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Liquid Culture of the Entomogenous Nematode *Steinernema feltiae* with Its Bacterial Symbiont[1]

EDWARD J. BUECHER[2] AND IRENE POPIEL[3]

Abstract: The insect-parasitic nematode, *Steinernema feltiae* Filipjev strain 42, was reared in liquid culture along with its bacterial symbiont, *Xenorhabdus nematophilus* Thomas & Poinar. First-stage juveniles developed into reproducing adults in a maintenance salts medium containing resuspended *Xenorhabdus* cells and the yeast *Kluyveromyces marxianus* (Hansen) van der Walt or cholesterol. Cultures with media depths greater than 4 mm required aeration. Nematode populations increased as bacterial density increased. An optimal culture system was obtained when the bacteria and nematodes developed in a semidefined medium containing tryptic soy, yeast extract, and cholesterol and were incubated on a rotary shaker at 25 ± 1 C. Under these conditions, up to 86% of the final population were infective juveniles.

Key words: biological control, entomogenous nematode, liquid culture, *Steinernema*, *Xenorhabdus*.

Entomogenous nematodes in the family Steinernematidae are intensively studied because of their control potential against insect pests (8,9). Although these nematodes are easily produced in vivo (5) or in vitro (2) on various complex semisolid organic media, the cost of mass production using these methods is a major constraint on nematode commercialization. A large-scale liquid culture system would constitute a more cost-effective approach.

The interaction between steinernematids and their bacterial symbiont is essential to their success as insect pathogens. Our objective in this study was to investigate the possibility of growing *Steinernema feltiae* Filipjev strain 42 (formerly *Neoaplectana carpocapsae* Weiser) in resuspended or growing cells of its bacterial symbiont *Xenorhabdus nematophilus* Thomas & Poinar strain 42 in shallow depths and in large volume, using various aeration methods to obtain sufficient gas exchange.

Materials and Methods

Bacterial preparation: The primary form of *Xenorhabdus nematophilus* strain 42 was isolated from homogenized surface-sterilized infective juveniles of *Steinernema feltiae* strain 42 (1) and stored in 17% glycerol in liquid nitrogen. A loopful of the bacteria was transferred to a 2-liter Fernbach flask containing 3% tryptic soy broth (Difco Labs, Detroit, MI) supplemented with 0.5% yeast extract (Difco Labs). Flask cultures were grown up to cell densities of 10^{10} cells/ml by aerating on a 200-rpm shaker for 24 hours. The cells were harvested, centrifuged at 3,000 rpm for 30 minutes, and then washed twice in buffer (0.5% sodium chloride, 0.68% potassium diphosphate, adjusted to pH 6.6 with 5 M sodium hydroxide). Cells were resuspended in S salts buffer (15) and counts were made using a Petroff Hausser counting chamber.

Nematode inoculum preparation: Egg-bearing female nematodes were dissected from infected *Galleria mellonella* L. larvae on day 5 after infection and suspended in an aqueous solution of 0.2 M sodium hypochlorite and 0.4 M sodium hydroxide. Within 10 minutes the females had disintegrated. The eggs were then washed by resuspending them in sterile M9 buffer solution (3). The eggs were allowed to hatch in the buffer overnight, and the resulting first-stage juveniles (J1) (10) were transferred by mouth pipet to Leighton tubes.

Third-stage infective juveniles (J3) were obtained from monoxenic nematode stock cultures maintained on dog food agar slants (11). The top 3 mm of a slant from a 14-day-old culture was suspended in 2.5 ml sterile water and poured under aseptic con-

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[1] Funds provided by BIOSYS.
[2] Department of Biology, Pacific University, Forest Grove, OR 97116.
[3] BIOSYS, 1057 East Meadow Circle, Palo Alto, CA 94303.
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ditions on four layers of Whatman #1 filter paper contained within the lid of a 9-cm-d petri dish. The lid was placed in a 15-cm-d petri dish containing 20 ml water. The dishes were then covered and left for 48 hours at 25 C, during which time the majority of infective J3 migrated from the inner plate into the water. They were removed, resuspended in S salts buffer, and counted. Suspensions of infective J3 were adjusted to the required concentration. The inoculum was added to Leighton tubes in drops by mouth pipet and to flask cultures as a 0.5-ml aliquot.

