The isolation and characterization of Kronos, a novel *Caulobacter* rhizosphere phage that is similar to lambdoid phages

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Abstract

Despite their ubiquity, relatively few bacteriophages have been characterized. Here, we set out to explore *Caulobacter* bacteriophages (caulophages) in the rhizosphere and characterized Kronos, the first caulophage isolated from the rhizosphere. Kronos is a member of the *Siphoviridae* family since it has a long flexible tail. In addition, an analysis of the Kronos genome indicated that many of the predicted proteins were distantly-related to those of bacteriophages in the lambdoid family. Consistent with this observation, we were able to demonstrate the presence of *cos* sites that are similar to those found at ends of lambdoid phage genomes. Moreover, Kronos displayed a relatively rare head and tail morphology compared to other caulophages but was similar to that of the lambdoid phages. Taken together, these data indicate that Kronos is distantly related to lambdoid phages and may represent a new *Siphoviridae* genus.

Introduction

Bacteriophages (phages) are the most diverse and abundant organisms within the entire biosphere. However, only a small percentage of phages have been isolated and characterized. As of February 2019, there are approximately 2200 completely sequenced phage genomes in the NCBI database, which amounts to about 1% of the completely sequenced bacterial genomes. Given that there are about 10 phages for every bacterium [13] and that phage-mediated horizontal gene transfer contributes to bacterial function and evolution [8], it is clear that the study of phages needs to be increased.

Understanding how phages interact with their hosts is innately linked to the way in which phages package their genomes, since phage genome packaging is dependent on host machinery. Although some phages have RNA or single-stranded DNA genomes, most phages have a double-stranded DNA genome. Three different mechanisms are commonly used by phages to package their DNA: headful packaging, *cos* site packaging, and T7-like packaging. Phages such as P1, T4, SPP1, P22 and the caulophage Cr30 employ a headful

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packaging system [10, 21], where packaging is initiated by a terminase at a *pac* site on the substrate genomic DNA concatemer, and a genome that is longer than unit length is packaged and completed by sequence-independent cleavage [5]. Upon infection the genome is circularized by recombination between the duplicated segments at the ends of the packaged DNA. The second mode, termed *cos* site packaging, is also initiated on concatemeric genomic DNA by terminase cleavage; however, packaging is completed at a sequence-specific *cos* site, generating a unit length monomer with the cohesive termini used for circularization [24]. In contrast to both headful and *cos* site packaging, T7-like packaging results in well-defined terminally redundant sequences that can be thousands of base pairs in length [14]. This form of DNA packaging is considered to be more complex because, unlike headful and *cos* site packaging, phages employing this form of replication reduce their reliance on host replication machinery by encoding most of their own replication proteins [25]. Recent studies have indicated that most phages that infect *Caulobacter* strains use the T7-like packaging method [14, 19].

Members of the genus *Caulobacter* were originally isolated from freshwater and were thought to be restricted to aquatic environments [22]. However, several recent studies [18, 23, 29] have demonstrated that caulobacters are present in the rhizosphere as well, and that some species are considered to be plant growth-promoting bacteria (PGPB). PGPB can increase plant growth in a variety of ways including: increased IAA production, increased ACC deaminase production, siderophore production, nitrogen fixation, and phosphate solubilization [12].

Despite the recent interest in rhizosphere caulobacters, there have been no reports of caulophages that have been isolated from the rhizosphere. This lack of rhizosphere phage research neglects potentially insightful information about the plant growth-promoting abilities of bacteria such as caulobacters since PGPB research is being conducted to thwart the imminent world-wide shortage of biofertilizers [34] and provide food for the rising world population. Currently, all of the known caulophages have been isolated from aquatic sources and most belong to the phiCbk *Siphoviridae* family, the *Myoviridae* family, or the *Podoviridae* family and include only phages that utilize either T7-like or headful DNA packaging mechanisms. Since phages are thought to be present in all environments, we have initiated a study of phages that infect rhizosphere caulobacters. We hypothesized that some rhizosphere caulophages would be significantly divergent from the currently characterized caulophages, since the typical rhizosphere environment drastically differs from most aquatic environments. Here we report the characterization of a novel rhizosphere caulophage that has *cos* sites at the termini of its genome and appears to be distantly related to the lambdoid family of phages.

