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Permalink
https://escholarship.org/uc/item/2xm5s26f

Journal
BMC genomics, 18(1)

ISSN
1471-2164

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Publication Date
2017-12-01

DOI
10.1186/s12864-017-4311-4

Peer reviewed
The insect pathogenic bacterium *Xenorhabdus innexi* has attenuated virulence in multiple insect model hosts yet encodes a potent mosquitocidal toxin

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**Abstract**

**Background:** *Xenorhabdus innexi* is a bacterial symbiont of *Steinernema scapterisci* nematodes, which is a cricket-specialist parasite and together the nematode and bacteria infect and kill crickets. Curiously, *X. innexi* expresses a potent extracellular mosquitocidal toxin activity in culture supernatants. We sequenced a draft genome of *X. innexi* and compared it to the genomes of related pathogens to elucidate the nature of specialization.

**Results:** Using green fluorescent protein-expressing *X. innexi* we confirm previous reports using culture-dependent techniques that *X. innexi* colonizes its nematode host at low levels (~3–8 cells per nematode), relative to other *Xenorhabdus-Steinernema* associations. We found that compared to the well-characterized entomopathogenic nematode symbiont *X. nematophila*, *X. innexi* fails to suppress the insect phenoloxidase immune pathway and is attenuated for virulence and reproduction in the Lepidoptera *Galleria mellonella* and *Manduca sexta*, as well as the dipteran *Drosophila melanogaster*. To assess if, compared to other *Xenorhabdus* spp., *X. innexi* has a reduced capacity to synthesize virulence determinants, we obtained and analyzed a draft genome sequence. We found no evidence for several hallmarks of *Xenorhabdus* spp. toxicity, including Tc and Mcf toxins. Similar to other *Xenorhabdus* genomes, we found numerous loci predicted to encode non-ribosomal peptide/polyketide synthetases. Anti-SMASH predictions of these loci revealed one, related to the *fcl* locus that encodes fabclavines and *zmn* locus that encodes zeamines, as a likely candidate to encode the *X. innexi* mosquitocidal toxin biosynthetic machinery, which we designated Xlt. In support of this hypothesis, two mutants each with an insertion in an Xlt biosynthesis gene cluster lacked the mosquitocidal compound based on HPLC/MS analysis and neither produced toxin to the levels of the wild type parent.

**Conclusions:** The *X. innexi* genome will be a valuable resource in identifying loci encoding new metabolites of interest, but also in future comparative studies of nematode-bacterial symbiosis and niche partitioning among bacterial pathogens.

**Keywords:** Virulence, Toxin, Symbiosis, Insect, Immunity, NRPS/PKS, Mosquito, Lipopeptide
**Background**

Nematodes in the genus *Steinernema* associate with *Xenorhabdus* bacteria in a mutually beneficial relationship that allows the pair to utilize insect hosts as a reproductive niche. *Steinernema* nematodes have a soil-dwelling stage, known as the infective juvenile (IJ) that carries *Xenorhabdus* bacteria into insect prey that will be killed and used for nutrients that support reproduction. Progeny IJs then emerge from the spent insect cadaver, carrying their *Xenorhabdus* partner, to begin the cycle again.

In general, the bacterial symbionts promote nematode fitness by helping kill insect hosts and by contributing to the degradation and protection of the host cadaver from competitors and predators [1]. Because they can be pathogenic to insects when injected without their nematode host, *Xenorhabdus* bacteria and their genes are being exploited for use in agricultural settings to help control important crop pests. For example, certain *X. nematophila* genes can confer resistance to insect pests when expressed transgenically in plants [2, 3]. The potential for insecticidal and crop protection can be associated with *S. scapterisci* IJs [10].

Generally, *Steinernema* bacterial symbionts are thought to benefit their hosts by contributing to insect death and degradation. Using aposymbiotic *S. scapterisci* nematodes and cultured *X. innexi* symbionts, Bonifassi et al. determined that neither was pathogenic towards *G. mellonella* individually, but were when combined [10]. This indicates that both partners are necessary to kill this insect, in contrast to *S. carpopcapsae* and *X. nematophila* each of which can kill insects without the other (see, for example [26, 27]). Later studies demonstrated that *S. scapterisci* could survive, parasitize, and reproduce aposymbiotically in *G. mellonella*, but with reduced overall fitness [24], and that the impact of different *Xenorhabdus* species on *S. scapterisci* fitness is directly correlated with their phylogenetic relatedness to *X. innexi* [13]. Generally, these studies support the idea that *X. innexi* specifically facilitates the establishment of *S. scapterisci* nematode infection and production of progeny IJs in insect hosts. However, Sicard et al. noted that *S. scapterisci* fared better in the absence of its symbiont than did the other nematode species examined, and that it was colonized by fewer bacterial symbionts (~0.07 CFU/IJ average, relative to 43.8 CFU/IJ for *X. nematophila*) as measured using a crushing and plating method [24]. Taken together, these reports suggest that *S. scapterisci* is trending toward decreased dependence of the nematode on its bacterial symbiont.

Although the findings reviewed above hinted that *X. innexi* may be less virulent, at least toward some insect hosts, than other *Xenorhabdus* species, an activity-screening approach revealed that it does secrete a peptide with insecticidal activity effective against the larvae of several mosquito species in the *Aedes*, *Anopheles*, and *Culex* genera [28]. Recent work has indicated the active compound is a lipopeptide,
dubbed Xenorhabdus lipoprotein toxin (Xlt) that can create pores in the apical surface of mosquito larval anterior midgut cells [29].

The experiments presented here were geared toward directly testing the nematode colonization and insect virulence properties of *X. innexi*, to provide further insights into the evolution of different symbiotic relationships among Steinernema-Xenorhabdus pairings. Further, we sought to identify distinctive virulence determinants that may be encoded by *X. innexi* relative to other Xenorhabdus species, predicted based on the specialization of the *X. innexi*-S. scapterisci symbiotic pair for crickets, and the production by *X. innexi* of a mosquitocidal toxin. To pursue these goals we established a laboratory model of *S. scapterisci*-*X. innexi* insect symbiosis. We assessed *X. innexi* virulence in several model insects, applied genetic tools to facilitate monitoring its presence and gene function, and used draft genome sequencing and analysis to explore its virulence potential.

**Results**

**S. scapterisci IJ receptacles are colonized by few X. innexi cells**

To assess the colonization levels of *X. innexi* in *S. scapterisci* nematodes, we added axenic nematodes (see Methods) to lawns of two *X. innexi* strains, one (HGB1681) isolated from *S. scapterisci* nematodes provided by Prof. Grover Smart (FL) and the other isolated from the *S. scapterisci* nematodes being used in this study (provided by Becker Underwood Inc.) (Table 1). IJs emerging from in vitro cultures such as those described above were surface sterilized and subjected to a grinding assay to calculate average colony-forming units (CFU) of bacteria per IJ. Both tested *X. innexi* strains colonized *S. scapterisci* at ~7 CFU/IJ (Table 2) and colonies were confirmed to be *X. innexi* based on distinctive phenotypic traits (catalase negative, characteristic brown color, and distinctive odor). No colonies grew from homogenates of axenic nematodes cultivated on *X. nematophila*, confirming the previous finding that *X. nematophila* does not colonize *S. scapterisci* nematodes (Table 2) [24].

The low colonization level we detected could be due to low frequency of colonization (few nematodes in the population are colonized) or low levels of colonization (the majority of nematodes are colonized by very few bacteria) or a combination of these phenotypes. To address this question, we generated *X. innexi* strains expressing green fluorescent protein (GFP) (Table 1) to facilitate their visualization within IJ receptacles (Fig. 1) [30]. As with non-GFP expressing strains, progeny IJs emerging from lawns of GFP-expressing *X. innexi* were colonized by an average of approximately 7–10 CFU/IJ, as determined by grinding assays (Table 2). Visualization by fluorescence microscopy revealed GFP-expressing bacterial cell colonization of the *S. scapterisci* IJs (Fig. 1b). *S. carpocapsae* and *S. scapterisci* had visible green-fluorescent bacteria at frequencies of 94.8 ± 0.007 and 92.7 ± 0.016, respectively (mean ± SD of three biological replicates of each nematode species). Like other Xenorhabdus spp., including *X. nematophila*, *X. innexi* localized to the receptacle region of the intestine posterior to the basal bulb. However, *X. innexi* appears distinct in that only a few cells (1–5 cells) were visible within the receptacles of individual colonized IJs (Fig. 1b), in contrast to the large number of *X. nematophila* occupying this region in *S. carpocapsae* nematodes (Fig. 1a). These observations support the quantitative data acquired by grinding, and indicate that *X. innexi* colonizes *S. scapterisci* IJs at a high frequency, but at very low levels compared to *X. nematophila* colonization of *S. carpocapsae* (typically ~40 CFU/IJ using this method) [31]. We next examined the growth characteristics of *X. innexi* in laboratory medium, compared to *X. nematophila* and *X. bovienii*, the symbiont of *S. jollieti*, two Xenorhabdus bacteria for which complete genomes exist [6]. We found that in LB medium, *X. innexi* displayed a longer lag, a significantly slower growth rate (Additional file 1), and a lower final OD_{600} compared to *X. bovienii* and *X. nematophila* (Fig. 1c).

*X. innexi* is avirulent at ecologically relevant doses

Given that an individual *S. scapterisci* nematode would inoculate an insect host with few cells of its *X. innexi* symbiont (Fig. 1) [24], we next assessed the contribution of *X. innexi* to the nematode-symbiont complex by injecting quantified doses into several potential insect hosts and the model insect *Drosophila melanogaster*. We compared this to the virulence of *X. nematophila*, the well-characterized bacterial symbiont of *S. carpocapsae*.

