APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1988, p. 188–196
0099-2240/88/010188-09$02.00/0

Vol. 54, No. 1

A Study of 33 Bacteriophages of *Rhizobium meliloti*

MICHEL WERQUIN,[1]* HANS-WOLFANG ACKERMANN,[2] AND ROGER C. LEVESQUE[2]

Laboratoire de Microbiologie Appliquée, Institut Agricole et Alimentaire de Lille, Université des Sciences et Techniques de Lille, 59655 Villeneuve d'Ascq, France,[1] and Department of Microbiology, Faculty of Medicine, Laval University, Quebec 10, Quebec, Canada G1K 7P4[2]

Received 25 June 1987/Accepted 28 October 1987

A total of 33 *Rhizobium meliloti* bacteriophages were studied. Of those, 21 were isolated in northern France from field soil in which *Medicago sativa* L. was grown. The other 12 phages were obtained by UV light and mitomycin C induction from 46 *R. meliloti* strains. Rhizobiophages were characterized by their morphology, host range, serological properties, restriction endonuclease patterns, DNA-DNA homologies, and DNA molecular weights. Five morphotypes were observed showing tailed phages with icosahedral heads. The categories of morphotypes included the *Myoviridae* (11 phages), *Siphoviridae* (3 morphotypes and 20 phages), and *Podoviridae* (2 phages). Type NM1 phage (*Siphoviridae*) is highly unusual because of the presence of transverse bars on the phage tail. Soil phages had broad host ranges, whereas phages isolated from bacterial cultures showed more or less narrow host ranges. Restriction endonuclease patterns and DNA-DNA hybridization experiments showed that the five phage type genomes were unrelated. Molecular weights of phage type DNAs were estimated, and they corresponded to values expected for capsid sizes, except for phage NM8. Type ΦM11S (*Siphoviridae*) did not correspond to any other described *Rhizobium* phages and represents a new species.

A number of soil microorganisms are able to use atmospheric nitrogen and to convert it into ammonia. Among those, *Rhizobium* and *Bradyrhizobium* bacteria form a specific interaction with plants of the family *Leguminosae* (legumes) (46), inducing the formation of root nodules in which they fix atmospheric nitrogen. Nodulated leguminous plants routinely fix between 30 and 300 kg of N per ha per year, depending on the plant species and environment. The fixed N is available to the nodulated plant, some is available to companion plants, and some is left in the field at harvest. There is a worldwide interest in increasing the input of biologically fixed N in agriculture. This increase might be achieved by several methods, such as by increasing nodulation in leguminous plants, selecting adapted rhizobia, or breeding new strains by genetic manipulations.

Strains of rhizobia are commonly added annually to agricultural soil to increase the nitrogen fixation of the host legume. In these new conditions, introduced strains must compete against highly adapted indigenous microorganisms and also against the harsh chemical, physical, and biological local soil conditions. Of the chemical and physical factors, temperature, water tension, salinity, and soil pH are the most important. Among biological factors, predators and bacteriophages affect the bacterial population of the soil. Protozoa play the key role in controlling bacterial numbers in the rhizosphere (10, 21). The presence of phages in soils was demonstrated as early as 1935 by Demolon and Dunez (11). They showed that phages destroyed *Rhizobium* bacteria in soils where lucerne crops had been grown for several years, resulting in a widespread lucerne sickness. In 1936, it was found that bacteriophages and bacteria occurred simultaneously in alfalfa root nodules (45). More recently, rhizobiophages were detected in soils and nodules on many occasions (5, 24, 27, 46). The presence of rhizobiophages in soils suggests that through selection or elimination of certain types of *Rhizobium* bacteria, the rhizobiophages influence the evolution of bacterial populations (47, 48).

In experiments done in vitro, bacteriophages reduced the population density of *Rhizobium trifolii*-susceptible strains on the root surface and, in nodulation, favored resistant, or even partially resistant, strains which were otherwise less able to form nodules (6, 14, 15). Since 1967, in vitro transduction experiments have been done with *R. meliloti*, showing that several auxotrophic markers can be transferred (25). Cotransduction of the *leu* gene and symbiotic activity was also described (26). In addition, general transduction was reported in *R. meliloti* (16, 34, 40) and *Rhizobium leguminosarum* (9). In contrast, the importance of the direct lytic action of phages, their mutagenic influence on nodule bacteria, or the genetic transfer levels occurring in soils are not well known. Only small amounts of data are available pertaining to environmental factors affecting both numbers of *Rhizobium* spp. and their bacteriophages (12, 13, 17, 18, 27, 29). Current research in *Rhizobium* genetics concentrates on extending the ability of N_2 fixation to other plant species and on enhancing N_2 fixation by *Rhizobium* spp. themselves. Bacteriophages could be useful cloning vectors for introducing DNA into *Rhizobium* species via specialized and generalized transduction.

Our interest was focused on morphological and physiological characterizations of *Rhizobium* bacteriophages as a prerequisite to their use in fundamental and applied research. In 1973, one of us (M.W.) described the morphology of eight *R. meliloti* phages isolated from soil (28). The phages were members of Bradley's basic morphological groups A, B, and C (8) and belonged to four different morphotypes. In the same year, Marantz et al. (33) described 17 *R. meliloti* phages; six of them were isolated from soil, and the others were isolated from lysogenic bacterial cultures. They contained double-stranded DNA and belonged to four morphotypes. The G+C content of some of these phages was determined. In 1978, *Rhizobium* phages were reviewed and

* Corresponding author.

