Variable virulence phenotype of *Xenorhabdus bovienii* (\(\gamma\)-Proteobacteria: Enterobacteriaceae) in the absence of their vector hosts

John G. McMullen II, Rebecca McQuade, Jean-Claude Ogier, Sylvie Pagès, Sophie Gaudriault and S. Patricia Stock

Abstract

*Xenorhabdus bovienii* bacteria have a dual lifestyle: they are mutualistic symbionts to many species of *Steinernema* nematodes and are pathogens to a wide array of insects. Previous studies have shown that virulence of *X. bovienii–Steinernema* spp. pairs decreases when the nematodes associate with non-cognate bacterial strains. However, the virulence of the *X. bovienii* strains alone has not been fully investigated. In this study, we characterized the virulence of nine *X. bovienii* strains in *Galleria mellonella* and *Spodoptera littoralis* and performed a comparative genomic analysis to correlate observed phenotypes with strain genotypes. Two *X. bovienii* strains were found to be highly virulent against the tested insect hosts, while three strains displayed attenuated insect virulence. Comparative genomic analyses revealed the presence of several clusters present only in virulent strains, including a predicted type VI secretion system (T6SS). We performed intra-species-competition assays, and showed that the virulent T6SS+ strains generally outcompeted the less virulent T6SS− strains. Thus, we speculate that the T6SS in *X. bovienii* may be another addition to the arsenal of antibacterial mechanisms expressed by these bacteria in an insect, where it could potentially play three key roles: (1) competition against the insect host microbiota; (2) protection of the insect cadaver from necrotrophic microbial competitors; and (3) outcompeting other *Xenorhabdus* species and/or strains when co-infections occur.

INTRODUCTION

Bacteria of the genus *Xenorhabdus* Poinar and Thomas (*\(\gamma\)-Proteobacteria: Enterobacteriaceae) have a dual lifestyle with two different invertebrate hosts. They are mutualistic symbionts of entomopathogenic nematodes in the genus *Steinernema* Travassos (Nematoda: Steinernematidae), and are pathogens to a wide array of soil-dwelling insects [1–4]. Because of their insecticidal ability, *Xenorhabdus–Steinernema* pairs are a successful biological control agent against certain agricultural insect pests [1–9].

*Xenorhabdus* spp. lack a free-living stage and rely on *Steinernema* nematodes for their dissemination from one insect host to another [10]. Specifically, it is the third-juvenile-stage nematode (also known as infective juvenile or IJ) that vectors *Xenorhabdus* from one insect host to another. This is the only free-living stage in the *Steinernema* life cycle, which is similar to the dauer juvenile in *Caenorhabditis elegans* Maupas (Nematoda: Rhabditidae), as it is a non-feeding stage and possesses a double cuticle that allows it to withstand harsh environmental conditions [11].

*Xenorhabdus* symbionts are harboured in a specialized receptacle in the most anterior portion of the intestine during the IJ stage, which protects them from the environment [12, 13]. Once IJs enter a susceptible insect host, they release *Xenorhabdus* bacteria into the insect haemocoel, leading to septicemia. *Xenorhabdus* spp. are known to produce numerous insecticidal toxins that also contribute to insect mortality [14, 15], making bacterial virulence a polygenic trait [16]. They also produce antimicrobial compounds that sterilize the cadaver and ward off necrotrophic competitors [17–19]. Therefore, the insect cadaver becomes a suitable environment that not only allows *Xenorhabdus* to multiply, but also permits *Steinernema* to mature and reproduce [20].
As food resources in the insect cadaver are depleted, Stei-
ernerema nematodes become IJs and re-associate with Xeno-
rhabdus before leaving the insect host [21].

Several studies have demonstrated that certain Xenorhabdus spp. are capable of killing an insect host population when applied alone, i.e. without their respective nematode vectors [22]. However, some species, such as Xenorhabdus bovienii (Akhurst) and Xenorhabdus poenarii (Akhurst), have been reported to have an avirulent or attenuated virulence phenotype when injected alone [23–28]. For example, Bisch et al. [27] showed that X. bovienii strain CS03, the symbiont of Steinernema weiseri Mrácek, Sturhan and Reid, was avir-
ulent against Galleria mellonella (L.) (Lepidoptera: Pyrali-
dae) and Spodoptera littoralis (Boisduval) (Lepidoptera: Noctuidae) when injected alone, but was virulent when associated with its natural nematode host [27]. This is the first case of a reported avirulence phenotype for X. bovienii. The inability of X. bovienii CS03 to infect the tested insects alone was attributed, in part, to its susceptibility to cationic antimicrobial peptides, which are produced by the insect host as a response to bacterial infection [27]. Additionally, it has been proposed that genes involved in pathogenesis and toxemia were eroded in the genome of this strain through pseudogenization, which is likely to contribute to the lack of pathogenicity observed [28]. These findings stress the important role that the nematodes can have in the infection process.

