SYMBIONT SPECIFICITY OF *STEINERNEMA PUNTAUVENSE* (NEMATODA: STEINERNEMATIDAE)

By

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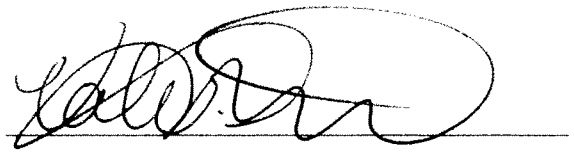
Signed: 

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Abstract

Entomopathogenic nematodes in the genus *Steinernema* (Nematoda: Steinernematidae) are mutualistically associated with Gram-negative bacteria in the genus *Xenorhabdus* (Gamma-Proteobacteria: EnterobacteriaceaIt is known that each *Steinernema* nematode species is associated with one *Xenorhabdus* spp. and several *Xenorhabdus* species are associated with more than one *Steienerma* nematode. The extreme example of this level of host promiscuity is that of *Xenorhabuds bovienii* which is currently known to be associated with nine *Steinernema* spp. In this study we focused on *Steinernema puntauvense* (Li 6 strain) one of the nematode host species associated with *X. bovienii* to assess symbiont colonization by non-cognate bacteria and its impact on nematode fitness. For this purpose nine *X. bovienii* strains including the cognate (natural) and eight non-cognate strains were considered. Results of this study showed that *S. puntauvense* was successfully colonized by eight of the nine *X. bovienii* strains. The *X. bovienii intermedium* strain was the only strain that did not colonize *S. puntauvense*. In all positive symbiont-host associations, IJs of *S. puntauvense* were able to mature and produce progeny. Another interesting finding of this study was that nematode fitness was apparently compromised in its capability to infect an insect host and either successfully reproduce and/or emerge from the hosts.

Introduction

Nematodes are non-segmented roundworms that belong to the phylum Nematoda. Approximately 25,000 species have been described, and 500,000 to 100,000,000 species are estimated to exist worldwide (Lambshead, 2004). Nematodes are ubiquitous, occupying numerous niches and existing in nearly every available habitat on every continent. Nematodes also have many trophic strategies and lifestyles including free-living, predatory, and parasitic. Many of the parasitic species of nematodes cause important diseases in humans, animals (both vertebrates and invertebrates) and plants. Among invertebrate parasites, there are 30 nematode families that are associated with insects and other invertebrates (Stock & Hunt, 2005). Seven of these families have the potential for being considered as biological control agents. Of these, the most widely studied group are the so-called "entomopathogenic nematodes," also known as EPN. Currently two nematode families: Steinernematidae and Heterorhabditidae are considered in this group. These nematodes are considered pathogens because of their mutualistic association with bacterial symbionts that enable them to rapidly kill their insect host, in circa 24-48 hours, (Adams, et al. 2006).

The life cycle of this mutualistic relationship between entomopathogenic nematodes and their bacterial symbionts begins with the non-feeding third-stage infective juveniles (IJs). In this stage the nematodes live in the soil and are highly resistant to environmental stressors and also do not feed and reproduce thus, are able to persist in this environment for extended periods of time. IJs search for a suitable insect host and once the nematodes find a suitable host, they penetrate the cuticle and enter into the host making their way to the insect's body cavity (i.e. haemocoel). Once in the haemocoel, the IJs release the bacterial symbiont. For the nematode, this stage is

called recovery, and molt to the last juvenile stage (J4). During this state the bacterial symbiont induce toxemia, immunodepression, and septicemia causing insect death. The bacteria also produce antimicrobial compound s and toxins (bacteriocins and others) to create a suitable environment that allows nematode growth to adult stages. Adult nematodes usually undergo one or two more generations within the insect cadaver. When food source is depleted, the new IJ generation abandons the host in search for another suitable host (Forst & Clark, 2002).

