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Characteristics and Comparative Study of Five *Rhizobium meliloti* Bacteriophages

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Abstract. Five *Rhizobium meliloti* bacteriophages isolated from soil or lysogenic bacteria and belonging to Bradley's group B or the *Siphoviridae* family of tailed phages were studied. They are of identical morphology, showing isometric heads and long, noncontractile tails with transverse bars. They are temperate and closely related by host range, DNA restriction endonuclease patterns and homology, DNA mass, serological properties, adsorption velocity, and latent period. However, the phages can be divided into three groups on the basis of burst size and frequency of lysogenization.

Rhizobium phages are of particular interest because of the ability of their hosts to fix atmospheric nitrogen. Some of them have transductional ability and may be used as DNA vectors in genetic engineering, possibly allowing the transfer of N_2-fixation genes [13–15, 28]. In addition, phages of the rhizosphere are important because their interaction with their hosts may result in selective effects on the ecology of N_2-fixing bacteria [4, 5, 9, 10].

Krsmanovic-Simic and Werquin [16] described four phages of *Rhizobium meliloti*. One of these, named NM1, had a highly unusual morphology, showing an isometric head and a long, noncontractile tail with transverse bars. The phage belonged to Bradley's group B [7] of the *Siphoviridae* family of tailed phages [20]. Phage NM1 was serologically unrelated to other *R. meliloti* phages [16] and represented a new species [1]. This paper describes a series of new NM1-like phages isolated from soil or lysogenic bacteria. Phage NM1 was re-examined for comparison.

Materials and Methods

Bacteriophages. Phages NM1, NM2, NM6, and NM7 were isolated from soil [4]. Phages ΦM20S was obtained by UV irradiation of *Rhizobium meliloti* strain M20S [12]. All phages were isolated and propagated on *R. meliloti* strain M9S. *R. meliloti* strains M20S and M9S were from our laboratory collection (Institut Agricole et Alimentaire de Lille). They were purified by three successive isolations of single plaques and were cultivated at 30°C by the double-layer technique [2] with 1.5% RC agar and a special soft layer described earlier [30]. After overnight incubation, phages were extracted with buffer and were filtered through membrane filters of 0.45 μm pore size (Millipore Corporation, Bedford, Massachusetts).

Host range. Phages were assayed on 52 strains of *R. meliloti*, 38 of which belonged to our collection (see above) and 14 of which were obtained from Lesley [17]. In addition, we tested 1 strain of *Agrobacterium tumefaciens*, 7 of *Bradyrhizobium japonicum*, 1 of *R. loti*, and 2 of *Galega* rhizobia (from our strain collection). Phages were used in tenfold routine test dilution (RTD), the lowest phage titer producing complete lysis of the propagating strain M9S [17].

Electron microscopy. Phages were sedimented for 90 min at 70,000 *g* in a Beckman L8-70M ultracentrifuge with a SW50.1 rotor (Beckman Instrument, Inc., Fullerton, California) and were washed twice with 0.1 *M* ammonium acetate (pH 7.0 or 5.0). Purified phages were deposited on carbon-coated Formvar grids and were stained with 2% phosphotungstate (pH 7.2) or 2% uranyl acetate (pH 4.5), containing bacitracin for wetting [11]. Specimens were observed in a Philips EM 300 electron microscope operated at 60 kV. Magnification was controlled with catalase crystals [18].

Restriction endonuclease patterns and DNA-DNA hybridization. Large quantities of phages were prepared as described earlier [30] and were concentrated with polyethylene glycol 8000 [31]. The final phage concentration and purification were done by ultracentrifugation in a three-step CsCl gradient at 110,000 *g* for 3.5 h [19], with the same ultracentrifuge and rotor as above. Phage DNA was extracted and deproteinized [22]. DNA aliquots of 1 μg were digested with various restriction endonucleases (Bethesda Research Laboratories, Inc., Gaitherburg, Maryland).

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Table 1. Serological relationships

	Antisera			
	NM1		CM1	MM1
Phages	K[a]	SC	K	L
NM1	246	1.00	—	—
NM2	206	0.83	—	—
NM6	240	0.97	—	—
NM7	208	0.84	—	—
ΦM20S	212	0.86	—	—

[a] K, neutralization constant; SC, similarity coefficient (K value divided by that of homologous or heterologous antiserum).

