Applied and Environmental Microbiology, Oct. 1986, p. 819–823 Vol. 52, No. 4
0099-2240/86/100819-05$02.00/0
Copyright © 1986, American Society for Microbiology

# Gram-Negative Versus Gram-Positive (Actinomycete) Nonobligate Bacterial Predators of Bacteria in Soil†

LAWRENCE R. ZEPH‡ AND L. E. CASIDA, JR.*

*Microbiology Program, The Pennsylvania State University, University Park, Pennsylvania 16802*

Received 5 May 1986/Accepted 14 July 1986

**The existence of nonobligate bacterial predators of bacteria in soil has been previously reported. Several additional predators were isolated from soil and tested for predation in situ in soil by use of the indirect bacteriophage analysis technique. The trials were conducted with nutritionally poor and nutritionally enriched soil. Certain of the predators that were gram negative were found to attack a range of both gram-positive and gram-negative host cell species, including at least some of the other predator bacteria, both gram positive and gram negative. The attack occurred in both the nutritionally poor and rich soils, but in some instances it was somewhat depressed in the rich soil. This may be due to the nonobligate nature of the predation. The gram-positive predators attacked a relatively narrow range of prey species, and the attack occurred only in the nutritionally rich soil. In addition, the gram-positive predators were subject to attack by certain of the gram-negative predators. These gram-negative predators therefore appeared to play a dominant role in the control of bacterial numbers in soil.**

Several bacterial predators of bacteria have been isolated from soil and studied (1–6, 8). They were nonobligate predators and grew on laboratory media in the absence of prey cells if a moderately nutritive medium, such as heart infusion agar made up at 0.1× or full strength, was used. The predator bacteria included both gram-negative and gram-positive (actinomycete) forms.

Instances of bacterial predator attack on bacterial predators in soil have been recorded (1, 3, 4). In addition, an attack-counterattack phenomenon for two bacterial predators was noted (1). Therefore, certain of these bacterial predators, by attacking other predators, seemed to be able to tie together food chains dominated by the individual predators.

As noted above, the predators were either gram positive or gram negative and were nonobligate. Therefore, the objectives of this study were twofold. One objective was to examine the effect of the nutritional level of the soil on nonobligate bacterial predation by both previously known predators and newly isolated ones. Preincubation with dried, ground alfalfa was used to raise the nutritional level of the soil. The other objective was to compare the predation activities, including prey cell ranges, of the gram-positive and gram-negative predators, both in the presence and absence of nutritionally preenriched soil. The indirect bacteriophage analysis technique (7) allows the monitoring of bacterial predation as it occurs naturally in situ in soil. This technique, with slight modification, was used for our study.

## MATERIALS AND METHODS

**Microorganisms and media.** The microorganisms and media used in this study are listed in Table 1. Preparations of bacteria to be used as prey were grown in 40 ml of the designated medium in 300-ml baffle-bottom flasks at 27°C on a reciprocal shaker. The cells were washed three times, suspended in 25 ml of sterile distilled water, and then added to the soil percolators to provide between $10^7$ and $10^9$ cells per g of soil-sand (1:1) mixture in the percolator (total amount of soil plus sand, 25 g). The actual number of cells depended on the number of cells that could grow in the media in the prescribed period listed in Table 1. For example, per gram of soil-sand, $10^7$ *Agromyces ramosus* cells were present, $10^8$ N-1 and *Azotobacter vinelandii* cells were present, and $7 \times 10^8$ *Escherichia coli* cells were present. These relatively high prey cell numbers were necessary to ensure that prey cells would be in the vicinity of the individual indigenous predator cells in the soil. In addition, it was necessary to have enough prey cells present in the soil for all of the indigenous soil predators that might attack the added prey cell species. This activity by other soil predators did not affect the evaluation of the activity of the predator of interest, because the evaluation depended on the high degree of specificity of the phage-host interaction. Suspensions of predator cells for plaque assay lawns were prepared as above, except that cell washing was not necessary. Heat-killed prey cell suspensions were prepared by autoclaving washed prey cells for 20 min. The cells were then washed once more after being autoclaved and suspended in 25 ml of distilled water. The *Ensifer adhaerens* strain used was a variant of ATCC 33212. It produced less slime during growth. Strain N-1 is ATCC 43291. All media components were obtained from Difco Laboratories, Detroit, Mich.

