APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1996, p. 676–679 Vol. 62, No. 2
0099-2240/96/$04.00+0

The Lactococcal Plasmid pNP40 Encodes a Third Bacteriophage Resistance Mechanism, One Which Affects Phage DNA Penetration

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Received 1 June 1995/Accepted 5 December 1995

The lactococcal plasmid pNP40 mediates insensitivity to φc2 by an early-acting phage resistance mechanism in addition to the previously identified abortive infection system, AbiF, in the *Lactococcus lactis* subsp. *lactis* MG1614 background. A second abortive infection determinant on pNP40, AbiE, does not confer resistance to φc2. The early-acting mechanism on pNP40 does not prevent phage adsorption nor does it appear to operate by restriction/modification. Phage DNA was not detected in pNP40-containing cells until 30 min following exposure to φc2 compared with 5 min in a sensitive host; however, electroporation of phage DNA into resistant hosts resulted in the release of phage progeny from a dramatically elevated number of cells compared with conventionally infected hosts. It appears therefore that pNP40 encodes a novel phage resistance mechanism which blocks DNA penetration specifically for φc2.

Bacteriophage resistance is a critically important trait required of starter cultures employed in dairy fermentations. In many instances, strains exhibiting superior insensitivity to phage have been shown to harbor specific resistance determinants, which are often located on plasmids (10). Frequently, these plasmids are conjugative, and a food-grade conjugal transfer strategy has been used to introduce these replicons into selected starter strains, thereby enhancing their phage resistance properties (9, 14). Since additive effects have sometimes been observed when two or more plasmids are present in a single strain (2, 16), the identification of complementary phage resistance mechanisms is central to the selection of plasmids which can be combined in strain improvement programs.

Three categories of phage resistance systems are currently recognized in lactococci (10). Adsorption inhibition describes mechanisms which prevent phage attachment to the cell surface and include determinants which direct the synthesis of exopolysaccharides which surround the cell, preventing access by the phage to the cell surface receptors. The second category is the restriction and modification (R/M) systems, which are composed of a restriction endonuclease which degrades incoming phage DNA and a methylation component which is responsible for protection of the host DNA from digestion. The third category, abortive infection, describes mechanisms which act late in the lytic cycle after the phage has established infection by injecting its DNA and effecting shut-down of the host's metabolism.

In a previous study, the lactococcal plasmid pNP40 was identified as encoding two distinct abortive infection systems, AbiE and AbiF (5). In the laboratory strain, *Lactococcus lactis* subsp. *lactis* MG1614, AbiE was active against the small isometric-headed phage φ712 but exhibited no activity against the prolate-headed phage φc2, while AbiF was effective against both phages. In this study, analysis of the pNP40-encoded resistance against φc2 has identified a third phage resistance system which does not conform to the criteria defining any of the three phage resistance categories recognized in lactococci to date. We propose therefore that it represents a fourth type of phage resistance, which we have called penetration blocking. This novel mechanism is expected to be complementary to all other known lactococcal phage resistance systems.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *L. lactis* subsp. *lactis* MG1614 (plasmid-free derivative of strain 712 [6]) and derivatives were grown at 30°C in M17 medium supplemented with 0.5% glucose (18). PG020 and MG1614/pNP40 are derivatives of MG1614 containing pCG1 (recombinant plasmid harboring *abiF* locus [5]) and pNP40 (native phage resistance plasmid from *L. lactis* subsp. *lactis* biovar diacetylactis DRC3 [12]), respectively. pCG1 was maintained in *L. lactis* with chloramphenicol at 10 μg/ml.

Phage DNA preparation. Lactococcal phage DNA was isolated by the method of Fitzgerald et al. (4) with the modifications described by Coveney et al. (3).

Electroporation of bacteria. Electroporation of lactococcal strains was executed according to the procedure of Holo and Nes (8) with a gene pulser apparatus (Bio-Rad Corp., Richmond, Calif.).

