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EXPERIMENTAL ARTICLES

Temperate Bacteriophage ZF40 of *Erwinia carotovora*: Phage Particle Structure and DNA Restriction Analysis

F. I. Tovkach[1]

Zabolotnyi Institute of Microbiology and Virology, National Academy of Sciences of Ukraine, ul. Zabolotnogo 154, Kiev, Ukraine

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Abstract—Structural organization of the temperate bacteriophage ZF40 of *Erwinia carotovora* was studied. Phage ZF40 proved to be a typical member of the *Myoviridae* family (morphotype A1). Phage particles consist of an isometric head 58.3 nm in diameter and a contractile 86.3-nm-long tail with a complex basal plate and short tail fibers (31.5 nm). Phage tail sheath, a truncated cone in shape, is characterized by specific packaging of structural subunits. The ZF40 phage genome is 45.8 kb in size, as determined by restriction analysis, and contains DNA cohesive ends. The ZF40 phage of *Erwinia carotovora* is assumed to be a new species of bacteriophages specific for enterobacteria.

Key words: *Erwinia carotovora*, temperate bacteriophage, phage particle structure, DNA restriction analysis.

Out of the overall number (4500) of different phages specific for the members of the family *Erwinia carotovora* at least 750 isolates have been characterized [1, 2]. However, none of the phages specific for such an important phytopathogen as *Erwinia carotovora* fills a place in modern bacteriophage classification [2]. Only two virulent phages were described: φKP and φM1 capable of mediating generalized transduction of genetic markers in some strains of *E. carotovora* subsp. *carotovora* and subsp. *atroseptica* [3, 4].

I have previously described some biological properties of a new temperate bacteriophage ZF40 of *Erwinia carotovora* [5]. Preliminary studies showed that phage ZF40 can be helpful for analyzing the intracellular restriction–modification systems and lysogeny in phytopathogenic erwinias.

In this work, we studied the particle structure and DNA restriction patterns of the temperate phage ZF40 of *Erwinia carotovora.*

MATERIALS AND METHODS

Bacteriophage ZF40 (ZF40-l) was titrated on the susceptible strain *E. carotovora* RC5297 described in [5]. *Escherichia coli* BE served as a host strain for the coliphage T4D provided by I.I. Serysheva (Institute of Biochemistry, Russian Academy of Science). Bacteria were grown on liquid or solid LB media. Both ZF40 and T4 phages were obtained at titers 2 to 4×10^{11} PFU/ml by the method of confluent lysis. Phage lysates were concentrated and purified by differential ultracentrifugation (rotor SW28, Spinco L8-70, 26000 rpm, 60 min), by ultracentrifugation in a discontinuous CsCl gradient (1.4 and 1.6 g/cm^3, rotor SW55, 40000 rpm, 5 h), and by the lysate clarification method [6] (K-24 centrifuge, 17000 rpm, 10 h).

Phage particles were dissolved and stored in either STM (NaCl, 50 mM; Tris–HCl, 50 mM, pH 7.5; $MgSO_4$, 20 mM) or STMG (STM + gelatin, 100 μg/ml) buffers. The ZF40 phage particles were additionally purified by gel-filtration on a column (8×200 mm) with Sepharose 2B.

The T4D phage particles with a tail length of 113 nm [7] served as reference particles to determine the size of phage particles, contrasted by 2% uranyl acetate and examined under a EMB 100BR electron microscope at a magnification of 36000 to 40000×. DNA isolation and restriction analysis were described previously [8]. The following endonucleases purchased from either Biopor, Fermentas, or Biolabs were used in this study: *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, *Hpa*I, *Kpn*I, and *Pvu*I.

