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Conjugative 40-Megadalton Plasmid in *Streptococcus lactis* subsp. *diacetylactis* DRC3 Is Associated with Resistance to Nisin and Bacteriophage†

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Streptococcus lactis subsp. *diacetylactis* DRC3 was examined for plasmid DNA and found to contain a previously unreported plasmid of 40 × 10^6 daltons. This plasmid, designated pNP40, was conjugally transferred to a plasmid-cured derivative of *S. lactis* C2. Transconjugants containing pNP40 acquired resistance to nisin produced by strains of *S. lactis* and to commercially available nisin when assay plates were incubated at 21, 32, and 37°C. In addition, c2 phage growth was completely restricted in transconjugants containing pNP40 at 21 and 32°C, but not at 37°C. This result suggests that pNP40 may be coding for a temperature-sensitive enzyme that restricts phage growth at 21 and 32°C, but not at 37°C. Eight consecutive transfers of a transconjugant containing pNP40 in Elliker broth at 37°C resulted in 100% loss of resistance to c2 phage when colonies were tested at 32°C. These phage-sensitive isolates had lost pNP40 and had also become sensitive to nisin. This result suggests that pNP40 may also be thermosensitive in its replication. The finding of a phage resistance determinant located on a conjugative plasmid should prove useful in constructing phage-resistant variants for dairy fermentation processes.

Plasmid biology is fast becoming an important area for research in bacteria used for milk, meat, and plant fermentation processes as well as for probiotics. It is now well established that lactic acid bacteria used in these processes harbor plasmids of diverse sizes (4, 7, 23, 28, 37). Further, it has been demonstrated that these organisms characteristically contain many plasmid species. The number observed ranges from 2 to 11, but most strains appear to contain 4 to 7 distinct plasmid sizes. Most of the plasmids observed in these organisms are cryptic, but some carry identifiable metabolic properties. In group N streptococci, phenotypic, physical, and genetic evidence has been obtained for plasmid-mediated lactose utilization (26, 42) as well as for production of a bacteriocin-like substance in *Streptococcus lactis* subsp. *diacetylactis* WM_4 (40). Phenotypic and physical evidence for plasmid linkage of metabolic traits in this group of streptococci has also been obtained for citrate (18), sucrose, glucose, mannose, and xylose utilization (23), galactose metabolism (5, 35), proteinase activity (28, 34), modification-restriction systems (38, 39), inorganic ion resistance (10), and nisin production (12, 21). Phenotypic and genetic evidence suggests that diplococcin production by some *Streptococcus cremoris* strains may be plasmid mediated (6). Due to the importance of plasmids in dairy fermentation processes, attempts to elucidate the functional properties of cryptic plasmids in food-fermenting microorganisms must continue. The results presented in this communication indicate that a 40-megadalton (Mdal) conjugative plasmid in *S. lactis* subsp. *diacetylactis* DRC3 carries genetic determinants for resistance to c2 phage at 21 and 32°C, but not at 37°C, as well as for nisin resistance.

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MATERIALS AND METHODS

Bacterial strains. All microorganisms used in this study were maintained by biweekly transfers at 32°C in M17 broth containing 0.5% glucose or lactose (43). Table 1 lists the strains of *S. lactis* and *S. lactis* subsp. *diacetylactis* used.

Conjugal matings. Solid surface matings were performed as previously described (29). Cells from the milk agar mating plate were harvested by pipette and plated in 0.2-ml samples on indicator agar containing the appropriate carbohydrate and selective agent. A second method of harvesting cells was to replica plate from the milk agar plate directly onto the appropriate indicator agar.

Nisin sensitivity. Nisin-producing (Nis^+) *S. lactis* DL16, 11454, and 354/07 were grown in M17-lactose broth at 32°C for 16 h. Cultures were diluted in 0.85% NaCl and surface plated on Elliker agar (11) plates. After incubation at 32°C for 22 to 29 h, the agar was flipped into the petri dish cover and overlaid with 7 ml of tempered Elliker agar (0.7%) containing 7% of the cells to be tested for sensitivity to nisin that had been grown in M17-lactose or M17-glucose broth at 32°C for 16 h. Overlaid plates were incubated at 32°C.

Resistance to nisin was also determined by the method of Novick and Roth (33), except Elliker agar adjusted to pH 6.3 and supplemented with 1% Tween 20 was used as the plating medium. Incorporation of Tween 20 facilitates the diffusion of nisin throughout the agar (16); additionally, we found that it aided the dispersal of cells in an agar medium containing nisin. Nisin (Aplin and Barrett Ltd., England) was prepared in 0.02 N HCl and filter sterilized through a 0.45-μm Nalgene filter unit.

