A comparative analysis of entomoparasitic nematodes *Heterorhabditis bacteriophora* and *Steinernema carpocapsae*

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ABSTRACT

***Heterorhabditis bacteriophora* and *Steinernema carpocapsae* are microscopic entomoparasitic nematodes (EPNs) that are attractive, organic alternatives for controlling a wide range of crop insect pests. EPNs evolved with parasitic adaptations that enable them to "feast" upon insect hosts. The infective juvenile, a non-feeding, developmentally arrested nematode stage, is destined to seek out insect hosts and initiates parasitism. After an insect host is located, EPNs enter the insect body through natural openings or by cuticle penetration. Upon access to the insect hemolymph, bacterial symbionts (*Photorhabdus luminescens* for *H. bacteriophora* and *Xenorhabdus nematophila* for *S. carpocapsae*) are regurgitated from the nematode gut and rapidly proliferate. During population growth, bacterial symbionts secrete numerous toxins and degradative enzymes that exterminate and bioconvert the host insect. During development and reproduction, EPNs obtain their nutrition by feeding upon both the bioconverted host and proliferated symbiont. Throughout the EPN life cycle, similar characteristics are seen. In general, EPNs are analogous to each other by the fact that their life cycle consists of five stages of development. Furthermore, reproduction is much more complex and varies between genera and species. In other words, infective juveniles of *S. carpocapsae* are destined to become males and females, whereas *H. bacteriophora* develop into hermaphrodites that produce subsequent generations of males and females. Other differences include insect host range, population growth rates, specificity of bacterial phase variants, etc.** This review attempts to compare EPNs, their bacterial counterparts and symbiotic relationships for further enhancement of mass producing EPNs in liquid media.

Keywords: Entomoparasitic Nematodes (EPNs); Heterorhabditis Bacteriophora; Steinernema Carpocapsae; Photorhabdus Luminescens; Xenorhabdus Nematophila; Symbiosis; Mass Production

1. INTRODUCTION

Entomoparasitic nematodes (EPNs) *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* are utilized as biocontrol agents against various insect pests of agricultural significance (**Figure 1**). EPN is an attractive organic alternative to chemical insecticides as they do not pose a threat to the environment. Additionally, EPNs are particularly safe for use around humans, livestock, and plants [1]. The close symbiotic relationship between EPNs and their bacterial counterparts contributes to the safety and efficacy of their use as biological control agents.

Figure 1. Brightfield micrograph depicting adults of *H. bacteriophora* under 40× magnification.

Symbiotic bacteria *Photorhabdus* spp. and *Xenorhabdus* spp. provide *Heterorhabditis* and *Steinernema* nematodes, respectively, with diverse services within the host insect: 1) produce virulence factors that neutralize and kill the insect host [2-4]; 2) bioconvert the host into nutritional components; 3) serve as the main food source for their nematode partners [5,6]; and 4) produce antimicrobials (**Figure 2**) which prevent putrification of the insect host by competing microbes [7-10]. These bacterial based services ultimately produce an optimal environment for nematode growth and reproduction [11].

These symbiotic associations are useful in managing insect pests that are destructive to many commercially viable plants and crops [12,13]. *Heterorhabditis* spp. usually performs better than *Steinernema* spp. [14]; which is a consequence of their predatory lifestyles. *Heterorhabditis bacteriophora* is considered to be a burrowing "cruiser" nematode that "seeks" out its insect host by borrowing into the soil. To the contrary, *Steinernema carpocapsae* is an "ambushing" nematode that attacks insect hosts that are "passing" by [14,15].

