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Characterization of the Lytic Bacteriophage phiEaP-8 Effective against Both *Erwinia amylovora* and *Erwinia pyrifoliae* Causing Severe Diseases in Apple and Pear

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Bacteriophages, bacteria-infecting viruses, have been recently reconsidered as a biological control tool for preventing bacterial pathogens. *Erwinia amylovora* and *E. pyrifoliae* cause fire blight and black shoot blight disease in apple and pear, respectively. In this study, the bacteriophage phiEaP-8 was isolated from apple orchard soil and could efficiently and specifically kill both *E. amylovora* and *E. pyrifoliae*. This bacteriophage belongs to the *Podoviridae* family. Whole genome analysis revealed that phiEaP-8 carries a 75,929 bp genomic DNA with 78 coding sequences and 5 tRNA genes. Genome comparison showed that phiEaP-8 has only 85% identity to known bacteriophages at the DNA level. PhiEaP-8 retained lytic activity up to 50°C, within a pH range from 5 to 10, and under 365 nm UV light. Based on these characteristics, the bacteriophage phiEaP-8 is novel and carries potential to control both *E. amylovora* and *E. pyrifoliae* in apple and pear.

Keywords **:** Bacteriophage, black shoot blight, *Erwinia amylovora*, *Erwinia pyrifoliae*, fire blight

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Erwinia amylovora and *E. pyrifoliae* are Gram-negative bacterial pathogens that cause the devastating diseases, fire blight and bacterial black shoot blight, in apple and pear, respectively. Since *E. amylovora* was first discovered in 1780 in the United States, it has been reported globally in Europe, North America, the Middle East and central Asia, and New Zealand (Van der Zwet et al., 2012). In 2015, this disease was reported in apple and pear orchards in South Korea (Myung et al., 2016; Park et al., 2016). In the case of *E. pyrifoliae*, it was first reported in pear orchards in South Korea in 1995 (Kim et al., 1999). These two pathogens are quarantine pathogens in South Korea and have caused severe economic loss due to extensive host eradication and difficulty of fruit export (Park et al., 2017).

So far, antibiotics and copper compounds have been mostly used for the control of fire blight and bacterial black shoot blight in apple and pear. However, the appearance of bacteria resistant to these chemicals have limited their use in the field (Manulis et al., 1998). As an alternative, lytic bacteriophages have been reconsidered as a tool for biological control (Loc-Carrillo and Abedon, 2011). Bacteriophages infect very specific target bacteria, and their host ranges are very narrow unlike antibiotics and copper compounds. They have two different life cycles: the lytic and the lysogenic cycles. During the lytic cycle, a bacteriophage actively infects host bacteria, multiplies inside the host, and kills the host to release progeny (Orlova, 2012). Due to this feature, lytic bacteriophages have been used for

phage therapy to control many bacterial pathogens causing disease in animals and plants (Buttimer et al., 2017; Doffkay et al., 2015).

Since the 1960's, many bacteriophages effective against *E. amylovora* have been reported, and their genomic and physiological features have been determined (Born et al., 2011; Esplin et al., 2017; Gill et al., 2003; Meczker et al., 2014; Müller et al., 2011; Yagubi et al., 2014). Based on the morphology of these bacteriophages, they belong to either the *Myoviridae* or *Podoviridae* family. Some bacteriophages with a broad host range have been applied for phage therapy to control *E. amylovora* (Meczker et al., 2014) and some of them have been commercialized (Buttimer et al., 2017). However, no bacteriophages effective against *E. pyrifoliae* or both *E. amylovora* and *E. pyrifoliae* have yet been reported.

