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# *Lla*FI, a Type III Restriction and Modification System in *Lactococcus lactis*

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**We describe a type III restriction and modification (R/M) system, *Lla*FI, in *Lactococcus lactis*. *Lla*FI is encoded by a 12-kb native plasmid, pND801, harbored in *L. lactis* LL42-1. Sequencing revealed two adjacent open reading frames (ORFs). One ORF encodes a 680-amino-acid polypeptide, and this ORF is followed by a second ORF which encodes an 873-amino-acid polypeptide. The two ORFs appear to be organized in an operon. A homology search revealed that the two ORFs exhibited significant similarity to type III restriction (Res) and modification (Mod) subunits. The complete amino acid sequence of the Mod subunit of *Lla*FI was aligned with the amino acid sequences of four previously described type III methyltransferases. Both the N-terminal regions and the C-terminal regions of the Mod proteins are conserved, while the central regions are more variable. An S-adenosyl methionine (Ado-Met) binding motif (present in all adenine methyltransferases) was found in the N-terminal region of the Mod protein. The seven conserved helicase motifs found in the previously described type III R/M systems were found at the same relative positions in the *Lla*FI Res sequence. *Lla*FI has cofactor requirements for activity that are characteristic of the previously described type III enzymes. ATP and $Mg^{2+}$ are required for endonucleolytic activity; however, the activity is not strictly dependent on the presence of Ado-Met but is stimulated by it. To our knowledge, this is the first type III R/M system that has been characterized not just in lactic acid bacteria but also in gram-positive bacteria.**

The susceptibility of *Lactococcus lactis* starter cultures to bacteriophage attack is one of the most enduring problems associated with industrial exploitation of such cultures. The most effective approach for combating bacteriophage infection has been based on a combination of a well-controlled fermentation process and the development of starter strains which are highly resistant to bacteriophage attack (8). A great deal of research on lactococci has been focused on identification and characterization of mechanisms that mediate bacteriophage resistance. Detailed characterization should ultimately allow rational construction of strains that exhibit high levels of bacteriophage resistance (20). Four host-directed bacteriophage resistance mechanisms in lactococci have been described. These mechanisms include adsorption inhibition, prevention of phage DNA penetration, host-controlled restriction and modification (R/M), and abortive infection (20).

R/M is the most common phage resistance mechanism found in bacteria. The infection cycle is halted at an early stage with no affect on the viability of the cells. The role of restriction is to cleave any invading DNA which has not been modified at a specific nucleotide sequence by the host methylation system. It is thought that the restriction enzymes in R/M systems confer phage resistance to the producing strains. Cloning of R/M systems into nonprotected strains should permit the construction of dairy starter cultures that exhibit improved phage resistance (31).

The following three distinct types of R/M systems are recognized based on their subunit compositions, cofactor requirements, and modes of DNA cleavage: types I, II, and III (4). Type III is the smallest class of restriction systems and contains only the following four well-studied members: *Eco*P1 from prophage P1 (18) and *Eco*P15 from the prophage P1-related plasmid p15B in *Escherichia coli* (1); *Hin*fIII from *Haemophilus influenzae* (19); and *Sty*LTI from *Salmonella typhimurium* (3). Type III R/M systems require at least two functional genes, *res* and *mod*. Mod is responsible for binding the DNA recognition sequence and also methylates DNA regardless of the presence of Res; Res is required for restriction and is not functional without Mod (4).

In a previous study (39) plasmid pND801 was isolated from *L. lactis* subsp. *lactis* LL42-1, and it was found that pND801 encoded an R/M system. The presence of pND801 in *L. lactis* reduced the efficiency of plating (EOP) of isometric phage $\phi$712 to $10^{-6}$. In this paper, we describe molecular cloning and characterization of the R/M system encoded by pND801 and show that it is a type III R/M system.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and culture conditions.** The strains and plasmids used in this study are listed in Table 1. *E. coli* cultures were incubated at 37°C in Luria-Bertani medium (35) or in M9 minimal medium (35) supplemented with glucose (0.2%, wt/vol). *L. lactis* strains were incubated at 30°C in M17 medium (41) supplemented with 0.5% (wt/vol) glucose. When appropriate, the following antibiotics were added: for *E. coli*, 50 μg of chloramphenicol per ml, 15 μg of tetracycline per ml, and 20 μg of kanamycin per ml; and for *L. lactis*, 5 μg of erythromycin per ml.

**Transposon Tn*5* mutagenesis.** A transposon Tn*5* mutagenesis analysis of cloned DNA segments was carried out by using the methods of de Bruijn and Lupski (11). Phage λ 476 (λ*b*221 *rex*::Tn*5* *c*I857 *O*am29 *P*am80), which was obtained from T. R. Klaenhammer, was used to infect *E. coli* HB101 containing pND805 ($Tc^r$ $Cm^r$ $R^+/M^+$) at a multiplicity of infection of 10. Transformants were selected on Luria-Bertani medium plates containing tetracycline and kanamycin.

