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An entomopathogenic *Caenorhabditis briggsae*

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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*.**

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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 (Kiontke 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 (Kiontke 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 insect host ranges, 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 either Steinernematidae or *Heterorhabditis*) 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 *Heterorhabditis* (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 two other 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 Imix-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 1. *Caenorhabditis* strains used in both molecular and behavioural assays

Strain	Species	Locality	Collected by	COII	ND5
DH1300	*C. briggsae*	Unknown	B. Zuckerman	n.a.	EU254725
DR1690	*C. briggsae*	Unknown	B. Zuckerman	EU254738	EU254726
AF16	*C. briggsae*	Ahmedabad, India	A. Fodor	EU254735	EU254724
HK104	*C. briggsae*	Okayama, Japan	H. Kagawa	EU254736	n.a.
HK105	*C. briggsae*	Sendai, Japan	H. Kagawa	EU245743	EU254727
JU725	*C. briggsae*	Yangshuo, Guangxi, China	M. A. Felix	EU254739	EU254728
JU726	*C. briggsae*	Sanjiang, Guangxi, China	M. A. Felix	EU254742	EU254729
PB800	*C. briggsae*	Dayton, OH, USA	S. Baird	EU254737	EU254730
PB826	*C. briggsae*	Hueston Woods State Park, OH, USA	R. M. Hampton and S. Baird	EU254740	EU254831
VT847	*C. briggsae*	Haena, Kauai, HI, USA	V. Ambros	EU254741	EU254733
KT0001	*C. briggsae*	Northwest Mpumalanga province, South Africa	M.J.	EU254734	EU245732
N2	*C. elegans*	Bristol, UK	L. N. Staniland		
PB4641	*C. remanei*	Inbred derivative of EM464	S. Baird		
JU727	*Caenorhabditis* sp. 5	Sanjiang, Guangxi, China	M. A. Felix		
CB5161	*C. brenneri*	Trinidad	A. Fodor		
EM464	*C. remanei*	Brooklyn, NY, USA	S. Baird		

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 Ampli*Taq* 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 rRNA gene of the associated bacterium was initially amplified and sequenced using 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

	Locus					
Strain	2852	2220	NA15	4228	2454	4404
AF16	483	487	352	408	470	490
KT0001	471	502	402	388	426	535
JU726	528	502	412	388	534	541
JU725	621	493	420	378	470	541
DH1300	450	520	412	394	450	511
DR1690	450	520	412	394	450	511
VT847	444	493	352	380	470	475
PB800	483	397	750	370	380	385
PB826	0	397	750	378	380	385
HK104	450	397	750	376	426	385
HK105	450	397	750	376	426	385

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 48 h culture of the bacterium on tryptone soy broth (TSB) skimmed milk agar and incubating at 28°C for 48 h (Kinsel, 1960). Degradation of starch was tested by streaking a 48 h culture of the bacterium on starch agar and incubating at 28°C for 48 h. 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 2 h and allowed to air dry for 1 h. 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 24 h 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 12–14 h 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 10–20 µl 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 titrations of *Serratia* sp. SCBI grown in LB broth for 12-14 h. Three *G. mellonella* were injected with 20 µl of each *Serratia* sp. SCBI titration; the stock solution of *Serratia* sp. SCBI was diluted for the titrations using sterile LB broth. The titrations of this experiment were: full concentration, and 1:10, 1:100, $1:10^3$, $1:10^4$, $1:10^5$, $1:10^6$, $1:10^7$, $1:10^8$, $1:10^9$ and $1:10^{10}$ dilutions. Titrations were spread onto untreated LB plates and incubated for 12–14 h 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 24 h 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; 20 µl 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 *Serratia* 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 dauers. Control conditions were identical to the experimental conditions without the addition of nematodes. Mortality was recorded at 24, 48, 72, 96 and 168 h. The experiment was terminated at 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 ND5 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

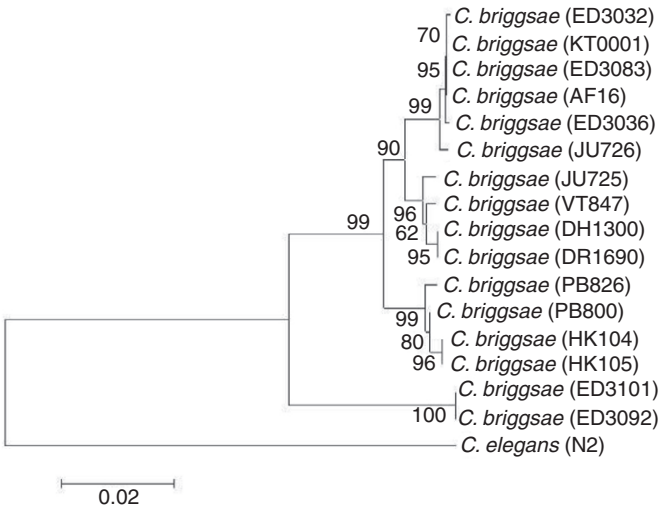

Fig. 1. Phylogenetic relationship of *Caenorhabditis briggsae* tropical strains based on 1381 bp of sequences of mitochondrial partial cytochrome oxidase II (COII) and NADH dehydrogenase subunit 5 (ND5) genes. The consensus tree was constructed using the neighbour-joining method with 1000 bootstraps. 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.

