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Characterization of Antimicrobial Activity of the Symbiotic Bacterium Xenorhabdus Szentirmaii

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CHARACTERIZATION OF ANTIMICROBIAL ACTIVITY OF THE SYMBIOTIC

BACTERIUM *XENORHABDUS SZENTIRMAII*

by

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ABSTRACT

CHARACTERIZATION OF ANTIMICROBIAL ACTIVITY OF THE SYMBIOTIC
BACTERIUM *XENORHABDUS SZENTIRMAII*

by

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Xenorhabdus species belonging to the Enterobacteriaceae family are gram negative

bacteria that engage in symbiotic associations with the entomopathogenic soil dwelling

nematodes of the family *Steinernematidae.* In this bacterium–nematode mutualistic interaction,

both partners benefit from the association. By entering and perforating the insect gut and

translocating to the body cavity of hemocoel, the nematodes gain access to the nutrient resource

of the insect hemolymph. *Xenorhabdus* spp are released in the hemolymph in which they

produce toxins and immunosuppressive compounds that kill the insect. During the infection

Xenorhabdus spp grow to high cell density in the hemolymph. As the nematode invades the

insect hemocoel gut microorganisms gain access to the hemocoel. Thus, *Xenorhabdus* spp

compete for space and nutrients with other microorganisms. *Xenorhabdus* spp produce an array

of antimicrobial compounds that act to suppress the growth of the gut microorganisms providing

a competitive advantage for growth in the hemolymph. *Xenorhabdus* spp possess genes that code

for non-ribosomal peptide synthetases (NRPS) that use specific amino acids to synthesize

peptide antibiotics. Synthesis of the antibiotics requires a prosthetic group that is attached by an

enzyme encoded by the *ngrA* gene. One species, *Xenorhabdus szentirmaii* was shown to produce

high levels of antibiotics. 10 different NRPS gene clusters have been identified in *X. szentirmaii*

that are potentially involved in synthesizing antibiotics. Mutant strains were previously constructed by insertional inactivation in each of the NRPS gene clusters. In the present study, antimicrobial activity of wild type, *ngrA* and the NRPS mutant strains *xsz3* and *xsz8* was examined. Antibiotic activity was assessed against *Staphylococcus saprophyticus*, *Aerococcus viridans*, and uncharacterized species of yeast, all isolated from the gut of tobacco horn worm (*Manduca sexta*). Amplification and sequencing of the 5.8S rRNA gene identified the uncharacterized yeast species as *Candida orthopsilosis*. Three different assays were used to assess the antibiotic activity in strains in which the NRPS genes *xsz3* and *xsz8* were inactivated. Antibiotic overlay assays showed that antibiotic activity in the *xsz3* and *xsz8* mutant strains was significantly reduced against *S. saprophyticus* and *C. orthopsilosis* whereas against the less sensitive *A. viridans* the antibiotic activity of the *xsz8* strain was more significantly reduced than the *xsz3* strain. In cell-free supernatant assays antibiotic activity of the *xsz8* strain was more noticeably reduced than the *xsz3* strain against *S. saprophyticus,* whereas against *C. orthopsilosis* antibiotic activity of the *xsz3* strain was more markedly reduced than the *xsz8* strain. In *ex vivo* competition experiments in Grace's insect medium with *S. saprophyticus* antibiotic activity of the *xsz3* strain was more markedly reduced than in the *xsz8* strain. In *ex vivo* competition experiments in YEPD medium, the *ngrA* strain that does not produce NRPS antibiotics suppressed the growth *C. orthopsilosis* as well as the wild type strain indicating that growth inhibition occurred in the absence of NRPS antibiotics. The significance of these findings will be discussed in this study.

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Introduction

Microorganisms employ several strategies to compete for space and nutrients [1]. The production of antimicrobial agents is one of the strategies by which they gain competitive advantage. Numerous bacteria can produce one of several different classes of antagonistic compounds that includes antibiotics, phage-derived bacteriocins and proteinaceous bacteriocins [2]. Most bacterial species produce a limited number of antagonistic compounds. Bacteria of the genus *Xenorhabdus* can produce three different classes of antagonists: small-molecule antibiotics, phage-derived R-type bacteriocins and colicin-type protein bacteriocins [3]. *Xenorhabdus* spp are associated with soil entomopathogenic nematodes (EPNs) of the family *Steinernematidae*. The bacteria–nematode complex has developed a sophisticated relationship in which both partners benefit from the mutualistic and parasitic associations. During the association of the two, the nematode-bacteria pair acts as a parasite to infect diverse insects. *Xenorhabdus* possesses two distinct stages in its life cycle [4,5]. *Xenorhabdus* spp are mutualists with steinernematid nematodes where they colonize a specialized region in the intestine of infective juvenile (IJ) stage of the nematode called the receptacle. The free-living IJ carrying the *Xenorhabdus* symbiont invade diverse soil insects, migrates to the insect midgut and perforate intestinal walls through which the IJ enters the hemocoel where the *Xenorhabdus* bacteria are released into the hemolymph of the insect [6]. The bacteria transition to the pathogenic stage by producing several toxins and exoenzymes, and compounds that inactivate or suppress the immune system of the insect. During the infection *Xenorhabdus* spp grow to high cell density in the hemolymph. Microorganisms derived from the insect gut also translocate to the hemocoel during nematode invasion. Some of these bacteria can proliferate in the insect hemolymph competing for nutrient resources and space [7,8]. Antimicrobial compounds produced by

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Xenorhabdus spp are presumed to suppress the growth of these microbial competitors [7]. The insect can be co-invaded by other species of EPNs which carries other closely associated bacteria like *Photorhabdus*. To compete against these species, *Xenorhabdus* spp utilizes phage-derived bacteriocins [3,9].

Of the numerous species of *Xenorhabdus* that have been identified, *Xenorhabdus nematophila* has been studied most extensively [4]. Several small molecule antimicrobial compounds produced by *X. nematophila* have been characterized. The genome of *X. nematophila* contains several gene clusters which code for non-ribosomal peptide synthetases (NRPS) and polyketide synthases (PKS) that generate secondary metabolites including antibiotics, virulence factors and immunosuppressive compounds [6]. NRPS enzymes consist of three domains; an adenylation domain (A) that binds specific amino acids, a peptidyl carrier protein transfer domain (PCP) that shuttles amino acids to the condensation domain (C) that accepts amino acids and catalyzes peptide bond formation [10]. The *X. nematophila* genome codes for seven NRPS and NRPS/PKS gene clusters and six discrete NRPS genes [7,8]. Seven compounds produced by NRPS and NRPS-PKS hybrid clusters have been described: Xenocoumacins, Xenematide, PAX peptides, Rhabdopeptides, Xenortide, Nematophin and Odilorhabdin (Fig.S1). While Xenocoumacins, PAX peptides, Nematophin and Odilorhabdin have been shown to possess antimicrobial activity, the mode of action has only been elucidated for Odilorhabdin that specifically binds to the 16S rRNA molecule of the small ribosomal subunit producing misincorporation mutations [11-13].

The enzyme phosphopantetheinyl transferase (PPTase) encoded by the *ngrA* gene adds a 4'phosphopantetheinyl (4'PP) moiety to the PCP domain of NRPS proteins. 4'PP contains a free sulfhydryl group that attaches to activated amino acids and transfers the amino acids to the

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condensation domain during the biosynthesis of antibiotics [14]. Inactivation of *ngrA* thus abolishes the synthesis of NRPS-derived secondary metabolites including antibiotics. Thus, unlike the wild-type strain, the *ngrA* strain does not suppress the growth of microbial species derived from the insect gut [8]. In addition, *ngrA*-dependent secondary metabolites produced by *X. nematophila* were required for optimal growth and development of the nematode partner in the insect [7,8]. NRPS-derived antibiotics are generally more active against non-related species. *Xenorhabdus* spp also produce phage tail-like R-type bacteriocins that kill related *Xenorhabdus* and *Photorhabdus* species [3,9].

The tobacco hornworm (*Manduca sexta)* has been used as a model insect to examine pathogenicity and interspecific competition involving *X. nematophila.* During invasions of *M. sexta* by the nematode *S. carpocapsae* several bacterial species and an unidentified yeast species were shown to translocate from the insect gut to the hemolymph [8]. The gut-derived bacterium *Staphylococcus saprophyticus* appeared in the hemolymph during the initial phase of infection and was eliminated early while *Enterococcus faecalis* proliferated in the hemolymph even when *X. nematophila* was present at high cell density [6,8]. *S. saprophyticus* was found to be sensitive to the antibiotics of *X. nematophila* while *E. faecalis* was relatively insensitive [7,15]. Antibiotic overlay assays revealed that *X. nematophila* also produced *ngrA*-dependent inhibitory activity against the unidentified yeast species [15]. In LB broth cultures of *S. saprophyticus* co-inoculated with the wild-type strain of *X. nematophila*, *S. saprophyticus* was eliminated. In-contrast, in co-cultures of *S. saprophyticus* and the *ngrA* strain, *S. saprophyticus* was able to grow indicating that the *ngrA*-dependent antibiotics were required to eliminate the competitor under LB broth conditions. When *S. saprophyticus* was injected into *M. sexta* it persisted at low levels. Unexpectedly, when *S. saprophyticus* was co-injected with either wild-type *X.*

nematophila or the *ngrA* strain, *S. saprophyticus* was eliminated suggesting that under natural biological conditions *X. nematophila* may utilize *ngrA*-independent antimicrobial compounds or other competitive mechanisms to eliminate some sensitive competitors [7,8]. The role of *ngrA*-dependent antibiotics of *X. nematophila* in interspecific competition in the insect hemolymph during natural infections remains to be determined.

Xenorhabdus szentirmaii, the symbiont of the nematode *Steinernema rarum*, was found to produce high levels of antibiotic activity [16]. Unlike most species of *Xenorhabdus,* the antibiotics of *X. szentirmaii* were active against other species of *Xenorhabdus* [17]. In competition experiments in insect cell culture medium (Grace's insect medium) co-inoculated with *X. szentirmaii* and *X. nematophila, X. szentirmaii* grew to higher levels while growth of *X. nematophila* was inhibited. In contrast, *X. nematophila* was able to grow when co-inoculated with the *ngrA* strain of *X. szentirmaii.* Likewise, *in vivo* competitions in *M. sexta* showed that *X. nematophila* was strongly inhibited by wild-type *X. szentirmaii* but not by the *ngrA* strain [18]. These findings indicate that the *ngrA*-dependent antibiotics of *X. szentirmaii* were required to inhibit growth of *X. nematophila* both *in vitro* and *in vivo* [18,19]. The precise NRPS and PKS clusters of *X. szentirmaii* involved in the biosynthesis of active antibiotics that are active against *X. nematophila* are not presently known.