Methods

Bacterial and caulophage isolation and growth

Dichotomous plant roots were extracted from riparian land on the banks of the Congaree River in Columbia, SC and brushed clean to remove remnant dirt. The roots were then soaked for 24 h in room-temperature sterilized tap water to allow the release of root-associated bacteria. In order to effectively isolate caulophages from the rhizosphere, we first

isolated a rhizosphere *Caulobacter* wild-type strain, since phage-host interactions are generally highly specific. Small amounts (20 μl) of water from the soaked root were pipetted onto PYE plates containing 2 μM of ampicillin, since Caulobacters are generally ampicillin resistant. The plates were incubated at 30°C for 24 h, and colonies with characteristics of *Caulobacter* strains were selected and suspended in 3 ml of PYE broth [11]. After overnight incubation at 30°C, the bacterial culture was visualized with a compound light microscope to observe cellular shape and motility. Presumptive *Caulobacter* strains were subjected to repeated single colony isolation to produce a pure culture. One strain, designated CBR1, was confirmed to be a species of *Caulobacter* by analyzing a portion of its 16S rRNA gene. CBR1 DNA was isolated using a Qiagen DNA isolation kit according to the manufacturer's instructions. The CBR1 16S rRNA gene was amplified by PCR using 5'-GGTTACCTTGTTACGACTT-3' and 5'-GTGCCAGCMGCCGCGGTAA-3' as forward and reverse primers, respectively. The nucleotide sequence of the amplified ~ 900 bp product was determined by Sanger sequencing, and the resulting 16S rDNA sequence was compared to other bacterial 16S rDNA sequences using BLASTn to verify that CBR1 was a *Caulobacter* isolate (Fig. S1).

To isolate a phage that infected CBR1, 10 ml of the root-soaked suspension was filtered using a 0.45 μm filter to remove bacteria and other particulate matter. The filtrate was then combined with 50 μl of a CBR1 culture, 2.5 μl of 5X PYE broth and 0.67 mg of ampicillin. The mixture was then incubated at 30°C on an orbital shaker for 24 h. The resulting phage enrichment was poured into a 15 ml sterile tube and centrifuged at 5,000 x g for 10 min. The supernatant was collected and 1 ml of chloroform was added, shaken, and left at room temperature for 30 min. Twenty microliters of the resulting supernatant (lysate) was used to perform a spot test for the presence of phages, and this plate was incubated overnight at 30°C. After observing phage lysis, serial dilutions were prepared and mixed with CBR1 in 3.5 ml of soft agar to obtain single plaques. A single plaque was stabbed with a sterile needle and suspended in 1 ml of PYE broth. To further purify the phage, the resulting phage suspension was diluted 1:100, and 100 μl of the diluted suspension was mixed with 100 μl of CBR1 in 3.5 ml of soft agar. After overnight incubation at 30°C, a single phage plaque was cut out from the soft agar overlay, placed in 1 ml of PYE broth and stored at 4°C overnight to allow the phage to diffuse out of the agar. The resulting phage solution was diluted 1:100 and again plated in a soft agar overlay with CBR1 to produce confluent lysis after overnight incubation at 30°C. The resulting phage particles were eluted with the addition of 7 ml of 1X PYE broth and incubated at room temperature for 24 h. The plates were then decanted, and the lysate was centrifuged at 5,000 x g for 10 min to remove residual bacteria. Approximately 1 ml of chloroform was added to the mixture and then vortexed for 10 s to kill any additional residual bacteria. Some of the lysate was used to make DNA plugs, and the remaining lysate was stored at −72°C.

Host range

The host range for Kronos was determined by mixing 100 μl of a selected *Caulobacter* species (i.e. CB1, CB2, CB3, CB13, CB15, *C. segnis*, *C. henricii* and AP07) with 3.5 ml of soft agar. Once the agar solidified, 20 μl of a 10^9 plaque forming units (PFU) ml^{-1} Kronos phage lysate was pipetted onto the surface of the agar plate: this was performed for each

Caulobacter species. Plates were incubated overnight at 30°C. After incubation, lysis was determined by a transparent zone of lysis at the site where the lysate was deposited.

To determine which phages infect CBR1, a volume of 100 μl was mixed with 3.5 ml of soft agar and poured onto a PYE agar plate. After the mixture solidified, 20 μl of a 10^9 PFU ml^{-1} phage lysate was pipetted onto the surface of the agar. The plates were then incubated overnight at 30°C. The following phiCbk phages were used for the host range experiments: phiCbk, BL-1, and BL-9. Lullwater and Cr30 were used as representatives of the *Podoviridae* family and *Myoviridae* family, respectively. Infection was measured by a transparent zone of lysis at the site where the 20 μl of phage lysate was placed.

Genome size determination

The size of the phage genome was determined using pulsed-field gel electrophoresis (PFGE). Agarose plugs containing phage DNA were made using the protocol of Dingwall et al. [9]. Briefly, a 1:1 ratio of phage lysate and 1% agarose in SBA was mixed in a microfuge tube and drawn up into a 1 cc syringe. After solidification, the resulting mixture was cut into 2 mm thick discs (plugs). The resulting plugs were incubated overnight at 50°C in 2 ml of lysis buffer (1.9 ml of 1% Sarkosyl in 0.5 M EDTA and 0.1 ml of proteinase K (20 mg ml^{-1})). After incubation, the plugs were washed twice with 2 ml of TE buffer and 30 μl of PMSF (17.4 mg ml^{-1}). The plugs were then washed an additional three times with 2 ml of TE buffer. After the completion of the plug washes, one of the plugs was subjected to PFGE at 6 V for 14 h with an initial switch time of 8 s and a final switch time of 20 s and compared to a lambda ladder (BioRad, Hercules, CA) to determine the size of the genome.