**DNA and molecular cloning techniques.** Lactococcal plasmid DNA was isolated by the method of Anderson and McKay (2). Plasmids were isolated from *E. coli* as described by Birnboim and Doly (5). Plasmid DNA was purified by cesium chloride-ethidium bromide density gradient centrifugation (35) and was desalted by dialysis in 1× TE buffer (10 mM Tris, 1 mM EDTA). Restriction digestion and molecular cloning were performed as described by Sambrook et al. (35). Restriction endonucleases and T4 DNA ligase were purchased from Boehringer, Mannheim, Germany, and were used as recommended by the manufacturer.

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TABLE 1. Bacterial strains, plasmids, and bacteriophages

| Bacterial strain, plasmid, or phage | Relevant characteristics[a] | Source or reference |
|---|---|---|
| Bacterial strains | | |
| *L. lactis* subsp. *lactis* | | |
| LL42-1 | Wild type harboring seven plasmids | Gist-Brocades Australia |
| MG1363 | Lac$^-$ Prt$^-$, plasmid-free derivative of *L. lactis* 712 | 14 |
| LM0230 | Lac$^-$ Prt$^-$, plasmid-free derivative of *L. lactis* C2 | 12 |
| *E. coli* HB101 | Pro$^-$ Leu$^-$ Thi$^-$ RecA$^-$, plasmid-free | 6 |
| Plasmids | | |
| pSA3 | 10.2 kb; hybrid vector encoding Cm$^r$ Tc$^r$ (*E. coli*) and Em$^r$ (*L. lactis*) | 9 |
| pND801 | 12 kb; encoding R/M obtained from LL42-1 via coelectroporation | 39 |
| pND805 | 22.2 kb; R/M, pND801 cloned into the pSA3 *Eco*RI site | This study |
| Bacteriophage φ712 | Small isometric-headed phage propagated on LM0230 and MG1363 | 14 |

[a] Lac$^-$, non-lactose fermenting; Prt$^-$, non-proteinase producing; Em$^r$, erythromycin resistance; Cm$^r$, chloramphenicol resistance; Tc$^r$, tetracycline resistance; Pro$^-$, proline requiring; Leu$^-$, leucine requiring; Thi$^-$, thiamine requiring; RecA$^-$, deficient in recombination protein.

DNA fragments were recovered from agarose gels with a QIAEX II gel extraction kit (QIAGEN, GmbH, Hilden, Germany). *L. lactis* was transformed by electroporation as described by Powell et al. (30). For *E. coli*, the $CaCl_2$ transformation method of Dagert and Ehrlich (7) was used without extended preincubation in $CaCl_2$.

**Nucleotide sequencing and analysis.** Both DNA strands were sequenced by using a model 377 DNA sequencer (Applied Biosystems, Foster City, Calif.) as recommended by the manufacturer. Sequencing of the phage-resistant determinant was initiated by using two primers designed on the basis of the sequence of plasmid pACYC184 (GenBank accession no. X06403), which is part of pSA3. Based on the sequences obtained, 20-mer oligonucleotide primers were then synthesized and used to walk along the DNA template. The nucleotide sequence was recorded and analyzed by using the AutoAssembler DNA sequence assembly software (Applied Biosystems) and the ANGIS software system operated by the Australian Genomic Information Center, University of Sydney. Amino acid sequences were compared with all of the sequences in the database by using the BLASTP program. A protein analysis was carried out by using the PEPSTATS program. The nucleotide sequence was searched for −10 and −35 sequences by using the Findpatterns program. A phylogenetic tree was drawn by using the program Growtree in Distances.

