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Role of symbiotic and non-symbiotic bacteria in carbon dioxide production from hosts infected with *Steinernema riobrave*

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ABSTRACT

Entomopathogenic nematodes of the family Steinernematidae and their mutualistic bacteria (*Xenorhabdus* spp.) are lethal endoparasites of insects. We hypothesized that growth of the nematode's mutualistic bacteria in the insect host may contribute to the production of cues used by the infective juveniles (IJs) in responding to potential hosts for infection. Specifically, we tested if patterns of bacterial growth could explain differences in CO_2 production over the course of host infection. Growth of *Xenorhabdus cabanillasii* isolated from *Steinernema riobrave* exhibited the characteristic exponential and stationary growth phases. Other non-nematode symbiotic bacteria were also found in infected hosts and exhibited similar growth patterns to *X. cabanillasii*. *Galleria mellonella* larvae infected with *S. riobrave* produced two distinct peaks of CO_2 occurring at 25.6–36 h and 105–161 h post-infection, whereas larvae injected with *X. cabanillasii* alone showed only one peak of CO_2, occurring at 22.8–36.2 h post-injection. *Tenebrio molitor* larvae infected with *S. riobrave* or injected with bacteria alone exhibited only one peak of CO_2 production, which occurred later during *S. riobrave* infection (41.4–64.4 h post-infection compared to 20.4–35.9 h post-injection). These results indicate a relationship between bacterial growth and the first peak of CO_2 in both host species, but not for the second peak exhibited in *G. mellonella*.

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1. Introduction

Entomopathogenic nematodes of the family Steinernematidae, which are lethal endoparasites of insects, form a mutualistic association with *Xenorhabdus* spp. bacteria (Thomas and Poinar, 1979; Akhurst, 1982a, 1983). These bacteria are carried by non-feeding infective juveniles (IJs) within a specialized intestinal receptacle (Forst and Nealson, 1996; Snyder et al., 2007). Infective juveniles actively search for and infect new insect hosts (Campbell and Lewis, 2002). Once an IJ enters the insect hemocoel, the symbiotic bacteria are released and overcome the host insect immune system, produce endo- and exotoxins that kill the host, and modify the environment to enhance nematode development (Boemare and Akhurst, 1988; Forst and Nealson, 1996; Walsh and Webster, 2003). Depending on the host/nematode/bacteria species combination, the host dies 24 h to 72 h post-infection due to toxemia or septicemia (Dowds and Peters, 2002) and nematodes develop and reproduce until crowding and decreasing nutrients lead to the formation of new IJs that emerge from host cadaver to seek out new hosts (Kaya and Gaugler, 1993).

Parasite–host interactions follow a series of steps, including host-habitat finding, host-finding, host-acceptance, and host-suitability (Salt, 1935; Laing, 1937; Doutt, 1964). For entomopathogenic nematodes, the processes of host-habitat finding (Rasmann et al., 2005) and host-finding (Campbell and Lewis, 2002) are better understood than the process of host-acceptance. However, host-acceptance is of critical importance in the infection process of entomopathogenic nematodes. After host encounter, IJs need to determine whether or not to infect the host. There are clear risks to making an incorrect choice, because once an IJ commits to infection and resumes development, the process is irreversible.

Entomopathogenic nematode species, host species, infection status of host, and time post-infection can influence the processes of host-finding and host-acceptance. Some studies have found increased attraction to hosts parasitized with conspecifics (Grewal et al., 1997; Lewis and Gaugler, 1994), but others have found no impact on attraction (Ramos-Rodríguez et al., 2007). Changes in

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infection behavior toward parasitized hosts have also been reported, with both decreases (Glazer, 1997) and increases (Campbell and Lewis, 2002) at specific time points after initial infection. Christen et al. (2007) reported that *Steinernema riobrave* Cabanillas, Poinar, and Raulston preferentially infected 24 h post-infection *Galleria mellonella* (L.) over an unparasitized *G. mellonella*. In contrast, with *Tenebrio molitor* (L.) as a host *S. riobrave* showed no infection preference for a host parasitized at any of the tested times previous to exposure.

Changes in attraction to, and infection of, a parasitized host could be due to changes caused by symbiotic bacteria and nematode population growth inside the host. Ramos-Rodríguez et al. (2007) reported variation among nematode and host species in how nematode density and stage distribution within a host changed over time. Density of *Xenorhabdus* spp. within the host also changes over the course of an infection and varies with nematode/bacteria symbiosis (Isaacson and Webster, 2002; Walsh and Webster, 2003).

It is likely that both volatile and non-volatile cues from parasitized hosts are dynamic with respect to time after infection and that the behavioral responses of IJs approaching the infection will be influenced. IJs have been shown to respond to symbiotic bacteria (Pye and Burman, 1981), therefore, bacteria-mediated cues may be used by IJs to assess host infection status. Previous studies have also shown that IJs respond to CO_2 (Gaugler et al., 1980; Thurston et al., 1994) in addition to other cues such as host excretory products (Schmidt and All, 1979; Gaugler et al., 1980; Byers and Poinar, 1982), temperature gradients (Byers and Poinar, 1982; Choo et al., 1989), and pH (Pye and Burman, 1981). Ramos-Rodríguez et al. (2006) found that CO_2 production from parasitized hosts varied over time, with up to two peaks of CO_2 release occurring, and hypothesized that the first peak was caused by exponential growth of the symbiotic bacteria, while the second peak was caused by nematode development. The level of nematode response to CO_2 from a host may depend on the concentration produced (Gaugler et al., 1980), but the timing of the CO_2 peaks did not correspond with increased IJ long-range attraction (Ramos-Rodríguez et al., 2007).

