Probing the tri-trophic interaction between insects, nematodes and *Photorhabdus*

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SUMMARY

Photorhabdus sp. are entomopathogenic bacteria which, upon experimental infection, interact with the insect immune system, but little is known about the roles of their symbiotic nematode partners *Heterorhabditis* sp. in natural infections. Here, we investigated the respective contributions of nematodes and bacteria by examining humoral and cellular immune reactions of the model lepidopteran insect *Manduca sexta* against *Heterorhabditis* carrying *Photorhabdus*, nematodes free of bacteria (axenic nematodes) and bacteria alone. Insect mortality was slower following infection with axenic nematodes than when insects were infected with nematodes containing *Photorhabdus*, or the bacteria alone. Nematodes elicited host immune responses to a lesser extent than bacteria. Transcription of certain recognition and antibacterial genes was lower when insects were naturally infected with nematodes carrying no bacteria compared to insects that received bacteria, either with or without nematodes. Axenic nematodes also did not elicit such high levels of phenoloxidase activity and haemocyte aggregates as did treatments involving *Photorhabdus*. By contrast, the phagocytic capability of host haemocytes was decreased by both axenic and bacteria-associated nematodes, but not by *Photorhabdus* alone. These results imply that both bacteria and nematodes contribute separately to the pathogenic modulation of host immune responses during natural infections by the mutualistic *Heterorhabdus-Photorhabdus* complex.

Key words: insect immunity, *Manduca*, *Heterorhabditis*, *Photorhabdus*, cellular response, humoral response.

INTRODUCTION

Photorhabdus are motile Gram-negative bacteria of the family Enterobacteriacae that form a mutualistic association with soil nematodes of the genus *Heterorhabditis*. Together this complex can infect a wide range of insects. The bacteria are normally found colonizing the gut of the free-living, soil-dwelling infective juvenile (IJ) stage of the nematode. The IJs actively seek and physically penetrate a potential insect host before migrating to the insect haemocoel, the body cavity that also constitutes the open circulatory system of the insect (Klowden, 2007; Nation, 2008). The symbiotic bacteria are then regurgitated directly into the haemocoel (Ciche and Ensign, 2003), where they multiply rapidly, converting the insect biomass into a nutrient 'soup' that supports nematode growth and development. *Photorhabdus* bacteria colonize specific tissues within the insect such as the midgut and fat body causing toxaemia and septicaemia in the host by the secretion of several toxins and virulence factors that kill the insect within 48 h (Silva *et al*. 2002). After several rounds of both bacterial and nematode replication, a new generation of nematodes selectively re-packages. *Photorhabdus* and the complex emerges from the carcass in search of a new insect host. Re-association between the IJ and its specific related strain of *Photorhabdus* is specific; therefore a bacterial strain representative of one species will not re-associate and re-colonize nematodes of another species, although nematode growth and development may be supported by *Photorhabdus* from another species (Watson *et al*. 2005; Joyce *et al*. 2006).

Insects activate their innate immune system through pattern recognition receptors (Yu *et al*. 2002), which leads to the expression of highly efficient immune mechanisms involving both humoral and cellular responses against microbial infections (Kanost *et al*. 2004; Lemaitre and Hoffmann, 2007). Humoral defences include the transcriptional activation of several genes that results in the production of peptides and effector molecules with both

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recognition and antimicrobial properties (Ferrandon *et al*. 2007). Cellular responses in insects are mediated by the activity of circulating blood cells, referred to as haemocytes, which participate in multiple immune functions including phagocytosis, the formation of multicellular haemocyte aggregates and nodules, encapsulation of invading bacteria, and the production of reactive nitrogen and oxygen radicals (Jiravanichpaisal *et al*. 2006). An important player of both arms of the insect immune system is the activation of phenoloxidase (PO) through a complex proteolytic cascade that is initiated upon recognition of non-self microorganisms leading to the regulation of haemolymph coagulation and melanization, wound healing, and the release of microbicidal reactive intermediates (Cerenius *et al*. 2008).

Bacterial pathogens have developed 2 main strategies to escape the systemic immune response: they avoid detection because they lack (or hide) immune elicitors on their cell surfaces or they suppress the immune response (Vallet-Gely *et al*. 2008). It has previously been shown that *Photorhabdus* bacteria are able to evade both the antimicrobial peptides and the professional phagocytes that also patrol the haemocoel. The bacteria can interfere with haemocyte phagocytosis (ffrench-Constant *et al*. 2003), block the production of highly reactive tyrosine metabolites with bacteriostatic actions (Eleftherianos *et al*. 2007*a*, 2009) and also destroy haemocytes using specific toxins (Daborn *et al*. 2002). However, in all these studies insects were injected directly with bacteria alone, which although it simplifies interpretation of the virulence role of the bacteria, does not provide information about that of the corresponding *Heterorhabditis* nematodes.

Here, we use the *Photorhabdus-Heterorhabditis-Manduca* tripartite model to investigate insect immune responses against nematodes carrying or lacking their symbiotic bacteria and bacteria alone. The goal was to examine whether the contributions to virulence of the partners in symbiosis are quantitatively equal, and whether heterorhabditid nematodes have their own distinct effects on host immunity compared to those of their associated bacteria.

MATERIALS AND METHODS

Insects and pathogens

Larvae of the Tobacco Hornworm, *Manduca sexta* (L.) (Lepidoptera: Sphingidae), were maintained individually on a wheat germ-based artificial diet at 25 °C and a photo-period of 17 h light:7 h dark (Reynolds *et al*. 1985). Newly moulted (day 0) fifth-stage larvae were used for all experiments. Insects were naturally infected by placing them on wet filter paper onto which a suspension of nematodes had been pipetted. The nematodes had either previously been allowed to re-associate with their own symbiotic bacteria, (*Heterorhabditis bacteriophora* carrying *Photorhabdus luminescens* subsp. laumondii strain TT01, *Heterorhabditis downesi* carrying *P. temperata* strain K122, *Heterorhabditis gerrardi* carrying *P. asymbiotica* strain ATCC43949), or were axenic (i.e. the nematodes were free of live bacteria – see below). Other insects were injected with *Photorhabdus* bacteria alone. One hundred 6-day-old nematodes were suspended in 50 μl of sterile phosphate-buffered saline (PBS; 0·15 M sodium chloride, 10 mM sodium phosphate buffer, pH 7·4) and the suspension was pipetted onto wet filter paper, which supported 2 *Manduca* caterpillars.

Bacterial cultures were prepared as previously described (Eleftherianos *et al*. 2006*a*, *b*). For insect injection, 50 μl of a PBS suspension containing an estimated 1×10^3 washed *Photorhabdus* cells were injected directly into the haemocoel of *Manduca* larvae using a 100 μl disposable syringe with a 30-gauge needle. The DH5α strain of *E. coli* was used as a control in all experiments (50 μl of PBS containing approximately 3×10^7 washed DH5α cells). PBS-injected insects served as negative controls. After treatment, experimental larvae were held at 28 °C for 96 h.

Axenicized IJ nematodes

Nematodes devoid of bacteria were prepared as described by Joyce and Clarke (2003). Briefly, *Heterorhabditis downesei* IJ nematodes were surface sterilized and inoculated onto Lipid Agar plates that were pre-seeded with either their natural symbiont *P. temperata* strain K122 or their non-natural symbiont *P. luminescens* strain TT01. Similarly, *Heterorhabditis bacteriophora* IJ nematodes were surface sterilized and inoculated onto Lipid Agar plates that were pre-seeded with either their natural symbiont *P. luminescens* strain TT01 or their non-natural symbiont *P. temperata* strain K122. Plates were incubated in the dark at 28 °C and after 3 weeks IJ stages were collected from the plates. The IJs recovered from all 4 source plates were surface sterilized. Ten individual nematodes from each source were chosen randomly and individually crushed using a motorized mortar and pestle in a 1·5 ml Eppendorf containing 100 μl of LB. The contents were then transferred to LB agar and the number of colony-forming units (CFU) was counted. These counts confirmed that nematode species grown using *Photorhabdus* from another species of nematode did not re-package any bacteria either in the nematode gut or in the intracuticular spaces. Those nematodes grown on their own natural mutualistic *Photorhabdus* therefore contained only their own species of *Photorhabdus*. The surface sterilized nematodes were diluted to 100 IJs per ml in sterile PBS and were subsequently used for insect

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infections. Control infections using *Galleria mellonella*, a host that requires a Lethal Dose 50% (LD_{50}) of only 1–3 colony-forming units (CFU) of *Photorhabdus*, irrespective of its species, were used as an internal control to test for the presence of *Photorhabdus* bacteria in the axenized and re-complexed nematodes (results not shown).