**Isolation and partial purification of enzyme extracts.** The method used to isolate and partially purify enzyme extracts was modified from the method described by Sugisaki and Kanazawa (40). The cells in a 5- to 10-ml overnight (16-h) culture were centrifuged at 8,000 × *g* for 5 min at room temperature, washed once with 1 ml of extraction buffer (50 mM Tris HCl [pH 7.6] containing 20 mM $MgCl_2$, 0.1 mM EDTA, and 0.01 M β-mercaptoethanol), and pelleted again by centrifugation at 8,000 × *g* for 5 min at 4°C. The pellet was resuspended in 1 ml of extraction buffer in a microcentrifuge tube. The cells were lysed with 1 g of acid-washed glass beads (diameters, 212 to 300 μm) by intermittent vortexing for 30 s, followed by 30 s on ice to cool the preparation, for 10 min. The microcentrifuge tube was quick-spun to settle the glass beads, and the upper liquid phase was transferred to a fresh tube. Following cell disruption, the cell debris was removed by centrifugation at 16,000 × *g* for 5 min at 4°C, and the supernatant was transferred to a new microcentrifuge tube. Streptomycin sulfate was then added to a final concentration of 1% (wt/vol), and the tube was placed in an ice bath for 30 min. The precipitated nucleic acids were removed by centrifugation (18,000 × *g*, 4°C, 5 min), and the clear supernatant was transferred to a new tube; 5 to 10 μl of this preparation was enough to perform a restriction endonuclease assay. The reaction mixtures (20 μl) contained 0.5 μg of λ DNA, 5 μl of cell extract (in extraction buffer containing 20 mM $Mg^{2+}$), 10 mM ATP with and without 5 μM S-adenosyl methionine (Ado-Met), and reaction buffer B (Boehringer). The ATP and Ado-Met concentrations used were chosen on the basis of similar assays performed by Kauc and Piekarowicz (19). After incubation at 37°C for the times indicated below, the reactions were stopped by heating the mixtures at 65°C for 5 min or by adding gel loading dye, and each mixture was applied to an 0.8% agarose slab gel for electrophoresis.

**Bacteriophage assays.** Both phage titers and cross-streaking were used to evaluate the phage resistance of cultures. Cross-streaking was performed as follows. A sterile cotton bud was dampened with a high-titer phage preparation ($10^9$ PFU $ml^{-1}$) and streaked in a straight line on plates containing M17 medium supplemented with 0.5% (wt/vol) glucose and 10 mM $CaCl_2$. A sterile stick was then used to streak bacterial colonies across the phage. When more accurate measurements were needed, the EOP was determined by plaque counting. The number of PFU was determined by standard plaque assays in which we used an overlay culture of *L. lactis* in M17 medium supplemented with 0.5% glucose and 0.6% agar. The plates were incubated for 24 h at 30°C, and the resulting plaques were counted.

**Nucleotide sequence accession number.** The GenBank accession number for the DNA sequence of the *Lla*FI gene encoding the R/M system from *L. lactis* LL42-1 is AF054600.

## RESULTS

**Cloning of pND801 into pSA3.** Previous work showed that pND801 conferred phage resistance through an R/M system (39). A physical map of pND801 is shown in Fig. 1. To characterize the genetic determinants of the R/M system, two strategies were used. First, pND801 was digested by *Eco*RI to generate three fragments. The three fragments were cloned separately into the *Eco*RI site of pSA3, but none of the fragments expressed phage resistance (Fig. 1). This suggested that the R/M system was inactivated when the DNA was cut with *Eco*RI. pND801 was then partially digested with *Eco*RI to linearize the plasmid and ligated into pSA3 digested with the same enzyme. The ligation mixture was transformed directly into *L. lactis* LM0230, and erythromycin-resistant transformants were screened for phage resistance by cross-streaking against φ712. One of the phage-resistant colonies was purified, and the plasmid harbored by the strain was designated pND805. Restriction analysis of pND805 indicated that the complete pND801 plasmid was inserted into pSA3. Expression

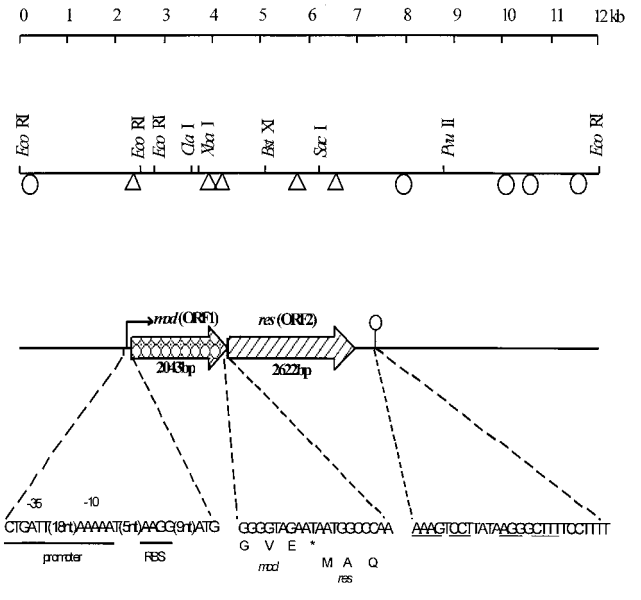


FIG. 1. Genetic organization of the type III R/M system in pND801. The 12-kb plasmid pND801 was cloned into pSA3 to generate pND805, which expressed resistance to φ712. Locations of the Tn*5* insertions in pND805 are shown (triangles, Tn*5* insertions which inactivated phage resistance; ellipses, Tn*5* insertions which did not affect the phage resistance phenotype). The locations of the *mod* gene and the *res* gene, the sequences of the putative promoter and the ribosome-binding site (RBS), and the position of the transcriptional terminator (ellipse with vertical line) are shown. The start codon of the *res* gene overlaps the stop codon of the *mod* gene.

