


May 2014

# The Role of Antimicrobial Compounds in the Life Cycle of the Symbiotic Bacterium, *Xenorhabdus Nematophila*

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**THE ROLE OF ANTIMICROBIAL COMPOUNDS IN THE LIFE CYCLE OF  
THE SYMBIOTIC BACTERIUM, XENORHABDUS NEMATOPHILA**

by

Swati Singh

A Dissertation Submitted in  
Partial Fulfillment of the  
Requirements for the Degree of

Doctor of Philosophy  
in Biological Sciences

at

The University of Wisconsin-Milwaukee

May 2014

# ABSTRACT

THE ROLE OF ANTIMICROBIAL COMPOUNDS IN THE LIFE CYCLE OF THE  
SYMBIOTIC BACTERIUM, *XENORHABDUS NEMATOPHILA*

by

**Swati Singh**

**The University of Wisconsin-Milwaukee, 2014  
Under the Supervision of Professor Steven A. Forst, Ph.D.**

The bacterium *Xenorhabdus nematophila* maintains a mutualistic relationship with the entomopathogenic nematode *Steinernema carpocapsae* and is also pathogenic towards insect larvae. *X. nematophila* possesses a large number of gene clusters potentially involved in antimicrobial production. Several antibiotics, including xenocoumacin (Xcn) produced at high levels in broth cultures, have been characterized. In this study I established that during nematode invasion of the insect body cavity (hemocoel) gut microbiota enter the hemocoel representing potential competitors for *X. nematophila*. As infection progressed some transient species, such as *Staphylococcus saprophyticus* disappeared early in infection, while other persistent species such as *Enterococcus faecalis* proliferated. *S. saprophyticus* was found to be highly sensitive towards *X. nematophila* antibiotics and *E. faecalis* was more resistant. *S. saprophyticus* was eliminated when co-injected with *X. nematophila* into the insect host, *Manduca sexta*. In contrast, *E. faecalis* proliferated when co-injected with *X. nematophila*. The induction of transcripts for cecropin, an insect antimicrobial peptide, by *E. faecalis* was suppressed by

the presence of *X. nematophila* suggesting that *E. faecalis* proliferation was due in part to a combination of immune suppression and relatively high antibiotic resistance. Injection of *E. faecalis* into *M. sexta* caused mortality suggesting that *E. faecalis* may contribute to, but is not required for, virulence in an insect infected with *X. nematophila*. The role of antibiotics in interspecies competition was assessed using various antibiotic-deficient strains of *X. nematophila* co-inoculated in LB broth with either *S. saprophyticus* or *E. faecalis*. Antibiotics are produced at high levels in LB broth. During the course of this study I discovered a new non-ribosomal peptide synthetase (NRPS) cluster (cluster F) that produced antibiotic activity. The elimination of *S. saprophyticus* required Xcn but not compound F. In contrast, elimination of *E. faecalis* was not dependent on either Xcn or compound F. When competitions were carried out in a more biologically relevant medium (Grace's medium) based on lepidopteran insect hemolymph, both the competitors grew better than *X. nematophila* due to lower production of antibiotics in Grace's medium and faster growth rate of the competitors. *S. saprophyticus* was eliminated when inoculated into growing cultures of either the *xcn* or F strains but grew in the presence of a strain (*ngrA*) completely devoid of antibiotic activity suggesting that antibiotics other than Xcn and compound F were required to eliminate the competitor. In contrast, *E. faecalis* was not eliminated in competition with any of the *X. nematophila* strains consistent with its relatively high antibiotic resistance. *S. saprophyticus* was eliminated when co-injected into *M. sexta* with either the *xcn* or *ngrA* strain while growth of *E. faecalis* was facilitated by co-inoculation with both of the mutant strains. Finally, when nematodes carrying the *ngrA* strain were used for natural infection of *M. sexta*, nematode reproduction was significantly reduced suggesting that NRPS-derived

compounds may function as developmental signals. Together, these findings establish the competitors for *X. nematophila* and the role of antimicrobials in differential competition and nematode reproduction.

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## ACKNOWLEDGEMENTS

I will always remember my journey as a Ph.D. student. I will cherish memories of these years, stories that I will like to retell. My stories will tell of successes, small joys, triumphs, laughter, with a sprinkling of unavoidable anxiety, hopelessness, and frustration. But my tales are not just mine, my experiences not mine alone. I will remember them because of people who have made them memorable. In these pages I will try my humble best, fumbling with words, stumbling to express my gratitude, to convey thanks to all the people who have made this journey possible.

I can already think of a story, of how I came to be in this place, this lab. It was made possible due to my graduate mentor, Dr. Steven Forst. He knows the story, but what he may not have heard mentioned enough is my gratitude to him, because it cannot be expressed enough. He has been the best mentor I could ask for, a wonderful teacher who guided me, encouraged me, and kindled in me the spark of ideas with numerous helpful discussions. He is incredibly calm, patient, a brilliant scientist, who continues to harbor the excitement and enthusiasm for science that is infectious, and helped me see patterns, stories, and light, when I could not see them. On a more personal note, I would like to thank him for being a father figure to me.

I am deeply grateful to the members of my graduate committee, Dr. Charles Wimpee, Dr. Mark McBride, Dr. Gyaneshwar Prasad and Dr. Daniel Sem. Thank you for your advice, comments, observations and time, all of which made possible the successful completion of my graduate dissertation.

In the years working towards my Ph.D. the network of people who have made my stay a wonderful experience are the members, past and present, of the Forst lab. My

heartfelt thanks go out to Dr. Ransome van der Hoeven and Dr. Dongjin Park, for being my teachers and friends. I learned a lot from them and they helped mould the initial phase of my graduate career. I would like to thank Jordan Reese, my friend, with whom I worked on many experiments. I thank other members of the lab, Nydia, Kristin, Mary, Kishore, John, for being really great, friendly, helpful people, who made the lab a great place to be. Thanks are also in order to Thomy, Andrea, David, Matt and Emmanuel, talented undergraduate students, who assisted me in my work. Mentoring them was an enjoyable experience.

I am grateful to the faculty and staff of the Department of Biological Sciences. My special thanks go to Dr. Jane Witten for providing insects for my work and to Dr. Sergei Kuchin for help with the statistical analysis. I would also like to thank Ching-Liu Wu and Thomas Schuck for always being ready to help on numerous occasions.

I would like to thank our collaborators, Dr. Heidi Goodrich-Blair and Ángel Casanova-Torres at the University of Wisconsin-Madison for contributing wonderful data to my work.

And finally, my stories will not be complete without the endless support and unconditional love of my families. Words will always be inadequate to express the entirety of my gratitude and love for my parents, who made this possible. I have always wanted this, I will admit unhesitatingly, more for you than for me. This success is for you. Thanks also to my brother, and my wonderful in-laws, for all your love and understanding. And my deepest thanks go to my husband, Joe, who has supported me in more ways than he realizes, for his continued faith in me, and for his enduring love.

# **Chapter One**

**Background and Significance: Antimicrobial Compounds and the Life**

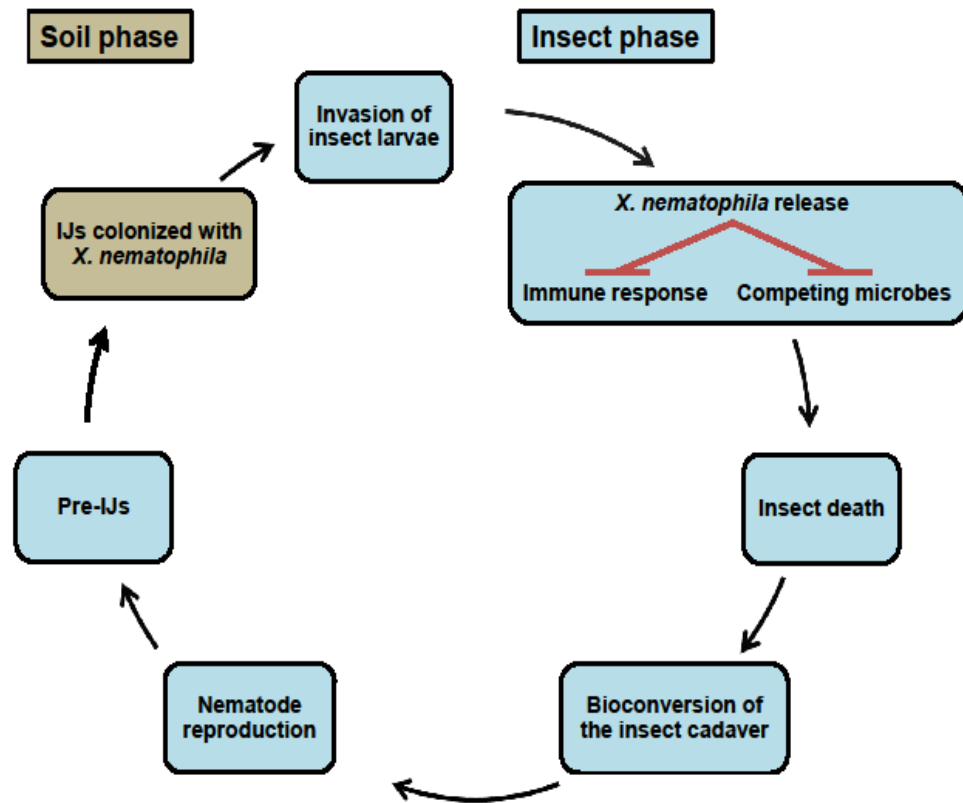
***Cycle of *Xenorhabdus nematophila****



## 1.0 Introduction

Bacteria exist in multispecies populations in which competition for resources and space drive community dynamics and evolutionary processes. Microbial communities that associate with animals and plants are widespread in nature. Pathogens that infect animal hosts confront a dual challenge of competing with other microbes in the environment and evading or suppressing activated immune responses of the host. In defensive mutualistic relationships the ability to produce antimicrobials to eliminate competitors and compounds to suppress the immune response can improve the fitness of a symbiotic partner. While competition under laboratory conditions has been extensively studied, much less is known about the competitive interactions in a host organism. The tripartite symbiosis involving the mutualistic-pathogenic bacterium *Xenorhabdus nematophila*, entomopathogenic nematode *Steinernema carpocapsae*, and susceptible insect hosts provides an excellent tractable model to study microbial competition and immune suppression in a natural biological environment.

In the dual-mode life cycle of *X. nematophila* the bacterium-nematode symbiont pair initiates infection of the insect host (FIG. 1.1). The bacterium *X. nematophila* colonizes a specialized region of the anterior intestine (receptacle) of the non-feeding juvenile stage of the nematode called the infective juvenile (IJ) that forages in the soil searching for susceptible insect hosts (1-3). The IJ invades insect larvae through natural openings such as the mouth or anus, punctures the midgut to enter the hemocoel (body cavity) and expels *X. nematophila* from the receptacle via the anus into the hemolymph where the bacteria transition to their pathogenic stage (2).



**FIG. 1.1.** Schematic diagram of the *X. nematophila* – *S. carpocapsae* life cycle highlighting the pathogenic phase

Once in the hemocoel *X. nematophila* functions as a pathogen. Part of being a successful insect pathogen is the ability to suppress the insect innate immune response, and another is host-killing, brought about by the insect toxins, cytotoxins, and hemolysins that *Xenorhabdus* secretes (4). Following insect death, bioconversion of the insect cadaver occurs due to exoenzymes produced by *X. nematophila* and the diverse antimicrobial compounds that it produces are believed to play a role in protection of the nutrient resources. The bacteria multiply using the now abundant nutrients and the nematodes feed on the bacteria and nutrients from the insect cadaver as they develop and reproduce. After 2-3 rounds of sexual reproduction, when nutrient sources are depleted, the second juvenile form develops into the specialized pre-IJ stage that is colonized by *X. nematophila*. Once colonized, the IJ leaves the cadaver in search of another insect host.

During this pathogenic phase, translocation of gut microbes into the hemocoel during nematode invasion, coupled with suppression of insect host immune response by *X. nematophila* may facilitate the growth of competitors in the insect hemocoel. The proliferation of gut-derived microbes in the hemocoel could in turn antagonize the reproduction of *S. carpocapsae* and suppress the growth of *X. nematophila* in the hemolymph. *X. nematophila* produces a plethora of antimicrobial compounds that are believed to participate in controlling competitor growth. Among the various antimicrobials produced by bacteria and fungi, three broad classes have been defined: small molecule antibiotics, peptide and protein bacteriocins, and contractile phage-tail bacteriocins. *X. nematophila* is unique among bacteria in producing antimicrobial compounds belonging to all three classes. Besides producing numerous small molecule antimicrobial compounds, *X. nematophila* produces phage-tail structures called

xenorhabdicins that bind to and kill related *Xenorhabdus* species and strains as well as the sister taxon *Photorhabdus luminescens* (5, 6). Xenorhabdicins provide a competitive advantage when an insect is co-invaded by more than one nematode species (6). *X. nematophila* also produces a protein bacteriocin called xenocin that displays broad antibiotic activity (7).

Although it has been assumed that antimicrobials are involved in interspecies competition and enhance the proliferation of *X. nematophila* and development of its nematode partner, such roles in the host have never been conclusively demonstrated. Most of the antimicrobial compounds have been studied in *in vitro* conditions which are completely different from the bacterium's actual natural environment. It is also possible that some of the compounds that exhibit *in vitro* antibiotic activity may have other functions in the host environment.

## **1.1. Challenges faced by *X. nematophila* in the insect host**

### **1.1.1. Insect immune response**

When *S. carpocapsae* invades the hemocoel microbiota from the insect gut translocate into the hemocoel and as yet unidentified signals induce pharyngeal pumping that expels *X. nematophila* into the hemolymph (2). Foreign microbes in the hemocoel are recognized by pattern recognition proteins (PRP) such as hemolin, peptidoglycan recognition protein (PGRP), and immunelectins (8). PRPs in the hemolymph bind conserved microbial-associated molecular pattern (MAMP) motifs on the surfaces of the foreign microbes. After the appropriate interactions between the PRPs and MAMPs, the insect innate immune response is activated. An example of a PRP is hemolin that binds

bacterial surfaces and causes protein complexes to form (9). Once activated, the immune response of insects consists of both cellular and humoral pathways. The cellular response involves activation of hemocytes that entrap microbial invaders in cell aggregates referred to as nodules. 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 expression of antimicrobial peptides (AMPs) such as cecropin (10, 11). Cecropin is a bacteria-inducible antimicrobial peptide that attacks bacterial cell membranes leading to cell lysis (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 formation of melanin that binds to the microbial cell surface functioning as an opsonin.

*X. nematophila* produces several compounds that suppress different components of the insect innate immune response. For example, the tyrosine-derived cell surface molecules (rhabduscin) that directly inhibit PO activity (12) and the monoterpenoid compound benzylideneacetone (13) that inhibits PLA2 activity, reduces AMP synthesis and blocks PO activity (10, 14, 15). Eight different secondary metabolites, including benzylideneacetone, that inhibit phenoloxidase and PLA2, were shown to be produced sequentially in broth cultures suggesting they act cooperatively to inhibit different stages of the immune response (16).

### 1.1.2. Microbial competitors in the infected insect hemolymph

It would be logical for the most encountered competing microorganisms in the insect hemocoel to be the insect's own gut microbiota. It has been assumed that microbes translocate from the insect gut into the hemocoel during nematode invasion. However, characterization of gut microbiota and especially monitoring its movement into the hemocoel during infection had not been reported.

Entomopathogenic nematodes have been shown to infect several Orders of insects, including Lepidoptera, Diptera, Coleoptera, Orthoptera, and Hymenoptera (17, 18). Different host organisms will harbor entirely different consortia of commensal microorganisms, indeed, variations have even been found among the gut microbiota of the same type of insects upon varying their diet. The insect gut microbiota represents the largest reserve of competitors to *X. nematophila* if they gain access to the insect hemocoel during a natural infection as the nematode breaches the intestinal barrier.

The model insects that have been most used to study the life cycle of entomopathogenic nematodes are the three lepidopterans, the tobacco hornworm (*Manduca sexta*), the wax worm (*Galleria mellonella*), and the common cutworm (*Spodoptera littoralis*). In *Galleria mellonella*, *Enterococcus* sp. were the most dominant bacteria isolated in three studies (19-21). In another analysis which identified only Gram-negative bacteria, *Salmonella*, *Pasteurella* and *Xanthomonas* were isolated from the gut of *G. mellonella* (22). Interestingly, *Enterococcus* sp. was also found in the gut of *M. sexta* when raised on the diet of tobacco leaves (23). When raised on a standard lab diet containing antibiotics, the *M. sexta* gut isolates included predominantly the Gram-positive bacteria, *Paenibacillus* and *Bacillus*, and the Gram-negative bacterium,

*Methylobacterium*. In contrast, when raised on standard antibiotic-free diet, the microbiota of *M. sexta* contained predominantly Gram-positive *Staphylococcus* and *Pediococcus* sp. and no Gram-negative bacteria (24). The microbiota of *Spodoptera* has not yet been analyzed. These findings reveal the enormous microbial diversity arising from the insect gut microbiota, which varies depending on the insects and even among insects, on the diet used.

Other sources of potential competitors are the non-symbiotic bacteria that might be carried between the cuticle and outer sheath of the IJs. Yet another type of competitive interactions that are feasible is competition between different *Xenorhabdus* species or between *Xenorhabdus* and *Photorhabdus*, both nematode-associated entomopathogens. This can occur when the insect host is co-invaded by two or more different *Steinernema* species carrying different *Xenorhabdus* species, or *Heterorhabditis* species that harbor *Photorhabdus*. In such cases, the effective antimicrobial defenses would include those that can target closely-related organisms, and include proteinaceous and phage-tail bacteriocins.

## **1.2. Antimicrobial compounds produced by *X. nematophila***

### **1.2.1. Small molecule antimicrobials**

Most small molecule antimicrobial compounds are usually synthesized by complex multi-enzyme systems consisting of non-ribosomal peptide synthetases (NRPS) and/or polyketide synthetases (PKS). NRPSs are modular enzymes composed of one or more adenylation (A) domains that bind a specific amino acid, a transfer or peptidyl carrier protein (T/PCP) domain that shuttles the activated amino acid, and the

condensation (C) domain that accepts the activated amino acid and catalyzes peptide bond formation (25). Five different classes of compounds synthesized by NRPS or NRPS-PKS biosynthetic clusters have been characterized to date (TABLE 1). The compounds have been isolated from *X. nematophila* cultures grown to stationary phase in nutrient-rich, complex media. Their activities were tested against indicator laboratory strains or clinical strains but not against biologically relevant microbial competitors. Three of the classes (xenocoumacin, xenematide and PAX peptides) have antibacterial and/or antifungal activity while the activities of the remaining two compound classes (rhabdopeptide and xenortide) remain unclear (FIG. A.1).

Xenocoumacins, the first antimicrobials isolated from *X. nematophila*, are water-soluble benzopyran-1-one compounds (26). *X. nematophila* produces two forms of xenocoumacin, Xcn1 and Xcn2. These compounds are structurally and pharmacologically similar to the amicoumacins produced by *Bacillus pumilus*. Both Xcn1 and Xcn2 are active against low G+C Gram-positive bacteria and some *E. coli* strains but are not active against other Gram-negative bacteria tested. Xcn1 is active against several fungal species but was inactive towards *Candida albicans*. Xcn2 does not display antifungal activity. Structural analysis predicted that leucine and arginine residues and several acetate units were utilized for synthesis of Xcn1 (26).

