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Effect of Increasing the Copy Number of Bacteriophage Origins of Replication, in *trans*, on Incoming-Phage Proliferation†

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Bacteriophage resistance mechanisms which are derived from a bacteriophage genome are termed Per (phage-encoded resistance). When present in *trans* in *Lactococcus lactis* NCK203, Per50, the cloned origin of replication from phage φ50, interferes with φ50 replication. The *per50* fragment was found to afford negligible protection to NCK203 against φ50 infection when present in a low-copy-number plasmid, pTRK325. A high-copy-number Per50 construct (pTRK323) dramatically affected φ50 infection, reducing the efficiency of plaquing (EOP) to 2.5×10^{-4} and the plaque size to pinhead proportions. This clone also afforded significant protection against other related small isometric phages. Per31 was cloned from phage φ31 and demonstrated to function as an origin of replication by enabling replication of *per31*-containing plasmids, in NCK203, on φ31 infection. A low-copy-number Per31 plasmid (pTRK360) reduced the EOP of φ31 on NCK203 to 0.3 and the plaque diameter from 1.5 to 0.5 mm. When this plasmid was cloned in high copy number, the EOP was further reduced to 7.2×10^{-7} but the plaques were large and contained Per31-resistant phages. Characterization of these "new" phages revealed at least two different types that were similar to φ31, except that DNA alterations were noted in the region containing the origin. This novel and powerful abortive phage resistance mechanism should prove useful when directed at specific, problematic phages.

Lactococcus species are used commercially as starter bacteria in dairy-food fermentations. A major problem that remains is their susceptibility to infection by lytic bacteriophages, which potentially can destroy the fermentation process. Development of phage resistance in desirable fermentative strains of lactococci is therefore a priority. Spontaneous phage-resistant mutants of lactococci are readily obtainable following infection of a bacterial population with a specific phage at high titer. These spontaneous mutants are not generally effective in practice since (i) the spectrum of their resistance is very narrow, (ii) they may acquire sensitivity to other phages, and (iii) their growth characteristics may be altered (reviewed in references 21 and 23). However, phage resistance mechanisms, which occur naturally, have been identified in many wild-type lactococcal strains (5, 9, 12, 24, 37). These mechanisms are often encoded on native conjugative plasmids and can be transferred to desirable strains, thus leading to the development of novel phage-resistant dairy starter strains (16, 38, 40).

Plasmid-encoded phage resistance mechanisms described to date for the lactococci can be divided into three groups, adsorption interference, restriction/modification, and abortive defense, the last of which is characterized by cell death following inhibition of phage development at some point after infection (26). The first abortive mechanism characterized to sequence level (AbiA) was encoded on pTR2030, and it was found to function by inhibiting phage DNA replication (18). The identical gene was later identified on a plasmid from another starter strain in Ireland (8). A number of distinct abortive genes have now been cloned and sequenced, but the mechanisms by which their gene products interfere with phage development remain unknown (7, 13, 14, 25).

Recently, a recombinant mechanism was designed to provide *Lactococcus lactis* NCK203 with resistance against an industrial phage, φ50 (19). Phage φ50 is a small isometric headed phage which has been morphologically and genetically described (1, 22). The φ50 origin of replication (*ori*50) was cloned onto the plasmid pSA3 and, when presented in *trans*, enabled plasmid replication on φ50 infection (19). This resulted in a false target for phage replication functions and correspondingly retarded replication of the phage genome, thus reducing the number of phage progeny. This phage resistance mechanism was termed Per, for phage-encoded resistance. Per is therefore an abortive type of phage resistance because the phage lytic cycle is disrupted after the early stages of phage development have ensued, specifically in this case at the point of phage DNA replication.

In this study, *per50* was cloned into high- and low-copy replicons, demonstrating that the Per phenotype is directly dependent on copy number. Per functions were also cloned from the cohesive-ended phage (φ31), demonstrating that it is feasible to direct this phage resistance concept at another bacteriophage. Characterization of the Per31 phenotype revealed that φ31 infection of NCK203 was severely impeded by the presence of *per31* on a high-copy-number plasmid. However, increasing the effectiveness of a single abortive phage resistance mechanism also provided selection pressure for the emergence, in this host background, of new phages that evolved by an unknown mechanism.

MATERIALS AND METHODS

Bacteria, bacteriophages, and plasmids. The bacteria, bacteriophages, and plasmids used in this study are listed in Table 1.