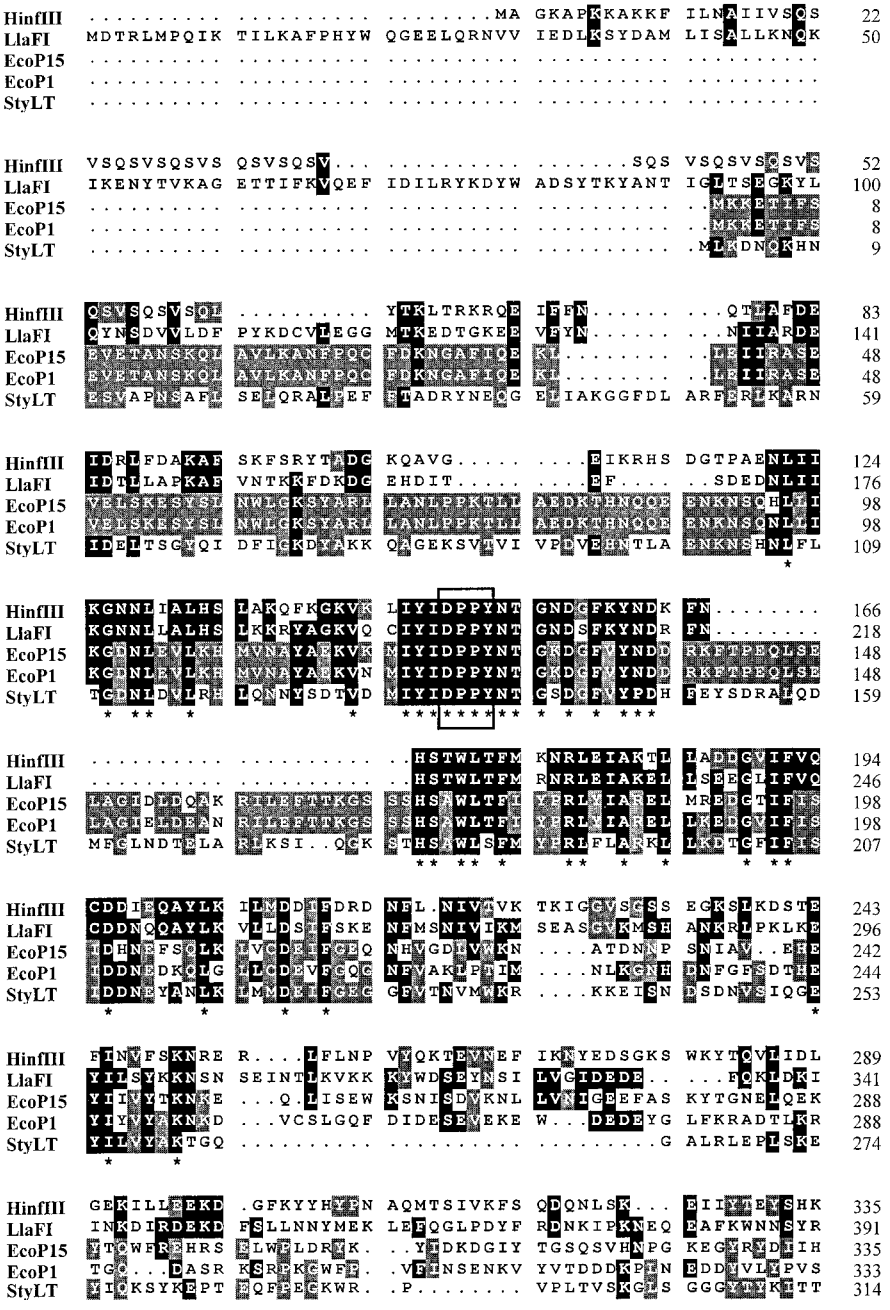


FIG. 2. Multiple alignment of the complete sequences of the Mod proteins of the *Hin*fIII, *Eco*P15, *Eco*P1, *Sty*LT, and *Lla*FI systems. Identity between two or more proteins is indicated by light shading. Darker shading indicates amino acid identity with *Lla*FI (one-letter code). Asterisks indicate completely conserved amino acids. The numbers on the right indicate the position of the rightmost amino acid of each line. The Ado-Met binding site common to all adenine methyltransferases is enclosed in a box.

of the phage resistance encoded by pND805 in *L. lactis* was investigated by challenging transformants that harbored pND805 with phage. pND805 and strains harboring the parent plasmid restricted plating of the isometric ϕ712 to similar extents (EOP, $\approx 10^{-6}$).

