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Molecular and physiological properties of bacteriophages from North America and Germany affecting the fire blight pathogen *Erwinia amylovora*

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Summary

For possible control of fire blight affecting apple and pear trees, we characterized *Erwinia amylovora* phages from North America and Germany. The genome size determined by electron microscopy (EM) was confirmed by sequence data and major coat proteins were identified from gel bands by mass spectroscopy. By their morphology from EM data, ϕEa1h and ϕEa100 were assigned to the *Podoviridae* and ϕEa104 and ϕEa116 to the *Myoviridae*. Host ranges were essentially confined to *E. amylovora*, strains of the species *Erwinia pyrifoliae*, *E. billingiae* and even *Pantoea stewartii* were partially sensitive. The phages ϕEa1h and ϕEa100 were dependent on the amylovoran capsule of *E. amylovora*, ϕEa104 and ϕEa116 were not. The *Myoviridae* efficiently lysed their hosts and protected apple flowers significantly better than the *Podoviridae* against *E. amylovora* and should be preferred in biocontrol experiments. We have also isolated and partially characterized *E. amylovora* phages from apple orchards in Germany. They belong to the *Podoviridae* or *Myoviridae* with a host range similar to the phages isolated in North America. In EM measurements, the genome sizes of the *Podoviridae* were smaller than the genomes of the *Myoviridae* from North America and from Germany, which differed from each other in corresponding nucleotide sequences.

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Introduction

Bacteriophages occur in many environments and may even outnumber their host cells. They need an appropriate receptor for infection, which restricts the host range. After docking to the cell surface, bacteriophages inject their genome to multiply inside the cell. At the end of their life cycle viral proteins lyse the host cells. Efficient destruction of a pathogen can be beneficial to prevent infections of the host tissue (Jones *et al*., 2007). Bacteriophages have been applied in controlling bacterial populations on plants such as onion, tomato and potato (McKenna *et al*., 2001; Obradovic *et al*., 2004; Lang *et al*., 2007). The Gram-negative bacterium *Erwinia amylovora* is the causal agent of fire blight, a necrotic disease that affects rosaceous plants and can lead to high commercial losses in production of the economically important fruit crops apple, pear and quince (Momol and Aldwinckle, 2000).

Bacteriophages have been classified by their electron microscopy (EM) morphotype, plaque morphology and restriction fragment pattern. MALDI-TOF mass spectroscopy (MS) was used to identify structural proteins. The genomes of five *E. amylovora* phages have been fully sequenced. Phage ϕEra103 (Accession No. EF160123; Vandenbergh and Cole, 1986), ϕEa1h and ϕEa100 (Müller *et al*., 2011) belong to the short-tailed *Podoviridae*, and phages ϕEa21-4 (Accession No. EU710883, Lehman *et al*., 2009) and ϕEa104 (Müller *et al*., 2011) to the *Myoviridae* with a long contractile tail structure. The *Podoviridae* package an EPS depolymerase into their coat (Bernhard *et al*., 1996), which degrades the amylovoran capsules of the pathogen and exposes *E. amylovora* to plant defence mechanisms (Kim and Geider, 2000). The enzyme expressed in plant cells under control of the strong 35S promoter reduced fire blight symptoms on apple (Flachowsky *et al*., 2008) and pear (Malnoy *et al*., 2005). Attempts have been described to apply bacteriophages for control of fire blight (Erskine, 1973; Schnabel and Jones, 2001), although details about their interaction with *E. amylovora* and bacterial populations in flowers are missing. We have concentrated our efforts to growth requirements of *E. amylovora* phages and symptom reduction in fire blight tissue, such as apple flowers and pear slices.

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Results

Morphology of four E. amylovora *phages and their genome size*

We have characterized four *E. amylovora* phages isolated in North America and three phages from Germany. The phages were allocated into morphotype groups according to Ackermann (2007). The American phages show an icosahedral head (size 60–73 nm, Table 1). The first group carries a short tail which was not seen in the contracted form (Fig. 1), and a second group of phages carries long tails of 114 nm (Table 1). This tail is contractile as shown in Fig. 1G for phage φEa104 and others. *Erwinia amylovora* phages φEa1h and φEa100 (Fig. 1A and D) are compact particles without an extended tail, whereas φEa104 and φEa116 have a well-visible tail. In negative staining of the phage particles small tail fibres were detected, and genes encoding tail fibre proteins were identified on the genomes.

The four American bacteriophages showed different lysis properties (Fig. 2). Phages φEa1h and φEa100 formed turbid plaques. φEa1h produced large plaques with a distinct turbid halo as described before by Ritchie and Klos (1977). φEa100 formed smaller plaques than φEa1h and no halos were observed. The phages φEa104 and φEa116 produced clear plaques of about 1 mm in diameter on *E. amylovora* strain Ea1/79Sm.

For measuring the size of their genome the phages were lysed on a grid. Phages φEa1h and φEa100 were close to 46 kb, whereas φEa104 and φEa116 were larger with a size of 85 kb (Table 1). EM measurements for the lengths of the genomes of φEa1h, φEa100 and φEa104 were in good agreement with sequence data (Table 1).

Genomic properties of bacteriophages φEa1h, φEa100, φEa104, φEa116

A striking difference of the phages φEa1h and φEa100 to the related phage φEra103 was an insertion within the DNA polymerase gene. The insertion was confirmed by resequencing this region twice in both directions. In an amino acid alignment both domains reconstitute a DNA polymerase similar to the enzyme of bacteriophage φEra103.

