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New Insights into the Role of Antimicrobials of Xenorhabdus in Interspecies Competition

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**NEW INSIGHTS INTO THE ROLE OF ANTIMICROBIALS OF *XENORHABDUS* IN
INTERSPECIES COMPETITION**

by

Kristin J. Ciezki

A Dissertation Submitted in

Partial Fulfillment of the

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ABSTRACT

NEW INSIGHTS INTO THE ROLE OF ANTIMICROBIALS OF *XENORHABDUS* IN INTERSPECIES COMPETITION

by

Kristin J. Ciezki

The University of Wisconsin – Milwaukee, 2017
Under the Supervision of Professor Steven A. Forst

Xenorhabdus spp. are symbionts of entomopathogenic nematodes and pathogens of susceptible insects. The nematodes penetrate the insect midgut to enter the hemocoel where *Xenorhabdus* bacteria are released, transitioning to their pathogenic stage. During nematode invasion microbes from the insect gut translocate into the hemocoel. In addition, different species of nematodes carrying specific strains of *Xenorhabdus* can invade a single insect. *Xenorhabdus* spp thereby engage in competition with both related strains and nonrelated gut microbes. In complex media *Xenorhabdus* spp produce diverse antimicrobial compounds whose functions in biological systems remain poorly understood. R-type bacteriocins are contractile phage-tail-like structures that are bactericidal towards related bacterial species. They are encoded by remnant P2-type prophage in which the C-terminal region of the tail fiber protein determines target-binding specificity. *Xenorhabdus nematophila* and *Xenorhabdus bovienii* produce R-type bacteriocins (xenorhabdicins) that are selectively active against different *Xenorhabdus* and *Photorhabdus* species. In this study we analyzed the P2-type remnant prophage clusters in the draft sequences of nine strains of *Xenorhabdus bovienii*. The C-terminal tail fiber region in each of the respective

strains was unique, consisting of mosaics of modular units. The intergenic regions between the main tail fiber gene (*xbpH*) and the sheath gene (*xbpS*) contained a variable number of genes encoding tail fiber fragment modules that were apparently exchanged between strains. Xenorhabdins purified from three different *X. bovienii* strains isolated from the same nematode species displayed distinct activities against each other. Competition experiments revealed that xenorhabdin activity was predictive of competitive outcomes between two of the strains while other determinants besides xenorhabdins were primarily involved in the competitive success between the other strains, indicating that several factors may be involved in determining the outcome of competitions between different strains of *X. bovienii*. Here we show that another species, *Xenorhabdus szentirmaii*, produced antibiotics that were active against both gut-derived microbes and several species of *Xenorhabdus* including the well-studied *Xenorhabdus nematophila*. Antibiotics of *X. nematophila* were not active against *X. szentirmaii*. In co-cultures of wild-type *X. szentirmaii* and *X. nematophila* in Grace's medium that mimics insect hemolymph *X. nematophila* was eliminated. An antibiotic-deficient strain of *X. szentirmaii* was created by inactivation of the *ngrA* gene. *X. nematophila* proliferated in co-cultures with the *ngrA* strain. In insects co-infected with wild-type *X. szentirmaii* and *X. nematophila* the later was eliminated while *X. nematophila* proliferated in insects co-infected with the *ngrA* strain indicating that wild-type *X. szentirmaii* produced sufficient levels of antibiotics to inhibit growth of a related competitor. MALDI-TOF analysis of hemolymph derived from insects infected with wild-type *X. szentirmaii* revealed the presence of two compounds (*m/z* 544 and *m/z* 558) that were absent in hemolymph infected with the *ngrA* strain. To our knowledge, this is the first study to directly demonstrate that antibiotics determine the outcome of interspecies competition in a natural host environment.

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Chapter One

Background and Significance

1.0 Introduction

Little is known about the role of antimicrobials in nature and in the lifecycles of the organisms that produce them. Historically antimicrobial research has been a pursuit to harness the potential of antimicrobial agents to prevent and treat disease. Comparatively few studies have investigated the maintenance, evolution, and expression of antimicrobial genes in producer organisms or how antimicrobials function in microbial communities in nature. Traditionally, antimicrobials have been perceived as antagonistic molecules that function as weapons employed by bacteria in competition for resources (1). Recent work has argued that in nature, antibiotics are mostly produced at subinhibitory concentrations (SIC) and subsequently their true functions are actions stimulated by SIC. The SIC of antibiotics induce shifts in bacterial gene expression *in vitro* (2–7), act as signals that may mediate species interactions (6, 8–11), and cause a range of global cellular responses, as all antibiotics exhibit pleiotropic effects (12). Experimental evidence coming from natural systems is lacking to support an antagonistic role or a signaling role for antibiotics and further, more rigorous studies are needed. Most of our current knowledge of antimicrobial production comes from studies utilizing highly artificial conditions and unnatural growth media. The organisms are studied under environmental conditions that are non-native, thus we are missing data about true phenotype and expression of genes under the optimal and natural conditions under which they evolved. The *Xenorhabdus* model system is uniquely conducive to investigating the evolution and role of antimicrobials in nature.

Competition for resources and favorable niches is a major driving force of evolution in the microbial world. Weapons employed in this competition include antibiotics, small molecule (ie colicins, pyocins) and phage-derived bacteriocins (13, 14). Acquisition of novel antimicrobial genes can confer a competitive advantage to producer organisms. Production of these agents requires a delicate balancing act of being antagonistic to competitors while

maintaining conditions that are not overly taxing to self and symbiont partners. The *Xenorhabdus* model system is a powerful tool for investigating the role of antimicrobials in host-pathogen and host-symbiont relationships, microbial community dynamics, and the evolution of microbial genomes.

Xenorhabdus species form symbiotic associations with entomopathogenic nematodes of the *Steinernematidae* family (15–17). These associations are non-obligate mutualisms. The symbiotic species can survive independently of one another under laboratory conditions. Cyclical colonization gives rise to the symbiosis. The *Steinernema* nematodes reproduce inside a diverse array of insects with the help of *Xenorhabdus* bacteria. It is in the insect cadaver that the juvenile nematode is colonized by *Xenorhabdus*, which occupies a specialized region of the gut called the receptacle (15, 18). When conditions inside the insect cadaver become limiting, unknown signals cause the juvenile nematodes to form an exterior cuticle, sealing the mouth and anus, resulting in the non-feeding infective juvenile stage. Infective juveniles will seek out new insect hosts to serve as an environment and nutrient source for reproduction. They will enter the new hosts via openings such as the respiratory spiracles, mouth, and anus. The infective juveniles will then migrate to the midgut and perforate the intestine as they enter the hemocoel, creating the opportunity for gut microbiota to be translocated to the hemocoel. Contact with the hemolymph in the hemocoel triggers the degradation of the cuticle and subsequent release of the *Xenorhabdus* bacteria by defecation. In the hemocoel *Xenorhabdus* transitions to its pathogenic phase, suppressing the insect's innate immune response and producing many biologically relevant compounds, including virulence factors (19) and insect toxins (20), which contribute to the rapid death of the host. Following adequate immune suppression the bacteria will reach high cell density and release exoenzymes (21) and antimicrobials (22–25). Exoenzymes such as

lipase and hemolysin bioconvert the carcass into a nutrient-rich food source (21) as antimicrobials are assumed to suppress competitor growth. *Xenorhabdus* bacteria may encounter two major types of competitors in the hemocoel, gut microbiota that was translocated when the nematode perforated the intestine, and other *Xenorhabdus* bacteria in the event of co-invasion of the insect host by more than one species of *Steinernema* nematodes.

1.1. Competitors of *Xenorhabdus*

Xenorhabdus survival depends on the ability to successfully compete for space and resources against diverse bacteria in the hemocoel. *Xenorhabdus* produce a plethora of soluble antimicrobials and phage-derived bacteriocins to theoretically inhibit the growth of bacterial competitors (26). Suppression of competitor growth allows *Xenorhabdus* to reach sufficient cell density to ensure reproductive success of its symbiotic nematode partner, which will carry it on to the next nutrient source. Gut competitors encountered by *Xenorhabdus* in the hemocoel varies with the insect host invaded and include gram-positive and gram-negative bacteria, and fungi (27). In the event of co-invasion by more than one nematode, *Xenorhabdus* may compete with other closely related species or strains (28–30).

There are currently over 20 *Xenorhabdus* and 50 *Steinernema* species that have been characterized. Each *Steinernema* nematode is colonized by only one cognate *Xenorhabdus* species, while a single *Xenorhabdus* species can colonize more than one *Steinernema* species. For example, *X. nematophila* strains colonizes three different *Steinernema* species; *S. carpocapsae*, *S. websteri*, and *S. anatoliense*, representing two separate clades (31). *X. bovienii* strains colonize at least 8 different *Steinernema* species across three clades (31).

Xenorhabdus genomes have evolved to encode a broad collection of diverse compounds with antimicrobial activity (32). Soluble antibiotics are traditionally thought to target species unrelated to producer organisms. The antimicrobials produced by *Xenorhabdus* are predominantly active against unrelated bacteria but have also been shown to inhibit growth of related strains of *Xenorhabdus* and *Photorhabdus* bacteria (32). The contribution of these antibiotics to competition against related strains is unknown. Defining the role of soluble antibiotics and R-type bacteriocins to inter-species and intra-species competition amongst *Xenorhabdus* strains could re-shape prevailing thought on target specificity of antimicrobial weapons.

1.2. *Xenorhabdus* antimicrobials

1.2.1. Bacteriocins

Bacteriocins are generally classified as either soluble proteinaceous antimicrobials produced by nearly all surveyed lineages of prokaryotes or phage-derived structures produced by a limited number of species (13). Bacteriocins have been implicated in intra-specific competition during times of nutrient limitation (13). It has been inferred that there is strong positive selection acting on enteric bacteriocins (13). They have traditionally been thought to target within a species or closely related species (13). The soluble bacteriocins of gram-negative bacteria are large, proteinaceous structures that can be encoded either on plasmids or on the chromosome. Recombination events between existing gram-negative bacteriocins appear to have given rise to new bacteriocins (33–36). The two methods of killing action that have been previously described are pore formation and nuclease activity (34). In contrast to gram-positive

bacteriocins, gram-negative bacteriocins have not evolved bacteriocin-specific regulation and rely on host regulatory networks.

The most well-studied proteinaceous bacteriocins are the colicins produced by *Escherichia coli*. They are encoded on plasmids and include toxin, immunity, and lysis genes. Their domain structure consists of the central domain that recognizes cell surface receptors and constitutes about 50% of the protein, the N-terminal domain whose function is translocation of the protein into the target cell, and the remaining portion of the bacteriocin encodes the killing domain, immunity region, and immunity binding protein (13). Consistent with early ideas about bacteriophage specificity, colicins have a narrow phylogenetic killing range. Colicins are induced under times of stress and are under control of the SOS regulon. They can also be induced using mitomycin C. Hypotheses surrounding the evolution of colicins are grouped around killing modality. Domain shuffling mediated by recombination is responsible for generating new pore-forming colicins (37, 38). The nuclease colicins are thought to evolve due to the action of strong positive selection for novel immunity and killing functions arising from mutation (13). The fitness of the producer strain is increased by the expanded immunity function while the novel colicin is capable of killing the ancestral strain.

Phage-derived bacteriocins encoded by P2-type phage are called R-type bacteriocins. Some R-type bacteriocins have a narrow killing range while some have a broad killing range and have been suggested as a primary mechanism for mediating microbial diversity (39). Mechanisms for mediating microbial diversity were recently described in a study that demonstrated a link between R-type bacteriocin production and spatial structuring (40). In this study, sympatric isolates of *Xenorhabdus bovienii* and *X. koppenhoeferi* from the same core soil sample showed resistance to bacteriocins produced by co-isolates, while being sensitive to

bacteriocins produced by conspecifics isolated just a few meters away (37). This spiteful behavior benefits kin of producer strains, promoting evolution of bacteriocins. The costly act of bacteriocin production persists as it provides opportunity for resistance to emerge, creating a balance maintenance system in well-mixed populations. Bacteriocin production and resistance mechanisms are energetically costly. Producer strains may derive little benefit from bacteriocin production, since bacteriocin action frees nutrients for kin and non-kin alike. Both producer and resistant strains may experience retardation in growth rate and population density compared to sensitive strains that did not invest any resources in resistance. Although sensitive strains may initially decrease in number, resistant strains can outcompete the producer strains, thus allowing sensitive strains, which do not expend energy or resources on resistance, to grow out and gain an advantage. This tripartite interaction creates a rock, paper, scissors model in which the producer strains outcompete sensitive strains, while resistant strains outcompete the taxed producer strains, allowing sensitive strains to reemerge and potentially become the dominant population (38, 41). The result of these complex interactions is the maintenance of existing strains and propagation of new strains.

An R-type bacteriocin that has been well characterized is xenorhabdycin of *X. nematophila*. Xenorhabdycin is produced *in vitro* under non-inducing conditions and can be highly induced by the addition of mitomycin C (42). The R-type bacteriocins produced by *X. nematophila* have a contractile tail sheath, tail tube, baseplate, and tail fibers (39). Tail fibers are likely to confer binding specificity. It has been previously demonstrated that xenorhabdycin is not expressed in strains with insertional disruption of tail fiber or tail sheath genes (14). Recent investigations using these mutants showed that xenorhabdycin is required for interspecies competition (14). Xenorhabdycin preparations displayed variable activity against diverse strains of *Xenorhabdus*

and *Photorhabdus* (14). The killing ability is not correlated with phylogenetic distance. A xenorhabdicolin-deficient strain was unable to outcompete the sensitive *Photorhabdus luminescens* TT01 species in the natural environment of the insect hemocoel (14). To date, this represents the only assessment of the *in vivo* contribution of xenorhabdicolin to competition, and it has only been examined at the genus level. TT01 does not produce bacteriocins or antibiotics that are active against *X. nematophila*, so the outcome of this competition is completely dependent on the xenorhabdicolin produced by *X. nematophila*. Antibiotics are not a dominant force in competition between TT01 and *X. nematophila*. However, it has yet to be examined if antibiotics are a dominating force in competition with other closely related species or strains. Investigations in *X. bovienii* - *S. jolietii* mutants deficient in xenorhabdicolin production revealed that xenorhabdicolin increased the competitive advantage of the producer strain, but competitive ability of the mutant strain was not completely lost when assayed against other *X. bovienii* strains *in vitro* (Forst lab, unpublished data). This was the first time that competition on the strain level had been assessed. Subsequent inquiries into the retention of competitive ability led to a novel discovery – soluble antibiotics can dominate over bacteriocins in certain competitions between strains of *X. bovienii*.

Several questions have yet to be addressed regarding these complex interactions. We are uniquely poised to characterize the contributions of xenorhabdicolin to intraspecies competition utilizing *X. bovienii* strains from *S. feltiae* isolated from different geographic regions, and to interspecies competition using *X. nematophila* and *X. szentirmaii*. *X. nematophila* mutants deficient in xenorhabdicolin production or NRPS-derived antibiotic production are available to elucidate the contributions of xenorhabdicolin versus antibiotics. Understanding the contribution of xenorhabdicolin to intragenus, interspecies, and intraspecies competition can shed light on the contribution of bacteriocins to nematode fitness in spatially structured communities.

Bacteriocins produced by *X. bovienii* and *X. koppenhoeferi* do not target kin, but have activity against other strains located meters away (37). This may be a mechanism for preserving the gene pool of kin (37). Nematodes often travel meters to seek out new insect hosts (43, 28). It is predicted that as distance between the original location increases, relatedness to newly encountered strains decreases, and the likelihood of bacteriocin sensitivity increases (44). Within the range of nematode movement there is genetic diversity and bacteriocin sensitivity amongst strain (44). With this in mind, it is reasonable to hypothesize that there is a selective pressure for the generation of divergent specificity.

The remnant P2-type clusters of *X. nematophila*, *X. bovienii* – *S. jollieti*, and *Photorhabdus luminescence* TT01 have been previously examined (45). It is likely that there was an ancestral acquisition within each genus followed by subsequent separate evolutionary histories (45). Comparison on the species level between *X. nematophila* and *X. bovienii* – *S. jollieti* revealed a high degree of conservation of bacteriocin tail structure genes while main tail fiber genes were significantly divergent (45). In addition to the main tail fiber genes, additional truncated tail fiber genes and transposase genes were found within an insert in the region flanking *funZ* between the baseplate assembly and tail structure genes (14). Insertions in the *funZ* location are common in P2 phage (46). It is possible that the inserted sequences may have been translocated by the transposases encoded within the insert. While divergence of tail fiber genes has been examined at the species level, it remains unexplored at the strain level.

Genomes are now available for nine strains of *X. bovienii* derived from nine different species of *Steinernema* nematodes. Comparative analysis revealed that all nine strains contain an ancestrally acquired remnant P2-type phage cluster. There is a high degree of conservation of the structural genes for making the baseplate, tail sheath, and tail tube, suggesting that the

remnant P2-type clusters are evolving with the chromosome. In contrast, the genes encoding the main tail fiber are highly divergent. While the baseplate assembly and tail structure genes have the same number of amino acids and a high percent identity, the main tail fiber genes differ in the number of amino acids and percent identity of the C-terminal region. Although the C-terminal region is highly divergent, there is a high degree of conservation of the N-terminal region. Interestingly, in most of the strains there has been an insertional event between the baseplate and tail structure genes. Tail fiber fragment and transposase genes constitute the bulk of these insertions. Previous work in *X. bovienii* – *S. jollieti* has revealed that at least one of these tail fiber fragment genes is being expressed (Forst lab, unpublished data). Previous work has also shown that the specificity of xenorhabdicolin produced by *X. nematophila* is very different from the specificity of the xenorhabdicolin produced by *X. bovienii* – *S. jollieti*, while the resolvable structures and expression patterns of key genes in the remnant clusters are homologous (2, 16, Forst lab unpublished data). Based upon this data, it is reasonable to hypothesize that specificity divergence is related to tail fiber divergence.