**Localization of the genetic determinants encoding the R/M activity of pND805 by Tn*5* mutagenesis.** Plasmid pND805 was introduced into *E. coli* HB101 and subjected to phage λ 476-mediated Tn*5* mutagenesis. Transformants ($Tc^r$ $Km^r$) were harvested, and the plasmid DNA was extracted collectively for electroporation into LM0230. $Em^r$ transformants of LM0230 were tested for phage sensitivity by cross-streaking. Of the 64 individual isolates tested, 30 were resistant to ϕ712 and 34 were sensitive to ϕ712. Plasmid DNA was then isolated from both phage-resistant and phage-sensitive isolates, transformed back into HB101, reisolated, and analyzed by restriction digestion. The positions of the Tn*5* insertions in the cloned fragment of pND805 and the corresponding phage sensitivities of the mutants are shown in Fig. 1. Insertions conferring phage resistance were clustered within a 7-kb region of pND801. We

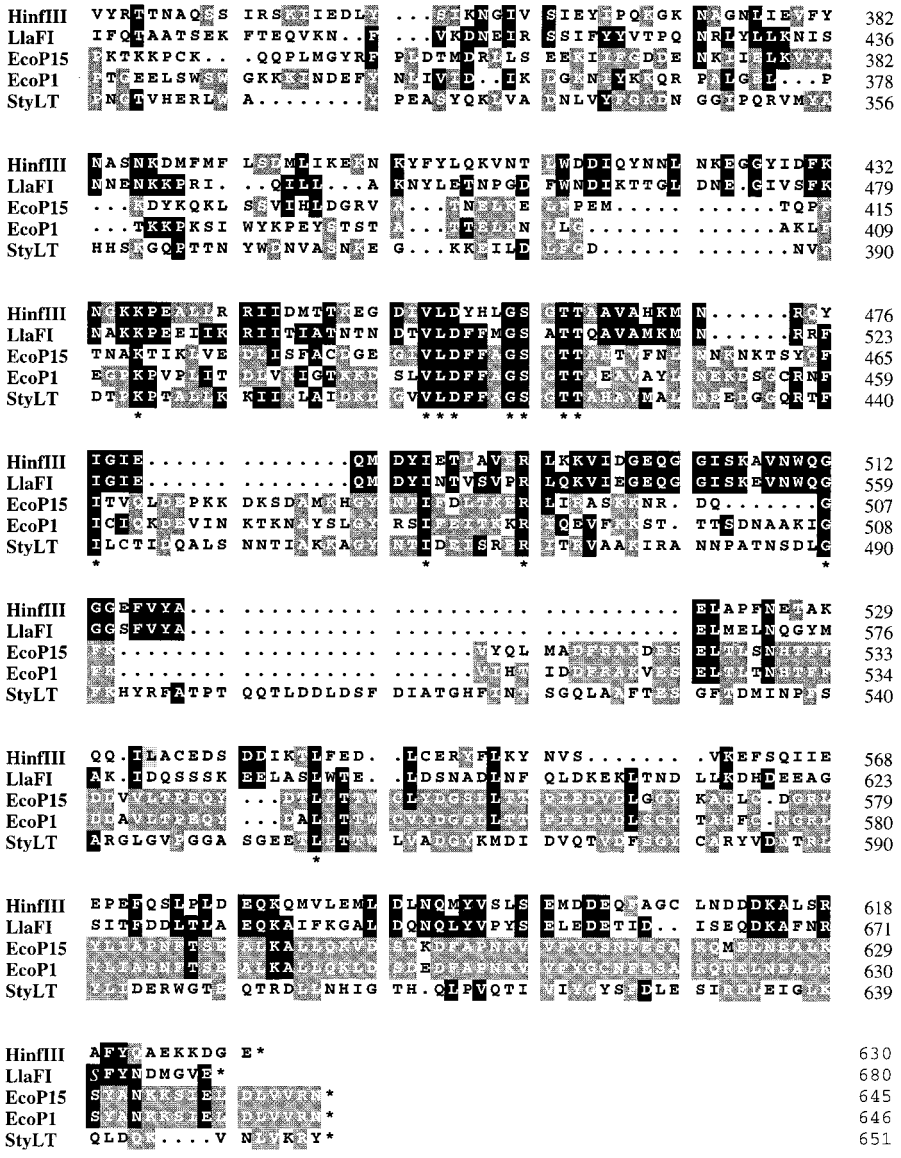


FIG. 2—*Continued.*

concluded that this region is essential for expression of the R/M phenotype.