Two NRPS-derived products (xenofuranones and szentiamides) isolated from broth cultures of *X. szentirmaii* did not exhibit antibiotic activity [20,21]. In a subsequent study using high-resolution electrospray ionization mass spectroscopy, six additional natural products were identified [22]. One NRPS-derived compound (xenematide) was shown to have antibiotic activity [23]. A draft genome sequence of *X. szentirmaii,* was published in 2014 [24]. The NRPS containing gene clusters for several of the natural products were identified [20,24]. In addition,

the gene cluster for the antimicrobial compound fabclavine was identified even though the compound was not previously isolated from broth cultures. Fabclavines are complex hybrid peptide-polyketide-polyamino natural products. They are composed of a core structure made up of 6 amino acids attached to a 36 carbon polyamino alkyl chain. The gene cluster containing the NRPS and PKS genes involved in fabclavine production has been identified and a biosynthetic pathway has been proposed [25]. Fabclavines were shown to also be produced by *X. budapestensis* and *X. cabanillasii.* The fabclavines of *X. cabanillasii* were active against the antibiotic-resistant dental root canal pathogen *Enterococcus faecalis* [26]. In addition, fabclavine enriched supernatant fractions of *X. budapestensis* were shown to cause feeding deterrence in several deadly mosquito vectors [27]. The feeding deterrent activity of the fabclavine fractions were like that of DEET, a widely used mosquito repellent compound.

Recent studies with *X. szentirmaii* showed that it also exhibits antifungal activity against fungal phytopathogens such as the soybean pathogen *Sclerotinia sclerotiorum* and the chestnut tree pathogen *Cryphonectria parasitica* [28,29]. Using a promoter-activated compound identification method it was shown that the fabclavines were the antifungal compound that conferred the inhibitory activity against *C. parasitica* [29]. Here in this study, a yeast species was isolated from the gut of insect host *M. sexta*, which was sensitive to the antibiotics produced by *X. szentirmaii*. The respective yeast strain was identified as *Candida orthopsilosis* by using PCR amplification of 5.8S rRNA. The final assembly of *C. orthopsilosis* genome contains 12.6 Mb on 8 chromosomes [30]. Out of several species of *Candida*, *C. orthopsilosis* was found in the digestive tract of plant associated beetles and other insects [31]. It is also considered as a human pathogen causing cellular damages [32]. Also, *X. szentirmaii* cell free supernatants were shown to synergize the effectiveness of commercial fungicides against fungal plant pathogens [33].

The goal of the present study is to elucidate NRPS gene clusters that were required to impart antimicrobial activity in *X. szentirmaii.*

Materials and Methods

Bacterial and yeast strains, media, and growth conditions

Bacterial and yeast strains used in this study are given in Table 1. All media storage and incubations were done in the dark. Liquid media used in this study are Lysogeny broth (LB), Grace's Insect cell culture (Gibco) and Yeast Extract Peptone Dextrose (YEPD). LB media was covered with dark cloth throughout the preparation. LB media consisted of 1.0% tryptone (Bacto), 0.5% yeast extract (Difco), 0.5% NaCl (JT Baker) and 1.2 mL/L 0.81M $MgSO_4$ (Fisher). Plate media was solidified with 1.5% agar (Difco). Grace's media (GM) was prepared per directions. For 500 mL of Grace's media, 22.85g of Grace's insect media powder was added to 500 mL distilled water and the pH was adjusted to 6.1. YEPD was made with Yeast extract (Difco) 10%, Peptone- 0.2%, Agar- 0.2%, and Dextrose- 40%. Initial bacterial cultures were streaked from -80 °C glycerol stocks onto either LB agar plates supplemented with ampicillin (50 µg/mL) for wild-type *X. szentirmaii* or ampicillin (50 µg/mL) and chloramphenicol (25 µg/mL) for mutants and LB plates without any antibiotics for *S. saprophyticus* and *A. viridans*. Plate cultures were grown at 28 °C for 24-48 h and stored at room temperature (22 °C) for up to one week. Grace's insect medium and YEPD was also used for dilution plating as indicated. Liquid cultures were incubated on a shaker at 30 °C.

TABLE 1. Bacterial and other strains used in this study

Strains	Nematode or description	Geographic location	Source
X. szentirmaii	*S. rarum*	Argentina	A. Fodor
Mutant strains			
ngrA::Cm	*S. rarum*	Argentina	Laboratory stock
xsz3::Cm			Laboratory stock
xsz7::Cm			Laboratory stock
xsz8::Cm			Laboratory stock
xsz9::Cm			Laboratory stock
xsz11::Cm			Laboratory stock
Insect gut isolates			
*Enterococcus faecalis**	*M. sexta* isolate		Laboratory stock
*Aerococcus viridans**	*M. sexta* isolate		Laboratory stock
*Staphylococcus saprophyticus**	*M. sexta* isolate		Laboratory stock
Yeast	*M. sexta* isolate		Laboratory stock

*Cm-chloramphenicol resistance. From Singh *et al*. [15].

Chromosomal preparation and amplification 5.8S rRNA gene to identify the yeast species isolated from insect gut

Genomic DNA was prepared by adding enough colonies from the plate containing yeast to a microcentrifuge tube with 200 µL of binding buffer and 100 µL of glass beads. The cells were vortexed at 4 °C for 10 mins, 200 µL of phenol was added and inverted 3-4 times. The tubes were spun at 10000 rpm at 4 °C, the aqueous layer was taken into a microcentrifuge tube and 200 µL equal volumes of chloroform: isoamyl alcohol solution was added at room temperature and inverted 3-4 times. The solution was centrifuged at 10000 rpm for 10 min, the top layer was taken into a new tube, 400 µL of binding buffer was added and solution was centrifuged at 10000 rpm for 2 mins. The flow-through was discarded, 500 µL of washing buffer added, tubes were centrifuged and flow through was discarded. DNA was eluted with 30-50 µL of 1X TE buffer. A nanodrop measurement was done to assess the concentration of the DNA fragments. Two PCR reactions of final volume 20 µL were made; PCR super mix (10 µL),

ddH$_2$0 (7 µL), forward primer ITS1 (1 µL), reverse primer ITS4 (1 µL) and genomic DNA (1 µL)

or (3 µL). The PCR conditions were 94 °C for 2 min, 94 °C for 30 sec, 48 °C for 30 sec, 65 °C

for 2 min, 65 °C for 10 min for 40 cycles. 10 kb ladder (Promega) was loaded into the first well.

The ITS/5.8S rDNA PCR products were examined by running 1 µL and 3 µL of each sample on

a 0.8% agarose gel at 150 V for 30 min. The PCR products were purified by adding 500 µL of

binding buffer, pipetting the solution onto a purification column that was spun at 10000 rpm for

1 min and then adding 500 µL of washing buffer after removing the flow through and

centrifuged again. DNA was eluted with 50 µL 1X TE by spinning at 10000 rpm for 2 mins.

Another 0.8% gel was run to confirm the PCR products. For DNA sequencing 5 µL of PCR

product, 5 µL of TE and 5 µL of ITS1/ITS4 primers were sent to Genewiz. Strain was identified

by querying the sequences in the nucleotide database of NCBI using BLASTN function.

Overlay assay for assessing antibiotic activity of wild-type *X. szentirmaii*, *ngrA* and mutants

For growth of *Xenorhabdus* strains, overnight cultures were prepared by scraping enough

colonies from the respective plates to 2 mL of LB media supplemented with ampicillin (50 µg/

mL) alone for wild-type and ampicillin (50 µg/mL) with chloramphenicol (25 µg/ mL) for

mutants (*ngrA, xsz3, xsz7, xsz8*, *xsz9* and *xsz11*) and grown for 18 h at 30 °C with shaking. After

incubation, 250 µL of the respective overnight culture was added to 5 mL of LB (1:20

subculture) and was grown to exponential phase at 30 °C for 4 h. These subcultures were grown

exponentially to an OD$_{600}$ (optical density at 600nm) of 0.2. Normalized 1 mL of subculture to

OD$_{600}$ =0.2, with LB using formula: $C_1V_1=C_2V_2$, (OD) (X) = (0.2) (1000) [X=volume in µL of

culture, LB= 1000-X µL]. 6 µL of the normalized subculture was spotted on LB agar. After 24 h

of incubation at 30 °C, cells were killed by exposing to chloroform vapors for 30 min, followed

by 30 min of air drying. The indicator strains *S. saprophyticus* and *A. viridans* were grown

overnight in 4 mL LB broth from glycerol stocks or plates at 30 °C plus shaking for 18 h. For growth of yeast, overnight cultures were made by growing in 4 mL YEPD media from glycerol stocks for 18 h. 1 mL of the overnight of tester strain was added to 12 mL top agar (LB with 0.7% agar) that was subsequently poured over the *X. szentirmaii* colonies (used wild-type, *ngrA*, *xsz3, xsz7, xsz8* and *xsz9* against *S. saprophyticus* and used wild-type, *ngrA*, *xsz3, xsz7, xsz8* and *xsz11* against yeast and *A. viridans*). Zones of inhibition were photographed and measured after 24 h of incubation. Three biological experiments were done with highly reproducible results. Mean and standard error was calculated. Statistical analysis was performed by two-tailed paired Student's t-test.

Cell-free supernatant antibiotic activity in LB Medium cultures

Overnight cultures (250 µL) of wild-type or *ngrA* strains or other mutants of *X. szentirmaii* were sub-cultured in 5 mL LB broth and grown at 30 °C. Supernatants were collected at 48 h by centrifugation at 5000 rpm for 2 mins. The resultant supernatants were transferred to fresh tubes and centrifuged again and they were filter sterilized with 0.2 µm pore size cellulose acetate syringe filters. Cell-free supernatant aliquots were stored at 4 °C until use. 250 µL of overnight cultures of the tester strains were inoculated into 5 mL respective media (YEPD for yeast and LB broth for *S. saprophyticus*) and grown for 18 h. The tester strains were diluted 1:200 in LB broth. Triplicate samples were loaded LB into 96 well plates. Each of the mixture contained 30 µL of LB, 40 µL of cell free supernatant and 200 µL of the diluted tester strain in microcentrifuge tubes. The mixtures were vortexed before aliquoting. OD_{600} values were taken and recorded at 0 h, 8 h and 24 h time points. The plate was incubated at 30 °C with shaking. Growth was assessed by examining OD_{600} values for each sample or by growth inhibition (% inhibition) which is calculated by the formula $1-(OD_{600}$ treated culture/OD_{600} untreated culture)

x100. Three biological experiments were done and mean, standard error was calculated from data points. Statistical analysis was performed by two-tailed unpaired Student's t-test.

Ex vivo competition between *X. szentirmaii* and *S. saprophyticus* in Grace's Insect Medium

Overnight cultures (2 mL) of wild-type *X. szentirmaii* (LB +Ampicillin), *X. szentirmaii*-mutants (LB +Ampicillin+ Chloramphenicol) and *S. saprophyticus* in LB broth were grown for 14 h. For the wild-type *X. szentirmaii* and mutant strains, 1 mL of the overnight culture was centrifuged for 1 min at 5000-6000 rpm, supernatants were removed, added fresh LB (1 mL) and vortexed to mix the pellet thoroughly and the cultures were normalized to an OD_{600} of 0.4. 250 µL of each of normalized cultures were added to 5 mL LB broth and grown at 30 °C with shaking for 3 h. Subcultures were normalized to an OD_{600} of 0.25. Control cultures were inoculated with 250 µL of normalized cells in 5 mL of Grace's insect medium. Competition cultures were inoculated with 250 µL of normalized wild-type *X. szentirmaii* and mutants with 125 µL of normalized *S. saprophyticus* in 5 mL of Grace's insect medium. At 4 h, 8 h, and 12 h, colony-forming units/mL (cfu/mL) were calculated by dilution in Grace's insect medium and plating on LB agar. Three biological experiments were done with highly reproducible results. Mean and standard error was calculated from data points. Statistical analysis was performed by two-tailed unpaired Student's t-test.