DNA fragments were separated by electrophoresis in a 0.8% agarose gel [21]. Ladder DNA (Bethesda Research Laboratories) was used as a standard molecular weight marker. DNA bands were stained for 15 min with 1 μg of ethidium bromide/ml (International Biotechnologies, New Haven, Connecticut), visualized in UV light, and recorded on Polaroid number 52 or 55 film (Polaroid Corporation, Cambridge, Massachusetts). DNA-DNA hybridization was done by the Southern-type gel hybridization technique [25]. Prehybridization [24] was followed by a hybridization performed with a phage DNA probe digested with *Sal*I endonuclease and labeled by nick translation [23] with deoxycytidine triphosphate (^{32}P-DCTP, Amersham Corporation, Oakville, Ontario, Canada). Autoradiography was done at −70°C, with Kodak X-Omat AR-2 film (Eastman Kodak Co., Rochester, New York). Exposure time varied from a few hours to several days. Positive and negative controls were included for each experiment.

Seroneutralization. Phages were tested with antisera against phages CM1, NM1, and MM1, prepared during an earlier investigation of *R. meliloti* phages [16]. Neutralization constants were calculated [2].

Adsorption velocity. A volume of 0.1 ml of lysage titering 2×10^8 PFU/ml was added to 0.9 ml of a log phase culture of the host bacterium (2×10^8 cells/ml; multiplicity of infection or MOI = 0.1) in the presence of 5×10^{-4} *M* $MgCl_2$ and 4×10^{-4} *M* $CaCl_2$ [29]. Samples were periodically withdrawn, diluted 100-fold in ice-cold broth containing 5% chloroform, and centrifuged for 5 min at 5,000 *g*. Supernatants were assayed for free virus particles. Adsorption rate constants were determined [27].

One-step growth experiments [2]. Bacteria grown to a density of 2×10^8 cells were infected with phages at a MOI of 0.1. After sufficient contact for adsorption of over 80% of phages (5 min under our conditions; see Results), 0.1-ml aliquots were transferred into 0.9 ml of RC broth diluted 100-fold and two times tenfold. Samples were withdrawn at different intervals and were plated in soft agar with an indicator strain. Plaques were counted the following day.

Lysogenization experiments. *R. meliloti* M9S cultures grown in TY broth [6] were infected with phages (MOI = 5). After allowing 20 min for adsorption, mixtures were diluted and inoculated onto RC agar. After 3–5 days at 28°C, isolated colonies were irradiated for 6 s with UV light (80 ergs/mm^2/s) (15 W germicid tube lamp; Mazda) and were inoculated onto a lawn of indicator bacteria. A lysed area indicated the liberation of phages and thus lysogenization. Noninfected M9S cultures did not produce plaques after UV irradiation.

Results and Discussion

Host range. All five phages were specific for *Rhizobium meliloti* and had identical, very narrow host ranges, lysing only two of 52 *R. meliloti* strains (M9S and M13S) and none of the other bacteria tested. Plaques were clear and had a diameter of 1.5–2.5 mm in soft agar.

Morphology. The phages were morphologically identical. Uranyl acetate-stained particles showed isometric heads of 70 nm in diameter and noncontractile, rigid tails of 110 × 8 nm (50 particles measured, Fig. 1a). Phosphotungstate-stained phages (20 particles measured, Fig. 1b) had nearly identical dimensions. The phages appeared about 40% larger than previously determined [16]. This is attributed to a more accurate calibration of magnification. Particles positively stained with uranyl acetate had shrunken heads (Fig. 1c) and were not measured. Phage heads were icosahedral, as shown by the observation of capsids with hexagonal and pentagonal outlines (Fig. 1d). Some phage heads showed capsomer-like structures (Fig. 1a). Tails possessed a highly unusual feature, namely, about 12 (6–13) transverse bars measuring 14.5 × 3.0 nm. These bars, whose usual periodicity was 11 nm, were more or less loosely attached to the tail, since repeated centrifugations resulted in their partial loss (Fig. 1b). In addition, tails showed six terminal, club-shaped prongs of 13.5 × 8.5 nm.

Serological and DNA relationships. All phages were rapidly and completely neutralized by NM1 phage antiserum and were thus closely related. K values varied between 206 and 246 (Table 1). In confirmation of our previous results [16], no neutralization by the heterologous CM1 and MM1 phage antisera was detected.

