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Isolation, characterization and genome analysis of the orphan phage Kintu infecting *Xanthomonas vasicola* pv. *musacearum*

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Abstract

Background *Xanthomonas vasicola* pv. *musacearum* is responsible for the widespread Banana Xanthomonas Wilt in banana cultivation regions across the globe. Biocontrol measures for disease management remain limited amidst increasing antimicrobial resistance and unsustainable conventional agricultural practices. The purpose of this study is to explore a viable alternative or adjunct strategy through the use of bacteriophages for disease management.

Results Kintu was isolated from sewage and displayed clear and circular plaques measuring 3 mm. Based on transmission electron microscopy, Kintu displays siphovirus characteristics, including an icosahedral head and a non-contractile tail. Kintu infects 78% (22 out of 28) Ugandan *Xvm* strains, has an optimal multiplicity of infection of 1, a 10 min adsorption and latent period, a 35 min burst period, and a burst size of 15 particles per bacterium. Phage titers remain stable for two and half months (75 days) in SM buffer at -20 °C and −40 °C but decrease significantly ($p \leq 0.0001$) at 4 °C. Kintu is active at pH 3 and 11, maintains viability at temperatures between 25 °C and 120 °C and tolerates UV irradiation for up to 2 min and 20 s. Kintu inhibits *Xvm* growth at MOI ratios of 0.1, 1 and 10. The genome is a double stranded DNA molecule that consists of 48,985 base pairs and a G + C content of 51.71%. Antibiotic resistance genes or genes associated with a lysogenic life cycle are absent. There is limited sequence similarity of Kintu with other phages, making it a novel phage belonging to an unclassified genus of the class *Caudoviricetes*.

Conclusion Kintu is a novel bacteriophage that infects and lyses *Xanthomonas vasicola* pv. *musacearum*, the causative agent for Banana Xanthomonas Wilt. Its stability across diverse temperatures and pH conditions highlights its potential as a biocontrol agent for managing the disease.

Keywords Bacteriophage, *Xanthomonas vasicola* pv. *musacearum*, Banana xanthomonas wilt, Biocontrol, Genome analysis, Biological analysis

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Background

Xanthomonas vasicola pv. *musacearum* (*Xvm*) is the causative agent of Banana Xanthomonas Wilt (BXW), a disease that remains a persistent threat to banana production in East and Central Africa [1]. It has devastating consequences for both the food security and livelihoods of banana farmers [1, 2]. The bacterium was initially identified in Ethiopia from a closely related wild banana plant species, *Ensente ventricosum*, and named *Xanthomonas musacearum* [3]. Later, the bacterium was found responsible for a wilting disease in cultivated banana plants, leading to its renaming as *Xanthomonas vasicola* pv. *musacearum* [4–6]. *Xvm* is transmitted by contaminated garden tools, planting materials, insect and animal vectors [7, 8]. Infected banana plants display a variety of symptoms including progressive yellowing and wilting of leaves, irregular ripening of premature fruit, fruit rot and the presence of a yellow discharge from the cut pseudostem [4, 9]. Conventional BXW management practices include debudding, tool sterilization, burning or burying infected plants, and the use of herbicides [10–12]. Despite these efforts, BXW remains persistent and challenging to eradicate [1]. This highlights the importance of exploring alternative and sustainable approaches through the use of naturally occurring bacteriophages for effective management of BXW.

Bacteriophages (phages) are natural predators of bacteria, capable of initiating lytic or lysogenic infection pathways upon infection [13–15]. Extensive studies conducted over the past decades have focused on understanding the nature of *Xanthomonas* phages and their role in combatting bacterial plant diseases [16, 17]. These microbes have been successfully isolated from various habitats including sewage, soil, water, and infected plant parts [18]. They exhibit a lytic life cycle, possess either a narrow, broad or polyvalent host range and can adapt to diverse environmental conditions [18].

The classification of phages has recently been revised by the International Committee on Taxonomy of Viruses (ICTV) [19]. Orphan phages, also known as undefined phages, are becoming increasingly common. These phages lack similarity with known phages and remain unassigned within existing classification systems [19]. According to the changes by ICTV, tailed phages are grouped within the class *Caudoviricetes*, encompassing siphoviruses with long and non-contractile tails, myoviruses with contractile tails, and podoviruses with short tail structures. Previously, Nakayinga and colleagues [18] reported a total of 168 *Xanthomonas* phages documented in literature and the NCBI database. However, in a recent study by Kizheva [20], only 134 have their genomes sequenced and deposited in the GenBank database. This discrepancy highlights the limited availability of genomic data for *Xanthomonas* phages, indicating that a significant portion remains uncharacterized. This observation highlights the need for genomic characterization of *Xanthomonas* phages in order to gain a more comprehensive understanding of their genetic makeup and potential biocontrol applications.

Initial biocontrol applications were documented for phages lytic against *Xanthomonas campestris* pv. *campestris*. These were isolated from a decomposing cabbage and successfully halted the spread of black rot disease [21]. Since then, *Xanthomonas* phages have been effectively used as either single or cocktail treatments for various plant diseases. In the case of bacterial leaf blight of rice, single phage treatment using *Xanthomonas oryzae* pv. *oryzae* phage, ϕXOF4, eliminated disease symptoms in seedlings grown from infected rice seeds [22]. A similar *Xanthomonas oryzae* pv. *oryzae* phage, NRO8, was also found to reduce the length of lesions compared to untreated plants [23]. Additionally, treatment with *Xanthomonas axonopodis* pv. *allii* phage, ϕ31, reduced bacterial leaf blight in welsh onions to 26.6% compared to the control plants at 67.5% [24].

Cocktail treatment with *Xanthomonas pruni* phages (Xp3-A and Xp3-I) reduced peach bacterial spot by 17 to 31%, compared to 96% disease incidence of the control group [25]. In a separate study, the application of a *Xanthomonas axonopodis* pv. *allii* phage cocktail (ϕ16, ϕ17A and ϕ31) showed limited efficacy in controlling bacterial leaf blight of welsh onions. It resulted in a 43.3% reduction compared to the 67.8% observed in the control group [24]. To enhance phage performance, phage solutions are incorporated with additives such as milk and sugar-based UV protectants, antibiotics, plant inducers and phage/bacteria mixtures [26–30]. These modifications, yielding promising results in plant disease control, suggest potential solutions for overcoming challenges associated with biocontrol applications. Collectively, these findings show promising potential of *Xanthomonas* phages as effective approaches to plant disease biocontrol.

In this study, we isolated phage Kintu from sewage, analyzed its host range and growth properties. Also, the effect of physiological factors on phage stability and in vitro efficacy were investigated, while the genome was annotated and analyzed.

Methods

Sample collection

The study was conducted at the Biological Sciences Research Laboratory, located within the Faculty of Science at Kyambogo University. Banana pseudostems infected with BXW were collected from gardens located in districts of Kampala (0.345749° N, 32.629173° E; 0.345592° N, 32.628994° E, Mukono (0° 39.426' N, 32° 51.098' E; 0 ° 39.530' N, 32° 51.559' E), Wakiso (0.422288° N, 32.629067° E; 0.422388° N, 32.629325° E) and Isingiro

(-1.396691° S, 30.162099° E; -1.381185° S, 30.193680° E). Untreated sewage samples for bacteriophage isolation were collected from the National Water Bugolobi Sewage treatment plant (0°19'086"N, 32 °36 '22.6"E).

