

Resistance against Industrial Bacteriophages Conferred on Lactococci by Plasmid pAJ1106 and Related Plasmids

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Plasmid pAJ1106 and its deletion derivative, plasmid pAJ2074, conferred lactose-fermenting ability (Lac) and bacteriophage resistance (Hsp) at 30°C to Lac⁻ proteinase (Prt)-negative *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *lactis* var. *diacetylactis* recipient strains. An additional plasmid, pAJ331, isolated from the original source strain of pAJ1106, retained Hsp and conjugative ability without Lac. pAJ331 was conjugally transferred to two *L. lactis* subsp. *lactis* and one *L. lactis* subsp. *cremoris* starter strains. The transconjugants from such crosses acquired resistance to the phages which propagated on the parent recipient strains. Of 10 transconjugant strains carrying pAJ1106 or one of the related plasmids, 8 remained insensitive to phages through five activity test cycles in which cultures were exposed to a large number of industrial phages at incubation temperatures used in lactic casein manufacture. Three of ten strains remained phage insensitive through five cycles of a cheesemaking activity test in which cultures were exposed to approximately 80 different phages through cheesemaking temperatures. Three phages which propagated on transconjugant strains during cheesemaking activity tests were studied in detail. Two were similar (prolate) in morphology and by DNA homology to phages which were shown to be sensitive to the plasmid-encoded phage resistance mechanism. The third phage was a long-tailed, small isometric phage of a type rarely found in New Zealand cheese wheys. The phage resistance mechanism was partially inactivated in most strains at 37°C.

Resistance to bacteriophage continues to be one of the principal criteria for the selection of lactococcal strains for use in cheesemaking, casein manufacture, and other milk fermentations. In recent years, several plasmid-encoded phage resistance mechanisms with various degrees of effectiveness against different types of phages have been reported. Plasmid pNP40 (13) conferred resistance against prolate phage C2. Plasmid pTR2030 (12) encoded resistance against a number of small isometric phages but only reduced resistance against prolate and large isometric phages (8, 17, 18). *Lactococcus lactis* subsp. *cremoris* transconjugants containing plasmid pTR2030 were also reported (16) to be resistant to all phages isolated from the field on parental recipient strains, although the morphology of the phages was not determined. Plasmid pC1750 (1) also conferred resistance against a small isometric phage (phage 712) and partial resistance against prolate phages.

Plasmid pAJ1106 (7), recently isolated in this laboratory, encoded a phage resistance mechanism which was effective against all prolate and most small isometric phages tested but was ineffective against a small isometric phage belonging to a DNA homology group not previously found in New Zealand wheys. Plasmid pAJ1106 is a cointegrate plasmid conferring both lactose utilization (Lac) and phage resistance (Hsp) phenotypes (7). Lac⁺ transconjugants obtained by transfer of pAJ1106 or its deletion derivative, pAJ2074, into Lac⁻ proteinase (Prt)-negative recipients were low in proteinase activity, producing acid slowly in milk. Transfer of the phage resistance mechanism into actively proteolytic Lac⁺ starter strains required a Lac⁻ donor to facilitate selection of transconjugants. As part of this investigation, a plasmid (pAJ331), which contained phage resistance genes and conjugative ability but not Lac genes, was isolated from the original source strain of plasmid pAJ1106. Transconjugants could then be selected as Lac⁺ phage-insensitive cells

in a Lac⁻ donor background (16). It was also considered that a Lac⁻ plasmid would transfer more readily than a Lac⁺ plasmid into Lac⁺ starter strains, in which there could be some plasmid incompatibility.

Several of the reported phage resistance mechanisms showed at least some heat sensitivity, and the degree of sensitivity was apparently related to the recipient strain (7, 16). The present study evaluated the effectiveness of the phage resistance mechanism in plasmids pAJ1106 and related plasmids against a large number of phages isolated from commercial casein or cheese wheys under temperature conditions which simulated casein or cheese-manufacturing conditions. In addition, phages which overcame the resistance conferred by pAJ1106 and related plasmids were compared with phages which were inhibited by these plasmid-encoded resistance mechanisms.

MATERIALS AND METHODS

Bacterial strains and plasmids. The sources and characteristics of donor and recipient strains are listed in Table 1. Plasmids used in this study were pAJ1106 (106 kilobases [kb]) and pAJ2074 (74 kb), both of which were conjugative and Lac⁺ and carried phage resistance genes, and pAJ331 (60 kb), which was conjugative, carried phage resistance genes, and was Lac⁻. Lac⁻ derivatives of strains which were originally Lac⁺ are indicated by the letter A; e.g., D6A is a Lac⁻ derivative of D6. Conjugal donors were strains 331 (pAJ331), T.4854 (pAJ1106), and T.4854 (pAJ2074).

