THE ROYAL SOCIETY

Received 12 November 2003
Accepted 22 December 2003
Published online 16 March 2004

Interference competition and parasite virulence

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Within-host competition between parasites, a consequence of infection by multiple strains, is predicted to favour rapid host exploitation and greater damage to hosts (virulence). However, the inclusion of biological variables can drastically change this relationship. For example, if competing parasite strains produce toxins that kill each other (interference competition), their growth rates and virulence may be reduced relative to single-strain infections. Bacteriocins are antimicrobial toxins produced by bacteria that target closely related strains and species, and to which the producing strain is immune. We investigated competition between bacteriocin-producing, insect-killing bacteria (*Photorhabdus* and *Xenorhabdus*) and how this competition affected virulence in caterpillars. Where one strain could kill the other, and not vice versa, the non-killing strain was competitively excluded, and insect mortality was the same as that of the killing strain alone. However, when caterpillars were multiply infected by strains that could kill each other, we did not observe competitive exclusion and their virulence was less than single-strain infections. The ubiquity and diversity of bacteriocins among pathogenic bacteria suggest mixed infections will be, on average, less virulent than single infections.

Keywords: *Photorhabdus*; bacteriocin; evolution of virulence; bacteria

1. INTRODUCTION

Within-host competition between parasite strains is predicted to result in more rapid host exploitation, hence greater disease-induced mortality (virulence) (Bremermann & Pickering 1983; Frank 1992, 1996; van Baalen & Sabelis 1995). However, empirical data for this, as well as all theoretical predictions for the evolution of virulence, are drastically lacking (Herre 1993, 1995; Taylor *et al.* 1998; Chao *et al.* 2000; Davies *et al.* 2002). This may be because inclusion of biological variables may drastically change this relationship (Chao *et al.* 2000; Brown *et al.* 2002; Schjørring & Koella 2003; West & Buckling 2003). We consider how anti-competitor toxins produced by parasites (spiteful behaviour or interference competition) determine the virulence of single- and mixed-strain parasitic infections. A simple prediction is that competing toxin-producing strains will kill each other, reducing the parasite population growth rate and hence reducing virulence. Thus, if toxins are important in mediating parasite competitive interactions, infections caused by genetically diverse parasites are predicted to be, on average, less virulent than infections caused by a single type (A. Gardner, A. S. West and A. Buckling, unpublished data).

Bacteriocins are among the most abundant range of antimicrobial compounds produced by bacteria and are found in all major bacterial lineages (Reeves 1972; Riley & Wertz 2002). They are a diverse family of proteins with a range of antimicrobial killing activities including enzyme inhibition, nuclease activity and pore formation in cell membranes (Riley & Wertz 2002). Unlike other antimicrobials, the lethal activity of bacteriocins is often (but not always) limited to members of the same or closely related species to the producer (Riley *et al.* 2003), suggesting a major role in competition with conspecifics. Clone mates are protected from the toxic effects of bacteriocins as a result of genetic linkage between the bacteriocin gene and an immunity gene that encodes a factor that deactivates the bacteriocin (Riley & Wertz 2002).

We investigated competition between bacteriocin-producing strains of *Photorhabdus* and *Xenorhabdus* spp., and how this affected the virulence of infections. *Photorhabdus* and *Xenorhabdus* spp. are insect-killing bacteria carried by entomopathogenic nematodes, which release bacteria after invasion of an insect (Forst *et al.* 1997; Forst & Clarke 2001). The bacteria produce other toxins (e.g. Mcf1 and Mcf2 (makes caterpillars floppy); Daborn *et al.* 2002; Waterfield *et al.* 2003) that kill the insect, allowing the bacteria and nematodes to replicate within the cadaver (Forst *et al.* 1997; Forst & Clarke 2001). Little is known about the frequency of mixed-strain infections (Forst *et al.* 1997; Forst & Clarke 2001); however, the release of antimicrobial toxins, especially proteinaceous bacteriocins, has been implicated as a major factor affecting the outcome of competitive interactions (Sharma *et al.* 2002).