In this study, to isolate bacteriophages with effective host specificity to both *E. amylovora* and *E. pyrifoliae*, 18 soil samples from apple and pear orchards at Jecheon, Chungju, and Yongin, South Korea were collected. After mixing soil with SM (sodium chloride-magnesium sulfate) buffer [50 mM Tris-HCl (pH 7.5), 10 mM NaCl, 10 mM $MgSO_4$] for 30 min, the mixed samples were centrifuged at 10,000 rpm, 4°C for 10 min and filtered with a 0.22 μm pore size filter (Sartorius, Gottingen, Germany). Then, bacteriophages were enriched through overnight incubation with 5ml of the supernatant, 10 ml of LB, and 500 μl of bacterial suspension (10^9 cfu/ml) of *E. amylovora* strain Ea-K1 isolated in South Korea at 26°C in a shaking incubator. The incubated samples were treated with chloroform (1% of the final volume) for 30 min, centrifuged at 3,000 g, 4°C for 15 min, and filtered with a 0.22 μm pore size filter. The presence of bacteriophages in the supernatant was confirmed with four *E. amylovora* strains and two *E. pyrifoliae* strains using a dotting assay (Kropinski et al., 2009; Yu et al., 2016). Then, in order to isolate individual bacteriophages, the overlay assay (Yu et al., 2016) was performed, and plaques with different sizes and shapes were picked separately. A total of 21 individual bacteriophages were picked based on their plaque sizes and shapes (Fig. 1). To determine whether isolated bacteriophages were separate isolates, the genomic DNAs from the 21 isolated bacteriophages were extracted using a phage DNA isolation kit (Norgen Biotek, Thorold, ON, Canada) and digested with restriction enzymes, *Eco*RI, *Bam*HI or both. According to DNA patterns, isolated bacteriophages were categorized into three groups. The bacteriophage phiEaP-8 isolated from apple orchard soil in Yongin, South Korea, where no fire blight or bacterial black shoot blight has been reported, represents one of the three groups and this bacteriophage was used for further characterization.

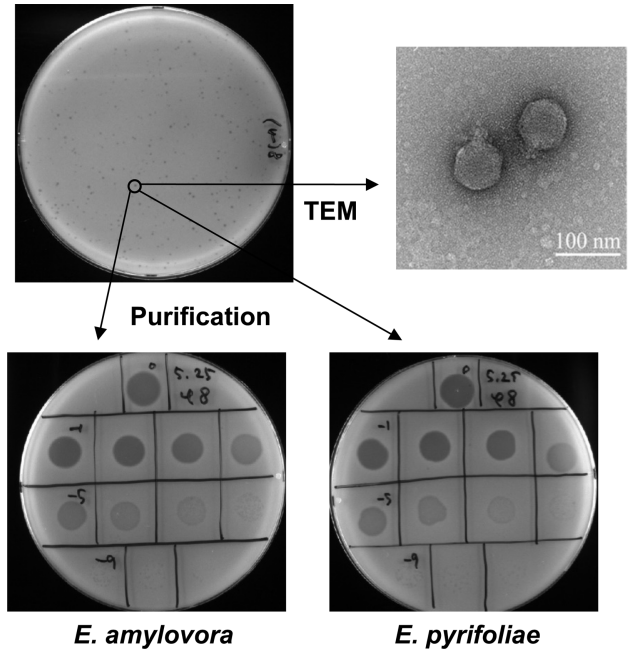

Fig. 1. Plaques from the overlay assay after extracting from soil (top left) and the dotting assay (bottom) with purified serially diluted phiEaP-8 against *E. amylovora* and *E. pyrifoliae*. Morphology of the bacteriophage phiEaP-8 (top right) was determined by transmission electron microscopy (TEM). Photos were taken at the KBSI (Korea Basic Science Institute).

To determine the shape of bacteriophage phiEaP-8, it was propagated by incubating with the Ea-K1 strain (OD_{600} = 0.5-1.0), as described previously (Kim and Ryu, 2011), and was then purified using CsCl gradient ultracentrifugation (Lim et al., 2013). After ultracentrifugation at 25,000 rpm, 4°C for 2 h, bacteriophages were collected and purified through dialysis twice for 1 h in SM buffer. The purified bacteriophage phiEaP-8 was observed by transmission electron microscopy at 120 kV after negative straining with 2% aqueous uranyl acetate (pH 4.0) on carbon-coated copper grids (Ackermann and Heldal, 2010; Brum and Steward, 2010). The bacteriophage phiEaP-8 belonged to the *Podoviridae* family based on its morphology (Fig. 1). The total length and head size are 95 nm and 75 nm, respectively.

Next, the host range of the bacteriophage phiEaP-8 was determined by an overlay assay with twenty *E. amylovora* and seven *E. pyrifoliae* strains isolated in Korea as well as other related bacteria such as *Pectobacterium carotovorum, Dickeya zeae* and three *Pantoea* strains. The bacteriophage phiEaP-8 could efficiently kill all tested strains of both *E. amylovora* and *E. pyrifoliae*, but it was not effective against other related bacteria (Table 1), indicating that this bacte-

