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Phenotypic variation and host interactions of *Xenorhabdus bovienii* SS-2004, the entomopathogenic symbiont of *Steinernema jolietii* nematodes

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Summary

Xenorhabdus bovienii (SS-2004) bacteria reside in the intestine of the infective-juvenile (IJ) stage of the entomopathogenic nematode, *Steinernema jolietii*. The recent sequencing of the *X. bovienii* genome facilitates its use as a model to understand host-symbiont interactions. To provide a biological foundation for such studies, we characterized *X. bovienii* in vitro and host-interaction phenotypes. Within the nematode host *X. bovienii* was contained within a membrane bound envelope that also enclosed the nematode-derived intravesicular structure. *S. jolietii* nematodes cultivated on mixed lawns of *X. bovienii* expressing green or DsRed fluorescent proteins were predominantly colonized by one or the other strain, suggesting the colonizing population is founded by a few cells. *X. bovienii* exhibits phenotypic variation between orange-pigmented primary form and cream-pigmented secondary form. Each form can colonize IJ nematodes when cultured *in vitro* on agar. However, IJs did not develop or emerge from *Galleria mellonella* insects infected with secondary form. Unlike primary-form infected insects that were soft and flexible, secondary-form infected insects retained a rigid exoskeleton structure. *X. bovienii* primary and secondary form isolates are virulent toward *Manduca sexta* and several other insects. However, primary form stocks present attenuated virulence, suggesting that *X. bovienii*, like *X. nematophila* may undergo virulence modulation.

Keywords

bacteria; microbe-higher organism interactions; symbionts; insect pathogen; nematode mutualist; virulence modulation; makes caterpillars floppy

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Introduction

Entomopathogenic nematodes in the genus *Steinernema* are mutualistically associated with Gamma-proteobacteria in the genus *Xenorhabdus*. Together, *Steinernema-Xenorhabdus* complexes infect, kill, and reproduce in the larval stage of a wide range of insect hosts, including those in the orders Lepidoptera, Diptera, and Coleoptera (Ehlers, 2001). The *Steinernema* nematode harbors *Xenorhabdus* bacteria in a modified portion of the intestine, and the bacteria are released by defecation when the nematode infects an insect and reaches the insect's body cavity (hemocoel) (Poinar, 1966; Martens et al., 2004; Snyder et al., 2007). After reproduction, nematode progeny become colonized by *Xenorhabdus* bacteria and emerge from the cadaver to seek a new host.

Since *Xenorhabdus* spp. are mutualists (of nematodes) and pathogens (of insects) they have become a model for studying both types of associations (Herbert and Goodrich-Blair, 2007; Richards and Goodrich-Blair, 2009) from cellular, molecular and evolutionary perspectives. Furthermore, unlike many current model systems to study host-microbe interactions, the mutualistic and pathogenic traits of *Xenorhabdus* bacteria are conserved among all members of the genus, facilitating comparative insights into the biology and evolution of these processes.

Twenty species of *Xenorhabdus* are currently recognized (Lee and Stock, 2010b; Tailliez et al., 2010) and the genomes of two, *X. nematophila* (ATCC 19061: genome accession #NC_014228, plasmid accession #NC_014170) and *X. bovienii* (SS-2004: accession #NC_013892), have been sequenced by our group. *X. nematophila* is known to colonize three nematode species from two clades, while *X. bovienii* strains appear to be more widely distributed, colonizing at least eight *Steinernema* spp. from three clades (Akhurst and Boemare, 1988; Fischer-Le Saux et al., 1998; Fischer-Le Saux et al., 1999; Spiridonov et al., 2004; Mráček et al., 2006; Emelianoff et al., 2008; Chapuis et al., 2009; Lee and Stock, 2010a). However, *X. bovienii* comprises multiple phylotypes (Fischer-Le Saux et al., 1998; Lee and Stock, 2010b) each of which may have a distinct host range. Indeed, variations have been observed in the ability of *X. bovienii* strains to colonize the nematode *S. feltiae* (Chapuis et al., 2009). The *Steinernema-Xenorhabdus* mutualistic association that has been most extensively studied at the molecular and cellular level is that between *X. nematophila* and *S. carpocapsae* (Goodrich-Blair, 2007). This study is focused on the relationship between *X. bovienii* and the nematode *Steinernema jolietii*.

Generally, both *Steinernema* nematodes and *Xenorhabdus* bacteria contribute to pathogenesis. The nematode produces a factor that can degrade inducible immune proteins (Götz et al., 1981) but most *Xenorhabdus* bacteria are pathogenic if injected alone (without the nematode host) directly into the hemocoel of a susceptible host (Akhurst and Boemare, 1990). *Xenorhabdus* spp. are able to suppress immunity and produce virulence factors, including toxins that contribute to rapid killing of the insect host (reviewed in (Herbert and Goodrich-Blair, 2007)). Nematode reproduction is promoted in the insect by the presence of the bacterial symbiont (Poinar and Thomas, 1966; Sicard et al., 2003; Mitani et al., 2004; Richards and Goodrich-Blair, 2010), with *X. nematophila* producing lipase and hemolysin activities necessary for nematode reproduction (Richards and Goodrich-Blair, 2010) and virulence in the insect (Cowles and Goodrich-Blair, 2005; Vigneux et al., 2007), respectively. In addition, *Xenorhabdus* promotes nematode development by protecting the insect cadaver from scavengers and other microbes (Zhou et al., 2002; Morales-Soto and Forst, 2011) through the production of a wide array of antimicrobials (Akhurst, 1982; Park et al., 2009; Fang et al., 2011; Fuchs et al., 2011) including bacteriocins that inhibit invasion of the cadaver by non-specific symbionts (Hawlena et al., 2010; Morales-Soto and Forst, 2011).