Phage Assay. *S. lactis* C2 lytic phage (c2) was prepared by adding phage to an actively growing, 32°C culture of C2 in phage broth (27). Incubation continued until lysis was complete. The lysate was filter sterilized by passing through a 0.45-μm Gelman Acrodisc filter unit.

TABLE 1. Bacterial strains

Strain	Plasmids (Mdal)	Description[a]
S. lactis C2	30, 18, 12, 5, 2, 1	Wild type, Lac$^+$ Prt$^+$
S. lactis LM2301	None	Lac$^-$ Prt$^-$ Str^r derivative of C2 (30)
S. lactis LM2302	None	Ery^r mutant of LM2301 (30)
S. lactis subsp. *diacetylactis* DRC3	52, 40, 34, 26.5, 5.5, 3.5, 3.2, 1.8	Wild type, Lac$^+$ Prt$^+$ Str^s
S. lactis subsp. *diacetylactis* D65	60, 40, 26.5, 5.5, 3.5, 3.2, 1.8	Lac$^+$ Prt$^-$ Str^s isolate of DRC3 (this study)
S. lactis K6528	60, 40	Lac$^+$ Str^r transconjugant of D65 × LM2301 (this study)
S. lactis D6528	40	Spontaneous Lac$^-$ isolate of K6528 (this study)
S. lactis KC5	52	Lac$^+$ Str^r transconjugant of DRC3 × D6528 (this study)
S. lactis KC7	52, 40	Lac$^+$ Str^r transconjugant of DRC3 × D6528 (this study)
S. lactis DL16	Not determined	Lac$^+$ Xyl$^+$ Nis$^+$ (23)
S. lactis 11454	Not determined	Lac$^+$ Nis$^+$ (23)
S. lactis 354/07	Not determined	Lac$^-$ Nis$^+$ (16)

[a] Lac$^+$, Lactose fermenting; Lac$^-$, lactose negative; Prt$^+$, proteinase positive; Prt$^-$, proteinase negative; Str^r, streptomycin resistant; Str^s, streptomycin sensitive; Ery^r, erythromycin resistant; Xyl$^+$, xylose fermenting; Nis$^+$, nisin producing.

Phage preparations from cells grown in 11% nonfat milk supplemented with 0.25% milk protein hydrolysate (NFM-MPH, pH 7.0, steamed 60 min) were obtained by acidifying the milk to pH 4.5 with 10% lactic acid followed by centrifugation at 9,000 × *g* for 10 min. The supernatant was retained, neutralized with 1 N NaOH, and centrifuged at 14,000 × *g* for 10 min. The resulting supernatant was filter sterilized as above.

The phage titer was determined by diluting in 0.85% saline and plaquing with the appropriate indicator strain on M17-glucose phage agar (43).

Phage adsorption. Adsorption of c2 lytic phage to *S. lactis* LM2301 and *S. lactis* D6528 was determined as described by Limsowtin and Terzaghi (24).

Activity test. An activity test that simulates the temperature changes during the manufacture of Cheddar cheese was employed (14). A 2% inoculum of C2 or K6528 was made into 11% NFM-MPH in the presence or absence of 9.0×10^3 PFU of c2 lytic phage per ml. Tubes were subjected to the test conditions described by Heap and Lawrence (13). After the 5-h test, pH determinations were made, and whey samples were obtained as described above. On each succeeding day, for 3 days, 2% fresh culture of K6528 was inoculated into NFM-MPH containing 10^2 PFU of c2 phage per ml and 0.1 ml of whey from the previous day's test, and the activity test was repeated. After the third growth cycle, all whey samples were assayed for phage.

The activity test was repeated in the absence of c2 lytic phage. After the sixth cycle, the culture was diluted and plated on bromcresol purple-lactose indicator agar (30). Individual colonies were picked, smeared on bromcresol purple-lactose agar, and spotted with 10 μl of a c2 phage stock solution (10^6 PFU/ml). Plates were incubated at 32°C for 16 to 24 h.

Appearance of phage-sensitive cells of K6528. The first method used consisted of eight successive transfers of K6528 in Elliker broth at 21°C for 24 h or at 32 or 37°C for 16 h. Before each transfer, the inocula were diluted 1/1,000 in 0.85% saline. After incubation at the appropriate temperatures, the eighth cycle of each temperature series was diluted in saline, plated on bromcresol purple-lactose agar, and incubated at 32°C. Individual colonies were picked and tested for phage sensitivity. The second method consisted of a 1% inoculum of K6528 (grown in NFM-MPH at 21°C for 16 h) into tubes of NFM-MPH containing 10^5 PFU of c2 lytic phage per ml. Tubes were separately incubated at 21°C for 16 h or 32 or 37°C for 6 to 8 h. Whey samples were obtained and pH determinations were made for each growth period. On the second and third days, a fresh 1% inoculum of K6528 was made into 9.9 ml of NFM-MPH containing 10^2 PFU of c2 phage per ml and 0.1 ml of whey from the previous incubation. All whey samples were assayed for phage.