RESULTS AND DISCUSSION

The particles of *E. carotovora* phage ZF40 proved to become unstable under various conditions. After a short-term storage in the STM buffer, a significant loss of titer of purified phage preparations was observed: about an order of magnitude over a month at 4°C. Storage in STMG buffer prevented, in part, spontaneous destruction of the phage particles. Phage preparations purified by differential centrifugation contained phage ghosts, i.e., phage particles whose heads lacked DNA as determined by electron microscopy (Fig. 1a). Phage particles were also unstable in 5 M cesium chloride, i.e., under conditions of osmotic shock. After centrifu-

[1]E-mail: feodorit@mail.ru

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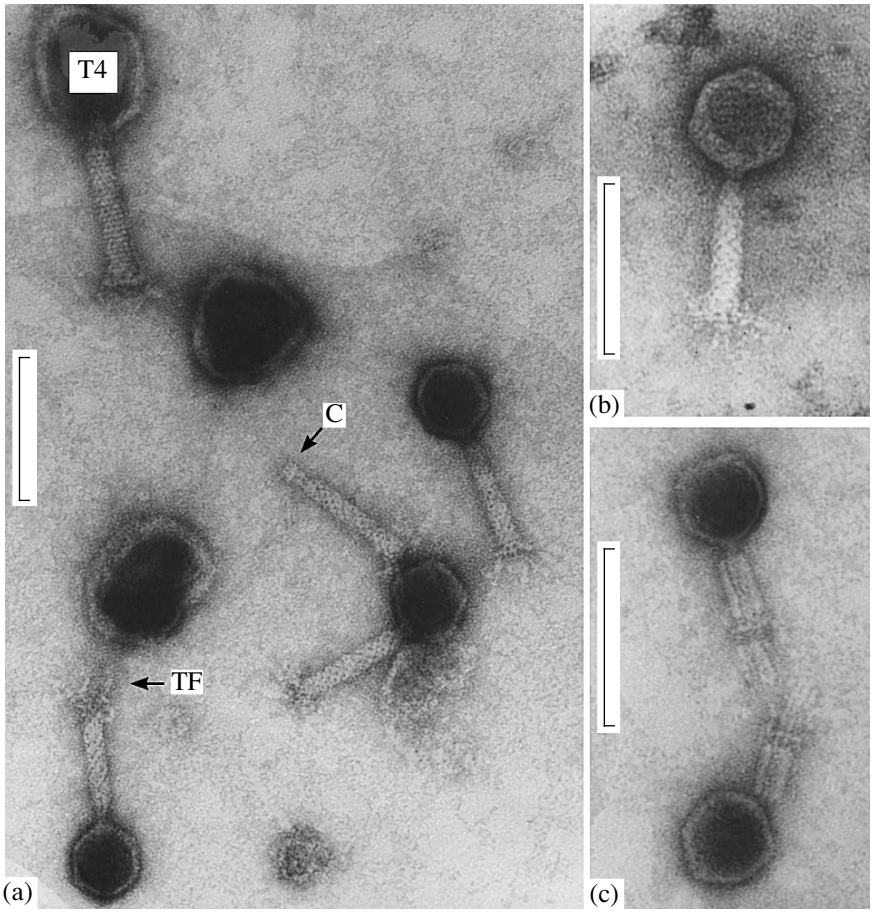

Fig. 1. Electron micrograph of the ZF40 phage particles: (a) phage ghosts; (b) normal particle of the ZF40 phage; (c) phage particles with contracted sheath. T4, a ghost of the T4D phage; C, connector; TF, tail fibrils. Bars correspond to 100 nm.

