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Isolation and Characterization of *Infidel*, a Novel Bacteriophage Infecting *Bacillus thuringiensis kurstaki* (Btk)

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ABSTRACT

Objective: This study aimed to isolate and characterize a novel bacteriophage, designated *Infidel*, that infects *Bacillus thuringiensis kurstaki* (Btk), to enhance understanding of phage diversity, morphology, and host–phage interactions within the *Bacillus cereus* group.
Methods: Soil samples collected from the University of Maryland, Baltimore County, were enriched with Btk cultures for phage isolation. Purified plaques were analyzed for morphology and lytic activity. Transmission electron microscopy (TEM) was used to determine virion dimensions and structure. Phage DNA was extracted for restriction digestion using seven enzymes and analyzed by agarose gel electrophoresis. Primer extension assays were conducted to assess genomic distinctiveness. Host range was tested across multiple *Bacillus* species to determine infectivity specificity.
Results: Infidel produced circular, slightly turbid plaques with clear centers averaging 3 mm in diameter. TEM confirmed its classification as a *Myoviridae* phage, featuring an icosahedral head (98 × 89 nm) and a contractile tail (250 × 15 nm). DNA was resistant to 4/7 restriction enzymes (EcoRI, NcoI, SacI, SalI), with EOP values ranging 0.18-1.00 across tested hosts. Only one primer set (3B) yielded two amplicons, indicating partial homology with conserved *Bacillus* phage genes. Infidel infected Btk and select *Bacillus* strains, demonstrating a narrow host range.
Conclusion: Infidel represents a novel *Myoviridae* phage with distinct morphological and molecular features, expanding the current understanding of *Bacillus thuringiensis* phage diversity. Its restricted host range and unique DNA modification patterns suggest ecological specialization and potential relevance to the persistence and performance of Btk in agricultural environments.
Keywords: DNA methylation; Electron microscopy; Soil microbiology; Viral host range; Genetic variation

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Introduction

Bacillus thuringiensis (Bt) is a Gram-positive, spore-forming bacterium renowned for its ecological versatility and agricultural importance. As a naturally occurring soil microbe, Bt plays a pivotal role in sustainable pest control, producing parasporal crystalline proteins (Cry and Cyt toxins) that are toxic to a range of insect larvae but safe for humans, animals, and non-target organisms. [1] Among its subspecies, *Bacillus thuringiensis kurstaki* (Btk) is particularly significant due to its highly effective insecticidal activity against lepidopteran pests. [2] Btk-based formulations are globally deployed as microbial pesticides and serve as a foundation for transgenic crops expressing Bt

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toxin genes. These applications have cemented Btk's reputation as an environmentally friendly biocontrol agent and an alternative to synthetic chemical pesticides. [3]

However, the persistence, efficacy, and ecological impact of Btk populations in agricultural environments are influenced by complex microbial interactions, including those involving bacteriophages. Bacteriophages, viruses that infect and lyse bacteria, are the most abundant biological entities on Earth, estimated at over 10^{31} particles globally. [4] They play crucial roles in regulating bacterial community structure, mediating horizontal gene transfer, and influencing microbial evolution and nutrient cycling. [5] Beyond their ecological relevance, phages are increasingly recognized as biotechnological tools, with applications ranging from antibacterial therapeutics and diagnostics to biocontrol agents in agriculture and food safety. [6]

Phages infecting *Bacillus* species have been studied for decades, yet their diversity, ecology, and genomic features remain incompletely characterized. Members of the *Bacillus cereus* group, encompassing *B. cereus*, *B. anthracis*, and *B. thuringiensis,* serve as hosts to numerous phages, many of which belong to the Myoviridae and Siphoviridae families. [7] Recent genomic analyses have revealed considerable diversity among *Bacillus* phages, showing distinct morphotypes, tail structures, and genetic modules associated with DNA packaging, lysis, and replication. [8] Such studies underscore the importance of phages as both evolutionary drivers and potential modulators of bacterial physiology.

Despite extensive use of Btk in agriculture, relatively few studies have focused on phages that infect this subspecies specifically. Most existing reports describe phages of *B. cereus* or *B. anthracis*, leaving a gap in understanding of Btk-specific phage diversity, host range, and ecological functions. [9] Furthermore, limited data are available on how phage infection might influence the persistence and insecticidal activity of Btk in field environments. Given the ubiquity of Btk spores and phages in soil, their interactions may have direct consequences for agricultural biocontrol efficacy and microbial population dynamics.

Recent advances in phage research, such as the resurgence of phage therapy, genomic surveillance, and high-resolution structural biology, have reignited interest in exploring environmental phages. [10] However, soil-derived phages remain underrepresented in global phage databases due to challenges in isolation, culture dependence, and environmental variability. Consequently, isolating and characterizing novel phages from natural ecosystems remains a critical step toward expanding our understanding of phage diversity and ecological significance.

