Mutational analysis of the antitoxin in the lactococcal type III toxin-antitoxin system AbiQ

Maxime Bélanger[1] and Sylvain Moineau[1*]

[1] Département de biochimie, de microbiologie et de bio-informatique & PROTEO, Faculté des sciences et génie, GREB & Félix d'Hérelle Reference Center for Bacterial Viruses, Faculté de Médecine Dentaire, Université Laval, Québec City, Québec, Canada, G1V 0A6

* Corresponding author. Mailing address: Groupe de recherche en écologie buccale, Faculté de médecine dentaire, Université Laval, Quebec City, Quebec, Canada, G1V 0A6
Phone: 418-656-3712. Fax: 418-656-2861. E-mail: Sylvain.Moineau@bcm.ulaval.ca

Abstract

The lactococcal abortive phage infection mechanism AbiQ was recently classified as a type III toxin-antitoxin system in which the toxic protein (ABIQ) is regulated following cleavage of its repeated non-coding RNA antitoxin (*antiQ*). In this study, we investigated the role of the antitoxin in anti-phage activity. The cleavage of *antiQ* by ABIQ was characterized using 5'RACE PCR and was located in an adenine-rich region of *antiQ*. Next, we generated a series of derivatives with point mutations within *antiQ* or with varying number of *antiQ* repetitions. These modifications were analyzed for their effect on the anti-phage activity (efficiency of plaquing) and on the endoribonuclease activity (Northern hybridization). We observed that increasing or reducing the number of *antiQ* repeats significantly decreased the anti-phage activity of the system. Several point mutations had a similar effect on the anti-phage activity and were associated with changes in the digestion profile of *antiQ*. Interestingly, point mutations in the putative pseudoknot structure of *antiQ* mutants led to an increased AbiQ anti-phage activity, thereby offering a novel way to increase the activity of an abortive infection mechanism.

Introduction

Lactococcus lactis is a Gram-positive bacterium used by the dairy industry to transform milk into fermented products such as cheese and yogurt. Many virulent phages specific to *L. lactis* strains have emerged over years of production and despite numerous control strategies, they still represent one of the major risks of productivity loss in cheese factories (1). The constant threat of phage infection led to the selection of strains with robust natural anti-phage systems. Anti-phage mechanisms can either prevent phage adsorption, block the entry of phage DNA, cleave foreign nucleic acids using restriction-modification systems or CRISPR-Cas systems, or abort infection through altruistic suicide (2). The latter group of anti-phage mechanisms are known as abortive infection systems (Abi). Globally, they act at various steps of the phage replication cycle, from DNA replication to bacterial lysis (3, 4), but their common characteristic is inducing cell death in phage-infected bacteria, seemingly to favour the survival of the bacterial population (3).

To date, an impressive number of distinct Abi systems have been identified in *L. lactis* (3-6). These 23 systems are effective, at various degrees, against some or all prevalent groups of lactococcal phages (936, c2, and P335) found in dairy plants (3). Nevertheless, only a few Abi systems have been characterized at the molecular level. In the lactococcal AbiD1 system, the phage protein ORF1 (bIL66) activates *abiD1* translation and the effective bacterial protein AbiD1 reduces transcription of a phage gene coding for a RuvC-like resolvase that is essential for replication and maturation of viral DNA (7-10). In the AbiK system, the AbiK protein has a template-independent reverse transcriptase activity that generates random complementary DNA fragments, which likely prevents viral protein translation (11, 12). In AbiP$^+$ cells, phage DNA

replication is stopped by the accumulation of early transcripts that prevent transcription of the middle/late phage genes (13). It has been suggested that this phenotype is caused by direct binding of the AbiP membrane protein to RNA and ssDNA (14). Like AbiP, AbiV causes a significant reduction in transcription of the middle- and late-expressed genes (15). During the infection, the phage protein SaV directly interacts with AbiV and inhibits the translational machinery of the cell (15, 16). Finally, AbiQ was recently identified as a type III toxin-antitoxin system (17).

A toxin-antitoxin (TA) system is typically a bicistronic operon that codes for a toxic protein and its cognate antitoxin which is more prone to degradation under stress conditions (18). Originally described for their role in post-segregational killing (plasmid stabilization) with the characterization of the CcdA/CcdB system (19), many other functions have now been associated to TA systems: protection against phages, persistence, biofilm formation, global cell regulation and stabilization of mobile genetic elements (20-25). TA are currently divided into five groups (type I-V) based on the nature of the antitoxin and the mode of regulation of its cognate toxin (18, 20).

Type I TA systems involve an antisense RNA that regulates translation of the toxic protein (26). In type II systems, an antitoxic protein interacts directly with a toxic protein to inhibit its activity (27). The type III systems involve a non-coding RNA that regulates the toxin through protein:RNA complex formation (25, 28). Such as type II systems, type IV systems involve two proteins but the antitoxin interacts with the target of the toxin, rather than the toxin itself, to prevent toxin activity (29, 30). Finally, the antitoxin of type V system has specific endoribonuclease activity that regulates toxic gene transcription (31).

Only a few type III TA systems have been characterized to date (17, 25, 28, 32). ToxIN, an Abi system from *Pectobacterium atrosepticum* ($ToxIN_{Pa}$) was the first to be studied and is the

model for type III systems (25). The antitoxin (ToxI) is a 5.5-repeat of a 36 nt non-coding RNA that is specifically cleaved by the toxin (ToxN) (25, 28). The mature small RNA fragment (one repeat of 36 nt) interacts directly with ToxN, forming a triangular hetero-hexameric (3ToxI:3ToxN) complex that inhibits toxicity (28). It has also been shown that the secondary structure (pseudoknot) of ToxI RNA is essential for the antitoxic activity (28). Under stress conditions, ToxN is free and can target essential mRNAs, leading to cell growth arrest and preventing phage replication (25, 28, 32).

The protein ToxN has 31% identity with the ABIQ protein from the *L. lactis* AbiQ system, suggesting a similar mode of action (25, 33). The antitoxin, *antiQ*, is a 2.8-repeat of 35 nt located downstream of a rho-independent terminator and the *abiQ* gene, respectively (17, 33). Similar to $ToxIN_{Pa}$, the toxic ABIQ protein is an endoribonuclease that specifically cleaves its cognate antitoxin (17). The superimposed 3D-structures of these two systems suggests that they share a similar mechanism of regulation (17). Interestingly, the endoribonuclease activity is not necessarily associated with the anti-phage activity, as key amino acid residues of ABIQ protein were different for both activities (17). AbiQ was first described as a defense mechanism against lactococcal phages of the c2 and 936 phage groups and its activity resulted in the accumulation of concatemeric viral DNA (33). It was recently shown that phage P008 (936 group) can become resistant to AbiQ through mutations within its *orf38* gene or ribosome binding site, suggesting that the phage protein ORF38 is playing an essential role in the activity of AbiQ (34).

