

Cloning and Analysis of the Restriction-Modification System *LlaBI*, 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 *LlaBI*, 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 *HindIII* 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 m⁵C methylases have been sequenced from the chromosomally encoded type II system *ScrFI* 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, *LlaDCHI*, recognizing 5'- ↓ GATC-3', has recently been cloned and sequenced (13). Two m⁶A methylases and one restriction endonuclease make up the system. Mayo et al. (12) have reported type II endonuclease activity, designated *LlaI* 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, *LlaAI* and *LlaBI*, 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 m⁶A methylase and three open reading frames (ORFs) involved in the *LlaI* 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, ex-

pressed type II activity (15). A restriction endonuclease, designated *LlaBI*, from *L. lactis* subsp. *cremoris* W56 was purified and its recognition sequence was determined (14).

In this study, the genes coding for the *LlaBI* 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 CaCl₂ 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 *LlaBI* 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 *ClaI* 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 *BclI* 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 *BglII* site in plasmid pJWC1, the transformant *L. lactis* subsp. *cremoris* MG1614(pJWE1) did not express any *LlaBI* 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		
<i>L. lactis</i> subsp. <i>cremoris</i> MG1614	Plasmid-free derivative of <i>L. lactis</i> subsp. <i>cremoris</i> 712	7, 21
<i>E. coli</i> XL1Blue	Transformation host; <i>mrr</i> ⁺	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 <i>Hin</i> PI pIL253 <i>erm</i>)	W. M. de Vos
pJWC1	pJW563 Ω (4.0-kb <i>cam</i> cassette from pVC5 [21]) in <i>Cla</i> I site; R ⁺ /M ⁺ ; Cm ^r	Anne Gravesen
pJWC2	pJWC1 Δ (1.2-kb <i>Bcl</i> I fragment); R ⁻ /M ⁻ ; Cm ^r	This study
pJWE1	pJWC1 Ω (1.1-kb <i>erm</i> cassette from pUC7, <i>erm</i>) in <i>Bgl</i> II site; R ⁻ /M ⁺ ; Cm ^r Em ^r	This study
pSNB1	pSA3 Ω (4.0-kb <i>Hind</i> III 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 *Hind*III fragment containing the *Bgl*II and *Bcl*I 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 *Hind*III 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 *Hind*III₁-*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 *Hind*III 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.

