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Cloning and Sequencing of *Lla*II Restriction/Modification Genes from *Lactococcus lactis* and Relatedness of This System to the *Streptococcus pneumoniae Dpn*II System

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The natural 7.8-kb plasmid pSRQ700 was isolated from *Lactococcus lactis* subsp. *cremoris* DCH-4. It encodes a restriction/modification system named *Lla*II. When introduced into a phage-sensitive *L. lactis* strain, pSRQ700 confers strong phage resistance against the three most common lactococcal phage species, namely, 936, c2, and P335. The *Lla*II endonuclease was purified and found to cleave the palindromic sequence 5′-GATC-3′. It is an isoschizomer of *Streptococcus pneumoniae Dpn*II. The plasmid pSRQ700 was mapped, and the genetic organization of *Lla*II was localized. Cloning and sequencing of the entire *Lla*II system allowed the identification of three open reading frames. The three genes (*llaIIA*, *llaIIB*, and *llaIIC*) overlapped and are under one putative promoter. A putative terminator was found at the end of *llaIIC*. The genes *llaIIA* and *llaIIB* coded for m^6A methyltransferases, and *llaIIC* coded for an endonuclease. The *Lla*II system shares strong genetic similarities with the *Dpn*II system. The deduced amino acid sequence of M.*Lla*IIA was 75% identical with that of M.*Dpn*II, whereas M.*Lla*IIB was 88% identical with M.*Dpn*A. However, R.*Lla*III shared only 31% identity with R.*Dpn*II.

Lactococcus lactis is used extensively worldwide in the manufacture of fermented dairy products (16). *L. lactis* cultures are normally inoculated into pasteurized or heat-treated milk to quickly start and control the fermentation. In this nonsterile milk environment, the added cells come into contact with the wild bacteriophage population that has survived pasteurization (7, 15, 20, 37). Although the natural phage concentration is low, their population can increase very rapidly if phage-sensitive cells are present in the starter culture. The consequent lysis of a large number of sensitive cells will retard the fermentation process. To cope with this natural phenomenon, the dairy industry has developed a series of solutions including the use of phage-resistant *L. lactis* strains (16).

In the last decade, extensive research has been conducted on interactions between lactococcal phages and their hosts (for a review, see reference 16). *L. lactis* was found to possess many plasmids coding for natural defense mechanisms against bacteriophages. More than 40 plasmids with phage defense barriers have been identified (16). Phage resistance systems are classified into three groups on the basis of their mode of action: blocking of phage adsorption, restriction/modification (R/M), and abortive infection. Phage-resistant *L. lactis* strains have been constructed by introducing these natural plasmids into phage-sensitive strains (55). The conjugative abilities of some of these plasmids were exploited to construct such resistant strains (14, 16, 23, 55, 61). However, after considerable industrial use of these strains, new phages capable of overcoming the introduced defense mechanism have emerged (1, 17, 41). Thus, the search for different natural phage barriers is still an ongoing objective for starter manufacturers.

Over the years, several studies have established the heterologous nature of the lactococcal phage population (for a review, see reference 22). On the basis of electron microscopy and DNA hybridization studies, 12 different lactococcal phage species have been identified (22). It has been proposed that the number be reduced to 10 because of the possible reclassification of the 1483 and T187 species into the P335 species (21, 51). Strong DNA homology is observed among members of the same species, but no homology is found between species (3, 22, 40, 48, 49). Although many species have been isolated, only three appear to be problematic for the dairy industry. Species 936 (small isometric head) and c2 (prolate head) have been, by far, the most disturbing lactococcal phage species worldwide (3, 40, 48, 49). Interestingly, phages from the P335 species (small isometric head) are now being isolated with increasing frequency from North American dairy plants (41). Two recent surveys in North American dairy plants revealed that all lactococcal phages isolated (45 phages) could be classified within one of these three species (39, 40). Therefore, from a practical point of view, industrial *L. lactis* strains should be resistant at least to the three most common phage species, namely, 936, c2, and P335. Because of the diversity of lactococcal phages, the need for phage defense mechanisms with broad activity (attacking many species) is becoming more meaningful. Because of their nature, R/M systems have the potential to fulfill this objective.

The phenomenon of R/M was first reported more than 40 years ago (33) and received a molecular explanation 10 years later (2, 12). The main biological activity of R/M systems is believed to be in degrading invading DNA. These gatekeepers are roughly the prokaryotic equivalent of the immune system (62). There are currently more than 2,400 known restriction enzymes, and over 100 have been cloned and sequenced (50, 52). There are several kinds of R/M systems, and they appear to have equivalent biological activities that are achieved in different ways. At least five types of R/M systems have been identified: I, II, IIs, III, and IV (for reviews, see references 2, 18a, 62, and 63). Of these, type II is the simplest and the most

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TABLE 1. Bacterial strains and bacteriophages used

Bacterial strain or phage	Relevant characteristics[a]	Source[b]
L. lactis subsp. *cremoris*		
DCH-4	Industrial strain, multiple plasmids, Lac^+	This study
UL8	Industrial strain, host for P335 phages, Lac^+	40
SMQ-87	UL8(pSRQ701); Lac^+ Em^r	This study
L. lactis subsp. *lactis*		
LM0230	Plasmid free, host for 936 and c2 phages; Lac^-	38
SMQ-16	LM0230(pSA3); Lac^- Em^r	This study
SMQ-17	LM0230(pSA3, pSRQ700); Lac^- Em^r	This study
SMQ-39	LM0230(pSRQ701); Lac^- Em^r	This study
SMQ-40	LM0230(pSRQ702); Lac^- Em^r	This study
SMQ-50	LM0230(pSRQ703); Lac^- Em^r	This study
SMQ-117	LM0230(pSRQ704); Lac^- Em^r	This study
SMQ-140	LM0230(pSRQ706); Lac^- Em^r	This study
E. coli		
DH5α	Transformation host	Gibco/BRL
SMQ-149	DH5α(pSRQ708); Ap^r	This study
Phages		
φp2	Small isometric headed, 936 species, 30.5 kb	L. L. McKay
φsk1	Small isometric headed, 936 species, 28.1 kb	L. L. McKay
φjj50	Small isometric headed, 936 species, 30.5 kb	24
φc2	Prolate headed, c2 species, 20.7 kb	54
φml3	Prolate headed, c2 species, 20.2 kb	W. E. Sandine
φeb1	Prolate headed, c2 species, 19.6 kb	L. L. McKay
φul36	Small isometric headed, P335 species, 28.8 kb	40
φQ30	Small isometric headed, P335 species, 37.0 kb	39
φQ33	Small isometric headed, P335 species, 29.6 kb	39

