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Ensifer adhaerens Predatory Activity Against Other Bacteria in Soil, as Monitored by Indirect Phage Analysis†

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An indirect phage analysis procedure was used to detect and follow the activity of the bacterial predator *Ensifer adhaerens* in situ in natural soil. The soil was percolated with an aqueous suspension of washed bacterial host cells so that the *E. adhaerens* cells naturally present in the soil would multiply in response to the host cells. The natural phage development which ensued against these multiplying *E. adhaerens* cells in the soil was then monitored by noting plaques which developed when the percolation fluid was plated with laboratory strains of *E. adhaerens* on laboratory media. The activities of the other members of the predation system that includes *E. adhaerens* (*Streptomyces* sp. strain 34 and a myxobacter) could not be monitored directly by phage analysis because phage were not found for them. Indirect monitoring was possible, however, because they were susceptible to attack by *E. adhaerens*. In general, the results were in agreement with previous observations by other methods of the predation sequence. *E. adhaerens* attacked *Micrococcus luteus*, *Streptomyces* sp. strain 34, and the myxobacter but did not attack several other possible species of hosts. It also did not respond to percolation of the soil with various nutrient solutions. *E. adhaerens* phage activity was not present in half of the soils percolated with *M. luteus* cells. This seemed to reflect too great a phage-host specificity for the technique as regards these soils, because *E. adhaerens*-like bacteria other than the strains used for plaquing were present in at least some of these soils. Although *E. adhaerens* did not attack *Escherichia coli* or *Pseudomonas aeruginosa* in soil, there was an overproduction of *E. adhaerens* phage if these bacteria were percolated simultaneously with *M. luteus* cells. The possibility is discussed that this represents an activation by *M. luteus* (or by a heat-extractable factor from it) of other bacterial predators that attack *E. coli* or *P. aeruginosa* and that these predators subsequently are themselves attacked by *E. adhaerens*.

Casida (4) has shown that *Micrococcus luteus* cells die rapidly when they are incubated in natural soil. Microscopic procedures were then used (5) to show that this death was caused by predatory bacteria. The initial attack was by a streptomycete (strain 34) that sought out the *M. luteus* cells and lysed them. This was quickly followed by multiplication of a previously unknown budding bacterium (strain A), now designated *Ensifer adhaerens* (6). *E. adhaerens* attached to the cells of both *M. luteus* and the streptomycete and lysed them. After a delay, a third bacterium multiplied in the soil, and it could also lyse *M. luteus*. It was a myxobacter, probably a *Myxococcus* species. The three predatory bacteria were isolated. As pure cultures on laboratory media, they sought out and destroyed *M. luteus* cells, but they were not obligate predators. This ability to grow in the absence of host cells made it difficult to define the breadth of potential host organisms for the predators. Thus, it was difficult to determine the precise environmental conditions, including background nutritive conditions (6), that turned on predation. Obviously, the precise conditions that were present in the soil were not known and hence could not be duplicated in the laboratory. For example, it was found that on laboratory media, *E. adhaerens* could attach to the streptomycete predator but did not lyse it. *E. adhaerens* also attached to *Escherichia coli* without lysing it. Finally, *E. adhaerens*, at least on laboratory media, was destroyed by the myxobacter predator.

Based on the above, it was apparent that a method was needed that would allow the interactions of the predatory bacteria and their host

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cells to be followed in situ in natural soil. The procedure would have to have a high degree of specificity. It seemed that in soil, one response that could be used in such a procedure was the natural production of bacteriophage for predatory bacteria during multiplication of the predatory bacteria as they attack their host cells. The phage provided the specificity, they responded only to multiplying cells, and they could be recovered from incubating soil for enumeration.

The object of this study was to use the natural production of bacteriophage in soil against naturally occurring predatory bacteria to follow the predators' activities as they responded to the presence of various potential host bacteria. *E. adhaerens* was chosen as the main predator for study because of its previously shown ability, at least in soil, to attack both added host cells (e.g., *M. luteus*) and the naturally occurring streptomycete predator that responded to *M. luteus* cells.

MATERIALS AND METHODS

Microorganisms and media. The microorganisms and media used in this study are listed in Table 1. The organisms were maintained on agar slants (Table 1) at 5°C. Unless otherwise indicated, growth of cells took place in shaken broth cultures in 30 to 50 ml of the appropriate growth medium (Table 1) at 25 to 27°C. All media components were from Difco Laboratories, Detroit, Mich.

Soils. A description and the characteristics of the soils used in this study are listed in Table 2. Each soil was stored at room temperature in a large polyethylene bag to prevent drying but still allow gaseous exchange with the atmosphere. However, soils 3 and 4 dried out during storage.

