Spring 5-2011

Characterization of the Microbial Associates of Nematodes Pathogenic to Myrmica rubra

Jonathan E. Dumont

CHARACTERIZATION OF THE MICROBIAL ASSOCIATES OF NEMATODES PATHOGENIC TO *MYRMICA RUBRA*

by

Jonathan E. Dumont

A Thesis Submitted in Partial Fulfillment
of the Requirements for a Degree with Honors
(Biology)

The Honors College

University of Maine

May 2011

Advisory Committee:

Eleanor Groden, Professor of Entomology, Advisor
Andrei Alyokhin, Associate Professor of Applied Entomology
David Lambert, Associate Professor of Plant Pathology
Stellos Tavantzis, Professor of Plant Pathology and Cooperating Professor of Biochemistry and Microbiology
François G. Amar, Associate Professor of Physical Chemistry

Abstract:

Pristionchus entomophagus is a necromenic nematode commonly associated with dung beetles (*Geotrupes spp.*) in Europe. Recently, it has been found in Maine emerging from cadavers of *M. rubra*, an ant native to Europe that is currently established and pestiferous in Maine (Groden and Stack 2011). Laboratory assays inoculating *M. rubra* with these nematodes caused significant mortality. This study aimed to characterize the bacterial associates of *P. entomophagus* in order to further understand its pathogenic relationship with *M. rubra*. Bacterial samples were collected from the cuticle and gut of the nematodes, and the hemolymph of infected hosts. Single spore isolates were established, cultured, and identified using bacterial 16s rRNA gene sequencing. Following BLAST search comparisons of 24 isolate sequences that I obtained, I identified 14 species of bacteria associated with *P. entomophagus* and its infected hosts. All of these species have been reported as associated with nematodes, insects, the rhizosphere of plants, fungi, or soil. Three species in particular, *Serratia marcescens*, *S. nematodiphila*, and *S. proteamaculans* have all been directly linked to insect mortality in previous studies (Zhang et al. 2009, Nishiwaki et al. 2007, Al Own et al. 2011). Furthermore, *S. proteamaculans* was found in association with *P. entomophagus* in the United Kingdom. In comparing the bacterial isolates found in association with the hemolymph of infected hosts to isolates gathered from *P. entomophagus*, two species, *Pseudomonas fluorescens* and *Delftia sp.* were found in both the hemolymph and in association with the external surface of the nematode. The potential mechanism of pathogenicity employed by *P. entomophagus* is discussed based on the similarities and differences between the species isolated from the nematode and infected hosts.

Dedication

I would like to dedicate this thesis to all of the people who helped me get to this point in my academic career and my personal life. First and foremost I would like to thank my parents for being supportive of my endeavors throughout the years. I would also like to thank my younger brother, Joe, for keeping me in line all these years and being my best friend. To all of the members of the SBFTD, you guys rock and I couldn't have made it through college sane without you. To all of the other friends I've made throughout my undergraduate career that made it truly special, AJC, MM, WC, ZG, JB, EP, KA, DB, DM, MC, RM, PM, DH, TS, and CT, thanks for the good times. The thesis process took me for a wild ride and you all helped me come out the other side successful, and for that I thank you.

Acknowledgements

I would like to thank all of the professionals that I have been involved with over the past four years that have had a hand in shaping my academic career. First, I would like to thank Dr. Eleanor Groden, whose guidance and commitment to my thesis was invaluable. I would like to thank the staff at the Maine Insect and Disease Laboratory for giving me my first experience with entomology, and the staff at The University of Maine who have kept me interested in entomology and have been invaluable as a support system and a positive influence on my academic career.

I would also like to thank the members of Dr. Groden's lab group at the University of Maine, Jennifer Lund and Tamara Levitsky, as well as the other individuals who aided me in the completion of this thesis, Rabern Simmons, Lee Beers, Frank Drummond, the University of Maine Sequencing Facility staff, and the members of my thesis committee.

Table of Contents

List of Figures and Tables

Introduction:

Myrmica rubra

Myrmica rubra (Linnaeus) (Hymenoptera: Formicidae), is an ant that is native to the much of the Palearctic ecozone in Europe and Asia, from Ireland in the west to Western Siberia in the east (Czechowski et al. 2000). *M. rubra* has been introduced into regions where it is non-native through unintentional human transport. Currently, *M. rubra* is established in North America, where it is largely invasive (NBII and ISSG, Arevalo et al. 2007). Established populations have been reported in Maine, Massachusetts, New York, Pennsylvania, New Jersey, Washington D. C., Rhode Island, New Hampshire in the US, and in Ontario, Québec, New Brunswick, Prince Edward Island, Newfoundland, and Nova Scotia in Canada (Wetterer and Ravchenko 2010). Within its native range it exists from approximately the 25°N latitude to the Arctic Circle (66°N) (Elmes et al. 1999). Based on this latitude range in its native habitat, it is believed that *M. rubra* may be able to subsist in various habitats from southern Florida to north of the Hudson Bay, Canada in North America (Arevalo et al. 2007).

The first reported case of *M. rubra* in the United States was in Massachusetts in 1908 (Wheeler 1908). In Maine, there are two confirmed cases before 1950, while other reports of stinging ants surfaced in the late 1960's and early 1970's (Groden et al. 2004, Groden et al. 2005). The stinging ants were confirmed as *M. rubra* years later in 1986. Since 1998, reports of *M. rubra* to the University of Maine Cooperative Extension have increased dramatically (Groden et al. 2005). *M. rubra* has been largely concentrated in humid regions along Maine's coast, including in Acadia National Park on Mt. Desert Island, however colonies established inland suggest the ant is able to survive in other environments throughout the state (Arevalo et al. 2007). In 2002, *M. rubra* had been

confirmed in more than twenty locations along the Maine coast in seven counties including Cumberland, Hancock, Kennebec, Knox, Waldo, Washington, and York (Groden et al. 2004).

The nests of *M. rubra* are not always apparent and may be difficult to spot at first. These ants do not form mounds in the soil, rather they usually burrow in places that maintain high humidity for the colony. These conditions are often found in soil near the roots of trees or shrubs, under various types of debris such as rocks, logs, or human debris, and within decaying logs (Groden et al. 2005).

Efforts to control *M. rubra* populations in the United States have been largely unsuccessful. Commercial pesticides tested by the University of Maine were unable to eliminate ant populations (Groden et al. 2004). At this time, attempting to prevent the spread of the ants by human means and altering the environment to make it less hospitable for the ants are the best methods of control (Arevalo et al. 2007). Finding suitable biological control agents for *M. rubra* in addition to chemical control and land-use practices are the best long term strategy for the management of *M. rubra*.

Research similar to that conducted by Evans et al. (2010) is likely to reveal potential biological control agents. In this project, three previously undocumented fungal pathogens, *Paraisaria myrmicarum* sp. nov., *Hirsutella stilbelliformis var. myrmicarum* var. nov., and *Hirsutella subramanianii* var.*myrmicarum* var. nov. were isolated from *M. rubra* cadavers gathered in the United Kingdom. Recently, a soil dwelling entomopathogenic nematode, *Pristionchus entomophagus*, was found to cause mortality when inoculating *M. rubra* (Groden et al. 2010). *P. entomophagus* has been documented as having a necromenic life history, where nematodes wait on the cuticle of

the host insect until death when they enter into the cadaver to feed and reproduce (Rae et al. 2008). Their recently discovered parasitism of *M. rubra* (Groden et al. 2010) suggests the potential for variable host associations, and provides the opportunity to study the mechanisms determining the type of association formed between the nematode and its insect host. This parasitic *P. entomophagus*/*M. rubra* interaction may hold potential for biological control of this pest.

Entomopathogenic Nematodes

Nematodes are among the most ubiquitous organisms on the planet. They inhabit virtually every ecosystem, and in doing so, form relationships with many hosts, including plants, vertebrates, and invertebrates. Overall, nematode-insect relationships can take one of four basic forms, phoretic, necromenic, facultative necromenic, or parasitic (Kiontke and Sudhaus 2006). Phoretic nematodes use their insect hosts only for transport between suitable habitats. Dauer stage (the free-living, non-reproductive stage) individuals attach to insect hosts in poor conditions and dislodge themselves in areas with adequate resources. Nematodes associated with carrion, such as *Caenorhabditis plicata* display this life history (Völk 1950). Conversely, necromenic nematodes rely on their insect host for food and reproduction. Dauer stage individuals attach to their insect hosts, often on the cuticle, but they never disembark, waiting for their insect host to die, at which point they feed and reproduce on the carcass (Rae et al. 2008).

There also exists an intermediate life history between these two types, referred to as facultative necromeny, where the species is able to propagate on the host (typical of necromenic species and absent in phoretic species) but can also disembark from the host

(typical of phoretic nematodes and absent in necromenic species). The model organism *C. elegans* has this type of life history (Kiontke and Sudhaus 2006).

Fourthly, parasitic nematodes, also referred to as entomopathogenic nematodes, cause direct mortality in their insect hosts. It should be noted that not all parasitic nematodes are entomopathogenic, as parasitic nematodes are regularly associated with vertebrate hosts (Kiontke and Sudhaus 2006), but all entomopathogenic nematodes employ a parasitic life history. The most commonly studied entomopathogenic nematodes are of the families Heterorhabditidae and Steinernematidae, largely due to their efficacy as biological control agents (Gaugler 2006). Other families with entomopathogenic species include Diplogasteridae, Mermithidae, Allantonematidae, and Sphaerulariidae (Tanada and Kaya, 1993).

The major factor responsible for the pathogenicity exhibited in *Heterorhabditis* and *Steinernema* entomopathogenic nematodes is their endosymbiosis with entomopathogenic bacteria. Other entomopathogenic nematodes of the families Mermithidae and Allantonematidae are much larger, and invariably cause insect mortality by simply proliferating inside and exiting the host (Gaugler 2006). In *Heterorhabditis* and *Steinernema* systems, *Xenorhabdus* and *Photorhabdus* bacteria are established internally in the nematodes, and are transported into an insect host during the nematode infection process. The bacteria are released into the hemolymph of the host, causing mortality in 24-48 hours in many cases (Gaugler 2006). The nematodes then feed on the bacteria, often completing multiple generations in a single host. Infecting stages of the nematode must obtain and maintain the bacteria to perpetuate the symbiotic relationship. Bacteria are only taken up by *Steinernema carpocapsae* during the third juvenile (IJ)

stage, the organism's only free living stage (Bird and Akhurst 1983). When host resources are adequate, the nematodes complete their life cycle. However, when conditions are unfavorable, i.e. when resources are depleted, the third-stage infective juveniles ingest the bacteria and disperse into the soil in search of another suitable insect host.

Recent research by Snyder et al. (2007) highlights the role of the bacterial receptacle in the development of the symbiotic relationship between the bacterium *X. nematophila* and its nematode host *S. carpocapsae*. The bacterial receptacle was shown to be a, "distended region of the anterior portion of the intestine" using DIC microscopy (Snyder et al. 2007). The part of the intestine closest to the mouth of the nematode contains this out-pocketed sac, the bacterial receptacle, which branches off at the esophagointestinal junction and extends down the length of the body alongside the intestinal tract. It should be noted that the bacterial receptacle is open at the proximal end at all times and at the distal end during the infection of the insect host, allowing for the colonization and release of the symbiotic bacteria. These two openings effectively establish the bacterial receptacle as a shunt in parallel to the intestinal tract where bacteria are exclusively colonized.

It was shown that *X. nematophila* colonizes the distal portion of the bacterial receptacle, associating with a "subcellular intravesicular structure that freely moves in the lumen of the vesicle" (Martens and Goodrich-Blair 2005, Snyder et al. 2007). The intravesicular structure provides amino acids and cofactors that facilitate bacterial growth within the bacterial receptacle (Martens et al. 2005). Initially, a small number of bacterial cells, perhaps as few as one, enter into the bacterial receptacle and propagate to fill the

bacterial receptacle up to, but not beyond the esophagointestinal junction at the proximal end (Synder et al. 2007). Snyder et al. (2007) showed that the amount of bacterial colonization of the bacterial receptacle was highly variable within the tested nematode community, with some specimens containing a high number of viable cells and others containing a considerably lower amount of bacterial cells.