Isolation and identification of *Xanthomonas vasicola* pv. *musacearum*

The bacterial isolation process was based on the method outlined in [31], with minor modifications. Briefly, pseudostems of banana plants exhibiting characteristic symptoms of BXW were cut with a sterile machete to a size of 10 cm, placed in rubber bags, labelled and transported to the laboratory in a cool box. Next, 100 µL of the exudate was suspended in 400 µL of sterile double distilled water, and 100 µL of the mixture was diluted 10-fold, and spread onto YPGA-CC plates (1% Yeast extract, 1% Peptone, 1% Glucose, 1.5% Agar, 100 mg/L Cephalexin, 150 mg/L Cycloheximide). The plates were incubated at 28 °C for 96 h.

Bacterial identification was achieved by visualization of colony phenotype on YPGA-CC media, gram stain and catalase examinations. PCR was used to confirm *Xvm* isolates using Xvm-specific primers, Xcm38_F (5′ CC GCCGGTCGCAATGTGGGTAAT 3′) and Xcm38_R (5′ CAGCGGCGCCGGTGTATTGAGTG 3′), resulting in a product size of approximately 650 base pairs [32]. The PCR reaction mixture (25 µL) included 12.5 µL of 1 x Bioneer master mix, 2 µL of 2 mM $MgCl_2$, 0.5 µL of 0.4 µM forward and reverse primer each, 1 µL of 50 ng template DNA and 8.5 µL nuclease-free sterile water. The reaction mixture was amplified for 35 cycles with the following steps: initial denaturation at 95 °C for 5 min, denaturation at 95 °C for 40 s, annealing at 58 °C for 40 s, extension at 72 °C for 40 s and final extension at 72 °C for 5 min. The amplified products were separated by gel electrophoresis at 100 V for 60 min and visualized using a UV transilluminator. The *Xvm* cultures were maintained in 30% glycerol and stored at -80 °C.

Bacteriophage isolation, detection and plaque morphology

Phages targeting *Xvm* were isolated using an enrichment method described by [33], with minor adjustments. A 40 mL sewage sample was mixed with an equal volume of double strength YPG broth containing 5 mM $CaCl_2$. Next, 1 mL of early log phase Xvm9-7 culture (OD_{600}=0.4) was added to the mixture and incubated for a week at 28 °C with shaking at 100 rpm. The lysate was centrifuged at 10,000 x *g* for 10 min, and the supernatant was filtered sterilized using a 0.22 µM membrane filter. The filtrate was stored at 4 °C for further analysis.

The spot test method was used to detect the presence of phages against *Xvm* strains. A 0.1 mL of early log phase Xvm9-7 culture was mixed with 5 mL of 0.7% YPG molten soft agar, supplemented with 5 mM $CaCl_2$ and poured onto solidified agar with the same $CaCl_2$concentration. Ten microliters of the filtrate were spotted onto the bacterial overlay, and incubated overnight at 28 °C with shaking at 100 rpm. The zone of lysis was removed using a sterile loop and suspended in 2 mL of SM buffer (5.8 g NaCI, 2 g $MgSO_4$, 50 mL 1 M Tris-HCL pH 7.5, 1 L ddH_2O), mixed by inversion and incubated at 4 °C for 30 min. Next, the mixture was added to 2 mL of YPG broth, which already contained 0.5 mL of Xvm9-7 overnight culture. The mixture was incubated overnight at 28 °C with shaking at 100 rpm, followed by centrifugation at 10,000 x *g* for 10 min, and filtration through a 0.22 µM membrane. The primary phage stock was used for further analysis.

The morphological details were investigated through the plaque assay method described by [34], with slight modifications. The phage stock was diluted 10-fold and 100 µL of the diluted sample was mixed with an equal volume of early log phase Xvm9-7 culture. Two hundred microliters of the mixture were suspended in 5 mL of 0.7% molten soft agar and poured onto YPG agar plates. The plates were allowed to set and incubated overnight at 28 °C. Individual plaques were selected using a pipette tip, suspended in 180 µL of SM buffer, mixed and incubated at 4 °C for 30 min. The plaque assay procedure was repeated five times to achieve a pure and stable plaque morphology. Transmission electron micrographs were performed as described by [35] at the Institute for Sustainable Plant Protection in Turin, Italy.

Phage amplification, harvesting and determination of titers

To amplify the pure phage particles, the plaque assay method was employed. Briefly, 100 µL of the purified phage was mixed with an equal volume of early log phase Xvm9-7 culture and incubated overnight at 28 °C with shaking at 100 rpm. After this, 200 µL of the mixture was added to 5 mL of 0.7% molten soft agar and poured onto agar plates. Once the plaques were visible, phage harvesting was achieved as previously described [36]. Briefly, the plates were flooded with SM buffer and incubated overnight at 4 °C. Following this, the SM buffer and the soft top agar were transferred to a clean falcon tube and incubated overnight at 28 °C. The supernatant was harvested by centrifugation at 10,000 x *g* for 10 min, filtered through a 0.22 µM membrane filter, and stored at 4 °C in a clean falcon centrifuge tube. Phage titers were determined using plaque assay and, the plaques were counted at the highest dilution. The data was then entered into the formula described by [37].

Biological characterization of Kintu

Host range

The host range of Kintu was determined using the spot test method and tested against Ugandan 28 *Xvm* strains as well as other bacteria from different genera. A volume of 100 µL of early log phase bacterial cultures were inoculated into 5 mL of 0.7% molten soft agar and the mixture was overlaid onto solidified YPG agar plates. A volume of 10 µL of Kintu with a titer of 1×10^9 PFU/mL was spotted onto the bacterial overlay, dried and incubated overnight at 28 ºC with shaking at 100 rpm. A clear zone on the bacterial lawn indicated a positive or lytic infection. As a negative control, each bacteria overlay was spotted with 10 µL of sterile SM buffer.

Evaluating the optimal multiplicity of infection

To determine the optimal multiplicity of infection (MOI), a three-day culture of Xvm9-7 strain incubated at 28 ºC was standardized to an OD_{600} of 0.4. One milliliter of log-phase Xvm9-7 culture was inoculated with the phage (3.4×10^8 PFU/mL) at different MOIs: 0.001, 0.01, 0.1, 1 and 10 PFU/CFU. The mixtures were incubated for 3.5 h at 28 °C with shaking at 100 rpm and centrifuged at 10,000 x *g* for 10 min. The supernatant was filtered through a 0.22 µm syringe filter (Millipore) and examined for phage titers using the plaque assay method. The MOI, representing the number of phage particles infecting a single bacterial cell was calculated [37]. Three independent experiments were conducted each consisting of three replicates.

Determination of duration for adsorption of phage on host bacteria

The adsorption time of Kintu onto Xvm9-7 was determined using the method outlined by [38]. One milliliter of early log-phase Xvm9-7 culture was inoculated with Kintu (1×10^9 PFU/mL) at MOI of 10 and incubated at 28 ºC at different time points i.e. 0 s, 30 s, 1 min, 5 min, 10 min, 15 min, 20 min, 30 min, 45 min and 60 min. After each time point, the phage/bacteria mixture was immediately chilled on ice to halt the adsorption process. The mixture was centrifuged at 10,000 x *g* for 10 min to sediment the phage-bound bacteria. The supernatants containing the unadsorbed phages were filtered through 0.22 µm syringe filter (Millipore) and titers determined using plaque assay. Three independent experiments were conducted each consisting of three replicates.

One-step growth curve

The procedure for the one-step growth curve were conducted as previously described [39] with minor adjustments. One milliliter of Xvm9-7 culture at early log-phase, was infected with Kintu (1×10^9 PFU/mL) at MOI of 1. The mixture was incubated at 28 ºC for 10 min to allow phage adsorption and centrifuged at 10,000 x *g* for 10 min. The resulting pellet was then re-suspended in fresh YPG broth, and a 1 mL aliquot was transferred to an eppendorf tube labeled with time points ranging from 0 s to 120 min. The samples were incubated at 28 ºC with shaking at 100 rpm. At each designated time point, the samples were removed, chilled on ice, centrifuged at 10,000 x *g* for 10 min and filtered through 0.22 µm syringe filter (Millipore). The phage particles were quantified by determining the phage titer in each time-point sample using plaque assay method described above. Three independent experiments were performed, each including three replicates.

