

# Expression and Site-Directed Mutagenesis of the Lactococcal Abortive Phage Infection Protein AbiK

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**Abortive infection mechanisms of *Lactococcus lactis* form a heterogeneous group of phage resistance systems that act after early phage gene expression. One of these systems, AbiK, aborts infection of the three most prevalent lactococcal phage groups of the dairy industry. In this study, it is demonstrated that the antiphage activity depends on the level of expression of the *abiK* gene and on the presence of a reverse transcriptase (RT) motif in AbiK. The *abiK* gene was shown to be part of an operon that includes two additional open reading frames, with one of these encoding a phage-related transcriptional repressor named Orf4. Expression of AbiK is driven by two promoters, *PabiK* and *Porf3*, the latter being repressed by Orf4 in vivo. Binding of the purified Orf4 to the *Porf3* promoter was demonstrated in vitro by gel retardation assays. The N-terminal half of the deduced AbiK protein possesses an RT motif that was modified by site-directed mutagenesis. Conservative mutations in key positions resulted in the complete loss of the resistance phenotype. These data suggest that an RT activity might be involved in the phage resistance activity of AbiK. A model for the mode of action of AbiK is proposed.**

Bacteria are used in a variety of fermentation processes because of their ability to convert a wide variety of substrates into complex products or specific molecules. Any process that relies on bacterial fermentation is vulnerable to bacteriophage infections. *Lactococcus lactis* strains are extensively used by the dairy industry for their fermentation abilities, and they are susceptible to virulent phages, which are ubiquitous in the dairy environment. Three genetically distinct groups of lactococcal phages (936, c2, and P335) are responsible for ruined milk fermentations, and one of the most effective way of controlling them is through the use of phage-resistant strains (49).

One common method to create a phage-resistant starter is to exploit lactococcal native plasmids and to introduce them into industrial phage-sensitive *L. lactis* strains (61). The genetic determinants of over 50 lactococcal phage resistance mechanisms have been cloned and sequenced (14). They can affect phage multiplication by inhibiting adsorption, blocking DNA entry in the cell, cutting the exogenous DNA (restriction/modification systems), or aborting infection after early phage gene expression. The last group comprises 22 abortive infection (Abi) mechanisms that rarely share amino acid sequence similarity. Although they are effective against similar groups of phages, their mode of action and regulation mechanisms seem quite different, suggesting that they interfere with different viral and/or cellular components (26).

The effects of these antiviral proteins on the course of the phage lytic cycle are usually determined to provide clues regarding their mode of action. Phage DNA replication, early and late transcription, and protein synthesis are modified or inhibited by Abi mechanisms (14). AbiA (20), AbiF (27), AbiK

(10), and AbiR (63) rapidly halt phage DNA replication by an unknown mechanism. Recently, it has been shown that AbiP disturbs both phage DNA replication and temporal control of early phage transcription (22). AbiG inhibits mRNA synthesis (54), whereas AbiB triggers the breakdown of phage transcripts through the induction of an RNase (57). AbiD1 likely blocks phage DNA replication or packaging through a decrease of a RuvC-like endonucleolytic activity (4). The transcription of genes encoding AbiA, AbiB, AbiL, AbiP, and AbiT was also found to be constitutive (7, 13, 18, 20, 22). AbiL and AbiT are encoded by two genes that are organized in an operon. Only two lactococcal Abi proteins have been mutated to study the roles of particular motifs in phage resistance. Nonconservative substitutions of regularly spaced leucine residues of AbiA lead to the loss of the phage resistance phenotype (21). Similarly, the substitution of a hydrophobic amino acid by a charged residue or a proline in the C-terminal region of AbiT abolished the effects of AbiT (7).

AbiK is an abortive infection system encoded by the lactococcal plasmid pSRQ800 (11). The AbiK protein has a predicted size of 599 amino acids and is effective against the 936-, P335-, and c2-like phages (25). It inhibits the DNA replication of phages of the P335 group but affects 936-like phages at a later step of the lytic cycle (10). The phage gene involved in the sensitivity to AbiK has recently been identified in several bacteriophages (9). These genes encode proteins related to single-strand annealing recombinases, but their role in the molecular mechanism of AbiK remains unknown. It has also been reported that the growth temperature and the gene copy number affect the efficacy of AbiK (25). In this study, the transcription and translation of the *abiK* gene were examined. Conserved residues in the reverse transcriptase (RT) motif of AbiK were also replaced by site-directed mutagenesis.

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics <sup>a</sup>	Source or reference
<i>E. coli</i> strains		
DH5 $\alpha$	<i>supE44</i> $\Delta$ <i>lacU169</i> ( $\phi$ 80 <i>lacZ</i> $\Delta$ M15) <i>hsdR17</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	Invitrogen
TOP10	F <sup>-</sup> <i>mcrA</i> $\Delta$ ( <i>mrr-hsdRMS-mcrBC</i> ) $\phi$ 80 <i>lacZ</i> $\Delta$ M15 $\Delta$ <i>lacX74</i> <i>recA1</i> <i>deoR</i> <i>araD139</i> $\Delta$ ( <i>ara-leu</i> )7697 <i>galU</i> <i>galK</i> <i>rpsL</i> (Str <sup>r</sup> ) <i>endA1</i> <i>nupG</i>	Invitrogen
<i>L. lactis</i> strains		
MG1363	Plasmid-free host for phage p2	28
LM0230	Plasmid-free host for phage p2	45
SMQ-20	LM0230(pSA3, pSRQ800), Em <sup>r</sup> , Abi <sup>+</sup>	25
SMQ-481	Plasmid-cured host for phage ul36	8
Plasmids		
pBAD-HisB	Expression vector, Ap <sup>r</sup> , 4.1 kb	Invitrogen
pBS	Cloning vector, Ap <sup>r</sup> , 2.9 kb	Stratagene
pGKH10	Encodes the <i>lacZ</i> gene of <i>E. coli</i> , Cm <sup>r</sup> , Em <sup>r</sup>	30
pLCF-1	PCR amplicon of the <i>orf4</i> gene in pBAD-HisB	This study
pMIG3	Shuttle vector, Cm <sup>r</sup> , 5.5 kb (intermediate-copy vector for <i>L. lactis</i> )	67
pNZ123	High-copy vector for <i>L. lactis</i> , Cm <sup>r</sup> , 2.8 kb	19
pSA3	Shuttle vector, Cm <sup>r</sup> , Tc <sup>r</sup> , Em <sup>r</sup> , 10.2 kb (low-copy vector for <i>L. lactis</i> )	17
pSRQ800	Native <i>L. lactis</i> plasmid encoding AbiK, Abi <sup>+</sup>	25
pSRQ817	3.7-kb EcoRI-PvuII of pSRQ800 in pMIG3, Cm <sup>r</sup> , Abi <sup>+</sup>	25
pSRQ818	2.2-kb PCR amplicon of the <i>abiK</i> operon in pMIG3, Cm <sup>r</sup> , Abi <sup>+</sup>	This study
pSRQ822	3.7-kb EcoRI-PvuII in pNZ123, Cm <sup>r</sup> , Abi <sup>+</sup>	This study
pSRQ840	PCR amplicon of <i>lacZ</i> in pMIG3, Cm <sup>r</sup>	This study
pSRQ841	PCR amplicon of <i>lacZ</i> in pSRQ818 (AbiK-LacZ fusion), Cm <sup>r</sup>	This study
pSRQ842	3.2-kb HaeIII-PvuII of pSRQ800 in pMIG3, Cm <sup>r</sup> , Abi <sup>+</sup>	This study
pSRQ843	3.0-kb NdeI-PvuII of pSRQ800 in pMIG3, Cm <sup>r</sup> , Abi <sup>+</sup>	This study
pSRQ851	3.7-kb EcoRI-PvuII in pSA3, Cm <sup>r</sup> , Tc <sup>r</sup> , Em <sup>r</sup> , Abi <sup>+</sup>	This study
pSRQ852	3.2-kb HaeIII-PvuII in pSA3, Cm <sup>r</sup> , Tc <sup>r</sup> , Em <sup>r</sup> , Abi <sup>+</sup>	This study
pSRQ853	3.0-kb NdeI-PvuII in pSA3, Cm <sup>r</sup> , Tc <sup>r</sup> , Em <sup>r</sup> , Abi <sup>+</sup>	This study
pSRQ854	0.5-kb EcoRI-HaeIII and 3.0-kb NdeI-PvuII in pSA3, Cm <sup>r</sup> , Tc <sup>r</sup> , Em <sup>r</sup> , Abi <sup>+</sup>	This study
pSRQ860	1.0-kb EcoRI-BglII in pNZ123, Cm <sup>r</sup>	This study
pSRQ871	2.2-kb PCR amplicon of the mutated <i>abiK</i> gene (D163E) in pMIG3, Cm <sup>r</sup>	This study
pSRQ872	2.2-kb PCR amplicon of the mutated <i>abiK</i> gene (Y168S) in pMIG3, Cm <sup>r</sup>	This study
pSRQ873	2.2-kb PCR amplicon of the mutated <i>abiK</i> gene (Y245F) in pMIG3, Cm <sup>r</sup>	This study
pSRQ874	2.2-kb PCR amplicon of the mutated <i>abiK</i> gene (Y245S) in pMIG3, Cm <sup>r</sup>	This study
pSRQ875	2.2-kb PCR amplicon of the mutated <i>abiK</i> gene (D247E) in pMIG3, Cm <sup>r</sup>	This study
pSRQ876	2.2-kb PCR amplicon of the mutated <i>abiK</i> gene (D248E) in pMIG3, Cm <sup>r</sup>	This study

