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Cloning and Analysis of the Restriction-Modification System *Lla*BI, a Bacteriophage Resistance System from *Lactococcus lactis* subsp. *cremoris* W56

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The genes coding for the type II restriction-modification (R/M) system *Lla*BI, which recognized the sequence 5′-C ↓ TRYAG-3′, have been cloned from a plasmid in *Lactococcus lactis* subsp. *cremoris* W56 and sequenced. The DNA sequence predicts an endonuclease of 299 amino acids (33 kDa) and a methylase of 580 amino acids (65 kDa). A 4.0-kb *Hin*dIII fragment in pSA3 was able to restrict bacteriophages, showing that the cloned R/M system can function as a phage defense mechanism in *L. lactis*.

In the dairy industry, species of the genus *Lactococcus* are widely used as starter cultures in the manufacture of cultured milk products. One of the major problems encountered is infection of the lactococcal cultures by lytic bacteriophages, which results in dead or slow cheese vats. Since it is very difficult to avoid contamination with phages, the starter cultures have to be phage resistant. On the basis of their mode of action, four categories of naturally occurring phage resistance mechanisms have been identified in lactococci: adsorption inhibition, penetration blocking (6), restriction-modification (R/M) systems, and abortive infection. Several R/M systems have been isolated from lactococci, but only a few have been cloned and characterized (11). Two m^5C methylases have been sequenced from the chromosomally encoded type II system *Scr*FI from *Lactococcus lactis* subsp. *cremoris* UC503. This system specifically recognizes 5′-CC ↓ NGG-3′ sequences, where N is any nucleotide (4, 20). A plasmid-encoded type II R/M system, *Lla*DCHI, recognizing 5′- ↓ GATC-3′, has recently been cloned and sequenced (13). Two m^6A methylases and one restriction endonuclease make up the system. Mayo et al. (12) have reported type II endonuclease activity, designated *Lla*I and recognizing the sequence 5′-CC ↓ WGG-3′ (W is A or T), from *L. lactis* subsp. *lactis* NCDO 497. The isolation and purification of two type II endonucleases, *Lla*AI and *Lla*BI, recognizing 5′- ↓ GATC-3′ and 5′-C ↓ TRYAG-3′, from *L. lactis* subsp. *cremoris* W9 and W56, respectively, have been described previously (14). A type IIS R/M system on plasmid pTR2030 from *L. lactis* subsp. *lactis* ME2 has also been sequenced (16). It consists of an m^6A methylase and three open reading frames (ORFs) involved in the *Lla*I restriction activity. Furthermore, plasmid pTR2030 codes for an abortive infection mechanism (11).

L. lactis subsp. *cremoris* W56 has previously been isolated from the Danish mixed cheddar starter TK5 (9). It was shown that *L. lactis* subsp. *cremoris* W56 harbors at least three plasmids, pJW563, pJW565, and pJW566, which encode distinct R/M systems (10). Only *L. lactis* subsp. *cremoris* W56 and the transformant *L. lactis* subsp. *cremoris* MG1614(pJW563), and not transformants harboring plasmid pJW565 or pJW566, expressed type II activity (15). A restriction endonuclease, designated *Lla*BI, from *L. lactis* subsp. *cremoris* W56 was purified and its recognition sequence was determined (14).

In this study, the genes coding for the *Lla*BI system from *L. lactis* subsp. *cremoris* W56 were cloned and their nucleotide sequences were determined. The bacterial strains and plasmids are listed in Table 1. Lactococcal strains were grown at 30°C in M17 medium (Oxoid) supplemented with 0.5% glucose (GM17) and with 5 mM $CaCl_2$ when phages were used. Transformations were performed as described by Holo and Nes (8), with the transformants selected on agar medium with 10 μg of chloramphenicol per ml or 2.5 μg of erythromycin per ml. The antibiotics were purchased from Sigma Chemical Co. *Escherichia coli* was grown at 37°C in LB broth (17) supplemented with chloramphenicol, erythromycin, tetracycline, or ampicillin at 10, 50 to 250, 12.5, or 100 μg/ml, respectively, when required. The isometric-headed phage jj50 (10) and the prolate-headed phage c2, both with double-stranded DNA (11), were propagated and titrated by the method of Terzaghi and Sandine (19). Plasmid DNA was extracted from *L. lactis* strains by the method of Andresen et al. (2). Subcloning and nucleotide sequencing were performed by standard DNA techniques (17).