If specificity is determined by tail fibers, the C-terminal sequence could be determining the evolutionary trajectory of the *X. bovienii* strains. Provided that specificity dictates the ability to compete, it will also determine niche colonization of the nematode. Colonization leads to geographic isolation and subsequent speciation. By examining the relationship between tail fiber sequence, specificity, and competitive success, we could identify gears that are driving the evolutionary machinery of the diverse *X. bovienii* strains.

1.2.2. *Xenorhabdus* antibiotics

As much as 7.5% of the *X. nematophila* genome is dedicated to the production of secondary metabolites including antibiotics (47). *X. nematophila* produces indole-derived nematophins (48), antibacterial peptides (49), and the benzopyran xenocoumacin (22–24, 50). Secondary metabolite antibiotics are synthesized by non-ribosomal peptide synthetases (NRPS) and polyketide synthetases (PKS). NRPSs and PKSs have several required and optional domains that function in tandem to assemble products. An NRPS/PKS hybrid cluster that includes two NRPS genes and three PKS genes produces xenocoumacin (51). The *X. nematophila* genome encodes seven NRPS and NRPS/PKS gene clusters (including the xenocoumacin cluster) and six individual NRPS genes. It is possible that one or more of these gene clusters encodes a secondary metabolite (compounds F and C) that could be responsible for the residual antibiotic activity displayed by mutants deficient in xenocoumacin production (52). Inactivation of the *ngrA* gene, which encodes a 4'phosphopantetheinyl required for active NRPS and PKS enzymes (53), eliminated NRPS/PKS secondary metabolite production in *X. nematophila*. The *ngrA* mutant is useful in assessing competition in the absence of NRPS/PKS derived antibiotics. A previous study demonstrated that wild-type *X. nematophila* eliminated the sensitive gut competitor *S. saprophyticus* when co-injected into *Manduca sexta* larvae, and in co-culture in LB broth (27). In broth culture, *S. saprophyticus* persisted when co-cultured with the *ngrA* mutant. When *S. saprophyticus* was co-injected into *M. sexta* with the *ngrA* mutant, *S. saprophyticus* was eliminated (27). *X. nematophila* may produce *ngrA*-independent antimicrobial compounds that were responsible for the activity against *S. saprophyticus*. These findings raise the question of whether *X. nematophila* produces inhibitory levels of *ngrA*-dependent antibiotics during *in vivo*

growth. Previous studies have suggested that secondary metabolites that displayed inhibitory activity at higher concentrations in *in vitro* assays may be produced at sub-inhibitory levels when the bacteria grow in their natural environment (12). In this regard, *X. szentirmaii*, a species that produces exceptionally high levels of antibiotic activity *in vitro* (32), was chosen to address this question.

The *X. szentirmaii* draft genome sequence is now available (54). Consistent with other sequenced *Xenorhabdus* genomes, there has been extensive expansion of NRPS genes and several candidate clusters may produce antibiotics. Thus far natural products isolated from *X. szentirmaii* broth cultures include xenofuranones A and B which display no antibacterial or antifungal activity (55), and szentiamide, which also displayed no antibacterial or antifungal activity but interestingly displayed activity against protists such as *Plasmodium falciparum* and against insect cells (56). In antibiotic overlay assays and cell-free supernatant assays *X. szentirmaii* displayed strong inhibitory activity against diverse *Xenorhabdus* species as well as competitors isolated from the gut of *M. sexta* (Forst lab, unpublished data). The compounds and gene clusters responsible for inhibitory activity against microbial competitors have yet to be identified. Given the high level of antibiotic production of *X. szentirmaii* and its ability to inhibit related competitors, it is the ideal bacterium for investigating the role of antibiotics under natural competitive conditions.

1.3. Dissertation objectives

In this study, we explore the role of R-type bacteriocins and NRPS-derived antibiotics in inter-species and intra-species competition across diverse *Xenorhabdus* strains. We define the relationship between R-type bacteriocin tail fiber sequence and specificity of strains of *X.*

bovieni to determine if killing ability is correlated to phylogenetic distance and if xenorhabdicens are being used as weapons in intra-species competition. Further, we demonstrate for the first time that antibiotics are produced *in vivo* at inhibitory concentrations and are required in a competition between two closely related species.

The first goal of this study is to characterize the genetic diversity of R-type bacteriocin tail fiber sequence in strains of the same species of *X. bovienii* and to assess if there is a correlation between sequence similarity and target specificity. Previous work had shown that the xenorhabdicens produced by two different species, *X. nematophila* and *X. bovienii* – *S. jollieti*, differed markedly in target specificity (45, Forst lab unpublished data). Whether xenorhabdicens from different strains of the same species exhibit diversity in target specificity had not been previously examined. To analyze the relationship between tail fibers and specificity, interrelatedness of tail fiber phylogenetic distance and specificity range of xenorhabdicens from select strains was assessed. The sequenced genomes of the *X. bovienii* strains from *S. oregonense*, *S. puntauvense*, *S. intermedium*, *S. jollieti*, *S. feltiae-Florida*, *S. feltiae-France*, *S. feltiae-moldova*, *S. krauseii-Quebec*, and *S. krauseii-Becker Underwood* are now available. Comparative genomic analysis of phage clusters across *X. bovienii* strains and *X. nematophila* as a comparative reference species was conducted and P2-like remnant phage clusters were identified. Conservation and divergence of major structural proteins of tail fiber and truncated tail fiber sequences were assessed. Select strains of *X. bovienii* were screened for bacteriocin production under induced and uninduced *in vitro* conditions. Bacteriocin specificity and activity against closely related strains was defined using PEG-precipitated bacteriocin preps. The role of bacteriocins in competition amongst strains of the same species isolated from the same nematode was assessed using *in vitro* assays.

The second aim of this study was to determine if NRPS-derived antibiotics are produced *in vivo* at inhibitory concentrations and function as a weapon targeting related and non-related natural competitors. Mutants deficient in NRPS-derived antibiotics (*ngrA*) and the R-type bacteriocin xenorhabdycin (*xspS*) were created to assess the contribution of each antimicrobial to inhibition of biologically relevant competitors under *in vitro* and *in vivo* conditions. Competitors displayed sensitivity to both xenorhabdycin and NRPS-derived antibiotics, confirmed by mutant analysis. *In vivo* competitions in *M. sexta* revealed that NRPS-derived antimicrobials are produced at sufficient concentrations to inhibit a competitor, and the outcome of the competition is determined by *X. szentirmaii* antibiotics. To our knowledge, this is the first study to directly demonstrate that antibiotics determine the outcome of interspecies competition in a natural host environment. MALDI-TOF mass spectrometry confirmed the presence of two compounds in the hemolymph of wild-type infected *M. sexta* that were absent in the hemolymph of *ngrA*-infected *M. sexta* supporting the hypothesis that *X. szentirmaii* antibiotics are produced under natural, *in vivo* conditions.

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Chapter Two

R-type bacteriocins in related strains of *Xenorhabdus bovienii*:

Xenorhabdycin tail fiber modularity and contribution to competitiveness

This chapter is a modified version of the published paper:

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2.0 Introduction

Bacteria employ several strategies to compete for resources and favorable niches. Interference competition is a direct antagonistic interaction in which organisms produce compounds to directly inhibit or kill competitors (1). Antagonistic compounds include small antibiotics, proteinaceous molecules such as colicins, and phage-derived bacteriocins (2–4). Several lines of evidence have demonstrated that bacteriophages and phage-derived particles can determine bacterial community structure and are major drivers of diversity of bacterial populations (5, 6). Compared with antibiotics and proteinaceous colicins, the role of phage-derived bacteriocins in competitive interactions has been less well studied. R-type bacteriocins are contractile phage-tail-like structures related to P2 phage encoded in remnant prophage clusters composed of tail sheath, tube, baseplate and fiber genes (7). The conserved N-terminal region of the tail fiber interacts with baseplate proteins while the divergent C-terminal end determines target-binding specificity (7). While antibiotics generally have a broader spectrum of activity, R-type bacteriocins are effective against more closely related species and strains.

Generation of tail fiber diversity in R-type bacteriocins has been demonstrated in *Erwinia carotovora* (carotovoricin) in which a DNA region encoding the C-terminal fiber module located adjacent to the main fiber gene inverts to create two distinct tail fibers (8). DNA inversion of tail fiber genes also occurs in *Photorhabdus luminescens*, the sister taxon of *Xenorhabdus* spp. (9). Recent investigation of the tail fibers of R-type bacteriocins of the mutualistic bacteria *X. nematophila* and *X. bovienii* suggested that modules encoding fragments of tail fibers were exchanged between the respective species (10).

Xenorhabdus species form mutualistic associations with soil-dwelling entomopathogenic nematodes of the *Steinernematidae* family (11, 12). Colonized nematodes invade an insect host

via natural openings and enter the hemocoel where they release *Xenorhabdus* into the insect hemolymph. The bacteria suppress the insect's innate immune response and produce antibiotics and colicin (xenocin) that inhibit the growth of sensitive gut-derived bacteria that enter the hemocoel during nematode invasion (13–16). Insect hosts may also be co-infected with more than one species of entomopathogenic nematode (17, 18). We showed previously that *in vivo* reproduction of aposymbiotic *Steinernema carpocapsae* (not colonized with its cognate symbiont, *X. nematophila*) was inhibited by a bacterial non-cognate symbiont isolated from another entomopathogenic nematode (4). The non-cognate competitor was eliminated and nematode reproduction was restored when wild-type *X. nematophila* was co-injected into the host. A mutant strain in which the tail sheath gene (*xnpS1*) of the remnant P2 phage cluster (*xnpI*) was inactivated did not restore nematode reproduction. *X. nematophila* also possesses an intact P2 phage cluster of *X. nematophila* that is neither expressed nor involved in interspecies competition. Similarly, a remnant P2 cluster (*xbpI*) in the *X. bovienii* strain isolated from *Steinernema jolietii* was expressed and required for xenorhabdicin production (10). These results suggest that xenorhabdicins are involved in the defensive mutualistic association between *Steinernema* nematodes and their *Xenorhabdus* symbionts.

Since strains of the same species are likely to occupy overlapping ecological niches, competition between strains is predicted to occur on a biological scale (6). Xenorhabdicin producer and non-producer isolates of *X. bovienii* were recently obtained from naturally coexisting *Steinernema* nematodes (19). Xenorhabdicin activity was implicated in conferring a competitive *in vivo* growth advantage to the producer cells but not the non-producer cells co-injected with a sensitive competitor. Recent genome sequencing of nine strains of *X. bovienii* isolated from six different species of *Steinernema* nematodes established genetic diversity at the

strain level (20). Further, in experiments using these strains an inverse correlation was noted between fitness of a *S. feltiae*-*X. bovienii* complex and phylogenetic distance of the test *X. bovienii* strain from the native symbiont of the nematode. Overall these data suggest that diversity at the strain level may underlie specific interactions with distinct nematodes to help maintain symbiotic associations (21). Since xenorhabdicens may provide fitness benefits to producer strains we examined remnant P2-like phage clusters and the C-terminal region of tail fibers in draft genomic sequences of nine *X. bovienii* strains.

2.1. Materials and methods

2.1.1. Bacterial strains and growth conditions

Strains used in this study are listed in Table S1. All strains were grown in lysogeny broth (LB) at 30°C. Green fluorescent protein (GFP)-producing *X. bovienii* strains were constructed as previously published (22, 23). Using the plasmid mini-Tn7-KSGFP (24), the GFP-encoding gene was introduced into the *attTn7* site of strains Xb-Sf-FL, Xb-Sf-FR, and Xb-Sf-MD to produce GFP-producing strains.

2.1.2. P2-type prophage bioinformatics analysis

The xenorhabdycin-encoding P2-type prophage clusters were identified using the BlastP algorithm in the MaGe MicroScope platform. Genes were annotated according to the results of the BlastP algorithms. The EMBL Clustal Omega and NCBI Align Sequences Protein Blast algorithms were used to identify conserved tail fiber sequences and calculate percent identity.

Table S1. Bacterial strains used in this study.

Strain	<i>Steinernema</i> nematode host	Geographic location	Source	Genome Accession Number
<i>X. nematophila</i> 19061 Xn-Sc	<i>S. carpocapsae</i>	USA	Laboratory stock	[EMBL:PRJNA13400] FN887742*
<i>X. nematophila</i> 19061:: Δ xnpS1	<i>S. carpocapsae</i>	USA	Laboratory stock	
<i>X. bovienii</i> Xb-Sf-FL	<i>S. feltiae</i>	Florida, USA	P. Stock	[EMBL:PRJEB4320]*
<i>X. bovienii</i> Xb-Sf-FR	<i>S. feltiae</i>	France	P. Stock	[EMBL:PRJEB4319]*
<i>X. bovienii</i> Xb-Sf-MD	<i>S. feltiae</i>	Moldova	P. Stock	[EMBL:PRJEB4321]
<i>X. bovienii</i> Xb-Si	<i>S. intermedium</i>	North Carolina, USA	P. Stock	[EMBL:PRJEB4327]*
<i>X. bovienii</i> Xb-Sj	<i>S. jollieti</i>	Monsanto		[EMBL:PRJEB4326]*
<i>X. bovienii</i> Xb-Sj-2000	<i>S. jollieti</i>	Monsanto		[EMBL:FN667741]
<i>X. bovienii</i> Xb-Sk-BU	<i>S. kraussei</i>	BeckerUnderwood	P. Stock	[EMBL:PRJEB4325]
<i>X. bovienii</i> Xb-Sk-CA	<i>S. kraussei</i>	Quebec, CA	P. Stock	[EMBL:PRJEB4324]*
<i>X. bovienii</i> Xb-So	<i>S. oregonense</i>	Oregon, USA	P. Stock	[EMBL:PRJEB4323]*
<i>X. bovienii</i> Xb-Sp	<i>S. puntauvense</i>	Costa Rica	P. Stock	[EMBL:PRJEB4322]*
<i>X. bovienii</i> Xb-Sf-FL-GFP	<i>S. feltiae</i>	Florida, USA	H. Goodrich - Blair	
<i>X. bovienii</i> Xb-Sf-FR-GFP	<i>S. feltiae</i>	France	H. Goodrich - Blair	
<i>X. bovienii</i> Xb-Sf-MD-GFP	<i>S. feltiae</i>	Moldova	H. Goodrich - Blair	

* From Murfin *et al.*, 2015a

2.1.3. Bacteriocin-enriched preparations

LB (100 mL) subcultures inoculated with a 50-fold dilution of overnight cultures were grown to an $OD_{600} = 0.3-0.4$ and induced with 5 $\mu\text{g/mL}$ mitomycin C for 15 hours. RNase A and DNase I were added to a final concentration of 1 $\mu\text{g/mL}$ each and cultures were incubated at 37° for 30 minutes. Cellular debris was removed by centrifugation and supernatants were sterile filtered. Bacteriocin-enriched fractions were obtained by precipitation at 4°C for 2 hours with polyethylene glycol 8000 (10% w/v final concentration) and NaCl (1M final concentration). Bacteriocin-enriched fractions were harvested by centrifugation at R_{max} of 13,776 x g at 4°C for 15 minutes, resuspended in 3 ml LB, and centrifuged again for 5 minutes. Supernatants containing xenorhabdicens were sterile filtered and stored at 4°C. For protein analysis of bacteriocin-enriched fractions 300 μL aliquots were ultracentrifuged at R_{max} of 287,582 x g at 4°C for 15 minutes, pellets were resuspended in 40 μL of Laemmli buffer and 3-6 μL were loaded on a 8-16% PAGE gel (Genscript). For transmission electron microscopy ultracentrifuged pellets were resuspended in 50 mM Tris-HCl (pH 8.7). 5 μL was placed on grids and negatively stained with 0.8% phosphotungstate for 2 minutes. The samples were examined using a Hitachi H-600 transmission electron microscope operated at 75 kV.

2.1.4 Microplate assay of xenorhabdicin activity

Subcultures (250 μL of overnight culture added to 5 mL LB) were grown to an $OD_{600} = 0.5 - 0.6$. Assays contained 100 μL of diluted subculture (1200-fold) and 50 μL of xenorhabdicin-enriched preparations in a 96 well plate and incubated for 24 hours. Percent inhibition was calculated by comparing OD_{600} of control cultures versus cultures treated with xenorhabdicin-enriched fractions.

2.1.5. *In vitro* competition assays

Subcultures of wild-type and GFP-labeled strains (250 μ L of overnight culture added to 5 mL of LB) were grown to mid-exponential phase. Cultures were normalized to an $OD_{600} = 0.25$ and 250 μ L of normalized cells were used to inoculate 5 ml of LB broth for control and competition cultures. Equal volumes (125 μ L) of each competitor were used in competitions. Colony forming units per mL at 0 and 24 hours were determined by dilution in Grace's insect medium and plating on LB agar. Competition experiments for both marked and unmarked strains were performed three times. Statistical significance was calculated by unpaired *t*-tests using GraphPad QuickCalcs.

