

## A Type IC Restriction-Modification System in *Lactococcus lactis*

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**Three genes coding for the endonuclease, methylase, and specificity subunits of a type I restriction-modification (R-M) system in the *Lactococcus lactis* plasmid pIL2614 have been characterized. Plasmid location, sequence homologies, and inactivation studies indicated that this R-M system is most probably of type IC.**

Restriction-modification systems (R-M) are an effective barrier to protect strains from phage infection. As expected for bacteria which are under strong selective pressure in the dairy environment, due to the presence of bacteriophages, a number of R-M systems have been found in *Lactococcus lactis* strains (18). However, only four have been studied in detail. Three are of type II (5, 9, 31, 33, 34, 41) and one, composed of three genes associated with restriction activity and a type II's methylase, is unclassified (35). We previously established that plasmid pIL2614 codes for both the Abi420 phage abortive infection and for an R-M activity (efficiency of  $10^{-4}$ ) (36). To characterize the R-M system, plasmid pIL2614, extracted from strain IL1403 (4), was sequenced by chromosome walking in cycle extension reactions, using appropriate primers, *Taq* polymerase, fluorescent dye-coupled dideoxynucleotides and an Applied Biosystems sequencer ABI-373. The DNA and protein sequences were analyzed with the Genetics Computer Group software (6), Genmark (3), and Blast (1) programs. A sequence of 8.6 kb (accession no. U90222), localized upstream of the *abi420* genes, revealed five open reading frames (ORFs). The organization of this sequence and the general features of the ORFs are summarized in Fig. 1.

The *orf1*-specified protein has 66 to 78% identity with replication proteins designated RepB from lactococcal plasmids pSK11 (19), pSL2 (20), pCI528 (29), pUCL22 (11), pSV40 (44), pCI305 (17), and pWV02 (22). All these plasmids belong to a family replicating via theta intermediates. They have structural and DNA sequence similarities at the replication origins (11). This origin is composed of two 10-bp repeats ([T/A]TATATTT) spaced by 3 bp and followed by an AT-rich core containing CG clusters. This core is followed by three 22-bp repeats (TATAn<sub>7</sub>AAAAAnCn<sub>2</sub>TG [where n stands for any base pair]) and one that is truncated (11). The -35 box of the promoter is located immediately downstream of this origin. All these features are present upstream of *orf1*. There are two 10-bp repeats (ATTATTATTTn<sub>3</sub> TTATATATTT), an AT-rich core, three 22-bp repeats (CTTATACCTAGAAAAAC AATG), one truncated repeat (CTTATACCTAGAAA), and a putative promoter sequence (TTGTATn<sub>17</sub>TATAAT). Therefore, *orf1* and the upstream DNA sequence are most probably involved in pIL2614 replication that proceeds as described previously for plasmid pUCL22 (11). RepB initiates replica-

tion by binding to the origin. This replication is bidirectional and would be under the control of RepB itself (11).

The *orf2*-specified protein has 53 and 57% identity with proteins encoded by *orfX* genes present downstream of *repB* on plasmids pUCL22 (11) and pJW563 (15), respectively. OrfX does not participate in plasmid replication, and its function remains unknown (11). The homology lies in two domains localized at the N and C terminal parts of the *orf2*-encoded protein. Between these two domains, this protein comprises a long helical domain that contains 10 repeats of 11 amino acids (aa) ([N/D]SLEDKQEKKA). These repeats are hydrophilic, and the protein is acidic (P<sub>i</sub> 4.5). Therefore, Orf2 is probably neither involved in DNA binding nor membrane anchored as expected for proteins with such repeats.

The *orf3*-specified protein has 29.8, 28.4, and 24% identity with the *Escherichia coli* EcoR124II (8), *Mycoplasmata pulmonis* (7), and *Haemophilus influenzae* (10) endonuclease (R) subunits of type IC R-M systems, respectively. This homology is particularly high at the level of seven helicase-like domains (14, 32) (Fig. 2). Conservation of these domains suggested that the R subunits of type I R-M enzymes may possess helicase activity, playing a role in local unwinding of DNA at the cleavage sites and in DNA translocation (14). Recently, Titheradge et al. (40) identified two additional domains (X and Y) which were well conserved among the four enzymes presented in Fig. 2. Moreover, a 10th domain, localized between domains IA and II, designated Z, is well conserved in R subunits of type IC (Fig. 2). Conserved domains and sequence homologies suggest that *orf3* codes for a type IC endonuclease subunit.

