Abortive phage infection and restriction/modification activities directed by pTR2030 determinants are enhanced by recombination with conjugal elements in lactococci

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The recombinant plasmid pTK6 is composed of a 13.6 kb fragment from pTR2030 encoding phage resistance determinants for restriction/modification (R^+/M^+) and abortive phage infection (Hsp^+) cloned into shuttle vector pSA3 (erythromycin resistance, Em^r). Conjugal matings were performed to mobilize pTK6-encoded markers from *Lactococcus lactis* subsp. *lactis* MMS362 and MG1363. Em^r transconjugants were recovered at 10^{-6} per input donor and harboured pTK6 or recombinant plasmids not found in either parental strain. The recombinant plasmids (pTRK78 and pTRK79) encoded Em^r , Hsp^+ and R^+/M^+ , and transferred at high frequency in second-round matings. Mobilization of pTK6 from the otherwise plasmid-free donor, *L. lactis* MG1363, confirmed the presence of a conjugal element in this strain. Phage resistance in transconjugants containing pTRK78 and pTRK79 was markedly enhanced over pTK6-directed Hsp^+ and R^+/M^+ . In *L. lactis* LM2345 transconjugants, a reduction in plaque size was accompanied by a significant decrease in the efficiency of plaquing for phages c2 (10^{-2} to 10^{-6}) and p2 ($< 10^{-9}$). *L. lactis* NCK203 transconjugants containing pTRK78 or pTRK79 exhibited an additional 100–1000-fold reduction in the plaquing efficiency of $\phi 48$ (10^{-4} to 10^{-5}) over pTK6 imposed restriction (10^{-2}). Increased resistance to phage was a consequence of the physical interaction of pTR2030-derived sequences on pTK6 with a conjugal element resident in the donor strains.

Introduction

Bacteriophages have long presented a problem in the manufacture of fermented dairy products. A number of plasmid-associated phage resistance mechanisms have recently been reported within *Lactococcus*. These include inhibition of phage adsorption, restriction/modification activities, and a mechanism which causes abortive phage infection (Klaenhammer, 1987; Sanders, 1988). After mobilization of lactose-fermenting ability from a phage-insensitive starter strain, *Lactococcus lactis* subsp. *lactis* ME2, Klaenhammer & Sanozky (1985) isolated a self-transmissible plasmid, pTR2030, that conferred resistance to bacteriophage attack. The efficacy of pTR2030-directed phage resistance was demonstrated by its conjugal transfer to a variety of lactococci, where it inhibited phage proliferation (Jarvis & Klaenhammer, 1986; Sanders *et al.*, 1986; Sing & Klaenhammer, 1986;

Steenison & Klaenhammer, 1985). More significantly, strategies were developed to introduce pTR2030 by conjugation into industrial starter culture strains without affecting desired fermentation characteristics (Sing & Klaenhammer, 1986; Sanders *et al.*, 1986). These pTR2030-containing transconjugants have been successfully used in commercial starter systems, where they exhibit improved resistance to bacteriophage attack (Klaenhammer, 1987; Sanders, 1988).

pTR2030 is a 46.2 kb plasmid that encodes both abortive phage infection (Hsp) and restriction/modification (R/M) activity (Hill *et al.*, 1989a). The Hsp^+ phenotype is defined as a reduction in plaque size accompanied by a less than 10-fold decrease in the efficiency of plaquing (e.o.p.) (Klaenhammer & Sanozky, 1985), and can be further characterized by its action on different phage species (Jarvis & Klaenhammer, 1986). In standard plaque assays, reduced plaque size at a lowered e.o.p. is the phenotype seen with prolate-headed phage c2. In contrast, small isometric-headed phages fail to form visible plaques on pTR2030-containing strains. R^+/M^+ is defined within the *L. lactis*

Abbreviations: e.o.c., efficiency of infective centre formation; e.o.p., efficiency of plaquing; Hsp , heat-sensitive phage resistance; m.o.i., multiplicity of infection; R/M , restriction/modification.

