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Involvement of the Major Capsid Protein and Two Early-Expressed Phage Genes in the Activity of the Lactococcal Abortive Infection Mechanism AbiT

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The dairy industry uses the mesophilic, Gram-positive, lactic acid bacterium (LAB) *Lactococcus lactis* to produce an array of fermented milk products. Milk fermentation processes are susceptible to contamination by virulent phages, but a plethora of phage control strategies are available. One of the most efficient is to use LAB strains carrying phage resistance systems such as abortive infection (Abi) mechanisms. Yet, the mode of action of most Abi systems remains poorly documented. Here, we shed further light on the antiviral activity of the lactococcal AbiT system. Twenty-eight AbiT-resistant phage mutants derived from the wild-type AbiT-sensitive lactococcal phages p2, bIL170, and P008 were isolated and characterized. Comparative genomic analyses identified three different genes that were mutated in these virulent AbiT-insensitive phage derivatives: *e14* (bIL170 [$e14_{bIL170}$]), *orf41* (P008 [$orf41_{P008}$]), and *orf6* (p2 [$orf6_{p2}$] and P008 [$orf6_{P008}$]). The genes $e14_{bIL170}$ and $orf41_{P008}$ are part of the early-expressed genomic region, but bioinformatic analyses did not identify their putative function. *orf6* is found in the phage morphogenesis module. Antibodies were raised against purified recombinant ORF6, and immunoelectron microscopy revealed that it is the major capsid protein (MCP). Coexpression in *L. lactis* of $ORF6_{p2}$ and $ORF5_{p2}$, a protease, led to the formation of procapsids. To our knowledge, AbiT is the first Abi system involving distinct phage genes.

Bacteriophages of *Lactococcus lactis* are among the most characterized virulent phages. The interest in these bacterial viruses arises from their detrimental impact in the milk fermentation industry. When virulent phages are present in sufficient concentration, they can infect and lyse a significant proportion of the starter bacterial cultures added to initiate the fermentation process (22). Members of three distinct lactococcal phage groups, 936, c2, and P335, are mainly responsible for slower fermentations, low-quality fermented products, or, less frequently, a complete fermentation failure (4, 33, 48). These three groups belong to the *Siphoviridae* family, as their genome is made of a linear double-stranded DNA molecule packaged into a capsid connected to a long noncontractile tail.

Over the last decades, diverse strategies have been established to control phage outbreaks. One avenue is to exploit natural phage resistance mechanisms (for a review, see reference 40). Some nonindustrial *L. lactis* strains are impervious to many phages and possess such antiviral barriers. These mechanisms can be transferred to an industrial phage-sensitive strain by conjugation or electroporation, thereby conferring a phage resistance phenotype (22, 47). These viral hurdles are grouped into several classes on the basis of their general mode of action. Some antiphage mechanisms block infection at the cell wall or membrane by interfering with either phage adsorption or phage DNA entry (40). Intracellular phage resistance mechanisms appear to be much more diverse and include restriction-modification systems, CRISPR-Cas systems (for reviews, see references 18 and 63), and abortive infection (Abi) mechanisms (for a review, see references 12 and 40). In contrast to other phage resistance mechanisms, infected Abi-positive (Abi^+) cells die while successfully fighting the phage infection. It has been suggested that phage-infected Abi^+ cells undergo a programmed cell death, although it is unclear whether bacteria are killed through the direct involvement of the Abi or by the initiation of the phage lytic cycle (12, 29).

To date, 23 distinct Abi mechanisms have been characterized in *L. lactis* (12, 28). Overall, Abi proteins are a heterogeneous group with low identity among themselves or with proteins of known functions (other than phage resistance) in databases. Most Abi proteins are constitutively expressed, and their antiphage activity is generally mediated by one or a few genes (12). The common effects of most Abi mechanisms on the phage lytic cycle have been demonstrated, but the molecular mechanisms underlying their mode of action remain largely undefined (12).

The lactococcal AbiT mechanism was previously isolated from an *L. lactis* strain recovered from a raw milk sample (5). AbiT is active against virulent phages belonging to two of the three main lactococcal phage groups (936 and P335) and against some lactococcal phages from rare groups such as Q54 (23), 1706 (26), and 949 (56). The AbiT phage resistance phenotype is due to two genes that are constitutively expressed in the bacterial cell: *abiTi* and *abiTii*. The products of these two genes have only weak homology with proteins of unknown function in public databases, and there-

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TABLE 1 Plasmids, bacterial strains, and phages used in this study

Plasmid, bacterial strain, or phage	Relevant characteristic	Reference or source
Plasmids		
pNZ123	*E. coli-L. lactis* shuttle vector	19
pMIG3	*E. coli-L. lactis* shuttle vector	62
pETG-20A	*E. coli* expression vector	A. Geerlof
pED109	pMIG3 + AbiT	5
pED209	pNZ123 + AbiT	5
pNZ8010	Cm^r; 5.0 kb; pNZ123 derivative carrying the β-glucuronidase gene (*gusA*) from *E. coli* transcriptionally fused to the *nisA* promoter and a multiple-cloning site	17
L. lactis strains		
MG1363	Plasmid-free host of phage p2	27
IL1403	Plasmid-free host of phages bIL170 and P008	11
NZ9000	Strain MG1363 with *nisRK* genes integrated in the chromosome; host strain for nisin-inducible vectors	36
SMQ-946	*L. lactis* MG1363(pED209)	This study
SMQ-1090	*L. lactis* IL1403(pNZ123)	This study
SMQ-1091	*L. lactis* IL1403(pED109)	This study
SMQ-1092	*L. lactis* IL1403(pED209)	This study
SMQ-1105	*L. lactis* MG1363(pNZ123)	This study
SMQ-1177	*L. lactis* NZ9000(pNZ8010::$orf6_{p2}$)	This study
SMQ-1178	*L. lactis* NZ9000(pNZ8010::*orf5-*$orf6_{p2}$)	This study
Bacteriophages		
p2	*Siphoviridae*, small isomeric capsid, 936 species	50
P008	*Siphoviridae*, small isomeric capsid, 936 species	44
bIL170	*Siphoviridae*, small isomeric capsid, 936 species	16

fore, their molecular mechanism still remains unknown. Some of the effects of AbiT were previously assessed on the lytic cycle of phages p2 (936) and ul36 (P335). For both cases, phage DNA replication is decreased. With phage p2, maturation of genomic DNA is not achieved, leading to reduced encapsidation (5). The fate of infected lactococcal AbiT positive ($AbiT^+$) cells was also examined by electron microscopy, showing that capsid formation was delayed and that only empty capsids were formed. It was also shown that transcription of a phage early-expressed gene (*orf34*) and a late-expressed gene (*orf11*) was not affected by AbiT (5). Finally, it was recently evidenced that P335-like phage evolution was greatly influenced by the presence of AbiT and of host prophages since the virulent phage ul36 could exchange as much as 70% of its genome with prophages to overcome AbiT (39).

