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Phenotypic and Genetic Characterization of the Bacteriophage Abortive Infection Mechanism AbiK from *Lactococcus lactis*

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The natural plasmid pSRQ800 isolated from *Lactococcus lactis* subsp. *lactis* W1 conferred strong phage resistance against small isometric phages of the 936 and P335 species when introduced into phage-sensitive *L. lactis* strains. It had very limited effect on prolate phages of the c2 species. The phage resistance mechanism encoded on pSRQ800 is a temperature-sensitive abortive infection system (Abi). Plasmid pSRQ800 was mapped, and the Abi genetic determinant was localized on a 4.5-kb *Eco*RI fragment. Cloning and sequencing of the 4.5-kb fragment allowed the identification of two large open reading frames. Deletion mutants showed that only *orf1* was needed to produce the Abi phenotype. *orf1* (renamed *abiK*) coded for a predicted protein of 599 amino acids (AbiK) with an estimated molecular size of 71.4 kDa and a pI of 7.98. DNA and protein sequence alignment programs found no significant homology with databases. However, a database query based on amino acid composition suggested that AbiK might be in the same protein family as AbiA. No phage DNA replication nor phage structural protein production was detected in infected AbiK$^+$ *L. lactis* cells. This system is believed to act at or prior to phage DNA replication. When cloned into a high-copy vector, AbiK efficiency increased 100-fold. AbiK provides another powerful tool that can be useful in controlling phages during lactococcal fermentations.

Lactococcus lactis is widely used in mesophilic milk fermentations to produce cheese, buttermilk, and sour cream. Due to the expanding activities in these industries, *L. lactis* starter cultures are under pressure to perform at industrial standards of consistency, efficiency, and speed. However, these starters can be inactivated by bacteriophages present in milk. Phages are the leading cause of fermentation failures during the manufacture of cultured dairy products (19, 28, 34). Since the identification of the causal agent in the mid-1930s, the dairy industry has learned to manage this natural phenomenon by developing various solutions such as better sanitation, process modifications, and use of phage-resistant cultures (19, 22). Extensive studies have been carried out on the innate phage exclusion mechanisms of *L. lactis* strains (for reviews, see references 19 and 22). Numerous native barriers against phages have been found, and most of them are encoded on plasmids. These antiphage systems are currently classified in four groups based on their mode of action (18, 19, 22): blocking of phage adsorption, blocking of phage DNA penetration, restriction/modification (R/M), and abortive infection (Abi). Commercial *L. lactis* strains with enhanced phage resistance have been constructed by introducing natural plasmids coding for such antiphage systems (50). The improved strains have already been successfully employed for large-scale dairy fermentations. Unfortunately, new phages capable of overcoming the introduced defense systems have emerged (1, 38). Thus, the search for novel phage resistance mechanisms is an ongoing objective for culture suppliers (39).

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Lactococcal phages are currently classified in 12 different species based on morphology and DNA homology (28). However, the most disturbing lactococcal phages in dairy plants worldwide have been, by far, members of the species 936 (small isometric heads), c2 (prolate heads), and P335 (small isometric heads) (28, 36). Numerous DNA-DNA hybridization studies have revealed the absence of significant DNA homology among the three species (28). The current consensus is that these three species are genetically distinct and that *L. lactis* starter cultures should be resistant to these phages (34).

Among the natural *L. lactis* phage resistance mechanisms, the Abi systems are believed to be the most powerful (51). In a classical abortive infection, the phage lytic cycle is terminated but only after phage adsorption, DNA injection, and early phage gene expression (32, 40, 51, 53). Generally, the host is also killed in the abortive process. This suicidal outcome traps the phages within the infected cells and limits their dissemination, which is demonstrated by a reduced plaque size (51). Many Abi systems have been identified in other bacterial genera (for reviews, see references 40 and 53). Some of these systems have been studied, but the molecular basis remains somewhat unclear. New evidence is now leading to more specific models for their action. Likewise, Abi systems in *L. lactis* are very poorly understood (51). To date, nine Abi systems have been characterized to the molecular level: AbiA (24), AbiB (6), AbiC (14), AbiD (32), AbiD1 (2), AbiE (17), AbiF (17), AbiG (42), and AbiH (46). These systems were isolated from different *L. lactis* strains. There is significant protein homology among AbiD, AbiD1, and AbiF, indicating the existence of a family of Abi proteins in *L. lactis* (2, 17). The absence of homology between the other *L. lactis* Abi mechanisms suggests a different mode of action and/or phage targets.

AbiA is believed to interfere with DNA replication of small isometric phages (10, 35). Parreira et al. (45) demonstrated that AbiB promotes the degradation of phage transcripts, start-

TABLE 1. Bacterial strains and bacteriophages used in this study

Bacterial strain or phage	Relevant characteristics[a]	Source[b]
L. lactis subsp. *lactis*		
LM0230	Plasmid free, host for 936 and c2 phages; Lac^-	31
W1	Multiple plasmids including pSRQ800; Lac^+	This study
SMQ-16	LM0230(pSA3); Em^r	39
SMQ-20	LM0230(pSA3, pSRQ800); Em^r Abi^+	This study
SMQ-37	LM0230(pSRQ801); Em^r Abi^-	This study
SMQ-38	LM0230(pSRQ802); Em^r Abi^+	This study
SMQ-39	LM0230(pSRQ701); Em^r R^+/M^+	39
SMQ-51	LM0230(pSRQ803); Em^r Abi^-	This study
SMQ-52	LM0230(pSRQ804); Em^r Abi^-	This study
SMQ-57	LM0230(pSRQ809); Em^r Abi^-	This study
SMQ-130	LM0230(pSRQ813); Cm^r Abi^+	This study
SMQ-138	LM0230(pSRQ701, pSRQ813); Em^r Cm^r R^+/M^+ Abi^+	This study
SMQ-143	LM0230(pSRQ815); Cm^r Abi^+	This study
SMQ-251	LM0230(pMIG3); Cm^r	This study
SMQ-252	LM0230(pNZ123); Cm^r	This study
SMQ-260	LM0230(pSRQ817); Cm^r Abi^+	This study
L. lactis subsp. *cremoris*		
UL8	Multiple plasmids, host for P335 phages; Lac^+	36
SMQ-86	UL8(pSA3); Em^r	This study
SMQ-88	UL8(pSRQ802); Em^r Abi^+	This study
E. coli		
DH5α	*supE44* Δ*lac U169* (f80 *lacZDM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Gibco/BRL
SMQ-92	DH5α(pSRQ810); Ap^r	This study
SMQ-116	DH5α(pSRQ812); Ap^r	This study
SMQ-129	DH5α(pSRQ814); Ap^r	This study
Bacteriophages		
p2	Small isometric headed, 936 species, 30.5 kb	L. L. McKay
sk1	Small isometric headed, 936 species, 28.1 kb	L. L. McKay
jj50	Small isometric headed, 936 species, 30.5 kb	29
c2	Prolate headed, c2 species, 20.7 kb	49
ml3	Prolate headed, c2 species, 20.2 kb	W. E. Sandine
eb1	Prolate headed, c2 species, 19.6 kb	L. L. McKay
ul36	Small isometric headed, P335 species, 28.8 kb	36
Q30	Small isometric headed, P335 species, 37.0 kb	34
Q33	Small isometric headed, P335 species, 29.6 kb	34