Mass production of *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* on a large scale is difficult and cumbersome either *in vivo* or *in vitro* due to various obstacles (**Figure 3**). Production of EPNs can be achieved *in vivo*; however, commercial scale production is impracticable due to high production costs and low nematode yields per gram of insect biomass [16,17]. EPN production with *in vitro* solid technology gives rise to higher nematode yields per gram of solid media when compared to *in vivo* technologies. However, costs associated with solid media technologies are much higher than *in vivo* technologies. The high production cost is mainly associated with labor, materials and storage area [18,19]. To many researchers, *in vitro* liquid technologies should be used in commercial production of EPNs for international markets because these technologies are considered to be

Figure 3. Mass production of *H. bacteriophora* in a 10 L bioreactor. Note the red pigmentation produced by its bacterial symbiont *P. luminescens*.

the most cost-efficient process when compared to other methods. Although mass production in submerged culture offers cost-efficiency, capital and technical expertise is still required [20]. Problems arising during nematode mass production are due to many different factors. Some of these factors include phase shifting of the bacterial symbiont, low percentages of nematode copulation, inoculum (bacterial/nematode) concentrations and fermentation parameters (oxygen concentration, pH, temperature, agitation, etc).

Due to the lack of biological knowledge of EPNs and their bacterial symbionts, optimization of liquid mass production technologies is hindered. Furthermore, the lack of knowledge involving the symbiotic relationship between EPNs and their respective bacterial symbionts poses more difficulty in establishing and optimizing standardized production protocols. Lastly, understanding the triangular relationship between EPNs, bacterial symbionts and host insects will further promote the development and optimization of media and fermentation parameters for maximizing nematode yields. This review will 1) identify the biological differences in the life cycles of *H. bacteriophora* and *S. carpocapsae*; 2) describe the nutritional relationship between EPNs and their bacterial symbionts; and 3) briefly describe potential biological processes occurring during host interaction that may benefit mass production processes.

2. NEMATODE BIOLOGY AND LIFE CYCLE

The third stage infective juvenile (IJ) of *Heterorhabdi-*

Figure 2. Antibiotic activity from permeate obtained from a culture of *P. luminescens*. *Moraxella* (*Branhamella*) *catarrhalis* is the test subject.

tis and *Steinernema* nematodes occurs free in the soil and their roles are to seek out and infect host insect larva. *Steinernema* nematodes typically enter the insect host through natural body openings (mouth, anus and spiracles). Furthermore, *Heterorhabditis* nematodes gain access to the insect host in a similar fashion as *Steinernema* spp.; however, *Heterorhabditis* spp. can also gain entry by penetrating the insect's cuticle utilizing a dorsal tooth. Within their anterior intestine, IJs carry a lethal dose of their bacterial symbiont and when stimulated within the insect host, are released by the nematode partner into the insect hemolymph (*i.e.*, initiation of recovery).

Upon bacterial release, bacterial proliferation occurs and as a result, secretion of insect toxins and degradating enzymes occur that kill and bioconvert the insect cadaver within 24 - 48 hours. As the bacterial population reaches stationary phase, production of secondary metabolites, in particular antimicrobials, and an unknown "food signal" is initiated. The secreted antimicrobials are speculated to be a defense mechanism used to ward off competing microbes that may cause the cadaver to putrefy [21,22]. Additionally, researchers suggest that unidentified food signals induce IJs to shed their protective sheaths and continue development to complete their life cycle [23,24]. After the sheaths are shed, IJs become feeding stage 3 nematodes (J3), develop to the fourth juvenile stage (J4) and ultimately to adulthood.

