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Research Letter

RESEARCH LETTER – VIROLOGY

Isolation and characterization of Hena1 – a novel *Erwinia amylovora* bacteriophage

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One sentence summary: This study reports a novel lytic *E. amylovora* bacteriophage Hena1, that could be classified as a member of *Myoviridae* subfamily *Vequintavirinae*.

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ABSTRACT

Fire blight, caused by plant pathogenic bacterium *Erwinia amylovora,* is one of the most important diseases of *Rosaceae* plants. Due to the lack of effective control measures, fire blight infections pose a recurrent threat on agricultural production worldwide. Recently, bacterial viruses, or bacteriophages, have been proposed as environmentally friendly natural antimicrobial agents for fire blight control. Here, we isolated a novel bacteriophage Hena1 with activity against *E. amylovora*. Further analysis revealed that Hena1 is a narrow-host-range lytic phage belonging to *Myoviridae* family. Its genome consists of a linear 148,842 bp dsDNA (48.42% GC content) encoding 240 ORFs and 23 tRNA genes. Based on virion structure and genomic composition, Hena1 was classified as a new species of bacteriophage subfamily *Vequintavirinae*. The comprehensive analysis of Hena1 genome may provide further insights into evolution of bacteriophages infecting plant pathogenic bacteria.

Keywords: *Erwinia amylovora*; bacteriophage; *Myoviridae*, *Vequintavirinae*

INTRODUCTION

Erwinia amylovora is a plant pathogen that causes fire blight, an important disease in *Rosaceae*. Current fire blight control strategies are based on a reduction of inoculum and preventive antimicrobial treatment (Nagy, Király and Schwarczinger 2012). Routine approaches, such as application of copper compounds and antibiotics, are limited by phytotoxic effects and development of bacterial resistance (Aćimović *et al.* 2015). In recent years, new solutions for *E. amylovora* control were proposed, including the use of bacteriophages as biocontrol agents (Erskine 1973; Schwarczinger *et al.* 2011; Nagy, Király and Schwarczinger 2012; Gusberti *et al.* 2015; Tancos *et al.* 2016).

To date, a substantial number of *E. amylovora* bacteriophages belonging to four families of *Caudovirales* (*Podoviradae*, *Myoviradae, Siphoviridae and Ackermannviridae*) have been isolated and characterized (Gill *et al.* 2003; Lehman *et al.* 2009; Boulé *et al.* 2011; Müller *et al.* 2011; Samoilova and Leclerque 2014; Lagonenko *et al.* 2015; Boyko *et al.* 2017; Schwarczinger *et al.* 2017; Buttimer *et al.* 2018; Sharma *et al.* 2018; Thompson *et al.* 2019).

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Table 1. Bacterial strains and isolates used in this study.

Strain/isolate	Origin, host, year of isolation	Reference/source
Erwinia amylovora		
1/79Sm	Germany, Spontaneous Sm-resistant mutant of 1/79, *Cotoneaster* sp., 1979	Dr. Klaus Geider
E2; E3; E4; E5; L3–6; L3–2	Belarus, isolates from *Malus sp.*; *Pyrus communis*, 2007–2009	BIM[a]
Pantoea agglomerans		
194; 197; 198; 208; 216; 219	Belarus, isolates from different plants, 1980–1981	Dr. S. Chernov, (Chernov and Fomichev 1981)
Pectobacterium carotovorum		
3–2	Belarus, isolated from *Solanum tuberosum*, 1978	CMBU[b]
Pectobacterium atrosepticum		
36A;	Belarus, isolated from *Solanum tuberosum*, 1978	CMBU
Pseudomonas syringae pv. *syringae*		
12.6, 14.5(1), 19.10, 20.1	Isolates; Belarus, *Pyrus communis*, 2007–2009	CMBU

BIM[a]—Belarusian collection of non-pathogenic microorganisms, Institute of Microbiology, National Academy of Science, Belarus.
CMBU[b]—Collection of Microbiology department, Belarusian State University.