Table 1. Host range of the bacteriophage phiEaP-8

No.	Bacterial species	Strain	Lytic activity*
1	*Erwinia amylovora*	Ea73	+
2	*Erwinia amylovora*	Ea74	+
3	*Erwinia amylovora*	Ea75	+
4	*Erwinia amylovora*	Ea76	+
5	*Erwinia amylovora*	Ea77	+
6	*Erwinia amylovora*	Ea78	+
7	*Erwinia amylovora*	Ea-K1	+
8	*Erwinia amylovora*	Ea80	+
9	*Erwinia amylovora*	Ea2016-1	+
10	*Erwinia amylovora*	Ea2016-2	+
11	*Erwinia amylovora*	Ea2016-3	+
12	*Erwinia amylovora*	Ea2016-4	+
13	*Erwinia amylovora*	YKB 12316 (TS 3128)	+
14	*Erwinia amylovora*	YKB 12317 (TS3133)	+
15	*Erwinia amylovora*	YKB 12318 (TS 3240)	+
16	*Erwinia amylovora*	YKB 12319 (TS 3241)	+
17	*Erwinia amylovora*	YKB 12320 (TS 3315)	+
18	*Erwinia amylovora*	YKB 12321 (TS 3325)	+
19	*Erwinia amylovora*	YKB 12322 (TS 3371)	+
20	*Erwinia amylovora*	YKB 12323 (TS 3373)	+
21	*Erwinia pyrifoliae*	Ep81	+
22	*Erwinia pyrifoliae*	EpK1/15	+
23	*Erwinia pyrifoliae*	YKB 12324 (TS 2743)	+
24	*Erwinia pyrifoliae*	YKB 12325 (TS 2744)	+
25	*Erwinia pyrifoliae*	YKB 12326 (TS 3239)	+
26	*Erwinia pyrifoliae*	YKB 12327 (TS 3340)	+
27	*Erwinia pyrifoliae*	YKB 12328 (TS 3342)	+
28	*Pectobacterium carotovorum* subsp. *carotovorum*	Pcc21	−
29	*Dickeya Zeae*		−
30	*Pantoea dispersa*		−
31	*Pantoea agglomerans*		−
32	*Pantoea stewartii*		−

*+, positive; −, negative

riophage is very likely specific to both *E. amylovora* and *E. pyrifoliae*.

To examine if the bacteriophage phiEaP-8 is homologous to known bacteriophages, whole genome sequencing was performed using the PacBio RS II platform (Pacific Biosciences, Menlo Park, CA, USA). Total genomic DNA of phiEaP-8 was isolated using a phage DNA isolation kit (Norgen Biotek, Thorold, ON, Canada), and 10 ug was used to generate a 10 kb SMRTbell™ template library. After sequencing, *de novo* assembly was performed using CANU v1.4 (Koren et al., 2017) and a single contig was generated. The bacteriophage phiEaP-8 carries a 75,929 bp genomic DNA (GenBank accession number, MH160392), and its G+C content is 46.8%. In order to compare the phiEaP-8 genome with other sequenced bacteriophages, which can infect *E. amylovora*, 42 sequenced genomes were obtained from GenBank database, and their genome were compared with BPGA (Bacterial Pan Genome Analysis) pipeline (Chaudhari et al., 2016) and USEARCH (Edgar, 2010). Specifically, 10 of them are *Podoviridae*, 29 of them are *Myoviridae*, and 3 bacteriophages are *Siphoviridae*. Based on a phylogenetic tree (Fig. 2, Supplementary Fig. 1), phiEaP-8 was closely related to five bacteriophages belonging to *Podoviridae*, which are vB_EamP_Rexella (GenBank accession number, KX098390), vB_EamP_Frozen (GenBank accession number, KX098389), Ea9-2 (GenBank accession number, KF806588), vB_EamP_Gutmeister (GenBank accession number, KX098391), and vB_EamP-S6 (GenBank accession number, HQ728266). At the DNA level, phiEaP-8 only has about 85% identity to these closely related bacteriophages.

Gene annotation to find coding sequences (CDS), tRNA, and rRNA genes was performed using Prokka (v1.12b). Gene annotation showed that the genome contains 78 CDSs and 5 tRNA genes, but no rRNA genes (Supplementary Table 1). The bacteriophage phiEaP-8 genome carries genes encoding putative holin and Rz/Rz1 spanin proteins, indicating that it is a lytic bacteriophage. Interestingly, this bacteriophage carries a gene homologous to *amsF* responsible for amylovoran biosynthesis in *E. amylovora* and also a gene encoding serine protease highly homologous to one in *Enterobacteriaceae* like *Escherichia coli* and *Salmonella enterica*. These results indicate that this bacteriophage might have obtained these genes from host bacteria during infection and genome multiplication.

