Characterization of the LlaCI methyltransferase from *Lactococcus lactis* subsp. *cremoris* W15 provides new insights into the biology of type II restriction–modification systems

Iwona Mruk, Magdalena Cichowicz and Tadeusz Kaczorowski

Department of Microbiology, University of Gdańsk, Kładki 24, 80-822 Gdańsk, Poland

The gene encoding the LlaCI methyltransferase (M.LlaCI) from *Lactococcus lactis* subsp. *cremoris* W15 was overexpressed in *Escherichia coli*. The enzyme was purified to apparent homogeneity using three consecutive steps of chromatography on phosphocellulose, blue-agarose and Superose 12HR, yielding a protein of *M*$_r$ 31 300 ± 1000 under denaturing conditions. The exact position of the start codon AUG was determined by protein microsequencing. This enzyme recognizes the specific palindromic sequence 5'–AAGCTT–3'. Purified M.LlaCI was characterized. Unlike many other methyltransferases, M.LlaCI exists in solution predominantly as a dimer. It modifies the first adenine residue at the 5' end of the specific sequence to N$_6$-methyladenine and thus is functionally identical to the corresponding methyltransferases of the HindIII (*Haemophilus influenzae* Rd) and EcoVIII (*Escherichia coli* E1585-68) restriction–modification systems. This is reflected in the identity of M.LlaCI with M.HindIII and M.EcoVIII noted at the amino acid sequence level (50 % and 62 %, respectively) and in the presence of nine sequence motifs conserved among N$_6$-adenine β-class methyltransferases. However, polyclonal antibodies raised against M.EcoVIII cross-reacted with M.LlaCI but not with M.HindIII. Restriction endonucleases require Mg$^{2+}$ for phosphodiester bond cleavage. Mg$^{2+}$ was shown to be a strong inhibitor of the M.LlaCI enzyme and its isospecific homologues. This observation suggests that sensitivity of the M.LlaCI to Mg$^{2+}$ may strengthen the restriction activity of the cognate endonuclease in the bacterial cell. Other biological implications of this finding are also discussed.

INTRODUCTION

Lactic acid bacteria (LAB) are probiotic micro-organisms that have been used for millennia in the processing of dairy products. It was shown recently that dairy establishments in Britain can be dated back to 5000 years BC (Copley *et al*., 2003). Manufacturing of food such as cheese and yogurt, but also sauerkraut and dill pickles, as well as the existence of some regional cuisine, is heavily dependent on lactic fermentation. LAB and food processed by them therefore have armies of admirers, but on the other side, these bacteria have also powerful enemies. Their enemies are not people who hate buttermilk, but virulent phages whose existence relies on propagation within bacterial cells. To combat phages, LAB have developed multiple molecular strategies. These measures can be divided into four groups: (i) inhibition of phage adsorption, (ii) blockage of phage DNA injection, (iii) abortive infection mechanisms, and (iv) restriction of phage DNA (Forde & Fitzgerald, 1999; Coffey & Ross, 2002). These defence mechanisms are often located on plasmids. That feature can facilitate their horizontal spread among LAB and when acquired affords them protection against phage invasion (Allison & Klaenhammer, 1998). Among the defence mechanisms listed above, restriction–modification (R–M) systems form the most diverse group. Based on their molecular structure and cofactor requirements, R–M systems can be divided into four distinct types. Except for type IV, members of other types of R–M systems have been found in LAB (Nyengaard *et al*., 1995; Roberts *et al*., 2003a). Of these, enzymes belonging to type II are most abundant and possess the simplest molecular structure. The classic type II R–M system is composed of two enzymes: an endonuclease (ENase) that recognizes and cleaves a specific short DNA sequence (4–8 bp), and a methyltransferase (MTase) that modifies the same sequence in order to protect the host DNA against the action of the cognate restriction enzyme. Despite the

**Abbreviations:** AdoMet, S-adenosyl-L-methionine; ENase, endonuclease; LAB, lactic acid bacteria; MTase, methyltransferase; R–M, restriction–modification.

**Nomenclature.** The nomenclature for restriction endonucleases and methyltransferases in this paper follows the recommendations of Roberts *et al*., 2003b).
fact that both components of this type II R–M system recognize the same specific sequence there is a clear lack of similarity between restriction ENases and their cognate MTases at the amino acid sequence level (Chandrasegaran & Smith, 1988). Apparently, the ENases and their cognate MTases are products of distinct evolutionary processes. Lack of significant identity at the amino acid sequence level is also observed between isoschizomers, i.e. restriction enzymes isolated from different bacteria that recognize the same specific sequence (Roberts et al., 2003b). On the other hand, DNA MTases, unlike restriction enzymes, are often very similar, which suggests that they have a common origin. Apart from a unified core structure which is composed of a mixed seven-stranded β-sheet (Martin & McMillan, 2002), DNA MTases contain several characteristic conserved sequence motifs whose distribution reflects the ability of a particular enzyme to modify a defined base (adenine or cytosine) within a specific site (Bujnicki & Radlińska, 1999; Gong et al., 1997; Malone et al., 1995; Pósai et al., 1989; Wilson, 1992). The linear arrangements of these motifs made it possible to group DNA MTases into three distinct classes: α, β and γ (Malone et al., 1995).