<sup>a</sup> Abi<sup>+</sup>, active Abi mechanism; Ap<sup>r</sup>, ampicillin resistance; Cm<sup>r</sup>, chloramphenicol resistance; Em<sup>r</sup>, erythromycin resistance; Tc<sup>r</sup>, tetracycline resistance; Tc<sup>s</sup>, sensitive to tetracycline.

## MATERIALS AND METHODS

**Bacterial strains and media.** The strains used in this study are listed in Table 1. *Escherichia coli* was grown at 37°C in LB medium, and *L. lactis* was grown at 30°C in M17 (Quélab) supplemented with 0.5% glucose (GM17). When needed, antibiotics were added to the medium at the following concentrations: 50  $\mu$ g/ml of ampicillin, 20  $\mu$ g/ml of chloramphenicol, or 10  $\mu$ g/ml of tetracycline for *E. coli* and 5  $\mu$ g/ml of chloramphenicol or erythromycin for *L. lactis*.

**Bacteriophage propagation and microbiological assays.** The two virulent bacteriophages used in this study are p2 (L. L. McKay, University of Minnesota), a member of the 936 species, and ul36, a *pac*-type member of the P335 species (51). They were propagated as described previously (51). The efficiency of plaquing (EOP) was calculated by dividing the phage titer on the tested strain by the titer on the sensitive control strain. One-step growth curves were determined as reported elsewhere (50). The burst size was determined by dividing the average titer after the exponential phase by the average titer before cells began to release virions.

**Plasmids, primers, and DNA manipulations.** The plasmids utilized in this study are described in Table 1. All primer sequences cited in this study are available upon request. *E. coli* plasmid DNA was isolated by the method of Birnboim (5). *L. lactis* plasmid DNA was obtained as described by O'Sullivan and Klaenhammer (55). All DNA manipulations were carried out as reported by Sambrook and Russell (60). When needed, pBS was first used to clone restriction fragments and to introduce deletions. The *abiK* operon was amplified using primers SM1 and SM2 to generate pSRQ818. The *E. coli lacZ* gene was amplified from pGKH10 with primers JB818a and JB818b to construct plasmids pSRQ840 and pSRQ841. The PCR products were digested and purified as

specified elsewhere (24). Site-directed mutagenesis of AbiK was made with the megaprimer PCR method (60). The *abiK* operon was amplified with primers SM1 and SM2, and site-directed mutagenesis was performed with primers Mut1, Mut2, Mut3, Mut4, Mut5, and Mut7. The mutations were confirmed by sequencing the cloned inserts. DNA sequencing was performed using an ABI Prism 3100 apparatus from the service center at the Université Laval or an ABI Prism 3700 apparatus from the genomic platform at the research center of the Centre Hospitalier de l'Université Laval. *E. coli* and *L. lactis* were transformed with the Gene Pulser II apparatus as indicated by the manufacturer and by Holo and Nes (32), respectively.

**RNA isolation and Northern blot analysis.** RNA of phage-infected *L. lactis* cells was isolated as described previously (7), and Northern blot analyses were carried out according to Sambrook and Russell (60). The probe corresponding to an internal segment of the *abiK* gene (coordinates 3988 to 4570 in pSRQ800, accession no. U35629) was amplified with primers IB800.14 and AbiK4. The probe covering the 3' end of *orf3* and the 5' region of *orf4* (coordinates 5405 to 5694) was amplified with primers IB800.12.1 and Orf4. The PCR products were labeled with [ $\alpha$ -<sup>32</sup>P]dATP (Amersham Pharmacia Biotech) by random priming.

**5'-RACE.** The transcription initiation site from the *abiK* promoter was determined by rapid amplification of cDNA ends (RACE) as described by Sambrook and Russell (60). The primer used for cDNA synthesis was PEabiK. PCR amplification was conducted with this primer and the poly(C) primer CB3 (12).

**Primer extension analysis.** The transcription initiation site of the 2.6-kb mRNA was confirmed by primer extension analysis using primer LCF-21 as described by Sambrook and Russell (60). Total RNA from *L. lactis* carrying plasmid pSRQ817 was isolated as reported previously (7).

