Bacteriophage Resistance Conferred on Lactic Streptococci by the Conjugative Plasmid pTR2030: Effects on Small Isometric-, Large Isometric-, and Prolate-Headed Phages†

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A series of reactions between phages, sensitive hosts, and transconjugants where the sensitivity of small isometric-, large isometric-, and prolate-headed phages to pTR2030-induced phage resistance was evaluated in *Streptococcus lactis* and *Streptococcus cremoris* strains. Phage-resistant transconjugants were constructed in the desired host by conjugal transfer of lactose-fermenting ability (Lac$^+$, pTR1040) and phage resistance (Hsp$^+$, pTR2030) from *S. lactis* TEK1. *S. lactis* and *S. cremoris* transconjugants harboring pTR2030 were resistant to all small isometric-headed phages examined. In contrast, prolate- and large isometric-headed phages were either not inhibited in the pTR2030 transconjugants or exhibited a reduction in plaque size without a reduction in the efficiency of plaquing. Small isometric-headed phages subject to pTR2030 induced inhibition shared no significant DNA homology with pTR2030, suggesting that phage immunity genes are not harbored on the plasmid or responsible for resistance. The general effectiveness of pTR2030 against small isometric-headed phages was highly significant since these are the phages which have been isolated most commonly from dairy fermentation plants.

Lactic streptococcal phages are highly significant to the dairy industry because they frequently attack bacterial strains used in cheesemaking. As a consequence, considerable effort has been directed to the isolation and use of phage-resistant starter cultures (for reviews, see references 6, 12, and 14). Phage-resistant mutants can be isolated readily after growth of bacterial strains in the presence of large numbers of lytic phage(s) which attack the culture in commercial situations (5, 6). Phage-resistant mutants are then used as replacement strains for multiple-strain starters upon appearance of phage active against any of the composite strains in the culture (14, 25). However, spontaneous mutations to phage resistance may be short lived. Host range mutations of the phage may occur, or the mutants may revert to phage sensitivity (8), particularly where repeated subculturing of starter cultures has been practiced.

In a recent study of phage resistance mechanisms in *Streptococcus lactis* ME2, Klaenhammer and Sanozky (13) described a 30-megadalton conjugative plasmid (pTR2030) that conferred a heat-sensitive phage resistance phenotype (Hsp$^+$) to *S. lactis* LM0230. Further studies demonstrated that pTR2030 could be transferred to five different strains of *Streptococcus cremoris* (21, 23). In each case the *S. cremoris* transconjugants were resistant to lytic phages which attacked the wild-type strains. It was highly significant that the pTR2030-induced mechanism was effective against six phages isolated independently from commercial cheese plants where *S. cremoris* M12 was employed (23). However, the phages were similar to one another in host range, and it was not determined whether they represented different phage groups. Moreover, in *S. cremoris* transconjugants the phage resistance imposed by pTR2030 was phenotypically different from Hsp$^+$ observed previously in

S. lactis transconjugants; efficiency of plaquing was less than 10^{-9}, and phage resistance was maintained through starter culture activity tests which included 40°C incubations. From these studies it was not clear whether differences between Hsp$^+$ phenotypes resulted from variability in host expression of pTR2030, susceptibility of different phages to the pTR2030-encoded resistance, or combinations of these effects (23).

Lactic streptococcal phages isolated from cheese plants belong to several morphological groups which are genetically distinct (9) and include small isometric-, large isometric-, and prolate-headed phages (7, 11, 15, 26). Therefore, it was important to determine whether pTR2030-induced phage resistance was directed against different lactic streptococcal phages and, further, against many phages within any single morphological group. The objective of this investigation was to define and characterize a series of phage-host relationships where the effect of pTR2030-induced phage resistance could be evaluated within different *S. cremoris* and *S. lactis* strains challenged with phage from several morphological groups. Characterization of phages used in previous studies (21, 23) and propagation of different phages on a series of *S. lactis* and *S. cremoris* hosts demonstrated that plasmid pTR2030 conferred resistance to all small isometric-headed phages studied. In contrast, the large isometric- and prolate-headed phages examined were either not inhibited or exhibited a reduction in plaque size without affecting the plaquing efficiency of the phage.