2.2. Results and Discussion

2.2.1. Remnant P2-like prophage clusters in *X. bovienii* strains

While xenorhabdicolin tail fibers of different species have been analyzed previously (10) comparison of tail fibers from different strains of the same species had not been examined. Here we analyze remnant P2-like prophage clusters in the draft genomes of nine strains of *X. bovienii* derived from 6 different *Steinernema* nematodes (Table S1). The organization of the primary structural genes between gene X and the T1 tube gene is identical in all strains (Fig. 1). The respective baseplate (V-I), sheath (S1) and tube (T1) proteins are highly conserved sharing >98% amino acid sequence identity (Fig 1, Tables S2 and S3). Other prophage proteins such as endopeptidase (*enp*) share approximately 90% amino acid identity. CI regulatory genes are present upstream of gene X in all of the P2-like prophage clusters (not shown). Additional conserved phage genes (purple arrows) and hypothetical genes (white arrows) are present between *xbpH1* and *xbpS1* (H1-S1 region). Finally, the sequences of the remnant P2-type

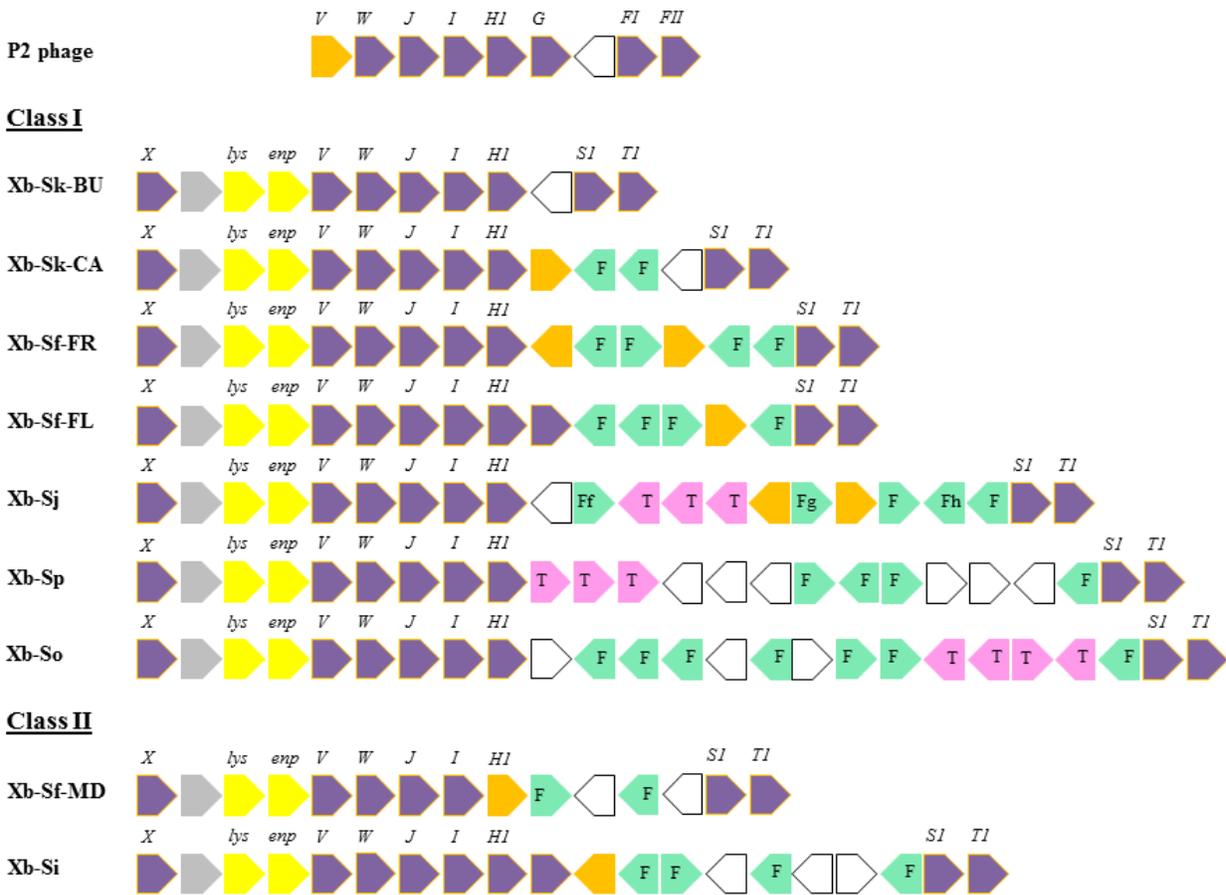


Figure 1. Remnant P2-type prophage of *X. bovienii* strains. Open reading frames are classified by color: tail synthesis structural proteins (purple), highly conserved unknown proteins (gray), lysis genes (yellow), truncated tail fibers (light green), transposases (pink), and other uncharacterized prophage encoded ORFs (white). The remnant prophage clusters were divided into two classes based on difference of the respective H1 tail fibers. A 210 amino acid sequence was absent in Class I fibers and was present in Class II fibers (see Fig 3). Open reading frames are not drawn to scale. P2-type prophage clusters were identified using XbpS1 of *X. bovienii* SS2004 as the query sequence and the MaGe platform (www.genoscope.cns.fr/agc/microscope/home/index.php).

Table S2. Percent identity of xenorhabdycin-encoding proteins as compared to Xb-Sj.

	<u>X</u>	<u>unk</u>	<u>lys</u>	<u>enp</u>	<u>V</u>	<u>W</u>	<u>J</u>	<u>I</u>	<u>S1</u>	<u>T1</u>
<u>Class I</u>										
Xb-Sj	100	100	100	100	100	100	100	100	100	100
Xb-Sf-FL	92.5	97.8	96.1	90.5	99.1	99.1	98.7	99.5	99.5	100
Xb-Sf-FR	92.5	97.8	96.1	90.5	99.1	99.1	98.7	99.5	99.5	100
Xb-So	98.5	97.8	93.9	90.4	97.2	99.1	99.0	99.5	99.7	100
Xb-Sk-CA	94.0	87.9	96.1	90.4	99.5	99.1	99.7	99.0	99.7	100
Xb-Sp	92.5	95.6	96.1	90.5	99.5	99.1	98.3	99.5	99.7	100
Xb-Sk-BU	92.5	97.8	96.7	89.9	99.1	98.3	99.0	99.0	99.5	99.4
<u>Class II</u>										
Xb-Si	97.0	98.9	95.5	89.9	99.1	99.1	99.3	100	99.7	100
Xb-Sf-MD	92.5	96.7	96.1	90.5	99.1	99.1	98.7	99.5	99.7	100
Xn-Sc	88.1	80.2	-	77.4	95.3	91.3	94.0	89.1	93.6	97.7

Table S3. Number of amino acid residues present in xenorhabdycin-encoding genes.

	<u>X</u>	<u>unk</u>	<u>lys</u>	<u>enp</u>	<u>V</u>	<u>W</u>	<u>J</u>	<u>I</u>	<u>S1</u>	<u>T1</u>
<u>Class I</u>										
Xb-Sj	68	92	180	169	212	116	303	203	391	173
Xb-Sf-FL	68	92	180	167	212	117	303	203	391	173
Xb-Sf-FR	68	92	180	167	212	117	303	203	391	173
Xb-So	68	92	180	167	212	117	303	203	391	173
Xb-Sk- CA	68	92	180	167	212	117	303	203	391	173
Xb-Sp	68	92	180	173	212	117	303	203	391	173
Xb-Sk- BU	68	92	180	167	212	117	303	203	391	173
<u>Class II</u>										
Xb-Si	68	92	180	173	212	117	303	203	391	173
Xb-Sf- MD	68	92	180	167	212	117	303	203	391	173
Xn-Sc	68	88	-	179	212	116	303	203	391	173

prophage of the Xb-Sj draft genome and the complete Xb-Sj-2000 (25) genome were identical providing evidence that the P2-type prophages of the nine draft genomes were accurately sequenced.

The H1-S1 regions in all strains except Xb-Sk-BU contain a variable number of genes that encode truncated tail fiber fragments (F) that share similarity with various regions of tail fiber genes present in the remnant P2-like prophages of *X. bovienii* strains. Transposon genes (T) are present in several of the H1-S1 regions suggesting that the accumulation of fiber fragments may occur via lateral transfer by transposition.

2.2.2. Comparison of the tail fiber fragments in the *xbpH1-xbpS1* region

To assess the generation of tail fiber diversity in different strains of *X. bovienii* we analyzed the H1-S1 region of three *X. bovienii* strains obtained from *Steinernema feltiae* nematodes isolated from different geographic locations (Fig 2). The Xb-Sf-FR and Xb-Sf-FL strains and their respective nematode partners belong to the same clades while the Xb-Sf-MD strain is more closely related to *X. bovienii* from *S. puntauvene* (21). We showed previously that R-type bacteriocins were induced by mitomycin C in the Xb-Sf-FL strain (10).

Fig 2 shows that genes 28 and 29 in Xb-Sf-FL are identical to genes 10 and 11 in Xb-Sf-FR and genes 25, 26 and 27 of Xb-Sf-FL are identical to genes 8, 7 and 6 of Xb-Sf-FR. In contrast, gene 30 in Xb-Sf-FL was not similar to any of the genes in the H1-S1 region of Xb-Sf-FR while the sequence between amino acids 15-357 of gene 30 was 100% identical to the C-terminal 341 amino acids of the tail fiber of Xb-Sf-FR (gene 12). Similarly, gene 9 in Xb-Sf-FR is 100% identical to the C-terminal 371 amino acids of XbpH1 of Xb-Sf-FL. These findings suggest that divergence of the tail fibers of these two strains may have been generated by

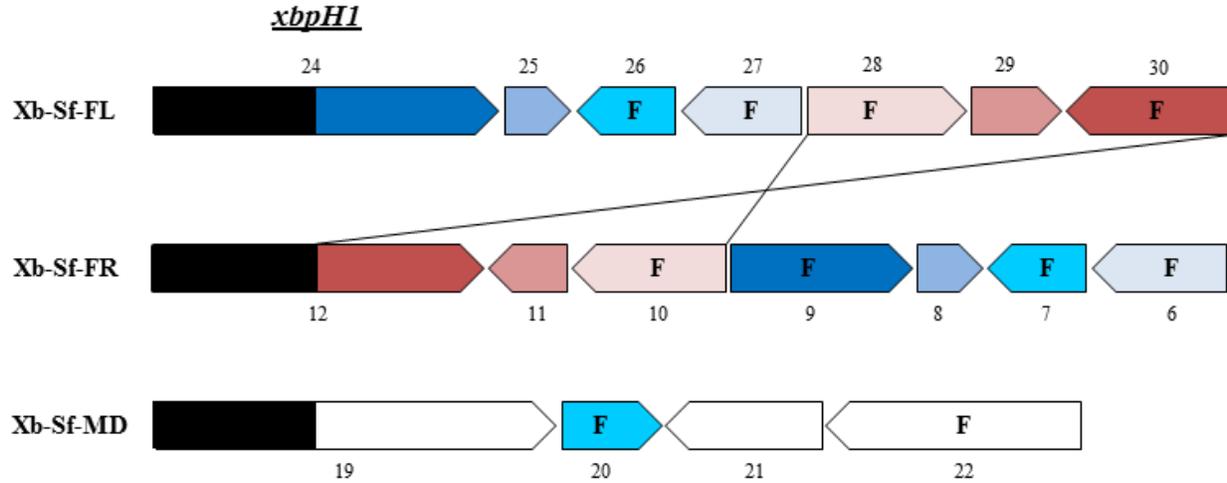


Figure 2. Mosaic modularity of XbpH1 tail fibers of three Xb-Sf strains. The *xbpH1-xbpS1* intergenic regions of Xb-Sf-FR, Xb-Sf-FL, and Xb-Sf-MD are shown. Numbers associated with each open reading frame represent gene labels derived from the respective draft genomes: Xb-Sf-FR (310006-3100012); Xb-Sf-FL (2380124-2380130); Xb-Sf-MD (1680019-1680022). Colors of the open reading frame indicate modules containing identical amino acid sequences. translocation and inversion of genes 28-30 of Xb-SF-FL such that the tail fragment gene 30 replaced the C-terminal region of the tail fiber (gene 24) producing the tail fiber of Xb-Sf-FR (gene 12).

translocation and inversion of genes 28-30 of Xb-Sf-FL such that the tail fragment gene 30 replaced the C-terminal region of the tail fiber (gene 24) producing the tail fiber of Xb-Sf-FR (gene 12).

The H1-S1 region in Xb-Sf-MD contains three genes, one of which (gene 20) is 79% identical to both gene 7 in Xb-Sf-FR and gene 26 in Xb-Sf-FL. The C-terminal region of XbpH1 in Xb-Sf-MD is unique and is not similar to the fiber fragments in either Xb-Sf-FR or Xb-Sf-FL. Thus, the H1-S1 cassette of Xb-Sf-MD was likely acquired independently and is consistent with the more distant phylogenetic relationship of Xb-Sf-MD relative to the Xb-Sf-FL and Xb-Sf-FR strains.

2.2.3. Mosaic organization of submodules in the XbpH1 tail fibers

The length of the H1 tail fibers in the nine strains is highly variable ranging from 485 (Xb-Sf-BU) to 922 (Xb-Si) amino acids (Fig 3). Six of the tail fibers contain unique sequences (white) not found in other tail fibers. Comparison of the amino acid sequence of the XbpH1 tail fibers revealed that the N-terminal 310 amino acids (black) are identical while the C-terminal regions consist of several different submodules and are highly divergent (Fig 3). A conserved 13 amino acid sequence (orange box) is located at the junction of the N-terminal domain and the C-terminal modules in all *X. bovienii* tail fibers suggesting it is involved in recombination events that generate diversity in the C-terminal domain. Extensive submodule exchange is apparent in the C-terminal domain. For example, the submodule between amino acids 401 and 627 of XbpH1 of Xb-Sf-FL (purple) is also present in the C-terminal region of Xb-Sj (Fig 3, Table S4). Likewise, a 123-residue submodule at the C-terminus of the tail fiber (brown) is also present in the tail fibers of Xb-Sk-CA and Xb-Si (Fig 3, Table S4). Similarly, the C-terminus of XbpH1 of

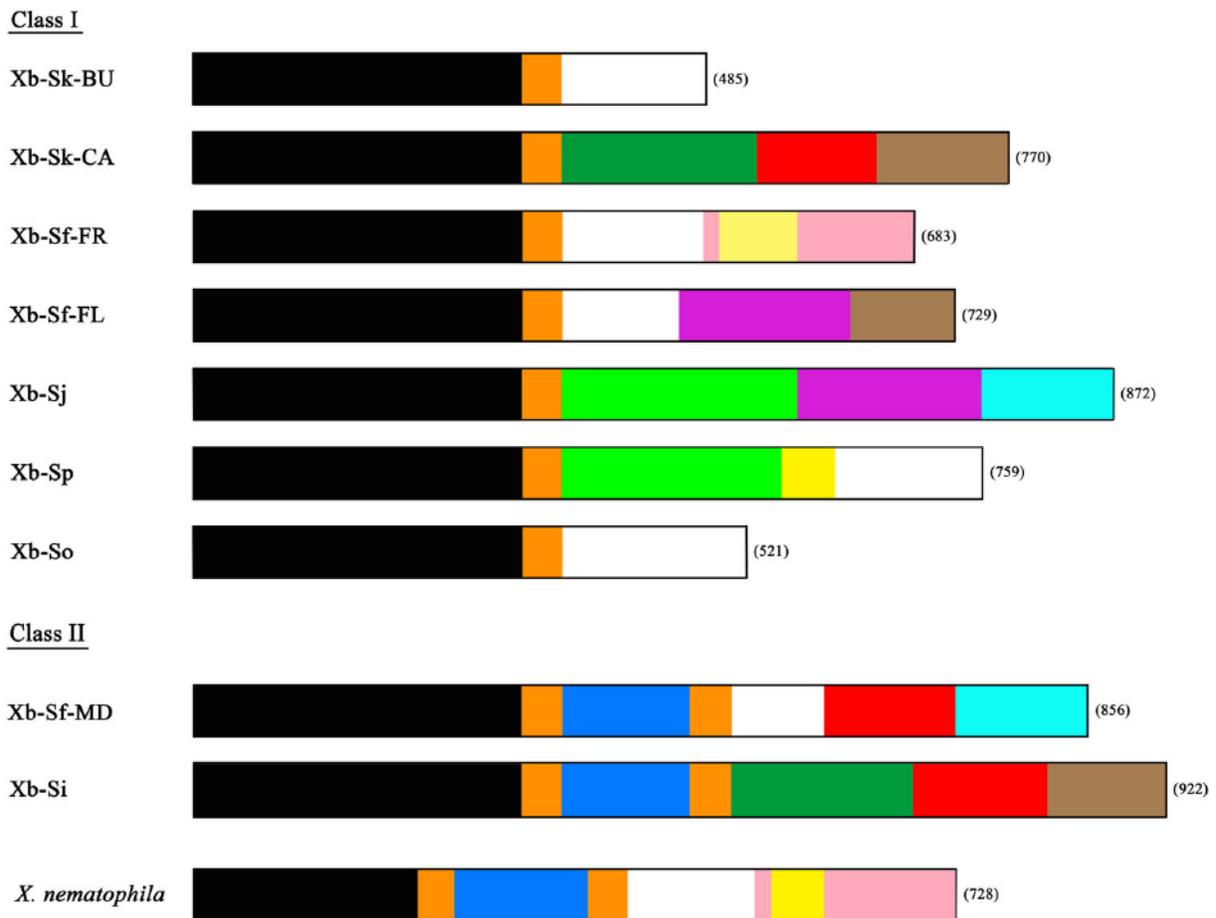


Figure 3. Submodules of the H1 tail fibers in the nine *X. bovienii* strains. Highly conserved submodules found in more than one strain are indicated by identical colors. All *X. bovienii* strains and *X. nematophila* 19061 share a conserved N-terminal region (black) followed by a linker region (orange) of variable size. Class II and *X. nematophila* fibers share an extended conserved N-terminal region (blue). Unique regions of tail fibers are shown in white. The various submodules and unique regions are drawn to scale. **S4.** Number of amino acids and percent identity of H1 tail fiber sequence modules as compared to *X. bovienii* – *S. feltiae* strains.