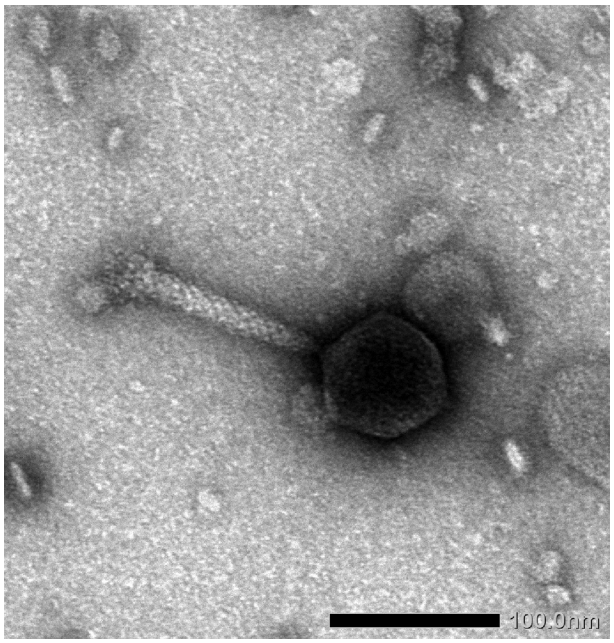

Figure 1. Transmission electron microscopy (TEM) of purified phage Hena1. Scale bar, 100 nm.

Importantly, some of the described *E. amylovora* bacteriophages have been shown to infect both epiphytic and pathogenic bacteria, such as *E. pyrifoliae*, *E. billingiae*, *Pantoea agglomerans*, *P. stewartii* and *P. vagans* (Gill *et al.* 2003; Müller *et al.* 2011; Lagonenko *et al.* 2015; Park *et al.* 2018; Sharma *et al.* 2019). This makes them an attractive tool for fire blight control, since phage-susceptible epiphytic bacteria like *P. agglomerans* could be potentially used for phage delivery during the disease treatment (Lehman 2007).

The objective of present study was to isolate, identify and characterize bacteriophages infecting *Erwinia amylovora*.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The bacterial strains and isolates used in this study (Table 1) were grown routinely in lysogeny broth (LB) medium or on lysogeny broth agar (LA) plates at 28°C (Bertani 1951).

Phage isolation and host range determination

E. amylovora 1/79Sm was used for bacteriophage isolation and propagation. Soil samples from an apple and pear orchard in Navagrudak, Belarus were used for phage isolation. Isolation procedure was performed using an enrichment protocol as described previously (Van Twest and Kropinski 2009). Phage was purified through three rounds of plaque purification using the soft agar overlay method (Adams 1959; Sambrook and Russell 2001). The host range was determined by performing *spot tests*. *Briefly, a total of* 10^7 cfu/mL of different bacterial strains in LB were mixed with melted semi-solid agar (0.7%) and poured over 1.5% solid agar. A 10 μL drop of bacteriophage stock solution (10^7 pfu/mL) was spotted onto each plate followed by incubation at 28°C for 20 h. Complete lysis of bacteria (plaque formation) under the phage spots was scored as a positive reaction. The results of the spot tests were further verified using the agar overlay method (Adams 1959).

Electron microscopy

Phages were propagated to obtain high titer (108–109 PFU/mL). Phage lysate was treated with DNAase and RNAase and concentrated by adding 10% (w/v) PEG. The resulting mixture was incubated at 4°C overnight. After incubation, phage particles were sedimented by ultracentrifugation (23 000 rpm, 2.5 h), resuspended in STM buffer (270 mM Sucrose, 10 mM Tris HCl, 1 mM MgCl2, pH 7.5,) and then stained using uranyl acetate (2% w/v) on collodion-coated copper grid (Tikhonenko 1970). Phages were visualized with a transmission electron microscope Jeol JEM-1400.