A restriction map of plasmid pJW563, which expressed *Lla*BI activity, was constructed (Fig. 1). When direct cloning of the entire R/M system was attempted in *E. coli* XL1Blue or HB101, only plasmids with deletions were found (data not shown). Thus, pJW563 was cloned only in *L. lactis* subsp. *cremoris* MG1614.

Because of difficulties in cloning the entire system in *L. lactis* subsp. *cremoris* MG1614, a decision was made to determine the location of the genes for the R/M system on plasmid pJW563. A chloramphenicol resistance cassette was inserted into one of the *Cla*I sites, resulting in plasmid pJWC1. *L. lactis* subsp. *cremoris* MG1614(pJWC1) expressed R/M activity similar to that of the wild-type plasmid. Deletion of the 1.2-kb *Bcl*I fragment from pJWC1 resulted in plasmid pJWC2, which showed neither endonuclease nor methylase activity (Fig. 1). When an erythromycin cassette was inserted into the unique *Bgl*II site in plasmid pJWC1, the transformant *L. lactis* subsp. *cremoris* MG1614(pJWE1) did not express any *Lla*BI endonuclease activity or phage resistance. However, phages propagated on *L. lactis* subsp. *cremoris* MG1614(pJWE1) were not restricted by *L. lactis* subsp. *cremoris* MG1614(pJW563), showing that pJWE1 expressed methylase activity. All these results indi-

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TABLE 1. Bacteria and plasmids used

Strain or plasmid	Relevant characteristics[a]	Reference or source
Strains		
L. lactis subsp. *cremoris* MG1614	Plasmid-free derivative of *L. lactis* subsp. *cremoris* 712	7, 21
E. coli XL1Blue	Transformation host; *mrr*$^+$	Stratagene Ltd.
Plasmids		
pJW563	Resident plasmid of W56; R^+/M^+; 11.5 kb	10
pSA3	Shuttle vector; Cm^r Tc^r Em^r; 10.2 kb	3
pBluescriptIISK$^+$	Cloning vector for sequencing; 3.0 kb; Ap^r	Stratagene Ltd.
pUC7,erm	pUC7 Ω(1.1-kb *Hin*PI pIL253 *erm*)	W. M. de Vos
pJWC1	pJW563 Ω(4.0-kb *cam* cassette from pVC5 [21]) in *Cla*I site; R^+/M^+; Cm^r	Anne Gravesen
pJWC2	pJWC1 Δ(1.2-kb *Bcl*I fragment); R^-/M^-; Cm^r	This study
pJWE1	pJWC1 Ω(1.1-kb *erm* cassette from pUC7, *erm*) in *Bgl*II site; R^-/M^+; Cm^r Em^r	This study
pSNB1	pSA3 Ω(4.0-kb *Hin*dIII fragment from pJW563)	This study

[a] Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Em^r, erythromycin resistance; Tc^r, tetracycline resistance.

cated that the endonuclease gene was located near the *Bgl*II site, whereas at least part of the methylase gene was located on the 1.2-kb *Bcl*I fragment.

The entire R/M system was then cloned in shuttle vector pSA3 on a 4.0-kb *Hin*dIII fragment containing the *Bgl*II and *Bcl*II sites, resulting in plasmid pSNB1. *L. lactis* subsp. *cremoris* MG1614(pSNB1) transformants expressed both *Lla*BI endonuclease and methylase activity (data not shown) and restricted phage jj50 with an efficiency of plaquing of 10^{-4} and phage c2 with an efficiency of plaquing of 10^{-3}. This demonstrates that the genes encode both the endonuclease and the methylase from the *Lla*BI R/M system and that they can be cloned and used to increase phage resistance in *Lactococcus* strains.

When the 4.0-kb *Hin*dIII fragment in pBluescriptIISK$^+$ was used to transform *E. coli* XL1Blue, only plasmids with deleted inserts were obtained, indicating that the endonuclease and/or methylase expression may be lethal to *E. coli* XL1Blue. Additionally, since the *Hin*dIII$_1$-*Bgl*II fragment was also difficult to clone, it is probably the methylase which caused this apparent lethality in *E. coli* XL1Blue, because of the presence of the Mrr restriction system, which restricts methylated DNA (22).

