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Assembly and Annotation of the Complete Genome Sequence of the *Paenibacillus* Bacteriophage phJNUCC32

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Abstract: A potential biocontrol agent for American foulbrood (AFB), the *Paenibacillus* bacteriophage phJNUCC32, was isolated from Baengnokdam in Halla Mountain. This study aimed to investigate its genomic characteristics through whole-genome sequencing. The genome of phJNUCC32 was found to be 62,871 base pairs in length, with a G + C content of 51.98%. Phylogenetic analysis classified phJNUCC32 within the unclassified Caudoviricetes bacteriophage category. The genome prediction confirmed the absence of virulence factors and antibiotic-resistance genes, ensuring its genetic safety. A total of 63 coding DNA sequences were identified, revealing a modular arrangement. Notably, the annotation of gene function indicates that phJNUCC32 harbors the holin/lysin system, suggesting significant potential for controlling bacterial infections in AFB and agriculture.

Keywords: *Paenibacillus* bacteriophage; whole-genome sequencing; American foulbrood; holin/lysin system

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1. Introduction

Bacteriophages, viruses infecting various microorganisms, were discovered by Twort and d'Herelle in 1915 and 1917 [1]. With unique host specificity, they combat bacterial infections and are extensively used in medicine, dentistry, and agriculture. Molecular biology advancements have deepened our understanding of bacteriophages, aiding in their application as antibiotic alternatives. Leveraging their specificity, self-replication, and low cost, they are widely employed in treating infections in humans, animals, plants, and environmental decontamination.

Some species, such as *Paenibacillus larvae*, *Paenibacillus apiarius*, and *Paenibacillus glabratella*, are pathogens to honeybees and other invertebrates, with *P. larvae* causing lethal intestinal infections [2,3]. *P. larvae* is a Gram-positive bacterium responsible for inducing American foulbrood, a significant affliction in apiculture [4]. AFB detrimentally affects honeybee larvae, exacerbates colony collapse disorder, and diminishes agricultural productivity. In particular, strains of *P. larvae* are increasingly exhibiting resistance to antibiotics [5]; bacteriophages targeting and lysing *P. larvae* present a potentially promising avenue for therapeutic intervention. In summary, reported *Paenibacillus* bacteriophages include the *P. larvae* phage phiIBB_Pl23 [6]; five *P. larvae* bacteriophages from soil [7]; nine *P. larvae* bacteriophages from soil, propolis, and infected bees [8]; and eighteen *P. larvae* phages from the western United States [9], etc. The results of the genomic analysis of 48 *P. larvae* bacteriophages reveal that all phage genomes display a conserved N-acetylmuramoyl-l-alanine amidase, serving as an endolysin [10].

The holin/lysin system in bacteriophages inhibits the impact of *P. larvae* on honeybee larvae through a collaborative mechanism [11]. Endolysin, primarily sourced from phages targeting Gram-positive bacteria [12], serves as a key component of the holin/lysin system; it participates in the bacteriophage's infection and cell lysis process by regulating the

localization of endolysins to the specific cleavage sites of "cross-links" within the peptidoglycan (PG) layer of bacterial cell walls through the accumulation of holins and creation of lesions in the cytoplasmic membrane, ultimately triggering host cell lysis at a specific time point [13–15].

In previous studies, the majority of bacteriophages utilize endolysin to enzymatically degrade the PG layer of the host bacterium, exhibiting anti-biofilm properties. This includes the natural lysis of *Salmonella* enteritidis by the endolysin of the bacteriophage vB_Sal-S-S10 [16], the endolysin Ply113 acting as a potent antibacterial agent against polymicrobial biofilms formed by *enterococci* and *Staphylococcus aureus* [17], and the endolysin from *Staphylococcus aureus* bacteriophage 52 showing anti-biofilm and broad antibacterial activity against Gram-positive bacteria [18]. Additionally, endolysins such as LysAB1245 targeting different capsular types associated with *Acinetobacter* exhibit extended lytic activity [19], while endolysins LysCPD2 and LysCPQ7 act as biocontrol agents against *Clostridium perfringens* [20,21], etc.

