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Conjugal transfer of lactose-fermenting ability from *Streptococcus lactis* C2 to *Leuconostoc cremoris* CAF7 yields *Leuconostoc* that ferment lactose and produce diacetyl*

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SUMMARY

Conjugation between lactose-fermenting (Lac^+) *Streptococcus lactis* C2 and Lac^- *Leuconostoc cremoris* CAF7 was performed. The frequency of Lac^+ transfer was $1.5 \cdot 10^{-2}$ per donor cell. Lac^+ *Leuconostoc* transconjugants could ferment lactose significantly faster than wild-type cells. When grown in litmus milk fortified with 0.2% yeast extract, Lac^+ transconjugants reached pH 4.68 within 24 h at 30°C and produced diacetyl. The identity of the transconjugants as *Leuconostoc* derivatives was confirmed by their resistance to phage c2 and to vancomycin (> 500 μg/ml), and by growth on selective medium containing azide. Plasmid profiles of 10 transconjugants showed two unique patterns. A novel enlarged plasmid was found. Southern blot hybridization revealed some homology with the 30 Md Lac^+ plasmid of donor, recipient and the transconjugants, as well as with some of the remaining plasmids of the donor.

INTRODUCTION

Group N streptococci and *Leuconostoc* are lactic acid bacteria widely used in milk fermentation. They are taxonomically and ecologically related. Group N streptococci are acid and/or flavor (diacetyl) producers, while leuconostocs are flavor producers. Similar to group N streptococci, *Leuconostoc* are fastidious, gram-positive bacteria of the family Streptococcaceae, found in fermenting vegetables, dairy products, wine and sugar refineries [5]. Members of the genus, especially *L. cremoris, L. dextranicum* and *L. lactis* [5,6], are important in the manufacture of dairy products. *Leuconostoc cremoris* and *L. dextranicum* ferment milk citrate and therefore are responsible for diacetyl production in buttermilk, sour cream, and cottage cheese [13]. *Leuconostoc* cannot ferment citrate unless the pH of milk is acidic [2]. Also, *Leuconostoc* grow poorly in milk due to a lack of the genes responsible for lactose fermentation and protein utilization [2]. Therefore, starter cultures used for fermented milk

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foods in North America contain both the lactic acid and aroma bacteria.

One way to overcome this inert characteristic of *Leuconostoc* would be to introduce gene(s) for lactose fermentation and/or proteolysis. This would allow *Leuconostoc* to become more useful dairy microorganisms, since phages for them may be less plentiful and more narrow in host range than for lactic streptococci [21]. Also, *Leuconostoc* are low in diacetyl reductase activity [20].

The conjugal transfer of genes between group N streptococci and *Leuconostoc* has received little attention. Hill et al. [8] reported the transfer of transposon Tn919 from *S. faecalis* into *L. cremoris* X2Sm. The frequency of transfer was the same as that of other group N *Streptococcus* strains. Tsai and Sandine [23] reported the transfer by conjugation of plasmids from *S. lactis* to *L. cremoris,* which enabled the *Leuconostoc* to produce nisin. *Leuconostoc* strains are naturally resistant to high levels of vancomycin [18], and its incorporation into media allows for their selection from mixed cultured backgrounds.

This paper documents the conjugal transfer of lactose-fermenting ability from lactic streptococci to *Leuconostoc*. Transconjugants are able to ferment milk lactose and citrate, resulting in diacetyl production.

MATERIALS AND METHODS

Bacterial strains. Table 1 lists the strains used in this study. *Streptococcus lactis* C2 was maintained by biweekly transfer at 30°C in M17 broth [22]. *Leuconostoc cremoris* CAF7 was maintained in MRS broth [3]; it produced gas when grown in 100-ml volumetric flasks of skim milk containing 0.5% yeast extract and overlaid with Vaspar (Vasoline-paraffin wax, 1:1) as evidenced by heating to 60°C after incubation at 30°C for 48–72 h using the method of Holmes et al. [9]. In contrast to *S. lactis* C2, it also grew on the *Leuconostoc* selective agar of Mayeux et al. [16]. *Escherichia coli* V517 [14] was obtained from E. Lederberg, Plasmid Reference Center, Stanford University School of Medicine, Stanford, CA, and propagated in brain-heart infusion broth at 37°C.