TABLE 1. Origins of *R. meliloti* strains

Strain(s)	Source
A16, FM1, LB1, LS2A, LS5B, TU20, VE26	Institut National de la Recherche Agronomique, Dijon, France
A145, A161	Rijksdienst voor de Ijsselmeer Polders, Kampen, The Netherlands
E12, MVII-1	Biology Section IV (Genetics) University of Bielefeld, Bielefeld 1, Federal Republic of Germany
L5-30	Department of General Microbiology, Marie Curie-Sklodowska University, Lublin, Poland
M5N1,[a] M2S, M3S, M5S, M9S, M10S, M11S, M12S, M13S, M14S, M15S, M18S, M19S, M20S, M22S, M23S, M26S, M27S, M28S, M29S	Institut Agricole et Alimentaire de Lille, Université des Sciences et Techniques de Lille, Villeneuve d'Ascq, France
M31, M1-5	Station Agronomique de Gembloux, Gembloux, Belgium
Rh21	Centro Nacional de Agricultura Tropical, Palmira, Colombia
U45	Division of Plant Industry, Commonwealth Scientific and Industrial Research Organisation, Canberra, Australia
Wu498, Wu499	Institute of Agriculture, University of Western Australia, Nedlands, Western Australia, Australia
444	Institut de Cytologie et de Biologie Cellulaire, Unité d'Enseignement et de Recherche Luminy, Marseille, France
2001, 2003, 2004, 2009, 2011	Rothamsted Collection of *Rhizobium*, Harpenden, Hertfordshire, United Kingdom
102F34	Department of Microbiology, University of California at San Diego, La Jolla, Calif.

[a] Nomenclature used for bacterial strains: M, *meliloti*; S, sauvage (wild type). M5N1 is a less-gummy derivative of strain M5S when cultured on *Rhizobium* complex medium supplemented with 55×10^{-3} M glucose.

were classified into 12 species (1). All had tails and, insofar as studied, contained double-stranded DNA.

Our study examined 26 new *R. meliloti* phages; 14 were from soil, and 12 were from bacterial cultures. These phages and several of our earlier isolates were characterized by their morphology, host range, serological properties, restriction endonuclease patterns, DNA-DNA homologies, and DNA molecular weights.

MATERIALS AND METHODS

Bacteria. A total of 46 *R. meliloti* strains were used throughout this study. Of those strains, 20 were from our laboratory, and the other 26 *R. meliloti* strains were of various origins (Table 1). All strains had been isolated from nodules of *Medicago sativa* L. They were able to fix atmospheric nitrogen in aseptic lucerne plants. Bacteria were grown aerobically at 30°C in *Rhizobium* complex medium (50). For induction experiments, the tryptone yeast broth of Beringer (7) was used.

Bacteriophages. A total of 21 bacteriophages were isolated by one of us (M.W.) from local *M. sativa* field soil, by the enrichment technique of Barnet (5). Seven phages, namely CM1, CM2, NM1, NM2, NM3, NM4, and MM1H, have already been described (34, 49) and were reexamined. In addition, 12 temperate phages were obtained by UV light and mitomycin C (Sigma Chemical Co., St. Louis, Mo.) induction of all of the *R. meliloti* strains listed in Table 1.

The 12 phages were designated by the name of their lysogenic host preceded by the letter Φ (22). Phages and propagating bacteria are listed in Table 2. All phages were purified by three successive isolations of single plaques. High-titer lysates were prepared by the double-layer technique of Adams (4) by using 1.5% rhizobium complex agar as a bottom layer and a special top agar described earlier (50).

TABLE 2. Phages and propagating bacteria

Propagating strains	Soil phages[a]	Phages from lysogenic bacteria[b]
L5-30		ΦA161, Φ2011, ΦM1-5
M3S		ΦM5N1, ΦM14S
M5S		ΦLS5B
M9S	NM1, NM2, NM6, NM7	ΦM20S
M11S	CM2, CM6, NM3, NM4, NM8, NM9	
M12S	CM1, CM3	
M13S	CM4, CM9	ΦM27S
M14S	CM7, CM8, MM1C, MM1H	
M18S	CM5, CM20, CM21	
M22S		ΦFM1
444		ΦM11S, ΦM23S, ΦM26S

[a] Phage nomenclature used for soil phages: (first letter) C, contractile tail; N, noncontractile tail; M, minus tail; (second letter) M, *meliloti*. For MM phages: C, clear plaque; H, plaque with a halo. Phages CM2, CM6, CM9, NM3, NM4, NM8, and NM9 have been described under the synonyms Φ9, Φ12, Φ11, Φ5[1], Φ6, Φ4, and Φ7, respectively (49).

[b] Phages were obtained by UV light induction, except ΦM20S, ΦM26S, and ΦM5N1, which were produced by mitomycin C induction.

Extracted phages were filtered through 0.45-μm-pore-size membrane filters (Millipore Corp., Bedford, Mass.). Phages were propagated at 30°C and were stored as lysates at 4°C or in 50% glycerol broth at −30°C. For large-scale preparation, phages were grown in fermentors as described by Werquin et al. (50) and were concentrated with polyethylene glycol 8000 (Sigma) according to the method of Yamamoto et al. (51). This procedure was followed by ultracentrifugation in a three-step CsCl gradient (32) at 110,000 × *g* for 3.5 h with a Beckman L8-70M ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.) and an SW50.1 rotor.

Induction experiments. Petri dishes containing 10 ml of *R. meliloti* culture in tryptone yeast broth medium (optical density of 0.2 at 600 nm) were exposed for variable periods (4 to 20 s) to UV light (80 ergs/mm^2 per s) (15-W germicide tube lamp; Mazda). The optimal irradiation time was about 6 s for all *R. meliloti* strains. After incubation for 15 h in the dark at 30°C, cultures were centrifuged at 12,000 × *g* for 15 min, filtered, and assayed. Phage induction was also attempted with mitomycin C with final concentrations of 0.1, 0.5, 1.0, 5.0, and 10.0 μg/ml for 1 h (Table 2).

Electron microscopy. Phages were sedimented at 70,000 × *g* for 90 min in a Beckman L8-70M ultracentrifuge with an SW50.1 rotor and were washed twice in ammonium acetate (0.1 M [pH 7.0 or 5.0]). They were then deposited on copper grids with carbon-coated Formvar films and were stained with uranyl acetate (UA) (4.7×10^{-2} M [pH 4.5]) or potassium phosphotungstate (PT) (7×10^{-3} M [pH 7.2]) alone or supplemented with 50 μg of bacitracin per ml as a wetting agent (19). Specimens were examined in a Philips EM 300 electron microscope. Magnification was monitored with catalase crystals (31). At least 20 particles per morphotype and stain type and at least 5 particles per phage were measured. Head diameters were measured between opposite apices. The tail width was determined on PT-stained particles.