Table S4. Number of amino acids and percent identity of H1 tail fiber sequence modules as compared to *X. bovienii* – *S. feltiae* strains.

	<u>Xb-Sf-FR</u>			
	<u>Pink</u>		<u>Yellow</u>	
	<u>% identity</u>	<u>Amino acids</u>	<u>% identity</u>	<u>Amino acids</u>
Xn-Sc	78	193	83	53

	<u>Xb-Sf-FL</u>			
	<u>Purple</u>		<u>Brown</u>	
	<u>% identity</u>	<u>Amino acids</u>	<u>% identity</u>	<u>Amino acids</u>
Xb-Sj	74	167		
Sb-Sk-CA			82	123
Xb-Si			82	123

	<u>Xb-Sf-MD</u>			
	<u>Red</u>		<u>Light blue</u>	
	<u>% identity</u>	<u>Amino acids</u>	<u>% identity</u>	<u>Amino acids</u>
Xb-Sk-CA	68	118		
Xb-Si	66	121		
Xb-Sj			54	150

Xb-Sf-FR consists of a submodule of 193 amino acids (pink) into which a 53-residue submodule (yellow) has been inserted (Fig 3). These submodules are also present in the C-terminal region of XnpH1 of *X. nematophila* (Fig 3, Table S4). The XnpH1 fiber possesses an approximately 210 amino acid submodule (blue) that is attached to the conserved N-terminal domain (black). This submodule is also present in the fibers of Xb-Sf-MD and Xb-Si (Class II fibers). The conserved 13-amino-acid sequence (orange) identified in Class I fibers (see above) is also present at the junction of the N-terminal domain (black) and 210-amino-acid submodule (blue) in Class II fibers. Finally, the tail fiber of Xb-Sf-MD possesses a 120 amino acid submodule (red) also present in the fibers of Xb-Sk-CA and Xb-Sj and a 150 amino acid submodule (light blue) found in the fiber of Xb-Sj (Table S4). Thus, the diversity of tail fiber sequence of *X. bovienii* strains isolated from distinct *S. feltiae* nematodes is apparently the result of extensive DNA transfer that generated unique mosaic organizations of submodules. These findings are reminiscent of gene mosaics of tail fibers of DNA bacteriophage in which horizontal gene transfer and DNA inversion were responsible for exchange of host-range determinants (26). Another example of variation of C-termini domains attached to conserved core N-terminal domains is evidenced in the Rhs proteins encoded in enterobacterial genomes. Homologous recombination of existing C-termini domains with other alternative domains carried on episomes was proposed as the mechanism driving C-termini variation (27).

2.2.4. Analysis of xenorhabdicin activity

The competitive outcome between *X. bovienii* strains is correlated with the relative activity and level of production of the xenorhabdicins of the respective strains (19). Since the activity of xenorhabdicins of different isolates of the same strain of *Xenorhabdus* has not been

previously analyzed we assessed the relative activity of xenorhabdicens from the three isolates of Xb-Sf. To this aim phage tail-enriched fractions were prepared from cell cultures induced with mitomycin C. The optical density of cultures 15 h after addition of mitomycin C was markedly lower than uninduced cultures indicating that cells had lysed due to prophage induction (unpublished data). The same amount of phage tail sheath protein (XbpS1) was present in the phage tail-enriched fractions of Xb-SF-FL (Fig 4, lane 3) and Xb-Sf-FR (lane 5). The amount of XbpS1 was similar in the phage tail-enriched fraction of Xb-Sf-MD (lane 7) though it migrated to a lower apparent molecular weight. XbpS1 was barely detectable in the uninduced preparations (lanes 4, 6, 8). Analysis of the phage tail-enriched fractions by transmission electron microscopy confirmed that R-type bacteriocins were present in the induced phage tail-enriched preparations of Xb-Sf-FL and Xb-Sf-FR while phage tail-enriched preparations Xb-Sf-MD appeared to have fewer typical R-type bacteriocins and more than one type of phage tail structure (Fig S1). Together, these findings indicate that R-type bacteriocins were induced by mitomycin C in the Xb-Sf strains.

We tested the growth inhibiting activity of each xenorhabdicin preparation against the two other strains (Table 1). Each xenorhabdicin displayed a different pattern of activity consistent with the high degree of variability of the C-terminal tail fiber regions. Xb-Sf-FR xenorhabdicin was highly active against Xb-Sf-MD that belongs to a different clade than Xb-Sf-FR and Xb-Sf-FL (21), but was inactive against the more closely related Xb-Sf-FL strain. The Xb-Sf-MD xenorhabdicin displayed the same moderate activity against both Xb-Sf-FR and Xb-Sf-FL. Finally, xenorhabdicin from Xb-Sf-FL was inactive against the other Xb-Sf strains.

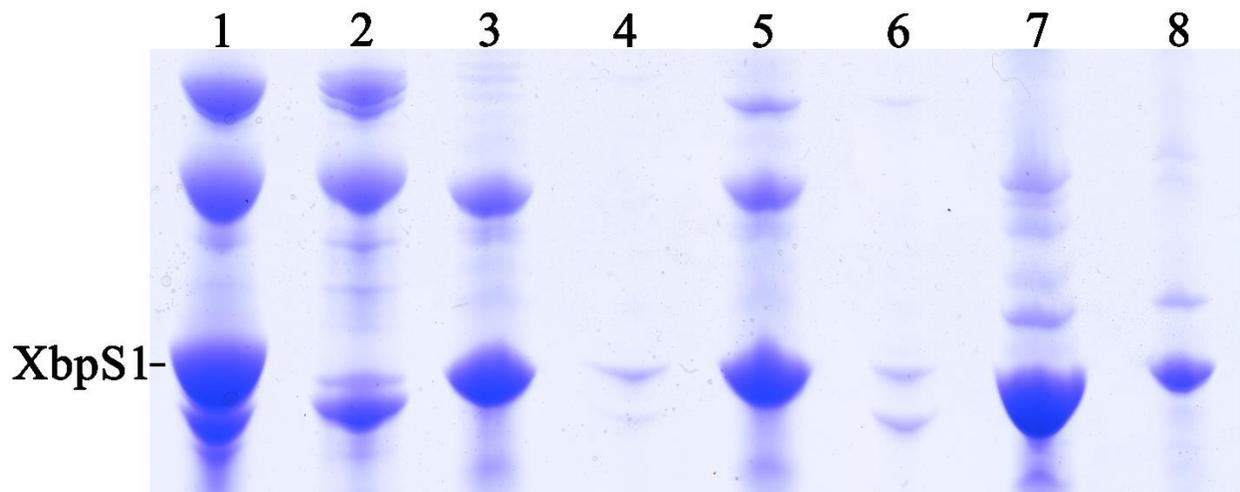


Figure 4. Analysis of tail sheath (XbpS1) levels in bacteriocin-enriched fractions of Xb-Sf isolates. Bacteriocin preparations from mitomycin C-induced (lanes 3, 5, 7) and uninduced (lanes 4, 6, 8) cultures were applied onto a 8%-16% PAGE gel. A xenorhabdicin fraction from wild-type *X. nematophila* (lane 1) and an *xnpS1*-mutant strain (lane 2) was used to mark the position of XbpS1 (43.5 kDa). Lanes 3 and 4, Xb-Sf-FL; lanes 5 and 6, Xb-Sf-FR; lanes 7 and 8, Xb-Sf-MD.

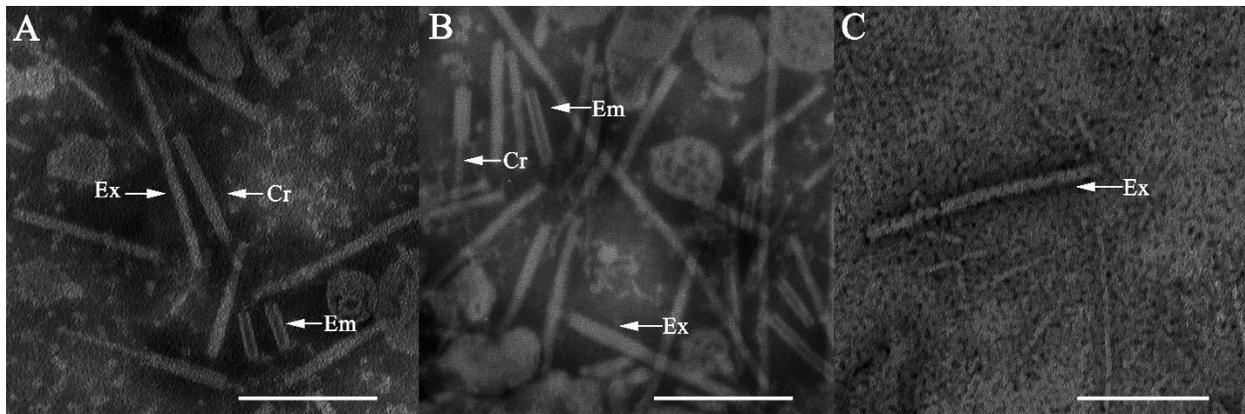


Figure S1. Transmission electron microscopic analysis of bacteriocin-enriched fractions of Xb-Sf isolates. Fractions were prepared as described in Methods. Panel A. Xb-Sf-FL. Panel B. Xb-Sf-FR. Panel C. Xb-Sf-MD. Arrows point to different R-type bacteriocin structures; extended sheath (Ex), contracted sheath (Cr), and empty sheath (Em). The number of bacteriocin particles per micrograph is representative of the relative concentration produced by each strain. Size bars represent 200 nm.

Table 1. Inhibitory activity of xenorhabdicolin from Xb-Sf strains.

<u>Target strain</u>	Xenorhabdicolin producer		
	Xb-Sf-FR	Xb-Sf-FL	Xb-Sf-MD
Xb-Sf-FR	-	3 (1.9)	39 (3.4)
Xb-Sf-FL	0 (0.3)	-	39 (4.4)
Xb-Sf-MD	89 (2.1)	3 (2.4)	-

Values represent mean percent inhibition and standard error in parentheses.
Data is derived from 4 experiments.

2.2.5. *In vitro* competition between Xb-Sf strains

To determine whether the different levels of xenorhabdicolin activity described above correlated with competitive success of the producer strain we carried out pairwise co-culture experiments. To distinguish between the Xb-Sf strains a GFP-encoding gene was inserted into the respective chromosomes and competitions were carried out with a GFP-marked strain and an unmarked strain. Inverse competitions were also conducted in which the strain marked with GFP was reversed. The outcome of the competition was determined by counting the number of fluorescent and non-fluorescent unmarked colonies using fluorescent and light microscopy. Before performing the competition experiments we confirmed that the individual growth rates of the GFP and unmarked versions of each strain, and the growth rates of each strain relative to the other strains, were comparable (unpublished data).

The strong inhibition of the MD strain by the xenorhabdicolin of the FR strain suggested the latter would display a competition advantage over the MD strain. To test this prediction the strains were co-cultured for 24 h and dilution plated. In the competition between GFP-MD and the unmarked FR strains the MD strain was completely eliminated (Fig 5A). In the inverse competition the GFP-FR strain outcompeted but did not completely eliminate the unmarked MD strain (Fig 5B; see below). Thus, the high level of activity of the FR xenorhabdicolin in the bacteriocin assay correlated with the ability to outcompete the MD strain. In contrast, the MD strain was resistant to the xenorhabdicolin of the FL strain while the MD xenorhabdicolin moderately inhibited the FL strain. Based on the relative activity of the respective xenorhabdicolins the MD was predicted to outcompete the FL strain. Unexpectedly, the GFP-FL strain outcompeted but did not eliminate the MD strain (Fig 5C). In the inverse competition the GFP-FL strain was able to outcompete the MD strain (Fig 5D) although the unmarked MD strain

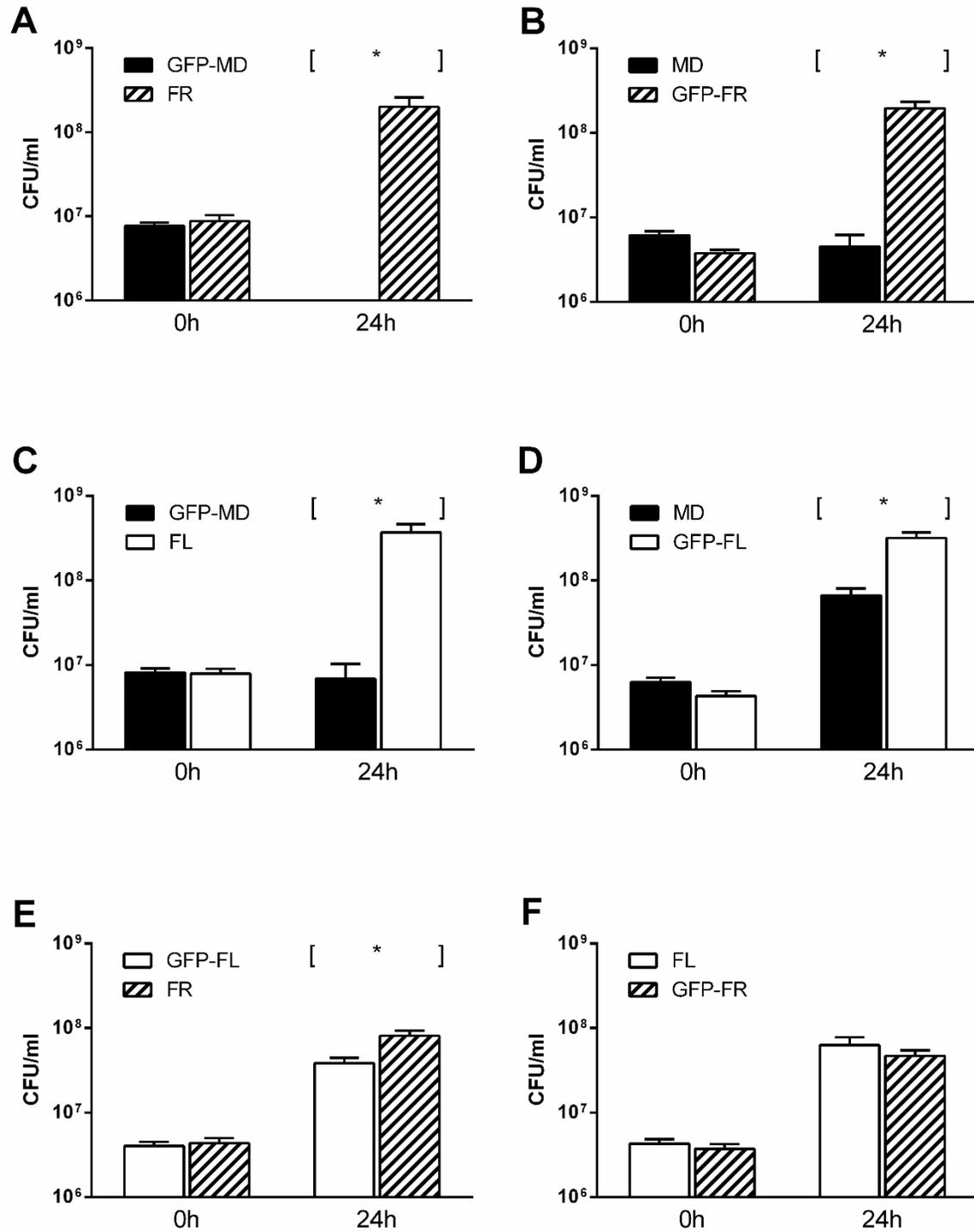


Figure 5. *In-vitro* pairwise co-culture competitions in LB broth amongst *X.bovienii*-*S.feltiae* strains. Competition outcomes at 24 hours were determined by dilution plating. Error bars show standard errors of the means. Asterisks indicate statistically significant differences ($P < 0.05$) as calculated by unpaired *t*-tests using the GraphPad QuickCalcs *t*-test calculator. Each competition was repeated at least four times.

grew to a higher level than in the GFP-MD versus FR competition (Fig 5C). These findings suggest that xenorhabdicolin was not a primary determinant in the outcome of this competition and that the FL strain produced antagonistic factors that were active against the MD strain.

The more closely related FR and FL strains were shown to be resistant to each other's xenorhabdicolin (Table 1) suggesting that neither would have a competitive advantage when grown together. In co-cultures with the GFP-FL and unmarked FR strains neither strain was inhibited although the FR strain grew to slightly higher levels (Fig 5E) while the unmarked FL strain grew to slightly lower levels in co-cultures with the GFP-FR strain (Fig 5F). Therefore the slight differences in growth in co-cultures with the closely related FR and FL strains were apparently not due to antagonistic factors but rather to fitness costs imposed by the expression of GFP. The apparent fitness cost was also discernible in the competition with the MD strain in which the marked FR and FL strains inhibited growth of the MD strain to a lesser extent than the unmarked strains.

2.3. Conclusions

We show that modular exchange and DNA inversion generate diversity and specificity in xenorhabdicolin tail fibers in related strains of *X. bovienii*. Xenorhabdicolins are primary determinants of competitive success between some strains of *X. bovienii* while different antagonistic factors may determine the competitive outcome between other strains. Under natural biological conditions the level of expression and activity of the various antagonistic factors, relative proportion of each competitor, differences in in-host growth rate and other variables interact to influence the competitive outcome. These interactions may in turn affect maintenance of community diversity (19).

