The lactococcal abortive infection protein AbiV interacts directly with the phage protein SaV and prevents translation of phage proteins

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Running title: AbiV and phage protein interaction prevent translation

Abstract

AbiV is an abortive infection protein that inhibits the lytic cycle of several virulent phages infecting *Lactococcus lactis* while a mutation in the phage gene *sav* confers insensitivity to AbiV. In this study, we have further characterized the effects of bacterial AbiV and its interaction with the phage p2 protein SaV. First, we showed that during phage infection of lactococcal $AbiV^+$ cells, AbiV rapidly inhibited protein synthesis. Among early phage transcripts, the *sav* gene transcription was slightly inhibited while the SaV protein could not be detected. Analyses of other phage p2 mRNAs and proteins suggested that AbiV blocks the activation of late gene transcription probably by a general inhibition of translation. Using size exclusion chromatography coupled with on-line static light scattering and refractometry, as well as fluorescence quenching experiments, we also demonstrated that both AbiV and SaV formed homodimers and they strongly and specifically interact with each other to form a stable protein complex.

Introduction

Bacteriophages infecting *Lactococcus lactis* strains during milk fermentation is a persisting problem in the dairy industry [1,47,49]. When growing in large industrial milk fermentation vats, host cells can succumb to attacks from members of many genetically-distinct lactococcal phage groups [15,18]. *L. lactis* strains have evolved numerous natural anti-phage barriers to protect themselves against a diverse population of virulent phages [42]. These defense mechanisms act at different steps of the phage lytic cycle such as blocking phage adsorption, DNA entry, DNA replication, or assembly [26,38,47]. Abortive infection mechanism (Abi) is a broad term used to identify anti-phage systems that inhibit phage multiplication after DNA entry but act before the release of phage progeny [32]. Abi systems also cause premature bacterial cell death upon phage infection [32]. Restriction-modification [1] and CRISPR [2] systems, which inhibit phage infection by cleaving the incoming nucleic acids, are excluded from the Abi group.

At least 23 distinct Abi mechanisms have been identified in *L. lactis* [23, 34]. They form a heterogeneous group usually encoded by a single gene though some Abi systems are encoded by two genes [7,16,17,28,52] or more [64]. Lactococcal Abi genes have a lower G+C content than the other host genes and they share limited amino acid sequence similarity [13]. They also vary significantly in their activity against various lactococcal phage groups [10,13,61].

Though Abi systems are considered the most efficient group of anti-phage systems, their industrial use has led to the emergence of Abi-insensitive phage mutants [47]. From an industrial point of view, there is therefore a recurrent need to either discover new phage resistance mechanisms [59] or to improve the efficacy of current systems. In order to improve or expand the efficacy of the known mechanisms, one needs to understand their mode of action.

Homologues of lactococcal Abi proteins exist in the databases, and are found in an impressive variety of microbes. Unfortunately, no homology with proteins of known detailed function (other than phage resistance) has been found, preventing any prediction to be made on their mode of action [4,6,13]. For most Abi systems, our knowledge is limited to their overall effect on the phage lytic cycle [13,22,61]. For example, AbiA, F, K, P, and T were shown to interfere with DNA replication [7,22,25,29,33] while AbiB, G, and U affected RNA transcription [7,14,16,17,28,52]. AbiC was shown to cause limited major capsid protein production [50] whereas AbiE, I, and Q affected phage packaging [24,28,60]. With this level of information it is difficult to separate the primary target of Abi from subsequent effects [4,6,13]. Comparative genome analyses of Abi-insensitive phage mutants have led to the identification of phage genes involved in the Abi phenotype [4,5,7-9,19-21,31,41,48]. Again, many of these phage genes code for proteins of unknown function thereby complicating prediction of the Abi system's mode of action.

Combined genetic and biochemical studies have provided insight into the mechanism of some lactococcal Abi systems [4,6,13]. AbiB was demonstrated to either induce or function as a RNase [53]. AbiD1 was found to be induced by a phage protein and to act on an essential phage RuvC-like endonuclease [4,6,13]. AbiZ causes premature cell lysis possibly by interacting with phage holin [23]. AbiK was found to possess a key reverse transcriptase motif [27] and a phage single-strand annealing protein is involved in its antiviral activity [54]. A deeper understanding of the molecular interactions between Abi mechanisms and phage components will certainly provide valuable information on the mode of action of Abi systems.

Recently, we isolated the chromosomally-encoded abortive infection mechanism AbiV [30] and reported that a phage protein (SaV) with anti-microbial properties is involved in AbiV sensitivity [31]. Here, we demonstrate that AbiV and SaV proteins interact directly and we show that AbiV prevents phage protein synthesis and late gene transcription.

Materials and Methods

Bacterial strains, phages, and growth conditions. Bacterial strains and phages used in this study are listed in Table 1. *E. coli* was grown at 37ºC in LB medium [56] or Turbo Broth for protein expression. *L. lactis* was grown at 30ºC in M17 [62] supplemented with 0.5 % glucose (GM17). In experiments with incorporation of radioactive uridine or methionine, *L. lactis* was grown in SA medium supplemented with 0.5 % glucose (GSA) [36] and with reduced methionine concentration (5 µg ml^{-1}) [39]. During phage infection experiments, bacterial growth and cell lysis were determined by cell density (OD_{450} in GSA medium and OD_{600} in GM17 medium) using a BioScreen C apparatus (Oy Growth Curves Ab Ltd). In phage infection experiments, 10 mM $CaCl_2$ was added to the medium. Propagation of phages [25] and phage titration [35] were performed as described previously. When needed, antibiotics were added as follows: for *E. coli*, 100 µg/ml of ampicillin, 34 µg/ml of chloramphenicol, and 25 µg/ml of kanamycin; for *L. lactis*, 5 µg/ml of chloramphenicol.

Determination of total RNA and protein synthesis in *L. lactis* cells. Exponentially growing cultures (OD_{600} = 0.5) of *L. lactis* JH-20 ($AbiV^+$) and *L. lactis* JH-54 ($AbiV^-$) were infected with phage p2 at a multiplicity of infection (MOI) of 5 while a non-infected *L. lactis* JH-20 culture served as control. Then, for labeling of RNA, 2.3 ml of culture was mixed with 2 µl of [^{14}C]uridine (50 µCi ml^{-1}) and 6 µl of 10 mM uridine to a final uridine concentration of 55 µM. Labeling of proteins was done by adding 5 µl of [^{35}S]methionine (15 mCi ml^{-1}) to 1.7 ml of culture (the concentration of methionine in the SA medium was reduced to 5 µg ml^{-1}). Samples (200 µl for RNA and 150 µl for protein) were taken from the labeled cultures at 5 min intervals, transferred to a tube with 3 ml of cold 5% trichloroacetic acid (TCA) and put on ice for 1 to 1.5 h.

