



Type 6 secretion system components *hcp* and *vgrG* support mutualistic partnership between *Xenorhabdus bovienii* symbiont and *Steinernema jolietii* host

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ABSTRACT

Xenorhabdus, like other Gram-negative bacteria, possesses a Type 6 Secretion System (T6SS) which acts as a contact-dependent molecular syringe, delivering diverse proteins (effectors) directly into other cells. The number of T6SS loci encoded in *Xenorhabdus* genomes are variable both at the inter and intraspecific level. Some environmental isolates of *Xenorhabdus bovienii*, encode at least one T6SS locus while others possess two loci. Previous work conducted by our team demonstrated that *X. bovienii* [Jolietti strain SS-2004], which has two T6SSs (T6SS-1 and T6SS-2), *hcp* genes are required for biofilm formation. Additionally, while T6SS-1 *hcp* gene plays a role in the antibacterial competition, T6SS-2 *hcp* does not. In this study, we tested the hypothesis that *vgrG* genes are also involved in mutualistic and pathogenic interactions. For this purpose, targeted mutagenesis together with wet lab experiments including colonization, competition, biofilm, and virulence experiments, were carried out to assess the role of *vgrG* in the mutualistic and antagonistic interactions in the life cycle of XBJ. Our results revealed that *vgrG* genes are not required for biofilm formation but play a role in outcompeting other *Xenorhabdus* bacteria. Additionally, both *vgrG* and *hcp* genes are required to fully colonize the nematode host. We also demonstrated that *hcp* and *vgrG* genes in both T6SS clusters are needed to support the reproductive fitness of the nematodes. Overall, results from this study revealed that in *X. bovienii* jolietii strain, the two T6SS clusters play an important role in the fitness of the nematodes in relation to colonization and reproduction. These results lay a foundation for further investigations on the functional significance of T6SSs in the mutualistic and pathogenic lifecycle of *Xenorhabdus* spp.

1. Introduction

Bacteria employ an array of secretion systems that deliver toxins and other proteins directly into target cells or into the environment (Green and Mecsas, 2016). Type 6 secretion systems (T6SSs) act as molecular syringes that deliver diverse effector proteins directly into other cells (Bingle et al., 2008; Cascales, 2008; Cotter, 2011; Filloux, 2009, 2013; Ho et al., 2014). Structurally, they resemble inverted phage tail-like cell puncturing nanomolecular machines. A functional T6SS usually consists of at least 13 structural proteins including the inner tube protein Hcp (also called TssD) and the needle tip protein VgrG (also called TssI), which are injected into target cells after contact, along with a payload of effectors (Filloux, 2009, 2013). Additionally, PAAR (Proline-Alanine-

Alanine-Arginine) repeat proteins, which sit on the top of VgrG and further sharpen the conical tip of T6SS complex (Shneider et al., 2013) also have additional and diverse functions including serving as toxins or as carriers for interacting toxins (Zheng et al., 2021).

T6SSs have been shown to play diverse functions targeting both prokaryotic and eukaryotic cells (Galán and Waksman, 2018; Russell et al., 2014; Zhong et al., 2019). Most characterized T6SSs appear to target bacterial competitors (Carruthers et al., 2013; Hood et al., 2010; Murdoch et al., 2011; Russell et al., 2014; Schwarz et al., 2010; Speare et al., 2018). In some cases, specific antibacterial effectors have been identified, along with “immunity proteins” expressed (but not secreted) that seem to play a role in preventing self-intoxication (Dong et al., 2013; Durand et al., 2014; Russell et al., 2011) or prevent the killing of

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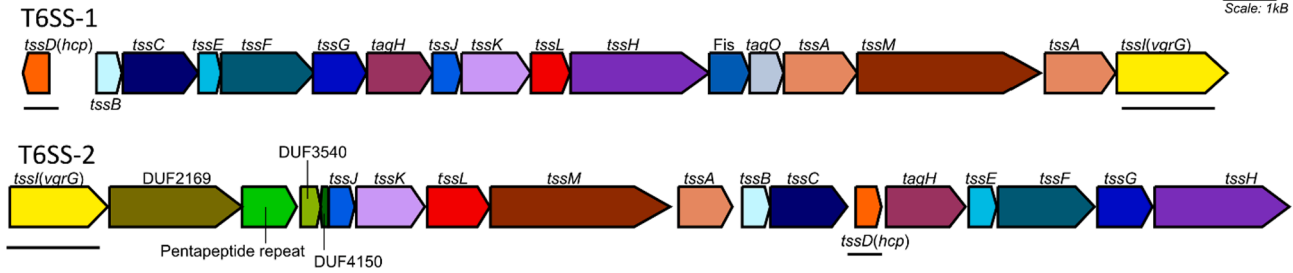
***Xenorhabdus bovienii* SS-2004 (XBJ)**

Fig. 1. Genetic organization of *Xenorhabdus bovienii* SS-2004 (XBJ) T6SS clusters. Same color indicates orthologous genes. Genes studied in this work are indicated with a black line underneath them.

sister cells (Basler and Mekalanos, 2012; Gallique et al., 2017; Unterweger et al., 2014). There is also growing evidence that suggests T6SSs may be involved in communication among closely related bacteria, especially in the context of biofilms (Decoin et al., 2015; Gallique et al., 2017; Tian et al., 2014).

Additionally, a few studies have shown this secretion system targets eukaryotic cells and has diverse roles, including virulence against a wide range of eukaryotic hosts (Blevess et al., 2014; Jiang et al., 2014; Lin et al., 2017; MacIntyre et al., 2010), metal ion acquisition, and anti-prokaryotic activity (Lennings et al., 2019; Schwarz et al., 2010). Moreover, in *Rhizobium etli* (Re) Mim1, T6SS has also been shown to promote prokaryote-eukaryote interactions as the bean plants inoculated with Re T6SS mutant strains performed poorly with fewer nodules and lower dry weight (Salinero-Lanzarote et al., 2019). Similarly, *vgrG* mutants of *Bradyrhizobium* sp. LmicA16 induced fewer nodules and resulted in smaller plants compared to the wild type strain (Tighilt et al., 2022).

Recently, our team identified a novel T6SS in *Xenorhabdus bovienii*, a Gram-negative enteric bacterium that has a dual life cycle. It is a pathogen of insects and a mutualist to *Steinernema* nematodes (Kochanowsky et al., 2020; McMullen et al., 2017). Specifically, *Xenorhabdus* live in a mutualistic symbiosis with *Steinernema* nematodes but are acutely pathogenic to a wide range of insects (Boemare, 2002; Boemare and Akhurst, 2006; Grewal and Georgis, 1999; Poinar, 1990; Stock, 2005). *Xenorhabdus* also produce antibacterial compounds in the insect host, which prevent the scavenging of the insect cadaver by soil saprobes and the other entomopathogens (Boemare and Akhurst, 2006; Forst et al., 1997; Forst and Nealon, 1996; Stock and Goodrich-Blair, 2008, 2012). Thus, the *Steinernema-Xenorhabdus* partnership is a useful and tractable model system to study mechanisms of mutualism and pathogenesis (Stock, 2005; Stock and Goodrich-Blair 2008, 2012).

T6SS was first identified in the genome of *Xenorhabdus bovienii*

(Jollieti, SS-2004 strain) (hereafter referred as XBJ) by Chaston et al. (2011) who recognized the presence of two loci. Subsequently, McMullen et al. (2017) discovered that the number of T6SS loci in *X. bovienii* isolates is variable (McMullen et al., 2017). The authors also demonstrated that strains encoding two T6SS loci (T6SS-1 and T6SS-2) outcompete those with only one locus, suggesting T6SS may play a role in intraspecific bacterial interactions. A recent study by Kochanowsky et al. (2020) revealed that in XBJ *hcp*-defective mutants, the two T6SS loci are involved in biofilm formation, but only one locus, T6SS-1, plays a role in antibacterial competition against closely related strains.