In this study, we investigated the role of the *antiQ* antitoxin region and the effect of mutations in this region on the anti-phage activity of the lactococcal abortive infection mechanism AbiQ.

Materials and Methods

Bacterial strains and phage propagation. Phage, bacteria, and plasmids used in this study are listed in Table 1. All *L. lactis* strains were grown at 30°C in M17 medium (Oxoid) supplemented with 0.5% glucose (GM17). *Escherichia coli* was grown in LB medium at 37°C with agitation. When needed, ampicillin (100 µg/ml) or chloramphenicol (5 µg/ml) was added to the media. For phage propagation, *L. lactis* IL1403 was grown at 30°C to an optical density 600 nm (OD_{600}, Spectronic 20) of 0.1 before the addition of 10^5-10^7 plaque forming units (PFU)/ml and $CaCl_2$ to a final concentration of 10 mM. After complete lysis, the clear lysate was passed through a 0.45 µm filter and kept at 4°C until use. Phage titer was determined by the double agar overlay plaque assay (35), in triplicate, on a lawn of *L. lactis* IL1403 on GM17 plates, and incubated overnight. The efficiency of plaquing (EOP) was calculated by dividing phage titer on a resistant strain ($AbiQ^+$) by phage titer on a sensitive strain ($AbiQ^-$). To increase titer, phages were purified on a discontinuous CsCl gradient (36).

Cloning and DNA manipulation. Primers used in this study are found in Supplementary Materials (Table S1). Plasmids (Table 1) were purified using a QIAprep spin Miniprep kit according to the manufacturer's instructions. For *L. lactis* strains, a lysozyme treatment (30 mg/ml in P1 buffer at 37°C for 20 minutes) was added to weaken the cell wall. To construct plasmids of interest, an endonuclease-based cloning strategy was carried out as described elsewhere (36). Briefly, DNA fragments were amplified by standard PCR, digested using restriction enzymes, ligated to a dephosphorylated restricted plasmid (O/N at 16°C) and then introduced into competent cells. Enzymes and commercial kits were used as recommended by the

manufacturers: restriction enzymes (Roche), Taq DNA polymerase (Feldan), Antarctic phosphatase (New Englands Biolabs), T4 DNA ligase (Invitrogen), and QIAquick PCR purification (Qiagen). DNA was sequenced at the Plateforme de séquençage et de génotypage des génomes of the CHUL center. Sequences were analysed using BioEdit (37), Staden (38), or Genious software (39).

5' Rapid amplification of cDNA ends (5' RACE PCR). Approximately 10^9 cells (O/N culture) of *L. lactis* IL1403 pNZ123-AbiQ were centrifuged and the pellets flash frozen (ice-cold isopropanol 80%) at -80°C. After pre-treating the cells with lysozyme (60 mg/ml in 25% sucrose solution) for 10 minutes at 37°C, total RNA was extracted using Trizol reagent, as recommended by the manufacturer (Invitrogen). The extracts were then treated with DNase I (Roche) and protected with RNase inhibitors (Roche) for 30 minutes at 37°C, before being purified again using RNA clean up protocols (RNeasy kit) as recommended (Qiagen). Total RNA was retro-transcribed (primer JS2) as per manufacturer's instructions using Superscript III (Invitrogen) and then treated with RNase H (Roche). The resulting double stranded complementary DNA (cDNA) was purified using a PCR cleanup kit (Qiagen). A 5' poly (G) tail was added using terminal transferase, as recommended (Invitrogen). The cDNA was amplified by PCR using primers PolyC/AbiQRev or AbiQFwd/AbiQRev (control) before being directionally cloned in pBluescript II KS+ (XhoI and EcoRI restriction sites), and transferred into *E. coli* XL1-blue for blue/white screening (40). Plasmids from clones were extracted and the insert regions sequenced (primer M13Fwd/M13Rev).

Site directed mutagenesis. Mutagenesis was carried out as described elsewhere (17). Briefly, the plasmids were isolated from *L. lactis* IL1403 and then transferred into *E. coli* MG1655 by

chemical transformation (36). Plasmid DNA was extracted before being amplified by PCR (16 cycles) using mutated reverse-complementary primers in combination with the high fidelity enzyme Pwo (Roche). The residual template plasmid was removed using DpnI (Roche) that cleaves methylated DNA. Amplified plasmids were introduced into the sub-cloning strain *L. lactis* MG1363 by electroporation (41), confirmed by sequencing (primer pNZ-F/pNZ-R) before being moved into *L. lactis* IL1403 by electroporation (41).

Bacterial growth and stationary phase mortality experiments. Bacterial growth was measured at an optical density of 630 nm (OD_{630}; Biotek Synergy 2 spectrophotometer). For each strain tested, eight experimental and three biological replicates were set up in 96-well plates. The growth rate (g) was calculated by manual determination of the slope in the exponential growth phase. In stationary phase mortality tests, bacteria were grown for six hours and then sampled at various times (t_{0h}, t_{1h}, t_{2h}, t_{4h} and t_{8h}). Samples were diluted in cold GM17 medium, spread on GM17 agar medium and grown overnight prior to counting colony forming units (cfu).

Northern hybridization. A time-course phage infection (non-infected, 2, 10, 20, 30 and 40 minutes) was carried out as described previously (17). Total RNA was first extracted and purified as described above (5' RACE PCR). The concentrations of total bacterial RNA extracts were determined using a NanoDrop 2000 and diluted to a concentration of 1 µg/µl. A total of 5 µg of purified RNA was added to formamide loading buffer (98% deionized formamide, 10mM EDTA pH 8.0, 0.025% xylene cyanol and 0.025% bromophenol blue) at a 1:1 ratio and separated on a 10% polyacrylamide/8 M urea denaturing gel. The RNA was electrophoretically-transferred to a nylon membrane (Roche) before being fixed by exposure to UV for 2 minutes (36). A DNA probe complementary to the *antiQ* antitoxin (GCTCCAATTTTATCAATTCCAACTATGGCTTGGATA) or the *abiQ* gene (GGGGTATTAATTCGCTGTCAGGAACTGGAATC) was radiolabelled with ^{32}P (Perkin-Elmer) using polynucleotide kinase (Roche), and purified using a Micro Bio-spin P-30 size-exclusion column (Bio-Rad). The radiolabeled probes were diluted to 1×10^6 cpm/ml with Beckman Coulter LS6500. Hybridization was carried out for 18 hours at 42°C, the filters were washed twice in SSC 2X+ SDS 0.05%, and revealed by autoradiography using BioMax XAR films (KODAK).