110 The precipitated macromolecules were collected on a membrane filter (0.45-µm pore size;
111 Schleicher & Schuell), washed twice with cold 5% TCA and once with boiling water, and left to
112 air-dry. The radioactivity on the filters was counted in a Packard Instant Imager [37].
113
114 **Effect of AbiV on phage mRNA.** Another set of exponentially growing cultures (OD_{600} = 0.5)
115 of *L. lactis* JH-20 ($AbiV^+$) and *L. lactis* JH-54 ($AbiV^-$) were concentrated 10-fold by
116 centrifugation, and resuspended in fresh medium. Then, the cells were infected with phage p2 at a
117 MOI of 5. Two milliliter samples were taken at 5 min intervals, quickly centrifuged, and snap-
118 frozen in -80ºC liquid ethanol. Infected-cell pellets were resuspended and incubated (37ºC, 15
119 min) in 100µl 0.5 M sucrose with 60 mg ml^{-1} lysozyme before being mixed with 1 ml TRIzol
120 reagent (Invitrogen). Total RNA was isolated according to the manufacturers' instructions and
121 the samples were treated with the DNase based TURBO DNA-*free* kit (Applied Biosystems)
122 before storing at -80ºC. Immediately before use, the RNA samples were thawed and 0.5 µg RNA
123 was added to 0.5 ml of a denaturing solution containing 10 mM NaOH and 1 mM EDTA. The
124 RNA samples were blotted onto Zeta-probe nylon membranes (Bio-Rad) by use of a Bio-Dot SF
125 slot blot apparatus (Bio-Rad). After a brief rinse in 2X SSC [56] plus 0.1% sodium dodecyl sulfate
126 (SDS) for 1 min at room temperature, the membrane was air-dried for 10 min and fixed by
127 exposure to UV-light for 2 minutes on each side. Membranes were prehybridized for minimum
128 2 h in UltraHyb-Oligo hybridization buffer (Ambion) before ^{32}P-labeled probe was added. After
129 hybridization overnight at 42°C, the membranes were washed three times at 42ºC in 2X SSC with
130 0,5 % SDS, for 30 min before being air-dried. Radioactivity was measured by overnight exposure
131 of Storage Phosphor Screens (Amersham) and subsequent detection in a STORM 860 scanner in
132 storage phosphor acquisition mode. Quantification of the radioactive signal was performed using
133 the software ImageQuant TL v.2003.03.

Primers used as probes for the detection of mRNA transcripts are listed in Table 2. The primers covered the following genes of lactococcal phage p2: *orf2* (terminase large subunit), *orf11* (major tail protein), *orf16* (baseplate protein), *orf25* (unknown function), *orf26* (SaV), *orf27* (unknown function), and *orf48* (holliday junction endonuclease). These oligonucleotides were labeled with [^{32}P]ATP (Easytides, Perkin Elmer) using polynucleotide kinase (Roche) and subsequent purification with NucAway spin columns (Ambion). Labelling efficiency was determined by quantification of 5 µl labeled probe using a Packard Instant Imager.

Intracellular detection of phage proteins during infection. First, anti-ORF26 (SaV) antibodies were produced by PickCell Laboratories BV while anti-ORF11 (major tail protein) and ORF16 (baseplate protein) antibodies were made by Davids Biotechnologie GmbH. Then, similar to phage mRNA analyses, exponentially growing cultures (OD_{600} = 0.5) of *L. lactis* JH-20 ($AbiV^+$) and *L. lactis* JH-54 ($AbiV^-$) were concentrated 10-fold and infected with phage p2 at a MOI of 5. Two milliliter samples were taken at 5 min time intervals, flash-frozen (-80°C), and analyzed for intracellular production of phage proteins by Western blots. Cell pellets were resuspended in 400 µl 10 mM Tris-Cl, pH 8.0, 1 mM EDTA, 0.3% SDS, and lysed. Twenty µl of the solution was mixed with 20 µl sample loading buffer and proteins were separated by SDS-PAGE electrophoresis [43] using 11 % gels for detection of ORF11 and ORF16 and 9 % gels for detection of SaV. The proteins were electroblotted (200 mA for 1 h) onto a PVDF membrane (Hybond P) using a 20% ethanol solution containing 25 mM Tris and 192 mM glycine pH 8.3 as transfer buffer in a Trans-Blot SD apparatus (Bio-Rad). The membranes were subsequently treated with blocking buffer (5 % non-fat dry milk in phosphate buffered saline supplemented with 0.1 % tween-20 (PBS-T)) for 1 h on an orbital shaker and then treated (1 h, shaking) with primary antibody diluted in blocking buffer. For the SaV, ORF11, and ORF16 antibodies, the following

158 dilutions were used: 1:75,000; 1:100,000; and 1:25,000; respectively. After three washes with
159 PBS-T the membrane was incubated (1 h, shaking) with secondary antibody (anti-rabbit IgG
160 alkaline phosphatase, Amersham) diluted 1:100,000 in blocking buffer. This was followed by
161 three washes with PBS-T and 10 min equilibration in PBS before the membrane was treated with
162 the ECF-substrate (Amersham) according to the manufacturers' instructions. The protein bands
163 were visualized using a STORM 860 scanner in the blue excitation (450 nm) fluorescence
164 acquisition mode. Quantification of the fluorescent signal was performed using the software
165 ImageQuant TL v.2003.03 (Amersham).
166
167 **Purification of proteins.** Genes coding for AbiV and SaV were cloned into the pQE-70 vector
168 (Qiagen) to create C-terminal His-tags on both proteins in *E. coli* M15 strains (strains JH-62 and
169 JH-65, respectively). Proteins were purified as follows: After an overnight induction at 25^{o}C with
170 0.5 mM IPTG, cells were harvested by centrifugation for 10 min at 4000 × g. The pellet was
171 resuspended in 40 ml of lysis buffer (Tris 50 mM, NaCl 300 mM, imidazole 10 mM, pH 8)
172 supplemented with 0.25 mg/ml of lysozyme, 20 ug/ml DNase, 20 mM $MgSO_4$, and EDTA-free
173 antiprotases (Roche), and frozen at –80^{o}C. After thawing and sonication, lysates were cleared by
174 centrifugation (30 min at 12,000 × g). The proteins were purified by nickel affinity
175 chromatography (His-Trap 5 ml column, GE Healthcare) on a FPLC (Pharmacia AKTA) using a
176 step-gradient of imidazole followed by a preparative Superdex 200 gel filtration (10 mM Tris,
177 300 mM NaCl, pH 8). Concentrations of the purified proteins were determined using NanoDrop
178 1000 (Thermo Scientific). A_{280} values were corrected for differences in absorption coefficient due
179 to amino acid composition of the protein monomers using the ProtParam tool
180 (http://www.expasy.org/tools/protparam.html). The p2 phage proteins SSB (single-strand binding
181 protein, ORF34) and Sak3 (ORF35) were purified as described elsewhere [57] .