The first generation of offspring depends on the nematode genus (*Heterorhabditis*, *Steinernema*). For *Heterorhabditis* spp., the first generation of offspring emerges as IJs [25]. These IJs were developed *in utero* of the parental hermaphrodite in a process known as *endotokia matricida*. *Endotokia matricida* (**Figure 4**) occurs as a result of self-fertilization; whereby fertilized eggs hatch into juveniles of Stage 1 (J1) within the hermaphroditic nematode [25]. After hatching, J1s feed upon the maternal nematode and continue to develop to Stage 2 (J2). During this developmental stage, nutrients become limited within the hermaphrodite that signals the J2 nematodes to develop into IJs. As development of IJs is completed, the IJs emerge from the maternal nematode by bursting through the hermaphroditic cuticle and into the protected environment. After their hermaphroditic emergence, IJs continue their life cycle due to the presence of residual food signals. This subsequent generation of IJs develop into adult nematodes that are sexual reproductive (male/females); however, if females do not mate with the opposite sex, offspring may be produced by parthenogenesis or hermaphroditically [17,26,27]. Furthermore, subsequent generations will continue within the insect cadaver until all nutrients and symbiotic bacteria are consumed. It is during this point in the reproductive life cycle that nutrient stress will induce J2 nematodes to develop and transition into IJs that will ultimately emigrate from the
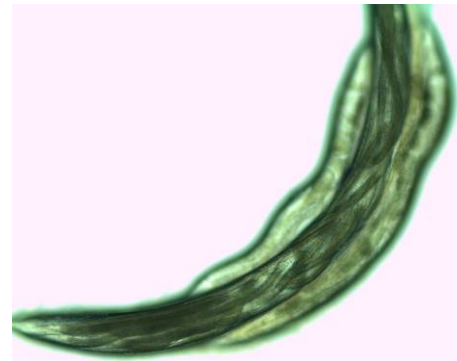

Figure 4. Hermaphrodite of *H. bacteriophora* exhibiting *endotokia matricida*. Nematodes seen in the body cavity will exit as IJs.

cadaver to search for new insect hosts [25,28].

In *Steinernema* spp., IJs undergo the same infective behavior as *Heterorhabditis* spp. with the exceptions of cuticle penetration and the initial round of recovery. In a population study conducted by Wang and Bedding, the researchers found that, upon recovery, IJs of *Steinernema* spp. develop into reproductive males and females (*i.e.* amphimictic reproduction) which also occurs in first and second generation offspring [25]. However, all eggs produced by third generation females were found to develop via *endotokia matricida*. Unlike *H. bacteriophora*, juvenile stages resulting from *endotokia matricida* in *S. carpocapsae* do not develop into IJs until they exit the maternal nematode. As a response to nutrient depletion, *endotokia matricida* occurs in *Steinernema* spp. due to cessation of egg-laying [29].

Differences in life cycle and reproductive biology influence the yield of the two genera in liquid culture. Heterorhabditids exhibit a "Y" or "λ" type copulation on solid media. In contrast to steinernematids, heterorhabditids are unable to attach to each other in liquid culture due to sheer caused by agitation and/or aeration [17]. Maximizing reproductive mating is a crucial factor that must be considered for mass production as the number of offspring produced from copulation is at least 10-fold higher than production by *endotokia matricida*. Optimizing mass production parameters for copulation can be achieved through bioreactor design and optimization of agitation and aeration [30]. However, maximizing mating has some limitations in *Heterorhabditis* spp. in liquid culture because the first generation is exclusively hermaphrodites where the amphimictic forms are not produced until subsequent generations [17].

Maximizing heterorhabditid yields in liquid culture will greatly depend on the concentration of nematode inoculum and the degree of recovery. Percentage recovery is found to be less in liquid media compared to *in vivo* conditions [23,31-34]. Additionally, recovery of EPNs is mostly dependent on the food signal secreted by

the associated bacterial symbiont during late exponential growth [23,35]. Maximum average yields of EPNs reported were in shake flask batch cultures were 300,000 and 320,000 IJs per ml for *H. bacteriophora* and *S. carpocapsae*, respectively [36]. Furthermore, in a recent study, fermentation modes (batch and fed-batch) for mass producing *S. carpocapsae* were compared and found that fed-batch modes produced an 8.8-fold higher IJ yield than batch modes [37].