DNA isolation and sequencing

DNA from phage particles was isolated as described by Anany *et al.* (2011), with some modifications. The modifications include removal of the cesium chloride (CsCl) gradient purification step and an additional step of purification using silica columns from the DNeasy Blood & Tissue Kit (QIAGEN, Cat#69 506, Hilden, Germany). DNA concentrations were measured using NanoPhotometer P330 (Implen, München, Germany). The DNA library for sequencing was prepared with the Ligation Sequencing Kit SQK-LSK109 (Oxford Nanopore Technologies, Oxford, United Kingdom) according to the manufacturer's recommendations.

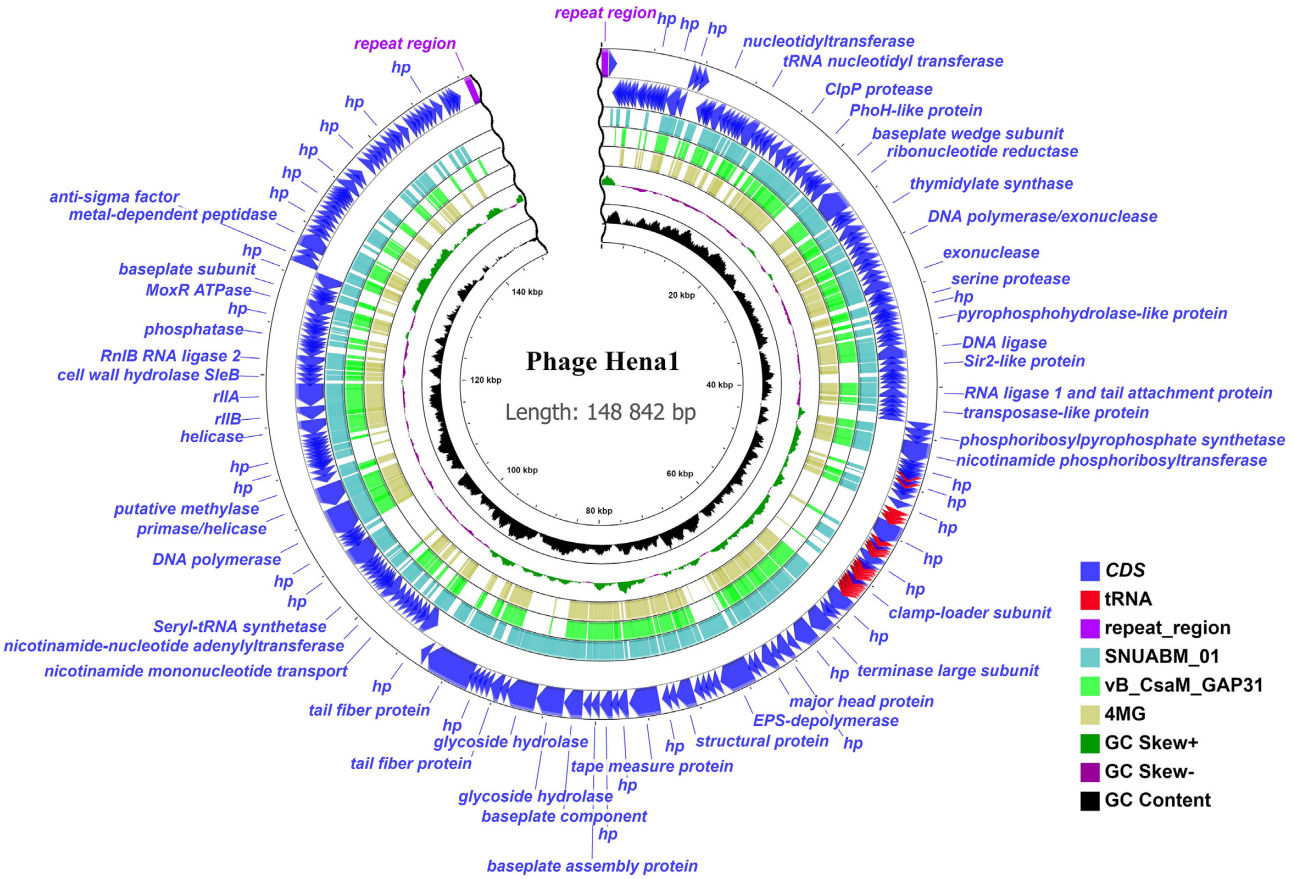

Figure 2. Genetic and physical map of phage Hena1 genome prepared using CGView. Two outer lanes correspond to predicted ORFs on the forward and reverse strands respectively (tRNA genes are indicated in red and predicted ORFs are indicated in dark blue). Cerulean, green and brown lanes in the middle represent tblastx analysis results for genomes of phages pEp_SNUABM_01, vB_CsaM_GAP31 and 4MG, respectively. The degree of sequence similarity to Hena1 is proportional to the height of the bars in each frame. Two innermost circles correspond to GC skew and GC plot, respectively.

Table 2. Discontiguous megablast comparisons of Hena1 genome against the complete genomes of bacteriophages classified as members of *Vequintavirinae* subfamily.

GenBank accession number	Phage name	Genus	Identity/coverage (%)
MN184887.1	*Erwinia pyrifoliae* phage pEp_SNUABM_01	Unclassified	79.48/87
MG250486.1	*Raoultella* phage Ro1	Unclassified	74.87/35
KY652726.1	*Klebsiella* phage vB_KpnM_BIS47	Unclassified	74.60/34
MK024806.1	*Proteus mirabilis* phage Mydo	Unclassified	74.60/34