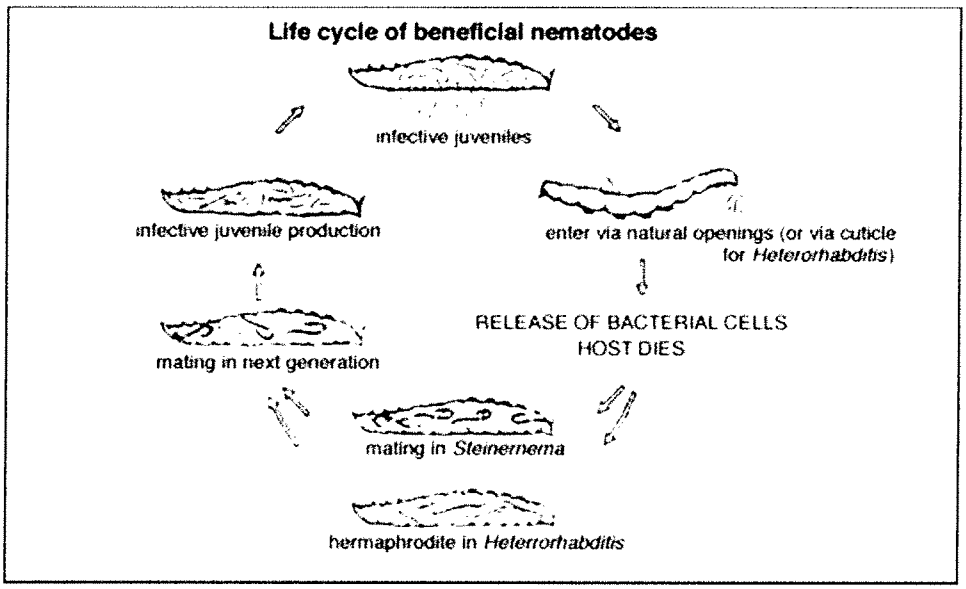


Figure 1. Life cycle of entomopathogenic nematodes
from http://www.nematodes.com/fact%20sheet.htm

Together, entomopathogenic nematodes and their bacterial symbionts are an insecticidal complex' against a wide variety of insect hosts. Interest in them began, mainly, because of this nematode-bacteria complex that had a potential for application in agriculture (Gaugler and Kaya, 1990; Kaya and Gaugler, 1993). Many researchers found that these entomopathogenic nematodes are ideal for applied research in agriculture because they are lethal towards many insect pests, but are harmless to humans, animals, and plants. Unlike chemicals and other

biological agents, such as the entomopathogenic bacterium *Bacillus thurigienesis*, that requires a high level of safety protocols, the use of entomopathogenic nematodes does not require use of safety equipment. Moreover, application of these nematodes into the soil does not contaminate groundwater or has residues, that potentially could harm beneficial organisms or non-target insects. Moreover, entomopathogenic nematodes can be mass produced, and many formulations are currently available, and any existing agricultural equipment can be used for their application (Adams and Nguyen, 2002).

Currently, research on this nematode-bacterial complex has expanded to other fields including medicine, pharmaceutical prospecting among others. Moreover, progress in these areas, along with the experimental tractability of these nematodes and their bacterial symbiont makes them ideal candidates for considering a model system studying mutualisms and also for basic research in ecology, evolution, biochemistry, molecular and genetics (Stock and Goodrich-Blair, 2008).

Steinernema-Xenorhabdus mutualism

Many scientists are currently researching the details of this nematode-bacterial complex, especially that of the *Steinernema* and *Xenorhabdus* spp. interaction. Each species of *Steinernema* has its own species of *Xenorhabdus* associated with it. In *Steinernema*, their *Xenorhabdus* spp. is located within a specialized intestinal receptacle (Flores Lara et al. 2007; Snyder et al., 2007). According to Forst and Clark (2002), it is likely that interaction between specific receptors on the intestinal epithelium of the respective nematodes and cell surface molecules of the bacteria facilitate tissue-specific and species–specific colonization. According to Lee and Stock (2010), the phylogenetic distance between cognate and non-cognate bacterial

symbionts seems to play a key part in the resultant levels of nematode fitness. In the *Steinernema-Xenorhabdus* life cycle, the nematode and their bacterial symbiont are diassociated for one to three generations within the insect cadaver. IJs take up their symbiont orally, and since *Steinernema* are amphimictic there is a possibility that their bacterial symbionts may not have originated from an immediate ancestor but from a conspecific one (Lee and Stock, 2010).

