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Isolation of naturally associated bacteria of necromenic *Pristionchus* nematodes and fitness consequences

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SUMMARY

Nematodes and bacteria are major components of the soil ecosystem. Many nematodes use bacteria for food, whereas others evolved specialized bacterial interactions ranging from mutualism to parasitism. Little is known about the biological mechanisms by which nematode–bacterial interactions are achieved, largely because in the laboratory nematodes are often cultured under artificial conditions. We investigated the bacterial interactions of nematodes from the genus *Pristionchus* that have a strong association with scarab beetles. *Pristionchus* has a different feeding strategy than *Caenorhabditis* and meta-genomic 16S sequence analysis of *Pristionchus* individuals showed a diversity of living bacteria within the nematode gut and on the nematode cuticle. Twenty-three different bacterial strains were isolated from three *Pristionchus*–beetle associations and were used to study nematode–bacterial interactions under controlled laboratory conditions. We show a continuum of bacterial interactions from dissemination, to reduction in brood size and nematode mortality caused by bacteria derived from insect hosts. Olfactory discrimination experiments show distinct chemoattraction and fitness profiles of *Pristionchus* nematodes when exposed to different bacteria. For example, *Pristionchus pacificus* avoids *Serratia marcescens* possibly because of pathogenicity. Also, *P. pacificus* avoids *Bacillus thuringiensis* and insect pathogenic bacteria but is resistant to the human pathogens *Staphylococcus aureus* and *Pseudomonas aeruginosa*, unlike *Caenorhabditis elegans*. *Pristionchus* specifically recognize and respond to bacteria that cause ill health. Bringing the nematode–bacterial interaction into the laboratory allows detailed functional studies, including the genetic manipulation of the interaction in both nematodes and bacteria.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/211/12/1927/DC1

Key words: *Pristionchus pacificus*, *Caenorhabditis elegans*, nematode–bacterial interactions, *Bacillus thuringiensis*, entomopathogenic bacteria.

INTRODUCTION

Nematodes and bacteria are among the most numerous organisms on Earth with numbers of nematodes thought to exceed 1 million per m^2 (Floyd et al., 2002) and numbers of bacterial cells in 1 g of soil thought to be approximately 10^{10} (Faegri et al., 1977). Many nematodes use bacteria only for food but some species have more specialized interactions, ranging from mutualism to parasitism. For example, entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis* exhibit a close symbiotic relationship with the bacteria *Xenorhabdus* and *Photorhabdus*, respectively (Forst et al., 1997) and rely on these bacteria to cause mortality to insect hosts. The slug parasitic nematode *Phasmarhabditis hermaphrodita* is thought to depend on the bacterium *Moraxella osloensis* to kill slugs and snails (Wilson et al., 1995a; Wilson et al., 1995b; Tan and Grewal, 2001; Tan and Grewal, 2002). Filarial nematodes such as *Brugia malayi* require the endosymbiotic bacterium *Wolbachia* for development, fertility and survival (Taylor et al., 2005). The combination of the pine wood nematode, *Bursaphelenchus xylophilus*, and strains of *Pseudomonas fluorescens* are thought to be responsible for causing an increase in mortality of pine trees (Han et al., 2003). Marine nematodes from the family Stilbonematinae have a mutualistic relationship with thiotrophic ectosymbiotic bacteria (Nussbaumer et al., 2004).

Although the interaction of the aforementioned nematodes with specific bacteria has been well documented, little is known about laboratory nematode model organisms, such as *Caenorhabditis elegans* and *Pristionchus pacificus*. *C. elegans* is a model organism for many areas of biology (see The *C. elegans* Research Community, 2005), whereas *P. pacificus* has been established as a satellite organism in evolutionary developmental biology (Hong and Sommer, 2006a). Both species have – at least in part – been selected as model organisms because they can be cultured in the laboratory using artificial *Escherichia coli* OP50 as food (Brenner, 1974; Sommer et al., 1996).

Recent studies started to investigate the environment in which *Pristionchus* can be found in nature. Several field studies revealed that *Pristionchus* nematodes have close associations with scarab beetles and the Colorado potato beetle (*Leptinotarsa decemlineata*) (Herrmann et al., 2006a; Herrmann et al., 2006b). For example, *P. pacificus* was isolated from the oriental beetle (*Exomala orientalis*) in Japan and the United States (Herrmann et al., 2007). Biological surveys of beetle-associated *Pristionchus* species have concentrated on Europe, North-America, Japan and South Africa. In total, more than 1200 *Pristionchus* isolates have been obtained from more than 15 000 surveyed beetles. These isolates fall into 18 distinct species with a specific biogeographic pattern (Mayer et al., 2007).

P. pacificus currently represents the only cosmopolitan species (Zauner et al., 2007).

Pristionchus nematodes show a high species specificity with certain beetles. For example, the two European species *Pristionchus maupasi* and *Pristionchus entomophagus* are found on cockchafers (*Melolontha* sp.) and dung beetles (*Geotrupes* sp.), respectively (Herrmann et al., 2006a). Similarly, the Colorado potato beetle, which lives in Europe and North America, is highly infested with *Pristionchus uniformis* (Herrmann et al., 2006b). Chemoattraction studies have shown that different *Pristionchus* species display unique chemoattraction profiles towards insect pheromones and plant volatiles (Hong and Sommer, 2006b) demonstrating the utility of such assays for probing the nematodes' environment under laboratory conditions. *Pristionchus* chemoattraction is highly diverse and is presumably involved in shaping the specific interaction with host beetles.

In general, nematode–insect associations can be categorized as phoretic, necromenic or parasitic (Kiontke and Sudhaus, 2006). *Pristionchus* has a necromenic association with beetles whereby the infective juvenile nematodes enter an insect, wait for the death of the host and then feed on bacteria and fungi that proliferate on the insect carcass. Necromenic associations are typically more specific than phoretic associations, in which nematodes use insects or other invertebrates for transport but not as food. It has been suggested that necromeny represents a pre-adaptation for the evolution of true parasitism because the nematode is exposed to low oxygen levels, high temperatures and toxic host enzymes (Weischer and Brown, 2000).

In the context of the different life-style of *Pristionchus* nematodes, it is important to note that these nematodes show major morphological and physiological adaptations with respect to feeding when compared with *C. elegans* and other rhabditids. *C. elegans* has a grinder in the terminal bulb of the pharynx, which disrupts food bacteria (such as *E. coli* OP50), and under laboratory conditions bacteria are completely lysed (Fig. 1A,B). By contrast, *Pristionchus* nematodes have a pharynx with a metacorpus and a terminal bulb typical for rhabditid nematodes but do not have a grinder (Fig. 1C) (Fürst von Lieven and Sudhaus, 2000; Chiang et al., 2006). *Pristionchus* worms do not completely lyse bacteria and intact cells can be found in the intestine as revealed by transmission electron microscopy (TEM; Fig. 1D). Interestingly, it has been suggested that *Pristionchus* nematodes might be actively involved in bacterial dissemination in the wild (Chantanao and Jensen, 1968; Poinar, 1983).

Here, we begin an analysis of the tritrophic interactions of *Pristionchus* nematodes with bacteria associated with beetles and soil. To gain insight into this relationship we chose the following aims: (1) to identify (using metagenomic and microbiological techniques) bacteria that are associated with *Pristionchus* nematodes isolated from soil and beetle hosts, i.e. *P. maupasi* (from cockchafers), *P. entomophagus* (from dung beetles) and *P. pacificus* (from the oriental beetle); (2) to investigate the effect these bacteria have on a range of *Pristionchus* species using chemotaxis, survival and fecundity assays; (3) to assess survival of *P. pacificus* to human (*Pseudomonas aeruginosa* and *Staphylococcus aureus*) and insect (*Xenorhabdus nematophila*, *Xenorhabdus* sp. and *Photorhabdus luminescens*) pathogens; (4) to examine whether physiological functions are variable between nematode species and are affected by bacteria.