Effect of storage on phage stability

The stability of Kintu in different storage conditions was assessed by monitoring its titer for 75 days. The phage, initially having a titer of 1×10^9 PFU/mL was stored in 30% glycerol at 4 ºC, -20 ºC, and −40 ºC. Aliquots were collected and the titer was determined using the plaque assay as described above. The resulting plaques were counted in order to determine the phage titer. The experiment was conducted three times independently, with each experiment consisting of three replicates.

Effect of pH on phage stability

The optimal pH for evaluating Kintu stability was determined by measuring its titer at various pH values, including 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, and 13, using a pH meter for verification. Different pH values were achieved by adjusting the phage suspensions with 1 M NaOH or HCL. A total of 100 µL of the phage sample (1×10^9 PFU/mL), was mixed with 900 µL of SM buffer and incubated at 28 ºC for 1 h. Following this, the plaque assay method was conducted as described above. The phage titer was determined by counting the resulting plaques. Three independent experiments were performed, each including three replicates.

Effect of temperature on phage stability

The effect of temperature on the stability of Kintu was examined by suspending 100 µL of phage stock (1×10^{10} PFU/mL) in 900 µL of YPG broth and incubating at different temperatures for one hour. The temperatures include 25 ºC, 28 ºC, 37 ºC, 40 ºC, 50 ºC, 60 ºC, 70 ºC, 80 ºC, 90 ºC, 100 ºC, 110 ºC, 120 ºC, 130 ºC and 140 ºC. The phage titers were determined at each temperature using the plaque assay previously described. Three experiment was conducted independently, each experiment consisting of three replicates.

Effect of ultraviolet irradiation on phage stability

To investigate the effect of ultraviolet (UV) irradiation on the stability of Kintu, 10 mL of phage solution with

an initial concentration of 1×10^9 PFU/ml was poured into a sterile petri dish. The dish was placed under a biosafety cabinet class II with a 60 cm distance from the UV light source that emitted light at a wavelength of 254 nm. At each time point, 100 µL of the exposed phage was removed and diluted 10-fold in YPG broth. The samples were subjected to the plaque assay method described earlier. Three independent experiments, each with three replicates were performed.

Bacterial growth inhibition by Kintu in liquid culture

To assess the phage's inhibitory effect on bacterial growth, 4 mL of the bacterial cultures at early log-phage were mixed with phage (1×10^9 PFU/mL) at MOI of 0.001, 0.01, 0.1, 1 and 10 in a sterile centrifuge tube (15 mL). Thereafter, the samples were transferred into cuvettes that were appropriately labeled and incubated at 28 ºC with shaking at 100 rpm. At regular intervals of 1 h, the absorbance of the samples in the cuvettes was measured at 600 nm using a spectrophotometer. This process was repeated for a duration of 24 h. As a control, a sample containing bacteria only was subjected to the same experimental conditions. Plain broth was used to calibrate the spectrophotometer. The experiment was conducted three times, with three replicates in each experiment.

Genome extraction and analysis

Phage DNA was extracted from the virions by adding 10 µL of 10 x DNase I buffer to 90 µL of the phage stock. The solution was filtered using a 0.45 µm ultrafiltration spin-column fitted in a new microcentrifuge tube and centrifuged at 2,500 x *g* for 1 min to remove bacterial cells. Then, 5 µL of DNase I (ThermoFisher Scientific) was added to the filtrate and incubated at 37 °C for 30 min to eliminate any bacterial DNA while viral DNA within the protein capsid remained intact. To deactivate the DNase I, 10 µL of 50 mM EDTA and 10 µL of 1% SDS were added to the filtrate. Subsequently, 5 µL of proteinase K was added, and the mixture was incubated at 55 °C for 45 min to digest phage capsids and release the phage DNA. A sequencing library of the phage DNA was prepared using the Nextera Flex kit (Illumina) and sequenced on an Illumina MiniSeq machine (2*150 bp reads) at KU Leuven (Belgium). The reads were trimmed using Trimmomatic v0.39 [40] and assembled using Unicycler v0.5.0 [41]. The phage was annotated automatically using the RASTtk pipeline [42] and manually curated using BLASTp. Phage lytic cycle was predicted by Phage. AI online tool [43].

Statistical analysis

The data from the experiments were entered into Microsoft Excel 2013 and then exported to GraphPad Prism software version 10 for analysis using Analysis of Variance (ANOVA). Tukey's Post-hoc multiple comparison test were computed to compare the means of different groups. Graphical representation was performed using GraphPad Prism software version 10. A $p<0.05$ was considered statistically significant.

Results

Characterization of *xvm* strains

BXW-infected banana plants displayed characteristic symptoms such as leaf yellowing and wilting, early fruit ripening and release of a yellowish exudate from cut pseudostems (Fig. 1A and B). Bacterial isolates obtained from the diseased pseudostems showed distinct characteristics of *Xvm* on YPGA-CC plates. These included yellowish, mucoid, convex colonies with an entire margin (Fig. 1C and D). Additional phenotypic analyses showed that the isolates were gram-negative and catalase positive. Among the isolates examined, 28 were confirmed as *Xvm* by PCR analysis, showing a product size of approximately 650 bp band on agarose gel (Fig. 1E). The observed bands correspond to the gene encoding the polymorphic membrane protein [32]. Twenty two of the *Xvm* isolates were from this study while the remainder were generously gifted by IITA.

Detection, plaque morphology and transmission electron microscopy

Among the eight sewage samples collected from National Water Bugolobi Sewage treatment plant, one sample exhibited clear plaques on an Xvm9-7 lawn (Fig. 2A). A consistent plaque morphology displaying a clear zone and circular shape with a diameter of 3 mm (Fig. 2B) was identified and assigned phage Kintu, accession number PP313117. Based on transmission electron micrographs, Kintu has a typical siphovirus morphology consisting of an icosahedral head and non-contractile tail (Fig. 2C). The titer of the isolated phage was 1×10^{10} PFU/mL.

Host range and efficiency of plating

The host range of Kintu was determined by the spot test method on 28 Ugandan *Xvm* strains. The phage lysed 78% (22 out of 28) of the tested *Xvm* strains (Table 1). There was no lysis in bacteria lawns formed by *Bacillus* and *Burkholderia* species, *Staphylococcus aureus*, *Salmonella typhi*, *Klebsiella pneumonia*, *Escherichia coli* and *Pseudomonas aeruginosa* (Table 1).

Optimal multiplicity of infection, adsorption rate, one-step growth curve and effect of storage

To determine optimal MOI, log-phase cultures of Xvm9-7 were inoculated with phage Kintu, at various MOIs, starting with an initial titre of 3.4×10^8 PFU/mL. After 3.5 h of incubation, the titers were determined.

