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In Vivo Restriction by *Lla*I Is Encoded by Three Genes, Arranged in an Operon with *llaIM*, on the Conjugative *Lactococcus* Plasmid pTR2030†

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The *Lla*I restriction and modification (R/M) system is encoded on pTR2030, a 46.2-kb conjugative plasmid from *Lactococcus lactis*. The *llaI* methylase gene, sequenced previously, encodes a functional type IIS methylase and is located ~5 kb upstream from the *abiA* gene, encoding abortive phage resistance. In this study, the sequence of the region between *llaIM* and *abiA* was determined and revealed four consecutive open reading frames (ORFs). Northern (RNA) analysis showed that the four ORFs were part of a 7-kb operon with *llaIM* and the downstream *abiA* gene on a separate transcriptional unit. The deduced protein sequence of ORF2 revealed a P-loop consensus motif for ATP/GTP-binding sites and a three-part consensus motif for GTP-binding proteins. Data bank searches with the deduced protein sequences for all four ORFs revealed no homology except for ORF2 with McrB, in three regions that coincided with the GTP-binding motifs in both proteins. To phenotypically analyze the *llaI* operon, a 9.0-kb fragment was cloned into a high-copy-number lactococcal shuttle vector, pTRKH2. The resulting construct, pTRK370, exhibited a significantly higher level of in vivo restriction and modification in *L. lactis* NCK203 than the low-copy-number parental plasmid, pTR2030. A combination of deletion constructions and frameshift mutations indicated that the first three ORFs were involved in *Lla*I restriction, and they were therefore designated *llaI.1*, *llaI.2*, and *llaI.3*. Mutating *llaI.1* completely abolished restriction, while disrupting *llaI.2* or *llaI.3* allowed an inefficient restriction of phage DNA to occur, manifested primarily by a variable plaque phenotype. ORF4 had no discernible effect on in vivo restriction. A frameshift mutation in *llaIM* proved lethal to *L. lactis* NCK203, implying that the restriction component was active without the modification subunit. These results suggested that the *Lla*I R/M system is unlike any other R/M system studied to date and has diverged from the type IIS class of restriction enzymes by acquiring some characteristics reminiscent of type I enzymes.

The biological role of restriction and modification (R/M) systems is to protect cells from invasion by foreign DNA, such as promiscuous plasmids or infecting bacteriophages. The restriction enzyme can discriminate between foreign and host DNA by the presence of methylation on certain nucleotides (reviewed in reference 4). Traditionally, these enzymes have been classified into three types. Type I enzymes consist of three subunits which are responsible for modification, restriction, and specificity. A complex of the three subunits is required to restrict DNA in the presence of Mg^{2+}, ATP, and *S*-adenosylmethionine. The site of cleavage is generally a large distance (often >1 kb) from the recognition site. Type II enzymes are the most common and the simplest, with over 1,000 different varieties identified (45). They consist of one subunit, require Mg^{2+} as a cofactor, and restrict at or near their recognition sites. Those that restrict outside their recognition sites are subdivided into type IIS enzymes. The type III enzymes have two subunits, one involved in recognition and modification and the other required for restriction; both are needed to restrict DNA. They require Mg^{2+} and ATP for activity and are stimulated by *S*-adenosylmethionine. Some restriction endonucleases that do not conform with any of the three classification types have been identified. *Eco*57I has a single subunit exhibiting both modification and restriction activities. A new class, type IV, has been proposed for this type of enzyme (21, 33). The McrBC enzyme from *Escherichia coli* is a very well studied enzyme that is sufficiently different to warrant a new classification. This multisubunit restriction enzyme restricts only cytosine-modified DNA and has an absolute requirement for GTP (34).

In the genus *Lactococcus*, the in vivo action of restriction endonucleases protects these widely used fermentation bacteria from attack by virulent bacteriophages. The first lactococcal type II enzyme characterized was *Scr*FI, and this enzyme recently was shown to confer phage resistance in vivo (8). R/M systems are very efficient early phage defense mechanisms, but upon prolonged use in fermentation environments, modified bacteriophage which are insensitive to the R/M systems present can emerge (reviewed in reference 24). Therefore, intensive effort goes into selecting strains with different resistance mechanisms against the prevalent phages in dairy fermentation environments. These highly evolving environments would tend to select for mobile phage resistance elements to increase the likelihood of their dissemination among the population. Indeed, numerous native conjugative phage resistance plasmids have been identified among *Lactococcus* strains used in dairy manufacturing. The first to be studied extensively, pTR2030, is a 46.2-kb conjugative plasmid encoding both an R/M system and an abortive infection mechanism (19).

The gene for the *Lla*I methylase (*llaIM*), on pTR2030, was

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previously identified by Hill et al. (18). It consisted of two separate domains, and a subclone encoding the N-terminal domain was capable of functional methylation, though not as efficiently as the full gene. Sequence comparison with the deduced protein sequence of another type IIS methylase, *Fok*I, showed 39% identity, indicating a close evolutionary lineage between these two genes. Another type IIS methylase gene, *stsIM*, was reported to be 35% identical to *llaIM* and 49% identical to *fokIM* at the deduced protein sequence level (25). The genes encoding the *Fok*I and the *Sts*I restriction endonucleases were 30% identical (at the deduced protein level), suggesting that even though they had diverged more than their cognate methylases, they had a recent common ancestral origin (25). In this study, we show that in contrast to its cognate methylase, the genetic organization encoding *Lla*I restriction is very different from the organization in these type IIS systems, suggesting a rapid evolutionary divergence.

It should be noted that the original *Lla*I designation for the pTR2030-encoded restriction enzyme was proposed by Hill et al. (18) following sequence characterization of the methylase gene. However, in a separate and unrelated instance, the *Lla*I designation was subsequently used to describe an observed type II endonuclease activity in another lactococcal strain (27). As numerous subsequent citations have referred to *Lla*I as the one proposed by Hill et al. (18), we will continue to do likewise.