Steinernema and *Xenorhabdus* reproduce within the insect cadaver until nutrient depletion and high nematode population density triggers development of the nematode into a non-feeding stage known as the Infective Juvenile (IJ) (Popiel et al., 1989). The IJs become colonized by the bacterial symbionts in the anterior intestinal lumen, a region known as the receptacle (Bird and Akhurst, 1983; Snyder et al., 2007). In *S. carpocapsae*, the receptacle comprises the lumen between the two most anterior intestinal cells, which are morphologically distinct from other intestinal cells (Martens and Goodrich-Blair, 2005; Flores-Lara et al., 2007; Snyder et al., 2007). The *X. nematophila* population in the *S. carpocapsae* receptacle is founded by 1–2 individual cells that grow to fill the space (Martens et al., 2003), where they adhere to a cluster of spheres termed the intravesicular structure (IVS) that is associated with a glycan-containing mucous material (Martens and Goodrich-Blair, 2005). Once formed, IJ progeny emerge from the spent insect cadaver to search for new hosts to infect (San-Blas et al., 2008).

All *Xenorhabdus* species reported to date undergo phenotypic variation characterized by the switching between two cell types known as primary and secondary forms. Although the phenotypic differences between primary and secondary forms can vary depending on strain and species (Akhurst and Boemare, 1990), typically the primary, but not secondary form cells are motile, pigmented, agglutinate red blood cells and produce fimbriae, hemolysins, proteases, antimicrobials, and crystalline inclusion bodies (Boemare and Akhurst, 1988; Volgyi et al., 1998; Forst and Clarke, 2002; Smits et al., 2006). *Xenorhabdus* bacteria isolated from nematode hosts typically are in the primary form, but in laboratory culture conditions some cells convert to the secondary form. Although again it varies depending on strain and species, some secondary form isolates can revert to the primary form, while others appear to be stable (Campos-Herrera et al., 2009; Goodrich-Blair, unpublished data). Studies to date indicate the mechanism underlying phenotypic variation does not involve DNA rearrangements (Akhurst et al., 1992) changes in plasmid content (Leclerc and Boemare, 1991), or recombination-dependent mechanisms (Pinyon et al., 2000). In *X. nematophila* the transcription factor *Lrp* is a positive regulator of primary form traits; *lrp* mutants are phenotypically secondary form (Cowles et al., 2007), suggesting phenotypic variation may be an epigenetic phenomenon.

The sequenced strain of *X. bovienii* (SS-2004) (Chaston, Suen *et al.*, in press) was isolated from the nematode *S. jolietii* (Spiridonov et al., 1991), but to date there is no published literature describing this strain. A primary goal of our research is to identify molecular mechanisms underlying the *X. bovienii*-*S. jolietii* association using the genome sequence as a resource. We anticipate that comparing this system to the more extensively studied *X. nematophila*-*S. carpocapsae* association and to other *X. bovienii* host associations will provide insights into the common and diverse features of *Steinernema*-*Xenorhabdus* interactions with each other and with insect hosts. Toward these goals, in this study we investigated general characteristics of *X. bovienii* SS-2004 as well as its interactions with its *S. jolietii* nematode and insect hosts.

Results and Discussion

To characterize *X. bovienii* (SS-2004) host-interaction phenotypes, in 2007 we isolated individual bacterial colonies from the same *S. jolietii* nematode host lineage, and compared them to *X. bovienii* stocks isolated from *S. jolietii* in 2000, at the time of sequencing. Both stocks contained two colony morphologies, cream and orange, on LB agar (Fig. 1). 16S rRNA sequencing confirmed that all isolates were *X. bovienii* (data not shown). When isolated cream and orange colonies were re-streaked, each gave rise to a mixed population of orange and cream colonies. For both orange and cream colonies, the stability of the colony type increased with increasing number of re-streaks. From each *X. bovienii* SS-2004 stock

individual stable orange and cream colonies were isolated and a variety of phenotypes were analyzed (Table 2). Small colony variants were observed in the 2007, but not the 2001 orange isolate after growth on LB medium (Fig. 1). Both cell types had similar growth rates in LB broth, defined medium, and hemolymph, and exhibited motility, antibiotic activity against *Bacillus subtilis*, and hemolysis toward rabbit and horse red blood cells. Orange but not cream colony isolates bound bromothymol blue dye, lysed sheep red blood cells, agglutinated horse red blood cells, and produced antibiotic activities against *Escherichia coli*. Conversely, cream colony isolates but not orange demonstrated lipase activity against Tween 20 substrate. Based on these tests we concluded that orange colony isolates are the primary form while cream color isolates are the secondary form (Akhurst, 1980; Boemare and Akhurst, 1988). Hereafter, isolates will be referred to by the stock from which they were isolated (2000 or 2007) and their phenotypic form (orange, primary [1°]; cream, secondary [2°]). The majority of stationary phase LB-grown primary and secondary cells were rod shaped, but 1° form cultures contained a higher frequency than secondary of long rods (Fig. 1).

***X. bovienii* (SS-2004) colonization of *S. jolietii* nematodes**

X. bovienii (2000-1°) was visualized within the *S. jolietii* colonization site in both in situ and extruded receptacles (Fig. 2). The *S. jolietii* colonization site shares several features in common with that of *S. carpocapsae*. In both nematodes, the bacteria colonize the nematode's intestinal receptacle (Fig. 2A–D). The receptacle of these two nematode species also possesses a cluster of nematode-derived spheres, termed the intravesicular structure (IVS) (Fig 2F and data not shown). However, the *S. jolietii* receptacle has a non-cellular, cellophane-like envelope or membrane that is not apparent in *S. carpocapsae*. We refer to this novel structure as the 'vesicle', by definition "a small sac." This vesicle is resistant to mechanical disruption and contains *X. bovienii* bacterial cells as well as the IVS (Fig. 2 D–F). Vesicle formation does not depend on the presence of bacteria, since it is present in axenically cultured *S. jolietii* nematodes (Fig. 2F) as well as in *S. feltiae* nematodes that are phylogenetically closely related to *S. jolietii* (Lee and Stock, 2010a). However, both the receptacle and the vesicle can stretch to accommodate the bacterial load (data not shown). At present there is no information about the chemical composition, physical nature, or function of this vesicle. We speculate it may serve to restrict bacterial growth, to facilitate timed release of bacteria into insect prey, or to protect symbiont bacteria from stress within the receptacle or insect hemolymph.

