Involvement of the LlaKR2I Methylase in Expression of the AbiR Bacteriophage Defense System in Lactococcus lactis subsp. lactis biovar diacetylactis KR2

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The native lactococcal plasmid, pKR223, from Lactococcus lactis subsp. lactis biovar diacetylactis KR2 encodes two distinct bacteriophage-resistant mechanisms, the LlaKR2I restriction and modification (R/M) system and the abortive infection (Abi) mechanism, AbiR, that impedes bacteriophage DNA replication. This study completely characterized the AbiR, revealing that it is the first Abi system to be encoded by three genes, abiRα, abiRβ, and abiRγ, arranged in an operon and that it requires the methylase gene from the LlaKR2I R/M system. An analysis of deletion and insertion clones demonstrated that the AbiR operon was toxic in L. lactis without the presence of the LlaKR2I methylase, which is required to protect L. lactis from AbiR toxicity. The novelty of the AbiR system resides in its original gene organization and the unusual protective role of the LlaKR2I methylase. Interestingly, the AbiR genetic determinants are flanked by two IS982 elements that have been shown to be present in other bacterial genomes. This novel AbiR system may contribute to the evolution of the LlaKR2I methylase toward a new and separate cellular function. This unique structure of both the LlaKR2I R/M system and the AbiR system may have contributed to the evolution of the LlaKR2I methylase toward a novel role comparable to that of the cell cycle-regulated methylase that encode Dam and CcrM methylases. This new role for the LlaKR2I methylase offers a unique snapshot into the evolution of the cell cycle-regulated methylases from an existing R/M system.

The mesophilic Lactococcus lactis bacterium is the principal starter culture used in the production of numerous fermented dairy products, such as cheese and fermented milks. This fermentation industry can be exposed to considerable economic hardships due to bacteriophage infection of the starter cultures, resulting in prolonged fermentation schedules, and, if not remedied, complete failure of the ongoing fermentation. This causes considerable variation in product quality, which is a further economic drain (1). Fortuitously, many L. lactis strains have evolved different sophisticated natural bacteriophage defense systems that can interfere with bacteriophage proliferation at different steps during the lytic cycle. These consist of mechanisms that impede the adsorption of the bacteriophage to the cell, mechanisms that block the injection of DNA into the cell, restriction and modification (R/M) systems, and numerous systems that abort the infection at various points in the replication cycle (9). Studies elucidating these latter defense mechanisms, termed abortive infection (Abi) systems, are shedding more light into the mechanisms by which bacterial defense systems evolve (7).

