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 completed the characterization of AbiR, revealing that it is the first Abi system to be encoded by three genes, *abiRa*, *abiRb*, and *abiRc*, 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 generating a likely transposable AbiR composite. This observation not only substantiated the novel function of the LlaKR2I methylase in the AbiR system but also illustrated 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 methylases that include 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). Fortunately, 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

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TABLE 1. Bacteria, plasmids, and bacteriophage used in this study

Strain, plasmid, or phage	Relevant feature(s)	Size (kb)	Source or reference
<i>Lactococcus lactis</i> strains			
LM0230	Plasmid-free derivative of <i>L. lactis</i> subsp. <i>cremoris</i> C2		14
IL1403	Plasmid-free derivative of <i>L. lactis</i> subsp. <i>lactis</i> biovar diacetylactis IL594		6
<i>Escherichia coli</i> strains			
HB101	F ⁻ <i>thi-1 hsdS20</i> (r _B ⁻ m _B ⁻) <i>supE44 recA13 ara-14 leuB6 proA2 lacY1 galK2 rpsL20</i> (Str ^r) <i>xyl-5 ml-1</i>		Promega
XL1-Blue	Δ(<i>mcrA</i>)183 Δ(<i>mcrCB-hsdSMR-mrr</i>)173 <i>endA1 supE44 thi-1 recA1 gyrA96 relA1 lac</i>		Stratagene
Plasmids			
pTRKH2	High-copy-number lactococcal shuttle vector, Erm ^r	6.9	27
pTRKL1	Low-copy-number lactococcal shuttle vector, Erm ^r	8.6	27
pCI372	High-copy-number lactococcal shuttle vector, Cm ^r	5.7	18
pMOD-2	Tn5-based transposon construction vector	2.5	Epicentre
pDOM1	pCI372 Cm ^r gene cloned in pMOD-2	4.2	This study
pGBK17	16.2-kb HpaII fragment from pKR223 cloned into HpaII site of pGB301	26.2	22
	Erm ^r Cm ^r R ⁺ /M ⁺ AbiR ⁺		
pDOT50	AbiR operon cloned into pTRKH2, Erm ^r	15.8	38
pDOT29	<i>llaKR2IM</i> gene cloned into pCI372, Cm ^r	7.2	This study
pDOU02	AbiR operon cloned into pTRKL1, Erm ^r	17.5	This study
pDOU012	BsgI deletion derivative of pGBK17, Erm ^r Cm ^r	20.5	This study
pDOM21	A 3.9-kb deletion derivative of pDOU012, Erm ^r	16.6	This study
pDOM27	A 5.4-kb deletion derivative of pDOU012, Erm ^r	15.1	This study
pDOY01	BbvCI/BtgI deletion derivative of pGBK17, Erm ^r	24.3	This study
pDOY02	A 6-kb deletion derivative of pDOU012, Erm ^r	14.5	This study
pDOY03	A 9-kb deletion derivative of pDOU012, Erm ^r	11.5	This study
pDOY04	A 600-bp deletion derivative of pDOU012, Erm ^r	19.9	This study
pDOY05	A 1.1-kb deletion derivative of pDOU012, Erm ^r	19.4	This study
Phage			
c2	Prolate-headed phage		L. L. McKay

atypical G+C content (less than 30%), which is significantly lower than the average G+C content over the *L. lactis* genome (35.4%). The characteristic G+C content of *abi* genes may be due to their function or may be an indication of their recent horizontal transfer from another source.

While all lactococcal Abi systems reported to date exhibit no significant similarities at the protein level, many have similar modes of action (7). AbiA, AbiF, AbiK, and AbiR inhibit bacteriophage DNA replication (5, 8, 16, 38), and AbiB, AbiG, and AbiU interfere with DNA transcription (10, 26, 29). Other Abi systems, AbiD1, AbiE, and AbiQ, interfere with bacteriophage maturation steps subsequent to replication and transcription (2, 13, 16). Interestingly, AbiP and AbiT were found to affect bacteriophage development at two different points in the lytic cycle. AbiP impedes both replication and transcription, while AbiT targets replication and encapsidation of phage DNA (4, 12). However, the mechanisms of action of other Abi systems, such as AbiH, AbiN, and AbiO, remain unknown (30, 31, 32).

