In Vivo Genetic Exchange of a Functional Domain from a Type II A Methylase between Lactococcal Plasmid pTR2030 and a Virulent Bacteriophage[†]

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The conjugative plasmid pTR2030 confers bacteriophage resistance to lactococci by two independent mechanisms, an abortive infection mechanism (Hsp⁺) and a restriction and modification system (R⁺/M⁺). pTR2030 transconjugants of lactococcal strains are used in the dairy industry to prolong the usefulness of mesophilic starter cultures. One bacteriophage which has emerged against a pTR2030 transconjugant is not susceptible to either of the two defense systems encoded by the plasmid. Phage nck202.50 (\$\$0) is completely resistant to restriction by pTR2030. A region of homology between pTR2030 and \$\$0 was subcloned, physically mapped, and sequenced. A region of 1,273 bp was identical in both plasmid and phage, suggesting that the fragment had recently been transferred between the two genomes. Sequence analysis confirmed that the transferred region encoded >55% of the amino domain of the structural gene for a type II methylase designated LlaI. The LlaI gene is 1,869 bp in length and shows organizational similarities to the type II A methylase FokI. In addition to the amino domain, upstream sequences, possibly containing the expression signals, were present on the phage genome. The phage ϕ 50 fragment containing the methylase amino domain, designated LlaPI, when cloned onto the shuttle vector pSA3 was capable of modifying another phage genome in trans. This is the first report of the genetic exchange between a bacterium and a phage which confers a selective advantage on the phage. Definition of the LlaI system on pTR2030 provides the first evidence that type II systems contribute to restriction and modification phenotypes during host-dependent replication of phages in lactococci.

The foundation of modern molecular biology was provided by the study of bacteriophage-host interactions. In addition, cellular defenses and bacteriophage counterdefenses provide a microcosm of evolutionary biology. The introduction or development of a bacteriophage resistance system within an organism imposes the evolutionary pressure for bacteriophage adaptation. One well-characterized resistance mechanism relies upon host-encoded restriction endonucleases to cleave incoming bacteriophage DNA. The host DNA is protected by companion methyltransferases which modify the recognition sequences of the host genome, rendering it insensitive to the endonuclease. Bacteriophage have developed a number of mechanisms to elude restriction (for a review, see reference 13). Some Escherichia coli bacteriophages produce a specific protein (ocr) which inhibits the host endonuclease. Other antirestriction mechanisms include modification other than methylation (e.g., glycosylation or the introduction of unusual bases) and selective reduction or elimination of the specific target sequences recognized by the host endonucleases (21, 28). In E. coli and Bacillus sp., a number of temperate bacteriophages have been identified which encode methylases that modify the host endonuclease target sequences (20, 22, 33). These bacteriophage methylases do not generally display significant relatedness to the host-encoded methylases, suggesting convergent evolution of function. A common, though distant, evolutionary origin has been suggested for the phage T4 dam and host dam genes, even though there is a complete lack of DNA-DNA homology (6). In this report, we describe a novel mechanism by which a lactococcal bacteriophage eludes restriction by the restriction system encoded by the 46.2-kb self-transmissible plasmid pTR2030 (7, 11).

Phage nck202.50 (ϕ 50) is small isometric-headed phage with a circularly permuted double-stranded DNA genome of 30 kb (1). This phage was isolated in a commercial fermentation process involving the industrial parent of Lactococcus lactis NCK204, a mesophilic cheese starter culture containing plasmid pTR2030 introduced by conjugation (25). Plasmid pTR2030 encodes resistance to phage via two independent mechanisms, an abortive infection mechanism (Hsp) and a restriction and modification (R/M) system (7, 11). Phage $\phi 50$ is unusual in that it is resistant to the action of the hsp gene product. In addition, regardless of the propagating host, $\phi 50$ is not restricted by NCK204, although it is susceptible to other R/M systems introduced to the same background. Southern hybridization experiments revealed a region of homology between pTR2030 and ϕ 50 (1). The region of homology on pTR2030 was localized to a region identified previously as the locus for the R/M system. Closer examination of the homologous regions by restriction mapping demonstrated a region of apparent identity between plasmid and bacteriophage. We present sequence data to confirm that $\phi 50$ has acquired a functional methylase domain of the type II A methylase gene, LlaI, on pTR2030 in an as yet undetermined fashion. The phage is therefore capable of self-methylation in any host background. This study pro-

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Strain or plasmid	Relevant characteristics	Source or derivation
L. lactis		
NCK202	Homologous host for ϕ 50 and ϕ 32	7
NCK203	R^{-}/M^{-} derivative of NCK202	7
NCK204	NCK203(pTR2030) Hsp ⁺ R ⁺ /M ⁺	7
NCK211	NCK203(pTK6)	7
NCK213	NCK203(pTRK103)	NCK203 transformant
NCK312	NCK203(pTRK140)	NCK203 transformant
NCK383	NCK203(pTRK179)	NCK203 transformant
E. coli DH5	Transformation host	Stratagene
Plasmids		C C
pBluescript	Cloning vector, Ap ^r	Stratagene
pSA3	Shuttle vector, Cm ^r Em ^r Tc ^r	4
pTK6	$Cm^r Em^r Hsp^+ R^+/M^+$	7
pTR2030	$Hsp^+ Tra^+ \dot{R}^+/M^+$	11
pTRK102	Ap ^r	pBluescript::4.5-kb ϕ 50 fragment
pTRK103	$Cm^r Em^r R^-/M^+ (LlaPI)$	pSA3::4.5-kb ϕ 50 fragment
pTRK140	$Cm^{r} Em^{r} R^{-}/M^{+} (LlaI)$	Hpal-Nrul deletion of pTK6
pTRK179	Cm ^r Em ^r	XbaI inversion of pTRK103

TABLE 1. I	Bacterial	strains	and	plasmids
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vides direct evidence that the R^+/M^+ phenotype conferred by pTR2030 involves at least one type II system.

MATERIALS AND METHODS

Bacteria, bacteriophage, and culture conditions. The bacterial strains used in this study are presented in Table 1. *L. lactis* strains were propagated at 30°C in M17 broth (32) or M17 broth with 0.5% glucose substituted for lactose (M17G) when appropriate. Bacteriophages were propagated and titrated as described previously (32). *E. coli* strains were grown in LB broth (29) at 37°C with shaking. When required for selection, the following antibiotics were added: for *E. coli*, ampicillin and chloramphenicol at 50 and 20 μ g/ml, respectively; for lactococci, erythromycin at 5 μ g/ml.

Isolation of a Hsp-resistant variant of ϕ 31. Phage nck202.31 ($\phi 31$) is a small isometric-headed phage which is subject to restriction by the R/M system encoded by the lactococcal plasmid pTR2030 (7). Phage ϕ 31 is also susceptible to the abortive infection mechanism (Hsp) directed by pTR2030. To eliminate the effect of the Hsp mechanism, a derivative of ϕ 31 was isolated which is insensitive to Hsp but remains susceptible to the R/M system. Phage ϕ 31 was propagated through a strain containing the recombinant plasmid pTRK70, an R^{-}/M^{+} derivative of pTK6 (7). The modified phage were subsequently titrated by plaque assays against NCK204, a homologous host containing pTR2030. Of 10¹³ phage used in a plaque assay on NCK204, only a single plaque was isolated. This phage isolate, nck202.32 (\$\$2), shows no significant difference in its restriction map compared with the parent phage ϕ 31, but it appears to be insensitive to the Hsp-directed resistance. Phages ϕ 31 and ϕ 32 are equally sensitive to the pTR2030 R/M system.