Similar to previous studies [32, 33], we found that *X. nematophila* is highly toxic to *G. mellonella* waxworm larvae, rapidly killing these insects even at low doses (Fig. 2a). *X. nematophila* quickly grew in waxworm larvae, reaching over 1 million colony-forming units (CFUs) in less than 24 h, regardless of the inoculating dose (Fig. 2b). We found similar results in adult *D. melanogaster*, where *X. nematophila* rapidly killed the adults and grew to over 1 million CFUs in less than 18 h (Fig. 2c-d; Additional file 2). In contrast to *X. nematophila*, *X. innexi* was nearly avirulent when injected into fruit fly adults (Fig. 2e). We found that all but the highest dose we tried, 100,000 CFUs, proved to have little to no effect on fruit fly survival. We plotted the growth of the bacteria in infected flies over time and found that *D. melanogaster* adults are highly resistant to *X. innexi* (Fig. 2f). The flies reduced bacterial...
growth and eventually cleared the bacteria from the system, even when given an initial dose of 10,000 CFUs (Fig. 2f). When we injected 100,000 CFUs, the bacterial cells were able to grow and kill the flies quickly, but this dose would require more than 14,000 nematode IJs to initiate infection and therefore is not ecologically relevant (Fig. 1) [24]. *X. innexi* was also avirulent in waxworm larvae, except when injected at 100,000 CFUs (Fig. 2g). In *Manduca sexta* larvae, an inoculum of 1000 CFU was sufficient for *X. nematophila* to cause death of 50% of insects by 48 h post-injection, while *X. innexi* only killed 10% of insects toward the end of the experiment (5 d post-injection) (Fig. 2h). The attenuated virulence of *X. innexi* in these various insects supports the idea that the *S. scapterisci*-*X. innexi* complex has a specialized host range. Further, we used the growth data from these experiments to calculate in vivo growth rates in *D. melanogaster*, which, similar to in vitro growth rates, are lower for *X. innexi* than for *X. nematophila* (Additional file 1).

| Table 1 | Strains and plasmids used in this study |
| --- | --- |
| **Strain** | Relevant characteristics | Source/Reference |
| HGB800 | Xenorhabdus nematophilis isolated from Steinernema carpocapsae All nematodes | ATCC19061 |
| HGB1053 | Xenorhabdus bovienii SS-2004 | [119] |
| HGB1681 | Xenorhabdus innexi isolated from Steinernema scapterisci nematodes from Grover Smart. Also called Xenorhabdus MT, deposited to ATCC in 2005. | G.C. Smart Jr. University of Florida; ATCC PTA-6826 |
| HGB1997 | Xenorhabdus innexi isolated in 2013 from Steinernema scapterisci nematodes obtained from Becker-Underwood | This study |
| HGB2171 | HGB1681 attTn7/Tn7-GFP (from pUJR25) | This study |
| HGB2172 | HGB1997 attTn7/Tn7-GFP (from pUJR25) | This study |
| HGB283 | Escherichia coli S17–1 lambda pir pUX-BF13 | [107] |
| HGB1262 | Escherichia coli BW29427 pUJR25, mini Tn7-GFP | B. Lies and D. Newman [108] |
| TOP10 | E. coli strain for general cloning | Thermo |
| **Plasmids** | | |
| pBluescript II SK (−) | General cloning | Stratagene |
| pKanWOR | pBluescript KS+ with Km cassette (1 kb) in BamHI site | H. Goodrich-Blair |
| pCR-Blunt II-TOPO | General cloning vector, Kanr | Thermo |
| pBlueXIS1_460109Up | XIS1_460109Up inserted in pBluescript SK- | This study |
| pBlueXIS1_460109UpDn | XIS1_460109Dn inserted in pBlueXIS1_460109Up | This study |
| pBlueXIS1_460109UpKanDn | Kan cassette from pKanWOR inserted in pBlueXIS1_460109UpDn | This study |
| pBlueXIS1_460115Up | XIS1_460115Up inserted in pBluescript SK- | This study |
| pBlueXIS1_460115UpDn | XIS1_460115Dn inserted in pBlueXIS1_460115Up | This study |
| pBlueXIS1_460115UpKanDn | Kan cassette from pKanWOR inserted in pBlueXIS1_460115UpDn | This study |
| pKR100 | oriR6K suicide vector, Cmr | H. Goodrich-Blair |
| pKRXIS1_460109 | XIS1_460109UpKanDn inserted in pKR100 | This study |
| pKRXIS1_460115 | XIS1_460115UpKanDn inserted in pKR100 | This study |

| Table 2 | *X. innexi* colonization of *S. scapterisci* nematodes |
| --- | --- |
| **Strain** | Relevant Characteristics | Avg. CFU/IJ ± SE a |
| HGB1681 | *X. innexi* (Smart) | 6.1 ± 1.1 |
| HGB1997 | *X. innexi* (BD) | 7.9 ± 1.4 |
| HGB2171 | *X. innexi* (Smart) GFP | 6.4 ± 1.4 |
| HGB2172 | *X. innexi* (BD) GFP | 6.7 ± 1.0 |
| HGB800 | *X. nematophila* | <0.005 |

 aAverage colony forming units (CFU) per infective juvenile (IJ) ± standard error (SE) from four independent experiments.
X. innexi supernatant does not suppress the Manduca sexta phenoloxidase cascade

A common activity associated with Xenorhabdus bacteria is the ability to suppress aspects of insect immunity, including the phenoloxidase (PO) system. PO is activated by the cleavage of proPO, which occurs as a result of a serine protease cascade [34]. Several metabolites secreted by X. nematophila such as rhabduscin can inhibit the activation of PO [35–37]. To determine if X. innexi also secretes immunosuppressive metabolites we isolated cell-free supernatant from it and X. nematophila as a control and assessed their abilities to inhibit the activation of PO when incubated with plasma extracted from M. sexta insects (Fig. 3). We found that as expected, X. nematophila produces heat-tolerant factor(s) that can reduce PO activation to 30% of control reactions. In contrast, X. innexi supernatants do not inhibit the activation of PO, indicating that when grown to stationary phase in laboratory culture this bacterium does not secrete immunosuppressive factors at levels sufficient for detection in this assay.

The X. innexi genome has a reduced complement of genes predicted to encode virulence determinants, compared to those of other Xenorhabdus spp

We have presented data that X. innexi is attenuated for virulence in several insect models and for the secretion of immunosuppressive factors. These data and previous publications support a model that S. scapterisci is less reliant than other EPNs on its symbiont for fitness. However, X. innexi does contribute to S. scapterisci success in some insect hosts [10, 13, 24] and also produces several factors of interest, including a mosquitoidal toxin [28]. We predicted that the genome of X. innexi might reveal a reduction in the canonical virulence determinants associated with Xenorhabdus and related species, while also potentially encoding novel virulence factors that contribute to its specialization for virulence in certain insect hosts.

To further investigate these ideas, we produced a draft genome sequence for X. innexi strain HGB1681 (Table 3) (Accession for the whole genome shotgun sequencing project: FTLG0000000.1). The XIS1 draft genome comprises 69 scaffolds (LT699767-LT699835) and 246 contigs (FTLG01000001-FTLG01000246). In total, the genome is similar in size (4,574,778 bp), GC content (43.68%), and coding potential (4418 CDS) and density (83%) to the complete genomes of X. nematophila and X. bovienii (Table 3) [6]. Due to the draft status of the sequenced genome, only one copy of 16S rRNA, one copy of 23S rRNA and two copies of 5S rRNA were successfully assembled while the completed genomes of both X. nematophila and X. bovienii have multiple copies of each rRNA gene. Since lower copy numbers of rRNA operons is associated with
lengthened lag phase and growth rate [38–40], phenotypes we have observed for X. innexi, it is possible that X. innexi does encode fewer rRNA gene copies, but this conclusion awaits further investigation. The draft genome of X. innexi encodes the same number (79) of tRNAs as do the complete genomes of X. nematophila and X. bovienii.

To investigate the virulence coding potential of the X. innexi genome, CDS protein sequences were analyzed using similarity to known virulence factors and conserved protein domains (Table 4, see Methods for details) [41]. In addition to these direct searches, we used the MicroScope Gene Phyloprofile tool [42] to identify sets of genes specifically absent in X. innexi genome (Additional file 3). We used loci present in the completely sequenced genome of the virulent strain X. nematophila (ATCC 19061) and identified those with homologs in the genomes of the virulent strains X. bovienii SS-2004 and X. doucetiae FRM16 [6, 7], but without homologs in the X. innexi HGB1681 genome.

Consistent with the reduction of virulence potential and absence of PO inhibition, the draft genome X. innexi lacked, or had a reduced complement of virulence

![Fig. 2 X. innexi is attenuated for virulence in three insect model hosts. Galleria mellonella (a, b, g), Drosophila melanogaster (c-f), or Manduca sexta (h) insects were injected with X. nematophila (a-d, h) or X. innexi (e-g, h) laboratory-grown bacterial cells at the level indicated in the symbol legend, or with controls as indicated. Over time after injection the insects were monitored for survival (a, c, e, g, and h) to assess bacterial virulence, or were destructively sampled for bacterial cell number (b, d, and f)](image-url)
factors typical of other Xenorhabdus genomes. For example, X. innexi does not encode Tc (or associated chitinases), Mcf, XaxAB, entire Rtx (see below), or Pir toxins [6, 43–46] or rhabduscin-encoding genes [37] (Table 4; Additional file 3).

In silico analysis of select X. innexi secretion systems and effectors
Bacteria encode numerous types of secretion systems, many of which allow delivery of virulence factors to the host environment and cells. As with other Xenorhabdus bacterial genomes [6], the genome of X. innexi lacks a Type III secretion system (T3SS) (determined using S. enterica T3S as a model; Additional file 4). Another class of secreted molecules that are often found in pathogens that lack T3SS is the MARTX (Multifunctional Autoprocessing Repeats-in-Toxin Toxins). These polymorphic toxins are very large and comprise an N-terminal region with conserved A and B repeats that appear necessary for delivery of the toxin into host cells, an effector domain region containing multiple modules with host-modulating functions, a CPD domain that processes the effector domains once in the host cell, and a C-terminal repeat domain necessary for secretion out of the bacterial cell through a Type 1 secretion system encoded by the rtxEDB operon and the unlinked tolc gene [47, 48] (Fig. 4).

Published literature has established that the X. nematophila and X. bovienii genomes each contain one complete MARTX-encoding gene, predicted to encode proteins of 4970 aa and 4716 aa respectively, each with canonical A repeats (A1-A10; A11-A14), B repeats (B1–38; B39–41) and C repeats (C1–2; C3–15), and a CPD domain. Both also contain the effector domains DUF1, RID, and MCF, but they are distinct in that X. nematophila includes a PMT C1/C2 (now known as RRSP) domain [49, 50], while that of X. bovienii encodes an ABH domain, both immediately following their respective RID domains [51, 52]. Consistent with the genomic context of other organisms, the MARTX-encoding genes of X. nematophila and X. bovienii are encoded adjacent to those predicted to encode Rtx activating and secretion functions.

