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Symbiont-mediated competition: *Xenorhabdus bovienii* confer an advantage to their nematode host *Steinernema affine* by killing competitor *Steinernema feltiae*

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Summary

Bacterial symbionts can affect several biotic interactions of their hosts, including their competition with other species. Nematodes in the genus *Steinernema* utilize *Xenorhabdus* bacterial symbionts for insect host killing and nutritional bioconversion. Here, we establish that the *Xenorhabdus bovienii* bacterial symbiont (Xb-Sa-78) of *Steinernema affine* nematodes can impact competition between *S. affine* and *S. feltiae* by a novel mechanism, directly attacking its nematode competitor. Through co-injection and natural infection assays we demonstrate the causal role of Xb-Sa-78 in the superiority of *S. affine* over *S. feltiae* nematodes during competition. Survival assays revealed that Xb-Sa-78 bacteria kill reproductive life stages of *S. feltiae.* Microscopy and timed infection assays indicate that Xb-Sa-78 bacteria colonize *S. feltiae* nematode intestines, which alters morphology of the intestine. These data suggest that Xb-Sa-78 may be an intestinal pathogen of the non-native *S. feltiae* nematode, although it is a nonharmful colonizer of the native nematode host, *S. affine.* Screening additional *X. bovienii* isolates revealed that intestinal infection and killing of *S. feltiae* is conserved among isolates from nematodes closely related to *S. affine*, although the underlying killing mechanisms may vary. Together, these data demonstrate that bacterial symbionts can modulate competition between their hosts, and reinforce specificity in mutualistic interactions.

Introduction

The defensive role of symbionts in the context of host disease is becoming increasingly recognized. For instance, microbial symbionts within hosts can interfere with invading parasites (Dillon *et al*., 2005; Koch and Schmid-Hempel, 2011). Symbionts can preempt infection by forming a protective physical barrier, drawing down available host resources (Donskey *et al*., 2000; de Roode *et al*., 2005; Caragata *et al*., 2013), modulating the host's immune system (Lysenko *et al*., 2010; Hooper *et al*., 2012; Abt and Artis, 2013) or directly attacking invaders (Jaenike *et al*., 2010; Hamilton *et al*., 2014). Here, we focus on a related but distinct form of symbiosis that likely overlaps in mechanism: the beneficial effects of a bacterial symbiont on the host's competitive ability.

We assessed symbiont-mediated competition using insect-parasitic nematodes in the genus *Steinernema.* These nematodes are well known for their use in biological control (Ehlers, 2001) and for their mutually beneficial symbiosis with Gammaproteobacteria in the genus *Xenorhabdus*, which enable their success as insect parasites (Supporting Information Fig. S1) (Herbert and Goodrich-Blair, 2007). In the soil environment, the nematodes exist as infective juveniles (IJs) that carry the bacterial symbionts between insects (Poinar and Thomas, 1966; Martens *et al*., 2003; Herbert and Goodrich-Blair, 2007). IJs locate and infect an insect and within the insect body cavity undergo a recovery process to release their bacterial symbiont and enter the reproductive life stages (Sicard *et al*., 2004a; Snyder *et al*., 2007; Baiocchi *et al*., 2017). The nematodes and bacteria then kill the insect host and grow within the cadaver [reviewed in (Herbert and Goodrich-Blair, 2007; Richards and Goodrich-Blair, 2010)]. After 2–3 rounds of reproduction,

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the progeny juvenile nematodes develop into IJs that will exit the insect cadaver to seek new hosts (Popiel *et al*., 1989; Richards and Goodrich-Blair, 2009). As multiple nematode species can infect a single host individual, a key venue for parasite competition is within the host (Peters, 1996; Spiridonov *et al*., 2007; Richards and Goodrich-Blair, 2009; Půža and Mrácek, 2010).

Steinernema nematodes are a good model system to examine symbiont-mediated competition for several reasons. First, the relationship between each nematode and bacterium pair is integrated and specific. The nematodes require their symbiont for successful reproduction and there is a specialized association between the partners, which ensures their co-transmission from host to host (Sicard *et al*., 2003; Snyder *et al*., 2007). However, within the insect host the nematode and bacteria can be physically separate and be free to associate with other coinfecting microbes and parasites. Second, in experimental pairings of non-native partners, there is an inverse correlation between the fitness of the pairing and the phylogenetic distance of the microbial partner from the native symbiont (Sicard *et al*., 2004b; Chapuis *et al*., 2009; Murfin *et al*., 2015b). However, the mechanisms responsible for this specificity are not well understood. Third, coinfection of insect hosts by more than one species of *Steinernema* nematodes likely occurs in nature as species overlap spatially in the soil and in host range (Spiridonov *et al*., 2007; Půža and Mracek, 2009). In most cases where coinfection between two *Steinernema* species has been experimentally observed, one species is competitively dominant, often fully suppressing reproduction of the other species (Kondo and Ishibashi, 1986; Koppenhofer and Kaya, 1996; Sicard *et al*., 2006; Bashey *et al*., 2016).

Competition between *Steinernema* nematodes can be direct or mediated by their symbionts (Fig. 1A) (Sicard *et al*., 2006). *Xenorhabdus* symbionts can influence competition between their nematode hosts by interacting with each other (bacteria–bacteria, Fig. 1A). For instance, *X. nematophila*, the symbiont of *S. carpocapsae*, produces an extracellular bacteriocin that kills the *Photorhabdus* bacterial symbiont of *Heterorhabditis* nematodes. In experimental infections, the *S. carpocapsae* nematode host successfully produced progeny in mixed infections with *Photorhabdus*, but only when the *X. nematophila* symbiont produced the bacteriocin (Morales-Soto and Forst, 2011). Bacteria–bacteria competition could also occur through exploitative competition, wherein faster growing bacteria promote the success of their nematode partner, excluding the other pair (Bashey *et al*., 2011; Bashey *et al*., 2013). Alternatively, *Xenorhabdus* symbionts could influence competition by negatively influencing growth and development of its nonpartner nematode, through direct bacteria–nematode interactions (Fig. 1A).

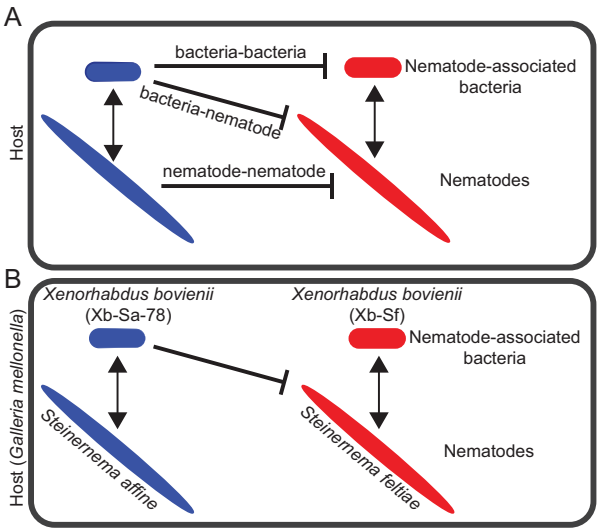


Fig. 1. Within-insect nematode competition.
A. Schematic representation of nematodes (blue and red oblong shapes) and their associated bacteria (blue and red rod shapes connected by arrows) competing within an insect host (black box). The lines indicate possible competition directly with other co-infecting nematodes (nematode–nematode) or with bacteria associated with co-infecting nematodes, which could mediate competition through bacteria–bacteria interactions or through interactions with the nematode directly (bacteria–nematode).
B. Schematic representation of competition between *Steinernema affine* (blue oblong) and *S. feltiae* (red oblong), which are hosts of *Xenorhabdus bovienii* symbiont strains Xb-Sa-78 (blue rod shape) and Xb-Sf (red rod shape) respectively. The experiments presented in this manuscript support the conclusion that Xb-Sa-78 directly inhibits (black line) production of *S. feltiae* during simultaneous infection of *Galleria mellonella* insects (black box) by infecting the target nematode's intestine.

For example, *X. bovienii* symbionts of *S. intermedium* have an incompatible interaction with *S. feltiae* nematodes that associate with a different *X. bovienii* bacterial strain. Specifically, when *S. feltiae* nematodes are coinjected into insects with the *X. bovienii* symbiont of *S. intermedium*, no *S. feltiae* progeny are produced, although several other *X. bovienii* bacterial strains are able to support *S. feltiae* nematode reproduction in insects (Murfin *et al*., 2015b).

Here, we investigate the potential for this type of interference competition between a microbial symbiont and a competitor of its host (Fig. 1B) by focusing first on competition between two nematode species that co-occur geographically, *S. feltiae* and *S. affine* (Emelianoff *et al*., 2008; Tarasco *et al*., 2014). We examine competitive outcomes between these two species using a series of experimental infections to establish the dependency of competition between the nematode species on the presence of the *Xenorhabdus bovienii* symbiont of *S. affine* (Xb-Sa-78). We then use microscopy of GFP-expressing strains to elucidate how the nematode–bacteria interaction differs when *S. feltiae* is exposed to Xb-Sa-78 versus its native *Xenorhabdus bovienii* symbiont (Xb-Sf). We

expand our approach by examining how *S. feltiae* nematodes interact with seven additional strains of *X. bovienii.* Some of these *X. bovienii* strains, such as the strain of *S. intermedium* discussed above and *S. affine*-derived strains, are associated with nematodes more distantly related to *S. feltiae*, while others are associated with nematodes closely related to *S. feltiae* [clade I vs. clade III (Lee and Stock, 2010a)]. Our work shows that clade I symbionts have diverged to be pathogenic to nonpartner nematodes, suggesting that symbiont-mediated competition among these parasites may alter their evolutionary as well as their ecological interactions.