Solid surface conjugation. A modification of the method of McKay et al. [17] for solid surface conjugation was performed. Cultures of the donor strain, positive for lactose fermentation, but sensitive to 10 μg/ml vancomycin (Lac^+ Vm^-), were grown overnight in M17 broth. Cultures of the recipient strain, negative for lactose fermentation but resistant to 10 μg/ml vancomycin (Lac^-Vm^+) were grown in MRS broth, and then transferred (2%) into fresh broth. Then they were incubated at 30°C for 3 h before mating. Mating was performed by mixing at a donor recipient ratio of 1:2 and spreading on Elliker agar [4] or passing through a membrane filter (0.45 μm pore size, Millipore). The membrane filters were then transferred to Elliker agar. The latter method was designated the membrane filter technique, the former was called the spread plate method. Plates were placed in a Gas-

Table 1

Plasmid profiles and description of bacterial strains used

Strain	Plasmid[a]	Description[b]
Streptococcus lactis C2	30, 17.5, 13, 9.4, 5.1, 2.3, 1.4	Wild-type, Lac^+Prt^+, donor
S. lactis C2 mutant	17.5, 13.9, 5.1, 2.3, 1.4	Lac^-Prt^+
Leuconostoc cremoris CAF7	36, 17.5, 10.5, 4.8	Wild-type, Lac^-Prt^-, recipient
L. cremoris HT6	58, 30, 19.1, 17.2, 9.4, 6.5	Transconjugant, Lac^+Prt^-
L. cremoris HT32	30, 24, 18.5, 17.2, 9.7, 6.5	Transconjugant, Lac^+Prt^-
Escherichia coli V517	35.8, 4.8, 3.7, 3.4, 2.6, 2.0, 1.8, 1.4	Source of reference plasmids

[a] Values of molecular masses of the plasmids are in megadaltons (Md).

[b] Lac = lactose fermenting ability; Prt = proteolytic ability; + = positive; − = negative.

Pak jar (BBL, Microbiology System, Cockeysville, MD) and incubated at 30°C for 16 h. After incubation, mating mixtures were suspended in peptone water (0.1%) and centrifuged at 9900 × *g* at 4°C for 12 min. The pellet was resuspended, diluted and plated on M17 agar containing 0.5% sodium glycerophosphate, 10 μg/ml vancomycin and 0.004% bromocresol purple (M17-Vm-BCP). Lac^+ transconjugants formed yellow colonies against a purple background within 48 h at 30°C. Yellow colonies were retransferred to modifed Rogosa Agar [19] containing 500 μg/ml vancomycin (MRA-Vm). Surviving organisms on MRA-Vm were transferred to MRS broth for later assays.

Donor strain was diluted and plated on M17 agar to determine total colony forming units (CFU) per ml and on M17-Vm-BCP agar to ensure vancomycin sensitivity of the donor. Recipient strains were also diluted and plated on MRS agar for enumeration and on M17-Vm-BCP agar for negative control. Control mating groups were done in the presence of DNAse (100 μg/ml), or by using cell-free filtrates of donor cells mixed with recipient cells so that transformation and transduction would be ruled out.

Lactose fermentation test broth (LFTB). Overnight cultures were inoculated (2.5%) into LFTB [18] and incubated at 30°C for 4 h.

pH in litmus milk with or without 0.2% yeast extract. Litmus milk was prepared by sterilizing 11% (w/v) reconstituted nonfat dry milk containing 0.075% litmus with or without 0.2% yeast extract. The litmus milk was inoculated with 1% overnight culture and incubated at 30°C, with pH measurements every 6 h.

pH in modified M17 broth. One percent overnight cultures were inoculated into M17 broth [22] with 0.5% or no sodium glycerophosphate. The cultures were kept at 30°C and/or room temperature (23°C) for 48 h and the pH was measured every 6 h.

King test. King's modification of Ritter's alpha-naphthol test [10] was performed as a qualitative test for diacetyl. *Streptococcus lactis* subsp. *diacetylactis* DRC1 was used as a positive control, while *S. lactis* C2 was a negative control.

Streptococcus lactis C2 phage assay and vancomycin resistance. Maintenance and particle assay of *S. lactis* C2 phage was performed as described by Terzaghi and Sandine [22]. The stock solution of vancomycin (Sigma) was prepared by dissolving the antibiotic in distilled water at 10 mg/ml and passing through a membrane filter (0.22 μm).

