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Cloning, Expression, and Sequence Determination of a Bacteriophage Fragment Encoding Bacteriophage Resistance in *Lactococcus lactis*†

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A number of host-encoded phage resistance mechanisms have been described in lactococci. However, the phage genome has not been exploited as a source of additional resistance determinants. A 4.5-kb *Bam*HI-*Hin*dIII fragment of phage nck202.50 (φ50) was subcloned in streptococcus-*Escherichia coli* shuttle plasmid pSA3 and introduced into *Lactococcus lactis* NCK203 and MG1363 by protoplast transformation. This cloned phage fragment directed a bacteriophage resistance phenotype designated Per (phage-encoded resistance). Both φ50 and a distantly related phage, nck202.48 (φ48), formed small plaques on strain NCK213 at a slightly reduced efficiency of plaquing on the Per$^+$ host. The *per* locus was further reduced to a 1.4-kb fragment through in vitro deletion analysis. The 1.4-kb fragment was sequenced, and the Per phenotype was found to be associated with a ca. 500-bp region rich in direct and inverted repeats. We present evidence that the Per region contains a phage origin of replication which, in *trans*, may interfere with phage replication by titration of DNA polymerase or other essential replication factors. It was demonstrated that the Per$^+$ phenotype is not a result of reduced adsorption or action of a restriction and modification system. Per$^+$ activity was not detected against six independent phages which were previously shown to be sensitive to the Hsp$^+$ mechanism. The mutually exclusive resistance mechanisms could be combined to confer resistance to both types of phages (Hsp resistant and Per resistant) in a single host. This is the first description in lactococci of a phage resistance phenotype, other than superinfection immunity, originating from a lactococcal phage genome.

Bacteriophage resistance in lactococci is generally a plasmid-encoded phenomenon. Plasmids have been described which direct resistance by inhibiting adsorption, by restricting phage DNA, or by aborting phage infection (for recent reviews see references 10 and 19). The presence of single plasmids encoding multiple resistance mechanisms or combinations of plasmids within a single strain can confer resistance phenotypes upon lactococcal strains. Frequently, the defense mechanisms succumb to the evolution of new phages. This has occurred in the case of pTR2030 transconjugants of the industrial strain NCK202. pTR2030 is a conjugative plasmid which confers resistance to phage via at least two independent mechanisms, abortive infection (Hsp) and restriction-modification (R/M). Phages have been recovered which can overcome either one or both of these mechanisms (T. Alatossava and T. R. Klaenhammer, unpublished data). The search for novel resistance determinants to add to the growing arsenal of available, independent genotypes has continued. In particular, defense mechanisms active against those phages that are insensitive to Hsp would provide a valuable adjunct to existing resistance genotypes.

One potential source of resistance mechanisms which has, to our knowledge, remained unexploited, is virulent phage genomes. One could envisage that cloned phage fragments could interfere with the normal lytic cycle by the overproduction or titration of essential regulatory signals necessary for the propagation of viable phage progeny. Antisense mRNA is a second possible mechanism by which phage DNA could inhibit homologous phage particles (8). Alatossava and Klaenhammer (unpublished data) have described two recently isolated phages which overcome the resistance of pTR2030 transconjugants of *Lactococcus lactis* NCK202. In this report, we describe the cloning and sequence analysis of a fragment from the terminally redundant lytic phage nck202.50 (φ50) that confers resistance (designated phage-encoded resistance [Per$^+$]) which is phenotypically analogous to the resistance encoded by the Hsp$^+$ recombinant plasmid pTK6 (7). It is highly significant that the Per$^+$ and Hsp$^+$ mechanisms are active against separate groups of phages. When Hsp$^+$ and Per$^+$ are combined in strain NCK203, resistance is directed against all of the phages isolated thus far for that host background.

MATERIALS AND METHODS

Bacteria, bacteriophages, and culture conditions. The bacterial strains used in this study are shown in Table 1. *L. lactis* strains were propagated at 30°C in M17 broth (23) or in M17 broth with 0.5% glucose substituted for lactose when appropriate. Bacteriophages were propagated and titers were determined as described previously (11). *Escherichia coli* strains were grown in LB broth (22) at 37°C with shaking. When they were required for selection, the following antibiotics were added: for *E. coli*, ampicillin (50 μg/ml) and chloramphenicol (20 μg/ml); and for lactococci, erythromycin (5 μg/ml).

Conjugation and transformation. Solid-surface conjugal matings and selection of Lac$^+$ transconjugants were performed as previously described (11, 17). Protoplasts of *L. lactis* NCK203 were transformed as described by Kondo and McKay (12), with some modifications (7). Transformants