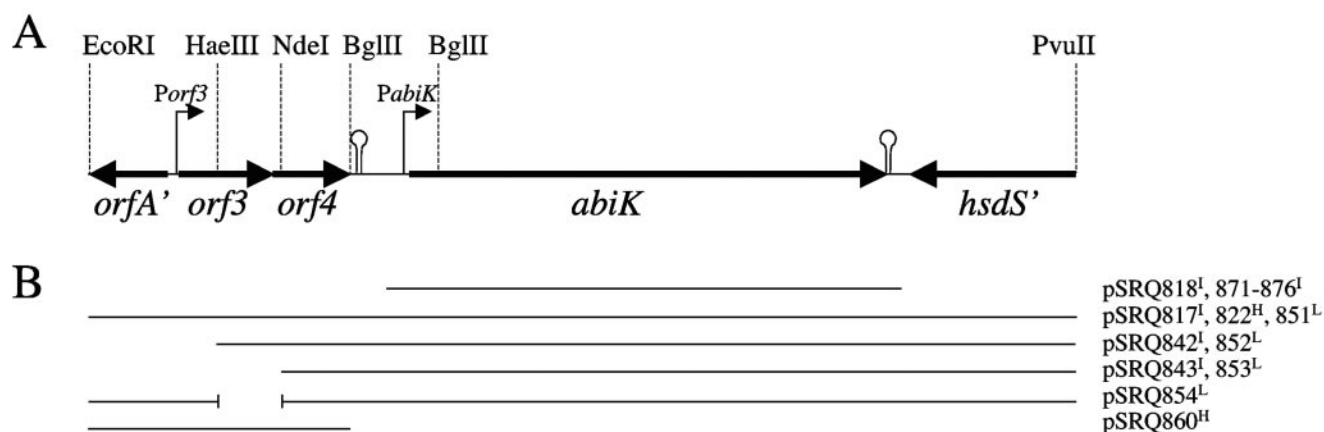


FIG. 1. Genetic organization of the DNA fragment of pSRQ800 that encodes phage resistance. Panel A: open reading frames, promoters (bent arrows), and rho-independent terminators (stem-loops) identified by bioinformatics analyses. Panel B: representation of the DNA fragments cloned into the different plasmids used in this study. The superscript letter at the end of the plasmid name represents the plasmid copy number in *L. lactis* (H = high; I = intermediate; L = low).

**$\beta$ -Galactosidase assay.**  $\beta$ -Galactosidase activity was measured as described by Boucher et al. (12). Briefly, cells were grown in LB (*E. coli*) or GM17 (*L. lactis*) medium at an optical density at 600 nm of 0.5, washed and resuspended in 0.5 ml of 50 mM sodium phosphate (pH 7.0), and lysed with glass beads. The lysate was cleared by centrifugation, and the protein concentration was measured with the Bio-Rad DC protein assay reagent. The  $\beta$ -galactosidase assay was performed as described by Miller (47).

**Cloning into pBAD-HisB and expression of the *orf4* gene product.** The *orf4* gene was cloned into the pBAD-HisB expression vector (Invitrogen) in order to create a C-terminal fusion of Orf4 with a tail of six histidine residues. The *orf4* gene was amplified by PCR from pSRQ800 by using primers LCF-11 and LCF-17. The LCF-17 primer included the codons for the six histidine residues followed by a stop codon. The NcoI-digested PCR product was ligated to the corresponding site in pBAD-HisB and cloned into *E. coli* TOP10 to create the pLCF-1 construct, which was confirmed by DNA sequencing using primers LCF-14 and LCF-15. Induction of the expression was conducted at 37°C for 2 h in the presence of 0.2% L-(+)-arabinose. The cleared lysate was used for purification of the fusion protein by nickel-chelating nitrilotriacetic acid affinity chromatography (Ni-NTA) as recommended by the manufacturer (Qiagen).

**Protein quantification and sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis.** Proteins were quantified using the Bio-Rad protein assay reagent and bovine serum albumin as a standard. The various protein fractions were analyzed by standard 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis run in Tris-glycine buffer (37).

**EMSA.** Electrophoretic mobility shift assay (EMSA) with the Ni-NTA-purified Orf4 protein was conducted as described elsewhere (39). The DNA fragments were labeled by incorporation of [ $\alpha$ - $^{32}$ P]dATP during PCR amplification and purified with Micro Bio-Spin P-30 chromatography columns (Bio-Rad). The PCR primers used were LCF-3 and LCF-6 for fragment A, LCF-4 and LCF-7 for fragment B, LCF-5 and LCF-8 for fragment C, and LCF-3 and LCF-23 for fragment D. The LCF-24 and LCF-25 complementary oligonucleotides were annealed to create a double-stranded DNA fragment of 41 bp used for competition assays. The binding buffer consisted of 10 mM HEPES (pH 7.9), 10% glycerol, 0.2 mM EDTA, 0.25 mM phenylmethanesulfonyl fluoride, 0.1  $\mu$ g poly(dI-dC), 50 mM KCl, and 5 mM MgCl<sub>2</sub> (39). The purified Orf4 was incubated in the binding buffer with 10,000 cpm of labeled DNA fragment. Binding was allowed to proceed at room temperature for 15 min with or without competitor DNA before analysis on a 5% native polyacrylamide gel (39).

## RESULTS

**The efficacy of *AbiK* is enhanced by neighboring genes in pSRQ800.** Previous bioinformatic analyses indicated the presence of a putative promoter (*PabiK*) and of a rho-independent terminator that were flanking the *abiK* gene on plasmid pSRQ800 (25). Thus, the *abiK* gene seemed to be part of a

monocistronic unit. In pSRQ800, another putative promoter (*Porf3*) and two short open reading frames (*orf3* and *orf4*) that have the typical G+C content of *L. lactis* are located upstream of the *abiK* operon (Fig. 1A). In this study, we investigated whether or not the presence of this second operon could influence the antiphage activity of *AbiK*.

The native plasmid pSRQ800 and DNA fragments from pSRQ800 subcloned into the shuttle vector pMIG3 confer protection against phages, with constant EOP values of  $10^{-6}$  (Fig. 1B; Table 2). The antiphage activity is further increased (EOP of  $\leq 1 \times 10^{-9}$ ) when the complete operon is cloned into the high-copy vector pNZ123 (Fig. 1B; Table 2). However, when restriction fragments of pSRQ800 are cloned in the low-copy vector pSA3, the antiphage phenotype is weaker and also variable, as EOP values ranged from  $10^{-2}$  to  $10^{-3}$  (Fig. 1B; Table 2). The EOP variations with the low-copy vector appear to depend on the absence or the presence of the putative *Porf3* promoter on the cloned fragment. Disrupting the *orf3-orf4* coding sequences without deleting the *Porf3* promoter (pSRQ854) (Table 2) did not alter significantly the EOP (pSRQ851) (Table 2). Thus, the EcoRI-HaeIII fragment harboring the *Porf3* promoter is responsible for the increased

TABLE 2. Effect of *Porf3* and *Orf4* on the activity of *AbiK* against phage p2

Plasmid	EOP <sup>a</sup>	Burst size <sup>a</sup>	EOP with pSRQ860 <sup>a</sup>
pSRQ800	$(6.8 \pm 2.5) \times 10^{-6}$	ND <sup>b</sup>	$(3.8 \pm 3.2) \times 10^{-2}$
pSRQ822	$\leq 1 \times 10^{-9}$	ND	ND
pMIG3	1.0	ND	ND
pSRQ817	$(1.7 \pm 1.7) \times 10^{-6}$	ND	ND
pSRQ818	$(2.1 \pm 1.8) \times 10^{-6}$	ND	ND
pSRQ842	$(2.6 \pm 0.9) \times 10^{-6}$	ND	ND
pSRQ843	$(3.1 \pm 1.2) \times 10^{-6}$	ND	ND
pSA3	1.0	83 $\pm$ 6	1.0
pSRQ851	$(3.1 \pm 2.3) \times 10^{-3}$	19 $\pm$ 5	$(1.2 \pm 0.2) \times 10^{-2}$
pSRQ852	$(5.5 \pm 0.8) \times 10^{-2}$	32 $\pm$ 4	$(3.0 \pm 1.1) \times 10^{-2}$
pSRQ853	$(6.1 \pm 4.6) \times 10^{-2}$	ND	$(3.8 \pm 2.6) \times 10^{-2}$
pSRQ854	$(2.0 \pm 1.3) \times 10^{-3}$	ND	ND

<sup>a</sup> Means and standard deviations from at least three experiments.