Recently, Murfin et al. [29] investigated the virulence and fitness of three different X. bovienii strains that are symbionts of Steinernema feltiae (Filipjev). The authors demon-
strated that the bacterial virulence of X. bovienii was negatively impacted when associated with a non-cognate S. feltiae host [29]. However, the virulence of these strains alone was not assessed. In this study, we investigated the viru-
ulence phenotypic trait in a selection of X. bovienii strains. Furthermore, we used a comparative genomics approach to identify candidate genes and/or genomic regions that may contribute to the variable virulence of X. bovienii strains.

METHODS

Isolation and rearing of bacterial cultures
Nine X. bovienii strains (XbFL, XbM, XbSN, Xb, Xbj, XbkBU, XbkCA, Xbp and Xbo) isolated from six different Steinernema spp. were used in this study. Geographic origins of the studied strains, including isolate code designations, are listed in Table 1. Additionally, Xenorhabdus nematophila (All strain) (Xn), the native symbiont of Steinernema carpocapsae (Weiser) (All strain), was used as a positive control for virulence assays since this species is consistently virulent [30, 31].

Nematodes were reared following procedures described by Kaya and Stock [32]. Bacterial isolates were obtained from either haemolymph from infected insect cadavers [33] or from fresh IJs [34]. Isolated bacteria were inoculated onto nutrient agar supplemented with 0.0025 % (w/v) bromothymol blue, 0.004 % (w/v) triphenyl-tetrazolium, and 0.1 % (w/v) sodium pyruvate (NBTA) and incubated for 2–3 days at 28±1 °C [34, 35]. A single blue colony was grown overnight (12–16 h) in 5 ml of Luria Bertani broth supplemented with 0.1 % (w/v) sodium pyruvate (LBP) [35, 36] in a 15 ml centrifuge tube with agitation at 28±1 °C and stored at –80 °C in 20 % (v/v) glycerol [33].

For all experiments, bacterial strains were grown overnight from frozen glycerol stocks in liquid culture and then plated onto NBTA the conditions described above. One blue col-
ylon of each Xenorhabdus strain was grown in liquid culture at 28±1 °C for various times depending on the assay (more detail below).

Insect virulence assays
Two Lepidoptera hosts with known differential susceptibil-
ity to entomopathogenic nematodes, the greater wax moth, Galleria mellonella (L.) (Pyralidae), and the Egyptian cotton leafworm, Spodoptera littoralis (Boisduval) (Noctuidae), were used to assess the virulence of X. bovienii strains. The rationale for this selection was based on the idea that testing a higher dose in S. littoralis would confirm observed differences in virulence with G. mellonella, a common laboratory model host for studying bacterial infections. Last instar lar-
vae of G. mellonella are commonly used in bioassays with many entomopathogens, including Xenorhabdus–Steiner-
nama pairs, and have also been regarded as highly suscepti-
ble to bacterial infections [37–39]. Larvae were obtained from Timberline Fisheries (Marion, IL) and stored at 15 ±1 °C in the dark until used. Only last instar larvae weighing between 0.20 and 0.40 g were used for virulence assays.

X. bovienii strains that were attenuated in G. mellonella were then tested in last instar larvae of Spodoptera littoralis, a host that is less sensitive to Xenorhabdus–Steiner-
nama infections [26, 27, 30]. Larvae were reared on an artificial medium [40] at 23±1 °C with a photoperiod of light to dark of 16 : 8 and 40±5 % relative humidity.

G. mellonella assays
Bacterial strains were grown following the methods described above. A single colony was selected for each culture and grown in LBP to an optical density of 0.8 at 600 nm (OD600 nm, mid-logarithmic phase). 1.5 ml of each bacterial culture was washed with sterile 1× PBS without calcium or magnesium (pH 7.4) twice by centrifugation, 16,000 g for 5 min. A final concentration of 10⁷ cells ml⁻¹ was obtained using a haemocytometer and four 1 : 10 serial dilutions were made from this sample to obtain the follow-
ing concentrations: 10⁶, 10⁵, 10⁴ and 10³ cells ml⁻¹.

To confirm bacterial concentration and cell variant phase, optical densities were checked and 50 µl of the 10⁷ cells ml⁻¹ concentration was plated onto NBTA twice. NBTA plates were incubated at 28±1 °C for 2–3 days and c.f.u. counted. X. nematophila was used as a positive control because this bacterium is known to be highly virulent to many insect hosts [30, 31]. Sterile PBS was used as a negative control.
**Table 1.** Bacterial and nematode strains used in bioassays and comparative genomic analyses