Size exclusion chromatography (SEC) with on-line multiangle laser light scattering, absorbance, and refractive index (MALS/UV/RI) detectors. SEC was carried out on an Alliance 2695 HPLC system (Waters) using a Superose S12 column eluted with buffer (50 mM Tris and 50 mM NaCl, pH 8.0) at a flow of 0.5 ml/min. Detection was performed using a triple-angle light scattering detector (Mini-DAWNTM TREOS, Wyatt Technology), a quasi-elastic light scattering instrument (DynaproTM, Wyatt Technology), and a differential refractometer (Optilab_rEX, Wyatt Technology). Molecular weight and hydrodynamic radius determination was performed by the ASTRA V software (Wyatt Technology) using a dn/dc value of 0.185 ml/g. Proteins were loaded at a final concentration of 0.22 mM and 0.33 mM for AbiV and SaV, respectively.

Fluorescence quenching experiments. Fluorescence experiments were carried out on a Varian Eclipse spectrofluorimeter using a quartz cuvette in a right-angle configuration as previously described [63]. Briefly, the light path was 1.0 and 0.4 cm for the excitation and emission, respectively. The interaction of AbiV with SaV was monitored by recording the quenching of the intrinsic SaV protein fluorescence upon addition of AbiV aliquots, which does not have an intrinsic fluorescence or absorbance at 285 nm. The excitation wavelength was 285 nm and the emission spectra were recorded in the range of 320 to 400 nm. The excitation slit was 5 nm while the emission slit was 10 nm for a SaV protein concentration of 0.5 µM. A moving-average smoothing procedure was applied, with a window of 5 nm. Titrations were carried out at room temperature with 0.24 µM of quencher protein in 10 mM Tris Buffer, 50 mM NaCl, pH 8. No correction of the fluorescence at the maximum level (341 nm) was needed since the fluorescence and absorbance levels of the buffer and the quencher protein were negligible. The affinity was

estimated by plotting the decrease of fluorescence intensity at the emission maximum as 100 - (I_i - I_{min})/(I_0 - I_{min}) x 100 against the quencher concentration; I_0 is the maximum of fluorescence intensity of the protein alone, I_i is the fluorescence intensity after the addition of quencher (i), and I_{min} is the fluorescence intensity at saturating concentration of quencher. The K_d values were estimated using Prism 3.02 (GraphPad Software Inc.) by nonlinear regression for a double binding site with the equation $Y = B_{max1} \bullet X/(K_{d1} + X)] + B_{max2} \bullet X/(K_{d2} + X)$, where B_{max} is the maximal binding, Kd_1 is the concentration of ligand required to reach half-maximal binding for the first binding site and Kd_2 is for the second binding site while X is the value of the concentration of binder added at each step. Additional controls were performed using the same protocol. Because the SSB protein of phage p2 (ORF34) does not possess any tryptophan and intrinsic fluorescence or absorbance at 285 nm, it was used in fluorescence quenching experiments with the p2 protein SaV. On the other hand, the Sak3 protein (ORF35) of phage p2, which possesses five tryptophan residues, was used in combination with the AbiV protein in fluorescence quenching assays.

Results

AbiV affects total RNA and protein synthesis during phage infection. We previously demonstrated that the dsDNA genome of the lactococcal phage p2 replicates in AbiV$^+$ *L. lactis* cells but the infection is aborted prior to the packaging of phage DNA [30]. To determine the effects of AbiV on the synthesis of other macromolecules, we measured the synthesis of RNA and proteins in both AbiV$^+$ (*abiV* is constitutively expressed) and AbiV$^-$ *L. lactis* cells during infection with the virulent phage p2.

Compared to the non-infected cells, the addition of phages (in both AbiV$^+$ and AbiV$^-$ cells) caused a rapid decline in RNA and protein synthesis (data not shown). Still, new RNA and proteins were synthesized in phage-infected AbiV$^-$ cells and they both increased until 26-28 minutes (Fig. 1), which coincided with the end of the phage lytic cycle and the release of new p2 virions. In phage-infected AbiV$^+$ cells, new RNA was also produced but at a reduced rate as compared to the AbiV$^-$ culture (Fig. 1). RNA synthesis continued throughout the experiment (50 minutes), due to the absence of complete cell lysis and it leveled off between 40 and 50 minutes. In contrast, protein synthesis was severely inhibited in the phage-infected AbiV$^+$ culture as compared to the phage-infected AbiV$^-$ culture (Fig. 1), and after 15 min, protein synthesis was stopped completely in the AbiV$^+$ culture.

AbiV affects transcription of middle and late phage genes. Knowing that the AbiV mechanism inhibits protein synthesis we wanted to address more specifically which part of the phage lytic cycle is targeted by AbiV. In another set of experiments, *L. lactis* JH-20 (AbiV$^+$) and *L. lactis* JH-54 (AbiV$^-$) were infected with virulent phage p2 and the synthesis of specific phage transcripts was investigated. Lysis of the sensitive AbiV$^-$ culture occurred 29 minutes after

infection and was accompanied by a rise in phage titer corresponding to a burst size of ca. 50 pfu per infected cell. To quantify the transcription level at different time-points during the phage lytic cycle, several radioactively labeled oligonucleotide probes covering early, middle, and late-expressed genes of phage p2 were used in a dot blot assay.

In the early-expressed region, three genes (*orf25*, *orf26*/*sav*, and *orf27*) were analyzed. The *sav* gene encodes a non-structural protein located in the early-expressed region of the phage and likely the target of AbiV [30,31]. In AbiV$^-$ cells, *orf25-27* all reached similar level of transcription, which peaked between 6 and 12 minutes after which they gradually decreased throughout the rest of the experiment (Fig. 2A). In AbiV$^+$ cells, transcription levels were also equal among the three genes. The highest level was reached at 6 minutes after infection followed by a gradual decrease of transcription. In AbiV$^+$ cells, the transcription level of the three analyzed genes (*orf25*, *orf26*/*sav*, and *orf27*) was between 60 to 75% of those found in infected AbiV$^-$ *L. lactis* cells.

Two other phage genes (*orf48* and *orf2*) had almost identical transcription patterns (Fig. 2B) and their burst of expression was mid-way through the phage cycle. In AbiV$^-$ cells, both middle-expressed transcripts increased until T23 whereas in the AbiV$^+$ cells they leveled off at T17 as well as reaching only 50% of the level observed in AbiV$^-$ cells.

