

Characterization of AbiR, a Novel Multicomponent Abortive Infection Mechanism Encoded by Plasmid pKR223 of *Lactococcus lactis* subsp. *lactis* KR2†

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The native lactococcal plasmid pKR223 encodes two distinct phage resistance mechanisms, a restriction and modification (R/M) system designated *LlaKR2I* and an abortive infection mechanism (Abi) which affects prolate-headed-phage proliferation. The nucleotide sequence of a 16,174-bp segment of pKR223 encompassing both the R/M and Abi determinants has been determined, and sequence analysis has validated the novelty of the Abi system, which has now been designated AbiR. Analysis of deletion and insertion clones demonstrated that AbiR was encoded by two genetic loci, separated by the *LlaKR2I* R/M genes. Mechanistic studies on the AbiR phenotype indicated that it was heat sensitive and that it impeded phage DNA replication. These data indicated that AbiR is a novel multicomponent, heat-sensitive, “early”-functioning Abi system and is the first lactococcal Abi system described which is encoded by two separated genetic loci.

Lactococci are widely used in the manufacture of numerous fermented dairy products. The nonsterile nature of many of these processes renders them particularly vulnerable to phage infection. Many strains have evolved natural phage resistance mechanisms and can be divided into four classes based on their mode of action: adsorption blocking, phage DNA penetration blocking, restriction and modification (R/M), and abortive infection (Abi) (reviewed in reference 15). While mechanisms of the first two classes act at the cell surface, R/M and Abi mechanisms act intracellularly. The complementary action of resistance mechanisms can provide strains with a significant barrier to phage infection.

Abi systems refer to any phage defense, other than R/M systems, which prevents phage proliferation after the phage DNA has entered the host cell intact. These systems encompass a diverse variety of mechanisms, which may act to prevent phage DNA replication, transcription, translation, processing, or assembly or lysis of the cell. Since 1984, when pNP40, which encodes an Abi system, was first reported (22), numerous native Abi-encoding plasmids have been reported. Currently, the genetic determinants of 17 Abi systems have been described, and all but two of them were encoded on native lactococcal plasmids (reviewed in reference 10). The majority of these Abi phage defense systems were found to be encoded by single genes, except for AbiE (13), AbiG (25), and AbiL (6), which are encoded by two genes. Comparisons of Abi sequences with existing database sequences have offered little insight into the molecular basis for their modes of action. In addition, com-

paring these sequences with each other has generally indicated evolutionary, diverse origins, presumably due to the multitude of potential mechanisms of action for this type of defense. The notable exceptions are AbiD (24), AbiDI (2), and AbiF (13), which exhibit significant similarity to each other, pointing to the presence of a family of Abi mechanisms. In addition, AbiA, which was identified as being encoded by pTR2030 (19) and also by pCI829 (5), shares homology with AbiK (9). A discernible molecular feature common to all lactococcal Abi systems characterized to date is that the G+C content of the encoding loci is significantly lower than the typical G+C content of lactococcal DNA.

The native lactococcal phage resistance plasmid pKR223 was isolated from *Lactococcus lactis* subsp. *lactis* bv. *diacetylactis* KR2 and was shown to mediate two distinct mechanisms of resistance to bacteriophage infection, an R/M system and an Abi system (20, 23). The sequence and genetic characterization of the R/M system (designated *LlaKR2I*) have been reported previously (31). The Abi system impeded proliferation of prolate-headed phage, and preliminary analysis indicated that the genetic determinants for its expression may not all be contained in one locus (23, 30). In this study we furthered the characterization of this novel phage defense system and determined the loci on the plasmid which are necessary for its expression.

Bacteria, plasmids, phage, and growth conditions. The *L. lactis* strains used in this study included *L. lactis* subsp. *lactis* bv. *diacetylactis* KR2, an industrial starter strain containing seven plasmids, including pKR223, which encodes the *LlaKR2I* R/M system and an Abi mechanism (20); *L. lactis* subsp. *cremoris* LM0230, a plasmid-free derivative of *L. lactis* subsp. *cremoris* C2 (7) (formerly called *L. lactis* subsp. *lactis*); and GBK17, an LM0230 transformant with a 19-kb *HpaII* fragment of pKR223 cloned into the *HpaII* site of pGB301 in a construct designated pGBK17 (20). *Escherichia coli* XL1Blue MRF' (Stratagene, La Jolla, Calif.) served as the host for the construction of pUC19 chimeras containing fragments of pGBK17 for sequencing. Plasmid pMN7 is an *SphI* deletion of pGBK17 and is R/M and Abi positive (23). Plasmid pMN5 is a partial *EcoRI* deletion of pGBK17 and is R/M positive and Abi negative (23). Plasmid