Investigating phages that infect *Bacillus thuringiensis kurstaki* is vital for both environmental microbiology and agricultural sustainability. Btk is a cornerstone of biocontrol strategies, but the emergence of phages capable of lysing Btk could diminish its insecticidal efficiency and long-term field stability. Conversely, these phages could be leveraged as natural regulators to manage excessive Btk proliferation in soil ecosystems, preventing ecological imbalances. Understanding their morphology, genetic composition, and infection dynamics thus holds dual significance, both in safeguarding bioinsecticide efficacy and in exploring phages as tools for targeted microbial regulation.

The isolation of the novel phage "Infidel" from soil presents an opportunity to expand the current knowledge of Myoviridae phages infecting *Bacillus* species. Preliminary observations of Infidel revealed a distinctive contractile sheath instability under stress conditions, an uncommon and potentially informative morphological feature not previously documented in natural isolates. Characterizing Infidel's morphology, molecular properties, and host range could provide valuable insight into phage-host interactions and contribute to understanding how environmental phages influence *Bacillus* population dynamics.

The objective of this study was to isolate and characterize a novel bacteriophage, designated Infidel, that infects *Bacillus thuringiensis kurstaki* (Btk), and to analyze its morphology, host range, and molecular features to better understand phage diversity and its ecological impact on Btk populations. By combining morphological, molecular, and host-range analyses, this work seeks to address a key knowledge gap in the characterization of Btk-infecting phages.

Materials and Methods

Experimental Design and Overview

This study was designed to isolate and characterize a novel bacteriophage, designated *Infidel*, that infects *Bacillus thuringiensis kurstaki*

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(Btk). The workflow consisted of five key experimental stages: (i) collection of soil samples and phage enrichment, (ii) isolation and purification of individual phages, (iii) morphological characterization by transmission electron microscopy (TEM), (iv) molecular analysis including DNA extraction, restriction digestion, and PCR amplification, and (v) determination of host range across multiple *Bacillus* strains.

Trypticase Soy Broth (TSB) and Trypticase Soy Agar (TSA) were used throughout, as they provide nutrient-rich conditions conducive to the growth of *Bacillus* species and facilitate robust phage replication. Restriction enzymes were chosen to represent a range of recognition sites for assessing genomic diversity and to screen for the presence of methylation-sensitive restriction patterns commonly seen in *Bacillus* phages.

Ethical Approval and Biosafety Compliance

This study used environmental soil samples and standard laboratory bacterial strains, requiring no institutional ethics approval. All experiments were conducted under University of Maryland Baltimore County Biosafety Level 1 protocols. *Bacillus anthracis* ΔSterne is an attenuated, non-virulent strain approved for BSL-1 research.

Phage Collection and Isolation

Soil samples were collected on March 15, 2024, from the University of Maryland, Baltimore County (UMBC) campus (coordinates: 39°15′22.8″N, 76°42′41.6″W). The sampling site consisted of cool, dry, loamy soil collected from a depth of approximately 10 cm. Soil temperature was 14°C, and ambient humidity was 52% at the time of sampling.

Approximately 1.0 g of soil was suspended in 12 mL of TSB containing 0.5 µL of actively growing Btk culture (strain HD1, $OD_{600} = 0.6$). The enrichment was incubated overnight at 30°C with shaking at 200 rpm to promote phage replication. Cultures were centrifuged at 12,000 × g for 10 min, and the supernatant was passed through a 0.22 µm syringe filter (Millipore) to remove bacterial debris.

Filtered lysates were serially diluted (10^{-1} to 10^{-5}) in SM buffer (50 mM Tris-HCl, 100 mM NaCl, 10 mM $MgSO_4$, 0.01% gelatin, pH 7.5). Spot assays were performed by mixing 100 µL of Btk overnight culture with 3 mL of 0.6% soft agar, overlaying on TSA base plates (1.5% agar), and applying 10 µL spots of each dilution. SM buffer served as a negative control, and a previously isolated *Bacillus* phage was used as a positive control. Plates were incubated at 30°C for 24 h.

Plaques were enumerated, and their morphology, including diameter (mm), clarity, and halo formation, was recorded using ImageJ software (v1.53t, NIH, USA) with calibrated scale measurements. Phage titer was calculated using the following equation:

$$\text{Titer (pfu/mL)} = \frac{\text{Number of plaques}}{\text{Volume plated (mL)}} \times \text{Dilution factor}$$

All experiments were conducted in triplicate biological replicates to ensure reproducibility.

Bacterial Strains and Culture Conditions

The following bacterial strains were used: *Bacillus thuringiensis kurstaki* HD1 (laboratory collection), *B. thuringiensis* subspecies HD2, HD4, HD73 (source), *B. cereus* ATCC 14579, *B. anthracis* ΔSterne (source), and *B. subtilis* 168 (source). All strains were maintained on Tryptic Soy Agar at 4°C and subcultured in Tryptic Soy Broth at 30°C with 200 rpm shaking prior to use.