[a] Abi^+, active abortive infection mechanism; Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Em^r, erythromycin resistance; Lac, lactose-fermenting ability; R^+/M^+, active restriction/modification enzymes.
[b] L. L. McKay, University of Minnesota, Minneapolis; W. E. Sandine, Oregon State University, Corvallis.

ing 10 to 15 min after infection. They proposed that an early phage product might stimulate an RNase activity in $AbiB^+$ cells. The endonucleolytic cleavage occurred preferentially at U/U, A/U, and U/A. AbiC does not prevent phage DNA replication but reduces the synthesis of structural phage proteins (14, 35). Bidnenko et al. (4) reported that AbiD1 protein interacted with a small isometric phage gene product (*orf1*) to prevent the translation of the phage *orf3* RNA.

In this study, we report the isolation and characterization of the 10th abortive infection mechanism from *L. lactis*. AbiK is efficient against phages of the 936 and P335 species. Although several Abi against phage 936 have been described, AbiK is only the second system which is efficient against the newly emerging P335 phage species. AbiK acts prior to or at the time of phage DNA replication and has no significant protein homology with previously isolated Abi from *L. lactis.*

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Strains and plasmids used in this study are listed in Tables 1 and 2, respectively. *Escherichia coli* was grown at 37°C in LB broth (48). *Lactococcus lactis* strains were grown at 30°C in M17 (54) supplemented with 0.5% glucose (GM17) or 0.5% lactose (LM17). When appropriate, antibiotics were added as follows: for *E. coli*, 50 μg of ampicillin per ml, 10 μg of tetracycline per ml, and 20 μg of chloramphenicol per ml; for *L. lactis*, 5 μg of chloramphenicol per ml and 5 μg of erythromycin per ml.

Bacteriophage propagation and assays. Bacteriophages used in this study are listed in Table 1. Bacteriophages c2, p2, sk1, and jj50 were a gift from T. R. Klaenhammer (North Carolina State University). All phages were isolated from a single plaque with a 1-ml sterile pipette as described previously (38). They were transferred to GM17 containing 10 mM $CaCl_2$ previously inoculated (1%) with an overnight culture of the host strain. They were incubated at 30°C until lysis and filtered through a 0.45-μm-pore-size filter (Acrodisc; Gelman Sciences, Ann Arbor, Mich.). High phage titers were then obtained by the method of Jarvis (27). Efficiency of plaquing (EOP) and adsorption assays were performed as described by Sanders and Klaenhammer (49). Cell survival was assayed by the method of Behnke and Malke (3) with a multiplicity of infection of 3. One-step growth and center of infection (COI) assays were performed as described previously (35). The phage burst size was determined as the titer of the phage at time 120 min divided by the titer of infecting centers at time 15 min. The latent period was estimated at the midpoint of the exponential phase of the one-step growth curve. The efficiency at which COI formed (ECOI) was obtained by dividing the number of COI from the resistant strain by the number of COI from the sensitive strain. The intracellular replication of phage ul36 DNA was also followed at time intervals after phage infection by the procedure of Hill et al. (23). The production of phage ul36 major capsid protein (MCP) was monitored at time intervals after phage infection with monoclonal antibodies and a sandwich enzyme-linked immunosorbent assay (ELISA) as described previously (33, 35).

DNA isolation and manipulation. Plasmid DNA from *E. coli* was isolated as described previously (39). Large amounts of *E. coli* plasmid DNA were isolated with the Qiagen (Chatsworth, Calif.) plasmid Midi or Maxi kit. Plasmid DNA

TABLE 2. Plasmids used in this study

Plasmid	Relevant characteristics[a]	Source
pBS	Cloning vector for sequencing, Ap^r, 2.9 kb	Stratagene
pMIG3	Shuttle vector, Cm^r, 5.5 kb	58
pNZ123	Shuttle vector, Cm^r, 2.5 kb	9
pSA3	Shuttle vector, Cm^r Tc^r Em^r, 10.2 kb	7
pSRQ701	7.0-kb *Eco*RI fragment from pSRQ700 cloned into pSA3; Cm^s Tc^r Em^r R^+/M^+	39
pSRQ800	Resident plasmid of W1, Abi^+, 8.0 kb	This study
pSRQ801	3.5-kb *Eco*RI fragment from pSRQ800 cloned into pSA3; Cm^s Tc^r Em^r	This study
pSRQ802	4.5-kb *Eco*RI fragment from pSRQ800 cloned into pSA3; Cm^s Tc^r Em^r	This study
pSRQ803	2.3-kb *Nco*I fragment from pSRQ800 cloned into pSA3; Cm^s Tc^r Em^r	This study
pSRQ804	5.7-kb *Nco*I fragment from pSRQ800 cloned into pSA3; Cm^s Tc^r Em^r	This study
pSRQ809	8.0-kb *Eco*RV fragment from pSRQ800 cloned into pSA3; Cm^r Tc^s Em^r	This study
pSRQ810	1.5-kb *Eco*RV-*Eco*RI fragment from pSRQ800 cloned into pBS; Ap^r	This study
pSRQ812	1.2-kb *Eco*RV-*Sca*I fragment from pSRQ802 cloned into pBS; Ap^r	This study
pSRQ813	4.5-kb fragment from pSRQ800 cloned into pMIG3; Cm^r	This study
pSRQ814	1.8-kb *Eco*RI-*Sca*I fragment from pSRQ802 cloned into pBS; Ap^r	This study
pSRQ815	4.5-kb *Eco*RI fragment from pSRQ802 cloned into pNZ123; Cm^r	This study
pSRQ817	3.8-kb *Eco*RI-*Pvu*II fragment from pSRQ800 cloned into pMIG3; Cm^r	This study
pSRQ819	1.5-kb *Eco*RI-*Eco*RV fragment from pSRQ800 cloned into pMIG3; Cm^r	This study

[a] Abi^+, active abortive infection mechanism; Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Cm^s, sensitive to chloramphenicol; Em^r, erythromycin resistance; Tc^r, tetracycline resistance; Tc^s, sensitive to tetracycline; R^+/M^+, active restriction/modification enzymes.

from *L. lactis* was isolated by the method of O'Sullivan and Klaenhammer (43). Large amounts of lactococcal plasmid DNA were obtained as described by Gonzalez and Kunka (20). Restriction endonucleases (Gibco/BRL, Grand Island, N.Y.) and T4 DNA ligase (Boehringer Mannheim, Indianapolis, Ind.) were used as recommended by the manufacturer. All DNA manipulations and clonings were carried out essentially as described by Sambrook et al. (48).