Fig. 1. Circular restriction map of pTK6. The bold line denotes the 13.6 kb fragment from pTR2030; the open line denotes the pSA3 part of the plasmid. See text for explanation of abbreviations.

NCK203 background, where phage ϕ nck202.48 (ϕ 48), which is capable of circumventing Hsp, is restricted at a level of 10^{-3} (Hill *et al.*, 1989*a*). The locations of the *hsp* and *r/m* genetic determinants were identified on pTR2030 and subcloned onto the shuttle vector pSA3 (Hill *et al.*, 1989*a,b*). The resulting plasmid, designated pTK6, is 23.8 kb in size and contains a 13.6 kb fragment from pTR2030 encoding Hsp⁺ and R⁺/M⁺ (Fig. 1). Phage resistance conferred by pTK6 was less than pTR2030 in that the plaque size for phage c2 was not reduced to the same extent, plaque formation by small isometric-headed phages was not completely inhibited, and restriction of ϕ 48 was reduced 10-fold. The deletion in pTR2030 identifying the *hsp r/m* region had no effect on conjugal transfer functions (Hill *et al.*, 1989*b*), indicating that pTK6 is unlikely to be self-transmissible. The utility of conjugal strategies for the transfer of plasmid-encoded phage resistance within lactococci prompted us to examine the ability of indigenous conjugal elements to mobilize pTK6. This study describes the mobilization of pTK6 via a recombination process and the subsequent isolation of pTK6-derived cointegrate plasmids that exhibit phage resistance enhanced over the pTK6 and pTR2030 phenotypes. The results also suggested the presence of a transposable element residing within the cloned pTR2030 fragment and a potential role for recombination in the dissemination of phage resistance within the lactococci.

Methods

Bacterial strains, plasmids and phages. The *L. lactis* strains, plasmids and phages used in this study are listed in Table 1. *L. lactis* cultures were propagated at 30 °C in M17 broth (Terzaghi & Sandine, 1975) supplemented with 0.5% (w/v) glucose (M17G) or lactose (M17L). Unless otherwise noted, antibiotics were added to the following concentrations (in µg ml⁻¹): chloramphenicol (Cm), 5; erythromycin (Em), 10; rifampicin (Rif), 50; spectinomycin (Spc), 300; streptomycin (Str), 1000. *Escherichia coli* strains were propagated in LB broth (Silhavy *et al.*, 1984). *E. coli* HB101 containing pSA3 or pTK6 (Hill *et al.*, 1989b) was propagated in media containing Cm (20 µg ml⁻¹) or tetracycline (Tc; 10 µg ml⁻¹) and served as a source of DNA for transformation experiments.

Phage propagation and plaque assays. Standard plaque assays and preparation of phage lysates were as described by Terzaghi & Sandine (1975). All assays were performed at 30 °C. The number of infective centres was determined using an adaptation of the method described by Keogh (1973). Briefly, strains were propagated to an optical density (OD_{600}) of 0.7–0.9 at 30 °C. Phages were adsorbed to cells in 5 ml culture at a multiplicity of infection (m.o.i.) of 0.1 or less, in the presence of 50 mM-CaCl₂, and incubated for 15–20 min at room temperature. Cells were recovered by centrifugation at 4000 *g* for 10 min, washed twice in 25 ml 10% (v/v) M17G containing 50 mM-CaCl₂, and resuspended in 5 ml of the same solution. Appropriate dilutions were combined with a sensitive indicator strain and plated as in standard plaque assays. The efficiency of infective centre formation (e.o.c.) was calculated as the number of infective centres on the test strain divided by the number of infective centres on the sensitive indicator. The results of standard plaque assays and infective centre determinations were averaged from at least two independent trials.

R/M activity was detected using the phage-host pair $\phi 48$ /NCK203 since pTK6 and pTR2030 do not confer the ability to restrict phages c2 and p2 in the LM2301/LM2345 background (Hill *et al.*, 1989*a*). Derivatives of *L. lactis* NCK203 containing pTK6 or pTR2030 restrict the small isometric-headed phage $\phi 48$ at levels of 10^{-2} to 10^{-3} , respectively. Phage $\phi 48$ escapes Hsp interference; therefore, plaque size is not affected.

