JOURNAL OF BACTERIOLOGY, July 1991, p. 4363–4370
0021-9193/91/144363-08$02.00/0
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Vol. 173, No. 14

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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Received 22 February 1991/Accepted 14 May 1991

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 (ϕ50) is completely resistant to restriction by pTR2030. A region of homology between pTR2030 and ϕ50 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 *Lla*I. The *Lla*I gene is 1,869 bp in length and shows organizational similarities to the type II A methylase *Fok*I. In addition to the amino domain, upstream sequences, possibly containing the expression signals, were present on the phage genome. The phage ϕ50 fragment containing the methylase amino domain, designated *Lla*PI, 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 *Lla*I 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 (ϕ50) 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 ϕ50 is unusual in that it is resistant to the action of the *hsp* gene product. In addition, regardless of the propagating host, ϕ50 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 ϕ50 (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 ϕ50 has acquired a functional methylase domain of the type II A methylase gene, *Lla*I, 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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† Paper FSR91-11 of the journal series of the Department of Food Science, North Carolina State University, Raleigh, NC 27695-7624.

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or derivation
L. lactis		
NCK202	Homologous host for φ50 and φ32	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		
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^+ R^+/M^+	11
pTRK102	Ap^r	pBluescript::4.5-kb φ50 fragment
pTRK103	Cm^r Em^r R^-/M^+ (*Lla*PI)	pSA3::4.5-kb φ50 fragment
pTRK140	Cm^r Em^r R^-/M^+ (*Lla*I)	*Hpa*I-*Nru*I deletion of pTK6
pTRK179	Cm^r Em^r	*Xba*I inversion of pTRK103

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 μg/ml, respectively; for lactococci, erythromycin at 5 μg/ml.

Isolation of a Hsp-resistant variant of φ31. Phage nck202.31 (φ31) is a small isometric-headed phage which is subject to restriction by the R/M system encoded by the lactococcal plasmid pTR2030 (7). Phage φ31 is also susceptible to the abortive infection mechanism (Hsp) directed by pTR2030. To eliminate the effect of the Hsp mechanism, a derivative of φ31 was isolated which is insensitive to Hsp but remains susceptible to the R/M system. Phage φ31 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 10^{13} phage used in a plaque assay on NCK204, only a single plaque was isolated. This phage isolate, nck202.32 (φ32), shows no significant difference in its restriction map compared with the parent phage φ31, but it appears to be insensitive to the Hsp-directed resistance. Phages φ31 and φ32 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 10^4 to 10^5 per μg of plasmid (pSA3) were routinely achieved under these conditions. Transformants were selected on 1.5 μg 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 *Lla*I. *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

φ50 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

Phage[a]	Relevant plasmid in:		Efficiency of plaquing
	Propagating host	Plaquing host	
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}
φ32.NCK383	pTRK179	pTK6	6.1×10^{-3}
φ32.NCK312	pTRK140	pTK6	2.0×10^{-1}
φ50.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 (φ31) in the NCK203 background, in addition to the Hsp$^+$ response (7). Phage nck202.32 (φ32) was isolated from a single plaque appearing after challenge of NCK204 (containing pTR2030) with 10^{13} PFU of modified phage φ31 per ml. The propagation conditions for modification of phage φ31 and isolation of φ32 are described in Materials and Methods. Phage φ32 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 φ32 is resistant to Hsp-directed abortive infection since this phenotype is not subject to host-dependent modification or restriction (11). Phage φ32 was used in subsequent plaque assays to assess R/M activities, independent of Hsp responses, in the course of this study.

In contrast to φ32, neither the plaque size nor efficiency of plaquing of the small isometric-headed phage nck202.50 (φ50) 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 φ50. pTRK68 is a native R/M plasmid which resides in NCK202 (the parental strain from which NCK203 is derived; 7). Phage φ50 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 φ50 to pTK6 is not a generalized phenomenon, as might be expected if φ50 relied upon the incorporation of unusual bases, or a postreplicational modification such as glycosylation, to elude restriction.

Cloning and mapping the φ50-pTR2030 homologous region. pTR2030 possesses a region homologous to φ50 (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 φ50 was shotgun cloned in the *E. coli* cloning vector pBluescript on a 4.5-kb *Bam*HI-*Hin*dIII 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 φ50 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 *Lla*I.