Electroporation. *E. coli* cells were prepared and electroporated as described previously (38). *L. lactis* was grown in GM17 supplemented with 0.5 M sucrose and 1% glycine and electroporated by the procedure of Holo and Nes (26) as modified previously (38).

Sequencing. The 4.5-kb *Eco*RI insert from pSRQ800 was segmented into three smaller fragments by use of the unique *Nco*I and *Eco*RV sites and subcloned into pBS. After introduction into *E. coli* DH5α, recombinants were selected by blue or white screening and restriction mapping (48) and the resulting clones were named pSRQ810, pSRQ812, and pSRQ814 (Table 2). Nested deletions were generated in both directions of each insert with an ERASE-A-BASE kit (Promega, Madison, Wis.). Plasmid DNA was extracted from the deletion mutants with the Qiagen plasmid kit and used directly for sequencing. The reactions were performed with the DyeDeoxy Terminator *Taq* sequencing kit, and products were separated on the model 373A automated DNA sequencing system (Applied Biosystems, Foster City, Calif.). The T7 and T3 primers were used as sequencing primers. Specific primers were also used to determine the contiguous regions between the fragments encoded by pSRQ810, pSRQ812, and pSRQ814.

DNA and protein analysis. The DNA sequence was analyzed with the Genetics Computer Group (Madison, Wis.) sequence analysis software (8). Homology to the *abiK* gene and the deduced protein were searched with the BLAST and FASTA family of programs (p, n, x, tn, and tx). The program PROPSEARCH was also used to determine if AbiK belongs to a putative protein family (25). The free energy for potential rho-independent terminator structures was determined with the program FoldRNA (59). The putative ribosome binding sites were identified by alignment with 3′ end of *L. lactis* 16S rRNA (3′-UCUUUCCUC CA) (30), and the free energy was calculated by the method of Freier et al. (15). Putative protein AbiK was searched for known amino acid motifs with the Genetics Computer Group program Motifs and the Prosite dictionary.

Nucleotide sequence accession number. The complete sequence of 4,467 bp was deposited in the GenBank database and is available under the accession number U35629.

RESULTS

Isolation of a plasmid encoding phage resistance. *L. lactis* subsp. *lactis* W1 was chosen for this study because of its resistance against many lactococcal phages of industrial relevance (data not shown). Total plasmid DNA from W1 was coelectroporated along with the shuttle vector pSA3 into *L. lactis* LM0230 at a plasmid-to-DNA mass ratio of 10:1. Erythromycin-resistant colonies (i.e., carrying pSA3) were isolated and tested for phage resistance by a spot assay (10^4 PFU/spot). All phage-resistant transformants isolated contained, in addition to pSA3, a plasmid of about 8.0 kb, which was named pSRQ800 (Fig. 1). One phage-resistant transformant was selected and named *L. lactis* SMQ-20.

Effectiveness of pSRQ800 on three lactococcal phage species. *L. lactis* SMQ-20 was tested for its resistance against three small isometric-headed phages of the 936 species as well as three prolate-headed phages of the c2 species (Table 1). EOPs ranging from 10^{-6} to 10^{-7} were obtained for the 936-type phages p2, sk1, and jj50, whereas EOPs of 10^{-1} were obtained with prolate phages c2, ml3, and eb1 (Table 3). The activity of pSRQ800 was also tested against P335 phages. Since host strain LM0230 cannot replicate P335 phages, pSRQ802, a functional derivative of pSRQ800 (Table 2), was introduced into *L. lactis* UL8 by electroporation, and the transformant was named SMQ-88. The EOPs of three P335 phages (ul36, Q30, and Q33) on SMQ-88 were variable, ranging from 10^{-5} to 10^{-8} (Table 3). All of these results showed that the phage resistance mechanism encoded on pSRQ800 is effective against small

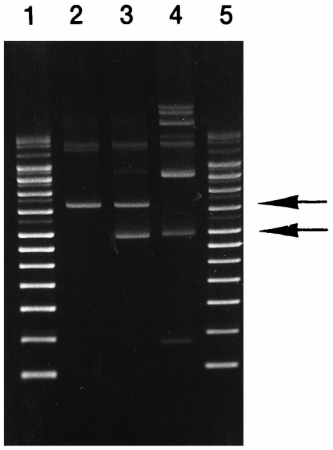

FIG. 1. Identification of the plasmid encoding phage resistance mechanism in *L. lactis* subsp. *lactis* W1. Lanes: 1 and 5, supercoiled DNA ladder (2.1, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 10.1, 12.1, 14.2, and 16.2 kb; Gibco/BRL); 2, *L. lactis* SMQ-16(pSA3); 3, *L. lactis* SMQ-20(pSA3 + pSRQ800); 4, *L. lactis* W1. Plasmids pSRQ800 and pSA3 are identified with arrows.

TABLE 3. EOPs of lactococcal phages at 30°C on *L. lactis* strains harboring pSRQ800

Phage species	Phage	EOP
936[a]	p2	4.6×10^{-6}
	sk1	7.5×10^{-7}
	jj50	6.0×10^{-6}
C2[a]	c2	2.3×10^{-1}
	ml3	3.3×10^{-1}
	eb1	2.7×10^{-1}
P335[b]	ul36	3.2×10^{-6}
	Q30	3.0×10^{-5}
	Q33	2.7×10^{-8}

[a] The EOPs of the 936 and c2 phages were tested on *L. lactis* SMQ-20. The EOP of these phages is 1.0 on *L. lactis* LM0230.

[b] The EOPs of the P335 phages were tested on *L. lactis* SMQ-88. The EOP of these phages is 1.0 on *L. lactis* SMQ-86.

isometric phages of the 936 and P335 species but has a very limited effect on prolate phages of the c2 species.

Effect of temperature on phage resistance mechanism. The effect of temperature on the phage resistance activity of pSRQ800 was tested with phage p2. Similar EOPs (ca. 10^{-6}) were obtained when cells were infected at 21 and at 30°C. However, the EOP of phage p2 increased from 4.6×10^{-6} to 6.7×10^{-2} when infections were carried out at 38°C, indicating that the phage resistance mechanism encoded on pSRQ800 is heat sensitive.