The *orf4*-specified protein has 36.9, 36.9, and 35.5% identity with the *E. coli* EcoR124II (21) and *prr* (42) and *M. pulmonis* (7) methylase (M) subunits respectively. This identity is in agreement with those (32% [38]) usually found for M polypeptides, thus indicating that Orf4 could be part of a type IC R-M system. An alignment of the sequences is shown in Fig. 3 (because of the identity between EcoR124II- and *prr*-encoded M subunits, only that from EcoR124II is shown). Two sequence motifs conserved in the adenine methyltransferases (MTases), motif CMI ([D/E/S]X[F/A]XGXG) and motif CMII ([L/I/V/M/A/C]X[D/N]PP[Y/F]) (24, 28, 45) are present in Orf4 (Fig. 3). Motif CMI, found in both N and C MTases is the binding site for the cofactor S-adenosylmethionine. This has been shown by mutational analysis of EcoKI N<sup>6</sup>-adenine MTase (45) and by crystallographic studies of C<sup>5</sup>-cytosine HhaI MTase (23). Motif II probably plays a role in catalysis (45). The aromatic residue has been shown to be essential for methyl group transfer (45). Moreover, the nature of the conserved amino acid residue preceding PP(Y/F) is characteristic for different classes of MTases and correlates with the base

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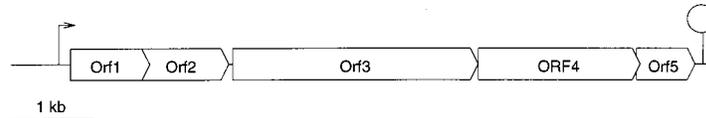


FIG. 1. (Top) Organization of the pIL2614 sequenced fragment. A putative promoter sequence is indicated by an arrow above a vertical line. (Bottom) Features of the ORFs. Start and stop numbers refer to positions in the sequence, the corresponding codons are shown in parentheses. The putative ribosome binding site and the beginning of the ORFs are shown in capital letters.

Gene designation	Start	Stop	Product size (aa)	Similarities	Translation start
1	593 (AUG)	1753 (UGA)	386	RepB	AAGGAGcaacttctc ATG GAA ATT
2	1750 (AUG)	2628 (UGA)	292		AAAGGcaGAtttatc ATG AGT GAA
3	2639 (AUG)	5716 (UAA)	1025	HsdR	AGGgGaatcaa ATG AGT CAT
4	5716 (AUG)	7311 (UAA)	531	HsdM	GGAAaGaaGaatata ATG GCG ACA
5	7301 (AUG)	8518 (UAG)	405	HsdS	AGGcGgcoatg ATG AGT AAA

methylation specificity of the enzymes (39, 45). Therefore, depending on the nature of this amino acid (D, N, or S) and on consensus sequences at two additional conserved motifs (CMIs and CMIII), the subdivision of the *N*<sup>4</sup>-cytosine and *N*<sup>6</sup>-adenine MTases into five classes has been proposed (39). The presence of the NPPY motif together with sequence conservation at the two other domains (Fig. 3) suggests that Orf4 may be an *N*<sup>6</sup>-adenine MTase (N12 class). Moreover, for the three en-

zymes compared in Fig. 3, the CMIs motif defined by Timinkas et al. (39) can be extended to the 22 upstream amino acids.