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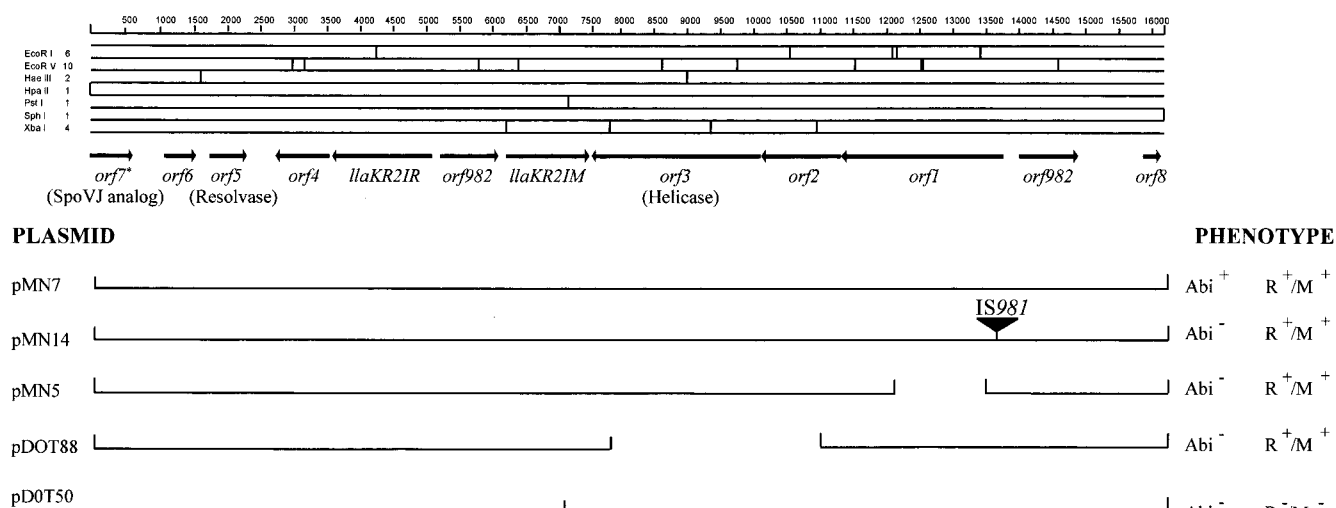


FIG. 1. Restriction map and gene organization of the 16,174-bp phage resistance region of plasmid pKR223. The organization and sequence of the *LlaKR2I* R/M system between base positions 3653 and 7537 have previously been reported (31). Predicted functions for ORF3, ORF5, and ORF7 based on sequence comparisons are indicated. Below the gene map, the organization of the deletion and insertion plasmids used in the Abi characterization is represented, together with their respective phenotypes. *, a 5' truncated ORF.

pDOT64 is the *LlaKR2I* R/M system cloned in pCI372 (31). Plasmid pTRKH2 is a 6.9-kb *E. coli/Lactococcus* shuttle cloning vector (27). The prolate-headed bacteriophage ϕ c2, which was obtained from the culture collection of L. L. McKay, was used for monitoring the Abi phenotype encoded by pKR223 and its derivatives. Growth conditions for lactococci and *E. coli*, as well as for phage assays, were as described previously (31).

Confirmation of the pKR223 Abi phenotype. Previous studies indicated that a 19-kb region of pKR223 encoded an R/M system and another phage defense system which did not impede absorption of the phage to the cells. As it also resulted in an approximately twofold reduction in the burst size of the phage, it was termed an Abi system (20). To exclude the possibility that this system might be impeding injection of the phage DNA into the cell, cell survival studies were undertaken. Unlike Abi systems, which undergo cell death following phage infection, DNA injection blocking systems do not result in cell death (14). The effect of the Abi mechanism on cell survival was evaluated by infecting a mid-log-phase suspension of *L. lactis* LM0230(pGBK17) in M17G containing 5 mM CaCl₂ with ϕ c2, at a multiplicity of infection of >1, and monitoring the survival of cells over a 3-h period. As *L. lactis* LM0230(pGBK17) infected with methylated ϕ c2 resulted in 100% cell death, it can be concluded that the phage defense system is an Abi mechanism.