Characterization of the phage resistance mechanism on pSRQ800. Adsorption experiments indicated that phages adsorbed to the same level (approximately between 90 and 95%) on the phage-sensitive strain *L. lactis* LM0230 and on the phage-resistant strain *L. lactis* SMQ-20, ruling out the adsorption-blocking mechanism. To determine if the mechanism was an R/M system, *L. lactis* SMQ-20 was challenged with a high concentration of phage p2 (10^{10} PFU/ml). Phages capable of overcoming the pSRQ800-encoded defense system in *L. lactis* SMQ-20 were isolated and tested again on the same host. Some of them were still inhibited by the defense mechanism, while others remained unaffected. These insensitive phages were further propagated on the sensitive host LM0230 and then tested again on *L. lactis* SMQ-20. They remained unaffected by pSRQ800, indicating a permanent modification. In a separate experiment, cell extracts prepared from SMQ-20 were incubated at 30°C for 2 h with genomic DNA from phage p2 or *Streptococcus mutans*. DNA was analyzed on agarose gel, and no endonucleolytic activity was observed (data not shown). These results ruled out the presence of host-controlled modifications such as R/M systems. When challenged with phage p2 or ul36, *L. lactis* harboring pSRQ800 exhibited a reduced plaque size phenotype. Cell survival studies showed that virtually all of the pSRQ802-containing cells died after phage infection (see Table 5). Based on the current classification of phage defense mechanisms (40, 51, 53) and all of the results described above, this system was classified as an abortive infection mechanism.

Localization of the *abi* gene on pSRQ800. The plasmid pSRQ800 was mapped by use of single, double, and triple endonuclease digestions. The results are presented in Fig. 2. The following endonucleases did not cut pSRQ800: *Apa*I, *Ava*I, *Bal*I, *Bam*HI, *Hpa*I, *Nru*I, *Pst*I, *Sal*I, *Sca*I, *Sma*I, *Sph*I, *Sst*I, *Xba*I, and *Xho*I. To localize the genetic determinant responsible for the Abi phenotype, pSRQ800 was digested with *Eco*RI, *Eco*RV, and/or *Nco*I and then ligated to shuttle vector pSA3. Ligation mixtures were introduced into *E. coli* DH5α by electroporation, and clones were selected by restriction mapping of the purified plasmid. The proper plasmids were reintroduced into *L. lactis* LM0230 by electroporation. Transformants were confirmed by restriction mapping and tested for phage resistance. Relevant clones (pSRQ801, pSRQ802, pSRQ803, pSRQ804, and pSRQ809) are presented in Fig. 2. Most of the clones (pSRQ801, pSRQ803, pSRQ804, and pSRQ809) conferred no resistance (EOP, 1.0) against phage p2. Only the clone pSRQ802 gave phage resistance similar to

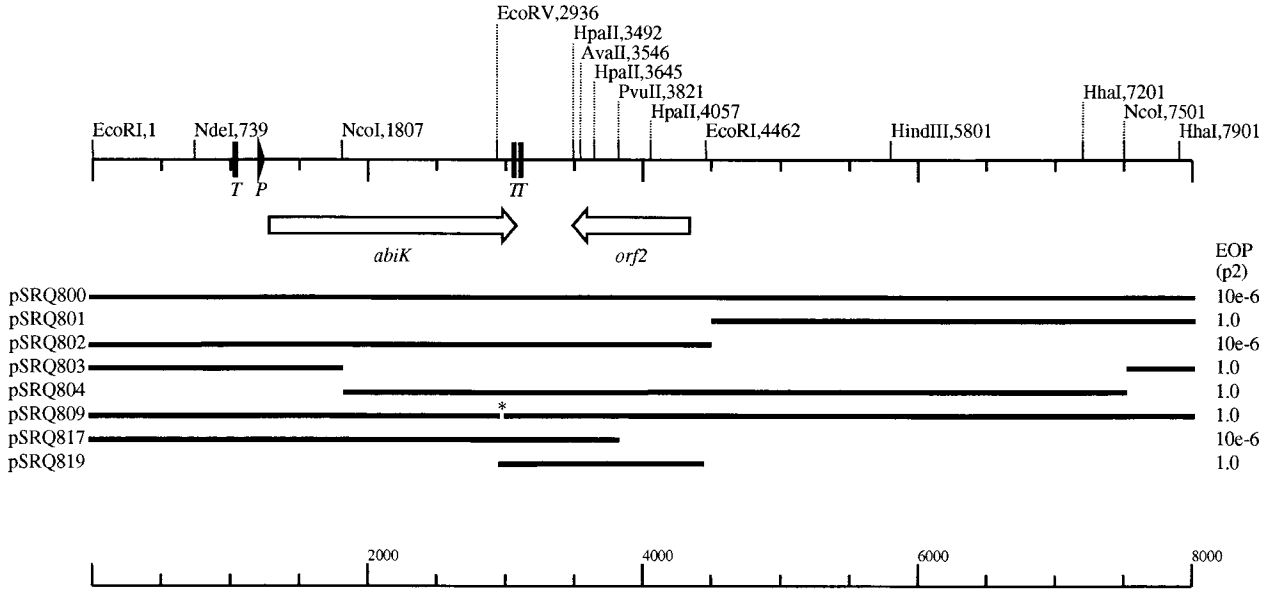

FIG. 2. Restriction map of pSRQ800 (8,000 bp) and deletion mutants used to localize the Abi determinants. The deletion mutants are represented by horizontal lines below the map, and the corresponding Abi phenotype (EOP) on phage p2 is indicated on the right. The asterisk represents the unique *Eco*RV site which was insertionally inactivated. Putative ORFs, promoter (P), and terminators (T) shown on the restriction map were inferred from sequence analysis.