Blockage of bacteriophage attachment to the bacterial cell (35) and inhibition of DNA injection (17) are the two first lines of defense against bacteriophage infection. R/M systems target bacteriophage DNA following injection into the cell and are the most characterized of all bacterial bacteriophage defense systems and numerous systems identified for different strains of L. lactis. The methylase component of R/M systems protects the host DNA from the restriction endonuclease that can digest the unmethylated bacteriophage DNA and terminate infection. The majority of the characterized R/M systems from L. lactis are plasmid encoded (15). However, strains do harbor chromosomally encoded systems as revealed in the genome sequence for L. lactis IL1403, which harbors one type I R/M system (3). All other bacteriophage defense systems target various steps in the bacteriophage intracellular development cycle and are collectively termed Abi systems. Abis are phenotypically characterized by a reduced plaque size, caused by a reduction in the burst size of the bacteriophage, and may or may not have a reduction in the efficiency of plaquing (EOP) of the bacteriophage. Since pNP40 and pTR2030, the first isolated plasmid-encoded Abi systems (24, 21), numerous native lactococcal Abi-encoding plasmids have been reported. While the majority of Abi systems are plasmid encoded, two chromosomally encoded Abi systems, AbiH and AbiN, have been characterized (30, 31). A single gene encodes the majority of Abi systems. However, five systems, AbiE, AbiG, AbiL, AbiT, and AbiU, are encoded by two genes (4, 10, 11, 16, 26), and AbiR was found to be encoded by at least three genes (38). A distinctive feature shared by most abi genes to date is their...
Relevant feature(s) | Size (kb) | Source or reference  
--- | --- | ---  
Plasmid-free derivative of *L. lactis* subsp. *cremoris* C2 | 14 |  
Plasmid-free derivative of *L. lactis* subsp. *lactis* biovar diacylactis IL594 | 6 |  
F′ thi-1 hsdS20(γ m5 + ) supE44 recA13 ara-14 leuB6 proA2 lacY1 galK2 rpsL20(StrR) rgy-5 mlc-1 | 20.5 | Promega  
Δ(mercI)183 Δ(mercIB-hsdSMR-nrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac | 27 | Stratagene  
High-copy-number lacoccal shuttle vector, Erm' | 6.9 |  
Low-copy-number lacoccal shuttle vector, Erm' | 8.6 |  
High-copy-number lacoccal shuttle vector, Cm' | 5.7 |  
Ta3-based transposon construction vector | 2.5 | Epicentre  
pGBK17 16.2-kb HpaII fragment from pKR223 cloned into HpaII site of pGB301 | 26.2 |  
Erm' Cm' R'/M' AbiR' | 22 |  
pDOT50 AbiR operon cloned into pTRKH2, Erm' | 15.8 |  
pDOT29 llaKR2IM gene cloned into pCI372, Cm' | 7.2 |  
pDOU02 AbiR operon cloned into pTRKL1, Erm' | 17.5 |  
pDOU012 Bigl deletion derivative of pGBK17, Erm' Cm' | 20.5 |  
pDOM21 A 3.9-kb deletion derivative of pDOU012, Erm' | 16.6 |  
pDOM27 A 5.4-kb deletion derivative of pDOU012, Erm' | 15.1 |  
pDOY01 BwCl/Bsgl deletion derivative of pGBK17, Erm' | 24.3 |  
pDOY02 A 6-kb deletion derivative of pDOU012, Erm' | 14.5 |  
pDOY03 A 9-kb deletion derivative of pDOU012, Erm' | 11.5 |  
pDOY04 A 600-bp deletion derivative of pDOU012, Erm' | 19.9 |  
pDOY05 A 1.1-kb deletion derivative of pDOU012, Erm' | 19.4 |  
Prolate-headed phage |  |  
L. M. McKay  

**MATERIALS AND METHODS**  

**Bacteria, plasmids, bacteriophage, media, and growth conditions.** The bacteria, plasmids, and bacteriophage used in this study are listed in Table 1. *Lactococcus lactis* strains were grown in M17 medium (Difco, Detroit, MI) supplemented with 0.5% glucose (M17G) at 30°C without shaking. *Escherichia coli* was grown in Luria-Bertani medium (34) at 37°C with agitation (225 rpm). Where necessary, the antibiotics chloramphenicol and erythromycin were added to the medium at a final concentration of 5 μg/ml for *L. lactis* strains and 20 μg/ml and 150 μg/ml, respectively, for *E. coli* strains. Ampicillin was used for plasmid selection in *E. coli* at 100 μg/ml.

**Plasmid transfer into *E. coli* and *Lactococcus lactis*.** Plasmids were introduced into *E. coli* using standard chemical transformation procedures (34). Electroporation was used to introduce plasmids into *L. lactis*. Electroporompted cells were prepared by subcooleding an overnight culture at 1% into M17G supplemented with 0.5 M sucrose and 2.3% and 2.5% glycine for *L. lactis* subsp. *lactis* IL1403 and *L. lactis* subsp. *lactis* LM0230, respectively, for 18 to 20 h at 30°C. Cells were pelleted by centrifugation and prepared as described by Holos and Nes (19). Electroporation was performed in a 2-mm gap electroporation cuvette at 2.45 kV.
using an Eppendorf 2100 electroporator apparatus (Eppendorf Scientific Inc., Madison, WI). Immediately after electroporation, 1 ml of M17G, containing 0.5 M sucrose, 10 mM CaCl$_2$, and 10 mM MgCl$_2$, was added to the cuvette, and the mixture was incubated at 30°C for 2 h. One hundred microliters of the culture was plated onto a M17G plate containing either erythromycin or chloramphenicol and incubated at 30°C for 48 h.

