Spring 2013

The Investigation of Microbe-Nematode Interactions

Chelsea K. Crepeau
University of New Hampshire - Main Campus, ckj23@wildcats.unh.edu

SENIOR THESIS

The Investigation of Microbe-Nematode Interactions

The University of New Hampshire

By Chelsea Crepeau

5/16/2013

Abstract

The interactions between nematode and microbe species is a very interesting, yet misunderstood relationship. Although there are many types of nematode-bacteria relationships that are currently known, they are not well understood as to why or how they occur. In attempts to define these relationships more firmly, a variety of nematodes and bacterial strains were evaluated. *Caenorhabditis briggsae* along with symbionts, *Escherichia coli* OP50 and *Serratia* sp. strain SCBI, was evaluated for an entomopathogenic association and for cold tolerance. Heterorhabditis bacteriophora along with its symbiont, *Photorhabdus temperata* NC19, was also evaluated for cold tolerance. *C. briggsae* associations proved to have a pathogenic relationship with its *Serratia* sp. strain SCBI symbiont. The *C. briggsae* species was also capable of cold tolerance when associated with both *E. coli* OP50 and *Serratia* sp. strain SCBI. *H. bacteriophora* with its bacterial symbiont, however, demonstrated that the nematode-microbe model was difficult to work with as the cold tolerance was not effectively proven.

Background

A select few bacterial strains were selected for investigation along with their nematode counterparts. The bacterial strains involved in the investigation were *Serratia* sp. strain SCBI, *Escherichia. coli* OP50, and *Photorhabdus temperata* NC19. The nematode strains that were paired with these bacteria were *Caenorhabditis briggsae* and *Heterorhabditis bacteriophora*. The SCBI and OP50 strains of bacteria were used in the investigation of *Caenorhabditis briggsae* and NC19 was used in the investigation of *Heterorhabditis bacteriophora*. These symbiotic relationships between the various bacterial strains and their nematode counterparts were assessed independently of one another.

The symbioses of bacterial and nematode strains are of particular interest in molecular biology because of the microbe-nematode entomopathogenic characteristic. Entomopathogenic nematodes are capable of forming a close symbiotic relationship with the bacteria that allows them to effectively penetrate insect larva while the bacteria infect the larva causing death. The nematodes then reproduce rapidly through digesting the insides of the insect cadaver (Dillman and Chaston, 2012). After the nematodes and cadavers have had their period of incubation, the nematodes emerge from the insect cadaver through a rehydration process. Each symbiosis must be analyzed separately from existing symbioses due to the molecular differences between bacterial species and between nematode species.

All of the bacterial symbionts involved in the investigation of nematode-microbe interactions belong to the bacterial family Enterobacteriaceae. Enterobacteriaceae are characteristically gram negative, bacillus (rod-shaped) bacteria. They are facultative anaerobes, which allows for the molecular breakdown of sugars into other molecular byproducts. Often the members of Enterobacteriaceae have pathogenic associations with the variety of genera belonging to the family.

E. coli strains are mostly non-pathogenic, commensals to humans except of in the case of the strain O157:H7, which can cause food poisoning. Some gut *E. coli* may also cause urinary tract infections. Enterobacteriaceae are also facultative anaerobes which breakdown sugars to form lactic acid along with other molecular byproducts. The *E. coli* OP50 strain is used in the maintenance and growth of *Caenorhabditis elegans*, which is a popular model organism and relative species to *C. briggsae*. *C. briggsae* was grown on OP50 as a control throughout nematode investigation. OP50 is a uracil auxotroph that has limited growth patterns on NGM

agar plates, which is a beneficial feature for easy observation of nematodes and provides faster and more efficient mating between nematodes (Stiernagle, 2006).

The bacteria *Serratia* sp. strain SCBI (South African *Caenorhabditis briggsae* Isolate) species is a rare type of *Serratia* that has been isolated from soil samples in South African provinces (Abebe and Akele, 2011). The isolated bacterial strain was used as the known symbiont to *C. briggsae* and analyzed for its symbiotic characteristics. SCBI is also grown onto NGM plates in the nematode maintenance alongside the OP50 cultures.

The bacteria *Photorhabdus temperata* is a specialized bacterium belonging to the Enterobacteriaceae family. *Photorhabdus* species are bioluminescent bacteria that are symbiotic to entomopathogenic nematodes. The genus of *Photorhabdus* is a relatively small genus containing only three distinguished species that have been discovered. The bacterium contains a number of toxin encoding genes that are involved in the infection process of entomopathogenic nematodes.

