

Isolation and evaluation of a lytic *Escherichia coli* phage as a potential food biocontrol agent: Biological characterization and independent genomic analysis

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ABSTRACT

This study characterized a lytic *Escherichia coli* phage isolated from Nem chua and evaluated its potential as a candidate for food biocontrol by integrating biological characterization, phylogenomic analysis, and genomic safety assessment. Twelve distinct lytic phages were successfully isolated based on the formation of clear plaques (2.0–2.5 mm) on *E. coli* lawns. Among the isolated phages, PHAGE_ECOL05 showed the broadest qualitative spot-test activity, producing visible clearing on the lawns of 8 of 19 tested *E. coli* strains. The multiplicity of infection (MOI) influenced the suppression of bacterial turbidity. Among the tested conditions, an initial MOI of 1 produced the greatest reduction in OD₆₀₀, with values decreasing to near-baseline levels within 4–6 hours. Transmission electron microscopy revealed that PHAGE_ECOL05 possessed an icosahedral head and a contractile tail, consistent with the morphological characteristics of phages within the class *Caudoviricetes*. DNBSEQ-G99 paired-end sequencing of DNA extracted from the plaque-purified PHAGE_ECOL05 preparation generated two viral contigs. No assembly connection was observed between the two contigs; therefore, they were analyzed independently. A total of 204 predicted open reading frames were identified across the two contigs. Comparative phylogenomic analyses placed Contig 1 within the family *Chaseviridae* and genus *Carltongylavirus*, whereas Contig 2 was placed within the family *Chaseviridae* but could not be confidently assigned to a currently recognized genus based on the available genomic comparisons. Neither contig contained predicted virulence-associated or antibiotic resistance-associated genes. Overall, the biological characteristics of PHAGE_ECOL05 and the absence of predicted virulence genes and antibiotic resistance-associated genes in the recovered viral contigs provide preliminary evidence supporting further investigation of the PHAGE_ECOL05 preparation as a potential food biocontrol candidate.

1. INTRODUCTION

Escherichia coli is a Gram-negative bacterium classified within the family Enterobacteriaceae and represents one of the most extensively studied and well-characterized microbial species [1]. Most warm-blooded animals, including humans, harbor *E. coli* as a commensal bacterium in their intestinal tract [2]. Most *E. coli* strains are non-pathogenic and coexist with their hosts without any problems. Nevertheless, some do carry pathogenic variants capable of causing diarrhea, urinary tract infections, and even neonatal meningitis and infections of the bloodstream [3]. What is especially worrisome is the case of enteropathogenic and enterohemorrhagic *E. coli*, which can

cause severe complications from foodborne illness. Epidemiological data indicate that nearly 2,961 deaths occur within 30 days of the onset of sepsis caused by *E. coli* infections, highlighting the extensive public health issues associated with this bacterium [4]. Pathogenic *E. coli* strains can also trigger significant economic losses due to food contamination, product recalls, and treatment costs.

Furthermore, the risk of *E. coli* has been intensified recently due to the rise in the global prevalence of antimicrobial resistance (AMR), which makes conventional antibiotics less effective [5]. AMR was associated with an impact on an estimated 4.95 million deaths in 2019, 1.27 million of which are a direct result of infections originating from antibiotic-resistant bacteria [6]. In India, 50%–80% of hospital isolates of *E. coli* and *Klebsiella pneumoniae* in 2021 exhibited resistance to beta-lactams, fluoroquinolones, or cephalosporins [7]. The genetic basis of antibiotic resistance is particularly evident in multidrug-resistant bacteria associated with livestock production and clinical settings. The indiscriminate use of antibiotics creates strong selective

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pressure for resistant bacteria, and resistance genes can subsequently spread rapidly through horizontal gene transfer [6]. These strains are becoming increasingly difficult to treat using existing antibiotics.

Biologically based strategies, including bacteriophage-mediated biocontrol, are increasingly being explored as alternatives to conventional antimicrobial approaches for improving food safety and supporting sustainable food production. Bacteriophages (phages) are viruses that target and destroy selected specific bacterial cells and represent a promising alternative to conventional antibiotics [8]. Apart from being economical, phages are also biodegradable and safe for use in agriculture and medicine. There is a growing body of research that demonstrates the effectiveness of phages in controlling pathogenic *E. coli* under a wide range of conditions [9,10]. The phage vB_EcoM-UFV13, for instance, is effective in treating *E. coli* infections associated with mastitis in cows [11], and phage vB_EcoStr-FJ63A shows potent bacteriolytic activity against colistin-resistant *E. coli* from animal sources [12]. In another study, phage vB_EcoS_PJ16 reduced the viability of avian pathogenic *E. coli* *in vitro* and significantly decreased chick mortality in an experimental colibacillosis model [13]. A combination of phages, SPR02 and DAF6, has demonstrated its application in poultry populations against *E. coli* respiratory infections [14]. The regulatory status of phage preparations varies depending on the product, target bacterium, intended application, and jurisdiction. In its supplemental response dated January 12, 2024, the US Food and Drug Administration (FDA) noted that it had previously raised no questions regarding Intralytix, Inc.'s conclusion that a preparation of lytic phages specific to Shiga toxin-producing *E. coli* is generally recognized as safe for use as an antimicrobial on meat, poultry, fruits, vegetables, dairy products, fish, and seafood. FDA likewise had no questions regarding its use in grain and grain products at the same maximum level of 10^8 PFU/g of food [15]. These conclusions apply only to the specified preparation and conditions of use and should not be generalized to other phage products, target bacteria, applications, or jurisdictions.

Despite increasing interest in phage-based food biocontrol, lytic *E. coli* phages recovered from food-associated microbial environments remain insufficiently characterized, particularly with respect to their genomic features and safety profiles. Given the occurrence of pathogenic and AMR *E. coli* in the food supply chain and the growing need for alternative biocontrol strategies, this study aimed to isolate and biologically characterize a lytic *E. coli* phage from Nem chua and investigate the genomic characteristics and safety features of viral contigs obtained from its plaque-purified preparation. Because the phage was isolated from Nem chua, a traditional Vietnamese fermented pork product and a food-associated microbial environment, its characterization provides preliminary information relevant to the exploration of phage-based biocontrol strategies for food-associated *E. coli*. This work therefore provides preliminary evidence for the identification of phage candidates warranting further investigation for the biocontrol of food-associated *E. coli*.

2. MATERIALS AND METHODS

2.1. Bacterial Isolation and Phenotypic Characterization

2.1.1. Isolation of *E. coli*

The *E. coli* strains used in this publication were recovered from similar Nem chua samples that were obtained at traditional markets in Vinh Long Province, Vietnam. For this purpose, the method of isolation by Lee *et al.* [16] was used, with slight modifications. Each sample of around 10 g was put in 250 ml Erlenmeyer flasks with buffered peptone water (HiMedia, India), followed by incubation

at $37^\circ\text{C} \pm 1^\circ\text{C}$ for 12 hours while shaking (120 rpm) for bacterial enrichment. Afterward, 100 μl from the enriched culture was spread on MacConkey agar plates (MCA, Himedia, India) and incubated at $37^\circ\text{C} \pm 1^\circ\text{C}$ for 24 hours. Typical *E. coli* were isolated and purified using the streak plating technique [17]. Further identification was done through standard biochemical tests, confirming that the sample was catalase-positive and oxidase-negative. The quality control strain *E. coli* ATCC® 25922 was employed as a reference throughout the isolation and identification process.

2.1.2. Antibiotic susceptibility analysis

The isolates were evaluated for antibiotic resistance against 12 antibiotics using the disk diffusion technique on Tryptone Soya Agar (TSA) medium following the procedure by the Kirby-Bauer protocol [18]. The determination of resistance, intermediate resistance, and sensitivity was carried out according to the Clinical and Laboratory Standards Institute guidelines (2020) [19]. The multiple antibiotic resistance (MAR) index for every isolate was calculated using the formula $\text{MAR} = \text{R}/\text{E}$ [20], with R being the resistant antibiotics and E the total antibiotics evaluated.

2.2. Phage Isolation and Characterization

2.2.1. Isolation of bacteriophages

Phages were isolated using the double-layer agar plaque technique, adapted from Duc *et al.* [21], with minor procedural adjustments. In short, 50 g from the “Nem chua” sample was mixed with 100 ml of LB broth with 10 mM CaCl_2 , then left to sit for a duration of 30 minutes, at which point 100 μl of *E. coli* from the overnight sample was added. This was left for 18–24 hours, during which the temperature was maintained at $37^\circ\text{C} \pm 1^\circ\text{C}$.

Following incubation, 100 μl of the enriched culture was serially diluted in Saline Magnesium (SM) buffer (comprising Tris-HCl 50 mM, NaCl 100 mM, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 8 mM, gelatin 0.01% [w/v], pH 7.5). Each diluted phage suspension was mixed with 100 μl of the host bacterial culture and 4 ml of molten soft agar (0.4% LB agar) and then poured onto the surface of (TSA, Himedia, India) plates. The formation of plaques was noticed after the plates were incubated for 24 hours at $37^\circ\text{C} \pm 1^\circ\text{C}$ [21].

Each plaque was collected and diluted in sterile microcentrifuge tubes containing 1 ml of SM buffer. The suspensions were mixed at full speed, and after 30 minutes of vigorous shaking, the tubes were placed at 4°C for 24 hours in an attempt to disassociate the phage particles. To remove bacterial fragments, 10 μl of chloroform was added, followed by centrifugation at 4°C and 6,000 rpm for 5 minutes. Fresh microcentrifuge tubes were then filled with the supernatants. To ensure similarity in morphology and the size of plaques, the purification process was performed repeatedly. The purified phage lysates were stored in SM buffer at 4°C for further analysis.

2.2.2. Qualitative assessment of phage activity by spot assay

The phage infectivity spectrum was evaluated using the double-layer agar technique with a completely randomized design and three replications. Tryptic Soy Broth (TSB, Himedia, India) plates containing 1.7% agar were prepared, sectioned, and labeled. Then, 10 ml of soft agar (consisting of TSB with 0.6% agar) was mixed with 100 μl of each bacterial suspension, which had been adjusted to an optical density at 600 nm (OD_{600}) of 0.5. The plates were left to dry for 15–20 minutes [22]. Subsequently, 2 μl of each phage suspension was applied

to the designated labeled sections on the plates and incubated at 37°C $\pm$ 1°C for 24 hours to facilitate observation of plaque development [23]. The incubation process helped determine the host ranges of the test subject based on the presence or absence of “plaques.”