**DNA isolation, enzymes, and cloning.** Plasmid DNA from *E. coli* was isolated and purified with the Qiagen plasmid mini kit (Qiagen Inc., Chatsworth, CA). Routine plasmid isolation from *L. lactis* was achieved using the mini-prep procedure of O’Sullivan and Kleinhammer (28). Purified plasmid preparations from *L. lactis* were obtained using the Qiagen plasmid mini kit with the following modifications: 10 ml of an overnight culture of *L. lactis* was pelleted; the cells were resuspended in 300 ml of buffer P1 (50 mM Tris-HCl, 10 mM EDTA pH 8.0) supplemented with 100 mg/ml of RNase A and 30 mg/ml of lysozyme and incubated at 37°C for 30 min. Thereafter, the procedure outlined for *E. coli* plasmid isolation was followed. Restriction enzymes, Klenow fragment from DNA polymerase I, and T4 DNA ligase were supplied by New England Biolabs (Beverly, MA) and Gibco BRL (Grand Island, N.Y.) and used according to the manufacturers’ instructions. Enzymes used in the Erase-a-Base strategy were provided by Promega (Madison, WI) and used as directed. E2:TN transposon was used in vitro transposition was supplied by Epicentre (Madison, WI). Two strategies for obtaining plasmid deletion derivatives were used. The first strategy involved digesting 1 mg of DNA with the restriction enzymes necessary to remove the desired fragment and ligating the mixture overnight. The ligation mixture was heated to 65°C for 10 min to inactivate the ligase, then digested by the same restriction enzyme(s) used in the deletion strategy, and finally transformed into *L. lactis* LM0230 by electroporation. The other deletion strategy involved the Erase-a-Base system. Reactions were carried out for 12 min at 30°C, and aliquots were taken every minute. Subsequent manipulations were performed as directed by the manufacturer. Ethyrenezymic-resistant transformants were selected, and deletion derivatives were identified by restriction analysis of plasmid DNA as well as by PCR amplification and sequence analysis.

**In vitro transposition.** A chloramphenicol-resistant (*Cm*) E2:TN transposon was constructed in plasmid pDOM1. The Cm’ E2:TN transposon was obtained from pDOM1 either by digestion with PstI or by PCR amplification using the pMOD <MCS> forward primer (5'-ATT CAG GCT GCC CAA CTG T-3') and reverse primer (5'-GTC AGT GAG CGA GAG AGA AG-3') that anneal to each end of the Cm’ E2:TN transposon. In vitro insertion of the Cm’ E2:TN was performed in a final reaction volume of 10 ml, containing 20 ng of pDOM1 reaction buffer (0.5 M Tris-acetate [pH 7.5], 1.5 M potassium acetate, 100 mM magnesium acetate, and 40 mM spermidine), 1 mg of target plasmid DNA, a molar equivalent of the Cm’ E2:TN, and 1 unit of E2:TN transposase. The reaction was carried out at 37°C for 2 h and then stopped by the addition of 1 ml E2:TN 10x stop solution (1.5% sodium dodecyl sulfate), followed by incubation at 70°C for 10 min. Five microliters of the mixture was used for electroporation into *L. lactis* LM0230. Chloramphenicol-resistant plasmid transformants were selected, insertion derivatives were identified by restriction analysis of plasmid DNA, and sequence analysis was used to precisely map specific insertions.

**PCR.** All PCR’s were carried out with a Robocycler gradient 40 temperature cycler (Stratagene, La Jolla, CA). Taq DNA polymerase (Promega, Madison, WI) was used for routine PCR applications.

**Bacteriophage assays.** The AbiR phenotype of lactococcal hosts was monitored by plaque assays using the bacteriophage c2 as previously described (10). Bacteriophage c2 was first purified from a single plaque and propagated on the host LM0230 in M17G to obtain high-titer lysates. To determine the titers of the plaque, 100 ml of the relevant plaque dilution was added to 3 ml of molten M17G (45°C) containing 300 ml of a growing (an optical density at 600 nm of 0.5) culture of the appropriate host and 10 mM CaCl$_2$. The contents were mixed and poured onto M17G agar plates containing 10 mM CaCl$_2$. The EOP of the plaque was defined as the titer of the plaque on the test host divided by the titer of the plaque on a nonrestrictive host (37).