Media and growth conditions. *Lactococcus* strains were cultured at 30°C in M17 broth (Difco Laboratories, Detroit,

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TABLE 1. Bacteria, bacteriophages, and plasmids used in this study

Strain, phage, or plasmid	Relevant characteristics	Source or reference
L. lactis		
NCK203	Sensitive host for all P335 phages used in this study	20
NCK612	NCK203 containing pTRK323	This study
NCK611	NCK203 containing pTRK325	This study
NCK619	NCK203 containing pTRK360	This study
NCK620	NCK203 containing pTRK361	This study
E. coli		
DH5α	*thi-1 recA1 relA1 gyrA96 hsdR17*, α-*lac* complementation	15
XL-blue	*thi recA1 hsdR17 relA1 gyrA94* Tc^r, α-*lac* complementation	2
MC1061	*ara leu lacX74 galU galK hsdB hsdM strA*	4
Bacteriophages		
φ50[a]	Small isometric, P335 species, terminally redundant *pac* phage, 29.8-kb genome	1
φ31	Small isometric, P335 species, cohesive ended, 31.9-kb genome	1
φA1, φB1, φCS, φD1	Small isometric, P335 species	10
Plasmids		
pTRK102	4.5 kb Per50$^+$ fragment from φ50 cloned in pBLUESCRIPT, Ap^r	19
pTRKH1	Shuttle cloning vector; Cm^r Em^r, G(−)*ori*-p15A, G(+)*ori*-pAMβ1, low copy number in *Lactococcus* spp.	31
pTRK323	pTRKH1 with 1.4-kb *per50* fragment	This study
pTRK325	pTRKL1 with 1.4-kb *per50* fragment	This study
pSA3	Shuttle cloning vector; Cm^r Tc^r Em^r, G(−)*ori*-p15A, G(+)*ori*-GB305, low copy number in *Lactococcus* spp.	11
pTRK354	pSA3 + 6.0-kb φ31 fragment; Per$^-$	This study
pTRK355	pSA3 + 1.6-kb φ31 fragment; Per$^-$	This study
pTRK356	pSA3 + 2.5-kb φ31 fragment; Per$^-$	This study
pTRK357	pSA3 + 2.5-kb φ31 fragment; Per$^-$	This study
pTRK358	pSA3 + 6.5-kb φ31 fragment; Per$^-$	This study
pTRK359	pSA3 + 10.0-kb φ31 fragment; Per$^+$	This study
pTRK360	pSA3 + 4.5-kb φ31 fragment; Per$^+$	This study
pTRKH2	Shuttle cloning vector; Em^r *lac*, G(−)*ori*-p15A, G(+)*ori*-pAMβ1, high copy number in *Lactococcus* spp.	31
pTRK361	pTRKH2 with 4.5-kb *per31* fragment	This study

[a] Unlike the other P335 bacteriophage used in this study, φ50 is unstable during long-term storage in GM17 broth at 4°C; this leads to reductions in PFU per milliliter and to variable plaquing strengths on NCK203 and its *per50* derivatives.

Mich.) containing 0.5% glucose (GM17). *Escherichia coli* strains were grown in LB medium (34) at 37°C. Tetracycline and chloramphenicol were used at 10 and 30 μg/ml, respectively, as required with *E. coli*. Erythromycin was used at 2 μg/ml for lactococci and 200 μg/ml for *E. coli*. However, for a clean erythromycin selection in *E. coli*, brain hearth infusion (BHI) medium (Difco) was used instead of LB. Isopropylthio-β-D-galactoside (IPTG; 250 μg/ml) and 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal; 30 μg/ml) were used as required; both were obtained from Gold Biotechnology, St. Louis, Mo.

Electroporation procedure. Electrocompetent *Lactococcus* cells were prepared by growth to the late log phase in GM17 broth supplemented with 20 mM DL-threonine followed by four washes in a solution of 0.5 M sucrose and 10% glycerol at 4°C. Cells were concentrated 100-fold (of initial volume) in the wash solution and stored at −70°C. Electroporation of plasmid DNA into *Lactococcus* cells was conducted by combining 50 μl of thawed, ice-cold electrocompetent cells with 2 μl of a salt-free DNA solution in a cold (−20°C) gene pulser cuvette with a 0.2-cm electrode gap (Bio-Rad Laboratories, Richmond, Calif.). This was then pulsed with a Gene Pulser (Bio-Rad) set at 25 μF and 2.45 kV and a Pulse Controller set at 200 Ω. One milliliter of an outgrowth medium (GM17 supplemented with 100 g of sucrose per liter, 2 mM $MgCl_2$, and 2 mM $CaCl_2$) was added to the cuvette and incubated for 1 to 2 h at 30°C, and then the contents were plated on outgrowth medium containing the required selective antibiotic.

Bacteriophage assays. Standard plaque assays were conducted as described previously (36, 43). Efficiency of plaquing (EOP) was obtained by dividing the phage titer on the test strain by the titer on the phage-sensitive parent strain. One-step growth curves and center-of-infection (COI) assays were performed as described previously (41). The efficiency of COI production (ECOI) was obtained by dividing the number of COI from the test strain by the number of COI produced on the parent (phage-sensitive) host. The burst size for a phage was defined as [(phage titer at the end of a burst − initial titer)/initial titer].

Visualization of intracellular phage DNA replication. The procedure as described by Hill et al. (19) was used to monitor intracellular phage DNA replication.