Bacteriophage monitoring and isolation. Bacteriophage for *E. adhaerens* were isolated from soil by three different techniques. For the classic phage isolation technique of Adams (1), 30 to 50 ml of nutrient broth (NB) was inoculated with 1 ml of a 24- to 48-h NB culture of *M. luteus* or *E. adhaerens* strains A, SA, or 7A, and 1 g of a sieved (1 mm) soil sample was added. This was shaken at 27°C. At daily intervals, 5 to 10 ml of sample was withdrawn and centrifuged at 480 × *g* for 5 min to remove soil debris. The supernatant was filtered through a 0.3-μm membrane filter (Millipore Corp., Bedford, Mass.) and then assayed for the presence (or absence) of phage (see below).

The incubated soil bottle technique involved incubating 10 g of sieved soil with or without added cells of strains A, SA, or 7A and with or without added nutrient amendments. The soil was adjusted to 50 to 60% of moisture-holding capacity and was in sterile 1-oz. (ca. 29.6 ml) screw-cap bottles. Incubation was at 27°C. When used, the nutrient amendment for the bacterial predator *E. adhaerens* was an appropriate host (e.g., *M. luteus*) or, unless stated otherwise, nutrient broth at 0.8 mg/g of soil. Bottles were withdrawn at various time intervals, and the soil from each bottle was washed into a 90-ml sterile tap water dilution blank (i.e., 1:10 dilution), shaken for 15 to 20 s, and allowed to sit for 10 s. Samples (5 to 10 ml each) were withdrawn from approximately the center of the dilution bottle and centrifuged for 5 min at 480 × *g*. The supernatant fluid was filtered through a 0.3-μm membrane filter then assayed for phage.

The third phage technique was the soil-percolation phage-enrichment procedure of Germida and Casida (8). This procedure involves the percolation of 30 to 40 ml of water or nutrient amendment (either a suspension of the predator's host cells or a nutrient solution)

TABLE 1. Microorganisms and media used

Microorganism	Medium[a]		Reference
	Maintenance	Growth	
Arthrobacter globiformis ATCC 8010	GYENA	NB	7, 8
Arthrobacter soil isolate SPI-1	GYENA	NB	7, 8
Arthrobacter soil isolate GSI-5	GYENA	NB	7, 8
Azotobacter vinelandii ATCC 12837	BA	B	
B. subtilis PSU 46a	NA	NB	
E. coli PSU 106	NA	NB	9, 10
E. adhaerens ATCC 33212 (strain A)	1/10 HIA	NB	5, 6
E. adhaerens SA	1/10 HIA	NB	5, 6
E. adhaerens ATCC 33499 (strain 7A)	1/10 HIA	NB	5, 6
M. luteus	1/10 HIA	NB	4–6
Myxobacter soil isolate strain 8	CMA	CM	5
Streptomycete soil isolate strain 34	1/10 HIA	NB	5
P. aeruginosa PSU 191	NA	NB	
R. leguminosarum PSU 201	YEMA	TY	14
R. meliloti PSU 204	YEMA	TY	14
R. meliloti NRG 185	YEMA	YEM	14

[a] GYENA, 0.1% glucose, 0.1% yeast extract, 0.8% NB, 1.5% agar; NB, 0.8% nutrient broth; NA, NB + 1.5% agar; B, 2.0% sucrose, 0.008% K_2HPO_4, 0.02% KH_2PO_4, 0.04% $MgSO_4 \cdot 7H_2O$, 0.03% $CaCl_2$, 0.01% $Na_2MoO_4 \cdot 7H_2O$, 0.07% $FeSO_4 \cdot 7H_2O$; BA, B + 1.5% agar; 1/10 HIA, 1/10 strength heart infusion broth + 1.5% agar; CM, 1.0% casitone, 0.2% $MgSO_4 \cdot 7H_2O$; CMA, CM + 1.5% agar; YEM, 0.05% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.01% NaCl, 1% mannitol, 0.04% yeast extract; YEMA, YEM + 1.5% agar; TY, 0.5% tryptose, 0.3% yeast extract, 7 mM $CaCl_2$.

TABLE 2. Soils

Soil no.	Soil name	Location	Description	pH[a]	% Moisture-holding capacity[b]
1	Hagerstown silty clay loam	State College, Pa.	Grass field	6.1	30
2	Cazenovia silt loam	Auburn, N.Y.	Garden	7.0	29
3	Dresden silt loam	Evansville, Wis.	Garden	6.7	3
4	Webster silty clay loam	Monona, Iowa	Corn field	5.6	4
5	Hagerstown silty clay loam	State College, Pa.	Grass field	5.3	50
6	Dresden silt loam	Evansville, Wis.	Under pine tree	5.7	ND
7	Dresden silt loam	Evansville, Wis.	Garden	ND[c]	ND
8	Tuxford clay loam (dark brown)[d]	Weyburn, Sask.	Summerfallow	7.2	30
9	Yorkton loam (deep black)	Watson, Sask.	Summerfallow	6.9	29
10	Waitville loam (grey)	Glaslyn, Sask.	Summerfallow	6.7	24
11	Elstow loam (dark brown)	Harris, Sask.	Summerfallow	6.8	18
12	Oxbow loam (black)	Fenwood, Sask.	Summerfallow	7.8	18
13	Oxbow loam (black)	Whitewood, Sask.	Summerfallow	7.8	29
14	Melfort silty clay loam (black)	Melfort, Sask.	Summerfallow	6.9	17
15	Waitville (grey)-Whitewood loam-clay loam (dark grey)	Leoville, Sask.	Summerfallow	7.0	20
16	Whitewood loam (grey black)	Shell Lake, Sask.	Summerfallow	7.4	27