Our research is focused on the nature of the isospecificity phenomenon among type II R–M systems, where we are especially interested in finding out what these enzymes have in common. As a model in our study we decided to use a group of systems isospecific to HindIII, an R–M system from Haemophilus influenzae Rd (Roy & Smith, 1973; Old et al., 1975). This group consists of over 30 R–M systems isolated from different bacteria, all of them recognizing the same specific palindromic sequence 5’-AAGCTT-3’ (Roberts et al., 2003a). To date, except for HindIII (Nwankwo et al., 1994), only two other systems, EcoVIII from Escherichia coli E1585-68 and LlaCI from Lactococcus lactis subsp. cremoris W15, have been cloned and sequenced (Kaczorowski & Szybalski, 1998; Mruk et al., 2001; Madsen & Josephsen, 1998).

In the present study, we investigated the properties of the M.LlaCI. The presence and distribution of nine highly conserved amino acid sequence motifs and a putative target recognition domain in the enzyme structure suggests that this enzyme belongs to the Nε-adenine β-class MTases. These motifs can be grouped into three clusters which are responsible for three principal functions: (i) sequence-specific DNA recognition (TRD, target recognition domain); (ii) binding of the methyl group donor – S-adenosyl-L-methionine (AdoMet) (motifs X, I and II); and (iii) catalysis of the methyl group transfer (motifs III, IV, V, VI, VII and VIII). The same motif organization was also observed in the case of M.HindIII and M.EcoVIII, both being isospecific to the M.LlaCl enzyme (Nwankwo et al., 1994; Mruk & Kaczorowski, 2003). Biochemical characterization of M.LlaCI led us to discover its unusual sensitivity to Mg²⁺. This particular property is shared with a few other MTases (e.g. M.EcoRI, Hanish & McClelland, 1988; M.FokI, Kaczorowski et al., 1999). We argue that this finding has far-reaching biological consequences. Most probably, the intracellular concentration of Mg²⁺ inhibits M.LlaCI activity without affecting the potency of the cognate restriction ENase. These circumstances clearly promote DNA restriction over DNA methylation, a scenario which seems to be profitable for bacteria fighting with invading DNA. However, it must be stressed that the observed inhibition of the modifying enzyme apparently does not affect the cell’s viability. In this article, we also argue that observed sensitivity of some MTases to Mg²⁺ can encourage the process of genetic recombination. Involvement of R–M enzymes in this process was raised in several reports (Chang & Cohen, 1977; McKane & Milkman, 1995; Kobayashi et al., 1999, 2001).

METHODS

Bacterial strains, plasmids and media. The bacterial strains used in this study were E. coli DH5α, MM294 (Sambrook et al., 1989) and BL21(DE3) (Studier & Moffatt, 1986). Bacteria were cultured in Luria–Bertani (LB) or TY medium (Sambrook et al., 1989). When required, antibiotics were added to media at the following concentrations: ampicillin (Ap), 100 μg ml⁻¹; chloramphenicol (Cm), 30 μg ml⁻¹. The following plasmids were used in this work: pSX1 (Ap⁺, LlaCI R⁻/M⁺; provided by Dr Jytte Josephsen, The Royal Veterinary and Agricultural University, Frederiksborg, Denmark; Madsen & Josephsen, 1998) as a source of the llaCIM gene; pGEM3Zf(+) (Ap⁺, Promega) as a vector in cloning experiments; pT7-6 (Ap⁺, Tabor & Richardson, 1985) as a vector in construction of the plasmid overexpressing llaCIM; and pLysS (Cm⁺, Studier, 1991) for M.LlaCI overproduction. M.EcoVIII and M.HindIII enzymes were overproduced using plasmids pT7-EcoVIII and pT7-6-M.HindIII, respectively (Mruk & Kaczorowski, 2003).

DNA isolation and manipulation. Molecular cloning experiments were performed by standard techniques (Sambrook et al., 1989). After cloning, hybrid plasmids were transformed into an appropriate E. coli strain. Recombinant plasmids were examined by restriction analysis and automated DNA sequencing by the dideoxy chain-termination method using Applied Biosystems ABI Prism BigDye Terminators and an ABI Prism 310 Genetic Analyser. Restriction ENases and DNA-modifying enzymes were purchased from either New England Biolabs or MBI Fermentas. Enzyme reactions were carried out under conditions suggested by the suppliers. PCR reactions were performed with DyNAzyme II DNA polymerase from Thermus brockianus (Finnzymes).