2.4. Acknowledgements

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Chapter Three

Antibiotics determine the outcome of competition between *Xenorhabdus* species in a natural host environment

This chapter is a modified version of the submitted paper:

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3.0 Introduction

Microorganisms exist in diverse communities in almost every natural habitat. To compete for space, nutrients, and other resources, microbes employ an array of strategies to gain a competitive advantage (1). One strategy, interference competition, involves the production of a wide range of antagonistic compounds and molecules to inhibit growth of related and non-related species. Bacteria can produce several classes of antagonistic agents. Small molecule antibiotics usually possess broad spectrum activity that inhibit growth of more distantly related species. While antibiotics have been extensively exploited as therapeutic agents, their role in interspecies competition in ecological settings remains poorly understood (2–4). R-type bacteriocins are phage-tail-like structures that suppress growth of related strains by specifically binding to the cell envelope of susceptible cells and can mediate interspecies competition in microbial communities. For example, R-type bacteriocins (R-pyocins) of *P. aeruginosa* are potentially involved in the modulation of bacterial populations during host colonization (5). The production of antagonistic molecules varies widely in bacterial populations. The symbiotic bacterium *Xenorhabdus nematophila* produces both small compound antibiotics and R-type bacteriocins.

Xenorhabdus nematophila engages in a species-specific mutualistic relationship with the entomopathogenic nematode *Steinernema carpocapsae*, where it resides in a specialized region of the anterior intestine of the infective juvenile (IJ) stage of the nematode (6–9). The IJs invade soil-dwelling insects, migrate to the midgut and perforates the intestinal wall to enter the body cavity (hemocoel) where *X. nematophila* is released, transitioning to its pathogenic phase. As a pathogen, *X. nematophila* produces compounds that suppress the host immune response (10, 11) and secretes several insect toxins and exoenzymes that participate in killing the host (12). Perforation of the insect gut allows microbiota to translocate to the hemocoel where they may

proliferate and compete for resources (8). Some transient competitors, such as antibiotic-sensitive *Staphylococcus saprophyticus*, are eliminated early while other more resistant competitors like *Enterococcus faecalis*, grow in the hemolymph as *X. nematophila* proliferates to high cell density (8). In addition, more than one species of steinernematid nematode can invade the insect and subsequently release their respective *Xenorhabdus* symbionts into the hemocoel (13–16). Thus, in the infected hemocoel, *Xenorhabdus* species compete against both related strains and nonrelated gut microbes.

X. nematophila produces numerous antimicrobials that can reach high concentrations when grown in complex laboratory media. The genome of *X. nematophila* encodes many non-ribosomal peptide synthetases (NRPS) and polyketide synthases (PKS) that synthesize secondary metabolites including antimicrobial compounds (7). The NRPS gene clusters and stand-alone NRPS genes that produce the antibiotic molecules xenocoumacin (17, 18), xenematide (19), nematophin (20), and compounds F and C (21, 22) have been identified. The antimicrobial compounds of *X. nematophila* are active against diverse microbial targets, but are generally not active against other *Xenorhabdus* species (23) (unpublished data). A requisite 4'phosphopantetheinyl (4'PP) moiety is required by NRPS and PKS proteins for enzymatic activity (6). Phosphopantetheinyl transferase, encoded by the *ngrA* gene, attaches the 4'PP moiety to the transfer domains of NRPS and PKS enzymes. Inactivation of *ngrA* thereby eliminates biosynthesis of NRPS and PKS-derived secondary metabolites. We have shown that *S. saprophyticus* persisted when injected alone into the insect hemocoel, but was eliminated when co-injected with the antimicrobial-deficient *ngrA* strain of *X. nematophila*, indicating that under natural biological conditions, *ngrA*-dependent compounds were not required to eliminate a sensitive competitor (21). These findings raised the question of whether NRPS-derived

antimicrobials were produced at inhibitory concentrations by *X. nematophila* during growth in the insect. On the other hand, *S. carpocapsae* reproduction in the insect was markedly reduced in the absence of *ngrA*-dependent compounds, suggesting that *X. nematophila* produces secondary metabolites that may be involved in nematode growth and development (21).

To compete against more closely related bacteria, *X. nematophila* produces R-type bacteriocins (xenorhabdins) that are active against a range of *Xenorhabdus* and *Photorhabdus* strains (24–27). *Photorhabdus* spp., symbionts of heterorhabdid nematodes that also invade and kill insects, are the sister taxon of *Xenorhabdus*. We found that xenorhabdin was required to eliminate *Photorhabdus luminescens* from co-infected insects while *P. luminescens* proliferated in insect co-infected with a xenorhabdin-deficient strain of *X. nematophila* (26). Furthermore, *S. carpocapsae* nematodes could not reproduce in co-infected insects in the absence of xenorhabdin, indicating that R-type bacteriocins were essential to eliminate related competitors that prohibit reproduction of the nematode partner. The level of induction and spectrum of activity of xenorhabdins of different *Xenorhabdus* species and strains can vary considerably (28).

In the present study, we investigate the role of the antimicrobials of *Xenorhabdus szentirmaii* in interspecies competition in the natural environment of insect hemocoel. *X. szentirmaii*, the symbiont of the nematode *Steinernema rarum* (29), produces antibiotic activity against diverse bacteria including *X. nematophila* (23) and is a potent insect pathogen (30). While the genome of *X. szentirmaii* possesses many NRPS and PKS genes (31), antibiotic compounds have not yet been isolated from this bacterium. We show that *X. szentirmaii* primarily uses antibiotics rather than R-type bacteriocins to suppress *X. nematophila* in co-

infected insects. These findings directly demonstrate that antibiotics determine the outcome of interspecies competition in a natural host environment.

3.1. Materials and Methods

3.1.1 Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Xenorhabdus* and *Escherichia coli* strains were routinely grown in Lysogeny broth (LB; 1% Bacto tryptone, 0.5% NaCl, 0.5% yeast extract, and 0.01 mM MgSO₄) or on solid media with the addition of 15 g/L agar, at 30°C or 37°C, respectively. For growth of *Xenorhabdus* strains, overnight cultures were supplemented with ampicillin (50 µg/mL), chloramphenicol (25 µg/mL), or kanamycin (30 µg/mL) when indicated and 250 µL of the overnight culture was added to 5 mL of LB broth (1:20 subculture). Growth was monitored by optical density at 600 nm (OD₆₀₀). Grace's Insect Medium (Gibco) (Grace's) was used for dilution plating and insect inocula.

3.1.2. Overlay assay for antibiotic activity

Overnight cultures of *Xenorhabdus* and insect gut strains were diluted 1:20 in LB broth and grown to exponential phase. 6 µL of subculture normalized to OD₆₀₀ = 0.2 were spotted on LB agar. After 24 h of incubation at 30°C, cells were killed by exposure to chloroform vapors for 30 min, followed by 30 min of air drying. A thin layer of 1 mL of indicator strain per 12 mL molten top agar (LB with 0.7% agar) was poured over the *Xenorhabdus* colonies. Zones of inhibition were photographed and measured after 24 h of incubation. The results from four assays were nearly identical.

TABLE 1 Bacterial strains and plasmids used in this study.

Strains and plasmids	Nematode or description	Geographic location	Source
<i>X. szentirmai</i>	<i>S. rarum</i>	Argentina	A. Fodor
<i>ngrA</i> ::Cm	<i>S. rarum</i>	Argentina	This study
<i>xspS</i> ::Cm	<i>S. rarum</i>	Argentina	This study
<i>X. nematophila</i>			
19061	<i>S. carpocapsae</i>	USA	Laboratory stock
AN6	<i>S. carpocapsae</i>	USA	Laboratory stock
	<i>S. anatoliense</i>	Jordan	P. Stock
	<i>S. websteri</i>	Peru	P. Stock
<i>X. bovienii</i>			
Xb-Sf-FL	<i>S. feltiae</i>	Florida, USA	P. Stock
Xb-Sj-2000	<i>S. jolietii</i>	Monsanto	
Xb-Sp	<i>S. puntauvense</i>	Costa Rica	P. Stock
<i>X. cabanillasii</i>	<i>S. riobrave</i>	USA	P. Stock
<i>X. japonica</i>	<i>S. kushidai</i>	Japan	DMS 16522
<i>X. poinarii</i>	<i>S. glaseri</i>	USA	DSM 4768
<i>P. luminescens</i> subsp. <i>Laumondii</i> TT01	<i>H. bacteriophora</i>	Trinidad & Tobago	T. Ciche
<i>Enterococcus faecalis</i>	<i>M. sexta</i> isolate		Laboratory stock
<i>Aerococcus viridans</i>	<i>M. sexta</i> isolate		Laboratory stock
Yeast	<i>M. sexta</i> isolate		Laboratory stock
<i>Staphylococcus saprophyticus</i>	<i>M. sexta</i> isolate		Laboratory stock
<i>E. coli</i> NovaBlue	<i>endA1 hsdR17</i> ($r_{K12}^- m_{K12}^+$) <i>supE44 thi-1 recA1 gyrA96 relA1 lac F</i> '[<i>proA</i> ⁺ <i>B</i> ⁺ <i>lacI</i> ^q <i>ZΔM15</i> ::Tn10] (Tet ^R)		Novagen
<i>E. coli</i> S17-1 (<i>λpir</i>)	<i>recA, thi, pro, hsdR</i> -M+. RP4-2Tc::Mu Km::Tn7 in the chromosome		Laboratory stock
pSTBlue-1	Cloning vector; Amp Km		Novagen
pKnock-Cm	Broad-host-range suicide vector; Cm RP4 <i>oriT oriR6K</i>		D. Saffarini
pKnock- <i>ngrA</i>	Internal fragment of <i>ngrA</i> cloned into pKnock-Cm		This study
pKnock- <i>xspS</i>	Internal fragment of <i>xspS</i> cloned into pKnock-Cm		This study

Amp, ampicillin resistance; Km, kanamycin resistance; Cm, chloramphenicol resistance; Tet, tetracycline resistance.

* From Singh *et al.*, 2014

3.1.3. Cell-free supernatant antibiotic activity from LB and Grace's Medium cultures

Overnight cultures of wild-type or *ngrA* strains of *X. szentirmaii*, or *X. nematophila* AN6 were subcultured in either Grace's Insect Medium or LB broth and grown at 30°C with shaking. Supernatants were harvested at 24 h and 48 h by centrifugation at 14,000 rpm for 2 min and sterile filtered with 0.2 µm pore size cellulose acetate syringe filters. Aliquots of cell-free supernatants were frozen and stored at 4°C until the time of use. To assess the inhibitory activity of antibiotic supernatants, 250 µL of overnight LB broth cultures of indicator strains was added to 5 mL of LB broth, grown to exponential phase, and subsequently diluted 1:200 in LB broth. 200 µL of diluted indicator strain was mixed with 25 µL of cell-free supernatant in a 96-well microtiter plate and incubated for 24 h at 30°C with shaking. Inhibition of growth (% inhibition) was calculated by dividing the OD₆₀₀ value of treated samples by the OD₆₀₀ value of untreated samples. Inhibition assays were repeated at least three times and mean and standard error were calculated from 8-9 individual data points.

3.1.4. Bioinformatics analysis of *xspS* gene cluster

The *xspS* gene cluster was located and genes were annotated using the BlastP algorithm in the MaGe MicroScope platform.

3.1.5. Construction of the *ngrA* and *xspS* mutant strains

The *ngrA* and *xspS* genes of *X. szentirmaii* were located based on sequence homology using the BlastP algorithm of MaGe Microbial Genome Annotation and Analysis Platform (<http://www.genoscope.cns.fr/agc/microscope/mage/index.php>). The *ngrA* and *xspS* mutant strains were constructed via insertional inactivation of the respective genes. 410 base pair (*ngrA*)

and 458 base pair (*xspS*) internal fragments located near the 5' end of the genes were amplified using gene-specific primers (Table S1) and *X. szentirmaii* genomic DNA as the template. PCR products were purified with GeneClean Turbo kit (MP Biomedicals), followed by end conversion and blunt-end ligation into the EcoRV site of pSTBlue-1 vector (Novagen). Successful cloning events were confirmed by colony PCR of recombinant colonies using primers for the T7 and SP6 promoters that flank the EcoRV site. Colonies containing inserts of the correct size were grown overnight and plasmids were extracted and purified using the QIAprep Spin Miniprep Kit (Qiagen). The plasmids were subsequently digested with PstI and XbaI and fragments containing the internal gene fragments were gel purified using the QIAquick Gel Extraction Kit (Qiagen). The fragments were then ligated into the conjugal suicide vector pKnock-Cm (Alexeyev, 1999). The recombinant plasmids were transformed into electrocompetent *Escherichia coli* S17-1 λ pir and conjugally transferred into wild-type *X. szentirmaii*. Exconjugants were plated on ampicillin and chloramphenicol to select for pKnock-containing colonies. Successful chromosomal insertion of the pKnock plasmid into the respective gene by single-crossover homologous recombination was confirmed by PCR analysis using upstream gene-specific and pKnock-specific primers.

3.1.6. Polyethylene glycol precipitation of uninduced and mitomycin C-induced cultures.

Overnight cultures of wild-type, *ngrA* and *xspS* strains of *X. szentirmaii* were diluted 50-fold into 100 mL of LB broth or Grace's Insect Medium and grown at 30°C with shaking. Mitomycin C (MMC, final concentration 5 μ g/mL) was added to induce subcultures at an OD₆₀₀ of 0.5 to 0.6. The uninduced subcultures were not treated with mitomycin C. Both conditions were subsequently incubated at 30°C with shaking for 18 h. Preparations were treated with

TABLE S1 Primers used in this study.

Gene or Vector	Primer	Sequence (5'-3')	Use
<i>ngrA</i>	XSR1v1_80075 F	CACCGACCTACACATAGGTTAC	mutant construction
	XSR1v1_80075 R	CAGCATCCTTGAGAATACTGGCTG	mutant construction
	XSR1v1_80075 OF	CATGTTTACCCAAGCCGTTGC	mutant screening
	XSR1v1_80075 OR	GAGTGTAACGCCTGCTCG	mutant screening
<i>xspS</i>	XSR1v1_360005 F	CCATCACTACCGTTAGCACTGC	mutant construction
	XSR1v1_360005 R	GCGATACCCGATGACATCTTCG	mutant construction
	XSR1v1_360005 OF	GCACAAGAATATCATCACGGAGTCC	mutant screening
	XSR1v1_360005 OR	GCAACCCCAGAGCATAAGC	mutant screening
pKnock	pKnock-F	ACACAGGAACACTTAACGGCTGAC	mutant screening
	pKnock-R	TGCGAAGTGATCTTCCGTCAGAG	mutant screening
pST-Blue-1	SP6	ATTTAGGTGACACTATAG	mutant screening
	T7	CTAATACGACTCACTATAGGG	mutant screening

1 $\mu\text{g}/\text{mL}$ RNase A and DNase I for 30 min at 37°C then centrifuged and sterile filtered with 0.45 μm pore size filters to remove cellular debris. Xenorhabdicolin-enriched fractions were precipitated by polyethylene glycol 8000 (10% w/v final concentration) and NaCl (1M final concentration) for 2 h at 4°C. Xenorhabdicolin-enriched fractions were harvested by centrifugation at R_{max} of 13,776 $\times g$ at 4°C for 15 min, resuspended in 3 mL of LB broth, and centrifuged again for 5 min to remove insoluble material. Supernatants containing xenorhabdicolins were sterile filtered and stored at 4°C. To prepare SDS-PAGE samples, 300 μL aliquots were ultracentrifuged at R_{max} of 287,582 $\times g$ at 4°C for 15 min. Pellets were resuspended in Laemmli buffer and 5 μL were loaded on a 8-16% PAGE gel (Genscript). Bands were visualized by Coomassie blue staining.

3.1.7. Microplate assay of xenorhabdicolin activity

X. nematophila AN6 was subcultured in LB broth and grown to an OD_{600} of 0.5 to 0.6. Assays of 100 μL of diluted (1200-fold) subculture mixed with 50 μL of 1X or 0.5X PEG-precipitated xenorhabdicolin were incubated in a 96-well microplate for 24 h with shaking. Inhibition of growth was calculated by comparing OD_{600} of control versus treatment wells at 24 h. Assays were repeated at least four times.

3.1.8. *Ex vivo* competitions in LB broth and Grace's Insect Medium.

Overnight cultures of *X. szentirmaii* and *X. nematophila* AN6 were separately subcultured in LB broth and grown at 30°C with shaking to an OD_{600} exceeding 0.25. Subcultures were normalized to an OD_{600} of 0.25 and competitions were established by mixing 1:1 ratios of *X. nematophila* AN6 and *X. szentirmaii*. Control and competition cultures were

inoculated with 250 μL of normalized cells in 5 mL of LB broth or Grace's insect medium. At 0, 24 h, and 48 h colony-forming units/mL were calculated by dilution in Grace's insect medium and plating on LB agar. The experiments were performed three times with highly reproducible results. Statistical significance was calculated by Wilcoxon matched-pairs signed rank test using GraphPad Prism.

3.1.9. Cultivation of *M. sexta* larvae

M. sexta eggs were obtained from Carolina Biological Supply and larvae were reared on North Carolina State University Insectary diet until fourth-instar stage as described previously (8).

3.1.10. *In vivo* competitions in *M. sexta*

Overnight cultures of *Xenorhabdus* strains were subcultured in LB broth and grown at 30°C with shaking to an OD₆₀₀ exceeding 0.25. Subcultures were normalized to an OD₆₀₀ of 0.25 by dilution in Grace's insect medium. Competitions were established by mixing 1:1 ratios of *X. nematophila* AN6 with *X. szentirmaii*. Insects were injected with 50 μL containing 10³ CFU of respective cultures using 1-mL Sub-Q syringes (0.45-mm by 16-mm; Becton Dickinson Co.) mounted on a Stepper repetitive dispensing pipette (Dymax Corp.). Prior to injection, fourth-instar larvae were anesthetized for 30 min on crushed ice and the area surrounding the horn was cleaned with 70% ethanol. Larvae were placed in plastic cups until their hemolymph was harvested by making a cut with a sterile scissors immediately below the horn and draining the hemolymph into sterile 1.5 mL tubes. Hemolymph from at least 3 insects per timepoint was pooled, diluted in Grace's insect medium, and duplicate plated on LB agar. The experiments

were performed four times with reproducible results. Statistical significance was calculated by Wilcoxon matched-pairs signed rank test using GraphPad Prism.