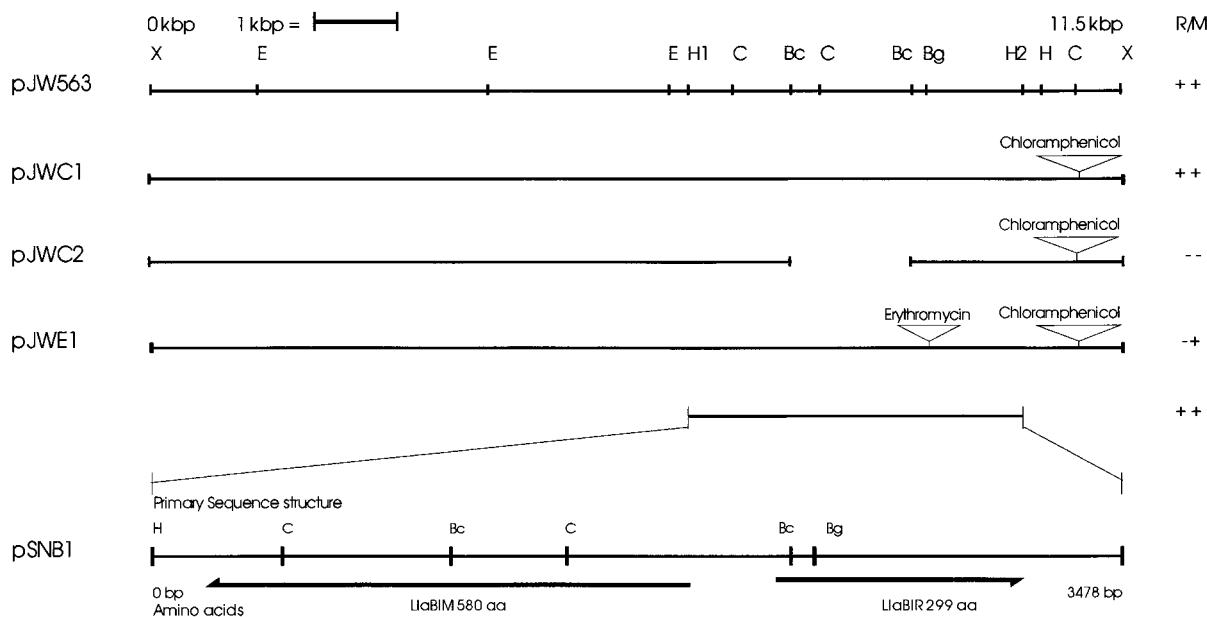


FIG. 1. Maps, R/M activities of pJW563 and its derivatives, and the products of the *Lla*BI genes. Plasmids are shown linearized by cleavage at an *Xho*I site; pJW563 is a wild-type plasmid. The positions of the putative ORFs and the direction of transcription are indicated (arrows). The putative sizes (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, *Hind*III; X, *Xho*I.

