Assessment of Pathogenic Bacteria Transfer From Pristionchus Entomophagus (Nematoda: Diplogasteridae) to the Invasive Ant Myrmica Rubra and Its Potential Role in Colony Mortality in Coastal Maine

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Abstract

Background:

Necromenic nematode *Pristionchus entomophagus* has been frequently found in nests of the invasive European ant *Myrmica rubra* in coastal Maine, United States. The nematodes may contribute to ant mortality and collapse of colonies by transferring environmental bacteria. *M. rubra* ants naturally hosting nematodes were collected from collapsed wild nests in Maine and used for bacteria identification. Virulence assays were carried out to validate acquisition and vectoring of environmental bacteria to the ants.

Results:

Multiple bacteria species, including *Paenibacillus spp.*, were found in the nematodes’ digestive tract. *Serratia marcescens*, *Serratia nematodiphila*, and *Pseudomonas fluorescens* were collected from the hemolymph of nematode-infected *Galleria mellonella* larvae. Variability was observed in insect virulence in relation to the site origin of the nematodes. *In vitro* assays confirmed uptake of RFP-labeled *Pseudomonas aeruginosa* strain PA14 by nematodes. Bacteria were highly concentrated in the digestive tract of adult nematodes, a small amount of bacteria were observed in the digestive tract of juveniles with a more significant amount on their cuticle, and none on the cuticle of adults. RFP-labeled *P. aeruginosa* were not observed in hemolymph of *G. mellonella* larvae, indicating an apparent lack of bacterial transfer from juvenile nematodes to the insects despite larval mortality.

Host species was the primary factor affecting bacterial community profiles. *Spiroplasma* sp. and *Serratia marcescens* sequences were shared across ants, nematodes, and nematode-exposed *G. mellonella* larvae. Alternative to the idea of transferring bacteria from environment to host, we considered whether nematode-exposure might disorder or depauperate the endobiotic community of an insect host. While total bacterial diversity was not statistically lower in nematode-exposed *G. mellonella* larvae when compared to controls, 16 bacterial sequence variants were less abundant in nematode-exposed larvae, while three were increased, including *Serratia, Pseudomonas*, and *Proteus*.

Conclusions:

This study suggests that transfer of bacteria from nematodes to ants is feasible, although largely serendipitous, and may possibly contributed to ant death and/or collapse of wild colonies in Maine. Hypothetically, the use of an engineered biological control, such as nematodes carrying specifically-seeded bacterial species, may be effective, especially if the pathogenic bacteria are normally found in soil ecosystems and represents a low risk for biosafety control.

Background
*Myrmica rubra* (Linnaeus) (Hymenoptera: Formicidae), commonly known as the European fire ant, is native to much of the Palearctic ecozone in Europe and Asia, stretching from Ireland in the west to Western Siberia in the east [1], and from approximately the 25°N latitude in the south to the 66°N latitude in the Arctic Circle [2]. *M. rubra* has been introduced into regions where it is non-native through unintentional human transport, including in North America where it is considered invasive [3, 4]. Since the early 1900s, established populations have been reported along the east coast in the U.S.; in Maine, Massachusetts, New York, Pennsylvania, New Jersey, Washington D.C., Rhode Island, and New Hampshire, and in Canada; in Ontario, Québec, New Brunswick, Prince Edward Island, Newfoundland, and Nova Scotia [5]. Based on the latitude range of its native habitat, it is believed *M. rubra* may be able to subsist in various habitats in North America, from southern Florida, US, to north of the Hudson Bay, Canada [6].

European invasive ants are aggressive, sugar-loving ants that feed on a variety of carbohydrates and protein sources, including nectar, honeydew, other arthropods, and animal debris [7]. These ants demonstrably alter biological community dynamics by negatively impacting native ant species [8, 9], altering seed dispersal by native ants [10], influencing distribution and abundance of Homopteran insects [11], and lowering arthropod abundance [12] and diversity [9]. Moreover, in the United States, other invasive species of ants cause heavy losses to biological diversity and agricultural production, and raise concerns for human health [13] as ant stings can be painful or toxic to sensitive individuals [14].

European ants were first observed in Maine in the late 1960s to early 1970s [14, 15], and since 1998, reports of *M. rubra* have increased dramatically [7]. *M. rubra* has been largely concentrated in humid regions along Maine’s coast [15], including in Acadia National Park on Mount Desert Island [14]; however, colonies established inland suggest the ant is able to survive in other environments throughout the state [6].

In other ecosystems, previous attempts to control invasive ants used chemical pesticides for eradication [16, 17], but commercial pesticides have been unable to eliminate populations in Maine [15]. More recently, control efforts have focused on reducing the spread of ants by human activities which inadvertently transport them, altering the environment to make it less hospitable for the ants [6], and investigating biological controls including insect-parasitizing nematodes. Many nematode species have been described as parasites of ants, evidence of which dates back to fossil records [18], and several are demonstrated to kill hosts and reduce the impact of invasive ant species [13, 17]. The majority of research on the use of nematodes as biological control agents against invasive ants focuses on entomopathogenic nematodes [13], which have an obligate symbiotic relationship with pathogenic bacteria that are lethal to the insect hosting the nematode.

However, other soil-inhabiting nematodes with different life cycles may also infect ants and may have a role in their biological control. Necromenic nematodes are usually free-living microbivores that associate in a tight phoretic (temporary and commensal) relationship with insect hosts, feeding on them after they die of natural causes [19]. Nematodes in the genus *Pristionchus*, which are characterized as necromenic
[20], may be facultatively virulent towards ants and beetles. *Pristionchus spp.* enter their insect host through natural openings such as the mouth or anus [21], and persist in dauer diapause, in which larvae are in stasis until conditions become favorable for growth. Once the host dies from other causes, dauer-stage nematode larvae detect favorable conditions and re-engage the reproductive life cycle, becoming J4 juveniles. Inside the intestines, the nematodes mature to adults and proliferate, rupturing the digestive tract wall and entering into the hemolymph [22], and eventually emerging from the host to continue their life cycle in soil [20, 23].

Adult *Pristionchus* nematodes feed selectively on bacteria and fungi proliferating in and on the insect carcass, during which ingestion of pathogenic bacteria can negatively impact nematode fitness or cause death [20]. Unlike entomopathogenic types, these nematodes do not have an obligate relationship with specific bacteria [19], and may host a diverse bacterial community of common inhabitants of soil, water, and other insects [20, 24]. Members of the nematode family Diplogasteridae, such as *Pristionchus spp.*, have specialized stoma morphology, including shorter, broader mouthparts with no grinder, which allow them to ingest whole bacteria without crushing them and prevent them from being regurgitated after ingestion [22, 25]. *Pristionchus* nematodes may sporadically infect insect hosts with intact pathogenic bacteria picked up from the environment [20, 26, 27].

There is evidence that mortality in wild *M. rubra* ant colonies in Maine is a result of *P. entomophagus* nematode infection, which was recreated in a laboratory setting [28]. Further, nematodes gathered from *M. rubra* cadavers collected from various sites on Mount Desert Island, Maine and used to inoculate *M. rubra* in laboratory reinfection assays revealed differential induced mortality, suggesting that there are inherent differences in the pathogenicity of *P. entomophagus*, likely due to their site of origin and local soil bacteria [28].