Host range. Phages were assayed on 33 *R. meliloti* strains (see Table 4), along with 11 various additional bacterial strains, including strains of *Agrobacterium tumefaciens* (1), *Bradyrhizobium japonicum* (7), *Rhizobium loti* (1), and *Galega* rhizobia (2). It was of importance that the correct number of phage particles be applied to the assay plates for each phage type. This was commonly accomplished by using the lowest phage dilution required to produce confluent lysis in an assay on the indicator strain, the routine test dilution. The routine test dilution varied with each phage and was dependent on plaque size and the presence or absence of a halo. As shown by Lesley (30), assays with *R. meliloti* must be carried out with a phage concentration 10-fold higher than the routine test dilution. All reactions were repeated three times.

Cross-neutralization tests. Selected phages were tested against previously prepared antisera for phages CM1, NM1, and MM1H (28). Neutralization constants were determined according to the method of Adams (4).

Extraction of phage DNA. Large volumes of lysate were prepared as described above. Phage DNA was deproteinized with a mixture of phenol-chloroform, dialyzed against TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]), precipitated with ethanol, and dissolved again in TE buffer (37).

Determination of DNA molecular weight. Restriction endonucleases *Bam*HI, *Bst*EII, and *Sal*I were from Bethesda Research Laboratories, Inc., Gaithersburg, Md., and they were used as suggested by the manufacturer. The reaction was usually performed in a 20-μl volume containing 1 μg of DNA, the appropriate buffer, and a threefold excess of endonuclease units to give complete cleavage after 1 h at 37°C. DNA fragments were then separated by electrophoresis in a 0.8% agarose gel in Tris borate buffer (36). This electrophoresis was done at 2 V/cm in a horizontal slab gel apparatus (Dankar Corp., Reading, Mass.) for 18 h or until the bromophenol blue tracking dye neared the end of the gel. Bands were stained with 1 μg of ethidium bromide per ml (International Biotechnologies, New Haven, Conn.) for 15 min, visualized with a UV transilluminator, and recorded on Polaroid number 52 or 55 film (Polaroid Corporation, Cambridge, Mass.). *Hin*dIII-digested lambda DNA and ladder DNA (Bethesda Research Laboratories) were used as molecular size standards. The sizes of DNA fragments and molecular weights of phage genomes were calculated by a least-squares fit with a second-order polynomial curve (39).

DNA-DNA hybridization. A probe of approximately 1 μg of phage DNA, digested with *Sal*I endonuclease, was labeled with [α-^{32}P]dCTP (Amersham Corp., Oakville, Ontario, Canada) by nick translation to 1×10^6 to 2×10^6 cpm (38). DNA fragments separated by gel electrophoresis as described above were transferred onto nitrocellulose membranes by a modification of the Southern transfer procedure (42) with formamide as described below. The filters were baked at 80°C for 2 h in a vacuum oven and were prehybridized for 1 to 2 h at 42°C in 10 ml of 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–50% formamide–5× Denhardt solution–50 mM phosphate buffer (pH 5.6)–1 μg of sonicated salmon sperm DNA per ml (41). The prehybridization solution was then removed, and 10 ml of the same reagents plus denatured phage probe DNA (10^6 cpm) was added. Stringency conditions selected to give 90 to 95% DNA homology included incubation at 42°C with 50% formamide, followed by washes at 65°C. After three serial washes, moist filters were mounted on Whatman 3MM paper (Whatman, Inc., Clifton, N.J.), covered with a plastic wrap, and autoradiographed at −70°C with Kodak X-Omat AR-2 film (Eastman Kodak Co., Rochester, N.Y.). Exposure times varied from a few hours to several days, depending on the intensity of labeling desired, and were monitored by including positive and negative controls.

RESULTS AND DISCUSSION

Morphology. All phages studied had isometric heads and tails which were contractile, long and noncontractile, or very short, thus corresponding to Bradley's basic morphological types A, B, and C (8) and to the *Myoviridae*, *Siphoviridae*, and *Podoviridae* families of phages with tails (35). The phages belonged to five morphotypes, represented by phages CM1, ΦM11S, NM1, NM8, and MM1H, respectively. Heads were icosahedral, as indicated by the presence of capsids with hexagonal and pentagonal outlines (Fig. 1d, 2a, and 2d). Main dimensions of the phages are given in Table 3. Previous observations on the morphology of phages CM1, NM1, and MM1H (28) were confirmed. Dimensions of phages stained with UA and PT were in good agreement. The only exception was the diameter of type CM1, which appeared larger with PT staining than with UA. This finding can be explained by some flattening of phage heads in PT staining. Phages positively stained with UA always had shrunken heads (3) and were not measured.

The *Myoviridae* family (Bradley's group A) was represented by 11 morphologically identical phages with large heads and rigid, contractile tails (Fig. 1). The latter were complex, consisting of a neck of 8 nm, a sheath with 22 striations measuring 40 by 24 nm upon contraction, a base

