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A Triggered-Suicide System Designed as a Defense against Bacteriophages†

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A novel bacteriophage protection system for *Lactococcus lactis* based on a genetic trap, in which a strictly phage-inducible promoter isolated from the lytic phage φ31 is used to activate a bacterial suicide system after infection, was developed. The lethal gene of the suicide system consists of the three-gene restriction cassette *Lla*IR$^+$, which is lethal across a wide range of gram-positive bacteria. The phage-inducible trigger promoter (φ31P) and the *Lla*IR$^+$ restriction cassette were cloned in *Escherichia coli* on a high-copy-number replicon to generate pTRK414H. Restriction activity was not apparent in *E. coli* or *L. lactis* prior to phage infection. In phage challenges of *L. lactis*(pTRK414H) with φ31, the efficiency of plaquing was lowered to 10^{-4} and accompanied by a fourfold reduction in burst size. Center-of-infection assays revealed that only 15% of infected cells released progeny phage. In addition to phage φ31, the φ31P/*Lla*IR$^+$ suicide cassette also inhibited four φ31-derived recombinant phages at levels at least 10-fold greater than that of φ31. The φ31P/*Lla*IR$^+$-based suicide system is a genetically engineered form of abortive infection that traps and eliminates phages potentially evolving in fermentation environments by destroying the phage genome and killing the propagation host. This type of phage-triggered suicide system could be designed for any bacterium-phage combination, given a universal lethal gene and an inducible promoter which is triggered by the infecting bacteriophage.

Many important industrial bioprocesses and food fermentations are susceptible to attack by bacteriophages. Control of phage problems in the commercial arena requires prevention of phage contamination, employment of strains that are resistant to phage infection, and minimization of opportunities for the appearance of new virulent phages. With the diversity of fermentation systems, it is not always possible to operate an aseptic fermentation or, if available, to maintain its integrity. Therefore, the longevity of any highly specialized strain in a commercial situation depends on the bacterium's relative phage resistance or sensitivity and how quickly defiant phages appear and proliferate against it. Modern biotechnology continues to provide increasing opportunities to create specialized strains with valuable processing or product characteristics. In dairy fermentations, which are plagued by phage attacks more often than any other bioprocessing industry, this problem has been traditionally approached with varying success by isolating phage-resistant mutants and incorporating the derivatives into the starter culture formulations. Over the last decade, a number of naturally occurring plasmid-encoded defenses in *Lactococcus* species have been discovered which prevent phage adsorption, prevent DNA injection, restrict unmodified phage DNA by resident restriction and modification (R/M) systems, or abort the phage infection following the early stages of phage development (21, 27). By using conjugation or transformation approaches, these phage defenses can now be genetically introduced into bioprocessing strains by pyramiding selected defense systems (16, 26). Bacteria which are recalcitrant to phage infection can be derived. After extended use, however, new phages virulent against new or improved strains inevitably appear as selection pressures compromise defenses or the phages define genetic routes to circumvent them (23, 35).

It is the rare appearance and then unchecked proliferation of new virulent phages that threaten most fermentation industries. Phage protection strategies should not only provide resistance, but also design mechanisms that will capture an emerging phage and prevent its proliferation to disruptive population levels (23, 53). Diverse pathways exist for the cell to halt phage development postinfection, leading concurrently to cell death. The variety of abortive infection (Abi) systems illustrates the importance of these natural mechanisms that limit phage proliferation. Essentially, Abi mechanisms are recognized as natural self-imposed suicide systems that limit phage development. It appears that cell death occurs as an aftermath of lasting infection after the host machinery has been redirected irreversibly towards phage functions. Rather than directing defense against phage per se, in this study we have used a genetically engineered form of Abi and evaluated an entirely new approach that could be used to control phages in industrial bioprocesses. A phage-specific inducible promoter was employed to sense the infection and trigger expression of a lethal gene designed to simultaneously kill the host and the infecting phage in a process that mimics programmed cell death. Sacrificing infected cells through phage-induced suicide strategies establishes an altruistic goal of protecting the bacterial population as a whole by enticing new virulent phages into individual cells armed with genetic traps that destroy the potential of the infecting phage and bacterial host to proliferate new progeny phages. We envisioned that a phage-inducible suicide system would require an appropriate lethal gene, a phage-inducible promoter triggered only after phage infection, and a suitable vector that would provide ample copies of the suicide cassette in the bacterial host. A number of lethal genes

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TABLE 1. Plasmids used or constructed in this study

Plasmid	Relevant characteristics	Reference or origin
pBluescript II KS$^+$	2.96 kb; Ap^r	Stratagene
pTRKL2	6.4 kb; *lacZ*; Em^r	41
pTRKH2	6.9 kb; *lacZ*; Em^r	41
pTRK370	15.9 kb; encodes complete *llaI* operon; Em^r; R$^+$/M$^+$	43
pTRK394	6.6 kb; Ap^r; pBluescript II KS$^+$ with 3.7-kb *Pvu*II-*Cla*I DNA fragment from pTRK370 that contains the promoterless *llaI.1-llaI.2-llaI.3* cassette	12
pTRK395	9.8 kb; Em^r; pTRKL2 with a 3.7-kb *Pvu*I-*Hin*dII DNA fragment from pTRK394 that contains the promoterless *llaI.1-llaI.2-llaI.3* cassette	12
pTRK397	10.3 kb; Em^r; pTRKH2 with 3.7-kb *Pvu*I-*Xho*I DNA fragment from pTRK394 that contains the promoterless *llaI.1-llaI.2-llaI.3* cassette	12
pTRK414L	10.1 kb; Em^r; the 239-bp *Fsp*I-*Bam*HI ϕ31 promoter fragment from pTRK391 (44) added to pTRK395	This study
pTRK414H	10.6 kb; Em^r; the 239-bp *Fsp*I-*Bam*HI ϕ31 promoter fragment from pTRK391 (44) added to pTRK397	This study
pTRK400	7.9 kb; Km^r Cm^r; pNZ18 (10) with 2.4-kb fragment from pTRK370, encoding *llaIC* with the native promoter from the *llaI* operon	12

