Conjugal Transfer from *Streptococcus lactis* ME2 of Plasmids Encoding Phage Resistance, Nisin Resistance and Lactose-fermenting Ability: Evidence for a High-frequency Conjugative Plasmid Responsible for Abortive Infection of Virulent Bacteriophage

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*Streptococcus lactis* ME2 exhibits at least three mechanisms which confer resistance to virulent bacteriophage. These include plasmid-induced interference with phage adsorption, host-controlled restriction and modification activities, and a heat-sensitive mechanism which suppresses development of virulent phage. Conjugal mating experiments were done with *S. lactis* ME2 to determine if phage-defence mechanisms present in this strain could be mobilized, associated with plasmid DNA elements and phenotypically characterized in transconjugants. Agar-surface matings of *S. lactis* ME2 with *S. lactis* LM0230 demonstrated that lactose-fermenting ability (Lac*) was transferred in a conjugation-like process at frequencies of $10^5$ per donor cell and was associated with a 40 MDal plasmid designated pTR1040. Resistance to nisin (Nis*) was acquired or lost simultaneously with Lac+, indicating that pTR1040 carried determinants for both phenotypes. Lac+ Nis transconjugants that carried a 30 MDal plasmid (pTR2030) exhibited a heat-sensitive phage-defence mechanism (Hsp+) which limited the burst size and plaque size of phage c2 without altering the efficiency of plaquing (e.o.p.) or the level of adsorption. The ability of phage c2 to initiate plaquing at an e.o.p. of 1-0 indicated that DNA injection and early viral gene expression are not affected in the Hsp+ transconjugants. We suggest, therefore, that the Hsp+ phenotype may result from plasmid-induced abortive infection of phage dependent on the presence of pTR2030. Hsp+ transconjugants carrying pTR2030 also promoted high-frequency conjugal transfer of Lac+ Nis associated with pTR1040 (> $10^{-1}$ per donor cell). It was concluded that Hsp+ and determinants for conjugal transfer ability (Tra+) are located on pTR2030.

INTRODUCTION

Milk fermentations rely on the growth and acid-producing ability of lactic streptococci to impart the desired flavour, texture and preservative qualities of cheese and cultured dairy products. The process occurs in open vats under non-aseptic conditions and is, therefore, highly susceptible to bacteriophage contamination and failure due to phage-induced lysis of the starter culture. Regardless of the precautions taken to select and prepare starter cultures, the fermentation bacteria cannot be protected from bacteriophage contamination or proliferation in the cheese vat under the existing conditions of these processes. Consequently, lactic streptococci that have mechanisms to resist bacteriophage attack and remain resistant under the dynamic phage pressures imposed by the fermentation process are of enormous practical significance to the dairy industries (for review see Klaenhammer, 1984b).

A variety of phage-defence mechanisms in group N streptococci have been identified and associated with plasmid DNA elements. These include restriction and modification activities (Sanders & Klaenhammer, 1981; Chopin et al., 1984), and plasmid-induced interference with
bacteriophage adsorption (Sanders & Klaenhammer, 1983; de Vos et al., 1984). McKay & Baldwin (1984) reported that plaquing ability of virulent phage was completely eliminated in transconjugants of Streptococcus lactis LM0230 that had acquired a nisin resistance plasmid, pNP40. The phage-defence mechanism did not prevent adsorption of phage and was inoperative at higher growth temperatures (37 °C) where plaquing efficiencies were unrestricted. A similar heat-sensitive mechanism of phage defence was described during studies with S. lactis ME2 when other barriers to phage infection, including phage adsorption and host-dependent phage replication, were negated (Sanders & Klaenhammer, 1984).

Plasmid involvement in phage-defence systems present in lactic streptococci is significant in two respects. First, genetic instability of plasmids encoding phage-defence systems provides a genetic mechanism to explain the rapid accumulation of phage-sensitive variants within starter cultures (Sanders & Klaenhammer, 1980, 1981, 1983; de Vos et al., 1984; McKay & Baldwin, 1984). Secondly, plasmid involvement in phage-defence mechanisms may be most advantageous in the development of phage-resistant strains, because of the ease of isolation, manipulation and transmission of these characters when located on extrachromosomal elements. In this regard, continuing developments in the genetics of group N streptococci (McKay, 1983; Gasson, 1983) and conjugal transfer of phage-defence mechanisms (Chopin et al., 1984; McKay & Baldwin, 1984) are highly significant.

In this report we describe the conjugal transfer of plasmids from S. lactis ME2 that encode lactose-fermenting ability (Lac⁺), nisin resistance (Nis⁺) and a heat-sensitive mechanism which reduces the burst size of infecting phage (Hsp⁺).