In this study, we furthered investigations on the role of XBJ T6SSs to assess the function of another important structural component, the needle tip protein VgrG (also called TssI), which is injected into target cells after contact, along with effector proteins. We hypothesize that VgrG is also involved in both mutualistic and pathogenic interactions in the life cycle of this bacterium. A combination of targeted mutagenesis and wet lab experiments (i.e. colonization, competition, biofilm, and virulence experiments) were carried out to assess the role of *vgrG* in the mutualistic and antagonistic interactions in the life cycle of XBJ.

2. Materials and methods

2.1. Nematode and bacterial cultures

Steinernema jollieti (S-2004 strain) was reared *in vivo* with the last instar of *Galleria mellonella* (Lepidoptera: Pyralidae) larvae following procedures described by Kaya and Stock (1997). Emerging IJs were harvested, washed twice with deionized water, and stored at 15 °C in 250 ml tissue culture flasks at a concentration of 3000 IJs/ml.

XBJ wild type and mutant strains were cultured on Luria-Bertani (LB) agar plates or in LB broth supplemented with 0.1 % sodium pyruvate

Table 1

Bacterial strains used in this study.

Species	Strain	Characteristics	Abbreviation	Antibiotic marker	Source
<i>Xenorhabdus bovienii</i>	Jollieti	Wild type	XBJ	Not applicable	H. Goodrich Blair
<i>Xenorhabdus bovienii</i>	Jollieti	<i>hcp1</i> disruption	<i>hcp1</i>	Chloramphenicol	Kochanowsky et al., 2020
<i>Xenorhabdus bovienii</i>	Jollieti	<i>hcp2</i> disruption	<i>hcp2</i>	Chloramphenicol	Kochanowsky et al., 2020
<i>Xenorhabdus bovienii</i>	Jollieti	<i>hcp1</i> disruption, pBMT- <i>hcp1</i>	<i>hcp1 comp</i>	Chloramphenicol	Kochanowsky et al., 2020
<i>Xenorhabdus bovienii</i>	Jollieti	<i>hcp1</i> disruption, <i>hcp2</i> deletion	<i>hcp1/hcp2</i>	Chloramphenicol+ Kanamycin	Kochanowsky et al., 2020
<i>Xenorhabdus bovienii</i>	Jollieti	<i>vgrG1</i> deletion	<i>vgrG1</i>	Kanamycin+ Ampicillin	This study
<i>Xenorhabdus bovienii</i>	Jollieti	<i>vgrG2</i> deletion	<i>vgrG2</i>	Kanamycin	This study
<i>Xenorhabdus bovienii</i>	Jollieti	<i>vgrG1/vgrG2</i>	<i>vgrG1/vgrG2</i>	Kanamycin + Chloramphenicol+ Ampicillin	This study
<i>Xenorhabdus bovienii</i>	Jollieti	<i>vgrG1</i> deletion/ <i>hcp1</i> disruption	<i>vgrG1/hcp1</i>	Chloramphenicol + Kanamycin	This study
<i>Xenorhabdus bovienii</i>	Jollieti	<i>vgrG1</i> deletion/ <i>hcp2</i> disruption	<i>vgrG1/hcp2</i>	Chloramphenicol + Kanamycin	This study
<i>Xenorhabdus bovienii</i>	Oregonense	GFP-labeled	XBO	Rifampicin	McMullen et al., 2017
	pCVD442	suicide plasmid	-	Ampicillin	Addgene
<i>Escherichia coli</i>	EC49	mating strain	-		

Table 2
vgrG Primers.

Primer	Oligonucleotide Sequence (5'-3')	Purpose
1	TCTTCTAGAGGTACCGCATGCTGGTCTACCTGTCCCAGTCC	Upstream of vgrG1
2	CGATCAACGTCTCATTTCGCGGGTTTCAGGTACGAGAGC	Upstream of vgrG1
3	CTTAAACGCCTGGTTGCTACGTTAATGTGCTTTGCCAGAA	Downstream of vgrG1
4	GTGGAATTCGCGGGAGAGCTCGGGTCACTCATGGTCAATCC	Downstream of vgrG1
5	TCTTCTAGAGGTACCGCATGCTGAGAACGAGCATTTCATTGAT	Upstream of vgrG2
6	TATGAGTCAGCAACACCTTCTTGTGTGAATAAGGAGGAAGGT	Upstream of vgrG2
7	TCTTCGCTATTACGCCAGCTCTGCGCTTAAGTCTGTACATA	Downstream of vgrG2
8	GTGGAATTCGCGGGAGAGCTTTCAGCCACTCTTTGTACATA	Downstream of vgrG2
9	GCTCTCGTACCTGAAAACCGGGCGAAAATGAGACGTTGTGCG	Chloramphenicol vgrG1
10	TTCTGGGCAAAGCACATTAACGTAGCAACAGGCGTTTAAG	Chloramphenicol vgrG1
11	ACCTTCTCCCTTATTACACAAGAAGGTGTGCTGACTCATA	Kanamycin vgrG1
12	TGTAGCAGACTTAAGCGCAGAGCTGGCGTAATAGCGAAGA	Kanamycin vgrG2
13	CTTTTGAAGAGCACGGTATCC	Screening for vgrG1 mutant
14	TGAAGGTGTAGTCCCTTGAGCTG	Screening for vgrG1 mutant
15	CAACAGATAAGAATGATCACA	Screening for vgrG2 mutant
16	TAGGATTGTTGTAGTACTCG	Screening for vgrG2 mutant
17	AGAAAATAAGAATTGACGGATGAA	Screening for hcp1 mutant
18	TCACGGAATGAGCACTTACA	Screening for hcp1 mutant
19	CCTATTATTGGTGGGAAAATAACCTCCC	Screening for hcp2 mutant
20	TTAAAACGTGTTCTGCAATCAACATTATTCA	Screening for hcp2 mutant
21	GCTGTCTCATCAGGGATATG	Positive control (gyrA)
22	AGAGGATGGTATCGTGTGTA	Positive control (gyrA)

(LBP) in the dark at 28 °C. Liquid cultures were incubated by shaking at 160 rpm according to Stock and Goodrich-Blair (2012). The media were supplemented with 100 µg/mL ampicillin, 10 µg/mL chloramphenicol, 50 µg/mL kanamycin, and 10 µg/mL rifampicin when needed.

2.2. XBJ T6SS hcp and vgrG knockouts

The hcp and vgrG mutants considered in this study were generated by Kochanowsky et al (2020) and maintained as glycerol stocks in PI Stock's laboratory (University of Arizona). Prior to their use, mutants were verified for the hcp gene disruptions by PCR with an initial denaturation at 95 °C for 3 min; 30 cycles of (denaturation at 95 °C for 30sec, annealing at 60 °C for 30sec, extension at 72 °C for 2 min); and a final extension at 72 °C for 10 min.