have been used successfully for programmed cell death in eukaryotes or prokaryotes and include genes encoding general nucleases, proteases, and membrane- or cell wall-active agents, etc. (1, 29, 33, 39, 54). In our study, the restriction component of the lactococcal *Lla*I R/M system was evaluated as a potential lethal gene for use in a suicide cassette.

In lactococci, R/M systems are widely distributed and are likely to provide at least two important roles, first as barriers to heterologous (unmodified) phage infection (27) and second as possible potentiators of plasmid retention (30, 38, 59). In the latter case, it is noteworthy that lactococcal plasmids which direct vital physiological properties (e.g., lactose metabolism, proteolytic activity, conjugative ability, bacteriocin production, and other complementary phage defenses) routinely encode R/M systems as well. The *Lla*I R/M system is naturally encoded on the lactococcal conjugative plasmid pTR2030 (22). The *Lla*I R/M system, which is a novel class exhibiting characteristics of both type IIS and type I, is encoded by a six-gene operon composed of a regulatory gene, *llaIC*; a methylase gene, *llaIM*; three genes essential to restriction activity (*llaI.1*, *llaI.2*, and *llaI.3*, designated *Lla*IR$^+$); and a 3′ open reading frame of unknown function (42, 43). The three-gene *Lla*IR$^+$ cassette has been subcloned away from its corresponding methylase into three shuttle vectors (pBV5030 [7] and pTRKL2 and pTRKH2 [41]) downstream from a constitutive *Lactobacillus* promoter, P6 (13), that is functional in both gram-positive and gram-negative bacteria (12). The P6/*Lla*IR$^+$ cassette was successfully assembled in *Escherichia coli* and exerted no restriction activity in this host, presumably due to inefficient translation or improper assembly of the restriction complex. The restriction cassette was functional in *Lactococcus* species, as well as across a wide range of gram-positive bacteria, and could not be transformed into these hosts without first inactivating one of the three essential reading frames (12). Therefore, if expressed under the control of a suitable promoter, the *Lla*IR$^+$ cassette could function as the lethal component of the phage-inducible suicide system.

The final critical element required for assembly of this system was an inducible phage promoter that is not expressed by the bacteria in the absence of a phage infection. Many such promoter elements are responsible for temporal expression and have been described for well-characterized bacteriophages which attack *E. coli* and *Bacillus subtilis* (15, 18, 25, 31). In addition, several phage-inducible promoters in lactococcal bacteriophages were mapped following the partial or complete sequencing of phage genomes (24, 32, 45). However, only one inducible element has been thoroughly characterized to date for a lytic lactococcal bacteriophage. A middle, phage-inducible promoter from the P335 species lactococcal bacteriophage ϕ31 was initially cloned on an 888-bp fragment (44). Molecular characterization and primer extension analysis of the promoter-containing region revealed four transcription start sites. Detailed characterization of the promoter element will be presented elsewhere. For the present study, a 239-bp fragment containing two tandem transcription starts which are expressed only after phage infection was subcloned. The suicide system was assembled by placing the phage ϕ31-specific trigger promoter (designated ϕ31P) 5′ of the *Lla*IR$^+$ cassette. The ability of this inducible suicide system to provide phage protection to *Lactococcus lactis* was evaluated in this study.

MATERIALS AND METHODS

Bacteria, bacteriophage, and plasmids. *E. coli* MC1061 (8) was used as the primary transformation host throughout the study. *E. coli* MC1061 derivatives harboring plasmid pTRK414L or pTRK414H were designated NCK785 and NCK781, respectively. *L. lactis* NCK690 was the sensitive host for bacteriophage ϕ31. This strain is a derivative of *L. lactis* NCK203 (22) spontaneously cured of all plasmids except a 7.5-kb plasmid (15a). *L. lactis* NCK690 harboring pTRK414L or pTRK414H was designated NCK786 and NCK782, respectively. Phage ϕ31 is a small, isometric, P335 species, cohesive-ended lactococcal bacteriophage with a double-stranded DNA genome of 31.9 kb. This phage is sensitive to both AbiA-mediated abortive resistance and *Lla*I restriction (2). Recombinant phages ϕ31.1, ϕ31.2, ϕ31.7, and ϕ31.8 are derivatives of phage ϕ31 that are unaffected by the ϕ31 origin of replication (*per31*) presented in *trans* (17, 40). Plasmids used or constructed in this study are listed in Table 1.