To address the importance of interference competition through bacteriocin production on virulence, we chose three strains that showed variation in the effects of their bacteriocins. Two strains produced bacteriocins that could kill the other two strains, whereas all strains were resistant to the bacteriocins produced by the third strain. Assuming that bacteriocin activity is an important determinant in the outcome of competitive interactions and that bacterial population growth rate positively correlated with virulence in hosts, we made some simple predictions. First, mixed infections of the two strains that could kill each other (symmetric killing) would be less virulent than both single-strain infections. Furthermore, they might coexist. Second, virulence of mixed infections with asymmetric killing should result in virulence levels equal to

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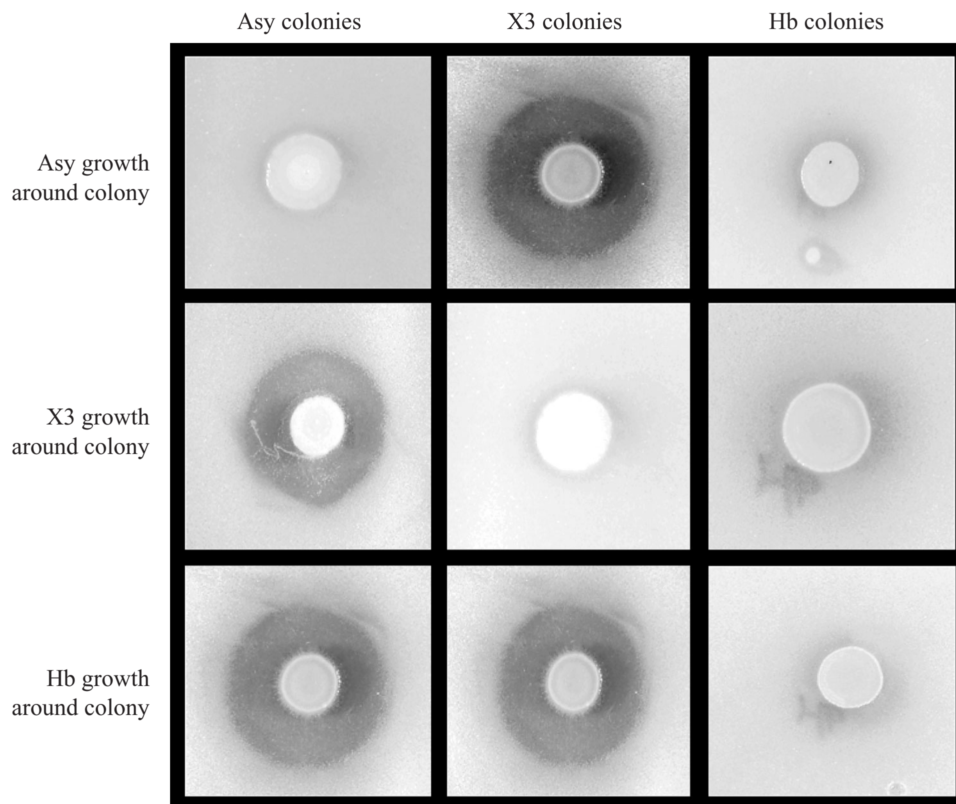

Figure 1. Bacteriocin-mediated growth inhibition of the bacterial strains Asy, X3 and Hb. Photographs of inhibition of growth of susceptible strains around a bacteriocin-producing colony of bacteria. Asy is inhibited by X3, X3 is inhibited by Asy and Hb is inhibited by both Asy and X3.

single-strain infections of the killing strain, as a result of competitive exclusion of the non-killing strain.

It is important to note that the virulence theory discussed above refers to intra- rather than interspecies competition. This is because different strains of the same species are more likely to occupy the same ecological niche, and hence undergo more intense competition than different species. We have used three different species because of the obvious variation in their bacteriocin activity. However, they occupy the same ecological niche (symbiotically within nematodes and parasitically within insects) and may come into direct competition in natural populations, hence the results should be relevant to intraspecific parasite interactions as well as interspecific interactions, where similar niches are occupied. Furthermore, bacterial taxonomy is often arbitrarily derived and it has been suggested that the specific ecological niches bacteria occupy are a more useful definition of bacterial species (Cohan 2002).

2. MATERIAL AND METHODS

(a) *Bacterial strains*

We used three strains in this study: *Photorhabdus asymbiotica* ATCC43949 (Asy); *Xenorhabdus nematophilus* DSM3370 (X3); *Photorhabdus luminescens* sp. *luminescens* ATCC29999 (Hb).

(b) *Growth inhibition assays*

Bacterial strains were grown for 48 h at 28 °C shaking (300 r.p.m.) in 10 ml of proteose peptone (2% PP3) broth in a 30 ml glass universal. An aliquot of 5 μl of the culture was spotted (producer-strain) onto the surface of a PP3 agar plate and allowed to grow at 28 °C for 48 h. Molten soft-top PP3 agar (0.6% agar) containing 50 μl of an overnight-bacterial solution (tester-strain) was poured over the surface of the agar plate containing the spot of bacterial growth. The plate was incubated for a further 48 h, at which time the inhibition of growth of the bacteria in the soft agar around the spot of bacterial growth could be visualized as a clear zone of inhibition.

