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Growth and Virulence of *Steinernema glaseri* Influenced by Different Subspecies of *Xenorhabdus nematophilus*[1]

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Abstract: Three *Xenorhabdus nematophilus* subspecies influenced *Steinernema glaseri* growth profiles and growth rates, but this was not necessarily because of different bacterial growth rates. Virulence of dauer nematodes in larval *Galleria mellonella* varied with the number of dauers retaining bacteria and the bacterial subspecies. Virulence was least for dauers grown on *X. nematophilus* subsp. *bovienii* because of the lack of retained bacteria. Virulence was subsequently restored by culturing these nematodes on *X. nematophilus* subsp. *poinari*.

Key words: Steinernema glaseri, Xenorhabdus nematophilus, Galleria mellonella, dauer juveniles, bacterial retention, virulence.

Dauer juveniles of the genus *Steinernema* carry the Gram negative bacterium *Xenorhabdus nematophilus* within an intestinal vesicle (7) and void them into the host insect's hemolymph (22,23). The nematodes and their bacteria tolerate the nonimmune insect's humoral and cellular resistance systems (10,11). Septicemia ensues, and the nematodes consume the insect cadaver and (or) bacteria, grow, and reproduce (1).

The bacteria are dimorphic, existing as both primary and secondary forms (1). The primary form is carried by the dauer nematode, produces antibiotics, takes up bromothymol blue (2,3), and best supports nematode growth and reproduction (1).

Steinernema glaseri (= *Neoaplectana glaseri* [30]) has shown substantial promise as a biological control agent of economically important cryptic insects (5,14,15,19,20, 24,26,27). Glaser (15) described an in vitro nematode culture system using *Popillia japonica*. Recently Bedding (5,6) developed an economically efficient in vitro production method, utilizing either pork kidney and beef fat or chicken offal homogenized and dispersed on crumbed polyurethane sponge and inoculated with the *X. nematophilus* subspecies (e.g., *X. nematophilus* subsp. *poinari* [25]) prior to inoculation with *S. glaseri* dauer juveniles.

Akhurst (2) documented the retention of primary form bacteria by *S. glaseri* dauer juveniles and the enhanced nematode growth on primary *Xenorhabdus*. Akhurst and Brooks (4) described the isolation of another *S. glaseri* strain and the production of antibiotics by the primary *X. nematophilus* subsp. *poinari*. The previous isolates of the bacteria did not produce antibiotics (4). The bacterial nutritional requirements of steinernematids is relatively nonspecific in that many of the steinernematids grow and reproduce to varying degrees by utilizing the bacteria of related steinernematids but not those of heterorhabditids (2,27). *Xenorhabdus* subspecies vary in their virulence for a given host (2).

In this study *S. glaseri* was reared on three primary *X. nematophilus* subspecies with the objectives of enhancing dauer juvenile virulence and yield of nematodes.

Materials and Methods

Nematodes: Steinernema glaseri (from W. Brooks, University of North Carolina, Raleigh), the Mexican strain of *S. feltiae* (from G. O. Poinar, Jr., University of California, Berkeley), and the T335 strain of *S. bibionis* (from R. A. Bedding, CSIRO, Hobart, Tasmania) were maintained in monoxenic culture on fortified lipid agar (29) that had been inoculated 24 hours earlier with the corresponding strain of primary *Xenorhabdus nematophilus*. Nematode stock cultures were subcultured biweekly. Nematode inocula were surface sterilized in 0.1% merthiolate for 15 minutes (27) before being added to fresh medium. Once a month the dauer juveniles were collected in White's water traps (28) and passed through larvae of *Galleria mellonella*; the emerging dauer juveniles were used to initiate new stock cultures.

Axenic *S. glaseri* cultures were prepared according to Dunphy and Webster (10) and

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maintained on sterile bovine liver homogenate in 1.5% (w/v) agar. All nematode cultures were incubated at 25 C.

Bacteria: Primary *X. nematophilus* subsp. *poinari* (Xnp), *X. nematophilus* subsp. *nematophilus* var. *mexicanus* (Xnn) and *X. nematophilus* subsp. *bovienii* (Xnb) were isolated from dauer juveniles of *S. glaseri, S. feltiae* (Mexican strain), and *S. bibionis,* respectively. Surface-sterilized dauer juveniles were incubated in 2 ml Grace's insect tissue culture medium (16) supplemented with 20% (v/v) heat-inactivated fetal calf serum (Grand Island Biological Company, Burlington, Ontario) at 25 C for 12 hours. The cultures were streaked on tergitol-7-agar (Difco, Detroit, Michigan) containing triphenyltetrazolium chloride (= TTC medium [1]). Pure cultures of primary bacteria were obtained after 48 hours incubation at 25 C. Culture purity was tested by subculturing colonies on TTC medium. Bacteria were routinely subcultured every 2 weeks on TTC medium. All bacterial cultures were incubated at 25 C.