Phage assays. Adsorption of phage to host cells was determined by adding 0.7 ml of a late-log-phase culture and 50 μl of 1 M $CaCl_2$ to 0.7 ml of phage (10^5 PFU/ml). Following incubation for 15 min at room temperature, the phage-host mixture was centrifuged for 10 min, and the supernatant was assayed for phage. The percentage adsorption was calculated as [(control titer − residual titer) (control titer)$^{-1}$] × 100.

The efficiency of the center of infection (ECOI) was determined by the method of Sing and Klaenhammer (15). Cells were infected with phage at a multiplicity of infection of 0.1 and incubated for 10 min to permit phage to adsorb to hosts. The ECOI was calculated as (the number of PFU of the infected resistant host) (then number of PFU of infected sensitive host)$^{-1}$.

Cell survival was assayed as described by Behnke and Malke (1). Surviving cells were enumerated as CFU. The percentage of cell death was calculated as [(CFU/ml in cultures without phage − CFU/ml in cultures with phage) (CFU/ml in cultures without phage)$^{-1}$] × 100.

Burst sizes were measured as described by Klaenhammer and Sanozsky (11). One-milliliter samples were removed at time zero and at increasing time intervals and assayed directly for phage by using plasmid-free MG1614 as a sensitive host.

Phage DNA replication. Intracellular phage DNA replication was monitored by the method of Hill et al. (7). DNA samples were digested with *Eco*RI and electrophoresed on 0.7% agarose gels. The DNA was subsequently transferred to a Hybond-N$^+$ nylon membrane (Amersham, Amersham, United Kingdom) by capillary blotting and probed with φc2 DNA by using the enhanced chemiluminescence gene detection system.

Determination of ECOI following electroporation of phage φc2 DNA into hosts. Phage φc2 DNA was electroporated into *L. lactis* subsp. *lactis* MG1614 and its derivatives and assayed for infective centers by plaquing the electropo-

TABLE 1. Parameters for φc2 proliferation in *L. lactis* subsp. *lactis* MG1614, PG020, and MG1614/pNP40

Strain	Resistance	% Adsorption	% Cell death	ECOI	Burst size
MG1614	None	99.4	100	1.0	161
PG020	AbiF	99.1	94	0.77	13
MG1614/pNP40	Total	98.6	10	0.0004	NP[a]

[a] NP, not possible to evaluate.

rated cells on a lawn of the sensitive host MG1614. The ECOI was calculated as (PFU of electroporated host) (PFU of electroporated sensitive host)$^{-1}$.

Electron microscopic analysis. A phage φc2 preparation was mixed with late-log-phase sensitive and resistant cultures at a multiplicity of infection of 10 in the presence of 10 mM $CaCl_2$ for 5 min. Phage-host mixtures were negatively stained with 2% uranyl acetate and examined with a JEOL (London, United Kingdom) 1200 EX transmission electron microscope at an accelerating voltage of 80 kV.

RESULTS

Resistance to φc2-encoded AbiF and pNP40. Two abortive infection mechanisms from pNP40, AbiE and AbiF, have been described previously (5). Although effective against φ712, AbiE mediated no resistance to φc2, while AbiF was active against both φ712 and φc2 in the *L. lactis* subsp. *lactis* MG1614 background. The effects of AbiF on φ712 proliferation, e.g., ECOI and burst size, have been determined previously (5). In this study, the resistance of cells containing AbiF to φc2 infection was found to be similar to that displayed against φ712. As expected for an abortive infection mechanism, a high proportion of cells (94%) died following φc2 infection as shutdown of the host metabolism had been initiated prior to operation of the abortive mechanism. The ECOI data indicated that the majority (77%) of infected cells released phage φc2 particles while the burst size was reduced dramatically from 161 for a sensitive host to only 13 in the pCG1-containing host (Table 1).