Cell lysis and agarose gel electrophoresis. The modified lysis procedure of Anderson and McKay (2) was used to obtain cleared lysates of the strains used in this study. Cleared lysates containing plasmid DNA were subjected to agarose gel electrophoresis as previously described (20).

RESULTS

Strain derivations and plasmid content. The parental strain, *S. lactis* subsp. *diacetylactis* DRC3, was previously shown to contain plasmids of 52, 34, 26.5, 5.5, 3.5, 3.2, and 1.8 Mdal (29). With the improved lysis procedure of Anderson and McKay (2), an additional plasmid of 40 Mdal was identified in DRC3 (Fig. 1). A spontaneous Lac$^+$ proteinase-negative (Prt$^-$) derivative of DRC3, designated D65, was isolated which contained plasmids of 60, 40, 26.5, 5.5, 3.5, 3.2, and 1.8 Mdal (Fig. 1). The 40-Mdal plasmid (designated pNP40) may have been responsible for the formation of recombinant Lac plasmids when DRC3 was used as a donor in conjugal matings and appeared to be involved in mobilization of the 60-Mdal Lac plasmid when D65 was used as a donor in conjugal matings (unpublished data). K6528 is a Lac$^+$ streptomycin-resistant (Str^r) transconjugant of a mating between Lac$^+$ Str^r D65 (donor) and LM2301 (recipient), which is a

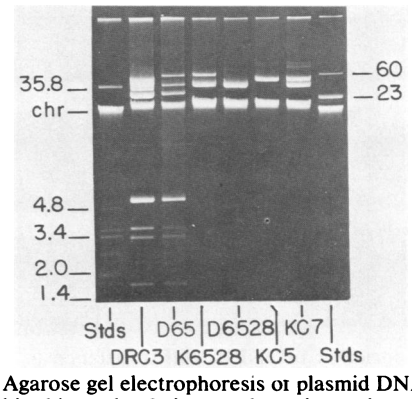

FIG. 1. Agarose gel electrophoresis of plasmid DNA detected in strains used in this study. *S. lactis* subsp. *diacetylactis* DRC3 is the Lac$^+$ Prt$^+$ parental culture, and D65 is a Lac$^+$ Prt$^-$ derivative of DRC3. K6528 is a Lac$^+$ transconjugant of a mating between D65 and *S. lactis* LM2301, a plasmid cured derivative of *S. lactis* C2. D6528 is a Lac$^-$ clone of K6528. KC5 and KC7 are Lac$^+$ transconjugants from a mating between DRC3 and K6528. Plasmids in *E. coli* V517 (far left) and *Salmonella typhimurium* LT2 (60 Mdal) and *E. coli* J53 (23 Mdal) on the right were used as molecular mass standards as previously described (20).

plasmid cured Lac$^-$ Str^s derivative of *S. lactis* C2. K6528 contained the 60-Mdal plasmid and pNP40, whereas a spontaneous Lac$^-$ clone (D6528) of K6528 possessed only pNP40 (Fig. 1). Matings between Lac$^+$ Str^s DRC3 and Lac$^-$ Str^r D6528 yielded Lac$^+$ Str^r transconjugants. The profiles of two such transconjugants are shown in Fig. 1. KC7 contained the 52-Mdal Lac plasmid from DRC3 and pNP40 carried by D6528. KC5 also acquired the 52-Mdal plasmid, but had lost pNP40.

Plasmid association of nisin resistance. We attempted to conjugate xylose-fermenting ability (Xyl$^+$) from Str^s erythromycin-sensitive (Ery^s) *S. lactis* DL16 into xylose-negative (Xyl$^-$) erythromycin-resistant (Ery^r) Str^r LM2302 or Xyl$^-$ Str^r D6528. Growth on the milk agar conjugation plates was replica plated onto bromcresol purple-xylose plates containing 15 μg of erythromycin per ml for LM2302 matings and bromcresol purple-xylose plates containing 600 μg of streptomycin per ml for D6528 mating. After incubation of the indicator plates, no Xyl$^+$ transconjugants were observed for either mating. However, no lawn of Xyl$^-$ background growth was observed when using LM2302 as the recipient, whereas a nearly confluent Xyl$^-$ background of D6528 cells was present. Since *S. lactis* DL16 produces nisin (23), this result suggested that the plasmid-cured LM2302 recipient cells were sensitive to the nisin produced by DL16 and that pNP40 may have conferred resistance to nisin when transferred into LM2301 cells to obtain D6528.