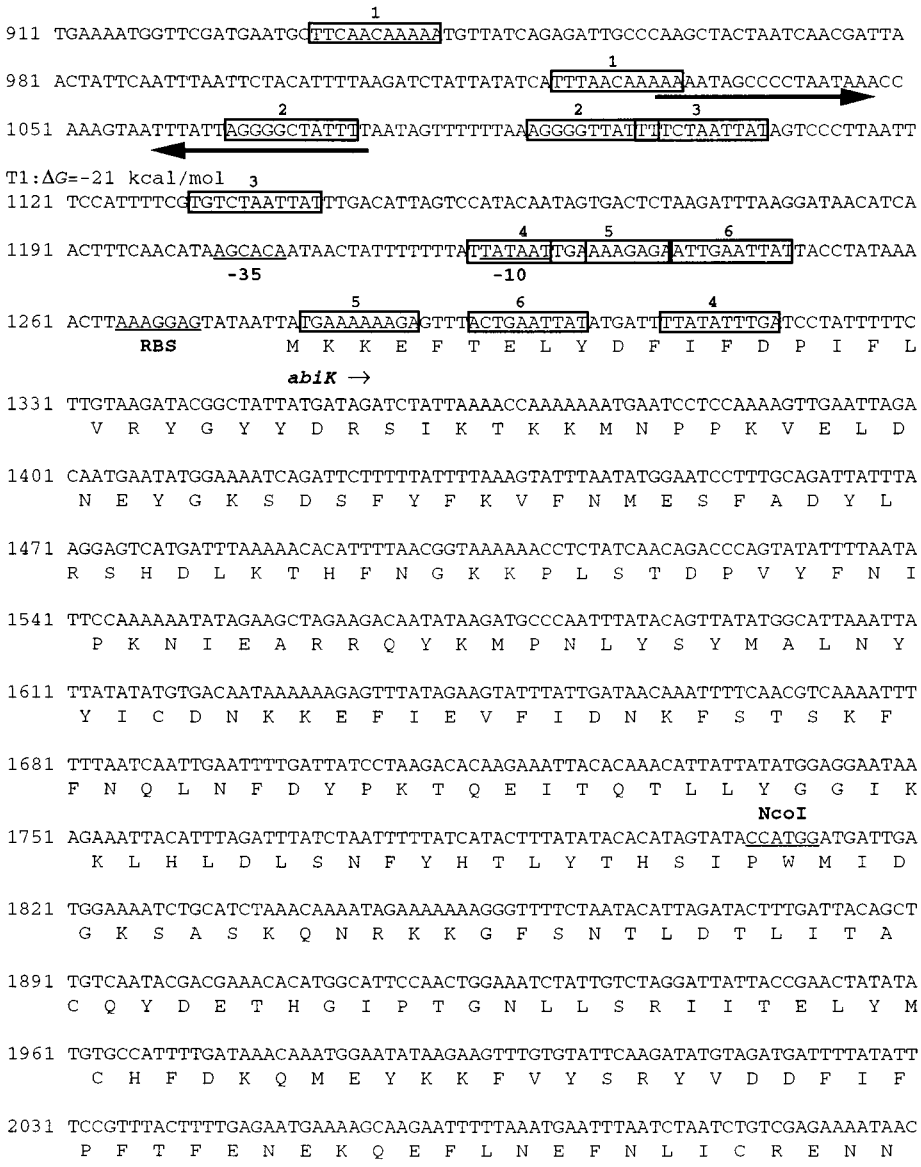

FIG. 3. Nucleotide sequence of the Abi locus fragment from pSRQ800. The deduced amino acid sequence of *abiK* is presented as a single-letter code. The putative promoter (−10 and −35 boxes), ribosome binding site (RBS), and endonuclease sites are underlined. Inverted repeats are indicated by arrows under the corresponding sequence, and their free energy values are given. Free energy was calculated by the method of Freier et al. (15) based on the *L. lactis* 3′ end of 16S rRNA (30). The pentapeptide FFENI repeated twice in the sequence of AbiK is boxed. Direct repeats localized around the *abiK* promoter are boxed, and each pair of repeats is identified by the number above the box. Nucleotide numbers correspond to the numbering of the 4,467-bp sequenced fragment submitted to GenBank.

that of pSRQ800 (EOP, 10^{-6}) (Fig. 2). Thus, the Abi phenotype was preliminarily localized on a 4.5-kb *Eco*RI fragment.

DNA analysis and deduced amino acid sequence. The 4.5-kb fragment carrying the determinants conferring the Abi phenotype was sequenced on both strands and found to contain 4,467 bp. This fragment had an overall G+C content of 29.5%. Two large open reading frames (ORFs) were identified (Fig. 2). *orf1* was oriented in one direction from position 1279 to 3075. It encodes a putative protein of 599 amino acids. *orf2* was on the complementary strand from position 4345 to 3491. It encodes a putative protein of 285 amino acids. The clones pSRQ804 and pSRQ809, which contained a full *orf2* but a disrupted *orf1*, exhibited no resistance (EOP, 1.0) against phage p2 (Fig. 2). pSRQ817, which contained a complete *orf1* but a disrupted *orf2*, conferred a phage resistance phenotype similar to that of pSRQ800 against phage p2 (reduced plaque size and an EOP of 10^{-6}) and phage P335 (data not shown) (Fig. 2). These results clearly show that only *orf1* is necessary to produce the Abi phenotype. *orf1* was named *abiK*, and the translated putative protein was named AbiK.

The complete *abiK* gene contained 1,797 bp with a very low GC content of 23.9%. A putative ribosome binding site (AA AGGAG; $\Delta G = -8.3$ kcal/mol) was found 8 bases upstream of the *abiK* start codon (Fig. 3). A putative promoter region including a −10 sequence (TATAAT) separated by 16 bp from a −35 sequence (AGCACA) was found upstream of the translation start codon (Fig. 3). This putative promoter is in good agreement with previously published lactococcal promoters (55, 57) and with *E. coli* and *Bacillus subtilis* promoter consensus (21, 41). *abiK* seemed to be part of a monocistronic operon since transcriptional terminator-like structures were found upstream and downstream of the *abiK* gene (Fig. 3). The first