It has been hypothesized that *Pristionchus entomophagus* may have caused the collapse of invasive ant colonies on Mount Desert Island by actively transporting pathogenic bacterial species from the environment into the ants [28]. To test if *P. entomophagus* nematodes may have driven *M. rubra* ant colony mortality on Mount Desert Island, in coastal Maine, in this study we isolated bacteria cultured from nematodes emerging from *M. rubra* cadavers, and assessed the ability of the nematodes to acquire environmental bacteria and their subsequent transfer to an insect host. We also identified bacteria which were potentially transferred from nematodes to infected ant nests on the island using bacterial community similarity and sequence tracking methods.

**Results**

**Identification of bacteria from nematodes emerged from *M. rubra* ant cadavers**

Ants were collected from colonies at the coastal Mount Desert Island sites COA and MGH, as these sites exhibited high mortality and emergence of nematodes from ant cadavers. Emerged nematodes were used
to culture a total of 45 bacterial isolates (Table S1; originally printed as Table 1 in [23]; Figure S1). Twenty-eight isolates were recovered from the nematodes’ cuticle, and were grouped into 12 unique bacterial morphotypes based on culture and cell morphology. Eight isolates were recovered from the nematodes’ digestive tract resulting in two distinct morphotypes. Nine bacterial isolates yielding five unique morphotypes were identified from the hemolymph of *Galleria mellonella* larvae exposed to nematodes harvested from ant cadavers. Of these, 32 isolates, representing all the observed morphotypes, were selected for molecular identification. Nearly full-length 16S rRNA gene sequences were obtained for 24 isolates (Table S2). Genetic distance comparison of query sequences with those in the NCBI BLAST database revealed 13 species in eight genera (Table 1; Fig. 1). Various species of cultured bacteria were recovered from the cuticle of nematodes. Three sequenced isolates collected from the nematodes’ digestive tract were identified as *Paenibacillus spp*. Five isolates were identified from the hemolymph of nematode infected *Galleria mellonella* larvae. These bacteria were identified as *Serratia marcescens*, *Serratia nematodiphila*, and *Pseudomonas fluorescens*. 
Table 1
Taxonomic identification for bacteria isolated from the external cuticle (E) and digestive tract (I) of *Pristionchus entomophagus* and from the hemolymph (H) of *Galleria mellonella* larvae co-cultured with this nematode.

| Isolate ID | Isolate source | Length (nt) | Best match                                      | Coverage (%) | Identity (%) | Accession of best match |
|------------|----------------|-------------|------------------------------------------------|--------------|--------------|-------------------------|
| PO50       | H              | 1420        | *Serratia marcescens* str. FY                  | 100          | 100          | CP053378                |
| PO52       | E              | 1436        | *Sphingobacterium multivorum* str. NBRC 14087  | 99           | 99.9         | AB680559                |
| PO54       | I              | 1445        | *Paenibacillus odorifer* str. DSM 15391        | 99           | 99.7         | CP009428                |
| PO56       | stock          | 1424        | *Escherichia coli* str. EcPF5                  | 100          | 99.9         | CP054236                |
| PO60       | H              | 1449        | *Pseudomonas protegens* str. SN15-2            | 100          | 100          | CP043179                |
| PO61       | H              | 1445        | *Pseudomonas protegens* str. SN15-2            | 100          | 100          | CP043179                |
| PO62       | H              | 1424        | *Serratia nematodiphila* str. BAB-6783         | 100          | 99.9         | MF319860                |
| PO63       | H              | 1422        | *Pseudomonas protegens* str. SN15-2            | 100          | 100          | CP043179                |
| PO64       | E              | 1420        | *Delftia acidovorans* str. B208 16S           | 99           | 100          | KJ781879                |
| PO65       | E              | 959         | *Stenotrophomonas maltophilia* str. yy01       | 100          | 100          | MN177222                |
| PO66       | E              | 1437        | *Pseudomonas putida* str. JCM 13063            | 100          | 100          | LC507960                |
| PO67       | E              | 1441        | *Bacillus mycoides* str. BF1-5                 | 99           | 99.9         | MT078667                |
| PO68       | E              | 1423        | *Bacillus mycoides* str. 2861                  | 100          | 99.9         | MT586023                |
| PO71       | I              | 1443        | *Paenibacillus* sp. FSL H7-0737               | 100          | 99.5         | CP009279                |
| PO72       | I              | 1429        | *Paenibacillus contaminans* str. CKOBP-6       | 99           | 99.9         | NR_044325               |
| PO74       | E              | 1420        | *Delftia lacustris* str. MB38                  | 100          | 99.9         | MH675503                |
| PO75       | E              | 1419        | *Delftia lacustris* str. MB38                  | 99           | 99.9         | MH675503                |
| PO76       | E              | 1025        | *Serratia quinivorans* str. 5619               | 100          | 99.8         | MT256279                |
| PO77       | E              | 1428        | *Pseudomonas protegens* str. SN15-2            | 100          | 100          | CP043179                |
| Isolate ID | Isolate source | Length (nt) | Best match                          | Coverage (%) | Identity (%) | Accession of best match |
|------------|----------------|-------------|-------------------------------------|--------------|--------------|-------------------------|
| PO78       | E              | 1426        | *Pseudomonas protegens* str. SN15-2  | 100          | 100          | CP043179                |
| PO79       | E              | 872         | *Serratia* sp. RS7                   | 100          | 99.9         | MN006027                |

**Nematode uptake of environmental bacteria and vectoring to other insect hosts**

**Insect virulence varied by site of origin of nematode isolates**

Nematodes cultures similarly sourced from ants at three collected sites, BNR and VC in Mount Desert Island (coastal), and OR in Orono (inland), exhibited varying virulence against *Galleria mellonella* larvae after 14 days (Fig. 2). Virulence significantly varied by site ($X^2_{2,8} = 64.91, p = 0.02$); overall, nematodes collected from OR had the highest virulence, followed by BNR, VC did not have significantly higher mortality compared to controls. There was not a significant difference in mortality between two tested doses of nematode exposure for any field site.

**Nematodes may uptake bacteria from their environment with varying survival**

Five percent of live adult nematodes (2/40) were able to uptake RFP-labeled *Escherichia coli* HB101 from culture media, i.e. environmental acquisition (Figure S2a). This *E. coli* strain is known to be non-pathogenic to nematodes. No bacteria were observed in dead nematodes, positive controls (nematodes exposed to non-labeled *Paenibacillus* sp. previously isolated from nematodes, or negative controls (no bacterial exposure). Nematode survival rates were not significantly different ($p > 0.05$) at two days post-exposure (87% survival +/- 4 SE), and when compared to controls exposed to no bacteria. However, after 5 days post exposure, survival fell to 69% ± 16 SE for *E. coli*-treated nematodes. There was no change in survival for nematodes exposed to *Paenibacillus* sp. (87% survival ± 0.1 SE) when compared to controls (86% survival ± 0.4 SE). Five days after exposure, survival was at 68% ± 05 SE for *Paenibacillus* sp.-treated nematodes, and 80% ± 0.3 SE for control nematodes. Overall, mortality at day 5 was significantly higher in both bacterial treatment groups, as compared to no-bacterial controls ($F_{1,6} = 7.08, p = 0.04$).