Ex vivo competition between *X. szentirmaii* and yeast in YEPD

Overnight cultures (2 mL) of wild-type *X. szentirmaii* (LB +Ampicillin), *ngrA* (LB+ Ampicillin+ Chloramphenicol) and yeast in YEPD broth were grown for 14 h. For wild-type *X. szentirmaii* and *ngrA*, 1 mL of the overnight culture was centrifuged for 1 min at 5000-6000 rpm. Supernatants were removed, fresh LB (1 mL) was added and the pellet was vortexed thoroughly and the cultures were normalized to OD_{600} of 0.4. For wild-type *X. szentirmaii* and *ngrA*, used

individual normalized overnight culture to set up subcultures (1:20) by taking 250 µL of normalized overnight culture into 5 mL LB broth and grown at 30 °C for 3 h. For yeast, to set up subcultures (1:20) 250 µL of normalized overnight culture was added into 5 mL YEPD broth and grown at 30 °C with shaking for 3 h. Normalized all cultures to an OD_{600} of 0.25. For yeast, took out 250 µL of subculture, spun and discarded the supernatant. Then added 260 µL of fresh YEPD and vortexed the pellet to make it concentrated. Control and competition cultures were inoculated with 250 µL of normalized cells in 5 mL of YEPD. At 0 h, 4 h, and 8 h, colony-forming units/mL (cfu/mL) were calculated by dilution in YEPD medium and plating on LB agar. Three biological experiments were done with highly reproducible results. Mean and standard error were calculated from data points. Statistical analysis was performed by two-tailed unpaired Student's t-test.

Construction of NRPS mutant strains

The mutants used in this study were previously constructed by using insertional inactivation mediated by a plasmid [19]. NRPS genes of *X. szentirmaii* were identified using key word searches of the draft genome of *X. szentirmaii* with BlastP algorithm of MaGe Microbial Genome Annotation and Analysis Platform (http://www.genoscope. cns.fr/agc/microscope/ mage/ index. php) [24]. Ten NRPS mutant strains and two siderophore strains were constructed (Table 2). Strains in which NRPS genes XSR1v1_320044 and XSR1v1_ 630001 were inactivated are referred to as the *xsz3* and *xsz8* mutant strains respectively. To construct the mutant strains, a 400-500 bp fragment near the 5' end of the genes was amplified, the purified PCR product was blunt-end ligated into the *Eco*RV site of pSTBlue-1 and PstI and XbaI sites of the resulting recombinant plasmid were used to generate the fragment for further cloning. The gel purified fragments were ligated into the conjugal suicide vector pKnock-Cm which has Cm

resistant gene as well as oriR6K. The pKnock suicide vector can replicate in *Escherichia coli*

S17-1 (λ*pir*) that provides the π protein required for replication while pKnock is not able to

replicate in *X. szentirmaii* [34]. The recombinant plasmids were first transformed into competent

E. coli S17 cells followed by conjugal transfer into wild-type *X. szentirmaii*. pKnock inactivated

NRPS genes were selected on LB-Amp-Cm agar and correct inactivation was confirmed by PCR

[18,19].

TABLE 2. NRPS genes inactivated in *X. szentirmaii*.

MaGe Gene number	NCBI Gene number	Mutant names	Known NRPS products
XSR1v1_120064	WP_051462298.1	*xsz1*	
XSR1v1_210038	WP_084616209.1	*xsz2*	
XSR1v1_320044	WP_099139143.1	*xsz3*	
XSR1v1_420022	WP_099139142.1	*xsz4*	
XSR1v1_580001	WP_244590081.1	*xsz5*	
XSR1v1_800001	WP_156935919.1	*xsz6*	
XSR1v1_210002	WP_051462365.1	*xsz7*	PaxA
XSR1v1_630001	WP_099139155.1	*xsz8*	
XSR1v1_10216	WP_038233794.1	*xsz9*	Yersiniabactin
XSR1v1_300022	WP_038238739.1	*xsz10*	Aerobactin (hydroxamate)
XSR1v1_700001	WP_099139157.1	*xsz11*	Fabclavine
XSR1v1_720001	WP_038241744.1	*xsz12*	Fabclavine

Results

Previous studies indicated that *X. szentirmaii* produced higher antimicrobial activity than the more well-studied *X. nematophila*. *S. saprophyticus* was shown to be highly sensitive to the antibiotics produced by *X. szentirmaii* [18,19]. The uncharacterized yeast was moderately sensitive while *E. faecalis* and *A. viridans* were less sensitive. In addition, *X. szentirmaii* antibiotics were active against all strains of *X. nematophila* tested, though the sensitivity of the various strains was variable [19].

Assays used to study antimicrobial activity of *X. szentirmaii*

The loss of antimicrobial activity in NRPS mutant strains was analyzed with three different assays. Differences in the assay parameters could affect the type and concentration of antibiotics produced and detected.

In the antibiotic overlay assay the producer species forms a colony on solid agar and the indicator species mixed in soft agar is poured on top and allowed to solidify. Antibiotics that diffuse from the producer colony inhibit the growth of sensitive indicator strains forming a zone of inhibition. The diameter of the zone of inhibition is related to the concentration, diffusibility and potency of the various antibiotics and the sensitivity of the indicator strain used. Cells in the center of the colony are older and grow to higher cell density while cells at the edge of the colony are younger and have greater accessibility to nutrients of the solid media. Thus, the colony contains a heterogeneous mixture of cells in different physiological states. Since cells can grow to high density the amount of antibiotics produced around the colonies can reach to high concentrations. In addition, antimicrobial compounds that are more diffusible such as polar molecules will generally produce a wider zone of inhibition. Previous analyses of NRPS mutant strains of *X. szentirmaii* were performed by directly spotting overnight cultures on LB agar

(Fig.S3). In that case, antibiotics that accumulate in stationary phase cultures and other products that may be released from cell lysis are also included in the culture spotted on the solid surface. In the present study the overnight cultures were diluted in fresh LB broth and grown for 4 h. Spotting the exponentially growing cells more accurately assesses the production of antimicrobial compounds by cells growing on the solid surface.

In the cell-free supernatant assay, cultures are grown with shaking for 48 h. In this case, *X. szentirmaii* strains are grown under more aerobic conditions, there is less heterogeneity in the culture and diffusion in the solid medium is not a primary factor in assessing antibiotic activity. In *ex vivo* competition assay, the *X. szentirmaii* strains are grown in the presence of the competitor strain. Release of molecules by the competitor may provide signals for production of antimicrobials that would not be produced when cells are cultured alone. In previous *ex vivo* competition assays overnight cultures of *X. szentirmaii* were directly mixed with the competitor strains. In the present study, *X. szentirmaii* cultures were centrifuged to remove antibiotic compounds and other inhibitory molecules that were present in the stationary phase cultures. Pellets were resuspended in fresh medium and normalized to the same optical density.

Identification of uncharacterized yeast species

The antibiotic activity of *X. szentirmaii* was previously tested against bacteria and an uncharacterized yeast species isolated from the gut of *M. sexta*. To identify the uncharacterized yeast species the region containing the 150 bp 5.8S rRNA gene and flanking variable DNA intervening internal transcribed spacer (ITS) regions was amplified. The ITS1 forward primer (5'-TCCGTAGGTGAACCTGCGG-3') and reverse primer ITS4 (5'- TCCTCCGC TTATTGATATGC-3') were used to PCR amplify a 512 bp fragment that includes 5.8S rRNA and flanking region. The primers ITS1 and ITS4 are in upstream and downstream flanking

regions of 5.8S rRNA respectively (Fig.S2). This region has been widely used for molecular-based analysis for the identification and characterization of fungi [35-38]. 5.8S rRNA present in eukaryotic ribosomes are involved in synthesizing proteins and they also have a relatively conserved nucleotide sequences in fungi [36,37].

Genomic DNA was purified and the PCR reaction was performed using two different volumes (1 μL and 3 μL) of the genomic DNA. The size of the PCR product is expected to be 512 bp (Fig.S2). Analysis of PCR products showed a single band on the gel (Fig.1). The products were column purified and sent for sequencing to Genewiz (South Plainfield, New Jersey). The resulting sequences were trimmed for quality and used to identify the organism by querying the nucleotide (nt) database of NCBI using BLASTN for both the primers ITS1 and ITS4 (Table S1). BLASTN results showed percentage identities of 98.14% and 98.7% respectively for both the primer sequences (Table 3) and an E-value of 0 and this corresponds to the organism *Candida orthopsilosis* (Accession numbers: MW358890.1 and KC777376.1).
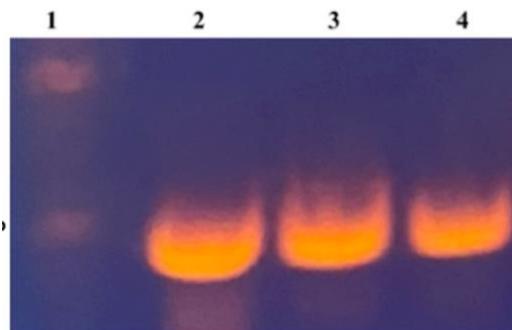
Figure. 1 - PCR products on 0.8% agarose gel at 150 V for 30 min. Lane 1, 10kb ladder. Lanes 2- 4, amplified 5.8S rDNA of *C. orthopsilosis* using 1 μL of DNA.

TABLE 3. NCBI BLASTN results for the ITS1 and ITS4 primer sequence. First two hits with more than 98% identities are listed.

Description and Scientific name	Maximum score	Total score	Query cover	E value	Percentage identity	Accession length	Accession Number
Results from ITS1 1. *Candida orthopsilosis isolate CP_6518-3*	763	763	100%	0.0	98.14%	518	MW358890.1
2. *Candida orthopsilosis strain Ortho/H-152*	761	761	100%	0.0	98.14%	525	KC777376.1
Results from ITS4 1. *Candida orthopsilosis strain Ortho/H-152*	822	822	98%	0.0	98.70%	525	KC777376.1
2. *Candida orthopsilosis isolate CP_6518-3*	819	819	98%	0.0	98.49%	518	MW358890.1

Analysis of NRPS mutant strains of *X. szentirmaii*

It was previously shown that *X. szentirmaii* displayed antibiotic activity against several strains of *X. nematophila* as well as microbes isolated from the gut of *M. sexta*. To further assess the production of antibiotics, overlay assay was performed against several species identified from the insect gut. The sensitivity of gut-derived microbes to antibiotics of *X. szentirmaii* also showed variations; *S. saprophyticus* was highly sensitive, *C. orthopsilosis* was moderately sensitive while *A. viridans* was less sensitive [19]. The enzyme encoded by the *ngrA* gene is responsible for adding the 4'PP moiety required for NRPS enzyme function. To address whether NRPS enzymes were involved in antibiotic production a *ngrA* mutant strain was created.

Inactivation of *ngrA* resulted in loss antibiotic activity suggesting that NRPS genes were involved in antibiotic production [18,19].

