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Stability and Activities of Antibiotics Produced during Infection of the Insect *Galleria mellonella* by Two Isolates of *Xenorhabdus nematophilus*

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***Xenorhabdus nematophilus* subsp. *dutki*, an entomopathogenic bacterium, is vectored by steinernematid nematodes into insects, where it produces broad-spectrum antibiotics. The use of the nematode-bacterium complex against soil-dwelling pest insects could introduce antibiotics into the soil via the dead insect fragments during the emergence phase of the nematodes. Studies on the stability and activities of these antibiotics produced in the insect *Galleria mellonella* may contribute to assessing the possible impact of antibiotics on soil bacteria. Two isolates of *X. nematophilus* subsp. *dutki* (isolates GI and SFU) produced xenocoumacins 1 and 2 in cadavers of *G. mellonella* larvae in a 1:1 ratio. Total xenocoumacin 1 and 2 production was 800 ng/200 mg (wet weight) of insect tissue for the GI isolate. Antibiotic activity of water extracts from insects that had been infected with *X. nematophilus* was stable at 60°C for 1 h and after repeated freeze-thaw cycles. The antibiotic titer of extracts held at 27°C declined by day 10. The spectrum of bacterial species killed by antibiotics produced in insect cadavers varied with the isolate of *X. nematophilus*. Levels of antibiotic activity were greater in vivo than in tryptic soy broth, which may represent a nutrient effect. The bacterial isolate, culture condition, and presence of nematodes influenced the total antibiotic production in vivo. However, the levels of activity were not correlated with bacterial levels in the different growth environments. Insect cadavers with antibiotic activity transiently lowered the numbers of the bacteria in the soil, the extent of decline varying with the strain of *X. nematophilus* and the time of sampling.**

Insect pathogenic nematodes of the genera *Steinernema* and *Heterorhabditis* are used to control pest insects of forests and agricultural crops (16). The infective juvenile (IJ) stage of the nematode enters the insect's body through the orifices (11) and voids the mutualistic species of *Xenorhabdus* or *Photorhabdus* (*Enterobacteriaceae*) into the hemolymph (2, 4, 6). The bacteria initiate septicemia, killing the insect and establishing conditions conducive to nematode reproduction by providing nutrients and producing antibiotics (16–19). After several generations in vivo, newly formed IJs emerge from the cadaver, extensively perforating the insect and releasing the cadaver's contents into the environment (21).

When cultured in vitro, *Xenorhabdus* and *Photorhabdus luminescens* (*Xenorhabdus luminescens* [8]) strains occur in two forms, phase one and phase two (1), which are distinguished by morphology, dye uptake, and antibiotic production (17, 18). Antibiotic production occurs during phase one for most species of *Xenorhabdus* and *P. luminescens*, the activity spectrum of the antibiotics varying with the species and strain of bacterium (3, 18). Three types of antibiotics are produced: (i) indole and stilbene derivatives by *Xenorhabdus bovienii* and *P. luminescens*, respectively (21, 24); (ii) organically soluble dithiolopyrrolones, xenorhabdins, by *Xenorhabdus* sp. strain Q, *X. bovienii*, and strains of *Xenorhabdus nematophilus* (4, 21); and (iii) water-soluble benzopyran derivatives, xenocoumacins, by *Xenorhabdus* sp. strain Q and the All strain of *X. nematophilus* (19).

In view of the broad antimicrobial activity of the antibiotics of *X. nematophilus* (19) and their production in infected insects (1), concerns have been raised regarding the possible impact of such agents released from the fragmenting of insects on soil microorganisms when *Steinernema carpocapsae* is used as an inundative insecticide against soil-dwelling insects. The present study investigates some factors influencing the production and stability of the antibiotics produced by *X. nematophilus* and the impact of these antibiotics on soil bacteria.

MATERIALS AND METHODS

Insects. Larvae of the greater wax moth *Galleria mellonella* were reared to the sixth instar by the method of Dutky et al. (10). Larvae weighing 200 mg each were used in the experiments.

Nematodes. Two different populations of IJ nematodes of *S. carpocapsae* (DD136 strain, originally supplied by Biosys Inc., Palo Alto, Calif.) were used in these studies: (i) a population from previously parasitized *G. mellonella* larvae (GI isolate) and (ii) a monoxenic population cultured with its mutualist, *X. nematophilus* subsp. *dutki*, on lipid-fortified nutrient agar for more than 24 months at Simon Fraser University (SFU isolate). GI nematodes were stored at 12°C on open-cell polystyrene foam with minimal water. IJ nematodes of the SFU group were isolated by allowing them to migrate through a 200-μm-pore-size mesh screen into distilled water. Aqueous suspensions of both groups of nematodes were surface disinfected with 0.2% thimerosal (Sigma) (1) and adjusted to 1,000 nematodes per ml of phosphate-buffered saline (PBS) (10) prior to injection into the insects.

To assess the contribution of the nematodes to antibiotic production, axenic juveniles from both populations were produced by a modification of the method of Popiel et al. (23).