Results

Inhibition by Xb-Sa-78 influences competition between S. feltiae *and* S. affine

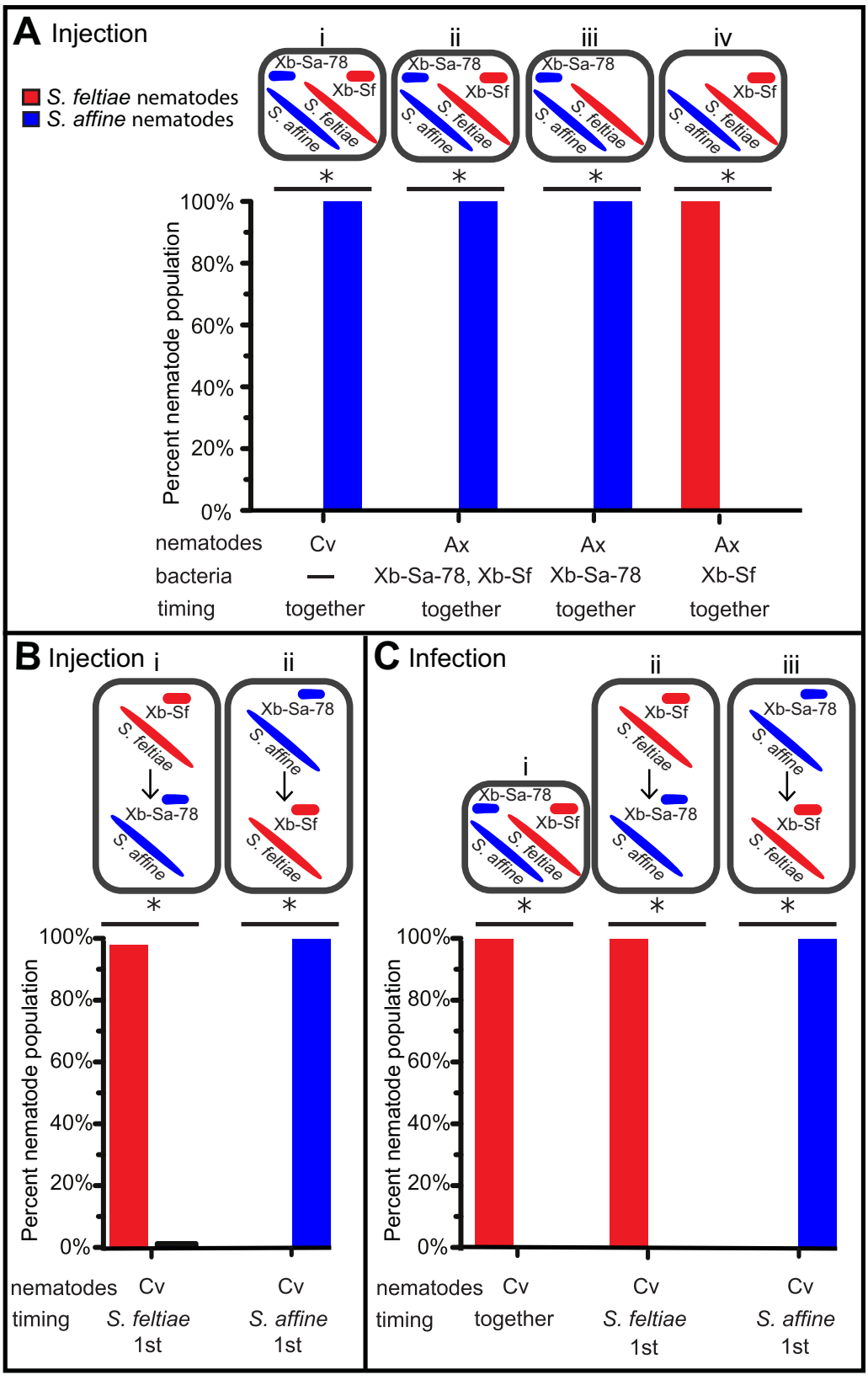
To consider the possibility that bacterial symbiont inhibition of non-native nematodes can provide a competitive advantage to the symbiont's native nematode host, we conducted co-infection assays with two species of *Steinernema: S. feltiae* and *S. affine.* This pairing was chosen because they co-occur geographically (Emelianoff *et al*., 2008; Tarasco *et al*., 2014) and because *S. affine* is closely related to *S. intermedium*, which has a symbiont incompatible with *S. feltiae* (Lee and Stock, 2010a; Murfin *et al*., 2015b). The nematodes were injected into *Galleria mellonella* insects, and the species identities of the resulting IJ progeny were assessed. For injection, nematodes were reared on agar plates either with their native bacterial symbionts (hereafter referred to as conventionally reared), which resulted in colonized IJs, or in the absence of bacteria (hereafter referred to as axenic), which resulted in uncolonized, bacteria-free IJs. When equal numbers of conventionally reared nematodes of each species (*S. affine* and *S. feltiae*) were injected at the same time, all of the resulting progeny IJs, were *S. affine*, indicating this nematode has a competitive advantage in direct competition (Fig. 2Ai). To determine if Xb-Sa-78 is mediating this competitive advantage, we performed co-injection assays of axenic nematode hosts with different combinations of the bacteria that had been separately cultured in laboratory media (Fig. 2Aii–iv). As expected, co-injections of both nematode hosts with both symbionts or both nematode hosts with only Xb-Sa-78 resulted in all *S. affine* progeny (Fig. 2Aii, iii). However, co-injections of both nematode hosts with only Xb-Sf bacteria resulted in the production of only *S. feltiae* progeny (Fig. 2Aiv). As injections of axenic nematodes alone do not yield progeny, competition in the absence of both symbionts cannot be determined (data not shown, (Murfin *et al*., 2015b)). These data indicate that the symbiont of Xb-Sa-78 promotes a competitive advantage of *S. affine* over *S. feltiae.*

To assess if the timing of injection affects the outcome of competition, we performed sequential injections of conventionally reared nematodes (Fig. 2B). When *S. feltiae* was injected before *S. affine*, almost all resulting progeny were *S. feltiae* (Fig. 2Bi), and when *S. affine* was injected before *S. feltiae*, all resulting nematode progeny were *S. affine* (Fig. 2Bii). These data indicate a priority effect (Hoverman *et al*., 2013; Natsopoulou *et al*., 2015; Quigley *et al*., 2017), whereby the first nematode to establish an infection has a relative advantage over the other. The priority effect was somewhat asymmetric, with *S. feltiae* failing to completely prevent the emergence of *S. affine* nematodes (Fig. 2Bi).

In the assays described above, nematodes and bacteria were directly injected into insects. To assess competition after a natural route of infection, we tested conventionally reared symbiotic nematodes in a sand trap assay, which requires that the nematodes locate and infect the insect host (Fig. 2C). In contrast to data from simultaneous injection, when *S. affine* and *S. feltiae* were added to sand at the same time, all resulting progeny were *S. feltiae* (Fig. 2Ci). This indicates that *S. feltiae* has a competitive advantage during the infection process that is bypassed by direct injection. A possible explanation for this infection-route specific competitive advantage is that *S. feltiae* may infect faster and gain a priority effect.

Consistent with our data from the injection route of infection, sequential infections during the natural route of infection also resulted in priority effects (Fig. 2Cii, iii): the first nematode species to be exposed to the insect host had a competitive advantage over the other species and was the sole species occurring among progeny nematodes. To assess if the *S. affine* priority effect under natural conditions is due to behavioural inhibition of *S. feltiae* infection (i.e., the *S. feltiae* do not invade an insect that has already been infected with *S. affine*), we monitored the invasion of *S. feltiae* nematodes within insects using their GFP-expressing symbiont (Xb-Sf-GFP) as a proxy (Supporting Information Fig. S2). Insects that were alive, freeze-killed or killed by *S. affine* or *S. feltiae* nematodes were exposed to *S. feltiae* nematodes carrying Xb-Sf-GFP and monitored for fluorescence. In all insect cadavers exposed to *S. feltiae* nematodes, fluorescence was detected by four days post exposure, indicating that *S. feltiae* nematodes invade previously infected insect hosts. There were no significant differences in the amount of fluorescence detected in the insects that were dead at the time of *S. feltiae* invasion, regardless of the presence or absence of *S. affine* and Xb-Sa-78. This indicates that *S. affine* and its symbiont do not have a direct negative influence on the bacterial symbiont Xb-Sf within the first four days of infection.

Previous reports of competition among nematode bacterial symbionts demonstrate that bacteria–bacteria



competition can influence the outcome of nematode host reproduction by killing competing bacteria through the production of antimicrobials (Sicard *et al*., 2004b–2006; Morales-Soto and Forst, 2011; Bashey *et al*., 2013). Therefore, to confirm that bacteria–bacteria inhibition is not occurring, we performed bacteriocin and cross-streaking assays, which indicated that Xb-Sf and Xb-Sa-78 are not sensitive to each other's bacteriocins or antibiotics (Supporting Information Table S2). Together, these data suggest that bacteria–bacteria competition is not affecting competitive outcomes of *S. affine* and *S. feltiae* simultaneous infections.

Another way in which Xb-Sa-78 may provide a competitive advantage to its *S. affine* host is through direct inhibition of the competing *S. feltiae* nematodes within the insect cadaver (Fig. 1). Therefore, we examined the ability of Xb-Sa-78 to directly inhibit *S. feltiae* growth and development. To distinguish inhibition from an inability to support nematode growth and development, we grew bacteria and nematodes together in permissive conditions [liver kidney agar (LKA)] that allow *S. feltiae* nematodes to grow in absence of their natural symbiont or any bacteria. Addition of axenic IJs to either plain LKA plates or lawns of Xb-Sf on LKA plates resulted in growth and development of the nematodes into adults by five days postaddition, and nematode reproduction to the next generation of progeny IJs occurred by day 14 (Supporting Information Table S3). In contrast, addition of nematodes to bacterial lawns of Xb-Sa-78 resulted in no growth or development of IJ nematodes into adults or progeny, indicating that incompatibility is due to inhibition rather than lack of nutritional support (Supporting Information Table S3). Taken together our data indicate that Xb-Sa-78 prevents co-infection by directly preventing growth of *S. feltiae* nematodes within the insect cadaver rather than preventing invasion, inhibiting the bacterial symbiont directly or failing to support the nutritional needs of the nematode.