MATERIALS AND METHODS

Bacterial strains and phages. Sources and characteristics of bacterial strains used in genetic experiments are listed in Table 1. Phages and their propagating strains are listed in Table 2.

Phage methods. Preparation of phage stocks, concentration and purification of phages, and extraction of phage DNA were carried out as described earlier (9). Sensitivities of

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TABLE 1. Bacterial strains and plasmids

Strain	Phenotype[a]	Appropriate plasmids	Description	Source or reference
S. lactis T-EK1	Lac⁺ Hsp⁺ Str^s	pTR1040, pTR2030	Conjugal donor	(21)
S. lactis T-RS1a	Lac⁻ Hsp⁺ Str^r	pTR2030	Source of pTR2030	(13)
S. lactis LM0230	Lac⁻ Hsp⁻ Str^r	Plasmid cured	Conjugal recipient	(18)
S. lactis L2FA	Lac⁻ Hsp⁻ Str^r	Multiple	Conjugal recipient	This study
S. lactis T-L2F1	Lac⁺ Hsp⁺ Str^r	pTR2030	Transconjugant	This study
S. lactis T-L2F2	Lac⁺ Hsp⁺ Str^r	pTR2030	Transconjugant	This study
S. lactis T-L2F6	Lac⁺ Hsp⁻ Str^r	pTR1040	Transconjugant	This study
S. cremoris KHA2	Lac⁻ Hsp⁻ Str^r	Multiple	Conjugal recipient	(21)
S. cremoris T-KH1	Lac⁺ Hsp⁺ Str^r	pTR1040, pTR2030	Transconjugant	(21)
S. cremoris HPA4	Lac⁻ Hsp⁻ Str^r	Multiple	Conjugal recipient	(21)
S. cremoris T-HP14	Lac⁺ Hsp⁺ Str^r	pTR1040, pTR2030	Transconjugant	(21)
S. cremoris M43a	Lac⁻ Hsp⁻ Str^r	Multiple	Conjugal recipient	(23)
S. cremoris T2r-M43a	Lac⁺ Hsp⁺ Str^r	pTR1040, pTR2030	Transconjugant	(23)

[a] Lac⁺, Lactose fermenting; Lac⁻, lactose negative; Str^r, resistant to streptomycin; Str^s, susceptible to streptomycin; Hsp⁺, heat-sensitive phage resistance; Hsp⁻, phage susceptible.

bacterial strains were determined by phage titration and spotting phage lysates onto bacterial lawns in M17 agar (24).

Electron microscopy. Phages (10^{11} to 10^{12} PFU/ml) purified in cesium chloride gradients were absorbed to carbon- and collodion-coated copper grids, washed twice successively in buffer (10 mM Tris hydrochloride [pH 7.0], 10 mM NaN_3, 1 mM $MgCl_2$), and stained with 2% uranyl acetate (Michel Wurtz, personal communication). Grids were photographed in a JEOL 100S electron microscope at 50,000× magnification.

Plasmid analysis. Plasmid extraction and detection on agarose gels were carried out by the method of Anderson and McKay (1) as modified by Steenson and Klaenhammer (23).

Gel electrophoresis. Undigested plasmid DNA, and HindIII digests of phage and plasmid DNA were fractionated on a horizontal gel apparatus in 0.65% agarose in Tris-acetate buffer (40 mM Tris, 20 mM sodium acetate, 2 mM EDTA, pH 7.8) at 6 V cm⁻¹ for 2.5 h. HindIII digests of bacteriophage lambda or preparations of *Escherichia coli* V517 plasmids (16) were used to provide molecular weight

TABLE 2. Phages and propagating strains

Phage	Morphology[a]	Source or reference[b]	Propagating strains				
			S. cremoris			*S. lactis*	
			KH	HP	M12	L2F	C2
kh	SI	NCSU (21)	+				
1405	SI	NZDRI	+				
mcb	SI	ML	+				
mkh	SI	ML	+				
m29	SI	ML	+				
hp	SI	NCSU		+			
1367	SI	NZDRI		+			
sg1	SI	ML (23)			+		
da	SI	ML (23)			+		
31	SI	ML				+	
35	SI	ML				+	
36	SI	ML				+	
949	LI	NZDRI (9)				+	
923	P	NZDRI (9)	+				
c2	P	NCSU (19)	+				+
643	P	NZDRI					+