2.2.3. Effect of initial multiplicity of infection (MOI) on bacterial growth

The experiment was designed to be completely randomized with three replicates. Phages used in this assay were selected based on the host range results. For 24 hours, we cultured bacteria and phages separately in liquid TSB and later diluted them to 10⁸ CFU/ml and 10⁸ PFU/ml. Infection was done at PFU-to-CFU ratios of 0.01, 0.1, and 1. After the bacteria and phages were mixed, they were placed in an incubator at 37°C $\pm$ 1°C, and later on, OD₆₀₀ was measured at specific time intervals (0, 2, 4, 6, 20, 22, 24, and 48 hours) to assess their growth and to determine whether phages had successfully lysed them.

2.2.4. Transmission electron microscopy (TEM) of bacteriophage

The morphological characteristics of the isolated phages were examined via TEM at the National Institute of Hygiene and Epidemiology in Hanoi, Vietnam.

2.3. Genomic and Bioinformatic Analyses

2.3.1. Genome sequencing and bioinformatic analysis

Phage preparation was performed using a modified protocol adapted from Soleimani-Delfan *et al.* [24]. Phages were propagated in Mueller-Hinton (MH) broth (Himedia, India) medium (OD₆₀₀ = 0.5) and incubated at 37°C with shaking at 110 rpm for 6 hours, during which they reached a concentration of 10⁸ PFU/ml. To eliminate remnants of bacterial DNA or RNA in the lysate, the phage suspension was treated with DNase I or RNase A (10 μ l/ml) at 37°C $\pm$ 1°C for 30 minutes. The enzyme reaction, in this case, stopped after 20 μ l of 0.5 M ethylenediaminetetraacetic acid was added. The precipitation of protein was conducted with 4 ml of the phage lysate, which was vortexed and mixed with 16 ml of acetone (pH 5.5) for 2 minutes. The mixture was then centrifuged for 2 minutes at 10,000 $\times$ g. After the supernatant was discarded, the ambient temperature was adjusted to allow the acetone to evaporate. The pellet subsequently obtained was used for DNA isolation by following the instructions of the QIAamp DNA Mini Kit (Qiagen). Qubit fluorometry was utilized to quantify the concentration of DNA in addition to determining the purity of the sample by the OD₂₆₀/OD₂₈₀ ratio. The concentration of DNA was also corroborated through the process of 1% agarose gel electrophoresis.

Genomic DNA was fragmented using NEBNext dsDNA Fragmentase, and genomic libraries were constructed using the NEBNext Ultra II DNA Library Prep Kit for Illumina (NEB, USA) according to the manufacturer's instructions. The Illumina-adapter libraries were subsequently converted to a DNBSEQ-compatible format using the MGIEasy Universal Library Conversion Kit (App-A) according to the manufacturer's instructions. Library concentration was quantified fluorometrically, and the mean fragment size was determined using an Agilent Bioanalyzer. According to the official sequencing service report, qualified libraries were sequenced on the DNBSEQ-G99 platform (MGI) in paired-end 150-bp (PE150) mode.

Paired-end reads generated on the DNBSEQ-G99 platform were processed with *fastp* v0.23.1 to remove adapters and low-quality bases and to trim and filter duplicates [25]. Clean reads were assembled de novo using *metaSPAdes* v3.15.5 [26]. To further assess the assembly,

pairwise BLAST comparison between the assembled contigs, independent mapping of quality-filtered reads to each contig, and assembly graph visualization using *Bandage* v0.8.1 were performed. Both assembled contigs were identified as viral sequences based on *BLAST* v2.13.0 analysis. *CheckV* v1.0.1 was used to evaluate the completeness of each viral contig [27]. Gene annotation was completed in *Pharokka* v1.4.1, employing a phage relational database for coding and functional feature predictions [28]. Each predicted open reading frame (ORF) amino acid sequence was individually analyzed using the NCBI SmartBLAST web service (accessed on 10 July 2025). SmartBLAST performs BLASTP-based searches against the landmark and nonredundant protein databases. Matches with an *E*-value of $\leq 1.0E-5$ were considered supported. Pairwise nucleotide similarity between the assembled viral contigs and reference phage genomes was calculated using *VRIDIC* v1.1 with default parameters to support genome-based taxonomic classification.

The *ABRicate* v1.0.1 tool was used with the CARD database (<https://card.mcmaster.ca/>) to screen for antibiotic resistance genes in the assembled contigs, with the thresholds set to 80% coverage and identity [29]. Virulence factor identification was done using the Virulence Factor Database first and was later annotated with *Bakta* v1.6.1. [30,31].

2.3.2. Genomic mapping and phylogenetic analysis

Genome maps were generated separately for each viral contig using Proksee (<https://proksee.ca/>). Comparative phylogenomic analyses were performed independently for each viral contig using ViPTree (<https://www.genome.jp/viptree/>) based on whole-proteome data [32]. Results were visualized as a circular proteomic tree.

The genome similarity score (S_g) was calculated using ViPTree [33], based on normalized tBLASTx scores of whole-proteome comparisons. A higher S_g value indicates greater proteomic similarity between phage genomes. An S_g threshold of 0.15 was applied to resolve viruses at the genus level. Identical genomes would each score a 1, while a score of 0 means that the comparisons using tBLASTx did not show any detectable similarity [32]. The output has been organized such that the branches form a rectangular tree structure, with each branch representing a phage.

2.4. Statistical Analysis

One-way analysis of variance was used to evaluate the data. If a difference was detected, then post-hoc mean comparisons using Tukey's test were done to identify the treatments that yielded significantly different response means at a significance level of $p < 0.05$.

3. RESULTS

3.1. Results of Isolation and Antibiotic Sensitivity Testing of *E. coli*

A total of 19 *E. coli* strains, designated Eco-01 to Eco-19, were successfully isolated. On MCA medium, *E. coli* colonies typically appeared pink with diameters ranging from 1 to 2 mm. The colonies were circular, smooth, convex, shiny, and had entire margins.

Antibiotic susceptibility tests of *E. coli* isolates revealed that strain Eco-07 had the highest resistance, with resistance to 9 out of 12 antibiotics tested. Across all isolates, the MAR index ranged from 0.08 to 0.75, with Eco-07 exhibiting the highest MAR value (Table 1).

Table 1. Antibiotic susceptibility profiles and multidrug resistance indices of isolated *E. coli* strains.

Isolate	Antibiotic resistance profile	Number of antibiotics tested (E)	Number of antibiotics resisted (R)	MAR index (R/E)
Eco-01	AMP, CHL, DOX, LEV, STR, TET, SXT	12	7	0.58
Eco-02	AMP, CHL, STR, TET, SXT	12	5	0.42
Eco-03	AMP, DOX, STR, TET	12	4	0.33
Eco-04	AMP, CHL, DOX, STR, TET, SXT	12	6	0.50
Eco-05	CHL, DOX, TET, SXT	12	4	0.33
Eco-06	AMP, CHL, CIP, DOX, STR, TET, SXT	12	7	0.58
Eco-07	AMP, CHL, CIP, DOX, GEN, OFX, STR, TET, SXT	12	9	0.75
Eco-08	AMP, DOX, TET	12	3	0.25
Eco-09	AMP	12	1	0.08
Eco-10	AMP, CHL, DOX, STR, TET	12	5	0.42
Eco-11	AMP, GEN	12	2	0.17
Eco-12	AMP, CHL, STR, TET, SXT	12	5	0.42
Eco-13	AMP, CHL, CIP, DOX, STR, TET	12	6	0.50
Eco-14	AMP, CHL, CIP, DOX, GEN, TET	12	6	0.50
Eco-15	CIP, GEN, STR, TET	12	4	0.33
Eco-16	AMP, CHL, CIP, TET, SXT	12	5	0.42
Eco-17	AMP, DOX, GEN, STR, TET, SXT	12	6	0.50
Eco-18	GEN	12	1	0.08
Eco-19	AMP, CHL, CIP, DOX, GEN, STR, TET, SXT	12	8	0.67

Ampicillin (AMP, 10 µg/disk), ampicillin/sulbactam (SAM, 20 µg/disk), ceftazidime (CAZ, 30 µg/disk), chloramphenicol (CHL, 30 µg/disk), ciprofloxacin (CIP, 5 µg/disk), doxycycline (DOX, 30 µg/disk), gentamicin (GEN, 10 µg/disk), levofloxacin (LEV, 5 µg/disk), ofloxacin (OFX, 5 µg/disk), streptomycin (STR, 10 µg/disk), tetracycline (TET, 30 µg/disk), and trimethoprim/sulfamethoxazole (SXT, 25 µg/disk).

3.2. Results of Phage Isolation and Characterization

3.2.1. Bacteriophage isolation results

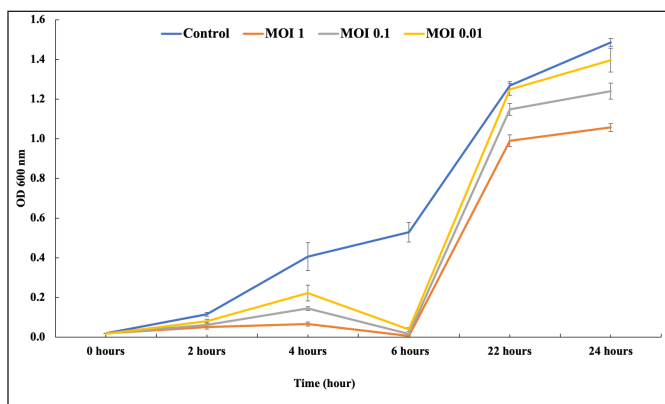
A total of 12 phages were isolated from Nem chua and referred to as PHAGE_ECOL01 to PHAGE_ECOL12. Each phage underwent purification at least three times to obtain clear and uniform plaques. All isolated phages generated circular plaques with clear centers and cloudy edges, with average diameters ranging from 2.0 to 2.5 mm. These results corroborate other work on phages such as UE-S5b, UE-M3, and UE-M6 derived from *E. coli*, which formed plaques 2.0 ± 0.2 mm in diameter on average [34]. Additionally, the plaques in this study were larger than the ones previously published in Grami *et al.* [35], wherein phage fBC-Eco01 formed plaques averaging 1.8 ± 0.2 mm in diameter.

3.2.2. Qualitative spot-test activity

The isolated *E. coli* strains were utilized as indicator strains to delineate the host-range spectrum of the phage. A summary of the results is

Table 2. Lytic spectrum of each bacteriophage against *E. coli* strains.