DNA manipulations. Procedures for *E. coli* plasmid isolation, endonuclease restriction, ligations, DNA hybridizations, and plasmid transformations were performed essentially as described by Sambrook et al. (34). Bacteriophage DNA was isolated as described previously (29), with some modifications. Briefly, 1 μl of RNase-DNase (1 mg/ml) was added to 750 μl of cleared lysate in an Eppendorf tube. Following incubation at room temperature for 10 min, 100 μl of an SDS solution (0.25 M EDTA, 0.5 M Tris-HCl [pH 9.0], 2.5% sodium dodecyl sulfate [SDS]) was added and the tube was incubated at 65°C. After 30 min, 125 μl of 8 M potassium acetate was added, and the contents of the tube were mixed by vortexing and centrifuged for 15 min at 4°C. Without delay, 640 μl of the cleared supernatant was transferred to a new tube containing 400 μl of 7.5 M ammonium acetate with 0.5 mg of ethidium bromide per ml. Phenol-chloroform (500 μl) was then added and vortexed. Following centrifugation at room temperature for 5 to 10 min, the upper phase was transferred to a new tube, mixed with 0.6 volume of isopropanol at room temperature, and centrifuged for 15 min. The ensuing DNA pellet was washed with 70% ethanol and resuspended in TE buffer, usually 40 μl. Lactococcal plasmid DNA was prepared by a new rapid miniprep procedure outlined by O'Sullivan and Klaenhammer (30).

RESULTS

Cloning of *per50* on high- and low-copy-number plasmid replicons. Per50 is the cloned φ50 origin of replication, which, when present in *trans*, affords measurable resistance to the host against φ50 infection (19). To evaluate the effect of copy number on the phenotype, *per50* was cloned in high-

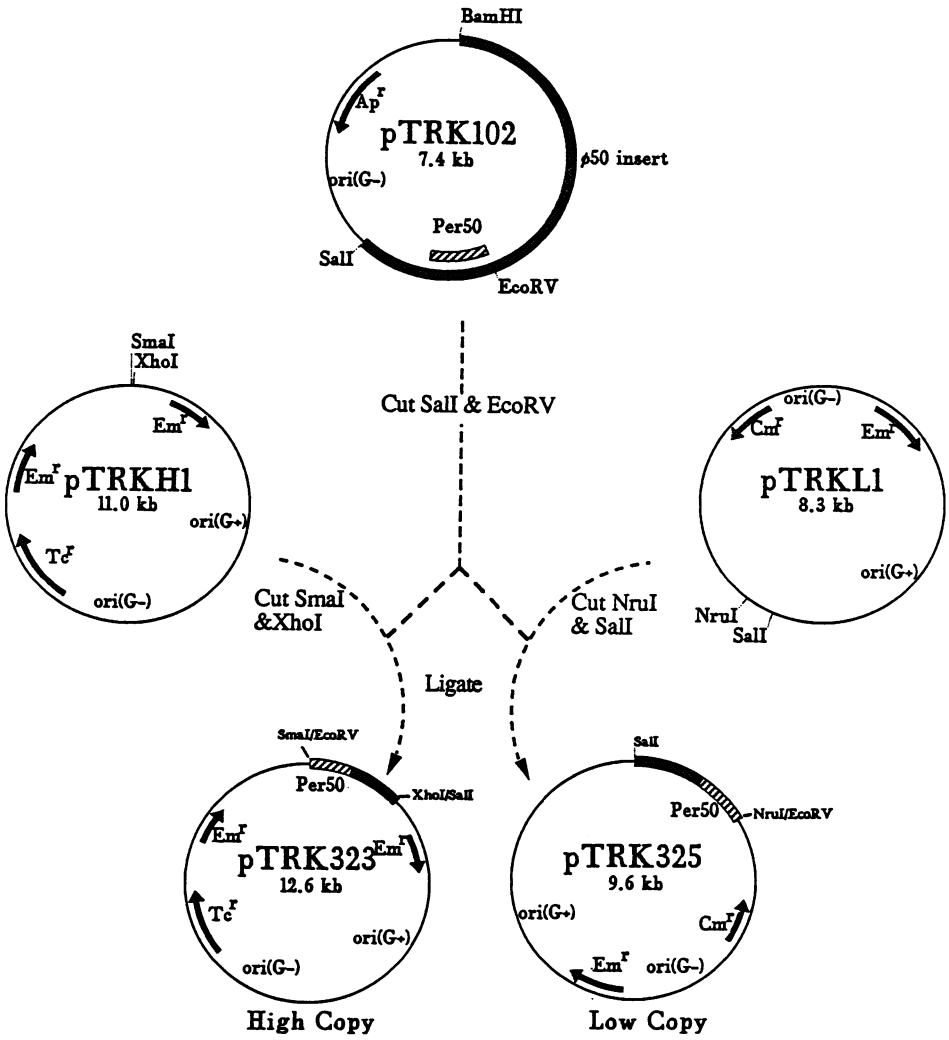

FIG. 1. Construction of the high- and low-copy-number Per50 clones. Only relevant restriction enzyme sites are indicated. Abbreviations: ori(G−), gram-negative plasmid origin from p15A; ori(G+), gram-positive plasmid origin from pAMβ1.