<table>
<thead>
<tr>
<th>Xenorhabdus representative species</th>
<th>Strain code</th>
<th>Steinernema representative species</th>
<th>Geographic origin</th>
<th>Bacterial genome accession no.</th>
<th>Nematode 12S/28S rDNA GenBank accession no.*</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>X. bovienii</td>
<td>XbFL</td>
<td>S. feltiae</td>
<td>FL, USA</td>
<td>PRJEB4320</td>
<td>GU569030/GU569049</td>
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<td>S. feltiae</td>
<td>Moldova</td>
<td>PRJEB4321</td>
<td>KF437815/KF437816</td>
<td>B. Adams</td>
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<td>S. feltiae</td>
<td>France</td>
<td>PRJEB4319</td>
<td>GU569031/GU569050</td>
<td>P. Stock</td>
</tr>
<tr>
<td>X. bovienii</td>
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<td>S. intermedium</td>
<td>SC, USA</td>
<td>PRJEB4327</td>
<td>AY944014/AF331909</td>
<td>P. Stock</td>
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<td>S. kraussei</td>
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<td>GU569034/GU569053</td>
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<td>S. kraussei</td>
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<td>KF437817/KF437818</td>
<td>Becker-Underwood</td>
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<td>X. bovienii</td>
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<td>S. jolielli</td>
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<td>FN667741</td>
<td>GU569032/GU569051</td>
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<tr>
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<td>S. jolielli</td>
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<td>Monsanto</td>
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<tr>
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<td>S. oregonense</td>
<td>OR, USA</td>
<td>PRJEB4323</td>
<td>AY944021/AF331891</td>
<td>P. Stock</td>
</tr>
<tr>
<td>X. bovienii</td>
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<td>PRJEB4322</td>
<td>GU569037/GU569056</td>
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<td>X. bovienii</td>
<td>XbCS03</td>
<td>S. wessleri</td>
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<td>GU569040/GU569059</td>
<td>Z. Mráček</td>
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<td>Xin</td>
<td>S. carpocapae</td>
<td>USA</td>
<td>FN887742</td>
<td>AY944007/AF331900</td>
<td>P. Stock</td>
</tr>
</tbody>
</table>

*The sequences used for S. wessleri are from a different isolate of this species.

The following Xenorhabdus doses were considered: 10^2, 10^3, 10^4, 10^5, 10^6 and 10^7 cells per insect. The rationale for the selection of these doses followed a protocol developed by Bucher et al. [41] to determine bacterial pathogenicity. Prior to injection, all insect larvae were anesthetized on ice. A 10 µl inoculum (for each strain and concentration tested) was injected into the haemocoel, using a microinjector with a 27-gauge needle, between the second and third proleg on the dorsal side. A total of 30 larvae for each bacterial strain and concentration tested were used in each replicate setup. The experimental setup was repeated twice independently.

Injected G. mellonella larvae were maintained at 25±1 °C in the dark. Insect mortality was checked daily for seven consecutive days and corrected using Abbott’s formula if control mortality was greater than 10% [42]. Controls consisted of larvae injected with PBS (negative control) and X. nematophilus (positive control). The PBS control group mortality was always less than 20% for each setup.

**S. littoralis assays**

Inoculum dose for each bacterial strain (10^3 cells per insect) and injection procedures followed protocols developed by Bisch et al. [27]. Briefly, 20 insects were injected between the second and third proleg with a Hamilton syringe on the dorsal side of the insect. X. nematophilus was used as the positive control, while LB broth was used as the negative control. Larvae were incubated at 23±2 °C with a photoperiod of light to dark of 16 : 8 for 48 h.

Insect mortality was monitored at 19, 22, 26, 32, 44 and 48 h post-injection, based on observations from natural infestations with Steinernema nematodes [20]. If strains were shown to cause less than 50% mortality in the injected population, we repeated the setup in order to confirm our observations.

**Bacterial intrinsic growth rates**

Growth curves were generated for all X. bovienii strains used in bioassays to assess their growth rates following methods described by Jubelin et al. [43]. Briefly, all strains were grown overnight from primary phase colonies (grown on NBTA) in LB broth. All strains were diluted (1:500) to obtain a final volume of 200 µl and added to a flat-bottom, 96-well plate (Greiner, NC) in triplicate. This diluted samples to concentrations that were initially undetectable through absorbance spectroscopy. Cultures were incubated at 28±1 °C with agitation on an Infinite M200 microplate reader (Tecan, Switzerland), and the optical density at 600 nm was read every 30 min for 18 h (beginning of the stationary phase).

**Comparative genomic analyses**

**Selected genomes**

Available genomes of all X. bovienii strains used in this study [28, 29, 44]. Genome accession numbers are listed in Table 4. All Xenorhabdus genomes were obtained and analysed using the online platform MicroScope [45].

**Phenotype-genotype correlation**

We conducted a protein-coding gene content comparison using MicroScope software (Gene Phyloprofile program) to identify ORFs that were specific to virulent strains (Xbi and Xbj) and absent in attenuated strains (XbfFL, XbfSN and Xbo). Parameters for analysis were at least 30% amino acid identity over at least 80% alignment coverage. For this analysis, the Xbj genome was used as the reference source. Candidate insect virulence ORFs were annotated using BLAST2GO to compare their general functions [46]. Biological pathways were assessed at level 4 and cellular compartment at level 2.
Variable genome module comparison
We examined the conservation of 244 modules defined in regions of genomic plasticity (RGPs) in nine X. bovienii genomes following settings defined by Ogier et al. [47]. The core and variable portions of the X. bovienii pan-genome were based on strain SS-2004. For each genome, module conservation was assessed by counting the number of syntenic genes represented out of the total genes found in the X. bovienii SS-2004 module. Conservation parameters were as follows: $\geq$75% are highly conserved modules, 25–75% are partially conserved modules, and $<25\%$ are low conserved or absent modules (similar to [47]).

