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# Spiteful interactions between sympatric natural isolates of *Xenorhabdus bovienii* benefit kin and reduce virulence

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## Abstract

Spite occurs when an individual harms itself in the act of harming others. Spiteful behaviour may be more pervasive in nature than commonly thought. One of the clearest examples of spite is the costly production and release of bacteriocins, antimicrobial toxins noted for their ability to kill conspecifics. A key question is to what extent these toxins provide a fitness advantage to kin of the producer cell, especially in natural communities. Additionally, when bacteria are involved in parasitic relationships, spiteful interactions are predicted to lower bacterial densities within a host, causing a reduction in parasite-induced virulence. Using five sympatric, field-collected genotypes of the insect pathogen *Xenorhabdus bovienii*, we experimentally demonstrate that bacteriocin production benefits kin within the host, and that it slows the mortality rate of the host. These results confirm that spite among naturally coexisting bacterial clones can be a successful kin-selected strategy that has emergent effects on virulence.

## Introduction

The view of spite held by evolutionary biologists has undergone a transition in recent years. This social behaviour, whereby both the actor and recipient are harmed, was long viewed as theoretically possible, but unlikely to occur in nature (Hamilton, 1970; West & Gardner, 2010). To evolve, spite requires conditions that are more restrictive than those needed to favour altruism, as the link between the costly action and the benefit gained by kin is indirect (Foster *et al.*, 2001). Recent theory and experimental work have shown that the feasibility of this link may not be so unlikely. Greenbeard mechanisms allow for nonkin to be targeted as the recipients of spiteful acts (Gardner & West, 2010), whereas spatial structure resulting in local competition allows for kin of the actor to benefit (Gardner & West, 2004).

One of the clearest examples of spite is the production of allelopathic or anticompetitor toxins, such as bacteriocins (Gardner & West, 2010; West & Gardner, 2010). Bacteriocins are distinguished from other antimicrobial compounds by their ability to kill even closely related strains of the same species (Riley *et al.*, 2003). Clone mates of the producing cell are not susceptible to the bacteriocin, either due to the constitutive production of an immunity protein or due to the lack of the appropriate target site (Feldgarden & Riley, 1999). Bacteriocin production can be considered spiteful as bacteriocin-producing cells harm themselves in the act of harming susceptible cells. Lysis is often required for release of the toxin (Riley & Wertz, 2002), but even when cell death is not required, toxin production can be energetically costly (Wloch-Salamon *et al.*, 2008), and it can outweigh any direct benefit to the producer cell (Inglis *et al.*, 2011). Because bacteriocins or similar allelopathic compounds are produced in all microbial lineages, much excitement has been generated about the possibility of spite in nature (Hawlena *et al.*, 2010b) and its role in maintaining intraspecific diversity (Kerr, 2007). Furthermore, these spiteful interactions can potentially affect other members of the community as, for example, when the dynamics of a bacterial population have emergent effects on the survival of their host (Gardner *et al.*, 2004).

The evolution of bacteriocin production depends on whether the kin of the bacteriocin-producing cell can benefit from the spiteful behaviour. Elegant laboratory studies have shown that, in well-mixed populations, the

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resources freed by the death of sensitive competitors are available to nonkin and that costly toxin production may provide little benefit to the producing clone (Chao & Levin, 1981; Greig & Travisano, 2008). Although spatial structure increases the likelihood that toxin production is favoured (Chao & Levin, 1981; Inglis *et al.*, 2011), lowered densities can override this effect (Greig & Travisano, 2008). Additionally, resource levels may affect the costs and benefits of bacteriocin production (Frank, 1994) or change the facultative expression of production or susceptibility (Wloch-Salamon *et al.*, 2008). Thus, to understand the evolution of bacteriocin production in nature, it is necessary to examine its indirect fitness benefits in an ecologically relevant competitive arena.

When bacteria are involved in symbiotic interactions, the relevant competitive arena is the within-host environment. Nevertheless, *in vivo* studies showing a kin benefit of bacteriocin production are exceedingly rare. In fact, many studies on *Escherchia coli* questioned the functional role of bacteriocins due to the lack of supporting *in vivo* data (reviewed in Kirkup, 2006), which has only been obtained relatively recently (Kirkup & Riley, 2004). *In vivo* studies also provide an opportunity to examine the emergent effects of bacteriocin production. The cost of bacteriocin production and its lethal effects may lower the total bacteria population within the host, which, when bacteria are pathogenic to their host, may result in reduced virulence (Gardner *et al.*, 2004; Inglis *et al.*, 2009). A reduction in host-mortality rate associated with bacteriocin activity has been shown in two different insect pathogenic bacteria (Massey *et al.*, 2004; Vigneux *et al.*, 2008; Inglis *et al.*, 2009). Most compellingly, Inglis *et al.* (2009) also found that this reduction in virulence was accompanied by lowered within-host population growth rates due to bacteriocin activity.

