^+$ Hsp$^+$ single-colony isolate of T-KH1	pTR1040, pTR2030	0.17
T-KH1-5	Lac$^-$ Hsp$^+$ derivative of T-KH1	pTR2030	0.06
T-KH1-27	Lac$^+$ Hsp$^-$ derivative of T-KH1	pTR1040	0.24
T-KH1-1	Lac$^-$ Hsp$^-$ derivative of T-KH1		0.12
KHA2	Lac$^-$ Hsp$^-$ recipient		0.13
HP	Parent		0.31
T-HP14	Lac$^+$ Hsp$^+$ transconjugant of T-EK1 × HPA4	pTR1040, pTR2030	0.32
T-HP14-3	Lac$^+$ Hsp$^+$ single-colony isolate of T-HP14	pTR1040, pTR2030	0.29
T-HP14-2	Lac$^-$ Hsp$^+$ derivative of T-HP14	pTR2030	0.20
T-HP1	Lac$^+$ Hsp$^-$ transconjugant of T-EK1 × HPA4	pTR1040	0.31
HPA4	Lac$^-$ Hsp$^-$ recipient		0.21

[a] Lac$^-$ strains were cultured in 11% RSM–1% glucose.
[b] Change in adsorbance at 340 nm as measured by an *o*-phthaldialdehyde spectrophotometric assay (3) after 22 h at 22°C.

TABLE 6. Acid production by *S. cremoris* parents and phage-resistant transconjugants during starter culture activity tests[a] in the presence of phage

S. cremoris strain	Phage	pH		PFU/ml	
		Initial	Final[b]	Initial	Final[c]
KH		6.5	5.6		
KH	kh	6.5	6.4	3.0×10^6	1.3×10^{10}
T-KH1		6.5	5.4		
T-KH1	kh	6.5	5.4	3.0×10^6	4.0×10^3
HP		6.4	5.8		
HP	hp	6.4	6.4	3.1×10^6	4.9×10^9
T-HP14		6.5	5.8		
T-HP14	hp	6.5	5.9	3.1×10^6	1.9×10^3
924		6.4	5.7		
924	924	6.4	6.0	6.7×10^5	1.8×10^8
T-9249		6.5	5.8		
T-9249	924	6.5	5.8	6.7×10^5	9.5×10^4
TDM1		6.5	4.9		
TDM1	18	6.5	6.4	5.2×10^6	3.0×10^9
TM-TDM1R3		6.5	5.2		
TM-TDM1R3	18	6.5	5.2	5.2×10^6	7.0×10^6

[a] Strains were inoculated into 11% RSM and incubated sequentially for 100 min at 30°C, 190 min at 40°C, and 100 min at 30°C.
[b] Final pH was determined after 6.5 h of incubation.
[c] Final PFU per milliliter was determined after 6.5 h of incubation.

strated higher levels of proteolytic activity than Lac$^-$ Hsp$^+$ and Lac$^-$ Hsp$^-$ derivatives. Furthermore, *S. cremoris* T-HP14 (Lac$^+$ Hsp$^+$) and *S. cremoris* T-HP1 (Lac$^+$ Hsp$^-$) demonstrated higher levels of proteolytic activity than *S. cremoris* T-HP14-2 (Lac$^-$ Hsp$^+$) and *S. cremoris* HPA4 (Lac$^-$ Hsp$^-$). These data provided evidence that in addition to Lac$^+$ and Nis^r (12) pTR1040 may carry a proteolytic determinant.

Starter culture activity tests. Acid production of parents and phage-resistant transconjugants was compared in the presence and absence of lytic phage during starter culture activity tests (Table 6). Phage proliferated on *S. cremoris* KH, HP, 924, and TDM1 cultures and severely retarded acid production. In contrast, acid production by Lac$^+$ Hsp$^+$ transconjugants was not inhibited in the presence of phage and compared favorably to parental strains grown in the absence of phage. Phage populations did not increase significantly on any of the *S. cremoris* Lac$^+$ Hsp$^+$ transconjugants. Most importantly, phage resistance conferred by pTR2030 was effective throughout the starter culture activity test, which included a 190-min incubation step at 40°C.