5' AAGCTTTCGGTTTCTAGTATTATGCTCGGTCCTGGAGTTTGGCAAGCTCTATGTTGCTACGCTTCGTTGGCGCAAAACGACCTTGTGGGGGAGTGT
 3' TTCGAAAGCCAAAGATCATAATACGAGCCAGAACCTCCAAACCGTTTCGAGATACAAACAGATCGGAGCACCAGCGTTTGGTGAACAACCCCTCCACAA
 100
 CACCTCCCCGAAACCCCTTAAAAACTGTCAAACGTAGCCGTTTGTATTAAAAAGATCAGCAGGAGAAGCCAGCTGATCTTTTAAATA TAG
 GTGAAGGGGCTTTGGGGAAATTTTTGACAGTTTTTCATCGGCAAAACATAATTTTTCTAGTGTCTCTTCGGGTGCGACTAGAAAAAATTAT ATC
 198
 TTC TTC GAA CAC AAT TCG TTT ATT CAT ATA CTC TTC ATA ATT TAT ATT TAA TTG GTA TTT TTC AAT TAA ATA TAA
 AAG AAG CTT GTG TTA AGC AAA TAA GTA TAT GAG AAG TAT TAA ATA TAA ATT AAC CAT AAA AAG TTA ATT TAT ATT
 E E F V I R K N M Y E E Y N I N L Q Y K E I L Y L
 273
 ATC AAG CTC TCT TTT AGA ATC GCA ATT TTT AAT AAA AGT TAA TTC GTC TTT TTC AAA ATG TGG AAT TGA AAA ATT
 TAG TTC GAG AGA AAA TCT TAG CGT TAA AAA TTA TTT TCA ATT AAG CAG AAA AAG TTT TAC ACC TTA ACT TTT TAA
 D L E R K S D C N K I F T L E D K E F H P I S F N
 348
 TTT AAG ATA TTT TTT TTG AAA GCA AAA GTA TCC TCC GCC ATA CAT ATA ACT AGT GTT TTC AAT ATA ATA TTT CAT
 AAA TTC TAT AAA AAA AAC TTT CGT TTT CAT AGG AGG CCG GAT GTA TAT TGA TCA CAA AAG TTA TAT TAT AAA GTA
 K L Y K K Q F C F Y G G G I M Y S T N E I Y K M
 423
 AAT AAC TGA GTT CAA TAT CTT AGC TAA AAT GTC TAA ATC GAT ACT TTC TAC TGA ATT TTT TAC TCC ATA AAT TGC
 TTA TTG ACT CAA GTT ATA GAA TCG ATT TTA CAG ATT TAG CTA TGA AAG ATG ACT TAA AAA ATG AGG TAT TTA ACG
 I V S N L I K A L I D L D I S E V S N K V G Y I A
 498
 ATA TCC ATT ATT AAA AAG AGC ATA ATC TGT AAA ATA TAC AAA GTT TGG ATT CAA AGA ATT TGT AGG AAA AAT TAT
 TAT AGG TAA TAA TTT TTC TCG TAT TAG ACA TTT TAT ATG TTT CAA ACC TAA GTT TCT TAA ACA TCC TTT TTA ATA
 Y G N N F L A Y D T F Y V F N P N L S N T P F I I
 573
 TTT AGG TAC ATG GCT ATT CAA TGC TTG AGA TCG CCC ATA TTC ATA CCA AAT GTT AAC GGT TGG TTT CCC AGC AAT
 AAA TCC ATG TAC CGA TAA GTT ACG TCT AGG GGG TAT AAG TGT TTA CAA TTG CCA ACC AAA GGG TCG TAA
 K P V H S N L A Q S R G Y E Y W I N V T P K G A N
 648
 GCG TTT ACT GAG CTC GTC TTT AAT AGC AAT AAA ATA ATT TAA AGT ATT AGG GAA TTT TTC CTT CAT TGA GAC AAT
 CGC AAA TGA CTC GAG CAG AAA TTA TCG TTA TTT TAT TAA ATT TCA TAA TCC CTT AAA AAG GAA GTA ACT CTG TTA
 R K S I L E D I F Y N L T N P F K E K M S V I
 723
 GCT GAT TGG TAC AGC ATT GCC GTT CAT ATT TTC ATA AGG ATA TAT TAT TCG AIT AAA TTC GTA AAA ATT ATT GTT
 CGA CTA ACC ATG TCG TAA CGG CAA GTA TAA AAG TAT TCC TAT ATA ATA AGC TAA TTT AAG CAT TTT TAA TAA CAA
 S I P V A N G N M N E Y P Y I I R N F E Y F N N N
 798
 AGT ATT AAC TTT TTT TTC TCC AGA TCC TTT AAT AAT AGG AAT AGT TAT TTC TTT CTC TAT GAG AAA AGG TGT GTC
 TCA TAA TTG AAA AAA AAG AGG TCT AGG AAA TTA TTA TCC TTA TCA ATA AAG AAA GAG ATA CTC TTT TCC ACA CAG
 T N V K K E G S G K I I P I T I E K E I L F P T D
 873
 ATT ATA CTT TTT CAC GAA ATA TTC TTT ATC ATT ATT AAC TTC TTT TTT AGT ATA GTC TAT TAA ATA AAG TTT ATC
