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THE EVOLUTION OF SPITE: POPULATION STRUCTURE AND BACTERIOCIN-MEDIATED ANTAGONISM IN TWO NATURAL POPULATIONS OF *XENORHABDUS* BACTERIA

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Spite occurs when an individual harms itself in the act of harming other individuals. Such behaviors were once assumed to be of limited evolutionary importance, as the conditions for the evolution of spite were thought to be too restrictive. Recent theoretical work, however, suggests that spatial population structure, which allows local competition among genotypes, could favor the evolution of spite. One of the clearest examples of spite is the costly production and release by bacteria of toxins (called bacteriocins) that can kill unrelated strains of the same species. Here, we establish the existence of spatial structure in two natural populations of bacteriocin-producing bacteria. Specifically, relatedness decreased with increasing spatial distance between the field isolates. In addition, toxin-mediated inhibitions were found only between isolates that were collected more than 1 m apart and that were generally less than 80% similar in their genomic fingerprints. Taken together, the results suggest that the bacteria are spatially structured, with mixing of genotypes and spiteful interactions at the boundaries between demes.

KEY WORDS: Bacteriocins, entomopathogenic bacteria, genetic variation, population structure.

Spiteful behaviors are those that are harmful to both the actor and the recipient. As these behaviors reduce the direct fitness of the actor, it is difficult to explain why they should be favored by natural selection. Hamilton (1963, 1964) introduced the concept of inclusive fitness, and he showed that spiteful behaviors would be favored by natural selection when they result in a net increase in the actor's genes in the population. This could occur when the recipient is less related to the actor than expected by chance, which has been called "negative relatedness" (Gardner and West 2004; West and Gardner 2010). However, because negative relatedness was only thought to occur in small populations, it was assumed that spite, although possible, was not a general phenomenon contributing to adaptive evolution (Keller et al. 1994; Hamilton 1996).

This view on the importance of spite has been challenged in recent years. Theoretical studies have recently shown that spatial population structure might allow spite to evolve by increasing the mean relatedness in the social arena, and thus increase the likelihood that the few nonrelated recipients will be negatively related to the abundant genotype (reviewed in Wloch-Salamon et al. 2008; Inglis et al. 2009; Gardner and West 2010). In particular, Gardner et al. (2004) demonstrated in a mathematical model that local competition among genotypes could favor spiteful behavior, provided that there are self/nonself recognition mechanisms, perhaps due to the existence of greenbeard genes (Gardner and West 2010). In addition, spite can be favored among dispersing individuals, even in the absence of self/nonself recognition, if dispersers are less related than average to their social partners (El Mouden and Gardner 2008). In both cases, it is mixing of genotypes in a local competitive arena that increases the likelihood of negative relatedness between actors and recipients, and may allow spite to evolve due to indirect fitness benefits (i.e., fitness benefits to the

actor's relatives) from a competitive release (Gardner and West 2010).

At present, the role for spatial structure in the evolution of spite is mainly supported by theoretical models and laboratory experiments (Chao and Levin 1981; Frank 1994; Czaran et al. 2002; Kerr et al. 2002; Czaran and Hoekstra 2003; Gardner and West 2004, 2010; Gardner et al. 2004; Wloch-Salamon et al. 2008; Inglis et al. 2009). Here, we establish the importance of spatial structure in two natural populations of spiteful bacteria that use bacteriocins as weapons. Bacteriocins are toxins produced by bacteria that can kill closely related strains of the same species. They provide one of the clearest examples of costly spite (West et al. 2006). In a previous study, we found evidence that bacteriocin-mediated interactions occur in a natural population of *Xenorhabdus bovienii*, where bacteriocin-producing and bacteriocin-sensitive genotypes co-exist within a scale of only a few meters (Hawlena et al. 2010). Here, we test the hypothesis that *X. bovienii*, as well as a second species (*X. koppenhoeferi*), consist of structured subpopulations, where relatedness decreases, and the probability of bacteriocin-mediated inhibitions increases, with increasing distance between natural isolates.