MATERIALS AND METHODS

Nematodes, bacteria strains and culture conditions

Nematode strains used were: *Pristionchus pacificus* (PS0312), *P. entomophagus* (RS0144), *P. maupasi* (RS5015) and *Caenorhabditis elegans* (Bristol N2). Nematodes were grown at 20°C on nematode growth medium (NGM) seeded with *E. coli* OP50 before use in experiments. Bacteria were isolated from beetle nematodes and soil nematodes. Bacteria were maintained on LB agar plates at 30°C. In all experiments bacteria were grown overnight in LB broth at 30°C. Bacteria used in this study were: *Pseudomonas aeruginosa* PA14, *Staphylococcus aureus* Newman, *Photorhabdus luminescens* TT01Tn7GFP (associated with the nematode *Heterorhabditis bacteriophora*), *Xenorhabdus* sp. (associated with *Steinernema scapterisci*), *Xenorhabdus nematophila* (associated with *Steinernema carpocapsae*). Phase variation in *X. nematophila*, *Xenorhabdus* sp. and *P. luminescens* was monitored using NBTA agar (nutrient agar supplemented with Bromothymol Blue and triphenyltetrazolium chloride) and only phase 1 bacteria were used, which are the most virulent. All equipment was sterilized before use and bacteria were only isolated under a laminar flow. To rule out any airborne contaminants 10 LB plates were placed in the laminar flow for 5–8 h and then sealed and stored at 30°C overnight and monitored for colonies the next day. No bacterial colonies or contaminants were recorded, proving that all bacteria isolated were solely from the nematodes.

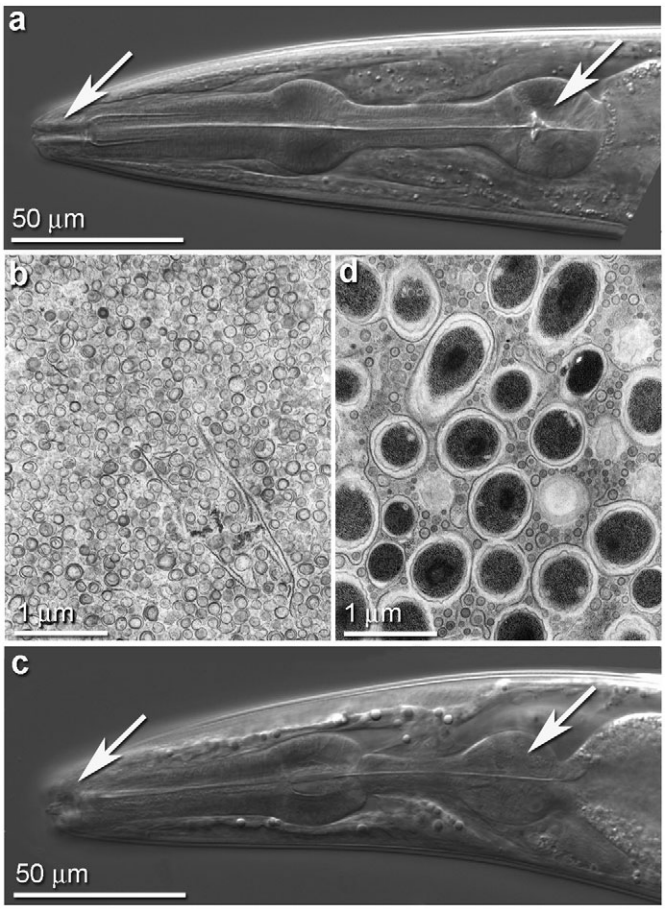


Fig. 1. Pharynx morphology of *Pristionchus pacificus* and *Caenorhabditis elegans*. (A) *C. elegans* pharynx with grinder and long, narrow mouth-like suction pump. (B) *Escherichia coli* OP50 crushed with the *C. elegans* grinder. (C) *P. pacificus* pharynx with no grinder and shorter, broader mouthparts. (D) *E. coli* OP50 is not completely disrupted after passage through the pharynx of *P. pacificus*.

Isolation of bacteria and nematodes from beetles

Cockchafers (*Melolontha hippocastani*) were collected from Karlsruhe, Germany, Oriental beetles (*Exomala orientalis*) were

collected from Carver, Massachusetts and dung beetles (*Geotrupes* sp.) were collected from the Schönbuch forest, near Tübingen, Germany. Beetles were cut in half transversely with scissors and then placed on 6 cm NGM plates and stored at room temperature. Beetles were inspected daily for 7–14 days for nematodes moving or reproducing on the cadavers. Any nematodes that resembled *Pristionchus* were removed, washed in M9 buffer for 1–2 min and placed on separate LB plates and incubated at room temperature. After 48 h the individual nematodes were removed, lysed and sequenced (see below for methods) to confirm whether the nematodes were the correct species of *Pristionchus* (according to Mayer et al., 2007). Bacteria growing on LB plates were isolated, subcultured and then prepared for sequencing for species identification using PCR amplification of 16S ribosomal RNA genes (Lane, 1991). The bacteria isolated from nematodes from beetles will be referred to as 'beetle-derived' bacteria, hereafter.

We also extracted one adult *P. entomophagus* from a soil sample from the Schönbuch forest, Tübingen and isolated the bacteria that were subsequently excreted from the nematode gut and removed from the nematode cuticle and grew them on an LB plate. The bacteria were then identified (as described below) and will be referred to as 'soil-derived' bacteria.

Bacteria and nematode DNA extraction and PCR amplification

Bacteria of each species were grown overnight in LB broth and DNA was extracted using Aqua Pure genomic DNA kit (Bio-Rad, Hercules, CA, USA). Polymerase chain reaction (PCR) amplification of bacterial 16S rRNA genes was carried out in 20 μl reactions using primer set 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5′-TACGGYTACCTTGTTACGACTT-3′) (Lane, 1991). Thermal cycling 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 8 min at 72°C. A typical reaction contained 2 μl 10× PCR buffer, 2 μl 2 mmol l^{-1} dNTPs, 1 μl 10 μmol l^{-1} 27f, 1 μl 10 μmol l^{-1} 1492r, one unit of Taq DNA polymerase, 12.8 μl H_2O and 1 μl of bacterial DNA. PCR amplicons were visualized by standard agarose gel electrophoresis (Sambrook et al., 1989) and bands were excised using a clean scalpel. DNA was extracted from bands using QIAquick gel extraction kit (Qiagen, Valencia, CA, USA).

