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The lactococcal abortive infection protein AbiP is membrane-anchored and binds nucleic acids

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Abstract

AbiP, a lactococcal abortive phage infection system, has previously been shown to arrest phage bIL66M1 DNA replication around 10 min after infection and to inhibit the switch off of phage early transcripts. We report here the functional characterization and implication in the abortive infection phenotype of two domains identified in the AbiP sequence. We show that AbiP is a protein anchored to the membrane by an N-terminal membrane-spanning domain. Our results further suggest that membrane localization may be required for the anti-phage activity of AbiP. The remainder of the protein, which contains a putative nucleic acid binding domain, is shown to be located on the cytosolic side. Purified AbiP is shown to bind nucleic acids with an approximately 10-fold preference for RNA relative to ssDNA. AbiP interaction with both ssDNA and RNA molecules occurs in a sequence-independent manner. We have analyzed the effect of substitutions of aromatic and basic residues on the surface of the putative binding fold. *In vitro* and *in vivo* studies of these AbiP derivatives indicate that the previously reported effects on phage development might be dependent on the nucleic acid binding activity displayed by the membrane-bound protein.
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Introduction

Bacteria have developed a plethora of mechanisms to escape bacteriophage attack. The molecular characterization of some of these systems has provided important insights on phage–host interactions, which contributed to a better understanding of the biology of both participants. Although these studies have mainly been carried out in *Escherichia coli* (for reviews, see Molineux, 1991; Snyder, 1995), most of the reported phage defense mechanisms have been identified in *Lactococcus lactis* (reviewed by Allison and Klaenhammer, 1998; Forde and Fitzgerald, 1999; and more recently by Chopin et al., 2005). Milk fermentation by lactococci, conducted in large dairy plants under non-aseptic conditions, is highly susceptible to infection by bacteriophages. The large number of lactococcal cells used in these processes and the long-term challenge with diverse bacteriophages seem to have contributed to the selection of varied and efficient mechanisms of phage resistance in these bacteria (Coffey and Ross, 2002). Among these, abortive infection (Abi) is characterized by a normal start of phage infection followed by an interruption of the lytic development, leading to the release of few or no progeny particles and cell death. Abi mechanisms have been found to interfere with different steps of phage development, including DNA replication, transcription, translation, packaging and assembly of phage particles and the host lysis (Allison and Klaenhammer, 1998; Chopin et al., 2005; Durmaz and Klaenhammer, 2007; Forde and Fitzgerald, 1999). The panoply of phenotypes associated with abortive infections has hampered a clear separation between the primary cause of phage exclusion and its

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subsequent effects. Molecular models have only been drawn for some of the Abi systems active in *E. coli*. The more extensively studied have been shown to act on highly conserved cellular components essential for cell survival. The PrrC nuclease and the Lit protease are directed against the translational apparatus (Amitsur et al., 1987; Bingham et al., 2000; Copeland and Kleanthous, 2005), whereas the Rex system and F-mediated exclusion seem to act at the cytoplasmic membrane level (Cheng et al., 2004; Parma et al., 1992).

Accordingly, the current knowledge of lactococcal Abi systems also seems to indicate that they interfere with conserved cellular functions (Chopin et al., 2005). For instance, AbiD1 seems to act by limiting the activity of an essential phage endonuclease (Bidnenko et al., 1995, 2002), and the toxicity of the encoded protein suggests that it may also affect cellular DNA metabolism. Similarly, while AbiR was proposed to inhibit host chromosomal DNA replication unless its toxicity is alleviated by the LlaKR2I methyltransferase (Yang et al., 2006), the complex control of AbiK expression suggests that a proper balance of its constitutive synthesis might be crucial (Fortier et al., 2005). It was not possible to transform *L. lactis* with a high copy-number plasmid harboring *abiK* under the control of a strong promoter (Emond et al., 1997), suggesting that AbiK might also be toxic. Finally, overexpression of AbiB, AbiN and AbiO has also been shown to be toxic (Chopin et al., 2005; Prévots and Ritzenthaler, 1998; Prévots et al., 1998).

AbiP, the lactococcal Abi system studied in this work, is effective against some phages of the 936 group (Deveau et al.,