gation in a discontinuous CsCl gradient, the phage particles formed a small band at a density of 1.50 g/cm^3, although phage concentration in the band was lower than the initial concentration. Clarification of lysates [6], when phage particles were pelleted at a low rotor speed, proved to be the most adequate procedure of phage purification. Additional purification of the pelleted phage particles by gel-filtration through Sepharose 2B provided native phage preparations. The ZF40 phage particle was found to have an isometric head which appeared as a regular hexagon in a plane. A three-dimensional phage head was a regular icosahedron, whose facets were visible only in normal particles (Fig. 1b). Both ghosts and normal phage particles contained the thick cone-shaped tail bound to a portal head apex through a connector (Figs. 1a and 1b). The connector was separated from the tail sheath by a thin neck. The tail sheath was contractile (Fig. 1c) and, upon contraction, two structural components could be seen that were sometimes invisible in normal particles. These were the hollow tail stem and basal plate. In phage ZF40, the tail stem seemed to be the least stable, since it was seldom encountered in preparations of destroyed particles. In tails of both normal phage particles and ghosts, at least four fibers (fibrils) were observed (Figs. 1a and 1b).

Seventy individual particles of phage ZF40 were examined to estimate the quantitative parameters of its constituents. The icosahedron head was measured between the opposite apexes (DH_1) and the opposite sides (DH_2) of its hexagonal projection (third order axis of symmetry). The complete tail length was measured from the portal apex to the end of the basal plate. The sheath thickness was measured near the tail neck (DT_1) and near the basal plate (DT_2). The basal plate and tail fiber parameters were measured only in those phage particles in which these components were clearly discernible. Parameters of both normal ZF40 phage particles and ghosts can be seen in Table 1. The data obtained allowed us to infer several conclusions. The difference between the DH_1 and DH_2 values comprised 9% and was close to the expected value [9]. In ghosts, both DH_1 and DH_2 values were 8% lower than in normal virions. In phage ZF40, the average diameter (DH_1), accepted as the standard for isometric phages [1, 2], was equal to 58.3 nm. The overall tail length of the phage including the neck (about 10 nm) and basal plate (but without tail fibers) was similar in normal

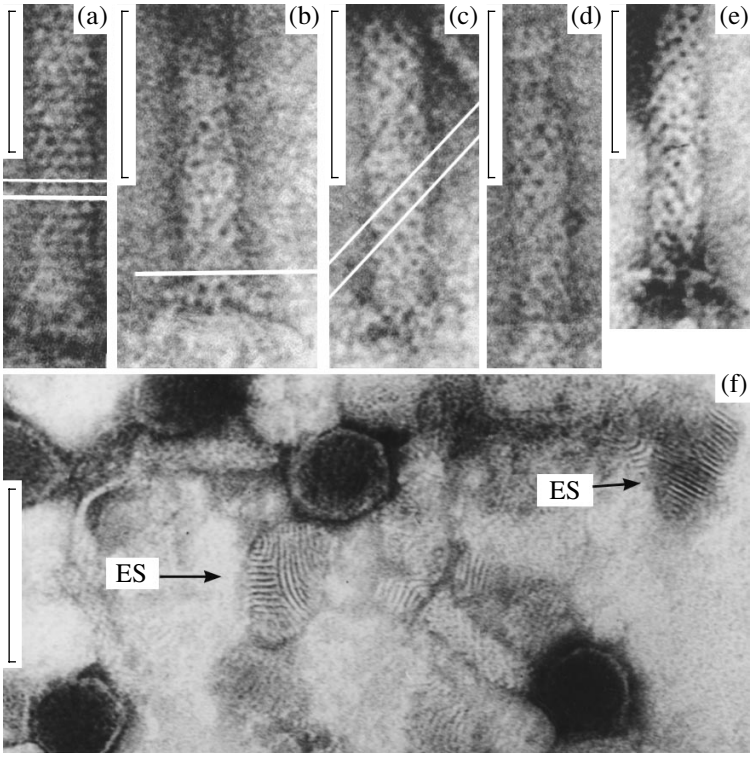

Fig. 2. Tail sheath structure of phage ZF40 (b, c, d, e) and products of its destruction (f). (a) T4 phage tail; (b, c, d) tail of phage ZF40 ghost; (e) phage tail of a normal particle. Transverse discs of the T4 phage sheath (a), inclined discs of the sheath (c), and basal plate with tail fibers (b) of phage ZF40 are indicated. ES, ellipsoid structures. Bars correspond to 40 nm (a, b, c, d, and e) or 100 nm (f).

phage particles and phage ghosts and comprised 86.3 nm on average. In both types of phage particles, the extended tail sheath was 76.1 nm long, whereas the contracted sheath measured 39.4 nm long. The diameter of a contracted tail sheath was 19.8 nm.