Culture conditions and bacteriophage assays. *E. coli* strains were grown at 37°C in Luria-Bertani medium (50) or brain heart infusion (BHI) medium (Difco Laboratories, Detroit, Mich.). Erythromycin (100 μg/ml) was added for selective propagation of *E. coli* in broth where appropriate. Agar plates (1.5% granulated agar; BBL Microbiology Systems, Cockeysville, Md.) were prepared from BHI medium. Erythromycin-resistant (Em^r) *E. coli* transformants were selected on BHI agar with 200 μg of erythromycin per ml. *L. lactis* was propagated at 30°C in M17 broth (Difco) containing 0.5% glucose (GM17). Erythromycin (2.5 μg/ml) or chloramphenicol (3.0 μg/ml) was used for antibiotic selection in lactococci. Bacteriophage plaque assays were conducted as described previously (56). Center of infection (COI) assays, one-step growth curves, and burst size determinations were all performed at 30°C as described previously (53).

Plasmid and phage DNA isolation and molecular cloning. Rapid isolation of *E. coli* plasmid DNA was done by an alkaline lysis method (6). Large-scale isolation of *E. coli* plasmid DNA was accomplished, using a kit (QIAGEN, Inc., Chatsworth, Calif.) according to the manufacturer's instructions. Isolation of plasmid DNA from lactococci was performed by an alkaline lysis protocol as described previously (7). Phage DNA was isolated by using a large-scale protocol as described elsewhere (47). Standard techniques were used for endonuclease restriction, dephosphorylation, and ligation of bacterial DNA (50). Ligation of phage DNA was performed as described by Sambrook et al. (50),

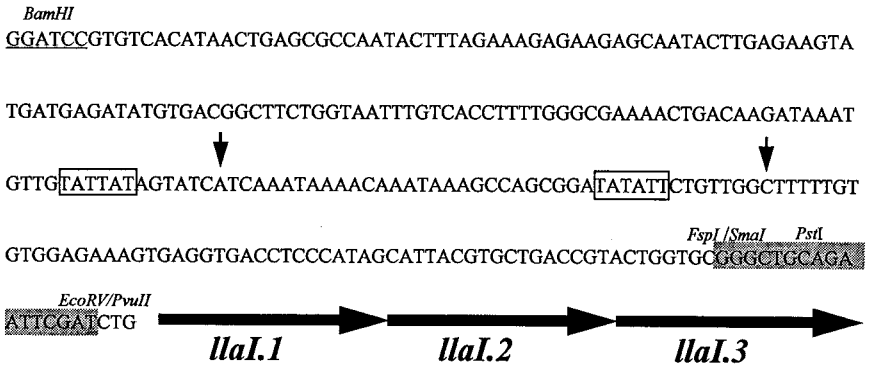

FIG. 1. Nucleotide sequence of the 239-bp ϕ31P promoter fused to the *Lla*IR$^+$ restriction cassette of *llaI.1*, *llaI.2*, and *llaI.3* (ϕ31P/*Lla*IR$^+$). The transcription start sites at nucleotides 703 and 744 (44) (vertical arrows), the −10 consensus promoter regions (boxes), and a portion of the multiple cloning site from pBluescript II KS$^+$ (shaded region) are indicated. Junctions between the multiple cloning site and ϕ31P and *llaI.1* are represented by *Fsp*I/*Sma*I and *Eco*RV/*Pvu*II, respectively. The *Bam*HI site was introduced by the oligonucleotide primer used for PCR subcloning of ϕ31P.

with the following modifications: phage DNA fragments were heated at 65°C for 15 min before addition of vector DNA, and the ligation mixture was heated at 65°C for an additional 10 min and then slowly cooled to 30°C before addition of ligation buffer and T4 DNA ligase. All DNA used in cloning reactions was first purified in SeaKem GTG agarose (FMC BioProducts, Rockland, Maine) and extracted by using a QIAEX DNA extraction kit (QIAGEN, Inc.) according to the manufacturer's instructions.

Bacterial transformation. Electrocompetent *E. coli* cells were prepared as described previously (14) and electroporated with a Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, Calif.). *L. lactis* cells were electrotransformed as described elsewhere (12).

RESULTS

Construction of suicide cassette. The promoterless *Lla*IR$^+$ restriction cassette was first cloned as a 3.7-kb *Pvu*II-*Cla*I fragment from the R/M plasmid pTRK370 into pBluescript II KS$^+$ (pTRK394). The *Lla*IR$^+$ cassette was then subcloned into the low- and high-copy-number shuttle vectors pTRKL2 and pTRKH2 to generate pTRK395 and pTRK397, respectively. To prevent transcription from a *lacZ* promoter, the restriction cassette was positioned in an orientation opposite to *lacZ* in all three plasmids. A 239-bp *Bam*HI-*Fsp*I DNA subfragment encoding a phage-inducible promoter with tandem transcription starts was isolated from the 888-bp fragment originally cloned into pTRK391 (44). The ϕ31 promoter element, designated ϕ31P (Fig. 1), exhibits no detectable activity in the absence of a ϕ31 infection (58). The ϕ31P fragment was cloned 5′ of the *Lla*IR$^+$ cassette encoded on pTRK395 and pTRK397, and pTRK414L (low-copy-number) and pTRK414H (high-copy-number) plasmids were recovered in *E. coli* MC1061 (Fig. 2). The two plasmids were electroporated into *L. lactis* NCK690. No differences in transformation frequencies were observed when *E. coli* or *L. lactis* cells were transformed with pTRK414L and pTRK414H, compared to pTRK395 and pTRK397 encoding the promoterless *Lla*IR$^+$ cassette. As *Lla*I (R$^+$/M$^-$) plasmids are lethal in *L. lactis* (43), it was evident that the ϕ31P promoter was not recognized in these *L. lactis* transformants in the absence of a phage infection. The plasmids were stable during repeated subculture under antibiotic selection.