(c) *Inoculation of caterpillars*

The caterpillars *Galleria mellonella* were purchased from Livefood UK (www.livefood.co.uk). Eighty caterpillars per strain and pairs of strains (a total of six treatments) were injected into the abdomen with 10 μl of fresh broth containing 10^2 bacterial cells using a Hamilton syringe. The inoculated insects were then incubated at 28 °C for 24 h. Insect death was evaluated by touching the head of the insect and assessing for reflexive movement.

(d) *Assessment of bacterial frequency after competition*

Oligonucleotide primers were designed based on sequence differences between the 16S ribosomal subunit of the three bacterial species:

Asy forward: GGTTTGGCCTGAAGAGGGTTAA,
Asy reverse: GTTCCCACCTCAACGTGCTGG,
Hb forward: GGTTCAGCTTGAACAGAGCTGG,
Hb reverse: GTTCCCGCCATTACGCGCTGG,
X3 forward: GTAAGTCTGAACAGGGCTTACG,
X3 reverse: TGAGTTCCCACCCGAAGTGCT.

These primers were used in polymerase chain reaction (PCR) reactions to identify individual colonies of the three bacterial

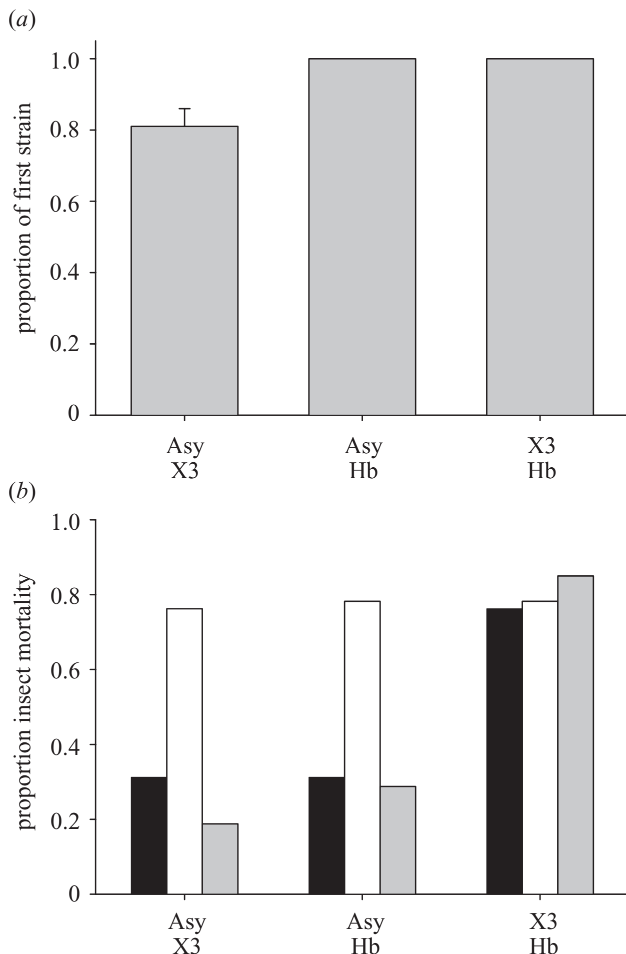

Figure 2. Outcome of mixed infections. (*a*) Proportion of the first strain listed after 24 h growth of the three mixtures. (*b*) Percentage mortality of *G. mellonella* (80 insects per treatment) after 24 h following inoculation of one or two strains. The black and white bars are the first and second listed single-strain infections, respectively, and the grey bars are the mixed infections.

species. After the death of the caterpillars they were individually homogenized and the resulting solution diluted and plated on PP3 agar. Twenty colonies from each of four insects (80 in total) per competition were selected and added directly to a PCR reaction containing one of each of the three sets of primers. The result of the PCR reaction was analysed on a 0.8% agarose gel.

(e) In vitro *homogeneous and heterogeneous competitions*

To address the importance of spatial structure for the coexistence of bacterial strains, we performed *in vitro* competitions in both spatially homogeneous and heterogeneous environments. Competitions in the homogeneous *in vitro* environments were performed by inoculating 10 ml of PP3 broth in a 30 ml glass universal with 10^4 cells of each strain. After incubation for 48 h at 28 °C shaking (300 r.p.m.) the cultures were diluted and plated onto PP3 agar plates. Twenty colonies from four replicates of the competition were analysed by PCR as described above to determine the competition outcome. The heterogeneous environment was created by the addition of 10^4 cells of each strain to molten (50 °C) semi-solid PP3 agar (0.3% agar). The agar was allowed to set and the plates were incubated at 28 °C for 48 h. The outcome of the competition could be