 TAA TAT GAA AAA GTG CTT TAT AAG AAA TAG TAA TAA TTG AAG AAA AAA TCA TAT CAG ATA ATT TAT TTC AAA TAG
 N Y K K N V F E K D N N V E K K T Y D I L Y L K D
 948
 TTT TTG AGT AGC AAT ACC AGT AGA TAT ATT CAG TGT AAA AGG TTG ATT TTC TAT TTT ATT TAT ATT TAA TAA TTC
 AAA AAC TCA TCG TTA TGG TCA TCT ATA TAA GTC ACA TTT TCC AAC TAA AAG ATA AAA TAA ATA TAA ATT ATT AAG
 K Q T A I G T S I N L T F P Q N E I K N I N L L E
 1023
 AAT TTC ATC TAA CAA ATT AAT AGA TTC AGG ATT AAC ATC ATC ATA CCT AAT TTG ATC AAA CTT GTT TTT TAA TTC
 TTA AAG TAG ATT GTT TAA TTA TCT AAG TCC TAA TAG TAG TAT GGA TTA AAC TAG TTT TTA AAA AAA ATT AAG
 I E D L L N I S E P N V D D Y R I Q D F K N A K L E
 1098
 TTT TTT CAT TGA TAC ACT ATT ACT ATT CGA CTG TAT ATT TTT ATA TAA TAT ATG ACT TTT TTC ACT TTT GTC TAA
 AAA AAA GTA ACT ATG TGA TAA TGA TAA GCT GAC ATA TAA AAA TAT ATT ATA TAC TGA AAA AAG TGA AAA CAG ATT
 K K M S V S N S N S I N K Y L I H S K E S K D L
 1173
 AAA TAA TAT AGC AGA ATA AGT TTG AGC ATT TGA AAA AAG TTG ATT ATC TTT AAA ATC TAT TAC TTT GTA TAT TGA
 TTT ATT ATA TCG TCT TAT TCA AAC TCG TAA ACT TTT TTC AAC TAA TAG AAA TTT TAG ATA ATG AAA CAT ATA ACT
 F L I A S Y T Q A N S F L Q N D K F D I V K Y I S
 1248
 TCT AGA ATC TAC TAA AAG AGC CCG CAA ACC AAA AGC AGA TTT CAT TTT TAA AAG GTG ATT TGG AAC AAT ATA ACC
 AGA GTT TAG ATT TTT TCG GGC GTT TCG TCT AAA GTA AAA ATT TTC CAC TAA ACC TTT TTA TAT TGG
 R S D V L L A R L G F A S K M K L L H N P V I Y G
 1323
 AAT CTT TCC ATT TTC AGA AAG AAT TTA TAA ACT TAA TTC TAT AAA TGC GTA AAA TAA ATT ATA GCT CCC AGA TTT
 TTA GAA AGG TAA AAG TCT TTC TTA TAA ATT TGA ATT TTT ACG TAT TTT ATT TAA TAT CGA GGG TCT AAA
 I K G N E S L I N L S L E I F A Y F L N Y S G S K
 1398
 GCA AGA CAT ATA ATG TTG TTG TAA ATA CTT TTT TTG ATT GGA GGA GAG TTC TTG TAT TTT TAC ATA TGG AGG ATT
 CGT TCT GTA TAT TAC AAC AAC ATT TAT GAA AAA AAC TAA CCT CCT CTC AAG AAC ATA AAA ATG TAT ACC TCC TAA
 C S M Y H Q L Y K Q N S S L E Q I K V Y P P N
 1473
 ACC GAT AAT AAA ATC GAT TAA AGA AAG GCT TGG TAT TAT ATG TTT AGC ATA CTT TAA TGC CGA TAG TAG AAA TTC
 TGG CTA TTA TTT TAG CTA ATT TCT TTC CGA ACC ATA ATA TAC AAA TCG TAT GAA ATT ACG GCT ATC ATC TTT AAG
 G I I F D I L S L S P I I H K A Y K L A S L L F E
 1548
 GCC ACA CCC AGA AGA AAA ATC ACC AAT GGA GCT TTT TTT ATT AAC TGA CTT TAA AGT TTC TTC AAC TAT AAA GTC
 CGG TGT GGG TGT TCT TTT TAG TGG TTA CCT CGA AAA TAA TAA TTG ACT GAA ATT TCA AAG AAG TTG ATA TTT CAG
 G C G G C S F D G I S S K K N V S K L T E V I F D
 1623
 TGA AAC TAG TGA GGG AGT ATA TAC GAT TCC ATT TTC TTT CTT TGA GTT CTC ACT CAA ACT AGC ATA TAA AAA TTC
 ACT TTG ATC ACT CCC TCA TAT ATG CTA AGG TAA AAG AAA GAA ACT CAA GAG TGA GTT TGA TCG TAT ATT TTT AAG
 S V L S P T Y V I G N E K Y S N E S L S A Y L F S E