X. bovienii SS-2004 primary and secondary form colonization of *S. jolietii* nematodes was monitored in IJ progeny from nematodes cultivated on bacterial lawns (*in vitro* co-cultivation). To assess colonization levels, we initially used a method developed for *X. nematophila*, in which the average number of bacteria per IJ is calculated by sonicating and dilution plating approximately $\sim 10^4$ surface sterilized IJs. However, we found that this method yielded a very low (~ 4 – 7) average CFU/IJ of *X. bovienii* from *S. jolietii* nematodes. Since numerous bacteria within individual *S. jolietii* IJs were visible by microscopy (Fig. 2) we considered the possibility that the larger size of *S. jolietii* IJs relative to *S. carpocapsae* (Spiridonov et al., 1991; Flores-Lara et al., 2007), or the presence of the thick-walled vesicle, might reduce the efficiency of bacterial release during sonication. Efficiency was improved by limiting the number of sonicated nematodes to 100. Using this revised method, we determined that primary and secondary *X. bovienii* SS-2004 isolates each colonized *S. jolietii* nematodes at similar levels to each other (2000-1°, 59.7 ± 28.2 ; 2000-2°, 55.3 ± 22.4 average CFU/IJ $\pm$ SEM, $P > 0.05$, $n = 3$), similar to the average *X. nematophila* CFU/ *S. carpocapsae* nematode IJ obtained using the sonication method (Martens et al., 2003). It has been reported that *X. bovienii* strains do not colonize its nematode hosts at as high a level as *X. nematophila*, as determined by crushing nematodes and plating the resulting suspension

(Emelianoff et al., 2008; Chapuis et al., 2009). Our data suggest that *X. bovienii* loads may be higher than estimated by this method.

To calculate the distribution of *X. bovienii* SS-2004 within *S. jolietii* populations (colonization efficiency), *X. bovienii* SS-2004 form variants were engineered to express the green fluorescent protein (GFP) (see Materials and Methods) (Fig. 3). The average CFU/IJ (determined by sonication and plating) achieved by the fluorescent strains (Fig. 3) was not significantly different from their parental strains ($P>0.05$). For each variant, approximately 200 (2 sets of 100) nematodes per experiment were randomly selected and the frequency of colonization (indicated by fluorescence within the colonization site) was determined. Primary and secondary form cells were not significantly different ($P=0.18$, Student's t-test) in their frequency of occurrence within nematodes: *X. bovienii* 2000-1°/GFP colonized $97.3 \pm 1.2\%$ of nematodes and 2000-2°/GFP cells were visible in $71.5 \pm 41.3\%$ of nematodes (Fig. 3) and did not switch forms within the IJ (data not shown). Within the vesicle, the bacteria are tightly packed together longitudinally (Fig. 3). In contrast, in *S. carpocapsae* nematodes, *X. nematophila* cells are more loosely packed within the receptacle and are not bound by a vesicle (Snyder et al., 2007).

Since the IJ is a non-feeding stage, the events necessary for intestinal colonization initiation must precede its development. Experimental evidence suggests that 1–2 *X. nematophila* bacterial cells initiate colonization in *S. carpocapsae* then grow within the receptacle after IJ formation (Martens et al., 2003; Martens et al., 2005). To begin to assess the colonization process in the *S. jolietii*-*X. bovienii* mutualism we determined the monoclonal and bi-clonal colonization frequency of IJs cultured on mixed lawns of 2000-1° *X. bovienii* expressing either GFP or DsRed fluorescent protein. As in *X. nematophila* (Martens et al., 2003), expression of DsRed significantly lowered the frequency of colonization ($P<0.05$, Student's t-test), with only $8 \pm 2.6\%$ and $1.5 \pm 0.9\%$ of nematodes being colonized by DsRed-expressing primary and secondary form cells (Fig. 3). Furthermore, DsRed expressing cells did not pack the receptacle as tightly as GFP-expressing cells, and appeared filamentous (Fig. 3). Despite the dramatically low frequency of colonization, the average DsRed expressing *X. bovienii* SS-2004 CFU/IJ was not significantly different from either *X. bovienii* SS-2004 without fluorescent protein, or expressing GFP. One possible explanation for this result is that in some nematodes the bacteria are no longer expressing DsRed and are not readily apparent by microscopy. In addition, the maximum CFU/IJ we obtain with our modified sonication method may be a gross under-estimate of the actual number of bacteria in nematodes colonized by wild type cells (see above), and is insufficient to detect differences in colonization level for *X. bovienii* SS-2004.

Although the DsRed-expressing strains are at a colonization disadvantage relative to GFP-expressing strains, we visually determined the frequency of nematodes colonized by GFP, DsRed, or both, after cultivation on a mixed (50:50) lawn of green and red primary form cells (Fig. 3F). Of the colonized nematodes derived from such lawns, $90.4 \pm 8.2\%$ fluoresced either all green (72.4%) or all red (18.0%), while nematodes visibly colonized by both types of bacteria represented only 9.6% of the population. Based on this frequency we calculated that, like *X. nematophila* (Martens et al., 2003), the final population of *X. bovienii* SS-2004 within an individual infective juvenile nematode is derived from 1–2 individual cells (see experimental procedures).

The colonization assays described above were conducted *in vitro* (i.e. on agar plates rather than within insects). Although colonization trends of *S. carpocapsae* IJs cultured *in vitro* on lawns of *X. nematophila* bacteria generally mirror those from *insecta* cultivations (Cowles and Goodrich-Blair, 2008), *S. carpocapsae* IJs that develop within insects have higher bacterial loads than those that develop on bacterial agar lawns (Goetsch et al., 2006; Flores-

Lara et al., 2007). To determine if *in vivo* cultivation within a host influences *X. bovienii* SS-2004 colonization of *S. jolietii* nematodes, we injected *Galleria mellonella* insects with *X. bovienii* SS-2004 bacteria and *S. jolietii* nematodes, and calculated average CFU/IJ for emerging progeny nematodes. *X. bovienii* 2000-primary colonized nematodes cultured in *G. mellonella* at 19.7 ± 10.9 CFU/IJ $\pm$ S.D. (n=2). Nematode IJs did not emerge from insects infected with secondary-phase cells, even after 4 weeks of incubation (data not shown). Dissection of insects at various time points after injection revealed that nematodes developed in both primary- and secondary-injected cadavers (Fig. 4A), but that IJ formation occurred only in the former (Fig. 4 C and E), not in the latter (Fig. 4 B and D) indicating that specifically in insects (not *in vitro* on lipid agar plates), secondary form cells lack an activity necessary for, or produce an activity inhibitory to development of infective juveniles.