Bacteriophage	<i>E. coli</i> strains lysed	Total
PHAGE_ECOL01	Eco-01, Eco-03, Eco-10	3
PHAGE_ECOL02	Eco-02	1
PHAGE_ECOL03	Eco-02	1
PHAGE_ECOL04	Eco-01, Eco-03, Eco-04, Eco-16, Eco-17	5
PHAGE_ECOL05	Eco-01, Eco-03, Eco-05, Eco-07, Eco-09, Eco-11, Eco-16, Eco-17	8
PHAGE_ECOL06	Eco-01, Eco-03, Eco-04, Eco-08, Eco-16, Eco-17	6
PHAGE_ECOL07	Eco-01, Eco-04, Eco-05, Eco-06, Eco-09	5
PHAGE_ECOL08	–	0
PHAGE_ECOL09	Eco-01, Eco-04, Eco-05, Eco-06, Eco-09, Eco-13, Eco-18	7
PHAGE_ECOL10	Eco-12, Eco-13	2
PHAGE_ECOL11	Eco-12, Eco-13	2
PHAGE_ECOL12	Eco-12, Eco-13	2

**Figure 1.** Lytic activity of PHAGE_ECOL05 against *Escherichia coli* strain Eco-07. Note: Blue line = Control; Orange line = MOI 1; Gray line = MOI 0.1; Yellow line = MOI 0.01.

presented in Table 2. Out of the different strains, Eco-01 was the most susceptible, being lysed by multiple phages; Eco-03, Eco-04, and Eco-13 were less susceptible. Their findings coincide with the bulk of the existing literature. Keomany *et al.* [36], for example, reported that four broad host range phages (P.DH.MHH6, P.DH.MH1, P.D.MHH3, and P.D.MHH4) were able to lyse 23, 21, 21, and 12 out of 36 tested *E. coli* strains, respectively. An *et al.* [37] also documented the phages PD6, N10, PD11, PD14, and PN15, where the host ranges were 6, 8, 7, 5, and 6 strains, respectively, out of 32 *E. coli* strains isolated. Rahmani *et al.* [38] also noted phage TPR10 to be active against 7 out of 10 *E. coli* strains tested.

3.2.3. Effect of initial MOI on bacterial turbidity

The effect of different initial MOIs on bacterial turbidity was evaluated by monitoring OD₆₀₀ over 48 hours. We selected the phage, PHAGE_ECOL05, and the most resistant bacterial strain, Eco-07 (with the highest MAR index; Table 1), for the experiment. All treatments significantly reduced bacterial growth when compared to the control treatment ($p < 0.05$) (Fig. 1). Generally, increasing the initial MOI from 0.01 to 1 resulted in greater suppression of bacterial turbidity during the first 6 hours.

At time 0, the control OD₆₀₀ value of 0.02 increased to 0.11 after 2 hours of incubation and continued to 0.44 after an additional 4 hours ($p < 0.05$). The OD₆₀₀ values of the MOI 1, 0.1, and 0.01 treatments slightly increased, with MOI 1 showing the lowest rise (from 0.05 to 0.07), which was significantly different from the other treatments ($p < 0.05$). After 6 hours, the OD₆₀₀ of the control continued to increase, whereas the OD₆₀₀ values in the MOI 1, 0.1, and 0.01 treatments

decreased to 0.01 ± 0^b , 0.02 ± 0^b , and 0.04 ± 0.01^b , respectively. However, after 22 hours of incubation, the OD₆₀₀ values increased markedly in all treatments. The control treatment and the MOI 0.01 treatment exhibited the highest OD₆₀₀ values, which were significantly higher than those of the other treatments ($p < 0.05$). At 24 hours, OD₆₀₀ values were 1.49 (control), 1.40 (MOI 0.01), 1.24 (MOI 0.1), and 1.04 (MOI 1).

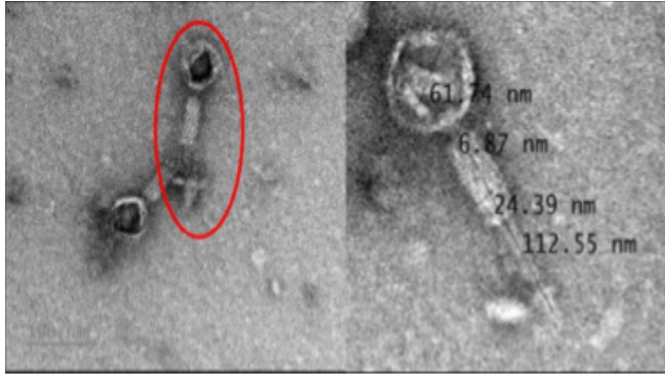


Figure 2. Transmission electron microscopy image of bacteriophage PHAGE_ECOL05 (scale bar: 100 nm).

3.2.4. Electron microscopy of PHAGE_ECOL05

TEM revealed that PHAGE_ECOL05 possessed an icosahedral head (61.74 nm in diameter) and a long contractile tail (112.55 nm long and 24.39 nm in diameter), consistent with the morphology of members of the class *Caudoviricetes* (Fig. 2). TEM imaging revealed that PHAGE_ECOL05 morphology was similar to that of phage vB_EcoM-4HA13, which infects *E. coli*, with a head diameter of 53.2 ± 2.6 nm, tail width of 11.9 ± 1.6 nm, and tail length of 116.5 ± 2.5 nm [39]. Based on the head and tail dimensions, PHAGE_ECOL05 was smaller than phage MLP1, which has a head diameter of 66 ± 0.4 nm and a contractile tail of 135 ± 0.9 nm [40]. Dien *et al.* [41] described the isolation of pAh6.2TG; it possesses an icosahedral head measuring 59.6 ± 2.5 nm and a contractile tail that is 137 ± 10.2 nm long and 20.2 ± 2.7 nm wide.

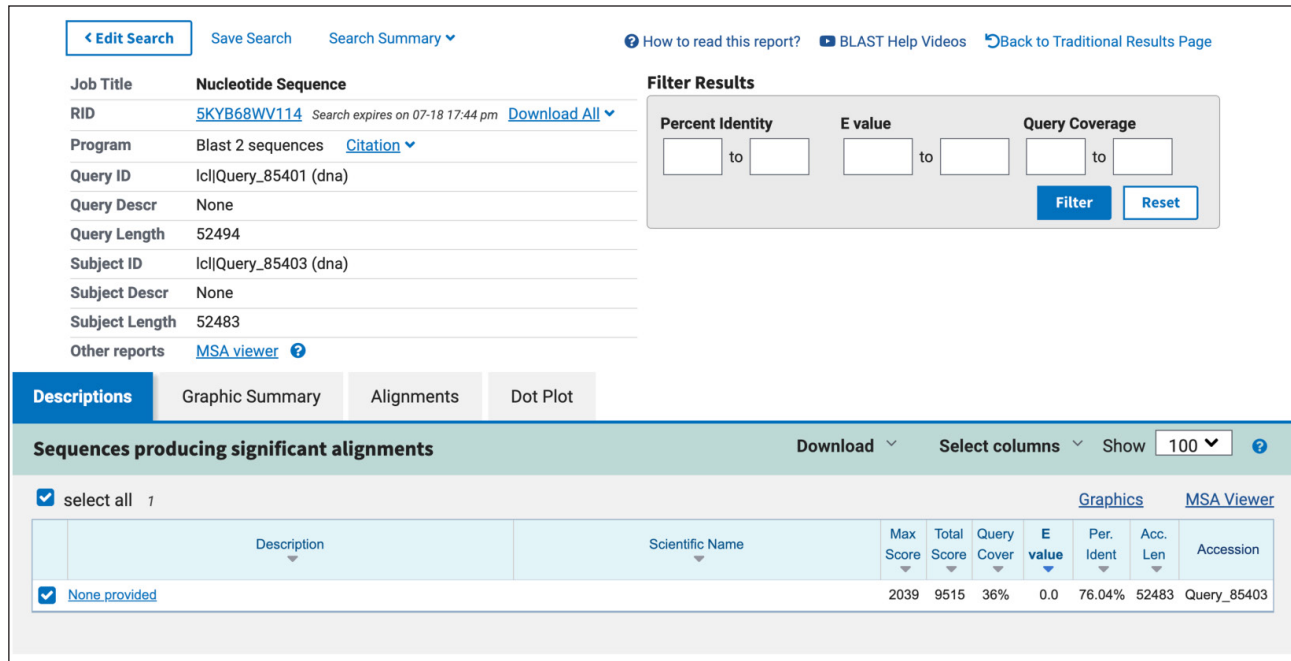


Figure 3. Pairwise BLAST comparison between the two assembled contigs showing 36% query coverage and 70.64% nucleotide identity.

Column 1	Column 2	Column 3	Column 4	Column 5	Column 6	Column 7	Column 8	Column 9
#rname	startpos	endpos	numreads	covbases	coverage	meandepth	meanbaseq	meanmapq
Contig1_length_52494_cov_997.307195	1	52494	554083	52494	100	1580.85	36.1	60
Contig2_length_52483_cov_75.320211	1	52483	41727	52483	100	119.054	36.3	60

Figure 4. Read-mapping statistics for the two assembled contigs showing 100% breadth of coverage and mean sequencing depths of 1,580.85× and 119.05× for Contig 1 and Contig 2, respectively.