The measurement error was 1 to 3% (Table 1). It can also be seen from the table that diameters of both normal and contracted sheaths, as well as the widths of the basal plates, showed large deviations from average values (from 5 to 25%) (Table 1). To a large extent, the rea-

Table 1. Parameters of phage particles of the temperate bacteriophage ZF40 *E. carotovora*

Structural components of a particle	Particles with normal head			Particles with empty head, phage ghosts		
	N	Average size, nm	Measurement error, %	*N*	Average size, nm	Measurement error, %
Head						
DH_1	13	58.3	2.5	9	49.2	2.6
DH_2	12	54.5	1.0	8	45.2	2.0
Tail length	18	86.3	3.0	14	86.2	1.7
Tail sheath						
length	5	76.1	1.8	8	77.6	2.0
DT_1	9	13.1	15	9	12.5	25
DT_2	9	16.8	8	9	14.7	8
Contracted sheath						
length	–	–	–	7	39.4	1.7
diameter	–	–	–	7	19.8	5.0
Width of basal plate	5	33.8	13	3	22.6	–
Length of tail fibers	6	31.5	4	–	–	–

Note: *N* is the number of measurements; "–" means that measurements were either impossible or not performed. See text for other designations.

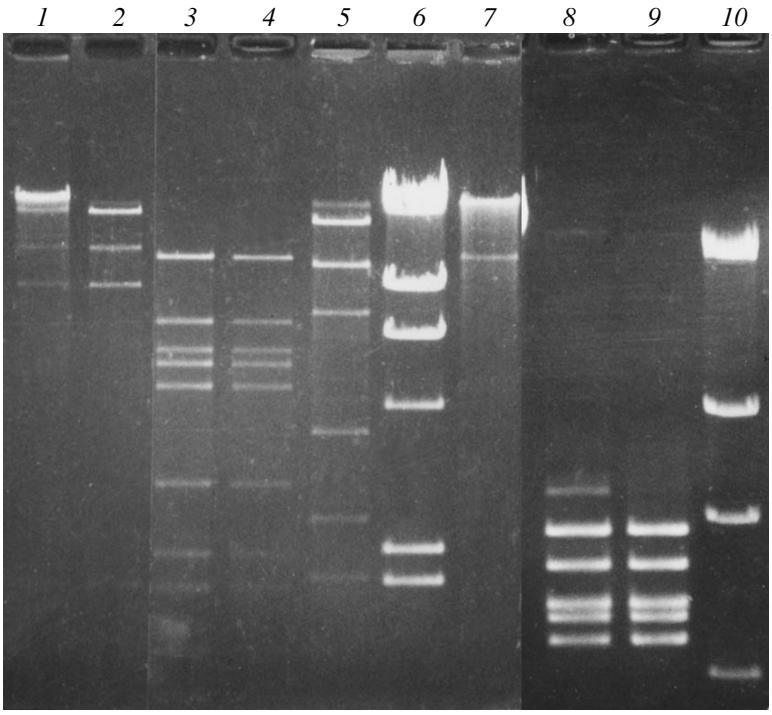

Fig. 3. Electrophoresis of fragments obtained by hydrolysis of the ZF40 phage DNA with endonucleases (*1* and *2*) *Bam*HI; (*3* and *4*) *Hpa*I; (*5*) *Kpn*I; (*7*) *Bgl*II; (*8* and *9*) *Eco*RI. *6* and *10*, *Hin*dIII-fragments of the λ phage; *2*, *4*, and *9*, fragments obtained after heating the incubation mixture at 80°C for 2 min followed by immediate cooling to 0°C.

