APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1981, p. 1389-1393
0099-2240/81/061389-05$02.00/0
Vol. 41, No. 6

Isolation of *Arthrobacter* Bacteriophage from Soil†

JAMES J. GERMIDA AND L. E. CASIDA, JR.*
Department of Microbiology, The Pennsylvania State University, University Park, Pennsylvania 16802

Received 18 December 1980/Accepted 19 March 1981

Soil was percolated with water and various nutrient solutions, and then the percolates were analyzed for bacteriophages which produced plaques on various *Arthrobacter* strains. The water percolates did not contain detectable phage. In contrast, phages for *A. globiformis* strains ATCC 8010 and 4336, and for several recent *Arthrobacter* species soil isolates, were easily detected in nutrient broth, soil extract, and cation-complete medium percolates. These percolates did not contain phage that produced plaques on *A. oxydans* and a recent *Arthrobacter* species soil isolate. Percolation with a selective nicotine-salts solution was required for demonstrating phage for these bacteria. None of the percolates contained phage for five additional named *Arthrobacter* species. In addition, phages were not detected for *A. crystallopoietes* in a 2-hydroxypyridine percolate of soil. Based on their lytic spectra, the phage isolates from this soil were relatively host specific.

Arthrobacter species are pleomorphic bacteria found in soil (7, 8, 10, 16) and other natural habitats (20). Although the ecology of *Arthrobacter* species has been the subject of much study, relatively little is known concerning the interaction of these bacteria with bacteriophage and other predators in nature. Phage for various *Arthrobacter* species have been isolated and partially characterized (3, 5, 11, 14, 17, 19). In addition, the use of phage to type soil *Arthrobacter* species has been investigated (3, 9).

The techniques available for phage isolation from soil have not worked well for *Arthrobacter* phage. To remedy this situation, Casida and Liu (5) developed a unique soil enrichment technique that did not involve addition of potential host cells to the soil. This technique allowed the isolation of a variety of phages for *A. globiformis*. However, these workers did not try to use their technique for isolating phages for other *Arthrobacter* species. Brown et al. (3) were not successful in isolating *Arthrobacter* phages from river water and sewage. They did, however, find small numbers of phage when they concentrated their preparations. There were phage for several *Arthrobacter* soil isolates in the concentrates, but none that were active against the named *Arthrobacter* species tested. The phages from the concentrates showed some cross-reactions among the host *Arthrobacter* soil isolates. These authors did not use the Casida and Liu procedure. As a result, they were unable to detect *Arthrobacter* phage in either unconcentrated or concentrated extracts of soil.

The present study used a modification of the Casida and Liu technique to show the presence (or absence) in soil of phage active against various named *Arthrobacter* species and recent *Arthrobacter* species soil isolates. We realized that host specificity might be a factor in the ease or difficulty of demonstrating phage in soil. Therefore, the host specificity ranges among various *Arthrobacter* strains were determined for some of the *Arthrobacter* phages isolated from soil.

MATERIALS AND METHODS

Bacterial cultures. The named species of *Arthrobacter* used in this study were obtained from the American Type Culture Collection. These species were *A. globiformis* strains 8010 and 4336, *A. oxydans* strain 14358 (yellow and white biotypes), *A. crystallopoietes* strain 15481, *A. simplex* strain 6946, *A. tumescens* strain 6947, *A. ramosus* strain 13727, and *A. atrocyaneus* strain 13752. Five *Arthrobacter* species soil isolates (13) also were studied. These isolates were GSI-1, GSI-5, GSI-6, GSI-10, and SPI-1. Isolate SPI-1 was isolated from soil that had been percolated with nicotine-salts solution (see below).

Bacteriophage isolation. Hagerstown silty clay loam soil was used for these studies. It was stored at room temperature in large polyethylene bags to prevent drying but still allow gaseous exchange with the atmosphere. The initial experiments were conducted after approximately 1 year of storage, and the rest of the experiments were done after 3 years of storage. During this time, the soil remained at 31 to 34% of moisture-holding capacity.