To address the role of symbiotic bacteria in producing cues used to make infection decisions, we evaluated the role of *Xenorhabdus* spp. in producing the peaks of CO_2 reported by Ramos-Rodríguez et al. (2006). Initially, we determined growth dynamics of bacteria in the host, both symbiotic *Xenorhabdus cabanillasii* Tailliez, Ginibre, and Boemare (Tailliez et al., 2006) and other non-symbiotic bacteria, using *G. mellonella* and *T. molitor* larvae infected with *S. riobrave* IJs or injected with primary phase *X. cabanillasii*. Then we measured differences in temporal pattern of CO_2 production from *G. mellonella* and *T. molitor* larvae infected with *S. riobrave* IJs or injected with *X. cabanillasii*.

2. Materials and methods

2.1. Nematode and host species

Steinernema riobrave was used for all experiments [originally obtained from Harry K. Kaya (University of California-Davis)]. Using methods described by Kaya and Stock (1997), nematodes were reared in *G. mellonella* at 25 °C. After emergence, nematodes were stored at room temperature (25 °C) in capped 275 mL flasks (NUNC™ EasY, Daigger®, Vernon Hills, IL, USA). For each block, different nematode infection batches were used and nematodes were less than 2 weeks old.

Two host species representing two different orders were used in the experiments, late instar larval *G. mellonella* (0.2–0.25 g) and *T. molitor* (0.1–0.15 g). Larvae of the greater wax moth, *G. mellonella* were obtained from Webster's Waxie Ranch (Webster, WI, USA) and larvae of *T. molitor*, the yellow mealworm, from Southeastern Insectaries (Perry, GA, USA). In all experiments, host larvae were exposed individually to nematode or bacteria treatments and thus each larva was considered a replicate.

2.2. Bacterial growth curves—S. riobrave infection

Prior to beginning the experiment, *G. mellonella* or *T. molitor* larvae were infected with *S. riobrave*. Seventy larvae of each host species were exposed individually to 100 infective juveniles in 1.5 mL plastic microcentrifuge tubes (Daigger®, Vernon Hills, IL, USA) with three small holes (~0.6 mm diameter) in the lid and one hole in the bottom. Nematodes were added in 50 μL of deionized water to a 3.5 × 1.5 cm piece of grade 360 filter paper (Baxter Scientific Products, McGaw Park, IL, USA) placed inside the microcentrifuge tube. Individual host larvae were then added to each tube. Larvae were held in an incubator set at 25 °C and dark conditions. At each time point (0, 12, 24, 36, 48, 72, 96, 120, 144, or 168 h post-infection), five larvae of each host species were randomly selected for examination. Experiment was repeated (2 blocks) for a total of 10 replicates of each insect species and time post-infection.

For surface sterilization, insects were grouped by species in 50 mL NUNC™ centrifuge tubes (Daigger®, Vernon Hills, IL, USA) and processed using the following procedure: 1 min in 0.05% sodium hypochlorite solution, 1× rinse with sterile water, 1 min in 70% ethanol, and 3× rinse with sterile water (Zurek et al., 2000). Sterile, disposable, 7 mL transfer pipets (Fisher Scientific Co., Pittsburgh, PA, USA) were used to remove excess solution between steps. Individual surface sterilized *G. mellonella* or *T. molitor* larvae were placed in sterilized 1.5 mL microcentrifuge tubes (Daigger®, Vernon Hills, IL, USA) containing 200 μL sterilized PBS and homogenized with a sterilized Kontes pellet pestle (Fisher Scientific). Total volume was brought up to 1000 μL and serially diluted.

From each dilution, 100 μL was spread in triplicates on NBTA [1 L nutrient agar, 0.04 g triphenyltetrazolium chloride, 0.025 g bromothymol blue (Kaya and Stock, 1997)] to allow for differentiation of *Xenorhabdus* spp. from other bacterial species. NBTA plates were incubated at 25 °C and dark conditions for 3–5 days. Number of *X. cabanillasii* colony forming units (CFUs), based on uptake of bromothymol blue, was counted. Because other bacterial colonies were observed on the plates, digital photos were taken of each plate in the second block and later the number of CFUs of non-*Xenorhabdus* species was also determined from these photos.

2.3. Bacterial growth curves—injection of primary phase X. cabanillasii

Primary *X. cabanillasii* cells were cultured from *T. molitor* infected with *S. riobrave*. At 24 h post-infection, individual larvae were surface sterilized, homogenized, and plated as previously described. After 5 days, individual *X. cabanillasii* colonies were streaked on NBTA. Pure primary *X. cabanillasii* was maintained by making new NBTA streak plates every week. Twenty-four hours prior to the beginning of the experiment, cells were harvested from a NBTA plate and used to inoculate 75–100 mL of sterile YS broth [0.5 g K_2HPO_4; 0.5 g $NH_4H_2PO_4$; 0.2 g $MgSO_4 \cdot 7H_2O$; 5.0 g NaCl; 5.0 g yeast extract; 1 L H_2O (Akhurst, 1980)]. Flasks containing inoculated YS broth were stored at room temperature (25 °C) on a Daigger® OR25 shaker (Daigger®, Vernon Hills, IL, USA) set 115 rpm. Total counts of bacterial cells present were made using a Petroff–Hausser bacterial counter (Arthur H. Thomas Co., Philadelphia, PA, USA).