3. NUTRITIONAL RELATIONSHIP BETWEEN NEMATODES AND THEIR BACTERIAL SYMBIONTS

Mass production in liquid media, regardless of the culturing vessel, requires the nematode culturing media to be conditioned [38]. Conditioning of the liquid medium refers to the inoculation of the appropriate bacterial symbiont. This step is crucial as the bacterial symbionts: 1) convert the complex medium into easily accessible components for both itself and partner nematodes; 2) secrete necessary metabolites, (food signals, antibiotics, pigments, etc.); band 3) serves as the main food source for the developing nematodes [38]. The nutritional relationship is highly specific for *Heterorhabditis*, because these nematodes cannot be cultured under axenic conditions or on other bacteria. On the other hand, *Steinernema* are less fastidious can reproduce in the absence of their symbiotic partner; however, nematodes yields are severely decreased [24]. The successful development of *Steinernema* spp. in axenic, *in vivo* conditions or plated on non-symbiotic bacteria (*i.e. Escherichia coli*) have been reported [6,24,39-41]. Additional research has been performed that shows *Steinernema* spp. is unable to develop when given *Photorhabdus luminescens* as the bacterial food source [39]. Furthermore, this finding suggests possible nematicidal properties of *Photorhabdus* spp.; which may also indicate that *Xenorhabdus* spp. may also exhibit similar properties.

There are two forms or variants of entomopathogens (*Xenorhabdus* spp. and *Photorhabdus* spp.) that exhibit different phenotypes and metabolic profiles based upon several factors [42]. These variants can either be isolated as primary wild type (Phase I) or secondary form (Phase II) [43,44]. EPNs require Phase I forms as these variants are extremely metabolically active and produce a battery of different substances and traits (enzymes, antimicrobials, insect toxins, bioluminescence, etc). The phase II form is commonly seen in the laboratory on routine culturing media. This transitioning between Phase I and Phase II states is known as phase variation. This biological phenomenon ensures the survival of a bacterial cell and/or population in unfavorable conditions [38]. Phase variation naturally occurs in many enteric bacteria such as *E. coli* and *Salmonella* spp. The role of phase variation

and the genetic mechanisms involved with it in *Photorhabdus* and *Xenorhabdus* have not been identified. Furthermore, researchers suggest that deteriorating environmental conditions (pH, nutrient exhaustion, osmolarity, etc.) may be responsible for triggering this effect [45].

Reports indicate the presence of two types of proteinaceous crystalline inclusions in the cytoplasm of both bacterial symbionts *X. nematophila* and *P. luminescens* [46,47]. Although the functions of the inclusion bodies are unknown, it is hypothesized that these proteins may be involved in nematode nutrition or insect pathogenicity as they represent 40% of the total cellular protein [48,49]. The genes responsible for producing inclusion bodies have been identified (cipA and cipB). Further research shows that the inactivation of these genes alters the characteristics of the Phase I variant of *P. luminescens* thereby rendering the symbiont incompetent to support nematode growth and reproduction [49,50]. Additional research has shown that *Steinernema* nematodes can feed, develop and reproduce on *E. coli* cultures that express at least one of the Cip proteins from *Xenorhabdus* spp. [49]. However, in a similar experiment, *E. coli* cultures expressing *Xenorhabdus* Cip proteins did not support the development or reproduction of *H. bacteriophora* [40]. These results suggest that nutrient requirements are different for *Heterorhabditis* spp. and *Steinernema* spp. [49].

4. STABILITY OF NEMATODES AND BACTERIAL SYMBIONTS DURING *IN VITRO* CULTURE

Trait deterioration is a major concern to industrial producers of entomopathogenic nematodes. Bilgrami *et al*. reported trait changes as a result of continuous subculturing in *S. carpocapsae* and *H. bacteriophora* [11]. These investigators studied trait stability of *P. luminescens* and *X. nematophila* after serial *in vitro* subculturing and demonstrated that phase variation (Phase I to Phase II) in *P. luminescens* and *X. nematophila* strains occurred within ten subculturing cycles [11]. Furthermore, phenotypic variation was controlled in *X. nematophila* strains by selection of primary variants; however, trait change was not detected after prolonged culturing. When phenotypic variation in *P. luminescens* was controlled, changes in the primary variant were observed. These observations include cellular morphology, size of inclusion bodies, and prevalence of inclusion bodies [51].