The LlaCI MTase assay. The M.LlaCI protection assay was performed in a 20 μl reaction mixture containing 0.5 μg λ DNA, 80 μM AdoMet, 10 mM Tris/HCl (pH 7.0), and 2 μl of column fractions (1 h, 37 °C). The reaction was stopped by heating the mixture (60 °C, 10 min). Following cooling, 2 μl NEB10 x reaction buffer (New England Biolabs) and 5 units HindIII were added (1 h, 37 °C), and the DNA was analysed by 0.8% agarose gel electrophoresis. One unit of the modification activity was defined as the minimum amount of MTase that conferred complete resistance to cleavage by the cognate restriction ENase. These circumstances clearly promote DNA restriction over DNA methylation, a scenario which seems to be profitable for bacteria fighting with invading DNA. Most probably, the intracellular concentration of Mg²⁺ inhibits M.LlaCI activity without affecting the potency of the cognate restriction ENase. These circumstances clearly promote DNA restriction over DNA methylation, a scenario which seems to be profitable for bacteria fighting with invading DNA. However, it must be stressed that the observed inhibition of the modifying enzyme apparently does not affect the cell’s viability. In this article, we also argue that observed sensitivity of some MTases to Mg²⁺ can encourage the process of genetic recombination. Involvement of R–M enzymes in this process was raised in several reports (Chang & Cohen, 1977; McKane & Milkman, 1995; Kobayashi et al., 1999, 2001).
trichloroacetic acid. The sample was centrifuged (10 000 g, 10 min). The pellet was washed with 1 mL 70% ethanol, centrifuged, and dried. Scintillation counting was used to estimate the incorporated radioactivity.

Protein purification. The DNA MTases M.EcoVIII and M.HindIII used in this study were purified to apparent electrophoretic homogeneity using procedures described previously (Mruk & Kaczorowski, 2003).

Purification of M.LlaCI from recombinant E. coli. The enzyme was prepared from E. coli BL21(DE3)pLysS transformed with pLlaMet3. This plasmid was constructed by cloning into a pT7-6 vector, previously linearized with BamHI and SmaI, a 1.15 kb DNA fragment carrying the M.LlaCI gene that was obtained in a PCR reaction followed by BamHI digestion and T4 polynucleotide kinase phosphorylation. The forward and reverse primers were 5'-GA-GGATCCGACCATTTA-3' and 5'-ATGAACTCAGTTTGAT-3', respectively (the BamHI site is underlined). Plasmid pSX1 (LlaCI K/R M7; Madsen & Josepshans, 1998) was used as a template in the PCR reaction. In recombinant plasmid pLlaMet3 the start codon of the llaCIM gene is located 164 nt downstream from the 30 promoter of phage T7. Bacteria carrying the overproducing plasmid were cultivated at 37°C in TY-broth (1 litre) supplemented with Ap and Cm to an OD600 of 0.3. At this time overproduction of the M.LlaCI was induced by adding IPTG to the culture to a final concentration of 1 mM. Induction proceeded for 2.5 h. Cells were harvested by centrifugation and stored at -70°C. For enzyme purification, frozen cells (2.5 g) were resuspended in 10 mL buffer P (10 mM potassium phosphate pH 7.0, 50 mM KCl, 1 mM EDTA, 10 mM 2-mercaptoethanol and 5% v/v glycerol) supplemented with PMSF (0.1 mM 2-mercaptoethanol and 5% v/v glycerol) and stored at -80°C. After sonication (60 bursts of 10 s at an amplitude of 12 μm), all procedures were carried out at 4°C. Cellular debris was removed by centrifugation (14 000 g, 40 min), and clarified lysate was applied to a 2.5 x 5 cm P-11 column (Whatman) equilibrated with buffer P. Proteins bound to the column were eluted with a 150 mL linear gradient of KCl (0.05-1 M) in buffer P. Fractions enriched in M.LlaCI were collected and applied directly to a 1 x 5 cm blue-agarose column (Pharmacia) equilibrated with buffer B (10 mM potassium phosphate pH 7.0, 1 mM EDTA, 10 mM mercaptoethanol and 5%, v/v glycerol). Protein was eluted with a 100 mL linear gradient of KCl (0-1 M) in buffer B. The final step was chromatography on Superose 12HR (FPLC, Pharmacia) equilibrated with buffer S (10 mM potassium phosphate pH 7.0, 150 mM KCl, 1 mM EDTA, 10 mM mercaptoethanol and 2.5%, v/v glycerol). Fractions with the highest M.LlaCI activity were dialysed overnight against buffer E (10 mM potassium phosphate pH 7.5, 50 mM KCl, 10 mM mercaptoethanol, 0.5 mM EDTA and 50%, v/v glycerol) and stored at -20°C.

M, determination. A purified preparation of the M.LlaCI was analysed by SDS-PAGE (Laemmli, 1970) in order to estimate purity and M, under denaturing conditions. After electrophoresis, protein position was visualized by Coomassie brilliant blue R 250 staining. The M, of the M.LlaCI was calculated using a calibration curve obtained with the following standard proteins (Pharmacia): phosphorylase b (M, 94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), trypsin inhibitor (20 100) and α-lactalbumin (14 400).

N-terminal amino acid sequencing. A 300 pmol sample of homogeneous preparation of M.LlaCI was separated by SDS-PAGE, electrobotted onto PVDF membrane and then analysed using an Applied Biosystems 491 gas-phase protein sequencer. The phenylthiohydantoin (PTH)derivatives of the amino acids were identified with an Applied Biosystems 140C PTH-analysler connected to the sequencer. The first 10 PTH-amino acids were unambiguously identified.