Xenorhabdus bovienii and *X. koppenhoeferi* are insect-killing bacterial species carried by entomopathogenic nematodes in the genus *Steinernema* (Tailliez et al. 2006). The nematodes release the bacteria after infecting an insect host. Following their release, the bacteria grow rapidly, killing the arthropod host within a few days. The nematodes then rapidly reproduce 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 two weeks after infection, each carrying an inoculum of bacteria. Importantly, the nematodes may travel a few meters in search of new insect hosts (Schroeder and Beavers 1987; Alatorre-Rosas and Kaya 1990). As multiple nematodes must enter an insect host for a successful reproduction (because the sexes are separate), different bacteria genotypes may mix within a single insect host; however, bacterial mixing should be limited by the maximum distance that the nematodes can move. We therefore expected to find a negative relationship between spatial distance and the overall genetic similarity of the bacteria (Green and Bohannan 2006). Moreover, as bacteriocin-mediated interactions involve self/nonself recognition mechanisms (e.g., actors are immune to their own toxins; Riley and Chavan 2007), we predicted that inhibitions occur nonrandomly, mainly between individuals that differ in their spiteful locus. Assuming that genetic similarity in the spiteful locus is correlated with overall genetic similarity, we expected to find a positive relationship between spatial distance and the probability of inhibition as the distance between natural isolates increased from centimeters to meters.

We conducted inhibition assays and estimated the genetic relatedness between pairs of either *X. bovienii* or *X. koppenhoeferi* colonies that were collected within centimeters, meters, or tens of meters of each other. Our findings support the theoretical predictions that spiteful interactions may evolve between nonkin individuals in populations characterized by local dispersal.

Methods

BACTERIA ISOLATES

A nested-sampling design was employed with 10 soil samples collected at each of six sites along a hill at the Indiana University Research and Teaching Preserve, Moore's Creek, Monroe County, Indiana (sites 1–6 in Fig. 1). The sites were located in intervals of 60 m and in each site, soil samples were uniformly collected along a 15 m line transect. Each soil core was 8 cm in diameter by 5 cm in depth. We brought the soil samples to the laboratory and placed them into separate Petri dishes (140 × 25 mm). We then placed 3-moth larvae (*Galleria mellonella*) into each dish. Hosts were kept at 22°C, and as soon as a host died (approximately 48 h post exposure), we replaced it by a live larvae, and transferred the dead host to a modified White trap for collection of emerging nematodes (Bashey and Lively 2009). Nematodes began to emerge approximately 14 days after infection and were stored in dH_20 at 8°C for less than two months. Approximately 2000 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_20 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 1 solid pellet of sodium hydroxide, 0.004% (w/v) triphenyltetrazolium chloride, and 0.0025% (w/vol) bromothymol blue (NBTA). Two colonies were isolated from each insect host sample, preserved in 20% glycerol at −80°C, and used in the growth-inhibition assays described below.

Two bacteria species, *X. bovienii* and *X. koppenhoeferi*, were identified by sequencing of 16S rDNA (Tailliez et al. 2006). *Xenorhabdus bovienii* colonies were isolated from 13 insect larvae, which were infected by seven soil samples (sites 4–6; Fig. 1), whereas *X. koppenhoeferi* colonies were isolated from 10 insect larvae, which were infected by five soil samples (sites 1, 2, and 4; Fig. 1). This experimental design resulted in 26 field isolates of *X. bovienii* and 20 field isolates of *X. koppenhoeferi*, and allowed us to examine intraspecific bacteriocin-mediated antagonism between pairs of bacterial colonies that are naturally located within a distance scale of centimeters (colonies that were isolated from a single insect larvae or from two hosts that were infected by exposure to the same soil sample), few meters (colonies that were isolated from two hosts that were infected by exposure to soil samples collected within a site, i.e., 1–15 m apart), or tens of meters (colonies that were isolated from two hosts that were infected