and low-copy-number gram-positive replicons. For this purpose, the *E. coli*/gram-positive shuttle cloning vectors pTRKL1 and pTRKH1 were used. These were chosen because they were derived from the gram-positive vectors pIL252 and pIL253, both of which contain the pAMβ1 replicon but have been estimated to exist in *Lactococcus* cells at 6 to 9 and 45 to 85 copies, respectively (39). The construction of the high- and low-copy-number Per50 clones is detailed in Fig. 1. The resulting constructs, pTRK323 and pTRK325, were selected for in *E. coli* MC1061 by using Tc^r for pTRK323 selection and Cm^r for pTRK325 selection. Both contained the same precise piece of ϕ50 DNA, a 1.4-kb fragment which has been completely sequenced and contains *per50* (19). These plasmids were introduced into *L. lactis* NCK203 via electroporation, using the erythromycin resistance (Em^r) marker for selection. The authenticity of the plasmids in the Em^r electrotransformants was confirmed by miniprep isolation and restriction enzyme analysis (data not shown).

Phage resistance phenotypes of pTRK323 and pTRK325. Phage ϕ50 infection of *L. lactis* NCK203 and derivative strains was monitored by standard plaque assays. The low-copy-number Per50 construct (pTRK325) was found to have a negligible effect on ϕ50 proliferation in NCK203 (Table 2). In contrast, the high-copy-number construct (pTRK323) had a marked effect, reducing the EOP by almost 4 log units and the plaque size to pinhead proportions. The cloning vectors pTRKH1 and pTRKL1 had no effect on phage infection of NCK203 (data not shown). Like ϕ50, phages ϕA1, ϕB1, ϕCS, and ϕD1 are recent isolates from industrial plants in which NCK203-based starter cultures were used. They are all small isometric headed phages of the P335 species, and each is genetically distinct on the basis of endonuclease restriction enzyme analysis (10). Infection of NCK203 with these lytic phages was not visibly retarded by the presence of pTRK325 (Table 2). However, the high-copy-number construct pTRK323 significantly reduced both the EOP and plaque size. Infection of NCK203 by another small isometric headed phage, ϕ31, was not affected by either of the *per50* containing clones.

Cloning of Per31. The results obtained with Per50 implied that a similar Per phenotype could be obtained for other

TABLE 2. Reactions of various phages on *L. lactis* NCK203 and derivative strains

Strain[a]	Phage	EOP	Plaque size (mm)
NCK203	φ50	1.0	1.5
	φA1	1.0	1.5
	φB1	1.0	1.5
	φCS	1.0	1.5
	φD1	1.0	1.5
	φ31	1.0	1.5
NCK203(pTRK325) /LC	φ50	0.8	1.2
	φA1	1.0	1.5
	φB1	1.0	1.5
	φCS	1.0	1.5
	φD1	1.0	1.5
	φ31	1.0	1.5
NCK203(pTRK323) /HC	φ50	2.5×10^{-4}	0.1
	φA1	3.2×10^{-3}	0.2
	φB1	1.5×10^{-3}	0.2
	φCS	7.5×10^{-2}	0.5
	φD1	5.0×10^{-2}	0.5
	φ31	1.0	1.5

[a] /LC, low-copy-number plasmid; /HC, high-copy-number plasmid.

phages by cloning their origins of replication. To investigate this hypothesis, we used φ31. This phage was isolated from an industrial cheese plant and has been morphologically and genetically characterized (1, 22). Its major structural difference from φ50 is that its genome contains cohesive ends; the φ50 genome is circularly permuted, with terminally redundant ends (1). Phage φ31 DNA was digested singly with *Eco*RV or doubly with *Sal*I and *Bam*HI and ligated into correspondingly digested pSA3. Clones were selected for Cm^r in *E. coli* DH5α. With this cloning strategy, seven clones were obtained, which represented more than 90% of the phage genome (Fig. 2). These clones were then electroporated into *L. lactis* NCK203 to determine whether they conferred any resistance against φ31 infection. Electrotransformants were not obtained for three of the clones, pTRK354, pTRK356, and pTRK357. Two clones afforded resistance against φ31 infection (Fig. 2). This was manifested as a slight reduction in EOP and a reduced plaque size, which is comparable to the φ50 reaction of NCK203 harboring Per50 cloned in pSA3 (19). Since the 4.5-kb *Sal*I-*Bam*HI fragment is contained within pTRK359 and pTRK360 (Fig. 2), this indicated that the Per31 phenotype was contained in this region. NCK203 containing pTRK360 exhibited no resistance to infection by φ50.

This *per31* fragment, cloned in pSA3, represented a low-copy-number construct in *Lactococcus* spp. To determine the effect of copy number on its phenotype, we cloned it as a *Sal*I-*Bam*HI fragment from pTRK360 into similarly digested pTRKH2. Selection was made in *E. coli* for Em^r and a Lac^- phenotype on LB agar plates containing erythromycin, IPTG, and X-Gal. This cloning vector is a derivative of pTRKHI and contains blue and white selection capability to facilitate ease of cloning in *E. coli* (31). The ensuing high-copy-number Per31 construct (pTRK361) was electroporated into NCK203, and Em^r electrotransformants were confirmed by restriction enzyme analysis of plasmid miniprep DNA.