The effect of pNP40 on φc2 proliferation was more marked than that of pCG1 (AbiF). The presence of pNP40 in MG1614 completely eliminated φc2 plaque formation while pCG1 reduced the efficiency of plaquing of φc2 by only 10^4 with a reduced plaque size. Cell survival was increased in MG1614/pNP40 to 90%, a feature that cannot be explained by an abortive infection mechanism alone, which inevitably has little effect on cell survival. Therefore, an additional phage resistance mechanism in pNP40 operational against φc2, one that acted at an earlier stage in the lytic cycle, was indicated (Table 1). Furthermore, ECOI data revealed that very few cells containing pNP40 release viable phage progeny (ECOI, 0.0004) compared with pCG1-containing cells (ECOI, 0.77). This striking contrast also suggested that pNP40 encoded at least two phage resistance mechanisms against φc2: AbiF plus an early-acting mechanism.

Investigation of adsorption inhibition or R/M involvement in pNP40-encoded resistance to φc2. A number of assays were conducted to assess whether the enhanced level of pNP40-encoded resistance against φc2 was mediated by adsorption blocking or by R/M, the only early-acting mechanisms recognized to date in lactococci. Adsorption assays using *L. lactis* subsp. *lactis* MG1614 and MG1614 containing pNP40 revealed that over 98% of φc2 particles adsorbed to both hosts, indicating that the early-acting mechanism did not operate by classical adsorption blocking (Table 1). The possibility that nonspecific adsorption was occurring was discounted by examining φc2 adsorption by electron microscopy. The phage particles were shown to adsorb in a tail-first orientation to both hosts, confirming that pNP40 did not operate by interfering with phage binding to the host and that the adsorption assay results did not conceal nonspecific adsorption by the phage (Fig. 1).

R/M is a second early-acting phage resistance mechanism commonly encountered in lactococci (for a review, see reference 10). Through the use of mutant φc2, capable of plaquing on cells containing AbiF, and of two alternative pNP40-containing strains and their homologous phages, no R/M activity was detected (unpublished data).

Effect of AbiF and pNP40 on φc2 DNA replication. AbiF was previously shown to reduce the rate of phage φ712 DNA replication (5). When the effect of AbiF on φc2 DNA replication was monitored over time, a similar response was observed (Fig. 2). The increase in the φc2 DNA content of cells following infection was considerably delayed in PG020 compared with the sensitive host. A high intracellular φc2 DNA concentration was not detected until 30 min following infection as opposed to 10 min in the sensitive host, supporting the view that *abiF* encodes a mechanism which depresses the rate of φc2 DNA replication. A remarkable difference, however, was observed in cells containing pNP40. Phage c2 DNA could not be detected in the cell until 30 min after infection and even then the concentration was comparatively low. This finding could reflect a delay in phage DNA internalization or, more likely, a reduction in the number of cells into which the phage DNA was injected. In the latter case, the phage DNA detected at 60 and 90 min postinfection would represent replication of the few phage genomes which were internalized, and the subsequent reduction in phage DNA concentration at 120 min could therefore reflect lysis of a proportion of these cells. This hypothesis supports earlier evidence which suggested that an early-acting mechanism against φc2 is operational in cells containing pNP40.

Electroporation circumvents the early-acting mechanism. Electrotransformation of φc2 DNA into pNP40-containing cells effectively permits the phage to bypass the adsorption and DNA injection stages of infection. To investigate whether the putative early-acting mechanism could be circumvented in this manner, the ECOI of φc2 following electroporation, which indicates the relative proportion of transformed cells that gives rise to infective centers, was compared with the ECOI determined following a conventional infection (Table 2). Following phage DNA electroporation, the ECOI for φc2 on MG1614/pNP40 differed by approximately 10^3 from the ECOI for a conventional infection. Since adsorption blocking was discounted, this ECOI difference suggests that pNP40-mediated resistance to φc2 operates by blocking phage DNA penetration into the cell in addition to aborting phage DNA replication through the AbiF-mediated system.