XBJ mutants lacking one or both of vgrG genes and XBJ mutants depleted with either hcp1 of T6SS-1 or hcp2 of T6SS-2 and vgrG1 of T6SS-1 or vgrG2 of T6SS-2 genes were generated and evaluated in this study (Fig. 1 and Table 1). To construct single vgrG mutants, three fragments, upstream and downstream regions of the specific target gene and integration cassettes such as kanamycin or chloramphenicol were generated. Each fragment was amplified by appropriate primer sets including specific enzyme sites and complement sequences (Table 2). Three fragments were fused and amplified by PCR (initial denaturation at 95 °C for 3 min; 30 cycles of (denaturation at 95 °C for 30sec, annealing at 60 °C for 30sec, extension at 72 °C for 3 min); and final extension at 72 °C for 10 min). PCR products were cloned into the suicide vector pCVD442. Completed constructs were transformed into XBJ. vgrG deletion mutants were screened by respective antibiotic selection and colony PCR with internal primer binding to vgrG gene (Table 2). vgrG gene insertion mutant was generated through recombination process with pKNOCK suicide plasmid containing specific region of vgrG gene. To generate double vgrG1/vgrG2 mutant, vgrG1 insertion XBJ clone and E. coli EC49 strain with vgrG2 deletion construct were prepared. Two strains were mated and vgrG2 deletion mutant clones were selected using antibiotic selection marker (Huang and Wilks, 2017). Finally, double mutants were confirmed using vgrG2 internal primers (Table 2).

2.3. Bacterial competition assays

To assess the role of T6SS in anti-bacterial activity, standard competition bioassays were conducted between wild and mutant XBJ strains against another X. bovienii strain that is the mutualist of

Steinernema oregonense (XBO) as the "prey" bacterium (Table 1). The prey bacterium (XBO) was GFP-labelled according to procedures described by McMullen et al (2017). Strains were grown in 5 ml LBP broth with appropriate antibiotics separately overnight. Then, the cultures were normalized to OD₆₀₀ = 1.0 and a final volume of 1 ml of the cultures were centrifuged at 3000 rpm for 5 min at room temperature. The pellets were washed with 1 ml of fresh LBP by centrifuging at 3000 rpm for 5 min at room temperature. The pellets were resuspended in 500 µl of fresh LBP by vortexing briefly. The prey (100 µl) and the predator (wild type or mutant) (100 µl) were mixed thoroughly by pipetting up and down and vortexing. A 5 µl of the mixture was spotted on a filter paper disc placed on the LBP agar plates and incubated in dark for 24 h. After 24 h, bacterial growth around the paper disc was collected, suspended in 2 ml LBP broth, and serially diluted to 10⁻⁷. From each dilution, 100 µl was plated on LBP agar plate and another 100 µl was plated on LBP agar plate supplemented with rifampicin (LBP + rif), selective for prey. Colony forming units (CFUs) on each plate were counted and reported as the proportion of prey CFUs to total CFUs, indicating the survival of prey population in the presence of the wild type or mutant. Each experiment was conducted with three independent clones and repeated three times for each mutant strain. Results were analyzed using one-way ANOVA and pair-wise significance among the mutants was determined by Tukey's test.

2.4. Biofilm formation assays

Biofilm assays were carried out following methods described by Kochanowsky et al. (2020). Briefly, wild type and mutant strains (listed in Table 1) were cultured as explained above with respective antibiotics. Overnight grown saturated cultures were normalized to OD₆₀₀ = 1.0 and then subcultured in individual wells of 24-well plates by diluting at 1:100 ratio in 2 ml of fresh LBP without antibiotics. LBP broth without bacteria was used as a negative control. The plates were incubated at 28 °C in the dark for 48 h without shaking. After 48 h, cultures were discarded, and plates were washed twice by immersing in deionized water and allowed to dry by turning them over for 1 h or until the wells were completely dry. The dried wells were filled with 1 % crystal violet solution and incubated for 15 min at room temperature. Then, the solution was discarded, and plates were washed thrice by immersing in deionized water. The bounded crystal violet was dissolved in 2 ml of 30 % acetic acid solution and 150 µl of the solution was pipetted into a 96-well plate. The absorbance of samples was quantified at 550 nm in a

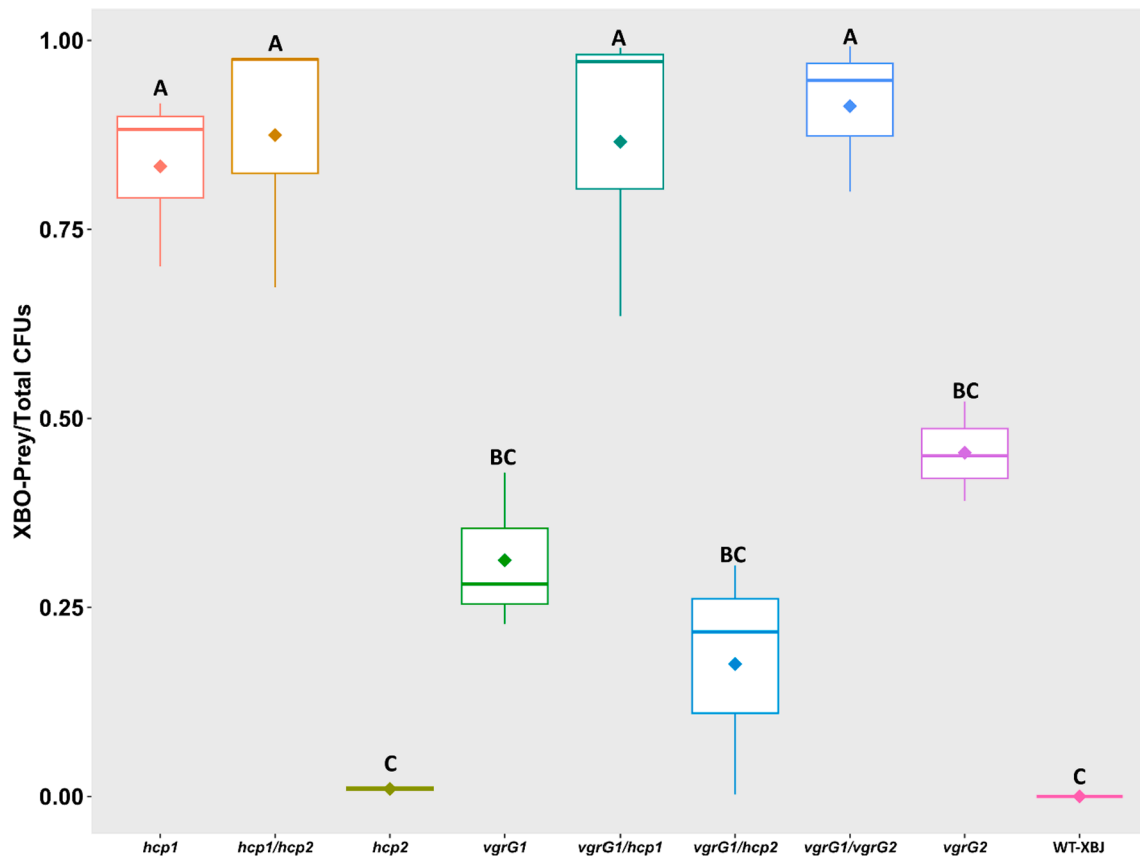


Fig. 2. Both the T6SSs in XBJ play a role in antiprokaryotic activity against intraspecific bacteria: XBJ T6SS mutants and XBJ wild type (WT) were competed against the prey bacteria, GFP-labelled *X. bovienii* strain oregonense (XBO) in 1:1 ratio on agar plate. The survival of prey bacteria was reported as proportion of prey colony forming units (CFUs) to total CFUs. Lower and upper box boundaries represent 25th and 75th percentiles, respectively, line inside box indicate the median, lower and upper error lines represent 10th and 90th percentiles, respectively. Letters indicate significant difference among XBJ mutants ($n = 9$, $p < 0.05$, Tukey's test).

BioTek Synergy HTX plate reader. Each experiment was conducted with three independent clones and repeated three times for each mutant strain. Data were analyzed using ANOVA one-way and significance was tested using Tukey's test.