To further examine the possible relationship between nisin resistance and pNP40, Nis$^+$ colonies of *S. lactis* DL16, 11454, and 354/07 were overlaid with various test strains

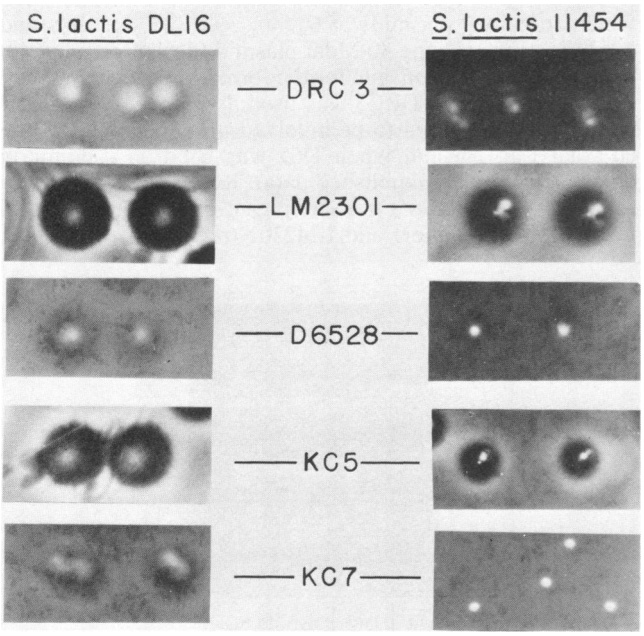

FIG. 2. Association of pNP40 with resistance to nisin. Nisin-producing cultures of *S. lactis* DL16 or *S. lactis* 11454 were diluted and surface plated on Elliker agar plates. After incubation at 32°C for about 25 h to obtain individual colonies, the agar from separate plates was flipped into the petri dish covers and overlaid with soft agar inoculated with various test strains containing or lacking pNP40. Strains possessing pNP40 (DRC3, D6528, and KC7) grew uniformly throughout the agar overlay above 11454 colonies and showed reduced growth above DL16 colonies. LM2301 and KC5, which do not harbor pNP40, exhibited large zones of growth inhibition above Nis$^+$ colonies of DL16 and 11454.

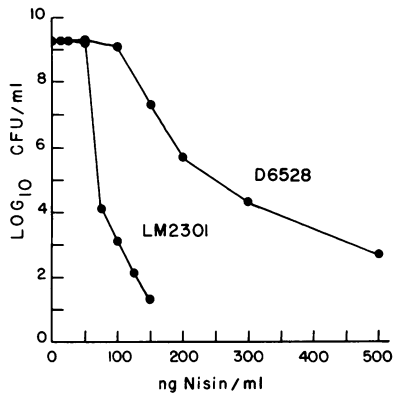

FIG. 3. Survival of colony-forming ability of *S. lactis* LM2301 and *S. lactis* D6528 as a function of nisin concentration.

containing or lacking pNP40. Those strains containing pNP40 (DRC3, D6528, and KC7) all grew uniformly throughout the agar overlay above 11454 colonies (Fig. 2) and 354/07 colonies (data not shown). A slight reduction in cell growth was visible directly above the Nis$^+$ colonies of DL16 (Fig. 2). The two strains that do not harbor pNP40 (LM2301 and KC5) exhibited large zones of inhibition of growth above Nis$^+$ colonies of DL16 and 11454 (Fig. 2) as well as above 354/07 Nis$^+$ colonies (data not shown).

A resistivity curve illustrating the difference in nisin sensitivity between a strain harboring pNP40 (D6528) and one lacking this plasmid (LM2301) is shown in Fig. 3. In the presence of 50 ng of nisin per ml, the cell populations for both strains remained at levels approximately equal to cells plated in the absence of nisin (about 10^9 CFU/ml). However, a concentration of 100 ng of nisin per ml reduced the population of LM2301 to 1.2×10^3 CFU/ml, whereas D6528 remained at about 10^9 CFU/ml. LM2301 cell numbers declined to 19 CFU/ml in the presence of 150 ng of nisin per ml, whereas it took 1,500 ng of nisin per ml for D6528 cells to decline to this population level (data not shown).