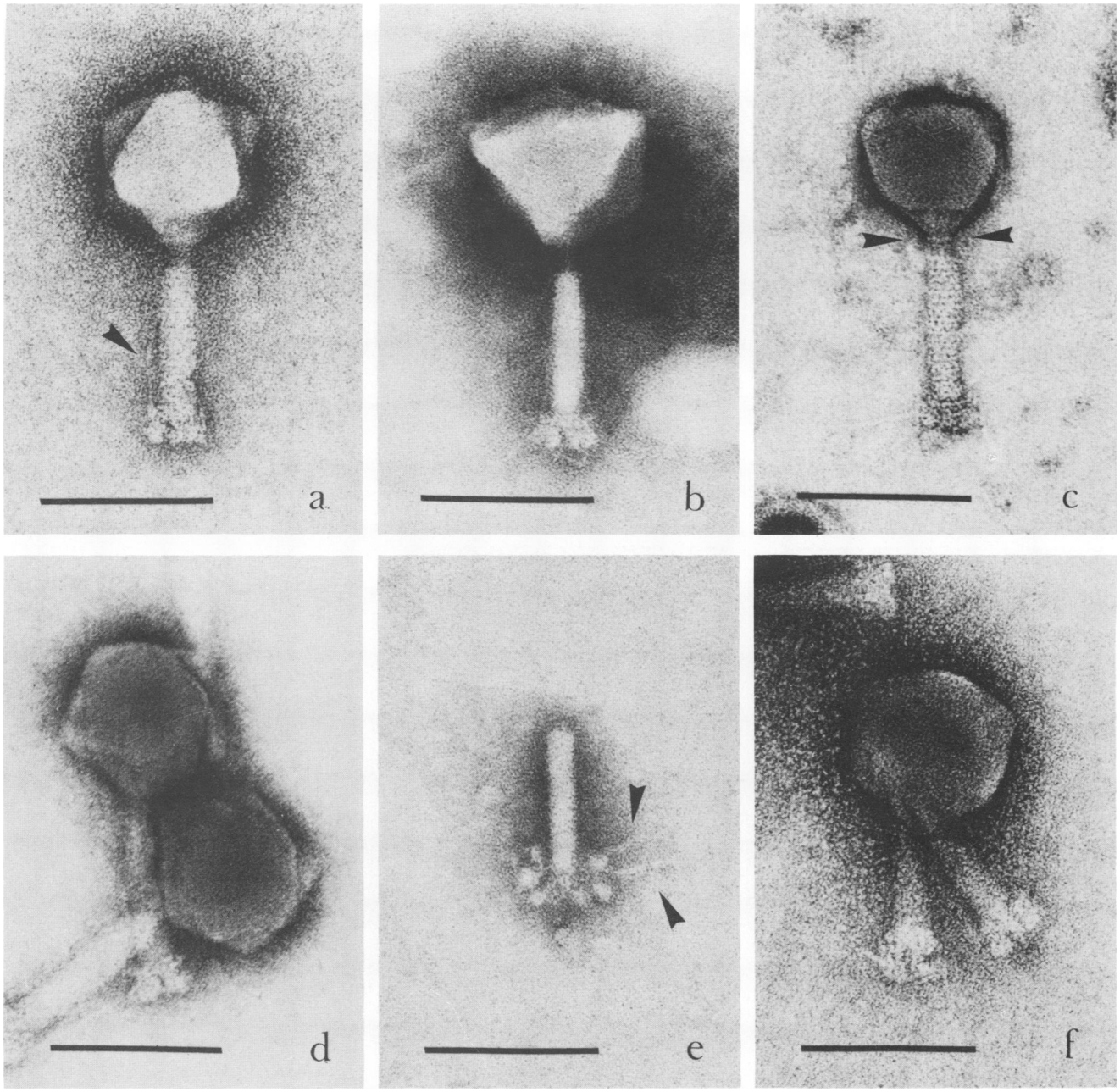

FIG. 1. *Myoviridae*, type CM1. (a) UA-stained phage with hexagonal head, extended tail, and folded tail fiber (arrow); (b) typical PT-stained phage showing a more or less triangular head; (c) UA-stained particle with conspicuous tail striations and collar (arrows); (d) UA-stained phages with pentagonal heads indicative of icosahedral shape; (e) PT-stained isolated tail with club-shaped spikes and thin fibers (arrows); (f) UA-stained exceptional particle with two tails; phage types CM1 (c and e), CM3 (a, d, and f), and CM20 (b) are represented. Bars, 100 nm.

plate measuring 34 by 3 nm, six club-shaped spikes measuring 12 by 7 nm, and at least two straight fibers measuring 64 by 2 nm. The neck showed a tiny collar measuring 13 by 2 nm. Fibers of extended tails were normally folded along the tail sheath. On one occasion, a particle with two tails was observed (Fig. 1f).

The *Siphoviridae* family (group B) included three types of phages of similar size. Three phages belonged to type ΦM11S (Fig. 2a). Their tails, which appeared more or less rigid with UA staining and flexible with PT staining showed base platelike structures of 27 nm width at their tips. Tail striations were tightly packed, and there were about 38. Type NM1 (Fig. 2b and 2c) consisted of five phages. With both UA and PT staining, when preparations were relatively slightly colored, in areas with high numbers of phage particles and bacterial debris concentrations, capsomerlike structures were frequently observed (3). As in type ΦM11S, tails were rigid with UA staining and flexible with PT staining. They were characterized by the presence of 12 to 13 transverse bars measuring 14 by 3 mm and six club-shaped spikes

