Microbial Population Dynamics in the Hemolymph of *Manduca sexta* Infected with *Xenorhabdus nematophila* and the Entomopathogenic Nematode *Steinernema carpocapsae*

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Xenorhabdus nematophila engages in a mutualistic association with the nematode *Steinernema carpocapsae*. The nematode invades and traverses the gut of susceptible insects. *X. nematophila* is released in the insect blood (hemolymph), where it suppresses host immune responses and functions as a pathogen. *X. nematophila* produces diverse antimicrobials in laboratory cultures. The natural competitors that *X. nematophila* encounters in the hemolymph and the role of antimicrobials in interspecies competition in the host are poorly understood. We show that gut microbes translocate into the hemolymph when the nematode penetrates the insect intestine. During natural infection, *Staphylococcus saprophyticus* was initially present and subsequently disappeared from the hemolymph, while *Enterococcus faecalis* proliferated. *S. saprophyticus* was sensitive to *X. nematophila* antibiotics and was eliminated from the hemolymph when coinjected with *X. nematophila*. In contrast, *E. faecalis* was relatively resistant to *X. nematophila* antibiotics. When injected by itself, *E. faecalis* persisted ($\sim 10^3$ CFU/ml), but when coinjected with *X. nematophila*, it proliferated to $\sim 10^9$ CFU/ml. Injection of *E. faecalis* into the insect caused the upregulation of an insect antimicrobial peptide, while the transcript levels were suppressed when *E. faecalis* was coinjected with *X. nematophila*. Its relative antibiotic resistance together with suppression of the host immune system by *X. nematophila* may account for the growth of *E. faecalis*. At higher injected levels (10^6 CFU/insect), *E. faecalis* could kill insects, suggesting that it may contribute to virulence in an *X. nematophila* infection. These findings provide new insights into the competitive events that occur early in infection after *S. carpocapsae* invades the host hemocoel.

Bacteria exist in multispecies populations in which competition for resources and space drive community dynamics and evolutionary processes. Pathogens confront a dual challenge of competing with other microbes in the environment and evading or suppressing activated immune responses. While competition under laboratory conditions has been studied extensively, much less is known about the competitive interactions in a host organism. The tripartite symbiosis involving the pathogenic bacterium *Xenorhabdus nematophila*, an entomopathogenic nematode, and an insect host provides a tractable model to study microbial competition and immune suppression in a natural biological environment.

Xenorhabdus nematophila exhibits a bimodal life cycle: it establishes a species-specific mutualistic relationship with the entomopathogenic nematode *Steinernema carpocapsae* and launches a pathogenic attack on susceptible insect larvae (1–5). The infective juvenile (IJ) stage of the nematode invades insect larvae through natural openings such as the mouth or anus, punctures the midgut to enter the hemocoel (body cavity), and releases *X. nematophila* into the hemolymph (2). *X. nematophila* is not detected in the hemolymph 5 h postinvasion, while by 12 h, it colonizes the connective tissue surrounding the anterior midgut (6). In the hemocoel, *X. nematophila* functions as a pathogen by suppressing the host immune system and secreting insect toxins, cytotoxins, and hemolysins that participate in killing the host (4).

An initial step toward mounting an insect immune response is recognition of foreign microbes by pattern recognition proteins (PRP), such as hemolin, peptidoglycan recognition protein (PGRP), and immunoglobins (7, 8). PRPs bind conserved microbial-associated molecular pattern (MAMP) motifs and initiate the im-

mune response. The immune response comprises humoral and cellular pathways. Humoral immune responses include stimulation of phospholipase A2 (PLA2) activity that releases arachidonic acid from membrane phospholipids, resulting in the production of eicosanoids that activate hemocytes and induce the expression of antimicrobial peptide (AMP) genes (9). Cecropin is a bacterium-inducible AMP that disrupts bacterial cell membranes, leading to cell lysis (10). Cellular immune responses use circulating hemocytes to bring about phagocytosis, aggregation, and encapsulation or nodulation (11). A central response of the innate immune system is the conversion of prophenoloxidase (ProPO) to the active phenoloxidase (PO), involved in quinone synthesis, and the formation of melanin that binds to the microbial cell surface, functioning as an opsonin. *X. nematophila* produces several compounds that suppress aspects of the insect innate immune response. These include tyrosine-derived cell surface molecules (rhabduscin), which directly inhibit PO activity (12), and the monoterpene compound benzylideneacetone (13), which inhibits PLA2 activity, reduces AMP synthesis, and blocks PO activ-

Received 6 March 2014 Accepted 28 April 2014

Published ahead of print 9 May 2014

Editor: C. R. Lovell

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AEM.00768-14>.

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doi:10.1128/AEM.00768-14

ity (9, 14). Interestingly, benzylideneacetone itself has antimicrobial activity (13).

The suppression of insect host immunity may benefit *X. nematophila* but can also facilitate the growth of competitors in the insect hemocoel, and it is therefore important to understand the broader microbial ecology of an *X. nematophila*-infected host. The tobacco hornworm, *Manduca sexta*, is a model insect commonly used to study *X. nematophila* pathogenicity and suppression of host immune responses (11). The intestinal microbiota of *M. sexta* has been characterized in insects grown on different diets. In insects raised on the natural diet of tobacco leaves, *Enterococcus* spp. were the predominant species isolated from the gut (15). *Enterococcus faecalis* is a common gut microbe isolated from lepidoptera (16) and several other orders of insects (17). Recently, it was shown that the injection of a clinical strain of *E. faecalis* into the hemocoel of *M. sexta* caused insect death, whereas when this strain was introduced into the gut, it persisted without overt damage to the host. However, when *E. faecalis*-colonized insects were also fed the pore-forming insecticidal *Bacillus thuringiensis* (Bt) toxin, *E. faecalis* translocated into the hemocoel, causing insect immune response induction and death (18).