RNA extraction and RT-PCR

At 18 h after infection, insects were chilled on ice for 15 min, surface sterilized with 70% ethanol and then dissected to collect the fat body tissue (approximately 100 mg), as previously described (Eleftherianos *et al*. 2006*a*, *b*). To isolate total RNA, extracted tissues were homogenized in TRI reagent (Sigma, UK). Single-step reverse transcription (RT)-PCR was performed with the 'OneStep' RT-PCR kit (Qiagen, UK). Each reaction was carried out in a 50 μl volume containing 0·6 μM of forward and reverse gene primers and 2 μg of RNA template (Eleftherianos *et al*. 2006*a*, *b*). Primers were gene-specific and their sequences have been given before (Eleftherianos *et al*. 2006*a*, 2007*a*). Amplifications were performed on a PTC-100 thermal controller (MJ Research, USA) under cycling conditions as previously stated (Eleftherianos *et al*. 2006*a*, 2006*b*). RT-PCR control reactions for ribosomal protein S3 (rpS3) (Jiang *et al*. 1996) were performed as before (Eleftherianos *et al*. 2006*a*, *b*). The control experimental treatment was fat body tissue from insects injected with PBS. An RT-PCR control without template was also included. In both of these controls, the levels of gene transcripts of the immune-related genes were too low to detect (not shown). Gene transcription levels were assessed in duplicate for each treatment and the reactions were repeated 3 times.

Numbers of haemocytes and bacteria

Day 0 fifth-stage *Manduca* larvae were infected as previously described and 18 h later were chilled on ice, cut at the midpoint of the dorsal horn and bled into pre-chilled sterile polypropylene tubes. A standard volume of haemolymph (500 μl) was extracted from each caterpillar. Aliquots of haemolymph (50 μl) were immediately added to pre-chilled Grace's Insect Medium (GIM – Sigma, UK) (450 μl). Haemocyte numbers were counted at 100× using a Neubauer haemocytometer.

To determine the number of surviving bacteria, serial dilutions of the haemolymph were plated onto Petri dishes containing 2·5% Luria-Bertani (LB) and 1·5% agar (Difco Laboratories, USA) and the number of *E. coli* CFU was recorded 24 h later. The average number of 2 counts per insect of 9 insects per treatment was calculated and the experiment was replicated 3 times.

Haemocyte viability

Manduca larvae were chilled at 18 h after infection, surface sterilized and bled into pre-chilled GIM and cell density was adjusted accordingly to 1×10^6/ml using a haemocytometer. Haemocyte suspensions (100 μl) were seeded onto each cover-slip (10 mm in diameter) and left for 30 min at room temperature to allow haemocytes to attach. The monolayers were washed 3 times with GIM then incubated in single wells of a 24-well plate (Nunc, UK) in 400 μl of fresh GIM and kept at room temperature. To assess haemocyte viability, monolayers were removed from the wells, washed 3 times with GIM, stained with 0·2% (w/v) Trypan blue (Sigma, UK) in PBS for 10 min at room temperature, washed 3 times with PBS and then fixed with 4% paraformaldehyde in PBS. Trypan blue-stained and total cell counts were visualized and recorded with a light microscope at 200× magnification. The mean of 2 monolayers per insect of 9 insects per treatment for 3 independent experiments was estimated.

Phenoloxidase activity

Insects were infected and bled as before. Phenoloxidase (PO) activity in the haemolymph of day 0 fifth-stage *Manduca* larvae was quantified by using a microplate enzyme assay as described previously (Eleftherianos *et al*. 2006*b*). Briefly, a reaction mixture containing 115 μl of 50 mM PBS buffer (pH 6·5), 10 μl of diluted haemolymph plasma, and 2 μl of *E. coli* LPS (5 mg/ml) (Sigma, UK) was left for 1 h on a shaker at slow speed (1 rpm) and at room temperature to allow the activation of prophenoloxidase (PPO) to its active form, and the reaction started by adding 25 μl of 20 mM 4-methyl catechol (Sigma, UK). The change in absorbance was read at 490 nm for 1 h at room temperature with a reading taken every 1 min. Eight insects were used per treatment and the experiment was repeated 3 times.

Haemocyte phagocytosis

E. coli bacteria were stained using the 'BacLight' green bacterial stain kit (Molecular Probes, UK) as previously described (Eleftherianos *et al*. 2007*b*). Insects were infected as before and 2 h later injected with 1×10^3 green fluorescent *E. coli* cells. Haemolymph samples were extracted after 18 h. Haemocyte monolayers were prepared as previously described (Eleftherianos *et al*. 2007*b*). Monolayers were incubated overnight at 4 °C in hybridoma supernatant containing the monoclonal antibody MS77 (Willott *et al*. 1994), without dilution. Cells were then incubated with secondary antibody solution (TRITC-conjugated goat anti-mouse IgG) (Sigma, UK) at 1 : 100 (v/v) dilution in 0·3% bovine serum albumin (BSA) in PBS. Cover-slips were finally mounted at

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room temperature in 5 μl of Mowiol-4-88 solution (Calbiochem, UK). Control slides were incubated with the secondary antibody only. The phagocytic competence of haemocytes from infected day 0 fifth-stage *Manduca* larvae and the numbers of ingested *E. coli* cells per haemocyte were examined using a Zeiss LSM-510 laser scanning confocal microscope. Nine insects were used for each treatment and the experiment was replicated 3 times.

Nodule formation

Day 0 fifth-stage *Manduca* larvae were infected with *Heterorhabditis* with or without *Photorhabdus* and with bacteria alone, and nodule formation was assessed after 18 h as described before (Eleftherianos *et al*. 2007*b*). Melanized nodules within the haemocoel were observed using a stereomicroscope and nodule numbers were manually counted with the help of a tally counter. Nine insects per treatment were examined and the experiment was repeated 3 times.

Haemocyte aggregation

Insects were infected as before and 18 h later were bled (100 μl haemolymph) into 1·5 ml polypropylene tubes containing *Manduca* buffered saline solution (MBS; 4 mM NaCl, 40 mM KCl, 18 mM $MgCl_2$·6H20, 1·5 mM Na_2HPO_4, 1·5 mM NaH_2PO_4, 192·8 mM sucrose, pH 4·5). Haemocytes were centrifuged at 200 ***g*** for 10 min at 4 °C and the cell pellets were resuspended in 200 μl of cold GIM. Cell density of the suspensions was adjusted to 1×10^6 cells/ml using a haemocytometer, samples were transferred to individual wells of a 96-well microtitre plate and the micro-aggregates were counted using a Nikon TMS-F inverted microscope. Microaggregates of 5 or more cells were counted as incipient nodules. The mean of 9 larvae per treatment was calculated and the assay was replicated 3 times.

Statistical analysis

All values were expressed as means ± standard deviation. Statistics were performed using the commercial computer software package GraphPad Prism version 5·01 for Windows (GraphPad Software, San Diego California, USA). For data analyses, means were compared using a one-way analysis of variance (ANOVA) with a Tukey *post-hoc* test for multiple comparisons. The effect of infection with *E. coli* on the ability of these bacteria to survive in *Manduca* haemolymph, compared to pre-treatment with PBS, was determined using the unpaired two-tailed Student's *t*-test. Comparison between survival curves (fraction death) was conducted using a Log-rank (Mantel-Cox) test. *P* values below 0·05 were considered statistically significant.