A preliminary characterization of phage DNAs was done with a battery of restriction endonucleases. *Cla*I, *Hind*III, *Kpn*I, *Sma*I, and *Who*I gave few DNA fragments, while *Ava*I, *Bam*HI, *Bst*EII, *Eco*RI, *Sal*I, and *Sst*I gave several fragments. Patterns were identical for all five phages. *Bam*HI, *Bst*EII, and *Sal*I were retained for further studies, since their patterns were easily visualized in agarose gels (Fig. 2a). Restriction patterns were

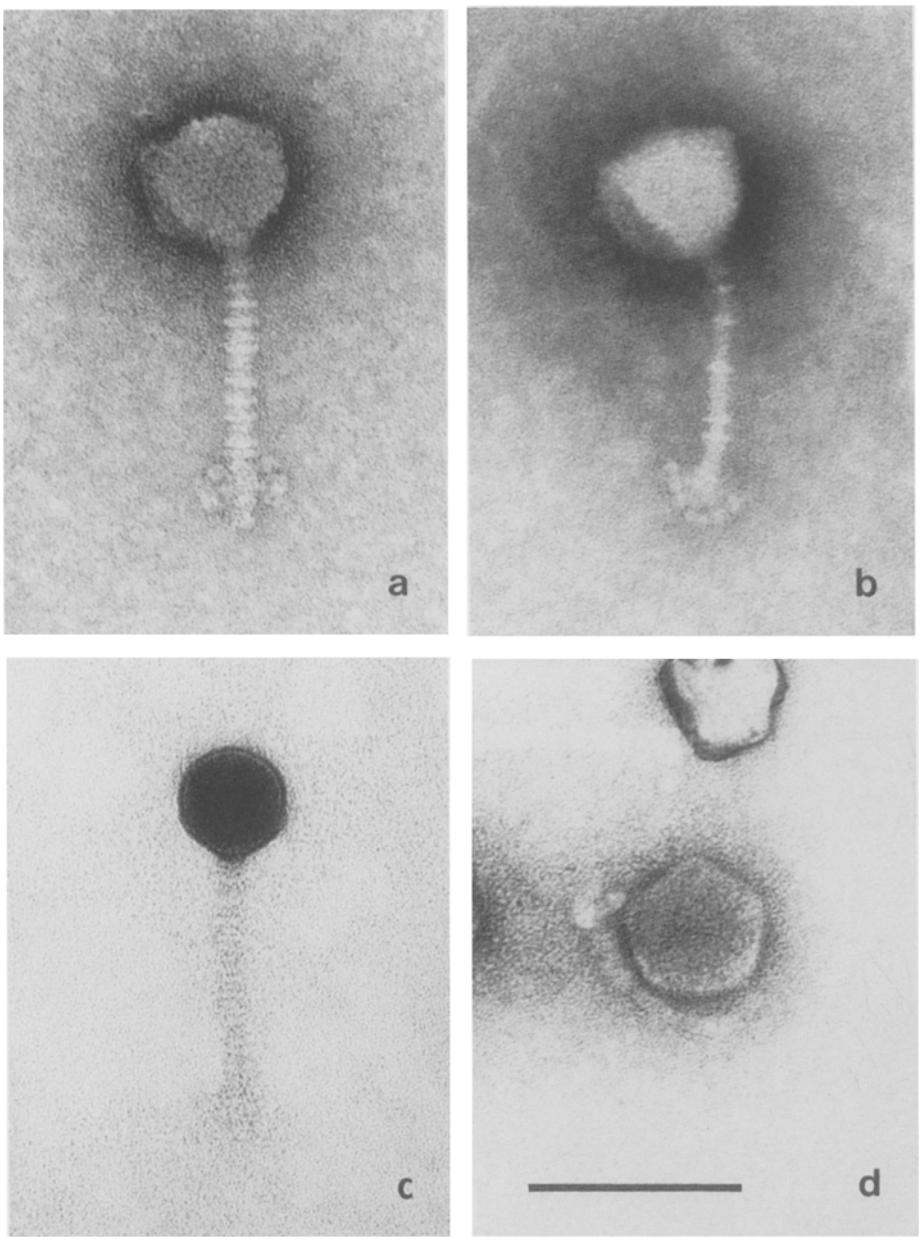

Fig. 1. Morphology of phages. (a) Normal phage NM1, showing capsomers and rigid tail with 12 transverse bars; (b) A particle of the same phage with five irregularly spaced tail bars; (c) Positively stained phage NM1 with strongly stained head and barely visible tail; (d) Isolated head of phage NM6 with pentagonal outline; (a, c, and d), uranyl acetate; (b) phosphotungstate. Bar indicates 100 nm.