The number of *X. cabanillasii* cells to be injected was estimated based on individual *Steinernema carpocapsae* IJs that initially have approximately 145 CFUs of *Xenorhabdus nematophilus* (Lewis et al., 1995) and an average of 20 *S. riobrave* IJs initiate an infection (Christen et al., 2007), which yields an estimated average of 2900

bacterial cells released per host. Surface sterilized larvae of each host species were injected with 0.75 μL bacterial suspension (YS broth + 2900 *X. cabanillasii* cells) or 0.75 μL sterile YS broth (control) using a Hamilton Model 7000 modified 5.0 μL syringe (Fisher Scientific), which was rinsed in sterile water before each use. Injected larvae were placed in 1.5 mL microcentrifuge tubes with three small holes (~0.6 mm diameter) in the lid and one hole in the bottom and contained a 3.5 × 1.5 cm piece of grade 360 filter paper (Baxter Scientific Products, McGaw Park, IL, USA) to which 50 μL of deionized water was added. Host larvae were held in an incubator set at 25 °C and dark conditions. At each time point (0, 12, 24, 36, 48, 72, 96, 120, 144, or 168 h post-infection), three larvae of each host species were selected for examination. Larvae were surface sterilized, homogenized, and plated as described above. *X. cabanillasii* colony forming units were counted after 5 days of incubation. Due to the presence of other bacterial species on the plates, digital photos were taken of each plate for both blocks and the number of CFUs of non-*Xenorhabdus* species was also determined. Experiment was repeated (2 blocks) for a total of 6 replicates (individual larvae) for each insect species and time post-infection.

2.4. Identification of bacterial species

Morphologically different single colonies from *S. riobrave* infection plates were isolated on trypticase soy broth agar (Difco, Detroit, MI, USA) to establish pure cultures and stored at 4 °C. Bacterial genomic DNA was extracted from pure cultures using DNeasy® Tissue Kit (Qiagen, Valencia, CA, USA) and the 16S rRNA gene was amplified using polymerase chain reaction (PCR) with universal eubacterial forward primer UF: 5′ AGA GTT TGA TYM TGGC 3′ (position 8–23) and reverse primer UR: 5′ GYT ACC TTG TTA CGA CTT 3′ (position 1492) (Barbieri et al., 2001). Amplification was performed using a Peltier Thermal Cycler (MJ Research, Waltham, MA, USA). PCR solutions (total volume = 25 μL) contained 12 μL PCR master mix (composed of 50 U/mL of TaqDNA Polymerase in buffer (pH 8.5), 400 μM dATP, 400 μM dGTP, 400 μM dCTP, 400 μM dTTP, 3 mM $MgCl_2$), 1 μL each of forward and reverse primers, 9.5 μL nuclease-free water, and 1 μL template DNA (except template DNA, all from Promega, Madison, WI, USA). Amplification was carried out using the following protocol: 94 °C for 2 min followed by 30 cycles consisting of 90 s at 94 °C, 90 s at 50 °C, and 60 s at 72 °C followed by final extension at 72 °C for 10 min and 4 °C hold (Barbieri et al., 2001). Resulting PCR products were purified using GFX PCR DNA and Gel Band Purification Kit (Amersham Bioscience, UK), and visualized by UV light on 1.5% agarose gel (Fisher Scientific) containing 0.05% ethidium bromide. Sequences were determined using the same universal primers mentioned above on an ABI 3700 DNA Analyzer at the DNA Sequencing Facility located in the Department of Plant Pathology at Kansas State University (Manhattan, KS, USA). Sequences were aligned manually with CodonCode Aligner (Dedham, MA) and consensus sequence was analyzed for similarity to known sequences at the NCBI GenBank database using BLAST (Basic Local Alignment Search Tool) (Altschul et al., 1990).

To sequence the 16S rRNA gene of *S. riobrave* symbiont, the 16S rDNA PCR product from the dark blue colonies on NBTA was purified with GFX PCR DNA and Gel Band Purification Kit (Amersham Bioscience) and cloned into the pGEM®-T Easy Vector and transformed into competent *Escherichia coli* cells (JM109) as recommended by the manufacturer (Promega). Clones were screened for the 16S rDNA insert by restriction enzyme EcoRI. Two clones with the proper insert were cultured overnight in Luria–Bertani broth with ampicillin (100 μg/mL) and the vector was extracted with Quickclean 5M Miniprep Kit (GenScript Corp, Piscataway, NJ, USA). Sequencing was carried out with M13 forward and reverse primers on ABI 3700 DNA Analyzer at the DNA Sequencing Facility located in the Department of Plant Pathology at Kansas State University.

2.5. CO_2 measurements

Changes in carbon dioxide release were measured with the Micro-Oxymax v5.12 respirometer (Columbus Instruments International Corporation, Columbus, OH, USA) using methods described in Ramos-Rodríguez et al. (2006). Percentage O_2 and CO_2 gas levels are taken from closed test chambers, which means that air from the test chamber is pumped through the gas sensors and back to the test chamber. Measurements were taken every 2 h from 0 h up to 280 h. Since this instrument has a maximum of ten sample chambers, two blocks of 10 replicates each were performed.

Late instar *G. mellonella* and *T. molitor* were exposed to three different treatments: YS broth injection (control), *X. cabanillasii* bacteria injection, and IJ infection. For injection treatments, individual surface sterilized larvae were injected with 0.80 μL of sterile YS broth or 0.80 mL bacterial suspension (YS broth + *X. cabanillasii*). After injection, larvae were placed in 1.5 mL microcentrifuge tubes and 50 μL of deionized water were added to the filter paper insert. For *S. riobrave* infection, individual surface sterilized larvae were exposed to 100 IJs in microcentrifuge tubes (1.5 mL). Nematodes were added in 50 μL of deionized water to a filter paper insert inside the tube and then the larvae were added. All microcentrifuge tubes contained 16 holes (~0.6 mm diameter) on the sides (4 columns of 4), three holes on top, and one hole on the bottom to allow for gas exchange.