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TABLE 1. Bacteria used to determine the activity spectrum of antibiotics produced by *X. nematophilus* subsp. *dutki*

Organism	Strain	Source
A. chroococcum	MCD1	K. Tibelius[a]
B. japonicum	31, 11	D. Smith[b]
P. mirabilis	MAC773	D. Niven[a]
Proteus vulgaris	MAC774	D. Niven
B. thuringiensis subsp. *kenyae*		Ren Gai-Xin[c]
B. sphaericus		Ren Gai-Xin
Alcaligenes faecalis	ATCC 8750	McGill University[a]
Bacillus cereus	ATCC 3329	McGill University
B. subtilis	ATCC 3329	McGill University
Enterobacter aerogenes	MAC438	McGill University
E. coli	K-12 (ATCC 9001)	McGill University
Pseudomonas aeruginosa	ATCC 9026	McGill University
S. marcescens	MAC21	McGill University

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Two hundred microliters of *X. nematophilus* subsp. *dutki* suspension was swabbed onto lipid-fortified nutrient agar. Plates were then incubated at 27°C for 24 h. IJs disinfected with thimerosal were placed on the bacterial lawns and incubated until gravid female nematodes were observed. The nematodes were washed from the agar surface with distilled water and suspended in 1 ml of sterilizing solution (0.4 M NaOH, 0.2 M NaHOCl) for 10 min. The suspension was centrifuged (11,000 × *g*, 3 min, 25°C), and the eggs were collected by aseptically pipetting the milky white top layer of the supernatant. The eggs were suspended in 25 ml of sterile MOPS (morpholinepropanesulfonic acid)-buffered saline (25 mM MOPS, 125 mM NaCl [pH 7.5]) and incubated at 25°C for 18 h, and the nematodes were collected and centrifuge washed several times in PBS prior to injection into the insects. Preliminary results established the absence of *Xenorhabdus* infection in these axenic juveniles and the presence of the bacteria in the juvenile nematodes of each isolate cultured with the bacteria.

Bacteria. An isolate of *X. nematophilus* subsp. *dutki* was obtained from each nematode isolate and labelled in the same way as the nematode isolate. The bacterial GI isolate was freshly obtained from *G. mellonella* larvae infected 24 h previously by the IJs. The SFU isolate, although initially obtained from parasitized insects, had been continuously cultured in vitro without its nematode symbiont for more than 24 months. The GI isolate, unless stated otherwise, was the main isolate used in the present study, and the SFU isolate was used for comparative purposes. Both isolates were maintained on tergitol-7 agar supplemented with 2,3,5-triphenyltetrazolium chloride (1). Bacterial species used to assay the activity spectrum of the antibiotics of *X. nematophilus* represented microorganisms commonly detected in soil (Table 1). All test bacteria were maintained on tryptic soy agar, except *Bradyrhizobium japonicum*, which was cultured on yeast salts agar supplemented with 5% (wt/vol) glycerol (14). The test bacteria were subcultured biweekly and incubated at 25°C in darkness.

Inocula of both isolates of *X. nematophilus*, unless otherwise stated, were prepared by growing the bacteria in 5 ml of tryptic soy broth in 20-ml culture tubes on a gyratory shaker (150 rpm) for 24 h at 27°C. The bacteria were centrifuge washed (11,000 × *g*, 20°C, 3 min) twice in PBS and adjusted to 5.4×10^7 cells per ml before use in subsequent experiments. The test bacteria (with the exception of *B. japonicum*) were grown in 5 ml of tryptic soy broth (150 rpm, 27°C, 24 h) and directly plated onto tryptic soy agar (10 ml per plate) with cotton swabs. The yeast salts medium with glycerol supplementation was used for *B. japonicum*.

Antibiotic extraction and bioassay. Water-soluble antibiotics were extracted from insect larvae killed by *X. nematophilus* by homogenizing the insects in distilled water (1 ml/g [wet weight] of tissue). Debris were removed by centrifugation (11,000 × *g*, 3 min, 25°C). Aliquots of the supernatant (100 μl; 25 μl in all subsequent assays) were placed on 10-mm-diameter disks of filter paper (Whatman no. 3) and air dried. The dried disks were placed on 10 ml of tryptic soy agar freshly inoculated with cotton swabs containing the test microorganisms. *Bacillus thuringiensis*, which is sensitive to antibiotics from *X. nematophilus* (3), was used to determine the relationship between the size of the zones of inhibited bacterial growth and the concentration of the antibiotic. The bacteria were also used to determine the levels of antibiotic activity in selected solvents that had been used to extract antibiotics from spent culture media. Studies of antibiotic activity established that *Bacillus subtilis* was most sensitive to the antibiotics of *X. nematophilus*. *B. subtilis* was used in all bioassays subsequent to the experiments on the efficiency of solvents for antibiotic extraction.

Zones of inhibition were measured after incubation of the plates for 18 h at 27°C. Measurements were taken from the edge of the antibiotic disk to the margin of the zone of inhibition. Antibiotic activity was expressed as units of activity per gram (wet weight) of insect tissue, where 1 U was defined as a 1.0-mm annular clearing around the antibiotic disk. For comparisons between test microorganisms or between media with a given bacterial species, the size of the zone of inhibition served as a measure of bacterial sensitivity or antibiotic titer, respectively.

To confirm the assumption that changes in the size of the zones of inhibition (expressed as units of activity per gram of insect tissue) represented changes in antibiotic concentrations, extracted antibiotics were diluted with water or concentrated by lyophilization and resuspension in fixed volumes of distilled water. The antibiotic levels, based upon dilution or concentration of the antibiotic extract, ranged from 1/10 to twice the concentration found in the insects. The relationship of the $\log_{10}$-transformed concentration of the antibiotic relative to the level in the unmodified extract (which was assigned a value of 1.00) (antibiotic levels of 0.37, 1.00, 1.29, 1.56, and 2.00 [log transformations of −0.43, 0.00, 0.11, 0.19, and 0.30, respectively]) to the diameter of the zone of inhibition (0.00, 13.8, 21, 31, and 37.5 mm) revealed a linear correlation ($r = 0.981$; $P > 0.05$). Similarly, $\log_{10}$-transformed concentrations of both antibiotics (1:1 ratio) (antibiotic concentrations of 0.5, 1.5, 2.5, and 2.0 μg/ml [log-transformed concentrations of −0.30, 0.21, 0.30, and 0.40, respectively]) produced inhibition zones (14.7, 22.3, 29.9, and 38.6 mm) that were linearly correlated ($r = 0.974$; $P > 0.05$). Thus, both procedures validated the use of the size of the zone of inhibition as an indicator of antibiotic concentration.