identical and suggested that all phages were closely related. NM1 DNA digested with *Sal*I and labeled with ^{32}P by nick translation was hybridized with that of the other phages. Multiple homologous segments were evident (Fig. 2b), indicating the presence of numerous common DNA sequences and close relationships between the five phages. Gel electrophoresis of DNA fragments indicated a total DNA molecular weight of 51.9 kb or 34.5×10^6 daltons.

Growth characteristics. Phage adsorption was very rapid, since 82%–90% of phages were adsorbed within 5 min (Table 2). About 100% of phages were adsorbed after 60 min. Adsorption rate constants were higher than those observed in phages of *R. trifolii* [26] and were three times higher than those reported in phage RL1 of *R. leguminosarum* [8]. The five phages had approximately identical latent periods, varying between 120 and 160 min; however, when phage ΦM20S was induced by UV irradiation, its latent period was about 4 h. Burst sizes varied between 11 and 19 for soil phages NM1, NM2, NM6, and NM7, but were much higher for phages ΦM20S. The latter had nearly identical burst

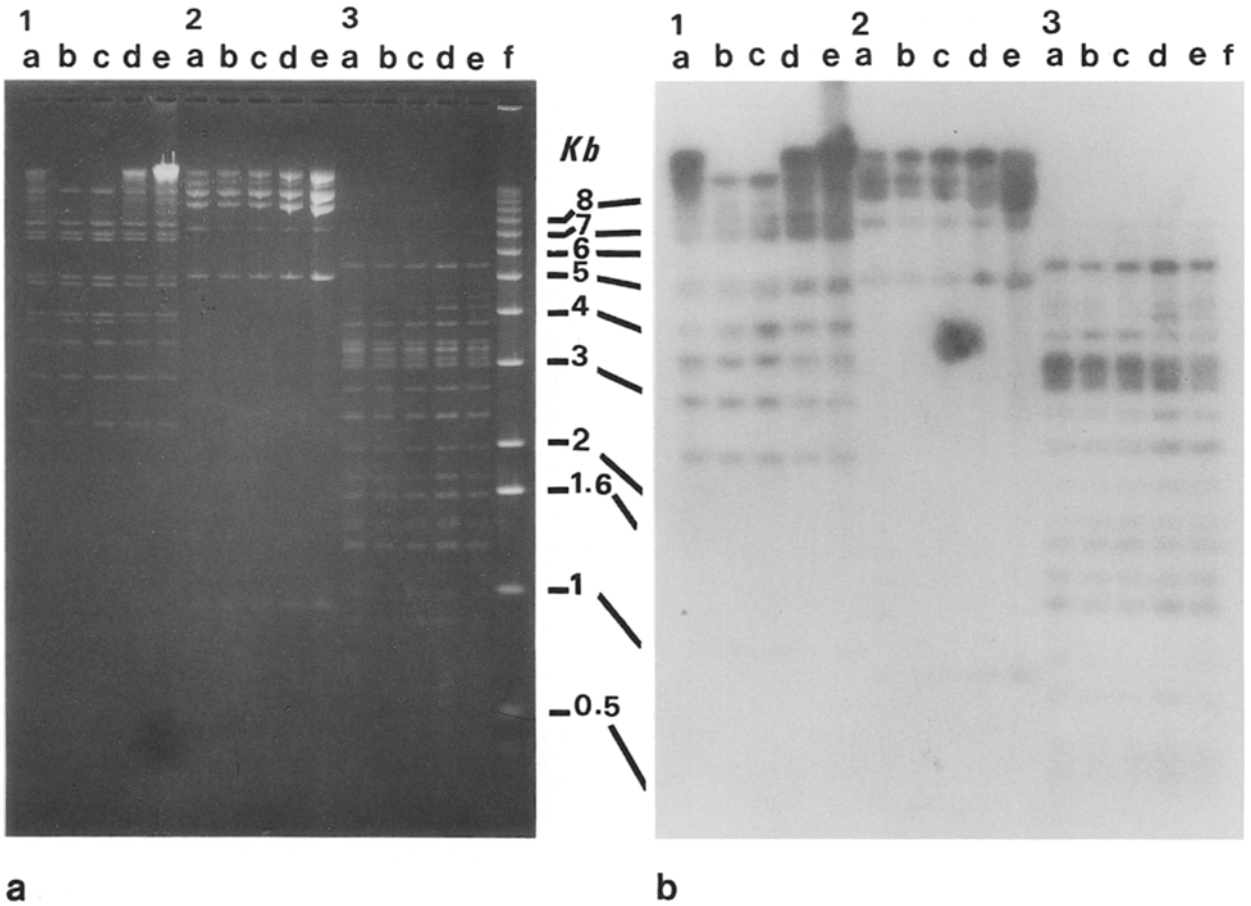

Fig. 2. (a) Agarose gel electrophoregrams of phage DNAs digested with *Bam*HI, *Bst*EII, and *Sal*I. Lanes la to le, *Bam*HI digests of NM6, NM2, NM7, NM1, and ΦM20S DNAs; lanes 2a to 2e and 3a to 3e, *Bst*EII and *Sal*I digests of the same, respectively; lane f, ladder DNA; fragments sizes in kb are indicated at the right margin. (b) Autoradiogram of Southern transfer of DNA from agarose gels in panel (a), followed by hybridization with ^{32}P-labeled NM1 DNA digested with *Sal*I.

Table 2. Growth characteristics