Individual centrifuge tubes were placed inside a 50 mL respirometer test chamber and placed in a 25 °C water bath. For each block, two replicates of nematode infection, two replicates of bacterial injection, and one replicate of YS injection control were performed for each insect species. Therefore, at the end of the second block, each insect species had a total of four replicates of nematode infection, four replicates of bacterial injection, and two replicates of YS injection. At the completion of the experiment, parasitized larvae were dissected to confirm the presence of nematodes. Injected larvae were also dissected to assess the condition and appearance of host tissues in comparison to larvae infected with *S. riobrave*.

Following the same procedure, an additional block of 10 replicates using only *G. mellonella* as a host was also performed to supplement previous experiments. Treatments consisted of two replicates of each of the following: YS broth injection, primary phase *X. cabanillasii* injection, *Enterobacter* sp. injection, heat killed primary phase *X. cabanillasii* injection, and live, uninfected larvae. Heat killed primary phase *X. cabanillasii* cells were obtained by autoclaving for 15 min at 121 °C. *Enterobacter* sp. cells were obtained from *T. molitor* larvae infected with *S. riobrave* following the same procedure to isolate primary phase *X. cabanillasii* cells. CO_2 measurements were taken every 2 h for up to 210 h for each treatment.

Before injection, all insects were surface sterilized except for the uninfected larvae controls. When switching injection treatments, the syringe was rinsed with 70% ethanol followed by sterile deionized water. At the completion of the experiment, larvae were dissected to note the appearance of host tissues. Additionally, one larva from both the heat killed primary phase *X. cabanillasii* injection and *Enterobacter* sp., originally isolated from *S. riobrave* infected *T. molitor*, injection was homogenized and plated on NBTA to confirm either the absence of *X. cabanillasii* or presence of *Enterobacter* sp. for the respective treatments.

2.6. Statistical analysis

Data for bacterial growth curves and CO_2 measurements are presented as the mean ± standard error of the mean. Slopes

generated by linear regression of the exponential growth phase exhibited by *X. cabanillasii* were compared between host species (Zar, 1984). For *S. riobrave* infection, four time points were used for comparison (12, 24, 36, and 48 h post-infection), while five time points were used for bacterial injection (0, 12, 24, 36, and 48 h post-injection). At the conclusion of exponential growth, which was at 60 h post-infection or post-injection, growth curve data were analyzed using the General Linear Models Procedure (GLM) with means separated using Tukey's test (SAS Institute, 2003) to determine if there were significant differences in number of bacteria between host species and when those differences occurred. Based on the results of the previous analysis, 72 h post-infection or post-injection was selected for further analysis as it produced the highest number of colony forming units (CFUs), or was not significantly different from the highest time point. Differences between host species in regard to the number of CFUs at 72 h were analyzed using the GLM procedure (SAS Institute, 2003). Differences between CO_2 release and time of release for each treatment were analyzed using the GLM procedure with means separated using Tukey's test (SAS Institute, 2003).

3. Results

3.1. Bacterial growth curves—S. riobrave infection

Insect mortality was observed between 24 and 48 h post-infection for *G. mellonella* and between 48 and 72 h post-infection for *T. molitor*. *X. cabanillasii* associated with *S. riobrave* was first detected at low levels 12 h post-infection for both host species (*G. mellonella* = $8.51 \times 10^3 \pm 1.85 \times 10^3$ CFU/larva; *T. molitor* = $8.04 \times 10^2 \pm 2.58 \times 10^2$ CFU/larva) (Fig. 1A). Exponential growth lasted up to 72 h for *G. mellonella* ($4.44 \times 10^8 \pm 1.86 \times 10^8$ CFU/larva) and 60 h for *T. molitor* ($8.87 \times 10^8 \pm 1.24 \times 10^8$ CFU/larva). Then, the number of bacteria decreased for both species with some fluctuation until the end of the experiment at 168 h post-infection. During exponential growth (12–48 h post-infection), there was no significant difference in slopes between species (comparison of slopes: $t = -0.5993$, $t_{0.05(2),4} = 2.776$). Comparison between host species of the number of bacteria at the peak (72 h post-infection) showed no significant difference in the number of CFUs/larva (GLM; $F = 0.29$, d.f. = 1, $p = 0.5993$).

In addition to *X. cabanillasii*, several other bacterial species were detected in both host species and were present from the beginning to the end of the experiment (Fig. 1B). Number of non-*Xenorhabdus* spp. bacteria (combining all different morphological types) in *G. mellonella* ranged from $6.70 \times 10^4 \pm 2.25 \times 10^4$ CFU/larva (0 h post-infection) to a maximum of $1.06 \times 10^9 \pm 9.87 \times 10^8$ CFU/larva (96 h post-infection). Low levels of non-*Xenorhabdus* spp. were observed in *T. molitor* starting at 0 h post-infection ($1.88 \times 10^4 \pm 1.87 \times 10^3$ CFU/larva), and increased to a maximum at 96 h post-infection ($1.95 \times 10^9 \pm 7.20 \times 10^8$ CFU/larva). After reaching the maximum number of bacteria in both host species, a decline was observed with some fluctuation until the conclusion of the experiment.

