

Development of Giant Bacteriophage ϕ KZ Is Independent of the Host Transcription Apparatus

Pieter-Jan Ceyssens,^{a*} Leonid Minakhin,^b An Van den Bossche,^a Maria Yakunina,^c Evgeny Klimuk,^{c,d,e} Bob Blasdel,^a Jeroen De Smet,^a Jean-Paul Noben,^f Udo Bläsi,^g Konstantin Severinov,^{b,c,d,e} Rob Lavigne^a

Division of Gene Technology, KU Leuven, Heverlee, Belgium^a; Waksman Institute of Microbiology, Rutgers University, New Jersey, USA^b; St. Petersburg State Polytechnical University, St. Petersburg, Russia^c; Institutes of Molecular Genetics and Gene Biology, Russian Academy of Sciences, Moscow, Russia^d; Skolkovo Institute of Science and Technology, Skolkovo, Russia^e; Biomedical Research Institute, Hasselt University, Diepenbeek, Belgium^f; Max F. Perutz Laboratories, University of Vienna, Vienna, Austria^g

ABSTRACT

Pseudomonas aeruginosa bacteriophage ϕ KZ is the type representative of the giant phage genus, which is characterized by unusually large virions and genomes. By unraveling the transcriptional map of the ~280-kb ϕ KZ genome to single-nucleotide resolution, we combine 369 ϕ KZ genes into 134 operons. Early transcription is initiated from highly conserved AT-rich promoters distributed across the ϕ KZ genome and located on the same strand of the genome. Early transcription does not require phage or host protein synthesis. Transcription of middle and late genes is dependent on protein synthesis and mediated by poorly conserved middle and late promoters. Unique to ϕ KZ is its ability to complete its infection in the absence of bacterial RNA polymerase (RNAP) enzyme activity. We propose that transcription of the ϕ KZ genome is performed by the consecutive action of two ϕ KZ-encoded, noncanonical multisubunit RNAPs, one of which is packed within the virion, another being the product of early genes. This unique, rifampin-resistant transcriptional machinery is conserved within the diverse giant phage genus.

IMPORTANCE

The data presented in this paper offer, for the first time, insight into the complex transcriptional scheme of giant bacteriophages. We show that *Pseudomonas aeruginosa* giant phage ϕ KZ is able to infect and lyse its host cell and produce phage progeny in the absence of functional bacterial transcriptional machinery. This unique property can be attributed to two phage-encoded putative RNAP enzymes, which contain very distant homologues of bacterial β and β' -like RNAP subunits.

Transcription is driven by DNA-dependent RNA polymerases (RNAPs), which synthesize RNA from DNA templates (1). RNAPs can be classified into two unrelated families: small single-subunit enzymes (ssRNAPs), encoded by some bacteriophages and also found in mitochondria and chloroplasts, and large multisubunit cellular enzymes (msRNAPs), transcribing genes in bacterial, archaeal, and eukaryal genomes. The catalytic activity of enzymes from both families is accomplished through a common two-metal-ion mechanism. The canonical bacterial msRNAP is a 400-kDa complex consisting of five core subunits ($\alpha_2\beta\beta'\omega$) which are directed to specific promoter sequences by a variety of σ factors (2). The two largest RNAP subunits, β and β' , contain conserved double-psi beta-barrel (DPBB) domains that together form the active center (3–5). Members of the ssRNAP family rely on different catalytic domains and amino acid motifs for catalysis of the RNA polymerization reaction and are related to DNA polymerases and reverse transcriptases (6, 7). Bacterial RNAPs are inactivated by the antibiotic rifampin (Rif), which acts by binding to the β -subunit pocket deep inside the active-site channel, preventing synthesis of RNA sequences longer than 3 to 4 nucleotides (nt) (8).

Bacterial RNAPs play a key role during the infection of bacterial cells by bacteriophages. Most known phages do not encode their own RNAP but redirect the host transcription machinery to viral promoters by relying on very strong promoters recognized by host RNAP and/or modifying the host RNAP specificity by phage-encoded factors (9, 10). Some phages, like T7, N4, Xp10, and ϕ EcoM-GJ1, depend on their own single-subunit RNAPs to transcribe a subset of viral genes (11–15). In these phages, two basic transcriptional strategies have been described. First, some phages,

like T7, recruit the bacterial RNAP at the onset of infection to transcribe the phage-encoded ssRNAP gene from an early promoter. Upon completion of this task, the bacterial RNAP is inactivated using posttranslational modifications or RNAP-binding proteins (16, 17), and the viral RNAP proceeds to transcribe middle and late phage genes. The N4-like viruses have evolved an inverse transcriptional strategy. An ssRNAP is carried within their capsids and is injected with phage DNA upon infection to transcribe early phage promoters (17–19). A second N4-encoded ssRNAP, a product of an early gene, transcribes middle phage genes. While early and middle N4 transcription is independent of bacterial transcription machinery, N4 recruits the host RNAP at the late stage of infection through the action of N4 single-stranded DNA-binding protein (20). A third strategy may be used by the giant *Bacillus subtilis* phage PBS2 (21). Forty years ago, the devel-

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Address correspondence to Konstantin Severinov, severik@waksman.rutgers.edu, or Rob Lavigne, rob.lavigne@biw.kuleuven.be.

P.-J.C. and L.M. contributed equally to this work.

* Present address: Pieter-Jan Ceyssens, Scientific Institute of Public Health (IIV-VSP), Brussels, Belgium.

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opment of this phage was reported to be independent of the addition of Rif, suggesting that host RNAP is not involved in viral transcription throughout the infection. In follow-up studies, the purification of a highly unusual multisubunit Rif-resistant RNAP complex from PBS2-infected cells was reported (22, 23). However, further study of this interesting phage and its transcription strategy has been hampered by the absence of its genome sequence.

Giant ϕ KZ-like bacteriophages form a very distant branch of myoviruses (24). The type virus, ϕ KZ, possesses an unusually large 280-kb genome, which displays little evolutionary relatedness to other genera. When the ϕ KZ genome was first reported in 2002, a function could be assigned to only 19% of its 306 predicted genes (25). The ϕ KZ virion is composed of no less than 50 different proteins (26, 27). Intriguingly, three virion proteins, gp178, gp80, and gp180, were found to resemble bacterial RNAP β and β' subunits. It was hypothesized that these proteins form a highly unusual virion RNAP, are co-injected with the phage DNA, and participate in early transcription, possibly through recruitment of host-encoded factors (26–29). Two other ϕ KZ proteins, Gp73 and Gp74, also resemble β/β' -like subunits but are not associated with virions, suggesting that the phage encodes an additional, nonvirion RNAP (nvRNAP) (25).

In this paper, we uncover the unique transcriptional scheme of giant phage ϕ KZ. We demonstrate that phage development and transcription of its genes is not sensitive to rifampin, proving that virus-encoded transcription enzymes are involved in phage gene expression. We also map conserved motifs located upstream of ϕ KZ genes belonging to different temporal expression classes. These motifs are distinct from promoters recognized by host RNAP. We propose that they are recognized by phage-encoded RNAPs which, based on our analysis, contain additional subunits that were not revealed by earlier work. The ϕ KZ transcription scheme likely is conserved and characteristic for other phages of the giant phage genus.

MATERIALS AND METHODS

Bacteriophages, bacterial strains, and growth conditions. In all experiments, the *P. aeruginosa* strain PAO1 was grown in standard LB medium, in some cases supplemented with rifampin (400 μ g/ml) or chloramphenicol (Cm; 100 μ g/ml). Bacteriophage ϕ KZ was amplified from confluent lysed agar plates, purified by 0.45- μ m filtration, and concentrated after incubation in 10% polyethylene glycol 8000 (PEG 8000)–1 M NaCl according to standard procedures (30). *P. aeruginosa* and its derivative strains were grown overnight in 5 ml LB medium to reach saturation. Cells were diluted 1:100 in 2 to 500 ml fresh medium, grown at 37°C, and, in infection experiments, infected at a multiplicity of infection (MOI) of 5 at an optical density at 600 nm (OD₆₀₀) of 0.3. Cell growth (or phage infection) was halted at the indicated time points by rapid cooling in ice water or by methods specific for RNA sequencing (RNA-seq) sampling (see below). Cells were harvested by centrifugation (6,000 \times g), flash-frozen, and stored at –80°C until use. The efficiency of infection was always checked by cell counts of the infected culture 5 min postinfection, which should contain <5% surviving cells compared to the noninfected culture.