Yeast cell preparation: A loopful of the yeast *Kluyveromyces marxianus* (Hansen) van der Walt (ATCC strain #8601) was transferred to a 250-ml Erlenmeyer flask containing potato dextrose broth (Difco Labs) and incubated on a reciprocal shaker at 25 C for 24 hours. Cells were centrifuged at 3,000 rpm for 30 minutes and resuspended in S salts buffer. Cell counts were made using a Petroff Hausser counter.

Maintenance salts medium containing resuspended cells: Initial cultures were established using dixenic and monoxenic systems. In the dixenic system, cells of *X. nematophilus* and *K. marxianus* were each suspended at a concentration of 2×10^8 cells/ml in S salts buffer, pH 6.7. In the monoxenic system, *X. nematophilus* cells were suspended in S salts medium, pH 6.7, containing 40 μg/ml cholesterol (15). Cholesterol was prepared as a saturated stock solution in 100% ethanol. Cod liver oil was added to the medium after mixing it with 100% ethanol. Leighton tubes containing 0.6 ml medium were inoculated with 25 J1. The effect of bacterial cell density (range: 4.4×10^8 to 4.4×10^{10}) on nematode growth and reproduction was also evaluated in Leighton tube cultures initiated with 25 J1. Each culture was tested in duplicate and then repeated to give a total of 4–6 culture replicates.

For small-volume flask cultures, infective J3 were used as the nematode inoculum because they were less labor intensive to produce in large quantities. Medium depths ranging from 3 to 16 mm were evaluated using 3×10^{10} bacterial cells/ml medium in 25 ml and 50 ml cotton-plugged Erlenmeyer flasks. These and all subsequent cultures were initiated with 100–500 infective J3/ml medium. Each culture system was retested to give a total of 8–17 culture replicates.

Aerated large-volume cultures (27–60-mm depths) containing 3×10^{10} bacterial cells/ml were initiated in several standard vessels, including 250-ml and 500-ml Wheaton magnetic Celstir flasks and paddle Celstir flasks (Wheaton Scientific, Millville, NJ), 200-ml round-bottom centrifuge tubes (Nalgene, Rochester, NY), 250-ml screw cap flasks, and 1-liter flat-bottomed jars. Vessels without a stirrer were maintained on a reciprocal shaker at 140–150 rpm or by bubbling filter sterilized air at 500–900 ml/minute through an open-ended tube inserted down to the bottom of round-bottom centrifuge cups. Each culture system was retested to give a total of 6–12 culture replicates.

Semidefined medium containing growing bacterial cells: Maintenance salts medium containing resuspended cells was supplemented with 0.5% yeast extract and tested in Leighton tubes, each inoculated with 25 J1.

A semidefined bacterial growth medium was compounded with 3% tryptic soy broth, 0.5% yeast extract, and 40 μg/ml cholesterol. Twenty-four-hour log phase cells of *X. nematophilus* (10^{10} cells/ml) in the semidefined medium were inoculated with 250 infective J3/ml. Small-volume cultures were set up in either 60-mm petri dishes containing 3 ml medium (static) or 25-ml cotton-plugged Erlenmeyer flasks containing 6 ml medium agitated on a Junior Orbitol rotary shaker (VWR Scientific, San Francisco, CA) at 140 rpm. Large-volume cultures were set up in either 250-ml cotton-plugged Erlenmeyer flasks containing 60 ml medium agitated at 150 rpm or 250-ml round-bottom centrifuge cups containing 100 ml medium sparged with 400–900 cc air/minute.