To further assess whether NRPS genes were involved in the synthesis of antibiotics, 10 NRPS mutant strains and 2 siderophore mutant strains were created (Table 2). The antibiotic activity of these strains was initially assessed in the overlay assays using overnight cultures that were not normalized for optical density. Inactivation of *xsz1, xsz2, xsz4, xsz5, xsz6* and *xsz10* genes showed minimal loss of antibiotic activity. In contrast, inactivation of *xsz3, xsz7, xsz8, xsz9, xsz11* and *xsz12* genes showed marked loss of antibiotic activity (Fig.S3). Based on BLASTN analysis, *xsz7* was expected to encode the PaxA enzyme involved in synthesis of the PAX peptide antibiotic (Table 2). *xsz9* was predicted to be involved in synthesis of a NRPS-derived siderophore similar to yersinibactin while *xsz10* was expected to be involved in the synthesis of a hydroxamate-type siderophore. *xsz11* and *xsz12* were predicted to be involved in the synthesis of the antibiotic fabclavine (Table 2). Blast analysis of the *xsz3* and *xsz8* genes did not reveal homologous NRPS genes suggesting they may be involved in the synthesis of novel products.

In the present study the mutant strains were analyzed using an overlay assay in which exponentially growing cultures that were normalized for optical density were spotted on LB agar plates. The mutant strains were first tested against *S. saprophyticus* (Fig.2). Wild-type *X. szentirmaii* inhibited growth of *S. saprophyticus* resulting in a zone of inhibition of 24 mm (Fig. 2A,B). The *ngrA* strain, as expected, did not inhibit growth of *S. saprophyticus* hence there wasn't a detectable zone of inhibition. The antibiotic activity of mutants *xsz3, xsz7, xsz8* and *xsz9* was significantly reduced but displayed residual activity unlike the *ngrA* strain in which antibiotic activity was not detected (Fig.2A,B). These findings suggest that the *xsz3, xsz7* and

xsz8 NRPS genes are involved in the production of antibiotics that are active against *S. saprophyticus*. In addition, it appears that the NRPS-type siderophore may play a role in inhibiting growth of *S. saprophyticus*.

Figure 2. Antibiotic overlay assay of the wild-type, *ngrA*, and mutant strains of *X. szentirmaii* against *S. saprophyticus*. A- Zones of inhibition surrounding each colony in an overlay assay. The zone of inhibition is proportional to the sensitivity of the respective indicator strain used. The results shown are representative of three independent biological overlay experiments with 10 replicates in each experiment. **B-** Graphical representation of zone of inhibition in mm against different mutants tested. Means were calculated for 10 data points for each experiment. Error bars show standard errors of the means. Statistical significance is ($p < 0.05$) indicated by asterisks *. Statistical analysis was performed using paired Student's t-test.

The antibiotics of wild-type *X. szentirmaii* were less active against *A. viridans* producing a zone of inhibition of 6 mm (Fig.3A, B). As expected, the *ngrA* strain did not inhibit growth of *A. viridans*. Inactivation of the *xsz8* genes markedly reduced antibiotic activity against *A. viridans* whereas mutant strains *xsz3 and xsz7* showed zones of inhibition slightly less than wild-type *X. szentirmaii.* Interestingly, inactivation of *xsz11* did not reduce antibiotic activity against *A. viridans* (Fig.3A, B). Taken together, these results indicate as shown previously that *A. viridans* is less sensitive to antibiotics produced by *X. szentirmaii* and that the antibiotic produced by NRPS *xsz8* gene is the more active against *A. viridans* than the other antibiotic produced by *X. szentirmaii*.
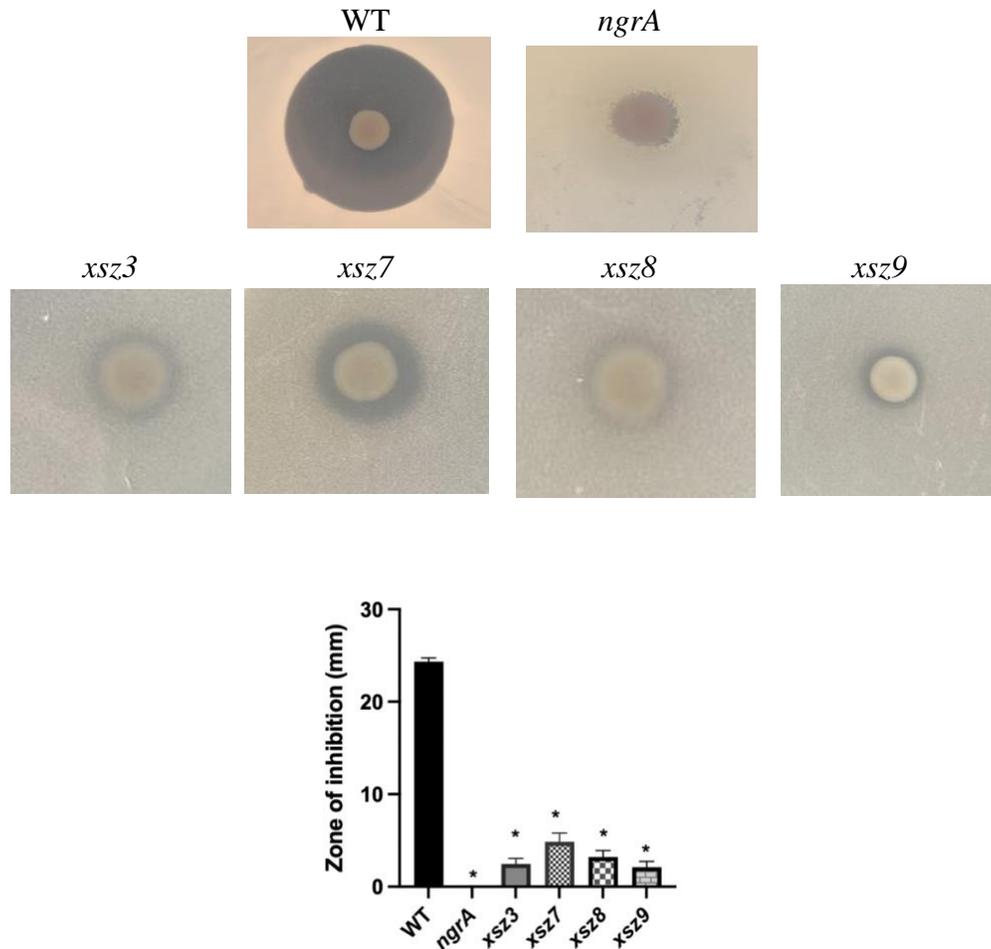
Figure 3. Antibiotic overlay assay of the wild-type, *ngrA*, and mutant strains of *X. szentirmaii* against *A. viridans*. A- Zones of inhibition surrounding each colony in an overlay assay. The zone of inhibition is proportional to the sensitivity of the respective indicator strain used. The results shown are representative of three independent biological overlay experiments with 10 replicates in each experiment. **B-** Graphical representation of zone of inhibition in mm against different mutants tested. Means were calculated for 10 data points for each experiment. Error bars show standard errors of the means. Statistical significance is (P<0.05) indicated by asterisks *. Statistical analysis was performed using paired Student's t-test.

In previous experiments, *C. orthopsilosis* showed moderate sensitivity to antifungals produced by wild-type *X. szentirmaii* [19]. To further evaluate which NRPS genes may be involved in antifungal activities overlay assays were performed with the NRPS mutant strains *xsz3, xsz7, xsz8* and *xsz11* (Fig.4A,B). The zone of inhibition produced by wild-type *X.*

szentirmaii against *C. orthopsilosis* was 13.7 mm. As expected, the *ngrA* strain did not inhibit

growth of *C. orthopsilosis*. Individual inactivation of the *xsz3, xsz7, xsz8* or *xsz11* NRPS genes

markedly reduced antifungal activity resulting in zones of inhibition of 0.9 mm, 1.9 mm, 1.7 mm

and 1.2 mm, respectively (Fig.4B). These results are similar to those obtained with *S.*

saprophyticus and suggests that compounds produced by the *xsz3, xsz7* and *xsz8* NRPS genes

produce compounds with both antibacterial and antifungal activities.
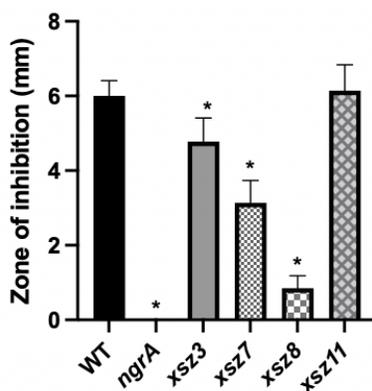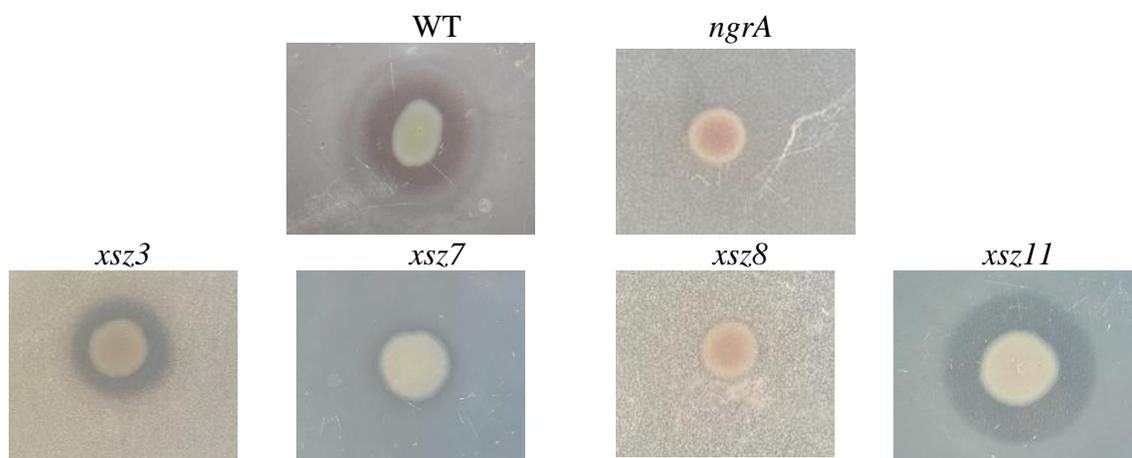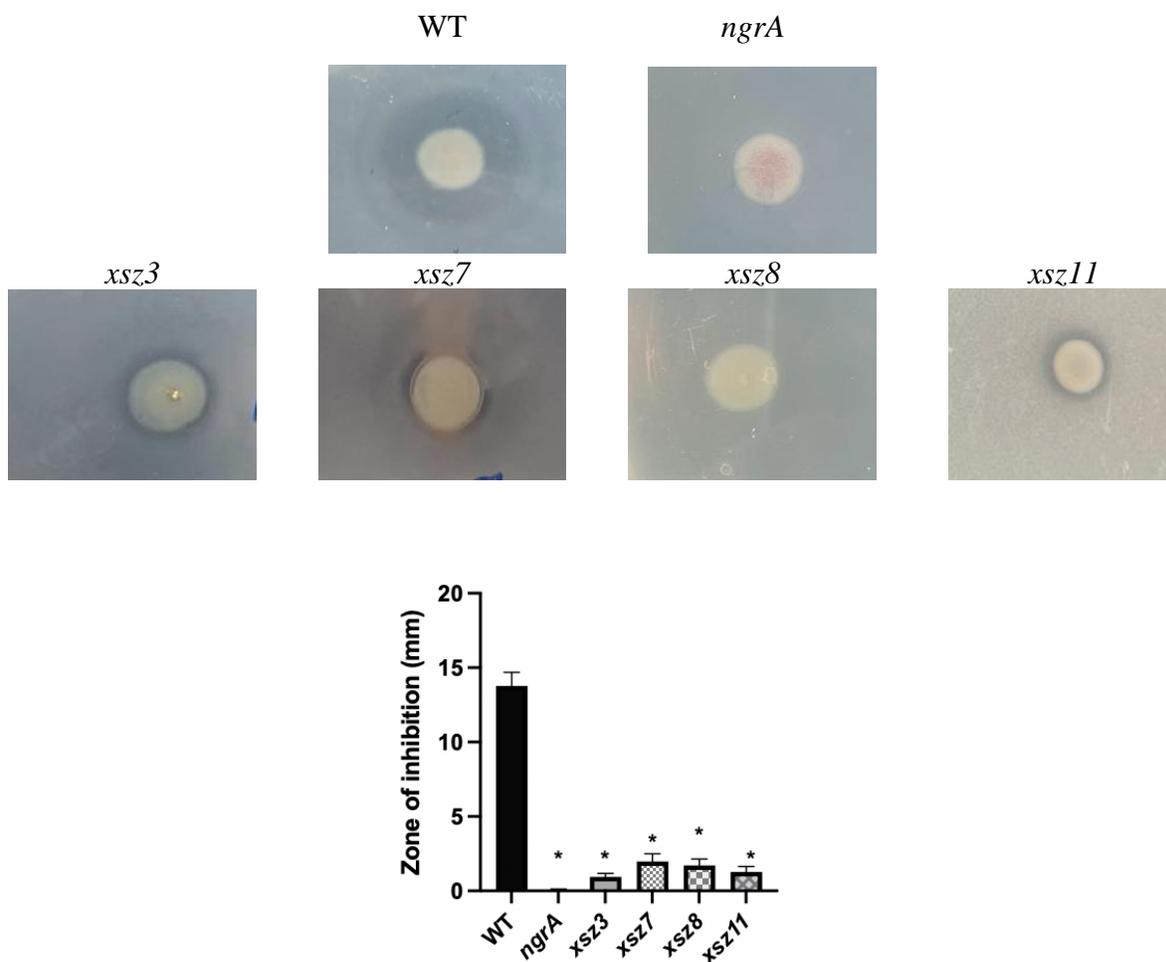