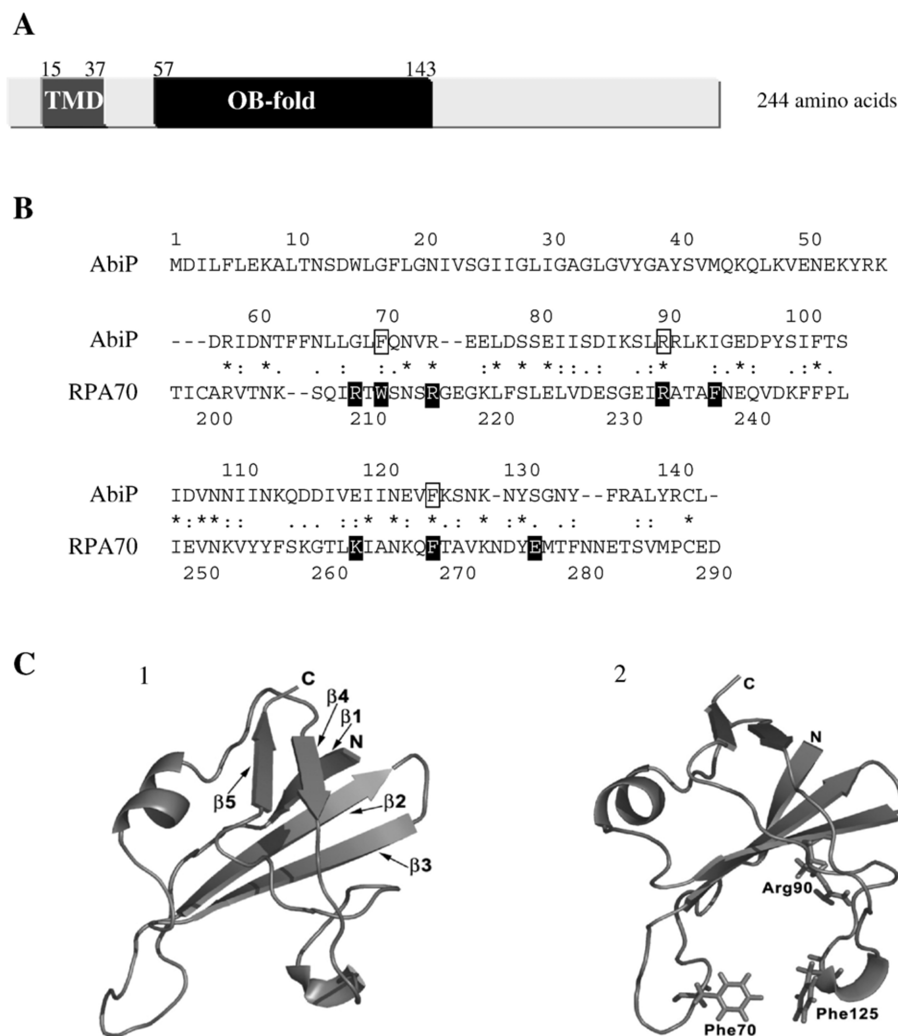

Fig. 1. Predicted domains and their relative position in the AbiP protein. (A) Schematic representation of AbiP showing the relative position of the predicted TMD (residues 15–37)—represented by a grey box and of the putative OB-fold (residues 57–143)—black box. Prediction of the TMD was carried using the TMHMM method (Krogh et al., 2001; Sonnhammer et al., 1998) and the OB-fold hit was highlighted with the SSEARCH software (Pearson, 1991; Smith and Waterman, 1981). (B) Alignment of residues spanning the OB-fold hit of AbiP with the domain A of human RPA70 (residues 198–291) done with CLUSTALW software (Thompson et al., 1994). Residues that are conserved between AbiP and RPA70 are indicated by "*", conservative substitutions are indicated by ":" and weakly similar residues by ".". The black boxes indicate the residues known to contact DNA in human RPA70 (Bochkarev et al., 1997). Residues mutated in the putative OB domain of AbiP are boxed. (C) Hypothetical three-dimensional structural model of the AbiP segment 57–143. (1) The typical five-stranded β-barrel capped by an α-helix is shown. In well-characterized OB-fold proteins, DNA binds across the face of the fold (Theobald et al., 2003). (2) Indication of aromatic (Phe70 and Phe125) and basic (Arg90) residues within the putative nucleic acid binding surface, which were altered into alanine.

2006). The resistance determinant is encoded by a single *orf* and its presence has been shown to arrest phage DNA replication 10 min after infection and to inhibit the temporal transcription switch off of the early genes (Domingues et al., 2004a). In an attempt to elucidate the molecular mode of action of AbiP, we report here the study of two AbiP functional domains that could be predicted from its sequence analysis: a transmembrane domain (TMD) in its N-terminal region and an OB(oligomer binding)-fold—a domain often implicated in nucleic acid binding. We show that AbiP faces the cytoplasm, being anchored to the membrane by its N-terminal end. AbiP is also shown to bind double- and single-stranded nucleic acids in a sequence-independent manner, exhibiting a preference for RNA. *In vitro* and *in vivo* studies of a series of AbiP mutant derivatives further correlate its membrane localization and nucleic acid binding activity with the Abi phenotype. To our knowledge, this is the first report of an Abi system that combines membrane localization with the ability to interact with nucleic acids.

Results

AbiP domains

The AbiP protein has no significant sequence similarity to any other protein of known function. However, analysis of its primary sequence revealed two putative domains. First, a TMHMM transmembrane helix prediction program (Krogh et al., 2001; Sonnhammer et al., 1998) predicted a TMD at the N-terminus of AbiP (Fig. 1A), which was also suggested to display an N-out/C-in topology. Secondly, a search in the 3D sequence database from the Protein Data Bank using the SSEARCH software (Pearson, 1991; Smith and Waterman, 1981) highlighted a region of AbiP (residues 57 to 143) sharing homology (20% identity, 37.5% similarity) with a single-stranded DNA binding domain (domain A) of the largest subunit of the human replication protein A (RPA70). This domain corresponds to an OB-fold, which consists of a five-stranded β-sheet coiled to form a β-barrel (Murzin, 1993). It is a common fold (with a fold-related binding face), adopted by a wide variety of protein sequences. Therefore, despite the structural identity, there is very little amino acid sequence homology among different OB-fold carrying proteins. Interestingly, most of the RPA70 amino acids shown to interact with ssDNA (Bochkarev et al., 1997) appear conserved or present conservative substitutions in AbiP (Fig. 1B). By using 3D modeling as described (see Materials and methods), this AbiP segment was predicted to adopt an OB-fold-like conformation (Fig. 1C).

AbiP is a membrane-anchored protein

The presence of a putative TMD (Fig. 1A) suggested that AbiP could be membrane-anchored. This prediction was tested by cell fractionation studies. Samples of the particulate (membrane) and soluble (cytoplasm) fractions, as well as of total protoplast lysate, were analyzed by SDS–PAGE and Western immunoblotting. Lactococcal aminopeptidase PepC (Chapot-Chartier et al., 1993; Neviani et al., 1989), known to be cytoplasmic, served as a control. AbiP was only detected in the membrane fraction, confirming its membrane association (Fig. 2A, lanes 1 to 3).

Two AbiP specific bands were detected. This two-band pattern was invariably observed with protoplasts whereas only the upper band was detected after Western blotting performed with AbiP expressed and purified from *E. coli* (data not shown). Taking into account that the first 14 AbiP residues were predicted to be outside the cell (see Fig. 1B), one possible explanation is that the process of cell wall removal for protoplast preparation would cause some cleavage of this extracellular region. The mass expected for a protein deleted from this N-terminal extension (27 kDa) is close to that of the lower migrating band. Furthermore, the upper band seems to disappear after protoplasts incubation with proteinase K (see below).

To check whether the predicted N-terminal TMD was responsible for the membrane localization of AbiP, we constructed a truncated form of the protein lacking its first 42 amino acids (named AbiP$_{\Delta N\text{-term}}$). In contrast to AbiP, which was only detected in the membrane fraction, a considerable amount of AbiP$_{\Delta N\text{-term}}$ was found in the cytoplasmic fraction (Fig. 2A). However, about one third of AbiP$_{\Delta N\text{-term}}$ was still observed in