Results

ABIQ cleaves its antitoxin (*antiQ*) in an adenine-rich region. When the non-coding RNA antitoxin, *antiQ*, is cleaved by its cognate toxin ABIQ, a specific digestion profile is produced (17). To further characterize this RNA maturation process *in vivo*, we used 5'RACE PCR to determine the cleavage site and also the transcription initiation site of the AbiQ operon. We extracted total RNA from *L. lactis* IL1403 AbiQ$^+$ and retro-transcribed it into cDNA before carrying out PCR amplification using the added 5'poly guanine tail as guide for the primer (Fig. 1). The RACE-AbiQ PCR products included a band at 450 bp, surrounded by two bands of lower intensity, approximately 50 bp apart. These cDNA fragments corresponded to the expected sizes of digested *antiQ* RNA fragments (350-450 bp) and the addition of 5' non-complementary primer nucleotides (49 bp). This PCR extract was cloned into the vector pBS-KS, transferred into *E. coli*, and the clones screened for ampicillin resistance and PCR products with the expected insert length (400-500 bp). Twelve positive clones were sequenced (Fig. 2).

Point mutations were observed in some of the sequences and are thought to be caused by the error rate of the reverse transcriptase (42) combined with the error rate of the Taq polymerase. In each case, the 5'end of the sequenced fragment was represented by the polycytosine sequence confirming the clones (Fig. 2). Five clones (#6-10) demonstrated the specific ABIQ cleavage site within the *antiQ* sequence. In all cases, the cleavage site was between adenine 26 and adenine 27 (A/AAA) of the first or second repeats. None of the clones showed cleavage in last repeat (0.8 repeats), suggesting no or a much lower frequency of cleavage even if the sequence of the last repeat is identical to the other two repeats up to nucleotide 29.

Transcription of the AbiQ operon. Analysis of the sequencing results of clones #1-5 (Fig. 2) indicates that transcription of the AbiQ operon starts at one of two adjacent nucleotides: T at the -7 position or A at the -6 position, relative to the first nucleotide of the first *antiQ* repeat. Bioinformatics analysis suggested similar results by predicting the transcription start site (TSS) at the same T with a 99% degree of confidence (Neural Network Promoter Prediction – prokaryote, http://www.fruitfly.org/seq_tools/promoter.html). This also agrees with the -10 and -35 bacterial promoter boxes identified respectively at -8 (TATAAT) and -34 (TTGCAT) (not shown).

***antiQ* is cleaved less frequently in the last repeat (0.8 repeat).** The above cleavage and transcription start sites were then used to associate mature (cleaved) fragments of *antiQ* to the specific bands previously observed (17) in the *in vivo* digestion profile as visualized by Northern hybridization (Fig. 3). Of the theoretical possible small RNA fragments, only the 106 nt fragment (transcription start site to last cleavage site (0.8r)) and the 74 nt fragment (first cleavage site to last cleavage site (0.8r)) appeared to be rare or absent, respectively. In both cases, the formation of these fragments is dependent on cleavage by ABIQ within the last repeat and they support the above cloning results that suggested a low frequency of cleavage within this 0.8 repeat.

The number of repeats in *antiQ* is important to the anti-phage activity. In a previous study, we investigated the effect of point mutations in the gene coding for the toxin protein, ABIQ (17). In the process of generating these mutations, we isolated a few clones that had acquired or lost repeat sequences in the *antiQ* region (0.8 to 3.8 repeats). These mutants were also detected here as two clones (#11-12) lost two complete repeats. Interestingly, one of the clones (#11) lost two complete repeats without any AbiQ mutations, suggesting that the variation in the number of repeats is not inevitably associated with mutation in the AbiQ operon (Fig. 2).

We used directed mutagenesis to construct mutants in *antiQ* that varied in the number of repeats by bringing back the wild type sequence of *abiQ*. Mutants containing 1.8 and 3.8 repeats were easily obtained. However, we were unable to isolate clones with only the 0.8 repeat, suggesting that a 0.8 repeat is not enough to avoid the toxic effects of ABIQ. We then compared the anti-phage activity of these clones against phage P008 by evaluating the EOP (Fig. 3). Deleting (1.8r) or adding (3.8r) one repeat to the wild-type 2.8 repeats led to a significant reduction (3 to 5 logs) in the phage resistance phenotype. Moreover, in contrast to phage escape mutants obtained on wild-type AbiQ (34), none of the phage from plaques picked on the two mutated *antiQ* plates had mutations in the *orf38* gene (data not shown).

We also performed Northern hybridization experiments using a probe complementary to one repeat of *antiQ*. As expected, the cleavage pattern of *antiQ* was affected by the number of repeats, as more RNA fragments were observed with the added repeat while a much simpler profile was obtained with the 1.8 repeats (Fig. 3). However, the expected relative abundance of each fragment was not affected (Fig. 3). Then, we infected AbiQ-containing strains (Mut 1.8r, Mut 3.8r and AbiQ-wt) with P008 and performed Northern hybridization experiments targeting *antiQ* or *abiQ*. We observed no differences between strains containing variable repeats, demonstrating that the reduction in anti-phage activity was not caused by differences at the level of transcription of either *antiQ* or the toxic gene *abiQ* (Fig. S1). Taken together, these results suggest that the wild type length of *antiQ* (2.8 repeats) is critical for optimal anti-phage activity.

More than one mature *antiQ* fragment plays a role in the regulation of ABIQ toxin. To further characterize the *antiQ* gene coding for the antitoxin, we tested the effect of point mutations on AbiQ anti-phage and endoribonuclease activities (Fig. 3). The mutations were introduced specifically in the first repeat, leaving a wild-type sequence of 1.8 repeats previously

shown to be sufficient for cell survival (see above). The mutations mostly surrounded the cleavage site (A26/A27) in the *antiQ* repeat (A24C, T25C, A26C and A28C). The A13C mutation was used as a control as we observed no differences in phage EOPs and *antiQ* digestion profile as compared to the wild type *antiQ* (Fig. 3). Similar results were obtained for mutation T25C, located only two nucleotides from the cleavage site. This nucleotide is likely not essential for ABIQ recognition and/or cleavage, or that it is permissive to pyrimidine bases at this site.

Conversely, mutations A24C, A26C and A28C resulted in significant changes to the digestion profile. The A24C mutation partially reduced the amount of the 97 nt fragment (first cleavage site to terminator) and similarly increased the amount of the 106 nt fragment (transcription start site to last cleavage site (0.8r)). A similar pattern was obtained for mutation A26C, but the transition between these two fragments was more complete. Based on the digestion profile described in Figure 3, these two mutations partially (A24), or completely (A26), prevented cleavage in the first repeat, which likely leads the protein ABIQ to target the 0.8 repeat region. The clone containing the A28C mutation carried a duplication of the mutated repeat. The digestion profile suggested partial inhibition of cleavage for A28C, similar to A24C. We performed bioinformatics analysis (RNAfold software) to compare the predicted secondary structures of wild type and mutated *antiQ* RNA (Fig. S2). The wild type *antiQ* sequence had free access to the first ABIQ cleavage site, unlike the A24 and A28 mutants, where this site was likely sterically hindered. The A26 mutant resulted in no predicted modification to the general structure, suggesting that this nucleotide is essential for recognition and/or cleavage by ABIQ. In all three cases (A24C, A26C and A28C), the single point mutation led to a phage EOP of almost 1, indicating a significant loss of anti-phage activity for these mutated AbiQ systems. These results confirm that the number, and relative abundancy, of ABIQ-generated *antiQ* RNA fragments play a role in the control of ABIQ toxicity and anti-phage activity.

