CURRENT MICROBIOLOGY Vol. 40 (2000), pp. 110–113
DOI: 10.1007/s002849910021

Current Microbiology
An International Journal

Morphology and General Characteristics of Phages Specific for *Astragalus cicer* Rhizobia

Sylwia Wdowiak, Wanda Małek, Małgorzata Grządka

Department of General Microbiology, M. Curie-Sklodowska University, 19 Akademicka St., 20-033 Lublin, Poland

Received: 21 July 1999 / Accepted: 25 August 1999

Abstract. Three newly isolated phages, K1, K2, and C1, specific for *A. cicer* rhizobia were characterized by their morphology, host range, rate of adsorption, restriction endonuclease patterns, and DNA molecular weights. All three phages were classified to the morphological group B of Bradley's (*Siphoviridae* family) on the basis of presence of hexagonal in outline heads and long noncontractile tails. Phages K1, K2, and C1 are related by host range and restriction endonuclease patterns. The molecular weights of phage DNAs estimated from restriction enzyme digests were in the range from 64.6 kb to 68.5 kb.

Rhizobiophages are of particular interest because of the ability of their bacterial hosts to fix nitrogen in symbiosis with leguminous plants. Some of them have transductional ability and may act as vehicles for genetic exchanges occurring in the soil and also as DNA vectors in genetic engineering. Additionally, the quite narrow specificity of rhizobiophages allows to use them as markers in ecological studies. Rhizobiophages are typical DNA phages with polyhedral (isometric or oblate) head and generally long, contractile or noncontractile tails; however phages with short tails were also found [1]. Up to now, the morphology and some properties of phages active against *Rhizobium leguminosarum* bv. *trifolii*, *viciae*, *phaseoli* [5, 17], *Sinorhizobium meliloti* [13, 17, 19, 20], *Mesorhizobium loti* [16], *Bradyrhizobium japonicum* [11], *Bradyrhizobium* sp. (*Lupinus*) [12], and *Mesorhizobium huakuii* [15] have been described.

The present report describes the morphology and certain biological characteristics of a virulent phages attacking rhizobia specific for *Astragalus cicer*.

Materials and Methods

Bacterial strains and phages. Bacterial strains and phages used in this study are listed in Table 1.

Media and growth conditions. For growth of bacteria and phage propagation liquid, solidified (1.4%) and soft (0.7%) agar media 79 were used [4].

Correspondence to: S. Wdowiak

Isolation of phages. Phages active against *A. cicer* rhizobia were isolated from the rhizosphere of *A. cicer* (cicer milkvetch) in Poland following the method of Staniewski and Kowalski [18]. They were purified by subsequent plating and picking up from a single plaque.

Electron microscopy study. Phages for electron microscopy were negatively stained with 2% (w/v) potassium phosphotungstate as described earlier [13].

Lytic specificity. Lytic specificities were determined according to the method of Adams [3].

Inactivation of phage. Phage propagated on its native bacterial host and diluted to $3 \sim 5 \times 10^3$ plaque-forming units (PFU)/ml was mixed with an equal volume of bacterial cells in SM buffer (5×10^5 bacterial cells/ml) or a lipopolysaccharide fraction (50 µg/ml) from the native bacterial host suspended in SM buffer. The mixture was incubated without shaking at 30°C for 60 min, then centrifuged and tested for nonadsorbed phages with native bacterial host as an indicator culture [3]. For phage adsorption studies bacteria heated at 100°C for 1 h were also used.

Preparation of lipopolysaccharide fraction (LPS) from rhizobial strains. LPS was obtained by the method of Westphal and Jann [21].

Buffer. SM and Tris-borate buffer was prepared according to Maniatis et al. [14].

DNA isolation. Rhizobiophages were propagated on native bacterial hosts using the double agar layer technique [3]. The phages were pelleted by means of twice repeated ultracentrifugation at 28,000 rpm for 1 h. Phage DNAs were isolated using the method of Maniatis et al. [14].