Movement of bacteria out of the bacterial receptacle, through the intestine, out of the anus, and into the hemolymph is triggered by nematode exposure to the hemolymph of the host itself (Snyder et al. 2007, Poinar and Thomas 1967). This process occurs in as little as 2 hours after nematode exposure to insect hemolymph. Bacterial cells move forward within the bacterial receptacle towards the esophagointestinal junction and into the intestinal tract where they are eventually passed through the anus, as well as posteriorly out of the distal end of the bacterial receptacle toward the anus (Snyder et al. 2007). The bacteria then aid in overcoming host immune defenses, eventually causing host death. Post-mortem, bacterial proliferation occurs and nematodes are able to complete their life cycle using the nutrients provided by the insect cadaver. Nematodes in the family Diplogasteridae are considered to have a less highly evolved symbiotic association with specific bacterial species. However, these associations have received very limited study.

In 2003 and 2004, nematodes were observed exiting from *M. rubra* cadavers collected from colonies in Maine, and in 2003, a similar nematode of the family Diplogasteridae was observed from colonies in England (Groden et al. 2010). In 2007 and 2008, colonies of *M. rubra* gathered at multiple sites in Maine were parasitized with nematodes, the pathogenicity of the nematodes was confirmed with reinfection assays,

and molecular and morphological characterizations determined that the nematodes were *Pristionchus entomophagus* (Groden et al. 2010).

Pristionchus entomophagus

Pristionchus nematodes belong to the family Diplogasteridae, a monophyletic clade in the order Rhabditida that includes over 300 species (Sudhaus and Fürst von Lieven 2003, Dietrich and Sommer 2009).

The life cycle of *Pristionchus pacificus,* which has been described in detail (Dieterich and Sommer 2009, Hong and Sommer 2006b) is typical of the life cycle of a necromenic nematode (Weller et al. 2010). When in ideal conditions, *P. pacificus* can go through an uninterrupted life cycle from the egg stage to mature adults (Dietrich and Sommer 2009). Felix et al. (1999) found that Diplogastridae only have three post-embryonic stages as opposed to the normal four found in most nematodes (Fürst von Lieven 2005). The first stage (J1) juveniles develop and molt within the egg (this is considered to be an embryonic stage) in contrast to other members of the order Rhabditida outside of the Diplogasteridae family, and hatch as J2 stage juveniles (Dietrich and Sommer 2009). After hatching, the juveniles undergo four successive molts and become hermaphroditic adults, completing the life cycle. In the laboratory this can be completed within 3-4 days when carried out at 20°C. In less favorable environmental conditions, *Pristionchus spp*. are capable of producing a juvenile form, known as the dauer or resting stage (Poinar 1969). This switch has been found to be genetically linked to the *daf-16* gene in *P. pacificus* (Ogawa et al. 2011). This genetic link has similarly been found in *C. elegans,* suggesting that there is a gene-by-environment interaction responsible for the formation of the dauer stage larvae. This dauer stage, a reproductively

inactive phase, allows *Pristionchus spp*. to endure periods of poor conditions away from a suitable host (insect carcass). However, this is the stage that infects the host.

Pristionchus spp. are soil dwelling organisms who are associated with a wide variety of beetle species. Herrmann et al. (2006a) showed a relationship between these nematodes and the Colorado potato beetle as well as a number of scarab beetles. After sampling some 15,000 beetles and identifying 1,200 *Pristionchus* isolates, it was discovered that each *Pristionchus* species shows a high degree of species specificity in laboratory testing and in the wild. Two of the major European species, *P. maupasi* and *P. entomophagus* were closely associated with cockchafers (*Melolontha sp*.) and dung beetles (*Geotrupes sp.*) respectively (Hermman et al. 2006a). During sampling in the United States, *P. entomophagus* was also found in association with scarab beetles (*Phyllophaga sp.)* in Ohio (Herrmann et al. 2006b). In classifying the *Pristionchus spp*. phylogenetically, Mayer et al. (2007) postulated that because a number of *Pristionchus spp*, including *P. entomophagus* were clustered together, forming a European branch within the phylogram, it is possible that *P.entomophagus* samples in North America originated in Europe and were spread either by beetles or by human transport.

A study by Hong and Sommer (2006a) attempted to shed light on the actual mechanism by which these nematodes find and colonize their host. In comparisons between the two model organisms *Pristionchus pacificus* and *Caenorhabditis elegans*, it was found that chemoattraction differs between the two species. After further testing comparing *P. pacificus*, *C. elegans*, and other *Pristionchus spp*, differences between *Pristionchus spp*. suggested that the olfaction of *Pristionchus spp*. is highly diverse and is likely tied to the development of the nematode-host interaction. In an attempt to gather a

species-specific attraction profile, Hong and Sommer subjected two highly related pairs of nematodes (two species closely related phylogenetically), including *P. entomophagus*, to eleven known semiochemicals involved in plant-insect communication. It was found that even between these two pairs of phylogenetically similar *Pristionchus* species, *P. entomophagus* and *P. uniformis*, and *P. pacificus* and *P. maupasi*, there were dramatically different chemoattraction profiles (Hong and Sommer 2006b). It is likely that semiochemicals found attractive by nematodes of the genus *Pristionchus* are closely linked with the life cycle of the host organism they are associated with. Results showed that *P. entomophagus* had the strongest attraction to isopentylamine, which smells similar to decaying material. Isopentylamine can also be used to trap dung beetles (*Geotrupes spp*.), the common host of *P. entomophagus* in Europe. This high attraction to a chemical closely linked to a common host in consistent across the test data, with *P. maupani*, a leaf beetle, linked closest with plant derived compounds such as linalool (Hong and Sommer 2006). Furthermore, the chemical structure of isopentamyl contains a branched carbon group similar to the sex pheromone of June beetles (*Phyllophaga lanceolata*), the genus of beetle *P. entomophagus* was found in association with in Ohio (Herrmann et al. 2006b).

The point of entry into the host for *Pristionchus spp*. is a natural opening such as the mouth or anus (Poinar 1972). Once an infected host is dead, *Pristionchus spp*. dauer larvae detect favorable conditions and re-engage the reproductive life cycle, becoming J4 juveniles. Once inside the intestines, the nematodes mature to the adult form and are able to proliferate, rupturing the gut wall and entering into the hemolymph (Poinar 1969). At this point they continue to reproduce and feed selectively on the bacteria and fungi

proliferating in and on the carcass. The exact time scale over which this process occurs has not been determined. It is important that *Pristionchus spp*. are able to distinguish between pathogenic and non-pathogenic bacteria in and on the cadaver in order to prevent losses in brood size, development time, and even death as a result of ingesting the wrong type of bacteria (Rae et al. 2008).

Members of the family Diplogasteridae, including *Pristionchus spp*., have different buccal and gut cavity morphologies than *Steinernema* and *Heterorhabditis* nematodes. Their stoma morphology consists of a muscular, median esophageal bulb with valve plates and a glandular basal bulb (Poinar 1969). They have shorter, broader mouthparts with no grinder, a characteristic that is present among other Rhabditid nematodes such as the model organism *C. elegans* (Rae et al. 2008). These characteristics in addition to flaps that surround the stoma allow *Pristionchus spp*. to ingest bacteria whole without crushing them and prevent them from being pushed out of the buccal cavity after ingestion (Lieven and Sudhaus 2000). Furthermore, some *Pristionchus* species have developed a phenotypic plasticity among stoma morphologies, affording two distinct stomal morphotypes, stenostomatous and eurystomatous (Furst von Lieven and Sudhaus 2000). The differences between the two morphologies include buccal cavity width and depth, as well as the presence and orientation of teeth within the cavity. This plasticity affords *Pristionchus spp*. the ability to specialize their different feeding habits based on the buccal cavity morphology (Hong and Sommer 2006b).

Pristionchus spp. have developed a number of characteristics that are commonly referred to as pre-adaptations for parasitism (Weller et al. 2010), including their necromenic association with beetles where infective juveniles enter an insect host, wait

for host death, and then feed on the bacteria and fungi that grow within the carcass (Rae et al. 2008). Their ability to tolerate the harsh conditions inside a larva (high toxicity due to hemolymph composition, low oxygen concentration), in addition to the ability to form dauer larvae (resting and infective stage) during periods of environmental disturbance lends support to *Pristionchus spp*. being on the evolutionary path to parasitism (Weller et al. 2010).

The dauer stage may also provide a mechanism of ingress for the bacteria that cause mortality in insect hosts. Bacteria have been isolated from between the two cuticular layers that ensheath the dauer stage larvae, that is between the internal cuticle of the J3 dauer stage and the external J2 cuticle that they retain from the preceeding juvenile stage (Gaugler, 2002). Bonifassi et al. (1999) found bacteria in between the two cuticles in *S. scapterisci*. Unlike Steinernema species, Pristionchus dauer stage juveniles do not retain the J2 cuticle as a protective sheath for the free living stage. However, these nematodes do carry associated bacteria on their cuticle (Rae et al. 2008) that are likely introduced to the host during the infection process.

Poinar (1969), however, suggests that the stage of nematode invading the host may be important relative to the resulting lethality of *Pristionchus* infections. He found that dauer stage *Pristionchus* larvae caused significantly fewer lethal infections when applied to *Galleria mellonella* in comparison with samples containing a mix of dauer stage larvae and normal juvenile stages. The cause of insect mortality was not linked solely to the presence of nematodes, but rather the establishment of the nematodes. Once established within the insect host, Poinar (1969) reported that *P. uniformis* caused mortality in all subjects. Many of the inoculated wax worm larvae (*G. mellonella*) were

able to successfully void the nematodes, presumably passing dauer stage larvae through their digestive tracts before they could establish.

There is evidence that the bacteria associated with *P. entomophagus* can be pathogenic to insects (Al Own et al. 2011). The transport of bacteria from the external surface of nematodes into an infected host has been shown for the endospore-forming bacteria, *Paenibacillus nematophilus*, on the cuticle of an entomopathogenic nematode host, *H. megidis* (Enright and Griffin 2005). While the actual role of the dauer stage larvae in infectivity is somewhat unclear, it is known that they are capable of carrying bacteria, and that bacteria associated with entomopathogenic nematodes is critical for virulence.

Groden et al. (2010) have recent evidence that *P. entomophagus* induced mortality in *M. rubra* populations appears to be related to the origin of the *P. entomophagus* population. Nematodes gathered from *M. rubra* cadavers collected from various sites on Mt. Desert Island, ME were mass-reared in the laboratory and used to inoculate *M. rubra* in laboratory reinfection assays. The differential induced mortality in these tests suggests that there are inherent differences in the pathogenicity of *P. entomophagus*, likely due to their site of origin. Furthermore, studies in Europe have shown this nematode to harbor a complex of bacterial species in or on its body (Rae et al. 2008) although it does not appear to have a specialized receptacle to harbor specific endosymbiotic species. This suggests that the variability seen in *P. entomophagus* induced mortality may be a function of the particular bacterial associates that the nematode is carrying, likely due to the presence of specific species of bacteria being present in the habitat of the *P. entomophagus* population. Identifying the species of

bacteria associated with virulent populations of *P. entomophagus* nematodes is the focus of this study. I hypothesize that bacterial species found in the hemolymph of infected hosts will also be among the species found on either the external or internal surfaces of the infecting *P. entomophagus* nematodes. To test this hypothesis, I isolated and identified the bacteria on the external cuticle and internally in the digestive tract of *P. entomophagus* and compared it to bacteria species that I isolated from the hemolymph of insects hosts infected with *P. entomophagus*.

Materials and Methods:

Myrmica rubra colonies:

Prior to experimentation, *Myrmica rubra* colonies were collected from different sites in Acadia National Park in September 2010 and maintained in overwintering conditions in a cold chamber set at 4°C. Multiple colonies from each site were transferred to nest boxes approximately two weeks prior to use for these experiments and labeled by site and nest number (e.g. COA 4). Each colony was provided with a small portion of a cardboard egg carton for covering brood and sugar, water, and tuna were provided for sustenance. Colonies were inspected for dead individuals on a regular schedule (2-3 times per week). Cadavers were removed using sterile tweezers and surface sterilized by submerging in a 0.1% zephiran chloride solution for thirty seconds (Roberts 1973) before being transferred to two rinses in sterile distilled water and placed on clean absorbent paper. After drying, cadavers from each nest were held in individual wells of 48 well microtitre plates, with cadavers from each colony maintained in their own separate plate. Plates were stored at ambient temperature (ca. 21°C) inside plastic bags containing damp paper towels to maintain high humidity (Evans et al. 2010).