To determine if phiEaP-8 can be used for phage therapy against both *E. amylovora* and *E. pyrifoliae*, its lytic activity under diverse environmental conditions was examined. For this, 10^5 PFU/ml of bacteriophages were treated for 1 h at 30, 40, 50, and 60°C, at pH range 3 to 12, or under 365 nm UV light, and its lytic activity was measured by the dotting assay. This bacteriophage retained lytic activity stable against *E. amylovora* and *E. pyrifoliae* up to 50°C, within a pH range from 5 to 10, and under 365 nm UV light (Fig. 3).

Bacteriophages are typically isolated from soil, water, and plants surrounding infected trees (Doffkay et al., 2015; Müller et al., 2011). Interestingly, the apple orchard in Yongin, South Korea where phiEaP-8 was isolated has never been infected with either *E. amylovora* or *E.*

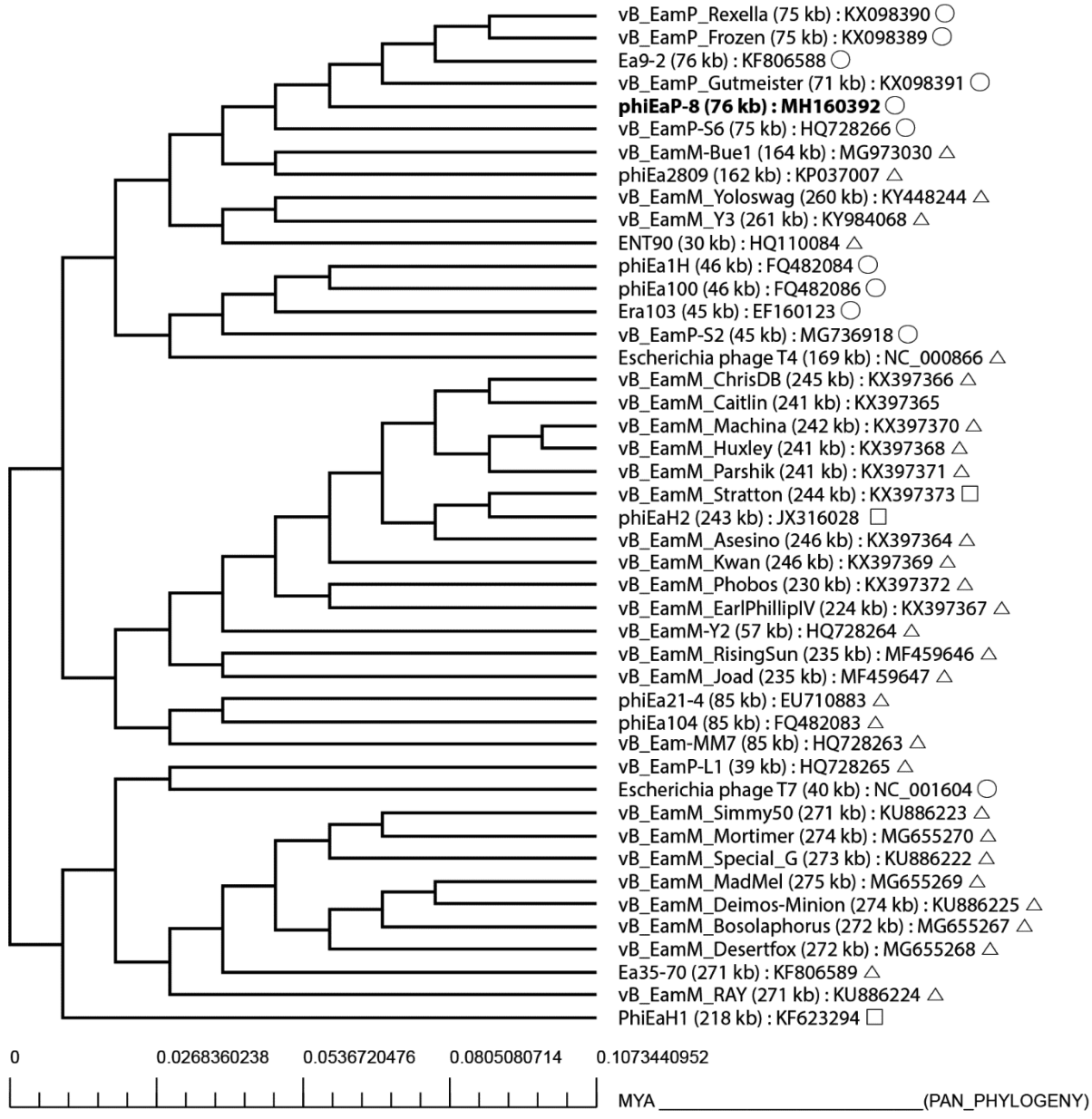

Fig. 2. Phylogenetic tree with phiEaP-8 and other 42 *Erwinia amylovora* bacteriophages. The genome sequences were obtained from GenBank database, and their names, sizes, and accession numbers were stated in the figure. The tree was generated with BPGA pipeline and USEARCH tool. *Escherichia* phages, T4 and T7 bacteriophages, were used as an outgroup. Circle, *Podoviridae*; triangle, *Myoviridae*; square, *Siphoviridae*.