Functional characterization of the ϕ31P/*Lla*IR$^+$ cassette. Standard plaque assays were used to evaluate the efficiency at which phage ϕ31 infected *L. lactis* harboring the restriction cassettes (Table 2). When *L. lactis* NCK690 containing either pTRK395 or pTRK397, both encoding the promoterless restriction cassette, was challenged, phage ϕ31 plaqued at full efficiency. However, in the presence of pTRK414L, containing the ϕ31P/*Lla*IR$^+$ cassette on a low-copy-number replicon, the efficiency of plaquing (EOP) was reduced to 0.5, with no notable changes in plaque size or morphology. In the presence of pTRK414H, containing the ϕ31P/*Lla*IR$^+$ cassette on the high-copy-number replicon pTRK414H, the EOP for ϕ31 was reduced dramatically to 2.2×10^{-4} (Table 2) and the plaques were very small and aberrant in size (Table 3). Therefore, the ϕ31P/*Lla*IR$^+$ cassette reduced the plaquing efficiency of phage ϕ31 to levels that correlated with the high or low copy number of the replicon on which it was cloned.

L. lactis NCK690 and NCK690(pTRK414H) were also challenged with 10^5, 10^6, and 10^7 PFU of phage ϕ31 per ml in broth cultures (Fig. 3). The presence of the high-copy-number plasmid did not alter the growth rate of *L. lactis* NCK690 (Fig. 3A). At multiplicities of infection (MOI) ranging from 0.001 to 0.1, phage ϕ31 readily lysed *L. lactis* NCK690 cells (Fig. 3B). In contrast, lactococcal cells harboring the suicide system on pTRK414H did not suffer phage-induced cell lysis and continued to grow normally (Fig. 3C). When the suicide system was presented on the low-copy-number replicon, pTRK414L, phage ϕ31 was still able to lyse the culture when challenged with 10^5 to 10^7 PFU/ml. These results correlated with the higher EOP observed for phage ϕ31 on *L. lactis* NCK690 harboring pTRK414L (Table 2).

Evaluation of the efficiency of the ϕ31P/*Lla*IR$^+$ restriction cassette. One-step growth curves and COI assays for phage ϕ31 were conducted with *L. lactis* NCK690(pTRK414H) harboring the ϕ31P/*Lla*IR$^+$ suicide cassette (Fig. 4). Cells were propagated until the optical density at 600 nm reached 0.4 and were then infected with 10^8 PFU of phage ϕ31 per ml (MOI = 5). The number of infective centers (PFU per milliliter) formed initially on *L. lactis* NCK690(pTRK414H) was 5.4×10^4, 85% lower than that for *L. lactis* NCK690 (3.6×10^5) (Fig. 4). Only 15% of infected cells harboring pTRK414H released progeny phages, and phage development was notably retarded over the course of the one-step growth experiment. Phage burst sizes in *L. lactis* NCK690 with and without pTRK414H were estimated at 41 and 161, respectively. Therefore, the ϕ31P/*Lla*IR$^+$ suicide cassette encoded on pTRK414H reduced the burst size of phage ϕ31 fourfold and severely limited the efficiency at which infections were completed.

Phages present in plaques formed on lawns of the restrictive host *L. lactis* NCK690(pTRK414H) were evaluated for their sensitivity or resistance to restriction by the *Lla*I R/M system. Several small plaques were recovered from *L. lactis* NCK690(pTRK414H) and suspended by vortexing in 2 ml of GM17. The samples were then plaqued on *L. lactis* NCK690

