Response in Soil of *Cupriavidus necator* and Other Copper-Resistant Bacterial Predators of Bacteria to Addition of Water, Soluble Nutrients, Various Bacterial Species, or *Bacillus thuringiensis* Spores and Crystals†

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Soil was incubated with various species of bacteria, *Bacillus subtilis*, or *Bacillus thuringiensis* spores and crystals. These were added to serve as potential prey for indigenous, copper-resistant, nonobligate bacterial predators of bacteria in the soil. Alternatively, the soil was incubated with soluble nutrients or water only to cause potential indigenous prey cells to multiply so the predator cells would multiply. All of these incubation procedures caused excessive multiplication of some gram-negative bacteria in soil. Even greater multiplication, however, often occurred for certain copper-resistant bacterial predators of bacteria that made up a part of the gram-negative response. Incubation of the soil with copper per se did not give these responses. In most cases, the copper-resistant bacteria that responded were *Cupriavidus necator*, bacterial predator L-2, or previously unknown bacteria that resembled them. As was the case for *C. necator* and L-2, these new bacteria did not use glucose, had white colonies, produced copper-related growth initiation factor (GIF), and attacked *B. thuringiensis* spores on laboratory media. The results were different, however, when *B. thuringiensis* spores and crystals per se were added to the soil. The copper-resistant bacterial response in the soil did not, to any extent, include *C. necator*-like bacteria. Instead, the main copper-resistant bacterial predators that developed had yellow colonies and did not resemble *C. necator* or L-2 in other ways. They were not seen before, and they did not develop on the addition of *B. subtilis* spores to soil. Apparently, they could not produce a *C. necator*-like GIF. Nevertheless, they did respond very quickly to *B. thuringiensis* spores and crystals in soil, as if a GIF of some sort were involved. These results suggest that, under various conditions of soil incubation, gram-negative bacterial predators of bacteria multiply and that several copper-resistant types among them can be detected, counted, and isolated by plating dilutions of the soil onto media containing excess copper.

*Cupriavidus necator*, which was originally designated strain N-1 (5, 6, 9), is a gram-negative, nonobligate bacterial predator of bacteria in soil. Among its prey are some other predatory bacterial species (2, 11). In fact, Zeph and Casida (11) have suggested that *C. necator* and another bacterial predator designated L-2 might be at the top of the hierarchy of bacterial predators in soil (6). *C. necator* is resistant to high levels of copper (5, 9). In fact, its growth initiation responds strongly to copper; *C. necator* makes a peptide growth initiation factor (GIF) that aids it in obtaining the copper for its growth initiation. Casida (5) has suggested that *C. necator* might also use this copper GIF for delivering toxic levels of copper to its prey cells. L-2, the other bacterial predator mentioned above, is also resistant to high levels of copper and makes the GIF. The relation of L-2 and *C. necator* to copper predators that the predatory activities of these bacteria in soil might be followed without the addition of extra copper to the soil. Instead, the numbers of these bacteria in soil would be determined by plating the soil onto copper agar, after their activities within the soil were completed. This, of course, assumes that soil does not contain more than minimal numbers of other bacteria that might be resistant to high copper levels.

Petras and Casida (10) have reported that 90% of *Bacillus thuringiensis* spores added to soil died relatively quickly, although the remaining 10% survived for prolonged periods of time. *C. necator* and L-2 are known to attack *B. thuringiensis* and *Bacillus subtilis* spores in soil (11). In contrast, the non-copper-resistant predatory bacteria that were tested did not attack either kind of spores. It is not known whether soil might contain other copper-resistant bacteria, in addition to *C. necator* and L-2, that can attack these spores.

The present study was undertaken to evaluate the responses of some indigenous, copper-resistant bacterial predators in soil to indigenous or added prey cells or nutrients. It was also of interest to determine whether soil might contain additional types of copper-resistant predatory bacteria that are able to attack *B. thuringiensis* and *B. subtilis* spores. It was hoped that the activities of these various copper-resistant bacteria could be monitored by plating dilutions of the soil onto copper-containing medium, even though copper was not added to the soil.