Two consensus sequences are found in all type II A methylases (16, 31). A manual search of the predicted protein sequence of *Lla*I revealed the presence of both the consensus 15-amino-acid sequence and the 10-residue DPPY (asparagine-proline-proline-tyrosine) consensus sequence within the *Lla*I 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 *Fok*I

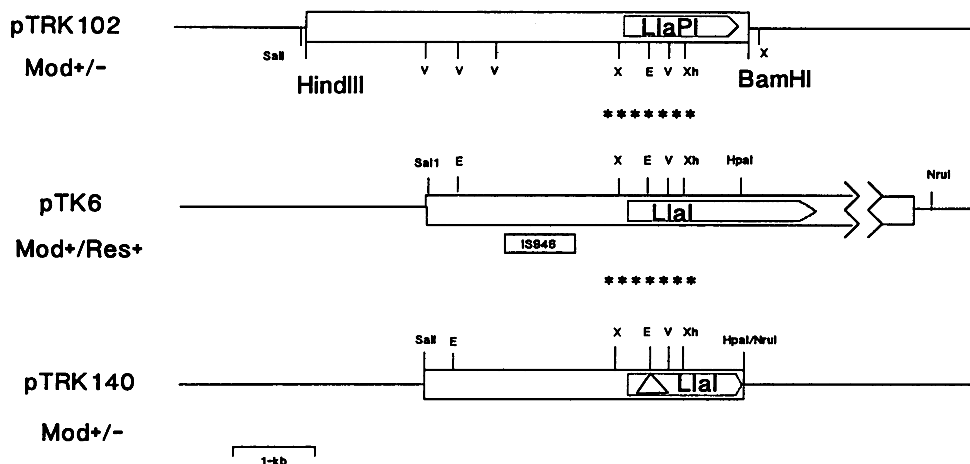

FIG. 1. Physical maps of pTRK102, pTK6, and pTRK140, aligned to show the region of identity (***) between plasmid and phage fragments. Open boxes represent the φ50 region cloned on pTRK102 (4.5 kb) and the pTR2030 region cloned on pTK6 (13.6 kb). The position of the insertion sequence IS*946* within pTK6 is also indicated (23). Restriction endonuclease sites: E, *Eco*RI; V, *Eco*RV; X, *Xba*I; Xh, *Xho*I.