Whereas *X. nematophila* produces diverse antimicrobial products in culture, the role they play in suppressing microbial competitors during infection remains poorly understood. Xenocoumacin (Xcn) is the major soluble antibiotic produced by *X. nematophila* in broth culture (19) and has been detected in the infected wax worm, *Galleria mellonella* (20). Xcn1, the most active form of xenocoumacin, is produced at high levels and subsequently converted to the less active compound, Xcn2, to avoid self-toxicity (21).

Very little is known about microbial competition during the early stages of invasion of the insect hemocoel by *S. carpocapsae*. In the present study, we address several unanswered questions. Do microbes translocate from the insect gut into the hemolymph when the nematode invades the hemocoel? Do gut microbes proliferate in the hemocoel? What are the population dynamics of competitors and *X. nematophila* during the early stage of infection? And, do the competitors exhibit different sensitivities to the antimicrobial products of *X. nematophila*?

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Xenorhabdus nematophila* AN6/1 (phase I, opaque colonies) was used as the wild-type strain. The *E. faecalis* human clinical strain OG1RF (22) was kindly provided by R. van der Hoeven. All bacteria used in this study were grown at 30°C either in Luria-Bertani (LB) broth (23) or on LB agar plates (15 g/liter agar). After preparation, medium was maintained in the dark. Strains grown overnight in LB broth (supplemented with 50 µg/ml ampicillin for *X. nematophila*) were subcultured (1:20) in 5 ml of fresh LB broth, and growth was monitored by turbidity using a Klett-Summerson colorimeter or by measuring optical density at 600 nm (OD₆₀₀). The final bacterial cultures were normalized using OD₆₀₀ values. Grace's insect culture medium (Gibco) was used to dilute cultures for insect injections and dilutional plating.

Sources, treatments, and rearing of *Manduca sexta* larvae. Unless otherwise mentioned, *M. sexta* eggs were obtained from the insect colony at the University of Wisconsin—Milwaukee. Eggs were placed in clean plastic cups along with diet and incubated in an insect incubator with a 16-h/8-h light/dark photoperiod at room temperature. After hatching, larvae were moved to clean boxes and provided fresh diet. Boxes were cleaned daily, and larvae were fed regularly. The fourth-instar stage was used for all experiments. Commercial premixed diet (North Carolina State University Insectary, referred to as NCSU diet hereinafter) without

added antibiotics was the primary initial diet used, later prepared from individual ingredients. The diet was prepared according to supplier instructions (24). For some experiments, the commercially available gypsy moth diet (high wheat germ diet; MP Biomedicals), prepared according to the manufacturer's instructions and without added antibiotics, was used. The diets were swabbed and homogenized and then plated on LB agar plates and shown to be devoid of microbial contamination.

Gut dissections and isolation of gut microbes. Fourth-instar larvae anesthetized on crushed ice for 15 to 20 min were surface sterilized by submerging them in 70% cold ethanol. Dissecting implements were sterilized with ethanol, and rubber gloves were worn during the procedure. The insect gut was exposed by dorsal incision, and the dissected gut was placed in a sterile 1.5-ml tube containing between 200 and 500 µl of LB broth. The tissue was homogenized by grinding for 2 min using a Kontes pellet pestle microgrinder (Kimble Chase). Serial dilutions of the suspension were made in LB broth and plated on LB agar, followed by incubation at 30°C for 48 h. The resulting colonies were categorized based on colony morphology, pigmentation, and surface properties. Representative colonies of each type were patched to fresh plates and used for colony PCR to amplify 16S rRNA genes. Briefly, a small portion of the colony was resuspended in 3 µl of nuclease-free water and boiled for 4 min. PCR amplification (25-µl final volume) was performed using the GoTaq green master mix kit (Promega) with 1 µl each, at 10 µM, of the universal 16S rRNA (bacterial) gene primers 11F (5'-GTTTGATCCTGGCTCAG-3'), and 1512R (5'-ACGGYTACCTTGTTACGACTT-3'), obtained from Integrated DNA Technologies, Inc. The PCR was carried out for 30 s at 94°C, 30 s at 50°C, and 2 min at 72°C for 30 cycles. If direct colony PCR did not yield products, DNA extracted from overnight cultures using the PurElute bacterial genomic kit (Edge BioSystems) was used in the PCR. The PCR product was checked on an agarose gel and purified using the GeneClean Turbo kit (MP Biomedicals). Nucleotide sequence analysis was performed at the University of Chicago Cancer Research DNA Sequencing and Genotyping Facility. Trimmed sequences were used for BLASTN analysis for genus and species identification. For *E. faecalis*, *Staphylococcus saprophyticus*, and *Aerococcus viridans*, the sequences were at least 1,000 nucleotides long and showed 99% identity along the entire length. In cases in which PCR amplification was unsuccessful, microscopic analysis was performed using a wet mount to identify large oval, nucleated, and budding cells characteristic of yeasts.