RESULTS

Insect survival decreases upon infection with axenic Heterorhabditis

We compared survival between insects injected with a standard dose of *Photorhabdus*, bacteria alone, and insects naturally infected with either axenic *Heterorhabditis* or nematodes carrying their own symbiotic bacteria (Fig. 1). In each case, there were large differences in percentage survival between *Heterorhabditis*-infected larvae and those challenged with bacteria alone or the nematode-bacterium complex from 36 h or 48 h and up to 96 h after infection. In particular, we found significant differences in survival curves between axenic and non-axenic nematode treatments (for example comparison between the Hb and Hb + TT01 survival curves: Mantel-Cox test, $\chi^2 = 8{\cdot}77$, $P < 0{\cdot}05$). However, there were no differences in survival curves between the *Photorhabdus* (TT01, K122 or ATCC43949) and the *Heterorhabditis* + *Photorhabdus* (Hb + TT01, Hd + K122 or Hg + ATCC43949) treatments (Mantel-Cox test, $P > 0{\cdot}05$). Negative controls including injection of PBS or a suspension of *E. coli* in PBS caused no mortality (results not shown).

Immune gene mRNA levels are lower in insects infected with axenic nematodes than with bacteria-associated nematodes

In order to determine the mRNA transcription of 3-pattern recognition protein genes (*haemolin*, *immulectin-2* and *peptidoglycan recognition protein-1A*), the precursor of the phenoloxidase enzyme (*prophenoloxidase-2*) and 1 antibacterial effector gene (*cecropin*), we used semi-quantitative RT-PCR on RNA extracted from fat body of day 0 fifth-stage *Manduca* larvae infected with axenic nematodes, nematodes carrying symbiotic *Photorhabdus* or bacteria alone (Fig. 2). We found that 18 h after challenge with axenic nematodes immune genes were either transcribed at levels too low to detect in our experiments, or were transcribed at lower levels (*immulectin-2*) compared to the other treatments. Control insects injected with PBS showed undetectable levels of all 5 transcripts (results not shown). Transcription of the ribosomal protein S3 gene (*rpS3*) was used as a loading control.

Infection with axenic nematodes suppresses Manduca PO activity, nodulation and haemocyte aggregation

We also measured PO activity within haemolymph extracted from 18 h infected *Manduca* (Fig. 3A). PO levels were significantly higher in cell-free haemolymph plasma from insects infected by axenic *Heterorhabditis*, the *Heterorhabditis-Photorhabdus* complex or the pathogenic bacteria alone compared to the PBS control (one-way ANOVA, D.F. = 9,70,

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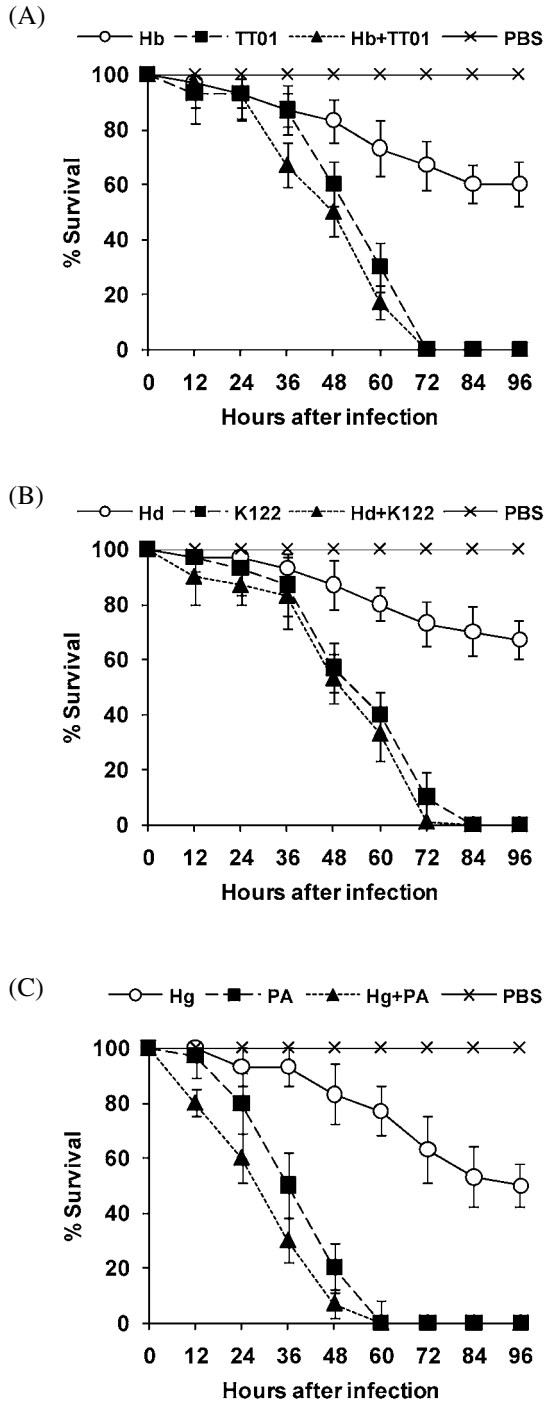


Fig. 1. Insect survival following challenge with axenic nematodes, nematodes carrying symbiotic bacteria and pathogenic bacteria alone. Day 0 fifth-stage *Manduca* larvae were infected with 100 six-day-old axenic *Heterorhabditis bacteriophora* (Hb), *Heterorhabditis downesi* (Hd) or *Heterorhabditis gerrardi* (Hg) nematodes, 1×10^3 cells of *Photorhabdus luminescens* strain TT01, *Photorhabdus temperata* strain K122, or *Photorhabdus asymbiotica* (PA) strain ATC43949, or 100 nematodes carrying their cognate symbiotic bacteria (Hb + TT01, Hd + K122 and Hg + ATCC43949). Insect mortality was then monitored for 96 h. Note that nematodes lacking bacteria symbionts decrease insect survival, but they are less able to kill insect larvae compared to pathogenic bacteria and *Heterorhabditis* carrying *Photorhabdus*.

$F = 33{\cdot}650$; $P < 0{\cdot}0001$; Tukey *post-hoc* test, $P < 0{\cdot}05$). In all cases, PO values were lower than for the *E. coli* treatment (one-way ANOVA, D.F. = 9,70, $F = 56{\cdot}487$; $P < 0{\cdot}0001$; Tukey *post-hoc* test, $P < 0{\cdot}05$). It should be noted, however, that the number of *E. coli* cells initially injected was considerably greater than for *Photorhabdus*, so that the difference between these bacteria is not necessarily functionally significant, but the *E. coli* treatment nevertheless serves as a positive control. This result indicates that PO activity is less than maximal in insects infected with nematodes alone. The reason for this is not known, but indicates either that nematodes are not recognized by the PO-activating system, or that they produce inhibitor(s) of PPO activation or of activated PO. There were no differences between different strains of *Heterorhabditis* (one-way ANOVA, $P > 0{\cdot}05$), implying that all are recognized to the same extent.

PO activity was also submaximal (i.e. less than following *E. coli*) in insects infected with all 3 *Photorhabdus* sp. (one-way ANOVA, D.F. = 3,28, $F = 58{\cdot}641$; $P < 0{\cdot}0001$; Tukey *post-hoc* test, $P < 0{\cdot}05$), despite the fact that all the bacterial treatments would have led to proliferation of bacteria within the insect's haemocoel with ultimately lethal effects. This may reflect the fact that *Photorhabdus* is known to produce inhibitors of activated PO (Eleftherianos *et al*. 2007*a*, 2009).

Interestingly, PO values in haemolymph from larvae naturally infected with axenic *H. downesi* nematodes were significantly lower than PO values from insects infected with the same nematodes carrying *P. temperata* strain K122 bacteria or *P. temperata*-injected individuals (one-way ANOVA, D.F. = 2,21, $F = 36{\cdot}170$; $P < 0{\cdot}0001$; Tukey *post-hoc* test, $P < 0{\cdot}05$), whereas this was not the case for the other 2 *Photorhabdus* species. This may reflect a lower PO-inhibitory ability of this *Photorhabdus* species, or a different rate of proliferation of the bacterium within the host. No significant differences in haemolymph PO activity were noted among insects infected with the other pathogens (one-way ANOVA, $P > 0{\cdot}05$).

At 18 h post-infection we also estimated the extent of nodulation within dissected caterpillars (Fig. 3B). Again, we found that both nematodes and symbiotic bacteria of all 3 species pairs elicited higher numbers of nodules than the PBS control treatment, implying that both are recognized by the nodule forming system. As before, we found that a significantly smaller number of melanotic nodules was formed in tissues of *Manduca* infected with *Heterorhabditis* associated with *Photorhabdus*, axenic *Heterorhabditis*,

Bars show means ± standard deviations (S.D.) ($n = 3$ replicated per point, 10 insects were used per replicate). PBS injection was used as a negative control.

