PREREQUISITES FOR PARASITISM IN RHABDITID NEMATODES

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ABSTRACT: To evaluate their potential for survival in a vertebrate host, dauer larvae from 7 species of rhabditid nematodes were subjected to in vitro conditions designed to emulate those of a vertebrate digestive tract. Dauer larvae from 3 of the 7 species, selected for their ability to survive elevated temperatures and low pH, and representing differing types of phoretic associations with invertebrate hosts, were fed to frogs to examine their ability to survive passage through a vertebrate digestive system. The degree of invasiveness of the phoretic association that dauer larvae had with their invertebrate hosts did not correspond to patterns of in vitro survivorship for any of the experimental conditions. When consumed with a prey item, dauer larvae from all 3 species were recovered from frogs 72 hr postexposure, and no differences for in vivo survivorship were observed among the 3 species. The contention that invasiveness or facultative parasitism within an invertebrate host is a beneficial or necessary step toward vertebrate parasitism by rhabditid nematodes was not supported by the survivorship data.

The nematode order Rhabditida possesses species with a diversity of life histories (Bovien, 1937) including entirely free-living representatives. Some species attach as dauer larvae (third-stage juveniles) either superficially, e.g., *Turbatrix aceti* coiled on the legs of *Drosophila* spp. (Clark, 1994), or invasively, e.g., *Rhabditis stammeri* in the gut and genitalia of Necrophorus vespilloides (Richter, 1993), to insects as a means of dispersal. There also are facultative parasites, e.g., *Rhabditis maupasi* in the earthworm (Völk, 1950), and obligate parasites such as species of *Rhabdias* and *Strongyloides* (Dogiel, 1964). This diversity of life histories often has been viewed as a continuum, and it has been suggested that the evolution of parasitism in this group has tracked this continuum through progressive adaptation to the host environment, with phoresy serving a transitional role in the process (Rühm, 1956; Clark, 1994).

As an alternative, Osche (1962a, 1962b, 1965) proposed that the saprobiotic environments in which free-living rhabditids were found (generally decaying plant matter or dung) predisposed them to survival in the host environment. Osche (1962a, 1962b, 1965) viewed the phenomenon of preadaptation (the “exaptation” of Gould and Vrba, 1982) as requiring both a platform and a bridge for an organism to colonize a novel habitat. The platform consists of physiological traits that facilitate survival of an organism in both its historical and novel habitats, whereas the bridge consists of ecological characters that allow an organism to function as a member of 2 different ecosystems, and create the potential for connecting the 2 systems. Osche (1952) recognized that the environment of saprobiotic nematodes was quite similar to that which would be encountered within a vertebrate host. Specifically, saprobiotic nematodes are tolerant of low pH, low oxygen availability, elevated temperatures, and exposure to digestive enzymes released by bacteria (Grewal and Siddiqi, 1993). Thus, the physiological platform envisioned by Osche (1962a, 1962b, 1965) is already in place for free-living rhabditids.

Decomposing organic habitats are spatially and temporally discontinuous, requiring rhabditid nematodes to make use of invertebrates to transport them from habitat to habitat in a manner that is analogous, or perhaps homologous, to the transmission of parasites between hosts. In terms of Osche’s (1962a, 1962b, 1965) bridge, phoretic associations with insects also make it possible for attached dauer larvae to be consumed by insectivorous predators, creating a facile link between disparate habitats, and ensuring the propagation of surviving nematodes by depositing them as part of a nutrient rich fecal package. The recovery of free-living rhabditid nematodes as “accidental” parasites of humans (Chandler, 1938; Stempell, 1938; Osche, 1952), and the ability of small numbers of *Rhabditis* (*Pellioditis*) *pello* to survive in frogs and salamanders (Örley, 1886) lends credence to this suggestion.

The difficulty associated with addressing questions of preadaptation is that conclusions generally are drawn ex post factum (Osche 1962a). A comparison of the physiological tolerances, and ability to survive the host digestive tract, among rhabditid nematodes from varying points along the continuum of phoretic insect associations would shed light on the relative importance of adaptation to a saprobiotic environment, and progressive adaptation to phoretic hosts, in terms of promoting survival in a vertebrate host. The present investigation compares the survivorship of various free-living rhabditid nematode species in a variety of physiological conditions designed to emulate the environment of the vertebrate digestive tract, and compares the survival rates of a subset of these nematodes during passage through the gut of a leopard frog (*Rana pipiens*). Thus, the potential for extant free-living species to survive the vertebrate host environment will be evaluated as a function of their current physiological and ecological characters.