T6SS ORF annotation
ORFs encoding putative T6SS components were annotated by using InterPro, BLASTp, and the classification scheme implemented in Shyntum et al. [48]. Each T6SS ORF was aligned to homologous S. marcescens ORFs – previously described to have type A T6SS loci [49] – using Muscle [50] to obtain percent identity support for a putative function in X. bovienii. Mauve v.2.3.1 was used to align genomic regions to identify rearrangements, duplications and insertions/deletions between Serratia and Xenorhabdus loci in Generous R8.1.7 [51].

Intra-bacterial competition assay
GFP and kanamycin resistant genes were inserted into the chromosomes of T6SS$^+$ strains (XbFL, XbSN, XbCA and Xb) chromosomes using the triparental Tn7 conjugation method described by Martens et al. [52]. The donor strain was E. coli strain BW29427 Tn7 GFP [53] and the helper strain was E. coli strain BW29427 [54]. X. bovienii strains were selected on LBP agar with 50 µg ml$^{-1}$ kanamycin after conjugation. Exconjugants were confirmed as X. bovienii through dye absorption on NBTA and lack of catalase activity [33].

Competition assays were performed to correlate bactericidal activity with the putative T6SS identified in the gene content comparison analysis. Procedures followed those described by Murdoch et al. [55] with some modifications. Briefly, bacterial cultures were grown as mentioned above overnight (12–16 h) in LBP broth from a single blue colony. Cultures were normalized to OD$^{600\ nm}$ 0.5. Liquid cultures were combined as either 5 : 1 or 1 : 1, virulent T6SS$^+$ served or absent modules (similar to [47]). The strain treatment was confirmed as a positive control for each experiment, X. nematophila was used as a positive control since this bacterium is known to be competitive against X. bovienii due to bacteriocin activity [56]. For each competition, four 25 µl aliquots were spotted onto LBP agar plates and incubated for 12 h at 28±1 °C. Each spot was then suspended in 1 ml of LBP broth, serially diluted and spread on LBP agar with 50 µg ml$^{-1}$ of kanamycin to quantify surviving attenuated T6SS$^-$ bacteria. Competitiveness was expressed as the ratio of surviving T6SS$^-$ bacteria to the initial input of T6SS$^-$ bacteria, normalized to the negative control. All samples were plated in duplicate and incubated at 28±1 °C for 2–3 days before counting c.f.u. The limit of detection was 10 c.f.u. Two independent experiments were performed.

Phylogenetic analyses
Xenorhabdus phylogeny
Phylogenetic analysis of X. bovienii strains was carried out to generate a framework to address the evolution of virulence phenotypes. MEGA version 6 [57] was used for this purpose. Concatenated sequences composed of 16S rDNA and five housekeeping gene sequences (recA, dnaN, gltX, gyrB and infB) with a total length of 10 315 bp (see Table 1 for accession numbers) were aligned using Muscle [50]. A maximum-likelihood analysis was performed using a TN93 +G+1 model of evolution with 1000 bootstrap replicates to generate a Xenorhabdus–Xenorhabdus tanglegram.

Steinernema phylogeny
A phylogenetic tree for Steinernema spp. was generated considering mitochondrial 12S and nuclear 28S rDNA (1403 bp) genes following procedures similar to those described above (see Table 1 for accession numbers). A GTR+G model of evolution was used to generate a species tree for the Xenorhabdus–Xenorhabdus tanglegram.

T6SS phylogeny
We assessed the evolutionary relationships of Xenorhabdus T6SSs by comparing amino acid sequences of the tube sheath proteins, TssB and TssC (712 sites), between seven strains of this genus and four S. marcescens strains [Db11 (accession number: HG326223), FG194 (accession number: CP003942), FS14 (accession number: CP005927) and WW4 (accession number: CP003959)]. Francisella tularensis FSC147 (accession number: CP000915) was considered as the outgroup taxon to root the tree, which is commonly done for T6SS phylogenetic analyses [58]. An LG+G model of evolution was used to generate the T6SS phylogenetic tree.

In addition, a species tree was reconstructed following the Xenorhabdus species tree to compare T6SS evolution and speciation events among the studied strains. As before, the genes 16S rRNA, recA, dnaN, gltX, gyrB and infB (10 541 bp) were concatenated and assessed under a TN93 +G+1 model of evolution.