FIG. 2. Complete nucleotide sequence of the 3,476 bp of the 4.0-kb *Hind*III fragment containing the *Lla*BI R/M enzyme genes. Nucleotide positions are numbered consecutively starting at the left *Hind*III 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.

1698
TTC AAT ATT TTT AAG AGA AAA ATG AAG CTC ATT TTC TTC AAT ATA ATT TCT TAT GTC TAA ATT TGA GTA GCC TAA
AAG TTA TAA AAA TTC TCT TTT TAC TTC GAG TAA AAG AAG TTA TAT TAA AGA ATA CAG ATT TAA ACT CAT CGG ATT
E I N K L S F H L E N E E I Y N R I D L N S Y G L
1773
TAG CTC GTT TAT CAA ACT ATT TTT AAG GGA TTC TAT GGG TAT TTT TTT TTC GGT GAA GTA ATT TCT TAT TAT CTC
ATC GAG CAA ATA GTT TGA TAA AAA TTC CCT AAG ATA CCC ATA AAA AAA AAG CCA CTT CAT TAA AGA ATA ATA GAG
L E N I L S N K L S E I P I K K E T F Y N R I I E
1848
GCT TAA TAT TTC TTT GCT TGA ATA TTT ATC GAG TAT TTT TTT TAT AAA CTC TAT ATT TGT TTG TTT ATC TAT AAC
CGA ATT ATA AAG AAA CGA ACT TAT AAA TAG CTC ATA AAA AAA ATA TTT GAG ATA TAA ACA AAC AAA TAG ATA TTG
S L I E K S S Y K D L I K K I F E I N T Q K D I V
1923
M LlaBI S.D. -10 -35 ORFX M I I F
CTC AAG CAT AATGACACCTCATTTTTTATTTAATTATAACTCCTAGGGTTATAAAAAGTCAAGTGGAAAGGAGTAACATT ATG ATT ATT TTT
GAG TTC GTA TTATCGTGGAGTAAAAATAAATTAATATTTAGGATCCCAATATTTTCAGTTCCCTTTCTCATTGTAA TAC TAA TAA AAA
E L M
2013
V L N E R L K E L N I S Q N K F A K Q S H I R P I
GTT CTT AAC GAA CGG CTA AAA GAA CTA AAT ATA TCA CAA AAT AAG TTT GCG AAG CAA TCA CAT ATT AGG CCG ATA
CAA GAA TTG CTT GCC GAT TTT CTT GAT TTA TAT AGT GTT TTA TTC AAA CGC TTC GTT AGT GTA TAA TCC GGC TAT
2088
Q ***
CAA TAA ATGATATCTGCAATAACAGTACTAAAAGAATAGAAAGTTTCAACTATCAACAAAATACTAATTCAATTAATAAGATAGGTATTCGTAATA
GTT ATT TACTATAGACGTTATTGTTCATGATTTTCTTATCTTCAAAGTTGATAGTTGTTTATGATTAAGTTAATTTATCTATCCATAGGATTTAT
-35
R LlaBI
2185 -10 S.D. M N I D Q V A N K M K R
CTCTATTGAAGACATAATAAAAATAAGCATGAATAAAGGAGATTTTCAT ATG AAT ATA GAT CAA GTT GCA AAT AAA ATG AAA AGG
GAGATAAATCTCTGATTTATTTTATATTTTCTTACTTTTCTCTAAAAGTA TAC TTA TAT CTA GTT CAA CGT TTA TTT TAC TTT TCC
2270
D L E L A I T D Q I V D G S K V N K K G K L F L N
GAT TTA GAA CTA GCT ATT ACT GAT CAA ATA GTT GAC GGT TCT AAA GTA AAT AAA AAA GGG AAA TTA TTT TTA AAT
CTA AAT CTT GAT CGA TAA TGA CTA GTT TAT CAA CTG CCA AGA TTT CAT TTA TTT TTT CCC TTT AAT AAA AAT TTA
2345
G A E A K Q S L I R S S K L I N Y V H E F V K H E
GGA GCA GAA GCA AAA CAA TCT TTA ATT AGA TCT AGT AAA CTT ATT AAT TAT GTT CAC GAG TTT GTA AAA CAT GAA
CCT CGT CTT CGT TTT GTT AGA AAT TAA TCT AGA TCA TTT GAA TAA TTA ATA CAA GTG CTC AAA CAT TTT GTA CTT
2420
L I R N S V E E S L I F P P L G Q T N P E I K L T
CTA ATA AGA AAT AGT GTT GAA GAA TCT CTG ATA TTC CCC CCA TTA GGT CAG ACA AAC CCT GAA ATA AAA CTT ACT
GAT TAT TCT TTA TCA CAA CTT CTT AGA GAC TAT AAG GGG GGT AAT CCA GTC TGT TTG GGA CTT TAT TTT GAA TGA
2495
G M F K Q K D Q D V C V K P Q G V L P E R T L I G