The X. innexi genome contains 4 contigs with regions that have similarity to MARTX-encoding genes, based on a BLASTp search with XNC1_1381 (Fig. 4). In the assembly, only one gene (XIS1_650005) encodes a full suite of A, B, and C repeats and the effector domains. However, based on alignment with the X. nematophila and X. bovienii MARTX proteins this protein lacks A repeats 4 through 8 (of 14) (Fig. 4 and Additional file 5). In addition to XIS1_650005, we identified another five regions with one or more MARTX protein-encoding domains. Of these, two (XIV1_640001 and XIS1_650001) are predicted to encode an A domain region that, like

Table 3 Draft genome statistics

|                     | X. innexi HGB1681 | X. nematophila ATCC 19061 | X. bovienii SS-2004 |
|---------------------|-------------------|--------------------------|---------------------|
| Size of chromosome (bp) | 4,575,778         | 4,432,590                | 4,225,498           |
| G+C content, %        | 43.68             | 44.15                    | 44.97               |
| Coding sequences      | 4418              | 4648                     | 4406                |
| Number of scaffolds   | 69                | 1                        | 1                   |
| Number of contigs     | 246               | 1                        | 1                   |
| Average CDS length (bp) | 885.8            | 850.81                   | 849.48              |
| Average intergenic length (bp) | 179.85 | 163.62 | 158.03 |
| Protein coding density % | 82.93      | 82.65                    | 84.07               |
| tRNAs                | 4                 | 29                       | 29                  |
| tRNAs                | 79                | 79                       | 83                  |

Table 4 Numbers of X. nematophila and X. innexi genes encoding known virulence factors

| Gene family | X. innexi HGB1681 | X. nematophila ATCC 19061 |
|-------------|-------------------|--------------------------|
| Chitinases  | 0                 | 2                        |
| HIP57 (GroEL) | 1                  | 3                        |
| MARTX       | 3                 | 1                        |
| Mcf         | 0                 | 1                        |
| Pir toxins  | 0                 | 2                        |
| PrtA        | 1                 | 1                        |
| Rhabduscin  | 0                 | 3                        |
| Tc toxins (A) | 0                | 6                        |
| Tc toxins (B) | 0                 | 3                        |
| Tc toxins (C) | 1                 | 3                        |
| TPS-Fha     | 2                 | 0                        |
| TPS-Hemolysin | 2                 | 1                        |
| Xenocin     | 0                 | 1                        |

*a indicates at least one fragment

Fig. 3 X. innexi supernatant does not suppress prophenoloxidase activation. Percent proPO system activation ± SEM in hemolymph incubated with control medium (dotted bars) or cell-free supernatant from X. innexi or X. nematophila that was either untreated (white bars) or boiled for 10 min. at 95 °C (black bars). Different letters indicate significant difference (p < 0.05, one-way ANOVA Friedman test followed by Dunn’s post test) was observed between strains when compared for proPO inhibition.
XIS1_650005, lack repeats 4 through 8, as well as a truncated B domain region (extending up to repeat 16 of 38) that lack repeats B1–B6 (Fig. 4 and Additional file 5). Additional B repeats (corresponding to ~B24 through B33) are found encoded by XIS1_630001. Finally, XIS1_640004 and XIS1_6200012 both encode B repeats (38–41) and an effector domain region with the same composition as that of X. bovienii (Fig. 4 and Additional file 5). In sum, consistent with the attenuated virulence of X. innexi, the genome appears not to encode a complete MARTX protein (since internal repeats appear to be missing from the A domain of XIS1_650005). It will be of interest to determine the functional significance of the absence of these repeats, assuming that the missing repeats are verified and not due to assembly issues.

Another secretion system with implications for virulence are the two-partner secretion (TPS) systems (also known as Type 5 or autotransporters), which encode both the toxin (e.g. hemolysin) and its transport system (Tables 4 and 5) [53]. In these systems one protein forms a beta-barrel pore that facilitates translocation of an exoprotein across the outer membrane. Typically, the beta-barrel protein and the exoprotein are encoded adjacent to each other. A conserved feature of these systems is the TPS domain encoded in the N-terminal 250 aa of the A exoprotein, which is necessary for translocation and contains a conserved NPNL-35aa-NPNGI motif. Generally, this region is conserved across types of secreted exoproteins, while the remaining portions of the protein are distinct. Previous phylogenetic analyses of whole TpsA sequences [6] revealed that Tps proteins are divided into three clusters. The first cluster contains CdiA exoproteins, which are involved in the contact-dependent inhibition systems, playing important roles in inter-strain competition and self/nonself discrimination. CDI systems are mainly distributed among pathogenic Gram-negative bacteria [54–57], and recently described in the entomopathogenic bacterium Xenorhabdus doucetiae FRM16 [58].
The second cluster is comprised of active hemolysins, such as PhlA from *Photorhabdus luminescens* and XhlA from *X. nematophila* [59, 60]. A third cluster contains TpsA proteins with unknown functions, which are characterized by the presence of a DUF637 domain.

In the draft *X. innexi* genome we identified a total of five genes predicted to encode proteins with an N-terminal TPS domain including conserved NPNL and NPNGI domains (Table 5). The genomic contexts of these suggest five independent loci encoding Tps systems. XIS1_1110029 and XIS1_1600026 genes encode proteins with TPS domains that belong to the hemolysin phylogenetic cluster (Fig. 5; Additional file 6). The XIS1_1110029-encoding protein displays 67% identity over its entire length (1468 aa) to the *X. nematophila* XhlA hemolysin (XhlA xn) [59] and was therefore named XhlAxi.

The genomic locus XIS1_1110029-1 encoding protein displays identity with functionally characterized CdiA proteins (36% identity with the *X. doucetiae* FRM16 CdiA and 34% identity with the *E. coli* EC93 CdiA) and has a VENN domain, which usually separates the conserved N-terminus from the variable C-terminus in many CdiA proteins [61]. Moreover, the adjacent genes XIS1_680062 and XIS1_680060 encode a CdiB ortholog and a potential immunity protein CdiI (based on location of the gene and the small size of the encoded protein), respectively. We therefore hypothesize that this locus is a cdiBAI locus.

The fifth tps genomic locus we identified includes XIS1_680061 and XIS1_260017 encode proteins with TPS domains that belong to the Cdi phylogenetic cluster (Fig. 5). The XIS1_680061-encoding protein displays identity with functionally characterized CdiA proteins (36% identity with the *X. doucetiae* FRM16 CdiA and 34% identity with the *E. coli* EC93 CdiA) and has a VENN domain, which usually separates the conserved N-terminus from the variable C-terminus in many CdiA proteins [61]. Moreover, the adjacent genes XIS1_680062 and XIS1_680060 encode a CdiB ortholog and a potential immunity protein CdiI (based on location of the gene and the small size of the encoded protein), respectively. We therefore hypothesize that this locus is a cdiBAI locus.

**Table 5** *X. innexi* two partner secretion pathway loci

| XIS1_ | Gene       | Length (aa) | Predicted Function | TPS cluster or relevant features |
|-------|------------|-------------|--------------------|----------------------------------|
| 1110029 | xhlBxi  | 558         | Beta-barrel        | Cluster II (hemolysin)            |
| 1110029 | xhlAxi  | 1468        | Exoprotein         | Cluster II (hemolysin), TPS motif |
| 1600025 | xhlB2xi | 558         | Beta-barrel        | Cluster II (hemolysin)            |
| 1600026 | xhlA2xi (part 1) | 656 | Exoprotein fragment | Cluster II (hemolysin), TPS motif |
| 1600027 | xhlA2xi (part 2) | 801 | Exoprotein fragment | Cluster II (hemolysin)            |
| 680062  | cdiBxi   | 569         | Beta-barrel        | Cluster I (Cdi)                   |
| 680061  | cdiAxi   | 4029        | Exoprotein         | Cluster I (Cdi), TPS motif and VENN domain |
| 680060  | cdiB-likexi | 4029   | Immunity protein   | Cluster I (Cdi), putative cdiB-likexi |
| 260016  | cdiB-likexi | 571   | Beta-barrel        | Cluster I (Cdi)                   |
| 260017  | cdiA-like (part 1) | 1157   | Exoprotein fragment | Cluster I (Cdi), TPS motif, lacks VENN motif |
| 270001  | cdiA-like (part 2) | 180    | Exoprotein fragment | Cluster I (Cdi), lacks TPS motif and VENN domain, contains beta barrel region, |
| 280001  | cdiA-like (part 3) | 4062   | Exoprotein fragment | Cluster I (Cdi), lacks TPS motif, starts with the beta barrel region, includes VENN domain |
| 1500009 | tpsBxi   | 566         | Beta-barrel        | Cluster III (DUF637 domain)       |
| 1500008 | tpsAxi   | 1907        | Exoprotein         | Cluster III (DUF637 domain), TPS motif and DUF637 domain |
| 1500007 | tpsIxi   | 110         | unknown            | Potential immunity protein        |

The second cluster is comprised of active hemolysins, such as PhlA from *Photorhabdus luminescens* and XhlA from *X. nematophila* [59, 60]. A third cluster contains TpsA proteins with unknown functions, which are characterized by the presence of a DUF637 domain.

In the draft *X. innexi* genome we identified a total of five genes predicted to encode proteins with an N-terminal TPS domain including conserved NPNL and NPNGI domains (Table 5). The genomic contexts of these suggest five independent loci encoding Tps systems. XIS1_1110029 and XIS1_1600026 genes encode proteins with TPS domains that belong to the hemolysin phylogenetic cluster (Fig. 5; Additional file 6). The XIS1_1110029-encoding protein displays 67% identity over its entire length (1468 aa) to the *X. nematophila* XhlA hemolysin (XhlA xn) [59] and was therefore named XhlAxi.

The genomic locus XIS1_1110029-1 encoding protein displays identity with functionally characterized CdiA proteins (36% identity with the *X. doucetiae* FRM16 CdiA and 34% identity with the *E. coli* EC93 CdiA) and has a VENN domain, which usually separates the conserved N-terminus from the variable C-terminus in many CdiA proteins [61]. Moreover, the adjacent genes XIS1_680062 and XIS1_680060 encode a CdiB ortholog and a potential immunity protein CdiI (based on location of the gene and the small size of the encoded protein), respectively. We therefore hypothesize that this locus is a cdiBAI locus.