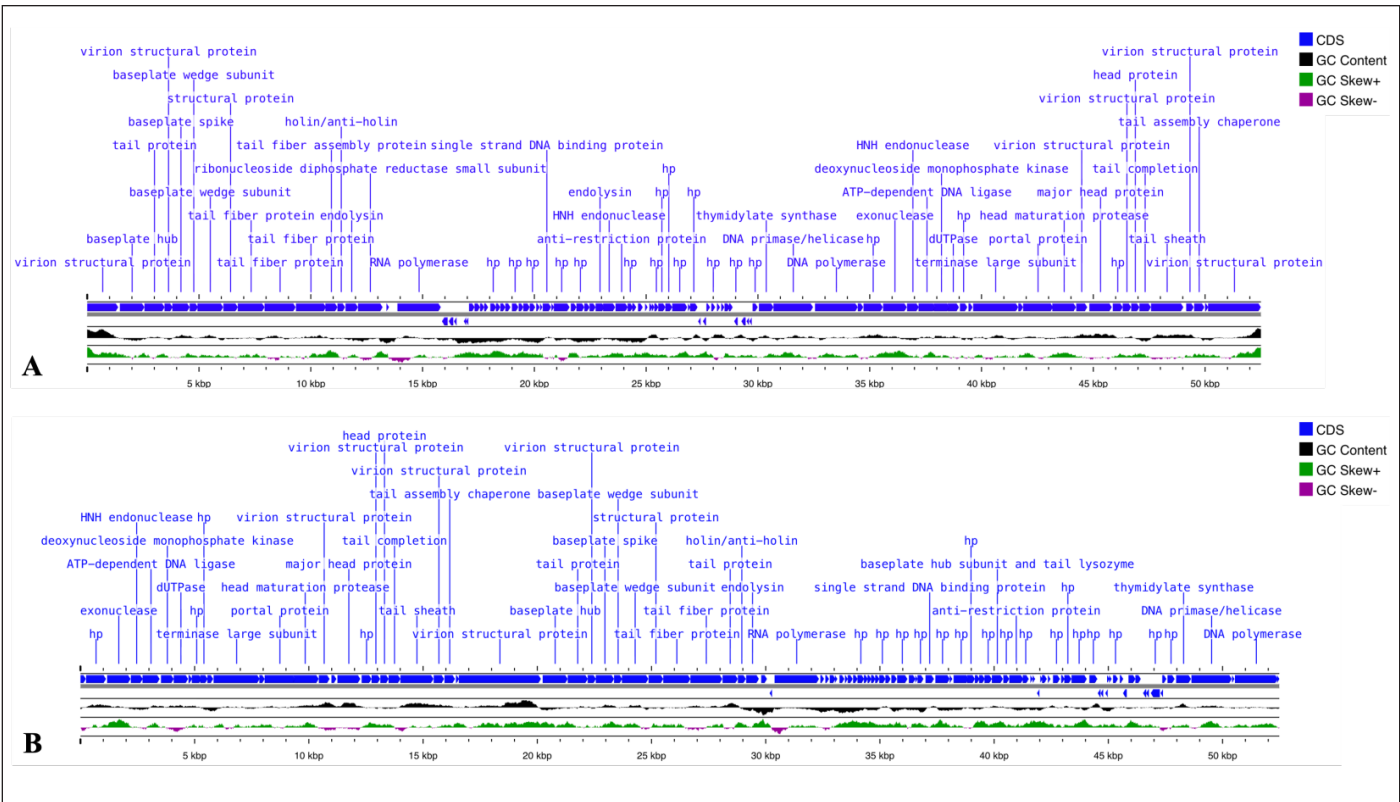


Figure 5. Comparative genome maps of the two assembled contigs of PHAGE_ECOL05. (A) Contig 1 (52,494 bp). (B) Contig 2 (52,483 bp).

Table 3. Annotation of ORFs and predicted functions in phage PHAGE_ECOL05.

ORFs	Start	End	Length (amino acids)	Molecular weight (kDa)	pI	Predicted function	Closest protein match identified using NCBI SmartBLAST	E-value
C1_ORF1	76	1383	435	46.56	5.35	virion structural protein	virion protein [<i>Escherichia</i> phage BSBP01] (XFC50498.1)	0.0E+00
C1_ORF13	11193	11534	113	12.55	7.81	holin/anti-holin	holin [<i>Escherichia</i> phage MLP1] (YP_010844446.1)	1.0E-75
C1_ORF15	12113	13213	366	42.06	4.79	ribonucleoside diphosphate reductase small subunit	MULTISPECIES: class Ia ribonucleoside-diphosphate reductase subunit beta [<i>Enterobacteriaceae</i>] (WP_000332037.1)	0.0E+00
C1_ORF18	15868	16122	84	9.87	6.38	hypothetical protein	hypothetical protein HWC55_gp82 [<i>Escherichia</i> phage Mangalitsa] (YP_009850552.1)	6.0E-16
C1_ORF28	18282	18473	63	6.8	4.5	hypothetical protein	EF-1 alpha-binding zinc finger protein Zpr1 [<i>Schizosaccharomyces pombe</i>] (NP_594291.1) ^b	9.5E-02 ^a
C1_ORF35	20086	20238	50	6.02	9.7	hypothetical protein	hypothetical protein QNH04_gp18 [<i>Escherichia</i> phage vB_EcoM_SA91KD] (YP_010844962.1)	3.0E-28
C1_ORF44	22740	23135	131	15.06	9.13	endolysin	MULTISPECIES: peptidoglycan L-alanyl-D-glutamate endopeptidase CwIK [<i>Bacillus</i>] (WP_009966473.1)	1.6E-02 ^a
C1_ORF45	23074	23616	180	20.5	9.83	HNH endonuclease	Integrase-type DNA-binding superfamily protein [<i>Arabidopsis thaliana</i>] (NP_195688.1) ^b	1.0E-08
C1_ORF54	25545	25862	105	12.36	9.15	hypothetical protein	long-chain acyl-CoA synthetase 2 [<i>Arabidopsis thaliana</i>] (NP_001321632.1) ^b	3.7E-01 ^a
C1_ORF66	28701	28871	56	6.36	6.52	Rz-like spanin	Rz1-like protein [Yersinia phage PYps23T] (YP_010844612.1)	3.0E-30
C1_ORF72	30047	30697	216	24.8	5.99	thymidylate synthase	MULTISPECIES: FAD-dependent thymidylate synthase [<i>Thermotoga</i>] (WP_004081517.1)	3.0E-29
C1_ORF73	30698	32464	588	66.16	5.86	DNA primase/helicase	Disease resistance protein (CC-NBS-LRR class) family [<i>Arabidopsis thaliana</i>] (NP_001319213.1) ^b	2.4E-02 ^a

ORFs	Start	End	Length (amino acids)	Molecular weight (kDa)	pI	Predicted function	Closest protein match identified using NCBI SmartBLAST	E-value
C1_ORF80	37857	38564	235	26.85	8.74	deoxynucleoside monophosphate kinase	Disease resistance protein (CC-NBS-LRR class) family [Arabidopsis thaliana] (NP_201492.1) ^b	3.6E-02 ^a
C1_ORF81	38485	38991	168	17.86	7.68	dUTPase	MULTISPECIES: dUTP diphosphatase [Enterobacteriaceae] (WP_000976070.1)	1.0E-25
C1_ORF90	45872	46315	147	16.47	5.53	hypothetical protein	MULTISPECIES: PBSX family phage terminase large subunit [Bacillus] (WP_003229917.1)	3.2E-02 ^a
C1_ORF91	46315	46692	125	14.14	4.59	virion structural protein	tail completion protein [Yersinia phage PYps3T] (YP_010844556.1)	3.0E-73
C1_ORF93	47061	47576	171	19.53	5.54	tail completion	tail tube protein [Yersinia phage PYps3T] (tail tube protein [Yersinia phage PYps3T])	6.0E-121
C1_ORF98	50135	52492	786	85.54	5.19	virion structural protein	hypothetical protein DDB_G0269080 [Dictyostelium discoideum AX4] (XP_646949.1) ^b	1.2E-01 ^a
C2_ORF7	4086	4712	208	21.95	6.4	dUTPase	MULTISPECIES: dUTP diphosphatase [Streptomyces] (WP_003973153.1)	2.0E-28
C2_ORF10	5241	5570	109	12.35	9.7	hypothetical protein	hypothetical protein phiEcoMGJ1_gp45 [<i>Escherichia</i> phage phiEcoM-GJ1] (YP_001595440.1)	1.0E-64
C2_ORF20	12747	13121	124	14.21	4.55	virion structural protein	virion structural protein [<i>Escherichia</i> phage phiEcoM-GJ1] (YP_001595450.1)	2.0E-70
C2_ORF24	15466	15921	151	16.46	5.22	virion structural protein	virion structural protein [<i>Escherichia</i> phage ST32] (YP_009790723.1)	5.0E-90
C2_ORF30	22207	22569	120	13.6	6.24	virion structural protein	virion structural protein [<i>Escherichia</i> phage flopper] (YP_009855587.1)	5.0E-58
C2_ORF37	28111	28779	222	24.02	6.5	tail protein	prophage tail fiber N-terminal domain-containing protein [<i>Escherichia coli</i>] (WP_000268883.1)	4.2E-02 ^a
C2_ORF38	28779	29132	117	12.92	6.04	holin/anti-holin	holin/anti-holin [<i>Escherichia</i> phage vB_ EcoM_4HA13] (YP_009884139.1)	1.0E-58
C2_ORF39	29145	29702	185	20.7	10.02	endolysin	endolysin [<i>Escherichia</i> phage vB_ EcoM_4HA13] (YP_009884140.1)	4.0E-81
C2_ORF63	36995	37366	123	13.71	4.89	single-strand DNA binding protein	MULTISPECIES: single-stranded DNA- binding protein SSB1 [Gammaproteobacteria] (WP_000168305.1)	1.9E-02 ^a
C2_ORF69	39344	39592	82	9.24	8.8	hypothetical protein	hypothetical protein [<i>Escherichia</i> phage FXie- 2024a] (WWE96996.1)	4.0E-16
C2_ORF79	42099	42305	68	7.8	9	hypothetical protein	hypothetical protein [<i>Escherichia</i> phage Ecp_ YSF] (WMM91860.1)	2.0E-04 ^a
C2_ORF83	43075	43383	102	11.34	8.93	hypothetical protein	hypothetical protein HOR42_gp29 [Pectobacterium phage PP101] (YP_009788044.1)	4.0E-35
C2_ORF102	47969	48619	216	24.7	6.18	thymidylate synthase	MULTISPECIES: FAD-dependent thymidylate synthase [Thermotoga] (WP_004081517.1)	6.0E-31
C2_ORF103	48638	50401	587	66.2	5.58	DNA primase/helicase	replicative DNA helicase [<i>Clostridioides difficile</i>] (WP_021373712.1)	9.0E-05 ^a
C2_ORF105	50543	52414	623	71.93	7.27	DNA polymerase	DNA polymerase [<i>Escherichia</i> phage vB_EcoM_ JL1] (WPF71427.1)	0.0E+00

Superscript a indicates E-value > 1.0E-5, that is, above the predefined threshold for supported SmartBLAST matches (E-value ≤ 1.0E-5); these hits are retained only as low-confidence observations and must not be described as supported homology or used independently to assign function. No separate percent-identity or query-coverage threshold was applied. Superscript b indicates a taxonomically incongruent eukaryotic top hit that was not used to infer function.

3.3. Genomic Characterization of Viral Contigs Recovered from the PHAGE_ECOL05 Preparation

DNBSEQ-G99 paired-end sequencing was performed on DNA extracted from a plaque-purified PHAGE_ECOL05 preparation. *De novo* assembly generated two viral contigs, which were subsequently evaluated and analyzed independently. The two contigs were

deposited in GenBank under accession numbers PV904263 and PV904264, respectively. Pairwise BLAST comparison between the two contigs showed 36% query coverage and 70.64% nucleotide identity, indicating limited sequence similarity between the contigs (Fig. 3). Together with the independent read-mapping results and the absence of an assembly graph connection, these findings did not support treating the two contigs as a single assembled genome.