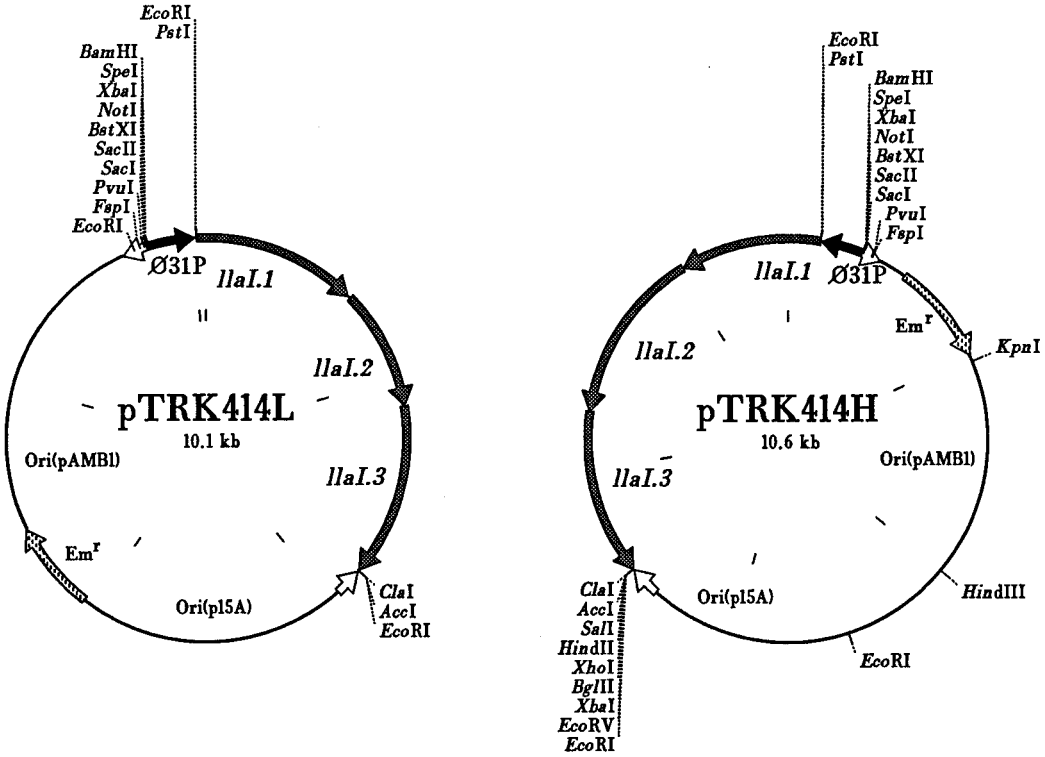

FIG. 2. The pTRK414L (low-copy-number) and pTRK414H (high-copy-number) plasmids encoding the ϕ31P/*Lla*IR$^+$-based bacterial suicide cassette. The *llaI.1*, *llaI.2*, and *llaI.3* genes encode the restriction endonuclease activity of the *Lla*I R/M system. ϕ31P, the 239-bp *Bam*HI-*Fsp*I phage DNA fragment from pTRK391 that encodes a phage ϕ31-specific middle, trigger promoter; Ori(pAMβ1), gram-positive replication origin; Ori(p15A), gram-negative replication origin; Em, erythromycin resistance gene; *lacZ*, β-galactosidase gene that allows blue or white selection in α-complementing *E. coli* strains (only flanking 5′ and 3′ sequences of the gene are present on pTRK414L and pTRK414H vectors). Only relevant restriction sites are shown.

(R$^-$/M$^-$), *L. lactis* NCK690(pTRK370) (R$^+$/M$^+$), and *L. lactis* NCK690(pTRK414H) (R$^+$/M$^-$). Approximately 10^7 PFU/ml was obtained for *L. lactis* NCK690. Plaques were detected on *L. lactis* NCK690 bearing pTRK370 or pTRK414H at EOPs of 6.7×10^{-4} and 1.3×10^{-5}, respectively. Therefore, progeny phages released from cells bearing pTRK414H remained fully sensitive to restriction by *Lla*I. These results are consistent with the appearance of small aberrant ϕ31 plaques on *L. lactis* NCK690(pTRK414H). Phage ϕ31 is limited in burst size, and progeny phage continue to be severely restricted during subsequent infections of surrounding cells in the lawn.

C · LlaI enhances the ϕ31P/*Lla*IR$^+$ suicide system. The *llaIC* gene product, encoded upstream of the *llaIM* methylase gene in the *Lla*I R/M operon, enhances the restriction activity of the *Lla*I system (12, 42). The effects of C · LlaI on the efficiency of the phage-inducible restriction cassette were evaluated. Plasmid pTRK400, encoding *llaIC*, was introduced into *L. lactis* NCK690, *L. lactis* NCK690(pTRK414L), and *L. lactis* NCK690(pTRK414H). The pTRK400 transformants were challenged with 10^6 PFU of phage ϕ31 per ml (MOI of 0.01). The results in Table 2 show that the EOP for ϕ31 on *L. lactis* NCK690(pTRK414H and pTRK400) was 2.0×10^{-5}, 10-fold lower than the EOP of 2.2×10^{-4} obtained for NCK690 bearing pTRK414H alone. No enhancement due to the presence of *llaIC* was observed in lactococcal cells harboring pTRK414L (Table 2). This likely reflects the low baseline level of restriction activity encoded on the low-copy-number vector. At functional levels of restriction activity, the C · LlaI protein markedly enhanced the effectiveness of the ϕ31P/*Lla*IR$^+$ cassette.

Effect of the ϕ31P/*Lla*IR$^+$ restriction cassette against related phages. We evaluated whether the ϕ31P/*Lla*IR$^+$ suicide cassette would confer resistance to phages which were closely related to ϕ31 (P335 species) or totally unrelated, representing the other two major virulent phage species that attack lactococci (c2 and 936 species). Table 3 shows that phages c2 and

TABLE 2. EOP for phage ϕ31 on *L. lactis* NCK690 harboring the phage-inducible *Lla*IR$^+$ restriction cassette

Plasmid content	Relevant characteristic(s)	EOP
None	Sensitive host	1.0
pTRK395	Low-copy-number *Lla*IR$^+$	1.0
pTRK397	High-copy-number *Lla*IR$^+$	1.0
pTRK414L	Low-copy-number ϕ31P/*Lla*IR$^+$	0.5
pTRK414H	High-copy-number ϕ31P/*Lla*IR$^+$	2.2×10^{-4}
pTRK400	High-copy-number C · LlaI	1.0
pTRK414L + pTRK400	Low-copy-number ϕ31P/*Lla*IR$^+$ + C · LlaI	0.6
pTRK414H + pTRK400	High-copy-number ϕ31P/*Lla*IR$^+$ + C · LlaI	2.0×10^{-5}

TABLE 3. EOPs of lactococcal phages on *L. lactis* hosts encoding pTRK414H

Phage		EOP[a]
Species	Strain	
P335	φ31	2.2×10^{-4}[b,c]
	φ31.1	5.3×10^{-6}[b,c]
	φ31.2	1.9×10^{-5}[b,c]
	φ31.7	3.0×10^{-5}[b,c]
	φ31.8	3.7×10^{-5}[b,c]
P335	ul36	1.0[c]
	mm210	1.0[c]
c2	c2	1.0[d]
936	sk1	1.0[d]

[a] EOPs are the averages of three independent experiments.
[b] Plaques were small (<0.1 mm) and variable in size.
[c] The plaquing host was *L. lactis* NCK203(pTRK414H).
[d] The plaquing host was *L. lactis* MG1363(pTRK414H).