Effect of solvents on antibiotic extraction. Xenocoumacins are readily soluble in water and marginally soluble in organic solvents (18). To aid in the identification of the xenocoumacins, the insects killed by *X. nematophilus* were extracted in 2 ml of the following individual solvents: distilled water, 95% ethanol, 100% ethyl acetate (which extracts xenorhabdins [24]), and chloroform. Antibiotic activities of the extracts and the solvent controls were assayed as described above. Further confirmation of the identity of the antibiotics and the ratio of xenocoumacin 1 and 2 was based on the isolation of the antibiotics by the chromatographic procedures of McInerney

et al. (18) coupled to analytical reverse-phase C-18 high-performance liquid chromatography (HPLC). In the latter case, the mobile phase consisted of methyl cyanide-ammonium acetate (35:65, vol/vol; 0.2 M; pH 4.5) at a flow rate of 0.8 ml/min. Detection was based on A_{254}. Purified xenocoumacins 1 and 2 (donated by Biotech Australia Pty., Ltd., Roseville, Australia) were used as standards.

Effect of temperature on antibiotic activity. The numerous insect species against which the bacterial-nematode complex can be used occupy soils which may be subject to temperature fluctuations (depending on the geographic location and time of the season in which the IJs are used), and these fluctuations could influence the stability and persistence of the antibiotics in the field. Constraints associated with the efficiency of antibiotic extraction and antibiotic degradation from nonsterile soils precluded experiments assessing the effects of temperature on xenocoumacins in soil (the preferred method). Temperature effects were, therefore, examined using the antibiotics in aqueous insect extracts. Aliquots (100 μl) of aqueous extracts from larvae killed by *X. nematophilus* 36 h postinjection, were incubated in 1.5-ml microcentrifuge tubes at temperatures and time regimes of (i) a −20 to +20°C freeze-thaw cycle repeated daily for 8 days, (ii) 60 and 100°C for 1 h, and (iii) 121°C for 20 min. Negative controls consisting of extracts from larvae injected with PBS were similarly treated, while positive controls were neither chilled nor heated. Following treatment, the antibiotic extracts from each of four replicates were assayed for activity against *B. subtilis.*

Effect of growth conditions on production and activity of antibiotics. Antibiotic production has been detected for *X. nematophilus* in vivo and in culture media (2, 3). However, because medium composition influences the production of antimicrobial agents by *P. luminescens* (20), it was necessary to determine whether different growth conditions would change the bactericidal spectrum of antibiotics of *X. nematophilus*. The growth media used consisted of hemolymph in vivo and tryptic soy broth. Uninoculated media and larvae served as negative controls. Larvae were extracted with water, and the antibiotic sensitivity disks were prepared by using 25 μl of antibiotic extract. Antibiotic activity was tested against the microorganisms listed in Table 1. To test whether quantitative differences in antibiotic potency of the spent media were attributable to differences in the levels of the *X. nematophilus* isolates, total bacterial concentrations in both insect extracts and culture media at 36 h were determined with a Petroff-Hauser counter. Viable plate counts of *X. nematophilus* were done on the yeast salts medium of Dunphy and Webster (10).

Influence of nematodes on antibiotic production in insects. Insects can be induced to produce proteins lethal to *X. nematophilus*; however, *S. carpocapsae* effectively ensures the survival of the bacteria in these immunized insects by selectively destroying the antibacterial proteins (15). The nematodes might also influence antibiotic production and/or stability in vivo. To assess the impact of nematodes on antibiotic levels, both the GI and SFU isolates were used. Larvae were injected with 10 μl of PBS containing either (i) *X. nematophilus*, (ii) surface-disinfected IJ nematodes, or (iii) axenic nematodes at 1,000 nematodes per ml. Larvae were incubated at 27°C and extracted for antibiotics at death and every 12 h thereafter until 72 h postinjection.

Long-term stability of the antibiotics in insects. To test the long-term stability of the antibiotics, surface-disinfected IJ nematodes of both the GI and SFU populations were separately injected into larvae. This approach was a close approximation to the infection of insects in a field situation. Control insects were injected with PBS. Insects were incubated at 27°C for 0, 2, 6, 10, 15, 24, and 66 days after death before the antibiotics were extracted. All treatment experiments consisted of four replicates and a control group of five replicates, each of which contained five larvae.

Interaction of antibiotics with bacteria in soil. Soil (pH 5.73; organic matter, 2.7%; clay, 4%; silt, 18%; and sand, 78%) was collected from the top 10 cm of grassland (Simon Fraser University, Burnaby, British Columbia, Canada), air dried, and sieved (no. 30 sieve, 600-μm pore size). Larvae previously injected with 5 μl of bacterial suspension (5.0×10^6 bacteria per ml) of the *Xenorhabdus* isolates and which had been dead for 48 h were punctured with a needle to mimic the emergence stage of the nematodes from the cadavers and the subsequent release of antibiotics. The insects were encased in dialysis tubing at densities of one and five larvae (representing 250 mg and 1.25 g of larval tissue, respectively) per bag and buried in 10 g of soil that had been saturated with 7.5 ml of distilled water in a petri plate. The density of insects was chosen to represent numbers commonly encountered for soil-dwelling pest insects (for examples, see references 15 and 16) and could represent the potential release of 0.5 to 4.5 mg of antibiotics into the soil. To further assist antibiotic diffusion into the soil, distilled water (100 μl per insect) was added to each bag. The petri dishes were sealed with Parafilm and incubated at 24°C for 8 days, and the bacterial levels in the soil were determined every 2 days. Preliminary studies based upon changes in methylene blue-coated strips of filter paper established that the conditions in the soil would not be anaerobic by day 8. Soil controls consisted of dialysis tubing containing distilled water and larvae killed by extensive puncturing with a needle or by freezing followed by extensive puncturing of the cadaver. Killing by both methods produced similar viable plate count results for the soil bacteria.