**Sequence alignment.** The nucleotide sequences of both the *L. lactis* IL1403 and bacteriophage c2 genomes were obtained from the GenBank database, with accession numbers NC_002662 and NC_001706, respectively. The sequence alignment program CLUSTAL X was used to align the ori of *L. lactis* IL1403 (coordinate 2,564,567; 1001 bp) with the ori of bacteriophage c2 (coordinate 21,141; 1001 bp).

**RESULTS**

**Illustrating the AbiR phenotype.** Previous studies had indicated that a 16.2-kb fragment from pKR223 encoded the LlaKR21 R/M system and an abortive infection defense system, designated AbiR (25, 37, 38). While the LlaKR21 R/M system was found to be effective against both prolate- and small isometric-headed bacteriophage, AbiR was effective only against prolate-headed bacteriophage. AbiR results in the inhibition of phage DNA replication, which leads to fewer phage progeny, causing a dramatic reduction in the size of plaques obtained on AbiR-containing hosts.

**Phenotypic characterization of the three-genre AbiR operon.** A Sphi/PstI fragment, containing the three-genre AbiR operon from pGBK17, was previously cloned and cloned into the high-copy-number vector pTRKH2. This construct was designated pDOT50 and was first introduced into *E. coli* at a high frequency. Purified pDOT50 DNA from *E. coli* was then electroporated into *L. lactis* LM0230, and transformants exhibited an AbiR$^{-}$ phenotype (38). However, it was noted that the frequency of transfer of pDOT50 into *L. lactis* was several hundredfold lower than that of the parent vector, suggesting that it may have some toxicity when present at high gene dosage. To further investigate this, a BamHI/Sall fragment from pDOT50 (isolated from *E. coli*) containing the three-genre AbiR operon was cloned into the low-copy-number vector, pTKR11, to minimize toxicity effects in *L. lactis* (Fig. 1). This construct was designated pDOU02 and introduced into *L. lactis* LM0230. Surprisingly, pDOU02 was also difficult to electroporate into *L. lactis* (>100x lower frequency than that of the parent vector), and few transformants that were obtained exhibited an AbiR$^{-}$ phenotype. These data suggested that the three-genre AbiR operon was toxic and that more DNA was required for the expression of the AbiR phenotype in *L. lactis*.

**Deletion analysis of pGBK17.** The 16,174-bp HpaI fragment encompassing the AbiR and the LlaKR21 R/M bacteriophage defense systems was previously sequenced (38) and is depicted in Fig. 2. The region upstream of the three-genre AbiR operon contains an IS982 element, and previous work by McKay et al. (25) ruled out the involvement of this region in the expression of the AbiR phenotype. The LlaKR21 R/M system is encoded downstream of the abiR operon and is followed by three complete genes, orf4, orf5, and orf6. It was previously suggested that orf4 may be the most likely candidate for involvement in AbiR expression as its low G+C content is consistent with that of abi genes (38). The LlaKR21R and llaKR21M genes have previously been shown to work independently of the other orf genes (37) and were deemed unlikely candidates for expression of AbiR.

A BglII deletion within the 16.2-kb DNA fragment encompassing both the LlaKR21 R/M system and the AbiR system resulted in pDOU012 (Fig. 2). This deletion derivative plasmid had the entire orf4 deleted and the llaKR21R gene and orf5 partially deleted. Surprisingly, this clone harbored the AbiR$^{-}$ phenotype, which ruled out the involvement of these three genes in the AbiR mechanism. The only orf genes remaining on pDOU012 downstream of the abiR operon were llaKR21M, orf6, and the IS982 transposase gene. The involvement of orf6 in AbiR was investigated by digesting pGBK17 with BbvC1/ BglII, which removed orf5 and orf6, resulting in pDOY01. Surprisingly, this clone harbored the AbiR phenotype, which ruled out the contribution of orf6 in the expression of this bacteriophage defense mechanism. These deletion clones effectively
ruled out the involvement of all the remaining ORFs on the plasmid except for the LlaKR2I methylase gene and the IS982 transposase gene. This suggested a very unusual organization of the AbiR bacteriophage defense system.