Anti-phage activity of AbiQ can be significantly increased by a point mutation in the putative pseudoknot structure of *antiQ*. It has been described, for the type III toxin-antitoxin system $ToxIN_{Pa}$, that specific point mutations in the RNA antitoxin can reduce cell survival, mostly by affecting pseudoknot secondary structure of RNA (28). Based on the model, the free toxin causes cell death and prevents phage replication. We generated a G32A mutant, which would significantly modify the predicted secondary structure (pseudoknot) of the *antiQ* RNA (Fig. S3). Two different mutants containing G32 mutation were obtained with either 2.8 or 3.8 repeats (duplication of the mutated repeat). Both clones were characterized for their anti-phage activity and endoribonuclease digestion profile (Fig. 3).

Interestingly, the anti-phage efficiency of ABIQ was significantly increased (3 logs) with a mutated *antiQ* G32A as compared to the with-type *antiQ* (from an EOP of 10^{-5} to an EOP 10^{-8}). Remarkably, the mutated *antiQ* G32A with 3.8 repeats also increased ABIQ efficacy by 6 logs as compared to the wild-type *antiQ* with 3.8 repeats (from an EOP of 10^{-1} to an EOP 10^{-7}). No difference in the relative abundance of the RNA fragments was observed between the two clones (other than the extra repeat). However, the 97 nt fragment (first cleavage site to terminator) increased relative to all other bands in the pattern. It is still not clear if this modification plays a role in AbiQ regulation but it suggests that the number of inhibitory RNA fragments is important. To further characterize the mutated *antiQ* G32A, we analyzed the bacterial fitness of these clones. The comparison between wild-type (2.8r) and mutated (G32A-2.8r) revealed no differences between generation times (60 minutes, data not shown). Stationary phase mortality assays also demonstrated no differences between the strains tested (data not shown), strongly suggesting that the *antiQ* G32A mutation does not affect fitness while increasing anti-phage efficiency. Taken together, our results showed the role of the G32A mutation within *antiQ* for optimization of complete anti-phage activity.

Discussion

Described as an efficient abortive infection system against prevalent dairy phage groups (936 and c2) (33), the AbiQ mechanism has recently been reported as a type III TA system (17). To understand the molecular basis of the mechanism, two previous studies focused on the role of specific phage proteins in AbiQ anti-phage activity (34) and on the specificity of the toxic protein ABIQ within the system (17). Here, we investigated the role of the antitoxin (*antiQ*, 2.8 repeats of 35 nt), emphasizing the effects of mutations on the anti-phage activity.

Within the AbiQ system, the toxic protein (ABIQ) is a sequence-dependent endoribonuclease that specifically cleaves its cognate non-coding RNA antitoxin (17). Our 5' RACE experiments showed that the cleavage site was located within the repeat between adenine 26 and adenine 27 in an adenine rich region (A/AAA), one nucleotide away from *in silico* ABIQ-*antiQ* cleavage site prediction (/AAAA) (43). Interestingly, the toxin of the two other type III TA systems also cleaved RNA fragments in adenine-rich regions ($ToxIN_{Pa}$: AA/AU and $ToxIN_{Bt}$: A/AAAA) (32). In *antiQ*, this poly-adenine sequence is found in the complete repeats (R1 and R2) and also in the last 0.8 repeat (R3). Our data showed that ABIQ seems to cleave at same frequency between the first and the second repeat. However, cleavage in the last repeat is either rare or absent. This may be explained by the presence of a stem-loop structure (transcriptional terminator) next to the last repeat that could sequester the cleavage site. Moreover, secondary structure prediction of *antiQ* RNA suggests that the poly adenine region interacts with poly uracil residues of the terminator sequence, increasing the total length of the stem-loop structure. In a few cases, this extension of the stem-loop structure (rho-independent terminator) can lead to reduced termination efficiency (44). It is also possible that nucleotides 30 to 35 (absent in the last repeat) are important for recognition and/or cleavage by ABIQ.

Using 5' RACE PCR, the transcription start site of *abiQ* was determined to be 6 or 7 nucleotides upstream of the start of the first repetition, more specifically at a thymine or adenine residue, respectively. This double transcription site phenomenon has been described in the characterization of transcription start sites in *E. coli* (45). Interestingly, thymine and adenine were identified as transcription start sites in 35% (7 nt upstream) and 31% (6 nt upstream) of cases. We also investigated the role of specific modifications within the *antiQ* region. Our data showed that 1.8 repeats (*antiQ*) are enough to enable cell survival (and neutralize ABIQ) because clones were obtained, which was not possible with only a 0.8 repeat. In comparison, $ToxIN_{Pa}$ needs 2.5 repeats (out of 5.5 repeats) to prevent ToxN toxicity *in vivo* (46). However, when the protein (ToxN) is expressed in *trans*, only 1.5 repeats are necessary (25). Presumably, the key would be to have at least one complete mature repeat fragment (from cleavage site to cleavage site), this fragment playing a critical role in toxin regulation within the type III TA systems (28, 32).

Two clones with either Mut 1.8 or Mut 3.8 repeats were used to see the effect on anti-phage activity of ABIQ. In both cases, the EOP of phage P008 was reduced by only 1 or 2 logs when compared to the phage titer on the sensitive strain without AbiQ. This value is far from the EOP of 10^{-5} obtained with the wild-type AbiQ system. Interestingly, phages replicating on the mutated strains (Mut 1.8r and Mut 3.8r) do not have mutations in *orf38*, in contrast with AbiQ (2.8r) where escaping P008 phages were shown to be mutated in that gene (34). This indicates that modification in the number of repeats (*antiQ*) leads to reduced efficiency of AbiQ that enables wild type phage to directly bypass the system, rather than selecting phage-escaping mutant. It is plausible that one additional repeat (Mut 3.8r) leads to an increased number of regulatory RNA fragments, thereby regulating the toxin ABIQ, increasing cell survival and decreasing anti-phage activity. Conversely, it is unclear how Mut 1.8r reduces the anti-phage activity, since the deletion did not cause a change at the level of *antiQ* or *abiQ* transcription.