Phages for *Arthrobacter* species were isolated from this soil by a modification of the Casida and Liu (5) enrichment technique. The soil was passed through a 1.19-mm sieve, and then 50 g was layered upon a 0.5-

† Paper no. 6148 in the journal series of the Pennsylvania Agriculture Experiment Station.

cm-deep glass wool plug at the base of a glass column (3 by 20 cm) which was designed to serve as an air-lift percolator (4). Nutrient solution or water (50 to 60 ml) was then percolated over the soil. The nutrient solutions were nutrient broth, soil extract, cation-complete medium (13), nicotine-salts solution (6), and 2-hydroxypyridine solution (12). At daily intervals, samples of percolate were withdrawn from each column and passed through a sterile 0.3-μm-pore-size membrane filter. This filtrate was then diluted in fresh, sterile percolation fluid and assayed for plaque-forming units (PFU) on various *Arthrobacter* species. For this assay, 1.0 or 0.1 ml of diluted filtrate and 0.5 ml of a 24- to 48-h nutrient broth culture (shaken at 27°C) of the host bacterium to be tested were added to 2.5 ml of nutrient agar (0.75% agar). This was then applied as an overlay on a nutient agar (1.5% agar) basal layer. The plates were incubated 48 h at 27°C before counting the plaques. Each bacteriophage selected for study was purified by performing seven consecutive transfers of phage from individual plaques to new bacterial cell lawns.

Concentrated phage suspensions were made by suspending the surface agar overlays from confluent lysis plates into nutrient broth to give a 5- to 10-ml total volume. The residual cells and agar were removed by centrifugation (10,000 × *g* for 10 min at room temperature). The supernatant fluid containing the phage was passed through a sterile 0.3-μm-pore-size membrane filter, titered, and refrigerated.

Phage typing. The lytic spectra of the phage isolates were determined by the spot plate method (2). Three milliliters of nutrient agar (0.75% agar) was seeded with 1 ml of a 24- to 48-h nutrient broth culture of the test bacterium. This was applied as an overlay on a nutrient agar (1.5% agar) basal layer. The resulting culture plate was then spotted with 0.1 ml of a freshly prepared phage lysate (approximately 10^6 to 10^8 PFU/ml). Usually, six to eight different phage isolates were tested on each culture plate. The lytic reactions were recorded after incubation at 27°C for 24 to 72 h.

RESULTS

Nutrient broth, cation-complete medium, and soil extract percolates of soil, but not water percolates, contained phages for *A. globiformis* strains 8010 and 4336 and for four *Arthrobacter* soil isolates (GSI-1, GSI-5, GSI-6, and GSI-10). The phage numbers in these percolates varied with the nutrient and host used, but usually at least 10^2 to 10^3 PFU/ml were obtained. Table 1 shows the phage numbers for *A. globiformis* strain 8010. In contrast to these results, no phages for *A. crystallopoietes* strain 15481, *A. oxydans* strain 14358 (yellow and white biotypes), *A. simplex* strain 6946, *A. tumescens* strain 6947, *A. ramosus* strain 13727, and *A. atrocyaneus* strain 13752 were detected in the percolates. Likewise, no phages for *A. crystallopoietes* were detected when the soil was percolated with 2-hydroxypyridine medium. Interestingly, no phages for *Pseudomonas facilis* or soil *Pseudomonas* isolate RI-2 (15) were detected in the nutrient broth percolate of soil (other percolates were not tested), although this percolate did contain *Bacillus subtilis* phage (1.9×10^3 PFU/ml).

Although phages for *A. oxydans* strain 14358 (yellow and white biotypes) and isolate SPI-1 were not present in the above percolates, phages were present, and were isolated, when a selective nicotine-salts solution was used as the percolation fluid. In fact, the natural population of nicotine-utilizing bacteria in the soil increased during the percolation such that the surface of the soil became covered with a blue-pigmented layer of cells.

Based on plaque morphology, the total phage population in the percolate appeared to be homogeneous when the cation-complete medium percolate was analyzed on *Arthrobacter* soil isolate GSI-1 or GSI-6. In contrast, heterogeneous phage populations for these bacteria were present in the nutrient broth percolate. The total phage population which produced plaques on strain 8010 or 4336 was heterogeneous regardless of the nutrient used.