Pulldown experiments. For the analysis of the bacterial RNAP complex upon phage infection, we used an available *P. aeruginosa* PAO1 *rpoA*::streptocinase (31). For the identification of protein interaction partners of the structural β -like subunit gp178, its gene was cloned with a 6 \times His tag coding sequence into the expression vector pHERD20T (32). This plasmid was electroporated to freshly prepared electrocompetent *P. aeruginosa* PAO1 cells. The obtained cells were tested for growth and sensitivity to ϕ KZ infection upon overexpression of the protein by 1% arabinose.

Infected *P. aeruginosa* cells were harvested by centrifugation and resuspended in 8 ml resuspension buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 0.1% [vol/vol] NP-40, 2.5 mM Pefabloc SC [Merck]) containing 1 mg/ml lysozyme (Sigma). After one freeze-thaw cycle, the mixture was supplemented with 800 μ l 10 \times BugBuster protein extraction reagent (Novagen) and 10 μ l Benzonase nuclease (Novagen) and incubated at room temperature for 15 min with gentle agitation. For His₆-mediated purification, the soluble fraction was loaded on a Bio-Rad Poly-Prep chromatography column containing 1 ml prewashed nickel nitrilotriacetic acid (Ni-NTA) agarose beads (Qiagen). The beads were washed with two successive wash steps of 10 ml and 5 ml resuspension buffer containing 25 mM and 50 mM imidazole, respectively, and eluted with 5 ml resuspension buffer containing 250 mM imidazole. The eluted fractions were pooled and concentrated by ultrafiltration (Amicon Ultra 3K; Millipore), heat denatured for 5 min at 95°C, and loaded on a 10% SDS-PAGE gel. The Coomassie-stained gel (Simply Blue safe stain; Invitrogen) was cut into slices, which were subjected to trypsin digestion previous to mass spectrometry analysis.

LC-MS/MS analysis and data analysis. An Easy-nLC 1000 liquid chromatograph (LC) (Thermo Scientific) was coupled on-line to a mass-calibrated LTQ-Orbitrap Velos Pro (Thermo Scientific) via a Nanospray Flex ion source (Thermo Scientific) using sleeved, 30- μ m-inner-diameter stainless steel emitters. The SpeedVac dried tryptic peptide mixture was dissolved in 20 μ l buffer A (0.1%, vol/vol, formic acid in Milli-Q water) and then loaded, concentrated, and desalted on a trapping precolumn (Acclaim PepMap 100 C18; Thermo Scientific) at a buffer A flow rate of 5 μ l/min for 5 min. The peptide mixture was separated on an Acclaim PepMap rapid separation liquid chromatography (RSLC) C₁₈ column (Thermo Scientific) at a flow rate of 250 nl/min of buffer A with a linear gradient in 40 min of 0 to 70% buffer B (0.1%, vol/vol, formic acid in acetonitrile [Biosolve]). Mass spectrometry (MS) data were acquired in a data-dependent mode under direct control of the Xcalibur software (version 2.2.SP1.48), selecting the fragmentation events based on the top six precursor abundances in the survey scan (350 to 2,000 Th). The isolation window for tandem MS (MS/MS) fragmentation was set to 2 Th, and the normalized collision energy, Q value, and activation time were 30%, 0.25, and 10 ms, respectively.

The analysis of the mass-spectrometric RAW data was carried out using Proteome Discoverer software v.1.3 (Thermo Scientific) with build-in Sequest v.1.3.0339 and interfaced with an in-house Mascot v.2.4 server (Matrix Science). MS/MS spectra were searched against a database containing all *P. aeruginosa* PAO1 proteins and all “stop-to-stop” protein sequences in all six frames of phage ϕ KZ. Peptide scoring for identification was based on the following search criteria: enzyme, trypsin; maximum missed cleavages, 2; precursor mass tolerance, 10 ppm; fragment mass tolerance, 0.5 Da.

RNA-seq analysis. Cells intended for RNA sequencing were rapidly suspended in 1/10 volumes of ice-cold stop solution (10% phenol in ethanol) and chilled to inhibit RNA transcription and degradation at the indicated time points. Cells then were collected by centrifugation (6,000 \times g), and total RNA was extracted from the resulting cell pellet by resuspending it in TRIzol (Ambion) and performing a classical phenol-chloroform extraction followed by ethanol precipitation. Remaining DNA was removed using TURBO DNase (Ambion), the efficiency of which was checked by the absence of reporter PCR products of both bacterial and phage genes. Subsequently, the samples were processed into directional cDNA libraries with the TruSeq stranded mRNA sample preparation kit and run on an Illumina HiSeq sequencer. For each of our comparative analyses, we used the total gene reads mapped to the host (*P. aeruginosa* PAO1) and viral (*Pseudomonas* phage ϕ KZ) annotated gene features using the CLC Genomics Workbench 6.0.2. software. The comparisons of relative levels of expression for gene features between different time points after infection within both the phage and host genomes were made with the negative binomial distribution test using the DESeq Bioconductor package in R.

Primer extension. *In vivo* primer extension reactions were done essentially as described elsewhere (33). *P. aeruginosa* PAO1 cells were infected with ϕ KZ (MOI, 5) at an OD₆₀₀ of 0.3 and harvested at time points specified for the experiments performed. When necessary, rifampin and chloramphenicol were added to the infected culture at designated time points at final concentrations of 150 μ g/ml and 100 μ g/ml, respectively. Total RNA was extracted using TRIzol reagent (Life Technologies) according to the manufacturer's procedure. For each primer extension reaction, 10 μ g of total RNA was reverse transcribed with 100 U of Moloney murine leukemia virus (MMLV) reverse transcriptase (Life Technologies) in the presence of 10 pmol of γ -³²P-end-labeled primer. The reaction mixtures were treated with RNase H, precipitated with ethanol, and dissolved in formamide loading buffer. To precisely identify the 5' ends of the primer extension products, DNA sequencing reaction mixtures containing both the corresponding PCR-amplified ϕ KZ genome fragments and end-labeled primers used for the primer extension reaction were performed. The reaction products were resolved on 6 to 8% (wt/vol) polyacrylamide sequencing gels and visualized using a PhosphorImager (Molecular Dynamics).

Time-lapse microscopy. For microscopic recordings, the infected culture was diluted a thousand times, spotted on an LB agar pad, and recorded in real time for 5 h. For this, we used a temperature-controlled (Okolab Ottaviano) Ti-Eclipse inverted microscope (Nikon) equipped with a TI-CT-E motorized condenser and a CoolSnap HQ2 FireWire charge-coupled device (CCD) camera. Images were acquired using the NIS-Elements AR 3.2 software (Nikon) as described previously (34).

GEO series accession number. All raw RNA-seq data were submitted to the Gene Expression Omnibus (GEO) repository under series record [GSE58494](#).

RESULTS

The transcriptional map of ϕ KZ. To investigate transcription regulation during ϕ KZ infection, we first acquired a blueprint of the transcriptional scheme of this giant phage by performing RNA-seq of total RNA prepared from cells collected at different times postinfection. This approach allowed us to globally detect phage transcriptional units belonging to different temporal classes at single-nucleotide resolution and to study the impact of phage infection on the *P. aeruginosa* transcriptome in a single experiment.

DNA-free total RNA was extracted from *P. aeruginosa* PAO1 cells immediately before (0 min) and 5, 15, and 35 min after infection. In the course of infection, the host cells marginally increased in size but acquired a rugby-ball shape (Fig. 1A). The amount of total intracellular RNA extracted from cells in the later stages of infection increased over 5-fold compared to the level in noninfected cells (Fig. 1B). This increase in total RNA was not due to host transcription, since a reverse transcription-PCR (RT-PCR) control of two household genes, *oprL* and 5S rRNA, showed a significant decrease in transcript abundance 35 min postinfection (Fig. 1C). RNA-seq analysis was performed on total RNA samples (in duplicate, with 5×10^6 reads/sample). In addition, early (10 min) and late (35 min) transcription was analyzed by deeper (nondirectional) RNA sequencing (5×10^7 reads/sample) (see Table S1 in the supplemental material). Reads originating from the phage and the host next were mapped to the respective genome sequences. A drastic accumulation of phage mRNA as the infection proceeded was observed. Late in infection, 36.9% of all reads mapped to the phage genome, which represented up to 98.5% of all non-rRNA and non-tRNA host RNA reads.