The fifth tps genomic locus we identified includes XIS1_150008, encoding a 1907 aa protein with a TPS-domain and a DUF637 domain, placing it in the third phylogenetic cluster (Fig. 5), for which no function is described to date. XIS1_150009 encodes a TpsB ortholog. Interestingly, XIS1_150007 displays features of immunity genes due to its location and its small size although the
The tpsA gene does not fall in the Cdi phylogenetic cluster (Table 5).

In summary, the X. innexii genome displays tps clusters in each of the three phylogenetic clusters, which sets it apart from other Xenorhabdus genomes. For instance, in the genome of the highly virulent Xenorhabdus nematophilus ATCC19061 strain, only hemolysin and DUF637 domain clusters are represented (Fig. 5; Additional file 6).

Another class of secretion systems that can be involved in virulence is the T6SS. These are bacterial nanomachines comprising 13 conserved structural proteins, which deliver toxic effectors into eukaryotic or prokaryotic organisms in a one-step firing mechanism [62]. T6SSs often are associated with roles in virulence and inter-bacterial competition, providing a selective advantage against competitors [63]. To analyze the T6SS content in the draft genome of X. innexii, we used a combination of the Magnifying Genomes server (MaGe) and NCBI Conserved Domain Database and identified three T6SS clusters, T6SS-1,2, and 3 (named in order of their appearance in the draft genome) (Fig. 6a, Additional file 7).

T6SS-1 appears to be incomplete, as it lacks the tssJ, tssG, and clpV components. Although ClpV is dispensable for some T6SSs, TssJ and TssG are required [62] suggesting the

![Fig. 5 Phylogenetic analysis of putative TpsA proteins of X. innexi.](image)
X. innexi T6SS-1 system may not be functional. The T6SS-2 cluster lacks tssG, but contains all other core components as well as potential effector-immunity (E-I) protein pairs. Additionally, T6SS-2 contains both tagH, a forkhead-associated domain-containing protein, and ppkA, a transmembrane threonine kinase. TagH and PpkA are components of the threonine phosphorylation pathway (TPP), a post-translational regulatory mechanism for T6SS activity [64]. The T6SS-3 cluster appears complete and includes a duplication of the baseplate protein, tssA, and a potential E-I protein pair. Genes encoding putative T6SS E-I pairs can be found clustered with the T6SS structural genes or scattered about the genome, often linked to a T6SS chaperone. One such group of T6SS effectors is the polymorphic toxins, Rhs proteins.

Rhs proteins containing PAAR domains have been reported as T6SS-dependent antibacterial effectors that mediate both intra- and inter-species competition [65]. Rhs proteins, in human pathogens such as Pseudomonas aeruginosa and Enterobacter cloacae, mediate bacterial competition under in vitro conditions. For these pathogens, Rhs proteins may play an important role in virulence by establishing a suitable niche for survival during infection of the host [66]. Genes encoding known or
putative T6S effectors, including Rhs proteins, are often found near vgrG genes and require the cognate Vgr for T6S secretion [67]. X. innexi strain HGB1681 encodes ten YD-repeat (PF05593) proteins, five of which are putative Rhs proteins based on the presence of characteristic YD-repeats, a rhs core domain flanked by conserved motifs, and a variable C-terminal ‘tip’ [68] (Fig. 6b). These five genes can be further categorized into two groups based on their genomic context. Two genes, Xi_rhs-1 and Xi_rhs-2 encode an N-terminal PAAR motif (PF05488), though Xi_rhs-2 is annotated as a truncated ORF, missing its C-terminal-encoding domain. The other three (Xi_rhs-3 through rhs-5) lack the PAAR domain but are encoded next to small open reading frames with a PAAR-like domain, DUF4150/PF13665. Furthermore, the Xi_rhs-1 and Xi_rhs-2 are encoded downstream of a DUF1795-containing protein and putative vgrG gene, both of which were necessary for Rhs protein translocation in Serratia marcescens [69]. The other three rhs genes are also encoded downstream of putative vgrG genes. The genomic contexts of these three rhs genes are distinct from those of rhs-1 and rhs-2 in that these gene clusters also encode DUF2169 and DUF4150 domain-containing proteins, which in Agrobacterium tumefaciens are demonstrated accessory proteins required for secretion of their cognate T6SS toxin [67].

The highly variable C-terminal domains (CTDs) of Rhs proteins contain the toxic effector activity. An analysis of the CTDs of X. innexi Rhs-family proteins (except Xi_rhs-2 which lacks a CTD) revealed no recognizable CTD function in rhs-3, −4, and −5. In contrast rhs-1 contains PF14437, a MafB19 deaminase domain [70]. This domain occurs in the CTDs of several classes of polymorphic toxins, including the recently recognized Neisseria MafB toxins, and the Rhs protein putative toxin E1IMF [70]. While our data demonstrate that in several insect hosts, X. innexi displays attenuated virulence relative to other Xenorhabdus spp. it remains an associate of S. scapterisci nematodes (Fig. 1, Table 2), and successfully reproduces within crickets, where it may encounter competing microbes (Additional file 8). Together with the T6SS, the Rhs family proteins encoded by X. innexi may play a role in any one of these activities.

### Table 6 NPRS, PKS, and NPRS-PKS hybrid clusters in the X. innexi genome

| Location         | Size (bp) | Type        | Number of A or AT domains |
|------------------|-----------|-------------|---------------------------|
| XIS1_300014      | 5139      | PKS         | 1 AT domain               |
| XIS1_250010      | 13,755    | NRPS        | 6 A domains               |
| XIS1_400005 - XIS1_60002 | 21,138  | NPRS        | 4 A domains               |
| XIS1_170001 - XIS1_190004 | 21,111  | NPRS        | 6 A domains               |
| XIS1_370002 - XIS1_370004 | 15,468  | NPRS        | 3 A domains               |
| XIS1_390007 - XIS1_400001 | 15,519  | NPRS        | 5 A domains               |
| XIS1_450016      | 2997      | NPRS        | 1 A domain                |
| XIS1_460014      | 7323      | NPRS        | 2 A domains               |
| XIS1_460105 - XIS1_460116 | 44,193  | Hybrid      | 6 A domains and 2 AT domains |
| XIS1_480023 - XIS1_480027 | 23,055  | Hybrid      | 3 A domains and 1 AT domain |
| XIS1_600036      | 3060      | NPRS        | 1 A domain                |
| XIS1_660020 - XIS1_660029 | 21,609  | Hybrid      | 3 A domains and 1 AT domain |
| XIS1_1050019 - XIS1_1050019 | 3366  | NPRS        | 1 A domain                |
| XIS1_1690009 - XIS1_1690010 | 23,319  | NPRS        | 7 A domains               |
| XIS1_1700078     | 11,811    | NPRS        | 3 A domains               |
| XIS1_1750018 - XIS1_1750021 | 44,826  | NPRS        | 13 A domains              |

a A domain; Adenylation domain, AT domain; Acyltransferase domain predicted by antiSMASH
b PKS; Polyketide synthase
c NRPS; Non-ribosomal peptide synthetase
d Hybrid; NRPS-PKS hybrid gene
further examined by analyzing their amino acid sequences through AntiSMASH or Conserved Domain searches to identify NRPS and PKS domains and to confirm the number of adenylation (A) and acyltransferase (AT) domains, which are responsible for selection and loading of amino acids or carboxylic acids, respectively for incorporation into the product (Table 6).

Identification of the Xlt-encoding NRPS/PKS hybrid gene cluster

_X. innexi_ secretes a small lipopeptide named Xlt with toxicity toward mosquitoes [28, 29]. Previous structural data suggested that Xlt is a cyclic lipopeptide composed of six amino acids and two fatty acids [28] and we hypothesized that it may be synthesized by a hybrid NRPS/PKS cluster [72]. Based on this hypothesis, we predicted that the locus would encode six A- and two AT-domains respectively. Among the three identified hybrid genes in _X. innexi_ genome, only one gene cluster, XIS1_460105 to _460116_ (present in the center of a single contig) has two AT-domains and six A-domains that correspond to the number of fatty acids and amino acids identified in Xlt [28].

The candidate gene cluster encodes 12 ORFs with predicted NRPS or PKS functions based on BLASTp analysis (Table 7). Eight of these had predicted PKS or PKS-related functions: _XIS1_460105 (NRPS/glutamate racemase), _XIS1_460107 (NRPS), and _XIS1_460106 (condensation protein).

The arrangement of genes from _XIS1_460105 to _460116_ is similar to those of the fabclavine synthesis loci found in _Xenorhabdus budapestensis_ DSM 16342 and _X. szentirmaii_ DSM 16338 (fcl), and prezeamine synthesis loci from _Serratia plymuthica_ AS 9 (zmn) [73, 74]. We compared the _X. innexi_ genes predicted to encode Xlt biosynthesis machinery to fcl and zmn sequences from _X. szentirmaii_ and _S. plymuthica_ respectively (the sequences of _X. budapestensis_ fcl were not available). BLASTp analysis indicated that the predicted function of each gene in Xlt biosynthesis gene cluster is very similar to both Fcl and Zmn coding genes (Table 8). We noted two differences in coding content, both on the flanking edges of the _X. innexi_ cluster, relative to _X. szentirmaii_: the first gene in the _X. szentirmaii_ locus (fclA, predicted to encode a NUDIX hydrolase) is absent in the Xlt-encoding cluster (Tables 7 and 8) [74]. Instead, the flanking genes are predicted to encode a TonB homolog and a cardiolipin synthase. Also, _X. szentirmaii_ has cluster genes fclM and fclN, predicted to encode ABC transporters, immediately downstream of the last condensation domain gene [74]. In contrast, in _X. innexi_ the gene following the last condensation domain is predicted to encode an acyl-CoA thioesterase (XIS1_460105). This difference may reflect a distinct release mechanism of the final Xlt product relative to fabclavine and zeamine. Acyl-CoA thioesterases are involved in the release of fatty acids [75] and are most active on myristoyl-CoA but also display high activities on palmitoyl-CoA, stearoyl-CoA and arachidoyl-CoA [76–78]. Therefore, it is possible that in _X. innexi_ the second PKS module produces 3-oxo-saturated fatty acids of the chain length from C14 to C20, consistent with the description of preliminary fatty acid structure data for Xlt [28]. The presence of a distinctive acyl-CoA thioesterase encoding gene within the putative Xlt-biosynthetic cluster provides further support that this cluster is involved in the synthesis of Xlt and that Xlt may have unique characteristics relative to zeamine/fabclavine.

