An entomopathogenic Caenorhabditis briggsae

Eyualen Abebe1,*, Miriam Jumba2, Kaitlin Bonner3, Vince Gray2, Krystalynne Morris3 and W. Kelley Thomas3

1Department of Biology, Elizabeth City State University, 1704 Weeksville Road, Jenkins Science Center 421, Elizabeth City, NC 27909, USA, 2School of Molecular and Cell Biology, University of the Witwatersrand, Republic of South Africa and 3Hubbard Center for Genome Studies, University of New Hampshire, 35 Colovos Road, Durham, NH 03824, USA

*Author for correspondence (ebabebe@mail.ecsu.edu)

Accepted 27 May 2010

SUMMARY

Caenorhabditis elegans is a premier model organism upon which considerable knowledge of basic cell and developmental biology has been built. Yet, as is true for many traditional model systems, we have limited knowledge of the ecological context in which these systems evolved, severely limiting our understanding of gene function. A better grasp of the ecology of model systems would help us immensely in understanding the functionality of genes and evolution of genomes in an environmental context. Consequently, there are ongoing efforts to uncover natural populations of this model system globally. Here, we describe the discovery of a Caenorhabditis briggsae strain and its bacterial associate (Serratia sp.) that form an entomopathogenic complex in the wild. Laboratory experiments confirm that this nematode and its natural bacterial associate can penetrate, kill and reproduce in an insect host and that the bacterial associate can induce this insect pathogenic life cycle in other Caenorhabditis species, including C. elegans. Our findings suggest that this life history may be widespread in nature and critical to the understanding of the biology of this important model organism. Caenorhabditis–insect interaction could be a key factor in our quest for a better grasp of gene functionality in this important model species. The discovered association, consequently, would provide an ecological framework for functional genomics of Caenorhabditis.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/213/18/3223/DC1

Key words: insect parasite, nematode, symbiosis, nematode–bacterial association, African Caenorhabditis briggsae.

INTRODUCTION

Linking the complete genome sequences of an organism to its development and behaviour remains a major goal in biology (Kamath et al., 2003). Progress towards that goal has traditionally relied on model organisms such as the nematode Caenorhabditis elegans upon which considerable knowledge of cell and developmental biology has been built (Wood, 1988) (Wormbook, http://www.wormbook.org). Interest in the development of alternative models for understanding host–pathogen interactions has resulted in the use of Caenorhabditis and a variety of microorganisms (Couillault and Ewbank, 2002; Schulenburg and Ewbank, 2004; Pradel et al., 2007; Kim, 2008). Unfortunately, C. elegans, like most model organisms, was chosen for its laboratory prowess (e.g. short generation time, tractable development), and not because we had a detailed understanding of its ecology (Wood, 1988; Feder and Mitchell-Olds, 2003; Kinotke and Sudhaus, 2006). Consequently, as our catalogue of genes and the tools to explore function for organisms like C. elegans has become extraordinarily rich, the absence of an ecological context has become a major limitation to understanding biological function (Caswell-Chen et al., 2005).

A concerted effort is now underway to obtain a greater understanding of C. elegans outside of the laboratory including the first studies focusing on genetic diversity in natural populations of C. elegans and related species (Barrière and Félix, 2005; Fitch, 2005; Sivasundar and Hey, 2005; Cutter et al., 2006; Haag et al., 2007). Rather than being a truly free-living soil nematode as it is often portrayed, Caenorhabditis is thought to be associated with other animals, most often invertebrates (Caswell-Chen et al., 2005), in either a phoretic relationship travelling on the host between food sources, or a necromenic relationship where the nematode waits for the demise of its ‘host’ and feeds on the resulting decay (Kinotke and Sudhaus, 2006). In both cases, it is the dauer or resistant larval stage that is associated with the host. Phoretic and necromenic associations are two of many known associations between nematodes and other animals. Hitherto, none of the known Caenorhabditis are reported to be entomopathogenic.