Plasmid association of phage resistance. Results obtained in our laboratory indicated that when *S. lactis* LM2301 acquired pNP40, it was no longer susceptible to lysis by c2 lytic phage. A plaque assay was then performed with various indicator strains. Table 2 shows that the c2 phage readily replicated on strains that did not harbor pNP40 (LM2301 and KC5), but no plaques were observed on strains containing pNP40 (DRC3, D6528, and KC7). To determine whether the absence of plaques was due to the inability of the phage to adsorb to strains carrying pNP40, LM2301 and D6528 were examined for phage adsorption. LM2301 cells adsorbed 99.3% of the available c2 phage, and D6528 cells adsorbed 98.9% of the available c2 phage (Table 2). These results indicated that inability to adsorb phage was not responsible

TABLE 2. Titer and adsorption of *S. lactis* C2 lytic phage on various indicator strains

Strain	PFU/ml	% Phage adsorption
S. lactis subsp. *diacetylactis* DRC3	0	ND[a]
S. lactis LM2301	7.0×10^7	99.3
S. lactis D6528	0	98.3
S. lactis KC5	3.0×10^7	ND
S. lactis KC7	0	ND

[a] ND, Not determined.

TABLE 3. Effect of laboratory activity test on pH and appearance of lytic phage against *S. lactis* C2 and *S. lactis* K6528 when K6528 was challenged with c2 lytic phage

No. of growth cycles	pH		PFU/ml[a]	
	− Phage	+ Phage	C2[b]	K6528[c]
1	5.16	5.33	9.2×10^7	0
2	5.15	5.49	8.5×10^9	0
3	5.15	5.72	2.3×10^9	0

[a] The phage titer was determined only from K6528 cells which were challenged with c2 lytic phage.
[b] Whey samples were plaqued on *S. lactis* C2 at 32°C.
[c] Whey samples were plaqued on *S. lactis* K6528 at 32°C.

for the absence of plaques on D6528.

Laboratory activity test. To determine whether modified lytic phage would rapidly appear against K6528, a New Zealand activity test was performed (14). In this test, phage-resistant strains are propagated repeatedly through milk in the presence of phage to determine the number of growth cycles that could be achieved before lytic phage would appear against the test strain. During the activity test, the temperature cycles from 32 to 39°C and back to 32°C over a 5-h period and simulates the temperature changes during Cheddar cheese manufacture. The pH after 5 h should be 5.0 to 5.2 for an active culture. When *S. lactis* C2 was grown in the presence of c2 lytic phage, a pH of 6.31 was achieved after one growth cycle, whereas the control C2 culture in the absence of phage reached pH 5.09. The former result is indicative of acid production being limited by a high phage titer. After one growth cycle, K6528 reached a pH of 5.16, and K6528 challenged with c2 phage attained a pH of 5.33 (Table 3). After the second and third growth cycles of K6528 with phage challenge, however, the pH obtained was higher, suggesting the buildup of lytic phage against K6528 in the milk (Table 3). To investigate this possibility, titers of phage against C2 and K6528 were determined in whey samples from each cycle (Table 3). A titer of 9.2×10^7 PFU/ml was obtained after one growth cycle of K6528 cells challenged with phage when C2 was used as the plaquing organism. The initial c2 phage titer was 10^3 PFU/ml. Whey samples from cycles two and three also demonstrated high phage titers on C2. Surprisingly, however, no plaques were detected when the whey samples were tested on K6528 cells. These results suggested that rather than the phage being modified, phage-sensitive derivatives of K6528 were appearing during the growth cycle treatments.

Appearance of phage-sensitive cells of K6528. The activity test described above was repeated in the absence of c2 lytic phage through six growth cycles. When individual colonies were isolated and treated with c2 phage, 24.9% of the K6528 isolates were sensitive to the phage. Sanders and Klaenhammer (38, 39) suggested that the heat treatment included during the activity test facilitated the loss of phage restriction systems, leading to the production of a modified lytic phage, and that this phenomenon was related to loss of restriction plasmids during growth at the elevated temperatures. Hence it was possible that K6528 was losing pNP40 when propagated at the elevated temperature. To determine the effect of various temperatures on the appearance of phage-sensitive (ϕ^s) cells, K6528 was successively transferred eight times in Elliker broth at 21, 32, and 37°C, followed by dilution and plating on BCP-lactose indicator agar. Lac^+ and Lac^- colonies were isolated from the 21 and 32°C transfers, and only Lac^- colonies were isolated from the 37°C transfers (Table 4). A total of 70 Lac^- and 7 Lac^+ colonies from the 21°C transfers were tested with c2 phage; all 7 Lac^+ and 61 Lac^- colonies were phage resistant (ϕr), whereas 9 Lac^- colonies (11.6%) were ϕ^s. At 32°C all 10 Lac^+ colonies examined were ϕ^r, and of 92 Lac^- colonies examined, 89 were ϕ^r and three (2.9%) were ϕ^s. All 90 Lac^- colonies (100%) examined from platings of 37°C transfers were ϕ^s. Since the extent of loss of lactose-fermenting ability and phage resistance seemed unusually high after eight transfers in Elliker broth at 37°C, we determined loss of both phenotypes after one transfer at 32 or 37°C. After the 16-h incubation at 32°C, 12% of the K6528 cells were Lac^-. All Lac^- and Lac^+ colonies tested (12 and 88, respectively) were ϕ^r. Forty-three percent of the K6528 cells incubated at 37°C for 16 h were Lac^-. Of 43 Lac^- colonies and 57 Lac^+ colonies examined, 42 Lac^- and 56 Lac^+ colonies were still ϕ^r. Clearly, successive transferring at high temperature was increasing the loss of phage resistance.