The relationship between entomopathogenic nematodes and their bacterial symbiont have varying levels of associations. *Steinernema* species are mutualistically associated with one *Xenorhabdus* spp. However, species of *Xenorhabdus* are symbionts of multiple species of *Steinernema* (Koppenhöfer, 2007). Specifically, *Xenorhabdus bovienii* is the symbiont of nine species of *Steinernema* (Lee & Stock, 2010). Therefore, for this purpose we considered the nematode *Steinernema puntauvense*, which is one of the nematode hosts of *X. bovienii*. Particularly, the goal of this study was to assess nematode fitness and symbiont colonization considering cognate (natural) and non-cognate symbiotic partners. With the apparent flexibility in nematode hosts, *X. bovienii* and one of its nematode associations, was chosen to further elucidate the level of specificity of this mutualistic interaction.

Materials and Methods

1 – Nematode and bacterial strains considered

The entomopathogenic nematode *S. puntauvense* Li 6 isolate, a host of *Xenorhabdus bovienii* was considered for this study. Nine *X. bovienii* strains, including the cognate symbiont of *S. puntauvense* were considered. Table 1 provides a detailed description of bacterial strain origin including nematode hosts and geographic precedence.

Table 1. List of *X. bovienii* cultures considered in this study

Isolate name	**Nematode host**	**Origin**
SN	*S. feltiae*	France
FL	*S. feltiae*	Florida, USA
Moldova	*S. feltiae*	Moldova
OS-10	*S. oregonense*	Oregon, USA
Li6	*S. puntauvense*	Costa Rica
Type isolate	*S. intermedium*	South Carolina, USA
Monsanto	*S. jollieti*	USA
Nemasys	*S. kraussei*	England
Quebec	*S. kraussei*	Quebec, Canada

2 – Culture of Aposymbiotic Nematodes

One of the key steps in this experiment was to obtain aposymbiotic (i.e. symbiont-free) infective juvenile nematode stages. As described above this stage is the one that associates and harbors *Xenorhabdus* bacteria. For this purpose nematodes were grown in vivo in last instar *Galleria melonella* (Lepidoptera: Pyrallidae) larvae according to procedures described by Kaya and Stock (1997). Briefly the technique is as follows: Two discs of filter paper (5cm diam, Whatman #1) were placed on a 5cm petri dish that was inverted so that the top contained the filter paper. One milliliter of *S. puntauvense* inoculum (concentration of 1,000 IJ/ml) was added onto the filter paper. Five *G. melonella* larvae were sterilized using 95% ethanol subsequently rinsed with distilled water, and then placed on the filter paper. After approximately 2-4 days insects died from the nematode infection. Cadavers were dissected to recover fertilized female nematodes. Females were collected and placed in a watch glass with M9 buffer solution. A 1% axenizing bleach solution was added to the harvested female nematodes to dissolve their cuticle and release their eggs. Bleach kills the females but the eggs which have a thick layer are not affected by the axenizing solution. Eggs were collected and placed into 1.5 ml Eppendorf tubes and centrifuged at 6,000 rpm for 6 min. Supernatant (950 µl) was removed and 950 µl of 3% bleach solution was added. Sample was vigorously vortexed for 1 min and the tube centrifuged for 3 additional minutes. Supernatant was removed and 1 mL of 1X PBS was added. This step was repeated twice. Solution containing axenized eggs was transferred to a sterile watch glass containing sterile distilled water. Approximately 400 µl of and the eggs suspension was seed on liver-kidney agar plates. Nematodes were allowed to grow on the agar for approximately within one to two weeks.

