

RESEARCH LETTER – Virology

Characterization of a new ViI-like *Erwinia amylovora* bacteriophage phiEa2809

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Tel: +375 (17) 209-58-48; Fax: +375 (17) 209-58-08; E-mail: lagonenkoal@mail.ru**One sentence summary:** In this study, we investigated morphologic and genomic features of the new *Erwinia amylovora* bacteriophage phiEa2809 isolated from plant sample collected in Belarus.

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ABSTRACT

Erwinia amylovora is a Gram-negative plant pathogenic bacteria causing fire blight disease in many *Rosaceae* species. A novel *E. amylovora* bacteriophage, phiEa2809, was isolated from symptomless apple leaf sample collected in Belarus. This phage was also able to infect *Pantoea agglomerans* strains. The genome of phiEa2809 is a double-stranded linear DNA 162 160 bp in length, including 145 ORFs and one tRNA gene. The phiEa2809 genomic sequence is similar to the genomes of the *Serratia plymutica* phage MAM1, *Shigella* phage AG-3, *Dickeya* phage vB DsoM LIMEstone1 and *Salmonella* phage ViI and lacks similarity to described *E. amylovora* phage genomes. Based on virion morphology (an icosahedral head, long contractile tail) and genome structure, phiEa2809 was classified as a member of *Myoviridae*, ViI-like bacteriophages group. PhiEa2809 is the firstly characterized ViI-like bacteriophage able to lyse *E. amylovora*.

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

INTRODUCTION

Fire blight, which is caused by plant pathogenic bacterium *Erwinia amylovora*, is a very destructive and economically significant disease of apples, pears and many other *Rosaceae* plants. This pathogen may be controlled by the application of antibiotics, copper compounds and/or microbial antagonists (Psallidas and Tsiantos 2000; Roselló et al. 2013). *Erwinia amylovora* specific bacteriophages may play an important role in epidemiology of fire blight and have a great potential for disease control (Erskine 1973).

A number of different phages infecting *E. amylovora* has been isolated from soil or plant material (Erskine 1973; Ritchie and Klos 1976; Schnabel and Jones 2001; Gill et al. 2003; Born et al. 2011). Based on virion morphology (TEM) and RFLP analysis,

most of them were placed into different subtypes of families *Siphoviridae*, *Myoviridae* and *Podoviridae* (Gill et al. 2003). Some of the described bacteriophages were highly specific towards *E. amylovora*, while others could also infect related epiphytic and pathogenic bacteria, such as *E. pyrifoliae* and *Pantoea agglomerans*. During last years, several complete genomes of *E. amylovora* bacteriophages became available (Erskine 1973; Lehman et al. 2009; Born et al. 2011; Boulé et al. 2011; Dömötör et al. 2012; Meczker et al. 2014; Yagubi et al. 2014).

ViI-like bacteriophages belong to the *Myoviridae* family and group members can infect *Salmonella* (ViI, SFP10), *Shigella* (phiS-boM), *Serratia* (MAM1), *Escherichia* (CBA120, PhaxI) and *Dickeya* (LIMEstone) (Anany et al. 2011; Hooton et al. 2011; Kutter et al. 2011; Adriaenssens et al. 2012b; Park et al. 2012; Matilla and

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Salmond 2012; Shahrababak et al. 2013; Czajkowski et al. 2014). They have virions very similar in morphology and size. Genomes of these phages are similar in size (152–158 kb) and share a large degree of DNA homology. Based on those, and several other criteria, Adriaenssens and coauthors proposed to classify ViI-like phages into a new genus, named ‘Viunalikevirus’ (Adriaenssens et al. 2012a).

In this study, we investigated morphologic and genomic features of the new *E. amylovora* bacteriophage phiEa2809 isolated from plant sample collected in Belarus.

MATERIALS AND METHODS

Strains and growth conditions

The bacterial strains and isolates used in this study (Table 1) were grown routinely on Luria–Bertani (LB) agar or LB broth. The bacterial strain *E. amylovora* 1/79Sm was used for bacteriophage isolation and propagation.

Phage isolation and host range determination

Samples of aerial portions of trees were collected from apple orchards in Smolevichi region of Belarus. Isolation procedure was performed as described previously (Gill et al. 2003). The host range was determined by performing spot tests; 10^7 cfu ml⁻¹ of different bacterial strains were mixed with melted semi-solid agar and poured over 1.5% solid agar. Bacteriophage stock solution (10^8 pfu ml⁻¹) was spotted onto each plate followed by incubation at 28°C for 20 h. Complete lysis of bacteria under phage spots was scored as positive reaction.