Among the most interesting associations within the phylum Nematoda are those of the entomopathogenic nematodes (EPNs). EPNs represent the collaboration between a nematode and bacterium where, as in necromenia, the nematode feeds on bacteria using the insect as a carbon source (Burnell and Stock, 2000; Rae et al., 2008); however, in a bacterial/EPN complex, the bacteria actively kills the insect after gaining access with the aid of the nematode partner. Among species of the genus Caenorhabditis, reports show that C. briggsae is necromenic, C. japonica is a facultative necromenic, and C. elegans and C. remanei are primarily phoretic with some questionable necromenic associations (Kinotke and Sudhaus, 2006). Sudhaus (Sudhaus, 1993) suggested the two well-known EPN genera, i.e. Heterorhabditis and Steinernema, most probably evolved from necromenic nematodes through the association with an entomopathogenic bacterium.

Entomopathogenesis appears to have evolved at least twice in nematodes (Poinar, 1993; Blaxter et al., 1998): once in the lineage giving rise to the Heterorhabditis and its bacterial associate Photorhabdus and once in the lineage leading to the Steinernematidae.
(Steinernema) and its bacterial associate Xenorhabdus. These EPNs have broad host range, and are used as agents of biological control and to study the evolution of the mutualistic relationships between nematodes and bacteria (Adams et al., 2006). The collection of EPNs from nature is traditionally by the use of Galleria traps, which are wax moth larvae (caterpillars) buried in the soil (Bedding and Akhurst, 1975). The Galleria cadavers are collected and nematodes (virtually always Steinernematidae or Heterorhabditidae) are collected. The EPN families have global distributions with apparent co-evolution of nematode and bacterium (Adams et al., 2007). These nematodes can routinely be cultured on agar plates with bacteria as a food source but in nature are considered to always be involved in entomopathogenic life cycles. In Galleria trap experiments conducted globally, only rarely are other nematodes reported (Rueda et al., 1993; Ye et al., 2010). Recently, Galleria trap experiments and direct isolation methods have, however, revealed nematodes other than members of the Steinernematidae and Heterorhabditidae (Young-Keun et al., 2007; Zhang et al., 2008). Moreover, Zhang and colleagues (Zhang et al., 2009) reported a new species of Serratia that they found symbiotically associated with a new entomopathogenic nematode genus discovered earlier (Zhang et al., 2008).

Here we report our findings, an entomopathogenic Caenorhabditis and its bacterial associate, from a recent Galleria trapping experiment that included several independent Galleria traps in soils from three provinces (North West, Mpumalanga and Kwa Zulu-Natal) in South Africa.

**MATERIALS AND METHODS**

**Isolation of nematodes and their bacterial associate**

Soil samples for the study were collected from three sites at farms in three provinces of South Africa; North West, Mpumalanga and Kwa Zulu-Natal. Entomopathogenic nematodes were isolated in the laboratory from the three soil samples using late instar larval Galleria mellonella L. (Bedding and Akhurst, 1975). Initial isolates were maintained on LB agar plates without any addition of an external bacterial food source. After the initial isolation of the bacterium associated with our nematode strain, nematode cultures were maintained using three different methods. One set of cultures was maintained by the infection cycle using G. mellonella to reflect possible natural conditions. The other two cultures were reared under normal C. elegans laboratory conditions using nematode growth media (NGM) agar with one set seeded with E. coli OP50 and the other set seeded with the isolated associated bacterium, Serratia sp. SCBI (South African Caenorhabditis briggsae isolate). The culture maintained on E. coli OP50 was initially bleached twice following a C. elegans culture contaminant cleaning protocol (Stiernagle, 2006) to eliminate its bacterial associate – Serratia sp. SCBI. Frozen stock cultures of nematode and associated bacterium in glycerol stored at –80°C were established and are maintained at the Hubbard Center for Genome Studies at the University of New Hampshire.

**Establishing nematode identity**

We studied nematodes morphologically at 1000× magnification using an Olympus IX81 inverted compound microscope with a differential interference contrast option. We also studied nematodes using an Amray 3300FE field emission scanning electron microscope (SEM) with PGT Immix-PC microanalysis system to evaluate whether bacteria were attached to the nematode cuticle surface externally.