In the present study, we provide an experimental demonstration that spiteful interactions benefit kin *in vivo* and cause a reduction in the mortality rate of the insect host. Uniquely, our study focuses on sympatric natural isolates of a single species of insect pathogenic bacteria, *Xenorhabdus bovienii* that were collected at a spatially relevant scale. We identified two isolates that produce a bacteriocin, which can inhibit the growth of a third isolate in a noncompetitive, *in vitro* assay, as well as two other isolates that do not (Hawlena *et al.*, 2010a). We first tested the hypothesis that the ability to produce an inhibitory bacteriocin *in vitro* confers a fitness benefit to kin *in vivo* by examining the fitness of the two inhibitory and noninhibitory isolates relative to the focal isolate in paired competition assays. To control for differences among the isolates not due to bacteriocin activity, we also grew each competitor isolate alone as well as in competition against a resistant strain of a second *Xenorhabdus* species. Finally, we tested the hypothesis that spiteful interactions lower virulence by comparing the rate of insect-host death in single-isolate and mixed-isolate infections. Our results suggest that spiteful interactions are favoured because they provide a competitive benefit to kin in within-host competition and confirm that these interactions can significantly delay host mortality.

## Methods

### Study system

*Xenorhabdus bovienii* is an insect-killing bacterial species carried by entomopathogenic nematodes in the genus *Steinernema* (Tailliez *et al.*, 2006). The nematodes carry the bacteria from one insect host to the next. Upon finding a new host, the nematodes penetrate the insect midgut and release *Xenorhabdus* into the insect hemolymph (Sicard *et al.*, 2004). Both the nematodes (Goetz *et al.*, 1981; Burman, 1982; Simoes, 2000) and bacteria (Boemare & Akhurst, 1988; Dunphy & Webster, 1988) produce factors to overcome the immune system of the insect, and insect death ensues within a few days of infection. The nematodes and bacteria live independently within the insect, each reproducing until the resources of the dead insect are used up, at which time the bacteria and the nematodes reassociate and leave the insect. Each transmission-stage nematode carries only one or two clonal lineages of bacteria (Martens *et al.*, 2003). As successful transmission requires that multiple nematodes enter the hemocoel and reproduce sexually inside the insect, infection may involve multiple *Xenorhabdus* genotypes. Nematodes can persist in the soil for months and may travel a few metres in search of new insect hosts (Schroeder & Beavers, 1987; Strong, 2002).

*Xenorhabdus bovienii* carries the genes that encode xenorhabdicin, a phage-tail-like particle similar to *Pseudomonas* R-type pyocins. Xenorhabdicin produced by *Xenorhabdus nematophila* has been found to have a narrow killing breadth (Boemare *et al.*, 1992; Thaler *et al.*, 1995), and gene inactivation has demonstrated its importance in interspecific competition (Morales-Soto & Forst, 2011). These high molecular weight particles are thought to be costly to produce and are assumed to be released by lysis (Morales-Soto & Forst, 2011).

### Bacteria isolates

The five isolates of *X. bovienii* used in this study were obtained from separate soil samples collected in 2007 from Indiana University Research and Teaching Preserve, Moore's Creek, Monroe County, Indiana as described in Hawlena *et al.* (2010a). Briefly, each soil sample was baited with a larva of the greater wax moth (*Galleria mellonella*), and a single bacteria colony was isolated from nematodes emerging from each caterpillar carcass. Each bacteria colony was described to species by sequencing of the 16S rRNA gene and identified as a distinct genomic fingerprint type by banding patterns based on PCR amplification of enterobacterial repetitive intergenic

consensus sequences (Tailliez *et al.*, 2006; Hawlena *et al.*, 2010a).