**MATERIALS AND METHODS**

**Organisms.** *Micrococcus luteus*, *Ensifer adhaerens* SA ATCC 33212, and *Arthrobacter globiformis* ATCC 8010 were grown in nutrient broth. *Agromyces ramosus* ATCC 25173 was grown in full-strength heart infusion broth. All media were products of Difco Laboratories (Detroit, Mich.). The flasks were shaken at 27°C until the late-logarithmic phase of growth. The cells were then washed 3 times by centrifugation in sterile distilled water and, finally, were suspended in distilled water.

Washed spore and crystal suspensions of *B. thuringiensis*...
fields and at different times from a farm near University Park, Pa. The soil pH values were 4.9 and 5.5, respectively. Soils OC (pH 6.4) and MW (pH 7.4) were garden soils from Oakland, Calif., and Madison Wis., respectively. Soil CG (pH 6.8) was from a barnyard in Cottage Grove, Wis. All soils were stored in polyethylene bags and were not allowed to dry.

Soil incubations. Soils were incubated in various ways. The 28-day incubations of soil with added nutrient broth were conducted in bottles. Soil that was passed through a 3-mm-pore-size sieve was added (10 g) to sterile 1-ounce (ca. 29.6-ml) screw-cap bottles. Sterile nutrient broth (1.2 ml) was added to bring the soil to 55 to 65% of its moisture-holding capacity. The caps were left partially loose during incubation. Water loss from the soil was counteracted by adding 0.3 ml of sterile distilled water at 4, 8, and 21 days of incubation. Three-day incubations of soil, with water added instead of nutrient broth, were conducted in a similar manner.

Soils were shaken for 24 h with added water or with heart infusion broth (0.01 strength), bacterial cultures, spores, copper, or zinc. For these trials, 1 g of soil was placed in a sterile tube with 9 ml of sterile distilled water. In some cases, 2 drops of a washed cell suspension or washed spores were added. Alternatively, the 9 ml of water was replaced with 9 ml of 0.01-strength heart infusion broth, aqueous 0.01% CuCl2 · 2H2O, or ZnCl2. The soil plus water and other additions in the tube were then mixed thoroughly (vortex Genie Mixer; American Hospital Supply Corp., Evanston, Ill.) and poured aseptically into a sterile 500-ml Erlenmeyer flask. This was shaken for 24 h at 27°C. Further dilutions for plating were then prepared from this 10^{-1} soil dilution. Two variations on this soil incubation procedure were used. In one case, the flask was shaken for 4 days instead of 1 day. The other variation was designed to give microaerophilic to anaerobic growth conditions. Soil plus water or 0.01-strength heart infusion broth was prepared in tubes as described above. The tubes were then allowed to sit stationary for 3 h (to wet the soil) before the tube contents were gently mixed by rolling the tube between the hands. Further stationary incubation was done for 21 h (24 h total). Flasks were not used for this; i.e., this 10^{-3} dilution tube was then thoroughly mixed in a vortex mixer as preparation for further dilution.

Microbial enumeration. Soil dilutions were made as 10-fold increments in tubes containing 9 ml of sterile distilled water. Each tube was thoroughly mixed in a vortex mixer before 1 ml was removed for preparing the next dilution of the sequence. The dilutions were plated on various media. Colony numbers for copper-resistant bacteria and fungi were determined on 0.1-strength heart infusion agar containing 0.01% CuCl2 · 2H2O (pH 6.5). Colony numbers for total bacterial counts, whether copper resistant or not, were determined by using a medium containing 0.01-strength heart infusion broth, 0.01% L-alanine, 0.07% MgCl2 · 6H2O, and 0.6% Gelrite (7) to solidify the medium. It was important that the MgCl2 · 6H2O is dissolved completely before the Gelrite is added. The medium was poured at 60°C into petri dishes. Gram-negative bacteria, whether copper resistant or not, were enumerated on a similar medium but with crystal violet added to give 4.9 μM before adding the Gelrite.