**METHODS**

*Bacteria and culture conditions.* The Streptococcus lactis, Streptococcus cremoris and Escherichia coli strains used in this study are listed in Table 1. Unless otherwise noted, group N streptococci were propagated in M17 broth at 30 °C as described by Terzaghi & Sandine (1975). Lactose-negative (Lac⁻) streptococci were propagated in M17-glucose broth (Sanders & Klaenhammer, 1981). E. coli V517 was propagated at 37 °C in brain-heart infusion broth (Difco). All cultures were maintained at −76 °C as frozen stocks in the appropriate broth medium plus 10% (v/v) glycerol. Frozen stock cultures were thawed at 25 °C and transferred twice through broth prior to use in experiments.

*Conjugation.* Conjugal matings were done on the surface of glucose/milk agar plates as described by McKay et al. (1980). In all mating experiments transconjugants were scored by selection of Lac⁺ transconjugants on lactose indicator agar (McKay et al., 1972) containing either 1 mg streptomycin ml⁻¹ for S. lactis LM0230 recipients or 15 μg erythromycin ml⁻¹ and 1 mg streptomycin ml⁻¹ for S. lactis LM2302 recipients used in high-frequency matings (Walsh & McKay, 1981).

Control mating experiments were done in the presence of DNAase (100 μg ml⁻¹) or by using cell-free filtrates of donor cells mixed with recipient cells, to rule out transformation or transduction, respectively, as potential mechanisms of gene transfer. Donor filtrates were obtained from 4 h cultures of S. lactis ME2; cells were removed by centrifugation and the supernates passed through a sterile 0.22 μm membrane filter.

*Plasmid analysis.* Plasmid purification and detection on agarose gels were done as described previously (Klaenhammer, 1984a), with the modifications noted for use with group N streptococci (Sanders & Klaenhammer, 1983). In all experiments group N streptococci were propagated in M17 or M17-glucose to obtain cells for plasmid extraction. However, pTR1040 in S. lactis ME2 was not detectable when cells from M17 broth cultures were used (see Results). In order to detect pTR1040 in S. lactis ME2, cells were grown in Elliker broth (Difco) and subjected to plasmid purification.

*Nisin resistance.* Scoring for nisin resistance or sensitivity was done initially in Elliker broth or Elliker agar (1-5% (w/v) agar, pH 6-5) containing 0·1% (v/v) Tween 20 and 100 ng nisin ml⁻¹ (Aplin and Barrett Ltd, Trowbridge, Wilts., UK). Nisin stock solutions (50 μg ml⁻¹) were prepared in 0-02 M-HCl as described by McKay & Baldwin (1984) and were diluted to the desired concentration in 10% (w/v) Elliker broth. For survival curves, cultures were propagated for 4-6 h at 30 °C in M17 or M17-glucose broth, diluted in 10% (w/v) Elliker broth and plated on Elliker agar plus 0-1% Tween 20 containing various concentrations of nisin (0-800 ng ml⁻¹). Colony-forming units (c.f.u. ml⁻¹) were determined using a Spiral Plater (Spiral System Instruments, Bethesda, Md., USA).

*Phage propagation, plaque assays and adsorption.* Bacteriophage propagation and plaque assays were done as described by Terzaghi & Sandine (1975) using the S. lactis C2 phage, c2. For plaque assays conducted at 30 °C or 40 °C, indicator cells were propagated in broth for 4-6 h at the indicated temperature prior to challenge with phage. Agar plates for these plaque assays were then further incubated at the stated temperature.
Conjugation in Streptococcus lactis

Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description*</th>
<th>Plasmid content (MDal)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptococcus lactis ME2</td>
<td>Wild-type; Lac⁺ Nis⁺ Hsp⁺</td>
<td>40,† 34, 30 (pME0030), 20, 16, 7-5, 5-8, 3-6, 2-6, 2-0, 1-9, 1-7, 1-6</td>
<td>Sanders &amp; Klaenhammer, 1983</td>
</tr>
<tr>
<td>LM0230</td>
<td>Lac⁻ Nis⁻ Hsp⁻ Str⁻</td>
<td>None</td>
<td>McKay et al., 1972</td>
</tr>
<tr>
<td>LM2302</td>
<td>Lac⁻ Nis⁻ Hsp⁻ Ery⁺ Str⁻</td>
<td>None</td>
<td>Walsh &amp; McKay, 1981</td>
</tr>
<tr>
<td>Streptococcus cremoris 14365</td>
<td>Sensitive indicator for assay of nisin</td>
<td>Unknown</td>
<td>ATCC 14365</td>
</tr>
<tr>
<td>Escherichia coli V517 S. lactis</td>
<td>Source of reference plasmids</td>
<td>35, 5-1, 3-5, 3-0, 2-2, 1-7, 1-5, 1-2</td>
<td>Macrina et al., 1978</td>
</tr>
<tr>
<td>T-RS3</td>
<td>Lac⁺ Nis⁺ Hsp⁻</td>
<td>40 (pTR1040)</td>
<td>This paper</td>
</tr>
<tr>
<td>T-RS33</td>
<td>Lac⁺ Nis⁺ Hsp⁻</td>
<td>40, 25 (pTR3025)</td>
<td>This paper</td>
</tr>
<tr>
<td>T-RS15</td>
<td>Lac⁺ Nis⁺ Hsp⁻</td>
<td>40, 2-6 (pTR4002)</td>
<td>This paper</td>
</tr>
<tr>
<td>T-RS1</td>
<td>Lac⁺ Nis⁺ Hsp⁺</td>
<td>40, 30 (pTR2030)</td>
<td>This paper</td>
</tr>
<tr>
<td>T-RS7</td>
<td>Lac⁺ Nis⁺ Hsp⁺</td>
<td>40, 30, 2-6</td>
<td>This paper</td>
</tr>
<tr>
<td>T-RS17</td>
<td>Lac⁺ Nis⁺ Hsp⁺</td>
<td>40, 30, 25, 2-6</td>
<td>This paper</td>
</tr>
</tbody>
</table>