The uptake of RFP-labeled *Pseudomonas aeruginosa* strain PA14 from culture media by the nematodes was visually confirmed (Figure S2, Fig. 3), and was highest in the digestive tract of adult nematodes. No bacteria were observed on the cuticle of adults. Contrarily, bacteria were confirmed on the external cuticle of juveniles, with a small amount of fluorescence observed in their digestive tract (Fig. 3). The greatest difference in proportion of overall fluorescence, as well as peak fluorescence, was between the digestive.
tract of adults (95%) and the cuticle of juveniles (30%) on day 10 ($p < 0.001$; Fig. 3). Fluorescence overall varied over time ($p < 0.001$), with a general increase in the digestive tract of adults, but a steady and significant decrease in the proportion of juveniles with fluorescence (external and internal) over time ($p = 0.038$). Survival in nematodes exposed to RFP-labeled *P. aeruginosa* strain PA14, known to be virulent to nematodes, was not significantly different (44.9% ± 7 SE survival) when compared to nematodes with their natural microflora (50.8% ± 4 SE survival) after two days of exposure ($t_7 = 0.75$, $p > 0.05$).

Nematode vectoring of *P. aeruginosa* to *G. mellonella* larvae

Larvae mortality increased when exposed to juvenile nematodes carrying the RFP-labeled *P. aeruginosa* strain PA14 (92% mortality; $X^2_{1,11} = 53.32$, $p < 0.001$), in comparison to control larvae not exposed to nematodes (36% mortality). Larvae mortality likewise increased when exposed to juvenile nematodes reared on nematode growth agar without bacteria (96% mortality; $X^2_{1,11} = 65.77$, $p < 0.001$), in comparison to control larvae. However, *Galleria mellonella* larvae exposed to nematodes carrying bacteria did not exhibit significantly higher mortality compared to larvae exposed to nematodes without bacteria ($X^2_{1,11} = 1.92$, $p > 0.05$). Microscopic observations did not reveal the presence of RFP-labeled *P. aeruginosa* in *G. mellonella* larvae hemolymph, indicating a lack of bacterial transference from juvenile nematodes to larvae despite larval mortality.

**Microbial community profiling of host-associated bacteria**

Host species was the primary factor affecting bacterial communities

A total of 317 bacterial sequence variants (SVs) were present in field-collected ant samples, that harbored nematodes in the wild, including multiple SVs identified as *Spiroplasma* sp., *Serratia marcescens*, *Entomoplasma* sp., *Enterococcus* sp., *Cutibacterium acnes*, among others (Fig. 4). A total of 274 unique SVs were present in field-collected nematodes which emerged from ant cadavers, including multiple SVs identified as *Spiroplasma* sp., *Serratia marcescens*, *Entomoplasma* sp., *Pandorea* sp., *Flavobacterium* sp., *Cutibacterium acnes*, among others (Fig. 4). Four SVs were shared between ant and nematode samples at an abundance of 1% and a prevalence of 70% (Table 2). Three of these SVs were variants of *Serratia marcescens*. A random forest analysis (77% accuracy) identified 13 SVs with differential abundance between ant and nematode samples (Figure S3), and demonstrated multiple SVs identified as *Spiroplasma* or *Pandorea* that were host-species specific.
Table 2

Core bacterial sequence variants (SVs) between Myrmica rubra ants, Pristionchus entomophagus nematodes, and Galleria mellonella larvae co-cultured with nematodes.

Core was defined at 70% prevalence across samples, with 1% minimum relative abundance for ant:nematodes or nematode:larvae comparisons, or 1% and prevalence of 60% for ant:larvae comparisons.

| Shared between                                      | Core bacteria     | Number of SVs |
|-----------------------------------------------------|-------------------|---------------|
| Ants and nematodes                                  | *Spiroplasma*     | 1             |
|                                                     | *Serratia marcescens* | 3             |
| Nematodes and Nematode-exposed larvae               | *Spiroplasma*     | 1             |
|                                                     | *Serratia marcescens* | 1             |
|                                                     | *Pandoraea*       | 1             |
|                                                     | *Pedobacter*      | 1             |
| Ants and nematode-exposed larvae                    | *Spiroplasma*     | 1             |
|                                                     | *Serratia marcescens* | 1             |
|                                                     | *Bacillus pumilus* | 1             |
|                                                     | *Pseudomonas*     | 2             |
|                                                     | *Delftia acidovorans* | 1             |

Table 3

GPS coordinates of Myrmica rubra colony collection sites.

| Location                      | GPS coordinates | Experiments these samples were used in |
|-------------------------------|-----------------|---------------------------------------|
| Mount Desert Island, Maine    |                 |                                       |
| Woodchip (WC)                 | 68°15’23”W 44°22’37”N | 1                                     |
| Otter Cliff Road (MGH)        | 68°12’01”W 44°19’45”N | 1                                     |
| Visitors Center (VC)          | 68°14’53”W 44°24’37”N | 2                                     |
| Sports Park (SP)              | 68°12’12”W 44°22’52”N | 1,3                                   |
| Eden St. South (COA)          | 68°13’21”W 44°23’41”N | 1,3                                   |
| Old Farm Road (OFR)           | 68°11’42”W 44°22’24”N | 1,3                                   |
| Breakneck Road (BNR)          | 68°15’22”W 44°22’39”N | 2                                     |
| Orono, Maine                  |                 |                                       |
| Orono (OR)                    | 68°40’3”W 44°53’14”N | 2                                     |
Observed SV richness did not vary significantly between ants and nematodes \((F_{1,10} = 0.375, p = 0.554)\), whereas ant samples had higher evenness than nematode samples \((F_{1,10} = 7.81, p = 0.019)\). Neither observed richness or evenness varied significantly between field sites for ant \((F_{2,3} = 0.035, p = 0.966; F_{2,3} = 3.57, p = 0.161, \text{respectively})\) or nematode samples \((F_{2,3} = 0.37, p = 0.963; F_{2,3} = 0.527, p = 0.636, \text{respectively})\).

A total of 563 bacterial SVs were present across all *G. mellonella* samples, and many commonly occurring bacterial SVs were identified as members of the genera *Alcaligenes*, *Cellvibrio*, *Entomoplasma*, *Flavobacterium*, *Pandoraea*, *Pedobacter*, *Pseudomonas*, *Serratia marcescens*, and *Spiroplasma* (Fig. 4). *G. mellonella* larvae, including controls and those exposed to field-collected nematodes, shared 4 SVs at an abundance of 1% and prevalence of 70%, including three *Pseudomonas*, and two *Pedobacter*. There was no consistent pattern differentiating the most abundant taxa found in nematode-exposed versus control samples. Within the nematode-exposed *G. mellonella* larvae, there appears to be little consistency between abundant bacterial taxa by field site.

Across all samples, bacterial communities clustered (Fig. 5a) based on host species (permanova, \(F_{2,20} = 1.68859, R^2 = 0.16129, p < 0.001\)) but not by field site \((p > 0.05)\). For the field collected ants and nematodes, and the nematode-exposed larvae, there was no host x field site interaction, nor was the effect of the host stronger when compared within the field site (Fig. 5b).

**Testing the hypothesis of bacterial transfer by nematodes**

There were two SVs shared across all ants, nematodes, and nematode-exposed *G. mellonella* larvae \((n = 18)\), at an abundance of 1% and prevalence of 70%; *Spiroplasma* sp. and *Serratia marcescens* (Fig. 1). When this was disaggregated by field site of the original ant colonies, hosts at COA \((n = 6)\), as well as at OFR \((n = 6)\), shared only the same *S. marcescens* SV, and hosts at SP \((n = 6)\) shared both those same *Spiroplasma* sp. and *Serratia marcescens* SVs, at an abundance of 1% and prevalence of 99% per site.