Nematode development was monitored daily by microscopic examination. Nema-

TABLE 1. Development to adult stage of *Steinernema feltiae* strain 42 in supplemented S salts medium containing 2 × 10^8 cells/ml *Xenorhabdus nematophilus* strain 42.

Supplement to medium	Adult nematodes at 6 days (mean %)	Range (%)
2 × 10^6 yeast cells/ml	19	16–24
40 μg/ml cholesterol	55	32–84
50 mg/ml cod liver oil	0	
None	0	

Data represent six culture replicates for each supplement tested. Each Leighton tube culture contained 0.6 ml medium and was inoculated with 25 J1.

TABLE 2. Fourteen-day growth response of *Steinernema feltiae* strain 42.

Bacterial cell density/ml	Mean nematode density/ml	Population range/ml
4.4 × 10^8	210	155–245
4.4 × 10^9	1,900	570–5,040
4.4 × 10^{10}	21,800	16,400–35,100

Data represent four culture replicates for 4.4 × 10^8, four culture replicates for 4.4 × 10^9, and six culture replicates for 4.4 × 10^{10} cell densities. Each Leighton tube culture contained resuspended bacterial cells in 0.6 ml S salts medium with 40 μg/ml cholesterol and was inoculated with 25 J1.

tode population size was determined by performing dilution counts on samples removed from the cultures.

RESULTS

Cultures in maintenance salts medium with resuspended cells: Cells of *X. nematophilus* strain 42 and *K. marxianus* resuspended in S salts buffer supported the development of *S. feltiae* strain 42 from J1 to adults in thin-film Leighton tube cultures (Table 1). More J1 developed to adults in S salts medium containing cells of *X. nematophilus* and cholesterol. Cod liver oil was not a suitable substitute for the yeast cells.

Populations of *S. feltiae* increased in Leighton tube cultures when the number of resuspended bacteria was increased from 4.4 × 10^8 to 4.4 × 10^{10}/ml (Table 2). First-stage juveniles developed into adult females and males within 5 days. The progeny of these adults developed to a new generation of adults; however, the eggs produced by the new generation females did not develop.

In static tube and flask cultures containing resuspended cells, depths of the medium > 3 mm had an adverse effect on nematode development and population size. Although normal adults developed in medium 4 mm deep, at 6 days the nematode density was only 70/ml compared with more than 4,000/ml in the 3-mm deep cultures. At a depth of 6 mm, 50% of the nematodes developed to normal sized adults, but they did not reproduce. At depths > 9 mm, none of the nematodes developed to adults.

In most of the large-volume culture systems tested, the nematodes became trapped in spaces within the bottom of the vessels and development was poor. However, when a constant flow of air was delivered by bubbling sterilized air through an open-ended tube inserted in the bottom of round-bottom centrifuge cups, the air turbulence kept the nematodes in suspension and populations up to 58,000/ml were obtained after 2 weeks. These cultures could be sustained by periodically decanting the spent medium and adding fresh medium. This system, however, yielded populations with less than 20% infective J3.

Cultures in semidefined media containing growing bacterial cells: When 0.5% yeast extract was added to the defined basal salts medium containing resuspended cells of *X. nematophilus*, females measuring up to 9 mm long and containing up to 2,000 eggs were observed. Also, observations of the bacterial cells indicated possible new cell formation in the presence of the yeast extract. When nematodes were inoculated into a semidefined medium containing tryptic soy broth, yeast extract, cholesterol, and 24-hour log phase cells of *X. nematophilus* (10^{10} cells/ml), the nematode population density reached a peak at 14 days with mean populations ranging from 14,300 nematodes/ml under small-volume, static conditions to 47,000 nematodes/ml under large-volume, aerated conditions (Table 3). Infective J3 formation reached a peak at 17 days with greater than 60% of the mean final

TABLE 3. Development of *Steinernema feltiae* strain 42 infective J3 inoculated into a semidefined medium containing growing bacterial cells.