[a] Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Em^r, erythromycin resistance; Lac, lactose-fermenting ability.
[b] L. L. McKay, University of Minnesota; W. E. Sandine, Oregon State University.

common. Many R/M systems have been characterized at the protein level. Restriction enzymes are generally dissimilar, suggesting an independent evolution and the absence of a common ancestor (2, 62, 63). In contrast, extensive similarities occur among the methyltransferases (MTases) (2, 25, 31, 36, 62, 63). They can be grouped into three classes corresponding to the modification types: m^4C, m^5C, and m^6A (for a review, see references 62 and 63). m^4C and m^6A can be further divided into two (α and β) and three (α, β, and γ) subclasses, respectively, on the basis of their amino acid sequences (25, 31).

A number of plasmids encoding for R/M have been identified in *Lactococcus* spp. (for a review, see reference 16). Surprisingly, only a handful have been partially characterized. The *Lla*I R/M system encoded on the conjugative plasmid pTR2030, isolated from *L. lactis* subsp. *lactis* ME2, was the first analyzed at the sequence level (17). The methylase gene of the pTR2030 system has been sequenced, and the deduced protein was found to share similarities with the type IIs MTase (class m^6A), M.*Fok*I (17). The endonuclease genes have also been sequenced, and four open reading frames were identified (46). Only the first three open reading frames were involved in the *Lla*I activity. Recent data have provided evidence for a new class of multisubunit endonucleases (46). The restriction complex, however, has yet to be purified, and its recognition sequence is unknown.

*Scr*FI was the first classical type II restriction enzyme isolated from *L. lactis* and is the only one available commercially (13). *Scr*FI recognizes the sequence 5′-CCNGG-3′, where N is any of the nucleotides. Two methylase genes from the *L. lactis* subsp. *lactis* UC503 chromosome have been cloned and sequenced (9, 58). They both coded for an m^5C MTase. The endonuclease gene has yet to be identified. Mayo et al. (35) isolated a type II endonuclease (also named *Lla*I) from *L. lactis* subsp. *lactis* NCDO497 which recognized the sequence 5′-CCWGG-3 (W is A or T), but the R/M genes have not been cloned. Recently, Nyengaard et al. (44) isolated two plasmid-encoded type II endonucleases from *L. lactis* subsp. *cremoris* W9 and W56 which are isoschizomers of *Mbo*I and *Sfc*I. The molecular organization of these two R/M systems is still unknown.

This report describes the isolation and characterization of plasmid pSRQ700 from *L. lactis* subsp. *cremoris* DCH-4. This 7.8-kb plasmid encoded an R/M system, named *Lla*II, which conferred strong resistance against phages of the 936, c2, and P335 species. The *Lla*II endonuclease was isolated, and this type II enzyme recognized the sequence 5′-GATC-3′. It is an isoschizomer of *Dpn*II and *Mbo*I. All of the *Lla*II genes were cloned and sequenced, and the gene order was established. The molecular organization showed two methylases and one endonuclease. The *Lla*II methylases are highly homologous to *Dpn*II methylases. To our knowledge, *Lla*II is the first R/M system from *L. lactis* for which recognition site and genetic structure are known.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The strains and plasmids used in this study are listed in Tables 1 and 2. *Escherichia coli* was grown at 37°C in Luria-Bertani broth (53). *L. lactis* strains were grown at 30°C in M17 (57) supplemented with 0.5% glucose (GM17) or 0.5% lactose (LM17). When appropriate, antibiotics were added as follows: for *E. coli*, 50 μg of ampicillin per ml, 10 μg of tetracycline per ml, and 20 μg of chloramphenicol per ml; for *L. lactis*, 5 μg of erythromycin per ml.

Bacteriophage propagation and assays. The bacteriophages used in this study are listed in Table 1. Bacteriophages were propagated and titrated by the method of Jarvis (19). Efficiency of plaquing (EOP) assays were performed as described by Sanders and Klaenhammer (54). Bacteriophages c2, p2, sk1, and jj50 were supplied by T. R. Klaenhammer (North Carolina State University).

DNA isolation and manipulation. Plasmid DNA from *E. coli* was isolated as described previously (42). Large quantities of *E. coli* plasmid DNA was isolated with a Qiagen (Chatsworth, Calif.) plasmid Midi or Maxi kit. Plasmid DNA from *L. lactis* was isolated as described by O'Sullivan and Klaenhammer (45). Large quantities of lactococcal plasmid DNA were obtained by the Leblanc and Lee

TABLE 2. Plasmids used in this study

Plasmid	Relevant characteristics[a]	Source or reference
pSA3	Shuttle vector, Cm^r Tc^r Em^r, 10.2 kb	8
pBS KS(+)	Cloning vector for sequencing, Ap^r, 2.9 kb	Stratagene
pSRQ700	Resident plasmid of DCH-4, R^+/M^+, 7.8 kb	This study
pSRQ701	7.0-kb *Eco*RI fragment from pSRQ700 cloned into pSA3; R^+/M^+ Cm^s Tc^r Em^r	This study
pSRQ702	5.3-kb *Nco*I-*Eco*RI fragment from pSRQ700 cloned into pSA3; R^-/M^+ Cm^s Tc^r Em^r	This study
pSRQ703	6.6-kb *Nco*I fragment from pSRQ700 cloned into pSA3; R^-/M^+ Cm^s Tc^r Em^r	This study
pSRQ704	7.8-kb *Eco*RV fragment from pSRQ700 cloned into pSA3; R^+/M^+ Cm^r Tc^s Em^r	This study
pSRQ706	3.0-kb *Nru*I-*Eco*RV fragment from pSRQ700 cloned into pSA3; R^+/M^+ Cm^r Tc^s Em^r	This study
pSRQ708	3.0-kb *Nru*I-*Eco*RV fragment from pSRQ700 cloned into pBS; R^+/M^+ Ap^r	This study

[a] Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Cm^s, chloramphenicol sensitive; Em^r, erythromycin resistance; Tc^r, tetracycline resistance; Tc^s, tetracycline sensitive; R^+/M^+, active restriction/active modification enzymes.

procedure (32) as modified by Gonzalez and Kunka (13a). Restriction endonucleases (Gibco/BRL, Grand Island, N.Y.) and T4 DNA ligase (Boehringer Mannheim, Indianapolis, Ind.) were used as described in the manufacturer's instructions. When needed, DNA fragments were obtained from low-melting-point agarose with a Qiaex gel extraction kit.