Transformation and electroporation. Protoplasts of *L. lactis* NCK203 were transformed as described by Kondo and McKay (12), with some modifications (8). Alternatively, plasmids were introduced to *L. lactis* by electroporation essentially as described by Luchansky et al. (18) except that cells were washed and resuspended in doubly distilled water prior to electroporation. A voltage of 12,500 V/cm was applied to the cell-DNA suspension. Transformation frequencies of 10⁴ to 10⁵ per μ g of plasmid (pSA3) were routinely achieved under these conditions. Transformants were selected on $1.5 \ \mu g$ of erythromycin per ml.

Molecular cloning techniques. Plasmid isolation, restriction, ligation, and transformation in E. coli DH1 were performed as described by Maniatis et al. (19). Lactococcal phage DNA was isolated as previously described (9). Lactococcal plasmid DNA was isolated by the procedure of Anderson and McKay (2).

Sequence determination and analysis. Nucleotide sequences of both strands were determined by using the dideoxy-chain termination method (26) and the Sequenase enzyme (Stratagene, La Jolla, Calif.), using either the recombinant M13 single-stranded templates or pBluescript (Stratagene) clones. Short fragments (200 to 400 bp) were directionally cloned in M13mp18 and M13mp19 and sequenced by using commercially available primers. Synthetic oligonucleotide primers (17-mers) were synthesized in those instances when a subclone was too large to be fully sequenced from commercially available primers. The pBluescript clones were sequenced by using double-stranded templates. The facilities of the University of Wisconsin Genetics Computer Group were used to analyze the sequence information. The sequence alignments were performed by the COMPARE and BESTFIT programs.

Overexpression of *LlaI. E. coli* bearing pTRK144 or pBluescript was grown overnight at 37°C with shaking in LB supplemented with 20 mM isopropyl- β -D-thiogalactopyranoside (IPTG). Cells were adjusted to an optical density at 600 nm of 0.5 by the addition of fresh LB and lysed by the addition of protein solvent at a 1:1 ratio according to the method of Laemmli (14). Samples were boiled for 5 min prior to loading on 15% sodium dodecyl sulfate-polyacrylamide gels. Gels were stained with Coomassie blue.

Nucleotide sequence accession number. DNA sequence information is available in GenBank through accession number M35638.

RESULTS

 ϕ 50 is insensitive to the pTR2030 R/M system. The recombinant plasmid pTK6 contains both bacteriophage resistance mechanisms associated with the conjugative lactococcal

TABLE 2. Phage reactions on L. lactis NCK203 and derivatives

Dha and	Relevant pl	asmid in:	Efficiency
Phage ^{**}	Propagating host	Plaquing host	of plaquing
φ32.NCK203	None	None	1.0
φ32.NCK203	None	pTK6	1.3×10^{-4}
φ32.NCK211	pTK6	pTK6	1.0
φ32.NCK203	None	pTRK103	1.0
φ32.NCK213	pTRK103	pTK6	1.1×10^{-1}
632.NCK383	pTRK179	pTK6	6.1×10^{-3}
632.NCK312	pTRK140	pTK6	2.0×10^{-1}
650.NCK203	None	pTK6	1.0
φ50.NCK203	None	pTRK68	1×10^{-3}

^a The suffix indicates the last host upon which the phage was propagated.

plasmid pTR2030 (7, 8). pTK6 directs a high level of restriction against the small isometric-headed bacteriophage nck202.31 (\$\$) in the NCK203 background, in addition to the Hsp⁺ response (7). Phage nck202.32 (ϕ 32) was isolated from a single plaque appearing after challenge of NCK204 (containing pTR2030) with 10^{13} PFU of modified phage $\phi 31$ per ml. The propagation conditions for modification of phage ϕ 31 and isolation of ϕ 32 are described in Materials and Methods. Phage ϕ 32 is susceptible to the R/M system (Table 2), but after propagation on a pTK6-containing host, this phage is not inhibited (efficiency of plaquing or plaque size) during a subsequent infection of the pTK6-bearing host NCK211 (Table 2). This finding suggests that ϕ 32 is resistant to Hsp-directed abortive infection since this phenotype is not subject to host-dependent modification or restriction (11). Phage ϕ 32 was used in subsequent plaque assays to assess R/M activities, independent of Hsp responses, in the course of this study.

In contrast to $\phi 32$, neither the plaque size nor efficiency of plaquing of the small isometric-headed phage nck202.50 ($\phi 50$) was affected by the presence of pTK6, regardless of the plasmid complement of the propagating host (Table 2). A number of other R/M systems were evaluated for activity against $\phi 50$. pTRK68 is a native R/M plasmid which resides in NCK202 (the parental strain from which NCK203 is derived; 7). Phage $\phi 50$ is restricted by NCK202, at levels similar to those for other phages homologous for this background (Table 2). The same outcome was observed with another R/M system encoded by pTN20 introduced to the NCK203 background (1). These data suggested that the resistance of ϕ 50 to pTK6 is not a generalized phenomenon, as might be expected if ϕ 50 relied upon the incorporation of unusual bases, or a postreplicational modification such as glycosylation, to elude restriction.

Cloning and mapping the ϕ 50-pTR2030 homologous region. pTR2030 possesses a region homologous to ϕ 50 (1). This region was localized by restriction mapping and Southern hybridization to the R/M locus of pTR2030 as defined previously by deletion analysis (7). The homologous region from $\phi 50$ was shotgun cloned in the E. coli cloning vector pBluescript on a 4.5-kb BamHI-HindIII fragment to create plasmid pTRK102. The recombinant plasmid was physically mapped with restriction enzymes and aligned with the pTR2030 R/M locus (Fig. 1). A perfect alignment of restriction sites could be detected within the region of homology. In no instance was a restriction site located within the plasmid region of homology which could not be similarly located within the phage DNA. This suggested that plasmid pTR2030 and phage ϕ 50 shared an identical or closely related fragment.

Sequence analysis of the pTR2030 homologous region. The homologous region from pTR2030 was subcloned and sequenced from M13 cloning vectors. The complete nucleotide sequence, determined from both strands, is presented in Fig. 2. An open reading frame (ORF) analysis of the sequenced region revealed a single large ORF which extends beyond the 3' end of the homologous region (Fig. 2). The ORF is 1,869 bp in length and encodes a predicted protein with a molecular mass of 72.5 kDa. This ORF was designated *LlaI*.

Two consensus sequences are found in all type II A methylases (16, 31). A manual search of the predicted protein sequence of *LlaI* revealed the presence of both the consensus 15-amino-acid sequence and the 10-residue DPPY (asparagine-proline-proline-tyrosine) consensus sequence within the *LlaI* predicted protein sequence. Interestingly, each consensus sequence was found twice within the predicted protein (Fig. 2). The most significant level of similarity was found with the atypical type II A methylase *FokI*



FIG. 1. Physical maps of pTRK102, pTK6, and pTRK140, aligned to show the region of identity (***) between plasmid and phage fragments. Open boxes represent the ϕ 50 region cloned on pTRK102 (4.5 kb) and the pTR2030 region cloned on pTK6 (13.6 kb). The position of the insertion sequence IS946 within pTK6 is also indicated (23). Restriction endonuclease sites: E, *Eco*RI; V, *Eco*RV; X, *Xba*I; Xh, *Xho*I.