After isolation of bacteria, nematodes were removed from the LB plate and identified using the small subunit rRNA gene. Genomic DNA from single nematodes was isolated using the NaOH digestion method of Floyd et al. (Floyd et al., 2002). Briefly, single worms were added to 20 μl of 0.25 mol l^{-1} NaOH and incubated at 25°C overnight. The worm mixture was then heated to 99°C for 3 min before the addition of 4 μl of 1 mol l^{-1} HCl, 10 μl of 0.5 mol l^{-1} Tris-HCl (pH 8.0) and 5 μl of 2% Triton X-100. The mixture was then heated to 99°C for 3 min, frozen to –20°C and then heated for a further 3 min at 99°C. Two microlitres of the extract were then used for PCR. DNA was amplified using the primers SSU18A (5′-AAAGATTAAGCCATGCATG-3′) and SSU26R (5′-CATTCTTGGCAAATGCTTTCG-3′). PCR was carried out in 25 μl reactions containing 2.5 mmol l^{-1} $MgCl_2$, 0.16 mmol l^{-1} each deoxynucleoside triphosphate, 0.5 μmol l^{-1} each primer, 2 μl lysate, 2 units Taq DNA polymerase (Amersham Biosciences, Piscataway, NJ, USA). The mixture was then subjected to the following PCR conditions: 2 min at 95°C, 35 cycles including, 15 s at 95°C, 15 s at 50°C, 2 min at 72°C, followed by 7 min at 72°C. PCR products were then diluted 10–20-fold and added to the Big Dye terminator sequencing mix (Applied Biosciences, Foster City, CA, USA), which contained the sequencing primer SSU9R (5′-AGCTGGAATTACCGCGGCTG-3′). For bacteria we required a minimum length of 200 base pairs for the query sequence (16S rRNA). Gene sequences of nematodes and bacteria were aligned using Seqman (DNA Star, Madison, WI, USA), compared with GenBank database sequences using Blastn searches using sequence similarity matches at 90%.

Metagenomic analysis of bacteria in the *Pristionchus* gut and cuticle

Soil samples were taken from the Schönbuch forest, Tübingen and were added to 9 cm NGM plates and stored at room temperature. The plates were then checked for presence of nematodes every day for the next 7 days. In total, four *P. entomophagus* and four *P. lheritieri* individuals were isolated, washed in M9 buffer and placed in single worm lysis buffer. The resultant suspension was then used for nematode identification (as described above) and bacterial cloning. Bacterial DNA was amplified using conditions and reagents as described above and was then cloned using Topo cloning kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's guidelines. The full-length gene was then ligated into pCR4-TOPO (Invitrogen) and transformed into Top 10 chemically competent bacterial cells for sequence analysis. In total, 683 clones were picked and screened for bacterial inserts using PCR primers T7 (5′-TAATACGACTCACTATAGGG-3′) and T3 (5′-ATTAACCCTCACTAAAGGGA-3′). Bacterial inserts were then sequenced using the same methods as described above.

Chemotaxis assays

Chemotaxis assays were modified from previous studies (Zhang et al., 2005; Hong and Sommer, 2006b). Briefly, 25 μl of overnight bacterial suspension was placed 0.5 cm away from the edge of a 9 cm Petri dish filled with NGM medium. The same amount of *E. coli* OP50 was placed on the opposing side and acted as the counter attractant. Approximately 50–200 J4/adult stage *Pristionchus* individuals were placed between the two bacterial spots. All nematodes used were previously fed on *E. coli* OP50. Plates were then sealed with Parafilm® and stored at room temperature in the dark. After 24 h the number of nematodes found in each bacterial spot was recorded. A chemotaxis index was used to score the response of the nematodes, which consisted of: number of nematodes in the test bacteria – numbers of nematodes in control bacteria/total number of nematodes counted (Zhang et al., 2005). This gave a chemotaxis score ranging from –1.0 (total revulsion from test bacteria) to 1.0 (total attraction towards test bacteria). A score of around 0 means there were equal numbers of nematodes in each bacterial spot. Five plates were used per replicate, and the procedure was repeated five times for each bacterium (a total of 25 individual assays).

Chemotaxis experiments were as follows: (1) *E. coli* OP50 *versus* soil-derived bacteria; (2) *E. coli* OP50 *versus* insect-derived bacteria; (3) *B. thuringiensis* or *Bacillus* sp. 1 *versus* insect-associated bacteria – this was used to examine the effect of removing *E. coli* from the analysis and using more ecologically relevant controls; (4) *E. coli* OP50 *versus* insect and human pathogenic bacteria; (5) *P. luminescens versus* human and insect pathogenic bacteria.

Survival of *P. pacificus* exposed to bacteria

Liquid cultures of all insect- and soil-derived bacteria as well as human and insect pathogens were grown overnight at 30°C. Bacterial suspensions (200 μl) were spread evenly on 6 cm Petri dishes with NGM medium and incubated overnight. Twenty J4 *P. pacificus* were added to each plate and stored at 25°C. Survival of worms was

monitored daily for 8 days. Nematodes were transferred every 2 days to fresh plates to prevent misidentification of original worms from offspring. Mortality was determined by prodding worms with a metal pick and nematodes that did not respond were considered dead. One hundred *P. pacificus* were exposed to each of the soil-derived and insect-derived bacteria as well as the human pathogens (*P. aeruginosa* and *S. aureus*), the insect pathogens (*X. nematophila* and *Xenorhabdus* sp. and *P. luminescens*) and *E. coli* OP50 was used as the control. *C. elegans* was also exposed to each bacterium as a comparison to *P. pacificus*. Nematodes were only exposed to bacteria at the phase 1 stage which is the most virulent and were transferred to fresh plates every 2 days to ensure nematodes would be only exposed to phase 1. Nematodes were well fed on *E. coli* OP50 before addition to pathogenic bacteria to avoid starving. Any mortality observed was due to bacterial pathogenicity and no starvation or bagging behaviour was observed.

Fecundity experiments

Twenty microlitres of overnight bacteria suspension was placed on separate Petri dishes with NGM agar and left to dry. Five single virgin hermaphrodites of *P. pacificus* were individually placed on separate dishes in the bacterial spot and were stored at 25°C. The numbers of live offspring produced by each worm was recorded daily. Worms were transferred to fresh plates daily for 4–5 days.

Defecation and residence time assays

Liquid cultures of *E. coli* OP50, *Xenorhabdus* sp. and *Bacillus* sp. 2 were cultured overnight at 30°C. Bacteria were mixed with red fluorescent 0.5 μm carboxylate-modified polystyrene latex beads (Sigma Aldrich, St Louis, MO, USA) to a final bead concentration of 0.8%. Standard 6 cm NGM agar plates were seeded with 20 μl of the bacteria–bead mixture and when dry, ten J4 *P. pacificus* or *C. elegans* larvae were added to separate dishes and allowed to feed overnight at 20°C. Cycle length was recorded by timing the intervals between defecations using a dissecting microscope with a halogen light source. For each species, ten defecations of ten individual worms were recorded.

Residence time was assessed by allowing worms to feed overnight on the bacteria–bead mixture (using methods as described above). Individual worms were transferred to fresh bacterial plates and the total time taken to clear the intestine of the fluorescent bead mixture was recorded as well as the total number of defecations. We took fluorescent images of worm faeces to determine the number of defecations with beads still present (until no further fluorescence could be observed). Ten worms were analyzed for each treatment.

Statistical analysis

Chemotaxis scores, fecundity and survival (after 7 days) were compared using one way analysis of variance (ANOVA) and differences between treatments were determined using the Bonferroni multiple comparison test using Small Stata, 9.2 (StataCorp, College Station, TX, USA). Mean defecation cycles and residence times were compared using Student's *t*-test for means. The effect of bacteria over time was analyzed using Kaplan–Meier and log rank tests.