Figure 4. Antibiotic overlay assay of the wild-type, *ngrA*, and mutant strains of *X.*
***szentirmaii* against *C. orthopsilosis*.** **A-** Zones of inhibition surrounding each colony in an
overlay assay. The zone of inhibition is proportional to the sensitivity of the respective indicator
strain used. The results shown are representative of three independent biological overlay
experiments with 10 replicates in each experiment. **B-** Graphical representation of zone of

inhibition in mm against different mutants tested. Means were calculated for 10 data points for each experiment. Error bars show standard errors of the means. Statistical significance is (P<0.05) indicated by asterisks *. Statistical analysis was performed using paired Student's t-test.

Cell-free supernatant assay for identifying antibiotic activity in LB Medium cultures

Since *A. viridans* was less sensitive to antibiotics produced by *X. szentirmaii*, further analysis using the cell-free supernatant assay was performed with *S. saprophyticus* and *C. orthopsilosis*. Wild-type *ngrA* , *xsz3* and *xsz8* were grown in LB broth for 48 h, cultures were centrifuged and supernatants were filtered using a 0.2 μm filter. Cell-free supernatants were added to dilute cultures of the indicator strain and optical density of the cultures was measured at 0h, 8 h and 24 h. Growth inhibition percentage was calculated using the formula 1- (OD_{600} value of treated sample / OD_{600} value of untreated sample) x 100.

The untreated culture of *S. saprophyticus* grew to an OD_{600} of 0.5 by 8 h and to OD_{600} of 1.3 by 24 h (Fig.5A). Addition of wild-type cell-free supernatant suppressed growth of *S. saprophyticus*.

Figure 5. Cell free supernatant assay of wild-type *X. szentirmaii*, *ngrA*, *xsz3* and *xsz8* against *S. saprophyticus*. The cultures were grown for 48 h. **A**-Growth was assessed by OD_{600} values of each sample at three time points. **B**-Inhibitory activity of cell-free supernatants. The percent inhibition against *S. saprophyticus* was calculated by the formula 1−(OD_{600} treated culture/OD_{600} untreated culture) x 100. The assay was performed three times with three samples for each strain at each time points. Error bars indicate standard errors of the means. Statistical significance is (P<0.05) indicated by asterisks *. Statistical analyses were done by two tailed-unpaired Student-t test.

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In contrast, *S. saprophyticus* treated with *ngrA* cell-free supernatants grew to levels slightly less than that of untreated cultures suggesting that most of the activity in the cell-free supernatants was derived from NRPS antibiotics. The cell-free supernatants of *xsz3* and *xsz8* strains displayed antibiotic activity similar to the wild-type strain at 8 h (Fig.5B). At 24 h time point, the supernatants of the *xsz8* strain displayed less inhibition (43%) than the supernatants of the *xsz3* strain (67%), Thus, unlike the overlay assay in which the *xsz3* and *xsz8* strains both displayed similar low levels of antibiotic activity, in the cell-free supernatant assay the level of inhibition was less pronounced and the *xsz3* strain appeared to inhibit growth of *S. saprophyticus* to a greater extent than the *xsz8* strain indicating that the compound produced by *xsz8* NRPS gene is involved in suppressing the growth of *S. saprophyticus*.

When *C. orthopsilosis* was used as the target organism, the OD_{600} values of the untreated culture was 0.17 at 8 h and 0.38 at 24 h (Fig.6A). Addition of wild-type cell-free supernatant suppressed the growth of *C. orthopsilosis* mostly at 24 h. When *ngrA* supernatant was used, *C. orthopsilosis* grew to level marginally less than the untreated culture indicating that *ngrA*-dependent compounds were involved in suppressing growth of *C. orthopsilosis*. The percent inhibition of the *xsz3* cell-free supernatant was less than the *xsz8* cell-free supernatant at 24 h (Fig.6B). The percent inhibition of the *xsz3* supernatants was 15% as compared to 34% for *xsz8* strain suggesting that the compound produced by the *xsz3* NRPS gene is involved in suppressing growth of *C. orthopsilosis*.
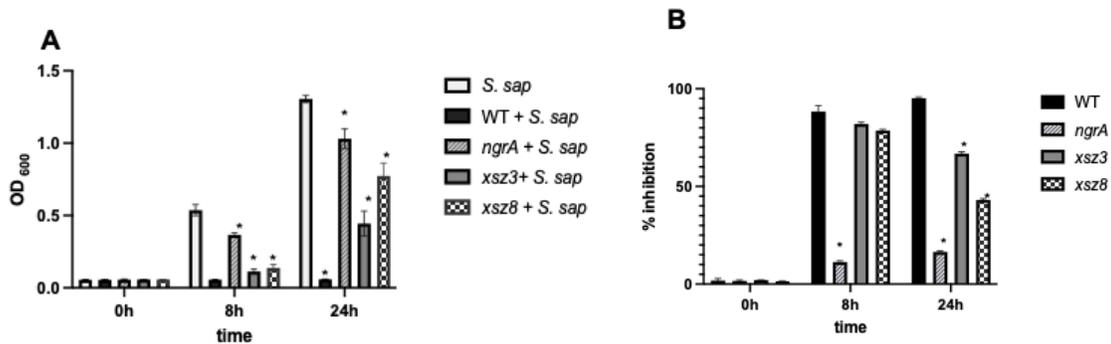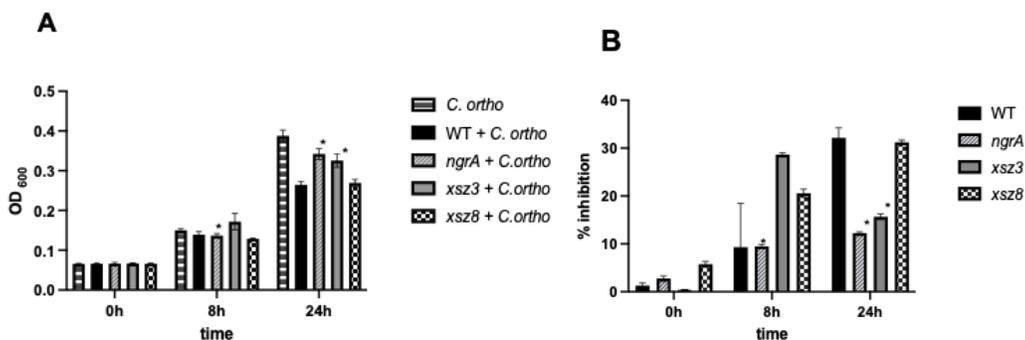
Figure 6. Cell free supernatant assay of wild-type *X. szentirmaii*, *ngrA, xsz3* and *xsz8* against *C. orthopsilosis*. The cultures were grown for 48 h. **A**-Growth was assessed by OD_{600} values of each sample at three time points. **B**-Inhibitory activity of cell-free supernatants. The percent inhibition against *C. orthopsilosis* was calculated by the formula $1-(OD_{600}$ treated culture$/OD_{600}$ untreated culture$)$ x 100. The assay was performed three times with three samples for each strain at each time points. Error bars indicate standard errors of the means. Statistical significance is $(P<0.05)$ indicated by asterisks *. Statistical analyses were done by two tailed-unpaired Student-t test.

These results are also in contrast with that of overlay assay in which both *xsz3* and *xsz8* displayed significant loss of antifungal activity against *C. orthopsilosis*.

***Ex vivo* competition between *X. szentirmaii* and *S. saprophyticus* in Grace's Insect Medium**

To analyze the effect of antibiotics *ex vivo*, both *X. szentirmaii* strains and *S. saprophyticus* were grown together in Grace's insect medium that mimics the hemolymph of the insect. At 4 h, 8 h and 12 h post-inoculation aliquots were taken and dilution plated on LB agar. Colonies were counted manually and expressed as cfu/mL. The colonies were distinguished based on color, size and morphology. *X. szentirmaii* colonies were large and brownish whereas that of *S. saprophyticus* was small and whitish. The cfu/mL was similar for wild-type and *S. saprophyticus* at 4 h. But the cfu/mL of wild-type *X. szentirmaii* at 8 h and 12 h was much higher than *S. saprophyticus* (Fig.7A) suggesting that during growth, *X. szentirmaii* produced antibiotics that suppressed the growth of *S. saprophyticus*.

