Microbiological and molecular impacts of AbiK on the lytic cycle of Lactococcus lactis phages of the 936 and P335 species

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The lactococcal abortive infection mechanism AbiK was previously shown to be highly effective against the small isometric-headed bacteriophage ul36 of the P335 species, as evidenced by an efficiency of plaquing (e.o.p.) of 10−5, a 14-fold reduction in the burst size and an efficiency at which centres of infection form (e.c.o.i.) of 0.5%. No phage DNA was detected in the infected AbiK+ cells [Émond, É., Holler, B. J., Boucher, I., Vandenberghe, P. A., Vedamuthu, E. R., Kondo, J. K. & Moineau, S. (1997). Appl Environ Microbiol 63, 1274–1283]. Here, the effects of AbiK are compared on the small isometric-headed phages p2 and P008 (936 species) and on the phage P335 (P335 species). The microbiological impacts of AbiK on p2 were relatively similar to those reported for ul36, with an e.o.p. of 10−5, an 11-fold reduction in the burst size and an e.c.o.i. of 5%. Contrary to phage ul36, replication of phage p2 DNA was observed in the AbiK+ cells. Only immature forms (concatemeric and circular DNA) of phage p2 DNA were found, indicating that the presence of AbiK prevented phage DNA maturation. These distinct molecular consequences of AbiK were also observed for phages P335 and P008, two phages that propagate on the same host. To the knowledge of the authors, this is the first time that different phage responses towards an Abi system have been reported.

Keywords: bacteriophages, abortive infection mechanism, Lactococcus

INTRODUCTION

Lactococcus lactis is a Gram-positive lactic acid bacterium used to manufacture fermented dairy products such as sour cream, buttermilk and cheeses. The acidification process in large-scale milk fermentation can be delayed due to lytic phages present in pasteurized milk and in the manufacturing environment (Dinsmore & Klaenhammer, 1995). Lactococcal phages are classified into 12 genetically distinct groups (Jarvis et al., 1991), but only three of them (936, P335 and c2 species) are repeatedly isolated in dairy plants worldwide (Moineau et al., 1992, 1996). The three species are members of the Siphoviridae family (with a non-contractile tail), which also includes the coliphage lambda (Pringle, 1996). Phages of the 936 and P335 species have small isometric heads, whereas the c2-like phages have a prolate head. Many bacterial strains carrying defence systems against these phages have been identified (Allison & Klaenhammer, 1998).

Abortive infection mechanisms (Abi) form a particular class of natural anti-phage systems. These phage resistance mechanisms are generally plasmid-encoded and act after phage adsorption, DNA penetration and early gene expression. Their intracellular activities result in reduced burst size, efficiency of plaquing (e.o.p.) and efficiency at which centres of infection form (e.c.o.i.). One of the distinctive aspects of Abi systems is the massive cellular death observed in infected cells (Durmaz et al., 1992; Geis et al., 1992; Sing & Klaenhammer, 1990). To date, 17 Abi systems have been cloned and sequenced in L. lactis.

The first lactococcal Abi system, AbiA, was isolated (Klaenhammer & Sanozky, 1985) from the highly

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Abbreviations: c.o.i., centres of infection; e.c.o.i., efficiency at which centres of infection form; e.o.p., efficiency of plaquing.
resistant industrial strain *L. lactis* ME2 (Klaenhammer, 1989; Hill et al., 1990). In P335-infected AbiA+ cells, phage DNA replication and major capsid protein production are absent (Hill et al., 1991; Moineau et al., 1993). Site-directed mutagenesis of a leucine repeat motif in AbiA showed that this structure is essential to the phage resistance phenotype (Dinsmore et al., 1998). A specific DNA fragment isolated from a mutant phage, insensitive to AbiA, reduced the resistance phenotype towards the wild-type phage when provided in trans in the AbiA+ cells (Dinsmore & Klaenhammer, 1997). A similar phenomenon was observed for AbiD1 (Bidnenko et al., 1995), which contains two helix–turn–helix motifs in its sequence (Anba et al., 1995). A helix–turn–helix motif was also identified at the N-terminal end of AbiJ (Deng et al., 1997). Amino acid motifs characteristic of DNA-binding functions were not detected in AbiD and AbiI. However, homologies were found between AbiD1 and AbiF (47% identity), between AbiD and AbiD1 (28% identity), and between AbiD and AbiF (26% identity) (Garvey et al., 1995; McLandsborough et al., 1995).