Natural infections, isolation of insect hemolymph, and determination of microbial composition. For natural infections, *Steinernema carpocapsae* infective juveniles (IJs) carrying wild-type *X. nematophila* were used. IJs washed and resuspended in sterile water were pipetted onto wet filter paper lining the bottom of a plastic cup, at 200 IJs/insect. Several fourth-instar larvae were added to each cup. To extract hemolymph at various times, larvae were anesthetized on ice, placed in a bath of 70% ethanol for 30 s, and air dried. A cut was made just below the last proleg, and hemolymph was drained into sterile 1.5-ml tubes. Hemolymph was isolated from individual infected larvae and subsequently pooled for each time point. Hemolymph was also obtained from uninfected larvae raised on the respective diets. The control hemolymph plated on LB agar did not produce bacterial colonies. For culture-dependent determination of the microbial composition of hemolymph, serial dilutions of the extracted hemolymph were plated in triplicate on LB agar plates that were incubated at 30°C for 48 h. Colonies were grouped according to colony morphology, color, shape, and surface properties. All species could be distinguished by colony morphology and pigmentation, with the exception of *E. faecalis* and *Aerococcus viridans*, which were combined in a single group. At least three colonies of each type were patched on LB agar and stored as glycerol stocks at -80°C. From the patch plates, colony PCR and BLASTN analysis was performed and used for species identification. Colonies in which 16S rRNA gene amplification was not successful were analyzed microscopically. Microscopic analysis using wet mounts revealed yeast cells. From the same hemolymph samples, culture-independent analysis was carried out using 1 ml of pooled hemolymph that was centrifuged at 8,000 rpm at

4°C for 10 min. DNA was extracted from the bacterial pellet using the PurElute bacterial genomic kit (Edge BioSystems). The amplified 16S rRNA gene sequence was cloned into *Escherichia coli* using the pGEM-T easy vector kit (Promega). At least 20 positive clones were picked for each time point, and colony PCR using SP6 and T7 primers was performed to amplify the cloned 16S rRNA genes. The PCR products were sequenced and characterized using BLASTN analysis as described above. The experiments were performed at least twice, with reproducible results. The experiments were performed at similar times of the day, with similar feeding cycles for the insects.

Antibiotic overlay assay. Subcultures of *X. nematophila* were grown to exponential phase, and 6- μ l samples of the culture were spotted on LB agar plates and incubated for 24 h. The bacteria were exposed to chloroform fumes for 30 min, followed by air drying for 30 min. One milliliter of overnight culture of the indicator bacterial strain was added to 12 ml top agar (LB with 0.7% agar), which was then poured to form a thin layer over the *X. nematophila* colonies (1, 25). The plates were incubated at 30°C for 48 h. Zones of inhibition were measured in millimeters. The overlay assays were performed four times, with nearly identical results.

In vivo competitions. *X. nematophila*, *E. faecalis*, and *S. saprophyticus* were subcultured in LB, grown to exponential phase, normalized, and diluted in Grace's medium. For the competition experiments, three different ratios were used, (i) a 1:1 mixture of 10^4 CFU/insect of the appropriate bacterial cultures, (ii) a mixture consisting of 10^4 CFU/insect *X. nematophila* and 10^5 CFU/insect *E. faecalis* (1:10 ratio), and (iii) a mixture consisting of 10^5 CFU/insect *X. nematophila* and 10^4 CFU/insect *E. faecalis* (10:1 ratio). Fifty-microliter amounts of the mixtures were injected per insect using 1-ml, 0.45-mm by 16-mm BD syringes with SubQ needles (Becton, Dickinson Co.) mounted on a Stepper repetitive dispensing pipette (Dymax Corp.). Fourth-instar *M. sexta* larvae were anesthetized by placing them on crushed ice for 15 to 20 min, and the area around the horn was cleaned using 70% ethanol before each injection. Grace's medium was injected as a negative control. The insects were placed in plastic cups, and hemolymph was collected at designated time points, serially diluted, and plated on LB agar as described above. Three to four larvae were used per time point, and the experiment was performed at least twice, with reproducible results. The larvae used were obtained from the insect colony and were raised on the NCSU diet prepared from individual ingredients. Again, the experiments were performed at similar times of the day, with similar feeding cycles for the insects.

Immunosuppression by *X. nematophila* in the presence of natural competitors derived from *M. sexta* gut microbiota. Two fifth-instar larvae of *M. sexta* were injected with $\sim 10^4$ CFU/insect of the following: *X. nematophila*, *E. faecalis*, *S. saprophyticus*, *X. nematophila* plus *E. faecalis*, or phosphate-buffered saline (PBS; as negative control). Insect fat body tissue (a major site for immune protein expression) was dissected 16 h postinjection, followed by total RNA extraction using TRIzol reagent (Invitrogen). For reverse transcription, 5 μ g of total RNA was treated with RQ1 RNase-free DNase I (Promega). Reverse transcription was performed using the Mg primer (5'-CGGGCAGTGAGCGCAACGTTTTTTT TTTTTT-3') (Integrated DNA Technologies) and AMV reverse transcriptase (Promega). cDNA was used as the template for quantitative PCR (qPCR). qPCR was performed with Bullseye EvaGreen (MidSci) on a Bio-Rad iCycler. The transcript levels of cecropin were measured and normalized against the transcript levels of rpS3 using the following primers: cecropin-forward (5'-GGTCAAAGGATTCGTGACGC-3'), cecropin-reverse (5'-TTTGATTGTCCTTTGAAAATGGCG-3'), rpS3-forward (5'-ACTTCTCAGGCAAGGAGTGC-3'), and rpS3-reverse (5' GT CACCAGGATGTGGTCTGG-3'). Data were analyzed as described previously (26). Briefly, the cycle threshold (C_T) values were normalized by calculating the ratio between the reference gene and the gene of interest and are presented as the ratio between the values for infected and PBS-injected larvae. Data were statistically analyzed using mixed-effect analysis of variance (ANOVA) with a Tukey's *post hoc* test for multiple compari-

sons on normalized C_T values (SAS Software). The experiment was performed four times.