The holin/lysin system offers a sustainable and environmentally friendly approach to selectively eradicate target pathogens without harming beneficial microorganisms [22]. Operating through physical mechanisms that disrupt bacterial cell membranes and walls rather than chemical agents, it minimizes the likelihood of inducing resistance, making it promising for controlling drug-resistant pathogens [23]. Its mechanism of action leads to rapid lysis and dissolution of bacterial cells, releasing numerous bacteriophage particles, thus swiftly reducing pathogen populations and aiding in disease control [24]. Compared to chemical pesticides, it reduces reliance on chemical substances and minimizes environmental pollution, aligning with principles of sustainable agriculture and environmental protection.

This study isolated a potential strain, *Paenibacillus* bacteriophage phJNUCC32, from Baengnokdam in Halla Mountain and conducted whole-genome sequencing. Through sequence comparisons, gene function annotation of the bacteriophage, as well as predictions of virulence factors and antibiotic-resistance genes, were conducted, resulting in the identification of genes with potential therapeutic effects against American foulbrood.

2. Materials and Methods

2.1. Bacterial Isolation

The Paenibacillus bacteriophage phJNUCC32 was isolated from Baengnokdam, Mt. Halla, South Korea, in September 2019. Soil samples (0.5 g) were suspended in 0.45 mL of 0.1% tris-buffer and shaken (180 rpm, 30 °C, 1 h). After serial dilution (10^{-5} to 10^{-9}), 100 µL of the suspension was spread onto MRS medium (pH 6.5). Routine culture involved aerobic growth on LB solid/liquid medium at 30 °C for 1 day, with storage in 20% glycerol at −80 °C [25].

2.2. Sequencing and De Novo Assembly

The strain phJNUCC32's DNA was extracted using a QIAGEN genomic-tip and sequenced with PacBio RSII and Illumina at Macrogen, Inc. (Seoul, Republic of Korea). K-mer analysis was performed to estimate the genome size of the sample. The k-mer distribution of the genome was analyzed using Jellyfish (v2.2.10), and GenomeScope 2.0. HGAP (v3.0) first assembled PacBio long reads. Then, Illumina reads were used to refine genome sequence accuracy with Pilon (v1.21). Finally, subreads were mapped against contigs to generate consensus sequences with coverage depth data.

2.3. Genome Annotation and Phylogenetic Tree

We used the online tool PHASTEST (PHAge Search Tool with Enhanced Sequence Translation) (https://phastest.ca/, accessed on 5 March 2024) to predict functional annotations of the genome of the bacteriophage phJNUCC32. Antibiotic-resistance gene prediction was performed by submitting the genome of the bacteriophage phJNUCC32 to the online database (http://arpcard.mcmaster.ca, accessed on 21 March 2024). Virulence gene pre-

diction was conducted by submitting the genome of the bacteriophage phJNUCC32 to the online tool (http://www.mgc.ac.cn/VFs/search_VFs.htm, accessed on 21 March 2024). For the construction of phylogenetic trees, we employed full-length amino acid sequences of the terminase large subunit (TerL). Sequence alignment was performed using MAFFT. Sequence trimming was performed using trimAl, with commands to retain conserved regions. Phylogenetic tree construction was performed using IQ-TREE 2.0, which automatically determined the optimal model, utilizing the maximum likelihood method and 1000 bootstrap replicates. [26]. The resulting phylogenetic trees were visualized using TVBOT (https://www.chiplot.online/tvbot.html, accessed on 7 March 2024).

3. Results and Discussion

3.1. Genome Characteristics of the Paenibacillus Bacteriophage phJNUCC32

Jellyfish generated a 21-mer count histogram from a subset of short DNA reads. GenomeScope then used this histogram to estimate genome size, k-mer coverage, and heterozygosity. The graph was plotted with the coverage and frequency of k-mers. The genome size can be estimated using total k-mer number and volume peak (Figure 1). The genome of the phage JNUCC32 has a size of 62,871 base pairs, with a G + C content of 51.98%, a k-mer coverage of 33.9, and low heterozygosity of 0.022% (below 0.05%). Genome scanning identified 63 coding DNA sequences (CDSs) in the genome, with no RNA sequences detected. The genomic features of the phage JNUCC32 are summarized in Figure 2. The circular genome map was generated using Prokka (v1.12b). The draft genome sequence of the phage JNUCC32 has been submitted and deposited in the NCBI GenBank database under the accession ID CP062261.