3 – Harvest of Aposymbiotic Nematodes

Modified White traps (Kaya and Stock 1997) were used to collect aposymbiotic IJs. Briefly, the procedure was as follows: Petri dish containing the liver-kidney agar with developed nematodes was placed in) in a larger sterile Petri dish 10cm). Approximately 20 mL of distilled autoclaved water was added to the larger dish. Nematodes are attracted to water, so after a few hours IJs can be collected from the clean water and stored for future assays. IJ suspension was rinsed three times by allowing the nematodes to decant in a 250 ml beaker. Water was carefully removed and replaced with sterile distilled water. The IJ suspension was then stored in tissue culture flasks (250 ml) at room temperature (22-25 °C) Tissue culture flask contained approximately 50-70 ml of IJ suspension at a concentration of a 1,000 IJs/mL

4 – Counting Nematodes

A concentration of 10,000 IJs/mL was needed for this experiment to guarantee that there was a generous amount of living IJs to interact and potentially reassociate with the specific strain of *Xenorhabdus*. A concentration of 10,000 IJs/mL was collected by the following calculation:

$$X = \#of\ Nematodes/mL$$

$$500\ \mu l\ per\ plate\ x\ \#\ of\ plates = Total\ \#\ of\ mL\ with\ IJs\ needed$$

$$\frac{y\ \#of\ IJs\ needed}{X_{avg}\frac{IJs}{mL}} = mL\ to\ use\ from\ Aposymbiotic\ Nematode\ Mix$$

Cultures of *S. puntauvense* Li 6 were poured into a large Erlenmeyer flask. The nematodes were continuously mixed using a stirring plate to homogenize the suspension and allow even distribution of nematodes in the suspension. One mL of the aposymbiotic nematode aliquoted and poured on a hemocytometer for counting. Three counts were performed and then averaged.

$$L\ of\ Aposymbiotic\ Nematode\ Mix\ needed$$

Aposymbiotic nematodes were surfaced sterilized to reduce contamination prior to plating them onto the lipid agar plates.

5 – Isolation and Rearing of *X. bovienii* Strains

Except for the bacterial symbiont of *S. puntauvense*, all other bacteria strains considered in this study were retrieved from glycerol stocks maintained at -70 °C in Dr. P. Stock's laboratory (Dept. Entomology, University of Arizona). Isolation of *X. bovienii* from *S. puntauvense* nematodes was done considering the grinding method described by Lee and Stock (2010). Briefly, IJs were surfaced sterilized using 1% bleach solution, followed by two rinses with 1X PBS. IJs were then recovered and added to 2 mL of LB broth and sonicated for two minutes. 30 µl of the sonicated suspension was then spread onto nutrient bromothymol blue agar (NBTA) plates.

Bacterial strains were retrieved from the frozen bacterial stock cultures. Using sterile technique, 5mL of LB and Pyruvate broth and 30 µl of the frozen stock cultures were combined. They were grown for 18-24 h at 30°C in an incubator rotating at 150 rpm. After 18-24 h, 100 µl of the bacterial liquid culture was plated as a bacterial lawn on 5 cm lipid agar Petri dishes to ensure sufficient growth of the *X. bovienii* strain. As a control for contamination, 30 µl of the bacterial liquid culture was plated on NBTA to ensure that the *X. bovienii* strain was uncontaminated.

6 – Confirmation of *Xenorhabdus* spp. Identity

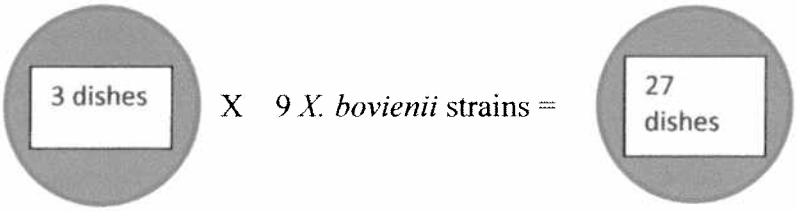
Colonies of *Xenorhabdus bovienii* were identified considering morphological characteristics as described by Koppenhöfer (2007). Cells occur in two phases: I and II. Only phase I of the bacteria was exclusively used to *Xenorhabdus* spp. During this phase, the cells absorb dyes producing blue colonies on NBTA. *Xenorhabdus* spp. is facultative anaerobic and is negative for oxidase and catalase. Using hydrogen peroxide, catalase tests were performed on the *Xenorhabdus* spp. colonies (bubbling indicates a catalase positive reaction whereas no bubbling is indicative of negative catalase reaction) (Koppenhöfer, 2007).