Four additional domains, conserved among DNA methylases of type II R-M systems, have been identified by structure-guided analysis (30). These domains, not clearly apparent in the MTases compared in Fig. 3, could be absent from type I MTases. Two other motifs are well conserved among the three MTases aligned on Fig. 3: GOEX<sub>4</sub>TXNLARMNX<sub>2</sub>L located

RpIL2614	MSHSEQMIE--NQFIQLSEKENQW---YRPLDKSEELQCNFRGHLNRINLAVLEBQL	RpIL2614	RSVVKKFKVKNQFPFMSAILLTTHSIAQAKHYIRLTKEMKDNQ---TLLNGRQFDRHRLLID
RpIL2614	MTQHTHTIAESNNFIVLDKRYKARPTGDSVQSESDLERELIQDLRNGG---YEFISVKSQ-	RpIL2614	-----TFPGSKGFNAMLAVSVDAARAYATPKRLQREANKSATYKPLRIATIPFSAAN
RpIL2614	MSRNELLYE---KEFVDDLKVNQKY---VKLDIKNEEKIFELIPENIGRLNINIELTQQ-	RpIL2614	-----FTNNRN--YNSIIAPDTIQDALTFYDEPY---KMDVG---DIFATPIFSSYSN
RpIL2614	MLNENDIEQLTLQRLQSLGWEYRYGKDLVPHGKFAFGDLGSGVVFVQELREAVRKLNPQ-	RpIL2614	-----SQE-RLLELAADFVQHFARNEVVDVDS--RAMVVS---SRQICVDVLYNQIIA
RpIL2614	LFDKPEKQVQVVEFSRLTCTPPLASQWLKQ---ENGVAQVLLEREDGKRVTLKFAFRND	RpIL2614	KDFPRVAIFSTNPDRLKNEQDDE--LVELIMKAYQPDASPSYQDQKLYNQINIKRLAK
RpIL2614	--SAMLAVREQLQNLANGVFNDSERRRFTQGYLDNFSDGLLKRKTHIDYICDFIFDD	RpIL2614	EQWALGELISDEFTDTSAMDSSAKPELDAARIEYNSHFXTMFTDSS--NFGQVYRLDAQ
RpIL2614	--NILDLRELLSNGSASSVRFPAQYLAG---PDTVKIYKNDGPKKIKLRFKVDWE	RpIL2614	EE-----KNEKFPNLKHEKHEK---ILKRYEKFNTSKRVEG---FDKYMVDQWR
RpIL2614	PRSAVDSVVKSATKSDZGLDVLVRRNQTFYK---LLRDGVRVEYQTQNGEQKIEHVRLLVD	RpIL2614	LH-----PEWHSNDINEGAIK---IVMTGSASDTPMEQKH--IYSQKQKQLLRR
RpIL2614	-----ISGG---TSSYEVVHVQVVP---DSSRVDRG-DVSLILINGLPIIHLELQKES-	RpIL2614	-----X-----
RpIL2614	ERLGNLYLIDKMKLNMRKVNQIQOPEQ---AGSHANRY-DVTLVINGLPLVQLELKRK--	RpIL2614	-----V-----
RpIL2614	-----NAN---NEFYVLQFPTRDAANNMGRF-DSLILINGLPLVLEFKDK--	RpIL2614	-----VI-----
RpIL2614	-----FEHWG---NNRFVAVVQLEIR---SRGGKRIPDIIIGFVNGLPLVLEKLNPLR	RpIL2614	EKQYQSDGQWLDVFIIVDRLLTGFDSFPAITLYIDRENNYQKLLQAFSRTNRIYTG--K