The 14 gene cluster that encodes enzymes required for production of Xcn1 and Xcn2 and the biosynthetic pathway have been characterized (27, 28). The *xcn* biosynthetic cluster contains two NRPS genes (*xcnA*, *xcnK*) and three PKS genes (*xcnF*, *xcnH*, *xcnL*). A mechanism to prevent self-toxicity to *X. nematophila* from Xcn has also



**TABLE 1. Known antimicrobial compounds produced by *X. nematophila***

Name	Class	Activity	NRPS	PKS	References
Xenocoumacin	Benzopyran	Antibacterial Antifungal	2	3	McInerney, 1991 Park, 2009 Reimer, 2009
Xenematide	Cyclic Non-polar	Antibacterial	1	0	Lang, 2008 Crawford, 2011
PAX peptides	Lysine-rich cyclolipopeptide	Antifungal Antibacterial	3	0	Gualtieri, 2009 Fuchs, 2011
Rhabdopeptide	Linear Non-polar	Antiparasitic Cytotoxic	4	0	Reimer, 2013
Xenortide	Di-amino acid Non-polar	Unknown	-	-	Lang, 2008

been described (27). The penultimate genes in the *xcn* cluster, *xcnM* and *xcnN*, encode enzymes that are involved in an unusual reaction in which the guanidinium group of arginine is removed resulting in a cyclic pyrrolidine structure forming the less active Xcn2 (28). Prolonged incubation of an *xcnM* mutant strain resulted in accumulation of Xcn1 and a reduced viability possibly due to self-toxicity from high concentration of Xcn1 (27).

Studies with the wax worm, *Galleria mellonella* infected with *X. nematophila* suggested that Xcn1 and Xcn2 were produced *in insecta*. Water extracts of macerated *G. mellonella* cadavers infected with *X. nematophila* displayed antibiotic activity against Gram-positive species while the activity against Gram-negative species was more variable (29). HPLC analysis identified the presence of Xcn1 and 2 in a ratio of 1:1 in these extracts. Antibiotic activity was not recovered by extraction of *X. nematophila*-infected *G. mellonella* with organic solvents.

Another antibacterial compound produced by *X. nematophila* is a cyclic depsipeptide (Thr-Trp-Trp- $\beta$ -Ala) called xenematide that is active against some Gram-positive and Gram-negative bacteria (30). It is produced by a stand-alone NRPS (XNC1\_2713) that contains four adenylation modules (31).

*X. nematophila* also produces a group of lysine-rich cyclolipopeptides called PAX (Peptide-Antimicrobials-*Xenorhabdus*) that have high activity against various human and plant fungal pathogens, lower activity against Gram-positive bacteria and minimal activity against Gram-negative bacteria (32). Thirteen different PAX compounds have been identified to date (33). The biosynthetic cluster that produces the PAX compounds

consists of three NRPS genes, the first of which contains one adenylation domain while the second and third NRPS genes contain three adenylation domains each.

Linear, NRPS-derived peptides called rhabdopeptides were recently identified by an *in vivo* expression technology (IVET) approach (34). The biosynthetic cluster for rhabdopeptides consists of three NRPS genes each containing one adenylation domain. Six different rhabdopeptides were isolated from broth cultures. Interestingly, rhabdopeptides were active against parasites such as *Trypanosoma brucei* and *T. cruzi* while their activity against bacteria and fungi was not reported. Rhabdopeptides were produced in *G. mellonella* infected with *X. nematophila* reaching optimal levels 10 days post-injection when the insect bioconversion or nematode reproduction stages are occurring.

The fifth class of compounds are two dipeptides called xenortides (30). Xenortides were not active against bacterial and fungal indicator stains tested and did not possess cytotoxic activity.

Four other NRPS and NRPS-PKS gene clusters have been identified in the genome of *X. nematophila*. Three of the clusters contain only NRPS genes. These include XNC1\_2299-30 (two NRPS genes), XNC1\_2038-40 (three NRPS genes), and XNC1\_2464-67 (four NRPS genes). In addition, a mixed hybrid cluster containing three NRPS genes (XNC1\_1762-64) and two PKS genes (XNC\_ 1756-57) has been identified. A stand-alone gene, XNC1\_2022 (*xtpS* - NRPS with four adenylation domains), encodes xenotetrapeptide (35). Other than XNC1\_2022, the compounds encoded by these clusters have not yet been identified.

Several small non-polar compounds possessing antibiotic activity but that have not yet been associated with any genes have been isolated from broth cultures of *X. nematophila* (FIG. A.2) (13, 36, 37). Two related indole derived compounds isolated from stationary phase cultures were active against low G+C Gram-positive bacteria, members of the Enterobacteriaceae and *Pseudomonas* sp. Nematophin, a novel indole-type compound, was active against *Bacillus* and *Staphylococcus* sp. (38). Finally, benzylideneacetone that possesses immune suppression activity as described below was shown to be active against some species of Gram-negative plant pathogens (13). At present, the modes of action of the numerous antimicrobial compounds produced by *X. nematophila* are not known.

### **1.2.2. Phage-tail bacteriocins and xenocin**

Microbial competitors other than those derived from the insect gut can gain access to the hemocoel when an insect host is co-invaded by different species of entomopathogenic nematodes. Thus, competition can occur between different species and strains of *Xenorhabdus* and *Photorhabdus* in a host co-infected by their respective nematode partners (39-41). Small molecule antimicrobial compounds are generally not active against closely related species. Bacteria can produce phage tail-like structures that bind to and kill more closely related species. R-type bacteriocins are contractile phage tail structures that resemble the tail portion of defective bacteriophages. R-type bacteriocins have been extensively studied in *P. aeruginosa* where binding to the cell surface of sensitive related bacteria causes contraction of the tail sheath and the penetration of the tail tube through the outer membrane resulting in depolarization of the cytoplasmic

membrane and increased permeability of the cell envelope (42, 43). *X. nematophila* produces R-type bacteriocins referred to as xenorhabdins (5, 6, 44, 45). Xenorhabdin was shown to have variable activity against various *Xenorhabdus* and *Photorhabdus* strains, and also demonstrated intraspecies activity (6).

Finally, *X. nematophila* produces a 64 kDa bacteriocin called xenocin that possesses endonuclease activity and is induced under  $\text{Fe}^{3+}$  depleted conditions that may exist in the insect hemolymph. Xenocin was shown to be active against gut bacteria isolated from *Helicoverpa amerigera* (cotton boll worm) larvae (7). The xenocin-immunity protein complex is secreted through the flagella secretion system (46). In the extracellular environment xenocin is believed to dissociate from the immunity protein and enter target cells where it degrades cellular nucleic acids.

### **1.3. Diverse functions of small molecule antimicrobial compounds**

The overall percentage of the *X. nematophila* genome dedicated to secondary metabolism is 7.5% as compared to 4.5% for *Streptomyces coelicolor* (47). It would appear that there is a strong selection for secondary metabolites that confer the ability to effectively compete against a broad spectrum of microbes that *X. nematophila* may encounter. However, the antimicrobial compounds were isolated from cultures of *X. nematophila* grown in nutrient-rich complex media and tested against laboratory and clinical strains in *in vitro* assays. Whether these compounds are produced in insects at sufficient levels to suppress growth of potential competitors remains to be determined. It is possible that several of the compounds play a role in immune suppression, nematode development, biofilm formation or other processes yet to be identified.

The most intensely studied antimicrobials are the small molecule antibiotics that have been exploited for their usefulness as therapeutics and additives in animal feed. These secondary metabolites are usually most active against distantly related species but may also be active against more closely related species and strains. Antibiotic production has been studied mostly under laboratory culture conditions in which antibiotics can reach high levels. Furthermore, the levels needed for an antimicrobial effect may be higher than the concentration of compound produced under natural biological conditions (48-52) Thus, compounds characterized as an antimicrobial under assay conditions may in fact have other functions, such as signaling molecules, in a natural biological environment.

A large number of antimicrobial compounds are derived from *Streptomyces* species. A well studied example of a role for antimicrobial compounds in nature is a *Streptomyces* species (S4) involved in the mutualism between leaf-cutting attine ants and fungus cultivated by them for food (53). The fungal garden can be invaded by a co-evolved fungal pathogen, *Escovopsis* sp. *Streptomyces* S4 colonizes a specialized structure on the cuticle of the ant and helps to protect the food source. In broth cultures *Streptomyces* S4 produces two antifungal compounds, candicidin and antimycin, that are active against *Escovopsis*. It was recently shown that a mutant strain deficient in both of these antifungal compounds was still able to inhibit growth of *Escovopsis* suggesting that the ant-associated *Streptomyces* is able to produce other antifungal compounds. The genome of the *Streptomyces* species was shown to contain several unassigned NRPS and PKS biosynthetic clusters that may produce antimicrobials. Whether antifungal

compounds are produced at high enough levels in nature to protect the fungal gardens remains to be determined.

Cumulative data supports the idea that in nature most antimicrobial compounds are produced at sub-inhibitory concentrations (SIC). The concentrations of antimicrobials produced in soil environments are unlikely to reach levels seen under broth culture conditions (54). Numerous studies have shown that antimicrobials can cause a differential response depending on concentration. This phenomenon is referred to as hormesis. For example, using promoter-*lux* reporter libraries of *Salmonella typhimurium* as many as 5% of the promoters were modulated by exposure to SIC of either erythromycin or rifampicin (49). The genes affected encoded diverse functions such as transport, virulence and DNA repair. Furthermore, the so-called antibiotics may have different physiological and ecological effects. Phenazines produced by *Pseudomonas aeruginosa* not only have antibiotic activity but also are involved in the transfer of electrons when oxygen is unavailable (55). Likewise, isopropylstilbene, a major antibiotic compound produced by *Photobacterium luminescens*, also inhibits insect immune responses and serves as a developmental signal for the nematode partner, *Heterorhabditis bacteriophora* (56, 57). Finally, a large number of clinically relevant antibiotics induce biofilm formation in a variety of bacteria exposed to SIC of the antibiotic (48).

The antimicrobial activity of *X. nematophila* was discovered over 30 years ago (1, 36). Since then numerous antimicrobial compounds and biosynthetic gene clusters have been characterized. While it has been assumed that these compounds play a role in interspecies competition very little is known about the production of antimicrobials in the host and whether they function in other aspects of the life cycle of *X. nematophila*.

#### 1.4. Dissertation objectives

*X. nematophila* engages in a defensive mutualistic relationship with a nematode partner and functions as a pathogen in the insect host. During the infectious phase of its life cycle *X. nematophila* faces competition with microbes growing in the hemolymph and an activated insect immune response. It has never been conclusively established that the actual biological competitors for *X. nematophila* are the insect gut microflora. In this study we explore the microbial population dynamics that occur in the infected host hemolymph, which will help shed light on the interactions of *X. nematophila* with its competitors, and whether these interactions involve secondary metabolite antimicrobials. Numerous antimicrobial compounds have been isolated from broth cultures of *X. nematophila* but their production and role in competition has never been studied in more biologically relevant media or host systems. Also, despite the large field of study of small molecule antibiotics by bacteria, our knowledge of whether they are produced in natural environments is limited. Their role as antimicrobial agents has also been tested only against lab strains and never biologically relevant competitors. Finally, secondary metabolites may serve as developmental signals for the nematode and function in as yet unidentified processes in the life cycle of *X. nematophila*. The goal of this study is to determine the microbial competitors to *X. nematophila* and the relative contributions of *X. nematophila* antimicrobials and insect immune response to the microbial population dynamics in the insect hemolymph. Using biologically relevant competitors, we



determine the effect of growth and assay conditions on antimicrobial determination, the differential competition tactics of *X. nematophila* against different competitors, and the role of an unidentified NRPS gene cluster. By creating mutant strains deficient in antimicrobial production we suggest other possible functions of antimicrobial compounds in the life cycle of *X. nematophila*.

## 1.5. References

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## Chapter Two

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

The text of this chapter is a slightly modified version of the accepted paper:

**Singh S, Reese JM, Casanova-Torres ÁM, Goodrich-Blair H, Forst S.** 2014. Microbial population dynamics in the hemolymph of *Manduca sexta* infected with *Xenorhabdus nematophila* and the entomopathogenic nematode, *Steinernema carpocapsae*. *Appl. Environ. Microbiol.* **80**: In press.

## 2.0. Introduction

Bacteria rarely exist in isolation and are usually found in multispecies populations in which competition for resources and space becomes a prime factor in driving community dynamics and evolutionary processes. For pathogens there exists a dual challenge of competing with other microbes in the environment and evading or suppressing activated immune responses. Competition under laboratory conditions has been extensively studied but much less is known about the competitive interactions in a host organism. The tripartite system involving the symbiotic-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 post-invasion 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 towards mounting an insect immune response is recognition of foreign microbes by pattern recognition proteins (PRP) such as hemolin, peptidoglycan



recognition protein (PGRP), and lectins (7, 8). PRPs bind conserved microbial-associated molecular pattern (MAMP) motifs and initiate the immune 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 expression of antimicrobial peptide (AMP) genes (9). Cecropin is a bacteria-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 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) that directly inhibit PO activity (12) and the monoterpene compound benzylideneacetone (13) that inhibits PLA2 activity, reduces AMP synthesis and blocks PO activity (9, 14). Interestingly, benzylideneacetone itself has antimicrobial activity (13).

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 a *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 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 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? Do the competitors exhibit different sensitivities to the antimicrobial products of *X. nematophila*?

## 2.1. Materials and Methods

### 2.1.1. Bacterial strains and growth conditions

*Xenorhabdus nematophila* AN6/1 (phase 1, 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 in either Luria-Bertani broth (LB) (23) or on LB agar plates (15 g/l agar). After preparation, media were 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 via optical density measurement at 600 nm (OD<sub>600</sub>). Final bacterial cultures were normalized using OD<sub>600</sub> values. Grace's insect culture medium (Gibco) was used to dilute cultures for insect injections and dilutional plating.

### 2.1.2. 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 at 16:8 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, hence referred to as NCSU) 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 manufacturer's instructions without added antibiotics was used. The diets were swabbed as well as homogenized then plated on LB agar plates and were shown to be devoid of microbial contamination.

### **2.1.3. Gut dissections and isolation of gut microbes**

Fourth instar larvae anaesthetized on crushed ice for 15-20 min were surface sterilized by submerging 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 200-500  $\mu$ l of LB broth. The tissue was homogenized by grinding for 2 min using a Kontes pellet pestle micro grinder (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 $\mu$ l of nuclease-free water and boiled for 4 min. PCR amplification (25  $\mu$ l final volume) was performed using the GoTaq® Green Master Mix kit (Promega) with 1  $\mu$ l each of the 10 $\mu$ M universal 16S rRNA (bacterial) gene primers, 11F (5'-GTTTGATCCTGGCTCAG-3') and 1512R (5'-ACGGYTACCTTGTTACGACTT-), obtained from Integrated DNA Technologies, Inc. The PCR reaction 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 reaction. The PCR product was checked on an agarose gel and purified using the GENECLAN® 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*, *S. saprophyticus*, *A. viridans*, the sequences were at least 1000 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 yeast.

#### **2.1.4. 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 on 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 anaesthetized 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 larvae and subsequently pooled for each time point. For culture-dependent determination of 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. 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 8000 r.p.m. at 4°C for 10 min. DNA was extracted from the bacterial pellet using the PurElute™ Bacterial Genomic Kit (Edge BioSystems). 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.

#### **2.1.5. Antibiotic overlay assay**

Subcultures of *X. nematophila* were grown to exponential phase and 6 µl 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.

### **2.1.6. *In vivo* competitions**

*X. nematophila*, *E. faecalis* and *S. saprophyticus* were subcultured in LB broth, grown to exponential phase, normalized and diluted in Grace's medium. For the competition experiments three different ratios were used: a) 1:1 mixture of  $10^4$  CFU/insect of the appropriate bacterial cultures, b) mixture consisting of  $10^4$  CFU/insect *X. nematophila* and  $10^5$  CFU/insect *E. faecalis* (1:10 ratio), and c) mixture consisting of  $10^5$  CFU/insect *X. nematophila* and  $10^4$  CFU/insect *E. faecalis* (10:1 ratio). Fifty microliters of the mixtures were injected per insect using BD 1ml Sub-Q, 0.45 mm x 16 mm syringes (Becton Dickinson Co.) mounted on a Stepper™ Repetitive Dispensing Pipette (Dymax Corp.). Fourth instar *M. sexta* larvae were anaesthetized by placing on crushed ice for 15-20 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. Again, the experiments were performed at similar times of the day, with similar feeding cycles for the insects.

### **2.1.7. 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*+*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 post-injection, followed by total RNA extraction using TriZol reagent (Invitrogen). For reverse transcription, 5 µg of total RNA were treated with RQ1 RNase-free DNase I (Promega). Reverse transcription was performed using the Mg primer: 5'-CGGGCAGTGAGCGCAACGTTTTTTTTTTTTTT-3' (Integrated DNA Technologies) and AMV Reverse Transcriptase (Promega). cDNA was used as template for quantitative real time PCR (qRT-PCR). qRT-PCR was performed with Bullseye EvaGreen (MidSci) on a Bio-Rad iCycler. Transcript levels of cecropin were measured and normalized against rpS3 using the following primers: cecropin-forward (5'-GGTCAAAGGATTCGTGACGC-3') and cecropin-reverse (5'-TTTGATTGTCCTTTGAAAATGGCG-3'), rpS3-forward (5'-ACTTCTCAGGCAAGGAGTGC-3') and rpS3-reverse (5'-GTCACCAGGATGTGGTCTGG-3'). Data were analyzed as previously described (26). Briefly, Ct values were normalized by calculating the ratio between the reference gene and the gene of interest and presented as a ratio between infected versus PBS injected larvae. Data were statistically analyzed using Mixed Effect ANOVA with a Tukey's post-hoc test for multiple comparisons on normalized Ct values (SAS Software). The experiment was performed four times.

#### **2.1.8. Virulence comparison of gut and human OG1RF clinical strains of *E. faecalis***

Eggs obtained from Carolina Biological Supply Company raised on NCSU diet were used. Both strains of *E. faecalis* along with *X. nematophila* were grown overnight then subcultured in LB. Exponential phase cultures were normalized to obtain similar



CFUs and these cultures were diluted in Grace's medium for injection. Fifty microliters of diluted culture were injected per larva in varying doses:  $10^4$  CFU/insect for *X. nematophila*, and  $10^4$ ,  $10^5$ ,  $10^6$  CFU/insect for both *E. faecalis* strains. Injections were performed as described above, using a BD 1ml Sub-Q, 0.45 mm x 16 mm syringe (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.

#### **2.1.9. GenBank accession numbers**

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

## **2.2. Results**

### **2.2.1. 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 3). In larvae

**TABLE 2. Published diversity of gut microbiota of *Manduca sexta***

<b>Insect Diet</b>	<b>Antibiotics Added</b>	<b>Egg Source</b>	<b>Egg Treatment</b>	<b>Major genera identified</b>	<b>Method of identification</b>	<b>Reference</b>
<b>Natural diets</b>						
<i>Solanum dulcamara</i> (Nightshade)	None	Lab colony	None	<i>Bacillus</i> , <i>Serratia</i> , <i>Candida</i>	Culture-dependent	Toth-Prestia, 1988
<i>Nicotiana tabacum</i> (Tobacco)	None	Lab colony	None	<i>Enterococcus</i>	Culture-independent	Brinkmann, 2008
<b>Artificial diets</b>						
NCSU Insectary	None	Lab colony	Bleach	<i>Pediococcus</i> <i>Micrococcus</i> , <i>Staphylococcus</i>	Culture-dependent	van der Hoeven, 2008
	Kanamycin, Streptomycin	Lab colony	Bleach	<i>Paenibacillus</i> , <i>Microbacterium</i> , <i>Bacillus</i> , <i>Methylobacterium</i>	Culture-dependent	
USDA, Hamden formula <sup>a</sup>	None	Carolina Biological Supply	Tween 80, Bleach	<i>Enterobacter</i> , <i>Klebsiella</i>	Culture-independent	Broderick, 2004
	Penicillin, Gentamicin, Rifampicin, Streptomycin	Carolina Biological Supply	Tween 80, Bleach	None	Culture-independent	

<sup>a</sup> Contains Chlortetracycline

raised on nightshade, *Bacillus* spp., *Serratia* spp. and yeast (*Candida*) were the dominant microbes isolated (27). In *M. sexta* larvae raised on the natural diet of tobacco leaves *Enterococcus* spp. were the dominant species isolated from the intestine (15). In contrast, a range of Gram-positive and Gram-negative bacteria were isolated from the intestines of larvae derived from bleached eggs raised on different artificial diets (16, 28). Addition of antibiotics to the diet dramatically altered or eliminated the microbial gut community.