The nucleotide sequence of 3,476 bp in the 4.0-kb *Hin*dIII fragment containing the *Lla*BI R/M genes was determined and analyzed as shown in Fig. 2. ORF1 was 1,740 bp and predicts a protein of 580 amino acids (65 kDa), while ORF2 was 897 bp and predicts a protein of 299 amino acids (33 kDa). The inactivated *Bgl*II site in plasmid pJWE1 (Fig. 1) was located in ORF2, showing that ORF2 codes for the endonuclease R · *Lla*BI. The deleted 1.2-kb *Bcl*I fragment (positions 1305 to 2520) in plasmid pJWC2, spanning 856 bp downstream in ORF1, shows that ORF1 codes for the methylase M · *Lla*BI. A secondary structure indicating a putative terminator structure with a 13-bp inverted repeat sequence ($\Delta G = -16.9$ kcal [ca.

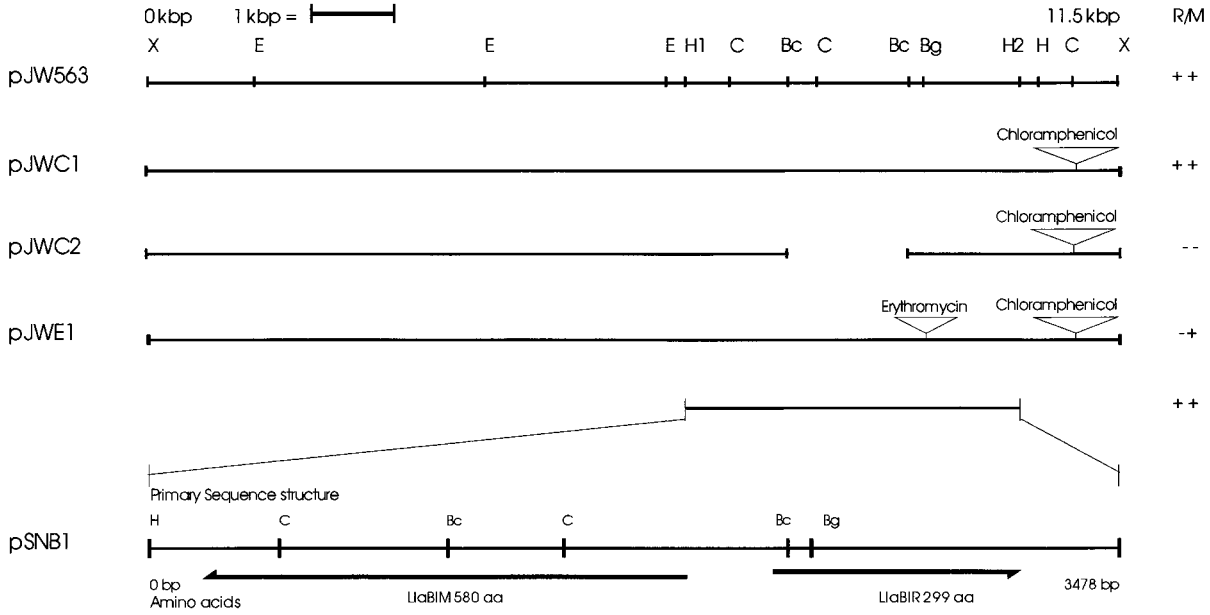

FIG. 1. Maps, R/M activities of pJW563 and its derivatives, and the products of the *Lla*BI genes. Plasmids are shown linearized by cleavage at an *Xho*I site; pJW563 is a wild-type plasmid. The positions of the putative ORFs and the direction of transcription are indicated (arrows). The putative sizes (in amino acids) are indicated for the methylase and the restriction endonuclease. Bc, *Bcl*I; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; X, *Xho*I.