Statistics. Values, unless otherwise stated, represent the means ± the standard errors. Statistical differences between means were determined by the Student-Newman-Keul test ($P = 0.05$), unless stated otherwise. Viable plate count data, which had a Poisson distribution, were analyzed by the 95% confidence limits overlap procedure. Correlation analyses of antibiotic levels and the zones of inhibition were based upon $\log_{10}$ transformation of the antibiotic dilution factors.

RESULTS

Effect of solvents on antibiotic extraction. Of the solvents used to extract antibiotics from the cadavers containing the GI isolate, only the distilled water extract showed antibiotic activity (20.7 ± 5.8 U/g). The HPLC analysis confirmed the presence of xenocoumacins 1 and 2 in a ratio of 1:1 in these water extracts. Collectively, the yield of these two antibiotics was 800 ng/200 mg of insect tissue. The antibiotic levels in cadavers containing the SFU isolate and in tryptic soy broth containing either bacterial isolate were not determined.

Effect of temperature on antibiotic activity. The activity of the total antibiotic extract declined with increasing temperature (unheated, 34.4 ± 0.6 U/g; 60°C, 29.2 ± 0.1 U/g; and 100°C, 16.2 ± 0.1 U/g), with some activity present after autoclaving (15.4 ± 0.3 U/g). Although heating initiated the precipitation of extracted insect proteins, coprecipitation of the proteins and antibiotics did not occur, since insect protein precipitation induced by 10% (vol/vol) trichloroacetic acid did not lower antibiotic activity (30.5 ± 0.3 U/g) below that of the extract which received distilled water in lieu of acid (31.7 ± 0.6 U/g). Freeze-thawing cycles did not decrease antibiotic levels (27.8 ± 2.8 U/g) below those of the unheated control samples and samples heated to 60°C.

TABLE 2. Activity spectra and titers of antibiotics produced by the GI and SFU isolates of *X. nematophilus* subsp. *dutki* in the insect *G. mellonella* and tryptic soy broth

Test bacterium	Antibiotic activity (U/ml)[a] in:			
	Insect extract		Tryptic soy broth	
	GI	SFU	GI	SFU
B. subtilis	328.0 ± 6.0[b]	344.0 ± 8.0	55.0 ± 2.0	54.0 ± 1.0
A. chroococcum	266.0 ± 0.0	286.0 ± 6.0	0.0 ± 0.0	0.0 ± 0.0
B. cereus	214.0 ± 4.0	262.0 ± 4.0	9.0 ± 1.0	18.0 ± 9.0
B. sphaericus	206.0 ± 6.0	272.0 ± 2.0	11.0 ± 1.0	12.0 ± 1.0
B. thuringiensis	192.0 ± 8.0	244.0 ± 4.0	20.0 ± 0.0	2.0 ± 0.0
P. aeruginosa	146.0 ± 10.0	0.0 ± 0.0	25.0 ± 2.0	2.0 ± 0.0
E. coli	144.0 ± 6.0	ND[c]	20.0 ± 0.0	ND
E coli K-12	94.0 ± 12.0	158.0 ± 4.0	15.0 ± 1.0	6.0 ± 1.0
E. aerogenes	122.0 ± 2.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
P. vulgaris	26.0 ± 10.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
P. mirabilis	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
A. faecalis	38.0 ± 2.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
S. marcescens	22.0 ± 6.0	52.0 ± 2.0	0.0 ± 0.0	0.0 ± 0.0
B. japonicum 31	184.0 ± 8.0	80.0 ± 4.0	4.0 ± 1.0	0.0 ± 0.0
B. japonicum 11	116.0 ± 16.0	26.0 ± 6.0	2.0 ± 0.0	0.0 ± 0.0

[a] Comparison of antibiotic activities within a growth environment against different test bacteria is indicative of bacterial sensitivity. Comparison between media with a given test bacterium indicates antibiotic titers. 1 U = 1 mm of annular clearing around the test microbe.
[b] Mean ± standard error, $n = 3$.
[c] ND, not done.

Effects of growth conditions on the antibiotic activity spectra of xenocoumacins. The bactericidal spectrum and relative activities of the antibiotic extract varied with the growth conditions and between the two isolates of *X. nematophilus* (Table 2). Inhibition of both gram-positive and -negative bacteria was seen, with the greatest sensitivity shown by gram-positive bacteria, including the insect pathogens *Bacillus sphaericus* and *B. thuringiensis*. *B. subtilis* was the most sensitive of the bacteria tested and *Proteus mirabilis* was the least sensitive.

Generally, after allowing for dilution effects, spent broth media showed less antibiotic activity, and had more restricted activity, than the extracts of insects containing either isolate of *X. nematophilus*. Differences between isolates were most pronounced with the insect extracts; the antibiotics of the SFU isolate were more active against *B. sphaericus*, *B. thuringiensis*, *Escherichia coli* K-12, and *Serratia marcescens* than were those from the GI isolate. However, the antibiotics of the GI isolate were more effective than those of the SFU isolate against the remaining gram-negative bacteria.

There was no statistical difference in viable plate counts between the two bacterial isolates in the same growth environment (tryptic soy broth: SFU isolate, $2.1 \times 10^8 \pm 5.2 \times 10^6$ CFU/ml, and GI isolate, $1.7 \times 10^8 \pm 5.3 \times 10^6$ CFU/ml [$P > 0.05$]; and insect extract: SFU isolate, $1.3 \times 10^9 \pm 3.6 \times 10^8$ CFU/ml, and GI isolate, $1.1 \times 10^9 \pm 3.9 \times 10^8$ CFU/ml [$P > 0.05$]). However, the insect growth environment supported 10-fold greater bacterial numbers and substantially higher antibiotic titers (Table 2).

