# Natural History Note

## Spiteful Interactions in a Natural Population of the Bacterium *Xenorhabdus bovienii*

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abstract: An individual behaves spitefully when it harms itself in the act of harming other individuals. One of the clearest potential examples of spite is the costly production and release of toxins called bacteriocins. Bacteriocins are toxins produced by bacteria that can kill closely related strains of the same species. Theoretical work has predicted that bacteriocin-mediated interactions could play an important role in maintaining local genetic and/or species diversity, but these interactions have not been studied at biologically relevant scales in nature. Here we studied toxin production and among-strain inhibitions in a natural population of *Xenorhabdus bovienii*. We found genetic differences and inhibitions between colonies that were collected only a few meters apart. These results suggest that spite exists in natural populations of bacteria.

*Keywords:* bacteriocins, spatial scale, genotypic variability, coinfection, entomopathogenic bacterium.

### Introduction

Spiteful behaviors are those that are harmful to both the actor and the recipient. It has generally been assumed that the conditions required for spite to evolve are too restrictive (reviewed in Hamilton 1970, 1996; Keller et al. 1994), but this assumption has been challenged in recent years by a handful of examples of spite from social insects (reviewed in Foster et al. 2001; Gardner and West 2004*b*, 2006). One such example involves worker policing in hymenopteran societies. Although typically unable to mate, workers in most hymenopteran societies have functioning ovaries that allow them to produce males from unfertilized eggs. However, some nonreproductive workers prevent other workers from producing male progeny through aggression or by eating their eggs. The workers' behavior is considered to be spiteful because they invest time and energy to kill other workers' sons with no direct reproductive benefit to themselves (Ratnieks 1988; Foster et al. 2001). As in this example, most examples in social insects are cases of "weak spite," because the spiteful individuals usually have little to lose with respect to direct fitness (Hamilton 1971). "Strong spite," a behavior that demands high costs for the actor, is more difficult to explain (Hamilton 1971). The best-documented example for strong spite comes from the sterile soldier caste of polyembryonic parasitic wasps in the hymenopteran family Encrytidae. In some (but not all) species, some of the wasp larvae develop precociously as soldiers, giving up their own future reproduction in order to kill competitors, including opposite-sex siblings. Soldiers are capable of recognizing their kin, and they preferentially attack nonkin larvae (Gardner and West 2004*a*; Gardner et al. 2007).

The production and release of bacteriocins provides another potential example of strong spite (West et al. 2006). Bacteriocins are extracellular toxins, produced by almost all bacteria, that can kill other strains of bacteria. Bacteriocin-mediated interactions can be considered spiteful because the actor cells die or pay substantial costs to produce the toxins (Riley and Wertz 2002). Theoretical work has suggested that bacteriocin-mediated interactions could play an important role in nature because they promote local species (or genetic) diversity (Kerr 2007). For example, bacteriocins might promote local diversity when producer, sensitive, and resistant strains are engaged in a version of the rock-paper-scissors game (i.e., the producer can kill the sensitive strain, the resistant strain outcompetes the producer, and the sensitive strain outcompetes the resistant strain) in a spatially structured environment (e.g., Kerr et al. 2002; Kirkup and Riley 2004). Alternatively, if there are multiple producers, each producer might occupy a certain area and prevent the invasion of the other producer (e.g., due to inherent bistabilities in the population dynamics; Pagie and Hogeweg 1999; Czaran et al. 2002; Johnson and Seinen 2002).

At present, most of the empirical evidence for bacteriocin-mediated interactions has been documented from inhibition assays between strains that were isolated from

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a variety of host species and/or locations against indicator lab strains (Booth et al. 1977; Gaston et al. 1989; Farias et al. 1992; Gordon et al. 1998, 2007; Lima et al. 2002; Nigutova et al. 2005; Lux et al. 2007; Nes et al. 2007). However, it is not enough for bacteriocin-producer and bacteriocin-susceptible strains to exist in order for spite to evolve; the strains also need to coexist over biologically relevant scales (Gardner and West 2010). Here, we establish the existence of these interactions at biologically relevant spatial scales in nature, using the entomopathogenic bacterium *Xenorhabdus bovienii*. We studied toxin production and among-strain antagonism in a natural population of *X. bovienii* by conducting inhibition assays for pairs of bacterial colonies that were isolated from locations that were centimeters to meters apart. We found toxin-mediated inhibitions between *X. bovienii* colonies that were collected only a few meters apart. We also found genetic differences among these colonies. Given that bacteriocins are produced in almost all lineages of bacteria, these results suggest that spiteful behavior may be more common in nature than previously thought.