Electron microscopy

A 100 μl aliquot of a 10^9 PFU ml^{-1} phage lysate was mixed with 100 μl of a log phase culture of CBR1 for 30 min at 30°C. The mixture was then centrifuged and resuspended in 100 μl of autoclaved, purified water. A total of 20 μl of the resulting mixture was combined with 20 μl of 2% uranyl acetate. A formvar/carbon 300 mesh, copper grid was then floated on the mixture for í30 s. Free and adsorbed phages were then observed in a JEOL 200CX transmission electron microscope.

DNA isolation

Phage genomic DNA was prepared from 3-5 ml of a concentrated, high-titer (> 10^9 PFU ml^{-1}) phage lysate using a Qiagen DNA isolation kit (Germantown, MD) according to the manufacturer's instructions.

Genome sequence analysis and assembly

The nucleotide sequence of the phage DNA was determined at the Delaware Bioinformatics Institute using a PacBio RSII single-molecule sequencer. The resulting sequence reads were assembled using HGAP 3 and was polished using Quiver. The consensus sequence was then annotated using the Rapid Annotation using Subsystem Technology (RAST) [4]. The annotated sequence was analyzed in Artemis [28] and trimmed to remove duplicate sequences. The annotated genes were analyzed using BLASTn, BLASTp, and the NCBI CD-search tool. The ends of the genome were determined by digesting a phage plug

containing DNA in a mixture that consisted of 5 µl of the restriction endonuclease *Sac*II, 10 µl of NEB Cutsmart buffer, and 55 µl of autoclaved deionized water (diH$_2$O) in a 37°C water bath overnight. The fragments of digested DNA were then separated and visualized by subjecting the plug to PFGE with a pulse time of 1 s for 14 h at 14°C. *Cos* sites were identified by using Sanger sequencing to determine the nucleotide sequence of the ends of the genome using Kronos genomic DNA as the template and the following primers: 5'-CGAGAAGAACACGGAAGCCAT-3' and 5'-AGCCGACTTGCCTTGATGGTCTG-3', to sequence each end of the genome. The NCBI accession number for the Kronos genome is MH884648.

Lysogen detection

Bacterial colonies were selected from phage-bacteria overlay plates containing Kronos plaques. Bacteria from the center of a plaque were streaked onto a PYE plate and incubated at 30°C for 48 h. Isolated colonies were then selected from the streak plate and restreaked onto another PYE plate and were incubated at 30°C for 48 h. The resulting streak plate was then transferred to a velveteen template and then onto a PYE plate containing 3.5 ml of soft agar and 100 µM of CBR1. Lysogens would have been detected by the presence of phage lysis.

Burst size

Phages were added at a final concentration of 10^7 PFU ml^{-1} to a 10 ml of host bacteria (CBR1) at a concentration of 5×10^8 colony forming units (CFU) ml^{-1}. After 30 min of incubation at 30°C, the sample was diluted 1:1000 in 3 ml of PYE broth and plated immediately. Every 15 min for 240 min, a 100 µl sample of this dilution was mixed with 100 µl of CBR1 and 3.5 ml of soft agar and plated on a PYE plate. After solidification, the plates were incubated overnight at 30°C. Plaques were counted, and a one-step growth curve was generated by plotting the PFU over time. The assay was repeated three times, and the average was calculated to generate a representative burst size.

Adsorption rate

Phages were added at a final concentration of 10^5 PFU ml^{-1} to a 10 ml culture of CBR1 at a concentration of 5×10^8 CFU ml^{-1} and was vortexed for 15 s. The mixture was incubated at 30°C, and aliquots of 100 µl were pipetted into a mixture containing 1 ml of chloroform and 9 ml of diH$_2$O at 15 min intervals. The diluted samples were shaken vigorously for 10 s and allowed to rest until the chloroform and diH$_2$O separated. Once the chloroform settled to the bottom of the tube, 100 µl of each sample was mixed with 100 µl of CBR1 and 3.5 ml of soft agar and poured onto a PYE plate and incubated overnight at 30°C. The experiment was performed three times, with the adsorption rate and adsorption rate constant averaged from the three experiments.

Results and Discussion

Phage isolation and characterization

A phage designated Kronos was isolated from plant roots anchored on the banks of the Congaree River in Columbia, SC in August of 2017. To date, Kronos has been shown to

infect only the rhizosphere *Caulobacter* CBR1. Kronos failed to infect *C. crescentus* isolates including CB1, CB2, CB3, CB13, and CB15 as well as members of other *Caulobacter* species such as *C. segnis* and *C. henricii*. Interestingly, Kronos also failed to infect AP07—a soil derived *Caulobacter* species isolated from the roots of a poplar tree [30]. The specificity exhibited by Kronos for its host could be the result of a unique receptor present on the cell surface of CBR1, since not only did Kronos fail to infect other *C. crescentus* strains, but other *C. crescentus* phages including several phicbkviruses, Podoviridae phage Lullwater, and myophage Cr30 failed to infect CBR1.