The native lactococcal bacteriophage resistance plasmid pKR223 was isolated from the commercial starter culture *L. lactis* subsp. *lactis* biovar diacetylactis KR2 and was shown to encode two distinct bacteriophage resistance mechanisms, an R/M system and an Abi system (22, 25). The nucleotide sequence of a 16,174-bp HpaII segment of pKR223 encompassing both the R/M and Abi systems was obtained, allowing the characterization of the R/M system, designated LlaKR2I (37), and partial characterization of the multigene Abi system, designated AbiR (38). In the latter communication, mechanistic studies of the AbiR system revealed that it was effective against the prolate bacteriophage c2 by impeding its DNA replication

and that the AbiR phenotype was abolished when *L. lactis* was grown at high temperatures. This HpaII fragment contains the LlaKR2I restriction and methylase genes separated by an IS982 element, followed by a three-gene AbiR operon and another IS982 element and directly preceded by three open reading frames (ORFs) and a 5' truncated ORF at the 5' end of the fragment. The AbiR system presents originality in its genetic organization as it is encoded by a three-gene operon in addition to at least one more gene located on this 16,174-bp HpaII fragment (38). This current study uncovers another role for the LlaKR2I methylase in the functioning of AbiR, making the LlaKR2I R/M system the first characterized functional R/M system in which the methylase component has a dual role within the cell.

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*. Electrocompetent cells were prepared by subinoculating 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 Holo and Nes (19). Electroporation was performed in a 2-mm gap electroporation cuvette at 2.45 kV

using an Eppendorf 2510 electroporator apparatus (Eppendorf Scientific Inc., Madison, WI). Immediately after electroporation, 1 ml of M17G, containing 0.5 M sucrose, 10 mM CaCl₂, and 10 mM MgCl₂, 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 Klaenhammer (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 µl of buffer P1 (50 mM Tris-HCl, 10 mM EDTA [pH 8.0]) supplemented with 100 µg/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. EZ::TN transposon used for in vitro transposition was supplied by Epicentre (Philadelphia, PA). Two strategies for obtaining plasmid deletion derivatives were used. The first strategy involved digesting 1 µg 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 redigested 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. Erythromycin-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^r) EZ::TN transposon was constructed in plasmid pDOM1. The Cm^r EZ::TN was obtained from pDOM1 either by digestion with PvuII or by PCR amplification using the pMOD<MCS> forward primer (5'-ATT CAG GCT GCG CAA CTG T-3') and reverse primer (5'-GTC AGT GAG CGA GGA AGC GGA AG-3') that anneal to each end of the Cm^r EZ::TN transposon. In vitro insertion of the Cm^r EZ::TN was performed in a final reaction volume of 10 µl, containing EZ::TN reaction buffer (0.5 M Tris-acetate [pH 7.5], 1.5 M potassium acetate, 100 mM magnesium acetate, and 40 mM spermidine), 1 µg of target plasmid DNA, a molar equivalent of the Cm^r EZ::TN, and 1 unit of EZ::TN transposase. The reaction was carried out at 37°C for 2 h and then stopped by the addition of 1 µl EZ::TN 10× stop solution (1% 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 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 PCRs were carried out with a Robocycler gradient 40 temperature cyclor (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 phage, 100 µl of the relevant phage dilution was added to 3 ml of molten M17G (45°C) containing 300 µl of a growing (an optical density at 600 nm of ~0.50) culture of the appropriate host and 10 mM CaCl₂. The contents were mixed and poured onto M17G agar plates containing 10 mM CaCl₂. The EOP of the phage was defined as the titer of the phage on the test host divided by the titer of the phage 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 2,364,567; 1,001 bp) with the *ori* of bacteriophage c2 (coordinate 21,141; 1,001 bp).

RESULTS

Illustrating the AbiR phenotype. Previous studies had indicated that a 16.2-kb fragment from pKR223 encoded the

LlaKR2I R/M system and an abortive infection defense system, designated AbiR (25, 37, 38). While the LlaKR2I 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-gene AbiR operon.

A SphI/PstI fragment, containing the three-gene *abiR* operon from pGBK17, was previously klenowed 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/SalI fragment from pDOT50 (isolated from *E. coli*) containing the three-gene *abiR* operon was cloned into the low-copy-number vector, pTRKL1, 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* (>100× lower frequency than that of the parent vector), and the few transformants that were obtained exhibited an AbiR⁻ phenotype. These data suggested that the three-gene *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 HpaII fragment encompassing the AbiR and the LlaKR2I R/M bacteriophage defense systems was previously sequenced (38) and is depicted in Fig. 2. The region upstream of the three-gene *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 LlaKR2I 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 *llaKR2IR* and *llaKR2IM* genes have previously been shown to work independently of the other *orf* genes (37) and were deemed unlikely candidates for expression of AbiR.