JN882284.1	*Cronobacter sakazakii* phage vB_CsaM_GAP31	*Se1virus*	75.88/36
JX181824.1	*Salmonella phage* SSE121	*Se1virus*	75.95/33
NC_02 2968.1	*Escherichia coli* phage 4MG	*Se1virus*	73.44/36

Fragmentation step was omitted in this library prep. Sequencing was performed using a MinIon sequencer (Oxford Nanopore Technologies [ONT]) at the Center of Analytical and Genetic Engineering Research (Minsk, Belarus). Guppy v3.2.4 software was used for basecalling, yielding a total of 56.3 Mbp distributed in 9383 reads. Reads with a Q score of > 25 were used for further analysis. The Nanopore reads were assembled with Flye assembler v2.6 (Kolmogorov *et al.* 2019). Since Nanopore sequencing results in a relatively high error, the same DNA sample was additionally sequenced with an Illumina MiSeq platform using a MiSeq reagent kit v3 (2 × 300 bp). The paired-end library for sequencing was prepared with the MuSeek library preparation kit (Thermo Fisher, Cat#K1361, Waltham, USA) according to the manufacturer's recommendations. Raw fastq files were quality-filtered with Trimmomatic (Bolger, Lohse and Usadel 2014). A total of 451 028 high-quality reads (Q > 20, length 55–263 bp) were used for further work. The Illumina reads were used to correct Nanopore errors in assembly using Bowtie2 v2.3.5.1 and Pilon v1.23 software (Langmead and Salzberg 2012; Walker *et al.* 2014). Default parameters were used for all software. All read sequences were deposited in the Sequence Read Archive under the accession numbers SRR11217904 и SRR11217905.

Sequence analysis

Hena1 genome annotation was performed using Artemis software (Rutherford *et al.* 2000). Open reading frames in the genome were predicted using Glimmer 2.0 and RAST (Salzberg *et al.*