Kronos plaques were transparent and uniform in size (< 1 mm). This exceptionally small plaque size produced by Kronos counters the typical inverse relationship of plaque size to genome size among caulophages. For example, giant caulophages that possess larger genomes such as phiCbk (205 kbp) and Colossus (297 kbp) produce small plaques (1 mm) [3, 14]. In contrast, caulophages with small genomes (40-50 kbp) and small capsid sizes (< 60 nm), such as Lullwater [20] produce variable-sized larger plaques (1-3.5 mm). Although not entirely understood, plaque size is most likely associated with a combination of diffusivity, burst size, adsorption efficiency and phage morphology [2].

The Kronos phage particle consists of a 50 nm icosahedral head with a 150 nm flexible tail (Fig. 1). This size and morphology is relatively rare among caulophages, but it is similar to that of Ccr1 [15]. However, Ccr1 was lost when a −70°C freezer malfunctioned, and further analyses could not be conducted. Interestingly, Kronos possesses a head and tail morphology strikingly similar to those of the lambdoid phages such as λ phage, HK97, and N15 [6]. Based on the head and tail morphology, we concluded that Kronos is a member of the *Siphoviridae* family.

Phage host interactions

Kronos binds to CBR1 at apparently random locations similar to the binding of Cr22 and Cr30, which adsorb independently of the host flagellum and is in contrast to the polar adsorption method used by the Phicbkviruses (Fig. 2) [15]. The adsorption rate of Kronos was also relatively fast, with 82% of the phages adsorbed after 30 min with an adsorption rate constant of ~ 5.4×10^{-9} ml min^{-1} (Fig. 3).This adsorption rate was consistent with that of other rhizosphere phages [31], but was in stark contrast to the adsorption rate of the transducing phage Cr30, which was previously shown to adsorb at a rate that was 2.3-fold slower [11]. In addition, Kronos exhibited an eclipse period of 55 min with a burst size of 28 PFU per adsorbed phage (Fig. 4). This eclipse period of Kronos is similar to that of lambdoid phages such as λ phage and HK97 which have an eclipse period of 45 min and 53 min, respectively [35]. In contrast, Cr30 had an extended eclipse period of 90 min [11]. Moreover, despite multiple attempts to detect the ability of Kronos to form a prophage in CBR1, no lysogens were detected. However, some phages have been shown to require hyper-specific, unfavorable physiochemical interactions to induce lysogeny and others have genetic mutations that render lysogenic capabilities ineffective [1].

Kronos genome

The Kronos genome nucleotide sequence contains 42,424 bp in a linear chromosome. Terminal repeats were not observed when the raw Kronos sequence data was analyzed with the Tablet graphical genome viewer. The N50 read length for the data was ~26 kbp with a read count of ~90 kbp and coverage of ~178X. The absence of double coverage sequence reads in the sequencing data indicated the absence of terminal repeats. In order to verify the sequence and assembly of the Kronos genome, plugs containing Kronos DNA were digested with the restriction endonuclease *Sac*II and were subjected to PFGE. The resulting band pattern did not match the predicted band pattern (4,366 bp and 38,058 bp), since only a single ~42 kbp band was present (Fig. 5a, lane 1). However, undigested plugs containing Kronos DNA produced a ladderlike band pattern similar to that of plugs containing λ phage DNA, implying the presence of *cos* sites that could anneal to each other (Fig. 5b). Since the presence of *cos* sites would cause the termini of the ~4 kbp and the ~38 kbp bands to reanneal, we decided to heat the plugs to 70°C for 60 min to denature any annealed *cos* sites, as demonstrated previously with *Bst*EII digested λ DNA [27]. After heating the plugs and subjecting them to PFGE, the band sizes matched the predicted 4,366 bp and 38,058 bp band sizes (Fig. 5a, lane 2). Additionally, when Kronos DNA was extracted in liquid form, heated to 70°C, and then mixed at a 1:1 ratio with molten 1% agarose, the resulting plugs produced a single ~42 kbp band, which confirmed that the *cos* sites had been separated (Fig. 5b, lane 2). We also confirmed previous data showing that the formation of the ladder is concentration dependent [36], since an increased DNA concentration increased the ladder appearance (Fig. 5c).

Visual inspection of the annotated Kronos genome with Artemis revealed a 13 bp region (5'-GGGGCAGAGCACT-3') located at the 5' end of the genome, approximately 1 kbp upstream from the terminase genes. This presumptive *cos* site is similar to those of phages such as HK97, HK022, and λ phage, since these phages have *cos* sites that are located at the start of their genomes, are 10-14 bp long and are located approximately 1 kbp upstream of the terminase genes. In addition, they have a GGGGC sequence at positions 1-5 and either CT or CG at the terminal two bases [16, 32, 33]. To verify the presumptive *cos* site, we sequenced the ends of the genome by Sanger sequencing and observed the sequencing reads ended at the *cos* site. Based on the similarities to other *cos* sequences and our end-sequencing results, we concluded that this 13 bp sequence is the Kronos *cos* site, and the Kronos genome was arranged so that this sequence was at the beginning.