Protein cross-linking with glutaraldehyde. In order to determine the state of aggregation in solution, M.LlaCI enzyme was subjected to chemical cross-linking, which was performed by using a previously described protocol (Schlossman et al., 1984). An aliquot of M.LlaCI enzyme (2.5 μg) was incubated at 30°C for 20 min in 30 μl reaction mixtures in standard buffer. Glutaraldehyde (1.9 μl of a 2.5% solution) was added to the reaction mixture (2 min, 30°C), followed by sodium borohydride treatment (2.6 μl of a 1 M solution, 20 min, 4°C). The reaction was terminated by the addition of Tris/HCl pH 7.5 (9 μl of a 1 M solution) and incubation of the sample for 5 min at 4°C. Proteins were analysed using SDS-PAGE (5%) (Weber & Osborn, 1969), and visualized by silver staining (Heukeshoven & Dernick, 1985).

Determination of the methylated base. To determine the base methylated by M.LlaCI the method employing type II restriction ENases was used (Pösfai & Szybalski, 1988). In order to obtain appropriate templates two synthetic DNA fragments carrying the M.LlaCI recognition site overlapped by the MboII cut site (5'-AATTTCGAAGATCGATCA-3'/3'-GCTTCCTA GCTAGTTCCA and 5'-AAATCTGAAGATCGATCA-3'/3'-GCTTCCTAGCTAGTTCCA-5') were cloned into plasmid pGEM3Zf (+) previously double-digested with HindIII and EcoRI, resulting in plasmids pL1M and pL2M, respectively. These plasmids differ by one base-pair in the region between the LlaCI and MboII sites. Plasmid pL3M without the LlaCI site was used as a control. This plasmid was constructed by digestion of plL1M with R.HindIII followed by the filling-in of the 5' protruding ends with Klenow fragment in the presence of dNTPs. After ligation and transformation into E. coli MM294 cells, recombinants were checked by DNA sequencing analysis. To determine M.LlaCI methylation pattern, 365 bp (pL1M), 365 bp (pL2M) or 370 bp (pL3M) DNA fragments were amplified using a pair of primers complementary to pGEM3Zf (+) vector (ADE1, 5'-TTACCGCA-GCTGGGCAAG-3' and ADE2, 5'-CATTAAAGCAGTGGCGACC-3'). DNA fragments carrying synthetic oligonucleotides were examined from PCR reactions where either plL1M (365 bp) or plL2M (365 bp) served as template, were methylated by M.LlaCI. In the control experiment a 370 bp DNA fragment without an LlaCI site was used and plasmid plL3M was used as a control. Methylation was performed in a 30 μl reaction mixture containing 0.5 μg of a particular DNA fragment (365, 366 or 370 bp), 10 mM Tris/HCl pH 7.0, 3·7 x 105 Bq [15N]H3AdoMet (3·1 x 107 Bq mmol-1, Amersham) and 50 ng M.LlaCI (1 h, 37°C). In the experiment investigating the effect of cleavage by the MboII enzyme, the 15N-labelled DNA fragment was cut either between adenine and adenosine (366 bp DNA fragment) or between adenine and guanine (365 bp DNA fragment) within the M.LlaCI recognition site. After electrophoresis, particular DNA fragments were extracted from 1·5% agarose gel using a method employing DEAE-cellulose membrane (Kaczorowski et al., 1993). Scintillation counting was used to estimate the radioactivities of the DNA fragments.

Western blot analysis. Homogeneous preparations of the enzymes (M.LlaCI, M.EcoVIII and M.HindIII) were separated by SDS-PAGE (10%) and then electrobotted onto nitrocellulose membrane. After blocking with 3% skimmed milk, the membrane was incubated with a 1:25 dilution of anti-M.EcoVIII rabbit polyclonal antibodies, which were prepared according to standard procedures (Harlow & Lane, 1988). The primary antibodies were tagged successively with a 1:10 000 dilution of goat anti-rabbit polyclonal antiserum and 5-bromo-4-chloro-3-indolyl phosphate (Sigma). All antibodies were diluted in phosphate-buffered saline (Sambrook et al., 1989). Reactive bands were visualized by using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as the alkaline phosphatase substrate and nitroblue tetrazolium (NBT) as the colour development reagent. The activity of M.LlaCI and its isomethylomers was measured in a reaction buffer (10 mM Tris/HCl pH 7.0) containing divalent metal ions, and 50 ng of the enzyme in a 1:25 dilution.
ions (Mg$^{2+}$, Mn$^{2+}$, Ca$^{2+}$, Zn$^{2+}$) at different concentrations. Enzyme activity of M.LlaCI and its isospecific homologues (M.EcoVIII and M.HindIII) was assayed by measuring incorporation of $^3$H-labelled methyl groups from [methyl-$^3$H]AdoMet into 2:5 pmol of 29 bp double-stranded oligonucleotide (5'-TGCAG-TCGCGAAGCTTGGACCCAGTGAACTCCGT-3'; the LlaCI site is underlined) using 35 ng of the enzyme (30 min, 37°C). Each experiment was repeated at least three times. For each cation the value of a 50% inhibitory concentration (IC$50$) was determined.