Temperature-dependent growth of c2 phage on K6528. To assess the increase in phage titer due to the appearance of phage-sensitive mutants at 21, 32, and 37°C, three successive transfers were made in NFM-MPH in the presence of c2 phage or c2 phage plus 0.1 ml of whey from a previous transfer. The data in Table 5 for the 21 and 32°C transfers indicate that the phage were unable or only slightly able to replicate, since the phage titers on C2 cells were at the expected levels based on the titer of c2 phage added to the milk samples. However, a minimal increase was observed after the third transfer. The pH values paralleled these observations. The data for transfers at 37°C indicated a dramatic increase in phage titer on C2 cells, coupled with a decreased ability of K6528 to produce acid. The phage from these whey samples were unable to form plaques on K6528 when assayed at 32°C (Table 3). Since K6528 cells appeared to become sensitive to lysis by c2 phage at 37°C, c2 phage was plaqued on C2 and K6528 cells; one set of plates was incubated at 32°C, and another was incubated at 37°C. At 32°C, no plaques were observed on K6528, whereas a titer of 7.2×10^5 PFU/ml was obtained with C2. However, at 37°C, the c2 phage formed plaques on both strains. The titer was 1.7×10^4 PFU/ml for K6528 and 8.9×10^5 PFU/ml for C2 cells.

To confirm the temperature-dependent restriction of c2 phage growth by pNP40, host cells were grown at 21, 32, or 37°C and used to titer the phage on LM2301 or D6528 at 21, 32, or 37°C (Table 6). The results indicate that c2 phage is restricted by the cells harboring pNP40 at 21 and 32°C, but not at 37°C. The efficiency of plating was generally lower than that on LM2301 even at the permissive temperature (0.06 to 0.33), and complete restriction of c2 phage growth at 21 and 32°C was observed.

Since the above data indicated a temperature-dependent relationship for phage resistance, we reexamined the effect

TABLE 4. Effect of temperature on appearance of Lac^- or ϕ^s (or both) derivatives from Lac^+ ϕ^r K6528

Incubation temp (°C)	Total no. of colonies examined[a]	No. of colonies			
		Lac^+ ϕ^r	Lac^+ ϕ^s	Lac^- ϕ^r	Lac^- ϕ^s
21	77	7	0	61	9
32	102	10	0	89	3
37	90	0	0	0	90

[a] *S. lactis* K6528 was successively transferred eight times in Elliker broth at the indicated temperature before plating for colony isolation.

TABLE 5. Changes in pH and phage titer when *S. lactis* K6528 was challenged with c2 lytic phage at three different temperatures

No. of transfers	21°C			32°C			37°C		
	pH	PFU/ml[a]		pH	PFU/ml[a]		pH	PFU/ml[a]	
		C2	K6528		C2	K6528		C2	K6528
1	4.6	1.4×10^5	0	4.7	1.0×10^5	0	6.0	1.4×10^{10}	0
2	4.6	3.2×10^3	0	4.8	7.2×10^3	0	6.6	4.4×10^8	0
3	4.7	3.5×10^4	0	4.9	2.6×10^4	0	6.2	4.0×10^9	0

[a] The phage titer was determined after each incubation by plaquing on *S. lactis* C2 or *S. lactis* K6528 and incubating the assay plates at 32°C.

of temperature on nisin resistance conferred by pNP40. Unlike the temperature-dependent prevention of phage proliferation, strains harboring pNP40 were resistant to nisin at 21 and 32°C as well as at 37°C (data not shown).

Linkage of ϕ^r to pNP40. When K6528 was successively transferred at 21, 32, and 37°C in Elliker broth, three types of isolates were obtained with respect to lactose-fermenting ability and phage sensitivity (Lac^+ ϕ^r, Lac^- ϕ^r, and Lac^- ϕ^s). A fourth derivative (Lac^+ ϕ^s) was isolated from the growth cycle experiments. The plasmid profiles of these four phenotypes are shown in Fig. 4. The Lac^+ ϕ^r clones all contained the 60-Mdal Lac plasmid and pNP40, i.e., K6528. Lac^- ϕ^r cells contained only pNP40, Lac^+ ϕ^s derivatives possessed only the 60-Mdal plasmid, and Lac^- ϕ^s isolates had lost both plasmids. Representative strains of the four phenotypes were also tested for sensitivity to nisin produced by DL16. In all cases, retention of pNP40 correlated with resistance to nisin, but loss of pNP40 was coupled with nisin sensitivity.