3.2. Bacterial growth curves—injection of primary phase X. cabanillasii

Host mortality in *X. cabanillasii*-injected larvae occurred at the same time periods as exhibited in *S. riobrave* infection. Initially, 2.90×10^3 CFU/larva were injected and this was used for 0 h post-injection counts. At 12 h post-injection, $3.30 \times 10^3 \pm 2.22 \times 10^3$ CFU/larva were observed for *G. mellonella* and $1.06 \times 10^5 \pm 7.88 \times 10^4$ CFU/larva for *T. molitor* (Fig. 1C). Maximum

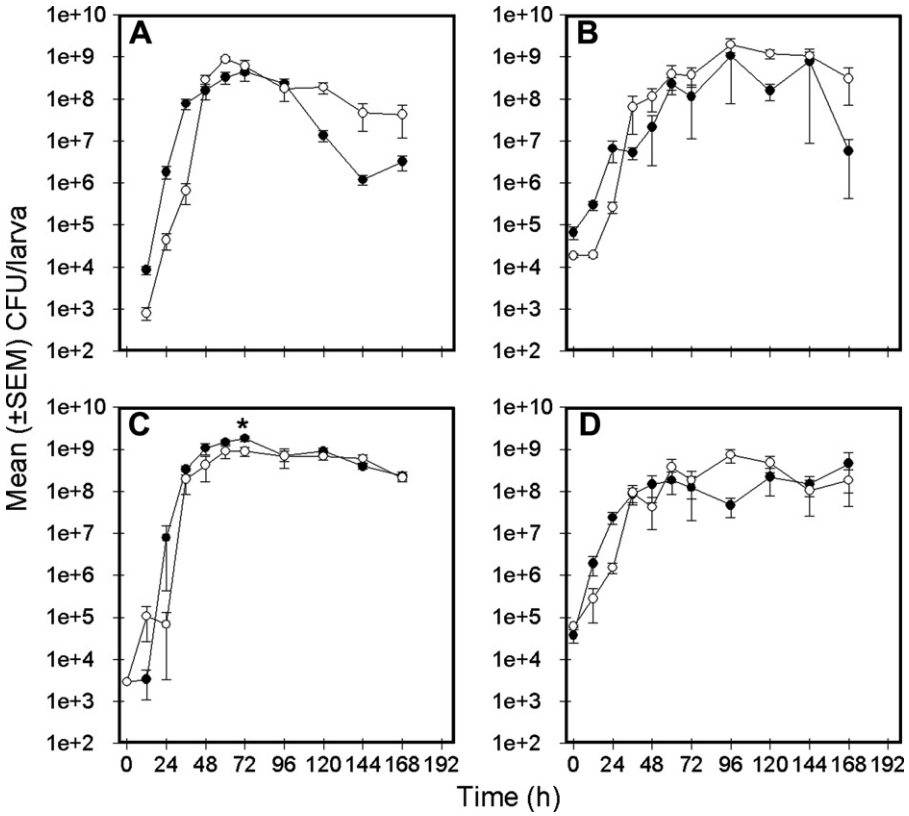

Fig. 1. *Xenorhabdus cabanillasii* (A,C) and non-*Xenorhabdus* species (B,D) growth curves (mean ± SEM CFU/larva) for *S. riobrave* infection (A,B) and bacteria injection (C,D) of *Galleria mellonella* (black circle) and *Tenebrio molitor* (white circle). *Indicates time point in which the number of CFU/larva is significantly different between host species.

number of bacteria occurred at 72 h post-injection for *G. mellonella* ($1.80 \times 10^9 \pm 2.85 \times 10^8$ CFU/larva) and at 60 h post-injection for *T. molitor* ($8.98 \times 10^8 \pm 2.91 \times 10^8$ CFU/larva). Comparison of slopes from 0 to 48 h post-injection between host species showed no significant difference ($t = 1.481$, $t_{0.05(2),6} = 2.447$). Using the GLM procedure, a significant difference in the number of CFUs/μL was detected between host species at 72 h post-injection (time of peak bacteria numbers) ($F = 6.26$, d.f. = 1, $p = 0.0313$).

Non-*Xenorhabdus* spp. bacteria were persistent throughout the experiment for both host species injected with *X. cabanillasii* (Fig. 1D). Number of bacteria observed in *G. mellonella* started at $3.76 \times 10^4 \pm 1.36 \times 10^4$ CFU/larva (0 h post-injection), reached $1.44 \times 10^8 \pm 9.08 \times 10^7$ CFU/larva at 48 h post-injection and fluctuated around this number until 168 h post-injection when the maximum number of bacteria was reached ($4.55 \times 10^8 \pm 3.65 \times 10^8$ CFU/larva). Levels of non-*Xenorhabdus* spp. bacteria started at 0 h post-injection ($6.09 \times 10^4 \pm 4.28 \times 10^3$ CFU/larva) and increased to a maximum of $7.31 \times 10^8 \pm 2.54 \times 10^8$ CFU/larva at 96 h post-injection in *T. molitor*. After reaching the maximum number, a decrease in number of bacteria was observed until 168 h post-injection where a slight increase occurred.

3.3. Identification of bacterial species

Based on CFU morphology, a number of other bacterial colonies were present on the selective NBTA media in addition to *X. cabanillasii* (Table 1). BLAST results of 16S rDNA sequences, with manual sequence alignment, produced matches at the species level. All matched sequences showed high similarities (⩾99%) to known strains in GenBank (Table 1). The dark blue colonies (isolate G3-B) which were identified and counted as *X. cabanillasii* in previous analyses, was 99% similar to the partial sequence for *X. cabanillasii* for *S. riobrave* found in the database.