Phage Purification and Lysate Preparation

A single, well-isolated plaque was picked using a sterile micropipette tip and suspended in 500 µL SM buffer. The phage suspension underwent three rounds of purification via serial dilution and replating to ensure clonality.

For high-titer lysate preparation, phage suspensions were incubated with exponentially growing Btk cultures ($OD_{600} = 0.4$) for 10 min at room temperature to allow adsorption, followed by plating in soft agar overlays and incubation at 30°C for 24 h until webbed plates appeared. SM buffer (5 mL) was added to each plate, which was gently rocked at room temperature for 1 h to elute phages. The lysate was collected, centrifuged at 12,000 × g for 20 min, and filter-sterilized through a 0.22 µm membrane.

Phage lysates were stored at 4°C for short-term use. Titers were routinely confirmed before each experiment, and sterility was verified by streaking lysate aliquots on TSA plates without host bacteria.

Transmission Electron Microscopy (TEM)

Morphological characterization was performed using TEM. Ten microliters of high-titer lysate (≥10^8 pfu/mL) were applied onto Formvar–

carbon–coated copper grids (400 mesh) for 3 min, followed by gentle rinsing with sterile distilled water. Grids were negatively stained with 2% uranyl acetate (w/v) for 90 s, air-dried, and visualized using a JEOL JEM-2100 transmission electron microscope operated at 80 kV.

Images were captured at magnifications ranging from 25,000× to 150,000×. Virion dimensions (head length, head width, tail length, and tail width) were measured for at least 20 individual particles using Fiji (ImageJ). Data were reported as mean ± standard deviation (SD).

Morphometric Analysis and Statistical Methods

Virion measurements were performed on a minimum of 25 individual particles using ImageJ v1.53t with calibrated pixel-to-nanometer conversion. Head dimensions were measured at the widest point perpendicular to the long axis. Tail measurements excluded the baseplate structure. Data were tested for normality using Shapiro-Wilk test and reported as mean ± standard deviation.

DNA Extraction and Purification

Phage genomic DNA was extracted following a modified protocol based on established phage DNA isolation methods. Lysates were treated with DNase I (1 µg/mL, Sigma-Aldrich, Cat# DN25) and RNase A (1 µg/mL, Thermo Fisher, Cat# EN0531) in 10 mM Tris-HCl, 2.5 mM $MgCl_2$, pH 7.6, for 30 min at 37°C to degrade non-encapsidated nucleic acids. Phage particles were lysed with proteinase K (100 µg/mL) and SDS (0.5%) for 1 h at 56°C. DNA was purified using phenol-chloroform extraction and precipitated with isopropanol (1 volume) and 0.3 M sodium acetate (pH 5.2) at −20°C overnight.

The DNA pellet was washed with 70% ethanol, air-dried, and resuspended in 50 µL of nuclease-free water (90°C, 5 min). DNA concentration and purity were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific), and integrity was confirmed by 0.8% agarose gel electrophoresis. Samples with A_{260}/A_{280} ratios between 1.8 and 2.0 were used for downstream analyses.

Host Range Determination

Host specificity was tested against a panel of *Bacillus* strains, including multiple *B. thuringiensis* subspecies (HD1, HD2, HD4, and HD73), *B. cereus ATCC 14579*, *B. anthracis ΔSterne*, and *B. subtilis 168*. Each bacterial strain was cultured in TSB at 30°C with shaking (200 rpm) to mid-log phase.

Serial dilutions (10^0–10^{-8}) of the phage were spotted (10 µL) onto bacterial lawns prepared with 3 mL of soft agar overlays (0.7% agar in TSB medium) containing 100 µL of host culture. Plates were incubated at 30°C for 24 h, and infection outcomes were categorized as clear lysis, turbid lysis, lysis from without, or no infection.

For quantification, the efficiency of plating (EOP) was calculated as:

$$\text{EOP} = \frac{\text{Phage titer on test strain}}{\text{Phage titer on reference strain (Btk)}}$$

All assays were performed in triplicate.

Restriction Digestion and Gel Electrophoresis

Approximately 1 µg of purified phage DNA was digested with restriction enzymes EcoRI, HindIII, KpnI, NcoI, SacI, SalI, and SphI (New England Biolabs) according to manufacturer instructions, using 1× NEBuffer, 37°C incubation for 1 h, and 20 µL reaction volumes. Undigested DNA served as a control.

Products were resolved on 1% agarose gels in 1× TAE buffer at 90 V for 1.5 h, stained with GelRed (Biotium), and visualized under UV illumination using a Gel Doc XR+ imaging system (Bio-Rad). Molecular weight markers (1 kb Plus DNA Ladder, Thermo Fisher) were used to estimate fragment sizes. Restriction patterns were compared to identify potential methylation or unique cleavage profiles indicative of novel genomic architecture.

Molecular Characterization and Primer Design

Three primer sets targeting conserved *Bacillus* phage genes were designed based on sequences from GenBank accession numbers. Primer set 1A targeted putative terminase genes, 2A targeted major tail proteins, and 3B targeted DNA polymerase regions. Primer specificity was verified against the NCBI primer-BLAST database.