The percolation trials with cation-complete medium and soil extract were repeated after the soil had been stored for 2 years at room temperature. The soil had not changed in moisture content during the storage. Although the phage numbers for strain 8010 produced in soil extract percolate did not decrease during storage (Table 2), the numbers in the cation-complete medium percolate decreased dramatically. A similar decrease (from 2.2×10^4 to 20 PFU/ml at 2 days of percolation) occurred for *A. globiformis* strain 4336. Again (Table 2), no phages were present in these percolates for *A. oxydans* and isolate SPI-1.

A number of purified bacteriophage isolates from this soil were typed (Table 3) against various *Arthrobacter* strains. Phages GAP-14, -15, and -16, which were isolated on *A. globiformis* strain 8010, did not produce plaques on most of the other *Arthrobacter* strains. In general, the other phages showed only minimal, if any, cross-reactivity. However, phage isolated from plaques on the white and yellow biotypes of *A. oxydans*

TABLE 1. *Production in percolated soil of phages producing plaques on A. globiformis strain 8010*

Percolation fluid	PFU/ml of percolation fluid			
	0[a]	1	2	3
Cation-complete	0	4.2×10^2	4.2×10^5	ND[b]
Soil extract	0	20	80	ND
Nutrient broth	0	1	7	44

[a] Days of percolation. For day 0, samples were taken after 3 h of percolation.

[b] ND, Not determined.

TABLE 2. *Production in percolated soil of phages producing plaques on various Arthrobacter strains*

Bacterial host[a]	PFU/ml of percolation fluid							
	Cation-complete medium				Soil extract			
	0[b]	1	2	3	0	1	2	3
A. globiformis 8010	0	33	40	0	0	24	63	85
A. globiformis 4336	0	1	20	1	0	0	5	6
A. oxydans 14358	0	0	0	0	0	0	0	0
Arthrobacter soil isolate								
SPI-1	0	0	0	0	0	0	0	0
GSI-1	0	1.0×10^3	5.5×10^2	9	0	7.1×10^2	3.3×10^2	7.5×10^3
GSI-5	0	7.0×10^2	3.8×10^3	1.4×10^3	0	3.2×10^2	3.3×10^3	$>3.0 \times 10^3$
GSI-6	0	2.5×10^2	7.1×10^2	0	0	18	53	5.0×10^2
GSI-10	0	5.2×10^2	9.4×10^2	5	0	26	1.1×10^2	$>3.0 \times 10^3$

[a] Water percolates of soil did not contain phages for any of the host strains tested.
[b] Days of percolation. For day 0, samples were taken after 3 h of percolation.

TABLE 3. *Lytic spectra of 11 Arthrobacter phage isolates tested against 5 Arthrobacter soil isolates and 8 named strains of Arthrobacter*[a]

Phage[b]	Lytic reaction with *Arthrobacter* host strain[c]						
	8010	4336	SPI-1	14358	GSI-5	GSI-1	GSI-6
GAP-15	++++	−	−	−	−	−	−
GAP-16	++++	++	−	−	−	−	−
GAP-14	++++	++++	++++	−	−	−	−
GAP-40	−	−	++++	−	−	−	−
GAP-41	−	−	−	++++	−	−	−
GAP-42	−	−	−	+++	−	−	−
GAP-33	−	−	−	−	++++	−	−
GAP-32	−	−	−	−	++++	++++	−
GAP-31	−	−	−	−	−	++++	−
GAP-30	−	−	−	−	−	++++	++++
GAP-34	−	−	−	−	−	−	++++

[a] The table shows only the *Arthrobacter* strains on which a reaction occurred; the other strains which did not react were *A. crystallopoietes* 15481, *A. simplex* 6946, *A. tumescens* 6947, *A. ramosus* 13727, *A. atrocyaneus* 13752, and soil isolate GSI-10.
[b] The original host on which the phage was isolated agrees, in most instances, with the sensitive host reported in the table. Phages GPA-14 and -16 were isolated on strain 8010, GAP-32 on GSI-5, and GAP-30 on GSI-1.
[c] ++++, Yields confluent lysis on the propagating strain within 24 to 48 h; +++, lysis within 48 to 72 h; ++, lysis within 48 to 72 h but not confluent; −, no lysis.

strain 14358 did cross-react for these biotypes. None of the phages tested formed plaques on any of the *Arthrobacter* strains for which no phages were detected in the nutrient percolates of soil.