3.4. CO_2 measurements

There were significant differences in both peak height (GLM; $F = 22.76$, d.f. = 3, $p < 0.0001$; means separated using Tukey's test) and timing of the first CO_2 peak (GLM; $F = 10.25$, d.f. = 3, $p = 0.0013$; means separated using Tukey's test) among treatments (*G. mellonella*–*S. riobrave* infection, *G. mellonella*–bacteria injection, *T. molitor*–*S. riobrave* infection, and *T. molitor*–bacteria injection).

Galleria mellonella larvae infected with *S. riobrave* exhibited two peaks of CO_2 production (Fig. 2A). These peaks varied from approximately 6.60 to 10.40 μL/min for each insect host. The mean CO_2 release for the first and second peak was 7.46 ± 0.30 μL/min and 8.69 ± 0.59 μL/min, respectively. Timing of the first peak ranged from around 25.6 to 36 h post-infection with a mean of 28.90 ± 2.43 h, and the second peak ranged from approximately 105 to 161 h post-infection with a mean of 124.80 ± 12.32 h. In contrast, *G. mellonella* larvae injected with primary phase *X. cabanillasii* cells exhibited only one peak of CO_2 production (Fig. 2A). Individual insect host production of CO_2 for this peak ranged from approximately 6.50 to 8.70 μL/min and occurred between 22.8 and 36.2 h post-injection. This peak occurred at a mean of 27.58 ± 3.14 h post-injection and had a mean CO_2 release of 7.80 ± 0.52 μL/min. There were no significant differences in peak height between *G. mellonella* larvae infected with *S. riobrave* and larvae injected with primary phase *X. cabanillasii*. The *G. mellonella* larvae injected with sterile YS broth (control) exhibited a small discernable peak of CO_2 production (1.0–4.0 μL/min) (Fig. 2A).

Unlike infected *G. mellonella*, larvae of *T. molitor* infected with *S. riobrave* showed only one distinct peak of CO_2 production (Fig. 2B). Peak CO_2 production for individual insect hosts ranged from 3.70 to 4.80 μL/min and occurred between 41.4 and 64.4 h post-infection. This peak occurred at a mean of 51.24 ± 4.95 h post-infection and had a mean CO_2 release of 4.49 ± 0.26 μL/min. In some cases there was a suggestion of a second peak, but it was generally low and broad. The *T. molitor* larvae injected with primary phase *X. cabanillasii* cells also exhibited only one peak of CO_2 production. Individual insect host peak CO_2 production ranged between 4.00 and 5.30 μL/min (mean of 4.78 ± 0.32 μL/min) and from 20.40 to 35.90 h post-injection. Mean CO_2 release was similar between *S. riobrave* infection and bacteria injection treatments; however, the timing of this peak occurred at approximately 28.81 ± 3.25 h post-injection, which was significantly earlier than the timing observed for *S. riobrave* infection. There were no signif-

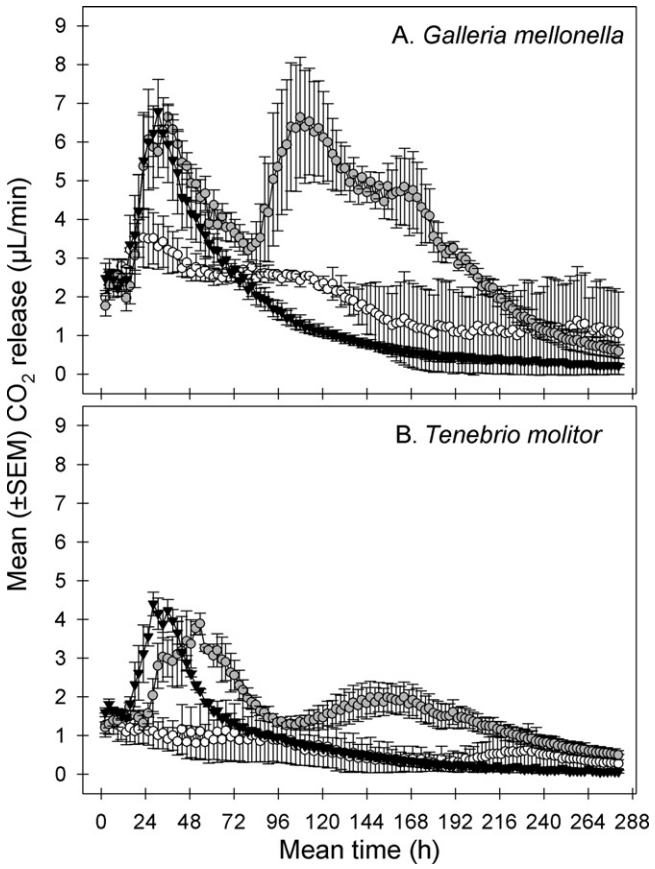

Fig. 2. Mean (±SEM) CO_2 production from *Steinernema riobrave* infected larvae (filled gray circle), primary phase *Xenorhabdus cabanillasii*-injected larvae (filled black triangle), and YS broth-injected larvae (filled white circle) of *Galleria mellonella* (A) and *Tenebrio molitor* (B).