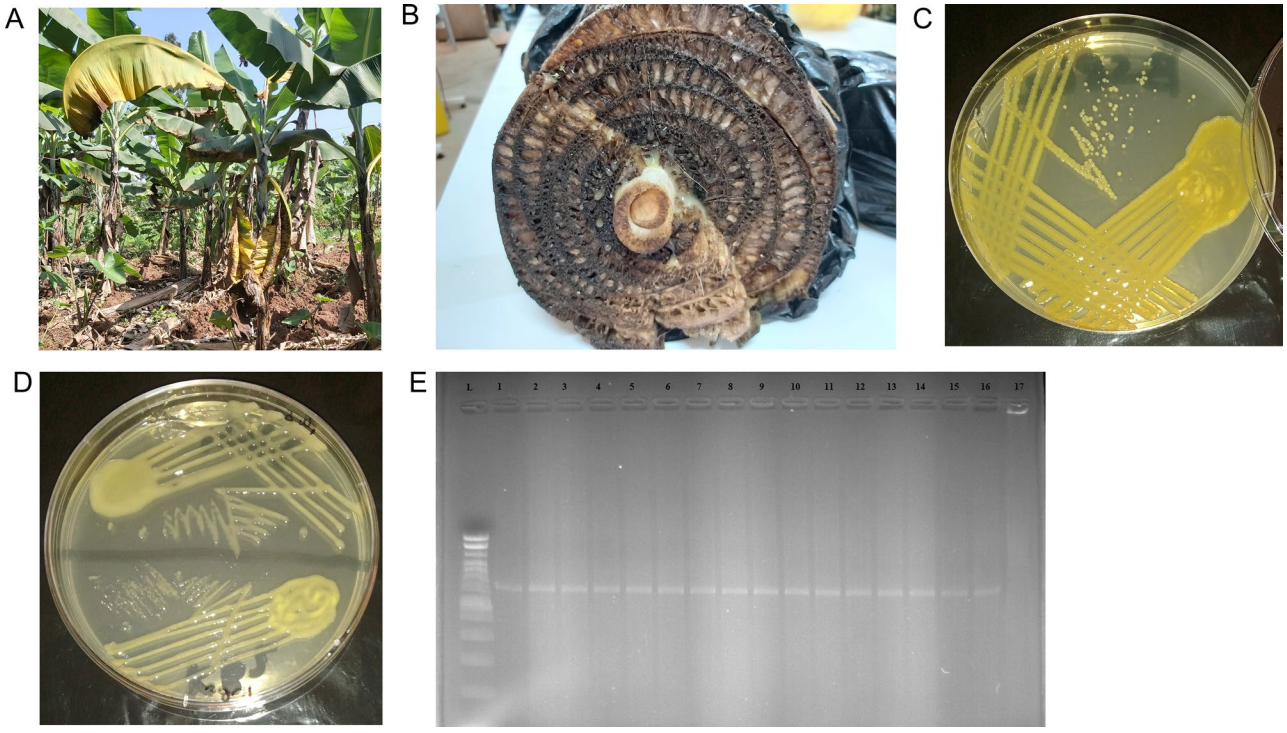

Fig. 1 BXW-infected banana plant, pseudostem and bacteria isolates on YPGA-CC plates. **A**. Yellowing and wilting of leaves as symptoms for BXW. **B**. Pseudostem with yellowish exudate. **C**. Bacteria isolates with small yellow colonies situated at the center of the agar plate. **D**. Bacteria isolate that appears as a yellowish mucoid colony after four days of incubation. **E**. Confirmation of *Xvm* by PCR. Lane L: 1000 bp ladder, Lane 1–17: Xvm9-7, Xvm1-4, BS17-5, BS17-6, BS8b2, Ooze 3b, L, P1, B6 , AB14, B3 7, BCC280, U40L2, BCC 278, U09L3b, KB1, E3

The highest production of phage progeny was observed at a MOI of 1 (2.4×10^{11} PFU/mL), followed by MOI 0.1 (1.3×10^{10} PFU/mL), MOI 10 (8.9×10^{9} PFU/mL), MOI 0.01 (2.1×10^{9} PFU/mL) and MOI 0.001 (2.3×10^{8} PFU/mL) (Fig. 3A). Phage titres at MOI 1 were significantly different from titers obtained at MOI 0.01 ($p=0.0049$) and MOI 0.001 ($p=0.0002$) (Fig. 3A). Therefore, the optimal MOI for Kintu is 1. The adsorption time of Kintu to Xvm9-7 cells was assessed by infecting them at an MOI of 10 and incubation at 28 ºC. At 10 min, 90% of the phages were adsorbed onto the host strain marked with a significant ($p\leq0.0001$) reduction of initial phage titres (1×10^{9} PFU/mL) in the mixture. There was no significant ($p=0.9965$) decrease in phage titres beyond 10 min (Fig. 3B). From the one-step growth curve, Kintu exhibited a latent period of 10 min, succeeded by a burst period lasting 35 min (Fig. 3C). The burst size was calculated to be 15 viral particles per infected cell.

To investigate the impact of storage temperature, the experiments were conducted at 4 ºC, -20 ºC and -40 ºC, for 75 days. At an initial titer of 1×10^{9} PFU/mL, there was no statistically significant reduction in phage titres during storage at -40 ºC for 49 days (7 weeks) ($p=0.1482$), at -20 ºC for 35 days (5 weeks) ($p=0.3403$) and then at 4 ºC for 7 days (1 week) ($p=0.9999$). By day 75, the phage titres significantly decreased by 1.66 log units at -40 ºC ($p=0.0156$) and by 2.26 log units at -20 ºC ($p=0.0007$). However, at 4 ºC, the phage particles were completely lost after 75 days (10 weeks) of storage ($p\leq0.0001$) (Fig. 3D).

Kintu stability under pH, temperature and ultraviolet irradiation

The stability of Kintu was evaluated under various in vitro physiological conditions. Based on pH results, phage titers remained stable between pH 3 and 11 with highest activity at pH 7 (1.5×10^{9} PFU/mL) and 8 (1.49×10^{9} PFU/mL). However, a slight but significant decrease in phage activity was observed at pH 11 ($p=0.0124$) compared to pH 7. Complete inactivation was observed, with significant effects when the pH dropped below 3 ($p\leq0.0001$) or above 11 ($p\leq0.0001$) (Fig. 4A). The temperature experiment revealed that the initial phage titers of Kintu (1×10^{10} PFU/mL) showed no significant reduction at 25 ºC (1.6×10^{10} PFU/mL, $p=0.1416$) and 40 ºC (3.6×10^{9} PFU/mL, $p=0.1024$). However, a gradual decline was observed up to 70 ºC, resulting in a reduction of 1.71 log10 units (3.6×10^{8} PFU/mL, $p\leq0.0001$). Furthermore, at 120 ºC, there was significant reduction of phage titres by 8.46 log10 units (4.8×10^{1} PFU/mL, $p\leq0.0001$). Complete phage viability was lost beyond this temperature (Fig. 4B). Upon exposure to UV irradiation at a wavelength of 254 nm, the initial phage titer (1×10^{9} PFU/ml)