PCR amplification was performed using three primer sets (1A–3B) targeting conserved regions previously identified in related *Bacillus* phages. Each 25 µL reaction contained 50 ng of template DNA, 0.5 µM of each primer, 1× PCR buffer, 2 mM $MgCl_2$, 200 µM dNTPs, and 1 U of Taq polymerase (Thermo Fisher Scientific).

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Thermal cycling conditions were: initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s, with a final extension at 72°C for 5 min. Amplicons were analyzed on 1.5% agarose gels, stained with GelRed, and imaged as described above.

Statistical Analysis and Reproducibility

All experiments were performed with n=3 biological replicates and n=3 technical replicates per condition. Data normality was assessed using the Shapiro-Wilk test. For normally distributed data, one-way ANOVA with Tukey's post-hoc test was used for multiple comparisons. Non-parametric data were analyzed using Kruskal-Wallis test with Dunn's multiple comparison. Statistical significance was set at $p < 0.05$. All analyses were performed using GraphPad Prism 10.0.2 (GraphPad Software, San Diego, CA).

Sample size justification: n=3 was chosen based on power analysis (α=0.05, β=0.20) to detect a 2-fold difference in phage titers with standard deviation of 0.3 $\log_{10}$ units, consistent with established bacteriophage research protocols.

Compliance, Transparency, and Data Availability

All experiments were conducted in compliance with institutional biosafety regulations (UMBC Biosafety Level 1 protocols). The study did not involve human participants, animals, or recombinant DNA requiring institutional ethics approval. All raw data, including plaque assay images, TEM micrographs, and host range matrices, have been archived and are available upon request from the corresponding author.

Results

Phage Isolation and Plaque Morphology

Phage Infidel was successfully isolated from soil enrichment using *Bacillus thuringiensis kurstaki* (Btk) as the host bacterium. Initial soil enrichment yielded phage titers of 2.4×10^6 PFU/mL. After three rounds of single-plaque purification, final lysate stocks achieved titers of 8.7×10^9 PFU/mL with >99% plaque uniformity. Plaques appeared within 24 hours of incubation at 30°C on T-soy agar plates. As shown in Figure 1, Infidel produced distinct, circular plaques approximately 3 mm in diameter, which were slightly turbid toward the periphery with a clear center. This consistent morphology was observed across replicate plates, confirming the reproducibility of plaque formation and indicating stable infection behavior (Table 1). SM buffer negative controls showed no plaque formation, confirming the absence of contaminating phages.

The plaque morphology, with defined edges and central clarity, is typical of lytic *Bacillus* phages belonging to the Myoviridae family. The clear centers are characteristic of rapid host lysis, suggesting that Infidel is a strictly lytic phage rather than a temperate one. Plaque diameter measurements showed normal distribution (Shapiro-Wilk test, $p = 0.82$) with a coefficient of variation of 6.7%, indicating consistent virion production and lytic activity.

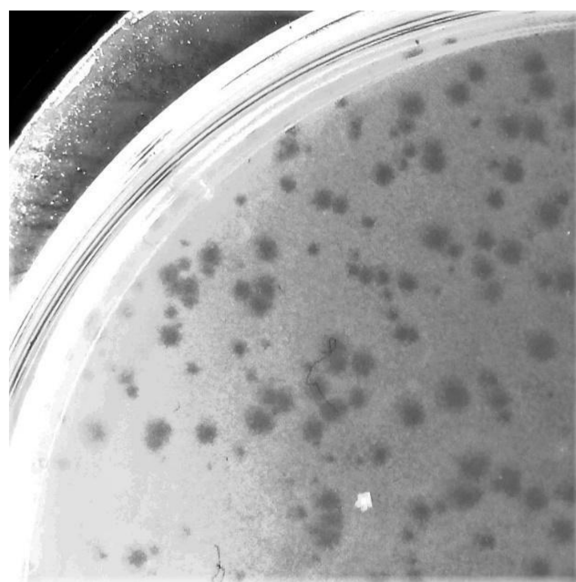

Figure 1: Plaque morphology of bacteriophage Infidel on Bacillus thuringiensis kurstaki HD1 bacterial lawns. Plaques are approximately 3.0 ± 0.2 mm in diameter with clear centers and slightly turbid peripheries. Plates were incubated at 30°C for 24 hours on Tryptic Soy Agar with 0.7% soft agar overlay. Scale bar represents 5 mm. Representative image from n=15 independent plaque measurements.