3.1.11. Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI TOF-MS) analysis of infected hemolymph

M. sexta hemolymph samples from 6 insects per condition were collected 24 h post-injection as described in the *in vivo* competition experiment section above. Samples were then batched, centrifuged 5 min at 13,500 rpm at 4°C, and sterile-filtered with 0.45 µm pore size filters to remove bacteria and hemocytes. To each 1 mL aliquot, 80 µL/mL glutathione was added to prevent agglutination. 1 µl of the hemolymph sample was placed on the steel MALDI target plate (Shimadzu Scientific, Fleximass targets TO-431R00) in triplicate and 1µl of matrix solution was added. The matrix solution was prepared by dissolving 10mg of α-cyano-4-hydroxycinnamic acid in 1ml of 70% acetonitrile in 0.1% trifluoroacetic acid. The sample-matrix mixture on the MALDI plate was allowed to dry at room temperature. The MALDI mass spectra were recorded using a Shimadzu Scientific MALDI 7090 TOF-TOF mass spectrometer equipped with an ultrafast 2 kHz UV laser. The data were acquired by scanning in positive ion reflectron mode with a mass range of 150-3000 Da. The mass spectra were generated by accumulating 500 laser shots across the sample spot. Samples from two separate experiments were assessed for each condition. Each sample was analyzed in triplicate by loading sample on three different spots on the MALDI plate to assess the reproducibility of the results.

3.2. Results

3.2.1. Analysis of antibiotic activity of *X. szentirmaii*

Here we address the unresolved question of whether antibiotics play a role in interspecies competition between related species in a natural host environment. *X. szentirmaii* was shown previously to produce antibiotic activity against *X. nematophila* 19061 while neither antibiotics nor R-type bacteriocins of *X. nematophila* were active against *X. szentirmaii* (23, 26, unpublished data). To further characterize the antibiotic activity of *S. szentirmaii*, overlay assays were performed with several strains of *X. nematophila* and microbes isolated from the gut of *Manduca sexta*. *X. szentirmaii* antibiotics were active against all strains of *X. nematophila*, although the sensitivity of the various strains varied as indicated by differences in the zones of inhibition (Fig 1A). The sensitivity of gut-derived microbes to antibiotics of *X. szentirmaii* also varied. *Staphylococcus saprophyticus* (Ss) was highly sensitive, while *Aerococcus viridans* (Av) and *Enterococcus faecalis* (Ef) were less sensitive (Fig 1B).

To determine whether NRPS and PKS enzymes were involved in the biosynthesis of antibiotics of *X. szentirmaii*, we created an *ngrA* strain. Inactivation of *ngrA* almost completely eliminated antibiotic activity against both *X. nematophila* and *S. saprophyticus* (Ss) as well as other target strains tested (Fig 1A and B; also see below). We identified >30 NRPS genes and 2 PKS genes in the genome of *X. szentirmaii*, indicating that most secondary metabolites and antibiotics are synthesized by NRPS enzymes in this species.

To date, secondary metabolites have been isolated from *Xenorhabdus* species grown in complex laboratory medium such as LB broth. To assess the level of antibiotic production under more biologically relevant conditions, *X. szentirmaii* was grown in medium that mimics lepidopteran hemolymph (Grace's Insect Medium) as well as in LB broth. To determine the level

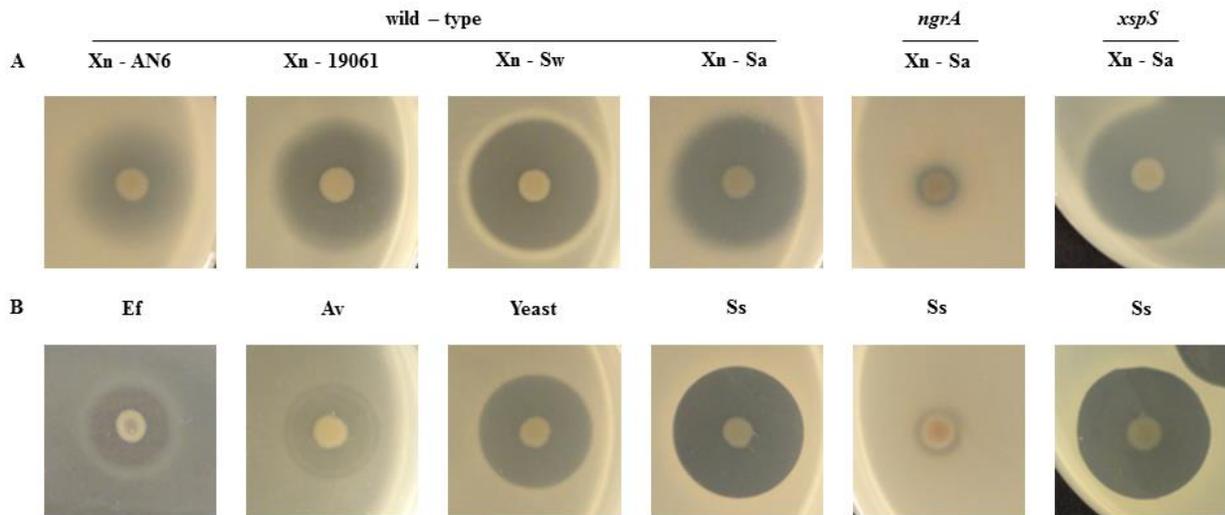


Figure 1. Antibiotic overlay assay of the wild-type, *ngrA*, and *xspS* strains of *X. szentirmaii*. Antibiotic activity was assessed against *X. nematophila* strains (Xn-AN6, Xn-19061, Xn-Sw and Xn-Sa) and microbes isolated from the gut of *M. sexta* (*Enterococcus faecalis*, Ef; *Aerococcus viridans*, Av; unidentified yeast; *Staphylococcus saprophyticus*, Ss). Zones of inhibition surrounding each colony is proportional to the sensitivity of the respective indicator strains.

of antibiotic activity produced under different conditions, filtered soluble supernatants obtained from cultures grown for 24 and 48 h were added to dilute cultures of the target microbe, and the inhibition of growth relative to the untreated culture (% inhibition) was measured. Supernatants from cultures of wild-type *X. szentirmaii* grown in LB broth for 24 h and 48 h strongly inhibited growth of all strains of *X. nematophila* (Fig 2A). The 24 h supernatants also exhibited high antibiotic activity against gut-derived microbes, while the 48 h supernatants were less active against the more resistant species, *E. faecalis* and *A. viridans* (Fig 2C). Soluble supernatant of the *ngrA* strain was inactive against all gut-derived microbes except the highly sensitive *S. saprophyticus*, which was partially inhibited (unpublished data). The antibiotic activity of wild-type and *ngrA* soluble supernatants was further analyzed against diverse *Xenorhabdus* species and the related *Photorhabdus luminescens*. The wild-type supernatants exhibited high levels of antibiotic activity against several strains of *X. bovienii* as well as *X. poinarii*, and were moderately active against *X. japonica*, *X. cabanillasii* and *P. luminescens* (Fig. S1). In contrast, supernatants of the *ngrA* strain of *X. szentirmaii* exhibited low levels of antibiotic activity against the *Xenorhabdus* species and *P. luminescens*. Culture supernatants of *X. nematophila* AN6 exhibited low levels of antibiotic activity against the *Xenorhabdus* species and *P. luminescens*.

The supernatants from *X. szentirmaii* grown in Grace's possessed lower levels of antibiotic activity against the *X. nematophila* strains and the sensitivity of the strains to the supernatants was more variable (Fig 2B). Supernatants were not active against the more resistant gut microbes, *E. faecalis* or *A. viridans*, but were highly active against yeast and *S. saprophyticus* (Fig 2D). Thus, the spectrum of antibiotic compounds and/or their concentrations produced under more biologically relevant conditions of Grace's medium differed from antibiotic production in LB broth.

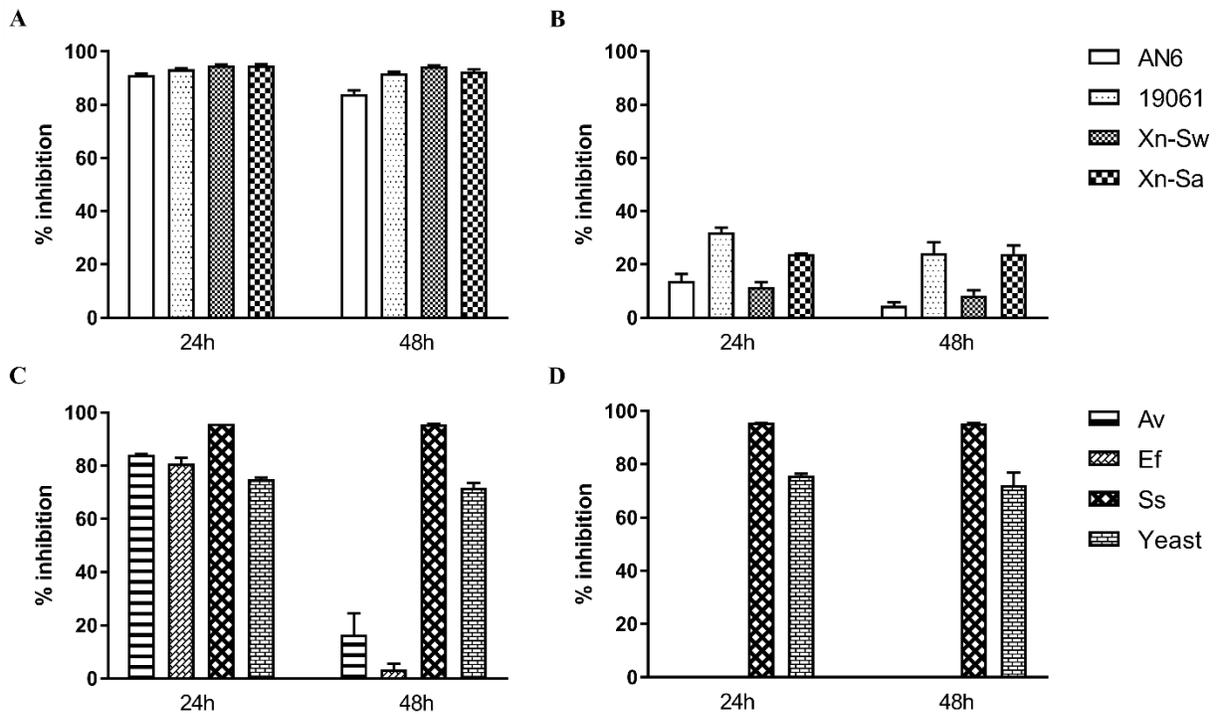


Figure 2. Inhibitory activity of filtered supernatants obtained from *X. szentirmaii* cultures grown for 24 h and 48 h. Cultures were grown in either LB broth (A and C) or Grace's medium (B and D). Supernatants were assayed against *X. nematophila* strains (Xn-AN6, Xn-19061, Xn-Sw and Xn-Sa) and microbes isolated from the gut of *M. sexta* (*Enterococcus faecalis*, Ef; *Aerococcus viridans*, Av; unidentified yeast; *Staphylococcus saprophyticus*, Ss). The % inhibition was calculated by dividing the optical density of treated cultures by the optical density of the untreated culture at each time point.

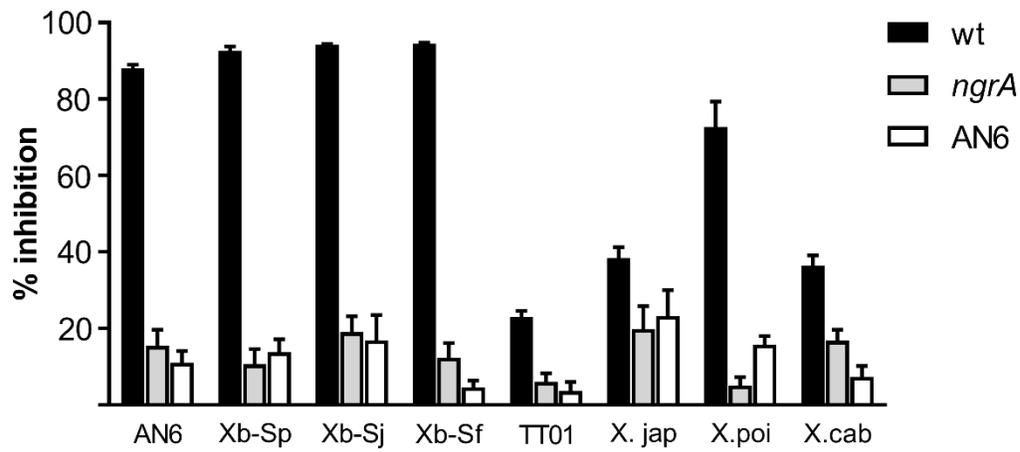


Figure S1. Inhibitory activity of filtered culture supernatants obtained from *X. szentirmaii* wild-type and *ngrA* strains and *X. nematophila* AN6 grown for 24 h in LB broth. Supernatants were assayed against *X. bovienii* strains (Xb-Sp, Xb-Sj, Xb-Sf), *X. poinarii* (X. poi), *X. japonica* (X. jap), *P. luminescens* TT01 (TT01) and *X. cabanallasii* (X. cab).

3.2.2. Xenorhabdicolin production in *X. szentirmaii*

That *X. szentirmaii* antibiotics were active against *X. nematophila* suggested the possibility that both antimicrobials and R-type bacteriocins (xenorhabdicolins) could be involved in competition between *Xenorhabdus* species. To address this possibility, we identified the remnant P2-type prophage in the genome of *X. szentirmaii* that contained the tail sheath (*xspS*), tube and baseplate genes but lacked capsid and replication genes (Fig S2). We had shown previously that inactivation of the tail sheath gene (*xnpS*) in *X. nematophila* abolished production of xenorhabdicolin (26). Xenorhabdicolin production in *X. nematophila* is strongly induced by exposure to mitomycin C. To confirm that the remnant P2 prophage of *X. szentirmaii* was required for xenorhabdicolin production, we created a strain in which the *xspS* sheath gene was inactivated. Xenorhabdicolin preparations were obtained from the wild-type and *xspS* strains by precipitation with polyethylene glycol (see Materials and Methods). We found that xenorhabdicolin preparations from both mitomycin C induced (Fig 3, lane 1) and uninduced (lane 2) cultures contained comparable levels of XspS. Thus, unlike with *X. nematophila*, mitomycin C did not induce higher levels of xenorhabdicolin production in *X. szentirmaii*. We also showed that xenorhabdicolin preparations from the *xspS* strain lacked XspS (lane 5 and 6), confirming that the remnant P2-type prophage cluster encoded xenorhabdicolin. In addition, we confirmed that inactivation of *ngrA* did not affect xenorhabdicolin production (compare lanes 1 and 3). Finally, we showed that inactivation of *xspS* did not alter antibiotic production (Fig 1A and B).

The activity of the xenorhabdicolin preparations from wild-type and *xspS* strains of *X. szentirmaii* was assessed against *X. nematophila* AN6 (Table 2). The inhibitory activities of preparations from the uninduced and induced wild-type strains were comparable. This result was consistent with the above findings that XspS levels in uninduced cells and mitomycin-induced

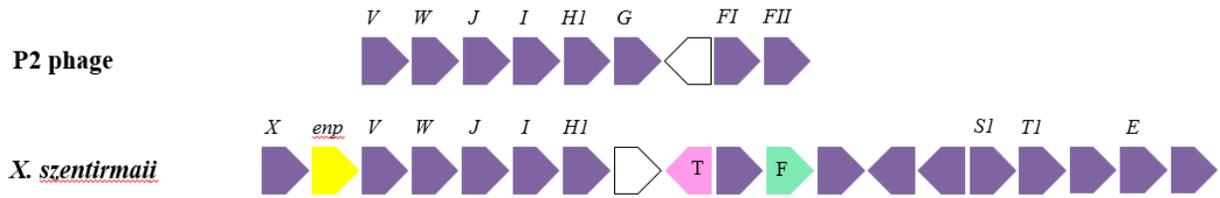


Figure S2. Remnant P2-type prophage of *X. szentirmaii*. Open reading frames are classified by color: tail synthesis structural proteins (purple), lysis genes (yellow), truncated tail fibers (light green), and other uncharacterized prophage encoded ORFs (white). The *xspS1* gene is designated as S1. Open reading frames are not drawn to scale.

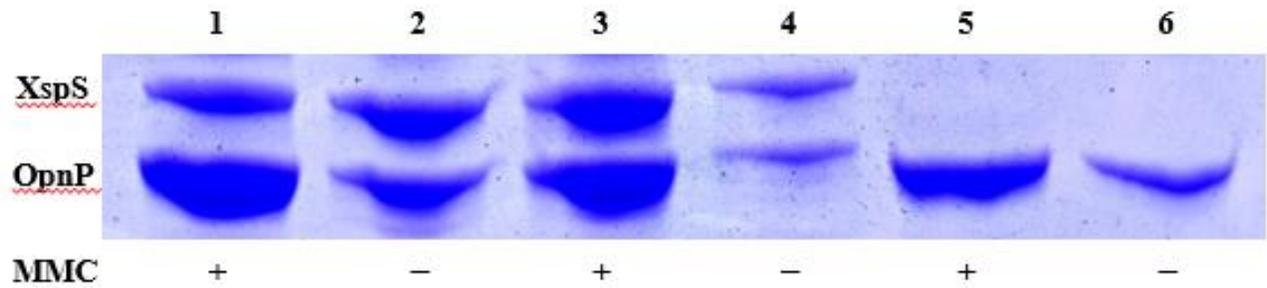


Figure 3. SDS-polyacrylamide gel electrophoresis analysis of xenorhabdysin preparations from *X. szentirmaii* strains. Xenorhabdysin preparations were obtained from either uninduced (-) or mitomycin (MMC)-induced (+) cultures of the wild-type (lanes 1 and 2), *ngrA* (lanes 3 and 4), and *xspS* (lanes 5 and 6) strains grown in LB broth. XspS indicates the major xenorhabdysin sheath protein and OpnP indicates the outer membrane porin P that co-precipitates with xenorhabdysin.