pyrifoliae. In a previous paper (Lagonenko et al., 2015), phiEa2809 was isolated from the leaves of an apple tree without fire blight symptoms in an apple orchard where fire blight was never detected. The genus *Erwinia* is classified to the *Enterobacteriaceae* family, which includes both pathogenic and non-pathogenic bacteria in genera such as *Erwinia*, *Enterobacter*, *Pantoea*, *Pectobacterium*, and *Brenneria* (Kado, 2006). Presence of bacteriophages in an apple orchard, in which fire blight or bacterial black shoot blight have never been detected, might be explained by the thought that bacteriophages could exist owing to the presence of some of these bacteria.

Born et al. (2011) reported eight bacteriophages effective against *E. amylovora*. Interestingly, some of them were effective against other related bacteria such as *Erwinia billingiae*, *Pantoea agglomerans*, and *Pantoea ananatis*, indicating the presence of wide host-range bacteriophages. The bacteriophage phiEaP-8 looks specific to both *E.*

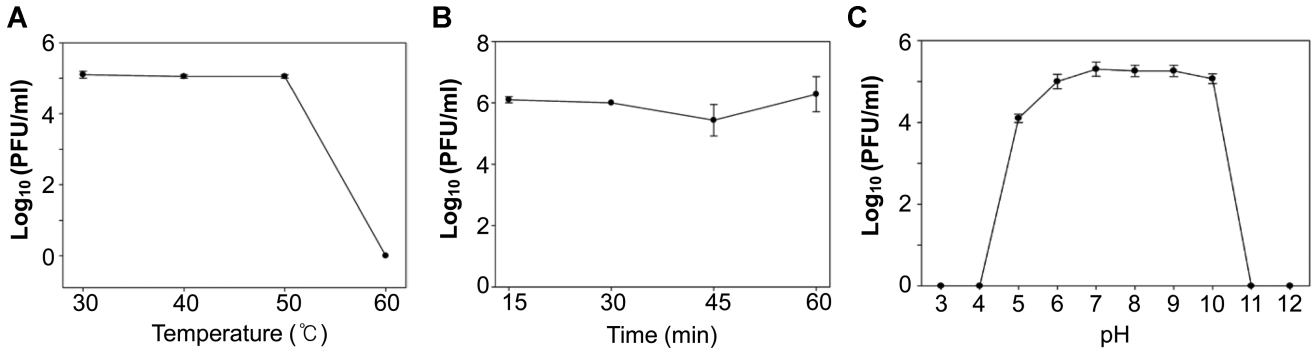

Fig. 3. Stability of the bacteriophage phiEaP-8 under diverse environmental conditions. (A) Temperature. (B) 365 nm UV light. (C) pH. Bacteriophages were counted as PFU/ml by a dotting assay. All tests were repeated at least three times. Error bars indicate standard errors.

amylovora and *E. pyrifoliae*, which indicates that this is a somewhat narrow host-range bacteriophage. However, this bacteriophage could be very useful because both pathogenic bacteria can co-exist in apple and pear orchards in Korea.