<sup>b</sup> ND, not determined.

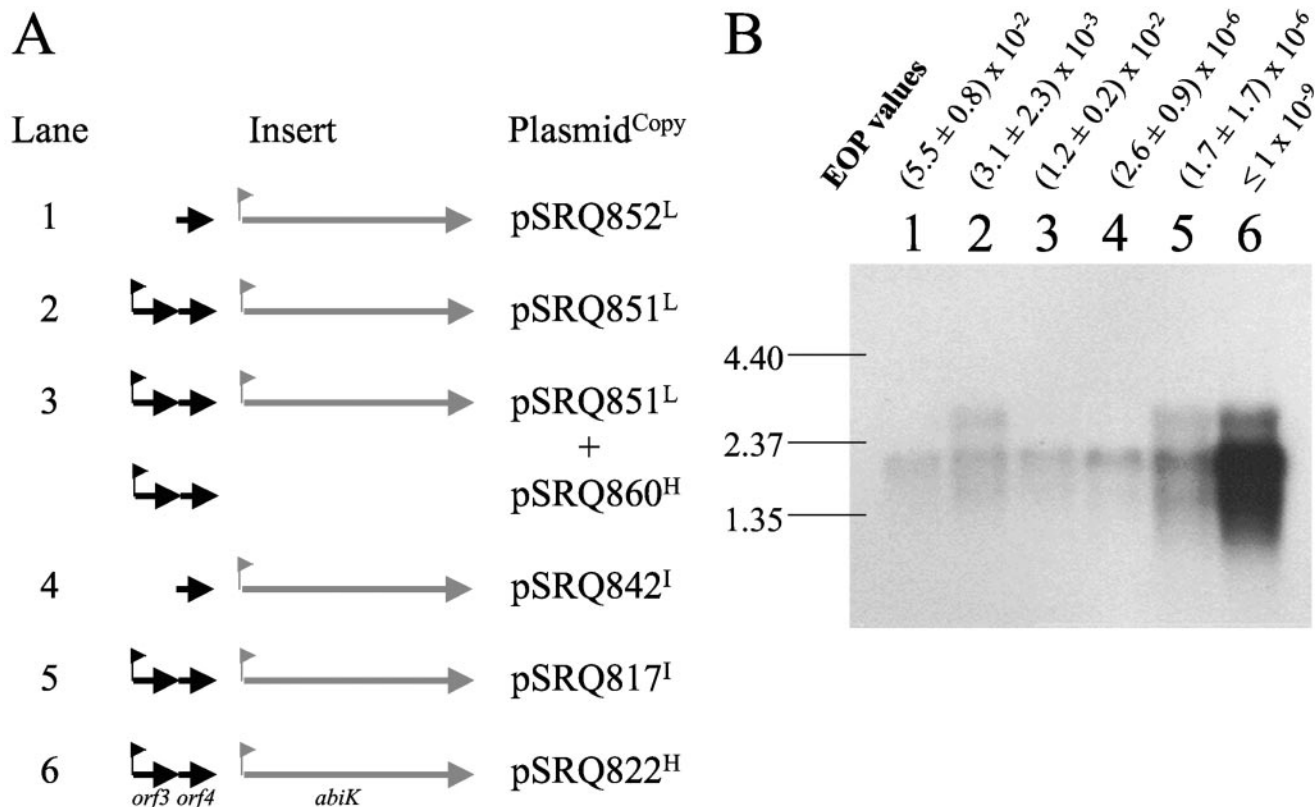


FIG. 2. Transcription of the *abiK* operon. Panel A shows the plasmids carried by the six strains used, along with a schematic representation of the cloned insert. The corresponding plasmid copy number in *L. lactis* MG1363 is indicated by a superscript letter at the end of the plasmid name (see the legend to Fig. 1). The pNZ123 vector was cointroduced as a control in addition to the indicated plasmids in lanes 1 and 2. Panel B presents the Northern blot analysis with a probe corresponding to the *abiK* gene. To facilitate the comparison of the different constructs, some EOP values from Table 2 (without pSRQ860) are shown above the blot. Bands of the molecular weight marker (in kbp) are indicated on the left.

antiphage activity (EOP from  $10^{-2}$  to  $10^{-3}$ ) with the low-copy vector pSA3 (Table 2). This DNA fragment does not affect phage multiplication (EOP of 1) when cloned without the *AbiK* system (pSRQ860) (Table 2).

Furthermore, when the *orfA'-orf3-orf4* operon (Fig. 1A) was provided in *trans* on the high-copy plasmid pNZ123 (pSRQ860), the enhancement of the antiphage activity was eliminated (Table 2). The presence of plasmid pSRQ860 also drastically reduced the phage resistance phenotype conferred by the native plasmid pSRQ800 (from  $10^{-6}$  to  $10^{-2}$ ) (Table 2). The presence of the high-copy vector pNZ123 (as a control) did not affect the antiphage phenotype of pSRQ800 (EOP of  $10^{-6}$ ). Finally, the abortive infection phenotype was not affected when pSRQ860 was provided in *trans* in *L. lactis* strains containing low-copy constructs with a deleted *Porf3* (pSRQ852 and pSRQ853) (Table 2). Taken altogether, these data suggest that the *abiK* gene could be expressed from another promoter (*Porf3*) located upstream from *PabiK*. Also, a putative protein product encoded by the EcoRI-BglII fragment could interfere with the *AbiK* antiphage activity. The *orf4* gene product is the only possible candidate, since the transcription start site downstream of *Porf3* is located inside the start codon of *orf3* (see below) and there is no putative ribosome-binding site up-

stream of this ATG. A series of experiments were carried out to confirm these hypotheses.