[a] Soil pH was determined on a 1:20 soil-distilled water suspension equilibrated for 20 min at room temperature.
[b] Soil moisture as a percent of moisture-holding capacity.
[c] Not determined.
[d] Zonal designation used in Saskatchewan.

through a soil sample. To increase aeration and percolation rate (maintained at about 3 to 5 ml of fluid per min), 25 g of sieved soil and 25 g of sand were thoroughly mixed and placed in a soil percolation column (8). The plastic membrane support of a Nuclepore filter apparatus was used instead of a glass wool plug to support the soil-sand mixture. Nutrient solutions or water were then percolated over the soil-sand mixture. When washed host cells (washed three times with distilled water) were percolated, 10-ml portions of pure or mixed species suspensions were poured over about one-third of the soil-sand mixture, and then another one-third of soil-sand was layered, followed by 10 ml of additional cells. This procedure was repeated until the entire soil sample had been "saturated" with host cells.

At various time intervals (usually 24 h), samples of percolation fluid were withdrawn from the soil column, passed through a sterile 0.3-μm membrane filter (Millipore), and assayed for the presence of phage. Unless otherwise stated, the phage assay (8) consisted of a nutrient agar (1.5%) basal layer and soft nutrient agar (0.75%) overlay. All results represent duplicate or triplicate assays of duplicate samples. Bacteriophage selected for study were purified, and concentrated phage suspensions were prepared as described previously (8).

Phage typing and morphology. The lytic spectra of the phage isolates were determined by the spot plate method (1) as described previously (8). A soft nutrient agar (0.75%) overlay was seeded with 1 ml of a 24- to 48-h NB culture of the test bacterium and applied to a nutrient agar (1.5%) basal layer. This culture plate was then spotted with 0.05 ml of a freshly prepared phage lysate (approximately 10^8 to 10^{10} PFU/ml). The lytic reactions were recorded after incubation at 27°C for 24 to 48 h. The morphology of each phage isolate was determined by transmission electron microscopy for negatively stained preparations (1% uranyl acetate; 1 min).

Isolation and counting of other bacterial predators. For some soil samples, the unfiltered percolation fluid was analyzed for total bacteria, *E. adhaerens*-like bacteria, protozoa, and other bacterial predators. Total bacteria were counted (10) on total plate count agar. Protozoa were detected by direct plating with double-layer plates (9) and verified by microscopic observation. *E. adhaerens*-like bacteria were isolated in two ways. The first procedure involved plating dilutions of unfiltered percolation fluid from an *M. luteus*-treated soil column on MacConkey agar. Based on the growth patterns of known *E. adhaerens* strains, slimy, transparent, pinkish (due to medium) or slightly white tinted colonies were picked and purified by streaking. The second procedure involved spreading dilutions of this same (or other) percolation fluid over the surfaces of fully developed *M. luteus* lawns grown on heart infusion agar made up at 1/10 strength (5). After suitable incubation, suspected *E. adhaerens* colonies (5) were picked and purified. *E. adhaerens*-like isolates were confirmed by activity against *M. luteus* cells (5), phage typing, and cell morphology. The spreading of percolation fluids over *M. luteus* lawns also allowed enumeration and isolation of other lytic microorganisms.

Heat-killed cells and extracts. *E. coli* or *M. luteus* cells were washed three times, suspended in 30 to 40 ml of distilled water, and autoclaved for 15 min. This was used as is, or it was centrifuged. The supernatant fluid (the hot water extract) was passed through a 0.3-μm membrane filter (Millipore) before use. In some cases, the cell pellet described above received two additional washings with cool distilled water before final suspension in distilled water.

Broth culture interactions. *E. coli*, *M. luteus*, and *E. adhaerens* strains A and 7A inocula were grown as

TABLE 3. Production in percolated soils of phage producing plaques on various *E. adhaerens* strains

Soil no.[a]	Percolation fluid (PFU/ml)[b]		
	Strain A	Strain SA	Strain 7A
1	1.8×10^4	1.2×10^4	2
2	5.0×10^4	5.0×10^4	2.9×10^4
3	3.7×10^3	4.0×10^3	1.2×10^3
4	11	11	0
5	2	0	8
8	9	5	1
9	0	0	1.9×10^2
10	3	2	2

[a] Soils were percolated with 30 to 40 ml of a washed *M. luteus* cell suspension in water.

[b] Represents highest average titer observed during 7-day percolation period.

shaken cultures at 27°C in nutrient broth. Erlenmeyer flasks (500 ml) with Klett sidearms, containing 40 ml each of heart infusion broth made up at 1/10 strength, received 0.1 ml of inoculum for each organism added. Extract of heat-killed *M. luteus* or *E. coli* cells was added at 0.5 ml. The flasks were shaken at 27°C, and periodic observations for turbidity were made.