Plasmid isolation and characterization. Plasmid DNA was isolated as described by Anderson & McKay (1983). Plasmids from *E. coli* V517 and J53 were used as reference mobility standards. pSA3 and pTK6 DNA, used in transformation experiments, was isolated from *E. coli* as described by Rodriguez & Tait (1983).

Nucleic acid was transferred from agarose gels onto MSI Magnagraph nylon membranes (Micron Separations, Westboro, Massachusetts, USA) as described by Southern (1975). [³²P]dCTP-labelled probes were prepared using a Multiprime DNA Labelling Kit (Amersham). Radiolabelled probes were separated from unincorporated nucleotides using Sephadex G50 spin columns (Boehringer Mannheim Biochemicals). Hybridization reactions were performed using an Omni-blot apparatus (American Bionetics, Emeryville, California, USA) as described by Luchansky *et al.* (1989).

Conjugation. Solid-surface conjugal matings on M17 agar were performed as described by McKay *et al.* (1980). Frequencies of conjugal transfer were calculated as the number of transconjugants recovered per input donor cell. *L. lactis* MMS362 is recombination-deficient (Rec⁻) and contains the 48·2 kb self-transmissible plasmid pRS01 (Anderson & McKay, 1984b).

Transformation. *L. lactis* protoplast transformation was performed as described by von Wright *et al.* (1985). Antibiotic-resistant transformants were selected on M17 regeneration agar supplemented with Em ($5 \mu\text{g ml}^{-1}$).

Table 1. Bacterial strains, plasmids and phages

Strain, plasmid or phage	Relevant characteristics*	Description (reference)
<i>L. lactis</i>		
LM2301	<i>str-1</i> , plasmid-free	Conjugation host (Snook & McKay, 1981) derived from NCDO 2031 (Davies <i>et al.</i> , 1981)
LM2345	<i>spc-4 rif-5</i> , plasmid-free	Conjugation host (Anderson & McKay, 1984b)
MG1363	Plasmid-free	Conjugation host (Gasson, 1983) derived from NCDO 712 (Davies <i>et al.</i> , 1981)
MMS362	<i>str-14 Rec⁻</i> pRS01	Rec ⁻ conjugation host (Anderson & McKay, 1984b)
T-WS1	Hsp ⁺ pTR2030	LM0230/pTR2030 (Sing & Klaenhammer, 1986)
NCK203	<i>str-15</i>	Conjugation host (Hill <i>et al.</i> , 1989a)
NCK6	pTK6	MG1363/pTK6 (Hill <i>et al.</i> , 1989b)
NCK161	<i>str-1</i> , pTK6	LM2301/pTK6
NCK166	<i>str-14, Rec⁻</i> pRS01, pSA3	MMS362/pSA3
NCK167	<i>str-14, Rec⁻</i> pRS01, pTK6	MMS362/pTK6
NCK178	<i>spc-4 rif-5</i> , pTRK78	LM2345/pTRK78; transconjugant from NCK167 × LM2345 mating
NCK179	<i>spc-4 rif-5</i> , pTRK79	LM2345/pTRK79; transconjugant from NCK6 × LM2345 mating
NCK222	<i>str-15</i> , pTRK78	NCK203/pTRK78; transconjugant from NCK178 × NCK203 mating
NCK223	<i>str-15</i> , pTRK79	NCK203/pTRK79; transconjugant from NCK179 × NCK203 mating
<i>E. coli</i>		
V517	54.2, 7.3, 5.5, 5.2, 4.1, 3.0, 2.7, 2.1 kb	Source of plasmid size standards (Macrina <i>et al.</i> , 1978)
J53	34.5 kb	Source of plasmid size standard (Klaenhammer <i>et al.</i> , 1978)
Plasmids		
pSA3	Em ^r Cm ^r Tc ^r , 10.2 kb	Shuttle vector (Dao & Ferretti, 1985)
pTK6	Em ^r Hsp ⁺ R ⁺ /M ⁺ Cm ^r , 23.8 kb	pSA3 containing 13.6 kb pTR2030 fragment (Hill <i>et al.</i> , 1989b)
pTRK28	Em ^r Cm ^r Tc ^r , 11.0 kb	pSA3::iso-ISS1 (Romero & Klaenhammer, 1990)
pRS01	Tra ⁺ Clu [±] , 48.2 kb	Resident plasmid of MMS362 (Anderson & McKay, 1984)
pTRK78	Em ^r Hsp ⁺ R ⁺ /M ⁺ Clu ⁺ Tra ⁺	pTK6::pRS01 from NCK178
pTRK79	Em ^r Hsp ⁺ R ⁺ /M ⁺ Clu ⁺ Tra ⁺	pTK6::conjugal element MG1363 (from NCK179)
pTR2030	Hsp ⁺ R ⁺ /M ⁺ Tra ⁺ , 46.2 kb	(Klaenhammer & Sanozky, 1985)
Phages		
c2	Prolate	Homologous for <i>L. lactis</i> LM2301/LM2345/MG1363/MMS362/T-WS1
p2	Small isometric	Homologous for <i>L. lactis</i> LM2301/LM2345/MG1363/MMS362/T-WS1
φnck202.48	Small isometric	Homologous for <i>L. lactis</i> NC203