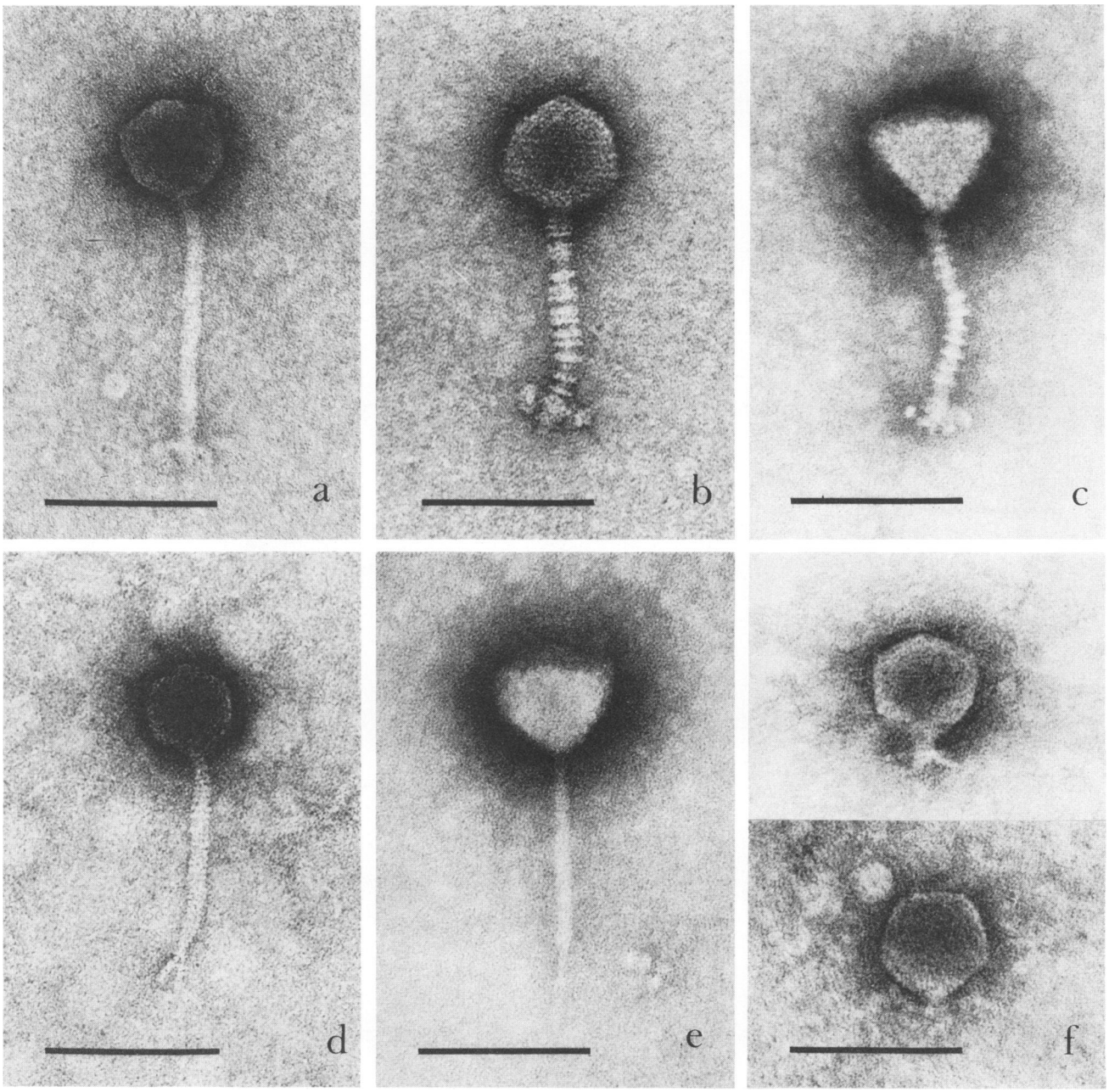

FIG. 2. *Siphoviridae*. (a) UA-stained type Φ2011; (b) UA-stained type NM1; (c) PT-stained type NM1; (d) UA-stained type NM8; (e) PT-stained type NM8. The relatively small size of the phage head (a) is due to partial positive staining. (f) *Podoviridae*, type MM1H, UA-stained particles with short tails and hexagonal or pentagonal heads. Bars, 100 nm.

measuring 14 by 9 nm. The bars had an average periodicity of 11 nm but were somewhat irregularly spaced. They were partially removed by centrifugation in a CsCl density gradient and seemed to be loosely attached. Type NM8 included 12 phages with rigid, tapering tails (Fig. 2d) showing 24 striations with a periodicity of 3 nm and up to three terminal fibers measuring 17 by 3 nm.

The *Podoviridae* (group C) were represented by two apparently identical phages, of the MM1H type (Fig. 2f). Tails had at least two club-shaped spikes measuring 12 by 7 nm.

Host ranges. Two principal types of host ranges were observed. Phages from soil, in particular, all phages of the CM1 type and certain members of the NM8 type, had a broad lytic activity, whereas most phages that had been isolated from bacteria lysed only a few strains (Table 4). Both phages and bacteria could be grouped by host range, but differences between phages of the same morphotype were often minor and seemed to reflect slight variations in phage titers, plaque size, and phage sensitivity of the individual bacteria tested. Only the NM1 type was completely homogeneous, its five members lysing only 2 of 44 bacterial strains. All other groups could be subdivided. The CM1 type included six subgroups. Five of them had similar host

TABLE 3. Main dimensions of phages[a]

Family (type)	Member(s)	Dimension of phage stained with:					
		UA			PT		
		N^b	Head (nm)	Tail (nm)	N^b	Head (nm)	Tail (nm)
Myoviridae (CM1)	CM2, CM3, CM4, CM5, CM6, CM7, CM8, CM9, CM20, CM21	65	98	103 by 17	50	107	96 by 16
Siphoviridae							
ΦM11S	ΦM23S, Φ2011	25	65	127 by 10	20	64	120 by 9
NM1	NM2, NM6, NM7, ΦM20S	50	70	110 by 8	45	72	114 by 8
NM8	NM3, NM4, NM9, ΦA161, ΦFM1, ΦLS5B, ΦMI-5, ΦM5N1, ΦM14S, ΦM26S, ΦM27S	75	64	111 by 8	30	64	109 by 8
Podoviridae (MM1H)	MM1C	20	59	12 by 8	5	56	13 by 8

[a] Limit of error, 2 to 3%.
[b] *N*, number of particles measured.