* Lac⁺, lactose fermenting; Lac⁻, lactose negative; Nis⁺, resistant to nisin; Nis⁻, sensitive to nisin; Hsp⁺, heat-sensitive phage-resistance; Hsp⁻, phage sensitive; Str⁻, resistant to streptomycin; Ery⁺, resistant to erythromycin.
† This paper.

Levels of phage adsorption and efficiency of plaquing (e.o.p.) were determined as described previously (Sanders & Klaenhammer, 1980).

One-step growth curves and burst size. Cells (1 ml) from 3-5 h cultures propagated at 30 °C were added to a sterile Eppendorf microfuge tube (1-5 ml). After centrifugation for 3 min the supernate was discarded, the pellets resuspended in 1 ml fresh M17-glucose broth and 30 μl 1 M-CaCl₂·7H₂O and 100 μl phage c2 (10⁸-10⁹ p.f.u. ml⁻¹) were added. The broth was mixed gently and incubated for 5 min at 30 °C to allow for adsorption of phage. After centrifugation for 4 min, the supernate was aspirated and the cells resuspended in 1 ml M17-glucose broth plus calcium (50 μl 1 M-CaCl₂·7H₂O in 10 ml M17-glucose). This cell suspension was diluted to 10⁻⁴ in M17-glucose broth plus calcium. The diluted, phage-infected cell suspensions were incubated at 30 °C for 45 min. Samples (1 ml) were removed at intervals and placed in microfuge tubes containing 100 μl chloroform to kill the cells and halt phage propagation. Cell lysis did not occur upon addition of chloroform. Chloroform and cells were removed from each sample by centrifugation for 3 min. The phage titres of the supernates were measured by standard plaque assays.

For burst size determinations, phage-infected cells were prepared at both 30 °C and 40 °C and incubated at 30 °C or 40 °C as above. At 0 min and 45 min, 1 ml samples were removed and immediately subjected to plaque assay without treatment with chloroform. Burst size was calculated as the number of progeny phage at 45 min divided by the total number of infective centres at time 0.

RESULTS

Conjugal transfer of lactose metabolism and nisin resistance

During agar matings of S. lactis ME2 and S. lactis LM0230, lactose-fermenting ability was conjugally transferred at a frequency of 2.8 × 10⁻⁶ per donor cell (Table 2). Control experiments using donor cells treated with chloroform, using donor cell filtrates and in which matings were conducted in the presence of DNAase confirmed that the mechanism of genetic transfer was a conjugation-like process, not transduction or transformation.

Lac⁺ transconjugants selected from matings between S. lactis strains ME2 and LM0230 were also resistant to nisin (Table 2). Survival curves were constructed to assess the levels of nisin resistance expressed by S. lactis ME2 and a Lac⁺ transconjugant, T-RS3 (Fig. 1). Both ME2 and T-RS3 were more resistant to nisin than the standard nisin-sensitive indicator, S. cremoris.
Table 2. Conjugal transfer of lactose metabolism and nisin resistance from S. lactis ME2 to S. lactis LM0230

The recipient for each mating was LM0230. Donor cells were \(1.3 \times 10^8\) c.f.u. ml\(^{-1}\). All mating pairs were mixed at a donor:recipient ratio of 1:2 as described by McKay et al. (1980). Samples of either ME2 or LM0230 alone gave no recombinants (\(<7.6 \times 10^{-9}\) recombinants per donor). Recombinants were selected on lactose-indicator agar containing 1 mg streptomycin ml\(^{-1}\).

<table>
<thead>
<tr>
<th>Donor in mating</th>
<th>No. per ml</th>
<th>Frequency per donor</th>
<th>Percentage nisin resistant*</th>
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<tr>
<td>S. lactis ME2</td>
<td>369</td>
<td>2.8 (\times) (10^{-6})</td>
<td>100</td>
</tr>
<tr>
<td>Cell-free filtrate from S. lactis ME2</td>
<td>1</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>S. lactis ME2 + DNAase†</td>
<td>526</td>
<td>4.0 (\times) (10^{-6})</td>
<td>NA</td>
</tr>
<tr>
<td>S. lactis ME2 + chloroform‡</td>
<td>0</td>
<td>&lt;7.6 (\times) (10^{-9})</td>
<td>0</td>
</tr>
</tbody>
</table>

NA, Not applicable, spontaneous Str\(^r\) mutant of donor; ND, not determined.