Virulence comparison of an *M. sexta* gut-derived strain and a human clinical strain (OG1RF) of *E. faecalis*. *M. sexta* eggs obtained from Carolina Biological Supply Company and raised on NCSU diet were used. Both strains of *E. faecalis*, along with *X. nematophila*, were grown overnight and then subcultured in LB broth. Exponential-phase cultures were normalized to obtain similar CFU, and these cultures were diluted in Grace's medium for injection. Fifty microliters of diluted culture were injected per larva in various doses: 10^4 CFU/insect for *X. nematophila* and 10^4 , 10^5 , and 10^6 CFU/insect for both *E. faecalis* strains. Injections were performed as described above, using a 1-ml, 0.45-mm by 16-mm BD syringe with SubQ needle (Becton, Dickinson Co.) mounted on a Stepper repetitive dispensing pipette (Dymax Corp.). Injected larvae were put in cups along with some food and moved to the insect incubator, where they were observed for mortality for up to 69 h. Six larvae were used per condition, and the experiment was performed twice with reproducible results.

GenBank sequence accession numbers. The GenBank accession numbers for 16S rRNA sequences are as follows: *E. faecalis*, KF709388; *S. saprophyticus*, KF709389; and *A. viridans*, KF709390.

RESULTS

Translocation of gut microbiota to the hemolymph during natural infection. To address the question of whether or not insect gut microbiota translocate into the hemocoel during *S. carpocapsae*-*X. nematophila* infection, we first characterized the gut microbial community of our laboratory colony of *M. sexta*. Since the gut microbiota of *M. sexta* can vary widely depending on the diet used to raise the insects (Table 1), we characterized the microbial communities of *M. sexta* larvae grown on two different commercially available artificial diets, North Carolina State University (NCSU) diet and gypsy moth diet. We performed two independent experiments with insects raised on the NCSU diet (experiments 1 and 2) and two independent experiments with insects raised on the gypsy moth diet (experiments 3 and 4). In each experiment, a portion of the population was used to dissect the intestine to analyze the gut microbial community and a portion was exposed to *S. carpocapsae* to assess the microbial population in the hemolymph of infected larvae during the early phase of infection (Table 2).

In insects raised on NCSU diet, *Klebsiella oxytoca* and *Staphylococcus saprophyticus* were the dominant gut microbes in experiment 1, while *E. faecalis* and *S. saprophyticus* were dominant in experiment 2. In insects raised on gypsy moth diet, *Enterococcus mundtii*, *S. saprophyticus*, and a yeast were the major gut microbes in experiment 3, while the yeast was the major gut microbe isolated in experiment 4. Thus, *Enterococcus* spp. and *S. saprophyticus* were dominant in insects raised on both diets, while the yeast was more prevalent in insects raised on the gypsy moth diet.

To determine whether gut microbiota can translocate from the gut to the hemocoel during natural infection, hemolymph was dilutionally plated and the resulting colonies were characterized as described above. In all experiments, the major isolates identified in the gut were also microbes that were dominant in the hemolymph early in infection (Table 2). In control experiments, no colonies were obtained from hemolymph collected from uninfected insects. These findings indicate that gut microbes are translocated into the hemocoel when the invading nematode penetrates the intestine of *M. sexta*.

Microbial population dynamics in hemolymph of *M. sexta* infected with *S. carpocapsae* IJs. In experiment 2, we used both culture-dependent and culture-independent methods to charac-

TABLE 1 Published diversity of gut microbiota of *Manduca sexta*

Insect diet	Antibiotics added	Egg source	Egg treatment	Major genera identified	Method of identification	Reference
Natural						
<i>Solanum dulcamara</i> (nightshade)	None	Laboratory colony	None	<i>Bacillus</i> , <i>Serratia</i> , <i>Candida</i>	Culture dependent	27
<i>Nicotiana tabacum</i> (tobacco)	None	Laboratory colony	None	<i>Enterococcus</i>	Culture independent	15
Artificial						
NCSU Insectary	None	Laboratory colony	Bleach	<i>Pediococcus</i> <i>Micrococcus</i> , <i>Staphylococcus</i>	Culture dependent	28
	Kanamycin, streptomycin	Laboratory colony	Bleach	<i>Paenibacillus</i> , <i>Microbacterium</i> , <i>Bacillus</i> , <i>Methylobacterium</i>	Culture dependent	
USDA Hamden formula ^a	None	Carolina Biological Supply	Tween 80, bleach	<i>Enterobacter</i> , <i>Klebsiella</i>	Culture independent	16
	Penicillin, gentamicin, rifampin, streptomycin	Carolina Biological Supply	Tween 80, bleach	None	Culture independent	

^a Contains chlortetracycline.