The Kronos genome codes for 58 genes with > 68% of the genes located on the forward strand (Table 1 and Fig. 6). Interestingly, no nucleotide or predicted amino acid sequence matches were identified between any Kronos gene and any previously characterized caulophage gene. In addition the gene arrangement for Kronos was consistent with the gene arrangement of the lambdoid phages with the terminase genes situated within the first couple thousands bases relative to the start of the 5' plus strand upstream and relatively close to the major capsid genes [7, 16]. This general similarity is consistent with the *cos* site packaging mechanism and suggests that the Kronos DNA replication mechanism may be similar to that of lambdoid phages; however, this genome arrangement is not entirely exclusive to lambdoid phages, considering other virulent siphophages such as coliphage T1 and Salmonella phage

Chi also possess genomic structural modules similar to that of λ phage despite their terminase genes being located ~10,000 bp upstream from the start of 5' end of the plus strand [17, 26]. Nonetheless, the top BLASTp match for the Kronos small subunit terminase (TerS) was with the TerS gene of the *E. coli*-infecting phage P27, which is a lambdoid phage that is capable of lysogeny [19]. Similarly, BLASTn analyses identified a ~ 1 kbp region in the Kronos TerL gene that is homologous with the TerL gene of the virulent *Halomonas* phage QHHSV-1. *Halomonas* phage QHHSV-1 possesses a phage Cro repressor and a putative integrase that is homologous to λ phage; but due to mutations in its recombination cassette, *Elalomonas* phage QHHSV-1 has repressed lysogenic activities [1]. Moreover, the Kronos major capsid protein had the most significant BLASTp match with the lambdoid phage HK97. These data combined with the strikingly similar PFGE ladder band pattern between λ phage and Kronos (Fig. 5) strengthens the idea that Kronos uses a *cos* site DNA packaging mechanism, which is similar to the one used by λ phage.