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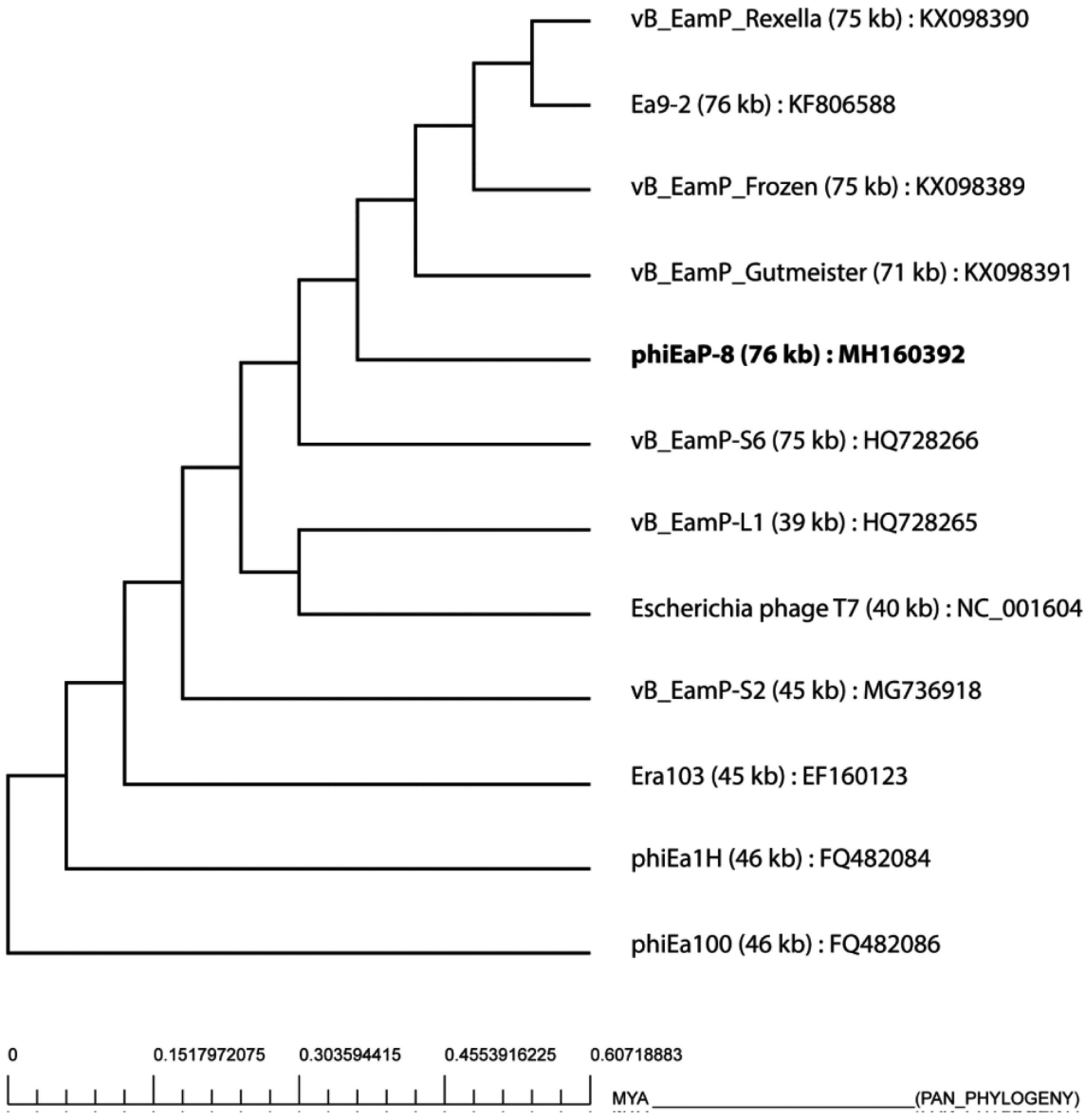

Supplementary Fig. 1. Phylogenetic tree with phiEaP-8 and other 10 *Erwinia amylovora* bacteriophages only belonging to the *Podoviridae* family. The genome sequences were obtained from GenBank database, and their names, sizes, and accession numbers were stated in the figure. The tree was generated with BPGA pipeline and USEARCH tool, and *Escherichia* phage T7 was used as an outgroup.