Induction of xenocoumacins in *G. mellonella* larvae. Antibiotic induction profiles and titers varied with the bacterial isolate and the presence or absence of the corresponding isolates of nematodes (Fig. 1). In all samples, antibiotic activity was detected after the demise of the insect. The GI isolate of *X. nematophilus* initiated antibiotic production sooner than did the bacteria in the presence of monoxenic nematodes. The antibiotic titer for larvae containing the bacteria with the monoxenic nematodes was comparable to that for larvae with bacteria alone, with maximum titers occurring at 36 h postinjection. Larvae injected with monoxenic nematodes and bacteria exhibited a plateau from 36 to 72 h. There was no discernible antibiotic activity in extracts of insects injected with axenic nematodes or with heat-killed bacteria or IJs. Antibiotic activity in freeze-killed larvae injected with bacteria or monoxenic nematodes was similar to activity in live insects (data not shown).

The SFU isolate of *X. nematophilus* and of the respective

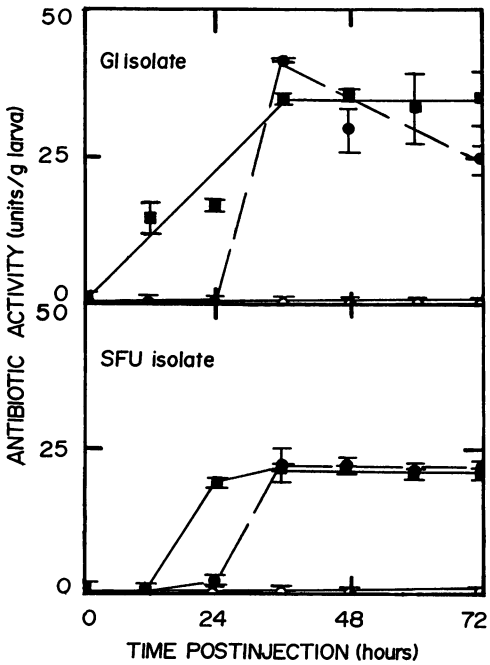

FIG. 1. Antibiotic activity developing in larvae of *G. mellonella* inoculated with the GI and SFU isolates of *X. nematophilus*. Insects were injected with axenic *S. carpocapsae* (DD136 strain) (○), monoxenic *S. carpocapsae* (●), and *X. nematophilus* (■). Larvae injected with PBS had no antibiotic activity (data not shown).

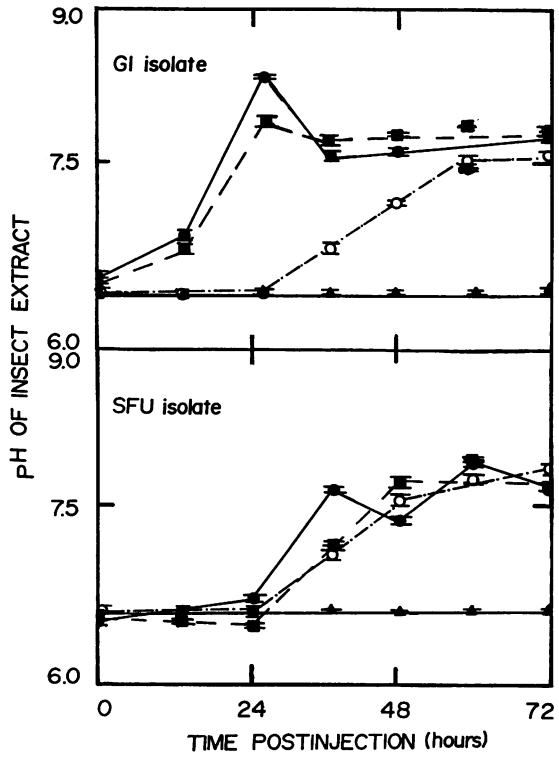

FIG. 2. pH of *G. mellonella* larval extracts from cadavers containing the GI and SFU isolates of *X. nematophilus* and *S. carpocapsae* (DD136 strain) following treatment with PBS (▲), bacteria, (●), SFU monoxenic nematodes (■), and axenic nematodes (○).

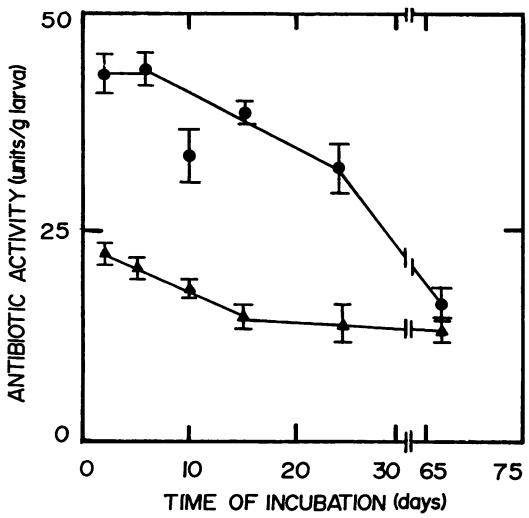

FIG. 3. Levels of antibiotics in cadavers of *G. mellonella* containing the GI (●) and SFU (▲) isolates of *S. carpocapsae* (strain DD136) after incubation at 27°C over time.

monoxenic nematodes produced less antibacterial activity in the insects than did their GI counterparts. Antibiotic activity was lowest in larvae injected with the SFU isolate of monoxenic nematodes during the initial 24 h. However, by 36 h the levels of antibiotics were comparable to those for insects infected by bacteria alone. The 24-h lag phase in antibiotic activity was evident in larvae with both the GI and SFU populations of monoxenic nematodes.