TABLE 4. Host ranges of phages grouped by morphotype[a]

Bacteria	Lysis pattern[b] of the following phage morphotype:																			
	CM1						ΦM11S		NM1	NM8									MM1H	
	CM1, CM21	CM3, CM6, CM20	CM2, CM9	CM7, CM8	CM5	CM4	ΦM11S, ΦM23S	Φ2011	NM1, NM2, NM6, NM7, ΦM20S	NM3	NM4, NM8	NM9	ΦA161, ΦM1-5, ΦM26S	ΦFM1	ΦM5N1, ΦM14S	ΦM27S	ΦLS5B	MM1C	MM1H	
LS18, M20S	v	v	v	v	v															
M2S	CL	CL	v	v																
M3S	CL	CL													CL					
LS2A		CL	CL		CL	CL				CL	CL	CL					CL			
M5S	CL	CL	CL	CL	v	v				v	v						CL		v	
M11S	CL	CL	CL	CL	v	CL				CL	CL	CL							v	
M19S, M27S	CL	CL	CL	CL	v	v	CL			CL	CL	v							v	
444	CL	CL	CL	CL	CL	v	CL	CL		CL	CL	CL	CL		v	v	v	v	v	
L5-30	CL	CL	CL	CL	CL	CL	v	CL		CL	v		CL	v					v	
M13S	CL	CL	CL	CL	v		v		CL	CL	CL	CL				CL	v		v	
M9S	CL	CL	v	v	v				CL	v									v	
M22S, Rh21, U45		v	v	v	v					CL	v	v		CL					v	
M14S	CL	CL	CL	CL	CL													CL	CL	
A161, E12, MVII-1, M5N1, M12S, M18S, M26S, M28S, 102F34, 2009	CL	CL	CL	CL	v															
Origin of phages	S	S	S	S	S	S	B	B	S or B	S	S	S	B	B	B	B	B	S	S	

[a] No lysis was observed on *R. meliloti* M1-5, M10S, M15S, M23S, M29S, and 2004 or on *A. tumefasciens* (1 strain), *B. japonicum* (7 strains), *R. loti* (1 strain) and *Galega* rhizobia (2 strains).
[b] Abbreviations: CL, confluent lysis; v, variable lysis (isolated plaques to semiconfluent lysis); S, soil; B, bacteria.

TABLE 5. Serological relationships

Type	Phage(s)	K or SC for antisera type[a]:					
		CM1		NM1		MM1H	
		K	SC	K	SC	K	SC
CM1	CM1, CM3, CM5, CM7, CM8, CM20, CM21	167–186	0.89–1.0	<0	<0	<0	<0
	CM4	90	0.49	<0	<0	<0	<0
	CM2, CM6	4.5–10	<0	<0	<0	<0	<0
NM1	NM1, NM2, NM6, NM7, ΦM20S	<0	<0	206–246	0.83–1.0	<0	<0
MM1H	MM1C, MM1H	<0	<0	<0	<0	37–39	0.96–1.0
ΦM11S	ΦM11S	12.3	<0	14.5	<0	3.2	<0
NM8	NM8	7.6	<0	1.6	<0	1.0	<0

[a] Abbreviations: K, neutralization constant (4); SC, similarity coefficient (K value of heterologous phage divided by K value of homologous phage).

ranges; however, phage CM4 differed in only lysing a few strains. Type NM8 appeared to be very heterogeneous, consisting of a group of phages isolated from soil (NM3, NM4, NM8, and NM9) and a series of isolates with distinctive host specificities. The two short-tailed phages of type MM1H differed greatly in their lytic activity. Phage MM1H caused partial lysis on numerous strains and phage MM1C lysed only a few strains, suggesting the presence of different receptors or one or more restriction-modification systems in strains sensitive to MM1H. The phages lysed only *R. meliloti* and none of the 11 other bacteria tested. This result was in agreement with previous observations on the narrow specificity of *Rhizobium* phages (43).

Serological relationships. Neutralization tests with previously prepared antisera (28) against phages CM1, NM1, and MM1H confirmed morphological and host range data. Three phage groups could be established, each corresponding to a different antiserum, and no cross-neutralization between them was observed (Table 5). In addition, phages NM8 and ΦM11S, for which no specific antiserum was available, were either not neutralized by the antisera mentioned above or were neutralized only at nonspecific levels. The CM1 group appeared to be heterogeneous, consisting of phages that were completely, partly, or not at all neutralized by CM1 antiserum. These results are in agreement with the general observation that morphologically different phages are serologically unrelated, whereas phages with the same morphology may or may not have antigenic relationships (2).

DNA relationships and molecular weights. Preliminary experiments with selected phages had shown that restriction endonuclease *Sal*I produced numerous DNA fragments which were easily separated and visualized by agarose gel electrophoresis. When digested with *Sal*I, the DNAs of phages CM1, ΦM11S, NM1, NM8, and MM1H showed entirely different patterns (Fig. 3a), suggesting that their genomes were unrelated. The DNA of phage CM1 was not cleaved by *Sal*I and appeared as a single band. Phage MM1H had produced two bands in CsCl density gradients, both of which contained viable phages which were propagated on the same host strain. *Sal*I digests of their DNAs showed identical patterns, suggesting that the MM1H population was heterogeneous and included two varieties with different buoyant densities. DNA fragments of phage NM1 were then labeled with [^{32}P]dCTP by nick translation and were hybridized with the various DNA fragments from phage NM1 and the others shown in Fig. 3a. Phage NM1 DNA hybridized only with itself (Fig. 3b) and the DNA of the other four NM1-like phages (manuscript in preparation). Similar results were obtained in large, well-conducted studies of enterobacterial and streptococcal phages of different morphotypes, indicating a close correlation between DNA relationships and morphologies (20, 23). The DNA molecular weight of phages ΦM11S, NM1, NM8, and MM1H was estimated to be 42.2, 51.9, 28.9, and 40.5 kilobases, respectively (28.1×10^6, 34.5×10^6, 19.3×10^6, and 27.0×10^6 daltons, respectively). Except for phage NM8, these data correspond

