Isolation and Characterization of the Bacteriophage Yucatan Specific to Bacillus Licheniformis

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Abstract

Bacteriophages have been proposed as a biological alternative for reducing bacterial pathogens in diverse fields, including the food industry, healthcare, and agriculture, considered as promising tools to counteract antibiotic resistance. In this study, we present//provide the morphological and genetic characterization of a novel bacteriophage isolated from the digestive system of *Bemisia tabaci* (whitefly) Biotype A, collected from *Capsicum chinense* (habanero pepper). Its bacteriolytic activity, thermostability, pH tolerance, and host range were assessed. Host range analysis revealed that among the bacteria tested, *Bacillus licheniformis* served as the host for this bacteriophage. Transmission electron microscopy images showed virions with a head–tail structure, characterized by a long, contractile tail and an icosahedral head. Characterization results indicated that bacteriolytic activity of the phage begins at the first hour of contact with its host. Among the evaluated parameters, the optimal temperature for maximum activity was 20°C, while the optimal pH was 7.0. Lytic activity was observed exclusively against *Bacillus licheniformis* strains. The newly identified phage possesses a double-stranded DNA genome of 147,395 bp, predicting a total of 266 protein-coding sequences, with a GC content of 38.98%. It lacks host virulence-modifying genes and antibiotic resistance genes and exhibits a strictly virulent life cycle. Based on viral taxonomy criteria, this phage was classified as a novel species within the family *Herelleviridae*, with its closest relative being phage SIOphi. The proposed name for this isolate is *Bacillus phage Yucatan*.

Introduction

Insects represent the animal group with the greatest number of species worldwide. Owing to their wide distribution, they are commonly associated with diverse microorganisms, including bacteria, viruses, fungi, protozoa, nematodes, and multicellular parasites (Mondal et al. 2023). Permanent associations between bacteria and higher organisms are referred to as symbiosis, with the type of symbiosis depending on the host, the symbiont, and the environmental conditions (Arora et al. 2017). Thus, bacterial identification is important to determine roles related to hosts' biology including nutrition, development, defense against pathogens, community interactions, and survival in hostile environments through toxin metabolism (Rupawate et al. 2023).

Bemisia tabaci is one of the world's most important pests, as it directly attacks agricultural products and is the main vector of plant viruses, such as Geminiviruses. *B. tabaci* is a species complex with genetically distinct lineages, host types, and rapid spread. Biotype A is found in the Americas, while biotypes B (MEAM1) and Q (MED) are present worldwide (De Barro et al. 2011). Therefore, biotype A is of paramount importance for studies related to endemic species in the Americas, specifically Mexico and *Capsicum annuum* plants. Several studies have isolated and identified bacterial symbionts from *Bemisia tabaci*. Ateyyat et al. (2023) isolated and identified culturable bacteria reporting *B. licheniformis* in both nymphs and adults. Similarly, Pujar et al. (2024) isolated bacterial colonies from *Bemisia tabaci* where the predominant bacterial species identified were *Bacillus licheniformis*, *Bacillus pumilus*, and *Bacillus*

safensis, among others. The examples suggest that *B. licheniformis* is an important component of the *Bemisia tabaci* microbiome.

Bacillus licheniformis belongs to the family *Bacillaceae*, whose members are characterized by their ability to form endospores, conferring high resistance to heat, radiation, chemicals, and desiccation, and enabling survival under adverse conditions for extended periods (He et al. 2023). *B. licheniformis* is predominantly found in soil and is a rod-shaped, Gram-positive, motile, and facultatively anaerobic species (Logan et al. 2015). It is part of the *Bacillus subtilis* group, which comprises two subspecies (*B. subtilis* subsp. *subtilis* and *B. subtilis* subsp. *spizizenii*) and twelve species (*B. mojavensis*, *B. valismortis*, *B. amyloliquefaciens*, *B. atrophaeus*, *B. pumilus*, *B. licheniformis*, *B. sonorensis*, *B. aquimaris*, *B. oleronius*, *B. sporothermodurans*, *B. carboniphilus*, and *B. endophyticus*) (Mandic-Mulec et al. 2015). Although *B. licheniformis* isolates are mainly derived from soil, their sources of isolation are diverse, including insects, bird feathers, internal plant tissues, animal leather, milk, paper, and cardboard (Logan et al. 2015). In insects, *B. licheniformis* has been isolated from the digestive systems of both nymphs and adults of whitefly species (*Bemisia tabaci*, *B. argentifolii*) (Ateyyat et al. 2023; Davidson et al. 2000; Pujar et al. 2024). Some studies suggest that, due to the characteristics of the insect microbiota, including *B. licheniformis*, host survival against insecticides is enhanced by the production of pesticide-degrading enzymes, the microbiome acting as a type of defense (Fan et al. 2025). Once the symbiotic bacteria of the insect are identified and their importance in biological development is recognized, one strategy that has been employed to disrupt insect development is the elimination of these bacteria through antibiotic treatment, resulting in reduced fertility and survival rates (Bai et al. 2019; Koga et al. 2007). However, the emergence of antibiotic resistance among bacteria poses a significant challenge for their management, highlighting the need for alternative approaches. One such alternative, used for over a century and now being widely reconsidered, is phage therapy (Sawa et al. 2024).

Bacteriophages, or phages, are viruses that specifically infect and replicate within bacterial cells. They are recognized as the most abundant biological entities on Earth, exhibiting diverse sizes, morphologies, and genomic organizations (Kasman et al. 2022). Tailed phages are considered key components in shaping the structure, dynamics, and interactions of microbial communities across different habitats. They also exert a significant impact on human health and the food industry (Nami et al. 2021). Structurally, phages consist of nucleic acid enclosed within a protein shell, the capsid, which protects the genetic material and mediates its delivery into the host cell (Rees et al. 2024). Phages are highly specific, often infecting only a single bacterial species or even particular strains within that species. Although phages represent a promising alternative for biocontrol, it is essential to characterize them both biologically and genetically. Such characterization allows the determination of host range, resistance to environmental stresses, and verification of the absence of undesirable genes in their genomes, particularly those associated with host virulence modification or antibiotic resistance. This knowledge is fundamental for understanding the potential of phages to regulate bacterial populations and for tailoring their application to be both precise and effective. Furthermore, molecular analysis provides a robust foundation for their taxonomic classification (Elois et al. 2023; Jo et al. 2023). In this study, we present the morphological and genomic characterization of a novel bacteriophage related to SIOphi, isolated

from the digestive system of *Bemisia tabaci* (whitefly) Biotype A, collected from habanero. This phage demonstrated *in vitro* effectiveness in controlling cultured *Bacillus licheniformis*.