Partial DNA sequences of the small and large subunit ribosomal genes, internal transcribed spacer and mitochondrial genes cytochrome oxidase II (COII) and NADH dehydrogenase subunit 5 (ND5) were amplified and sequenced from the nematode strain using the primers in supplementary material Table S1. Once the species level identity of the strain was established as C. briggsae (Dougherty and Nigon 1949), we conducted further comparisons between our strain, i.e. C. briggsae KT0001, and 10 other wild-type isolates of C. briggsae obtained from the Caenorhabditis Genetics Center (Table 1) using six microsatellite loci amplified with primers listed in supplementary material Table S1.

**Microsatellite analysis**

Microsatellite loci were analysed to further investigate the relationship among the wild-type isolate strains of C. briggsae and the putative entomopathogenic C. briggsae KT0001. The published genome sequence of the C. briggsae strain AF16 was used for microsatellite development. The perl script copyByMsLoc.pl was used to search and isolate all microsatellites found in the published

<table>
<thead>
<tr>
<th>Strain</th>
<th>Species</th>
<th>Locality</th>
<th>Collected by</th>
<th>COII</th>
<th>ND5</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH1300</td>
<td>C. briggsae</td>
<td>Unknown</td>
<td>B. Zuckerman</td>
<td>n.a.</td>
<td>EU254725</td>
</tr>
<tr>
<td>DR1690</td>
<td>C. briggsae</td>
<td>Unknown</td>
<td>B. Zuckerman</td>
<td>EU254738</td>
<td>EU254726</td>
</tr>
<tr>
<td>AF16</td>
<td>C. briggsae</td>
<td>Ahmedabad, India</td>
<td>A. Fodor</td>
<td>EU254735</td>
<td>EU254724</td>
</tr>
<tr>
<td>HK104</td>
<td>C. briggsae</td>
<td>Okayama, Japan</td>
<td>H. Kagawa</td>
<td>EU254726</td>
<td>n.a.</td>
</tr>
<tr>
<td>HK105</td>
<td>C. briggsae</td>
<td>Senda, Japan</td>
<td>H. Kagawa</td>
<td>EU254743</td>
<td>EU254727</td>
</tr>
<tr>
<td>JU725</td>
<td>C. briggsae</td>
<td>Yangshuo, Guangxi, China</td>
<td>M. A. Felix</td>
<td>EU254739</td>
<td>EU254728</td>
</tr>
<tr>
<td>JU726</td>
<td>C. briggsae</td>
<td>Sanjiang, Guangxi, China</td>
<td>M. A. Felix</td>
<td>EU254742</td>
<td>EU254729</td>
</tr>
<tr>
<td>PB800</td>
<td>C. briggsae</td>
<td>Dayton, OH, USA</td>
<td>S. Baird</td>
<td>EU254737</td>
<td>EU254730</td>
</tr>
<tr>
<td>PB826</td>
<td>C. briggsae</td>
<td>Hueston Woods State Park, OH, USA</td>
<td>R. M. Hampton and S. Baird</td>
<td>EU254740</td>
<td>EU254831</td>
</tr>
<tr>
<td>VT847</td>
<td>C. briggsae</td>
<td>Haena, Kaauli, HI, USA</td>
<td>V. Ambros</td>
<td>EU254741</td>
<td>EU254733</td>
</tr>
<tr>
<td>KT0001</td>
<td>C. briggsae</td>
<td>Northwest Mpumalanga province, South Africa</td>
<td>M.J.</td>
<td>EU254734</td>
<td>EU254732</td>
</tr>
<tr>
<td>N2</td>
<td>C. elegans</td>
<td>Bristol, UK</td>
<td>L. N. Staniland</td>
<td>n.a.</td>
<td></td>
</tr>
<tr>
<td>PB4641</td>
<td>C. remanei</td>
<td>Inbred derivative of EM464</td>
<td>S. Baird</td>
<td>n.a.</td>
<td></td>
</tr>
<tr>
<td>JU727</td>
<td>C. briggsae</td>
<td>Sanjiang, Guangxi, China</td>
<td>M. A. Felix</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CB5161</td>
<td>C. brenneri</td>
<td>Trinidad</td>
<td>A. Fodor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EM464</td>
<td>C. remanei</td>
<td>Brooklyn, NY, USA</td>
<td>S. Baird</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All strains other than KT0001 were provided by the Caenorhabditis Genetics Center. GenBank Accession numbers are given for mitochondrial genes COII and ND5 sequences for C. briggsae isolates.