I characterized the microbial community of *M. sexta* larvae grown on two different commercially available artificial diets, North Carolina State University (NCSU) diet and Gypsy moth diet. 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 3). Following dilution plating, colonies were grouped based on morphology and pigmentation. Representative isolates were subjected to 16S rRNA gene sequencing for species identification. All species could be distinguished by colony morphology and pigmentation with the exception of *E. faecalis* and *Aerococcus viridans* that were combined in a single group.

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

**TABLE 3. Transfer of microbiota from the gut to the hemolymph during natural infection of *Manduca sexta***

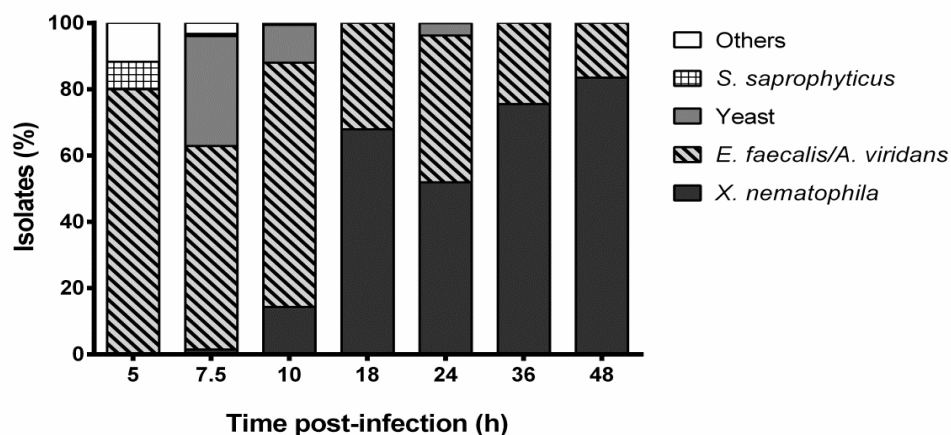
Experiment	Insect diet	Gut	Hemolymph	
		Major isolates	Major isolates	CFU/ml <sup>a</sup>
1	NCSU Insectary	<i>K. oxytoca</i> <i>S. saprophyticus</i>	<i>K. oxytoca</i> <i>S. saprophyticus</i>	$3.7 \times 10^5$ $1.0 \times 10^4$
2	NCSU Insectary	<i>E. faecalis</i> <i>S. saprophyticus</i>	<i>E. faecalis/A.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

<sup>a</sup> All CFU/ ml values measured at 7.5 h post-infection except Experiment 3 for which it was at 18

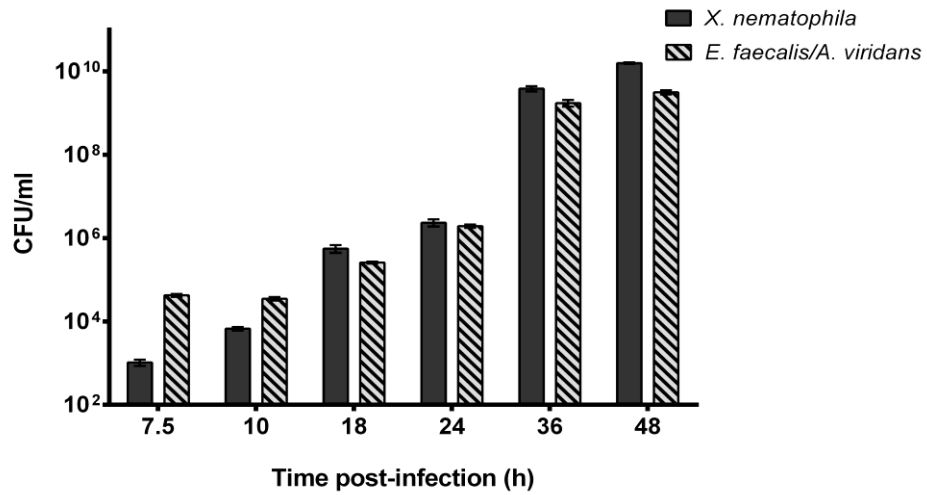
To determine if gut microbiota can translocate from the gut to the hemocoel during natural infection, hemolymph was dilutionally plated and the resulting colonies were characterized as described above. In all experiments the major isolates identified in the gut were also microbes that were dominant in the hemolymph early in infection (Table 3). 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*.

### **2.2.2. Microbial population dynamics in hemolymph of *M. sexta* infected with *S. carpocapsae* IJs**

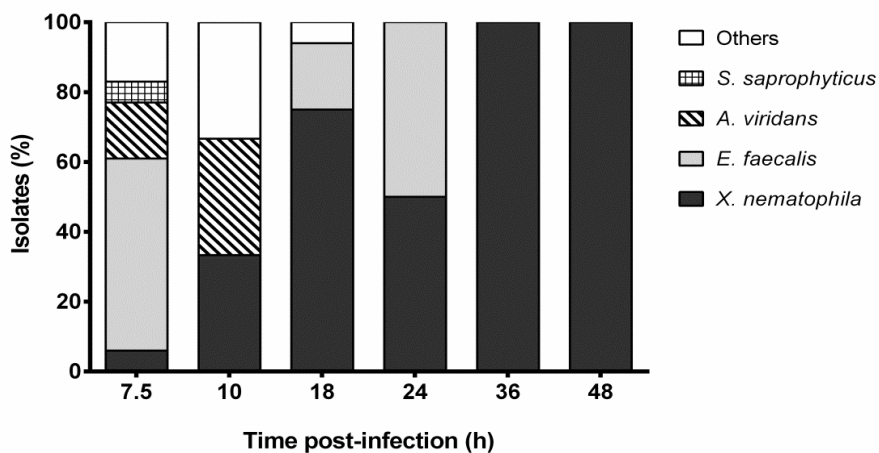
To assess temporal fluctuations in microbial populations in the hemocoel in *M. sexta* naturally infected with *S. carpocapsae* in Exp. 2 hemolymph was obtained at various times post-infection and microbial species were identified as described above. The microbial population was diverse early in infection at 5 h and 7.5 h (FIG. 2.1). *E. faecalis/A. viridans* were dominant while *S. saprophyticus*, other minor species and 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 (Exp. 2) by determining colony forming units per ml (CFU/ml) of



**FIG. 2.1. Microbial population dynamics in hemolymph of *M. sexta* naturally infected with *S. carpocapsae*.** *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 were: *X. nematophila* (black bars), *E. faecalis/A. viridans* (light gray striped bars), yeast (dark gray bars), *S. saprophyticus* (white hatched bars), and other minor bacteria (white bars), including small percentages of *Pseudomonas* spp. and *Brachybacterium* spp. Data are represented as the percentage of each species relative to the total colonies counted at each timepoint.



**FIG. 2.2. Microbial population dynamics in hemolymph of *M. sexta* naturally infected with *S. carpocapsae*.** Total colony forming units per ml of hemolymph (CFU/ml) of *X. nematophila* (black bars) and *E. faecalis/A. viridans* (light gray striped bars) obtained at each time point shown in FIG. 2.1.



**FIG. 2.3. Microbial population dynamics in hemolymph of *M. sexta* naturally infected with *S. carpocapsae*.** 16S rRNA gene clones obtained from total microbial genomic DNA in infected insect hemolymph (see FIG 2.1) were sequenced and species were identified by BLASTN analysis. Species identified were *X. nematophila* (black bars), *E. faecalis* (light gray bars), *A. viridans* (white striped bars), *S. saprophyticus* (white hatched bars), and other bacteria (white bars). “Others” represent minor percentages of: *Brachy bacterium* spp., *Klebsiella* spp., *Paracoccus* spp., and *Pediococcus* spp. Data are represented as percentage of clones of each species relative to the total clones at each time point.



hemolymph (FIG. 2.2). At 7.5 h the population of *E. faecalis/A. viridans* had reached  $4 \times 10^4$  CFU/ml while *X. nematophila* was present at  $10^3$  CFU/ml. By 18 h *X. nematophila* had reached  $4 \times 10^5$  CFU/ml while the population of *E. faecalis/A. viridans* increased at a slower 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 slower rate than *X. nematophila* resulting in the higher percentage of *X. nematophila* seen in FIG. 2.1. 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 if the culture-dependent analysis of microbial population dynamics was representative and not biased against unculturable species I carried out culture-independent analysis of the microbial community in the hemolymph of infected insects in the same experiment (Exp. 2, FIG. 2.3). 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.

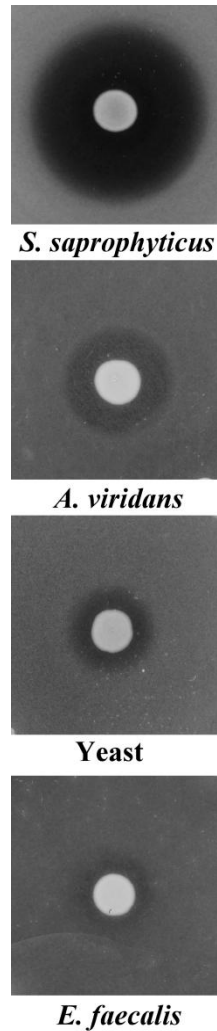
The natural infection experiment was repeated and temporal fluctuations in microbial populations in the hemocoel were monitored as described above. The results were similar to Exp. 2 except that *S. saprophyticus* was not detected during the early phase of infection and the level of yeast was noticeably higher. By 18 h *X. nematophila* was dominant, *E. faecalis/A. viridans* continued to persist and yeast had disappeared. Together, these findings establish that diverse gut microorganisms translocate into the hemocoel during invasion, with *E. faecalis/A. viridans* dominating early in infection. By 18 h *X. nematophila* becomes the dominant species, *E. faecalis/A. viridans* persist (FIG. 2.2) while other species disappear.

### **2.2.3. Sensitivity of competitors to *X. nematophila* antibiotics**

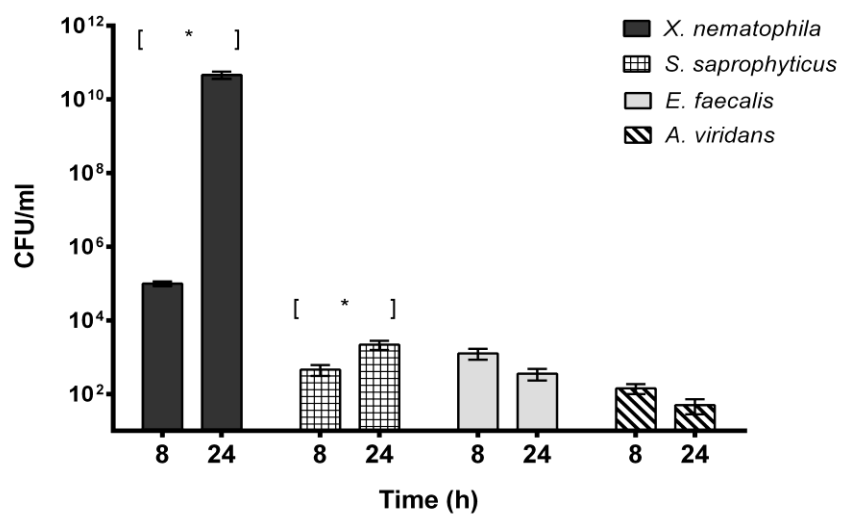
To assess the possibility that antibiotics produced by *X. nematophila* contributed to the population fluctuations observed during infection, the sensitivities of *S. saprophyticus*, *E. faecalis*, *A. viridans* and yeast were analyzed by diffusion overlay assays (FIG. 2.4). *S. saprophyticus* was the most sensitive (27 mm zone of inhibition) to antibiotics produced by *X. nematophila*, *A. viridans* was moderately sensitive (16 mm) and yeast was somewhat less sensitive (12 mm). 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.

### **2.2.4. *In vivo* competition in *M. sexta***

During natural infection competitors such as *S. saprophyticus*, *A. viridans* and yeast disappeared early in infection while *E. faecalis* persisted (FIG. 2.1, 2.2, 2.3). To



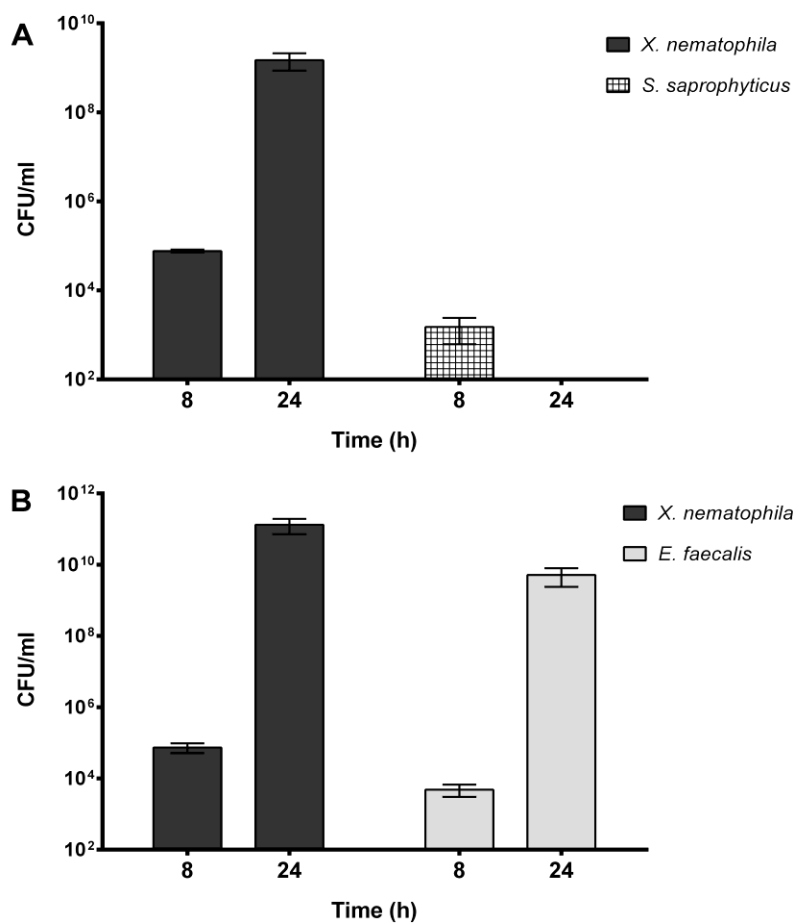
**FIG. 2.4. Antibiotic activity of *X. nematophila* against the microbes present in the insect hemolymph.** Antibiotic overlay assays were performed with *X. nematophila* against the following strains obtained from infected insect hemolymph: *S. saprophyticus*, *A. viridans*, yeast, *E. faecalis*. The diameter of the zone of inhibition is proportional to the sensitivity of the competitors to *X. nematophila* antibiotics.



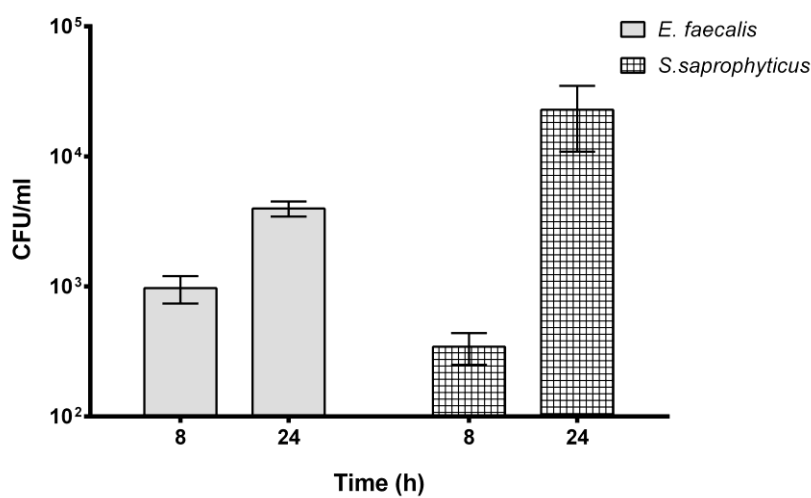
**FIG. 2.5. *In vivo* growth of isolates in *M. sexta* hemocoel after injection.** Insects were injected with  $10^4$  CFU/insect of *X. nematophila* (black bars), *E. faecalis* (light gray bars), *S. saprophyticus* (white hatched bars) or *A. viridans* (white striped bars). Hemolymph recovered at 8 and 24 h post-injection was dilutionally plated. Data are represented as CFU/ml of each bacterial species at 8 and 24 h. Statistically significant differences ( $P < 0.05$ ) between the two timepoints in a group are indicated by an asterisk. Statistical analysis was performed using a two-tailed paired Student's t-test.

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 if 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* and growth was monitored for 24 h. *S. saprophyticus* persisted at 8 h and the cell density increased at 24 h (FIG. 2.5). *E. faecalis* persisted at 8 h while cell density decreased 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, with *S. saprophyticus* able to survive better than either *E. faecalis* or *A. viridans*.

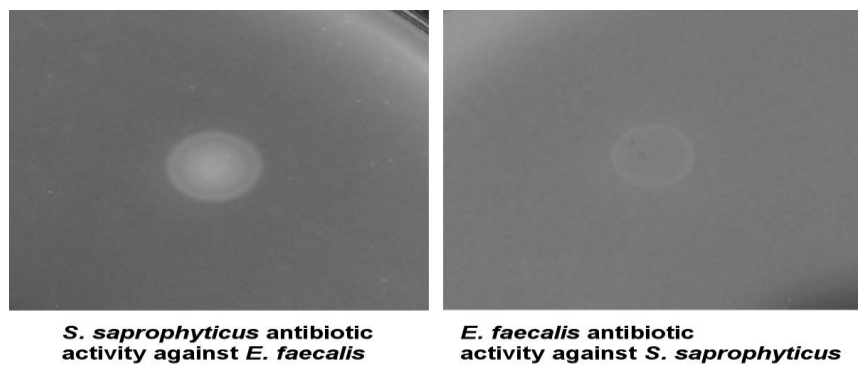
To evaluate the competitive interactions between *S. saprophyticus* and *X. nematophila*, both bacteria were co-injected into *M. sexta* (FIG. 2.6. A). *S. saprophyticus* was eliminated by 24 h when co-injected with *X. nematophila*. In contrast, *S. saprophyticus* was not eliminated by 24 h when co-injected with *E. faecalis* (FIG. 2.7). *S. saprophyticus* and *E. faecalis* also did not display any antibiotic activity against each other in overlay assays (FIG. 2.8). When *E. faecalis* was co-injected into *M. sexta* (FIG. 2.6. B) it proliferated to high levels in the presence of *X. nematophila* while it did not grow significantly better when co-injected with *S. saprophyticus* (FIG. 2.7). These findings are consistent with the population dynamics we observed during natural infection and suggest that 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 the suppression of the host immune system by *X. nematophila* which might aid *E. faecalis* growth (see below).



**FIG. 2.6.** *In vivo* growth after 1:1 co-injections of *X. nematophila* with *S. saprophyticus* or *E. faecalis* into *M. sexta*. **A.** Insects were co-injected with a 1:1 mixture of *X. nematophila* (black bars) and *S. saprophyticus* (white hatched bars).  $10^4$  CFU/insect of each species were injected. **B.** Insects were co-injected with a 1:1 mixture of *X. nematophila* (black bars) and *E. faecalis* (light gray bars).  $10^4$  CFU/insect of each species were injected. Hemolymph recovered at 8 and 24 h post-injection was dilutionally plated. Data are represented as CFU/ml of each bacterial species at 8 and 24 h.