The late-expressed transcripts specific to *orf11* (encoding the major tail protein (MTP) of phage p2) and *orf16* (encoding a baseplate protein of phage p2 [58]) peaked toward the end of the phage cycle in the AbiV$^-$ cells (Fig. 2C). The expression of *orf16* was slightly delayed compared to *orf11*. For both genes, their transcription in the AbiV$^+$ cells ceased at T17 concomitant with the middle transcripts and reached only 10 % of the level found in the AbiV$^-$ cells.

Taken altogether, the above transcription data shows that phage mRNAs are produced in the presence of AbiV. However, transcription leveled off at T17 which affected the levels of early, middle and late transcripts unequally. Early-expressed phage genes were the least affected by

AbiV, followed by the middle-expressed transcripts (50 % of wild type level). The late phage transcripts were almost completely inhibited in the presence of AbiV.

AbiV inhibits translation of phage proteins. During the above phage infection experiments, samples were also taken for phage protein analyses. Using Western blotting and antibodies specific to the phage proteins ORF26/SaV, ORF11, and ORF16, we followed the production of these proteins during the phage p2 lytic cycle within $AbiV^-$ and $AbiV^+$ cells. In the $AbiV^-$ cells, SaV production increased throughout the infection until cell lysis (Fig. 3), whereas no significant production of SaV could be detected in the $AbiV^+$ cells during the experiment. A similar pattern was observed for the structural proteins ORF11 and ORF16. Production of the two proteins increased in $AbiV^-$ cells from midway in the lytic cycle and throughout the experiment (Fig. 3). The timing of expression of the three proteins in the $AbiV^-$ cells was thus in agreement with mRNA synthesis. In the $AbiV^+$ cells however, no production of the phage structural proteins ORF11 and ORF16 occurred. A low and constant level of ORF11 and ORF16 was observed, which was most likely due to the presence of the two structural proteins in the p2 virions used for the infection.

Taken altogether, Western blots showed that translation of both early and late phage proteins was severely inhibited in the presence of AbiV. For SaV, no protein production was observed while its gene was significantly transcribed (Fig. 2A) thus suggesting that AbiV inhibits translation of phage proteins early in the lytic cycle.

AbiV and SaV interact with each other. *In vitro* chemical cross-linking assays indicated a direct protein interaction between AbiV and SaV (data not shown). In order to determine if the

proteins do interact directly to confer the anti-phage phenotype, we used two different approaches: SEC-MALS/UV/RI and fluorescence quenching experiments.

To determine the stochiometry of the AbiV and SaV homodimers, and the size of the complex AbiV/SaV, we used SEC-MALS/UV/RI (Fig. 4A). The MALS/UV/RI analysis gave a measured mass of 47,550 Da and 36,000 Da for AbiV and SaV, respectively. Because the theoretical masses of AbiV and SaV are 22.7 kDa and 15.3 kDa, the measured mass indicates that both proteins form homodimers (Fig. 4A). When both proteins are injected together, the chromatogram shows a single major peak and a second peak corresponding to excess of unbound SaV. The measured mass (71,410 Da) corresponded to a complex consisting of $AbiV_2SaV_2$. The theoretical mass of such complex was calculated at 75,600 Da. The above data supports our previous observations that both AbiV and SaV probably are native dimers [30,31]. The hydrodynamic rayon (R_h) of AbiV and SaV was estimated 3.0 nm and 3.2 nm, respectively, while the R_h of the complex is 4.0.

Since AbiV has no tryptophan, it was possible to measure the dynamics of the AbiV-SaV association using SaV tryptophan fluorescence quenching. Addition of AbiV quenched fluorescence emission of SaV tryptophans when excited at 285 nm. A slightly better fit between the experimental data and the theoretical curve could be obtained assuming two binding sites instead of one: a high affinity one with a Kd_1 of 19 ± 1.6 nM, indicating a strong interaction between both proteins (Fig. 4B), and a low affinity site with a Kd_2 of 500 nM, reflecting probably additional non-specific interactions often observed at high concentration. The specificity of the high affinity interaction was assessed by testing SaV or AbiV interaction with the phage p2 proteins SSB (ORF34) and Sak3 (ORF35), respectively. The choice of proteins was made on their availability and their composition in tryptophan residues (the SSB has no trp while Sak3 has 5 trp). For both protein couples (SaV-SSB or Sak3-AbiV), the Δ value obtained, which

315 correspond to the percentage of the difference between the maximum and the minimum of
316 fluorescence intensity at the maximum wavelength, was 3.5 to 3.9 %. This indicates that there is
317 no interaction between those proteins. Thereby, it confirms the specificity of the SaV-AbiV
318 interaction, which showed a decrease in fluorescence (Δ value) of 17% (Fig. 4C).

Discussion

Recently, we isolated the lactococcal abortive infection mechanism AbiV [30] and also identified the phage protein SaV as being necessary for the abortive phenotype [31]. Here, we demonstrate a direct protein-protein interaction between the host protein AbiV and the phage protein SaV by SEC-MALS/UV/RI and fluorescence quenching assays (Fig. 4). AbiV and SaV likely form a complex consisting of 2 AbiV and 2 SaV molecules. The strength of interaction was significant (K_d value of 19 ± 1.6 nM) and is, to our knowledge, the first demonstration of a direct interaction between an Abi protein and a phage protein. Together with our previous demonstration that a functional SaV is needed for the Abi phenotype [31], this finding suggests that the $AbiV_2$-SaV_2 complex is responsible for the Abi phenotype.

Previous transcription analyses of the lactococcal phage sk1, which shares 96% nucleotide identity with phage p2 (data not shown), have revealed that early transcripts appear 2-5 minutes after the beginning of infection whereas middle transcripts are observed after 7-10 minutes and late transcripts after 15 minutes [11]. Our phage p2 transcriptional analysis revealed a continuous mRNA production both in $AbiV^+$ and $AbiV^-$ phage-infected cells (Fig. 2). However, the presence of AbiV reduced the transcription of early- (*orf25*, *orf26*/*sav*, and *orf27*)**,** middle- (*orf2, orf48*), and late-expressed genes (*orf11, orf16*). The decrease was most evident for the late transcripts probably due to a general cessation of transcription around T17. In the $AbiV^-$ cells, early phage p2 transcripts started decreasing after 12 minutes due to a known switch-off mechanism [11,22,53]. In the $AbiV^+$ cells, these early phage p2 transcripts decreased as well but earlier and their overall level was lower as we observed a 25-40 % reduction compared to the $AbiV^-$ cells. For the middle- and late-expressed phage genes, the decrease in transcription was approximately 50 % and 90 %, respectively in phage-infected $AbiV^+$ cells. It was previously demonstrated for

lactococcal phages that the switch of transcription from early- to middle-expressed phage genes is mediated by an early translated product activating a middle promoter [5,12,40]. This activator is probably not fully expressed in AbiV$^+$ cells thereby causing the observed partial inhibition of middle gene transcription (Fig. 2B). Similarly, it was previously demonstrated that a middle-expressed phage gene codes for an activator of late transcription [11,40]. This domino effect of transcription inhibition likely prevented the synthesis of late phage transcripts.