In this study, we selected three different phages of the 936 group to explore the AbiT mode of action: phage p2 (58) infecting *L. lactis* subsp. *cremoris* MG1363 and phages P008 (45) and bIL170 (16) infecting *L. lactis* subsp. *lactis* IL1403. Our main objective was to determine which phage genes/proteins are involved in the sensitivity to AbiT.

MATERIALS AND METHODS

Bacterial strains, phages, and plasmids. The biological materials used in this study are listed in Table 1. *L. lactis* strains were grown at 30°C in M17 broth (60) supplemented with 0.5% glucose (GM17). When needed, chloramphenicol was added at a final concentration of 5 μg/ml. All phages used in this study were obtained from the Félix d'Hérelle Reference Center for Bacterial Viruses (www.phage.ulaval.ca). Phages were propagated as previously described (28). The efficiency of plaquing (EOP) was measured by dividing the phage titer on the resistant strain by the phage titer on the sensitive strain, and the results are the means of at least three assays (49). When needed, phages were purified on a CsCl gradient as described elsewhere (38).

DNA isolation and sequencing strategy. Phage DNA was isolated as previously described (21). The genomes of three AbiT-resistant mutants, one derived from each of phages P008, bIL170, and p2, were sequenced with an ABI Prism 3700 apparatus by primer walking using phage genomic DNA as the template. All the sequencing was done at the Plateforme de Séquençage et de Génotypage des Génomes, Centre de Recherche du CHUL (CHUQ). The mutations in the other phages were identified using specific primers and PCR to amplify the target region. The PCR products were sequenced and compared to the sequence of the wild-type phage genome. The genome sequence of the wild-type AbiT-sensitive phages was also confirmed by sequencing the corresponding region.

Bioinformatics. Complete phage genome sequences were assembled using the Staden package (59) and aligned using the Clustal W2 (41) and BioEdit (30) programs. Translated open reading frames (ORFs) were analyzed with the National Center for Biotechnology Information (NCBI) PSI-BLAST program (1). The searches for conserved domains and functions were conducted using different approaches with the following software/websites: Jalview (14), conserved domain search (46), InterProScan (65), Protein Homology/analogY Recognition Engine (PHYRE) (2), and SMART (42).

Purification of ORF6. The *orf6* of phage p2 was cloned in the Gateway (Invitrogen)-compatible destination vector pETG-20A. The following primers have been used for the initial PCR: SiL270 (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCT**TA*GAAAACCTGTACTTCCAGGGTAATAAACCTGATTTAATCGAAAA***-3′) and SiL251 (5′-GGGGACCACTTTGTACAAGAAAGCTGGGT***TCATCA*TTATGAAACTGTAATTACTGCACC**-3′). The *attB* sequence is in bold, while the tobacco etch virus (TEV) protease recognition sequence is displayed in italic and the stop codons are underlined and in italics. The underlined TAs are supplementary bases to allow an in-frame gene fusion. This protocol fuses a 6×His tag and thioredoxin (Trx) to the N terminus of the protein. Constructs were trans-

ferred into the *Escherichia coli* T7 Express I^q competent pLysS expression strain (NEB). Prior to induction, *E. coli* was grown with shaking at 37°C in Turbo Broth medium (AthenaES) to an optical density at 600 nm (OD_{600}) of 0.8. Protein expression was induced by adding 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) and incubating overnight at 25°C. Cells were harvested by centrifuging for 10 min at 4,000 × *g*, and the pellet was resuspended in 40 ml lysis buffer (50 mM Tris, 500 mM NaCl, 10 mM imidazole, pH 8) per liter of culture supplemented with 0.25 mg/ml lysozyme, 20 μg/ml DNase I (Roche), 20 mM $MgSO_4$, and antiproteases (Roche), before being frozen at −80°C. Cells were thawed and lysed by sonicating on ice: 3 times for 30 s each time (output control = 5, duty cycle = 80%). The lysate was clarified by centrifuging for 30 min at 12,000 × *g* and by filtering through a 0.45-μm-pore-size filter. The proteins were purified by nickel affinity chromatography on a 5-ml HiTrap nickel affinity column (GE Healthcare) preequilibrated with 50 mM Tris, 500 mM NaCl, 10 mM imidazole, pH 8. ORF6 was eluted using a step gradient of imidazole (10 mM, 50 mM, and 250 mM). To reduce the amount of imidazole, the buffer was changed using a HiPrep 26/10 desalting column (GE Healthcare) preequilibrated with 50 mM Tris, 500 mM NaCl, 10 mM imidazole, pH 8. Then, the 6×His–thioredoxin tag was cleaved with TEV protease at a ratio of 1:10 (wt/wt) and incubated overnight at 4°C. The cleaved protein was recovered in the flowthrough of a 5-ml HiTrap nickel affinity column (GE Healthcare). Finally, the protein was further purified using a preparative HiLoad Superdex 200 gel (GE Healthcare) with 10 mM Tris, 500 mM NaCl, pH 8.