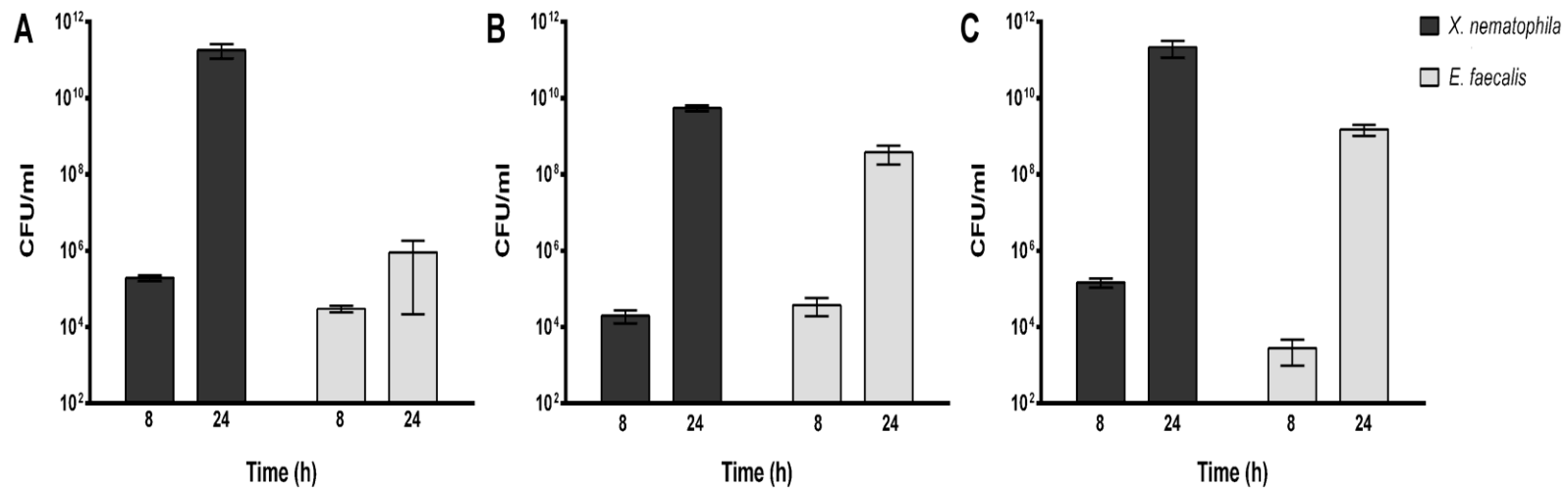


**FIG. 2.7. In vivo growth after 1:1 co-injection of *E. faecalis* and *S. saprophyticus* into *M. sexta*.** Insects were co-injected with a 1:1 mixture of *E. faecalis* (light gray bars) and *S. saprophyticus* (white hatched bars).  $10^4$  CFU/insect of each species were injected. Hemolymph recovered at 8 and 24 h post-injection was dilutionally plated. Data are represented as CFU/ml of each bacterial species at 8 and 24 h.



**FIG. 2.8.** Antibiotic overlay assay testing mutual activity of *E. faecalis* and *S. saprophyticus*. The lack of a zone of inhibition indicates that *E. faecalis* and *S. saprophyticus* do not possess antibiotic activity against each other.



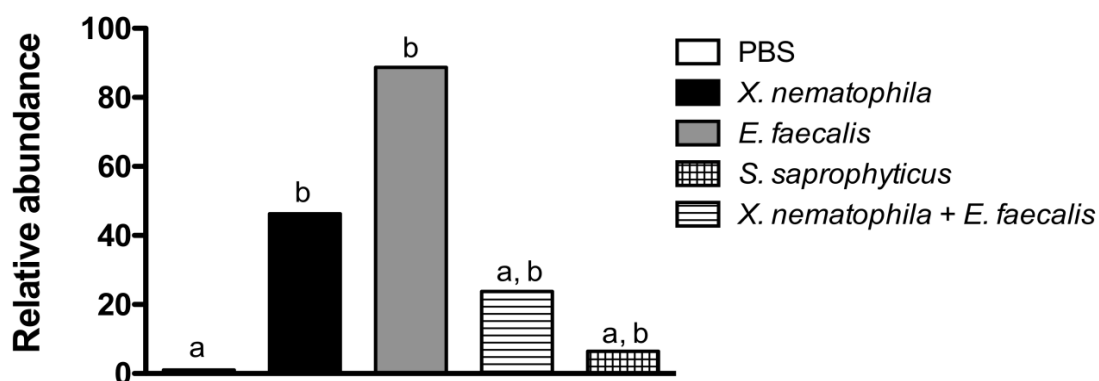


**FIG. 2.9. *In vivo* competition between *X. nematophila* and *E. faecalis* in *M. sexta* co-injected at varying ratios. **A.** Insects were injected individually with 10<sup>5</sup> CFU/insect of either *X. nematophila* (black bars) or *E. faecalis* (light gray bars). **B.** Insects were co-injected with a 1:10 mixture of *X. nematophila* (10<sup>4</sup> CFU/insect) and *E. faecalis*, (10<sup>5</sup> CFU/insect). **C.** Insects were co-injected with a 10:1 mixture of *X. nematophila* (10<sup>5</sup> CFU/insect) and *E. faecalis* (10<sup>4</sup> CFU/insect). Hemolymph recovered at 8 and 24 h post-injection was dilutionally plated. Data are represented as CFU/ml of each bacterial species at 8 and 24 h.**

During the early phase of natural infection the levels of *E. faecalis* were significantly higher than those of *X. nematophila* (FIG. 2.1, 2.2, 2.3). To more closely mimic the relative cell density that occurs during natural infection, *M. sexta* was co-injected with a 10:1 ratio of *E. faecalis*/*X. nematophila* (FIG. 2.9. B). As expected, *E. faecalis* proliferated in the presence of *X. nematophila*. Since the cell density of *X. nematophila* was significantly higher than *E. faecalis* at later times in infection, I also co-injected *M. sexta* with 10-fold more *X. nematophila* than *E. faecalis* (FIG. 2.9. C). 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 FIG. 2.5 with FIG. 2.6 and 2.9) even when the latter was present at 10-fold higher levels.

#### **2.2.5. Induction of antimicrobial peptide transcripts by *E. faecalis* is suppressed by *X. nematophila***

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, qRT-PCR analysis was performed to determine relative transcript levels of cecropin in insects injected with either *X. nematophila* or *E. faecalis*, or co-injected with both bacteria. Cecropin transcript was detectable in insects injected with *X. nematophila* and was induced to high levels in insects injected with *E. faecalis* relative to PBS-injected controls (FIG. 2.10). In insects co-injected with both bacteria the transcript level of cecropin was similar to or less than those injected with *X. nematophila* alone. These results support the idea that *X. nematophila* suppresses AMP gene expression

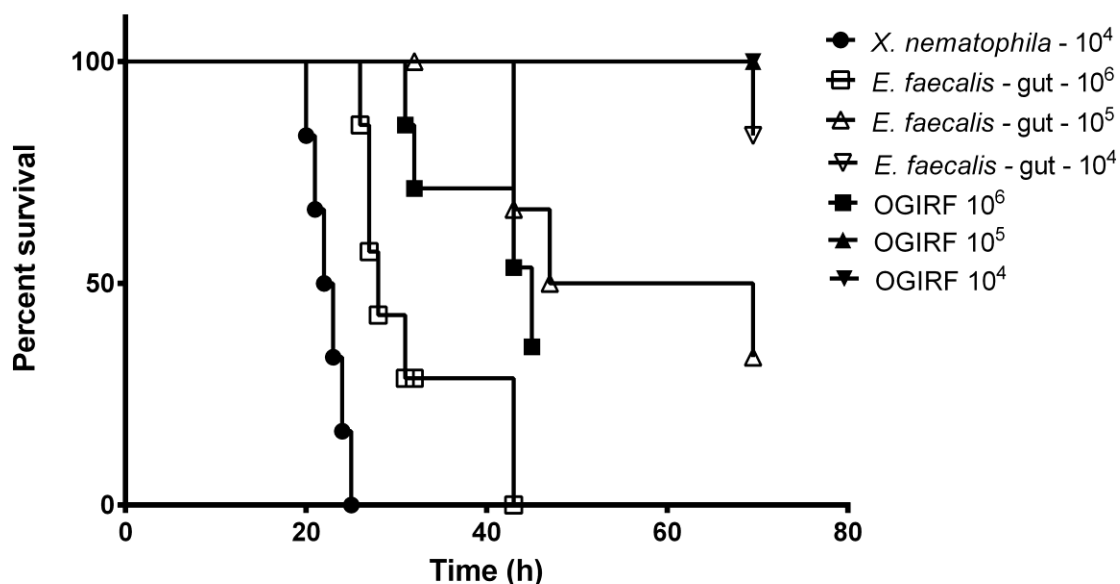


**FIG. 2.10. Relative cecropin transcript levels in insects injected with *E. faecalis* and *S. saprophyticus* alone, and co-injected with *E. faecalis* and *X. nematophila*.** Fifth instar *M. sexta* larvae were injected with *X. nematophila* (black bars), *E. faecalis* (gray bars), *S. saprophyticus* (white hatched), or co-injected with both *X. nematophila* and *E. faecalis* (white horizontal striped bars). Insects injected with PBS (white bars) served as a negative control for immune activation. RNA extracted at 16 h post-injection 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.

induced by the presence of *E. faecalis*. I also found that the ability of *S. saprophyticus* to survive in the hemolymph when injected alone (FIG. 2.5) could be attributed, in part, to the lack of induction of cecropin transcripts relative to the control (FIG. 2.10).

#### **2.2.6. *E. faecalis* isolated from the gut is pathogenic towards *M. sexta***

The persistence of *E. faecalis* during the early phase of infection and virulence of a clinical strain of *E. faecalis* towards *M. sexta* (18) raised the possibility that *E. faecalis* isolated from the insect gut would be pathogenic towards *M. sexta*. To explore this possibility, virulence of the gut strain was compared to the clinical strain of *E. faecalis* and *X. nematophila* (FIG. 2.11). At 22 h, 50% of the insects had died (LT<sub>50</sub>, 22 h) when injected with *X. nematophila* at a dose of 10<sup>4</sup> CFU/insect while all of the insects were dead by 25 h (LT<sub>100</sub>, 25 h). The gut strain of *E. faecalis* was also virulent at a higher dose of 10<sup>6</sup> CFU/insect (LT<sub>50</sub>, 27 h; LT<sub>100</sub>, 43 h) while the clinical strain was less virulent (LT<sub>50</sub>, 43 h) and was not able to kill 100% of the injected insects. In contrast, *S. saprophyticus* injected at a dose of 10<sup>6</sup> 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*.



**FIG. 2.11.** Comparison of the virulence of *E. faecalis* (gut isolate), *E. faecalis* (OG1RF), and *X. nematophila* towards *M. sexta*. Insects were injected with *E. faecalis* at a dose of either  $10^6$  CFU/insect (squares),  $10^5$  CFU/insect (upright triangles) or  $10^4$  CFU/insect (inverted triangle). The *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.

### 2.3. 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 intestinal 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 I characterize the gut microbiota of *M. sexta* and the translocation of microbes into the hemocoel during the early phase of infection by *S. carpocapsae*.

I show that gut microbes were translocated into the hemocoel of *M. sexta* 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 yeast (11, 31). Within hours after injection of bacteria, activated hemocytes engulf bacterial invaders and pattern recognition proteins are induced. Microaggregation of hemocytes has been observed 4 h after injection of *M. sexta* with *E. faecalis* (18) and *Salmonella enterica* (32), and AMP genes were induced 9 h after injection of *Salmonella enterica* (32). In *Spodoptera exigua* numerous immune response genes were induced 8 h after 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 a possibility 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 density 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 2 and 3). *Enterococcus* species were major isolates identified in Exp. 2 and Exp. 3, consistent with previous observations in *M. sexta* 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. 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). I show 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* co-injected with *E. faecalis* and *X. nematophila*. I also found that *E. faecalis* was relatively resistant to antibiotics produced by *X. nematophila*. Additionally, the transcript level of the AMP cecropin was suppressed by *X. nematophila* when it was co-injected with *E. faecalis*, which by itself induced the up-regulation of this gene (FIG. 2.10). These findings suggest that the combined effect 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. 2.11). 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 mortality of *M. sexta* lacking *E. faecalis* was similar to when it was present (data not shown).

During later phases of infection (e.g. 18 h) *X. nematophila* was dominant and *E. faecalis* cell density had increased while strains such as *S. saprophyticus*, *A. viridans* and yeast 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 suggest that 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 media. 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 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 of cross-species signaling that may induce 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 in both determining the host range of *S. carpocapsae* and 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, co-inoculation of some species of *Xenorhabdus* with axenic *S. carpocapsae* did not alter nematode reproduction while other species prevented reproduction (38). Similarly, different gut microbiota may have either neutral or antagonistic effects on nematode reproduction. How 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 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.

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## **Chapter Three**

**Differential Role of Antibiotics in the Life Cycle of**

***Xenorhabdus nematophila***

### 3.0. Introduction

Competition among microorganisms often occurs in multispecies populations, commonly for space and nutrients, where bacteria employ various strategies to affect the outcome in their favor. Normally, competition for limiting factors can be divided into two general categories: exploitative competition, and interference competition (1). In exploitative competition, the limiting nutrients are quickly utilized without direct interaction between competitors. Interference competition, on the other hand, makes use of direct, antagonistic interactions. One of the effectors of interference competition by bacteria is antimicrobial compounds. *Xenorhabdus nematophila*, a symbiotic, entomopathogenic bacterium, is a known producer of a plethora of antimicrobial compounds that are believed to assist in competition. Several of its secondary metabolite antimicrobial compounds have been characterized and several have as yet unknown activities, while there is genetic potential for the production of still more that are undiscovered. Whether these antimicrobial compounds play a role in interspecies competition, which could directly or indirectly affect symbiotic interactions with the nematode partner or virulence towards an insect host, is still unknown. Our tripartite model system involving the pathogenic bacterium *Xenorhabdus nematophila*, an entomopathogenic nematode and an insect host provides a tractable model to identify biologically relevant competitors, and study the role of *X. nematophila* antibiotics in competition.

*Xenorhabdus nematophila* exhibits two distinct roles in its life cycle (2-5). In its first role as a symbiotic partner it maintains a species-specific mutualistic relationship with the entomopathogenic nematode *Steinernema carpocapsae*. *X. nematophila* resides

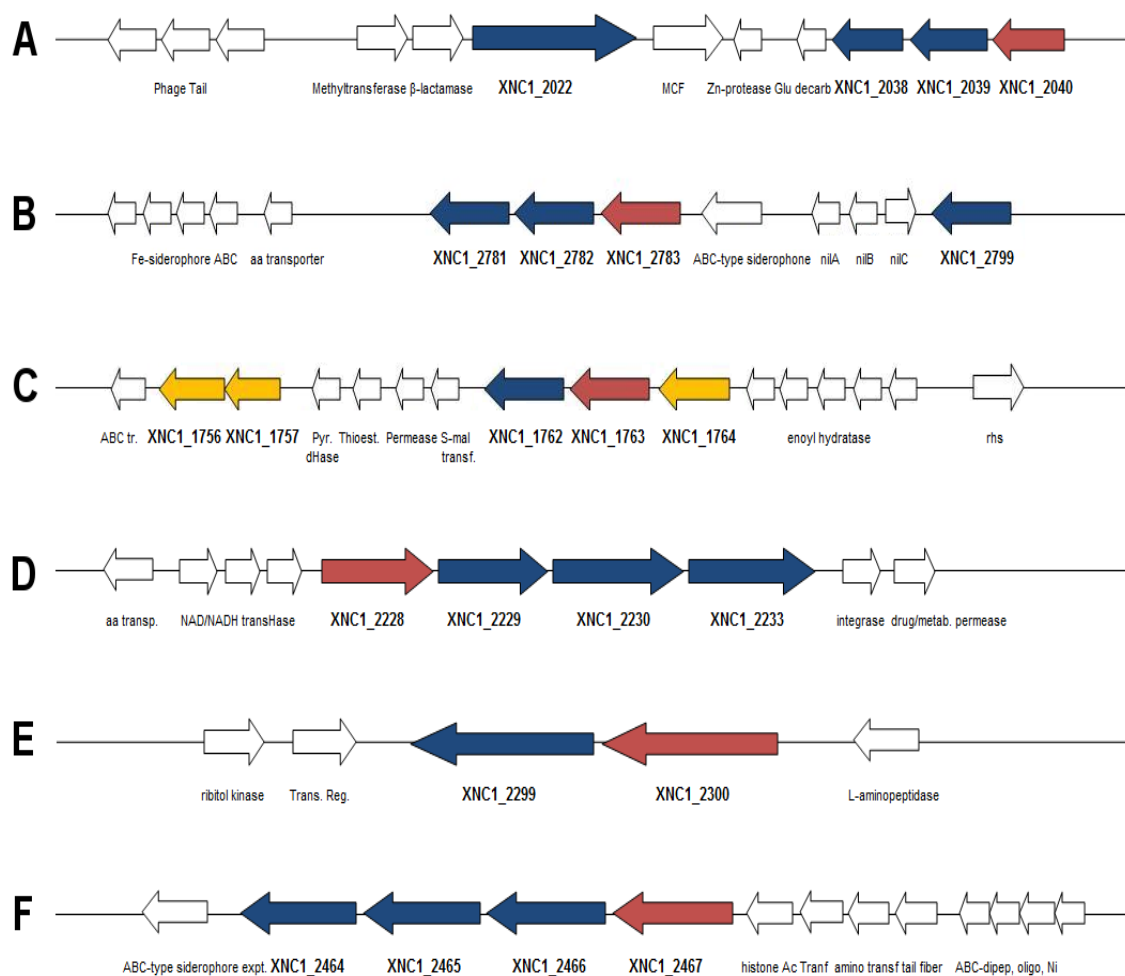
in a specialized region of the anterior midgut of the infective juvenile (IJ) stage of the nematode (6). The IJs invade soil-dwelling insect larvae through natural openings such as the mouth or anus, and enter the body cavity (hemocoel) of the insect. Herein lies the second role of *X. nematophila*, an insect pathogen. Once in the hemocoel, the bacteria are released and *Xenorhabdus* brings about the death and bioconversion of the insect larva by suppressing the insect immune response and producing toxins, cytotoxins and hemolysins (4).

The most well studied antimicrobial compounds produced by bacteria are small molecule antibiotics. Antibiotics are secondary metabolites and are often produced by multi-enzyme assemblies called non-ribosomal peptide synthetases (NRPS) and/or polyketide synthetases (PKS). Both of these enzyme assemblies are multi-modular and function using various domains (FIG. A.3). The major antimicrobial compound produced by *X. nematophila* in nutrient-rich broth cultures is the antibiotic xenocoumacin (Xcn), a water-soluble, benzopyran-1-one compound, which is a product of a 14 gene NRPS-PKS hybrid cluster (7, 8). Xenocoumacin exists in two forms: Xcn1, the more active form that possesses antibacterial (against both Gram-positive and -negative bacteria) and antifungal activities, and the less active form, Xcn2, which lacks antifungal activity (7). In the 14 gene biosynthetic cluster, there are 2 NRPS genes (*xcnA*, *xcnK*) and 3 PKS genes (*xcnF*, *xcnH*, *xcnL*). The genes *xcnM* and *xcnN* are responsible for conversion of Xcn1 into Xcn2 (8-10). Xcn has been shown to be produced in the wax worm, *Galleria mellonella*, where water extracts from macerated larvae infected with *X. nematophila* were shown to contain both Xcn1 and Xcn2 (11). But the significance of xenocoumacin in the life cycle, especially in the respective host organisms, has never been demonstrated.



Several other NRPS gene clusters have been identified in the *X. nematophila* genome. We had arbitrarily assigned these clusters letter designations A-F (FIG. 3.1). The products of two of these clusters (B and D) have since been characterized. Cluster B consists of 3 NRPS genes that produce lysine-rich cyclolipopeptides called PAX peptides (peptide antibiotics-*Xenorhabdus*) that possess antifungal activity against human and plant fungal pathogens, and low activity against Gram-positive and -negative bacteria (12, 13). Cluster D consists of 3 NRPS genes that produce 6 linear, non-polar peptides (rhabdopeptides) that possess antiparasitic activity against protozoan parasites and cytotoxic activity against insect hemocytes (14). Rhabdopeptides were shown to be produced in the infected insect *G. mellonella*, in later stages of infection. In addition, the cyclic depsipeptide xenematide, which has activity against some Gram-positive and Gram-negative bacteria, was shown to be synthesized by a large stand-alone NRPS gene. (TABLE 1) (15, 16). Four other NRPS and NRPS-PKS gene clusters with the potential to produce antimicrobial compounds have been identified in the genome of *X. nematophila* (TABLE 4). These include cluster A (XNC1\_2038, 2039, 2040), cluster E (XCN1\_2299, 2300) and cluster F (XNC1\_2464, 2465, 2466, 2467) and the hybrid NRPS-PKS cluster C containing two NRPS genes (XNC1\_1762, 1763) and three PKS genes (XNC1\_1756, 1757, 1764). These NRPS clusters and genes have not been studied so far for their biosynthetic capacity. In addition, the large stand-alone NRPS gene, XCN1\_2022 (*xtpS*), is known to encode xenotetrapeptide but its function remains unknown (17).