All incubations of soils and plates were done at 27°C. The plates were incubated for 5 to 6 days.

The numbers of C. necator ATCC 43291 and L-2 in soil were estimated by the method of Makkar and Casida (8).

Testing for GIF activity and predation. Production and assay of N-1-like GIF in culture filtrates of the copper-resistant isolates were done as described by Casida (3). The medium used for the production of GIF was modified slightly to contain 0.01% KH2PO4, 0.1% NH4Cl, 0.02% Na2SO4, 0.02% NaCl, 0.02% MgCl2 · 6H2O, and 0.05% L-glutamic acid; the pH was adjusted to 7.0. The flasks were shaken at 27°C for periods of up to 114 h to obtain good growth. The filtrates were assayed directly without being concentrated. A 1-ml portion of the filtrate was added before autoclaving to the medium in each Klett flask used for the N-1 GIF assay (5). The assay medium was made up of 0.1% KH2PO4, 0.1% NH4Cl, 0.02% Na2SO4, 0.02% NaCl, and 0.1% L-alanine (pH 7.0). The isolates were isolated from the pure GIF if the response was greater than or equal to that for an N-1 culture filtrate produced as described above for the other copper-resistant bacterial isolates. All of the active isolates produced at least this amount.

The 0.01-strength heart infusion agar and 1.5% Noble agar in water were poured into plates and then allowed to dry for 2 days at room temperature. Potential prey cells were applied as a single-streak line onto the agar surface. Any excess moisture along this streak was allowed to dry into the agar. The copper-resistant isolates (one per plate) were then applied so that, although the beginning of the streak was distant from the prey streak, the last half of the streak was superimposed on (mixed with) the prey cell streak. Thus, the two organisms on the plate were applied as a Y, with each upper arm of the Y being a nonmixed pure culture. The plates were incubated for 7 days at 27°C. They were then observed macroscopically, and growth on the plates and the B. thuringiensis crystals were observed as wet mounts by phase microscopy or as crystal violet stains of smears. In all instances, monocultures and overgrown areas on the plates were observed and compared.

RESULTS

The microbial counts that were made for the various soils were for total bacteria, gram-positive and gram-negative bacteria, copper-resistant and zinc-resistant bacteria, and the bacterial predators C. necator and L-2. The 0 h (no incubation) indigenous counts for these organisms in the various soils are given in Table 1. Numerical determinations of copper-resistant fungi in the soils were made, but the numbers of these fungi (approximately 1 × 10^5 to 5 × 10^5) did not respond to the experimental treatments of the soils and therefore are not given. The zinc-resistant bacteria (Table 1) also did not respond to treatments. Therefore, they are not referred to again.

Additions to soil. Soil was moistened with water and incubated without the addition of any nutrients or prey cells. Under these conditions, indigenous, nonobligate predatory bacteria in the soil, including the copper-resistant ones, should multiply (5, 9). They should use other bacteria in the soil as prey cells. For these trials, soils in excess water was shaken for 1 day (five different soils were tested) or 4 days, or was incubated stationarily for 1 day. In addition, soil at 60% of its moisture-holding capacity was incubated for 3 days.
During these trials (Table 2), the total bacterial count increased only minimally. The numbers of gram-negative bacteria and copper-resistant bacteria in soil, however, increased to a much greater degree. The increase in numbers of C. necator and L-2 was yet greater. Even with this increase, however, the C. necator and L-2 cells made up only 4.0% of the copper-resistant bacteria. The copper-resistant bacteria were always gram negative, and their numbers often increased at a faster rate than did the overall gram-negative population. Neither the gram-negative nor the copper-resistant populations were made up of heterogeneous mixtures of organisms. In fact, each of these populations usually contained only approximately two to, at most, five different species (determined by colony and cellular morphology and Gram stain). The copper-resistant bacteria did not necessarily show up on the counting plates for the gram-negative bacteria, because they were present in lower numbers than the rest of the gram-negative bacteria.