Detection of phage-resistant transconjugants in the absence of antibiotics. Matings between *S. lactis* T-EK1 and Lac$^-$ *S. cremoris* recipients were conducted to recover phage-resistant transconjugants in the absence of antibiotics. Neither donor nor recipients carried streptomycin markers, nor were antibiotics used as a selective agent to recover recombinants. When this strategy was applied, Lac$^+$ transconjugants were detected at frequencies of 7.9×10^{-3} and 3.1×10^{-5} for *S. cremoris* M43 and HP, respectively (Table 7). Lac$^+$ transconjugants of *S. cremoris* KH and 924 were not detected due to interference by donors not effectively eliminated by c2 phage. Spontaneous phage-resistant mutants of T-EK1 appeared at low dilutions of cell mating mixtures plated on Reddy agar, creating large zones of neutralization which prevented detection of the smaller, Lac$^+$ *S. cremoris* colonies. Donor colonies did not interfere with recovery of Lac$^+$ transconjugants of *S. cremoris* HPA and M43 because transconjugants appeared on Reddy agar plates at higher dilutions of the cell mating mixture. Selected *S. cremoris* HPA and M43 Lac$^+$ transconjugants did not allow detectable plaque formation when challenged with high-titer phage preparations. Evidence for the presence of pTR2030 in these phage-resistant strains was provided by hybridization of ^{32}P-labeled pTR2030 probes to plasmid DNA preparations from Lac$^+$ Hsp$^+$ *S. cremoris* transconjugants immobilized on nitrocellulose filters. Typical data are shown in Fig. 3, where a pTR2030-labeled probe hybridized strongly to the 30-MDa position of plasmids isolated from phage-resistant transconjugants of *S. cremoris* HPA. In comparison, substantially less homology was detected at the 30-MDa position of the conjugal recipient, HPA. Homology with other plasmids in *S. cremoris* HPA was observed at similar levels in HPA and the phage-resistant transconjugants. This nonspecific hybridization might be expected, noting the size of the probe (30 MDa) used in these experiments and the probability of similar sequences occurring throughout plasmids harbored by lactic streptococci.

DISCUSSION

Conjugal transfer of plasmid-encoded mechanisms of phage resistance into phage-sensitive lactic streptococci offers the dairy fermentation industry a novel and effective strategy for culture development programs. Although conjugation has been successfully used to transfer phage resistance determinants (2, 12, 17, 27), the practical usefulness of phage-resistant transconjugants remains questionable because of (i) plasmid instability and loss of resistance during growth at the elevated temperatures encountered in cheesemaking (12, 17), (ii) inadequate fermentative activity (27), and (iii) an obvious concern over acceptance by dairy industry and federal regulatory agencies of starter cultures which harbor antibiotic resistance markers created for selection of phage-resistant recombinants in conjugation experiments. The results from this investigation demonstrated that conjugation could be employed to introduce pTR2030 into different strains of *S. cremoris*. The resulting phage-resistant transconjugants exhibited acceptable fermentation activities, were stable to phage challenge in starter culture activity tests, and could, in two *S. cremoris* strains, be constructed without use of antibiotic resistance markers in conjugation experiments.

TABLE 7. Conjugal transfer of lactose metabolism and phage resistance into *S. cremoris* and selection on Reddy differential agar in the absence of antibiotics

Mating pair		Lac$^+$ Transconjugants	
Donor[a] (*S. lactis*)	Recipient[b] (*S. cremoris*)	Frequency/ donor	Phage resistance
T-EK1	M43	8.4×10^{-3}	+
T-EK1	HPA	3.1×10^{-5}	+
T-EK1	924EB	Not detected	ND[c]
T-EK1	KHA	Not detected	ND

[a] Lac$^+$ *S. lactis* donors.
[b] Lac$^-$ *S. cremoris* recipients.
[c] ND, Not determined.