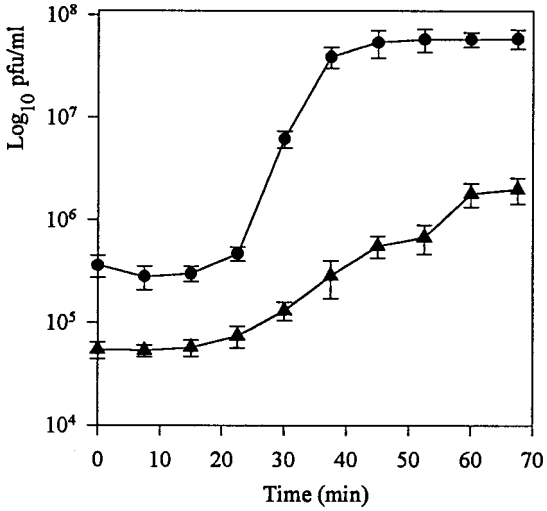

FIG. 4. One-step growth curves for phage φ31 infection of *L. lactis* NCK690 (●) and *L. lactis* NCK690(pTRK414H) (▲).

sk1 were unaffected by the presence of pTRK414H in *L. lactis* MG1363. Similarly, ul36 and mm210, which are P335 species phages different from φ31, were fully lytic on NCK690 (pTRK414H). Only phages that were closely related to phage φ31 were affected by the presence of the φ31P/*Lla*IR$^+$ cassette. Phages φ31.1, φ31.2, φ31.7, and φ31.8 are *per31*-resistant derivatives of phage φ31 that have acquired 8 kb of chromosomal DNA from NCK203 (17, 40). These derivatives have recently been found to encode the φ31P promoter near the right *cos* end of the phage (44). Interestingly, these recombinant phages were restricted by the φ31P/*Lla*IR$^+$ suicide cassette at levels which were at least 10-fold greater than that of phage φ31 (Table 3). Plaques formed by the recombinant phages were substantially smaller than φ31 plaques, and their appearance was altered (data not shown). The lower EOPs observed for φ31-derived recombinant phages indicated that they were more sensitive to the lethal activity of the φ31P/*Lla*IR$^+$ cassette than phage φ31. Of the φ31-derived recombinant phages, phage φ31.1 was the most sensitive to restriction by the φ31P/*Lla*IR$^+$ suicide cassette.

L. lactis NCK690(pTRK414H) broth cultures were also challenged with φ31-derived recombinant phages. Four cultures at an optical density at 600 nm of 0.15 to 0.2 were individually infected with 10^7 PFU of the recombinant phages φ31.1 and φ31.8 per ml (MOI of 0.1), and cell growth was monitored for 320 min (Fig. 5). Cultures harboring pTRK414H were unaffected by the phage challenge and continued to grow normally.

DISCUSSION

In this study, we have developed a novel bacteriophage protection strategy by genetic engineering of a mechanism that aborts the phage infection and intentionally kills the propagation host. The killing capacity of a three-gene restriction cassette extracted from the *Lla*I R/M operon was exploited to develop a novel bacterial suicide system triggered by a phage infection. The phage φ31-inducible promoter used to drive expression of the *Lla*IR$^+$ cassette did not exert detrimental effects on the host in the absence of a phage infection. How-