n.a., not applicable.
strain of *C. briggsae* AF16 (W. Sung, personal communication). Information on dimer and trimer repeats was extracted from the genome, including the sequence (±1000 bp) surrounding the microsatellites. Six microsatellite loci and the surrounding sequence, including three dimers and three trimers, ranging in size from 57 to 60 repeats for dimers and 39 to 70 repeats for trimers in the reference genome were chosen (supplementary material Table S2). Primers were developed for each microsatellite using the open-source web program, Primer3 (Rozen and Skaletsky, 2000). Primers and corresponding PCR conditions are listed in supplementary material Table S2.

All microsatellites were amplified under gradient PCR using AmpliTaq polymerase (Applied Biosystems, Inc., New England Biolabs, Ipswich, MA, USA) to determine optimum annealing conditions. When optimum conditions were confirmed, all strains were evaluated for all microsatellites using PCR following the visualization and purification protocol stated above. At each microsatellite locus, DNA from each strain of *C. briggsae* was amplified using fluorescent dye-labelled primers, and genotyped with fragment analysis protocol on a Beckman Coulter Genetic Analyzer CEQ 8000 (Durham, NH, USA) using a 600 bp fragment size ladder.

The fragment analysis results were binned to the nearest even integer for dimers and nearest multiple of three for trimers. All results were visually verified for appropriate bin assignments (Table 2). All contig locations referenced are from http://wormbase.org, version 181, *C. briggsae* build 3.

Comparison with tropical strains of *C. briggsae*
Using a total of 1381 bp of sequences of partial COII and ND5 genes, we compared *C. briggsae* KT0001 with other *C. briggsae* strains from tropical regions: two Kenyan strains (ED3092, ED3101), two from Taiwan (ED3036, ED3032), and one from Johannesburg, South Africa (ED3083).

Establishing bacterial identity
The associated bacterium *Serratia* sp. SCBI was isolated as a monoculture from *C. briggsae* KT0001 on untreated LB agar plates. An ~600 bp portion of the small subunit RNA gene of the associated bacterium was initially amplified and sequenced with the primers shown in Table S1 (supplementary material) and the complete SSU fragment was subsequently sequenced as part of the *Serratia* sp. SCBI genome sequencing.

### Table 2. Fragment analysis results for all *C. briggsae* strains

<table>
<thead>
<tr>
<th>Locus</th>
<th>Strain</th>
<th>2852</th>
<th>2220</th>
<th>NA15</th>
<th>4428</th>
<th>2454</th>
<th>4404</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AF16</td>
<td>483</td>
<td>487</td>
<td>352</td>
<td>408</td>
<td>470</td>
<td>490</td>
</tr>
<tr>
<td></td>
<td>KT0001</td>
<td>471</td>
<td>502</td>
<td>402</td>
<td>388</td>
<td>426</td>
<td>535</td>
</tr>
<tr>
<td></td>
<td>JU726</td>
<td>528</td>
<td>502</td>
<td>412</td>
<td>388</td>
<td>534</td>
<td>541</td>
</tr>
<tr>
<td></td>
<td>JU725</td>
<td>621</td>
<td>493</td>
<td>420</td>
<td>378</td>
<td>470</td>
<td>541</td>
</tr>
<tr>
<td></td>
<td>DH1300</td>
<td>450</td>
<td>520</td>
<td>412</td>
<td>394</td>
<td>450</td>
<td>511</td>
</tr>
<tr>
<td></td>
<td>DR1690</td>
<td>450</td>
<td>520</td>
<td>412</td>
<td>394</td>
<td>450</td>
<td>511</td>
</tr>
<tr>
<td></td>
<td>VT847</td>
<td>444</td>
<td>493</td>
<td>352</td>
<td>380</td>
<td>470</td>
<td>475</td>
</tr>
<tr>
<td></td>
<td>PB800</td>
<td>483</td>
<td>397</td>
<td>750</td>
<td>370</td>
<td>380</td>
<td>385</td>
</tr>
<tr>
<td></td>
<td>PB826</td>
<td>0</td>
<td>397</td>
<td>750</td>
<td>378</td>
<td>380</td>
<td>385</td>
</tr>
<tr>
<td></td>
<td>HK104</td>
<td>450</td>
<td>397</td>
<td>750</td>
<td>376</td>
<td>426</td>
<td>385</td>
</tr>
<tr>
<td></td>
<td>HK106</td>
<td>450</td>
<td>397</td>
<td>750</td>
<td>376</td>
<td>426</td>
<td>385</td>
</tr>
</tbody>
</table>

All strains were homozygous at every locus. Allele sizes are binned to the nearest appropriate repeat length. Locus IDs are as given in supplementary material Table S2.