The genetic determinants of AbiB (Cluzel et al., 1991), AbiH (Prévots et al., 1996) and AbiN (Prévots et al., 1998) are encoded on the chromosome. The action of AbiB is possibly induced by an early phage transcript and leads to the degradation of phage mRNAs 10–15 min after infection (Parreira et al., 1996). For AbiE (Garvey et al., 1995), AbiG (O’Connor et al., 1996) and AbiL (Deng et al., 1999), a DNA region containing two ORFs is associated with the phage resistance phenotype. The requirement of the two peptides to generate full resistance has yet to be established in the case of AbiE. Two putative integral membrane-spanning helices were identified near the N-terminal end of AbiC (Durmaz et al., 1992). This system caused a reduction in the synthesis of the major capsid protein of a P335 phage (Moineau et al., 1993). The AbiQ mechanism appears to result in defective assembly of 936 and c2 phages (Emond et al., 1998). Three other Abi genes were also sequenced, namely, Abi1 (Su et al., 1997), AbiO (Prévots & Ritzenthaler, 1998) and AbiP (GenGank accession no. U90222).

In a previous study, we showed that plasmid pSRQ800 coded for the phage abortive infection system AbiK and conferred strong resistance against small isometric phages of the 936 and P335 species (Emond et al., 1997). A database query, based on amino acid composition, suggested that AbiK and AbiA might be in the same protein family. Furthermore, as with AbiA, replication of phage u136 (P335 species) DNA and production of its major capsid protein were not observed in the AbiK+ infected cells (Emond et al., 1997). The characterization of Abi systems is generally performed using only one phage species and it is generally assumed that the observations will be similar towards the other phage species. Here, we report distinct observations on the lytic cycle of 936 and P335 phages resulting from the presence of AbiK.

**METHODS**

**Bacterial strains, phages and media.** The bacterial strains and phages used in this study are listed in Table 1. *Lactococcus* strains were grown at 30 °C in M17 broth containing 0·5 % glucose (GM17) and supplemented, when required, with 5 μg erythromycin or chloramphenicol ml−1.

**Bacteriophage propagation and assays.** High phage titre was obtained by the method of Jarvis (1978). Phage p2K, a variant of phage p2, was isolated from a single plaque (Moineau et al., 1994) obtained by infecting AbiK+ cells with a m.o.i. of 5. e.o.p. was assessed as described by Sanders & Klaenhammer (1980). One-step growth curves and centres of infection (c.o.i.) were assayed as described previously (Moineau et al., 1993). The phage burst size was estimated as the ratio between the phage titre at two consecutive latent phases on the growth curves. The c.e.o.i. was calculated by dividing the number of c.o.i. on the resistant strain by the number of c.o.i. on the sensitive strain × 100. In coinfection assays, phages p2 and p2k were simultaneously added at a m.o.i. of 1.

**DNA isolation and manipulation.** Routine DNA manipulations were carried out as described by Sambrook et al. (1989). Phage DNA was isolated according to the method of Chibani Azaiez et al. (1998) with the following modifications: concentrated phages were treated with proteinase K (1 mg ml−1) for 1 h at 37 °C. Phenol/chloroform (24:1, v/v) extractions were repeated until a white interface was absent. DNA was precipitated using 2 vols ethanol and dissolved in 50 μl H2O. Purified DNA was digested with restriction enzymes as recommended by the manufacturer (Boehringer Mannheim).

**Phage p2 restriction map.** Phage p2 DNA was digested with the restriction enzymes EcoRI, EcoRV, HindIII, Ncol, SalI, SeaI and SphI in single and double digestions. Restriction fragments were separated by electrophoresis on agarose gel, stained with ethidium bromide and photographed under UV illumination. The profiles were used to establish the EcoRV restriction map.

**Phage DNA replication.** Intracellular phage DNA replication was monitored by the method of Hill et al. (1991). In short, total DNA was isolated from cultures infected at a m.o.i. of 1, digested with EcoRV and heated at 65 °C for 10 min. The heat treatment prior to migration on gel allowed the identification of the fragments carrying the cos sites. The fragments were then electrophoretically separated on a 0·8 % agarose gel. After ethidium bromide staining, DNA was visualized under UV and photographed. For phage p2, DNA was transferred to Hybond-N nylon membranes (Amersham) by capillary blotting (Southern, 1975). A probe was prepared by labelling the EcoRV fragments from the phage p2 genome with the DIG-High Prime Kit (Boehringer Mannheim). Prehybridization, hybridization and posthybridization washes as well as detection were performed as directed by the manufacturer. The standard hybridization buffer (50% formamide) and CSPD detection were performed as directed by the manufacturer.