Restriction digests of the *Podoviridae* genomes with BamHI, BglII, ClaI, HindIII, SpeI and XbaI agreed with the expected positions. For EcoRI a 1.7 kb band instead of a 1.3 kb fragment was detected on a 1% agarose gel.

Both *Podoviridae* may use direct repeats for replication as concatemers, also proposed for the related *Escherichia coli* phage T7. The 54-mer 'GTCTATAGGTAGGC CCAGGTTATCCAGGTCTATAGGTAGGCCCAGGTTATC CAG' is repeated twice and could serve as required direct repeat. However, an *in silico* tested alternative start of the

Table 1. Genome features and morphology of the *E. amylovora* phages investigated.

	Podoviridae				*Myoviridae*		
	φEa1h[a]	φEa100[b]	φEaJ08T	φEaK08T	φEa104[c]	φEa116	φEaJ08C
Size from EM measurement (bp)	45 741 ± 880	45 642 ± 1 340	39 600 ± 629	39 865 ± 518	84 522 ± 1 014	85 578 ± 755	84 189 ± 1 117
Genome size by sequencing (bp)	45 522	45 554			84 564	70% sequenced	
GC content (%)	49.7	49.7			43.9		
Predicted ORFs	50	50			118		
Predicted tRNAs	0	0			24		
Head diameter (nm)	59.89 ± 1.49	61.42 ± 2.14	59.35 ± 1.39	59.83 ± 3.03	71.56 ± 2.20	73.36 ± 1.89	72.98 ± 3.14
Tail length (nm)					114.42 ± 2.51	114.62 ± 2.28	115.54 ± 5.17
Tail diameter (nm)					17.75 ± 1.58	20.41 ± 1.06	22.42 ± 2.08

Accession numbers of complete genomes:
a. FQ482084;
b. FQ482086;
c. FQ482083.

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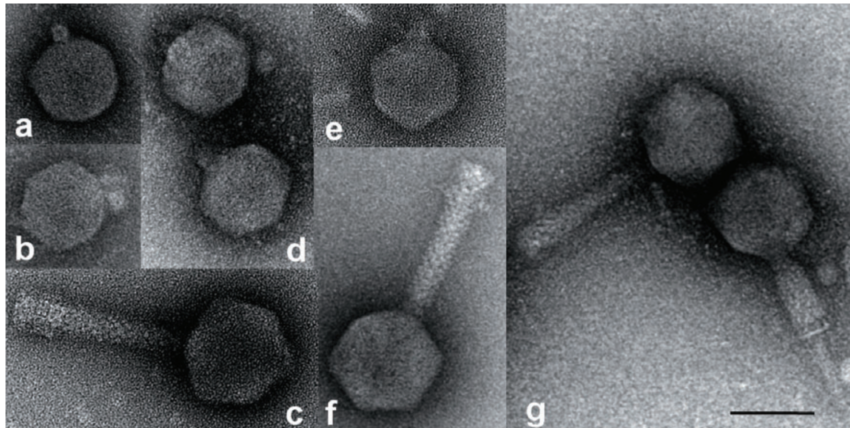

Fig. 1. Electron microscopic visualization of *Erwinia amylovora* phages. (A) ϕEa1h; (B) ϕEaJ08T; (C) ϕEaJ08C; (D) ϕEa100; (E) ϕEa08KT; (F) ϕEa116; (G) ϕEa104. The bar represents a size of 60 nm.

Podoviridae genomes with the 26-mer 'GCAAGGTAATG GCTAGGCTATGTCCC' was in full agreement with the EcoRI digest.

About 70% of the genome size of ϕEa116 as determined by EM were obtained by shotgun sequencing and primer walking using the complete genome sequence of the related phages ϕEa104 and ϕEa21-4 for primer design. We assume strong viral promoters to cause lethal effects by cloning some insertions in *E. coli*. The relationship of ϕEa116 to ϕEa104 indicated a genome length of 85 kb, which is in agreement with EM data for the contour length of DNA from ϕEa116. The positions of the six contigs obtained are outlined in Fig. 3 and Table 2, with respect to the genome of the related phage ϕEa104. The contigs show 80–90% identity to the corresponding sequences of ϕEa104. Their annotation corresponds to parts of the annotated ϕEa104 genome. No EPS depolymerase gene was identified in the genome of both phages, which could indicate their EPS-independent action for growth on *E. amylovora* strains without an amylovoran capsule.

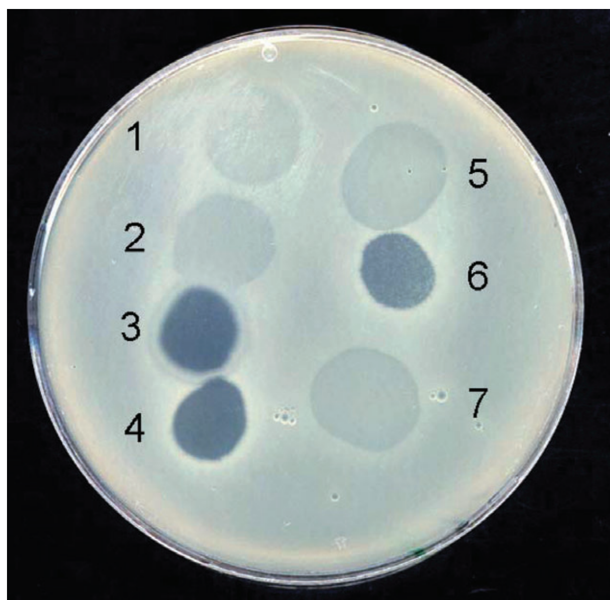

Fig. 2. Bacteriophage drop test with American and German phages on *E. amylovora* strain Ea1/79Sm: 1: ϕEa1h, 2: ϕEa100, 3: ϕEa104, 4: ϕEa116, 5: ϕEaK08T, 6: ϕEaJ08C, 7: ϕEaJ08T.