In four independent experiments, primary cell-infected insects reproducibly had a soft and spongy texture 3–5 days post-infection, while the secondary-form infected insects maintained a rigid exoskeleton (Fig. 4F), even at 14 d post-infection, relative to a time of emergence of 8–10 days from insects infected with primary form cells. One possible explanation for this finding is that secondary form *X. bovienii* SS-2004 may lack one or more activities (e.g. secreted enzymes) necessary for degradation of the insect cadaver exoskeleton. If so, this activity appears to be expressed specifically in the presence of the nematode and only by primary form cells, since insect cadavers injected with primary or secondary bacteria alone (in the absence of nematodes) were rigid (Fig. 4G), even at high bacterial loads (Fig. 4H).

Candidates for a bacterial factor contributing to the flaccid cadaver phenotype are the "Makes caterpillars floppy" (Mcf) toxins, Mcf1 (PLU4142, NP_931332) and Mcf2 (PLU3128, AAR21118), discovered in the entomopathogenic nematode symbiont *Photorhabdus luminescens*. *E. coli* expressing *mcf1* is toxic when injected into *M. sexta* larvae, and the larval cadavers lose body turgor and become "floppy" (Daborn et al., 2002). Mcf2 has an N-terminal truncation relative to Mcf1, but is similar in its toxicity (Waterfield et al., 2003). *Pseudomonas fluorescens* encodes an *mcf1* homolog (*fitD*), deletion of which decreases toxicity, melanization, and "floppiness" in *Manduca sexta* and *G. mellonella* insects (Pechy-Tarr et al., 2008). The *X. bovienii* SS-2004 genome encodes one *mcf* homolog (XBJ1_2410, YP_003468304, 2533 aa) in contrast to *X. nematophila* which encodes two: *mcf1* (XNC1_2028, YP_003712268) and *mcf2* (XNC1_2265, YP_003712501). XBJ1_2410 and *P. luminescens* Mcf2 share 76% and 31% identity in the N- and C-terminal thirds of the proteins, respectively. However, the middle third of XBJ1_2410 is distinct from *P. luminescens* Mcf2 and contains a cysteine peptidase domain (aa945–1100) (pfam11713, E-value: $8.5e-34$) (Marchler-Bauer and Bryant, 2004; Marchler-Bauer et al., 2009; Marchler-Bauer et al., 2011). This domain is also present in *X. nematophila* *mcf2*. It will be of interest to determine if XBJ1_2410 expression phenotypically varies and if its expression or product activity is influenced by the presence of the nematode, as would be expected if Mcf2 is responsible for the flaccid phenotype observed in insects injected with primary-form *X. bovienii* and *S. jolietii* nematodes.

Our data are also consistent with the possibility that the nematode produces one or more activities necessary for insect bioconversion, but only in the presence of primary form bacteria. Indeed, *S. carpocapsae* expresses proteases specifically in the infective juvenile stage that have activity against insect tissues (Jing et al., 2010; Toubarro et al., 2010). Expression of these proteases may be triggered by primary form bacteria.

***X. bovienii* (SS-2004) virulence in *M. sexta* and other agricultural insect pests**

Virulence of *X. bovienii* (SS-2004) variants was measured as the percent survival of 4th instar *M. sexta* larvae after injection with approximately 100 cfu of bacteria (Fig. 5A). *X.*

bovienii 2000-2°, 2007-1°, and 2007-2° each killed ~59% of insects by 96 h post-injection. However, 2000-1° cells killed only 20% of insects by that time, and both the percent mortality and time to death of insects injected with this strain were significantly different from that caused by the other three strains ($p < 0.0001$). We considered the possibility that we had inadvertently chosen an attenuated variant from the 2000 stock and therefore examined the virulence of ten additional 2000-1° colonies. None were able to kill insects to the same levels as 2000-2°, 2007-1°, and 2007-2° (Fig. 5B). These data indicate that *X. bovienii* primary form cells can become attenuated for virulence, and that the majority of, if not all the primary form cells in the *X. bovienii* 2000 stock, but not the 2007 stock, have done so. Recently, it was reported that variants of *X. nematophila* arise within a population that are heritably and reversibly attenuated for virulence and immune suppression (Park et al., 2007). These virulence modulated (vmo) strains arise in laboratory stocks of *X. nematophila* but the mechanism underlying the modulation and the conditions that trigger modulation are unknown (Park et al., 2007). It is possible that the 2000 stock of *X. bovienii* was exposed to conditions (e.g. prolonged freezer storage) that promoted virulence modulation. Alternatively, some other phenomenon, such as a genetic mutation that altered virulence, may have occurred. The latter possibility is less likely since the genetic mutation would have had to selectively impact only the primary form cells in the population (secondary form colonies are virulent). We therefore favor the idea that laboratory-derived conditions promoted virulence modulation of the *X. bovienii* 2000 stock, and the intriguing corollary that primary form cells are more susceptible than secondary form cells to this modulation.

We also tested the oral toxicity of primary forms of both *X. bovienii* and *X. nematophila* against a number of known insect pathogens of plants (Table 3). *X. bovienii* exhibited a broader host range of secreted toxicity, with concentrated supernatants (>10 kDa) causing mortality in the Western Tarnished Plant Bug, Corn Ear Worm, and Western Corn Rootworm. In contrast, *X. nematophila* supernatants caused mortality only in Corn Ear Worm, and at lower levels (11% mortality) than that caused by *X. bovienii* (87%). In light of these results it is curious that the *X. bovienii* genome encodes a narrower repertoire of insecticidal Tc toxins than does the *X. nematophila* genome. Tc toxins are large molecular weight complexes of A, B, and C subunits (Ffrench-Constant and Waterfield, 2006). *X. bovienii* encodes 3 A subunits, 2 B subunits, and 2 C subunits, while *X. nematophila* encodes 7, 3, and 3 of each type respectively (see supplemental Text S5 in (Chaston et al.)). The apparently broader host range of *X. bovienii* may be due to broader target specificity of its TC toxins (Sergeant et al., 2003). Alternatively, *X. bovienii* may encode one or more additional toxins that expand its target host range.