Phages	Adsorption rate (ml/min × 10^{-8})	Adsorption (% in 5 min)	Latent period (min)	Burst size
NM1	0.196	83.0	120–140	16
NM2	0.250	89.5	120–140	19
NM6	0.188	81.8	140–150	18
NM7	0.220	86.6	140–160	11
ΦM20S[a]	0.206	85.2	135–150	130
ΦM20S[b]			235	120

[a] Strain M9S infected with phage ΦM20S.
[b] UV-induced strain M9S carrying the ΦM20S prophage.

sizes regardless of whether it was induced by UV light or propagated in agar double layers (Table 2). Latent periods of about 2 h and burst sizes of 120–150 are common in *Rhizobium* phages [3]. To our knowledge, burst sizes of 10–20 have not yet been reported in phages of this bacterial genus.

Lysogenization experiments indicated that all phages tested were temperate and also showed further differences between them. M9S clones lysogenized by phages NM1, NM2, NM6, NM7, and ΦM20S, respectively, were lysed by phages NM2 only (Table 3). Lysogenization of strain M9S by

Table 3. Lysogenization experiments

R. meliloti strains[a]	Phages				
	NM1	NM2	NM6	NM7	ΦM20S
M9S	+[b]	+	+	+	+
M9S(NM1)	−	+	−	−	−
M9S(NM2)	−	+	−	−	−
M9S(NM6)	−	+	−	−	−
M9S(NM7)	−	+	−	−	−
M9S(ΦM20S)	−	+	−	−	−
Lysogenization (%)	57	1	42	32	30

[a] Prophages are indicated in parentheses.
[b] +, lysis; −, no lysis.

phages NM2 was inefficient and affected only 1% of M9S colonies, thus explaining the apparent sensitivity of M9S(NM2) clones against superinfecting phage NM2. By contrast, phages NM1, NM6, NM7, and ΦM20S lysogenized 30%–57% of M9S colonies.

In conclusion, the five phages studies are identical or closely related by host range, morphology, DNA restriction endonuclease patterns and homology, serological properties, and several growth characteristics. They can be subdivided into three groups by burst size and frequency of lysogenization.