Detection of ORF6 by one-dimensional gel electrophoresis and Western blot assays during infection of *L. lactis* by phage p2. Cells of *L. lactis* SMQ-1105 (AbiT negative [$AbiT^-$]) and *L. lactis* SMQ-946 ($AbiT^+$) were grown in GM17 to an OD_{600} of 0.5 at 30°C, and each strain was infected with either the wild-type phage p2 or the AbiT-resistant mutant p2.t3 at a multiplicity of infection (MOI) of 1. Cells were sampled at 5-min intervals and flash-frozen at −80°C. Cell pellets were thawed and resuspended in SDS-PAGE loading buffer (62.5 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.05% bromophenol blue). An equivalent of 5×10^7 cells was loaded on 15% SDS-polyacrylamide gels. The proteins were electrotransferred (30 V) overnight (15 h) at 4°C onto a polyvinylidene difluoride membrane (Hybond P; GE Healthcare) using a Transblot apparatus (Bio-Rad Laboratories) and Tris-glycine-methanol buffer (25 mM Tris, pH 8.3, 192 mM glycine, 10% methanol). The efficiency of transfer was verified by staining the membrane with Ponceau S (0.1% [wt/vol] Ponceau S, 5% acetic acid). ORF6 was detected using protein A-purified polyclonal antibody raised against ORF6 in rabbits (Davids Biotechnologie GmbH). The membrane was blocked using phosphate buffer with 0.1% Tween 20 (PBST) and 5% (wt/vol) nonfat dry milk (NFDM) for at least 1 h with gentle shaking. The primary antibody, anti-ORF6, was diluted in blocking buffer (1:100,000, vol/vol) and incubated with the membrane with gentle shaking for 1 h. Unbound antibody was removed in three successive 15-min washes in PBST with gentle shaking. The secondary antibody (goat horseradish peroxidase-labeled anti-rabbit IgG; Rockland Immunochemical) was added at a concentration of 1:100,000 (vol/vol) to detect the primary antibody. After washing, membrane-bound signals were developed according to the manufacturer's instructions using an ECL-Plus detection kit (GE Healthcare) and detected on Biomax XR autoradiography film (GE Healthcare).

Detection of ORF6 by 2-DE during infection of *L. lactis* by phage p2. *L. lactis* SMQ-1105 ($AbiT^-$) and *L. lactis* SMQ-946 ($AbiT^+$) were grown in GM17 supplemented with 10 mM $CaCl_2$ until an OD_{600} of 0.2 was reached, and then phage p2 was added at an MOI of 5. Samples were taken at 5-min intervals and flash-frozen (−80°C). ORF6 was revealed by two-dimensional gel electrophoresis (2-DE) as previously described elsewhere (57).

Immunoelectron microscopy. For each step, the grids were incubated for 30 min at room temperature and excess liquid was removed using the side of a Whatman blotting paper. Immunoelectron microscopy was carried out with CsCl-purified phage p2 at 1×10^{12} PFU/ml. Fifteen microliters of 10-fold-diluted phage (20 mM Tris-HCl, 75 mM NaCl, 0.5% bovine serum albumin [BSA], 0.1% Tween 20, pH 8.0) was deposited on a nickel-Formvar-coated grid (200-mesh; Ted Pella). Then, the grids were blocked with 1% BSA and the primary antibody (1:32,500) was added: 10 μl of a 10-fold dilution or a 50-fold dilution for the anti-ORF11 and the anti-ORF6 antibodies, respectively. The grids were then washed by immersion in 30 ml of dilution buffer (3 times for 1 min each time), and 15 μl of immunoconjugate gold-labeled protein A (10 nm; Ted Pella), 10-fold diluted, was deposited on the grid. The grids were again extensively washed in dilution buffer, followed by distilled water. Finally, the samples were stained with 2% phosphotungstic acid (pH 7.0) and were observed with a JEOL 1230 transmission electron microscope.

Expression of ORF5 and ORF6. Genes encoding a protease (*orf5*) and the major capsid protein (*orf6*) of phage p2 were PCR amplified with their respective ribosome-binding site using high-fidelity *Pwo* DNA polymerase (Roche) and cloned into the nisin-inducible vector pNZ8010 (17). Plasmids pNZ8010::$orf6_{p2}$ and pNZ8010::$orf5\text{-}orf6_{p2}$ were introduced into *L. lactis* NZ9000 by electroporation (32). Bacterial strain NZ9000 containing either plasmid pNZ8010::$orf6_{p2}$ or pNZ8010::$orf5\text{-}orf6_{p2}$ was grown in 400 ml of GM17 until an OD_{600} of 0.5 was reached. Nisin was then added to a final concentration of 5.0 ng/ml, and incubation was prolonged for 3 h. Cells were harvested by centrifugation and resuspended in 30 ml of ice-cold 50 mM Tris-HCl, pH 8.5, 5 mM EDTA. Prohead preparations were produced as described elsewhere (20). Lysozyme was added (50 μg/ml), and the mixture was incubated for 1 h at 37°C. Following lysis, $MgSO_4$ (7.5 mM) and DNase I (20 g/ml) were added and the mixture was incubated for 1 h at 37°C. Cell debris was removed by centrifugation at 10,000 × *g* for 10 min. Proheads were precipitated after incubation in 6% polyethylene glycol 8000 and 0.5 M NaCl at 4°C overnight, followed by low-speed centrifugation at 10,000 × *g* for 10 min. The pellet was then resuspended in 4 ml of 1× phage buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 8 mM $MgSO_4$) for 1 h at 4°C. The procapsid suspension was then ultracentrifuged at 35,000 rpm (210,000 × *g*) in a swinging bucket rotor (SW41Ti; Beckman) on a CsCl gradient consisting of 5 steps of varying density (2, 7, 12, 17, and 22%). After ultracentrifugation, the pellet corresponding to the procapsids was kept and dialyzed three times against phage buffer. The procapsid preparation was stored at 4°C prior to electron microscopy observation. Preparations were observed by electron microscopy as described above.

RESULTS

Isolation and characterization of AbiT-resistant phage mutants. Lactococcal phage mutants resistant to AbiT were isolated in the laboratory from the wild-type phages p2, bIL170, and P008 that are sensitive to AbiT (Table 2). In total, 28 AbiT-resistant phage mutants were randomly selected for further studies, including 20 derived from bIL170 [bIL170.t(*x*)], 3 from p2 (p2.t1 to p2.t3), and 5 from P008 (P008.t1 to P008.t5). All phage mutants isolated in this study were resistant to AbiT, with EOPs ranging from 0.1 to 0.8 (Table 2). The genomes of three randomly selected phage mutants (p2.t3, P008.t3, and bIL170.t4) were sequenced. Those genomes were compared to the wild-type phage genomes, and the phage determinant(s) involved in the insensitivity to AbiT was identified.