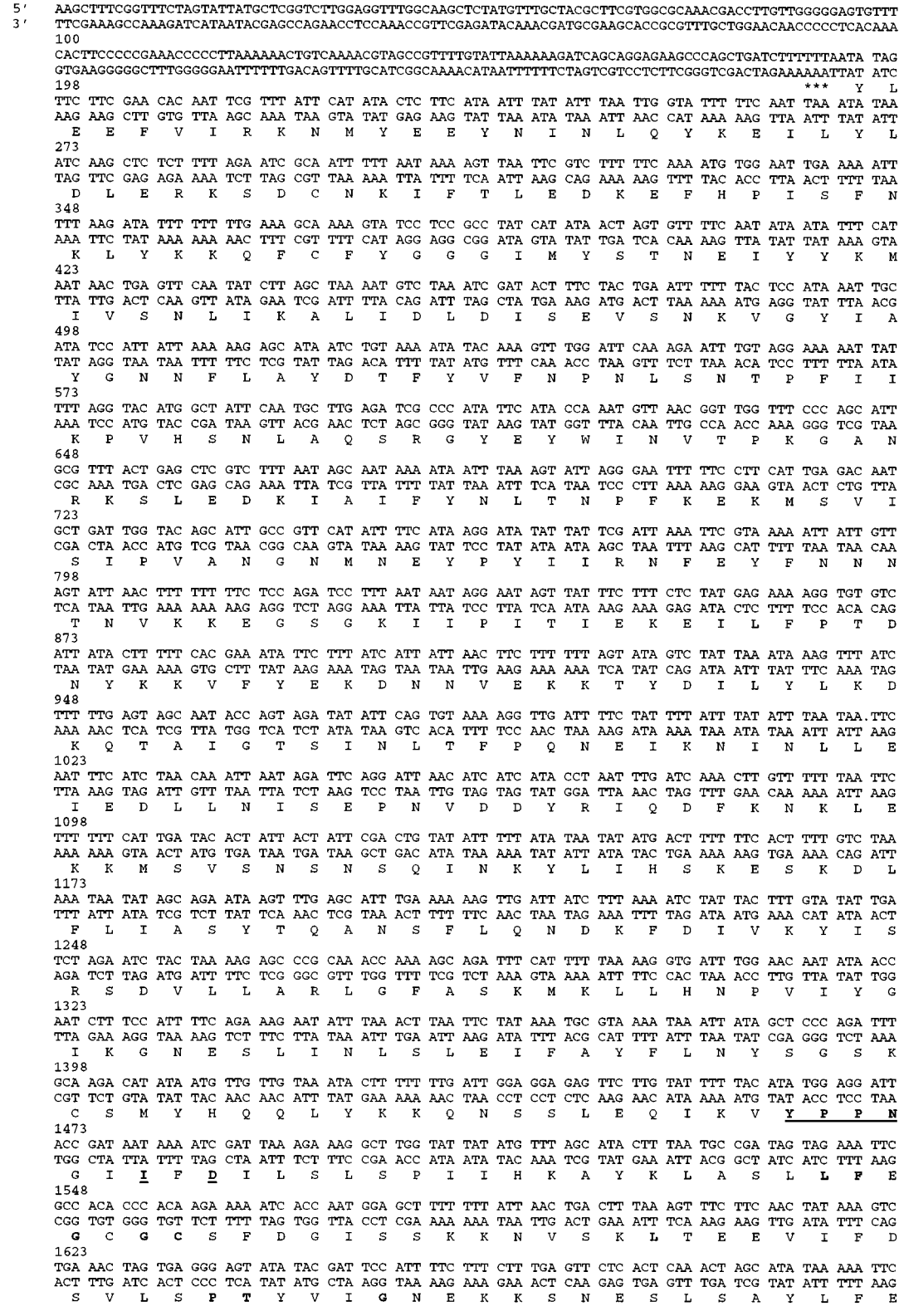

FIG. 2. Complete nucleotide sequence of the 3,476 bp of the 4.0-kb *Hin*dIII fragment containing the *Lla*BI R/M enzyme genes. Nucleotide positions are numbered consecutively starting at the left *Hin*dIII site (H1, Fig. 1). The restriction endonuclease (nucleotides 2234 to 3131) is encoded by the top strand; the modification enzyme (nucleotides 191 to 1931) is encoded by the bottom strand. Promoter sequences (−10 and −35), Shine-Dalgarno sequences (S.D.) (underlined), stop codons (*), and translation start codons (boldface and gene designations) are indicated. The deduced amino acid sequences for both proteins are also shown. The amino acids are aligned with the first nucleotide of each codon. Motifs I (boldface) and IV (underlined boldface letters) are also indicated.