Various domain analysis programs were used to verify the predicted biosynthetic activities and specificities of the candidate Xlt synthesis gene cluster (see Methods). As expected based on the similarities noted above, the number of A-domains found from _XIS1_460105 to _460116_ was the same as observed for fcl and zmn gene clusters [73, 74]. In fact, the predicted Stachelhause codes from Xlt coding genes were nearly identical to that of Fcl and Zmn coding genes (Fig. 7b, Table 2 in [79] and Table 8 in [80]). The peptide moiety incorporated by A-domains in Xlt coding genes closely resembled both Fcl and Zmn synthesis genes, and this further suggested that the candidate Xlt biosynthesis gene

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**Table 7** Gene location, size and putative function of the candidate Xlt biosynthesis gene cluster from _X. innexi_.

| Gene location | Size (aa) | Putative function |
|---------------|----------|------------------|
| XIS1_460116   | 338      | Membrane protein of unknown function |
| XIS1_460115   | 1974     | Type-I PKS |
| XIS1_460114   | 1471     | Beta-ketoacyl synthase |
| XIS1_460113   | 948      | PfaD family protein |
| XIS1_460112   | 255      | 3-oxoacyl-(acyl-carrier-protein) reductase |
| XIS1_460111   | 412      | Thioester reductase/polyketide synthase |
| XIS1_460109   | 539      | Condensation protein/peptide synthase |
| XIS1_460107   | 142      | Acyl-CoA thioesterase/acetyl-CoA thioester hydrolase |
cluster is homologous to the Fcl and Zmn clusters. In *X. innexi*, NRPSpredictor2 predicted A1 through A6 to be A1: serine, A2: phenylalanine, A3: asparagine, A4: asparagine, A5: threonine, and A6: valine (Fig. 7a and b). Also, some programs predicted an epimerization domain, which may indicate that the A3-domain incorporates a D-asparagine/aspartic acid. Refinement with Stachelhause codes indicated 90% probability that A3 and A4 are asparagine and A5 is threonine (Fig. 7b). However, consistent with the fact that spectral analysis between 260 and 280 nm indicates Xlt lacks phenylalanine (J.C. Ensign, unpublished data), the nearest neighbor scores for this amino acid (as well as serine and valine) were low.

In silico analysis of NRPS and PKS modules in the gene cluster from *XIS1_460105* to _460116_ provided a

### Table 8

| Locus tag     | Identity (%) to *X. szentirmaii* fcl locus | Identity (%) to *S. plymuthica* zmn locus |
|---------------|------------------------------------------|------------------------------------------|
| XIS1_460116   | N/A                                      | SerAS9_4283                             |
| XIS1_460115   | FdC                                      | SerAS9_4282                             | 43.03%                   |
| XIS1_460114   | FdD                                      | SerAS9_4281                             | 59.15%                   |
| XIS1_460113   | FdE                                      | SerAS9_4280                             | 58.37%                   |
| XIS1_460112   | FdF                                      | SerAS9_4279                             | 75.53%                   |
| XIS1_460111   | FdG                                      | SerAS9_4278                             | 70.20%                   |
| XIS1_460110   | FdH                                      | SerAS9_4277                             | 62.72%                   |
| XIS1_460109   | FdI                                      | SerAS9_4276                             | 65.12%                   |
| XIS1_460108   | FdJ                                      | SerAS9_4275                             | 50.79%                   |
| XIS1_460107   | FdK                                      | SerAS9_4274                             | 59.94%                   |
| XIS1_460106   | FdL                                      | SerAS9_4273                             | 55.06%                   |
| XIS1_460105   | N/A                                      | SerAS9_4272                             | 48.05%                   |

Amino acid identities* of predicted proteins encoded by *X. innexi* putative Xlt-biosynthetic locus and fabclavine and prezeamine biosynthetic clusters encoded by *X. szentirmaii* DSM 16338 and *S. plymuthica* AS9 respectively

*Based on the protein blast (BLASTp) analysis

*N/A- Not available

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**Fig. 7** Predicted NRPS and PKS domains between *XIS1_460105* and _460116_, and the analysis of adenylation domains. Domains were identified by analyzing translated sequences of each ORF using the Conserved domain search and AntiSMASH. Panel a displays the domain annotation based on the AntiSMASH analysis and Conserved Domain search. Aminotransferase (AT) domain containing ORFs are highlighted in orange and adenylation (A) domain containing ORFs are highlighted in blue. Panel b represents the predicted amino acid substrate of each adenylation domain from the candidate Xlt synthesis NRPS-PKS hybrid cluster from *X. innexi*. The amino acid substrate prediction was made based on the extracted Stachelhause code by NRPSpredictor2 [120]. KS: ketoacyl-synthase, AT: acyl-transferase, KR: ketoreductase, AM: aminotransferase, NAD: NAD(P)-binding amidase, A: adenylation, T: thiolation/peptide carrier protein, E: epimerization
strong rationale that the selected gene locus is the likely candidate to produce Xlt. This prediction is largely consistent with the preliminary structural analysis of the mosquitocidal toxin, which indicated the presence of serine, asparagine, glycine and at least one oxo-fatty acid of C8 to C20 [28]. The presence of certain amino acid residues of Xlt, including histidine and 2,3-diaminobutyric acid (DAB), could not be explained by the in silico analysis conducted in this study. However, the structural analysis of fabclavine, which is produced by a homologous gene cluster from X. szentirmaii and X. budapestensis, showed a replacement of phenylalanine by histidine as well as the presence of 2,3-diaminobutyric acid (DAB) in its peptide moiety. The structure of fabclavines, which corresponds to the preliminary structural data of Xlt, provided further support that the selected gene cluster should produce Xlt. Based on our prediction, we next tested if mutation of the gene cluster from XIS1_460105 to 460116 would disrupt mosquitocidal toxin activity in X. innexi.

Site-directed mutagenesis at XIS1_460115 or XIS1_460109 resulted in phenotypic changes

To further explore the possibility that the candidate gene cluster is involved in Xlt mosquitocidal toxin biosynthesis, we used site-directed mutagenesis to mutate two independent genes within the locus: XIS1_460115 and XIS1_460109. Each was individually replaced with a kanamycin cassette (see Methods) and supernatants from the resulting XIS1_460115:kan (ΔXIS1_460115) and XIS1_460109:kan (ΔXIS1_460109) mutants were analyzed with MALDI-TOF MS. Consistent with previous preliminary data, which indicated that Xlt has a molecular weight range between 1182 and 1431 Da, with the difference in molecular weights ascribed to varying lengths of fatty acids [28], our MALDI-TOF MS analysis of wild type X. innexi (HGB1681) supernatant revealed major peaks between 1348 and 1402 Da (Additional file 9). In contrast the supernatants of ΔXIS1_460115 and ΔXIS1_460109 did not have peaks in this region and rather showed either one major peak at 751 Da or three major peaks between 1182 and 1210 Da, respectively (Additional file 9).

Bioassays were conducted to examine if the mutation of XIS1_460115 or XIS1_460109 resulted in reduction or loss of the mosquito larvicidal activities, as predicted if the candidate gene cluster locus is necessary for Xlt biosynthesis. Of the mosquito larvae exposed to wild type X. innexi supernatant, 100% mortality was observed, up to 25% dilution of the supernatant (Fig. 8). Exposure to dilutions of 12.5% and 6.25% of supernatant resulted over 70% of mortality in 48 h (Fig. 8). However, both ΔXIS1_460115 and ΔXIS1_460109 culture supernatants were inactive at dilutions of 25% or lower (Fig. 8), and
only the undiluted supernatants from these mutants resulted in 100% mortality (Fig. 8).

Discussion
Xenorhabdus bacteria, symbionts of Steinernema nematodes, are increasingly exploited for novel products that may be useful in pharmaceutical, agricultural, and industrial settings [81]. Further, exploration of the biology of Xenorhabdus-Steinernema associations is yielding new insights into molecular and cellular biology and evolutionary and ecological principles underlying parasitism (e.g. [1, 82, 83]). In this study, we used X. innexi and its nematode host S. scapterisci, which specializes in parasitism of crickets, to expand our knowledge of potential virulence determinants produced by Xenorhabdus bacteria and to discern how X. innexi may be impacted by specialization. Our findings that X. innexi is an ineffective pathogen of several insects tested, that it does not secrete immunosuppressive factors, and that the X. innexi genome lacks many of the canonical virulence determinants encoded by its sister species may indicate that specialization in crickets has led to an erosion of virulence coding potential. However, the specificity of S. scapterisci for colonization by X. innexi, and our identification of several loci predicted (e.g. T6SS/Rhs) or confirmed (e.g. xlt) to be necessary for production of secreted factors indicate that X. innexi remains an actively transmitted and biologically active symbiont.

Relative to the well-characterized entomopathogenic nematode symbiont X. nematophila, X. innexi is attenuated for virulence and reproduction in the lepidopteran hosts G. mellonella and M. sexta, as well as the dipteran D. melanogaster. Unpublished data suggests X. innexi is also avirulent towards honeybees (Apis mellifera) and Colorado potato beetles (Leptinotarsa decemlineata) [29]. This suggest that the toxicity of the S. scapterisci-X. innexi pair either relies on the nematode or on an emergent synergism that we did not detect when using the bacteria alone [11].

Common genomic features of Xenorhabdus species with attenuated virulence phenotypes
X. innexi joins a growing list of Xenorhabdus species that displays attenuated virulence relative to other members of the genus. Other examples include X. poinarii G6, which is attenuated for virulence when injected into Spodoptera littoralis and G. mellonella insects. Its genome is smaller (3.66 Mbp) than that of either X. nematophila (ATCC19061) or X. bovienii (SS-2004) and lacks hemolysins, T5SS, Mcf, NRPS, and TA systems [7], suggesting a streamlining of the genome. In contrast, we report here that the genome of X. innexi is of similar size (slightly larger) as those of X. nematophila and X. bovienii, and while it lacks Tc toxins, Mcf, and other canonical Xenorhabdus virulence determinants, it does contain genes predicted to encode hemolysins and other T5SS genes and non-ribosomal small molecule biosynthetic machinery, including a locus necessary for production of an extracellular mosquitoicidal small molecule. Although caution is necessary when interpreting data based on a draft genome, we propose that in contrast to X. poinarii, the X. innexi attenuated virulence is due not to genome reduction, but rather to the presence of a distinct repertoire of genes.