Restriction endonuclease digests. One microgram of DNA was added to 20 µl of reaction mixture containing buffers specified by the manufacturers of the restriction enzymes. Digests were analyzed using horizontal 1% (w/v) agarose gel electrophoresis in Tris-borate buffer. PegGOLD Ladder-mix DNA (PEQLAB Biotechnologie GmbH) was

Table 1. Bacterial strains and bacteriophages used in this study

Strains and phages	Relevant characteristics	Source
A. cicer rhizobia		
ACMP 9, 18	Nod^+Fix^+ wild type	Małek
Mesorhizobium huakuii		
38, Pl-52, S-52, A-106	Nod^+Fix^+ wild type	Chen
B3	Nod^+Fix^+ wild type	Murooka
Mesorhizobium loti		
HAMBI 1129, 1633	Nod^+Fix^+ wild type	Lindström
NZP 2235	Nod^+Fix^+ wild type	Legocki
Mesorhizobium ciceri		
UPM $Ca7^T$	Nod^+Fix^+ wild type	Normand
Mesorhizobium mediterraneum		
CP92	Nod^+Fix^+ wild type	Normand
Sinorhizobium meliloti		
13, L5-30, L 54	Nod^+Fix^+ wild type	Kowalski
SU47	Nod^+Fix^+ wild type	Singer
Sinorhizobium fredii		
USDA 1-6, 16-1, 440	Nod^+Fix^+ wild type	Chen
R. leguminosarum bv. *trifolii*		
21, 24, 24V	Nod^+Fix^+ wild type	Staniewski
ANU 843	Nod^+Fix^+ wild type	Skorupska
R. leguminosarum bv. *viciae*		
1, 2, 3, 33, 36	Nod^+Fix^+ wild type	Staniewski
R. galegae		
HAMBI 1141, 1185, 1155	Nod^+Fix^+ wild type	Lindström
B. japonicum		
USDA 110	Nod^+Fix^+ wild type	USDA[a]
Bradyrhizobium sp. (*Lupinus*)		
USDA 3045	Nod^+Fix^+ wild type	Legocki
Phages		
K1	Virulent phage	Małek, Wdowiak
K2	Virulent phage	Małek, Wdowiak
C1	Virulent phage	Małek, Wdowiak
H1	Virulent phage	Małek, Murooka

[a] USDA, United States Department of Agriculture, Beltsville, Md.

used as a standard molecular weight marker. Restriction enzymes were purchased from Fermentas, Lithuania.

Results

Phage K1, K2, and C1 active against *A. cicer* rhizobia were virulent and formed detectable plaques on the host lawn after 24 h. Plaques were clear with sharp regular edges and an average diameter of 2–3 mm, which did not increase after longer incubation (data not presented). All studied phages had isometric heads and noncontractile, long tails (Fig. 1A, 1B, 1C). Heads of *A. cicer* rhizobiophages were icosahedral, as indicated by the presence of capsids with hexagonal outlines. The dimensions of phage heads and tails are given in Table 2. Phages K1, K2, and C1 represent the Bradley's group B of viruses (*Siphoviridae* family) with long, noncontractile tails. The majority of rhizobiophages described up to now have been classified to three morphological groups A, B, and C according to Bradley's (*Myoviridae*, *Siphoviridae*, *Podoviridae* families, respectively) with hexagonal outline of heads and long contractile, long noncontractile and short tails, respectively [2, 6]. No rhizobiophages with heads hexagonal in outline and without tails as well as in the form of long flexible filaments have been described.

Phages K1, K2, and C1 were also submitted to more detailed studies. Their host range, rate of adsorption, as well as the size of phage DNA was determined.

The lytic activity of the studied phages was determined using 33 strains representing four rhizobium genera (*Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, and *Bradyrhizobium*). Phages C1 and K2 lysed three bacterial strains, i.e., ACMP18-microsymbiont of *A. cicer*, *M. ciceri* UPMCa 7^T, and *M. mediterraneum* CP 92 (Table 3). Phage K1 exhibited a broader lytic activity and besides the native bacterial strain ACMP18 also lysed *M. ciceri* UPMCa 7^T, *M. mediterraneum* CP 92, *R. galegae* HAMBI 1141, *R. galegae* HAMBI 1185, and *S. meliloti* 13. The sensitivity of *M. ciceri* and *M. mediterraneum* to phages specific for *A. cicer* rhizobia was not surprising. It is known that microsymbionts of *Astragalus* sp. are phenotypically and genotypically closely related to *M. ciceri* and *M. mediterraneum* and belong to the same genus *Mesorhizobium* [10]. The plating efficiency of phage K1, K2, and C1 on native bacterial hosts (ACMP 9, ACMP 18) was higher than on other rhizobia sensitive to them and changed from 99.6% to 69.8% (Table 4). Rhizobium cells killed by temperature (100°C for 60 min) adsorbed phages at a similar level as the viable cells (Table 4). The adsorption of the studied phages to their native bacterial hosts was not inhibited by their preincubation with purified lipopolysaccharide fraction, suggesting that LPS was not the cell surface phage receptor (data not presented). Little information is currently available on the mechanism of adsorption of rhizophages onto the host cells. Barnet and Vincent [6] documented that the receptor sites for three studied phages were associated with O-antigen of *R. leguminosarum* bv. *trifolii*. The phages specific for *R. leguminosarum* bv. *viciae* have their receptor in exopolysacchcarides [7], receptors for phage 1P *R. leguminosarum* bv. *trifolii* 24SM and phage NM8 lysing *S. meliloti* M11S cells reside in the LPS [8, 9, 22].