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or derivation	Reference
L. lactis strains			
MG1363	Plasmid free, host for φp2, φsk1, and φc2		3
NCK1	MG1363(pTR2030, pTR1040) Hsp^+ R^+/M^+ Lac^+	This study	
NCK202	*str15*, host for φ50, φ48, and φ31		7
NCK203	R^-/M^- derivative of NCK202		7
NCK204	NCK203(pTR2030) Hsp^+ R^+/M^+		7
NCK213	NCK203(pTRK103) Per^+	Transformant, this study	
NCK214	NCK203(pTRK104) Per^+	Transformant, this study	
NCK215	NCK203(pSA3)	Transformant, this study	
NCK216	NCK203(pTRK106)	Transformant, this study	
NCK219	MG1363(pTRK103)	Transformant, this study	
NCK298	NCK203(pTR2030, pTRK104, pTR1040) Hsp^+ R^+/M^+ Per^+ Lac^+	Transconjugant, this study	
NCK294	NCK203(pTRK136) Per^+	Transformant, this study	
NCK295	NCK203(pTRK137) Per^-	Transformant, this study	
NCK296	NCK203(pTRK138) Per^+	Transformant, this study	
E. coli DH1	Transformation host		4
Plasmids			
pBluescript	Cloning vector, Ap^r	Stratagene, La Jolla, Calif.	
pSA3	Shuttle vector, Cm^r Em^r Tc^r		1
pSA34	*ori* selection vector, Cm^r Em^r Tc^r		—[a]
pTRK102	Ap^r	pBluescript::4.5-kb φ50 fragment	
pTRK103	Cm^r Em^r Per^+	pSA3::4.5-kb φ50 fragment	
pTRK104	Cm^r Em^r Per^+	*Eco*RV deletion of pTRK103	
pTRK105	Ap^r	pBluescript::2.5-kb φ50 fragment	
pTRK106	Cm^r Em^r Per^-	pSA3::2.5-kb φ50 fragment	
pTRK136	Cm^r Em^r Per^+	pTRK104, opposite orientation	
pTRK137	Cm^r Em^r Per^-	pTRK104, *Asp* 718 deletion	
pTRK138	Cm^r Em^r Per^+	pTRK104, *Sal*I-*Xho*I deletion	
pTRK141	Cm^r Em^r	pSA34::4.5-kb φ50 fragment	
pTR2030	Hsp^+ Tra^+ R^+/M^+		11

[a] Sanders and Schultz, European patent application 88118362.8.

were selected on plates containing 1.5 μg of erythromycin per ml.

Phage adsorption and center of infection determination. Phage adsorption assays were conducted as described by Sanders and Klaenhammer (20). Center-of-infection data were calculated as described by Klaenhammer and Sanozky (11). Growth curves were determined as described below. A 10-ml portion of M17 broth with 0.5% glucose substituted for lactose, 50 μl of 1 M $CaCl_2$, and 200 μl of an overnight culture were added to a sterile 15-ml cuvette. The cuvettes were placed in a 30°C incubator without aeration, and the optical density at 600 nm was monitored over time. After 1 h, phage were added to a final concentration of 10^4 or 10^6 phage per ml.

Molecular cloning techniques. Plasmid isolation, restriction, ligation, and transformation in *E. coli* DH1 were performed as described by Maniatis et al. (14). Lactococcal phage DNA was isolated as previously described (9).

Sequence determination and analysis. Nucleotide sequences of both strands were determined by the dideoxy chain termination method (21), using the Sequenase enzyme (Stratagene) and either recombinant M13 single-stranded templates or pBluescript clones. Synthetic oligonucleotide primers (17-mers) were synthesized when a subclone was too large to be fully sequenced from commercially available primers. The facilities of the University of Wisconsin Genetics Computer Group were used to analyze the sequence information.

Analysis of phage φ50 replication. Elsewhere we will describe a rapid method for the detection of phage DNA replication (C. Hill, I. J. Massey, and T. R. Klaenhammer, unpublished data). Briefly, sensitive or resistant cells were freshly grown to an optical density at 600 nm of 0.5. $CaCl_2$ (final concentration, 10 mM) was added, and this was followed by the addition of the test phage strain at a multiplicity of infection of more than 1.0. Infected cells were incubated at 30°C. At various times 1 ml of cells was harvested in an Eppendorf centrifuge, and the resulting pellet was frozen rapidly by immersion in ultracold ethanol (−70°C). After thawing on ice, the pellet was suspended in 400 μl of ice-cold lysis solution (6.7% sucrose, 50 mM Tris, 1 mM EDTA, pH 8.0). Lysozyme (20 μl of a 10-mg/ml stock solution) was added, and incubation on ice was continued for 20 min. Sodium dodecyl sulfate (50 μl of a 10% stock solution) was added. Proteinase K (20 μl of a 20-mg/ml stock solution) was added, and the lysate was incubated at 65°C for 20 min. The resulting clear suspension was extracted once with TE-saturated phenol; this was followed by a chloroform-isoamyl alcohol (24:1) extraction. Then 0.1 volume of 3 M sodium acetate and 2 volumes of cold 95% ethanol were added to precipitate the total DNA. The DNA pellet was finally suspended in 100 μl of distilled water; 5 μl was generally sufficient for restriction analysis. Restricted DNA samples from various time points were electrophoresed and stained for the appearance of intense bands, which corresponded to replicating phage DNA.

Nucleotide sequence accession number. The sequence data have been deposited in GenBank under accession number M35639.