Insects: G. mellonella were reared according to Dutky et al. (13), and sixth-instar juveniles, weighing 125 ± 7 mg were used for nematode passaging.

Nematode and bacterial growth: Lawns of the three bacterial subspecies were prepared by growing the bacteria in lipid broth (29) at 25 C for 16 hours in shake culture (100 rpm). The concentrations of bacterial subspecies were not significantly different at that time (1.2×10^9 bacteria/ml, $P > 0.05$). Two milliliters bacterial suspension were pipetted onto 30 ml solidified lipid agar. The petri dishes were swirled to ensure uniform bacterial dispersion over the agar surface. Lawns were visible after incubation at 25 C for 24 hours.

One milliliter sterile distilled water containing 425 axenic dauer juveniles of *S. glaseri* was added to the bacterial lawns. Nematode inocula were swirled to disperse the juveniles before inverting the petri dishes and incubating the monoxenic nematode cultures at 25 C.

Five plates from each of the three culture types were flooded with 10 ml distilled water at 72-hour intervals. Cultures were incubated at 25 C for 10 minutes to dislodge juvenile nematodes from the agar–bacteria matrix. A rubber policeman was used to complete nematode removal. The resulting suspension contained randomly dispersed nematodes, a few nematode aggregations, and some ruptured females containing developing juveniles. Complete nematode dispersion was achieved by briskly pipetting the nematode suspensions through silanized Pasteur pipettes. The number of nematodes per plate was estimated by averaging the nematode numbers in five samples containing 100-µl aliquots from each suspension.

To determine whether different nematode growth rates reflect different bacterial growth rates, the population doubling times of the bacterial subspecies in lipid broth were determined using total bacterial counts (10). The procedure was repeated with bacterial cultures containing 100 axenic *S. glaseri* dauer juveniles. Fifteen bacterial samples from the axenic bacterial cultures and nematode–bacterial cultures were examined at 2-hour intervals.

LD_{50} determination: Dauer juveniles of *S. glaseri* were exposed to larval *G. mellonella,* and the level of larval insect mortality was determined for groups of 10 juveniles per dose after 96 hours (12). Three samples were used for each dauer dosage. Probit-mortality regression slopes and LD_{50} were determined according to Hewlett and Plackett (17).

Bacterial retention: Dauer juveniles cultured on different bacterial subspecies were examined for bacterial retention using four procedures: phase contrast microscopical examination of squashed nematodes (2), fluorescent microscopical examination of nematodes stained with Hoechst 33258 (9), differential interference contrast microscopy (7), and viable plate counts of bacteria released into culture medium.

The last technique consisted of adding individual surface-sterilized monoxenically grown dauer juveniles to 2 ml Grace's medium in individual sterile containers. At 30-minute intervals, 100 µl medium was aseptically diluted with 100 µl Ringer's insect saline and plated on yeast–salts agar (1). The number of colony forming units per milliliter was determined after incubation at 25 C for 72 hours. This procedure facilitated determination of bacterial growth rates based upon viable plate counts. Control bacterial growth rates were