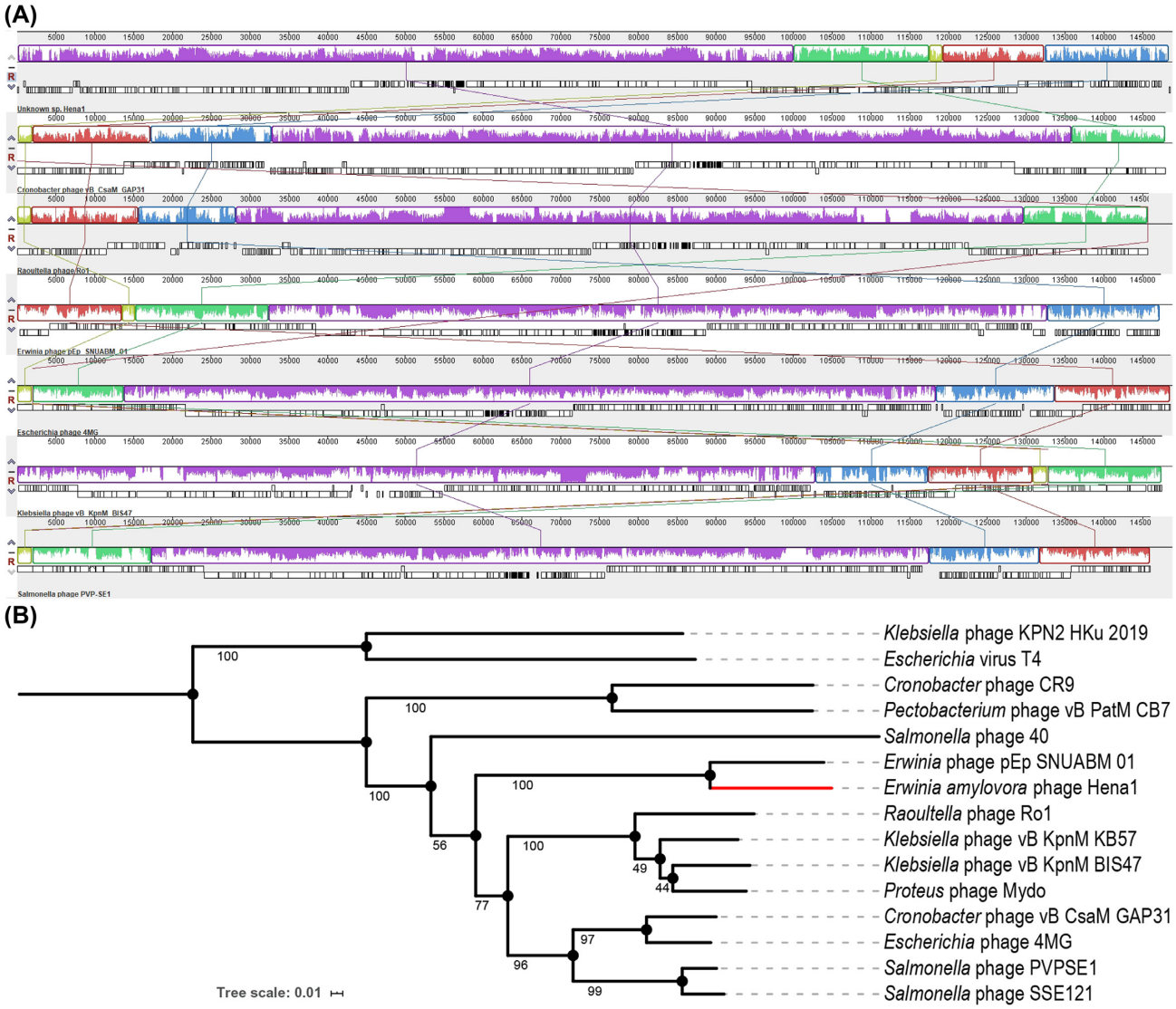

Figure 3. Comparative genomic analysis of Hena1 and related bacteriophages. A. ProgressiveMauve alignment of Hena1, pEp_SNUABM_01, Ro1, vB_KpnM_BIS47, vB_CsaM_GAP31, PVP-SE1 and 4MG genomes. Connection lines indicate similar regions between genomes. The blocks of similar colo for each phage indicate regions of DNA sequence relatedness; the degree of sequence similarity is indicated by the intensity of the colored region. The contiguous box-like diagrams indicate gene positions. **B.** Phylogenomic analysis of bacteriophages at the amino acid level using VICTOR. The recommended VICTOR tree (formula *d6*) is shown. The numbers above the branches represent Genome-BLAST Distance Phylogeny (GBDP) pseudo-bootstrap support values from 100 replications, given that branch support exceeds 50%.