Large-scale plasmid DNA isolation, CsCl-EtBr purification, and agarose gel electrophoresis. The methods were as described in Maniatis et al. [15]; the plasmid DNA was isolated using the alkali-SDS lysis procedure. The plasmid content of donor, recipient and transconjugants was analyzed.

Recovery of 30 Md plasmid of S. lactis C2 from agarose gel. Pure 30 Md plasmid of *S. lactis* C2 was isolated in order to make ^{32}P-radioactive probe. After running electrophoresis on 0.7% low-melting-temperature agarose (Type V11, Sigma) containing 0.5 μmg/ml of ethidium bromide (EtBr), a 30 Md plasmid was localized using a long-wavelength UV lamp and was excised. The gel slice was melted by heating at 65°C for 20 min, and then cooled at 37°C. An equal volume of phenol, preheated to 37°C, was added. This mixture was shaken and spun down at 13 000 rpm (Microfuge 11, Beckman) for 1 min. The aqueous layer was collected and re-extracted with phenol. After the aqueous layer was transferred to a new tube, NaCl was added to 0.1 M final concentration. The top phase was removed by centrifugation at room temperature and was extracted three times with ether which was evaporated for 15 min under the hood. Plasmid DNA was precipitated by adding 1/10 volume of 3 M sodium acetate and 2 volumes of 100% ethanol. At least 50% covalently closed circular DNA was recovered.

Southern blot hybridization. Plasmid DNA of donor, recipient, two transconjugants and *E. coli* V517 were run on 0.7% agarose gel in Tris-acetate buffer (0.04 M Tris-acetate and 0.001 M EDTA). These plasmid DNAs were then transferred to a nitrocellulose filter as described by Maniatis et al. [15]. It took about 21 h for the DNA to transfer. The dried filter was baked for 2 h at 80°C under vacuum. Prehybridization was performed at 42°C for 2 h with a prehybridization mix solution consisting of 20 × SSC (3 M sodium chloride, 0.3 M

sodium citrate), 20 × Denhardt's solution, deionized formamide (DIF) and denatured sonicated salmon sperm DNA (sssDNA). Hybridization was performed overnight at 42°C in a hybridization mix solution containing 20 × SSC, 20 × Denhardt's solution, DIF, 50% dextran sulfate, and sssDNA. A probe consisting of nick-translated 30 Md plasmid was added to the hybridization solution.

Probe of 30 Md plasmid DNA was prepared by nick-translation with (α-^{32}P)dATP (New England Nuclear). The specific activity of the probe was 25 · 10^6 CPM/μg DNA. The probe was collected by centrifugation through Sephadex G-50 packed in a disposable syringe.

After hybridization, the filter was washed at 42°C for 15 min three times in a solution consisting of 2 × SSC and 0.1% SDS (sodium dodecyl sulfate) and twice in 0.1 × SSC and 0.1% SDS. The filter was placed on Kodak XAR-5 Xomat film at −70°C for 5 h.

RESULTS

Conjugation

Donor cells (27 · 10^8 CFU/ml) grew on M17 agar but there was no growth on M17-Vm-BCP agar. This selective agar was exclusive for recipient cells and transconjugants. Recipient cells, however, could not form yellow colonies on M17-Vm-BCP agar. The average number of yellow colonies produced on the selective agar plate at the 10^{-7} dilution was four for the spread plate method, and three for the membrane filter technique. Therefore, the frequency of conjugation was 1.5 · 10^{-2} and 1.1 · 10^{-2}, respectively. No transconjugants were detected in mating studies with cell-free filtrates or heat-treated donor cells when mixed with recipients (less than one colony out of 20 plates at the 10^{-7} dilution – Table 2).

Lactose fermentation broth test

Transconjugants produced acid from lactose faster than the wild-type. During 4 h of incubation at 30°C, the transconjugants lowered the pH of broth to 4.5 while the wild-type only lowered the pH to 5.2. Acid production from lactose also was evident by the color change of the broth from purple to yellow.

pH in litmus milk

Although the pH difference between wild-type and transconjugants was about 0.5 unit after incubation at 30°C for 48 h, this difference was magnified to 1.4 units when litmus milk was fortified with 0.2% yeast extract (Fig. 1B). The milk was coagu-

Table 2

Conjugal transfer of lactose fermenting genes from *Streptococcus lactis* C2 to *Leuconostoc cremoris* CAF7