Physiological characterization of *Serratia* sp. SCBI
The physiological tests included Gram stain, spore stain, oxidase test, hydrogen sulphide production, degradation of casein and starch, iron/sulphur oxidation and antimicrobial production. Degradation of casein was tested by streaking a 48h culture of the bacterium on tryptone soy broth (TSB) skimmed milk agar and incubating at 28°C for 48h (Kinsel, 1960). Degradation of starch was tested by streaking a 48h culture of the bacterium on starch agar and incubating at 28°C for 48h. The bacterium was grown in ferric sulphate–basal salts medium for the iron oxidation test (Manning, 1975; Akhurst, 1982). To test for antimicrobial activity, nutrient agar plates were spot inoculated with bacteria, incubated for 3 days, then killed by exposure to chloroform for 2h and allowed to air dry for 1h. Afterwards, the plates were covered with soft sterile agar at 45°C, and seeded with a culture of *Bacillus thuringiensis* (McInerney et al., 1991a; McInerney et al., 1991b). The plates were then incubated for 24h at 28°C.

Testing for the entomopathogenicity of *Serratia* sp. SCBI
The entomopathogenic properties of *Serratia* sp. SCBI were assayed through topical exposure and haemocoelic injection of *Serratia* sp. SCBI into *G. mellonella.*

Injection experiments
All injection experiments were carried out on moistened filter paper (Whatman No. 1) in 6 cm Petri dishes at 25°C in dark conditions, with injections administered using a 26 gauge 5/8 1 ml sterile syringe. Prior to the start of the experiment, *E. coli OP50* and *Serratia* sp. SCBI were grown separately in LB broth for 12–14h at 37°C and 20°C, respectively. For each injection control experiment five *G. mellonella* were placed in a 6 cm Petri dish without any physical or chemical exposure to *Serratia* sp. SCBI. The first set of controls were undisturbed *G. mellonella*; second set was exposed to a simple penetration injection without solution; and the final set was of five *G. mellonella* that were injected with 10–20μl of M9 buffer solution, LB broth only, or LB incubated with *E. coli OP50* (N=five for each injection set). The experimental injections were completed using tiritations of *Serratia* sp. SCBI grown in LB broth for 12-14h. Three *G. mellonella* were injected with 20μl of each *Serratia* sp. SCBI titration; the stock solution of *Serratia* sp. SCBI was diluted for the tiritations using sterile LB broth. The titrations of this experiment were: full concentration, and 1:10, 1:100, 1:104, 1:106, 1:108, 1:109 and 1:1010 dilutions. Titrations were spread onto untreated LB plates and incubated for 12–14h at 37°C, and colonies were counted to determine the colony-forming units (c.f.u.) for each titration. Percent mortality of *G. mellonella* was evaluated at 24h intervals, and the experiment was terminated after 14 days.

For experiments where *E. coli OP50* replaced *Serratia* sp. SCBI, nematodes were used 2 weeks after seeding on NGM plates. We did not quantify the amount of *E. coli* used for the injection experiment, because the extremely high concentration we used was reached by cultivating the bacteria in LB agar for 3 days and by concentrating the bacteria through centrifugation to reach high density; 20μl of concentrate was used for the experiment.

Nematode penetration and reproduction in *Galleria*
Evaluation of the entomopathogenic behaviour of *Caenorhabditis* strains was twofold. The first set of experiments tested the capacity of *Caenorhabditis* strains to reproduce inside *G. mellonella*, and the second set of experiments tested the ability of each nematode strain to gain entry into the insect larvae haemolymph. Insect larvae
used for all experiments were late instar *G. mellonella* from Connecticut Valley Biological Supply Company (Southampton, MA, USA). All experiments were completed at 25°C and in the dark.