Random forest feature selection comparing bacterial communities between ants and nematode-exposed *G. mellonella* larvae identified 21 SVs with differential abundance between the sample groups, with a model accuracy of 75% (Figure S4). Several ant samples were characterized by a high abundance of taxa within the genus *Serratia*. In comparison, most nematode-exposed larvae were characterized by the genera *Pedobacter* and *Pseudomonas*. Six SVs were identified as shared between ant and larvae samples at an abundance of 1% and prevalence of 60% (Table 2). Two of these SVs were variants within the genus *Pseudomonas*.

Random forest feature selection comparing nematode to nematode-exposed *G. mellonella* larvae identified 20 SVs differentiating the sample groups, with an accuracy of 83% (Figure S5). Four SVs were shared between nematode and nematode-exposed larvae at an abundance of 1% and prevalence of 70% (Table 2). Each of these SVs were from different genera.
Nematode-exposed *G. mellonella* larvae were not significantly different from controls (*p* > 0.05), although samples were visually trending towards separate clustering (Fig. 5c). Random forest feature prediction similarly showed little alteration of the collective bacterial state, with a model accuracy of only 66%.

**Testing the hypothesis of bacterial community disruption by nematodes**

Alternative to the idea that nematodes transfer bacteria from the environment to their ant host, we considered whether nematode exposure might disorder or depauperate the endobiotic community of insect hosts. Random forest feature selection identified 9 SVs which were more abundant in *G. mellonella* larvae controls than any sample which was field-collected or field-associated (i.e. nematode-exposed larvae), with a model accuracy of 86% (Fig. 6).

While total bacterial diversity visually appeared to be lower in nematode-exposed larvae compared to controls (Figure S6) this was not significant for bacterial SV observed richness (*W* = 2, *p* = 0.092), evenness (*W* = 7, *p* = 0.714), or Shannon diversity (*W* = 3, *p* = 0.167). However, 16 bacterial SVs were found in lower abundance (*p* < 0.05) in nematode-exposed larvae as compared to controls (Fig. 7), while three were increased; SVs identified as *Serratia, Pseudomonas,* and *Proteus.*

**Discussion**

In this study we tested the ability of the necromenic nematode, *P. entomophagus,* to vector bacteria to ants, and assessed their role in the mortality and subsequent collapse of *M. rubra* invasive ant colonies on Mount Desert Island, in coastal Maine. For this purpose, we isolated bacteria from nematodes that emerged from *M. rubra* cadavers, and microscopically assessed the ability of the nematodes to acquire and transfer environmental bacteria to another insect host during nematode infection. We also identified the bacteria potentially transferred from nematodes to ants from collapsed nests on the island using bacterial community similarity and sequence tracking. If demonstrated that infection by *P. entomophagus* leads to *M. rubra* mortality, this may represent an avenue for the biological control of this invasive ant in coastal Maine. While the use of nematodes as a biological control vector has clear logistical constraints, discussed below, it represents a more targeted, and safer, approach than direct application of a chemical agent, which may be non-specific and fatal to native insects.

**Nematode-carried bacteria and their acquisition from the environment**

In the present study, only two (BNR, OR) of the three isolated nematode cultures showed significant insect mortality. These data suggest that differences may exist between *P. entomophagus* populations from different sites, with one hypothesis being that bacteria associated with *P. entomophagus* vary between sites due to environmentally selected bacterial communities in different locations. Unlike many entomopathogenic nematodes (Steinernematidae and Heterorhabditidae), *Pristionchus* species are not
associated with specific symbiotic bacteria but are capable of ingesting and carrying a diversity of bacteria [22, 25], including species of *Pseudomonas*, *Serratia*, *Enterobacter* and *Bacillus* [20]. It has been demonstrated that *Pristionchus* nematodes sporadically infect insect hosts with pathogenic bacteria acquired from the environment [20, 26, 27].

In the present study, *Paenibacillus* was isolated from the digestive tract of adult nematodes that emerged from ant cadavers (discussed more thoroughly in [24]), and most adult nematodes readily consumed fluorescently-labelled *Pseudomonas aeruginosa* from culture plates and maintained them in their gut environment. Adults that emerged from ant cadavers carried a variety of bacterial species on their cuticles but were unable to acquire and vector *P. aeruginosa*.

Conversely, juvenile nematodes appeared to seldom consume *P. aeruginosa*, and harbor them on their cuticles. In dauer third stage juveniles, the second stage cuticle is retained in the third instar dauer juvenile state [29], thus, RFP-labeled *P. aeruginosa* may have been contained between the two cuticle layers, and potentially remain between the cuticles during sampling. Adult and juvenile *P. entomophagus* rarely acquired RFP-labeled *E. coli* HB 101 from culture media, suggesting this strain is not an ideal bacterial transfer candidate as it is infrequently consumed by nematodes. This may be because of a lack of chemoattraction, or perhaps the bacteria were unable to adhere to the cuticle. Enhancement of virulence in juvenile nematodes, specifically, is necessary for insect virulence, as these nematodes may only infect ants hosts during the nematode’s juvenile stage. Even if juvenile nematodes could frequently carry bacteria on the external cuticle, without their internal harborage, the bacteria may not be able to survive and multiply, making them poor vectors.

The life stages of nematodes, and their respective morphology, may play a role in the ability of environmental bacteria to attach to nematode surfaces, to be retained as the cuticle is shed from one stage to the next, and of insect hosts to void nematode larvae before they establish and mature. For example, Poinar showed that dauer stage *Pristionchus* juveniles caused significantly fewer lethal infections when applied to *G. mellonella* larvae in comparison with samples containing a mix of dauer stage larvae and juvenile stages [22]. Once established within the insect host, *P. uniformis* caused mortality in all subjects, yet many of the inoculated larvae were able to successfully void the nematodes, presumably passing dauer stage larvae through their digestive tracts before they could establish and mature [22]. In addition, the proteins and other secretions of other species of parasitic nematodes are known to be cytotoxic to their hosts [30]. In our study, we found *G. mellonella* mortality was significantly higher when they were exposed to nematodes, regardless of whether those nematodes were carrying the RFP-labeled *P. aeruginosa* strain PA14 and an apparent lack of transfer of bacteria to larvae hemolymph. It is possible that larvae mortality was driven by other nematode-specific factors, or this possibly contributed to the putative bacterial-transferred effects on the host.

While bacteria have been isolated from the space between the two cuticular layers of the dauer stage juveniles, *Pristionchus* dauer stage juveniles do not retain the J2 cuticle as a protective sheath for the free-living stage [31]. Collectively, these morphological differences between nematode species and
lifestages may moderate the transfer of bacteria from nematodes to insects, and the subsequent mortality of the insect host, potentially explaining why *Pristionchus* nematode infections in ant colonies are not necessarily fatal.

Olfaction and chemoattraction profiles of *Pristionchus* spp. are highly diverse, allow nematodes to find and colonize specific insect hosts [32], and to find or avoid particular microorganisms. Even among phylogenetically similar *Pristionchus* species; *P. entomophagus*, *P. uniformis*, *P. pacicus*, *P. maupasi*, there are dramatically different chemoattraction profiles to insects and plants that are also linked to their native habitats [32]. In particular, *P. entomophagus* has been shown to have the strongest attraction to isopentylamine [32], which smells similar to decaying material. Isopentylamine is produced by *P. mirabilis* [33], a common environmental bacterium which ferments maltose. While not previously demonstrated in *M. rubra* ants, it is possible that carriage of *P. mirabilis* bacteria by ants may draw nematode infection, rather than nematode infection of ants being the source of transfer of *P. mirabilis* to ants. In chemotaxis assays, *P. entomophagus* nematodes have been demonstrated to be strongly attracted to *P. vulgaris* bacteria [20]. This may be a critical factor when designing biological control measures for *M. rubra* ants. In the present study, *P. mirabilis* and another *Proteus* sp. were not differentially abundant by host or by site (data not shown).