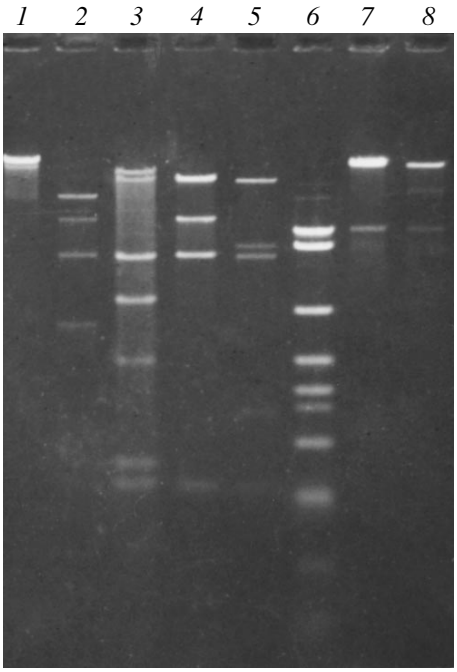

Fig. 4. Restriction analysis of the ZF40 phage DNA using endonucleases (*1*) *Hin*dIII, (*4*) *Bam*HI, (*7*) *Bgl*II and products of doubled hydrolysis of ZF40 DNA using (*2*) *Hin*dIII/*Bam*HI; (*5*) *Bam*HI/*Bgl*II; and (*8*) *Hin*dIII/*Bgl*II. *3* and *6*, *Hin*dIII and *Cla*I fragments of phage λ DNA.

son for this remains unclear. However, further comparative analyses showed that the tail sheath of the *E. carotovora* phage ZF40 may differ in the structure from that of the T4 phage of *E. coli* (Figs. 1a and 1b; Figs. 2a to 2e). In the T4 sheath, the structural subunits resemble regular discs located transversely to the longitudinal axis of the phage tail (Fig. 2a). Conversely, in the ZF40 phage sheath, cross striation is faint (Figs. 2b to 2e). In this bacteriophage, sheath subunits form helices which are clearly discernible and appear as inclined discs (Fig. 2c). These structural differences in the phage tails may be explained by the fact that, whereas the T4 sheath discs are composed of six subunits [11], the transverse discs in the ZF40 phage sheath consist of a lower number of subunits. In the latter phage, the sheath structural subunits are, probably, larger in size than those of the T4 phage, and this may account for the helical shape of the ZF40 sheath. The ZF40 tail sheath is a truncated cone in shape (Figs. 1a and 1b), whereas that of the T4 phage resembles a cylinder [11]. The reason for the conical shape of the ZF40 phage sheath is probably the presence of additional subunits near the basal plate, whereas near the neck, the number of subunits is lower. This was confirmed by electron microscopic examination of the ZF40 phage particles disrupted by an osmotic shock (5 M CsCl). Such a treatment of phage particles resulted in a specific disruption of the sheaths: they swelled and then slid off from the stem to form finally irregular ellipsoid structures. The two poles of these structures differed in width (Fig. 2f).

The sheath subunits in the ellipsoid structures appeared as arcs or straight lines. In my opinion, the ellipsoid structures were formed due to sheath disruption along one of its helices (inclined discs, Fig. 2c).

Although the ZF40 phage particles undoubtedly contain basal plates (Figs. 1b, 1c, and 2b), it is still impossible to determine exactly the size and structural organization of the latter. It is possible that in the extended phage tail, the basal plate is a truncated cone in shape, which appears as a trapeze in a plane (Fig. 2b). In phage ZF40, tail fibers are fivefold shorter than in the T4 phage and measure 31.5 nm (Table 1). In phage ZF40, the tail fibers are directly connected to the structural subunits of the basal plate, as can be seen from Fig. 2b.