Sequence analysis of the pKR223 Abi system. Previous deletion studies of this 19-kb region of pKR223 indicated that the R/M and Abi systems were located on a 16.2-kb *HpaII/SphI* fragment (23). This fragment was subcloned in a variety of fragments in pUC19, and the nucleotide sequence of both DNA strands was determined. This segment included a 4.6-kb *EcoRV/XbaI* fragment, which encodes the *LlaKR2I* R/M system comprising an endonuclease gene (*llaKR2IR*) and a methyltransferase gene (*llaKR2IM*) which are divergently transcribed with respect to each other, with a complete copy of the lactococcal insertion sequence IS982, located in the intergenic region (31). Sequencing reactions were performed with an ABI Prism dye terminator cycle sequencing kit using *AmpliTaq* DNA polymerase, and the products were separated using an

ABI 377 automatic sequencer (Applied Biosystems, Foster City, Calif.). DNA sequences were compiled and analyzed using DNASTAR software (DNASTAR, Madison, Wis.) and were compared to database sequences using the BLAST suite of programs (1).

In addition to the *LlaKR2I* R/M genes and two IS982 elements, the sequence organization revealed seven additional open reading frames (ORFs) and a 5' truncated ORF (Fig. 1). The truncated ORF, ORF7, spanned the *HpaII* site used in the construction of pGBK17, and the deduced truncated protein sequence is homologous to members of the CBXX/CBQX superfamily of proteins with greatest identity (44% over 214 amino acids) to the C-terminal end of SpoVJ from *Bacillus subtilis* (11). The deduced protein sequence of ORF6 shared similarity with a gene of unknown function located on the 3' end of an operon encoding the *LlaI* R/M system on pTR2030 (28), with 30% identity observed over 168 amino acids. Based on homologies to known protein sequences, ORF5 was predicted to belong to the resolvase family of site-specific recombinases, which have a role in recombination, either between inverted repeat sequences contributing to the inversion of a DNA segment or with repeat sequences in different DNA molecules permitting either cointegration or excision events to occur. The deduced resolvase protein shares 92% identity with a putative protein from the lactococcal antibiotic-resistant plasmid pK214 (29). The largest ORF, ORF3, was over 2.5 kb in length and was capable of encoding a putative SNF-like helicase homolog. The greatest identity (41% over 569 amino acids) was observed with a deduced protein from *Bacillus cereus* (26). Members of the SNF2 family of proteins have been implicated in a variety of cellular processes including transcriptional regulation, recombination, and a variety of DNA repair mechanisms. All members of the SNF2 family of proteins contain sequence motifs characteristic of the DNA and RNA helicase protein families. These related families are characterized by a number of conserved sequence motifs, including a special version of the B motif of ATP-binding proteins which is present as either DEAD, DEAH, or DEXH (3, 4, 17). The presence of characteristic helicase motifs (including a DEVH box) in the C-terminal end of ORF3 suggests that this protein