* Abbreviations: Cm, chloramphenicol; Em, erythromycin; Rif, rifampicin; Spc, spectinomycin; Str, streptomycin; Tc, tetracycline; Hsp, abortive phage infection; R/M, restriction/modification; Tra, conjugal proficiency; Rec, host recombination; Clu, cell clumping ability (aggregation); r, resistant; (+), positive; (-), negative.

Results

Conjugal mobilization of pTK6

To determine if pTK6 was self-transmissible, *L. lactis* NCK161 [LM2301 (pTK6)] and NCK6 [MG1363 (pTK6)], were used as conjugal donors of Em^r in matings with *L. lactis* LM2345. Em^r was not transferred from NCK161, indicating that pTK6 was not self-transmissible. Em^r transconjugants were recovered from NCK6 × LM2345 matings at a frequency of 5×10^{-6} . Transconjugants contained either pTK6 or a large plasmid (Fig. 2, lane F) not found in either of the parental strains. The latter transconjugants all displayed a clumping phenotype in broth and transferred Em^r at high efficiency (about 1) in second-round matings. This suggested a physical linkage between pTK6 and a conjugal element residing within MG1363. A representative transconjugant, designated NCK179, was selected for further study.

To examine pTK6 mobilization further, pSA3 and pTK6 were transformed into the Rec⁻ strain *L. lactis* MMS362, which contains the self-transmissible plasmid pRS01 (Anderson & McKay, 1984b). The transformants were used as conjugal donors of Em^r in matings with *L. lactis* LM2345. Transfer of Em^r from NCK167 [MMS362 (pTK6)] occurred at frequencies of 2×10^{-5} , whereas no Em^r transconjugants were recovered from NCK166 [MMS362 (pSA3)] donors. The inability of pRS01 to mobilize pSA3 indicated that the cloned 13.6 kb fragment was necessary for conjugal transfer of pTK6. Em^r transconjugants contained either pTK6 or a large plasmid suspected to be a pTK6::pRS01 cointegrate (Fig. 2, lane D). The latter exhibited either a clumping (Clu⁺) or non-clumping (Clu⁻) phenotype. In second-round matings, transconjugants containing the putative cointegrate transferred Em^r at frequencies of about 1 (Clu⁺) and 10^{-5} (Clu⁻). These results corresponded to transfer frequencies associated with the clumping phenotype encoded by pRS01 (Anderson & McKay, 1984b).

Fig. 2. Plasmid profiles (a), and corresponding autoradiogram (b) probed with ^{32}P -labelled pSA3, of *L. lactis* strains used in pTK6 mobilization experiments. Lane A, pTK6 (from *E. coli* HB101); lane B, MMS362; lane C, MMS362/pTK6; lane D, NCK178; lane E, MG1363/pTK6; lane F, NCK179; lane G, *E. coli* V517 (5.4, 7.3, 5.5, 5.2, 4.1, 3.0, 2.7 and 2.1 kb) and J53 (34.5 kb) plasmid standards. Additional bands seen in lanes A, C, and E represent open circular or linear forms of pTK6 generated upon isolation.