To investigate the involvement of the methylase gene in the AbiR phenotype, it was necessary to obtain deletions in this gene. Initially, the combination of unique restriction enzymes was used to try to delete the LlaKR2I methylase gene in
plasmid pDOU012. Digestion of pDOU012 with the restriction enzymes SacI and BsgI removed the methylase gene and the IS982 element. However, following ligation and selection of the deletion plasmid in L. lactis, unexpected extra deletions occurred beyond the targeted gene into the AbiR operon. Several other attempts to remove the LlaKR21 methylase gene were attempted using different combinations of restriction enzymes: PstI/BstEII, SacI/BstEII, PstI/BsgI, and PstI/BsrBI. Surprisingly, in all instances, the recovery of L. lactis clones was very low, and all exhibited deletions extending beyond the methylase gene into the three-gene abiR operon. Plasmids pDOM21 and pDOM27 are examples of clones in which the deletion extended into the abiRc gene, leaving only abiRa, abiRb, and orfb intact (Fig. 2).

Another strategy used to attempt to disrupt LlaKR21M was to introduce a frameshift mutation within the gene by restriction with SacI and blunting the ends with T4 DNA polymerase prior to ligation and selection in L. lactis. However, all clones obtained in L. lactis displayed extra deletions extending into the AbiR operon. These data suggested that deletion of the LlaKR21 methylase gene may cause toxicity problems in L. lactis from the AbiR operon.

Erase-a-Base deletion analysis of the LlaKR21 methylase gene. To precisely evaluate how much DNA could be deleted without toxicity problems, the Erase-a-Base method was used to progressively delete DNA from upstream of LlaKR21M through the gene, and the deletion derivatives in L. lactis were tested to assess the AbiR phenotype. Plasmid pDOU012 was digested with BsgI and BstEII as they introduced a 3' extension and a 5' end overhang, respectively. Numerous clones in L. lactis were obtained that showed progressive deletions through the IS982 element and again through the abiR gene. However, not one deletion terminating within the LlaKR21M gene was obtained. Plasmids pDOY04 and pDOY05 are examples of clones with deletions progressively extending into the IS982 element, while pDOY02 and pDOY03 are clones with deletions extending beyond the methylase gene into the abiR operon. The deletion extended to the 5' end of the abiRc gene in pDOY02 and extended further in pDOY03, leaving only the abiRa gene intact. Phenotypic analysis showed that those two deletion derivatives were AbiR negative and that pDOY04 and pDOY05 were AbiR positive. This confirmed that the methylase gene was critical for expression of the AbiR phenotype, as it was the only ORF on the plasmid that was not inactivated. It also substantiated the growing hypothesis that its role was to prevent toxicity from AbiR.

In vitro transposition analysis of pDOU012. A custom EZ::TN transposon was constructed using the transposon construction vector pMOD-2. The chloramphenicol resistance gene was obtained on an MsiI/EcoRI fragment from pC1372 and was cloned into SmaI/EcoRI-digested pMOD-2, resulting in plasmid pMOD1. The Cm' EZ::TN transposon was then obtained from pDOM1 and used in vitro to insert into pDOU012. A total of 327 independent clones containing the Cm' EZ::TN were obtained, and restriction analysis indicated that insertions occurred randomly all over the plasmid. Nineteen clones were sequenced to precisely locate the insertion (Fig. 3). Restriction mapping localized the approximate Cm' EZ::TN insertion for 146 clones. Among these, 79 clones had the Cm' EZ::TN located upstream of the LlaKR21M gene while 67 clones had the Cm' EZ::TN located downstream of the gene. Notably, the only gene in which an insertion was not obtained was
The methylase requirement in the AbiR phage defense