Phages and phage methods. *Streptococcus salivarius* subsp. *thermophilus* phage ST455 was kindly supplied by M. Teuber. Other phages were from the New Zealand Dairy Research Institute collection or were isolated during the course of this study. Phage methodology has been reported previously (5). The morphology of phages was determined by electron microscopy as described earlier (9).

Plasmid analysis. Extraction of plasmids and gel electro-

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TABLE 1. Donor and recipient strains and plasmids

Strain	Phenotype ^a	Source or reference
<i>L. lactis</i> subsp. <i>lactis</i> var. <i>diacetylactis</i> 4942	Lac ⁺ Hsp ⁺	7
<i>L. lactis</i> subsp. <i>lactis</i> var. <i>diacetylactis</i> 4942A	Lac ⁻ Hsp ⁺	This study
<i>L. lactis</i> subsp. <i>lactis</i> LM2302	Lac ⁻ Sm ^r Em ^r Hsp ⁻	13
<i>L. lactis</i> subsp. <i>lactis</i> 331(pAJ331) ^{b,c}	Lac ⁻ Sm ^r Em ^r Hsp ⁺	This study
<i>L. lactis</i> subsp. <i>cremoris</i> T.4854(pAJ1106) ^c	Lac ⁺ Fu ^r Hsp ⁺	7
<i>L. lactis</i> subsp. <i>cremoris</i> T.4854(pAJ2074) ^c	Lac ⁺ Fu ^r Hsp ⁺	7
<i>L. lactis</i> subsp. <i>lactis</i> H1	Lac ⁺ Hsp ⁻	NZDRI ^d
<i>L. lactis</i> subsp. <i>lactis</i> ML3	Lac ⁺ Hsp ⁻	NZDRI
<i>L. lactis</i> subsp. <i>lactis</i> var. <i>diacetylactis</i> D6A	Lac ⁻ Hsp ⁻	This study
<i>L. lactis</i> subsp. <i>lactis</i> var. <i>diacetylactis</i> D7A	Lac ⁻ Hsp ⁻	This study
<i>L. lactis</i> subsp. <i>lactis</i> var. <i>diacetylactis</i> D10A	Lac ⁻ Hsp ⁻	This study
<i>L. lactis</i> subsp. <i>lactis</i> var. <i>diacetylactis</i> DRC2A	Lac ⁻ Hsp ⁻	This study
<i>L. lactis</i> subsp. <i>cremoris</i> 484	Lac ⁺ Hsp ⁻	NZDRI
<i>L. lactis</i> subsp. <i>lactis</i> 4262	Lac ⁻ Hsp ⁻	ML3 derivative
<i>L. lactis</i> subsp. <i>lactis</i> 4862	Lac ⁻ Hsp ⁻	H1 derivative

^a Lac, Lactose utilization; Hsp, phage resistance; Sm^r, streptomycin resistance; Em^r, erythromycin resistance; Fu^r, fusidic acid resistance.

^b Derived at T.LM2302(pAJ331). Transconjugant strains are indicated by the letter T, which is followed by the number of the recipient strain and then the name of the plasmid (in parentheses).

^c Conjugal donor strains.

^d NZDRI, New Zealand Dairy Research Institute collection.

phoresis were carried out as described by Steenson and Klaenhammer (18).

DNA-DNA hybridization. Protocols for labeling of DNA with [³²P]CTP, hybridizations, and autoradiography were as described earlier (14).

Isolation of Lac⁻ recipient strains. Lac⁻ isolates of five *S. lactis* subsp. *lactis* strains, D6, D7, D10, D13, and DRC2, were identified by growth on lactose indicator agar after curing with novobiocin (7). These strains were shown by growth on modified KCA medium (20) to have retained citrate production; they were low in proteinase activity and were sensitive to the phages which propagated on the parent strains. Plasmid profiles (data not shown) showed the loss of a large plasmid, and in some instances there was a loss of smaller plasmids.

Conjugation. Donors and recipients were combined in a ratio of 1:2 to 1:5, and matings were performed on glucose-milk agar (14). Cells were harvested from mating plates in 2 ml of M17 broth. Transconjugants obtained by transfer of pAJ1106 or pAJ2074 from an *L. lactis* subsp. *cremoris* donor to Lac⁻ *L. lactis* subsp. *lactis* or *L. lactis* subsp. *lactis* var. *diacetylactis* strains were selected on lactose indicator agar (15) at 37°C. At this temperature, *L. lactis* subsp. *cremoris* donor cells did not grow.