7 – Experimental Procedure

As described above nine *X. bovienii* strains were considered for this study. One of the strains was the natural symbiont (cognate) of the nematode *S. puntauvense*. The remaining eight isolates were non cognate symbionts (Table 1). Using the above techniques, all nine *X. bovienii* strains mentioned were cultured from the frozen bacterial Stock cultures overnight for 18-24 h in LB supplemented with pyruvate broth. After the specified time period, the bacterial liquid cultures were plated on 5cm lipid agar plates (27 plates total) and NBTA plates (9 plates total). Three lipid agar and one NBTA plates were used for each strain of *X. bovienii*. After 48 h of growth, aposymbiotic *S. puntauvense* IJs were surface sterilized and plated equally onto each of the three dishes. The plates were observed every two days. Observation occurred for ~1 month. During this period IJs were monitored for growth and development and emergence of the IJs (progeny production). If IJs were observed crawling up the side of the dishes then the dishes were transferred to White traps to allow nematodes to move into the water. When a large quantity of IJs were observed in the water they were harvested and stored in 250 mL tissue

culture flasks (approx. 1 flask/nematode-bacteria pair was collected). One week from the initial harvest, a sample of IJ suspension from each nematode-bacteria pair was taken. The IJ suspension was surface sterilized and ground and plated onto 5 cm NBTA Petri dishes to confirm if nematodes had been successfully been colonized by the *X. bovienii*. Plates were incubated at 30°C for 48 h. After this period, the plates were checked for bacterial growth. To confirm bacteria identification, the catalase test was performed A second IJ grind was performed after 15 days of the initial harvest following the same procedure explained above. A selection of positively colonized IJs were considered to assess their virulence and reproductive fitness *in vivo* considering last instar larvae of the greater was moth, *Galleria mellonella*. Selected pairs were: *a) S. puntauvense-X. bovienii OS-10, b) S. puntauvense-X. bovienii – Monsanto and c) S. puntauvense-X. bovienii –Nemasys.* IJs Infections were done as described in the Materials and Methods section. As a positive control *S. puntauvense* with its cognate symbiont pair were also considered. Five *G. mellonella* cadavers per nematode-bacteria pair reassociation were considered. Infections were done following procedures described in section 2.

***Steinernema puntauvense* Li6**								
X. bovienii S. feltiae SN France	*X. bovienii S. feltiae* FL	*X. bovienii. S. feltiae* Molova	*X. bovienii. S. oregonense*	*X. bovienii. S. puntauvense*	*X. bovienii. S. kraussei* Quebec	*X. bovienii. S. kraussei* Nemays L	*X. bovienii. S. intermedium*	*X. bovienii. S. jollieti*



Results

A. Nematode development

Our observations indicate that all *X. bovienii* strains allowed growth and development of *S. puntauvense*. Growth of the plated IJs on bacterial loans was markedly visible. IJs matured to first generation males and females in approximately 2 to 4 days. All the positive reassociations generated IJs.

B. Symbiont colonization

As previously stated, all positive associations which, developed into IJs were ground to assess if cognate and non-cognate bacterial symbionts had colonized the IJs. Examples of grinding results are shown in Figure 2.