**Soil.** A Hagerstown silty clay loam soil, pH 6.1, from a grass field was used in these studies. The roots were removed after collection. Soil was stored at room temperature in large polyethylene bags to allow gas exchange while keeping moisture loss to a minimum. The soil was sieved through a 2-mm sieve prior to its addition to soil percolator columns. Soil nutritionally preenriched with dried, ground alfalfa was prepared as described previously (1).

**Indirect phage analysis.** The soil percolator columns and plaque assays for indirect phage analysis were as described by Byrd et al. (1). Significant bacteriophage cross-reaction between individual bacterial predator strains did not occur. This would have been evident as consistent, simultaneous

* Corresponding author.

† Paper no. 7395 in the journal series of the Pennsylvania Agriculture Experiment Station.

‡ Present address: New York University, 1009 Main Building, Washington Square, New York, NY 10003.

TABLE 1. Microorganisms and media used

| Microorganism[a] | Growth medium[b] | Time (days)[c] | Plaque assay medium[b] |
|---|---|---|---|
| *Actinomyces humiferus* ATCC 25174 | HIB | 4 | |
| *Agrobacterium tumefaciens* PSU21B | NB | 2 | |
| *Agromyces ramosus* ATCC 25173 | HIB | 3 | AR |
| *Arthrobacter globiformis* ATCC 8010 | NB | 2 | |
| *Azotobacter vinelandii* ATCC 12837 | BB | 2 | |
| *Azotobacter vinelandii* cysts | BA | 2 | |
| *Bacillus subtilis* PSU46a | 1/10HIA | 14 | |
| *Bacillus thuringiensis* | HIM | 7 | |
| *Bacillus stearothermophilus* ATCC 12980 | NYT | 7 | |
| *Ensifer adhaerens* ATCC 33212 | NB | 2 | NA |
| *Escherichia coli* PSU106 | NB | 2 | |
| *Micrococcus luteus* PSU221 | NB | 2 | |
| *Nocardia salmonicolor* ATCC 21243 | HIB | 2 | |
| *Rhizobium leguminosarum* PSU201 | HIB | 2 | |
| *Rhizobium meliloti* ATCC 10310 | HIB | 2 | |
| *Salmonella typhosa* PSU210 | NB | 2 | |
| *Staphylococcus aureus* PSU241F | NB | 2 | |
| N-1 | NB | 1 | NA |
| L-2 | NB | 2 | HIC |
| M-8 | NB | 2 | HIC |
| 5 | NB | 1 | NA |
| L-3 | NB | 2 | HIC |
| B | NA | 3 | NA |
| 34 | NB | 2 | PYE |
| W1 | NB | 2 | PYE |
| W3 | NB | 2 | PYE |
| C2 | HIB | 2 | PYE |

[a] Isolates N-1, L-2, M-8, 5, L-3, and B were gram-negative rods; isolates 34, W1, W3, and C2 were *Streptomyces* species.

[b] Abbreviations: BB, Burk broth (contained per liter, $K_2HPO_4$, 0.64 g; $KH_2PO_4$, 0.16 g; $MgSO_4 \cdot 7H_2O$, 0.20 g; $CaSO_4 \cdot 2H_2O$, 0.05 g; NaCl, 0.20 g; $NaMoO_4 \cdot 2H_2O$, 0.00025 g; and ferric citrate, 0.0018 g [pH 7.2]); BA, Burk agar; HIA, full-strength heart infusion broth; 1/10HIA, heart infusion agar with heart infusion made up at 1/10 of recommended strength; HIB, full-strength heart infusion broth; HIC, 1/10-strength heart infusion agar containing 1.0 g of $Ca(NO_3)_2$ per liter; HIM, 1/10-strength heart infusion agar with 0.01% $MnSO_4 \cdot H_2O$; AR, *Agromyces ramosus* broth (contained, per liter, peptone, 10.0 g; glycerol, 10.0 g; disodium glycerophosphate, 3.0 g [pH 6.8]); NA, nutrient agar; NB, full-strength nutrient broth; NYT, full-strength nutrient agar containing 0.1% yeast extract, 1.0% tryptone, and 0.005% $MnSO_4 \cdot 4H_2O$; PYE, peptone-yeast-extract medium [contained, per liter; peptone, 5.0 g; yeast extract, 3.0 g; $Ca(NO_3)_2 \cdot 4H_2O$, 1.0 g]. These media were for the predator bacteria. For the rest of the bacteria, the prey organisms, possible phage production in soil was monitored with nutrient agar.