Transconjugants containing pAJ331 were obtained by mating Lac⁻ donor *L. lactis* subsp. *lactis* 331(pAJ331) with phage-sensitive Lac⁺ *L. lactis* subsp. *lactis* or *L. lactis* subsp. *cremoris* strains, and they were selected by a method similar to that of Sanders et al. (16). Cells harvested from mating plates and from control plates containing recipient cells only were each diluted 10⁻¹ to 10⁻⁴. Cells (0.15 ml) were incubated for 10 min at room temperature with 0.15 ml of a phage lysate active against the recipient strain (approximately 10⁹ PFU/ml) and 50 µl of 1 M CaCl₂ and then were plated on lactose indicator agar. When a strain was known to propagate both prolate and small isometric phages, both types of phage were used together in the selection procedure. Control plates containing recipient cells with phage indicated the number of naturally occurring phage-resistant colonies. Lac⁺ colonies were purified by replating three times on lactose indicator agar, and the colonies were checked for phage sensitivity. Phage-insensitive isolates were examined for the presence of plasmid pAJ331 by plasmid analysis and Southern blot hybridization or by colony hybridization. Intended donors which had acquired Lac plasmids from the intended recipient strains were differentiated by plasmid analysis from recipient strains which had received plasmid pAJ331.

Cheese starter activity tests. The cheese starter activity test, described as the modified activity test (MAT) by Heap and Lawrence (4), was carried out as follows. Cultures were grown overnight at 22°C in autoclaved 9.5% reconstituted skim milk supplemented with 0.2% Trypticase for Prt⁻ cultures. Reconstituted skim milk (9.6-ml portions) with Trypticase, where specified, was inoculated (0.2% inoculum) in duplicate with the overnight cultures. Phage suspension (0.2 ml) was added to the duplicate tube, and both cultures were incubated through a 5-h cheesemaking temperature profile. The phage suspension consisted of lysates of 80 phages from the New Zealand Dairy Research Institute collection. The test was repeated for four more consecutive days, with whey from tube 2 on one day being added to tube 2 on the following day in each experiment. The pHs of control and phage-containing cultures were determined at the end of each incubation. Whey from the phage-containing tube of the last experiment (day 5) was spotted onto a lawn of the culture being tested and examined for the presence of phage after overnight incubation. A culture was considered to have passed a MAT test if there was no inhibition in acid production in the presence of phage and if no phage was detected from spotting of wheys.

Casein starter activity tests. A similar test was carried out to screen potential starters for use in casein manufacture. The phage mixture consisted of wheys collected from casein and cheese plants during the last three manufacturing seasons. In addition, 0.2 ml of phage lysate (10⁹ PFU/ml) which propagated on the parent recipient strain was added to the phage tube in each experiment. Incubation in this test was for 16 h at 26°C to simulate a bulk coagulation silo.

RESULTS

Isolation of phage resistance plasmid pAJ331. *L. lactis* subsp. *lactis* var. *diacetylactis* 4942 was cured of some of its plasmids with novobiocin (0.8 µg/ml), and Lac⁻ colonies were identified on lactose indicator agar. The majority of Lac⁻ isolates had lost the three largest plasmids, i.e., those of 60, 48, and 40 kb. Screening with phages showed that these isolates had become sensitive to the prolate phages 643 and C2. By testing 40 Lac⁻ 4942 isolates for sensitivity to

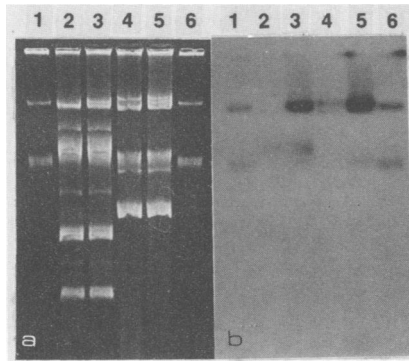


FIG. 1. (a) Plasmid profiles of recipient and transconjugant *L. lactis* strains. (b) Autoradiogram prepared after hybridization with ^{32}P -labeled pAJ331. Lanes 1, pAJ331; lanes 2, ML3; lanes 3, T.ML3(pAJ331); lanes 4, H1; lanes 5, T.H1(pAJ331); lanes 6, pAJ331.

phage 643, 1 phage-resistant isolate, 4942A, was detected. Plasmid analysis of this strain showed the presence of a 60-kb plasmid, which was similar in size to the largest plasmid in strain 4942. Strain 4942A was conjugally mated with *L. lactis* subsp. *lactis* LM2302, and transconjugants were selected by growth in the presence of phage C2 on lactose indicator agar containing erythromycin, which selected against the donor. Plasmid analysis and Southern blot hybridizations (not shown) demonstrated that transconjugant strains resistant to phage C2 contained a 60-kb plasmid which showed homology with plasmids pAJ1106 and pAJ2074. This was in agreement with earlier findings (7) that plasmid pAJ1106 is a cointegrate plasmid formed by recombination of the 60- and 48-kb plasmids of *L. lactis* subsp. *lactis* var. *diacetylactis* 4942. Plasmid pAJ2074 is a deletion plasmid of plasmid pAJ1106 (7). One of the transconjugant strains resistant to phage C2 was selected as *L. lactis* subsp. *lactis* 331, and the 60-kb plasmid was designated pAJ331.