Other *X. nematophila* compounds with known antimicrobial activity but that do not yet have a gene cluster associated with them are indole-derivatives (active against both Gram-positive and -negative bacteria) and benzylideneacetone (antibacterial against



**FIG. 3.1. NRPS gene clusters in the genome of *X. nematophila*.** NRPS genes are colored red and blue and the PKS genes are colored yellow. The genes in red were inactivated for mutant construction.

**TABLE 4. NRPS and PKS clusters in *X. nematophila* with unidentified activity**

<b>Cluster</b>	<b>Type</b>	<b>Number of genes</b>	<b>Genes</b>
A	NRPS	3	XNC1_2040, 2039, 2038
C	NRPS	2	XNC1_1763, 1762
	PKS	3	XNC1_1764, 1757, 1756
E	NRPS	2	XNC1_2299, 2300
F	NRPS	4	XNC1_2764, 2765, 2766, 2767
-	NRPS	1	XNC1_2022

five plant pathogenic strains tested) (18-20). Nematophin, another indole-derived compound, has antibacterial activity against *Bacillus* sp., *Staphylococcus* spp. and some antifungal activity, and is not known to be synthesized by NRPS or PKS genes (21). Other small molecules include the non-polar di-peptide derivatives xenortide A and B that lack antibacterial, antifungal, and insecticidal activity against the strains tested (15).

While the activities of some of these compounds have been elucidated, it largely remains unclear what their role is in natural biological conditions. Antibiotic production is mostly studied in laboratory culture conditions, often in nutrient-rich media, when antibiotics are produced in high concentrations. Additionally, these compounds are tested against a chosen set of indicator organisms, which may not have any biological relevance to the producer organism. It has been proposed that antibiotics are usually produced in sub-inhibitory concentrations in natural environments and under such conditions can participate in signaling (22-26). Therefore, these compounds purported to have antimicrobial activity, might not be present in high enough concentrations in the natural environment, and might demonstrate altogether different functions. A link that connects all the NRPS, PKS clusters together is the enzyme phosphopantetheinyl (Ppant) transferase. This enzyme, the product of the *ngrA* gene, attaches the PPant moiety to the transfer (PCP) domain of NRPS and PKS enzymes (FIG. A.4). A mutant in this gene created in the related bacterium *Photobacterium luminescens* was shown to lack antibiotic activity and was unable to support nematode growth (27). This finding suggests that the products of these gene clusters can have other functions, such as a developmental signal for the nematode partner.

We have previously demonstrated that the gut microbiota of the insect host *Manduca sexta* translocates to the hemocoel during natural infection with the nematode *Steinernema carpocapsae* harboring *X. nematophila*, and are potential competitors (28). During early infection some competitors were eliminated such as *Staphylococcus saprophyticus* that was sensitive to *X. nematophila* antimicrobials. Another gut microbe, *Enterococcus faecalis* that was relatively resistant to antimicrobials, was dominant during early stage infection and proliferated in the hemolymph along with *X. nematophila*. In the present study we address several unanswered questions. What is the role of xenocoumacin in competition against the biologically relevant competitors *E. faecalis* and *S. saprophyticus*? Do any of the as yet uncharacterized NRPS, PKS clusters produce antimicrobial compounds? Does the outcome of the competition change depending on growth conditions? To address these questions we created NRPS mutant strains and a *ngrA* mutant defective in synthesis of all NRPS, PKS-derived antimicrobials. Additionally, we addressed the question of whether these compounds have one or more functions in the natural host environment, which might affect the symbiotic or pathogenic relationships of *X. nematophila*.

### **3.1. Materials and Methods**

#### **3.1.1. Bacterial strains and growth conditions**

All strains and plasmids used in this study are listed in TABLE 5. Cells were routinely grown at 30°C in either Luria-Bertani broth (LB) or on LB agar plates (15 g/l

**TABLE 5. Bacterial strains and plasmids used in this study**

<b>Strain or plasmid</b>	<b>Relevant genotype, phenotype or characteristic(s)</b>	<b>Source or Reference</b>
<b>Strains</b>		
<i>X. nematophila</i>		
AN6/1	Wild-type, phase 1 variant; Amp <sup>r</sup>	Laboratory stock
$\Delta xcnKL$	AN6/1 $\Delta xcnKL::Km^r$	D. Park
F	AN6/1 2467::Cm <sup>r</sup>	This study
<i>ngrA</i>	AN6/1 1028::Cm <sup>r</sup>	This study
$\Delta xcnKL:A$	AN6/1 $\Delta xcnKL::Km^r$ 2040::Cm <sup>r</sup>	This study
$\Delta xcnKL:B$	AN6/1 $\Delta xcnKL::Km^r$ 2783::Cm <sup>r</sup>	This study
$\Delta xcnKL:C$	AN6/1 $\Delta xcnKL::Km^r$ 1763::Cm <sup>r</sup>	This study
$\Delta xcnKL:D$	AN6/1 $\Delta xcnKL::Km^r$ 2228::Cm <sup>r</sup>	This study
$\Delta xcnKL:E$	AN6/1 $\Delta xcnKL::Km^r$ 2300::Cm <sup>r</sup>	This study
$\Delta xcnKL:F$	AN6/1 $\Delta xcnKL::Km^r$ 2467::Cm <sup>r</sup>	This study
<i>E. coli</i> S17- $\lambda$ pir	<i>recA</i> , <i>thi</i> , <i>pro</i> , <i>hsd</i> (R-M+). RP4-2Tc::Mu Km::Tn7 in the chromosome	Laboratory stock
<i>E. faecalis</i>	<i>Manduca sexta</i> gut isolate	
<i>S. saprophyticus</i>	<i>Manduca sexta</i> gut isolate	
<b>Plasmids</b>		
pSTBlue-1	Cloning vector: Amp <sup>r</sup> Km <sup>r</sup>	Novagen
pKnock-Cm <sup>r</sup>	Broad-host-range suicide vector; Cm <sup>r</sup> RP4 <i>oriT</i> <i>oriR6K</i>	D. Saffarini
pKnock-A	Internal fragment of 2040 cloned into pKnock-Cm <sup>r</sup>	This study
pKnock-B	Internal fragment of 2783 cloned into pKnock-Cm <sup>r</sup>	This study
pKnock-C	Internal fragment of 1763 cloned into pKnock-Cm <sup>r</sup>	This study
pKnock-D	Internal fragment of 2228 cloned into pKnock-Cm <sup>r</sup>	This study
pKnock-E	Internal fragment of 2300 cloned into pKnock-Cm <sup>r</sup>	This study
pKnock-F	Internal fragment of 2467 cloned into pKnock-Cm <sup>r</sup>	This study

agar) (29). After preparation, media were maintained in the dark. Strains grown overnight in LB broth (supplemented with ampicillin, chloramphenicol and kanamycin to a final concentration of 50, 25 and 30 mg/ml respectively, when required) were subcultured (1:20) in 5 ml of fresh LB broth and growth was monitored by turbidity via optical density measurement at 600 nm ( $OD_{600}$ ). Final bacterial cultures were normalized using  $OD_{600}$  values. Grace's insect culture medium (Gibco) was used to dilute cultures for insect injections and dilutional plating.

### **3.1.2. Construction of the NRPS and *ngrA* mutant strains**

The approach of insertional inactivation of the appropriate genes was utilized to create the mutant strains. Briefly, for each gene primers were designed to amplify a 200–800 bp internal fragment located near the 5' end of the gene. The amplified products were purified with GeneClean Turbo kit (MP Biomedicals), followed by end conversion and subsequent blunt end-ligation into the EcoRV site of pSTBlue-1 vector (Novagen). Several of the resulting recombinant colonies were selected and analyzed by colony PCR using a T7 and SP6 primer pair from the regions flanking the EcoRV site of pSTBlue-1 to confirm the size of the cloned fragment. A colony having the desired plasmid was grown overnight and the plasmids containing the inserts were purified using the QIAprep Spin Miniprep kit (Qiagen). A PstI–XbaI fragment containing either 2040, 2783, 1763, 2228, 2300, 2467, or 1028 (*ngrA*) internal gene fragment was gel-purified and ligated into the conjugal suicide vector pKnock-Cm (30). The resultant recombinant plasmids were transformed into electrocompetent *E. coli* S17- $\lambda$ pir and conjugally transferred into the wild-type strain of *X. nematophila*. The 2040, 2783, 1763, 2228, 2300, 2467 plasmids

were also conjugally transferred to the  $\Delta xcnKL$  strain of *X. nematophila* to generate double mutants in the xenocoumacin deletion background strain. Selection on ampicillin and chloramphenicol identified the mutants in which the recombinant pKnock-Cm had integrated into the chromosome within the respective gene by single-cross-over homologous recombination, leading to gene disruption, which was confirmed by PCR. The primers used in this study are mentioned in TABLE 6.

### **3.1.3. Antibiotic overlay assay**

Six microliter volume of exponential phase subcultures of *X. nematophila* were spotted on LB agar plates and incubated. After 24 h of growth, the bacteria were exposed to chloroform fumes for 30 min followed by air drying for 30 min. Five hundred microliters of overnight culture of the indicator bacterial strain was added to 6 ml top agar (LB with 0.7% agar) which was then poured to form an overlay on the *X. nematophila* colonies (2, 31). The plates were incubated at 30°C for 48 h. Zones of inhibition were measured in millimeters. The overlay assays were performed twice, with nearly identical results.

### **3.1.4. *In vitro* competitions in LB and Grace's**

The appropriate *X. nematophila* strains, *E. faecalis* and *S. saprophyticus* subcultures were set up in either LB or Grace's medium, depending on the condition chosen. Cultures were allowed to grow till exponential phase and were normalized based on O.D.<sub>600</sub>. The competitions were set up by inoculating (1:20) fresh 5 ml LB or Grace's



**TABLE 6. Primers used in this study**

<b>Gene, cluster or vector</b>	<b>Primer</b>	<b>Sequence (5'-3')</b>	<b>Use</b>
<i>ngrA</i>	XN_1028 F	CATGTCATGCTGGCCTATTTTG	Mutant construction
	XN_1028 R	CAAATAGTGTCCAGGCCAGATTGG	Mutant construction
Cluster A	XNC1_1028 OF	ACCTCTACTCAAGTCACTAC	Mutant screening
	XNC1_2040 F	TCTCTCAGTACGACTCAGCAAG	Mutant construction
	XNC1_2040 R	CTATCTTGTCTGTAGCGTTGTGAG	Mutant construction
Cluster B	XNC1_2040 OF	TCATGCAATCGTATCAACAACG	Mutant screening
	XNC1_2783 F	GCCAAACGATTAGAAGAAGCTCTC	Mutant construction
	XNC1_2783 R	ACAGTGAATTGTGCAGGATCTTG	Mutant construction
Cluster C	XNC1_2783 OF	TCAAATTGACCCTGACAAACG	Mutant screening
	XNC1_1763 F	CACTTACGGATATGCAGCAGG	Mutant construction
	XNC1_1763 R	GTGTTGTTGCTGCCAATTGAC	Mutant construction
Cluster D	XNC1_1763 OF	TTTGGGAAAACATTTCTGATGTTG	Mutant screening
	XNC1_2228 F	ACAGCATACCGCCAGAATTG	Mutant construction
	XNC1_2228 R	TGAACCTTTAGCACTGGCAAT	Mutant construction
Cluster E	XNC1_2228 OF	TCAATGAGGCGTTAAATCAGGG	Mutant screening
	XNC1_2300 F	CATCATGACAATATACCACCGGAAC	Mutant construction
	XNC1_2300 R	AAGTTCATCCAATGCTCTGTG	Mutant construction
Cluster F	XNC1_2300 OF	CTTTAAGCCAAGGAGTTACCTTG	Mutant screening
	XNC1_2467 F	GACAGGCTCTCAATGGCAC	Mutant construction
	XNC1_2467 R	GGACTCTGTCACTTTGGTTGATAC	Mutant construction
pKnock	XNC1_2467 OF	TGCCATAAAAGAGACGCTCCAG	Mutant screening
	pKnock-F	ACACAGGAACACTTAACGGCTGAC	Mutant screening
	pKnock-R	TGCGAAGTGATCTTCCGTCAGAG	Mutant screening
pSTBlue-1	SP6	ATTTAGGTGACACTATAG	Mutant screening
	T7 promoter	CTAATACGACTCACTATAGGG	Mutant screening

with 1:1 mixtures of *X. nematophila* and competitor. The flasks were incubated at 30°C with shaking and dilution plating using the same medium at 0, 9, 18, 24 h was performed to obtain CFU/ml of the bacteria. The experiments were performed at least twice, yielding highly reproducible results.

### **3.1.5. *In vitro* competitions in LB with pre-incubated *X. nematophila***

Overnight cultures of the required *X. nematophila* strains were subcultured 1:20 in 5 ml of Grace's and incubated at 30°C for 10-11 h. Subcultures of the competitors *E. faecalis* and *S. saprophyticus* set up in 5 ml Grace's were allowed to grow till exponential phase and were normalized based on O.D.<sub>600</sub>. These were added to the pre-inoculated 10-11 h *X. nematophila* cultures to start the competition. Dilutions were made in Grace's medium and plated on LB agar to determine the relative CFU/ml of the different strains at 0 and 24 h. The experiments were performed twice, with highly similar results.

### **3.1.6. Antibiotic activity in cell-free supernatants from LB and Grace's cultures**

Overnight cultures of wild-type *X. nematophila* were subcultured 1:20 in 5 ml of LB and Grace's. The cultures were grown at 30°C, shaking, and at 6, 9, 12, 24 h post-inoculation 1 ml of cultures were withdrawn from the flasks. This volume was centrifuged at 14,000 r.p.m for 1.5 min. The cell pellet thus obtained was discarded and the supernatant was sterilely filtered through 0.2 µm pore size filters (Millipore) attached to 3 c.c. syringe (B.D.). The sterile supernatants were frozen until used in the antibiotic assay. The O.D.<sub>600</sub> measurement was taken and dilution plating in the respective medium was also performed at all the timepoints. For the antibiotic assay, 200 µl of a 1:50

dilution of the overnight culture of *S. saprophyticus* was used in a 96-well microtitre plate (BD) to which 20 µl of 1X and 0.5X diluted *X. nematophila* sterile supernatants were added. The microtitre plate was incubated at 30°C shaking, and 0 and 24 h O.D.<sub>600</sub> measurements were made to determine the levels of inhibition of *S. saprophyticus*.

### **3.1.7. Sources, treatments and rearing of *Manduca sexta* larvae**

*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 at 16:8 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, hence referred to as NCSU) without added antibiotics was the diet used, prepared from individual ingredients. The diet was prepared according to supplier instructions (32).

### **3.1.8. *In vivo* competitions**

*X. nematophila* ( $\Delta xcnKL$  and *ngrA*), *E. faecalis* and *S. saprophyticus* were subcultured in LB broth, grown to exponential phase, normalized and diluted in Grace's medium. For the competition experiments a 1:1 mixture of 10<sup>4</sup> CFU/insect of the appropriate bacterial cultures was prepared in Grace's medium.. Fifty microliters of the mixtures were injected per insect using BD 1ml Sub-Q, 0.45 mm x 16 mm syringes (Becton Dickinson Co.) mounted on a Stepper™ Repetitive Dispensing Pipette (Dymax

Corp.). Fourth instar *M. sexta* larvae were anaesthetized by placing on crushed ice for 15-20 min and the area around the horn was cleaned using 70% ethanol prior to 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, followed by dilution plating on LB agar plates. Three to four larvae were used per time point and the experiment was performed three times, with reproducible results. The experiments were performed at similar times of the day, with similar feeding cycles for the insects.

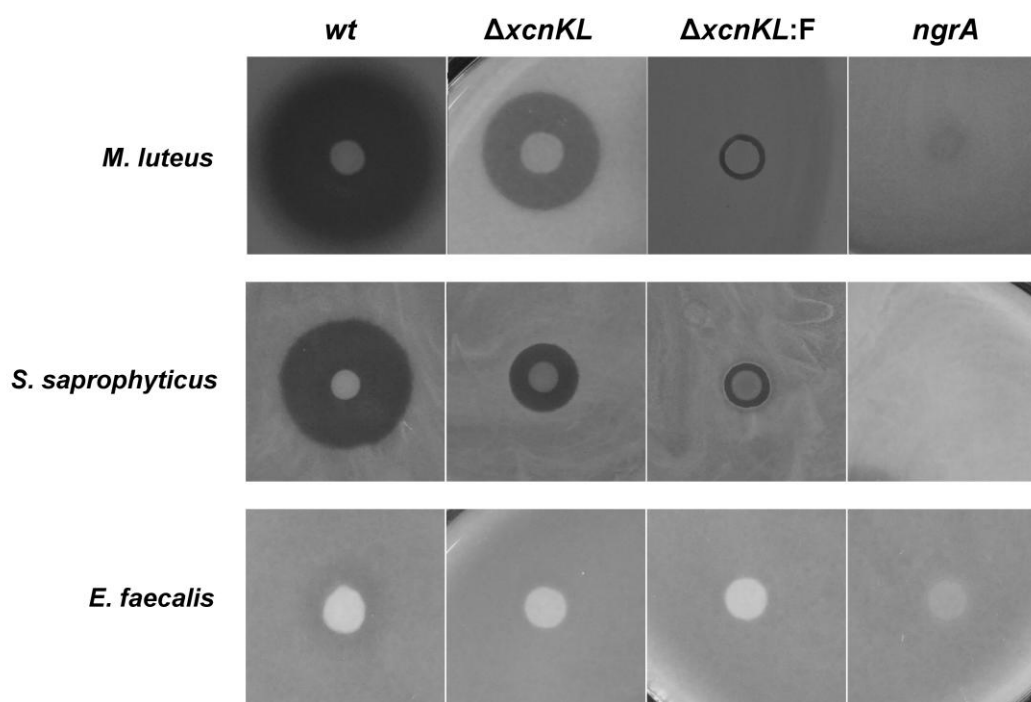
### **3.1.9. Nematode reproduction**

Twelve fourth instar *M. sexta* larvae per strain were used. The larvae were naturally infected by using *S. carpocapsae* IJs carrying either the wild-type AN6 or the *ngrA* strain of *X. nematophila*. Briefly, IJs were washed using sterile water. Six larvae were placed on top of a moistened filter paper in a clean plastic cup. The appropriate volume of the IJ suspension resulting in 200 IJs/larvae was pipetted in random drops on the filter paper and the cup was placed in the insect incubator. Following insect death within 48 h, larvae were transferred to modified White water traps (33) containing 25 ml sterile distilled water (4 insects per trap). The emerging nematodes were counted in the trap until Day X after emergence and the average number of IJs per milliliter was determined. Four independent experiments were conducted, all yielding highly similar results. Data from a representative experiment is shown.