MATERIALS AND METHODS

Bacteria, bacteriophages, and plasmids. *Lactococcus lactis* subsp. *lactis* NCK203 was the sensitive host for all bacteriophages used in this study. *E. coli* MC1061 (6) and XL1-blue (5) were used as cloning hosts. Phage φ31 is a small isometric, P335 species, cohesive ended, lactococcal bacteriophage with a double-stranded DNA genome of 31.9 kb and is sensitive to both *Lla*I restriction and AbiA-mediated abortive resistance (1). Phage φ31.1 is an AbiA-resistant derivative of φ31 with a genome size of 30 kb (28). Plasmid pTR2030 is a native 46.2-kb conjugative lactococcal plasmid encoding the *Lla*I R/M system and the AbiA abortive phage resistance phenotype (23). Plasmid pTK6 consists of a 13.6-kb region of pTR2030, encoding *Lla*I and AbiA, cloned into pSA3 (20). It encodes chloramphenicol resistance and erythromycin resistance (Em^r) in *E. coli* and Em^r in lactococci. Plasmid pTRKH2 is a 6.9-kb high-copy-number shuttle cloning vector for lactococci, with blue/white screening capability in *E. coli* and expressing Em^r in both hosts (30). It replicates by theta replication in lactococci. Plasmid pBluescript (Stratagene) is a 3.2-kb *E. coli* cloning vector based on the ColE1 replication origin and expressing ampicillin resistance.

Culture conditions and bacteriophage assays. Lactococcal strains were grown at 30°C in M17 broth (Difco laboratories, Detroit, Mich.) containing 0.5% glucose. Strains of *E. coli* were propagated at 37°C in LB medium (38) or brain heart infusion medium (Difco). For selection in *E. coli*, chloramphenicol (30 μg/ml), ampicillin (50 μg/ml), and erythromycin (150 μg/ml) were used. Brain heart infusion medium was used specifically for selection with erythromycin. For selection in lactococci, erythromycin was used at 2 μg/ml. Bacteriophage plaque assays were conducted as described previously (46).

Molecular cloning and sequence determination. Standard techniques were used for *E. coli* plasmid isolation, endonuclease restriction, ligations, exonuclease III deletions, and plasmid transformations (38). Nucleotide sequence information was obtained from pBluescript clones, using the Sequenase 2.0 enzyme (United States Biochemical Corporation, Cleveland, Ohio) and standard dideoxy sequencing as described by the manufacturers. PCR-generated clones were used in some cases, but in every case, sequence was obtained from at least two independently generated clones to ensure that no mutations were incorporated. Commercial pBluescript primers or synthetic oligonucleotides were used for priming sequence.

RNA manipulations. Total RNA was isolated from lactococcal strains at mid-log phase of the growth cycle, using standard techniques. Briefly, cells were pelleted and immediately frozen in −70°C ethanol. Following thawing on ice, pellets were resuspended in cold 25% sucrose and treated on ice with lysozyme (30 μg/ml) for 40 min. Sodium dodecyl sulfate (1.5%) was then added, and samples were put at 100°C for 2 min before two extractions with preheated phenol (>50°C) equilibrated with 50 mM sodium acetate (pH 5.4). Following a chloroform-isoamyl alcohol extraction, RNA was precipitated with 2 volumes of ethanol at −20°C and was subsequently treated with RNase-free DNase I (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Spectrophotometric measurements and gel electrophoresis were used to evaluate quantity and purity. Formaldehyde gel electrophoresis, Northern (RNA) transfer, and hybridizations were accomplished by using standard techniques (38). The *llaIM*-specific probe was obtained by using PCR to generate a 506-bp fragment from the 5′ region of the gene, using primers ATGGGAATGACTGATGAGG and GCCTGATAA GTCCCAGAC. PCR was also used to generate a 196-bp probe specific to open reading frame 2 (ORF2) (from bp 1520 to 1716 in Fig. 1), a 970-bp probe specific to both ORF3 and ORF4 (from bp 2784 to 3754 in Fig. 1), and a 762-bp *abiA*-specific probe. Probes were gel purified and labeled by using a multiprime labeling kit (Amersham International, Amersham, England), using [α-^{32}P]dCTP as instructed by the manufacturer.

PCR and electroporation techniques. PCRs were carried out by using *Taq* DNA polymerase (Perkin Elmer-Cetus, Norwalk, Conn.) as described by the manufacturer with an Ericomp (San Diego, Calif.) thermal cycler. If the PCR product was for cloning, suitable restriction enzyme sites were incorporated in the 5′ ends of the primers, at least three bases from the end. Electroporation of plasmid DNA into lactococci was carried out as described previously (28).

Plasmid copy number measurements. Total DNA was isolated from strains of interest, and equal quantities of DNA were digested with suitable restriction enzymes. Following electrophoresis and Southern transfer, blots were probed with *llaI*-specific probes, and the resulting signals were comparatively analyzed.

Plasmid mutagenesis. Specific translation frameshifts were incorporated into each ORF in the *llaI* operon by selectively restricting desired restriction enzyme sites, blunting the cohesive ends with Klenow enzyme and deoxynucleoside triphosphate, and religating. *Eco*RI sites were chosen for the frameshifts in *llaIM*, ORF1, and ORF2. This resulted in the addition of 4 bp, thus destroying the particular *Eco*RI site and creating a new *Xmn*I site. This feature was used to confirm that the desired frameshift had occurred. Filling in a *Nar*I site in ORF3 resulted in a 2-bp addition, which inactivated the *Nar*I site and created an *Asc*I site. Blunting a *Bst*XI site in ORF4 removed 4 bp and fortuitously created an *Mun*I site. An in-frame deletion in ORF1 and ORF2 was constructed by deleting a 920-bp fragment between an *Eco*RI site in the 3′ end of ORF1 and a *Stu*I site in ORF2.

Nucleotide sequence accession number. The sequence shown in Fig. 1 has been assigned GenBank accession number U17233.

RESULTS

A 13.6-kb region of pTR2030, cloned in pTK6, was previously shown to encode an R/M system and an abortive infection phage resistance mechanism, AbiA (previously designated Hsp [20]). Subsequent analysis identified the methylase gene, *llaIM*, positioned directly downstream from an IS*946* insertion element and ~5 kb upstream from the *abiA* gene (18). A deletion of the DNA downstream from *llaIM* abolished the restriction phenotype. A spontaneous R^-/M^- derivative of pTK6 was previously shown to have an ~3.5-kb deletion consisting of the *llaIM* gene and downstream DNA (20). From these data, the *Lla*I restriction phenotype was localized to the region between *llaIM* and *abiA*.