Our interest also focused on the genetic material of the studied rhizobiophages, which appeared to be double-strand DNA sensitive to restriction modification systems as in the case of other known rhizobiophages [19, 20]. The restriction endonucleases *Eco*RI, *Hind*III, *Pst*I, and *Sal*I produced numerous DNA fragments separated and visualized by agarose gel electrophoresis (Fig. 2, Fig. 3). These studies also included phage H1, specific for *M.*

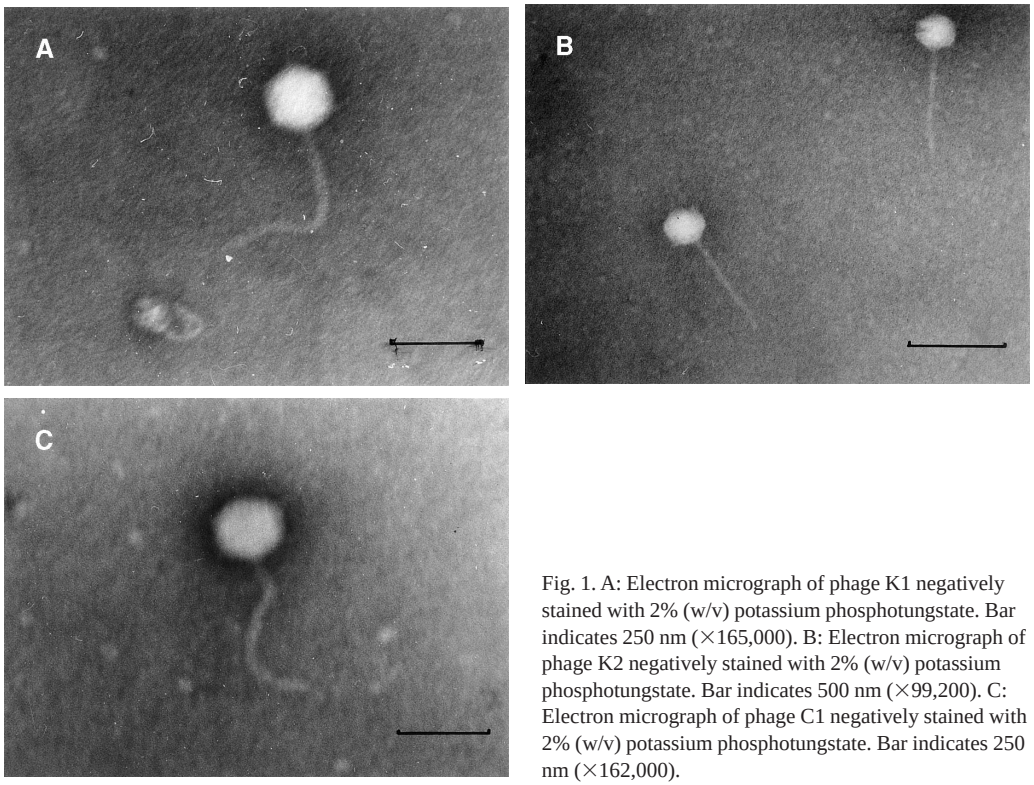

Fig. 1. A: Electron micrograph of phage K1 negatively stained with 2% (w/v) potassium phosphotungstate. Bar indicates 250 nm (×165,000). B: Electron micrograph of phage K2 negatively stained with 2% (w/v) potassium phosphotungstate. Bar indicates 500 nm (×99,200). C: Electron micrograph of phage C1 negatively stained with 2% (w/v) potassium phosphotungstate. Bar indicates 250 nm (×162,000).

