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1 **Effect of the abortive infection mechanism and type III toxin/antitoxin system AbiQ**

2 **on the lytic cycle of *Lactococcus lactis* phages**

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12

13 # Abstract

14

15 To survive in phage-containing environments, bacteria have evolved an array of anti-
16 phage systems. Similarly, phages have overcome these hurdles through various means. Here, we
17 investigated how phages are able to circumvent the *Lactococcus lactis* AbiQ system, a type III
18 toxin-antitoxin with antiviral activities. Lactococcal phage-escaping mutants were obtained in the
19 laboratory and their genome sequenced. Three unrelated genes of unknown function were
20 mutated in derivatives of three distinct lactococcal siphophages: *orf38* of phage P008, *m1* of
21 phage bIL170, and *e19* of phage c2. One-step growth curve experiments revealed that the phage
22 mutations had a fitness cost while transcriptional analyses showed that AbiQ modified the early-
23 expressed phage mRNAs profile. The *L. lactis* AbiQ system was also transferred into *E. coli*
24 MG1655 and tested against several coliphages. While AbiQ was efficient against phages T4
25 (*Myoviridae*) and T5 (*Siphoviridae*), escaping mutants of only phage 2 (*Myoviridae*) could be
26 isolated. Genome sequencing revealed a mutation in gene *orf210*, a putative DNA polymerase.
27 Taken altogether, different phages genes or genes products are targeted or involved in AbiQ
28 phenotype. Moreover, this antiviral system is active against various phages families infecting
29 Gram-positive and Gram-negative bacteria. A model for the mode of action of AbiQ is proposed.

30

Introduction

Bacteriophages are ubiquitous in most environments, including foods. Some virulent phages will thrive during food manufacturing processes that rely on rapid bacterial growth or metabolic activities. *Lactococcus lactis* is a gram-positive bacterium used in the production of several fermented dairy products. These milk-based cultures can be lysed by a plethora of distinct virulent phages (1), leading to variations in product quality. Numerous anti-phage hurdles have been devised over the past decades to cope with this risk (reviewed in (2-5)). Yet, some phages will persist or emerge in dairy environs (6). *L. lactis* phages belong to the *Caudovirales* order (1, 7). Their dsDNA genomes are within an icosahedral capsid connected to a short (*Podoviridae* family) or a long non-contractile tail (*Siphoviridae*). They are also divided in at least ten genotypes but three of them, the 936, c2 and P335 groups, contains hundreds of known members and are mostly associated with failed milk fermentations worldwide (1, 8). Complete genome of at least one member of the 10 genotypes is available, with more genomes determined for the most common groups (9). Progress has been made in the structural aspects of the interaction of these phages with their hosts (reviewed in (10)). However, many phage genes coding for non-structural proteins have unknown function and our knowledge of their roles in phage biology is limited.

When infecting bacterial cells, phages may face barriers that will hamper their amplification. These hurdles can prevent the phage adsorption process, inhibit the phage genome ejection into the cell, cut the invading genome or simply abort another step of the lytic cycle (11). Over 20 lactococcal abortive infection systems (Abi) have been reported (12-15). While some Abi systems stop the replication of several phage genotypes, others inhibit only a few groups (12-15). Of note, not every member of a phage group is sensitive to an Abi system at similar levels (14, 16, 17). Lactococcal Abis systems have been reported to block phage DNA replication, transcription, translation, maturation and/or lysis, but the mechanistic details are still elusive.

55 The characterization of Abi-escape phage mutants has led to some mechanistic
56 information. A mutated Orf1 of phage bIL66 (936 group) is no longer able to induce AbiD1 (18-
57 20). The wild-type Orf1 binds to an mRNA secondary structure to activate AbiD1 expression
58 (18). AbiD1 interferes with the phage RuvC-like endonuclease to inhibit replication (20, 21). To
59 resist AbiK, lactococcal phages have evolved mutations in genes named *sak,* which code for
60 single-strand annealing proteins (17, 22-25). AbiK polymerizes an untemplated DNA molecule
61 via its reverse-transcriptase motif to confer phage resistance (26, 27). Phage bIL66M1 (936)
62 needs to acquire a gene (*e6*) of unknown function from phage bIL170 (936) to avoid abortion by
63 AbiP (28). *L. lactis* AbiP inhibits the switch off from early to middle phage genes transcription
64 (29). Lactococcal phages of the 936 group can become insensitive to AbiV due to mutations in
65 the *sav* gene (30). The interaction between SaV and AbiV leads to a general inhibition of protein
66 synthesis (31). Finally, AbiT-escaping mutants derived from distinct phages (p2, P008, bIL170 /
67 936 group) resist due to mutations in different genes, indicating a possible phage-dependent
68 activity (32). While two of these phage genes (*e14*/bIL170 and *orf41*/P008) have no known
69 function, the gene coding for the major capsid protein (*orf6*/p2) plays a role in the AbiT
70 phenotype (32), which affects DNA replication and encapsidation (16).

71 The AbiQ system is active against members of the common 936 and c2 groups (33) as
72 well as rare lactococcal phage groups with EOPs of $<10^{-8}$ for phages Q54 (34), P087 (35), and
73 949 (9). Recently, it was demonstrated that AbiQ is also a type III toxin-antitoxin (TA) system
74 with the antitoxin being a RNA molecule (2.8 repeats of 35 nucleotides) and the AbiQ toxin
75 being a protein (172 amino acids, 20.3 kDa) with an endoribonuclease activity (36, 37). AbiQ is
76 also related to another Abi named ToxIN found in *Pectobacterium atrosepticum* (37). In this
77 study, we further characterized AbiQ through the analysis of phage escape mutants.

78

79 ## Materials and Methods

80

81 **Bacteria and phage propagation.** Phage and hosts used in this study are listed in Table 1. *L.*
82 *lactis* strains were grown in M17 media supplemented with 0.5% glucose (GM17, Oxoid) at
83 30°C. When needed, 5 µg/ml of chloramphenicol or erythromycin were added for plasmid
84 maintenance and 10 mM $CaCl_2$ was added for phage propagation. *E. coli* strains were grown in
85 LB or Trypticase soy broth (TSB) media and incubated at 37°C with agitation. Chloramphenicol
86 (20 µg/ml) was added to the media when necessary. For phage amplification, bacteria were
87 grown until an $O.D._{600}$ of 0.2 prior to the addition of approx. 10^4 phages per ml and incubated
88 until lysis. When the culture was completely clear, the phage lysate was filtered (0.45µm) and
89 stored at 4°C. The efficiency of plating (EOP) was calculated by dividing the phage titer on an
90 $AbiQ^+$ strain by the phage titer on an isogenic $AbiQ^-$ strain. To obtain a concentrated phage
91 sample, 1L of phage lysate was purified on a discontinuous cesium chloride gradient as described
92 previously (38).