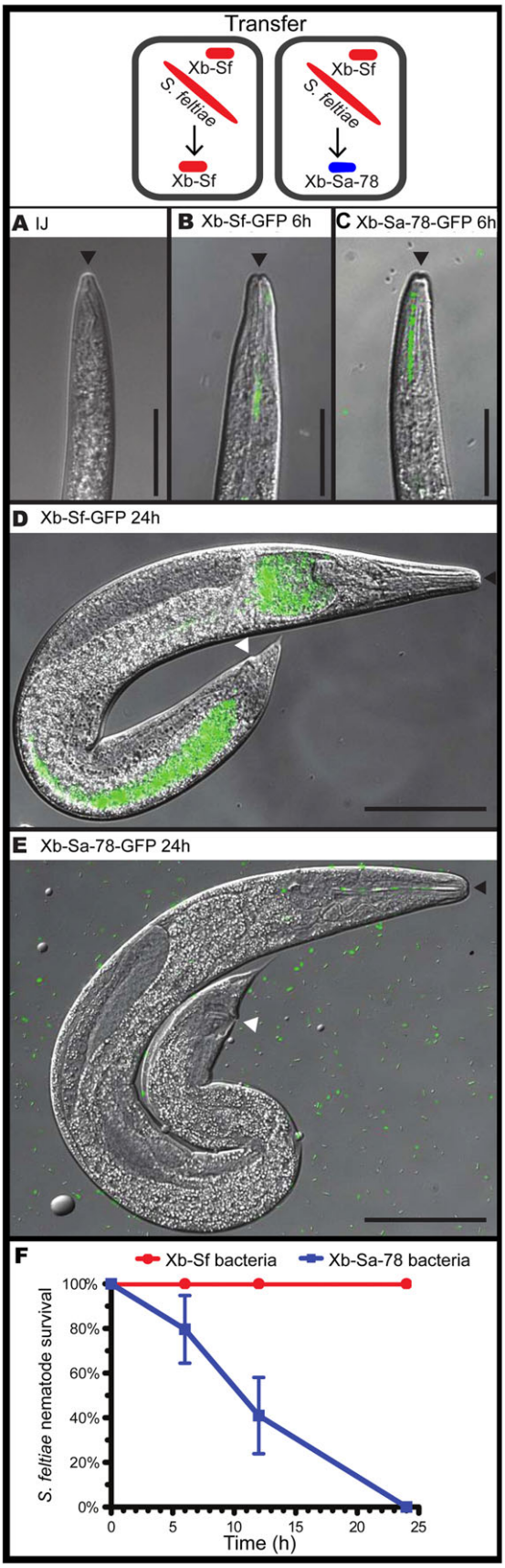
Xb-Sa-78 causes an intestinal infection in S. feltiae

To further investigate the direct effects of Xb-Sa-78 on *S. feltiae* nematodes, we used microscopy to examine contact between the bacteria and nematodes and to determine the stage at which Xb-Sa-78 might be inhibiting *S. feltiae.* These experiments were performed on Lipid Agar (LA), which supports growth of nematodes in the presence of the bacterial symbiont. Without the bacterial symbiont, nematodes will survive for several days on this medium. We first assessed the ability of *S. feltiae* IJs to recover (i.e., transition between the IJ and reproductive life stages) and survive in the presence of Xb-Sa-78. To facilitate visualization of bacteria, conventionally reared *S. feltiae* IJ nematodes were placed onto lawns of GFP-expressing Xb-Sf (Xb-Sf-GFP) or Xb-Sa-78 (Xb-Sa-78-GFP) (Fig. 3A–E). At 6 h postexposure, live nematodes on both bacterial lawns had morphology consistent with initiation of recovery (i.e., open mouths and GFP-expressing bacteria present within the esophagus and intestine, Fig. 3B and C). By 24 h postexposure, 99.97% of nematodes on Xb-Sa-78-GFP were dead, while 0.03% of nematodes on Xb-Sf-GFP were dead. However, on either lawn, nematodes showed development indicative of full recovery (i.e., nematodes had an open mouth, anus and intestine, Fig. 3D and E). In most nematodes, bacteria were also visible within the open intestine (Fig. 3D). However, in some recovered nematodes on Xb-Sa-78, bacteria did not appear to be present within the intestine (Fig. 3E). These data indicate that while Xb-Sa-78 allows recovery of *S. feltiae* IJs and initial stages of development, it causes nematode death prior to or early during adulthood.

To further investigate the effects of Xb-Sa-78 on *S. feltiae* nematodes, we performed survival assays using young adult *S. feltiae* nematodes transferred from lawns of Xb-Sf to lawns of either Xb-Sf or Xb-Sa-78 (Fig. 3F). All nematodes transferred to Xb-Sa-78 lawns were dead within 24 h, whereas nematodes transferred to lawns of native symbiont survived, indicating that *S. feltiae* nematodes are killed by Xb-Sa-78 (Fig. 3F).

To determine if Xb-Sa-78-mediated nematode death is due to alteration in the growth environment (e.g., secretion of a toxin or altered pH), we performed survival assays of adult nematodes on plates preconditioned by Xb-Sf and Xb-Sa-78 (Supporting Information Table S4). All nematodes transferred onto each of these plates survived, indicating that under these conditions any changes to the environment by Xb-Sa-78 are insufficient or too transient to mediate killing of *S. feltiae* nematodes. These experiments do not eliminate the possibility that Xb-Sa-78 may

Fig. 2. *S. feltiae* and *S. affine* competition experiments.
Conventional (Cv), reared with symbiont or axenic (Ax), reared with no bacteria, *S. feltiae* and *S. affine* nematodes (represented by red and blue oblongs, respectively, in schematic diagrams) were co-injected (A and B) or infected (C) with and without their symbionts (Xb-Sa-78 or Xb-Sf, represented by red and blue rod shapes, respectively, in schematic diagrams), either simultaneously (A and C) or sequentially (*S. feltiae* 1st or *S. affine* 1st) (B and C). In sequential infections, insects were exposed to one nematode species for three days followed by three days of exposure to the second nematode species. Bar graphs show the percentage of the progeny population that was *S. feltiae* (red) or *S. affine* (blue). Measurements are an average of three blocks with progeny counts from at least four insects per treatment within each block. In Bii, the error bars indicating standard error are too small to show. In all other cases, there was no error to display, as the progeny nematodes were 100% one of the species. *indicate a significant difference between the progeny percentages, indicating a competitive advantage for one species (student's *t*-test, $P < 0.05$).



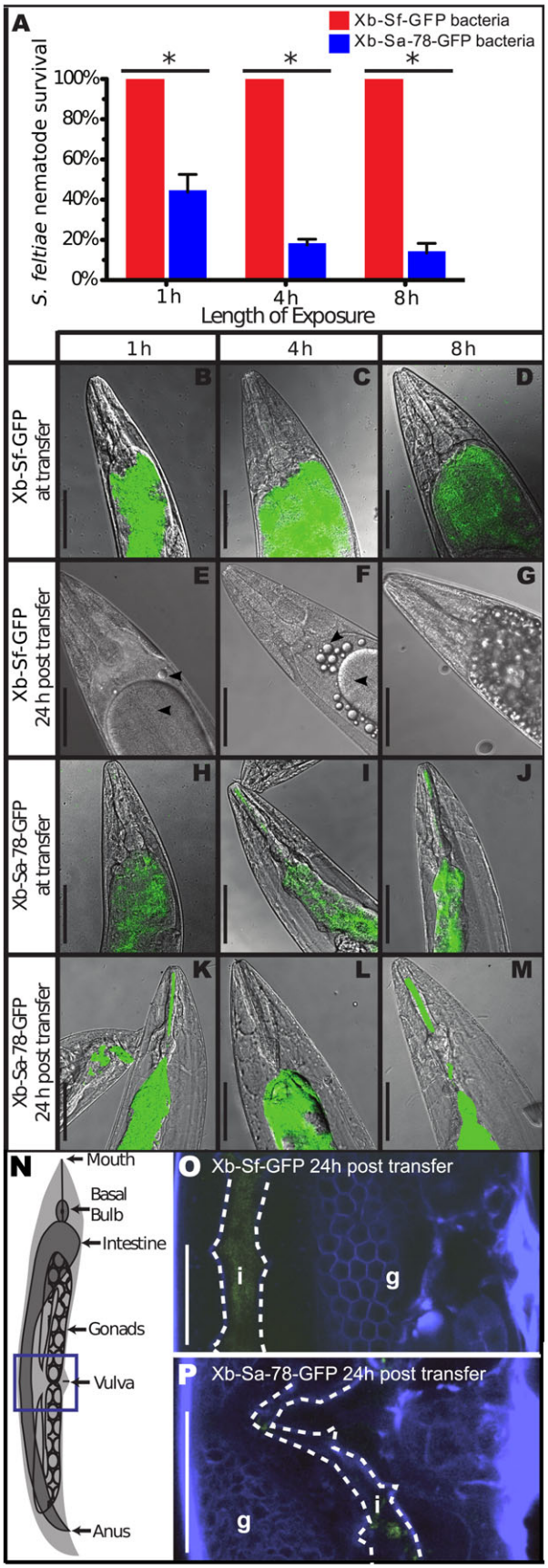
produce a toxin molecule specifically in the presence of *S. feltiae* or use secretion systems to directly inject a toxin into the host.

To assess if Xb-Sa-78 killing is due to an infection-mediated process, we performed nematode shifting assays, where adult nematodes were transferred from lawns of Xb-Sf to lawns of Xb-Sf-GFP or Xb-Sa-78-GFP for 1, 4 or 8 h before transferring onto plain LA plates where they were monitored for survival over time (Fig. 4). Nematodes exposed to Xb-Sa-78-GFP for as little as 1 h showed significantly decreased survival compared to nematodes exposed to Xb-Sf-GFP (Fig. 4A), consistent with the idea that Xb-Sa-78-GFP cells are capable of rapid infection-mediated killing of *S. feltiae* nematodes. Also consistent with this idea, during shifting assays, both bacterial strains were observed primarily in the intestines of living nematodes rather than on other surfaces. However, gravid female nematodes were occasionally observed with Xb-Sa-78-GFP bacteria localized internally to the area surrounding the eggs (Supporting Information Fig. S3). These bacteria may have escaped the intestine or invaded through the vulva.