DISCUSSION

Bacteriophages for *A. globiformis* strains 8010 and 4336 were present in various nutrient, but not water, percolates of soil. The respective host bacteria were not added to the soil for these percolations (or for the other percolations that were studied). These observations confirm the results obtained by Casida and Liu (5). In addition, however, the present study shows that a modification of the Casida and Liu technique allows the isolation from soil of phages for *A. oxydans* strain 14358 (yellow and white biotypes) and for five *Arthrobacter* soil isolates (isolated from the soil used in this study). Use of this technique, however, did not allow isolation of phages for five other named *Arthrobacter* species. Assuming that phage production in soil reflects the growth of indigenous host cells, then failure to isolate a phage for these *Arthrobacter* species could indicate their absence from the soil used. It is possible, however, that they were present, but that the nutrients percolated through the soil were not selective enough to allow growth (and phage production) of these bacteria from very low cell numbers in soil when they were competing with the other soil bacteria

for nutrients. In this regard, phages for *A. oxydans* and soil isolate SPI-1 were not detected in nutrient broth, soil extract, and cation-complete medium percolates, although these bacteria grew well on these media as pure cultures in the laboratory (13). Also, these bacteria were not detected when dilutions of natural nonpercolated soil were plated on nicotine agar, although they can use nicotine as their only source of carbon and nitrogen. They were easily isolated, however (e.g., isolate SPI-1), from nicotine-salts solution percolates of soil (or the percolated soil itself), and the percolates contained phage for them. If this reasoning is correct, it can be concluded that *A. crystallopoietes* was not present in our soil. Percolation of the soil with 2-hydroxypyridine did not produce phage for this bacterium, and pigment (11) did not accumulate at the soil surface. It is not known, however, whether 2-hydroxypyridine might inactivate phage for this bacterium. Achberger and Kolenbrander (1) used a phage for *A. crystallopoietes*, but did not say how they obtained it. It is unclear from the publications of Brown et al. (3) and Ostle and Holt (18) whether they actually had a phage for this bacterium.

It is possible that soil extract, which is of low nutritional value but does contain various salts extracted from the soil, may have merely eluted indigenous phage from the soil. As an argument against elution, no phages were detected after a 3-h percolation of the soil. In addition, the numbers of PFU per milliliter tended to increase with time.

Phages for *A. globiformis* strains 8010 and 4336 were detected in soil to which no host cells had been added. It is not known whether these particular bacterial strains were present in the soil under study. Also, it is not known why their phage numbers in cation-complete medium percolates, but not in soil extract percolates, decreased upon prolonged storage of the soil. It is possible that the phage production in soil was due to the presence not of strains 8010 or 4336, but of other closely-related *Arthrobacter* strains or species. The lytic spectra obtained for the phage isolates from our study demonstrated good specificity for their hosts. Similar observations have been made in other laboratories (3, 9, 11). In our studies only *Arthrobacter* soil isolate SPI-1 supported the multiplication of a phage (GAP-14) which was isolated on *A. globiformis* strain 8010. This observation indicated the presence in soil of *Arthrobacter* strains or species genetically related to the type strain of *A. globiformis*. However, a bacteriophage (GAP-40) isolated on SPI-1 failed to replicate on *A. globiformis* strain 8010. Thus, the lytic spectra of *Arthrobacter* phage do not necessarily provide a true indication of the diversity of these bacteria in nature.

As noted previously, other investigators have reported only limited success in isolating bacteriophage for *Arthrobacter* species. Use of the soil percolation procedure that we describe should increase the numbers and types of phage that can be obtained from soil. It may be necessary, however, to select percolation fluids that favor the growth of the particular *Arthrobacter* species of interest.

ACKNOWLEDGMENTS

This work was supported by grant DAAG29-79-G-0043 from the U.S. Army Research Office.