The levels of *X. nematophilus* at 36 h postinjection varied with the treatments: those insects injected with axenic nematodes and monoxenic nematodes did not contain *X. nematophilus* detectable by plating at 36 h. However, larvae injected with the bacterial isolates contained concentrations of bacteria similar to one another by 36 h postinjection (GI isolate, $9.6 \times 10^8 \pm 1.3 \times 10^7$ CFU/ml; and SFU isolates, $1.2 \times 10^9 \pm 0.5 \times 10^8$ CFU/ml [$P > 0.05$]). By 72 h, there was no evidence of culturable *X. nematophilus* in any of the extracts. There was no correlation between antibiotic titer (Fig. 1) and the level of either bacterial isolate at 36 or 72 h postinjection.

Larvae infected with the monoxenic nematodes of each isolate were prone to liquefaction and cuticular rupture. Insects exposed to the different bacterial and nematode isolates (but not those containing axenic nematodes) contained small, rod-shaped, gram-negative bacteria that were catalase positive and formed blue-grey colonies on tergitol-7 agar with tetrazolium chloride and, thus, were not regarded as *X. nematophilus*. These bacteria occurred independently of liquefaction and increased in concentration over time (pooled data at 24 h postinfection, $1.0 \times 10^6 \pm 0.2 \times 10^5$ CFU/ml; and at 72 h postinfection, $2.7 \times 10^9 \pm 0.3 \times 10^7$ CFU/ml).

Injections of each isolate of *X. nematophilus* and monoxenic nematodes into the insects elevated the insect extracts to a pH slightly greater than 8 by 24 h postinjection for the SFU isolate and to pH 7.7 by 60 h for the GI isolate (Fig. 2). Thereafter, the pH remained essentially constant. Axenic nematodes from both sources elevated the pH of the cadavers but at rates lower than those injected with bacteria alone and lower than those of insects with the monoxenic nematodes of the GI population. There was no correlation between extract pH and antibiotic activity. The use of buffers at pH 8.0 and 8.5 in lieu of extracts established that localized pH changes on the agar test plates did not produce inhibition zones.

Prolonged incubation of cadavers containing the GI isolate of monoxenic nematodes established antibiotic levels (based on zones of inhibition) to be stable for up to 6 days, declining thereafter to 60% of the initial level by day 66 (Fig. 3). Qualitative HPLC analysis detected xenocoumacin 1 and 2 in cadavers 66 days after death. Titers of the antibiotics with the SFU isolate exhibited a continuous decline from 14 h, leveling off at day 15 at approximately 60% of the original level.

Impact of antibiotics on soil bacteria. One larva and a group of five larvae without either isolate of *X. nematophilus* served as the soil nutrient augmentation controls and essentially did not elevate bacterial counts above the distilled water (negative-control) levels (Table 3). Soil containing one larva killed by each of the bacterial isolates transiently reduced bacterial levels below the nutrient augmentation control levels ($P < 0.05$). However, only the GI isolate lowered bacterial numbers below the distilled water control levels. This effect persisted for up to 6 days. Similarly, five larvae killed by both bacterial isolates transiently lowered the numbers of bacteria from days 2 to 8, the effect being most pronounced for the GI strain.

DISCUSSION

Soil-dwelling insects and the overwintering stages of aerial phytophagous insects experience a range of temperatures while in and on soil. The temperatures vary with the geographical location, insect species, time within the season, and soil type and may represent a collective range of −10 to 60°C (16). Antibiotic activity of the GI isolate was stable over this temperature range including the range covering the early

TABLE 3. Numbers of bacteria in soil (CFU × 10^8/g) exposed to antibiotics of the GI and SFU isolates of *X. nematophilus* cultured in killed larvae of *G. mellonella*

Treatment	Isolate	No. of bacteria (CFU × 10^8/g)[a] after days:			
		2	4	6	8
1 cadaver, + bacteria	SFU	75 ± 12 (b)	60 ± 7 (b)	55 ± 4 (b)	96 ± 11 (b)
5 cadaver + bacteria	SFU	131 ± 10 (c)	62 ± 7 (b, d)	48 ± 2 (b, d)	61 ± 3 (b, d)
1 cadaver − bacteria	SFU	95 ± 12 (b, c, e)	102 ± 9 (e)	84 ± 5 (e)	92 ± 15 (b, e)
5 cadavers − bacteria	SFU	219 ± 14 (f)	138 ± 10 (e, g)	104 ± 8 (e, g)	124 ± 16 (b, e, g)
1 cadaver + bacteria	GI	89 ± 10 (b, e, h)	52 ± 5 (b, d, i)	47 ± 3 (b, d, i)	72 ± 9 (b, d, e, i)
5 cadavers + bacteria	GI	95 ± 7 (e, j, h)	46 ± 5 (b, d, i, k)	41 ± 3 (b, d, i, k)	53 ± 3 (b, d, i, e, k)
1 cadaver − bacteria	GI	110 ± 7 (c, h, i, l)	124 ± 5 (l)	114 ± 6 (l)	89 ± 11 (b, d, i, l)
5 cadavers − bacteria	GI	197 ± 10 (m)	155 ± 11 (l, m)	132 ± 9 (l, m)	137 ± 12 (m)
Distilled water (pooled controls)		177 ± 12 (m, n)	123 ± 11 (l, n)	98 ± 4 (e, g, l, o)	115 ± 9 (b, e, m, n)

[a] Mean ± standard error of the mean, $n = 10$. Means with the same letters (b, c, d, e, f, g, h, i, j, k, l, m, n, or o) are not statistically different ($P > 0.05$).

spring freeze-thaw cycles range found in Québec, Canada. The detection of activity after incubation at higher temperatures confirmed the heat stability of the antibiotics reported by Akhurst (3).