2.5. XBJ virulence assays

Wild type and mutant cultures were grown as described in Section 2.1. Saturated cultures were standardized to $OD_{600} = 1.0$ using fresh LBP. Last instar *Galleria mellonella* larvae were considered for these assays following procedures described by McMullen et al. (2017). Briefly, larvae were surface sterilized with 70 % ethanol and anesthetized on ice for 10 min. The bacteria suspension was loaded onto a syringe and 10 μ l was injected into each larva on the ventral side between second and third prolegs at a flow rate of 0.3 μ l/s using a microinjector (Tritech Research, CA). Injected insects were kept individually in 12-well plates and checked for mortality for every 3 h. Each experiment consisted of 12 insects per each of the tested mutant clone and each experiment was repeated four times. The data were analyzed using survival analysis and the survival times of insects injected with various mutants were compared using Log-rank test.

2.6. Nematode-bacteria symbiosis assays

2.6.1. Rearing of aposymbiotic *S. jolietii* IJs

Aposymbiotic (symbiont-free) *S. jolietii* IJs were generated as described previously (McMullen and Stock, 2014). Briefly, gravid female nematodes were collected from *G. mellonella* cadavers infected with *S. jolietii* IJs. The females were processed by treating them with

axenizing solution. The sterilized eggs were seeded on liver-kidney agar plates (LKA) (Stock and Goodrich-Blair, 2012) and incubated in dark at 28 °C. Upon emergence of IJs, the bottom of LKA plate was transferred to a modified White trap set with sterile water. The emerging IJs were rinsed twice and stored in a sterile culture flask at 15 °C until further use. The absence of bacteria from the aposymbiotic nematodes was confirmed by grinding IJs in LB broth and plating the solution on NBTA plates (Akhurst, 1980).

2.6.2. Colonization of aposymbiotic *S. jolietii* IJs with wild and mutant strains

Wild and mutant strains were grown as explained above. Bacterial pellets were resuspended in 500 μ l of fresh LBP by pipetting up and down and vortexing the suspension. A 60 μ l suspension was plated on modified lipid agar plates supplemented with 0.1 % of sodium pyruvate (Akhurst, 1980; Vivas and Goodrich-Blair, 2001; Xu and Hurlbert, 1990). Lipid agar plates were modified by pouring a thin layer of LB agar on top of the solidified lipid agar medium to ensure the uniform spread of the bacteria on the plates. The plates were incubated in dark at 28 °C for 24 h. An inoculum of 1000 sterilized aposymbiotic IJs was seeded on each plate, sealed them with parafilm, and incubated in dark at 28 °C. The emerged IJs were harvested and stored as explained above for aposymbiotic nematodes. The mutant strains were confirmed by PCR.

2.6.3. Nematode colonization efficiency assays

The colonization efficiency (bacterial load) of *S. jolietii* IJs was measured as the number of CFUs per 100 IJs (Snyder et al., 2007). Nematodes grown on lawns of wild and mutant strains were harvested and surface sterilized with 1 % NaOCl solution. IJs colonized with the

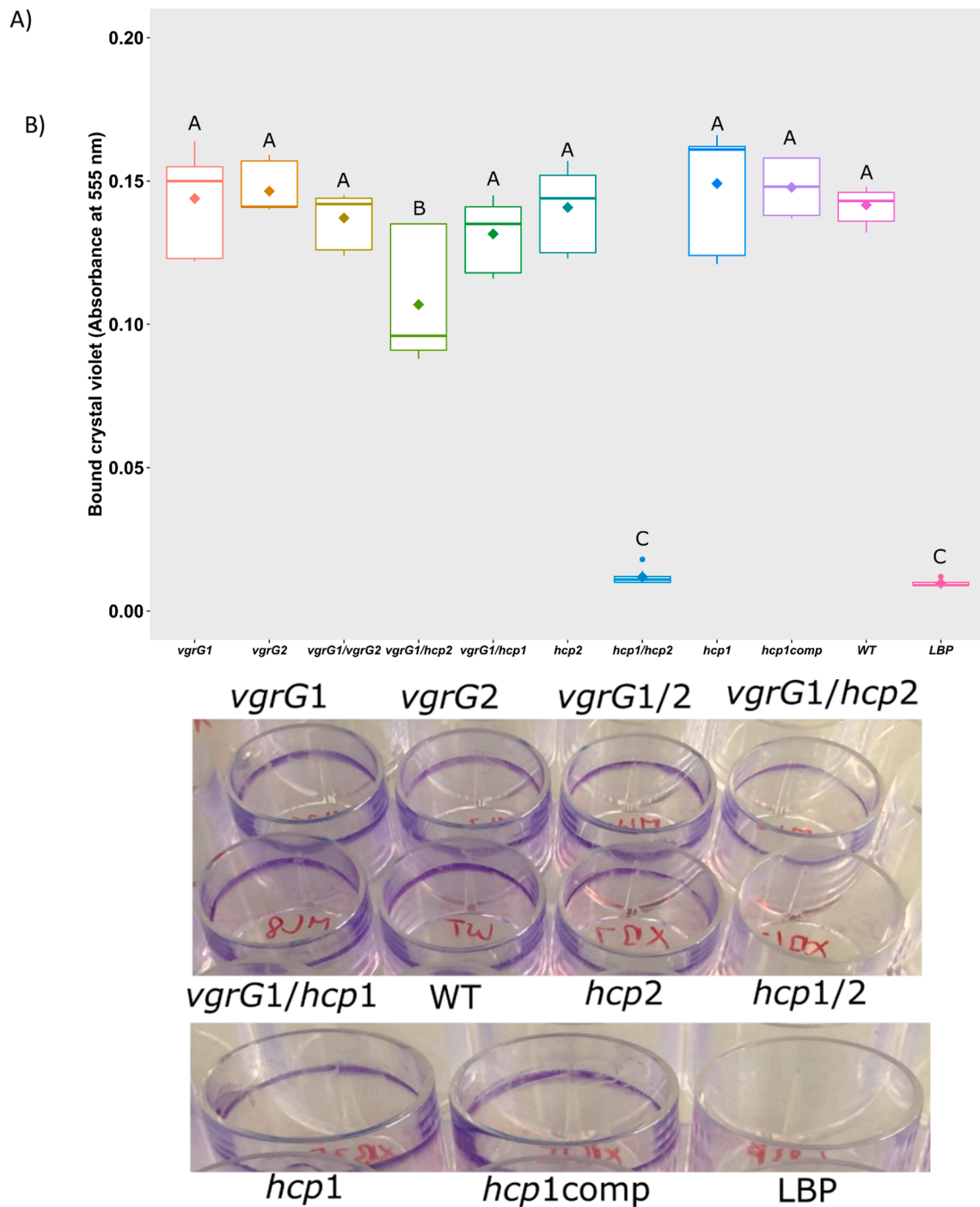


Fig. 3. Biofilm formation in XBJ is mediated by *hcp* genes: Wild type and XBJ T6SS mutants were grown in LBP broth without shaking for 48 h in 24-well plate to allow the biofilm formation. Bounded biofilm was stained with crystal violet and plates were imaged (A) and quantified the absorbance at 550 nm by solubilizing in acetic acid (B). Lower and upper box boundaries represent 25th and 75th percentiles, respectively, line inside box indicate the median, lower and upper error lines represent 10th and 90th percentiles, respectively. Letters indicate significant difference among XBJ mutants (n = 9, p < 0.05, Tukey's test). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

wild XBJ strain were used as the control. Sterilized nematodes were ground with a handheld motorized rotor in 50 µl of LBP. Following procedures described by McMullen et al. (2017), the suspension was plated onto NBTA plates and incubated in dark at 28 °C for 24 h. Then, the CFUs were counted and expressed as the total number of CFUs per 100 IJs. Each experiment was conducted with three independent clones and repeated four times for each mutant strain. The results were analyzed using negative binomial distribution.