Electron microscopy

Phage suspensions were concentrated by centrifugation (36 600 g for 1 h), washed with 0.1 M ammonium acetate and then stained using phosphotungstic acid (2% w/v) on Formvar-coated copper grid. Phages were observed with a transmission electron microscope JEM-100CX.

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

Strain/isolate	Origin, host, year of isolation	Reference/source
<i>E. amylovora</i> LMG2024	United Kingdom, type strain, <i>Pyrus communis</i> , 1959	Prof. R.I. Gvozdyak
1/79Sm	Germany, Spontaneous Sm-resistant mutant of 1/79, <i>Cotoneaster</i> sp., 1979	Dr Klaus Geider
Ea659	Poland, isolated from <i>Malus</i> sp., 1986	Prof. P. Sobiczewski
E1; E2; E3; E4; E5; L3-5; L3-6; L3-8; L13; IK-2309; L3-2	Belarus, isolates from <i>Malus</i> sp.; <i>Pyrus communis</i> , 2007–2009	Lagonenko et al. (2008), Lagonenko, Kudzina and Evtushenkov (2011)
<i>P. agglomerans</i> 194; 196; 198; 209; 216; 219; 220; 223; 224; 227; 228; 229; 230; 231; 232; 234; 235; 236; 238; 239; 240; 242; 246; 247;	Belarus, isolates from different plants, 1980–1981	Dr S. Chernov (Chernov and Fomichev 1981)
<i>E. chrysanthemi</i> 3937	Type strain, isolated from <i>Saintpaulia</i> plants	Dr V. Shevchik (Glasner et al. 2011)
<i>Pe. carotovorum</i> 3–2	Belarus, isolated from <i>Solanum tuberosum</i> , 1978	CMBU ^a
2.18; 26/1; VM29; 550	Belarus, isolates from <i>Solanum tuberosum</i> , 2007–2011	Dr V. Myamin
<i>Pe. atrosepticum</i> 21A	Belarus, isolated from <i>Solanum tuberosum</i> , 1978	Nikolaichik et al. (2014)
36A	Belarus, isolated from <i>Solanum tuberosum</i> , 1978	CMBU
<i>Ps. syringae</i> pv. <i>syringae</i> A13; A14	Isolates; Belarus, <i>Pyrus communis</i> , 2007–2009	CMBU
<i>Ps. syringae</i> pv. <i>Lachrymans</i> BIM-695; BIM-1059	Belarus, isolated from cucumber, 2011	Dr V. Myamin; BIM ^b
<i>Ps. corrugate</i> BIM-86.2	Belarus, isolated from <i>Solanum lycopersicum</i> , 2011	Dr V. Myamin; BIM
Pc37.1; Pc25; Pc20; Pc19; 3'M; B-9069	Belarus, isolates from <i>Solanum lycopersicum</i> , 2009–2011	Dr V. Myamin

CMBU^a – Collection of Microbiology department, Belarusian State University.

BIM^b – Belarusian collection of non-pathogenic microorganisms, Institute of Microbiology, National Academy of Science, Belarus.

DNA isolation and sequencing

DNA from phage particles was isolated as described by Anany et al. (2011) without the stage of cesium chloride (CsCl) gradient purification. A paired-end library for sequencing was prepared with the Nextera XT kit. The genome was sequenced using Illumina MiSeq platform to 23-fold coverage. The resulting high-quality reads were used as the input for the SPAdes ver. 3.1. assembler (Alekseyev and Pevzner 2012). The initial assembly was verified by mapping the raw reads to the contigs using Bowtie 2 (Langmead and Salzberg 2012).

Sequence analysis

Annotation of PhiEa2809 genome was performed using Artemis software (Rutherford et al. 2000). Open reading frames (ORFs) in genome were predicted using Glimmer 2.0 and RAST (Salzberg et al. 1998; Aziz et al. 2008). For final gene prediction, the presence of sequences resembling the ribosome-binding site upstream of the start codon was taken in account. 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). A search for tRNA genes was done with tRNAscan-SE (Schattner et al. 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 phiEa2809 genome were constructed using CGView (Stothard and Wishart 2005). For core genes determination in phage genomes, CoreGenes ver. 3.5 was used (Turner et al. 2013).

Nucleotide sequence accession number

The complete annotated genome sequence of *E. amylovora* phage phiEa2809 has been deposited in GenBank under the accession number KP037007.

RESULTS AND DISCUSSION

Phage phiEa2809 was isolated from leaves of apple tree without fire blight symptoms, collected from Smolevichi orchard (Minsk region, Belarus). Interestingly, fire blight has not ever been detected in this region. PhiEa2809 produced plaques of variable diameter (0.5–2 mm) with irregular margins.