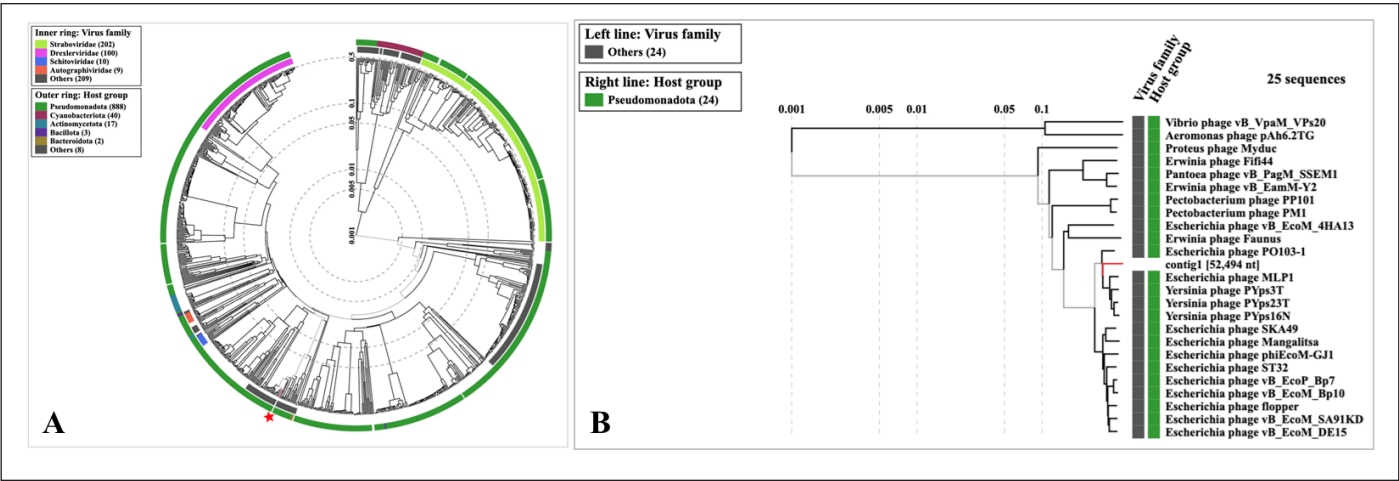


Figure 6. Whole-proteome phylogenetic analysis of contig1 using ViPTree. (A) Proteomic tree showing the phylogenetic position of contig1 (52,494 bp) among reference viral genomes. The inner ring indicates viral families, whereas the outer ring represents the bacterial host phyla. (B) Enlarged phylogenetic tree showing Contig 1 and the 24 closest Pseudomonadota-infecting reference bacteriophages (25 sequences in total).

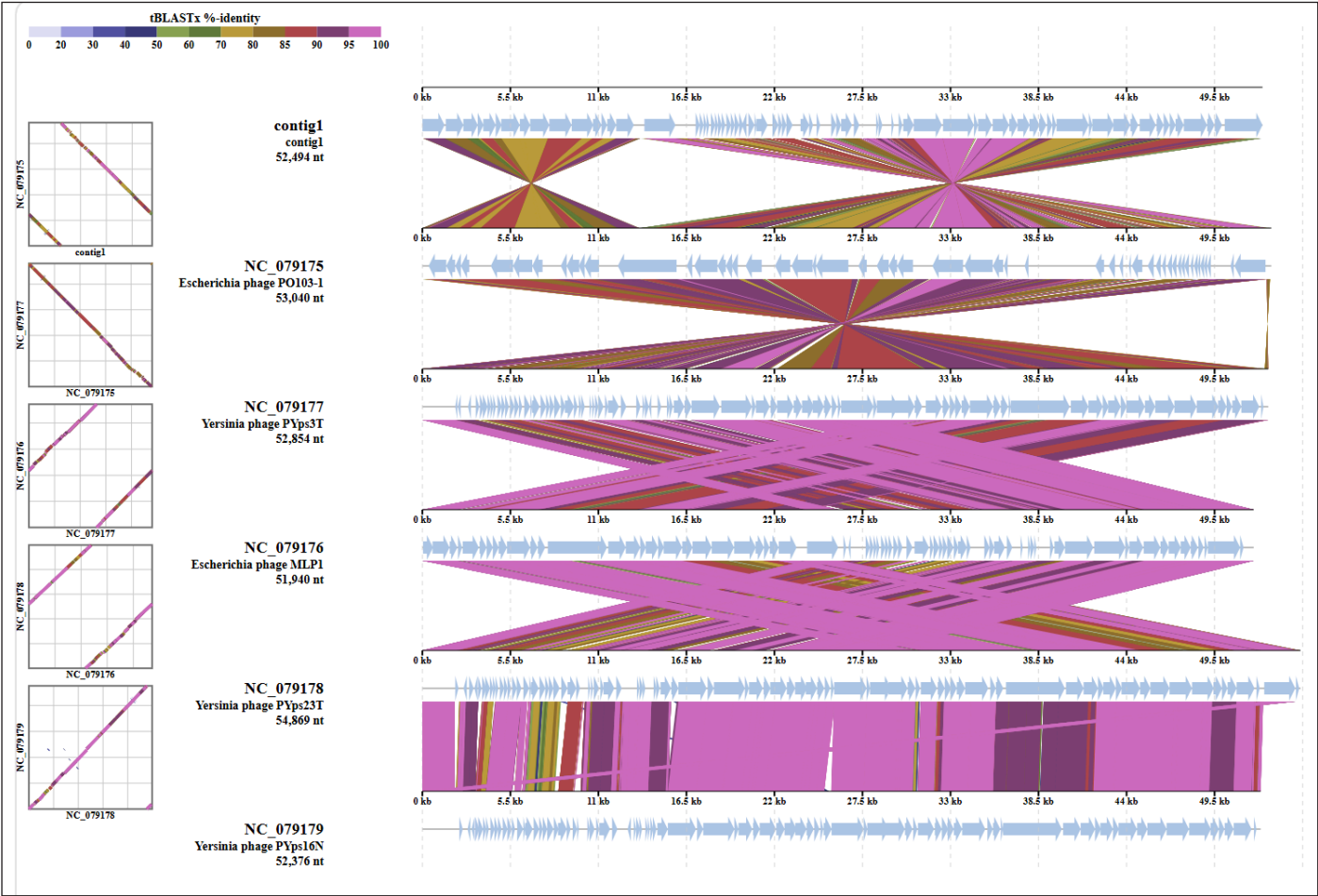


Figure 7. Whole-genome comparison of contig1 with closely related bacteriophages using tBLASTx. Homologous genomic regions identified by tBLASTx are connected by colored blocks. The color scale indicates the percentage of sequence identity, with purple representing the highest sequence similarity and lighter colors representing lower levels of sequence similarity.

In addition, quality-filtered paired-end reads were independently mapped to each contig, demonstrating 100% breadth of coverage with average sequencing depths of 1,580.85× and 119.05× for

Contig 1 and Contig 2, respectively (Fig. 4). Furthermore, assembly graph visualization using *Bandage* revealed no graph connection or bridging path between the two contigs. *CheckV* estimated 100%

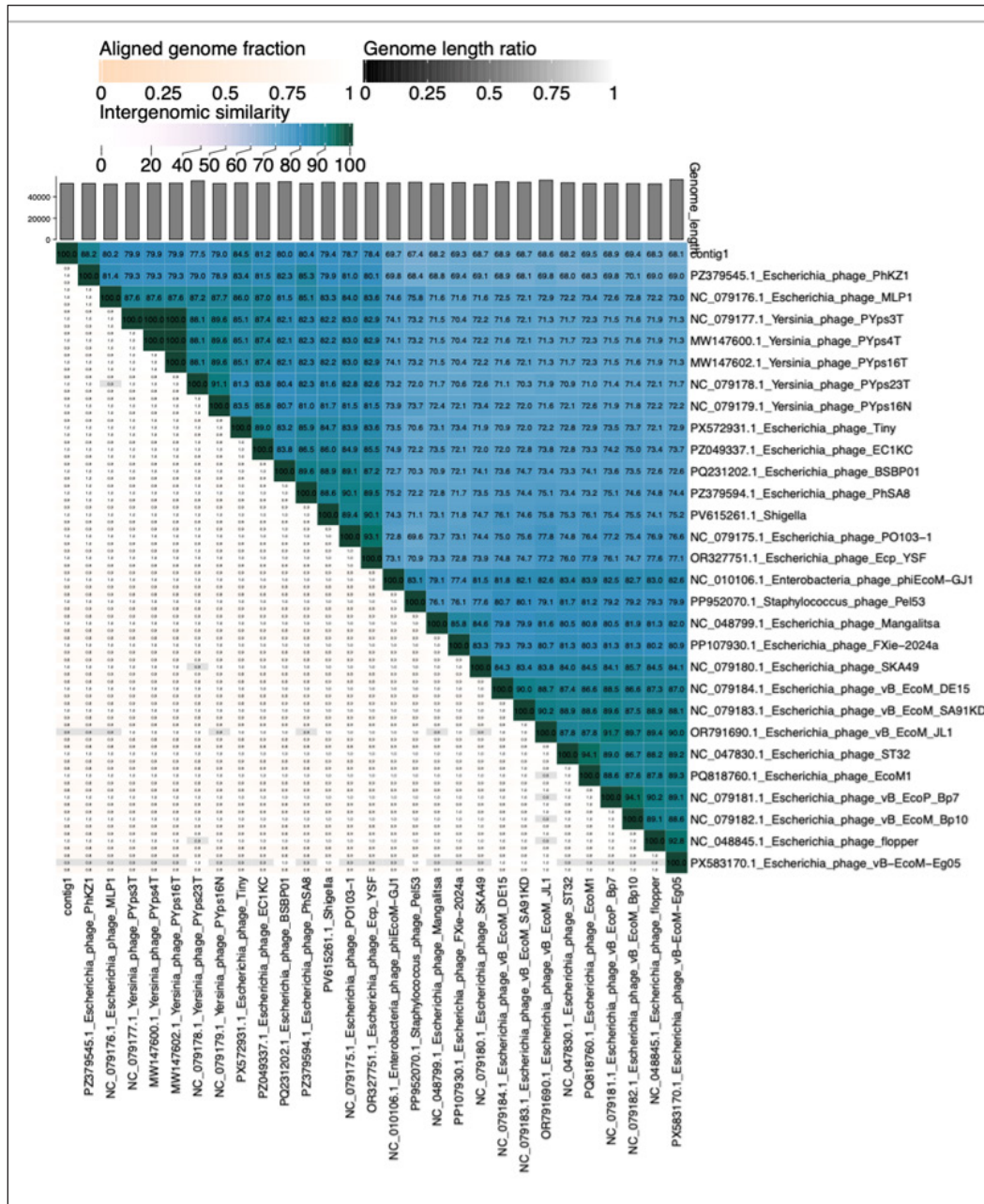


Figure 8. Heatmap showing the intergenomic similarity of Contig 1 and reference bacteriophages generated using VIRIDIC.

completeness for each contig at the individual-contig level and classified both sequences as high-quality viral sequences. These estimates do not indicate that either contig represents the complete PHAGE_ECOL05 genome or that the two contigs together constitute a single complete genome.