RESULTS

Purification of the M.LlaCI enzyme overproduced in E. coli cells

When we tried to overexpress the llaCIM gene in a heterologous system we found that overproduction of M.LlaCI was toxic to E. coli BL21(DE3) cells, most probably due to the background transcription of the lacUV5 promoter which controls expression of the gene encoding T7 RNA polymerase. On the other hand, no toxicity was observed when E. coli MM294 was used as a host for overproducing plasmid pLlaMet3. This strain does not contain a copy of the T7 RNA polymerase gene. In E. coli BL21(DE3)[pLlaMet3] small amounts of T7 RNA polymerase permitted expression of the llaCIM gene and in consequence inhibition of E. coli growth. The level of basal expression of the T7 RNA polymerase gene in the uninduced E. coli BL21(DE3) cells was extensively investigated by Dubendorff & Studier (1991), who also suggested appropriate countermeasures for controlling it (Studier, 1991; Dubendorff & Studier, 1991). To overcome this problem we used, as proposed by Studier (1991), plasmid pLysS encoding T7 lysozyme, which is a potent inhibitor of T7 RNA polymerase. The overexpression of the M.LlaCI in E. coli BL21(DE3)[pLysS, pLlaMet3] is shown in Fig. 1(b). One hour after induction (1 mM IPTG), a distinct band of M.LlaCI was observed on SDS-PAGE (Fig. 1b, lane 2). The amount of the M.LlaCI enzyme increased significantly with time, reaching its maximum at 4–6 h after induction (data not shown). As a negative control we used E. coli BL21(DE3) cells carrying plasmid pLysS alone (Fig. 1a, lanes 1–3).

The standard purification procedure consisted of three chromatographic steps. The final enzyme preparation did not show the presence of nonspecific nucleases. From 2.5 g of bacteria we were able to obtain 0.75 mg of homogeneous enzyme preparation with an overall yield of 22%. The purified preparation of the M.LlaCI enzyme was analysed by SDS-PAGE (Laemmli, 1970) in order to estimate its purity and $M_r$ under denaturing conditions. On Coomassie-blue-stained gels the enzyme was found to

![Fig. 1. Overproduction of the M.LlaCI in E. coli BL21(DE3). SDS-PAGE (10%) of cellular proteins from 200 μl cultures of bacteria. (a) E. coli BL21(DE3)[pLysS]: lane 1, before induction; lane 2, 1 h after induction; lane 3, 2 h after induction (1 mM IPTG). (b) E. coli BL21(DE3)[pLysS, pLlaMet3]: lanes 1–3 correspond to the treatments described in (a). (c) Coomassie-blue-stained SDS-PAGE (10%) of purified M.LlaCI enzyme: lane 1, M.LlaCI preparation after chromatography on a Superose 12HR (FPLC, Pharmacia) column (2.0 μg protein). $M_r$ values of protein standards (see Methods, lane M) are indicated on the right.](3334)
be at least 95% pure (Fig. 1c, lane 1). Relative to the standards of known Ms, a value of $M_\text{s}$, 31 300 ± 1000 was calculated for M.LlaCI, which is close to the 33 896 deduced from the nucleotide sequence of the llaCIM gene. The value obtained is in agreement with the $M_\text{s}$ values of isospecific homologues: M.HindIII (35 549) and M.EcoVIII (33 930). The purified preparation of M.LlaCI was subjected to N-terminus microsequencing. The first 10 amino acids of the M.LlaCI were found to be MKIDTIYNEN. The sequence obtained corresponds to that predicted from the nucleotide sequence of the llaCIM gene (Madsen & Josephsen, 1998). M.LlaCI microsequencing also enabled us to determine the exact position of the start codon (AUG) of the llaCIM gene.

**Chemical cross-linking of M.LlaCI**

The state of aggregation of the M.LlaCI enzyme was tested using a glutaraldehyde cross-linking reaction under optimal conditions (10 mM potassium phosphate pH 7-0, 80 μM AdoMet and 10 mM EDTA) with M.LlaCI at 2-7 μM (2-5 μg). Chemical intermolecular cross-linking, catalysed by glutaraldehyde, is strongly dependent on the presence of lysine residues in the protein structure. A molecule of M.LlaCI contains 21 lysine residues as predicted from the nucleotide sequence of the llaCIM gene. As a result of a cross-linking experiment, we have found that M.LlaCI exists in solution predominantly as a dimer (see Fig. 2, lane 2). A well-known complex-forming protein, E. coli DnaK, was used as a control. Upon cross-linking, DnaK, which is known to produce multimeric forms (Liberek et al., 1990), formed three distinct bands corresponding to DnaK monomers, dimers and trimers (Fig. 2, lane 1). Most of the type II restriction ENases exist in solution as dimers. On the other hand, type II DNA MTases usually function as monomers. M.LlaCI makes an exception to this rule, existing in solution predominantly as a dimer. We have shown previously that an isomethylomer of M.LlaCI, M.EcoVIII, exists in solution as a monomer (Mruck & Kaczorowski, 2003). A few other MTases possess dimeric structure: e.g. M.RsrI (Kaszubska et al., 1989), M.DpnA, M.DpnM (de la Campa et al., 1987), and M.KpnI (Bheemanaik et al., 2003). Structural elements required for dimer formation remain unclear. Only in the case of the M.RsrI enzyme, crystallographic analysis resulted in the proposition that the unusual fold of the putative DNA binding domain is most probably involved in the formation of M.RsrI dimers (Scavetta et al., 2000). However, it is unclear how such a state of oligomerization can affect enzyme function. Detailed biochemical analysis of the M.KpnI enzyme did not provide any clues for understanding why a dimeric form could be superior when such factors as DNA binding preferences (unmethylated, hemimethylated DNA) or kinetic parameters were taken into consideration (Bheemanaik et al., 2003).