LITERATURE CITED

1. **Achberger, E. C., and P. E. Kolenbrander.** 1978. Isolation and characterization of morphogenetic mutants of *Arthrobacter crystallopoietes*. J. Bacteriol. **135**:595–602.
2. **Adams, M. K.** 1959. Bacteriophages. Interscience Publishers Inc., New York.
3. **Brown, D. R., J. G. Holt, and P. A. Pattee.** 1978. Isolation and characterization of *Arthrobacter* bacteriophages and their application to typing of soil *Arthrobacter*. Appl. Environ. Microbiol. **35**:185–191.
4. **Bryner, L. C., J. V. Beck, D. B. Davis, and D. G. Wilson.** 1954. Microorganisms in leaching sulfide minerals. Ind. Eng. Chem. Anal. Ed. **46**:2587–2592.
5. **Casida, L. E., Jr., and K.-C. Liu.** 1974. *Arthrobacter globiformis* and its bacteriophage in soil. Appl. Microbiol. **28**:951–959.
6. **Casida, L. E., Jr., and R. Rosenfield.** 1957. Bacterial oxidation of nicotine: formation of gama-aminobutyric acid. J. Bacteriol. **75**:474–479.
7. **Conn, H. J.** 1928. A type of bacteria abundant in productive soils but apparently lacking in certain soils of low productivity. N.Y. State Agric. Exp. Station Tech. Bull. **138**:3–26.
8. **Conn, H. J.** 1948. The most abundant group of bacteria in soil. Bacteriol. Rev. **12**:247–273.
9. **Conn, H. J., E. J. Bottcher, and C. Randall.** 1945. The value of bacteriophage in classifying certain soil bacteria. J. Bacteriol. **49**:359–373.
10. **Conn, H. J., and I. Dimmick.** 1948. Soil bacteria of the genus *Arthrobacter*. Soil Sci. **65**:349–351.
11. **Einck, K. H., P. A. Pattee, J. G. Holt, C. Hagedorn, J. A. Miller, and D. L. Berryhill.** 1973. Isolation and characterization of a bacteriophage of *Arthrobacter globiformis*. J. Virol. **12**:1031–1033.
12. **Ensign, J. C., and S. C. Rittenberg.** 1963. A crystalline pigment produced from 2-hydroxypyridine by *Arthrobacter crystallopoietes* n. sp. Arch. Microbiol. **47**:137–153.
13. **Germida, J. J., and L. E. Casida, Jr.** 1980. Myceloid growth of *Arthrobacter globiformis* and some other *Arthrobacter* species. J. Bacteriol. **144**:1152–1158.
14. **Gillespie, D. C.** 1960. Isolation of bacteriophage for *Arthrobacter globiformis*. Can. J. Microbiol. **6**:447–478.
15. **Labeda, D. P., K.-C. Liu, and L. E. Casida, Jr.** 1976. Colonization of soil by *Arthrobacter* and *Pseudomonas* under varying conditions of water and nutrient availability as studied by plate counts and transmission electron microscopy. Appl. Environ. Microbiol. **31**:551–561.
16. **Lochhead, A. G., and F. E. Chase.** 1943. Qualitative studies of soil microorganisms. V: Nutritional requirements of the predominant bacterial flora. Can. J. Microbiol. **3**:35–42.
17. **Mulder, E. G.** 1964. *Arthrobacter*, p. 254. *In* H. Heukelekian and N. C. Dondero (ed.), Principles and appli-

cation in aquatic microbiology. John Wiley & Sons, Inc., New York.
18. **Ostle, A. G., and J. G. Holt.** 1979. Elution and inactivation of bacteriophages on soil and cation exchange resin. Appl. Environ. Microbiol. **38**:59–65.
19. **Schippers-Lammertse, A. F., A. O. Muijsers, and K. B. Klatser-Oedekerk.** 1963. *Arthrobacter polychromogenes* nov. spec., its pigment, and a bacteriophage of this species. Antonie van Leeuwenhoek J. Microbiol. Serol. **29**:1–15.
20. **Veldkamp, K.** 1970. Saphrophytic coryneform bacteria. Annu. Rev. Microbiol. **24**:209–241.