Sequence of the region between *llaIM* and *abiA*. Figure 1 outlines the sequence of 4,800 bp between *llaIM* and *abiA*. This sequence was obtained from both strands by using subclones in pBluescript and clones with nested deletions mediated by exonuclease III. Where suitable subclones were not available, synthetic oligonucleotides were used for primer walking. Bases 1 to 90 were previously reported with the 3′ end of the *llaIM* gene (18), and bases 3714 to 4813 were reported with the 5′ end of the *abiA* sequence (17). Analysis of the sequence for ORFs showed four consecutive ORFs, all with potential ribosome binding sites and ATG start codons. These ORFs were positioned directly downstream from *llaIM* and organized in an apparent operon structure. The A+T content of the sequence is 65%, which is in good agreement with the reported A+T content for lactococci of 64% (22). The four ORFs encode potential proteins with calculated molecular masses of 38.3, 37.8, 54.7, and 47.5 kDa, respectively. These deduced proteins are largely hydrophilic and are not predicted to be membrane associated. A histidine-rich region in the ORF4 deduced protein is homologous to a motif in a family of DNA relaxases involved in *oriT* nicking for conjugal plasmid transfer (32). A potential ATP/GTP-binding site was detected at the N terminus of the deduced protein sequence for ORF2, using the PROSITE program of PC-GENE sequence analysis software (IntelliGenetics, Inc., Mountain View, Calif.). This site is ho-

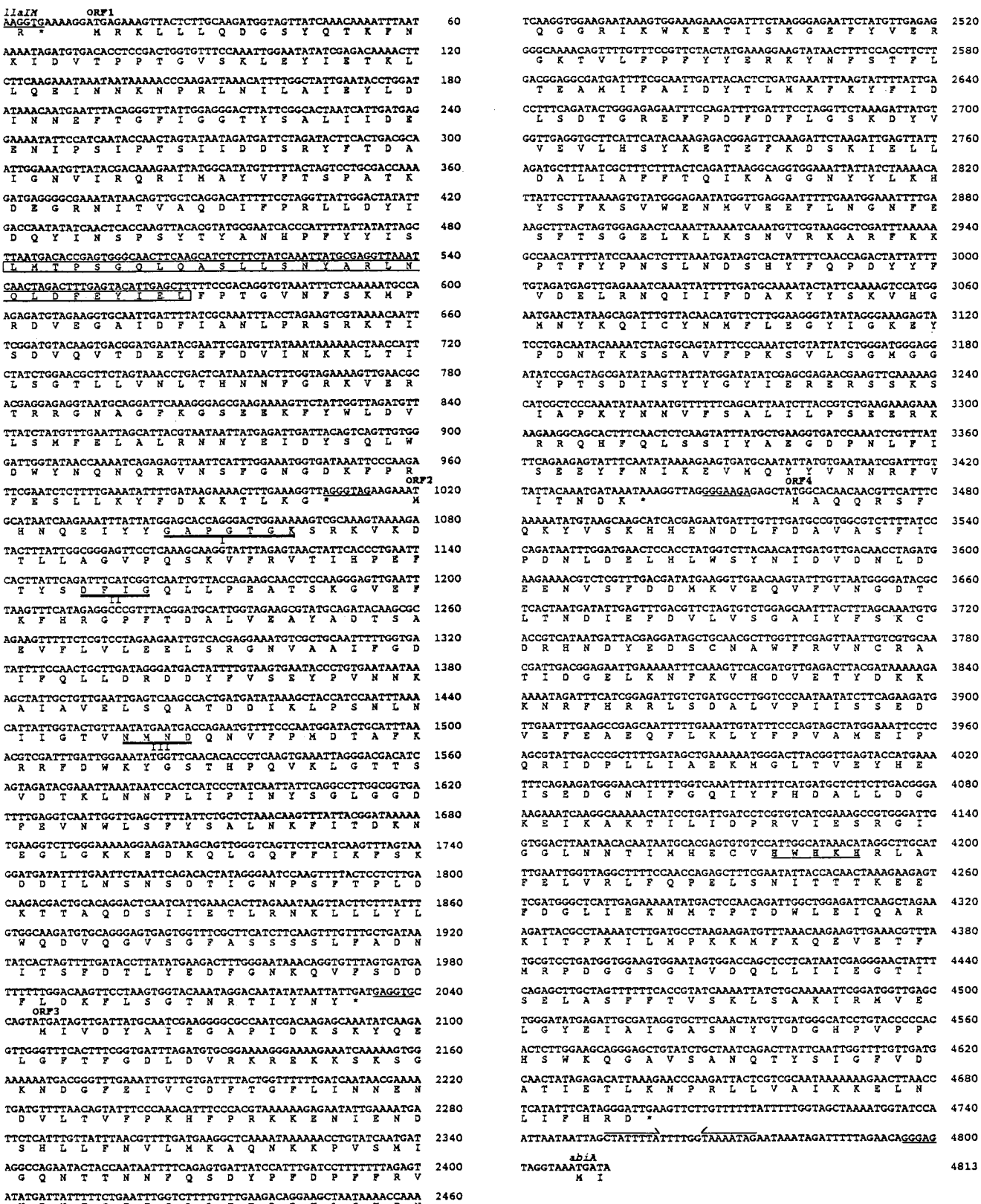

FIG. 1. Nucleotide sequence of the region in pTR2030 between *llaIM* and *abiA*. The deduced protein sequences of four ORFs are shown in single-letter code. A putative ribosome binding sites is underlined prior to the ATG start codon for each ORF. An inverted repeat structure is indicated by opposing arrows at the 3′ end of each sequence. Double underlining represents parts I, II, and III of a GTP-binding motif (9) present in the deduced amino acid sequence for ORF2. A histidine-rich motif in the deduced protein sequence for ORF4 is underlined, and a putative leucine zipper motif in the deduced protein sequence for ORF1 is boxed.