Lactose-fermenting ability and phage-resistance, encoded by pTR1040 and pTR2030, respectively (12), were successfully transferred by conjugation into four different *S. cremoris* strains. However, the *S. cremoris* recipients varied considerably in their ability to accept or express Lac^+ and Hsp^+ determinants. *S. lactis* T-EK1 demonstrated high-frequency transfer of Lac^+ and Hsp^+ into *S. lactis* LM2302 and *S. cremoris* M43a (10^{-2} recombinants per donor). Neither of these strains exhibited restriction and modification activities against phage (22; L. R. Steenson and T. R. Klaenhammer, submitted for publication). Alternatively, *S. cremoris* KH exhibits significant restriction and modification activity (23) and was accordingly found to accept Lac^+ at the lowest conjugation frequencies (10^{-6} recombinants per donor). The transconjugant T-KH1 was missing the 10-MDa plasmid (Fig. 1, lane H) shown previously to encode restriction and modification activity in *S. cremoris* KH (23). We conclude from these observations that whereas *S. lactis* T-EK1 exhibits high-frequency conjugal ability, formation of recombinants was to a large extent dependent on levels of restriction and modification activity present in the recipients. Restriction and modification activities are common to lactic streptococci (9). Consequently, genetic strategies to improve dairy starter cultures may often suffer from low-frequency recombination events if these systems are not negated.

In matings between *S. lactis* T-EK1 and *S. cremoris* TDM1, Hsp^+ transfer was not detected in Lac^+ transconjugants. Switching to *S. lactis* ME2 as a donor resulted in the isolation of Lac^+ Hsp^+ transconjugants of *S. cremoris* TDM1 that had acquired 40- and 30-MDa plasmids corresponding to pTR1040 and pTR2030, respectively. These observations emphasize the importance of a specific donor-recipient combination in conjugation experiments. However, the events responsible for the successful transfer and expression of phage resistance from ME2, or lack thereof from T-EK1, to *S. cremoris* TDM1 are not clear.

Introduction of pTR2030 into all four *S. cremoris* strains yielded Hsp^+ transconjugants resistant to the lytic phages which normally attack these strains. High-titer phage preparations failed to form plaques on the transconjugants in standard plaque assays. This phage resistance phenotype was identical to that described previously for the pTR2030 transconjugant, *S. cremoris* T2r-M43a (27), but strikingly different from Hsp^+ exhibited by pTR2030 transconjugants of *S. lactis* LM0230, where burst size was affected without a reduction in the plaquing efficiency of the phage. Recent results from our laboratory have now demonstrated that the phages used against *S. cremoris* transconjugants in this study and the study of Steenson and Klaenhammer (27) were small isometric-headed phages which are highly susceptible to pTR2030-induced phage resistance (8). The Hsp^+ phenotype was exhibited in *S. lactis* T-RS1 (12) in response to a prolate phage (c2) which was less susceptible to inhibition by pTR2030 (8). Therefore, in the five different *S. cremoris* strains where pTR2030 was introduced, small isometric phages were completely inhibited.

The *S. cremoris* phage-resistant transconjugants constructed in this investigation demonstrated acceptable levels of acid production, coagulation ability, and proteolytic activity when propagated in milk. The presence of pTR2030 did not suppress the overall fermentative acitivity of phage-resistant transconjugants. Evidence was obtained suggesting that pTR1040 harbored a proteolytic determinant (Tables 4 and 5) in addition to the Lac^+ and Nis^r previously correlated with this plasmid (12). Conjugal acquisition of pTR1040 by proteolysis-deficient strains KHA2 and HPA4 resulted in parental levels of proteolytic activity exhibited by the transconjugants. Alternatively, in a previous study the presence of pTR1040 in the phage-resistant transconjugant *S. cremoris* T2r-M43a did not overcome the Prt^- character of the recipient strain, *S. cremoris* M43a (27). Expression of Prt^+ determinants on pTR1040 thus appears to be dependent on the specific strain harboring the plasmid. Nevertheless, the presence of pTR2030 did not significantly suppress the fermentative activity of phage-resistant transconjugants constructed in this study. Through judicious selection of conjugal recipients, active fermentative properties should not be attenuated by the addition of pTR2030 during construction of phage-resistant transconjugants.