RESULTS

***M. luteus* host cells.** Except with soil no. 8, the classic soil enrichment technique for phage and the incubated soil bottle technique yielded few or no phage active against *E. adhaerens* strains A, SA, or 7A when soils were incubated with these strains or with *M. luteus*. With the soil enrichment technique, soil no. 8 with added strain SA cells yielded 0, 3.4×10^3, and 7.6×10^3 PFU/ml for strains A, SA, and 7A, respectively. As a check on the efficiency of the soil bottle technique, 10% of *E. adhaerens* phage added to soil at 5×10^2 to 5×10^4 PFU/g could be recovered immediately. Recovery was 2.5% when 10^8 PFU/g was added. The percent phage recovered decreased during incubation and with increased numbers of original phage added. Thus, the recovery for 5×10^2 PFU/g added initially was 3% at 14 days. For 10^8 PFU/g, the recovery was 0.05%.

The soil percolation technique yielded *E. adhaerens* phage for several soils. Sixteen different soil samples (Table 2) were percolated with an aqueous suspension of washed *M. luteus* cells (approximately 1.0×10^8 cells per ml). Half of these soils produced phage that could be detected in the percolate (Table 3), although the titers for three of the soils (no. 1 to 3) were considerably greater than for the others. For soil no. 1 (the only one tested), a 5-fold decrease in the numbers of *M. luteus* cells caused a 100-fold decrease in the numbers of strain A and SA (7A was not tested) phage detected in the percolates. Interestingly, soil no. 4 yielded phage, even though it had been stored for 11 years and was quite dry. Also, soil no. 6 contained no detectable phage in the percolate, although previously (4) it was shown to contain *E. adhaerens*-like cells during the die-out of *M. luteus* cells in incubated soil bottles.

Figure 1 shows the time sequence of *E. adhaerens* phage production for soil samples 1, 2, and 3 during percolation with *M. luteus* cells or water. Several of the *E. adhaerens*-like bacteria in these percolates were isolated and shown by phage typing to be related to the *E. adhaerens* type strain ATCC 33212 (Table 4). During these percolations with *M. luteus*, phage were also produced that were active against an *Arthrobacter* sp. soil isolate GSI-5 (8). As noted later, phage for this bacterium were also produced in response to percolation of the soil with soluble nutrients. Tests were not made for phage against other bacterial species. The maximum phage titers (in PFU per ml) against GSI-5 for soil percolated with *M. luteus* cells or water, respectively, were 10^4 and 0.0 for soil no. 1, 13 and 0.0 for soil no. 2, and 10^3 and 0.0 for soil no. 3.

Percolation of soil no. 1 with nutrient solution instead of *M. luteus* cells yielded few or no phage for *E. adhaerens* A, SA, and 7A. For example, percolation with full-strength heart infusion broth or cation-complete medium (7) con-

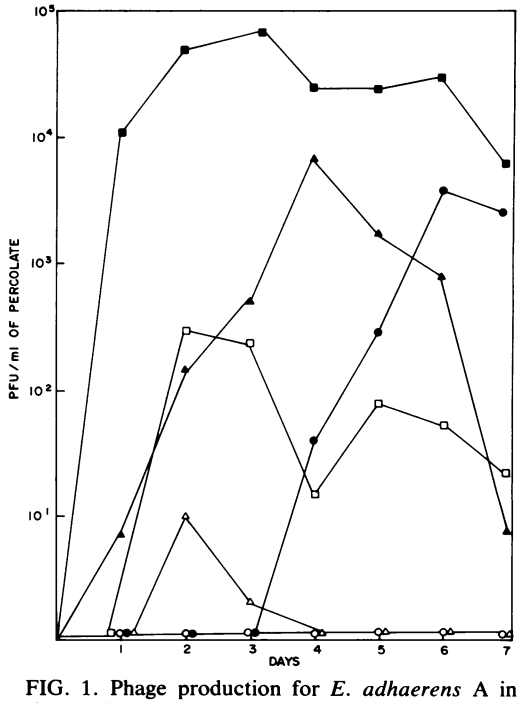

FIG. 1. Phage production for *E. adhaerens* A in various soils percolated with washed *M. luteus* cells (closed symbols) or water (open symbols). Circles, soil no. 1; squares, soil no. 2; triangles, soil no. 3.