Statistical analyses
Survival analyses were performed using R, version 3.1.2 [59]. The G. mellonella lethal time data for the selected dose (i.e. 10 cells per insect) was compared using a Cox mixed-effects model ($\alpha=0.05$) using the ‘coxme’ package [60]. Xn was used as the control strain in this analysis, which allowed for comparing the rate of mortality between Xn and each X. bovienii strain. The X. bovienii strain treatment was considered as a fixed effect in this model and the experimental replicate setup was used as a random effect to explain how much of the variation in this model could be explained by differences due to experiment repetition artifacts. Similarly,
the S. littoralis data was analysed using the ‘coxph’ function in the ‘survival’ package [61]. This function was implemented instead of the other ‘coxme’ method because there was only a single trial conducted and therefore no need for random effects in the model. If there was no mortality induced, one death was added at 48 h to allow for statistical comparisons, otherwise the model becomes unstable.

Lethal time data for both insect hosts were plotted using the ‘survfit’ function in the ‘survival’ package [61]. The lethal time to kill 50% of the injected population (i.e. median survival time or LT50) and the restricted mean survival time (i.e. area under the curve from time 0 to the time when insects were right censored) were calculated from this plot.

A two-way ANOVA was performed with JMP version 11 software (SAS Institute, Inc.) to assess the growth of attenuated T6SS− strains in co-culture with virulent T6SS+ strains. T6SS+ strains and T6SS− strains were used as the main fixed effects in this model, which included T6SS+−T6SS− as the interaction term. Each T6SS+:T6SS− ratio (i.e. 1 : 1 and 5 : 1) was analysed separately with each of their replicate experimental setups combined. A post hoc Tukey–Kramer test was used to discriminate between all strains (α=0.05). For each treatment and control, the relative fold change in growth of the attenuated T6SS− strain is reported. This is calculated as the output c.f.u. divided by the input c.f.u. of the T6SS− strain, which was normalized to the average ratio of the control to limit setup variation.

**RESULTS**

**Characterization of bacterial virulence**

We first characterized the virulence of X. bovienii strains in G. mellonella larvae. Specifically, we tested the following nine strains: XbfFL, XbfM, XbFSN, Xbi, Xbj, XbkBU, XbkCA, Xbo and Xbp (Table 1). The highly virulent symbiont Xenorhabdus nematophila (Xn) was included as a positive control (Table 1). The lowest inoculum (10 bacterial cells per insect) exhibited the most variation in mortality (Table 2). At this concentration, percent mortality ranged from as low as 13.0% for XbkCA to as high as 98.3% for Xbi (Table 2). In general, doses greater than 10 cells per insect did not show substantial differences in mortality between the X. bovienii strains (Table 2). We used this dose to categorize virulence phenotypes of X. bovienii strains in relation to the control strain (Xn).

At this dose, the lethal time (LT50) also varied (Table 3). For example, strains Xbi and Xbj were able to kill 50% of the insect population within 48 h, similar to the control strain (Xn) (Table 3, Fig. S1a, available in the online Supplementary Material). The LT50 for strains XbfFL, XbFSN, XbkBU and Xbp was 72 h (Table 3). The remaining strains, XbfM, XbkCA and Xbo, killed less than 50% of the injected population during the 7-day study period; therefore, we report their LT50 as greater than 168 h (Table 3, Fig. S1a).

Xbi, Xbj and Xn were able to kill most or all of the injected insect population by 48 h (Fig. S1a). This time range is comparable to the time it takes natural Xenorhabdus–Steinernema pairs to kill insects, which is typically 24–48 h [20]. The infection kinetics among these strains appear to be similar to one another, with Xbi being 1.2 times more likely and Xbj 1.5 times less likely than Xn to induce mortality, as estimated by hazard ratios (Table 4). The infection kinetics of the remaining strains (i.e. XbfFL, XbfM, XbSN, XbkBU, XbkCA, Xbo and Xbp) were significantly different from Xn and their respective death rates varied from 5.3 to 41.7 times slower than Xn (Table 4).

We then further characterized the virulence of these nine strains by screening insect mortality in S. littoralis at a dose of 1000 cells per insect, which is an established and robust method for identifying attenuated virulence strains of Xenorhabdus spp. [26, 27]. In the S. littoralis experiment, five out of the nine X. bovienii strains, XbfM, Xbi, Xbj, XbkBU and Xbp, exhibited an LT50 that ranged between 19 and 32 h and reached 95–100% mortality in the injected population within a 48 h study period (Table 3). Analysis of the infection kinetics of these strains in comparison to Xn showed more variability between strains during a 48 h period than they did in G. mellonella (Table 4); this may be due to the shorter intervals between sampling times, the higher dose tested and/or differences in the insect host. Xbi and XbkBU had similar infection kinetics to Xn (Table 4), while XbfM, Xbj and Xbp were significantly different from Xn and displayed death rates ranging from 2.2 to 5.7 times slower than Xn (Table 4). The remaining strains, XbfFL, XbSN, XbkCA and Xbo, had an LT50 that exceeded 48 h and exhibited lower mortality, ranging between 0 and 10% (Table 3, Fig. S1b). As expected, their infection kinetics differed significantly from Xn and they were 250–333.3 times less likely to prompt insect mortality than Xn (Table 4). The attenuated virulence of XbfFL, XbSN, XbkCA and Xbo against S. littoralis was not because of a slower intrinsic
Table 3. Summary of lethal time plots for Xenorhabdus spp. in G. mellonella and S. littoralis

LT50 (i.e. median survival time), RMST (restricted mean survival time), and final mortality were calculated from the data in the survival plot generated in Fig. S1.