[c] Incubation time for cell production.

phage increases for two (or more) predators. Careful checking of predator bacteria responses showed that no two phage increases could be correlated in this way.

## RESULTS

Various species of bacteria were added as potential prey cells to soil in soil column percolators. Water was then percolated through the soil. Both nonenriched soil and soil previously enriched by incubation with ground alfalfa were used. Attack on the added prey cells in the soil by nonobligate, indigenous bacterial predator species was monitored by the indirect phage analysis technique. Thus, the predator bacteria multiplied if they attacked susceptible prey cells, but this was followed by attack on the multiplying predator cells by specific indigenous soil bacteriophage. The increase in the number of these phage was monitored; it reflected both the attack of bacterial predator on bacterial prey and the attack of specific phage on the predator cells. This increase of phage usually occurred 1 to 3 days after percolation was started. A summary of the indigenous predator bacterium responses to various added prey species is presented in Table 2, in which no differentiation is made between results for nonenriched and preenriched soil. It should be noted that some of the predator bacteria, in addition to being predators, served as prey for some of the other predator bacteria.

For each of the predator bacteria in Table 2, an arbitrary judgment was made of what constituted a positive predator response, i.e., the minimum number of phage produced per milliliter of percolate that would be accepted as being representative of an increase in phage titer. In most cases, the numbers of phage already present in the soil were quite low and did not present a problem. If these phage were present, their numbers were subtracted. The minimum acceptable phage titer representing a positive response for the gram-positive actinomycete predators was lower than for the gram-negative predators. For the work shown in Table 2, 200 PFU per ml of percolate was accepted as the minimum response for the actinomycete predators, and 1,000 PFU per ml was considered to represent extensive multiplication of these predators. Typically, the response was several hundred to several thousand phage per ml of percolate (see Table 3). For the work shown in Table 2, 1,000 PFU per ml of percolate was used as the minimum acceptable response for the gram-negative predators. These bacteria were capable of producing phage in excess of $10^4$ PFU per ml of percolate if they were attacking the proper prey bacteria.

As a control in these experiments, percolation of soil containing added heat-killed prey bacteria did not cause more than slight predator multiplication. Less than 100 PFU per ml of percolate for predator-specific phage were produced under these conditions. Distilled water percolation of the soil, with no prey cell amendments, did not result in increases in phage titers. Thus, the predator bacteria were not growing in the absence of prey.

Titers of phage specific for the indigenous predator bacteria were used to detect attacks of the predator bacteria on added prey bacteria (Table 2). Titers of phage specific for the added prey cells, instead of the predator cells, could not be monitored. The prey cells were attacked quickly by predator bacteria before extensive attack on the prey cells by indigenous phage could occur. Thus, these titers were 10 PFU or less per ml of percolate.

Of the predators, strains N-1 and L-2 were capable of attacking the largest number of different prey species (Table 2). Predators 5 and L-3 also attacked a relatively wide variety of prey species. These predators are all gram negative. In contrast, streptomycete predators W1, C-2, and 34 were able to multiply in response to only a few of the prey tested. When tested, no predators, either gram positive or gram negative, were found that attacked *Rhizobium meliloti*, *R. leguminosarum*, *Nocardia salmonicolor*, *Actinomyces humiferus*, or spores of *Bacillus stearothermophilus* or a *Bacillus* soil isolate. In some instances, the predators that should have attacked them may themselves have been