Xb-Sf and Xb-Sa-78 bacterial strains each were visible in nematode intestines at the time of transfer (Fig. 4B–D and H–J). By 24 h post-transfer to blank agar plates, Xb-Sf-GFP cells were no longer visible within examined nematode intestines (Fig. 4E–G). However, Xb-Sa-78-GFP cells persisted within examined nematode intestines (Fig. 4K–M). *S. feltiae* nematodes exposed to their native symbiont, Xb-Sf-GFP, had obvious open and empty intestines by 24 h (with air bubbles or lipid granule droplets apparent, Fig. 4E–G), whereas at 24 h the majority of those nematodes exposed to Xb-Sa-78-GFP had intestines filled with GFP-expressing bacteria (Fig. 4K–M).

During exposure to Xb-Sa-78-GFP, the intestine of the nematode appears narrower than when the nematodes are reared on Xb-Sf-GFP (Compare Fig. 4 panels B–G to panels H–M). To further characterize the morphological differences in the nematode intestine and intestinal localization of the bacteria, we performed confocal microscopy on adult nematodes exposed to Xb-Sf-GFP or Xb-Sa-78-GFP for 8 h (Fig. 4N–P). Nematodes exposed to

Fig. 3. Xb-Sa-78 kills *S. feltiae* nematodes rather than inhibiting development.
IJ nematodes (A) were assessed for recovery at 6 h (B, C) and 24 h (D, E) postaddition to bacterial lawns of the GFP-expressing symbionts, Xb-Sf-GFP (B, D) or Xb-Sa-78-GFP (C, E). For both strains, nematodes showed morphology consistent with recovery (i.e., shedding of the chitin cuticle, opening of the mouth and anus and bacteria present within the esophagus and intestine). Bacteria are in green and overlayed on nematode phase contrast images. The black arrowheads point to the nematode mouths, and the white arrowheads point to anuses. The scale bars represent 25 μm in A–C and 50 μm in D–E. Survival (± 1 s.e.) of *S. feltiae* nematodes reared on the different bacterial strains (F) was significantly different over 24 h using log-rank analysis ($P < 0.05$).



Xb-Sf-GFP displayed a normal intestinal morphology (i.e., open, smooth intestines; e.g., Fig. 4O) throughout the total length of their body. However, nematodes exposed to Xb-Sa-78-GFP had frequent narrow spots along the length of the intestine and a constricted, ruffled appearance (e.g., Fig. 4P). Localization of the bacteria to the intestine and altered intestinal morphology suggests that killing of *S. feltiae* is due to an intestinal infection by Xb-Sa-78.

X. bovienii *symbiont isolates exhibit varying effects on* S. feltiae *nematode mortality and intestinal morphology*

Previous studies demonstrated that *X. bovienii* symbiont isolates from other clade III nematodes can support *S. feltiae* growth and development *in vivo*, while the symbiont of the clade I nematode *S. intermedium* did not support growth and development of *S. feltiae* (Murfin *et al*., 2015b). This suggests that support of *S. feltiae* by clade III nematode symbionts and inhibition of *S. feltiae* by clade I nematode symbionts might be a conserved phenomenon. To examine this possibility, we assessed the impact on *S. feltiae* survival of six other *X. bovienii* symbionts, two (Xb-Sa-52 and Xb-Sa-66) from isolates of clade I *S. affine* nematodes and four (Xb-Sk-44, Xb-Sk-47, Xb-Sk-59 and Xb-Sk-95) from isolates of clade III *S. kraussei* nematodes (Supporting Information Tables S1 and S5). The clade III *Steinernema* symbionts supported growth of *S. feltiae* nematodes with one exception: Xb-Sk-44 did not support *S. feltiae* nematodes in one of three biological replicates (Supporting Information Table S5). In contrast, clade I *Steinernema* symbionts Xb-Sa-52, Xb-Sa-66 and Xb-Si (as reported in (Murfin *et al*., 2015b)) did not support *S. feltiae* nematodes: Xb-Sa-66 failed to support

Fig. 4. Xb-Sa-78 killing of *S. feltiae* is infection mediated.
S. feltiae adult nematodes were transferred from native symbiont bacterial lawns (nonfluorescent Xb-Sf) onto GFP-expressing native symbiont (Xb-Sf-GFP) or the symbiont of *S. affine* (Xb-Sa-78-GFP) and exposed for 1 (B, E, H, K), 4(C, F, I, L) or 8 (D, G, J, M) h. The nematodes were then collected and transferred onto blank agar plates. Nematodes were monitored for survival at 32 h postinitial exposure (A), and nematodes exposed to Xb-Sa-78-GFP (blue) showed significantly less survival than nematodes exposed to Xb-Sf (red) (student's *t*-test, $P < 0.05$). Graphs show averages (±1 s.e.) of 3 blocks of 100 nematodes each. Nematodes were visually inspected by phase contrast and fluorescence microscopy (overlaid images are shown) at the time of transfer (B–D, H–J) and at 24 h post-transfer (E–G, K–M) for morphology and the presence and location of Xb-Sf-FL (B–G) or Xb-Sa-78 (H–M) GFP-expressing bacteria (green). Black arrowheads indicate air bubbles or lipid droplets present within the nematode intestine after transfer. Nematode intestinal shape after exposure to Xb-Sf-GFP (O) or Xb-Sa-78-GFP (P) was examined by confocal microscopy. The blue–purple box in the schematic (N) represents the area seen in the micrographs (O, P). Nematode tissues were stained using Alexa 633 phalloidin (false colored purple) and bacteria were expressing GFP. The white dotted lines outline the intestine to show intestinal narrowing in P relative to O, and nematode morphological features are indicated with letters: intestine (i) and gonad (g). All scale bars indicate 50 μm.

S. feltiae nematodes in three of four biological replicates while Xb-Sa-52 failed to support *S. feltiae* nematodes in all four biological replicates (Supporting Information Table S5). In composite with published data (Murfin *et al*., 2015b), these results support the idea that *X. bovienii* from clade I, but not those from clade III *Steinernema* hosts, have a negative impact on the development or survival of the clade III nematode *S. feltiae.*

To determine if similar to Xb-Sa-78, other clade I *Steinernema* symbionts can kill *S. feltiae* young adult nematodes through intestinal infection, we performed shifting assays and analyzed nematode appearance. We found that overall survival of *S. feltiae* nematodes varied significantly when exposed to clade I *Steinernema* symbionts (Fig. 5A). At 12 h postexposure, Xb-Sa-66 killed more nematodes (39%) than did the other clade I symbionts (2%–8%). However, by 24 h postexposure, Xb-Sa isolates killed more nematodes than did Xb-Si (84%–91% vs. 27%, Fig. 5A). These results suggest that under these conditions Xb-Si is less toxic than Xb-Sa-78, Xb-Sa-66 or Xb-Sa-52 towards *S. feltiae* nematodes. In addition, although Xb-Sa-66 begins killing *S. feltiae* nematodes earlier, by 24 h postexposure Xb-Sa-66, Xb-Sa-78 and Xb-Sa-52 kill a similarly high percentage of *S. feltiae* nematodes.

We examined the appearance of *S. feltiae* nematodes during infection with the different strains of *X. bovienii.* In addition to the constricted intestine phenotype observed in nematodes exposed to Xb-Sa-78 (Fig. 4I, J and P), we observed two additional phenotypes (Fig. 5B). Some *S. feltiae* nematodes had intestines that appeared to be clear, possibly due to gut content clearance or nonfeeding. We termed this as the transparent intestine phenotype. Other *S. feltiae* nematodes have expanded intestines that filled most of the nematode body cavity causing other nematode organs such as the gonads to be displaced (Fig. 5B). We termed this as the swollen intestine phenotype. The frequency of these intestine phenotypes differed based on the clade I symbiont to which *S. feltiae* nematodes were exposed (Fig. 5C; Supporting Information Fig. S4). Across multiple experiments, compared to *S. feltiae* nematodes exposed to other clade I symbionts, those exposed to Xb-Sa-78 exhibited higher frequency of the constricted intestine phenotype while those exposed to Xb-Si exhibited higher frequency of the transparent intestine phenotype (Fig. 5C, Supporting Information Fig. S4). *S. feltiae* nematodes exposed to Xb-Sa-52 displayed a higher frequency of a normal intestine phenotype compared to those exposed to Xb-Sa-78 but not to those exposed to Xb-Sa-66 and Xb-Si (Fig. 5C, Supporting Information Fig. S4). *S. feltiae* nematodes exposed to Xb-Sa-66 or Xb-Sa-52 also exhibited a higher frequency of the swollen intestine phenotype compared to other clade I *Steinernema* symbionts but this difference in frequency was not statistically significant (Fig. 5C, Supporting Information Fig. S4). Together these data show

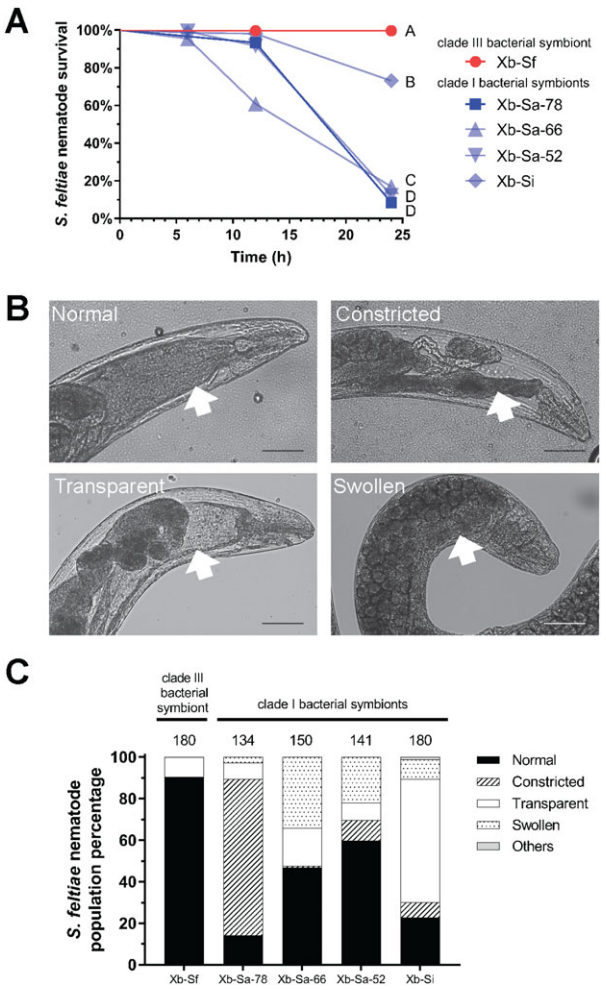


Fig. 5. Clade I *X. bovienii* symbionts infection and killing of *S. feltiae* nematodes.