Restriction enzyme digest analysis of the ZF40 bacteriophage DNA was conducted by using seven restriction endonucleases: *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, *Hpa*I, *Kpn*I (Figs. 3 and 4), and *Pvu*I to estimate the number of yielded DNA fragments and their sizes in kb. The *Bgl*II I and *Hin*dIII endonucleases proved to digest phage DNA at a single site each. DNA treatment by endonucleases *Eco*RI, *Hpa*I, or *Pvu*I yielded more than 10 fragments in each case. With the above enzymes and under conditions used (1% agarose gels), we failed to estimate accurately the size of phage DNA (Table 2). The task was solved by using endonucleases *Bam*HI and *Kpn*I, which yielded four and six fragments, respectively (Figs. 3 and 4; Table 3). To verify the results obtained, the fragments yielded by doubled DNA digestion with the enzyme pairs *Bam*HI/*Hin*dIII, *Bam*HI/*Bgl*III, and *Bgl*II/*Hin*dIII were also analyzed. Our calculations showed that the genome of the temperate phage ZF40 specific for *Erwinia* was, on average, 45.8 kb in size which corresponds to a 29.8 mDa molecular mass.

Additional fragments with lower molar concentration than that of the major fragments were found among DNA fragments yielded by enzymatic hydrolysis of DNA stored at 4°C for 3 to 4 months (Fig. 3, lanes *1, 5*, and *3*, *Bam*HI, *Kpn*I, and *Eco*RI). These fragments were absent after digestion of the freshly prepared DNA by restriction endonucleases. The submolar fragments disappeared after heating the incubation mixture for 2 min at 80°C (Fig. 3, lanes *2* and *9; Bam*HI and *Eco*RI). The submolar fragments yielded by treatment with endonucleases *Eco*RI, *Pvu*I, and *Kpn*I were 7.3, 11.2, and 22.5 kb in size, respectively. Note that the *Kpn*I submolar fragment is equal in length to the sum of A and D fragments (18.7 and 3.8 kb, respectively), whereas the *Pvu*I submolar fragment is equal in length to the sum of A and B fragments (8.9 and 2.3 kb, respectively) (Tables 2 and 3). Each of the three paired combinations of fragments can add up to the size of the *Eco*RI submolar fragment (Table 2).

The results obtained suggest that spontaneous formation of a coiled DNA molecule of phage ZF40 occurred after the interaction between the complementary single-stranded cohesive ends. Hydrolysis of the coiled DNA yielded submolar DNA fragments. Mapping the restriction sites for endonucleases *Bam*HI, *Bgl*III, *Hin*dIII, and *Kpn*I on the phage DNA showed that the *Bam*HI fragments (Table 3) were located in the CBDA order. The submolar fragment yielded by DNA hydrolysis with this endonuclease (Fig. 3) was equal in size to the sum of the terminal fragments A and C. Although special studies should be undertaken to confirm the presence of cohesive ends in the phage genome, the restriction endonuclease digest analysis is a rather accurate method of revealing these structures. Our results suggest that the cos-mechanism is involved in DNA replication in the ZF40 bacteriophage, and, like other temperate phages, ZF40 may contain a genetic system responsible for prophage integration and excision [12].

Table 2. Restriction analysis of the ZF40 phage DNA performed using endonucleases *Hpa*I, *Eco*RI, and *Pvu*I

Fragment	Fragment size (kb)		
	Endonuclease		
	*Hpa*I	*Eco*RI	*Pvu*I
A	11.89	6.46**	8.97*
B	7.00	5.79***	5.22
C	5.89	5.31	3.63
D	5.50	5.13	3.02
E	4.79	4.85****	2.88
F	3.04	2.85	2.75
G	2.29	2.49****	2.34*
H	2.00	1.84	2.14
I	1.60	1.58**	1.89
J	1.35	1.41	1.71
K	1.12	1.17	1.50
L	0.93	0.79**	1.37
M	0.75	–	1.21
N	–	–	0.89
O	–	–	0.75
P	–	–	0.60
DNA size	48.15	39.67	40.84

* Marks terminal DNA fragments; **, ***, **** mark three possible paired combinations of fragments that add up to the size of a submolar 7.3-kb *Eco*RI fragment (see the text).