Since the Kronos genome organization resembles that of lambdoid phage genomes and has significant BLASTp homologies to lambdoid proteins, the ~11 kbp section of genes located on the reverse strand may include a collection of genes functioning in lysis and possibly lysogeny. Recent analyses have demonstrated that lambdoid repressors that induce lysogeny and repress the lytic phase have highly variable amino acid sequences and are often not recognizable as homologous [6]. which may explain some of the amino acid sequence dissimilarities with lambdoid proteins throughout the Kronos genome, particularly on the minus strand. In fact, only 45% of the predicted amino acid sequences in the Kronos genome returned statistically significant (E $\leq$ 10^{-5} and query coverage $\geq$ 40%) BLASTp/BLASTcd matches with phage proteins (Table 1 and Table S1). Since 76% of the genes encoded either hypothetical proteins or proteins with an unknown conserved domain, the overall gene function for most of the genes in the genome remains unknown. Taken together, our data support the idea that Kronos is distantly related to, but genetically distinct from the lambdoid family of phages, and may represent a new genus of *Siphoviridae* cos phages.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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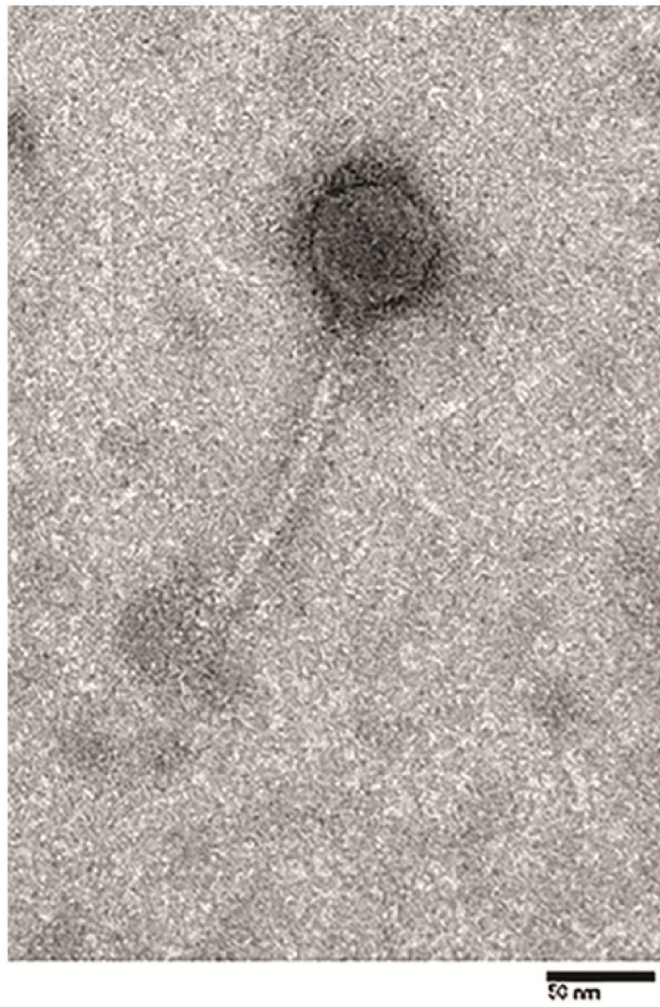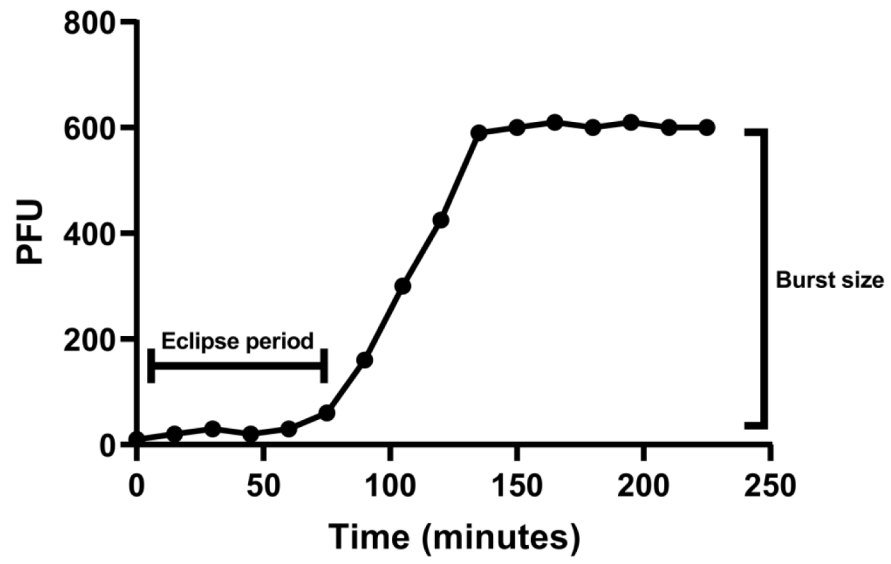
Figure 1.
Electron micrograph of Kronos. Bar = 50 nm.
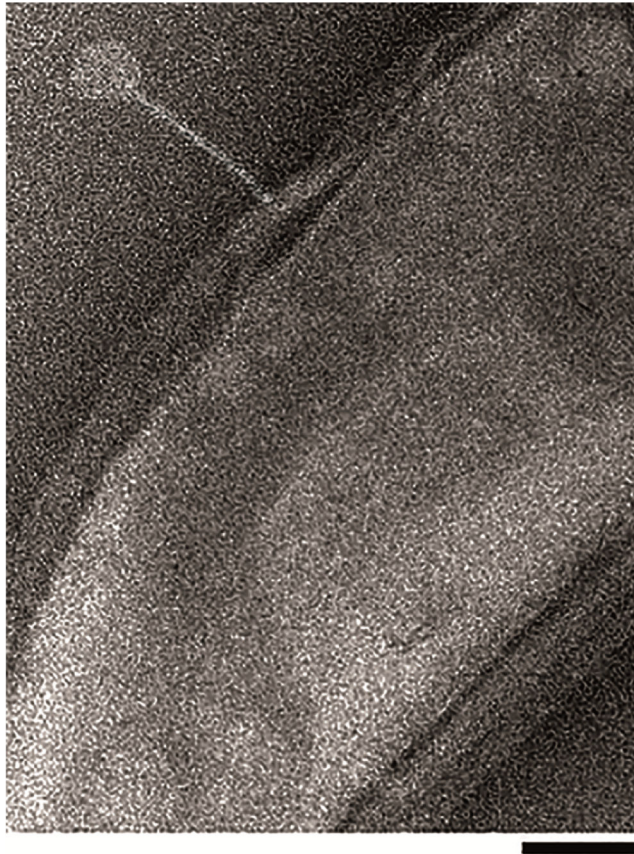
Figure 2.
Electron micrograph of phage Kronos binding to a CBR1 host cell. Bar = 100 nm.
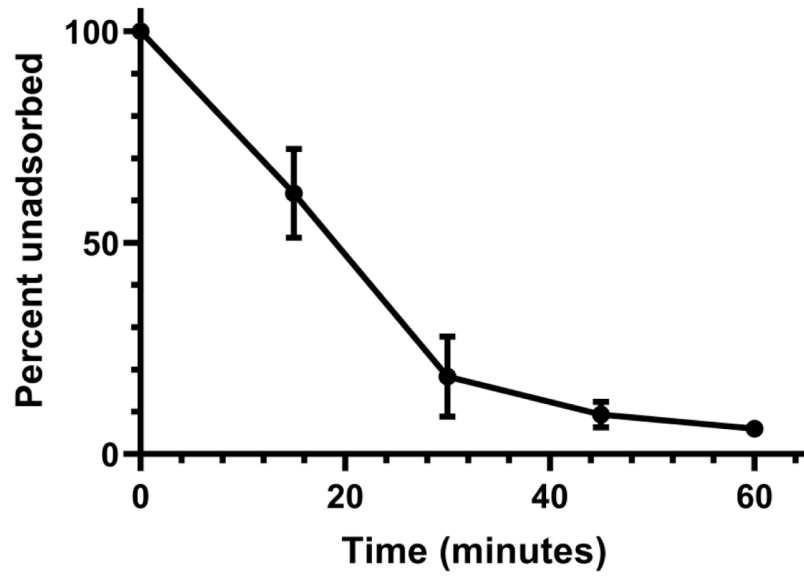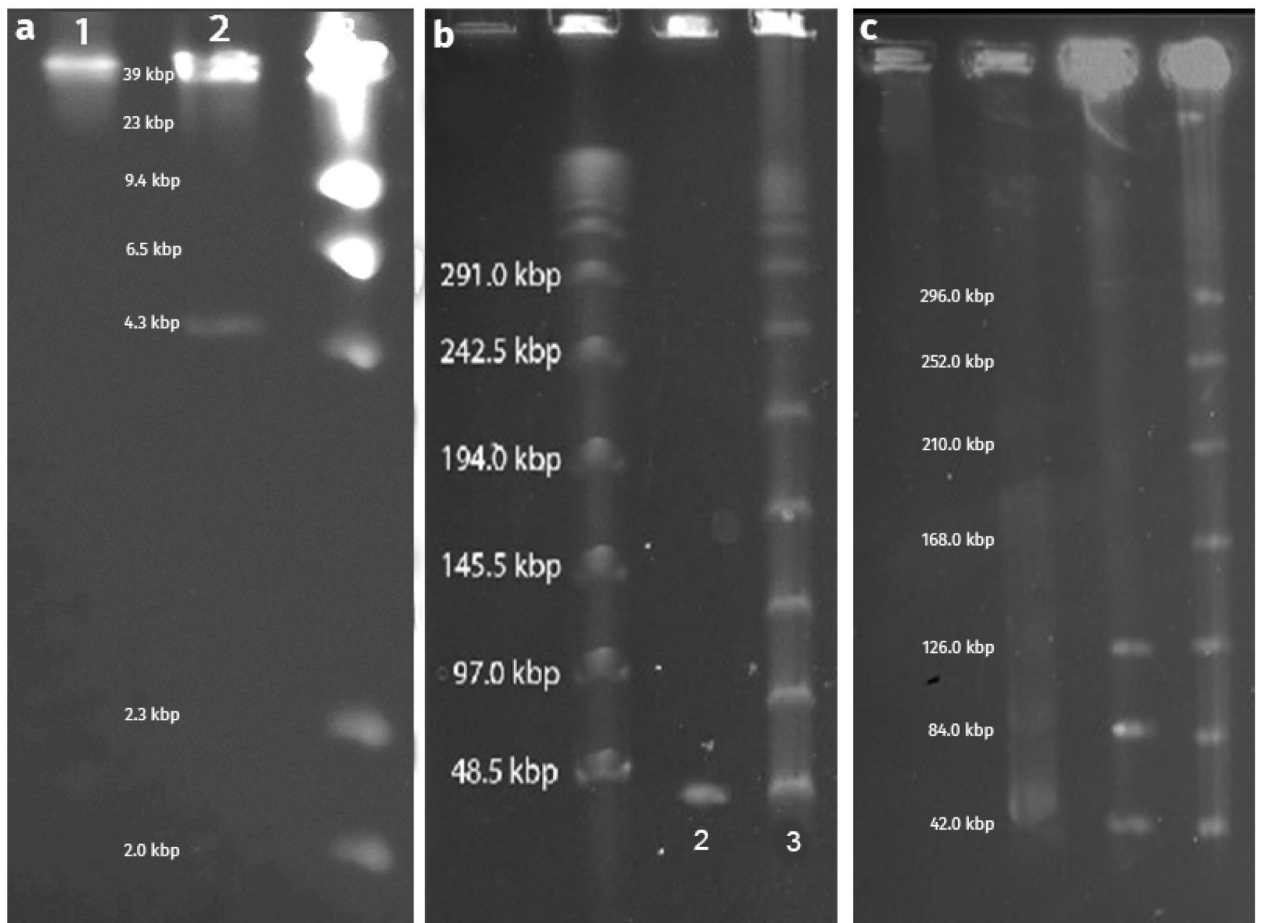
Figure 3.
Kronos adsorption efficiency. Each point represents the average of three experiments. The error bars represent the range of each data point.
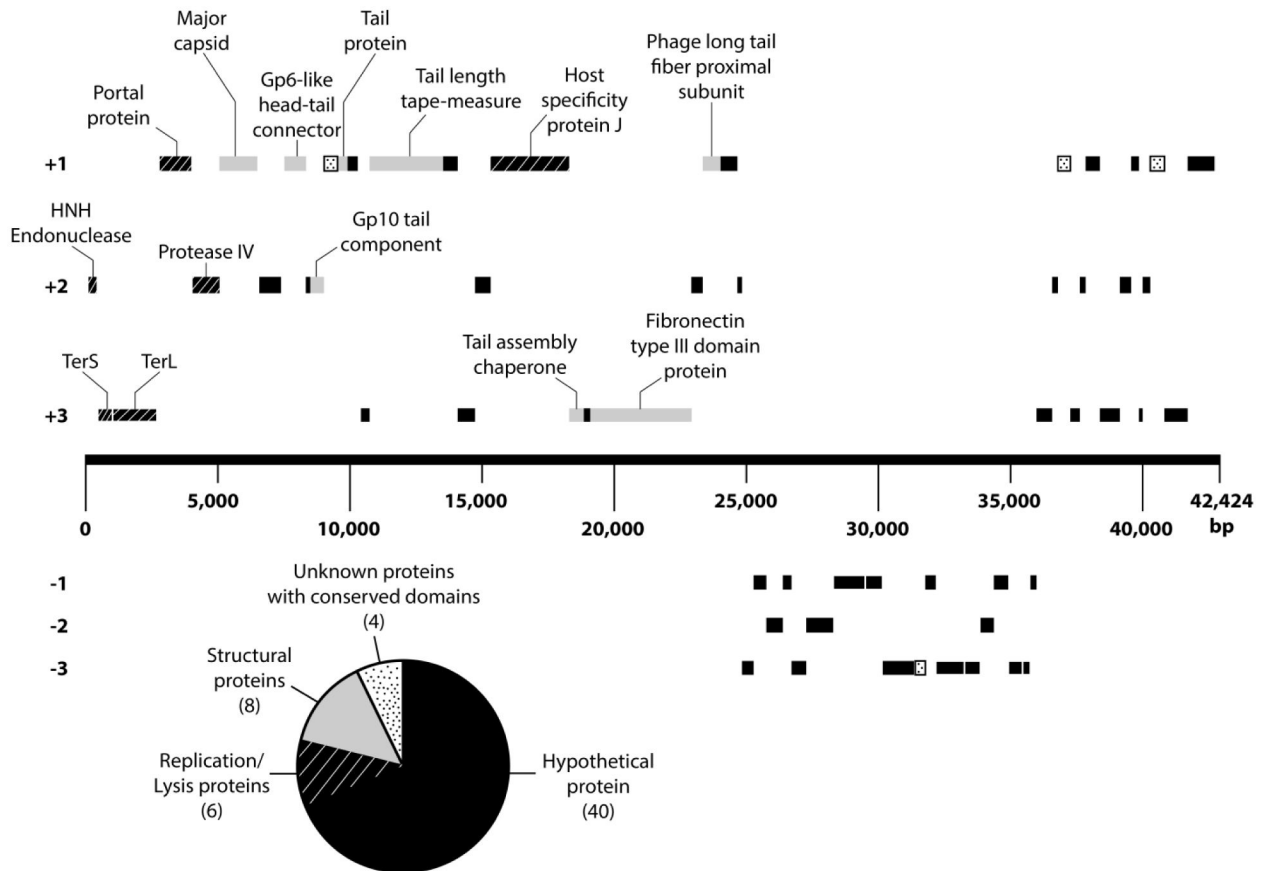
Figure 4.
A representative one-step growth curve of Kronos to determine burst size.