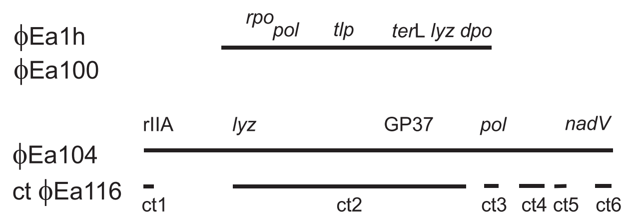

Fig. 3. A map for the genomes of ϕEa1h and ϕEa100 and position of contigs of phage ϕEa116 aligned to the genome of ϕEa104. Abbreviations: *rpo*, RNA polymerase; *pol*, DNA polymerase; *tlp*, tail tubular protein; *ter*L, large terminase subunit; *lyz*, lysozyme; *dpo*, EPS depolymerase; rIIA, membrane integrity protector; GP37, gene product 37; *nadV*, nicotinamide phosphoribosyl transferase.

Table 2. Size and positions of ϕEa116 contigs (ct) within the genome of ϕEa104.

ct	Size (in bp)	Position of ϕEa116 cts in the genome of ϕEa104	Nucleotide identity (%)
1	1 978	1 to 1 979	85
2	41 982	16 565 to 58 154	87
3	2 566	61 463 to 64 028	88
4	4 302	67 670 to 71 976	80
5	2 208	74 049 to 76 256	89
6	3 033	81 533 to 84 565	89

Identity on nucleotide level is indicated.

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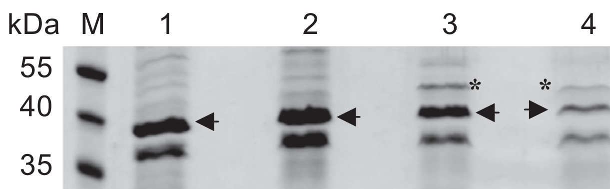

Fig. 4. SDS-PAGE of *E. amylovora* phage protein preparations. The proteins from bands marked with arrows and asterisks were identified by MALDI-TOF MS analysis. The major capsid protein is marked for all four phages by an arrow, a structural protein for ϕEa104 and ϕEa116 by an asterisk. Lanes 1–4: ϕEa1h, ϕEa100, ϕEa104, ϕEa116 respectively; M: pre-stained protein ladder (Fermentas, St. Leon-Rot, Germany).

Identification of phage proteins by MALDI-TOF MS analysis

Protein preparations of the bacteriophages ϕEa1h, ϕEa100, ϕEa104 and ϕEa116 were analysed on an SDS gel (Fig. 4). A size of 39–40 kDa was estimated for a dominant protein band, possibly the major capsid protein. MALDI-TOF MS analysis indicated a nominal molecular mass of 40.0 kDa for the ϕEa1h and ϕEa100 protein and 40.8 kDa for the protein of ϕEa104 and ϕEa116, with intermediate to high sequence coverage of the cleavage peptides. A high amino acid similarity was found to the capsid proteins of *E. amylovora* phages ϕEra103 (gi 125999997) for ϕEa1h and ϕEa100 and of ϕEa21-4 (gi 219681311) for ϕEa104 and ϕEa116. The molecular weight of the major capsid proteins calculated for ϕEa1h/ϕEa100 from the genome sequences is 39.95 kDa and for ϕEa104 and ϕEa116 40.79 kDa. In addition, a structural protein was identified for ϕEa104 and ϕEa116. This protein has similarity to a conserved structural protein from ϕEa21-4 (gi 219681316). A band below 40 kDa may represent a bacterial protein.

Phage growth and propagation on various E. amylovora *strains and other bacteria*

The four American bacteriophages were tested for their lysis efficiency on various *E. amylovora* strains, as well as on other *Erwinia* and *Pantoea* strains (Table 3). Apart from *E. amylovora* fruit tree isolates, two raspberry isolates were tested. The *Podoviridae* were not able to lyse all *E. amylovora* strains. The *Myoviridae* exhibited a broader host range than the *Podoviridae*. Strong lytic activity was

Table 3. Host range of bacteriophages from North America and Germany on *Erwinia* and *Pantoea* host strains.