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>G. mellonella (dose: 10 cells per insect, n=60)</th>
<th>S. littoralis (dose: 1000 cells per insect, n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LT50 (h)</td>
<td>RMST±SE (h)</td>
</tr>
<tr>
<td>XbFL</td>
<td>72</td>
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<tr>
<td>XbM</td>
<td>&gt;168</td>
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<td>XbkCA</td>
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<td>Xn</td>
<td>48</td>
<td>46.4±0.672</td>
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</tbody>
</table>

growth rate; all strains displayed similar growth kinetics in vitro, except for XbkCA which grew more slowly than the other strains (Fig. S2).

Considering results from both host bioassays, we classified Xbi and Xbj as the most virulent strains and XbFL, XbSN and Xbo as the least virulent ones tested. In both of the tested insect hosts, Xbi and Xbj were able to induce insect mortality within the entire, or almost entire, injected population within a 48h period, while XbFL, XbSN and Xbo were not (Fig. S1). Although XbkCA does appear to exhibit an attenuated virulence phenotype, we excluded it from this classification scheme due to its slow inherent growth kinetics (Fig. S2). The remaining strains, XbM, XbkBU and Xbp, were considered to have an ‘intermediate’ virulence phenotype because they were unable to kill all G. mellonella larvae, which are highly susceptible to infection, at a low inoculum dose within a 48h period, but were able to do so when they were injected into S. littoralis at a higher dose.

Comparative genomic analyses

We used a multi-locus sequence analysis approach to compare virulence phenotypes and Xenorhabdus phylogeny. Our analysis suggests the attenuated virulence phenotype is a derived phenotypic trait that appears multiple times in the evolutionary trajectory of X. bovienii (Fig. 1). Similarly, there appeared to be no cluster of Steinernema spp. in the host phylogeny to suggest that the attenuated virulence phenotype correlates by nematode taxa. Multiple comparative genomic analyses were performed to identify common insect virulence-associated genes and gene clusters lost in these attenuated virulence strains.

First, we generated a list of ORFs present within the genomes of highly virulent strains (Xbi and Xbj), but absent

Table 4. Infection kinetics of X. bovienii strains and X. nematophila in G. mellonella and S. littoralis

Hazard ratios for each X. bovienii strain were generated from the time-to-death data at (a) the dose of 10 cells per insect in G. mellonella and at (b) the dose of 1000 cells per insect in S. littoralis. Xn was used as the reference strain to compare each X. bovienii strain to this virulent bacterium. Hazard ratios indicate the likelihood of an X. bovienii strain to cause mortality in comparison to Xn. (a) Cox mixed-effect model: integrated X^2=370.82, df=10, P<0.0001. (b) Cox proportional hazard model: X^2=278, df=9, P<0.0001.

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>(a) G. mellonella (dose: 10 cells per insect, n=60)</th>
<th>(b) S. littoralis (dose: 1000 cells per insect, n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Coefficient±SE</td>
<td>Hazard ratio</td>
</tr>
<tr>
<td>Xn</td>
<td>−2.019±0.214</td>
<td>0.133</td>
</tr>
<tr>
<td>XbFL</td>
<td>−2.655±0.243</td>
<td>0.070</td>
</tr>
<tr>
<td>XbM</td>
<td>−2.195±0.223</td>
<td>0.111</td>
</tr>
<tr>
<td>XbSN</td>
<td>0.160±0.212</td>
<td>1.173</td>
</tr>
<tr>
<td>Xb</td>
<td>−0.379±0.217</td>
<td>0.685</td>
</tr>
<tr>
<td>XbkBU</td>
<td>−1.682±0.204</td>
<td>0.186</td>
</tr>
<tr>
<td>XbkCA</td>
<td>−3.730±0.330</td>
<td>0.024</td>
</tr>
<tr>
<td>Xbo</td>
<td>−2.799±0.291</td>
<td>0.061</td>
</tr>
<tr>
<td>Xbp</td>
<td>−1.675±0.204</td>
<td>0.187</td>
</tr>
</tbody>
</table>
in the attenuated virulence strains (XbfFL, XbfSN and Xbo). At least 122 ORFs (Xbi had multiple copies of some ORFs) were identified (Table S1). Annotated ORFs (52%) were identified to be involved in known gene ontology (GO) biological pathways including: organic acid (3%), small molecule (3%), amino acid (3%), aromatic compound (9%), macromolecule (7%) and nitrogenous compound metabolism (22%) (Fig. 2). The majority of these sequences (86%) were associated with the bacterial membrane (data not shown). Further investigation of the identified ORFs revealed a T6SS (Table S1), which comprised 11% of the candidate insect virulence ORFs.