RESULTS

Cloning *per* from nck202.50. Phage nck202.50, designated φ50, is a 29.8-kb, virulent, small, isometric-headed phage

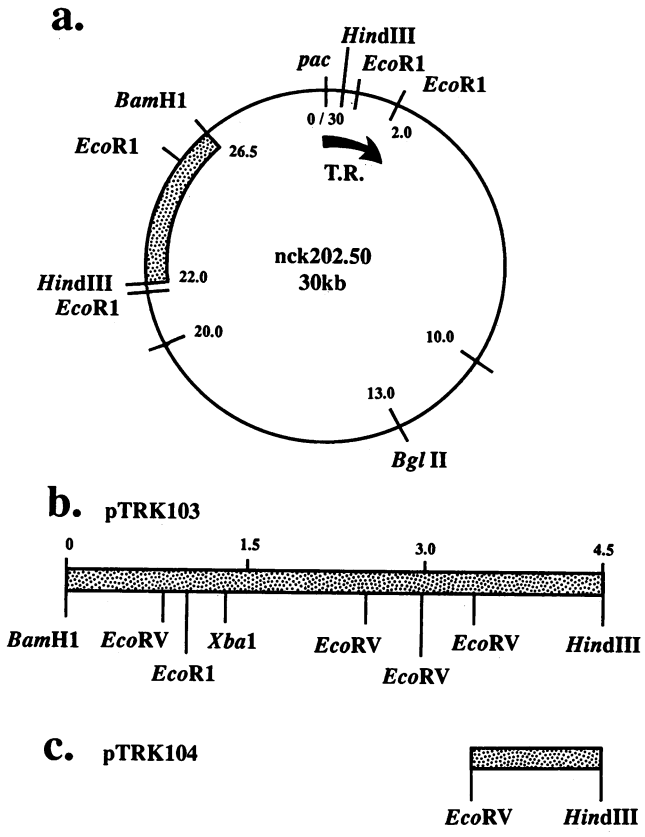

FIG. 1. (a) Restriction endonuclease map of phage ϕ50. The phage genome is shown as a circular molecule. Only 2 of the 13 *Hin*dIII sites are shown. T.R., Terminal redundancy; *pac*, packaging site. The arrow indicates the direction of packaging. (b) The box represents the 4.5-kb *Bam*HI-*Hin*dIII phage fragment cloned in pTRK103 (Per$^+$). (c) The 1.4-kb fragment cloned in pTRK104.

described originally by Alatossava and Klaenhammer (unpublished data). It possesses a unique *Bam*HI site in addition to at least 13 *Hin*dIII sites which have not been mapped on the phage genome (Fig. 1a). Phage ϕ50 DNA was digested with *Bam*HI and *Hin*dIII and shotgun cloned into similarly digested pBluescript. Recombinant plasmids containing both possible *Bam*HI-*Hin*dIII inserts were recovered from transformants. The 4.5-kb insert from one of the plasmids, pTRK102, was subcloned as a *Bam*HI-*Sal*I fragment in the streptococcus-*E. coli* shuttle plasmid pSA3 (1) to create pTRK103 (Fig. 1b). pTRK103 was introduced into *L. lactis* NCK203 via protoplast transformation. NCK203 is a derivative of NCK202 that was cured of a native R/M plasmid, pTRK68 (6). One NCK203 transformant, NCK213, was verified as possessing intact pTRK103 by plasmid profile analysis (data not shown). The plasmid was isolated from NCK213, retransformed into *E. coli* DH1, and subjected to restriction enzyme analysis. No rearrangements in pTRK103 were detected (data not shown).

pTRK103 is Per$^+$. The effect of pTRK103 on ϕ50 was investigated by using standard plaque assays. When present in NCK203, pTRK103 strongly inhibited the ability of ϕ50 to form plaques. Plaques were pinpoint in size and difficult to enumerate; the efficiency of plaquing (EOP) was estimated to be 0.35 (Table 2). On NCK203 harboring shuttle vector pSA3 without an insert, ϕ50 gave normal plaques having a diameter of 1.6 mm and an EOP of 1.0 (Table 2).

TABLE 2. Phage ϕ50, ϕ48, and ϕ31 reactions with *L. lactis* NCK203 and derivatives

Phage	Host (plasmid)	EOP	Plaque size (mm)
ϕ50	NCK203	1.0	1.6
ϕ50	NCK215(pSA3)	1.0	1.6
ϕ50	NCK213(pTRK103)	0.35	0.2
ϕ48	NCK203	1.0	1.5
ϕ48	NCK213(pTRK103)	0.5	0.2
ϕ31	NCK203	1.0	1.5
ϕ31	NCK213(pTRK103)	1.0	1.5
ϕ50	NCK216(pTRK106)	1.0	1.6
ϕ48	NCK216(pTRK106)	1.0	1.5
ϕ50	NCK214(pTRK104)	0.42	0.2
ϕ48	NCK214(pTRK104)	0.5	0.2
ϕ50	NCK298(pTR2030, pTRK104)	0.44	0.2
ϕ31	NCK298(pTR2030, pTRK104)	$<10^{-8}$	No plaques

NCK203(pTRK103) also inhibited plaque formation by small isometric-headed phage nck202.48 (ϕ48). The plaque size was reduced, and an EOP of 0.5 was estimated for this phage (Table 2). However, NCK203(pTRK103) did not inhibit plaque formation by small isometric-headed phages nck202.31 (ϕ31), nck202.35 (ϕ35), and nck202.36 (ϕ36) (data for ϕ31 in Table 2, others not shown). pTRK103 was introduced into *L. lactis* MG1363 by protoplast transformation, and one transformant, strain NCK219, was challenged with three phages that were lytic for that host. The presence of pTRK103 did not affect the plaque size or the EOP of prolate phage ϕc2 or small isometric phages ϕp2 and ϕsk1. In contrast, Hsp$^+$ plasmid pTK6 retards normal plaque formation for ϕc2, ϕp2, and ϕsk1 in MG1363 (7) and for ϕ31, ϕ35, and ϕ36 in NCK203 (6). The Hsp$^+$ activity directed by pTK6 did not inhibit either ϕ48 or ϕ50 in the NCK203 background (data not shown).