**Transcription of the *abiK* gene.** *L. lactis* MG1363 derivatives carrying different combinations of *abiK*- and *Porf3*-containing plasmids were selected for Northern blot experiments (Fig. 2A). Two transcripts (1.9 and 2.6 kb) hybridized with the *abiK* probe. The smaller mRNA has the size of the predicted *abiK* operon, and its abundance in the different strains was correlated with the plasmid copy number (Fig. 2). A probe covering *orf3* and *orf4* also hybridized with the 2.6-kb transcript and with a 0.5-kb mRNA present at a higher concentration (data not shown). Therefore, the 2.6-kb transcript covers the two operons. This also indicates that the putative terminator located downstream of *orf4* (Fig. 1A) is partially effective. Interestingly, the 2.6-kb transcript is absent if *Porf3* is deleted (Fig. 2, lanes 1 and 4) or if *orf4* is provided in *trans* on pSRQ860 (Fig. 2, lane 3). Judging by the relative concentrations of the 1.9- and 2.6-kb transcripts (Fig. 2, lanes 2, 5, and 6), the *orf4* gene product, when provided in a sufficient amount in *cis* (pSRQ822) (Fig. 2, lane 6) or in *trans* (pSRQ860) (Fig. 2, lane 3), can repress the expression from *Porf3*.

The 5' ends of the two transcripts were determined by 5'-RACE and primer extension analyses. Sequencing of the am-

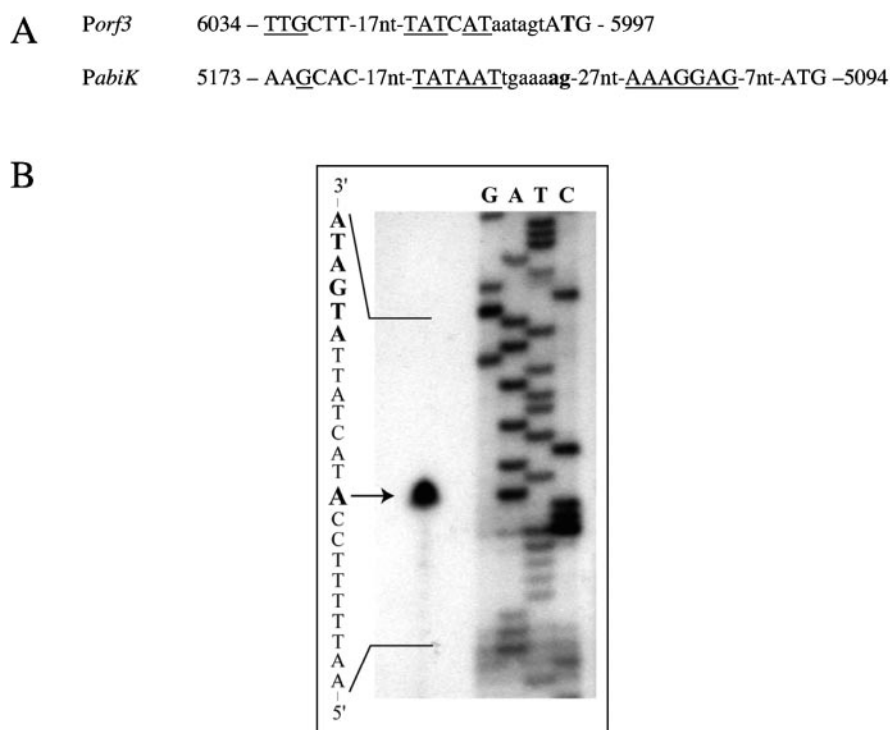


FIG. 3. Promoter determination. Panel A: DNA sequence and transcription initiation sites (in boldface) of the *Porf3* and *PabiK* promoters. The  $-35$  and  $-10$  regions, the ribosome binding site of *abiK*, and the start codons of *orf3* and *abiK* are in capital letters. Nucleic acids that are identical to the consensus sequence are underlined. The coordinates correspond to the DNA sequence of pSRQ800 (accession no. U35629). Panel B: primer extension analysis of the *Porf3* promoter. Total RNA from an *L. lactis* MG1363 strain carrying the pSRQ817 plasmid was purified, and primer extension was performed as described in Materials and Methods. The lower-strand DNA sequence shown on the left was obtained with the same primer in a T7-based sequencing reaction. The  $-10$  box is highlighted in boldface. The transcription initiation start site is indicated by an arrow.

plicon obtained by RACE with a primer downstream of the *abiK* promoter indicated that there are two possible initiation sites from this promoter (Fig. 3A). The putative *Porf3* promoter was analyzed by primer extension analysis of total RNA isolated from strains carrying pSRQ817 and pSRQ860. The extension products confirmed the functionality of the *Porf3* promoter and the location of the transcription initiation site (Fig. 3).

**Binding of Orf4 to the *Porf3* promoter.** The Orf4 putative protein possesses a helix-turn-helix (HTH) motif (cd00093, pfam01381.9) and is similar to several regulatory proteins (11). One of its homologues, the repressor (cI) of the coliphage 434, belongs to a group of phage transcriptional regulators with similar structures and low levels of sequence similarity (59, 69). Two structure programs (SAM-T99 and PROF) (34, 56) predicted that Orf4 has the five  $\alpha$ -helices and the four turns of this family of proteins. These comparisons strongly suggested that Orf4 could act as a transcriptional repressor.

To test this prediction, the Orf4 gene product was overexpressed in *E. coli* as a His tag fusion and purified to near homogeneity by Ni-NTA affinity chromatography (see Materials and Methods). This protein preparation was used in a series of EMSA experiments to confirm its DNA-binding activity (see below).

Different promoter fragments were amplified and labeled by PCR (Fig. 4A). Incubation of fragments B (192 bp) and C (210 bp) with the Orf4 protein extract did not show any retardation

in EMSA experiments (Fig. 4B). On the other hand, fragments A (306 bp) and D (215 bp) were completely bound and retarded in the presence of Orf4 (Fig. 4B, complex  $C_1$ ). Addition of antihistidine antibodies into the binding reaction mixture resulted in a supershifted complex of higher molecular mass (Fig. 4B, complex  $C_2$ ). This confirmed that the protein causing the retardation of the labeled fragments was indeed Orf4 and not a contaminating protein from *E. coli*. Up to 2.0  $\mu$ g protein was tested in EMSA, and even at the highest protein concentration, only one complex was detected (data not shown). This suggests the presence of a single Orf4 binding site in the *Porf3* promoter sequence. A 10-bp inverted repeat (10-6-10) was found between the  $-10$  and  $-35$  boxes of the *Porf3* promoter and could represent the target of Orf4 (Fig. 4A). A 41-bp DNA fragment covering this region was created by annealing complementary oligonucleotides (Fig. 4A, fragment E). When used in competition assays with fragment D as the labeled probe, the  $C_1$  complex gradually disappeared as the amount of unlabeled competitor DNA increased (Fig. 4B). This confirmed that Orf4 binds to the *Porf3* sequence and that the inverted repeat might be the targeted structure.