The results allowed us to identify 134 ϕ KZ operons, spanning an average of 2,075 bp and containing an average of 2.7 genes per

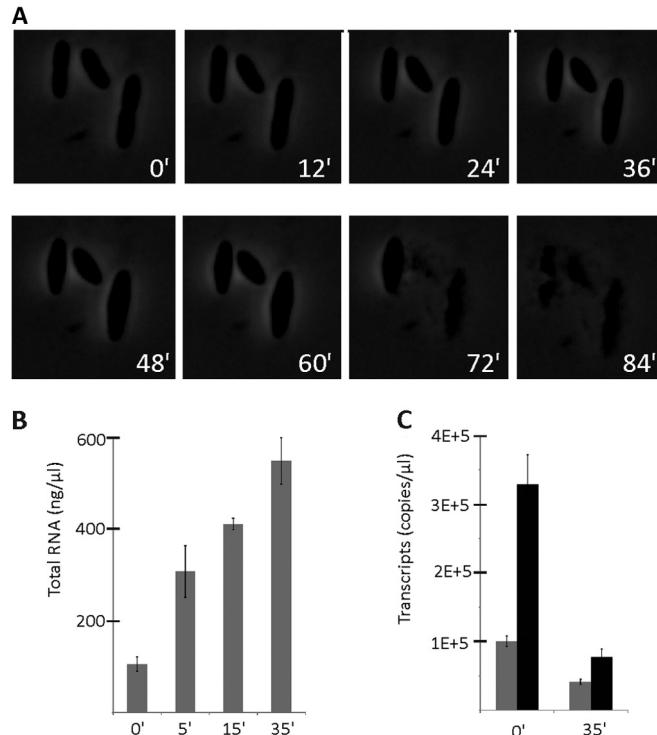


FIG 1 Total RNA extraction from ϕ KZ-infected cells. (A) Time-lapse recording of three *P. aeruginosa* cells infected with ϕ KZ at time zero. (B) Total RNA was extracted in triplicate from 200 μ l infected cells at the indicated times postinfection and quantified spectrophotometrically. (C) Transcript abundance of 5S rRNA (black) and *oprL* (gray) per μ l extracted RNA, as determined by quantitative RT-PCR with three biological and technical replicates.

operon (Fig. 2; also see Table S2 in the supplemental material). When comparing these transcriptional units to the original genome annotation (25), many events of intergenic transcription initiation were observed. We used this information to expand the predicted gene feature content of the genome from the original 306 to 369 coding sequences (+20.5%), removing the annotations of ORF24 and ORF117 due to a lack of sense transcripts and updating start codon positions of 27 open reading frames (ORFs) (see Table S3). The resulting updated ϕ KZ annotation file (GenBank accession number [AF399011.1](#)) corresponds to only 74% of the initial version submitted in 2002. Most of the newly identified ORFs display clear Shine-Dalgarno sequences. The majority of the newly annotated genes (72%) also have a counterpart in the closely related ϕ KZ-like viruses PA7 and ϕ PA3 (35), although we propose an alternative start codon for 48% of these genes. Many of the newly predicted proteins contain structural features, such as hydrophobic stretches (e.g., ORF 117.1), signal peptides (e.g., ORF 166.1), or conserved domains (e.g., PAAR domain in ORF 163.1; Pfam entry 05488; E = 1.96e-04), hinting at the functional importance of these proteins in the infection cycle (see Table S3). We also observed cases of antisense transcription, for example, involving genes encoding structural proteins 29 and 30 (see Fig. S1). The function of these antisense RNAs (asRNA) might be to regulate expression levels by interfering with sense RNA transcription (36), protect the primary transcript by masking single-stranded binding sites of endoribonuclease E (37), or by blocking translation by occluding ribosome binding sites (38).

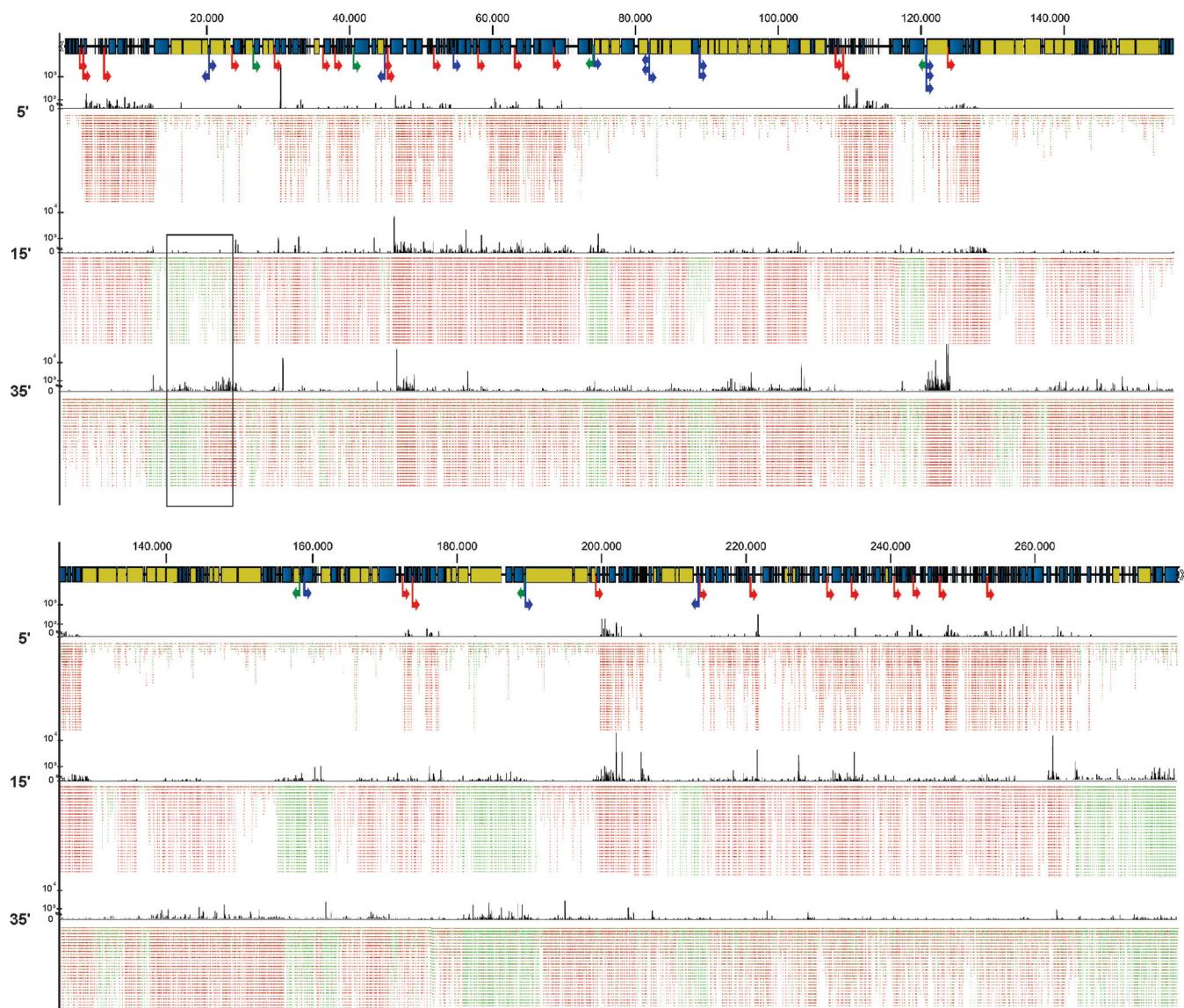


FIG 2 RNA-seq analysis of the φKZ transcriptome. Genome-wide overview of reads mapped to the sense (red) or antisense (green) strand of the φKZ genome at samples taken 5, 15, and 35 min after infection. Phage genes encoding structural proteins are indicated in yellow. Early, middle, and late promoters examined in more detail are indicated as red, green, and blue arrows, respectively. An example of the antisense transcription in more detail can be found in Fig. S1 in the supplemental material.

Differential gene expression during phage infection. When comparing transcripts at 10 and 35 min postinfection, we observed that, as expected, the majority of virion-associated structural gene transcripts were significantly upregulated at the later time point. The accumulation of phage transcripts coincides with a clear pattern of general decrease in abundance of bacterial transcripts (Fig. 3). Remarkably, halfway through infection, only 1.5% of mRNAs in the infected cells are of bacterial origin. To find out whether the phage employs any host transcription shutoff mechanism associated with factor-dependent inhibition of host RNAP activity, we affinity purified the bacterial RNAP from cells collected at various times postinfection (31). Although the composition of the bacterial RNAP changed subtly as the infection proceeded, no φKZ proteins were copurified alongside the complex at any time point. Moreover, enzyme purified from infected cells was

as active as control enzyme purified from control uninfected cells (data not shown), suggesting the absence of phage-encoded host RNAP inhibitors and/or modifications.