DISCUSSION

Three categories of naturally occurring phage resistance mechanisms are recognized in lactococci: adsorption inhibition, R/M, and abortive infection (10). On the basis of the results of this study, we propose a fourth category to account for the enhanced resistance against φc2 (relative to *abiF* alone) mediated by the lactococcal plasmid pNP40 which does not conform to the criteria defining any of these mechanisms. Although not specifically a host-encoded phage resistance mechanism, a similar phenomenon was observed in *Salmonella typhimurium* in which a prophage, P22, prevented superinfection by a number of other phages by inhibiting injection of their DNA into the lysogen (17).

The results presented here indicate that the lactococcal plasmid pNP40 encodes an early-acting phage resistance mecha-

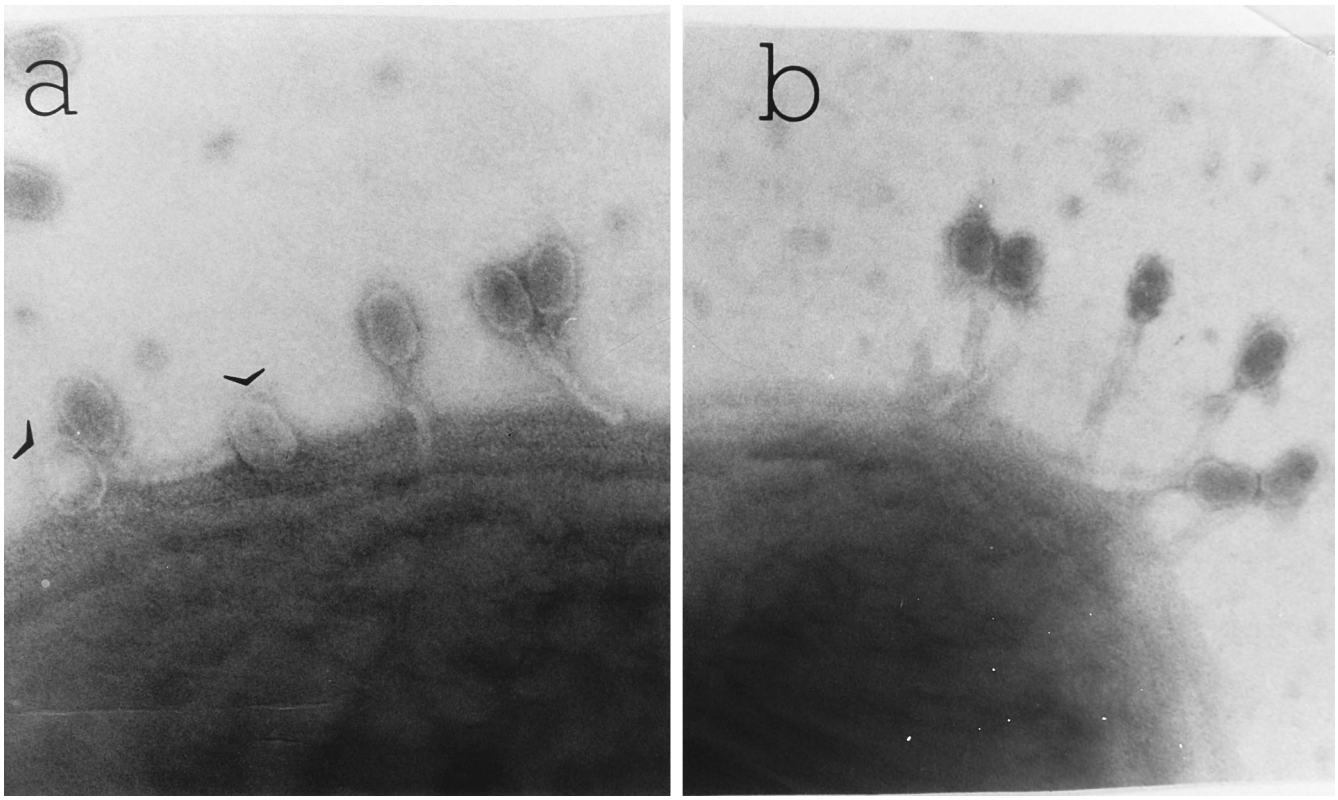

FIG. 1. Electron micrographs of phage ϕc2 adsorption to *L. lactis* subsp. *lactis* hosts, plasmid-free MG1614 (a) and MG1614/pNP40 (b).

nism, but no evidence of either classical adsorption blocking or R/M was detected. Following classical adsorption studies and analysis by electron microscopy, it appears that phage adsorb normally to pNP40-containing