Antibacterial activity was greatest against bacteria of the family *Bacillaceae*, moderate against those of the family *Pseudomonadaceae*, and least against the family *Enterobacteriaceae*. The bactericidal spectrum and antibiotic activity titers varied with the isolate of *X. nematophilus* and the growth environment. Akhurst (2, 3), Boemare and Akhurst (7), and Chen (9) described semiquantitive variation in antibiotic production by *X. nematophilus*, and Nealson et al. (20) reported that the type of medium influenced antibiotic production by *P. luminescens*.

Antibiotic production in the present study was detected after the death of the insects, thereby precluding antibiotics as factors that enhance bacterial virulence against insects. The titer may have represented the combined effects of xenocoumacins and the nonspecifically induced antibacterial proteins, i.e., cecropins and attacins, released from the living insect's fat body tissue (12). Although *X. nematophilus* is susceptible to lysis by these proteins (15), in this study induction of immune proteins is unlikely because (i) the dose of bacteria required to kill the larvae within 72 h does not induce antibacterial proteins (10, 15) and the dosage used in this study exceeded the dose causing the insects to die within 24 h, (ii) the bacterial dosage induced the rapid destruction of the fat body and hemocyte cells within 2 h of infection, reducing the probability of antibacterial protein synthesis (which requires 6 to 8 h postinfection for induction by avirulent bacteria), (iii) the antibiotic induction profiles and titers produced in living larvae containing the GI isolate of *X. nematophilus* and its monoxenic symbiont were comparable to those produced in dead larvae, (iv) dead *X. nematophilus* cells did not induce antibacterial activity, and (v) Chen (9) did not detect antibiotic activity in larvae infected with the antibiotic-negative phase two variant of the bacteria. The actual titer varied with the isolate of pathogen and medium and was influenced by the presence of nematodes but only in terms of initial antibiotic production. The different antibiotic titers from the different growth environments may represent different levels of xenocoumacin 1 and 2 production. It has been suggested that the antibiotics in insects preclude adventitious contamination of the insect's hemocoel by microorganisms introduced by the IJ stage of nematodes (4). Barbercheck and Kaya (5) reported that depending on the sequence of contact of insects with the nematodes and other pathogens in multiple infections and the temperature of infection, the antibiotics of *Xenorhabdus* and *Photorhabdus* spp. may preclude the development of other insect pathogens. However, in the present study the IJs of both the GI and SFU populations did not totally prevent infection by bacteria other than *X. nematophilus*. The bacterial contaminants may represent bacteria in the insect gut that invaded the hemocoel. The gut itself exhibits extensive degeneration of the muscularis during infection by the monoxenic nematodes. These contaminating bacteria were resistant to the antibiotics, increasing in numbers when the antibiotic titers were high. Thus, the later stages of nematode development in the insect do not occur as previously suggested in an environment composed entirely of its symbiont (4).

Both Chen (9) and we observed the absence of *X. nematophilus* by 72 h postinfection, using either direct microscopic examination or viable plate counts. Despite this, the antibiotic titers remained high. It is possible that the bacteria became attached to insect tissues, perhaps in a viable but nonculturable form. This raises the questions of where nematodes acquired phase one *X. nematophilus* when the IJ stage was formed and whether the bacteria are directly available as nutrients for the nematodes or whether they indirectly provide nutrition.

Generally, antibiotic production in soil is limited by nutrient restriction (25). However, in view of the production of high levels of antibiotics in the nutrient-rich insect cadaver, it is possible that the long-lasting antibiotics would have substantial impact on soil microorganisms. Although vegetative *B. thuringiensis* was very sensitive to the antibiotics, it is not likely that the bacterium will be harmed in the soil, since its vegetative form rarely exists in the soil. However, the antimicrobial agents might influence the recycling of *B. sphaericus* and the free-living nitrogen-fixing bacterium *Azotobacter chroococcum*. Both *Xenorhabdus* isolates transiently and marginally reduced the numbers of soil bacteria below those of the water controls, but the effect varied in intensity with the number of insects (and thus antibiotic concentration) and with the bacterial isolate. The effects persisted for a few days, during which the antibiotic levels in the larvae in the soil declined by approximately 60% (present study; 9). Chen (9) reported higher bacterial numbers after 8 days; however, it remains to be determined whether the increase or the plateau levels shown in the present study represent proportionate changes for the different functional groups of bacteria or selection of specific bacterial groups. Although these antibiotics could affect soil microorganisms such as those in the rhizosphere, their influence in the soil in the present study is limited. The impact of the antibiotics on the total area of application would be additionally restricted by the inherently clustered distribution of the insects and the binding of the antibiotics to soil particles.

Chemical or biological inactivation reported for antibiotics added to soils may further reduce the already marginal effects of xenocoumacins on soil bacteria.