93 Phage growth curves was performed at 30°C during 70 minutes with a starting
94 multiplicity of infection (MOI) of 0.05 as reported elsewhere (39). Growth curves were made at
95 least three times and all the sampling dilutions were plated in triplicates. The burst size was
96 calculated by dividing the average phage titer after the exponential phase by the average titer
97 before the infected cells began to release new virions. The efficiency to form center of infection
98 (ECOI) was done in triplicate as described previously (33, 39) with phages P008 and P008-Q12
99 at a MOI of 0.2 and with the phage-sensitive strain IL1403 (pNZ123) and an AbiQ-containing
100 derivative (pSRQ928). The ECOI was calculated using the formula: number of COI on the
101 resistant strain divided by number of COI on the sensitive strain and the result multiplied by 100.

102

103 **Phage-escaping mutants.** Phage plaques from multiple lysates were isolated on a plate
104 containing a lawn of $AbiQ^+$ cells infected with wild-type phages. Each escaping-phage plaque
105 was purified three times on a lawn of $AbiQ^+$ cells. Then, phage amplifications were performed in
106 liquid media until a titer of at least 10^9 pfu/ml was obtained. To isolate mutants derived from the
107 wild-type AbiQ-sensitive phage P008 (936 group), the strain *L. lactis* IL1403 containing the
108 AbiQ plasmid pSRQ928 was used. Mutants from wild-type phage bIL170 (936) were isolated on
109 *L. lactis* IL1403 containing the AbiQ plasmid pSRQ925. Wild-type c2 phage was propagated on
110 *L. lactis* LM0230 harboring pSRQ928 (33). AbiQ-insensitive coliphages were obtained on *E. coli*
111 MG1655 with the vector pNZ123-AbiQ. To improve phage plaque visualization, 0.5% glycine
112 was added to top agar (40), and/or agarose 0.4% was used to replace agar. The temperature of
113 incubation was also reduced from 30°C to 25°C for *L. lactis* and 37°C to 30°C for *E. coli*.
114
115 **DNA manipulations and analyses.** Phage DNA was isolated using a Qiagen Maxi Lambda
116 DNA preparation kit with the modifications described previously (41). The genome of phages
117 P008-Q1, P008-Q12, c2, and c2-Q3 DNA were sequenced at the Plateforme the séquençage et de
118 génotypage des génomes of the CHUQ-CHUL. The genome of phages bIL170, bIL170-Q22, 2,
119 and 2Q4 DNA were sequenced at the Plateforme d'analyses génomiques de l'IBIS (U. Laval).
120 Genomes were assembled using the Staden package (http://staden.sourceforge.net/) and edited
121 with Bioedit (http://www.mbio.ncsu.edu/bioedit/bioedit.html). To find ORF functions, protein
122 sequences were analysed with BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), ACLAME
123 (http://aclame.ulb.ac.be/), FASTA (http://www.ebi.ac.uk/Tools/sss/fasta/), Conserved Domain
124 Database (42), PHYRE 2 (43), PDB (44), InterProScan
125 (http://www.ebi.ac.uk/Tools/pfa/iprscan/), and SMART (45) tools. To determine protein
126 properties, ProtParam was used (http://web.expasy.org/protparam/). To evaluate the codon usage

127 of phage *orf*, the codon usage of *L. lactis* was used for comparison
128 (http://www.kazusa.or.jp/codon/). For promoters, the consensus -35 box (TTGACA) followed by
129 the -10 box (TATAAT) were visually searched. Stretch of Ts preceded by a hairpin structure
130 (MFOLD, http://www.idtdna.com/SciTools/SciTools.aspx and http://mobyle.pasteur.fr/cgi-
131 bin/portal.py?#forms::mfold) suggested the presence of rho-independent terminators.

132

133 **Recombination assay**. A PCR product of the *orf38* or *orf38*M was cloned in the pTRKH2 shuttle
134 vector using the phage P008 and P008-Q12 DNA as template. These plasmids were sub-cloned in
135 *E. coli* XL1-Blue and transformed in *L. lactis* IL1403 containing pNZ123 or IL1403 containing
136 pSRQ928. All constructs were confirmed by sequencing. The EOPs of phages P008 and P008-
137 Q12 were calculated by dividing the titer of the phage on the tested strain by the titer of the phage
138 on the control strain phage-sensitive *L. lactis* IL1403 harboring the empty cloning vectors
139 pNZ123 and pTRKH2. To try to obtain null (two stop codons at positions 5 and 6 of *orf38*) and
140 frameshift (addition of one nucleotide at the 5th codon) mutations in *orf38*, site-directed
141 mutagenesis of the vector pTRKH2-*orf38* was used to introduce the targeted modification (36).
142 Primers used were: orf38nulA / ATGTACACAGCATAATAAAGAGAGCAAATCATCG,
143 orf38nulB / CGATGATTTGCTCTCTTTATTATGCTGTGTACAT, orf38fsA /
144 ATGTACACAGCAAGAAGAGAGAGAGCAAATCAT, and orf38fsB /
145 ATGATTTGCTCTCTCTCTTCTTGCTGTGTACAT. Resulting plasmids were transformed in *E.*
146 *coli* DH5-α at first, then in *L. lactis* IL1403 + pSRQ928, and confirmed by sequencing. Ten
147 plaques of phage P008 isolated on *L. lactis* IL1403 containing pSRQ928 and on *L. lactis* IL1403
148 + pSRQ928 with pTRKH2-*orf38*M, pTRKH2-*orf38*null or pTRKH2-*orf38*fs were purified and
149 amplified on IL1403 containing pSRQ928. Then, the *orf38* gene of each phage was PCR
150 amplified and the PCR products sequenced to identify the mutation.

151 **Detection of ORF38 by mass spectrometry.** *L. lactis* IL1403 was grown in 10 ml of GM17 at
152 30°C until an OD_{600} of 0.5. Then, phage P008 was added at a MOI of 5 and the infection was
153 followed for 15 minutes. Cells were rapidly pelleted and frozen at - 80°C. After thawing on ice,
154 cells were re-suspended in SLB-1X buffer, sonicated, and proteins were separated on a 15%
155 SDS-PAGE gel. Protein bands of the size expected for the ORF38 protein (8 kDa) were cut and
156 sent for LC-MS/MS analysis at the Proteomic platform of the Quebec genomic center (CHUQ-
157 CHUL). The peptide identification results were compared with the phage P008 ORF database and
158 the identification confidence was determined with Scaffold 3 software.
159
160 **Northern Blot.** A time course infection of *L. lactis* IL1403 (phage-sensitive) or IL1403
161 containing pSRQ928 (AbiQ+) was performed with phages P008 and P008-Q12 at a MOI of 5 as
162 described (36). Samples were taken at the following times: non-infected (NI), 2, 10, 20, 30, and
163 40 minutes post-infection. Total RNA was purified with Trizol as described by the manufacturer
164 (Invitrogen) with the addition of a lysozyme pre-treatment (60 mg/ml lysozyme for 10 minutes at
165 37°C) to increase bacterial lysis. RNA samples were treated with DNase-I (Roche) to eliminate
166 residual DNA, protected with RNA inhibitor (Roche), and the RNA concentration was estimated
167 with a NanoDrop 2000 (Thermo-scientific). Aliquots of 5 μg of RNA were migrated on 1%
168 formaldehyde-agarose denaturating gels and transfer on nylon membranes (38). Northern blot
169 experiments were performed as described previously (34) with ^{32}P-radiolabelled oligonucleotides
170 probes (sense or anti-sense orientation) to specific genes of phage P008: *orf33*, *orf36*, *orf37*,
171 *orf38*, *orf39*, *orf40*, *orf41*, *orf45*, *orf52,* and *orf53* (see supplementary material).
172
173 **Coliphage 2 accession number.** The genome of coliphage 2 was deposited in GenBank
174 (KC690136).