pTR2	030																														
1	TCIT	TAC	IGC	CIG	aag	TOC	TGA	TAT	AAT	AGT	TCI	ATA	TTT	ATA	GAA	TAA	ATT	TCC	T												
<i>a</i> 50																			TA	AAA	ATG	GAA	TGG	AGG	AAC	TCA	ATG	MGI	TTT	NG	90
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91	CIAT	GAA	CAT	CIT	<u>TGG</u> -3	GA	CAG	GCT	TTA	777	AAG	77 <u>7</u>	<u>ATA</u> -1	<u>AA</u> T 0	AAA	A CC	GCA	TTA	OGA	GAT	ATG	GCT	GGA	ATA	ACT	aat	ICE	NCG	GIC	IC.	180
181	Xbal TAGA	GIC	тст	CIG	GAT	TG	GAC	GGT	стс	TAT	GGA	GGC	ATT	GGC	ANG	AAT	atg	TGA	AGC	ACT	002	CTG	CGG	GTT	AGA	AGA	TAT	IGI	TGA	AT	270
271	ATAA	ACT	TGA	aaa	GAA	T <u>OG</u> F	igaa BS	TGA	CIG	atg M	AGG R	TAT Y	TTA L	G G	AAC N	AAA K	ACT T	AAT N	TTA L	TTG L	AAT N	TTC F	ATA I	CAA Q	cana Q	GTT V	ATA I	AAA K	AAA K	CA H	360 21
361	TGAT	ATA	CAA	GGT	CAG	ACZ	TT	GCA	GAC	TTA	TTT	ĢCG	GGA	ACA	GGT	TCT	GIC	GGC	GAC	TAC	TTC	aaa	GGT	GAA	TAC	N CC	GIT	стс	TCA	AA	450
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451	TGAT	TAT	ATG	TAT	TTT	TC	AAG	GIC	ATT	AGT	GAA	GCA	888	TTG	E TTG	<u>oo</u> r Aat	1 TCA	GIG	GAG	COG	AAG	TTT	GAT	AGT	TTT	GTA		AGA	TAT	aa	540
	D	Y	M	Y	F	S	к	v	I	S	E	A	K	L	L	N	s	E	K	P	K	F	D	s	F	v	K	R	Y	G	81
541	GANA	XCA	CCT	TTT	CAA	TGG	TIG	AAO	GAA	CGA	GAA	TAT	ACA	$\overline{\alpha}$	AAT	GAT	GGA	TAT	TTT	GTT	TAT	AAC	AAC	TAT	ACT	cca	OGT	GCT	GAA	N G	630
	K	т	P	r	Q	w	Ъ	N	E	R	Е	¥	т	Р	N	D.	G Eco	RV	F	v	¥	N	N	Y	т	P	R	A	Е	R	111
631	AATG M	TAT Y	TTG L	ACT T	GAA E	GAN E	N N	GCT A	CTT L	AAA K	ATT I	GAT D	GGA G	ATG M	R	L	GAT	ATC	GAG E	GAAG E	TTG L	TTT F	CAG Q	GAA E	GGA G	OTT V	ATT I	TCA S	AAA K	GC A	720 141
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901	AAAT	ACA	AAT	AGA	TTG	GIT	AGG	GAA	ATT	TCC	GGA	GAT	ATT	GCI	TAT	ATT	GAT	007	сст	TAT	ACA	ATT	ACC	CAA	TAT	ACT	AAT	TCG	TAT	CA	990
	N	т	N	R	L	V	R	Е	I	s	G	D	I	λ	Y	I	D	P	P	Y	T	I	Т	Q	Y	Т	N	S	Y	H	231
991	V	L	GAG E	ACA T	ATC I	OCA A	R	TAT Y	GAT: D	AAT N	P	GAA E	TIG L	TTT F	GGT	K	ACT T	GCC A	AGA R	AGA R	OTT V	AAG K	COG R	GAG E	TTT F	TCG S	GGG G	TAT Y	TCA S	AA N	1080 261
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FIG. 2. Nucleotide sequences from both pTR2030 and ϕ 50. The identical 1,273 bp from nucleotides 56 to 1329 is shown once, in boldface for clarity. Individual plasmid and phage sequences are labeled. The predicted amino acid sequences of both *LlaI* and *LlaPI* are also shown and numbered. The amino acid consensus regions described in the text are highlighted and underlined. The putative expression signals are underlined and labelled. Restriction sites used to construct subclones for sequencing are indicated.

(Fig. 3) (10, 17). The two protein products are similar along their entire length, with 39.4% identity and 61.4% similarity. However, phage or plasmid DNA modified by LlaI was not protected from digestion by FokI (data not shown). The consensus sequences described by Lauster (16) are highly conserved in LlaI (Fig. 3). The LlaI DNA and predicted protein sequences were compared with sequences in the GenBank and NBRF data bases, using the algorithms of Wilbur and Lipman (34). A high level of identity was found between the predicted protein sequences of LlaI and the DNA adenine methylase from bacteriophage T4 (27). The carboxyl terminus of LlaI showed significant identity (33%) to the entire T4 dam protein (data not shown). A second alignment could also be made between the amino domain of LlaI and T4 dam and the entire T4 dam with 26% identity. We conclude from these data that LlaI is a type II A methylase with two functional domains.

We have previously presented evidence that a protein product of approximately 70 kDa is produced when pTK6 is used as a template in in vitro transcription and translation experiments (8). This product is eliminated by a deletion which removes functional methylase activity (7).

Sequence analysis of the phage ϕ 50 homologous region. The complete nucleotide sequence of a 1,581-bp region of ϕ 50 was determined from both strands (Fig. 2). The phage and plasmid sequences were aligned (Fig. 2). A region of 1,273 bp was found that was identical in both plasmid and phage sequences. The flanking divergent sequences showed no similarity. The upstream region of homology extended for 243 bp 5' to the predicted start codon and included sequences which resemble the canonical -35 and -10 regions associated with promoter activity (4 of 6 consensus bases at both -35 and -10 positions; Fig. 2). In addition, a putative ribosome binding site is present at the correct spacing (6