PhiEa2809 was tested for ability to infect different *E. amylovora* strains and other plant-associated bacteria such as *P. agglomerans*, *Pectobacterium carotovorum*, *Pe. atrosepticum*, *E. chrysanthemi*, *Pseudomonas syringae*, *Ps. corrugata* and *Ps. fluorescens*. Based on the host range study, the phiEa2809 phage infected 14 strains of *E. amylovora* and 24 strains of *P. agglomerans*, while all other bacterial isolates and strains did not produce lysis spots.

Transmission electron microscopy was used to determine bacteriophage phiEa2809 morphology. Electron micrographs revealed that phiEa2809 particle has an icosahedral head with a long contractile tail and therefore could be placed into the morphotype A1 of the family *Myoviridae* according to the morphological classification of Bradley (1967) (data not shown).

The genome of phage phiEa2809 is double-stranded linear DNA 162 160 bp in length with a GC content of 51.21%. For annotation, genome was opened upstream of the rIIA gene. The phiEa2809 genomic sequence is similar to the genomes of the

Serratia plymutica phage MAM1 (Genbank Acc. No. JX878496; 71% identity, query coverage 45%), *Shigella* phage AG-3 (Genbank Acc. No. FJ373894; identity 69%, query coverage 43%) and *Dickeya* phage vB DsoM LIMestone1 (Genbank Acc. No. HE600015; identity 69%, query coverage 40%), that belongs to ViI-like bacteriophages group.

ORFs equal or longer than 100 bp were predicted using Glimmer. For final prediction of potential genes, the presence of putative ribosome-binding site upstream of start codon was taken into account. An analysis of phiEa2809 genome revealed 145 ORFs and only one Lys (TTT) tRNA gene (Fig. 1). A total of 67 ORFs (46%) were annotated as encoding hypothetical proteins and most of them showed the similarity to the *S. plymutica* phage MAM1. A total of 78 ORFs were annotated as functional genes. Of those 35 of them were found to encode proteins involved in nucleic acid metabolism, modification, DNA replication and recombination (exonuclease A, ribonuclease H, HsdR family deoxyribonuclease, deoxycytidylate deaminase, dUTP diphosphatase, RuvC, UvsX/Y, thymidylate synthase, ribonucleotide reductase subunits, nucleoside triphosphate pyrophosphohydrolase, DNA topoisomerase subunits, DNA ligase, DNA primase, sliding clamp and subunits of clamp loader, DNA polymerase, single-stranded DNA-binding protein).

A total of 39 ORFs were predicted to encode structural proteins for head, tail, baseplate and proteins involved in virion morphogenesis, including terminase, prohead protease, baseplate and tail assembly proteins. Protein encoded by ORF55 was identified as baseplate lysozyme with domains similar to the Gp5 N-terminal OB domain and to the CHAP domain, which is possibly involved in peptidoglycan cleaving. It is assumed that all phages with double-stranded nucleic acid genomes affect host lysis with a holin–endolysin system, but no corresponding genes have been detected in the phiEa2809 genome. Interestingly, all current published genome sequences of similar bacteriophages also lack the holin gene (Anany et al. 2011; Hooton et al. 2011; Kutter et al. 2011; Adriaenssens et al. 2012b; Czajkowski et al. 2014). The protein encoded by ORF62 contains N-terminal putative peptidoglycan-binding domain so that it can possibly act as an endolysin.

Only two predicted ORFs in phiEa2809 genome seem to be involved in host recognition. ORF121 and ORF125 encode, respectively, putative tailspike protein and putative EPS-depolymerase, similar to *E. amylovora* AmsF, which is involved in amylovan biosynthesis. Interestingly, these two genes are located in a genome region with a very low homology to genome sequences of MAM1, SboM-AG3 and ViI. This region (127 833–139 603) also includes ORFs that were predicted to encode a putative tail fiber protein and a tail fiber assembly protein (Fig. 1). Another low-homology region (13 850–20 167) contains eight ORFs, including ORFs that were predicted to encode STEC autoagglutinating adhesin, putative alpha hydrolase, HsdR family type I site-specific deoxyribonuclease and a putative deoxycytidylate deaminase.

Additionally, several potential regulatory genes were also identified. ORF74 encodes a protein with significant similarity to the T4 Gp55 sigma factor involved in the transcription of late phage genes. ORF49 was annotated as coding for a small protein with N-terminal zinc ribbon domain which belongs to FmdB family of putative regulatory proteins. Two genes (ORF93 and ORF71) encode putative translational regulators with similarity to RegA and RegB, which control the expression of phage early genes.