Electroporation. *E. coli* was grown, electroporated, incubated, and plated as described previously (42). *L. lactis* was grown in GM17 supplemented with 0.5 M sucrose (SGM17) and 1% glycine and electroporated as described by Holo and Nes (18). The Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, Calif.) was set at 25 μF and 2.45 kV, and the pulse controller was set at 200 Ω. Plasmid DNA was mixed with 40 μl of cells in a chilled cuvette (0.2 cm). After electroporation, *L. lactis* cells were resuspended in SGM17, incubated for 2 h at 30°C, plated on GM17 supplemented with erythromycin (5 μg/ml), and incubated for 2 days at 30°C.

Sequencing. The entire *Lla*II system (3-kb *Nru*I-*Eco*RV fragment from pSRQ700) was cloned into *E. coli* pBluescript. The resulting clone was named pSRQ708. Nested deletions were made in both orientations from pSRQ708 with an ERASE-A-BASE kit (Promega, Madison, Wis.). For the first set of deletions, the exonuclease III digested the inserted DNA from a 5′ protruding *Xba*I site. The adjacent sequencing primer site was protected from digestion by a 4-base 3′ overhang *Sst*I site. The restriction pair *Kpn*I-*Dra*II was used to obtain the nested deletions in the other orientation. Plasmid DNA was extracted from the nested clones with Qiagen and used directly for sequencing. The sequencing reactions were performed with the DyeDeoxy Terminator Taq sequencing kit for use on the model 373A automated DNA sequencing system (Applied Biosystems, Foster City, Calif.). The T7 and T3 primers were used for annealing.

Restriction enzyme purification. *L. lactis* SMQ-17 was grown in 2 liters of GM17, concentrated by centrifugation (10,000 rpm, 15 min), and washed twice in saline. The cells were then resuspended in 30 ml of PME buffer (10 mM NaH_2PO_4 [pH 7.4], 0.1 mM EDTA, 10 mM β-mercaptoethanol). Cells were lysed by 15 bursts (30 s each, followed by a 1-min rest) with glass beads and a bead beater (BIOSPEC, Bartlesville, Okla.). After centrifugation to remove cell debris and glass beads, the supernatant (crude extract) was used for digestion (see Fig. 2) and for ion-exchange chromatography. Successive chromatographies were performed on phosphocellulose (Whatman P11) and DEAE cellulose (Whatman DE52) with a salt gradient in PME buffer. Restriction endonuclease activity was found in the fractions around 0.5 M NaCl. Lactococcal phage ul36 DNA (40–42) was used as the substrate, and the digestions were performed at 37°C for 2 to 4 h with the following buffer: 50 mM Tris-HCl [pH 8.0]–10 mM $MgCl_2$–50 mM NaCl. DNA samples were analyzed as described by Sambrook et al. (53) in 0.7% agarose gels in TAE (0.04 M Tris-acetate, 0.001 M EDTA).

DNA and protein analysis. The DNA sequence was analyzed with the Genetics Computer Group (Madison, Wis.) sequence analysis software. The SwissProt (release 29, June 1994) sequence was searched for homology to all three deduced *Lla*II amino acid sequences.

Nucleotide sequence accession number. The GenBank accession number of the nucleotide sequence of *Lla*II system is U16027.

RESULTS

Isolation of pSRQ700. For many years, *L. lactis* subsp. *cremoris* DCH-4 has performed very well during industrial buttermilk and sour cream production. One reason for continued good performance is the natural resistance of DCH-4 to lactococcal bacteriophages. The objective of this study was to identify and understand the mechanism(s) responsible for the phage resistance of DCH-4. The total plasmid DNA of DCH-4 was isolated and coelectroporated with the vector pSA3 into the phage-sensitive, plasmid-free strain *L. lactis* LM0230. The latter strain was selected because it can propagate phages from two species, 936 and c2. The DNA ratio of DCH-4/pSA3 used for electroporation was about 10:1. Erythromycin-resistant colonies were tested for phage resistance by spot assay (10^3 to 10^4 PFU of φp2 per spot). A few phage-resistant colonies were obtained, analyzed, and found to contain pSA3 and a 7.8-kb plasmid which was named pSRQ700 (Fig. 1). The transformant containing pSRQ700 was named *L. lactis* SMQ-17. Plasmid pSRQ700 was also electroporated into *L. lactis* UL8, which can propagate phages from P335 species. The transformant was named *L. lactis* SMQ-87.

Effectiveness of pSRQ700 on lactococcal phage species. *L. lactis* SMQ-17 and SMQ-87 were tested for phage resistance against a total of nine phages belonging to three species (three phages per species). Phages p2, sk1, and jj50 were selected as representatives of the 936 species (Table 1). The lactococcal phage species c2 was represented by the phages c2, ml3, and eb1. These six phages were tested individually on SMQ-17, and their EOPs are presented in Table 3. The new, emerging P335 species was represented by the phages ul36, Q30, and Q33. They were tested separately on SMQ-87, and their EOPs are also presented in Table 3. All three 936 phages had similar EOPs in the range of 10^{-6}. More variability was observed with the c2 species, where EOPs ranged from 10^{-3} to 10^{-4}. The P335 phages were the most affected by pSRQ700, since EOPs of 10^{-7} were observed (Table 3). Identical results were obtained when phage resistance was tested at 21, 30, and 38°C (data not shown). These results indicated that the phage resistance mechanism encoded on pSRQ700 is temperature insensitive.