Materials and Methods

Sampling

Adult specimens of the whitefly *Bemisia tabaci* were collected from habanero pepper (*Capsicum chinense* Jacq.) in greenhouses at the Scientific Research Center of Yucatán (21°01'47" N, 89°38'20" W). The sampled whiteflies corresponded to biotype A, as reported before (Bravo-Pérez et al. 2024). Specimens were collected in small jars with mesh lids containing 70% ethanol and transported to the laboratory for processing. The jars were stored at 4°C and processed on the same day.

Isolation of intestinal bacteria

To ensure that only intestinal bacteria were obtained and to prevent external contamination, insects were first cooled at 4°C for 15 minutes. They were then washed in a Petri dish under a stereoscope within a laminar flow hood using 70% ethanol for 5 minutes, with gentle agitation every minute. Subsequently, the insects were rinsed three times in phosphate-buffered saline (PBS, pH 7.4). From the final rinse, 100 µL of buffer was spread onto trypticase soy agar (TSA; BIOXON®) plates to confirm the absence of residual bacteria. In addition, sanitized whole insects were deposited on TSA plates to verify the absence of bacteria or other microorganisms.

The abdomens of 50 insects were carefully removed and placed in a 1.5 mL microcentrifuge tube containing 200 µL of sterile PBS, then macerated with a sterile pestle for 30 seconds (Apte-Deshpande et al. 2012). From the macerated mixture, 100 µL were taken to prepare dilutions (10^{-1} to 10^{-2}), which were plated on TSA medium by spread plating: 100 µL of the 10^{-1} dilution (two replicates), 100 µL of the 10^{-2} dilution (two replicates), and 100 µL of the initial macerate (one replicate). Plates were incubated at 37°C for 48 h. Colonies were selected based on differential morphology, suspended in 100 µL of sterile distilled water, and individually spread onto TSA plates. After 72 h, colonies were cultured in 5 mL of trypticase soy broth (TSB; BIOXON®) for subsequent DNA extraction. Bacterial growth was monitored by spectrophotometry, until an optical density of 0.8 at 600 nm was reached.

Bacterial DNA Extraction

DNA extraction was carried out using a cetyltrimethylammonium bromide (CTAB)-based protocol (Sambrook and Russell 2001; Tito et al. 2015). DNA concentration and purity were assessed with a Nanodrop 2000c spectrophotometer (Thermo Scientific, USA), while integrity was confirmed by visualization on a 1.5% agarose gel under UV illumination.

PCR amplification, cloning and sequencing

Bacterial DNA was amplified by PCR using a T100™ thermal cycler (Bio-Rad, Germany). Degenerate primers 1494r (5'-TAC GGY TAC CTT GTT AGC ACT T-3') and 27f (5'-AGA GTT TGA TCM TGG CTC AG-3') were used//employed to amplify a ~ 1542 bp fragment corresponding to the near complete 16S rRNA gene region. PCR reactions were performed in a final volume of 25 µL, containing 17.7 µL of water, 1 µL of $MgCl_2$ (50 mM), 0.5 µL of dNTPs (10 mM), 0.6 µL of each primer (20 pmol), 0.1 µL of GoTaq® DNA Polymerase (Promega), and 2 µL of DNA template. Cycling conditions were as follows: initial denaturation at 94°C for 6 min (1 cycle); 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 45 s, and extension at 72°C for 1 min, followed by a final extension at 72°C for 4 min. Amplified products were analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide and visualized under UV illumination. PCR products (25 µL) were subsequently purified using the Wizard® SV Gel and PCR Clean-Up System kit according to the manufacturer's instructions.

The PCR products were ligated into the pGEM-T Easy cloning vector (Promega®) according to the manufacturer's instructions. The ligation products were subsequently used to transform competent *Escherichia coli* TOP10 cells. Five clones from each candidate strain were selected and submitted to Macrogen (South Korea) for sequencing.

Sample enrichment

Bacteria isolated from the intestinal microbiota of whiteflies were individually cultured on TSA plates. A single colony from each isolate was subsequently inoculated into TSB and incubated for 24 h at 37°C. After incubation, each isolate was processed, and 1 mL of each isolate was combined in 50 mL Corning tubes to create a bacterial pool consisting of *Bacillus licheniformis*, *Streptomyces nigra*, *Solibacillus silvestris*, *Solibacillus isronensis*, and *Paenibacillus lautus*. Each isolate was inoculated with 5 mL of insect macerate and incubated under aerobic conditions at 37°C for 18–24 h. After incubation, the tubes were vortexed and centrifuged at 13,800 rpm for 10 min at 4°C. The supernatant was collected and filtered through nitrocellulose membranes with a pore size of 0.22 µm; this filtrate was designated as the "lysate."

Identification of bacteriophages by the "spot" test

To detect the presence of lytic bacteriophages against each bacterial isolate, a spot test was performed using the double agar layer technique with the lysates obtained from each sample. Briefly, 1 mL of bacteria in the exponential growth phase was mixed with 3 mL of 0.8% (w/v) TSB-agarose, preheated and liquefied at 45°C, and the mixture was poured onto TSA plates. After drying, 5 µL of each lysate was applied to the soft agar layer. Once the plates had dried, they were inverted and incubated at 37°C for 18–24 h. This procedure was repeated for each bacterial isolate and corresponding lysate in which bacteriophage presence was suspected. The appearance of bacterial cell clearance zones in lysis

plaques at the site of inoculation indicated the presence of lytic bacteriophages against the tested bacteria (Clokie and Kropinski 2009).