Heterorhabditis bacteriophora is a species of nematodes that are known to be entomopathogenic in nature. The *H. bacteriophora* species is an insect parasite that is used in combination with *Photorhabdus* bacterial strains in the biological control of insects. The relationship the nematode has with the *Photorhabdus* species is to provide the bacteria with an obligate host. The transmission of bacteria occurs in the infective juvenile (IJ) stage of nematode life cycle, and is essential for successful parasitism of insects as well as nematode reproduction within insect cadaver (Ciche, 2007). The relationship is characterized as an obligate vector-borne disease of insects based on the clear symbiotic association and infection methods. The species was discovered in 1975 as a new nematode species that in the IJ stage, much like the dauer stage

of *C. elegans*, transports a gram negative bacterium to the hemocoel (interconnected tissue spaces through which blood circulates) of insect hosts. The IJ stage of the life cycle is an arrested stage of development where there is no feeding. This particular point in the life cycle of *H. bacteriophora* is the only stage in which the nematodes are able to freely swim outside of a host in nature.

The entomopathogenic cycle occurs based on the infective capabilities of the IJ nematodes. When the *H. bacteriophora* IJs are freely swimming through soil, containing a bacterial monoculture within their intestine, they are able to search for a host organism. Once a host organism is found the IJ will effectively enter the hemocoel of the insect and regurgitate the bacterial monoculture into the hemocoel. The insects die rapidly once the bacteria have entered the hemocoel, being the cause of mortality in the insect host. The nematodes are known to not be the mortality cause because the bacterial strain is needed for nematode reproduction (Ciche, 2007). After insect death, the nematodes grow rapidly for 2-3 generations before developing into the DJ, the stage in which they disperse from the cadaver.

Caenorhabditis briggsae are soil-dwelling nematodes that are of interest in scientific investigations because of its close relationship to the model organism *C. elegans*. The two species of *Caenorhabditis* share a large amount of their morphology, as well as hermaphroditic life cycle and ease of cultivation. Although the species are known to have diverged from each other many years ago, their morphology and behaviors are almost identical, allowing *C. briggsae* to provide an alternative model organism. After the whole genome of *C. briggsae* was sequenced it was comparatively analyzed and found to have 62% of protein-encoding genes orthologous to *C. elegans* (Gupta, Johnsen, Chen, 2007). *Caenorhabditis briggsae* is of particular interest in molecular biology because of the potential for entomopathogenicity.

Literature Review

The microbe-nematode interaction between *Caenorhabditis briggsae* and *Serratia* SCBI has been of increasing interest in the most recent years. Since the new *Serratia* species was isolated from South African soils the relationship between the nematodes and bacteria have been experimentally analyzed. The largest analysis has been regarding the entomopathogenic nature of nematodes. Since the bacteria species of SCBI was isolated from the nematode strain in question, the possibility for infection of insects seems like a plausible theory.

In a study by Abebe and Jumba (2010), the two species were analyzed for an entomopathogenic relationship. The premise of the paper was to gain a new understanding of the ecology of *Caenorhabditis* as a model organism. The article focuses on the ability of nematodes to live off hosts during the dauer (resistant) larval stage of the nematode life cycle. It was known that *Caenorhabditis* use insect hosts in two ways: a phoretic relationship in which the nematode uses the host as a transportation agent between food sources and a necromenic relationship in which the nematode waits for host demise and feeds on the decay (Abebe and Jumba, 2010). Given the known associations, however, it had not been determined that any *Caenorhabditis* members were entomopathogenic.

The authors investigated the entomopathogenesis of *C. briggsae* with its bacterial associate by obtaining three soil samples from three different farm sites in South Africa. The farm sites were in North West, Mpumalanga, and Kwa Zulu-Natal. The nematodes were isolated from samples through the use of late instar larval *Galleria mellonella* (Abebe and Jumba, 2010). The nematode cultures were maintained in three varieties: infection cycle to reflect possible natural conditions, on *E. coli* OP50, and on *Serratia* SCBI. The entomopathogenicity was tested

by topical exposure and hemocoelic injection of *Serratia* SCBI into *G. mellonella*. According to the authors the ability for *Caenorhabditis* to become entomopathogenic is possible when cultured on *Serratia* SCBI. This was defined as entomopathogenic based on the ability to reproduce inside *G. mellonella*, penetration of the insect host, killing of the insect, and emergence from the cadaver as infective dauer stage juveniles (Abebe and Jumba, 2010).