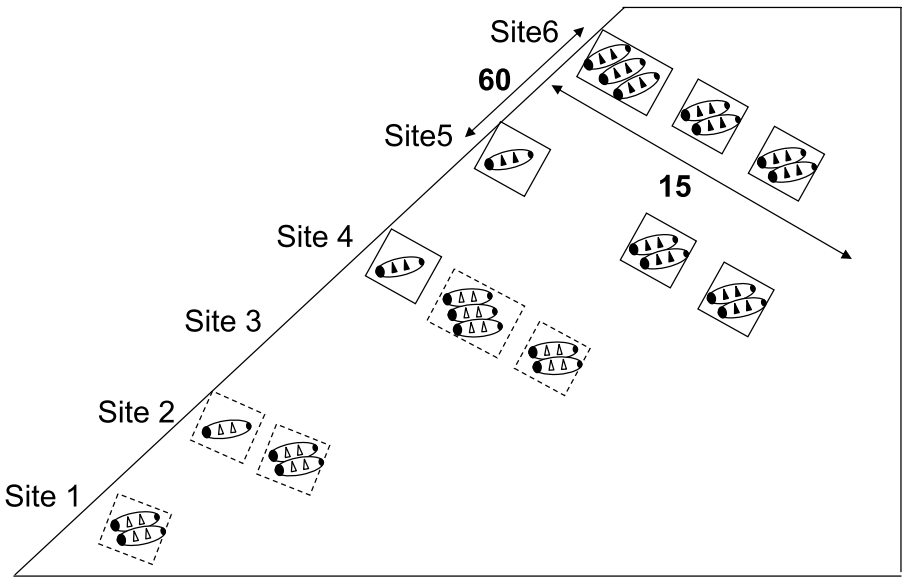

Figure 1. Schematic representation of *X. bovienii* (filled triangles) and *X. koppenhoeferi* (open triangles) bacterial isolates from 23 caterpillar hosts that were infected by 12 soil samples (solid and dashed squares for *X. bovienii* and *X. koppenhoeferi*, respectively) at five sites across a hill.

by exposure to soil samples collected in two sites, i.e., 60–180 m apart).

INHIBITION ASSAYS

We performed 1030 growth-inhibition assays to determine the pairwise relationships between colonies that were isolated (1) from the same insect host (26 pairs for *X. bovienii* and 20 pairs for *X. koppenhoeferi*); (2) from two hosts that were infected by exposure to the same soil sample (56 pairs for *X. bovienii* and 48 pairs for *X. koppenhoeferi*); (3) from two hosts that were infected by exposure to soil samples collected within a site (192 pairs for *X. bovienii* and 64 pairs for *X. koppenhoeferi*), and from two hosts that were infected by exposure to soil samples collected from sites that are (4) 60 m apart (320 pairs for *X. bovienii* and 48 pairs for *X. koppenhoeferi*); (5) 120 m apart (56 pairs for *X. bovienii* and 120 pairs for *X. koppenhoeferi*); and (6) 180 m apart (none for *X. bovienii* and 80 pairs for *X. koppenhoeferi*).

We estimated the probability of a false-positive response (i.e., the probability of finding an inhibition where there is none) based on self-, and negative-control tests. In the 46 self-tests (26 *X. bovienii* and 20 *X. koppenhoeferi*), where the same isolate was used both as the recipient and as the actor, no inhibition should be observed, as bacteriocin-producing clones carry immunity to their own toxin (Riley and Chavan 2007). In the 92 negative control-tests (52 *X. bovienii* and 40 *X. koppenhoeferi*), we tested each isolate's response as a recipient by applying (at two different occasions) a 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 168 positive-control tests that were performed at the same time and according to the same protocol that was 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 *X. nematophila* (strain HGB 801 ATCC 19061) as the actor (H. Hawlena, unpubl. data).

We employed a modified version of the Pugsley and Oudega (1987) method, which uses mitomycin C, to induce the production of bacteriocins in each field isolate. The release of bacteriocins has been implicated as a major factor affecting the outcome of competitive interactions within the host (Massey et al. 2004; Sicard et al. 2005; Vigneux et al. 2008). 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% of repeatability; H. Hawlena, unpubl. 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.