***L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* transconjugants containing pAJ331.** Conjugal transfer of plasmid pAJ331 into 2 *L. lactis* subsp. *lactis* strains (H1 and ML3) and 10 *L. lactis* subsp. *cremoris* strains (AM1, 112, 164, 484, 584, 2002, 2046, 2150, 2162, and 2164) was attempted. Lac^+ colonies of donor origin and naturally occurring phage-resistant recipient cells were differentiated by plasmid analysis from the desired phage-resistant Lac^+ transconjugants in the recipient strain background. Transconjugants were obtained for both *L. lactis* subsp. *lactis* strains and only one *L. lactis* subsp. *cremoris* strain (strain 484) at the rate of approximately 1 of 20 phage-insensitive colonies from the mating mix, as indicated by plasmid profile and Southern blot hybridizations.

Figure 1a shows the plasmid profiles of recipient strains ML3 and H1 and of transconjugant strains T.ML3(pAJ331) and T.H1 (pAJ331). Hybridization with probe pAJ331 (Fig. 1b) confirmed the presence of this plasmid in the transconjugant strains. Transconjugant strains *L. lactis* subsp. *lactis* T.H1(pAJ331) and T.ML3(pAJ331) and *L. lactis* subsp. *cremoris* T.484(pAJ331) were all insensitive (efficiency of plating, $<10^{-8}$) to the phages which propagated on the parent recipient strains (Table 2).

***L. lactis* subsp. *lactis* and *L. lactis* subsp. *lactis* var. *diacetylactis* transconjugants containing plasmids pAJ1106 or pAJ2074.** Five *L. lactis* subsp. *lactis* var. *diacetylactis* strains, D6A, D7A, D10A, D13A, and DRC2A, were used as recipients in conjugal matings with *L. lactis* subsp. *cremoris*

TABLE 2. Phage reactions of recipient and transconjugant strains

Strain	Description	Phage ^a	Phage morphology	Reaction ^b
H ₁	Recipient	623, 923	Prolate	S
T.H ₁ (pAJ331)	Transconjugant	623, 923	Prolate	R
ML3	Recipient	643	Prolate	S
T.ML3(pAJ331)	Transconjugant	643	Prolate	R
484	Recipient	1039	Prolate	S
T.484(pAJ331)	Transconjugant	1039	Prolate	R
D6A	Recipient	1459	Small isometric	S
T.D6A(pAJ1106)	Transconjugant	1459	Small isometric	R
T.D6A(pAJ2074)	Transconjugant	1459	Small isometric	R
D7A	Recipient	1492	Small isometric	S
T.D7A(pAJ1106) ^c	Transconjugant	1492	Small isometric	S
T.D7A(pAJ2074)	Transconjugant	1492	Small isometric	R
D10A	Recipient	1784	Small isometric	S
T.D10A(pAJ1106)	Transconjugant	1784	Small isometric	R
T.D10A(pAJ2074)	Transconjugant	1784	Small isometric	R
DRC2A	Recipient	1768	Small isometric	S
T.DRC2(pAJ2074)	Transconjugant	1768	Small isometric	R
4262	Recipient	643	Prolate	S
T.4262(pAJ2074)	Transconjugant	643	Prolate	R
4862	Recipient	1039	Prolate	S
T.4862(pAJ1106)	Transconjugant	1039	Prolate	R

^a Phage lysates, 10^9 to 10^{10} PFU/ml.

^b R, Efficiency of plating, $<10^{-8}$; S, sensitive.

^c Lac genes apparently integrated into chromosome. No additional plasmid evident.

donor strains T.4854(pAJ1106) and T.4854(pAJ2074). Transconjugants were obtained with four of the five strains (Table 2). Plasmid analysis of Lac^- *L. lactis* subsp. *lactis* var. *diacetylactis* D6A and transconjugants T.D6A(pAJ1106) and T.D6A(pAJ2074) (Fig. 2a) and hybridization with pAJ331 (Fig. 2b) confirmed the transfer of pAJ1106 and pAJ2074 into strain D6A. Similar results (data not shown) were obtained for other transconjugant strains, except for T.D7A (pAJ1106). Plasmid analysis of this strain did not detect the presence of any additional plasmids, and it appeared that *lac* genes were integrated into the chromosome. T.D7A (pAJ1106) retained sensitivity to the phage which propagated on the parent strain D7.