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	v	N	T	P	Α	D	Е	L	I	R	N	v	Е	Q	I	v	т	R	Y	S	L	Е	K	K	G	K	Ε	A	F	N	44
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TR203 621 T	0 0 R	CGA	XAAA XOGJI R	AGA CGA1 D	ICA1	IGAA ITAA Y	laac Daac N	etgi Eaat N	FTT IGA E	ICTZ SGAV	VICA Q	AAAZ AACZ T	AGG AGG P	GAZ ATT	VAAG CAAC N			XACI T	TTC L	laat N	I AT	ritat Y	ric: s	rTR F	Q	AA N	PAT	ACTO R	30G	AIT F	171 47
TR203 621 T	10 0 0 R	ITIC L	AAA COGI R	AGA GA1 D	ICA1 H	IGAA ITAA Y	LAAC N	FIG DAA N	FITI IGAL E	ICTZ SGAV E	VIC VCA	AAC7	4007 P	GAZ ATT	VAAG CAAC N	GAI CI7 L	ц т т х	ZACI T	L	aan N	I	ritati Y	ric: S	rrn F	Q	AA N	EAT I	ACTV R	30G	AIT F	171 47
IR203 621 T IR203 711 C	0 COSC R 0 ZAAI	CAP	XAAA XOG71 R VGC74	AGA GAI D	ITTI ICAI H	IGAA ITAA Y	LAAC N CAAC	FIG FAA1 N FAC2	FITI IGAC E	ICIZ SGAJ E DATI		AAAZ AACZ T	VAGG VCCP P	GAA ATTI I GAA	VAAG CAAC N	GAI CIP L AAI		T T	TICL	aat N	IATI I I	ritati Y DAG2	FIC: S	FTTO F	Q	AAA N	EAT I IGT	ACTO R TAC	SOCI L	AIT F AGC	171 47 180
FR203 621 T FR203 711 C	IAA OGC R AAI N	CAP	AAA R R GC2 A	AGA D AAAC K	ITTI ICAI H H K	IGAA TIAI Y VIAC	LAAC N CAAC N	FIG DAAI N DACI T	FITT FIGAC E ACC: P	ICIZ SGAJ E IATTI I	VIC VCA Q ICC G	AAAZ T T TAAA N	VAGG P EAA1 N	GAA ATTI I GAA E	LAAG TAAC N VITT	CTA L ZAAI N		T T G	TTIC L VIAI Y	eaad N Daal K	IATT I I GAT D	TIAI Y IAGJ	FIC: S VATZ	FTR F	Q Q R R	AA N PTT F	EAT I IGI V	ACTO R TAC	SOGI L AOGI R	AIT F AGC A	171 47 180 50
TR203 621 T TR203 711 C	1744 1000 R 100 2741 N	CAP	AAA R R GC7 A	AGA D AAAC K	ICAI H SAA/ K	TIAI Y Y YIAC Y	DAAC N CAAC N	FIGE DAAT N DACZ T	FITI IGA E ACC. P	ICIZ SGAJ E IATT I	VICA Q IGG G	AAC T T DAAC N	AGG P DAAI N	GAA I I GAA E	LAAC N VITI F	CTP L XAAI N		T T G	TTIC L VIAO Y	iaat N K	IATI I VGAI D	TIAI Y DAGI R	FIC: S VATZ I	F F AC: T	Q Q R R	N N FTT F	DAT I IGI V	ACTV R TAC	SOGI L AOGI R	ATT F AGC A	171 47 180 50
TR203 621 T TR203 711 C TR203 801 T	IAA OCC R DOCC R DOCC A	CAP CAP Q	AAA R KGCP A	AGA D AAAC K AAAC	ITTI ICAJI H KAAI K	IGAA TIAI Y AIAC Y 303J	LAAC N CAAC N CAAC	DAAT N DACZ T 3007.	SITT IGA E ACC: P IAG	ICIX SGAJ E IATI I CIA		AAAZ T TAAC N AGO	AGG P P IAAI N	I ATTI I CAA E	LAAC N VITI F	CIP L XAAI N XAAI		T T G G TAN	TTIC L VIAI Y	SAAT N CAAJ K	I I VGAC D	TIAC Y R R CAO	I S AATZ I	FTR F AC: T	Q Q R R	AAA N FTT F	IAT I IGT V IGA	ACTO R TAC	SOGI L AOGI R	AIT F AGC A	171 47 180 50
IR203 621 T IR203 711 C IR203 801 T	IAA OCC R OAAI N O P	CCAP L CAP Q CAP	AAA R KGC7 A VGTC V	AGA D AAA K XGAC E	ICAN H SAAA K SATC M	IGAA TIAI Y XIAC Y SOGI	CAAC N CAAC N CAAC N L	FIG DAAI N DACI T G	IGAC E ACC. P IACC	ICIZ SGAJ E IATT I STAT	VICA VCA Q ICC G ITC S	AAAZ T IAACJ N AGO A	AGG P IAAI N I	GAA ATTI I GAA E ZAATI N	LAAC N VITI F	CIA L XAAI N XAAI N		T T YOGF G SIAI Y	TTIC L VIAI Y IGAI D	n N K K D	IATI I VGAC D IGAO D	TIAN Y R R CAO		TTR F AAC. T STT.	CCAJ Q R R TICA Y	AAA N FTT F TTT F	eat I Igt V Iga D		icia L Acci R Acci P	ATT F AGC A ATA Y	171 47 180 50 189
TR203 621 T 711 C TR203 801 T	IAA OCCC R OCCC AAI N OCCA P OCCA	CGA L CAA Q GAA E	AAA R KGC2 A VGTC V	AGA D AAA K CAA E	ICAN H SAA K SATC M	TIAI Y Y Y Y Y Y Y Y Y Y	IAAA N CAAA N CAAA N TTTTC L	FIG FAAT N FAC T G G	FIT IGA E ACC: P IAG S	CTA CTA I TAT Y	VICA VCA Q ICC G ITIC S	AACZ T TAAC N AGOO A	AGG P IAAJ N SATI	KATI I KATI E XAAI N	TAAC N N TTT F	CCIF L TAAT N TAAC N	LL LL LL LL LL LL LL LL LL LL LL LL LL	XACI T G SIAI Y	TTIC L ATAI Y IGAI D	iaai N K Iga: D	I I VGAU D I GAU D	TIAN Y R R CAOC T	FIC: S AATZ I OGTO	FTTC F AAC. T STT. F	CCAA Q ROGS R TTCAY Y	AAA N FTT F TTT F	PAT I IGT V IGA D	ACT R IAC T C C P	SOGI L AOGI R AOGI P	AIT F AGC A AIA Y	171 47 180 50 189 50
TR203 621 T TR203 711 C TR203 801 T TR203 891 1	0 CGC R 0 ZAAI N 0 CCA P 0 CCA	CGA TTC L CAA Q GAA E CGA	AAA R GCFI R GCF A GCCF A C C C C C C C C C C C C C C C C C	AGA GAU D VAAC K GAC	NTTI ICAI H SAAA K SATIO M	IGAA TIAI Y ATAC Y SOGI R IGGI	AAA TAAA N CAAA N TTTO L	FIG TAAT N TAC G G TAA	FITT IGAX E ACC: P IACC S CGAX	ICIX E DAT I CIA: Y IGG	VICA VCA Q ICCC G ITIC S ITIC	AAAA T TAAA N AGOO A GOOT	AGG P IAAI N SATI I IGGI	KATI I KATI I KAAT N TTT	NAAG N N N TTT F TTT F	CCIP CAL RAAI N RAAC N	LL LL LL LL LL LL LL LL LL LL	XACI T G STAI Y SGAI	TTA L ATAJ Y IGAJ D	laan N K Igaa D Ggaa	I I VGAU D I GAA D	TIAI Y R CAO T CAO	FIC: S AATZ I COSTO V AGC	TTIC F AAC: T SITI: F	CCAJ Q ICCS R ITIA Y ICCI	AAA N FTT F F TTT F	IGI IGI V IGA D AAA	ACTV R TACC T COCC P ATA	ictiv	AITT F AGC A AITA Y CAC	171 47 180 50 189
TR203 621 T TR203 711 C TR203 801 T TR203 891 1	0 COCC R 0 CAAI N CCA P 0 CCA P 0 CCA D CCA	CAN Q CAN Q CAN Q CAN Q CAN Q CAN Q CAN Q CAN Q CAN Q CAN Q CAN Q CAN Q CAN	AAA R GC7 A GC7 A C C A C T	AGA GAI D AAA K CAA E VAC2 T	ICAI H SAAA K SAT(C M SAT(C A C A SCI A	IGAA TIAI Y AIAA Y SOGI R IGGI G	IAAX IAAX N IAAX N ITTIC L ITTAY Y	FIG FAAN N FAC T G G TAAM N	JITI IGA E ACC P IACC S CGA D	ICIA BCAA E IAT I I I I I I I I I I I G G	VICA VCA Q ICOG G ITIC S ITAA K	AAAAA T TAAA AGOO A GOOT R	AGG P PAAI N SATI I GGI G	GAA I I CGAA E ZAAA N FTTT F	AAG TAAC N ATTI F TTTI F CGAI D	CIF GAI L AAI N AAA N CGGF G	LL LL LL LL LL LL LL LL LL LL LL LL LL	XACI T G SIXI Y SGAI D	TTIC L ATAY Y IGAY D IGOX A	N N K IGA: D CGA: E	I GAN I VGAN D IGAA D VGAA Q	TIACI Y R CACC T SGAV E	TIC: S VATE I OGTO V AGCI A	FTTC F AAC. T STT. F	Q Q ROG R TIA Y ICTU L	AAA N FTT F TTT F CTT L	IGI V IGA D AAAA K	ACT R IAC T OCC P ATA	SOGI L AOG R AOC P ICTI L	AITT F AGC A AITA Y CAC T	171 47 180 50 185 53 196 56