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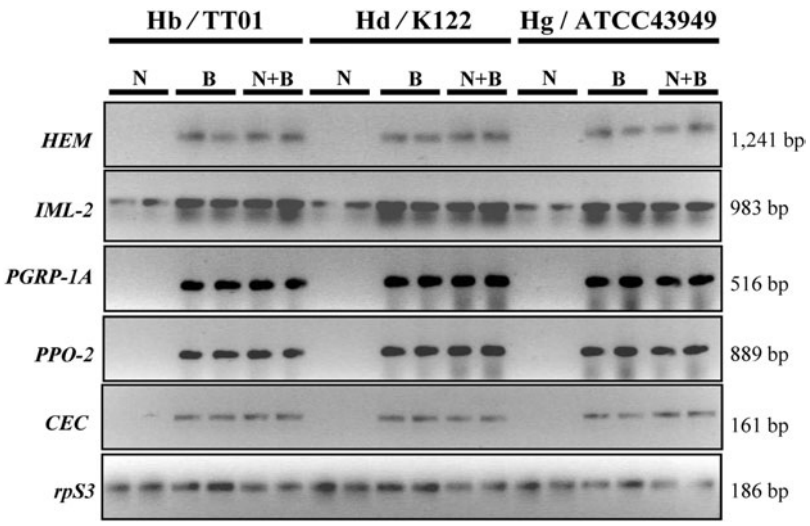


Fig. 2. Immune gene transcription in day 0 fifth-stage *Manduca* larvae. Genes tested were: *hemolin* (*HEM*), *immulectin-2* (*IML-2*), *peptidoglycan recognition protein-1A* (*PGRP-1A*), *prophenoloxidase-2* (*PPO-2*) and *cecropin* (CEC). Insects were infected with axenic *Heterorhabditis* (N), *Photorhabdus* alone (B) or *Heterorhabditis* carrying symbiotic *Photorhabdus* (N + B). Abbreviations as in Fig. 1. Semi-quantitative RT-PCR was used to assess levels of mRNA in fat body tissue 18 h following the experimental infection. Note that transcription of immune genes decreases in insects infected with axenic nematodes. Sizes of the PCR products are indicated. PCR amplifications used 35 cycles for each combination of primers and templates. A loading control (*Manduca sexta* ribosomal protein S3 – *rpS3*) was used to ensure equal loading in each lane. Each RT-PCR experiment was performed in duplicate.

or injected only with symbiotic bacteria, than in tissues of larvae injected with non-pathogenic *E. coli* (one-way ANOVA, D.F. = 9,80, $F = 74{\cdot}503$; $P < 0{\cdot}0001$; Tukey *post-hoc* test, $P < 0{\cdot}05$). Again, this probably reflects the larger number of *E. coli* cells that had been injected, but also clearly indicates that the nodulation response is submaximal following infection by all 3 *Heterorhabditis-Photorhabdus* species pairs. Notably, for all 3 *Photorhabdus* species, the number of dark nodules was significantly less in tissues of insects infected with axenic nematodes compared to nematodes with bacteria, or bacteria alone (one-way ANOVA, D.F. = 8,72, $F = 30{\cdot}815$; $P < 0{\cdot}0001$; Tukey *post-hoc* test, $P < 0{\cdot}05$). This indicates either that nematodes are much less well recognized than bacteria, or despite being recognized are much less efficient in provoking a nodulation response. In evaluating this difference, it should be borne in mind that the *Photorhabdus* bacteria would have multiplied in number within the host during the 18 h since infection, while the nematodes would not. There was no difference in the extent of nodulation in insects infected with *P. temperata* K122, compared to the other two *Photorhabdus* species (one-way ANOVA, $P > 0{\cdot}05$), which implies that all 3 bacteria are equally well recognized, and suggests that the previously observed strain-related difference in PO activity may be due to a lesser ability of K122 to inhibit PO activation or activity.

Next, we examined the *in vivo* ability of washed haemocytes to form aggregates around naturally infected *Heterorhabditis* nematodes or injected *Photorhabdus* bacterial cells (Fig. 3C). This is commonly taken to be a measure of nodule formation that is distinct from melanization (Miller and Stanley, 1998). Again, we noticed that the number of haemocyte micro-aggregates in insects infected with nematodes, with or without symbiotic bacteria, or *Photorhabdus* alone, was significantly greater than for the PBS controls (one-way ANOVA, D.F. = 9,80, $F = 8{\cdot}399$; $P < 0{\cdot}0001$; Tukey *post-hoc* test, $P < 0{\cdot}05$). Although the number of micro-aggregates following infection with axenic *Heterorhabditis* was somewhat lower than the other treatments, which involved *Photorhabdus* bacteria with or without nematodes, these values did not differ significantly (one-way ANOVA, $P > 0{\cdot}05$). These results clearly show that both nematodes and their symbiotic bacteria are recognized by the system that initiates haemocyte aggregation. The number of micro-aggregates was, however, lower in all cases than following injection of *E. coli* (one-way ANOVA, D.F. = 9,80, $F = 29{\cdot}393$; $P < 0{\cdot}0001$; Tukey *post-hoc* test, $P < 0{\cdot}05$). As previously noted, this result is not necessarily significant in itself, since the number of *E. coli* bacteria injected was much greater than the number of either nematodes or *Photorhabdus*, but does indicate that haemocyte aggregation is submaximal for all 3 *Heterorhabditis-Photorhabdus* species pairs.

The Heterorhabditis-Photorhabdus *complex reduces haemocyte viability and phagocytosis in* Manduca

We recorded the total numbers of circulating haemocytes recovered from *Manduca* fifth-stage larvae infected with axenic nematodes, nematodes

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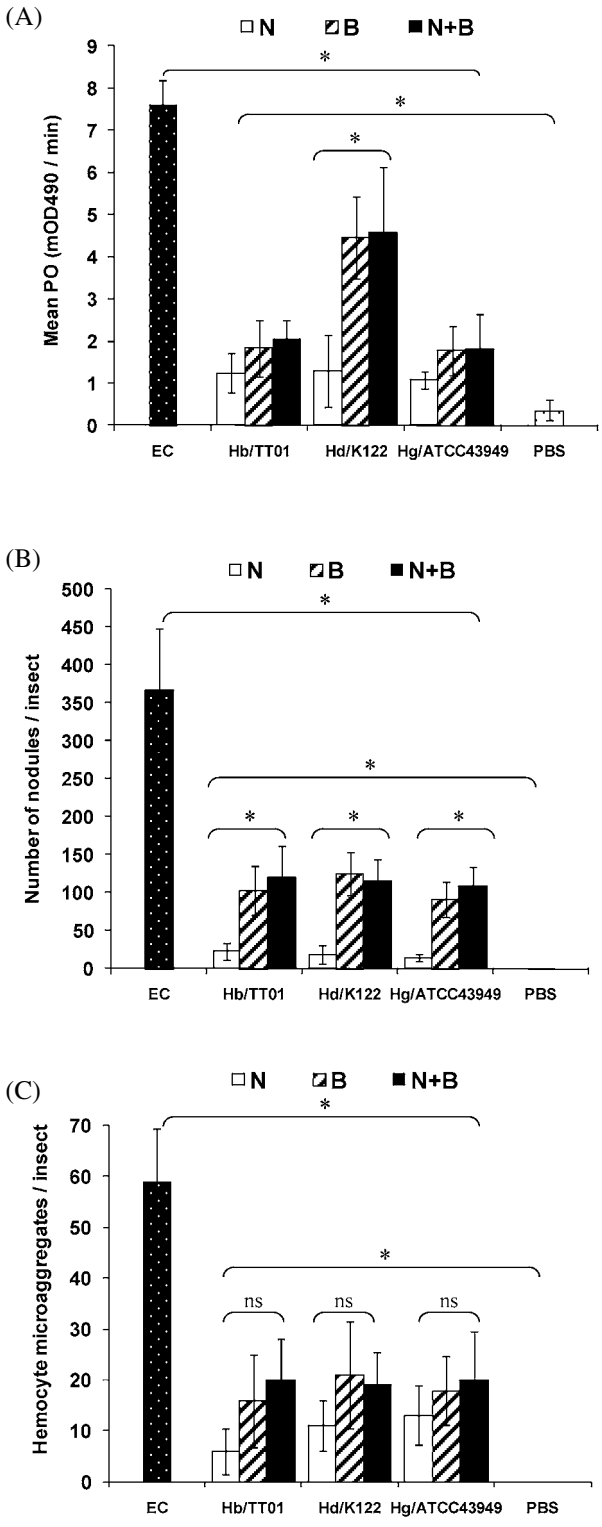