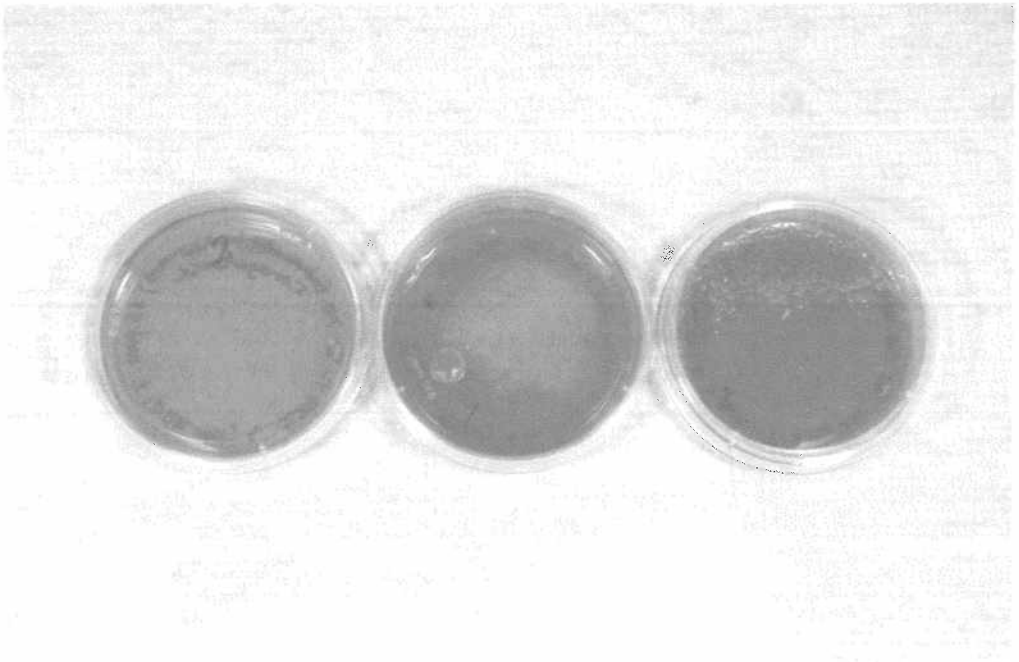


Figure 2. Grinding results of (from left to right) S. puntauense IJ reassociated with *X. bovienii-OS-10, X. bovienii-* Nemasys, and *X. bovienii-Monsanto*

Table 2. Summary of IJ growth/development and bacterial colonization

Bacterial Strain	**Growth and Development**	**IJ Emergence**	**Colonization**	**IJ Colonization**
SN France	Good	Yes	+	No
FL	Good	Yes	+	No
Moldova	Good	Yes	+	No
OS-10	Good	Yes	+	Yes
Li 6	Good	Yes	+	Yes
Intermedium-Type	No growth IJs moved away from bacteria	No	-	N/A
Monsanto	Good	Yes	+	No
Nemasys	Good	Yes	+	Yes
Quebec	Good	Yes	+	No

C. Nematode Fitness

As indicated before, we tested three *S. puntauvense - X. bovienii* reassociations*: a) S. puntauvense-X. bovienii OS-10, b) S. puntauvense-X. bovienii – Monsanto and c) S. puntauvense-X. bovienii –Nemasys. S. puntauvense-X.bovienii-Li6* was used as a positive control. Two consecutive IJ harvests were tested. The four tested nematode-bacteria reassociations were virulent resulting in insect host death. Cadavers were transferred to White traps for IJ emergence. After ~1 month in the White trap only one of the four reassociations had IJ emergence. At this time, cadavers were dissected to assess fate of the nematodes. Cadavers of the *S. puntauvense X. bovienii*-Monsanto combination showed no visible signs of nematodes. The reassociation of *S.*

puntauvense-X. bovienii-NEMASYS revealed presence of 2nd generation adults and juveniles. Nematodes were dead at the time of the dissection. The S. *puntauvense-X. bovienii-OS-10* reassoications revealed the presence of live IJs in all cadavers. It is worth noting that for all nematode-non-cogante symbiont combination, infected insects had variable level of fungal contamination. Lastly, the *S. puntauvense- X. bovienii-* Li 6 (its cognate symbiont) resulted in IJs emerging from the cadavers.

Table 3. Summary of In Vivo Infections with *G. mellonella*

Bacteria species involved in the reassociation	+/- Reassociations	Insect Host Death: Yes/No	Emergence (1st & 2nd Harvests)	Comments
Xb. S. feltiae SN France	N/A	N/A	N/A	N/A
Xb. S. feltiae FL	N/A	N/A	N/A	N/A
Xb. S. feltiae Moldova	N/A	N/A	N/A	N/A
Xb. S. oregonense OS-10	+	Yes	No	All cadavers had IJs
Xb. S. puntauvense Li 6	+	Yes	Yes	IJs visible in water
Xb. S. intermedium	N/A	N/A	N/A	N/A
Xb. S. jollieti	-	Yes	No	No sign of IJs
Xb. S. kraussei Nemasys L	+	Yes	No	All cadavers had developing 2nd generation IJs
Xb. S. kraussei Quebec	N/A	N/A	N/A	N/A