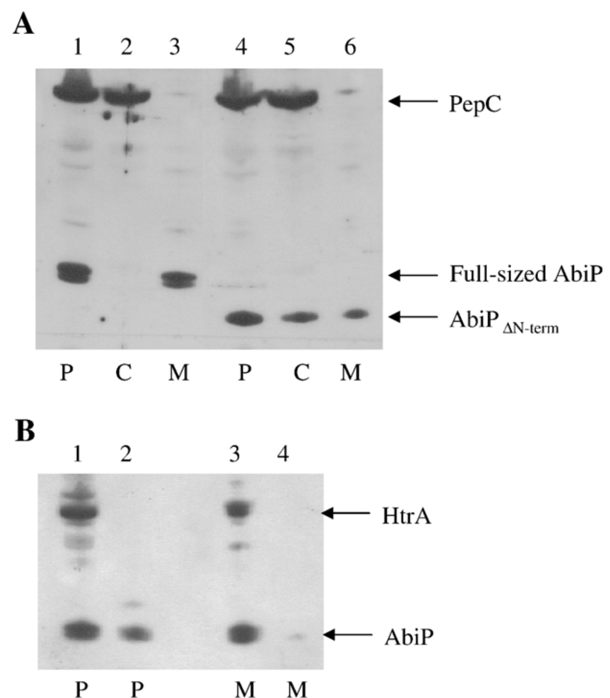

Fig. 2. AbiP cellular localization and topology. (A) Membrane localization of AbiP. Logarithmically growing *L. lactis* cells containing full-sized AbiP (28 kDa; lanes 1 to 3), or AbiP$_{\Delta Nterm}$—a truncated form of AbiP, lacking the first 42 amino acids (24 kDa; lanes 4 to 6) were protoplasted and fractionated. The whole protoplast lysate (P), cytoplasmic fraction (C) and membrane fraction (M) were analyzed by Western blotting simultaneously using antibodies against AbiP and the lactococcal aminopeptidase (PepC). (B) AbiP topology. Logarithmically growing *L. lactis* cells containing AbiP were protoplasted and fractionated. Protoplast (P) and membrane (M) samples were treated with proteinase K (lanes 2 and 4) and analyzed by Western blotting simultaneously using antibodies against AbiP and the surface protease HtrA. Lanes 1 and 3: control without incubation with proteinase K. Note that a small amount of AbiP is digested before cell lysis (lane 2). This might be due to lysis of some protoplasts during incubation with proteinase K.

the membrane fraction. This suggested that AbiP$_{\Delta N\text{-term}}$ retained some membrane affinity or that the protein structure was modified, resulting in its aggregation and subsequent precipitation. To discriminate between these two possibilities, we treated the membrane pellet with 0.1% Triton X-100. This detergent concentration, used during AbiP purification to release the protein from the membrane (see Materials and methods), was not expected to solubilize protein aggregates. Under these conditions, AbiP was readily released from the membrane fraction, whereas AbiP$_{\Delta N\text{-term}}$ retained in the membrane fraction was barely released (data not shown). These results indicate that the remnant of the truncated protein still found in the membrane pellet was likely an insoluble form. Thus, the N-terminus is the main determinant for membrane anchorage and the removed segment contains the membrane anchor. The fact that about one third of protein precipitated probably means that during the protein folding, some intermediates do not follow the folding process to the native form but alternatively participate in intermolecular associations leading to the formation of aggregates and precipitation.

The prediction of a C-in topology for AbiP was also tested by cell fractionation analysis followed by proteolysis with proteinase K and Western blotting. The lactococcal protease HtrA (Rigoulay et al., 2004), an extracellular membrane protein anchored by an N-terminal TMD (I. Poquet, unpublished results), was used as a control. As shown in Fig. 2B, HtrA was digested when the protoplast fraction was treated with proteinase K, while AbiP was only completely digested after cell lysis, demonstrating that AbiP faces the interior of the cell.

The relevance of the AbiP cellular localization for the Abi phenotype was also studied. For this purpose, phage bIL66M1, an AbiP-sensitive phage, was enumerated on strains IL1403 (pIL253::*abiP*) and IL1403 (pIL253::*abiP*$_{\Delta N\text{-term}}$). IL1403 (pIL253) was used as a control. Both phage titer and plaque morphology were identical in the strain carrying AbiP$_{\Delta N\text{-term}}$ and in the control, indicating that the truncated protein is no longer able to confer the Abi phenotype (data not shown).

DNA binding characterization of AbiP

Previous studies on the effect of AbiP on phage bIL66M1 development have shown that, in cells expressing AbiP, phage DNA replication stops abruptly early after infection and that transcription of phage early genes is not normally switched off (Domingues et al., 2004a). The fact that AbiP contains a putative OB-fold domain, which is often implicated in protein–nucleic acid interactions (Theobald et al., 2003), led us to examine the ability of AbiP to bind nucleic acids *in vitro*. For this purpose, AbiP was purified to more than 95% homogeny following over expression in *E. coli* as described in Materials and methods.

First, in order to analyze the DNA binding activity of AbiP and to determine its ability to bind double-stranded DNA (dsDNA) and/or single-stranded DNA (ssDNA), gel mobility-shift assays were performed using purified AbiP and a mix of ssDNA and dsDNA fragments. The 325-bp DNA segment used covered the region located between the early- and middle-expressed phage bIL66M1 genomic regions (GenBank accession number: L35175; coordinates: 263–588). This segment contains the first early promoter (pE1) and the middle promoter (pM) divergently located (Bidnenko et al., 1995; Domingues et al., 2004a). This region, highly conserved among phages of the 936 group, is most probably implicated in controlling the switch from early to middle transcription and could conceivably be a target for AbiP. The dsDNA fragment was obtained by PCR amplification and used as a template to prepare the corresponding ssDNA fragment by linear DNA amplification. Both fragments, end-labeled with ^{32}P, were used simultaneously as substrates in gel retardation assays carried out in the presence of increasing amounts of AbiP. Purified single-stranded DNA binding protein from *Bacillus subtilis* (SSB$_{Bs}$) (Bruand et al., 2005) was used as a control in these experiments. As expected, only the ssDNA fragment was shifted by SSB$_{Bs}$ (Fig. 3A, lanes 2 to 4). In contrast, AbiP shifted both ssDNA and dsDNA (lanes 6 to 8), exhibiting a preferential interaction with ssDNA. This result was confirmed by performing the same experiment with each substrate alone (data not shown). Increasing concentrations of AbiP led to the formation of high molecular weight nucleoprotein complexes, suggesting the simultaneous interaction of several AbiP molecules with the substrate.

The affinity of AbiP for ssDNA was characterized using several 80-mer ssDNA oligonucleotides covering both DNA strands along the entire 325-bp bIL66M1 region and an 80-mer oligonucleotide with an unrelated sequence derived from the phage M13 genome. Each oligonucleotide was end-labeled with ^{32}P and separately used as substrate in gel mobility-shift assays carried out in the presence of increasing amounts of purified AbiP. Our results showed that AbiP interacted similarly with all ssDNA probes used with an average K_d value of 217±64 nM. Under these conditions, no determinants of specificity were found. To confirm that AbiP is causing the band shift, we revealed protein–DNA complexes using either a radiolabeled oligonucleotide (Fig. 3B-1) or a cold oligonucleotide and an anti-AbiP antibody (Fig. 3B-2). As previously observed, high molecular weight nucleoprotein complexes with an upper smear were visible on Fig. 3B-1. The use of anti-AbiP antibody revealed that AbiP alone forms high molecular weight complexes (Fig. 3B-2, lane 6). This is in agreement with results from gel filtration analysis of the protein, where AbiP eluted as an oligomer of about 350 kDa (data not shown). Even though differences in the migration of the large protein complex when bound to an 80-mer oligonucleotide could be hard to detect, the migration profile of AbiP with and without ssDNA is different. Protein alone forms smaller complexes than in presence of ssDNA. Moreover, at identical protein concentration, the amount entering the gel is higher in absence of ssDNA (Fig. 3B-2, lanes 6 and 4). This difference most probably reflects the formation of large nucleoprotein complexes unable to enter the gel. Thus, the different migration profile observed for AbiP with and without ssDNA indicates that AbiP is responsible for ssDNA binding shift.