Remarkably, only a single bacterial operon, including genes PA0718 to PA0728, shows significant and easily discernible up-regulation during infection (Fig. 3). These genes encode proteins of the filamentous prophage Pfl, raising the intriguing possibility that Pfl tries to escape the infected cell before lysis occurs. To test this idea, we performed quantitative PCR on DNA extracted from φKZ-infected cells to determine whether new copies of the Pfl genome are produced during infection. However, we did not see a significant rise in Pfl genome copies compared to the level of the housekeeping gene *oprL*; in contrast, we saw a 57-fold average increase in φKZ genome copies (data not shown).

Mapping of early, middle, and late φKZ promoters. All φKZ-

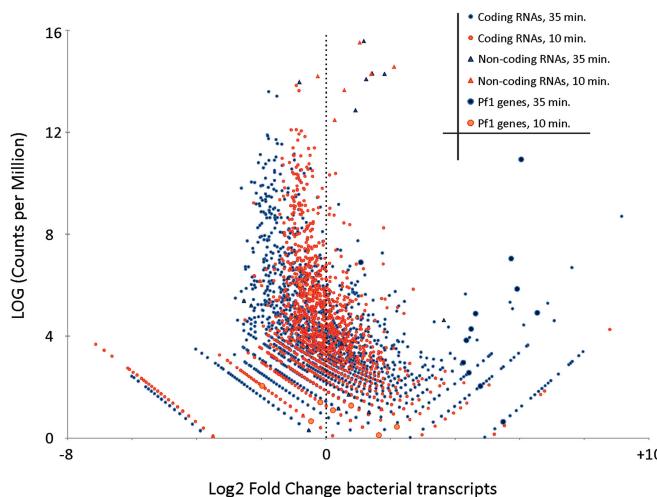


FIG 3 Impact of phage infection on bacterial transcriptome. Volcano plot of the *P. aeruginosa* transcriptome compared to the uninfected sample, with each dot representing an ORF, 10 (blue) and 35 (red) min postinfection. Triangles indicate structural RNAs, and large circles indicate genes which are located in the Pf1 operon.

related viruses are characterized by the presence of similar AT-rich intergenic motifs (5'-TATATTAC-3' in ϕ KZ) that are unidirectionally distributed throughout their genomes. Inspection of the ϕ KZ genome sequence revealed 28 such highly conserved motifs. These motifs have been assumed to act as phage-specific promoters (25, 29, 39–41). Indeed, our RNA-seq data clearly indicate that after 5 min of ϕ KZ infection, all transcription is performed on the leading strand and is exclusively associated with these motifs (Fig. 2, red arrows, and 4, with alignment of the corresponding sequences), supporting their likely function as early promoters. Comparison of sequences surrounding this core motif revealed an additional upstream motif of lower conservation (5'-TTTaA-3'; the lowercase letter represents a lower level of conservation) (Fig. 4). The early ϕ KZ promoters mostly drive transcription of blocks of short genes with unknown function. Primer extension analysis of total RNA purified at 0, 10, and 30 min of ϕ KZ infection using primers annealing downstream of three randomly chosen early promoters confirmed RNA-seq data. Each primer extension product appeared after 10 min of infection and did not decline afterwards, suggesting that the early transcripts are stable over the course of infection or that early promoters are not fully turned off at middle and late stages of infection and reach a steady state, because the level of early transcription is balanced by degradation. (Fig. 4). The 5' ends of primer extension products, which should correspond to the transcription start sites, are located ~10 bp downstream from the center of the core TATATTAC motif and are associated with an additional conserved 5'-TG-3' motif.

In contrast to early transcription starts, no common sequence motifs were found in front of 5' ends of middle or late ϕ KZ transcript 5' ends. For several non-early transcripts, primer extension analysis using total RNA purified 10, 25, and 40 min postinfection was performed. The results are shown in Fig. 2 (blue and green arrows) and 4. The middle promoters are distributed throughout the genome but are located on both strands. They are united by only a weak AT-rich motif (5'-AAannTAC-3'; lowercase letters represent a lower level of conservation) centered at position –24

with respect to the transcription start site (Fig. 4). Primer extension products corresponding to middle ϕ KZ transcripts appear 10 min postinfection and peak at around 25 min. These transcripts decrease in abundance late in infection, suggesting differences in half-lives compared to early phage mRNAs or stronger downregulation of middle transcription at a late stage of infection.

Late transcription is associated predominantly with structural and lysis-associated genes, which are distributed throughout the genome. Late transcripts are most abundant 40 min postinfection (Fig. 2 and 4). No sequence conservation upstream of 5' ends of late transcripts could be detected apart from a 5'-TATG-3' motif overlapping the transcription start site (Fig. 4). Transcription of several operons is initiated from multiple late promoters. In particular, the operon encoding the major capsid protein (gp120; operon 52 in Table S2 in the supplemental material) is initiated from no less than three promoters.

ϕ KZ infection is resistant to rifampin. With the exception of giant *Bacillus* phage PBS2 (21–23), all currently known phages require the bacterial host transcriptional machinery at least during part of the infection. However, none of the ϕ KZ transcripts have sequences resembling bacterial promoters upstream of their 5' ends. To test if host RNAP is involved in ϕ KZ infection, we analyzed the sensitivity of infection to Rif. Infection with *P. aeruginosa* phage LUZ19 was used as a control. LUZ19 encodes a T7-like ssRNAP but requires host RNAP for early transcription (42). The addition of 400 μ g/ml Rif to bacterial cultures prior to LUZ19 infection completely abolished progeny phage production. In contrast, ϕ KZ-infected cultures produced similar amounts of progeny in both the presence and absence of Rif (Fig. 5A).

To confirm that transcription from all ϕ KZ promoters is indeed Rif resistant, primer extension reactions described above were repeated on total RNA extracted from *P. aeruginosa* cells infected with the phage in the presence of Rif. As can be seen (Fig. 5B), the presence of Rif from the onset of infection did not change the levels of early (P_{54}), middle (P_{152}), or late (P_{153}) ϕ KZ transcripts but completely abolished a transcript from host promoter P_{rpoB2} . These observations show that ϕ KZ has the capacity to initiate and complete the infection cycle in the absence of transcription by host RNAP, strongly suggesting reliance on Rif-independent phage-encoded RNAP(s). However, it cannot be totally excluded that the phage virion contains a factor(s) eliminating the Rif sensitivity of the host RNAP and somehow directs it to phage DNA only.

Early transcription does not require protein synthesis. To investigate which temporal classes of phage transcription require newly synthesized phage proteins, we purified total RNA from ϕ KZ-infected *P. aeruginosa* cells supplemented with Cm before or during ϕ KZ infection. Primer extension analysis showed that the overall kinetics of early phage transcription does not depend on newly synthesized phage proteins (Fig. 5C). With Cm being added at different time points, the profiles of early transcript abundances changed slightly, likely showing that new phage proteins somehow attenuate transcription from early promoters and/or participate in their processing or decay (Fig. 5C). In contrast, transcription from middle (P_{50}) and late (P_{29}) phage transcripts is clearly translation dependent. Indeed, both P_{50} - and P_{29} -initiated transcripts were completely absent if Cm was added prior to phage infection (Fig. 5C). The partial transcription activity from P_{50} observed with Cm added shortly after infection may be explained by incomplete blockage of protein translation. When protein synthesis was