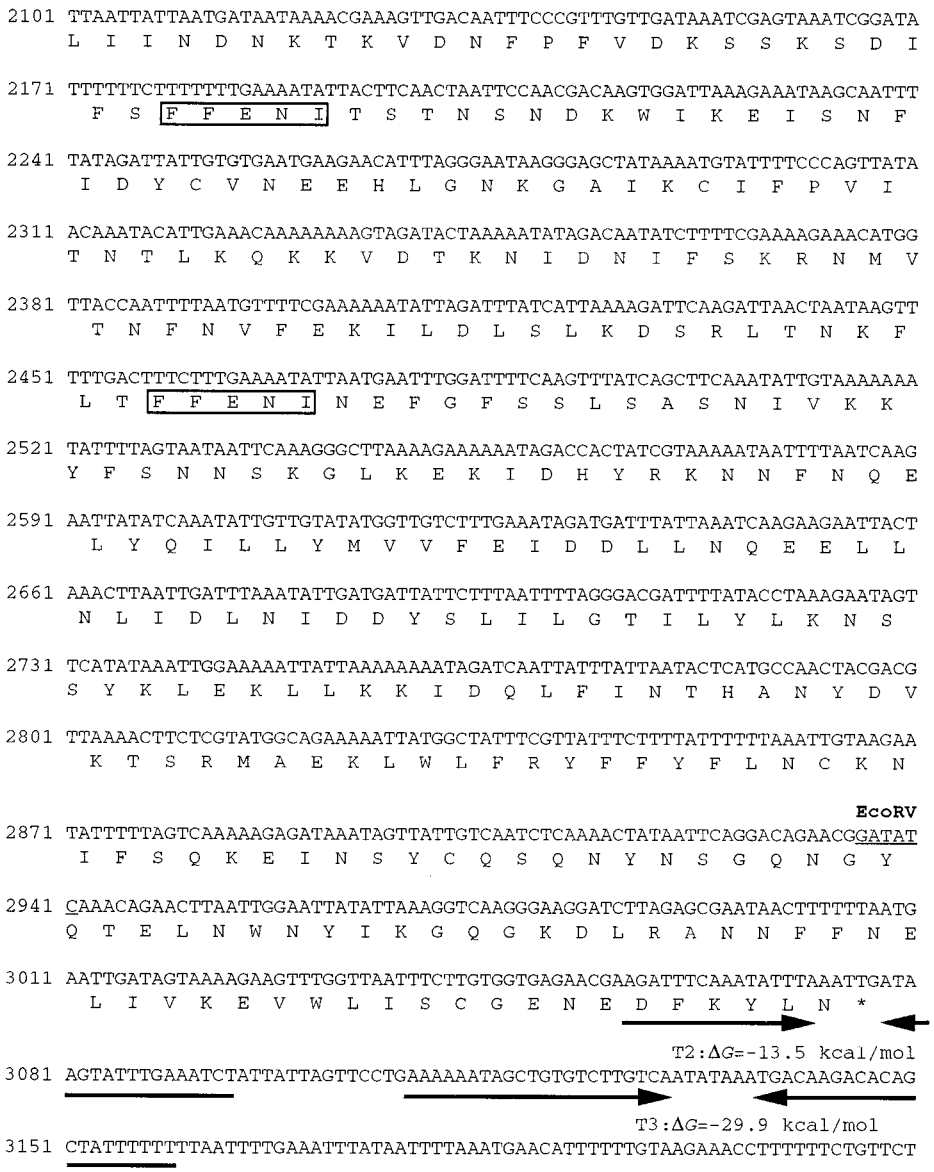

FIG. 3—*Continued.*

inverted repeat of 18 bp (T1; $\Delta G = -21.8$ kcal/mol) was found 127 bp upstream of the AbiK putative promoter. A second inverted repeat of 16 bp (T2; $\Delta G = -13.5$ kcal/mol) overlapping the *abiK* stop codon was found at the 3′ end of the gene. Finally, a third strong inverted repeat of 22 bp (T3; $\Delta G = -29.9$ kcal/mol) was found 30 bp downstream of the *abiK* stop codon. We also identified several direct repeats in the putative promoter region of *abiK* (Fig. 3), suggesting that the expression of the operon could be regulated.

The predicted AbiK protein contained 599 amino acids with an estimated molecular size of 71,416 Da and a pI of 7.98. No transmembrane helix or secretory signals were identified, suggesting that AbiK is most likely a cytoplasmic protein. Interestingly, the amino acid sequence FFENI was repeated twice in the AbiK protein, from amino acid 301 to 305 and from amino acid 394 to 398. No helix-turn-helix structure could be identified within the protein by using the matrix of Dodd and Egan (12). The Abi phenotype was totally abolished with the clone pSRQ809, where the last 42 amino acids were missing from AbiK. This result suggested that the C terminus of the AbiK protein might be critical to the activity of the protein. However, sequences from the vector may contribute additional codons leading to extra amino acids fused to the C-terminal end of AbiK, which could be responsible for its altered function.

The *abiK* gene and the deduced AbiK protein were compared with nucleotides, translated nucleotides, and proteins from databases (GenBank release 96.0, EMBL release 48.0, NBRF-PIR release 50.0, and Swiss-Prot release 33.0) by use of the BLAST and FASTA families of programs. These comparisons revealed no significant sequence homology at the DNA or the amino acid level. Also, AbiK showed no homology to protein motifs found in release 13.0 of the Prosite dictionary. Even though programs based on alignment methods failed, homology with a high level of reliability (99.6%) was found between AbiK, AbiA (24), and a *Haemophilus influenzae* pro-

TABLE 4. EOP of phage p2 and c2 on *L. lactis* strains harboring various phage resistance mechanisms

Strain	EOP of p2	EOP of c2
LM0230	1.0	1.0
SMQ-16(pSA3)	1.0	1.0
SMQ-20(pSA3 + pSRQ800)	4.6×10^{-6}	2.3×10^{-1}
SMQ-38(pSRQ802)	2.6×10^{-6}	6.9×10^{-1}
SMQ-251(pMIG3)	1.0	1.0
SMQ-130(pSRQ813)	1.8×10^{-5}	1.0
SMQ-252(pNZ123)	1.0	1.0
SMQ-143(pSRQ815)	3.8×10^{-8}	ND[a]
SMQ-39(pSRQ701)	1.9×10^{-6}	ND
SMQ-138(pSRQ701 + pSRQ813)	1×10^{-9}	ND

[a] ND, not determined.

TABLE 5. Specific effects of AbiK on the lactococcal phage ul36

Assay	Values[d] for:	
	SMQ-86	SMQ-88
EOP[a]	1.0	$(3.15 \pm 1.63) \times 10^{-6}$
Cell survival[a] (%)	0.06 ± 0.06	2.65 ± 0.85
ECO1[b]	1.0	$(5.13 \pm 3.57) \times 10^{-3}$
Burst size[a]	504 ± 164	36 ± 27
Latent period[a] (min)	75	90
DNA replication[c]	+++	−
MCP production[a] (%)	100	1

[a] Average of five trials.
[b] Average of 10 trials.
[c] +++, strong; −, none.
[d] Some values are given as means ± standard deviations.

tein of unknown function (56) when a program (Propsearch) which is based on amino acid composition and several other nonalignment parameters (25) is used.

Enhancement of AbiK efficiency. The copy number of pSRQ800 was not determined but was estimated to be roughly the same as that of the low-copy-number vector pSA3 (Fig. 1). To determine the effect of gene copy number on Abi phenotype, the *Eco*RI 4.5-kb fragment was introduced into the high-copy-number plasmid pNZ123. Previously, it was estimated that pSA3 was present at 5 to 10 copies (10) and pNZ123 was found at 50 to 100 copies in *L. lactis* (9). The resulting high-copy-number clone and transformant were named pSRQ815 and SMQ-143, respectively. The EOP of phage p2 on SMQ-143 was 10^{-8}, which is 100-fold lower than the EOP on SMQ-20. These results indicated that multiple copies of the *abiK* gene increased resistance against phage p2. We also attempted to improve the Abi phenotype by cloning *abiK* on a high-copy-number plasmid under the control of a strong constitutive lactococcal promoter (data not shown). Surprisingly, the construct could be transformed into *E. coli* but not into *L. lactis.*

Another way of increasing the efficiency of the Abi system is to supply in *trans* a complementary phage resistance mechanism (52). Previously, we isolated an *L. lactis* R/M system named *Lla*DCHI (formerly *Lla*II) that was encoded on the plasmid pSRQ700 (39). This plasmid was isolated from the industrial strain *Lactococcus lactis* subsp. *cremoris* DCH-4. The genetic determinants for *Lla*DCHI were previously cloned into pSA3 and named pSRQ701 (39). Phage p2 had an EOP of 10^{-6} on *L. lactis* LM0230 harboring pSRQ701 (SMQ-39), which is similar to the EOP on *L. lactis* harboring pSRQ800 (SMQ-20) (Table 4). To complement AbiK with *Lla*DCHI, the 4.5-kb fragment encoding the *abiK* gene was cloned into a chloramphenicol selection vector (pMIG3) and named pSRQ813. Plasmids pSRQ701 and pSRQ813 were introduced into *L. lactis* LM0230, and the resulting Em^r Cm^r transformant was named SMQ-138. The combined use of pSRQ813 and pSRQ701 reduced the EOP of phage p2 on SMQ-138 to $<10^{-9}$, indicating a full phage resistance phenotype (Table 4). The EOP of phage ul36 was also $<10^{-9}$ on another *L. lactis* strain harboring both plasmids (data not shown). These results showed that the AbiK and *Lla*DCHI systems, when supplied in *trans*, can have a synergistic effect which leads to an enhanced phage resistance.