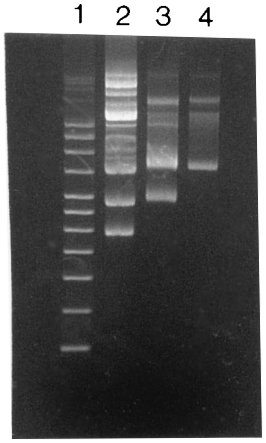

FIG. 1. Plasmid analysis of *L. lactis* strains. Lanes: 1, supercoiled DNA ladder (Gibco/BRL); 2, *L. lactis* DCH-4; 3, *L. lactis* SMQ-17(pSA3, pSRQ700); 4, *L. lactis* SMQ-16(pSA3).

TABLE 3. Comparison of the EOPs of lactococcal phages and the numbers of *Mbo*I sites in the phage genomes

Phage	EOP	No. of *Mbo*I sites[a]
936 species[b]		
φp2	1.7×10^{-6}	11
φsk1	2.5×10^{-6}	9
φjj50	2.0×10^{-6}	10
c2 species[b]		
φc2	1.0×10^{-4}	3
φml3	6.1×10^{-3}	2
φeb1	5.5×10^{-3}	2
P335 species[c]		
φul36	2.7×10^{-7}	13
φQ30	5.2×10^{-6}	12
φQ33	1.3×10^{-7}	15

[a] Only the number of fragments that were >0.5 kb was determined.
[b] The EOP of the 936 and c2 phages was tested on *L. lactis* SMQ-17. The EOP of these phages is 1.0 on *L. lactis* LM0230.
[c] The EOP of the P335 phages was tested on *L. lactis* SMQ-87. The EOP of these phages is 1.0 on *L. lactis* UL8.

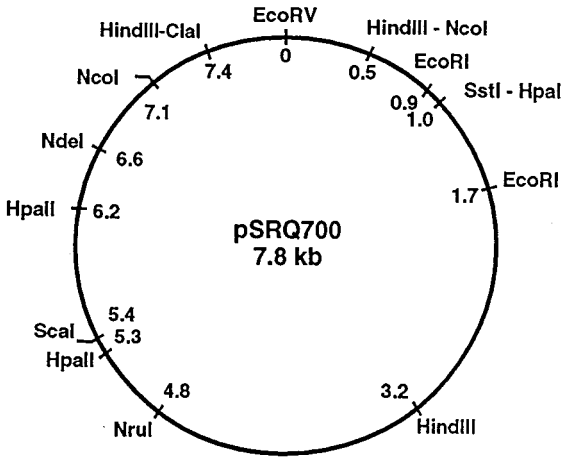

FIG. 3. Restriction map of lactococcal plasmid pSRQ700. Site positions are indicated in kilobases.

Identification of the phage resistance mechanism on pSRQ700. Phages capable of overcoming the defense mechanism encoded on pSRQ700 were isolated. These phages had EOPs of 1.0 on *L. lactis* SMQ-17. When these resistant (modified) phages were propagated back on their original host, they became sensitive (restricted) to pSRQ700 at the same previous level (data not shown). This temporary host-specific immunity demonstrates the presence of a classical R/M system encoded on pSRQ700. The R/M system was named *Lla*II.

Isolation of the restriction endonuclease. DNA from the well-characterized lactococcal phage ul36 (40–42) was digested with *Lla*II crude extract. The digestions were conducted overnight at 37°C since the R/M encoded on pSRQ700 is temperature insensitive (up to 38°C). Defined DNA fragments were identified on agarose gels with the crude extract without any purification steps (Fig. 2). Unexpectedly, the DNA restriction pattern obtained corresponded to the *Mbo*I restriction pattern (Fig. 2). Nevertheless, the nonspecific nucleases were removed after ion-exchange chromatographies were performed on phosphocellulose (Whatman P11) and DEAE cellulose (Whatman DE52) with a salt gradient in PME buffer (data not shown). No attempts were made to determine the number of activity units in the collected fractions nor the percentage of recovery from the crude supernatant. Attempts to cut pSRQ700 with *Mbo*I were unsuccessful. It was concluded that the R/M system present on pSRQ700 was similar to the *Mbo*I system which recognized the sequence 5′-GATC-3′.

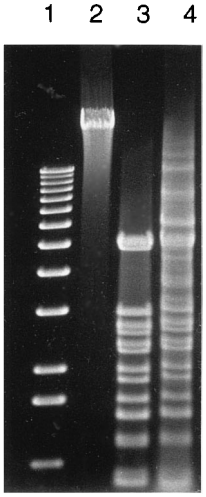

FIG. 2. Digestion of lactococcal phage ul36 DNA. Lanes: 1, 1-kb ladder (Gibco/BRL); 2, uncut ul36 DNA; 3, ul36 DNA cut with *Mbo*I; 4, ul36 DNA cut overnight at 37°C with 1 μl of crude extract from SMQ-17 (*Lla*II).

Mapping of pSRQ700. Single, double, and triple digestions were performed with endonucleases to obtain a map of pSRQ700. The results are presented in Fig. 3. The following endonucleases did not cut pSRQ700: *Apa*I, *Ava*I, *Ava*II, *Bal*I, *Bam*HI, *Hpa*I, *Mbo*I, *Pst*I, *Pvu*II, *Sal*I, *Sma*I, *Sph*I, *Xba*I, and *Xho*I.

Localization of the *Lla*II system on pSRQ700. The *Lla*II R/M system was cloned entirely into *E. coli* by use of the *E. coli-L. lactis* shuttle vector pSA3 (Fig. 4). Since appropriate unique restriction sites were present on pSA3 and pSRQ700, total plasmid DNA from *L. lactis* SMQ-17 was used directly for cloning. Plasmid DNA from SMQ-17 was digested with selected endonucleases, phenol extracted, ethanol precipitated, and ligated, and the ligation mixture was electroporated in *E. coli* DH5α. This strategy was very effective because expected clones were obtained rapidly. The clones were electroporated into *L. lactis* LM0230, and phage resistance was determined. The relevant clones with deleted regions are presented in Fig. 4. The entire R/M system of pSRQ700 was localized on a 3-kb *Nru*I-*Eco*RV fragment. The pSA3 clone containing this 3-kb fragment was named pSRQ706. Similar EOPs were obtained with pSRQ700 and pSRQ706 (Fig. 4).