A. Survival of insect-derived *S. feltiae* nematodes when exposed to clade III symbiont Xb-Sf (red line) versus clade I symbionts Xb-Sa-78, Xb-Sa-66, Xb-Sa-52 and Xb-Si (blue lines). Letters indicate statistically significant differences in rate and/or overall survival as determined by two-stage procedure analyses.

B. Representative micrographs for different intestine morphologies of insect-derived *S. feltiae* nematodes after exposure to clade I symbionts: normal intestine, constricted intestine, transparent intestine and swollen intestine. Arrows point to the nematode intestines and scale bar indicates 250 μm.

C. Total population frequency distribution of intestine morphology for all time points of insect-derived *S. feltiae* nematodes exposed to clade I symbionts. Total numbers of nematodes assessed are shown on top of each bar. (See Supporting Information Fig. S4 for statistical tests.)

that clade I *Steinernema* symbionts cause varying survival of and intestinal phenotypes in *S. feltiae* nematodes, which suggests that the strains may utilize distinct mechanisms of infection and killing.

Discussion

Defensive symbionts protect their hosts from predators and disease through which they indirectly alter their

hosts' competitive abilities (Clay *et al*., 2005). In this study, we extend this framework to describe a related phenomenon, a symbiont that directly interferes with its host's competitors (Fig. 1B). By examining competition between the nematode hosts *S. affine* and *S. feltiae* with different combinations of their symbionts, we show that the bacterial *X. bovienii* symbiont Xb-Sa-78 allows *S. affine* to outcompete *S. feltiae* (Fig. 2A). Further, we present data demonstrating that Xb-Sa-78 colonizes the intestine of *S. feltiae* and causes nematode death (Figs 3 and 4). To our knowledge, this is the first study to identify a bacterial symbiont affecting the competitive success of its host through infection and direct inhibition of a competing host. Moreover, our study also demonstrates that this symbiont effect correlates with nematode host species: symbionts associated with nematodes in the same clade as *S. affine* also show this killing effect (Fig. 5), while those associated with nematodes in the same clade as *S. feltiae* do not (Supporting Information Table S5). Finally, as all the bacterial isolates we describe here are within the same species (*X. bovienii*), our work highlights the facility with which bacterial symbionts can alter their ecological roles to behave as mutualists or pathogens depending on the environmental context.

Competition among parasites and other microbes within a host individual, and the consequences of within-host competition for parasite diversity and host health has been the focus of much attention (Brown *et al*., 2009; Mideo, 2009; Bashey, 2015). Bacterial symbionts can affect parasitic success and competition through indirect methods, such as stimulating the host immune system to overcome parasitic infection (Moreira *et al*., 2009; Weiss and Aksoy, 2011) or inhibiting the bacterial symbiont of the parasite (Morales-Soto and Forst, 2011; Bashey *et al*., 2013). Here, we show a third type of competitive interaction in which the bacterial symbiont of one nematode competitor is pathogenic to another nematode competitor (Fig. 1B). This finding is similar to insect defensive symbioses, wherein a bacterial symbiont that is not harmful to its host is directly harmful to an invading eukaryotic parasite (Jaenike *et al*., 2010; Oliver *et al*., 2010). However, in the case presented here, the symbiont is targeting a competitor within the same genus as its own nematode host.

In competitive interactions between *S. affine* and *S. feltiae*, the outcome of the competition (i.e., which nematode is able to produce progeny) depends on the presence or absence of Xb-Sa-78 (Fig. 2A), which allows *S. affine* to outcompete *S. feltiae.* This competitive advantage can prevent successful *S. feltiae* co-infection of *S. affine* infected cadavers (Fig. 2C and B). Our data suggest that the mechanism responsible for this competitive inhibition is pathogenic colonization of the intestine of *S. feltiae* by Xb-Sa-78 (Figs 3 and 4). This is perhaps surprising, given that the *X. bovienii* strain Xb-Sa-78 that is pathogenic toward *S. feltiae* nematodes mutualistically associates with its own nematode host, *S. affine.* The underlying mechanism of this differential susceptibility to Xb-Sa-78 infection awaits further investigation. Another intriguing observation presented here is that the pathogenic and intestinal infection phenotypes of *X. bovienii* strains in *S. feltiae* are common among those strains that are mutualists of clade I *Steinernema* nematodes (such as *S. affine*), but is not prevalent among those that are mutualists of clade III *Steinernema* nematodes (such as *S. feltiae*; Fig. 4; Supporting Information Table S5). Similarly, Chapuis *et al*. (2009) reported that *S. feltiae* did not reproduce well when reared with the *X. bovienii* symbionts of *S. affine* and *S. intermedium*, as well as another member of clade I, *S. sichuanense* (Mrácek *et al*., 2006; Lee and Stock, 2010a). One possibility consistent with these findings is that clade I nematodes have evolved resistance to clade I symbiont pathogenesis, allowing them to maintain their symbiont despite its pathogenic activities.

In nature, clade I and clade III nematode species have been isolated in close proximity to one another (Spiridonov *et al*., 2007; Emelianoff *et al*., 2008; Půža and Mracek, 2009; Tarasco *et al*., 2014). Similar to our competition experiments previous reports indicate that *S. affine* (clade I) outcompetes *S. kraussei* (clade III) (Půža and Mracek, 2009). Taken together with our work, these data suggest that inhibitory effects of *X. bovienii* symbionts from clade I nematodes may be a conserved phenomenon that provides clade I nematodes a competitive advantage over clade III nematodes. However, it is important to keep in mind that other factors may affect the outcome of competitive interactions in nature. For example, the timing of infection was sufficient to change the outcome of within-host competition between *S. affine* and *S. feltiae* (Fig. 2). Additionally, differences in foraging strategies and insect host use may facilitate the coexistence of competing nematodes (Koppenhofer and Kaya, 1996).

Although inhibition of clade III nematodes by clade I symbionts is a conserved phenomenon, our data suggest that the precise virulence mechanisms utilized by the clade I symbionts may be distinct, even among isolates from the same nematode species. These differences could be explained by divergent identities or regulation of nematicidal mechanisms. It also is possible that the observed phenotypes are due to variation of responses of nematode populations to the same mechanism. Differences across *X. bovienii* isolates in the pathogenic effects they elicit in nematode competitors may reflect the dynamic nature of their ecological roles, such as maintaining mutualism with distinct nematode partners, growing within a variety of insect hosts or encountering

divergent competitors. Such context-dependency and evolutionary lability of microbial interactions with eukaryotes increasingly has been recognized in field based and experimental studies of symbiosis (Heath and Tiffin, 2007; Oliver *et al*., 2008; Sachs *et al*., 2011; King *et al*., 2016). Additionally, pathogenic effects on nonpartner nematodes may serve to maintain specificity and suggest that clade I *X. bovienii* are unlikely to switch to a clade III host, a pattern consistent with current phylogenies (Lee and Stock, 2010a).

In conclusion, we describe a competitive interaction that occurs between *S. affine* and *S. feltiae* nematodes that is modulated by the *X. bovienii* bacterial symbiont, Xb-Sa-78, associated with *S. affine.* Our work demonstrates that Xb-Sa-78 can modulate competition through direct infection and killing of the competing *S. feltiae* nematodes. Additionally, our study highlights that bacterial strain differences should be considered when assessing ecological phenotypes, as the two *X. bovienii* bacterial isolates studied here (Xb-Sa-78 and Xb-Sf), although the same species, have very different impacts on *S. feltiae* nematodes. Overall, this study demonstrates that competition between hosts can be modulated by microbial symbionts and that understanding these types of interactions is important for our understanding of the ecology and evolution of natural systems.

Experimental procedures

Nematode and bacterial strains

Bacterial strains were isolated through sonication or crushing of surface sterilized nematode hosts, and species identity was confirmed by analysis of 16S rRNA (Supporting Information Table S1) (Lee and Stock, 2010b; Chaston *et al*., 2011). Bacterial strains were stored in Lysogeny Broth (LB) supplemented with 20% glycerol frozen at –80°C. Unless otherwise noted, bacterial strains were grown in LB with aeration or on LB agar supplemented with 0.1% pyruvate at 30°C in the dark (Xu and Hurlbert, 1990).

Nematode isolates of *S. feltiae* were obtained from the laboratories of Dr. S. Patricia Stock (*S. feltiae* isolated in FL, USA). *S. affine* was isolated in Indiana, United States as in (Hawlena *et al*., 2010). Species identity of the nematodes were verified through sequencing of the 12S and 28S genes (Lee and Stock, 2010a). As previously described (Martens *et al*., 2003), nematodes were propagated through *Galleria mellonella* larvae kept at room temperature or at a controlled 25°C. Nematodes were stored at room temperature or at 25°C at a density less than 5 IJs/μl at a volume less than 60 ml in 250 ml tissue culture flasks or 10 ml in 50 ml tissue culture flasks (BD Falcon, Franklin Lakes, NJ, USA), until used directly in assays (insect-derived) or to generate conventional and axenic IJs.