*P. entomophagus* was significantly more attracted to non-pathogenic specific soil- and beetle-derived bacteria than *E. coli* OP50, an artificial strain used in laboratory culturing of nematodes [20]. Similarly, nematodes can recognize and avoid some entomopathogenic bacteria that negatively impact their fitness, including *Bacillus thuringiensis* and other bacilli, that are nematicidal or reduce fecundity in *Pristionchus* spp. [20, 34–36]. *P. entomophagus* nematodes have been shown to be moderately attracted to *Serratia marcescens*, and variably attracted to *Pseudomonas* strains [20]. In the present study, *Serratia* and *Pseudomonas* were isolated from nematodes that emerged from *M. rubra* ant cadavers, a strain of *Pseudomonas* was acquired from the environment to nematodes, and strains were shared in the bacterial community of field-collected ants and nematodes, as well as *G. mellonella* larvae exposed to these nematodes.

**Capacity to transfer bacteria from nematodes to insects**

Several strains of *Serratia marcescens*, *S. nematodiphila*, and *Pseudomonas protegens* were isolated from the hemolymph of *G. mellonella* exposed to *P. entomophagus* cultures. We did not attempt to isolate bacteria from the larvae which were not exposed to the nematodes. In the present study we were unable to visually determine if nematode transferred *Pseudomonas aeruginosa* contributed to *G. mellonella* larvae mortality (discussed more thoroughly in [37]). The high virulence of strain PA14 in *G. mellonella* and other insects [38] indicates it would have likely contributed to mortality if transfer was successful.

There are many potential reasons for a failure to transfer, including bacterial community competition in the hosts, insufficient conditions for growth or survival, or insufficient bacteria present on the cuticle of juveniles and a low rate of transference. In this study, fluorescent microscopy suggested that *P. aeruginosa* in the digestive tract of nematode juveniles was rare. Thus, successful transfer of bacteria to
the insect larvae relied on the presence of the bacteria on the external cuticle of the nematodes. If labeled bacteria were present between the second and third stage juvenile cuticles rather than externally, its physical constraint may preclude transfer to larvae. Additionally, *P. aeruginosa* may have been washed off or mechanically removed as the nematodes moved through the sand in the experimental arena.

*Serratia marcescens* has previously been detected as part of the bacterial communities of other ant species such as *Camponotus japonica* [39], *Formica cinerea* [40], *Anoplolepis gracilipes* [41], and *Myrmica scabrinodis* [42]. In the present study, we observed *Serratia marcescens* in the hemolymph of nematode-infected *G. mellonella* larvae, and *S. quinivorans* from the external cuticles of nematodes collected from ant cadavers. *Serratia marcescens* has been shown to have insecticidal[43], and nematicidal [21] activity. Furthermore, *S. marcescens* has been used in the biological control of *A. gracilipes* ant colonies in Australia[41]. *Serratia marcescens* has also been shown to cause mortality in *G. mellonella* larvae, but only when transferred via entomopathogenic nematodes and not by the bacteria alone[44]. Sequencing of the bacterial community revealed that *S. marcescens* was among the top 10 SVs present in most ant and nematode bacterial communities, and three variants of *S. marcescens* were detected in the core microbiome shared by ant and nematode samples.

While our results suggest the possibility of transfer of *S. marcescens* to ants via nematode infection, ants may acquire this pathogen from elsewhere in the environment. Bacteria within the genus *Serratia* occur frequently in the environment and at low abundances in healthy insects [45]. In some systems, ant acquisition of *Serratia* is hypothesized to occur through the diet, where ants ingest aphids that have secondary endosymbioses with *Serratia* [39]. Our results showed that ants’ bacterial communities were characterized by a higher abundance of *S. marcescens* than were nematode bacterial communities. (Fig. 1). The low abundance of *S. marcescens* in some nematode samples may indicate that ants acquire the pathogen from diet or the environment, or that the transfer of the pathogen from nematodes to ants is efficient. Infesting nematodes may also provide a means of ingress for bacteria harbored outside the hemocoel, but inside the ant’s body. Ants are known to collect a diversity of microorganisms acquired with feeding and grooming in pellets with the buccal chamber in their heads [46]. These pellets are held for a period of time before being expelled into refuse areas outside the nests [47]. Bacteria recovered within these pellets include *Serratia* and *Pseudomonas* species amongst others [48, 49].

Results from our bacterial culturing experiment identified *Pseudomonas fluorescens* in the hemolymph of infected *G. mellonella* larvae and on the external cuticle of the nematodes. Bacterial community sequencing revealed that *Pseudomonas* SVs occurred in the top 10 genera of both ant and nematode samples, but were in fairly low abundances in all but one ant sample from site OFR. *Pseudomonas* have previously been reported to be pathogenic to insects [50–52], as it produces the secondary metabolite hydrogen cyanide [53], which 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. Infection by *Pseudomonas* species can be fatal to other genera of nematodes [51], but did not impact survival of *Pristionchus* nematodes [20]. Further, *Pristionchus* were attracted to *Pseudomonas* in culture [20]. *Pseudomonas* are commonly found in soil [54], so it is unclear if the occurrence of *Pseudomonas* in ant
and nematode samples is related to intra-specific transfer of the bacteria, or due to acquisition of the bacteria from the environment.

Other patterns in bacterial community structure in the field-collected samples include the common occurrence of *Spiroplasma* in nematode samples. *Spiroplasma* are often found in association with insect hosts [55], and can be a maternally-transferred symbiont of *M. rubra* [56]. A specific strain of *Spiroplasma poulsonii* has been shown to confer resistance of *Drosophila neotestacea* to infection by the parasitic nematode *Howardula aoronymphium* [57]. In this study, nematodes had higher occurrences of *Spiroplasma* than ants had, but the core microbial community of ants and nematodes did include a *Spiroplasma* SV. Given the known relationship between *Spiroplasma* and *M. rubra*, and the defensive role that *Spiroplasma* can take against nematodes, this study’s finding of *Spiroplasma* in higher abundance in nematodes than ants is unexpected. However, the presence of *Spiroplasma* in the bacterial communities of both ant and nematode samples suggests the potential transfer of this taxa from ants to nematodes.

*Delftia sp.*, which was cultured in the present study from the cuticle of nematodes and the hemolymph of nematode-exposed *G. mellonella* larvae, are common soil-dwelling bacteria that have not been demonstrated to cause insect mortality. *Delftia spp.* has been identified previously in the hemolymph of insects [58]. It is possible that *Delftia sp.* may be ingested by the insect host, where they are fed upon by nematodes, and stick to the cuticle of the nematode as it exits the host.