Fig. 3. (A) Phenoloxidase (PO) activity, (B) number of melanotic nodules and (C) number of haemocyte micro-aggregates in day 0 fifth-stage *Manduca* larvae 18 h after infection with axenic nematodes alone (N), bacteria alone (B) or nematodes with symbiotic bacteria (N + B). Abbreviations as in Fig. 1. Injections with *E. coli* (3×10^7 washed DH5α cells) and PBS were used as positive and negative controls, respectively. Note the reduced PO activity, nodulation and haemocyte aggregation in insects infected with axenic nematodes compared to the other treatments. Bars show means ± S.D. ($n = 3$ replicates). Significant differences are indicated with an asterisk (ANOVA, $^*P < 0{\cdot}0001$); ns denotes non-significant differences between treatments.

carrying bacterial symbionts, or *Photorhabdus* alone (Fig. 4A); for all 3 species pairs, we found no significant differences between all these treatments at 18 h after the infection (one-way ANOVA, $P > 0{\cdot}05$). Although total haemocyte counts in insects challenged with axenic *Heterorhabditis*, non-axenic nematodes, or *Photorhabdus* were not different from the PBS control (one-way ANOVA, $P > 0{\cdot}05$), they were significantly lower than in those insects subjected to *E. coli* injection (one-way ANOVA, D.F. = 9,80, $F = 14{\cdot}812$; $P < 0{\cdot}0001$; Tukey *post-hoc* test, $P < 0{\cdot}05$). We previously noted (Au *et al.* 2004) that in *Manduca*, total haemocyte numbers increase after injection of *E. coli*, although the time-course was different to that seen here. Au *et al.* (2004) also observed that injections of *P. temperata* K122 and *P. luminescens* W14 were followed by falls in the total number of haemocytes. It is not clear why this did not happen here.

In the same experiment, we counted numbers of viable haemocytes (i.e. those able to adhere to a glass surface and form a monolayer) (Fig. 4B); the results revealed that *Manduca* larvae naturally infected with any of the 3 species of *Heterorhabditis* carrying their own species of *Photorhabdus*, or injected with *Photorhabdus* alone, yielded significantly fewer viable haemocytes than did larvae infected only with axenic nematodes (one-way ANOVA, D.F. = 8,72, $F = 23{\cdot}909$; $P < 0{\cdot}001$; Tukey *post-hoc* test, $P < 0{\cdot}05$). This is consistent with the previous finding that *Photorhabdus* bacteria produce toxins that have adverse effects on haemocyte viability (Au *et al.* 2004; Vlisidou *et al.* 2009).

We also estimated the number of phagocytic haemocytes in treated insects. To do this, we pre-treated insects with the various pathogens, and subsequently injected them with a standard dose of fluorescent-labelled *E. coli* and assessed the number of labelled bacteria that could later be seen inside haemocytes. Strikingly, for all 3 *Heterorhabditis-Photorhabdus* species pairs, we found that the number of haemocytes containing fluorescent bacterial cells (Fig. 4C) was significantly lower in insects that had been pre-infected with axenic *Heterorhabditis*, or with nematodes carrying *Photorhabdus*, compared to the number of phagocytic haemocytes in larvae previously injected with *Photorhabdus* alone (one-way ANOVA, D.F. = 8,72, $F = 78{\cdot}972$; $P < 0{\cdot}0001$; Tukey *post-hoc* test, $P < 0{\cdot}001$). The number of phagocytic haemocytes did not differ between those insects that had been pre-treated with any of the 3 species of *Photorhabdus* alone, and the 2 controls that had been pre-injected with either *E. coli* or PBS (one-way ANOVA, $P > 0{\cdot}05$). This clearly indicates that all

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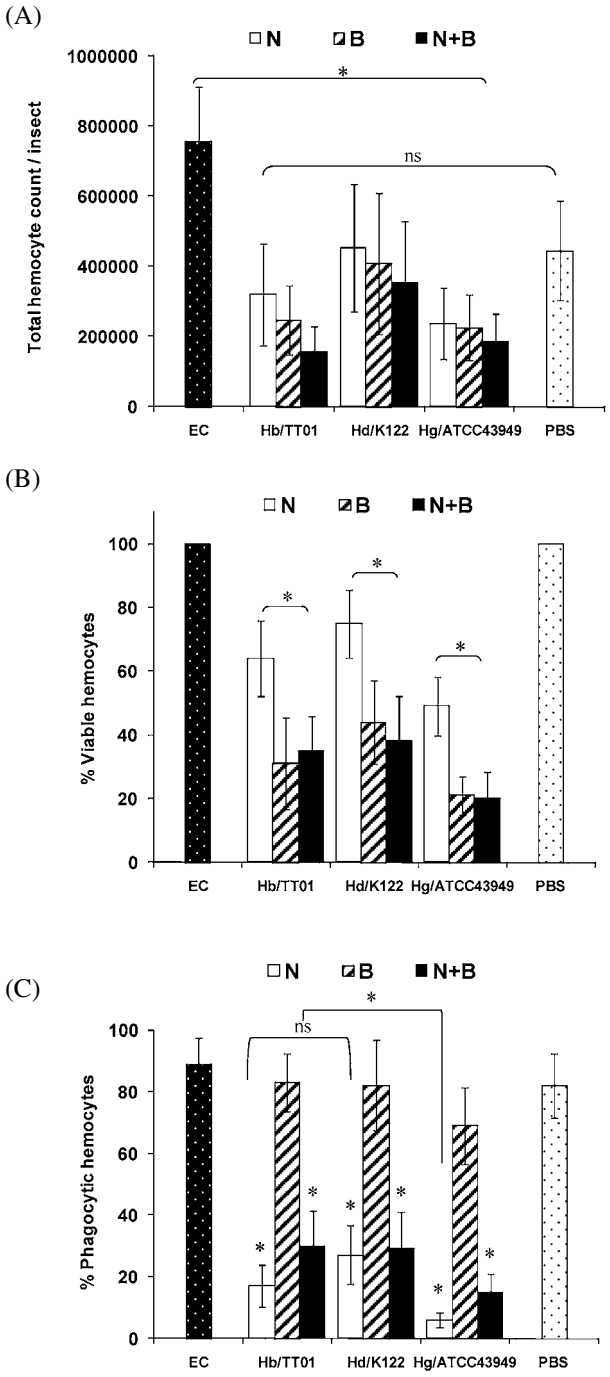


Fig. 4. (A) Total numbers of haemocytes, (B) viable haemocytes and (C) percentage of phagocytic haemocytes recovered from *Manduca* larvae 18 h post-infection with axenic nematodes alone (N), bacteria alone (B) or nematodes with symbiotic bacteria (N+B). Abbreviations as in Fig. 1. As before, injections with *E. coli* and PBS were the positive and negative controls, respectively. Note the increase in free haemocytes after *E. coli* injection and the decrease following infection with *Photorhabdus*. Also, note the decrease in the number of viable haemocytes following infection with *Photorhabdus* alone or with nematodes carrying pathogenic bacteria, and the decrease in numbers of phagocytic haemocytes in insects infected with axenic nematodes or nematodes carrying *Photorhabdus*. Bars show means ± S.D. ($n=3$ replicates). The asterisks show a value that is significantly different from the other treatments (ANOVA, $*P<0{\cdot}0001$) and ns denotes non-significant differences between treatments.

3 heterorhabditids, but not their associated *Photorhabdus* spp., are able to depress the phagocytic capability of the insect host. The number of phagocytic cells was also significantly lower in insects pre-infected by axenic *H. gerrardi* nematodes compared to the other 2 axenic nematode treatments (one-way ANOVA, D.F. = 2,24, $F=19{\cdot}808$; $P<0{\cdot}0001$; Tukey *post-hoc* test, $P<0{\cdot}5$). This may indicate that this species of *Heterorhabditis* is particularly potent in suppressing phagocytosis.