Purification of bacteriophage

For bacteriophage propagation, the double agar layer technique was used. The strain *Bacillus licheniformis*, isolated from the abdomen of whiteflies, produced lysis plaques and was therefore used as the host bacterium for bacteriophage isolation. This strain was cultured in TSB at 37°C for 24 h and evaluations were conducted using *B. licheniformis* in the logarithmic growth phase (5 h). Subsequently, 1 mL of bacterial culture and 100 µL of lysate containing bacteriophages were mixed with 3 mL of 0.8% (w/v) TSB-agarose, preheated and liquefied at 45°C. The mixture was homogenized, poured onto TSA plates, allowed to dry, and incubated at 37°C for 18–24 h. After incubation, plaques were selected based on size and clarity and transferred to 1.5 mL Eppendorf tubes containing 1 mL of 1.5% meat extract (BIOXON®). The double agar layer and single-plaque recovery procedure was repeated four times to obtain unique and purified phages (Clokie et al. 2009).

Propagation of bacteriophages

For bacteriophage propagation, the methodology described by Carey-Smith et al. (Carey-Smith et al. 2006) was followed with modifications. Briefly, 1 mL of the host strain in the exponential growth phase was mixed with 100 µL of purified bacteriophage in a test tube containing 3 mL of 0.8% (w/v) TSB-agarose liquefied at 45°C. The mixture was poured onto TSA plates and allowed to dry. Plates were incubated at 37°C for 18–24 h. Once bacteriophage replication was evident, the soft agar layer was recovered using a sterile cell scraper and transferred to 1.5 mL tubes containing meat extract. The final eluate was centrifuged at 8,500 rpm for 10 min at 4°C to remove bacterial cells and culture residues. The supernatant was then filtered through nitrocellulose membranes with a pore size of 0.45 µm and stored at 4°C, protected from light.

Concentration and titration of bacteriophage

For phage concentration and titration, 40 mL of bacteriophage propagated in the previous step was centrifuged at 40,000 rpm for 2 h. The supernatant was discarded, and the pellet was resuspended in 10 mL of meat extract buffer, followed by filtration through a 0.22 µm AcrodiscR syringe filter. Phage titers were determined using a standard plaque assay method (Clokie and Kropinski, 2009). Briefly, serial dilutions (10^{-1} to 10^{-10}) of the concentrated bacteriophage were prepared in 1.5% meat extract buffer, and the double agar layer technique was performed. Dilutions yielding between 30 and 300 countable plaques were selected, and the phage concentration was calculated accordingly.

Transmission Electron Microscope

Phage morphology was examined by transmission electron microscopy (TEM) using a Hitachi H7500 instrument (Hitachi Ltd., Tokyo, Japan) operated at 100 keV to visualize uranile acetate stained preparations (Luo et al. 2012). Purified phage particles were suspended in 2% (w/v) phosphotungstic acid (pH 7.2) and applied onto the surface of a carbon-coated, glow-discharged copper grid (200 mesh).

Host range test

The host range was determined using the spot test (Clokie and Kropinski, 2009). For this evaluation, five bacterial species isolated from the digestive system of the whitefly were tested.

Bacteriolytic activity

The bacteriophage infection kinetics assay was performed following the methodology described by Haq et al. (2012). Briefly, 1 mL of a *Bacillus licheniformis* culture was inoculated into flasks containing 50 mL of TSB broth, to which 1 mL of bacteriophage suspension in PBS (titer: 7×10^8 pfu/mL) was added. Cultures were incubated at 25°C with continuous shaking. A control group without bacteriophage inoculation was included. Optical density at 600 nm (OD_{600}) was measured at hourly intervals for 9 h. All assays were performed in triplicate.

Thermal and pH stability

Thermal stability tests were performed to evaluate the heat resistance of the phage at pH 7, with room temperature (~23°C) serving as the control. Phage stability was assessed using a hot plate with a beaker of water and a thermometer. Briefly, phage stocks (1×10^8 pfu/mL in 1.5% meat extract buffer) were incubated separately for 60 min at temperatures ranging from 20 to 60°C, with intervals of 10ºC between each point. For pH stability, phage suspensions (1×10^8 pfu/mL) were added to meat extract buffer adjusted to different pH values (range 3–10, with intervals of 1 pH unit between each point) and incubated at 37°C for 24 h. Following incubation, surviving phages were quantified using the double agar layer method. All experiments were conducted in triplicate.

Phage DNA extraction

DNA extraction from bacteriophages was performed using the methodology described by Sambrook and Russell (2001), with modifications. Briefly, 1 mL of (titer: 8×10^8 pfu/mL) phage suspension was placed in a 1.5 mL microcentrifuge tube, to which 2 U of DNase I (Sigma-Aldrich, USA) and 2 U of RNase A (Sigma-Aldrich, USA) were added. The mixture was incubated at 37°C for 30 min. Following incubation, 40 µL of EDTA (0.5 M, pH 8.0; Sigma, USA), 25 µL of proteinase K (20 mg/mL; Qiagen, Germany), and 50 µL of sodium dodecyl sulfate (SDS; 10%; Sigma, USA) were added, and the tubes were mixed by inversion 5–10 times. Samples were then incubated at 56°C for 2 h. After incubation, an equal volume of phenol (Sigma-Aldrich, USA) was added and mixed by inversion until fully emulsified, followed by centrifugation at 3,500 rpm for 10 min at 25°C. The aqueous phase was transferred to a fresh 1.5 mL microcentrifuge tube, and an equal volume of phenol-chloroform (1:1, v/v; Sigma-Aldrich, USA) was added. Centrifugation was repeated under the same conditions three times. The final aqueous phase was transferred to a new 1.5 mL tube, and 200 µL of 3 M sodium acetate, together with absolute ethanol were added until the tube

was full. Samples were incubated at -20°C overnight. The resulting pellet was centrifuged at 13,000 rpm for 30 min, the supernatant discarded, and the pellet washed with 70% ethanol. A second centrifugation was performed at 13,000 rpm for 15 min. The supernatant was removed, and the pellet was air-dried at room temperature before resuspension in 100 µL of nuclease-free water. DNA concentration and purity were assessed.