AbiP binds preferably to RNA

Proteins carrying an OB-fold motif are often able to bind different types of nucleic acids (Theobald et al., 2003). Moreover,

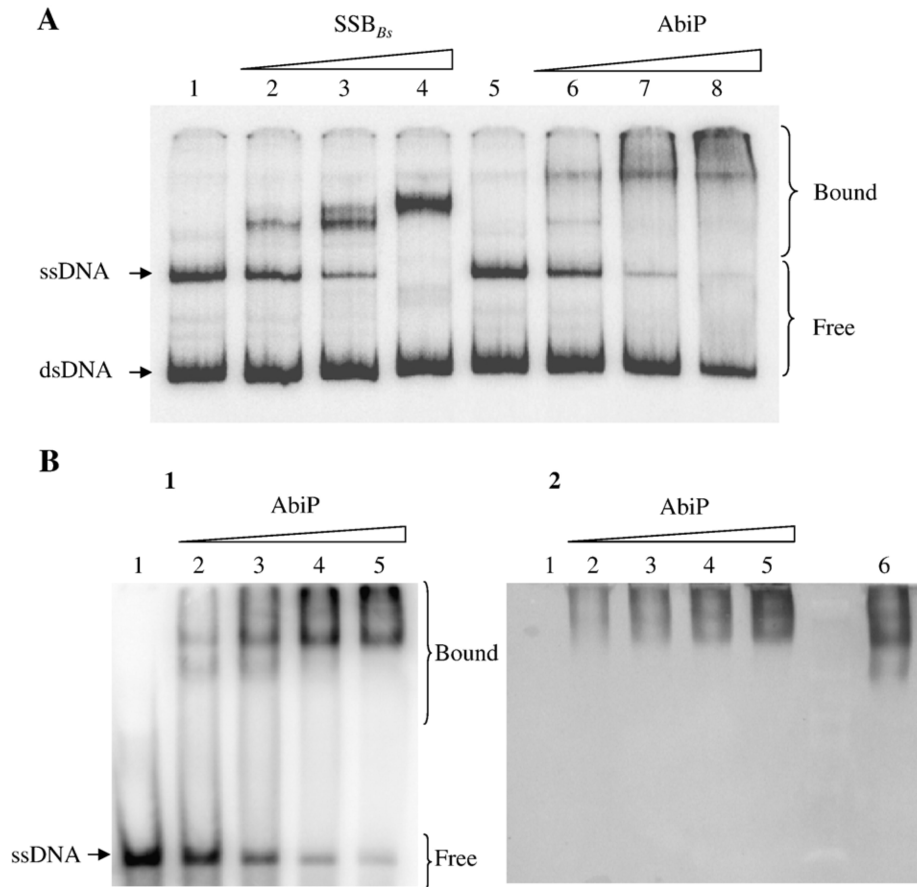

Fig. 3. AbiP interaction with DNA. (A) Binding of AbiP to a mixture of ssDNA (325 nt) and dsDNA (325 bp). The mixture, containing 0.3 nM of each radiolabeled substrate, was incubated with either SSB$_{Bs}$ or AbiP (as indicated on top). Nucleoprotein complexes were resolved on a native 5% polyacrylamide gel as described in Materials and methods. Lanes 1 and 5: dsDNA/ssDNA mixture without protein. Lanes 2 to 4: 5, 10 and 20 nM SSB$_{Bs}$, respectively. Lanes 6 to 8: 80, 160 and 330 nM of AbiP, respectively. (B) Binding of AbiP to the 80-mer oligonucleotide –sd133. (1) Radiolabeled substrate (1 nM) was incubated with increasing amounts of protein and resolved in a native 5% polyacrylamide gel as described in Materials and methods. Lane 1: sample without AbiP. Lanes 2 to 5: 100, 200, 300 and 400 nM of AbiP, respectively. Lane 2: immunodetection of AbiP, following transfer of a gel identical to the one reported in B-1 but performed with 1 nM of cold substrate. Lanes 1–5: samples identical to lanes 1–5 in gel B-1. Lane 6: 300 nM of AbiP incubated without substrate.

given that no specific interaction was observed with DNA and considering the switch-off inhibition of several phage bIL66M1 early transcripts in the presence of AbiP (Domingues et al., 2004a), a direct interaction of AbiP with RNA was very plausible. Hence, the RNA binding activity of AbiP was investigated using four different RNA molecules. Three RNA transcripts consisted of the first 80 nt of phage bIL66M1 early transcripts *e1*, *e2* and *e3*, whose switch off is inhibited in AbiP$^+$ cells (Domingues et al., 2004a). The fourth one, used to assess the ability of AbiP to discriminate phage transcripts, consisted of the first 80 nt of *L. lactis* IL1403 *fruR* mRNA (Barrière et al., 2005). The 5′ end of the transcripts was chosen taking into account that specificity determinants are most probably located in this region, which carries the translation signals. Our data showed that all RNA molecules tested were bound by AbiP with approximately the same affinity (average K_d value = 22 ± 5 nM). An example of RNA shift by AbiP is presented in Fig. 4A. The 10-fold difference in the K_d value for AbiP binding to RNA (22 nM) *versus* ssDNA (217 nM) confirms the preferential binding of AbiP to RNA as compared to ssDNA. Under these conditions, no difference was observed between the four different transcripts, indicating that the interaction of AbiP with RNA occurs in a sequence-independent manner.

The abortive infection phenotype is affected by mutations in the putative nucleic acid binding surface

To determine whether the putative OB-fold participates in nucleic acid binding, we performed site-directed mutagenesis of AbiP. On the basis of our structural model, amino acid residues F70, F125 and A90 are localized within the groove of the putative OB-fold and could be involved in interactions with nucleic acids (Fig. 1C). Moreover, these amino acids are conserved or present conservative substitution with amino acids shown to interact with ssDNA in RPA70 (Fig. 1B). These amino acids were exchanged to alanine individually (F70A, F125A and R90A) and in pairs (covering every possible combination). Site-directed mutagenesis was performed in *E. coli* and the plasmid constructs carrying the mutant versions of *abiP* were then transferred to *L. lactis* IL1403. Cell fractionation studies were subsequently performed on each strain expressing a mutant protein, as described above for AbiP. Analysis of the different