DNA sequence analysis of the *Lla*II system. The 3-kb *Nru*I-*Eco*RV fragment containing the *Lla*II genes was sequenced in both directions and found to contain 2,987 bp (Fig. 5). This fragment was 65.6% A+T rich, typical of lactococcal genes (60). Three overlapping open reading frames were found, and the genes were named *llaIIA*, *llaIIB*, and *llaIIC*. The gene *llaIIA* was localized from position 97 to position 948 and coded for a protein of 284 amino acids with an estimated mass of 33,032 Da. The gene *llaIIB* was localized from position 941 to position 1747 and coded for a protein of 269 amino acids with an estimated mass of 30,904 Da. The gene *llaIIC* was localized from position 1740 to position 2651 and coded for a protein of 304 amino acids with an estimated mass of 34,720 Da. An EOP of 1.0 for phage p2 on *L. lactis* harboring pSRQ702 or pSRQ703 suggested that *llaIIC* coded for the endonuclease

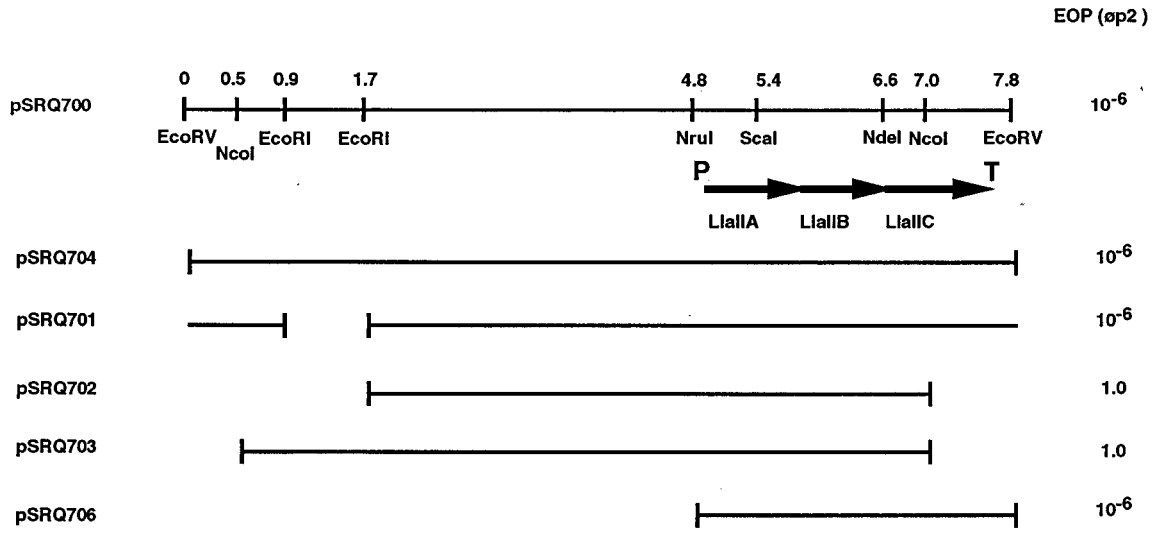

FIG. 4. Cloning of *Lla*II from pSRQ700 into pSA3. Clones were electroporated into LM0230. Transformants were tested for phage resistance against φp2.

(Fig. 4). No putative ribosome binding site (RBS) was found for *llaIIA* and *llaIIB*. A putative RBS (AAAGGAG) was found preceding *llaIIC*. Atypical RBSs have been identified for the *Dpn*II methylases which are similar to those of *Lla*II (Fig. 5). They were not found in the *Lla*II system. Atypical RBSs may be related to translational control of the methylase gene expression (30). All three genes appear to be under the control of the same promoter. However, no definite consensus *E. coli* −10 and −35 promoter sequences could be identified. Because EOPs were the same in pSRQ700, pSRQ701, and pSRQ706 (Fig. 4), it is believed that the promoter was present in the 3.0-kb fragment. A search for palindromic sequences identified two perfect inverted repeats of 19 bp, typical of a strong rho-independent terminator, at the very end of *llaIIC* (Fig. 5). Interestingly, the stop codon of *llaIIC* was within the beginning of the stem-loop structure. Finally, three GATC sites were found in the 3-kb fragment from pSRQ700. Since this plasmid is not cut by *Mbo*I, this provides specific evidence that the GATC sites are protected by the *Lla*II methylases.

Protein analysis. Homology searches showed that the deduced protein coded by *llaIIA* was 75.4% identical to *Dpn*II methylase (34), 41.5% identical to *Mbo*I methylase (59), and 30.1% identical to the Dam methylase of *E. coli* (4). It was concluded that *llaIIA* codes for a methylase, which was named M.*Lla*IIA. All three methylases (M.*Dpn*II, M.*Mbo*A, and Dam) homologous to *Lla*IIA are N-6 adenine MTases (m^6A MTases). The most conserved amino acid sequence motifs among the m^6A MTases are F-G-G (motif I) and DPPY (motif II). Their organization in the protein allowed the division of the m^6A MTases into three subclasses (α, β, and γ). In the m^6A MTase subclass α, the motif I is found close to the N terminus, followed by a variable region of 100 to 200 amino acids, and the motif II is close to the C terminus. The reverse situation is found in the subclass β, where the motif II appears before the motif I. M.*Lla*IIA has all the characteristics of a subclass α m^6A MTase: an F-G-G motif, a 146-amino-acid variable region, and a DPPY motif (Fig. 6). The F-G-G motif probably contained the *S*-adenosylmethionine binding site, and DPPY might be involved in the methylation of exocyclic amino acids (25). The deduced protein coded by *llaIIB* was found to be 88.9% identical to the second methylase of *Dpn*II (5), 50.2% identical to the second methylase of *Mbo*I (59), and 43.6% identical to the *Hin*fI methylase (6). It was concluded that *llaIIB* also codes for a methylase, which was named M.*Lla*IIB. All three methylases (M.*Dpn*A, M.*Mbo*C, and M.*Hin*fI) homologous to *Lla*IIB are m^6A MTases but of subclass β. M.*Lla*IIB has all the subclass β characteristics: a DPPY motif, a 175-amino-acid variable region, and an F-G-G motif. Interestingly, Fig. 6 also showed amino acid comparisons between two sets of four m^6A MTases isolated from two gram-positive and two gram-negative bacteria. These enzymes methylate the same 5′-GATC-3′ sequence. Despite the various origins, about 20 and 28% of the amino acids are conserved, respectively, in the four α and β methylases studied. Interestingly, almost all tryptophan residues are conserved in the methylases studied (Fig. 6).