The phage p2 genome contains 27,595 bp and 49 ORFs (GenBank accession number GQ979703 [58]). The phage P008 genome possesses 28,538 bp and 58 ORFs (GenBank accession number DQ054536 [45]), while the phage bIL170 genome is 31,754 bp long and encodes 64 ORFs (GenBank accession number AF009630 [16]). Each phage mutant had only a single nucleotide mutation, but these were in three different nonhomologous phage genes: *e14* (bIL170 [$e14_{bIL170}$]), *orf41* (P008 [$orf41_{P008}$]), and

TABLE 2 Characteristics of wild-type and mutant phages

Bacterial host strain	Phage[a]	Frequency (no. of phages/ total no. tested)	Mutation		EOP[b]
			Gene	Substitution(s)	
MG1363(pED209)	p2				$(3.7 \pm 0.7) \times 10^{-7}$
	p2.t2	1/3	*orf6*	T221I + P341S	0.5 ± 0.2
	p2.t3	2/3	*orf6*	P341T	0.3 ± 0.1
IL1403(pED109)	P008				$(2.8 \pm 1.3) \times 10^{-6}$
	P008.t2	3/5	*orf41*	I95N	0.8 ± 0.1
	P008.t3	2/5	*orf6*	A291V	0.4 ± 0.2
IL1403(pED209)	bIL170				$(4.2 \pm 0.6) \times 10^{-3}$
	bIL170.t22	1/20	*e14*	M1I (ATG-ATA)	ND[c]
	bIL170.t4	4/20	*e14*	E6G	0.5 ± 0.1
	bIL170.t13	2/20	*e14*	V14M	0.1 ± 0.1
	bIL170.ct8	2/20	*e14*	D23G	0.2 ± 0.0
	bIL170.t30	1/20	*e14*	V35A	0.1 ± 0.0
	bIL170.ct5	1/20	*e14*	P38L	0.1 ± 0.0
	bIL170.t5	3/20	*e14*	D40G	0.5 ± 0.1
	bIL170.t6	3/20	*e14*	E44K	0.4 ± 0.1
	bIL170.t20	2/20	*e14*	E44G	0.1 ± 0.0
	bIL170.t18	1/20	*e14*	A59D	0.1 ± 0.1

[a] Phages in boldface are those for which the genomes were completely sequenced.
[b] EOP values are the means of at least 3 replicates.
[c] ND, not determined.

orf6 (p2 [$orf6_{p2}$]). The mutations in the 25 other phage mutants were in the same three genes, as determined by sequencing these specific phage genomic regions. All three p2 phage mutants and two out of five P008 phage mutants had a mutation in the *orf6* gene ($orf6_{p2}$ and $orf6_{P008}$). This gene belongs to the late-transcribed module and likely codes for a phage structural protein (9). The other three P008 mutants had changes in the *orf41* gene ($orf41_{P008}$), which codes for a protein of unknown function produced early in phage infection (8). Finally, all mutations (20/20) found in phage bIL170 were in the *e14* gene ($e14_{bIL170}$), another early-expressed gene coding for a protein of unknown function. All mutations led to an amino acid change (Table 2).

Bioinformatic characterization of early-expressed protein $E14_{bIL170}$ and $ORF41_{P008}$. The phage proteins E14 of bIL170 and ORF41 of P008 are small polypeptides of 71 amino acids (aa) and 108 aa, respectively. The $e14_{bIL170}$ gene is highly conserved in all phages of the 936 species and is found in the proposed core genome for these lactococcal phages (55). On the other hand, the $orf41_{P008}$ gene is less conserved and is present in only 5 (P008, 712, p2, sk1, and jj50) of the 12 936-like phage genomes analyzed (55). No putative function was attributed to the gene products $E14_{bIL170}$ and $ORF41_{P008}$. Bioinformatics analyses gave no significant hit for $ORF41_{P008}$. In contrast, the PHYRE structure/function prediction for E14 points toward a DNA transposase (31) but with a weak estimated precision of 10%. PHYRE also detected homology with the rotavirus NSP3 protein (52) (5% precision), a protein involved in mRNA translation that also has an RNA binding motif. The precision of these predictions was too low to determine the exact function of E14. However, the position of the gene in the phage genome suggests that it may be involved in DNA metabolism or transcription regulation.

Bioinformatic characterization of $ORF6_{p2}$. The $orf6_{p2}$ gene codes for a 393-aa protein (44 kDa, pI 5.5) also included in the core genome for the lactococcal phages of the 936 species (55) and is highly conserved (>80% sequence identity). Others have previously identified this protein as a phage structural protein (13, 34, 35), but its location in the virion structure is not clear. PSI-BLAST analysis of the ORF6 protein detected weak homology with the coliphage HK97 major capsid protein (MCP; gp5). This is in agreement with the conserved domain search, which identified a relationship with the MCP/gp5 protein superfamily (SSF56563). These analyses suggest that ORF6 is the major capsid protein of phage p2. However, previous studies annotated the ORF11 as the MCP of 936-like lactococcal phages (13, 34, 35). This annotation was primarily based on the prominence of ORF11 in the structure of the lactococcal phage F4-1. A recent study on the structural proteome of lactococcal phage p2 also showed that ORF11 is a major structural protein, while ORF6 was found in multiple minor bands (58).

It was previously shown that the morphogenesis module of lactococcal phages of the 936 species can be successfully aligned with the bacteriophage lambda structural and packaging genes (9). The comparison of the genome organization of phage p2 with that of phage lambda and HK97 revealed that $orf11_{p2}$ occupied the same position as the gene coding for the major tail protein of phage lambda (Fig. 1). On the basis of the observations made above and its location in the genome, $orf6_{p2}$ likely encodes the major capsid protein of lactococcal phage p2 (Fig. 1).

ORF6 is a capsid protein of phage p2. To assess if $ORF6_{p2}$ was indeed the MCP, we conducted immunoelectron microscopy assays using specific rabbit polyclonal antibodies raised against purified $ORF6_{p2}$. Figure 2 clearly shows that anti-ORF6 antibodies target the capsid of phage p2, while the antibodies raised against $ORF11_{p2}$ are binding the tail of the phage. Similar to *E. coli* phage HK97 (54), the capsid protein of phage p2 does not appear as a major protein band on SDS-PAGE (58). Detailed analysis of the *orf6* gene sequence showed the presence of two ATG start codons, both preceded by conserved ribosome-binding sites. These pro-