	Podoviridae				*Myoviridae*		
Strain	ϕEa1h	ϕEa100	ϕEaJ08T	ϕEaK08T	ϕEa104	ϕEa116	ϕEaJ08C
E. amylovora							
CFBP1430	++	+++	+++	+++	+++	+++	+++
CFBP1430Sm-amsD	–	–	–	–	+++	+++	+++
PMV6076	++	+++	+++	+++	+++	+++	++(+)
Ea1/79	++	++	++	++	++	++	++
Ea1/79Sm	++	+++	++	++	+++	+++	+++
Ea1/79del100	–	–	–	–	+++	+++	++(+)
Ea322A	–[a]	–[a]	–[a]	–[a]	+++	+++	+++
EaDS05	+++	+++	+++	+++	++(+)	+++	++(+)
EaDS08	++	++	++	++	++(+)	++(+)	++
EaRW06	++	++(+)	+(+)	+(+)	+++	+++	++(+)
EaOR1/07	++[a]	++[a]	++[a]	++[a]	++(+)	++(+)	++(+)
EaOR2/07	(+)[a]	(+)[a]	(+)[a]	(+)[a]	+++	+++	+++
IL6	+++	+++	+++	+++	++	++	+
MR1	+(+)	+(+)	++	++	++	++	+(+)
E. pyrifoliae							
Ep1/96	(+)[a]	+[a]	+[a]	+[a]	++	++	+
Ep2/97	(+)[a]	+[a]	+[a]	+[a]	++	++(+)	+(+)
Ejp557	–	–	–	–	+	+	(+)
Ejp617	(+)	(+)	(+)	(+)	(+)	+	–
E. billingiae							
Eb661	–	+	+	+	+	+	+
E. tasmaniensis							
Et1/99	–	–	–	–	–	–	–
P. stewartii							
DC283	+(+)	+	+++	+++	+++	+++	++(+)
P. agglomerans							
C9-1	–	–	(+)	(+)	+	+(+)	(+)
MB96	–	+	+	+	+	+	(+)

a. Strains grown on MM2C minimal agar; *Pantoea* strains were grown on LBglc agar.
The phages were assayed in soft agar drop tests. Lysis efficiency is rated from very good (+++) to no lysis (–). A tendency to a lower category is indicated by parentheses.

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Table 4. Multiplication of bacteriophages with *E. amylovora* and other bacteria on filter disks.

Strain	Bacteriophages			
	ϕEa1h	ϕEa100	ϕEa104	ϕEa116
E. amylovora				
EaDS05	1.0×10^9	2.0×10^9	5.0×10^9	2.0×10^9
EaRW06	2.0×10^9	5.0×10^8	2.0×10^9	2.3×10^8
EaOR1/07	2.0×10^8	2.0×10^8	5.0×10^8	1.0×10^9
EaOR2/07	5.0×10^9	1.0×10^{10}	1.8×10^{10}	1.5×10^{10}
PMV6076	5.5×10^9	2.2×10^{10}	5.0×10^{10}	2.0×10^{10}
Ea1/79 del100	2.2×10^5	3.3×10^2	2.0×10^8	2.5×10^8
Ea322A	1.1×10^9	2.4×10^8	2.9×10^8	4.3×10^5
E. billingiae				
Eb661	1.0×10^4	0	7.0×10^3	3.5×10^3
P. stewartii				
DC283[a]	3.5×10^7	0	5.0×10^3	5.0×10^5

a. Grown on LBglc agar

visible for all *E. amylovora* and some *Erwinia pyrifoliae* strains. *Erwinia pyrifoliae* isolates from Japan were less sensitive than the Korean strains. *Erwinia billingiae* was slightly sensitive, but *Erwinia tasmaniensis* was resistant to all four *E. amylovora* phages. A low sensitivity was seen for *Pantoea agglomerans* MB96 and C9-1, whereas *Pantoea stewartii* strain DC283 was highly sensitive to ϕEa104 and ϕEa116, but less to ϕEa1h and ϕEa100.

Propagation of the four American *E. amylovora* phages was also assayed on nitrocellulose filters with wild type *E. amylovora* strains isolated from 2005 to 2007. They multiplied up to 10^{10} pfu per disk with a slight decrease for EaOR1/07 (Table 4). The non-pathogenic *hrp* mutant PMV6076 was also a good host strain, whereas Ea322A, a *hrp* mutant of a French strain, was less efficient for ϕEa116 in three independent assays. As seen in drop tests on lawns of *E. amylovora*, the EPS mutants Ea1/79-del100 and CFBP1430Sm-amsD did not propagate the phages ϕEa1h and ϕEa100, in contrast to ϕEa104 and ϕEa116. No increase of phage titre was found with *E. billingiae* strain Eb661. Only phage ϕEa1h grew to a high titre on *P. stewartii* strain DC283, in contrast to the low phage sensitivity of this strain in drop tests (Table 3).

EPS synthesis and phage sensitivity

The amount of EPS produced by *E. amylovora* strains from France, Germany and North America significantly differed (Table 5). The French strain CFBP1430 and its corresponding *hrp* mutant PMV6076 produced high amounts of EPS, similar to the Sm-resistant strain Ea1/79Sm. The three German isolates EaRW1/06, EaDS05 and EaDS08 produced less EPS than the former strains. Two strains from Oregon synthesized low amounts of EPS, close to the EPS-negative *ams* deletion mutant Ea1/79-del100.