For the first set of experiments cultures of each nematode strain were switched to growth on SCBI after standard bleaching and starvation of L1 (Kemphues, 2005). Each nematode strain was subsequently maintained on NGM plates seeded with *Serrata* sp. SCBI for 2 weeks prior to the start of the experiment. All cultures were maintained at 20°C until use. Nematodes and associated bacteria were isolated in buffered saline solution and known concentrations of nematodes in a 50 μl aliquot were injected into 10 larval *G. mellonella*. Each experiment was completed in triplicate. The insect larvae were incubated in 6 cm Petri dishes with moistened filter paper (Whatman No. 1) at 25°C in the dark for 24 h. After incubation the insect larvae were transferred to modified White traps (White, 1927; Caroli et al., 1996; Kaya and Stock, 1997; Glazer and Lewis, 2000). Emergent nematodes were collected on day 9 for each strain. A second experiment to test the ability of each *Caenorhabditis* strain to penetrate and kill the *G. mellonella* followed procedures modified from Glazer and Lewis (Glazer and Lewis, 2000) and Caroli et al. (Caroli et al., 1996). Ten late instar *G. mellonella* were placed in a prepared 6 cm Petri dish, with three Petri dishes per strain of nematode; the experiment was done in triplicate. One thousand nematodes (per strain) collected from the White trap experiment above were added to each prepared Petri dish. Although we did not quantify the various stages collected through White traps, we observed that most of the nematodes collected were dauer stage juveniles. However, we did not attempt to exclude non-dauer stages when they occurred, mixed with dauer. Control conditions were identical to the experimental conditions without the addition of nematodes. Mortality was recorded at 24, 48, 72, 96 and 168 h. After 168 h, at which point approximately 100% mortality of the insect occurred, harvested nematodes within each insect cadaver was counted. Our data on the mortality was confirmed at 24, 48, 72, 96 and 168 h. The experiment was terminated at 168 h. After 168 h, at which point approximately 100% mortality of the insect larvae exposed to the nematodes was reached. After 168 h the insect larvae were collected from each Petri dish and rinsed with sterile water to remove any nematodes on the insect cuticle. Rinsed insect larvae were stored at –20°C for subsequent dissection and pepsin digestion.

For the injection experiments, after digestion, the number of nematodes within each insect cadaver was counted. Our data on the penetration experiments were qualitative: we only made sure the total number of nematodes harvested from the insect cadavers was higher than the number we initially added to the Petri dish. In all cases more nematodes were harvested than were initially added. Determining the efficacy of entomopathogenicity of the various strains quantitatively is beyond the goal and focus of this work, though further work in this line is going on in other labs.

### RESULTS AND DISCUSSION

GenBank accession numbers for the mitochondrial sequences we generated for the *Caenorhabditis* strains are given in Table 1. DNA sequences for the SSU and ITS ribosomal fragments were identical to those of *C. briggsae* AF16. We compared DNA sequences with all other known nematode orthologues in GenBank and at the nematode tree of life project (http://Nematol.unh.edu) and found them to be identical or nearly identical to homologues from *C. briggsae*. Results of the microsatellite fragment analysis and locus details are given in Table 2 and supplementary material Table S2. Morphological analysis of the nematode strain KT0001 was also consistent with its identification as a *Caenorhabditis*. Based on mitochondrial gene sequences and several microsatellite loci, this new EPN was found to be a unique strain of *C. briggsae* (KT0001).

A more specific comparison of KT0001 with other tropical *C. briggsae* strains using a total of 1381 bp of sequences of partial COII and NDS genes (Fig. 1) revealed KT0001 to be similar to the Johannesburg strain from South Africa and the two Taiwan strains. This result of our analysis was similar to that of Howe and Denver (Howe and Denver, 2008) in that the Kenyan strains were distinct from all of the other strains including our strain KT0001.

The bacterial strain SCBI that we isolated with *C. briggsae* KT0001 is aerobic, rod shaped, gram negative, spore forming and oxidase positive, and shows antimicrobial activity when tested against *B. thuringiensis* (var. Kurstaki). Sequencing of the 1445 base SSU ribosomal RNA gene gave a sequence that is 99% identical to *Serratia marcescens* DB11. Genome sequencing of *Serratia* sp. SCBI is complete and large-scale sequence analysis of the entire genome confirmed the general similarity to *S. marcescens* (DB11) at thousands of loci (F. Abebe-Akele, personal communication).

