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Competition and reproduction in mixed infections of pathogenic and non-pathogenic *Bacillus* spp.

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Abstract

Diamondback moth, *Plutella xylostella*, larvae were infected with a primary pathogen, *Bacillus thuringiensis kurstaki* (Btk) in single strain and mixed infections. Mixed infections comprised Btk and a non-pathogenic isolate, either *Bacillus thuringiensis tenebrionis* (Btt) or *Bacillus cereus* (Bc). All strains reproduced in larval cadavers, but there was evidence of competition between different isolates within hosts. Non-pathogenic isolates (Btt, Bc) had growth rates that were faster than Btk *in vivo*, whereas Btk outcompeted Btt *in vitro*. Passage through insects increased the *in vitro* competitive ability of Btk against Btt.

Keywords: *Bacillus cereus*; *Bacillus thuringiensis*; Co-infection; Competition; Mixed infection; Reproduction

1. Introduction

Bacillus thuringiensis (Bt) is a spore forming Gram-positive bacterium. On sporulation, Bt produces crystalline parasporal inclusion bodies. These crystals consist predominantly of δ-endotoxin proteins that are known to have insecticidal activity (de Maagd et al., 2001). Although different toxins have been found to be active against a wide range of insects, each toxin is generally restricted in action to a few species within one insect order (de Maagd et al., 2001). Bt is very closely related to *Bacillus cereus*, the latter species is comprised of a specialist pathogenic clade that is associated with human food-poisoning (emetic *B. cereus*) as well as a number of paraphyletic clades that group taxonomically with Bt, but fail to produce crystal toxin (Priest et al., 2004). These non-pathogenic strains of *B. cereus* are believed to reproduce primarily as a soil saprophytes or gut symbionts in insects (Jensen et al., 2003).

Recombination during mixed infections is fundamental to the evolution and diversity of *B. thuringiensis* toxins (de Maagd et al., 2001; Vilas-Boas et al., 1998). Mixed infection of pathogenic and non-pathogenic strains also has important evolutionary implications for the maintenance of toxin-based virulence. When virulence factors are metabolically costly, competition with avirulent strains within hosts may lead to selection for increased replication at the price of decreased virulence (Brown et al., 2002). Despite the importance of mixed infection in Bt evolution there has been little investigation of the basic ecological interactions within hosts between diverse strains of Bt or between Bt and strains of *B. cereus* that do not produce toxins (Broderick et al., 2000; Vilas-Boas et al., 1998). In this study, we tested whether non-pathogenic Bt strains or *B. cereus* can reproduce as opportunistic pathogens in mixed infections; whether multiple strains competed for resources within hosts and *in vitro*; and tested if passage in a mixed infection in a host could select for improved competitive ability.

2. Materials and methods

2.1. Bacterial and insect strains

Three strains of bacteria were used: *Bacillus thuringiensis kurstaki* HD1 (Btk rif^R) isolated from the commercial

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biopesticide preparation, DiPel WP (Valent Biosciences); *Bacillus thuringiensis tenebrionis* (Btt spec^R) donated by DJI Thomas (HRI, Wellesbourne, UK) and *B. cereus* 6A4 (Bacillus Genetic Stock Centre, Bc strep^R). The *B. cereus* isolate has been placed in the ST33 clade using MLST and is therefore closely related to Btk (Richard Ellis, pers. comm.). All strains had been selected for resistance on *B. cereus* selective agar plates (BcSA, Oxoid, UK) with gradually increasing doses of antibiotic such that inclusion of different antibiotics (rifampicin, spectinomycin, or streptomycin at 100 μg ml^{-1}) in media ensured selective growth of the resistant strain. The stability of resistance was confirmed after 10 plate transfers. Selection for resistance to rifampicin did not affect the pathogenicity of Btk, although it has reduced its ability to grow within insects (B. Raymond unpublished data). A Btk susceptible population of diamondback moth, *Plutella xylostella* L., (supplied by Exosect, Southampton, UK) was maintained at 25 °C on 4–5 week old greenhouse-grown Chinese cabbage, *Brassica pekinensis* cv. "One Kilo S.B.". Neither the Btt spec^R nor Bc strep^R strain can kill or reproduce in *P. xylostella* in single strain infections.