**Nutrient additions to soil.** The addition of soluble nutrients to soil should cause nonpredatory multiplication of the indigenous nonobligate bacterial predators (5, 6, 9). However, it should also cause multiplication of the bacterial population in soil in general, resulting in the exhaustion of the added nutrients. When the added nutrients become exhausted, the predatory bacteria may switch their activities to a predatory attack on the other bacteria, which have themselves increased in number in response to the nutrients. As a last resort, the predatory bacteria can stop feeding. Therefore, the predatory bacteria should multiply extensively by using as nutrients either (or both) the added soluble nutrients or the other bacteria, which themselves have multiplied.

### TABLE 1. Zero-hour bacterial counts for various soils

<table>
<thead>
<tr>
<th>Soil designation</th>
<th>Total count</th>
<th>Gram positive</th>
<th>Gram negative</th>
<th>Copper resistant</th>
<th>Zinc resistant</th>
<th>C. necator</th>
<th>L-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>RS85</td>
<td>$3.3 \times 10^7$</td>
<td>$3.2 \times 10^7$</td>
<td>$6.3 \times 10^5$</td>
<td>$1.1 \times 10^4$</td>
<td>$6.2 \times 10^4$</td>
<td>$2.3 \times 10^2$</td>
<td>$1.3 \times 10^2$</td>
</tr>
<tr>
<td>RS86</td>
<td>$1.8 \times 10^7$</td>
<td>$1.6 \times 10^7$</td>
<td>$2.0 \times 10^6$</td>
<td>$\leq 1.0 \times 10^5$</td>
<td>$2.3 \times 10^2$</td>
<td>$1.3 \times 10^2$</td>
<td></td>
</tr>
<tr>
<td>OC</td>
<td>$6.2 \times 10^5$</td>
<td>$6.0 \times 10^5$</td>
<td>$1.8 \times 10^4$</td>
<td>$6.0 \times 10^3$</td>
<td>$6.0 \times 10^3$</td>
<td>$6.0 \times 10^3$</td>
<td></td>
</tr>
<tr>
<td>MW</td>
<td>$5.4 \times 10^4$</td>
<td>$5.3 \times 10^4$</td>
<td>$5.4 \times 10^3$</td>
<td>$8.0 \times 10^2$</td>
<td>$9.2 \times 10^4$</td>
<td>$9.2 \times 10^4$</td>
<td></td>
</tr>
<tr>
<td>CG</td>
<td>$1.5 \times 10^4$</td>
<td>$1.5 \times 10^4$</td>
<td>$1.1 \times 10^4$</td>
<td>$9.2 \times 10^4$</td>
<td>$9.2 \times 10^4$</td>
<td>$9.2 \times 10^4$</td>
<td></td>
</tr>
</tbody>
</table>

* Difference between total and gram-negative counts.
* Resistant to 0.01% CuCl$_2$ · 2H$_2$O or ZnCl$_2$.

Various soils were incubated by either shaking or remaining stationary, with 0.01-strength heart infusion broth (Table 3). Shaking of the soil aerated it and allowed greater interaction of predatory and prey cells. The total count increased for the shaken soils. The gram-negative and copper-resistant bacterial counts, however, increased to a distinctly greater degree. As with the water additions to soils (Table 2), the responding population of the gram-negative and copper-resistant bacteria represented only a narrow spectrum of species types. The copper-resistant bacteria as a percentage of the gram-negative bacteria increased for all of the soils tested except CG. This was also true for CG soil (Table 2), to which only water was added. Apparently, this is because the 0-h, copper-resistant bacteria component of the gram-negative bacteria was already high (compared with those of the other soils) for this nutritionally rich (CG) soil from a barnyard.