We induced bacteriocins from cells in their log-phase after 5–6 h of growth in LB (5 h for the faster growing *X. koppenhoeferi* and 6 h for *X. bovienii*) by incubating the cells with mitomycin C (0.5 μg/mL) at 28°C. After an additional 5 h, 67 μL 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 μm HT Tuffryn membrane and stored it at 4°C less than 14 days before use; bacteriocins of these species can be stored for more than one year without decaying at that temperature (H. Hawlena, unpubl. data). To test the sensitivity of an isolate, stationary-phase liquid culture (2% v/v) was added to molten soft (MS) agar (0.6% agar). Then, 10 μL of supernatant of actor isolates was spotted onto the surface of an MS agar plate containing the potential sensitive isolate. Plates were incubated for 48 h, at which time inhibition could be visualized as a clear zone on the recipient lawn.

To quantify the repeatability of the inhibition assays, we randomly repeated 134 of them (12.5%). Repeatability of the 134 inhibition assays was high as 90% of the inhibitions assays between the bacterial isolates showed the same result as before.

Actor extracts that killed the recipient cells could contain bacteriocins or bacteriophage. To distinguish between the two causes of inhibition, two assays were carried out: extracts were frozen at −80°C and serial dilutions (dilution factors: 10^1, 10^3, 10^6) were performed for the actors that identified as bacteriocin producers. Appearance of individual plaques and inactivation by freezing indicate that the extract contains bacteriophage, whereas zones of clearings, becoming increasingly opaque and no effect of freezing indicate bacteriocin production (Gordon and O'Brien 2006). All dilution series showed the latter responses, and we thereby concluded that the observed inhibitions are due to bacteriocin production.

GENOTYPIC CHARACTERIZATION OF STRAINS

Enterobacterial repetitive intergenic consensus sequence (ERIC) PCR fingerprints were determined for each isolate. Genomic DNA was amplified using the primers ERIC1R and ERIC2 (Tailliez et al. 2006). PCR reactions were carried out in a final volume of 25 μL, containing 1× of BioMix Red (Bioline), 0.5 μL genomic DNA, 0.3 μM of each primer and 1 mM $MgCl_2$. PCR reactions consisted of an initial denaturation at 95°C for 5 min, and 30 cycles of 95°C for 1 min, annealing at 48°C for 1 min, elongating at 72°C for 3 min, and a final extension at 72°C for 5 min. To achieve strong bands, a nested PCR approach was required for in which a second PCR reaction using 0.5 μL of the initial PCR product was set up identically to the first. The resulting PCR products were visualized on ethidium-bromide-stained 1% agarose gels. Two replicate fingerprints were generated for each isolate. Genetic similarity was calculated based on seven bands that were most reliable in the two species, using the mean presence/absence of two replicate runs.

STATISTICAL ANALYSIS

For each pair of field isolates, we calculated the probability of inhibition (0 = no inhibition, 1 = at least one isolate inhibits the other), the phenotypic similarity (the proportion of identical outcomes [inhibition/no inhibition] that two isolates shared both as actors and as recipients), and the genetic similarity (the proportion of ERIC bands shared by two isolates).

We performed Mantel tests to determine the correlation between the inhibition and genetic similarity matrices to the corresponding distance matrix, and between the inhibition and phenotypic similarity matrices to the corresponding genetic similarity. All statistical tests were two-tailed.

Results

There was only one false negative (0.6%) and no false positives in the growth inhibition assays, indicating their reliability. Our experimental design resulted in a total of 1030 actor–recipient tests (excluding self-tests), from which 296 (28.7%) were detected as inhibitions.

The high correlation between the genotypic and phenotypic similarities between pairs of field isolates supports our assumption that there is a close association between relatedness at the spiteful locus and at the rest of the genome in *Xenorhabdus* bacteria, most likely due to low horizontal transmission and/or mutation rates (Mantel test: $r = 0.74$, and $r = 0.89$ for *X. bovienii* and *X. koppenhoeferi*, respectively; $P < 0.005$ for both; Fig. 2). The fingerprinting data are therefore a good predictor for the relatedness at the spiteful locus of interest.