**Protein expression analysis with an *AbiK*-LacZ fusion.** The *abiK* gene is constitutively transcribed from *PabiK* and *Porf3*, but its translation could be regulated. The *PabiK* promoter, ribosome-binding site and first 178 codons of the *abiK* gene were fused to the last 1,015 codons of the *E. coli lacZ* gene and introduced into the shuttle vector pMIG3 to generate

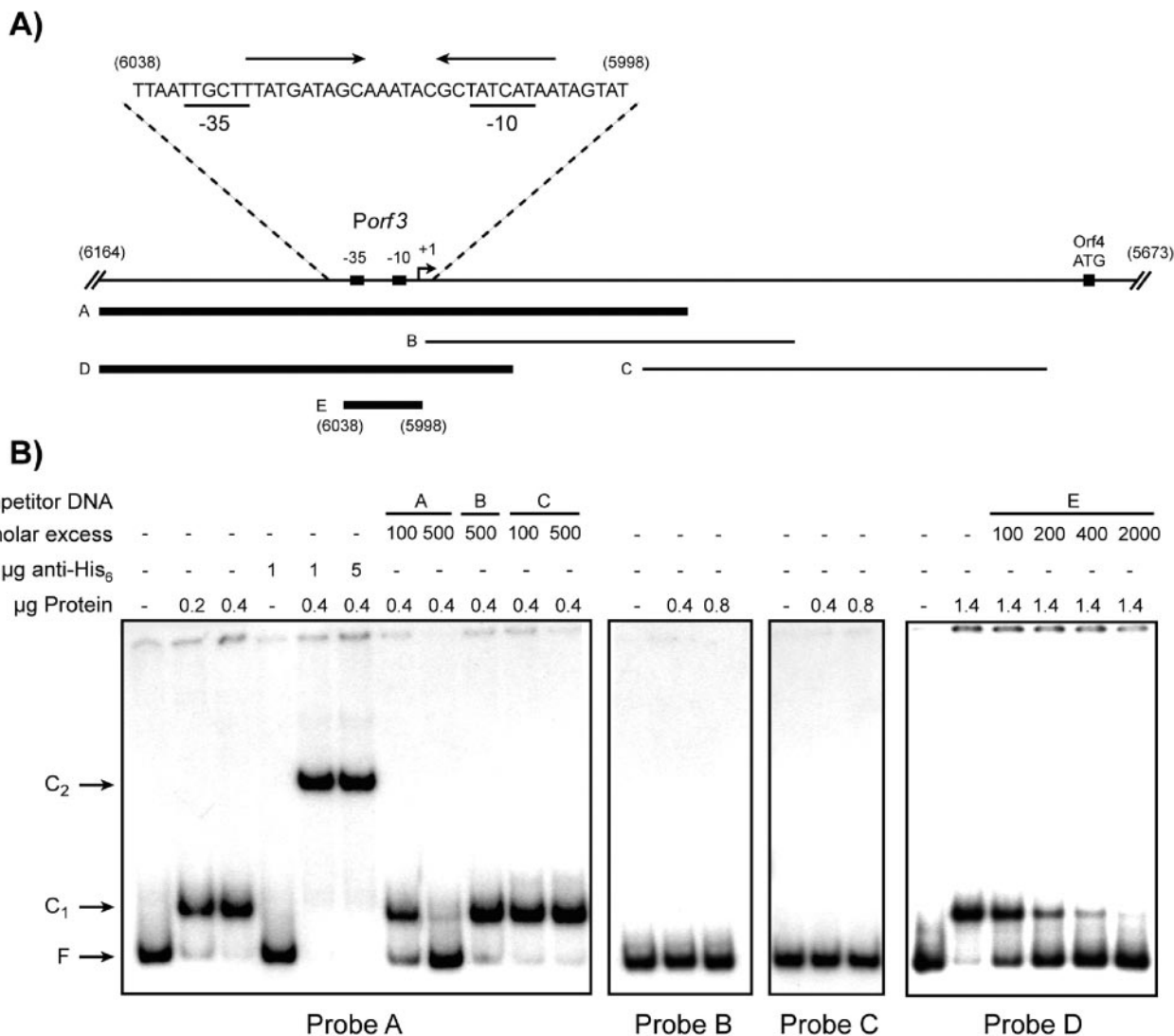


FIG. 4. Gel retardation assays (EMSA) with recombinant Orf4. Panel A shows the different fragments (indicated by letters A to E) used as labeled probes (10,000 cpm/reaction) or unlabeled competitor DNA. Thick lines represent the fragments retarded by Orf4 in EMSA, whereas thin lines represent unbound fragments. The DNA sequence corresponding to fragment E is shown above, along with the -10 and -35 boxes (underlined characters). The inverted repeats are indicated by arrows above the sequence. Some of the coordinates are shown in parentheses and correspond to the pSRQ800 sequence (GenBank accession no. U35629). The transcription initiation start site (+1) is indicated by a bent arrow. Panel B, EMSA with the various fragments shown in panel A. Unlabeled competitor DNA fragments are indicated by letters (corresponding to fragments in panel A). F, free probe; C<sub>1</sub>, Orf4-DNA complex; C<sub>2</sub>, anti-His<sub>6</sub>-Orf4-DNA complex.

pSRQ841 (Table 1). The β-galactosidase activity conferred by pSRQ841 was measured in *E. coli* and in two *L. lactis* strains at 30 and 37°C. The promoterless *lacZ* gene cloned in pMIG3 to generate pSRQ840 was used as a negative control. The fusion protein was constitutively expressed in the three hosts (Fig. 5). In *L. lactis*, the expression was approximately twice as high at 30°C, which is the optimal growth temperature of this microorganism. This could explain the reduced antiphage activity of AbiK at 37°C (compared to 30°C) and its so-called heat sensitivity (25). The β-galactosidase activity was also measured during infection by phage ul36 and was not found to be significantly different. This result demonstrates that the expression of AbiK is not induced following phage infection (Fig. 5).

**Site-directed mutagenesis of AbiK.** The N-terminal half of the AbiK protein (300 amino acids) contains a motif found in RTs (pfam 00078), as shown by the alignment with AbiA protein, which also possesses a similar motif (Fig. 6). Few amino acids are conserved in all RTs (70). To determine if this RT motif of AbiK is important for phage resistance, five conserved amino acids of the motif were replaced by site-directed mutagenesis and the antiphage activity of mutated AbiK proteins was assayed in *L. lactis* MG1363 with phage p2. The substitution of an aspartic acid (D163, D247, and D248) by a glutamic acid or of a threonine (Y168 and Y245) by a serine abolished the resistance phenotype (EOP of 1). In some active RTs, the tyrosine in position 245 is replaced by a phenylalanine (70).