To assess whether *ngrA*-dependent antibiotics were required for suppressing the growth of *S. saprophyticus,* the *ngrA* mutant strain was grown together with *S. saprophyticus* (Fig.7B). At 8 h and 12 h, both *ngrA* mutant and *S. saprophyticus* grew to similar cell density (7x 10^8 cfu/mL). The growth rate of *ngrA* was comparable to that of wild-type *X. szentirmaii.* In contrast to the wild-type strain, the *ngrA* strain failed to suppress the growth of *S. saprophyticus* due to its lack of antibiotics rather than the slow growth rate indicating that *ngrA*-dependent antibiotics were necessary to suppress growth of *S. saprophyticus* under competition conditions.
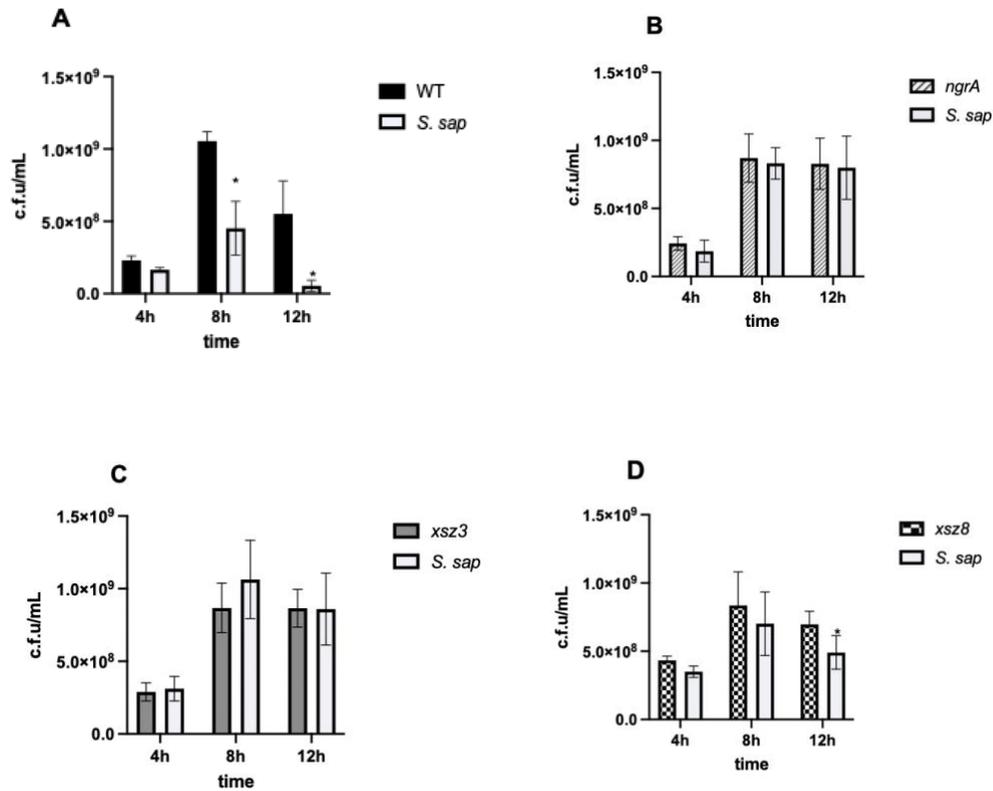
Figure 7. *Ex vivo* **competition between** *X. szentirmaii* **strains and** *S. saprophyticus*. **A**. wild-type strain. **B.** *ngrA* strain. **C.** *xsz3* strain. **D.** *xsz8* strain. Cells were grown in Graces Insect Medium at 4 h, 8 h and 12 h. Competition outcome was determined by dilution plating at 4 h, 8 h and 24 h. Data is shown in terms of colony forming units/ml (cfu/mL). The assay was performed three times for each dilution. Error bars indicate standard errors of the means. Statistical significance is (P<0.05) indicated by asterisks * Statistical analyses were done by two tailed-unpaired Student-t test.

The *xsz3* strain was co-inoculated with *S. saprophyticus* to assess the ability to suppress growth of the competitor. At 8 h the cfu/mL of *S. saprophyticus* was higher than that of the *xsz3* strain and the levels remained high at 12 h. These findings indicate that the compound produced by the *xsz3* NRPS gene was required to suppress growth of *S. saprophyticus*. The *xsz8* strain was also co-inoculated with *S. saprophyticus* to assess the ability to inhibit growth of the competitor (Fig.7D). At 8 h and 12 h the cfu/mL of *S. saprophyticus* was lower than that of the *xsz8* strain. Thus, the *xsz8* strain was able to inhibit growth to a greater extent than the *xsz3* strain.

Ex vivo competition between *X. szentirmaii* and *C. orthopsilosis* in YEPD medium

X. szentirmaii was able to inhibit growth of *C. orthopsilosis* in the overlay assay and to a lesser extent in the cell-free supernatant assay. To assess the inhibition of *C. orthopsilosis* under competition conditions strains were grown in YEPD medium that is more optimal for growth of yeast relative to Grace's medium. The wild-type, *ngrA* strains and *C. orthopsilosis* were first grown individually (Fig.8A). The cfu/mL of both the wild-type and *ngrA* strains reached considerably higher levels by 12 h than that of *C. orthopsilosis* indicating that the doubling time of the yeast is slower than that of *X. szentirmaii*.

In competition between wild-type *X. szentirmaii* and *C. orthopsilosis* at 4 h, wild-type *X. szentirmaii* reached 2x 10^9 cfu/mL whereas *C. orthopsilosis* had only few colonies. By 8 h, wild-type *X. szentirmaii* grew to higher levels while *C. orthopsilosis* was barely detectable (Fig.8B). In competition between the *ngrA* strain and *C. orthopsilosis,* the cfu/mL of the *ngrA* strain was 2 x 10^9 cfu/mL at 4 h while only a few colonies of *C. orthopsilosis* were present. At 8 h the *ngrA* strain continued to grow while *C. orthopsilosis* was barely detectable (Fig.8C). These findings suggest that under competition conditions *ngrA-* dependent compounds were not required to inhibit growth of *C. orthopsilosis*. Other *ngrA-* independent compounds may therefore function

to inhibit *C. orthopsilosis* under these conditions which is yet to be examined. Since *ngrA*-dependent activity was not required to suppress growth in *ex vivo* competition experiments competition assays with the *xsz3* and *xsz8* strains were not performed.

The level of inhibition caused by wild-type, *ngrA*, *xsz3* and *xsz8* strains of *X. szentirmaii* against three indicator organisms assessed in three different assays is depicted in Table 4.
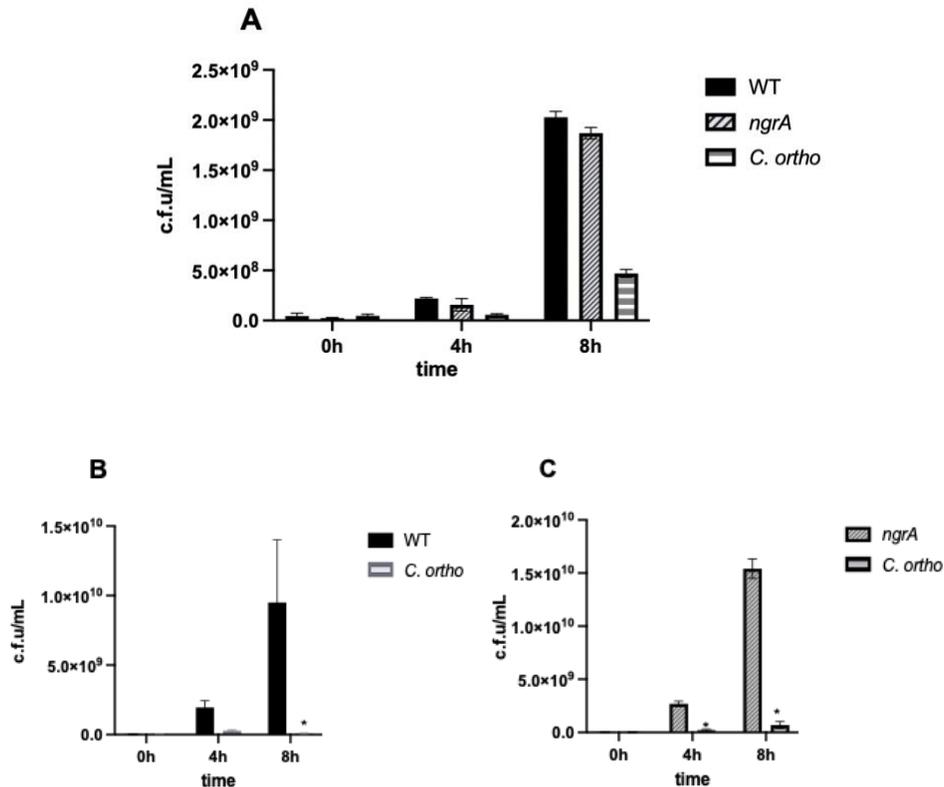
Figure 8. *Ex vivo* **experiment with wild-type and** *ngrA* **strains of** *X. szentirmaii* **and** *C. orthopsilosis* **in YEPD. A**- *Ex vivo* control experiment. **B**- *Ex vivo* competition experiment with wild-type *X. szentirmaii* and *C. orthopsilosis* in YEPD. **C-** *Ex vivo* competition experiment with *ngrA* strain of *X. szentirmaii* and *C. orthopsilosis* in YEPD. cfu/mL were calculated at 0 h, 4 h and 8 h for all the three experiments. The assay was performed three times for each dilution. Error bars indicate standard errors of the means. Statistical significance is (P<0.05) indicated by asterisks *. Statistical analyses were done by two tailed- unpaired Student-t test.

TABLE 4. Summary of relative antimicrobial activity of different mutants tested against different indicator strains. Level of activity is indicated by +. No detectable activity indicated by -.

	S. saprophyticus	*A. viridans*	*C. orthopsilosis*
Overlay assay			
WT	+++++	+++	++++
ngrA	-	-	-
xsz3	+	++	+
xsz8	+	+	+
Cell free supernatant assay[1]			
WT	+++++		+++
ngrA	+		+
xsz3	+++		+
xsz8	++		+++
***Ex vivo* competition**[2]			
WT	+++++		++++
ngrA	-		+++
xsz3	-		
xsz8	+		

[1]Data taken from 24 h time point. [2]Data taken from 12 h time point.

Domain analysis of peptides encoded by the *xsz3* and *xsz8* NRPS genes

Inactivation of the *xsz3* gene significantly reduced antibiotic activity against *S. saprophyticus*, *A. viridans* and *C. orthopsilosis* in overlay assays. Assuming this NRPS gene is expressed in the insect hemolymph it could play a major role in interspecies competition.

The *xsz3* gene (WP_099139143.1) is 25950 nucleotides long encoding a NRPS protein of 8650 amino acids (Fig.9). Computer algorithms are available to predict the subdomain structure

of enzymes that produce secondary metabolites [39]. The A domain or the adenylation domain of *xsz3* is variable like every A domain of NRPS which usually has 500 amino acids. There is a total of 8 A domains for *xsz3* and each of them specifically binds with amino acids as the differences in binding pocket of each A domain. The condensation domain or the C domain is a conserved domain which has 450 residues. To the peptidyl carrier protein transfer domain (PCP) which is the smallest NRPS containing 70-90 amino acids, the 4'phosphopantetheinyl (4'PP) moiety is added. The sulfhydryl group of 4'PP moiety is attached to activated amino acids and shuttles them to the condensation domain eventually synthesizing antibiotics [11,40]. The subdomains A-2 and A-7 showed similar residues in the binding pockets and the predicted first amino acid is to be asparagine. Also, subdomains A-3 and A-6 exhibited similar residues and the anticipated first amino acid is serine.
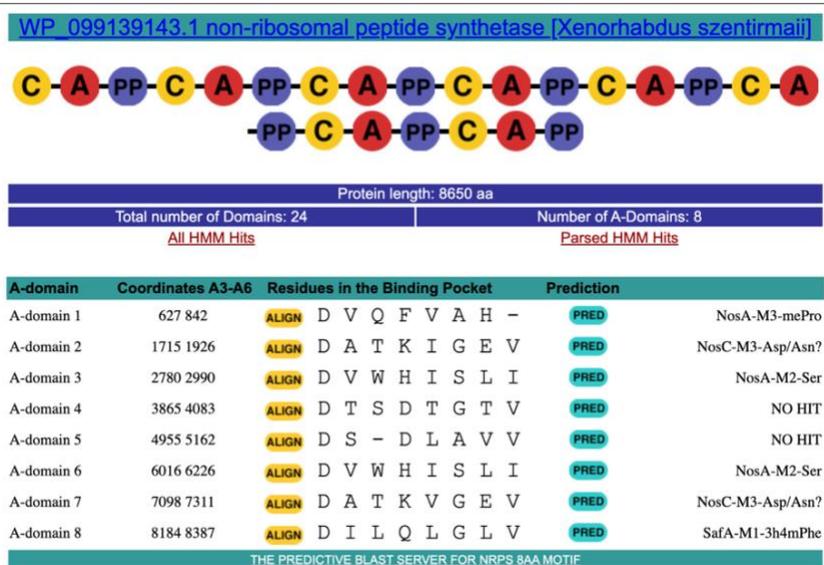
Figure 9. The predicted subdomains of the NRPS encoded by *xsz3* using a NRPS prediction server (http://nrps.igs.umaryland.edu).