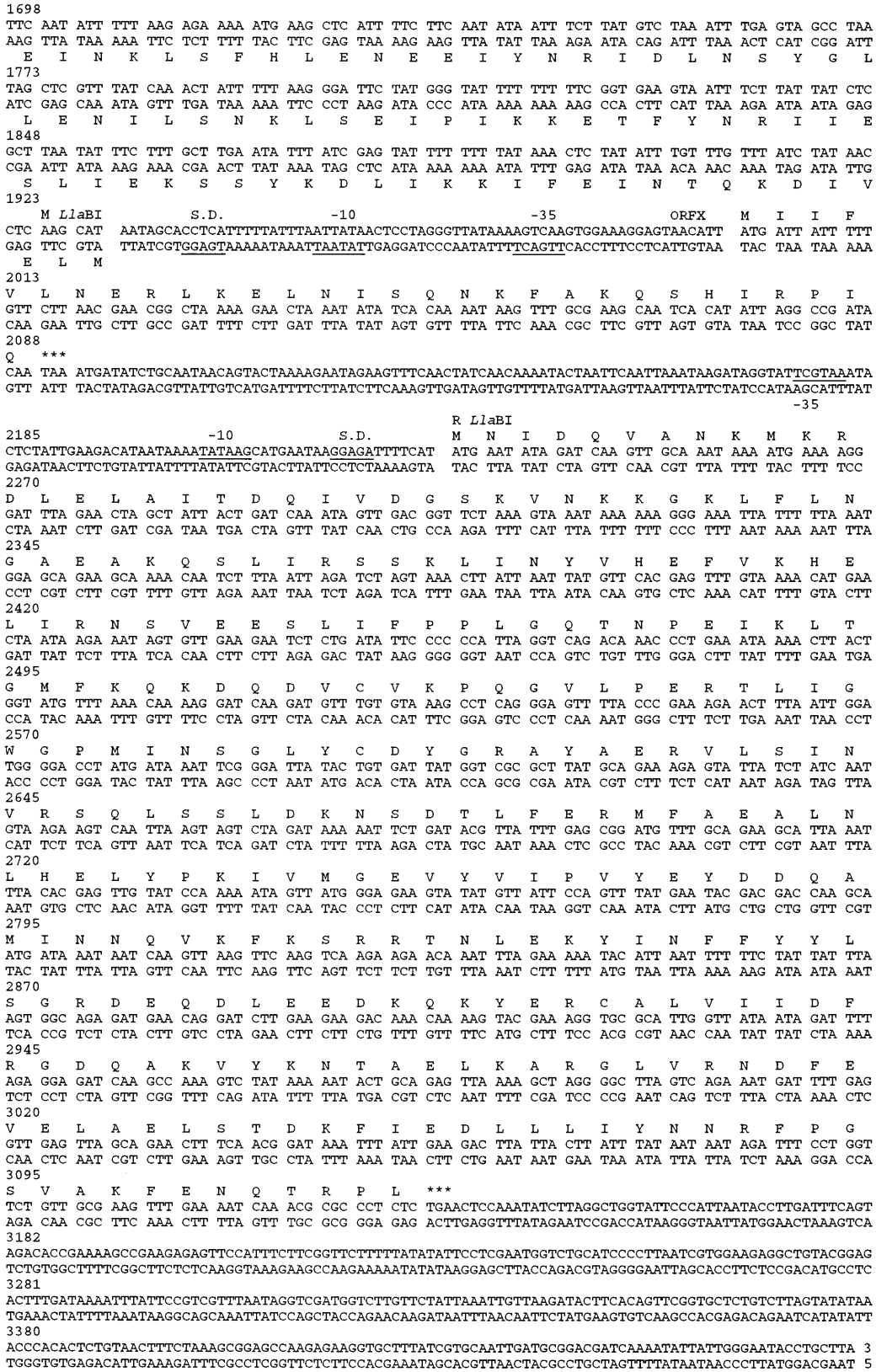

FIG. 2—*Continued.*

−70.7 kJ]) was found downstream of the methylase gene with a 2-bp overlap. The results show that the *Lla*BI R/M system consists of two divergently transcribed genes encoding a methylase, M · *Lla*AI, and an endonuclease, R · *Lla*AI.