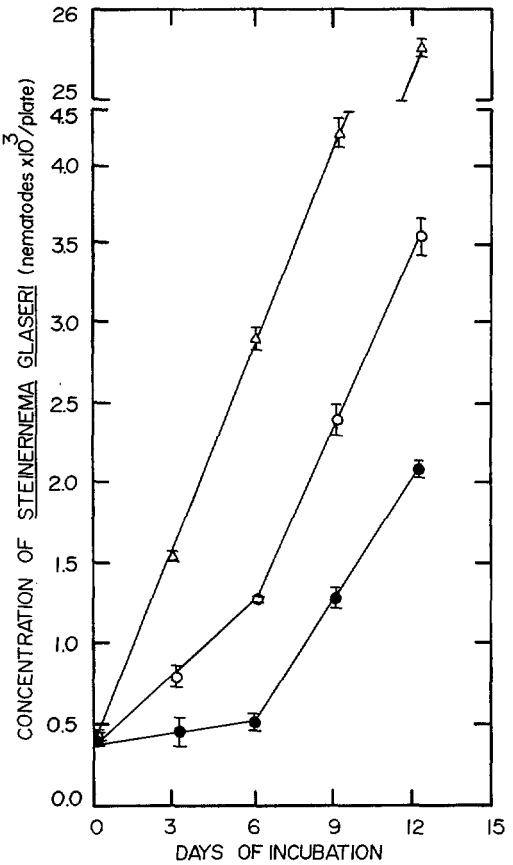

FIG. 1. Growth of *Steinernema glaseri* on fortified lipid agar at 25 C inoculated with either *Xenorhabdus nematophilus* subsp. *bovienii* (△), *X. nematophilus* subsp. *nematophilus* var. *mexicanus* (○) or *X. nematophilus* subsp. *poinari* (●).

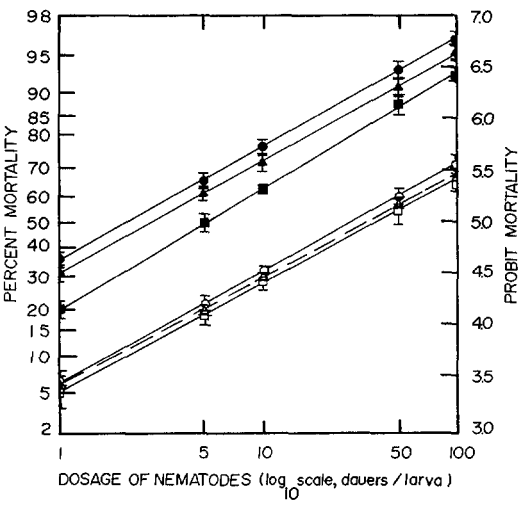

FIG. 2. Probit-mortality regressions of *Steinernema glaseri* dauer juveniles from different cultures of *Xenorhabdus nematophilus* subspecies for sixth instar *Galleria mellonella* at 25 C for 96 hours. Dauer juveniles from cultures of *X. nematophilus* subsp. *bovienii* series one (○), series two (□); *X. nematophilus* subsp. *poinari* (●); *X. nematophilus* subsp. *nematophilus* var. *mexicanus* (■); *X. nematophilus* subsp. *bovienii* and transferred to *X. nematophilus* subsp. *poinari* (▲); and axenically grown dauer juveniles (△).

determined for bacteria added to Grace's medium to produce the same bacterial level as those initially released from the dauer juveniles.

Three groups of 50 samples, each containing one dauer, were examined using the first three microscopical procedures. In the last procedure, five groups containing 100 samples with one dauer per sample were used to determine the number of bacteria per dauer juvenile and the percentage of dauer juveniles with bacteria. Bacterial growth rates were based on viable plate counts from 15 samples.

Statistical analysis: All data, except the percentage data, are represented as the mean ± standard error. The percentage data represent decoded means with 95% confidence limits of 2 $\arcsin\sqrt{p}$ transformed data. With the exception of the probit-mortality data, all data were analysed using Student's *t*-test.

RESULTS

Steinernema glaseri growth profiles varied with the bacterial subspecies (Fig. 1). Nematodes from Xnb cultures grew rapidly with a constant population doubling time of 3.0 ± 0.2 days. Nematodes on cultures of Xnp and Xnn possessed biphasic growth profiles. The initial doubling time of *S. glaseri* from the Xnp bacterial culture (21.9 ± 1.4 days) was greater than the nematode doubling time in the Xnn bacterial culture (6.9 ± 0.3 days; $P < 0.05$). These doubling times persisted for 6 days after which they declined to 3.7 ± 0.3 days for Xnp and 3.5 ± 0.2 days for Xnn. These accelerated growth rates were not different from that of *S. glaseri* on Xnb ($P > 0.05$). Subculturing the nematodes from the rapid growth phase to fresh, corresponding bacterial lawns always produced the same biphasic growth profiles.

The doubling times of Xnb, Xnp, and Xnn were not statistically different from each other or in the presence (0.9 ± 0.2 hours, 1.1 ± 0.2 hours, and 0.9 ± 0.1 hours, respectively; $P > 0.05$) or absence (0.8 ±

TABLE 1. Retention of selected subspecies of *Xenorhabdus nematophilus* by *Steinernema glaseri* dauer juveniles.