Table 2. Effects of pTR2030, pTK6 and pTK6-cointegrates (pTRK78 and pTRK79) on the e.o.p. and plaque size of phages c2 (prolate) and p2 (small isometric) attacking *L. lactis*

Strain	Plasmid	Phage c2*		Phage p2*	
		E.o.p.	Plaque size (mm)	E.o.p.	Plaque size (mm)
LM2345	None	1.0	2.0-2.5	1.0	1.0
NCK178	pTRK78	10^{-2}	<0.2†	< 10^{-9}	—
NCK179	pTRK79	10^{-6}	<0.2†	< 10^{-9}	—
T-WS1	pTR2030	0.57	0.5-1.0	< 10^{-9}	—
NCK161	pTK6	0.33	0.5-1.0	0.82	0.5

* Starting phage titre approximately 10^9 p.f.u. ml $^{-1}$.

† Mutant phage producing plaques of 1.0 mm diameter appeared at frequencies of 10^{-4} (NCK178) and 10^{-6} (NCK179).

and indicated a physical linkage between pTK6 and pRS01. A representative *Clu*⁺ transconjugant, designated NCK178, containing a putative pTK6::pRS01 cointegrate, was selected for further study.

Phage resistance phenotype of transconjugants NCK178 and NCK179

The phage resistance phenotypes of NCK178 and NCK179 were compared to those of isogenic strains containing pTK6, pTR2030, or no plasmid (sensitive indicator). Results of standard plaque assays using phages c2 (prolate-headed) and p2 (small isometric-headed) are shown in Table 2. Phage resistance in NCK178 and NCK179 was markedly greater than that of strains containing pTK6 or pTR2030. Inhibition of phage c2 was seen as a reduction in e.o.p. to 10^{-2} (NCK178) and 10^{-6} (NCK179) accompanied by a reduction in plaque size to less than 0.2 mm. At frequencies of 10^{-4} (NCK178) and 10^{-6} (NCK179),

plaques of 1 mm diameter began to appear. Plaque assays with phage purified from these large plaques indicated that they were mutant c2 phage capable of circumventing the inhibition imposed by pTRK78, pTRK79 and pTR2030. For phage p2, NCK178 (pTRK78) and NCK179 (pTRK79) completely inhibited plaque formation. This is in contrast to NCK161 (pTK6), where only a slight reduction in e.o.p. and a reduced plaque size for phage p2 were observed. Therefore, the level of phage resistance in NCK178 and NCK179 had returned to levels equal to or greater than that imposed by pTR2030 in T-WS1.

Linkage of *Emr* and phage resistance to pTRK78 and pTRK79

The plasmid profiles of NCK178, NCK179 and parental strains are shown in Fig. 2(a). The pTK6-recombinant plasmids, pTRK78 and pTRK79, are shown in lanes D and F. In rapid plasmid preparations of NCK178 and

Table 3. Effects of *pTR2030*, *pTK6* and *pTK6*-cointegrates (*pTRK78* and *pTRK79*) on the e.o.p. and e.o.c. of phages c2 (prolate) and p2 (small isometric)

Strain	Plasmid	Phage c2*		Phage p2*	
		E.o.p.	E.o.c.	E.o.p.	E.o.c.
LM2345	None	1.0	1.0	1.0	1.0
NCK178	<i>pTRK78</i>	10^{-2}	0.05	$<10^{-7}$	0.09
NCK179	<i>pTRK79</i>	$<10^{-6}$	0.01	$<10^{-7}$	0.08
T-WS1	<i>pTR2030</i>	0.28	0.21	$<10^{-7}$	0.07
NCK161	<i>pTK6</i>	0.50	0.40	0.49	0.22

* Starting phage titre approximately 10^6 – 10^7 p.f.u. ml⁻¹.