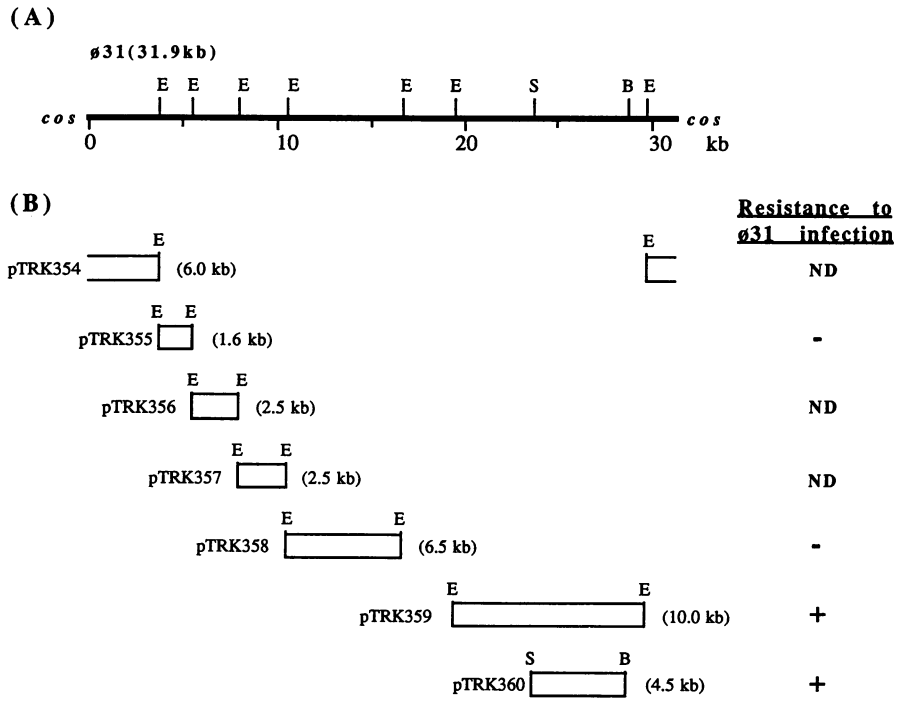

FIG. 2. (A) Restriction map of φ31 as described by Alatossava and Klaenhammer (1). (B) Fragments from φ31 which were subcloned into pSA3. The size and map position of each subcloned fragment are indicated. The column to the right indicates whether the subcloned fragment afforded any resistance to NCK203 from φ31 infection. Symbols: ND, not determined; −, EOP = 1.0; +, EOP = 0.3; E, *Eco*RV; S, *Sal*I; B, *Bam*HI.

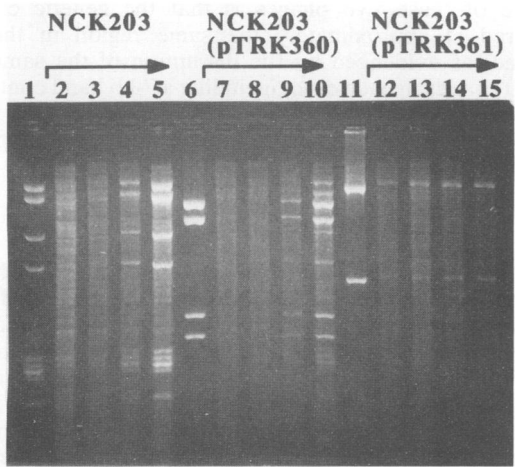

FIG. 3. Monitoring intracellular ϕ31 DNA replication. All lanes are digested with *Hin*dIII. Lanes: 1, purified ϕ31 DNA; 2, total DNA from *L. lactis* NCK203 prior to ϕ31 infection (time zero); 3, total DNA after 20 min of ϕ31 infection; 4, ϕ31/NCK203 infection at 40 min; 5, ϕ31/NCK203 infection at 60 min; 6, purified DNA of pTRK360 (low-copy-number Per31 clone); 7, total DNA from NCK203(pTRK360) prior to infection with ϕ31 (time zero); 8, ϕ31/NCK203(pTRK360) infection at 20 min; 9, ϕ31/NCK203 (pTRK360) infection at 40 min; 10, ϕ31/NCK203(pTRK360) infection at 60 min; lane 11, purified DNA of pTRK361 (high-copy-number clone); 12, total DNA from NCK203(pTRK361) prior to infection with ϕ31 (time zero); 13, ϕ31/NCK203(pTRK361) infection at 20 min; 14, ϕ31/NCK203(pTRK361) infection at 40 min; 15, ϕ31/NCK203(pTRK361) infection at 60 min.

