A Phage Protein Confers Resistance to the Lactococcal Abortive Infection Mechanism AbiP

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Phage bIL66M1 is sensitive to the lactococcal abortive infection mechanism AbiP. No spontaneous AbiP-resistant variant could be obtained at a frequency of <10⁻¹⁰. However, AbiP-resistant variants were readily obtained during infection with both bIL66M1 and the highly homologous AbiP-resistant phage bIL170. Gain of AbiP resistance was due to the acquisition of the e6 gene from bIL170.

Dairy lactococci have evolved an outstanding panoply of mechanisms enabling them to resist bacteriophage infection. In particular, they frequently possess bacteriophage abortive infection mechanisms (Abi) (4, 6, 15, 28) that interrupt an intracellular step of phage multiplication, leading to death of the infected cell and survival of the bacterial population (23, 27). Functional studies of these mechanisms have revealed a great diversity in their modes of action (14).

Lactococcal phages fall into three prevalent groups of DNA homology (19). Two of these groups (c6A and 936) are composed of virulent phages, and the third (P335) is composed of both temperate and virulent phages (20). In a group of phages, not all individuals are sensitive to a given Abi system. In some instances, sensitive phages have been reported to give rise to resistant variants. P335 phages have been shown to acquire resistance to Abi by either exchange of a DNA segment with a resident prophage or point mutations (4, 5, 12, 13, 22). By contrast, virulent phages of the 936 group, which have no homologous prophage on the host chromosome, evolved resistance to Abi only by point mutations (2–4, 13, 15). The mechanisms by which these variants bypass lactococcal Abis are unknown. It has, however, been established that phage bIL66 most likely activates AbiD1 following infection, whereas mutants resistant to AbiD1 are unable to do so (3).

AbiP has recently been shown to block phage bIL66M1 DNA replication 10 min after infection and to prevent the early transcription switch-off (14). Here we show that the early expressed phage bIL170 e6 gene (e6bIL170) confers resistance to AbiP (AbiP⁺) and is readily transferred to AbiP-sensitive (AbiP⁻) phages during coinfection.

AbiP is active on lactococcal phages of the 936 group (19), bIL41, bIL66M1, and sk1 but not on bIL170 (14). Phages of this group share homology over 90 to 92% of their genome length (9). In order to determine the phage region(s) involved in sensitivity or resistance to AbiP, we first tried to isolate spontaneous AbiP⁺ mutants from phage bIL41, bIL66M1, or sk1. Phages, AbiP⁻ plasmid-free L. lactis strains IL1403 (8) and MG1363 (16), AbiP⁺ derivatives IL1403(pIL2617) and MG1363(pIL2617), and growth conditions were previously described (14). AbiP⁺ phages form barely visible turbid plaques (typical of the sensitivity to an Abi mechanism), while AbiP⁻ phages form easily recognizable clear plaques. No mutant was obtained at a frequency of <10⁻¹⁰, suggesting that no single mutation can confer resistance to AbiP. We therefore used a second strategy, aimed at selecting hybrid phages having gained resistance to AbiP by recombination with a highly homologous AbiP⁺ phage. Comparison of the genome of the hybrids to the genome of the parents would allow identification of the region(s) involved in resistance to AbiP.

In a first experiment, IL1403(pIL1201, pIL352), expressing both AbiB (10, 25) and AbiP (14), was coinfectected with phages bIL41 (AbiB⁺ and AbiP⁻) and bIL170 (AbiB⁻ and AbiP⁺). Progeny phages formed plaques on the same strain. Under these conditions, both parental phages formed barely visible turbid plaques. By contrast, hybrid phages resistant to both Abi mechanisms formed clear plaques at a frequency of approximately 10⁻⁴ (24; A. M. Crutz-Le Coq, unpublished observations). Ten phages were isolated from clear plaques and propagated on the AbiB⁺ AbiP⁺ IL1403(pIL1201, pIL352) strain. This purification step was repeated twice. Purified phages demonstrated the same efficiency of plating on IL1403 and on the AbiB⁺ AbiP⁺ strain IL1403(pIL1201, pIL352). The structure of phage genomes was compared to that of the parent phages by restriction analysis. Two phages representative of the population of recombinant phages were further characterized. This was done by amplifying hybrid and parental phage DNAs in seven slightly overlapping PCR fragments covering the whole genome. DNA amplification was carried out as described previously (14). The oligonucleotides used (Table 1) were complementary to bIL170 sequence (11), which was expected to be very homologous to that of bIL41. Each segment was digested separately using the indicated restriction enzymes (Fig. 1).

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The involvement of the phage middle region in the resistance to AbiP was unlikely, since the adverse effect of AbiP on phage development can be detected before phage middle genes are expressed (14). Therefore, to localize more precisely the region conferring AbiPr, we modified the experimental procedure by replacing phage bIL41 and AbiB by phage bIL66M1 (2) and AbiD1 (1). The rationale for this change is that the resistance of bIL66M1 to AbiD1 is due to a mutation in one middle expressed gene (2) and that this region would likely be conserved in the hybrid progeny. Another advantage is that the DNA sequence of this region of bIL66M1 is known as compared to phage-encoded e6bIL170. The involvement of the phage middle operon is not required for resistance to AbiP. By contrast, the restriction patterns of segment F from all hybrid phages were compared in more detail. This allowed the identification of one hybrid phage, called h7, which contained less bIL170 DNA than the others. The structure of the F segment of h7 is shown in Fig. 2. From these data, h7 is likely to result from two homologous recombination events, leading to the exchange of a 1.5-kb region between bIL66M1 and bIL170. These 1.5-kb regions are very similar in both phages. The most striking difference is the presence of an additional open reading frame in bIL170, which has no counterpart in bIL66M1.