175 # Results

176

177 **AbiQ-escaping P008 phage mutants.** In order to identify the target of AbiQ, six wild-type
178 AbiQ-sensitive virulent phages of the 936 group (P008, bIL170, jj50, sk1, 712, and p2) and one
179 phage of the c2 group (c2) were used to infect AbiQ-containing *L. lactis* cells. All these phages
180 belong to the *Siphoviridae* family. No escaping phage mutants (no plaque) could be observed on
181 plates containing AbiQ-expressing *L. lactis* cells and high population (>10^8) of wild-type phages
182 jj50, sk1, 712, and p2. These data suggest that AbiQ strongly inhibits the multiplication of these
183 phages. On the other hand, AbiQ^R phages were isolated from phages P008, bIL170, and c2.

184 The EOP of phage P008 on *L. lactis* IL1403 carrying AbiQ (pNZ123 + AbiQ) is 10^{-5}.
185 Twenty-two P008-derived mutants able to propagate in an AbiQ-containing strain were randomly
186 selected, purified, and characterized (Table 2). The EOP of these mutants was measured by
187 dividing the titer of each phage lysate on the *L. lactis* strain IL1403 with AbiQ by the titer of the
188 lysate on the same strain without AbiQ. The EOP of these escaping phage mutants was 10^{-2}, an
189 increase of 3-logs as compared to the wild-type phage P008. These results also indicated that
190 these phage mutants are not completely escaping AbiQ (EOP of 1), but are significantly less
191 affected. The complete genome (28,538 bp) of two of these AbiQ-escaping mutants (Q1 and
192 Q12) was sequenced and compared to the wild-type genome (GenBank accession number
193 DQ054536 (46)). Both AbiQ-escaping phages had only one nucleotide mutation, which was
194 located in the *orf38* gene (P008-Q12) or in the ribosome binding site (RBS) located upstream of
195 its start codon (P008-Q1). The genomic region containing *orf38* was amplified and sequenced for
196 the other twenty P008-derived AbiQ-escaping mutants and similarly, all of them had a mutation
197 in the *orf38* gene or in its RBS, confirming the importance of this gene for the AbiQ activity.
198 ORF38 has 71 amino acids, a predicted molecular weight of 8.3 kDa and a pI of 4.5.

199 Detailed analyses of these 22 AbiQ-escaping phage mutants revealed 11 distinct
200 mutations including two (phage P008 mutants Q1 and Q14) in the RBS preceding the *orf38*
201 (Table 2), suggesting that the level of expression of ORF38 is important to bypass AbiQ. In
202 support of this observation, a mutation in phage-escaping P008-Q17 led to a substitution of the
203 methionine at position 1 by a leucine (Met1Leu) (Table 2). While, the leucine residue could serve
204 as an alternative translational initiation codon, it is used four times less frequently in *L. lactis* as
205 compared to the traditional methionine codon. Similarly in phage P008-Q5, the silent mutation
206 Thr3Thr should not change the intrinsic propriety of the protein, but the mutated codon (ACA) is
207 used twice less frequently then the one (ACC) found in the wild-type phage P008. The other
208 mutations led to amino acid changes distributed along the protein and could affect either its
209 conformation or its activity. One mutation also led to a truncated protein (Trp30Stop). Our
210 bioinformatics analyses failed to identify a function. However, this protein is well conserved
211 (>90% identity) in other lactococcal phages of the 936 group. The *orf38* is localized in a gene
212 cluster that is expressed early in the phage infection and is likely involved in DNA replication.
213
214 **AbiQ-escaping bIL170 phage mutants.** Using a similar approach, we isolated AbiQ-escaping
215 mutants derived from the wild-type AbiQ-sensitive virulent lactococcal phage bIL170 (936
216 group). The genomes of phages bIL170 and P008 share 77.8% identity at nucleotide level and
217 they both infect *L. lactis* IL1403 (47). A total of nine AbiQ-escaping bIL170 mutants were
218 characterized. The EOP of these 9 phage mutants was increased from 10^{-6} (for the wild-type
219 phage) to 10^{-1} on AbiQ-containing cells (Table 2). The complete genome (31,754 bp) of the
220 phage escaping mutant bIL170-Q22 was sequenced and compared to the wild-type genome
221 (GenBank AF009630 (48)). Again, a single nucleotide mutation was observed. The mutation was
222 located in the *m1* gene and not in the *e14* gene (homolog of the P008 *orf38* gene). The *m1* region

223 was amplified and sequenced for the other escaping mutants. Each had a mutation in *m1* but only
224 two distinct mutations were observed leading to amino acid changes Met1Phe and Gln5Stop. In
225 both cases, it probably leads to a defect in M1 protein production. No function could be attributed
226 to M1 (42 amino acids, 4.8 kDa, pI 4.2) but this protein is well conserved (>90% identity) in
227 lactococcal phages of the 936 group. It shares no similarities with ORF38 of P008. The *m1* gene
228 is located in a gene cluster that is starting to be expressed in the middle of the phage lytic cycle.
229

230 **AbiQ-escaping c2 phage mutants.** We also analyzed AbiQ-escape mutants from another group
231 of lactococcal phages, namely c2. Five phage mutants escaping AbiQ (EOP ~1) were isolated
232 (Table 2) and the genome (22,172 bp) of one mutant was compared to the wild-type (GenBank
233 L48605 (49)). One nucleotide change was found in the early-expressed *e19* phage gene leading to
234 a Glu17Asp substitution in the protein. The same mutation was found in the four other phage
235 mutants. E19 (107 amino acids, 12.5 kDa, pI 4.6) has no known function but is conserved (gp33,
236 94% aa identity) in the other phage (bIL67) of the c2 group for which the genome is available.
237

238 **AbiQ-escaping *E. coli* phage mutants.** We also tested whether AbiQ could be effective against
239 a phage that infects *E. coli*. We introduced the plasmid pNZ123-AbiQ into *E. coli* MG1655 and
240 measured the EOPs of 10 coliphages (Table 3). Four phages (T4/*Myoviridae* family,
241 RB69/*Myoviridae*, phage 2/*Myoviridae*, and T5/*Siphoviridae*) were strongly inhibited by AbiQ
242 with an EOP value reduced by 5-logs, while the six other phages (T1, T3, lambda vir, HK97, Mu,
243 pilHα) were not affected (Table 3). To our knowledge, this is the first lactococcal Abi system to
244 work against *E. coli* phages. Moreover, AbiQ could inhibit phages of the *Myoviridae* family.