The deduced protein coded by *llaIIC* was 34 and 31% identical to *Mbo*I (60) and *Dpn*II (11) endonucleases, respectively. These results confirmed that *llaIIC* coded for an endonuclease, which was named R.*Lla*II. Conserved amino acid motifs were observed among the three isoschizomers, but their functionality is unknown.

DISCUSSION

L. lactis subsp. *cremoris* DCH-4 harbors a 7.8-kb plasmid (pSRQ700) coding for a temperature-insensitive R/M system similar to *Dpn*II (30) and *Mbo*I (59). These systems recognize the nonmethylated DNA sequence 5′-GATC-3′, where the endonuclease cleaves before the guanine (30, 59). The plasmid pSRQ700 is probably one reason for the strong phage resistance shown by DCH-4 over the years. Any phage containing the nonmethylated GATC sequence in its genome will be restricted when infecting an *L. lactis* strain containing pSRQ700.

Members of the three most common lactococcal phage species were strongly restricted by pSRQ700 as shown by their reduced EOPs (Table 3). The small-isometric-headed phages of the P335 and 936 species were particularly affected by pSRQ700. This is due in part to their larger genomes. The average genome sizes for the P335, 936, and c2 phages used in this study were 31.8, 29.7, and 20.2 kb, respectively. However, the most important factor was the number of *Lla*II sites in the phage genome. Three *Lla*II sites in the prolate φc2 genome were enough to restrict its EOP by 4 logs on *L. lactis* SMQ-17

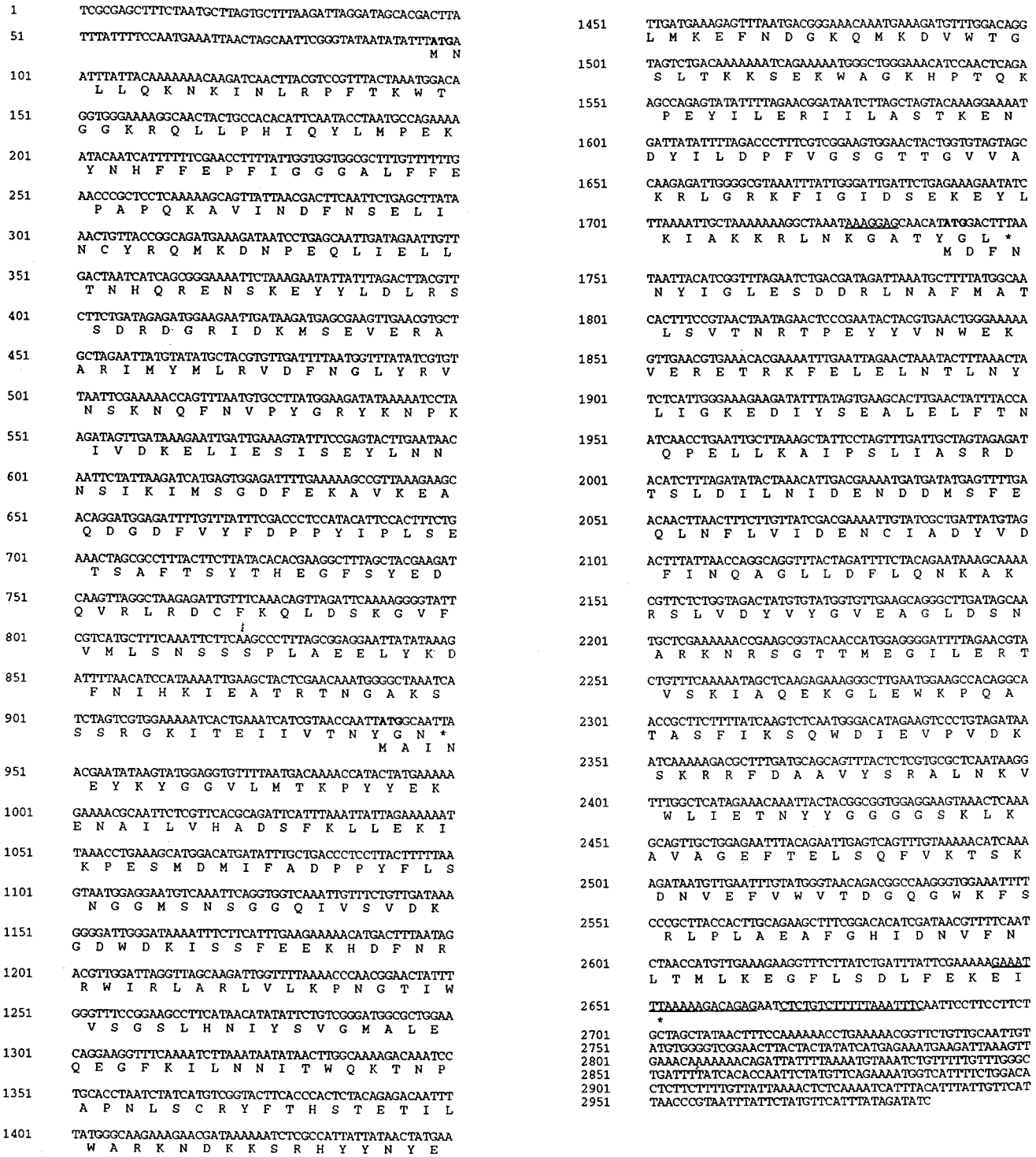

FIG. 5. Nucleotide sequence of the 3-kb *Nru*I-*Eco*RV fragment from pSRQ700. The deduced amino acid sequence of the three open reading frames is presented. The putative terminator and RBS are underlined. The first codon of each open reading frame is in bold.