A second comparative analysis examined the variable portion of each bacterial genome to distinguish differences in hypervariable regions and horizontal gene transfer events [47]. A total of 244 such modules, as previously defined in the reference genome of the virulent strain Xb SS-2004 [27, 47, 62], were compared with the nine X. bovienii strains used in this study (Table S2). Fifteen of the 244 modules were found to be fully or partially conserved in the genomes of the virulent strains (Xbi and Xbj) but were absent in the attenuated virulence strains (XbfFL, XbfSN and Xbo) (Table S3). These modules have diverse predicted functions ranging from phage components to roles in environment interaction. In particular, two of the 15 modules (RGP32bis_XB_b and RGP48_XB_a) were predicted to be involved in host interaction. RGP32bis_XB_b contains the same T6SS locus identified in the gene content analysis and is fully conserved in both strains. RGP48_XB_a includes a series of hypothetical proteins surrounded by transposases as well as invasin and lipase ORFs, however this region is only partially conserved in both strains (Table S3).

A pan-genomic analysis was further conducted with all available X. bovienii genomes to investigate the identified T6SS locus. It was found to be present in the genomes of other X. bovienii strains including: Xb SS-2004, XbfM, XbkBU and Xbp. However, this region is absent in the genomes of XbCS03 and XbkCA (Table S4). When examining surrounding genes, it became apparent that these ORFs were part of a larger locus containing 18 to 19 ORFs (~26 kb), depending on the X. bovienii strain examined (Fig. 3, Table S4). These ORFs were generally flanked on at
Phylogenetic analysis of the conserved TssBC sheath proteins revealed that all Xenorhabdus type A T6SS form a monophyletic cluster sister to the Serratia clade (Fig. 4). The virulent strains Xbi and Xbj grouped together with Xb SS-2004, another virulent strain from the same nematode host isolate as Xbj [27, 62]. The other three T6SS+ X. bovienii strains with intermediate virulence (i.e. XbfM, XbkBU and Xbp) formed a separate cluster. The T6SS phylogeny is congruent with the species phylogeny and suggests that strains lacking this locus lost it over their evolutionary trajectory (Fig. 4).

Intra-bacterial competition assays

As T6SSs are commonly involved in interbacterial competition [64, 65], we tested the ability of T6SS+ and T6SS− strains to compete with each other in co-culture. Competition between Xn and X. bovienii T6SS− strains was considered as a positive control since X. nematophila is known to produce bacteriocins active against X. bovienii [19]. Individually, all X. bovienii strains displayed similar growth kinetics, expect for XbCA (Fig. S2).

Most of the T6SS+ (depicted with either high or moderate virulence) strains outcompeted the T6SS− strains (Fig. 5). When inoculated at a 5 : 1 ratio, all T6SS+ strains were able to significantly displace T6SS− populations (Fig. 5a), with Xbi and Xbj causing the greatest reductions, similar to Xn.
The highly virulent strains, Xbi and Xbj, also retained their ability to outcompete T6SS− strains when inoculated at a 1:1 ratio (Fig. 5b), whereas the intermediate virulence strains, XbfM, XbkBU and Xbp, had little to no effect on displacing T6SS− strains at this ratio (Fig. 5b).

DISCUSSION

In this study, we investigated the virulence phenotype of nine different isolates of X. bovienii in the absence of their vectoring Steinernema host and examined their genomes for common genotypes that may contribute to their virulence. We demonstrated that there is striking variation in the virulence of X. bovienii strains toward two different lepidopteran hosts when the bacteria are inoculated in the absence of their vector nematode hosts. Specifically, two strains, Xbi and Xbj, were highly virulent in both hosts. Contrastingly, strains XbfFL, XbfSN and Xbo showed attenuated virulence. These results indicate that virulence in X. bovienii varies at the intraspecific level in two different insect hosts. They also suggest that differences in the host range of Xenorhabdus–Steinernema pairs could be due, at least in part, to differences in insecticidal activity of the bacteria. These findings highlight the possibility of fine-scale, strain-to-strain variation in the Xenorhabdus–Steinernema system. In this regard, there is growing evidence for the extent of such strain-to-strain variation within individual bacterial species. Studies such as this highlight how generalizations about bacterial virulence (or other phenotypic traits) cannot be made from a single isolate of a species, as isolates vary in their ability to kill different insects. It also reflects the diversity of the virulence phenotype across the X. bovienii ecological range, which is triggered by the variation in selective pressures and genetic drift these isolates have experienced during their interactions with their invertebrate hosts and the soil environment.

By definition, genetic differences between strains of a bacterial species are relatively small, facilitating identification of the genes contributing to phenotypic differences. In this study, 122 genes were found to be present in two of the most virulent X. bovienii strains but absent in the three least virulent strains. Many of these genes appear to encode metabolic functions. Several studies have shown that, although not classic virulence factors, unique or flexible metabolic capabilities can contribute to the ability of bacteria to colonize a host and therefore contribute to disease [66–68]. In this respect, we speculate that differences in metabolic pathways could reflect specialization of X. bovienii for certain hosts – both nematode and insect – and could also contribute to the relative success of virulent strains.