TABLE 4. Lytic spectra of 13 *E. adhaerens* bacteriophage isolates typed against various *E. adhaerens* strains

Phage isolate[a]	Lytic reaction with *E. adhaerens* host strain[b]								
	A	SA	7A	DS-2	ML-D	ML-B	ML-C	ML-F	DS-1
A-11/ML1	++++	++++	++++	++++	++++	+	+	++	−
A-10/ML2	++++	++++	++++	++++	++++	+	−	−	−
A-9/34/2	++++	++++	++++	++++	++++	−	−	−	−
SA-8/34/1	++	++	++	++	++	−	+	−	−
SA-7/ML/2	++++	++++	++++	++++	++++	+	++	−	+
SA-6/34/2	++++	++++	++++	++++	++++	−	−	−	−
7A-5/ML/1	++++	++++	++++	++++	++++	−	−	−	−
7A-4/34/1	++++	++++	++++	++++	++++	−	−	−	−
7A-3/34/1	+	++	++++	+	+	+	+	+	+
7A-2/ML/2	++++	++++	++++	++++	++++	−	−	+	−
7A-1/34/2	++++	++++	++++	++++	++++	−	−	−	−
DS-1/ML/3	−	−	−	−	−	−	−	−	++++
DS-2/ML/1	+	+	−	++++	+	++	−	−	−

[a] Phage isolates were coded according to the original host on which the phage was isolated, the host cells used to percolate soil for the phage, and the soil sample. For example, 7A-1/34/2 = isolate no. 1 of phage isolated on strain 7A from soil no. 2 percolated with strain 34 cells.

[b] *E. adhaerens* A, SA, and 7A have been described; strains DS-1 and DS-2 were picked from colonies on MacConkey agar plates and strains ML-B, ML-C, ML-D, and ML-F from colonies on *M. luteus* lawns whose surface had been spread with percolates of soil treated with *M. luteus* cells. ++++, Confluent lysis on the propagating strain within 24 h; ++, lysis, but not confluent, within 24 h; +, faint lysis within 24 to 48 h; −, no lysis.

taining 1% glucose yielded a maximum titer of 1 to 3 PFU/ml. No *E. adhaerens* phage were detected for percolations with nutrient broth or with heart infusion broth made up at 1/10 strength. In contrast, the above percolations produced maximum phage titers of 10^2 to 10^4 PFU/ml active against *Arthrobacter* species GSI-5. Percolation of soil no. 8 instead of no. 1 with nutrient broth yielded from 1 to 200 PFU/ml against the *E. adhaerens* strains.

Other species as host. Species of bacteria other than *M. luteus*, when percolated with soil no. 1, stimulated phage production for *E. adhaerens* (Table 5). Strain 34 is the *Streptomyces* species predator, and strain 8 is the myxobacter predator of *M. luteus* described by Casida (5). *E. adhaerens* and strain 34 in these percolates and in percolates of other soils could be detected by plating after 48 h. To verify the results, a strain 34-like streptomycete from the counting plates for percolation of soil no. 8 was added to fresh soil no. 8 for percolation; it elicited an *E. adhaerens* phage response as described above. Also, on laboratory media, it lysed *M. luteus*. For all of the host bacteria listed in Table 5, *E. adhaerens* phage were initially detected between 1 and 4 days. Usually, the phage production stimulated by strains 8 and 34 preceded by 2 to 3 days that caused by *M. luteus* cells. In these trials, the percolates were plated on the various *E. adhaerens* strains. When the same percolates were plated on the respective bacterial species that were actually percolated in the soil, no phage were detected.

Percolation of *E. adhaerens* A cells in soil no. 1 (no hosts or other bacteria added) did not

TABLE 5. Production in soil no. 1, percolated with various host cells, of bacteriophage producing plaques on various *E. adhaerens* strains[a]

Host cells	Percolate (PFU/ml)[b]		
	Strain A	Strain SA	Strain 7A
M. luteus	1.8×10^4	1.2×10^4	2
Strain 34[c]	1.6×10^2	1.4×10^2	1.4×10^2
Strain 8[c]	1.1×10^3	1.2×10^3	0
Arthrobacter globiformis 8010	0	2	0
Arthrobacter sp. strain SPI-1	6	9	0
Arthrobacter sp. strain GSI-5	2	3	0

[a] Soils were percolated with 40 ml of washed host cells.

[b] Represents the average highest titer observed during 7-day percolation period; no titer was produced with water-only percolates. Phage titer was determined on lawns of *E. adhaerens* A, SA, or 7A.

[c] Added as vegetative cells.