terize the population dynamics in naturally infected *M. sexta*. For this reason, temporal fluctuations in microbial populations in the hemocoel in *M. sexta* insects naturally infected with *S. carpocapsae* were examined in experiment 2. Hemolymph was obtained at various times postinfection, and microbial species were identified as described above. The microbial population was diverse early in infection, at 5 h and 7.5 h (Fig. 1A). *E. faecalis*/*A. viridans* were dominant, while *S. saprophyticus*, other minor species, and the yeast were present at lower levels. *X. nematophila* was detectable at 7.5 h. By 18 h, *X. nematophila* became the dominant species, *E. faecalis*/*A. viridans* persisted, and the other species disappeared. The relative levels of *E. faecalis*/*A. viridans* increased at 24 h, while *X. nematophila* was dominant at later times. The growth of *X. nematophila* and *E. faecalis*/*A. viridans* in the hemolymph at each time point was monitored in the same experiment (experiment 2) by determining CFU/ml of hemolymph (Fig. 1B). At 7.5 h, the population of *E. faecalis*/*A. viridans* had reached 4×10^4 CFU/ml, while *X. nematophila* was present at 10^3 CFU/ml. By 18 h, *X. nematophila* had reached 4×10^5 CFU/ml, while the population of *E. faecalis*/*A. viridans* increased at a lower rate during this period but began to increase more rapidly by 24 h. At later times, the population of *X. nematophila* increased rapidly, while *E. faecalis*/*A. viridans* continued to increase at a lower rate than *X. nematophila*, resulting in the higher percentages of *X. nematophila* seen in the results in Fig. 1A. In these experiments, it was difficult to reliably obtain CFU/ml data after 48 h due to degradation of insect tissues and increased viscosity of the hemolymph.

To determine whether the culture-dependent analysis of mi-

crobial population dynamics was representative and not biased against unculturable species, we carried out culture-independent analysis of the microbial community in the hemolymph of infected insects in the same experiment (Fig. 1C, experiment 2). This approach also allowed us to determine the relative levels of *E. faecalis* and *A. viridans* in the population. The culture-independent analysis identified the same pattern of population fluctuation as was observed with the culture-dependent approach. The microbial population was diverse early in infection (7.5 h). At this time, *E. faecalis* was dominant, while other species were present at lower levels. By 18 h and 24 h, *X. nematophila* had become the dominant species, *E. faecalis* persisted, and the other species, including *A. viridans* and *S. saprophyticus*, had disappeared. *X. nematophila* was the only species isolated at later times in the culture-independent analysis, most likely due to the limited number of 16S rRNA gene clones sequenced in this experiment.

The natural infection experiment was repeated, and temporal fluctuations in microbial populations in the hemocoel were monitored as described above. The results were similar to those of experiment 2 except that *S. saprophyticus* was not detected during the early phase of infection and the levels of the yeast were noticeably higher (see Fig. S1 in the supplemental material). By 18 h, *X. nematophila* was dominant, *E. faecalis*/*A. viridans* continued to persist, and the yeast had disappeared.

Together, these findings establish that diverse gut microorganisms translocated into the hemocoel during invasion, with *E. faecalis*/*A. viridans* dominating early in infection. By 18 h, *X.*

TABLE 2 Transfer of microbiota from the gut to the hemolymph during natural infection of *Manduca sexta*

Experiment	Insect diet	Major isolates (CFU/ml ^a) in:	
		Gut	Hemolymph
1	NCSU Insectary	<i>K. oxytoca</i> , <i>S. saprophyticus</i>	<i>K. oxytoca</i> (3.7×10^5), <i>S. saprophyticus</i> (1.0×10^4)
2	NCSU Insectary	<i>E. faecalis</i> , <i>S. saprophyticus</i>	<i>E. faecalis</i> / <i>A. viridans</i> (4.2×10^4), yeast (2.2×10^4), <i>S. saprophyticus</i> (5.0×10^2)
3	Gypsy moth	<i>E. mundtii</i> , yeast, <i>S. saprophyticus</i>	<i>E. mundtii</i> (2.5×10^7), yeast (4.3×10^6), <i>S. saprophyticus</i> (1.0×10^4)
4	Gypsy moth	Yeast	Yeast (1.6×10^4)

^a All CFU/ml values were measured at 7.5 h postinfection except in experiment 3, for which the measurement was at 18 h.

FIG 1 Microbial population dynamics in hemolymph of *M. sexta* naturally infected with *S. carpocapsae*. (A) *M. sexta* larvae were exposed to *S. carpocapsae* for various times over a 48-h period. At indicated times, hemolymph was extracted and serially diluted, and resulting colonies were grouped by morphology and pigmentation. Species were identified by 16S rRNA gene sequencing. The species isolated are indicated; “Others” represents minor bacteria including small percentages of *Pseudomonas* spp. and *Brachy bacterium* spp. Data are represented as the percentage of each species relative to the total colonies counted at each time point. (B) Total CFU/ml of hemolymph of *X. nematophila* and *E. faecalis/A. viridans* obtained at each time point shown in panel A. Error bars show standard errors of the mean. (C) 16S rRNA gene clones obtained from total microbial genomic DNA in infected insect hemolymph (see panel A) were sequenced, and species were identified by BLASTN analysis. The species identified are indicated; “Others” represents minor percentages of *Brachy bacterium* spp., *Klebsiella* spp., *Paracoccus* spp., and *Pediococcus* spp. Data are represented as the percentage of clones of each species relative to the total clones at each time point.

nematophila had become the dominant species and *E. faecalis/A. viridans* persisted (Fig. 1B), while other species had disappeared.