RpIL2614	AKDGFMAQYQIQR--YAEDGFFKQ---IYATQIMVTPNKVDTRYRPAESDTEAAYARKM	RpIL2614	VK-NQ-D--IDLLIVVGMFLTGFDAPTLNTLFDKRLRYHGLMQAFSRTNRIYDAT--K
RpIL2614	-GVAIREAFNQIHR--YSKESFNSENSLFKYLQLEVISNGTDTRYFAN---TTKRDKNFS	RpIL2614	PKEYNSENNSSIDIVIVDMLLTGFDSFRTNTLYINKEIKHNLIIQAFSRTNRLSDYS--K
RpIL2614	-SENINKAINQIDESYRGSVLNKG---IFRFIQLIGSNPEEVKFLAN---NKRTNNNK	RpIL2614	FKDPN-DP--LKVIVDRMMLTGFDAPOCCWYMLDCKPKGHNLLQALARVNRVFAKNSR
RpIL2614	ESADLLQAFNQFET--YKDEIAE---LFFVNGALIISDGIVARIGLSL---ADFQRFPTW	RpIL2614	-----
RpIL2614	KELFNMRTEFN--QTVSDLDFTRTVLRIIPDAHELISQYITLIVDDPKNPKFIMALRPFQIH	RpIL2614	DSGLLIVSFRKFPMTKENVQNTFRPLPSNEN---QNFDQLIP---REYEEVKKEFTECS
RpIL2614	DFTMWAKSDN--TLIKLDKDFATCFQKHTLNAVLYNYSVFDSSQ---TILVMRPFYQIA	RpIL2614	TFGNIVTFRDLERSTIDAITLPGDKNTKVVLEKSYTEYMEGFTDAATGEAKRGMFTVVS
RpIL2614	ILSGFTWSENGGSSKELIKDFLAKNALLEYLRYVVFQSSKDEK---TILRPFYQQR	RpIL2614	KRGIIVNFSLEEQSINDAFKIYANSSDKE---IQQLVYGE---KYEQVVEDPFINPWN
RpIL2614	KVDEKNSARLYPDDLEQLSGLANGMLQPEDLLDIYRYVLFERDSVGRTKIKTAAYQY	RpIL2614	NGGLIVDYVGLAKELRAATQYVNTS'GKG---QLAEDVQSVFFKMKQLEFIRTLFAI
RpIL2614	-----I-----	RpIL2614	-----
RpIL2614	AIRKIRPKAA-----QHGGFIWHTGSGKTTIS--FVATKLLAQAIGAIVDRTVM	RpIL2614	LYKQSEADLSNP---HDKMTMIAQVSAYQKLGKSYKAFPSYDQYED-----
RpIL2614	ATERILWTKRSSFTA--KNWSKPESSGGYIWHHTGSGKTLTS--FKAAELATELDF--IDKVFV	RpIL2614	ELQRFPDPTLSESEKEDFKLFGFELRANLQVDFEATLRAKQID--LSDPVA
RpIL2614	AIKKAINFVEKQLTNDLAKHNINNAIYIWHHTGSGKTLTS--YKIAEELSKNSD--IDHVVV	RpIL2614	SLKISKSNYDEKN--NEIFNLSLENKKKYLKNSLQVSNIPSLKTFEYIG--KNEK--
RpIL2614	GVNEAVDSTIWTATSE---KDRRGVMWHTGSGKTSMLFYAGKLLAQPELKNPTIVV	RpIL2614	PIEGKTFDVQAALE--KDNPNDLMAIRPAAHILSLDQLSPDGKAHQWHPNKKETEPR--
RpIL2614	-----IA-----	RpIL2614	-----
RpIL2614	VVDRDLDAQTQDEFKFASEYHTGQTTGNSVANTLIVGKKNQQLARNLLSKNNNTIL	RpIL2614	THKVDVDSFYINQLLKAIQLNAGAVEKPEKEIQQ---KDPQIQMYYHTLKDQLVNT
RpIL2614	VVDRKLDYQTKKEYQRFSS---PDSVN---GSENTAGLKNRNL--KDDNKII	RpIL2614	SPTDMDVVEVDLKSQENLNDYILGLTFEHNRNKNG--KGEIMEVKKRLIRSSLGNR