343 Characterization of *antiQ* point mutants also gives key information on AbiQ mechanism.
344 Our data showed the importance of specific nucleotides in the cleavage of *antiQ* and suggested
345 that the secondary structure of RNA is also important in the cleavage. Three mutations (A24C,
346 A26C and A28C) led to significant reductions in anti-phage activity, and were associated with
347 changes in the digestion profile of *antiQ* by ABIQ. Blocking cleavage in the first repeat brings
348 ABIQ to cleave in the last repeat and changed the ratio of specific fragments, increasing the 106
349 nt fragment (transcription start site to last cleavage site (0.8r)) and lowering the 97 nt fragment
350 (first cleavage site to terminator). Experiments assessing the EOPs of phage P008 suggest that
351 this 106 nt fragment is implicated in the regulation of ABIQ toxin, preventing its release,
352 favouring cell survival and leading to normal replication of the phage. This fragment could be
353 another inhibitory RNA like the mature repeat (cleaved RNA-36 nt) identified for the type III TA
354 $ToxIN_{Pa}$ (28) that is probably also involved in regulation in the AbiQ system. On the other hand,
355 it is also possible that the 97 nt fragment (first cleavage site to terminator) acts as an anti-
356 inhibitory fragment and that reduction of this fragment leads to this phenomenon.
357 It has been shown that the pseudoknot structure of type III antitoxin RNA is essential for
358 preventing toxicity of the cognate toxin (28). However, it had not been shown that mutations
359 affecting this structure could increase anti-phage activity. Our data showed that a single specific
360 mutation (G32A) led to a significant increase (3 logs) in anti-phage activity. Even the G32A-3.8r
361 mutant led to a remarkable 6-log EOP reduction as compared to the 3.8r mutant. Surprisingly,
362 this G32A mutation also increased AbiQ anti-phage activity against the previously isolated (34)
363 AbiQ-resistant phage P008-Q12 (EOP of 10^{-4}, data not shown). We propose that the G32A
364 mutation inactivated the inhibitory fragments, thereby preventing phage replication. Interestingly,
365 the G32A mutants did not reduce the fitness of AbiQ (G32A)-containing bacteria. Taken
366 altogether, a single specific mutation in *antiQ* can increase the anti-phage activity of AbiQ.

In summary, AbiQ is a complex molecular system that provides phage resistance. Our results suggest that different inhibitory RNA fragments regulate ABIQ. Under stress conditions (phage infection), a specific phage protein can destabilize the regulatory complex by interacting with the antitoxin, or by changing ABIQ activity. Freeing the toxin would enable it to cleave phage and cellular mRNA, cause growth arrest and prevent phage replication. Also, our data showed that we can optimize the AbiQ system, through a single mutation in the pseudoknot structure. Better comprehension of anti-phage systems is the key to limit rapid phage evolution, where phages often find a strategy to avoid the bacterial barrier (47).

Acknowledgements

We would like to thank Barbara-Ann Conway for editorial assistance as well as Julie Samson and Alex Hynes for discussion. This work was supported through a strategic grant from the Natural Sciences and Engineering Research Council of Canada. M.B. is the recipient of a scholarship from Quebec Protein Structure, Function and Engineering Research Network (PROTEO). S.M. holds a Tier 1 Canada Research Chair in Bacteriophages.

References

1. **Rousseau GM, Moineau S.** 2009. Evolution of *Lactococcus lactis* phages within a cheese factory. Appl. Environ. Microbiol. **75:**5336-5344.
2. **Labrie SJ, Samson JE, Moineau S.** 2010. Bacteriophage resistance mechanisms. Nat. Rev. Microbiol. **8:**317-327.
3. **Chopin M-C, Chopin A, Bidnenko E.** 2005. Phage abortive infection in lactococci: variations on a theme. Curr. Opin. Microbiol. **8:**473-479.
4. **Durmaz E, Klaenhammer TR.** 2007. Abortive phage resistance mechanism AbiZ speeds the lysis clock to cause premature lysis of phage-infected *Lactococcus lactis*. J. Bacteriol. **189:**1417-1425.
5. **Holubova J, Josephsen J.** 2007. Potential of AbiS as defence mechanism determined by conductivity measurement. J. Appl. Microbiol. **103:**2382-2391.
6. **Haaber J, Moineau S, Fortier L-C, Hammer K.** 2008. AbiV, a novel antiphage abortive infection mechanism on the chromosome of *Lactococcus lactis* subsp. *cremoris* MG1363. Appl. Environ. Microbiol. **74:**6528-6537.
7. **Bidnenko E, Chopin MC, Ehrlich SD, Anba J.** 2002. *Lactococcus lactis* AbiD1 abortive infection efficiency is drastically increased by a phage protein. FEMS Microbiol. Lett. **214:**283-287.
8. **Bidnenko E, Ehrlich D, Chopin M-C.** 1995. Phage operon involved in sensitivity to the *Lactococcus lactis* abortive infection mechanism AbiD1. J. Bacteriol. **177:**3824-3829.
9. **Bidnenko E, Ehrlich SD, Chopin M-C.** 1998. *Lactococcus lactis* phage operon coding for an endonuclease homologous to RuvC. Mol. Microbiol. **28:**823-834.

10. **Curtis FA, Reed P, Sharples GJ.** 2005. Evolution of a phage RuvC endonuclease for resolution of both Holliday and branched DNA junctions. Mol. Microbiol. **55:**1332-1345.
11. **Fortier L-C, Bouchard JD, Moineau S.** 2005. Expression and site-directed mutagenesis of the lactococcal abortive phage infection protein AbiK. J. Bacteriol. **187:**3721-3730.
12. **Wang C, Villion M, Semper C, Coros C, Moineau S, Zimmerly S.** 2011. A reverse transcriptase-related protein mediates phage resistance and polymerizes untemplated DNA in vitro. Nucleic Acids Res. **39:**7620-7629.
13. **Domingues S, Chopin A, Ehrlich SD, Chopin M-C.** 2004. The lactococcal abortive phage infection system AbiP prevents both phage DNA replication and temporal transcription switch. J. Bacteriol. **186:**713-721.
14. **Domingues S, McGovern S, Plochocka D, Santos MA, Ehrlich SD, Polard P, Chopin M-C.** 2008. The lactococcal abortive infection protein AbiP is membrane-anchored and binds nucleic acids. Virology **373:**14-24.
15. **Haaber J, Samson JE, Labrie SJ, Campanacci V, Cambillau C, Moineau S, Hammer K.** 2010. Lactococcal abortive infection protein AbiV interacts directly with the phage protein SaV and prevents translation of phage proteins. Appl. Environ. Microbiol. **76:**7085-7092.
16. **Haaber J, Rousseau GM, Hammer K, Moineau S.** 2009. Identification and characterization of the phage gene *sav*, involved in sensitivity to the lactococcal abortive infection mechanism AbiV. Appl. Environ. Microbiol. **75:**2484-2494.
17. **Samson JE, Spinelli S, Cambillau C, Moineau S.** 2013. Structure and activity of AbiQ, a lactococcal endoribonuclease belonging to the type III toxin-antitoxin system. Mol. Microbiol. **87:**756-768.
18. **Yamaguchi Y, Park JH, Inouye M.** 2011. Toxin-antitoxin systems in bacteria and archaea. Annu. Rev. Genet. **45:**61-79.