## 3.2. Results

### 3.2.1. Analysis of NRPS gene clusters for antibiotic activity

We had shown previously that inactivation of the *xcnA* gene for xenocoumacin synthesis significantly reduced but did not eliminate antibiotic activity in an overlay assay using *Micrococcus luteus* as the indicator strain (8). The residual activity could be a product of additional NRPS gene clusters. The NRPS gene clusters for the PAX peptides and rhabdopeptides whose products were shown to possess antifungal and cytotoxic activity, respectively, had been previously identified (12-14). The genome of *X. nematophila* contains four additional NRPS gene clusters, referred to as clusters A, C, E and F. The compounds synthesized by these clusters have not yet been identified (TABLE 4). To determine whether the additional gene clusters produced antimicrobial compounds the first NRPS gene of each cluster was inactivated. We also inactivated the first NRPS gene in the PAX and the rhabdopeptide clusters. In addition, the *ngrA* gene that encodes the phosphopantetheinyl transferase required for activity of NRPS and PKS enzymes was inactivated. The antibiotic activity of the individual mutant strains was analyzed in an overlay assay using *M. luteus* as the indicator strain. Antibiotic activity was not detectably reduced in any of the NRPS mutant strains (data not shown). As expected, antibiotic production was completely eliminated in the *ngrA* strain (FIG. 3.2). Since antimicrobials produced by the NRPS gene clusters may be masked by the high level of xenocoumacin detected in the overlay assay we created double mutant strains in a xenocoumacin-deficient background in which *xcnK* and part of *xcnL* were deleted ( $\Delta xcnKL$  strain). The antibiotic activity of the  $\Delta xcnKL$  strain was reduced but not eliminated as shown previously with the *xcnA* strain (FIG. 3.2). Inactivation of the first



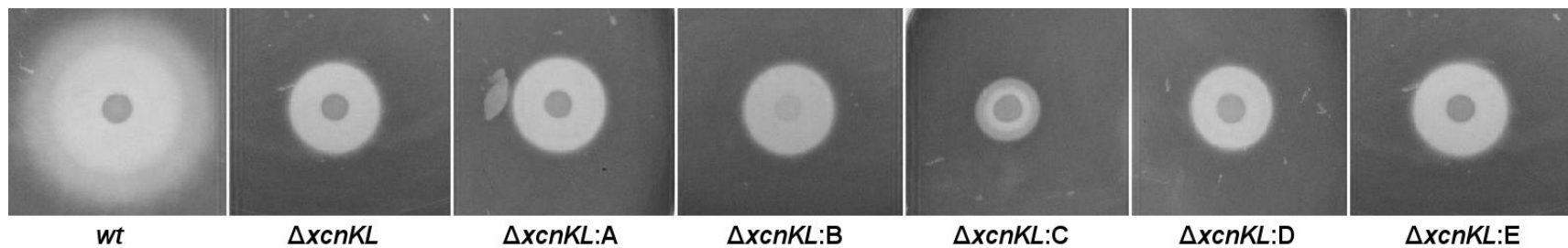
**FIG. 3.2.** Antibiotic overlay assay demonstrating activity of xenocoumacin against *M.luteus*, *E. faecalis* and *S. saprophyticus*; of compound F against *M. luteus* and *S. saprophyticus*; and complete lack of activity of the *ngrA* mutant. Antibiotic overlay assays were performed with *X. nematophila* wild-type and mutant strains ( $\Delta xcnKL$ ,  $\Delta xcnKL:F$ , *ngrA*) against the common indicator organism *M. luteus* and relevant competitors isolated from the insect host gut: *S. saprophyticus* and *E. faecalis*. The diameter of the zone of inhibition is proportional to the sensitivity of the competitors to *X. nematophila* antibiotics.

NRPS gene in cluster F in the  $\Delta xcnKL$  background resulted in almost complete loss of a zone of inhibition indicating that compound F possessed antibiotic activity against *M. luteus*. The  $\Delta xcnKL:C$  strain also displayed a markedly reduced zone of inhibition indicating that cluster C also possessed antimicrobial activity (FIG. 3.3). For the other double mutant strains the zone of inhibition was the same as that for the  $\Delta xcnKL$  strain indicating that these clusters did not produce antibiotic activity against *M. luteus*.

We had previously isolated *Staphylococcus saprophyticus* and *Enterococcus faecalis* strains from the gut of *Manduca sexta* and showed these microbes translocate into the hemocoel during nematode invasion (28). In overlay assays, *S. saprophyticus* was highly sensitive to *X. nematophila* antibiotics while *E. faecalis* was more resistant. We used these biologically relevant isolates to assess the antibiotic activity of the  $\Delta xcnKL$  and  $\Delta xcnKL:F$  strains. The antibiotic activity of the  $\Delta xcnKL$  strain was significantly reduced against *S. saprophyticus* and was undetectable in the assay against *E. faecalis* (FIG. 3.2). Antibiotic activity against *S. saprophyticus* was further reduced in the  $\Delta xcnKL:F$  strain and completely lost in the *ngrA* strain (FIG. 3.2). These findings show that xenocoumacin is active against both the biologically relevant competitors *S. saprophyticus* and *E. faecalis*, and it is the major antibiotic activity against *E. faecalis*. Compound F was shown to possess activity against *S. saprophyticus*. The  $\Delta xcnKL$ , F,  $\Delta xcnKL:F$ , and *ngrA* mutant strains were used for further analysis.

### **3.2.2. Competition of *S. saprophyticus* and *E. faecalis* with *X. nematophila***

The above results suggested that xenocoumacin and compound F are active against microbial competitors that enter the hemocoel during early stage infection. Since



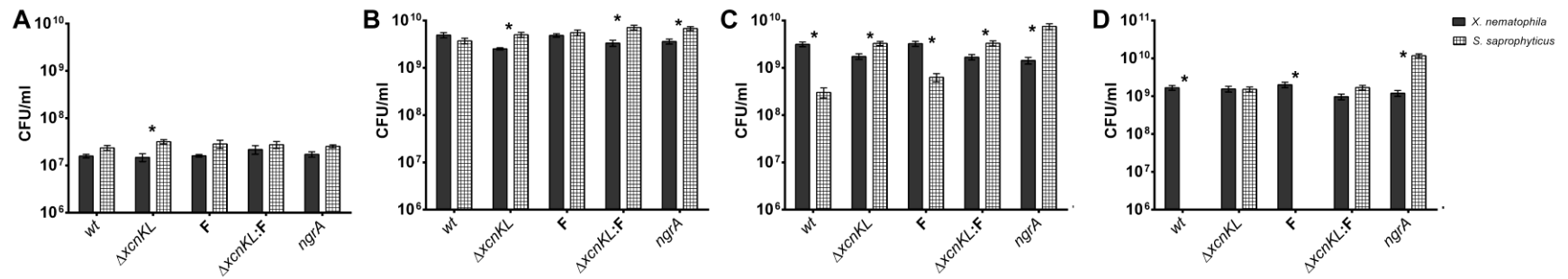
**FIG. 3.3. Antibiotic overlay assay demonstrating antibiotic activity of compound C and lack of activity of compounds A, B, D and E.** Antibiotic overlay assays were performed with *X. nematophila* wild-type and mutant strains ( $\Delta xcnKL$ ,  $\Delta xcnKL:A$ ,  $\Delta xcnKL:B$ ,  $\Delta xcnKL:C$ ,  $\Delta xcnKL:D$ ,  $\Delta xcnKL:E$ ) against the common indicator organism *M. luteus*. The diameter of the zone of inhibition is proportional to the sensitivity of the competitors to *X. nematophila* antibiotics.



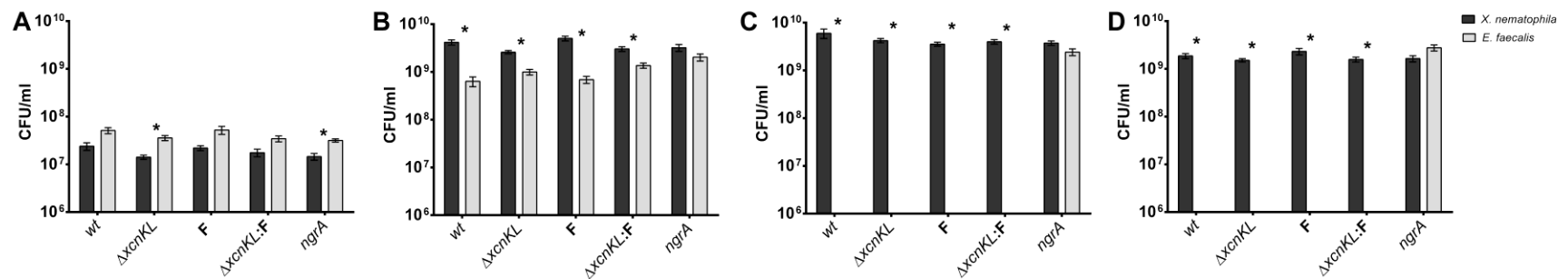
xenocoumacin has been shown to be produced in nutrient-rich medium (LB broth), competition experiments were first performed between wild-type and mutant strains of *X. nematophila* and either *S. saprophyticus* or *E. faecalis* in LB broth (FIGS. 3.4 and 3.5). Exponentially growing *X. nematophila* and competitor cultures were co-inoculated in a 1:1 ratio and grown for 24 h. Culture samples were dilutionally plated at 9 h, 18 h and 24 h to determine the relative cell density of the respective strains. By 9 h the cell density of the *X. nematophila* strains and *S. saprophyticus* had increased over 100-fold (FIG. 3.4). The ratio of *S. saprophyticus* to *X. nematophila* was higher in the  $\Delta xcnKL$ ,  $\Delta xcnKL:F$  and *ngrA* co-cultures. At 18 h the cell density of *S. saprophyticus* was significantly reduced in co-cultures with the wild-type and F strain, while the *S. saprophyticus* was eliminated by 24 h. In contrast, the levels of *S. saprophyticus* remained high at 24 h in co-cultures with the  $\Delta xcnKL$  and  $\Delta xcnKL:F$  strains and was significantly higher in the *ngrA* strain. These results indicated that xenocoumacin but not compound F was required to eliminate *S. saprophyticus* in LB broth co-cultures.

Co-culture competition experiments were also carried out with *E. faecalis* (FIG. 3.5). At 9 h the cell density of all *X. nematophila* strains except the *ngrA* strain was higher than the level of *E. faecalis*. At 18 h and 24 h *E. faecalis* was eliminated in all co-cultures except for those with the *ngrA* strain. These findings indicate that neither xenocoumacin nor compound F were required to eliminate *E. faecalis*, and that other NRPS-derived compounds were active against this competitor.

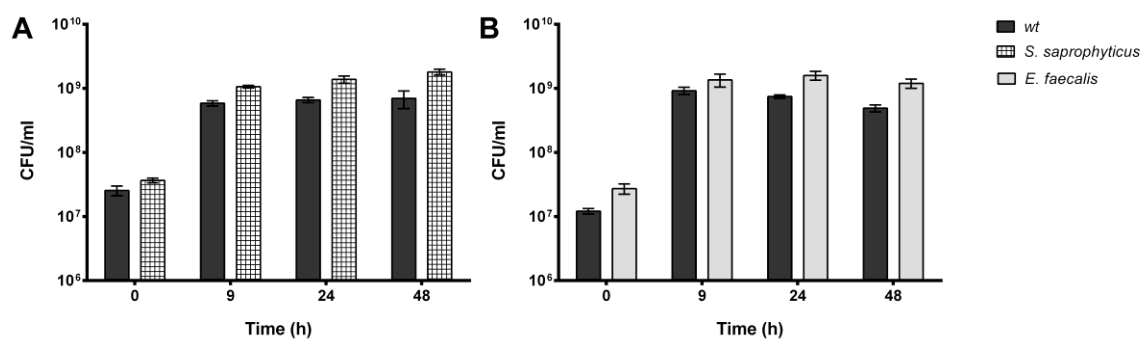
To more closely mimic the biological conditions that occur during natural infection, competition experiments were performed in Grace's medium, a defined medium based on lepidopteran hemolymph (FIG. 3.6). In co-cultures with *S.*



**FIG. 3.4. *In vitro* competition between *X. nematophila* mutant strains and *S. saprophyticus* in LB.** Subcultures of *X. nematophila* wild-type and mutant strains ( $\Delta xcnKL$ , F,  $\Delta xcnKL:F$ , *ngrA*) (dark gray bars) were mixed in a 1:1 ratio with *S. saprophyticus* (white hatched bars) and then used to inoculate fresh LB broth. Competition outcome was determined by dilution plating 0 (A), 9 (B), 18 (C), and 24 h (D). Graphs depict colony forming units/ml (CFU/ml) of both bacteria. Asterisks depict statistically significant differences (P < 0.05) as calculated by multiple paired t-tests using GraphPad Prism 6.



**FIG. 3.5. *In vitro* competition between *X. nematophila* mutant strains and *E. faecalis* in LB.** Subcultures of *X. nematophila* wild-type and mutant strains ( $\Delta xcnKL$ , F,  $\Delta xcnKL:F$ , *ngrA*) (dark gray bars) were mixed in a 1:1 ratio with *E. faecalis* (light gray bars) and then used to inoculate fresh LB broth. Competition outcome was determined by dilution plating at 0 (A), 9 (B), 18 (C), and 24 h (D). Graphs depict colony forming units/ml (CFU/ml) of both bacteria. Asterisks depict statistically significant differences ( $P < 0.05$ ) as calculated by multiple paired t-tests using GraphPad Prism 6.



**FIG. 3.6. *In vitro* competition of wild-type *X. nematophila* with *S. saprophyticus* (A) or *E. faecalis* (B) in Grace's.** Subcultures of wild-type *X. nematophila* were mixed in a 1:1 ratio with either *S. saprophyticus* (white hatched bars) or *E. faecalis* (light gray bars) and then used to inoculate fresh Grace's media. Competition outcome was determined by dilution plating at times 0, 9, 24, and 48 h. Graphs depict colony forming units/ml (CFU/ml) of both bacteria. No statistically significant differences between *X. nematophila* and the competitors were found at any of the time points.

*saprophyticus* the cell density of wild-type *X. nematophila* at 9 h was  $\sim 5.8 \times 10^8$  CFU/ml, in contrast to the  $\sim 10$ -fold higher level that was reached in LB broth. Unlike in the co-cultures in LB broth the cell density of *S. saprophyticus* was actually higher than *X. nematophila* at 24 h and remained at high levels at 48 h (FIG. 3.6.A). The same results were obtained in co-cultures with *E. faecalis* (FIG. 3.6.B). Thus, in Grace's medium the cell density of wild-type *X. nematophila* was  $\sim 10$ -fold less than the level reached in LB broth and the amount of antimicrobials produced were not sufficient to eliminate either *S. saprophyticus* or *E. faecalis*. In addition, we found that when grown individually both *S. saprophyticus* and *E. faecalis* grew much faster than *X. nematophila* in Grace's medium during early growth phase. At 6 h the cell density of *S. saprophyticus* and *E. faecalis* was  $\sim 8$ -fold and 105-fold greater, respectively, than *X. nematophila* (TABLES 7 and 8).

### **3.2.3. Comparison of antibiotic activity in LB broth and Grace's medium culture supernatants**

To compare the relative levels of antibiotic activity produced by wild-type *X. nematophila* grown in LB broth and Grace's medium, sterile cell-free supernatants prepared from cultures grown for 6 h, 9 h and 12 h were assayed in a microplate format using *S. saprophyticus* as the indicator strain (TABLE 9). Results were expressed as % inhibition of growth comparing the cell density (measured as optical density, O.D<sub>600</sub>) of the treated culture with the untreated culture of *S. saprophyticus*. At 6 h antibiotic activity was detectable at low and variable levels with either LB broth or Grace's medium supernatants. At 9 h the undiluted LB broth supernatants displayed 90% growth inhibition while the 0.5X diluted sample gave 34% growth inhibition. At 12 h the

**TABLE 7. Growth comparison between *X. nematophila* and *S. saprophyticus* in Grace's medium**

Time (h)	<i>X. nematophila</i>		<i>S. saprophyticus</i>		<i>S. sapro/X. nem</i>
	CFU/ml x 10 <sup>8</sup>	Fold increase <sup>a</sup>	CFU/ml x 10 <sup>8</sup>	Fold increase <sup>a</sup>	Fold increase
0	0.12 (0.01)	-	0.28 (0.01)	-	-
6	2.88 (0.50)	24.00	53.70 (11.29)	191.79	7.99
9	19.65 (2.69)	163.75	93.90 (23.74)	335.36	2.05
12	15.87 (4.03)	132.25	59.13 (11.14)	211.18	1.60
24	9.28 (0.48)	77.33	23.35 (2.65)	83.39	1.08

<sup>a</sup> CFU/ml relative to that at 0 h

**TABLE 8. Growth comparison between *X. nematophila* and *E. faecalis* in Grace's medium**

Time (h)	<i>X. nematophila</i>		<i>E. faecalis</i>		<i>E. faec/X. nem</i>
	CFU/ml x 10 <sup>7</sup>	Fold increase <sup>a</sup>	CFU/ml x 10 <sup>8</sup>	Fold increase <sup>a</sup>	Fold increase
0	0.11 (0.03)	-	0.11 (0.02)	-	-
6	7.10 (1.35)	64.55	746.25 (136.55)	6784.09	105.10
9	78.43 (13.95)	713.00	1291.93 (598.31)	11744.82	16.47
12	87.70 (6.82)	797.27	543.13(90.22)	4937.55	6.19
24	53.38 (12.75)	485.27	30.90 (3.17)	280.91	0.58

<sup>a</sup> CFU/ml relative to that at 0 h

**TABLE 9. *X. nematophila* antibiotic activity in cell-free supernatants**

	Time (h)	% Inhibition	
		1X	0.5X
LB broth	6	10.3 (3.7)	8.4 (3.3)
	9	89.9 (3.8)	33.5 (2.2)
	12	96.3 (0.7)	84.0 (2.3)
Grace's medium	6	10.2 (1.3)	14.1 (1.1)
	9	20.1 (0.6)	7.8 (3.7)
	12	96.9 (0.6)	4.2 (2.2)



antibiotic activity had increased in the LB broth supernatants, and was present at high levels in both undiluted and diluted supernatants. In contrast, the antibiotic activity in the supernatant from Grace's medium was barely detectable at 9 h. At 12 h the undiluted supernatants from Grace's medium displayed ~ 97% growth inhibition while little activity was present in the diluted supernatants. The low level of production in Grace's medium correlated with lower cell density relative to the LB broth levels (FIG. 3.7). These findings show that antibiotics were produced later and at lower levels in Grace's medium as compared to LB broth cultures.