RESULTS

Meta-genomic sequencing of individual *Pristionchus* reveals a diverse bacterial flora

A total of eight *Pristionchus* individuals (four *P. entomophagus*, four *P. lheritieri*) were isolated from soil samples from the Schönbuch forest, Tübingen (Germany). From those animals, we sequenced a total of 683 bacterial clones and identified 292 unique sequences with at least 40 different bacterial 16S sequences per individual nematode. The majority of these bacteria belonged to the Pseudomonadales, Burkholderiales, Flavobacteria and Xanthomonadales and also included the plant pathogens *Erwinia* and *Agrobacterium* and human pathogens such as *Bordetella*, *Burkholderia* and *Microbacterium* (see Tables S1 and S2 in supplementary material). Other spore-forming bacteria, such as *Bacillus* sp., cannot be detected with this approach because bacterial spores escape single worm lysis. This non-saturated bacterial sequencing approach clearly indicates that *Pristionchus* nematodes ingest an enormous diversity of bacteria. However, from this type of analysis it remains unknown if the nematodes can digest all of these bacteria as a food source.

A set of 23 bacteria from the *Pristionchus* intestine

We grew beetle- and soil-derived *Pristionchus* nematodes on rich medium and isolated a total of 23 bacterial strains (Fig. 2). Specifically, we obtained bacteria derived from each nematode–beetle system, i.e. bacteria from *P. pacificus* from the oriental beetle (collected in Carver, MA, USA), *P. maupasi* from the cockchafer (Karlsruhe, Germany) and *P. entomophagus* from dung beetles (Tübingen, Germany). Together, these bacterial isolates represent all major groups previously identified in the metagenomic sequencing approach. Bacterial isolates were sequenced for species designation and represent single species isolates.

Pristionchus prefers soil-derived bacteria to *E. coli* in chemotaxis assays

In chemotaxis experiments using soil-derived bacteria (from adult *P. entomophagus*) we found that *Pristionchus* species were equally attracted to all available *Pseudomonas* strains ($P>0.05$; Fig. 3A). *Pristionchus* species were weakly attracted to the *Pseudomonas* strains and scored chemoattration indices of 0.1–0.3. By contrast, *Bacillus* sp. 1 was highly unattractive to the *Pristionchus* nematodes. More specifically, *P. pacificus* was significantly more averse to *Bacillus* sp. 1 than *P. maupasi* and *P. entomophagus* ($P<0.001$). When exposed to *Bacillus* sp. 2, the response of the *Pristionchus* nematodes varied ($P<0.001$). *P. entomophagus* was more attracted to this bacterium than *P. pacificus* and *P. maupasi,* which showed aversion.

Chemoattraction of *Pristionchus* species exposed to beetle-derived bacteria

Next, we analyzed the chemotaxis behaviour of three *Pristionchus* species exposed to beetle-derived bacteria from three settings. In the first system (cockchafer and *P. maupasi*) we found no significant differences between the chemotaxis index scores of *P. pacificus*, *P. maupasi* and *P. entomophagus* when exposed to *Pseudomonas fluorescens*, *Pseudomonas aurantiaca*, *Bacillus megaterium*, *Stenotrophomas maltophila*, *Serratia* sp. or *Bacillus* sp. ($P>0.05$; Fig. 3B). However, when exposed to *Bacillus thuringiensis*, *Enterobacter* sp. and *Pseudomonas chlororaphis* there were significant differences between each nematode species ($P<0.05$). Most strikingly, *P. pacificus* was strongly averse to *B. thuringiensis*. From the second system (dung beetle and *P. entomophagus*), we isolated five bacteria that are usually found in animal and insect faeces (Holt et al., 2000). *P. entomophagus* was significantly more attracted to *Serratia* sp., *Enterobacter amnigenus* and *Proteus vulgaris* than *P. pacificus* and *P. maupasi* ($P<0.05$) (Fig. 4A). By contrast, *P. maupasi* and *P. pacificus* responded poorly to the *Serratia* sp., *Ochrobactrum* sp., *Enterobacter* sp. and *P. vulgaris*.

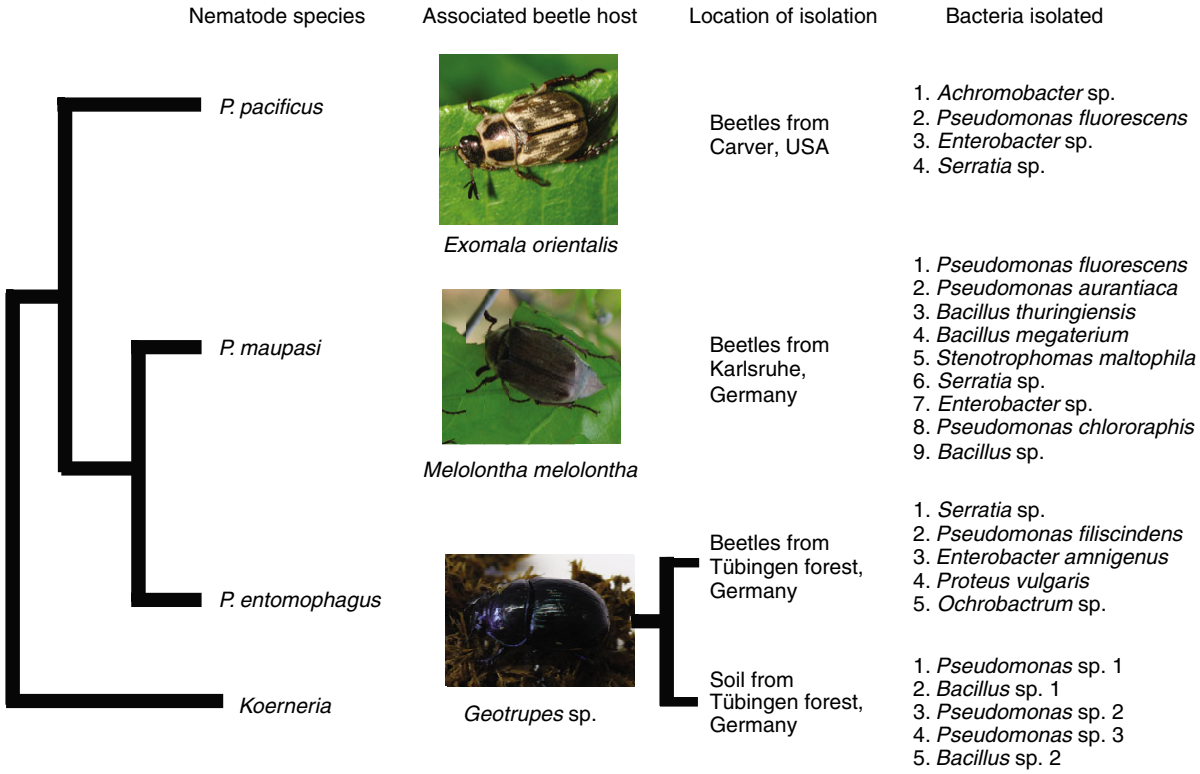


Fig. 2. Tritrophic interactions between *Pristionchus* nematodes, beetle and bacteria. Phylogeny of *Pristionchus* nematodes with associated beetle hosts detailing bacterial species isolated and used in this study.

In the third system (oriental beetle and *P. pacificus*) four bacterial species were isolated from *P. pacificus* emerging from oriental beetles including *Serratia* sp., *Achromobacter* sp., *Pantoea agglomerans* and *P. fluorescens*. These bacteria are commonly found in soil, water and the digestive tract of insects (Holt et al., 2000). All nematodes responded poorly to oriental beetle-derived bacteria, except for *P. fluorescens*, which was similarly attractive to all *Pristionchus* species (Fig. 4B). Taken together, the chemotaxis studies indicate that (1) *Pristionchus* nematodes never favour one bacterial strain completely over all others, (2) they strongly avoid certain *Bacillus* strains and (3) there is only a low *Pristionchus* species-specificity of bacteria in the chemotaxis assays.