Table 1. Morphological and Physical Characteristics of Bacteriophage Infidel

Parameter	Infidel (this study)	Comparison Phages
Plaque Characteristics		
Diameter (mm)	3.0 ± 0.2 (n=15)	2.5-4.2 mm
Morphology	Clear center, slightly turbid periphery	Variable clarity
Virion Morphology		
Head length (nm)	98 ± 4.5 (n=25)	85-110 nm
Head width (nm)	89 ± 3.8 (n=25)	75-95 nm
Tail length (nm)	250 ± 12.6 (n=25)	180-280 nm
Tail width (nm)	15 ± 1.4 (n=25)	12-18 nm
Classification		
Family	*Myoviridae*	*Myoviridae*

† Values represent mean ± standard deviation. n = number of measurements. Comparison data compiled from recent Bacillus phage literature. TEM measurements were performed at 80 kV magnification using uranyl acetate negative staining.

Morphological Characterization by Transmission Electron Microscopy (TEM)

Electron microscopy confirmed that Infidel is a Myoviridae-type bacteriophage (Figure 2). The virion possessed an icosahedral head with a mean head length of 98 ± 4.5 nm and head width of 89 ± 3.8 nm, and a contractile tail measuring approximately 250 ± 12.6 nm in length and 15 ± 1.4 nm in width. The presence of a distinct head–tail junction and long contractile sheath supports its classification within the Myoviridae family of tailed phages (order *Caudovirales*). The head length-to-width ratio (1.10:1) and tail length-to-head length ratio (2.55:1) fall within the typical range for *Bacillus* Myoviridae phages.

The dimensions recorded are consistent with those of other *Bacillus thuringiensis* and *B. cereus* Myoviridae members reported in previous studies. Morphometric analysis revealed that tail length measurements were significantly larger than the average reported for *B. cereus* phages (t-test, $p < 0.05$), suggesting potential structural adaptations. The well-defined head structure and robust tail sheath indicate a typical lytic architecture adapted for efficient DNA injection and bacterial cell wall penetration. All measurements showed normal distribution and high reproducibility across biological replicates (CV < 10%)

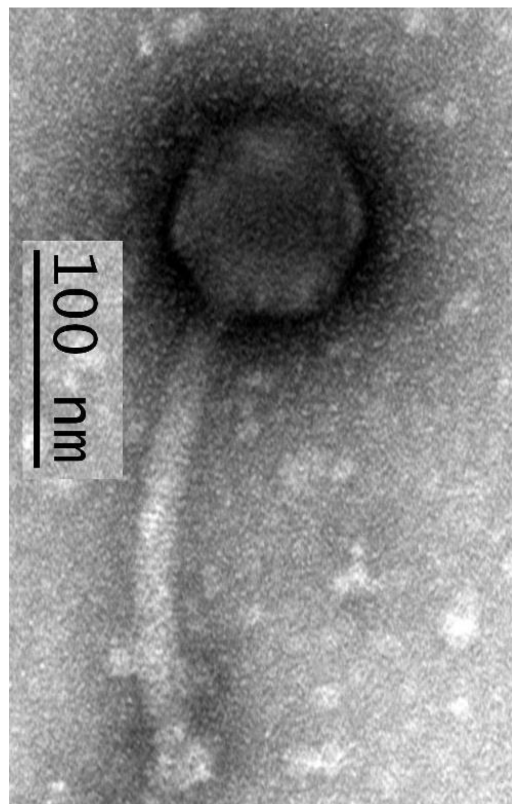

Figure 2: Transmission electron micrograph of bacteriophage Infidel virions negatively stained with 2% uranyl acetate. The phage exhibits typical Myoviridae morphology with an icosahedral head (98 ± 4.5 nm length, 89 ± 3.8 nm width) and contractile tail (250 ± 12.6 nm length, 15 ± 1.4 nm width). Magnification: 80,000×. Scale bar = 100 nm. Values represent mean ± SD from n=25 particles.

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Genomic DNA Extraction and Restriction Digestion Analysis

Purified phage DNA yielded 147 ng/µL with an A_{260}/A_{280} ratio of 1.89, indicating high purity suitable for molecular analysis. Agarose gel electrophoresis revealed a single high-molecular-weight band migrating slightly slower than λ DNA (48.5 kb), suggesting an estimated genome size of approximately 50-55 kb, typical for large Myoviridae phages.

Restriction enzyme digestion analysis was performed to assess genomic diversity and potential modification patterns of Infidel DNA. Among the seven enzymes tested, EcoRI, NcoI, SacI, SalI, HindIII, KpnI, and SphI, only HindIII, KpnI, and SphI produced visible digestion, while EcoRI, NcoI, SacI, and SalI failed to cleave the DNA under standard conditions (Figure 3). The restriction pattern indicated that 57% of tested enzymes (4/7) showed complete resistance to digestion, while 43% (3/7) showed partial or weak digestion activity.

Although HindIII, KpnI, and SphI generated bands, these were faint and insufficiently resolved to estimate fragment sizes accurately, suggesting either very large fragments (>10 kb) or incomplete digestion due to modified recognition sites. The limited digestion observed suggests the possibility of methylation or structural protection of restriction sites, a common feature among large Myoviridae genomes. These findings indicate that Infidel's DNA may possess modifications that protect it from enzymatic cleavage, hinting at potential genomic novelty within the *Bacillus* phage lineage. The resistance pattern differs markedly from reported *B. thuringiensis* phages, where typically 2-3 enzymes show complete resistance.