[a] SI, Small isometric; LI, large isometric; P, prolate.
[b] NCSU, North Carolina State University; NZDRI, New Zealand Dairy Research Institute; ML, Miles Laboratories, Inc.

standards for linear and covalently closed circular DNA. Gels were stained with 4 μg of ethidium bromide per ml and photographed under UV light.

Preparation of [^{32}P]DNA. Phage and plasmid DNA were labeled with [^{32}P]CTP by a modification of the random primer method (9). Labeled probes of pTR2030 were prepared from *S. lactis* T-RS1a plasmid DNA which had been purified once through cesium chloride-ethidium bromide density gradients.

DNA-DNA hybridization. The Southern blot technique (22) was used to transfer DNA from nitrocellulose filters of 0.45-μm pore size (BA 85; Schleicher & Schuell, West Germany). The protocols for hybridizations and autoradiography were described earlier (9), except hybridizations were carried out at 65°C.

Conjugative experiments. Lac⁻ streptomycin-resistant strains to be used as conjugal recipients were selected as described by Steenson and Klaenhammer (23). Conjugal matings were performed on the surface of glucose-milk-agar plates, and transconjugants were scored by selection of Lac⁺ colonies on lactose indicator agar containing 1,000 μg of streptomycin per ml as described by McKay et al. (18). Lac⁺ transconjugants were purified by replating on the same medium and tested for phage sensitivity (13).

RESULTS

Phage host range. Phages used in this and earlier studies were examined by electron microscopy, and host range data were determined. Table 2 shows the reactions of small isometric-, large isometric-, and prolate-headed phages on *S. cremoris* HP, KH, and M12R and *S. lactis* C2 and L2F. Phages representative of the three morphological types are shown in Fig. 1. None of the strains tested was attacked by phages of all three types, but *S. cremoris* KH propagated small isometric- and prolate-headed phages, and *S. lactis* L2F propagated small isometric- and large isometric-headed phages. In addition *S. cremoris* HP, KH, M43a and *S. lactis* C2 and L2F were attacked by phages isolated from United States and New Zealand cheese plants.

Conjugal transfer of pTR2030. Phage-resistant transconjugants T-KH1 and T-HP14 obtained by mating *S. lactis* T-EK1 with *S. cremoris* KHA2 and HPA4 were available (21). To confirm that these transconjugants contained pTR2030, plasmids from recipient strains *S. cremoris* KHA2 and HPA4, transconjugants T-KH1 and T-HPA4, and the conjugal donor (T-EK1) were separated on agarose gels (Fig.

2a). The DNA was transferred by Southern blotting to a nitrocellulose filter. Hybridization of the filter with ^{32}P-labeled pTR2030 DNA demonstrated the presence of pTR2030 in transconjugant T-KH1 (Fig. 2b, lane C), where there was no corresponding homology in the recipient strain KHA2 (lane B). Recipient HPA4 (Fig. 2a, lane E) contained a resident plasmid of 30 megadaltons, which masked the presence of pTR2030 in transconjugant T-HP14 (Fig. 2a, lane F). The presence of pTR2030 in T-HP14 was demonstrated in the autoradiogram (Fig. 2b, lane F) as a strongly labeled plasmid, whereas the HPA4 resident 30-megadalton plasmid

FIG. 2. (a) Agarose gel electrophoresis of plasmids from recipient strains S. cremoris KHA2 and HPA4 and their Lac$^+$ Hsp$^+$ transconjugants. (b) Autoradiogram prepared after hybridization with ^{32}P-labeled pTR2030 prepared from S. lactis T-RS1a. Lanes: A, T-EK1 (Lac$^+$ Hsp$^+$); B, KHA2 (Lac$^-$ Hsp$^-$); C, T-KH1 (Lac$^+$ Hsp$^+$); D, T-RS1a(pTR2030); E, HPA4 (Lac$^-$ Hsp$^-$); F, T-HP14 (Lac$^+$ Hsp$^+$); lane G, T-EK1. The positions of pTR1040 and pTR2030 are indicated.

showed substantially less hybridization with the pTR2030 probe (Fig. 2b, lane E).