A more profound effect was observed on protein synthesis in phage-infected AbiV$^+$ cells. Total protein synthesis was severely inhibited from the beginning of phage infection and ceased completely after 15 minutes (Fig. 1). It has been shown previously for the closely related lactococcal phage sk1 that a decrease of early protein production prevented translation of most middle transcripts and all late transcripts [11]. This was confirmed by the absence of the production of two phage structural proteins ORF11 (major tail protein) and ORF16 (baseplate protein) in phage-infected AbiV$^+$ cells (Fig. 3). Interestingly, the production of SaV could not be detected in AbiV$^+$ cells (using Western blotting) though *sav* mRNA was observed (Fig. 2A). A previous study has shown that the SaV protein is necessary for the Abi phenotype [30]. Thus, apparently the method used here was not sensitive enough to detect the limited amount of SaV needed to induce the Abi phenotype. Since AbiV caused almost complete inhibition of SaV translation while only minimally affecting its transcription and at the same time severely inhibited total protein synthesis, we suggest that the AbiV$_2$-SaV$_2$ complex inhibits the cellular translation apparatus.

The exact function of SaV in the phage lytic cycle is still unknown. Structural prediction of SaV using PHYRE software [3] revealed homology with the structure of the conserved region 2 of σ^{70} factor [46] . The region 2 is the most conserved in σ factors and this region is crucial for binding of σ factors to core RNA polymerase [44,45]. A Sav-like protein (E11) was identified previously in the virulent lactococcal phage c2 and also shown to be involved in AbiV activity [31]. Function

prediction of E11 using PHYRE is pointing toward an anti-sigma factor (FlgM) from *Aquifex aeolicus* [55]. It is thus tempting to speculate that the phage protein SaV, though not being essential is involved in phage transcription.

In conclusion, we have analyzed the interaction between the lactococcal phage resistance mechanism AbiV and the phage protein SaV [31]. Our current working hypothesis is that in phage-infected AbiV$^+$ cells, phage DNA replication occurs [30] as well as early-phage gene expression, including the expression of *sav*. But the transcription of phage middle- and in particular late-expressed genes (coding among others for ORF11 and ORF16) is inhibited probably due to the absence of activation. A small amount of SaV is produced early and rapidly interacts with the host AbiV protein to form an active complex that inhibits the translational machinery of the cell and hence also the phage encoded proteins including activators of the middle and late genes. However, the mechanistic details used by AbiV/SaV complex to abort the phage infection are still unresolved.

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571 **Figure legends:**

572 **Fig. 1.** Total incorporation of [^{14}C]-uridine and [^{35}S]-methionine in *L. lactis* during phage
573 infection. Total RNA synthesis in phage-infected AbiV$^-$ cells is represented by open squares
574 (AbiV$^-$ RNA). Total RNA synthesis in phage-infected AbiV$^+$ cells is represented by closed
575 squares (AbiV$^+$ RNA). Protein synthesis in phage-infected AbiV$^-$ cells is represented by open
576 diamonds (AbiV$^-$ protein). Protein synthesis in phage-infected AbiV$^+$ cells is represented by
577 closed diamonds (AbiV$^+$ protein). Vertical arrow represents time of lysis of the sensitive AbiV$^-$
578 culture.

579

580 **Fig 2:** Detection of early (A), middle (B) and late (C) phage p2 transcripts during infection of
581 AbiV$^+$ *L. lactis* cells (closed symbols) and of AbiV$^-$ *L. lactis* cells (open symbols).

582

583 **Fig. 3:** Western blotting for detection of ORF26/SaV, ORF11, and ORF16 during phage infection.
584 (A) Temporal phage protein production in AbiV$^-$ *L. lactis* cells and in AbiV$^+$ cells. Numbers
585 represent minutes in relation to time of infection (T0). Lane NI: non-infected cells. SaV: purified
586 SaV (p2). CsCl purified p2 virions. (B) Graphical presentation of the expression levels obtained
587 in (A). Open and closed symbols represent AbiV$^-$ and AbiV$^+$ cells, respectively.

588

589 **Fig. 4**: SEC-MALS/UV/RI analysis and fluorescence quenching assay. A) SEC-MALS/UV/RI
590 analysis. The abscissa indicates the time scale of the HPLC injection. The ordinate indicates the
591 molar mass in (Da). B) Fluorescence quenching assay fitting curve using a nonlinear regression
592 with a double binding site equation. The initial concentration of SaV was 0.5 μM followed by a
593 progressing addition of different concentrations of AbiV (calculated for the monomeric forms).

C) Fluorescence spectrum obtained for SaV-AbiV (i), SaV-SSB (ii) and Sak3-AbiV (iii). The Δ values obtained, which correspond to the percentage of the difference between the maximum and the minimum of fluorescence intensity at the maximum wavelength, were 17%, 3.9%, and 3.5%, respectively.

598 TABLE 1. List of bacterial strains and phages used in the study.

Bacteria or phages	Relevant characteristics	Source
Bacterial strains		
JH-20	*L. lactis* subsp. *cremoris* MB112 (pJH2); Cam^R, $AbiV^+$	30
JH-54	*L. lactis* subsp. *cremoris* MB112 (pLC5), Cam^R, $AbiV^-$	30
JH-62	*E. coli* M15 (pQE70::*abiV*), Amp^R, Km^R	30
JH-65	*E. coli* M15 (pQE70::*sav*); Amp^R, Km^R	31
Phages		
p2	Small isometric headed, 936 species	51
sk1	Small isometric headed, 936 species	12