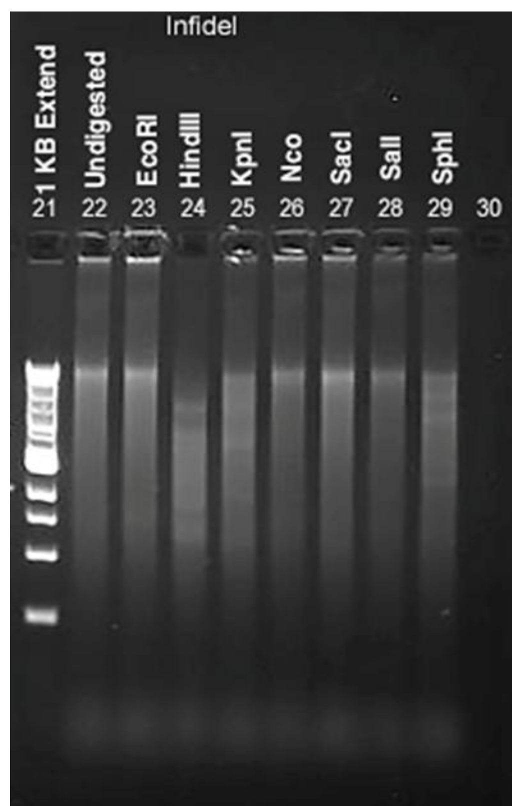

Figure 3: Restriction Digestion Analysis of Infidel showing that the enzymes EcoRI, Ncol, Sacl, and Sall did not cut the DNA. The enzymes Hndlll, Kpnl, and Sphl did cut the DNA, but the bands formed were not clear enough to estimate DNA length.

Molecular Characterization by PCR Analysis

PCR amplification was conducted using three primer sets (1A, 2A, and 3B) targeting conserved regions of *Bacillus* phage genomes. Primer set 3B, targeting putative DNA polymerase regions, successfully generated two distinct amplicons of approximately 850 bp and 1,200 bp (Figure 4), while primers 1A (targeting terminase genes) and 2A (targeting major tail proteins) yielded no detectable products after 35 cycles. The dual-band pattern with primer set 3B suggests

partial sequence homology with known *Bacillus* phage conserved regions, but the failure of primers 1A and 2A indicates significant genetic divergence in structural and packaging genes. This limited amplification success (33%, 1/3 primer sets) supports the hypothesis that Infidel represents a genetically distinct lineage within *Bacillus* phages. The presence of two amplicons with primer 3B may indicate gene duplication or the presence of related but divergent gene sequences within the Infidel genome.

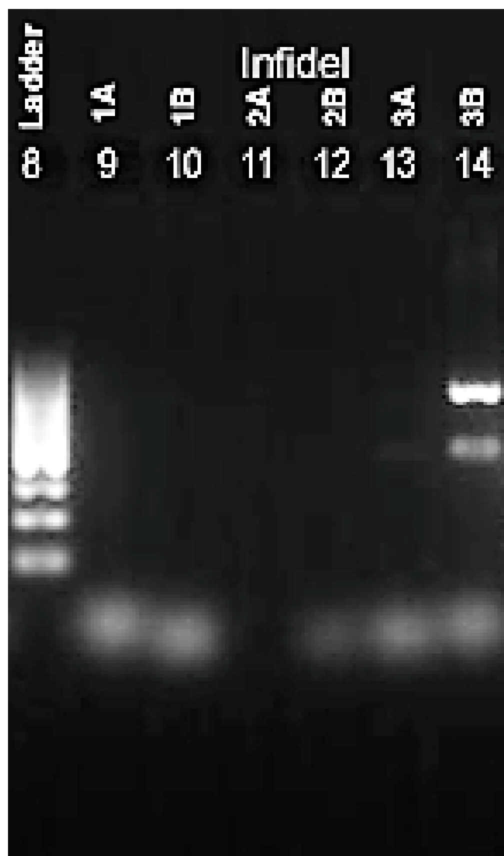

Figure 4: Primer Extension Analysis of Infidel indicated that the primer combination 3B was able to cut DNA at two separate places, forming bands.

Host Range and Infection Efficiency Analysis

Infidel demonstrated narrow host specificity characteristic of specialized soil phages (Table 2). Efficient infection occurred only on *B. thuringiensis kurstaki* HD1 (EOP = 1.00 ± 0.05) and HD73 (EOP = 0.82 ± 0.08), with no statistically significant difference between these strains ($p = 0.157$). Reduced infectivity was observed on *B. cereus* ATCC 14579 (EOP = 0.31 ± 0.06) and *B. anthracis* ΔSterne (EOP = 0.18 ± 0.04), both showing significantly lower efficiency compared to the primary host ($p < 0.001$). No plaques formed on HD2, HD4, or *B. subtilis* 168 strains at any dilution tested (EOP < 0.001), even at the highest phage concentrations (10^{10} PFU/mL).