2.6.4. Nematode virulence assays

Procedures described by McMullen et al. (2017) were followed to assess the virulence of *S. jolietii* IJs colonized with XBJ mutant strains. Briefly, *G. mellonella* last instar larvae were individually placed in each well of a 12-well plate lined with two filter paper (Whatman #1) discs and infected with 50 IJs colonized with each of the mutant strains. IJs colonized with the wild strain were used as control. The time to insect death was recorded every 6 h for a total of 10 days. Twelve larvae were considered per treatment and experiments were repeated four times.

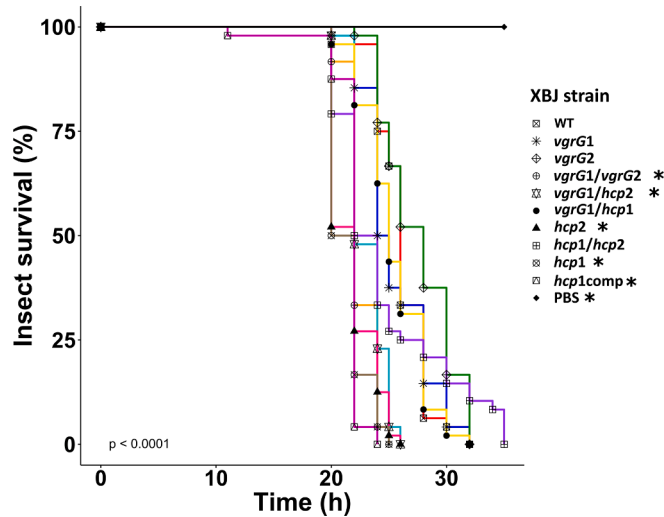


Fig. 4. T6SS is not responsible for XBJ's insect virulence when injected: Standardized XBJ wild type and XBJ T6SS mutants grown in LBP broth were injected into the *Galleria mellonella* larvae and the time to mortality was scored for every 3 h. Although significant differences were observed between some treatments, 100 % mortality was recorded in all the treatments (n = 48, p < 0.0001, log-rank test). The treatments with * are significantly different from other treatments.

Dead larvae with signs of nematode infection were individually placed in modified White traps to collect emerging IJ progeny as per Kaya and Stock (1997).

2.6.5. Nematode reproductive fitness assays

Steinernema jolietii IJs colonized with *hcp* and *vgrG* XBJ mutant strains were assessed for their reproductive fitness considering two parameters: a) number of productive infections and b) total IJ progeny/insect cadaver. As explained above, *G. mellonella* cadavers were monitored daily and for up to 15 days to record IJ emergence. Cadavers with a progeny of at least 100 IJs were considered 'productive', and productive infection was calculated as the proportion of cadavers with IJs progeny to the total number of insect cadavers, expressed as a percentage. IJ progeny from each cadaver was collected and total number of IJs were counted by serial dilution as described by Kaya and Stock (1997).

2.7. Characterization of *vgrG* genes of two T6SSs in XBJ

The sequence similarity of two *vgrG* genes was assessed with pairwise alignment of amino acid sequences of VgrG1 of T6SS-1 (WP_012986891.1) and VgrG2 of T6SS-2 (WP_012988566.1) using EMBOSS-needle program with default parameters (Madeira et al., 2022). The sequence homology of VgrG1 and VgrG2 was performed by BLASTP against NCBI nr database (non-identical protein sequence database) excluding *Xenorhabdus* to prevent self-hits. The conserved domains in VgrG1 and VgrG2 were searched by analyzing the amino acid sequences in NCBI-CDD (Marchler-Bauer et al., 2017).

3. Results

3.1. Bacterial competition

Results from this experiment showed that XBJ *hcp1*, *hcp1/hcp2*,

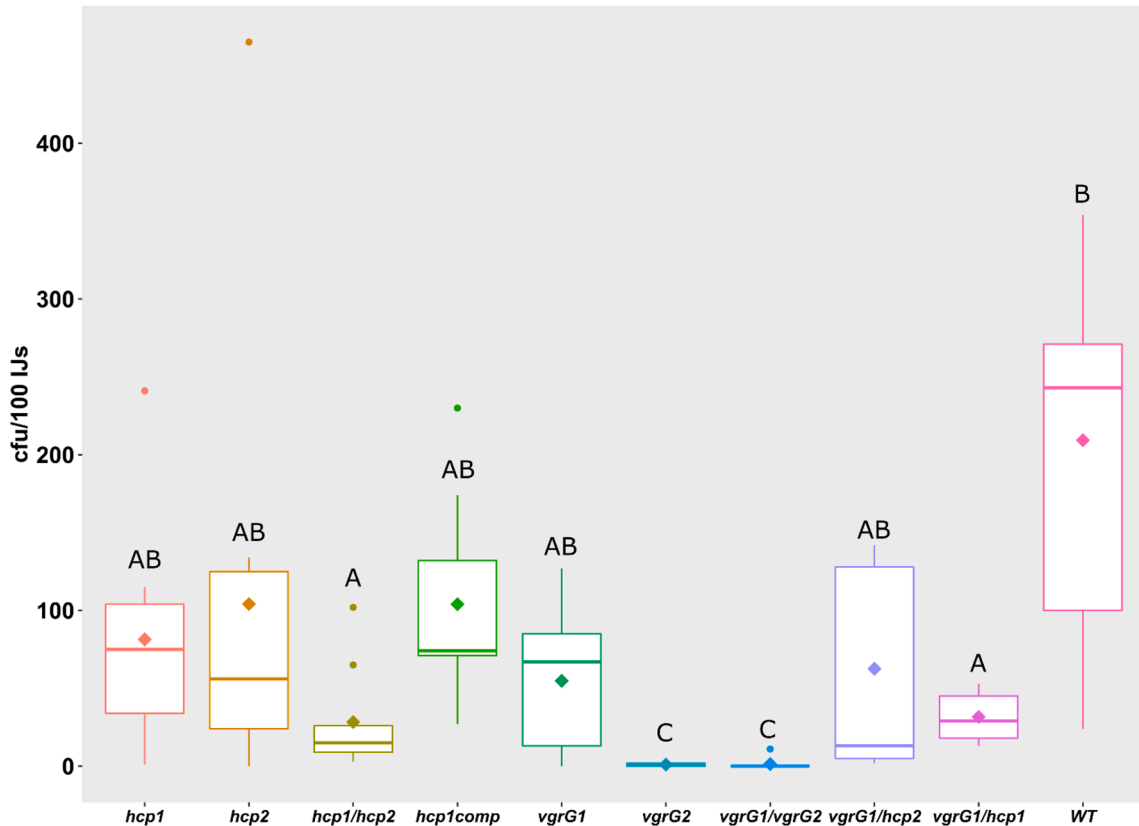


Fig. 5. XBJ requires an intact T6SS to fully colonize its nematode host. Wild type (WT) and the T6SS mutants of XBJ were reared on Lipid-Agar and allowed to colonize aposymbiotic *Steinernema jolietii*. Resulted infective juveniles (IJs) were harvested, crushed, plated and Colony forming units (CFU) were counted and represented as cfu per 100 IJs. Lower and upper box boundaries represent 25th and 75th percentiles, respectively, line inside box indicate the median, lower and upper error lines represent 10th and 90th percentiles, respectively. Letters indicate significant difference among XBJ mutants. n = 9, negative binomial model: p < 0.05.