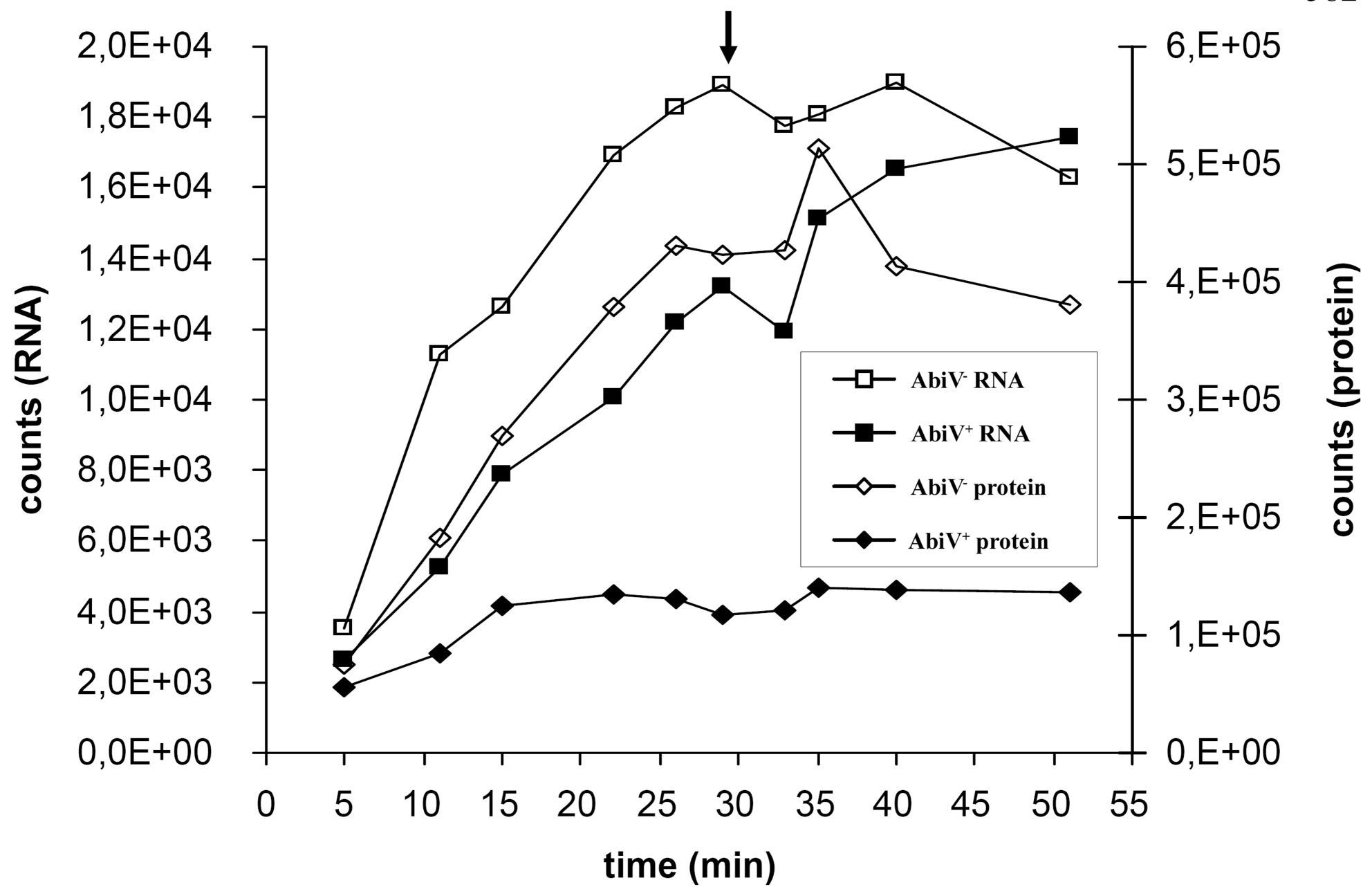
599 Amp^R, ampicillin resistance; Cam^R, chloramphenicol resistance; Km^R, kanamycin resistance;
600 $AbiV^+$, phage resistance phenotype (*abiV* is constitutively expressed from pHJ2); $AbiV^-$, phage
601 sensitive phenotype (empty expression vector).
602