**Electron microscopy.** Phage morphology was observed as described previously (Moineau et al., 1992). The lytic cycle was monitored by electron microscopy according to the following protocol. The sensitive and resistant hosts were grown in GM17 to an optical density of 0·5. Calcium chloride
**Table 1.** Bacterial strains, bacteriophages and plasmids used in this study

<table>
<thead>
<tr>
<th>Bacterial strain, bacteriophage or plasmid</th>
<th>Relevant characteristics*</th>
<th>Source†</th>
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<tbody>
<tr>
<td>L. lactis subsp. cremoris</td>
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<tr>
<td>LM0230</td>
<td>Plasmid-free, host for phage p2</td>
<td>McKay et al. (1972)</td>
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<tr>
<td>SMQ-16</td>
<td>LM0230(pSA3), Tc- Em- AbiK+</td>
<td>Moineau et al. (1995)</td>
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<tr>
<td>SMQ-38</td>
<td>LM0230(pSRQ802), Tc- Em- AbiK+</td>
<td>Emond et al. (1997)</td>
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<tr>
<td>L. lactis subsp. lactis</td>
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<tr>
<td>F4/2</td>
<td>Host for phages P008 and P335</td>
<td>H. W. Ackermann†</td>
</tr>
<tr>
<td>SMQ-463</td>
<td>F4/2(pSRQ817), Cm- AbiK+</td>
<td>This study</td>
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<tr>
<td>SMQ-501</td>
<td>F4/2(pSRQ830), Cm- AbiK-</td>
<td>This study</td>
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<tr>
<td>Bacteriophages</td>
<td></td>
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<tr>
<td>p2</td>
<td>936 species, AbiK+</td>
<td>T. R. Klaenhammer*</td>
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<tr>
<td>p2K</td>
<td>936 species, variant of phage p2, AbiK+</td>
<td>This study</td>
</tr>
<tr>
<td>P008</td>
<td>Type phage, 936 species, AbiK+</td>
<td>H. W. Ackermann†</td>
</tr>
<tr>
<td>P335</td>
<td>Type phage, P335 species, AbiK+</td>
<td>T. R. Klaenhammer*</td>
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<tr>
<td>Plasmids</td>
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<tr>
<td>pSRQ802</td>
<td>4·5 kb EcoRI fragment from pSRQ800 cloned into pSA3, Tc- Em- AbiK+</td>
<td>Émond et al. (1997)</td>
</tr>
<tr>
<td>pSRQ817</td>
<td>3·8 kb EcoRI–PvuII fragment from pSRQ800 cloned into pMIG3, Cm- AbiK+</td>
<td>Émond et al. (1997)</td>
</tr>
<tr>
<td>pSRQ830</td>
<td>Frameshift mutation (BstEII/Klenow) of abiK in pSRQ817</td>
<td>This study</td>
</tr>
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</table>

*TC, tetracycline; Em, erythromycin; Cm, chloramphenicol; AbiK, abortive infection mechanism.
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was then added at a final concentration of 10 mM, followed by phage p2 at a m.o.i. of 1. The infected cells were incubated at 30 °C. At various time points, 1·7 ml culture was transferred to an Eppendorf tube and cells were fixed using a modification of the method of Strausbauch et al. (1985). The cell pellets were resuspended in a glutaraldehyde solution (2% in 0·1 M sodium cacodylate, pH 7·3) and incubated at room temperature for 1·5 h. The cells were centrifuged and the pellets were embedded in 3% agar dissolved in 0·1 M sodium cacodylate. After gelification on ice, samples were cut into 1–2 mm3 pieces with a razor blade, rinsed five times in cacodylate buffer and post-fixed in 1% OsO4 in water for 1·5 h at room temperature. The samples were rinsed in water, gradually dehydrated in increasing concentrations of ethanol (10–100%) and embedded in Spurr resin. After sectioning, the specimens were observed with a transmission electron microscope Phillips 420 at 80 kV.

**RESULTS**

**Effects of AbiK on phage development**

Three parameters (e.o.p., e.c.o.i. and burst size) were used to evaluate the effectiveness of this anti-phage system on the lactococcal phage p2 of the 936 species (Table 2). AbiK had a strong inhibitory effect on the p2 lytic cycle as the values of all parameters tested were dramatically reduced. In the presence of this abortive infection mechanism, the release of progeny phages by the infected cells (e.c.o.i.) was decreased 20-fold and the burst size was lowered more than 11-fold. These effects led to an e.o.p. of 10−6. A variant of phage p2, p2K, which showed a strongly reduced sensitivity to AbiK was also isolated. Phage p2K had the same morphology and DNA restriction pattern as the wild-type phage p2 (data not shown). The e.o.p. and burst size of this mutant phage were similar to those of the wild-type phage on the sensitive strain SMQ-16 (Table 2). The release of progeny phages by the AbiK+ strain was seven times greater for phage p2K than for p2. To determine if...
the mutated allele in p2K was dominant or recessive, a coinfection assay was conducted. An e.c.o.i. of 23·8±1·1% was obtained when coinfecting an AbiK+ strain with phages p2 and p2K, as compared to e.c.o.i. values of 4·8±1·3% and 35·2±2·5% with single infections of p2 or p2K, respectively. Therefore, these alleles are codominant.