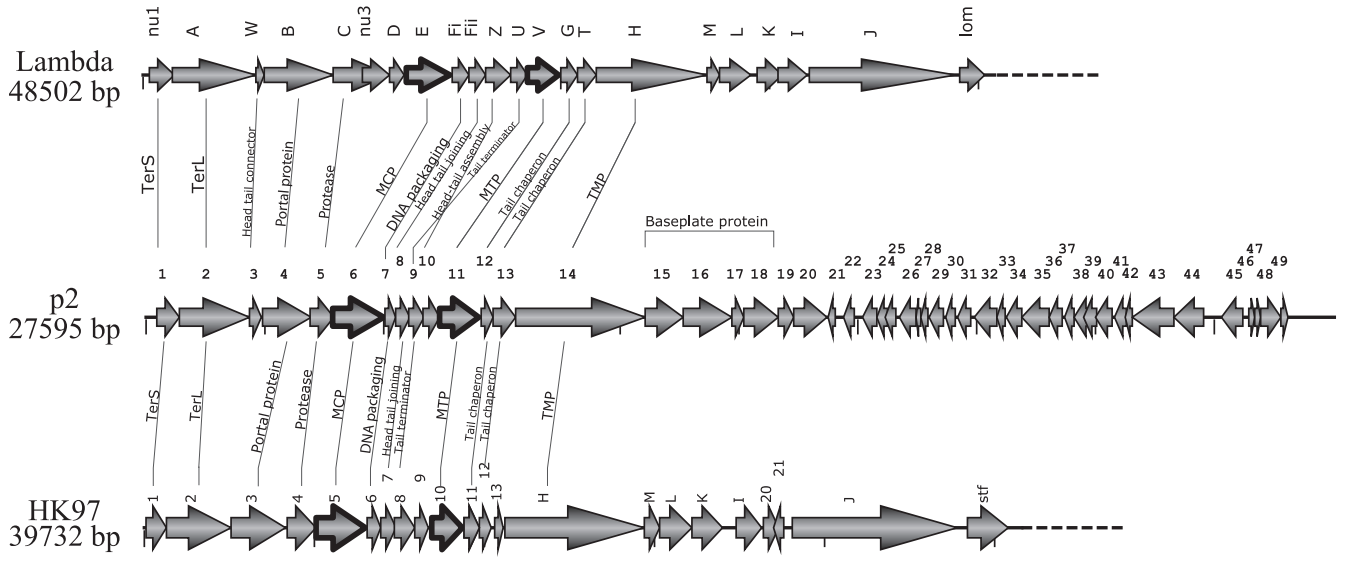

FIG 1 Alignment of phages lambda, p2, and HK97 on the basis of the genomic synteny of these phages. Arrows in bold represent ORF6 and ORF11 of phage p2 as well as respective orthologs. TMP, tape measure protein; TerS, terminase small subunit; TerL, terminase large subunit; MTP, major tail protein.

teins would correspond to truncated (ORF6Δ75, 35-kDa, pI 5.9) and full-length (44-kDa, pI 5.5) versions of ORF6. Of note, only the truncated version (ORF6Δ75) of the protein was previously detected in *L. lactis* cells infected with phage F4-1 (35). However, a phage genome fragment cloned into *E. coli* and *L. lactis* led to the production of two forms (35). We also carefully analyzed the phage genome sequence for the presence of a frameshift that could lead to different variants of the capsid protein, as previously observed in phage A2 (25), but we could not identify any slippery sequences.

Expression of ORF6$_{p2}$ during infection of *L. lactis* cells. Polyclonal antibodies raised against purified ORF6$_{p2}$ were used to detect the intracellular production of this protein in p2-infected *L. lactis* cells during a time course experiment. Western blot analysis detected at least five different forms of ORF6$_{p2}$ during the infection of the phage-sensitive strain *L. lactis* MG1363 (Fig. 3A). Three forms were predominant (33 kDa, 39 kDa, and 50 kDa), while two others were at lower concentrations (35 kDa and 48 kDa). The same time course experiment was performed with phage-infected AbiT-containing cells, and production of ORF6$_{p2}$ could not be detected (Fig. 3B). Interestingly, the purified ORF6$_{p2}$ overexpressed in an *E. coli* culture yielded two forms (39 kDa, 50 kDa; Fig. 3).

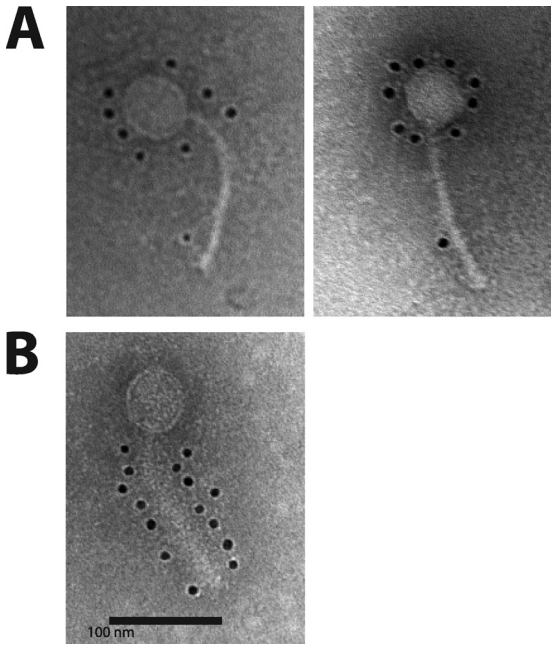

FIG 2 Immunoelectron microscopy of phage p2 with antibodies specific for the major capsid protein (ORF6) (A) and the major tail protein (ORF11) (B).

Then, we performed a similar experiment but performed it using the AbiT-resistant mutant p2.t3, which has a mutation in *orf6* leading to an amino acid change in the C-terminal part of the capsid protein (P341T). Again, the five forms of ORF6 were detected during the infection of wild-type strain *L. lactis* MG1363 (Fig. 3C). Interestingly, the five forms of ORF6 were also detected at similar levels during a p2.t3 infection of AbiT-containing cells (Fig. 3D). Of note, the mature phage p2 contains only one variant of the ORF6 protein, corresponding to the band of approximately 35 kDa (Fig. 3C and D).

Finally, the production of ORF6$_{p2}$ was also investigated using 2-DE analysis during the infection of the phage-sensitive strain *L. lactis* MG1363. Five different spots not present in noninfected *L. lactis* cells were identified as different variants of ORF6$_{p2}$ by Western blotting (Fig. 4), and three of these spots were confirmed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Fig. 4, spots 1 to 3). Peptide coverage analysis of spot 1 (estimated size, 48 kDa; pI 5.7) indicates that the protein is complete, while spots 2 (37 kDa, pI 6.0) and 3 (30 kDa, pI 6.0) contain peptides partially covering the protein sequence, suggesting that the translation may begin at two initiation sites or that ORF6 undergoes a posttranslational cleavage.