A total of 204 ORFs were predicted across the two viral contigs, with 98 ORFs identified in Contig 1 and 106 in Contig 2. To facilitate genome annotation and comparison, ORFs in Contig 1 were designated C1_ORF1 to C1_ORF98, whereas those in Contig 2 were designated C2_ORF1 to C2_ORF106 (Fig. 5). Contig 1 was 52,494 bp in length with a GC content of 43.83%, whereas Contig 2 was 52,483 bp with a GC content of 45.44%. No tRNA or rRNA genes were identified in either contig. Functional annotation assigned the

predicted ORFs to five major functional categories, including the early, middle, and late transcriptional regions, the lysis module, and hypothetical protein-coding regions. Among the 204 predicted ORFs, 121 (59.31%) were annotated as hypothetical proteins, including 56 in Contig 1 and 65 in Contig 2. Of these, only nine hypothetical proteins (five in Contig 1 and four in Contig 2) produced SmartBLAST matches to proteins with annotated functions, whereas the remaining hypothetical proteins either matched uncharacterized proteins or lacked supported functional annotation. Most ORFs were predicted to initiate with the canonical ATG start codon (82.35%), followed by TTG (9.80%) and GTG (7.35%). C2_ORF1 was the only ORF predicted to utilize the rare CTG start codon (0.49%). In total, 33 ORFs produced SmartBLAST matches; 22 met the predefined *E*-value threshold ($\leq 1.0E-5$), whereas 11 were retained

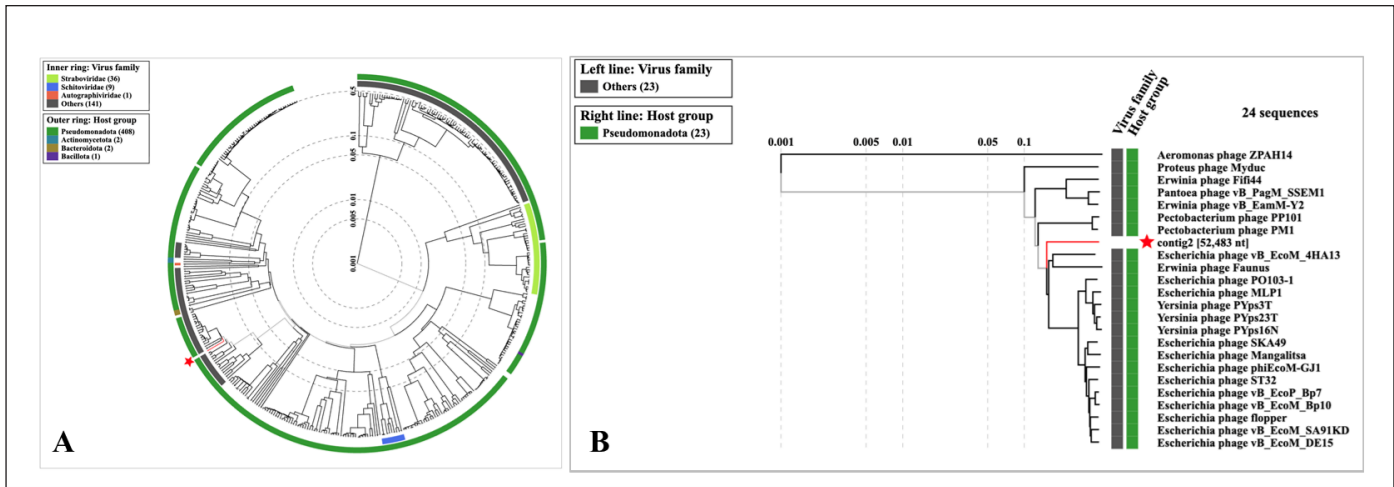


Figure 9. (A) Proteomic tree showing the phylogenetic placement of Contig 2 (52,483 bp) within the ViPTree reference database. The inner ring indicates viral families, whereas the outer ring represents the bacterial host phyla. (B) Expanded phylogenetic tree of the 24 closest bacteriophages infecting members of the phylum Pseudomonadota, showing the phylogenetic position of Contig 2 (highlighted in red).

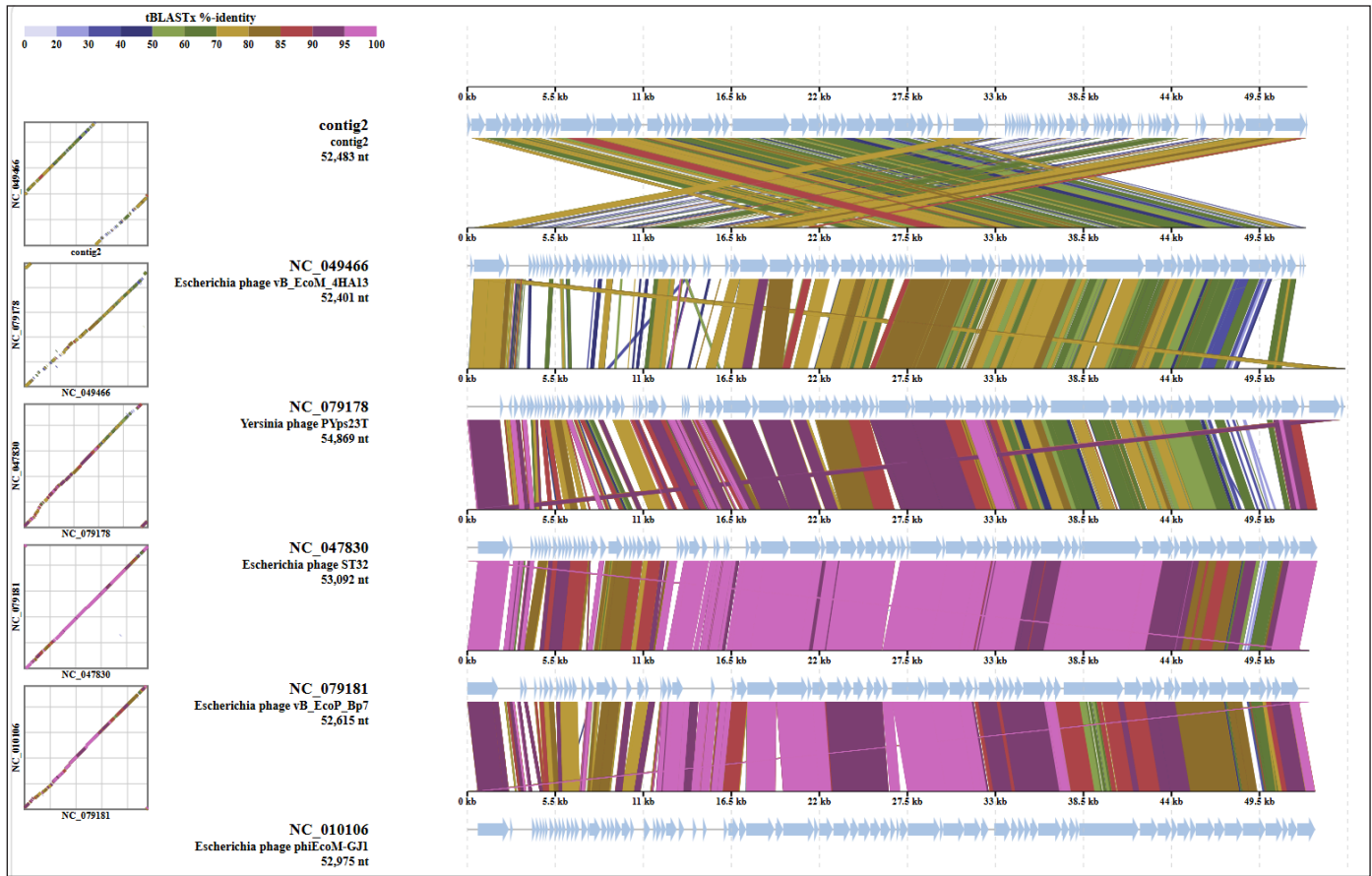


Figure 10. Whole-genome comparison of Contig 2 with closely related bacteriophages using tBLASTx. Homologous genomic regions identified by tBLASTx are connected by colored blocks. The color scale indicates the percentage of sequence identity, with purple representing the highest sequence similarity and lighter colors representing lower levels of sequence similarity.

only as low-confidence observations (Table 3). The predominance of hypothetical proteins highlights the substantial proportion of phage genetic content that remains functionally uncharacterized, a feature commonly observed in bacteriophage genomes.