GGT ATG TTT AAA CAA AAG GAT CAA GAT GTT TGT GTA AAG CCT CAG GGA GTT TTA CCC GAA AGA ACT TTA ATT GGA
CCA TAC AAA TTT GTT TTC CTA GTT CTA CAA ACA CAT TTC GGA GTC CCT CAA AAT GGG CTT TCT TGA AAT TAA CCT
2570
W G P M I N S G L Y C D Y G R A Y A E R V L S I N
TGG GGA CCT ATG ATA AAT TCG GGA TTA TAC TGT GAT TAT GGT CGC GCT TAT GCA GAA AGA TTA TCT ATC AAT
ACC CCT GGA TAC TAT TTA AGC CCT AAT ATG ACA CTA ATA CCA GCG CGA ATA CGT CTT TCT CAT AAT AGA TAG TTA
2645
V R S Q L S S L D K N S D T L F E R M F A E A L N
GTA AGA AGT CAA TTA AGT AGT CTA GAT AAA AAT TCT GAT ACG TTA TTT GAG CGG ATG TTT GCA GAA GCA TTA AAT
CAT TCT TCA GTT AAT TCA TCA GAT CTA TTT TTA AGA CTA TGC AAT AAA CTC GCC TAC AAA CGT CTT GCA AAT TTA
2720
L H E L Y P K I V M G E V Y V I P V Y E Y D D Q A
TTA CAC GAG TTG TAT CCA AAA ATA GTT ATG GGA GAA GTA TAT GTT ATT CCA GTT TAT GAA TAC GAC GAC CAA GCA
AAT GTG CTC AAC ATA GGT TTT TAT CAA TAC CCT CTT CAT ATA CAA TAA GGT CAA ATA CTT ATG CTG CTG GTT CGT
2795
M I N N Q V K F K S R R T N L E K Y I N F F Y Y L
ATG ATA AAT AAT CAA GTT AAG TTC AAG TCA AGA AGA ACA AAT TTA GAA AAA TAC ATT AAT TTT TTC TAT TAT TTA
TAC TAT TTA TTA GTT CAA TTC AAG TTC AGT TCT TCT TGT TTA AAT CTT TTT ATG TAA TTA AAA AAG ATA ATA AAT
2870
S G R D E Q D L E E D K Q K Y E R C A L V I I D F
AGT GGC AGA GAT GAA CAG GAT CTT GAA GAA GAC AAA CAA AAG TAC GAA AGG TGC GCA TTG GTT ATA ATA GAT TTT
TCA CCG TCT CTA CTT GTC CTA GAA CTT CTT CTG TTT TTC ATG CTT TCC ACG CGT AAC CAA TAT TAT CTA AAA
2945
R G D Q A K V Y K N T A E L K A R G L V R N D F E
AGA GGA GAT CAA GCC AAA GTC TAT AAA AAT ACT GCA GAG TTA AAA GCT AGG GGC TTA GTC AGA AAT GAT TTT GAG
TCT CCT CTA GTT CGG TTT CAG ATA TTT TTA TGA CGT CTC AAT TTT CGA TCC CCG AAT CAG TCT TTA CTA AAA CTC
3020
V E L A E L S T D K F I E D L L L I Y N N R F P G
GTT GAG TTA GCA GAA CTT TCA ACG GAT AAA TTT ATT GAA GAC TTA TTA CTT ATT TAT AAT AAT AGA TTT CCT GGT
CAA CTC AAT CGT CTT GAA AGT TGC CTA TTT AAA TAA CTT CTG AAT AAT GAA TAA ATA TTA TTA TCT AAA GGA CCA
3095
S V A K F E N Q T R P L ***
TCT GTT GCG AAG TTT GAA AAT CAA ACG CGC CCT CTC TGAACCTCAAATATCTTAGGCTGGTATTCCTTAAATACCTTTGATTTTCAGT
AGA CAA CGC TTC AAA CTT TTA GTT TGC GCG GGA GAG ACTTGAGGTTTATAGAAATCCGACCATAAGGTTAATTTATGGAATAAGTCA
3182
AGACACCGAAAAGCCGAAGAGAGTTCCATTTCTTCGGTTCTTTTTATATAATTTCTCGAATGGTCTGCATCCCCCTTAATCGTGGAAAGGCGTGTACGGAG
TCTGTGGCTTTTTCGGCTTCTCAAGGTAAAGAAGCCAAAGAAAATATATAAGGAGCTTACCAGACGTAGGGGAATTAGCACCTTCTCCGACATGCCTC
3281
ACTTTGATAAAAATTTATTCGGTCTTTAATAGGTCGATGGTCTTGTCTTATTAATTTGTTAAGATACTTCACAGTTCCGGTGTCTGTCTTAGTATATAA
TGAAACTATTTTAAATAAGCGAGCAAATTTCCAGCTACCAGAACAGATAATTTAACAATTTCTATGAAGTGTCAAGCCACGAGACAGAATCATATATT
3380
ACCCCACTCTGTAACCTTTCTAAAGCGGAGCCAAAGAGAAGGTGCTTTATCGTGAATTTGATGCGGACGATCAAAAATATTATTGGGAATACCTGCTTA 3'
TGGGTGTGAGACATTTGAAAGATTTTCGCTCGGTTCTTCTCCACGAAATAGCACGTTAACTACGCTGCTAGTTTATATAAACCTTTATGGACGAAT 5'