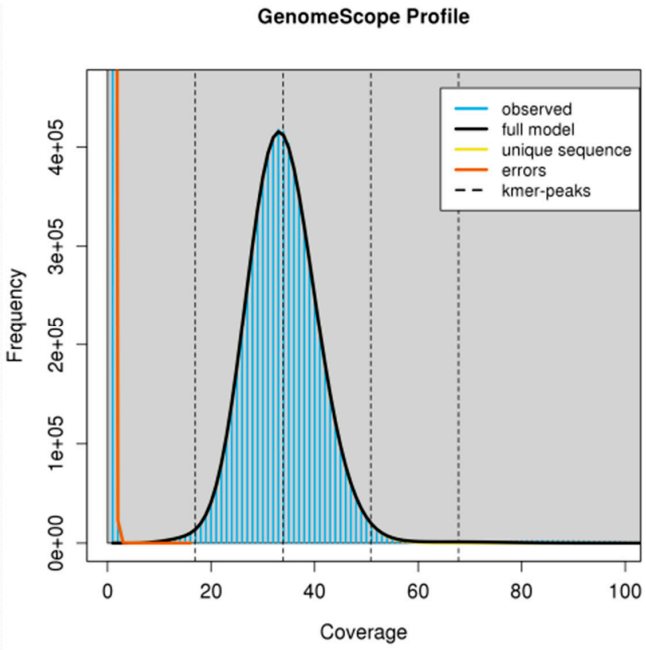

Figure 1. K-mer analysis of the genome.

3.2. Phylogenetic Analysis

To further elucidate the evolutionary relationships among bacteriophages, a phylogenetic tree was constructed by comparing conserved and evolutionarily significant sequences within these viruses. The sequence of the terminase large subunit (ORF2), a conserved region within bacteriophages, was utilized for this purpose, allowing for comparison of genetic relationships among different bacteriophages. As shown in Figure 3, phylogenetic analysis of the terminase large subunit of the *Paenibacillus* phage phJNUCC32 reveals a close genetic relationship with the *Bacillus* phage phBC6A51. This proximity within the evolutionary tree suggests their classification within the unclassified Caudoviricetes bacteriophage

category. In summary, phylogenetic linkages provide a framework for understanding the evolutionary history, taxonomy, and functional diversity of bacteriophages. These insights are crucial for advancing phage research, including phage therapy, biotechnology, and microbial ecology.