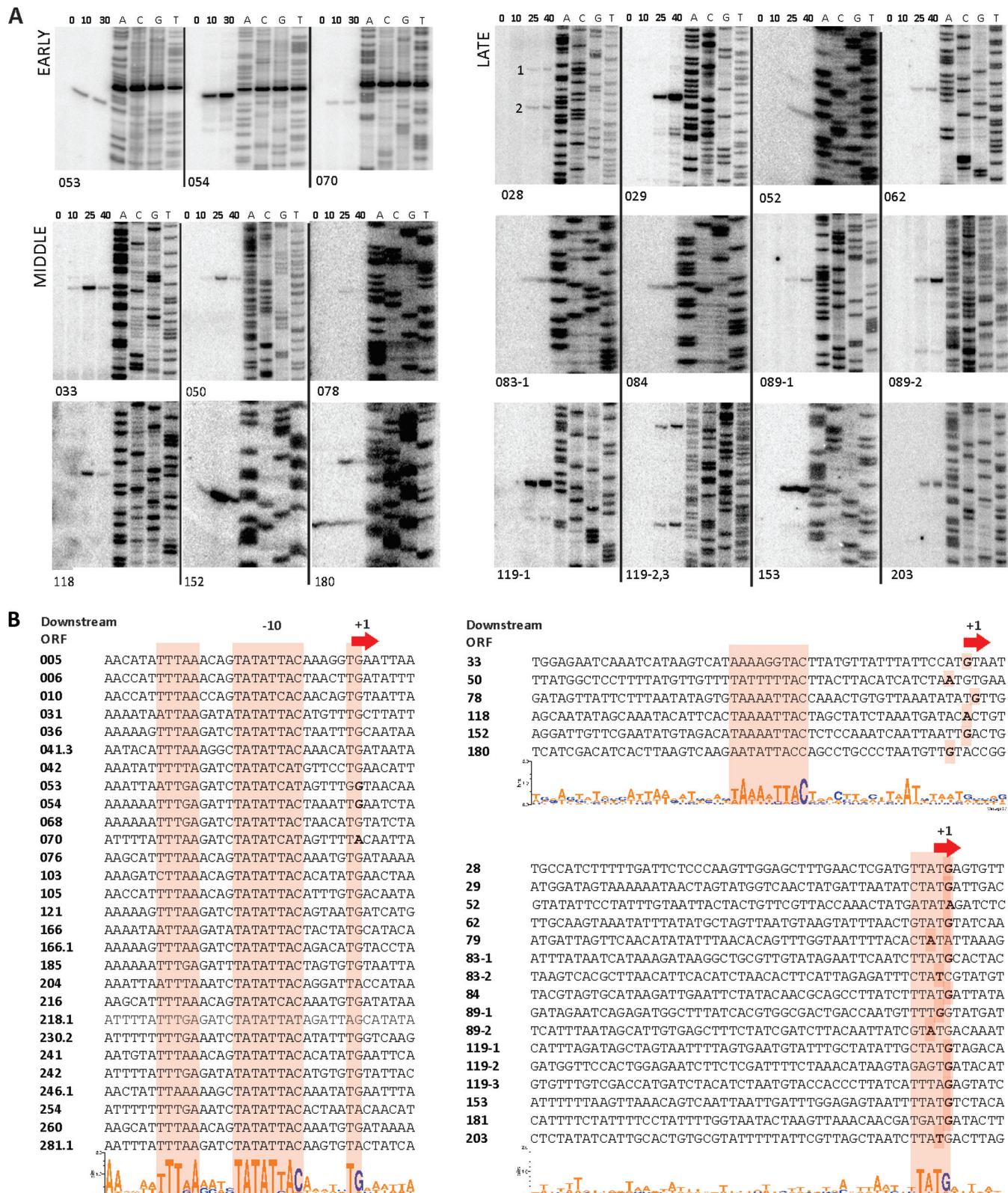


FIG 4 Mapping the ϕ KZ promoters. (A) Kinetics of accumulation of selected early, middle, and late ϕ KZ transcripts, as revealed by primer extension assay, is shown. Numbers of genes located downstream of primer extension endpoints and time points when infected cells were collected and processed for RNA purification are indicated. (B) Alignments of ϕ KZ promoter sequences. Consensus sequences of early, middle, and late promoters derived from 28, 6, and 16 sequences, respectively. Experimentally defined transcription start sites are in boldface. The corresponding sequence logos are depicted below the alignments. Red bars delineate conserved promoter elements.

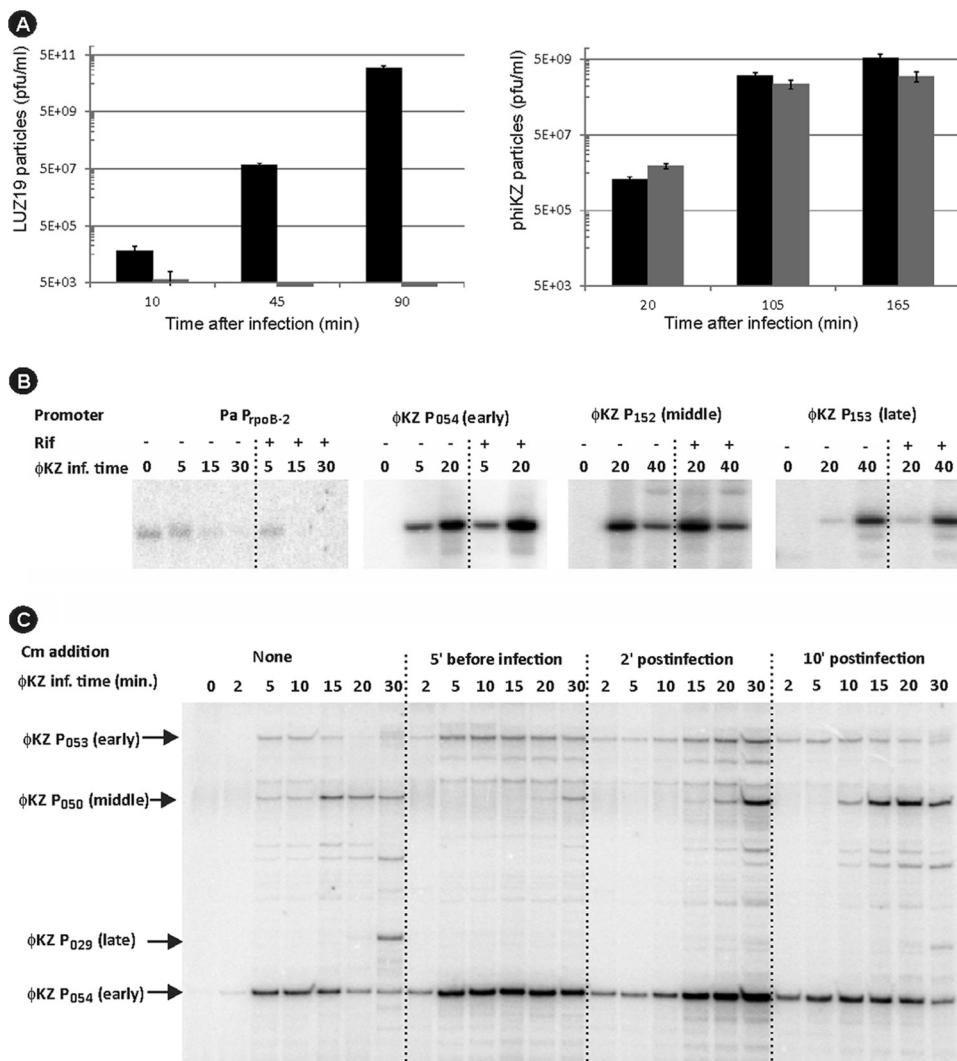


FIG 5 ϕ KZ requires translational but not transcriptional machinery of its host. (A) Phage titers of the culture supernatant of LUZ19 and ϕ KZ in the absence (black) and presence (gray) of 400 μ g/ml Rif, supplied to *P. aeruginosa* cell cultures 5 min before the addition of phage (MOI, 1). The graphs represent the average titers obtained from three independent experiments. (B) Results of primer extension analysis of phage transcripts from early (P_{54}), middle (P_{152}), and late (P_{153}) ϕ KZ promoters, performed on total RNA extracted from cells infected (inf.) in the presence (+) or absence (-) of Rif. (C) Results of primer extension analysis of phage transcripts from selected early, middle, and late ϕ KZ promoters on total RNA from ϕ KZ-infected cells grown in the presence of Rif and chloramphenicol (Cm), added at different time points. A primer extension reaction was performed simultaneously for several phage promoters of different temporal classes, including two early promoters, P_{53} and P_{54} , one middle promoter, P_{50} , and one late promoter, P_{29} .

ceased 10 min postinfection, transcription from middle and late promoters was not affected. Taken together, the data clearly demonstrate that ϕ KZ transcription is performed by Rif-resistant phage-encoded RNAP. Early transcription must be performed by an enzyme originating from the virion, while middle and late transcription must be performed by an enzyme that is fully or partially synthesized *de novo* after infection.

Two sets of split RNAP β and β' -like proteins are encoded by all known giant phage genomes. Careful *in silico* reanalysis of the ϕ KZ genome merged with previously published mass spectrometric data (26, 27) indicated that ϕ KZ encodes four β/β' -like virion-associated proteins (gp178, gp149, gp180, and gp80) that constitute viral RNAP (vRNAP). Some of these proteins (e.g., gp149) show barely detectable resemblance to known bacterial β or β' RNAP subunits, and identification of similarities is hindered fur-

ther by interspersions by mobile genetic elements (Fig. 6; also see Table S4 in the supplemental material). All putative vRNAP subunits are products of middle and/or late genes. In contrast, four additional proteins with similarities to fragments of bacterial β/β' subunits, gp123, gp71/73, gp74, and gp55, are the products of early genes; therefore, they should be part of the vRNAP complex (Fig. 6; also see Table S4 in the supplemental material). These eight RNAP subunits are conserved in all known ϕ KZ-related phages infecting a wide range of Gram-negative species belonging to the *Pseudomonas*, *Salmonella*, *Erwinia*, *Vibrio*, *Cronobacter*, and *Yersinia* genera (Fig. 6; also see Table S4).