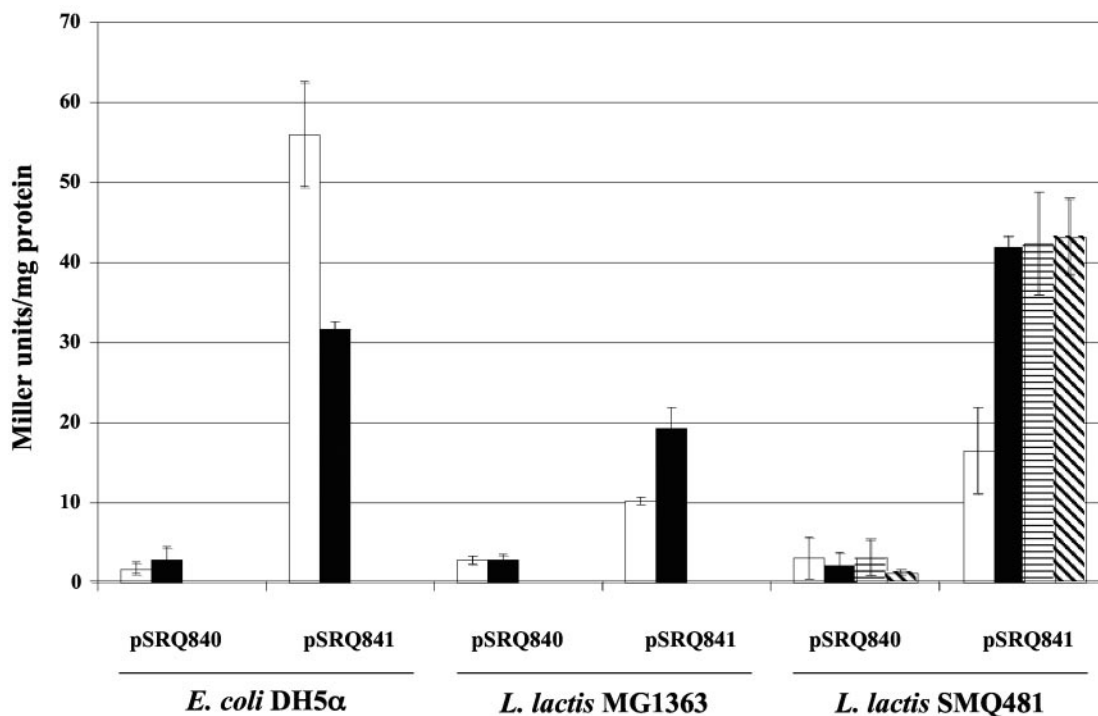


FIG. 5.  $\beta$ -Galactosidase activity in various *E. coli* and *L. lactis* strains at 37°C (white bars), at 30°C (black bars), and during infection by phage ul36 (horizontally striped bars, after 15 min; diagonally striped bars, after 30 min). These data represent the means and standard deviations from at least three experiments.

This substitution reduced slightly the efficacy of *AbiK* (EOP of  $1.4 \times 10^{-5}$  instead of  $2.1 \times 10^{-6}$ ).

## DISCUSSION

**Effect of the *orf4* operon on *AbiK* expression.** The *abiK* gene is transcribed from the promoter previously identified by DNA sequence analysis (25) and from another promoter (*Porf3*) that is not located in the AT-rich region of pSRQ800 that encodes *AbiK* (this study). Results from Northern blot experiments revealed that the transcription from this second promoter, *Porf3*, is repressed when *Orf4* is provided, in *cis* or in *trans*, at high levels relative to *AbiK*. Thus, *Orf4* acts as a repressor by inhibiting transcription from *Porf3*, resulting in a decreased expression of *abiK* and a weaker phage resistance phenotype in *L. lactis*.

The *Orf4* protein possesses the HTH motif found in many bacteriophage regulatory proteins and numerous transcriptional regulators (69). Secondary-structure prediction indicated the presence of helices 2 and 3, which bind and recognize DNA, and of helices 4 and 5, which are involved in dimerization (31). Many members of the HTH family of bacterial regulators bind their consensus sequence as homodimers or homotetramers (59). Two putative helices that could play a role in dimer formation were found in the C-terminal part of *Orf4*. Also, inverted repeats are often part of target promoter/operator regions and can be bound by multimeric forms of transcriptional regulators containing HTH DNA-binding motifs. As examples, two homodimers of the ferric uptake regulator (*Fur*) from *Bacillus subtilis* bind to the *Fur* box composed of a

9-1-9 inverted repeat (2), and four homodimers of the Zn(II)-sensing metal-regulated repressor *SmtB* from *Synechococcus* strain PCC7942 bind to a 40-bp oligonucleotide containing a single 12-2-12 inverted repeat (64). Interestingly, the presence of a 10-6-10 inverted repeat inside the 41-bp region bound by the purified *Orf4* suggests that this repressor might indeed act as a homodimer. Only one complex was observed in EMSA, over a wide range of *Orf4* concentrations, indicating that only one binding site is occupied by a single protein complex. Otherwise, we could have expected retarded bands of intermediate mobility depending on the protein-DNA molar ratio, corresponding to multiple *Orf4* complexes composed of monomers or dimers bound to one or more binding sites (41, 58). The nature of the *Orf4*-binding complex is unknown and remains to be investigated.

Other *Abi* systems have been shown to be associated with potential regulatory proteins. In *L. lactis*, the antiphage activity of *AbiU* is increased (EOP from  $10^{-2}$  to  $10^{-4}$ ) when the putative regulatory gene *abiU2* is deleted (16). The *abiU2* gene seems to be involved in downregulation of *abiU1* or its gene product, *AbiU1*, which confers phage resistance. This regulation scheme resembles that of *PifA*, an *E. coli* protein encoded by plasmid F that aborts T7 infection. The *PifC* protein negatively controls the expression of the three genes of a polycistronic operon (*pifC*, *pifA*, and *pifB*). Inactivation of *pifC* increases the expression of the *Abi* protein (15, 46).

In the case of *AbiK*, the *orf3-orf4* regulatory operon improves the efficacy of the system, since the *abiK* gene is expressed from an additional promoter. However, the impact of the upstream *Porf3* promoter is variable, depending on the