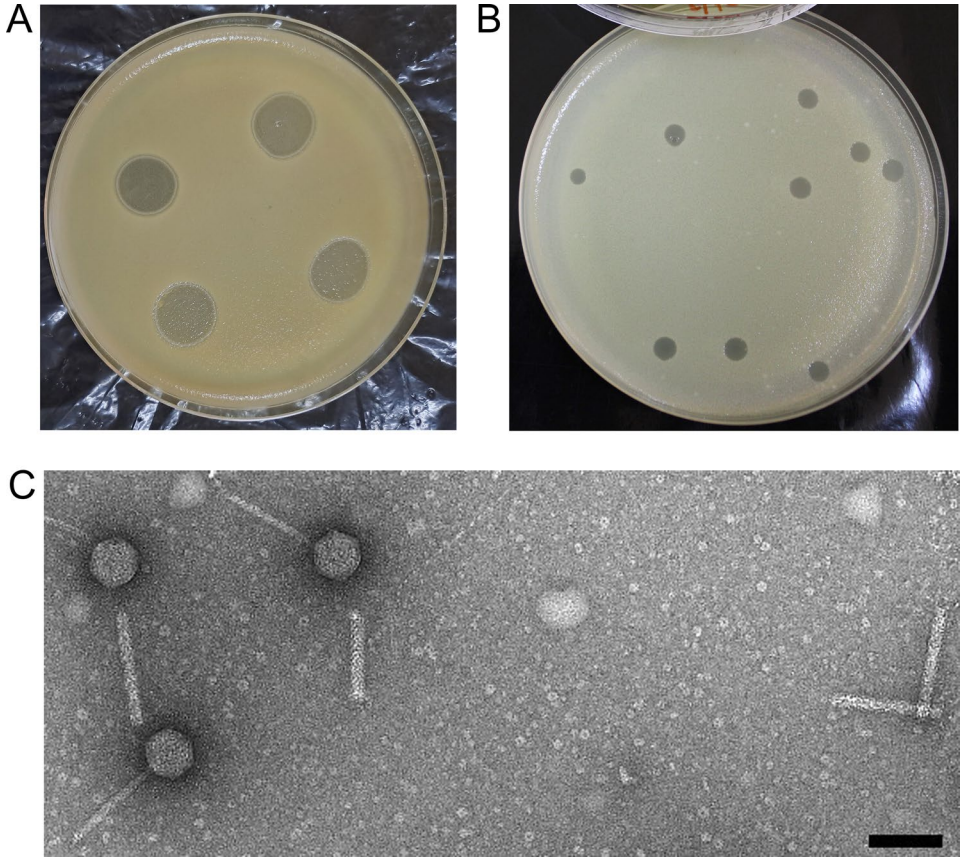

Fig. 2 Phage detection and phenotypic characterization. **A**. Zone of lysis by sewage filtrate on an Xvm9-7 lawn. **B**. Circular and clear plaques with a diameter of 3 mm on YPG agar plates. **C**. Transmission electron micrograph of Kintu displaying a siphovirus morphology. Scale bar represents 100 nm

declined significantly (6×10^6 PFU/ml, $p \le 0.0001$), corresponding to a 2.8 log10 unit reduction at 110 s. A further significant decline in phage titers (3.1×10^1 PFU/ml, $p \le 0.0001$) was observed at 140 s. Beyond this point, no phage titre was recorded (Fig. 4C).

Evaluation of bacterial inhibition by Kintu

A host growth inhibition assay was conducted to evaluate the ability of Kintu to halt bacterial culture growth. The bacterial cultures were mixed with Kintu and incubated at 28 ºC for 24 h. Kintu significantly reduced ($p \le 0.0001$) all tested bacteria strains (Xvm 1-4, BS17-5, BS8b2) at MOI 0.1, 1, and 10, but had no effect on growth at MOI 0.001 and 0.01 (Fig. 5). Importantly, at an MOI of 0.1, an increase in the turbidity in the BS8b2 culture after 12 h suggests the potential emergence of resistant strains (Fig. 5). There was a significant increase in the optical density of bacteria strains BS17-5 and BS8b2 ($p \le 0.0001$) beyond that of the control between 10 and 12 h at phage MOI 0.01 and 0.001 (Fig. 5).

Genome sequencing and annotation of phage Kintu

Kintu has a dsDNA genome of 48,985 bp and a G+C content of 51.71%. A BLASTn analysis revealed limited sequence similarity to known phages in the NCBI database. The highest hit showed a sequence coverage of 41% and a sequence identity of 80.7% with the uncultured siphovirus NHS-Seq1 (MH029512). According to Viptree analysis [44], Kintu showed no closely related phages at the proteome level. To classify phage Kintu taxonomically, a VIRIDIC analysis [45] was run to compare Kintu to the BLASTp hits. The highest intergenomic distance was observed between Kintu and uncultured phage NHS-Seq1 (MH029512). However, with 45%, this is far below the 70% genus threshold. Therefore, Kintu represents a new phage species within an unclassified genus of the class *Caudoviricetes*.

The genome was arbitrarily opened at the terminase gene and structurally annotated, displaying 81 coding sequences and two tRNAs. The coding sequences were functionally annotated with BLASTp, revealing 26 genes encoding a known protein function. These include ten structural proteins: a HK97 family protein, a tail terminator protein, a tail protein, the tail assembly chaperone, tail tape measure protein, two minor tail proteins, a tail protein peptidase, the tail assembly protein and a host specificity protein. The non-structural elements are DNA and metabolism-associated proteins: three HNH

Table 1 Host range and host specificity of Kintu

Bacteria strains	Xvm strain	District of origin	Lytic activity of Kintu against bacteria strains
Xvm9-7	Yes	Isingiro	+
Xvm1-4	Yes	Isingiro	+
BS17-5	Yes	Kampala	+
BS17-6	Yes	Kampala	+
BS8b2	Yes	Kampala	+
KFS3	Yes	Kampala	-
Ooze 3b	Yes	Wakiso	+
L	Yes	Mukono	+
P_0	Yes	Mukono	+
P_1	Yes	Mukono	+
P_5	Yes	Mukono	+
B6 (2)	Yes	Mukono	+
B4 (5)	Yes	Mukono	+
B3 (7)	Yes	Mukono	+
B6 (1)	Yes	Mukono	+
B8 (5)	Yes	Mukono	+
B13 (2)	Yes	Mukono	-
B13 (4)	Yes	Mukono	-
B14 (4)	Yes	Mukono	+
B14 (8)	Yes	Mukono	+
AB14	Yes	Mukono	+
E3	Yes	Mukono	-
U40L2	Yes	Mukono	-
BCC278	Yes	Mukono	-
U09L3b	Yes	Mukono	+
KB_1	Yes	Mukono	+
$B_1T_1KX_1$	Yes	Mukono	+
6	Yes	Mukono	+
7HRB8; *Bacillus* sp	No	Mukono	-
IDRB1; *Burkholderia* sp	No	Mukono	-
Pseudomonas aeroginosa	No	Kampala	-
Escherichia coli	No	Kampala	-
Klebsiella pneumoniae	No	Kampala	-
Salmonella typhi	No	Kampala	-
Staphylococcus aureus	No	Kampala	-

The lytic activity on *Xvm* strains was indicated by (+) for lysis and (-) for no lysis

endonucleases, an endonuclease, a ssDNA-binding protein, a recombinase, a DNA adenine methyltransferase, a transcriptional regulator, a deoxyuridine 5' triphosphate nucleotide hydrolase, a DNA binding protein and a methyltransferase. Finally, Kintu encodes some known lysis- and packaging-associated proteins: an endolysin, a spanin, holin and the large and small subunits of the terminase. In addition, 68% of the coding sequences remain unknown (Fig. 6). The life cycle was predicted as lytic.

Discussion

Xanthomonas-associated diseases of important crops are becoming increasingly common, leading to a growing interest in the isolation and characterization of *Xanthomonas* phages for biological control applications. Specifically, genomic characterization provides information on evolution, diversity, and interactions with bacteria, which can inform the development of effective therapies against related diseases. A number of phages targeting the genus *Xanthomonas* remain unexplored and inadequately studied [18]. In this study we isolated and investigated the biological and genomic characteristics of Kintu targeting *Xanthomonas vasicola* pv. *musacearum*.

Sewage constitutes a significant reservoir for phages lytic against the phytopathogen *Xanthomonas* [46–48]. For instance, phage Pg125 lyses several *Xanthomonas* species of agricultural importance [46] while ϕRS lyses *Xanthomonas phaseoli*, the causative agent of common bean blight [47]. Phage Kintu was isolated from the National Water Bugolobi Sewage treatment plant, which receives effluent and run-off water from open food markets within Kampala city center and Nakivubo channel area. Bananas from these markets may carry *Xvm* and probably washed off during cleaning or by rain. These bacteria can enter the run-off water and make their way to the sewage plant. The isolation of phages infecting *Xvm* from sewage indicates that sewage is a potential habitat for *Xvm*-specific phages. The ability of *Xvm*-specific phages to infect only *Xvm* suggests that these phages avoid infecting non-*Xanthomonas* bacteria present in sewage. The bacterial strains used in this study were identified and confirmed through PCR analysis using specific primers, Xcm_38 forward and reverse primers, targeting the polymorphic membrane protein unique to *Xvm* [32].