3.4. Phylogenetic Analysis Based on Whole-Genome Proteome

Whole-proteome phylogenetic analyses were performed independently for Contig 1 (52,494 bp) and Contig 2 (52,483 bp) using ViPTree to investigate their taxonomic positions and

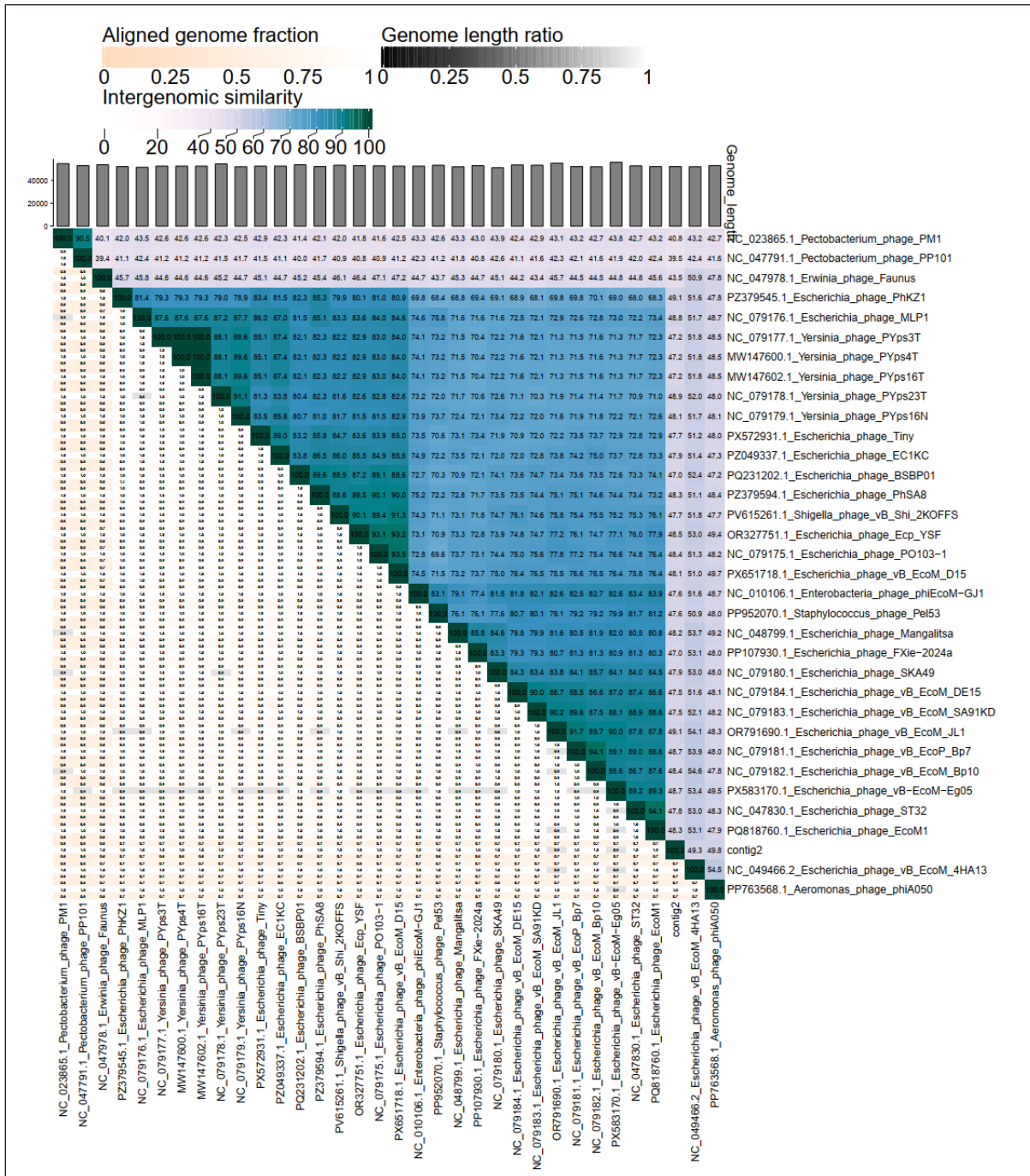


Figure 11. Heatmap showing the intergenomic similarity of Contig 2 and reference bacteriophages generated using *VIRIDIC*. Pairwise intergenomic similarities are shown as percentages.

evolutionary relationships (Figs. 6, 9). In the proteome-based phylogenetic tree (Figs. 6A, 9A), both contigs clustered with bacteriophages infecting bacterial hosts belonging to the phylum *Pseudomonadota*, indicating their affiliation with phages infecting Gram-negative bacteria. Both contigs were consistently placed within the family *Chaseviridae*, supporting their taxonomic assignment to this family.

A refined phylogenetic tree including representative phages with similarity scores of $S_g > 0.15$ further resolved the evolutionary relationships of the two contigs (Figs. 6B, 9B).

Contig 1 clustered most closely with *Escherichia* phage PO103-1 ($S_g = 0.7654$), followed by *Yersinia* phage PYPs3T ($S_g = 0.7646$), *Escherichia* phage MLP1 ($S_g = 0.7576$), *Yersinia* phage PYPs23T ($S_g = 0.7545$), and *Yersinia* phage PYPs16N ($S_g = 0.7503$), forming a well-supported clade corresponding to the genus *Carltongylsvirus*. tBLASTx comparison between Contig 1 and these closely related phages (Fig. 7) revealed extensive sequence synteny, with sequence identities exceeding 90% across conserved genomic regions despite several localized genomic rearrangements. These findings indicate that Contig 1 shares a highly conserved genome organization with members of the

same evolutionary lineage. The *VIRIDIC* intergenomic similarity analysis further supported the phylogenomic relationships (Fig. 8). Contig 1 shared 68%–88% intergenomic similarity with representative members of the genus *Carltongylesvirus*, with the highest similarities observed for *Escherichia* phage PO103-1, *Escherichia* phage Ecp_YSF, and several closely related *Yersinia* phages. Together with its phylogenetic placement, these results support the assignment of Contig 1 to the genus *Carltongylesvirus*. The highest *VIRIDIC* intergenomic similarity between Contig 1 and the currently available reference phages was 88%, which was below the 95% species-level demarcation threshold. Therefore, Contig 1 may represent a distinct species-level lineage within the genus *Carltongylesvirus*. However, this interpretation remains provisional and does not constitute formal taxonomic recognition by the ICTV.

In contrast, Contig 2 formed a sister lineage to the *Carltongylesvirus* clade within the family *Chaseviridae* (Fig. 9B). It exhibited the highest proteome similarity to *Escherichia* phage vB_EcoM_4HA13 ($S_g = 0.5040$), followed by *Yersinia* phage PYps23T ($S_g = 0.4968$), *Escherichia* phage ST32 ($S_g = 0.4836$), *Escherichia* phage vB_EcoM_Bp7 ($S_g = 0.4833$), and *Escherichia* phage phiEcoM-GJ1 ($S_g = 0.4828$). tBLASTx comparison between Contig 2 and these reference phages (Fig. 10) demonstrated extensive conservation of homologous genomic regions, with high sequence identities across most coding regions. However, differences in the distribution and orientation of homologous blocks indicate substantial genome rearrangements while maintaining an overall genome organization consistent with this evolutionary lineage. The *VIRIDIC* intergenomic similarity analysis further supported the observed genomic divergence (Fig. 11). Contig 2 shared only 40%–55% intergenomic similarity with its closest reference phages, with the highest similarity observed for *Escherichia* phage vB_EcoM_4HA13. This relatively low intergenomic similarity indicates that Contig 2 is substantially divergent from currently recognized members of the genus *Carltongylesvirus*. Although its phylogenetic position indicates a close evolutionary relationship with members of *Chaseviridae*, the observed intergenomic similarity was insufficient to support confident assignment of Contig 2 to a currently recognized genus. Therefore, Contig 2 was conservatively left unassigned at the genus level.

Collectively, the independent proteome-based phylogenetic and intergenomic similarity analyses supported the placement of both contigs within the family *Chaseviridae*. Contig 1 was consistent with assignment to the genus *Carltongylesvirus*, whereas Contig 2 showed substantial divergence from currently recognized genera within *Chaseviridae* and could not be confidently assigned to a recognized genus based on the present data.

4. DISCUSSION

Phages create transparent lytic zones, known as plaques [42]. In this study, the isolated phages produced circular plaques on susceptible *E. coli* strains. These plaques were characterized by transparent centers and diffuse, turbid margins, with mean diameters ranging from 2.0 to 2.5 mm. Lai *et al.* [43] showed that the morphology of plaques with a clear center and a diffuse, turbid halo may result from the depolymerization of bacterial exopolysaccharides or host cell surface polysaccharides into oligosaccharide units during the infection process. This phenomenon occurs because bacteriophages produce polysaccharide depolymerase enzymes that diffuse beyond the lytic zone, degrading extracellular or host surface polysaccharides and generating a halo surrounding the plaque. In addition, variations in

halo appearance may be influenced by reduced phage lytic activity associated with the aging of host bacterial cells [44]. In addition, plaque size typically increases over time as bacterial cells continue to be lysed. Conversely, a slow increase in plaque diameter may reflect the slow growth of the host bacteria, indicating that the culture has entered a stationary phase [45].

PHAGE_ECOL05 was able to lyse 8 strains of *E. coli* out of 19 tested and thus is the phage with the broadest host range among those evaluated in this study and a potential candidate for further investigation as a food biocontrol agent against *E. coli*. In contrast, PHAGE_ECOL02 and PHAGE_ECOL03 were able to lyse 1 out of 19 strains each, indicating that they are specific to phages with a narrow host range. All the above demonstrate that the phages collected do exhibit some breadth of parasitic range in comparison to one another. One single bacterium can be a host to different phages, while a single phage can infect multiple bacterial strains [46]. The observation indicated that several phages could infect a single bacterial strain, whereas a single phage could also infect multiple bacterial strains. In many of its applications, a phage that has a broad-host range is of great importance [47]. In phage therapy, a broad-host-range phage relates to a phage that attacks several strains of a bacterium. Such a phage works like a broad-spectrum antibiotic. At present, a number of phage products are manufactured in the form of cocktails in order to broaden the range of the phages as well as to improve the effectiveness of the treatment [48].

These results support the findings of Lin *et al.* [39] and Balcão *et al.* [49], which reported greater levels of bacterial inhibition with increased MOI levels. As explained by Abedon [50], the MOI is the ratio of infecting phage particles to the number of host bacterial cells. It can be adjusted by changing the relative cell densities [51]. A decrease in optical density due to cell lysis is one of the earliest indicators of phage infection in culture. OD began to decline after 2 hours of incubation, suggesting effective *in vitro* bacterial killing by the phages shortly after infection. Lysis efficiency largely depends on phage concentration. At MOI 1, the decline in optical density was steep, lysis was complete in 4–6 hours, and OD was at baseline, suggesting active lysis and high phage-host contact rates [52]. This suggests that phages can readily bind to host receptors, initiate infection, and cause bacterial lysis, resulting in the release of progeny phages. During the study's course, OD₆₀₀ fell significantly in the MOI 1, 0.1, and 0.01 treatments after 6 hours. Further investigations assessing the effectiveness of phages have yielded similar outcomes [53]. In the research of Malik *et al.* [53], it was noted that single phages inhibited bacterial growth for only 6 to 8 hours, while phage cocktails inhibited the growth of *E. coli* for 12 hours. Similarly, Liao *et al.* [22] showed a complete 5.5-log reduction of *E. coli* O45:H16 within 6 hours at 25°C, followed by regrowth 24 hours after. The decline in antibacterial activity observed after 6 hours may be partly associated with the emergence of phage-resistant bacterial subpopulations [54]. Consistent with this possibility, Yang *et al.* [55] reported high-density bacterial regrowth after 12–24 hours following treatment with phages PAO1 and PA1, suggesting the possible emergence of phage-resistant bacterial populations. However, this possibility was not investigated in the present study, and alternative mechanisms, including phenotypic tolerance [56] or bacterial persistence [57], may also have contributed to the observed bacterial regrowth. Therefore, the application of phage cocktails containing phages with different host ranges or receptor specificities may represent a potential strategy to delay the development of phage resistance and improve antibacterial efficacy.