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                I                                          II
LlaI.2:    9 GAPGTGK---SRKVKDTLLAGVPQSKVFRVTIHPEFTYSDFIG   9   GVEFKFHRGPFTDALVEAYADTSA
             G+PG+GK   +R++   L ++ +  +V  V++H +++Y+DFI        GV+F+   G F +   +A  +++
McrB:    207 GPPGVGKTFVARRLAYLLTGEKAPQRVNMVQFHQSYSYEDFIQ   5   GVGFRRKDGIFYNFCQQAKEQPEK
                I

                                                       III
            EVFLVLEELSRGNVAAIFGDIFQLLDRDDYF   23   LPSNLNIIGTVNMNDQNVFPMDTAFKRRFDW 166
            + +++++E++R+N++ +FG+++ L+++D+           +P+N+ IIG++N+ D+++  +D A++RRF +
            KYIFIIDEINRANLSKVFGEVMMLMEHDKRG   18   VPENVYIIGLMNTADRSLAVVDYALRRRFSF 358
                                 II                      III
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FIG. 2. Alignment of three consecutive regions from McrB (10) with the corresponding homologous regions from the deduced protein sequence of ORF2, as determined from a data bank search using the BLASTP program (2). Double underlining represents the three parts of the GTP-binding motif present in the ORF2 deduced sequence and the same motif in McrB, as proposed by Dila et al. (10). See text for details. The number of amino acids in each gap is indicated. Amino acid identities are indicated, and conservative changes are represented by +.

mologous to the glycine-rich P-loop class of ATP/GTP-binding sites which have been detected in many different energy-requiring proteins (39). This site also conforms to the first part of a consensus motif for GTP-binding proteins proposed by Dever et al. (9). The other two parts to this consensus are also present (Fig. 1). The spacing between parts I and III, 131 amino acids, is consistent with the proposed consensus. The spacing between parts I and II is 29 amino acids, which is lower than the consensus value of 40 to 80. However, the positioning of part II relative to parts I and III does vary in some GTP-binding proteins and is thought not to be a critical part of the general consensus (9).

A computer search of the recent data banks at the National Center for Biotechnology Information (National Institutes of Health, Bethesda, Md.), using the BLASTP program (2), did not reveal any significant homologies to the deduced protein sequences for ORF1, ORF3, and ORF4. The deduced protein sequence for ORF2, however, did show homology to three regions of the *E. coli* McrB protein. The three homologous regions are closely linked in both sequences and are depicted in Fig. 2. McrB is one subunit of the GTP-dependent, multisubunit McrBC restriction enzyme (reviewed in reference 34). The homologous regions contain the proposed GTP-binding domains on this protein as depicted by Dila et al. (10). The spacing between parts I and III of this proposed GTP-binding site in McrB is 122 amino acids, which is close to the corresponding spacing in the proposed site in ORF2. It is intriguing that the positioning of part II in McrB differs from the general consensus. The high degree of homology of the ORF2 deduced protein with the proposed GTP-binding region of McrB and its homology to the GTP-binding consensus motif suggest that this region of the ORF2 deduced protein may bind GTP.

A putative leucine zipper motif, which is involved in protein-protein interactions through hydrophobic bonding (31), was noted within the deduced protein sequence for ORF1. Interestingly, the *E. coli* McrC protein also contains a leucine zipper motif (10). The middle leucine of a consensus of five evenly spaced leucines is missing from this motif in ORF1, and this mismatch (asparagine for leucine) is not a conservative change of a hydrophobic amino acid. However, two extra leucine residues just preceding the asparagine residue may possibly have a role in the central part of this putative hydrophobic zipper motif. A recent study indicated that unevenly spaced hydrophobic residues can also function in the formation of protein hydrophobic zippers (14). A DNA-binding basic region, which is generally found upstream of zipper motifs in DNA-binding proteins, is not present upstream of the ORF1 deduced protein motif.

Northern analysis. The sequence organization depicted in Fig. 1 suggested that *llaIM* and the four downstream ORFs were organized in an operon structure of 7.0 kb. To investigate this, total RNA was isolated from *L. lactis* NCK203 and NCK203 containing pTK6. Northern hybridization of these RNAs with an *llaIM*-specific probe or a probe specific to ORF2 hybridized to a large RNA species of ~7 kb (Fig. 3). A probe specific to ORF3 and ORF4 also hybridized to this large RNA species and others (Fig. 4a). A weak background hybridization signal to the 16S and 23S RNAs can occur with some probes. However, RNA from NCK203 did not show any signal above the 2.5-kb position, confirming that hybridization detected in this region with RNA from NCK203(pTK6) was specific to the *llaI* operon. Previous work had demonstrated that *abiA*, which begins 110 bp downstream from ORF4, was transcribed from its own promoter and that its expression was not affected by upstream sequences (17). It is therefore probable that termination of transcription occurs in the untranslated region between ORF4 and *abiA*. Northern analysis of RNA from NCK203 containing pTK6 with an *abiA*-specific probe substantiated this concept, as it hybridized to a smaller RNA species than the probes from the upstream ORF3 and ORF4 (Fig. 4). An inverted repeat which could potentially form a stem-loop structure was identified in the 3′ untranslated region of the sequence in Fig. 1. The free energy value for the formation of this stem-loop structure is −5.6 kcal/mol (ca. −23.4 kJ/mol), and it is therefore unlikely to function as a factor independent transcriptional terminator.

Construction and phenotypic analysis of an *Lla*I R/M subclone. To analyze the *Lla*I phenotype, in isolation from AbiA, *Lla*I was cloned on a 9.0-kb *Sal*I-*Xho*II fragment from pTK6

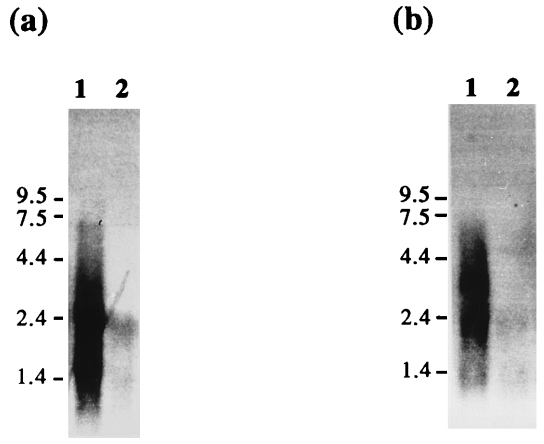

FIG. 3. Northern analysis of total RNA isolated from *L. lactis* NCK203-(pTK6) (lanes 1) and NCK203 (lanes 2) hybridized with an *llaIM*-specific probe (a) and an ORF2-specific probe (b). See Materials and Methods for details of probes used. The molecular masses of RNA standards are indicated in kilobases.

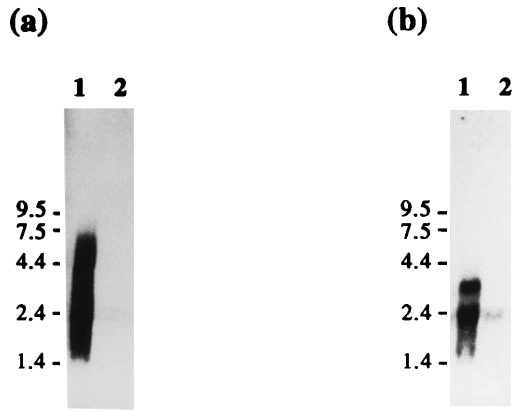

FIG. 4. Northern analysis of total RNA isolated from *L. lactis*(pTK6) (lanes 1) and NCK203 (lanes 2) hybridized with an ORF3- and ORF4-specific fragment (a) and an *abiA*-specific fragment (b). See Materials and Methods for details of probes used. The molecular masses of RNA standards are indicated in kilobases.