The results in Table 3 are for short-term trials with excess fluid (nutritive) added to the soil. Table 4 shows the results for soil incubated for 28 days with nutrient broth used initially to adjust the soil moisture content to 60% of the moisture-holding capacity (no extra fluid present), and additional water (no nutrients) was added at intervals to prevent the drying out of the soil. In general, the responses of the various bacterial populations were like those for which the results are given in Table 3, but with one major exception. After 28 days the gram-negative bacterial population was composed almost entirely of copper-resistant bacteria. The bacterial predators C. necator and L-2 accounted for 10.2% of this copper-resistant population. The numbers of these predators increased dramatically over those at time zero in this soil (Table 4).

### TABLE 2. Relative increases in cell numbers for various bacterial populations during incubation of soil with water

<table>
<thead>
<tr>
<th>Soil designation</th>
<th>Soil treatment$^a$</th>
<th>Total count (fold$^b$)</th>
<th>Gram-negative bacteria (fold$^c$)</th>
<th>Copper-resistant bacteria (fold$^d$)</th>
<th>Copper-resistant bacteria as % of gram-negative bacteria$^e$</th>
<th>C. necator (fold$^f$)</th>
<th>L-2 (fold$^f$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RS85</td>
<td>A</td>
<td>2.1</td>
<td>9.7</td>
<td>14.4</td>
<td>2.8 (1.5)</td>
<td>1,000.0</td>
<td>1,769.2</td>
</tr>
<tr>
<td>RS86</td>
<td>A</td>
<td>1.9</td>
<td>5.4</td>
<td>620.0</td>
<td>5.3 (&lt;0.05)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OC</td>
<td>A</td>
<td>0.9</td>
<td>17.2</td>
<td>666.7</td>
<td>12.9 (0.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MW</td>
<td>A</td>
<td>29.6</td>
<td>107.4</td>
<td>287.5</td>
<td>4.0 (1.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CG</td>
<td>A</td>
<td>10.7</td>
<td>72.7</td>
<td>18.5</td>
<td>2.1 (0.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RS85</td>
<td>B</td>
<td>0.3</td>
<td>0.7</td>
<td>280.0</td>
<td>20.0 (&lt;0.05)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RS85</td>
<td>C</td>
<td>0.3</td>
<td>3.0</td>
<td>1.6</td>
<td>0.7 (1.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RS85</td>
<td>D</td>
<td>1.8</td>
<td>6.0</td>
<td>3.8</td>
<td>0.9 (1.4)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ A, Shaken for 24 h in a flask; B, shaken for 4 days in a flask; C, stationary for 24 h in a tube; D, stationary for 3 days in a bottle.
$^b$ Number of fold increase over the value at 0 h.
$^c$ Values in parentheses were those at 0 h.
$^d$ Except for the C. necator and L-2 determinations, values are averages for five experiments conducted at different times. Standard deviations were, from left to right, respectively, beginning with the column "Total count," 1.2-fold, 2.8-fold, 10.6-fold, 3.2%, and 0.2%.
$^e$ Values are averages from two experiments.
TABLE 3. Relative increases in cell numbers for various bacterial populations during 24 h of incubation of soil with 0.01-strength heart infusion broth

<table>
<thead>
<tr>
<th>Soil designation</th>
<th>Soil treatment</th>
<th>Total count (fold*)</th>
<th>Gram-negative bacteria (fold*)</th>
<th>Copper-resistant bacteria (fold*)</th>
<th>Copper-resistant bacteria as % of gram-negative bacteria*</th>
</tr>
</thead>
<tbody>
<tr>
<td>RS86</td>
<td>shaken in flask</td>
<td>94.4</td>
<td>425.0</td>
<td>45,000.0</td>
<td>5.3 (&lt;0.05)</td>
</tr>
<tr>
<td>OC</td>
<td>shaken in flask</td>
<td>15.5</td>
<td>205.6</td>
<td>7,666.7</td>
<td>12.4 (0.3)</td>
</tr>
<tr>
<td>CG</td>
<td>shaken in flask</td>
<td>40.0</td>
<td>154.5</td>
<td>119.6</td>
<td>6.5 (8.3)</td>
</tr>
<tr>
<td>RS85</td>
<td>stationary in tube</td>
<td>0.3</td>
<td>8.6</td>
<td>46.7</td>
<td>7.8 (14.1)</td>
</tr>
</tbody>
</table>

* Number of fold increase over the value at 0 h.
* Values in parentheses were those at 0 h.