Conclusions

Although *X. bovienii* (SS-2004) and *X. nematophila* (ATCC 19061) colonize nematodes from different clades, we found many aspects of their physiology and host interactions to be similar. Both form mono- or bi-clonal populations within the nematode, undergo phenotypic variation of multiple activities, and exhibit heritable virulence variability. Also, the secondary forms of both strains express more lipase and lecithinase activities than their primary counterparts, are virulent in insects and colonize nematodes. Characteristics that distinguish *X. bovienii* from *X. nematophila* include that *X. bovienii* primary form colonies and cultures are pigmented orange, while those of *X. nematophila* are cream. Also, *X. bovienii* exhibits antimicrobial activities against *E. coli* and *B. subtilis*, and only the former is subject to phenotypic variation. In contrast only *X. nematophila* activity against *B. subtilis* is subject to phenotypic variation. The *X. nematophila* *xcn* locus is necessary for the production of the antibiotic xenocoumacin, which has activity against *Micrococcus luteus* (Park et al., 2009). The genome of *X. bovienii* lacks *xcn* gene homologs, indicating *X. bovienii* produces antibiotics that are distinct from those of *X. nematophila*. A further

distinction between *X. bovienii* and *X. nematophila* is that the secondary form of the former, but not the latter, has a defect in supporting infective juvenile development within insect cadavers, and also fails to cause cadaver flaccidity. It remains to be determined whether or not these two defects are related, and what the molecular bases are for these phenotypes. Finally, a striking difference exists in the colonization site occupied by these two species of bacteria: when colonizing the nematode *X. bovienii* cells are bound within an envelope structure (vesicle) while *X. nematophila* are freely distributed in the receptacle. The fact that the host nematode tissues with which *X. bovienii* and *X. nematophila* cells interact suggests that the molecular requirements necessary to persist in this environment may differ between the two symbionts.

Experimental Procedures

Organisms and growth conditions

Strains used are listed in Table 1. The *X. bovienii* strain used in this study was deposited on Jun. 28, 2000 with the Agriculture Research Culture Collection (NRRL) International Depository Authority at 1815 North University Street, in Peoria, Ill. 61604 U.S.A., according to the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedures and was designated as NRRL-30311. Permanent stocks of all cultures were stored in Luria-Bertani (LB) broth (Miller, 1972) supplemented with 20% glycerol at -80°C . Unless otherwise stated, cultures were grown at 30°C , in LB broth that had not been exposed to light or on LB agar that was supplemented with 0.1% pyruvate (Xu and Hurlbert, 1990). When appropriate, media were supplemented with antibiotics, including ampicillin (150 $\mu\text{g/ml}$), kanamycin (50 $\mu\text{g/ml}$), chloramphenicol (30 $\mu\text{g/ml}$), erythromycin (200 $\mu\text{g/ml}$), or tetracycline (15 $\mu\text{g/ml}$) were added as indicated. *X. bovienii* (SS-2004), like other *Xenorhabdus* spp. is catalase negative and is resistant to ampicillin, but is sensitive to the other antibiotics listed above. However, the genome of *X. bovienii* does encode a homolog of chloramphenicol transacetylase that confers resistance to 30 $\mu\text{g/ml}$ chloramphenicol when present in high copy (data not shown). Lipid agar (LA), used to co-culture all strains of nematodes on respective bacterial lawns was prepared as previously described (Vivas and Goodrich-Blair, 2001). All nematode strains were propagated as previously described (Martens et al., 2003) through last instar *Galleria mellonella* larvae. *In vitro*, aposymbiotic cultivation of nematodes to produce uncolonized IJs of both *S. jolietii* and *S. carpocapsae*, was performed using liver-kidney agar as previously described (Sicard et al., 2003; Martens and Goodrich-Blair, 2005). *X. bovienii* growth rates in insect hemolymph were performed by harvesting *Manduca sexta* hemolymph as previously described (Orchard and Goodrich-Blair, 2004), performing a 1:100 subculture of rinsed *X. bovienii* cells into insect hemolymph and growing the cells at 30°C with shaking. The optical density (OD_{600}) of the hemolymph culture was recorded every hour for 24 hours using a Beckman Coulter DTX880 Multimode Detector. Similarly, *X. bovienii* growth rates in defined medium broth were performed by subculturing 1:100 rinsed *X. bovienii* cells into defined medium broth made as previously described (Orchard and Goodrich-Blair, 2004) except that amino acids and agar were omitted and 0.68 g $\text{MgCl}_2(\text{H}_2\text{O})_6 \text{ L}^{-1}$ and 0.91% glucose were added after autoclaving and growing the cells at 30°C with shaking. The optical density (OD_{600}) of the defined medium broth culture was recorded every hour for 24 hours.

Phenotypic assays

X. bovienii colony color and morphology were observed after growth for 24–36 h at 30°C in or on LB medium. Haemolysin activity (Rowe and Welch, 1994) against mammalian erythrocytes (Colorado Serum Company, Denver, CO), Tween 20 lipolytic activity (Sierra, 1957), lecithinase activity (Boemare, 1997; Thaler et al., 1998), proteolytic activity

(Boemare et al., 1997), haemagglutination (Moureaux et al., 1995), motility (Vivas and Goodrich-Blair, 2001), antibiotic activity (Akhurst, 1982; Herbert et al., 2007) and dye binding assays (Boemare and Akhurst, 1988) were conducted as described in the literature.

Determination of 16S rDNA sequences

Crude DNA extracts from fresh overnight bacterial cultures were used as templates in ExTaq (Takara, Otsu, Shiga, Japan) polymerase chain reactions (PCR) with the universal primers 27F: 5'-AGAGTTTGATCATGGCTCAG-3', and 1492R: 5'-TACGGTTACCTTGTACGACTT-3' (Lane, 1991). Amplified products were then sequenced at UW-Madison Biotechnology Center using Big Dye version 3.1 (Applied Biosystems, Foster City, CA) with the primers above in addition to Xn850R: 5'-CATTTGAGTTTAAACCTTGCG-3'. Sequence similarity to 16S rRNA sequences in the national database were determined using BLAST (Altschul et al., 1997).