**DISCUSSION**

The bacteriophage defense mechanism AbiR from *L. lactis* subsp. *lactis* KR2 is encoded by three genes, *abiRa*, *abiRb*, and *abiRc*, organized in an operon that is involved in the expression of the AbiR phenotype (38). However, the presence of the *abiR* operon alone failed to exhibit an AbiR phenotype in *L. lactis*, implying that extra DNA was necessary to complete the expression of the AbiR phenotype. The findings of this study surprisingly demonstrate that the extra DNA required for expression of AbiR is the methylase gene from the LlaKR2I R/M system, which is located downstream of the AbiR operon. To date, almost 500 R/M systems have been characterized at the gene level (http://rebase.neb.com/cgi-bin/statlist), and the role of the methylase in each case is to protect the bacterial DNA from digestion by the cognate restriction enzyme. The LlaKR2I methylase gene was previously shown to be an active component of the LlaKR2I R/M system (37), and in this study, it was shown to protect *L. lactis* from the toxic effects of AbiR (Fig. 4 and 5). This latter protective role for the LlaKR2I methylase differs significantly from its protective role in the LlaKR2I R/M system. The sole role of a methylase in an R/M system is to protect the host genome from restriction by the cognate restriction enzyme, as methylated DNA would successfully escape the restriction system. Unlike an R/M system, AbiR terminates the proliferation of both unmethylated and methylated bacteriophage DNA. In addition, mechanistic studies have shown that AbiR acts by impeding bacteriophage DNA replication (38). This substantiates a novel role for the methylase from the LlaKR2I R/M system in *L. lactis* since besides protecting the chromosome from the LlaKR2I restriction enzyme, it also protects *L. lactis* from the toxicity of the AbiR operon.
To uncover the extra DNA necessary to express the AbiR mechanism in _L. lactis_, numerous deletion and insertion strategies were attempted. Digestion with single-cut restriction enzymes allowed targeted deletions in all ORFs on the plasmid whose function was not known. Of these, ORF5 and -6 contained a G+C content of 36.4% and 34.5%, respectively, similar to the _L. lactis_ typical range, while ORF4 contained a significantly lower G+C content of 30.5%, consistent with atypical _abi_ genes. Surprisingly, deletion of ORF4, -5, or -6 had no effect on the AbiR phenotype in _L. lactis_ (Fig. 2). The only other genes present on the plasmid were the IS982 transposase genes and the LlaKR2I restriction and modification genes. The IS982 element positioned directly upstream from the _abiR_ operon was disrupted by in vitro transposition, and the downstream IS982 element was successfully deleted without affecting the AbiR phenotype (Fig. 2 and 3). The LlaKR2I restriction gene was also successfully deleted without altering the AbiR phenotype (Fig. 2), leaving the LlaKR2I methylase gene as the only possible candidate gene on the plasmid to be investigated for involvement in the expression of the AbiR phenotype. However, numerous attempts to delete or introduce mutations specifically in _llaKR2IM_ either failed or resulted in further unintended deletions in the _abiR_ operon. The culmination of these data indicated that removal of the LlaKR2I methylase gene could not occur while the _abiR_ operon was intact, suggesting that the establishment of this operon in _L. lactis_ required this methylase gene. It is proposed that AbiR inhibits the host chromosomal DNA replication in a mechanism similar to phage DNA replication and that the presence of the LlaKR2I methylase is necessary to shield _L. lactis_ from AbiR effects. Confirmation of the protective role for the LlaKR2I methylase gene in the AbiR phenotype was obtained by trans-complementation of these two components in _L. lactis_, whereby the AbiR operon alone was toxic to the cell, and the _llaKR2IM_ gene was necessary to protect the cell from the AbiR toxicity and allow expression of the AbiR phenotype in _L. lactis_ (Fig. 4 and 5). This observation uncovered not only an original makeup for an Abi bacteriophage defense system for _L. lactis_ but also a novel protective role for the LlaKR2I methylase gene besides its traditional role to protect the host DNA from its cognate restriction enzyme.
Interestingly, the LlaKR21 methylase protects L. lactis from AbiR while it does not protect bacteriophage DNA replication. Indeed, replication of unmethylated and methylated bacteriophage DNA is affected identically by AbiR (38). Although how the LlaKR21 methylase works in protecting the host from AbiR is unclear, it is possible that the LlaKR21 methylase prevents AbiR from impeding the host DNA replication by methylating 5′-GATC-3′ sites in the origin of replication region. Interestingly, sequence analysis of the bacteriophage c2 genome shows only two 5′-GATC-3′ sequences (9,543 bp and 20,487 bp) that are neither contained in the bacteriophage ori region nor located close to it. Although the precise location of the ori of L. lactis IL1403 is unknown, it was mapped approximately in a region containing conserved elements (3). Sequence analysis shows 10 5′-GATC-3′ sequences localized within a 4.1-kb DNA stretch (coordinate 2,363,401; 2,100 bp) that includes the L. lactis ori. Alignment of these 5′-GATC-3′ sequences, extended by 10 bp on either side, with the corresponding 5′-GATC-3′ sites on bacteriophage c2 did not reveal any other sequence identities (Table 2). Similarly, sequence alignment of a 2-kb DNA region containing the bacteriophage ori with the corresponding region from the L. lactis IL1403 genome did not reveal any significant sequence identities or relevant motifs (data not shown). However, ori regions have elaborate secondary structures, and it is feasible that AbiR may bind DNA via specific secondary structures rather than sequence motifs. As AbiR impedes bacteriophage replication, and possibly the host DNA replication in the absence of the LlaKR21 methylase, it is likely that the LlaKR21 methylase protects L. lactis DNA either by physically interfering with the binding of the AbiR complex to the ori or by hindering its action due to methylation.