A second article by Abebe and Akele (2011) focused on the same entomopathogenic relationship, as multiple authors were the same. The article focuses on the comparison of the relationship to the archetypical entomopathogenic nematode-bacteria complexes of *Steinemema-Xenorhabdus* and *Heterorhabditis-Photorhabdus*. The authors claim that the *Caenorhabditis-Serratia* complex is identical to these systems (Abebe and Akele, 2011). The article also focuses on the question of whether the relationship between *Caenorhabditis* and *Serratia* is an obligatory or facultative relationship. The authors notate that by the narrow definition of EPN interactions, the *Caenorhabditis-Serratia* complex does not fit because the relationship involves a transient association rather than a highly specific co-evolving association (Abebe and Akele, 2011).

The results of the two studies do not provide a stable understanding for the entomopathogenic relationship between *Caenorhabditis* and *Serratia* SCBI. Although the particular lab involved seems to have come up with an association the reproduction of results has been lacking. Prior to beginning the following research, members of the lab in which the experiments were done had not been able to reproduce the above results.

In an article by Dillman and Chaston (2012) it is pointed out that according to the EPN definition, *C. briggsae* may not be an EPN. This is due to the fact that IJs from *G. mellonella* were able to reinfect new hosts, but are not as virulent a complex than if the bacterium associated

is directly injected alone. These results suggest that there may be an inefficient release of pathogen or antagonism by nematode vector. According to these results it is unclear what the relationship between *C. briggsae* and *Serratia* SCBI can be defined as: necromenic, entomopathogenic, or an inefficient nascent entomopathogen. These results support the need for more entomopathogenic testing and reproduction of results from prior studies involving the "entomopathogenic" relationship of *C. briggsae* and *Serratia* SCBI.

In relation to *Heterorhabditis bacteriophora* and its ability to tolerate freezing protocols there are three papers to consider. The first paper is a protocol developed for *H. bacteriophora* and to *Steinernema* species. The second paper is a protocol for the cryopreservation of various *Heterorhabditis* species. The third paper is a novel freezing protocol involving the use of host insects to serve as a layer of protection in cryopreservation.

The first paper by Curran, Gilbert, and Butler (1992) focuses on preincubation of nematodes in glycerol cryoprotectant, filtration of nematodes with a washing step, incubated for a short period, resuspended, sedimented, and aliquoted into cryovials to be frozen by liquid nitrogen. The results of the paper have an average viability rate of 68% for *H. bacteriophora* that ranged from 30 to 87. The second paper by Nugent, O'Leary, and Burnell (1994) focuses on the ability of *Heterorhabditis* species to tolerate freezing had a similar protocol in which the nematodes were preincubated for a variety of days in a variety of liquid cryoprotectants, filtered and washed, incubated for a short period, resuspended, sedimented and aliquoted into cryovials to be frozen by liquid nitrogen. The differences in this paper are that the variety of cryoprotectants included more than just varying glycerol solutions and the necessity of application on filter paper before cryovials were frozen. The paper results state that DMSO as a

cryoprotectant yield results comparable to that of glycerol solutions across the *Heterorhabditis* species.

The third paper by Lewis and Shapiro-Ilan (2002) focuses on the ability to use the EPN relationship of *H. bacteriophora* to freeze the nematodes while inside the insect host. After infecting the insect host with the EPN, survival was checked after 24 hours with varying incubation times within the cadaver host. When cadavers were frozen at early stages of infection few IJs emerged post-freezing, but when frozen during later stages of infection the results seemed to show that there were no negative effects on IJ production. *H. bacteriophora* also showed improved survival when frozen inside the host than when directly exposed to freezing.

Hypothesis

The goal of the research and experimentation is to define *Caenorhabditis briggsae* on the scale of entomopathogenesis, to develop and test *C. briggsae* freezing conditions, and to test for efficient freezing protocols of *Heterorhabditis bacteriophora*. The first portion is to experimentally test the theory that *C. briggsae* is an entomopathogenic nematode (EPN) based on the association with the SCBI bacterial isolate found in South Africa. The EPN will be tested only using *Serratia* SCBI grown on NGM agar plates and *Caenorhabditis briggsae*, the two species found together in natural isolation in South Africa.

The two freezing protocol portions are a goal to provide an efficient storage method of nematodes. This storage step could allow the maintenance of many nematodes in stock without having to grow them on media plates and transfer every two weeks. The amount of space necessary for maintenance of nematodes would decrease significantly since C. *briggsae* are

grown and maintained on NGM plates at room temperature and *H. bacteriophora* are stored in refrigerators in screw-top containers after their growth period.