Donor in mating[a]	Number of yellow colonies[b]		Frequency per donor[c]	
	SP	MF	SP	MF
Donor	4	3	1.5 · 10^{-2}	1.1 · 10^{-2}
Cell-free filtrate of donor	<0.05	<0.05	NA	NA
Donor in the presence of DNAse[d]	4.3	3.5	1.6 · 10^{-2}	1.3 · 10^{-2}
Heat-treated donor[e]	<0.05	<0.05	NA	NA

[a] At a donor:recipient ratio of 1:2.
[b] Average number from 20 of 10^{-7} plates.
[c] Calculated from 27 · 10^8 CFU/ml donor cells.
[d] 100 μg/ml.
[e] 100°C for 10 min.

SP, spread plate; MF, membrane filter technique; NA, not applicable.

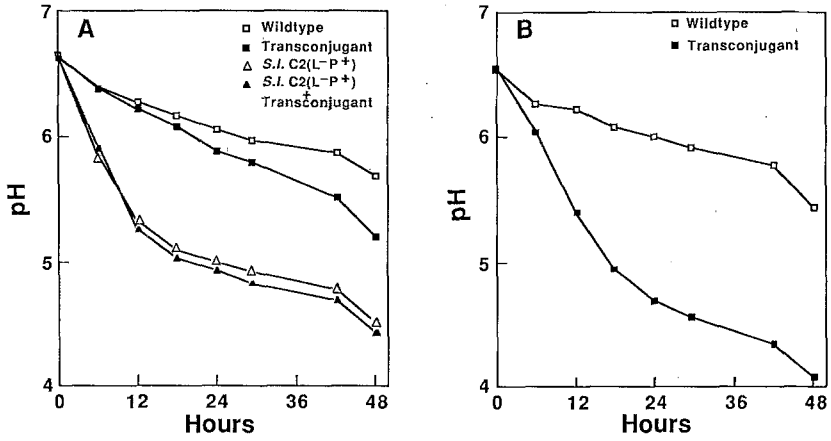

Fig. 1. (A) pH value reached over time at 30°C when wild-type *L. cremoris* CAF7 (1%), transconjugant *L. cremoris* HT6 (1%), L^-P^+ mutant of *S. lactis* C2 (1%), and transconjugant (0.5%) plus L^-P^+ mutant of *S. lactis* C2 (0.5%) were inoculated in litmus milk. (B) pH value reduced over time at 30°C when wild-type *L. cremoris* CAF7 (1%) and the transconjugant *L. cremoris* HT6 (1%) were inoculated in litmus milk fortified with 0.2% yeast extract.

lated after incubation at 30°C for 24 h. If a Lac^-Prt^+ mutant of *S. lactis* C2 was inoculated together with transconjugants, the pH of milk was lower than the Lac^-Prt^+ inoculum alone (Fig. 1A).

pH in modified M17 broth

To reduce the buffering capacity, M17 broth was prepared with 0.5% SGP instead of 1.9%. With this modified broth, the pH was 4.55 when incubated for 12 h at 30°C, or for 18 h at 23°C. The difference was 1.7 units between wild-type and transconjugant cells (Fig. 2).

King test

When the transconjugant, *L. cremoris* HT6, was incubated for 24 h either at 30°C or at 23°C, the King test was positive. If it was incubated only for 16 h at 23°C, the King test was negative because the

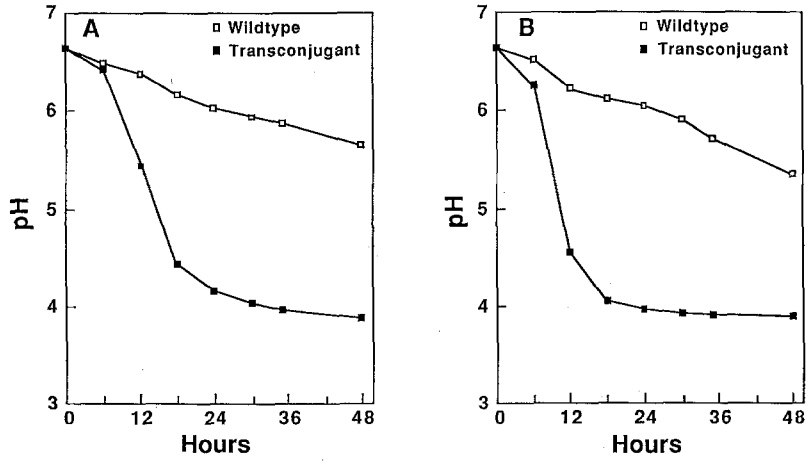

Fig. 2. pH vs. incubation time in M17 with 0.5% sodium glycerophosphate. pH value reduced over time when 1% wild-type *L. cremoris* CAF7, and 1% transconjugant *L. cremoris* HT6 were inoculated in M17 with 0.5% sodium glycerophosphate and incubated at 25°C (A), and at 30°C (B).