**Suitability of Pristionchus entomophagus nematodes as vectors**

Our data suggest *Pristionchus entomophagus* nematodes are potential candidates, due to their ability to acquire and transfer bacteria, and their association with ants. *Pristionchus* nematodes are necromenic and proliferate inside insect cadavers, where they survive the low oxygen conditions, high toxicity of host hemolymph, and the presence of potentially toxic soil bacteria (e.g. *Bacillus* spp.). This is achieved partially through their capacity to form a dauer state, which is automatically triggered during periods of environmental disturbance, which supports the theory that these nematodes are on the evolutionary path to parasitism [59]. *Pristionchus* nematodes demonstrated differential chemoattraction to bacteria based on host association, and have been shown to be able to suppress spore germination in their intestine, as well as using pathogenic spore forming (e.g. *Bacillus*) and non-spore forming pathogenic bacteria (e.g. *Pseudomonas*) as a food source [36]. *Pristionchus* nematodes are rarely directly exposed to insecticidal crystal proteins and functionally can be resistant to them. Thus, *Pristionchus* are viable candidates to transfer insecticidal bacteria to insect hosts and act as biological control agents. However, there are several considerations and limitations to their use.

Dissemination of bacteria from nematode to host likely occurs through ingestion and defecation of intact bacteria [60] and potentially through transfer of bacteria adhered to the cuticle or the hemolymph [24]. The lack of bacterial transfer to the insect host in this study may indicate the importance of internal harborage, as bacteria adhered to the external cuticle may be a more serendipitous and/or transient association, or an insufficient population for infection. The absence of RFP-labeled *P. aeruginosa*, an
experimental proxy for environmental bacteria, in the digestive tract of juveniles and high prevalence in
the digestive tract of adults suggests different nutritional preferences, but there is currently no
information on this. This provides a potential explanation for the decrease in juveniles with external
fluorescence over time, as fewer environmentally located bacteria would be present to adhere to the
cuticle as adults consume them.

The putative lack of consumption of *P. aeruginosa* by juveniles poses a challenge to using *P.
entomophagus* for biological control vectors, as the juvenile stage is needed for host infection to occur.
Moreover, a number of mechanistic questions remain to be answered, including the dynamics and
repeatability of bacterial acquisition from the environment, repeatability and dosage of successful
vectoring to ants, and whether suitable pathogens to ants could be identified which would not introduce
contaminating bacteria into the environment.

**Conclusions**

The work presented demonstrates the complexity of the interactions between nematodes, insects and
bacteria. The experiments and field observations reported here suggest that the transfer of bacteria from
*Pristionchus entomophagus* nematodes to ants is feasible and was possibly a contributor to ant death
and colony collapse in wild colonies in Maine. While a single, clear, common causative agent of colony
mortality did not emerge, *Serratia marcescens* and *Spiroplasma* SVs were present in ants, nematodes and
waxworm larvae, and *Serratia* were in increased abundance in waxworms infected by nematodes, relative
to controls. The use of an engineered biological control against invasive ant species, such as nematodes
carrying specifically-seeded bacterial species, is highly desirable, especially if the pathogenic bacteria is
normally found in soil ecosystems and represents a low risk for biosafety control.

**Methods**

*Myrmica rubra* colonies were collected at multiple sites in Acadia National Park Mount Desert Island,
Maine (Table 3). Nests were located under natural (logs, rocks, etc.) and human (boards, plant pots, etc.)
debris and exposed colonies were collected with an aspirator and transferred into plastic nest boxes (11
cm x 26 cm x 9 cm) or 19 L buckets with their nest soil. Colonies collected in buckets were stored at 4°C
until used for experiments, when they would be transferred into nest boxes and held at 21°C. Each nest
box contained a small portion of a cardboard egg carton covering a 3 x 3 x 3 cm³ piece of moistened
sponge. The sponge was remoistened, and ants were fed ca. 2 g of sugar and tuna diet mix every 2 - 4
days.

*Collection of nematodes:* Ant colonies were inspected for dead individuals every 2-3 days. Dead worker
ants were removed using sterile tweezers and surface sterilized by submerging in a 0.1% zephiran
chloride solution for 30 sec [61], followed by two rinses in sterile dH₂O, and drying 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 [62]. Cadavers were monitored for nematode emergence every other day. As nematodes emerged, they were pipetted from the wells and transferred to 50 mL centrifuge tubes with dH$_2$O held at 10° C.

New *M. rubra* colonies were collected from field sites for each set of experiments to assure that the bacterial communities associated with the nematodes did not drift with repeated culturing. Nematode infections levels and successful recovery from different sites varied between collections. Nematodes recovered from colonies and locations that resulted in moderate to high ant mortality and high emergence of nematodes were chosen for each set of experiments.

**G. mellonella infections and hemolymph collection:** Last instar *Galleria mellonella* (Lepidoptera: Pyraidae) larvae were purchased from Petco (Bangor, ME) or Grubco Inc. (Fairfield, OH), and held at 5-10°C until used for nematode infections and/or bleeding experiments. For infection, larvae were placed in 100 mL cups or 100 mm diameter Petri dishes with 20 g sterilized sand moistened with sterile dH$_2$O. Nematodes in dH$_2$O solution were pipetted directly onto the sand and/or the dorsal cuticle of the larvae, which were incubated without food at ambient temperature (approximately 21°C) and monitored daily for signs of infection and death. White traps [63,64] were used to harvest nematodes from dead larvae.

The hemolymph of larvae, infected and non-infected, was sampled for the presence of bacteria by bleeding caterpillars under aseptic conditions in a laminar flow hood. Caterpillars were removed from rearing/ infection cups, rinsed with dH$_2$O, and surface sterilized with 0.1% zephrin chloride as described previously [61] or with 70% ethanol and 1% sodium hypochlorite (NaClO) as described previously [63]. After surface sterilization, a single proleg was cut with sterile, micro-dissecting scissors, and a microcapillary tube was used to draw the hemolymph that welled up from the wound. Slight pressure was applied to the body with soft forceps to exude as much sample as possible. Hemolymph samples were stored briefly at 4°C until bleeding of all specimens were completed, with plating or DNA extractions immediately following this process.

**Isolation and identification of associated bacteria from nematodes emerged from *M. rubra* ant cadavers**

Additional detail for experiment 1 can be found elsewhere [24]. The conceptual schematic for this work is visualized in Figure S7.

**Preparation of ant colonies**

Multiple *M. rubra* colonies collected from different sites (Table 3) in September 2010, were maintained at 4°C for approximately 2 weeks prior to being transferred to nest boxes for use in bacterial culturing experiments. Colonies were maintained and monitored for dead ants and emergence of nematodes from ant cadavers. Colonies from two sites experiencing high levels of mortality and emergence of many
nematodes were selected for use in this study, and nematodes were harvested from their corresponding cadaver plates.

Collection of externally located bacteria from nematodes

Approximately 50 μL of sterile distilled water was added to wells containing *M. rubra* cadavers and emerged nematodes. Nematodes from each well were pipetted into a 1.5 mL microcentrifuge tube filled with 1 mL of 1% Tween. Tubes were vortexed gently to mix and centrifuged for 10 sec at 13,000 RPM to concentrate the nematodes in the bottom of the tube. This stock solution was serial diluted four times, using a 1:10 ratio of rinse solution to distilled water. Three 300 μL aliquots of each 1×10⁻⁴ dilution were plated onto Trypticase Soy Agar (TSA). Plates were incubated at 29°C for 48 hr, after which, colonies were observed at 1-100X under a dissecting microscope and unique morphotypes were identified based on colony size, color, shape, and surface characteristics (Table S1; Figure S1a). Individual colony forming units of all unique morphotypes within a sample were transferred to fresh TSA plates. After two days of growth, monoculture plates were stored at 4°C.