Conjugation experiments were carried out between S. lactis T-EK1 and S. lactis L2FA to introduce pTR2030 into a host where the resistance or sensitivity to large isometric- and small isometric-headed phages could be evaluated (Table 1). Lactose-fermenting ability was transferred to S. lactis L2FA at a frequency of 2×10^{-6} per donor cell. Of 38 Lac$^+$ transconjugants tested, 31 (82%) were resistant to phage 31, a small isometric-headed phage isolated originally on S. lactis L2F. Plasmid analysis of the recipient strain L2FA and phage-resistant (T-L2F1 and T-L2F2) and phage-susceptible (T-L2F6) transconjugants showed a 30-megadalton plasmid in all four strains (Fig. 3a, lanes C through F). ^{32}P-labeled pTR2030 showed strong homology with the phage-resistant transconjugants T-L2F1 and T-L2F2 at a position corresponding to 30 megadaltons (Fig. 3b, lanes D and E). On the other hand, the recipient strain L2FA and the phage-susceptible transconjugant TL2F6 contained plasmids of similar size, but these showed only weak homology with pTR2030 (Fig. 3b, lanes C and F). These data provided evidence for the acquisition of pTR2030 in the phage-resistant transconjugants of S. lactis L2FA. Transconjugants
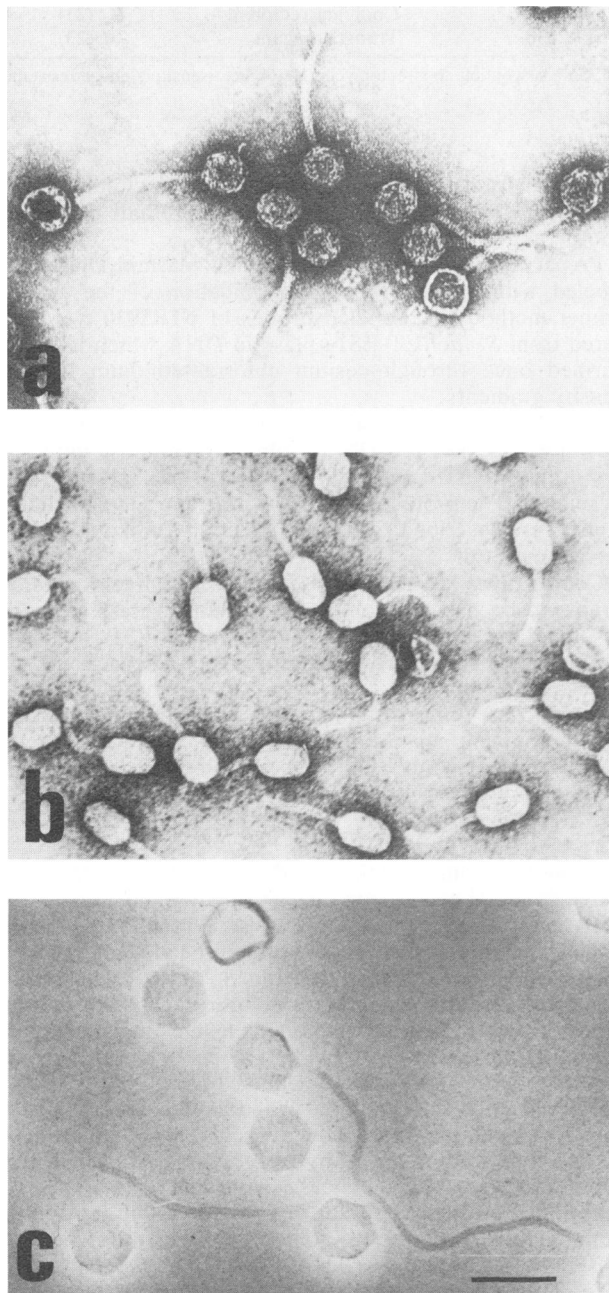