If gradual adaptation to the host environment is a requirement for the transition to parasitism in rhabditid nematodes, the degree of invasiveness that can be attributed to phoretic associations with invertebrates should be the best predictor of the ability to survive the vertebrate digestive tract. If, however, rhabditid nematodes are preadapted for such passage, the best predictor of survivorship should be the tolerance to the previously outlined physiological parameters as the result of the similarity between the present habitat of the nematodes and the vertebrate host habitat.

MATERIALS AND METHODS

Throughout 2003 and 2004, 50-ml samples of cattle dung and plant compost were collected, and dung beetles were captured in pitfall traps in Lyon County, Kansas. Dung beetles were dissected and examined with the aid of a stereomicroscope. Dissected beetles, and dung and compost samples, were macerated separately in 0.06% saline solutions, and nematodes extracted from each sample with the use of a modified
Figure 1. Ranked within-treatment in vitro survivorship of third-stage juvenile nematodes of 7 rhabditid species exposed to a solution of 0.5% trypsin and 0.5% pepsin at varying levels of pH and temperature. Lower rankings indicate higher in vitro survivorship. (A) Normoxic conditions. (B) Hypoxic conditions.
Baermann apparatus (MacInnis and Yoge, 1970). Dauer larvae collected by dissection or extraction from the dung beetles, and individual gravid female nematodes extracted from dung and compost samples, were transferred to individual wells of 6-well tissue culture plates containing cornmeal agar. Earthworms (Lumbricus terrestris) were purchased from a local bait shop, washed in distilled water, and cut into sections that were placed in individual wells of 6-well tissue culture plates containing cornmeal agar. All nematode cultures were subcultured at 2-wk intervals by transferring individual gravid females to separate wells of 6-well tissue culture plates containing cornmeal agar. Adult nematodes were collected from established cultures, fixed in glacial acetic acid, cleared in lactophenol, and identified from glycerin mounts with the use of Goodey (1963).

In vitro survivorship tests were conducted by transferring 30–70 dauer larvae to individual wells of 6-well tissue culture plates and exposing the nematodes to 3 ml of 0.5% trypsin and 0.5% pepsin at a pH of 2, 4, or 6, at 7 C, room temperature, or 30 C, with ambient oxygen levels, or hypoxic conditions induced by bubbling nitrogen through the solution for 1 min and sealing the wells with Parafilm®. In vitro survivorship was quantified by unstimulated motility assay every 2 hr for the first 12 hr, and every 12 hr thereafter. Three trials were conducted, each with 3 replicate 6-well plates for each experimental condition created for each nematode species in each trial. In vitro survivorship data were analyzed with the use of an accelerated time failure model (Fox, 1998) programmed with SAS 9.0 software (SAS Institute, Carey, North Carolina).

For each of the 3 phoretic strategies represented by nematodes exposed to the in vitro testing (superficial attachment, attachment under beetle elytra, and invasive), the nematode species that demonstrated the highest survivorship in normoxic conditions at 30 C and a pH of 2 was used for in vivo survivorship testing, which involved feeding dauer larvae to leopard frogs (Rana pipiens) purchased form Carolina Biological Supply (Burlington, North Carolina). For each nematode species, 50 dauer larvae were fed in 0.06% saline to each of 8 female leopard frogs using a feeding needle. To simulate ingestion while associated with a phoretic host, laboratory-reared grain beetles (Tenebrio molitor) were exposed individually to single cultures of the noninvasive nematode species for 24 hr, and then fed to 8 frogs (1 beetle per frog) for each nematode species. Attachment of dauer larvae to the beetles was confirmed by dissection of the remaining exposed beetles, and the attached dauer larvae quantified to estimate the number of dauer larvae frogs were exposed to by this method.

For the invasive nematode species (Rhabditis maupasi), 2 earthworms were cut into 5 equal sections. Eight frogs were fed 1 section of earthworm each. Infections of the worms were confirmed, and estimates of frog exposure generated, by placing the remaining sections in cornmeal agar, and quantifying the dauer larvae that colonized the culture.