* Growth on Elliker agar containing 0.1% Tween 20 and 50 ng nisin ml\(^{-1}\).
† Matings done in the presence of 100 \(\mu\)g DNAase ml\(^{-1}\).
‡ Donor cells were treated with 10% (v,v) chloroform for 30 min at 25 \(^\circ\)C. Following complete removal of chloroform the treated cells were used in conjugal matings (Neve et al., 1984).

Table 2 shows the conjugal transfer of lactose metabolism and nisin resistance from S. lactis ME2 to S. lactis LM0230. The recipient for each mating was LM0230. Donor cells were \(1.3 \times 10^8\) c.f.u. ml\(^{-1}\). All mating pairs were mixed at a donor:recipient ratio of 1:2 as described by McKay et al. (1980). Samples of either ME2 or LM0230 alone gave no recombinants (\(<7.6 \times 10^{-9}\) recombinants per donor). Recombinants were selected on lactose-indicator agar containing 1 mg streptomycin ml\(^{-1}\).

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* Growth on Elliker agar containing 0.1% Tween 20 and 50 ng nisin ml\(^{-1}\).
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‡ Donor cells were treated with 10% (v,v) chloroform for 30 min at 25 \(^\circ\)C. Following complete removal of chloroform the treated cells were used in conjugal matings (Neve et al., 1984).

14365, and the recipient, S. lactis LM0230. Moreover, the Lac\(^+\) transconjugant, T-RS3, was unaffected by nisin concentrations up to 200 ng ml\(^{-1}\), whereas ME2 was moderately affected in this range.

These data indicate that Lac\(^+\) and Nis\(^r\) were cotransferred during conjugation experiments where Lac\(^+\) was used as the selection marker for detection of recombinants. To confirm concomitant transfer of Lac\(^+\) and Nis\(^r\), attempts were also made to select Nis\(^r\) recombinants directly on agar containing nisin. However, spontaneous Nis\(^r\) mutants of the recipient occurred readily when LM0230 was plated alone on nisin plates. The appearance of spontaneous Nis\(^r\) mutants prevented direct selection or quantification of Nis\(^r\) recombinants in mating mixtures.

Resistance of transconjugants to bacteriophage

Lac\(^+\) Nis\(^r\) transconjugants generated from matings of S. lactis ME2 with LM0230 were further examined to determine their sensitivity to the recipient's homologous lytic phage, c2. When challenged with phage in standard plaque assays conducted at 30 \(^\circ\)C, 4% of the transconjugants exhibited plaques that were smaller than those routinely observed on LM0230. Plaque development was restored when the transconjugants were propagated at 40 \(^\circ\)C and assayed for plaque formation at the higher temperature. The alterations in plaque morphology of phage c2 that were typical on these selected Lac\(^+\) Nis\(^r\) transconjugants are shown for strain T-RS1 in Fig. 2.

Phage adsorption, e.o.p. and burst size were examined for phage c2 on LM0230, T-RS1 and a second Lac\(^+\) Nis\(^r\) transconjugant, T-RS3, that exhibited normal plaque formation when challenged with phage c2 at 30 \(^\circ\)C (Table 3). Phage adsorption and e.o.p. were similar in the three strains, indicating that these parameters were not responsible for the smaller plaque size observed for phage c2 on T-RS1 at 30 \(^\circ\)C. However, the burst size of phage c2 on T-RS1 was reduced to levels approximately eightfold below those observed for either LM0230 or T-RS3 when the experiment was conducted at 30 \(^\circ\)C. The burst size of phage c2 from T-RS1 increased from 5 at 30 \(^\circ\)C to 51 at 40 \(^\circ\)C, suggesting that the phage resistance exhibited by T-RS1 (designated Hsp\(^+\)) was labile at the higher temperature. The changes in burst size correlated well with the alterations in plaque morphology observed at 30 \(^\circ\)C and 40 \(^\circ\)C in Fig. 2; both plaque and burst sizes were increased at the higher temperature. One-step growth curves constructed for phage c2 on T-RS1, T-RS3 and LM0230 showed no difference in latent periods, but confirmed that the amount of phage released from T-RS1 during a single growth cycle at 30 \(^\circ\)C was reduced substantially below that seen for T-RS3 and LM0230 (Fig. 3). These data indicated
Conjugation in *Streptococcus lactis*

**Fig. 1.** Survival curves of *S. lactis* ME2 (○), *S. lactis* LM0230 (■), *S. cremoris* 14365 (□) and the transconjugant *S. lactis* T-RS3 (●) at various concentrations of nisin.

![Survival curves](image)

**Fig. 2.** Plaque morphology of phage c2 on its homologous host, *S. lactis* LM0230, at 30 °C (a) and on a Lac⁺ Nis⁺ Hsp⁺ transconjugant T-RS1 (generated from matings of *S. lactis* strains ME2 and LM0230), at 30 °C (b) and 40 °C (c). The small plaque morphology exhibited by T-RS1 cells when challenged with phage at 30 °C was designated Hsp⁺.