FIG. 1. Morphology of phage types: (a) small isometric-headed phage 1405; (b) prolate-headed phage 923; (c) large isometric-headed phage 949. Bar, 100 nm.
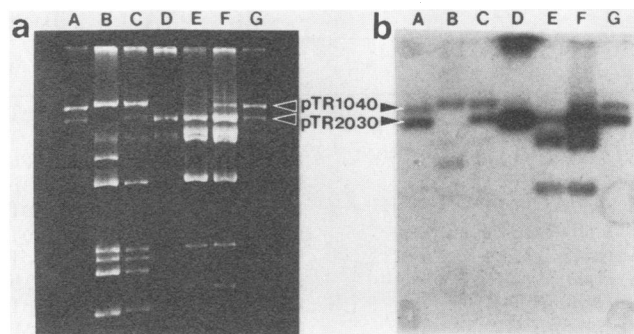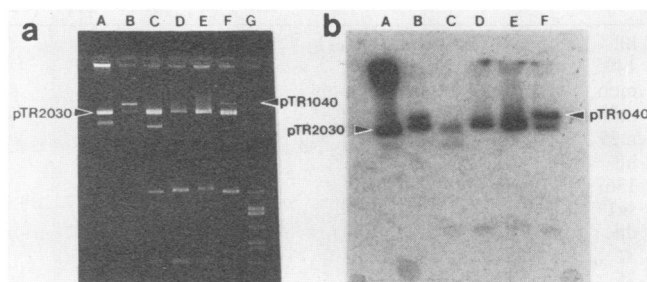

FIG. 3. (a) Agarose gel electrophoresis of plasmids from recipient strain S. lactis L2FA and its Lac$^+$ Hsp$^+$ and Lac$^+$ Hsp$^-$ transconjugants. (b) Autoradiogram prepared after hybridization with ^{32}P-labeled pTR2030. Lanes: A, T-RS1a(pTR2030); B, T-EK1 (Lac$^+$ Hsp$^+$); C, L2FA (Lac$^-$ Hsp$^-$); D, T-L2F1 (Lac$^+$ Hsp$^+$); E, T-L2F2 (Lac$^+$ Hsp$^+$); F, T-L2F6 (Lac$^+$ Hsp$^-$); G, E. coli V517 plasmids (molecular masses of 35, 5.1, 3.5, 3.0, 2.2, 1.7, 1.5, 1.2 megadaltons). The positions of pTR1040 and pTR2030 are indicated.

TABLE 3. Phage reactions of *S. cremoris* and *S. lactis* recipients and pTR2030 transconjugants

Phage[a]	Phage morphology[b]	Recipients			Transconjugants		
		Strain	Titer (PFU/ml)	Plaque size (mm)	Strain	Titer (PFU/ml)	Plaque size (mm)
kh	SI	*S. cremoris* KHA2	3.6×10^{10}	1.0–2.0	*S. cremoris* T-KH1	<10	
1405	SI		5.1×10^{10}	1.0–2.0		<10	
mcb	SI		5.0×10^{9}	1.5–2.0		<10	
mkh	SI		3.0×10^{8}	1.0–1.5		<10	
m29	SI		6.0×10^{9}	1.5–2.0		<10	
923	P		5.0×10^{10}	2.5–3.0		5.1×10^{10}	2.5–3.0
c2	P		4.4×10^{5}	0.2–0.6		<10	
31	SI	*S. lactis* L2FA	1.0×10^{10}	1.0–1.5	*S. lactis* T-L2F1	<10	
35	SI		2.6×10^{9}	0.5–1.0		<10	
36	SI		1.8×10^{9}	1.0–2.0		<10	
949	LI		5.0×10^{10}	0.4–0.5		9.0×10^{9}	0.1–0.2
hp	SI	*S. cremoris* HPA4	3.4×10^{11}	2.5–3.0	*S. cremoris* T-HP14	<10	
1367	SI		3.9×10^{10}	2.5–3.0		<10	
sg1	SI	*S. cremoris* M43a	3.2×10^{10}	1.0–2.0	*S. cremoris* T2r-M43a	<10	
da	SI		4.6×10^{9}	1.0–2.0		<10	
c2	P	*S. lactis* LM0230	1.7×10^{10}	2.5–3.0	*S. lactis* T-EK1	1.5×10^{10}	1.0–2.0
643	P		9.4×10^{9}	2.5–3.0		5.0×10^{9}	1.0–2.0