Discussion and Conclusions

Steinernema nematodes are insect pathogens that are able to kill a wide range of insects. Their pathogenic nature is mostly due to their association with enteric bacteria in the genus *Xenorhabdus*. It is known that each *Steinernema* nematode species is associated with one *Xenorhabdus* sp. However several *Xenorhabdus* species are associated with more than one *Steienerma* nematode. The extreme example of this level of host promiscuity is that of *Xenorhabuds bovienii* which is currently known to be associated with nine *Steinernema* spp. In this study we focused on *Steinernema puntauvense* (Li 6 strain), one of the nematode host species associated with *X. bovienii.* The goal of this study was to assess symbiont colonization by non-cognate bacteria and its impact on nematode fitness. For this purpose nine *X. bovienii* strains including the cognate (natural) and eight non-cognate strains were considered.

Sicard et al. (2004) conducted a similar study but their focus was to assess colonization of *S. carpocapsae* with three different *Xenorhabdus* species. Results from his study showed variable degrees of association that were characterized into three categories: 1) mutualistic bacteria that increased both the infestation success and offspring production of the nematode 2) 'neutral' bacteria that have a slightly beneficial or no impact on the fitness of the nematode 3) pathogenic or even lethal bacteria that reduce or even suppress offspring production of the nematode. For this study, the reassociations appear to be related to either the second or third categories described above. As found in this study, Sicard et al. (2004) explain that the classification of a bacterial strain into one of these three classes seems to depend on its evolutionary history. As in Sicard et al.'s (2004) study, this suggests that the native symbiont is more beneficial than the

other strains and reassociations are possible with *Xenorhabdus* species depends on its genetic distance to the native symbiont. However, in a different study conducted by Sicard et al. (2005), suggests that specialization levels can vary between bacterial symbionts and their nematode host.

As mentioned before, our study focused on assessing bacterial colonization and nematode fitness considering different bacterial strains of *X. bovienii*. Contrarily to Sicard et al (2004), our study tested intraspecific symbiont variation in nematode colonization and nematode fitness. Results from our study showed that *S. puntauvense* was successfully colonized by eight of the nine *X. bovienii* strains. The *X. bovienii* intermedium strain that is the natural symbiont of another *Steinernema* sp., *S. intemedium*, was the only strain that did not colonized *S. puntauvense*. Interestingly, this nematode belongs to a different evolutionary clade (Clade I) than the one *S. puntauvense* belongs to. We speculate this phylogenetic distance between nematode hosts may be the cause for preventing the bacterial symbiont to colonize another nematode host. All other *Xenorhabdus* strains tested in this study belong to nematode Species that are either sister taxa or close-related taxa to *S. puntauvense* and they all belong to the same phylogenetic clade (Clade III). In all positive symbiont-host associations, IJs of *S puntauvense* were able to mature and produce progeny in vitro assays. However, an interesting finding of this study was that nematode fitness was apparently compromised in its capability to either successfully infect an insect host and/or mature and produced an IJ generation that could emerge from the host. The findings of this study correlate to describe the inherent relationship of *Xenorhabdus* bacteria and their *Steinernema* hosts. Additional research should be performed considering other nematode hosts of *X. bovienii*. This will provide further insights into how this binary mutualism is achieved and the evolutionary qualities shared amongst different *Steinernema* spp.

The difference in the degree of specialization observed in this study and others is perhaps the result of genetic determinants. Further research on this field may reveal specific factors that contribute to the degree of specificity between *Steinernema* and *Xenorhabdus* that potentially translate into physiological adaptations contributing to nematode fitness and transmission (S.P. Stock, pers. comm.). This could possibly explain why the IJs from infections using the positive reassociations did not emerge from the cuticle. In addition, further full genome analysis of different *X. bovienii* strains could give further insights to the co-evolutionary relationships between the mutualistic relationship between *Steinernema* and *Xenorhabdus*. The association of *Steinernema* and *Xenorhabdus* and the evolution of their mutualistic interactions provide a fertile ground for further explorations of this experimental system to describe the underlying principles that contribute to fundamental aspects of mutualism.