Construction and visualization of fluorescent-protein expressing *X. bovienii* strains

To generate *X. bovienii* strains expressing GFP or DsRed, pURR25 (mini-Tn7-KSGFP) or pBK-mini-Tn7(Gm)PA1/04/03-DsRed respectively were conjugated from BW29427, with the helper strain BW29427 (pUX-BF13) (Bao et al., 1991) according to previously described methods (Vivas and Goodrich-Blair, 2001), except the donor and helper strains were eliminated by selection of colonies on media without diaminopimelic acid (80 µg/ml) and *X. bovienii* exconjugants were selected on pyruvate (0.1%), ampicillin (150 µg/ml), and kanamycin (50 µg/ml). The resulting colonies were confirmed to be *X. bovienii* based on 16S rRNA sequencing.

Microscopy

Phase-contrast, differential interference contrast, and fluorescence microscopy were performed using a Nikon Eclipse TE300 inverted microscope. Fluorescence microscopy was performed using fluorescein isothiocyanate, tetramethyl rhodamine isocyanate (FITC), tetramethyl rhodamine isothiocyanate (TRITC) and triple-band DAPI (4,6-diamidino-2-phenylindole)-FITC-TRITC filter sets (Chroma, Brattleboro, Vt.; items 31001, 31002, and 82000, respectively). Images were recorded using an ORCA digital camera (model C4742-95-10R; Hamamatsu, Hamamatsu City, Japan) and Metamorph version 4.5r6 software (Universal Imaging Corporation, West Chester, PA). Additional images were obtained using a Zeiss LSM 510 Axioplan II confocal microscope (Zeiss, New York, NY). For cell morphology analysis, bacterial cells were immobilized on 3% agarose pads and viewed using DIC microscopy at 100× magnification. When visualizing *X. bovienii* (SS-2004) within whole nematodes, nematodes were paralyzed and immobilized with 1% levamisole in agarose as previously described (Martens et al., 2003) using confocal microscopy between 400× and 1000×. For microscopy of progeny nematodes, nematodes were immobilized as mentioned above and viewed using phase contrast microscopy under 40× and 400× magnification. To visualize the receptacle and vesicle (Fig. 2), nematode intestines were extruded by cutting the nematode head with a razor blade, then were maintained in M9 buffer and stained with 0.001% Neutral Red in M9 buffer. The photograph shown in Fig. 2 was taken with Olympus BX51 equipped with digital camera. Extended focal length images were taken and stacked by using Microsuite image analyzing software.

Photography and *in vivo* imaging

Additional photography for colony morphology and insect cadavers was done using a Canon Powershot camera. Scale bars were inserted using Metamorph version 4.5r6 software (Universal Imaging Corporation, West Chester, PA). Seven days after injection, 1 to 10

insects per experiment were visualized by using an IVIS Imaging System 200 (Xenogen Corp., Alameda, CA). Fluorescence was quantified by using Living Image software v2.6 (Xenogen).

Host interaction assays

Axenic *S. jolietii* eggs were isolated from adult female nematodes as previously described for *S. carpocapsae* (Cowles and Goodrich-Blair, 2004) and were stored in 5 ml LB broth supplemented with ampicillin, kanamycin, and chloramphenicol. Axenic eggs were collected and rinsed with sterile LB broth prior to inoculation onto LA plates (in vitro cultivation) or injection into *G. mellonella* (in vivo cultivation). *X. bovienii* co-cultivation with *S. jolietii* nematodes on lipid agar plates was as described for *X. nematophila* and *S. carpocapsae* co-cultivations (Martens et al., 2005). For in vivo colonization assays ~700 µl of fresh overnight bacterial cultures was mixed with ~500–1500 axenic nematode eggs and/or J1 juveniles and 12.5 µl of the mixture was injected into the haemocoel of *Galleria mellonella* larvae (Vanderhorst Wholesale, NJ) with a 30-gauge syringe (Hamilton, Reno, NV). Approximately 15 *G. mellonella* larvae were injected per bacterial treatment and once injected, the insects were placed in modified White traps as previously described (Kaya and Stock, 1997).

Collected nematodes were assessed by sonication for *X. bovienii* colonization as previously described for *X. nematophila* (Heungens et al., 2002) except that *S. jolietii* were surface sterilized for 3 min. with 0.5% NaOCl. Approximately 10^2 surface sterilized nematodes were sonicated (Branson Ultrasonics, Danbury, CT) for 60–90 seconds each and the sonicate solution was diluted and plated to enumerate colonies. The number of individual cells that are the founders of the final population of *X. bovienii* bacteria within a single *S. jolietii* IJ was calculated from the percentage of nematodes carrying either green or red fluorescence (90.4%) using the mathematical model described by Wollenberg and Ruby (Wollenberg and Ruby, 2009): $p(\text{red})^x + p(\text{green})^x = p(\text{single})$. In this formula, x is the initiating cell number, $p(\text{single})$ is the probability of observing a nematode with only red or green fluorescence, and $p(\text{red})$ and $p(\text{green})$ are the initial proportions of each bacterium in the input lawn (0.5 each). Based on our observations that 90.4% of *S. jolietii* nematodes were colonized by either red or green fluorescent bacteria (but not both): $0.5^x + 0.5^x = 0.94^x$ and $x = 1.1$.

X. bovienii injection virulence assays in *Manduca sexta* insect larvae were performed as previously described (Herbert et al., 2007). For feeding toxicity screens against multiple insects, *X. bovienii* and *X. nematophila* (ATCC19061) were streaked onto LB agar plates from frozen glycerol stocks and incubated for 16 h at 25°C. Individual colonies were used to inoculate 4 × 2 ml TB media each and were grown on a roller drum incubator for 45 h at 25°C. After pelleting the bacteria, supernatants from each strain were combined (8 ml total) and concentrated in an Amicon 10 MWCO to 1.2 ml (6×, protein fraction). The flow-through was also collected. Both fractions were tested in a feeding assay for toxicity against 20–24 insects. The flow-through did not cause mortality in any insect. Artificial diet feeding assays were conducted as described by (Baum et al., 2007) except that assays were run for 5 days. Feeding assays were conducted with neonate larvae (<24 hr post hatch) of the coleopteran species *Diabrotica virgifera virgifera* (Western corn rootworm, WCR) and *Diabrotica undecimpunctata howardii* (Southern corn rootworm, SCR) (both obtained from Crop Characteristics, Inc.; Farmington, MN) using corn rootworm artificial diet and the lepidopteran species: *Agrotis ipsilon* [Hufnagel] (black cutworm, BCW) and *Helicoverpa zea* [Boddie] (corn earworm, CEW) (both obtained from Benzon, Inc.; Carlisle, PA) using Multiple Species diet (Southland, Lake Village, AK). Feeding assays with the hemipteran species *Lygus hesperus* were based on a 96 well micro-titer plate format using a sachet system as previously described (Habibi et al., 2002). The *Lygus hesperus* (Western tarnished plant bug, WT) artificial diet was obtained from Bio-Serv® (Bio-Serv® Diet F9644B,