Cadavers from all sites in Acadia National Park were monitored for emergent nematodes. Colonies from two sites experiencing high levels of nematode induced mortality (College of the Atlantic (COA) and Miller Greenhouse (MGH)) were selected for use in this study, and nematodes were harvested from their corresponding cadaver plates.

Isolation of Bacterial Associates:

External Surface of Nematodes: Bacteria harbored externally on the nematodes were collected through thorough rinses of the live nematodes. Emergent nematodes in two wells from COA and two wells from MGH were used to isolate associates from the external surface of the nematodes. Approximately 50 μL of sterile distilled water was added to wells with high numbers of emergent nematodes. Leaving the *M. rubra* cadaver in the well, nematodes from each well were pipetted into their own 1.5 mL microcentrifuge tube filled with 1 mL of 1% Tween. After gently vortexing, each solution was centrifuged for approximately 10 seconds at 13,000 RPM to concentrate the nematodes in the bottom of the tube. Four fold serial dilutions of this stock solution were made by adding 100 μL of the rinse solution to 900μL of distilled water, and repeating three additional times. Three 300 μL aliquots of each 1×10^{-4} dilution were then plated onto Trypticase Soy Agar (TSA). Plates were incubated at 29°C for 48 hours, after which morphologically unique individual colony forming units were identified using a dissecting microscope and transferred onto fresh TSA plates. After two days of growth, monoculture plates were stored at 4°C.

Internally Harbored in Nematode: Bacteria harbored internally in the digestive tract of the nematodes were sampled by surface-sterilizing live nematodes to remove

external bacteria, then transferring the nematodes to TSA plates. Nematodes feeding and tunneling on the agar gave rise to colonies of bacteria excreted from the nematode gut. This procedure was initiated by adding 50 μL of sterile distilled water to each chosen well in the 48 well plates housing the *M. rubra* cadavers. After gently mixing, three 5 μL aliquots were taken from each well and plated onto a contrasting black surface for counting. The number of nematodes in each aliquot was counted and averaged across each of the three aliquots for each site. The remaining 40 μL of each nematode solution was equilibrated to ½ the concentration of the least concentrated solution by adding sterile distilled water to each solution in the appropriate amount.

After standardization of nematode concentration, 50 μL of nematode solution was gently loaded onto a concavely folded piece of vacuum filter paper. The filter paper was loaded into an appropriately sized Buchner Funnel and attached to the laboratory vacuum system. Nematodes were continuously surfaced sterilized for 2-3 minutes by pipetting 1% bleach solution onto them, making sure not to spill the nematodes off of the filter paper. After surface sterilization, the nematodes were rinsed with sterile distilled water in the same manner for 2-3 minutes. The filter paper was then loaded into a small Petri dish and flooded with sterile distilled water to dislodge the nematodes from the filter paper. After nematode presence was confirmed using a dissecting microscope, 300 μL aliquots of the nematode filled solution was plated onto TSA agar.

To assure inoculation of a sufficient number of nematodes, individual nematodes were pipetted out of the remaining solution using a 200 μL pipette tip and added to the TSA plate with the nematode solution. It was determined that holding nematodes at room

temperature until pipetting was best, as nematodes tended to stick to the surface of the Petri dish if refrigerated for long periods.

Hemolymph Samples: A total of 60 *Galleria mellonella* (waxworm) larvae were inoculated with nematodes from selected COA and MGH wells. Nematodes were harvested from six individual ants each from both COA and MGH in wells with large amounts of emerged nematodes. Nematodes from each well were transferred to a set of five waxworm larvae resulting in twelve sets of inoculated larvae (six per site). Each of the six sets of larvae for each site were placed in a 100 mL cup with 1 cm sand and 50 μL sterile distilled water. Nematodes were pipetted directly on to the dorsal cuticle of the larvae. Cups were covered to retain moisture and maintained at ambient temperatures (ca. 21°C) in the laboratory. Larvae were monitored daily and dead were collected and surface sterilized by submerging in a 0.1% zephiran chloride solution for thirty seconds before transferring to two rinses of sterile distilled water. Sterilized cadavers were placed in individually marked Petri dishes for each set of five larvae. All cadavers were stored for 24-72 hours at 4°C until hemolymph was gathered *en masse*.

Hemolymph samples were collected from each cadaver by cutting one leg off close to the body with sterile, micro-dissecting scissors, and using a microcapillary tube to draw hemolymph from the wound. All gathered hemolymph from each set of larvae was pooled and placed into a 1.5 mL microcentrifuge tube filled with 1mL of sterile distilled water. Four-fold serial dilutions were made and quadrant streaks of each 1×10^{-4} dilution were plated onto two Trypticase Soy Agar (TSA) plates for each of the twelve samples. Plates were incubated at 29°C for 48 hours, after which unique individual colony forming

units were identified using a dissecting microscope and transferred onto fresh TSA plats. After two days of growth, all samples were stored on the clean plates at 4°C.

DNA Extraction, PCR, and Sequencing:

Due to time and monetary restrictions, a subset of the bacterial isolates was chosen to be identified to species using molecular tools. All isolates were grown overnight in LB Broth. Due to the unknown status of the cultured organisms, a salt concentration of 5 g/L was used as it fell at the low end of the published range of salt concentrations from 5 g/L to 10 g/L (Formedium). Samples were grown in 3 mL of LB broth in 14 mL test tubes (VWR, Radnor, PA, USA) at 29°C and 90 RPM overnight, typically for 14-18 hours. After the initial centrifugation of 1 mL of overnight culture, additional 1 mL aliquots were added and re-centrifuged for samples with low bacterial density, as evidenced by a small initial pellet after centrifugation. Bacterial DNA was extracted using the Promega Wizard® Genomic DNA Purification Kit Cat no. A1120 (Promega, Madison, Wisconson, USA). Upon completion of DNA Extraction, all samples were stored in 1.5 mL microcentrifuge tubes at -20°C.

Gel electrophoresis was run on each sample to determine the presence of DNA using 0.8% Agarose gels made using 30 mL of TAE Buffer, 3 µL GelStar® GelStain (Lonza, Rockland, ME, USA) and 0.24 g Agarose (VSB Company, Cleveland, OH, USA). Samples were loaded using 5 µL of DNA and 1 µL of 6x loading dye (Gilbert). The Lambda HindIII ladder (Promega) was used as the standard and samples were run at 90V for approximately one hour. Gels were visualized on a UV transilluminator and recorded with a remote shooting camera. For post DNA extraction gels, the mere presence of a band was taken to be a successful extraction.

Polymerase chain reaction (PCR) amplification of bacterial 16s rRNA was conducted using the primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-ACGGGCGGTGTGTAC-3') (Lane 1991). Thermocycler conditions were as follows: 3 min at 95°C followed by 35 cycles of 15 s at 95°C, 30 s at 55°C, 1.5 min at 72°C, and a final step of 6.5 min at 72°C (Rae et al. 2008). Reactions were carried out at a volume of 20 µL. The PCR master mix recipe was derived from the original reaction conditions listed by Rae et al. (2008). After modifications, the final reaction mix included 9.6 µL H_2O, 4 µL 5x PCR Buffer, 1.2 µL 25 mM $MgCl_2$, 2 µL 2 mM dNTP's, 1 µL 10 µM 27f, 1 µL 10 µM 1492r, 0.2 µL GoTaq DNA Polymerase (Promega), and 1 µL of bacterial DNA.

Gel electrophoresis was run for all samples to determine the presence of PCR amplicons using 1.2% Agarose gels (30 µL of TAE Buffer, 3 µL GelStar® GelStain (Lonza, Rockland, ME, USA) and 0.36 g Agarose). Invitrogen Low DNA Mass Ladder Cat. # 10068-013 (Invitrogen, Carlsbad, CA, USA) was used as a standard to detect the expected fragment length of 1465 bp. Five microliters of low mass ladder and 1 µL of 6x loading dye was used for the ladder (L. Beers, personal communication, March 2011). Three microliters of PCR product and 2 µL of 2x loading dye was mixed and loaded for each sample. Gels were visualized on a UV transilluminator and recorded with a remote shooting camera. A fragment at the correct base pair size was taken to be a successful PCR run.

Each PCR sample was purified using a QiaQuick PCR Purification Kit (Qiagen, Venlo, Netherlands) and prepared for sequencing as instructed by the University of Maine Sequencing Facility. Ten microliters of the remaining 17 µL of PCR product was

purified following the Qiagen QiaQuick protocol. Samples were stored in sterile 1.5 mL microcentrifuge tubes at -20°C.

The quantity and quality of DNA in the purified samples was determined on a Nanodrop 1000 with version 3.3 software (ThermoScientific, Waltham, MA, USA). DNA concentrations were adjusted as necessary for sequencing by the University of Maine Sequencing Center. Samples were prepared for sequencing by placing 100 ng of DNA into a sterile tube and diluting the concentration to 10 ng/μL. Nanodrop results for DNA concentration were used to calculate the amount of PCR sample needed to obtain 100 ng of purified DNA, and sterile DiH_2O was added appropriately to total 10 μL, yielding a concentration of 10 ng/μL. Approximately 1 μL of 27f and 1492r primers per sample was delivered to the sequencing center at a concentration of 5 μM.

Identification of the species of bacterial isolates:

Sequenced and edited bacterial 16s rRNA sequences were obtained from the University of Maine Sequencing Facility. Using the Basic Local Alignment Search Tool (BLAST) online database provided by the United States National Center for Biological Information, edited sequences were referenced against the current database of bacterial samples (NCBI, accessed 2011). The quality of the match, quantified by query coverage and maximum identity (percent match) were used to distinguish good matches from poor matches. Matches with good query coverage and percent match were further investigated by exploring accession numbers and determining the origin of the sample. Matches with sources such as soil, insects, and nematodes were targeted due to their relevance to our study.

Results:

We isolated a large number of bacterial samples from the internal and external surfaces of the nematodes, as well as the hemolymph of the infected *G. mellonella* larvae (Figure 1).

Figure 8: Original 10^{-4} dilution from external rinse of nematodes from the COA C8 sample plated on TSA.

Figure 9: Unique morphotypes of bacteria isolated from the external surface of nematodes from the MGH E3 sample after 72 hours of colony growth. (40X magnification) Left: Isolate 1, Right: Isolate 3

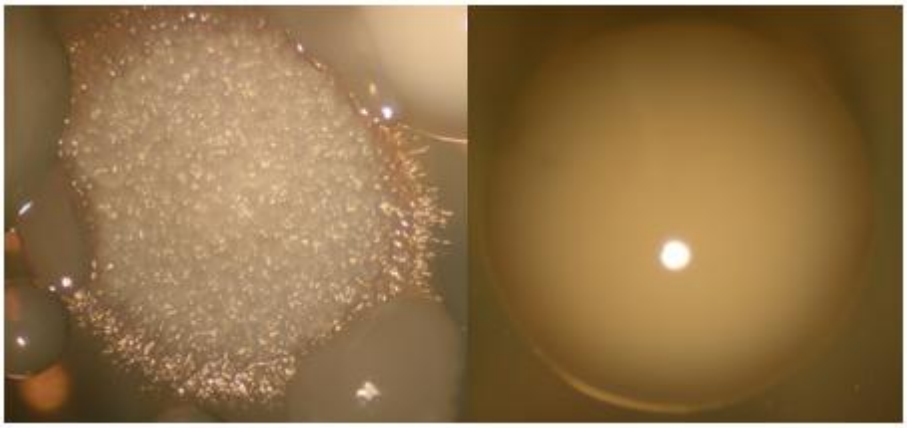


Figure 10: Unique morphotypes of bacteria isolated from the external surface of nematodes. (400X magnification) Left: MGH E3 Isolate 1, Right: COA C7 Isolate 6

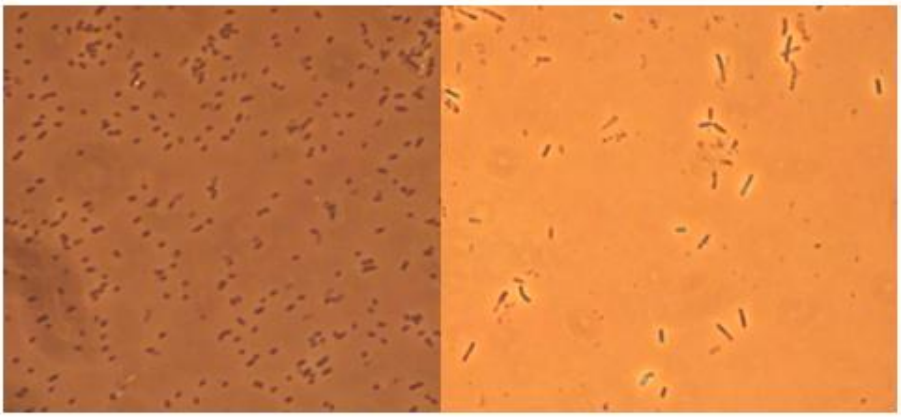


Figure 11: Plated surface sterilized nematodes from the MGH E3 sample. Cultured excrement yielded internal nematode isolates.