Inactivation of the *xsz8* gene also considerably reduced antibiotic activity against *S. saprophyticus*, *A. viridans* and *C. orthopsilosis* in overlay assays. Presumptively if this NRPS gene is expressed in the insect hemolymph it could play a major role in interspecies competition. The *xsz8* gene (WP_099139155.1) is 10965 nucleotides long encoding a NRPS protein of 3655 amino acids [39]. Out of the 10 domains of *xsz8*, it has 3 A domains which specifically binds to the amino acids which is being incorporated. The prediction of all the three A domains showed same binding sequence but the specific amino acid could not be identified, thus showing no hits. It also has a thioesterase domain which is the final module of the NRPS cluster. To release the peptide and free the NRPS enzyme for another round of synthesis, the activity of a thioesterase domain is required [39,40].
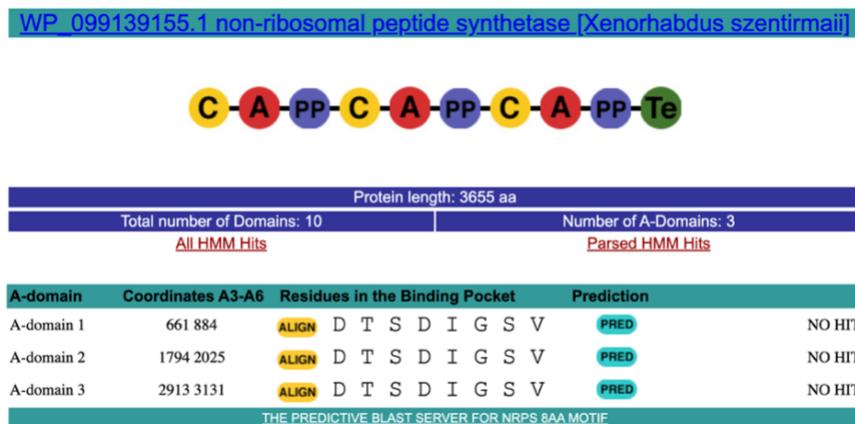
Figure 10. The predicted subdomains of mutant *xsz8* using NRPS prediction server.

Discussion

Xenorhabdus species produce both small molecule antibiotics and R-type bacteriocins (xenorhabdicins) for competition with both related and non-related species during infection in the insect hemocoel. Previous studies showed that *ngrA*-dependent antibiotics of *X. nematophila*

were active against non-related species but not related *Xenorhabdus* and *Photorhabdus* species [8]. In competition between related species R-type bacteriocins were shown to be necessary for *X. nematophila* to outcompete *Photorhabdus luminescens* in both broth cultures and in the insect host [9]. *X. szentirmaii* has been shown to produce high levels of antibiotic activity against both unrelated and related bacteria [18,19]. It was demonstrated recently that *X. szentirmaii* primarily used *ngrA*-dependent antibiotics to outcompete *X. nematophila* both *in vitro* and in insect [18]. R-type bacteriocins produced by *X. szentirmaii* were involved to a lesser extent than the *ngrA*-derivatives [3]. *ngrA*-dependent products were also required for full virulence of *X. szentirmaii* towards *M. sexta* [19].

The *X. szentirmaii* genome contains a usually large number of NRPS gene clusters that are potentially involved in producing an array of antibiotic compounds. To identify NRPS gene clusters which produce antibiotics, 10 different NRPS gene clusters were inactivated creating mutant strains *xsz1-xsz8*, *xsz11* and *xsz12*. Two additional strains were created in putative siderophore producing genes; *xsz9* and *xsz10*. Earlier experiments showed that mutant strains *xsz1, xsz2, xsz4, xsz5, xsz6* and *xsz10* produced antibiotic activity similar to the wild-type strain indicating that the NRPS genes inactivated in these mutant strains were not involved in the synthesis of antibiotics as measured by overlay assay against *S. saprophyticus*. Furthermore, inactivation of NRPS genes *xsz3, xsz7, xsz8, xsz11* and *xsz12* results in reduced antibiotic activity in overlay assays (Fig.S3). Genomic analysis of *X. szentirmaii* revealed that the *xsz7* NRPS gene belonged to a gene cluster involved the synthesis of PAX peptide antibiotics while the *xsz11* and *xsz12* NRPS genes belonged to a gene cluster involved in synthesis of the fabclavine antibiotic (Table 2). Blast analysis of the *xsz3* and *xsz8* NRPS genes did not reveal significant similarities

to known NRPS antibiotic clusters suggesting they may be involved in producing novel antibiotic compounds.

When the gram-positive gut competitors were used as tester strains in the antibiotic overlay assay, *S. saprophyticus* was highly susceptible, *C. orthopsilosis* was moderately susceptible while *A. viridans* was least susceptible. It was also observed that the zones of inhibition produced by *X. szentirmaii* in overlay assay against *A. viridans* and *C. orthopsilosis* were hazy because there could be bacteriostatic effect for *X. szentirmaii* antibiotics that is slowing down bacterial growth rather than being bactericidal. The *ngrA* strain showed lack of activity with all the indicator strains used pointing to the fact that antibiotic activity under overlay conditions are *ngrA*-dependent. To explicitly identify which *ngrA*-dependent compounds are necessary, further investigation is done with mutants *xsz3* and *xsz8* specifically because the compounds synthesized by these mutants have not been characterized before. The experimental results showed that *xsz3* and *xsz8* were to exhibit loss of activities both in *S. saprophyticus* and *C. orthopsilosis,* while for already less sensitive *A. viridans, xsz3* showed no loss of activity and *xsz8* showed high loss of activity (Fig.2B,3B,4B). Altogether from the overlay experiment it is speculated that the compound produced by both *xsz3* and *xsz8* have both antibacterial and antifungal properties.

In the cell-free supernatant assay, the aliquots devoid of living cells of *X. szentirmaii* are harvested at 48 h. Percent inhibition caused by *xsz3* was higher when used against *S. saprophyticus* than in *C. orthopsilosis* (Fig 5B and 6B). These results suggest that the compound produced by *xsz3* in cell-free supernatant assay has antifungal activity against *C. orthopsilosis*. Percent inhibition caused by *xsz8* was higher when used against *C. orthopsilosis* than in *S.*

saprophyticus indicating that *xsz8* is contributing highly towards the antibiotic activity in *S. saprophyticus* but assessment in *in vivo* conditions is yet to be determined.

Evaluation of the competitive interactions between *S. saprophyticus* and *X. szentirmaii* in a partially biologically relevant condition was done by *ex vivo* competitions in Grace's insect medium which simulates growth conditions as that of the insect hemolymph. At 12 h, cfu/mL produced by *xsz3* and *S. saprophyticus* were same. This shows that *xsz3* was not capable of inhibiting the growth of *S. saprophyticus* at higher time points (Fig.7C). This finding implies that the compound produced by *xsz3* is adding more towards the activity against *S. saprophyticus* unlike in cell-free supernatant assay. At 12 h, cfu/mL produced by *xsz8* was higher than *S. saprophyticus* suggesting that *xsz8* could be involved but not as much of *xsz3*.

In *ex vivo* control experiment, yeast was growing slower than wild-type *X. szentirmaii* and *ngrA* strains where the doubling time of yeast is lengthier compared to others. As expected, in *ex vivo* competition, wild-type *X. szentirmaii* did suppress the activity of *C. orthopsilosis* at 8 h time point (Fig 8A). Strikingly, in *ex vivo* competition between the *ngrA* strain and *C. orthopsilosis*, it didn't show any loss of activity even at early time point (Fig. 8B). From this finding it is speculated that there could be involvement of *ngrA*- independent processes or compounds that inhibits the growth of *C. orthopsilosis*. This possibility needs further investigations both *ex vivo* and in insect because in interspecies competition that occurs during natural infection by the nematode is highly complex and can be influenced by other members of the microbial community present in the insect gut, the immune response of the insect and several other environmental and biotic factors.

Altogether in the present study it is shown that specific activities of *xsz3* and *xsz8* compounds can be distinguished in different assays. Antibiotic overlay assay is a qualitative

assay where it allows the screening of different mutants against different indicator strains by means of inhibition zones. The concentration of antibiotic around the colony is higher and the cells are in a heterogenous state thus the difference in the effect of compounds produced by gene clusters is hard to distinguish at higher concentrations. In case of cell-free supernatant assay, the cultures were grown in a larger volume of media making the concentration of antibiotics lower than in overlay assay. In *ex vivo* assays, both competitors are allowed to grow together utilizing the same growth conditions provided. It has been shown that bacteria use quorum sensing mechanism to activate antibiotics which help them in competing for limited resources in mixed bacterial communities [41,42]. In quorum sensing, there could be inadequate concordance between signals and receptors signifying widespread social interactions between cells called eavesdropping where cells respond to signals that they do not produce [41].

The antibiotic fabclavine produced by the NRPS gene cluster *xsz11* is shown to possess broad-spectrum bioactivity against Gram-positive and Gram-negative bacteria, fungi, and protozoa [25]. Most of the antibiotics hinder with either DNA synthesis like the quinolone class of antimicrobials which interferes with DNA gyrase or inhibition of RNA synthesis by rifamycins. Antibiotics of the class β-lactams cause either cell death or merely inhibit the growth of bacteria by suppressing cell wall synthesis. Some impede with protein synthesis by inhibiting the two subunits of ribosome [43]. The cumulative findings of the present study contemplate that the compounds produced by the NRPS clusters *xsz3* and *xsz8* are novel and unique as it possesses activities against both bacteria and yeast. The mode of action many antibacterial agents inhibit steps important for the formation of peptidoglycan, the essential component of the bacterial cell wall. Unlike, majority of antifungal compounds aim either the formation of ergosterol, an important element of the fungal cell membrane. However, there are important

counterparts between the mechanisms by which fungi develop resistance to ergosterol biosynthesis inhibitors and bacteria develop resistance to anti-cell wall agents [44].