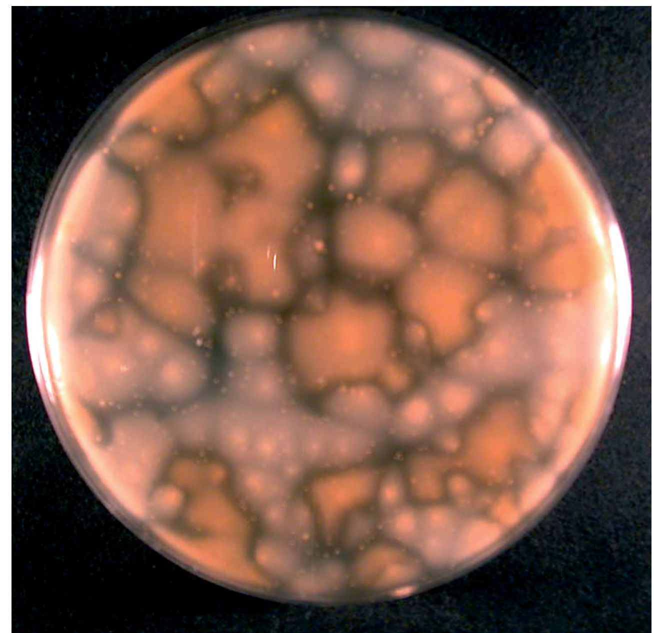

Figure 3. Coexistence of Asy and X3 in a spatially structured environment. Photograph of semi-solid agar plate in which Asy and X3 were inoculated and allowed to grow. The coexistence of the two strains is visible because of their differential pigmentation under these growth conditions. Zones of inhibition exist between each zone of growth, illustrating their antagonistic interaction.

visualized as a result of the differential pigmentation of the two bacterial species when grown under these specific conditions (figure 3). A comparable number of cells grew under both homogenous and heterogenous conditions.

3. RESULTS AND DISCUSSION

We examined the ability of three strains of entomopathogenic bacteria to kill each other through toxin production *in vitro*. Figure 1 shows the growth inhibition of each of the strains on each of the other strains. From this it is clear that Asy and X3 can kill each other and Hb, whereas Hb cannot kill either Asy or X3.

We then determined the outcome of competition between these strains in caterpillars, and how this affected virulence relative to single-strain infections. The data were consistent with our predictions based on the bacteriocin activities. Both Asy and X3 competitively excluded Hb (figure 2*a*; Wilcoxon test of median of lowest-frequency strain less than 1: $n = 4$, $p = 0.05$, in both cases), whereas Asy and X3 did not competitively exclude each other (figure 2*a*; Wilcoxon test: $n = 4$, $p > 0.2$). Virulence of mixed Asy + X3 infections was less than both single Asy and X3 infections (figure 2*b*; Fisher's exact test: $p < 0.05$, in both cases), whereas the Asy + Hb and X3 + Hb mixtures resulted in the same rate of host mortality as single Asy and X3 infections, respectively (figure 2*b*; Fisher's exact test: $p > 0.2$, in both cases).

The lack of competitive exclusion of either Asy or X3 within the same host may be dependent on spatial structure within the caterpillar. Spatial structure allows different strains to dominate in different localities, impeding competitive exclusion (Frank 1994; Durrett & Levin 1997; Pagie & Hogeweg 1999; Czárán *et al.* 2002; Kerr

et al. 2002; Czárán & Hoekstra 2003). By contrast, removing spatial structure effectively creates a single population, where one strain is likely to dominate. To address the role of spatial structure to the outcome of competition, we competed the strains in both homogeneous (shaken tubes) and heterogeneous (static agar plates) *in vitro* environments. In a homogenous environment, Asy competitively excluded X3 in all cases (Wilcoxon test: $n = 4$, $p > 0.2$). By contrast, in a heterogeneous environment Asy and X3 dominated in different localities (figure 3), suggesting spatial structure is important for the coexistence of bacteriocin-producing strains.

We have demonstrated the crucial role of bacteriocins in mediating competitive interactions, and how this can result in lower virulence in mixed- compared with single-strain infections. Lower virulence in single- compared with mixed-strain infections is inconsistent with much theory (Bremermann & Pickering 1983; Frank 1992, 1996; van Baalen & Sabelis 1995), but such theory has not considered the specific biological details of interacting pathogens, such as the production of antimicrobial toxins (A. Gardner, S. A. West and A. Buckling, unpublished data), as investigated here, or the cooperative production of extracellular molecules necessary for successful growth (Turner & Chao 1999; Brown *et al.* 2002; West & Buckling 2003). Given the ubiquity of bacteriocins, reduced virulence in mixed- compared with single-strain bacterial infections is likely to be common.

This work was funded by a Royal Society Merit Award to R.ff.-C.; A.B. is funded by a Royal Society University Research Fellowship.

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