In general, the *Myoviridae* were not influenced by presence or absence of amylovoran capsules of the host cells (Table 3). The rubus strains IL6 and MR1 and also the German strain Ea1/79 showed an intermediate sensitivity. The French strains CFBP1430 and PMV6076 were highly susceptible to ϕEa1h and ϕEa100, as *E. amylovora* strain EaDS05 isolated in Germany. The German strain Ea1/79 with low EPS synthesis showed an intermediate sensitivity to the phages ϕEa1h and ϕEa100. The increased EPS synthesis of the spontaneous mutant Ea1/79Sm enhanced phage sensitivity for the *Podoviridae*. Generally, a correlation between high EPS production and enhanced phage sensitivity was noted for the *Podoviridae*.

Erwinia pyrifoliae strains, except Ejp557, showed high EPS synthesis, as well as the *E. billingiae* strain Eb661 and the *P. stewartii* strain DC283. Nevertheless, they were slightly sensitive to the *Podoviridae*, which might be due to unsuited phage receptors on the cell surface. *Erwinia tasmaniensis* Et1/99 without detectable EPS synthesis was insensitive to the phages. These strains were not or only partially sensitive to the *Myoviridae*.

Reduction of disease symptoms on apple flowers and immature pears

The *Podoviridae* had a weak effect on growth reduction of *E. amylovora* in flowers (Fig. 5A). ϕEa1h and ϕEa100 reduced the recovered pathogen by 40% compared with the control flowers. The *Myoviridae* ϕEa104 and ϕEa116 reduced the recovered cells by 90%.

Symptoms on pear slices treated with a single phage were reduced by half compared with the control without phage treatment. A significant difference among *Podoviri-*

Table 5. EPS synthesis of suspension cultures of various *Erwinia* strains and the *P. stewartii* strain DC283.

Strain	EPS produced ($\mu g\ ml^{-1}$)
CFBP1430	38.3
PMV6076	71.6
Ea1/79	0.8
Ea1/79Sm	46.0
Ea1/79del100	0.4
Ea322A	0.7
EaRW1/06	1.2
EaOR1/07	0.6
EaOR2/07	0.2
EaDS05	6.7
EaDS08	2.9
Ep1/96	551.1
Ep2/97	479.6
Ejp557	0.4
Ejp617	17.1
Eb661	7.4
Et1/99[a]	1.3
P. stewartii DC283[a]	97.7

a. Grown in LBglc medium.
The height of EPS production was determined with the CPC assay and is expressed in $\mu g\ ml^{-1}$

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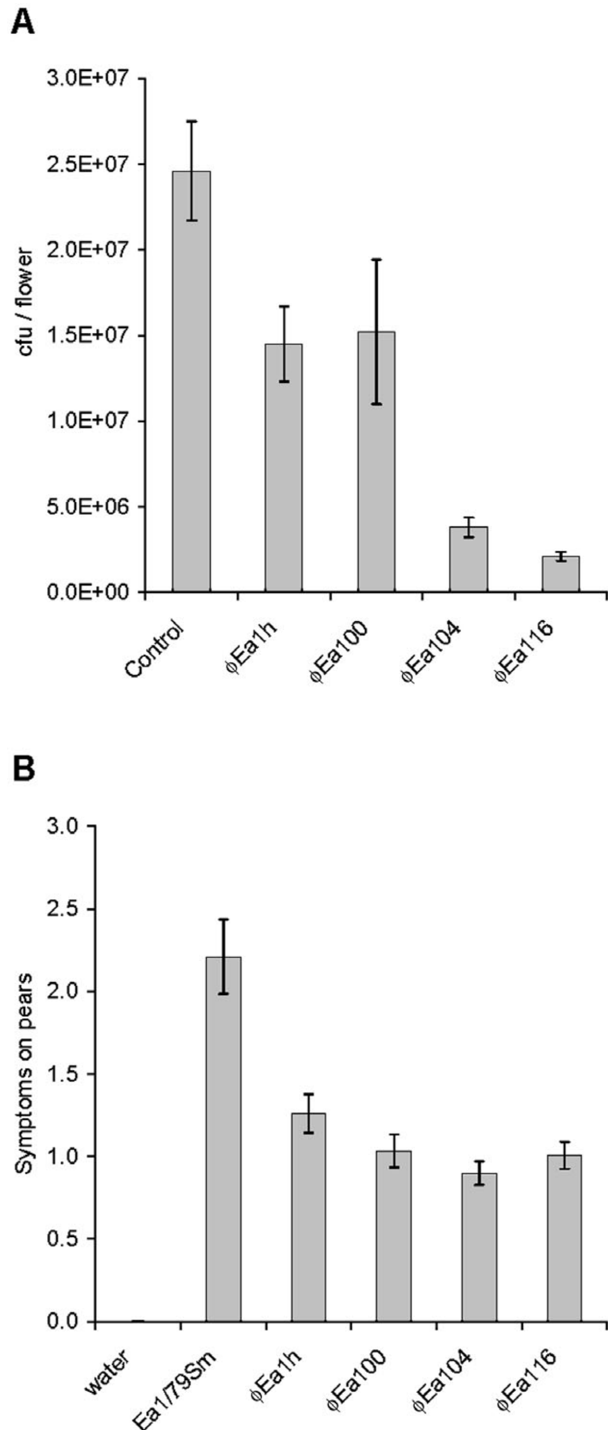

Fig. 5. Growth reduction of *E. amylovora* by phage treatment on apple flowers and immature pears.
A. Flower assays with Ea1/79Sm using single bacteriophages. Control flowers were inoculated only with Ea1/79Sm cells.
B. Fire blight symptoms on pear slices. Strain Ea1/79Sm applied without and with single bacteriophages.

dae and *Myoviridae* was not observed for immature pears. Due to high ooze production of *E. amylovora* on pear slices, the *Podoviridae* may find a favourable environment for interference with the pathogen (Fig. 5B).