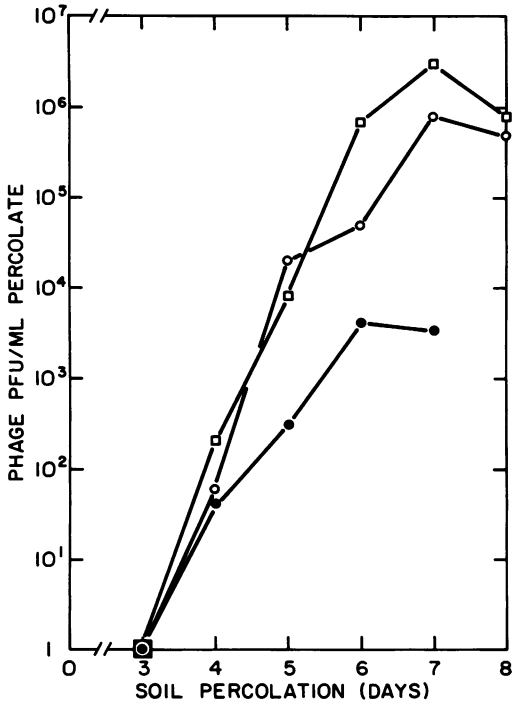

FIG. 2. Phage production for *E. adhaerens* A, SA, and 7A in soil no. 1 percolated with mixed cultures of *M. luteus* and *E. coli*. Closed circles, soil percolated with only *M. luteus* and plated on strains A or SA; open circles, soil percolated with a mixture of *M. luteus* and *E. coli* and plated on strains A or SA; open squares, soil percolated with a mixture of *M. luteus* and *E. coli* and plated on strain 7A. There was virtually no phage for strain 7A produced during percolation with a monoculture of *M. luteus*. Values plotted are averages of means of two experiments. Average percentage variation of means plotted is 30%.

cause production of phage plaquing on *E. adhaerens* A, SA, or 7A. *E. coli*, *Azotobacter vinelandii*, *Rhizobium meliloti* (PSU 204), *Rhizobium leguminosarum*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, strain 8 myxospores, and two unidentified soil isolates (a gram-negative rod and a gram-positive coccoid form), when percolated in soil no. 1, also failed to cause phage production against *E. adhaerens* A, SA, or 7A. In addition, no *E. adhaerens* phage were detected in percolates from soil no. 8 treated with *R. meliloti* NRG 185 cells.

The effect of percolating a mixture of *E. coli* and *M. luteus* cells on *E. adhaerens* phage production in soil no. 1 is shown in Fig. 2. Mixing of *P. aeruginosa* with *M. luteus* cells gave approximately the same results as the *E. coli-M. luteus* mixture as regards phage production for *E. adhaerens* A and SA, but only a delayed production on strain 7A (4×10^2 PFU/ml at 7 days and 9×10 at 8 days). For the mixture of *E. coli* (or *P. aeruginosa*) and *M. luteus* cells, as compared to *M. luteus* alone, there was a 100-fold increase in phage for strains of *E. adhaerens*, although, as noted previously, phage were not produced when only *E. coli* was percolated in soil. During the first 4 days, before the phage buildup, protozoan numbers increased (Table 6) but then decreased dramatically during the phage buildup for *E. adhaerens*. The increase in protozoan numbers occurred more quickly when *E. coli* and *M. luteus* were both present than with *E. coli* alone, but the eventual total numbers of protozoa were the same. The numbers of *E. coli* decreased concurrently with the initiation of the phage buildup. Filtrates of samples put through 0.5-μm pore size membrane filters did not produce *Bdellbovibrio* species plaques on *E. coli* lawns.

Heat-killed cells. Percolation in soil no. 1 of an autoclaved suspension of prewashed *M. luteus* cells caused production of *E. adhaerens* phage (about 5×10^2 PFU/ml each for strains A, SA, and 7A). Although this was about 100-fold less than for percolation with live cells, the phage production was detected about 2 days earlier than with the live cells. Separate experiments

TABLE 6. Numbers of protozoa and *E. coli* in soil no. 1 percolates

Percolation (days)	Soil percolated with *E. coli*		Soil percolated with *E. coli* plus *M. luteus*	
	E. coli/ml[a]	Protozoa/ml[b]	*E. coli*/ml[a]	Protozoa/ml[b]
0	1.4×10^9	ND[c]	1.4×10^9	ND
1	1.5×10^9	ND	1.6×10^9	ND
2	8.2×10^8	20	3.9×10^8	80
3	4.7×10^8	11	3.5×10^8	320
4	8.4×10^6	300	5.6×10^5	0
5	4.1×10^5	130	1.9×10^4	10
6	2.3×10^5	10	7.7×10^4	110
7	1.1×10^5	0	3×10^4	0

[a] Determined by plate counts on MacConkey agar (10).
[b] Determined by unfiltered percolates on *E. coli* as the host cells.
[c] ND, Not determined.

showed that the phage response was due to a heat-extractable material in the *M. luteus* cells. For example, the suspension water in which the prewashed *M. luteus* cells had been autoclaved, but not these same dead cells after they had been washed further, stimulated *E. adhaerens* phage production as described above. In contrast, percolation of soil with autoclaved suspensions of prewashed cells of strain 34 and several *Arthrobacter* species (including isolate GSI-5) in the coccoid growth stage did not to any extent stimulate phage production for any of the *E. adhaerens* strains.

In broth growth experiments with heart infusion broth at 1/10 strength in the absence of soil, the hot water extract of *M. luteus* cells caused a slight increase (1.4-fold) in the growth rate for *E. adhaerens* A (but not for strain 7A) when it was the only organism present. A similar extract of *E. coli* had no effect. Initial inoculation of broth with *E. adhaerens* (A or 7A) plus *E. coli* showed no effect of *E. adhaerens* on *E. coli* in the presence or absence of either extract or with both extracts present simultaneously, and the above response of *E. adhaerens* to *M. luteus* extract did nor occur. In fact, the growth of *E. adhaerens* was poor, probably because rapid initial growth of *E. coli* removed available nutrients.