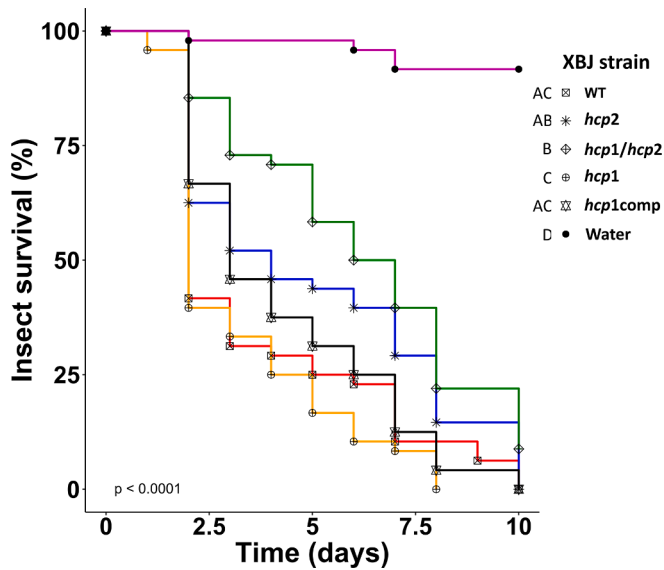


Fig. 6. *hcp* mutations delayed *S. jolietii*-XBJ virulence against *G. mellonella* larvae when infected: Each larva was infected with 50 *S. jolietii* ijs colonized with XBJ *hcp* mutants and mortality was scored for every 6 h. The XBJ mutant lacking both *hcp* genes (*hcp1/hcp2*) took longer time to kill the insect larvae compared to wild type (WT) and other mutants ($n = 48$, $p < 0.0001$, log-rank test). Letters indicate significant differences among XBJ mutants.

vgrG1/hcp1, and *vgrG1/vgrG2* mutant strains outcompeted prey (XBO) at significantly higher levels when compared to the wild strain (Fig. 2). XBJ *vgrG1* or *vgrG2* mutants exhibited moderate (0.3 to 0.5) competing ability against the prey population (Fig. 2). The XBJ *hcp2*, *vgrG1*, *vgrG1/hcp2* mutant strains exhibited competing abilities that were similar to the wild type against the prey population (Fig. 2).

3.2. Biofilm formation

Results from the biofilm assays revealed that the XBJ *hcp* double mutant (*hcp1/hcp2*) was unable to form biofilm. The XBJ *vgrG1/hcp2* mutant formed significantly less biofilm when compared to the wild-type, *vgrG1*, *vgrG2*, *vgrG1/vgrG2*, *vgrG1/hcp1*, *hcp2*, *hcp1*, *hcp1* mutant strain complemented with *hcp1* (*hcp1 comp*) mutants (Fig. 3).

3.3. Insect virulence

The virulence of all tested XBJ mutant strains was similar to that observed in wild type strain. However, when assessing time to death, XBJ mutants *vgrG1/vgrG2*, *vgrG1/hcp2*, *hcp2*, *hcp1*, and *hcp1comp* (Fig. 4) was significantly lesser (20–25 h) when compared to the wild type and other mutant strains including *vgrG1*, *vgrG2*, *vgrG1/hcp1*, *hcp1/hcp2* (28–35 h). But, the differences were few hours (Fig. 4).

3.4. Nematode-bacteria symbiosis assays

3.4.1. Nematode colonization efficiency

IJs colonized with *vgrG* double mutant (*vgrG1/vgrG2*) (mean 1.33 CFUs/100 IJs) and *vgrG2* single mutant (mean 1 CFU/100 IJs) exhibited the lowest bacterial load ($P < 0.0001$) when compared to the wild strain (mean 209.3 CFUs/100 IJs). Similarly, IJs colonized with double mutant strains *hcp1/hcp2* (28.33 CFUs/100 IJs, $P = 0.0035$) and the *vgrG1/hcp1* (mean 31.67 CFUs/100 IJs, $P = 0.0081$) had significantly fewer CFUs when compared to the wild type strain (Fig. 5). Nematode colonization of mutants *hcp1*, *hcp2*, *vgrG1*, *vgrG1/hcp2*, and the *hcp1* complemented strain was similar to that observed for the wild type ($P > 0.05$) (Fig. 5).

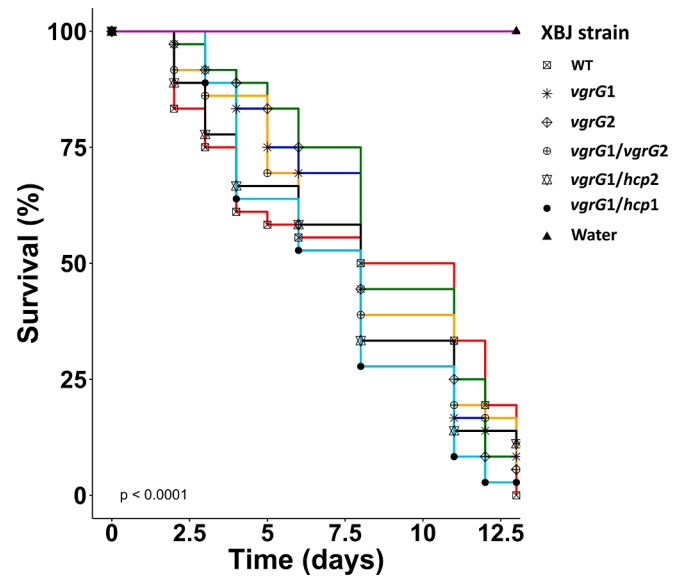


Fig. 7. *vgrG* gene has no role in *S. jolietii*-XBJ virulence against *G. mellonella* larvae when infected: Each larva was infected with 50 *S. jolietii* ijs colonized with XBJ *vgrG* mutants and mortality was scored for every 6 h. The XBJ *vgrG* mutants retained full virulence compared to wild type (WT) against the insect larvae ($n = 48$, $p < 0.0001$, log-rank test).

3.4.2. Nematode virulence assays

Our results showed that the time to death of insects infected with *S. jolietii* IJs associated with XBJ double *hcp* (*hcp1/hcp2*) mutant strain was significantly longer when compared to that observed in insects infected with IJs colonized with the wild, mutant *hcp1*, and *hcp1comp* strains. The time to death of larvae infected with IJs colonized with the single *hcp* mutants, *hcp1* and *hcp2* was comparable to that observed for the wild-type strain (Fig. 6). However, the virulence (expressed as percent mortality) of all tested XBJ mutant strains was not significantly different than that observed for the wild strain (Figs. 6 and 7).

In contrast, time to death of *G. mellonella* infected with *S. jolietii* IJs associated with XBJ *vgrG* mutants and wild type of strain were not significantly different (Fig. 7).

3.4.3. Nematode reproductive fitness

The percentage of cadavers yielding progeny was variable across the tested mutant strains (Fig. 8). A significant difference was observed with respect to the IJs colonized with *hcp* mutants. For example, a reduced percentage of productive infections was observed in cadavers infected with XBJ double *hcp* mutant strain (*hcp1/hcp2*) when compared to IJs colonized with *hcp1*, *hcp2*, and *hcp1comp* mutants and the wild-type strain (Fig. 8).

With respect to *vgrG* mutants, a significant reduction in productive infections was observed from cadavers infected with IJs colonized by *vgrG1*, *vgrG2*, *vgrG1/vgrG2*, *vgrG1/hcp2*, and *vgrG1/hcp1* when compared to the wild-type strain (Fig. 8).

Cadavers infected with mutant strains *hcp1/hcp2*, *vgrG1*, *vgrG2*, *vgrG1/vgrG2*, *vgrG1/hcp2*, *vgrG1/hcp1* exhibited significantly lesser yields of IJ progeny when compared to those insects infected with IJs colonized with wild type strain (Fig. 9). Contrastingly, the progeny of cadavers infected with IJs colonized with *hcp* single mutants, *hcp1* and *hcp2* were similar to wild type and *hcp1comp* strains.