TABLE 2 Inhibitory activity of xenorhabdicolin of *X. szentirmaii* on *X. nematophila* AN6

<u>Strain</u>	Uninduced		Induced	
	% inhibition*		% inhibition*	
	<u>1X</u>	<u>0.5X</u>	<u>1X</u>	<u>0.5X</u>
wild-type	89 (0.01)	24 (0.07)	80 (0.01)	30 (0.08)
<i>xspS</i>	91 (0.00)	25 (0.03)	90 (0.01)	49 (0.12)

* Numbers represent the OD₆₀₀ value of the treated culture divided by the OD₆₀₀ value of the untreated culture.

cells were similar. Unexpectedly, the inhibitory activity of preparations from the *xspS* strain was indistinguishable from that of the wild-type strain. This finding suggested that a non-xenorhabdicolin activity accounted for the inhibition of *X. nematophila*. We further assessed xenorhabdicolin preparations from the wild-type and *xspS* strains against *S. saprophyticus* (Table S3). Both preparations strongly inhibited *S. saprophyticus*, indicating that *X. szentirmaii* grown in LB broth produced an uncharacterized non-xenorhabdicolin inhibitory activity against both *X. nematophila* and *S. saprophyticus* that was precipitated with polyethylene glycol. This type of inhibitory activity had not been observed in xenorhabdicolin preparations of sheath mutant strains of other *Xenorhabdus* species (26, 28, unpublished data). As shown below, this inhibitory activity did not contribute to the outcome of interspecies competition in Grace's medium.

3.2.3. Ex vivo competition between *X. szentirmaii* and *X. nematophila* in LB broth

The availability of the *ngrA* and *xspS* strains allowed us to assess the relative contribution of antibiotics and xenorhabdicolin to the outcome of interspecies competition between *X. szentirmaii* and *X. nematophila*. Initially, competitions were carried out in LB broth co-cultures containing *X. nematophila* AN6 and either the wild-type, *ngrA*, or *xspS* strain of *X. szentirmaii*. We first established that the respective *X. szentirmaii* strains and *X. nematophila* grew to comparable cell concentrations in LB broth (Fig S3). Cultures co-inoculated with the respective *X. szentirmaii* strains and *X. nematophila* were incubated for 24 h and 48 h, at which time aliquots were diluted and plated. *X. szentirmaii* and *X. nematophila* are clearly distinguished by distinctive pigmentation properties. By 24 h *X. nematophila* was eliminated in co-cultures with either the wild-type, *ngrA*, or *xspS* strains (Fig 4). By 48 h, *X. nematophila* remained inhibited in the co-cultures with the wild-type and *xspS* strains while low levels were detected in co-cultures

TABLE S2 Inhibitory activity of xenorhabdicolin of *X. szentirmaii* on *S. saprophyticus*

	Uninduced		Induced	
	% inhibition*		% inhibition*	
	<u>1X</u>	<u>0.5X</u>	<u>1X</u>	<u>0.5X</u>
<u>LB</u>				
wild-type	95 (0.00)	96 (0.00)	95 (0.00)	95 (0.00)
<i>xspS</i>	95 (0.00)	95 (0.00)	95 (0.00)	95 (0.00)
<u>Grace's</u>				
wild-type	95 (0.00)	ND	95 (0.00)	ND
<i>xspS</i>	95 (0.00)	ND	95 (0.00)	ND

* Numbers represent the OD₆₀₀ value of the treated culture divided by the OD₆₀₀ value of the untreated culture.

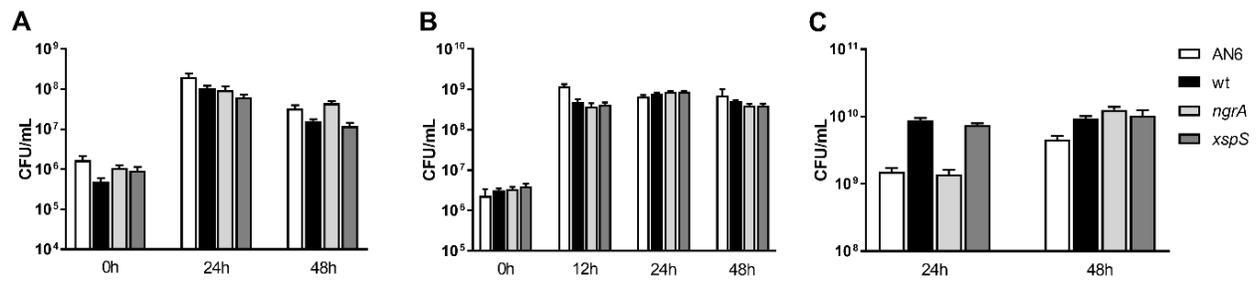


Figure S3. Growth of individual *X. szentirmaii* strains and *X. nematophila* AN6 used in competition experiments. Strains were grown in either LB broth (A), Grace's medium (B), or directly in *M. sexta* (C).

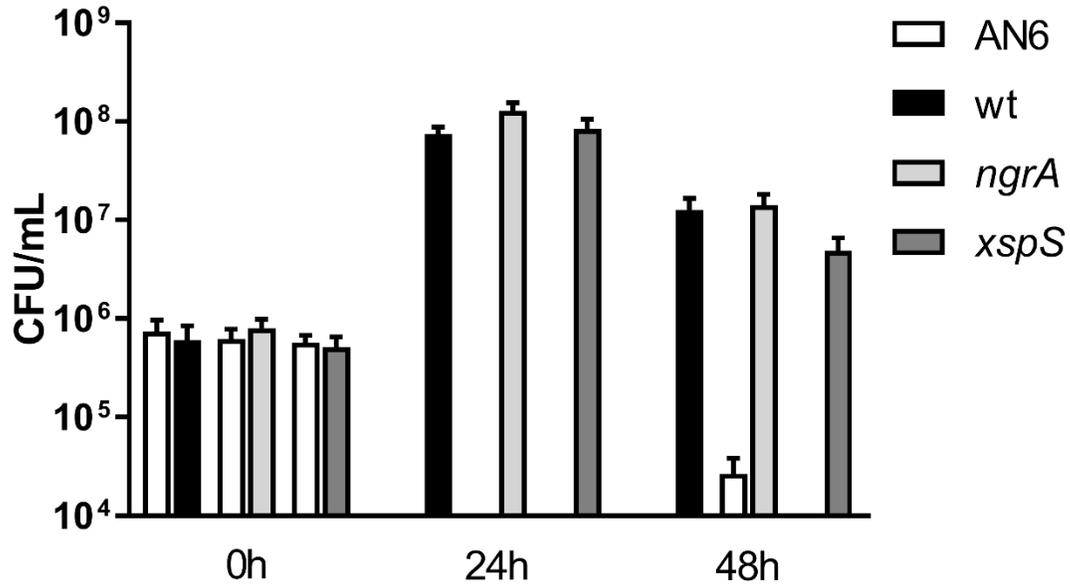


Figure 4. Pairwise *in-vitro* competition between *X. szentirmaii* strains and *X. nematophila* AN6 in LB broth. Co-cultures were established by mixing 1:1 ratios of *X. nematophila* AN6 and the respective *X. szentirmaii* strains and then inoculating LB broth. Competition outcome was determined by dilution plating at 24 h and 48 h. The initial ratio of each competition is shown at time 0. Data is reported as colony forming units/ml (CFU/ml).

with the *ngrA* strain, suggesting that the production of soluble antibiotics were required to continually inhibit growth of *X. nematophila*. Inhibition of *X. nematophila* in co-cultures with the *ngrA* strain that lacked detectable antibiotic activity suggested that other inhibitory components such as xenorhabdicolin and/or uncharacterized non-xenorhabdicolin inhibitory activity were produced at sufficient quantities in LB broth to suppress growth of *X. nematophila*.

3.2.4. *Ex vivo* competition between *X. szentirmaii* and *X. nematophila* in Grace's medium

To determine whether under more natural biological conditions antibiotics were required to inhibit growth of *X. nematophila*, Grace's medium was co-inoculated with *X. nematophila* and one of the respective *X. szentirmaii* strains. By 12 h, the cell density of both *X. nematophila* and *X. szentirmaii* strains increased in all co-cultures (Fig 5A). It was also apparent that the ratio of *X. nematophila* in co-cultures with either the wild-type or *xspS* strains decreased relative to the initial cell ratio at time 0, while the ratio of *X. nematophila* in the co-culture with the *ngrA* strain increased in comparison to the initial ratio (Fig 5B). By 24 h, the cell density and cell ratio of *X. nematophila* in co-culture with the either the wild-type or *xspS* strain decreased dramatically (Fig 5A and B). In marked contrast, the cell density and ratio of *X. nematophila* in co-cultures with the *ngrA* strain had not decreased. Similarly, at 48 h the cell density and ratio of *X. nematophila* in co-culture with either the wild-type or *xspS* strain continued to decrease to barely detectable levels, while *X. nematophila* was not inhibited in co-culture with the *ngrA* strain. These findings indicate that in Grace's medium, soluble antibiotics of *X. szentirmaii* were primarily responsible for suppression of growth of *X. nematophila*. In the absence of antibiotics, *X. nematophila* was not inhibited.

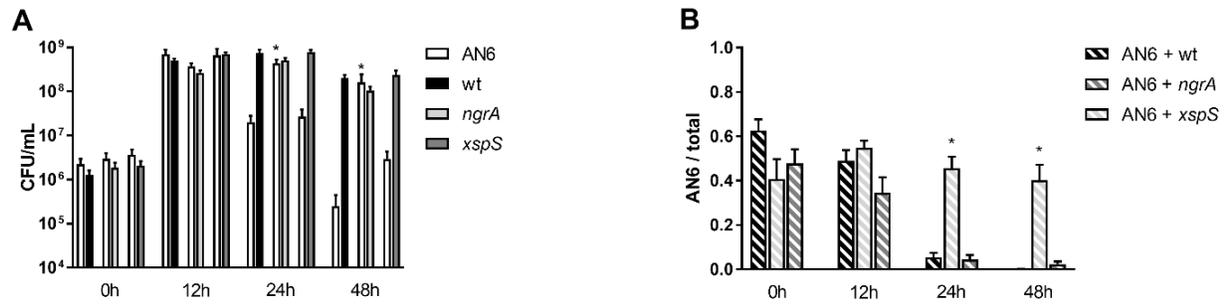


Figure 5. Pairwise *in-vitro* competition between *X. szentirmaii* strains and *X. nematophila* AN6 in Grace's medium. Co-cultures were established by mixing 1:1 ratios of *X. nematophila* AN6 and the respective *X. szentirmaii* strains and then inoculating Grace's medium. Competition outcome was determined by dilution plating at 12 h, 24 h and 48 h. The initial ratio of each competition is shown at time 0. Data is reported as CFU/ml (A) and the ratio of *X. nematophila* AN6 relative to the total colonies counted (B). Asterisks depict statistically significant differences ($P < 0.05$) as calculated by Wilcoxon matched-pairs signed rank test using GraphPad Prism.

3.2.5. *In vivo* competition between *X. szentirmaii* and *X. nematophila* in *M. sexta*

The above results raised the possibility that antibiotics were primarily involved in competition between *Xenorhabdus* species in the natural environment of the insect hemocoel. To address this possibility, co-mixtures of *X. nematophila* and the various *X. szentirmaii* strains were injected into the hemocoel of *M. sexta* and the relative amount of each strain was determined in hemolymph at 24 h and 48 h post-injection (Fig 6). The initial ratio of *X. nematophila* relative to the total number of cells injected is shown in Fig 6A. At 24 h, all *X. szentirmaii* strains were present at low levels in the hemolymph, while *X. nematophila* was barely detectable (Fig 6B). The lower levels of *X. nematophila* were likely due to a slower initial rate of growth in the hemolymph (unpublished data). By 48 h, the cell density of wild-type *X. szentirmaii* continued to increase while the growth of *X. nematophila* was inhibited. In marked contrast, in insects co-injected with the *ngrA* strain the cell density of *X. nematophila* had increased, while the cell density of *X. szentirmaii* had decreased. Thus, in the absence of antibiotics, *X. szentirmaii* was not only unable to inhibit *X. nematophila*, but was outcompeted by *X. nematophila*. We also found that the cell density of the *xspS* strain increased between 24 h and 48 h, but to a lesser extent than the wild-type strains, while *X. nematophila* was not fully inhibited. This indicates that xenorhabdicolin contributed to the full inhibition of *X. nematophila* under natural conditions of the insect hemolymph.

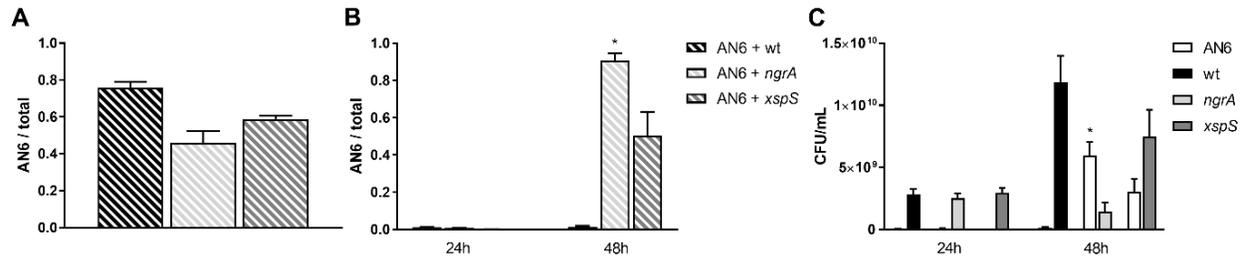


Figure 6. Pairwise *in-vivo* competition between *X. szentirmaii* strains and *X. nematophila* AN6 in *M. sexta*. (A) Co-cultures of *X. nematophila* and *X. szentirmaii* strains injected into *M. sexta* larvae at the ratios indicated at time 0. The competition outcome was determined by dilution plating of hemolymph obtained at 24 h and 48 h post-injection. (B) Data reported as the ratio of *X. nematophila* AN6 relative to the total colonies counted and (C) the number of colony forming units (CFU)/ml. Asterisks depict statistically significant differences ($P < 0.05$) as calculated by Wilcoxon matched-pairs signed rank test using GraphPad Prism.

3.2.6. MALDI-TOF analysis of infected hemolymph

To detect NRPS-derived compounds produced in the infected insect, we collected hemolymph from insects injected with either the wild-type or *ngrA* strain of *X. szentirmaii*, or co-injected with the wild-type strain and *X. nematophila* AN6, and subjected these samples to MALDI-TOF analysis (Fig. 7 A, B, C, respectively). In addition, hemolymph collected from insects injected with either *X. nematophila* alone or Grace's medium was analyzed. Two species, *m/z* 544 and *m/z* 558, were reproducibly detected in hemolymph from wild-type infected insects but were not present in hemolymph from insects infected with the *ngrA* strain. These two compounds were also present in hemolymph from insects co-infected with the wild-type strain and *X. nematophila*, but were not present in hemolymph from insects injected with either *X. nematophila* alone (Fig. 7D) or with Grace's medium (Fig 7E). The masses of these compounds were consistent with other peptide antibiotics isolated from *Xenorhabdus* species (32). These findings confirmed that *X. szentirmaii* produced NRPS-derived compounds in insects co-infected with *X. nematophila* and suggest that these compounds may function as antibiotics that inhibit growth of *X. nematophila* in co-infected insects.