Remarkably, no recognizable homology to the bacterial RNAP assembly α subunit or to the promoter-specific σ subunit is predicted in any of the giant phage genomes. One would assume that these phage-encoded β/β' subunits recruit the bacterial RNAP α

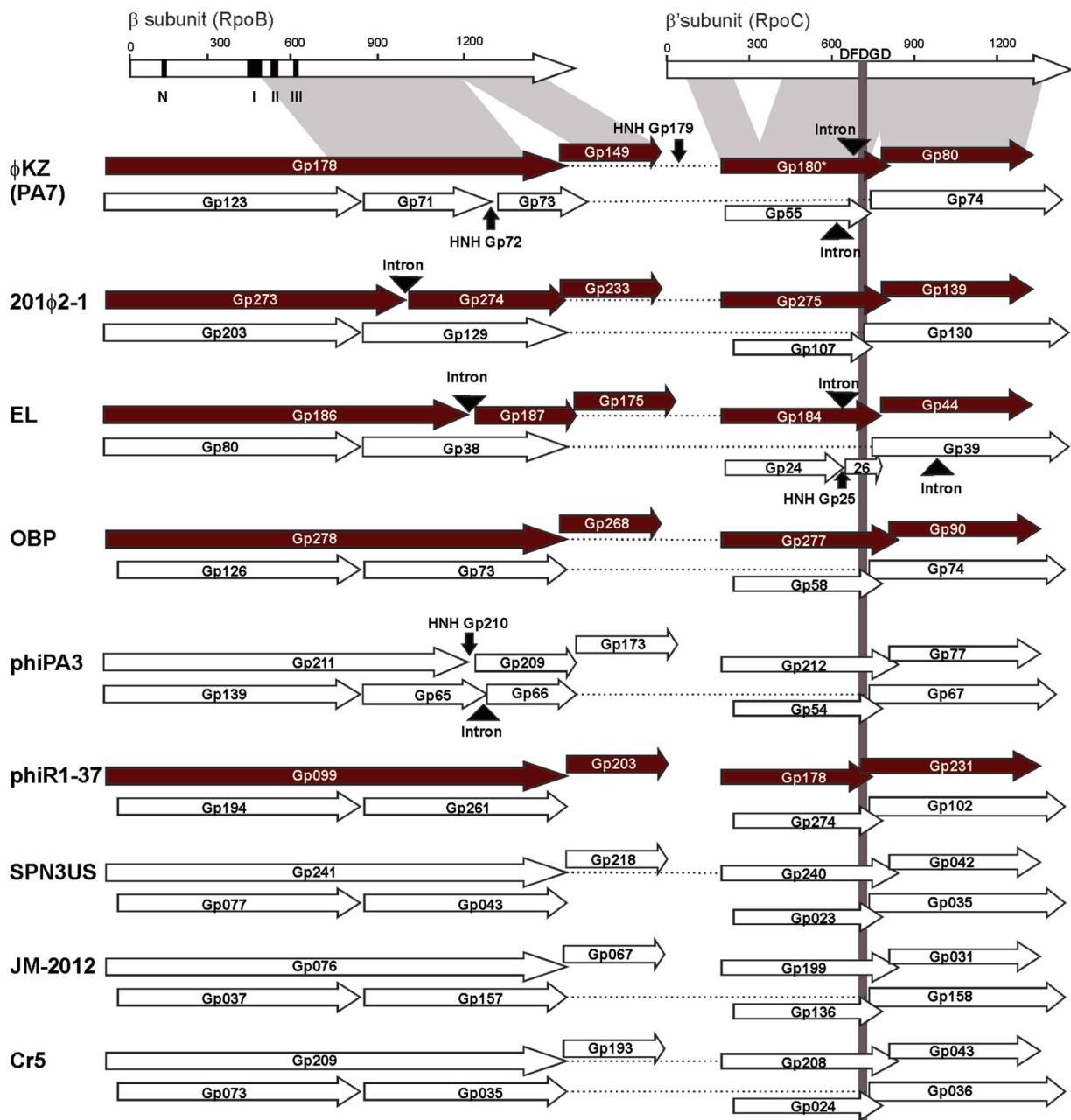


FIG 6 β/β' RNAP-like subunits of ϕ KZ family viruses. Subunits identified as being part of the virion are colored brown. The corresponding gene numbers are shown. The position of the metal-binding catalytic motif of the β' subunit is indicated. Introns are indicated by black triangles and arrows. Black boxes (labeled N, I, II, and III) indicate the four clusters where Rif^r mutations have been identified (46). In addition to ϕ KZ, included in the alignment are *Cronobacter* phage CR5 ([NC_021531.1](#)), *Yersinia* phage ϕ R1-37 ([NC_016163.1](#)), *Erwinia* phage ϕ EaH2 ([NC_019929.1](#)), *Pseudomonas* phage OBP ([NC_016571.1](#)), *Pseudomonas* phage EL ([NC_007623.1](#)), *Pseudomonas* phage 201 ϕ 2-1 ([NC_010821.1](#)), *Vibrio cyclitrophicus* phage JM-2012 ([NC_017975.1](#)), and *Salmonella* phage SPN3US ([JN641803.1](#)). More information on mutual similarities can be found in Table S4 in the supplemental material.

subunit as an assembly platform (2, 3). However, the coimmuno-purification experiment described above demonstrated that purification of bacterial RNAP complex from infected cells through a tag positioned on the α subunit failed to identify phage RNAP subunits. This suggests that the bacterial α subunit does not form a hybrid(s) with any of the phage-encoded RNAPs subunits.

In a reciprocal experiment, we expressed 6 \times His-tagged gp178 in *P. aeruginosa* cells and infected the resulting strain with ϕ KZ. After 10 and 25 min of infection, we copurified the tagged

gp178-His₆. As a negative control, noninfected cells were used. Because of the low-stringency wash conditions, large amounts of background proteins were identified in both noninfected and infected samples (see Table S5 in the supplemental material). However, while in the presence of gp178-His₆ spectral counts for three other predicted vRNAP subunits (gp80/gp149/gp180) were markedly increased, host bacterial RNAP and nvRNAP subunits were present in negligible amounts (see Table S5). This result indicates that the phage-encoded RNAP subunits from virion and nonvir-

riion sets do not form a hybrid(s) with host RNAP subunits or with each other.

DISCUSSION

Pseudomonas phage ϕ KZ is the type virus of a genus of remarkably large *Myoviridae* that are only marginally related to other known phages. Although they were originally uniquely associated with *Pseudomonas*, ϕ KZ-related phages have been discovered that target a variety of Gram-negative bacteria (26–29, 39–41). A remarkable feature of these phages is that they all contain two sets of proteins distantly related to fragments of bacterial multisubunit RNAP β and β' subunits. Proteins from one set were found in the virion for several giant phages (26–29). Here, we show that the entire ϕ KZ genome, comprising ~280 kb and encoding 369 genes grouped into 134 operons, can be transcribed entirely by virus-encoded RNAP. Host RNAP does not seem to contribute significantly to ϕ KZ gene expression, since phage infection proceeds normally in the presence of high concentrations of rifampin.

Global RNA profiling of ϕ KZ allows us to come up with a general view of ϕ KZ transcription. The vRNAP is injected in the bacterial cell and initiates transcription from 28 early promoters, characterized by a highly conserved AT-rich consensus element and a less conserved upstream motif. All early promoters are located on the same strand and are distributed throughout the circular permuted genome of ϕ KZ (Fig. 2). Phage protein synthesis is not needed for early transcription to occur, indicating that all determinants of early transcription are provided from the virion. Transcription of middle and late promoters requires phage protein synthesis. At least one of these promoter classes must be recognized by nvRNAP, whose subunits are encoded by early genes. Consensus elements for middle and late promoters are distinct but not very well conserved. Since middle and late transcripts clearly have different temporal kinetics and different promoter consensus elements, a question arises as to how the differential expression is achieved. In principle, two scenarios are possible. First, middle transcription can be catalyzed by vRNAP modified by a product(s) of an early gene, with late transcription performed by nvRNAP. A second possibility is that both middle and late transcription is catalyzed by nvRNAP, whose promoter specificity is modified by a middle gene product at the onset of the late stage of infection. Purification and characterization of ϕ KZ RNAPs from infected cultures at different time points of infection will be needed to determine which scenario actually takes place.