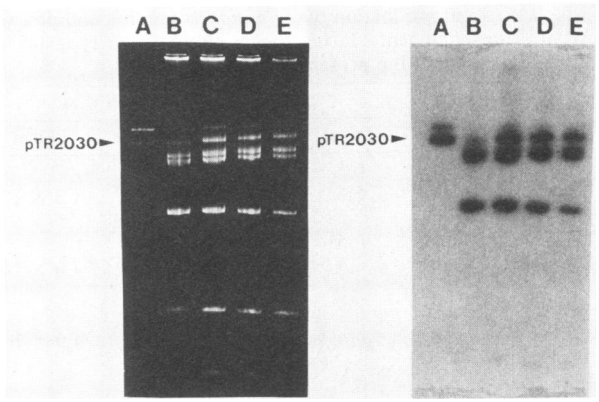

FIG. 3. Agarose gel electrophoresis (left) of plasmids from recipient strains *S. cremoris* HPA (Lac^- Str^s) and the Lac^+ Hsp^+ transconjugants and autoradiogram (right) prepared after hybridization with ^{32}P-labeled DNA from pTR2030. Lanes: A, T-EK1 (Lac^+ Hsp^+); B, HPA (Lac^- Hsp^-); C, T-HPS-1 (Lac^+ Hsp^+); D, T-HPS-2 (Lac^+ Hsp^+); E, T-HP14 (Lac^+ Hsp^+).

Previous studies have documented that phage defenses encoded by plasmids can be inactivated or genetically destabilized (or both) during growth of lactic streptococci at the elevated temperatures (37 to 40°C) encountered in cheesemaking (9, 12, 17, 22, 25, 27). During starter culture activity tests conducted in the presence of phage, acid production by *S. cremoris* Lac^+ Hsp^+ transconjugants was not inhibited, and phage did not proliferate. Therefore, pTR2030-induced phage resistance was not significantly destabilized by exposure to the 190-min incubation period at 40°C. As observed for *S. cremoris* T2r-M43a (27), *S. cremoris* transconjugants T-KH1, T-HP14, T-9249, and TM-TDM1R3 do not grow at 40°C and therefore will not provide a replicating host for phage during high-temperature incubations in the starter culture activity test. Nevertheless, if pTR2030 were destabilized at 40°C or its Hsp^+ gene product(s) were irreversibly inactivated, it would be expected that some phage proliferation would occur during the subsequent 30°C incubation period of the activity test. Phage proliferation was not detected, suggesting that pTR2030 was relatively stable in the *S. cremoris* transconjugants and Hsp^+ remained active at 30°C throughout the time-temperature course of the starter culture activity test.

A strategy was developed in this study to circumvent the use of antibiotic markers for the selection and detection of recombinants from conjugal mating mixtures. Reddy agar was used to differentiate the Lac^+ Hsp^+ *S. lactis* donor from *S. cremoris* Lac^- recipients and Lac^+ transconjugants. A high-frequency conjugal donor, *S. lactis* T-EK1, was employed to enhance conjugation events, but transfer frequen-

cies also were dictated by the recipient and its apparent restriction and modification activities. In all cases transfer frequencies were low enough that spontaneous phage-resistant mutants of the Lac^+ donor interfered with detection of Lac^+ *S. cremoris* transconjugants on Reddy agar plates at lower dilutions. Therefore, only for those recipients ammenable to higher transfer frequencies (*S. cremoris* M43a and HPA4, Table 2) could Lac^+ recombinants be recovered. In spite of these difficulties, phage-resistant transconjugants of *S. cremoris* M43 and HPA were isolated from matings with *S. lactis* T-EK1 and shown to carry pTR2030 determinants by hybridization. Therefore, conjugation of pTR2030 can be accomplished and transconjugants can be detected without use of antibiotic markers to select recipient strains from mating mixtures; however, further efforts are needed to refine these strategies. We are now investigating alternative methods for construction and selection of phage-resistant transconjugants of lactic streptococci. Considering the effectiveness of the phage defense mechanism encoded by pTR2030 and its demonstrated ability for transfer to numerous lactic streptococcal strains, this conjugative plasmid offers a distinct advantage for genetic strategies used in the development of phage-resistant starter cultures for dairy fermentations.

ACKNOWLEDGMENTS

This work was supported in part by Miles Laboratories, Inc., Biotechnology Group, Elkhart, Ind., and by the U.S. Department of Agriculture under agreement 85-CRCR-1-1727.

We also thank E. G. Kleeman for her efforts in the development of *S. lactis* T-EK1.

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