4. Discussion

Several studies have shown that T6SS is involved in bacterial competition (Carruthers et al., 2013; Hood et al., 2010; Murdoch et al., 2011; Russell et al. 2014; Schwarz et al. 2010; Speare et al., 2018) and mediates intraspecific competition (Speare et al., 2018). For example,

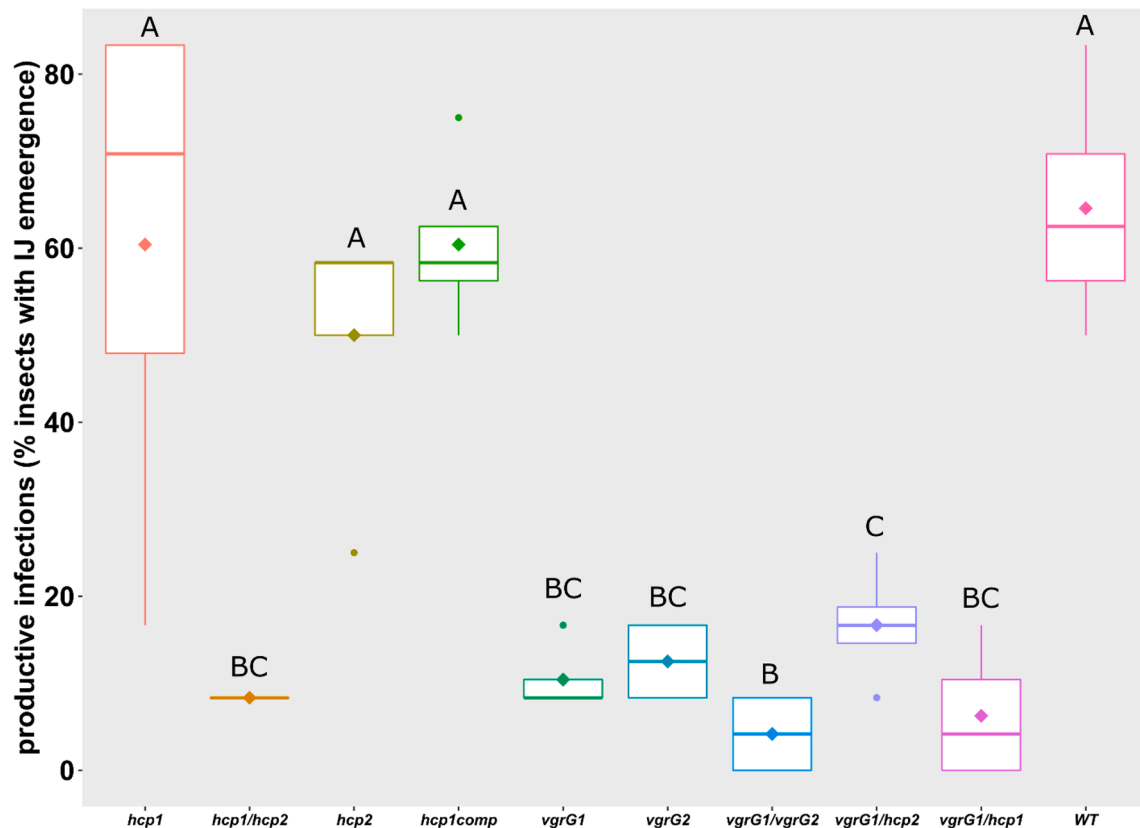


Fig. 8. XBJ requires an intact T6SS to support ij emergence from infected insect cadavers. The *G. mellonella* larvae infected with *S. jolietii*-XBJ T6SS mutants were white trapped individually and observed for ij progeny emergence. The productive infections represent the percent insect cadavers with at least 100 ij emergence to the total insect cadavers white trapped from each treatment. Lower and upper box boundaries represent 25th and 75th percentiles, respectively, line inside box indicate the median, lower and upper error lines represent 10th and 90th percentiles, respectively. Letters indicate significant difference among XBJ mutants. $n = 4$, negative binomial model: $p < 0.05$.

Hood et al. (2010) demonstrated that *Acinetobacter baumannii* strain M2 uses T6SS to outcompete *Escherichia coli*. Similarly, lethal strains of *Vibrio fischeri* use T6SS for intraspecific elimination of non-lethal strains of *V. fischeri* when they cooccur (Speare et al., 2018). Recently, we reported that in *X. bovienii*, *hcp1* (T6SS-1) but not *hcp2* (T6SS-2) is required to suppress the prey bacterial populations (Kochanowsky et al., 2020). In this study, we observed that *vgrG* genes (*vgrG1/vgrG2*) are required to outcompete the prey strain XBO suggesting that both genes are involved in intraspecific bacterial competition. These results provide further support to a previous study by McMullen et al (2017) who indicated that *X. bovienii* strains lacking T6SS were outcompeted by other strains that possess these genes. These findings can also be correlated with the promiscuous association *X. bovienii* has with over 10 different *Steinernema* nematodes (Lee and Stock, 2010). In this respect, we hypothesized that *X. bovienii* strains, through the utilization of T6SS, developed the ability to colonize different nematode species in their evolutionary trajectory.

The VgrG protein has been demonstrated as an integral component of T6SS facilitating the assembly of the T6SS machinery, puncturing the cell walls of target cells, and harboring the C-terminal domains acting as effectors and/or conducting effectors (Dong et al., 2013; Flaughnatti et al., 2016; Pukatzki et al., 2007). As noted in other bacteria, the two *vgrG* genes present within the two T6SS clusters in the genome of XBJ differ in their sequence length due to diverse C-terminal regions and associated domains (Hachani et al., 2011; Robinson et al., 2021) (Fig. 10). The pairwise sequence alignment of VgrG1 and VgrG2 revealed that they had only 16.5 % identity (Madeira et al., 2022). Similarly, NCBI-BLASTP-homology search identified that VgrG1 shares homology with VgrG protein of *Photorhabdus bodei* (NDL01399.1–62.84 % amino acid

identity), while VgrG2 shared homology with VgrG of *Erwiniaceae bacterium* PD-1 (QKJ86483.1–44.21 % amino acid identity). NCBI Conserved domain analysis predicted that both VgrG proteins harbor regions belonging to VI_Rhs_Vgr superfamily (cl37255, E-value: $0e + 00$) in their N-terminal ends. In addition, VgrG1 was predicted to contain autolysin/endolysin family (cd00737, E-value: $7.94e-49$) domain with three conserved catalytic residues while VgrG2 had a domain of five superfamily/Gp5_C (cl33691, E-value: $6.93e-03$) in their C-terminal regions. The bacteriophage endolysins have been characterized with antibacterial activity (Ding et al., 2020; Li et al., 2016) and the Gp5 domain is prevalent among bacteriophage T4 tail lysozyme protein GP5 and bacterial VgrG proteins, forming the membrane-puncturing needle-like structure (Arisaka et al., 2003; Pukatzki et al., 2007) (Fig. 10). Altogether the sequence analysis of VgrG1 and VgrG2 suggest that the two proteins may be functionally diverse and may act as effectors by themselves or may recruit the distantly located T6SS effector chaperons and contribute to antibacterial activity in XBJ (Pukatzki et al., 2007; Jurénas et al., 2021).