The *Caenorhabditis briggsae* freezing protocol, modeled after the protocol developed for *C. elegans*, should be an efficient method to freeze the species based on the behavioral similarities to *C. elegans*. The *Heterorhabditis bacteriophora* protocol that should prove to be an efficient method will involve using the entomopathogenic nature of the nematode to freeze during their regenerative stage within insect cadavers. The accepted method of *H. bacteriophora* freezing should be tested alongside the novel protocol for comparative purposes to ensure that the recovery of viable nematodes is equivalent, or nearly so, to the existing protocol.

Methods

Maintenance and Growth of *Caenorhabditis briggsae*

A *C. briggsae* strain was obtained from Sheldon Hurst IV in the Louis Tisa Laboratory at the University of New Hampshire. *E. coli* OP50 and *Serratia* sp. strain SCBI strains were also obtained from the Tisa Laboratory, and kept as stocks on LB plates: 5g tryptone, 2.5g yeast extract, 5g NaCl, and 7.5g BactoAgar in 500mL solution. The bacterial streak stock plates were grown at 37˚C overnight and parafilmed to be stored at room temperature. The streak plates were re-streaked every 2-3 weeks to maintain fresh bacterial stocks throughout experimentation. The *C. briggsae* strain was grown on prepared NGM plates by protocol from Stiernagle (2006), but altered by cutting the recipe in half to be made in 500mL batches. The plates were poured using sterile technique by flaming.

The bacterial strains were prepped for application onto NGM plates by using a sterile wood stick to obtain bacteria from stock LB plate colonies. The bacteria were grown in LB media in a shaker overnight. The bacterial strains were aliquoted in 100μL volumes onto the NGM plates prepared and grown in a 37˚C incubation room overnight. The nematodes were seeded onto the fresh NGM plates by needle picking method until about 10-15 nematodes were transferred. The *C. briggsae* strain was seeded onto new plates about every 3-5 weeks depending on how quickly the strain utilized the bacterial food source.

Maintenance and Growth of *Heterorhabditis Bacteriophora*

A surface sterilized *H. bacteriophora* strain was obtained from Sheldon Hurst IV in the Tisa Laboratory at the University of New Hampshire. The bacterial strain of *P. temperata* NC19 was also obtained from the Tisa Lab. The *P. temperata* NC19 strain was obtained as a stock streak and was re-streaked to maintain healthy and unmutated forms of the bacteria often. The bacteria were grown in a 28˚C incubator for two days and kept at room temperature until re-streaking. The *H. bacteriophora* strain was grown on prepared lipid agar plates. The recipe for the lipid agar plates used contains a media step and CS Mix solution step. In a 1000mL flask, 2g nutrient broth, 3.75g BactoAgar, 1.25g yeast extract, 222.5mL diH_2O, and 2.5mL $MgCl_2 \cdot 6\ H_2O$ were added. CS Mix was made in 200mL screw-top bottle: 7mL dark corn syrup and 89mL diH_2O. The two solutions were autoclaved on liquid setting for 30 minutes (about a full hour cycle). After autoclaving, 24mL of CS Mix and 1mL of corn oil were added to the media mixture that was in constant stirring on a stir plate. The plates were poured using sterile technique.

The bacterial strains were prepped for application onto lipid agar plates by using a sterile wood stick to pick up bacteria from a colony on the stock plate and grown in an LB media tube

in a shaker overnight. The bacterial strain was aliquoted onto the lipid agar plates in 800µL volumes and distributed through swirling technique. The plates were grown in a 28˚C incubator for 2-3 days before seeding of nematodes. Nematodes were seeded from stocks in the lab refrigerator in 10-20µL volumes depending on amount of nematodes in those volumes (at least 10 nematodes to be seeded). Seeded nematode-lipid agar plates were grown in the 28C incubator for about 12-14 days. Once nematodes were adequately grown they were pipetted off using water and put into a filtration system overnight with additional (a petri plate with a Falcon filter) to ensure that only live nematodes were to be stored. Once live nematodes filtered out they were aliquoted into a conical tube and allowed to settle, excess water was pipetted off, and clean water was added to fill half of the conical tube. The conical tube was dated and stored as a *H. bacteriophora* stock in the lab refrigerator until needed for experimentation.