Procapsid assembly. To understand the origin of the different ORF6$_{p2}$ variants detected, *orf6*$_{p2}$ was cloned in *L. lactis* expression

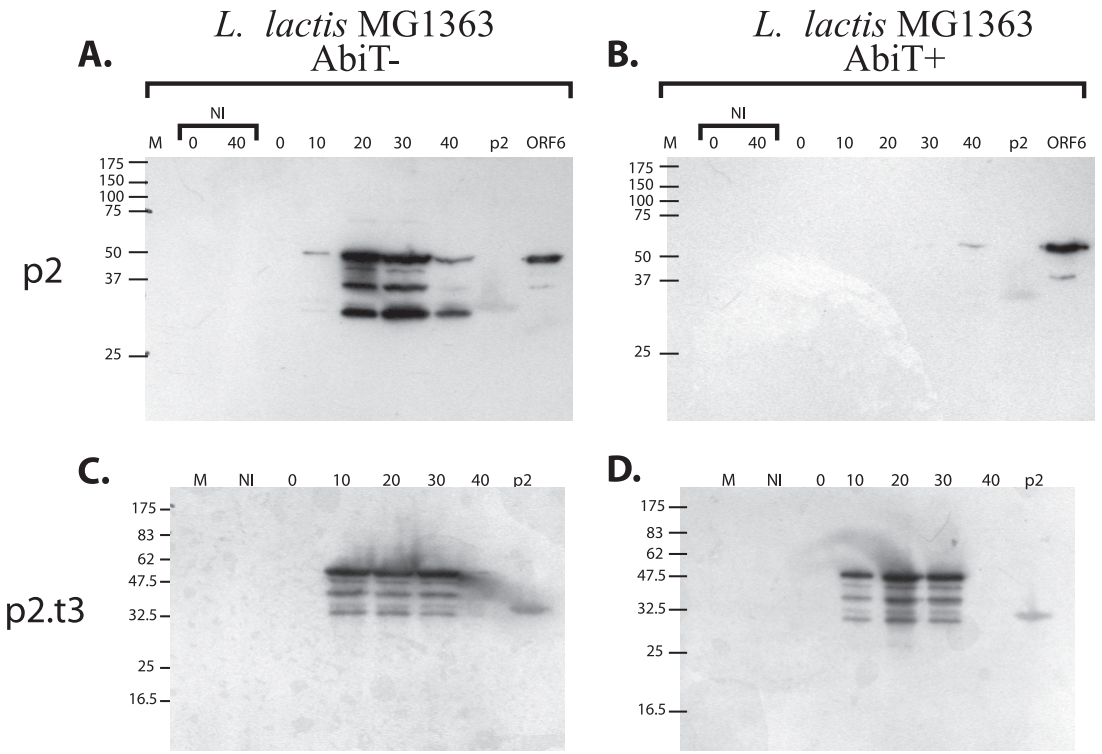

FIG 3 Western blot detection of ORF6 during time course infection of 40 min (lanes 0 to 40). (A) Phage p2 without AbiT; (B) phage p2 with AbiT; (C) phage p2.t3 without AbiT; (D) phage p2.t3 with AbiT. Lanes: M, molecular size marker (in kilodaltons); NI, not infected; p2, CsCl-purified p2; ORF6, purified ORF6.

vector pNZ8010. In parallel, *orf6* was also cloned with the gene *orf5*, which is a putative protease involved in the maturation and assembly of procapsids (64). When ORF6 is expressed alone, two bands are detected by Western blot analysis of the cell extracts (Fig. 5A). These two bands correspond to the full length of the protein (43.5 kDa) and the shorter version of the protein that is translated from the dual start at position 226 in the gene sequence (ORF6Δ75). When gene *orf6* is clone with *orf5* and coexpressed, one more band corresponding to the cleaved protein with an estimated molecular mass of 33 kDa is detectable by Western blotting. According to the peptide coverage, the protein is shorter by approximately 125 aa (Fig. 5B).

The cell extracts containing the overexpressed ORF6 and ORF5-ORF6 were purified on a cesium chloride gradient. The fractions were observed under an electron microscope, and spherical procapsids were mainly observed in the purified fraction when ORF6 was coexpressed with the protease ORF5 (Fig. 5D). Some procapsids could be observed in the purified fraction containing only ORF6, but they were more sparsely distributed (Fig. 5C).

DISCUSSION

Abortive infection mechanisms are one of the many strategies that bacteria use to fight phage infections. Despite their widespread distribution in numerous bacterial genera and their relative efficacy in controlling phage populations (39), their mode of action still remains poorly understood. The aim of this study was to shed further light on the lactococcal phage resistance mechanism AbiT by identifying phage genes involved in the sensitivity. Previous studies have shown that AbiT affected phage DNA replication and delayed the production of structural proteins without disturbing transcription (5) and that the target or activator of AbiT might be located in the late-expressed gene module (39).

AbiT-resistant phage mutants were first isolated using three different phage-host systems. Following phage genome sequencing and comparative analyses, three distinct phage genes involved in AbiT activity were identified: *orf6* of p2 and P008, *e14* of bIL170, and *orf41* of P008. The identification of three distinct phage genes in different phage genomic regions was unexpected. A similar approach used to characterize phage mutants resistant to another abortive infection system, namely, AbiK, led to the identification of a phage gene (named *sak*) localized in the same genomic region in different phages (6, 53). Similar findings were also obtained when characterizing phage mutants resistant to AbiV, as all mutations were in homologous genes (29). Strikingly, the three gene products involved in the sensitivity to AbiT are different: $e14_{bIL170}$ and $orf41_{P008}$ are located in the early-expressed modules, whereas $orf6_{p2}$ and $orf6_{P008}$ are found in the morphogenesis module and is expressed later during the phage infection. The functions of $E14_{bIL170}$ and $ORF41_{P008}$ are still unknown, but the former protein is highly conserved among lactococcal phages of the 936-like species, while the latter is not found in all species members. As only three p2 AbiT-resistant mutants were isolated and characterized, it is still possible that another gene(s) could be involved in the AbiT mechanism. In contrast, all 20 bIL170 AbiT-resistant mutants isolated have a mutation in the *e14* gene; thus, it is probably the sole phage gene involved.