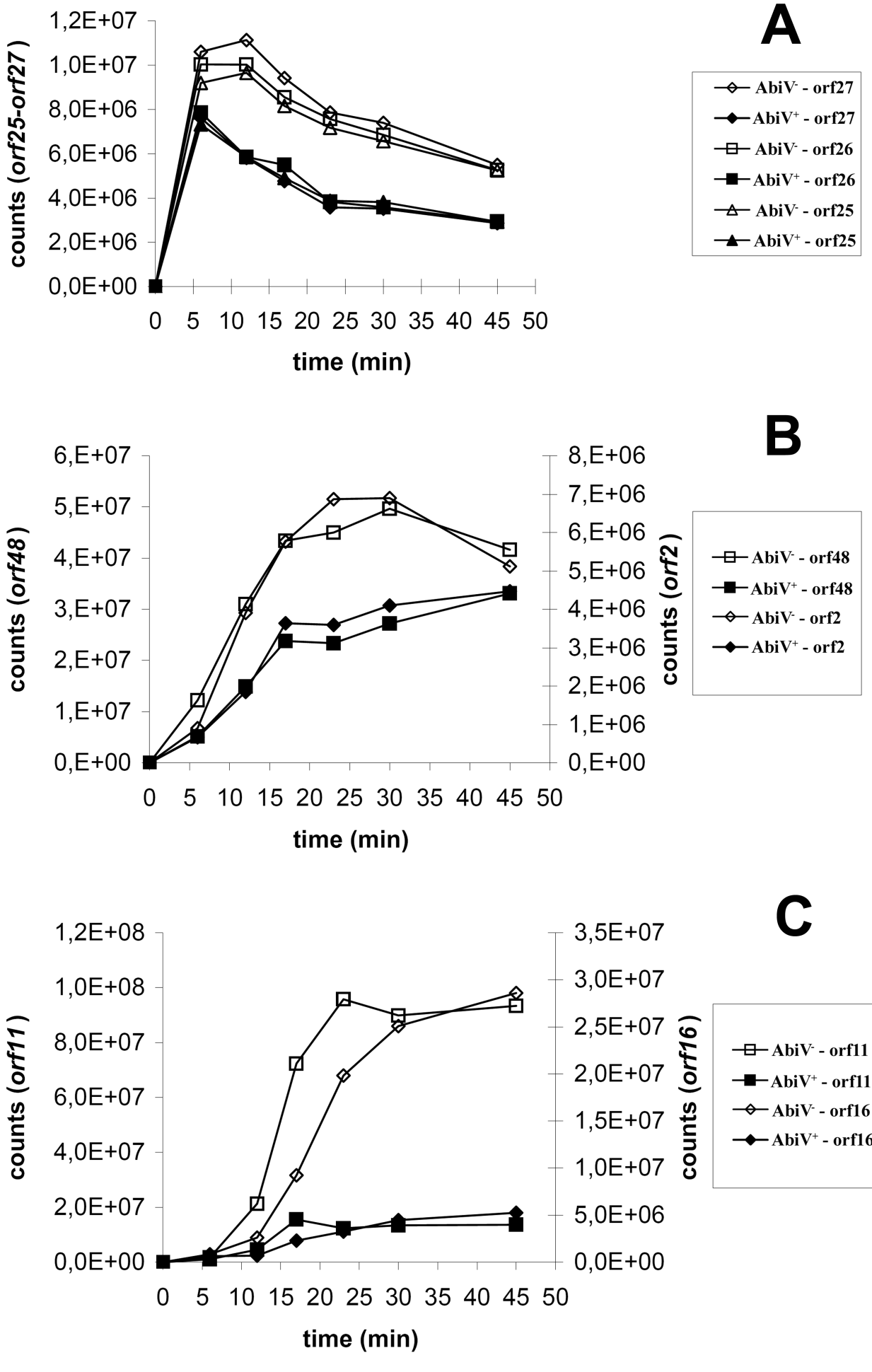
603

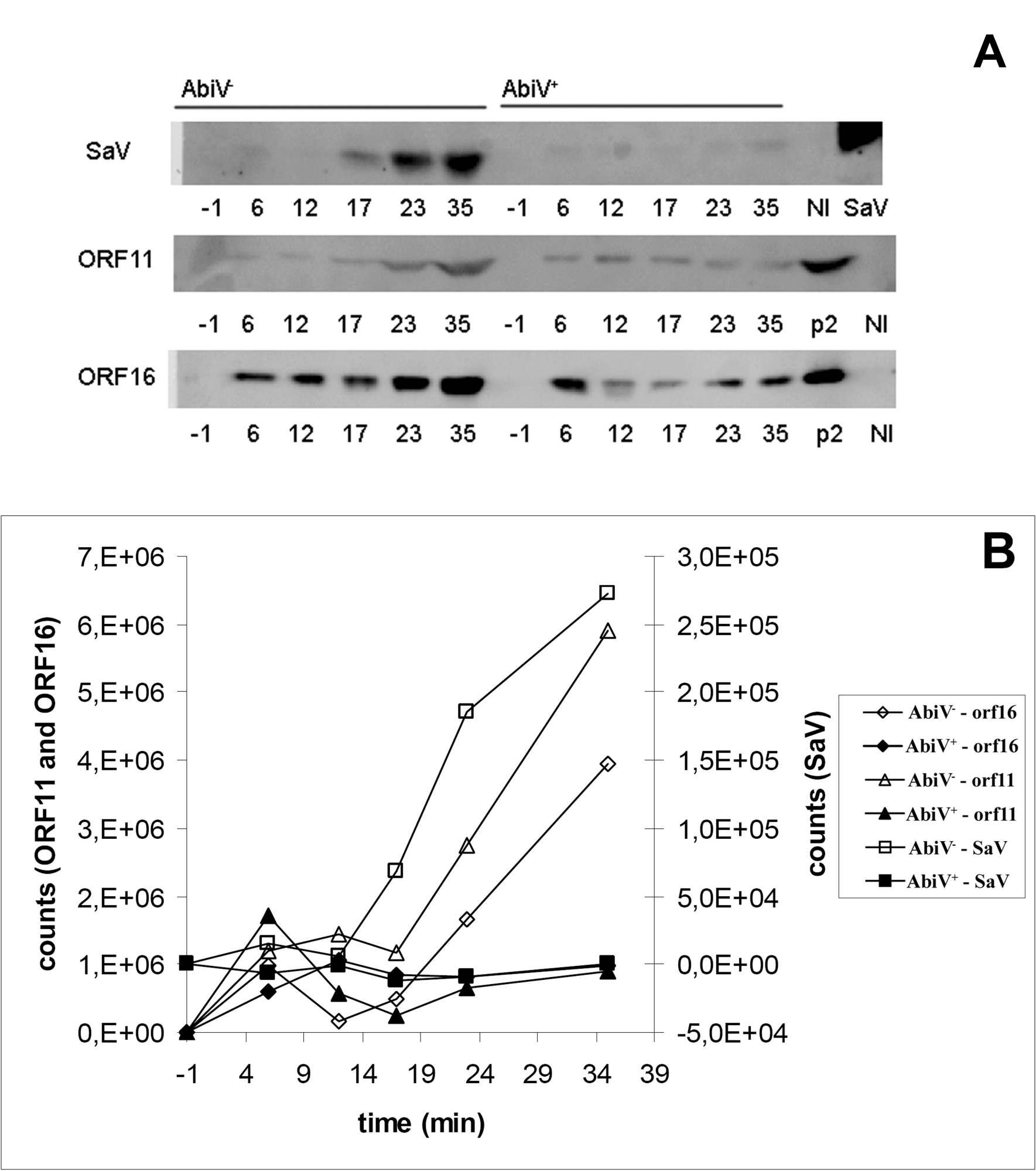
TABLE 2. List of primers used in the phage transcription analyses.

Primers	Sequence (5' - 3')
orf2 (*terL*)	GCCACTTAGGGACACTGCCAATAAGAGGTAAAGCC
orf11 (*mcp*)	GCGATAACCGTCGACAAATTCCCCTGTAACT
orf16	GCTGATGAAGTGTAAAGATAGCCTGTTTCCCACAG
orf25	GACAGGTCTACCATTATCAAGCCACCTGATGACTG
orf26 (*sav*)	GAATTAACTTTAGACCTCTTCAATAAATTCCAAGTATC
orf27	GTTAGATGTTACCCCCAATCCATGTAATAAGCAACG
orf48	GCCTGCAATTGAACCGACTACATACTCATTTGTCAA

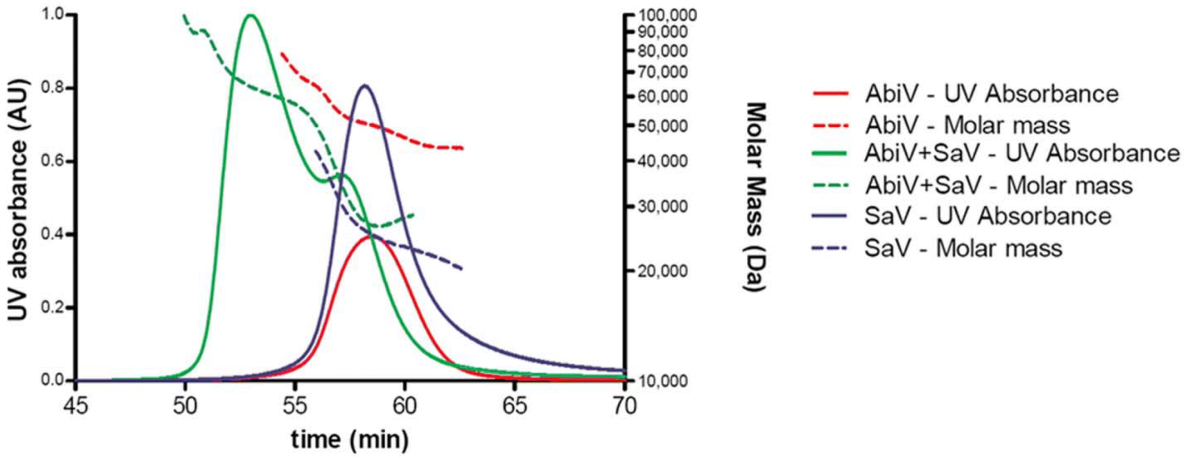
604


2,0E+04
1,8E+04
1,6E+04
1,4E+04
1,2E+04
1,0E+04
8,0E+03
6,0E+03
4,0E+03
2,0E+03
0,0E+00
counts (RNA)
6,E+05
5,E+05
4,E+05
3,E+05
2,E+05
1,E+05
0,E+00
counts (protein)
0
5
10
15
20
25
30
35
40
45
50
55
time (min)
AbiV- RNA
AbiV+ RNA
AbiV- protein
AbiV+ protein