A BsgI deletion within the 16.2-kb DNA fragment encompassing both the LlaKR2I R/M system and the AbiR system resulted in pDOU012 (Fig. 2). This deletion derivative plasmid had the entire *orf4* deleted and the *llaKR2IR* 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 *llaKR2IM*, *orf6*, and the IS982 transposase gene. The involvement of *orf6* in AbiR was investigated by digesting pGBK17 with BbvCI/BtgI, 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

FIG. 1. Construction of plasmids pDOT50 (28) and pDOU02 containing the three-gene *AbiR* operon. The numbers refer to the *orf* genes sequenced on the 16.2-kb phage resistance fragment of plasmid pGBK17, where 1, 2, and 3 are the *abiR* gene operons and M and E are the determinants of the *LlaKR2I* R/M system.

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 *llaKR2IM* methylase gene in

FIG. 2. Restriction map and gene organization of the 16,174-bp bacteriophage-resistant region of pKR223. Organized below the gene map are the deletion plasmids used to investigate the involvement of the genes, other than the three-gene operon (*abiRa*, *abiRb*, and *abiRc*), in the expression of the AbiR mechanism. *, a 5' truncated *orf*. Dashed lines indicate deleted regions.

plasmid pDOU012. Digestion of pDOU012 with the restriction enzymes *SacI* and *BglI* 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 *LlaKR2I* methylase gene were attempted using different combinations of restriction enzymes: *PstI*/*BstEII*, *SacI*/*BstEII*, *PstI*/*BglI*, 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 *orf6* intact (Fig. 2).

Another strategy used to attempt to disrupt *llaKR2IM* 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 *LlaKR2I* methylase gene may cause toxicity problems in *L. lactis* from the AbiR operon.

Erase-a-Base deletion analysis of the *LlaKR2I* 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 *llaKR2IM* through the gene, and the deletion derivatives in *L. lactis* were tested to assess the AbiR phenotype. Plasmid pDOU012 was digested with *BglI* 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 *llaKR2IM* 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 *MslI*/*EcoRI* fragment from pCI372 and was cloned into *SmaI*/*EcoRI*-digested pMOD-2, resulting in plasmid pDOM1. The *Cm^r* 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^r* 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^r* EZ::TN insertion for 146 clones. Among these, 79 clones had the *Cm^r* EZ::TN located upstream of the *llaKR2IM* gene while 67 clones had the *Cm^r* EZ::TN located downstream of the gene. Notably, the only gene in which an insertion was not obtained was

FIG. 3. Organization of the random *Cm^r EZ::TN* insertions in pDOU012. The map shows the precise location of the *Cm^r EZ::TN* transposon for 19 clones and the resulting *AbiR* phenotype. Numbers over the black arrowheads refer to the number of transposons mapped at this point. The arrows at the bottom indicate the approximate location of 79 *Cm^r EZ::TN* insertions located upstream of the *LlaKR2I* methylase gene and 67 *Cm^r EZ::TN* insertions located downstream of it. No insertion occurred in the methylase gene.

llaKR2IM. Figure 3 illustrates the precise and approximate locations of the *Cm^r EZ::TN* insertions in pDOU012 in addition to the *AbiR* phenotype of the clones. Disruption of the three-gene *abiR* operon abolished the *AbiR* phenotype, while *Cm^r EZ::TN* insertions downstream of the *llaKR2IM* gene did not affect the bacteriophage defense system. The in vitro transposon insertion data substantiated the results obtained earlier from the gene deletion strategy. The failure to delete or mutate the methylase gene *llaKR2IM* in *L. lactis* with the *AbiR* operon intact supported the hypothesis that the methylase gene was needed to prevent toxicity from *AbiR*.

***AbiR* toxicity in *L. lactis* and the protective role of the *LlaKR2I* methylase gene.** The difficulty of transforming the *abiR* operon in *L. lactis* at both high and low gene dosages suggested toxicity from *AbiR*. In addition, the data obtained from the deletion strategy and the in vitro transposon analysis highly supported the hypothesis that *AbiR* was toxic without the methylase gene in *L. lactis* and suggested that it was required to alleviate toxicity from *AbiR*. To investigate the role of the *LlaKR2I* methylase gene in the expression of *AbiR* in *L. lactis*, a *trans*-complementation cloning strategy was used with *L. lactis* LM0230 and *L. lactis* IL1403 serving as bacterial hosts.