Genome sequencing and analysis

DNA sequencing was conducted at the Centro de Investigación en Alimentación y Desarrollo (CIAD), Mazatlán Unit, using the MiniSeq sequencing system (Illumina, Inc.) with a 2 × 150 bp paired-end protocol (300 cycles). Libraries were prepared using Nextera adapters, barcoded, and pooled with additional samples, and sequenced on a MiniSeq Mid Output Kit (300 cycles). Raw FASTQ reads were quality-trimmed using fastp v0.22.0 with the default settings (Chen et al., 2018), and *de novo* assembly was performed with SPAdes v3.15.5 (Bankevich et al. 2012). A minimum coverage cutoff of 10x was applied for contig retention, resulting in a single contig with an average coverage of 193.7x.

Genome annotation was conducted using Pharokka v1.7.3 (Bouras et al. 2023) to identify structural and functional features, which was also employed for genome annotation and for the identification of virulence genes in the VFDB database and antibiotic resistance genes in the CARD database. Manual curation (BLASTp and domain-based validation) was then performed to confirm and refine Pharokka functional assignments; conflicts were resolved by prioritizing domain-supported hits and phage hallmark gene context, while low-confidence proteins were annotated as hypothetical. Bacphlip v0.9.6 (Hockenberry and Wilke 2021) was used to predict the lifestyle (virulent or temperate) of the isolated phage, while Taxmyphage v0.3.3 (Millard et al. 2024) was applied to determine phage taxonomy. A heat map was generated with VIRIDIC v1.0 (Turner et al. 2021) to refine the phage classification obtained from the phylogenetic tree. VIRIDIC was used to calculate the intergenomic similarity matrix with the most closely related phages reported in GenBank belonging to *Siophivirus* genus. In addition, phage sequences from the closest nine genus of *Bastillevirinae* subfamily were used to determine their taxonomic placement.

Results

Bacterial identification

A total of nine bacterial colonies were isolated based on their color, shape, and size. Five colonies exhibited irregular circular morphology, with colors ranging from whitish to creamy gray, a convex surface, and a dry appearance after several days. Colonies labeled MB9 and MB10 were translucent and transparent, respectively, displaying irregular growth patterns. *Streptomyces nigra* formed colonies with a grayish-white, powdery appearance that hardened over time and developed a reddish-brown pigmentation around the colony. The remaining isolates included *Solibacillus silvestris* and *Solibacillus isronensis*, which produced dark beige circular colonies with convex surfaces and rounded edges, as well as *Paenibacillus lautus*, which formed light yellow circular colonies with convex morphology.

Based on sequence analysis, five colonies were identified as *Bacillus licheniformis*, while the remaining isolates corresponded to *Streptomyces nigra*, *Solibacillus silvestris*, *Solibacillus isronensis*, and *Paenibacillus lautus* (Table 1). All isolates shared > 96% nucleotide identity with previously reported sequences.

Table 1
Identification of the bacteria isolated from the digestive system of *Bemisia tabaci*

Sample	Organism	Percentage of nucleotide identity	Lysis plaques	GenBank Accession No.
MB2	*Bacillus licheniformis*	98.84	+	PV399969
MB3	*Streptomyces nigra*	98.30	-	PV399970
MB4	*Bacillus licheniformis*	99.32	+	PV399971
MB7	*Bacillus licheniformis*	96.08	+	PV399972
MB8	*Solibacillus silvestris*	97.70	-	PV399973
MB9	*Bacillus licheniformis*	99.64	+	PV399974
MB10	*Bacillus licheniformis*	99.46	+	PV399975
MB11	*Solibacillus isronensis*	99.41	-	PV399976
MB12	*Paenibacillus lautus*	97.48	-	PV399977

Phage identification

Among the bacterial isolates obtained from the digestive system of whiteflies (*B. licheniformis*, *S. nigra*, *S. silvestris*, *S. isronensis*, and *P. lautus*), only *B. licheniformis* tested positive for the presence of lysis plaques in the spot test (Table 1). Three lysates were evaluated: the first derived from macerated whitefly insects, the second from sooty mold on host leaves, and the third from macerated whitefly egg masses. Bacteriophages were detected exclusively in the first lysate (Fig. 1a). Transmission electron microscopy revealed that the phage virions possess an icosahedral head (Fig. 1b).

Host range

Host range analysis was performed on *B. licheniformis*, *S. nigra*, *S. silvestris*, *S. isronensis*, and *P. lautus* (Table 1). The phage produced visible plaques on all five *B. licheniformis* strains (MB2, MB4, MB7, MB9, and MB10), indicating that these strains were susceptible to infection. The plaques were round, translucent, and exhibited well-defined boundaries on double-layer agar plates. In contrast, no lysis plaques were observed on the other bacterial species isolated from the digestive system of whiteflies.

Bacteriolytic activity

The growth kinetics of *Bacillus licheniformis* revealed a lag phase during the first hour, followed by an exponential (logarithmic) phase that extended until the fifth hour. The stationary phase was maintained throughout the 9-hour evaluation period. In contrast, when assessing the bacteriolytic activity of the isolated phage (7×10^8 PFU/mL) over the same period, the lag phase of *B. licheniformis* was prolonged until the fourth hour. Notably, a stationary phase was not observed; instead, bacterial growth began to decline at the eighth hour, immediately following the exponential phase (Fig. 2).

Thermal and pH stability

The results demonstrated that the Yucatan phage retained 98.38% activity relative to the control at 20°C for 60 min, but progressively decreased with increasing temperature, reaching approximately 50% at 60°C (Fig. 3A). The phage remained stable when stored at 4°C and for up to three months at -20°C and −80°C (data not shown). Maximum activity was observed at pH 7; however, phage viability declined under more acidic or alkaline conditions. At pH 3, no activity was detected, while at pH 4, activity was reduced to 25%, and at pH 10, activity decreased to 20% (Fig. 3B).

Genomic characterization

A 147,395 bp phage genome was obtained through sequencing (GenBank accession number PV261948). BLAST analysis revealed that the phage with the highest coverage (97%) and nucleotide identity (96.57%) was phage SIOphi. The highest average nucleotide indentity value was observed between our phage and SIOphi (94.6%), followed by phages KKP_4047 and KKP_4048 (92.5%), KKP_4050 (92%), and KKP_4049 (91.8%) (Fig. 4). These results and taxmyphage program classified *Bacillus* phage vB_Blim_Yucatan as a new species within the genus *Siophivirus*, subfamily *Bastillevirinae*, family *Herelleviridae* from class *Caudoviricetes*. Members of this family are bacterial viruses that infect hosts belonging to the *Firmicutes* phylum (Barylski et al. 2020).