(Table 3). Two *Lla*II sites in the ϕml3 and ϕeb1 genomes were still enough to reduce the EOP by 3 logs. These data are in agreement with the single-hit kinetic of the R/M system and shows that restriction at one site is enough to prevent phage proliferation (63). For the small isometric phages which had more *Lla*II sites in their genome, the presence of 9 to 12 sites gave a 6-log reduction in EOP, whereas 13 to 15 sites were needed for a 7-log reduction. As reported previously, the EOP decreases logarithmically as the number of sites in the phage genome increases (63).

One criterion that should be addressed while studying the phage resistance mechanism is its potential long-term effec-

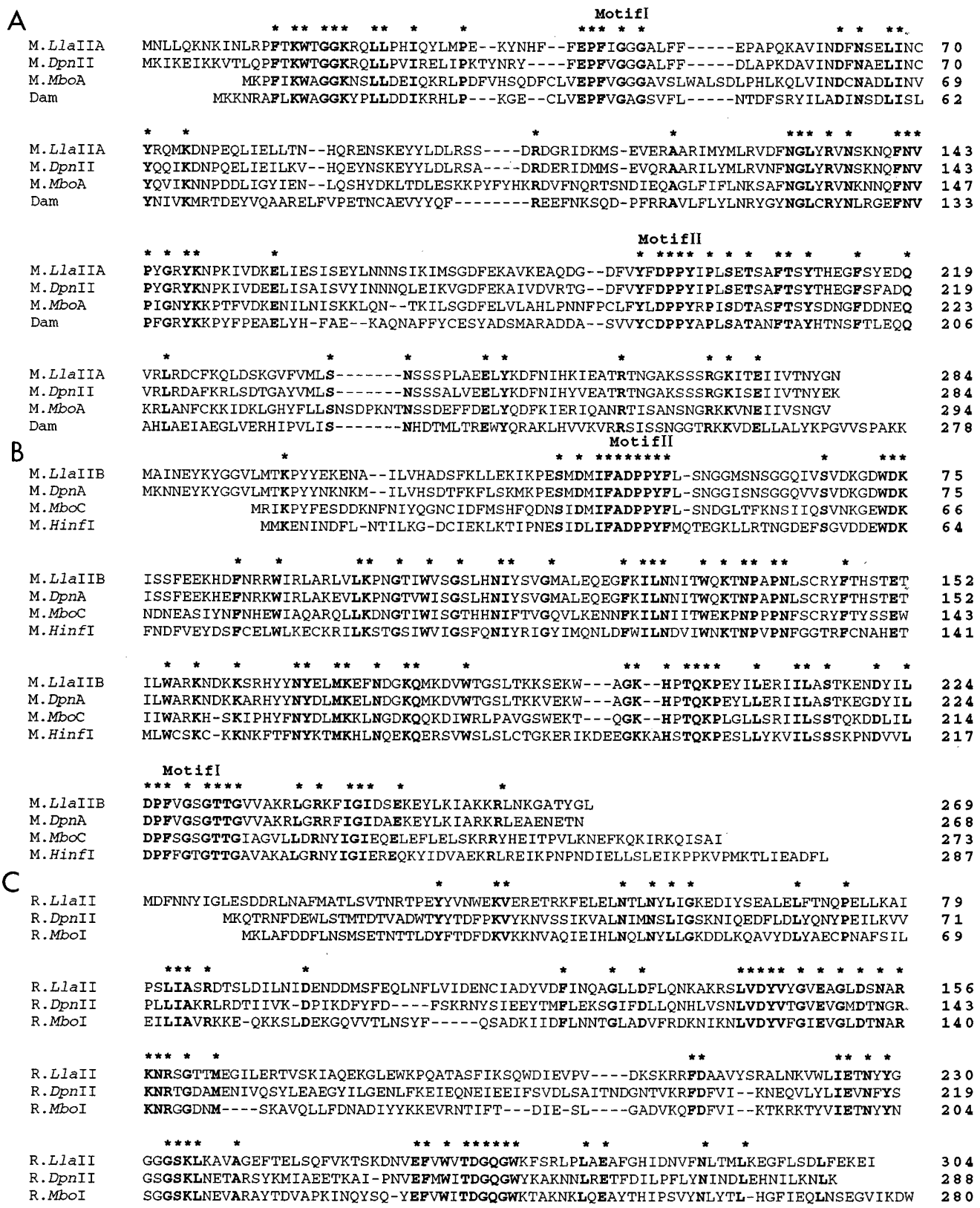

FIG. 6. Comparison of amino acid sequences between M.*Lla*IIA, M.*Dpn*II, M.*Mbo*A, and *E. coli* Dam methylases (A); M.*Lla*IIB, M.*Dpn*A, M.*Mbo*C, and M.*Hin*fI (B); and R.*Lla*II, R.*Dpn*II, and R.*Mbo*I (C). Asterisks indicate conserved amino acids. Dashes indicate gaps in the aligned sequences.

tiveness. It is widely believed that phages will evolve in response to pressure from host R/M systems. Phage antirestriction systems have been identified in many bacterial genera (26, 56). The elimination (by base mutation) of restriction sites is a likely phage evolutionary mechanism in response to such restrictive pressure (26, 56). Lactococcal phages characterized at the DNA level have already shown a paucity of restriction sites for many endonucleases purified from other bacterial genera (3, 22, 40, 41, 48, 49). However, phage resistance conferred by the *Lla*II R/M system encoded on pSRQ700 was still substantial against members of the three lactococcal phage species tested. Even if in situ activity of *Lla*II is related to promoter strength and transcription or translation efficiencies, trends were established between the effectiveness of pSRQ700, the number of *Lla*II sites in the phage genome, and the EOPs. Obviously, the higher the number of *Lla*II sites in the phage genome, the lower the EOP and the higher the phage resistance conferred by pSRQ700. However, when the number of genomic *Lla*II sites is low, as for prolate phages, the elimination of one *Lla*II site would reduce the efficacy of pSRQ700 by about 1 log. In contrast, when the number of *Lla*II sites is high (>10), as for small isometric phages (936 and P335 species), the elimination of one or two sites would have no influence on pSRQ700 effectiveness (Table 3). Thus, the *Lla*II R/M system encoded on pSRQ700 could potentially be very effective against small isometric phages for a fairly long period.