Supplementary Table 1. List of ORFs in the genome of the bacteriophage phiEaP-8

Gene_ID	Start	End	aa[X]	Product	aa Identity (%)[Y]	Homolog[Z]
phiEaP-8_01	441	761	106	hypothetical protein	68	*Xanthomonas citri* phage CP2
phiEaP-8_02	825	1511	228	hypothetical protein	67	Ea9-2 phage Ea92_88
phiEaP-8_03	1441	3096	551	terminase large subunit	79	Ea9-2 phage Ea92_87
phiEaP-8_04	3104	3808	234	hypothetical protein	65	Ea9-2 phage Ea92_86
phiEaP-8_05	3813	4901	362	putative tail-spike protein	35	Ea9-2 phage Ea92_85
phiEaP-8_06	4911	7094	727	putative cellulase or glycoside hydrolase	46	*Erwinia* phage PEp14
phiEaP-8_07	7108	9516	802	putative glycoside hydrolase	56	*Erwinia* phage vB_EamP-S6
phiEaP-8_08	9585	9920	111	putative tail-length tape measure protein	63	*Erwinia* phage vB_EamP_Frozen / Ea9-2 phage Ea92_82
phiEaP-8_09	9905	10150	81	putative holin	63	Ea9-2 phage Ea92_81
phiEaP-8_10	10137	10742	201	putative N-acetylmuramidase / lytic transglycosylase	83	Ea9-2 phage Ea92_80 / *Erwinia* phage vB_EamP_Frozen
phiEaP-8_11	10747	11304	185	putative Rz/Rz1 spanin protein	56	Ea9-2 phage Ea92_79 / *Erwinia* phage vB_EamP_Frozen
phiEaP-8_12	11264	13576	770	portal protein	76	Ea9-2 phage Ea92_78
phiEaP-8_13	13587	13919	110	hypothetical protein	56	Ea9-2 phage Ea92_77
phiEaP-8_14	13919	15166	415	putative tape measure protein	52	Ea9-2 phage Ea92_76 / *Erwinia* phage vB_EamP_Frozen
phiEaP-8_15	15179	16408	409	major capsid protein	88	Ea9-2 phage Ea92_75
phiEaP-8_16	16464	17075	203	hypothetical protein	64	Ea9-2 phage Ea92_74
phiEaP-8_17	17120	18100	326	putative structural protein	55	Ea9-2 phage Ea92_73 / *Erwinia* phage vB_EamP_Frozen
phiEaP-8_18	18105	20783	892	hypothetical protein	54	Ea9-2 phage Ea92_72
phiEaP-8_19	20795	21262	155	hypothetical protein	69	Ea9-2 phage Ea92_71
phiEaP-8_20	21272	23263	663	putative structural protein	34	Ea9-2 phage Ea92_70 / *Erwinia* phage vB_EamP_Frozen
phiEaP-8_21	23361	33887	3508	virion-associated RNA polymerase	50	Ea9-2 phage Ea92_69
phiEaP-8_22	33922	34158	78	hypothetical protein		No homology
phiEaP-8_23	34148	34285	45	hypothetical protein	46	Ea9-2 phage Ea92_66
phiEaP-8_24	34295	36094	599	putative exopolysaccharide (amylovo-ran) biosynthesis protein/AmsF	61	*Erwinia* phage vB_EamM_Yoloswag
phiEaP-8_25	36457	36538		tRNA-Tyr		
phiEaP-8_26	36732	36807		tRNA-Ile		
phiEaP-8_27	36946	37020		tRNA-Asp		
phiEaP-8_28	37027	37103		tRNA-Glu		
phiEaP-8_29	37110	37185		tRNA-Asn		
phiEaP-8_30	37462	37989	175	hypothetical protein	46	Ea9-2 phage Ea92_64
phiEaP-8_31	37989	38546	185	holliday junction resolvase	88	Ea9-2 phage Ea92_63
phiEaP-8_32	38546	39289	247	putative ssDNA-binding protein	51	Ea9-2 phage Ea92_62
phiEaP-8_33	39351	40079	242	polynucleotide kinase	83	Ea9-2 phage Ea92_61
phiEaP-8_34	40128	42287	719	DNA primase	78	Ea9-2 phage Ea92_60
phiEaP-8_35	42284	43261	325	putative exonuclease	65	Ea9-2 phage Ea92_59
phiEaP-8_36	43328	43606	92	hypothetical protein	81	Ea9-2 phage Ea92_58
phiEaP-8_37	43599	43982	127	hypothetical protein	36	Ea9-2 phage Ea92_57
phiEaP-8_38	44078	46237	719	hypothetical protein	51	*Erwinia* phage vB_EamP-S6
phiEaP-8_39	46234	46566	110	hypothetical protein	45	*Erwinia* phage vB_EamP-S6