Frenchtown, NJ) and prepared as previously described (Habibi et al., 2001). Samples were prepared by mixing one hundred microliters of the test sample with one hundred microliters of blended diet (1:1). A sheet of Parafilm® (Pechiney Plastic Packing, Chicago, IL) was placed over a vacuum manifold designed for 96-well format (Analytical Research Systems, Gainesville, FL) and a vacuum of approximately -20 millimeters mercury was applied, sufficient to cause extrusion of the Parafilm® into the wells. Twenty or forty microliters of test sample were added to the Parafilm® wells. A sheet of Mylar film (Clear Lam Packaging, Inc., Elk Grove Village, IL) was placed over the Parafilm® and sealed gently with a tacking iron (Bienfang Sealector II, Hunt Corporation, Philadelphia, PA). The Parafilm® sachets were placed over a flat-bottom 96-well plate containing *Lygus* eggs suspended in a 0.19% agar solution. Upon hatching, *Lygus* nymphs were allowed to feed on the sachet diet for 5 days. Stunting and mortality scores were determined on day 5 and compared to the untreated control. Data were analyzed using JMP4 statistical software.

Statistical analysis

Colonization assays were analyzed using a paired, two-tailed Student's t-test assuming unequal variance. Survival curves of *M. sexta* injected with different treatments were analyzed using a proportional hazards model and the final survival values were analyzed with a logistic regression model.

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Figure 1.

Colony and cell morphology of *X. bovienii* primary and secondary variants. When streaked on LB agar, *X. bovienii* primary form colonies produce an orange pigment, causing both colonies and the surrounding agar to appear orange, while secondary form colonies are cream colored (size bars: 2 mm). Cultures were grown overnight in LB broth, and DIC microscopy revealed rod shaped cells, with primary form variant cultures containing a higher frequency of elongated cells (insets) (size bars: 10 μ M).

Figure 2.

A. *S. carpocapsae* (A–B) and *S. jolietii* (C–F) intestinal receptacle. A *in situ* colonized *S. carpocapsae* receptacle; B. Extruded colonized *S. carpocapsae* receptacle; C. *in situ* colonized *S. jolietii* receptacle; D. Extruded receptacle of *S. jolietii* showing colonized vesicle with thick membrane; E. Extruded colonized vesicle of *S. jolietii*; F. Uncolonized vesicle with thick membrane and IVS, extruded from an *S. jolietii* IJ cultured on liver-kidney agar in the absence of bacteria. .References: Bb; basal bulb, EIJ: esophago-intestinal junction, R: receptacle, V: vesicle, Xn: *X. nematophila* bacteria, Xb: *X. bovienii* bacteria, IVS: intra vesicular structure. Scale bar for all images as in A: A–C= 20 μ m; D–E= 7 μ m; F= 15 μ m.

Figure 3.

X. bovienii colonization frequency. *X. bovienii* primary (1⁰) or secondary (2⁰) cells expressing green fluorescent protein (GFP) or DsRed protein (DsRed) were visualized after (A) growth in liquid culture or (B and C) within nematode intestines using an epifluorescence or confocal microscope respectively. In (B) confocal images overlay DIC images. Size bars for A, B, and C represent 5 microns. (D) Nematodes were cultivated on lawns of each bacterial strain, and the average CFU per infective juvenile (CFU/IJ) was calculated by sonication and plating and (E) the percentage of colonized nematodes (% colonized nematodes) were determined by microscopy. The experiment was conducted three independent times. Standard error of the mean and range (in parentheses) are provided. (F)

Mixed lawns of *X. bovienii* expressing GFP or DsRed were cultivated with nematodes. Nematodes colonized by GFP-expressing cells, DsRed-expressing cells, or both were apparent within the population. Shown are confocal images of each type, with the size bar representing 5 microns.

Figure 4.

Nematode-infected *G. mellonella* with primary form, but not secondary form *X. bovienii* produce infective juveniles and are flaccid and liquefied.

At 7 d post-infection with nematodes and secondary form *X. bovienii* (HGB1054) *G. mellonella* insect cadavers (white arrow) are filled with non-emergent juvenile nematodes that can be seen in the water surrounding the cadaver upon dissection (an individual nematode among many is indicated by a white arrowhead) (A). However, phase contrast microscopy revealed that none of these is in the IJ stage (B and D) as indicated by body morphology and the presence of an open mouth (arrow). In contrast, juveniles from primary-form infected cadavers (C and E) are infective juveniles with closed mouths (arrow). Scale

bars in B and C are 200 microns, and in D and E, 100 microns. *G. mellonella* insects 3 d post-infection with nematodes and bacteria (F) are flaccid if the injected bacteria are primary, but rigid if the injected bacteria are secondary. The flaccid insect cadaver phenotype associated with primary-form infection requires the presence of nematodes, since *G. mellonella* insects injected with either primary or secondary form bacteria, but without nematodes, are rigid (G). Both primary and secondary form bacteria are able to reproduce within the cadaver, as indicated by in vivo imaging system visualization of fluorescence in cadavers 7 days post-injection with secondary (HGB1245) or primary (HGB1263) *X. bovienii* strains expressing the green fluorescent protein (H). Images were taken using GFP-appropriate excitation and emission filters. Color corresponds to varying levels of emitted fluorescence, with a color spectrum of corresponding fluorescence levels shown on the right. Control insects without bacteria expressing green fluorescent protein showed no detectable fluorescence at these settings (not shown).

Figure 5.