DISCUSSION

In 1974 Kozak et al. (21) presented phenotypic data suggesting that nisin production was plasmid mediated in *S. lactis*. In *S. lactis* 11454 there appeared to be an association of Nis^+ and sucrose-fermenting phenotypes to a 28-Mdal plasmid (23). Davey and Pearce (6) suggested that the *nis* genes of *S. lactis* could be chromosomal or plasmid mediated. A plasmid responsible for nisin resistance, however, had not previously been reported. A conjugative plasmid of 40 Mdal found in *S. lactis* subsp. *diacetylactis* DRC3, designated pNP40, carries genetic determinants for nisin resistance. This finding may have several practical implications. First, in the development of cloning vectors applicable to microorganisms used in dairy and food fermentation processes, traditional antibiotic selection markers may be unacceptable due to possible transmission of the drug resistance plasmid. Alternative markers must be used, and the nisin resistance trait on pNP40 may be useful as a selective marker for construction of cloning vehicles applicable to the food fermentation industry. Second, pNP40 may be useful in developing nisin-resistant strains for use in conjunction with Nis^+ strains to control butyric blowing of Emmental cheeses (25).

Host-controlled restriction of entering phage DNA is a common bacterial defense mechanism that functions by recognition and degradation of the unmodified DNA (3). Phage that escape destruction are modified during development to produce lytic phage that are immune to restriction by the host organism. The presence of modification-restriction systems in dairy streptococci was reviewed by Lawrence et al. (22). Loss of restriction activity in metabolizing cells of *S. cremoris* TR and KH after a heat challenge from 40 to 50°C was reported by Sanders and Klaenhammer (38). They suggested that the loss of plasmids carrying restriction enzymes during growth at elevated temperatures generated variants susceptible to phage. They observed the presence of a 10-Mdal plasmid in KH, but not in the majority of variants which were partially deficient in modification-restriction activities. This provided the first physical evidence implicating involvement of plasmids in modification-restriction systems among lactic streptococci. The results presented in this study indicated that pNP40, in addition to carrying nisin-

TABLE 6. Temperature-dependent restriction of c2 phage growth by pNP40

Growth temp (°C)[a]	Plaquing temp (°C)	PFU of c2 phage per ml plated on *S. lactis* strains:	
		LM2301 ($\times 10^9$)	D6528
21	21	2.5	0
21	32	2.7	0
21	37	2.7	1.6×10^8
32	21	2.2	0
32	32	2.9	0
32	37	2.2	5.0×10^8
37	21	1.5	0
37	32	2.3	0
37	37	3.0	1.0×10^9

[a] Cells were grown at respective temperatures in Elliker broth at 21, 32, or 37°C for 16, 6, and 4 h, respectively. A 0.1-ml sample of this culture was added to soft agar at 45°C, and 1.0 ml of appropriately diluted phage was added, mixed, and plated. Plates were incubated overnight at indicated temperatures, and plaques were counted.

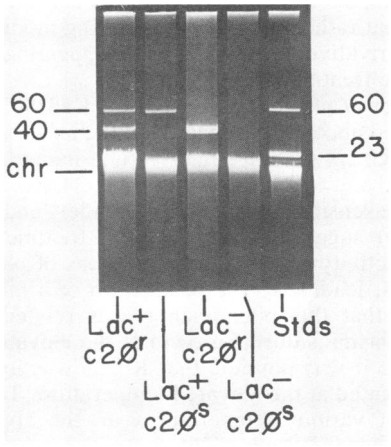

FIG. 4. Agarose gel electrophoresis of plasmid DNA detected in K6528 (Lac^+ and resistant to lysis by c2 phage [ϕ^r] at 21 and 32°C, but not at 37°C) and Lac^+ phage-sensitive (c2ϕ^s), Lac^- c2ϕ^r, and Lac^- c2ϕ^s derivatives of K6528. Molecular masses of plasmids were determined as in Fig. 1.

resistance determinants, also carried genetic determinants for phage resistance. Although transconjugants harboring pNP40 restricted c2 phage growth at 21 and 32°C, modified phage did not appear. The c2 phage were able to grow on transconjugants harboring pNP40 at 37°C. However, even after phage replication at 37°C on strains containing pNP40, the phage were unable to form plaques at 32°C when assayed on a host carrying pNP40. It therefore appears that we are not dealing with a typical modification-restriction system for the observed phage resistance.