Axenic IJs were produced *in vitro* on LKA similar to previously described methods (Sicard *et al*., 2003; Martens and Goodrich-Blair, 2005). The protocol was modified to produce axenic *S. affine.* Instead of direct addition of nematodes eggs to LKA, nematode eggs were incubated in LB supplemented with 150 μM kanamycin for three days. Additionally, LKA plates were also supplemented with 150 μM kanamycin and 100 μM ampicillin.

Genetic modification of the bacterial strains to express GFP was performed as previously described (Murfin *et al*., 2012; Chaston *et al*., 2013). Briefly, the pURR25 Tn7 delivery vector including the gene encoding GFP was transferred from *Escherichia coli* into recipient *Xenorhabdus* strains using triparental mating conjugations (Teal *et al*., 2006) and a helper plasmid (Bao *et al*., 1991). GFP-expressing exconjugants in which the Tn*7* had inserted into the *att*Tn*7* site were selected using LB supplemented with pyruvate and 50 μM kanamycin.

Injections in Galleria mellonella

Axenic or conventional nematodes were surface sterilized and mixed with log-phase bacterial culture in order to inject 50 IJ nematodes. For injections of bacteria, overnight cultures were subcultured, grown to ~0.6 OD and diluted to inject 100 CFU of log phase bacterial cells. In co-injections, bacteria were grown separately and combined prior to injection in order to inject 100 CFU of log phase bacterial cells for each strain. For competition injections, nematodes were mixed and 50 IJs of each species were co-injected. For sequential injections, 50 IJs of the first species were injected and 50 IJs of the second species were injected after 72 h. At 7 days postfirst-injection, the insect cadavers were placed in a water trap, and nematodes emerged within 10 days posttrapping. For co-injections that did not display nematode emergence, cadavers were dissected to confirm that no nematodes were present. For quantification of progeny populations, 100 nematodes from each water trap were scored for species identify based on size and morphology. For each treatment, five insects were injected and scored as technical replicates and averaged. Three blocks were performed. In all injections, at least four insect cadavers produced progeny.

Natural infections

Sand traps were performed similarly to previously described (Grewal *et al*., 1994; Murfin *et al*., 2015b). Briefly, 1 g of sand was added to wells of a 12 well plate. Approximately 50 IJs in 100 μl of water were added to

the well and allowed to equilibrate for 1 h. For direct competition, 50 IJs of each species in 100 μl of water was added together in one well. One *G. mellonella* larva was added to each well and allowed to infect for 7 days. For sequential natural infections, the first species was allowed to infect for 3.5 days and then insect cadavers were transferred to new sand trap wells with the second species for an additional 3.5 days. 7 days postinitial infection, the insect cadavers were placed in a water trap and nematodes emerged within 10 days of trapping. For infections from which no nematodes emerged, cadavers were dissected to confirm that no nematodes were present. For quantification of progeny populations, 100 nematodes from each water trap were scored for species identify based on size. For each treatment, five insects were infected and scored as technical replicates. Three blocks were performed and averaged. At least four insect cadavers per treatment produced progeny.

Nematode recovery assays

Conventional IJ nematodes were added to LA plates with bacterial lawns, grown as described above. Nematodes were rinsed off of LA plates at various time points using 1 ml of PBS and collected in microcentrifuge tubes. Nematodes were rinsed three times in PBS: nematodes were allowed to settle, PBS was removed and replaced with fresh PBS. Nematodes and GFP-expressing bacteria were viewed by microscopy using a Nikon Eclipse TE300 inverted microscope under 10x, 20x and 40x magnification. Images were taken using a Hamamatsu digital camera (Hamamatsu City, Japan; model C4742-95-10NR) and analyzed using the Metamorph software v4.5r6 (Universal Imaging Corporation, West Chester, PA, USA). Three blocks of two technical replicates were performed, and at least 25 nematodes were observed per technical replicate.

In vitro *nematode growth and survival assays*

To test growth of IJs, conventional, axenic or insect-derived IJ nematodes were added directly to test bacterial strain lawns on LKA or LA plates. Briefly, 600 μl of overnight bacterial culture of the native symbiont was spread on 6 cm LKA or LA plates and allowed to grow at 25°C. Approximately 2000 surface sterilized nematodes in 200 μl LB was added to the bacterial lawn, and the nematodes were allowed to develop at 25°C. Plates were viewed under 4x magnification daily to assess the production of adults and juvenile progeny. At least three blocks of two technical replicates were performed.

In vitro testing of young adults was performed by transferring nematodes from native symbiont growth plates onto test bacterial strains. Briefly, 600 μl of overnight bacterial culture of the native symbiont was spread on 6 cm LA plates agar plates and allowed to grow at 25°C for 48 h. Approximately 5000 IJ nematodes in 500 μl of LB was added to the bacterial lawn on LA, and the nematodes were allowed to grow at 25°C for 48 h. During nematode growth, test plates were set up by adding 600 μl of overnight bacterial culture of test strains to LKA or LA and allowing growth at 25°C for 48 h (Sicard *et al*., 2003; Martens and Goodrich-Blair, 2005). After growth was complete, adult nematodes were transferred from growth plates to test plates. Nematodes were collected in PBS and rinsed 3 times. Approximately 200 rinsed nematodes in 200 μl of PBS were added to each test plate and air dried in a hood. For growth assays, nematodes were monitored every 24 h for seven days. For growth assays on plates preconditioned by bacterial growth, a sterile filter paper was added to the surface of the test plate prior to addition of the test bacterial strain. Before nematode addition, the filter paper containing the bacterial lawn was removed. Two technical replicates of transfer plates were observed for qualitative growth for each condition, and three blocks were performed.

Survival assays were set up the same way as growth assays using the transfer method of adult nematodes. Nematode survival was monitored at 6, 12 and 24 h, and 20–40 nematodes were scored at each time point. Nematodes were scored as dead when they no longer moved or responded to gentle prodding with a thin wire. Two or three technical replicates from separate transfer plates were counted per condition and three blocks were performed.

For frequency counts of nematode intestinal phenotypes, assays were set up the same as survival assays. Nematodes were collected off transfer plates at 6, 12 and 24 h using PBS. Samples were suspended in PBS + 2% final concentration paraformaldehyde and fixed for at least 18 h. Samples were washed at least three times in PBS and stored at 4°C prior to double-blind analyses. Nematodes were observed at 4x and 10x using Nikon Eclipse TE3000 inverted microscope and based on intestine morphology, categorized into normal, constricted, swollen, transparent or others. Up to 20 nematodes were categorized per time point and three blocks were performed. Representative images were taken using Hamamatsu digital camera (Hamamatsu City, Japan; model C4742-95-10NR) and processed using ImageJ.

Testing of bacterial localization was done using *in vitro* transfer methods as described for growth assays. *S. feltiae* adult nematodes were transferred from native symbiont LA plates onto test LKA or LA plates with GFP-expressing bacterial cultures. Nematodes were collected in PBS after 4, 8, 12 and 24 h post-transfer. Nematodes were rinsed in PBS three times and observed using epifluorescent and phase contrast microscopy on a Nikon Eclipse TE300 inverted microscope. Three blocks of two

technical replicates were performed, and at least 25 nematodes were observed per technical replicate.

Confocal imaging of nematodes

Fixing, permeabilizing and staining of nematodes was performed using previously described protocols (Murfin *et al*., 2012; Chaston *et al*., 2013). Nematodes were stained using Alexa 633 phalloidin. Representative images were taken on a Zeiss LSM 510 confocal microscope with an Axioplan2 imaging system and processed using LSM image software v2.1 (Zeiss, New York, NY, USA).

In vivo *imaging*

Injection or infection of insects was done as described above. For nematode superinfection assays, axenic *S. feltiae* nematodes were added to LKA plates containing lawns of Xb-Sf-GFP set up as described in recovery assays. Seven days postaddition of nematodes to LKA plates, the plates were water trapped and IJs carrying GFP expressing symbiont were collected 7–14 days later. Insects preinjected with PBS or bacterial strains or nematodes were used in a natural infection assay with the nematodes carrying GFP-expressing symbionts was done four days postinjection. For all insect cadavers images were taken every 24 h for 5 days, and optimum time points (i.e., most fluorescence without oversaturation) was determined to be 4 days postinjection or infection. Analysis was done using an IVIS Imaging System 200 (Xenogen, Alameda, CA, USA). Fluorescence was quantified by using Living Image software v2.6 (Xenogen). Five insects were imaged as technical replicates and average, and three biological replicates were performed.

Bacterial competition

Bacterial strain competition was measured by bacterial cross streaking and bacteriocin assays. Bacterial cross streaking (Anderhub *et al*., 1977) was done similarly to previously described methods. For bacterial cross streaking, the test strain is applied in a single line onto an LB supplemented with 0.1% pyruvate agar plate and allowed to incubate at 30°C for 24 h. The bacteria are then removed with a glass slide or pipette tips, and the remaining bacteria are chloroform killed for 30 min. After airing the plates for an additional 30 min, the indicator strain is applied in a single line perpendicular to the test strain. Plates were allowed to grow at 30°C for 24 h. Plates were then observed for growth. An obvious zone of clearing around the test strain was scored as positive inhibition of growth of the indicator strain. If the indicator strain could grow up to the edge of the test strain streak, it was scored as negative. In these experiments, self-testing and LB controls were done as negative controls and *Escherichia coli, Micrococcus luteus* and *Bacillus subtilis* were used as positive controls, as they are known to be sensitive to antibiotics produced by Xb-Sf (Murfin *et al*., 2015a).