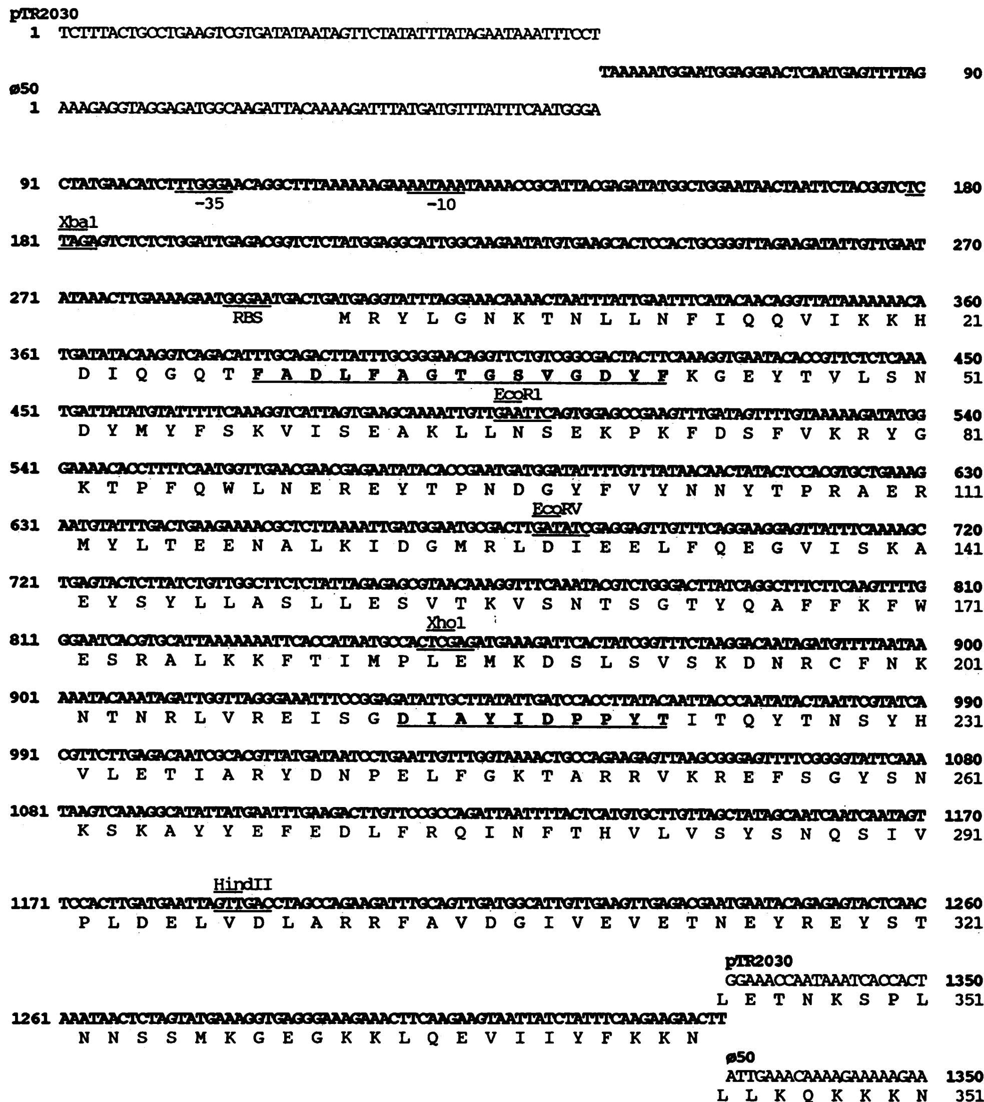

FIG. 2. Nucleotide sequences from both pTR2030 and ϕ50. The identical 1,273 bp from nucleotides 56 to 1329 is shown once, in boldface for clarity. Individual plasmid and phage sequences are labeled. The predicted amino acid sequences of both *Lla*I and *Lla*PI are also shown and numbered. The amino acid 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 *Lla*I was not protected from digestion by *Fok*I (data not shown). The consensus sequences described by Lauster (16) are highly conserved in *Lla*I (Fig. 3). The *Lla*I 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 *Lla*I and the DNA adenine methylase from bacteriophage T4 (27). The carboxyl terminus of *Lla*I 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 *Lla*I and T4 *dam* and the entire T4 *dam* with 26% identity. We conclude from these data that *Lla*I 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 ϕ50 homologous region. The complete nucleotide sequence of a 1,581-bp region of ϕ50 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

```
pTR2030
1351 AAACTATGCCGGAAGTAAAGACGATGTGATTCCTAGAATAGTTAAGCTATTACCTAAACATGTGACAACTTTTGTTGATGCAATGGGAGG 1440
      N  Y  A  G  S  K  D  D  V  I  P  R  I  F  K  L  L  P  K  H  V  T  T  F  V  D  A  M  G  G  381

ø50
1351 CGTAGAAAGCACGAAACAAAAGAAGAACGTAGGTTGCGAAGAAATATCAGAGCGCAAATGCCAATCGAAAACTCAAGAAAAGAAGAATTA 1440
      V  E  S  T  K  Q  K  K  N  V  G  C  E  E  I  S  E  R  K  C  Q  S  K  T  Q  E  K  K  N  *  380

pTR2030
1441 AGCGTTCAATGTGGGTGCGAATCGAACTGCATTGAACAAAGTAGTGTATAACGAATACCATCCATTTGTCTTTGAAATGATGCAGATGAT 1530
      A  F  N  V  G  A  N  R  T  A  L  N  K  V  V  Y  N  E  Y  H  P  F  V  F  E  M  M  Q  M  I  411

ø50
1441 GGATTATAGCAATCCAAAATTACCTGAATTAATAAGAGAGGACACGAAAAATGACTAAGTTTGAAACAGCGAACGAATTAATATCTTTTG

pTR2030 HpaI
1531 TGTTAACACTCCGGCAGATGAGTTGATTAGAAATGTTGAGCAGATCGTAACTCGATATTCATTGGAGAAGAAAGGGAAAGAAGCGTTTAA 1620
      V  N  T  P  A  D  E  L  I  R  N  V  E  Q  I  V  T  R  Y  S  L  E  K  K  G  K  E  A  F  N  441

ø50                                          BamH1
1531 TTAAGGAAAAAGATTTGAAAAGTGGTTTCTATCAAAAAGGGAAAAGGATCC

pTR2030
1621 TCGCTTGCGTGATCATTATAATAATGAGGAACAAACACCAATTAACCTATACACTTTGAATATTTATTCTTTCCAAAATATACTGCGATT 1710
      R  L  R  D  H  Y  N  N  E  E  Q  T  P  I  N  L  Y  T  L  N  I  Y  S  F  Q  N  I  R  L  F  471
pTR2030
1711 CAATCAAGCAAAGAAATACAATACACCTATTGGTAATAATGAATTTAATGAAGGATATAAAGATAGAATAACTCGTTTTGTTACACGAGC 1800
      N  Q  A  K  K  Y  N  T  P  I  G  N  N  E  F  N  E  G  Y  K  D  R  I  T  R  F  V  T  R  A  501
pTR2030
1801 TCCAGAAGTGGAGATGCGTTTGGGTAGCTATTCAGCGATTAATTTTAACGAGTATGATGATGACACCGTGTTTTATTTTGACCCACCATA 1890
      P  E  V  E  M  R  L  G  S  Y  S  A  I  N  F  N  E  Y  D  D  D  T  V  F  Y  F  D  P  P  Y  531
pTR2030