A mixture of *Podoviridae* and *Myoviridae* applied with the pathogen for inoculation of apple flowers and pear slices did not significantly change symptom formation compared with treatment with a single myovirus (data not shown).

Partial characterization of new E. amylovora *phages*

From apple orchards in Germany three *E. amylovora* phages were isolated in 2008 and were investigated by negative staining for their morphological properties with EM. ϕEaJ08T and ϕEaK08T (Fig. 1B and E) belonged to the *Podoviridae* and resembled ϕEa1h and ϕEa100 shown in Fig. 1A and D. The head capsules of ϕEaJ08T and ϕEaK08T were 60 nm in diameter (Table 1), comparable to the particle size of ϕEa1h and ϕEa100. Their genome length was determined by EM and found to be 40 kb. ϕEaJ08C was identified as member of the *Myoviridae* based on its morphology in the EM (see Fig. 1C). Its head capsule was 73 nm in diameter and its contractile tail carrying short tail fibres was 116 nm in length. Its genome size determined by EM was approximately 84 kb, similar to the American *Myoviridae* (Table 1).

On *E. amylovora* host strains they formed either turbid (T) or clear plaques (C). In host range assays, ϕEa1h, ϕEa100, ϕEaJ08T and ϕEaK08T were related as were ϕEa104, ϕEa116 and ϕEaJ08C (Table 3). The lysis pattern of the German strains resembled those of the other *Podoviridae* and *Myoviridae* respectively.

PCR primer pairs were designed from the genome sequences of the four American phages and applied to the German phages. The German phages ϕEaJ08T and ϕEaK08T showed no or weak signals for primer pairs designed from ϕEa1h, ϕEa104 or ϕEa116. For ϕEaJ08C partial sequences were recovered, which showed high similarity to phage ϕEa116 (Table S1) and less to ϕEa104. Most mismatches were found in two sequences of a putative structural protein.

Discussion

We have characterized several *E. amylovora* phages from North America and from Germany for their virion morphology, interaction with host strains and properties of their genomes. They belong to the order *Caudovirales* and were classified according to their morphotypes C1 and A1 as *Podoviridae* or *Myoviridae* respectively (Ackermann, 2001; 2007). The particle size of 60 nm for ϕEa1h was in agreement with previous data (Ritchie and Klos, 1979). The head capsules of the *Myoviridae* had a similar size as

shown for the related *Salmonella* phage Felix O1 (Kuhn *et al*., 2002) and the closely related *E. amylovora* phage ϕEa21-4 (Lehman *et al*., 2009). The genome structure of the American *Myoviridae* is completely different from that of the *Podoviridae*. The *Myoviridae* genome encodes 24 tRNA genes, one HNH endonuclease gene in contrast to five for the *Podoviridae*, where also a DNA-directed RNA polymerase gene was predicted. The *Myoviridae* lack a small terminase subunit gene, and they do not carry an EPS depolymerase gene. This supports our results that they are not dependent on an amylovoran capsule for host infection, unlike the *Podoviridae*. *Podoviridae* and *Myoviridae* can be classified by their protein sequence similarity with a BLASTP tool (Lavigne *et al*., 2008; 2009). Due to the high similarity of our sequenced bacteriophages on nucleotide and protein level to ϕEra103, ϕEa1h and ϕEa100 belong to the newly formed subfamily of *Autographivirinae* (SP6-like phages). We observed that ϕEa1h and ϕEa100 lack the large terminal repeat described for ϕEra103. Phages ϕEa104 and ϕEa116 are related to ϕEa21-4 and belong to the newly suggested genus of Felix O1-like phages (Lavigne *et al*., 2009).

The genomes of ϕEa1h and ϕEa100 are similar to the genome of ϕEra103 with 45 455 bp (Vandenbergh and Cole, 1986). HNH endonucleases, encoded in the phage genomes as one or more homologues, can cleave double-stranded DNA similar to colicin E9 from *E. coli* (Pommer *et al*., 2001). In a previous report the genome size of ϕEa100 and ϕEa116 was estimated from PFGE analysis to be 34 kb and 75 kb respectively (Schnabel and Jones, 2001). These sizes were corrected by our sequencing data and the EM measurements of the DNA contour lengths.

To test the ability of *E. amylovora* phages to control fire blight, they were applied to liquid cultures of a pathogenic *E. amylovora* strain and the decrease of the optical density was measured (Schnabel and Jones, 2001). Similar assays were performed by Gill and colleagues (2003), who also analysed the host range of *E. amylovora* phages and observed considerable differences. In our experiments the host range of *Podoviridae* and *Myoviridae* phages diverged. The former depend on EPS capsules of *E. amylovora* cells (Billing, 1960; Ritchie and Klos, 1977), as shown for ϕEa1h (Ayers *et al*., 1979; Ritchie and Klos, 1979; Bernhard *et al*., 1996). *Erwinia amylovora* strains without amylovoran synthesis are not sensitive to the *Podoviridae*. The EPS depolymerase enables them to degrade the amylovoran capsule (Kim and Geider, 2000), which results in the formation of expanding halos (Ritchie and Klos, 1979; Hartung *et al*., 1988). The EPS depolymerase genes of ϕEa1h and ϕEa100 differ by three bases resulting in three different amino acids. This could explain the lack of halo formation for ϕEa100.