With respect to biofilm formation, a few studies have demonstrated that this is a crucial aptitude in the symbiotic phase of another entomopathogenic bacterium, *Photorhabdus* (An and Grewal, 2011; Somvanshi et al., 2010, 2012). For example, in *Photorhabdus temperata*, *purL* gene (purine synthesis gene) mutations lead to reduced biofilm formation and decreased persistence of the bacterium in its nematode host *Heterorhabditis bacteriophora* (An and Grewal, 2011). However, the functional role of biofilm in *Xenorhabdus* lifecycle is not yet fully understood. Earlier studies by Drace and Darby (2008) showed that in *Xenorhabdus nematophila*, 4-gene *hmsHFRS* mutants failed to form biofilm, but this did not affect the ability of the bacteria to colonize the

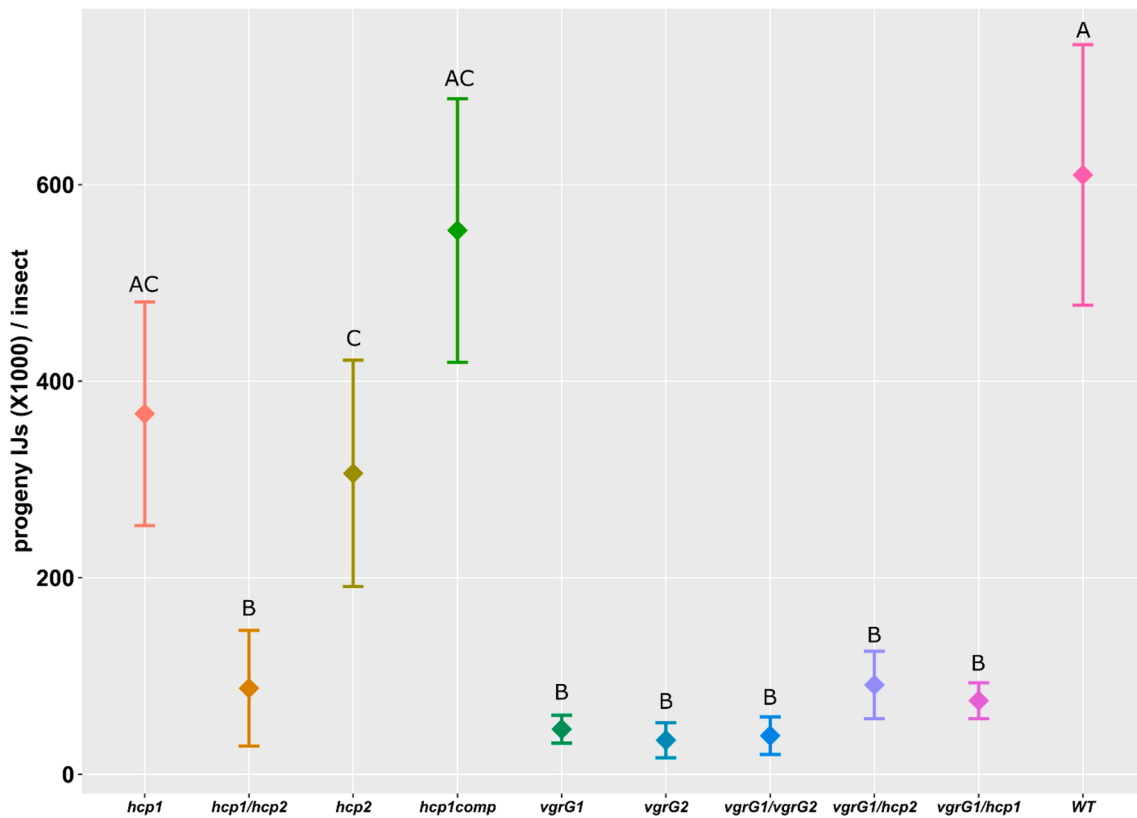


Fig. 9. XBJ requires an intact T6SS to support *S. jolietii* reproduction. The *G. mellonella* larvae infected with *S. jolietii*-XBJ T6SS mutants were white trapped individually and counted the emerged ij progeny. The data were represented as progeny count (X 1000) on Y-axis. Error bars indicate the standard errors. Letters indicate significant difference among XBJ mutants. (n = 36, ANOVA, p < 0.05).

nematode host or had an impact on the nematodes' reproductive fitness. Interestingly, the authors reported that the hmsHFRS gene locus is absent in the genome of *X. bovienii*. In another study, An and Grewal (2016) reported that in *X. koppenhoeferi*, several genes with predicted functions in biofilm formation were downregulated during the symbiotic phase of this bacterium, which indicated the lack of biofilm formation by *X. koppenhoeferi* when associated with its nematode host.

In this study, our results suggest that biofilm formation in XBJ is severely impacted by the absence of both *hcp* genes but not either *hcp* gene alone under *in vitro* conditions, suggesting T6SS regulates the biofilm formation in XBJ. Although biofilm formation in XBJ is yet to be fully characterized, we hypothesized that at least in XBJ, T6SS-mediated biofilm formation is important in the attachment of XBJ with its nematode host, like the partnership between *Vibrio fischeri* and *Euprymna scolopes*, in which the biofilm formation is required for symbiosis (Yip et al., 2005).

A robust biofilm formation in many bacteria has been shown to promote symbiotic colonization of their hosts (Cao et al., 2017; Yip et al., 2005). In this study, we demonstrated that all the *hcp* mutant strains except the double *hcp* mutant strain (*hcp1/hcp2*) were able to retain their ability to form biofilm. Concomitant with our hypothesis about the biofilm formation, our nematode colonization experiments revealed that the double *hcp* mutant strain did not colonize *Steinernema* nematodes. Together, these results indicate a redundant role for two T6SSs in the biofilm formation of XBJ. Additionally, at least regarding *hcp*, the attachment of XBJ cells to its nematode host is governed by the T6SS mediated biofilm formation.

In contrast, the double *vgrG* mutant, *vgrG1/hcp1*, and *vgrG2* mutants were not able to colonize the nematode host to levels comparable to those observed in the wild type strain although they retained the ability to form biofilm. From these observations, it appears that both *vgrG* genes are critical in promoting the colonization of nematode host by XBJ.

Although T6SS effectors promoting symbiotic association are not yet characterized, we speculate that the VgrG proteins may act as and/or recruit effector proteins that attenuate the host defense response promoting the host colonization (Tighilt et al., 2022). In support of this speculation, several T3SS effectors promoting the nodule formation and suppression of host defenses have been characterized in *Bradyrhizobium* and *Sinorhizobium* species targeting several legume plants (Berrabah et al., 2018; Jiménez-Guerrero et al., 2015; Ratu et al., 2021; Songwattana et al., 2021; Xin et al., 2012). Together, the results of biofilm and nematode colonization assays indicate that nematode colonization by XBJ may be governed by multiple mechanisms that needs further investigations.

Various components of T6SSs including *vgrG* have been implicated in pathogenic activity against eukaryotes (reviewed by Monjarás and Valvano, 2020). For example, *Vibrio cholera* mutants lacking *vgrG* were unable to infect amoebas or mammalian macrophages (Pukatzi et al., 2007). Similarly, *vgrG* was found to be responsible for the pathogenic activity of extra-intestinal pathogenic *E. coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Burkholderia pseudomallei*, *B. thailandensis*, *Aeromonas hydrophila* against their hosts (reviewed by Monjarás and Valvano, 2020).

In this study, we showed that, when injected alone, the virulence of all tested T6SS mutants against *G. mellonella* larvae was not affected. These findings are consistent with previous results reported by Kochanowsky et al. (2020). We also showed a longer time to death of cadavers infected with IJs paired with XBJ *hcp* double mutants when compared to that observed in the wild-type and the *hcp1* complemented strains. These observations suggest that biofilm through T6SS in XBJ may indirectly affect nematode colonization and/or multiplication in the insect host, thus compromising its virulence.

In this study, we also demonstrate that T6SS plays a role in the reproductive fitness of *Steinernema* nematodes. Our data revealed the

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