Sensitivity of competitors to *X. nematophila* antibiotics. To assess the possibility that antibiotics produced by *X. nematophila* contributed to the population fluctuations observed during infection, the sensitivities of *S. saprophyticus*, *E. faecalis*, *A. viridans*, and

FIG 2 Antibiotic activity of *X. nematophila* against the microbes present in the insect hemolymph. Antibiotic overlay assays were performed with *X. nematophila* against the indicated organisms obtained from infected insect hemolymph. The diameter of the zone of inhibition is proportional to the sensitivity of the competitor to *X. nematophila* antibiotics.

the yeast were analyzed by diffusion overlay assays (Fig. 2). *S. saprophyticus* was the most sensitive (27-mm zone of inhibition) to antibiotics produced by *X. nematophila*, *A. viridans* was moderately sensitive (16-mm zone), and the yeast was somewhat less sensitive (12-mm zone). Interestingly, *E. faecalis* was the most resistant strain (9-mm, hazy zone), suggesting that its persistence during later stages of infection may be due, in part, to its resistance to *X. nematophila* antibiotics.

In vivo competition in *M. sexta*. To further dissect the competitive events that occur early in infection, we assessed the ability of individual species to compete with *X. nematophila*. To first determine whether the individual species were able to persist and proliferate alone in the hemocoel, *S. saprophyticus*, *E. faecalis*, and *A. viridans* were injected individually into *M. sexta* insects and growth was monitored for 24 h. *S. saprophyticus* persisted at 8

FIG 3 *In vivo* growth of isolates in *M. sexta* hemocoel after injection. Insects were injected with 10^4 CFU/insect of one of the indicated bacteria. Hemolymph recovered at 8 and 24 h postinjection was dilutionally plated. Data are represented as CFU/ml of each bacterial species at 8 and 24 h. Error bars show standard errors of the means. Statistically significant differences ($P < 0.05$) between the two time points in a group are indicated by an asterisk. Statistical analysis was performed using a two-tailed paired Student's *t* test.

h, and the cell density increased slightly at 24 h (Fig. 3). *E. faecalis* persisted at 8 h, while the cell density decreased slightly at 24 h. *A. viridans* was present at low levels at 8 h and was detectable at lower levels at 24 h. Thus, all strains persisted at 24 h, while the cell densities did not increase significantly relative to the levels seen at 8 h, with *S. saprophyticus* able to survive better than either *E. faecalis* or *A. viridans*.

To evaluate the competitive interactions between *S. saprophyticus* and *X. nematophila*, both bacteria were coinjected into *M. sexta* insects (Fig. 4A). *S. saprophyticus* was eliminated by 24 h when coinjected with *X. nematophila*. In contrast, *S. saprophyticus* was not eliminated by 24 h when coinjected with *E. faecalis* (see Fig. S2 in the supplemental material). *S. saprophyticus* and *E. faecalis* also did not display any antibiotic activity against each other in overlay assays (see Fig. S3). When *E. faecalis* was coinjected into *M. sexta* (Fig. 4B), it proliferated to high levels in the presence of *X. nematophila*, while it did not grow significantly better when coinjected with *S. saprophyticus* (see Fig. S2). These findings are consistent with the population dynamics we observed

during natural infection and suggest that its relative antibiotic resistance and possible syntrophic effects when present along with *X. nematophila* allowed *E. faecalis* to grow in the insect hemocoel. Another possibility could be that the suppression of the host immune system by *X. nematophila* might aid *E. faecalis* growth (see below).

During the early phase of natural infection, the levels of *E. faecalis* were significantly higher than those of *X. nematophila* (Fig. 1). To more closely mimic the relative cell density that occurs during natural infection, *M. sexta* insects were coinjected with a 10:1 ratio of *E. faecalis*/*X. nematophila* (Fig. 5B). As expected, *E. faecalis* proliferated in the presence of *X. nematophila*. Since the cell density of *X. nematophila* was significantly higher than that of *E. faecalis* at later times during infection, we also coinjected *M. sexta* with 10-fold more *X. nematophila* than *E. faecalis* (Fig. 5C). Again, *E. faecalis* was able to proliferate in the presence of *X. nematophila*. These findings show that the growth of *E. faecalis* in the hemolymph was enhanced by the presence of *X. nematophila* (compare the results in Fig. 3 with those in Fig. 4 and 5) even when the latter was present at 10-fold-higher levels.

Induction of antimicrobial peptide transcripts by *E. faecalis* is suppressed by *X. nematophila*. The proliferation of *E. faecalis* in the presence of *X. nematophila* suggested that this strain might benefit from suppression of the host immune response by *X. nematophila*. To explore this possibility, qPCR analysis was performed to determine the relative transcript levels of cecropin in insects injected with either *X. nematophila* or *E. faecalis* or coinjected with both bacteria. The expression of cecropin is highly upregulated when insects are injected with nonpathogenic bacteria but suppressed when nonpathogenic bacteria are coinjected with *X. nematophila*. As predicted, cecropin transcripts were detectable in insects injected with *X. nematophila* and were induced to high levels in insects injected with *E. faecalis* relative to the levels in PBS-injected controls (Fig. 6). In insects coinjected with both bacteria, the transcript levels of cecropin were similar to or less than the levels in insects injected with *X. nematophila* alone. These results support the idea that *X. nematophila* suppresses the AMP gene expression induced by the presence of *E. faecalis*. We also found that the ability of *S. saprophyticus* to survive in the hemolymph when injected alone (Fig. 3) could be attributed, in part, to

FIG 4 *In vivo* growth after 1:1 coinjection of *X. nematophila* with *S. saprophyticus* or *E. faecalis* into *M. sexta*. (A and B) Insects were coinjected with a 1:1 mixture of *X. nematophila* and *S. saprophyticus* (A) or *E. faecalis* (B). An amount of 10^4 CFU/insect of each species was injected. Hemolymph recovered at 8 and 24 h postinjection was dilutionally plated. Data are represented as CFU/ml of each bacterial species at 8 and 24 h. Error bars show standard errors of the means.