Table 3

King test for C4 compounds

Strains	Culture preparations			
	incubation (h)	temperature (°C)	supplement[d]	results
(1) *Leuconostoc cremoris* CAF7[a]	24	30	0.2% YE	−
(2) *Leuconostoc cremoris* CAF7	24	23	0.2% YE	−
(3) *Leuconostoc cremoris* CAF7	16	23	None	−
(4) *L. cremoris* HT6[b]	24	30	0.2% YE	+ + +
(5) *L. cremoris* HT6	24	23	0.2% YE	+
(6) *L. cremoris* HT6	16	23	None	−
(7) *Streptococcus lactis* C2	16	23	None	−
(8) *S. lactis* subsp. *diacetylactis* DRC1[c]	16	23	None	+ + + +
(9) (3) + (7)	16	23	None	+ + + +
(10) (6) + (7)	16	23	None	+ + + +
(11) (7) + (8)	16	23	None	+ + + +

[a] Recipient; [b] transconjugant; [c] positive control organism; [d] in litmus milk.

−, negative; + + + +, 100% absolute color intensity; + + +, 75% relative color intensity; +, 25% relative color intensity.

23°C, room temperature; YE, yeast extract.

acid produced was not sufficient to allow *L. cremoris* HT6 to produce diacetyl. All cultures of wild-type cells were negative for the King test. However, when acid-producing *S. lactis* C2 was added to these cultures, the King test became positive (Table 3).

Table 4

Streptococcus lactis C2 phage assay and vancomycin resistance for Lac$^+$ donor, recipient and transconjugants

Host cell	c2 phage titer (PFU/ml)	Vancomycin resistance (μg/ml)
Streptococcus lactis C2[a]	$2 \cdot 10^7$	< 10
Leuconostoc cremoris CAF7[b]	< 10	> 500
L. cremoris HT6[c]	< 10	> 500
L. cremoris HT32[c]	< 10	> 500

[a] Donor; [b] recipient; [c] transconjugant.

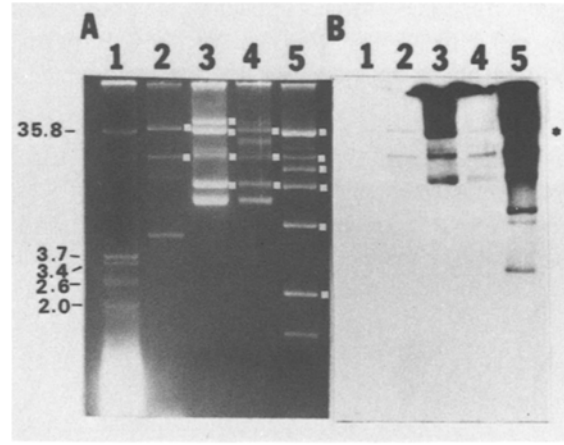

Fig. 3. Agarose gel electrophoresis and DNA-DNA hybridization of plasmids. (A) Agarose gel (0.7%) electrophoresis of plasmid DNA from: lane 1, *E. coli* V517; lane 2, recipient *L. cremoris* CAF 7; lane 3 and 4, transconjugants *L. cremoris* HT6 and HT32, respectively; lane 5, Lac$^+$ plasmid donor *S. lactis* C2. (B) Autoradiogram of Southern blots corresponding to (A). The probe (*) was the ^{32}P-labeled 30 Md plasmid from the donor organism. The white square identifies the plasmids homologous to the probe.

Streptococcus lactis C2 phage assay and vancomycin resistance

The donor cells, *S. lactis* C2, were sensitive to both phage c2 and to vancomycin (10 μg/ml). However, the recipient cells, *L. cremoris* CAF7, and transconjugants, *L. cremoris* HT6 and HT32, were totally resistant both to phage c2 and to > 500 μg/ml vancomycin (Table 4).