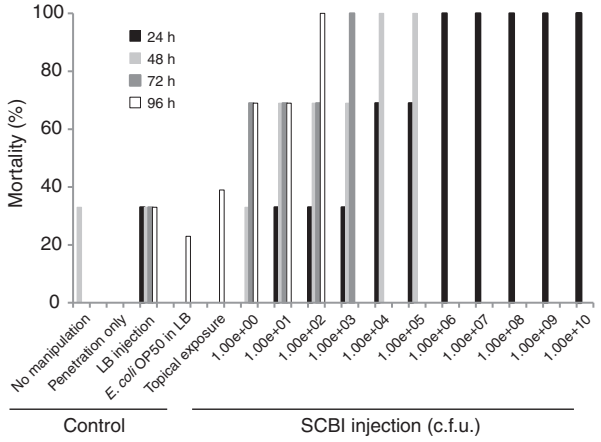

Fig. 2. Analysis of the pathogenicity of *Serratia* sp. SCBI. Summary of *Galleria mellonella* mortality at 24 h intervals from 24 to 96 h post-injection and with varying concentrations of *Serratia* sp. SCBI. At all time intervals *G. mellonella* mortality was greater in the injected treatments compared with the control treatments. Control treatments include: no manipulation (NGM plates with no bacterial lawn present, *N*=5), penetration only, injection of LB, injection of *E. coli* OP50 in LB, and cutaneous or topical exposure experiments which consisted of late instar larval *G. mellonella* placed on NGM plates seeded with *Serratia* sp. SCBI (*N*=10). Mortality was monitored at 24 h intervals for 20 days after which time the experiment was terminated. The absence of a bar in controls means no insect mortality.

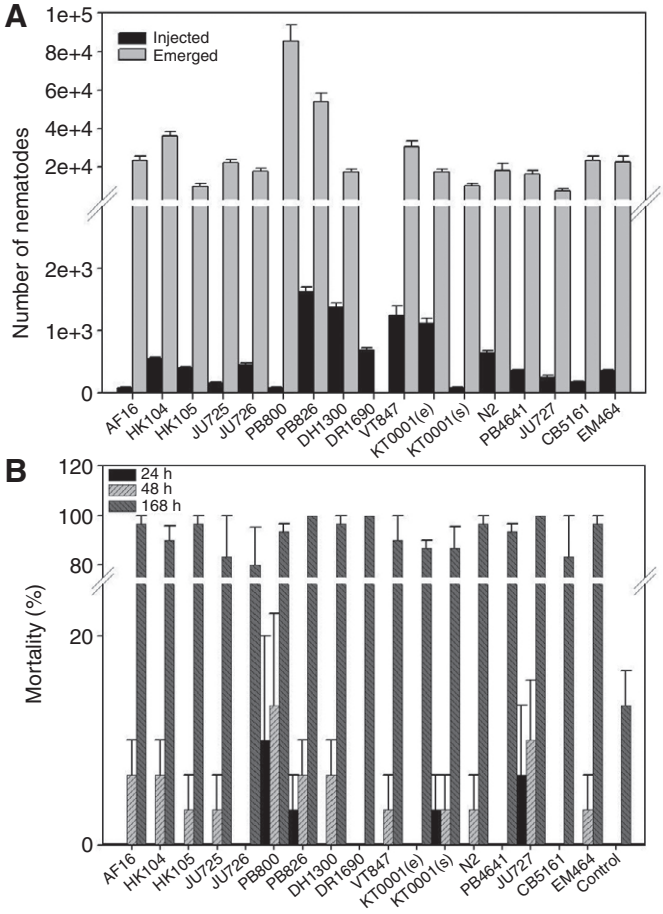

Fig. 3. Experimental evaluation of the ability of *Caenorhabditis* species and *Serratia* sp. SCBI to kill and reproduce within the insect larvae of *G. mellonella*. (A) Comparison of the ability of 16 *Caenorhabditis* strains to reproduce inside *G. mellonella*. In each case, the number of nematodes injected into late instar *G. mellonella* is compared with the number emerged after day 9. (B) Late instar *G. mellonella* larvae mortality at 24, 48 and 168 h post-exposure to strains of nematodes previously emerged from *G. mellonella* cadavers in A. Because DR1690 did not produce detectable nematodes in A after 9 days, DR1690 nematodes for the mortality assay were collected after several weeks. In both experiments, two cultures of KT0001 were used: one, KT0001(e), that had previously been cultured on *E. coli* OP50 and a second, KT0001(s), that had been maintained only on *Serratia* sp. SCBI. The control for this experiment was insect larvae not exposed to nematodes.

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 48 h 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 48 h 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 system, 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; Kemphues, 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.

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