The observation of putative necromenic associations of *Caenorhabditis* with insects is common (Kiontke and Sudhaus, 2006). Necromenia differs from entomopathogenesis only in the role of the bacteria, where in the case of true EPNs it hastens the death of the host and moves with the nematode from host to host. We tested the effectiveness of *Serratia* sp. SCBI as an insect pathogen by direct injection of *Serratia* sp. SCBI into the insect larvae haemocoel. While direct injection of a small number of bacteria (<1000 c.f.u.) is 100% lethal in less than 72 h, topical exposure of insect larvae to *Serratia* sp. SCBI and injection controls with buffered saline solution, sterile LB and *E. coli* in LB, or piercing with the syringe did not significantly increase insect mortality, confirming that the penetration of a relatively small number of these bacteria is essential and sufficient to cause the rapid death of the insect (Fig. 2).

We then demonstrated that reproduction of *C. briggsae* KT0001 and the proliferation of *Serratia* sp. SCBI occurs within the insect larvae (Fig. 3A) and that dauer juveniles of *C. briggsae* KT0001

![Phylogenetic relationship of Caenorhabditis briggsae tropical strains](image-url)

Fig. 1. Phylogenetic relationship of *Caenorhabditis briggsae* tropical strains based on 1381 bp of sequences of mitochondrial partial cytchrome oxidase II (COII) and NADH dehydrogenase subunit 5 (ND5) genes. The consensus tree was constructed using the neighbour-joining method with 1000 bootstraps. Strains included in the analysis are two Kenyan strains (ED3092, ED3101), two strains from Taiwan (ED3036, ED3032), and one strain from Johannesburg, South Africa (ED3083). The list of remaining strains is given in Table 1.
can complete the cycle of parasitism by penetrating, reproducing and killing the new insect host (Fig.3B). These events parallel observations in well-studied entomopathogenic nematodes and their associated bacterial species, demonstrating that this association between C. briggsae KT0001 and Serratia sp. SCBI is potentially entomopathogenic and that both the nematode (for penetration) and the bacterium (for insect pathogenesis) appear to be necessary and sufficient to effect insect death.

Our current understanding of Caenorhabditis life history is limited to very few direct observations of nematode–invertebrate associations [see discussion in Caswell-Chen et al. (Caswell-Chen et al., 2005)], and because of the way most strains are collected we would not know whether an EPN life cycle is widespread in the group. In fact, the first step in the culture of most Caenorhabditis is to eliminate associated bacteria and to switch the culture to a standard laboratory culture of E. coli (OP50). Consequently, we know very little about the possible natural bacterial associates of Caenorhabditis and we do not claim that Serratia sp. SCBI are always associated with all C. briggsae strains. Nevertheless, in the light of past extensive efforts to find Caenorhabditis in nature, the inability to recover C. elegans from soil and its extensive association with invertebrates, a shift in paradigm seems to be a necessary step towards future understanding of Caenorhabditis ecology (Caswell-Chen et al., 2005).

To explore the possibility that the capacity to be an EPN is not limited to this specific strain of C. briggsae, we tested 10 wild isolates of C. briggsae (AF16, HK104, HK105, JU725, JU726, PB800, PB826, DH1300, DR1690 and VT847) and 5 other Caenorhabditis species (C. elegans N2, C. remanei PB464, C. remanei EM464, C. brenneri CB5161, Caenorhabditis sp. 5 JU727) for the ability to become entomopathogenic when coupled with Serratia sp. SCBI. While all strains showed no ability to kill insects within 48h when cultured on E. coli OP50, when cultured on Serratia sp. SCBI all but one of the C. briggsae strains and all other Caenorhabditis species were transformed to become entomopathogenic: they were competent to reproduce inside the insect host (Fig.3A) and penetrated the insect host, killed the insect and emerged from the cadaver as infective dauer stage juveniles. A closer look at insect mortality 48h post-exposure to nematode strains demonstrated to have the ability to reproduce in insect larvae in injection experiments revealed that three strains – C. brenneri CB5161, C. remanei PB4641 and C. briggsae JU726 – are poor insect pathogens.