1891 TTTGGTCACAACAGCTGGTTATAACGATGGTAAGCGTGGTTTTGATGGATGGGATGCCGAACAGGAAGCAAGTCTCTTAAAATATCTCAC 1980
      L  V  T  T  A  G  Y  N  D  G  K  R  G  F  D  G  W  D  A  E  Q  E  A  S  L  L  K  Y  L  T  561

pTR2030
1981 CGAATTGGATTCAGCAGGCAAAAAATTTATGTTATCAAATGTTCTTGAGCATAAAGGAAAAACTAATCACTTGTTAATGGAGTGGATTCA 2070
      E  L  D  S  A  G  K  K  F  M  L  S  N  V  L  E  H  K  G  K  T  N  H  L  L  M  E  W  I  Q  591
pTR2030
2071 ACACCATGGCTTTAATGTAAATACAATTGGTGAAACAGGGATTAAATATCCAAGACGGGAAATTTTAGTAACAAACTATAATACTTTTGA 2160
      H  H  G  F  N  V  N  T  I  G  E  T  G  I  K  Y  P  R  R  E  I  L  V  T  N  Y  N  T  F  E  621
pTR2030
2161 AAGGTGAAAAGGATGAGAAAGTTACTCTTGCAAGATGGTAGTTATCAAACAAAATTTAATAAAATAGATGTGACACCTCCGACTGGTGTT 2250
      R  *                                                                                     622
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nucleotides) from the ATG start codon. The phage φ50 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 *Lla*PI. The 4.5-kb φ50 fragment cloned in pTRK102 was subcloned as a *Bam*HI-*Sal*I 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 φ32 (Table 2). However, φ32 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 φ32 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 *Xba*I 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 *Lla*PI (Fig. 2). Phage φ32 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 *Lla*I is functionally active. A plasmid was constructed in which the amino domain of *Lla*I was separated from the carboxy domain and tested for in vivo methylase activity. Plasmid pTK6 was digested with *Hpa*I (nucleotide 1532) and *Nru*I 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 φ32 from restriction by pTK6 (Table 2). The degree of protection closely approximates that provided by pTRK103 (*Lla*PI) and confirms that the amino domain of *Lla*I is sufficient to encode a functional methylase enzyme.

DISCUSSION

Phage φ50 is a natural isolate which was detected initially after the construction and release of lactococcal starter

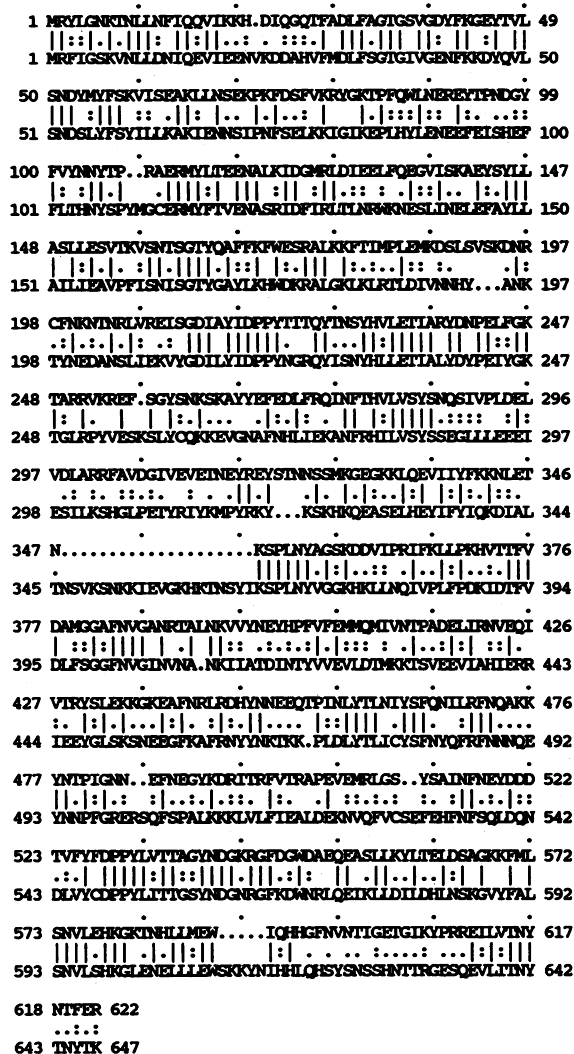

FIG. 3. Alignment of the amino acid sequences of *Lla*I and *Fok*I. Identical (|) and related (:) residues are indicated. Gaps in the aligned sequences are also indicated (.). The degree of identity is 39.4%, and the degree of relatedness based on evolutionarily related residues is 61.4%. The 15- and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