## Methods

### *Study System*

*Xenorhabdus bovienii* is an insect-killing bacterial species carried by entomopathogenic nematodes from the genus *Steinernema* (Tailliez et al. 2006). The nematodes release the bacteria after infecting an insect host. Following their release, the bacteria grow rapidly, and the arthropod host dies within a few days. The nematodes reproduce rapidly within the dead host for one or more generations, consuming the bacteria and host tissue. Thousands of transmission-stage juvenile worms leave the host approximately 2 weeks after infection, each carrying an inoculum of bacteria (Burnell and Stock 2000). *Xenorhabdus* bacteria are known to release toxins (bacteriocins) that inhibit the growth of susceptible strains of the same species. Evidence suggests that lysis (suicide) is required in *Xenorhabdus* sp. in the process of releasing the toxin (Thaler et al. 1997). But the production and secretion of these toxins would carry some cost, even if the cell were not lysed (Gardner and West 2004*b*; Gardner et al. 2004).

### *Bacteria Isolates*

We collected two soil samples (4 m apart) at the Indiana University Research and Teaching Preserve, Moore's Creek, Monroe County, Indiana, using a soil corer. Each soil core was 8 cm in diameter and 5 cm in depth. We brought the soil samples to the lab and placed them into separate petri dishes (140 mm × 25 mm). We then placed two moth larvae (*Galleria mellonella*) into each dish (hosts 1 and 2 and hosts 3 and 4 were placed on soil samples 1 and 2, respectively). Infected hosts were kept at 22°C; as soon as they died (up to 48 h postinfection), we transferred the hosts to modified White traps for collection of emerging nematodes (following Bashey et al. 2007). Nematodes began to emerge approximately 12–28 days after infection. Two months after the host died, approximately 2,000 nematodes were crushed to extract bacteria from each insect host sample.

Nematodes were surface sterilized in 2% NaOCl for 3 min and rinsed four times with sterile $H_2O$ before being crushed with a Kontes Pellet Pestle and sterile sand. The extracted bacteria were diluted in lysogeny broth medium (LB) and plated on nutrient agar supplemented with one solid pellet of sodium hydroxide, 0.004% (w/v) triphenyltetrazolium chloride, and 0.0025% (w/vol) bromothymol blue (NBTA). Nine colonies were isolated from each insect host sample, preserved in 20% glycerol at −80°C, and used in the growth-inhibition assays described below. *Xenorhabdus bovienii* colonies were identified by sequencing of 16S rDNA (Tailliez et al. 2006). This experimental design resulted in 36 field isolates of *X. bovienii* and allowed us to examine bacteriocin-mediated antagonism between pairs of bacterial colonies that are likely to interact when they encounter the same insect host.

### *Inhibition Assays*

We performed 1,260 growth-inhibition assays to determine the pairwise relationships between colonies that were isolated (1) from the same insect host ($N = 288$), (2) from two hosts that were infected by exposure to the same soil sample (host 1 vs. host 2, host 3 vs. host 4; $N = 324$), or (3) from two hosts that were infected by exposure to soil samples collected 4 m apart (host 1 vs. hosts 3 and 4, host 2 vs. hosts 3 and 4; $N = 648$; fig. 1).

We estimated the probability of a false-positive response (i.e., the probability of finding an inhibition where there is none) based on self-tests and negative-control tests. In the 36 self-tests, where the same isolate was used both as the recipient and as the actor (diagonal in fig. 1), no inhibition should be observed, as bacteriocin-producing clones carry immunity to their own toxin (Riley and Chavan 2007). In the 108 negative-control tests, we tested each isolate's response as a recipient by applying (at three different occasions) an actor supernatant that was produced at the same time and according to the same protocol (see below) but without the addition of bacteria. We estimated the probability of a false-negative response (i.e., the probability of failing to find a true inhibition) based on 160 positive-control tests that were performed at the same time and according to the same protocol that was