In both 4-polypeptide sets of phage β/β' -like subunits, one pair of proteins jointly forms a counterpart of a full-sized cellular RNAP subunit homolog (Fig. 6). Remarkably, the split of the β -like phage proteins roughly coincides with the natural split in the β counterpart from archaeal RNAP, while the split of the β' -like phage subunits is analogous to the subdivision of the β' counterpart in cyanobacterial RNAPs (43, 44). Such splits also are tolerated by *Escherichia coli* RNAP and occur at surface-exposed loops of RNAP. The two largest subunits of bacterial RNAPs jointly form the catalytic center of the enzyme and are held together by a dimer of α subunits, which are strictly required for RNAP assembly but not for promoter melting (45). Remarkably, ϕ KZ does not encode a recognizable α homolog. Moreover, the affinity purification data as well as the analysis of virion polypeptide content suggest that the phage RNAP subunit fragments do not associate with the host α subunit. These observations suggest that ϕ KZ and, by extension, other giant phage RNAPs use a novel

protein for assembly of large RNAP subunit fragments or assembly occurs without an α homolog or analog. To experimentally investigate this interesting question, as well as to determine which factors, if any, determine promoter specificity of viral RNAPs, purification of these enzymes from infected cells and ϕ KZ virions will be required.

To date, the giant phages PBS2 (infecting Gram-positive bacilli) and ϕ KZ (infecting Gram-negative pseudomonads) are the only phages known that are independent of the host transcription apparatus. Both giant phages encode Rif-resistant, multisubunit RNAPs (22 and this work). Comparison of the numbers of subunits (five in PBS2 and four predicted in ϕ KZ) as well as their approximate sizes suggests that both RNAP complexes of these giant phages are related. If this conjecture is true, then giant phages will be unique in their ability to infect both Gram-negative and Gram-positive hosts. Independence from the host transcription apparatus could be an important trait that contributes to such versatility. The determination of the PBS2 genomic sequence ongoing in one of our laboratories should help answer this question and may shed more light on the highly unusual multisubunit bacteriophage RNAPs.

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REFERENCES

- Cramer P. 2002. Multisubunit RNA polymerases. *Curr. Opin. Struct. Biol.* 12:89–97. [http://dx.doi.org/10.1016/S0959-440X\(02\)00294-4](http://dx.doi.org/10.1016/S0959-440X(02)00294-4).
- Darst SA. 2001. Bacterial DNA polymerase. *Curr. Opin. Struct. Biol.* 11:155–162. [http://dx.doi.org/10.1016/S0959-440X\(00\)00185-8](http://dx.doi.org/10.1016/S0959-440X(00)00185-8).
- Lane WJ, Darst SA. 2010. Molecular evolution of multisubunit RNA polymerases: structural analysis. *J. Mol. Biol.* 395:686–704. <http://dx.doi.org/10.1016/j.jmb.2009.10.063>.
- Lane WJ, Darst SA. 2010. Molecular evolution of multisubunit RNA polymerases: sequence analysis. *J. Mol. Biol.* 395:671–685. <http://dx.doi.org/10.1016/j.jmb.2009.10.062>.
- Iyer LM, Koonin EV, Aravind L. 2003. Evolutionary connection between the catalytic subunits of DNA-dependent RNA polymerases and eukaryotic RNA-dependent RNA polymerases and the origin of RNA polymerases. *BMC Struct. Biol.* 28:1. <http://dx.doi.org/10.1186/1472-6807-3-1>.
- Cermakian N, Ikeda TM, Miramontes P, Lang BF, Gray MW, Cedergrren R. 1997. On the evolution of the single-subunit RNA polymerases. *J. Mol. Evol.* 45:671–681. <http://dx.doi.org/10.1007/PL00006271>.
- Cheetham GM, Steitz TA. 1999. Structure of a transcribing T7 RNA polymerase initiation complex. *Science* 286:2305–2309. <http://dx.doi.org/10.1126/science.286.5448.2305>.
- Campbell EA, Korzheva N, Mustaev A, Murakami K, Nair S, Goldfarb A, Darst SA. 2001. Structural mechanism for rifampicin inhibition of bacterial RNA polymerase. *Cell* 104:901–912. [http://dx.doi.org/10.1016/S0092-8674\(01\)00286-0](http://dx.doi.org/10.1016/S0092-8674(01)00286-0).
- Nechaev S, Severinov K. 2003. Bacteriophage-induced modifications of host RNA polymerase. *Annu. Rev. Microbiol.* 57:301–322. <http://dx.doi.org/10.1146/annurev.micro.57.030502.090942>.
- Nechaev S, Severinov K. 2008. The elusive object of desire—interactions of