245 Despite numerous assays and conditions tested, we could not isolate AbiQ-escaping
246 mutants from T4, T5, and RB69. However, we were able to isolate five AbiQ-resistant mutants

247 derived from coliphage 2, a phage which is also infecting *E. coli* O157:H7 strains (50). As the
248 complete genome of the wild-type phage 2 was not available prior to this study, we sequenced it
249 (GenBank KC690136, 136,910 bp) and additional information are available in supplemental
250 material (Table S2, Fig. S1). The genome of one escaping mutant (phage 2-Q4) was compared to
251 the wild-type and bioinformatics analyses revealed a mutation in a gene coding for a putative
252 protein of 925 amino acids (106.7 kDa). The four other phage mutants had a mutation in the same
253 gene but at different position (Table 4). The deduced protein, named ORF210, has multiple
254 domains and some similarities with DNA polymerases. It has a putative polynucleotidyl
255 transferase of ribonuclease H domain localized in the N-terminal part of the protein (amino acids
256 3 to 217) and a DNA polymerase A palm domain in the C-terminal (amino acids 607 to 819). The
257 mutations found in the escaping-phage mutants 2Q4 (Glu298Gly) and 2Q5 (Met290Val) were
258 close to the polynucleotidyl transferase domain, while the mutations in the phages 2Q1
259 (Lys331Glu) and 2Q3 (Asn426Lys) are between the two functional domains and the 2Q2
260 mutation (Val681Ala) is in the putative catalytic site (amino acids 634 to 815).

261 No conserved features could be found from these 4 phage genes (P008/*orf38*, bIL170/*m1*,
262 c2/*e9*, 2/*orf210*), except that they may be involved in functions related to nucleic acids.

263

264 **Mutated ORF38 confer insensitivity to AbiQ.** A recombination assay was designed to confirm
265 that a mutated *orf38* from the lactococcal phage P008 was responsible for the insensitivity to
266 AbiQ. The plasmid pSRQ928 ($AbiQ^{+}$) was co-transformed with the plasmid pTRKH2-*orf38*
267 wild-type or pTRKH2-*orf38*M (mutated from P008-Q12) in *L. lactis* IL1403. Of note, *orf38* and
268 *orf38*M are not transcribed when cloned into the pTRKH2 vector (data not shown). The presence
269 of the wild-type *orf38* or its mutated version had no effect on the EOPs of both phages in the
270 absence of AbiQ (Table 5). Moreover, the wild-type *orf38* gene did not change the phage EOPs

271 in the presence of AbiQ (Table 5). However, the presence of pTRKH2-*orf38*M increased the
272 EOP of the wild-type phage P008 by one-log in a strain carrying AbiQ. Ten P008 plaques were
273 purified and their *orf38* gene was PCR-amplified and sequenced. The 10 isolated AbiQ-resistant
274 phages had the same mutation in the *orf38* as the one found on plasmid pTRKH2-*orf38*M,
275 strongly suggesting that recombination occurred. A similar experiment was performed with *L.*
276 *lactis* cells containing only pSRQ928 (AbiQ$^+$) and only 2 of the 10 phage mutants had acquired
277 this specific mutation. These data confirm that the *orf38* is involved in the AbiQ phenotype and a
278 mutation in this gene allows phages to partially circumvent the antiviral system.
279
280 **Orf38 is an essential gene.** While no function could be attributed to ORF38, we tested whether
281 *orf38* was an essential gene for P008. Of note, no genetic tool is currently available to generate
282 virulent lactococcal phage mutants. Thus to construct a null allele of *orf38*, we first introduce two
283 stop codons at the 5th ad 6th positions of *orf38* (orf38null) and in a different construct, a
284 frameshift (fs) in *orf38* fifth codon. In both cases, these mutations were generated by site-directed
285 mutagenesis on the pTRKH2-*orf38* vector. The vectors were introduced into a *L. lactis* strain
286 carrying AbiQ. Then, we used the above recombination assay and AbiQ selective pressure to try
287 to generate P008 mutants with a null allele of *orf38*. Ten plaques of P008 infecting these strains
288 (IL1403 + pSRQ928 + pTRKH2-*orf38*null or *orf38*fs) were purified and PCR-sequenced in the
289 *orf38* gene. No mutant has recombined with the plasmid to acquire any of both modifications,
290 strongly suggesting that *orf38* is an essential phage gene.
291
292 **Production of ORF38 during the phage infection.** Since *orf38* encodes for a well-conserved
293 small protein (8.3 kDa), we tested if this protein was produced during phage infection. An
294 intracellular cell extract from a sample of *L. lactis* IL1403 infected with P008, 15 minutes after

295 the beginning of the infection, was migrated on a SDS-PAGE and bands of the expected size for
296 ORF38 was cut and sent for mass spectrometry analysis (LC-MS/MS). The ORF38 protein was
297 detected in the sample with a confidence of 95% (data not shown).
298

299 **Effects of AbiQ on the growth of P008 and P008-Q12.** To determine the effect of the mutation
300 in the *orf38* on the phage multiplication, we performed growth curve assays of phages P008 and
301 P008-Q12 in the presence or absence of AbiQ. The burst size of P008 in the absence of AbiQ
302 was estimated at 310 ± 67 new phages per infected cell and its latent period was 39 ± 1 minute.
303 We could not estimate those parameters for P008 in AbiQ-containing cells as the phage infection
304 aborted. The burst size of phage P008-Q12 in the absence of AbiQ was 230 ± 47 virions per cells
305 and its latent period was 43 ± 2 minutes, suggesting that the mutation in *orf38* had a small fitness
306 cost on the escape mutant. The burst size of P008-Q12 was limited to only 9 ± 4 phages per
307 infected AbiQ-containing cells and the latent period was 47 ± 1 minute, confirming that the
308 mutation in *orf38* did not confer a complete insensitivity to AbiQ. The efficiency to form center
309 of infection (ECOI) of both phages was also determined on *L. lactis* IL1403 strains with or
310 without AbiQ. About half (47.6 ± 5.5%) of P008-Q12-infected AbiQ-containing cells released
311 new virions, which was similar (54.3 ± 12.1%) to P008 infecting the same strain. These results
312 showed that P008-Q12 is not infecting an AbiQ-containing strain with a maximum efficiency,
313 which is consistent with its EOP of $\sim 10^{-2}$, its low burst size and its increased latent period.
314 Surprisingly, phage P008 could also replicate in some AbiQ-containing cells.
315