**Nucleotide sequencing of pND805.** Tn*5* mutagenesis indicated that the region encoding phage resistance included the 0.3-kb *Eco*RI fragment (Fig. 1). This fragment was sequenced first, and based on its sequence more primers were designed and used to sequence the entire DNA region encoding R/M activity. Examination of the sequence revealed the presence of two large open reading frames (ORFs) on the same strand reading in the same direction. The first ORF (ORF1) was 2,043 bp long, began with an ATG start codon, ended at a TAA stop codon, and had the potential to encode a 680-amino-acid protein with a predicted molecular mass of 78,916 Da and an isoelectric point of 4.88. The second ORF (ORF2) began with an ATG codon which overlapped the stop codon of the first ORF by 1 bp (Fig. 1). This ORF was 2,622 bp long and was capable of encoding an 873-amino-acid protein with a predicted molecular mass of 101,630 Da and an isoelectric point of 5.84. The protein encoded by ORF2 contained a higher percentage of basic amino acid residues than the protein encoded by ORF1 contained. Both proteins were hydrophilic, with only a few small hydrophobic regions. No transmembrane regions were found, from which we inferred that the two proteins were located in the cytoplasm.

Examination of the DNA sequence for transcriptional and translational regulatory sequences revealed a putative promoter region upstream of ORF1 (Fig. 1). Nine base pairs upstream from the ATG codon was a sequence (AAGG) that resembled the Shine-Dalgarno sequences that have been reported for *L. lactis* (16). This putative ribosome binding site had a free energy of binding of $-8.4$ kcal mol$^{-1}$ with the 3′ end of the 16S rRNA of *L. lactis*. The nucleotide sequence was compared with $-10$ and $-35$ sequences found in members of the genus *Lactococcus* (16). Upstream of the putative Shine-Dalgarno sequence were putative $-10$ and $-35$ sequences which were similar to consensus *E. coli* and *Bacillus* promoters. Three of six nucleotides in the $-35$ region and four of the six nucleotides in the $-10$ region were the same as the nucleotides

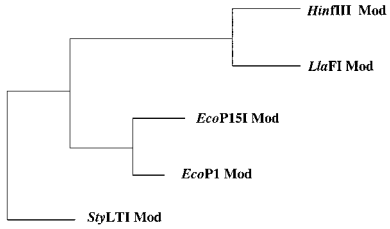


FIG. 3. Evolutionary relationship of the *Lla*FI Mod subunit sequence to previously described type III Mod subunit sequences: Growtree phylogram of Mod sequences determined by using the distance matrix method and the deduced amino acid sequences. Since the phylogram option was used, the branch lengths reflect the calculated distances.

in consensus sequences. Located 306 bp downstream of the stop codon of ORF2 was a region of dyad symmetry that could form a stem-loop structure up to 20 bp long, followed by a run of T residues (TCCTTTT). This region was similar to a rho-independent transcription terminator. Therefore, we assumed that the two ORFs are arranged in an operon.

**Comparative analysis of the amino acid sequences.** BLASTP analysis of the proteins encoded by ORF1 and ORF2 revealed significant homology to the Mod and Res subunits, respectively, of type III R/M systems encoded by *E. coli* plasmid p15B (*Eco*P15), *E. coli* prophage P1 (*Eco*P1), *H. influenzae* Rf (*Hin*fIII), and *S. typhimurium* (*Sty*LTI). Based on this homology, the R/M system of *L. lactis* LL42-1 was designated *Lla*FI in accordance with the nomenclature proposed by Smith and Nathans (38). The complete amino acid sequence of the Mod subunit of *Lla*FI was aligned with the amino acid sequences of the previously described type III methyltransferases (Fig. 2). Both the N-terminal regions and the C-terminal regions of the five proteins were conserved; certain regions scored as high as 71% (74/104) identity, 90% (94/104) similarity with a high probability ($4.2e^{-140}$). The central regions were less homologous, which reduced the overall similarity. The overall levels of identity between the *Lla*FI Mod subunit and the previously described Mod proteins ranged from 25.8 to 38.2%, and the overall levels of similarity ranged from 49.5 to 58.7%. The Asp-Pro-Pro-Tyr motif, a putative Ado-Met-binding motif present in all adenine methyltransferases, was found in the N-terminal conserved region of all five Mod proteins (Fig. 2, box). A phylogenetic tree relating the deduced Mod subunit amino acid sequence to the previously described type III Mod subunit sequences is shown in Fig. 3. *Lla*FI Mod is most closely related to *Hin*fIII Mod.

Using the previously described type III systems, we performed an amino acid homology search by comparing the *Lla*FI Res protein with the two previously described type III Res proteins (*Eco*P1 and *Sty*LTI) (SWISS-PROT database). The Res subunit sequences of *Eco*P15 and *Hin*fIII are not available yet in any of the databases. The levels of similarity between the *Lla*FI Res protein and the previously described Res proteins were 42 to 45%.