FIG. 2—Continued.

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

No primary sequence similarities between the restriction endonuclease R · *LlaBI* and the corresponding methylase M · *LlaBI* or other type II restriction endonucleases were found. As shown in Fig. 2, the deduced amino acid sequence of M · *LlaBI* has all the characteristics of subclass γ of the m⁶A 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 *LlaBI* system, it was found that subclones of pJW563 containing fragments of the *LlaBI* methylase were not resistant to *PstI* restriction, although plasmid pJW563 was resistant to digestion by the *PstI* restriction endonuclease (data not shown). The *PstI* endonuclease recognizes 5'-CTGCA ↓ G-3' and cuts as indicated by the arrow. *LlaBI* can recognize the same sequence, 5'-C ↓ TGCAG-3' (and 5'-C ↓ TATAG-3'), but cuts the recognition sequence at a different position (*LlaBI* generates 5' overhangs while *PstI* yields 3' overhangs). This indicates that the adenine in the *PstI* recognition sequence has been methylated by the *LlaBI* methylase. This reinforces the assumption that M · *LlaBI* is an m⁶A 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 *PvuII* R/M system (1).

The high C+G content in the recognition sequences of *ScrFI* (5'-CC ↓ NGG-3') and *LlaI* (5'-CC ↓ WGG-3'), in contrast to those of *LlaAI* or *LlaDCHI* (5'- ↓ GATC-3') and *LlaBI* (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 *LlaBI* genes is 27.8% (31.5% for R · *LlaBI* and 25.7% for M · *LlaBI*), 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 *LlaBI* 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

- Adams, G. M., and R. M. Blumenthal. 1995. Gene *pvuIIW*: a possible modulator of *PvuII* endonuclease subunit association. *Gene* **157**:193-199.
- Andresen, A., A. Geis, U. Krusch, and M. Teuber. 1984. Plasmidmuster milchwirtschaftlich genutzter Starterkulturen. *Milchwissenschaft* **39**:140-143.
- 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.
- Davis, R., D. van der Lelie, A. Mercenier, C. Daly, and G. F. Fitzgerald. 1993. *ScrFI* restriction-modification system of *Lactococcus lactis* subsp. *cremoris* UC503: cloning and characterization of two *ScrFI* methylase genes. *Appl. Environ. Microbiol.* **59**:777-785.
- 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.
- 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.
- 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.
- 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.
- 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.
- 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.
- 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.
- Mayo, B., C. Hardisson, and A. F. Brana. 1991. Nucleolytic activities in *Lactococcus lactis* subsp. *lactis* NCDO 497. *FEMS Microbiol. Lett.* **79**:195-198.
- Moineau, S., S. A. Walker, E. R. Vedamuthu, and P. A. Vandenberg. 1995. Cloning and sequencing of *LlaII* restriction/modification genes from *Lactococcus lactis* and relatedness of this system to the *Streptococcus pneumoniae DpnII* system. *Appl. Environ. Microbiol.* **61**:2193-2202.
- Nyengaard, N., F. K. Vogensen, and J. Josephsen. 1993. *LlaAI* and *LlaBI*, 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.
- Nyengaard, N., F. K. Vogensen, and J. Josephsen. 1995. Restriction-modification systems in *Lactococcus lactis*. *Gene* **157**:13-18.
- O'Sullivan, D. J., K. Zagula, and T. R. Klaenhammer. 1995. In vivo restriction by *LlaI* is encoded by three genes, arranged in an operon with *llaIM*, on the conjugative *Lactococcus* plasmid pTR2030. *J. Bacteriol.* **177**:134-143.
- 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.
- 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.
- Terzaghi, B. E., and W. E. Sandine. 1975. Improved medium for lactic streptococci and their bacteriophages. *Appl. Microbiol.* **29**:807-813.
- Twomey, D. P., C. Daly, and G. F. Fitzgerald. 1993. Sequence of the gene encoding a second *ScrFI* m³C methyltransferase of *Lactococcus lactis*. *Gene* **136**:205-209.
- von Wright, A., S. Wessels, S. Tynkynen, 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.
- 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.
- Wilson, G. G. 1992. Amino acid sequence arrangements of DNA methyltransferases. *Methods Enzymol.* **216**:259-279.