TABLE 1. Assay for *Lla*I restriction of phage DNA in *L. lactis* NCK203 hosts

Phage	Plasmid in propagating host	Plasmid in plaquing host	EOP
φ31		pTRKH2	1.0
		pTR2030	$<1.0 \times 10^{-10}$
		pTK6	2.0×10^{-2}
		pTRK370	5.0×10^{-6}
	pTRK370	pTRK370	1.0
φ31.1		pTRKH2	1.0
		pTR2030	5.0×10^{-2}
		pTK6	2.5×10^{-2}
		pTRK370	1.0×10^{-6}
	pTRK370	pTRK370	1.0
	pTR2030	pTR2030	1.0
	pTR2030	pTRK370	8.0×10^{-5}

into pTRKH2. The resulting construct, pTRK370 (Fig. 5), when present in NCK203, exhibited a significantly higher level of restriction against φ31 than did pTK6 (Table 1), even though pTK6 also encodes AbiA. The increased phage resistance level of pTRK370 can be attributed to the copy number of the plasmid; pTRK370 exists in the NCK203 background at ~7 to 10 times more copies than pTK6 (data not shown). Phage φ31.1, an AbiA-resistant derivative of φ31, more accurately reflected the difference in *Lla*I restriction between pTRK370, pTK6, and the original parental plasmid, pTR2030 (Table 1). These data demonstrated that levels of *Lla*I expres-

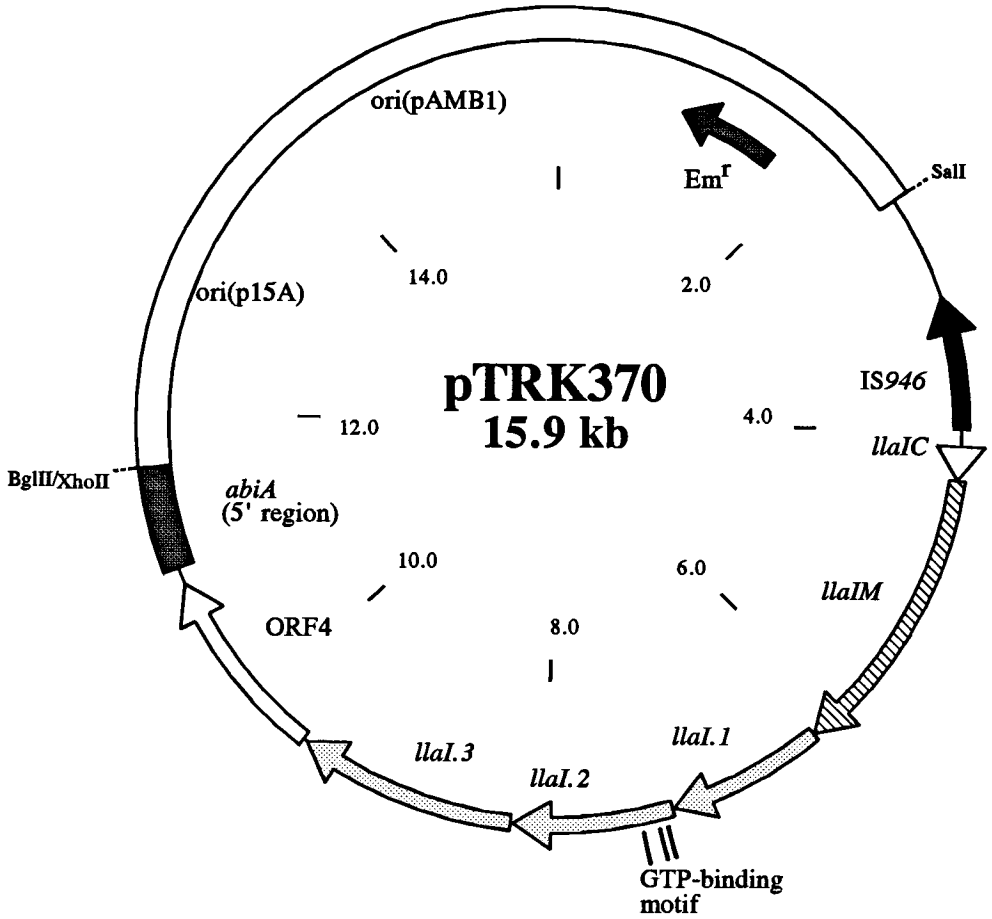

FIG. 5. Schematic representation of the R/M subclone, pTRK370, detailing the organization of the *llaI* operon and upstream IS*946* insertion element, which was described previously (35). The *llaIM* gene encodes a type IIS methylase (18), and *llaI*C encodes a regulatory protein for the *Lla*I R/M system (29). The designations *llaI.1*, *llaI.2*, and *llaI.3* refer to ORF1, ORF2, and ORF3 on the sequence in Fig. 1. See text for description of these genes.