Recently, Sanders and Klaenhammer (Abstr. Annu. Meet. Am. Dairy Sci. Assoc. 1983, abstr. D7, p. 57–58) presented evidence for involvement of a 36-Mdal plasmid in genetically determining phage adsorption in a strain of *S. lactis*. This plasmid, present in phage-resistant strains, appeared to code for a function that prevented phage adsorption. This was not the mechanism for phage resistance in the strain carrying pNP40, since it adsorbed 98% of the available phage.

Investigations on the effects of temperature on the interactions of lactic streptococcal phages and their hosts have revealed that the elevated temperatures used in the cooking of Cheddar cheese can effect phage proliferation. For example, Mullan et al. (31) reported on the interaction of 23 phages with lactic streptococcal strains at 30, 38, and 40°C. They found that eight of the phages exhibited extensive proliferation at all three temperatures, four replicated at 30 and 38°C, but not at 40°C, and 11 phages replicated at 30°C, but not at 38 or 40°C. This inability of certain phages to replicate at 37 and 40°C has also been noted in other investigations (19, 32, 41). In contrast to the temperature-sensitive phage replication observed by these workers, Pearce (36) observed that bacteriophage 643 formed plaques on *S. lactis* BA and on *S. cremoris* Z_8 at 37°C, but not at 30°C, and Hull and Brooke (15) noted that certain raw milk phages were active in milk only at temperatures above 30°C. In our study, strains harboring pNP40 were able to replicate the c2 phage at 37°C, but not at 21 or 32°C. It therefore appears that pNP40 is a plasmid that confers upon its host the ability to restrict c2 phage growth at 21 and 32°C, but not at 37°C. These observations are similar to those described by Ishaq and Kaji (17) for Rts1, a plasmid that confers upon *Escherichia coli* the ability to resist T_4 phage growth at 32°C, but not at 42°C. The temperature sensitivity was not at the step of T_4 phage adsorption to the cells, since even at temperatures nonpermissive for T_4 phage replication (32°C) the T_4 phage were bound to cells harboring Rts1 to almost the same extent as to cells missing the plasmid (9). c2 phage also exhibited similar adsorption efficiency at 32°C on strains with or without pNP40 (Table 2). Ishaq and Kaji (17) suggested that the plasmid Rts1 coded for a thermosensitive enzyme that specifically degraded T_4 DNA, resulting in the reduction of T_4 phage growth at 32°C, but not at 42°C. Whether a similar mechanism exists when c2 phage are propagated on strains carrying pNP40 is currently under investigation. This would represent another plasmid-linked mechanism by which lactic streptococci could become resistant to phage.

In addition to finding that strains carrying pNP40 allowed c2 phage replication at 37°C, but not at 21 or 32°C, it was noted that this plasmid was lost when the host cells were propagated at elevated temperatures. These cells, cured of pNP40, could then serve as the host for c2 phage replication. The high incidence of pNP40 loss when cells harboring this plasmid were propagated at 37°C also suggests that pNP40 is thermosensitive in its replication, as is the Rts1 plasmid from *E. coli* (8).

In conclusion, a conjugative plasmid of 40 Mdal found in *S. lactis* subsp. *diacetylactis* coded for nisin resistance and for resistance to phage c2. Independent genetic loci for these traits are suggested by data showing that resistance to nisin was conferred at 21, 32, and 37°C, whereas resistance to phage was only observed at 21 and 32°C. The specificity and range of phage resistance conferred by pNP40 are currently under investigation. The increasing evidence for association of plasmids with phage resistance in lactic streptococci provides a genetic mechanism to explain the rapid development of phage-sensitive dairy starter cultures and as suggested previously (7, 38), the manipulation of these plasmids by genetic engineering techniques could be one approach for obtaining phage-resistant mutants for commercial purposes. It may be of value to combine the different genetic loci for phage resistance into a single strain and to stabilize the phage-resistant phenotype by integrating the phage resistance genes into the chromosome or into a high-copy-number, thermostable plasmid. It may now be possible to develop resistant strains by conjugally transferring the appropriate plasmid or plasmids to selected phage-sensitive strains of dairy streptococci as well as to other lactic acid bacteria. Although pNP40 confers phage resistance to LM2301 when challenged with phage c2 at 21 and 32°C, it is interesting to note that DRC3, the original source of pNP40, is sensitive to lytic phage drc3 at 21, 32, and 37°C (unpublished data). Therefore, either the protective influence conferred by pNP40 is nonfunctional in DRC3 or drc3 phage is able to overcome the protective mechanism of pNP40. The construction of strains containing pNP40, in addition to other phage resistance mechanisms, could result in bulk culture preparation at 21 to 27°C without the expense of purchasing phage-resistant media for cell propagation.

Although it remains to be determined, one possible mechanism for nisin resistance in strains harboring pNP40 is the production of nisinase. This nisin-destroying enzyme has been found in some strains of *Streptococcus thermophilus* (1).

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