No primary sequence similarities between the restriction endonuclease R · *Lla*BI and the corresponding methylase M · *Lla*BI or other type II restriction endonucleases were found. As shown in Fig. 2, the deduced amino acid sequence of M · *Lla*BI has all the characteristics of subclass γ of the m^6A methylases: a motif I, a short variable region (14 amino acids), and a motif IV with asparagine in the first position (NPPY) (23).

During the cloning of the *Lla*BI system, it was found that subclones of pJW563 containing fragments of the *Lla*BI methylase were not resistant to *Pst*I restriction, although plasmid pJW563 was resistant to digestion by the *Pst*I restriction endonuclease (data not shown). The *Pst*I endonuclease recognizes 5′-CTGCA ↓ G-3′ and cuts as indicated by the arrow. *Lla*BI can recognize the same sequence, 5′-C ↓ TGCAG-3′ (and 5′-C ↓ TATAG-3′), but cuts the recognition sequence at a different position (*Lla*BI generates 5′ overhangs while *Pst*I yields 3′ overhangs). This indicates that the adenine in the *Pst*I recognition sequence has been methylated by the *Lla*BI methylase. This reinforces the assumption that M · *Lla*BI is an m^6A methylase.

Preceding the *LlaBIR* gene, and in the same direction, several putative small ORFs were found. ORFX, of 30 codons (Fig. 2), was the largest and may code for an inhibitory peptide preventing endonuclease subunit association, as found for the *Pvu*II R/M system (1).

The high C+G content in the recognition sequences of *Scr*FI (5′-CC ↓ NGG-3′) and *Lla*I (5′-CC ↓ WGG-3′), in contrast to those of *Lla*AI or *Lla*DCHI (5′- ↓ GATC-3′) and *Lla*BI (5′-C ↓ TRYAG-3′), may have a practical implication in the use of these systems as phage defense mechanisms, since lactococcal phage DNA has <40% C+G (11).

The average G+C content of the *Lla*BI genes is 27.8% (31.5% for R · *Lla*BI and 25.7% for M · *Lla*BI), which is much lower than the 34 to 43% G+C content normally found in lactococci as measured by determination of the melting temperature (18). This may indicate that the *Lla*BI R/M system originates from a genus other than *Lactococcus* or that it is an inherent feature of particular R/M systems. Genes coding for the phage resistance mechanism abortive infection in *L. lactis* also have been found to have a low G+C content (26 to 29%) (5).

Nucleotide sequence accession number. The nucleotide sequence (3,476 bp) of pSNAI has been deposited in the EMBL Nucleotide Sequence Database under accession no. X97263.