TABLE 2. Indigenous predator bacterium responses to various bacteria tested as prey in soil[a]

| Predator[b] | Potential prey bacteria[c] | | Species attacked (% of total) |
|---|---|---|---|
| | Attacked | Not attacked | |
| N-1 | AR, AG, AV, BS, BT, EA, EC, ML, SA, 34, C2 | AT, NS, ST, B, 5, L-2, M-8 | 61 |
| L-2 | AR, AT, AG, AV, BS, BT, EC, ST, SA, M-8, 34, C2 | EA, ML, B, N-1 | 75 |
| 5 | AR, AG, AV, EC, ST, SA, 34, C2 | EA, ML, NS, N-1, M-8, L-2, B | 53 |
| L-3 | AR, AG, ST, 34, C2 | AT, AV, BS, BT, SA, M-8, N-1, L-2 | 38 |
| B | SA, N-1, 34, C2 | AT, AR, AV, BS, EC, ML, NS, ST, L-2, M-8 | 29 |
| *Ensifer adhaerens* | AR, ML, SA, 34, C2 | AH, AT, AG, AV, BS, BST, BT, EC, RL, RM, ST, W1, N-1, 5, B | 25 |
| *Agromyces ramosus* | AV,[d] N-1, 34, C2 | AH, AT, AV, AG, BS, BI, BT, EA, ML, RL, RM, ST, SA, W1, L-2, M-8 | 20 |
| M-8 | AV, ST, SA | AT, AR, AG, BS, BT, EA, EC, ML, NS, L-2, N-1, C-2, 34 | 19 |
| C2 | AV,[d] ML, SA | AH, AT, AR, AG, AV, BI, RL, RM, ST, L-2, M-8, N-1, 5, B | 18 |
| 34 | ML, SA | AT, AG, AV, BS, BI, BT, RL, RM, ST, N-1, L-2 | 15 |
| W1 | ML | AG, AV, BS, BI, BT, EC, RL, RM, ST, SA, N-1, L-2, 5 | 7 |

[a] Results include soil percolations done with both preenriched and nonenriched soil. The *bacillus* species were added to the soil as spores.
[b] The predator Gram reactions are shown in Table 3.
[c] Abbreviations: AH, *Actinomyces humiferus*; AT, *Agrobacterium tumefaciens*; AR, *Agromyces ramosus*; AG, *Arthrobacter globiformis*; AV, *Azotobacter vinelandii*; BST, *Bacillus staerothermophilus*; BS, *Bacillus subtilis*; BI, *Bacillus* soil isolate; BT, *Bacillus thuriengiensis*; EA, *Ensifer adhaerens*; EC, *Escherichia coli*; ML, *Micrococcus luteus*; NS, *Nocardia salmonicolor*; RL, *Rhizobium leguminosarum*; RM, *Rhizobium meliloti*; ST, *Salmonella typhosa*; SA, *Staphylococcus aureus*.
[d] Attacked only in preenriched soil.

attacked by other predators. For example, *Agromyces ramosus* should have attacked the *Rhizobium* species (5), but instead (Table 2) was itself subject to attack by N-1 and other predators (see below). If attacked by another predator while attacking *Rhizobium* species, *Agromyces ramosus* would not grow enough to allow extensive phage multiplication for detection by the indirect phage analysis technique.

Table 2 also lists predator-on-predator interactions. In most instances, these interactions involved a gram-positive predator that was attacked by one or more gram-negative predators. All of the gram-negative predators, plus *Agromyces ramosus*, could attack at least three other predators species. Streptomycete strains 34 and C2, along with *Agromyces ramosus*, were those most commonly attacked by the gram-negative predators. Strains N-1, M-8, and *Ensifer adhaerens* represented gram-negative predators that were attacked by other predators, either gram negative or gram positive. Two predator bacteria, N-1 and *Agromyces ramosus*, demonstrated an attack on each other when one or the other was added individually as prey to soil. This phenomenon was described by Byrd et al. (1) as an attack-counterattack relation.

The predator bacterium response was dependent on the nutrient status of the soil that was used. Soil that had been nutritionally enriched by incubation with ground alfalfa before the trials and nonenriched soil represented a high-nutrient and a low-nutrient soil, respectively. Only in preenriched soil did the gram-positive actinomycete predator bacteria (C2, 34, W1, W3, and *Agromyces ramosus*) multiply to a sufficient degree to enable a phage response to be detected (Table 3). There was, however, a low-level growth response to the increased background soil nutrient level provided by the preenrichment. This occurred without addition of prey cells. Since this did not involve predation, the phage PFU on day 0 associated with it were subtracted to give the values in Table 3.

The gram-negative predators often multiplied in response to certain prey organisms to a lesser degree in preenriched than in nonenriched soil (Table 3). For example, strain L-2 cell multiplication in response to *Salmonella typhosa* or *Azotobacter vinelandii* was significantly lower (greater than 100-fold difference in phage yields) in preenriched soil than in nonenriched soil. Several examples of this phenomenon can be seen in Table 3.