2.2. Competition within larvae

Bc strep^R, Btk rif^R, and Btt spec^R were grown for 5 days on BcSA plates at 30 °C, at which time sporulation is nearly complete. Spores and crystals were recovered from plates and subjected to three rounds of washing and centrifugation (at 12000*g*) in sterile saline (0.85% w/v NaCl) before spore density was measured with a haemocytometer. Chinese cabbage leaf discs (40 per treatment) were inoculated by dipping leaves into spore suspensions for 10 s using standard techniques (Sayyed et al., 2000). The three treatments (Btk alone, Btk + Bc, and Btk + Btt) used suspensions with 2000 spores μl^{-1} of Btk and, in mixed infection treatments, 500 spores μl^{-1} of either Btt or Bc. After leaves had dried, five 3rd instar *P. xylostella* larvae were confined on each leaf disc in a 50 mm Petri dish at 20 °C. After 7 days cadavers from each Petri dish were pooled, homogenised in sterile saline (0.85% NaCl), and pasteurized (45 min at 70 °C). Previous work has established that at this time (7 days after insects have been added to inoculated leaves) bacterial growth is complete in all the resulting cadavers (B. Raymond unpubl. dat). We plated serial dilutions of each sample (three aliquots of 20 μl per dilution) onto BcSA containing rifampicin and, in addition, replica plated mixed infection samples onto BcSA containing spectinomycin (for Btt spec^R) or streptomycin (for Bc spec^R). Colonies were counted after incubation at 30 °C for 24 h and again after 48 h.

2.3. In vitro competition

We isolated non-clonal stocks of Btk rif^R and Btt spec^R from cadavers in the Btk + Btt treatment in previous experiment. Stocks were transferred from the original insect homogenates plates onto BcSA containing the appropriated antibiotic in order to remove contamination from other bacterial species. We refer to these stocks as 'passaged Btk' and 'passaged Btt'. Both passaged strains and the original cultures of the Btk and Btt strains were grown overnight at 28 °C in LB broth and the density of vegetative cells then assessed with a haemocytometer. The competitive ability of the different strains was measured in mixed inoculations of LB broth culture (6 ml LB in a 30 ml glass universal) such that each universal had an initial density of 6.5×10^3 cells ml^{-1} of each strain. Four treatments were used: (1) both original strains; (2) original Btk + passaged Btt; (3) passaged Btk + original Btt; (4) both passaged strains. Treatments were replicated four times. Cultures were incubated for 14 days at 28 °C with shaking (120 rpm), and final spore density was assessed by replica plating 200 μl of diluted broth onto LB plates containing rifampicin or spectinomycin and incubating for 48 h.

3. Results and discussion

All three strains displayed substantial reproduction in larval cadavers (Fig. 1a) demonstrating that non-pathogenic strains of *Bacillus* can reproduce opportunistically in cadavers killed by pathogenic isolates. Mortality in the single strain infection was 96% and was very similar in the mixed infection treatments (Btk + Bc 98%, Btk + Btt 96%). Reproduction of co-infecting strains was positively correlated within samples (Btk rif^R and Bc strep^R $R^2 = 0.32$, $F_{1,37} = 19.24$, $P < 0.001$, Fig. 1b; Btk rif^R and Btt spec^R $R^2 = 0.49$, $F_{1,37} = 38.65$, $P < 0.001$, Fig. 1c) suggesting that variation in growth conditions between replicates affected pathogenic and non-pathogenic strains similarly.

Btk competed with co-infecting strains: its reproduction was reduced when non-pathogenic strains were present (one-way ANOVA $F_{1,115} = 24.64$, $P < 0.001$) (Fig. 1a). Since genetically distinct members of the Bt/*B. cereus* complex are found together at very local spatial scales (Collier et al., 2005), mixed infections are likely to be common in the field and should impose strong selection pressure on competitive ability. The *B. cereus* isolate grew faster than Btk rif^R when they competed within hosts (paired $t = 8.08$, df = 39, $P < 0.001$, Fig. 1a). Comparisons between the two mixed infection treatments (Btk + Bc, Btk + Btt) shows that *B. cereus* produced more spores than Btt spec^R ($t = 4.53$, df = 78, $P < 0.001$, Fig. 1a), as might be expected from a strain that does not invest in crystal toxin production. Increased growth rates did not translate to more severe competition as the effects of Bc strep^R and Btt spec^R on Btk reproduction were indistinguishable (Fig. 1a); statistical model simplification with Btk reproduction as a response variable showed that pooling the two mixed infection treatments did not result in a significant loss of explanatory power ($F_{1,117} = 0.92$, $P = 0.33$).