For each species of nematode, and each feeding method (needle or consumption with prey), 2 frogs were killed by double-pithing at 1, 12, 24, and 72 hr postinfection (PI), and the intestines removed and examined by dissection in 0.06% saline with the use of a stereomicroscope. Frogs were housed separately until they were killed, at which point their digestive tract and any feces present were examined for the presence of nematodes. Nematodes recovered at each time point were standardized by the known (feeding needle exposure) or estimated (prey exposure) number of dauer larvae to which frogs were exposed. The relationship between in vivo survival and time PI was examined with the use of Spearman’s product moment correlation. Differences in in vivo survivorship among the nematode species and between exposure types were analyzed with a 2-factor Kruskal–Wallis analysis with the use of SAS 9.2 software (SAS Institute). Differences in the proportion of exposed frogs that retained living nematodes upon necropsy were examined among species and between exposure types with the use of 3-dimensional contingency table analysis.

RESULTS

Six nematode species were recovered from compost samples, dung samples, and dung beetles, collected from Lyon County, Kansas in 2003 and 2004. Three of the collected nematode species utilize superficial attachment to their phoretic hosts (Cruzennia tripartitum, Rhabditis longicaudata, and Protorhabditis oxyroides), and 3 (Diploscapter coronatus, Diploscapter lycostoma, and Stomachorhabditis fastidiosa) attach beneath the elytra of dung beetles. Rhabditis maupasi is invasive, and was collected from the body cavity of the dissected earthworms.

Ranked (within treatment) in vitro survivorships are shown in Figure 1 for the dauer larvae of all 7 species tested for all
combinations of temperature and pH under normoxic (Fig. 1A) and hypoxic (Fig. 1B) conditions. In vitro survivorship of the dauer larvae varied significantly among species \( \chi^2 = 4,224.89; P < 0.0001 \) and was significantly affected by pH \( \chi^2 = 2,018.42; P < 0.0001 \), temperature \( \chi^2 = 1,436.47; P < 0.0001 \), and oxygen availability \( \chi^2 = 45.90; P < 0.0001 \). There were significant interactions between the effects of temperature and pH \( \chi^2 = 2,039.38; P < 0.0001 \); the effects of oxygen availability and pH \( \chi^2 = 902.37; P < 0.0001 \); and the effects of temperature, pH, and oxygen availability \( \chi^2 = 1,028.15; P < 0.0001 \) on in vitro survivorship of the dauer larvae.

Overall in vitro survivorship of the dauer larvae is shown for each of the 7 species in Figure 2. Examination of the 95% confidence limits for the survivorship estimates (not shown) indicated 2 distinct groups, with *P. oxyuroides*, *R. maupasi*, and *C. tripartitum* comprising the group with the highest in vitro survivorship, and the remaining 4 species exhibiting relatively low in vitro survivorship.

In vivo survivorship for the 3 nematode species (Fig. 3) was not correlated with time PI for any nematode species introduced to frogs by feeding needle \( r_S \) ranging from 0.516 to 0.314; \( P \) ranging from 0.190 to 0.449) or introduced with a prey item \( r_S \) ranging from 0.481 to 0.447; \( P \) ranging from 0.228 to 0.785). Pooled survivorships (across species) also were not correlated with time PI for exposure of frogs with a feeding needle \( r_S = -0.195; P = 0.362 \) or a prey item \( r_S = -0.062; P = 0.774 \). Two of the 24 frogs exposed to nematodes by feeding needle produced feces during the interval prior to necropsy, and live nematodes were recovered from both fecal samples (*C. tripartitum* at 24 hr PI and *R. maupasi* at 72 hr PI). Only 1 of the 24 frogs exposed to nematodes with prey items produced feces during the interval prior to necropsy (at 72 hr PE), and live *C. tripartitum* were recovered from those feces. No other helminths were recovered from any of the frogs during necropsy.

Differences in in vivo survivorship among the 3 nematode species (Fig. 4A) approached significance \( F = 3.20; P = 0.051 \), but there was no significant effect of method of exposure \( F = 0.01; P = 0.917 \), and no significant interaction between nematode species and exposure method \( F = 0.68; P = 0.510 \). The proportion of successful exposures in frogs (Fig. 4B) also was independent of nematode species exposed, and the means of exposure \( \chi^2 = 4.83; P > 0.75 \).