A comparison of the phiEa2809, MAM1, AG-3 and ViI proteomes using CoreGenes detected 114 common protein

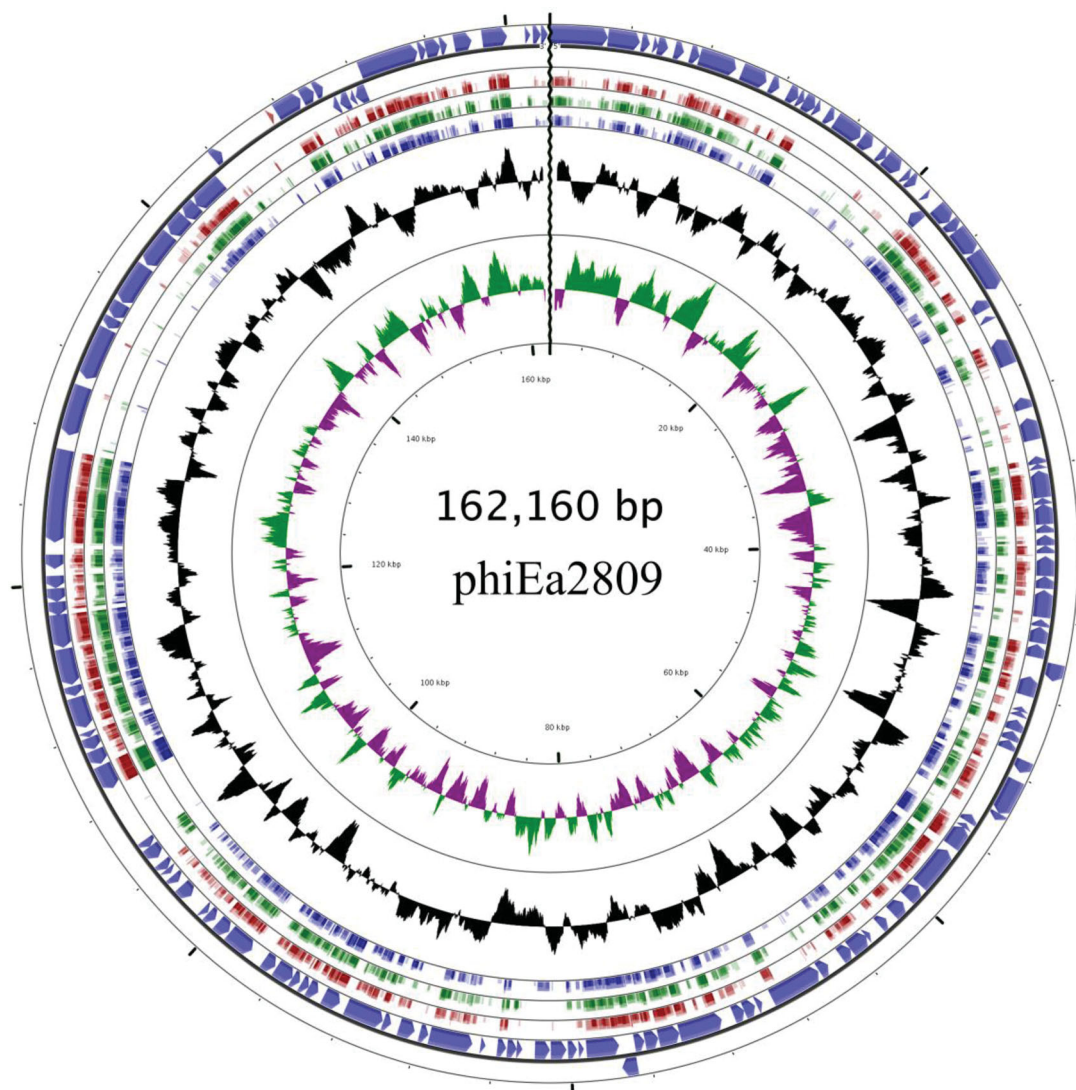


Figure 1. Genetic and physical map of phage phiEa2809 genome prepared using CGView. The genome was opened upstream of the rIIA gene. The outer lane and the next lane correspond to genes on the forward and reverse strands, respectively (Lys tRNA gene is indicated in red and predicted ORFs are indicated in dark blue); the next three lanes represent tblastx analysis results for genomes of phages MAM1, SboM-AG3 and ViI (the degree of sequence similarity to phiEa2809 is proportional to the height of the bars in each frame). Final two circles correspond to GC plot and GC skew, respectively.

homologs. Whole-genome comparisons showed similar genome organization and strongly conserved gene order for these phages (Fig. 2). The genome of phiEa2809 contains 31 ORFs that are not present in other analyzed genomes. Most of them, except above-mentioned ORF125, were annotated as encoding hypothetical proteins. These data allow us to classify phiEa2809 as belonging to the ViI-like group of bacteriophages.