NCK179, only a single plasmid band was visible, suggesting the cointegrate to be the predominant plasmid form. Plasmid DNA was transferred to a nylon membrane and probed with ³²P-labelled pSA3 (Fig. 2*b*). Specific hybridization to *pTK6* (both covalently closed circular and linear forms) was seen in the control (lane A) and donors NCK167 (lane C) and NCK6 (lane E). The predominant signal in transconjugants NCK178 and NCK179 corresponded to the respective presumptive cointegrates indicated in Fig. 2(*a*) confirming the presence of pSA3 sequences in these plasmids. A hybridizing band, comigrating with *pTK6*, was also visible in NCK179 (Fig. 2*b*, lane F), suggesting the presence of free *pTK6*. No hybridization was observed to the plasmid complement of MMS362 (lane B) or to chromosomal DNA isolated from MG1363 (data not shown).

To establish plasmid linkage of Em^r Hsp⁺ Clu⁺ to *pTRK78* and *pTRK79*, NCK178 and NCK179 were subjected to consecutive propagation at 37 °C in the absence of selective (Em) pressure. After ten consecutive passages (about 60 generations), 1 of 50 colonies (2%) examined from NCK178 and 1 of 50 colonies examined from NCK179 were Em^s. The Em^s derivatives were also Hsp⁻ and Clu⁻. *pTRK78* and *pTRK79* were not detected in rapid plasmid DNA preparations of either of the Em^s Hsp⁻ Clu⁻ isolates, consistent with plasmid linkage of these phenotypes.

Phage resistance encoded by *pTRK78* and *pTRK79* is Hsp

The formation of infective centres was examined to ascertain the possible mechanism of phage resistance conferred by *pTRK78* and *pTRK79*. Table 3 shows a comparison between the efficiency of plaquing and the efficiency at which infective centres were formed in strains containing *pTR2030* or *pTK6*-derived plasmids.

For phage c2, NCK178 gave an e.o.c. value of 0.05, which corresponds to an e.o.p. of 10^{-2} . For NCK179, an e.o.c. of 0.01 indicated that release of viable c2 phage was reduced by 99% to 10^{-2} . In contrast, no visible plaques were observed in the standard plaque assays run in parallel (e.o.p. $<10^{-6}$). This suggested that, although 1% of the infected NCK179 cells produced viable c2 progeny, an insufficient number of phage were released to cause visible plaque formation in standard plaque assays. The e.o.c. levels for NCK178 and NCK179 with phage c2 were reduced approximately 10-fold compared with T-WS1 (*pTR2030*) and NCK161.

With phage p2, NCK178 and NCK179 gave e.o.c. values of 0.09 and 0.08, respectively. Despite the complete inhibition of plaque formation in standard plaque assays, less than a 100-fold decrease in e.o.c. was observed. As described above for phage c2, it is probable that an insufficient number of p2 phage progeny were released by successfully infected cells to produce a visible plaque in standard plaque assays. The e.o.c. levels for NCK178 and NCK179 were comparable to T-WS1 and about 40% lower than NCK161.

These data indicated that for phages c2 and p2, *pTRK78* and *pTRK79* resulted in a 91–99% reduction in the number of successful phage infections which released viable phage. These levels are typical of *pTK6*- and *pTR2030*-encoded Hsp reactions on phages c2 and p2.

Restriction/modification activity of *pTRK78* and *pTRK79*

To determine if the cointegrate plasmids possessed R/M activities, *pTRK78*- and *pTRK79*-encoded Em^r were conjugally transferred into *L. lactis* NCK203. In this host background, R/M activity can be evaluated independently of Hsp using phage ϕ 48 (Hill *et al.*, 1989*a*). Em^r transconjugants were recovered and a representative isolate from each mating was tested for phage resistance. The transconjugants, NCK222 (*pTRK78*) and NCK223

(pTRK179), restricted $\phi 48$ at e.o.p. values of 10^{-4} and 10^{-5} , respectively. These levels of restriction are 10–1000 times higher than those elicited by pTK6 (10^{-2}) and pTR2030 (10^{-3}). To confirm R/M activity, a cycle of host-dependent phage replication was performed. After growth through NCK222 (pTRK78) or NCK223 (pTRK79), phage from single plaque isolates were no longer restricted by hosts containing pTK6, pTRK78 or pTRK79, indicating that a similar R/M system was encoded by all three plasmids. Following propagation through NCK203, thereby removing any pTK6-, pTRK78- or pTRK79-encoded modification, $\phi 48$ was again restricted by hosts containing these plasmids, to the original degrees.