A second clone was constructed to evaluate the possibility that the resistance encoded by pTRK103 is a general phenomenon of phage fragments distributed around the ϕ50 genome. Phage ϕ50 DNA was digested with *Hin*dIII and shotgun cloned into *Hin*dIII-digested pBluescript. A bank of clones with inserts representing the phage genome was obtained. One recombinant plasmid, pTRK105, which contained an insert of approximately 2.5 kb, was randomly selected, and the insert was subcloned in pSA3 as a *Bam*HI-*Xba*I fragment. The resultant recombinant plasmid, pTRK106, was introduced into strain NCK203 by protoplast transformation. The pTRK106 transformant was challenged with ϕ48 and ϕ50. This randomly selected phage fragment had no effect on the plaque size or the EOP of either phage (Table 2).

Subcloning the *per* region. pTRK103 contains five *Eco*RV sites, four of which are internal to the ϕ50 insert (Fig. 1b). pTRK103 was digested with *Eco*RV, religated, and transformed into *E. coli* DH1. Cm^r transformants were recovered containing plasmids from which one or more of the *Eco*RV fragments had been deleted. These plasmids were transformed into strain NCK203 and examined for their effect on ϕ48 and ϕ50. All of the derivatives directed the same Per$^+$ resistance phenotype as pTRK103 did. This localized the resistance determinant to a 1.4-kb *Eco*RV-*Hin*dIII fragment which remained undisturbed in all of the in vitro deletions (Fig. 1c). This fragment was present in all deletion derivatives because there is no *Eco*RV site to the right of this

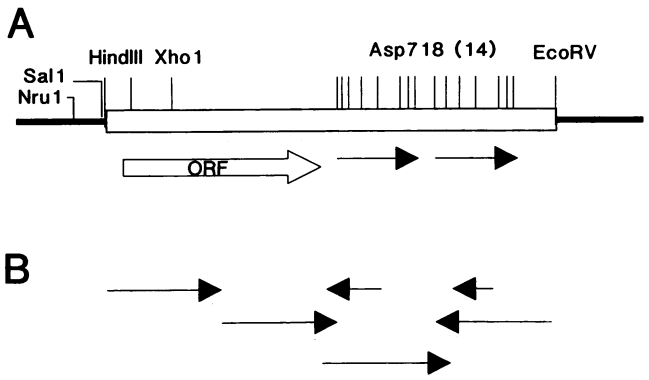

FIG. 2. (A) Physical map of the 1.4-kb insert in pTRK104. The positions of the ORF and direct repeats are indicated. (B) Sequencing strategy for the pTRK104 insert. Only the repeated sequence was determined from both strands.

region. The Per$^+$ phenotype was encoded by a plasmid containing only this 1.4-kb region, pTRK104 (Fig. 2A). The 1.4-kb insert in pTRK104 was inverted by digesting the plasmid with *Eco*RV and *Nru*I, which cut on either side of the insert (Fig. 2A). After religation, a recombinant plasmid, pTRK136, was recovered, in which the insert was present in the opposite orientation. pTRK136 directed Per$^+$ at a level identical to that of pTRK104, indicating that any information necessary for *per* activity was located within the fragment.

Sequence and analysis of *per*. The 1.4-kb insert in pTRK104 was subcloned in M13mp18 and mp19 as an *Eco*RV-*Cla*I fragment and was sequenced by the strategy shown in Fig. 2. The sequence analysis revealed a single 701-bp open reading frame (ORF) (Fig. 3). An unexpected result was the presence of a second *Hin*dIII site 69 bp from the pSA3-derived *Hin*dIII site used in the original cloning protocol. This small fragment either was inserted fortuitously during the ligation reaction or was a contiguous ϕ50 fragment which was included as a result of a partial digestion. This region includes the potential start codon for the 701-bp ORF contained within the fragment. The 3′ end of the insert proved to be difficult to sequence and was resolved finally by conducting the polymerase reactions at 0°C, as recommended by the manufacturer for regions rich in secondary structure. The resulting unambiguous sequence revealed a 236-bp direct repeat separated by a 28-bp region (Fig. 3). This extremely large direct repeat was almost 99% identical, with only three mismatches in the 236-bp repeat. Within the direct repeat, a number of inverted repeats capable of forming stable stem-loop structures could be detected (Fig. 4). These stable structures are typical of sequences associated with rho-independent termination. The 10-bp sequence GGTACCGAGG was also repeated four times within each larger direct repeat (Fig. 3). In addition, the palindromic sequence GGTACC was repeated 14 times overall in this 500-bp region.