RpIL2614	VVDRNLDQTSQTFFQKLSMS---SSKNEK--IDPLNODTSKLDYEIFLKKKELI	RpIL2614	YLENLDFNKKIKSKYNNRNLVYEDTLESDIKHQLTKNNYNOGKINQKSYEIFLLVQVK
RpIL2614	VVDRNLDGQLFQTFSSGK-----DLIKQTP--QVVEDRDLRQLLAAQ--NEVGGVF	RpIL2614	LVNQTVYSBGVLDLFDLLEKQPQIGLSLSEEFQTVKNSPTKMLVWSAMERYLASEIKVK
RpIL2614	-----Z-----	RpIL2614	-----
RpIL2614	VFTIQKLSAAMRQAQSEEEKGNOFEKLRQEHIVPVIDEAHRAVDEEMKR-----	RpIL2614	TEEIDVAQ-----LKETS-----IQNEI-----QRLL--QKEAREBFGLSF
RpIL2614	VFTIQKLNLMK---AESDLPVN---QVVFVDECHRSQGFGEAQN-----	RpIL2614	AREGLVVD-----FIQOT-----NLDDLDPKASIIDAFPTFAREQ--QREABALIKE
RpIL2614	VFTIQKLNLLSSYKNEKLEIFLN---KRFVFIIDECHRSNAGLMGRK-----	RpIL2614	KNEIKNFF-----IKKDK-----SLDEKEFIDYGRKILKSVFKVKNQIEAMWLEKIL
RpIL2614	VFTIQKFLALNEB---ESRFPILE---RNNIIVLDEAHRHSYQFTKHLNGKFTQG	RpIL2614	SGTNLTQKDFERRKLEALNQYHNNHLLVVEILDLPKMSQDFQERIALGKLLGTLKEEL
RpIL2614	-----III-----	RpIL2614	-----
RpIL2614	----IKKILPNSTWFGLTGTFIFEANKKQENQFARTTSQQYQGLHLSYTTKNAMDDGAV	RpIL2614	DFLOQAM-NEYQGDKKAIPYLTHLSDMTLS-----KEEPEKAGE
RpIL2614	----LKKPKRYYQFGFTGTFIPFN---ALGSETTASVFGREHLSYVITDARDEIV	RpIL2614	NLNEDAA--KRYIRTSLKREYATENG'ELNET-----LPKLSPIN--PQ
RpIL2614	----IKDFLANNISIMIGSFTGTFEEN---NDRFQKTIQNEIDSYNKKDAILDKVNI	RpIL2614	HYGHGIN--NDQIRKDKWRKINDKDLDEKSE-----FIKKWSRRSEVVDKDIIDK
RpIL2614	VARHLRDLAPNATIGFTGTFISLEED---KD--TQDFVGRYVSYDLDQAVDEGAT	RpIL2614	AFYEAALSQNSAKLMDGDEVLSKLAKEITETLRKSVTIDQYKAEVRAIRILLVRRALQK
RpIL2614	-----Y-----	RpIL2614	-----
RpIL2614	LGQFVYHSLISEEDLEIVTQLNKGKLPGDALQQEELLPAELYEKDEHIRTMLQKIFNR	RpIL2614	YRRRPKV--LEERLRQNFQIQKWKBEL---
RpIL2614	LKFKVDYNDV---PQFKSLETETDEKLLSAAENQQAFLHPMRIQETIYILNFRQKTRH	RpIL2614	YVTK---QAVFRKSSRLRLSLKA---
RpIL2614	LGQF-----KVMYQYQETRIFFENN---NSNLGKIKSIIINVIKSKHLD	RpIL2614	LSIEYKESTETFLDEPKMKKLESKI---
RpIL2614	VFIYVDDAR-----QIRLKRKHDFALFAEID-----ALLEEPPSTSLRLREKLLG	RpIL2614	YKYPPDK--QBAVTVVYKQAEIADLTLG