Survival and fecundity of *P. pacificus* exposed to insect- and soil-derived bacteria

P. pacificus grown on *Bacillus* sp. 1 and *Bacillus* sp. 2 (from soil-derived nematodes) reduced brood size significantly compared to *E. coli* OP50 ($P<0.001$; Fig. 3A). This is also true for *B. thuringiensis*, *P. aurantiaca* and *Serratia* sp. from cockchafers ($P<0.05$; Fig. 3B), *Ochrobactrum*, *P. vulgaris* and *Serratia* isolated from the dung beetle ($P<0.05$; Fig. 4A) and *S. marcescens*, *P. agglomerrans* and *Achromobacter* sp. from oriental beetle ($P<0.05$; Fig. 4B).

The only bacteria that affected the survival of *P. pacificus* were *Serratia* sp. from cockchafers, and *S. marscens*, *P. agglomerrans* and *Achromobacter* sp. from the oriental beetle ($P<0.05$).

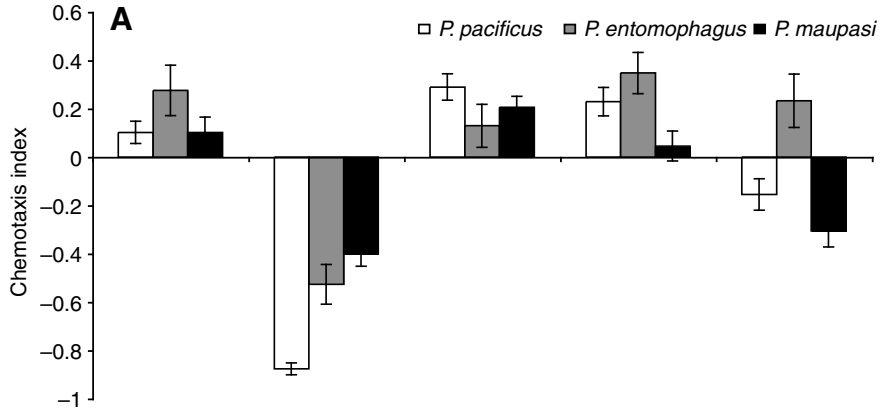
P. pacificus avoids *Bacillus* species

Next, *P. pacificus* was given the choice of either *E. coli* OP50, *B. thuringiensis* or *Bacillus* sp. 1 as a counter attractant to a range of insect-associated bacteria as an attractant. The nematode consistently avoided both *Bacillus* sp. 1 and *B. thuringiensis* and scored chemotaxis indices between 0.96–0.99 and 0.85–0.96, respectively (Fig. 5). The response to the *Bacillus* species was significantly different from the response to the *E. coli* OP50 control ($P<0.001$). These results demonstrate the revulsion *Pristionchus* has to these *Bacillus* species and provides a first indication that these nematodes might be able to avoid bacteria that have a potential harmful effect on their fitness.

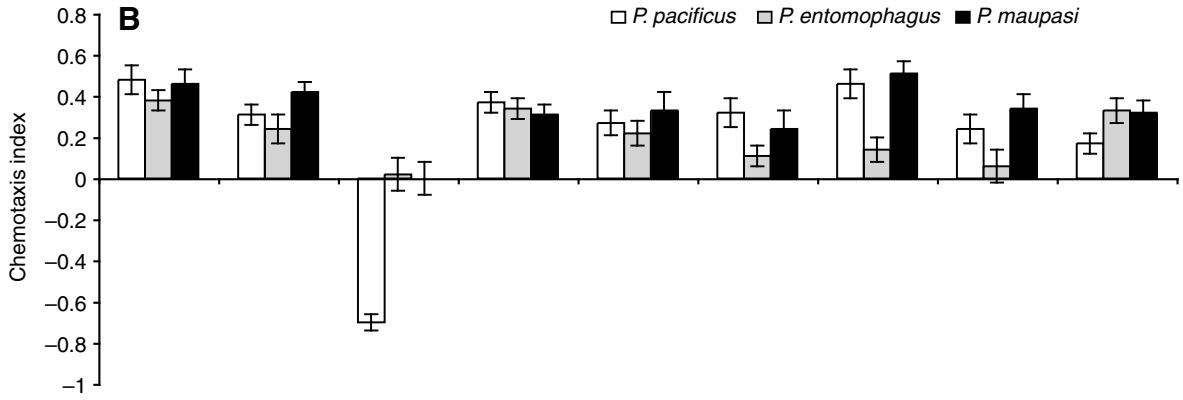
P. pacificus is susceptible to insect but not human pathogenic bacteria

We assessed survival of *P. pacificus* and *C. elegans* to human and insect pathogens. *P. pacificus* was highly susceptible to *P. luminescens*, *X. nematophila* and *Xenorhabdus* sp. but was not susceptible to *S. aureus* and *P. aeruginosa* (Fig. 6A). After 24 h, only 1±0.55 *P. pacificus* were alive compared with 12±0.87 and 19±0.19 *P. pacificus* exposed to *X. nematophila* and *Xenorhabdus* sp., respectively. *P. luminescens* is therefore the most pathogenic of the entomopathogenic bacteria to *P. pacificus*. The number of surviving *P. pacificus* exposed to *S. aureus* and *P. aeruginosa* was similar to the numbers surviving *E. coli* OP50 exposure ($P>0.05$). *C. elegans* was highly susceptible to both human and insect pathogens ($P<0.001$; Fig. 6B) which is in stark contrast to the immunity *P. pacificus* has to *S. aureus* and *P. aeruginosa*.

In chemotaxis assays *P. pacificus* was exposed to either *E. coli* OP50 (as counter-attractant) or *X. nematophila*, *Xenorhabdus* sp., *P. aeruginosa* or *S. aureus* in separate experiments. We found that *P. pacificus* strongly avoided the killer species of bacteria (*X. nematophila* and *Xenorhabdus* sp.) and the human pathogens (*S. aureus* and *P. aeruginosa*; Fig. 6C). There was no difference between the responses of the nematode to these bacteria ($P>0.05$).



Bacteria	*Pseudomonas* sp. 1	*Bacillus* sp. 1	*Pseudomonas* sp. 2	*Pseudomonas* sp. 3	*Bacillus* sp. 2	*E. coli* OP50
No. of progeny	171.2±20.5	14.8±6.8**	140.0±26.0	114.0±12.2	3.6±2.2**	123.6±6.2
Survival	17.0±0.7	17.0±0.6	16.0±0.9	18.0±0.3	16.0±0.9	17.0±0.7



Bacteria	*P. fluorescens*	*P. aurantiaca*	*B. thuringiensis*	*B. megaterium*	*S. maltophila*	*Serratia* sp.	*Enterobacter* sp.	*P. chlororaphis*	*Bacillus* sp.	*E. coli* OP50
No. of progeny	109.8±12.4	49.4±6.4**	68.2±6.4*	120.0±5.6	94.6±15.3	3.6±1.9**	135.5±17.1	118.4±11.7	33. 5±8.8**	138.5±3.8
Survival	11. 6±1.3	11.2±1.0	11.2±0.6	14.6±0.6	12.2±0.7	6.6±1.4**	14.4±0.5	13.8±1.1	13.8±1.2	14.2±0.7

Fig. 3. Chemotaxis results of *Pristionchus* exposed to soil- and insect-derived bacteria. (A) Chemotactic response of *P. pacificus*, *P. entomophagus* and *P. maupasi* after 24 h exposure to five different soil-derived bacteria isolated from adult *P. entomophagus*. Significant differences between number of live progeny and survival are shown for *P. pacificus* only (*$P \leqslant 0.05$ and **$P \leqslant 0.001$ using one way analysis of variance). Bars represent ±1 s.e.m.
(B) Chemotactic response of *P. pacificus*, *P. entomophagus* and *P. maupasi* exposed to cockchafer-associated bacteria (isolated from *P. maupasi*). Significant differences between number of live progeny and survival are shown for *P. pacificus* only (*$P \leqslant 0.05$ and **$P \leqslant 0.001$ using one way analysis of variance). Bars represent ±1 s.e.m.