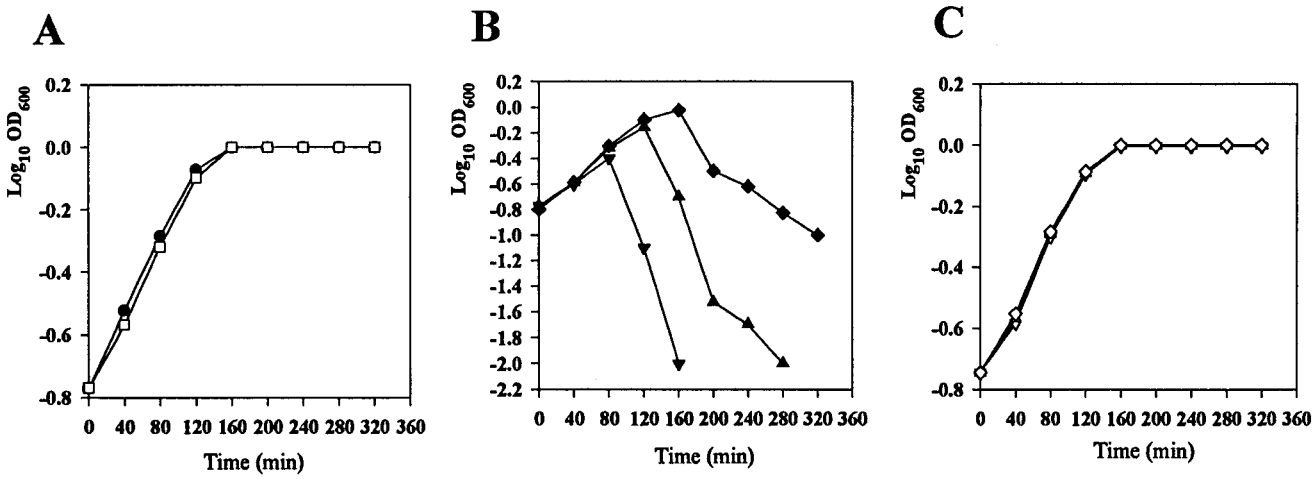

FIG. 3. Effect of phage φ31 on growth of *L. lactis* NCK690 and *L. lactis* NCK690(pTRK414H). (A) Growth curves for *L. lactis* NCK690 (●) and *L. lactis* NCK690(pTRK414H) (□) in the absence of phage φ31. (B) Growth curves for *L. lactis* NCK690 infected with 10^5 (◆), 10^6 (▲), and 10^7 (▼) PFU of phage φ31 per ml. (C) Growth curves for *L. lactis* NCK690(pTRK414H) infected with 10^5 (◇), 10^6 (△), and 10^7 (▽) PFU of phage φ31 per ml. OD_{600}, optical density at 600 nm.

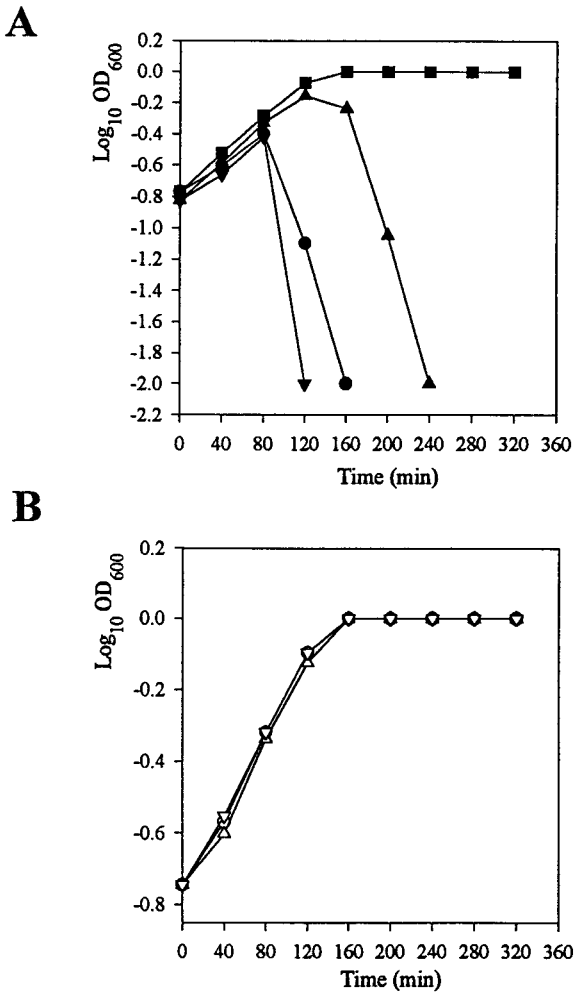

FIG. 5. Effect of phage φ31-derived recombinant phages on growth of *L. lactis* NCK690 and *L. lactis* NCK690(pTRK414H). (A) Growth curves for *L. lactis* NCK690 in the absence of phage (■) and after infection with 10^7 PFU of phage φ31 (●), φ31.1 (▲), and φ31.8 (▼) per ml. (B) Growth curves for *L. lactis* NCK690(pTRK414H) when infected with 10^7 PFU of phage φ31 (○), φ31.1 (△), and φ31.8 (▽) per ml. OD_{600}, optical density at 600 nm.

ever, when transcription of the restriction cassette was initiated by phage infection, the plaquing efficiencies of φ31 and closely related phages were limited to very low levels of 10^{-4} to 10^{-6}. Moreover, phage progeny that escape restriction are not modified and will continue to suffer full restriction in subsequent attempts to infect host cells carrying φ31P/*Lla*IR$^+$. As a result, any phage development within liquid cultures is severely retarded, since each new host infected is triggered to commit suicide and destroy the incoming-phage genome.

Both components of this novel bacterial suicide system, the type of lethal gene and the inducible expression signal, are unique. First, the promoter and restriction cassette are of lactococcal origin and therefore could be considered within a GRAS (generally recognized as safe) category for food-grade applications (11). The GRAS status of this novel phage protection system would allow deployment of the φ31P/*Lla*IR$^+$ cassette in starter cultures used in dairy fermentations. Second, to our knowledge, this is the first attempt to employ a restriction endonuclease component of an R/M system as a lethal gene in a conditional-suicide system. The *Lla*IR$^+$ restriction endonuclease is functional and lethal across a wide range of gram-positive bacteria when expressed from a constitutive promoter (12). Therefore, *Lla*IR$^+$-based suicide systems could be widely exploited as a phage defense strategy if combined with a suitable phage-inducible promoter. Numerous types of conditional-suicide systems in which potent killing genes are coupled to inducible expression signals have been developed (37). The combinations are designed not to interfere with normal growth, and expression occurs under very specific conditions defined by the physical or chemical composition of the environment. Environmental signals used to trigger expression of bacterial suicide systems have included isopropyl-β-D-thiogalactopyranoside (IPTG) (5, 28, 29), poor nutrient conditions (55), limitation of phosphate (51) or tryptophan (36), and availability of aromatic compounds in the environment (9). Conditional-suicide systems in bacteria have been developed for a single purpose, to prevent release of recombinant strains outside a controlled environment. The signals most successfully applied are those directing plasmid transfer (3, 20, 49, 57). The lethal genes studied most extensively in these applications are the two-component toxin-antidote *E. coli* suicide systems involved in postsegregational killing of plasmid-free cells. Suicide systems of this kind include members of the *gef* gene family, such as the *hok-sok* gene pair responsible for the maintenance of plasmid R1 (20, 36) and *hok-sok* homologous chromosomal loci *relF* (19, 29) and *gef* (46), *ccd* loci of sex factor F (3), *parDE* of RP4 plasmid (49), and *pem* of R100 (57). Potent killing genes used to design several suicide systems have also included the following: gene *E* from φX174 (28), the phage φT7 lysozyme gene (51), gene *S* from φλ (55), the *B. subtilis sacB* gene (48), the barnase gene from *Bacillus amyloliquefaciens* (54), and endonuclease genes from *Serratia marcescens* (4) and *Staphylococcus aureus* (11). In contrast to these suicide systems, the φ31P/*Lla*IR$^+$ cassette is designed to provide protection against a bacteriophage by creating a genetic trap that is triggered after a phage infection, destroying both the phage genome and the bacterial host.