Cross streaks (5) of *E. adhaerens* (A or 7A) in various combinations with *E. coli* and *M. luteus* on heart infusion agar made up at 1/10 strength did not show a growth response of *E. adhaerens* to *E. coli* or destruction of *E. coli* by *E. adhaerens*.

Phage specificity. During the course of these experiments, six additional strains of *E. adhaerens* were isolated (Table 4). In addition, 13 strains of *E. adhaerens* phage (Table 4) were isolated from soils no. 1, 2, and 3 percolated with either *M. luteus* or *Streptomyces* sp. strain 34. The host ranges of the phage strains on all of the *E. adhaerens* strains (old and new) are shown in Table 4.

Transmission electron microscopy of eight of the phage isolates placed five of them in Bradley's group B (2) and the rest in group C. Representative morphologies are shown in Fig. 3.

DISCUSSION

Populations of *E. adhaerens* bacteriophage developed in some of the soils that were being

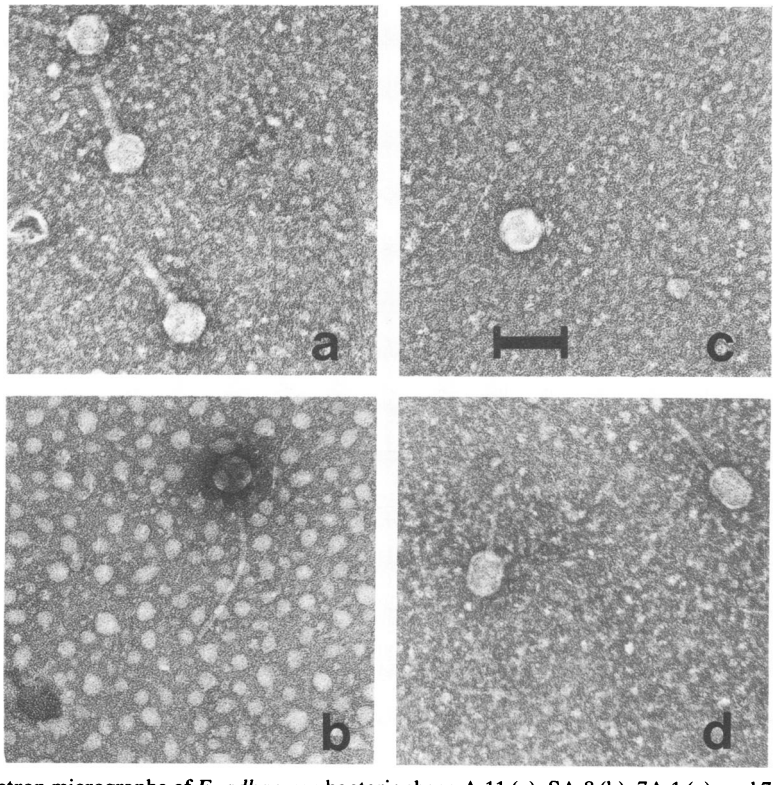

FIG. 3. Electron micrographs of *E. adhaerens* bacteriophage A-11 (a), SA-8 (b), 7A-1 (c), and 7A-2 (d). Phage 7A-1 belongs to Bradley's group C; the others belong to group B. Bar, 100 nm.

percolated with a suspension of washed cells of *M. luteus* or *Steptomyces* sp. strain 34. *E. adhaerens* cells were not added to these soils. Percolation of soil with added *E. adhaerens* cells, but without added *M. luteus* or strain 34 cells, did not yield *E. adhaerens* bacteriophage. Finally, percolations of soil with various nutrient solutions, without adding cells of any species, did not yield *E. adhaerens* phage. It would appear, therefore, that in soil the indigenous population of *E. adhaerens* multiplied in response to the presence of host cells (*M. luteus* or strain 34) but did not multiply in response to other sources of nutrients. These results are in agreement with the observations of Casida (4, 5) that *M. luteus* cells added to soil (our soil no. 1) die out rapidly, and that this death is due mainly to attack by *E. adhaerens* (and strain 34). Casida also noted that, at least in soil, *E. adhaerens* could attack strain 34, which explains the *E. adhaerens* phage production when we percolated strain 34 in soil.

A myxobacter predator was also mentioned by Casida (5) as multiplying belatedly in soil to which *M. luteus* cells had been added. On laboratory media, *E. adhaerens* succumbed to attack by this myxobacter. In soil, however, the picture apparently was reversed, with phage for *E. adhaerens* building up when the soil was percolated with the vegetative cells, but not the myxospores, of the myxobacter.

The time required for appearance of *E. adhaerens* phage in soil also agrees with the above conclusions. Thus, phage for *E. adhaerens* appeared 1 to 2 days earlier when the soil was percolated with strain 34 or the myxobacter than when soil was percolated with *M. luteus*. That is, the multiplication of *E. adhaerens* responded quickly to the presence of strain 34 and the myxobacter, using them as host cells, and did not have to wait while strain 34 was destroying *M. luteus* (decreasing the available host cells) and simultaneously multiplying its own mycelium (to provide additional host cells). A further proof of the above conclusions would be had if phage production in soil for strain 34 and the myxobacter could be followed. However, we have not yet detected any phage for these organisms in these soils.