FIG. 2. Alignment of the predicted amino acids of the pIL2614 HsdR peptide with the R subunits of *EcoR124II* (8), *M. pulmonis* (7), and *H. influenzae* (10). Sequence accession numbers are U90222, X13145, L25415, and L45919, respectively. Helicase-like domains I to VI (14) and X and Y domains (40) as well as the additional conserved domain Z are shown in boldface letters. Conserved amino acids and conservative or semiconservative substitutions are indicated by an asterisk and a period, respectively.

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MLlac2614 MA-----TGLNQLQWASADILRGKMDASEYKNVLLGLIFPKYLSDAQLREVVEQENGK
MEcoR124II MKMTSQQRAELHRQTKTANDVRSVGVGDFKQVVLGALFVYFISEN--PSSVYLEAGDD
MMycoPlasm MS----NSKELTAVKTKCDQLRSKRVETVRYIYIMHLEFFKYLSE-QSKNFSEPKER

MLlac2614 TDTPPERSTQYAGFMWEYEDKDDLEENIQPKQGVYIQPDQLFYSYRIKADNYEENLTDL
MEcoR124II SICVARKLDDSVIT-----DDIKDDAIKT---KGVFTYPSQLPCNVAARKINDRLNADL
MMycoPlasm VD-YIK---YSEFDENHEQFKIKELIINQDDDFFLAYKYSFQNVVDMQKGVNVIPTI

MLlac2614 QAGFNELERQGEFFS-----GLFADIDLNSKILGSMALLRNVTITEVLRALDEIDL-
MEcoR124II NSIPVAIESSAYGVYSEAD--IKGLFADFDTTNSRLGNVTKDKNARLAAVLKGVEGLKLG
MMycoPlasm EESFNKIESINSELNDEKKEFFKDLFTNIDFSNKNLGNIDEEKKTQLIITKEINTLNLS

CMI
MLlac2614 -P-EHNGDVIQDAVEYLIGEFASSAGKKAAGEFYTPQAVSKIMSEITSIGQETRAPPHIVD
MEcoR124II DFNEHQIDLFGDAVEFLISNYAANAGSGGEGFFTPQHVSKLIAQLAMHQTHVN--KIYD
MMycoPlasm --MDEVDFHGMTEYELLSEFASDTGKKAAGEFYTPSKVSELLVKIVSHGKNKIN--KAYD

CMI
MLlac2614 PAMSGSLMLNTR--VLNPPQVYHHQQLLNTTTEENLARMNLILHGDKERMYLANAGDPL
MEcoR124II PAAGSGLLLQAKKQFDNHIIEEGFPGQINHTTYNLRMMPLHNIINVDKFDIKLGNL
MMycoPlasm PACGSGSLILKLANKGVKY---NKIYQGVKTKATYNLRMMNLILGVFSPKLDLRSQDPL

CMI
MLlac2614 -DADWPEEPYQPDVSVCMNPPYSAKMSAADQ--FLSDPRFRFGKILAPSKADFAFLHGG
MEcoR124II TEPHFPRDEKP--PDAIVSNPFFYKMGSDDEPTLINDERFAPAGVLAPEKADFAFLVLA
MMycoPlasm INP-LHIEEGSFGDVIKAVNPFYKGMNPTQE--LSKDRYNSYPSGLAPSKADFAFLVLA

CMII
MLlac2614 FYHL-KEGTMGIIVLPHGVLFPGA--AEGTIRQALLEMGDAIVGLPANIFFGSPKPT
MEcoR124II LNYL-SARGRALIVCFPGIYRGG--AQKIRQYLVNDDNYVETVLSLANLFFGPTIAVN
MMycoPlasm LPHVNDKDGILIASVFLSILSRSKSPABEDIRKYIDKNIYIDTIIFFPNLFFNTSIESC

MLlac2614 VIIKRNRS--RRDVLFDASQDFEKRNQNVLLDEHIDKIVSIHKREDIERVAHVAF
MEcoR124II ILVLSKHT--DTNVQFIDASELFKETTNNLITDAHIEQIMQVFAKEDVAHLAKSVAF
MMycoPlasm IIVARKNKPTNDKRIEMINATKEIQNAKQNTLSDENINRIFSAAKKEKEEMNFSKYISY

MLlac2614 DEIQENDENLNIIPRY-VDTPEEE-EPVDLIVANNLLKINEELVQQGVLLSMIDNFAES
MEcoR124II ETWVANDYNSVSSY-VEAKDNK-EIIDIABLNAELKTTVSKIDQLKIDDAIVAEIEG
MMycoPlasm EDIVKNEYSLSMRFYDLNDPESEEDIDIDFVSEIVKINEBLLKYENEFKMLNPELN-
EDIVKNEYSLSMRFYDLNDPESEEDIDIDFVSEIVKINEBLLKYENEFKMLNPELN-

MLlac2614 EENQALIESMRLRLRGHDE
MEcoR124II EVOK-----
MMycoPlasm -----KKN
    
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	CMI	CMI	CMI	CMI
N12 class	G-ffTP--aschhhp-h	hLnPscCGsGsha-sh	FdhahsNPPF	h---hphLpsGG-LshahP
All	--s-P--Lhp-hhphh	hLnPfhGsh-sh	hDhhhgPPY	h--h-p-LpsgG-hhhh-s