## DISCUSSION

Of the predator bacteria that we studied, the gram-negative predators seemed to be more important than the gram-positive (actinomycete) predators as agents of prey cell destruction in soil. In fact, it would seem that certain of the gram-negative predators are dominant over many of the other species of both gram-positive and gram-negative bacteria in soil. Of the gram-negative predators, strains N-1, L-2, and 5 were particularly effective at attacking a variety of prey cell species, as well as attacking other gram-positive and gram-negative predator species. The gram-positive predators, such as *Agromyces ramosus* and the streptomycetes C-2, 34, W3, and W1, had relatively narrow prey ranges in soil and were themselves subject to attack by various gram-negative predators. The gram-positive predators did not attack other gram-positive or gram-negative predators, except for the attack-counterattack relationship of *A. ramosus* and N-1 (1). Therefore, multiplication of the gram-

TABLE 3. Comparison of maximum phage responses for predators in nonenriched versus preenriched soil

| Prey | Predator | Predator Gram reaction | Max phage titers (PFU/ml) (day) for: | |
|---|---|---|---|---|
| | | | Nonenriched soil | Preenriched soil |
| *Azotobacter vinelandii* | *Agromyces ramosus* | + | 0[c] | $2.7 \times 10^2$ (4) |
| | C2 | + | 0 | $3.0 \times 10^2$ (1) |
| | N-1 | − | $2.8 \times 10^6$ (3) | $8.0 \times 10^4$ (1) |
| | L-2 | − | $3.1 \times 10^5$ (2) | $1.2 \times 10^3$ (2) |
| *Micrococcus luteus* | W1 | + | 0 | $3.3 \times 10^3$ (2) |
| | 34 | + | 0 | $2.0 \times 10^3$ (3) |
| | W3 | + | 0 | $2.9 \times 10^3$ (2) |
| | C2 | + | 0 | $5.1 \times 10^2$ (1) |
| | N-1 | − | $1.1 \times 10^5$ (4) | $3.8 \times 10^5$ (1) |
| | *Ensifer adhaerens* | − | $2.6 \times 10^4$ (4) | $1.9 \times 10^4$ (3) |
| *Salmonella typhosa* | W1 | + | ND[d] | 0 |
| | 34 | + | ND | 0 |
| | L-2 | − | $2.1 \times 10^5$ (1) | $1.7 \times 10^3$ (1) |
| | L-3 | − | 0 | $8.8 \times 10^2$ (4) |
| | M-8 | − | 0 | $2.2 \times 10^5$ (4) |
| | 5 | − | $5.9 \times 10^4$ (3) | $6.7 \times 10^3$ (2) |
| *Staphylococcus aureus* | C2 | + | 0 | $1.5 \times 10^2$ (1) |
| | 34 | + | 0 | $2.6 \times 10^4$ (2) |
| | N-1 | − | $4.1 \times 10^5$ (2) | $4.7 \times 10^5$ (1) |
| | L-2 | − | $9.2 \times 10^4$ (3) | $4.4 \times 10^4$ (1) |
| | 5 | − | $1.3 \times 10^6$ (3) | $4.6 \times 10^3$ (1) |
| | M-8 | − | 0 | $3.2 \times 10^4$ (1) |
| | *Ensifer adhaerens* | − | 0 | $9.2 \times 10^4$ (4) |
| | B | − | $1.1 \times 10^3$ (3) | $3.1 \times 10^2$ (1) |
| *Agromyces ramosus* | N-1 | − | $9.9 \times 10^6$ (4) | $4.7 \times 10^5$ (4) |
| | L-2 | − | $1.6 \times 10^4$ (5) | $2.0 \times 10^2$ (5) |
| | 5 | − | $3.9 \times 10^4$ (5) | 0 |
| | *Ensifer adhaerens* | − | $9.5 \times 10^2$ (1) | 0 |

[c] 0, No PFU detected.
[d] ND, Not determined.

positive predators in soil and their concomitant attack on their prey cells may be controlled by members of the gram-negative predator group. Gram-positive rods and cocci from soil were not evaluated as predators in our study, because our isolations of predatory bacteria from soil (C. E. Sillman and L. E. Casida, Jr., Can. J. Microbiol., in press) have not yielded predatory strains of these organisms.