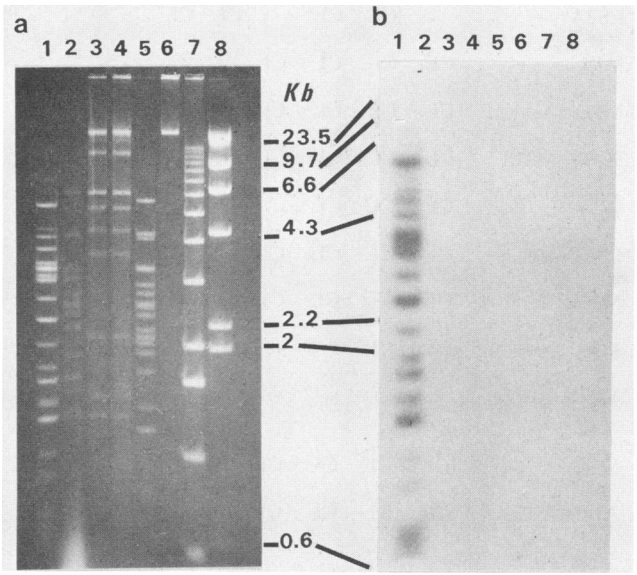

FIG. 3. Restriction endonuclease patterns and DNA relationships. (a) Agarose gel electrophoresis of *Sal*I-digested phage DNAs. Lane 1, phage NM1; lane 2, phage NM8; lane 3, phage MM1H, lower CsCl band; lane 4, phage MM1H, upper CsCl band; lane 5, phage ΦM11S; lane 6, phage CM1; lane 7, ladder DNA; lane 8, lambda DNA digested with *Hin*dIII. (b) Autoradiogram obtained after Southern transfer of DNA from the agarose gels in panel a, followed by hybridization with ^{32}P-labeled NM1 DNA digested with *Sal*I. Kilobases (kb) are indicated at the sides of gels.

to values expected from capsid sizes. The DNA content of phage NM8 appeared to be too low for its head size, suggesting that some DNA fragments were not resolved. The mass of CM1 DNA could not be determined with certainty.

In conclusion, the phages studied belong to five distinct groups, differing from each other in their morphology, DNA homology, and, insofar as tested, serological properties. Differences are also evident in their host ranges, which correlate with other properties and allow further subdivision of these phages.

The phages belong to common morphological groups, but all five phage types described here correspond to different species. Two of them, CM1 and NM1, are already known and have been described in a recently revised classification scheme of *Rhizobium* phages (H.-W. Ackermann and M. S. DuBow, ed., *Viruses of Prokaryotes*, in press). A phage of type CM1, ΦM12S, is known as a general transductant (16). Types NM8 and MM1H may belong to phage species 317 and Φ2042, respectively, whereas type ϕM11S does not correspond to any other *Rhizobium* phage and represents a new species.

It is remarkable that all of the phages with contractile tails were isolated from soil and have comparatively wide host ranges. In contrast, most phages isolated from bacteria have long, noncontractile tails and a relatively narrow host range. Except for NM1 and its relatives, the phages have no particular distinguishing morphological characteristics. Type NM1 is highly unusual because of the presence of transverse bars on the phage tail; this is a rare property which has only been found in a few morphologically unrelated phages of *Acinetobacter*, *Dactylosporangium*, *Streptomyces*, and *Vibrio* spp. (44).