Table 1
Bacteria isolated from *Galleria mellonella* (G) and *Tenebrio molitor* (T) infected with *Xenorhabdus cabanillasii* and *Steinernema riobrave* nematodes

Isolate	Identification	Sequence length (bp)	Similarity (%)
G3-B	*Xenorhabdus cabanillasii*	1506	99
G3-A	*Enterobacter aerogenes*	1418	99
T1-A	*Enterobacter aerogenes*	1417	99
T1-E	*Enterobacter aerogenes*	1413	99
T2-A	*Enterobacter hormaechei*	1361	99
T3-A	*Enterobacter cancerogenus*	1385	99
T1-F	*Staphylococcus succinus*[*]	860	100
G-A	*Stenotrophomonas maltophilia*	1434	99
T3-B	*Stenotrophomonas maltophilia*	1433	99
T4-A	*Pseudomonas fulva/parafulva*[**]	1428	99

Identification based on sequencing of 16S rDNA.
[*] Identification based on forward primer sequence only.
[**] Species indistinguishable (same number of mismatches).

icant differences in peak height between *T. molitor* larvae infected with *S. riobrave* and larvae injected with *X. cabanillasii*. No peak was observed for larvae injected with sterile YS broth (control), with CO_2 levels being close to 1.0 μL/min throughout the course of the experiment (Fig. 2B).

Peak height of *S. riobrave* infection and *X. cabanillasii* injection of *G. mellonella* was significantly higher than *S. riobrave* infection and primary phase *X. cabanillasii* injection of *T. molitor*. Timing of the first peak of CO_2 for *T. molitor* infected with *S. riobrave* was significantly later than that of all other treatment combinations.

In the additional experimental run using only *G. mellonella* larvae, larvae injected with primary phase *X. cabanillasii* showed similar results to the previous experiment (timing of peak = 32.9 h, CO_2 production = 8.24 μL/min) (Fig. 3C). None of the additional controls produced any distinct peaks of CO_2. The levels of CO_2 from live uninfected and YS broth-injected larvae were between 2.0 and 4.0 μL/min and between 3.0 and 5.0 μL/min, respectively, throughout the course of the experiment (Fig. 3A and B). CO_2 production from larvae injected with heat killed *X. cabanillasii* cells started at 0.75 μL/min, increased to approximately 3.0 μL/min at 56 h post-injection, and decreased to an ending level around zero (Fig. 3D). The *G. mellonella* larvae injected with *Enterobacter* sp. had CO_2 levels that began at 1.80 μL/min, increased to 3.72 μL/min at 32.89 h post-injection, decreased to values around zero by 72 h post-injection, and remained at such levels until the end of the experiment (Fig. 3E).

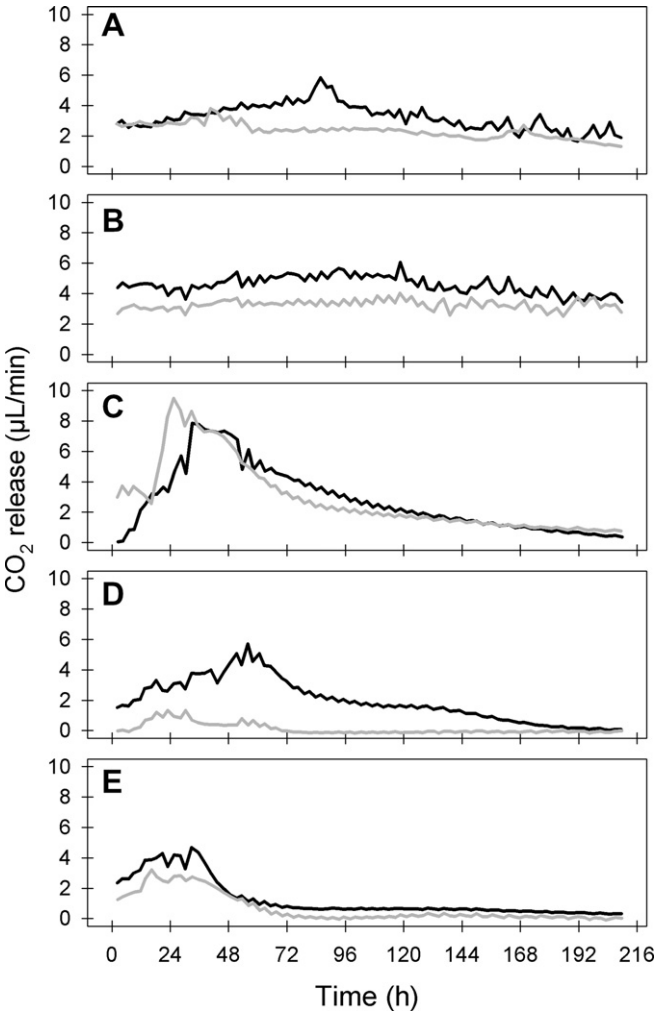

Fig. 3. CO_2 production from *Galleria mellonella* larvae with following treatments: (A) Uninfected, (B) YS injected, (C) *Xenorhabdus cabanillasii*-injected, (D) heat killed *X. cabanillasii*-injected, and (E) *Enterobacter* sp.-injected. Lines represent two different insect replicates.

4. Discussion

Growth curves obtained for *X. cabanillasii*, regardless of treatment (i.e., *S. riobrave* infection or bacterial injection), exhibited typical exponential and stationary growth phases. At 24 h post-infection or post-injection, which is when IJ infection was greatest in *G. mellonella* and when the first peak of CO_2 was produced, the bacteria are undergoing exponential growth. The timing of this exponential growth phase is similar to that of previous studies with this and other entomopathogenic nematode/bacterial species (Götz et al., 1981; Isaacson and Webster, 2002; Walsh and Webster, 2003). Decline in number of colony forming units observed in *G. mellonella* hemolymph starting 72 h post-infection and the lack of such decline in the bacteria-injected hosts may be the result of nematodes feeding on the bacteria, competing for resources, and IJs harboring bacteria in specialized receptacles (Forst and Nealson, 1996; Flores-Lara et al., 2007). A similar, but less dramatic, decline was seen in infected *T. molitor*.