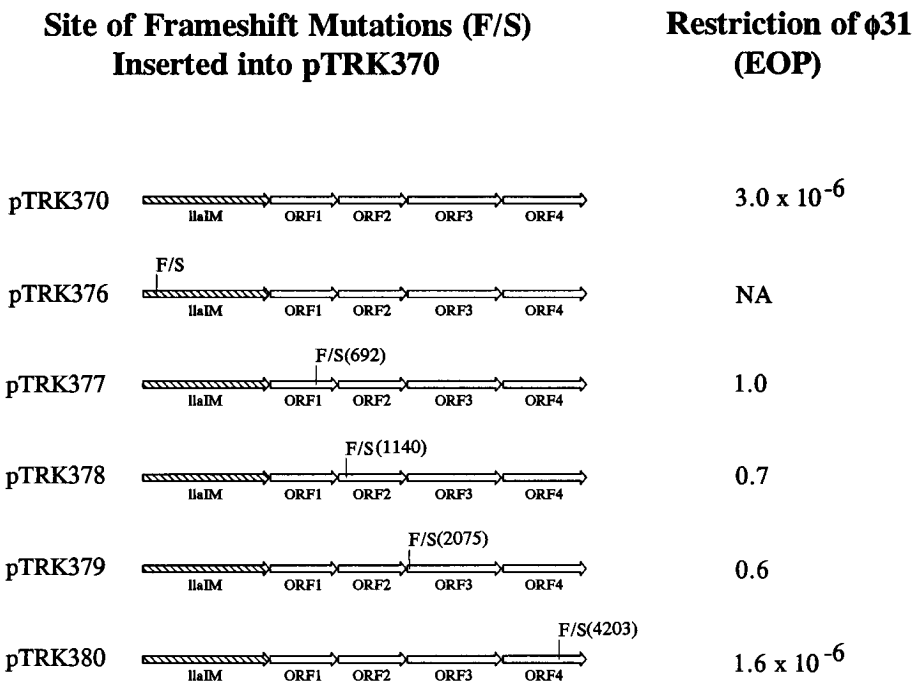

FIG. 6. Evaluation of in vivo *Lla*I restriction of ϕ31 DNA by pTRK370 and derivative mutant constructs in *L. lactis* NCK203. F/S represents the site of the incorporated frameshift mutation, and each number after F/S indicates the base pair position of the mutation according to the sequence in Fig. 1.

sion from pTR2030 and pTK6 are comparable and that the increased restriction from pTRK370 can be attributed to the higher copy number of this plasmid. In contrast, comparing ϕ31 reactions on pTK6 and pTR2030, the AbiA phenotype was surprisingly much higher from pTR2030 than from pTK6. Copy number comparisons showed that pTR2030 was present at slightly fewer copies per cell than pTK6 in this background (data not shown). This result suggested that either the expression of *abiA* is higher from pTR2030 or something else on pTR2030 contributes to the increased level of AbiA-induced resistance.

Full modification of both ϕ31 and ϕ31.1 was obtained following passage through NCK203 bearing pTRK370 (Table 1), confirming that the pTRK370-encoded resistance phenotype is due entirely to the *Lla*I R/M system. Alternatively, passage of ϕ31.1 through NCK203 containing pTR2030 offered only partial protection from *Lla*I restriction by NCK203(pTRK370). These data, therefore, suggest that *Lla*I modification is not an all-or-nothing event and that a greater degree of methylation is required to protect against increased levels of *Lla*I restriction.

Functional characterization of ORF1 to ORF4. To evaluate the contribution of each ORF to the *Lla*I restriction phenotype of pTRK370, translational frameshift mutations were selectively introduced at specific restriction sites (Fig. 6). Analysis of the mutants for *Lla*I restriction activity in the NCK203 background showed that ORF1, ORF2, and ORF3 were important for the restriction phenotype, but a mutation in ORF4 had no apparent effect (Fig. 6). It was noticeable that the mutations in ORF2 and ORF3 did not fully abolish the restriction phenotype, but a variable plaque phenotype (Fig. 7) remained along with a slight reduction in efficiency of plaquing (EOP). *Lla*I-modified phage ϕ31 did not exhibit these reactions on the pTRK370 mutants in ORF2 and ORF3, indicating that the variable plaque phenotype was due to *Lla*I restriction. This phenotype did not occur during restriction of ϕ31 by NCK203(pTRK370), suggesting that it may be a feature of inefficient *Lla*I restriction. A mutation in ORF1 completely abolished all restriction, indicating that it was an integral part of the restriction component of *Lla*I. This observation was further substantiated by an in-frame deletion in ORF1 and ORF2, leaving only ORF3 intact, which fully quenched the restriction phenotype (Fig. 8). A mutation in *llaIM* proved lethal to NCK203. The R^+/M^- construct could not be introduced into this *Lactococcus* background, providing evidence that the *Lla*I restriction component was active in isolation from the methylase component. In addition, translational frameshifts introduced into *llaIM* did not unduly affect translation of downstream ORFs. The *llaIM* mutant of pTRK370 was, however, stable in *E. coli*, suggesting that the restriction components were not expressed or properly assembled in this background. This observation substantiated previous findings that the *Lla*I R/M system encoded on pTK6 offered no protection to *E. coli* from numerous bacteriophages (20).

Deletion derivatives of pTRK370 confirmed that ORF4 had no effect on the in vivo *Lla*I restriction phenotype in the NCK203 background under the conditions used (Fig. 8). However, deletions extending into ORF2 and ORF3 effectively quenched activity to a level similar to frameshift mutations in these ORFs, yielding a variable plaque phenotype and a slight reduction in EOP.

From the combined mutation and deletion analysis, it was concluded that ORF1, ORF2, and ORF3 are intimately involved in the in vivo *Lla*I restriction phenotype. To adopt the accepted nomenclature for restriction endonucleases (44), these ORFs are accordingly designated *llaI.1*, *llaI.2*, and *llaI.3*. Even though ORF4 is part of the *llaI* operon, there is no direct evidence for a role in *Lla*I restriction. A possible role under different conditions or with different phages cannot, however, be ruled out.

DISCUSSION

The conjugative phage resistance plasmid pTR2030 encodes a type IIS methylase, designated M · *Lla*I (18). Its deduced