FIG. 3. Alignment of the predicted amino acids of the pIL2614 HsdM peptide with the M subunits of *EcoR124II* (21) and *M. pulmonis* (7) (GenBank accession no. U90222, X13145, and L25415, respectively). Conserved amino acids and conservative or semiconservative substitutions are indicated by an asterisk and a period, respectively. Conserved motifs (CMI, CMI, CMI, and CMI) are shown in boldface letters, and the proposed consensus for the N12 class and all MTases (39) are indicated below. Different groups of amino acids are indicated as follows: p, polar (D, E, N, H, K, R, S, Q, and G); h, hydrophobic (W, F, I, L, M, V, A, P, Y, C, and T); n, negatively charged (D and E); f, aromatic (F, W, Y, and H); a, aliphatic (I, L, V, and M); c, charged (D, E, K, R, and H); and s, small, nonbulky (G, A, S, T, D, N, P, and V).

downstream of CMI and LAPKSKADFAF located just upstream of CMIII. They could be significant in relation to special properties of MTases of the type IC R-M systems.

The *orf5*-specified protein has 26% identity (45% homology) with the putative specificity (S) subunit of a *Spiroplasma citri* type I R-M system (27). Moreover, Orf5 presents structural organization characteristics of S subunits. Two repeats of 38 aa (designated A and A'), present in the central and the C-terminal part of the protein, respectively, have 87% identity. Parts of these repeats are homologous to the repeats (24 aa) identified in all S subunits from type I restriction enzymes (21) (Fig. 4). Two split repeats (designated D and D'), characteristic of type IC S subunits (25), are present in the N-terminal and the central parts of Orf5. Homologies between the central conserved domain and sequences near the N and C termini were proposed to favor a circular organization of the domains of the S subunit, which provides the required symmetry for interactivity with the M subunits and the target DNA sequence (40).

These sequence homologies and gene structure suggest that *orf5* codes for an S subunit which is part of an R-M system including the R (Orf3) and M (Orf4) subunits described above. Based on amino acid identities observed for both R and M, this system must be of type IC. However, the *L. lactis* HsdS protein lacks the TAEI direct repeats characteristic of S subunits of

type IC enzymes (37), the number of which has been shown to determine the length of the nonspecific spacer between the specific domains of the recognition sequence (2). Nevertheless, these repeats are absent in the S subunit of the type IC R-M system of *M. pulmonis* (7) as well as in S subunits of other type I enzymes.

In order to confirm that the region from Orf3 to Orf5 confers the R-M phenotype, a 5,358-bp *EcoRV-SacI* segment, from position 2968 (309 bp downstream of the start codon of *hsdR*) to position 8326 (192 bp upstream of the stop codon of *hsdS*), was deleted from plasmid pIL2614. This segment was replaced by a chloramphenicol resistance cassette recovered from plasmid pGKV259 (43) and previously cloned in pBlue-script plasmid (pIL1388) (1a). The construct was designated pIL1032. Phage bIL170 propagated on strain IL1403 showed efficiencies of plating of  $3 \times 10^{-3}$  and 1 when plated on strains IL1403(pIL2614) and IL1403(pIL1032), respectively. Phages picked up from plaques formed on the pIL2614-harboring strain were no longer restricted by this strain. In contrast, phages picked up on the pIL1032-harboring strain were still restricted by IL1403(pIL2614) with an efficiency of plating of  $5 \times 10^{-3}$ . The loss by pIL1032 of the aptitude to restrict and/or modify the growth of phage bIL170 indicated that the region from Orf3 to Orf5 confers the R-M phenotype. In contrast, pIL1032 still conferred the Abi420 phenotype active on the phage bIL41 (35).