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REFERENCES

1. **Akhurst, R. J.** 1980. Morphological and functional dimorphism in *Xenorhabdus* spp., bacteria symbiotically associated with the insect pathogenic nematodes *Neoaplectana* and *Heterorhabditis*. J. Gen. Microbiol. **121**:303–309.
2. **Akhurst, R. J.** 1982. A *Xenorhabdus* sp. (Eubacteriales: Enterobacteriaceae) symbiotically associated with *Steinernema kraussei* (Nematoda: Steinermatidae). Rev. Nematol. **5**:277–280.
3. **Akhurst, R. J.** 1982. Antibiotic activity of *Xenorhabdus* sp., bacteria symbiotically associated with insect pathogenic nematodes of the families Heterorhabditidae and Steinernematidae. J. Gen. Microbiol. **128**:3061–3065.
4. **Akhurst, R. J., and M. E. Boemare.** 1990. Biology and taxonomy of *Xenorhabdus*, p. 75–92. *In* R. Gaugler and H. K. Kaya (ed.), Entomopathogenic nematodes in biological control. CRC Press, Inc., Boca Raton, Fla.
5. **Barbercheck, M. E., and H. K. Kaya.** 1990. Interactions between *Beauveria bassiana* and the entomogenous nematodes, *Steinernema feltiae* and *Heterorhabditis heliothidis*. J. Invertebr. Pathol. **55**:225–234.
6. **Bleakley, B., and K. N. Nealson.** 1988. Characterization of primary and secondary forms of *Xenorhabdus luminescens* strains HM. FEMS Microbiol. Ecol. **53**:241–250.
7. **Boemare, N. E., and R. J. Akhurst.** 1988. Biochemical and physiological characterization of colony form variants in *Xenorhabdus* spp. (Enterobacteriaceae). J. Gen. Microbiol. **134**:751–761.
8. **Boemare, N. E., R. J. Akhurst, and R. G. Mourant.** 1993. DNA relatedness between *Xenorhabdus* spp. (*Enterobacteriaceae*), symbiotic bacteria of entomopathogenic nematodes, and a proposal to transfer *Xenorhabdus luminescens* to a new genus, *Photorhabdus* gen. nov. Int. J. Syst. Bacteriol. **43**:249–255.
9. **Chen, G.** 1992. Effect of antibiotics produced by *Xenorhabdus* spp. on soil bacteria. M.S. thesis. Simon Fraser University, Burnaby, British Columbia, Canada.
10. **Dunphy, G. B., and J. M. Webster.** 1986. Influence of the Mexican strain of *Steinernema feltiae* and its associated bacterium *Xenorhabdus nematophilus* on *Galleria mellonella*. J. Parasitol. **72**:130–135.
11. **Dutky, S. R., J. V. Thompson, and G. E. Cantwell.** 1962. A technique for mass rearing of the greater wax moth (Lepidoptera: Galleridae). Proc. Entomol. Soc. Wash. **64**:56–58.
12. **Faye, I., and D. Hultmark.** 1993. The insect immune proteins and the regulation of their genes, p. 25–53. *In* N. E. Beckage, S. N. Thompson, and B. A. Federici (ed.), Parasites and pathogens of insects, vol. 2. Pathogens. Academic Press, Inc., Toronto.
13. **Forschler, B. T., and W. A. Gardner.** 1991. Parasitism of *Phyllophaga hirticula* (Coleoptera: Scarabaeidae) by *Heterorhabditis heliothidis* and *Steinernema carpocapsae*. J. Invertebr. Pathol. **58**:396–407.
14. **Foustaci, J. M., I. Sangwan, and M. R. O'Brian.** 1991. Aerobic growth and respiration of a δ-aminolevulinic acid synthase (*hemA*) mutant of *Bradyrhizobium japonicum*. J. Bacteriol. **173**:1145–1150.
15. **Götz, P., A. Boman, and H. G. Boman.** 1981. Interactions between insect immunity and an insect-pathogenic nematode with symbiotic bacteria. Proc. R. Soc. Lond. Ser. B Biol. Sci. **212B**:333–350.
16. **Jausson, R. K., S. H. Lecrone, and R. Gaufler.** 1992. Comparison of single and multiple release of *Heterorhabditis bacteriophora* Poinar (Nematoda: Heterorhabditidae) for control of *Cylas formicarius* (Fabricius) (Coleoptera: Apionidae) J. Biol. Control **1**:320–328.
17. **Klein, M. G.** 1990. Efficacy against soil-inhabiting insect pests, p. 195–214. *In* R. Gaugler and H. K. Kaya (ed.), Entomopathogenic nematodes in biological control. CRC Press, Boca Raton, Fla.
18. **McInerney, B. V., R. P. Gregson, M. J. Lacey, R. J. Akhurst, G. R. Lyons, S. H. Rhodes, and D. R. J. Smith.** 1991. Biologically active metabolites from *Xenorhabdus* spp. Part 1. Dithiolopyrrolone derivatives with antibiotic activity. J. Nat. Prod. (Lloydia) **54**:774–784.
19. **McInerney, B. V., W. C. Taylor, M. J. Lacey, R. J. Akhurst, and R. P. Gregson.** 1991. Biologically active metabolites from *Xenorhabdus* spp. Part 2. Benzopyran-l-one derivatives with gastroprotective activity. J. Nat. Prod. **54**:785–795.
20. **Nealson, K. H., T. M. Schmidt, and B. Bleakley.** 1990. Physiology and biochemistry of *Xenorhabdus*, p. 271–284. *In* R. Gaugler and H. K. Kaya (ed.), Entomopathogenic nematodes in biological control. CRC Press, Inc., Boca Raton, Fla.
21. **Paul, V. J., S. Frautschy, W. Fenical, and K. H. Nealson.** 1981. Antibiotics in microbial ecology: isolation and structure assignment of several new antibacterial compounds from the insect symbiotic bacteria *Xenorhabdus* spp. J. Chem. Ecol. **7**:589–597.
22. **Poinar, G. O., Jr.** 1991. Biology and taxonomy, p. 23–62. *In* R. Gaugler and H. K. Kaya (ed.), Entomopathogenic nematodes in biological control. CRC Press, Inc., Boca Raton, Fla.
23. **Popiel, I., D. L. Grove, and M. J. Friedman.** 1989. Infective juvenile formation in the insect parasitic nematode *Steinernema feltiae*. Parasitology **88**:77–81.
24. **Richardson, W. H., T. M. Schmidt, and K. H. Nealson.** 1988. Identification of an anthraquinone pigment and a hydroxystilbene antibiotic from *Xenorhabdus luminescens*. Appl. Environ. Microbiol. **54**:1602–1605.
25. **Williams, S. T., and J. C. Vickers.** 1986. The ecology of antibiotic production. Microb. Ecol. **12**:43–52.
26. **Wouts, W. M.** 1981. Mass production of the entomogenous nematode *Heterorhabditis heliothidis* (Nematoda: Heterorhabditidae) on artificial media. J. Nematol. **13**:467–469.