Repeated sequences are responsible for Per. pTRK104 was digested with *Sal*I and *Xho*I and religated. The resulting construct, pTRK138, no longer possesses the putative start codon for the ORF. pTRK138 was introduced into *L. lactis* NCK203 and challenged with ϕ50 and ϕ48. The Per$^+$ phenotype was observed (data not shown), indicating that the ORF is not involved in the phage resistance phenotype. A second derivative was constructed to assess the effect of the repeated sequence on the Per phenotype. The palindromic repeat GGATCC, present 14 times within the repeated regions, is the recognition site for the type II restriction endonuclease *Asp* 718 and its isoschizomer, *Kpn*I. pTRK104 was digested with *Asp* 718 and religated to form pTRK137. This plasmid has lost approximately 400 bp, including most of the large direct and inverted repeats present in pTRK104. When introduced into strain NCK203, pTRK137 did not direct any resistance against either phage ϕ48 or ϕ50. We conclude that an intact repeated sequence is essential for the Per$^+$ phenotype.

Effect of pTRK104 on ϕ50. The growth of strain NCK203 containing pTRK104 (strain NCK214) at 30°C in the presence and absence of ϕ50 was examined (Fig. 5A). NCK214 grew normally in the presence of ϕ50 (initial levels, 10^4 phage per ml), whereas the control strain NCK215, containing the cloning vector pSA3, was rapidly lysed under the same conditions. The mechanism of action of *per* was further examined by conducting phage adsorption and center-of-infection studies with strain NCK214. Adsorption studies were performed at 30°C with NCK203 derivatives harboring either pSA3 (strain NCK215) or pTRK104 (strain NCK214). NCK214 adsorbed ϕ50 at a level of 87%, compared with a level of 84% for the sensitive host, NCK215. However, the number of infective centers formed on NCK214 was 43% of that on NCK215. Standard plaque assays conducted at a high temperature (39°C) revealed that the Per mechanism is not heat sensitive. Similar plaque sizes and EOPs were obtained at both 39 and 30°C (data not shown). Phage were inhibited to the same extent by pTRK104 irrespective of whether the phage was first propagated on a host bearing pTRK104. Therefore, phage surviving through a Per$^+$ host are incapable of circumventing Per in subsequent infections.

Per$^+$ affects phage DNA replication. The internal replication of ϕ50 DNA was followed by using a rapid DNA isolation procedure in which phage-infected cells are harvested at various times and examined for phage DNA (Fig. 6). At 40 min after phage infection, the appearance of ϕ50 DNA could be clearly detected in the propagating host, NCK203 (Fig. 6, lane 4). Phage-directed replication of the ϕ50 genome prior to cell lysis was reflected in the accumulation of ϕ50-specific DNA fragments. With increasing time after infection, phage DNA became more evident, suggesting continued replication (lanes 5 and 6). In the NCK203 derivative containing pTRK138, strain NCK296 (Per$^+$), phage DNA replication over the identical time course was less pronounced, suggesting that Per interferes with phage DNA replication. It is significant that the plasmid bearing Per, pTRK138, increased in copy number after infection (Fig. 6, lanes 9 through 11). An increase in the copy number of pTRK138 was not apparent during ϕ31 infection of strain NCK296, nor was there any decrease in ϕ31 DNA replication (data not shown). We speculate that a plasmid bearing the phage origin of replication (*ori*) should increase in copy number as a consequence of phage infection. It is noteworthy that the increased amount of pTRK138 evident in ϕ50-infected NCK296 was not present in a covalently closed circular form. Uncut DNA preparations do not show increased amounts of the covalently closed circular plasmid, suggesting that pTRK138 is amplified as a concatemeric form during phage infection (data not shown). These data indicate that the Per region contains a phage origin of replication.

The pSA3 cloning vector, contained in strain NCK215, was not amplified after phage ϕ50 infection. Therefore, the presence of the repeated ϕ50 DNA sequence is required for amplification of pTRK138. The 4.5-kb insert from ϕ50, contained in pTRK103, was subcloned into pSA34, a derivative of pSA3 from which the gram-positive origin of repli-