The path of entrance of C. briggsae KT0001 into the Galleria larvae is not currently known. Caenorhabditis do not have the anatomical features that would enable them to readily penetrate the insect cuticle to reach the haemocoel. We speculate that the most likely route of entrance into the insect host for this strain would be through natural openings such as the mouth and anus. Nevertheless,
this warrants further investigation for those Caenorhabditis able to produce insect mortality within 48 h post-exposure. A closer look at how KT0001 might carry Serratia sp. SCBI did not produce a definite answer. Our extensive light microscopic (LM) and SEM studies of KT0001 and other C. briggsae strains cultured on Serratia sp. SCBI did not reveal any bacteria on the worm surface (data not shown). LM study of the intestine of live KT0001 showed actively moving bacterial cells with a gradual decline in activity towards the rectum. Nonetheless, although very small in proportion, we did observe active bacterial cells even close to the rectum. We use the term ‘associated’ because the nematode feeds on the bacteria, and carries the bacteria in its gut as it penetrates the insect host.

One of the typical characteristics of the two major groups of entomopathogenic nematodes is that the only life stage found outside the host cadaver is the third-stage infective juvenile. Our efforts to find adult C. briggsae through direct culturing (spreading soil on NGM) and the use of rotting fruits from the soils where this strain was found did not produce any results. This may not be conclusive evidence of the absence of a free-living stage of this strain in the soil, but it clearly points towards the true entomopathogenic nature of the strain. Based on these observations it would seem that the capacity to become entomopathogenic is widespread in Caenorhabditis and that an entomopathogenic life history can be driven by the insect pathogenic nature of the bacterium. However, it is not our intention to suggest that all Caenorhabditis are always EPNs as is thought to be the case for Steinernema and Heterorhabditis. Nevertheless, it is possible that the necromenic life cycle is an ideal pre-adaptation to an entomopathogenic life history in the presence of an insect pathogenic bacterium, and because the collection of Caenorhabditis almost always includes the elimination of contaminating bacteria, it remains possible that this EPN life history is much more widespread among these species than previously considered. The only evidence that Caenorhabditis are not all EPNs is the fact that no other Caenorhabditis has ever been reported from Galleria traps.

Despite the extensive use of Caenorhabditis as an important model system, the natural life history of these species is little known (Kiontke and Sudhaus, 2006) and finding these organisms in nature has been a serious challenge for the scientific community (Barrière and Félix, 2005). Clearly, in association with the bacterium Serratia sp. SCBI, these Caenorhabditis can become an entomopathogenic species, a complex life history not assayed in normal experimental manipulations of C. elegans. The details of this association will be interesting and enlightening with regard to the evolutionary and ecological capacity of this premier model system and may expand our functional understanding of its genome. In this context it is interesting to consider the fact that, as with all model organisms, a large fraction of the C. elegans genome remains functionally uncharacterized, the number of essential genes under normal laboratory conditions is small (<30%) compared with the total number of genes encoded and evolutionarily conserved, and a large fraction of the functional knockouts have no detectable phenotype in typical laboratory culture (Kamath et al., 2003; Kempheus, 2005). Genetic redundancy and incomplete phenotypic characterization can explain some fraction of the uncharacterized genome. However, it is not difficult to speculate that numerous genes in C. elegans may have been critical to their ability to function in an entomopathogenic system and have no testable function in the context of normal laboratory culture.

The goal of linking the DNA sequence of an organism to its development and behaviour is best achieved through a better understanding of the ecological context in which these genes and their interactions evolved. The knowledge of life history strategies such as that reported here should serve as a route to a better understanding of the functionality and evolution of the genes in this important model system, and their specific roles in the evolution of this nematode in the context of its natural environment and ecology.

ACKNOWLEDGEMENTS
Eyualbe Abele was supported in part by the US Army Research Laboratory and US Army Research Office under Contract number W911NF-08-1-0402, and US National Science Foundation Award Number 0808632.

REFERENCES


THE JOURNAL OF EXPERIMENTAL BIOLOGY