Bacteriocin assays were performed similarly to those described in previously (Hawlena *et al*., 2012). Briefly, log-phase cultures were induced with mitomycin C (0.5 μg/ml) and were incubated overnight at 28°C. Cell-free supernatant was obtained from each culture by centrifugation (15 min at 4500 g) followed by filtering via 0.45 μm HT Tuffryn membrane and stored at 4°C. The bacteriocin activity of the supernatant was tested by spotting 10 μl onto a nutrient soft agar (0.5% agar) sowed with 2% (v/v) of stationary-phase liquid culture of a recipient colony. Plates were incubated for 48 h at 28°C, at which time inhibition could be visualized as a clear zone on the recipient lawn. Supernatant from blank LB cultures treated with mitomycin and self tests, whereby the both supernatant and the recipient colony were of the same strain, were used as negative controls. A known sensitive strain (Xb-Sk-44) was used as a positive control. Two independent inductions were performed for each strain.

Statistics

Statistical analyses were performed in R (Team, 2013) or Prism (GraphPad). For survival curves analyzing Xb-Sa-78 killing, log rank analyses were done. In log-rank analysis, all data points from the three blocks were combined. Each experimental block was also analyzed separately, and trends remained the same. For comparison of survival at single time points and population percentage differences, ANOVA was done on the average of the experimental blocks to determine if the values were significantly different ($df = 2$). Each experimental block was also analyzed separately, and trends remained the same. For survival assays comparing Xb-Sa-78, Xb-Sa-66, Xb-Sa-52 and Xb-Si killing, two-stage procedure analyses were done using R package TSHRC version 0.1–4 with bootstrap samples set at 1000 and eps set at 0.01. *P*-values were corrected for multiple comparisons using the Bonferroni method. All data points from the three blocks were combined. Trends remained the same when log-rank or Gehan–Breslow–Wilcoxon tests in Prism were used for statistical analyses. For comparison of intestine morphology, means of population percentage from three blocks were calculated and analyzed using randomized block one-way ANOVA and Tukey's Multiplicity Comparison test in Prism.

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References

Abt, M.C., and Artis, D. (2013) The dynamic influence of commensal bacteria on the immune response to pathogens. *Curr Opin Microbiol* **16**:4–9.

Anderhub, B., Pitt, T.L., Erdman, Y.J., and Willcox, W.R. (1977) A comparison of typing methods for *Serratia marcescens. J Hyg (Lond)* **79**:89–102.

Baiocchi, T., Lee, G., Choe, D.H., and Dillman, A.R. (2017) Host seeking parasitic nematodes use specific odors to assess host resources. *Sci Rep* **7**:6270.

Bao, Y., Lies, D.P., Fu, H., and Roberts, G.P. (1991) An improved Tn*7*-based system for the single-copy insertion of cloned genes into chromosomes of gram-negative bacteria. *Gene* **109**:167–168.

Bashey, F. (2015) Within-host competitive interactions as a mechanism for the maintenance of parasite diversity. *Philos Trans R Soc Lond B Biol Sci* **370**:20140301.

Bashey, F., Reynolds, C., Sarin, T., and Young, S.K. (2011) Virulence and competitive ability in an obligately killing parasite. *Oikos* **120**:1539–1545.

Bashey, F., Hawlena, H., and Lively, C.M. (2013) Alternative paths to success in a parasite community: within-host competition can favor higher virulence or direct interference. *Evolution* **67**:900–907.

Bashey, F., Sarin, T., and Lively, C.M. (2016) Aging alters interspecific competition between two sympatric insect-parasitic nematode species. *Ecol Evol* **6**:3750–3759.

Brown, S.P., Fredrik Inglis, R., and Taddei, F. (2009) Evolutionary ecology of microbial wars: within-host competition and (incidental) virulence. *Evol Appl* **2**:32–39.

Caragata, E.P., Rances, E., Hedges, L.M., Gofton, A.W., Johnson, K.N., O'Neill, S.L., and McGraw, E.A. (2013) Dietary cholesterol modulates pathogen blocking by *Wolbachia. PLoS Pathog* **9**:e1003459.

Chapuis, E., Emelianoff, V., Paulmier, V., Le Brun, N., Pages, S., Sicard, M., and Ferdy, J.B. (2009) Manifold aspects of specificity in a nematode-bacterium mutualism. *J Evol Biol* **22**:2104–2117.

Chaston, J.M., Suen, G., Tucker, S.L., Andersen, A.W., Bhasin, A., and Bode, E. (2011) The entomopathogenic bacterial endosymbionts *Xenorhabdus* and *Photorhabdus*: convergent lifestyles from divergent genomes. *PLoS One* **6**:e27909.

Chaston, J.M., Murfin, K.E., Heath-Heckman, E.A., and Goodrich-Blair, H. (2013) Previously unrecognized stages of species-specific colonization in the mutualism between *Xenorhabdus* bacteria and *Steinernema* nematodes. *Cell Microbiol* **15**:1545–1559.

Clay, K., Holah, J., and Rudgers, J.A. (2005) Herbivores cause a rapid increase in hereditary symbiosis and alter plant community composition. *Proc Natl Acad Sci USA* **102**:12465–12470.

Dillon, R.J., Vennard, C.T., Buckling, A., and Charnley, A.K. (2005) Diversity of locust gut bacteria protects against pathogen invasion. *Ecol Lett* **8**:1291–1298.

Donskey, C.J., Chowdhry, T.K., Hecker, M.T., Hoyen, C.K., Hanrahan, J.A., Hujer, A.M., *et al*. (2000) Effect of antibiotic therapy on the density of vancomycin-resistant enterococci in the stool of colonized patients. *N Engl J Med* **343**:1925–1932.

Ehlers, R.U. (2001) Mass production of entomopathogenic nematodes for plant protection. *Appl Microbiol Biotechnol* **56**:623–633.

Emelianoff, V., Le Brun, N., Pages, S., Stock, S.P., Tailliez, P., Moulia, C., and Sicard, M. (2008) Isolation and identification of entomopathogenic nematodes and their symbiotic bacteria from Herault and Gard (Southern France). *J Invertebr Pathol* **98**:211–217.

Grewal, P.S., Selvan, S., and Gaugler, R. (1994) Thermal adaptation of entomopathogenic nematodes: niche breadth for infection, establishment, and reproduction. *J Thermal Biol* **19**:245–253.

Hamilton, P.T., Leong, J.S., Koop, B.F., and Perlman, S.J. (2014) Transcriptional responses in a *Drosophila* defensive symbiosis. *Mol Ecol* **23**:1558–1570.

Hawlena, H., Bashey, F., and Lively, C.M. (2010) The evolution of spite: population structure and bacteriocin-mediated antagonism in two natural populations of xenorhabdus bacteria. *Evolution* **64**:3198–3204.

Hawlena, H., Bashey, F., and Lively, C.M. (2012) Bacteriocin-mediated interactions within and between coexisting species. *Ecol Evol* **2**:2521–2526.

Heath, K.D., and Tiffin, P. (2007) Context dependence in the coevolution of plant and rhizobial mutualists. *Proc Biol Sci* **274**:1905–1912.

Herbert, E.E., and Goodrich-Blair, H. (2007) Friend and foe: the two faces of *Xenorhabdus nematophila. Nat Rev Microbiol* **5**:634–646.

Hooper, L.V., Littman, D.R., and Macpherson, A.J. (2012) Interactions between the microbiota and the immune system. *Science* **336**:1268–1273.

Hoverman, J.T., Hoye, B.J., and Johnson, P.T. (2013) Does timing matter? How priority effects influence the outcome of parasite interactions within hosts. *Oecologia* **173**:1471–1480.

Jaenike, J., Unckless, R., Cockburn, S.N., Boelio, L.M., and Perlman, S.J. (2010) Adaptation via symbiosis: recent spread of a Drosophila defensive symbiont. *Science* **329**: 212–215.

King, K.C., Brockhurst, M.A., Vasieva, O., Paterson, S., Betts, A., Ford, S.A., *et al*. (2016) Rapid evolution of microbe-mediated protection against pathogens in a worm host. *ISME J* **10**:1915–1924.

Koch, H., and Schmid-Hempel, P. (2011) Socially transmitted gut microbiota protect bumble bees against an intestinal parasite. *Proc Natl Acad Sci USA* **108**:19288–19292.

Kondo, E., and Ishibashi, N. (1986) Infectivity and propagation of entomogenous nematodes, *Steinernema* spp., on the common cutworm, *Spodoptera litura* (Lepidoptera: Noctuidae). *Appl Entomol Zool* **21**:95–108.

Koppenhofer, A.M., and Kaya, H.K. (1996) Coexistence of two steinernematid nematode species (Rhabditida:Steinernematidae) in the presence of two host species. *Appl Soil Ecol* **4**:221–230.

Lee, M.M., and Stock, S.P. (2010a) A multilocus approach to assessing co-evolutionary relationships between *Steinernema* spp. (Nematoda: Steinernematidae) and their bacterial symbionts *Xenorhabdus* spp. (gamma-Proteobacteria: Enterobacteriaceae). *Syst Parasitol* **77**:1–12.

Lee, M.M., and Stock, S.P. (2010b) A multigene approach for assessing evolutionary relationships of *Xenorhabdus* spp. (gamma-Proteobacteria), the bacterial symbionts of entomopathogenic *Steinernema* nematodes. *J Invertebr Pathol* **104**:67–74.

Lysenko, E.S., Lijek, R.S., Brown, S.P., and Weiser, J.N. (2010) Within-host competition drives selection for the capsule virulence determinant of *Streptococcus pneumoniae*. *Curr Biol* **20**:1222–1226.

Martens, E.C., and Goodrich-Blair, H. (2005) The *Steinernema carpocapsae* intestinal vesicle contains a subcellular structure with which *Xenorhabdus nematophila* associates during colonization initiation. *Cell Microbiol* **7**:1723–1735.

Martens, E.C., Heungens, K., and Goodrich-Blair, H. (2003) Early colonization events in the mutualistic association between *Steinernema carpocapsae* nematodes and *Xenorhabdus nematophila* bacteria. *J Bacteriol* **185**: 3147–3154.

Mideo, N. (2009) Parasite adaptations to within-host competition. *Trends Parasitol* **25**:261–268.

Morales-Soto, N., and Forst, S.A. (2011) The *xnp1* P2-like tail synthesis gene cluster encodes xenorhabdicin and is required for interspecies competition. *J Bacteriol* **193**: 3624–3632.