**Determination of the methylation specificity of the M.LlaCI enzyme**

The base methylated by M.LlaCI was identified by using the method which employs class II restriction ENases (Pösfai & Szybalski, 1988). We used two kinds of DNA fragments with the LlaCI site next to the recognition site of the MboII enzyme. Both sites were located in such a way as to ensure cleavage between two adenine residues (Fig. 3a) or between adenine and guanine residues (Fig. 3b) within the LlaCI recognition site. After methylation of a 366 bp DNA fragment with M.LlaCI in the presence of [methyl-3H]AdoMet as methyl groups donor we found that both fragments (A and B) produced by MboII digestion were 3H-labelled (Fig. 3a, d). This suggested that the base being modified by M.LlaCI is the first adenine residue in the sequence 5'-6mAGCTT-3'. This was confirmed when a 365 bp fragment derived from plM2 was used as a substrate in a methylation reaction (Fig. 3b, d). In a control experiment a 370 bp DNA fragment lacking a LlaCI site failed to be a substrate for M.LlaCI modification activity (Fig. 3c, d). The observed difference in scintillation counts between products derived from plasmids plM1 and plM2 was a result of the presence of traces of exonucleolytic activity contaminating the commercial preparation of the MboII enzyme. This problem was encountered by us earlier, as bacteria belonging to the genus Moraxella are especially abundant in nucleases (Sektas et al., 1992). The results obtained demonstrate that M.LlaCI is an isomethyl-omer of M.HindIII, an enzyme from H. influenzae Rd (Roy & Smith, 1973).
Immunological analysis of M.LlaCI

Based on the similarities noted at the level of amino acid sequences we attempted to determine if these enzymes share common epitopes. Thus, the ability of antiserum prepared against M.EcoVIII to cross-react with isospecific enzymes (M.LlaCI, M.HindIII) was examined using the immunoblotting method (Western blotting). Despite the observed homology between all three isospecific MTases, we noted the cross-reaction of antibodies raised against the M.EcoVIII enzyme only with the M.LlaCI protein, not the M.HindIII protein (Fig. 4b, lanes 2 and 3). A homogeneous preparation of the M.EcoVIII enzyme was used as the control (Fig. 4b, lane 1). This experiment demonstrated that M.LlaCI possesses substantial antigenic similarity to M.EcoVIII, suggesting that both proteins may have identical epitopes. M.LlaCI and M.EcoVIII are 62% identical at the amino acid sequence level (Mruk & Kaczorowski, 2003). The M.EcoVIII and M.HindIII amino acid sequences are identical at a much lower degree – 50% (Mruk & Kaczorowski, 2003).

Effect of divalent cations on the activity of M.LlaCI in vitro

We found that divalent metal ions are strong inhibitors of the M.LlaCI enzyme (IC_{50} 0.35 mM Mg^{2+}). The IC_{50} values obtained for other divalent cations were as follows: Ca^{2+}, 0.3 mM; Mn^{2+}, 0.2 mM; and Zn^{2+}, 0.03 mM. (100% of the enzyme activity corresponded to the conditions when the inhibitory factor was not present.) We also observed that Mg^{2+} exerts a strong inhibitory effect on M.EcoVIII (IC_{50} 2.0 mM) and M.HindIII (IC_{50} 2.0 mM), both being isospecific to M.LlaCI. The results obtained are summarized in Table 1 and Fig. 5.

DISCUSSION

In this article we present a characterization of the M.LlaCI, which is a part of the type-II R–M system from L. lactis subsp. cremoris W15. This enzyme shares the same specificity with M.HindIII from H. influenzae Rd (Roy & Smith, 1973) and M.EcoVIII from E. coli E1585-68 (Mruk...
& Kaczorowski, 2003). All three enzymes are highly homologous at the level of their amino acid sequences (Mruk & Kaczorowski, 2003). In general, type II DNA MTases form a homogeneous group of proteins possessing a common building plan with easily distinguishable conserved amino acid sequence motifs, in contrast to restriction ENases. In our study we were interested not only in biochemical analysis of M.LlaCI but also in finding out whether similarities between analysed homologues noted at the amino acid sequence level can also be observed in their tertiary structure. Thus the ability of antiserum prepared against M.EcoVIII to cross-react with isospecific enzymes (M.LlaCI, M.HindIII) was examined by immunoblotting. The results obtained show that despite the fact that all of the three analysed MTases share a substantial level of homology they do not cross-react in an identical manner with polyclonal antibodies raised against M.EcoVIII.

We observed positive cross-reaction only in the case of M.LlaCI but not M.HindIII. Thus our experiment revealed substantial differences in the tertiary structure of the enzymes analysed, despite significant identity noted at the amino acid sequence level. The data obtained indicate that M.EcoVIII and M.LlaCI may have a similar architecture, since antigenic closeness is generally correlated with three-dimensional similarity.