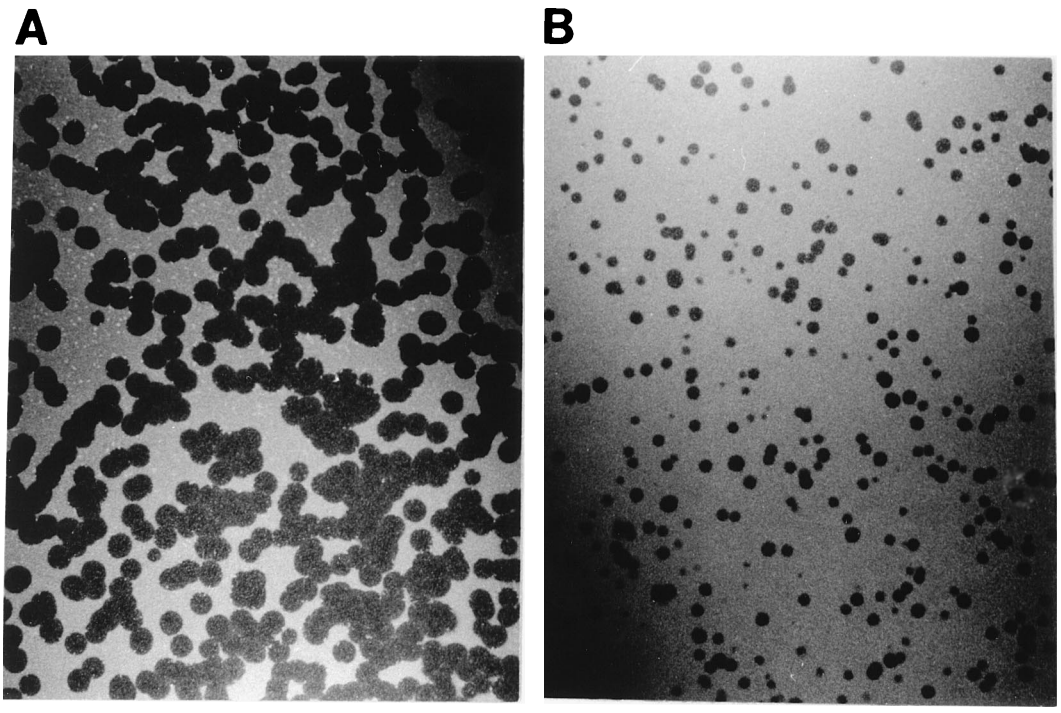

FIG. 7. Plaques of ϕ31 on a lawn of *L. lactis* NCK203 (a) and NCK203(pTRK382; ORF3 mutant) (b). The photographic magnification factor in each case is the same.

protein sequence displays a very high sequence conservation with two other type IIS R/M systems, *Fok*I and *Sts*I. Because the genetic organization and deduced protein sequences of restriction endonucleases *Fok*I and *Sts*I were very similar (25), it was expected that the genetic organization of *Lla*I would show some similarity with these systems. This was not the case, however, as three genes, *llaI.1*, *llaI.2*, and *llaI.3*, were required for *Lla*I restriction, and all were encoded on a large complex operon with *llaIM*.

The mutagenesis and deletion analysis of the cloned *Lla*I R/M system on pTRK370 clearly demonstrated that *llaI.1*, the first downstream ORF from *llaIM* (Fig. 5), was crucial for any restriction activity (Fig. 6 and 8). Deletion or mutagenesis of either *llaI.2* or *llaI.3* almost eliminated the restriction phenotype, but a slight restriction phenotype, primarily manifested by a variable plaque phenotype, remained (Fig. 7). M · *Lla*I-modified ϕ31 did not exhibit this phenotype, establishing that it was due to an inefficient *Lla*I restriction activity (data not shown). These data suggest that the presumptive protein encoded by *llaI.1* is involved at the "cutting edge" of the restriction complex. Without both *llaI.2* and *llaI.3*, however, its efficiency is almost negligible. A pTK6 deletion construct was previously observed to exhibit a similar phenotype (16). From the sequence data in Fig. 1, this construct was deleted in *llaI.2* and downstream DNA; *llaI.1* was the only functional restriction ORF remaining. As the pTRK370 deletion clones are present at ~7 to 10 times more copies than the pTK6 derived clones, it appears that the observed restriction phenotype encoded by *llaI.1* is not increased with a higher copy number of the gene. These data, therefore, suggest that *llaI.2* and *llaI.3* are not required merely to enhance the restriction phenotype of *llaI.1* but rather to achieve full *Lla*I restriction.

Noting the two functional domains within some type IIs methylases, Szybalski et al. suggested by analogy that their cognate restriction endonucleases might have two cutting domains, one for each strand (45). Furthermore, recent evidence on the mechanism of action of the type IIS *Fok*I restriction enzyme suggests that the cleavage functions are separate for both strands (42). In accordance with its probable type IIS origin, it is possible that *Lla*I restricts double-stranded DNA on one strand near its recognition site but cleaves the other strand at a large distance away, thus diverging from the atypical type IIS enzymes. One possible explanation for the phenotype encoded by *llaI.1* in the NCK203 background is that this gene product may restrict only one strand of the phage DNA. Increasing the *llaI.1* gene dosage alone did not result in an improved *Lla*I restriction phenotype. The increase in restriction was obtained only in the presence of intact *llaI.2* and *llaI.3*, suggesting that these genes may be required to enable restriction of the second strand of phage DNA, perhaps at some distance from the recognition site. This latter feature is reminiscent of type I enzymes which require energy to translocate the DNA through the enzyme to enable cleavage at a distant site (reviewed in reference 13). The putative GTP-binding site on *llaI.2* may suggest an energy requirement for translocation of the second strand to enable restriction at the putative distal site. This model would explain the failure to observe specific in vitro cleavage of DNA substrates from extracts of NCK203 (pTK6) (40). It is therefore intriguing to speculate that the *Lla*I endonuclease may have evolutionary origins with both type IIS and type I restriction enzymes. Evidence for an unusually high rate of intraspecific divergence of type I enzyme genes has been provided, and this is speculated to be largely due to the selection pressure imposed by bacteriophage (41).

Data bank searches with the deduced protein sequence of *llaI.2* revealed its three highest homologies with three consecutive regions of the *E. coli* McrB subunit of the McrBC restriction enzyme. As it transpired, these regions corresponded with the three parts of the GTP-binding consensus of Dever et al. (9), present in both *Lla*I.2 and McrB. In vitro evidence has