'ViI-like viruses' is a new genus of the T4 superfamily, most members of which are virulent for *Salmonella*, *Shigella*, *Klebsiella* and *Serratia* species (Anany et al. 2011; Hooton et al. 2011; Kutter et al. 2011). Recently, three ViI-like bacteriophages (vB_DsoM_LIMEstone1, vB_DsoM_LIMEstone2 and phiD5) of plant pathogenic *Dickeya* were described (Adriaenssens et al. 2012b; Czajkowski et al. 2014). Published genome sequences of *E. amylovora* bacteriophages that belong to Myoviridae family show similarity to *Salmonella* Felix O1-like viruses (phages phiEa21–4

and M7), Phikzlikevirus genus (phage Ea35–70), *Salmonella* phage SPN3US (phage PhiEaH2) and *E. coli* phage phiEcoM-GJ1 (phage Y2) (Lehman et al. 2009; Born et al. 2011; Dömötör et al. 2012; Yagubi et al. 2014). So we could conclude that phiEa2809 is the first described ViI-like phage of *E. amylovora*.

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Conflict of interest statement. None declared.

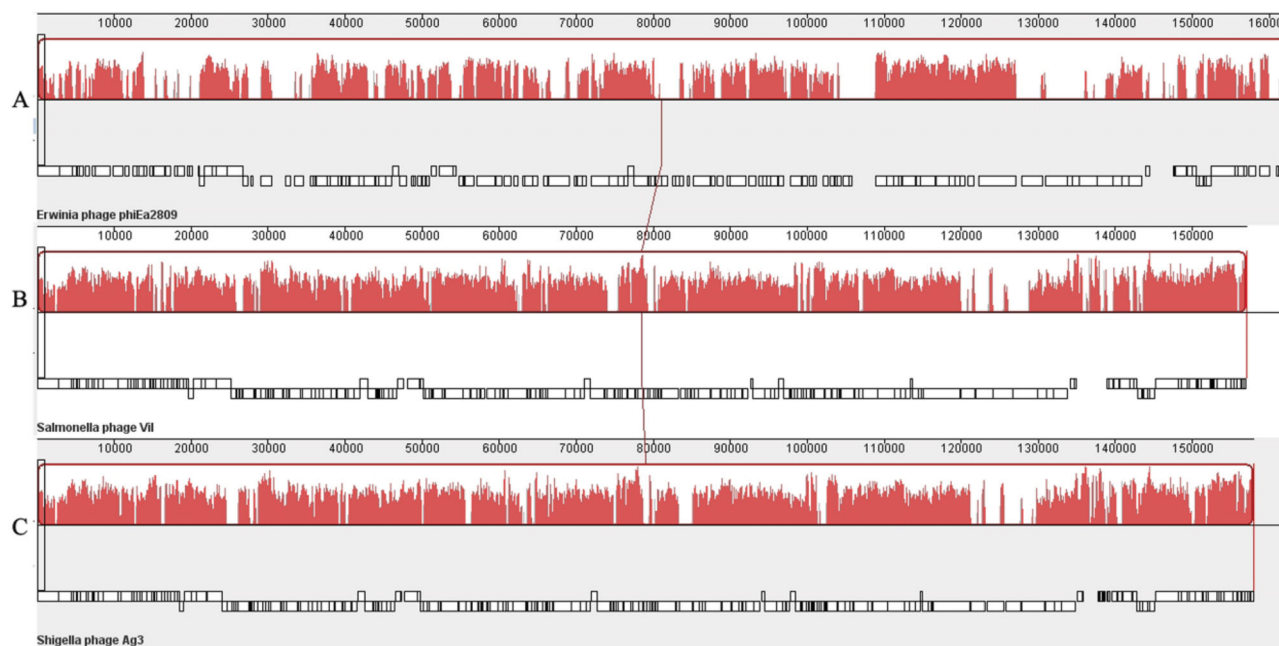


Figure 2. Alignment of phiEa2809 (A), ViI (B) and AG-3 (C) genomes generated using the progressive MAUVE algorithm (ver. 2.3.1). Connection lines indicate similar regions between phiEa2809, ViI and AG-3 genomes. The degree of sequence similarity is indicated by the intensity of the red-colored region. The contiguous box-like diagrams indicate gene positions.

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