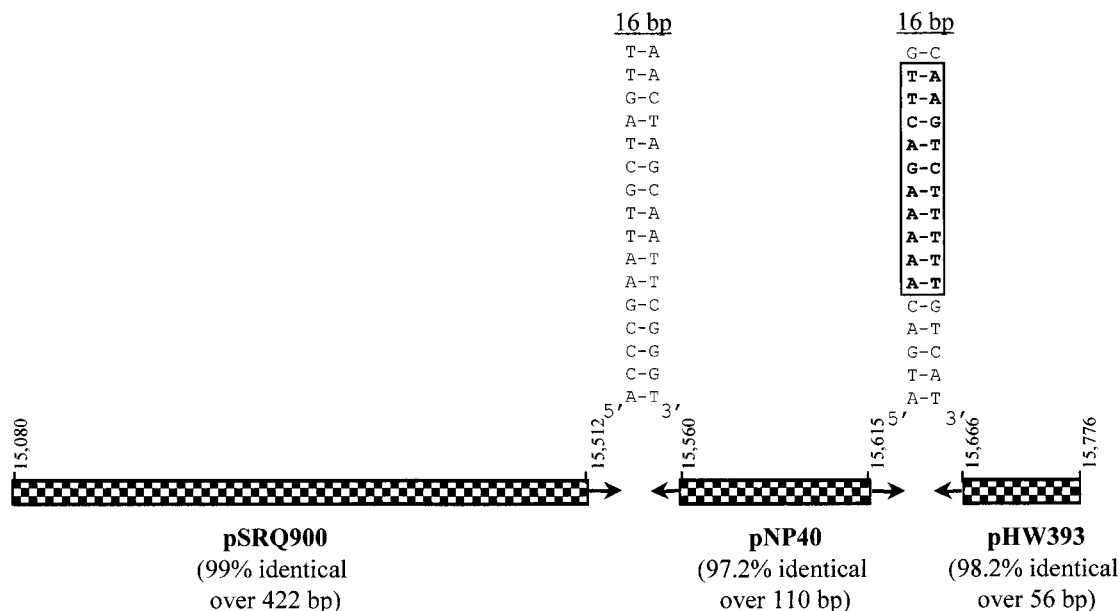


FIG. 2. Modular organization of DNA segments from the intervening region between IS982 and ORF8 which are homologous to segments from different plasmids and which are separated by inverted repeat structures. Portions of the plasmids pSRQ900 (8), pNP40 (12), and pHW393 (21) which share homology with pKR223 are represented by checkered boxes. The 16-bp inverted repeat located between segments similar to fragments of pSRQ900 and pNP40 are identical to the left and right termini of IS982. A perfect 16-bp inverted repeat located between segments similar to fragments of pNP40 and pHW393 shared similarities with the putative transcriptional terminator structure (10 bp highlighted in boldface and boxed) located after the nisin resistance gene (12). The nucleotide positions of the homologous segments in the 16,174-bp *Hpa*II/*Sph*I fragment of pKR223 are indicated above the segments.

may be regarded as a DEXH-box ATP-dependent helicase. ORF2 (C-terminal region) exhibited the greatest sequence similarity (23% over 212 amino acids) to Pip (phage infection protein), which is a chromosomally encoded transmembrane protein in *L. lactis* that is required for ϕ c2 infection (16). However, the region of Pip similar to the predicted ORF2 protein does not correspond to the putative transmembrane region but to the large central region of the protein, which is theoretically oriented outside the membrane. As the biological function of Pip is not currently known, it is not possible to predict functional analogies for the predicted ORF2 protein. The deduced protein sequences of ORF1, ORF4, and ORF8 did not reveal any significant homologies.

ORF1 to ORF3 are codirectionally transcribed, with no significant secondary structure observed within these three ORFs. A putative rho-independent transcriptional terminator was located between ORF3 and the countertranscribed methyltransferase gene, *llaKR2IM*. Upstream of ORF1, a second copy of IS982 was observed which was 98.8% identical to the IS element located between the *LlaKR2I* restriction and modification genes and which contained identical 16-bp perfect inverted repeats at its left and right termini. The region between IS982 and ORF8 contained a number of significant secondary structures, including 16-bp perfect inverted repeats (separated by 16 nucleotides), which are characteristic of the left and right termini of IS982 elements (Fig. 2). An additional 17-bp perfect inverted repeat structure with similarities to the putative rho-independent terminator structure located after the plasmid-borne nisin resistance gene, *nsr* (12), was located 55 bp downstream. These two sets of inverted repeat structures appear to form the boundaries between DNA fragments from a variety of sources, as DNA segments bordering these two sets of inverted repeat structures displayed significant identity to segments from different plasmids. This modular organization suggests that the inverted repeat structures are associated with a num-

ber of DNA recombination events in this region. It is interesting to speculate that the putative resolvase encoded by ORF5, which belongs to a family of site-specific recombinases known to promote DNA arrangements between inverted repeat sequences, may be responsible for some of these arrangements.

Experimental analysis of ORFs involved in the Abi phenotype. Previous work by McKay et al. (23) found that during transformation of *L. lactis* LM0230 with pGBK17, an unexpected transformant was observed in which pGBK17 had acquired an extra piece of DNA and had lost the Abi phenotype. This extra piece of DNA was linked to the presence of an insertion sequence, IS981 (30), and its precise insertion point corresponds to a site within ORF1, indicating that either the disruption of this ORF or possible polar effects on the codirectionally transcribed ORFs, ORF2 and ORF3, accounted for the loss of the Abi phenotype (Fig. 1). Precise mapping of the deletion plasmid pMN5 substantiated the likely involvement of ORF1, as the deletion in this plasmid was contained within ORF1 and resulted in the abolition of the Abi phenotype. To determine if ORF2 and ORF3 may be involved in the Abi phenotype, pGBK17 was partially digested with *Xba*I and religated. This resulted in the isolation of deletion plasmid pDOT88, which had a deletion extending over ORF2 and ORF3 (Fig. 1). As this construct was Abi negative, it indicated that either one or both ORFs were implicated in the phage resistance mechanism.