We demonstrated by immunoelectron microscopy that $ORF6_{p2}$ is a capsid protein of phage p2. Bioinformatic analysis revealed that $ORF6_{p2}$ had low homology to the MCP/gp5 of coliphage HK97 (data not shown). The gp5 protein self-assembles

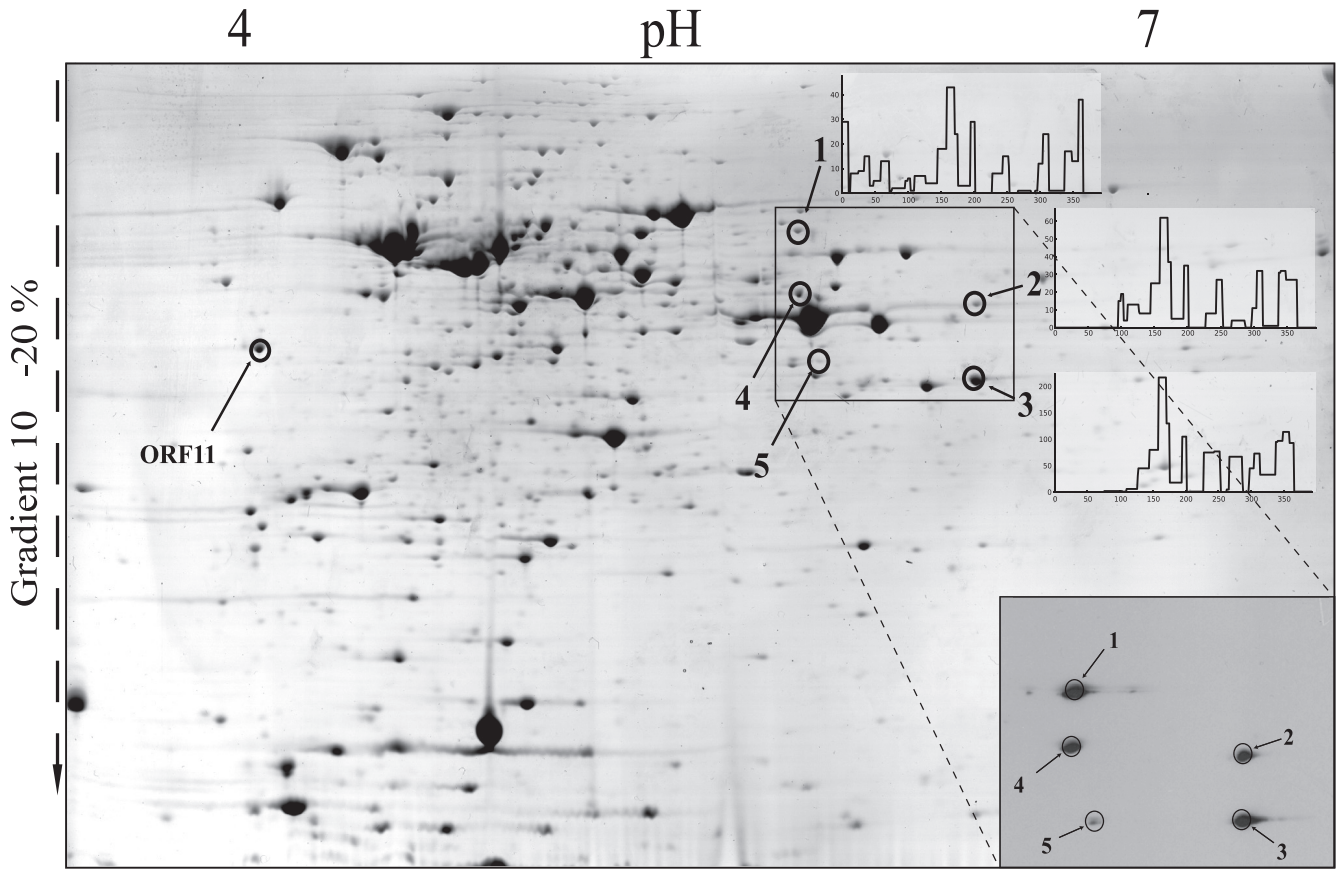

FIG 4 2-DE analysis of cells infected with phage p2 after 15 min of infection without AbiT. ORF6 was identified in five different spots (1 to 5). The plots presented as insets show the peptide coverage of the protein found in the spot. (Inset, lower right) Western blot analysis of the 2-DE gel using anti-ORF6 antibodies.

via covalent cross-linking to form the procapsid (54). The procapsid then matures via proteolytic cleavage performed by the gp4 protease. When analyzed by SDS-PAGE, gp5 of HK97 did not appear to be a major phage structural component, as observed for other lambda-like phages (37, 38, 43). While a large amount of gp5 failed to enter the gel, it also appeared as several minor bands (54). Similarly, ORF6$_{p2}$ was also found in minor bands. As MCP is often the most abundant protein in the phage structure, this led to an incorrect annotation of this protein in the early 1990s (34). The function of MCP was previously attributed to *orf11* for 936-like lactococcal phages, since its gene product was the major constituent detected by SDS-PAGE. We confirmed by immunoelectron microscopy using anti-ORF11$_{p2}$ antibodies that ORF11 is, in fact, the major tail protein.

Capsid assembly has been studied for many double-stranded DNA phages. The basic structure of the capsid of these phages consists of an icosahedral assembly of many copies of a unique major protein and few minor subunits (20). The assembly of the capsid often requires the presence of scaffolding proteins (7), but phage HK97 does not require scaffolding proteins. The gp5 MCP has been shown to self-assemble using its amino terminus (delta domain, the 102 residues from the N-terminal domain), which facilitates and guides the assembly of procapsids (prohead-I) (15, 61). Procapsid-I undergoes maturation following removal of the delta domains by the protease gp4 to form procapsid-II, which is metastable. We demonstrated that ORF6 of phage p2 can also self-assemble when expressed in *L. lactis*. Thus, the N-terminal domain also probably acts as a scaffolding protein. As it was difficult to find assembled procapsid by electron microscopy, ORF6 subunits alone are likely unstable or dissociated during purification. Conversely, coexpression of ORF6 and ORF5 led to formation of numerous and seemingly more stable capsid-like structures.