TR203 621 T TR203 711 C TR203 801 T TR203 801 T	0 VOGC R 0 XAAI N 0 XAAI N 0 XCAA P 0 XCAA P 0 XCAA N 0 XCAA N 0 XCAA	CAP CAP Q CAP E CAP CAP CAP CAP	AAA R AGC/P A AGC/P A CAC/P T	AGP D AAAC K CGAC E T	NTTI H H SAAV K SAT(M SAT(A SAT(A	IGAA TTAC Y SOGI R IGGI G	AAA AAA N CAAA N TTTC L L TTAA Y	FIGU FAAN N FACI T G G FAAN N	IGAA E ACC: P IAGC S CGAA D	CTA E TAT T G G	VICA VCA Q IOGG G ITIC S IAA K	AAC7 T IAA1 N AGOO A GOO <u>T</u> R	AGG P IAAAI I IGGI G	ATTI I E ZAAT N F	NAAC N N TTT F TTT T T D	CIP GAI L AAAI N AAA N COGP G	TAC Y SAV E SAV E	T T G SIA Y SGAY D	TTA L Y IGAI D IGO A	YAAN N K IGA D CGA E	I GAU I GAU D I GAU D A CAU Q	TIAI Y R CAO T SCA E	TIC: S AATZ I OGTV V AGCI A	TTC F AAC. T SIT. F	Q Q R TIA Y L L	AAA N FTT F TTT F CTT L	IGT IGT V IGA D AAAA K	ACTC R IAC T COC P ATA	ACG R ACG P ICT L	ATT F AGC A ATA Y CAC T	171 47 180 50 185 53 196
IR203 621 T IR203 711 C IR203 801 T IR203 891 T	0 VOGC R 0 AAAI N 0 VOCA P 0 VOCA D U U 0 COA	CAN CAN CAN E CAN CAN CAN CAN CAN CAN CAN CAN CAN CAN	AAAA R AGCA AGTIC V XACZ T	AGA D AAA K CGA E VAC T	ICAI H SAAM K SAT(M SAT(A C	IGAA TIAN Y AIIA Y SOGI R IGGI G	TAAC N CAAC N TTTC L TTAC Y	FIGU IAAA N IACI T G G IAAA N	IGAC E ACC: P IACC S CGAC D	CIA E EAT I CIA Y G	VICA Q ICCC G ITIC S IAA K	AAA2 T TAA1 N AGOO A GOOT R	AGG P TAAI N SATI I G G	GAM ATTI I CAM E ZAATI N TTTT F	AAAC N N TTTT F TCAA D	CIP L AAI N AAI N CGG G	TAC Y SAM E SAM E SAM W	T T G SIAN Y SGAN D	TTAL L Y IGAI D IGCO A	SAAD N K IGA: D CGAJ E	I I VGAU D I GAU D VGAU Q	TIAN Y R CAOO T SGAA	FIC: S AATZ I COTIC V AGCI A	FTTC F T F F F STT F	Q Q R TIA Y L	AAA N FTT F TTT F CTT L	IGT IGT V IGA D AAAA K	ACTC R IACC T COCC P ATA	SOGI L AOGI R AOCI P ICT L	ATT F AGC A T CAC T	171 47 180 50 189 53 196 56
TR203 621 T TR203 711 C TR203 801 T TR203 891 1 TR203 981 C	0 COCC R COCC R COCC R COCC R COCC R COCC R COCC R COCC R COCC R COCC R COCC R COCC R COCC R COCC R COCC R COCC COCC COCC COCC COCC COCC COCC COCC COCC COCC COCC COCC COCCC COCC COCCC COCCC COCCC COCCC COCCC COCCC COCCC COCCC COCCC COCCC COCCC COCCCC COCCCC COCCCC COCCCCC COCCCCC COCCCCCCCC		AAAA R R AGC7 A AGC7 V T T CAC7 T	AGA D AAAC K CGAC E T T	ICAI H SAAA K SATC M SATC A SCI A	IGAA TIAI Y XIAA Y SOGJ R IGGJ G	CAAC N CAAC N CAAC L CAAC Y CAAC	FIGU PAAN N PACI T SOG G TAAM N N AAAA	IGAX E ACC: P IAGC S CGAX D	CIA E E EAT I CIA Y G G IAT	VICA VCA Q ICCC G ITIC S ITIC S ITIC S ITIC	AAAC T TAAT N AGOO R GOOT	AGC P TAATI I G G AAATI	CAN I CAN E TAATI F TAATI F	TAAC N N TTT F CAN D	CCIP L PAAT N PAAC N CCCP C		T T G SIAN Y SGAN D	L L VIAI V IGAI D IGO A	IAAA N K IGAA D CGAA E SAAA	I I VGAU D I GAU D VGAU D VGAU	TIAI Y R CAO T SCA E IAA	FIC: S AATZ I OGTO V AGC: A	FTTC F T STT. F STT. F	CCAU Q IOG5 R ITEA Y ICEN L STE	AAA N FTT F TTT F CTT L	IGAT I IGT V IGA D AAAA K GGA	ACTC R IACC T OCC P ATA S IO GIG	SOGI L AOGI R AOCI P ICTL L SAT	ATT F AGC A ATA Y CAC T	171 47 1800 50 1899 53 1998 56 207
IR203 621 T IR203 711 C IR203 801 T IR203 891 1 IR203 981 C	0 CGC R 0 AAI N 0 CAA P 0 CAA P 0 CAA P 0 CAA P 0 CAA P 0 CCA P 0 CCA P 0 CCA P 0 CCA P 0 CCA P 0 CCA P 0 CCA P 0 CCA P 0 CCA P 0 CCA P 0 CCA P 0 CCA P 0 CCA P 0 CCA P 0 CCA P 0 CCA P 0 CCA P 0 CCA P 0 CCA P CCA P CCA P CCA CCA P CCA CCA	CAP L CAP Q CAP E CAP E CAP L	AAAA R R AGC7 A AGC7 V CAC7 T CAC7 T	AGA D AAAC K CAAC E T T T CIC2 S	NTTI H SAAM K SAT(M SAT(A SAT(SAT(IGAA TIAI Y XIIAG Y SOGI R G G AGGG G	CAAC N CAAC N CAAC L CAAC Y CAAC K	FIGU PAAN N PACI T SOGG G N N N AAAA K	IGA E ACC: P IAG S OGA D ATT	CEA E E TAT I CTA Y G G I AT M	VICA Q Q IGG G IIIC S IIAA K SIII L	AAC T TAAT N AGO A GO C R ATC S	AGG P TAATI I G G AAAAI N	CAN I CAN E TAAT N F TTT: F	NAAG N N TTT F NGAI D NCTI L	CCIP CAN L PAAL N TAAC N CCCF C CCAC E	TAC Y CAN E CAN E CAN E CAN H	CACI T G SIAI Y SGAI D IAAA K	L L VIAI V IGAI D IGCO A G G	SAAA N K IGAA D CGAA E SAAA K	I I VGAU D I GAU D VGAU D VGAU D T	TIAI Y R CAO T CAO T CAO N	FIC: S AATZ I OGTO V AGCI A CAA	TTIC F AAC: T SIT: F SIT: S CTIC L	CAN Q R TIAN Y L STTE L	AAA N FTT F TTT F CTT L AAT M	IGI IGI V IGA IGA K GGA E	ACTO R IACC T T OCC P ATA S IC GIG	SOGI L AOGI R AOCI P ICT L SAT	ATT F AGC A ATA Y CAC T ICA Q	171 47 180 50 189 53 196 50 207 59
IR203 621 T IR203 711 C IR203 801 T IR203 891 T IR203 891 1 IR203 891 C	0 CGC R 0 AAI N 0 CAAI P 0 CAAI CAAI E 0 CGAA E 0 0 CGAA	CAP L CAP Q CAP E CAP E CAP CAP CAP CAP CAP CAP CAP CAP CAP CAP	AAAA R AGC7 A AGTC A V XAC7 T T CAAT	AGA GAN D AAA E XAAC T T TTC2 S	ICAI H SAAM K SATO M GCCI A GCCI A	IGAA Y Y XITAC Y SOGJ R G G AGGC G	TAAC N CAAC N TTTC L TTTA Y CAAC	FIGE DAAD N DACD T G G DAAA N AAAA K	IGA E P IAG S OGA D	CEA E E TAT I CTA Y G G I AT M	VICA Q Q IGG G TIC S TAA K SITI L	AACZ T TAAT N AGOO A GOOT R ATCO	AGG P P AAAI N SATI G G N AAAI N	ATI I CAN E YAAI F TTT F	TAAC N TTT F GAI D CCT L	CIP L PAAT N TAAC N CGAC E	L L L L L L L L L L L L L L	CACI T G SIAI Y SGAI D K	TTAL L Y TGAI D TGOC A G	N K K CGA: D CGA: E SAAA K	I I VGAU D I GAA D VGAU D I GAA D I GAA T	TACI Y R CAOO T SCAI E IAAA N	I S AATZ I S S T S S S S S S S S S S S S S S S S	TTIC F AAC: T SIT: F SIT: S CITIC L	Q Q R TIX Y L STE L	AAA N FTT F CTT L AAT M	IGI IGI V IGA D AAAA K GGA E	ACTO R IACC T T O P ATA S GIG	CCC L ACCC R ACCC P ICTL L SAT I	ATT F AGC A ATA Y CAC T ICA Q	173 47 180 50 188 53 196 56 207 59