When *E. coli* OP50 was replaced with *P. luminescens* as the counter-attractant, *P. pacificus* was attracted to the human pathogens. The nematode found it difficult to distinguish between *X. nematophila* and *P. luminescens*, as well as *Xenorhabdus* sp. and *P. luminescens*, which was expected as the nematodes were faced with a choice of two unpleasant toxic species (Fig. 6B).

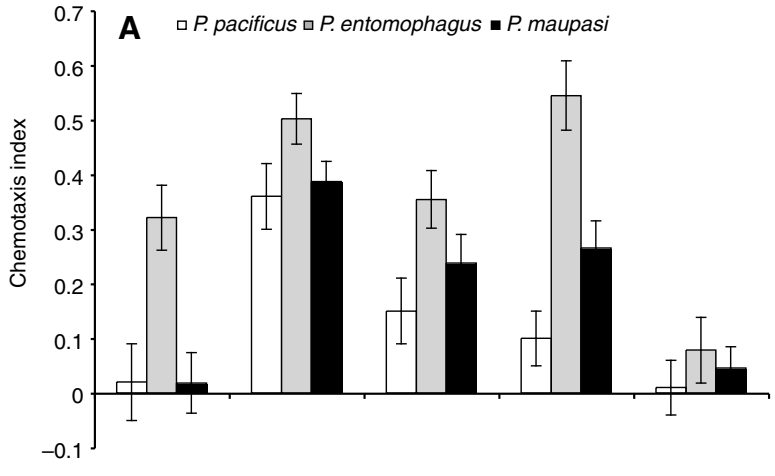
Morphological differences in nematodes influence defecation cycle and residence time of *P. pacificus*

We wanted to determine whether the absence of the grinder in *P. pacificus* affected defecation time and residence time compared with those in *C. elegans*. When *P. pacificus* is fed on *E. coli* OP50 the mean defecation time was 106±6.7 s compared to 48±1.9 s for *C. elegans* ($P<0.001$; Fig. 7A). When *P. pacificus* was fed on *Bacillus* sp. 2 the defecation cycle was significantly longer than when fed *E. coli* OP50 ($P<0.001$). There was no difference in defecation cycle when fed toxic *Xenorhabdus* sp. or *E. coli* OP50 ($P>0.05$). Conversely, the defecation cycle of *C. elegans* did not alter when fed *Bacillus* sp. 2 but was longer when fed *Xenorhabdus* ($P<0.05$). Videos demonstrating physiological differences in defecation cycles, as well as step-by-step muscle contractions can be seen in Movie 1 and Fig. S1 in supplementary material.

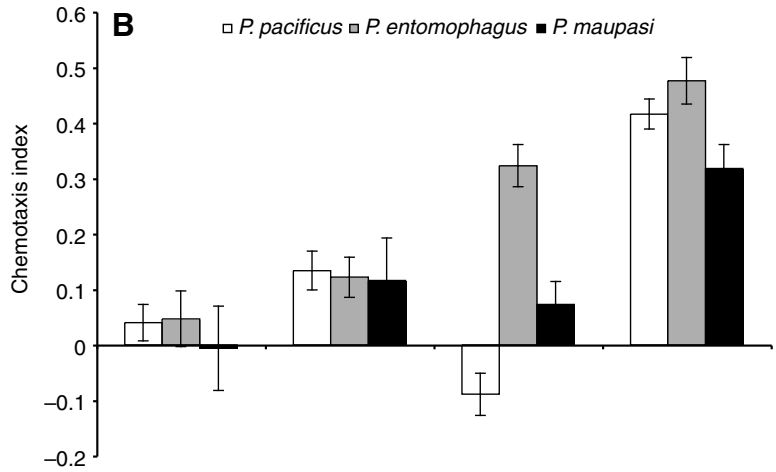
We defined the residence time as the total time recorded for a feeding worm to clear the gut of the OP50–fluorescent bead mixture after being transferred to a bacterial spot without beads. When *E. coli* OP50 was replaced with *Bacillus* sp. 2 the residence time for *P. pacificus* was significantly longer ($P<0.05$), whereas the residence time for *C. elegans* did not differ significantly ($P>0.05$; Fig. 7B).

DISCUSSION

We began an analysis of the tritrophic interactions of *Pristionchus* nematodes with bacteria associated with beetles and soil. We identified bacteria from *Pristionchus* nematodes using metagenomics and microbiological techniques and investigated the



Bacteria	*Serratia* sp.	*P. filicindens*	*E. amnigenus*	*P. vulgaris*	*Ochrobactrum* sp.	*E. coli* OP50
No. of progeny	95.0±10.8*	121.4±10.8*	135.4±7.1	84.0±12.1*	75.8±7.3**	164.7±15.4
Survival	16.8±1.1	18±1.5	19.2±0.5	18.2±0.7	17.0±0.8	18.2±0.7



Bacteria	*P. agglomerans*	*Achromobacter* sp.	*S. marcescens*	*P. fluorescens*	*E. coli* OP50
No. of progeny	21.8±7.4**	51.2±4.4**	52.0±11.1**	106.0±8.6	123.2±8.1
Survival	1.8±0.6**	9.2±1.7**	7.6±0.4**	15.6±1.2	17.8±0.9

Fig. 4. Chemotaxis results of *Pristionchus* exposed to insect-derived bacteria. (A) Chemotactic response of *P. pacificus*, *P. entomophagus* and *P. maupasi* exposed to dung beetle-associated bacteria (isolated from *P. entomophagus*) after 24 h. Significant differences between number of live progeny and survival are shown for *P. pacificus* only (*$P \leq 0.05$ and **$P \leq 0.001$ using one way analysis of variance). Bars represent ±1 s.e.m. (B) Chemotactic response of *P. pacificus*, *P. entomophagus* and *P. maupasi* exposed to oriental beetle-associated bacteria (isolated from *P. pacificus*) after 24 h. Significant differences between number of live progeny and survival are shown for *P. pacificus* only (*$P \leq 0.05$ and **$P \leq 0.001$ using one way analysis of variance). Bars represent ±1 s.e.m.

effect these bacteria have on a range of *Pristionchus* species using chemotaxis, survival and fecundity assays. We assessed survival of *P. pacificus* to human and insect pathogens and examined whether physiological functions are affected by bacterial species.

The interactions between *Pristionchus* and bacteria must be considered in the context of the necromenic life-style of these nematodes. On the death of the beetle infected with *Pristionchus* dauer larvae, bacteria and fungi rapidly colonize the beetle's body. This in turn creates a toxic 'beetle soup' consisting of large numbers of competing microorganisms originally present in the beetle intestine and the local environment. Around this time *Pristionchus* is thought to exit from the resistant dauer stage and develop into the J4 stage and then into adult form, with the main purpose to feed and reproduce. By distinguishing between an array of pathogenic and non-pathogenic bacteria and choosing the correct species to feed on, *Pristionchus* can lower the possibility of eating bacteria that cause low brood size, slow development and mortality. Once the food supply is depleted, the remaining nematodes turn into dauer larvae and search for potential beetle hosts in the surrounding soil. It is not known whether *Pristionchus* retains the bacteria it encounters before turning into the dauer form. Insect parasitic nematodes such as *S. carpocapsae* store their symbiotic bacteria *X. nematophila* in an intestinal vesicle (Martens et al., 2003) whereas *H. bacteriophora* stores *P. luminescens* in the intestine (Ciche and Ensign, 2003). Fedorko and Stanuszek (Fedorko and Stanuszek, 1971) observed *P. uniformis* dauer larva carrying bacteria cells in the gut but this observation has yet to be confirmed for *Pristionchus* species living on scarab beetles.