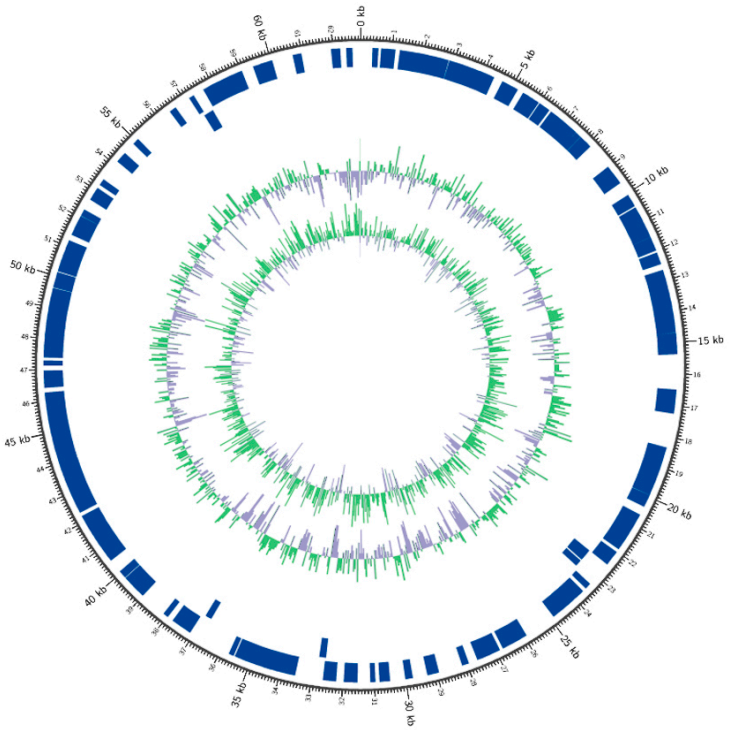

Figure 2. Circular genome map of the phJNUCC32 chromosome. The genomic characteristics are illustrated from the outer to the central region, including forward-strand CDS, reverse-strand CDS, GC content, and GC skew. Forward CDS: Regions containing forward CDS are represented, with non-CDS regions indicated as blank. Reverse CDS: Areas with reverse CDS are presented, and non-CDS regions are denoted as blank. GC content: Regions with a higher GC percentage than the average are depicted in an exterior light green peak. GC skew: Calculated by $(G - C)/(G + C)$, a positive value indicates G dominance, while a negative value indicates C dominance. The exterior light green peak signifies regions with higher G content, while the interior lavender peak represents regions with higher C content.

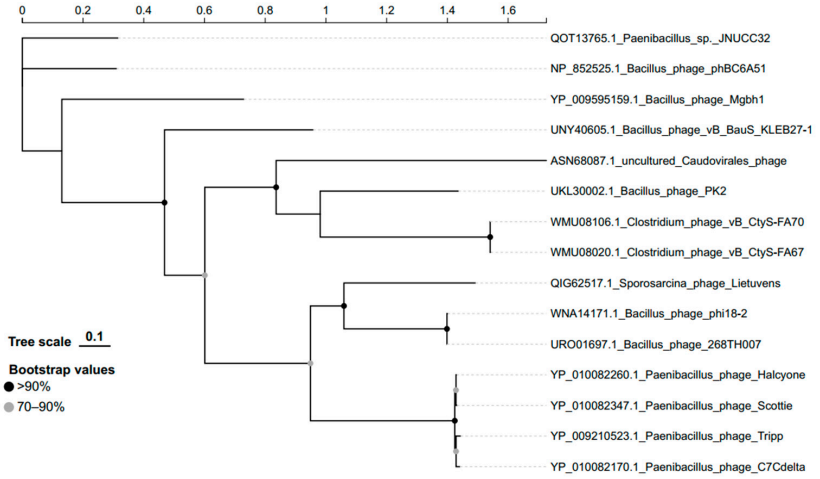

Figure 3. Maximum likelihood phylogeny of the phage terminase large subunit (TerL) proteins of the *Paenibacillus* bacteriophage phJNUCC32.

3.3. Functional Annotation

In the *Paenibacillus* bacteriophage phJNUCC32 genome, a total of 97 open reading frames (ORFs) have been identified. Of these, 63 are CDSs. Among the CDSs, 45 are predicted to encode proteins with known functions, constituting 71.4% of the total CDSs. (Figure 4, Table 1). The gene distribution of phJNUCC32 demonstrates a characteristic modular pattern, comprising various functional modules such as hypothetical protein, portal protein, head protein, tail protein, phage-like protein, plate protein, fiber protein, holin, regulatory protein, replication protein, DNA helicase, crossover junction protein, and endonuclease.