Bacterial additions to soil. Various predatory (E. adhaerens and Agromyces ramosus) and nonpredatory (M. luteus, Arthrobacter globiformis, and B. thuringiensis) spores and crystals) bacteria were added to soil. The added numbers per gram of soil were, respectively, $3.3 \times 10^8$, $1.1 \times 10^8$, $6.4 \times 10^7$, $1.0 \times 10^8$, and $3.9 \times 10^8$. The soil was then shaken for 24 h (Table 5). Although the total bacterial count response was relatively low in most cases, good responses occurred for the gram-negative bacteria, the copper-resistant bacteria, and C. necator and L-2. The latter two predators made up approximately 100% of the copper-resistant bacterial count, except for a value of 35% when Agromyces ramosus was added to the soil and 0.008% when B. thuringiensis spores and crystal were added to the soil. At time zero the value for C. necator and L-2 was 4%. C. necator and L-2 were not necessarily destroyed by the addition of B. thuringiensis to the soil. Rather, these two predators did not multiply to any extent in response to B. thuringiensis. Instead, other copper-resistant bacteria multiplied.

Neither autoclaved B. thuringiensis spores and crystals nor B. subtilis spores, either autoclaved or not, added to the soil elicited a copper-resistant bacterial response (data not shown). The increase in copper-resistant bacteria occurred even when B. thuringiensis spores and crystals (nonautoclaved) were added at low concentration, e.g., at $2.2 \times 10^7$ g of soil. Thus, 10-fold dilutions from $2.2 \times 10^4$ to $2.2 \times 10^7$ of soil (the lowest concentration tested) provided copper-resistant bacterial increases of $3.667\times 10^7$, $7.5\times 10^6$, and $4.4\times 10^6$, and 133-fold, respectively. The value was 13-fold when the spores were not added.

The response of copper-resistant bacteria in soil to B. thuringiensis spore and crystal addition (but not to various other additions) occurred quite rapidly. A soil that, when shaken for 24 h with water, increased 32-fold in copper-resistant bacteria increased 3,273-fold when shaken for a similar time with B. thuringiensis spores and crystals. However, by the end of the initial 1 h of incubation, the count had already increased 545-fold. This was due mainly to the yellow colony types. Shaking of the soil with B. subtilis spores had no effect at either 1 or 24 h of incubation.

Stationary instead of shaken incubation of B. thuringiensis spores and crystals with soil for 24 h gave only a minimal response. In fact, the response was similar to that presented in Table 3 for stationary incubation of soil with 0.01-strength heart infusion broth for 24 h.

B. thuringiensis vegetative cells (instead of spores) had <1% survival when they were incubated for 3 days in soil at 60% of the moisture-holding capacity. The reason for their death is not known. Although the indigenous gram-negative soil bacteria responded by multiplying 825-fold, the copper bacteria component of this soil increased only 83-fold.

Incubation of soil for 24 h with shaking and with 0.01% CuCl$_2$ · 2H$_2$O added gave microbial counts that were the same as those when the soil was shaken only with water. The addition of 0.01% CuCl$_2$ · 2H$_2$O also had no effect on the results when the addition was made simultaneously with the addition of B. thuringiensis spores.

Copper resistance versus predation. Various known strains (11) of nonobligate bacterial predators were checked for their growth on 0.1-strength heart infusion agar containing 0.01% CuCl$_2$ · 2H$_2$O (pH 6.5). Growth occurred for C. necator N-1 and L-2. No growth occurred for predators C-2, L-3, and 34; E. adhaerens A, SA, and 7A (3); or Agromyces ramosus ATCC 25173 and PSU35 (4).