References cited

Adams, B.J., Fodor, A., Koppenhöfer, H.S., Stackebrandt, E., Stock ,S.P., and Klein M.G. (2006). Reprint of "Biodiversity and systematics of nematode–bacterium entomopathogens." Biological Control 37: 32–49.

Adams, B.J., and Nguyen, K.B. (2002). Taxonomy and Sytematics. In R. Gaugler (Eds.). *Entomopathogenic Nematology* (pp. 1-2). Oxon: CAB International Publishing.

Flores-Lara, Y., Renneckar, D., Forst, S., Goodrich-Blair, H., and Stock S.P. 2007. Influence of nematode age and culture conditions on morphological and physiological parameters in the bacterial vesicle of *Steinernema carpocapsae* (Nemaroda: Steinernematidae). *Journal of Invertebrate Pathology* 95: 110-118.

Forst, S. and Clarke, D. 2002. Bacteria-Nematode Symbiosis. In Gaugler R. (Eds.). *Entomopahtogenic Nematology* (pp. 57-73). Wallingford: CAB International.

Gaugler, R., and Kaya, H.K. 1990. *Entomopathogenic Nematodes in Biological Control.* CRC Press, Raton, Florida. pp. 6.

Kaya, H. K., and Gaugler, R. 1993. Entomopathogenic Nematodes. *Annual Review of Entomology* 38: 181-206.

Kaya, H.K., and Stock, S. P. 1997. Techniques in insect nematology. In Lacey, L.A. (Eds.). *Manual of Techniques in Insect Pathology* (pp. 281-234). London: Academic Press.

Koppenhöfer, H.S. 2007. Bacterial Symbionts of Steinernema and Herorhabditis. In K.B. Nguyen D.J. & Hunt (Eds.), *Entomopathogenic Nematodes: Systematics, Phylogeny and Bacterial Symbionts: Nematology Monographs & Perspectives* (Vol. 5, pp. 735-808). Boston: Brill Academic Publishers.

Lee, M.M. and Stock, S.P. (2010). A multilocus approach to assessing co-evolutionary relationships between *Steinernema* spp. (Nematoda: Stenirenematidae) and their bacterial symbionts *Xenorhabdus* spp. (γ-Proteobacteria: Enterobacteriaceae). Syst. Parasitol 77: 1-12.

Sicard, M., Ramone, H., Le Burn, N., Pagés, S., Moulia, C. (2005). Specialization of the Nematode *Steinernema scapterisci* with its Mutualistic *Xenorhabdus* Symbiont. Naturwissencshaften 92: 472-476.

Sicard, M., Ferdy, J.B., Pagés, S., Le Brun, N., Godelle, B., Boemare, N., and Moulia, C. (2004). When mutualists are pathogens: an experimental study of the symbiosis between *Steinernema* (entomopathogenic nematodes) and *Xenorhabdus* (bacteria). Journal of Evolutionary Biology. 17: 985-993.

Snyder, M., Hinsinger, J., Le Brun, N., Pages, S, Boemare, M., and Moulia, C. 2007. New insights into colonization and release process of *Xenorhabdus nematophila* and the morphology and ultrastructure of the bacterial receptacle of its nematode host, *Steinernema carpocapsae. Applied and Environmental Microbiology*, in press.

Stock, S.P. and Goodrich-Blair, H. (2008). Entomopathogenic nematodes and their bacterial symbionts: The inside out of a mutualistic association. *Symbiosis* 46: 65-75.

Stock, S.P. and Goodrich-Blair, H. (2007). Nematode-bacterium symbioses: Crossing kingdom and disciplinary boundaries. *Symbiosis* 46: 61–64.

Stock, S.P. and Hunt, D.J. 2005. Nematode morphology and systematics. In Grewal, P. S., Ehlers, R.U., and Shapiro-Ilan, D.I. (Eds.). Publishing.*Nematodes as Biological Control Agents* (pp. 3-43). Wallingford: CAB International Publishing.