Entomopathogenic nematodes and symbiotic bacteria affect phagocytosis and clearance of E. coli *in vivo*

We looked at the ability of infected insects to remove bacteria from the circulation. To do this we first counted numbers of fluorescence-labelled *E.coli* cells in phagocytic haemocytes extracted from *Manduca* that had been previously infected with the pathogens (Fig. 5A). We found that the number of *E. coli* cells contained in haemocytes from larvae that had been pre-infected with nematodes carrying or lacking symbiotic bacteria was significantly lower than the number of bacterial cells in haemocytes from insects pre-injected with pathogenic *Photorhabdus* alone (one-way ANOVA, D.F. = 8,72, $F=36{\cdot}151$; $P<0{\cdot}0001$; Tukey *post-hoc* test, $P<0{\cdot}05$). No statistically significant differences were observed between numbers of ingested labelled *E. coli* in haemocytes from insects that had been pre-injected with any of the *Photorhabdus* strains alone, with *E. coli*, or with PBS (one-way ANOVA, $P>0{\cdot}05$). These results indicate that all 3 nematode species inhibit the extent of phagocytosis by host haemocytes, whether or not their symbiotic *Photorhabdus* bacteria are present, and that *Photorhabdus* alone does not have this effect. This is consistent with the result of the previous experiment showing that nematodes (but not bacteria) reduce the number of phagocytic cells.

Finally, we determined the influence of infection with nematodes and symbiotic bacteria on the ability of harmless bacteria to survive in *Manduca* hemolymph. To do this, we pre-infected insects with nematodes, symbiotic bacteria, or both, and 2 h later injected a large number of *E. coli* cells. We then estimated the number of viable cells recoverable from the haemolymph of insects as colony forming units (CFU) (Fig. 5B). As we previously found (Eleftherianos *et al.* 2006*a*), we noticed that pre-treatment of the insects with *E. coli* caused a significant decrease in the survival of bacteria that were injected later, compared to pre-treatment with PBS (Unpaired *t*-test, D.F. = 16, $t=9{\cdot}976$; $P<0{\cdot}0001$). This is evidence for 'priming' of the insect's innate immune system.

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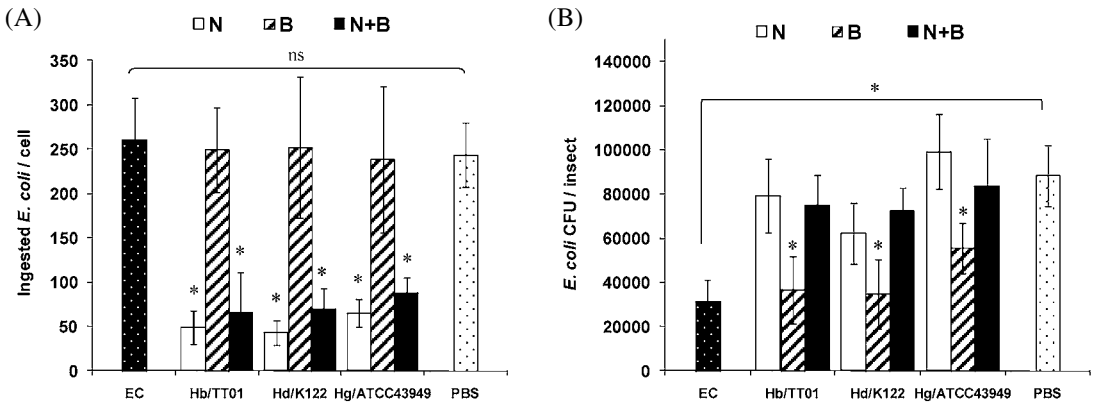


Fig. 5. (A) Numbers of *E. coli* ingested cells and (B) numbers of recoverable *E. coli* bacteria (CFU, colony forming units) from day 0 fifth-stage *Manduca* first infected with axenic nematodes (N), bacteria alone (B) or nematodes with symbiotic bacteria (N + B) and 2 h later challenged with *E. coli*-labelled bacteria. Larvae were bled 18 h following the first infection. Abbreviations as in Fig. 1. Injections with *E. coli* alone and PBS served as controls. Note the decrease in the number of engulfed *E. coli* cells and the increase in the number of recoverable *E. coli* bacteria in insects infected with axenic nematodes or nematodes carrying *Photorhabdus* compared to the treatment involving injection of pathogenic bacteria alone. Bars show means ± S.D. ($n = 3$ replicates, 20 haemocytes per insect were examined). The asterisk shows a value that is significantly different from the other treatments (ANOVA, $*P < 0{\cdot}0001$) and ns denotes non-significant differences between treatments.

When we treated the insects with components of the *Heterorhabditis-Photorhabdus* system either together or alone, however, we found that significantly greater numbers of live *E. coli* cells (Fig. 5B) were recoverable from haemolymph samples when the pre-treatment had involved natural infection by *Heterorhabditis*, with or without their bacterial symbionts, than from insects injected with *Photorhabdus* only (one-way ANOVA, D.F. = 8,72, $F = 17{\cdot}452$; $P < 0{\cdot}0001$; Tukey *post-hoc* test, $P < 0{\cdot}5$). Further, there were no significant differences between numbers of *E. coli* CFU recovered from insects pre-treated with *E. coli*, or with any of the pathogenic *Photorhabdus* strains alone (one-way ANOVA, $P > 0{\cdot}05$). Notably, all of these bacteria-only treatments led to a smaller number of CFU being recovered than in the case of the control pre-treatment with PBS alone (one-way ANOVA, D.F. = 4,40, $F = 28{\cdot}339$; $P < 0{\cdot}0001$; Tukey *post-hoc* test, $P < 0{\cdot}5$). These results indicate that both *Photorhabdus* and *E. coli* can 'prime' the *Manduca* immune system (as previously noted by Eleftherianos *et al.* 2006*a*), but that none of the 3 species of *Heterohabditis* nematode elicits priming in this way. Further, the simultaneous presence of nematodes evidently prevents priming by *Photorhabdus*; it might be conjectured that this is because the symbiotic bacteria are not exposed to recognition by the insect when in association with nematodes, as they would be if they were simply injected; however, this is not in accord with our other observations on host PO activity, which indicate that *Photorhabdus* can exert specific effects on host immunity whether it is associated with nematodes or not. A possible reconciliation is that in the PO experiments, a period of 18 h elapsed between pre-infection and the biological assay, whereas in the case of the observations on *E. coli* survival, there was only 2 h between pre-infection with the *Heterorhabditis-Photorhabdus* pair and the later injection of *E. coli*.

DISCUSSION

Symbioses between entomopathogenic nematodes of the genus *Heterorhabditis* and bacteria of the genus *Photorhabdus* are mutualistic associations in which both partners derive fitness benefits from each other in terms of their ability to cause disease in a third organism, generally an insect (Waterfield *et al.* 2009). This relationship has been extensively studied in terms of the pathogenic potential of the bacteria alone (Vallet-Gely *et al.* 2008), but the contribution of the nematode to pathogenesis is much less well understood. The goal of the present study was to address the question of the impact of *Heterorhabditis* on the outcome of insect infection, and to clarify the relative roles of the nematode and its symbiotic bacterium against the humoral and cellular immune responses of the host.

As in previous studies (Ciche and Ensign, 2003; Eleftherianos *et al.* 2006*b*), we found that a relatively small number of bacterial cells is sufficient to kill the host within a few days. Importantly, we also found that administering *Photorhabdus* together with their associated *Heterorhabditis* nematodes did not increase the speed or extent of mortality compared to bacteria alone. Thus, at least under the experimental infection conditions used, *Photorhabdus* bacteria clearly play a quantitatively predominant role in killing the host.

However, our results also showed that although natural infections using axenic (germ-free) nematodes alone were significantly less pathogenic than

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when bacteria were present, nematodes without bacteria were nevertheless able to cause significant mortality. Thus, the symbiotic nematode partner also contributes directly to virulence. Previous workers have also found that axenic *Heterorhabditis* can efficiently infect insects, but cause much less (or even no) insect mortality compared to symbiont-bearing infective juveniles, and are subsequently unable to reproduce in the host (Han and Ehlers, 2000; Hallem *et al*. 2007). On the other hand, axenic entomopathogenic nematodes of another genus (*Steinernema*) are known to produce toxins that are lethal to the host, even without their associated symbiotic bacteria (Brivio *et al*. 2005). All this suggested to us that heterorhabditid nematodes might produce factors that affect the host, even if they are not very effective (or even insufficient) in causing host mortality. We therefore studied the ways in which such factors might influence host immunity, and thus determine the outcome of the infection.