It is a common belief that R–M systems evolved to protect bacteria against lethal infections by phages. This notion is supported by the fact that some phages have developed multiple mechanisms to avoid antiviral activity exerted by R–M systems (Krüger & Bickle, 1983). As pointed out by some authors (Wilkins, 2002) this defensive character of R–M systems may facilitate bacteria which carry them to colonize a new habitat containing previously unencountered phages (Korona & Levin, 1993; Korona et al., 1993).

However, it must be stressed that the barrier raised by R–M systems is not absolute and the phages which evade it usually acquire protective methylation. However, this barrier can be raised higher when several R–M systems are present within the bacterial cell. Therefore, it is reasonable to assume that restriction enzymes exert selective pressure directed towards decreasing the number of restriction sites present in phage DNA. Computational analysis revealed significant avoidance of short palindromic sequences not only in DNA of bacteriophages but also in bacterial chromosomes (Karlin et al., 1992; Gelfand & Koonin, 1997; Rocha et al., 2001). In addition, it was shown that the sequences recognized by type II R–M enzymes present in the bacterium under scrutiny were among the most avoided palindromes in its genome (Gelfand & Koonin, 1997). In our opinion, the observed restriction site avoidance is a consequence of the aggressiveness of the ENases residing within the

![Fig. 4](image1.png)

**Fig. 4.** Immunological analysis of the M.LlaCI enzyme and its isomethylomers. (a) Purified preparations of M.EcoVIII (lane 1), M.LlaCI (lane 2) and M.HindIII (lane 3) separated by SDS-PAGE. (b) Immunological cross-reactivity of polyclonal antiserum against M.EcoVIII antibodies with preparations of purified proteins blotted on nitrocellulose membrane. Lanes in this panel correspond to those of (a). The same amount (2.0 μg) of each protein was loaded on the gel. Anti-rabbit alkaline phosphatase conjugate, 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) were used to detect specific complexes.

![Fig. 5](image2.png)

**Fig. 5.** Effect of Mg^{2+} on the activity of M.LlaCI (●) and its isospecific homologues M.EcoVIII (●) and M.HindIII (▲). Assay conditions are described in Methods.Means ± SD from three independent experiments are presented (error bars not shown where smaller than symbols).

### Table 1. Effect of divalent cations on activity of M.LlaCI and its isospecific homologues

<table>
<thead>
<tr>
<th>Cation</th>
<th>IC_{50} value (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M.LlaCI</td>
</tr>
<tr>
<td>Mg^{2+}</td>
<td>0.35</td>
</tr>
<tr>
<td>Mn^{2+}</td>
<td>0.2</td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>0.3</td>
</tr>
<tr>
<td>Zn^{2+}</td>
<td>0.03</td>
</tr>
</tbody>
</table>

http://mic.sgmjournals.org

3337
bacterial cell, whose restriction potential might be strengthened by particular properties of cognate MTases, a notion which we discuss below. Surprisingly, these critical conditions do not affect the viability of bacterial cells possessing a particular R–M system. This might be a result of the functioning of the precise repair mechanisms whose existence was proven recently (Heitman et al., 1999; Handa et al., 2000).

An important question still remains open: what molecular mechanisms are responsible for maintenance and evolution of R–M systems? It was proposed that these processes might be driven by phage-mediated selection (Sharp, 1986; Korona et al., 1993; Korona & Levin, 1993). A second mechanism might be based on involvement of R–M systems in genetic recombination. The role of restriction ENases in this process can be defined as the processing of large DNA molecules entering cells into smaller recombinogenic fragments. In the case of an E. coli genome, it was observed that its mosaic character might be associated with incorporation of short DNA fragments most probably processed by restriction enzymes (McKane & Milkmian, 1995; Milkmian, 1999). Considerable genome plasticity was noted not only in E. coli but in the structure of other bacterial chromosomes sequenced to date (Casjens, 1998). On the other hand, it was shown that the presence of a multitude of R–M systems in some bacteria (e.g. Helicobacter pylori) can prevent genome rearrangements by chromosomal DNA from competing strains which are non-isogenic (Aras et al., 2002). Lastly, R–M systems may have evolved as molecular parasites, a notion which has only recently received much attention (Naito et al., 1995).