A consistent plaque morphology, characterized by a clear and well-defined edge was observed, indicative of a lytic cycle. Similar plaque characteristics have been observed across multiple pathovars used for biocontrol including *Xanthomonas axonopodis* pv. *allii*, *Xanthomonas oryzae* pv. *oryzae*, *Xanthomonas oryzae* pv. *oryzicola*, *Xanthomonas euvesicatoria* etc. This indicates a common trend of using lytic phage for effective disease management [22, 24, 49–51]. A lytic cycle of infection is preferred for biocontrol applications due to its efficiency and effectiveness [52]. TEM analysis revealed a siphovirus phage type with an icosahedral head and non-contractile tail. This discovery contributes to the expanding collection of this phage type [19].

Kintu has a narrow host range, infecting and lysing 78% of Ugandan *Xvm* strains from different geographical locations. Also, the phage showed no lytic activity against bacteria present in the microflora of banana plants and some human pathogens. The specificity to *Xvm* strains reduces the risk of destroying beneficial bacteria, making it an ideal choice for biocontrol applications. Several *Xanthomonas* phages have shown both a narrow host range and specificity towards *Xanthomonas* species,

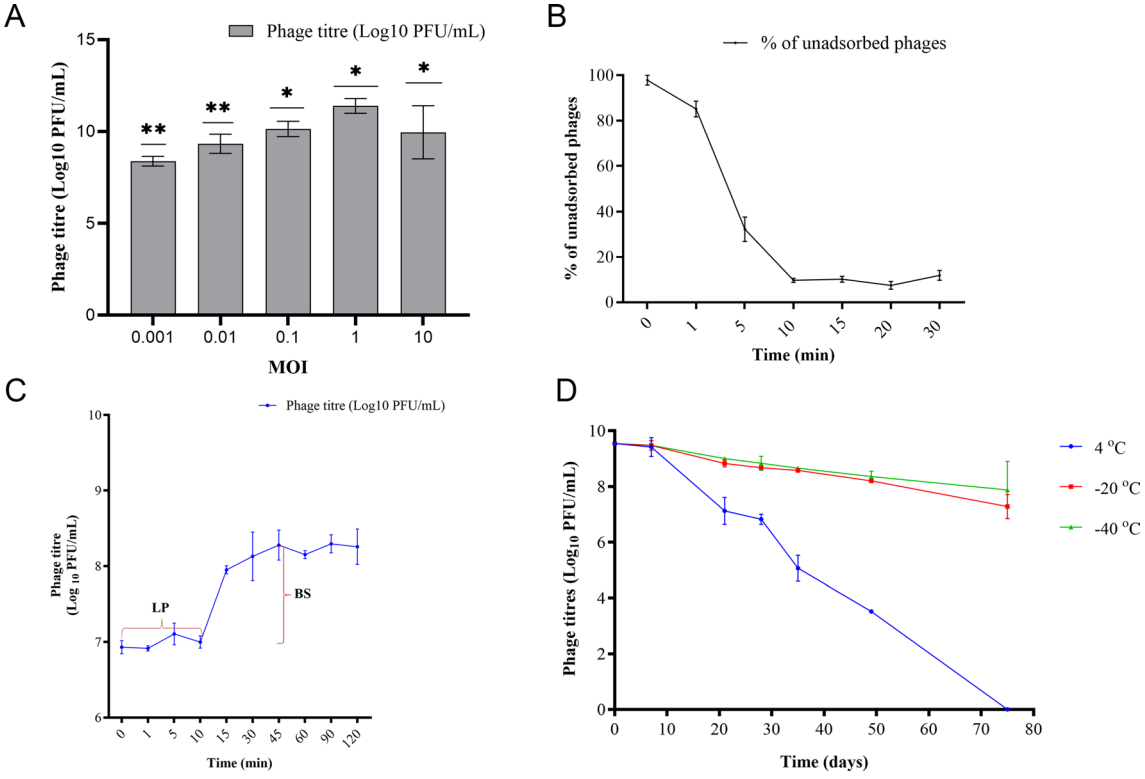

Fig. 3 Replication parameters and storage effects of Kintu phage at different temperatures. **A**. Shows MOI at ratios of 0.001, 0.01, 0.1, 1 and 10. ** indicates a statistically significant difference ($p < 0.05$), while * indicates no statistically significant difference ($p > 0.05$). Phage titers are expressed in PFU/mL. **B**. Percentage of unadsorbed phage over time. **C**. One-step growth curve showing a latent period (LP) of 10 min and a burst size (BS) of 15 PFU/cell. **D**. Phage stability at different storage temperatures (4 °C, -20 °C and -40 °C).Error bars represent the mean ± standard deviation from three independent experiments, each performed in triplicate

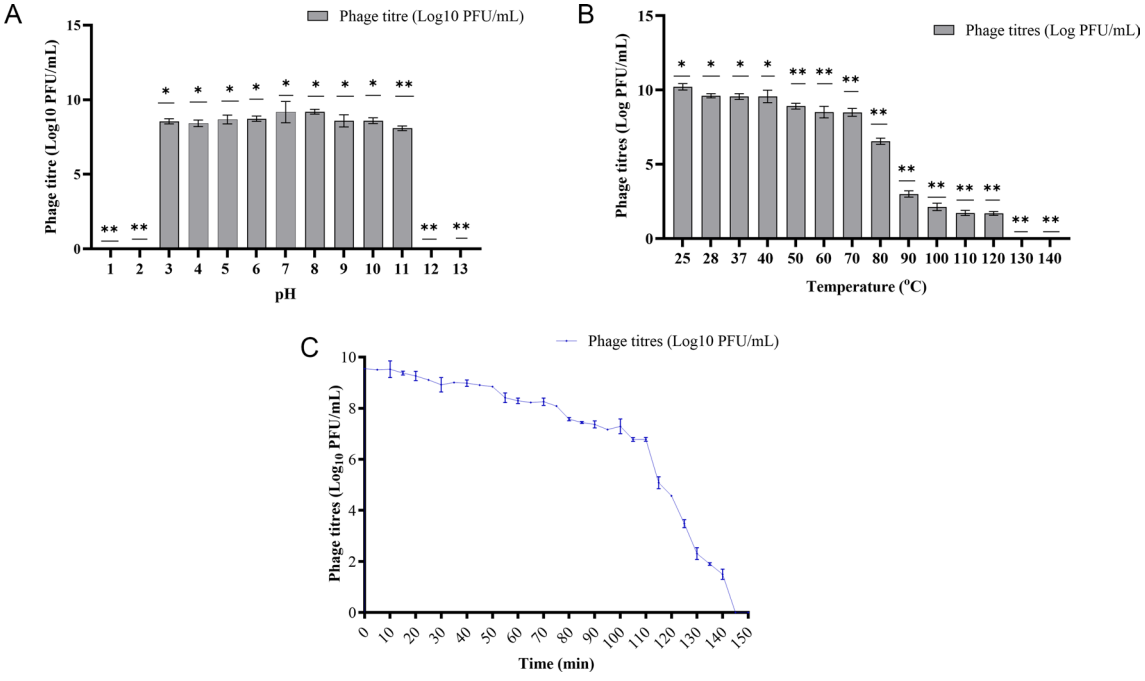

Fig. 4 In vitro physiological conditions of phage Kintu. **A**. Effect of pH (1 to 13) on phage viability. **B**. Effect of temperature (25 °C to 140 °C) on phage viability. **C**. Effect of UV irradiation at a wavelength of 254 nm. Seconds is represented as "s". Phage titers are expressed in PFU/mL. ** indicates a statistically significant difference ($p < 0.05$), while * indicates no statistically significant difference ($p > 0.05$). Error bars represent the mean ± standard deviation