In general, nematodes show an enormous range of feeding strategies that allows them to occupy innumerable ecological niches (Munn and Munn, 2002). Predatory and plant parasitic nematodes have stylets, whereas soil-dwelling rhabditids, such as *C. elegans*, have a grinder in the terminal bulb of the pharynx. In contrast to *C. elegans, P. pacificus* lacks a grinder. Under laboratory conditions, bacterial lysis in the gut is incomplete and bacteria can survive the passage through the *P. pacificus* gut. We speculate that in order to gain any nutrition from these bacteria *Pristionchus* will have to increase time taken for digestion. Also, *Pristionchus* might be actively involved in bacterial dissemination in the wild, a claim that has already been made several decades ago (Chantanao and Jensen, 1968; Poinar, 1983).

The metagenomic analysis revealed that *Pristionchus* harbours a huge diversity of bacteria within its gut and on its cuticle including plant pathogenic and opportunistic human pathogens. Although mechanisms of immunity have been discovered in *C. elegans* in response to human pathogenic bacteria, *C. elegans* is susceptible to a range of naturally occurring Gram-negative and -positive bacteria and fungi (Ewbank, 2002). We have shown that *Pristionchus* associates with many different pathogenic bacteria in nature and *P. pacificus* is resistant to a number of bacteria that *C. elegans* is susceptible to, such as *S. aureus*, *P. aeruginosa* and *P. fluorescens*. Although future studies will reveal the exact molecular basis behind this resistance, the analysis of the *P. pacificus* genome provides the first insight into potential underlying mechanisms.

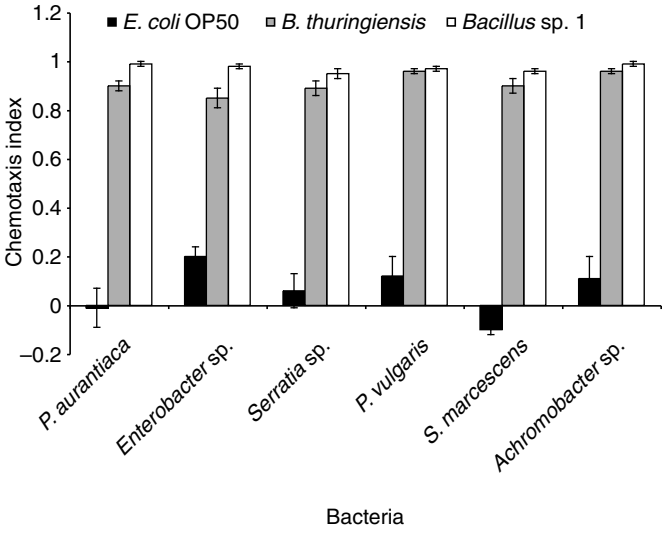
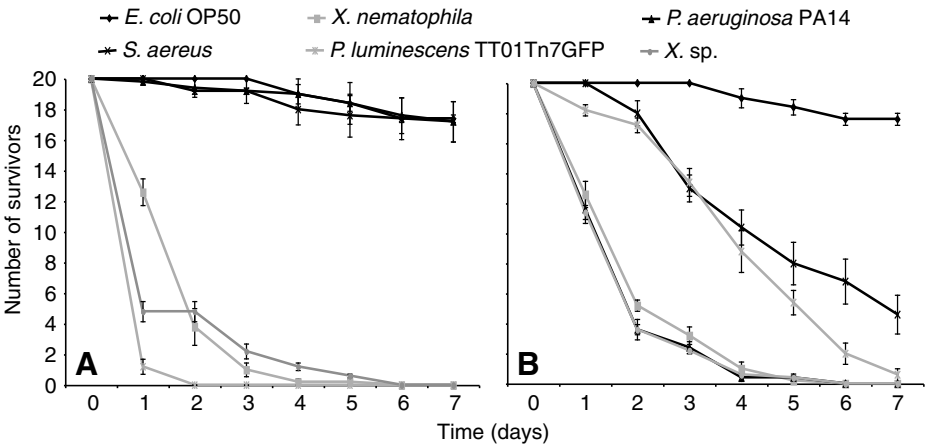


Fig. 5. Chemotaxis results of *P. pacificus* exposed to *Bacillus* species. Chemotactic response of *P. pacificus* exposed to a selection of cockchafer-, dung beetle- and oriental beetle-associated bacteria as attractants when assayed using *E. coli* OP50, *B. thuringiensis* and *Bacillus* sp. 1 as counter attractants. Bars represent ±1 s.e.m.

When compared to *C. elegans*, the *P. pacificus* genome shows a large expansion of genes encoding cytochrome P450 enzymes, glucosyl transferases, ABC transporters and other proteins that are thought to be involved in the degradation of xenobiotic compounds (C. Dieterich, S. W. Clifton, L. Schuster, A. Chinwalla, K. Delehaunty, I. Dinkelacker, R. Fulton, J. Godfrey, P. Minx, M. Mitreva et al., manuscript in revision). Future research will focus on genetic studies to elucidate methods of innate immunity, pathogenicity of associated bacteria and methods of detection and avoidance when in contact with pathogenic bacteria.

This study is one of the first to utilize both microbiological and metagenomic techniques to isolate bacteria from nematodes. Studies of other nematodes such as the slug parasitic nematode *P. hermaphrodita* have isolated 13 bacterial species from dauer larvae, culture medium and infected slugs (Wilson et al., 1995a; Wilson et al., 1995b). Bacterial communities associated with invertebrates have also been assessed using metagenomic tools. For example the bacterial communities in the hindgut paunch of a wood-feeding termite recorded 1750 bacterial 16S rRNA gene sequences that represented 12 phyla and 216 phylotypes (Warnecke et al., 2007). We did not expect to find a wealth of bacteria present in the nematode gut and on the cuticle. The function and relationship of many of these bacteria to *Pristionchus* remains unknown but from these results it can be seen that the nematodes associate with a huge diversity in nature.

In chemotaxis assays *P. pacificus* avoids species that cause ill health but the mechanism for sensing the causal properties of these bacteria currently remains unknown. We exploited this behaviour by using different *Bacillus* strains as a control and making other bacteria more attractive. Thus, the *P. pacificus* response to bacteria in chemotaxis assays differs from that of *C. elegans* in the type of



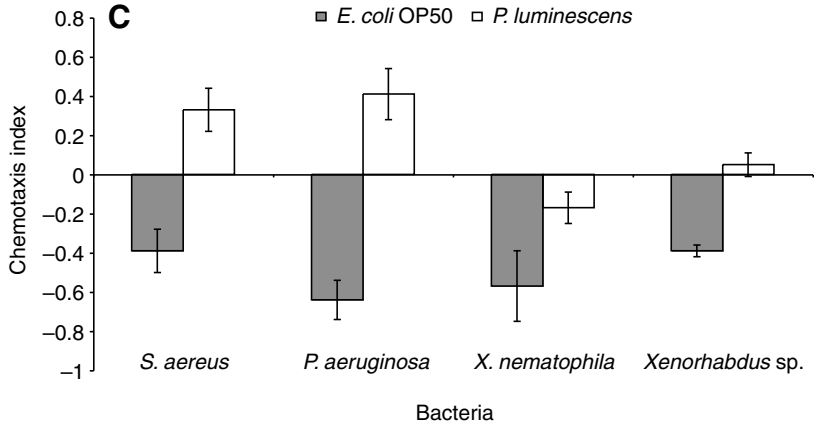


Fig. 6. Survival and chemotactic response of *P. pacificus* exposed to pathogenic bacteria. Survival of *P. pacificus* (A) and *C. elegans* (B) exposed to human and insect pathogens for 7 days. Bars represent ±1 s.e.m.
(C) Chemotactic response of *P. pacificus* exposed to human and insect pathogens with *E. coli* OP50 and *P. luminescens* as the control. Bars represent ±1 s.e.m.