The genetic organization of the LlaKR21 R/M system is unique for a type II R/M system, as it is encoded by an endonuclease gene (llaKR21R) and a methylase gene (llaKR21M) that are divergently transcribed and separated by a copy of the

There are two classes of methylases that do not have a cognate restriction enzyme and whose roles are involved in cell cycle replication and other cellular regulatory functions. These are the E. coli Dam methylase and the cell cycle-regulated methyltransferases, termed CcrM, which have been found in a number of gram-negative bacteria (23). Like the Dam methylase, the LlaKR21 methylase recognizes the DNA sequence 5′-GATC-3′. However, Dam methylates at the adenine residue while LlaKR21 methylates at the cytosine residue (37). Dam methylase plays an important role in regulation of several cellular functions, such as directing mismatch repair, coordinating the timing of DNA replication, and segregating chromosomal DNA. However, this global regulator is not essential to the survival of E. coli cells as Dam-negative mutants of E. coli can readily be obtained. In contrast, Dam was shown to be essential for the viability of Yersinia pseudotuberculosis and Vibrio cholerae (20). In fact, disruption of the dam genes in these two bacteria resulted in no growth unless a copy of the wild-type dam gene was present in trans. The importance of Dam methylation in Y. pseudotuberculosis and V. cholerae is analogous to the essential role of CcrM for viability of other gram-negative bacteria.

The role of the LlaKR21 methylase in AbiR-containing strains of L. lactis is also analogous, as it also cannot be disrupted. As the AbiR phenotype was shown to result from the inhibition of bacteriophage DNA replication (38), it is likely that it may be toxic to the host because of interference with DNA replication, and the role of the LlaKR21 methylase may be to prevent this in a manner similar to that of the CcrM methylases in gram-negative bacteria. Interestingly, as with the LlaKR21 methylase, CcrM also methylates at the cytosine residue in its recognition sequence, 5′-GANTC-3′ (36). In addition, it was demonstrated that CcrM methylation was involved in regulating the cell cycle and controlling gene expression and DNA replication of some 20 α-proteobacteria. The striking observation, in all instances, was that CcrM methylation was essential for cell viability (33). Although Dam is not essential for cell viability, except in a few cases, it has functional analogies with CcrM, suggesting that they may have evolved from an existing 5′-GATC-3′ R/M system into the global regulators they are now.