Data obtained in our laboratory indicate that the restriction activity can be regulated by divalent metal ions. Among cations investigated by us, Mg$^{2+}$ is the most abundant ion in bacterial cells, playing an important role in their metabolism. Cellular concentrations of the other divalent metal ions that we investigated are extremely low: Ca$^{2+}$, 10$^{-7}$ M (Gangola & Rosen, 1987); Zn$^{2+}$, 10$^{-12}$-10$^{-12}$ M; and Mn$^{2+}$, 10$^{-8}$ M (Outten & O'Halloran, 2001). Biochemical analysis carried out by us revealed that Mg$^{2+}$ is a strong inhibitor of M.LlaCI. We found that this property is shared with two isospecific MTases: M.EcoVIII and M.HindII. This is also true for a few other bacterial MTases, e.g. M.RsrI, M.BamHI, M.EcoRI, M.AluI, M.FokI, M.TaqI and M.EcoKDam (Kaszub ska et al., 1989; Hanish & McClelland, 1988; Kaczorowski et al., 1999). It is difficult to estimate whether the observed sensitivity to Mg$^{2+}$ is general among type II MTases. The survey performed by Hanish & McClelland (1988) was limited to only a few enzymes. The intracellular concentration of free Mg$^{2+}$ in E. coli cells was estimated to be in the range of 1–4 mM (Lusk et al., 1968; Alatossava et al., 1985) and it is very likely that this value is similar for other bacterial species, as common mechanisms are responsible for the accumulation of Mg$^{2+}$ inside bacterial cells (Moncrief & Maguire, 1999). These mechanisms appear to be omnipresent in prokaryotes (Kehres et al., 1998). Homologues of well-known Mg$^{2+}$ transporters such as CorA or MgtA were found in L. lactis (Seegers et al., 2000; Bolotin et al., 2001). This means that in L. lactis cells, the activity of M.LlaCI is strongly inhibited [IC$_{50}$ 0.35 mM Mg$^{2+}$] while the activity of the cognate restriction ENase is intact. This finding stresses the defensive character of type II R–M systems. For the bacterial cell, high activity of the restriction ENase seems to be profitable. Clearly, it promotes DNA restriction over DNA methylation. This is facilitated by the fact that type II DNA MTases, unlike solitary enzymes such as M.EcoKDam, modify DNA in a non-processive manner (Surby & Reich, 1996; Gowher & Jeltsch, 2000; Urig et al., 2002). Our results also explain experiments that have demonstrated the recombogenic role of restriction ENase EcoRI in vivo (Chang & Cohen, 1977; Stahl et al., 1983; Silverstein et al., 1993). It was shown previously that the activity of EcoRI MTase is strongly inhibited by Mg$^{2+}$ (Hanish & McClelland, 1988). Also, sensitivity of EcoKDam MTase to Mg$^{2+}$ could explain why only 50% of DNA molecules of phage $\lambda$ isolated from E. coli are methylated at dam sites (Dreiseikelmann et al., 1979). Our interpretation is different from concepts offered by other authors. Some of them have argued that unmethylation of dam sites is a result of a low level of Dam MTase in the E. coli cells (Szyf et al., 1984).

We also hypothesize that the fact that some type II R–M systems (e.g. ScrFI (Davis et al., 1993), BcnI (Vilkaitis et al., 2002), DpnII (de la Campa et al., 1987), LlaDCHI (Moineau et al., 1995), Mbol (Ueno et al., 1993)) possess two MTases with the same specificity can be linked to a possible sensitivity of one or both enzymes to the Mg$^{2+}$. In such circumstances, to ensure proper methylation of genomic DNA, it would make sense to employ in this process two enzymes with the same methylating activity. Additional evidence supporting this notion is an apparent lack of substantial identity between pairs of isospecific MTases at the amino acid sequence level (M.ScrFIA/M.ScrFIB, 24%; M.BcnI/M.BcnIB, 11%; M.DpnA/M.DpnM, 13%; M.LlaDCHIA/M.LlaDCHIB, 13%; M.MbolA/M.MbolB, 12%). This indicates that each MTase of a particular R–M system is not a product of gene duplication but was acquired/evolved independently. Recent studies on the diversity of R–M systems in Helicobacter pylori provide facts endorsing such a notion (Nobusato et al., 2000a, b; Lin et al., 2001). In vivo experiments with the BcnI R–M system show that M.BcnIB alone is sufficient to support the growth of E. coli cells carrying the BcnI ENase gene; this could not be demonstrated for M.BcnIA (Vilkaitis et al., 2002). In the light of our experiments, it is conceivable that this may be an effect of extreme sensitivity of M.BcnIA to Mg$^{2+}$. Verification of this hypothesis in the case of the MTases listed above will need further validation by biochemical analysis using purified proteins. Another argument endorsing our hypothesis might be derived from biochemical analysis of the FokI MTase, an enzyme which possesses two catalytic centres, each responsible for methylation of an adenine residue located on a different
strand of the asymmetric recognition site 5′-GGATG-3′/3′CCTAC-5′ (Landry et al., 1989). This suggests that the gene encoding M.FokI has most probably arisen by fusion of two non-homologous open reading frames. Physical separation of these two catalytic centres results in truncated derivatives which may function as independent MTases, each able to methylate only one strand within the complementary derivatives which may function as independent separations of these two catalytic centres results in truncated derivatives which may function as independent

The exceptional sensitivity of some type II MTases to Mg$^{2+}$ provides evidence that this particular property of the modifying enzymes can effectively modulate the flow of genes among bacteria by strengthening the restriction activity of cognate ENases. Analysis of the sequenced genomes supports the notion that the process of acquisition of genes from the environment is fundamental for the evolution of bacteria (Arber, 2000; Ochman et al., 2000).

**ACKNOWLEDGEMENTS**

We would like to thank Drs Lytte Josephsen, Elisabeth Raleigh, Richard J. Roberts and Stanley Tabor for bacterial strains and plasmids, and Dr Noreen E. Murray for critical reading of the manuscript. We appreciate stimulating discussion with Dr Slawek Sektas. A sample of an *E. coli* DnaK protein was a gift from Dr Krzysz Liberek. We are also grateful to Kasia Potrykus and Steven Hitchen for their excellent help at the manuscript editing stage. This work was supported by a grant from the Polish Committee for Scientific Research KBN 6P04B-027-18.

**REFERENCES**