1998; Aziz *et al.* 2008). Nucleotide and protein homology searches were performed using BLAST programs (Altschul *et al.* 1990). Predicted protein sequences were analyzed against Pfam database for conservative domain identification (Punta *et al.* 2012). tRNA genes were identified using tRNAscan-SE (Schattner, Brooks and Lowe 2005) and ARAGORN (Laslett and Canback 2004). For multiple genome alignment, Mauve software was used (Darling *et al.* 2004). Genetic and physical map of phage genome were constructed using CGView (Stothard and Wishart 2005). For whole-bacteriophage genome sequence phylogeny analysis (nucleotide- or amino-acid-based), the Virus Classification and Tree Building Online Resource (VICTOR) was used (Meier-Kolthoff and Goeker 2017). The resulting phylogenetic tree was visualized with iTOL (Letunic and Bork 2016). Average nucleotide identities (ANI) were calculated using ANI Calculator (Yoon *et al.* 2017).

Nucleotide sequence accession number

The complete annotated genome sequence of *E. amylovora* phage Hena1 has been deposited in GenBank under the accession number MN732867.

RESULTS AND DISCUSSION

E. amylovora is a plant pathogenic bacterium causing an economically important fire blight disease of apples and pears. Classical approaches like antibiotic treatment have been proven to be ineffective in long-term disease control (Aćimović *et al.* 2015). Phage therapy is an emerging and attractive alternative for fighting *E. amylovora* infection (Schwarczinger *et al.* 2011).

Phage Hena1 was isolated from soil samples in an apple and pear orchard (Navagrudak, Grodna district, Belarus) using

E. amylovora 1/79Sm as a host. Hena1 displayed small sized (under 1 mm) clear plaques on bacterial lawn, suggesting a lytic lifestyle. The phage was further tested for ability to lyse different *E. amylovora* strains and other plant associated bacteria such as *P. agglomerans, Pectobacterium carotovorum, P. atrosepticum, Pseudomonas syringae,* as well as *E. coli* strains. Hena1 showed a narrow host range, infecting only two strains of *E. amylovora*–1/79Sm and L3–6, while all other bacterial strains were resistant to phage infection. Phage host range is naturally limited by numerous active or passive resistance mechanisms incuding CRISPR/Cas and restriction-modification systems, loss/modification of receptors for phage adsorption, physical exclusion by the production of extracellular matrix (Brockhurst, Koskella and Zhang 2017). Additional studies are needed to explain the restricted host range of Hena1.

We further purified Hena1 phage particles and visualized them with transmission electron microscopy. As seen in Fig. 1, Hena1 virion consists of an icosahedral head (72,36 ± 5,38 nm in diameter) and a long contractile tail (126,28 ± 5,27 nm) with short tail fibers. Taken together, these observations allowed us to classify Hena1 as belonging to the family *Myoviridae* with an A1 morphotype.

Phage DNA sequencing revealed that Hena1 has a linear dsDNA genome of 148 842 bp with a GC content of 48.42%, encoding 240 putative ORFs and 23 tRNA genes (Fig. 2). Interestingly, the genome could be divided into 4 divergently oriented gene clusters. The first region (1188–42 687) includes 87 mostly leftward-transcribed ORFs that are involved in DNA packaging, nucleotide metabolism and cell lysis (e.g. DNA-ligase, Endo VII packaging and recombination endonuclease, endonuclease II, nucleotidiltransferase, endolysin). Among the 55 predicted ORFs in the second rightward oriented region (42 955–94 583), only 19 were annotated as functional genes. A total of 16 ORFs were predicted to encode structural proteins for head, tail, baseplate and virion morphogenesis (e.g. baseplate and tail fiber assembly, terminase large subunit, tail tape measure protein). We additionally identified 3 ORFs probably involved in cell lysis and/or host recognition. ORF160 and ORF161 encode putative cell wall hydrolases (family 5/PF00150 and 48/PF02011, respectively). Interestingly, ORF145 of Hena1 has a homolog in the genome of *E. amylovora* phage phiEa2809 and encodes a putative exopolysaccharide (EPS) depolymerase, which could be potentially involved in degradation of *E.* amylovora protective EPS coat, thus facilitating phage attachment to bacterial cell wall (Lagonenko *et al.* 2015). Furthermore, the third region includes 23 predicted tRNA genes. The third region (94 623–128 779) consists of 60 leftward-transcribed CDSs. While most of them were annotated as encoding hypothetical proteins, 18 genes were predicted to be involved in phage DNA replication and nucleotide metabolism (TableS1). The fourth rightward-transcribed region (128 917–148 813) includes 38 genes of unknown function, a putative anti-sigma factor and a putative DeoR/GlpR family transcriptional regulator.