Genomic analysis revealed that the Yucatan phage genome contains no virulence or antibiotic resistance genes. The Bacphlip v0.9.6 program predicted a lytic lifestyle with 95% confidence. Analysis with Pharokka identified 266 protein-coding sequences (CDS), of which 188 encode proteins with unknown or hypothetical functions. Based on predicted functions, the remaining 69 CDS encoding known proteins were classified into functional groups: transcription regulation (4 CDS), nucleotide and DNA/RNA metabolism (24 CDS), lysis (2 CDS), moron/auxiliary metabolic genes and host takeover (7 CDS), integration and excision (1 CDS), head and packaging (17 CDS), and others (9 CDS) (Fig. 5).

DISCUSSION

Members of the family *Herelleviridae* are bacterial viruses that infect hosts within the phylum *Firmicutes*. Their genomes consist of linear dsDNA of approximately 125 to 170 kb, and some members posses terminally redundant ends or terminal repeats; additionally, certain members encode tRNAs. Virions display a head-and-tail morphology characterized by an icosahedral head and a long, contractile tail.

Notably, the genome of *Bacillus* phage SPO1 contains thymidine replaced by 5-hydroxymethyluridine, and DNA modifications have also been reported in other members of the family (Barylski et al., 2020).

The Yucatan phage, virulent against *Bacillus licheniformis* and isolated in the present study, represents a new species within the *Herelleviridae* family. Its genome is 147,395 bp in length, predicting a total of 266 protein-coding sequences, and has a GC content of 38.98%. Comparative analysis revealed 94.6% homology with its closest relative, phage SIOphi, which according to ICTV is currently the only recognized species of the genus *Siophivirus*. Phage SIOphi possesses a genome of 146,698 bp, encodes 206 predicted unique protein-coding sequences, lacks tRNAs and terminal repeats, and infects *Bacillus subtilis* isolated from soil samples (Krasowska et al. 2015).

In addition, SIOphi was reported alongside three other phages infecting *B. subtilis*, with genome sizes ranging from 153,882 to 156,577 bp, encoding between 256 and 270 protein-coding genes, no tRNA genes, and GC contents of 38.6–38.7%, lower than that of their host *B. subtilis* (~ 43%) (Magness et al. 2023). By contrast, *B. licheniformis* has a GC content of ~ 46.2% (Rey et al. 2004), which is also higher than that of the Yucatan phage.

The *Herelleviridae* family comprises virions with isometric icosahedral heads measuring 85–100 nm in diameter. The heads display capsomeres, with capsid subunits arranged in pentons and hexons. Uncontracted tails range from 130 to 185 nm in length, featuring a base plate of approximately 60 nm and a small collar [40]. Electron microscopy revealed that the Yucatan phage is morphologically similar to phages SIOphi and fHSpt3 (Krasowska et al. 2015; Midha et al. 2024).

According to the morphological characteristics of this phage type and the latest classification by the International Committee on Taxonomy of Viruses (ICTV), the Yucatan phage exhibits the morphology typical of the class *Caudoviricetes*, which includes tailed phages. Within this class, the families with the highest number of sequences reported in GenBank are *Autographiviridae*, *Herelleviridae*, and *Demerecviridae*. Phages with this morphology are characterized by linear double-stranded DNA genomes (Zhu et al. 2022).

Although bacteriophage classification has traditionally relied on morphological criteria and only rarely on molecular data (Ackermann 2009), *Bacillus* phage Yucatan is described here both morphologically and molecularly, making it one of the few phages of the genus *Siophivirus* to be characterized in both ways. Temperature plays a crucial role in bacteriophage survival, attachment efficiency, and latency period duration (Olson et al. 2004). Phage Yucatan remained active at the highest temperature tested (60°C) for 60 min. These results are consistent with those reported by Melo, who observed that phage SIOphi retained approximately 43% activity at 60°C after 180 min, but lost activity at 70°C within 30 min and at 80°C within 2 min. In general, members of the former family *Myoviridae* (now *Herelleviridae*) (Barylski et al. 2020) and *Siphoviridae* are considered resistant to elevated temperatures (Jończyk et al. 2011).

The acidity and alkalinity of the environment are important factors influencing phage stability (Melo et al. 2019). The Yucatan phage exhibited its highest activity at pH 7. These results are consistent with those

reported by Krasowska et al. (2015), who found that phage SIOphi displayed maximum activity between pH 6 and 8, with no activity at pH 3 or 10. In contrast, the Yucatan phage retained 20% activity at pH 10.

The application of bacteriophages in biotechnological processes requires detailed knowledge of their biological characteristics, including host range, latency period, growth dynamics, and resistance to stress conditions such as temperature and pH. The evaluated properties of the Yucatan phage presented in this study demonstrate its potential to suppress *Bacillus licheniformis* populations.

Genomic analysis revealed two proteins involved in bacterial lysis: holin and endolysin, the holin-endolysin system is employed by most double-stranded DNA and tailed phages infecting Gram-positive hosts, facilitating the release of progeny virions during the final stages of the lytic cycle (Wu et al. 2021), and these proteins are also present in phage SIOphi. Holins are small membrane proteins that oligomerize to form pores in the cytoplasmic membrane, thereby enabling the release of endolysins into the periplasm (Wang et al. 2000; Young 2013). Endolysins are peptidoglycan hydrolases that degrade the bacterial cell wall, with lysis occurring due to osmotic pressure differences between the cell and its environment. During the lysis process, endolysins are synthesized and accumulate in the cytoplasm at the late stage of phage infection, when proteins cannot cross the cytoplasmic membrane (Schmelcher et al. 2012).

Although ICTV currently recognizes phage SIOphi as the sole species of the genus *Siophivirus*, Midha et al. 2024 described *Bacillus* phage Fhstp3 as a member of this genus. Phage Fhstp3 was isolated from wastewater, infects *B. subtilis*, and possesses a genome of 150,187 bp with 221 protein-coding sequences. It lacks virulence and antibiotic resistance genes and exhibits a virulent life cycle, with 78.97% confidence according to Phage AI (Tynecki et al. 2020).