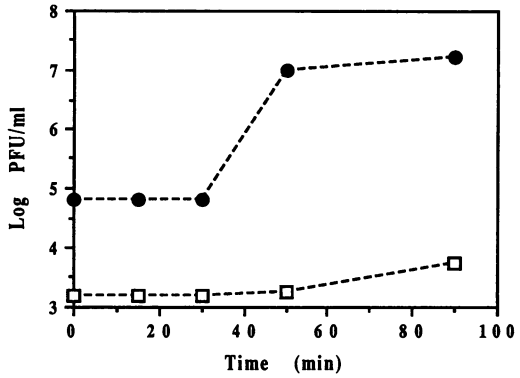

FIG. 4. One-step growth curves for ϕ31 infection of *L. lactis* NCK203 (●) and NCK203(pTRK361) (high-copy-number Per31 clone) (□).

Per31 is a phage origin of replication. Previous data showed that the *per50* fragment harbored a functional phage origin of replication, which could drive replication of pTRK133 (pSA3 containing *per50*) following superinfection with ϕ50 (19). To investigate whether the *per31* fragment harbored this ability, NCK203 strains containing pTRK360 or pTRK361 were infected with ϕ31 and intracellular phage DNA replication was monitored at regular intervals over a 1-h period. This was done by isolating total DNA postinfection and visualizing, following electrophoresis, its *Hin*dIII-digested banding pattern. Initiation of ϕ31 DNA replication in NCK203 could clearly be seen 20 min following ϕ31 infection and continued over the 1-h period (Fig. 3). In the case of ϕ31 infection of NCK203 containing pTRK360 (low-copy-number Per31 construct), replication of the phage DNA was considerably slowed, and after the 1-h period, the total phage DNA accumulated was greatly reduced (Fig. 3, lane 10). Before infection, pTRK360 could not be visualized (lane 7) since it was not present in sufficient copy number. However, a dramatic increase in its copy number was observed during the 1-h infection period (lanes 8 to 10). Since amplification of the plasmid does not occur without the ϕ31 DNA insert (data not shown), this indicates that *per31* is the origin of replication from ϕ31. Reduction of ϕ31 DNA replication was even more emphatic during its infection of NCK203(pTRK361) (a high-copy-number Per31 construct). In fact, over the 60-min period, no ϕ31 DNA replication was visualized by this procedure (lanes 12 to 15). Hybridization with ϕ31 DNA revealed that some ϕ31 DNA replication occurred after 20 min but did not increase after this time (data not shown). Unlike the explosion of pTRK360 replication from ϕ31 infection, much less amplification of the high-copy-number pTRK361 plasmid occurred (lanes 12 to 15).

Effect of pTRK361 on ϕ31 infection. Infection of ϕ31 on NCK203 with and without the high-copy-number Per31 construct (pTRK361) was monitored by COI assays. From the single-step growth curves obtained (Fig. 4), it is apparent that ϕ31 produced much smaller numbers of COIs on NCK203(pTRK361) than the control. The ECOI was calculated to be 0.024, indicating that ~2% of NCK203(pTRK361) cells produced viable COIs. The number of phages released during a single burst was also drastically reduced. When calculated after 50 min, the burst for ϕ31 infection of NCK203 was 162 compared with a burst of <1 for ϕ31 infection of NCK203(pTRK361). At 90 min, the total number of phages released per infected cell was calculated to be 6, compared with 276 for the control at this time point.

Cell survival experiments were used to examine the effect of the Per31$^+$ plasmids on the fate of NCK203 cells following an infection with ϕ31. With a high multiplicity of infection (> 10), the cell survival rate was estimated to be 4 to 7% for NCK203 and for NCK203 containing either pTRK360 or pTRK361. Therefore, even though ϕ31 proliferation on NCK203(pTRK361) was almost completely halted, there was no increase in cell survival. Since the Per phenotype does not influence phage adsorption to cells (19), these data indicated that infected cells were killed, irrespective of whether the infection progressed to completion.

Copy number effect on the Per31 phenotype. Standard plaque assays were used to evaluate the infection of ϕ31 on NCK203, NCK203(pTRK360), and NCK203(pTRK361). The control ϕ31-NCK203 reaction occurred with an EOP of 1.0 and produced a plaque diameter of ~1.5 mm on a lawn of NCK203 cells (Fig. 5). The low-copy-number construct, pTRK360, reduced the EOP to 0.2 and the plaque size to ~0.5 mm. This phenotype is reproducible on a second round of plaque formation, indicating that it is not modifiable; therefore, this is representative of an abortive type of phage resistance. The EOP of the ϕ31-NCK203(pTRK361) reaction was drastically reduced to 7.2×10^{-7} (Fig. 5). However, the plaque size was generally not reduced. This was surprising, since the burst for this infection was so low, barely detectable after 90 min (Fig. 4). Phages were isolated from the large plaques and were found to be Per31 resistant, giving an EOP of 1.0 for an infection of NCK203(pTRK361). Restriction enzyme analysis of the DNA isolated from two of these phages revealed some differences from each other and from