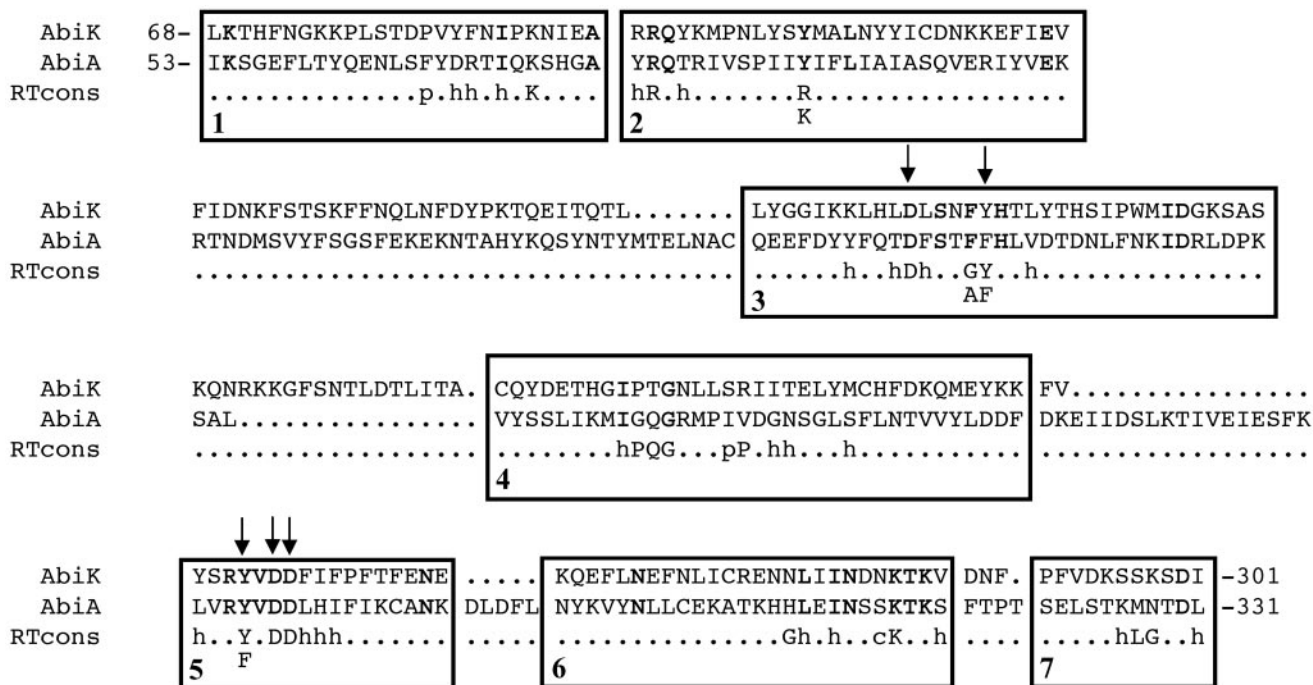


FIG. 6. Alignment of AbiK, AbiA, and the RT motifs of Xiong and Eickbush (70). The identical residues in the amino acid sequences of AbiK and AbiA are in boldface. h, hydrophobic residue; p, small polar residue; c, charged residue. The amino acids that were replaced by site-directed mutagenesis are identified by arrows.

copy number of the plasmid carrying the AbiK system. The exact function of AbiK in uninfected cells is yet unknown, but the *orf3-orf4* regulatory operon present on pSRQ800 might play a role in proper balancing of the constitutive expression of AbiK and Orf4.

From an evolutionary point of view, the similarity of Orf3 and Orf4 with putative phage proteins suggests that this operon might be of viral origin (11). Associations between Abi systems and foreign regulatory genes or sequences that were not initially associated with these Abi genes have been observed previously. For example, the *abiB* gene is transcribed from a promoter located in an Iso-ISS1 insertion sequence (13), and the two genes from the AbiT system appear to have been inserted into a preexisting operon (7). Similarly, the *orf3-orf4* operon might have been originally associated with other systems before being coupled with the *abiK* operon. Yet, the association of the *orf3-orf4* and *abiK* operons is undeniable with the native plasmid pSRQ800; when the *Porf3* promoter on pSRQ800 is repressed by Orf4 provided in *trans* from pSRQ860, the antiphage activity is reduced by more than 1000-fold.

**Putative RT activity of AbiK.** Despite a complex control of the expression of AbiK, the protein is present in the cell before infection and its expression is not affected by phage infection. The efficacy of AbiK against phages p2 (936 species) and ul36 (P335 species) clearly depends on the intracellular concentration of the protein. The plasmid copy number, the growth temperature that affects the expression of AbiK, or a second promoter can modify the efficacy of the system. At this time, it is not known if AbiK is expressed constitutively in an active form or if it must be activated by a phage component.

Mutagenesis experiments also showed that AbiK has an RT motif that is essential for phage resistance. The three aspartate residues that have been mutated in AbiK are all conserved in other RTs (70). They form a triad that can bind divalent metal ions and are directly involved in catalysis (36). Replacement of one of these aspartate residues by an asparagine, a glutamate, or a glutamine in the human immunodeficiency virus type 1 reverse transcriptase results in 1,000-fold reduction of the polymerase activity (42). This could explain the complete loss of phage resistance when the same conservative mutations were introduced in the AbiK protein. The replacement of one of the two tyrosine residues with a serine also reduces significantly the polymerase activity, probably because both mutations decrease the affinity of the enzyme for dTTPs (42, 44). However, when the tyrosine of the YXDD motif (Y245 in AbiK) is changed to a phenylalanine in human immunodeficiency virus type 1 or murine leukemia virus reverse transcriptases, the processivity of the enzyme is preserved, but several authors reported that the fidelity of DNA synthesis is either increased or reduced (1, 3, 33, 35, 40). The slight reduction of the antiphage activity caused by this mutation in AbiK is consistent with these data. To our knowledge, this is the first time that a link between reverse transcription and antiviral activity has been made.

Previously, it was shown that deletion of the last 46 amino acids of AbiK also eliminated the antiphage activity of AbiK (25). Although we cannot exclude a conformational change in the truncated AbiK to explain the lost of antiphage activity, this result also suggests that the C-terminal domain of the protein (amino acids 302 to 599) might have a second biochemical function that is also involved in resistance. Proteins with an



RT domain from viruses and retrotransposons of eukaryotes, but also from *E. coli*, possess an RNase H domain that cleaves the RNA strand of the DNA-RNA duplex (38, 43). RTs of group II introns, found in bacteria and in organelles of eukaryotes, are associated with maturase and endonuclease domains that seem to be involved in intron mobility (29, 48). The C-terminal region of AbiK does not share similarity with RNase, maturase, or endonuclease domains of proteins in the databases. Deletion and mutation analyses strongly suggest that the *abiK* gene could encode a novel bacterial reverse transcriptase with low levels of amino acid sequence similarity to other members of this ubiquitous family of proteins. More interestingly, specific mutations in the conserved RT motif suggest that such an activity is essential for phage resistance.

**Proposed mode of action of AbiK.** Many well-studied *E. coli* abortive infection systems involve activation of the Abi mechanism by a phage element which triggers blockage of an essential cellular function before virions are released. Macromolecule synthesis is often the target of the activated Abi system, leading to cell death (23, 52, 62). AbiK does not inhibit DNA and RNA synthesis by the lactococcal phage p2 (936 group) (6, 10). However, some mRNAs may not be translated, because procapsid structures were not observed in infected AbiK<sup>+</sup> cells (10). On the other hand, the action of AbiK prevents the DNA replication (10) and the late gene expression (6) of the lactococcal phage ul36 (P335 group). Taken altogether, the data indicate that blockage of protein synthesis is an interesting hypothesis to explain the mode of action of AbiK.

It is proposed that AbiK synthesizes a cDNA strand from a currently unknown template RNA through its RT activity. However, it is still uncertain if the RT activity of AbiK would require a primer (53, 65, 66, 68). If needed, this primer could be of viral or cellular origin. The former would suggest the activation of AbiK upon phage infection, while the latter would imply that the retrotranscription of the target RNA is constitutive and not detrimental to the cell, as noninfected AbiK<sup>+</sup> cells behave normally during growth. In these noninfected AbiK<sup>+</sup> cells, the RNA strand and the resulting single-stranded cDNA would be degraded by a cellular RNase and an exonuclease, respectively.

It was previously shown that phages can become insensitive to AbiK through a single-nucleotide mutation causing an amino acid substitution in a phage protein (Sak) related to single-strand annealing proteins (9). In infected AbiK<sup>+</sup> cells, the single-stranded cDNA released after degradation of the complementary template RNA could be annealed by Sak, thereby protecting it from degradation by cellular exonucleases. Subsequent base pairing of the single-stranded cDNA with a cRNA would sequester this RNA and prevent its further use. Phages harboring a mutation in the *sak* gene would have an altered activity, resulting in failure of AbiK. Experiments are currently under way to test these hypotheses.

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