The nematode species association of each bacteria isolate was determined by sequencing the 28S rRNA gene of their source nematodes (Stock *et al.*, 2001). Based on the 28S sequences, the nematodes may represent two previously undescribed species in the genus *Steinernema* (S. P. Stock, personal comunication). Each species clearly aligns to a different clade within the genus (Uribe-Lorio *et al.*, 2007). BLAST searches of 28S sequences place the species in Clade 1 as a sister taxon to *S. affine*, and the species in Clade 3 as a sister taxon to *S. kraussei*. The potential for spiteful interactions among the isolates was characterized by an *in vitro* assay, whereby each isolate was grown clonally and induced with mitomycin C. Cell-free supernatants from the induced cultures (actor) were then tested against potential sensitive cultures (recipient) by examining for growth inhibition by placing a drop of the actor supernatant on soft agar seeded with the recipient colony (Hawlena *et al.*, 2010a). SDS–PAGE analysis of these supernatants shows banding patterns characteristic of the sheath and tail fibre proteins of xenorhabdicin (S. Forst and F. Bashey, unpublished data).

## Experimental design

The five isolates were used in single and mixed infections. Four types of mixed infections were conducted, representing a paired infection of the focal isolate and one of four possible competitors (Table 1). Two competitors were found to produce a bacteriocin capable of inhibiting the growth of the focal isolate, and two were not. No bacteriocin production was detected from mitomycin C induction of the focal isolate, either in its original characterization (Hawlena *et al.*, 2010a) or in two subsequent inductions. We chose two replicates of each bacteriocin phenotype, one associated with the same species of nematodes as the focal isolate, and another associated with a different nematode species (Table 1). Consistent differences between the inhibitory and noninhibitory isolates despite differences due to their symbiont associations implicate bacteriocin activity. In addition, we examined the growth of each competitor isolate against a control strain involved in no bacteriocin activity with the competitor isolates either as an actor or as a recipient.

**Table 1** Characteristics of the *Xenorhabdus bovienii* isolates used in this study. All bacteria isolates were obtained from soil samples taken from a single hillside in 2007.

| | Inhibition of focal isolate | Inhibition of control strain | Nematode symbiont | Isolate name |
|---|---|---|---|---|
| Focal isolate | | | Clade 1 | B66a |
| Noninhibitory isolates | | | | |
| N1 | No | No | Clade 1 | B52b |
| N3 | No | No | Clade 3 | B44b |
| Inhibitory isolates | | | | |
| I1 | Yes | No | Clade 1 | B78a |
| I3 | Yes | No | Clade 3 | B59a |

Two replicate infection assays were conducted, consisting of each isolate used in a single infection, the four mixed infections and a PBS-injected control (1 × phosphate-buffered saline). In each infection assay, 20 caterpillars of *G. mellonella* (Vanderhost Wholesale Inc., St Marys, OH, USA) were infected in each treatment, for a total of 200 caterpillars per assay. To prepare the inoculating dose, bacteria were grown to stationary phase in lysogeny broth and diluted in PBS to total dose of $1 \times 10^4$ cells per caterpillar delivered in a total volume of 20 μL by a 30-gauge needle. Mixed infections were inoculated similarly by preparing an equal frequency mixture of the two isolates comprising a total dose of $1 \times 10^4$ cells per caterpillar. Initial doses were confirmed by diluting and plating each treatment as described below. Caterpillars were kept at 20 °C and monitored for mortality at regular intervals for 40 h post-infection. Host death was indicated when the caterpillar failed to move in response to probing.

To determine the outcome of within-host competition in each infection assay, six caterpillars were randomly sampled after their death in each mixed treatment. Each caterpillar was surface sterilized by placing it in a microcentrifuge tube filled with 1 mL of 70% ethanol and vortexing for 15 s. The caterpillar was then transferred to a new tube filled with 500 μL of 1 × PBS and homogenized with a pestle. The tube was then centrifuged at 800 *g* for 3 min, and 100 μL of a $1 \times 10^{-5}$ dilution of hemolymph was plated on NBTA containing 50 μg $mL^{-1}$ of ampicillin [nutrient agar supplemented with 0.0025% (w/vol) bromothymolblue and 0.004% (w/v) triphenyltetrazolium chloride, pH = 8] to exclude the gut bacteria of the caterpillars. Isolates were distinguished by also plating 100 μL onto NBTA concurrently with 100 μL of a selective bacteriocin. For each mixed infection, a nonselective plate and one or two selective plates were used. In addition to the selective effect of the *X. bovienii* bacteriocins, the focal isolate could be distinguished from N1, N3 and I3 due to their differential sensitivity to the bacteriocins produced by *Xenorhabdus koppenhoeferi* 43 and 46. Additionally, the focal isolate could be distinguished due to a distinct colour morphology (maroon vs. blue, green or turquoise). Colony counts were averaged based on three replicates of each plate type. To control for differences among the competitor isolates apart from their inhibitory effect on the focal isolate, hosts were also sampled in each single-infection treatment.