**Phage p2 replication assay**

The restriction map of the cos-containing phage p2 genome was established. Only the positions of the EcoRV sites are indicated in Fig. 1(c). Phage DNA replication was temporally monitored in the infected AbiK+ and AbiK− cells. The phage genome was rapidly replicated in the sensitive cells (Fig. 1a) as DNA appeared 20 min after infection, peaked at 30 min and then decreased after 40 min. When the cells burst, they release progeny phages which are not recovered during the centrifugation step. This explains the reduction in the total amount of phage DNA at 40 min and beyond. Cell lysis was also confirmed by a dramatic reduction in the optical density observed at 40 min (data not shown). The immature forms (circular and concatemer) of the phage genome with the cos sites covalently closed and the mature (linear single-length unit) form were simultaneously present in the sensitive cells. The immature form was associated with the presence of a 5·3 kb EcoRV restriction fragment corresponding to two smaller fragments of 1·3 and 4·0 kb, joined by their cos termini (Fig. 1). In the AbiK+ cells, phage p2 DNA also appeared 20 min post-infection and the amount of phage DNA increased continuously throughout the assay (Fig. 1b). No reduction in the optical density of the culture was observed (data not shown). Furthermore, only the immature forms of the phage genome were detected. These results indicated that phage p2 DNA replication did occur in the resistant cells and that the DNA was not processed to its mature form. These results are in contrast to a previous study where no phage ul36 DNA (P335 species) was detected in infected AbiK+ cells (Emond et al., 1997).
Effects of AbiK on 936 and P335 phages

Fig. 2. DNA replication of phages P008 and P335. Phage P008 DNA replication was followed during the infection of *L. lactis* F4/2 (a) and SMQ-463 (b) and phage P335 DNA replication was followed during the infection of *L. lactis* F4/2 (c) and SMQ-463 (d). Samples (1 ml) of infected cultures (m.o.i. = 1) were taken every 10 min and total DNA was isolated from the cellular fraction by the method of Hill *et al.* (1991). DNA was digested with EcoRV, heat-treated (65 °C for 10 min) and restriction fragments were separated on a 0–8% agarose gel. Numbers above the lanes indicate the time of sampling. 

C−, phage DNA digested with EcoRV; C+, phage DNA digested with EcoRV heated for 10 min at 65 °C; L, 1 kb DNA ladder (12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1 and 0 kb; Gibco-BRL); --, sample taken prior to infection.

**Phage P008 and P335 DNA replication assays**

The DNA replication assays of phage ul36 and p2 were done on distinct lactococcal hosts. To confirm that the observed differences were not due to host factors, DNA replication was temporally monitored for the type phages P008 (936 species) and P335 (P335 species), which are lytic for the same host. The e.o.p. of phage P008 and phage P335 on *L. lactis* F4/2 carrying AbiK (pSRQ817) were 10^{-1} and 10^{-3}, respectively. As observed for p2, phage P008 DNA was rapidly replicated in sensitive cells and the amount of DNA decreased after 40 min due to cell lysis (Fig. 2a). The two genomic forms of phage DNA were also detected. The immature form was associated with the presence of a 5 kb EcoRV fragment containing the covalently closed cos sites and the processed form corresponded to the dissociation of this fragment into two smaller fragments. In resistant cells, the processing of the phage DNA to its mature form was severely reduced and no reduction in the amount of DNA was observed at 40 min (Fig. 2b). For phage P335, the DNA replicative process was observed in the sensitive cells (F4/2) until cell lysis, which occurred 100 min following infection (Fig. 2c). In the resistant cells, no phage DNA was detected (Fig. 2d). These results are in agreement with the previous study on the effects of AbiK on phage ul36 (Emond *et al.*, 1997). DNA replication experiments using the host strain F4/2 carrying pSRQ830, a pSRQ817 derivative with a frameshift within *abiK*, ruled out the possibility that the observed molecular consequences could result from the expression of a different gene product from pSRQ817, or even the presence of a site on pSRQ817 that could interact with some phage protein (data not shown).