Table 2. Characteristics of phages specific for *A. cicer* rhizobia

Phage	Head diameter (nm)	Tail length × width (nm)	Type of tail	DNA size (kb)	Bradley's group
K1	66.7	164.1 × 9.49	Noncontractile	~68.5	B (*Siphoviridae*)
K2	61.6	146.3 × 7.69	Noncontractile	~64.6	B (*Siphoviridae*)
C1	69.8	164.0 × 7.81	Noncontractile	~65.7	B (*Siphoviridae*)

Table 3. Host range of phages specific for *A. cicer* rhizobia

	Phages		
Bacterial strains[a]	K1	K2	C1
ACMP 9	+	+	+
ACMP 18	+	+	+
M. mediterraneum CP 92	+	+	+
M. ciceri UPM Ca 7^T	+	+	+
S. meliloti 13	+	−	−
R. galegae HAMBI 1141	+	−	−
R. galegae HAMBI 1185	+	−	−

[a] The other strains listed in Table 1 were not sensitive to the studied rhizobiophages.

huakuii [14]. Phage H1 showed entirely different DNA patterns than phages K1, K2 and C1 when digested with restriction enzymes (Fig. 2, Fig. 3). Phages K2 and C1, which showed identical or similar DNA patterns when digested with *Eco*RI and *Pst*I, respectively, seem to be closely related (Fig. 2, Fig. 3). Summation of the

Table 4. Adsorption of rhizobiophages to bacterial cells

		% of adsorbed phages to bacterial cells	
Phages	Bacterial strains	Viable	Killed[a]
K1	ACMP 18	94.3	89.0
	R. galegae HAMBI 1141	69.8	63.3
	R. galegae HAMBI 1185	71.4	71.8
	S. meliloti SU 47	73.2	71.5
	M. ciceri CP92	90.0	74.05
	M. mediterraneum UPM $Ca7^T$	91.95	71.8
K2	ACMP 18	94.05	88.93
	M. ciceri CP92	83.4	83.0
	M. mediterraneum UPM $Ca7^T$	82.0	80.2
C1	ACMP 9	99.6	97.57

[a] Bacteria were killed as described in Materials and Methods. LPS fraction of *A. cicer* rhizobia (ACMP 9, ACMP 18) did not inactivate K1, K2 and C1 phages.

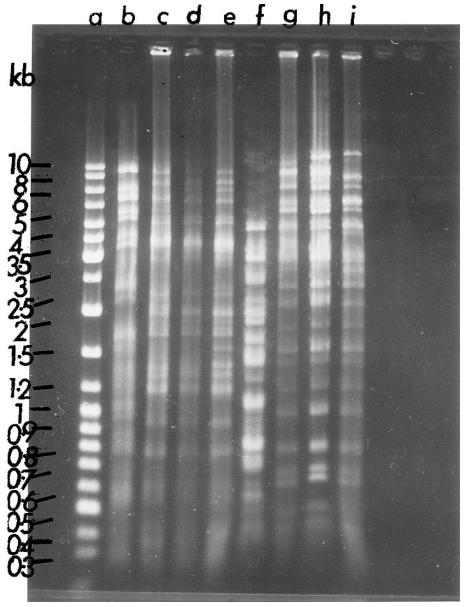

Fig. 2. Agarose gel electrophoregram of phage DNAs digested with *Pst*I and *Sal*I. Lanes b–e *Pst*I digests of H1, K1, K2, and C1 phage DNAs. Lanes f–i *Sal*I digests of DNAs of the same phage, respectively. Lane a ladder DNA. Fragment sizes in kb are indicated at the left margin.

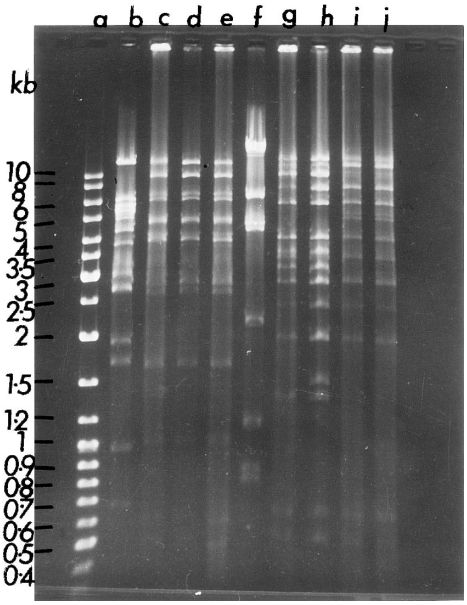

Fig. 3. Agarose gel electrophoregram of phage DNAs digested with *Eco*RI and *Hind*III. Lanes b–e *Eco*RI digests of H1, K1, K2, and C1 phage DNAs. Lanes f–i *Hind*III digests of DNAs of the same phage, respectively. Lane j *Hind*III digests of phage C1. Lane a ladder DNA. Fragment sizes in kb are indicated at the left margin.

restriction fragment sizes allowed to estimate the molecular weights of phage DNAs. They were in the range of sizes between about 64.6 kb and 68.5 kb (Table 2).