TR203 621 T TR203 711 C TR203 801 T TR203 891 T TR203 981 C TR203 981 C	0 0 0 0 0 0 0 0 0 0 0 0 0 0	CAP L CAP Q CAP E CAP E CAP CAP CAP CAP CAP CAP CAP CAP CAP CAP	AAAA R R AGC7 A AGTC V XAC2 T T CAC2 T T CAC2 T	AGA D AAA E AAC T T T S TTC	ITTI H K SAAM K SATIC M C SATIC A SATIC A SATIC A	IGAA Y Y XIIAG Y SOGJ R G G G IGIJ	CAAC N CAAC N CAAC N TTTO L TTTO Y CAAA K AAAA	FICE IPAAN N IPACI T SOGJ G IPAAN N AAAA K IPACI	IGA E P IAC S CGA D ATT F	ICEA E E IATT I CEA Y G G IATC M IGG	ATCA Q Q TTC G TTC S TAA K STT L ICA	AACZ T TAAT N AGOO A GOO R ATCO S AACO	AGG P P AAAI N SATTI G G AAAAI N AGGG	ATI I CAM E VAAI F TTT F COT V SAT	TAAC N N TTT F GAI D CCT L	CIP L ZAAI N ZAAC N ZAAC G G CGAC E		T T G SIAI D SGAI D K K AAGJ	TTAC L Y TCA D TCC A C C A C C A C C	N XAAN K CGA D CGA E SAAN K SGA	I I I I CAA D I CAA D I CAA Q A A CAT	TACI Y R CAO T SCAI E IAA N	TIC: S AATZ I OGTV AGCI A AGCI	TTO F AAC: T SIT: F S CITO L	Q Q R TIX Y ICTL J STE L	AAA N FTT F CTT I CTT I AAT M CTA	PAT I IGT V IGA D AAAA K GGA E IDAA	ACTO R TACC T COCC P ATAC S TAC S TAC	L ACCE R ACCE P ICTL L SAT I	AITT F AGC A AITA Y CAC T ICA	171 47 1800 50 1899 53 1966 56 207 59 216
TR203 621 T TR203 711 C TR203 801 T TR203 891 T TR203 981 C TR203 981 C	0 0 0 0 0 0 0 0 0 0 0 0 0 0	CAP CAP CAP CAP CAP CAP CAP CAP CAP CAP	AAAA R R GCCFI R GCCFI T CACCFI T CACCFI C G G	AG2 D AAAC E AAAC T T S T T S T T S	NTTI H SAAA K SATIC M SATIC A SATIC A SATIC N	IGAN TIAI Y Y Y Y Y Y Y Y Y Y Y Y Y Y Y Y Y Y	CAAC N CAAC N CAAC N TITIC L TITIC Y CAAC N	FICE FAAT N FAC2 T G G TAAA N AAAA K TACC T	JITI E ACC P IACC S S CGA D ATT F AATT I	ICEA E E IATT I CEA Y G INGG M INGG G	ATCA Q Q TTC G G TTC S TTA K STT L L CA E	AAAC T TAAA N AGCC A GCC R AACC S AAAC T	AGG P P AGC P P AAAI I G G AAAI N G G	CAN I E VAAT F TTT F COT V SAT	AAG N N ATTI F TTTI F NGAI D CCTI L L XAAZ K	CIP GAI L PAAI N TAAC N G G CGAC E	LIAC LIAC Y E SCAN E VICO W CAI H COY P	T T G SIAI D SGAI D K K AAGJ R	TTIC L YIAN D IGOX A G G XOX R	YAAM N K IGA D SAA K SAA K SGAA E	I I GAU D I GAU D I GAU D I GAU T I I	TIAI Y R CAO T E IAA N ITTII L	FICI S AATZ I OGTO V AGCI A H AGTI V	TTO F T SIT. F S CITO L MAC	CCAN Q ROGS R INCE L STEL L NAAN N	AAA N FTT F CTT L AAT M CTA Y	IAT I IGI V IGA IGA K GGA E IAA N	ACTO R TACC T COCC P ATAC S TAC T TAC	COCC L ACCC R ACCC P L L L L L L L L L L L L L L L L L	AITT F AGC A Y CAC T ICA Q ICA E	171 47 180 50 189 53 196 56 207 59 216 62
TR203 621 T TR203 711 C TR203 801 T TR203 891 T TR203 981 C TR203 981 C TR203 981 C	0 CCC R 0 AAI N 0 CCA P 0 CCA E 0 CCA H 0 CCA H 0	CAP CAP CAP CAP CAP CAP CAP CAP CAP CAP	AAAA R R GCCFI A CACC T T CACC T T CACC T C C C C C C	AG2 D AAA E AAAC T T S TTCI F	NTTI H SAAA K SATIC M SATIC A SATIC A SATIC N	IGAN TIAI Y ATAC Y SOGJ R G G G G IGIJ V	CAAC N CAAC N CAAC N TTTC L TTTC L Y CAAC N	FICE PAAN N FAC2 T SOG G TAAA N N AAAA K TAC2 T	JITI E ACC: P IACC S S CGA D ATT: F AATT I	ICIA E I I I I I I I I I I I I I I I I I I	VICA Q Q IGG G ITIC S ITIC S ITIC A E	AAAC T TAAA N AGCC A C C R AGCC R AATCI S C R T	AGG P P TAAT I G G AAAT N G G	CAN I E VAAT F TTT: F V SAT: I	TAAC N TAAC N TTT F TTT T T T T T T T T T T T T T T	CTIP GAT L PAAC N TAAC G G CGAC E VIAT	TAC Y SAM E SAM E SAM H CAN H	T T G SIAI D SIAI D K K R	TTIC L Y TGAI D IGOX A G G R	YAAM N K IGA D CGA E SAA K SGA E	I I AGAU D I GAU D I GAU D I CAU I I I	TAAT Y R CAOO T CAOO T E IAAA N ITTE L	FIC: S AATE I COTIC AGCI AGCI H AGCI V	F F T SIT F S C T C T C T	CCAN Q ROG R TIAN Y L STTL L AAA N	AAA N FTT F CTT L AAT M CTA Y	IAT I IGI V IGA D AAA K GGA E IAA N	ACTO R IACC T COCC P ATA S GIG GIG W TAC	COCC L ACCC P ICTU L SAT I F	ATT F AGC A ATA Y CAC T ICA Q ICA E	171 47 1800 50 1899 53 1966 56 2077 59 2166 62
VIR203 621 T VIR203 621 T VIR203 6801 T VIR203 981 C VIR203 981 C VIR203 981 C VIR203 2071 A VIR203 2071 A	0 CCC R 0 CCCC R 0 CCC R 0 CCCC R 0 CCCC R 0 CCCC R 0 CCCC R 0 CCCC R 0 CCCC R 0 CCCC R 0 CCCCCC R 0 CCCCC R 0 CCCCC R 0 CCCCC R 0 CCCCCCCC	CAN CAN Q CAN CAN CAN CAN CAN CAN L CAN H	AAAA R R WGC74 A WGTC7 T CAC74 T D CAC74 T D CAC74 C C CAC74 C C CAC74 C C C C C C C C C C C C C C C C C C	AGA D AAA K E XAAC T T T T T S T T S T T S	H H H H H H H H H H H H H H H H H H H	IGAN TIAI Y XIA Y XIA Y XIA Y XIA G G G G G G G G G G G G G G G G G G G	CAAC N CAAC N CAAC Y CAAA K AAAC N AAC	FICE FAAN N TACI G TAAA N AAAA K TACI T	FITI	ICIA E IAT I CIA Y I I I I I I I I I I I I I I I I I I	VICA Q Q ICCC G ITIC S ITIC S ITIC A CAA	AACZ T TAACI N AGOO A GOO S AACO T GATO	AGG P P TAAI N SATTI G G N N G G G G G G G G G G G	GAA I I GAA N F TTT F V GTT I V GTT I I MGT	TAAC N N TTT F TTT T D N CTT L L K K	CIPA CAA L YAAL N YAAC N G G CAA E VITAI Y YAA	TAC Y CAN E CAN E CAN H CAN H CCAN	CACI T G SIAI Y SGAI D K K AAGJ R AAAJ	LITAL VIAN VIAN VIAN VIAN VIAN VIAN VIAN VIAN	N K K CAAJ E SAAJ K SCAJ E SAAJ K SCAJ E	I I AGAN D I GAA D A CAA T I AAAT	TTATY Y R CACC T CACC T CACC T SCAL N I TTT L	FIC: S AATE I OGTV AGCC A AGCC H H AGTE V SATO	FTTC F AAC. T SIT. F SIT. L CITC L SIG	Q Q R TIA Y ICT L J ICT L L AAA N N ACA	AAA N FTT F CTT L AAT M CTA Y CTA	PAT IGT V IGA B AAAA K GGA E IAAA N COG	ACTO R IACC T COCC P ATA S GIG GIG TAC T ACT	SOCI L MOGE P MOCE P ICTL L SAT I TTT F SOT	ATT F AGC A ATA Y CAC T CAC T CAC T CAC T CAC	1711 47 1800 50 1899 53 1988 56 2007/ 59 2166 62 225