and pNP40-free hosts. In addition, analysis of at least three phage-host systems provided no evidence for the involvement of R/M. At no time during the analysis of this phage resistance mechanism were any modified phage capable of propagating on MG1614/pNP40 ever recovered, and no phage DNA was detected in pNP40-containing cells either in a normal or degraded state until 30 min following infection, results which also eliminate any role for R/M in this early-acting pNP40 mechanism.

The proposal that pNP40 encodes a novel penetration-blocking mechanism is supported by several lines of evidence: (i) phage ϕc2 particles adsorbed to sensitive hosts and to MG1614/pNP40 with equal efficiency, and electron microscopy showed that this attachment occurred in the normal tail-first orientation; (ii) only 10% of pNP40-containing cells died as a

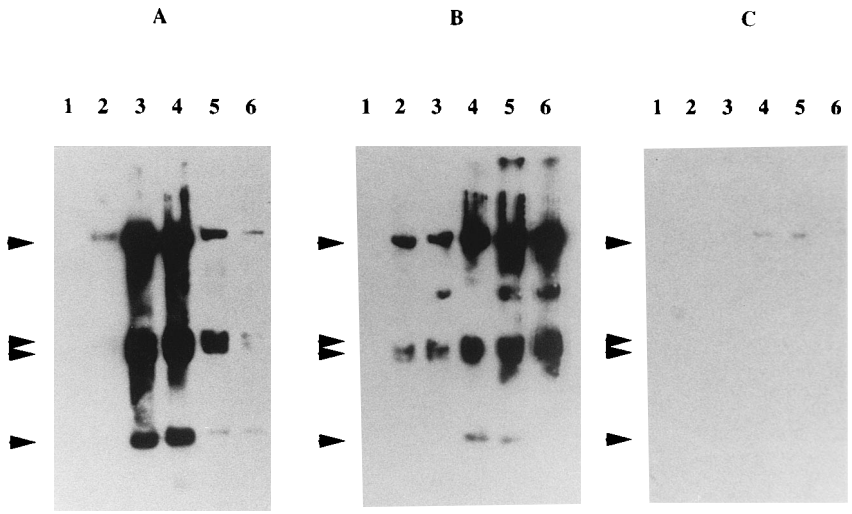

FIG. 2. Phage DNA content of *L. lactis* subsp. *lactis* MG1614 (A), PG020 (B), and MG1614/pNP40 (C) hosts following infection with ϕc2. Lanes: 1 to 6, *Eco*RI-digested DNA isolated from infected hosts at 0, 5, 10, 20, 40, and 60 min, respectively (A), and at 0, 5, 10, 30, 60, and 120 min, respectively (B and C).