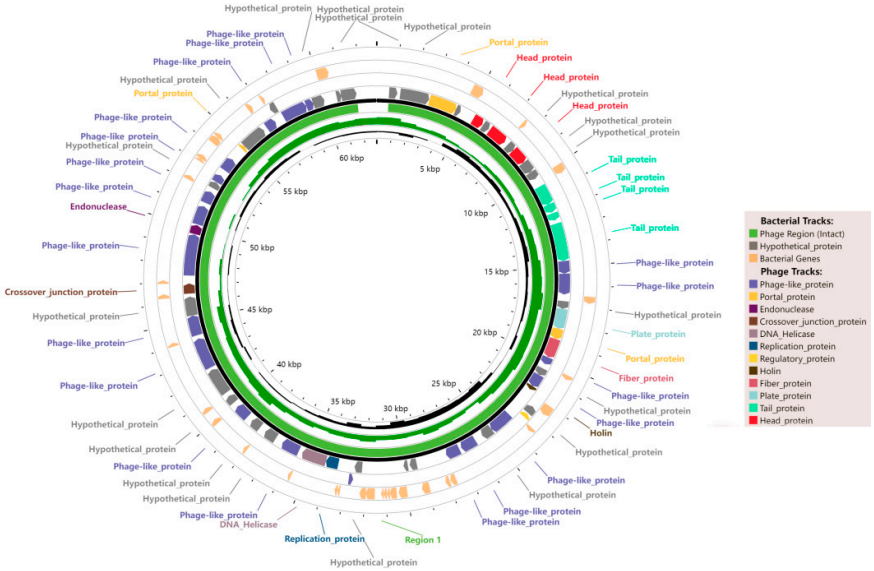

Figure 4. Gene functional annotation circle plot of the *Paenibacillus* phage phJNUCC32.

Table 1. General features of the CDSs predicted from the genome of the *Paenibacillus* phage phJNUCC32.

CDS	Position	Prediction Function	BLAST Hit	*E*-Value
1	658..1101	Helix–turn–helix protein	Bacill_phBC6A51_NC_004820	1.48×10^{-33}
2	1252..2823	Terminase large subunit	Bacill_phBC6A51_NC_004820	0.0
3	2845..4356	Portal Gp-6 family-like protein	Bacill_Mgbh1_NC_041879	8.21×10^{-154}
4	4349..4618	Hypothetical protein	PHAGE_Verruc_P8625_NC_029047	1.29×10^{-12}
5	5395..5970	Capsid protein-like protein	Bacill_Mgbh1_NC_041879	1.15×10^{-25}
6	6011..6385	Hypothetical protein	Bacill_Mgbh1_NC_041879	2.85×10^{-46}
7	6457..7482	Major capsid protein	Bacill_Mgbh1_NC_041879	1.42×10^{-131}
8	7689..8117	Hypothetical protein	Bacill_phBC6A51_NC_004820	4.01×10^{-13}
9	8114..8962	MuF-like minor capsid protein	Gordon_Sadboi_NC_048815	8.92×10^{-7}
10	8962..9603	Hypothetical protein	Paenib_Tripp_NC_028930	1.40×10^{-5}
11	9603..10037	HK97 gp10 family phage protein	Paenib_Tripp_NC_028930	1.97×10^{-5}
12	10513..11559	xkdK-like tail sheath protein	Clostr_phiCTC2B_NC_030951	8.04×10^{-23}
13	11575..12021	Putative tail core protein	Clostr_phiCDHM19_NC_028996	1.06×10^{-15}
14	12089..12472	Putative core tail protein	Lactob_jlb1_NC_024206	1.87×10^{-12}
15	12674..14686	Tail tape measure protein	Lister_LP_101_NC_024387	7.65×10^{-71}