Plasmid pDOT50 (*abiR* operon) was electroporated into *L. lactis* LM0230 and *L. lactis* LM0230 containing the methylase gene (pDOT29) as well as into the native *L. lactis* IL1403 and *L. lactis* IL1403 harboring pDOT29. Transformants were selected on M17G plates with erythromycin. The results are striking since without the presence of the *llaKR2IM* gene, only a few transformants were obtained while hundreds were obtained in the host harboring the methylase gene (Fig. 4). This observation suggested that the presence of the *LlaKR2I* methylase gene in *L. lactis* protected the cell from *AbiR* since higher transformation efficiency was observed when both plasmids were present in *L. lactis*. Phenotype analysis of colonies in Fig. 4 showed that those in panels A and C were *AbiR* negative and that those in panels B and D were *AbiR* positive (Fig. 5).

Reisolation of pDOT50 from the transformants in Fig. 5A and C and reelectroporation into *L. lactis* LM0230 and *L. lactis* IL1403, with and without pDOT29, exhibited similar transformation frequencies, suggesting that the *AbiR* operon on the reisolated pDOT50 was no longer active. This suggested that mutations occurred in pDOT50 following its transfer into these

hosts. Phenotype analysis of these transformants showed that they were all *AbiR* negative, confirming that the introduction of the *AbiR* operon into *L. lactis*, without the *LlaKR2I* methylase gene, can occur only following inactivation of *AbiR*. The combination of the data obtained from deletion, in vitro transposon insertion, and *trans*-complementation indicates that the expression of the *AbiR* bacteriophage defense mechanism in *L. lactis* depended on the three-gene *abiR* operon together with the *llaKR2IM* gene from the *LlaKR2I* R/M system.

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.

FIG. 4. Demonstration of the protective role of the LlaKR2I methylase in AbiR toxicity after introducing pDOT50 by electroporation into *L. lactis* LM0230 (A), *L. lactis* LM0230(pDOT29) (B), *L. lactis* IL1403 (C), and *L. lactis* IL1403(pDOT29) (D).

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.

FIG. 5. Measurement of the EOP of bacteriophage c2 on *L. lactis* containing the AbiR⁺ plasmid, pDU012, and the effect of the *llaKR2I* methylase gene on the expression of the AbiR phenotype by the three-gene *abiR* operon in pDOT50. The arrow refers to reisolated pDOT50 from *L. lactis* LM0230 that is reintroduced into *L. lactis* LM0230. Numbers 1 and 2 refer to the sequence that the plasmids were introduced into *L. lactis* LM0230.

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 LlaKR2I methylase recognizes the DNA sequence 5'-GATC-3'. However, Dam methylates at the adenine residue while LlaKR2IM 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 LlaKR2I 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 in 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 LlaKR2I methylase may be to prevent this in a manner similar to that of the CcrM methylases in gram-negative bacteria. Interestingly, as with the LlaKR2I 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 -pro-

teobacteria. 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 LlaKR2I 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 LlaKR2I methylase works in protecting the host from AbiR is unclear, it is possible that the LlaKR2I 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 LlaKR2I methylase, it is likely that the LlaKR2I 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 LlaKR2I R/M system is unique for a type II R/M system, as it is encoded by an endonuclease gene (*llaKR2IR*) and a methylase gene (*llaKR2IM*) that are divergently transcribed and separated by a copy of the

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*

GATC source (coordinate)	Sequence
c2 (9,543)	ATAGAGAAAA GATC GGTGAGGGT
c2 (20,487)	TAACCCATTA GATC TTATAGGCTT
IL1403 (546)	AGTCCGATT GATC ACAACGCGG
IL1403 (1,120)	TTTAAGTTCT GATC GTATTCCGA
IL1403 (1,153)	TAATTTGGAA GATC GCTTGGTTTC
IL1403 (1,504)	TTTCATTTTCA GATC TTGTTGGTCC
IL1403 (2,363,547)	GAAAGGAGGT GATC CAGCCGCACC
IL1403 (2,363,721)	GCGGCGTGCT GATC CGCGATTACT
IL1403 (2,364,652)	AGAGTTTAC GATC CGAAAACCTT
IL1403 (2,364,771)	CAATGTGGCC GATC ACCCTGTCAG
IL1403 (2,364,846)	TACAACGCGG GATC ATCTTTGAGT
IL1403 (2,365,067)	CCTGAGCCAG GATC AAACCTCTCAA

insertion element IS982, which is located within the intergenic region. The presence of the IS982 element between the two genes is ambiguous as the LlaKR2I 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 LlaKR2I methylase in AbiR expression but also illustrates the evolution of the LlaKR2I methylase from its role in the LlaKR2I R/M system toward a new and separate cellular function. The unique organization of the LlaKR2I R/M system may have contributed to the evolution of the LlaKR2I 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 LlaKR2I 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 LlaKR2I 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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