A
counts (orf25-orf27)
1,2E+07
1,0E+07
8,0E+06
6,0E+06
4,0E+06
2,0E+06
0,0E+00
0 5 10 15 20 25 30 35 40 45 50
time (min)
AbiV- - orf27
AbiV+ - orf27
AbiV- - orf26
AbiV+ - orf26
AbiV- - orf25
AbiV+ - orf25
B
counts (orf48)
6,E+07
5,E+07
4,E+07
3,E+07
2,E+07
1,E+07
0,E+00
8,E+06
7,E+06
6,E+06
5,E+06
4,E+06
3,E+06
2,E+06
1,E+06
0,E+00
counts (orf2)
0 5 10 15 20 25 30 35 40 45 50
time (min)
AbiV- - orf48
AbiV+ - orf48
AbiV- - orf2
AbiV+ - orf2
C
counts (orf11)
1,2E+08
1,0E+08
8,0E+07
6,0E+07
4,0E+07
2,0E+07
0,0E+00
3,5E+07
3,0E+07
2,5E+07
2,0E+07
1,5E+07
1,0E+07
5,0E+06
0,0E+00
counts (orf16)
0 5 10 15 20 25 30 35 40 45 50
time (min)
AbiV- - orf11
AbiV+ - orf11
AbiV- - orf16
AbiV+ - orf16

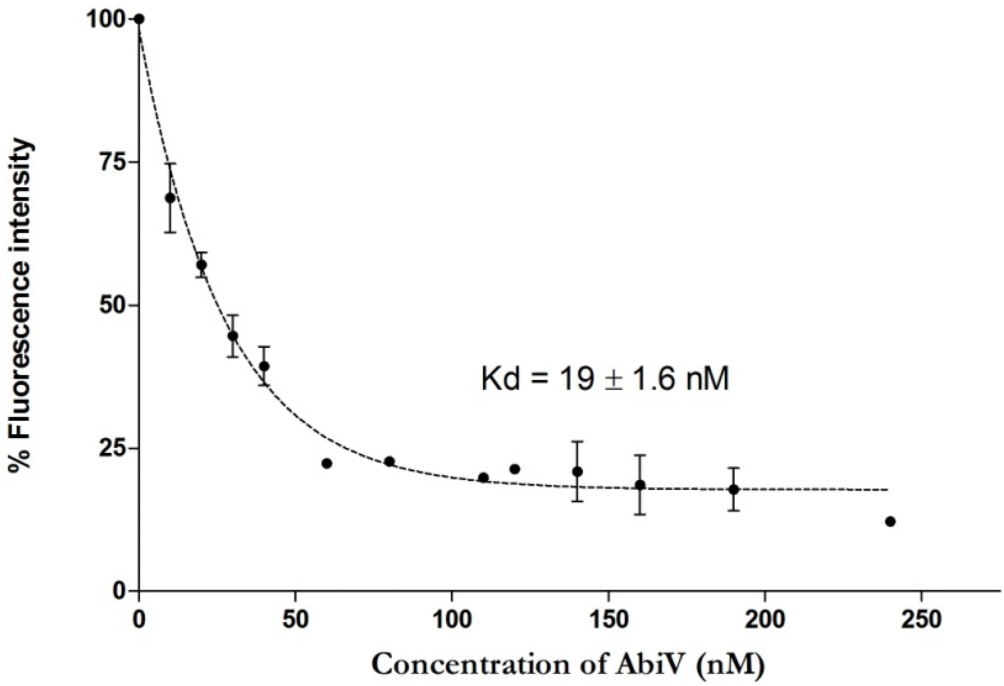

A
AbiV-
AbiV+
SaV
-1 6 12 17 23 35 -1 6 12 17 23 35 NI SaV
ORF11
-1 6 12 17 23 35 -1 6 12 17 23 35 p2 NI
ORF16
-1 6 12 17 23 35 -1 6 12 17 23 35 p2 NI
B
counts (ORF11 and ORF16)
7,E+06
6,E+06
5,E+06
4,E+06
3,E+06
2,E+06
1,E+06
0,E+00
3,0E+05
2,5E+05
2,0E+05
1,5E+05
1,0E+05
5,0E+04
0,0E+00
-5,0E+04
counts (SaV)
-1 4 9 14 19 24 29 34 39
time (min)
AbiV- - orf16
AbiV+ - orf16
AbiV- - orf11
AbiV+ - orf11
AbiV- - SaV
AbiV+ - SaV

A

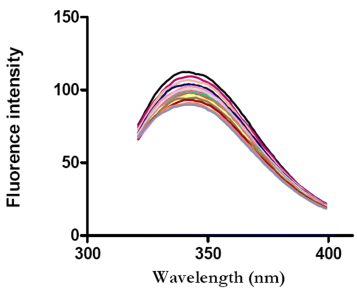


Masses (Da)	2AbiV	2SaV	2AbiV+2SaV
Theoretical	45,200	30,400	75,600
Measured	47,550	36,000	71,410

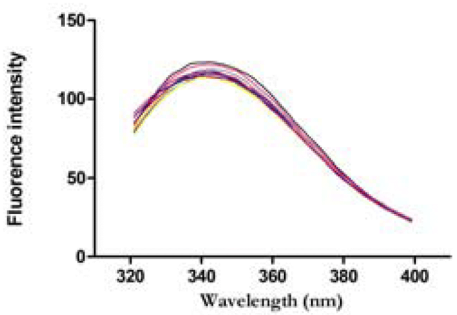
B



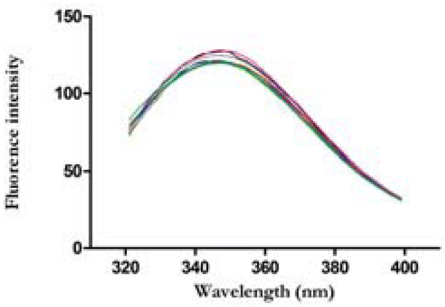
C



i) SaV-AbiV



ii) SaV-SSB



iii) Sak3-AbiV