LITERATURE CITED

1. **Ackermann, H.-W.** 1978. La classification des phages d'*Agrobacterium* et *Rhizobium*. Pathol. Biol. **26**:507–512.
2. **Ackermann, H.-W., A. Audurier, L. Berthiaume, L. A. Jones, J. A. Mayo, and A. K. Vidaver.** 1978. Guidelines of bacteriophage characterization. Adv. Virus Res. **23**:1–24.
3. **Ackermann, H.-W., P. Jolicoeur, and L. Berthiaume.** 1974. Avantages et inconvénients de l'acétate d'uranyle en virologie comparée: étude de quatre bactériophages caudés. Can. J. Microbiol. **20**:1093–1099.
4. **Adams, M. H.** 1959. Bacteriophages. Interscience Publishers, Inc., New York.
5. **Barnet, Y. M.** 1972. Bacteriophages of *Rhizobium trifolii*. I. Morphology and host range. J. Gen. Virol. **15**:1–15.
6. **Barnet, Y. M.** 1980. The effect of rhizobiophages on populations of *Rhizobium trifolii* in the root zone of clover plants. Can. J. Microbiol. **26**:572–576.
7. **Beringer, J. E.** 1974. R factor transfer in *Rhizobium leguminosarum*. J. Gen. Microbiol. **84**:188–198.
8. **Bradley, D. E.** 1967. Ultrastructure of bacteriophages and bacteriocins. Bacteriol. Rev. **31**:230–314.
9. **Buchanan-Wollaston, A. V.** 1979. Generalized transduction in *Rhizobium leguminosarum*. J. Gen. Microbiol. **112**:135–142.
10. **Danso, S. K. A., S. O. Keya, and M. Alexander.** 1975. Protozoa and the decline of *Rhizobium* populations added to soil. Can. J. Microbiol. **21**:884–895.
11. **Demolon, A., and A. Dunez.** 1935. Recherches sur le rôle du bactériophage dans la fatigue des luzernières. Ann. Agron. **5**:86–111.
12. **Dhar, B., B. D. Singh, R. B. Singh, J. S. Srivastava, and R. M. Singh.** 1980. Seasonal incidence of rhizobiophages in soils around Varanasi. Indian J. Exp. Biol. **18**:1168–1170.
13. **Dhar, B., B. D. Singh, R. B. Singh, J. S. Srivastava, V. P. Singh, and R. M. Singh.** 1979. Occurrence and distribution of rhizobiophages in Indian soils. Acta Microbiol. Pol. **23**:319–324.
14. **Evans, J., Y. M. Barnet, and J. M. Vincent.** 1979. Effect of a bacteriophage on colonisation and nodulation of clover roots by a strain of *Rhizobium trifolii*. Can. J. Microbiol. **25**:968–973.
15. **Evans, J., Y. M. Barnet, and J. M. Vincent.** 1979. Effect of a bacteriophage on colonisation and nodulation of clover roots by paired strains of *Rhizobium trifolii*. Can. J. Microbiol. **25**: 974–978.
16. **Finan, T. M., E. Hartwieg, K. LeMieux, K. Bergman, G. C. Walker, and E. R. Signer.** 1984. General transduction in *Rhizobium meliloti*. J. Bacteriol. **159**:120–124.
17. **Golebiowska, J., A. Sawicka, and J. Swiateck.** 1976. The occurrence of rhizobiophages in various lucerne plantations. Acta Microbiol. Pol. **25**:161–163.
18. **Golebiowska, J., A. Sawicka, and U. Sypniewska.** 1971. Occurrence and characteristics of rhizobiophages in lucerne fields. Pol. J. Soil Sci. **12**:131–136.
19. **Gregory, D. W., and B. J. S. Pirie.** 1973. Wetting agents for biological electron microscopy. I. General considerations and negative staining. J. Microsc. (Oxford) **99**:251–265.
20. **Grimont, F., and P. A. D. Grimont.** 1981. DNA relatedness among bacteriophages of the morphological group C3. Curr. Microbiol. **6**:65–69.
21. **Habte, M., and M. Alexander.** 1977. Further evidence for the regulation of bacterial populations in soil by protozoa. Arch. Microbiol. **113**:181–183.
22. **Jarvis, A. W.** 1978. Serological studies of a host range mutant of a lactic streptococcal bacteriophage. Appl. Environ. Microbiol. **36**:785–789.
23. **Jarvis, A. W.** 1984. Differentiation of lactic streptococcal phages into phage species by DNA-DNA homology. Appl. Environ. Microbiol. **47**:343–349.
24. **Kleczkowska, J.** 1957. A study of distribution and effects of bacteriophage of root nodule bacteria in the soil. Can. J. Microbiol. **3**:171–180.
25. **Kowalski, M.** 1967. Transduction in *Rhizobium meliloti*. Acta Microbiol. Pol. **16**:7–12.
26. **Kowalski, M., and J. Denarié.** 1972. Transduction d'un gène contrôlant l'expression de la fixation de l'azote chez *Rhizobium meliloti*. C.R. Acad. Sci. Ser. D **275**:141–144.
27. **Kowalski, M., G. E. Ham, L. R. Frederick, and I. C. Anderson.** 1974. Relationship between strains of *Rhizobium japonicum* and their bacteriophages from soil and nodules of field-grown soybeans. Soil Sci. **118**:221–228.
28. **Krsmanovic-Simic, D., and M. Werquin.** 1973. Etude des bactériophages de *Rhizobium meliloti*. C.R. Acad. Sci. Ser. D **276**:2745–2748.
29. **Lawson, K. A., Y. M. Barnet, and C. A. McGilchrist.** 1987. Environmental factors influencing numbers of *Rhizobium leguminosarum* biovar *trifolii* and its bacteriophages in two field soils. Appl. Environ. Microbiol. **53**:1125–1131.
30. **Lesley, S. M.** 1982. A bacteriophage typing system for *Rhizobium meliloti*. Can. J. Microbiol. **28**:180–189.
31. **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.
32. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual, p. 75–85. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. **Marantz, L. A., L. N. Moskalenko, and Y. I. Rautenstein.** 1973. Some biological properties of phages for *Rhizobium meliloti*. Mikrobiologiya **42**:1088–1094.
34. **Martin, M. O., and S. R. Long.** 1984. Generalized transduction in *Rhizobium meliloti*. J. Bacteriol. **159**:125–129.
35. **Matthews, R. E. F.** 1982. Classification and nomenclature of viruses. Fourth report of the International Committee on Nomenclature of Viruses. Intervirology **17**:1–199.
36. **Meyers, J. A., D. Sanchez, L. P. Elwell, and S. Falkow.** 1976. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol. **127**:1529–1537.
37. **Radloff, R., W. Bauer, and J. Vinograd.** 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.
38. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977.

Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237–251.

39. **Schroeder, J. L., and F. R. Blattner.** 1978. Least-squares method for restriction mapping. Gene **4**:167–174.
40. **Sik, T., J. Harwath, and S. Chatterjee.** 1980. Generalized transduction in *Rhizobium meliloti*. Mol. Gen. Genet. **178**:511–516.
41. **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1974. Experiments with gene fusions, p. 191–195. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
42. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
43. **Staniewski, R.** 1970. Typing of *Rhizobium* by phages. Can. J. Microbiol. **16**:1003–1009.
44. **Stuttard, C., and M. Dwyer.** 1981. A new temperate phage of *Streptomyces venezuelae*. Morphology, DNA molecular weight, and host range of SV2. J. Can. Microbiol. **27**:496–499.
45. **Vandecaveye, S. C., and H. Katznelson.** 1936. Bacteriophage as related to the root nodule bacteria of alfalfa. J. Bacteriol. **31**:465–477.
46. **Vincent, J. M.** 1970. A manual for the practical study of root nodule bacteria. *In* International Biological Programme handbook no. 15. Blackwell Scientific Publications, Ltd. Oxford.
47. **Vincent, J. M.** 1974. Root nodule symbiosis with *Rhizobium*, p. 265–341. *In* A. Quispel (ed.), Biology of nitrogen fixation. North-Holland Publishing Co., Amsterdam.
48. **Vincent, J. M.** 1977. *Rhizobium*: general microbiology, p. 277–366. *In* R. W. F. Hardy and W. S. Silver (ed.), A treatise on dinitrogen fixation. Section III. Biology. John Wiley & Sons, Inc., New York.
49. **Werquin, M., M. T. Ben Brahim, and D. Krsmanovic-Simic.** 1977. Etude des bactériophages de *Rhizobium meliloti*. C.R. Acad. Sci. Ser. D **284**:1851–1854.
50. **Werquin, M., C. Defives, L. Hassani, and M. Andriantsimiavona-Otonia.** 1984. Large scale preparation of *Rhizobium meliloti* bacteriophages by fermenter culture. J. Virol. Methods **8**:155–160.
51. **Yamamoto, K. R., B. M. Alberts, R. Benzinger, L. Lawhorne, and G. Treiber.** 1970. Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large-scale virus purification. Virology **40**:734–744.