For fire blight control a combination of bacteriophages and antagonistic bacteria was suggested (Svircev *et al*., 2006). This approach could not be realized with our potentially antagonistic strains. The two *Pantoea* strains tested by us did not support phage propagation. *Erwinia billingiae* Eb661 was weakly lysed without propagation of phages. For the fire blight antagonist *E. tasmaniensis* Et1/99 no lysis was observed. Cultivation of the *Myoviridae* on avirulent *E. amylovora* mutants increased the titre to 10^{10} pfu per disk. In laboratory and greenhouse trials *E. amylovora* mutants reduced symptom formation (Tharaud *et al*., 1997). The use of avirulent *E. amylovora* mutants for phage propagation is an alternative to the application of epiphytic bacteria, however, meaning the release of genetically modified organisms.

Growth of *E. amylovora* was well promoted on young flowers (Thomson and Gouk, 2003; Pusey and Smith, 2008) which were also applied in our fire blight control experiments. Phage treatment under lab conditions yielded in significant symptom reduction. Biocontrol of plant pathogens was attempted with *Pseudomonas* phages to cure bacterial spot on tomato (Iriarte *et al*., 2007). Bacteriophages were also applied for treatment of human bacterial diseases (Stone, 2002).

Three different phages were isolated during the fire blight season 2008 from blighted plant material in southern Germany. The isolation of bacteriophages from symptomless plant material was not successful, which is not uncommon (Ritchie and Klos, 1977). The soil around infected trees is assumed to be a reservoir for phages (Crosse and Hingorani, 1958; Gill *et al*., 2003). The *Podoviridae* or *Myoviridae* we isolated differed from the American phages in genome size and molecular properties. They may become enriched in *E. amylovora* populations of infected plants. Their ecology and persistence in nature is difficult to describe. The phages may migrate with spread of fire blight, or they may propagate in *E. amylovora* as a more sensitive host than other bacteria in their environment.

Experimental procedures

Bacterial strains and bacteriophages

Bacterial strains and bacteriophages used in the experiments are listed in Table 6. The bacteria were grown on StI agar (Merck AG, Darmstadt, Germany), in Luria–Bertani (LB) broth or in MM2C medium (Bereswill *et al*., 1998). For strains of the genus *Pantoea* and for *E. tasmaniensis*, 1% glucose was added to the LB medium.

Isolation of new bacteriophages

Bacteriophages were isolated in two apple orchards in southern Germany. Aerial plant material (approximately 0.5 g of

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Table 6. Bacterial strains and bacteriophages used in the assays.

Strain	Isolation, properties	Source/reference
E. amylovora		
CFBP1430	*Crataegus* sp.; France, 1972	CFBP
CFBP1430Sm-amsD	Sm^r mutant of CFBP1430, Tn*5* insertion in *ams*D	Lab collection
PMV6076	*hrp/dsp* deletion mutant CFBP1430	Barny *et al*. (1990)
Ea1/79	*Cotoneaster* sp.; Germany, 1979	Falkenstein *et al*. (1988)
Ea1/79del100	Cm^r, deletion of *ams* cluster	Bugert and Geider (1995)
Ea1/79Sm	Sm^r spontaneous mutant of Ea1/79	Bellemann *et al*. (1994)
Ea322A	CFBP1368, avirulent Tn*5* mutant of Ea322	Jock *et al*. (2000)
EaDS05	Quince; Germany, 2007	Lab collection
EaDS08	Apple; Germany, 2008	Lab collection
EaOR1/07	Apple; USA, 2007	Lab collection
EaOR2/07	Apple; USA, 2007	Lab collection
EaRW1/06	*Cotoneaster floccosus*; Germany, 2006	Lab collection
IL6	Raspberry; USA	Kim *et al*. (2001)
MR1	Raspberry; USA	Jock and Geider (2004)
E. pyrifoliae		
Ep1/96	Nashi pear; Korea	Kim *et al*. (1999)
Ep2/97	Nashi pear; Korea, *hrpL*	Jock *et al*. (2003)
Ejp557	Nashi pear; Japan	Kim *et al*. (2001)
Ejp617	Nashi pear; Japan	Kim *et al*. (2001)
E. billingiae		
$Eb661^T$	$NCPPB661^T$, apple tissue; England	Mergaert *et al*. (1999)
E. tasmaniensis		
$Et1/99^T$	Apple flowers; Tasmania	Geider *et al*. (2006)
P. stewartii		
DC283	Nal^r mutant of SS104, *Zea mays*; Illinois, 1967	Coplin *et al*. (2002)
P. agglomerans		
C9-1	Apple; USA	V. Stockwell
MB96	Corn; Germany, 1996	Lab collection
Bacteriophages		
ϕEa1h	Blighted Jonathan apple shoots; Michigan, 1975	Hartung *et al*. (1988)
ϕEa100	Soil sample; Michigan, 1996	Schnabel and Jones (2001)
ϕEa104	Soil apple orchard; Michigan, 1996	Schnabel and Jones (2001)
ϕEa116	Blighted apple tissue; Michigan, 1996	Schnabel and Jones (2001)
ϕEaJ08C	Blighted apple shoots; Germany, 2008	This work
ϕEaJ08T	Blighted apple shoots; Germany, 2008	This work
ϕEaK08T	Blighted Gala apple shoots; Germany, 2008	This work

CFBP = Collection Française de Bactéries Phytopathogènes; NCPPB = National Collection of Plant Pathogenic Bacteria (UK).

leaves or twigs) was washed in 10 ml of water for 10 min or directly added to 10 ml of LB medium containing 1% sorbitol and *E. amylovora* cells. The mixtures were grown overnight, a few drops of $CHCl_3$ were added to the culture supernatants and dilutions plated on LB agar with *E. amylovora* cells in the top agar.