[a] All phages were propagated on the recipient strain.
[b] See footnote a of Table 2.

from these matings were selected for lactose-fermenting ability and should harbor the Lac+ plasmid pTR1040. However, plasmid analysis of the L2FA Lac+ transconjugants (Fig. 3a, lanes D, E, and F) showed that although pTR1040 was visible in *S. lactis* T-L2F6, it was not evident in T-L2F1 or T-L2F2, despite repeated analysis. Hybridization of ^{32}P-labeled pTR1040 with plasmids from these strains showed significant homology with plasmids present in *S. lactis* L2F (Lac+ parent stain of the recipient) and in the Lac+ Hsp− transconjugant T-L2F6, but not in the Lac+ Hsp+ transconjugant T-L2F1 or Lac− recipient strain L2FA (data not shown). The data suggested that the Lac+ plasmid pTR1040 may have been integrated into the chromosome in *S. cremoris* T-L2F1 and T-L2F2.

Phage resistance in transconjugants. Comparisons of phage titers on recipients and transconjugants demonstrated that pTR2030 conferred resistance against all 15 small isometric phages tested (Table 3). These phages were isolated from commercial cheese plants in the United States and New Zealand. In contrast, the replication of the prolate phage 923 was unaffected by the presence of pTR2030 in *S. cremoris* T-KH1. No inhibition occurred under conditions where pTR2030-induced resistance was operational, since all four small isometric-headed phages that were active against *S. cremoris* KHA2 were completely inhibited in the T-KH1 transconjugant. The two other prolate-headed phages (c2 and 643) used in this study showed no reduction in the efficiency of plaquing on *S. lactis* T-EK1, but exhibited reductions in plaque size (Table 3). Phage c2 also propagated on *S. cremoris* KHA2, but phage action was not detected on the *S. cremoris* T-KH1 transconjugant. Phage c2 was subject to considerable restriction on *S. cremoris* KHA2 even after propagation through *S. cremoris* KH; plaque diameter was 0.2 to 0.6 mm, and lysates contained only 4×10^{5} PFU/ml (Table 3). Therefore, although the prolate phage was completely inhibited in the *S. cremoris* T-KH1 transconjugant, the poor replication of c2 phage in this host may have contributed to the level of phage resistance observed.

The large isometric-headed phage 949 propagated on *S. lactis* L2F was only slightly inhibited in transconjugant *S. lactis* T-L2F1 harboring pTR2030. There was no significant reduction in plaquing efficiency (Table 3), although plaque size was reduced from 0.4 to 0.5 mm to 0.1 to 0.2 mm. In contrast, three small isometric-headed phages, 31, 35, and 36, were completely inhibited in the pTR2030 transconjugant *S. lactis* T-L2F1.

Hybridizations between plasmid and phage DNA. It was considered possible that pTR2030 contained phage genes that conferred immunity to superinfecting phages. To test this possibility, DNA from the phages used in this study and pTR2030 were digested with *Hae*III, fractionated on agarose gels, transferred to nitrocellulose filters, and hybridized with ^{32}P-labeled probes of pTR2030. Under these conditions, homology was not detected between phage and plasmid DNA (data not shown), indicating that pTR2030 did not contain DNA sequences homologous to DNA isolated from phages inhibited by pTR2030.

DISCUSSION

Plasmids in lactic streptococci have been shown to encode for a variety of phage resistance mechanisms (2, 3, 17, 19, 20). Klaenhammer and Sanozky (13) conjugally transferred plasmid pTR2030 from *S. lactis* ME2 to *S. lactis* LM0230 and conferred a heat-sensitive phage resistance mechanism (Hsp+) which reduced the burst size and plaque size for phage c2. Conjugal transfer of pTR2030 to strains of *S. cremoris* (21, 23) resulted in complete inhibition of phages which grew on the recipient strains. Clearly, these findings may have significant implications for the dairy industry in the development of phage-resistant starter strains. The long-term usefulness of such a finding will be related to whether the phage resistance mechanism is effective and generalizes against a variety of phages.