Collection of digestive tract bacteria from nematodes (internal)

A total of 50 μL of sterile distilled water was added to selected wells in the 48-well plates housing the *M. rubra* cadavers and emerged nematodes. 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, a 50 μL aliquot of nematode suspension 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 suspension were 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 suspension. 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. Nematodes feeding and tunneling on the agar gave rise to colonies of bacteria excreted from the nematode digestive tract (Figure S1b). Plates were incubated at 29°C for 48 hr, after which trails of feeding nematodes and bacterial colonies were observable. Morphotypes were
identified and individual colony forming units of unique morphotypes were transferred, grown and stored as above.

Collection of bacteria in infected waxworm hemolymph

A total of 60 last instar *G. mellonella* larvae were inoculated with nematodes from the well plates of the two selected ant colonies/sites. Nematodes were harvested from six individual ants from each of the two colonies and transferred to individual infection dishes containing five larvae. Inoculated larvae were monitored daily and the dead were collected and surface sterilized in 0.1% zephiran chloride solution. 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 caterpillars could be bled for hemolymph *en masse*. Hemolymph collected from each set of five larvae was pooled for one sample and placed into a 1.5 mL microcentrifuge tube filled with 1mL of sterile dH$_2$O. Four-fold serial dilutions were made and quadrant streaks of each $1 \times 10^{-4}$ dilution were plated onto two Trypticase Soy Agar (TSA) plates for each of the 12 samples. Plates were incubated at 29°C for 48 hr, after which morphotypes were identified and individual colony forming units of unique morphotypes were transferred, grown and stored as above.

DNA extraction and sequencing of bacterial isolates

Of the 45 bacterial isolates cultured (Table S1), 32 were selected for further evaluation via 16S rRNA sequencing. Due to the morphological similarities of isolates derived from samples from the same location and culture source (internal, external, hemolymph), samples originally derived from one well from each location were selected as representative samples of the external bacterial associates for sequencing. Fewer isolates were obtained from the internal and hemolymph samples and all were prepared for sequencing (Table S2).

All isolates were grown for 14-18 hr in 3 mL LB Broth, with a salt concentration of 5 g/L (Formedium), in 14 mL test tubes (VWR, USA) at 29°C and 90 rotations per minute (RPM). Broth cultures were centrifuged to pellet cells. Bacterial DNA was extracted using the Promega Wizard® Genomic DNA Purification Kit Cat no. A1120 (Promega, USA). DNA extract was stored at -20°C. Gel electrophoresis was used to assess presence of DNA extract using 0.8% Agarose gels made using 30 mL of TAE Buffer (tris base, acetic acid, and EDTA), 3 μL GelStar® GelStain (Lonza, 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 1 hr. Gels were visualized on an ultraviolet (UV) transilluminator and recorded with a remote shooting camera.

Polymerase chain reaction (PCR) amplification of bacterial 16s rRNA was conducted using the primers 27F (5’-AGAGTTTGATCCTGGCTCAG-3’) and 1492R (5’-ACGCGGTGTGTTAC-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 H₂O, 4 μL 5x PCR Buffer, 1.2 μL 25 mM MgCl₂, 2 μL 2 mM dNTPs, 1 μL 10 μM 27f, 1 μL 10 μM 1492r, 0.2 μL GoTaq DNA Polymerase (Promega), and 1 μL of bacterial DNA.

PCR amplification was verified using 1.2% Agarose gels (30 μL of TAE Buffer, 3 μL GelStar® GelStain (Lonza, USA), and 0.36 g agarose). Invitrogen Low DNA Mass Ladder Cat. # 10068-013 (Invitrogen, USA) was used as a standard to detect the expected fragment length of 1465 bp; 5 μL of low mass ladder and 1 μL of 6x loading dye. 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. PCR products were purified using a QiaQuick PCR Purification Kit (Qiagen, Venlo, Netherlands) following the Qiagen QiaQuick protocol, and stored at -20°C.

Purified PCR product quality was determined on a Nanodrop 1000 with version 3.3 software (ThermoScientific, USA). DNA concentrations were adjusted to 10 ng/μL using sterile water, as needed. Sanger sequencing was performed by the University of Maine Sequencing Facility (Orono, Maine, USA), using primers 27f and 1492r primers and ABI model 3730 DNA Sequencer with the XL Upgrade.

Quality-edited bacterial 16S rRNA gene sequences were obtained from the University of Maine Sequencing Facility and identified using the Basic Local Alignment Search Tool (BLAST) online database provided by the National Center for Biological Information (NCBI, accessed 2011). Proofed sequences can be accessed on NCBI (Bioproject PRJNA646935, GenBank accessions MT797825 - MT797845). A phylogenetic tree was generated by aligning isolates and their closest matches in MEGA ver X [65] using the MUSCLE algorithm, and calculating branch lengths with Maximum Likelihood algorithm. Tree was visualized in the Interactive Tree of Life [66].

**Transfer potential of pathogenic bacteria from environment to nematodes to other insect hosts**

Additional detail for all methods used in experiment 2 can be found elsewhere [37].

**Harvest and storage of *Pristionchus entomophagus* nematodes**

In September 2012, approximately 200 specimens were collected from each of 16 *Myrmica rubra* colonies located in eight sites in Acadia National Park and Orono, ME (Table 3) where *Pristionchus entomophagus* infection has previously been confirmed. Colonies were maintained and nematodes were collected from all colonies.

**Virulence of *P. entomophagus* nematode populations**
Galleria mellonella larvae were exposed to nematodes collected from ant cadavers from different colonies and monitored for mortality to assay nematode virulence. For each nematode population, larvae were exposed to low (20 - 25) and high (200 - 250) numbers of nematodes and compared to a control group exposed to no nematodes. Per site and dose assay, four replicate plates with 5 larvae per plate were exposed. The larvae were inoculated and maintained in 100 mm diameter Petri dishes, and monitored daily for mortality for 14 days. Survival analysis was conducted using a general parametric model based on the Weibull distribution to examine difference in time to death between sites and nematode treatments (JMP, SAS Institute Inc. 2012). Dead larvae were removed and placed into white traps [63], and nematodes were harvested once per week over 10 weeks by pipetting from the white traps into 50 mL centrifuge tubes. These nematode stocks were stored at 10°C. To replace old and dying nematode stocks 12 weeks after initial harvesting, nematode populations from the BNR site were exposed to G. mellonella as described above and collected via white traps (Figure S8).

Assessing uptake of RFP-labelled bacteria by P. entomophagus nematodes

To assess the ability of P. entomophagus to ingest and carry bacteria from their environment (Figure S2), nematodes from the BNR were used in the following experiments in which the nematodes were transferred to and held on culture media plates containing various bacteria treatments. For the first experiment, bacteria treatments included: 1) Escherichia coli HB101 (p6TT1) bacteria expressing a red fluorescent protein [67], and which have not been documented to be pathogenic towards nematodes; 2) bacterial-control plates containing non-labelled Paenibacillus sp. previously isolated from P. entomophagus; and 3) no-bacterial control plates without any additional bacteria added. For the second experiment bacteria treatments included: 1) the bacterial-control, non-labelled Paenibacillus plates; 2) no-bacterial control plates; and 3) plates containing a red fluorescent protein (p66TT1 plasmid d-tomato) labeled Pseudomonas aeruginosa (strain PA14) shown to be highly virulent in Caenorhabditis elegans worms and G. mellonella larvae with an LD$_{50}$ of fewer than 10 bacteria [38], but not documented to be virulent in P. entomophagus nematodes.