Plasmid pAJ2074 was transferred conjugally also to *L. lactis* subsp. *lactis* 4262, and plasmid pAJ1106 was transferred to *L. lactis* subsp. *lactis* 4862. Transconjugant strains were insensitive (efficiency of plating, $<10^{-8}$) to the phages which propagated on the parent strains (Table 2). Plasmid analysis confirmed the presence of the plasmids in the transconjugant strains (data not shown).

Casein starter activity tests. Casein activity tests for *L. lactis* subsp. *lactis* var. *diacetylactis* D6 and the transconjugants T.D6A(pAJ2074) and T.D6A(pAJ1106) (Table 3) showed that acid production was inhibited when phage was added to the parent strain D6 but was unaltered for transconjugant strains T.D6A (2074) and T.D6A (1106). Casein

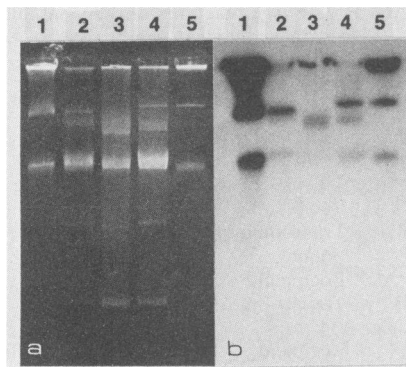


FIG. 2. (a) Plasmid profiles of *L. lactis* subsp. *lactis* var. *diacetylactis* recipient and transconjugant strains. (b) Autoradiogram prepared after hybridization with ^{32}P -labeled pAJ331. Lanes 1, pAJ2074; lanes 2, T.D6A(pAJ2074); lanes 3, D6A; lanes 4, T.D6A(pAJ1106); lanes 5, pAJ1106.

activity tests for these and eight other transconjugant strains are listed in Table 4, and the final pHs obtained after five cycles are shown. Transconjugants from *L. lactis* subsp. *lactis* var. *diacetylactis* D6, D7, and DRC2 and *L. lactis* subsp. *lactis* 4262, 4862, and ML3 remained phage insensitive, whereas H1 and D10 transconjugants failed the casein activity tests.

Cheese starter activity tests. The cheesemaking activity test (Table 4) was a more rigorous screening procedure than the casein activity test, with 3 of 10 transconjugants remaining phage insensitive. The casein and cheese starter activity tests differed in incubation time, temperature, and range of phages used. To determine whether one or all of these factors were responsible for the difference in the phage effect in these tests, a casein activity test was carried out on strains ML3, T.ML3(pAJ331), D6, and T.D6A(pAJ2074) by using MAT phages. Both recipient strains, D6 and ML3, were susceptible to MAT phages under casein activity test conditions. During the 5-day casein activity test, acid production by T.D6A(pAJ2074) was unaffected by the presence of MAT phages, whereas T.ML3(pAJ331) failed the activity test on cycle 5 (data not shown). Therefore, both the greater number of different phages and the higher temperatures were significant factors in the failure of strains to survive MAT tests.

TABLE 3. Casein activity tests on *S. lactis* subsp. *diacetylactis* D6 and transconjugant strains T.D6A (1106) and T.D6A (2074)

Strain	pH on day ^a :					Plate reaction ^b
	1	2	3	4	5	
D6						
With phage	4.55					
Without phage	5.83					+
T.D6A(pAJ1106)						
With phage	4.47	4.43	4.46	4.34	4.48	
Without phage	4.52	4.59	4.39	4.34	4.47	-
T.D6A(pAJ2074)						
With phage	4.50	4.46	4.38	4.57	4.36	
Without phage	4.50	4.40	4.40	4.62	4.42	-

^a pH after 18 h at 26°C in reconstituted skim milk, with Trypticase added to transconjugant cultures.

^b +, Positive phage reaction from spotting whey from previous test on lawn on cultures of strain being tested; -, no reaction.

TABLE 4. Cheese and casein activity tests on transconjugant strains

Strain	Casein activity test			Cheese activity test		
	Control pH ^a	Test pH ^a	Plate ^b reaction	Control pH	Test pH	Plate reaction
T.H1(pAJ331)	4.50	4.50	+ (4)	5.20	6.73	+ (2)
T.ML3(pAJ331)	4.25	4.34	-	4.97	5.95	+ (2)
T.484(pAJ331)	4.52	4.57	-	4.82 ^c	4.86	-
T.D6A(pAJ1106)	4.48	4.47	-	4.81 ^c	4.80	-
T.D6A(pAJ2074)	4.36	4.42	-	5.78	6.45	+ (4)
T.D7A(pAJ2074)	4.53	4.55	-	5.16	6.23	+ (2)
T.D10A(pAJ1106)	4.37	4.54	+ (3)	5.50	6.12	+ (2)
T.DRC2A(pAJ2074)	4.30	4.45	-	5.01	5.92	+ (3)
T.4262(pAJ2074)	4.42	4.46	-	4.69	6.13	+ (2)
T.4862(pAJ1106)	4.23	4.18	-	4.65 ^c	4.62	-

^a pH of final test in cultures without phage (control) and with phage (test).