cultures containing the conjugative phage resistance plasmid pTR2030 (1). Phage φ50 is completely resistant to the pTR2030-encoded R/M system, which we have designated *Lla*I. 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 φ50 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-ISS*1* insertion sequence (IS*946*) has been identified upstream of the *Lla*I methylase structural gene (Fig. 1; 23). IS*946* has been implicated in inter- and intramolecular rearrangements involving pTR2030 and its subclones (23). However, the point of identity between φ50 and pTR2030 sequences does not correspond to the known location of IS*946* within pTR2030. It is possible that IS*946* could have been involved in the formation of a φ50 precursor::pTR2030 intermediate which resolved to create φ50. 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 φ50, 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 *Lla*I and *Lla*PI 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, φ32, 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 *Lla*I, 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 *Lla*PI was similar to that encoded by a truncated *Lla*I. In any case, it is evident that φ50 possesses a gene, *Lla*PI, 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 φ50 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, φ50 may possess fewer recognition sites than φ32 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 φ50 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, *Scr*F1, isolated from *L. lactis* subsp. *cremoris* F, has not been shown to have a role in the in vivo restriction of phage φkh (5). *Lla*I 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, *Lla*I, is unusual in a number of respects. The size of *Lla*I (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 *Lla*I is composed of two functional methylase domains arranged in tandem. An analogous situation has been described for the *Fok*I A methylase, which also contains two functional domains within a single gene product (10, 17). The *Fok*I methylase shows a significant region of internal homology surrounding the DPPY motifs and in this regard is similar to the methyltransferases M · *PaeR7*I and *Pvu*IIM (31). *Fok*I 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 *Fok*I endonucleolytic cleavage. The striking genotypic and phenotypic similarities between *Fok*I and *Lla*I strongly suggest that this enzyme may also recognize a nonpalindromic sequence.

ACKNOWLEDGMENTS

We thank Tapani Alatossava and Thomas A. Trautner for helpful discussions. We also thank Ira Schildkraut for alerting us to the similarities between *Lla*I and *Fok*I. We acknowledge the primer synthesis conducted by Paul Bishop of the Biochemistry Department, North Carolina State University.

This work was supported by the Food Ingredients Division of the Haarmann & Reimer Corp., Elkhart, Ind., and the U.S. Department of Agriculture competitive grants program in Animal Molecular Biology (grant 87-CRCR-1-2547).

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