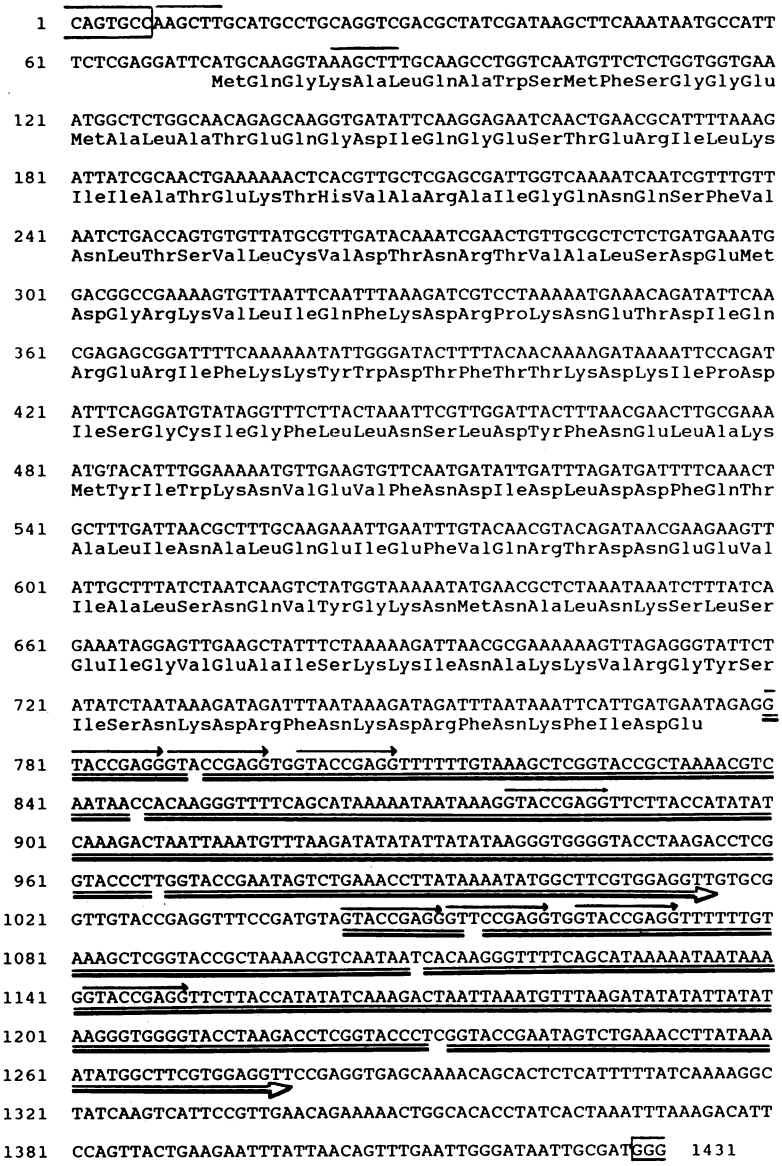

FIG. 3. Nucleotide sequence of the pTRK104 insert and deduced amino acid sequence of the ORF. The 236-bp direct repeat is indicated by the larger arrows; the 10-bp direct repeats are indicated by the smaller arrows. The positions of both *Hin*dIII sites are overlined. M13 flanking sequences are enclosed in boxes.

cation had been deleted (M. E. Sanders and J. W. Schultz, European patent application 88118362.8, 1989). We were unable to transform *L. lactis* NCK203 with the resulting construct pTRK141. Therefore, the putative phage φ50 *ori* is incapable of supporting viable plasmid replication in the absence of a concurrent phage infection.

Combining Hsp$^+$ and Per$^+$ in NCK203. pTR2030 was introduced into strain NCK214 by solid surface conjugation with donor strain NCK1. Transconjugants were selected initially for acquisition of the Lac$^+$ phenotype encoded by the nonconjugative plasmid pTR1040. Lac$^+$ (pTR1040) Em^r (pTRK104) transconjugants were subsequently screened for the presence of pTR2030. One transconjugant, strain NCK298, containing both pTR2030 and pTRK104, was selected and examined for combined resistance to phages φ50 and φ31 (insensitive to Hsp$^+$ and Per$^+$, respectively). Strain NCK298 was completely resistant to φ31 in standard plaque assays, a typical reaction for this phage on pTR2030-bearing hosts. The host bearing both plasmids also displayed the typical Per$^+$ response against φ50 (Table 2). The growth of strain NCK298 was not affected when both φ50 and φ31 were present at initial levels of 10^6 PFU/ml (Fig. 5B). Strains containing either pTR2030 or pTRK104 were inhibited by the presence of both types of phage. These data indicate that both resistance mechanisms can operate simultaneously in the NCK203 background and expand the phage resistance of this strain.

DISCUSSION

A number of phage resistance phenotypes have been identified and characterized in lactococci (reviewed in reference 10). Most resistance genotypes described thus far are plasmid linked. Strategies that will be useful for the con-

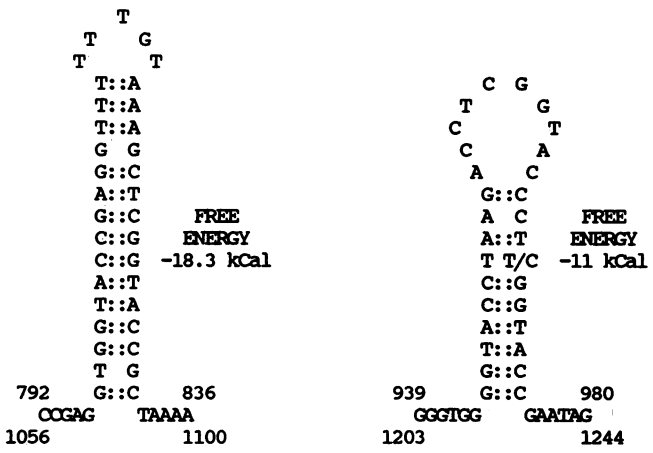

FIG. 4. Inverted repeats contained within the large direct repeats, shown as hairpin loops. The locations of the inverted repeats are also indicated. (1 kcal = 4,184 J.)

struction of phage-insensitive strains include the introduction of one or more resistance mechanisms within a single host and the introduction of a single plasmid containing more than one resistance mechanism (19). A prerequisite for the success of these strategies is that the resistance mechanisms should work in combination to prevent phage proliferation. The insensitivity of any constructed, or natural, isolate is probably a function of time, amount of use, and environmental conditions (13). In the case of NCK202-pTR2030 transconjugants, two apparently novel phages which can overcome either one or both resistance mechanisms encoded by this plasmid (Hsp [abortive infection] and R/M) have been detected during extended industrial use. One response to the evolution of Hsp-resistant phages is to identify complementary mechanisms which are effective against that group and to employ these mechanisms in combination with Hsp. It is not likely that all avenues of phage replication can be blocked in this stepwise manner, but adaptation and proliferation of new phages could be minimized and the longevity of the cultures could be extended.