For a phage to be considered suitable for phage therapy, host range is a critical characteristic. Ideally, a therapeutic phage should be specific to a single bacterial species to avoid off-target effects, yet broad enough within that species to infect multiple strains (Hyman 2019). The phage characterized in this study was evaluated against five independent *Bacillus licheniformis* isolated from the abdomen homogenate of the whitefly, and it exhibited lytic activity against all of them. Genomic analysis confirmed that the phage does not harbor antibiotic resistance genes, virulence factors, CRISPR elements, or tRNAs, all of which support its suitability as a potential biocontrol agent targeting *B. licheniformis* in the whitefly gut.

The Yucatan phage showed reduced activity under pH conditions outside the neutral range; however, it can still be considered a promising candidate for suppressing *B. licheniformis* populations in the digestive system of *Bemisia tabaci*. The pH of most insect digestive systems typically ranges from 6 to 8, although exceptions exist, for example, blue flies (*Calliphora*) exhibit highly acidic gut conditions, while lepidopterans maintain alkaline environments between pH 8 and 10 (Engel et al. 2013). Previous studies using oral antibiotic treatments to eliminate gut symbionts have reported suppression or elimination of these bacteria, resulting in higher mortality rates, slower growth, and reduced body size (Bai et al. 2019; Koga et al. 2007; Rupawate et al. 2023).

The use of bacteriophages to suppress bacterial populations within insect microbiomes as an alternative to antibiotics has been scarcely studied. Xu et al. (2016) evaluated phage BiBurk16MC_R against *Burkholderia* but obtained limited results, probably due to the blockage of the connection between the anterior and posterior abdominal regions, it suggests that the initial colonization by the symbiont programs the ontogeny of the midgut, providing a protected residence against microbial antagonists. Therefore, further evaluation of the Yucatan phage across different developmental instars of *Bemisia tabaci*, using both contact and ingestion methods, is necessary to determine its effectiveness as a biocontrol agent.

Declarations

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Data availability All raw data are available from the corresponding author upon request.

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Author Contribution

DBP, CCHQ and OAMV conceived the project, DBP performed the majority of the experiments, JPGG contributed to the bioinformatic analysis, YMG contributed to molecular whiteflies identification, ECL contributed to electron microscopy experiments, JPGG, LRP and CHZ contributed to the analysis of the data, DBP, OAMV and CHZ wrote the manuscript, and all authors contributed to the final version. All authors gave their final approval of the version to be published and pledge accountability for the quality and reliability of the work.

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Figures

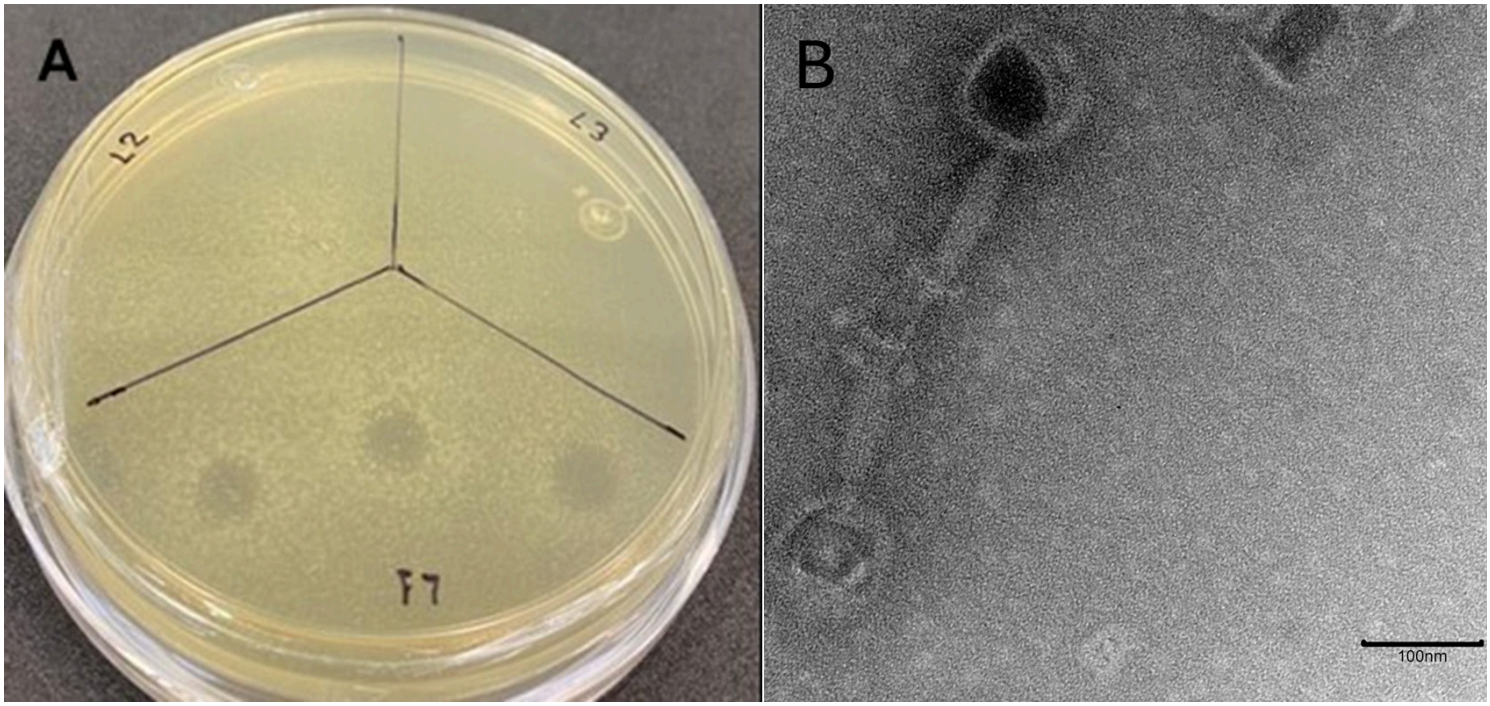


Figure 1

Phenotypic characterization of Yucatan phage. a) Lysis plaques from the spot test, b) Transmission Electron micrograph.