Supplementary Table 1. Continued

Gene_ID	Start	End	aa[X]	Product	aa Identity (%)[Y]	Homolog[Z]
phiEaP-8_40	46576	47247	223	hypothetical protein	75	*Erwinia* phage vB_EamP-S6
phiEaP-8_41	47244	47708	154	hypothetical protein	51	Ea9-2 phage Ea92_55
phiEaP-8_42	47785	50481	898	DNA polymerase	73	Ea9-2 phage Ea92_54
phiEaP-8_43	50493	50993	166	hypothetical protein	43	Ea9-2 phage Ea92_53
phiEaP-8_44	51001	52305	434	ATP-dependent DNA helicase	66	Ea9-2 phage Ea92_52
phiEaP-8_45	52348	52800	150	putative phosphoribosyl-ATP pyro-phosphohydrolase	64	Ea9-2 phage Ea92_51
phiEaP-8_46	52793	53125	110	hypothetical protein	71	Ea9-2 phage Ea92_50
phiEaP-8_47	53177	55051	624	putative rIIB-like protein	60	Ea9-2 phage Ea92_49
phiEaP-8_48	55048	57519	823	putative rIIA-like protein	42	Ea9-2 phage Ea92_48
phiEaP-8_49	57762	58712	316	thymidylate synthase ThyX	70	Ea9-2 phage Ea92_47
phiEaP-8_50	58712	58894	60	hypothetical protein	42	*Pectobacterium* phage vB_PatP_CB4
phiEaP-8_51	58894	59151	85	hypothetical protein	49	Ea9-2 phage Ea92_44
phiEaP-8_52	59161	60006	281	DNA adenine methylase	64	Ea9-2 phage Ea92_42
phiEaP-8_53	60035	60526	163	dCTP deaminase	63	Ea9-2 phage Ea92_40
phiEaP-8_54	60530	61663	377	putative metallopeptidase domain protein	59	Ea9-2 phage Ea92_39
phiEaP-8_55	61675	62724	349	putative ATPase	55	Ea9-2 phage Ea92_38
phiEaP-8_56	62787	62996	69	hypothetical protein		No homology
phiEaP-8_57	63062	64504	480	putative ATP-binding protein	84	Ea9-2 phage Ea92_37
phiEaP-8_58	64578	64946	122	hypothetical protein	50	Ea9-2 phage Ea92_36
phiEaP-8_59	64951	65529	192	hypothetical protein	41	Ea9-2 phage Ea92_35
phiEaP-8_60	65528	65876	115	HNH endonuclease	90	Ea9-2 phage Ea92_33
phiEaP-8_61	65873	66025	50	hypothetical protein		No homology
phiEaP-8_62	66063	66818	251	ATP-dependent Clp protease	76	Ea9-2 phage Ea92_31
phiEaP-8_63	66885	67808	307	serine protease	66	*Escherichia coli*/*Salmonella enterica*
phiEaP-8_64	68021	68554	177	capsid decorating protein		*Escherichia* phage EC1-UPM
phiEaP-8_65	68613	69017	134	hypothetical protein		
phiEaP-8_66	69099	70331	410	RNA polymerase 2	61	Ea9-2 phage Ea92_23
phiEaP-8_67	70355	70615	86	hypothetical protein		No homology
phiEaP-8_68	70612	71427	271	RNA polymerase 1	66	Ea9-2 phage Ea92_21
phiEaP-8_69	71450	71632	60	hypothetical protein	51	Ea9-2 phage Ea92_18
phiEaP-8_70	71632	71865	77	hypothetical protein	49	Ea9-2 phage Ea92_17
phiEaP-8_71	71865	72248	127	hypothetical protein	63	Ea9-2 phage Ea92_16
phiEaP-8_72	72313	72528	71	hypothetical protein	52	Ea9-2 phage Ea92_15
phiEaP-8_73	72529	72936	135	hypothetical protein	68	Ea9-2 phage Ea92_14
phiEaP-8_74	72815	73150	111	hypothetical protein		No homology
phiEaP-8_75	73152	73466	104	hypothetical protein	57	Ea9-2 phage Ea92_13
phiEaP-8_76	73463	73648	61	hypothetical protein		No homology
phiEaP-8_77	73645	73929	94	hypothetical protein	67	Ea9-2 phage Ea92_11
phiEaP-8_78	73926	74132	68	hypothetical protein	65	Ea9-2 phage Ea92_9
phiEaP-8_79	74183	74464	93	hypothetical protein	34	Ea9-2 phage Ea92_7
phiEaP-8_80	74457	74732	91	hypothetical protein		
phiEaP-8_81	74895	75296	133	putative RNAP1 subunit A	60	Ea9-2 phage Ea92_2 / *Erwinia* phage vB_EamP_Frozen
phiEaP-8_82	75386	75550	54	hypothetical protein		No homology
phiEaP-8_83	75601	75929	108	hypothetical protein	56	Ea9-2 phage Ea92_1

[X]The number of amino acids (aa)
[Y]Identity to a homolog at the amino acid level
[Z]Closest homolog based on BLASTP at the GenBank database