316 **Effect of AbiQ on the transcription of early and middle-expressed genes of P008 and P008-**
317 **Q12.** AbiQ is an endoribonuclease that cleaves its RNA antitoxin precursor *in vivo* to generate
318 small RNAs to neutralize the toxic effect of the protein (36). To test if AbiQ affects phage

319 transcription, a time course infection of *L. lactis* IL1403 containing or not AbiQ was performed
320 with phages P008 or P008-Q12. Samples were withdrawn at different times, the total RNA was
321 extracted, migrated on an agarose gel and a Northern blot was carried out using oligo ^{32}P-labeled
322 probes complementary to 10 phage genes (*orf33*, *orf36*, *orf37*, *orf38*, *orf39*, *orf40*, *orf41*, *orf45*,
323 *orf52,* and *orf53*). All these genes are presumably expressed early during the phage infection
324 process, except orf53, which is a so-called middle-expressed gene. Controls with anti-sense
325 probes did not detect any transcript (data not shown). The detected transcripts obtained by
326 Northern blots and the bioinformatics analyses were used to generate a transcription map of
327 P008. The results are presented in the Figure 1 and in supplementary material (Fig. S2).

328 Most of the detected P008 transcripts could be assigned to predicted transcripts by
329 bioinformatics. The transcription of the nine early-expressed phage genes was detected 2 minutes
330 after the beginning of the infection of the sensitive *L. lactis* cells, peaked at 10 minutes, and
331 decreased until the end of the sampling period (40 min). The transcription of the middle-
332 expressed gene *orf53* started and peaked at 10 minutes, followed by a decrease until the release of
333 phage particles. The transcription profile of these P008 genes was similar in the presence of
334 AbiQ. However, with a few probes (*orf33*, *orf38*, *orf39*, *orf40*, *orf41,* and *orf53*), additional small
335 transcripts, lower than 500 nt in size, were detected in phage-infected AbiQ-containing cells (Fig.
336 1 and Fig. S2), suggesting that AbiQ modified the transcription profile of the phage.

337 Small transcripts were also detected with probes targeting the *orf38*, *orf40* and *orf53*,
338 during the infection with phage P008-Q12 but their concentration was lower (Fig. 1C and Fig.
339 S2). Moreover, P008-Q12 transcription of *orf53* differed compared to those of P008 in the
340 presence of AbiQ. The transcription started at 10 minutes, but peaked later at 20 minutes
341 followed by a decrease over the remaining 20 minutes (Fig. 1C). This delay may explain the
342 longer latent period of P008-Q12. Taken altogether, AbiQ affects phage transcription profiles.

343 # Discussion

344 Whole genome sequencing of wild-type phage and escaping mutants is a powerful tool to
345 shed light on the biology of phage resistance mechanisms. Here, we identified four different
346 AbiQ targets/activators in phages infecting Gram-positive *L. lactis* and Gram-negative *E. coli*
347 strains. These genes code for proteins with no homology between them, but their genomic
348 context suggests that they are involved with nucleic acids.

349 The *orf38* of *L. lactis* phage P008 is located in the early-expressed gene cluster involved
350 in phage DNA replication and nucleotide metabolism. This protein is well conserved within 936-
351 like phage including those tested in this study. As we could not inactivate *orf38* and we could
352 detect ORF38 production during the infection process, these data strongly suggest that ORF38 is
353 essential for phage P008 replication. Interestingly, a homolog of *orf38*, gene *e14* of phage
354 bIL170, is involved in the activity of another lactococcal Abi mechanism, namely AbiT (32). The
355 AbiT system is made of two genes, which share no similarities with AbiQ and its molecular
356 mechanism is unknown (32). Unexpectedly, AbiQ-escaping mutants derived from bIL170 were
357 mutated in gene *m1* and not in *e14*. Accordingly, AbiQ-escaping P008 phage mutants are still
358 sensitive to AbiT while AbiT-escaping P008 mutants (mutated in *orf41*, see reference 32) as well
359 as AbiT-escaping bIL170 mutants (mutated in *e14*) are also sensitive to AbiQ (data not shown).
360 These data suggests that the genetic context influence Abi's activity.

361 The *m1* gene of phage bIL170 is localized in the middle-expressed gene cluster and
362 appears to have roles in DNA repair and recombination. It is also a conserved gene (>90%
363 identity in amino acid) among 936-like phages. A homolog of the *m1* gene found in phage
364 bIL166 (936 group), gene *orf1*, is critical to the activity of the lactococcal AbiD1 system (19, 20).
365 AbiQ and AbiD1 share no similarities other than providing phage resistance. It has been shown
366 that the wild-type Orf1 induces the expression of AbiD1, while a mutated Orf1 does not (18, 21).

367 It was also demonstrated that the C-terminal part of the Orf1 is essential for phage bIL66
368 replication (19). Considering that the mutations in *m1* of AbiQ-escaping bIL170 mutants likely
369 resulted in no M1 production, this gene does not seem essential to bIL170 replication. These data
370 illustrate the particularities of each phage, even if they belong to the same genotype (936 group).

371 Phage-escaping mutants from the 936 group were obtained only with those (P008 and
372 bIL170) infecting *L. lactis* strain IL1403. No AbiQ-escaping mutant could be obtained from 936-
373 phages (p2, sk1, jj50, 712) that infect *L. lactis* strain MG1363, suggesting that host factors might
374 also be involved. In support of this, the EOP of phage p2 on *L. lactis* MG1363 was previously
375 (33) shown to be below the detection limit ($<10^{-8}$) while that of P008 was 10^{-5} on *L. lactis*
376 IL1403. Moreover, the significant difference between ECOI values of P008 (54.3%) and p2
377 (0.8%) could explain why we were able to isolate AbiQ-escaping mutants with P008 but not p2
378 (33). Summary of phage genes involved in lactococcal Abi phenotypes is summarized in Table 6.

379 Analyses of AbiQ-escaping c2 mutants led to the identification of the *e19* gene, which is
380 located in the early-expressed gene cluster and is flanked by genes related to DNA replication.
381 Interestingly, the *e18* gene of phage c2 has homology with phage P008 *orf37*, suggesting that *e19*
382 (c2) and *orf38* (P008) may have an analogous function. Domain prediction (43) suggested that
383 E19 and ORF38 might have a DNA/RNA binding activity but with a low confidence.

384 While all AbiQ-sensitive wild-type lactococcal phages discussed above belong to the
385 *Siphoviridae* family, the AbiQ-sensitive coliphage 2 belongs to the *Myoviridae* family. Therefore,
386 AbiQ has a much broader range than previously reported (33). Nonetheless, a mutation in the
387 *orf210* of coliphage 2 led to insensitivity to AbiQ. This phage gene likely encodes for a DNA
388 polymerase, with similarities to the chain A of phage T7 DNA polymerase I (51, 52).