X. nematophilus subspecies	Percentage of dauers with bacteria*			
	Phase contrast microscopy†	Fluorescent microscopy†	Differential interference contrast microscopy†	Viable plate counts‡
bovienii	1.3 (0.25–0.13)	2.2 (0.31–0.26)	0.0	0.0
nematophilus var. *mexicanus*	11.1 (0.72–0.61)	2.8 (0.35–0.30)	12.7 (0.78–0.67)	11.0 (0.72–0.64)
poinari	65.4 (1.98–1.67)	22.0 (1.02–0.90)	42.1 (1.53–1.28)	48.1 (1.67–1.47)

* Decoded mean (with 95% confidence limits of 2 arcsin$\sqrt{p}$ transformed data).
† Three subsamples of 51 dauer samples.
‡ Fifteen samples, one dauer per sample.

0.1 hours, 1.0 ± 0.2 hours, and 1.2 ± 0.2 hours, respectively; $P > 0.05$) of axenic dauer juveniles.

Dauer *S. glaseri* LD_{50} varied with bacterial subspecies (Fig. 2). Dauer juveniles from Xnb cultures were the least virulent ($LD_{50} = 31.0 \pm 1.1$ dauer juveniles/insect), those from Xnp lawns were the most virulent ($LD_{50} = 1.4 \pm 0.2$ dauer juveniles/insect), and those from Xnn cultures were moderately virulent ($LD_{50} = 4.5 \pm 0.3$ dauer juveniles/insect). The regression slopes were equal regardless of the bacterial subspecies and averaged 0.30 probit-mortality units per log dose. Nematode growth rates were not correlated with LD_{50} ($r^2 = 0.37$, $P > 0.05$).

Each of the bacterial retention assay methods produced similar patterns but different values (Table 1). Significantly more dauer juveniles of *S. glaseri* retained Xnp than Xnn. Not all dauer juveniles retained the Xnp. No dauers retained viable Xnb. Incubating 10,000 surface-sterilized dauer juveniles from cultures of Xnb in 2.0 ml Grace's medium for 96 hours did not result in detection of bacteria. Dauer juvenile virulence was highly correlated with the number of juveniles retaining *Xenorhabdus* bacteria ($r^2 = 0.87$, $P < 0.05$).

Bacteria were detected in Grace's medium 2 hours after inoculating with *S. glaseri* dauer juveniles. The colony forming unit (CFU) counts remained constant until 30 minutes after the initial bacterial detection. Bacterial cell division was also confirmed using phase contrast microscopy. Thus it was possible to determine the number of bacteria per dauer juvenile for those nematodes retaining bacteria based on the CFU counts prior to bacterial multiplication. *S. glaseri* dauer juveniles from cultures of Xnn retained 1.0 ± 0.2 CFU/dauer and juveniles from Xnp retained 16 ± 2 CFU/dauer juvenile. Dauer juvenile virulence was highly correlated with the number of bacteria per juvenile ($r^2 = 0.96$, $P < 0.05$).

The growth rates of Xnb, Xnn, and Xnp in Grace's medium were not statistically different in the presence (1.1 ± 0.2 hours, 0.9 ± 0.1 hours, and 1.3 ± 0.2 hours, respectively; $P > 0.05$) or absence (1.0 ± 0.1 hours, 1.1 ± 0.1 hours, and 1.1 ± 0.2 hours, respectively; $P > 0.05$) of the nematodes. These growth rates were similar to their counterparts in lipid broth.

The data imply that nematode virulence could be enhanced by the retention of Xnp. To test this hypothesis, the LD_{50} of axenically cultured dauer juveniles, dauer juveniles from Xnb cultures and dauer juveniles from Xnp previously grown on cultures of Xnb were compared to *S. glaseri* dauer juveniles for Xnp. Axenically grown dauer juveniles had LD_{50} (36 ± 3 dauer juveniles/insect) equal to those from Xnb cultures (31 ± 2 dauer juveniles/insect; $P > 0.05$; Fig. 2).

Transferring the nematodes from Xnb cultures to Xnp lawns decreased the LD_{50} (2.1 ± 0.4 dauer juveniles/insect) to the LD_{50} of nematodes maintained on Xnp. The level of dauer juveniles with bacteria (43% [1.59–1.33]) and the bacterial number per dauer juvenile (14 ± 2) for the former nematodes equalled those from the latter culture.

DISCUSSION

Steinernema glaseri growth rates and profiles were influenced by associated bacterial subspecies. Growth was most rapid and uniform for *S. glaseri* on Xnb cultures as opposed to Xnp, the nematode's indigenous bacterium. Akhurst (2) reported,

however, that *S. glaseri* grew best on its own bacterial subspecies and grew well on Xnn. Studies preceding the present study established that marginal nematode growth occurred on Xnn (Rutherford, unpubl.). Such discrepancies may reflect differences in culture medium, nematode strains, and (or) assessment procedures. However, all of these studies establish that *S. glaseri* is capable of growth and reproduction on steinernematid bacterial cultures other than its own indigenous subspecies.