As noted above, the response of indigenous C. necator, L-2, and other copper-resistant bacteria in soil when various bacterial species were added to the soil is given in Table 5. In most cases, the response by the indigenous copper-resistant bacteria seemed to be due mainly to C. necator and L-2, which were a component of this population. Other copper-resistant bacteria could be very active, however, e.g., when Agromyces ramosus or B. thuringiensis spores and crystals

TABLE 4. Relative increases in cell numbers for various bacterial populations during 28 days of stationary incubation of soil RS85 in bottles with a single nutrient broth addition

<table>
<thead>
<tr>
<th>Incubation time (days)</th>
<th>Total count (fold*)</th>
<th>Gram-negative bacteria (fold*)</th>
<th>Copper-resistant bacteria (fold*)</th>
<th>Copper-resistant bacteria as % of gram-negative bacteria*</th>
<th>L-2 (fold*)</th>
<th>L-2 + C. necator (fold*)</th>
<th>C. necator (fold*)</th>
<th>L-2 + C. necator as % of copper resistant*</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>6.9</td>
<td>23.8</td>
<td>1,777.8</td>
<td>106.7 (1.4)</td>
<td>40.0</td>
<td>147.8</td>
<td>1,434.8</td>
<td>10.2 (3.3)</td>
</tr>
</tbody>
</table>

* Number of fold increase over the value at 0 h.
* Value in parentheses was that at 0 h.
* Zero-hour count = 1.3 × 10$^7$/g of soil.
* Zero-hour count = 2.3 × 10$^7$/g of soil.
* C. necator = 2.1%; L-2 = 8.1%.
TABLE 5. Relative increases in cell numbers for various indigenous bacterial populations during 24 h of shaking incubation of RS85 soil with some species of added bacteria

<table>
<thead>
<tr>
<th>Organism added</th>
<th>Relative increases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total count</td>
</tr>
<tr>
<td>H₂O only</td>
<td>3.9</td>
</tr>
<tr>
<td><em>Encephalophora adhaerens</em></td>
<td>14.4</td>
</tr>
<tr>
<td>Micrococcus luteus</td>
<td>20.0</td>
</tr>
<tr>
<td>Arthrobacter globiformis</td>
<td>12.5</td>
</tr>
<tr>
<td>Agromyces ramosus</td>
<td>156.2</td>
</tr>
<tr>
<td>Bacillus thuringiensis</td>
<td>26.2</td>
</tr>
</tbody>
</table>

* Residual of the added organism, or further multiplication of it, is not a component of the total count. Values are number of fold increase over the value at 0 h.
* At 0 h, the value was 1.4% for all organisms added to soil.
* Residual of added organism, or further multiplication of it, at the end of the experiment. The colonies could be distinguished on the plates.
* A repeat experiment gave a 6,333.3-fold increase.

were added to the soil. This existence of copper-resistant bacteria other than *C. necator* and L-2 was also apparent (Tables 2 and 4). In order to examine these other copper-resistant bacteria for predation and other characteristics, isolations of the dominant copper-resistant bacteria were made from the various plates containing copper medium used in these experiments. Although they were all gram negative, none of these isolates showed a cross-reaction with phage for *C. necator* or L-2.

The isolates were grouped as to whether they did or did not utilize glucose (*C. necator* and L-2 did not) and whether their colonies were white (as for *C. necator* and L-2) or yellow. The isolates were then grown in glutamate synthetic broth, and the resulting culture filtrates were tested for *C. necator* GIF activity. The seven isolates with yellow colonies, and 3 of the isolates with white colonies did not grow in the glutamate synthetic medium and, therefore, were not tested for *C. necator* GIF activity. Four of the seven yellow colony forms described above did not use glucose. It should be noted that the yellow colony forms tended to be the dominant types that appeared on the copper isolation plate for soils treated with *B. thuringiensis* spores and crystals, as opposed to the white colony forms for soils treated otherwise.