Genetic similarity decreased with increasing distance between the field isolates (Mantel test: $r = -0.21$, and $r = -0.87$ for *X. bovienii* and *X. koppenhoeferi*, respectively; $P < 0.05$ for both; Fig. 3). As the genetic similarity decreased, the probability of inhibition increased (Mantel test: $r = -0.59$, and $r = -0.74$ for *X. bovienii* and *X. koppenhoeferi*, respectively; $P < 0.005$ for both; Fig. 4). In both bacteria species, the probability of inhibition between two field isolates significantly increased with the distance between them (Mantel test: $r = 0.29$, and $r = 0.68$ for *X. bovienii* and *X. koppenhoeferi*, respectively; $P < 0.005$ for both). The isolates inhibited each other only if they were isolated from locations that were at least 1 m apart, and the probability of inhibition further increased for pairs of isolates that were more than 60 m apart (Fig. 3).

Discussion

We found that genetic relatedness decreased, and the probability of bacteriocin-mediated interactions increased, with spatial distance between isolates in two natural populations of *Xenorhabdus* bacteria. These results thus support the theoretical predictions that spatial structure favors the evolutionary maintenance of spiteful interactions.

A broad strand of experimental and theoretical work on the ecology of spiteful interactions highlights the importance of

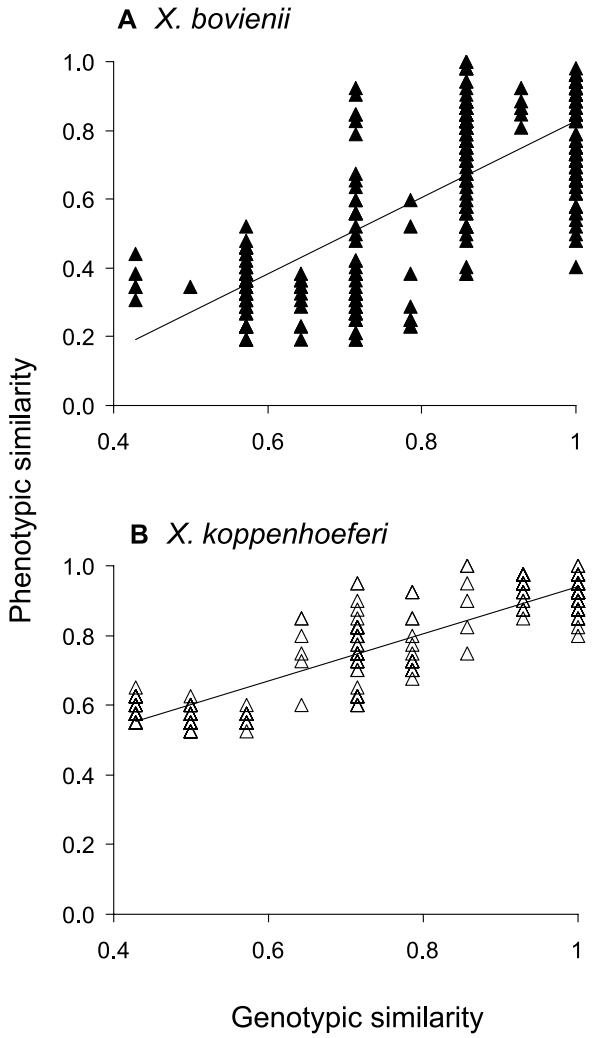

Figure 2. **The relationships between genetic similarity (based on ERIC fingerprints) and phenotypic similarity (based on inhibition/resistance profiles) between pairs of (A) *X. bovienii* ($r = 0.74$, $P < 0.005$) and (B) *X. koppenhoeferi* ($r = 0.89$, $P < 0.005$) isolates.**