| | | | Recipient isolate | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Soil sample 1 | | | | | | | | | | | | | | | | | | Soil sample 2 | | | | | | | | | | | | | | | | | |
| | | | Host 1 | | | | | | | | | Host 2 | | | | | | | | | Host 3 | | | | | | | | | Host 4 | | | | | | | | |
| Actor isolate | | | a | b | c | d | e | f | g | h | i | a | b | c | d | e | f | g | h | i | a | b | c | d | e | f | g | h | i | a | b | c | d | e | f | g | h | i |
| Soil Sample 1 | Host 1 | a | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I |
| | | b | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I |
| | | c | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I |
| | | d | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I |
| | | e | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I |
| | | f | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I |
| | | g | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I |
| | | h | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I |
| | | i | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I |
| | Host 2 | a | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I |
| | | b | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I |
| | | c | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I |
| | | d | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I |
| | | e | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I |
| | | f | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I |
| | | g | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I |
| | | h | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I |
| | | i | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I |
| Soil Sample 2 | Host 3 | a | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | b | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | c | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | d | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | e | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | f | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | g | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | h | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | i | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | Host 4 | a | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | b | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | c | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | d | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | e | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | f | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | g | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | h | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | i | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

**Figure 1:** Bacteriocin-mediated antagonism among *Xenorhabdus bovienii* isolates from four infected insect hosts. Hosts 1 and 2 were infected by the same soil sample, which was 4 m away from the soil sample used to infect hosts 3 and 4. "I" indicates inhibition of recipient growth by cell-free extracts of actor cultures, whereas "0" indicates interactions in which the recipient growth was not affected by cell-free extracts of actor cultures. The letters a–i represent different colonies that were isolated from each insect host.

applied in the other growth-inhibition assays. For these tests, we used two strains that had shown repeatable inhibitions in a prior study: a field isolate of *X. bovienii* as the recipient and *Xenorhabdus nematophila* (strain HGB 801 ACCT 19061) as the actor (H. Hawlena, unpublished data).

We employed a modified version of Pugsley and Oudega's (1987) method, which uses mitomycin C to induce the production of bacteriocins in each field isolate. By using induced toxins, as opposed to competition trials, we tested for the existence of antagonistic weapons rather than for the facultative release of the toxins. We chose this method over induction by heat or induction at stationary phase of the cultures as it (1) detects the largest number of bacteriocin producers (Riley et al. 2003), (2) has the highest repeatability (84%–100%; H. Hawlena, unpublished data), and (3) is the most common induction method employed in growth-inhibition assays (reviewed by Riley and Chavan [2007]), thus allowing us to compare the results to other studies.

Cells in log-phase growth were centrifuged and pellets resuspended in LB to a density of $5 \times 10^8$ cells/mL. We then incubated the cells with mitomycin C (0.5 $\mu$g/mL) at 28°C. After 5 h, 67 $\mu$L of chloroform was added to the

induced cultures, which were then centrifuged for 10 min at 13,000 rpm. We filtered the resulting supernatant through a 0.45-$\mu$m HT Tuffryn membrane and stored it at 4°C less than 20 days before use; bacteriocins of *X. bovienii* bacteria can be stored for more than 1 year without decaying at that temperature (H. Hawlena, unpublished data). To test the sensitivity of a clone, molten soft (MS) agar (0.6% agar) was sowed with 2% (v/v) of its stationary-phase liquid culture. Then, 10 $\mu$L of supernatant of actor isolates was spotted onto the surface of an MS agar plate containing the potential sensitive clone. Plates were incubated for 48 h, at which time inhibition could be visualized as a clear zone on the recipient lawn.

Actor extracts that killed the recipient cells could contain bacteriocins or bacteriophage. To distinguish between the two causes of inhibition, serial dilutions (dilution factors: $10^1$, $10^3$, $10^6$) were performed for the actors that could reliably be identified as bacteriocin producers (see "Results"). Appearance of individual plaques indicates that the extract contains bacteriophage, whereas zones of clearings, becoming increasingly opaque, indicate bacteriocin production (Gordon and O'Brien 2006). All dilution series showed the latter response, and we thereby concluded that the observed inhibitions are due to bacteriocin production.