^b Reaction when whey from previous cycle was spotted onto bacterial lawn. +, Phage reaction; -, no reaction. Numbers in parentheses indicate cycles in which test failed.

^c Cheese activity tests of strains which passed the initial tests were confirmed by repeating tests in triplicate.

To further examine the possible effect of the elevated MAT temperatures on transconjugant strains, all transconjugants were tested for their sensitivity at 37°C to the phages which propagated on the parent recipient strains as listed in Table 2. All transconjugants except T.D6A(pAJ1106), which passed the MAT test (Table 4), and T.DRC2A(pAJ2074) showed phage sensitivity at 37°C. Strain T.484(pAJ331) was not tested because *L. lactis* subsp. *cremoris* 484 did not grow at 37°C.

Phage 923, which grew on transconjugant strain T.H1(pAJ331) at 37°C, was plated again at 37°C and then replated to strain T.H1(pAJ331), and the plates were incubated at 30 and 37°C. Growth on the transconjugant strain at 37°C did not enable the phage to grow on the same strain at 30°C.

Characterization of MAT phages which grew on transconjugant strains. At the end of each activity test, wheys from the last tube containing culture plus phage were spotted onto lawn cultures of the strain being tested. Zones of lysis were replated to confirm the presence of phage. Phages growing on transconjugant strains T.H1(pAJ331), T.D10A(pAJ1106), and T.D7A(pAJ2074) were propagated and purified through CsCl gradients. Morphologically, the phages which grew on the three transconjugant strains were of two types (Table 5): prolate phages 296 and 335 and small isometric phage 293, which had a longer tail (280 nm) than the commonly isolated, small isometric phage (tail, 150 nm) commonly isolated in New Zealand. Phage 293 propagated on transconjugant strain T.D10(pAJ1106) but not on recipient strain D10A.

Gel electrophoresis of *EcoRI* digests of DNA from phages 296 (Fig. 3a, lane 6) and 335 (lane 7) showed some similarity. Fragment 1 of phage 296 DNA was equal in size to the sum of fragments 1 and 4 in phage 335 DNA, indicating that phage 296 DNA lacks one *EcoRI* cleavage site present in phage 335 DNA. Phages isolated on transconjugant strains were compared by DNA homology with phages representative of different DNA homology groups (5), including some phages which grew on the recipient strains. Phages 1496, 1483, and 1358 are small isometric phages belonging to different DNA homology groups. Phages C2, 1039, 296, and 335 are prolate phages belonging to one DNA homology group. Phages 293, ST455, and BK₅-T are long-tailed small isometric phages. ST455 is a *S. salivarius* subsp. *thermophilus* phage, and BK₅-T is a temperate phage isolated from *L. lactis* subsp.

TABLE 5. Characteristics of phages isolated on transconjugant strains during activity tests

Phage	Transconjugant propagating strain	Size (nm)		Titer		Plaque size (mm)	
		Head	Tail	Transconjugant	Recipient	Transconjugant	Recipient
335	T.H1(pAJ331)	55 by 44	103	3×10^8	5×10^8	1.0-2.0	2.5-4.0
293	T.D10A(pAJ1106)	59	280	4×10^{10}	$<10^1$	<0.5	ND ^a
296	T.D7A(pAJ2074)	56 by 45	101	6×10^8	1.4×10^9	1.5-2.0	2.5-3.0

^a ND, No plaques detected.

cremoris BK₅ and propagated on *L. lactis* subsp. *cremoris* H₂. Southern blot hybridizations with ³²P-labeled DNA from phages 296 (Fig. 3b) and 335 (not shown) demonstrated that both these phages belonged to the same DNA homology group as commonly found prolate phages, including C2 and 1039 (5), both of which were inhibited by the phage resistance mechanism in transconjugant strains containing pAJ1106 or its derivatives (Table 2) (7). In agreement with earlier findings (5), neither phage 296 nor phage 335 showed any homology with phages of different morphology, i.e., small isometric phages 1496, 1483 and 1358 or long-tailed small isometric phages 293, ST455, and BK₅-T.