19. **Ogura T, Hiraga S.** 1983. Mini-F plasmid genes that couple host cell division to plasmid proliferation. Proc. Natl. Acad. Sci. U S A **80:**4784-4788.
20. **Unterholzner SJ, Poppenberger B, Rozhon W.** 2013. Toxin-antitoxin systems: Biology, identification, and application. Mob. Genet. Elements **3:**e26219.
21. **Magnuson RD.** 2007. Hypothetical functions of toxin-antitoxin systems. J. Bacteriol. **189:**6089-6092.
22. **Lewis K.** 2010. Persister cells. Annu. Rev. Microbiol. **64:**357-372.
23. **Maisonneuve E, Shakespeare LJ, Jørgensen MG, Gerdes K.** 2011. Bacterial persistence by RNA endonucleases. Proc. Natl. Acad. Sci. U S A **108:**13206-13211.
24. **Wang X, Wood TK.** 2011. Toxin-antitoxin systems influence biofilm and persister cell formation and the general stress response. Appl. Environ. Microbiol. **77:**5577-5583.
25. **Fineran PC, Blower TR, Foulds IJ, Humphreys DP, Lilley KS, Salmond GP.** 2009. The phage abortive infection system, ToxIN, functions as a protein-RNA toxin-antitoxin pair. Proc. Natl. Acad. Sci. U S A **106:**894-899.
26. **Fozo EM, Hemm MR, Storz G.** 2008. Small toxic proteins and the antisense RNAs that repress them. Microbiol. Mol. Biol. Rev. **72:**579-589.
27. **Gerdes K, Christensen SK, Løbner-Olesen A.** 2005. Prokaryotic toxin-antitoxin stress response loci. Nat. Rev. Microbiol. **3:**371-382.
28. **Blower TR, Pei XY, Short FL, Fineran PC, Humphreys DP, Luisi BF, Salmond GP.** 2011. A processed noncoding RNA regulates an altruistic bacterial antiviral system. Nat. Struct. Mol. Biol. **18:**185-190.
29. **Masuda H, Tan Q, Awano N, Yamaguchi Y, Inouye M.** 2012. A novel membrane-bound toxin for cell division, CptA (YgfX), inhibits polymerization of cytoskeleton proteins, FtsZ and MreB, in *Escherichia coli*. FEMS Microbiol. Lett. **328:**174-181.

30. **Masuda H, Tan Q, Awano N, Wu KP, Inouye M.** 2012. YeeU enhances the bundling of cytoskeletal polymers of MreB and FtsZ, antagonizing the CbtA (YeeV) toxicity in *Escherichia coli*. Mol. Microbiol. **84:**979-989.

31. **Wang X, Lord DM, Cheng HY, Osbourne DO, Hong SH, Sanchez-Torres V, Quiroga C, Zheng K, Herrmann T, Peti W, Benedik MJ, Page R, Wood TK.** 2012. A new type V toxin-antitoxin system where mRNA for toxin GhoT is cleaved by antitoxin GhoS. Nat. Chem. Biol. **8:**855-861.

32. **Short FL, Pei XY, Blower TR, Ong SL, Fineran PC, Luisi BF, Salmond GP.** 2013. Selectivity and self-assembly in the control of a bacterial toxin by an antitoxic noncoding RNA pseudoknot. Proc. Natl. Acad. Sci. U S A **110:**E241-249.

33. **Emond E, Dion E, Walker SA, Vedamuthu ER, Kondo JK, Moineau S.** 1998. AbiQ, an abortive infection mechanism from *Lactococcus lactis*. Appl. Environ. Microbiol. **64:**4748-4756.

34. **Samson JE, Bélanger M, Moineau S.** 2013. Effect of the abortive infection mechanism and type III toxin/antitoxin system AbiQ on the lytic cycle of *Lactococcus lactis* phages. J. Bacteriol. **195:**3947-3956.

35. **Kropinski AM, Mazzocco A, Waddell TE, Lingohr E, Johnson RP.** 2009. Enumeration of bacteriophages by double agar overlay plaque assay, p. 69-76, Bacteriophages: Methods and Protocols, Volume 1: Isolation, Characterization, and Interactions. (eds) Humana Press.

36. **Sambrook J, Russel DW.** 2001. Molecular Cloning: A laboratory manual, Third Edition. (eds) Cold Spring Harbor Laboratory Press.

37. **Hall TA.** 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. **41:**95-98.

38. **Bonfield JK, Whitwham A.** 2010. Gap5--editing the billion fragment sequence assembly. Bioinformatics **26:**1699-1703.

39. **Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A.** 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics **28:**1647-1649.

40. **Ullmann A, Jacob F, Monod J.** 1967. Characterization by *in vitro* complementation of a peptide corresponding to an operator-proximal segment of the β-galactosidase structural gene of *Escherichia coli*. J. Mol. Biol. **24:**339-343.

41. **Holo H, Nes IF.** 1989. High-frequency transformation, by electroporation, of *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilized media. Appl. Environ. Microbiol. **55:**3119-3123.

42. **Potter J, Zhang W, Lee J.** 2003. Thermal stability and cDNA synthesis capability of SuperScript reverse transcriptase. Focus (Invitrogen) **25:**19-24.

43. **Blower TR, Short FL, Rao F, Mizuguchi K, Pei XY, Fineran PC, Luisi BF, Salmond GP.** 2012. Identification and classification of bacterial Type III toxin-antitoxin systems encoded in chromosomal and plasmid genomes. Nucleic Acids Res. **40:**6158-6173.

44. **Wilson KS, von Hippel PH.** 1995. Transcription termination at intrinsic terminators: the role of the RNA hairpin. Proc. Natl. Acad. Sci. U S A **92:**8793-8797.

45. **Mendoza-Vargas A, Olvera L, Olvera M, Grande R, Vega-Alvarado L, Taboada B, Jimenez-Jacinto V, Salgado H, Juárez K, Contreras-Moreira B, Huerta AM, Collado-Vides J, Morett E.** 2009. Genome-wide identification of transcription start sites, promoters and transcription factor binding sites in *E. coli*. PLoS One **4:**e7526.

46. **Blower TR, Fineran PC, Johnson MJ, Toth IK, Humphreys DP, Salmond GP.** 2009. Mutagenesis and functional characterization of the RNA and protein components of the toxIN abortive infection and toxin-antitoxin locus of *Erwinia*. J. Bacteriol. **191:**6029-6039.

47. **Samson JE, Magadán AH, Sabri M, Moineau S.** 2013. Revenge of the phages: defeating bacterial defences. Nat. Rev. Microbiol. **11:**675-687.

48. **Chopin A, Chopin M-C, Moillo-Batt A, Langella P.** 1984. Two plasmid-determined restriction and modification systems in *Streptococcus lactis*. Plasmid **11:**260-263.