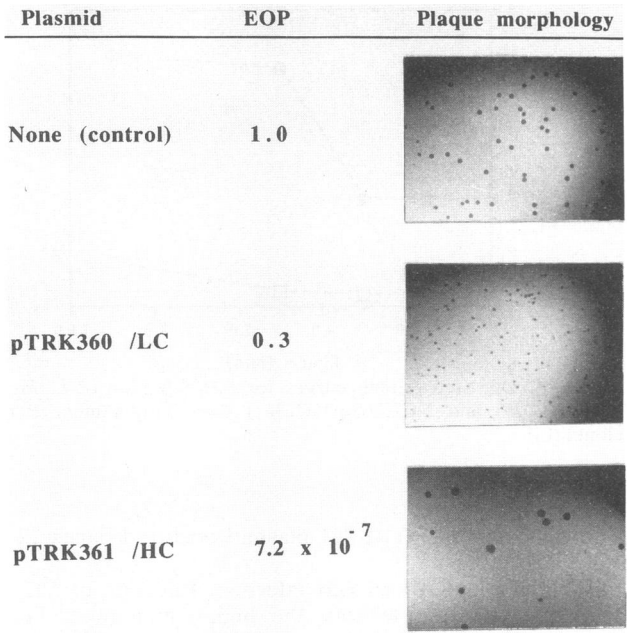

FIG. 5. Phage ϕ31 infection of *L. lactis* NCK203 and NCK203 containing pTRK360 or pTRK361. /LC, low copy number; /HC, high copy number. Plaque photographs were taken at equivalent magnifications.

ϕ31 (Fig. 6). The common bands observed were confirmed by hybridization experiments to be ϕ31, and the other bands displayed various degrees of homology to ϕ31 DNA (data not shown). The two altered phages (ϕ31.1 and ϕ31.2) acquired five and four new *Hin*dIII bands, respectively, which were different for each phage (Fig. 6). The ϕ31.1 genome was approximately the same size as that of ϕ31, whereas the ϕ31.2 genome was ~1 kb smaller. A common

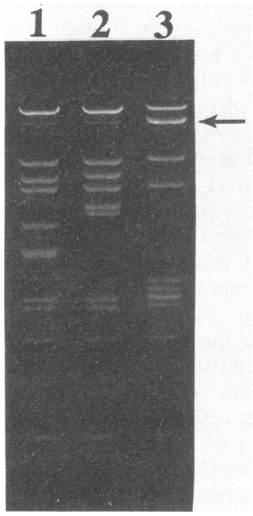

FIG. 6. *Hin*dIII restriction patterns of DNA from ϕ311 (lane 1), ϕ312 (lane 2), and ϕ31 (lane 3). The arrow indicates a 7-kb band in lane 3 which contains the ϕ31 origin of replication. The faint band (visible in lanes 1 and 2) which comigrates at this position results from partial denaturation of the larger 9-kb band at the *cos* site (data not shown).

feature of these two phages is that the genetic changes occurred at approximately the same region in the ϕ31 genome, as evidenced by the disruption of the same four *Hin*dIII bands in each case, including a 7-kb band containing the ϕ31 origin of replication. The genetic events which contributed to the appearance of the Per31-resistant phages remain to be elucidated.

DISCUSSION

Per is a nonnative phage resistance mechanism, which is artificially engineered and is phenotypically classified as an abortive-type system. It is based on providing a cloned phage origin of replication in *trans*, which can compete with superinfecting phages for replication functions, thus inhibiting the proliferation of the phages within the host (19). In this study, it was demonstrated, that the severity of the Per phenotype is essentially in direct proportion to the copy number of the cloned Per-containing fragment in the host (Table 2; Fig. 5).

A significant finding in this study was that Per50 also protected *L. lactis* NCK203 from infection with other phages, which were isolated from industrial cheese plants (Table 2). Recent evidence has strongly suggested that ϕ50 and these other industrial isolates (ϕA1, ϕB1, ϕCS, and ϕD1) are newly evolved phages which have emerged as a result of the selective pressures provided by their habitat (28). Another phage (ϕ48) was previously shown to be sensitive to Per50 (19). This phage also originated from dairy fermentations in which the parent of NCK203 was used as a starter culture. Interestingly, ϕ48 was isolated from a cheese plant in Utah and independently from a cheese plant in France (35). Although this may represent a case of cross-contamination (a notorious precedent, involving the same two countries, occurred during the discovery of the human immunodeficiency virus, the cause of AIDS [32]), it is more likely to represent a case of convergent bacteriophage evolution. In Quebec, Canada, another phage (ϕUL36) isolated from a cheese plant has been found to be Per50 sensitive (27). Interestingly, ϕUL36 was reported to be more sensitive than ϕ50 to Per50, giving an EOP of less than 10^{-6} upon infection of NCK203(pTRK323) (high-copy-number Per50 clone), which is approximately 2 log units lower than for ϕ50. All these Per50-sensitive phages are part of a proposed family of recently evolved phages from dairy fermentation environments (28). Therefore, development of a novel resistance mechanism for this class of newly emerging phages, such as Per50, may be very significant in preventing their proliferation in industrial dairy fermentations.