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REFERENCES

1. **Adams, G. M., and R. M. Blumenthal.** 1995. Gene *pvuIIW*: a possible modulator of *Pvu*II endonuclease subunit association. Gene **157:**193–199.
2. **Andresen, A., A. Geis, U. Krusch, and M. Teuber.** 1984. Plasmidmuster milchwirtschaftlich genutzter Starterkulturen. Milchwissenschaft **39:**140–143.
3. **Dao, M. L., and J. J. Ferretti.** 1985. *Streptococcus-Escherichia coli* shuttle vector pSA3 and its use in the cloning of streptococcal genes. Appl. Environ. Microbiol. **49:**115–119.
4. **Davis, R., D. van der Lelie, A. Mercenier, C. Daly, and G. F. Fitzgerald.** 1993. *Scr*FI restriction-modification system of *Lactococcus lactis* subsp. *cremoris* UC503: cloning and characterization of two *Scr*FI methylase genes. Appl. Environ. Microbiol. **59:**777–785.
5. **Garvey, P., G. F. Fitzgerald, and C. Hill.** 1995. Cloning and DNA sequence analysis of two abortive infection phage resistance determinants from the lactococcal plasmid pNP40. Appl. Environ. Microbiol. **61:**4321–4328.
6. **Garvey, P., C. Hill, and G. F. Fitzgerald.** 1996. The lactococcal plasmid pNP40 encodes a third bacteriophage resistance mechanism, one which affects phage DNA penetration. Appl. Environ. Microbiol. **62:**676–679.
7. **Gordon, J.-J., C. Delome, S. D. Ehrlich, and P. Renault.** 1992. Divergence of genome sequences between *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*. Appl. Environ. Microbiol. **58:**4045–4047.
8. **Holo, H., and I. F. Nes.** 1989. High-frequency transformation, by electroporation, of *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilized media. Appl. Environ. Microbiol. **55:**3119–3123.
9. **Josephsen, J., and E. W. Nielsen.** 1988. Plasmid profiles and bacteriophage sensitivity of bacteria of a cheddar starter used for five years without rotation. Milchwissenschaft **43:**219–223.
10. **Josephsen, J., and F. K. Vogensen.** 1989. Identification of three different plasmid encoded restriction/modification systems in *Streptococcus lactis* subsp. *cremoris* W56. FEMS Microbiol. Lett. **59:**161–166.
11. **Klaenhammer, T. R., and G. F. Fitzgerald.** 1994. Bacteriophages and bacteriophage resistance, p. 106–168. *In* M. G. Gasson and W. M. de Vos (ed.), Genetics and biotechnology of lactic acid bacteria. Blackie, London.
12. **Mayo, B., C. Hardisson, and A. F. Brana.** 1991. Nucleolytic activities in *Lactococcus lactis* subsp. *lactis* NCDO 497. FEMS Microbiol. Lett. **79:**195–198.
13. **Moineau, S., S. A. Walker, E. R. Vedamuthu, and P. A. Vandenbergh.** 1995. Cloning and sequencing of *Lla*II restriction/modification genes from *Lactococcus lactis* and relatedness of this system to the *Streptococcus pneumoniae Dpn*II system. Appl. Environ. Microbiol. **61:**2193–2202.
14. **Nyengaard, N., F. K. Vogensen, and J. Josephsen.** 1993. *Lla*AI and *Lla*BI, two type-II restriction endonucleases from *Lactococcus lactis* subsp. *cremoris* W9 and W56 recognizing, respectively, 5′-/GATC-3 and 5′-C/TRYAG-3′. Gene **136:**371–372.
15. **Nyengaard, N., F. K. Vogensen, and J. Josephsen.** 1995. Restriction-modification systems in *Lactococcus lactis*. Gene **157:**13–18.
16. **O'Sullivan, D. J., K. Zagula, and T. R. Klaenhammer.** 1995. In vivo restriction by *Lla*I is encoded by three genes, arranged in an operon with *llaIM*, on the conjugative *Lactococcus* plasmid pTR2030. J. Bacteriol. **177:**134–143.
17. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
18. **Schleifer, K. H., J. Kraus, C. Dvorak, R. Kilpper-Bälz, M. D. Collins, and W. Fischer.** 1985. Transfer of *Streptococcus lactis* and related streptococci to the genus *Lactococcus* gen. nov. Syst. Appl. Microbiol. **6:**183–195.
19. **Terzaghi, B. E., and W. E. Sandine.** 1975. Improved medium for lactic streptococci and their bacteriophages. Appl. Microbiol. **29:**807–813.
20. **Twomey, D. P., C. Daly, and G. F. Fitzgerald.** 1993. Sequence of the gene encoding a second *Scr*FI m^5C methyltransferase of *Lactococcus lactis*. Gene **136:**205–209.
21. **von Wright, A., S. Wessels, S. Tynkkynen, and M. Saarela.** 1990. Isolation of a replication region of a large lactococcal plasmid and use in cloning of a nisin resistance determinant. Appl. Environ. Microbiol. **56:**2029–2035.
22. **Waite-Rees, P. A., C. J. Keating, L. S. Moran, B. E. Slatko, L. J. Hornstra, and J. S. Benner.** 1991. Characterization and expression of the *Escherichia coli* Mrr restriction system. J. Bacteriol. **173:**5207–5219.
23. **Wilson, G. G.** 1992. Amino acid sequence arrangements of DNA methyltransferases. Methods Enzymol. **216:**259–279.