Culture system	Nematode density/ml at 14 days		Final population developing into infective juveniles at 17 days (%)	
	Mean	Range	Mean	Range
Small volume, static	14,300	3,600–29,600	35	28–50
Small volume, shaker	23,400	14,600–41,600	77	63–86
Large volume, shaker	16,700	12,000–23,000	65	52–86
Large volume, air sparge	47,000	24,300–63,600	63	54–74

Infective J3 were inoculated into 24-hour exponential cultures of their symbiont *Xenorhabdus nematophilus* strain 42. Data represent eight culture replicates for 3-ml static cultures, 17 culture replicates for small-volume 6-ml shaker cultures, 12 culture replicates for large-volume 60-ml shaker cultures, and six culture replicates for 100-ml air sparge cultures.

population consisting of this stage under aerated conditions. Fewer infective J3 formed under static conditions.

We observed that infective J3 did not exsheath for at least 2 days after inoculation. They could be added simultaneously with the bacterial inoculum or up to 2 days after bacterial inoculation without any effect on the nematode population density at 14 days. Also inoculation densities > 250 infective J3/ml did not enhance the nematode population density at 14 days.

Cholesterol was essential for normal development and reproduction. In its absence, the cultures terminated and contained disintegrating adults and developmentally arrested J2.

DISCUSSION

Nutritional studies on free-living nematodes have demonstrated the need for both a heme-containing molecule and a sterol for normal development (7,13). We have confirmed that *S. feltiae* does have a requirement for a sterol. Also, by replacing yeast with cholesterol, we have established a more practical culture system.

A major limitation for liquid axenic nematode cultures is inadequate gas exchange. In previous studies this was overcome by culturing nematodes in very thin layers of liquid in saturated glass wool fibers (6). Large-volume axenic cultures of several nematode species, including some steinernematids, were successfully produced if they were vigorously aerated (4). Further improvements were made by Skimming et al. (14) who developed a technique to produce, with vigorous aeration, large numbers of *Caenorhabditis elegans* (Maupas) Dougherty in resuspended cells of *Escherichia coli* (Migula) Castellani & Chalmers.

We have demonstrated that aeration and bacterial density are important components for development and reproduction of *S. feltiae* strain 42 in a liquid medium. The adverse effects of increased medium depth and poorly mixed media indicated that O_2 transfer was a potential limiting factor in large-volume cultures. Of particular concern was the possibility that the agitation resulting from adequate aeration of large-volume cultures would damage the nematodes or prevent them from mating. Nematode reproduction occurred in both rotary shaker and vigorously air-sparged systems, however, and the nematodes appeared normal in appearance. Further work is required to optimize aeration levels and to determine the limits of nematode shear sensitivity at the different life-cycle stages.

The monoxenic system we first used, which involved culturing and washing the bacteria and resuspending them in the basal medium together with the nematodes, is similar to the two-step system used for growing *Caenorhabditis elegans* (14). We found this approach time consuming and inefficient, and it seemed more logical to grow the bacteria and nematodes together. The finding that nematode development was enhanced by supplementing the basal

salts medium with yeast extract also led us in this direction. Thus, the basal medium was supplemented with tryptic soy and yeast extract to provide nutrients for bacterial growth. The method by which nematodes were inoculated into growing cultures of the symbiont provides a more efficient system for scale-up and mass production. Furthermore, growth of both organisms together more closely mimics development in vivo.

In our study, nematode population densities averaged up to 47,000/ml, turbidity of the medium was reduced, suggesting nutrient depletion, and infective J3 averaged > 60% of the final population. A quantitative investigation of nematode population density and starvation has since demonstrated the enhancing effects of these two factors on infective J3 formation (12). It remains to be seen if the efficiency of infective J3 formation in larger volume cultures is adequate, or if the culture conditions must be modified to ensure maximal induction of infective J3.

The monoxenic liquid culture system described here establishes a baseline for media and process optimization studies for *S. feltiae* and other steinernematid nematodes. It has paved the way for large-volume liquid culture in fermenters and the provision of low-cost high yields of nematodes required for the commercialization of this insect pest-control agent.

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