**Figure 3.** Relationship between survivorship of 3 species of nematodes introduced to the digestive tract of *Rana pipiens* and time postinfection. (A) Exposure by feeding needle. (B) Exposure with prey item.

**Figure 4.** Survival of 3 species of juvenile nematodes in *Rana pipiens* exposed by feeding needle, or by ingestion of prey. (A) Mean proportion surviving in each frog individual (pooled across times postinfection [PI]). Error bars are standard error. (B) Proportion of frogs with surviving nematodes (pooled across times PI). Error bars are 95% confidence limits.
DISCUSSION

The in vitro tests were designed to evaluate the potential for saprobiotic rhabditids to tolerate the conditions they would encounter in a vertebrate gut. Low pH and elevated temperatures had the strongest negative influences on survival of the dauer larvae. The effects of low pH are presumably direct, but elevated temperatures could exert influences on survivorship indirectly by increasing metabolic rates and exhausting the energy reserves of the dauer larvae, or by influencing the activity of the proteolytic enzymes present in the medium. Higher survivorship under hypoxic conditions also might be related to depletion of energy stores, as higher levels of ambient oxygen promote β oxidation of lipids in rhabditid dauer larvae (Cooper and Van Gundy, 1970; Holt and Riddle, 2003). Normoxic conditions also might decrease survivorship through the potentially damaging effects of aerobic oxidative metabolism (Holt and Riddle, 2003; Burnell et al., 2005). Overall, in vitro survivorship of dauer larvae was lowest at a pH of 2 at 30°C under normoxic conditions.

The degree of invasiveness did not correspond to patterns of in vitro survivorship under any of the experimental conditions. The nematode species with the highest overall in vitro dauer larva survivorship included 2 species that attach superficially to their phoretic hosts (P. oxyuroides and C. tripartitum), and R. maupasi, a facultative parasite of earthworms. Thus, it appears that selective pressures in the environment that the dauer larvae occupy are a better predictor of their physiological tolerances than the nature of their phoretic associations.

The lack of a correlation between in vivo survivorship of dauer larvae and time PI suggests that initial tolerance of exposure to the host environment is a key factor in establishment (sensu Read, 1972). The in vivo survivorship data are equivocal with regard to interspecific differences, due in large part to the small number of frog hosts used relative to the variation in survivorship. The 3 nematode species exposed to frogs were selected based on their ability to survive the harshest (based on the survivorship data) in vitro conditions, including temperatures within the range experienced by rain frogs, and a pH comparable to the range of 2.2–3.5 measured within rain stomachs (Vonk, 1929, 1941; Reeder, 1964) and the optimum pH range (1.4–1.9) for ranid pepsin (Vonk, 1929; Pjatnitzky, 1931). Thus, the data do not suggest any advantage in colonization of a vertebrate host for rhabditid nematodes with more invasive invertebrate associations.

Live individuals of both R. maupasi and C. tripartitum were recovered from the feces of leopard frogs exposed with the use of a feeding needle; live individuals of C. tripartitum were recovered from the feces of a prey-exposed leopard frog, demonstrating that these species are capable of surviving passage through the digestive tract of the leopard frogs. Survival of passage through the gut would deposit dauer larvae in a nutrient-rich environment for further development, similar to the circumstances that occur for the dauer larvae of R. maupasi upon the death of the earthworm host (Poinar and Thomas, 1975).

The present investigation does not support the contention that invasiveness or facultative parasitism within an invertebrate host is a necessary prerequisite for vertebrate parasitism by rhabditid nematodes. The saprobiotic habitat occupied by these nematodes appears to exert similar selective pressures to those that would be encountered in a vertebrate host, thereby allowing dauer larvae to survive exposure to the host environment, as initially postulated by Osche (1962a, 1962b). Although the invertebrate associations have obvious implications in terms of a preadaptive “bridge,” and might generate important selective pressures for the keystone adaptation of immune evasion (Zelmer, 1998), it does not appear that such associations contribute substantially to the preadaptive “platform.”

ACKNOWLEDGMENTS

Funding for this research was provided by a Sigma Xi Grant In Aid of Research and a Research and Creativity Grant from Emporia State University.

LITERATURE CITED