Bilgrami *et al*. noted the stability of the virulence in *S. carpocapsae* after subculturing for prolonged periods of time when compared to *H. bacteriophora* [11]. Gaugler *et al*. also reported the virulence stability in *S. carpocapsae* by correlating stability with numerous nematode passages in *G. mellonella* [52]. The basis for virulence stability in *S. carpocapsae* may lie in the phase stability

of its associated bacterial symbiont *X. nematophila* [11]. *In vitro* subculturing of *P. luminescens* resulted in vast changes including alterations in growth rates, cell size, number and size of inclusion bodies, virulence and pigmentation [11]. In comparison to *P. luminescens*, *X. nematophila* did not result in measurable changes to any of the traits tested [11]. However, Bilgrami *et al.* are critical to the findings of Wang and Grewal involving stress tolerance reduction, storage stability and reproduction in *H. bacteriophora* after three passages in *G. mellonella* [11,53]. These findings are noteworthy because present strains in mass production are subcultured many times before their adoption for commercial use. Such stability can make a difference between successful and unsuccessful production runs.

5. HOST INTERACTION

Steinernema spp. typically searches for insect hosts on or near the soil surface. This group of EPNs is usually referred to as "ambush" predators. They generally remain inactive until a mobile insect host passes by [54]. Ambushing in *S. carpocapsae* also consists of an unusual jumping behavior in which the IJ nictates, curls into a loop, and propels itself into the air. Jumping is unique to Steinernematids and is considered a specialized evolutionary adaptation that facilitates attachment to passing hosts [54]. *Heterorhabditis* spp. dwells into the soil in search of subterranean, sedentary hosts and commonly categorized as "cruisers". *Heterorhabditis* nematodes are highly mobile that can respond and target insect hosts over long-range chemical cues [55]. Host volatiles, such as CO_2, can stimulate both *H. bacteriophora* and *S. carpocapsae* [56-59]. Hallem *et al.* investigated the response of *H. bacteriophora* and *S. carpocapsae* IJs to host odors by using CO_2 exposure studies [60]. They found that IJs of both species were strongly attracted to increasing CO_2 concentrations. In the same study, Hallem *et al.* demonstrated that the BAG sensory neurons are required for CO_2 attraction [60]. BAG-ablated *H. bacteriophora* IJs do not chemotax towards the insect host *G. mellonella*, demonstrating a critical role of BAG neurons in host localization. Since BAG neurons are sensory neurons that detect CO_2, it seems that CO_2 is an essential cue for host attraction [61]. In contrast, ablation of the BAG neurons did not significantly affect the ability of *S. carpocapsae* IJs to jump in response to *G. mellonella* volatiles, demonstrating that other neurons besides BAG or possibly other host cues are sufficient to mediate host attraction [60].

6. CONCLUSION

Understanding the biology of both the nematodes and bacterial partner is important for mass production. The

differences in nematode life cycles and bacterial symbiosis play major roles in final nematode yields. The time and concentration of the nematode inoculum along with nematode recovery greatly affect final yield [62,63]. Mass production strategies involving *S. carpocapsae*, *H. bacteriophora* and their bacterial symbionts have been developed by many while studying characteristics of both symbiotic partners in liquid culture [37,38,64,65]. Inman III and Holmes have described the role of trehalose, a non-reducing sugar found in abundance within insect hemolymph that seems to aid in maintainence of Phase I variant of *P. luminescens* over extended periods of time [66]. Research is on-going to increase: 1) the stability of the bacto-helminthic complex; 2) final nematode yields; and 3) the cost-effectiveness of liquid mass production technologies.

7. ACKNOWLEDGEMENTS

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