TABLE 2. ECOI of phage c2 on *L. lactis* subsp. *lactis* MG1614, PG020, and MG1614/pNP40 after either conventional infection or electroporation of phage φc2 DNA

Strain	ECOI after:	
	Conventional infection	Electroporation
MG1614	1.0	1.0
PG020 (AbiF)	0.77	0.26
MG1614/pNP40	0.0004	0.11

result of infection, suggesting that a pNP40-encoded mechanism must operate prior to corruption of host functions or DNA degradation by the infecting phage; (iii) internalization of the genomes of the infecting phage into pNP40-containing cells was delayed or impaired as evidenced by the inability to detect bacteriophage-specific DNA within infected cells until at least 30 min following infection as opposed to 5 min in the case of a sensitive host; and (iv) a significant increase in ECOI was detected following circumvention of the early stages of infection by electroporation of phage DNA into resistant hosts, supporting the view that the early-acting pNP40-encoded resistance acts at the level of phage DNA penetration into the cell.

Monteville et al. (13) have shown that for φc2 infection of *L. lactis* subsp. *lactis* C2, a membrane protein Pip (phage infection protein) is essential for phage interaction with the host cell membrane and that a second 32-kDa membrane protein may also play a role in phage infection or translocation of phage DNA across the membrane. MG1614 is a derivative of *L. lactis* subsp. *lactis* 712, a strain closely related to C2, and it is probable that φc2 infection of MG1614 proceeds in a manner similar to that for strain C2 and that MG1614 possesses the necessary host functions for φc2 infection. It is likely therefore, on the basis of the results presented here, that an alteration at the level of the cell membrane prevents phage DNA penetration into the pNP40-containing host. It can be envisaged that a protein product, encoded by pNP40, could prevent production, membrane insertion, or activity of either the MG1614 Pip or 32-kDa protein equivalents and thus prevent the involvement of these proteins in phage DNA ejection from the phage head and translocation of DNA across the membrane.

The early-acting pNP40-encoded resistance mechanism is not active against the small isometric-headed phage φ712 since phage DNA could be detected inside pNP40-containing cells within 15 min following φ712 infection (5). This is consistent with evidence by Valyasevi et al. (19) that all phages do not have the same infection requirements with respect to the host cell membrane. The inferior resistance mediated by pNP40 to φc2 at elevated temperatures (37°C) also supports the concept of a mechanism operating at the level of the cell membrane whose fluidity at this temperature could significantly alter any interaction between the pNP40-encoded protein and the membrane components required for φc2 infection.

A penetration-blocking resistance mechanism is potentially a very powerful system since it not only allows the cell to survive despite exposure to high numbers of phage but also titrates phage out of the environment. One disadvantage is that it may be very specific, being effective, in this study, only against φc2. Assessment of the survival of other hosts containing pNP40 following exposure to their homologous phages would establish whether the activity of this resistance mechanism is confined to the φc2-MG1614 phage-host system. Nevertheless, it seems likely that at least four categories of phage resistance are operative in lactococci against phage: adsorption blocking, R/M, abortive infection, and a mechanism which inhibits phage DNA penetration. This new class of resistance mechanism is expected to be complementary to all known resistance systems, and it is anticipated that it will make a significant contribution in strain improvement when used in combination with other phage resistances.

ACKNOWLEDGMENTS

We thank Elke Arendt for performing electron microscopic analysis of φc2 adsorption.

P.G. was the recipient of a Teagasc Research Studentship. This work was supported by European Community BRIDGE and BIOTECHNOLOGY grants to G.F.F. (contract no. BIO-CT91-0263 and BIO2-CT94-3055, respectively) and by an Irish Department of Agriculture, Food and Forestry Non-Commissioned Food Research grant.