In the experiments reported here, the mRNA levels of certain pattern recognition proteins (Hemolin and PGRP-1A) in response to injected bacteria alone were much the same as when a similar number of bacteria were delivered through a natural nematode infection. In addition, we found that mRNA levels of a representative antimicrobial peptide (AMP) (Cecropin) were very low (undetectable under our conditions) in fat body of insects infected only with axenic nematodes. These results together imply that (i) nematodes are not recognized by the insect, or are much less efficiently recognized, than their symbiotic bacteria and (ii) nematodes do not markedly influence the host's recognition of, and response to, pathogenic bacteria.

The observed failure of heterorhabditid nematodes without symbiotic bacteria to provoke a host immune response agrees with previous findings. It has previously been shown that there is no defensin transcription in the fat body of *Armigeres subalbatus* in response to *Brugia malayi* (Lowenberger *et al*. 1996). More recently, axenic *Heterorhabditis* failed to induce significant expression of any of 7 AMP genes in *Drosophila* larvae (Hallem *et al*. 2007). Nevertheless, it is not yet clear whether the nematodes are able to interfere with host antibacterial activity (Richman and Kafatos, 1995). In the case of *Manduca*, the specific roles of Cecropins and other AMPs in targeting and/or disrupting the anti-parasite response remain thus far unknown, and will be the focus of future experiments.

In the present study we further show that natural infection with axenic *Heterorhabditis* nematodes fails to provoke transcription of PPO-2 in *Manduca* fat body. Because activation of the PO cascade is one of the earliest processes that can effectively prevent the growth and diffusion of potential pathogens (Zhao *et al*. 2007), inhibition of active PO may be conjectured to be a major contribution to successful parasitization. Brivio *et al*. (2002) observed a drastic PPO system inactivation in *Galleria mellonella* larvae following short-term infection with the entomopathogenic nematode *Steinernema feltiae* (Brivio *et al*. 2002); it was subsequently suggested (Brivio *et al*. 2004) that to ensure successful infection, PO depression must be achieved by the nematode before its symbiotic bacteria (*Xenorhabdus*) are released. Our observation of low levels of PO in *Manduca* infected by heterorhabditid nematodes, compared to levels in those given *Photorhabdus* K122 bacteria, is consistent with this idea. As explained in the results section, however, other strains of *Photorhabdus* also produce their own PO inhibitors, which would probably obscure the PO-inhibiting effects of the corresponding nematodes.

Besides humoral responses, insect hosts are also able to mount cellular immune reactions against parasitic nematodes and their symbiotic bacteria (Strand, 2008). It would therefore be expected that successful parasites and pathogens would possess adaptations to abrogate these defences. Here we show that *Photorhabdus* and *Heterorhabditis* (injected separately or together) reduce both total haemocyte numbers, and counts of viable haemocytes, and also suppress haemocyte aggregation. Interestingly, axenic *Heterorhabditis* do not cause such effects. This implies that the principal agent of these anti-haemocytic effects is the bacterial symbiont. By contrast, both axenic *Heterorhabditis* and nematodes carrying symbiotic *Photorhabdus*, but not bacteria alone, adversely affect phagocytosis (measured as the number of phagocytically active haemocytes, and the number of ingested bacterial cells) thus interfering with the ability of *Manduca* haemocytes to clear non-pathogenic *E. coli* from the haemolymph. Similarities of the cellular defences between bacterial and nematode infections may suggest that *Photorhabdus* bacteria, released from *Heterorhabditis*, could be responsible for the observed response. It is well documented that *Photorhabdus* secretes certain toxins that actively kill haemocytes or have an adverse effect on haemocyte function (Waterfield *et al*. 2009). However, immuno-evasion strategies employed by insect nematodes are often attributable to the mimetic properties of the parasite body surface rather than to the cells being damaged by the worm. For example, *G. mellonella* immunocompetent haemocytes are unable to recognize *S. feltiae* parasites as non-self (Brivio *et al*. 2004). Nevertheless, the nematobacterial complex *S. carpocapsae-X. nematophilus* produces unidentified molecules having toxic activity *in vitro* towards insect haemocytes (Ribeiro *et al*. 1999). Previously, it has been reported that *H. bacteriophora* is able to escape encapsulation in *Popillia japonica* larvae by an unknown mechanism (Wang *et al*. 1994, 1995). It remains now to be clarified whether *Heterorhabditis* produces

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anti-phagocytic factors that have direct and/or indirect pathological effects on *Manduca* haemocytes.

In conclusion, this study emphasizes the complexity found in similar interactions between mutualistic organisms. By studying systems based on entomopathogenic nematodes, we can investigate a diverse array of functions in insect innate immunity, as the insect defends against not only the nematode vector, but also against the bacterial symbiont. The present work could also give important insight into recognition mechanisms required to trigger a rapid and efficient anti-parasite immune response and use this information to identify specific molecular components involved in vector competence and disease transmission. From the practical point of view, since entomopathogenic nematodes represent an alternative to chemicals for insect pest control, it is crucial to elucidate the basis of infectivity of the nematode-bacterium complex and the interaction with the host immune system. Although the effects of *Photorhabdus* infection on insect immune responses have been extensively studied before (Waterfield *et al*. 2009), the role of *Heterorhabditis* in pathogenesis and its impact on host immune function are not fully established. The present study shows that the role of the nematode is quantitatively less important; however, this does not imply that the contributions of the two partners to the entire symbiotic life cycle are unequal. Important benefits may also be conferred by the nematode on its bacterial partner through enhancements in the efficiency of transmission and survival between infections (Goodrich-Blair and Clarke, 2007). Thus the symbiosis appears to be a division of labour, rather than an even-handed collaboration.

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REFERENCES

Au, C., Dean, P., Reynolds, S. E. and ffrench-Constant, R. H. (2004). Effect of the insect pathogenic bacterium *Photorhabdus* on insect phagocytes. *Cellular Microbiology* **6**, 89–95.

Brivio, M. F., Mastore, M. and Moro, M. (2004). The role of *Steinernema feltiae* body-surface lipids in host-parasite immunological interactions. *Molecular and Biochemical Parasitology* **135**, 111–121.

Brivio, M. F., Mastore, M. and Pagani, M. (2005). Parasite-host relationship: a lesson from a professional killer. *Invertebrate Survival Journal* **2**, 41–53.

Brivio, M. F., Pagani, M. and Restelli, S. (2002). Immune suppression of *Galleria mellonella* (Insecta, Lepidoptera) humoral defenses induced by *Steinernema feltiae* (Nematoda, Rhabditida): involvement of the parasite cuticle. *Experimental Parasitolgy* **101**, 149–156.

Cerenius, L., Lee, B. L. and Söderhäll, K. (2008). The proPO-system: pros and cons for its role in invertebrate immunity. *Trends in Immunology* **29**, 263–271.

Ciche, T. A. and Ensign, J. C. (2003). For the insect pathogen, *Photorhabdus luminescens*, which end of a nematode is out? *Applied Environmental Microbiology* **69**, 1890–1897.

Daborn, P. J., Waterfield, N., Silva, C. P., Au, C. P., Sharma, S. and ffrench-Constant, R. H. (2002). A single *Photorhabdus* gene, makes caterpillars floppy (*mcf*), allows *Escherichia coli* to persist within and kill insects. *Proceedings of the National Academy of Sciences, USA* **99**, 10742–10747.

Eleftherianos, I., Boundy, S., Joyce, S., Aslam, S., Marshall, J., Cox, R., Simpson, T., Clarke, D., ffrench-Constant, R. and Reynolds, S. (2007*a*). An antibiotic produced by an insect-pathogenic bacterium suppresses host defenses through phenoloxidase inhibition. *Proceedings of the National Academy of Sciences, USA* **104**, 2419–2424.

Eleftherianos, I., Gökçen, F., Felföldi, G., Millichap, P. J., Trenczek, T. E., ffrench-Constant, R. H. and Reynolds, S. E. (2007*b*). The immunoglobulin family protein Hemolin mediates cellular immune responses to bacteria in the insect *Manduca sexta*. *Cellular Microbiology* **9**, 1137–1147.