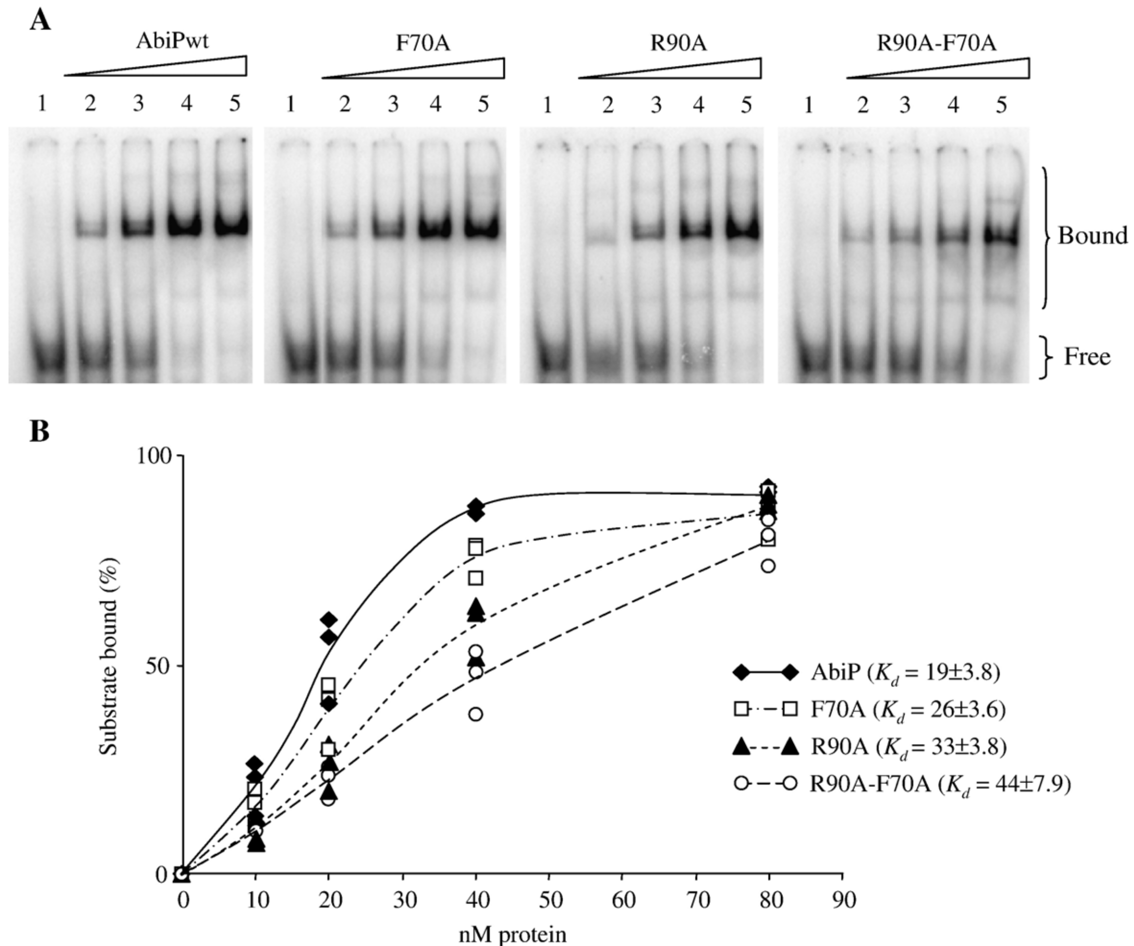

Fig. 4. Binding of AbiP and derivatives to the 5′-terminal 80 nt of bIL66M1 *e1* transcript. (A) Gel mobility-shift assays. Radiolabeled substrate (1 nM) was incubated separately with increasing amounts of each protein (as indicated on top) and resolved on a native 5% polyacrylamide gel as described in Materials and methods. Lane 1: sample without protein. Lanes 2–5: 10, 20, 40 and 80 nM of the respective protein were incubated with the substrate. (B) Binding curves and binding affinity of AbiP and derivatives to the 5′-terminal 80 nt of bIL66M1 *e1* transcript. The curves were derived from densitometric quantification of the autoradiograms (see Materials and methods). K_d values were calculated from these curves. Results are the mean of three independent assays.

fractions (protoplasts, cytoplasm and membrane), simultaneously using antibodies against AbiP and PepC, indicated that none of the mutations introduced in AbiP affected either the subcellular localization of the proteins or their expression level (data not shown).

To assess the impact of each mutation on the abortive infection phenotype displayed by AbiP, we studied its effect on phage bIL66M1 multiplication by enumerating bIL66M1 on *L. lactis* IL1403 expressing each mutant protein. The results are presented in Table 1. In the absence of AbiP, bIL66M1 formed clear plaques. In contrast, when plated on the strain carrying AbiP, it formed only barely visible turbid plaques with a strongly reduced efficiency of plating (EOP = 10^{-8}). The same EOP and plaque morphology were obtained when bIL66M1 was plated on strains expressing AbiP with single substitutions F70A or F125A, whereas on the strain carrying both substitutions the EOP raised to 10^{-1}. However, the plaques formed were still turbid, indicating that the abortive infection phenotype was not completely abolished. On the strain carrying the R90A mutation, the EOP of bIL66M1 slightly increased (10^{-7}). The additional presence of the F125A mutation had no significant effect on the phenotype, whereas on an R90A/F70A background the EOP increased by one order of magnitude (10^{-6}). A mix of turbid and clear plaques was observed on all strains carrying the R90A mutation. Phages isolated from the clear plaques were able to

Table 1
Development of phage bIL66M1 on IL1403 expressing AbiP or derivatives

Expressed protein in IL1403	bIL66M1 EOP [a]	Plaque morphology
Absence of AbiP	1	Clear
AbiP	10^{-8}	Turbid
F70A	10^{-8}	Turbid
F125A	10^{-8}	Turbid
F70A-F125A [b]	10^{-1}	Turbid
R90A	10^{-7}	Turbid + Clear
R90A-F70A	10^{-6}	Turbid + Clear
R90A-F125A	10^{-7}	Turbid + Clear

[a] Efficiency of plating (EOP) was calculated as the ratio between the titer of the phage in the presence of AbiP or derivatives and its titer in the control strain (in the absence of AbiP). The EOP values listed are the mean of at least three experiments.

[b] The F70A-F125A result is probably spurious (for details, see Discussion).

overcome AbiP$_{R90A}$ and thus corresponded to spontaneous-resistant mutants. Yet, these phages were still sensitive to the wild-type AbiP, which is in agreement with previous reports concerning the absence of spontaneous AbiP-resistant mutants (Domingues et al., 2004b). Altogether, these results provide evidence for the involvement of residues F70 and R90 in the anti-phage activity of AbiP.

Nucleic acid binding activity of the mutant proteins

Finally, the nucleic acid binding activity of the mutant proteins shown to have a stronger incidence on the abortive infection phenotype was investigated. For this purpose, proteins carrying the F70A, F70A-F125A, R90A and F70A-R90A mutations were overexpressed in *E. coli* and purified following the protocol described for AbiP. Under these conditions, the F70A-F125A double mutant was not recovered after protein precipitation with 20% ammonium sulfate, suggesting a substantial structural modification in the protein. The nucleic acid binding activity of the other mutant proteins was compared to that of the wild type using the previously described ssDNA and RNA templates. All the mutant proteins exhibited an approximately 10-fold preference for RNA (data not shown). However, their binding affinity was lower than that displayed by the wild-type protein. This is exemplified in Fig. 4A, where binding of AbiP and derivatives to the 80 nt of bIL66M1 *e1* transcript was examined. Binding curves, obtained from three independent assays, and the calculated K_d values from these curves are shown in Fig. 4B. Mutants F70A, R90A and F70A-R90A bound *e1* with 1.3-, 1.7- and 2.3-fold reduced affinity, respectively. Altogether, these results indicate that residues F70 and R90 are implicated in the nucleic acid binding activity of AbiP. Furthermore, these results are in agreement with the observed phenotype of the mutants *in vivo*, suggesting a correlation between the abortive infection phenotype and the presence of the putative OB-fold in AbiP.