X. bovienii virulence in *M. sexta* larvae.

Approximately 100 CFU of bacteria in late logarithmic phase growth ($OD_{600} \approx 0.8$) were injected into each 4th instar *Manduca sexta* larvae (10 insects per treatment) and the percent of insects surviving each treatment (% survival) was monitored for approximately 13 days post-injection (dpi). A) Insects were injected with primary (squares) or secondary (circles) *X. bovienii* isolates from 2000 (open symbols) or 2007 (closed symbols). B) Individual primary form colonies were isolated from the parent stock and each was injected into insects as above (open squares). Closed squares show the survival curve of insects injected with *X.*

bovieni 2007-1° (same as in A). Each experiment was conducted at least 3 independent times.

Table 1

Strains and plasmids used in this study

Lab Strain Number	Description/Use	Reference/Source
<i>Xenorhabdus</i> strains		
HGB800	<i>X. nematophila</i> ATCC 19061, wild-type	American Type Culture Collection (ATCC)
HGB1053	<i>X. bovienii</i> SS-2004 Jollieti 2000 stock	B. Goldman (Monsanto)
HGB1054	<i>X. bovienii</i> SS-2004 Jollieti 2000-2°	This study
HGB1055	<i>X. bovienii</i> SS-2004 Jollieti 2000-1°	This study
HGB1269	<i>X. bovienii</i> Jollieti 2007 stock	This study
HGB1267	<i>X. bovienii</i> Jollieti 2007-2°	This study
HGB1268	<i>X. bovienii</i> Jollieti 2007-1°	This study
HGB1245	HGB1054-GFP (2000-2°;GFP)	This study
HGB1263	HGB1055-GFP (2000-1°;GFP)	This study
HGB1281	HGB1054-DsRed (2000-2°;DsRed)	This study
HGB1285	HGB1055-DsRed (2000-1°;DsRed)	This study
HGB1282	HGB1267-GFP (2007-2°;GFP)	This study
HGB1283	HGB1268-GFP (2007-1°;GFP)	This study
HGB1286	HGB1267-DsRed (2007-2°;DsRed)	This study
HGB1287	HGB1268-DsRed (2007-1°;DsRed)	This study
Other bacterial strains		
HGB1006	<i>E. coli</i> K12 test strain for antibiotic	J. Imlay
HGB005	<i>E. coli</i> DH5a test strain for antibiotic	Bethesda Research Laboratory
HGB006	<i>E. coli</i> S17-1 λ pir test strain for antibiotic	(Simon et al., 1983)
BW29427	<i>E. coli</i> (<i>thrB1004 pro thi rpsL hsdS lacZAM15 RP4-1360</i> Δ (<i>araBAD</i>)567 <i>AdapA1341::[erm pir (wt)]</i>); Donor for conjugations: Strep ^R	K.A. Datsenko and B.L. Wanner
HGB1262	BW29427+pURR25; Strep ^R , Kan ^R	D. Lies and D. Newman
HGB1266	BW29427+pBK-mini-Tn7(Gm)PA1/04/03-DsRed; Strep ^R , Gm ^R	This study
HGB1284	BW29427+ pUX-BF13; Strep ^R , Amp ^R	D. Lies and D. Newman
HGB009	<i>Bacillus subtilis</i> AD623 test strain for antibiotic	A. Driks
Plasmids		
pURR25	MTn7 PA1/03/04gfpmut3*Mini-Tn7-KSGFP	D. Lies and D. Newman
pUX-BF13	Tn7 transposase, Amp ^R	(Bao et al., 1991)
pBK-mini-Tn7(Gm)PA1/04/03-DsRed	Mini-Tn7-DsRed	L. Lambertsen

Phenotypes of *X. bovienii* SS-2004

Table 2

Strain	Colony Color ^a	Dye binding (NBTA)	Growth Rate (h/generation) ^b				Motility ^c	Lipase ^d	Lecithinase ^e	Protease ^f	Hemolysin ^f		Hem-agglutination ^g	Antibiotic Production ^h	
			Luria Broth	Minimal	Hemolymph						Sheep/Rabbit/Horse	Sheep/Rabbit/Horse		Sheep/Rabbit/Horse	<i>E. coli</i>
2000-1°	Orange	+	1.27 +/- 0.09	5.7 +/- 1.8	4.9 +/- 0.6	+	-	-	-	+	+/+/+	+/+/+	-/-/+	+	+
2000-2°	Cream	-	1.27 +/- 0.07	5.2 +/- 1.1	4.4 +/- 1.2	+	+	+	+	+/-	-/+/+	-/+/+	-/-/-	-	+
2007-1°	Orange	+	1.16 +/- 0.10	6.4 +/- 2.5	4.1 +/- 1.1	+	-	-	-	+	NT	NT	NT	+	+
2007-2°	Cream	-	1.24 +/- 0.10	5.9 +/- 0.3	4.2 +/- 1.1	+	+	+	+	+/-	NT	NT	NT	-	+

^a Colony color was observed on LB plates after 24 h incubation at 30°C.

^b Growth rate in LB, minimal medium, and hemolymph presented as the slope of log10-transformed OD values from exponential part of growth curve.

^c Size of colony (mm), 24 h after inoculation on swim plates (0.25% agar).

^d Presence (+) or absence (-) of halo surrounding the bacterial colony, 3 days after inoculation on Tween 20-containing plates

^e Presence (+) or absence (-) of halo surrounding the bacterial colony, 4 days after inoculation on egg yolk-containing plates

^f Subjective evaluation of halo surrounding the bacterial colony, 3 days after inoculation on milk (protease) or blood (hemolysin) containing plates. NT; Not tested

^g Ability to keep red blood cells in suspension (+) or not (-). NT; Not tested

^h Presence (+) or absence (-) of halo surrounding the bacterial colony, 3 days after inoculation of the indicated tester bacterium.

Table 3

Toxicity of *X. bovienii* concentrated protein samples against selected insect pests of plants.

Insect	Percent Mortality ^a	
	<i>X. bovienii</i>	<i>X. nematophila</i>
Black Cutworm	0	0
Corn Earworm	87 ± 12	11 ± 9.6
Southern Corn Rootworm	0	0
Western Corn Rootworm	45.8 ± 12.3	0
Western Tarnished Plant Bug	100	0

^aPercent mortality ± standard deviation across three microtiter dish columns of each treatment