Eleftherianos, I., Marokhazi, J., Millichap, P. J., Hodgkinson, A. J., Sriboonlert, A., ffrench-Constant, R. H. and Reynolds, S. E. (2006*a*). Prior infection of *Manduca sexta* with non-pathogenic *Escherichia coli* elicits immunity to pathogenic *Photorhabdus luminescens*: roles of immune-related proteins shown by RNA interference. *Insect Biochemistry and Molecular Biology* **36**, 517–525.

Eleftherianos, I., Millichap, P. J., ffrench-Constant, R. H. and Reynolds, S. E. (2006*b*). RNAi suppression of recognition protein mediated immune responses in the tobacco hornworm *Manduca sexta* causes increased susceptibility to the insect pathogen *Photorhabdus*. *Developmental and Comparative Immunology* **30**, 1099–1107.

Eleftherianos, I., Waterfield, N. R., Bone, P., Boundy, S., ffrench-Constant, R. H. and Reynolds, S. E. (2009). A single locus from the entomopathogenic bacterium *Photorhabdus luminescens* inhibits activated *Manduca sexta* phenoloxidase. *FEMS Microbiology Letters* **293**, 170–176.

Ferrandon, D., Imler, J.–L., Hetru, C. and Hoffmann, J. (2007). The *Drosophila* systemic immune response: sensing and signalling during bacterial and fungal infections. *Nature Reviews Immunology* **7**, 862–874.

ffrench-Constant, R., Waterfield, N., Daborn, P., Joyce, S., Bennett, H., Au, C., Dowling, A., Boundy, S., Reynolds, S. and Clarke, D. (2003). *Photorhabdus*: towards a functional genomic analysis of a symbiont and pathogen. *FEMS Microbiology Reviews* **26**, 433–456.

Goodrich-Blair, H. and Clarke, D. J. (2007). Mutualism and pathogenesis in *Xenorhabdus* and *Photorhabdus*: two roads to the same destination. *Molecular Microbiology* **64**, 260–268.

https://doi.org/10.1017/S0031182010000508 Published online by Cambridge University Press

Hallem, E. A., Rengarajan, M., Ciche, T. A. and Sternberg, P. W. (2007). Nematodes, bacteria, and flies: A tripartite model for nematode parasitism. *Current Biology* **17**, 898–904.

Han, R. C. and Ehlers, R. U. (2000). Pathogenicity, development, and reproduction of *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* under axenic *in vivo* conditions. *Journal of Invertebrate Pathology* **75**, 55–58.

Jiang, H., Wang, Y. and Kanost, M. R. (1996). Primary structure of ribosomal proteins S3 and S7 from *Manduca sexta*. *Insect Molecular Biology* **5**, 31–38.

Jiravanichpaisal, P., Lee, B. L. and Söderhall, K. (2006). Cell-mediated immunity in arthropods: hematopoiesis, coagulation, melanization and opsonization. *Immunobiology* **211**, 213–236.

Joyce, S. A. and Clarke, D. J. (2003). A hexA homologue from *Photorhabdus* regulates pathogenicity, symbiosis and phenotypic variation. *Molecular Microbiology* **47**, 1445–1457.

Joyce, S. A., Watson, R. J. and Clarke, D. J. (2006). The regulation of pathogenicity and mutualism in *Photorhabdus*. *Current Opinion in Microbiology* **9**, 127–132.

Kanost, M. R., Jiang, H. and Yu, X. Q. (2004). Innate immune responses of a lepidopteran insect, *Manduca sexta*. *Immunological Reviews* **198**, 97–105.

Klowden, M. J. (2007). Circulatory systems. In *Physiological Systems in Insects* (ed. Klowden, M. J.), pp. 357–402. Academic Press, Boston, USA.

Lemaitre, B. and Hoffmann, J. (2007). The host defense of *Drosophila melanogaster*. *Annual Review of Immunology* **25**, 697–743.

Lowenberger, C. A., Ferdig, M. T., Bulet, P., Khalili, S., Hoffmann, J. A. and Christensen, B. M. (1996). *Aedes aegypti*: induced antibacterial proteins reduce the establishment and development of *Brugia malayi*. *Experimental Parasitology* **83**, 191–201.

Miller, J. S. and Stanley, D. W. (1998). The nodule formation reaction to bacterial infection: assessing the role of eicosanoids. In *Techniques in Insect Immunology* (ed. Weisner, A.), pp. 265–270. SOS Publications, Fair Haven, USA.

Nation, J. L. (2008). Circulatory system. In *Insect Physiology and Biochemistry* (ed. Nation, J. L.), pp. 339–365. CRC Press, Boca Raton, FL, USA.

Reynolds, S. E., Nottingham, S. F. and Stephens, A. E. (1985). Food and water economy and its relation to growth in 5th-instar larvae of the tobacco hornworm, *Manduca sexta*. *Journal of Insect Physiology* **31**, 119–127.

Ribeiro, C., Duvic, B., Oliveira, P., Givauda, A., Palha, F., Simoes, N. and Brehélin, M. (1999). Insect immunity–effects of factors produced by a nematobacterial complex on immunocompetent cells. *Journal of Insect Physiology* **45**, 677–685.

Richman, A. and Kafatos, F. C. (1995). Immunity to eucaryotic parasites in vector insects. *Current Opinion in Immunology* **8**, 14–19.

Silva, C. P., Waterfield, N. R., Daborn, P. J., Dean, P., Chilver, T., Au, C. P. Y., Sharma, S., Potter, U., Reynolds, S. E. and ffrench-Constant, R. H. (2002). Bacterial infection of a model insect: *Photorhabdus luminescens* and *Manduca sexta*. *Cellular Microbiology* **4**, 329–339.

Strand, M. R. (2008). The insect cellular immune response. *Insect Science* **15**, 1–14.

Vallet-Gely I., Lemaitre, B. and Boccard, F. (2008). Bacterial strategies to overcome insect defences. *Nature Review Microbiology* **6**, 302–313.

Vlisidou, I., Dowling, A. J., Evans, I. R., Waterfield, N., ffrench-Constant, R. H. and Wood, W. (2009). *Drosophila* embryos as model systems for monitoring bacterial infection in real time. *PLOS Pathogens* **5**, e1000518.

Wang, Y., Gaugler, R. and Cui, L. W. (1994). Variations in immune response of *Popillia japonica* and *Acheta domesticus* to *Heterorhabditis bacteriophora* and *Steinernema* species. *Journal of Nematology* **26**, 11–18.

Wang, Y., Campbell, J. F. and Gaugler, R. (1995). Infection of entomopathogenic nematodes *Steinernema glaseri* and *Heterorhabditis bacteriophora* against *Popillia japonica* (Coleoptera, Scarabaeidae) larvae. *Journal of Invertebrate Pathology* **66**, 178–184.

Waterfield, N. R., Ciche, T. and Clarke, D. (2009). *Photorhabdus* and a host of hosts. *Annual Review of Microbiology* **63**, 557–574.

Watson, R. J., Joyce, S. A., Spencer, G. V. and Clarke, D. J. (2005). The *exbD* gene of *Photorhabdus temperata* is required for full virulence in insects and symbiosis with the nematode *Heterorhabditis*. *Molecular Microbiology* **56**, 763–773.

Willott, E., Trenczek, T., Thrower, L. W. and Kanost, M. R. (1994). Immunochemical identification of insect haemocyte populations: monoclonal antibodies distinguish four major haemocyte types in *Manduca sexta*. *European Journal of Cellular Biology* **65**, 417–423.

Yu, X. Q., Zhu, Y. F., Ma, C., Fabrick, J. A. and Kanost, M. R. (2002). Pattern recognition proteins in *Manduca sexta* plasma. *Insect Biochemistry and Molecular Biology* **32**, 1287–1293.

Zhao, P., Li, J., Wang, Y. and Jiang, H. (2007). Broad-spectrum antimicrobial activity of the reactive compounds generated *in vitro* by *Manduca sexta* phenoloxidase. *Insect Biochemistry and Molecular Biology* **37**, 952–959.

https://doi.org/10.1017/S0031182010000508 Published online by Cambridge University Press