Different forms of ORF6$_{p2}$ were detected using various techniques. SDS-PAGE of purified phage p2 coupled with LC-MS/MS analysis led to the identification of four forms of ORF6$_{p2}$ (data not shown). Similarly, 2-DE of the intracellular content of phage-infected *L. lactis* cells and LC-MS/MS analysis identified ORF6$_{p2}$ in five new protein spots. The expression of ORF6$_{p2}$ during the infection was also monitored by Western blotting, and five ORF6$_{p2}$ variants could also be detected with polyclonal antibodies. The largest variant corresponds to the complete ORF6 (393 aa). A second band is a variant translated from the second methionine at positions 226 to 228 in the gene sequence, preceded by a conserved ribosome-binding site (ORF6Δ75 to 318 aa). To our knowledge, this is the first report of a dual-start motif for a capsid protein. Although we do not know the function of this dual-start motif, we suggest that it might be involved in the timing and the regulation of the capsid assembly. The third band is the shorter variant of the protein that has been cleaved by the protease. Two

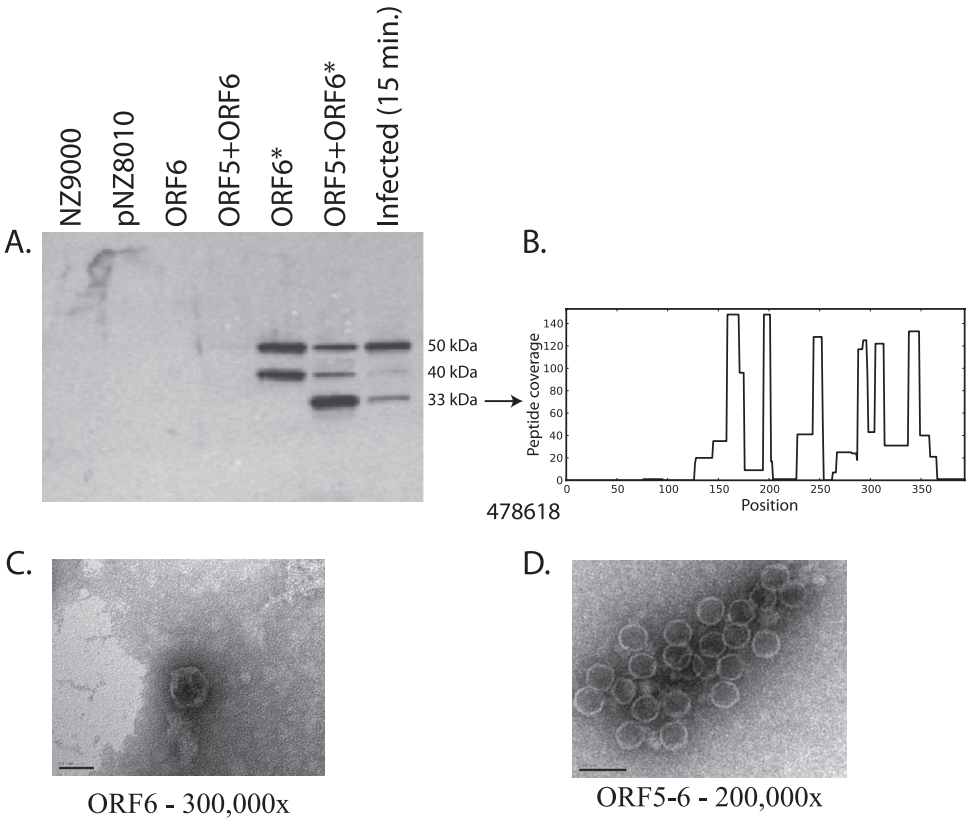

FIG 5 The major capsid protein ORF6$_{p2}$ is cleaved by the protease ORF5$_{p2}$. (A) A Western blot experiment was conducted with rabbit anti-ORF6$_{p2}$ antibodies and cytosolic proteins extracts of wild-type *L. lactis* NZ9000, *L. lactis* NZ9000 containing plasmids pNZ8010, pNZ8010::*orf6*$_{p2}$, or pNZ8010::*orf5 orf6*$_{p2}$ induced with 0.0 ng/ml or 5.0 ng/ml (*) of nisin A, and *L. lactis* NZ9000 infected with phage p2 for 15 min; (B) peptide coverage from mass spectrometry of the smallest form of ORF6 (33 kDa); (C) electron microscopy observation of purified ORF6 at ×300,000 magnification; (D) electron microscopy observation of purified ORF5 and ORF6 at ×200,000 magnification.

other variants of ORF6 are also observed during the infection of *L. lactis* by phage p2, but their presence cannot be explained to date. Of note, the mutations found in AbiT-resistant p2 phages are all in the C-terminal domain, which is conserved in the structure of the capsid. Finally, only one form of the ORF6 was detected by Western blot analysis of the mature purified phage p2. The discrepancy between the numbers of bands detected by Western blotting and by LC-MS/MS is also likely due to the higher sensitivity of the latter method. Moreover, as observed for gp5 of HK97, it is likely that many ORF6 proteins are cross-linked in large complexes that do not migrate in SDS-PAGE.

Two Abi systems from *E. coli*, Lit and PifA, involve the phage major capsid protein. Lit is encoded by a defective prophage in *E. coli* and is activated late in the infectious cycle by the 29-amino-acid Gol peptide, found in the major capsid protein (3). Once activated, Lit cleaves the elongation factor EF-Tu, stopping protein synthesis and leading to cell death and abortion of infection. The mutations found in the *orf6* gene in this study span a region of 120 aa, likely excluding the possibility that AbiT is activated by a peptide.

PifA is an F-plasmid-associated membrane protein that blocks infection of some coliphages (10). To overcome PifA, T3- and T7-like phages must have a mutation in the *gp1.2* gene or a double mutation in the *gp10* gene (51). The *gp1.2* gene codes for a dGTP triphosphohydrolase necessary for phage replication, whereas *gp10* codes for MCP (24). When activated, PifA severely reduces macromolecule synthesis, phage DNA replication is reduced, and the bacterial chromosome is degraded. Furthermore, membrane integrity is altered, leading to leakage of ATP and other molecules. It is noteworthy that AbiT also reduces phage replication as well as prevents phage genomic maturation (5).

In conclusion, three phage genes involved in the AbiT mode of action were identified. Two are early-expressed genes of unknown function, while the third one (*orf6*) codes for the major capsid protein. We have shown that ORF5 and ORF6 are involved in the formation of procapsids and that AbiT blocks capsid protein synthesis of the wild-type phage, while it does not affect the resistant phage p2.t3, mutated in the capsid gene.

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