389 All AbiQ-escaping phage mutants studied here were found to contain a single mutated
390 gene, which is a sharp contrast to ToxIN-escaping phages of *Pectobacterium atrosepticum*. AbiQ

is related to the type III toxin-antitoxin mechanism ToxIN found in *P. atrosepticum* (36, 37). *Pectobacterium* phage TE has acquired a pseudo antitoxin (pseudo-toxI), which is similar to the antitoxin (toxI) of ToxIN, but with fewer repetitions (1.5 repeats in the phage genome as compared to 5.5 repeats of 36 bp in the host bacteria) (53). To circumvent ToxIN system, escaping TE phage mutants either expanded their pseudo-toxI sequence (from 1.5 to 4.5-5.5 repeats) or recombined with the natural toxI sequence (53). These expanded pseudo-toxI mimic the natural antitoxin, neutralizing the toxin protein (ToxN) during a phage infection (53). Phage TE belong to the *Myoviridae* family and share similarities to coliphage rv5 as does the coliphage 2 characterized in this study. However, unlike phage TE, coliphage 2 does not appear to encode a pseudo *antiQ* in its genome, but both phages encoded the gene ORF210 (TE gp10, 53% amino acid identity). Thus, this study clearly shows that phages can bypass type III toxin-antitoxin systems using at least two different strategies. Interestingly, coliphages T4, Mu, and λvir tested here have also been tested for sensitivity to ToxIN (37). Phages Mu and λvir were found to be insensitive to ToxIN and AbiQ, while T4 is sensitive to AbiQ but not to ToxIN. Hence, these systems share common characteristics but have their own specificities.

We also investigated the impact of one of these AbiQ-escaping mutations on phage fitness. One-step growth curve experiments showed that lactococcal phage P008-Q12 took more time (10%) to complete its lytic cycle and its burst size was reduced by 26%, indicating that the mutation in *orf38* had a fitness cost. Transcriptional analyses supported the above microbiological parameters as gene expression was reduced for phage P008-Q12.

Northern blot experiments coupled with bioinformatic analyses led to a transcriptional map of phage P008 for its early- and middle-expressed genes when infecting *L. lactis* IL1403. The transcription of early-expressed genes started at 2 minutes, peaked at 10 minutes and decreased until the end of the infection. The transcripts for the middle-expressed gene appeared

415 and peaked at 10 minutes followed by a decrease until the end of sampling. This temporal profile
416 was similar to the one reported for the lactococcal phage sk1, another member of the 936 group
417 (54). In the presence of AbiQ, additional small transcripts were observed, likely attributed to the
418 endoribonuclease activity of AbiQ (36), although its specificity still needs to be established.

419 While the data above failed to provide the exact mode of action of AbiQ, they still offered
420 additional detail. In type III TA systems, the antitoxin molecule forms a pseudoknot structure of
421 three antitoxin repetitions bound to three toxin molecules leading to a hetero-hexamer triangular
422 structure (55, 56). It has been also demonstrated that the free toxin can cleave, through its
423 endoribonuclease activity, the cognate antitoxins (36, 54) as well as housekeeping bacterial RNA
424 molecules (55, 56), leading to cell death. During the phage infection process, this TA interaction
425 is likely disrupted leading to cell death and abortion of the phage infection. Since no phage
426 product cleaves the AbiQ antitoxin molecule *in vivo* (36) and very distinct phage genes and/or
427 gene products are involved in AbiQ phenotype, the interaction may be more functional rather
428 than physical. It is also possible that phage products bind antitoxins, thereby freeing the toxins.

429 In summary, AbiQ is a powerful resistance mechanism effective against two phage
430 morphotypes (*Siphoviridae* and *Myoviridae*), including those infecting Gram-positive and Gram-
431 negative bacteria. Its efficiency is likely due to the endoribonuclease activity on phage
432 transcripts. Phages can bypass this system through point mutation in four different phage
433 targets/activators, which demonstrated the complexity of this anti-phage system. Finally, a
434 general trend is starting to emerge that several lactococcal Abi systems (AbiD1, AbiK, AbiQ,
435 AbiT, AbiV) appear to target early-expressed genes as well as proteins involved in activities
436 related to nucleic acids. It is tempting to speculate that the use of multiple anti-phage mechanisms
437 in a single strain that are targeting the same phage genomic region may provide the coveted long-
438 term phage resistance to industrially relevant bacteria.

439

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441

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450

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452

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651

652 **Table 1.** Bacterial strains, plasmids, and phages used in this study.

Bacterial strains, plasmids and phages	Relevant characteristics	References
Escherichia coli		
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´ *proAB lacI*^{q}Z∆*M15* Tn*10* (Tet^{r})]	Stratagene
MG1655	*F^{-} λ^{-} ilvG- rfb-50 rph-1*, GenBank U00096	(57)
DH5-α	*supE44* Dlac U169 (f80 *lacZ*DM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Gibco/BRL
Lactococcus lactis		
IL1403	Plasmid free, host of P008 and bIL170, GenBank NC_002662	(58, 59)
MG1363	Plasmid free, host of p2, GenBank NC_009004	(60, 61)
LM0230	Plasmid free, host of jj50, sk1, 712, and c2	(62)
Plasmids		
pNZ123	High copy number vector, Cm^{r}, 2.8 kb	(63)
pTRKH2	High copy number vector, Em^{r}, 6.9 kb	(64)
pMIG3	Medium copy number vector, Cm^{r}, 5.5 kb	(65)
pSRQ928	pNZ123 + 2.2 kb fragment containing AbiQ	(33)
pSRQ925	pMIG3 + 2.2 kb fragment containing AbiQ	(33)
pNZ123-AbiQ	pNZ123 + AbiQ operon	(36)
pTRKH2-*orf38*	pTRKH2 + *orf38* of phage P008	This study
pTRKH2-*orf38M* (Q12)	pTRKH2 + *orf38* of phage P008-Q12	This study
pTRKH2-*orf38*null	pTRKH2 + *orf38* with two stop codon in the position 5 and 6 of the protein	This study
pTRKH2-*orf38*fs	pTRKH2 + *orf38* with a reading frame modification in the fifth codon	This study
Phages		
P008	*Siphoviridae*, 936 group, propagated on IL1403, GenBank DQ054536	(46)
bIL170	*Siphoviridae*, 936 group, propagated on IL1403, GenBank AF009630	(48)
jj50	*Siphoviridae*, 936 group, propagated on LM0230, GenBank NC_008371	(46)
sk1	*Siphoviridae*, 936 group, propagated on LM0230, GenBank NC_001835	(66)
712	*Siphoviridae*, 936 group, propagated on LM0230, GenBank NC_008370	(46)
p2	*Siphoviridae*, 936 group, propagated on MG1363, GenBank GQ979703	(67)
c2	*Siphoviridae*, c2 group, propagated on LM0230, GenBank NC_001706	(49)
T1	*Siphoviridae*, propagated on MG1655, GenBank NC_005833	(68)
T3	*Podoviridae*, propagated on MG1655, GenBank NC_003298	(69)
T4	*Myoviridae*, propagated on MG1655, GenBank NC_000866	(70)
T5	*Siphoviridae*, propagated on MG1655, GenBank NC_005859	(71)
Lambda vir	*Siphoviridae*, propagated on MG1655, GenBank NC_001416	(72)
RB69	*Myoviridae*, propagated on MG1655, GenBank NC_004928	(73)
HK97	*Siphoviridae*, propagated on MG1655, GenBank NC_002167	(74)
Mu	*Myoviridae*, propagated on MG1655, GenBank NC_000929	(75)
pilHα	*Leviviridae*, propagated on MG1655	(76)
2	*Myoviridae*, propagated on MG1655, GenBank KC690136	(50)

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654

655 **Table 2.** Characterization of lactococcal AbiQ-escape mutants derived from phages P008,

656 bIL170, and c2.