nucleotides) from the ATG start codon. The phage ϕ 50 ORF extends for an additional 33 codons beyond the point of divergence and encodes a protein with a predicted molecular mass of 45 kDa. We designate this ORF *Lla*PI, to take account of its phage origin and altered carboxy terminus compared with *Lla*I. *Lla*PI encodes a protein which is equivalent to the amino domain of *Lla*I, and it includes one copy of each consensus sequence.

Activity of LlaPI. The 4.5-kb \$\$\$ \$\$\$ \$\$\$ \$\$\$ fragment cloned in pTRK102 was subcloned as a BamHI-SalI fragment in the streptococcal-E. coli shuttle plasmid pSA3 (4) to construct pTRK103. This plasmid was introduced into NCK203 via electroporation, and pTRK103 transformants were analyzed for functional methylase activity. The presence of pTRK103 in NCK213 did not affect the plaquing ability or plaque morphology of ϕ 32 (Table 2). However, ϕ 32 propagated on NCK203(pTRK103) was able to plaque on NCK203(pTK6) at a frequency of 10^{-1} , 3 orders of magnitude over the level of ϕ 32 propagated on NCK203 (Table 2). Phage propagated on NCK213 were not protected against restriction by the NCK202 R/M plasmid pTRK68 (data not shown). A second plasmid, called pTRK179, was constructed in which a 1.3-kb XbaI fragment of pTRK103 was inverted by in vitro digestion and religation. In this construction, the upstream sequences are disrupted between the tentatively assigned promoter region and the structural gene for LlaPI (Fig. 2). Phage ϕ 32 propagated on NCK203(pTRK179) afforded only a low level of protection to restriction activities directed by pTK6 (Table 2). These data confirm that an actively expressed *Lla*PI gene is necessary for the protection afforded by pTRK103 in *trans* and demonstrate that the expression signals for *Lla*PI are upstream of the *Xba*I site at positions 179 to 184.