Entomopathogenic Test of *Caenorhabditis briggsae*

To test *C. briggsae* for an entomopathogenic relationship with *Serratia* SCBI freshly starved nematodes on NGM plates were used. Sterile H2O was applied to the freshly starved NGM plate and allowed to sit for 5-10 minutes. The water-nematode mixture was pipetted into a clean test tube and the nematodes were allowed to settle, excess water was pipetted off to obtain a concentrated solution of nematodes. The nematodes were tested for EPN relationship by two methods: a direct injection using a 26 5/8 gauge needle and 1mL syringe, and a natural infection method. The nematodes were also tested using two different types of insect host models: *Galleria mellonella* and *Manduca sexta*.

The *G. mellonella* infections involved placing ten insects into petri dishes with a No. 1 Whatman filter paper. The natural infection method entailed pipetting 1mL of concentrated *C.*

briggsae solution to the epidermis of each individual insect host in the form of a droplet and the rest of the solution was applied to the filter paper that the insects try to eat. *C. briggsae* were directly injected into *G. mellonella* by needle and syringe in about 20-40µL volumes per insect. One natural infection method and three direct infection methods were set up, one natural and one direct on a particular day, and two direct three days after the initial series. The two varying dates allow for variation in incubation time within the insect cadaver before testing for emergence. Both sets of infections were set up in white traps at the same time (nine days from the first series of infections and six days from the second series of infections). A white trap is a medium sized closed petri dish with water surrounding a small open-faced petri dish with damp filter paper and insect cadavers placed inside. The white trap is an EPN set up that allows for emerging nematodes to swim into water of the medium sized petri dish.

The *Manduca sexta* infections involved placing one insect into petri dishes with a No. 1 Whatman filter paper. The natural infection method entailed cutting a small cube of insect food from the stock and placing it in the petri dish with the insect, then 1mL of *C. briggsae* solution was injected into the food by 26 5/8 gauge needle and 3mL syringe. *C. briggsae* were directly injected into *M. sexta* by needle and syringe in the amount of 1mL distributed at various points along the insect, some of the solution dripping out of the insect at the injection points. The cadaver hosts were set up in white traps three days after infection series.

Freezing Protocol of *Caenorhabditis briggsae*

The protocol to test the ability of *Caenorhabditis briggsae* to tolerate freezing conditions was based off of the freezing protocol of *C. elegans* found in WormBook by Stiernagle (2007). The *C. briggsae* freezing test was carried out with *C. briggsae* on *E. coli* OP50 and *C. briggsae*

on *Serratia* SCBI. The S Buffer solution and S Buffer + 30% glycerin(v/v) solution were both made per protocol (Stiernagle, 2007). First 3.6mL of S Buffer solution was added to the surface of freshly starved *C. briggsae* NGM plates. The liquid was allowed to sit for about 3-5 minutes to elute nematodes into the liquid, and then pipetted off into a sterile test tube. An equal volume of S Buffer+30% glycerin was added into the test tube and mixed well by shaking gently. An aliquot of 1mL liquid mixture was pipetted into a round-bottom 1.8mL cryovial. The cryovial was labeled with the bacterial strain name and the date. Six cryovials were prepared following the protocol. The cryovials were placed into a cryovial freezer box and placed into the -80˚C freezer.

After one week two cryovials of nematodes were thawed and observed for viability, one cryovial of each bacterial strain the nematodes were grown on. The cryovials were thawed at room temperature until all of the ice has turned back into liquid form. The OP50 cryovial liquid contents were poured onto the *E. coli* OP50 lawn of a fresh NGM plate. The SCBI cryovial liquid contents were poured onto the *Serratia* SCBI lawn of a fresh NGM plate. Each plate was observed for viability under dissecting microscope.

Freezing Protocols for *Heterorhabditis bacteriophora*

H. bacteriophora was tested for tolerance to freezing conditions by manipulation of the EPN association. *H. bacteriophora* from a stock were tested using the natural infection EPN method into *G. mellonella*. Ten insects were placed into a petri dish with filter paper on the bottom, then a 1mL aliquot of *H. bacteriophora* and H_2O solution was distributed onto each insect hosts' epidermis in the form of a droplet and the remaining solution was applied to the filter paper which the insects eat. Once the *G. mellonella* were infected with *H. bacteriophora,*

they were allowed to incubate for four days after infection protocol, and were set up for freezing. One or two insects were placed into a round-bottom 1.8mL cryovial with 60% glycerol solution, enough solution to cover the insects completely. The cryovials labeled and dated with the day frozen and the day collected from lipid agar plates. The cryovials were then placed in a cryovial holding freezer box and placed into the -80˚C freezer.