We identified a novel mechanism of resistance, designated Per, which is phenotypically similar and complementary to Hsp. This resistance mechanism is unique among lactococcal phage resistance genotypes in that it is derived from a fragment isolated from a lytic phage genome (ϕ50). In addition to encoding resistance to the phage from which it originated, the Per mechanism is also active against another phage (ϕ48), which is also unaffected by Hsp. Both Per-sensitive phages were isolated on pTR2030-containing transconjugants and are resistant to the Hsp mechanism encoded by pTR2030 (Alatossava and Klaenhammer, unpublished data). In contrast, Hsp-sensitive phages ϕ31, ϕ35, and ϕ36 are resistant to the Per mechanism. In all cases, adsorption is not affected, but the numbers of infective centers formed on pTRK104-bearing cells are reduced significantly.

Phage-encoded resistance has been described for phage lambda in *E. coli* and is mediated by the autonomously replicating lambda-dv deletion derivatives of phage lambda (15, 16). In that instance it is believed that phage replication is inhibited by the presence of the repressor *tof* gene product expressed from the lambda-dv replicon, which acts to prevent transcription of the incoming phage operon. Our evidence strongly suggests that the Per region is the origin of replication for phage ϕ50. First, since the region involved in Per does not direct the expression of a protein intermediate, it is probable that the resistance encoded by Per reflects the titration of essential phage regulatory signals by plasmids containing this region and not the production of a repressor. Second, the Per region can be shown to have a negative

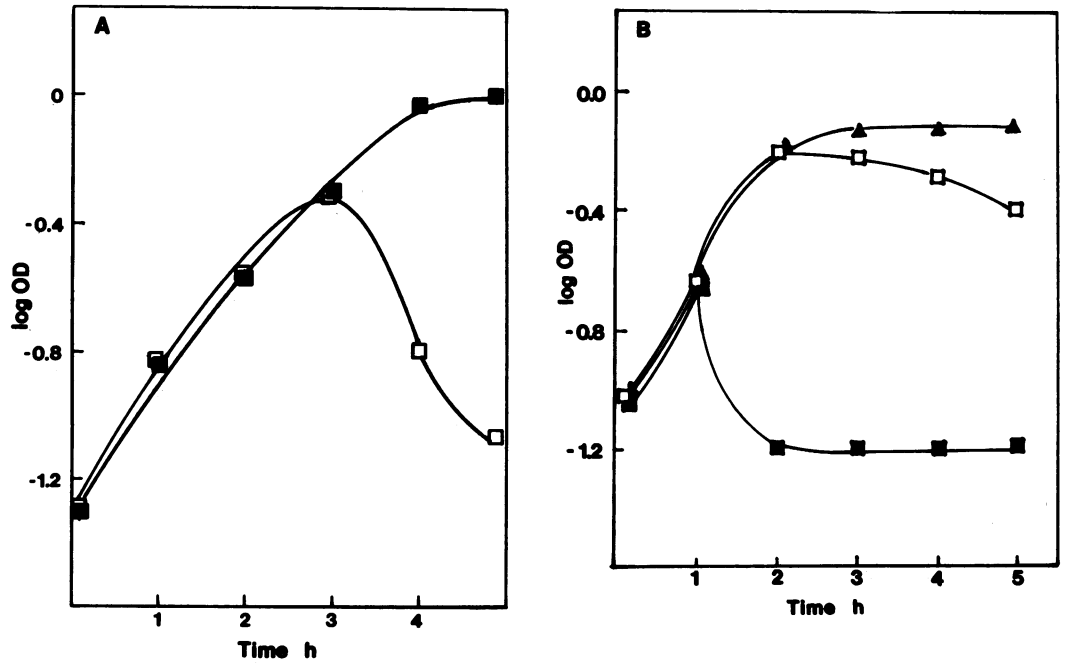

FIG. 5. Effect of lytic phages on the growth of NCK203 derivatives containing pSA3, pTRK104 (Per$^+$), and/or pTR2030 (Hsp$^+$ R$^+$/M$^+$). (A) Growth of NCK203(pSA3) (□) and NCK203(pTRK104) (●) in the presence of ϕ50. (B) Growth of NCK203(pTRK104) (■), NCK203(pTR2030) (□), and NCK203(pTR2030, pTRK104) (▲) in the presence of both ϕ50 and ϕ31. OD, Optical density.