Interestingly, the LlaKR21 methylase protects L. lactis from AbiR while it does not protect bacteriophage DNA replication. Indeed, replication of unmethylated and methylated bacteriophage DNA is affected identically by AbiR (38). Although how the LlaKR21 methylase works in protecting the host from AbiR is unclear, it is possible that the LlaKR21 methylase prevents AbiR from impeding the host DNA replication by methylating 5′-GATC-3′ sites in the origin of replication region. Interestingly, sequence analysis of the bacteriophage c2 genome shows only two 5′-GATC-3′ sequences (9,543 bp and 20,487 bp) that are neither contained in the bacteriophage ori region nor located close to it. Although the precise location of the ori of L. lactis IL1403 is unknown, it was mapped approximately in a region containing conserved elements (3). Sequence analysis shows 10 5′-GATC-3′ sequences localized within a 4.1-kb DNA stretch (coordinate 2,363,401; 2,100 bp) that includes the L. lactis ori. Alignment of these 5′-GATC-3′ sequences, extended by 10 bp on either side, with the corresponding 5′-GATC-3′ sites on bacteriophage c2 did not reveal any other sequence identities (Table 2). Similarly, sequence alignment of a 2-kb DNA region containing the bacteriophage ori with the corresponding region from the L. lactis IL1403 genome did not reveal any significant sequence identities or relevant motifs (data not shown). However, ori regions have elaborate secondary structures, and it is feasible that AbiR may bind DNA via specific secondary structures rather than sequence motifs. As AbiR impedes bacteriophage replication, and possibly the host DNA replication in the absence of the LlaKR21 methylase, it is likely that the LlaKR21 methylase protects L. lactis DNA either by physically interfering with the binding of the AbiR complex to the ori or by hindering its action due to methylation.

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**TABLE 2. DNA sequence alignment of 5′-GATC-3′ sequences from the bacteriophage c2 genome with 5′-GATC-3′ sequences from the L. lactis IL1403 ori**

<table>
<thead>
<tr>
<th>GATC source (coordinate)</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>c2 (9,543)</td>
<td>ATAGAGAAAA GTTC GGTTGAGGGT</td>
</tr>
<tr>
<td>c2 (20,487)</td>
<td>TAAACCCATT GTA GGTTAGGCTT</td>
</tr>
<tr>
<td>II1403 (5,546)</td>
<td>AGCTCAGTTT GTA AACACCCGCG</td>
</tr>
<tr>
<td>II1403 (1,120)</td>
<td>TTACTCTCTC GTA GGTCCGCAA</td>
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<td>GCCGGCGTCT GTA CGCCGATTAC</td>
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<tr>
<td>II1403 (2,364,771)</td>
<td>CAACTCAACGT GTA ACCCTTCAG</td>
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<td>TACAAACCCG GTA ACGGTGGAG</td>
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<td>II1403 (2,365,067)</td>
<td>CTTGACGGCAG GTA AACATCCAG</td>
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insertion element IS982, which is located within the intergenic region. The presence of the IS982 element between the two genes is ambiguous as the L.laK21 R/M system can operate without that element and with more efficiency. It is interesting to observe that the presence of the two IS982 elements, flanking the AbiR genetic determinants, generates a likely transposable AbiR composite. This striking observation not only substantiates the novel function of the L.laK21 methylase in AbiR expression but also illustrates the evolution of the L.laK21 methylase from its role in the L.laK21 R/M system toward a new and separate cellular function. The unique organization of the L.laK21 R/M system may have contributed to the evolution of the L.laK21 methylase toward a separate role comparable to that of Dam and CcrM methylases.

In this study, the three-gene AbiR operon was shown to require the L.laK21 methylase for expression in L. lactis. This is the first example in which a methylase gene that is associated with a cognate endonuclease gene has another role within the cell besides its traditional role of protecting the host DNA from its cognate restriction enzyme. It is now apparent that the L.laK21 methylase has evolved a novel cellular role independent from the R/M system that resembles the function of Dam and CcrM methylases. This may represent a snapshot in the evolution of the cell cycle-regulated methylases from an existing R/M system.

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REFERENCES