To investigate the Per concept in another phage-host system, we cloned Per31. The location of the *per31* fragment on the phage genome was between 23.5 and 28 kb (Fig. 2). This is similar to the location of *per50* between 22 and 23.6 kb on the map of the ϕ50 genome, deduced by Alatossava and Klaenhammer (1). Whether this positioning is coincidental or represents a common feature among P335 phages will remain unknown until more P335 phage origins are cloned and mapped. Per50 and Per31 are functionally distinct, as evidenced by the reaction of ϕ31 on NCK203(pTRK323) (Table 2). Furthermore, all the Per50-sensitive phages tested in this study were completely resistant to Per31 (data not shown). The use of the Per abortive resistance phenotype may therefore target specific subgroups of bacteriophages which use the same or similar replication functions.

The data presented in Fig. 3 strongly indicated that production of sufficient factors for dynamic replication is

dependent on an initial threshold level of phage replication. This is evidenced by the difference in amplification between pTRK360 and pTRK361 following phage infection and its correlation with the corresponding amount of phage DNA replication. This therefore suggests that the phage genes which encode the replication factors are still expressed during the early rounds of phage replication. Since genes coding for coliphage replication functions are generally expressed during the middle stages of the phage cycle and during the early rounds of DNA replication (reviewed in reference 3), it is probable that a similar timing for expression of replication functions occurs in the lactococcal P335 phages.

The mechanism by which lactococcal lytic phages kill the host cell is still under investigation. Recently Powell et al. (33) found that degradation of host DNA initiated after 4 to 6 min of φc6A infection of *L. lactis* C6 and suggested that this results in the eventual killing of the cell, even if the infection was prematurely aborted. Host cell DNA degradation by bacteriophage-encoded nucleases occurs for many other phages, for example the T7 coliphage (17). If similar host DNA degradation occurs on φ31 infection of NCK203, it is very slight and not visibly detectable even after 60 min of infection (Fig. 3). However, the survival experiments demonstrated that φ31 killed Per^+ host cells, even when the infection was aborted just after the initiation of DNA replication. Consequently, the host-killing mechanism used by φ31 is not reversible, irrespective of phage DNA replication. It is not possible to pinpoint when the cell-killing process initiates, since late-gene expression may still occur in the absence of replication. It has been shown for the *Bacillus subtilis* bacteriophage SPO1 that late-gene expression does occur, although at a much reduced rate, even if DNA replication is halted (42).

It was conclusively demonstrated that increasing the Per copy number concurrently increased the observed resistance to a superinfecting phage. This increase in resistance was different for each phage-host reaction (Table 2; Fig. 5), suggesting that different phages may produce different amounts of replication factors or may compete differently for a limited pool. However, the correlation between increased copy number and increased phage resistance held true for each phage-host combination examined. The effectiveness of Per31 on φ31 infection of an NCK203 host (Fig. 4 and 5) clearly highlighted the potential of increasing the efficiency of a single abortive phage resistance mechanism. It also demonstrated the disadvantage of relying exclusively on a single strong phage resistance mechanism, since it can provide a direct selection for new or altered phages to evolve. In this study, φ31.1 and φ31.2 were isolated following the infection of φ31 on NCK203(pTRK361) (high-copy-number Per31 clone). Phenotypically these phages were Per31 resistant, and genetically they both exhibited differences from the φ31 genome in a specific region (Fig. 6). Further studies are needed to determine the origin of these genetic changes in the phage genome. According to the published restriction map of φ31 (1), *per31* (as localized in Fig. 2) is contained within the second-largest *Hin*dIII fragment (7 kb). Since this fragment is altered in both φ31.1 and φ31.2, it suggests that the genetic changes which contributed to the emergence of both these new phages may have occurred in a region involving the origin. Therefore, they may have acquired another origin of replication from the NCK203 genome. A possible source of another origin may be a resident prophage in this host background, which has been shown to be homologous to phages of the P335 species (10). Further studies are required to clarify the source of these new phages. It should be noted, however, that the frequency of new phage emergence, when using a high-efficiency Per abortive resistance mechanism, may be much lower for different phage-host combinations.

Mechanistically, Per has been suggested to function by titrating out factors required for replication, thus retarding replication of the superinfecting phage genome (19). The copy number effect on the Per phenotype, reported here, fully substantiates this theory, since reducing the copy number effectively abolishes the phenotype and increasing the copy number dramatically enhances the observed phage resistance (Table 2). These data therefore indicate that a threshold number of copies of a *per* fragment is required to observe detectable phage resistance. Cloning and characterization of Per functions from another phage (Fig. 2 to 5) further substantiate this reasoning and demonstrate that the Per50 phenotype is not just an isolated incident associated with φ50 but, rather, is a general concept among phages that replicate via a replication origin. Superinfection of *E. coli* containing a cloned bacteriophage T7 origin of replication has also been shown to result in a dramatic amplification of the plasmid (6). It can therefore be concluded that use of a Per-mediated abortive infection mechanism can potentially be designed to target any bacteriophage-host reaction.

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