Literature Cited

1. Ackermann HW (1978) La classification des phages d'*Agrobacterium* et *Rhizobium*. Pathol Biol 26:507–512
2. Adams MH (1959) Bacteriophages. New York: Interscience Publishers Inc.
3. Atkins GJ (1973) Some bacteriophages active against *Rhizobium trifolii* strain W19. J Virol 12:149–156
4. Barnet YM (1972) Bacteriophages of *Rhizobium trifolii*. I. Morphology and host range. J Gen Virol 15:1–15
5. Barnet YM (1980) The effect of rhizobiophages on populations of *Rhizobium trifolii* in the root zone of clover plants. Can J Microbiol 26:572–576
6. Beringer JE (1974) R factor transfer in *R. leguminosarum*. J Gen Microbiol 84:188–198
7. Bradley DE (1967) Ultrastructure of bacteriophages and bacteriocins. Bacteriol Rev 31:230–314
8. Dhar B, Singh BD, Singh RB, Singh RM, Singh VP, Srivastava JS (1978) Isolation and characterization of a virus (RL1) infective on *Rhizobium leguminosarum*. Arch Microbiol 119:263–267
9. Evans J, Barnet YM, Vincent JM (1979a) Effect of a bacteriophage on the colonization and nodulation of clover roots by a strain of *Rhizobium trifolii*. Can J Microbiol 25:968–973
10. Evans J, Barnet YM, Vincent JM (1979b) Effect of a bacteriophage on the colonization and nodulation of clover roots by paired strains of *Rhizobium trifolii* Can J Microbiol 25:974–978
11. Gregory DW, Pirie BJS (1973) Wetting agents for biological electron microscopy. I. General considerations and negative staining. J Microsc 99:251–265
12. Hassani L (1983) La lysogénie chez *Rhizobium meliloti*. Thesis n°1059 Université des Sciences et Techniques de Lille Flandres-Artois 59655 Villeneuve d'Ascq Cedex France
13. Kowalski M (1967) Transduction in *Rhizobium meliloti*. Acta Microbiol Pol 16:7–12
14. Kowalski M (1976) Transduction of effectiveness in *Rhizobium meliloti*. In: P.S. Nutman (ed.) Symbiotic Nitrogen Fixation in Plants 63–67 Cambridge University Press London
15. Kowalski M, Denarie J (1972) Transduction d'un gène contrôlant l'expression de la fixation de l'azote chez *R. meliloti*. C R Acad Sci Ser D 275:141–144
16. Krsmanovic-Simic D, Werquin M (1973) Etude des bactériophages de *Rhizobium meliloti*. C R Acad Sci Ser D 276:2745–2748
17. Lesley SM (1982) A bacteriophage typing system for *Rhizobium meliloti*. Can J Microbiol 28:180–189
18. Luftig R (1967) An accurate measurement of the catalase crystal period and its use as an internal marker for electron microscopy. J Ultrastruct Res 20:91–102
19. Maniatis T, Fritsch EF, Sambrook JS (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory
20. Matthews REF (1982) Classification and nomenclature of viruses. Fourth Report of the International Committee on Nomenclature of Viruses. Intervirology 17:1–199
21. Meyers JA, Sanchez D, Elwell LP, Falkow S (1976) Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J Bacteriol 127:1529–1537
22. Radloff R, Bauer W, Vinograd J (1967) A dye-buoyant density method for the detection and isolation of closed circular duplex DNA: the closed circular DNA in HeLa cells. Biochemistry 57:1514–1521
23. Rigby PWJ, Dieckmann M, Rhodes C, Berg P (1977) Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J Mol Biol 113:237–251
24. Silhavy TJ, Bermann ML, Enquist LW (1974) Experiments with gene fusions. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory
25. Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503–517
26. Staniewski R, Kowalski M, Gorzkowska K (1963) The rate of phage adsorption on *Rhizobium* cells. Acta Microbiol Pol 12:184–187
27. Stent GS (1963) Molecular biology of bacterial viruses. San Francisco: Freeman
28. Svab Z, Kondorosi A, Orosz L (1978) Specialized transduction of a cysteine marker by *Rhizobium meliloti* 16-3. J Gen Microbiol 106:321–327
29. Werquin M, Ben Brahim T, Krsmanovic-Simic D (1977) Etude des bactériophages de *Rhizobium meliloti*. C R Acad Sci Ser D 284:1851–1854
30. Werquin M, Defives C, Hassani L, Andriantsimiavona-Otonia M (1984) Large scale preparation of *Rhizobium meliloti* bacteriophages by fermenter culture. J Virol Methods 8:155–160
31. Yamamoto KR, Alberts BM, Benzinger R, Lawhorne L, Treiber G (1970) Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large-scale virus purification. Virology 40:734–744