Intracellular effects of the AbiK system on the phage life cycle. The effects of the AbiK system on the phage ul36 life cycle by using the phage-sensitive *L. lactis* SMQ-86(pSA3) and phage-resistant *L. lactis* SMQ-88(pSRQ802) were tested. Phage ul36 was selected because it was previously used to characterize two other Abi systems, AbiA and AbiC (35). The replication of phage ul36 was severely inhibited on SMQ-88, as shown by the EOP of 10^{-6} (Table 5 and Fig. 4C). Plaques were reduced in size from 1 mm to pinpoint and difficult to enumerate (data not shown). The average percentages of surviving cells following phage infection (multiplicity of infection of 3) were 0.06% on SMQ-86 and 2.65% on SMQ-88 (Table 5). AbiK improved cell survival by approximately 50-fold, but a majority of infected cells still died.

The ECOI for phage ul36 on SMQ-88 was 0.0051, indicating that only 5 of 1,000 infected cells successfully release viable phages (Table 5). The burst size of these productive infections was also reduced from an average of 504 phages on the phage-sensitive SMQ-86 to 36 phages on SMQ-88 (Table 5). Furthermore, the phage latent period was longer on SMQ-88 than on SMQ-86 by approximately 15 min (Fig. 4C). These results indicated that ul36 took more time to complete its life cycle on an $AbiK^+$ host and that the number of released phages was reduced by almost 15-fold. The cumulative effects of AbiK on the cell survival, ECOI, burst size, and latent period were responsible for the severely reduced EOP of phage ul36 on SMQ-88 (Table 5).

The intracellular replication of ul36 DNA on SMQ-86 and SMQ-88 was monitored (Fig. 4A). No replication of ul36 could be detected in *L. lactis* SMQ-88, whereas significant replication could be observed in SMQ-86 starting at 45 min, followed by a decrease at 75 min, probably as a result of DNA packaging and release of progeny phages (23). The intracellular production of phage ul36 MCP was also monitored on SMQ-86 and SMQ-88 (Fig. 4B). The MCP is the most abundant structural protein of ul36 (33). By using an ELISA detection system, hardly any MCP protein was detected in infected SMQ-88 cells whereas a massive amount of MCP was detected in infected SMQ-88 cells. These results indicated that AbiK might act at or prior to phage DNA replication.

DISCUSSION

L. lactis subsp. *lactis* W1 harbors a 8.0-kb plasmid (pSRQ800) coding for a temperature-sensitive abortive phage infection mechanism. The genetic elements responsible for the Abi phenotype were cloned and sequenced. One gene is necessary for the Abi phenotype, and it was named *abiK*. AbiK inhibits the development of small isometric phages of the 936 and P335 species but has a very limited effect on the prolate c2 species. Thus, AbiK is effective against two genetically distinct phage species, including the newly emerging P335 species.

Our results show that AbiK expresses a typical abortive infection phenotype: (i) substantial cell death (97.5%) occurs upon phage abortion; (ii) very few infected $AbiK^+$ cells release