In this sense, the X. innexi genome may be more similar to X. bovienii CS03, the symbiont of S. weiseri, another attenuated virulent Xenorhabdus bacterium [4]. In this case, rather than genome reduction (as in X. poinarii) the attenuated virulence appears to be associated with a genome shift away from virulence determinants and towards inter-bacterial competition. Both X. bovienii and X. innexi have genomes that are larger than those of X. nematophila and X. bovienii (SS-2004). Bisch et al. [4] proposed that the X. bovienii (CS03) genome had been shaped by the selection for factors mediating intermicrobial competition. A similar phenomenon may be occurring in X. innexi, an idea supported by the presence of T6SS and Rhs homologs, which in other systems mediate inter-bacterial competition, concomitant with an absence of canonical insect virulence determinants.

Curiously, X. poinarii, X. bovienii CS03, and X. innexi all lack, or have degraded genes encoding Tc toxins [4, 7]. It should be noted that the production of Tc toxins is not a requirement for virulence, since the Clade C1 bacterium, X. doucetiae is virulent in both S. littoralis and G. mellonella, even though it does not produce Tc toxins [7]. Ogier et al. [7] suggested that the absence of Tc toxins encoded in genomes of members of this clade (C1) [84] is due to loss of an ancestral component [7]. In the X. innexi genome we did not find evidence of fragmented or pseudogenized copies of Tc-encoding genes, as are present in the X. bovienii CS03 genome [4]. As such, we propose that the apparent lack of these genes in the genome of X. innexi, a member of clade CIV, indicates a loss event, separate from that proposed to have occurred in clade C1. Interestingly, in the draft genome of another strain of the CIV clade (X. cabanillasii, accession number: GCA_000531755), Tc loci are incomplete, which supports the idea that recent deletions for Tc-encoding genes have occurred in this clade (unpublished data, S. Gaudriault). Regardless, our data combined with those of Ogier et al. [7] and Bisch et al. [4] indicate that the presence of Tc-encoding genes is not a uniformly present trait among Xenorhabdus species. It may be that Tc toxins are generalized insecticidal factors that are not of adaptive benefit to Xenorhabdus with narrow host ranges. Although not investigated for S. weiseri- X. bovienii CS03 pair, both S. scapterisci-X. innexi and S.
glaseri-X. poinarii symbiont pairs appear to have a restricted host range relative to other Steinernema-Xenorhabdus pairs [7, 19, 85–87].

A hybrid NRPS/PKS locus is necessary for X. innexi mosquitocidal toxicity

Despite the ineffectiveness of X. innexi as a pathogen when injected into members of various orders of insects, cell-free supernatants from X. innexi do exhibit toxicity specifically towards larvae of Aedes, Anopheles and Culex mosquitoes [28]. Our bioinformatic analysis of the X. innexi genome revealed a candidate hybrid NRPS/PKS for the biosynthesis of a secreted mosquitocidal toxin. This prediction is supported by our experimental data showing reduction of Xlt lipopeptide synthesis in and mosquitocidal toxicity of the XIS1_460115 or XIS1_460109 supermatants. Entomopathogenic bacteria, including X. innexi, produce a diversity of secondary metabolites including antibiotics, antifungal and other virulence factors [88] and it is possible that the loss of mosquitocidal toxicity in the XIS1_460115 and XIS1_460109 mutants is due to disruption an indirect impact on these other pathways. However, the combined bioinformatic and genetic evidence more strongly support a direct role for the Xlt biosynthetic machinery in the production of the mosquitocidal lipopeptide.

The Xlt biosynthesis gene cluster we have identified is homologous to fcl and zmn clusters in the genomes of X. szentirmaii and S. plymuthica that encode machinery for the synthesis of a class of lipopeptides known as fabclavines and zemaines. Xlt biosynthesis gene cluster also differed from fcl and zmn cluster by the presence of acyl-CoA thioesterase at the end of the cluster as well as the lack of NUDIX hydrolase gene in the N-terminus and ABC transporter genes in the C-terminus. This genetic similarity and difference, combined with the similar mass to charge ratios of Xlt and fabclavines (~1347) supports the idea that Xlt is a derivative within the fabclavine family. X. budapestensis and X. szentirmaii produce multiple forms of fabclavine, some of which are distinguished by the presence of either a histidine or phenylalanine moiety at the 2 position. Since Xlt does not absorb at 280 nm (J. Ensign, unpublished data) it is unlikely to contain phenylalanine and thus Xlt is a derivative of a fabclavine Ib [74].

Fabclavines, and the related zemaine have a broad spectrum of bioactivity against bacteria, fungi, nema-todes, oomycetes, apicomplexans, and protozoa [79, 80, 89–92]. Similarly, Xlt demonstrated antimicrobial activities towards a broad spectrum of bacteria including Pseudomonas aeruginosa, Salmonella spp., Escherichia coli, Listeria monocytogenes, and Bacillus cereus [28].

The results presented here expand the list of fabclavine targets to include mosquito larvae. The literature includes multiple reports of Steinernema-Xenorhabdus activities against mosquitoes, which our data suggest could be mediated by bacterially-produced fabclavine and fabclavine derivatives. For instance, S. carpocapsae (the nematode host of X. nematophila) triggers an immune response in and can kill the larvae of Aedes aegypti, a vector of many diseases of humans [73, 93]. Although the mechanism underlying this observation was not investigated, the authors of these studies suggested it could involve a secreted toxin. In support of this concept, recent studies demonstrated toxicity toward Ae. aegypti larvae of cell-free supernatants from X. nematophila, the symbiont of S. carpocapsae [74]. Mosquitoes are unlikely to be natural hosts of Steinernema-Xenorhabdus species complexes in nature, raising the question of what the biological function of Xlt may be in the X. innexi life history. One possibility may be that it acts in inter-microbial competition, since as a lipopeptide Xlt may be able to disrupt bacterial cell membranes through detergent-like action [94, 95]. Certain bacterial lipopeptides such as surfactins and cyclic lipopeptides (CLPs) from Bacillus subtilis have both insecticidal and antimicrobial activity [96–99], although their mode of action against insects is not well understood.

It should be noted that while fabclavines as a class clearly have a broad target spectrum, moiety substitutions within individual fabclavine derivatives could result in varying and specialized activities. In turn, if Xlt and other Xenorhabdus-produced fabclavines have non-discriminant broad-spectrum bioactivities, it will be of interest to determine how Steinernema nematode hosts associated with the fabclavine-producing Xenorhabdus symbionts survive exposure to this generally toxic compound.

Conclusions

As a basis for continued exploration of X. innexi in biological studies and biotechnological applications we examined some of its characteristics. We found that unlike other reported EPN/bacterial symbioses, S. scapterisci is colonized at very low levels and that X. innexi has attenuated virulence compared to other species of Xenorhabdus. We have sequenced a draft version of the X. innexi genome and reported detailed analyses of several families of known virulence factors. We found no evidence for several key Xenorhabdus spp. toxicity genes, including Tc toxins and “makes caterpillars floppy” (Mcf) toxins. However, we also found that the X. innexi genome contains two-partner secretion (TPS) system genes from all three TPS clusters, including CdiA exoproteins, active hemolysins, and TpsA proteins. Consistent with other Xenorhabdus spp. genomes, we found numerous loci predicted to encode non-ribosomal peptide synthetases, which we explored and identified a
locus that putatively encodes a fabclavine derivative with mosquitocidal activity. The X innexi genome will be a valuable resource in identifying loci encoding new metabolites of interest, but also in future comparative studies of nematode-bacterial symbiosis and niche partitioning among bacterial pathogens.

Methods
Bacterial strains and growth conditions
Strains and plasmids used in this study are listed in Table 1. Two X. innexi strains were tested. One, HGB1681 (a.k.a. PTA-6826), is a lab stock strain acquired by Prof. Jerry Ensign (UW-Madison) from Prof. Grover Smart (University of Florida), the other was isolated from S. scapterisci nematodes provided by BD Scientific. In both cases the primary form was isolated as blue colonies on NBTA plates [100]. Xenorhabdus strains were incubated at 30 °C in media not exposed to light, or supplemented with 0.1% pyruvate [101]. Permanent stocks of the cultures were stored in broth supplemented with 20% glycerol at −80 °C. Luria Bertani (LB) was used for standard growth, and lipid agar (LA) was used for nematode-bacterium co-culture [102]. When noted, media were supplemented with ampicillin (150 μg/ml), kanamycin (50 μg/ml), streptomycin (150 μg/ml), or diaminopimelic acid (DAP) (80 μg/ml).

To determine the in vitro growth rate of X. innexi, we subcultured overnight cultures to an OD600 of 0.1 in LB with limited light exposure and grew them in a 96 well plate (Sarstedt 82.1581.001), 200 μl/well with liquid only (no cultures) in the outermost wells. The plate was incubated in a BioTech plate reader at 30 °C every hour. OD600 was measured, and then the biological replicates were plotted with the technical replicates within the 96-well plate. The technical replicates within each technical replicate were averaged for each biological replicate, and the biological replicates were measured, each with three technical replicates within the 96-well plate. The technical replicates were averaged for each biological replicate, and then the biological replicates were plotted with the standard error of the mean. The in vivo growth rates of X. nematophila and X. bovienii were included for comparison. For each species, three biological replicates were measured, each with three technical replicates within the same species. The technical replicates were averaged for each biological replicate, and then the biological replicates were plotted with the standard error of the mean. The in vivo growth rates of X. nematophila and X. innexi in D. melanogaster were calculated using the number of CFU (N1) at time 0 (t1) and the number of CFU (N2) recovered at 6 HPI (t2), using the following formula ln(N2/N1) = k(t2-t1).