REFERENCES

1. **Behnke, D., and H. Malke.** 1978. Bacteriophage interference in *Streptococcus pyogenes*. 1. Characterisation of prophage-host systems interfering with the virulent phage A25. Virology **85:**118–128.
2. **Chopin, A., M.-C. Chopin, A. Moillo-Batt, and P. Langella.** 1984. Two plasmid-determined restriction and modification systems in *Streptococcus lactis*. Plasmid **11:**260–263.
3. **Coveney, J. A., G. F. Fitzgerald, and C. Daly.** 1987. Detailed characterization and comparison of lactic streptococcal bacteriophages based on morphology, restriction mapping, DNA homology, and structural protein analysis. Appl. Environ. Microbiol. **53:**1439–1447.
4. **Fitzgerald, G. F., C. Daly, L. R. Brown, and T. R. Gingeras.** 1982. *Scr*FI: a new sequence specific endonuclease from *Streptococcus cremoris*. Nucleic Acids Res. **10:**8171–8179.
5. **Garvey, P., G. F. Fitzgerald, and C. Hill.** 1995. Cloning and DNA sequence analysis of two abortive infection phage resistance determinants from the lactococcal plasmid pNP40. Appl. Environ. Microbiol. **61:**4321–4328.
6. **Gasson M. J.** 1983. Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. J. Bacteriol. **154:**1–9.
7. **Hill, C., I. J. Massey, and T. R. Klaenhammer.** 1991. Rapid method to characterize lactococcal bacteriophage genomes. Appl. Environ. Microbiol. **57:**283–288.
8. **Holo, H., and I. F. Nes.** 1989. High-frequency transformation, by electroporation, of *Lactococcus lactis* subsp *cremoris* strains grown with glycine in osmotically stabilized media. Appl. Environ. Microbiol. **55:**3119–3123.
9. **Kelly, W., J. Dobson, D. Jorck-Ramberg, G. F. Fitzgerald, and C. Daly.** 1990. Introduction of bacteriophage resistance plasmids into commercial *Lactococcus* starter cultures. FEMS Microbiol. Rev. **87:**P63.
10. **Klaenhammer, T. R., and G. F. Fitzgerald.** 1994. Bacteriophage and bacteriophage resistance, p. 106–168. *In* M. J. Gasson and W. M. de Vos (ed.), Genetics and biotechnology of lactic acid bacteria. Chapman & Hall, Glasgow, Scotland.
11. **Klaenhammer, T. R., and R. B. Sanozky.** 1985. 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. J. Gen. Microbiol. **131:**1531–1541.
12. **McKay, L. L., and K. A. Baldwin.** 1984. Conjugative 40-megadalton plasmid in *Streptococcus lactis* subsp. *diacetylactis* DRC3 is associated with resistance to nisin and bacteriophage. Appl. Environ. Microbiol. **47:**68–74.
13. **Monteville, M. R., B. Ardestani, and B. R. Geller.** 1994. Lactococcal phages require a host cell wall carbohydrate and a plasma membrane protein for adsorption and ejection of DNA. Appl. Environ. Microbiol. **60:**3204–3211.
14. **Sing, W. D., and T. R. Klaenhammer.** 1986. Conjugal transfer of bacteriophage resistance determinants on pTR2030 into *Streptococcus cremoris* strains. Appl. Environ. Microbiol. **51:**1264–1271.
15. **Sing, W. D., and T. R. Klaenhammer.** 1990. Characteristics of phage abortion conferred in lactococci by the conjugal plasmid pTR2030. J. Gen. Microbiol. **136:**1807–1815.
16. **Sing, W. D., and T. R. Klaenhammer.** 1991. Characterisation of restriction-modification plasmids from *Lactococcus lactis* ssp. *cremoris* and their effects when combined with pTR2030. J. Dairy Sci. **74:**1133–1144.
17. **Susskind, M. M., D. Botstein, and A. Wright.** 1974. Superinfection exclusion by P22 prophage in lysogens of *Salmonella typhimurium*. Virology **62:**350–366.
18. **Terzaghi, B. E., and W. E. Sandine.** 1975. Improved medium for lactic streptococci and their bacteriophage. Appl. Microbiol. **29:**807–813.
19. **Valyasevi, R., W. E. Sandine, and B. L. Geller.** 1991. A membrane protein is required for bacteriophage c2 infection of *Lactococcus lactis* subsp. *lactis* C2. J. Bacteriol. **173:**6095–6100.