In the present study, pTR2030-encoded phage resistance was effective against all small isometric-headed phages studied which attacked different strains of *S. lactis* and *S.*

cremoris. Although the investigation included phages isolated from cheese plants in both New Zealand and the United States, none of the small isometric-headed phages was able to proliferate in strains which harbored pTR2030. Small isometric-headed phages have constituted the most common morphological group isolated from cheese plants in New Zealand, comprising 80 to 85% of all phages detected (7). These observations emphasize the potential usefulness of pTR2030 to the cultured dairy product industries. It further suggests that since the majority of strains of lactic streptococci are attacked by small isometric-headed phages, genetic determinants on pTR2030 that are responsible for phage resistance are not widely dispersed or are repressed in most dairy starter strains. Despite emphasis here on small isometric-headed phages, it must be considered that although prolate-headed phages are less frequently found in cheese plants, they can exhibit a wider host range than do isometric-headed phages (4). Consequently, their significance to the dairy industry cannot be underestimated.

In this regard, the three prolate-headed phages examined in this study showed responses on pTR2030 transconjugants that were variable and remarkably different from those of small isometric phages. This difference was underscored in *S. cremoris* T-KH1; the prolate-headed phage 923 was unrestricted, whereas small isometric-headed phages were completely inhibited. A second prolate-headed phage, c2, showed no plaquing ability on T-KH1, but it is unclear whether this effect was related to poor phage growth and low titers of c2 phage when propagated on *S. cremoris* KHA2. Phage c2 on *S. lactis* T-EK1 or T-RS1 (13) showed a reduction in plaque size without restriction of the plaquing efficiency of the phages. A similar response was observed for the single large isometric-headed phage used in this study. When phage 949 was plaqued on *S. lactis* T-L2F1, only a reduction in plaque size was observed. On the same pTR2030 transconjugant, small isometric-headed phages were unable to form plaques. The small number of prolate- and large isometric-headed phages used in this study weaken generalizations that might be made concerning these two phage groups. However, when compared in host backgrounds where the transconjugants showed no susceptibility to small isometric-headed phages, the prolate- and large isometric-headed phages were relatively unaffected by pTR2030-induced phage resistance.

Previously, it was reported that pTR2030 in *S. lactis* LM0230 resulted in a reduction in burst size and plaque size for phage c2 (13), whereas when pTR2030 was introduced into *S. cremoris* M43a complete inhibition of six virulent phages was observed (23). In this study, characterization of c2 phage and the phages attacking *S. cremoris* M43a revealed a prolate-headed phage and small isometric-headed phages respectively. Therefore, it appears that variations in phenotypic responses in pTR2030 transconjugants resulted primarily from differences between phages rather than differences between the host strains.

The finding that the phage resistance mechanism encoded by plasmid pTR2030 was effective against all of the small isometric-headed phages tested is important when consideration is given to current methods commonly used to isolate phage-resistant starter strains. Phage-resistant derivatives are generally obtained by growth of strains in the presence of large numbers of phages (5, 6). In commercial use, such phage-resistant strains may be attacked by other phages which appear as a result of mutations in the original phages. If a strain has acquired phage resistance to small isometric-headed phages, which is effective against all phages of that type, then it might be less susceptible to attack by new phages.

The correlation reported here between morphology and susceptibility to the phage resistance mechanism suggested that the response to the mechanism was characteristic of the phage rather than the host. The variation in reaction between phage types was in agreement with an earlier report (9) that lactic streptococcal phages of different morphology groups were genetically distinct as determined by DNA-DNA homology. However, even phages of similar morphology may have considerable regions of nonhomology in their DNAs (10), and one would not necessarily expect all phages of one morphological type to react in the same way. One possible interpretation of this apparent correlation between morphology and sensitivity to the resistance mechanism is that some common step of phage infection or development for small isometric phages may be affected.

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