### *Genetic Characterization of Strains*

Enterobacterial Repetitive Intergenic Consensus Sequence (ERICs) and BOX-element PCR profiles were determined for two randomly selected isolates from each insect host. Two replicate DNA extractions were performed for each isolate, using Promega's genomic DNA purification kit. Fingerprints for each marker were generated using the primers ERIC1R and ERIC2 (Tailliez et al. 2006) or the primer BOXA1R (Koeuth et al. 1995). PCR reactions were carried out in a final volume of 25 $\mu$L, containing 1 × of BioMix Red (Bioline), 0.5 $\mu$L genomic DNA, 0.3 $\mu$M of each primer (ERIC) or 0.6 $\mu$M of the single primer (BOX), and 1 mM $MgCl_2$. The PCR reaction consisted of an initial denaturation at 95°C for 5 min and 30 cycles of 95°C for 1 min, annealing at 53°C (BOX) or 48°C (ERIC) for 1 min, 72°C for 3 min, and a final extension of 72°C for 5 min. To achieve strong bands, a nested PCR approach was required for the ERIC marker in which a second PCR reaction using 0.5 $\mu$L of the initial PCR product was set up identically to the first. Band sizing was done using Quanity One software (ver. 4.5; BioRad). Band intensities for the two replicates were averaged, and the similarity between each isolate based on the combined ERIC and BOX fingerprints was calculated using Pearson's product-moment correlation coefficient. Cluster analysis was performed by using the UPGMA algorithm (Rademaker et al. 2000; Ramette and Tiedje 2007).

## Results

There were no false negatives or false positives in the growth inhibition assays, indicating their reliability. Our experimental design resulted in a total of 1,260 actor-recipient tests (excluding self-tests), from which 648 (51.43%) were detected as inhibitions. The spatial distance between the actor and the recipient had a significant effect on the probability of inhibition (Fisher's exact test, $P < .0001$). There was no inhibition between pairs of isolates collected from the same insect host or from the same soil sample, but in all cases where actor and recipient were collected 4 m apart, they inhibited each other (i.e. the interactions were reciprocal; fig. 1). Colonies isolated from the two soil samples were distinguished by their ERIC- and BOX-based genomic fingerprints (fig. 2).

In order to examine the possibility that we by chance sampled the only two strains that inhibit each other, we collected an additional soil sample several meters away from the original sites (5 and 9 m away from soil samples 1 and 2, respectively). Using the same methods, we then placed two moth larvae on the third soil sample (i.e., hosts 5 and 6) and isolated two *Xenorhabdus bovienii* colonies from nematodes that were emerged from each host. This allowed us to conduct 76 additional inhibition assays, four from the same infected host, eight from two hosts that were infected by exposure to the same soil sample, and 64 from two hosts that were infected by exposure to the original soil samples collected several meters apart (fig. 3). Inhibition assays were done in the same way, except that we induced bacteriocins after 7 h of bacteria growth. Because of the slight change in the induction procedure, we also repeated 64 of the previous inhibition assays by randomly choosing two out of the nine bacterial isolates from hosts 1–4 and conducting inhibition assays between these different combinations (fig. 3). Repeatability of the 64 inhibition assays between colonies isolated from hosts 1–4 was high; 60 of the inhibition assays showed the same result as before (figs. 1, 3). In the other four cases, the new assay failed to show an inhibition that was previously detected, suggesting that the control of actor density in the first trial increased the power of the assays to detect inhibitions. As in the first set of inhibition assays, no inhibition was detected between colonies that were isolated from the same soil sample (either within or between hosts), whereas 34 inhibitions were detected between colonies that were meters apart (53%). These included inhibitions of the new isolates by isolates from the first soil sample and inhibitions of isolates from the second soil sample by isolates from the new soil sample (fig. 3). Colonies isolated from the new soil samples were distinguished from the other two samples by their ERIC- and BOX-based genomic fingerprints (fig. 2). The average genetic similarity between