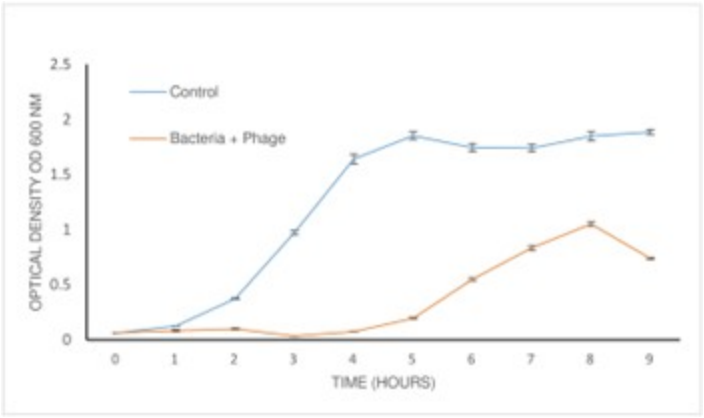
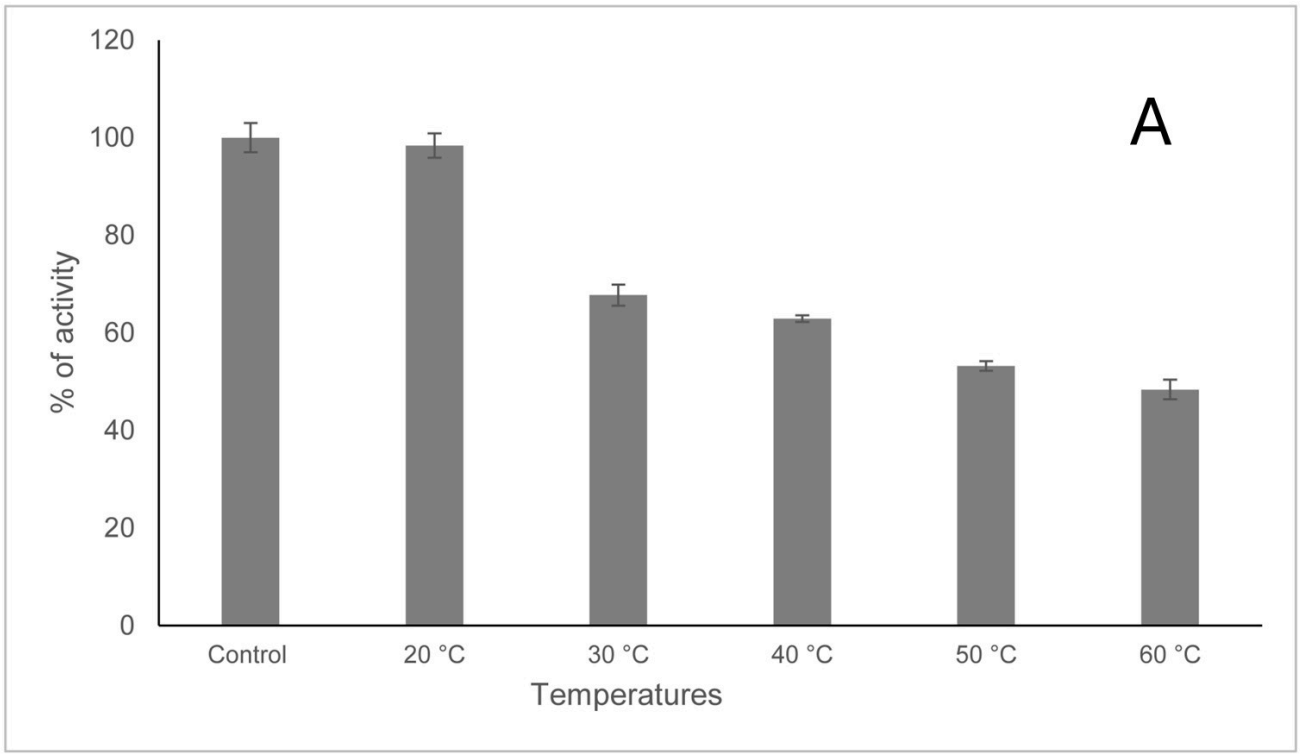


Figure 2

Growth kinetics of the Yucatan phage on the *Bacillus licheniformis* strain.