- bacteriophages and their hosts. *Curr. Opin. Microbiol.* 11:186–193. <http://dx.doi.org/10.1016/j.mib.2008.02.009>.
11. Steitz TA. 2004. The structural basis of the transition from initiation to elongation phases of transcription, as well as translocation and strand separation, by T7 RNA polymerase. *Curr. Opin. Struct. Biol.* 14:4–9. <http://dx.doi.org/10.1016/j.sbi.2004.01.006>.
 12. Kazmierczak KM, Davydova EK, Mustaev AA, Rothman-Denes LB. 2002. The phage N4 virion RNA polymerase catalytic domain is related to single-subunit RNA polymerases. *EMBO J.* 21:5815–5823. <http://dx.doi.org/10.1093/emboj/cdf584>.
 13. Jamalludeen N, Kropinski AM, Johnson RP, Lingohr E, Harel J, Gyles CL. 2008. Complete genomic sequence of bacteriophage phiEcM-GJ1, a novel phage that has myovirus morphology and a podovirus-like RNA polymerase. *Appl. Environ. Microbiol.* 74:516–525. <http://dx.doi.org/10.1128/AEM.00990-07>.
 14. Semenova E, Djordjevic M, Shraiman B, Severinov K. 2005. The tale of two RNA polymerases: transcription profiling and gene expression strategy of bacteriophage Xp10. *Mol. Microbiol.* 55:764–777. <http://dx.doi.org/10.1111/j.1365-2958.2004.04442.x>.
 15. Sousa R, Mukherjee S. 2003. T7 RNA polymerase. *Prog. Nucleic Acid Res. Mol. Biol.* 73:1–41. [http://dx.doi.org/10.1016/S0079-6603\(03\)01001-8](http://dx.doi.org/10.1016/S0079-6603(03)01001-8).
 16. Savalia D, Robins W, Nechaev S, Molineux I, Severinov K. 2010. The role of the T7 Gp2 inhibitor of host RNA polymerase in phage development. *J. Mol. Biol.* 402:118–126. <http://dx.doi.org/10.1016/j.jmb.2010.07.012>.
 17. Falco SC, Laan KV, Rothman-Denes LB. 1977. Virion-associated RNA polymerase required for bacteriophage N4 development. *Proc. Natl. Acad. Sci. U. S. A.* 74:520–523. <http://dx.doi.org/10.1073/pnas.74.2.520>.
 18. Gleghorn ML, Davydova EK, Rothman-Denes LB, Murakami KS. 2008. Structural basis for DNA-hairpin promoter recognition by the bacteriophage N4 virion RNA polymerase. *Mol. Cell* 32:707–717. <http://dx.doi.org/10.1016/j.molcel.2008.11.010>.
 19. Ceyssens PJ, Brabban A, Rogge L, Lewis MS, Pickard D, Goulding D, Dougan G, Noben JP, Kropinski A, Kutter E, Lavigne R. 2010. Molecular and physiological analysis of three *Pseudomonas aeruginosa* phages belonging to the “N4-like viruses.” *Virology* 405:26–30. <http://dx.doi.org/10.1016/j.virol.2010.06.011>.
 20. Cho NY, Choi M, Rothman-Denes LB. 1995. The bacteriophage N4-coded single-stranded DNA-binding protein, N4SSB, is the transcriptional activator of *Escherichia coli* RNA polymerase at N4 late promoters. *J. Mol. Biol.* 246:461–471. <http://dx.doi.org/10.1006/jmbi.1994.0098>.
 21. Price AR, Frabotta M. 1972. Resistance of bacteriophage PBS2 infection to rifampicin, an inhibitor of *Bacillus subtilis* RNA synthesis. *Biochem. Biophys. Res. Commun.* 48:1578–1585. [http://dx.doi.org/10.1016/0006-291X\(72\)90894-7](http://dx.doi.org/10.1016/0006-291X(72)90894-7).
 22. Clark S. 1978. Transcriptional specificity of a multisubunit RNA polymerase induced by *Bacillus subtilis* bacteriophage PBS2. *J. Virol.* 25:224–237.
 23. Clark S, Losick R, Pero J. 1974. New RNA polymerase from *Bacillus subtilis* infected with phage PBS2. *Nature* 252:21–24. <http://dx.doi.org/10.1038/252021a0>.
 24. Hendrix RW. 2009. Jumbo bacteriophages. *Curr. Top. Microbiol. Immunol.* 328:229–240.
 25. Mesyanzhinov VV, Robben J, Grymonprez B, Kostyuchenko VA, Bourkaltseva MV, Sykilinda NN, Krylov VN, Volckaert G. 2002. The genome of bacteriophage phiKZ of *Pseudomonas aeruginosa*. *J. Mol. Biol.* 317:1–19. <http://dx.doi.org/10.1006/jmbi.2001.5396>.
 26. Thomas JA, Weintraub ST, Wu W, Winkler DC, Cheng N, Steven AC, Black LW. 2012. Extensive proteolysis of head and inner body proteins by a morphogenetic protease in the giant *Pseudomonas aeruginosa* phage phiKZ. *Mol. Microbiol.* 84:324–339. <http://dx.doi.org/10.1111/j.1365-2958.2012.08025.x>.
 27. Lecoutere E, Ceyssens PJ, Miroshnikov KA, Mesyanzhinov VV, Krylov VN, Noben JP, Robben J, Hertveldt K, Volckaert G, Lavigne R. 2009. Identification and comparative analysis of the structural proteomes of phiKZ and EL, two giant *Pseudomonas aeruginosa* bacteriophages. *Proteomics* 9:3215–3219. <http://dx.doi.org/10.1002/pmic.200800727>.
 28. Thomas JA, Rolando MR, Carroll CA, Shen PS, Belnap DM, Weintraub ST, Serwer P, Hardies SC. 2008. Characterization of *Pseudomonas chlororaphis* myovirus 201varphi2-1 via genomic sequencing, mass spectrometry, and electron microscopy. *Virology* 376:330–338. <http://dx.doi.org/10.1016/j.virol.2008.04.004>.
 29. Skurnik M, Hyttiäinen HJ, Happonen LJ, Kiljunen S, Datta N, Mattinen L, Williamson K, Kristo P, Szeliga M, Kalin-Mänttäri L, Ahola-Iivarinen E, Kalkkinen N, Butcher SJ. 2012. Characterization of the genome, proteome, and structure of yersiniophage phiR1-37. *J. Virol.* 86:12625–12642. <http://dx.doi.org/10.1128/JVI.01783-12>.
 30. Ceyssens PJ, Lavigne R, Mattheus W, Chibeau A, Hertveldt K, Mast J, Robben J, Volckaert G. 2006. Genomic analysis of *Pseudomonas aeruginosa* phages LKD16 and LKA1: establishment of the phiKMV subgroup within the T7 supergroup. *J. Bacteriol.* 188:6924–6931. <http://dx.doi.org/10.1128/JB.00831-06>.
 31. Klimuk E, Akulenko N, Makarova KS, Ceyssens PJ, Volchenkov I, Lavigne R, Severinov K. 2013. Host RNA polymerase inhibitors encoded by phiKMV-like phages of *Pseudomonas*. *Virology* 436:67–74. <http://dx.doi.org/10.1016/j.virol.2012.10.021>.
 32. Qiu D, Damron FH, Mima T, Schweizer HP, Yu HD. 2008. PBAD-based shuttle vectors for functional analysis of toxic and highly regulated genes in *Pseudomonas* and *Burkholderia* spp. and other bacteria. *Appl. Environ. Microbiol.* 74:7422–7426. <http://dx.doi.org/10.1128/AEM.01369-08>.
 33. Sevostyanova A, Djordjevic M, Kuznedelov K, Naryshkina T, Gelfand MS, Severinov K, Minakhin L. 2007. Temporal regulation of viral transcription during development of *Thermus thermophilus* bacteriophage phiYS40. *J. Mol. Biol.* 366:420–435. <http://dx.doi.org/10.1016/j.jmb.2006.11.050>.
 34. Cenens W, Mebrhatu MT, Makumi A, Ceyssens PJ, Lavigne R, Van Houdt R, Taddei F, Aertsen A. 2013. Expression of a novel P22 ORFan gene reveals the phage carrier state in *Salmonella Typhimurium*. *PLoS Genet.* 9:e1003269. <http://dx.doi.org/10.1371/journal.pgen.1003269>.
 35. Monson R, Foulds I, Foweraker J, Welch M, Salmond GP. 2011. The *Pseudomonas aeruginosa* generalized transducing phage phiPA3 is a new member of the phiKZ-like group of “jumbo” phages, and infects model laboratory strains and clinical isolates from cystic fibrosis patients. *Microbiology* 157:859–867. <http://dx.doi.org/10.1099/mic.0.044701-0>.
 36. Toledo-Arana A, Dussurget O, Nikitas G, Sesto N, Guet-Revillet H, Balestroino D, Loh E, Gripenland J, Tiensuu T, Vaitkevicius K. 2009. The *Listeria* transcriptional landscape from saprophytism to virulence. *Nature* 459:950–956. <http://dx.doi.org/10.1038/nature08080>.
 37. Stasic D, Lindell D, Steglich C. 2011. Antisense RNA protects mRNA from RNase E degradation by RNA-RNA duplex formation during phage infection. *Nucleic Acids Res.* 39:4890–4899. <http://dx.doi.org/10.1093/nar/gkr037>.
 38. Kawano M, Aravind L, Storz G. 2007. An antisense RNA controls synthesis of an SOS-induced toxin evolved from an antitoxin. *Mol. Microbiol.* 64:738–754. <http://dx.doi.org/10.1111/j.1365-2958.2007.05688.x>.
 39. Cornelissen A, Hardies SC, Shaburova OV, Krylov VN, Mattheus W, Kropinski AM, Lavigne R. 2012. Complete genome sequence of the giant virus OBP and comparative genome analysis of the diverse phiKZ-related phages. *J. Virol.* 86:1844–1852. <http://dx.doi.org/10.1128/JVI.06330-11>.
 40. Hertveldt K, Lavigne R, Pleteneva E, Sernova N, Kurochkin I, Korchevskii R, Robben J, Mesyanzhinov V, Krylov VN, Volckaert G. 2005. Genome comparison of *Pseudomonas aeruginosa* large phages. *J. Mol. Biol.* 354:536–545. <http://dx.doi.org/10.1016/j.jmb.2005.08.075>.
 41. Dömöör D, Becságh P, Rákely G, Schneider G, Kovács T. 2012. Complete genomic sequence of *Erwinia amylovora* phage phiEaH2. *J. Virol.* 86:10899. <http://dx.doi.org/10.1128/JVI.01870-12>.
 42. Ceyssens PJ, Glonti T, Kropinski NM, Lavigne R, Chanishvili N, Kulakov L, Lashkhi N, Tedashvili M, Merabishvili M. 2011. Phenotypic and genotypic variations within a single bacteriophage species. *Virol.* 8:134. <http://dx.doi.org/10.1186/1743-422X-8-134>.
 43. Severinov K. 2000. RNA polymerase structure-function: insights into points of transcriptional regulation. *Curr. Opin. Microbiol.* 3:118–125. [http://dx.doi.org/10.1016/S1369-5274\(00\)00062-X](http://dx.doi.org/10.1016/S1369-5274(00)00062-X).
 44. Xie WQ, Jäger Potts KM. 1989. Cyanobacterial RNA polymerase genes rpoC1 and rpoC2 correspond to rpoC of *Escherichia coli*. *J. Bacteriol.* 171:1967–1973.
 45. Young BA, Gruber TM, Gross CA. 2004. Minimal machinery of RNA polymerase holoenzyme sufficient for promoter melting. *Science* 303:1382–1384. <http://dx.doi.org/10.1126/science.1092462>.
 46. Jatsenko T, Tover A, Tegova R, Kivisaar M. 2010. Molecular characterization of Rifr mutations in *Pseudomonas aeruginosa* and *Pseudomonas putida*. *Mutat. Res.* 683:106–114. <http://dx.doi.org/10.1016/j.mrfmmm.2009.10.015>.