Propagation of phages on nitrocellulose filters

Erwinia amylovora, *E. billingiae* or *P. stewartii* cells (100 μl, 1×10^8 cfu) were mixed with phages (1×10^4 pfu) and incubated overnight on nitrocellulose filters (25 mm in diameter, Whatman) placed on MM2C agar or LB agar with 1% glucose. Bacteria and phages were resuspended in 5 ml of MM2C medium by vortexing. The OD_{600} and EPS production were measured, and the phage titre was estimated by soft agar drop tests of dilutions.

Electron microscopy of bacteriophages and their DNA

Prior to EM assays, the bacteriophages were purified according to Boulanger (2009). Electron microscopic pictures were taken after negative staining with 2% uranyl acetate according to Steven and colleagues (1988). For DNA purification the phages were concentrated using a Beckman Airfuge followed by phenol extraction. The genome length of the phage DNAs was measured with the droplet method as described before (Spiess and Lurz, 1988), and circular DNA of plasmid RSF1010 (8684 bp) was used as an internal standard.

MALDI-TOF MS analysis of viral proteins

Bacteriophage protein solutions were precipitated with 10% TCA, desalted and the proteins dissolved by heating in SDS loading buffer. The denatured proteins were separated on a 12% SDS-PAGE for 120 min and stained with Coomassie Brilliant Blue G-250 (PageBlue Protein Staining Solution; Fermentas, St. Leon-Rot, Germany). As molecular marker the PageRuler Prestained Protein Ladder from Fermentas was used. Protein bands were excised and digested by trypsin. After tryptic digests of gel-separated proteins (Gobom *et al*., 2001) mass spectra were recorded on a Bruker Scout 384 Reflex II instrument (Bruker Daltonik).

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Protein identification by MALDI-TOF MS peptide mapping was accomplished with the search engine MASCOT (Matrix Science, UK).

DNA manipulation and sequencing

Purified bacteriophages were lysed with SDS and the solutions extracted with phenol : chloroform : isoamyl alcohol (25:24:1). After a final treatment with chloroform and ethanol precipitation, the DNA was digested with the fast-digest versions of enzymes BamHI, BglII, EcoRI and HindIII (Fermentas, St. Leon-Rot, Germany). Other restriction enzymes were from AGS/Hybaid, Heidelberg, Germany.

The partial sequence of phage ϕEa116 was determined by whole-genome shotgun sequencing. Two plasmid libraries with an average insert size of 1 and 2 kb were generated, as previously described (Kube *et al*., 2005). Sequencing was performed by using dye terminator sequencing on ABI3730XL capillary sequencers. Shotgun sequences were assembled using PHRAP (http://www.phrap.org) and GAP4 (http://www.gap-system.org). ORFs were identified by GLIMMER3 [Gene Locator and Interpolated Markov ModelER according to Delcher *et al*. (1999)].

After alignment of ϕEa116 sequences to the genomes of ϕEa104 and ϕEa21-4, missing or ambiguous DNA regions were sequenced from PCR fragments, applying freshly designed primers and primer walking.

The sequences for the contigs and their annotation of *E. amylovora* phage ϕEa116 have been submitted in the EMBL database under Accession No. FQ857195. Their corresponding positions in the genome of ϕEa104 (FQ482083) are given in Table 2.

Amylovoran and stewartan synthesis

The amount of EPS was measured with the CPC-turbidity assay (Bellemann *et al*., 1994).

Plant assays

Fully expanded apple flowers from greenhouse trees were placed on top of water-filled Eppendorf tubes. The stigmata of the flowers were inoculated with 5000 cfu of the streptomycin-resistant *E. amylovora* strain Ea1/79Sm and with 5×10^7 to 1×10^8 pfu of single or mixed *E. amylovora* phages in water. The flowers were incubated in a climate chamber with 24°C for day and 21°C for night conditions. After 5 days petals and stems of the flowers were removed, and the flowers were extracted in water for 10 min. From the extracts, 100 μl of a 10^{-4} dilution was plated on StI agar containing streptomycin (500 μg ml^{-1}) and cycloheximide (50 μg ml^{-1}).

Immature pears were cut in slices and soaked in a suspension of 5×10^8 pfu ml^{-1} of an *E. amylovora* phage or in water as a control. The briefly dried slices were inoculated with 500 cfu of the *E. amylovora* strain Ea1/79Sm and incubated in sealed Petri dishes for 5 days at 28°C. The pears were rated for symptoms from 0 (no symptoms) to 3 (browning and large drops of ooze).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Partial sequence analysis of the German isolate ϕEaJ08C amplified with primers from ϕEa104 and ϕEa116. The positions refer to the partial sequence of ϕEa116 (Accession No. FQ85719).

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