Using MinION long read sequencing platform allowed us to accurately and efficiently determine the location and structure of genomic termini. Phage Hena1 possesses gDNA with two 519-bp terminal repeat regions flanking its terminal ends. Different types of phage genome ends reflect various DNA replication strategies and specifically different terminase activities during DNA packaging (Casjens and Gilcrease 2009). Upon infection, Hena1 DNA likely forms a circular molecule which is then replicated via linear concatemer generation. The terminal repeat regions are duplicated during DNA packaging into Hena1 capsid (Weigel and Seitz 2006).

The whole genome discontiguous megablast analysis indicated that Hena1 shared high similarity (coverage 87%, identity 79.48%) with *Erwinia pyrifoliae* phage pEp_SNUABM_01. For these genomes the average nucleotide identity (OrthoANI) value was 78.63%. Apart from that, Hena1 genome was found to have similarity with bacteriophages annotated as members of *Vequintavirinae* subfamily (Table 2).

Recently, four new bacterial virus subfamilies Guernseyvirinae, Vequintavirinae, Tunavirinae and Bullavirinae were proposed by The International Committee on Taxonomy of Viruses (ICTV) (Krupovic *et al.* 2016). The myovirus subfamily Vequintavirinae *includes four* genera *Vequintavirus* virus (type species *Escherichia* virus V5), *Seunavirus* (*Salmonella* virus SE1), *Certrevirus* (*Cronobacter* virus CR3) and *Avunavirus* (*Escherichia* phage Av-05) (Korf *et al.* 2019).

The Mauve global alignment of Hena1 genome with related phage genomes (pEp_SNUABM_01, Ro1, vB_KpnM_BIS47, vB_CsaM_GAP31, PVP-SE1 and 4MG) demonstrated synteny in four conserved regions with significant rearrangements (Fig. 3A). Predominant number of Hena1 ORFs shared 53–94% and 33–71% amino acid sequence identity with pEp_SNUABM_01 and vB_CsaM_GAP31 phages, respectively. *Seunavirus* phages possess genomes of about 148 kb (45–46% G + C) that encode 242–269 proteins and 21–26 tRNAs. The members of this genus have an icosahedral head of 83–94 nm and a contractile tail of 107–121 by 18–19 nm (Santos *et al.* 2011; Abbasifar *et al.* 2012; Kim, Heu and Ryu 2014). As shown above, Hena1 capsid structure and genome properties are very similar to those of *Seunavirus* phages. However, the low level of DNA and protein identity does not allow attributing Hena1 to this genus (*Seunavirus* phages *Cronobacter* virus GAP31, *Escherichia* virus 4MG, *Salmonella* virus SSE121, *Salmonella* virus SE1 share 64–95% DNA sequence identity and 82–91% homologous proteins).

The amino-acid-based VICTOR analysis placed *Erwinia* phages Hena1 and pEp_SNUABM_01 in maximally supported separate cluster closely related to the group of *Vequintavirinae* phages (Fig. 3B).

Summarizing, this study reports a novel lytic *E. amylovora* bacteriophage Hena1. Based on whole-genome sequence comparisons, we propose that Hena1 is a novel species of the myovirus subfamily *Vequintavirinae*. Further phylogenomic analysis at the amino acid level showed that Hena1 together with closely related *E. pyrifoliae* phage pEp_SNUABM_01 could be separated as members of a novel genus within this subfamily. The complete genome of Hena1 provides additional data that may help to gain a better understanding of how the genomes of phytopathogenic bacteria-infecting phages evolve.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSLE* online.

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Conflicts of interest. None declared.

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