Although PHAGE_ECOL05 was isolated from a single purified plaque, sequencing of the plaque-purified preparation yielded two

viral contigs that were independently evaluated. Pairwise sequence comparison, read mapping, assembly graph analysis, and *CheckV* consistently supported the quality and completeness of each contig at the individual-contig level. Nevertheless, because only DNBSEQ-G99 short-read sequencing was performed, their biological relationship could not be conclusively resolved. The absence of an assembly graph connection, together with the limited sequence similarity between the contigs, did not provide sufficient evidence to regard the two contigs as a single assembled genome. Accordingly, the two contigs were analyzed independently in the present study. Similar situations have occasionally been reported for bacteriophage assemblies generated exclusively from short-read data, highlighting the need for long-read sequencing or targeted PCR to determine whether the contigs represent distinct viral genomes, unresolved genomic structures, or closely associated viral populations.

The phage preparation used for genome sequencing was purified by acetone precipitation to obtain sufficient viral particles for DNA extraction. Although this procedure may reduce phage infectivity under mildly acidic conditions, it was performed only after all biological characterization experiments had been completed. Therefore, it did not influence the phenotypic results reported in this study.

Genome annotation was performed independently for the two assembled contigs recovered from the PHAGE_ECOL05 preparation and revealed predicted functional modules that can be broadly categorized into transcription-, DNA replication/repair-, virion morphogenesis/packaging-, lysis-, and hypothetical protein-associated functions.

Several ORFs identified across the two contigs were predicted to be associated with transcription initiation and nucleotide or DNA metabolism [58]. C1_ORF17 and C2_ORF42 were predicted to encode RNA polymerase, suggesting possible autonomous transcription, although no corresponding database matches were identified. C1_ORF46 is predicted to encode an anti-restriction protein. C1_ORF72 and C2_ORF102 are predicted to encode thymidylate synthase, whereas C1_ORF73 and C2_ORF103 were annotated as DNA primase/helicase candidates. SmartBLAST searches of the predicted proteins returned strong matches for C1_ORF72 and C2_ORF102 to FAD-dependent thymidylate synthases from *Thermotoga* species (E-value = 3.0E-29 and 6.0E-31, respectively). By contrast, the top matches reported for C1_ORF73 (a disease-resistance protein from *Arabidopsis thaliana*; E-value = 2.4E-02) and C2_ORF103 (a replicative DNA helicase from *Clostridioides difficile*; E-value = 9.0E-05) exceeded the working E-value criterion for supported matches used in this study ($\leq 1.0E-5$) and were not used independently to support the predicted primase/helicase assignments.

Several ORFs were annotated as candidates involved in DNA replication, repair, or DNA metabolism. These included DNA polymerase (C1_ORF74 and C2_ORF105), ATP-dependent DNA ligase (C1_ORF79 and C2_ORF5), HNH endonuclease (C1_ORF45, C1_ORF78, and C2_ORF4), exonuclease (C1_ORF77 and C2_ORF3), and a putative host RecBCD inhibitor (C2_ORF8). C2_ORF105 showed a strong SmartBLAST match to the DNA polymerase of *Escherichia* phage vB_EcoM_JL1 (WPF71427.1; E-value = 0.0E+00). C1_ORF45, C1_ORF78, and C2_ORF4 were predicted as HNH endonucleases; however, the top match reported for C1_ORF45 was an integrase-type DNA-binding superfamily protein from *Arabidopsis thaliana* (E-value = 1.0E-08). Because this eukaryotic top hit is taxonomically incongruent, it was not used alone to assign function. The ATP-dependent DNA ligase and RecBCD-inhibitor predictions lacked corresponding matches in the reported database search and therefore remain annotation-based predictions requiring experimental confirmation.

Several ORFs across the two contigs were predicted to encode proteins associated with virion morphogenesis and DNA packaging [59]. These included putative major head, portal, head-maturation protease, tail-sheath, baseplate-hub, baseplate-spike, baseplate-wedge, tail-completion, and related structural proteins. The tail-fiber and baseplate-wedge functions were each assigned to five ORFs, whereas tail-protein functions were assigned to C1_ORF3, C2_ORF29, and C2_ORF37. C1_ORF93 showed a strong match to the tail-tube protein of *Yersinia* phage PYps3T (E-value = 6.0E-121). The reported match for C2_ORF37 to an *E. coli* prophage tail-fiber N-terminal domain-containing protein was weak (WP_000268883.1; E-value = 4.2E-02) and was therefore treated as low-confidence support rather than supported homology. Tail-fiber assembly protein C1_ORF12 and tail-assembly chaperones C1_ORF96 and C2_ORF25 were predicted, but no corresponding database matches were reported. The terminase large subunit (TerL) is central to packaging viral DNA into the capsid [60]. TerL candidates were predicted on both contigs, but no corresponding database matches were reported.

Several ORFs across the two contigs were predicted to encode proteins associated with bacterial cell-envelope degradation and phage-mediated lysis. Putative holin/anti-holin proteins encoded by C1_ORF13 and C2_ORF38 may regulate pore formation in the bacterial inner membrane [61]. C1_ORF13 showed strong similarity to the holin of *Escherichia* phage MLP1 (YP_010844446.1; E-value = 1.0E-75), whereas C2_ORF38 matched the holin/anti-holin of *Escherichia* phage vB_EcoM_4HA13 (YP_009884139.1; E-value = 1.0E-58). C1_ORF44 and C2_ORF39 were annotated as endolysin candidates. C1_ORF44 returned only a weak match to peptidoglycan L-alanyl-D-glutamate endopeptidase CwlK from *Bacillus* (WP_009966473.1; E-value = 1.6E-02), so that match alone does not support a confident functional assignment. C2_ORF39 instead showed strong similarity to the endolysin of *Escherichia* phage vB_EcoM_4HA13 (YP_009884140.1; E-value = 4.0E-81). An Rz-like spanin is expected to disrupt the outer membrane of Gram-negative bacteria during final lysis; among the predicted spanin-associated ORFs, C1_ORF66 showed strong similarity to the Rz1-like protein of *Yersinia* phage PYps23T (YP_010844612.1; E-value = 3.0E-30).

ORFs annotated as hypothetical proteins were distributed across the two viral contigs of bacteriophage PHAGE_ECOL05, indicating that a substantial proportion of the recovered viral genetic content remains functionally uncharacterized. Of the 204 predicted ORFs, 121 (59.31%) were annotated as proteins of unknown function. This high proportion may reflect the limited representation and functional characterization of genes associated with phages in existing databases. Across the two contigs, 33 ORFs yielded protein matches through NCBI SmartBLAST (Table 3). Of these, 22 met the working E-value criterion used for interpretation ($\leq 1.0E-5$), whereas the remaining 11 were retained only as low-confidence observations. Only nine hypothetical protein ORFs, including five in Contig 1 and four in Contig 2, matched proteins with assigned functions. The other matches involved uncharacterized proteins or lacked sufficient support for confident functional assignment. Weak or taxonomically incongruent matches, including top matches to eukaryotic proteins, were not used to infer function. These uncharacterized ORFs may represent genetic elements that are specific to phages or remain poorly characterized. However, their biological functions cannot be inferred from the present data.

TEM revealed that PHAGE_ECOL05 possessed an icosahedral head and a contractile tail, consistent with the general morphology of tailed phages within the class *Caudoviricetes*. Independently, phylogenomic analyses placed both recovered viral contigs within the family *Chaseviridae*. However, TEM does not link the observed phage

particle to either assembled contig, and the available short-read data do not establish a physical relationship between the two sequences. Therefore, the morphological and genomic evidence cannot be used to conclude that both contigs belong to a single PHAGE_ECOL05 genome.

Each contig was supported by 100% read coverage, substantial sequencing depth, and 100% estimated completeness by *CheckV*, indicating that both are individually well-supported viral sequences. Nevertheless, the absence of a connecting path in the assembly graph and the limited sequence similarity between them provide no evidence that they form a single continuous genome. Accordingly, the two contigs were analyzed and interpreted independently, and their combined length of 104,977 bp should be regarded only as the total length of the recovered viral sequences, rather than as the confirmed genome size of PHAGE_ECOL05. Long-read sequencing, targeted PCR, or other linkage-based approaches will be required to determine whether the two contigs originate from the same phage genome or represent distinct viral genomes in the plaque-purified preparation.

In conclusion, this study successfully isolated and characterized the lytic bacteriophage PHAGE_ECOL05 isolated from Nem chua and capable of infecting *E. coli*. PHAGE_ECOL05 exhibited the broadest host range among the isolated phages and showed the strongest *in vitro* lytic activity at an MOI of 1. Comparative phylogenomic analyses independently placed both viral contigs within the family *Chaseviridae*, with Contig 1 consistent with assignment to the genus *Carltongylsvirus* and Contig 2 showing substantial divergence from currently recognized genera within *Chaseviridae* and not being confidently assignable to a recognized genus based on the present data. The absence of predicted virulence-associated or antibiotic resistance-associated genes in either contig provides preliminary genomic safety information for the viral sequences recovered from the plaque-purified PHAGE_ECOL05 preparation. Overall, the biological characteristics of PHAGE_ECOL05 and the genomic features of the recovered viral contigs support further investigation of this phage preparation as a potential food biocontrol candidate. Further investigations using long-read sequencing and functional characterization are warranted to resolve the relationship between the two viral contigs and further assess the application potential of this phage preparation.

5. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be authors as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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7. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

8. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

9. DATA AVAILABILITY

All the data are available with the authors and shall be provided upon request.

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11. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors used ChatGPT (OpenAI, GPT-5.6 Sol) solely for English-language editing to improve the grammar, clarity, and readability of the manuscript. The AI-assisted tool was not used for study design, generation of experimental data, genomic or bioinformatic analyses, interpretation of results, formulation of scientific conclusions, or generation or manipulation of images. All AI-suggested language edits were critically reviewed, revised, and verified by the authors before inclusion in the manuscript. All scientific content, study design, data analyses, and interpretations were conducted entirely by the authors. The authors carefully reviewed and approved the final version and take full responsibility for the accuracy, integrity, and scientific content of the manuscript.

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