FIG 5 *In vivo* competition between *X. nematophila* and *E. faecalis* coinjected into *M. sexta* at various ratios. (A) Insects were injected individually with 10^5 CFU/insect of either *X. nematophila* or *E. faecalis*. (B) Insects were coinjected with a 1:10 mixture of *X. nematophila* (10^4 CFU/insect) and *E. faecalis* (10^5 CFU/insect). (C) Insects were coinjected with a 10:1 mixture of *X. nematophila* (10^5 CFU/insect) and *E. faecalis* (10^4 CFU/insect). Hemolymph recovered at 8 and 24 h postinjection was dilutionally plated. Data are represented as CFU/ml of each bacterial species at 8 and 24 h. Error bars show standard errors of the means.

the lack of induction of cecropin transcripts relative to the transcript level in the control (Fig. 6).

***E. faecalis* isolated from the gut is pathogenic toward *M. sexta*.** The persistence of *E. faecalis* during the early phase of infection and the virulence of a clinical strain of *E. faecalis* toward *M. sexta* (18) raised the possibility that *E. faecalis* isolated from the insect gut would be pathogenic toward *M. sexta*. To explore this possibility, the virulence of the gut strain was compared to those of the clinical strain of *E. faecalis* and *X. nematophila* (Fig. 7). At 22 h, 50% of the insects had died (median lethal time [LT₅₀], 22 h) when injected with *X. nematophila* at a dose of 10^4 CFU/insect, while 100% of the insects were dead by 25 h (LT₁₀₀, 25 h). The gut strain of *E. faecalis* was also virulent at a higher dose of 10^6 CFU/insect (LT₅₀, 27 h, and LT₁₀₀, 43 h), while the clinical strain was less virulent (LT₅₀, 43 h) and was not able to kill 100% of the injected insects. In contrast, *S. saprophyticus* injected at a dose of 10^6 CFU/insect did not result in mortality of *M. sexta* larvae (data not shown). These results, combined with the evidence of persistence of *E. faecalis* during infection, suggest the possibility that *E. faecalis* may contribute to pathogenicity during natural infection of *M. sexta*.

DISCUSSION

The role of gut microbiota in normal health, development, and disease susceptibility has been extensively examined in several animals (28–30). The movement of bacteria into and across intesti-

nal epithelial cells is a major source of diseases originating from the gastrointestinal tract. Whether native gut microbiota are translocated into the hemocoel during natural infections of *M. sexta* with entomopathogenic nematodes had not been previously studied. Here, we characterize the gut microbiota of *M. sexta* and the translocation of microbes into the hemocoel during the early phase of infection by *S. carpocapsae*.

We show that gut microbes were translocated into the hemocoel of *M. sexta* insects naturally infected with *S. carpocapsae*. During the early phase of infection, the initial population was diverse and reached cell densities of $\sim 10^5$ CFU/ml, while *X. nematophila* was barely detectable at this time. The relatively high microbial load was unexpected, since the innate immune response is rapidly induced in the presence of bacteria and yeasts (11, 31). Within hours after the injection of bacteria, activated hemocytes engulf bacterial invaders and pattern recognition proteins are induced. Microaggregation of hemocytes has been observed 4 h after the injection of *M. sexta* with *E. faecalis* (18) and *Salmonella enterica* (32), and AMP genes were induced 9 h after the injection of *Salmonella enterica* (32). In *Spodoptera exigua*, numerous immune response genes were induced 8 h after the injection of either *E. coli* or *Flavobacterium* (14). Several factors could account for the microbial load observed during the early phase of infection. Since AMPs are secreted into the insect intestine (11), it is possible that native microbiota may develop tolerance to the host immune response. They might also be able to avoid host immune mediators. Whatever the mechanism, the induction of the immune response is apparently not sufficient to prevent gut microbes from proliferating in the hemolymph. In addition, *X. nematophila*-derived antimicrobial compounds would not be present at appreciable levels during early infection, since this bacterium is present at low cell densities at that time. Antibiotic activity was not detected until 36 h after *G. mellonella* was injected with *S. carpocapsae* (20). Thus, during the early phase of infection, competitors benefit from an apparently insufficient immune response and minimal antimicrobial deterrence.

As shown previously, gut microbiota of *M. sexta* can vary and may be influenced by diet (Tables 1 and 2). *Enterococcus* species were major isolates identified in experiment 2 and experiment 3, consistent with previous observations in *M. sexta* insects raised on the natural diet of tobacco leaves (15). *E. faecalis* has been identified in the gut of gypsy moth larvae raised on 5 different diets (16). It was proposed that *E. faecalis* could modify the high alkalinity of the larval gut and influence the microbial gut community. *E.*

FIG 6 Relative cecropin transcript levels in insects injected with *E. faecalis* and *S. saprophyticus* alone or coinjected with *E. faecalis* and *X. nematophila*. Fifth-instar *M. sexta* larvae were injected with either PBS that served as a negative control for immune activation or *X. nematophila*, *E. faecalis*, or *S. saprophyticus* or were coinjected with both *X. nematophila* and *E. faecalis*. RNA extracted at 16 h postinjection from fat body tissue was converted to cDNA, and the cDNA was used to assess the relative transcript levels of the antimicrobial peptide cecropin (highly induced upon bacterial challenge). Statistically significant differences between two groups are indicated by different letters between the groups.