To check if the resistance of phage h7 to AbiP is linked to the open reading frame which was amplified using phage bIL170 DNA as a template and oligonucleotides 3 and 4. The PCR fragment was cloned at the SacI site of plasmid pIL5303, which has no counterpart in pIL871 (14). The resulting plasmid, pIL5303, was transferred into the AbiP+ IL1403(pIL2617) strain (14) using electroporation. Plasmids pIL2617 and pIL5303 were derived from phages bIL66M1 and bIL41, respectively. Therefore, expression in the phage-encoded e6bIL170 strongly decreases the AbiP efficiency but did not fully restore phage growth. This could result from an inappropriate level or timing of expression of the cloned e6bIL170 as compared to phage-encoded e6bIL170. Another explanation would be that an additional undetected phage genome region...
would be needed to confer full AbiP. To discriminate between these possibilities, we performed a directed insertion of e6bIL170 into the AbiP phage bIL66M1. For this, we took advantage of the capacity of Lactococcus lactis phages to recombine readily with a resident plasmid carrying a homologous phage segment (2). Plasmid pIL5304, harboring e6bIL170 flanked by e6bIL66M1 and e7bIL66M1, was constructed using three PCR fragments. e6bIL170 was amplified using oligonucleotides 5 and 6. e7bIL66M1 and the 5’/H11032 part of e8bIL66M1 were amplified with oligonucleotides 7 (with an SmaI extension) and 8 (with an extension complementary to the 3’/H11032 end of e6bIL170). e6bIL66M1 was amplified using oligonucleotides 9 (with a PstI extension) and 10 (with an extension complementary to the 5’/H11032 end of e6bIL170). The three PCR fragments were mixed and used as a template in a PCR with oligonucleotides 7 and 9. The final PCR product was cloned at the SmaI-PstI sites of the plasmid vector pIL253 (26). The recombinant plasmid pIL5304 was transferred into the AbiP/IL1403 strain. pIL2618 was obtained by cloning the abiP gene, amplified from plasmid pIL2617 (14) using oligonucleotides 1 and 2, at the HincII-PstI sites of pIL2608. Plasmid vector pIL2608 (M. C. Chopin and J. Anba, unpublished work) was constructed by ligating a 1.7-kb fragment containing the replication region of the natural lactococcal plasmid pIL105 (17) to a cassette containing the tetM gene of Tn51545 (21). An exponential-phase culture (optical density at 600 nm of 0.4) of the IL-1403(pIL2618, pIL5304) strain was infected with phage bIL66M1 and plated on the AbiP/strain IL1403(pIL2618). Nine clear plaques were observed among 10⁴ turbid plaques. In a control experiment, performed in the absence of pIL5304, no clear plaque was observed. Phages picked up from the nine clear plaques were propagated twice on IL1403(pIL2618). All demonstrated the same efficiency of plating on the AbiP/IL1403 and the AbiP/IL1403(pIL2618) strains. They were thus fully resistant to AbiP. PCR analysis confirmed that e6bIL170 had been transferred in each genome. Sequencing of relevant regions indicated that e6bIL170 was present at the expected position in all nine recombinant phage genomes (Fig. 3). These results indicate that e6bIL170 is the determinant of the AbiP phenotype. E6bIL170 (69 amino acids) shares no significant homology with proteins in the databases, precluding any hypothesis to be made about its function. By contrast to bIL66M1, the AbiP phages bIL41 (A. M. Crutz LeCoq, personal communication) and sk1 (7) possess a gene of the same length (encoding 70 and 60 amino acids, respectively), and at the same position that e6bIL170. Proteins encoded by these genes had some homology in the N- and C-terminal parts, flanking a variable central region. This variable protein might be dispensable in 936 phages, and not all orthologs can confer resistance to AbiP.

Our results show that virulent lactococcal phages from the 936 group can acquire AbiP by recombination with a homologous coinfetting phage. Given the high concentration of phages that may occur in a dairy environment, coinfection should not be a rare event and may represent a significant mode of evolution.

<table>
<thead>
<tr>
<th>Indicator strain</th>
<th>Characteristic(s)</th>
<th>Efficiency of plating of*:</th>
<th>bIL66M1</th>
<th>bIL41</th>
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<tr>
<td>IL1403</td>
<td>AbiP</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>IL1403(pIL2617, pIL5304)</td>
<td>AbiP, e6bIL170 in trans</td>
<td>10⁻⁷</td>
<td>10⁻⁸</td>
<td>10⁻⁸</td>
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<tr>
<td>IL1403(pIL2617, pIL5304)</td>
<td>AbiP, e6bIL170 in trans</td>
<td>10⁻³</td>
<td>10⁻³</td>
<td>10⁻³</td>
</tr>
</tbody>
</table>

* The efficiency of plating of phages bIL66M1 and bIL41 was determined on IL1403 AbiP* derivatives, in the presence or absence of E6bIL170 in trans. Shown is the number of PFU per milliliter formed on the indicator strain divided by the number of PFU per milliliter formed on the control strain IL1403.
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REFERENCES


FIG. 3. Insertion of e6bIL170 in bIL66M1 genome. The insertion was designed in order to mimic in bIL66M1(E6) the overlap that exists between e6bIL66M1 and e7bIL66M1.