Based on the evidence obtained, phage ZF40 was compared with some known morphotype A1 phages of the family *Myoviridae*, which are similar in the size of phage particles and DNA [3, 10, 13–16]. Table 4 shows that the ZF40 phage head is similar in diameter to those of phages φKP of *E. carotovora* subsp. *carotovora*, P278 of *E. coli*, and Spy-3 of *Bacillus polymyxa*. The diameter of an extended tail sheath was almost the same

Table 3. Restriction analysis of the ZF40 phage DNA performed using endonucleases *Bgl*II, *Hin*dIII, and *Kpn*I

Fragment	Fragment size (kb)						
	Restriction endonucleases						
	*Bgl*II	*Hin*dIII	*Bam*HI	*Kpn*I	*Bgl*II/*Hin*dIII	*Bgl*II/*Bam*HI	*Hin*dIII/*Bam*HI
A	33.4	29.8	21.6*	18.7*	–	21.6	–
a_0	–	–	–	–	17.4	–	–
b_0	–	–	–	–	16.0	–	–
a_1	–	–	–	–	–	–	16.0
B	12.4	16.0	13.1	11.5	12.4	–	13.1
b_1	–	–	–	–	–	9.9	–
C	–	–	9.1*	7.5	–	9.1	9.1
a_2	–	–	–	–	–	–	5.6
b_2	–	–	–	–	–	3.2	–
D	–	–	2.0	3.8*	–	2.0	2.0
E	–	–	–	2.7	–	–	–
F	–	–	–	1.6	–	–	–
DNA size**	–	–	45.8	45.8		45.8	45.8

Note: a_0,b_0, a_1,a_2, b_1,b_2 are fragments yielded by doubled DNA hydrolysis with *Bgl*II/*Hin*dIII, *Hin*dIII/*Bam*HI, and *Bgl*II/*Bam*HI, respectively; "–" means that a fragment was not detected.
* Terminal DNA fragments.
** DNA size determined in the experiment.

Table 4. Properties of some bacteriophages of the A1 morphotype [3, 10, 13–16]

Phage	Host bacterium	Dimensions, nm					Genomic size, kb
		head	tail	sheath length	sheath width	tail fiber length	
ZF40	*E. carotovora*	58.3	86.3	76.1	13–17	31.5	45.8
φKP	*E. carotovora*	60	120–135	–	–	–	46
Y64/(E2)	*E. herbicola*	51	109	–	24	–	–
Mu1	*E. coli*	54	–	100	18	–	38
P278	*E. coli*	58	102	–	16	–	87
P2	*E. coli*	50.7	137	–	16–17	40–50	33
Spy-3	*B. polymyxa*	59	127	–	–	–	45

Note: "–", to the author's knowledge, no evidence is available.

in phages ZF40, Mu1, P278, and P2. Phage ZF40 resembles phage Spy-3, the smallest one among the morphotype A1 phages which was found in gram-positive bacilli. These two bacteriophages proved to be similar in genome size and the size of the phage head, as well as in the structure of the contractile sheath, although they differed significantly in tail length (Table 4). No enterobacterial phages similar to phage ZF40 in the sheath structure and tail length have so far been described [2]. Thus, it is possible that the temperate bacteriophage ZF40 represents a new phage species specific for members of the family *Enterobacteriaceae* and may be of interest with regard to systematics of bacterial viruses.

The results of this study may serve as a basis for further molecular and genetic analysis of the new temperate phage ZF40 and of lysogeny in phytopathogenic pectinolytic *Erwinia*.

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