In addition to *X. cabanillasii*, 9 other bacterial strains were isolated from parasitized hosts and identified to species. The occurrence of other species suggests that *X. cabanillasii*, which is associated with *S. riobrave*, does not inhibit growth of other bacterial species as effectively as typically assumed for entomopathogenic nematode-bacteria symbioses (Maxwell et al., 1994; Walsh and Webster, 2003). Isaacson and Webster (2002) found that *X. cabanillasii* exhibited a rapid increase in antimicrobial activity starting at 72 h post-infection and reaching a maximum at 144 h post-infection, but the number of Gram-negative bacteria continued to increase. This study and others (Akhurst, 1982b; Maxwell et al., 1994) have shown that antibiotics produced by *Xenorhabdus* spp. are more effective against Gram-positive bacteria than Gram-negative. All isolated species in our study, except *Staphylococcus succinus* (Lambert), were Gram-negative bacteria. The continued growth in populations of non-symbiotic bacteria in apparently normal infections is unusual for entomopathogenic nematodes in general, but consistent with the findings for this species by Isaacson and Webster (2002).

Prior studies have hypothesized that the origin of non-*Xenorhabdus* species in an infected host may be from the nematode's gut or cuticular surface (Bonifassi et al., 1999; Isaacson and Webster, 2002), from the gut of the host (Bucher and Williams, 1967), or due to contamination during or after infection. Bucher and Williams (1967) found that *Enterococcus faecalis* (*hirae*) (Schleifer and Kilpper-Balz) was the dominant bacterial species in the gut of *G. mellonella*, but this species was not recovered in our study. However, one of the minor species, *Enterobacter aerogenes* (Hormaeche and Edwards), reported by Bucher and Williams (1967) was isolated in the current study. Thus, the presence of non-*Xenorhabdus* species at 0 h post-infection/injection in the current study supports the hypothesis that non-symbiotic bacteria were originally associated with the gut of the host insect.

Measurements of CO_2 production in *G. mellonella* and *T. molitor* larvae infected with *S. riobrave* showed similar results to those found by Ramos-Rodríguez et al. (2006). In that earlier study, the first peak occurred at 21.7 ± 0.6 h post-infection and the second peak at 97.7 ± 3.1 h post-infection, but in the current study, we found that the peaks occurred somewhat later at 28.90 ± 2.43 h and 124.80 ± 12.32 h post-infection. In both host species injected with *X. cabanillasii*, a single peak of CO_2 was produced and the mean CO_2 peak height was significantly different between host species. The timing of the first CO_2 peak corresponds with our

hypothesized optimal infection time, but Ramos-Rodríguez et al. (2007) found no changes in long-range attraction that corresponded with this peak. Christen et al. (2007) found enhanced infection behavior at this time point in choice bioassays, but decreased infection in no-choice bioassays. However, patterns of infection of bacteria-injected hosts were not the same as that of natural infections, even though an equivalent CO_2 peak was present. Therefore, the importance of elevated CO_2 production remains uncertain.

Control injection treatments with *G. mellonella* suggest that the trauma of injecting an insect may result in a small increase in CO_2 production, but such increases were much lower than that observed with the *X. cabanillasii* injections. This increase in CO_2 production could be a consequence of increased host immune system activity. Marris et al. (1999) found a similar response in *Lacanobia oleracea* (L.) pupae injected with *Pimpla hypochondriaca* (Retzius) venom; where initially low levels of CO_2 increased sharply 4 h post-injection, but then gradually decreased until the end of the experiment.

We conclude that bacterial growth is likely responsible—either directly or in combination with host related processes—for the first peak of CO_2 in both host species, but not for the second peak exhibited in *G. mellonella*. Degradation of host tissues may also contribute to the first peak of CO_2, although based on Ramos-Rodríguez et al. (2007) this contribution is likely to be minor. Since the second peak of CO_2 from infected *G. mellonella* occurs late in the infection, respiration by the large number of actively developing nematodes may be responsible. Between infected *G. mellonella* and *T. molitor*, which does not exhibit a second CO_2 peak, there is a relatively small difference in nematode numbers at the time when the second production peak occurs (Ramos-Rodríguez et al., 2007). The relationship between density of parasitic stages and CO_2 production needs further exploration. Smigielski et al. (1994) reported that stationary secondary phase bacteria maintain higher levels of respiratory enzymes than primary forms, which might contribute to a second peak of CO_2. However, we saw only primary phase *X. cabanillasii* in the host and only detected the secondary phase on NBTA plates after repeated sub-culturing.

Interactions with infected hosts are a dynamic process that may involve multiple cues from multiple sources, some of which may enhance or suppress infection behavior. It is likely that changes in CO_2 production observed here and the nematode response to these changes are moderated by other cues/factors. For example, removal of CO_2 did not completely eliminate host attraction (Gaugler and Campbell, 1991). Previous studies have shown that contact cues (Lewis et al., 1992, 1996) and host exudates (Kunkel et al., 2006) are also important in host selection, but the specific cues involved, concentrations needed to elicit responses, and how they interact are not well understood. To further understand the process of host-acceptance additional research is needed to identify the specific cues involved in host-acceptance and the sources of these cues.

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