A total of 45 isolates were identified as unique and isolated (Table 1, Table 2). Overall, there were 28 isolates gathered from the external surface of the nematodes, eight isolates from the digestive tract of the nematodes, and 9 samples gathered from the hemolymph of infected hosts (Table 2).

There were a number of common morphotypes present in the isolates collected from the external surface of the nematodes (Figures 2 and 3). Of the 28 isolates gathered from the external surface of the nematodes, morphological and microscopic evidence determined that nearly all were present in another sample, either in samples from the

same site, the opposite site, or both. A number of cream colored morphotypes were present in both the MGH and COA samples. Those that were not common to both sites were often found in both samples from the same site in Acadia National Park. This left approximately twelve unique-appearing isolates from the external surface of the nematodes. The examination of isolates gathered from the digestive tract of the nematodes (Figure 4) indicated that many of the isolates were similar. Morphologically, the colonies fell into two categories; cream colored and smooth, and cream colored with a distinct grainy appearance at the edge of the colony. Microscopic evidence was unable to discern differences within the groups. The isolates from the hemolymph had many similarities as well. Of the gathered isolates, nearly all had another sample with a similar morphotype. Of the nine samples gathered, it appears that there were four or five unique morphotypes.

Overall, there was a diverse bacterial complex (group/set of bacteria) found in association with the internal and external surfaces of the nematodes and the hemolymph of infected hosts. Based on analysis using primarily colony morphotypes with some microscopic evidence, it appears that as many as twelve unique isolates were found in relation to the external surface of the nematode, while two major morphotypes are apparent from the isolates associated with the internal surface of the nematode, and four or five morphotypes were isolated from the hemolymph of infected hosts.

Due to the large amount of overlap in samples, i.e. the appearance of the same morphotypes on a number of occasions, it also appears that the set of bacteria present within each type of sample (internal, external, hemolymph) is universal. Notable

exceptions include the presence of a unique yellow morphotype and a cream colored wiry morphotype in the COA samples that was not present in the MGH samples.

Because of the small number of morphotypes isolated from the hemolymph of infected hosts and the large amount of overlap present within each type of sample (external, internal, hemolymph), it is unclear whether or not the types of bacteria associated with the nematodes themselves (internal and external) are more diverse than the bacteria in the hemolymph.

Table 7: Bacterial morphotypes isolated from the internal and external surface of the nematodes and the hemolymph of infected hosts.

Source	Cadaver Sample	Isolate #	Colony appearance
External Rinse	MGH F2	1	large, round, cream colored. Wavy/bumpy surface texture.
External Rinse	MGH F2	2	small, irregularly shaped, resembles a fried egg. Very clear on the outside, slightly yellowish cloudy tint in center, striated edges
External Rinse	MGH F2	3	small, cloudy in middle with more solid off-white color around outside. Round with slightly wavy edges.
External Rinse	MGH F2	4	small, round with smooth edges. General cloudy white color. Shows some signs of having a lighter color in middle like isolate 3, but hemispheric shape suggests otherwise.
External Rinse	MGH F2	5	completely irregular. Cloudy. Generally ovular in shape with irregular edges and raised portions all over. Almost looks like a clovered-tongue.
External Rinse	MGH F2	6	irregular shape, heart like with wavy edges. Egg-like appearance with coloration different in middle. Possible resemblance to isolate 2 but this colony is much thicker and has more color.
External Rinse	MGH F2	7	small, round with slightly wavy edges. General cloudy white color. Shows some signs of having a fried egg appearance. Smooth surface

External Rinse	MGH E3	1	white, crystalline. Varies in size. Raises off surface of plate. Looks icy in a way
External Rinse	MGH E3	2	irregular in shape, slightly round with an invagination. Appears to have egg like appearance. Resembles isolates from mgh with egg appearance (2,7)
External Rinse	MGH E3	3	small, round with smooth edges. General cloudy white color. Very much resembles isolate MGH F2 #4
External Rinse	MGH E3	4	small, round with slightly wavy edges. Lighter white color. May be a younger version of isolate 3 from this set.
External Rinse	MGH E3	5	medium/large. Fried egg appearance. Cream color.
External Rinse	COA C8	1	large, round, cream colored. Wavy/bumpy surface texture. A virtual identical match to MGH F2 #1
External Rinse	COA C8	2	small, round, clearer that isolates 1 or 2 from MGH E3.
External Rinse	COA C8	3	yellow, round, slightly irregular edges, wavy surface
External Rinse	COA C8	4	Left side of photo, cream color, round with wavy edges, no apparent discoloration, slightly wavy color.
External Rinse	COA C8	5	Right side of photo. Small, round with smoother edges. Wavy surface appearance. Slightly transparent in middle, with solid color around outside, resembles MGH F2 #3
External Rinse	COA C8	6	big, white, round, smooth edges, no discoloration, resembles MGH E3 #3 and MGH F2 #4
External Rinse	COA C8	7	stringy, white, no crystalline appearance. Smooth looking with stringy appearance
External Rinse	COA C8	8	fungi? Fuzzy/hairy. Round conical colonies.
External Rinse	COA C7	1	small, irregularly shaped, resembles a fried egg. Very clear on the outside, slightly yellowish cloudy tint in center, striated edges. Resembles MGH F2 #2
External Rinse	COA C7	2	Left side of photo, small round, clear-ish appearance, smooth edges and surface. Resembles COA C8 #2
External Rinse	COA C7	3	Right side of photo, small round with slightly wavy edges. No overall discoloration. Resembles MGH E3 #4.

External Rinse	COA C7	4	mix between the bigger, white smooth/round colonies and the smaller, clearer smooth colonies (see COA C8 #6 and COA C7 #2)
External Rinse	COA C7	5	white, crystalline. Varies in size. Raises off surface of plate. Looks icy in a way. Closely resembles MGH E3 #1
External Rinse	COA C7	6	large, round, cream colored. Wavy/bumpy surface texture. A virtual identical match to MGH F2 #1
External Rinse	COA C7	7	yellow, round, slightly irregular edges, wavy surface. Similar to COA C8 #3
External Rinse	COA C7	8	small, clear with some cloudy white coloration. Smooth surface with a raised donut in the middle of the colony. Dissimilar to all other colonies.
Internal Culture	MGH E3	1	small, round, white color with slightly wavy edges. Similar color to big, white and round colonies such as COA C8 #6
Internal Culture	MGH E3	2	small, round, cloudy white color with no discoloration.
Internal Culture	COA B6	1 (Small)	small, round, no apparent color. After further analysis and streaking it appears that this may have been a raised water droplet or extra agar.
Internal Culture	COA D5	Int 1	irregular shape most likely caused by wet plate. Edges do seem to wavy though. White color, no discoloration
Internal Culture	COA D5	Int 2	somewhat round. Edges are very odd. They are irregular and appear to have bumps in them. Creamy color.
Internal Culture	MGH D3	Int 3	creamy white large colony with a small white dot in the middle.
Internal Culture	COA B6	2 (Big)	egg-shaped with raised circle in slender part of the colony. After looking at picture under closer scrutiny, it appears that this larger ovular colony may consist of two or three circular colonies. One appears to be a larger white colony which is at the right. another looks like the raised donut colony COA C7 #8.
Internal Culture	MGH E3	Rep 1	small white colonies. Appear to be somewhat "bumpy" looking." round with irregular edges.
Hemolymph Plating	COA 1		No Notes, colony present

Hemolymph Plating	COA 2		Smooth
Hemolymph Plating	COA 2		Fuzzy
Hemolymph Plating	COA 4		No Notes, colony present
Hemolymph Plating	COA 5		Dark
Hemolymph Plating	COA 5		Light
Hemolymph Plating	COA 6		No Notes, colony present
Hemolymph Plating	MGH 6		Raised, White, Big
Hemolymph Plating	MGH 6		Flat, Yellow, Odd Edge

Table 8: Summary of unique bacterial morphotypes isolated from the internal and external surfaces of nematodes and the hemolymph of infected hosts.

Source	Cadaver Sample	Isolated Morphotypes
External	COA C8	8
External	COA C7	8
External	MGH F2	7
External	MGH E3	5
Internal	MGH E3	2
Internal	COA B6	2
Internal	COA D5	2
Internal	MGH D3	1
Internal	MGH E3	1
Hemolymph	All COA	7
Hemolymph	All MGH	2

Of the 45 morphotypes cultured, 32 were selected for further evaluation; 15 total external samples; eight isolates from nematodes collected from one COA well and seven isolates from nematodes collected from one MGH well, all nine hemolymph isolates, and all eight isolates from internal nematode samples were used (Table 3). Due to the similarities between the MGH samples and the COA samples relative to their overall morphology and source (internal, external, hemolymph), samples derived from one of the

MGH wells (F2) and one of the COA wells (C8) were selected going forward as representative samples of the external bacterial associates (Table 3).

Table 9: Summary of isolates selected for further testing

Source	Plate/Well	Total Isolates
External Rinse	MGH F2	7
External Rinse	COA C8	8
Internal	MGH E3	2
Internal	COA B6	1
Internal	COA D5	2
Internal	MGH D3	1
Internal	MGH E3	2
Hemolymph	All COA	7
Hemolymph	All MGH	2

Figure 12:Electrophoresis gel of DNA extraction products

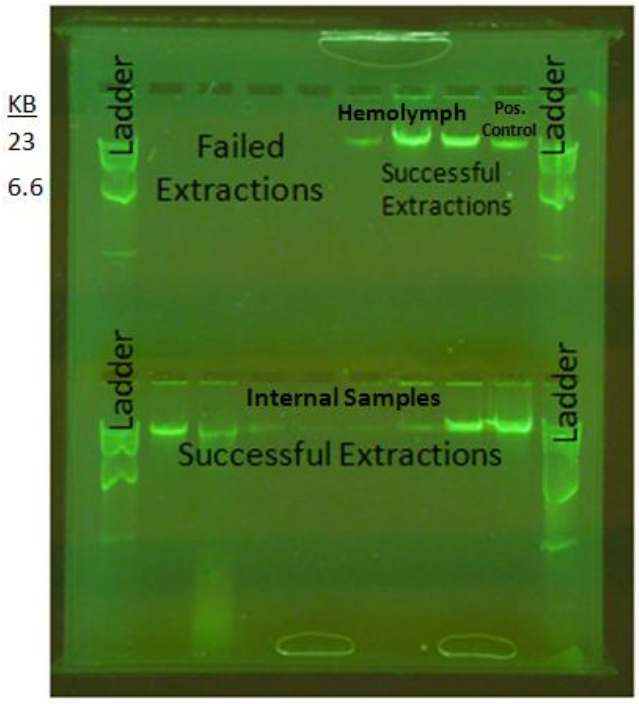
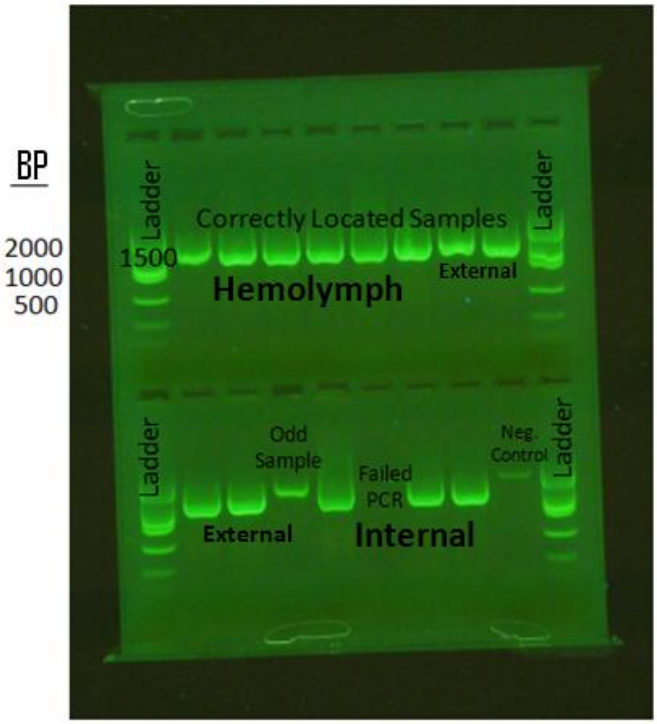