Phage 293 also was compared by DNA homology with phages belonging to different morphological and DNA homology groups. Considerable homology was detected between phage 293 and phage BK₅-T, which is a temperate phage with morphology similar to that of 293. A low level of homology was detected with phage 1483 (data not shown), which differs from phage 293 in having a shorter tail. Phage 1483 shows no DNA homology with any other phages with which it has previously been tested (6). Phage 1483 also differed from other small isometric phages in being unaffected by the phage resistance mechanism in pAJ1106 (7).

The phages isolated on transconjugants T.H1(pAJ331) and T.D7A(pAJ2074) produced markedly smaller plaques on the transconjugant strains than on the corresponding recipient strains (Table 5). This difference was observed whether the phage was propagated on the recipient or the transconjugant strain first. At 37°C, plaques in all strains were slightly larger than at 30°C, but the phages still produced smaller plaques on transconjugant than on recipient strains.

Plasmid stability. Cultures of transconjugant strains T.D6A(pAJ1106) and T.D6A(pAJ2074) were plated before

and after subjection to casein and cheesemaking temperatures for five successive cycles. No Lac⁻ colonies were detected on lactose indicator agar plates containing approximately 200 colonies of either T.D6A(pAJ1106) or T.D6A(pAJ2074), indicating that there was no detectable loss of plasmid pAJ1106 or pAJ2074.

Transconjugant strain T.ML3(pAJ331) was similarly grown under casein and cheesemaking temperatures, replated to colony hybridization filters, and probed with pAJ331. Filters also contained colonies of *L. lactis* subsp. *lactis* ML3 strains (negative control) and 331(pAJ331) (positive control). All colonies of transconjugant strains tested (80 isolates at the beginning and end of each experiment) and of strain 331(pAJ331) showed strong homology with pAJ331 (Fig. 3), whereas strain ML3 showed no homology. There was therefore less than a 1 in 80 (1.25%) loss of plasmid pAJ331.

DISCUSSION

The identification of plasmid-encoded mechanisms which confer phage resistance in lactic streptococci has practical implications for the development of phage-insensitive starter strains. For such starters to be useful in the dairy industry, they need to be fast acid-producing strains which retain insensitivity to a wide variety of phages when incubated at temperatures encountered in manufacturing. Plasmid pAJ1106 and its derivatives conferred phage resistance at 30°C against all the phages which grew on the original parent strains used in this study. All the transconjugant strains also retained phage insensitivity through one cycle of activity tests which simulated casein or cheese manufacturing temperature profiles. However, in order to be considered for use

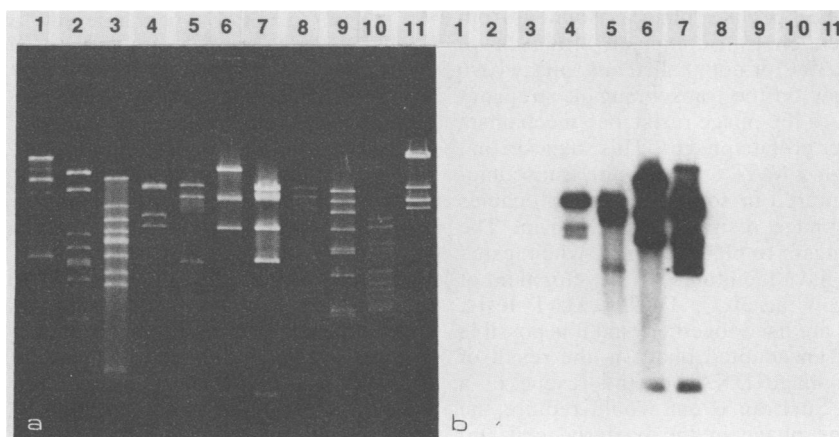


FIG. 3. (a) Restriction endonuclease digests of phage DNAs with *Eco*RI (lanes 1 to 7 and 11) or *Cfo*I (lanes 8 to 10). (b) Autoradiogram prepared after hybridization of DNAs with ³²P-labeled DNA from phage 296. Lanes 1, 1496; lanes 2, 1483; lanes 3, 1358; lanes 4, C2; lanes 5, 1039; lanes 6, 296; lanes 7, 335; lanes 8, 293; lanes 9, ST455; lanes 10, BK₅-T; lanes 11, lambda.

in the New Zealand starter system, strains were required to show no phage sensitivity through a 5-day activity test in the presence of a large number of phages isolated from cheese or casein plants. The majority (8 of 10) of transconjugant strains remained insensitive through a 5-day casein activity test, and 3 of 10 strains passed five cycles of the more stringent cheese activity test. All transconjugant strains were tested with a phage preparation containing a large number of different phages. The variation in results obtained suggested that the genetic background of the recipients was a major factor in determining the effectiveness of the plasmid in inhibiting phage.