Phage resistance is encoded by pTK6

NCK167 and NCK6 did not exhibit resistance to phages c2 and p2 beyond that seen with NCK161 (data not shown). However, it remained possible that a resistance mechanism other than pTK6-encoded Hsp or R/M was recovered or activated in the conjugation process which formed pTRK78 and pTRK79. To determine if a resistance mechanism residing on the conjugal elements was activated during mobilization, MMS362 and MG1363 were transformed with pTRK28 (pSA3::iso-ISS1; Romero & Klaenhammer, 1990). The transformants were then used as conjugal donors of Em^r in matings with LM2345 to generate cointegrates with the conjugal element residing in MMS362 and MG1363. Em^r transconjugants were obtained at frequencies comparable to pTK6. Randomly selected isolates containing pTRK28 and pTRK28-derived cointegrates remained fully sensitive to phages c2 and p2 (data not shown). Therefore, a resistance mechanism linked to the conjugal elements was not identified during the mobilization process.

Discussion

In the course of establishing plasmid linkage for Hsp⁺ with cointegrates pTRK78 and pTRK79, we discovered that the transconjugants *L. lactis* NCK178 and NCK179 harbouring these plasmids exhibited an enhanced phage resistance phenotype when compared to the parental donors containing pTK6. NCK178 and NCK179 possessed the most pronounced phage inhibition among the transconjugants tested. Several lines of experimental evidence indicated that the recombinant plasmids were formed via cointegration between pTK6 and resident conjugal elements in the donor strains, and selected following conjugation. pTK6 was not self-transmissible. Conjugal mobilization from *L. lactis* MMS362 and

MG1363 resulted in transconjugants that harboured large recombinant plasmids, represented by pTRK78 and pTRK79, not found in either parental strain. pTRK78 and pTRK79 conferred Hsp⁺ and R⁺/M⁺ typical of pTK6 or pTR2030, albeit at increased levels. Through second-round conjugal matings, standard plaque and e.o.c. assays, plasmid curing and hybridization experiments, the recombinant plasmids were shown to encode traits (Em^r Hsp⁺ R⁺/M⁺ Clu⁺ Tra⁺) associated with both pTK6 and the conjugal elements.

The ability of pTK6 to be mobilized from the plasmid-free strain *L. lactis* MG1363 is consistent with the suspected presence of a conjugal element in this strain. Similar observations have been made with the transfer of a deleted lactose plasmid, pMG820, and various *in vitro* constructed derivatives from MG1363 (van der Lelie, 1989). The progenitor for MG1363, *L. lactis* 712, has been reported to contain a conjugal element responsible for the mobilization of plasmid-borne lactose metabolic genes (Gasson, 1983; Fitzgerald & Gasson, 1988). The conjugal element is conjectured to be an episome, which may account for its low copy number and inability to be visualized in plasmid preparations. Isolation of pTK6::pRS01 recombinants following pTK6 mobilization from the Rec⁻ donor *L. lactis* MMS362 is consistent with a transposition or site-specific mechanism for cointegration similar to the transfer of lactose plasmid pSK08 (Anderson & McKay, 1984b). The inability to mobilize pSA3 indicated that the cloned pTR2030 fragment was necessary for recombination. We have recently identified the presence of an insertion sequence isogenic to ISS1, (Polzin & Shimizu-Kadota, 1987), residing on the cloned 13.6 kb fragment of pTR2030 present in pTK6 (Romero & Klaenhammer, 1990). As is the case with pSK08 conjugal transfer (Anderson & McKay, 1984b), this insertion sequence probably accounts for the mobilization of pTK6 by pRS01 in MMS362.