The initial nematode growth rates on fortified lipid agar may not have been caused by disproportionate bacterial growth rates because the doubling times of the three bacterial subspecies were the same in lipid broth during the first 16 hours of growth. Nematode adaptation cannot explain the biphasic growth profiles, since the profiles were duplicated by nematodes from the rapid growth phase transferred to new, corresponding bacterial cultures. The initial nematode inoculum from axenic culture did not produce colonies on tryptic soy agar or on TTC medium which confirmed inoculum sterility. Consequently, it was concluded that changes in bacterial species composition were not responsible for the biphasic growth profiles. It is possible that a medium modification requiring bacterial or nematode activity or both was not occurring at the same rates for the different monoxenic nematode cultures. Akhurst (3) has documented intra- and intersubspecies biochemical variations for Xnp and Xnb. Hotchkin and Kaya (18) detected soluble protein electrophoresis profile and isozyme differences between these two subspecies.

The percentage of dauer juveniles that retained *Xenorhabdus* varied with the bacterial subspecies, which agrees with Akhurst (2). As with Akhurst (2) and Bird and Akhurst (7), fewer than 50% of *S. glaseri* dauer juveniles retained their own bacteria. Although an unidentified *Steinernema* spp. is also known to have low retention for its own bacteria, bacterial retention by most steinernematids is substantially higher (2). In view of the *S. glaseri* growth and bacterial retention study, it appears that the nematode–bacterial relationship may not be as refined as those of other steinernematids, or that the populations of nematodes or bacteria may have heterogenous association mechanisms. The mechanism by which nematodes retain their specific bacteria is unknown (6).

The number of retained bacteria per dauer juvenile varied with the bacterial subspecies. The bacterial load of Xnp, as determined by the viable plate count procedure, was within the range reported for other steinernematids (21).

The microscopy techniques, although more rapid than the viable count method, did not permit accurate determination of bacterial numbers per dauer juvenile. The fluorescent stain procedure was difficult to interpret because of large numbers of stained nuclei caudal to the nerve ring and proximal intestinal vesicle. The irregular thickness of the vesicle wall and loosely arranged bacteria made scoring of the nematodes by differential interference contrast microscopy difficult, although it was the most facile of the optical procedures. Phase contrast microscopy was hindered by difficulties with dauer juvenile clean-up procedures and extraneous surface bacteria. Preliminary examination of surface-sterilized dauer juveniles revealed surface bacteria, but on the basis of viable plate counts of the juveniles prior to bacterial discharge, surface bacteria were considered dead. The viable count procedure determined both the number of dauer juveniles with *Xenorhabdus* and the number of viable bacteria the juveniles were capable of voiding. Although dead *Xenorhabdus* spp. destroy the cellular defenses of insects (1), viable bacteria are required to establish conditions necessary for nematode growth and reproduction (22). Viable counts are more in keeping with the in vivo situation and would be useful in assessing nematode virulence.

Dauer juvenile virulence was not correlated with bacterial growth rates in Grace's insect tissue culture medium. Because Grace's medium supports the physiological and biochemical functions of many tissue types of larval *G. mellonella* (Chadwick, pers. comm.), different bacterial growth rates may not occur in vivo. The nematodes did not influence bacterial growth rates in either lipid broth or Grace's medium. Dunphy and Webster (12) reported similar results with *S. feltiae* Mexican and DD136 strains and their corresponding bacteria.

Nematode virulence was correlated with the percentage of dauer juveniles retaining *Xenorhabdus* and the number of bacteria

per dauer juvenile. Surface-sterilized, monoxenically cultured dauer juveniles from Xnb were free of viable bacteria but were capable of killing *G. mellonella*. Axenic *S. feltiae* strains are known to be lethal to insects (8,12,22). The cause of death may be physiological starvation and (or) the accumulation of toxic metabolites produced either by the nematode or as a result of the nematode–insect association. Vectored bacteria enhanced dauer virulence in the present study.

The low regression slopes suggested that dauer juveniles did not harm *G. mellonella* by producing exotoxins. Dunphy and Webster (11,12) reported comparable results with *S. feltiae* strains. Thus, insect death induced by bacteria laden dauer juveniles may be due to septicemia (11,12).

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