Growth of strains in reconstituted skim milk would select against Lac⁻ cells and therefore exert a selective pressure in favor of transconjugants containing pAJ1106 or pAJ2074. Experiments with transconjugants containing pAJ331, in which there would not be a similar selective pressure, indicated that loss of plasmid was not the cause of phage sensitivity in activity tests. Six of the seven transconjugant strains which failed the MAT test allowed growth at 37°C but not at 30°C of the phages which propagated on the parent recipient strain. This indicates a need to test wheys on bacterial lawns at both temperatures at the conclusion of a MAT test. However, growth of a phage at 37°C on a transconjugant strain did not allow it to grow subsequently on the same strain at 30°C. This may indicate that the temperature-sensitive phage resistance mechanism was not a host-controlled modification-restriction system. Alternatively, if host-controlled restriction was inoperative at 37°C, it is possible that the phage would also not be modified at the higher temperature and therefore would be subject to restriction on subsequent propagation at 30°C. The plaque size seen in phage reactions on transconjugant strains, which was smaller than that of plaques on recipient strains, was not affected by the temperature of incubation or by growth of the phage on the transconjugant strain. This suggests that there may be two phage inhibitory mechanisms, of which one is temperature sensitive and causes a reduction in efficiency of plating, and one is not temperature sensitive and causes a reduction in plaque size, possibly as a result of reduced burst size (10).

Two of the phages (296 and 335) which were isolated from MAT tests on strains containing the phage resistance plasmids were similar in morphology and by DNA homology to commonly found prolate phages. However, these two phages were able to grow on the transconjugant strains at 30 and 37°C, whereas all the prolate phages isolated from factory wheys and tested on transconjugant strains have been able to grow only at 37°C or not at all. Thus, phages 296 and 335 were able to grow on the transconjugant strains at 30°C, at which temperature the phage resistance mechanism was effective against other prolate phages. This suggests that the phages grown through a MAT test with the transconjugant strains have been altered in some way which enables them to overcome the phage resistance mechanism. The sensitivity of a transconjugant to phage at 37°C, when tested on a bacterial lawn, may have facilitated the development of phages which could grow at 30°C. During MAT tests, approximately 80 phages are used together, and it is possible that the development of an adapted phage is the result of recombination between phage DNAs or the rescue of a lysogen by lytic phage. Such an event would require the growth of more than one phage in the bacterial cell and would therefore be more likely to occur when the strain under test was susceptible to phage at 37°C. Therefore, the use of a recipient strain which will yield a transconjugant

showing no phage sensitivity at 37°C when plated would be expected to increase the chances of producing a strain which will be useful as a phage-insensitive starter. As has been pointed out elsewhere (11), it is unlikely that starters with a single phage defense barrier will survive indefinitely in the dairy industry. A combination of plasmid-linked systems has been used (2, 3; T. R. Klaenhammer and R. B. Sanozky-Dawes, Program Abstr. 2nd Int. ASM Conf. Genet. Streptococci, abstr. no. 222, p. 25, 1986) to achieve a high level of phage resistance, and this may provide a strategy for the construction of long-lasting phage-insensitive starter strains.

Cointegrate plasmids pAJ1106 and the deletion plasmid pAJ2074 contain Lac genes but encoded only weak proteolytic activity when transferred into Lac⁻ recipients, producing strains which were low in proteinase activity. Propagation in combination with actively proteolytic strains resulted in more rapid growth of the transconjugant strains (data not given). *L. lactis* subsp. *lactis* var. *diacetyllactis* transconjugants low in proteinase activity could thus be useful in multiple casein starters without significant loss of activity (19).

For transconjugant strains to be of potential use as starters, it is necessary to select them without the use of antibiotic markers. In this study, this was achieved by the use of elevated temperature (37°C) for the selection of Lac⁺ transconjugants from matings of *L. lactis* subsp. *cremoris* donor cells with Lac⁻ *L. lactis* subsp. *lactis* or *L. lactis* subsp. *lactis* var. *diacetyllactis* recipients.

The isolation of plasmid pAJ331, which confers phage insensitivity but does not carry Lac genes, enabled us to use the strategy of Sanders et al. (16) for the construction of fast acid-producing strains, but this strategy was more successful for *L. lactis* subsp. *lactis* than for *L. lactis* subsp. *cremoris* strains. Work with other plasmids (11) has suggested that thermosensitivity of phage resistance mechanisms is less in *L. lactis* subsp. *cremoris* strains, and the finding that the only *L. lactis* subsp. *cremoris* transconjugant strain obtained in this study passed the cheese activity test agreed with this.

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