The gram-positive and gram-negative predator bacteria differed in another important way. The gram-negative predators were active in soil that had been previously incubated with dried alfalfa, but they were also active in nonenriched soil. The actinomycete predators, however, demonstrated extensive multiplication only in the enriched soil. The actinomycete predators tended to be dormant in soil when nutrients were not available. It would appear that our use of the nutritionally preenriched soil broke this dormancy and allowed at least the initial growth of these nonobligate predators. However, production of growth initiation factors (1) by other bacteria in the enriched soil also may have played a role.

There was evidence of a possible competitive interaction between the two predator groups when they were evaluted in soil that had been preenriched prior to testing. In some cases, the gram-negative predator activity was reduced somewhat in the preenriched soil compared with the nonenriched soil. During the preenrichment step, the gram-negative predators increased in number, as detected by an increase in phage PFU. Following this, in some instances, there was only a relatively small increase when prey cells were added to the soil columns. This meant that these gram-negative predators were not, to any extent, attacking the added prey cell species, nor were they attacking the indigenous gram-positive predators that were responding to the prey cells. It is possible that the gram-positive predators were depleting the supply of prey cells for the gram-negative predators. However, this would not explain why the gram-positive predators, in turn, were not being attacked by the gram-negative predators. A more likely explanation is that preenrichment of the soil increased the level of non-prey-cell available nutrients. As a result, certain of the gram-negative predators, being nonobligate for predation, no longer needed to attack prey cells.

*Agromyces ramosus* attacked *R. leguminosarum*, *R. meliloti*, *Agrobacterium tumefaciens*, and *Azotobacter vinelandii* when the interactions were studied with pure cultures in the absence of soil (5). However, study of these interactions in situ in soil by indirect phage analysis showed only the attack on *Azotobacter vinelandii*. In soil, *Agromyces ramosus* was itself rapidly attacked by indigenous strain N-1 cells (1) and by various other predators. This should stop the *Agromyces ramosus* attack on all three of the hosts. At present, we have no explanation why the *Agromyces ramosus* attack on *Azotobacter vinelandii* occurred while its attack on the other two species was prevented. However, we realize that the role of soil gram-positive rods and cocci, if any of them are predators, was not

considered in our study. A rapid attack by these bacteria on *Agrobacterium tumefaciens* or the *Rhizobium* species could decrease their numbers so that they would not be available as prey for *Agromyces ramosus*.


## ACKNOWLEDGMENTS

This work was supported by grants DAAG29-82-K-0055 and DAAG29-85-K-0084 from the U.S. Army Research Office.


## LITERATURE CITED


1. **Byrd, J. J., L. R. Zeph, and L. E. Casida, Jr.** 1985. Bacterial control of *Agromyces ramosus* in soil. Can. J. Microbiol. **31**:1157–1163.
2. **Casida, L. E., Jr.** 1980. Death of *Microsoccus luteus* in soil. Appl. Environ. Microbiol. **39**:1031–1034.
3. **Casida, L. E., Jr.** 1980. Bacterial predators of *Micrococcus luteus* in soil. Appl. Environ. Microbiol. **39**:1035–1041.
4. **Casida, L. E., Jr.** 1982. *Ensifer adhaerens* gen. nov., sp. nov: a bacterial predator of bacteria in soil. Int. J. Syst. Bacteriol. **32**:339–345.
5. **Casida, L. E., Jr.** 1983. Interactions of *Agromyces ramosus* with other bacteria in soil. Appl. Environ. Microbiol. **46**:881–888.
6. **Casida, L. E., Jr.** 1984. A growth initiation factor involved in magnesium utilization by certain soil bacteria. Can. J. Microbiol. **30**:824–829.
7. **Germida, J. J., and L. E. Casida, Jr.** 1983. *Ensifer adhaerens* predatory activity against other bacteria in soil, as monitored by indirect phage analysis. Appl. Environ. Microbiol. **45**:1380–1388.
8. **Liu, K.-C., and L. E. Casida, Jr.** 1983. Survival of myxobacter strain 8 in natural soil in the presence and absence of host cells. Soil Biol. Biochem. **15**:551–555.