Figure 5.
PFGE images depicting *Sac*II digested Kronos DNA and undigested DNA. Image (A) represents Kronos DNA digested with *Sac*II. Lane 1 depicts digested DNA that has not been heated, and lane 2 depicts DNA that has been heated (>70°C). Lane 3 is λ DNA digested with *Hin*DIII. Image (B) represents Kronos DNA that has been heated to denature the *cos* ends (lane 2) and Kronos DNA that has not been heated, leaving the *cos* ends annealed (lane 3). The λ ladder is appropriately labeled to illustrate relative band sizes. Image (C) illustrates the concentration dependency for concatemer formation of Kronos DNA. DNA concentration is increasing from left to right (~0.05 μg/μl to ~0.40 μg/μl).

Figure 6.
Genome map of Kronos. All genes that are not labeled code for either hypothetical proteins or proteins with a conserved unknown domain. A comprehensive list of all the genes and positions is available in Table S1.

Table 1.

Kronos genome summary table.

Genome size	42,424 bp
GC content percentage	66.22%
Average gene length	699 bp
Coding percentage	95.6%
Number of predicted genes	58
Number of non-hypothetical proteins	18
Hypothetical proteins	40
tRNA genes	0
Genes on forward strand	40
Genes on reverse strand	18
Genes with significant nucleotide homology to genes in the GenBank database	5
Proteins with significant amino acid homology to proteins in the GenBank database	26