**Helicase motifs in *Lla*FI.** The two previously described type III Res proteins contain seven conserved helicase motifs. These motifs were also identified in the Res protein encoded by *Lla*FI. The relative location of each motif was determined and compared to the locations of the motifs found in the Res proteins of *Eco*P1 and *Sty*LTI (Fig. 4). The motifs were searched by performing homology comparisons between the consensus sequences defined by Gorbalenya et al. (15) and the protein encoded by the *res* gene in *Lla*FI. Table 2 shows the actual motif sequences found in the Res subunits of *Lla*FI, *Eco*P1, and *Sty*LTI together with the consensus sequences. A DEAH motif with invariant D (asparate) and E (glutamate) residues, which is characteristic of the DEAD protein family (34), was found in motif II of all three subunits. Most of the conserved amino acid residues were the same in all three enzymes; in a number of cases amino acids were replaced by related amino acids.

**Characterization of the restriction activity of *Lla*FI.** Typically, type III enzymes are ATP and $Mg^{2+}$ dependent but do not have a stringent requirement for Ado-Met. To investigate the cofactor requirements of *Lla*FI, partially purified lysates were obtained from 10-ml overnight cultures of *L. lactis* LM0230 harboring pND805.

The restriction digests obtained in the presence and absence of cofactors are shown in Fig. 5. The pND805 endonuclease required ATP and $Mg^{2+}$ but did not require Ado-Met to breakdown DNA, although the reaction was stimulated by the presence of Ado-Met. Cleavage of λ DNA with the endonuclease encoded by pND805 resulted in a smear of fragments. The control reaction confirmed that this cleavage pattern was the result of endonuclease activity alone, which eliminated the possibility that it may have been due to a contaminating nucleases. Also, cleavage and smearing occurred only in the presence of ATP plus $Mg^{2+}$ and in the presence of ATP plus $Mg^{2+}$ plus Ado-Met. No effort was made to optimize the ATP, $Mg^{2+}$, and Ado-Met concentrations in this study. The incomplete digestion observed, which resulted in some DNA remaining almost full length, may be attributed to simultaneous methylase activity (19).

The results of the restriction enzyme assays indicated that the properties of the endonuclease encoded by pND805 are different from the properties of type I and type II enzymes and are identical to the properties of type III enzymes.

## DISCUSSION

On the basis of the nucleotide and deduced amino acid sequences and the homologies with the other type III *mod* genes and amino acid sequences, it appears that the R/M system encoded by pND805 is a type III R/M system. The Mod protein from pND805 is similar to the four other type III Mod proteins. Both the N-terminal regions and the C-terminal regions of the five proteins are partially conserved, while the central portions exhibit less homology. This is consistent with the hypothesis (17) that the central domain probably confers sequence specificity, while the two distal conserved blocks presumably are involved in Ado-Met binding interactions and in transmethylation reactions. This type of organization, consist-

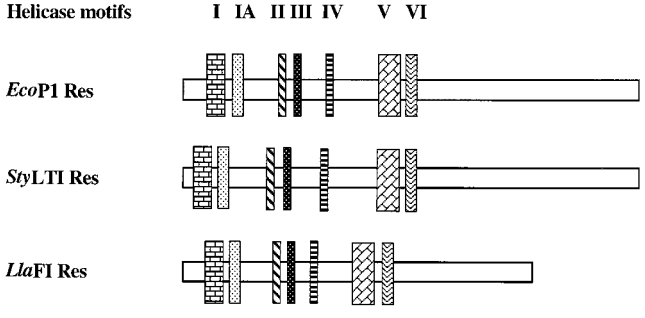


FIG. 4. Relative positions of the seven helicase motifs in the Res proteins of the *Eco*P1, *Sty*LTI, and *Lla*FI systems. The sizes of the genes are indicated by the sizes of the horizontal rectangles, and each vertical rectangle represents a helicase motif. ▩, ▨, ▧, ▩, ▬, ▩, and ▨, motifs I to VI, respectively.

FIG. 5. Agarose gel electrophoresis of λ DNA digested with partially purified enzyme preparations from LM0230(pND805), showing the effects of $Mg^{2+}$, ATP, and Ado-Met on pND805 endonuclease activity. Lane 1, λ *Hin*dIII standard marker; lanes 2 to 5, preparations containing partially purified enzyme extract from LM0230(pND805); lanes 6 to 9, preparations containing extract from LM0230(pSA3); lanes 2 and 6, extract plus $Mg^{2+}$; lanes 3 and 7, extract plus $Mg^{2+}$ plus ATP; lanes 4 and 8, extract plus $Mg^{2+}$ plus ATP plus Ado-Met; lanes 5 and 9, extract plus $Mg^{2+}$ plus Ado-Met. The reaction mixtures were incubated for 1 h at 37°C.