3.3. Discussion

Here we present the novel finding that antibiotics of *X. szentirmaii* determine the competitive outcome between related *Xenorhabdus* species in the natural environment of the insect host. In the insect hemocoel *X. nematophila* was not inhibited when co-injected with the *ngrA* strain, but was strongly inhibited when coinjected with wild-type *X. szentirmaii*. In addition, two compounds were produced in *M. sexta* infected with wild-type but not in insects infected with the *ngrA* strain. Whether these compounds possess antibiotic activity remains to be

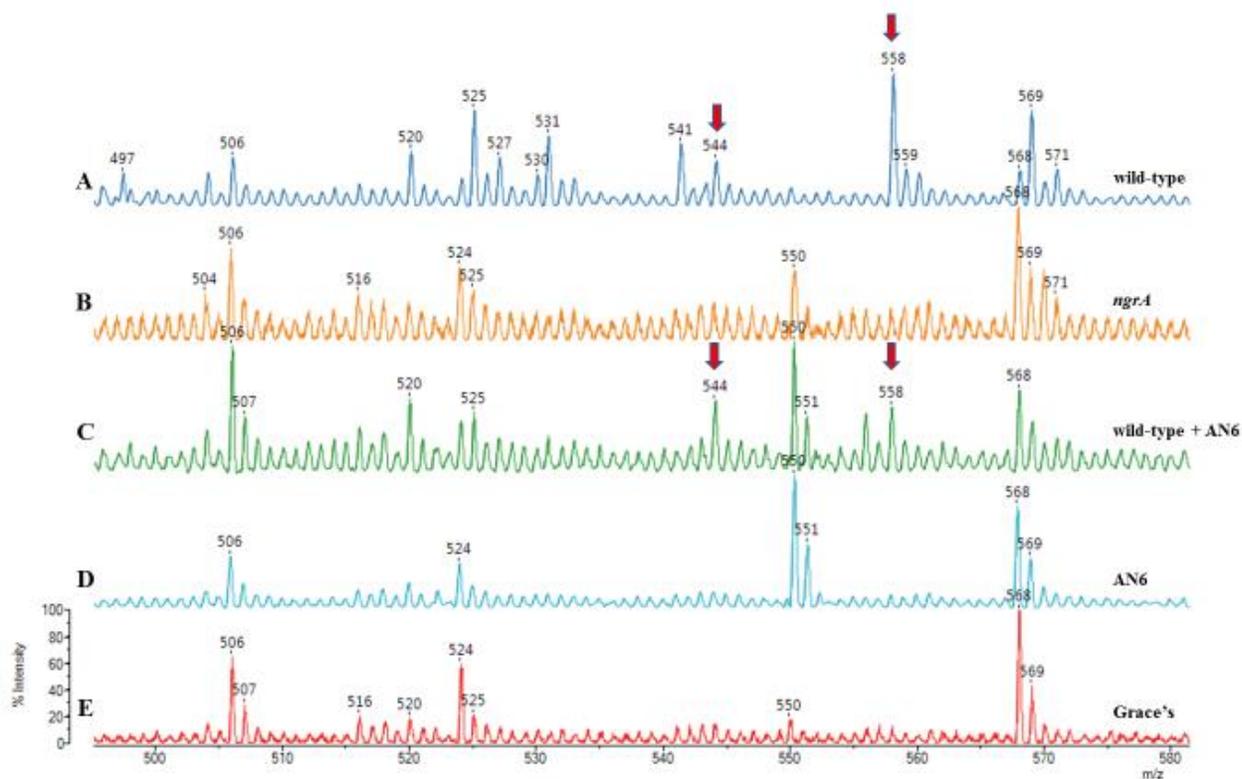


Figure 7. NRPS-derived compounds produced in hemolymph from infected insects detected by MALDI-TOF. Compounds detected in hemolymph of *M. sexta* injected with the following strains: (A) wild-type *X. szentirmaii*, (B) *ngrA* strain of *X. szentirmaii*, (C) wild-type *X. szentirmaii* and *X. nematophila*, (D) *X. nematophila* alone or (E) Grace's medium. Two species, *m/z* 544 and *m/z* 558, reproducibly detected in hemolymph from wild-type infected insects but were not present in hemolymph from insects injected with the *ngrA* strain, *X. nematophila* AN6 or Grace's medium, are indicated by arrows.

determined. Furthermore, in Grace's medium, *X. nematophila* grew in the presence the *ngrA* strain, but was inhibited when cocultured with the wild-type and *xspS* strains of *X. szentirmaii*. These findings support the idea that during natural infections of insect hosts, *X. szentirmaii* produces antibiotics at sufficient concentrations to inhibit growth of other species of *Xenorhabdus* and *Photorhabdus*, and presumably gut microbial competitors. To our knowledge, this is the first direct demonstration of the inhibitory role of antibiotics in interspecies competition in a natural host environment.

The question of whether inhibitory concentrations of antibiotics are produced in the hemolymph of infected insects has been addressed in the well-studied species *X. nematophila*. Maxwell *et al.* (33) demonstrated that extracts of homogenized *Galleria mellonella* killed by *X. nematophila* displayed antibiotic activity against a diverse range of bacteria. However, a subsequent report questioned whether *X. nematophila* antibiotics were produced at sufficient concentrations in infected *G. mellonella* to inhibit bacterial competitors (34). We found that *S. saprophyticus* was eliminated from the hemolymph of *M. sexta* co-injected with either wild-type or the *ngrA* strain of *X. nematophila*, indicating that the *ngrA*-dependent antibiotics were not required to inhibit growth of a sensitive bacterium (21). In addition, using mass spectrometry we have tried to identify the major antibiotic xenocoumacin in hemolymph of *M. sexta* infected with *X. nematophila*. So far, our attempts have been unsuccessful (unpublished data). Thus, the question of whether *X. nematophila* produces sufficient levels of antibiotics to inhibit potential competitors in the hemolymph remains unresolved. These findings raise the possibility that the production of antibiotics in infected insects may vary for different species, and strains of *Xenorhabdus* and may depend on the insect host in which the bacteria are growing.

The above findings support the hypothesis that *X. szentirmaii* and *X. nematophila* employ different strategies to compete against related species. *X. szentirmaii* appears to rely primarily on antibiotics to inhibit growth of *Xenorhabdus* and *Photorhabdus* species, while the contribution of Xsp xenorhabdycin to competitive inhibition was less pronounced. In contrast, Xnp xenorhabdycin is required to inhibit growth of *P. luminescens* in co-infected insects while *X. nematophila* antibiotics are not active against *Xenorhabdus* and *Photorhabdus* species. These findings suggest that *X. szentirmaii* produces potent antibiotics that may be expressed at high levels in the insect hemocoel. We have identified >30 NRPS genes in the *X. szentirmaii* genome, several of which may be involved in antibiotic synthesis. We identified NRPS genes for production of rhabdopeptides, linear nonpolar peptides with cytotoxic activity towards insect hemocytes (35), and the large NRPS gene for the synthesis of GameXPeptides produced by *Xenorhabdus* and *Photorhabdus* species (36). The function of GameXPeptide is not presently known. Interestingly, the production of GameXPeptide in *X. szentirmaii* was dramatically increased by ectopic production of the LeuO regulatory protein from *X. nematophila*. In *X. nematophila*, LeuO differentially regulates NRPS genes, while Lrp positively regulates NRPS genes (37). In addition, we have shown that NRPS genes are negatively regulated by OmpR (17). Whether elevated expression of NRPS genes in *X. szentirmaii* confers the ability to inhibit growth of related bacteria in the insect hemolymph remains to be determined. In this regard, comparative analysis of the regulatory circuitry of NRPS genes in *X. szentirmaii* and *X. nematophila* grown under natural conditions could help elucidate differences in the regulatory mechanisms that underlie the different competitive strategies of the respective bacteria.

Besides producing high levels of antibiotic activity, *X. szentirmaii* rapidly kills insect hosts such as *M. sexta* (unpublished data). In addition, the *S. rarum*-*X. szentirmaii* complex has

been shown to be highly virulent towards several agricultural insect pests (29, 30). A trade-off between host exploitation (virulence) and competitive ability has been demonstrated for related strains of *X. bovienii* (38). *X. bovienii* CS03 produced high levels of antibiotic activity against diverse bacteria, but was nonvirulent when injected into insects, while *X. bovienii* SS2004 gained a fitness advantage by effectively killing and exploiting the host, but did not produce antibiotics against most of the bacterial targets tested. *X. szentirmaii* represents an alternative strategy that includes both a high level of virulence and production of antibiotics that are active against both gut microbes and other species of *Xenorhabdus*. Whether trade-offs occur in other aspects of the *S. rarum*-*X. szentirmaii* life cycle, such as lower levels of nematode fitness (39) or reduced nematode colonization (40) remains to be determined.

The contribution of *ngrA*-derived antibiotics to competitive outcome was not detected in competition experiments in LB broth in which *X. nematophila* was eliminated in co-cultures with the *ngrA* strain. It appears that *X. szentirmaii* produced an *ngrA*-independent inhibitory activity in LB co-cultures that was not present in co-cultures in Grace's medium or in infected insects. We also found that antibiotic activity of supernatants from LB broth cultures was considerably greater than supernatants from cultures in Grace's medium. Similarly, we showed previously that total antibiotic activity and xenocoumacin levels in supernatants from LB cultures of *X. nematophila* were much higher than in supernatants from Grace's medium (21). Growth of *Xenorhabdus* species in nutrient-rich laboratory broth apparently enhances the production of secondary metabolites that facilitate isolation and identification of antibiotic compounds. On the other hand, the inhibitory activities produced in laboratory medium but not under more natural growth conditions may mask the contribution of antibiotics to interspecies competition. These findings point to the value of assessing interspecies competition and the production of antibiotics

in natural environments (41, 42). Furthermore, some antibiotics may be produced during growth in natural environments but not in laboratory medium, providing an opportunity to discover novel antibiotic compounds.

The role of antibiotics in a natural environment has been widely studied in the symbiosis involving the fungus-growing attine ant and actinomycetes bacteria (43–45). *Pseudonocardia* commonly associated with the attine ants produce a cyclic depsipeptide, dentigerumycin, that inhibits the *Escovopsis* fungal pathogen when grown on agar medium or in liquid broth (46). The NRPS-containing biosynthetic cluster for dentigerumycin biosynthesis has recently been identified (47). *Pseudonocardia* strains also produce antibacterial activity against gram-negative and gram-positive bacteria (48). In addition, ant-associated *Streptomyces* strains thought to be acquired from the nest environment produce the fungicidal agent nystatin that may also participate in interspecies competition (49). Thus, numerous types of antibiotic activities are potentially involved in sustaining the multipartite attine ant symbiosis. It is not yet known whether a few or several antimicrobial compounds are produced in sufficient quantities in nature to inhibit *Escovopsis* and other competitors. In this regard, further studies on the production and function of antibiotics of *Xenorhabdus* species in natural environments will help us better understand the role of antibiotics in interspecies competition in microbial communities.

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with statistical analyses. SF and KC conceived the study and designed the experiments. KC performed all the experiments and was primarily involved with the data analysis. SM performed and interpreted the MALDI-TOF experiments. SF and KC wrote the manuscript. All authors read and approved the final manuscript. The authors declare that they have no competing or conflicting interests.

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CURRICULUM VITAE

KRISTIN J. CIEZKI

Place of birth: Milwaukee, WI USA

Education

Ph.D. 2010-17. University of Wisconsin – Milwaukee, Milwaukee, WI

Molecular Microbiology

Thesis: New insights into the role of antimicrobials of *Xenorhabdus* in interspecies competition

M.S. 2006-08. University of Wisconsin – Milwaukee, Milwaukee, WI

Molecular Microbiology

Thesis: Molecular analysis of the xenocoumacin gene cluster of *Xenorhabdus nematophila*

B.S. 2002-05. University of Wisconsin – Milwaukee, Milwaukee, WI

Biological Sciences major, Chemistry teaching minor

Publications

- Kristin Ciezki, Kristen Murfin, Heidi Goodrich-Blair, Patricia Stock and Steven A. Forst. R type bacteriocins in related strains of *Xenorhabdus bovienii*: Xenorhabdycin tail fiber modularity and contribution to competitiveness. *FEMS Microbiol Lett.* 2017 Jan;364(1)
- Dongjin Park, Eliot Stanton, Kristin Ciezki, Daniel Parrell, Matthew Bozile, Daniel Pike, Steven A. Forst, Kwang Cheol Jeong, Renata Ivanek, Dörte Döpfer, and Charles W. Kaspar. Evolution of *stx2*-prophage in persistent Bovine *Escherichia coli* O157:H7 strains. *Appl Environ Microbiol.* 2013; 79(5): 1563-72
- Dongjin Park*, Kristin Ciezki*, Ransome van der Hoeven, Swati Singh, Daniela Reimer, Helge B. Bode, and Steven Forst. Genetic analysis of xenocoumacin antibiotic production in the mutualistic bacterium *Xenorhabdus nematophila*. *Molecular Microbiology* 2009; 73(5): 938-949

Posters

- **Second place poster prize.** Kristin Ciezki, Kishore Reddy Venkata Thappeta, Nydia Morales-Soto, and Steven Forst. The role of xenorhabdycin of *Xenorhabdus bovienii* in

reciprocal interspecies competition in a natural host environment. Perlman Symposium on Antibiotic Discovery and Development. April 12, 2013. Madison, Wisconsin.

- Kishore Reddy Venkata Thappeta, Kristin Ciezki, Nydia Morales-Soto, and Steven Forst. Comparative analysis of P2 phage-derived bacteriocins in *Xenorhabdus*. Perlman Symposium on Antibiotic Discovery and Development. April 13, 2012. Madison, Wisconsin.

Oral Presentation

“Weapons of bacterial warfare: *Xenorhabdus* antimicrobials target natural competitors”
Biological Sciences Colloquium, University of Wisconsin – Milwaukee, February 2016.

Research Experience

- Characterization of bacteriocin and small molecule antibiotic production, regulation, specificity, and contribution to competition in *Xenorhabdus* 2012-present
- Transmission electron microscopy studies of phage particles produced by natural variants of *E. coli* O157:H7 2011-13
- Isolation and characterization of algal phytochrome from *Micromonas pusilla* 2010-12
- PI-initiated and industry-sponsored primary research and clinical drug trials in pediatric gastroenterology 2008-10
- Genetic analysis of xenocoumacin antibiotic production in *Xenorhabdus nematophila* 2007-08
- Determination of boundaries of anticancer agent FK228 gene cluster 2006-07
- Assembly of GFP construct, measuring expression of bacterial motility genes 2006

Teaching Experience

- Teaching Assistant, TA Coordinator – Genetics 2010-present
University of Wisconsin – Milwaukee
- Teaching Assistant, Guest Lecturer - Anatomy and Physiology I, lab 2006-07,16
University of Wisconsin – Milwaukee
- Upward Bound Chemistry Teacher 2013
Marquette University – Milwaukee, Wisconsin
- Teaching Assistant - Anatomy and Physiology II, lab 2008
University of Wisconsin – Milwaukee

- Upward Bound Teacher and Tutor 2004-07
 Spanish instructor
 Tutor of Physics, Chemistry, Biology, Mathematics
 University of Wisconsin – Milwaukee
- High School Science Teacher 2005-06
 Bilingual Chemistry, Biology, Physical Science
 Loyola Academy – Milwaukee, WI
 Milwaukee Public Schools, Alternative High School
- Study Group Monitor and Tutor (Undergraduate Teaching Assistant) 2001-02
 Alverno College – Milwaukee, WI
 Foundations for Natural Science I (chemistry) and II (biology)

Clinical Research Experience

- A Multi-Center, Double-Blind Study To Evaluate The Safety, Efficacy And Pharmacokinetics Of The Human Anti-TNF Monoclonal Antibody Adalimumab In Pediatric Subjects With Moderate-To-Severe Crohn's Disease 2008-10
- A Multi-Center, Open-label Study of the Human Anti-TNF Monoclonal Antibody Adalimumab to Evaluate the Efficacy and the Long-Term Safety and Tolerability of Repeated Administration of Adalimumab in Pediatric Subjects with Crohn’s Disease Who Have Demonstrated a Clinical Response in the M06-806 Study 2008-10
- A Phase III, Randomized, Open-Label, Parallel-Group, Multi-Center Trial To Evaluate The Safety And Efficacy Of Infliximab (REMICADE®) In Pediatric Subjects With Moderately To Severely Active Ulcerative Colitis 2008-10
- A Multicenter International Study Of The Long-Term Safety Of Infliximab (REMICADE) In Ulcerative Colitis RESULTS UC: Remicade Safety Under Long-Term Study In Ulcerative Colitis 2008-10

- A Multi-Center, International, Observational Study Of The Long-Term Safety Of Infliximab (REMICADE) Results: Remicade Safety Under Long-Term Study 2008-10
- A Multi-Center, Prospective, Long-Term, Observational Registry Of Pediatric Patients With Inflammatory Bowel Disease 2008-10
- Pediatric (Inflammatory Bowel Disease) IBD Registry 2008-10
- Genetic Markers As Predictors Of Phenotypes In Pediatric Onset Crohn's Disease 2008-10
- An Efficacy and Safety Study of Reslizumab in the Treatment of Eosinophilic Esophagitis in Subjects Aged 5 to 18 Years 2008-10
- An Open-Label Safety and Efficacy Study of Reslizumab (CTx55700) for the Treatment of Pediatric Subjects with Eosinophilic Esophagitis Who Completed Study Res 5 0002 (Protocol Res 5 0004) 2008-10
- Predicting Response To Intravenous Corticosteroid Therapy In Pediatric Patients With Ulcerative Colitis 2008-10
- Risk Stratification And Identification of Immunogenetic and Microbial Markers Of Rapid Disease Progression In Children With Crohn's Disease 2008-10

Work Experience

- Medical College of Wisconsin – Milwaukee, WI 2008-10
 Department of Pediatrics, Gastroenterology
 Children’s Hospital of Wisconsin
 Clinical Research Coordinator
- Aurora St. Luke’s Medical Center – Milwaukee, WI 2006-07
 Inpatient Pharmacy Technician
- UWM TRIO and Pre-College Programs 2004-07
 University of Wisconsin – Milwaukee, Milwaukee, WI
 GEAR UP tutor
 Upward Bound tutor and teacher
- Alverno College Admissions Office 2001-04

Alverno College – Milwaukee, WI
Telecounseling Supervisor / Evening Office Supervisor
Receptionist, Telecounselor, Alverno Ambassadors
Coordinator

Professional Development

- CCMP Phytoplankton Culturing Techniques Course 2011

Honors and Awards

- Proposal accepted for sequencing the genomes of a *Xenorhabdus* species and an *Enterococcus Faecalis* strain as part of the Great Lakes Genomics Center next-gen sequencing pilot project, 2013.
- Louise Neitge Mather Scholarship
- Ruth Walker Graduate Award 2012, 2014
- UWM Chancellor's Award
- Rose Provasoli Scholar
- Roosevelt Scholar
- Harcourt Scholar
- Milwaukee Area Retired Teachers Association Scholarship

Leadership Experience

- Trained, co-mentored, and designed research projects for 10 undergraduate research students
- Managed campaigns for the Milwaukee Party (non-partisan UWM student government).
Member of the year, 2005
- Counseled Gear Up students and served as an in-class tutor at South Division High School
- Supervised the Upward Bound tutoring staff and mentored pre-college students
- Assisted in facilitating ESL sessions for Spanish-speaking immigrants
- Lead Discovery World workshops as an educator
- Organized a college community service organization and served as charter president