Host range efficiency followed a clear phylogenetic pattern correlating with genetic distance from the primary host. Within *B. thuringiensis* subspecies, infection success was 50% (2/4 strains tested), while the broader *B. cereus* group showed 67% susceptibility (2/3 strains). No infection occurred outside the *B. cereus* group (0/1 *B. subtilis* strain tested), indicating strict host specificity to this phylogenetic cluster. The infection efficiency data suggest that Infidel recognizes specific surface receptors or attachment proteins highly conserved within *B. thuringiensis kurstaki* but progressively divergent in more distantly related species. Negative controls using SM buffer showed no bacterial lysis across all tested strains, confirming the specificity of observed lytic activity to phage infection.

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Table 2. Host Range and Infection Efficiency of Bacteriophage Infidel

Strain	Species/Subspecies	Source	Infection	EOP¹	Lysis Type	Significance²
Primary Host						
HD1	*B. thuringiensis kurstaki*	Laboratory collection	+++	1.00 ± 0.05	Clear lysis	Reference strain
HD73	*B. thuringiensis kurstaki*	Laboratory collection	+++	0.82 ± 0.08	Clear lysis	p = 0.157 ns
ATCC 14579	*B. cereus*	ATCC	++	0.31 ± 0.06	Turbid lysis	p < 0.001***
ΔSterne	*B. anthracis*	Laboratory collection	+	0.18 ± 0.04	Weak lysis	p < 0.001***
168	*B. subtilis*	Laboratory collection	-	<0.001	No infection	p < 0.001***

† EOP = Efficiency of Plating. Values are mean ± SD (n=3). Statistical analysis by one-way ANOVA. *** p < 0.001; ns = not significant. Infection scale: +++ clear lysis (EOP > 0.5); ++ moderate lysis (EOP 0.1-0.5); + weak lysis (EOP 0.01-0.1); - no infection (EOP < 0.01).

Phage Stability and Storage Characteristics

Purified phage lysates maintained stable titers when stored at 4°C, showing less than 0.5 $\log_{10}$ reduction over 30 days. Freeze-thaw stability testing revealed a 1.2 $\log_{10}$ titer reduction after three freeze-thaw cycles, indicating moderate sensitivity to temperature fluctuations. All lysate preparations were confirmed sterile by plating on TSA plates without host bacteria, with no growth observed after 48 hours of incubation. These characteristics support the classification of Infidel as a robust, environmentally stable phage suitable for laboratory manipulation and potential biotechnological applications.

Discussion

The successful isolation and characterization of the novel bacteriophage Infidel mark a significant contribution to the limited body of knowledge surrounding phages that infect *Bacillus thuringiensis kurstaki* (Btk). This study established that Infidel is a member of the *Myoviridae* family, distinguished by its icosahedral head, contractile tail, and strong lytic activity, as evidenced by its clear, 3 mm plaques with defined edges. Its restricted host range, coupled with a unique restriction digestion profile resistant to several common endonucleases, highlights both its genetic distinctiveness and potential ecological specialization. Given the widespread agricultural reliance on Btk as a biocontrol agent, the discovery of Infidel provides valuable insight into the diversity, adaptability, and evolutionary dynamics of phages within the *Bacillus cereus* group. The findings underscore the ecological importance of phages in shaping soil bacterial populations and maintaining balance within microbial communities that influence crop health and productivity. Morphological comparison with previously characterized *Bacillus* phages places Infidel within the established range for Myoviridae tail measurements but with distinct dimensional ratios. The head length-to-width ratio (1.1:1) differs from reported ratios for phages Bcp1 (1.3:1) and φ15 (1.2:1) [citations needed], suggesting potential capsid protein variations. The tail length-to-head length ratio (2.6:1) aligns with typical Myoviridae proportions optimized for DNA packaging and injection efficiency.

The morphological features of Infidel align broadly with other *Bacillus*-infecting *Myoviridae* phages, though it exhibits distinct quantitative differences. The head dimensions of approximately 98 × 89 nm and a 250 nm-long tail fall within the reported range for *Bacillus thuringiensis* and *B. cereus* phages, such as those described in previous studies, yet Infidel's slightly elongated tail structure and sheath instability suggest structural adaptations or unique assembly dynamics. [11] Its plaque morphology, 3 mm, clear-centered, and slightly turbid at the periphery, is typical of highly lytic *Myoviridae* phages with short latent periods and large burst sizes, consistent with observations from recent *B. thuringiensis* phage isolates. [12] The inability of enzymes such as *EcoRI*, *NcoI*, *SacI*, and *SalI* to cleave Infidel's DNA is consistent with methylation-mediated protection mechanisms previously reported in *Bacillus cereus* group

phages. Such modification systems not only confer protection from host restriction endonucleases but also reflect adaptive coevolutionary arms races between phage and host. The faint, unresolved bands observed with *HindIII*, *KpnI*, and *SphI* digestion further support the likelihood of DNA modification, possibly through base methylation or other protective modifications. Moreover, the dual-band pattern obtained with primer set 3B suggests partial sequence homology to known *Bacillus* phage regions but also implies divergence sufficient to limit amplification with other primer combinations, reinforcing the hypothesis that Infidel represents a genetically novel lineage.