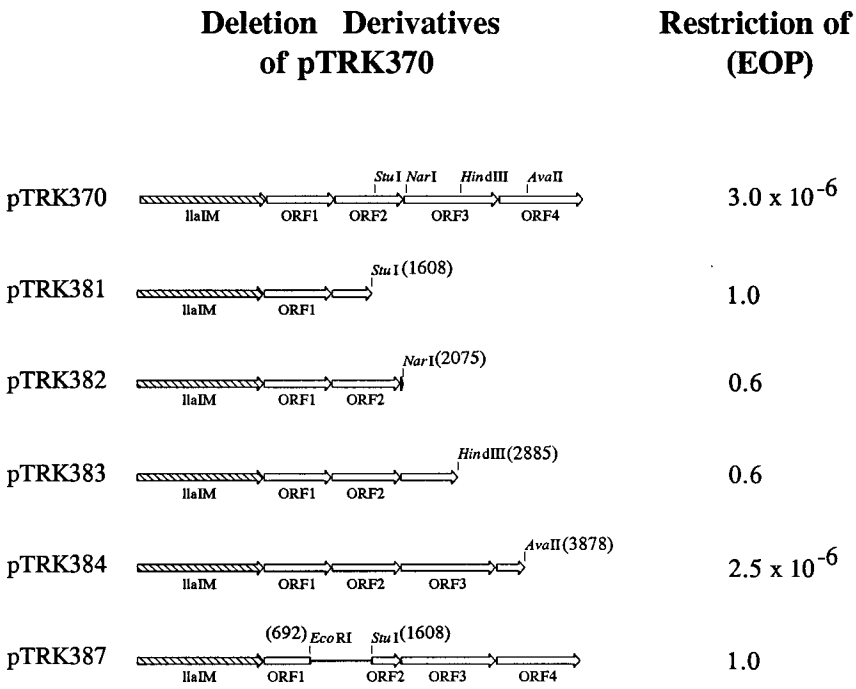

FIG. 8. Representation of EOPs of ϕ31 on *L. lactis* NCK203 hosts harboring pTRK370 and deletion derivatives thereof. The site of the deletion in each construct is represented by the corresponding restriction enzyme site in the unmutated constructed, and the numbers following each deletion refers to the base pair position of the deletion according to the sequence in Fig. 1. The line in pTRK387 represents an in-frame deletion between *Eco*RI and *Stu*I.

shown that McrBC requires GTP for activity, so it is conceivable that the *llaI.2* gene product is a GTP-binding protein in a class similar to McrBC. However, other than a putative leucine zipper motif in *Lla*I.1 and McrC, there are no similarities between *Lla*I and McrBC. The P-loop motif (39) on *Lla*I.2 suggests that it may also be capable of utilizing ATP for energy, whereas ATP inhibits McrBC activity (43). Moreover, McrBC only restricts cytosine-methylated DNA and has no cognate modification enzyme.

The 7-kb *llaI* R/M transcript did not appear to be very stable, as indicated by the Northern analysis (Fig. 3 and 4). The lack of any significant secondary structure in this operon may contribute to its instability, a feature which has been observed previously with other transcripts (7). Large transcripts frequently contain such processing sites which may function to differentially regulate the expression of genes within the transcript (26). Northern hybridization with an *abiA* probe indicated that the downstream *abiA* gene was part of a smaller transcript (Fig. 4b). Indeed, the *abiA* gene hybridized to two distinct RNA species, the largest of which was ~3.5 kb. As the *abiA* gene is <2 kb, it appears it is also part of an operon. The sequence of the 3′ region of the *abiA* gene does not show features reminiscent of a factor-independent transcriptional terminator (17), suggesting that another gene(s) may be transcribed from the *abiA* promoter. The Northern hybridization data in this study substantiate the hypothesis that termination of transcription from the *llaI* operon probably occurs in the 110-bp untranslated region before the start of *abiA*. As features typical of factor-independent terminators are not present in this region, it is conceivable that factor-dependent termination of transcription can occur. While there are as yet no published studies on the 3′ regions of lactococcal transcripts, the characteristic features of factor-independent prokaryotic terminators have been widely noted. A study of the probable transcription termination between the *llaI* operon and *abiA* would, therefore, be valuable to shed light on possible alternative transcription termination mechanisms in lactococci.

The function of the fourth ORF downstream from *llaIM* remains unclear. As part of the same operon with the *llaI* genes, it appears intimately associated with R/M functions. It is conceivable that it has a role in the restriction of some DNA substrates or facilitating restriction under different conditions. However, there is no evidence to link it to a hypothetical role promoting restriction flexibility of *Lla*I. The potential ORF4 gene product does encode a histidine-rich motif (Fig. 1) which is homologous to a motif found in DNA relaxases involved in conjugal transfer. This may suggest a possible role in promoting a high degree of lateral transfer to expedite options for evolutionary divergence of this R/M system. Type I systems rely on intra- and interspecific horizontal transfer for their high rates of recombination-mediated evolutionary divergence (15, 41).

The genetic organization outlined in this study for the *Lla*I R/M system is different from that of any other system studied to date; a comprehensive review on the genetic organization of R/M systems can be found in reference 47. The location of the modification and restriction genes on the same operon is especially unusual. This organization makes the differential regulation of each component very intriguing and raises questions as to how the cell sufficiently modifies itself prior to production of the active restriction component. Translation of *llaIM*, before the restriction genes, obviously gives the modification the initial advantage, especially as three genes need to be translated for production of the active endonuclease. Analysis of the transcripts hybridizing to *llaI*-specific probes (Fig. 3 and 4a) suggests that RNA processing may also play a role in modulating expression of the different *llaI* genes. Differential gene regulation by mRNA processing has been established for different polycistronic operons (12, 26). The *Sal*I R/M system was recently shown to be encoded on a two-gene operon, with the

salIR gene positioned before the *salIM* gene (3). However, a secondary promoter within the operon was found to enable M · *Sal*I to become established prior to the restriction enzyme (3). Secondary transcription starts within the *llaI* operon cannot, therefore, be ruled out. Evidence is also accruing for the regulation of *Lla*I expression by a small regulatory gene (*llaIC* on Fig. 5) located at the 5′ end of *llaIM* (29). Future studies on this regulatory protein, which is analogous to a class of regulatory proteins for type II R/M systems, will provide further insight into the regulation of *Lla*I expression in lactococci.

In conclusion, this study outlines the genetic organization of the genes encoding the cognate restriction endonuclease for the type IIS *Lla*I methylase. Using a classical in vivo assay, we determined that three genes were required to encode *Lla*I restriction and that they were organized in an operon with *llaIM*. This organization clearly shows a marked evolutionary divergence of *Lla*I from the type IIS class of endonucleases. The data presented in this study suggest that it has acquired some characteristics of type I enzymes, raising the possibility that it has evolutionary origins with both these two restriction enzyme classes. Its location on the large conjugative lactococcal plasmid pTR2030 suggests horizontal transfer capabilities which would facilitate this type of evolutionary divergence. Type I enzymes are frequently encoded on conjugative plasmids (4). The location of an IS*946* element directly upstream of the *llaI* operon provides further avenues for mobility of this R/M system. The functionality of this IS*946* element in transposition events has been established (36). A second IS*946* element, positioned ~5 to 10 kb downstream from *abiA* (35), raises the possibility that this R/M system may be part of a composite transposon; analogous insertion sequence elements frequently flank composite transposons (11, 37). The genetic location of this R/M system on pTR2030, therefore, suggests that it encountered ample opportunities for genetic diversification. The strong selective pressure for R/M diversification caused by bacteriophage (41) was also present, as pTR2030 comes from a dairy fermentation background. These factors combined might, therefore, provide some insight as to how an R/M system of a type IIS origin could evolve into a novel system, with characteristics of both type IIs and type I enzymes.

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