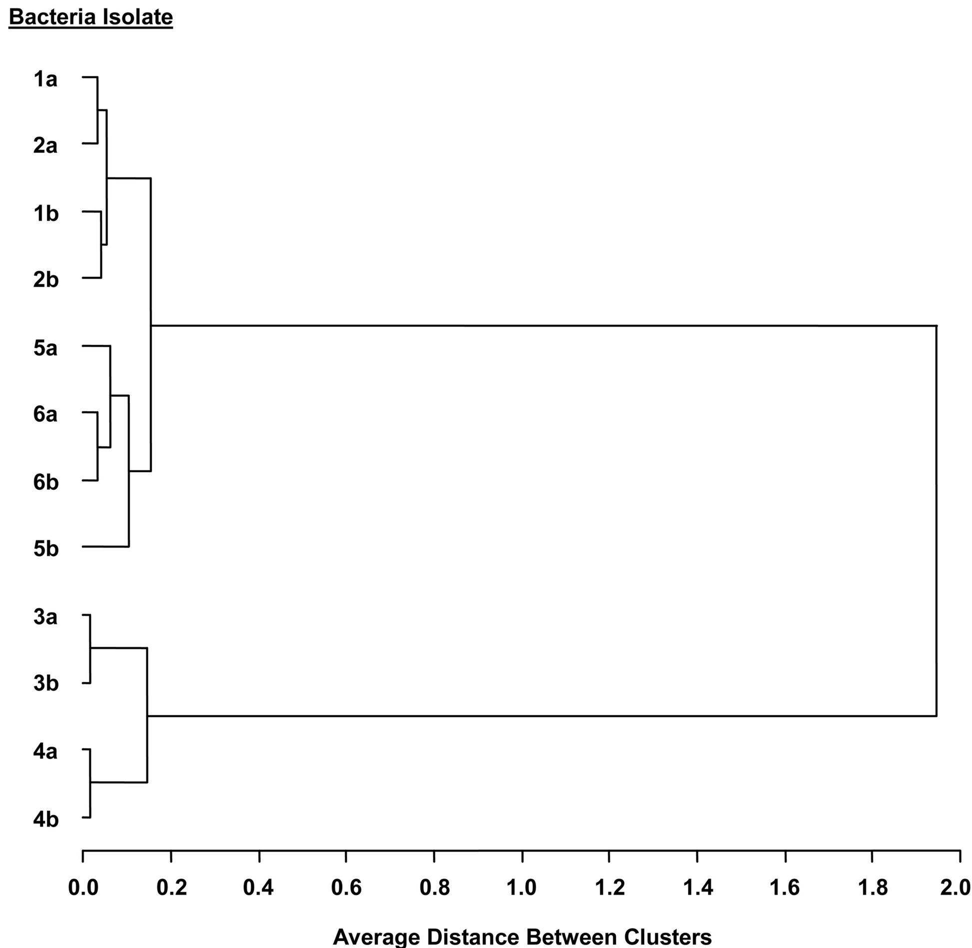


**Figure 2:** Distance tree showing the genetic relationships of 12 field isolates: colonies a and b from hosts 1–6, where hosts 1 and 2, 3 and 4, and 5 and 6 were isolated from soil samples 1, 2, and 3, respectively. For each isolate, the ERIC and BOX profiles were combined based on the presence and intensity of each band averaged over two replicates.

soil samples ($0.47 \pm 0.13$) was significantly lower than the average similarity within soil samples ($0.98 \pm 0.0001$; *t*-test, $P < .001$; fig. 2).

## Discussion

We found that isolates collected from the same soil sample of *Xenorhabdus bovienii* were genetically similar and that they did not inhibit each other. However, isolates that were collected several meters apart were genetically different and antagonistic toward each other. These results thus show that bacterial populations are spatially structured for toxin production, and they suggest that strong spite exists in natural populations.

*Xenorhabdus bovienii*, like other bacteria that are symbionts of entomopathogenic nematodes, can be carried by its nematode host for a few meters in search of new hosts (Schroeder and Beavers 1987; Alatorre-Rosas and Kaya 1990). It is therefore likely that different strains of bacteria from different nematodes, which were originally located a few meters away, would interact. The evidence for spiteful behavior between coexisting bacteriocin-producer and bacteriocin-sensitive strains is intriguing in light of theoretical predictions that the conditions for coexistence are restrictive (Kerr 2007; Gardner and West 2010). The persistence of the harming effect of bacteriocins may be due to the possibility for bacteria to evolve resistance to bacteriocins, which if costly can set up a balanced polymorphism between producer, resistant, and sensitive strains, reminiscent of the rock-paper-scissors game (Gardner and West 2010). For example, in our study the colonies isolated from soil sample 3 (hosts 5 and 6) resisted the toxin produced by colonies isolated from soil sample 2 (hosts 3 and 4), and the colonies that were isolated from soil sample 1