Plasmid profiles

Two types of plasmid profiles were observed among ten transconjugants examined. Some transconjugants had plasmids of 58, 30, 19.1, 17.2, 9.4 and 6.5 Md; these were designated type I (Fig. 3A, lane 3). Some transconjugants had plasmids of 30, 24, 18.5, 17.2, 9.7 and 6.5 Md and were designated type II. The ratio of type I to type II was 3:2 among ten transconjugants examined. The only difference between these two was that type I had a 58 Md plasmid while type II had a 24 Md plasmid (Fig. 3A). The phenotypic characteristics of all transconjugants were Lac^+Prt^-.

Homologous plasmids of donor, recipient and transconjugant cells

Using the 30 Md plasmid of *S. lactis* C2 as a probe, hybridization results showed that there were homologies in the remaining *S. lactis* C2 plasmids except for the 1.4 Md plasmid. In addition, this plasmid probe hybridized well with two plasmids of recipient cells and three and four plasmids of the transconjugants, HT32 and HT6, respectively (Fig. 3B).

DISCUSSION

Results indicate that the Lac^+Prt^- transconjugants are able to grow symbiotically in milk with protease positive strains. Only when nitrogen sources, such as yeast extract or casein degradation products produced by the Lac^-Prt^+ mutant, were supplied would they grow well and acidify milk. Klaenhammer et al. [11] suggested that 12.5 and 18 Md plasmids were involved in proteinase production by *S. lactis* C2. The transconjugants obtained during this study did not contain any plasmids near this molecular weight (Table 1).

The transconjugants were King-test-positive when grown in litmus milk containing 0.2% yeast extract, indicating that they produced acid by fermenting lactose; the resultant drop in pH then promoted flavor and aroma development. Wild-type *Leuconostoc* cells did not demonstrate this ability, even when yeast extract was added. Hence, they depend on acid-producing bacteria to produce aroma compounds. On the other hand, the transconjugants coagulated milk and produced diacetyl without requiring the presence of acid-producing strains.

The transconjugants evidently were derivatives of *L. cremoris* CAF7 because they were resistant to 500 μg/ml vancomycin to phage c2 and they grew on the selective azide medium for *Leuconostoc* described by Mayeau et al. [16]. While the nisin-producing *Leuconostoc* transconjugants described earlier [21] produced gas from glucose in MRS broth sealed with Vaspar, transconjugants HT6 and HT32, were negative when tested for gas in this manner. The recipient was also negative in this test but did produce small amounts of gas when tested by the procedure of Holmes et al. [9].

The Lac^+ donor, *S. lactis* C2, did not produce bacteriocin to inhibit recipient cells of *L. cremoris* CAF7. However, the recipient cells produced bacteriocin (data not shown) which would inhibit donor cells. Therefore, the conjugation needed to occur before *L. cremoris* CAF7 reached the mid-log growth phase. After conjugation, the prospective transconjugants had to be properly diluted in order to avoid overcrowding on the M17-Vm-BCP selective agar. If there were too many colonies, the purple background of the agar would turn yellow, making it difficult to judge which colonies were Lac^+ transconjugants.

Intrastrain and interspecies sequence homologies occurring between plasmids of bacteria have been reported [25]. The present study gives additional evidence that homologous plasmid regions occur between different genera, i.e. *Streptococcus* and *Leuconostoc*. Also high-frequency conjugal transfer of the lactose fermenting gene of group N

streptococci has been reported in *S. lactis* 712 [7], *S. lactis* ML3 [1,24], and *S. lactis* ME2 [12]. The present report is believed to be the first describing transfer of Lac from lactic streptococci to *Leuconostoc.*

The lactose fermenting gene of *S. lactis* C2 is located on a 30 Md plasmid [11]. Two plasmids of the Lac^- *L. cremoris* CAF7 were homologous with the C2 Lac plasmid probe. Therefore, these two resident plasmids are not responsible for the lactose fermenting ability. This indicates that the homologous segment of plasmid DNA of the recipient cells does not code for lactose metabolism in group N streptococci, so the Lac^+ phenotype must be due to the presence of other genes or insertion sequences. In contrast to the Lac^-Prt^- phenotype of recipient cells, transconjugants were Lac^+Prt^- because they fermented lactose. Also they had plasmids which hybridized with the probe.

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