As a second control, each competitor isolate was also placed in competition in a mixed infection with a control strain of *X. koppenhoeferi* S0. The control strain was isolated in the same field sampling as the *X. bovienii*

isolates and distinguished by a unique genomic fingerprint (Hawlena *et al.*, 2010a). Repeated inductions ($n = 4$) showed no bacteriocin production by the control strain nor were any of the competitor isolates able to inhibit the control strain. Infections with the control strain were performed and caterpillar hosts sampled as described earlier. Multiple dilutions of hemolymph were plated, and competitors were distinguished from the control strain by colony colour (Bashey *et al.*, 2011) and differential sensitivity to plates containing 50 μg $mL^{-1}$ of ampicillin.

### Statistical analyses

The performance of the competitor isolates relative to the focal isolate was calculated as the difference of the population growth rates of each clone: $m_{competitor} - m_{focal}$ (defined as the selection-rate constant, Lenski *et al.*, 1991). Where the growth rate (m) is defined as ln(final cell density/initial cell density) in each host over the course of the experiment. To determine whether performance differed between the inhibitory and noninhibitory isolates, we performed a mixed-model ANOVA for bacteria isolated from each nematode clade using SAS v. 9.2. Infection assay was included as a random effect. Similarly, we performed ANOVAs to compare the growth rate of the inhibitory and noninhibitory competitor isolates to the control strain. We performed Cox (proportional hazards) regressions using the PHREG procedure in SAS to test for differences in mortality rate between single and mixed infections for each pairing between the focal and competitor isolates.

## Results

The inhibitory isolates grew significantly better than the noninhibitory isolates when inoculated into a caterpillar with the focal isolate (Fig. 1a). This growth advantage was highly significant in isolates associated with either the same (Clade 1, $F_{1,21} = 63.30$, $P < 0.0001$) or different (Clade 3, $F_{1,21} = 157.91$, $P < 0.0001$) nematode species as the focal isolate, supporting the hypothesis that bacteriocin production confers a fitness advantage to kin in within-host competition. In contrast, when inoculated singly into caterpillars, the inhibitory isolates did not differ in growth from noninhibitory isolates (Clade 1, $F_{1,32} = 0.032$, $P = 0.85$; Clade 3, $F_{1,32} = 0.96$, $P = 0.3360$, Fig. 1b). Further, the inhibitory isolates had no growth advantage over the noninhibitory isolates when inoculated into a caterpillar simultaneously with the resistant control strain (Fig. 1c). In fact, whereas the Clade 3 pairing showed no difference in relative growth ($F_{1,18} = 0.68$, $P = 0.4220$), the Clade 1 pairing showed lower relative growth of the inhibitory isolate ($F_{1,22} = 78.90$, $P < 0.0001$).

Comparisons of the mortality rate of caterpillars infected with each isolate singly or in a mixed inoculation showed a significant difference between the inhibitory infections and the noninhibitory infections (Fig. 2). When the mixed infections occurred between the focal isolate and a noninhibitory competitor (Fig. 2a,b), the mortality rate of insects did not significantly differ from those infected with the competitor isolate alone (Table 2). In contrast, when mixed infections occurred between the focal isolate and an inhibitory competitor (Fig. 2c,d), insect mortality rate was significantly reduced by one-half or more (Table 2). These differences were