![Plaque morphology](image)

**Table 3.** Adsorption, burst size and e.o.p. of phage c2 on *S. lactis* LM0230 and Lac⁺ Nis⁺ transconjugants

<table>
<thead>
<tr>
<th><em>S. lactis</em> strain</th>
<th>Phenotype</th>
<th>Percentage adsorption</th>
<th>E.o.p.</th>
<th>Burst size at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>30 °C</td>
<td>40 °C</td>
</tr>
<tr>
<td>LM0230</td>
<td>Lac⁻ Nis⁻ Hsp⁻</td>
<td>99</td>
<td>1·0</td>
<td>40</td>
</tr>
<tr>
<td>T-RS3</td>
<td>Lac⁺ Nis⁺ Hsp⁺</td>
<td>96</td>
<td>1·0</td>
<td>41</td>
</tr>
<tr>
<td>T-RS1</td>
<td>Lac⁺ Nis⁺ Hsp⁺</td>
<td>98</td>
<td>0·83</td>
<td>5</td>
</tr>
</tbody>
</table>

that the small plaque size formed with phage c2 on T-RS1 at 30 °C resulted from a reduction in the burst size of the phage. Latent period, level of adsorption and e.o.p. for phage c2 were not affected in the Lac⁺ Nis⁺ Hsp⁺ transconjugant T-RS1.

**Plasmid analysis of transconjugants**

A 40 MDal plasmid (pTR1040) was common to all Lac⁺ Nis⁺ transconjugants examined, but additional plasmids of 30 MDal (pTR2030), 25 MDal (pTR3025) and 2·6 MDal (pTR4002) were also observed in various combinations (Fig. 4). Transconjugant T-RS3 (Lac⁺ Nis⁺ Hsp⁺)
Fig. 3. One-step growth curves at 30 °C for phage c2 on *S. lactis* LM0230 (○), the Lac⁺ Nis⁺ Hsp⁻ transconjugant T-RS3 (△) and the Lac⁺ Nis⁺ Hsp⁺ transconjugant T-RS1 (∆).

![Plasmid profiles of *S. lactis* ME2, T-RS3, T-RS1, T-RS15, T-RS7, and T-RS17](image)

Fig. 4. Plasmid profiles of Lac⁺ Nis⁺ Hsp⁻ transconjugants (T-RS3, T-RS15) and Lac⁺ Nis⁻ Hsp⁺ transconjugants (T-RS1, T-RS7, T-RS17) as compared to the donor, *S. lactis* ME2, and plasmid-cured recipient, *S. lactis* LM0230. Reference molecular masses of *E. coli* V517 plasmids and the positions of pTR1040, pTR2030, pTR3025 and pTR4002 in T-RS17 are given for comparison. Chr., chromosomal fragments present in all samples; o.c., the open-circular form of pTR4002.

harboured only pTR1040, providing further evidence that the 40 MDal plasmid carried determinants for both Lac⁺ and Nis⁻. In Lac⁺ Nis⁻ transconjugants exhibiting the Hsp⁺ character (T-RS1, T-RS7, T-RS17) both pTR1040 and pTR2030 were detected. In the Lac⁺ Hsp⁻ transconjugants T-RS3 and T-RS15, pTR1040 was detected but pTR2030 was not, suggesting that pTR2030 encodes the Hsp⁺ determinants. The presence or absence of pTR3025 and pTR4002 did not alter the lactose-fermenting ability, nisin-resistance or phage-resistance phenotypes expressed by the transconjugants.

Plasmid curing and instability of Lac⁺ Nis⁻ and Hsp⁺

Retention of Lac⁺, Nis⁻ and Hsp⁺ phenotypes in single-colony isolates of T-RS1 were examined following one passage of the culture through M17-glucose broth at 30 °C or 40 °C. Following propagation of T-RS1 at 30 °C, 7% of the isolates were Lac⁻ and 4% Hsp⁻ (Table 4).
Table 4. Instability of lactose metabolism and phage resistance in *S. lactis* ME2 and the transconjugant T-RS1

Cultures were propagated once through M17-glucose broth at 30 °C or 40 °C for 4 h and plated to isolate colonies.

<table>
<thead>
<tr>
<th><em>S. lactis</em> strain</th>
<th>Phenotype</th>
<th>Growth temperature</th>
<th>Lac- colonies/ colonies examined (percentage)*</th>
<th>Hsp- colonies/ colonies examined (percentage)+</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-RS1</td>
<td>Lac+Nis'Hsp+</td>
<td>30 °C</td>
<td>6/87 (6.9)</td>
<td>4/100 (4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40 °C</td>
<td>74/119 (62.2)</td>
<td>53/100 (53)</td>
</tr>
<tr>
<td>ME2</td>
<td>Lac+Nis'Hsp+</td>
<td>30 °C</td>
<td>1/100 (1.0)</td>
<td>ND (ND)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40 °C</td>
<td>18/114 (15.8)</td>
<td>ND (ND)</td>
</tr>
</tbody>
</table>

ND, Not determined.