49. **Wegmann U, O'Connell-Motherway M, Zomer A, Buist G, Shearman C, Canchaya C, Ventura M, Goesmann A, Gasson MJ, Kuipers OP, van Sinderen D, Kok J.** 2007. Complete genome sequence of the prototype lactic acid bacterium *Lactococcus lactis* subsp. *cremoris* MG1363. J. Bacteriol. **189:**3256-3270.

50. **Blattner FR, Plunkett III G, Bloch CA, Perna NT, Burland V, Riley M, Collado-Vides J, Glasner JD, Rode CK, Mayhew GF, Gregor J, Davis NW, Kirkpatrick HA, Goeden MA, Rose DJ, Mau B, Shao Y.** 1997. The complete genome sequence of *Escherichia coli* K-12. Science **277:**1453-1462.

51. **de Vos WM.** 1987. Gene cloning and expression in lactic streptococci. FEMS Microbiol. Rev. **46:**281-295.

52. **Mahony J, Deveau H, Mc Grath S, Ventura M, Canchaya C, Moineau S, Fitzgerald GF, van Sinderen D.** 2006. Sequence and comparative genomic analysis of lactococcal bacteriophages jj50, 712 and P008: evolutionary insights into the 936 phage species. FEMS Microbiol. Lett. **261:**253-261.

524 **Table 1.** Strains, plasmids and phages used in this study.

Bacterial strains, plasmids or phages	Specific characteristics	References
Strains		
Lactococcus lactis		
IL1403	Laboratory strain, plasmid free. Host of phage P008	(48)
MG1363	Laboratory strain, plasmid free. Cloning strain	(49)
Escherichia coli		
XL1-blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [*F' proAB lacI*^{q} *ZΔM15 Tn10* (Tet^R)]	Stratagene
MG1655	*F$^-$ λ$^-$ ilvG- rfb-50 rph-1*	(50)
Plasmids		
pBS-KS	Cloning vector suitable for blue-white screening, 3.0kb, Amp^R	Stratagene
pNZ123	Shuttle vector (*L. lactis* et *E. coli*), 2.5kb, Cm^R	(51)
pNZ123-AbiQ	AbiQ-wt operon cloned in pNZ123 at EcoRI site, Cm^R	(17)
pNZ-AbiQ (1.8r)	AbiQ operon in pNZ123, *antiQ* of 1.8 repeat (Mut 1.8r), Cm^R	This study
pNZ-AbiQ (3.8r)	AbiQ operon in pNZ123, *antiQ* of 3.8 repeat (Mut 3.8r), Cm^R	This study
pNZ-AbiQ (A13C)	AbiQ operon in pNZ123, mutation A13C in the first *antiQ* repeat, Cm^R	This study
pNZ-AbiQ (A24C)	AbiQ operon in pNZ123, mutation A24C in the first *antiQ* repeat, Cm^R	This study
pNZ-AbiQ (T25C)	AbiQ operon in pNZ123, mutation T25C in the first *antiQ* repeat, Cm^R	This study
pNZ-AbiQ (A26C)	AbiQ operon in pNZ123, mutation A26C in the first *antiQ* repeat, Cm^R	This study
pNZ-AbiQ (A28C)	AbiQ operon in pNZ123, mutation A28C in the two first *antiQ* repeats, 3.8 repeats, Cm^R	This study
pNZ-AbiQ (G32A-3.8)	AbiQ operon in pNZ123, mutation G32A in the two first *antiQ* repeats, 3.8 repeats, Cm^R	This study
pNZ-AbiQ (G32A)	AbiQ operon in pNZ123, mutation G32A in the first *antiQ* repeat, Cm^R	This study
Phages		
P008	*Siphoviridae*, 936 group, sensitive to AbiQ	(52)
P008-Q12	P008 mutated in ORF38 (Pro38Leu), resistant to AbiQ	(34)