| Actor isolate | | | Recipient isolate: Soil Sample1 | | | | Soil Sample 2 | | | | Soil Sample 3 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Host 1 | | Host 2 | | Host 3 | | Host 4 | | Host 5 | | Host 6 | |
| | | | a | b | a | b | a | b | a | b | a | b | a | b |
| Soil Sample 1 | Host 1 | a | 0 | 0 | 0 | 0 | I | I | I | I | I | I | I | I |
| | | b | 0 | 0 | 0 | 0 | I | I | I | I | I | I | I | I |
| | Host 2 | a | 0 | 0 | 0 | 0 | I | I | I | I | I | I | I | I |
| | | b | 0 | 0 | 0 | 0 | I | I | I | I | I | I | I | I |
| Soil Sample 2 | Host 3 | a | I | I | I | I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | b | 0 | I | I | 0 | 0 | 0 | 0 | 0 | 0 | I | 0 | 0 |
| | Host 4 | a | I | I | I | I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | b | 0 | I | I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | I | 0 |
| Soil Sample 3 | Host 5 | a | 0 | 0 | 0 | 0 | I | I | I | I | 0 | 0 | 0 | 0 |
| | | b | 0 | 0 | 0 | 0 | I | I | I | I | 0 | 0 | 0 | 0 |
| | Host 6 | a | 0 | 0 | 0 | 0 | I | I | I | I | 0 | 0 | 0 | 0 |
| | | b | 0 | 0 | 0 | 0 | I | I | I | I | 0 | 0 | 0 | 0 |

**Figure 3:** Bacteriocin-mediated antagonism among *Xenorhabdus bovienii* isolates from six infected insect hosts: colonies a and b from hosts 1–6, after the addition of the third soil sample, where hosts 1 and 2, 3 and 4, and 5 and 6 were isolated from soil samples 1, 2, and 3, respectively. "I" indicates inhibition of recipient growth by cell-free extracts of actor cultures, whereas "0" indicates interactions in which the recipient growth was not affected by cell-free extracts of actor cultures.

(hosts 1 and 2) resisted the toxin produced by colonies isolated from host soil sample 3 (fig. 3). It remains to be determined which of the strains with reciprocal inhibitions (A > B and B > A; e.g., isolated from soil samples 1 and 2) would dominate within coinfections of the same individual host. The outcome may depend on the presence of additional genotypes, on the magnitude of the effect each strain has on the other strains, and on the frequency of the strains in the insect host (May and Leonard 1975; Chao and Levin 1981; Johnson and Seinen 2002). For example, it is likely that the presence of strains from soil sample 3 changes the interaction between isolates from soil samples 1 and 2 because they are inferior to isolates from soil sample 1 but superior to isolates from soil sample 2.

We are aware of only two other studies that quantified antagonism among naturally co-occurring bacteria isolates at biologically relevant scales. In the first study, Vos and Velicer (2009) found a strong antagonism among colonies of *Myxococcus xanthus* that were isolated from the same 16 × 16-cm soil sample. The mechanistic basis of the developmental antagonisms and swarming incompatibilities observed in *M. xanthus* remains to be explored, but there is direct indication that it involves self-/non-self-recognition (Vos and Velicer 2009). In the second study, bacteriocin relationships were determined for 18 strains of *Bacteroides* isolated from the fecal sample of one human host on three different occasions (Booth et al. 1977). Booth at el. found that between 1% and 35% of the isolates produced bacteriocins that inhibited all other nonproducer colonies (Booth et al. 1977). Taken together, these studies, along with ours, support the notion that spiteful interactions occur in nature at local scales. Given that almost all lineages of bacteria produce bacteriocins (Riley and Wertz 2002), our evidence suggests that spiteful behavior may be more pervasive in nature than commonly thought.

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Indiana University Research and Teaching Preserve, where soil samples were collected. Photograph by Hadas Hawlena.