Close gene linkage is a feature of all R/M systems, and accordingly, *Lla*II genes are adjacent to each other (62, 63). The *Lla*II system is highly related to *Dpn*II (30). They share the same genetic structure, i.e., two methylase genes followed by an endonuclease gene (10). There is also gene overlapping in both systems. The most striking similarity is their methylases (5, 34). Amino acid comparisons showed 75% identity between M.*Lla*IIA and M.*Dpn*II and 88% between M.*Lla*IIB and M.*Dpn*A (Fig. 6). The M.*Dpn*II is a typical modification enzyme that methylates double-stranded DNA (34). M.*Dpn*II is an essential part of the *Dpn*II system which is involved in the restriction of infection by double-stranded DNA phages (30). Therefore, M.*Lla*IIA probably has an identical function in the *Lla*II system. The primary biological function of M.*Dpn*A is, interestingly, different (30). Indeed, M.*Dpn*A is active not only on double-stranded DNA but also on single-stranded DNA (5). It appears that DpnA is involved in facilitating the natural transfer of plasmid DNA between strains (30). In *Streptococcus pneumoniae*, transformed plasmid DNA enters the cell as a single strand, the other strand being degraded at the cell surface (30). Since a linear single strand of a plasmid cannot circularize and replicate itself, two complementary plasmid strands entering separately must interact to establish a plasmid (30). M.*Dpn*A would methylate the entering single-stranded DNA, and consequently, the newly formed double-stranded DNA would be protected from *Dpn*II endonuclease activity. Since M.*Dpn*A is highly homologous (88%) to M.*Lla*IIB, the same single-strand DNA methylation might be present in *L. lactis*. Interestingly, *L. lactis* DCH-4 harbors a 30-kb self-conjugative plasmid (data not shown). It is possible that single-stranded intermediates might be involved during conjugation in *L. lactis*.

The *Lla*II and *Dpn*II endonucleases also resemble each other, but to a much lesser extent than the methylases. Only 31% of the amino acids are identical between these two endonucleases. Interestingly, the *Lla*II endonuclease is slightly more homologous to *Mbo*I (34%) than to *Dpn*II. The homologous regions are probably involved in endonuclease specificity. This type of similarity is unusual among type II endonucleases that catalyze identical reactions (63). Although the evidence clearly suggests that the *Lla*II and *Dpn*II methylases have a common ancestor, the situation for endonucleases is unclear (2, 62, 63). Many type II R/M systems appear to have formed and evolved independently as partnerships between miscellaneous genes that were separated initially (2, 62, 63). They became linked because of a persistent selective advantage. In this case, one might suggest that these three R/M systems (*Mbo*I, *Dpn*II, and *Lla*II) might not have evolved independently.

The genetic similarities between *Lla*II and *Dpn*II might have important practical implications. The R/M systems in *S. pneumoniae* are unusual in some respects. The *Dpn*II system present in some *S. pneumoniae* strains acts only on the unmethylated 5′-GATC-3′ sequence. Some strains of *S. pneumoniae* that do not harbor *Dpn*II contain a complementary system that acts on methylated GATC sites (GmATC), namely, *Dpn*I. Thus, both systems are mutually exclusive. From a phage viewpoint, phages escaping the *Dpn*II system have methylated DNA and are susceptible to the *Dpn*I endonuclease. Phages that can propagate on *Dpn*I-containing strains are susceptible to the *Dpn*II endonuclease (28, 30). If a strain containing the *Dpn*I system is used in parallel with an isogenic strain containing the *Dpn*II system, it would provide an ideal starter culture containing two mechanisms working in concert to restrict phage infection. *S. pneumoniae* phages have EOPs of 10^{-6} on strains harboring *Dpn*I or *Dpn*II systems (43), which is similar to the EOPs of some lactococcal phages on *L. lactis* containing *Lla*II (Table 3).

If there is a lactococcal R/M system similar to *Dpn*II, one might think that there is a *Dpn*I system in *L. lactis*. Historically, phage resistance mechanisms in *L. lactis* have been identified on the basis of their action on reference phages propagated on laboratory strains that do not carry a known R/M system. That is why all known lactococcal R/M systems isolated thus far act only on unmethylated DNA. Use of modified phages to search for complementary resistance mechanisms could be an interesting perspective to increase phage protection bestowed by an R/M system. A *Dpn*I-like system would reduce the practical concern associated with the use of R/M as a phage defense mechanism, which is the selective propagation of modified phages.

The *Dpn*I and *Dpn*II systems are bordered by identical genes on either side in the pneumococcal chromosome (29). The genetic cassette arrangement ensures the mutually exclusive nature of the two systems. It also allows the exchange of one system for another by providing homologous regions adjacent to the cassettes (36). The presence of such adjacent genes for *Lla*II has not been investigated. Although *Lla*II is encoded on a plasmid, the presence of such genes could substantiate the likelihood of a *Dpn*I-like system in *L. lactis*. Other similarities between *L. lactis* and *S. pneumoniae* have been observed previously. For example, there is considerable amino acid similarity between the lysin of a lactococcal phage (936 species) and the N terminus of the autolysin from *S. pneumoniae* (47).

Finally, from a culture manufacturer standpoint, the introduction of the natural plasmid pSRQ700 into industrial *L. lactis* strains should confer strong phage resistance against phages of the 936 species and the newly emerging P335 species. Its effectiveness against the c2 species will be variable. The temperature-insensitive nature of *Lla*II (up to 38°C) makes this phage resistance mechanism amenable to various types of high-temperature dairy fermentations, especially cheese and yogurt. The use of pSRQ700 as part of a starter rotation scheme should improve the overall phage resistance of starter cultures.

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