Table 1. *Cont.*

CDS	Position	Prediction Function	BLAST Hit	*E*-Value
16	14699..15373	XkdP	BalMu_1_NC_030945	4.36×10^{-42}
17	15373..16485	Late control D protein	Brevib_Jimmer1_NC_029104	4.17×10^{-23}
18	16836..17252	DUF2634 domain-containing protein	Brevib_Abouo_NC_029029	1.36×10^{-20}
19	17252..18334	Baseplate J	Thermu_OH2_NC_021784	2.68×10^{-70}
20	18331..18894	Portal protein	Clostr_phiCT9441A_NC_029022	2.04×10^{-28}
21	18898..19938	Putative tail fiber protein	Bacill_BCD7_NC_019515	6.31×10^{-7}
22	19953..20351	Rhodanese-related sulfurtransferase	Thermu_OH2_NC_021784	1.04×10^{-24}
23	20607..20999	Hpothetical protein	Bacill_vB_BboS_125_NC_048735	3.69×10^{-12}
24	21003..21692	Endolysin	Bacill_Waukesha92_NC_025424	3.66×10^{-30}
25	21694..21912	Holin	Entero_AUEF3_NC_042134	1.48×10^{-6}
26	22530..22928	Hypothetical protein	Paenib_Tripp_NC_028930	5.86×10^{-21}
27	22981..23205	Helix–turn–helix transcriptional regulator	Bacill_BceA1_NC_048628	1.85×10^{-15}
28	23654..24877	DNA translocase FtsK	Bacill_BceA1_NC_048628	4.29×10^{-81}
29	24877..25479	Replication–relaxation family protein	Bacill_PfEFR_4_NC_048641	4.68×10^{-64}
30	25921..26772	Conserved phage protein	Bacill_WBeta_NC_007734	3.53×10^{-21}
31	26888..27655	Putative cobyrinic acid ac-diamide synthase	Brevib_Sudance_NC_028749	3.91×10^{-102}
32	29301..29675	Hypothetical protein	Tripp_NC_028930	1.29×10^{-12}
33	29777..30010	Hypothetical protein	Bacill_Staley_NC_022767	1.71×10^{-24}
34	32215..32625	Hypothetical protein	Paenib_Tripp_NC_028930	1.10×10^{-35}
35	32620..32838	Xre-like protein	Bacter_Lily_NC_028841	4.75×10^{-11}
36	33500..34174	DNA replication protein	Paenib_Tripp_NC_028930	8.27×10^{-79}
37	34178..35518	DNA helicase-like protein	Bacill_Mgbh1_NC_041879	1.12×10^{-137}
38	35720..36700	DNA primase	Paenib_Tripp_NC_028930	9.76×10^{-80}
39	37121..37783	Sigma-70 family RNA polymerase sigma factor	Bacill_Mgbh1_NC_041879	4.35×10^{-10}
40	38141..38707	Hypothetical protein	Paenib_Tripp_NC_028930	1.20×10^{-24}
41	39014..39790	Single-stranded DNA-binding protein	Paenib_Tripp_NC_028930	3.87×10^{-95}
42	40070..40516	Hypothetical protein KLEB271_gp57 Bacillus phage	Paenib_Tripp_NC_028930	9.99×10^{-49}
43	40737..42182	Hypothetical protein	Bacill_Mgbh1_NC_041879	6.27×10^{-19}
44	42311..43990	DNA polymerase	Paenib_Tripp_NC_028930	0.0
45	44173..45258	DNA polymerase	Paenib_Tripp_NC_028930	0.0
46	45264..46277	hypothetical protein	Paenib_Tripp_NC_028930	2.99×10^{-110}
47	46437..46979	Crossover junction endodeoxyribonuclease	Bacill_Mgbh1_NC_041879	5.54×10^{-48}
48	47398..49614	Ribonucleotide diphosphate reductase alpha subunit	Bacill_Eldridge_NC_030920	0.0
49	49634..50128	Putative HNH homing endonuclease	Bacill_BCP8_2_NC_027355	3.09×10^{-30}
50	50166..51197	Ribonucleotide diphosphate reductase beta subunit	Bacill_SP_15_NC_031245	1.95×10^{-109}
51	51394..52047	Phosphate starvation-inducible protein PhoH-like protein	Bacill_SP_10_NC_019487	2.71×10^{-56}
52	52274..52627	Hypothetical protein	Bacill_Blue_NC_031056	9.58×10^{-16}
53	52645..53076	dCTPase	Acinet_ZZ1_NC_018087	7.08×10^{-9}

Table 1. *Cont.*

CDS	Position	Prediction Function	BLAST Hit	*E*-Value
54	53407..54123	Thymidylate synthase	Bacill_Riggi_NC_022765	1.53×10^{-105}
55	54881..55084	Portal protein	Bacill_T_NC_024205	2.15×10^{-8}
56	55081..56412	Hypothetical protein	Paenib_Tripp_NC_028930	1.33×10^{-32}
57	56620..57171	ATPase-like protein	Paenib_Tripp_NC_028930	1.76×10^{-58}
58	57276..57605	Hypothetical protein	Paenib_Tripp_NC_028930	1.14×10^{-7}
59	57676..59025	Modification methylase	Bacill_SPbeta_NC_001884	1.24×10^{-92}
60	59025..59426	SigK-like protein	Paenib_Tripp_NC_028930	2.46×10^{-43}
61	59413..60039	Hypothetical protein	Paenib_Tripp_NC_028930	4.38×10^{-33}
62	60715..60981	Hypothetical protein	Paenib_Tripp_NC_028930	6.08×10^{-6}
63	60968..61792	Hypothetical protein	Paenib_Tripp_NC_028930	1.42×10^{-106}