#### **3.2.4. Competition in Grace's medium pre-inocubated with *X. nematophila* strains**

The combination of low level antibiotic production by *X. nematophila* and more rapid initial growth rates of *S. saprophyticus* and *E. faecalis* in Grace's medium resulted in the inability of *X. nematophila* to suppress growth of microbial competitors. To further assess the role of antimicrobial production in interspecies competition, wild-type and mutant strains of *X. nematophila* were first inoculated in Grace's medium and grown for 10.5 or 11.5 h before inoculating with either *S. saprophyticus* or *E. faecalis*. The co-cultures were dilutionally plated at 24 h to determine the cell density of the respective strains (TABLES 10 and 11). *S. saprophyticus* was eliminated in co-cultures with the wild-type and NRPS mutant strains but grew to high levels in co-culture with the *ngrA* strain. These findings indicate that NRPS-derived antibiotics other than xenocoumacin and compound F were required to eliminate *S. saprophyticus* in Grace's medium. In contrast, *E. faecalis* was not eliminated when co-cultured with any of the pre-inoculated *X. nematophila* strains. The relative resistance of *E. faecalis* to *X. nematophila* antibiotics

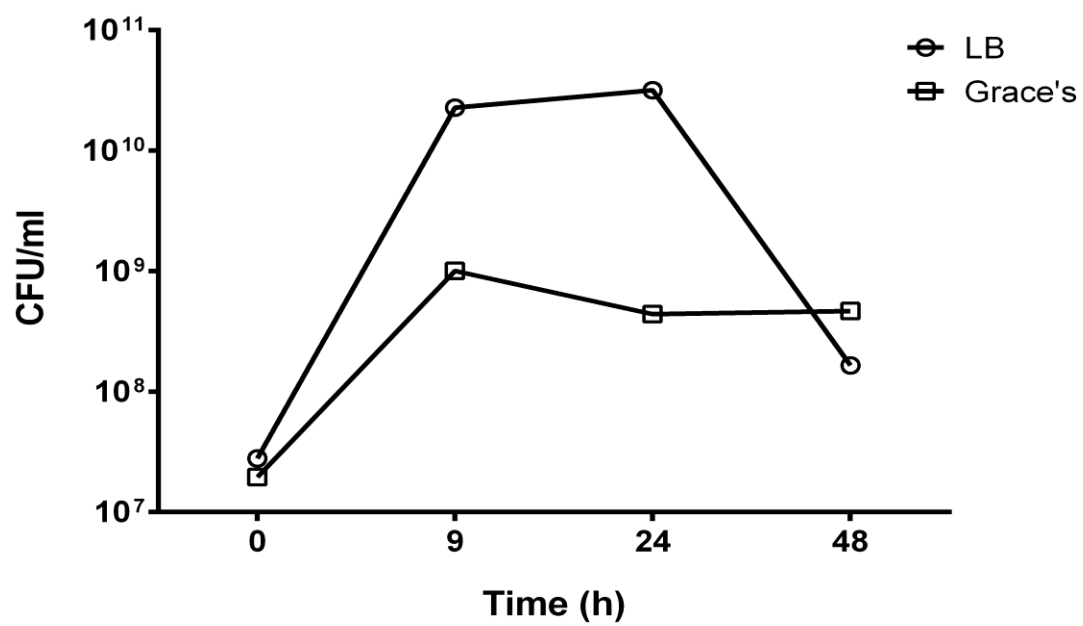


FIG. 3.7. Temporal cell density of *X. nematophila* in LB broth and Grace's medium

**TABLE 10. Competition of *S. saprophyticus* with pre-incubated *X. nematophila* in Grace's medium**

Strains	<i>X. nematophila</i>	<i>S. saprophyticus</i>
	Initial cell density <sup>a</sup>	
	CFU/ml x 10 <sup>8</sup> <sup>b</sup>	CFU/ml x 10 <sup>8</sup>
WT	25.5 (3.0)	1.1 (0.1)
$\Delta xcnKL$	21.7 (2.1)	1.1 (0.1)
F	31.9 (4.4)	1.1 (0.1)
$\Delta xcnKL:F$	32.7 (3.2)	1.1 (0.1)
<i>ngrA</i>	34.9 (4.7)	1.1 (0.1)

Strains	Cell density after 24 h <sup>c</sup>	
	<i>X. nematophila</i>	<i>S. saprophyticus</i>
WT	7.9 (1.7)	0.0
$\Delta xcnKL$	3.9 (0.8)	0.0
F	3.9 (0.4)	0.0
$\Delta xcnKL:F$	1.4 (0.3)	0.0
<i>ngrA</i>	6.9 (0.9)	3.8 (0.6)

<sup>a</sup> Cell density at start of competition after *X. nematophila* was pre-incubated for 10.5 h

<sup>b</sup> Values represent mean and standard error (parentheses)

<sup>c</sup> Cell density after 24 h of competition

**TABLE 11. Competition of *E. faecalis* with pre-incubated *X. nematophila* in Grace's medium**

Strains	<i>X. nematophila</i>	<i>E. faecalis</i>
	Initial cell density <sup>a</sup>	
	CFU/ml x 10 <sup>8</sup> <sup>b</sup>	CFU/ml x 10 <sup>8</sup>
WT	14.3 (1.2)	0.7 (0.0)
$\Delta xcnKL$	13.9 (1.4)	0.7 (0.0)
F	45.5 (5.3)	0.7 (0.0)
$\Delta xcnKL:F$	26.4 (2.2)	0.7 (0.0)
<i>ngrA</i>	31.6 (2.8)	0.7 (0.0)
	Cell density after 24 h <sup>c</sup>	
WT	9.1 (0.9)	0.7 (0.3)
$\Delta xcnKL$	5.6 (0.4)	0.1 (0.0)
F	19.3 (1.3)	0.9 (0.1)
$\Delta xcnKL:F$	3.0 (0.5)	0.4 (0.0)
<i>ngrA</i>	18.7 (2.8)	2.1 (0.4)

<sup>a</sup> Cell density at start of competition after *X. nematophila* was pre-incubated for 11.5 h

<sup>b</sup> Values represent mean and standard error (parentheses)

<sup>c</sup> Cell density after 24 h of competition

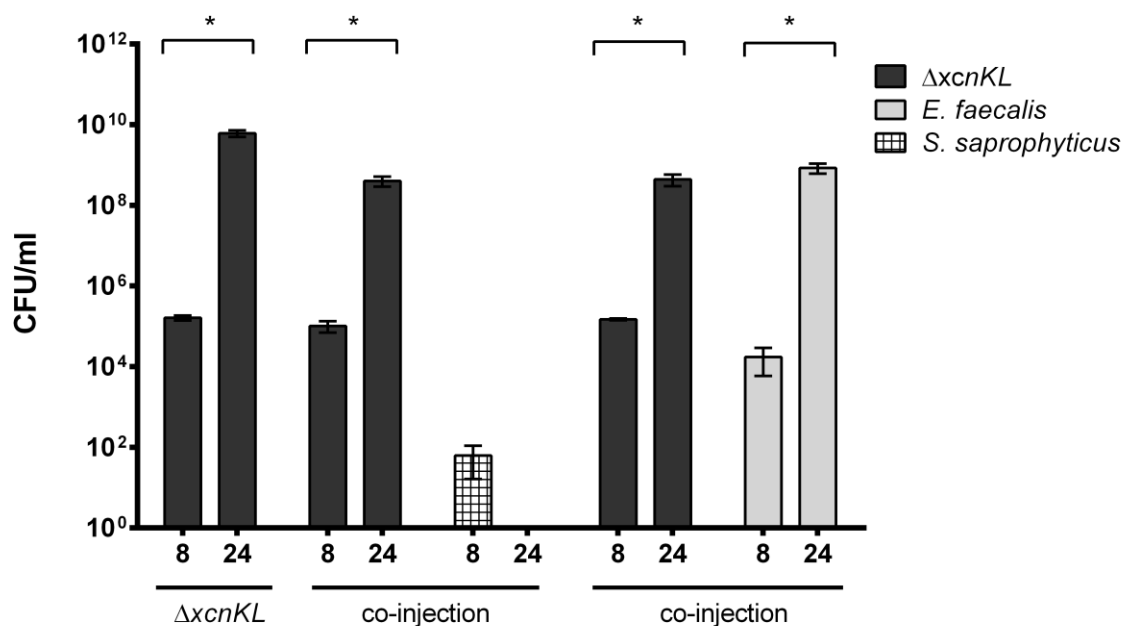
and the lower level of antibiotic production by *X. nematophila* in Grace's medium apparently accounts for the inability to eliminate this competitor.

### 3.2.5. Competition in the insect host, *Manduca sexta*

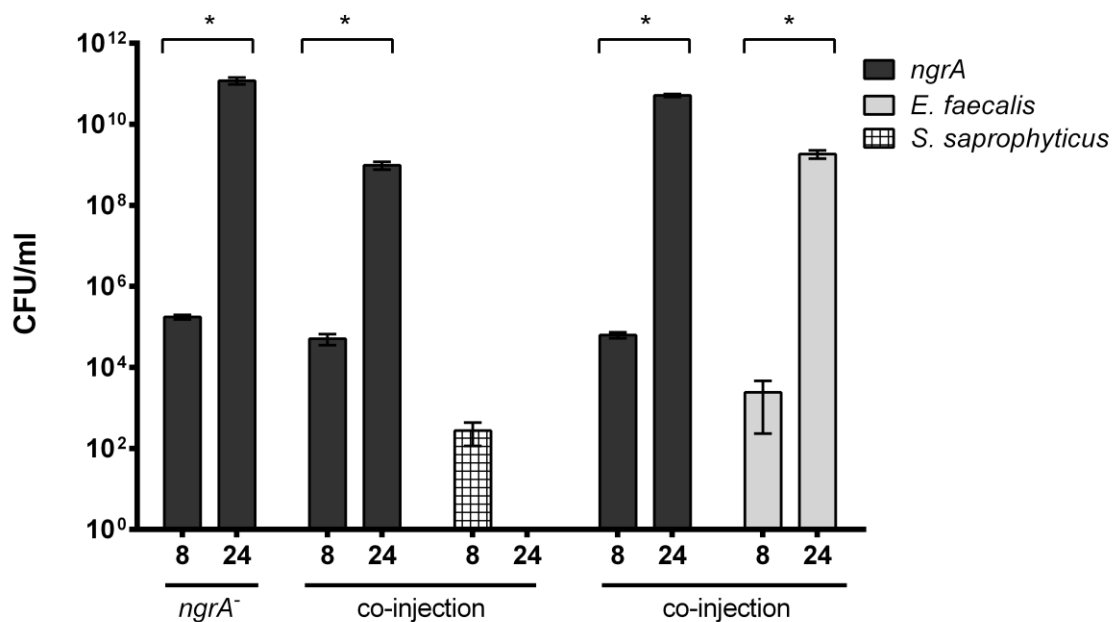
We had previously shown that when *S. saprophyticus* was injected into *M. sexta* it was present in the hemolymph at low levels ( $\sim 10^2$  CFU/ml) at 9 h and grew to slightly higher cell density ( $\sim 10^3$  CFU/ml) at 24 h (28). However, when *S. saprophyticus* was co-injected with wild-type *X. nematophila* it was eliminated by 24 h. This finding, together with the sensitivity of *S. saprophyticus* to *X. nematophila* antibiotics, suggested that antibiotic production could be involved in the elimination of *S. saprophyticus in vivo*. To address this question *S. saprophyticus* was co-injected into the hemocoel of *M. sexta* with either the  $\Delta xcnKL$  (FIG. 3.8) or the *ngrA* strain (FIG. 3.9) and hemolymph was collected and dilutionally plated at 8 h and 24 h post-injection. In both cases *S. saprophyticus* was present at 8 h and was eliminated by 24 h. This finding suggests that NRPS-derived antibiotics were not essential for elimination of *S. saprophyticus in vivo*. In contrast, *E. faecalis* growth was shown to be facilitated when co-injected with wild-type *X. nematophila* (28) and also was facilitated when co-injected with either the  $\Delta xcnKL$  (FIG. 3.8) or *ngrA* (FIG. 3.9) strain.

### 3.2.6. Natural infection and nematode reproduction in *M. sexta*

It has been proposed that sub-inhibitory concentrations of antibiotics and other secondary metabolites can function as signaling molecules (22-24, 34, 35). With this in mind we addressed the possibility that NRPS-derived compounds produced by *X.*



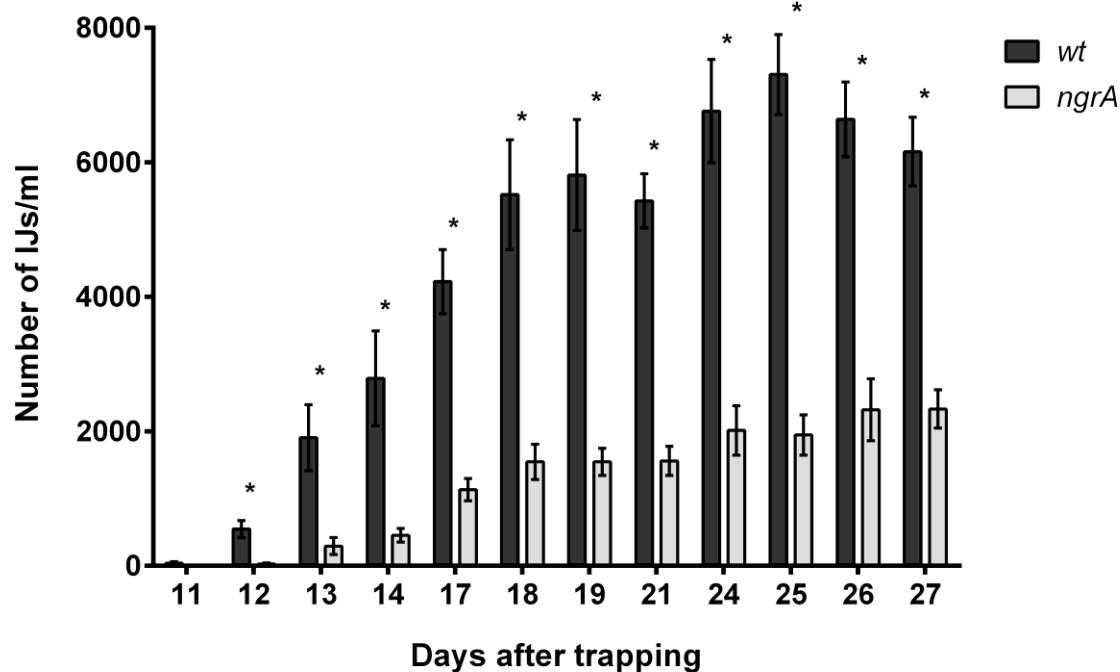
**FIG. 3.8.** *In vivo* growth after 1:1 co-injections of *X. nematophila*  $\Delta xc n K L$  strain with *S. saprophyticus* or *E. faecalis* into *M. sexta*. Insects were co-injected with a 1:1 mixture of *X. nematophila*  $\Delta xc n K L$  (dark gray bars) with either *S. saprophyticus* (white hatched bars) or *E. faecalis* (light gray bars). 10<sup>4</sup> CFU/insect of each strain were injected. Hemolymph recovered at 8 and 24 h post-injection was dilutionally plated. Data are represented as CFU/ml of each bacterial species at 8 and 24 h. Asterisks depict statistically significant differences (P<0.05) as calculated by multiple paired t-tests using GraphPad Prism 6.



**FIG. 3.9.** *In vivo* growth after 1:1 co-injections of *X. nematophila ngrA* strain with *S. saprophyticus* or *E. faecalis* into *M. sexta*. Insects were co-injected with a 1:1 mixture of *X. nematophila ngrA* (dark gray bars) with either *S. saprophyticus* (white hatched bars) or *E. faecalis* (light gray bars).  $10^4$  CFU/insect of each strain were injected. Hemolymph recovered at 8 and 24 h post-injection was dilutionally plated. Data are represented as CFU/ml of each bacterial species at 8 and 24 h. Asterisks depict statistically significant differences ( $P < 0.05$ ) as calculated by multiple paired t-tests using GraphPad Prism 6.

*nematophila* might play a role in *in vivo* reproduction of the nematode, *S. carpocapsae*. To assess this possibility, IJs harboring either the wild-type or *ngrA* strain of *X. nematophila* were used for natural infections of *M. sexta*. When the insects died 24-48 h post-injection the cadavers were transferred to water traps and the cumulative number of emergent progeny IJs was monitored over a 27 day period (FIG. 3.10). When infected with wild-type *X. nematophila* - carrying nematodes, progeny IJs began to appear in the water traps at day 11 and continued to accumulate for 25 days reaching a level of 7300 IJs/ml. In contrast, when infected with IJs colonized with the *ngrA* strain, progeny IJs appeared in much lower numbers and after 25 days only 1947 IJs/ml had accumulated in the water traps. In contrast, nematodes reproduced to a similar extent on lawns of wild type and *ngrA* strains (data not shown) suggesting that the *ngrA* strain was not defective in providing a nutrient base for nematode reproduction. The *ngrA* strain also grew as well as the wild type strain when injected into *M. sexta* (FIG. 3.9). Together, these findings suggest that NRPS-derived compounds are involved in stimulating optimal nematode reproduction and emergence *in vivo*.





**FIG. 3.10.** *In vivo* nematode reproduction using *S. carpocapsae* carrying *X. nematophila* wild-type and *ngrA* strains after natural infection of *M. sexta*. The total number of IJs emerging from insect cadavers infected with *S. sarpocapsae* harboring either wild-type (dark gray bars) or *ngrA* (light gray bars) *X. nematophila* strains were counted for a period of 27 days from the day of trapping. Three traps containing four insects each were used for both sets and IJs were counted in five 5  $\mu$ l drops ( $n = 15$ ). Except for the initial time point (Day 11), the mean values for the wild type and the mutant were significantly different at each time point (two-tailed  $P < 0.01$ ); when paired at each time point (including Day 11), the wild type and the mutant were also significantly different over the entire time scale examined (paired two-tailed  $P < 0.001$ ). Error bars represent standard errors.

### 3.3. Discussion

We showed previously that insect gut microbes translocate into the hemocoel during nematode invasion. Transient species such as *S. saprophyticus* disappeared early while persistent species such as *E. faecalis* proliferated in the presence of *X. nematophila*. In the present study we examined the possible role of NRPS-derived antimicrobials in early stages of interspecies competition. *X. nematophila* possesses seven NRPS-containing gene clusters and two large stand alone NRPS genes. Transfer of a phosphopantetheinyl group to the PCP domain by PPant transferase encoded by *ngrA* is required for NRPS function. Using NRPS and *ngrA* mutant strains and an overlay diffusion assay we found that xenocoumacin and an uncharacterized antimicrobial, compound F, accounted for most of the antimicrobial activity against *S. saprophyticus*. Xenocoumacin, a hydrophilic benzopyran compound, was shown previously to be active against *S. aureus* and *Streptococcus* species (7). The present study is the first to show that xenocoumacin is active against a potential competitor derived from the gut of an insect host. As predicted from previous results *E. faecalis* was relatively resistant to *X. nematophila* antibiotics.

Most of the *X. nematophila* antimicrobials and secondary metabolites characterized to date are hydrophobic. Since the overlay assay detects compounds that diffuse away from a bacterial colony grown on LB agar more hydrophobic compounds may exhibit limited activity in this assay. For this reason a liquid medium-based competition assay in which antibiotics disperse throughout the culture was used to examine the role of NRPS-derived compounds in interspecies competition. Wild-type and NRPS mutant strains were co-inoculated in LB broth with either *S. saprophyticus* or *E.*

*faecalis* and cultures were continually shaken during the assay. *S. saprophyticus* was not eliminated in co-cultures with either the  $\Delta xcnKL$  or  $\Delta xcnKL:F$  strains but was eliminated in co-cultures with the wild-type and F strains indicating that xenocoumacin was required to eliminate *S. saprophyticus*. In contrast, *E. faecalis* was eliminated in co-cultures with the  $\Delta xcnKL$  and  $\Delta xcnKL:F$  strains but not the *ngrA* strain indicating that NRPS-derived antibiotics other than xenocoumacin and compound F were produced at levels sufficient to eliminate *E. faecalis*.

To assess the contribution of antimicrobials under more natural biological conditions co-culture competition experiments were performed in Grace's insect medium. Under these conditions neither *S. saprophyticus* nor *E. faecalis* were eliminated suggesting that antimicrobials were produced at sub-inhibitory levels. Indeed, antibiotic activity in sterile cell-free supernatants from *X. nematophila* grown in Grace's medium was markedly lower than supernatants for cells grown in LB broth. Importantly, the cell density of the Grace's medium cultures was ~10-fold lower than in LB broth. In many microbial species studied robust antibiotic production involves quorum sensing in which accumulation of autoinducer molecules at higher cell densities activate the expression of secondary metabolite genes (22). The lower cell density and autoinducer concentrations in cultures in Grace's medium could result in reduced levels of antibiotic production and the inability to eliminate competitors. In addition, secondary metabolite production was induced by the addition of 50 mM L-proline to cultures of *X. nematophila* (36). Grace's medium contains only 3 mM L-proline. We found that addition of 50mM L-proline to Grace's cultures did not stimulate antibiotic production (unpublished data) suggesting that other environmental and metabolic signals may be involved in inducing NRPS genes

in *X. nematophila* in Grace's medium. In well studied antibiotic producers such as *Streptomyces coelicolor* numerous regulatory cascades and metabolic signals control antibiotic production (37). Taken together, the lower cell density of *X. nematophila* and possible suboptimal inducing conditions could account for the inability to eliminate competitors in Grace's medium.

We also found that the cell density of *S. saprophyticus* in Grace's medium increased 8-fold faster than *X. nematophila* at 6 h post-inoculation. Thus, competitor cell densities reached high levels before antibiotics have time to accumulate. To be able to detect antimicrobial activity in Grace's medium *X. nematophila* was first grown for ~10 h and *S. saprophyticus* was subsequently inoculated into the culture. Under these conditions *S. saprophyticus* was eliminated by the wild-type and NRPS strains but not the *ngrA* strain indicating that NRPS-derived antimicrobial besides xenocoumacin and compound F were able to inhibit growth of *S. saprophyticus*. Thus, detection of antimicrobial activity against *S. saprophyticus* was dependent on the type of assay employed. Xenocoumacin and compound F were detected in the overlay assay, xenocoumacin but not compound F in the LB competition assay and NRPS-derived antimicrobial activity besides xenocoumacin and compound F in the Grace's medium assay. Elucidation of the environmental and metabolic signals that control NRPS gene expression would shed light on the role of antimicrobial activity in interspecies competition in different host insects.