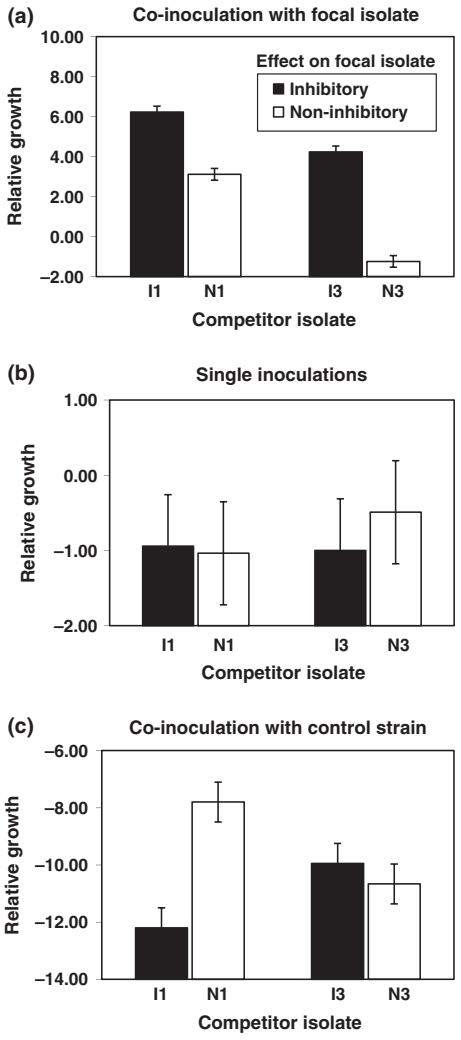


**Fig. 1** Growth of each competitor isolate relative to (a) the focal isolate when inoculated simultaneously into caterpillars, (b) the focal isolate when inoculated singly into caterpillars and (c) the control strain when inoculated simultaneously into caterpillars as a function of whether the competitor isolate can produce an inhibitory bacteriocin against the focal isolate (filled bars) or not (open bars). Relative growth is the growth rate [ln (final cell density/initial cell density)] of the competitor isolate minus the growth of the focal isolate (or control strain) over the course of the experiment. Error bars represent 1 SE.

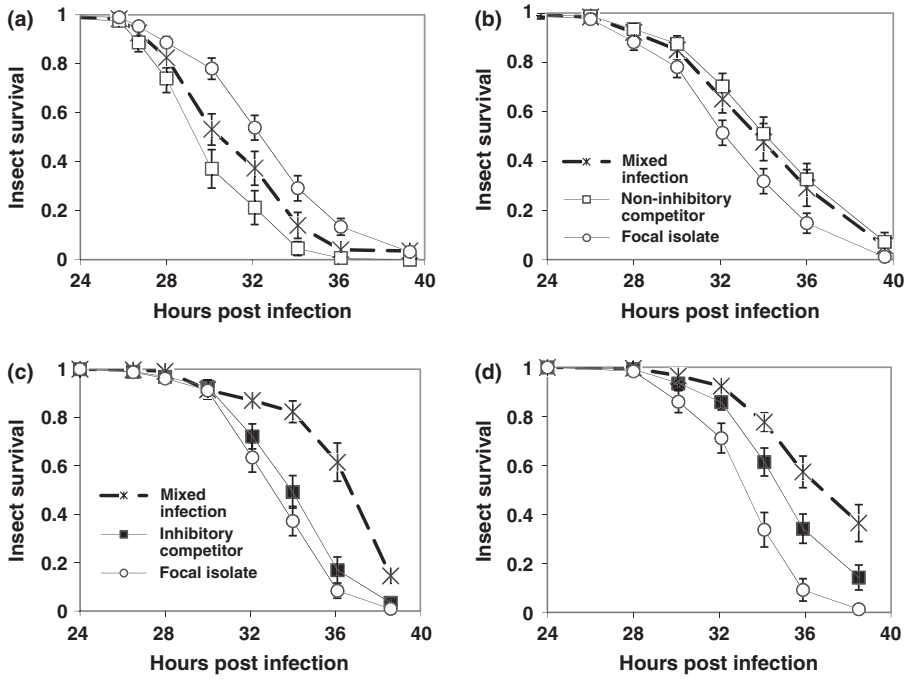


**Fig. 2** Insect survival probability (±1 SE) in single and mixed infections between the focal isolate (open circles) and one of four competitor isolates. Single infections with each noninhibitory competitor (open squares) and its mixture (x's) with the focal isolate are shown in (a) N1 isolate and (b) N3 isolate. Single infections with each inhibitory competitor (black squares) and its mixture (x's) with the focal isolate are shown in (c) I1 isolate and (d) I3 isolate. Proportions shown are pooled over two replicate infection assays.

**Table 2** Results of Cox regression models testing for differences in mortality rate between single (noninhibitory or inhibitory competitor isolates) and mixed (focal and competitor isolates) infections.

| | Assay 1 | | Assay 2 | |
|---|---|---|---|---|
| | Hazard ratio* | *P*-value | Hazard ratio* | *P*-value |
| Noninhibitory isolates | | | | |
| N1 | 1.014 | 0.9665 | 0.657 | 0.2524 |
| N3 | 0.700 | 0.2792 | 1.425 | 0.2730 |
| Inhibitory isolates | | | | |
| I1 | 0.388 | 0.0195 | 0.461 | 0.0410 |
| I3 | 0.337 | < 0.0001 | 0.530 | < 0.0001 |

*Hazard ratios are given for insects infected with a mixture of two isolates (focal and competitor isolates) relative to the single-isolate infections. Hazard ratios <1 indicate that insects infected with a mixture of two isolates took longer to die than insects infected with each isolate alone.

consistent across two replicate infection assays for each isolate and regardless of the nematode association.