Discussion

The aim of this work was to further characterize the lactococcal phage abortive infection protein AbiP. For this purpose, we carried out the analysis and functional characterization of two domains identified in the AbiP sequence: an N-terminal TMD and a central OB-fold motif.

Cell fractionation experiments and mutational analysis showed that the predicted N-terminal TMD of AbiP is responsible for its anchorage to the cell membrane. The remainder of the protein was shown to face the cytoplasm.

A putative OB-fold was predicted from primary sequence homology to the human replication protein RPA (Bochkarev et al., 1997) and structural modeling. Proteins containing this motif are frequently involved in nucleic acid binding (Theobald et al., 2003). We showed that this is also the case of AbiP, which interacts *in vitro* with both ds- and ss-nucleic acids, exhibiting a 10-fold preference for RNA. Electrophoretic mobility-shift assays performed with AbiP mutant derivatives indicate that the putative OB-fold is involved in the interaction of AbiP with both types of molecules. However, this interaction was not drastically affected by the introduced mutations, which is not unexpected since several amino acids of an OB-fold are usually involved in the interaction with nucleic acids (Theobald et al., 2003). Mutagenesis of additional residues would be necessary in order to further validate the structural model proposed for the putative OB-fold of AbiP. Under our experimental conditions, no specificity was detected either for phage DNA or phage transcripts.

The relevance of the cellular localization and nucleic acid binding activity of AbiP for the phage resistance phenotype was further investigated. We showed that AbiP$_{\Delta N\text{-term}}$, an AbiP derivative lacking its N-terminal TMD, was devoid of Abi activity. It is not clear whether the absence of phage resistance was strictly dependent on the cellular localization or was related with the correct folding of AbiP while anchored to the membrane. The latter hypothesis seems less likely. Although deletion of the membrane anchorage domain resulted in loss of solubility in about one third of the protein molecules, two thirds of the expressed protein were still soluble. The fact that protein solubility correlates with the correct folding, and conversely aggregation and precipitation are associated with protein misfolding (Wigley et al., 2001), suggests that about two thirds of the truncated protein have adopted the correct folding. Unless membrane localization is essential, this amount of soluble protein would likely still be enough to confer some degree of resistance. The complete absence of abortive infection phenotype strongly indicates that membrane location might be necessary. Moreover, AbiP was previously shown to block phage DNA replication (Domingues et al., 2004a). Although little is known about lactococcal phage replication and transcription, compelling results from the best studied models have indicated that replication of bacterial genomes, including resident plasmids and bacteriophages, occur at the cell membrane (for reviews, see Bravo et al., 2005; Firshein, 1989; Sueoka, 1998). In this context, it seems reasonable to assume that membrane anchorage would favor a model involving the interference of AbiP with phage DNA replication. Even though the molecular models have not been elucidated, several lactococcal Abi systems have been described that prevent phage DNA replication. It is the case of AbiA, AbiF, AbiK and AbiR (Emond et al., 1997; Garvey et al., 1995; Hill et al., 1991; Twomey et al., 2000). Nevertheless, none of the corresponding proteins seem to present membrane anchor domains. AbiA presents a leucine zipper motif, shown to be essential for the anti-phage activity (Dinsmore et al., 1998), whereas mutations in a reverse transcriptase motif identified in AbiK caused the loss of the resistance phenotype (Fortier et al., 2005). On the other hand, two lactococcal Abi proteins that are probably anchored to the membrane, AbiZ and AbiTi (Bouchard et al., 2002; Durmaz and Klaenhammer, 2007), do not seem to interfere with phage DNA replication. AbiZ causes premature cell lysis of infected cells (Durmaz and Klaenhammer, 2007) and AbiT was proposed to act at a late step of the phage lytic cycle, affecting phage morphogenesis and inducing premature cell death (Bouchard et al., 2002). No effect was observed on phage DNA replication in the presence of AbiC, a protein that shares 22% identity with AbiP and possesses two N-terminal TMD. The authors proposed that this Abi system would more probably target any step of phage transcription or translation (Moineau et al., 1993). Indeed, despite

few common features, the means by which each system causes Abi appears to be unique, as it seems to be the case of AbiP.

Mutations introduced in the putative OB-fold affected phage multiplication differently. Single F70A and F125A substitutions had no effect on phage EOP and plaque morphology. The drastic increase ($\times 10^7$) observed in the EOP of an AbiP-sensitive phage when plated on a strain carrying both mutations is probably an artifact. Structure modifications revealed during protein purification most probably account for such a decrease in the efficiency of AbiP. In contrast, the R90A mutation increased the EOP (10-fold) and allowed the appearance of mutant phages resistant to $AbiP_{R90A}$. The effect of the R90A mutation on both nucleic acid binding and phage multiplication is not very pronounced but is enhanced by the additional F70A change; the double mutant exhibits a 100-fold increase in EOP. Altogether, these results establish the importance of AbiP residues F70A and R90A for the Abi phenotype. They also suggest that the effects of AbiP on phage development might be dependent on its nucleic acid binding activity. More pronounced effects would perhaps be obtained by mutagenesis of additional residues. It is of interest to note that AbiP and AbiC share three regions of homology separated by an insertion (on AbiC) or by deletion (on AbiP) of two stretches of 34 and 36 amino acids (Domingues et al., 2004a,b). The 34 amino acids insertion on AbiC is located within the predicted OB-fold domain on AbiP and could participate in the different mode of action of the two proteins (Domingues et al., 2004a,b; Moineau et al., 1993). Another hypothesis would be that the remaining C-terminal part of the protein (101 amino acids) may also contribute to the Abi phenotype.