Genes of type I R-M systems of enterobacteria are arranged into two contiguous transcription units, with *hsdM* and *hsdS* forming an operon and *hsdR* being transcribed from its own promoter. The order of the two transcriptional units is different for different families (46), and this has been proposed as an additional evidence for a horizontal transfer of the *hsd* genes (40). This organization differs in *M. pulmonis*, in which the gene order is *hsdS hsdR hsdM*, with only one promoter upstream of *hsdS* and the expression of the genes being con-

		D
SLlac2614	MMSKSPQLRFEQFTDDWEERKFGVEVWVKSSERNLNLEYSPKQVLSVAQMKNLNSPDRNEQ	
SLlac2614	DDYMKTYNVLKKGDIAFEGNKKSKSFAFGFRVLDLDDQGVIVSHVFXYVRPICKMDTDFMIV	A
SLlac2614	YINNESVMKYLVLKATTKTLMMTTLNKTDKIVPKPLNPSLEEQKIGSFFKQLDAPIALH	D'
SLlac2614	QRKLDLLKEQKQYFQKMFPKNGAKVPELRFAGFADDWEDRKLGLASFGKNGYTKNDL	
SLlac2614	VEFGDPIILYGRLYTKYETVIEKVDTFVNKKDKSIIISGGSEVIVPASGESSEDISRASVV	
SLlac2614	GRSGIILGGDLNIIKIPVNIYDIFLALPITNSGQQKEMSKRAQKGSVVHLHNSDKQVNI	A'
SLlac2614	LVPKLEEQKIGSFFKQLDNTIVLHQRKLDLFLKEQKQFLQKMF*	
		Repeats of 24 amino-acids
	consensus	IP*ppI*EQ-rIV*d*1-al*d
Central domain	SEcoR124II NPEKSLAIQSEIVRILDKFTALTA	
	SMycoplasma LP--NLKIQSAILKIEPKEDLFF	
	SSpiroplasma IP--SLQEQTKIVNFFSIIIDRKE	
	SLlac2614 LP--SLEEQKIGSFFKQLDAPIA	
C terminal domain	SEcoR124II IPVFNINEQRIIVEILDKFDTLTN	
	SMycoplasma IP--NLKIQSAILKIEPKEDLFF	
	SSpiroplasma FVSLNYKQTKIANFSSIIDR-KIE	
	SLlac2614 LP--SLEEQKIGSFFKQLDAPIA	

FIG. 4. Predicted amino acid sequence of the pIL2614 HsdS peptide. Thirty-nine-amino-acid repeats (A and A') and split repeats (D and D') (25) are shown in boldface letters. The consensus for the 24-aa repeats present in all S peptides (21) is indicated below, together with sequences from *EcoR124II* (21), *M. pulmonis* (7), and *S. citri* (27).

trolled by inversion of a DNA element (7). In *L. lactis*, the absence of consensus sequences for a promoter upstream of *hsdR* and *hsdM* together with gene organization and the presence of a putative terminator structure downstream of *hsdS* (GCCCTAAGATCTAACCTTTATATCTTAGGGGCTATTT TTTT) suggests that the five genes identified could be transcribed from the promoter located upstream of *repB*. However, as weak promoters transcribing type I genes are difficult to spot in DNA sequences, functional analysis will be needed to identify transcriptional units. It has been proposed that autoregulation of the RepB protein could be under the control of heat-shock proteins (11). If this were true, *hsd* genes would be activated under stress conditions and therefore perhaps after phage infection.

Type I R-M systems are able to evolve rapidly. A single subunit that concomitantly confers sequence specificity to both restriction and modification facilitates the acquisition of new specificities. Moreover, an S polypeptide has two recognition domains, each specifying one component of the bipartite target sequence (2). In a given family of S polypeptides, the two variable recognition domains are separated by a conserved core sequence. It has been established *in vivo* (12) and *in vitro* (13, 16) that the *hsdS* genes can recombine at the level of the conserved domain, creating a functional R-M system with an entirely new specificity.

In conclusion, our report describes the second functionally characterized (7) and the third (47) type I R-M system described for gram-positive bacteria. Its location on a plasmid and probably under the control of its replication machinery could both increase plasmid stability by postsegregational killing of plasmid-free cells (26) and possibly allow activation of the R-M system by the stress due to phage infection. This, in addition to the facility to acquire new specificities, confers an obvious selective advantage. Therefore, plasmid-encoded type I R-M systems are likely to be widespread in the *L. lactis* species and possibly other bacteria exposed to phage-abundant environments.

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