Enhanced phage inhibition, relative to pTK6, was a result of a recombination event that was selected through conjugal mobilization. Initially, it could not be ruled out that enhanced resistance was due to the activation of a restriction system effective against phages c2 and p2. However, phage from single plaque isolates appearing on NCK178 and NCK179 were c2 phage mutants that did not follow host-dependent R/M patterns. Infective centre assays confirmed that phage inhibition was typical of the Hsp responses encoded by pTK6/pTR2030 (Table 3) and not due to an R/M system operating on phages c2 and p2. Furthermore, the possibility of a latent resistance mechanism associated with the conjugal elements and expressed following mobilization of pTK6 was excluded: pTRK28-cointegrates generated from *L. lactis* MMS362 and MG1363 did not confer phage

resistance. Due to the limited number of transconjugants that can be examined in this way, we cannot exclude the possibility that a phage resistance gene on the conjugal element was activated during the specific recombination events leading to the formation of pTRK78 and pTRK79; however, we believe this to be unlikely. Nevertheless, it is significant that expression of phage resistance derived from pTK6-cointegrates could be elevated to and beyond the original pTR2030 levels. In addition to effects on Hsp, R/M activities of pTRK78 and pTRK79, as detected in *L. lactis* NCK203 using phage ϕ 48, were also elevated over that of pTK6. This is consistent with the identification of an R/M system encoded on the 13.6 kb fragment of pTR2030 cloned on pTK6 which functions independently of Hsp (Hill *et al.*, 1989b).

Subcloning the genetic determinants from pTR2030 onto pSA3 resulted in lower levels of phage resistance. The return of 'full' phage resistance imposed by pTK6-cointegrates implies that the entire structural gene requirements for Hsp and R/M are present on pTK6. Although it is not clear why cointegration would cause elevated phage resistance, it is interesting to note that the physical structures of pTRK78 and pTRK79 resemble pTR2030, where the resistance determinants are physically linked to conjugative functions. Anderson & McKay (1984a) described a similar *in vivo* recombination event where the *lac* genes from pSK08 were fused to a higher copy number replicon in *L. lactis* ML3. This resulted in a twofold increase in *lac* gene dosage. However, the increase in the expression of phospho- β -D-galactosidase did not lead to an increased ability of cells harbouring the recombinant plasmid to produce lactic acid in milk. In *E. coli*, subcloning the *pif* phage resistance determinants from the F factor onto a pSC101 vector resulted in increased phage inhibition (Rotman *et al.*, 1983). The authors speculated that this elevation in resistance resulted from increased gene dosage due to the higher copy number of the cloning vector. Relative measurements have indicated that pTK6 was present in approximately 20–30 copies per cell, whereas pTR2030, pTRK78 and pTRK79 were estimated at about 1 copy per cell (unpublished results). This suggests that increased plasmid copy number does not play a role in the elevated phage resistance conferred by pTRK78 and pTRK79. Activation of Hsp and R/M did, however, require recombination since the presence of a conjugal element was not sufficient in itself to elevate phage resistance.

A variety of lactococcal plasmids encoding phage resistance that differ in size, types of resistances, and associated characteristics have been described (Klaenhammer, 1987; Sanders, 1988). The degree to which these plasmids interfere with phage development is

variable and ranges from partial to complete inhibition of plaquing ability. We have shown that genetic recombination with indigenous conjugal elements may result in enhanced levels of phage resistance affecting both abortive infection and R/M activities encoded on pTK6. This suggests that starter strains with improved resistance to bacteriophage attack may be constructed through *in vivo* genetic engineering of existing determinants. It is significant that elements that promote recombination, such as insertion sequences, are associated with traits such as phage resistance which are advantageous if they can be rapidly disseminated through the population. Furthermore, the R/M activity of pTR2030 may also play a role in promoting recombination events. Price & Bickle (1986) have conjectured that DNA cleavage caused by R/M systems can provide highly recombinogenic sites, thereby promoting genetic rearrangements. The ability to recombine, coupled with conjugative abilities, may therefore provide an efficient means to disseminate phage resistance in lactococci.

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