* Lac- colonies were scored on lactose-indicator agar. Lac- colonies were also Nis+.
† Hsp-, deficient in heat-sensitive phage resistance. Single colonies were purified from M17-glucose agar plates and scored for Hsp+ or Hsp- by challenging with phage c2 and examining plaque morphology at 30 °C.

When the growth temperature for the curing experiment was increased to 40 °C, loss of Lac+ and Hsp+ occurred within 62% and 53% of the population, respectively. Nis+ was lost concurrently with Lac+, but the Hsp+ phenotype segregated independently of Lac+ and Nis+, and Lac- Nis+ Hsp+ and Lac- Nis+ Hsp- variants were routinely isolated (data not shown). Fig. 5 compares the plasmid profiles of T-RS1 to Lac- Nis+ Hsp+ and Lac- Nis+ Hsp- variants of this transconjugant. The data showed that loss of Lac+ and Nis+ accompanied curing of pTR1040 and Hsp+ was lost upon curing of pTR2030. Growth at high temperature rapidly destabilized both pTR2030 and pTR1040, indicating that these plasmids are not maintained by LM0230 during growth at 40 °C. Therefore, elimination of Hsp+ in T-RS1 at 40 °C may be partially accounted for by the rapid appearance of Hsp- variants in the culture due to curing of pTR2030.

In plasmid profiles of the conjugation donor, *S. lactis* ME2, a 40 MDal plasmid could not be detected when cells were propagated in M17 broth (Figs 4 and 5). This suggested that either pTR1040 was a recombinant plasmid that was formed during conjugation and was similar to those described in *S. lactis* ML3 (Walsh & McKay, 1982; Anderson & McKay, 1984), or it was simply not detected in the *S. lactis* ME2 cultures under the growth and plasmid extraction conditions employed. In subsequent analysis a 40 MDal plasmid was detected in *S. lactis* ME2 cells propagated in Elliker broth instead of M17 broth (Fig. 6). Moreover, Lac- variants of ME2 occurred spontaneously in cultures propagated at 30 °C or 40 °C, albeit at lower frequencies than were observed for the T-RS1 transconjugant (Table 4). Lac- variants of ME2 were also Nis+ and lacked the 40 MDal plasmid (Fig. 6). These data defined a common plasmid determinant for Lac+ and Nis+ in *S. lactis* ME2 and suggested that a recombination event did not result in the formation of pTR1040 in the Lac+ Nis+ transconjugants.

High-frequency conjugation mediated by pTR2030

Six different Lac+ Nis+ transconjugants harbouring various combinations of pTR1040, pTR2030, pTR3025 and pTR4002 were examined for their ability to act as conjugal donors for transfer of Lac+ Nis+ and Hsp+ to a second plasmid-cured, Ery+ Str+ recipient, *S. lactis* LM2302 (Table 5). Lac+ Nis+ Hsp+ transconjugants T-RS1, T-RS7 and T-RS17 were able to transfer Lac+ Nis+ at frequencies of 10⁻¹ per donor cell. Of the Lac+ Nis+ Ery+ Str+ transconjugants generated from matings with T-RS1, 85% were Hsp+ and carried both pTR1040 and pTR2030, and 15% were Hsp- and carried pTR1040, but lacked pTR2030 (data not shown). Three Lac+ Nis+ Hsp- transconjugants lacking pTR2030 (T-RS3, T-RS15 and T-RS33) were unable to transfer Lac+ Nis+, even at low frequency (Table 5). Therefore, pTR1040 was unable to promote its own transfer, and pTR3025 or pTR4002 could not mobilize pTR1040 or the Lac+ Nis+ determinants located on pTR1040. High-frequency conjugation of Lac+ Nis+ phenotypes associated with pTR1040 occurred only with the presence of pTR2030. These data indicated...
Fig. 5. Plasmid profiles of *S. lactis* T-RS1 (Lac⁺ Nis⁺ Hsp⁺), and Lac⁻ Nis⁺ Hsp⁺ and Lac⁻ Nis⁺ Hsp⁻ variants isolated from T-RS1. The plasmid profiles of *S. lactis* strains ME2 and LM0230 are shown for comparison.

Fig. 6. Plasmid profile of *S. lactis* ME2 (Lac⁺ Nis⁺) as compared to a Lac⁻ Nis⁺ variant. Arrows denote the position of the 40 MDal plasmid correlated with Lac⁺ Nis⁺ phenotypes in ME2. Reference molecular masses of *E. coli* V517 plasmids are given for comparison.