The genomic analysis of *xsz3* displayed that there is a gene downstream which encodes 6 amino acids (Fig.11) but unlikely to be involved in synthesizing antibiotics. Both *xsz3* and *xsz4* are adjacent to each other (WP_099139143.1 and WP_099139142.1) confirming that *xsz4* is the gene located downstream and also the inactivation of *xsz4* did not show any reduction in antibiotic activity against both bacteria and in yeast (Dey, Forst, unpublished data). Analysis of the draft genome sequences of *X. szentirmaii* revealed 5 NRPS containing gene clusters and also, they identified 5 compounds (Xenematide, GameX peptide, Rhabdopeptide, Xenoamicin, Szentiamide) which are predicted to be synthesized by NRPS clusters [45]. Recently, global bioinformatic assessment of *X. szentirmaii* of the whole genome was done which contained at least 11 NRPS and 3 PKS genes. Out of the 11 NRPS genes, one codes for the enzymes for PAX peptide synthesis designated as *xsz7* (NCBI accession*:* WP_051462365) and another one that is a siderophore named yersinibactin (NCBI accession*:* WP_038233794) designated as mutant *xsz9* and two with fabclavine- *xsz11* and *xsz12* (NCBI accession*:* WP_099139157 and WP_038241744). Also marked reduction of antimicrobial activities are seen in two genes that are inactivated which codes for fabclavine and PaxA.
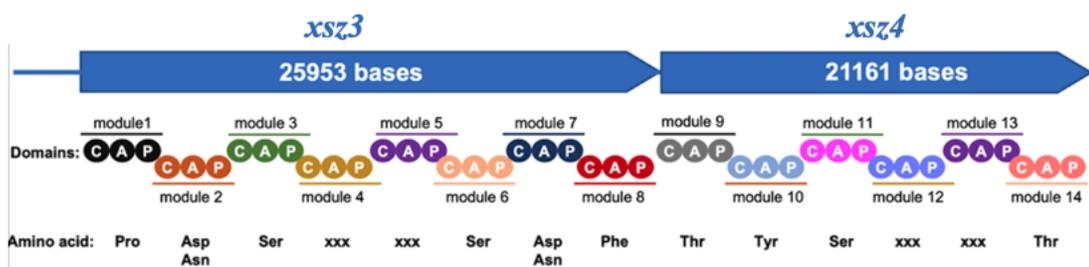
Figure 11. Two NRPS genes- *xsz3* and *xsz4* (WP_099139143..1 and WP_099139142.1) showing the catalytic domains that they encode, and the predicted amino acids incorporated by the respective modules (xxx- unpredicted) are shown. Domains: C- condensation; A, adenylation; P, peptidyl carrier protein transfer (Dey, Forst, unpublished data).

The future direction of this research is to purify and characterize the bioactive compounds produced by *xsz3* and *xsz8* genes. As far of now, the mode of action of only Oldilorhabdin is known, thus the mode of action and the structure of these compounds need to be characterized. Also, investigations in the host insect with co-injection of mutants and competitors is yet to be described. Furthermore, the activity of these compounds needs to be tested against several species of organisms which are of clinically and agriculturally important. Eventually, all these findings support to the assumption that the compounds produced by *Xenorhabdus* strains under natural conditions could pave for the discovery of novel antibiotics which in turn can make contributions to tackle the problem of antibiotic resistance.

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Appendix: Supplemental data

Table S1. Sequences of both forward and reverse primers used for querying in NCBI BLASTN function

| Primer	Sequence
ITS1	GAAAACTNNGCTTTGGTGGGCCCATGGCCTGCCAGAGATTAAACTC AACCAAATTTTTATTTAAGTCAACTGATTAACTAATAGTCAAAACTT TCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGA AATGCGATAAGTAATATGAATTGCAGATATTCGTGAATCATCGAAT CTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGT TTGAGCGTCATTTCTCCCTCAAACCTTCGGGTTTGGTGTTGAGCGAT ACGCTGGGTTTGCTTGAAAGAAAGGCGGAGTATAAACTAATGGAT AGGTTTTTTTCCACTCATTGGTACAAACTCCAAAATTCTTCCNAATT CGACCTCAAATCAGGTANGACTACCCGCTGAACTTAANCATATCAA TAANCCGGNGGAA
ITS4	CTGANTNAGGTCGATTTGGAAGATTTTGGAGTTTGTACCAATGAGT GGAAAAAAACCTATCCATTAGTTTATACTCCGCCTTTCTTTCAAGCA AACCCAGCGTATCGCTCAACACCAAACCCGAAGGTTTGAGGGAGA AATGACGCTCAAACAGGCATGCCCTTTGGAATACCAAAGGGCGCA ATGTGCGTTCAAAGATTCGATGATTCACGAATATCTGCAATTCATA TTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCGAGAACCAAGA GATCCGTTGTTGAAAGTTTTGACTATTGTTAATCAGTTGACTTAAAT AAAAATTTGGTTGAGTTTAATCTCTGGCAGGCCATGGGCCCACCAA AGCAAAGTTTTCAAAAAAAAAGAAAAACACATGTGTAAAAAAATG CAGTTAAGCACTTTCATTCTGTAATGATCCTTCCGCAGGNNNCCCTA CGGA
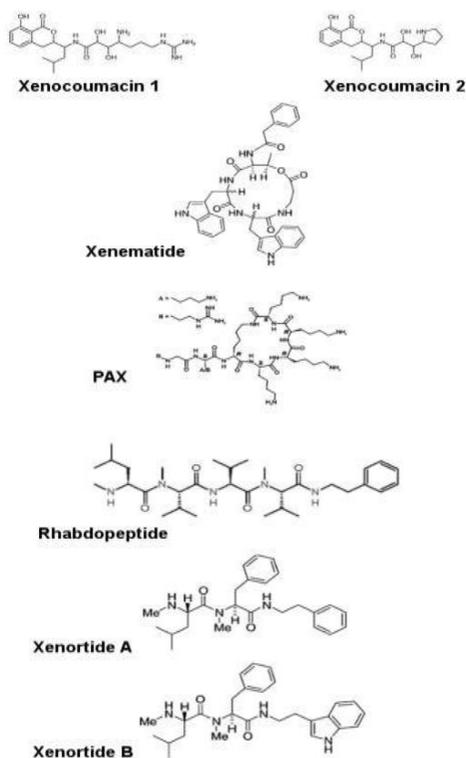
Figure S1. Known antimicrobial compounds produced by X. nematophila. (Sources: Xenocoumacin - McInerney BV, Taylor WC, Lacey MJ, Akhurst RJ, Gregson RP. 1991. Biologically active metabolites from Xenorhabdus spp., Part 2. Benzopyran-1-one derivatives with gastroprotective activity. J. Nat. Prod. 54:785-795; xenematide and xenortide - Lang G, Kalvelage T, Peters A, Wiese J, Imhoff JF. 2008. Linear and cyclic peptides from the entomopathogenic bacterium Xenorhabdus nematophilus. J. Nat. Prod. 71:1074-1077; PAX - Fuchs SW, Proschak A, Jaskolla TW, Karas M, Bode HB. 2011. Structure elucidation and biosynthesis of lysine-rich cyclic peptides in Xenorhabdus nematophila. Org. Biomol. Chem. 9:3130-3132; rhabdopeptide - Reimer D, Cowles KN, Proschak A, Nollmann FI, Dowling AJ, Kaiser M, Constant Rf, Goodrich-Blair H, Bode HB. 2013. Rhabdopeptides as insect-specific virulence factors from entomopathogenic bacteria. Chembiochem. 14:1991-1997).
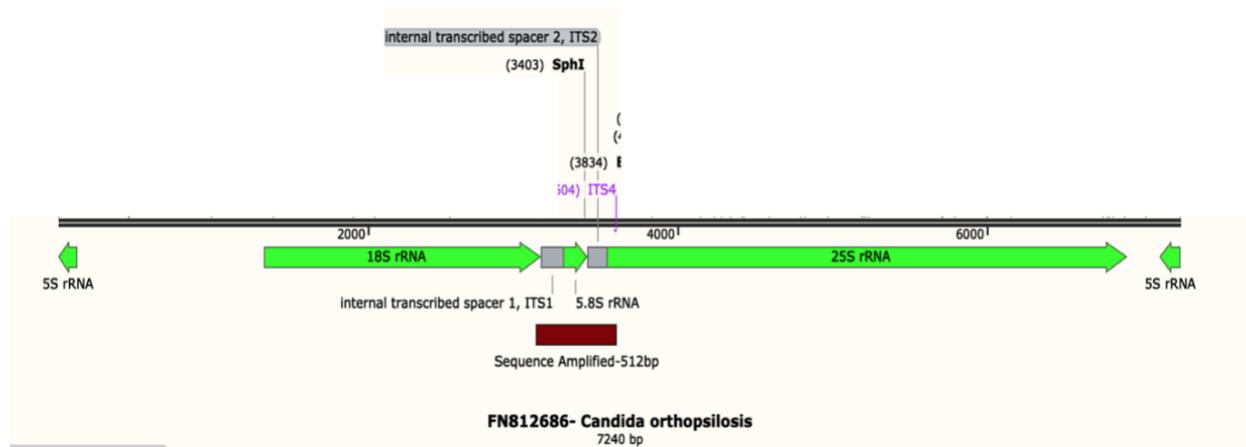
Figure S2: Map of 5.8S rDNA gene of *C. orthopsilosis* (Dey lab).

Figure 1:

Analysis of antibiotic mutant strains of
X. szentirmaii

Analysis of antibiotic mutant strains of
X. szentirmaii

Figure 1. Overlay assay of 12 NRPS mutant strains of *X. szentirmaii*. Zones of inhibition were determined using *S. saprophyticus* as the target species in the overlay agar. Values represent mean and standard error of 16 replicates for each mutant strain. Xsz11 and Xsz12 represent inactivation of the same NRPS cluster.
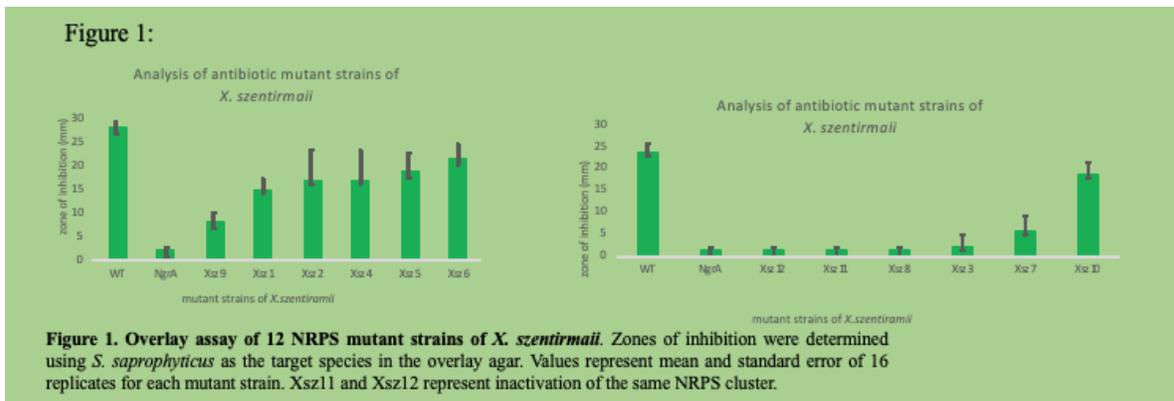
Figure S3. Data showing the antibiotic activities of different NRPS mutant strains obtained from *X. szentirmaii* against *S. saprophyticus* in antibiotic overlay assay (Unpublished data K. Jazayeri and S. Forst).