After about a 20 day period of being in the freezer the cryovials were taken out of the freezer and thawed using body temperature (by rubbing in between the hands) to thaw quicker than room temperature. Once the ice turned back into liquid solution it was poured off and the insects were rinsed with dionized H_2O. The *G. mellonella* were placed on a paper towel and allowed to dry. After the insects appeared to be dry, they were set up into two white traps: one for each stock date used (one stock that was a month and a half old and one stock that was two weeks old prior to freezing). The white traps were observed for emergence of nematodes for over a month.

A second freezing protocol was to be run, but was incomplete. A stock of *H. bacteriophora* was allowed to settle, the excess water was removed, and the remaining nematode suspension was divided into four 1.5mL centrifuge tubes for surface sterilization. Four cryoprotectants were made for testing: 1% NaCl, 0.5% NaCl, 17% glycerol, and 8% DMSO. The compiled list of cryoprotectants was taken from the Nugent protocol paper (1994). A small amount of cryoprotectant was added into the centrifuge tube to resuspend nematodes and the liquid solution was poured into a petri dish, the total volume of cryoprotectant in each petri dish was about 12mL. The *H. bacteriophora* petri dishes were left to incubate for 72 hours (last step reached, rest of protocol was unable to be done for lack of materials). After 72 hours, nematodes were to be isolated from cryoprotectants by suction filtration and washed in 70% ice cold

methanol. The filter paper was to be placed into a small petri dish on ice containing 3mL of ice cold 70% methonal and IJs resuspend by agitation and incubated on ice for seven minutes. The IJ nematodes were to be transferred into 1.5mL centrifuge tubes and centrifuged at 4000rpm for two minutes. The excess methanol was to be pipetted off until about 60µL of nematode pellet/suspension remained. The suspension was to then be transferred in 30µL aliquots to round-bottom cryovials and plunged into liquid nitrogen.

Results

Entomopathogenic Test of *C. briggsae*

The biological species of *C. briggsae* was tested for and entomopathogenic relationship with its bacterial symbiont *Serratia* sp. strain SCBI. The test was performed by using natural infection and direct infection protocols with two insect host strains, *G. mellonella* and *M. sexta*. Two sets of infections were performed with the *G. mellonella* host strain and one set of infections was performed with the *M. sexta* host strain.

After about six days in the white traps *C. briggsae* IJs were emerging from the *G. mellonella* insect host cadavers. In the first set of infections, *G. mellonella* white trap contained about 75-100 IJ nematodes per 10µL of solution pipetted directly off the wet filter paper that the IJs were clearly swimming on, they had yet to get out of the small petri dish. This pipetting was done directly next to a host insect cadaver. The natural infection from the first set of infections did not appear to have re-emerging IJs yet. The second set of infections was done with two direct injection protocols; both had re-emerging IJs swimming on the filter paper of the white trap.

Each filter paper was moistened and 10μL of nematode-water solution was analyzed for viable IJ nematodes and showed that each white trap sample contained about 20-40 viable re-emerging nematodes based on the area of the filter paper the sample was pipetted from.

In the infections utilizing *M. sexta* as the insect host for *C. briggsae*, two infections were set up, one direct injection and one natural infection. The results of the infection were recorded three weeks after the white traps were set up. The direct infection did not yield any IJ nematodes. The natural infection that utilized injecting *C. briggsae* into the food course of the insect did yield IJ nematodes that had re-emerged into the outer shell of the white trap. In about 150μL from the outer shell there were 5-10 viable IJ nematodes per liquid volume.

Freezing Protocol for *Caenorhabditis briggsae*

The freezing protocol for *C. briggsae* was modeled after the *C. elegans* protocol by Stiernagle (2007). The cryopreserved nematodes were initially analyzed for viability after a week of freezing in the -80C freezer. The nematodes that were frozen were very much viable, displaying signs of twitching and swimming readily in the liquid on top the NGM plates. After about 6 days of growth on the new NGM plates, new plates were seeded from the freezer stock to analyze reproduction capabilities. The newly seeded plates were allowed to grow for six days before being used for new seeding. The freezer stock showed massive capabilities to reproduce, and through microscopic analysis the SCBI nematodes showed slightly more reproduction and growth during the seven day period, displaying several layers of nematodes atop each other.

The strain was also observed post-freezing from three different freezing sessions that were used for comparative analysis, both for time frozen effects and for variation between bacterial species used as a food source for *C. briggsae*. In each freezing session there were about

50-70 viable nematodes in buffer solution prior to being placed in the freezer. The results, obtained on May 15th, are listed in Table 1 below.