Phage[a]	EOP[b]	Frequency	Mutated ORF	Amino acid substitution
P008	$1.5 \pm 1.3 \times 10^{-5}$	-	-	-
P008-Q1	$9.0 \pm 0.8 \times 10^{-2}$	1/22	RBS *orf38*[c]	-
P008-Q14	$8.1 \pm 1.1 \times 10^{-2}$	1/22	RBS *orf38*[d]	-
P008-Q17	$1.5 \pm 0.9 \times 10^{-2}$	1/22	ORF38	Met1Leu
P008-Q5	$3.4 \pm 0.7 \times 10^{-2}$	1/22	ORF38	Thr3Thr
P008H2-10	$1.2 \pm 0.4 \times 10^{-2}$	1/22	ORF38	Thr3Ile
P008H2-7	$2.6 \pm 1.9 \times 10^{-2}$	1/22	ORF38	Glu6Gly
P008-Q4	$2.5 \pm 0.7 \times 10^{-2}$	1/22	ORF38	Ser18Asn
P008-Q16	$3.9 \pm 1.3 \times 10^{-2}$	6/22	ORF38	Asp23Gly
P008-Q19	$4.0 \pm 0.6 \times 10^{-2}$	2/22	ORF38	Trp30Stop
P008-Q12	$3.3 \pm 1.2 \times 10^{-2}$	6/22	ORF38	Pro38Leu
P008-Q11	$4.1 \pm 1.3 \times 10^{-2}$	1/22	ORF38	Ser49Pro
bIL170	$< 10^{-6}$	-	-	-
bIL170-Q2	$1.7 \pm 1.0 \times 10^{-1}$	6/9	M1	Met1Phe
bIL170-Q22	$3.2 \pm 2.5 \times 10^{-1}$	3/9	M1	Gln5Stop
c2	$< 10^{-6}$	-	-	-
c2-Q3	1	5/5	E19	Glu17Asp

657 [a] Phages in bold indicate those for which the complete genome was sequenced.
658 [b] The EOP and standard deviation values were calculated for three assays done in triplicate.
659 [c] Consensus RBS in *L. lactis* : AGAAAGGAGGT, RBS of *orf38* in wild-type phage P008 :
660 AGAAAGTCGGT, RBS mutation: AGAAA<u>T</u>TCGGT (position 21,406).
661 [d] RBS mutation : AGAAAGTC<u>T</u>GT (position 21,403).
662

663 **Table 3.** Sensitivity of different *E. coli* phages to AbiQ.

Phage	Family	EOP[a]
T1	*Siphoviridae*	1.1 ± 0.1
T3	*Podoviridae*	1.1 ± 0.5
T4	*Myoviridae*	$< 1.2 \pm 0.3 \times 10^{-6}$
T5	*Siphoviridae*	$< 5.4 \pm 0.5 \times 10^{-7}$
Lambda vir	*Siphoviridae*	1.9 ± 0.1
RB69	*Myoviridae*	$< 3.5 \pm 1.8 \times 10^{-6}$
HK97	*Siphoviridae*	0.4 ± 0.3
Mu	*Myoviriadae*	1.2 ± 0.2
pilHα	*Leviviridae*	1.3 ± 0.3
2	*Myoviridae*	$2.0 \pm 0.6 \times 10^{-5}$

664 [a] The EOP and standard deviation values were calculated for three assays done in triplicate.

665

666 **Table 4.** Phage 2 mutants characterization.

Phage[a]	EOP[b]	Frequency	Mutated ORF	Amino acid substitution
2	$2.0 \pm 0.6 \times 10^{-5}$	-	-	-
2Q1	0.7 ± 0.1	1/5	ORF210	Lys331Glu
2Q2	0.5 ± 0.1	1/5	ORF210	Val681Ala
2Q3	0.6 ± 0.1	1/5	ORF210	Asn426Lys
2Q4	0.6 ± 0.1	1/5	ORF210	Glu298Gly
2Q5	0.7 ± 0.1	1/5	ORF210	Met290Val

667 [a] Phages in bold indicate those for which the complete genome was sequenced.
668 [b] The EOP and standard deviation values were calculated for three assays done in triplicate.
669

670 **Table 5.** EOPs of phages P008 or P008-Q12 on various *L. lactis* strains.

L. lactis IL1403[a]			EOP[b]	
abiQ *(pNZ123)*	*orf38* *(pTRKH2)*	*orf38M* *(pTRKH2)*	P008	P008-Q12
- [c, d]	-	-	1.0 ± 0.1	1.0 ± 0.1
- [d]	+	-	1.0 ± 0.1	0.8 ± 0.4
- [d]	-	+	0.8 ± 0.4	0.5 ± 0.4
+ [c]	-	-	$0.9 \pm 0.1 \times 10^{-5}$	0.1 ± 0.1
+	+	-	$1.3 \pm 0.3 \times 10^{-5}$	0.2 ± 0.1
+	-	+	$14.9 \pm 0.7 \times 10^{-5}$	0.1 ± 0.1

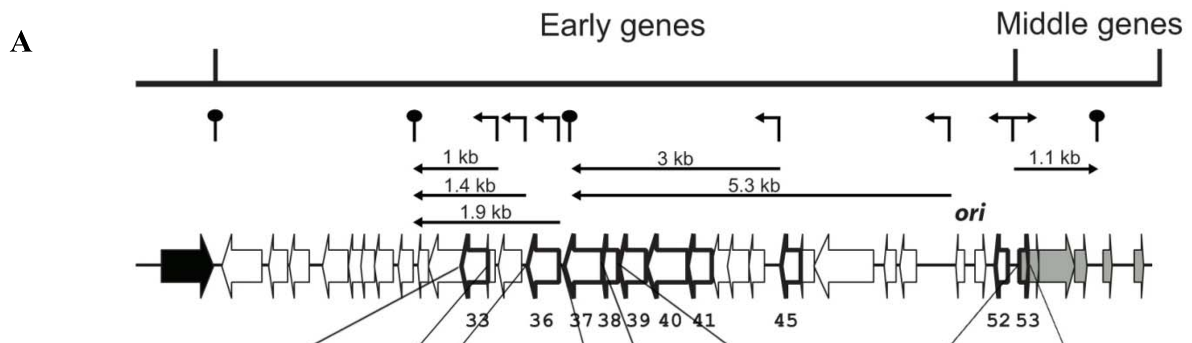
671 [a] The – and + indicate the presence or not of these genes in the various strains tested.
672 [b] The EOP and standard deviation values were calculated for three assays done in triplicate.
673 [c] The strains also contain pTRKH2 empty vector.
674 [d] The strains also contain pNZ123 empty vector.
675