In conclusion we can say that three phages specific for *A. cicer* rhizobia are closely related with regard to host range, morphology, and DNA restriction endonuclease patterns.

ACKNOWLEDGMENTS

This work was supported by the Polish Committee for Scientific Research grant number 6 P04B 029 14.

Literature Cited

1. Ackermann HW (1978) La classification des phages d'*Agrobacterium* et *Rhizobium*. Pathol Biol 26:507–512
2. Ackermann HW, Audurier A, Berthiaume L, Jones LA, Mayo JA, Vidaver AK (1978) Guidelines of bacteriophage characterization. Adv Virus Res 23:1–24
3. Adams MH (1959) The bacteriophages. New York: Interscience Publications
4. Allen ON (1959) Experiments in soil bacteriology, 3d ed. Minneapolis: Min Burgess Publ Co
5. Atkins GJ (1973) Some bacteriophages active against *Rhizobium trifolii* strain W19. J Virol 1:149–159
6. Barnet YM, Vincent JM (1970) Lysogenic conversion of *Rhizobium trifolii*. J Gen Microbiol 61:319–325
7. Bradley DE (1967) Ultrastructure of bacteriophages and bacteriocins. Bacteriol Rev 31:230–314
8. Dandekar AM, Modi VV (1978) Interaction between *Rhizobium japonicum* phage M-1 and its receptor. Can J Microbiol 24:685–688
9. Defives C, Werquin M, Hornez JP, Derieux JC (1993) In vivo morphogenesis and growth characteristics of phages CM (*Myoviridae*) virulent for *Rhizobium meliloti*. Curr Microbiol 27:307–310
10. De Lajudie P, Laurent-Fulele E, Willems A, Torck U, Coopman R, Collins MD, Kersters K, Dreyfus B, Gillis M (1998) *Allorhizobium undicola* gen. nov., sp. nov., nitrogen-fixing bacteria that efficiently nodulate *Neptunia natans* in Senegal. Int J Syst Bacteriol 48:1277–1290
11. Kowalski M, Ham GE, Frederick LR, Anderson IC (1974) Relationship between strains *Rhizobium japonicum* and their bacteriophages from soil and nodules of field-grown soybeans. Soil Sci 118:221–228
12. Lotz W, Mayer F (1972) Electron microscropical characterization of newly isolated *Rhizobium lupini* bacteriophages. Can J Microbiol 18:1271–1274
13. Malek W (1990) Properties of the transducing phage M1 of *Rhizobium meliloti*. J Basic Microbiol 30:43–50
14. Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory
15. Nuswantara S, Fujie M, Yamada T, Malek W, Inaba M, Kaneko Y, Murooka Y (1999) Phylogenetic position of *Mesorhizobium huakuii* subsp. *rengei*, a symbiont of *Astragalus cicer* cv. J J Biosc Bioeng 1:49–55
16. Patel JJ (1979) Morphology and host range of virulent phages of lotus rhizobia. Can J Microbiol 22:204–212
17. Staniewski R (1987) Morphology and general characteristics of phages active against *Rhizobium*. J Basic Microbiol 27:155–165
18. Staniewski R, Kowalski M (1965) Effect of lysogenization on variability of phage type in *Rhizobium meliloti*. Acta Microbiol Polon 14:231–236
19. Werquin M, Ackermann HW, Levesque RC (1988) A study of 33 bacteriophages of *Rhizobium meliloti*. Appl Environ Microbiol 1:188–196
20. Werquin M, Ackermann HW, Levesque RC (1989) Characteristics and comparative study of five *Rhizobium meliloti* bacteriophages. Curr Microbiol 18:307–311
21. Westphal D, Jann K (1965) Bacterial lipopolysaccharides. Extraction with phenol-water and further applications of the procedure. In: Whister RL (ed.) Method in carbohydrate chemistry. London: Acad Press, pp 5:83–91
22. Zając E, Russa R, Lorkiewicz Z (1975) Lipopolysaccharide as receptor for *Rhizobium* phage 1P. J Gen Microbiol 90:365–367