The efficiency of the φ31P/*Lla*IR$^+$ system was improved by two approaches. Presentation of the suicide cassette on a high-copy-number replicon dramatically increased the level of phage restriction. This construction was largely responsible for development of a functionally effective defense system. Noting this, genetic stabilization of the phage-inducible restriction cassette in the chromosome would likely require use of a stronger promoter, a promoter recognized earlier in the phage development cycle, and/or a more effective lethal gene. Second, the presence of C · LlaI increased restriction against phage φ31 10-fold. This regulatory protein appears to serve a bifunctional role in the native *Lla*I R/M operon by both repressing transcription and promoting restriction activity (42). In the latter role, the C · LlaI protein has been proposed to enhance RNA stability and facilitate translation of the three-gene restriction cassette (42a). The existing φ31P/*Lla*IR$^+$ system could be improved by incorporating *llaIC* within the cassette.

The *Lla*I restriction endonuclease is a novel class of restriction enzyme with characteristics of both type IIS and type I R/M systems and represents the first multisubunit endonuclease described for *L. lactis* (43). In accordance with its type IIS ancestry, the endonuclease is proposed to cleave one strand of double-stranded DNA near its recognition site and cut the other strand at a distal location. This type of DNA damage is more repairable than that caused by other nucleases and thus may allow some phage progeny to escape lethal restriction and yield productive COIs. Within the *Lla*IR$^+$ system, 15% of infected cells did produce progeny, and it is unknown whether this was one factor contributing to escaping phage. The frequency of restriction sites in the phage genome is also an

important factor that varies among phage species and dictates the effectiveness of restriction systems (34, 52). In this regard, it is interesting that recombinant derivatives of φ31, in which phage DNA was exchanged for 8 kb of lactococcal chromosomal DNA, were 10-fold more susceptible to φ31P/*Lla*IR$^+$ restriction. This may reflect a higher incidence of target *Lla*I restriction sites in the new DNA that was acquired by the recombinant phages from the NCK203 chromosome.

The φ31P trigger promoter selected and used in the design of the suicide system presents two inherent limitations, timing and tight recognition specificity. φ31P is a middle phage promoter that is transcribed approximately 20 min after the phage infection (44, 58). Considering the dynamics of the phage life cycle, activation of the φ31P/*Lla*IR$^+$ cassette at this late stage does allow some DNA to escape restriction, and progeny phage survive within 15% of the infected cells. The efficiency of the *Lla*I-based suicide system could be improved substantially if the restriction cassette was expressed from a phage-specific promoter triggered earlier during the phage infection. It is still critical that the earlier trigger promoters remain completely phage specific and not be recognized by the bacterial host in the absence of phage. Such phage-inducible trigger promoters are being sought but have not yet been defined in lactococcal phages. Therefore, in the design of the phage-induced suicide system, a fine balance between tight control of expression signals and the optimal stage of temporal phage development when those promoters are recognized must be sought. Lethal genes expressed too late in the cycle may serve only to facilitate cell death and promote release of assembled phage progeny. Also, because of the high specificity of phage expression signals, the φ31P/*Lla*IR$^+$-based suicide cassette can be triggered only by phage φ31 or related phages that contain similar promoter regions or encode transcriptional factors with similar promoter specificity. Attempts to induce the φ31P promoter with other phages from the P335 species, as well as phages from other lactococcal species (936 and c2), were not successful (data not shown). Due to this promoter specificity, the defense system would not be practically effective against the diverse combinations of phage strains and species that attack lactococci. To increase the general utility of this system, an important research target would be the definition of consensus promoter regions within phage species, and potentially across species, that are tightly regulated and inducible during temporal phage development. Whether such promoters exist or could be genetically designed is being investigated.

In conclusion, we have devised a novel bacteriophage defense strategy that programs cell death and intracellular destruction of phage DNA postinfection. The system is designed as a genetic trap to invite phage adsorption and DNA injection. In bacterial populations where every cell harbors a phage-inducible suicide cassette, infected cells hydrolyze the phage genome and undergo programmed cell death in an altruistic fashion designed after naturally occurring Abi mechanisms. Various combinations of lethal genes and promoter elements can be envisioned to design similar systems for virtually any bacterium. Alone or combined with other defense systems, phage-triggered intracellular-suicide defense systems are capable of trapping and eliminating phages, and their genetic potential, from fermentation environments.

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