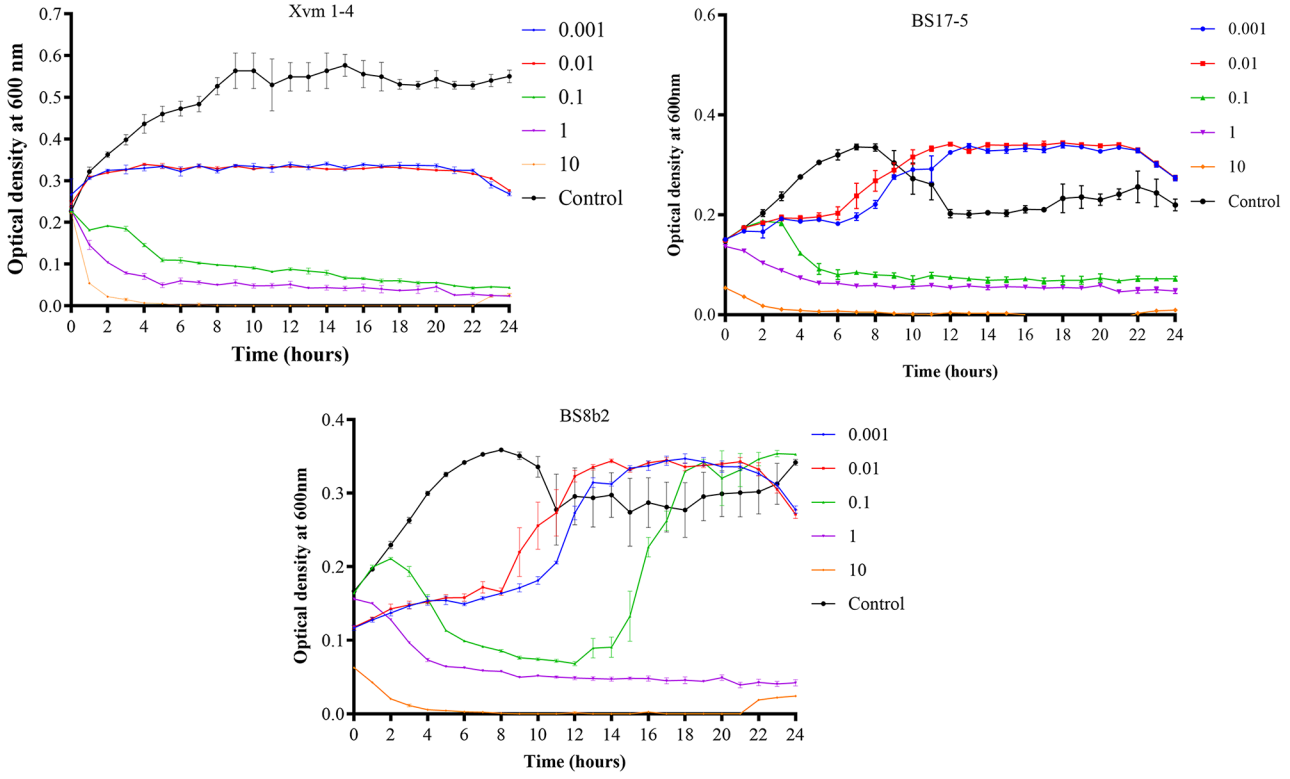

Fig. 5 Growth inhibition of Xvm 1-4, BS17-5, BS8b2 by Kintu at MOI 0.001, 0.01, 0.1, 1 and 10. The y-axis represents optical density (OD) at 600 nm, while x-axis represents time in hours. Error bars represent the mean ± standard deviation from three independent experiments, each performed in triplicate

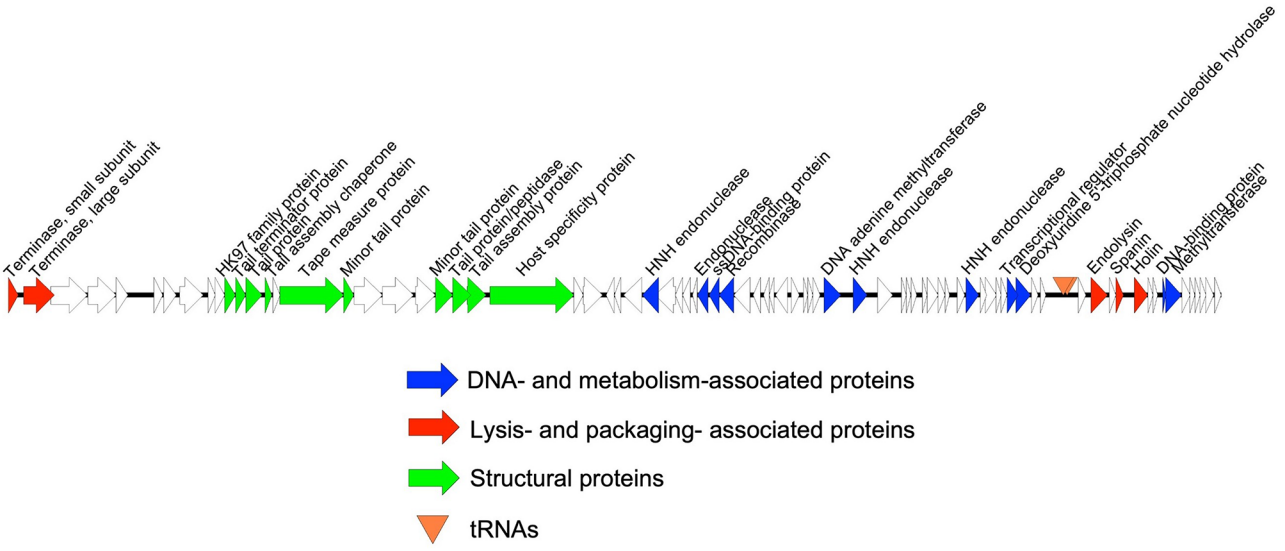

Fig. 6 Genome map of Kintu. Each arrow represents a coding sequence on the Kintu genome. The coding sequences were colored according to the function of the encoded protein: blue represents proteins associated with DNA replication and general metabolism; red represents proteins associated with lysis and packaging; green represents structural proteins; and white represents hypothetical proteins. Two tRNA genes were retrieved on the Kintu genome, displayed as an orange triangle

making them promising candidates for biocontrol application [18, 20, 23].

At MOI of 1, infection of Kintu resulted in the highest production of progeny, making it an effective dose for successful biocontrol treatment. With a short adsorption time of 10 min, a fast infection cycle is initiated, leading to a productive lytic cycle. The one step growth analysis revealed a 10 min latent period and a burst size of 15

virus particles per cell. The short latent period of Kintu is comparable to the latent periods of previously studied *Xanthomonas* phages, which have demonstrated the ability to control the growth of their host bacterial populations [22, 53]. The burst size of Kintu is comparable to phage Cp1, which also exhibits lytic activity against *Xanthomonas axonopodis* pv. *citri* [54]. Among the previously studied phages, the lowest burst size of 4.6 virus particles per cell was observed in Pg125, a polyvalent phage that was isolated from sewage [46].