Figure 13: Electrophoresis gel of PCR products



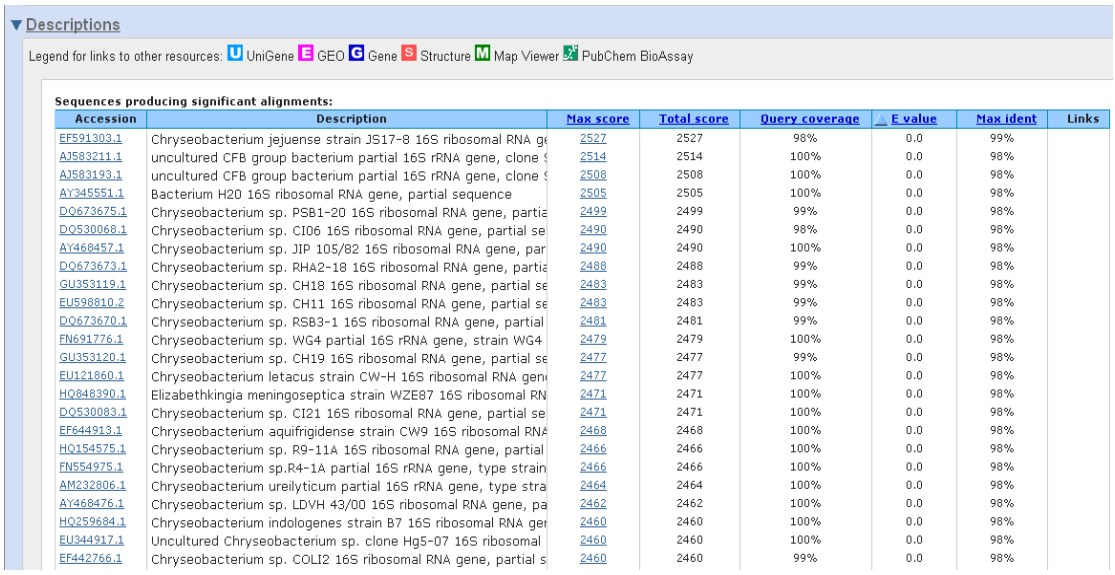
Of the 32 isolates processed for DNA extraction, most successfully yielded DNA for PCR amplification (Figure 5, Table 4). Extractions from two of the external rinse isolates were unsuccessful, and hence 30 total isolates were used for the PCR protocol. Of these samples, 26 successfully yielded PCR amplicons (Figure 6). I was unable to amplify three of the eight internal samples and one of the nine hemolymph samples. All of the 26 amplified samples yielded sufficient high quality DNA for sequencing, however two of the samples, one from the external surface of the nematodes and one from the internal surface of the nematodes, did not result in clean sequences that could be further analyzed (Table 4).

Table 10: Samples selected for further testing that did not yield final results

Source	Plate/Well	Test Not Passed
External	COA C8 1	DNA Extraction
External	MGH F2 1	DNA Extraction
Internal	MGH E3	PCR
Internal	MGH E3 1	PCR
Internal	MGH E3 2	PCR
Hemolymph	MGH 6 Rep 2/2 Flat, Yellow, Odd Edge	PCR
External	MGH F2 7	Sequencing
Internal	COA B6 Big Out	Sequencing

Each sample run through the BLAST procedure produced several catalogued samples that were good matches with the sequence generated by the University of Maine DNA Sequencing Facility.

Figure 14: BLAST results for sample P053 (COA C8 3)



Descriptions

Legend for links to other resources: UniGene GEO Gene Structure Map Viewer PubChem BioAssay

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
EF591303.1	Chryseobacterium jejuense strain JS17-8 16S ribosomal RNA ge	2527	2527	98%	0.0	99%	
AJ583211.1	uncultured CFB group bacterium partial 16S rRNA gene, clone	2514	2514	100%	0.0	98%	
AJ583193.1	uncultured CFB group bacterium partial 16S rRNA gene, clone	2508	2508	100%	0.0	98%	
AY345551.1	Bacterium H20 16S ribosomal RNA gene, partial sequence	2505	2505	100%	0.0	98%	
DQ673675.1	Chryseobacterium sp. PSB1-20 16S ribosomal RNA gene, partia	2499	2499	99%	0.0	98%	
DQ530068.1	Chryseobacterium sp. CI06 16S ribosomal RNA gene, partial se	2490	2490	98%	0.0	98%	
AY468457.1	Chryseobacterium sp. JIP 105/82 16S ribosomal RNA gene, par	2490	2490	100%	0.0	98%	
DQ673673.1	Chryseobacterium sp. RHA2-18 16S ribosomal RNA gene, partia	2488	2488	99%	0.0	98%	
GU353119.1	Chryseobacterium sp. CH18 16S ribosomal RNA gene, partial se	2483	2483	99%	0.0	98%	
EU598810.2	Chryseobacterium sp. CH11 16S ribosomal RNA gene, partial se	2483	2483	99%	0.0	98%	
DQ673670.1	Chryseobacterium sp. RSB3-1 16S ribosomal RNA gene, partial	2481	2481	99%	0.0	98%	
FN691776.1	Chryseobacterium sp. WG4 partial 16S rRNA gene, strain WG4	2479	2479	100%	0.0	98%	
GU353120.1	Chryseobacterium sp. CH19 16S ribosomal RNA gene, partial se	2477	2477	99%	0.0	98%	
EU121860.1	Chryseobacterium letacus strain CW-H 16S ribosomal RNA gen	2477	2477	100%	0.0	98%	
HQ848390.1	Elizabethkingia meningoseptica strain WZE87 16S ribosomal RN	2471	2471	100%	0.0	98%	
DQ530083.1	Chryseobacterium sp. CI21 16S ribosomal RNA gene, partial se	2471	2471	100%	0.0	98%	
EF644913.1	Chryseobacterium aquifrigidense strain CW9 16S ribosomal RNA	2468	2468	100%	0.0	98%	
HQ154575.1	Chryseobacterium sp. R9-11A 16S ribosomal RNA gene, partial	2466	2466	100%	0.0	98%	
FN554975.1	Chryseobacterium sp.R4-1A partial 16S rRNA gene, type strain	2466	2466	100%	0.0	98%	
AM232806.1	Chryseobacterium ureilyticum partial 16S rRNA gene, type stra	2464	2464	100%	0.0	98%	
AY468476.1	Chryseobacterium sp. LDVH 43/00 16S ribosomal RNA gene, pa	2462	2462	100%	0.0	98%	
HQ259684.1	Chryseobacterium indologenes strain B7 16S ribosomal RNA ger	2460	2460	100%	0.0	98%	
EU344917.1	Uncultured Chryseobacterium sp. clone Hg5-07 16S ribosomal	2460	2460	100%	0.0	98%	
EF442766.1	Chryseobacterium sp. COLI2 16S ribosomal RNA gene, partial s	2460	2460	99%	0.0	98%	

A number of different species were identified from the external surface of the nematodes. In all, there were species from eight different genera present, with as many as thirteen individual species. The BLAST results revealed species previously found in association with insects, the soil, the rhizosphere of plants and fungi (Table 6). The four samples that were isolated and sequenced from the internal digestive tract of the

nematodes all were identified as *Paenibacillus spp*. The matches in the BLAST database all classified this species as being associated with soil samples (Table 6). The isolates from the hemolymph of infected hosts were found to be *Serratia marcescens, Serratia nematodiphila*, *Pseudomonas fluorescens*, and *Delftia spp*. BLAST results linked these species to many different sources (Table 6). *S. nematodiphila* was found in association with the nematode *Heterorhabditidoides chongmingensis*, and *P. fluorescens* was found in association with the fungi *Hygrophorus russula*. *S. marcescens* was found to be associated with the crop of the predatory insect *Myrmeleon bore*, and was directly implicated in insect mortality. *Delftia spp*. were found to be related to soil.

Table 11: BLAST results for bacteria isolated from the external cuticle and internal digestive tract of *P. entomophagus*, and from the hemolymph of insects infected by this nematode.

Isolate	Source	Closest Match	Query %	% Match	Accession #	Targeted Gene	Fragment Length
MGH6 Rep 2/2 Raised, White, Big	Hemolymph	Serratia nematodiphila	100	100	EU036987.1	16s rRNA	1434
CDA 1 Rep 2/2	Hemolymph	Serratia marcescens	100	100	AB244453.1	16s rRNA	1435
CDA 2-F	Hemolymph	Delftia sp.	100	99	EU341200.1	16s rRNA	1430
CDA 2-S	Hemolymph	Serratia marcescens	100	99	EF627046.1	16s rRNA	1433
CDA 4	Hemolymph	Serratia marcescens	100	100	EF627046.1	16s rRNA	1432
CDA 5 Dark, Bumpy	Hemolymph	Pseudomonas fluorescens	100	100	FJ588704.1	16s rRNA	1432
CDA 5 Light, Recessed	Hemolymph	Serratia nematodiphila	100	99	EU914257.1	16s rRNA	1434
CDA 6	Hemolymph	Pseudomonas fluorescens	100	100	FJ588704.1	16s rRNA	1432
CDA C8 2	External	Sphingobacterium spp.	99	99	FM958445.1	16s rRNA	1436
CDA C8 3	External	Chryseobacterium sp. C06	98	98	DQ530068.1	16s rRNA	1413
CDA C8 4	External	Delftia sp.	100	99	HM234001.1	16s rRNA	1430
CDA C8 5	External	Stenotrophomonas maltophilia	99	98	AB008509.1	16s rRNA	1116
CDA C8 6	External	Pseudomonas sp. WMQ-7 (pu5da7)	100	100	EU807744.1	16s rRNA	1437
CDA C8 7	External	Bacillaceae bacterium QT26	99	99	GU385872.1	16s rRNA	1451
CDA C8 8	External	Bacillus sp. IMM01	100	99	FR727703.1	16s rRNA	1423
MGH F2 2	External	Delftia sp. Lp-1	100	99	GQ994938.2	16s rRNA	1430
MGH F2 3	External	Delftia sp. Hg4-10	99	99	EU304256.1	16s rRNA	1429
MGH F2 4	External	Serratia Proteamaculans	99	99	CP000826.1	16s rRNA	1111
MGH F2 5	External	Pseudomonas fluorescens	100	100	FJ588704.1	16s rRNA	1438
MGH F2 6	External	Pseudomonas fluorescens	100	100	FJ588704.1	16s rRNA	1436
CDA D5 Int 1 Rep 2	Internal	Paenibacillus sp. LX20	98	99	HQ660810.1	16s rRNA	1455
CDA D5 Int 2 In 2	Internal	Paenibacillus sp. 436-1	100	98	AY286989.1	16s rRNA	1451
MGH E3 Middle L, Normal	Internal	Paenibacillus sp. LX20	99	98	HQ660810.1	16s rRNA	1458
MGH D3 Int 1, Raised Circle	Internal	Paenibacillus curofinensis	99	94	EU257517.1	16s rRNA	1438
E. coli	Pos. Control	E. coli					1488

Query % is defined as how much of the sequence overlaps with the sequence in the BLAST database.