The amino domain of *LlaI* is functionally active. A plasmid was constructed in which the amino domain of *LlaI* was separated from the carboxy domain and tested for in vivo methylase activity. Plasmid pTK6 was digested with *HpaI* (nucleotide 1532) and *NruI* and religated to construct pTRK140 (Fig. 1). From the available sequence data of the pACYC184 moiety of pSA3 (24) and the data presented here, we are able to determine that a truncated protein of 421 amino acid residues would result from this fusion. pTRK140 was introduced to *L. lactis* NCK203 and tested for its ability to protect ϕ 32 from restriction by pTK6 (Table 2). The degree of protection closely approximates that provided by pTRK103 (*LlaPI*) and confirms that the amino domain of *LlaI* is sufficient to encode a functional methylase enzyme.

DISCUSSION

Phage $\phi 50$ is a natural isolate which was detected initially after the construction and release of lactococcal starter

1 MRYLCHKINLINFICOVIKKH. DIOGOTFADLFAGIGSVGDYFKGEYTVL 49 ύT. 50 50 SNDYMYPSKVISEAKLINSEKPKFDSFVKRYGKIPFQMINERETTFNDGY 99 ||| :||| :: .||: |.. |.|..: | |.|:::|:: |:. ..:: SNDSLYFSYTLLKAKTENNSTENISELKKIGIKEPLHYLENEEFEISHEF 100 148 ASLLESVIKVSNISGTYQAFTKFWESRALKKFTIMPLEMKOSLSVSKONR 197 198 CENRVINRLVREISGDIAYIDPPYTTTQVINSYHVLETIARVINPELFGK 247 .: |.:. |.: .: ||| ||||||. ||...|!: ||||| || |::|| 198 TYNEDANSLIEKVYCDILYIDEPPINGRQYISNYHLLETIALYDYPETYGK 247 297 VDLARRFAVDGIVEVEINEVREYSINNSSMRGEGKKLQEVIIYFKKNLET 346 347 N......KSPLNYAGSKODVIPRIFKLLPKHVTTFV 376 377 DAMSGAFNYGANRTALNKVVYNEYHPFVFEMISMIVNIPADELJENVEDI 426 | ::|:|||| | .| ||:: .:.::|.|::: : .|.:|:| ::|. DLFSGGFNVGINVNA.NKIIATDINTYVVEVLDIMKCTSVEEVLAHIERR 443 395 427 VIRYSLEKKCKEAFNRIRDHYNNEEOIPINLYTINIYSFONILRFNOAKK 476 YNIPIGNN. . EFNEGYKURITRFVTRAPEVEMRLGS. . YSAINFNEYDDD 522 ||.|:|.: :|...|.::. |:. .| :...: :. :||...|:: 493 XINNPFGRERSOFSPALKKLVLFIEALDEXNVOFVCSEFEHENFSOLDON 542 523 TVFYFDPPYLVTTAGYNDGKRGFDGNDAEOFASLLKYLITELDSAGKKIML 572 .:.| ||||:||:|||.||.:|: || .||..|..|: || || 543 DEVYCOPPYLITTICSYNDONREFKUMREOETKULDILDHUNSKGVYFAL 592 573 SNVLEHRGRINHLIMEN..... ICHHGENVNTIGETGIKYPRREILVINY 617 ||||.||| .|.||:|| |:| . . . :...: ...|:|:||| SNVLSHKGLENELLLEWSKKYNTHHIGHSYSNSSHNTTRGESOEVLITNY 642 618 NIFER 622 643 INYIK 647

FIG. 3. Alignment of the amino acid sequences of *Lla*I and *Fok*I. Identical (I) and related (:) residues are indicated. Gaps in the aligned sequences are also indicated (.). The degree of identity is 39.4%, and the degree of relatedness based on evolutionarily related residues is 61.4%. The 15- and 10-residue methylase consensus sequences are boxed in each case.

cultures containing the conjugative phage resistance plasmid pTR2030 (1). Phage $\phi 50$ is completely resistant to the pTR2030-encoded R/M system, which we have designated LlaI. A region of homology was detected between the phage and plasmid genomes and was subcloned, sequenced, and aligned. Sequence analysis demonstrated that a region, 1,273 bp in length, is present in both plasmid and phage. This sequence was 100% identical, strongly indicating a recent genetic exchange from plasmid to bacteriophage, the method of which is currently unknown. Since a precursor of $\phi 50$ without the plasmid fragment has not yet been identified, we cannot ascertain whether the exchange was the result of direct insertion or replacement recombination. It may be significant that an iso-ISS1 insertion sequence (IS946) has been identified upstream of the LlaI methylase structural gene (Fig. 1; 23). IS946 has been implicated in inter- and intramolecular rearrangements involving pTR2030 and its

subclones (23). However, the point of identity between $\phi 50$ and pTR2030 sequences does not correspond to the known location of IS946 within pTR2030. It is possible that IS946 could have been involved in the formation of a $\phi 50$ precursor::pTR2030 intermediate which resolved to create $\phi 50$. The sequences flanking the region of identity show no significant similarity between the phage and plasmid. If a direct insertion is assumed, no phage ORF was interrupted by the acquisition of the plasmid fragment.

The 1,273-bp fragment encodes over 55% of the 1,869-bp structural gene for *Lla*I. In ϕ 50, the structural gene (*Lla*PI) continues for 33 amino acids after the point of divergence as a result of a fortuitous fusion between the *Lla*I gene and a phage sequence devoid of stop codons for a short distance. The upstream 243 bp, included in the exchanged region, contains a putative promoter sequence. Disruption at the *Xba*I site located between the structural gene and the putative promoter results in a decrease in biological activity.

Both LlaI and LlaPI structural genes were subcloned in the E. coli-Streptococcus shuttle vector pSA3 and introduced into L. lactis NCK203 to assay for biological methylase activity. Activity was determined by the ability of the recombinant plasmids pTK6 and pTRK103 to confer protection to a phage, ϕ 32, which is subject to restriction by the plasmid pTR2030-encoded R/M system. The modification activity of the *Lla*PI gene was markedly lower (10-fold) than that of LlaI, but in both cases phage propagated in cells bearing these genes in trans afforded significant protection to φ32 against the pTR2030 R/M system. The lower activity encoded by LlaPI was similar to that encoded by a truncated *Lla*I. In any case, it is evident that ϕ 50 possesses a gene, LlaPI, which can confer significant resistance against restriction in trans to phage DNA, and that this gene was acquired from the plasmid pTR2030 in the host background.

Given the complete resistance of ϕ 50 to restriction by the pTR2030 R/M system, it may be that the *Lla*PI methylase is more efficient in *cis* or is produced in significantly higher concentrations from a bacteriophage location (final copy number prior to cell lysis, 100 to 150) than from the pSA3-cloned bacteriophage fragment (copy number, <10). Alternatively, ϕ 50 may possess fewer recognition sites than ϕ 32 and thus requires less methylation to be fully modified.

The results presented here are significant from a number of perspectives. Protection of fermentation bacteria from bacteriophage attack is a concern to the traditional bioprocessing industries and can be expected to emerge as a major problem in novel culture-based technologies. The adaptive response of the bacteriophage genome when confronted with powerful bacterial defense mechanisms is therefore of industrial concern, in addition to being an interesting fundamental study in evolutionary biology. Phage $\phi 50$ must have undergone a genetic exchange event with host DNA, which conferred a selective advantage in systems in which pTR2030 is the primary barrier to phage proliferation. To our knowledge, this is the first report of genetic exchange between a virulent phage and a plasmid that confers such a selective advantage upon the bacteriophage. This exchange took the form of an in vivo cloning of a gene which allows the phage genome to remain methylated regardless of the propagating background. This response is highly directed, conferring resistance against only a single R/M system. These results demonstrate a weakness in relying on R/M systems as a primary means of bacteriophage defense, or indeed any system in which the acquisition of a single small region of DNA by the bacteriophage genome negates the resistance mechanism.

R/M phenotypes are extensively distributed among lactococci (3). However, little is known about the genetic basis of these phenotypes. The type II restriction endonuclease, ScrF1, isolated from L. lactis subsp. cremoris F, has not been shown to have a role in the in vivo restriction of phage ϕ kh (5). LlaI provides the first direct evidence that in vivo restriction and modification activities, directed against bacteriophage in lactococci, can result from type II systems.

The gene identified in this study, LlaI, is unusual in a number of respects. The size of LlaI (72.5 kDa) is considerably larger than the range normally encountered with type II A methylases (usually between 30 and 50 kDa). Computer analysis, and limited genetic and biological evidence, strongly suggests that LlaI is composed of two functional methylase domains arranged in tandem. An analogous situation has been described for the FokI A methylase, which also contains two functional domains within a single gene product (10, 17). The FokI methylase shows a significant region of internal homology surrounding the DPPY motifs and in this regard is similar to the methyltransferases M · PaeR7I and PvuIIM (31). FokI recognizes a nonpalindromic sequence (5'-GGATG/3'-CCTAC) and functions as an asymmetric dimer to modify the complementary sequences on opposite strands (15, 17, 30). Individual domains are capable of protecting DNA from FokI endonucleolytic cleavage. The striking genotypic and phenotypic similarities between FokI and LlaI strongly suggest that this enzyme may also recognize a nonpalindromic sequence.

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