525

526

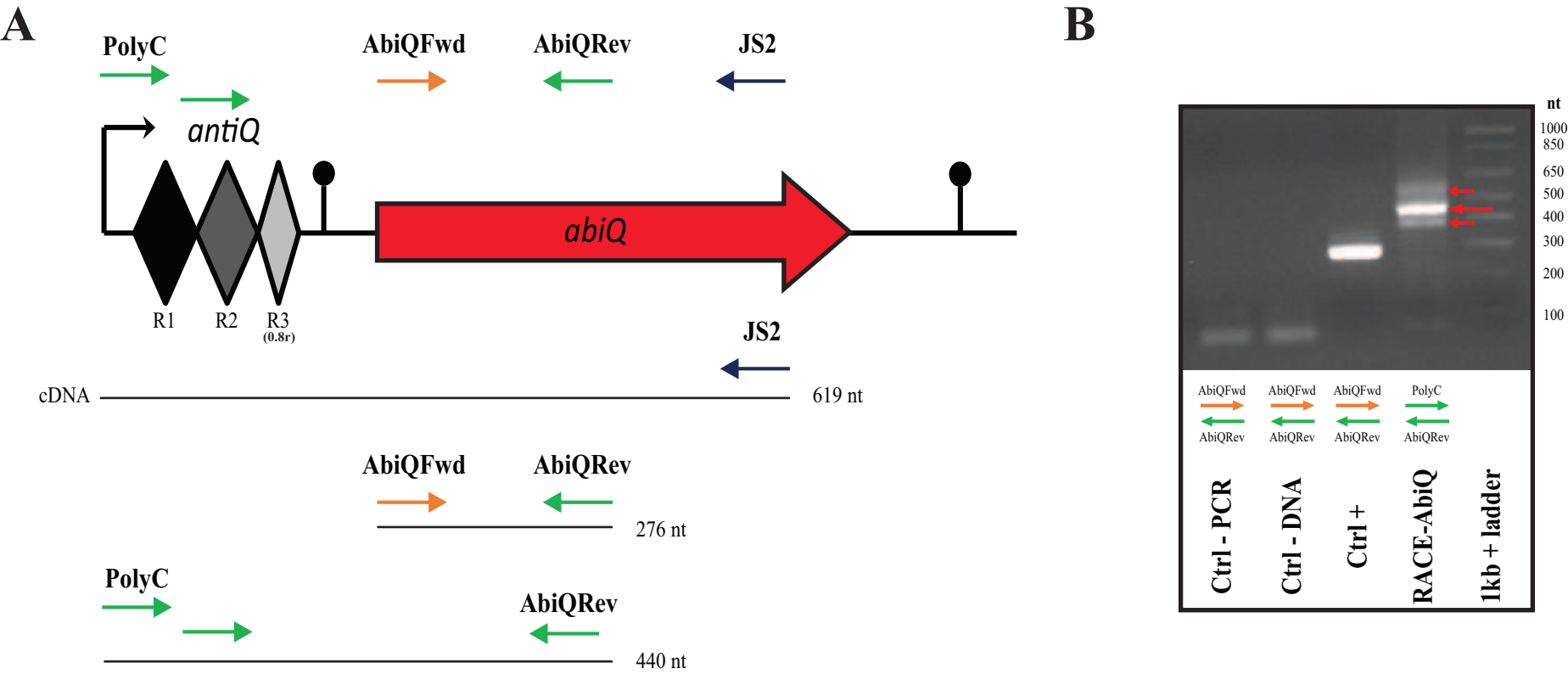
Legends of Figures

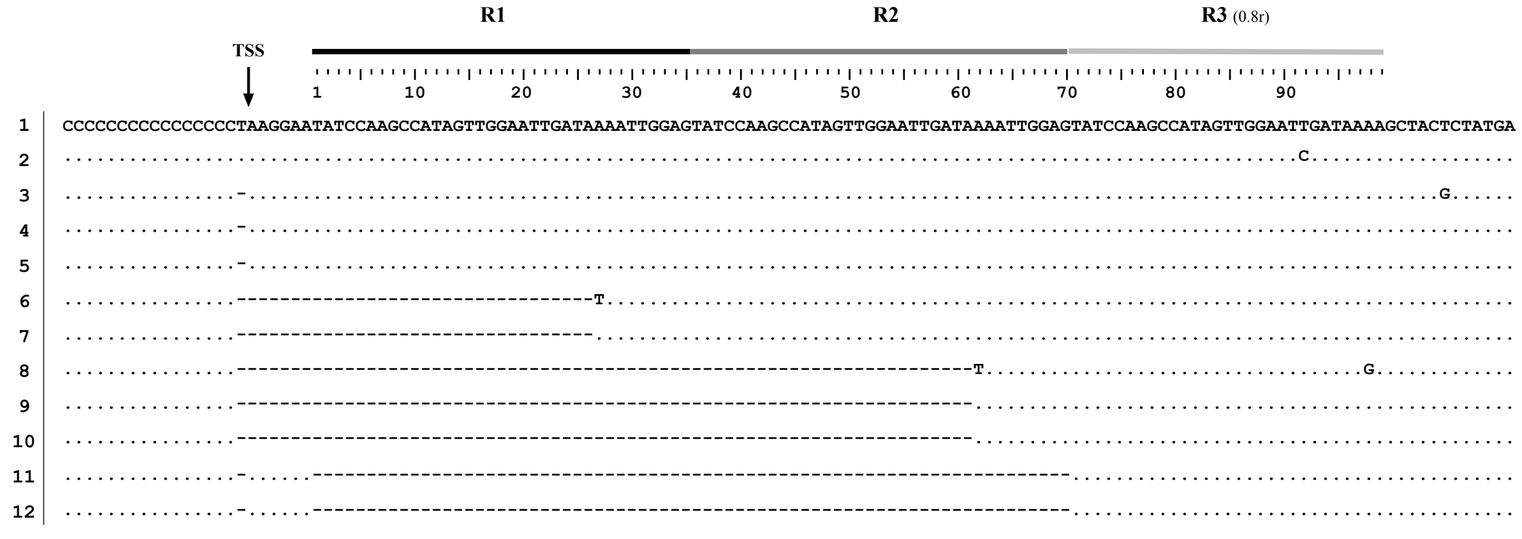
Figure 1. The AbiQ operon and PCR products of the 5'RACE assay. Panel A) The specific position (and orientation) of each primer (arrows) is represented in the schematic form of the operon AbiQ. The PCR products are: Ctrl-PCR (AbiQFwd/AbiQRev, water as template), Ctrl-DNA (AbiQFwd/AbiQRev, RNA without retro-transcription as template), Ctrl+ (AbiQFwd/AbiQRev, cDNA as template) and RACE-AbiQ (PolyC/AbiQRev, cDNA as template). Panel B) 5'RACE PCR products of the AbiQ operon separated on a 2.0% agarose gel. Red arrows are pointing out the three PCR product bands. The molecular weight standard was 1 kb Plus DNA Ladder (Invitrogen)

Figure 2. Sequences of the 12 pBS-KS::RACE-AbiQ clones. The repeats (R) and the transcription start site (position -7 / -6) are indicated over the first sequence (clone #1) with different shades of gray representing the three repeats. "." indicates identical sequence, "-" indicates the absence of the nucleotide in sequenced clone.

Figure 3. Efficiency of plaquing (EOP), size of lysis plaques and digestion profile of *antiQ* for wild-type and mutated AbiQ operons. One *antiQ* repeat (GCTCCAATTTTATCAATTCCAACTATGGCTTGGATA) was used as a probe to define the digestion profile for AbiQ mutants in Northern hybridization experiments. [a] EOP and standard deviation were calculated from at least three biological assays. [b] P008 infecting *L. lactis* IL1403 pNZ123 ($AbiQ^-$) produces lysis plaques of 3-5 mm.

550


A
PolyC
AbiQFwd
AbiQRev
JS2
antiQ
abiQ
R1
R2
R3
(0.8r)
JS2
cDNA
619 nt
AbiQFwd
AbiQRev
276 nt
PolyC
AbiQRev
440 nt
B
nt
1000
850
650
500
400
300
200
100
AbiQFwd
AbiQRev
AbiQFwd
AbiQRev
AbiQFwd
AbiQRev
PolyC
AbiQRev
Ctrl - PCR
Ctrl - DNA
Ctrl +
RACE-AbiQ
1kb + ladder


R1
R2
R3 (0.8r)
TSS
1 10 20 30 40 50 60 70 80 90
1 CCCCCCCCCCCCCCCCCTAAGGAATATCCAAGCCATAGTTGGAATTGATAAAATTGGAGTATCCAAGCCATAGTTGGAATTGATAAAATTGGAGTATCCAAGCCATAGTTGGAATTGATAAAAGCTACTCTATGA
2 ....C..........
3 ..........-..........G.....
4 ....-..........
5 ....-..........
6 ....----------T..........
7 ....----------..........
8 ..........----------T..........G.....
9 ....----------..........
10 ....----------..........
11 ..........-.....----------..........
12 ..........-.....----------..........

	AbiQ-wt	Mut 1.8r	Mut 3.8r	A13C	A24C	T25C	A26C	A28C (3.8)	G32A (3.8)	G32A
EOP[a]	$(2.4 \pm 1.1) \times 10^{-5}$	$(2.9 \pm 2.4) \times 10^{-2}$	0.2 ± 0.1	$(1.8 \pm 1.6) \times 10^{-4}$	0.3 ± 0.1	$(3.0 \pm 2.2) \times 10^{-5}$	1.0 ± 0.2	0.6 ± 0.2	$(1.2 \pm 1.9) \times 10^{-7}$	$(2.3 \pm 3.3) \times 10^{-8}$
Lysis plaques[b] (mm)	Pinpoint - 2	1	1	Pinpoint - 2	1.5	Pinpoint - 2	2.5	3	1	1