Match % is defined as the percent of bases that match between sequences.

Table 12: Summary of the habitat of bacteria isolates identified by BLAST as the close match of species isolated from *P. entomophagus* and their infected *G. mellonella* hosts.

	Number of Samples			
Habitat Association	Hemolymph	Internal	External	Total
Nematodes	1	0	0	1
Insects	4	0	4	8
Soil	1	4	3	8
Rhizosphere	0	0	3	3
Fungi	2	0	2	4

Discussion:

All cultured bacteria isolated from nematodes (external and internal) and from the hemolymph of infected hosts have previously been found in association with nematodes, insects, fungi, the rhizosphere of plants, or the soil. Of the species isolated and identified from the hemolymph of infected hosts, all have previously been found in association with insects and or nematodes. *Serratia nematodiphila* has been found symbiotically associated with the entomopathogenic nematode *Heterorhabditidoides chongmingensis* (Zhang et al. 2009) and is directly implicated in insect mortality (Zhang et al. 2008). In their study, *S. nematodiphila* was identified as the primary causal agent of host (*Galleria mellonella*) mortality with an LD_{50} of only 50 injected cells. All of the strains associated with *H. chongmingensis* that were tested, caused some level of insect mortality upon injection, however insects that were injected with *S. nematodiphila* turned red in color, the same as seen in *S. nematodiphila* cultures. Furthermore, it was found that axenic (non-symbiont carrying) *H. chongmingensis* juveniles could not successfully reproduce and survive. This finding suggests that the nematodes may depend on their symbionts not only for infectivity, but also for sustenance (Zhang et al. 2008). *Serratia marcescens* was

isolated from the crop of the ant lion, *Myrmeleon bore*, and was shown to cause insect mortality in *Spodoptera litura,* a common test insect (Nishiwaki et al. 2007). Although not isolated from the hemolymph directly in my study, other *Serratia spp*. have also been found associated with dung beetles, the common host of *P. entomophagus*, in Germany (Rae et al. 2008).

Pseudomonas fluorescens was found both in the hemolymph of infected hosts and on the external surface of the nematodes. *P. fluorescens* has also been found to cause direct insect mortality, making it a potential cause of mortality in this system. Devi and Kothamasi (2009) found that *P. fluorescens* has the ability to produce the secondary metabolite hydrogen cyanide. They proved that H-CN producing bacteria may be able to cause insect death via cyanide poisoning because cyanide inhibits the function of the enzyme cytochrome c oxidase (CCO), an integral part of the respiratory chain of the test organism *Odontotermes obesus* and other insects. Several other researchers have investigated the activity of insecticidal metabolites produced by *P. fluorescens* against a number of insects, including mosquitoes (Padmanahban et al. 2005). It has also been engineered to produce the insecticidal *Bacillus thuringiensis Cry* proteins, with recombinant *P. fluorescens* showing moderate success against a test insect (Stock et al. 2009).

Delftia sp., which was isolated in both the hemolymph of infected hosts and on the external surface of the nematodes, has recently been found in the hemolymph of the Glassy-Winged Sharpshooter (*Homalodisca vitripennis*) (Hail et al. 2011). In the past, *Delftia spp*. have shown the ability to, "degrade di-n-butylphthalate (DBP), an industrial pollutant and phthalate derivative, as a sole source of carbon and energy" (Hail et al.

2011, Neelakanteshwar et al. 2006). *Delftia spp*. have not been found to cause insect mortality, suggesting the presence of this bacteria could be used as sustenance by the nematodes. Hail et al. (2011) found *Delftia spp*. in the hemolymph. *Delftia sp*. may proliferate in the insect host, and upon death, be fed upon by the nematodes, and stick to the cuticle of the nematode as it exits the host. *Delftia spp*. are also common soil-dwelling organisms, with one strain showing potential as a plant-growth facilitator (Morel et al. 2011). The *Delftia sp*. isolated from the external surface of the nematode and the hemolymph of infected hosts may be a chance associate of *P. entomophagus*, picked up at random in the environment and deposited in the hemolymph of hosts upon infection.

The internal surface of the nematodes generated only one genus of bacteria, *Paenibacillus*, which was identified in four sequenced samples. It should be noted that the results of the BLAST search produced conclusive evidence that the samples were *Paenibacillus spp*., but all were listed as non-speciated isolates with no classified name to date. All samples were listed as being of soil origin in the BLAST results. *Paenibacillus nematophilus* has been linked to the entomopathogenic nematode *Heterorhabditis megidis*, which carries *Photorhabdus* spp. as its entomopathogenic bacterial symbiont (Enright and Griffin 2005). This study suggests that *P. nematophilus* has either neutral or detrimental effects on *H. megidis*. Factors such as survival rate of infective juveniles (IJ), the yield of emergent IJ's from an infected cadaver, and the size of IJ's were unaffected by the presence of *P. nematophilus*. Conversely, the infectivity (the number of IJ's entering a host) was cut nearly in half, and the motility of emergent IJ's was severely hampered in comparison to control (reared in the absence of *P. nematophilus*) nematodes.

Another species of *Paenibacillus*, *P. larvae*, is the major causal agent of American foulbrood disease in honeybees. The bacteria, if present in the diet of honeybee larvae of the first or second instar, causes an infection, with as little as 10 infectious spores causing mortality (Brodsgaard et al. 1998). With that being said, many *Paenibacillus spp*. are opportunistic in nature and are benign to insects (Qin et al. 2006).

Three species of bacteria isolated from the external cuticle of the nematodes are of note, *P. fluorescens*, *Delftia spp*., and *S. proteamaculans*. As previously mentioned, two isolates, *P. fluorescens* and *Delftia spp*., were found in both the hemolymph of infected hosts and in the external nematodes samples. *Serratia proteamaculans* was isolated from the hemolymph of nematode infected *G. mellonella* larvae exposed to English soils (Al Own et al. 2011). *Galleria mellonella* larvae were used as bait for potential entomopathogenic nematodes in soils from two locations in the United Kingdom. Using methods very similar to our own, Al Own et al. (2011) gathered infected *G. mellonella* and placed them on white-traps to collect emergent nematodes. Hemolymph samples from infected and uninfected larvae were collected in addition to internal nematodes samples which were gathered by surface sterilizing and crushing the nematodes. They found that the hemolymph of the majority of the infected insects contained a *S. proteamaculans* like bacteria. These bacteria were found to be associated internally with two species of *Rhabditid* nematodes and one species of *Pristionchus* nematode, but not with uninfected hemolymph (Al Own et al. 2011). This study also proved the ability of the nematodes to successfully feed and reproduce on the *S. proteamaculans* like strains, suggesting a possible symbiosis in nature.

It is interesting to note that our study found *S. proteamaculans* externally associated with nematodes while Al Own et al. (2011) found it associated with the hemolymph and the internal surface of the nematodes. One possibility for the difference is that the Al Own et al. (2011) study did not explicitly isolate external bacterial associates of the nematodes. *Serratia proteamaculans* may also be an external associate of *Pristionchus* and *Rhabditid* nematodes as our findings suggests. Another possibility is that due to the limited number of internal samples explored in our study, *S. proteamaculans* may have been missed, but is in fact an internal symbiont in our studied system as well.

In analyzing all of the bacterial species found in association with *P. entomophagus* and the hemolymph of infected hosts, a close internal association with entomopathogenic bacteria typical of widely studied entomopathogenic nematode systems (*Heterorhabditis*/*Steinernema* nematodes with *Xenorhabdus*/*Photorhabdus* bacteria) was not found. Conversely, a loose association between bacteria in the hemolymph of infect hosts was found with those on the external surface of *P. entomophagus*. The presence of *Serratia spp*. in both the hemolymph and on the external surface of *P. entomophagus* is the most likely explanation of *M. rubra* mortality by the infecting *P. entomophagus* nematodes.

Pristionchus entomophagus pathogenic associations may be relatively new. While *Pristionchus spp*. have been found in species specific necromenic associations with beetles in Europe for quite some time (Rae et al. 2008), and Poinar (1969) found the diplogasterid nematode *Pristionchus uniformis* to be pathogenic against *G. mellonella* in laboratory testing, the fact that *P. entomophagus* has recently been shown to be

pathogenic against *M. rubra* creates a situation which could represent a host shift to these invasive insects (although *P. entomophagus* may have a broader, undiscovered native host range that includes *M. rubra* among other species)

This new found parasitic and pathogenic relationship between *P. entomophagus* and *M. rubra* affords scientists the opportunity to study the possible mechanisms that allow for the evolutionary switch from a necromenic lifestyle to a predatory lifestyle by *P. entomophagus*. It also allows for the study of the actual mechanism of pathogenicity employed by *P. entomophagus*, which lacks a distinct morphological adaptation to facilitate the formation of a symbiotic relationship with entomopathogenic bacteria. Because *P. entomophagus* is different from other entomopathogenic nematodes of the genera *Heterorhabditis* and *Steinernema*, the mechanism of pathogenicity may be similar to what has been documented in the past within those systems, i.e. a close association with an entomopathogenic endosymbiont, or it may be very different.

Nematodes of the genera *Heterorhabditis* and *Steinernema* have long been used in pest control due to their entomopathogenic capabilities, mainly attributed to their endosymbiotic bacteria of the genera *Xenorhabdus* and *Photorhabdus*. Due to this large bank of evidence suggesting that internal bacterial symbionts are implicated in insect mortality, the fact that only four internal nematode samples were identified to species is a limitation of this study. The sampling of more internal bacterial isolates would likely give a more complete picture of the internal microbial community.

With that being said, the presence of *Serratia spp*., *P. fluorescens*, and *Delftia spp*. in both the samples isolated from the hemolymph of infected hosts and the external surface of the nematodes does suggest the potential role of external bacteria in insect

mortality. If, for example, a soil bacterium pathogenic to insect hosts was present at random in the environment, some nematodes would be exposed to the bacteria and pick up the bacteria externally while others would not. If this were the case, insect mortality between sites, and even within sites, could be variable based on the presence (or lack thereof) of the intermittently distributed external symbiont. There is some evidence of this type of process occurring in the nematode community. Poinar (1969) reported that the diplogasterid nematode *Mesodiplogaster lheritieri* directly caused insect mortality by rupturing the digestive tract of the insect, entering the hemocoel, and bringing in bacteria which caused septicemia. Furthermore, documented cases in the plant parasitic nematode "*Rathayibacter-Anguina* disease complex" affecting plants state that in wheat, *Anguina tritici* carries bacterium on its external body surface, acting as a vector for a bacterial infection of the plant in the wound it creates by feeding (Gupta and Swarup 1972). If these associations were at random, as suggested, this would explain the difference in mortality between sites documented by Groden et al. (2010) in preliminary testing of *P. entomophagus* on laboratory reared colonies of *M. rubra* from various sites in Acadia National Park. Unfortunately, evidence of this kind of interaction existing within the entomopathogenic nematode community is virtually non-existent.

Given the findings of this study and those of Al Own et al. (2010) regarding the association of *Serratia spp*. with *P. entomophagus*, more testing of both the internal and external surfaces of emergent nematodes within the Acadia National Park *M. rubra* community is warranted. Studies using axenic nematode and species specific bacteria for the inoculating of nematodes may help determine the actual method of pathogenicity employed by *P. entomophagus*.

Conclusion and Future Directions of the Study:

Following BLAST search comparisons of 24 isolate sequences which we obtained, we identified 14 species of bacteria associated with *P. entomophagus* and its infected hosts. All of these isolated bacterial species have previously been reported as associated with nematodes, insects, the rhizosphere of plants, fungi, or soil.

The presence of *Serratia spp*., bacterial species previously implicated in insect mortality, in the hemolymph of infected insect hosts is promising, and suggests a possible role of these bacteria in nematode-induced mortality of *M. rubra*. One species, *P. fluorescens* was found in both the hemolymph samples and on the external cuticle of the nematodes, and while it has been found to cause insect mortality in the past, it has not been found associated with nematodes infecting insects. Despite the identification in this study of several bacterial species previously implicated in insect mortality and related to entomopathogenic nematodes, further work is needed to determine the role of the bacterial species in the pathogenicity of these nematodes against *M. rubra*. Future studies are needed to determine the origin of the bacteria introduced into the insect host by the nematode, and the mechanism by which they are introduced in order to show a clear link between bacterial isolates in the hemolymph of infected insect hosts and isolates associated with the internal and external surfaces of the nematode.