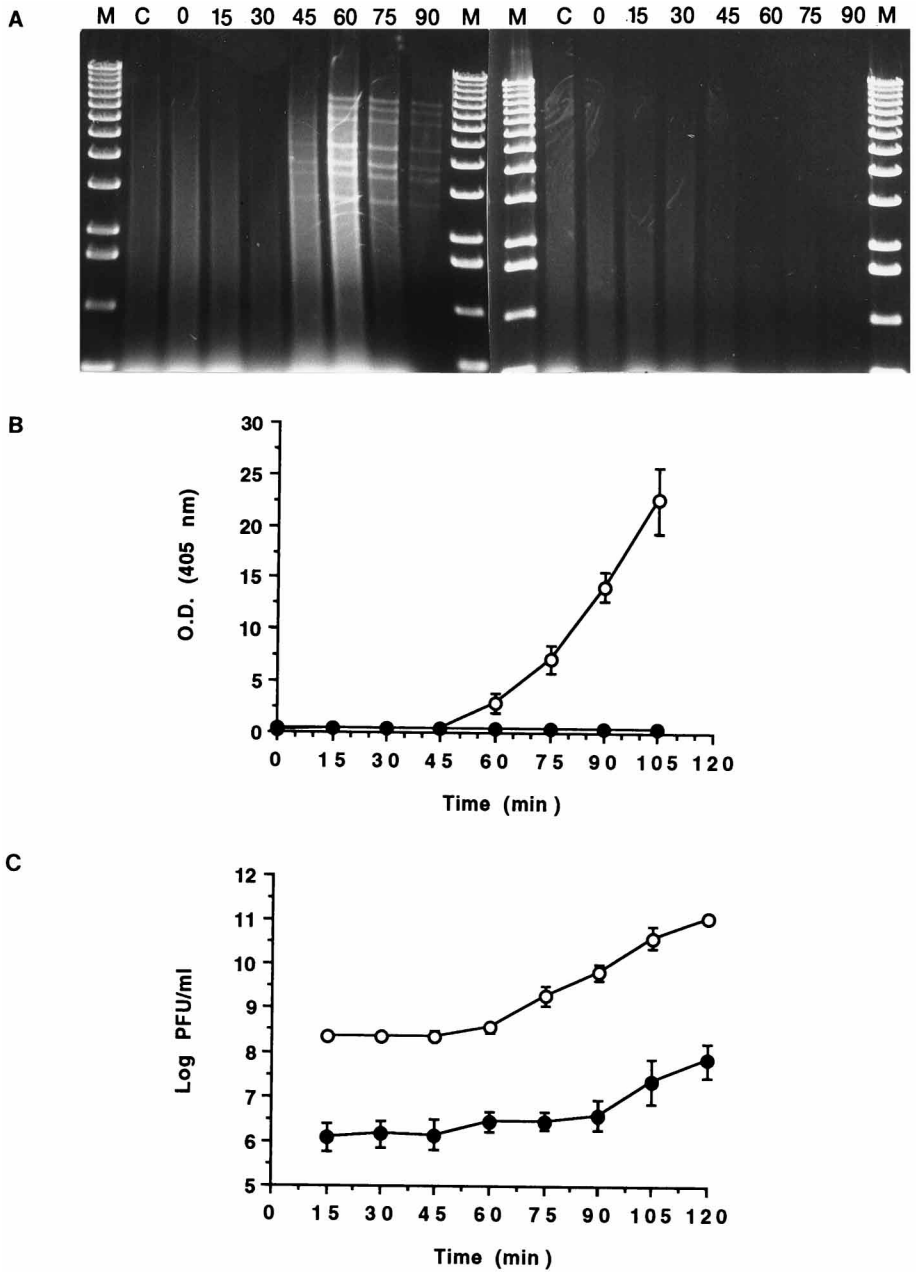

FIG. 4. (A) Phage ul36 DNA replication was monitored at time intervals during infection of *L. lactis* SMQ-86 (left 10 lanes) and SMQ-88 (right 10 lanes). One-milliliter samples were taken at time intervals, and the total DNA was isolated by the method of Hill et al. (23). Total DNA was cut with *Eco*RV, and a sample was run on a 0.7% agarose gel. Lanes: M, 1-kb ladder (0.5, 1.0, 1.6, 2.0, 3.0, 4.0, 5.1, 6.1, 7.1, 8.1, 9.2, 10.2, 11.2, and 12.2 kb; Gibco/BRL); C, DNA sample prior to phage infection; 0, 15, 30, 45, 65, 75, and 90, time intervals indicated in minutes. (B) Accumulation of the MCP of phage ul36 was monitored at time intervals during infection of *L. lactis* SMQ-86 and SMQ-88 with an ELISA detection system that was described previously (33, 35). An optical density (OD) of 0.389 ± 0.132 was obtained at 105 min with infected SMQ-88 cells. The background optical density level of noninfected cells was 0.296 ± 0.085. (C) One-step growth curves for ul36 on *L. lactis* SMQ-86 and SMQ-88. The phages were allowed to adsorb to the cells for 5 min, and the cells were washed twice to remove nonadsorbed phages, resuspended in LM17, and incubated at 30°C. Time 15 min corresponded to the first phage count after resuspension in LM17. Symbols: ○, SMQ-86(pSA3); ●, SMQ-88(pSRQ802). Error bar indicates the standard deviation for five trials.

progeny phages (0.5%); (iii) when productive infection occurs, the latent period is increased (by 15 min); and (iv) the burst size is reduced (15-fold). The overall effect of AbiK can be visualized as a reduction in number and size of phage plaques. Thus, AbiK acted internally to interfere with the lytic phage cycle.

As shown by other groups (5, 10), increasing the copy number of abortive infection mechanisms can increase the efficacy. We obtained similar results with the AbiK operon cloned into a high-copy-number vector. However, that we were unable to overexpress *abiK* constitutively in *L. lactis* suggests that this Abi could be tightly regulated. The presence of several direct repeats in the promoter region supports this hypothesis. We are currently investigating *abiK* expression under various conditions.

The genetic determinants of nine other *L. lactis* Abi systems

have been sequenced (2, 6, 14, 24, 32, 42, 46). In all cases but two, the Abi phenotypes rely on a single polypeptide. AbiE and AbiG seem to be depending on the presence of two peptides for the resistance phenotype (17, 42). The *abiK* gene had a low G+C content of 23.9%, which is below the average of 37.4% observed for other lactococcal genes (2). Interestingly, this characteristic is shared by all other Abi genes (2, 42, 46). This low GC content could reflect their common origin or the function performed by Abi proteins and the consequent need for certain amino acids mainly encoded by AT-rich codons (42).

The deduced proteins involved in the Abi phenotype have 628 (AbiA), 251 (AbiB), 344 (AbiC), 366 (AbiD), 351 (AbiD1), 287 (AbiEi), 298 (AbiEii), 342 (AbiF), 242 (AbiGi), 397 (AbiGii), and 346 (AbiH) amino acids. Thus, AbiK (599 amino acids; 71.4 kDa) would be closer to AbiA (628 amino acids; 73.8 kDa) based on protein size. Interestingly, a 70-kDa protein (PifA) is also responsible for the *E. coli* abortive infection mediated by the F plasmid (40, 47). The only known function of PifA is to inhibit the development of small isometric coliphages like T7 (16, 40).

To date, AbiK and AbiA are the only two Abi proteins efficient against both the 936 and P335 species (5, 10). They are also the only heat-sensitive systems (24). As with AbiK, the AbiA phage target is currently unknown, but it is believed to act prior to phage DNA replication, and no DNA replication or MCP production was observed in infected $AbiA^+$ cells (35). The absence of significant amino acid sequence homology between the two proteins does not allow us to speculate further on the potential relationship between the two proteins. It is noteworthy that the program Propsearch classified them in the same putative protein family based on amino acid composition, molecular weight, content of bulky residues, content of small residues, average hydrophobicity, and average charge as well as about 137 other properties not based on sequence alignment (25). The fact that phage p2 derivatives permanently insensitive to AbiK are still sensitive to AbiA (11) indicates that AbiK has a different target than AbiA.

Mutant phages permanently insensitive to Abi systems have been isolated in laboratories (2, 13, 38) as well as from industrial fermentations (1, 37). These mutants had point mutations (2, 13) or major genetic rearrangements (1, 38). This biological material is currently being studied to gain more knowledge on the Abi processes (3). When developing phage-resistant cultures, it is critical to consider methods that will prevent the emergence of mutant phages in industrial environs. One approach is to stack different phage resistance mechanisms within one strain (13, 52). In fact, some natural *L. lactis* plasmids like pTR2030 (24, 44) and pTN20 (14) already encode two distinct phage resistance mechanisms, an abortive infection and an R/M system. In these two plasmids, the Abi and R/M systems result in a synergistic effect which confers very strong phage resistance (EOP, $<10^{-9}$). AbiK and *Lla*DCHI are considered strong phage resistance mechanisms (39). However on their own, both systems are leaky and a high phage population ($>10^6$) can overpower them. In such an instance, mutant phages can potentially be opportunistic. When AbiK and *Lla*DCHI were introduced in *L. lactis* strains, a full resistance phenotype (EOP, $<10^{-9}$) was observed against 936 and P335 phages. These results demonstrated the beneficial effect of providing in *trans* an Abi and an R/M system. Similar added resistance has been observed previously with other *L. lactis* antiphage systems in *trans* (13, 52).

From a practical and current regulatory point of view, the introduction of the natural plasmid pSRQ800 into an industrial *L. lactis* strain is an attractive strategy to confer strong resistance against small isometric phages of the 936 and P335 species. The isolation of additional Abi systems and the elucidation of their molecular action may lead to new targeted strategies for the development of phage-resistant starter cultures that would minimize the appearance of mutant phages.

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