Table 5. High-frequency conjugation of lactose metabolism and nisin resistance from *S. lactis* transconjugants to *S. lactis* LM2302

<table>
<thead>
<tr>
<th>Donor strain</th>
<th>Phenotype</th>
<th>Plasmid content</th>
<th>Lac⁺ Nis⁺ Ery⁺ Str⁺ recombinants</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-RS1</td>
<td>Lac⁺ Nis⁺ Hsp⁺</td>
<td>pTR1040, pTR2030</td>
<td>9.6 × 10⁶</td>
</tr>
<tr>
<td>T-RS7</td>
<td>Lac⁺ Nis⁺ Hsp⁺</td>
<td>pTR1040, pTR2030, pTR4002</td>
<td>1.2 × 10⁷</td>
</tr>
<tr>
<td>T-RS17</td>
<td>Lac⁺ Nis⁺ Hsp⁺</td>
<td>pTR1040, pTR2030, pTR3025, pTR4002</td>
<td>1.1 × 10⁷</td>
</tr>
<tr>
<td>T-RS3</td>
<td>Lac⁺ Nis⁺ Hsp⁻</td>
<td>pTR1040</td>
<td>0</td>
</tr>
<tr>
<td>T-RS15</td>
<td>Lac⁺ Nis⁺ Hsp⁻</td>
<td>pTR1040, pTR4002</td>
<td>0</td>
</tr>
<tr>
<td>T-RS33</td>
<td>Lac⁺ Nis⁺ Hsp⁻</td>
<td>pTR1040, pTR3025</td>
<td>0</td>
</tr>
</tbody>
</table>

that in addition to the Hsp⁺ character, pTR2030 encodes determinants essential for conjugal transfer (Tra⁺). For transconjugants carrying pTR2030, donor frequencies for transfer of Lac⁺ Nis⁺ were five orders of magnitude over those observed initially with matings of *S. lactis* ME2 (frequency per donor of 2.8 × 10⁻⁷, Table 2). Strains capable of high-frequency conjugation did not show a clumping tendency in broth culture, or major alterations in the molecular mass of the transferred plasmids, as has been observed previously for other high-frequency conjugation systems in group N streptococci (Gasson & Davies, 1980; Walsh & McKay, 1981; Anderson & McKay, 1984).
DISCUSSION

Conjugal transfer of plasmid DNA in lactic streptococci is widespread and can encompass a variety of characteristics vital to the activity of these bacteria in dairy fermentations. Properties subject to conjugal transfer include lactose and sucrose metabolism (McKay et al., 1972, 1980; Snook & McKay, 1981; Gasson, 1983, 1984), bacteriocin production and resistance (Scherwitz et al., 1983; Neve et al., 1984; Gasson, 1984) and restriction and modification activity (Chopin et al., 1984). Recently, in work with Streptococcus diacetylactis DRC3, McKay & Baldwin (1984) reported that nisin resistance and a heat-sensitive mechanism of phage resistance were encoded on a conjugative plasmid (pNP40). Our observations made with S. lactis ME2 and its transconjugants showed gross similarities in plasmid-linked phenotypes. However, clear differences were evident in phenotype combinations, plasmid sizes and expression of phage resistance in the transconjugants.

Nisin resistance and lactose metabolism in S. lactis ME2 were correlated with the presence or absence of a 40 MDal plasmid. Acquisition of a 40 MDal plasmid in all Lac+ Nis' transconjugants generated from conjugal matings with S. lactis ME2 provided further evidence that this plasmid encoded both Lac+ and Nis' determinants. Similarities in the molecular mass, conjugal behaviour and phenotypes expressed by the 40 MDal plasmid in both S. lactis ME2 and the transconjugants suggested that this plasmid was not altered by the conjugation process. However, we did not compare restriction sites on the 40 MDal plasmid isolated from ME2 with pTR1040 because separation of the 40 MDal plasmid from other large molecular mass plasmids present in ME2 was not possible. Therefore, we did not ascertain whether or not these plasmids were identical, or if pTR1040 had incurred minor genetic rearrangements pursuant to conjugal transfer. Nevertheless, definition of a Nis' determinant in S. lactis ME2 may be practically significant in the development of nisin-resistant strains for selected dairy fermentations and cloning determinants for lactic acid bacteria (McKay & Baldwin, 1984). In the work described in this paper, direct use of nisin for selection of recombinants was routinely complicated by the appearance of spontaneous Nis' mutants. However, Nis' determinants could easily be employed as secondary markers in genetic or cloning experiments where direct selection of recombinants was first made by using another characteristic.

Conjugal transfer of Lac+ Nis' from S. lactis ME2 occurred at frequencies approaching $10^{-6}$ per donor cell. However, following the initial mating between ME2 and LM0230, transconjugants harbouring both pTR1040 (Lac+ Nis') and pTR2030 (Hsp+ Tra+) could transfer these phenotypes at frequencies approaching $10^{-1}$ per donor cell. High-frequency conjugal transfer of the Lac+ character in group N streptococci has been reported in both S. lactis 712 (Gasson & Davies, 1980) and S. lactis ML3 (Walsh & McKay, 1981; Anderson & McKay, 1984) where clumping ability and formation of large recombinant plasmids accompanied the high conjugal transfer frequencies. The high-frequency conjugation system described here for S. lactis ME2 did not show clumping tendencies or the formation of large recombinant Lac+ plasmids. Transconjugants harbouring only pTR1040 were unable to transfer Lac+ Nis', even at low frequency, in the absence of pTR2030. Therefore, the Lac+ Nis' plasmid (pTR1040) appeared to be transferred in the absence of permanent recombination events where Tra+ determinants could be secured. Consequently, the conjugal state imposed by pTR2030 on the high-frequency donors may be sufficiently effective to promote coincidental passage of intact, co-resident plasmids that are themselves deficient in transfer characteristics.