Different storage conditions for phages have been studied and their efficacy can vary significantly. This variation is dependent on factors such as the specific phage strain and composition of the storage medium [55]. Storage of purified phages at 4 ºC is generally considered optimum for short-term periods [56]. Our results align with the findings of the aforementioned author as the phage titer was inactivated after two and half months of storage probably due to protein denaturation. Consistent findings were previously reported for other *Xanthomonas* phages with a short shelf life when stored at 4 ºC [57, 58]. On the contrary, *Xanthomonas* phages can maintain stability at 4 ºC over long periods [20] indicating that there is no one-size-fits-all approach for phage storage shelf life. Phage stability at freezing temperatures has been reported previously for some *Xanthomonas* phages [20]. In this study, phage titres remained relatively stable, with only a slight decline observed at -40 ºC and - 20 ºC during the storage period. Overall, *Xanthomonas* phages have unique storage conditions and they should be investigated individually.

Xanthomonas phages are stable across a broad range of pH levels, from 5 to 11 [18, 20, 59]. The results of this study demonstrate that Kintu remains viable between 3 and 11 pH values. This broader range exceeds what has been reported in the aforementioned studies. Outside of this optimal pH, the phage becomes inactivated in highly acidic (pH 1 and 2) or highly alkaline (pH 12 and 13) conditions, which is consistent with the findings of previous studies [18, 20, 59]. Therefore Kintu has the ability to adapt to different pH range found in the rhizophere and phyllosphere, making it a promising candidate for use as a biocontrol agent.

The titres of Kintu remain stable across a wide temperature range, from 25 ºC to 120 ºC, differing from the ranges observed for other phages. For instance, a narrow temperature range between 4 ºC and 50 ºC is displayed by *Xanthomonas* phages like ϕXOF4 and NR08 [22, 23]. On the other hand, a broad temperature range between 35 ºC and 70 ºC is reported for *Xanthomonas euvesicatoria* phages, Kϕ1 to Kϕ15 [60]. This exceptional temperature adaptability of Kintu strongly suggests that the phage might possess unique mechanisms that enable the phage to adapt to diverse thermal conditions.

Phages, in general, are susceptible to destruction by UV irradiation, and *Xanthomonas* phages are not exempt from this vulnerability [61]. Kintu showed resistance against UV irradiation for a period of 2 min and 20 s when subjected to UV light from a biosafety cabinet lamp. Similarly, phages against *Xanthomonas euvesicatoria* and *Xanthomonas oryzae* are reported to exhibit short-term stability when exposed to UV irradiation. Unlike phage Kintu, which is a siphovirus, these are myoviruses i.e. Kϕ1, vb_XciM_LucasX and phage Murka [57, 59, 62].

The effectiveness of Kintu in eliminating different host bacteria was evaluated by analyzing the O. D values at 600 nm, following a 24-hour period of phage infection using different MOI ratios: 10, 1, 0.1, 0.01 and 0.001. Significant ($p \leq 0.0001$) reduction of bacteria, Xvm1-4, BS17-5 and BS8b2, was observed at MOI of 0.1, 1 and 10, over a 24 h period. These MOIs are appropriate for achieving the best treatment outcome. It is worth noting that BS8b2 growth resumed after 12 h at an MO1 of 0.1, possibly indicating growth of resistant strains. Overall these findings suggest that Kintu exhibits potency against susceptible bacterial strains across different MOI ratios. It is important to note that the emergence of resistant strains is a possibility during phage treatment, as similar studies have reported on this phenomenon [23, 63, 64]. Despite this, Kintu remains a promising candidate for biocontrol of BXW.

Kintu possesses a dsDNA genome that spans 48,985 base pairs in length, with a G+C content of 51.71%. These characteristics align with the range for base pair and G+C content of *Xanthomonas* genomes summarized in [18]. The structural and non-structural proteins encoded by the genome are believed to play a crucial role in initiating infection and facilitating the replication process [23, 65]. Lysis-associated proteins include endolysin, spanin and holin, potentially contributing to the lytic life cycle of Kintu. These proteins are also found in the genomes of other *Xanthomonas* phages, indicating their shared importance in infection (23, 57,). Kintu is a new species within the unclassified genus of the class *Caudoviricetes*, as no closely related phages were identified at genomic and proteomic level. The recent shift from morphology-based to genomic-based classification [19] enables the inclusion of new and previously uncharacterized phages.

Conclusion

The study isolated a novel phage from sewage with 3 mm-sized clear plaques exhibiting characteristics of a siphovirus. Kintu specifically targets and lyses Ugandan *Xvm* strains while sparing other bacteria that might have an important role in plant immunity. The narrow host range of Kintu makes it an ideal candidate for the biocontrol of

BXW. Kintu exhibits an optimal MOI of 1 along with a one-step growth curve characterized with a short adsorption, latent period and a small burst size. Kintu is highly versatile, tolerating a wide range of temperatures and pH levels. The recommended storage temperature is -20 °C and −40 °C and is inactivated upon exposure to UV light emitted by a biosafety cabinet lamp. For effective inhibition of *Xvm* growth a MOI 0.1, 1, and 10 is recommended. Kintu is the first *Xvm*-specific phage to have its entire genome sequenced. It possesses a dsDNA genome of 48,985 base pairs with a G+C content of 51.71%. The genome is devoid of genes associated with lysogeny and follows a lytic life cycle as predicted by phage AI. Kintu has limited similarity to known phages in the Genbank, indicating its status as a new phage against *Xvm* within an unclassified genus of the class *Caudoviricetes*. Due to its efficiency to lyse and control *Xvm* growth, Kintu emerges as a promising candidate for combatting Banana Xanthomonas Wilt.

Abbreviations

ICTV	International Committee on Taxonomy of Viruses
pv	Pathovar
mm	Millimeter
Xvm	Xanthomonas vasicola pv. Musacearum
MOI	Multiplicity of Infection
°C	Degrees centigrade
ds	Double stranded
DNA	Deoxyribonucleic acid
GC	Guanine-Cytosine
%	Percentage
bp	Base pairs
BXW	Banana Xathomonas Wilt
NCBI	National Center for Biotechnology Information
UV	Ultraviolet
N	North
E	East
S	South
cm	Centimeters
µL	Microliters
YPGA	CC-Yeast, Peptone, Glucose, Agar, Cephalexin, Cycloheximide
mL	Milliliters
mM	Millimolar
OD	Optical density
CFU/mL	Colony forming units per milliliter
g	Relative centrifugal force
µM	Micromolar
rpm	Rotations per minute
g	Grams
NaCl	Sodium chloride
$MgSO_4$	Magnesium sulfate
Tris	HCL-Tris hydrochloride
L	Liter
ddH_2O	Double distilled water
pH	Potential of Hydrogen
$CaCl_2$	Calcium chloride
PFU/mL	Plaque Forming Units per milliliter
min	Minutes
s	Seconds
hrs	Hours
p	Probability
M	Molarity
NaOH	Sodium hydroxide
HCL	Hydrochloric acid
nm	Nanometer
DNase I	Deoxyribonuclease I
EDTA	Ethylenediaminetetraacetic acid
SDS	Sodium dodecyl sulfate
v	Version
tRNA	Transfer ribonucleic acid
ssDNA	Single stranded deoxyribonucleic acid
TEM	Transmission electron microscopy

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Author contributions
R.N. conceptualized, designed the framework, performed experiments, data analysis, wrote the main manuscript text and proofread the manuscript. I.N. performed experiments and data analysis. J.W. performed genomic experiments and sequence analysis. M.V. performed the TEM analysis. Authors, I.N., J.W., R.K., A.K., J.K. proofread the manuscript. All authors reviewed and approved the final version of the manuscript.

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Data availability
Sequence data that support the findings of this study have been deposited in the NCBI GenBank repository with the primary accession number PP313117.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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