TABLE 2. Seven conserved helicase motifs found in the *Eco*P1, *Sty*LTI, and *Lla*FI Res subunits

| R/M subunit | Helicase motifs[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | I | IA | II | III | IV | V | VI |
| *Eco*P1 Res | IDVS**METGTGKT**YTYTk | NK**FIIIVPT1SI**KAGT | **FIIIDEpH** | IIRY**GATFS** | TLF**FIDdIEG** | **FIFSKWTLREGWDNPNVFQIC** | STT**SKLQEVGRGLR** |
| *Sty*LTI Res | IdVK**METGTGKT**YVYTr | FK**FVLVVPTpAI**KEGA | **VVIIDEpH** | IIrY**gATFS** | tLF**FIDdIEG** | **FIFsKWTLREGWDnPNVFVIA** | SES**SKIQEVGRAvR** |
| *Lla*FI Res | LLFN**MATGSGKT**MVMAS | QN**FIFLVnTdAI**IKKT | **VILGDEAH** | LLEF**SATIN** | TVE**sLVKYLK** | **VIFAvAKLNEGWDVLNLYDIV** | ATD**SEAQLVGRGAR** |
| Consensus | +++ **tg GKT** + | +++++**p r** + | **++++DEah** | ++++**sat** | +++**f t** | +++++**t** + **g** + + + ++ | **q** +**GR**+**ar** |

[a] Boldface type indicates amino acids which are either completely conserved or replaced by similar amino acids. Plus signs represent hydrophobic amino acid residues. The consensus residues determined by Gorbalenya et al. (15) are indicated.

ing of alternating conserved and variable regions, is also found in type I and type II methylases (32).

It seems to be a general phenomenon that the genes encoding the two subunits found in type III R/M systems overlap to some extent or are close to each other. Start codon ATG of the type III *res* gene found in *L. lactis* overlaps the stop codon of the *mod* gene, TAA, by 1 bp (A). The genes lie in the same direction of transcription in the order *mod-res*. *Eco*P1 and *Sty*LTI, like *Lla*FI, comprise two close ORFs that are 2 to 12 bp apart in the same order and orientation (37). This observation suggests that the genetic organization of the new R/M system is similar to the genetic organization of other type III R/M systems.

The Res subunits of type I and type III R/M systems exhibit low levels of sequence similarity and contain seven sequence motifs that are characteristic of DNA and RNA helicases belonging to superfamily II (15). The seven helicase motifs described previously for the *Eco*P1 and *Sty*LTI Res proteins are also found, at the same relative positions, in the *Lla*FI Res sequence. Helicase motifs play a classical role in unwinding of DNA during repair and recombination. Recently, several researchers have suggested that the helicase motifs identified in both type I and type III R/M systems are also involved in translocation of DNA (21, 23, 25).

The endonuclease encoded by *Lla*FI required ATP and $Mg^{2+}$, which is a characteristic of other type III restriction endonucleases (19). Like other type III systems, the R/M system encoded by pND805 did not require Ado-Met to break down DNA, but the reaction was stimulated by the presence Ado-Met (33, 44). Restriction reactions and modification reactions have been found to be competing reactions in the presence of ATP and Ado-Met because once a recognition site has been modified, it can no longer be cleaved (19). The role of Ado-Met in methylase binding is not clear, but it is thought that Ado-Met plays a role in the affinity of the enzyme for binding to a specific DNA sequence. The methylase binds to specific and nonspecific sequences, and it has been suggested that the presence of ATP greatly helps in discrimination of

these sequences (1). Type III systems characteristically recognize DNA sequences that are asymmetric, uninterrupted, and five or six nucleotides long. Cleavage occurs approximately 25 to 27 nucleotides downstream from the recognition sequence. Only one strand of the recognition site is methylated (44). Cleavage takes place only when two unmodified sites are present in the DNA in inverse orientations (4).

Cleavage and smearing were observed only in the presence of ATP plus $Mg^{2+}$ and in the presence of ATP plus $Mg^{2+}$ plus Ado-Met. The smearing, which implied that incomplete digestion occurred, and the finding that some DNA remained nearly full length may be attributed to simultaneous methylase activity (19). Type III enzymes rarely completely digest DNA, even in the absence of Ado-Met, for reasons that are not clear (4).

The existence of most R/M systems in lactococci has been deduced from the results of phage restriction studies. However, four systems have been characterized by enzyme purification and characterization of the DNA target (13, 22, 26, 27) or by cloning and sequencing of the corresponding genes (10, 24, 27–29, 42, 43). Three of these systems are type II systems, and one (29), comprising three genes associated with restriction activity and a type IIs methylase, has not been classified. Recently, type I R/M systems have been found on lactococcal plasmids (36). Here we describe an example of the least common class of R/M systems (type III) in *L. lactis*. The existence of all three types of R/M systems in lactococci demonstrates that many lactococci have developed collections of defense mechanisms, which presumably resulted from constant exposure to phages. The variety of defense mechanisms provides excellent biological material for constructing phage-resistant strains for the dairy industry.

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