**Monitoring of the lytic cycle by electron microscopy**

Sensitive and resistant cells were infected with phage p2 and samples were collected at intervals to observe the
intracellular course of the lytic cycle by electron microscopy (Fig. 3). At 30 min post-infection, most sensitive cells had phages adsorbed to their surface (Fig. 3c). A network of DNA filled a large part of the intracellular space (Fig. 3c). At 50 min, capsid-like structures were detected throughout the cytoplasm and the DNA network was no longer visible, an indication of the packaging process (Fig. 3d). Finally, the infected cells lysed and released headful progeny phages (Fig. 3e). In the AbiK\(^{+}\) strain, no such progression of the lytic cycle was observed (Fig. 3f, g and h). Phages adsorbed to the bacterial surface and the presence of empty heads indicated that DNA had entered the cells. No capsid structures were detected within the cytoplasm. However at later stages, a few head-like structures were observed around a DNA network, which is likely to comprise the accumulated phage DNA concatemers evidenced in the DNA replication assays. No cell lysis was noticed at any time in the AbiK\(^{+}\) cells.

**Effects of m.o.i. on e.c.o.i.**

Studies on phage–host interactions in cells harbouring anti-phage mechanisms are generally performed using non-standardized m.o.i.s. Since discrepancies could result from the use of various m.o.i.s, we measured their impact, ranging from 0·1 to 50, on the e.c.o.i. of cells carrying AbiK (Fig. 4). For m.o.i.s lower than 5, the e.c.o.i. remained constant, with around 5% of infected cells releasing progeny phages. Above a m.o.i. of 5, the e.c.o.i. values increased gradually with increasing m.o.i.s. Thus, a relationship exists between the e.c.o.i. and the m.o.i. in a *L. lactis* strain carrying AbiK.

**DISCUSSION**

Although several lactococcal Abi systems have been shown to be efficient on multiple phage species, mechanistic studies are generally conducted on a single species. Here, we showed that even if the resistance
mediated by AbiK on the phage p2 is relatively similar to that reported for the phage ul36 (Emond et al., 1997), the molecular impacts are clearly distinct. Moreover, the fact that the same differences were observed with phages P008 and P335 replicating on a single host demonstrates that the molecular impacts are related to the particularities of the two phage species, rather than on host factors.

The absence of accumulation of P335 phage DNA in infected cells suggests an interference of AbiK with an early stage of the lytic cycle. The fact that the concatenated DNA of 936 phages was not efficiently processed into mature genome suggests that AbiK halted the packaging process, possibly as a consequence of the disrupted early stages. These molecular consequences are likely to result from an interaction of AbiK with analogous phage components of both species, or with a host product necessary for a common early step of phage development. A better understanding of the molecular interactions between AbiK and phages of the 936 and P335 species will provide valuable information on the similarities and differences in the lytic cycle of these phages. The absence of the mature processed form of the 936 phage genome in the AbiK+ cell with multiple phages reduced the efficacy of this anti-phage system (data not shown). Therefore, it appears that the balance between the number of invading phages and the intracellular concentration of AbiK molecules is an important variable in experimental procedures on phage–host interactions. Our results showed that AbiK is potent enough to resist multiple phage infections, up to a threshold value, above which the resistance factor is most likely outnumbered and the proportion of viruses escaping AbiK is enhanced. Similarly, the codominance of phages p2 and p2K in the coinfection experiment supports the idea that competition occurs at the molecular level for factors needed for the AbiK phenotype.

In conclusion, AbiK has diverse consequences on the lytic cycle of 936 and P335 phages. This is the first report of such a dual effect of an Abi on two phage species. The molecular basis for the different kinds of interactions is not yet understood but further characterization of variant phages resistant to AbiK, such as p2K, could be a useful tool in investigating the AbiK mode of action. However, our results emphasize the importance of conducting mechanistic studies on both phage species. Finally, the determination of e.c.o.i. with extended m.o.i. values could represent an additional tool to evaluate the robustness of any given Abi system.

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We previously demonstrated that the impact of AbiK on phage resistance was substantially improved when cloned in a high-copy vector (Emond et al., 1997). Furthermore, the infection of an AbiK+ cell with multiple phages reduced the efficacy of this anti-phage system (data not shown). Therefore, it appears that the balance between the number of invading phages and the intracellular concentration of AbiK molecules is an important variable in experimental procedures on phage–host interactions. Our results showed that AbiK is potent enough to resist multiple phage infections, up to a threshold value, above which the resistance factor is most likely outnumbered and the proportion of viruses escaping AbiK is enhanced. Similarly, the codominance of phages p2 and p2K in the coinfection experiment supports the idea that competition occurs at the molecular level for factors needed for the AbiK phenotype.

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