Animal sources and husbandry
After purchase from a local vendor (Reptile Rapture, Madison, WI or PetSmart, Knoxville, TN) A. domesticus were stored in a large bucket and provided with apple slices and fresh spinach. S. scapterisci nematodes were obtained from Becker Underwood Inc. and BD Scientific and established in the laboratory through infection of Acheta domesticus house crickets. Typically, 20 crickets were used for infection with S. scapterisci nematodes, while 5 were left uninfected as controls. Crickets were infected within 1–2 days of purchase. Nematodes were propagated every 8 weeks. For infections a 100 mm diameter filter paper was placed in the top of an inverted 100 mm petri dish in which holes had been burned to allow airflow. The filter paper was soaked with 1 ml of S. scapterisci IJ stock from the previous infection round, stored in H2O. In each dish, 3–4 live crickets were placed and provided fresh spinach or apple slices. Infection with ~100 S. scapterisci IJs per individual A. domesticus cricket yielded 90 ± 0% mortality (n = 4; 10–20 insects per trial) within 2–3 d of exposure, and some within 1 d. This rapid host killing is a hallmark characteristic of EPNs [103] and reflects efficient release of the bacterial symbiont and/or the release of toxic factors by the nematodes themselves. Once crickets died, the cadavers were placed onto 60 mm filter paper in a 60 mm petri dish, which was then set in a water-filled 100 mm petri dish. After 2–3 days IJs were visible on cadavers and after an additional 4 days IJs emerged from the host and thousands of progeny migrated into the water trap. The nematodes were stored in H2O for up to 16 weeks. A Stereo Star dissection microscope was used to visually monitor A. domesticus infection and collect photos shown in Additional file 8.

Inbred laboratory Aedes aegypti (Rockefeller strain) larvae were reared at 26 °C under a 14 L: 10D photoperiod and provided with pellets of fish food [104]. Late 3rd instars were used to bioassay for the presence of mosquito larvicidal lipopeptide, Xlt.

Drosophila melanogaster Oregon-R strain used for infection experiments were kept in standard fly bottles containing dextrose medium (129.4 g dextrose, 7.4 g agar, 61.2 g corn meal, 32.4 g yeast, and 2.7 g tegosopt per liter; polypropylene round bottom 8 oz. bottles plugged with bonded dense weave cellulose acetate plugs, Genesee Scientific Cat #49–100) and were housed at 25 °C with 60% relative humidity and a 12 h light and 12 h dark cycle, as previously described [105].

Galleria mellonella waxworms used for infection experiments were purchased from CritterGrub (http://www.crittergrub.com/). Once received, any dead waxworms were discarded and the healthy individuals were kept at 15 °C in the dark until used for experiments. All waxworms were used for experimentation within 14 days.

Tobacco hornworm Manduca sexta larvae were raised from eggs (obtained from Carolina Biological Supply Company) on artificial diet (Gypsy moth wheat germ diet, MP Biomedicals, Aurora, OH) with a photoperiod of 16 h.

In vitro colonization assays
After overnight incubation, lawns of X. innexi were inoculated with 1 ml of S. scapterisci stock and incubated at
room temperature for 72 h or until a large number of adult nematodes were visible. Axenic eggs were isolated from these nematodes as previously described [30] and resuspended in 5 ml LB supplemented with ampicillin. The eggs were used immediately or allowed to hatch into J1 juveniles and stored at room temperature for up to 3 days. The absence of contamination was visually confirmed before use. Approximately 500–1000 axenic eggs and/or J1 nematodes were placed onto lipid agar plates with bacterial lawns and allowed to incubate at room temperature for 3–5 days before placement into White traps to capture emerging IJs [106]. To assess bacterial colonization of IJs, ~1000 IJs were prepared by – plates with bacterial lawns and allowed to incubate at room temperature for 24–48 h at 30 °C without exposure to light. Single colonies were picked grown overnight at 30 °C in liquid LB, with supplementation with 300 μM diaminopimelic acid for the helper and donor strain. Cells were subcultured into fresh medium and grown for an additional 4 h after which 900 μl of X. innexi (HGB1681 or HGB1997) and 300 μl each of the helper and donors strains were pelleted separately, washed and re-suspended at their original volumes. The three strains were then mixed together, and plated as a single spot onto a permissive LB pyruvate + DAP plate. After 24 h incubation an inoculation loop was dragged through the spot and the collected cells were re-suspended in LB and plated onto a selective LB pyruvate with ampicillin and kanamycin. After 24–48 h incubation at 30 °C the resulting colonies were analyzed for the expression of GFP with a Nikon Eclipse TE300 inverted fluorescent microscope.

Bacterial infection of insects

Injections into D. melanogaster adults were performed as previously described [105]. Briefly, different colony forming unit (CFU) doses were injected into CO2 anesthetized adult male flies aged 5–7 days old with control flies being injected with PBS. Each fly received a total volume of 50 nl injections in the anterior abdomen. Injections were performed using a MINJ-FLY high-speed pneumatic injector (Tritech Research, CA) and a pulled glass needle. After each injection all flies were maintained at 25 °C and 60% humidity. The bacteria were grown to log phase and then diluted to obtain the desired CFU count in a 50 nl volume. To determine CFUs in infected flies, individual flies were homogenized in 200 μl of PBS, diluted serially, and spotted 50 μl onto LB plates supplemented with 0.1% sodium pyruvate. Plates were kept overnight at 28 °C and total CFUs were then determined. For each virulence experiment we injected ≥60 flies, per dose of bacteria. Each experiment was repeated three times. For each in vivo growth assay, we injected and homogenized ≥10 flies, per dose at each time point. These experiments were repeated in triplicate.

Injections into G. mellonella larvae were performed as previously described [33]. Briefly, different colony forming unit (CFU) doses were injected into CO2 anesthetized 6th instar larvae. The larvae weighed between 0.19 and 0.30 g. We injected 10 μl in to the hindmost first proleg using a 27-gauge needle. After injections, all insects were kept in 60 mm petri dishes in the dark at 25 °C. Mortality was checked every 12 h. To determine CFUs in infected waxworms, we extracted approximately 10 μl of hemolymph from individual larvae and diluted this with 190 μl of PBS. The diluted hemolymph was then diluted serially, and 50 μl was spotted onto LB plates supplemented with 0.1% sodium pyruvate. Plates were kept overnight at 28 °C and total CFUs were then determined. For each virulence experiment we injected ≥10 larvae, per dose of bacteria. These experiments were repeated experiments in triplicate. For each growth assay, we injected and bled ≥10 larvae, per dose. These experiments were repeated in triplicate.

For injections into M. sexta, fifth-instar insect larvae were incubated on ice for approximately 10 min prior to injection. Ten microliters of the diluted culture were injected behind the first set of prolegs of each of 10 insect larvae per treatment using a 30-gauge syringe (Hamilton, Reno, NV). Dilution plating of the inoculum confirmed that for each treatment, an individual insect received 10^4 CFU.

Activation of the proPO system in insect plasma

Supernatants of X. innexi and X. nematophila strains were used to test their proPO inhibitory activity. Bacterial cultures were grown in LB broth for ~18 h at 30 °C and bacterial supernatant was isolated by spinning cells for 5 min at 8000 x g and filtering through a 0.2 μm syringe filter. Filtered supernatants were heat-treated for 10 min at 95 °C to inactivate heat-labile factors in the supernatant.
Hemolymph (plasma) from wounded fifth instar \textit{M. sexta} larvae was harvested as described previously [109]. In vitro activation of the proPO system was assessed by combining the following in a 96-well plate: 150 \(\mu\)l PBS (phosphate-buffered saline; 137 mM NaCl, 2.7 mM KCl, 10 mM \(\text{Na}_2\text{HPO}_4\), 1.8 mM \(\text{KH}_2\text{PO}_4\), pH 7.4), 10 \(\mu\)l plasma, and 20 \(\mu\)l of bacterial supernatant (filtered through a 0.20 \(\mu\)m syringe filter). Fresh LB was used as a negative control. This reaction was incubated at room temperature with constant shaking for 30 min to allow time for inhibition of proPO activation. Immediately following incubation, 20 \(\mu\)l of L-dihydroxyphenylalanine (L-DOPA) (4 mg/ml PBS) were added to the reaction. A microplate reader was used to monitor absorbance at 490 nm every min for 1 h. proPO activation was measured by calculating the rate of synthesis of dopachrome (a melanin intermediate) from L-DOPA. Data are presented as the percentage of each treatment against a negative control for proPO inhibition.

DNA extraction, genome sequencing and annotation

The \textit{X. innexi} genomic DNA was isolated using a standard protocol [110] and submitted for Roche (454) pyrosequencing and assembly at the University of Wisconsin Biotechnology Center. The assembled genome sequence was annotated using the Magnifying Genomes server (MaGe) from MicroScope Microbial Genome Annotation and Analysis Platform. Sequences are available through accession numbers: FTLG01000001-FTLG01000246.

Identification of putative toxin genes in \textit{X. nematophila} and \textit{X. innexi}

The \textit{X. nematophila} ATCC19061 genome was used as a reference to identify the various toxin gene families that we evaluated [104]. We determined the presence or absence of genes encoding putative toxins in \textit{X. innexi} in three ways: using \textit{X. nematophila} sequences as BLAST queries (E \leq 0.00005) [111], performing Pfam analyses to identify the presence of Pfam domains associated with the various toxin proteins, and using the MicroScope Gene Phyloprofile tool [42] to identify sets of genes specifically absent in \textit{X. innexi} genome. For BLAST analyses, we used the following \textit{X. nematophila} genes as queries: MARTX (XNC1_1376, 1377, 1378, 1380, 1381); Mef (XNC1_2265); Pir toxins (XNC1_1142, and XNC1_1143); PrtA (XNC1_4025); Tc toxins A (XNC1_2333 + 2334, XNC1_2560 + 2561, XNC1_2566, XNC1_2569, XNC1_3020 + 3021 + 3022 + 3023 + 3024, and XNC1_2187); B (XNC1_2186, XNC1_2335, XNC1_2568); and C (XNC1_2188, XNC1_2336, XNC1_2567); chitinases (XNC1_2562 and XNC1_2569); Txp40 (XNC1_1129); XaxAB (XNC1_3766 and XNC1_3767); Xenocin (XNC1_1221–1223). For Pfam searches we used hmmscan from the latest version of HMMER (3.0) software package, which implements probabilistic profile hidden Markov models. We set our threshold \(E\)-value criterion at \(10^{-6}\), to reduce the probability of false-positive matches. For MaGe analyses we used loci present in the completely sequenced genome of the virulent strain \textit{X. nematophila} (ATCC 19061) and identified those with homologs in the genomes of the virulent strains \textit{X. bovienii} SS-2004 and \textit{X. doucetiae} FRM16 [6, 7], but without homologs in the \textit{X. innexi} HGB1681 genome. The following homology constraints were used: bidirectional best hit, minimal alignment coverage of 0.8, and amino acid sequence identity of 30%.