The lack of specificity for nucleic acids of phage origin, observed under our experimental conditions, suggests that AbiP might bind cellular nucleic acids. However, this conclusion is in contradiction with the absence of toxicity of AbiP. The hypothesis of a protein turned on following phage infection into an active and toxic form would conciliate these two contradictory features. Phage infection would be halted as a consequence of the toxic activity of the system rather than by a direct action against the phage. This is the case of other known phage exclusion systems, which are triggered upon phage infection. Lit protease, which cleaves the elongation factor Ef-Tu, is activated by the short T4 Gol peptide (Bingham et al., 2000; Georgiou et al., 1998). Activation of PrrC nuclease is dependent on a small T4-encoded peptide (Stp) (Kaufmann et al., 1986). PifA, the single F gene responsible for T7 exclusion, is triggered by T7 gp1.2 or gp10 (Molineux et al., 1989). The nature of the Rex-triggering signal remains unknown. There are, however, some evidences that it may be a protein–DNA complex produced when the phage begins to replicate and recombine (Parma et al., 1992). Similarly, a lactococcal phage protein has been proposed to activate either AbiD1 expression or its activity (Bidnenko et al., 2002). Mutations in relevant phage proteins in these systems suppress the activation of the exclusion mechanism and allow normal phage development (Bidnenko et al., 2002; Copeland and Kleanthous, 2005; Kaufmann et al., 1986; Molineux et al., 1989). Thus, the hypothesis of a latent protein activated following phage infection would also allow an explanation for the appearance of $AbiP_{R90A}$-resistant mutant phages, which would fail to activate this AbiP derivative. It might also explain how phage bIL170 protein E6 confers resistance to AbiP (Domingues et al., 2004b). On the light of this hypothesis, it is tempting to speculate that the phage protein would inhibit AbiP by interfering with the activation of the latent form.

The data presented here strongly suggests that the anti-phage activity of AbiP depends on both its membrane anchorage and binding to nucleic acids. Our results are compatible with the previously established interference of AbiP on phage DNA replication and switch off of the phage early transcripts (Domingues et al., 2004a). However, further work is required in order to elucidate how these AbiP properties act in conjunction to arrest phage development.

Materials and methods

Bacterial strains, bacteriophages and growth media

L. lactis sp. *lactis* IL1403 (Chopin et al., 1984) and its derivatives were grown at 30 °C in M17 medium (Terzaghi and Sandine, 1975) in which lactose has been replaced by glucose (M17-G). When needed, 5 μg/ml erythromycin (Ery) was added to the culture medium. *E. coli* strains used in this work were NovaBlue (Novagen), XL1-blue (Stratagene) and Tuner(DE3) pLacI (Novagen). *E. coli* was cultivated in Luria Broth medium supplemented with 25 mg/ml thymine (LB-T) at 37 °C, except when differently specified. When required, this medium was supplemented with 50 or 100 μg/ml ampicillin (Amp), 15 μg/ml tetracycline (Tet), 34 μg/ml chloramphenicol (Cm), 70 μg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and 80 μM IPTG [isopropyl-(3-D-thiogalactoside)]. Phage bIL66M1 (Bidnenko et al., 1995) was enumerated on IL1403 and derivatives as previously reported (Terzaghi and Sandine, 1975).

DNA manipulations, enzymes and oligonucleotides

Standard DNA manipulations and cloning were carried out essentially as previously described (Sambrook et al., 1989). *L. lactis* was transformed by electroporation as described by Holo and Nes (1989). Transformation of *E. coli* was done by electroporation according to Sambrook et al. (1989). Plasmid DNA was extracted with the Wizard Plus Minipreps DNA Purification System (Promega). DNA amplification was performed with TaKaRa Ex Taq (TaKaRa Biomedicals), as recommended by the supplier, on GeneAmp PCR System 9700 (Applied Biosystems). PCR products were purified with the Wizard PCR Preps DNA Purification System (Promega). [α-^{32}P]UTP (800 Ci mmol^{-1}) and [γ-^{32}P]ATP (4000 Ci mmol^{-1}) were obtained from MPBiomedicals. Labeling reactions were carried out with T4 polynucleotide kinase (New England Biolabs) as described by the supplier. Enzymes used for DNA cloning were from New England Biolabs, Novagen or Roche and were used according to the supplier's instructions. DNA sequencing was done with the ABI PRISM BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and resolved on an automated DNA sequencer (ABI3700, Applied Biosystems). Sequences were analyzed by

GCG software (Devereux et al., 1984). Prediction of the TMD in the N-terminus of AbiP was done using the online TMHMM version 2 (http://www.cbs.dtu.dk/services/TMHMM; Krogh et al., 2001; Sonnhammer et al., 1998), and the OB-fold hit was found with the SSEARCH software (Pearson, 1991; Smith and Waterman, 1981). All oligonucleotide sequences used in this work are listed in Supplementary Table 1.

3D modeling of the AbiP segment (positions 57–143)

The hypothetical three-dimensional model of the AbiP segment (positions 57–143) was constructed using HOMOLOGY module (Accelrys) based on the average of five chosen structures as a template (the first OB-fold of the human replication protein A subunit RPA70, the telomere binding protein α, the RecG bacterial helicase, the human mitochondrial DNA binding protein and the pertussis toxin). Energy minimization *in vacuo* was performed (DISCOVER, Accelrys).

Plasmid constructions

abiP was amplified from pIL2617 (Domingues et al., 2004a) with primers sd101 and sd102, and the PCR product containing 3′dA overhangs was cloned in pETBlue-1 (Novagen) using *E. coli* NovaBlue. Site-directed mutagenesis of *abiP* was carried out with the QuickChange Site-Directed Mutagenesis Kit (Stratagene) as indicated by the supplier, using primer pairs sd152/sd153, sd154/sd155 and sd156/sd157. In each case, the entire gene sequence was checked to confirm that no other mutations had been introduced. After PCR amplification with primers sd158 and sd159, *abiP* and its derivatives were cloned in *L. lactis* IL1403 using the *Eco*RI–*Xba*I sites in pIL253 (Simon and Chopin, 1988). N-terminal deletion of *abiP* was achieved by amplifying *abiP* with primers sd159 and sd160 using pIL2617 as template. The purified PCR product was also cloned in pIL253 as described above.

Overexpression and purification of proteins

All protein manipulations and buffers were at 4 °C. AbiP and derivatives were expressed from pETBlue-1 as native proteins (i.e. without any added tag) using *E. coli* Tuner(DE3)pLacI as the expression strain. Cultures were grown in 1 L LB-T at 30 °C to an optical density (OD) (600 nm) of 1. Protein expression was induced for 8 h at 23 °C by the addition of 500 μM IPTG. Cells were harvested by centrifugation and stored at −20 °C until further use. Frozen cells were resuspended in 40 ml of cold lysis buffer (50 mM Tris–HCl pH 8, 500 mM NaCl) and broken by sonication (Vibracell 72408 sonicator from Bioblock) for 30 min (pulse 2 s, pause 9 s), amplitude 40%. The lysate was centrifuged for 30 min at 15300×*g* and the supernatant, containing mainly cytoplasmic soluble proteins, was discarded. The pellet (enriched with membranes and AbiP) was resuspended in lysis buffer supplemented with 0.1% Triton X-100 (to solubilize membrane-anchored proteins as AbiP) and centrifuged again in the same conditions. Soluble proteins were precipitated with 20% ammonium sulfate, resuspended in 10 ml of cold buffer R (50 mM Tris–HCl pH 8, 150 mM NaCl, 0.1% triton X-100, 1 mM dithiothreitol [DTT]) and centrifuged again in the same conditions. The supernatant was loaded onto a HiTrap Q column (Amersham Pharmacia Biotech). AbiP and derivatives were eluted with a linear NaCl gradient (0.15 to 2 M) in buffer R. Purified proteins were stored at −20 °C in 50% glycerol. AbiP identity was checked by N-terminal sequencing (Edman method) and the presence of the mutations in AbiP mutants was confirmed by mass spectroscopy (MALDI-TOF) following trypsinolysis.