In my study, I did not attempt to isolate bacteria from the hemolymph of uninfected insect hosts, as was done by Al Own et al. (2011). This would be necessary to confirm that the species detected in the hemolymph of the nematode infected hosts resulted from the nematode infection. However, past research has suggested that as little as 1% of the microorganisms in nature can be cultivated using conventional techniques such as culture media and naked-eye detection (Amann et al. 1995). In this study, I only

used culture-dependent techniques for the initial isolation of bacteria from nematodes and infected insect hosts. Furthermore, only one media, Trypticase Soy Agar, was used at a general growth temperature of 29°C. This is a very common agar and is generally used as an all-purpose medium in situations where many kinds of microorganisms are expected (Disckson 2000). Many bacteria need very specific growth conditions, including temperature, humidity, and nutrient availability (Kaiser 2006). It is generally accepted within the scientific community that culture dependent and culture independent techniques are necessary to fully characterize the bacterial diversity in soil (Hamaki et al. 2005, Liesack et al. 1997). While there is a potential for the expansion of culture techniques in order to capture novel species (Joseph et al. 2003), due to the general culture techniques used in this study, it is possible that some of the species involved in the tritrophic interactions between *P. entomophagus* and *M. rubra* were not cultured.

A potential complement to a study such as this would be the incorporation of culture independent techniques such as scorpion primers, pyrosequencing, and denaturing gradient gel electrophoresis (DGGE) with the goal of obtaining more samples and a more complete picture of the bacterial species involved in the relationship between *P. entomophagus* and *M. rubra*.

Ciancio et al. (2000) used a novel molecular tool, scorpion primers, to detect an unculturable nematode parasitic bacterium, *Pasteuria sp*. from the soil, suggesting the potential existence of unculturable nematode-related species. In this study, the scorpion primer was used to target a suspected species. The sequence of the scorpion primer must be written as the reverse complement of the target, so this tool is ineffective in surveying

samples for unknown unculturable bacterial species, but very specific for targeted species.

Pyrosequencing is a technique that is commonly used when the detection of species from a mixed sample such as in seawater or soil, is desired. Pyrosequencing works by fragmenting all captured DNA and sequencing each fragment enzymatically. Each nucleotide is added in sequence to the solution containing the fragments rather than all at once. The amount of each base pair being added to the sequence is quantified by the amount of light emitted. The group of fragments gathered is then assembled into complete genomes by a computer affording the identification of many species in a sample from the gathered "DNA soup" without culturing. In this study, the incorporation of pyrosequencing would allow for sampling directly from gathered hemolymph, and the internal and external rinses of nematodes rather than from cultured colonies.

Denaturing gradient gel electrophoresis (DGGE) also allows for the isolation of fragments of DNA from an uncultured sample. DNA from an unknown sample run in DGGE will fragment in a species specific manner. Furthermore, some DGGE techniques are capable of detecting differences between two sequences as little as 1 base pair. After fragments are excised from the gel, they can be sequenced and identified down to species.

Another approach that could prove useful for exploring the relationship between *P. entomophagus* and its bacterial associates in infection and pathogenicity is the development of axenic populations of the nematodes (nematodes with no bacterial associates). With the recent discovery of *S. proteamaculans* as an insect pathogenic bacteria associated with *P. entomophagus* in the United Kingdom, rearing of axenic nematodes on *S. proteamaculans* and subsequent exposure to *M. rubra* would provide

valuable comparative information to the work of Al Own et al. (2011). Axenic nematodes could also be inoculated with other bacterial species isolated from the nematodes emergent from *M. rubra* as well as the hemolymph of infected hosts. It is possible that some species may play a role that is non-pathogenic to the insect host. It has been documented that *P. nematophilus* has direct effects on the pathogenic potential of nematodes while having no role in insect mortality (Enright and Griffin 2005). It is possible that pathogenicity is only achieved if a specific combination of bacteria is associated with the nematode and transferred to the insect host. By testing each species of bacteria found in association with *P. entomophagus* in axenic nematodes, it could be discerned which species, if any, are involved in insect mortality and perhaps if pair-wise or group associations of bacteria are necessary for mortality.

References:

Al Own, F., Feil, E., Stock, S.P., Waterfield, N.R. 2010. *Serratia proteamaculans* strains associated with novel insect pathogenic nematodes in UK soils. Unpublished Abstr. Second NEMASYM Workshop, University of Arizona, Tucson, AZ, November 11-14, 2010.

Amann, R.I., Ludwig, W., Schleifer, K.H. 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation, Microb. Rev. 59, 143–169.

Attard, L.M., Carreno, R.A., Pare, J.A., Peregrine, A.S., Dutton, C.J., Mason, T.R, 2008. Mermithid Nematode Infection in a Colony of Blue-winged Grasshoppers (*Tropidacris collaris*). Journal of Zoo and Wildlife Medicine 39(3), 488-492.

Bird, A. F., Akhurst, R.J., 1983. The nature of the intestinal vesicle in nematodes of the family Steinernematidae, Int. J. Parasitol. 16, 511-518.

Bonifassi, E., Fischer-Le Saux, M. Boemare, N., Lanois, A., Laumond, C., Smart, G. 1999. Gnotobiological study of infective juveniles and symbionts of *Steinernema scapterisci*: a model to clarify the concept of the natural occurrence of monoxenic associations in entomopathogenic nematodes. J. Invertebr. Pathol. 74, 164-172.

Brodsgaard, C. J., Ritter, W., Hansen, Henrik, 1998. Response of in vitro reared honey bee larvae to various doses of *Paenibacillus larvae larvae* spores. Apidologie (Celle), 569–578.

Campbell, J.F., Lewis, E., Yoder, F., Gaugler, R., 1996. Entomopathogenic nematode (Heterorhabditidae and Steinernematidae) spatial distribution in turfgrass. Parasitology. 113 (5), 473-482.

Campbell J.F., Gaugler, R., 1993. Nictation behaviour and its ecological implications in the host search strategies of entomopathogenic nematodes (Heterorhabditidae and Steinernematidae). Behaviour. 126, 156–169.

Ciancio, A., Leonetti, P., Finetti Sialer, M.M., 2000. Detection of nematode antagonistic bacteria by fluorogenic molecular probes. EPPO Bulletin. 30 (3-4), 563–569.

Czechowski, W., Radchenko, A., Czechowska, W., 2000. The Ants (Hymenoptera, Formicidae) of Poland. Museum & Institute of Zoology, Warsaw.

Devi, K.K., Kothamasi, D., 2009. *Pseudomonas fluorescens* CHA0 can kill subterranean termite *Odontotermes obesus* by inhibiting cytochrome *c* oxidase of the termite respiratory chain. FEMS Microbiology Letters. 300 (2), 195-200.

Dieterich, C., Sommer, R.J., 2009. How to become a parasite-lessons from the genomes of nematodes. Trends Genet. 25 (5), 203-209.

Elmes, G.W., Wardlaw, J.C., Nielsen, M.G., Kipyatkov, V.E., Lopatina, E.B., Radchenko, A.G., Barr, 1999. Site latitude influences on the respiration rate, fat content and the ability of worker ants to rear larvae: A comparison of *Myrmica rubra* (Hymenoptera: Formicidae) populations over their European range. Eur. J. Entomol. 96, 117 - 123.

Enright, M.R., Griffin, C.T. 2005. Effects of *Paenibacillus nematophilus* on the entomopathogenic nematode *Heterorhabditis megidis.* J. Invertebr. Pathol. 88 (1), 40-48.

Evans, H.C., Groden, E., Bischoff, J.F., 2010. New Fungal pathogens of the red ant, *Myrmica rubra*, from the UK and implications for and invasions in the USA. Fungal Biol. 114 (5-6), 451-466.

Felix, M.A., Hill, R.J., Schwarz, H., Sternberg, P.W., Sudhaus, W., Sommer, R.J., 1999. *Pristionchus pacificus*, a nematode with only three juvenile stages, displays major heterochronic changes relative to *Caenorhabditis elegans*, Proc. R. Soc. London B. 266, 1617–1621.

Fürst von Lieven, A., Sudhaus, W., 2000. Comparative and functional morphology of the buccal cavity of Diplogastrina (Nematoda) and a first outline of the phylogeny of this taxon. J. Zoolog. Syst. Evol. Res. 38, 37-63.

Fürst von Lieven, Alexander, 2005. The embryonic moult in diplogastrids (Nematoda) – homology of developmental stages and heterochrony as a prerequisite for morphological diversity. Zool. Anz. 244 (1), 79-91.

Gaugler, R., Lewis, E., Stuart, R.J., 1997. Ecology in the service of biological control: the case of entomopathogenic nematodes. Oecologia. 109, 483–489.

Gaugler, Randy, 2002. Entomopathogenic Nematodes, first ed. CABI, Wallingford, Oxfordshire, UK.

Gilbert, Griffeth. Personal files and protocols, accessed 2011.

Groden, E., Drummond, F.A., Garnas, J., Franceour, A., 2005. Distribution of an Invasive Ant, *Myrmica rubra* (Hymenoptera: Formicidae), in Maine. J. Econ. Entomol. 98, 1774-1784.

Groden, E., S.P. Stock. 2010. Establishment of new host relationship with an invasive? Or hitchhiker across the Atlantic? Unpublished Abstr. Second NEMASYM Workshop, University of Arizona, Tucson, AZ, November 11-14, 2010

Gupta, P., Swarup, G., 1972. Ear-cockle and yellow ear rot disease of wheat, Il. Nematode bacterial association. Nematologiqa. 18, 320-324.

Hail, D., Lauziere, I, Dowd, S.E., Bextine, B., 2011. Culture Independent Survey of the Microbiota of the Glassy-Winged Sharpshooter (Homalodisca vitripennis) using 454 Pyrosequencing. Environ. Entomol. 40 (1), 23-29.

Hamaki, T, Suzuki, M., Fudou, R., Jojima, Y., Kaijura, T., Tabuchi, A., Sen, K., Shibai, H., 2005. Isolation of novel bacteria and actinomycetes using soil-extract agar medium. J. Biosci. Bioeng. 99 (5), 485-492.

Herrmann, M., Mayer, W.E., Sommer, R.J., 2006a. Nematodes of the genus *Pristionchus* are closely associated with scarab beetles and the Colorado potato beetle in Western Europe. Zoology (Jena). 109, 196-198.

Herrmann, M., Mayer, W.E., Sommer, R.J., 2006b. Sex, bugs and Haldane's rule: the nematode genus *Pristionchus* in the United States. Front. Zool. 3, 15 -31.

Hong, R.L., Sommer, R.J., 2006a. Chemoattraction in Pristionchus Nematodes and Implications for Insect Recognition. Curr. Biol. 16, 2359–2365.

Hong, R.L., Sommer, R.J., 2006b. Pristionchus pacificus: a well-rounded nematode. BioEssays 28, 651–659. Wiley Periodicals, Inc.

Joseph, S.J., Hugenholtz, P., Sangwan, P., Osborne, C.A., Janssen, P.H., 2003. Laboratory Cultivation of Widespread and Previously Uncultured Soil Bacteria. Appl Environ Microbiol. 69 (12), 7210–7215.

Lewis, E.E., Gaugler, R., Harrison, R., 1992. Entomopathogenic nematode host finding: response to host contact cues by cruise and ambush foragers. Parasitology. 105, 309–315.

Liesack, W., Janssen, P.H., Rainey, F.A., Ward-Rainey, N.L., Stackebrandt, E., 1997. Microbial diversity in soil: the need for a combined approach using molecular and cultivation techniques, in: Van Elsas, J.D., Trevors, J.T., and Wellington, E.M.H. (Eds.), Modern soil microbiology. Marcel Dekker Inc., New York. pp. 375–439.