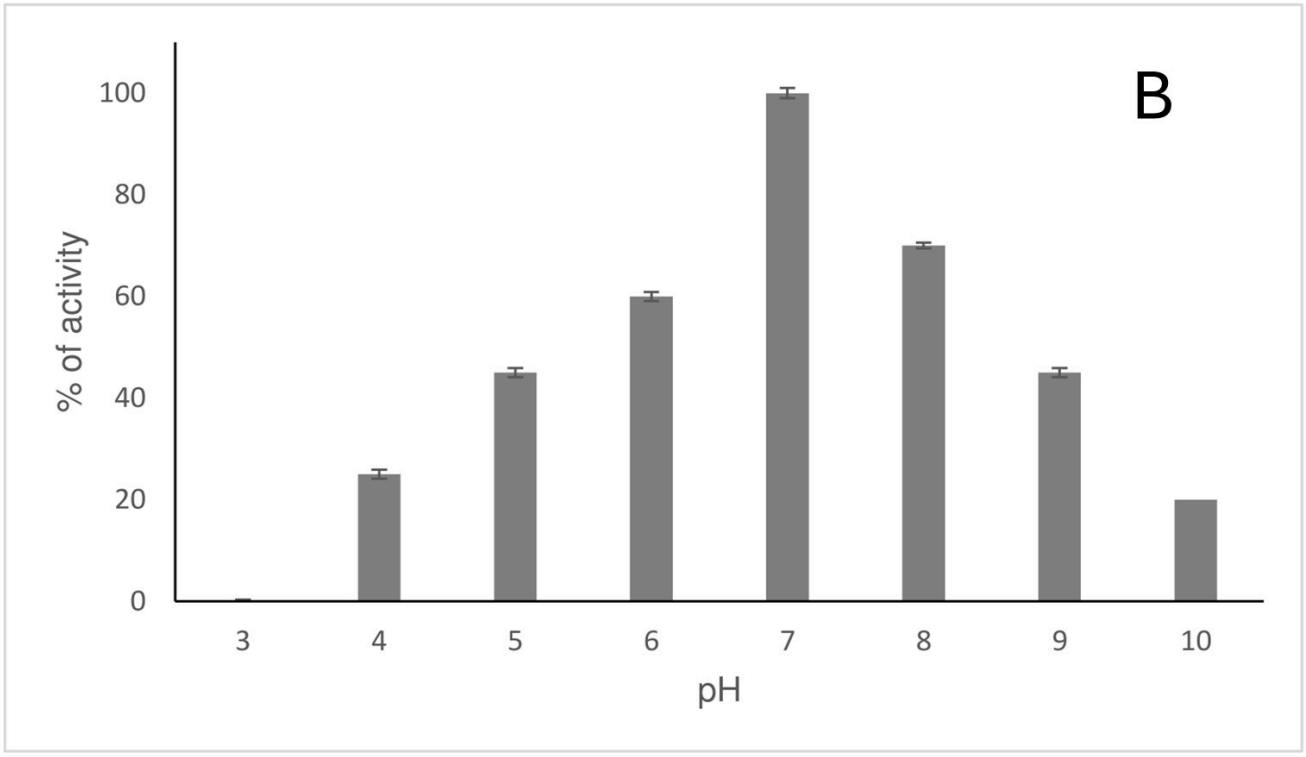


Figure 3

A. Stability of the Yucatan phage at different temperatures. B.Viability of Yucatan phage at different pH.

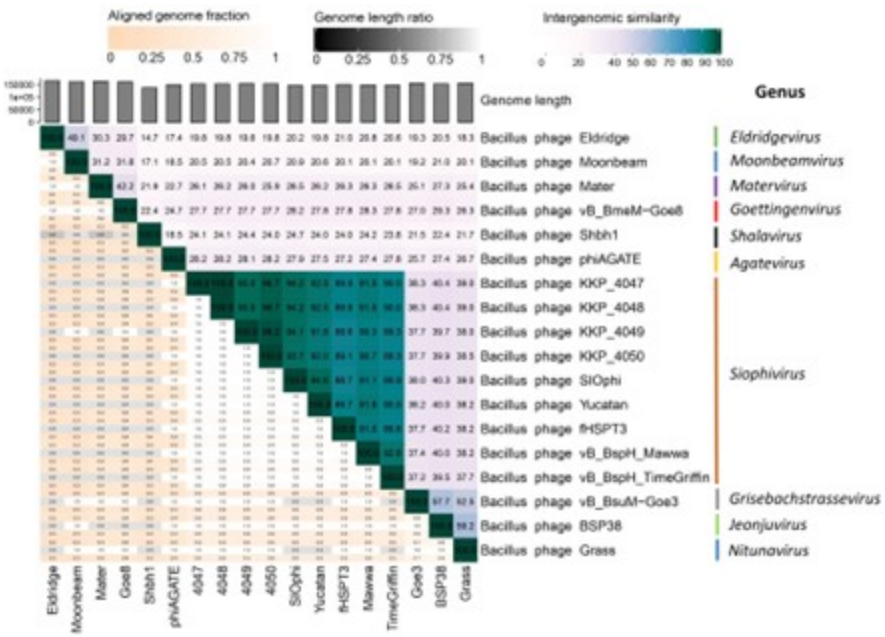


Figure 4

Heatmap of genomic sequence similarity (right) and alignment metrics (left) generated by VIRIDIC between *Bacillus* phage Yucatan, the phages with the highest similarity, and phages other genera.

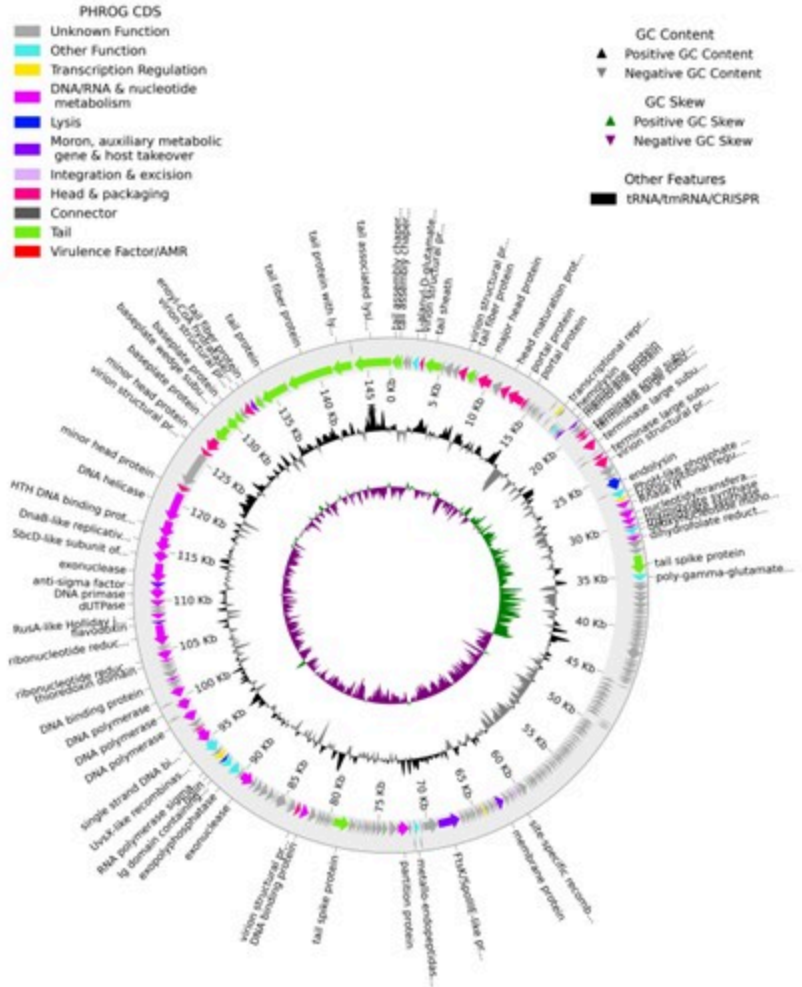


Figure 5

Genome map of the Yucatan phage. The phage genome was represented in a circular shape. Different colors represent different gene functions.