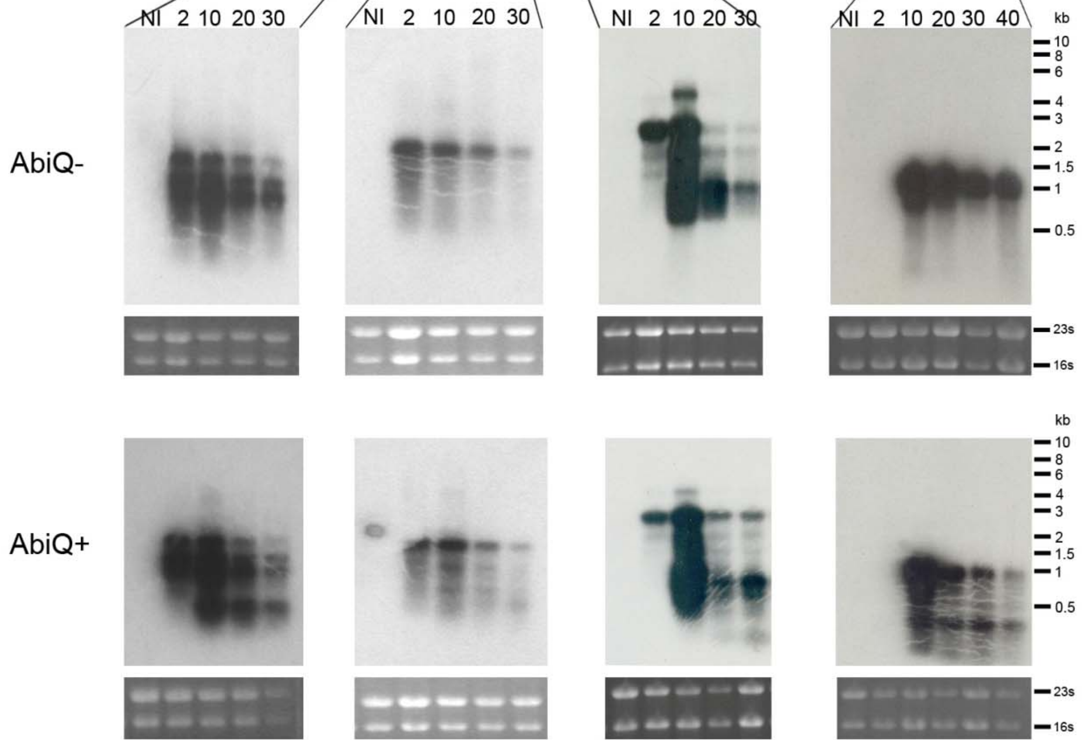
676 **Table 6.** Summary of the different lactococcal Abi's targets and their links in related 936 phages

Homolog phage genes[a]				Timing of expression	Function of gene products	Abi's target/activators
P008	bIL170	bIL66	p2			
orf6-orf7	*l6-l7*	nd	***orf6***	Late	Major capsid protein	AbiT
orf38[b]	***e14***[c]	*e14*	*orf33*	Early	Unknown	AbiQ and AbiT
orf40	*e12*	*e12*	***orf35***	Early	SSAP (Sak)	AbiK
orf41	-	*e11*	*orf36*	Early	HNH endonuclease	AbiT
orf53	***m1***[b]	***orf1***[d]	*orf46*	Middle	AbiD1 activator	AbiD1 and AbiQ

677 [a] *orfs* in bold indicate genes experimentally demonstrated to be involved in Abi phenotype.
678 [b] *orf involved in AbiQ*
679 [c] *orf involved in AbiT*
680 [d] *orf involved in AbiD1*

681





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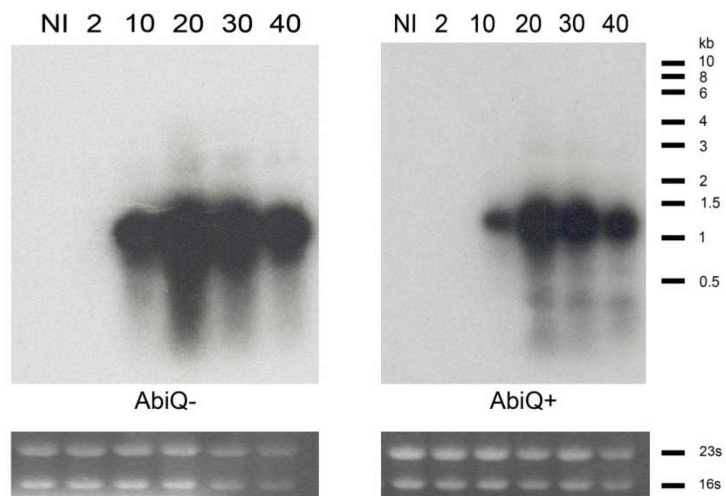

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685 **Figure 1.** Transcription map of early- and middle-expressed genes from wild-type phage P008
686 and phage-escaping mutant P008-Q12. Panel A) Summary of P008 partial transcription map
687 deduced from Northern blots and bioinformatics analyses. Detected phage P008 genes in the
688 different experiments are represented by arrows with thick lines and numbers (Fig. S2 for
689 supplementary results). The white arrows correspond to early-expressed genes, grey arrows to
690 middle-expressed genes, and the black arrow to a late-expressed gene. Promoters and terminators
691 determined by bioinformatics analyses are above the genome and are represented by broken thin
692 arrows and hairpins, respectively. Transcripts and their size detected by Northern blots and
693 confirmed by bioinformatics analysis are indicated by straight arrows below the promoters-
694 terminators. Panel B) Transcripts of P008 genes *orf33*, *orf36 orf38,* and *orf53* detected by
695 Northern blots during a time course infection in the absence (-) or in the presence (+) of AbiQ.
696 RNA samples (5 µg) at time NI (non-infected), 2, 10, 20, 30, and 40 minutes were migrated on
697 agarose gels and transfer on nylon membranes. Loading controls of the RNA 23S and 16S
698 migration are presented below each membrane. Northern blots were performed with ^{32}P-
699 oligonucleotides probes complementary to the *orfs*. Ladder size bands are indicated on the right
700 (0.5-10 kb ladder, Invitrogen). Panel C) Transcripts of P008-Q12 gene *orf53* detected by
701 Northern blots during a time course infection in the absence (-) or in the presence (+) of AbiQ.