We showed previously that *S. saprophyticus* persisted when injected alone into *M. sexta*, proliferated when co-injected with *E. faecalis* and was eliminated when co-injected with *X. nematophila*. Here we show that co-injection with either the  $\Delta xcnKL$  or *ngrA*

strains resulted in disappearance of *S. saprophyticus*. Thus, antimicrobial agents other than NRPS-derived compounds may be involved in the elimination of *S. saprophyticus* and presumably other transient species. *X. nematophila* produces indole derived compounds that are highly active against *B. cereus* and *M. luteus* (20), nematophin that is active against *S. aureus* strains (21) and benzylideneacetone that possesses both antimicrobial and immunosuppressive activities and is active against Gram-negative plant pathogens (18). *X. nematophila* may also produce additional as yet unidentified compounds that are active against transient species. Since gut microbiota may vary widely in different insect hosts the potential to synthesize a spectrum of antimicrobial compounds that are active against a variety of microbes confers competitive advantages in diverse host environments.

It remains unknown whether during early stages of infection *X. nematophila* produces antimicrobials at levels that can inhibit species growing in the hemolymph of *M. sexta*. Using an *in-vivo* expression technology approach NRPS genes encoding rhabdopeptide that possesses anti-parasite activity were shown to be expressed soon after *X. nematophila* was injected into *M. sexta* (14). Induction of other NRPS genes that encode known antimicrobial compounds was not detected by this approach. As discussed above, L-proline induces secondary metabolite synthesis in *X. nematophila* (36). The L-proline concentration in *M. sexta* is low (3 mM) relative to that in other lepidopterans such as *Galleria mellonella* (72 mM). Furthermore, *X. nematophila* was barely detectable at early stages of natural infection unlike the higher levels present when co-injected with *S. saprophyticus* (FIGS. 3.8 and 3.9). Thus, elimination of transient species during early stages of natural infection may be due initially to activation of immune responses. As *X.*

*nematophila* proliferates with concomitant suppression of immune responses increased antimicrobial levels could effectively inhibit growth of transient competitors. Presumably, when *X. nematophila* reaches higher cell density during later stages of infection elevated levels of antimicrobials prevent the reemergence of transient species.

The scenario for interactions between *X. nematophila* and a persistent species such as *E. faecalis* is distinctly different. *E. faecalis* is relatively resistant to xenocoumacin but is sensitive to other yet identified antimicrobials produced by *X. nematophila* grown in LB broth. In contrast, antimicrobial activity produced in Grace's medium was insufficient to eliminate *E. faecalis*. In addition, the increase in cell density of *E. faecalis* inoculated into Grace's was 105-fold greater than *X. nematophila* 6 h post-inoculation. Furthermore, rather than being eliminated, *E. faecalis* growth was facilitated by the presence of *X. nematophila in vivo*. We had shown that *E. faecalis* strongly induced cecropin transcription in *M. sexta* while transcript levels were reduced markedly when *E. faecalis* was co-injected with *X. nematophila*. Together, these findings suggest that the dominance and persistence of *E. faecalis* during natural infection could result from the combination of rapid growth rate after translocation into the insect hemolymph, relative resistance to antimicrobials produced in the hemolymph and suppression of the host immune response by *X. nematophila*.

Bacterial factors that influence *S. carpocapsae* growth and development *in vivo* had not been previously studied. Here we show that reproduction of nematodes in *M. sexta* naturally infected with IJs colonized with the *ngrA* strain was dramatically reduced relative to levels obtained with the wild-type strain. It was shown previously that nematodes reared on lawns of an *lrp* strain in which numerous phenotypic traits were lost

reproduced to a lesser extent than those raised on the wild-type lawns (38). The number of IJ progeny that emerged from the *lrp* lawns was ~85% of the number obtained with the wild-type strain. In *P. luminescens* inactivation of *ngrA* resulted in loss of antibiotic and siderophore production while other phenotypic traits examined were similar to the wild-type strain (27). The development of *Heterorhabditis bacteriophora* IJs to the J4 stage was significantly reduced when grown on lawns of the *ngrA* strain. Moreover, nematodes were unable to reproduce on the *ngrA* strain. Since a siderophore mutant was able to support nematode reproduction (39) it was concluded that the *ngrA* gene is involved in production of a signaling compound for nematode development. Likewise, recovery of *H. bacteriophora* IJs on a *P. luminescens* strain that was deficient in production of multipotent stilbene compounds was significantly reduced relative to that of nematodes grown on lawns of wild-type cells (40). Thus, in *P. luminescens* both *ngrA*-derived compounds and stilbenes are required for nematode reproduction *in vitro*. Whether these compounds are involved in growth and development *in vivo* remains to be determined. To our knowledge the findings of the present study with the *ngrA* strain are the first to establish a role for bacterial products for *S. carpocapsae* growth and development in an insect host.

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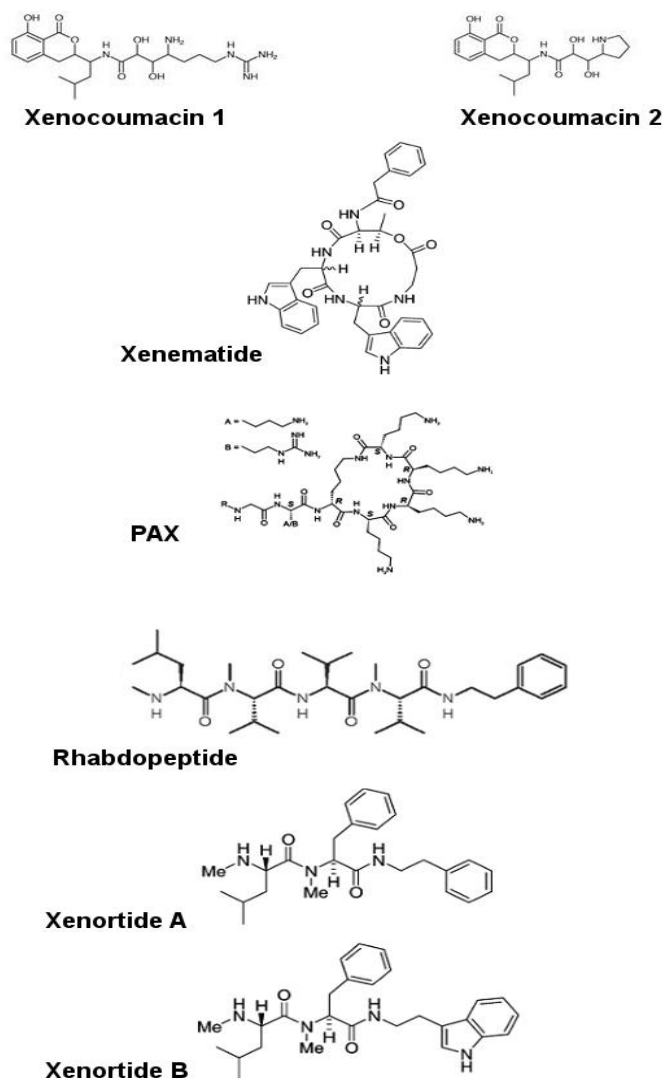
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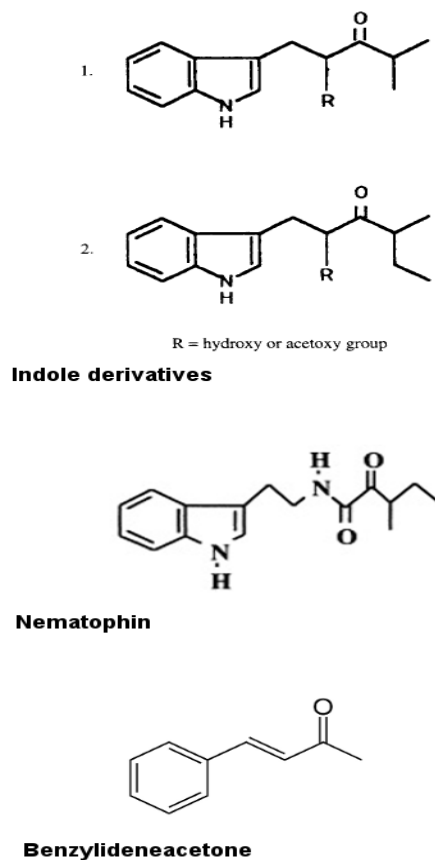
## **Appendix**

**Supplemental Figures: Structures of Compounds Produced by *X.***

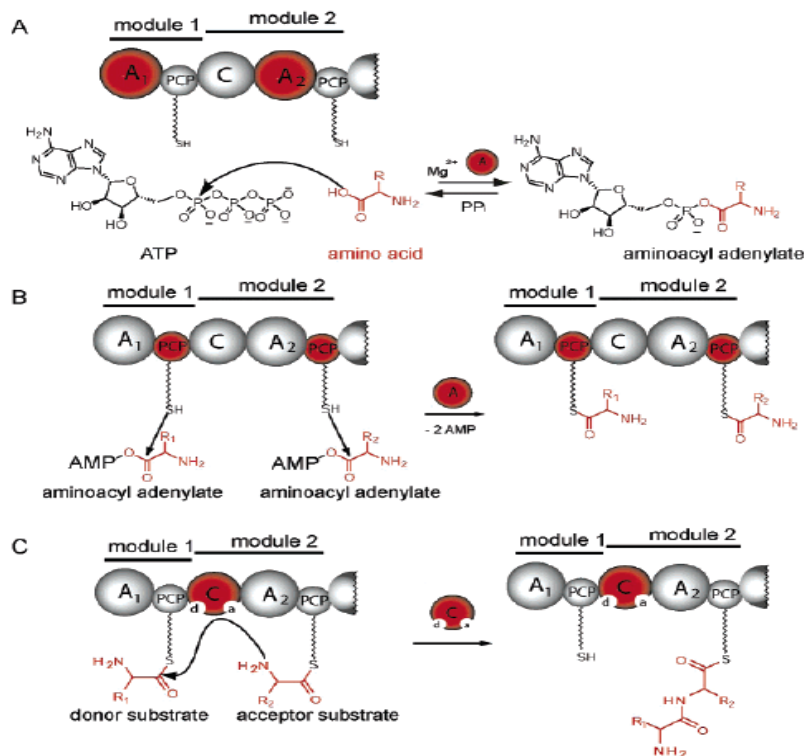
***nematophila* and Schematic Illustrations of NRPSs**



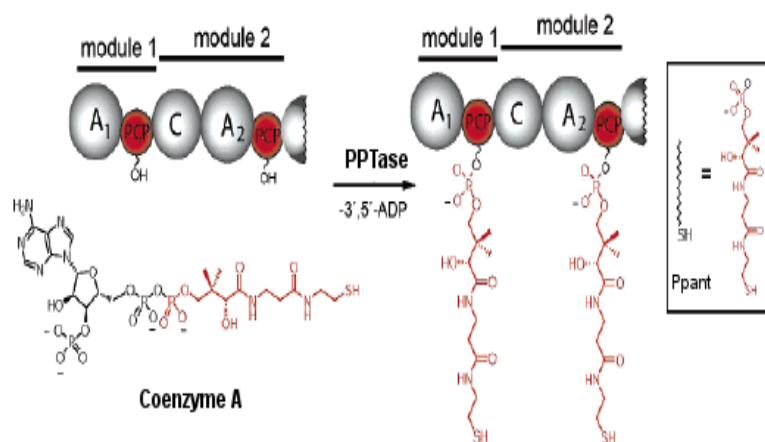
**FIG. A.1. Known antimicrobial compounds produced by *X. nematophila*.** (Sources: **Xenocoumacin** - McInerney BV, Taylor WC, Lacey MJ, Akhurst RJ, Gregson RP. 1991. Biologically active metabolites from *Xenorhabdus* spp., Part 2. Benzopyran-1-one derivatives with gastroprotective activity. *J. Nat. Prod.* 54:785-795; **xenematide** and **xenortide** - Lang G, Kalvelage T, Peters A, Wiese J, Imhoff JF. 2008. Linear and cyclic peptides from the entomopathogenic bacterium *Xenorhabdus nematophilus*. *J. Nat. Prod.* 71:1074-1077; **PAX** - Fuchs SW, Proschak A, Jaskolla TW, Karas M, Bode HB. 2011. Structure elucidation and biosynthesis of lysine-rich cyclic peptides in *Xenorhabdus nematophila*. *Org. Biomol. Chem.* 9:3130-3132; **rhabdopeptide** - Reimer D, Cowles KN, Proschak A, Nollmann FI, Dowling AJ, Kaiser M, Constant Rf, Goodrich-Blair H, Bode HB. 2013. Rhabdopeptides as insect-specific virulence factors from entomopathogenic bacteria. *Chembiochem.* 14:1991-1997)



**FIG. A.2. Other small molecules produced by *X. nematophila* with antibiotic activity.** (Sources: **Indole derivatives** – Paul VJ, Frautschy S, Fenical W, Neilson KH. 1981. Antibiotics in microbial ecology, isolation and structure assignment of several new antibacterial compounds from the insect-symbiotic bacteria *Xenorhabdus* spp. *J. Chem. Ecol.* 7:589-597 and Sundar L, Chang FN. 1993. Antimicrobial activity and biosynthesis of indole antibiotics produced by *Xenorhabdus nematophilus*. *J. Gen. Microbiol.* 139:3139-3148; **nematophin** - Li J, Chen G, Webster JM. 1997. Nematophin, a novel antimicrobial substance produced by *Xenorhabdus nematophilus* (Enterobacteriaceae). *Can. J. Microbiol.* 43:770-773; **benzylideneacetone** - Ji D, Yi Y, Kang GH, Choi YH, Kim P, Baek NI, Kim Y. 2004. Identification of an antibacterial compound, benzylideneacetone, from *Xenorhabdus nematophila* against major plant-pathogenic bacteria. *FEMS Microbiol. Lett.* 239:241-248)



**FIG. A.3. Reactions catalyzed by NRPS domains.** (A) The A domain recognizes and activates a dedicated amino acid, (B) the activated aminoacyl adenylate covalently attaches onto the free thiol group of the PCP-bound Ppant cofactor, (C) the C domain carries out peptide elongation by catalyzing an attack of the nucleophilic amine of the acceptor substrate onto the electrophilic thioester of the donor substrate. (Source: Sieber SA, Marahiel MA. 2005. Molecular mechanisms underlying nonribosomal peptide synthesis: approaches to new antibiotics. *Chem. Rev.* 105:715-738)



**FIG. A.4. Phosphopantetheinylation by the enzyme PPTase which is a product of the *ngrA* gene in *X. nematophila*.** The phosphopantetheine moiety of coenzyme A is covalently attached to the PCP domain by PPTase, a dedicated phosphopantetheinyl transferase. (Adapted from: Sieber SA, Marahiel MA. 2005. Molecular mechanisms underlying nonribosomal peptide synthesis: approaches to new antibiotics. Chem. Rev. 105:715-738)



## CURRICULUM VITAE

### SWATI SINGH

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### EDUCATION

<b>Ph.D. Microbiology</b> <i>August 2008 – May 2014</i>	University Of Wisconsin – Milwaukee.
<b>M.Sc. Biosciences</b> <i>August 2006 – May 2008</i>	Jamia Millia Islamia, New Delhi, India.
<b>B.Sc. (Honors) Microbiology</b> <i>August 2003 – May 2006</i>	Delhi University, New Delhi, India.

**DISSERTATION TITLE:** The role of antimicrobial compounds in the life cycle of the symbiotic bacterium, *Xenorhabdus nematophila*

### RESEARCH EXPERIENCE

- Research Assistant** in the laboratory of Dr. Steven Forst at the University of Wisconsin – Milwaukee.  
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### TEACHING EXPERIENCE

- Teaching Assistant** Department of Biological Sciences, University of Wisconsin – Milwaukee.  
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*General Survey of Microbiology.*  
*General Microbiology.*
- Undergraduate student training.**  
*May 2011 – May 2014*

## AWARDS/HONORS/FELLOWSHIPS

Selected for the “**Young Investigators Oral Presentation**” at the 113<sup>th</sup> General Meeting of the American Society for Microbiology, Denver, CO, May 2013.

**UWM Graduate School Travel Grant**  
2013

**UWM Distinguished Graduate Student Fellowship**  
2012-2013

**Ruth Walker Grant-in-Aid Graduate Student Award**  
April 2012

**Perlman Symposium – UW Madison – 2<sup>nd</sup> Prize Poster**  
April 2012

**Ruth Walker Grant-in-Aid Graduate Student Award**  
April 2011

**Chancellor’s Graduate Student Award**  
August 2008 – August 2013

## PUBLICATIONS

**Singh S**, Reese JM, Casanova-Torres AM, Goodrich-Blair H, Forst S. 2014. Microbial population dynamics in the hemolymph of *Manduca sexta* infected with *Xenorhabdus nematophila* and the entomopathogenic nematode, *Steinernema carpocapsae*. Appl. Env. Microbiol. 80: In press.

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Park D, Ciezki K, van der Hoeven R, **Singh S**, Reimer D, Bode H, and Forst S. 2009. Genetic analysis of xenocoumarin antibiotic production in the mutualistic bacterium *Xenorhabdus nematophila*. Mol. Microbiol. 73: 938-949.

“Differential role of antibiotics in the life cycle of the entomopathogen *Xenorhabdus nematophila*” (Abstract accepted) Young Investigators Oral Presentation at the 113<sup>th</sup> General Meeting of the American Society for Microbiology, Denver, CO, May 2013.

Proposal accepted for sequencing the genome of an *Enterococcus faecalis* isolate from the gut of the tobacco hornworm as part of the UWM Great Lakes Genomics Center’s pilot next-gen sequencing projects using the new Mi-Seq sequencer, School of Freshwater Sciences, University of Wisconsin-Milwaukee, Oct 2013.

## ORAL PRESENTATIONS

“Natural biology of antibiotics in the life-cycle of *Xenorhabdus nematophila*”  
Biological Sciences Colloquium, University of Wisconsin-Milwaukee, November  
2013.

“Differential role of antibiotics in the life cycle of the entomopathogen  
*Xenorhabdus nematophila*” Young Investigators Oral Presentation at the 113<sup>th</sup>  
General Meeting of the American Society for Microbiology, Denver, CO, May  
2013.

“New insights into the role of antibiotics in a natural host environment”  
Milwaukee Microbiology Seminar, School of Freshwater Sciences, University of  
Wisconsin-Milwaukee, December 2012.

“Genomic and biological analysis of the role of antibiotics in the life cycle of  
*Xenorhabdus nematophila*” NEMASYM 2011, Oregon State University, July  
2011.

## POSTER PRESENTATIONS

“Differential role of antibiotics in the life cycle of the entomopathogen  
*Xenorhabdus nematophila*” Perlman Symposium, University of Wisconsin-  
Madison, April 2013.

“The role of antimicrobial compounds in the natural biology of the  
entomopathogen *Xenorhabdus nematophila*” Perlman Symposium, University of  
Wisconsin-Madison, April 2012.

“Antimicrobial warfare: Defining the role of antibiotics in the life cycle of  
*Xenorhabdus nematophila*” Perlman Symposium, University of Wisconsin-  
Madison, April 2011.

“Antimicrobial warfare: Defining the role of antibiotics in the life cycle of  
*Xenorhabdus nematophila*” Biological Sciences Research Symposium, University  
of Wisconsin-Milwaukee, April 2011.

“Role of antimicrobial agents in the life cycle of *Xenorhabdus nematophila*”  
Raper Symposium, University of Wisconsin-Madison, August 2010.

“Role of antibiotics in interspecies competition in the life cycle of *Xenorhabdus  
nematophila*” Perlman Symposium, University of Wisconsin-Madison, April  
2010.