Intriguingly, one set of syntenic genes we identified as present in the virulent strains and absent in the attenuated ones appears to encode a T6SS. This secretion system has been shown to allow bacteria to inject proteins called effectors directly into other cells [64, 65]. It is also known to be implicated in a diverse range of symbiotic and pathogenic interactions with eukaryotic hosts, as well as in competition between bacteria, depending on the partners and effectors involved [64, 65].

**Fig. 4.** T6SS phylogeny phylogenetic reconstructions (maximum likelihood) of Xenorhabdus T6SS TssBC conserved sheath proteins and species tree (16S rRNA, recA, dnaN, gitX, gyrB and infB genes). Interior nodes with greater than 50% bootstrap support are displayed. T6SS TssBC phylogeny is scaled to amino acid changes per site. Sm, S marcescens; strain designations are provided in the tree. Ft, Francisella tularensis strain FSC147; outgroup taxon. See Table S6 for gene labels.
Bioinformatics analyses conducted in this study revealed homology between the *Xenorhabdus* T6SSs and loci found in *S. marcescens*, which are involved in bacterial competition [55]. This homology suggests that T6SS in *X. bovienii* function may also play a role in bacteria competition. In this respect, our competition assays support this possibility, as the virulent T6SS+ strains generally outcompeted the attenuated T6SS− strains (Fig. 5). Thus, we speculate that the T6SS in *X. bovienii* may be another addition to the arsenal of antibacterial mechanisms expressed by these bacteria in an insect, where it could potentially play one of three roles. First, it could target the insect’s gut microbiota, which is known to translocate into the haemocoel when the nematodes migrate from the insect gut into the haemocoel [69].

**Fig. 5.** Bacterial competition assays. (a) 5:1 T6SS+:T6SS− assay. (b) 1:1 T6SS+:T6SS− assay. For both (a) and (b), the mean fold change of growth from the attenuated strain is reported along with the SE. Fold change is relative to the LB negative control (i.e. no T6SS+ added). The y-axis is log_{10}-scaled. Letters indicate statistical ranking generated from a post hoc Tukey-Kramer test of T6SS+ “T6SS−” interaction from two-way ANOVA analysis. 5:1 experiment ANOVA: F_{27,196}=39.6419, P<0.0001; T6SS− effect: F_{3,196}=4.5746, P=0.0040; T6SS+ effect: F_{6,196}=170.2603, P<0.0001; T6SS+ “T6SS−” interaction effect: F_{18,196}=1.9471, P=0.0144. 1:1 experiment ANOVA: F_{27,191}=32.5807, P<0.0001; T6SS− effect: F_{3,191}=22.9927, P<0.0001; T6SS+ effect: F_{6,191}=112.6779, P<0.0001; T6SS+ “T6SS−” interaction effect: F_{18,191}=6.8877, P=0.0001. For pairwise-comparison results, see Tables S7 and S8.
Second, it could help protect the insect cadaver from necrotrophic microbial competitors, keeping it preserved until the *Xenorhabdus–Steinernema* life cycle is complete [70]. Third, it could play a role in competition with other *Xenorhabdus* species and/or strains when co-infections occur [71, 72]. This latter possibility seems especially plausible based on our competitions assays, which were intraspecific.

It can also be speculated that the T6SS itself contributes to insect virulence. Several studies have demonstrated roles for T6SSs in bacterial pathogenesis [64]. For example, it has been shown that the T6SS in *Vibrio cholera* Pacini (*γ-Proteobacteria: Vibrionaceae*) contributes to colonization of animal hosts, by either enhancing competition against the host’s normal microbiota [73] or by directly targeting eukaryotic host cells [64, 74].

Sudhaus [75] suggested that the evolution of the entomopathogenic lifestyle in *Steinernema* relied on the selection of highly virulent symbionts. However, this is not likely to be applicable to all species. For example, for *X. bovienii–S. weiseri* strain CS03, both the bacterium–nematode pair and the bacterium alone are less virulent when compared with other *X. bovienii–nematode* pairs [27]. Furthermore, Bisch *et al.* [28] showed that the transmission–virulence trade-off in this bacterium–nematode partnership is likely to be heavily reliant on the inhibition of competitors rather than pathogenicity toward insects. In relation to this, and based on our findings, we predict that the ability to outcompete other bacteria (including other *Xenorhabdus* species and/or strains) is also an important trait that played a key role in the perpetuation of *Xenorhabdus–Steinernema* partnerships. Further experimentation is required to characterize these T6SSs in the *Xenorhabdus* life cycle with gene knockouts and to examine their impact on different phenotypic traits that are critical for *Xenorhabdus–Steinernema* fitness.

Altogether, the results presented in this study highlight the genetic and phenotypic diversity of the *X. bovienii* species and suggest that a variety of approaches to mutualism and virulence are evident at a fine scale in the *Xenorhabdus–Steinernema* system.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

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