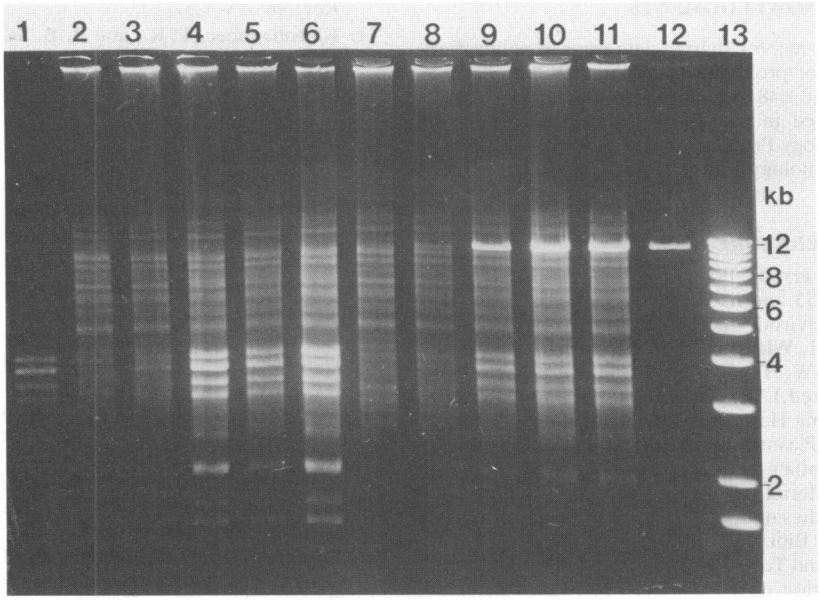

FIG. 6. Agarose gel electrophoresis of total DNA preparations isolated at various times after φ50 infection. Lane 13 contained lambda DNA size standards. Lane 1 contained φ50 DNA isolated from purified phage particles and digested with *Eco*RI. Lanes 2 through 6 contained total DNA from strain NCK203 isolated before infection (lane 2) and at 20, 40, 60, and 80 min after infection (lanes 3 through 6, respectively; lanes 7 through 11 contained total DNA from strain NCK296 isolated at the same time points, respectively. Lane 12 contained pTRK138. All DNA samples were digested with *Eco*RI.

impact on the replication of φ50 DNA, whereas there is no effect on the replication of an insensitive phage, φ31. Third, the presence of the Per region on plasmid pTRK139 leads to an increase in its replication rate after infection with φ50, although not after infection with φ31. Fourth, the Per region itself bears the hallmarks associated with phage replication origins, repeated and inverted repeats, and terminatorlike structures (2, 18). To our knowledge, this is the first description of a lactococcal phage *ori*.

A successful phage infection relies upon an interaction between a phage- or host-encoded DNA polymerase and the phage *ori*. The presence of the *ori* on a multicopy plasmid would be expected to interfere with the normal lytic process by titrating DNA polymerase and diverting replication toward the plasmid. This could be expected to result in the uncontrolled replication of plasmid DNA. This theory is also consistent with the finding that Per is effective only against phage with which it shares homology (φ50 and φ48). Those phages unaffected by Per differ in that they must utilize an alternative *ori* sequence or alternative replication factors and are therefore not susceptible to any titration effects on the phage-directed DNA polymerase.

The Per phenotype is not a generalized feature of cloned phage DNA. A randomly selected phage fragment did not affect the proliferation of φ50, despite a larger region of homology (2.5 kb) to the phage than that possessed by pTRK104 (1.4 kb) or its derivatives. We can therefore rule out homologous recombination and concomitant disruption of phage sequences as a cause of Per activity.

Given the similarity of the Per and Hsp phenotypes when both are cloned in vector pSA3, we suggest that these two phenotypes affect similar points in the phage lytic cycle. However, it is obvious that they are unrelated genotypically. The Hsp essential region has been cloned and sequenced and has been shown to be a large single gene (5), whereas the Per phenotype is the result of a noncoding region of repeated sequences. The Per region shares homology with both phages to which it encodes resistance, but the Hsp region does not (9; Alatossava and Klaenhammer, unpublished data). Finally, the Hsp mechanism is slightly heat sensitive in strain NCK203, whereas Per is not. Nonetheless, it is significant that the *hsp* gene product has been shown to interfere with phage DNA replication (Hill et al., unpublished data). It may be that the *hsp* gene product is also *ori* specific, but only against the φ31 type of *ori* and not that of phages φ48 and φ50.

It is noteworthy that a strain containing both pTR2030 and pTRK104 is insensitive to contaminating levels of phages representing both the Per-resistant (φ50) and Hsp-resistant (φ31) groups. Hsp^+ and Per^+ can work in concert to provide a more extensive range of insensitivity than either mechanism used in isolation. It remains to be determined whether phages which can be resistant to both Hsp and Per can evolve or whether all possible avenues of phage proliferation have been blocked. This question can be answered only by constructing strains containing both mechanisms and subjecting them to the kinds of pressures which led to the isolation of Hsp-insensitive phages φ48 and φ50.

Isolation of phage resistance mechanisms from lytic phage genomes may provide a wealth of alternative defenses that can be used to protect industrial strains against failure due to phage attack. The Per phenotype is not dependent on the production of repressors or on the transcription of antisense RNA. Because of this, no energy is expended by the resistant host, other than normal plasmid replication, to maintain the Per response to phage attack. It is important to note that the Per mechanism is effective against a phage (φ48) other than the source phage (φ50). The approach outlined in this report may also be useful for industrial strains other than lactococci which are subject to phage problems, particularly those for which plasmid-encoded defenses are not readily available.

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