The Comparitive Analysis of Freezing Stocks of *C. briggsae*		
Date Frozen	Bacterial Food Source	Average Number Viable Nematodes in 10μL solution
February 14th	*E. coli* OP50	20
	Serratia SCBI	30
May 4th	*E. coli* OP50	10
	Serratia SCBI	20
May 27th	*E. coli* OP50	40
	Serratia SCBI	30

Table 1: The number of viable nematodes post-freezing in S Buffer solutions on varying bacterial strains and after variable lengths of time.

Freezing Protocols for *H. bacteriophora*

A freezing protocol was performed utilizing the EPN association of *H. bacteriophora* with *Photorhabdus*. The insect host cadavers were to serve as a protective coating around the *Heterorhabditis* IJs so that the cryoprotectant was not directly contacting the nematode IJs. The insect host cadavers were frozen for about three weeks before being set up in white traps to analyze for re-emergence. After about three weeks of observation for re-emergence, it was clear that the protocol performed did not yield any viable nematode IJs. A second trail of the experiment protocol could not be performed.

A second freezing protocol was to be performed using liquid solution cryoprotectants that the IJ nematodes were directly exposed to. After a 72 hour incubation period in the cryoprotectants, the protocol was not continued. A second trial of the experiment protocol could not be performed.

Discussion

Entomopathogenic Test of *C. briggsae*

The species *C. briggsae* along with its bacterial symbiont *Serratia* sp. strain SCBI have proved to have an intimate relationship that can result in re-emergence of healthy and viable nematodes from an insect host. The protocol that was used involved a more natural technique than in previous testing attempts by Abebe and Jumba (2010). In the previous protocol the direct injections of nematodes into the insect host called for the use of buffered saline solution, but in the present technique the solution was purely a sterile H_2O solution. This approach was used as a more simple approach avoiding excess chemicals.

There were several issues involving the experimental protocol based on the amount of time that the research was being conducted. The white traps that were observed for entomopathogenic nematode activity were not able to be replicated because it was towards the end of research semester. The direct injection of *M. sexta* did not work as an EPN test, but all of the direct injections of *G. mellonella* did work as an EPN test. A reason that the *M. sexta* direct injection did not yield any IJ nematodes is that the insect used was a very small insect and was very fragile in trying to transfer to the white trap. Also there is a chance the small *M. sexta* insect

could have died from alternate causes in which case the nematodes would not necessarily infect and reproduce within the cadaver. In opposition to the direct inject results, the natural infection of *M. sexta* did work as an EPN test, but the natural infection of *G. mellonella* did not seem to work as an EPN test. A reason that *G. mellonella* did not yield positive EPN results during the infection process is because there was not enough time to observe the white trap. Another explanation for lack of IJ nematodes from the natural infection is that the *G. mellonella* hosts may not have actually been penetrated or infected by any of the *C. briggsae* nematodes.

The true relationship between the species involved is clearly a pathogenic relationship, but how this pathogenic relationship works is still very unclear to researchers. The *Serratia* sp. strain SCBI strain is clearly the pathogenic factor that kills the insects from the inside, but whether it is the nematodes delivering the bacteria by regurgitation or mere exposure cannot be determined. Since the *C. briggsae* epidermis is not as tough as that of *H. bacteriophora,* it cannot be surface sterilized before infection techniques are used. Without the surface sterilization step, the *C. briggsae* may still be carrying the SCBI strain on their epidermal layer, which would mean that the bacteria can infect through the mere transportation into the host and reproduce there. The reasons *Serratia* can be found in the gut of the nematodes is because it can still eat the bacteria as a food source inside of the insect cadaver.

Freezing Protocol for *C. briggsae*

In considering the effect of freezing conditions on *C. briggsae*, the nematode strain in question is reliably able to tolerate cold conditions. The nematode also shows signs of positive freezing regardless of the species of bacteria grown on as a food source. There were slight differences between the two bacterial species during the regeneration stage post-freezing,

however, that suggests there might be an increased ability for *C. briggsae* originally grown on strain SCBI to reproduce after freezing after being seeded on new NGM-bacterial plates. As this was an observation made by eye of the two different types and there was not any quantitative data that support the idea of increased activity of the SCBI strain, this theory cannot be accepted as true, but merely considered as a possibility. If there had been more time to continue the research, this association would have been a quality theory to test as it might give results indicating the strength of *C. briggsae* to associate with Serratia SCBI over other bacterial strains that could be used as potential food source in experimentation.