ORF2 in the DNA packaging module encodes the large subunit of the terminase enzyme. Terminase enzymes are essential for packaging phage DNA into the capsid during virion assembly, ensuring proper encapsulation of genetic material. These proteins play a critical role in phage replication and propagation [27].

Portal proteins are encoded by ORFs 3, 20, and 55. Portal proteins create a channel in the capsid for phage DNA injection into host cells during infection [28]. They facilitate early infection stages by aiding phage DNA entry into the host cell cytoplasm. Portal proteins are vital for phage infectivity, often conserved among different phage species. They are promising targets for phage therapy and genetic engineering.

Tail proteins are represented by ORFs 12 to 15. Tail proteins attach phage to host cell surfaces, recognize host receptors, and inject phage DNA into host cells [29]. They facilitate phage adsorption and penetration, initiating infection. Tail proteins exhibit diversity across phage types, reflecting host recognition specificity and range determination.

ORF22 encodes thiosulfate sulfurtransferase (TST), also known as rhodanese, pivotal in microbial metabolism, detoxifying thiosulfate by binding it with organic toxins, aiding their elimination [30]. TST also balances sulfur cycling by transferring sulfur atoms and may combat oxidative stress by neutralizing oxygen radicals [31]. In microorganisms, TST ensures detoxification, metabolic balance, antioxidation, and energy metabolism, vital for survival and adaptation.

ORF24 is predicted to encode the endolysin gene based on bioinformatic analysis, indicating that nucleotides 21,003–21,692 (690 bp) of ORF24 encoded amino acid endolysin and that the protein belongs to the N-acetylmuramoyl-L-alanine amidase CwlA family. Endolysins are phage-encoded peptidoglycan hydrolases (PGHs), also known as internal lysins or bacterial lysozymes. They exhibit high specificity and are involved in bacterial lysis at the end of the infection cycle. The bacteriophage perforin forms pores on the cell membrane, allowing endolysin to reach PG targets on the bacterial cell wall and cleave it hydrolytically, ultimately leading to bacterial lysis and death [32].

ORF25 of phJNUCC32 is annotated as the holin gene. Holin is a small transmembrane protein encoded by bacteriophages. Upon bacteriophage infection, holin forms non-specific pores on the host cell membrane within a specific time frame, thereby controlling the duration of the bacteriophage infection cycle and lysing the host cell at the optimal time point. Due to its role in regulating the bacteriophage infection cycle, holin is also referred to as the "clock" controlling bacteriophage infection [33].

ORF49 is predicted to encode the HNH endonuclease (H: Histidine, N: Asparagine, and H: Histidine), which participates in mediating the insertion of the bacteriophage genome into the host genome. The endonuclease can cleave specific sites in the host bacterium's DNA, followed by homologous recombination between the bacteriophage's single-stranded DNA and the host genome DNA, facilitated by host nuclease inhibitory proteins [34,35].

No known antibiotic-resistance genes or virulence factors were predicted in phJNUCC32, indicating the phage's safety at the genetic level.

4. Conclusions

In this study, we isolated and characterized the *Paenibacillus* bacteriophage phJNUCC32, which shows promising biocontrol activity. Whole-genome sequencing and functional analysis revealed the presence of the holin/lysin system, enabling selective infection and eradication of *P*. larvae, effectively controlling the spread of American foulbrood while preserving beneficial microorganisms, thereby contributing to ecosystem balance and biodiversity conservation. Moreover, phJNUCC32 exhibited genetic safety by lacking virulence factors and antibiotic-resistance genes, thus mitigating potential harm to the environment and ecosystems. Overall, the bacteriophage phJNUCC32 demonstrates significant potential for biocontrol and ecological safety, offering a novel approach for sustainable bee health management and beekeeping industry development.

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