The *E. adhaerens* phage production in response to *M. luteus* over a 7-day period in soils no. 1 and 3 was as would be predicted from the studies of Casida (5). Thus, soil no. 1 was stated to have all three of the predators operating in sequence, with *E. adhaerens* appearing after strain 34. This was evidenced in the present study by the occurrence of a 3-day lag period before phage production. The Casida study also pointed out that, for soil no. 3, *E. adhaerens* was responsible for the initial and main attack on *M. luteus*. This was shown in the present study by the rapid and steady rise in *E. adhaerens* phage. The rapid production of *E. adhaerens* phage in soil no. 2 percolated with water or host cells was not expected, however. This soil was an organically rich garden soil that had become quite dry during storage. Therefore, release of soil nutrients on rewetting of the soil (11) might be an explanation. However, this soil is known to contain another, as yet undescribed, bacterial predator of *M. luteus*, and it could affect the results in the manner discussed later for percolations of soil with *E. coli* plus *M. luteus*.

Half of the soils studied did not produce any *E. adhaerens* phage when percolated with *M. luteus* cells. This could mean that *E. adhaerens* was not present in these soils. However, it could also mean that *E. adhaerens* was present but that it was just different enough from the *E. adhaerens* strains used for plaquing that its phage would not plaque. Also, these soils might harbor another as yet unisolated species of *Ensifer*. For example, phage DS-1/ML/3 for *E. adhaerens*-like isolate DS-1 did not cross-react with strains A, SA, or 7A, and phage did not develop in soil no. 6, a soil known to contain *E. adhaerens*-like bacteria. Obviously, another explanation would be that recovery of phage from some soils may be difficult. Phage are known to adsorb to clay surfaces (3, 13) and are affected by soil pH (12). However, these factors did not seem to correlate with the characteristics for the soils not yielding *E. adhaerens* phage. We did find, however, that with the soil bottle technique, only 10% or less of *E. adhaerens* phage PFU added to soil could be recovered.

Percolation of soil with various *Arthrobacter* species and several other bacteria, including *E. coli* and *P. aeruginosa*, did not yield phage for *E. adhaerens*. Thus, these bacteria appear not to serve as hosts for *E. adhaerens* in soil. At least for *E. coli*, this is in agreement with Casida's original statement (5) that, for pure cultures viewed by microscopy, *E. adhaerens* could attach to *E. coli* cells but apparently could not lyse them. Cross streak experiments (5; this study), as well as broth interaction studies, showed no stimulation of *E. adhaerens* by *E. coli* or destruction of *E. coli* by *E. adhaerens*. Streaks of pure cultures of *E. adhaerens* through lawns of heat-killed *M. luteus* cells (5) showed no attack or extra *E. adhaerens* growth. Also, direct microscopy of soil to which heat-killed *M. luteus* had been added (no other host cells added) did not show development of the strain 34-*E. adhaerens* predation sequence. Finally, percolation of soil with nutrient solutions (host cells not added) did not elicit a phage response for *E. adhaerens*. Regardless of the above, however, in the present study, percolations of soil with *E.*

coli or *P. aeruginosa* produced *E. adhaerens* phage in excess if *M. luteus* was simultaneously percolated in the soil. An initial decrease in *E. coli* numbers in the soil seemed to be associated with an increase in protozoan numbers. However, this was followed by a rapid decrease in protozoan numbers to nondetectable levels plus the increase in *E. adhaerens* phage numbers noted above. There was an additional 10-fold decrease in the *E. coli* numbers during this time. It might be concluded, therefore, that *E. adhaerens* can attack *E. coli* (or *P. aeruginosa*) if *M. luteus* is also present and that protozoa are not involved in this attack.

Based on pure culture studies, it appeared that *M. luteus*, or its heat-extractable factor(s), did not directly mediate this attack. The effect of *M. luteus*, however, might be indirect by allowing the action of some other bacteiral predator(s) on *E. coli*, with this predator in turn being attacked by *E. adhaerens*. Unless the other predator was dormant, however, its activity on *E. coli*, followed by *E. adhaerens* activity on it, would not be easily detectable, because the activity would show up as a component of the *E. adhaerens* phage response whether or not *M. luteus* or its factor(s) were present. If, however, the other predator was dormant, with dormancy being broken by the *M. luteus* factor(s), then the results discussed above would be obtained. A possible example of the other predator might be myxobacter, as mentioned in this study. It is susceptible to *E. adhaerens* attack in soil and, based on studies not discussed here, produces myxospores in soil which do not germinate easily but do germinate if *M. luteus* is present. Although its myxospores do not germinate in response to *E. coli*, its vegetative cells can attack *E. coli*. Obviously, however, the other predator(s) might not be a myxobacter but instead a predator whose existence is still unknown.

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