Martens, E.C., Russell, F.M., Goodrich-Blair, H., 2005. Analysis of *Xenorhabdus nematophila*metabolic mutants yields insight into stages of *Steinernema carpocapsae* nematode intestinal colonization. Mol. Microbiol. 1, 28-45.

Martens, E.C., Goodrich-Blair, H., 2005. The *Steinernema carpocapsae* intestinal vesicle contains a subcellular structure with which *Xenorhabdus nematophila* associates during colonization initiation. Cell. Microbiol. 12, 1723-1735.

Meng, Xiao., 2000. The Hemolymph Pathology of Lepidopteral Larvae Infected with Entomopathogenic Nematodes. Chinese Journal of Biological Control, 16 (3), 114-117.

Morel, M.A., Ubalde, M.C., Braña, V., Castro-Sowinski, S., 2011. Delftia sp. JD2: a potential Cr(VI)-reducing agent with plant growth-promoting activity. Arch. Microbiol. 193 (1), 63-68.

Nishiwaki, H., Ito, K., Shimomura, M., Nakashima, K., Matsuda, K., 2007. Insecticidal bacteria isolated from predatory larvae of the antlion species *Myrmeleon bore* (Neuroptera: Myrmeleontidae). J. Invertebr. Pathol. 96 (1), 80-88.

Ogawa, A., Bento, G., Bartelmes, G., Dieterich, C., Sommer, R.J., 2011. *Pristionchus pacificus daf-16* is essential for dauer formation but dispensable for mouth form dimorphism. Development. 138, 1281-1284.

Padmanabhan, V., Prabakaran, G., Paily, K.P., Balaraman, K., 2005. Toxicity of a mosquitocidal metabolite of *Pseudomonas fluorescens* on larvae & pupae of the house fly, Musca domestica. Indian J. Med. Res. 121, 116-119.

Patil, N.K., Kundapur, R., Shouche, Y.S., Karegoudar, T.B.,2006. Degradation of a plasticizer, di-nbutylphthalate by *Delftia* sp. TBKNP-05. Current Microbiol. 52, 225–230.

Poinar, G. O. Jr., Thomas, G. M., 1967. The nature of *Achromobacter nematophilus* as an insect pathogen. J. Invertebr. Pathol. 9, 510-514.

Poinar, George, Jr., 1969. Diplogasterid Nematodes (Diplogasteridae: Rhabditida) and Their Relationship to Insect Disease. J. Invertebr. Pathol.13, 447-454.

Poinar, G.O. Jr., 1972. Nematodes as Facultative Parasites of Insects. Annu. Rev. Entomol. 17, 103-122.

Qin, X., Evans, J.D., Aronstein, K.A., Murray, K.D., Weinstock, G.M., 2006. Genome sequences of the honey bee pathogens *Paenibacillus larvae* and *Ascosphaera apis.* Insect Mol. Biol. 15, 715–718.

Rae, R., Riebesell, M., Dinkelacker, I., Wang, Q., Herrmann, M., Weller, A., Dieterich, C., Sommer, R.J., 2008. Isolation of naturally associated bacteria of necromenic *Pristionchus* nematodes and fitness consequences. J. Exp. Biol. 211, 1927–1936.

Roberts, L.S., 1973. Modifications in media and surface sterilization methods for *in vitro* cultivation of *Hymenolepis dimunuta*. J. Parasit. 59 (3), 474-479.

Snyder, H., Stock, S.P., Kim, S.K, Flores-Lara,Y., Forst, S., 2007. New Insights into the Colonization and Release Processes of *Xenorhabdus nematophila* and the Morphology and Ultrastructure of the Bacterial Receptacle of Its Nematode Host, *Steinernema carpocapsae.* Appl. Environ. Microbiol. 73 (16), 5338–5346.

Sommer, R.J., Carta, L.K., Kim, S.Y., Sternberg, P.W., 1996. Morphological, genetic and molecular description of Pristionchus pacificus sp. n. (Nematoda : Neodiplogastridae). Fundam. appl. Nematol., 19 (6), 511-521.

Stock, S.P., Glazer, I., Vandenberg, J., 2009. Insect Pathogens: Molecular Approaches and Techniques. CABI Publishing. Cambridge, MA, USA

Sudhaus, W., Fürst von Lieven, A., 2003. A phylogenetic classification and catalogue of the Diplogastridae (Secernentea, Nematoda). J. Nem. Morph. Syst. 6, 43-90.

Tanada, Y., Kaya, H., 1993. Insect Pathology. Academic Press, Inc., New York.

Volk, J., 1950. Die Nematoden der Regenwürmer und aasbesuchenden Käfer (Nematodes associated with earthworms and carrion beetles). Zool. Jahrb. Abt. Anat. Ontog. Tiere . 79 (1-2), 1-70.

Weller, A.M., Mayer, W.E., Rae, R., Sommer, R.J., 2010. Quantitative Assessment of the Nematode Fauna Present on *Geotrupes* Dung Beetles Reveals Species-Rich Communities with a Heterogeneous Distribution. J. Parasit. 96, 525-531.

Wetterer, J.K., Ravchenko, A.G.,2010. Worldwide spread of the ruby ant, Myrmica rubra (Hymenoptera : Formicidae). Myrmecological News. 14, 87-96.

Wheeler, W.M., 1908. A European ant (*Myrmica laevinodis*) introduced into Massachusetts. Journal of Economic Entomology 1, 336-339.

Zhang, C., Liu, J., Xu, M., Sun, J., Yang, S., An, X., Gao, G., Lin, M., Lai, R., He, Z., Wu, Y., Zhang, K., 2008. *Heterorhabditidoides chongmingensis* gen. nov., sp.nov. (Rhabditida: Rhabditidae), a novel member of the entomopathogenic nematodes. J. Invertebr. Pathol. 98 Issue 2. (2008), pp. 153-168.

Zhang, C.X., Yang, S.Y., Xu, M.S., Sun, J., Liu, H., Liu, J.R., Kan, F., Sun, J., Lai, R., Zhang, K.Y., 2009. *Serratia nematodiphila* sp. nov., symbiotically associated with entomopathogenic nematode *Heterorhabditidoides chongmingensis* (Rhabditida: Rhabditidae). Int. J. Syst. Evol. Microbiol. 59 (2009), pp. 1603-1608.

Websites:

Arevalo et al., 2007. European fire ant-Myrmica rubra Linnaeus, accessed 1.24.2001. http://entnemdept.ufl.edu/creatures/urban/ants/Myrmica_ruba.htm

Dickson, James S., 2000. Faculty, Microbiology, Iowa State University. Re: What bacterial results can grow out of (TSA) (CNA) & MacConkey petri dishes? MadSci Network: Microbiology. Accessed 4.11.2011. http://www.madsci.org/posts/archives/2000-02/951527342.Mi.r.html

Gaugler, R., 2006. Nematodes-Biological Control, accessed 4.11.2011. http://www.biocontrol.entomology.cornell.edu/pathogens/nematodes.html

Groden, E., Drummond, F.A., Stack, L.B., Graham, C., 2004. European Fire Ant*: A New Invasive Insect in Maine, accessed 2.24.2011. http://umaine.edu/publications/2550e/

http://www.formedium.com/uk/EscherichiaColi/EscherichiaColi_1.htm. accessed March 2011.

http://www.promega.com/tbs/tm050/tm050.pdf. accessed March 2011.

http://www.qiagen.com/Products/DnaCleanup/GelPcrSiCleanupSystems/QIAquickPCRPurificationKit.aspx?r=926#Tabs=t0. accessed March 2011.

http://www2.umaine.edu/dnaseq/order/. accessed March 2011.

https://www.vwrsp.com/index.cgi. accessed March 2011.

Kaiser, G., 2006. Factors that Influence Bacterial Growth. Doc Kaiser's Microbiology Home Page, accessed 4.11.2011. http://student.ccbcmd.edu/courses/bio141/lecguide/unit6/metabolism/growth/factors.html

Kiontke, K. and Sudhaus, W.**,** 2006. Ecology of *Caenorhabditis* species. In *WormBook* (ed. The *C. elegans* Research Community), accessed 4.11.2011. http://www.wormbook.org

Lautenslager, P. et al., 2003. Mermithid Nematodes. Accessed 4/14/2011. http://www.uoguelph.ca/pdc/Factsheets/PDFs/031MermithidNematode.pdf

National Biological Information Infrastructure (NBII) & IUCN/SSC Invasive Species Specialist Group (ISSG), 2009. *Myrmica rubra* (insect), accessed 4.12.2011. http://interface.creative.auckland.ac.nz/database/species/ecology.asp?si=1014&fr=1&sts=&%20ang=SC&ver=print&prtflag=false

Nguyen, Khoung B., (2005). Photographs showing how to rear entomopathogenic nematodes, accessed 2.23.2011. http://entnemdept.ufl.edu/nguyen/morph/rearwax.htm

Appendix A: Methods for mass production and storage of nematodes

In previous studies, collected nematodes were studied but not propagated for future study (Groden et al. 2010). In order to produce a sufficient number of nematodes to study and store for future use, *Galleria mellonella* (waxworm) larvae were inoculated with nematodes from all sites to propagate their numbers. A first trial was conducted using nematodes stored at 4°C from older testing. Nematodes from eight sites throughout Acadia National Park were used. Two waxworm larvae were placed in five separate Petri dishes for each site, totaling ten inoculated larvae. Larvae were monitored for time of death, decay pattern, and the emergence of nematodes. A second trial was run using fresh nematodes from *M. rubra* cadavers collected from colonies reared in the laboratory. Waxworm larvae to be inoculated were set up in two different environments, sand and soil. One hundred milliliter cups were set up with either 1 cm of sand or 2.5 cm of soil. Five waxworm larvae were added to each cup and inoculated with the emergent nematodes from one *M. rubra* cadaver. Cups were monitored daily, and cadavers were removed using forceps and placed in white traps (Nguyen, 2005). The appearance and density of nematodes was noted. Nematodes were collected and placed into 1.5 mL microcentrifuge tubes and stored at 4°C. Another subset of the harvested nematodes were plated onto Baby Food Agar (BFA) and stored at room temperature.

Appendix B: Initial attempts at isolation of bacteria from gut cavity of nematodes

Original Procedure: After plating aliquots of the external rinse solution, 1.5 mL microcentrifuge tubes containing nematodes were centrifuged and the supernatant was drawn off. One milliliter of 1% bleach solution was added to each tube and left to sit for 2-3 minutes, gently vortexing periodically. Afterwards, tubes were centrifuged at 13,000 RPM for one minute and the supernatant was drawn off, being careful to leave all nematodes at the bottom of the tube. Each tube of nematodes was rinsed with 1 mL of sterile distilled water three times, allowing the nematodes to sit in the water for 1-2 minutes while gently vortexing and centrifuging each rinse. Nematodes were then crushed using a pestle unit that fit tightly into the bottom of the 1.5 ml microcentrifuge tubes (National Scientific Supply Co., Claremont, CA, USA). Crushed nematode solutions were plated onto TSA in 300 µL aliquots and incubated at 29°C degrees for 48 hours. Unique isolate colonies were transferred to fresh TSA plates and grown at 29°C for 48 hours, after which samples were stored at 4°C.

Modified Procedure: After completing the original procedure, it was found that the nematodes were lost in the process. It was determined that the amount of pipetting involved in surface sterilizing and rinsing the nematodes within a 1.5 mL microcentrifuge tube was the main cause of lost nematodes. As a result, a different procedure, including a standardization of nematode concentration that was not previously done, was undertaken in an attempt to minimize the loss of nematodes.

Author's Biography:

Jonathan E. Dumont was born in Bangor, Maine on June, 2, 1989. He was raised in Oakland, Maine and graduated from Messalonskee High School in 2007. A member of the University of Maine's Class of 2011, Jonathan graduated in May 2011 with a Bachelor's of Science Degree in Biology and a minor in Psychology. He is a member of Phi Beta Kappa, Phi Kappa Phi, and the National Society of Collegiate Scholars. He has received various departmental awards in the School of Biology and Ecology for academic excellence.

Upon graduation, Jonathan plans to take time off before returning to his studies to pursue an advanced degree in nematology, entomology, or insect pathology.

An avid golfer, Jonathan participates in a number of intramural athletics and enjoys playing tennis and hockey with friends in his spare time.