FIG 7 Comparison of the virulence of *E. faecalis* (gut isolate), *E. faecalis* (OG1RF), and *X. nematophila* toward *M. sexta*. Insects were injected with *E. faecalis* at a dose of 10^6 CFU/insect, 10^5 CFU/insect, or 10^4 CFU/insect as indicated. *E. faecalis* (gut isolate) is represented with open symbols, and *E. faecalis* OG1RF is represented by closed symbols. Insects injected with *X. nematophila* (10^4 CFU/insect) are represented by closed circles. Survival was monitored over a period of time, and virulence is depicted as percent survival.

faecalis was also the dominant microbial species isolated from the gut of *G. mellonella* (33) and was the only bacterial species isolated from macerated *G. mellonella* (34). We showed that during natural infection, *E. faecalis* was present in the hemolymph by 5 h, persisted at 10 h, and subsequently increased as the cell density of *X. nematophila* increased. A similar pattern was observed in *M. sexta* insects coinjected with *E. faecalis* and *X. nematophila*. We also found that *E. faecalis* was relatively resistant to the antibiotics produced by *X. nematophila*. Additionally, the transcript levels of the AMP cecropin were suppressed by *X. nematophila* when it was coinjected with *E. faecalis*, which by itself induced the upregulation of this gene (Fig. 6). These findings suggest that the combined effects of immune suppression by *X. nematophila*, the relative antibiotic resistance of *E. faecalis*, and possible syntrophic interactions may create conditions for *E. faecalis* to proliferate. Furthermore, the native *E. faecalis* had, by 18 h, reached a cell density that was shown to be lethal to *M. sexta* (Fig. 7). Thus, in insects in which *E. faecalis* is present in the gut, invasion by entomopathogenic nematodes could result in its translocation to the hemocoel, where it may contribute to virulence. However, *E. faecalis* was not essential for virulence, since the mortality of *M. sexta* insects lacking *E. faecalis* was similar to the mortality when it was present (data not shown).

During later phases of infection (e.g., 18 h), *X. nematophila* was dominant and the *E. faecalis* cell density had increased, while organisms such as *S. saprophyticus*, *A. viridans*, and yeasts had disappeared. These findings correlated with sensitivity to the antibiotics of *X. nematophila*. In a previous study in which Gram-negative bacteria were sampled in the hemolymph of *G. mellonella* naturally infected with *S. carpocapsae*, *Pasteurella* sp. was the predominant species isolated at 6 h and 12 h, while it had disappeared by 18 h, when *X. nematophila* was the only species isolated (35). The reciprocal relationship between increasing cell density of *X. nematophila* and reduction of competitors suggests that the production of antimicrobial compounds may play a role in the population fluctuations in the infected host. *X. nematophila* produces more than 20 antimicrobial compounds when grown in pure culture in complex medium. However, little is known about antibiotic production in natural host environments. Proline, which is

present at high levels in the hemolymph of *G. mellonella*, was shown to stimulate the production of some secondary metabolites by *X. nematophila* grown in tryptone-yeast extract broth (36). The growth of *X. nematophila* in mixed cultures in the hemolymph also creates the potential for cross-species signaling that may induce the production of antibiotics not detected in pure cultures (37). Further studies in *M. sexta* naturally infected with *S. carpocapsae* and antibiotic-deficient strains of *X. nematophila* will provide greater insight into the role of antimicrobial compounds in natural host environments.

The present findings suggest that *S. carpocapsae* development and colonization is unlikely to occur in a monoculture of *X. nematophila*. The proliferation of competitors could play a role both in determining the host range of *S. carpocapsae* and the susceptibility of the host to infection. Also, since the gut microbiota can vary considerably, the types of competitors that are encountered in the hemolymph of different hosts may differentially affect the ability of *S. carpocapsae* to reproduce. For example, coinoculation of some species of *Xenorhabdus* with axenic *S. carpocapsae* did not alter the nematode's reproduction, while other species prevented its reproduction (38). Similarly, different gut microbiota may have either neutral or antagonistic effects on nematode reproduction. How the variability of the insect gut microbiota influences *S. carpocapsae* development and colonization and insect mortality remains to be determined. It is becoming increasingly apparent that the interspecies competition that occurs during natural infection by entomopathogenic nematodes is complex, influenced by the microbial community of the insect gut, insect immune response, temporal and environmental control of antimicrobial products, and other microbe-nematode interactions yet to be identified.

ACKNOWLEDGMENTS

We are grateful to Jane Witten for providing *M. sexta* eggs, Charles Wimpsee for providing sequences for the universal bacterial ribosomal primers, and Thomy Singh for help with the natural infection experiments. We thank Ransome van der Hoeven for his critically reading of and valuable suggestions for the manuscript. Special thanks go to the Forst laboratory members for the many beneficial discussions.

Swati Singh was supported by a UWM Distinguished Graduate Student Fellowship.

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