Following matings with S. lactis ME2, Hsp+ transconjugants carrying pTR2030 showed a reduction in burst size of the lytic phage c2. Restoration of normal burst and plaque sizes were observed when Hsp+ transconjugants were propagated and challenged with phage at 40 °C. When compared to the recipient or Hsp+ transconjugants, the level of adsorption, latent period and e.o.p. of phage c2 were not altered in Hsp+ transconjugants. Therefore, the resistance to phage c2 expressed by the Hsp+ transconjugants carrying pTR2030 could not be accounted for by defence mechanisms involving phage adsorption, DNA injection, or action of restriction endonucleases or generalized DNAases on injected phage DNA. These observations are consistent with the identification of a phage-defence mechanism in S. lactis ME2, distinct from restriction and modification or adsorption reactions, that was temperature sensitive and
inhibited phage proliferation (Sanders & Klaenhammer, 1984). Although the phenotypic behaviour of Hsp\(^+\) was similar in both \textit{S. lactis} ME2 and the transconjugants harbouring pTR2030, there were two significant differences in genetic properties. First, Hsp\(^-\) variants were not isolated from \textit{S. lactis} ME2 following growth at 40°C even though the Hsp\(^+\) character is inoperative at this temperature (Sanders & Klaenhammer, 1983, 1984); unlike pTR2030 in the Hsp\(^+\) transconjugants, Hsp\(^+\) determinants are stably maintained in \textit{S. lactis} ME2 at the higher temperature. Secondly, Hsp\(^+\) was not associated with a 30 MDal plasmid in \textit{S. lactis} ME2. Variants which had lost pME0030, a 30 MDal plasmid, retained the Hsp\(^+\) phenotype and restricted plaque size and burst size of phage at 30°C, but not 40°C (Sanders & Klaenhammer, 1984). These comparisons suggest that pTR2030 secured Hsp\(^+\) determinants which were not associated with the corresponding 30 MDal plasmid in \textit{S. lactis} ME2. The location of the Hsp\(^+\) determinants in \textit{S. lactis} ME2 and the events responsible for the formation of pTR2030 are under investigation.

The mechanism by which pTR2030 inhibits development of phage c2 in Hsp\(^+\) transconjugants is not presently known. However, the behaviour of pTR2030 and the associated Hsp\(^+\) phenotype are very similar to the processes of ‘plasmid-induced abortive infections’ as described by Duckworth et al. (1981). As occurs in plasmid-induced abortive infections, the phage infection process was inhibited in Hsp\(^+\) transconjugants even though phage adsorption, DNA entry and the early stages of viral gene expression seem normal. Although no direct evidence was provided in this study for normal DNA entry and viral gene expression in Hsp\(^+\) transconjugants, the ability of phage c2 to initiate plaque formation at a normal e.o.p. demonstrated that these processes are unaffected. Plasmid-induced abortive infections are also routinely associated with self-transmissible plasmids including the F plasmid and various R plasmids found in \textit{Enterobacteriaceae} (for review see Duckworth et al., 1981). Suppression of progeny phage in \textit{E. coli} K12 by autonomous replicating F or ColB results in reduced burst size, plaque size and e.o.p. of virulent phage W-31 (Watanabe & Okada, 1964). Lac\(^+\) Nis\(^+\) transconjugants carrying the Tra\(^+\) plasmid pTR2030 showed similar inhibition in both plaque and burst size of phage c2. Therefore, the Hsp\(^+\) phenotype may not be a phage-directed defence mechanism, but rather a manifestation of the conjugal transfer characteristics expressed by pTR2030 that result in plasmid-induced abortive infection of virulent phage c2. The phage-defence mechanism associated with pNP40 may function similarly, in the light of preliminary observations suggesting that this plasmid also carries mobility determinants (McKay & Baldwin, 1984).

Whether or not Hsp\(^+\) is a phage-directed mechanism or a coincidental property of the Tra\(^+\) phenotype encoded by pTR2030 is under investigation. Nevertheless, it is apparent that the ability of the Hsp\(^+\) character to suppress virulent phage development is substantial and, therefore, may be of enormous practical significance in the genetic development of phage-insensitive starter cultures for dairy fermentations. The transfer abilities associated with pTR2030 may be particularly useful for dissemination of Hsp\(^+\) in lactic streptococci, and permit study of its phage-inhibitory properties under a variety of phage-host interactions and in combination with additional phage-defence mechanisms.

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