Identification and analysis of Tps genes in \textit{X. innexi}

TpsA proteins sequences were aligned using the CLUSTAL W program implemented in SEAVIEW [112], and alignments were cleaned using Gblocks [113]. The phylogenetic trees were built by the maximum likelihood (ML) method using the LG substitution model, and branch support values, estimated by the aLRT (SH-like) method, are indicated at the nodes.

Search for type III secretion system homologs in \textit{X. innexi}

The Type III Secretion (T3S) genes (Additional file 4) of \textit{Salmonella enterica} (NCBI Reference Sequence NC_003197.2) were used to search for homologs in \textit{X. innexi}. The nucleotide sequence of the genes in Additional file 4 were used as query sequences in a nucleotide BLAST performed with the Magnifying Genomes server (MaGe) from MicroScope Microbial Genome Annotation and Analysis Platform. Consistent with other examined species of \textit{Xenorhabdus}, \textit{X. innexi} did not contain homologs for any T3S genes.

NRPS-PKS hybrid cluster domain analysis and identification of a candidate Xlt biosynthetic gene cluster in \textit{X. innexi}

\textit{X. innexi} genome was screened to locate NRPS, PKS and NRPS-PKS hybrid gene clusters. The initial screening was conducted by analyzing protein sequences of each coding DNA sequence (CDS) through a conserved domains search in National Center for Biotechnology Information (NCBI). If a conserved domain search recognized the candidate gene sequence as NRPS, PKS or NRPS-PKS hybrid, the number of A- or AT- domains were examined. NRPS, PKS and NRPS-PKS hybrid genes identified were further analyzed by submitting the corresponding protein sequences into the antibiotic and secondary metabolite analysis shell (AntiSMASH) to identify NRPS and PKS domains [114]. The data file generated by AntiSMASH analyses of the candidate gene cluster is available in Additional file 10.

One candidate gene cluster predicted to encode the \textit{Xlt} biosynthetic machinery was identified based on
preliminary chemical data on Xlt structure and composition. Additional in silico analyses were conducted to further test this prediction. Protein sequences of each ORF in the cluster were examined through protein BLAST to predict the putative function, and then analyzed through the conserved domain search to identify PKS, NRPS and non-PKS/NRPS domains. Protein sequences of A-domains in NRPS modules were analyzed through NRPSpredictor2 [115] and AT- domains identified in PKS modules were analyzed using I-TASSER server [116].

Construction of XIS1_460115 and XIS1_460109 mutants
To provide a functional test of the role of the candidate xlt gene cluster in Xlt biosynthesis, we used allelic exchange site-directed mutagenesis to replace the PKS (XIS1_460115) or NRPS (XIS1_460109) genes with a kanamycin cassette [117] and tested relevant phenotypes of the resulting mutants. Briefly, upstream and downstream regions of XIS1_460115 or XIS1_460109 were amplified using restriction-site-containing primers (Table 9). Amplified fragments were cloned individually into pBluescript SK (−) plasmids; the kanamycin resistant cassette from pKanWor plasmid was cloned into the BamHI site of pBlueXI-S1_460109UpDn or pBlueXIS1_460115UpDn (Table 1). The pBlueXIS1_460109UpDn or pBlueXIS1_460115UpDn construct was cloned into a pKR100 suicide vector; the resulting mutants were first verified by PCR amplification of nilB, which is a Xenorhabdus-specific gene [118]. The position of mutation was also confirmed by PCR amplification of the flanking regions of the inserted kanamycin cassette.

Mosquito larvicidal bioassays
Mosquito larval bioassays were conducted to determine if mutation at XIS1_460115 or XIS1_460109 resulted in the loss of mosquito larvicidal activity. WT X. innexi, ΔXIS1_460115 and ΔXIS1_460109 were grown in liquid LB media overnight at 30 °C. Samples of overnight cultures were transferred to fresh liquid LB media and were grown at 30 °C until they reached an optical density of 1.0 at 600 nm. Bacterial cultures were centrifuged at 6000 rpm for 10 min and only supernatants were used for bioassays. Various dilutions of the supernatants were made in water and then 2 ml of each dilutions were pipetted into 24- well plastic plates (Becton Dickinson Labware, Franklin Lakes, NJ). Five Ae. aegypti larvae were transferred into each well with four replications in each treatment. The experiment was repeated five times and the percent mortality in each concentration of the bacterial supernatant was calculated.

MALDI-TOF MS analysis of WT X. innexi, ΔXIS1_460115 and ΔXIS1_460109 culture supernatants: ΔXIS1_460115 and ΔXIS1_460109 were cultured in liquid LB media for 24 h at 30 °C, and then centrifuged at 6000 rpm to collect supernatants. Supernatants were submitted for Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis to examine the potential mass profile differences between WT X. innexi and mutants (Biotechnology Center, University of Wisconsin-Madison).

Additional Files

Additional file 1: Growth rates of X. innexi, X. nematophila and X. bovienii in vitro and in vivo (PDF 77 kb)
Additional file 2: Percent survival over 50 days of D. melanogaster flies injected with controls or X. nematophila. (PDF 590 kb)

Additional file 3: List of X. nematophila ATCC19061 genes present in X. novemis SS-2004, X. duculont FRM16, but absent in X. innexi HGB1681. (DLSX 42 kb)

Additional file 4: ORFs used for T3SS BLASTp analysis of X. innexi draft genome. (PDF 104 kb)

Additional file 5: Repeat domains in MARTX-like genes of X. innexi. (PDF 141 kb)

Additional file 6: Accession numbers of the sequences used in the phylogenetic analyses of TpsA proteins. (PDF 85 kb)

Additional file 7: X. innexi loci with genes predicted to encode T6SS components. (PDF 72 kb)

Additional file 8: A. domesticus infected with S. scapenteri. (PDF 5905 kb)

Additional file 9: MALDI-TOF MS of WT X. innexi, ΔXIS1_460109 and ΔXIS1_460115. (PDF 330 kb)

Additional file 10: AntiSMASH analysis of NRPS and PKS genes from XIS1_460105 to XIS1_460161. (ZIP 2696 kb)

Abbreviations
A-: Adenylation-; AA: Amino acid; AntiSMASH: Antibiotics and secondary metabolite analysis shell; AT-: Acyltransferase-; BLAST: Basic local alignment search tool; CDS: Coding DNA sequence; Cfu: Colony forming unit; CTDs: C-terminal domains; Da: Dalton; DAB: Diaminobutyric acid; DAP: Diaminopimelic acid; DNA: Deoxynucleobonic acid; EPN: Entomopathogenic nematode; GC: Guanine-cytosine; GFP: Green fluorescent protein; HPI: Hours post infection; HPLC/MS: High performance liquid chromatograph/mas spectrometry; IJ: Infective juvenile; LA: Lipid agar; LB: Luria agar; LR: Luria broth; L-DOPA: L-dihydroxyphenylalanine; MaGe: Magnifying genomes server; MARTX: Multifunctional autoprocessing repeats-in-toxin toxins; Msp: Msp1; ORF: Open reading frame; PBS: Phosphate buffered saline; PCR: Polymerase chain reaction; PKS: Polyketide synthase; PO: Phenoloxidase; Rpm: Revolutions per minute; rRNA: Ribosomal ribonucleic acid; SD: Standard deviation; T3S: Type III secretion; T3SS: Type V secretion system; T6SS: Type VI secretion system; TA: Toxin-antitoxin; Tissue Culture Center; TV: Tomato virus; TRF: Tryptophan repressor; TVP: Tomato virus protein; TPS: Tissue penetration system; U: Urd; UDA: Uridine diphosphate adenylic acid; UV-AB: Ultraviolet-activated blue; V: Val; VATs: Variable antigenic tags; Wt: Wild type; XIS1: X. innexi strain 1; Y: Tyr; L: Lys; B: Bina

Acknowledgements
The authors wish to thank Melinda Hauser and Xiaofei Bai for their assistance with microscopy.

Funding
Work in the Goodrich-Blair lab was supported by a grant (IOS-1353674) from the National Science Foundation, the UW-Madison University of States Department of Agriculture (USDA) Hatch Multi-state research formula fund (WS01582), and the University of Tennessee-Knoxville. TJM and AMCT were supported by a grant from the National Institutes of Health Research Service Award T32-GM07215 and Rhs comparisons and phenoloxidase assays. J-CO, and SG conducted comparisons and helped write and edit the manuscript. KH conducted T6SS and Rhs comparisons and phenoloxidase assays. J-CO, and SG conducted comparisons and helped write and edit the manuscript. WGG helped supervise I-HK at UW-Madison and edit the manuscript. JCE identified and completed preliminary characterization of the mosquitocidal toxin and acquired funding to obtain the genome sequence. HGB was responsible for supervision of the research at UW-Madison and UTK. Acquisition of data and articles, PLS, MARTX, and other genomic elements, and writing of the manuscript. ARD was responsible for supervising research at UC Riverside, acquisition of additional funding, writing the manuscript, and analyzing genomic content. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable. This study did not involve human subjects, human material, or human data.

Consent for publication
Not applicable. This manuscript does not include details, images, or videos relating to individual participants.

Competing interests
JCE declares pursuit of a patent pertaining to the activity of the mosquitocidal toxin (see bibliography for reference). All other authors declare that they have no competing interests.

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Authors’ contributions
I-HK carried out mosquitocidal toxin experiments and wrote portions of the manuscript. SKA conducted virulence assays in Drosophila and Gallenla, while AMCT and MPK conducted virulence assays in Manduca. DTA established X. innexi’s scapentrici rearing mechanisms in crickets and calculated Xenorhabdus colonization levels. EJM and TJM evaluated colonization phenotypes of X. innexi in S. scapentrici and TJM also conducted bacterial growth analyses and TSSs comparisons and helped write and edit the manuscript. KH conducted T6SS and Rhs comparisons and phenoloxidase assays. J-CO, and SG conducted comparisons of TPS and helped edit the manuscript. WGG helped supervise I-HK at UW-Madison and edited the manuscript. JCE identified and completed preliminary characterization of the mosquitocidal toxin and acquired funding to obtain the genome sequence. HGB was responsible for supervision of the research at UW-Madison and UTK. Acquisition of funding, analysis of TPS, MARTX, and other genomic elements, and writing of the manuscript. ARD was responsible for supervising research at UC Riverside, acquisition of relevant funding, writing the manuscript, and analyzing genomic content. All authors read and approved the final manuscript.

Received: 7 June 2017 Accepted: 16 November 2017 Published online: 1 December 2017

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