## Discussion

Although the ubiquity of bacteriocins and other antimicrobials compounds has long been viewed as testament to their importance in determining competitive outcomes, the functional roles of these compounds are quite diverse and are understudied in natural contexts (Martínez, 2008; Mlot, 2009). Here, we show that bacteriocin activity is associated with a competitive benefit to kin in an ecologically relevant competitive arena, the within-host environment. Further, by examining the performance of these isolates when inoculated singly into a caterpillar or simultaneously with a resistant control strain, we demonstrate that the within-host benefit of bacteriocin production is dependent on competition with a sensitive competitor. Finally, we found that bacteriocin activity resulted in a reduced mortality rate of the insect host. As our study focuses on sympatric natural isolates, these findings support the important role of bacteriocin-mediated interactions in natural communities of bacteria.

Our study also adds to the growing body of work suggesting that spiteful behaviour may be more common in nature than previously thought (Gardner & West, 2004, 2010). In the social insects, spite is widespread, and it has been shown to be important in maintaining nonreproductive workers, altering sex ratios and influencing queen genotypic diversity (Foster *et al.*, 2001). Additionally, segregation distorters that operate via lethal effects, such as the *Medea* gene in *Tribolium* or *Wolbachia*-mediated cytoplasmic incompatibilities, can be viewed as a form of spite (Hurst, 1991; Frank, 1995). In general, any mechanism of antagonism or interference competition can be viewed as spiteful behaviour, if the actor

receives no net direct benefit from the harming action. Bacteriocin-mediated interactions between sympatric *Xenorhabdus* isolates provide another example of spite; the phage-tail-like bacteriocins produced by *Xenorhabdus* are large and unlikely to be released without lysis or an otherwise net fitness cost to the producer cell. The experimental evidence we present here suggests that the high frequency of bacteriocin production observed in this population (Hawlena *et al*., 2010a) may be maintained by benefits received by kin of the spiteful producers.

Crucially, our study shows that these indirect benefits occur in a relevant competitive arena with genotypes that are likely to interact in nature. Moreover, by demonstrating that the growth advantage of the inhibitory isolates is conditional on interacting with susceptible competitors (compare Fig. 1a–c), the data suggest that the evolutionary dynamics of spiteful behaviour in this population will depend critically on the probability of interaction with specific genotypes. For example, we might expect an inhibitory genotype to replace a susceptible genotype, but that this dominance may not persist in the face of invasion by a resistant genotype. In fact, such fluctuating dynamics have been shown experimentally with otherwise isogenic bacteriocin producer, sensitive and resistant strains of *E. coli* (Kerr *et al*., 2002; Kirkup & Riley, 2004).

Inhibitory interactions between genotypes within a host may also affect the transmission dynamics in this population. We show that the mortality rate of infected insect hosts was dramatically reduced in the presence of inhibitory interactions (Fig. 2c,d). This delay in the timing of insect death could result in an unsuccessful infection. In experimental populations of the closely related bacteria *X. nematophila* and its nematode symbiont (Bashey & Lively, 2009), we have found that delayed insect mortality results in a significantly lower probability of successful parasite emergence (F. Bashey, unpublished data). Further, in a more resistant insect species, it is possible that reduced growth of the bacteria due to bacteriocin interactions could result in the insect host successfully clearing the infection. Our work thus strengthens the importance of including within-host interactions in our approaches to understanding disease dynamics.

In conclusion, our study demonstrates the importance of bacteriocins to within-host competitive success of sympatric isolates of *X. bovienii*. Inhibitory isolates had more than a two-fold greater growth advantage in within-host competition relative to the noninhibitory isolates. Although this kin benefit of bacteriocin production is predicted to lead to its increase in the population, we also demonstrate that this benefit disappears in the absence of a susceptible competitor. Further, we show that spiteful interactions within a host delay the virulent effects of the bacteria. In total, our work supports the view that spiteful interactions affect both intraspecific diversity and community dynamics in nature.

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