To establish if the three ORFs exhibited any Abi phenotype, an 8.9-kb *Pst*I/*Sph*I segment from pGBK17 was subcloned in pTRKH2 and introduced into *L. lactis* LMO230. This construct, pDOT50, also did not exhibit any detectable Abi phenotype (Fig. 1). As this subclone contained all the DNA from the right of the R/M system (as positioned in Fig. 1), this result indicated that while several and possibly all three of the contiguous ORFs are involved in the Abi phenotype, an additional

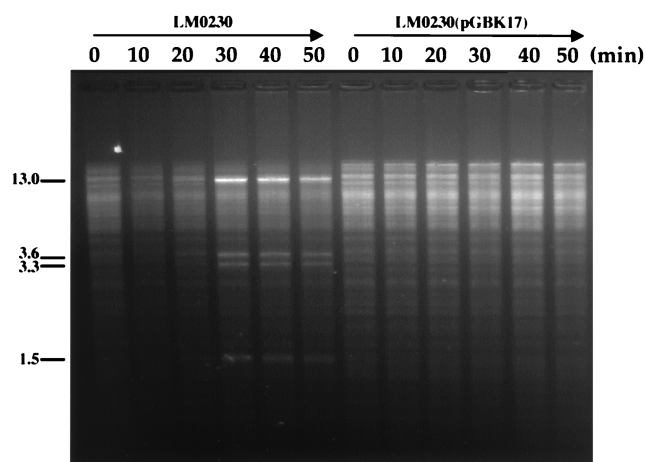


FIG. 3. Depiction of the replication of the ϕ c2 genome in *L. lactis* LM0230 and in the same strain containing pGBK17 over 50 min. All lanes are digested with *Eco*RI. The numbers on the left refer to the expected size of the ϕ c2 fragments, in kilobases.

element(s) from the left of the R/M system is also required for the Abi phenotype.

The overall sequence organization of the phage resistance region of pKR223 points to an Abi mechanism interrupted by an R/M system. If the R/M system did insert into pKR223 to divide the genes encoding the Abi mechanism, then this suggests that ORF4 may be involved in the Abi mechanism. Evidence in support of this is the fact that ORF4 is the only ORF located to the left of the *Lla*KR2I R/M system (as oriented in Fig. 1) that has a low G+C content (30.5%), which is a characteristic feature of lactococcal Abi systems. To date, all genes associated with Abi mechanisms have a G+C content that is atypically lower (24 to 31%) than the average lactococcal G+C content (37%). ORF1, ORF2, and ORF3 also have atypically low G+C contents (29.8, 32.5, and 31.6%, respectively). As all other ORFs have largely typical G+C contents (34.4 to 39.1%), ORF4 may be associated with the pKR322 Abi mechanism. However, this has yet to be investigated.

Mode of action. The mode of action of the pKR223 Abi mechanism was investigated essentially as outlined by Hill et al. (18). The bacteriophage ϕ c2 was first propagated on a lactococcal host harboring the methyltransferase gene, *lla*KR2IM, to protect the phage DNA from digestion by the *Lla*KR2I restriction endonuclease. Following infection of *L. lactis* LM0230(pGBK17) with this phage and subsequent isolation of total genome DNA at various times postinfection, it was found that relative to the plasmid-free phage-sensitive host, pGBK17 significantly reduced the replication of phage DNA (Fig. 3). Therefore, these data indicate that the pKR223 Abi system targets an early stage in the ϕ c2 developmental cycle, and the mechanism can therefore be classified as “early.”

Of the characterized Abi systems reported to date, this is the fourth one where the mechanism can be classified as “early.” As two of the other three (Abi A and K) were found to be ineffective during growth of *L. lactis* at 39 to 40°C, the pKR223 Abi phenotype was tested at the maximum growth temperature for strain LM0230, 39°C. At this temperature, there was essentially no significant Abi phenotype, as evidenced by infection with ϕ c2.

The results of this study clearly indicate that this Abi system is a novel, multicomponent phage resistance mechanism which is encoded by two genetic loci that are separated by *Lla*KR2I

R/M, and the system has been designated AbiR. It protects *L. lactis* cells from ϕ c2 infection by impeding its DNA replication. However, similar to many lactococcal phage defense systems, AbiR is relatively ineffective during growth at elevated temperatures. Understanding the molecular mechanisms for the temperature sensitivity of AbiR will allow strategies to be developed to overcome this limitation.

Nucleotide sequence accession number. The 16,174-bp nucleotide sequence presented here has been deposited in the GenBank database and has been assigned the accession number AF216814.

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