The positive results of the *C. briggsae* freezing protocol allow implications to be made on how the organism is stored, shipped, and maintained. Prior to testing the organism for cold tolerance the main way to obtain the species and to maintain the species involved the growth and transfer of nematodes onto fresh NGM plates often enough so as to not kill off the species. The ability to freeze nematodes will now allow for long term storage while the strain is not needed for experimentation. The strain can be more easily transported between locations as well since it can now be sent by dry ice in cryovials, rather than being transported in much bulkier containers capable of holding multiple NGM plates of nematodes.

Freezing Protocols for *H. bacteriophora*

The strain of nematodes, *H. bacteriophora*, proved to be the most difficult portion of research during the year. The *H. bacteriophora* strain was the hardest to grow, hardest to maintain, hardest to store, and hardest to test. There were many setbacks through the year involving both the strain of nematode and its *Photorhabdus* symbiont. *P. temperata* NC19 caused issues throughout the year because it has a rapid mutability rate and often would mutate

and contaminate, which would keep from *Heterorhabditis* growth and infection. There were probably about three times the NC19 strain that was used had to be disposed of because of mutation and contamination, requiring a new streak from stock which at times could take up to a week to get.

The strain of *H. bacteriophora* had many issues of its own throughout the year as well. *Heterorhabditis* has a long growth incubation period and does not regenerate quite as fast as *C. briggsae*, the other research strain in question. Given that the regeneration time is about two weeks, the ability to build up lots of nematodes for stock was difficult especially with the many bacterial strain setbacks. *H. bacteriophora* also did not grow well, if at all during some of the incubation times. Due to all of these factors only two groups of stock were able to be made throughout the semester, giving only a small amount of nematodes to work with for the freezing protocols.

The first protocol, involving the use of the EPN association, did not yield positive results. The white trap that was set up did not show re-emergence of IJ nematodes. There are a few potential reasons for this including: cryoprotectant type, length of nematode infection prior to freezing, and inability to alter the protocol. The cryoprotectant that was used in the procedure was 60% glycerin which is a high concentration cryoprotectant. In most cases of freezing *H. bacteriophora* the cold tolerance occurs at levels between 11-17%, but this is direct contact and the theory was that a high concentration would not affect the ability of nematodes to re-emerge because of the protective layer the insects served. If there had been enough stock of *H. bacteriophora* built up prior to the experiment it would have been redone with varying concentrations of cryoprotectants.

The second protocol, which utilized information from multiple procedures of freezing strains of *Heterorhabditis*, also did not yield any results. Although the experiment was begun, the filtration step was to occur after 72 hours, the day that this fell on the materials necessary could not be found and there was no supporting aids within the laboratory that day to help find the materials necessary. Given that the opportunity to filter and freeze had passed after 72 hours the procedure was not able to be finished. Also, given the small amount of *H. bacteriophora* stock that was built up throughout the year, there were no more nematodes left to attempt the procedure a second time. This failure of experiment was strictly experimenter-based and the protocol cannot be deemed as a failing protocol for this reason.

References

Abebe, E., et al. An entomopathogenic *Caenorhabditis briggsae. (*May 27, 2010). The Journal of Experimental Biology Vol. 213 (3223-3229). doi: 10.1242/jeb.043109.

Ciche, T. The biology and genome of *Heterorhabditis bacteriophora* (February 20, 2007), *WormBook*, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.135.1. http://www.wormbook.org.

Curran, J., Gilbert, C., and Butler, K. (1992). Routine Cryopreservation of Isolates of Steinernema and Heterorhabditis spp. Journal of Nematology Vol. 24 No. 2 (269-270).

Dillman, A.R., Chaston, J.M., and Sternberg, P.W. An Entomopathogenic Nematode by Any Other Name. (March 8, 2012). Public Library of Science Pathogens. doi: 10.1371/journal.ppat.1002527

Gupta, B.P., et al. Genomics and biology of the nematode *Caenorhabditis briggsae* (May 3, 2007), *WormBook*, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.136.1. http://www.wormbook.org.

Lewis, E.E. and Shapiro-Ilan, D.I. (September 2002). Host cadavers protect entomopathogenic nematodes during freezing. Journal of Invertebrate Pathology Vol. 81 No. 1 (25-32).

Nugent, M.J., O'Leary, S.A., and Burnell, A.M. (November 17, 1994). Optimised procedures for the cryopreservation of different species of *Heterorhabditis*. Fundamental Applied Nematology Vol. 19 No. 1 (1-6).

Stiernagle, T. Maintenance of *C. elegans* (February 11, 2006), *WormBook*, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.101.1. http://www.wormbook.org.