For the 14 white colony isolates that were tested, 10 produced *C. necator* GIF and 4 did not. Actually, one of the culture filtrates of the latter group was toxic to *C. necator*. Except for one isolate from each group, none of these isolates could use glucose. Paper chromatography controls showed that there was no residual glutamate carryover in any of the filtrates that might be misinterpreted as a GIF response.

Several of the isolates were tested on water agar and 0.01-strength heart infusion agar for predation against *Arthrobacter globiformis*, *M. luteus*, and *B. thuringiensis* spores and crystals. As evidenced by the disappearance of prey cells, predation was more prevalent on the water agar.

The occurrence of picket fence attachment (predator cell alignment on prey cells, as occurs with *E. adhaerens*) (3) did not necessarily indicate that prey cell destruction would occur. *Arthrobacter globiformis* was not attacked by any of the isolates tested. Seven of the isolates attacked *B. thuringiensis* spores without attacking *M. luteus*, and one attacked *M. luteus* without attacking the spores. These belonged to the white colony type that did not use glucose. Two isolates attacked both *M. luteus* and *B. thuringiensis*.

One was a white colony type that did not use glucose, and the other was a yellow colony type that did use glucose. Five isolates did not attack either organism. One of them was yellow that did not use glucose; another was white that did use glucose. The rest were white and did not use glucose. There was no correlation between the original source of the organism (or method of obtaining it) and its attack, or lack of attack, on *B. thuringiensis* spores or *M. luteus* cells. Those that did not attack either of them were not tested to find suitable alternative prey.

The *B. thuringiensis* spores did not germinate under the plate testing conditions described above. Attack on the spores was accompanied by attack on the crystals and vice versa (>50% reduction of both). Some of the residual crystals showed an eroded surface, so that the crystal appeared to be round.

**DISCUSSION**

Incubation of soil in various ways, but without the addition of copper, enriched the soil for gram-negative, copper-resistant bacteria. This apparently was because these copper-resistant bacteria, which made up only a relatively few species, actually were bacterial predators that were multiplying while they attacked other bacteria in the soil. The addition of copper to the soil did not further enrich for these bacteria. This probably was because they were already responding to the indigenous bacterial prey cells and, hence, could not respond to the copper as well. The only previously known bacteria that are both predators of other bacteria and copper resistant are *C. necator* and L-2 (5, 9). As was found in the present study, however, only in some cases did *C. necator* and L-2 constitute a major component of the copper-resistant bacterial predatory population.

The general picture for the soil incubations was as follows. The total count increased minimally, unless soluble nutrients were added. Even with nutrient addition, however, the relative increases of counts of some of the component populations were considerably greater than the overall increase in total count. For example, the gram-negative count increased more so than did the total count. However, it never became the total count; i.e., the gram-positive count was always greater.

The copper-resistant bacterial (always gram-negative) component of the gram-negative population usually increased at a faster rate than did the general gram-negative...
It appears that the copper-resistant bacteria (whether they have white or yellow colonies) that responded to *B. thuringiensis* spores and crystals in soil were responding to the living (not autoclaved) spores and not to the dead spores or vegetative cells. The response appeared to be specific and quite sensitive. Only a small number of spores was required, and *B. subtilis* spores were inactive. The specificity could come from the crystal protein, which also coats the spore (1). If this protein was involved, its activity for the predators would have to have been destroyed by heat. Nevertheless, it was shown that, at least on plates, the protein crystals themselves are eroded at the surface and are eventually destroyed by the copper-resistant bacteria.

The copper-resistant bacteria that responded to *B. thuringiensis* spores and crystals in soil did so very rapidly. There was a large apparent increase in numbers already after 1 h. This large early response was due primarily to the yellow colony types. The response was too rapid to be due to cellular multiplication, but it could have been due to growth initiation, i.e., a breaking of dormancy of a population(s) that was already in place but that was dormant. It appears, however, that the GIF for this, if that is how it works, may not be of the type for *C. necator* or L-2. Proof of this will have to await the finding of a suitable way to grow them so their culture filtrates can be tested for this GIF.

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LITERATURE CITED