Moreira, L.A., Iturbe-Ormaetxe, I., Jeffery, J.A., Lu, G., Pyke, A.T., Hedges, L.M., *et al*. (2009) A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, Chikungunya, and *Plasmodium*. *Cell* **139**:1268–1278.

Mrácek, Z., Nguyen, K.B., Tailliez, P., Boemare, N., and Chen, S. (2006) *Steinernema sichuanense* n. sp. (Rhabditida, Steinernematidae), a new species of entomopathogenic nematode from the province of Sichuan, east Tibetan Mts., China. *J Invertebr Pathol* **93**:157–169.

Murfin, K.E., Chaston, J., and Goodrich-Blair, H. (2012) Visualizing bacteria in nematodes using fluorescent microscopy. *J Vis Exp* (68): 4298.

Murfin, K.E., Whooley, A.C., Klassen, J.L., and Goodrich-Blair, H. (2015a) Comparison of *Xenorhabdus bovienii* bacterial strain genomes reveals diversity in symbiotic functions. *BMC Genomics* **16**:889.

Murfin, K.E., Lee, M.M., Klassen, J.L., McDonald, B.R., Larget, B., Forst, S., *et al*. (2015b) *Xenorhabdus bovienii* strain diversity impacts coevolution and symbiotic maintenance with *Steinernema* spp. nematode hosts. *mBio* **6**: e00076-15.

Natsopoulou, M.E., McMahon, D.P., Doublet, V., Bryden, J., and Paxton, R.J. (2015) Interspecific competition in honeybee intracellular gut parasites is asymmetric and favours the spread of an emerging infectious disease. *Proc Biol Sci* **282**:20141896.

Oliver, K.M., Campos, J., Moran, N.A., and Hunter, M.S. (2008) Population dynamics of defensive symbionts in aphids. *Proc Biol Sci* **275**:293–299.

Oliver, K.M., Degnan, P.H., Burke, G.R., and Moran, N.A. (2010) Facultative symbionts of aphids and the horizontal transfer of ecologically important traits. *Annu Rev Entomol* **55**: 247–266.

Peters, A. (1996) The natural host range of *Steinernema* and *Heterorhabditis* spp. and their impact on insect populations. *Biocontrol Sci Tech* **6**:389–402.

Poinar, G.O.J., and Thomas, G.M. (1966) Significance of *Achromobacter nematophilus* Poinar and Thomas (Achromobacteraceae: Eubacteriales) in the development of the nematode, DD-136 (*Neoaplectana* sp. Steinernematidae). *Parasitology* **56**:385–390.

Popiel, I., Grove, D.L., and Friedman, M.J. (1989) Infective juvenile formation in the insect parasitic nematode *Steinernema feltiae. Parasitology* **99**:77–81.

Půža, V., and Mracek, Z. (2009) Mixed infection of *Galleria mellonella* with two entomopathogenic nematode (Nematoda: Rhabditida) species: *Steinernema affine* benefits from the presence of *Steinernema kraussei*. *J Invertebr Pathol* **102**:40–43.

Půža, V., and Mrácek, Z. (2010) Mechanisms of coexistence of two sympatric entompathogenic nematodes, *Steinernema affine* and *S. kraussei* (Nematoda: Steinernematidae), in a central European oak woodland soil. *Appl Soil Ecol* **45**:65–70.

Quigley, B.J.Z., Brown, S.P., Leggett, H.C., Scanlan, P.D., and Buckling, A. (2017) Within-host interference competition can prevent invasion of rare parasites. *Parasitol* **145**: 770–774.

Richards, G.R., and Goodrich-Blair, H. (2009) Masters of conquest and pillage: *Xenorhabdus nematophila* global regulators control transitions from virulence to nutrient acquisition. *Cell Microbiol* **11**:1025–1033.

Richards, G.R., and Goodrich-Blair, H. (2010) Examination of *Xenorhabdus nematophila* lipases in pathogenic and mutualistic host interactions reveals a role for *xlpA* in nematode progeny production. *Appl Environ Microbiol* **76**: 221–229.

de Roode, J.C., Pansini, R., Cheesman, S.J., Helinski, M.E., Huijben, S., Wargo, A.R., *et al*. (2005) Virulence and competitive ability in genetically diverse malaria infections. *Proc Natl Acad Sci USA* **102**:7624–7628.

Sachs, J.L., Skophammer, R.G., and Regus, J.U. (2011) Evolutionary transitions in bacterial symbiosis. *Proc Natl Acad Sci USA* **108** (Suppl. 2): 10800–10807.

Sicard, M., Le Brun, N., Pages, S., Godelle, B., Boemare, N., and Moulia, C. (2003) Effect of native *Xenorhabdus* on the fitness of their *Steinernema* hosts: contrasting types of interaction. *Parasitol Res* **91**:520–524.

Sicard, M., Brugirard-Ricaud, K., Pages, S., Lanois, A., Boemare, N.E., Brehelin, M., and Givaudan, A. (2004a) Stages of infection during the tripartite interaction between *Xenorhabdus nematophila*, its nematode vector, and insect hosts. *Appl Environ Microbiol* **70**:6473–6480.

Sicard, M., Ferdy, J.-B., Pagès, S., Le Brun, N., Godelle, B., Boemare, N., and Moulia, C. (2004b) When mutualists

are pathogens: an experimental study of the symbioses between *Steinernema* (entomopathogenic nematodes) and *Xenorhabdus* (bacteria). *J Evol Biol* **17**:985–993.
Sicard, M., Ramone, H., Le Brun, N., Pages, S., and Moulia, C. (2005) Specialization of the entomopathogenic nematode *Steinernema scaptersci* with its mutualistic *Xenorhabdus* symbiont. *Naturwissenschaften* **92**:472–476.
Sicard, M., Hinsinger, J., Le Brun, N., Pages, S., Boemare, N., and Moulia, C. (2006) Interspecific competition between entomopathogenic nematodes (*Steinernema*) is modified by their bacterial symbionts (*Xenorhabdus*). *BMC Evol Biol* **6**:68.
Snyder, H., Stock, S.P., Kim, S.K., Flores-Lara, Y., and Forst, S. (2007) New insights into the colonization and release processes of *Xenorhabdus nematophila* and the morphology and ultrastructure of the bacterial receptacle of its nematode host, *Steinernema carpocapsae*. *Appl Environ Microbiol* **73**:5338–5346.
Spiridonov, S.E., Moens, M., and Wilson, M.J. (2007) Fine scale spatial distributions of two entomopathogenic nematodes in a grassland soil. *Appl Soil Ecol* **37**:192–201.
Tarasco, E., Clausi, M., Rappazzo, G., Panzavolta, T., Curto, G., and Sorino, R. (2014) Biodiversity of entomopathogenic nematodes in Italy. *J Helminthol* **89**:359–366.
Teal, T.K., Lies, D.P., Wold, B.J., and Newman, D.K. (2006) Spatiometabolic stratification of *Shewanella oneidensis* biofilms. *Appl Environ Microbiol* **72**:7324–7330.
Team, R.C. (2013) R: a language and environment for statistical computing. URL http://www.R-project.org/.
Weiss, B., and Aksoy, S. (2011) Microbiome influences on insect host vector competence. *Trends Parasitol* **27**:514–522.
Xu, J., and Hurlbert, R.E. (1990) Toxicity of irradiated media for *Xenorhabdus* spp. *Appl Environ Microbiol* **56**:815–818.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Bacterial strains, plasmids and primers used in this study.

Table S2. Assessment of bacterial–bacterial interactions mediated through antimicrobial production[a]

Table S3. *S. feltiae* nematode growth and reproduction *in vitro* on lawns of *X. bovienii* symbionts.

Table S4. *S. feltiae* adult nematodes survive on media preconditioned by Xb-Sa-78[a].

Table S5. Impact of *X. bovienii* symbionts of clade I and clade III nematodes on *S. feltiae* nematode survival and development into adults.

Figure S1. *Steinernema* nematodes and *Xenorhabdus* bacteria lifecycle. The schematic above shows the combined lifecycle of the nematodes (grey) and bacteria (red) in the insect host (tan). In the soil environment, IJ nematodes carry the bacteria in their intestine as they seek and invade insect hosts. Once inside the insect host environment, IJ nematodes recover into juveniles with open mouths and anuses and release their bacterial symbiont. The nematodes also begin consuming bacteria and nutrients. The nematodes and bacteria then kill the insect host and reproduce within the cadaver, including adult, egg and juvenile stages. Once nutrients are limiting the nematodes form the next generation of progeny IJs that exit the cadaver.

Figure S2. Superinfection of cadavers by *S. feltiae* nematodes. Insects that were alive, freeze killed or previously infected by *S. feltiae or S. affine* nematodes were exposed to *S. feltiae* nematodes carrying GFP-expressing symbiont. The relative fluorescence per insect cadaver is shown as fold change (± 1 s.e.) over insects not exposed to *S. feltiae* FL nematodes. The bar graph shows the average of three biological replicates with five technical replicates per condition. No significant differences were detected among insects dead at the time of nematode exposure.

Figure S3. Xb-Sa-78 localizes to *S. feltiae* nematode eggs. In some *S. feltiae* gravid female nematodes, Xb-Sa-78 bacteria were present near the eggs within the nematodes, either in the uterus or the nematode body cavity surrounding the uterus. Micrographs show the phase contrast (A), GFP (B), and overlay of phase contrast and GFP (C) of a female nematode with bacteria in this location. Abbreviations denote nematode anatomy: v (vulva) and g (gonad arm). The arrowhead points to an individual nematode egg within the uterus. Scale bars represent 50 μm.

Figure S4. *S. feltiae* nematode intestinal morphology after exposure to *X. bovienii* strains. Population frequency distribution for normal intestine, constricted intestine, transparent intestine, swollen intestine and others. Means between groups were compared using randomized block one-way ANOVA and Tukey's Multiplicity Comparison test, and letters denote statistical significance between means of the groups.