To create treatment plates, 2.5 mL aliquots of nematode growth media (NGM; Carolina Biological, US) were poured into 45 mm diameter Petri plates under sterile conditions, streaked with bacteria, and incubated at 37°C for 48 - 72 hr to allow for bacterial growth. Four replicates were produced per treatment for a total of 12 plates. Approximately 200 - 400 nematodes in 140 - 150 μL of dH$_2$O were pipetted onto each plate, and cultured at 20°C for 48 hr to allow nematode grazing, after which nematodes were sampled for mortality and the evidence of acquiring the fluorescent protein.

To determine mortality, total dead (indicated by straight, stiff or disintegrating nematodes) and living were counted in the entire plate (E. coli) or a subsample of ca. 50% of the plate sampling variable fields of view at 100X magnification under a dissecting microscope. To determine prevalence of uptake of environmental bacteria, 10 - 12 live juveniles and 10 - 12 live adults per plate with labeled E. coli or P.
Microbial community profiling of bacteria associated with ants, nematodes, and infected G. mellonella larvae to determine in situ bacterial transfer between hosts
The design for this experiment is visualized in Figure S10. Three invasive ant colonies were collected from different sites on Mount Desert Island, Maine, in September 2015, held in rearing boxes in the laboratory. Ant colonies were maintained with regular watering and food, and dead ants were removed every 2-3 days and set up for emergence of nematodes as described above. Any adult nematodes that emerged were collected from wells and transferred to Petri dishes with Pasteur pipettes, reared in Baby Food (BF) agar according to procedures described previously [68], and identified via molecular and morphological assessment [63,69].

Last instar *G. mellonella* larvae were inoculated with nematode cultures originating from each of the three ant colonies. Twenty-mL aliquots of nematode solution (7 nematodes/mL dH$_2$O) were applied directly onto the dorsal surface of each of five larvae per inoculation dish with 5 replicate dishes per nematode culture and 5 dishes of untreated larvae. Larvae were monitored daily and two dishes per nematode culture plus two dishes with untreated larvae were selected for hemolymph sampling 3 days post exposure when treated larvae showed signs of septicemia, but had not yet died (bloated, discolored, with only minor movement when prodded). Larvae were surface-sterilized and hemolymph was collected and pooled from the five larvae per dish to yield 75-100 mL samples for DNA extraction.

Whole ants and nematodes were sampled from their original colonies and cultures for bacterial community analysis. Two samples of 5 ants were randomly collected from each nest box, transferred to sterile 1.5 mL snap tubes, and frozen for 15 min at -80°C prior to transfer to extraction tubes. Nematodes were collected by pipetting and manual transfer with a bent probe until two tubes with concentrations of 100 mixed aged nematodes in 500 mL dH$_2$O were collected for each of the three nematode cultures. Neither ants nor nematode samples were surface sterilized.

**DNA extraction and sequencing of bacterial communities**

DNA was extracted from the whole ants, and whole nematodes samples, as well as from four individual whole *G. mellonella* larvae using the MoBio Soil Extraction kit (MoBio Laboratories, Inc., US) per the manufacturer’s protocol. DNA was extracted from hemolymph samples using QIAmp DNA Micro Kit for small sample volumes following the manufacturer’s protocols for Isolation of Genomic DNA from Small Volumes of Blood. A total of six nematode samples (2 per field site), six ant samples (2 per field site), and 13 *G. mellonella* larvae samples were selected for sequencing. Of the larvae samples, two were single whole larvae, two were single surface-sterilized larvae, and the remainder were hemolymph samples collected as described above from both nematode inoculated (infected, n = 6) and control (not inoculated, n = 2). Amplification of the 16S rRNA gene was done using eubacterial primers 27F (5’-AAGGTTTATCGATGACGCTAG-3’) [70] and 519R (5’-GTNTTACNGCGGCGGCGTCG-3’) [71], and a 30-cycle PCR protocol per Molecular DNA Lab (MR DNA, Stillwater, TX). PCR products were pooled in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, US). Resulting library preparations were sequenced using the Illumina platform following the manufacturer’s
protocols (MR DNA). Sequence data are publicly-available under BioProject Accession PRJNA646935, from the NCBI Sequence Read archive (SRA).

**Sequence data processing**

Raw data were denoised, barcodes were removed, and forward and reverse reads merged into contiguous sequences (i.e. 'contigs') by MR DNA. Resulting FASTQ files were processed in RStudio using R version 3.6.3 “Holding the Windsock” [72], following the DADA2 pipeline [73]. Sequence quality was measured and plotted using the ShortRead package [74] and base R plotting. The first 15 bases and last 50 bases of reads were removed due to low sequence quality (sequence quality scores < 20). Error rates for each sequencing run were calculated, sequence variants identified, and chimeric reads were removed using DADA2. Taxonomy was assigned using the Silva ver. 138 [75] taxonomic training database for DADA2, and any sequences which matched as eukaryotic mitochondria were removed using the dplyr package [76]. Sequencing runs, along with taxonomy and metadata, were merged into one object using phyloseq the package [77]. This phyloseq object was then subset into ant, nematode, and *G. mellonella* larvae groups, as needed for analytical comparisons. All subset groups were pruned to remove null samples or taxa (phyloseq) and rarefied to 5,000 SVs per sample for data analysis that included larvae only, or 10,000 SVs per sample for data analysis that included ants and nematodes.

Initial exploration of the data showed that two whole larvae samples that were prepared using surface sterilization prior to microbial DNA extraction had bacterial profiles that were distinct from the remainder of the larvae samples. These two samples were removed from the dataset along with a positive control sample that had been spiked with *Bacillus* bacteria.

Observed SV richness and evenness were calculated for all ant, nematode, and *G. mellonella* larvae samples in the package phyloseq. A Shapiro-Wilkes test was used to test if diversity data were normally distributed. Differences in observed richness and evenness between sample groups were tested using an ANOVA for normally distributed data, and a Wilcoxon test for those data that were not normally distributed. To determine differential abundance of bacteria SVs across sample groups, random forest classification algorithms were run using the rfpermute package [78]. Principal Coordinate Analysis (PCoA) was performed using Jaccard’s distance to explore similarity of samples based on host species identity and host infection status. PCoA was visualized for all samples, then separately for *G. mellonella* larvae samples. The taxa belonging to the core microbial community between ant and nematode samples at an abundance of 1% and a prevalence of 70%-90%, noted in the respective results, were identified using the microbiome package [79].

**List Of Abbreviations**

BLAST, Basic Local Alignment Search Tool

BNR, Breakneck Road sample site
Declarations

Ethics approval and Consent to participate

Not applicable.

Consent for publication

All authors have approved the final version of this manuscript and consent to publication.

Availability of data and material

Sequence data are publicly available under BioProject Accession PRJNA646935, from the NCBI Sequence Read archive (SRA).

Competing interests

No conflicts of interest to declare.

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Authors' contributions

SPS and EG conceived of the original project and performed the molecular and morphological characterization of the nematodes, and provided guidance for all subsequent work on this project. EG oversaw and directed sample collection and processing, isolations and maintenance of cultures, and infections and bioassays with students and technical staff in her laboratory. JM and EG oversaw infection and DNA extraction for community analysis. JD performed the culturing, bacterial isolation, and culture identification. AM performed virulence bioassays and visualization of labeled bacteria on and in nematodes and assessed transfer during infection, and SLI taught and oversaw DNA data analysis and contributed to writing and reviewing the manuscript. SS and AH performed data analysis and contributed to writing the manuscript. All authors reviewed and approved the manuscript.

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Authors’ Information

Not applicable.

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