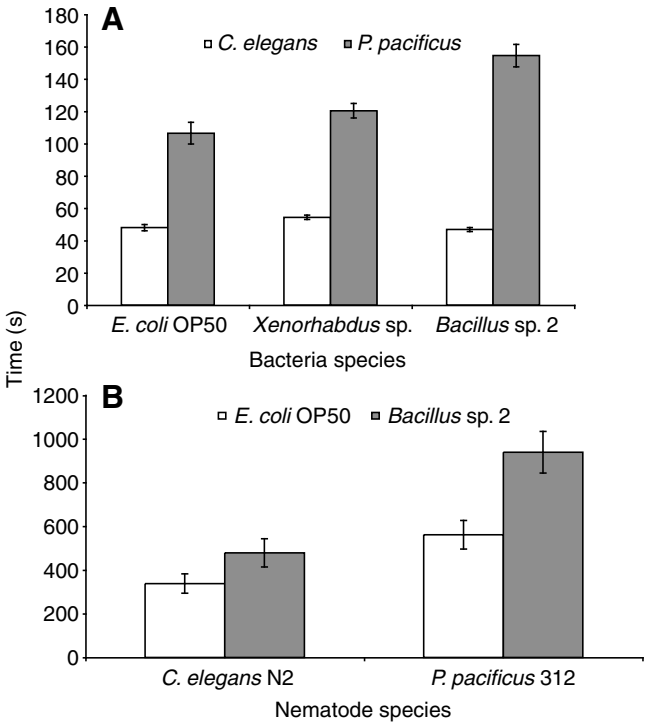


Fig. 7. Mean defecation cycle and residence time of *P. pacificus* and *C. elegans*. (A) Mean defecation time (seconds) of *C. elegans* and *P. pacificus* exposed to *E. coli* OP50, *Xenorhabdus* sp. and *Bacillus* sp. 2. Error bars represent ±1 s.e.m. (B) Mean residence time (seconds) of *E. coli* OP50 and *Bacillus* sp. 2 mixed with fluorescent beads, in *C. elegans* and *P. pacificus*. Bars represent ±1 s.e.m.

attraction (or repulsion). Similar differences have also been observed for the *P. pacificus* and *C. elegans* responses to pure insect-associated chemicals (Hong and Sommer, 2006b). Most likely, all of these differences are part of the adaptive forces that shape the tripartite interactions between bacteria, nematodes and beetle hosts in the wild.

C. elegans protects itself from potential pathogenic bacteria by avoidance behaviour and innate immunity pathways (Pujol et al., 2001; Nicholas and Hodgkin, 2004; Kurz and Ewbank, 2003). Avoidance behaviour of *C. elegans* has been recorded for *B. thuringiensis*, *S. marcescens*, *P. aeruginosa*, *P. luminescens* and *M. nematophila* using different molecular mechanisms (Pujol et al., 2001; Pradel et al., 2007; Yook and Hodgkin, 2007; Zhang et al., 2005; Beale et al., 2006; Schulenburg and Müller, 2004; Sicard et al., 2007; Hasshoff et al., 2007). In our experiments, bacteria that cause mortality to *P. pacificus* tend to score low in the chemotaxis index, ranging from –0.09±0.04 for *Serratia* sp. (from the cockchafer) to 0.37±0.05 for *S. marcescens* (from the oriental beetle). Unlike previous studies (Zhang et al., 2005) that demonstrated that *C. elegans* grown on a mixture of OP50 and *P. aeruginosa* or *S. marcescens* would avoid the two human pathogens in chemotaxis experiments, we have shown that OP50-raised *Pristionchus* species avoid *Bacillus* species without any training. Nematodes exposed to *Bacillus* species, particularly *Bacillus* sp. 1 and *B. thuringiensis* had significantly lower brood size than those grown on *E. coli* OP50. Previous studies have demonstrated that exposure of *P. pacificus* to purified Cry 5B crystal protein from *B. thuringiensis* causes a significant reduction in brood size and affects development (Wei et al., 2003).

P. pacificus is highly susceptible to *P. luminescens* and *X. nematophila* and *Xenorhabdus* sp. These bacteria are commonly found in entomopathogenic nematodes (*Steinernema* and *Heterorhabditis*) and are responsible for causing insect mortality 24–48 h after nematode penetration (Forst et al., 1997). As *Pristionchus* has a strong relationship with a number of beetle hosts that entomopathogenic nematodes can also infect, e.g. *Steinernema scarabaei,* which has been isolated from oriental beetles (Stock and Koppenhöfer, 2003), the chances of co-infection with *Pristionchus* and entomopathogenic nematodes and their associated bacteria are high. Our studies suggest that *P. pacificus* can recognize and avoid highly pathogenic bacteria such as *X. nematophila*. The ecological interaction between *Pristionchus* and entomopathogenic nematodes and bacteria clearly warrants further research.

P. entomophagus was significantly more attracted to dung beetle bacteria than to *P. maupasi* and *P. pacificus*. This was the only nematode–beetle system for which bacterial specificity was recorded, as generally the three *Pristionchus* species tested responded similarly when exposed to bacteria isolated from the respective beetle host. From this study the reasons behind nematode–beetle host specificity has not be discovered but it is not due to bacteria harboured in the beetle gut. As *Pristionchus* nematodes show a high species specificity with cockchafers, dung beetles and Colorado potato beetles (Herrmann et al., 2006a; Herrmann et al., 2006b) and display unique chemoattraction profiles towards insect pheromones and plant volatiles (Hong and Sommer, 2006b) other reasons apart from bacteria species must be considered. The behavioural response of the entomopathogenic nematode *S. carpocapsae* is correlated with nematode-induced mortality and number of infective juveniles produced on each host species (Lewis et al., 1996; Lewis, 2002). Other reasons for host specificity may include increased production of males, higher reproduction rate or better health. Also as well as bacteria living in the beetle, fungi and other parasitic or phoretic nematodes are present, perhaps these organisms contribute to nematode–beetle specificity. Further studies are needed to elucidate the exact mechanism behind these complex nematode–beetle associations.

Taken together, we have analyzed the tritrophic interactions of *Pristionchus* nematodes with various bacteria from soil and beetles. We found a range of different interactions from bacterial dissemination by the worm to reduction in brood size, longer defecation cycles and nematode mortality caused by certain bacterial strains. *Pristionchus* can recognize, respond to and avoid bacteria that cause poor health. The ability to discriminate between bacteria is important for success and survival in the soil ecosystem. Given the genetic and genomic toolkit available in *P. pacificus*, this nematode interaction with its living environment can in the future be investigated at the molecular level to provide mechanistic insight into the adaptive and non-adaptive forces that shape this nematodes ecosystem.

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