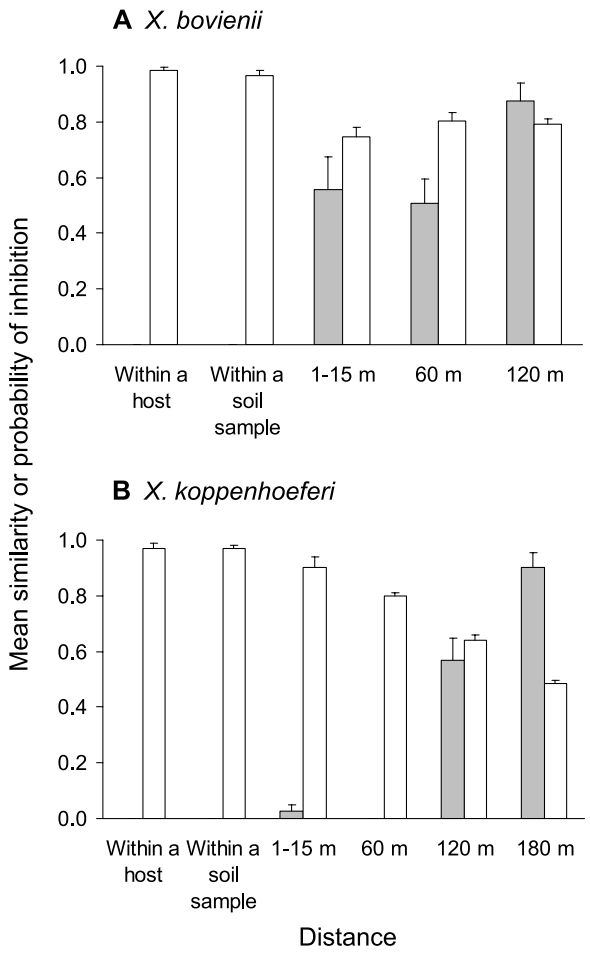

Figure 3. **Mean (±SE) genetic similarity (empty columns), and probability of inhibition (filled columns) between pairs of (A) *X. bovienii* and (B) *X. koppenhoeferi* isolates as a function of the distance between their location in nature. We included each field isolate only once to avoid pseudoreplication, by using the average genetic similarity or the average probability of inhibition of this isolate with all its partners at a given distance.**

spatial structure in mediating the outcome of interactions (reviewed in Gardner and West 2004, 2010; Wloch-Salamon et al. 2008; Brown et al. 2009). These studies suggest that local competition among individuals that are different in their spiteful locus promotes the invasion of spiteful actors by increasing their indirect fitness benefits. Here, we support the existence of such a spatial structure in natural populations of bacteria. Negative relationships between spatial distance and mean genetic similarity suggest that dispersal in *Xenorhabdus* bacteria is limited. Moreover, toxin-mediated inhibitions were found only between isolates that were collected more than 1 m apart and that were generally distinct in their genomic fingerprints and phenotypic profile (i.e., inhibition profile). Our results are thus consistent with a spatial structure of patches (or demes) of producer and sensitive genotypes with some mixing and spiteful interactions at the boundaries between them. On the boundaries, the spiteful action of producers may liberate nutrients and space for their close relatives. In vivo competitive assays between the different genotypes of the *Xenorhabdus* isolates are currently under way to determine whether producers indeed benefit indirectly from a competitive release.

In other systems, the spatial distance between interacting species has been found to be an important predictor of the strength of associations between them. This is because species that are located further apart, are less likely to interact and to adapt to each other (Hoeksema and Forde 2008). For example, increasing geographic distance between host and parasite origin was found to be correlated with a decrease in parasite reproductive success and virulence, indicating the parasite's lack of adaptation to novel host genotypes (Ebert 1994). Vos and Velicer (2009) found evidence that distance predicts the strength of social associations