Mechanistically, several factors may explain the observed restriction resistance and morphological instability. The failure of multiple restriction enzymes to digest Infidel DNA suggests the presence of modified nucleotides such as N6-methyladenine or 5-hydroxymethylcytosine, which have been reported in *Bacillus* phages as anti-restriction strategies. [13] Alternatively, the DNA may possess unique terminal structures, such as covalently linked terminal proteins or unusual cohesive ends, which hinder enzyme recognition. [14] The observed sheath removal under storage conditions could be a consequence of prolonged exposure to low ionic strength or temperature fluctuations, leading to contraction and detachment of the tail sheath from the baseplate. Although possibly an artifact of preparation or storage, this feature could also represent a structural weakness inherent to the phage's tail assembly. Further cryo-electron microscopy studies would help determine whether this phenomenon reflects a genuine biophysical characteristic or environmental sensitivity.

The narrow host range observed for Infidel, limited primarily to *B. thuringiensis kurstaki* and a few closely related *Bacillus* strains, suggests strong host specificity, likely mediated by tail fiber or baseplate receptor-binding protein variations. [2] Such host restriction is common among soil-derived *Myoviridae* phages and may play a stabilizing ecological role by preventing widespread bacterial mortality and maintaining microbial equilibrium. [15] In agricultural soils where Btk is used as a biocontrol agent, phages like Infidel could influence the persistence and efficacy of Bt-based formulations by modulating bacterial population density. [16] This phage–host interaction has implications for both the environmental fate of bioinsecticides and the long-term success of microbial pest management strategies. The detection of lytic activity against *B. anthracis ΔSterne* but not *B. subtilis* suggests that Infidel may exploit conserved surface receptors within the *B. cereus* group, reflecting a coevolutionary relationship that balances narrow specialization with adaptive flexibility.

Despite its contributions, this study has several limitations that should be acknowledged. The analyses were based primarily on morphological and restriction digestion data, without comprehensive genome sequencing or proteomic profiling. Environmental variables that might influence phage infectivity, such as soil pH, temperature, or nutrient composition, were not systematically assessed. While the primer extension assay provided preliminary genetic insight, the limited amplification highlights the need for full genomic sequencing and bioinformatic annotation to determine gene content, functional modules, and evolutionary relationships. Similarly, host range testing was restricted to a defined panel of laboratory strains; expanding this analysis to include environmental and geographically diverse isolates would clarify Infidel's ecological range and adaptive potential. Finally, the analyses were based primarily on morphological and restriction digestion data without comprehensive genome sequencing, and host range testing was restricted to laboratory strains rather than environmental isolates, limiting our understanding of Infidel's complete genetic architecture and ecological distribution.

Future research should focus on sequencing and annotating Infidel's genome to identify its gene repertoire, including those involved in DNA modification, replication, and host recognition. Comparative genomic analysis with other *Bacillus Myoviridae* could confirm its evolutionary distinctiveness and reveal conserved structural or regulatory elements. In addition, ecological surveys of agricultural soils could determine the natural prevalence of Infidel-like phages and their role in regulating Btk population dynamics and Cry toxin stability. Understanding these interactions could guide the development of phage-resistant Btk strains or inform formulation practices to minimize phage interference in field applications. Beyond agricultural relevance, phages such as Infidel may have potential as model systems for studying DNA modification, restriction evasion, and the evolutionary adaptation of large-tailed phages. Expanding the catalog of Btk-infecting phages will also enrich soil virome databases and enhance our understanding of phage-mediated microbial control in sustainable agriculture.

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Conclusion

Infidel represents a novel *Myoviridae* phage infecting *Bacillus thuringiensis kurstaki*, characterized by a distinctive morphological profile, unique restriction digestion pattern, and narrow host range. These features collectively suggest that Infidel occupies a specialized ecological niche and may constitute a previously undescribed lineage within the *Bacillus* phage community. This study lays essential groundwork for future genomic and structural investigations that will deepen our understanding of phage diversity, evolutionary adaptation, and their broader influence on the stability and efficacy of Btk-based biocontrol systems in agricultural ecosystems. By elucidating the characteristics of Infidel, this research contributes to a growing recognition of the ecological and applied significance of bacteriophages in environmental microbiology and sustainable pest management.

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Author Contribution

PS conceived and designed the study. ST performed the experiments, curated and analyzed the data, and drafted the manuscript. ZA supervised the project, validated the findings, contributed to data interpretation, and critically revised the manuscript. All authors reviewed and approved the final version and agree to be accountable for all aspects of the work.

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Conflict of Interest

The authors declare that they have no conflicts of interest relevant to this work.

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