Protoplasting and cell fractionation

The procedure used was based on the method described by Wu and Errington (1997). *L. lactis* cultures were grown to an OD (600 nm) of 0.4. Cells were harvested by centrifugation (generally 6 ml of culture was processed), resuspended in 2 ml of SMMM medium (0.5 M sucrose, 20 mM maleic acid, 20 mM $MgCl_2$ and M17-G 2×) containing 10 mg/ml lysozyme and incubated at 37 °C with gentle agitation for 2 h (more than 95% of the cells were protoplasted and no cell lysis was detected by microscopy examination). Protoplasts were pelleted by centrifugation and resuspended in the same volume of SMMM without lysozyme. Cell fractionation was usually carried out using 1 ml of the protoplast suspension. Protoplasts were harvested by centrifugation and resuspended in 50 μl of TM buffer (20 mM Tris–HCl pH 8, 5 mM $MgCl_2$) containing 2 mM phenylmethylsulfonyl fluoride (PMSF). The sample was incubated at room temperature for 30 min, checked by microscopy to ensure that most of the protoplasts had lysed, centrifuged for 15 min at 4 °C and washed twice with TM buffer. The supernatant (cytoplasmic fraction) was added to 50 μl of 2× sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) loading buffer, and the pellet (membrane fraction) was resuspended in 100 μl 1× SDS–PAGE loading buffer. When required, protoplast and membrane samples were previously treated with 50 μg/ml proteinase K for 20 min, at room temperature. The samples were subsequently analyzed by SDS–PAGE followed by Western immunoblotting.

Standard protein manipulations and Western blotting

Protein concentrations were estimated by the Bradford method (Bradford, 1976) using the Bio-Rad protein assay (Bio-Rad) as described by the supplier. Polyclonal antibodies for AbiP were prepared by Eurogentec following rabbit immunization with the corresponding band cut out from a polyacrylamide gel performed after protein purification. Electrophoresis of protein samples was carried out with NuPAGE Novex 10% or 4–12% Bis-Tris gels (Invitrogen) in MOPS buffer (Invitrogen). After electrophoresis, proteins were transferred to a Hybond PVDF membrane (Amersham Pharmacia Biotech) by electroblotting using a semi-dry transfer system (Bio-Rad). Membranes were probed with antisera containing AbiP (1/5000 dilution), PepC (1/2500 dilution) and HtrA (1/1000 dilution). Immunodetection was carried out as described in the BC Chemiluminescence Western Blotting Kit (Roche).

DNA and RNA substrates for binding studies

dsDNA, ssDNA and RNA used as substrates in gel mobility-shift assays were prepared by several means. The mix containing dsDNA and ssDNA substrates was prepared by PCR amplification of the bIL66M1 genomic region comprising P_{E1} and P_M, using primers sd125 and sd126. The 325-bp PCR product was then used as a template for a linear PCR with only primer sd126, previously labeled at the 5′ end with [γ-^{32}P]ATP. After purification with MicroSpin G-50 columns (Amersham Pharmacia Biotech), the mix was resolved on a 5% polyacrylamide (29:1) gel in 0.25× TBE buffer (22.5 mM Tris borate, 0.5 mM EDTA). The gel was dried and analyzed with a Storm Apparatus (Molecular Dynamics), and the DNA was quantified with the ImageQuant software (Molecular Dynamics), relatively to the known concentration of radiolabeled sd126.

Oligonucleotides sd129, sd130, sd131, sd132, sd133, sd134 and ocs2 were used as ssDNA substrates. These oligonucleotides were 5′ end-labeled with [γ-^{32}P]ATP, electrophoresed on a 10% polyacrylamide gel (29:1) in 0.25× TBE buffer and purified by passive elution (Maxam and Gilbert, 1977) with buffer E_1 (10 mM Tris–HCl pH 8, 1 mM EDTA, 0.3 mM NaCl, 0.2% SDS) overnight at 42 °C. DNA concentration was estimated by measuring absorbance at 260 nm, using a Biophotometer (Eppendorf) with Uvettes.

RNA substrates were prepared by *in vitro* transcription. DNA templates used in the transcription reactions were obtained by PCR amplification. *e1*, *e2* and *e3* were amplified from bIL66M1 lysate with primer pairs sd140/sd145, sd146/sd147 and sd148/sd149, respectively. *fruR* was amplified from *L. lactis* IL1403 DNA with primers sd150 and sd151. Oligonucleotides sd140, sd146, sd148 and sd150 contained the proper binding site for the T7 RNA polymerase. *In vitro* transcription was carried out using T7 RNA polymerase (Roche) essentially as recommended by the supplier. Reactions were performed in 25 μl using 10 U of T7 RNA polymerase, 40 U RNasin (Promega) and [α-^{32}P] UTP as the only rUTP source. The reaction mixtures were separated on a 10% polyacrylamide (29:1) gel in 0.25× TBE buffer. The transcripts were purified by passive elution in buffer E_2 (50 mM Tris–HCl pH 7.5, 0.5% SDS, 5 mM EDTA) for 2 h at 42 °C. RNA was recovered by ethanol precipitation, resuspended in 10 mM Tris–HCl (pH 8) and stored at −80 °C with 40 U RNasin. RNA concentration was estimated as described for ssDNA substrates.

Gel mobility-shift assays

Purified SSB_{Bs}, AbiP and AbiP mutant derivatives, previously diluted in dilution buffer (50 mM Tris–HCl pH 8, 50 mM NaCl, 1 mM DTT), were incubated with the radiolabeled substrates in 20 μl of binding buffer (50 mM Tris–HCl pH 7, 50 mM KCl, 1 mM DTT, 0.01% Triton X-100, 0.4 mg/ml bovine serum albumin [BSA]) for 15 min at 30 °C. Protein and substrate concentrations are indicated in the figure legends. Dilution buffer was added to the control samples (without protein). All assays with RNA were performed in the presence of 40 U RNasin. After the incubation step, the reaction mixtures were loaded on a native 5% polyacrylamide (29:1) gel. Electrophoresis was carried out in 0.25× TBE buffer for 2 to 4 h at 4 °C. Gels were dried under vacuum and revealed with a Storm Apparatus (Molecular Dynamics). Densitometric quantification was done using the ImageQuant software and the apparent K_d value was determined according to Riggs et al. (1970). Gel mobility-shift experiments presented in this work were performed at least three times with each substrate.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2007.11.004.

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