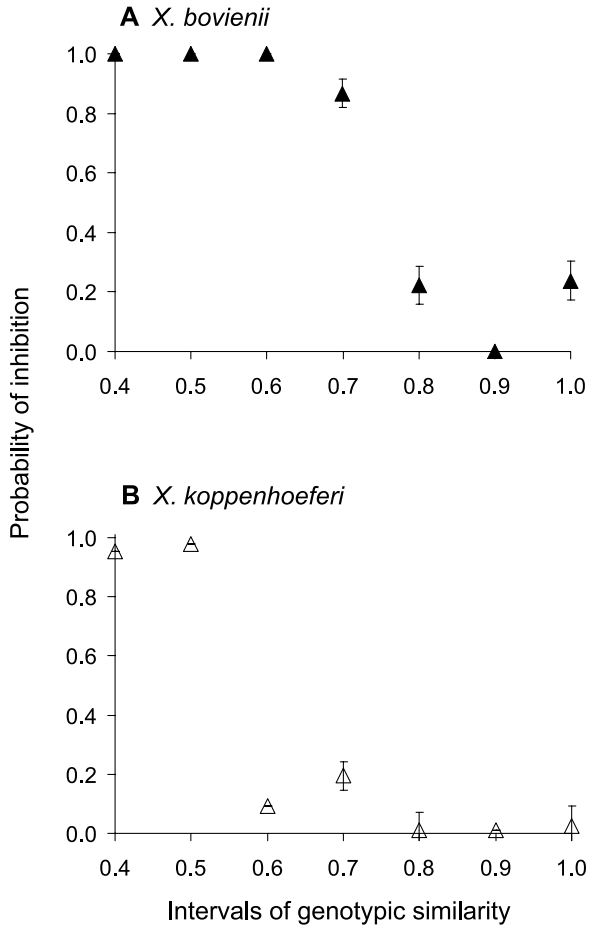

Figure 4. The relationships between genetic similarity (given in intervals, e.g., "0.4" is for the range of 0.40–0.49) and the mean probability of inhibition between pairs of (A) *X. bovienii* and (B) *X. koppenhoeferi* isolates. We included each field isolate only once to avoid pseudoreplication, by using the average genetic similarity and the average probability of inhibition of this isolate with all its partners.

between different genotypes of the same species as well. Greater disruptions to cooperative aggregation and sporulation behavior was found among global isolates (strains isolated from distant global locations) than among local strains (strains isolated from a centimeter-scale) of the bacterium *Myxococcus xanthus*. Here, we found evidence that even within the range of local interactions (centimeter-to meter scales), the spatial distance between genotypes is important. An increase of a few meters between the isolates was enough to significantly increase the probability of bacteriocin-mediated inhibitions between them (Fig. 3).

The probability of inhibition did not decrease between tens and hundreds of meters. This result is perhaps intriguing, as there would be little evolutionary pressure to harm genotypes that the spiteful bacteria do not often encounter. It is possible that the inhibition of spatially distant isolates is a byproduct of selection to harm spatially close isolates. Alternatively, the high probability of inhibitions between distant isolates may suggest a high cost of resistance, especially the cost of resistance to spatially distant producers, which are likely to possess different types of bacteriocins. Finally, some mixing may occur over larger spatial scales, which might be enough to benefit individuals that are able to inhibit rare intruders.

The genetic and social structure we found for both *Xenorhabdus* species is consistent with the movement pattern of their symbiotic nematodes; both genetic variability and spiteful interactions occur within the range of nematode dispersal. This suggests that the evolution of spite between a pair of genotypes can be strongly affected by interactions with another species. Nevertheless, spiteful interactions should not be restricted to symbiotically associated microbes. Recent evidence suggest that spatial structure formed by migration barriers is common in free-living microbial species as well, including those capable of forming resistant spores that disperse easily (reviewed by Green and Bohannan 2006; Whitaker 2009). The spatial structure combined with the presence of self/nonself recognition mechanisms in many microbial groups (Simms and Bever 1998; Pfaller et al. 2000; Gibbs et al. 2008; Ostrowski et al. 2008; Vos and Velicer 2009) may explain the widespread distribution of bacteriocins, and suggest that spiteful behavior may be more common in microbes than in macro-organisms (West et al. 2006).

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