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Quantifying the reproduction of *Bacillus thuringiensis* HD1 in cadavers and live larvae of *Plutella xylostella*

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Abstract

The *Bacillus cereus* group comprises a range of micro-organisms with diverse habits, including gut commensals, opportunistic pathogens and soil saprophytes. Using quantitative microbiological methods we tested whether *Bacillus thuringiensis* (*Bt*) could reproduce in cadavers of *Plutella xylostella* killed by *Bt*, or in the gut of live insects, or be transmitted vertically from females to their offspring. We also tested whether diverse *Bt* strains could grow in high nutrient broth at a pH similar to that in the larval midgut. Low levels of reproduction were found in insect cadavers but there was no evidence of vertical transmission, or of significant reproduction in live insects. Four strains of *B. thuringiensis* var. *kurstaki* and one of *B. thuringiensis* var. *tenebrionis* were found to be capable of growth at high pH. Greater spore recovery rates in frass were found in hosts that were resistant or tolerant of infection. We concluded that that spores recovered in frass represent, in general, an ungerminated fraction of ingested inoculum and that germination rates are reduced in unsuitable hosts.
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1. Introduction

There is a wide diversity of niches occupied by the *Bacillus cereus* complex (Jensen et al., 2003). The entomopathogen *Bacillus thuringiensis* (*Bt*) is considered to be part of this group, together with *B. cereus sensu stricto* and *Bacillus anthracis,* the causative agent of anthrax. Despite high levels of pathogenicity, the ability of *B. thuringiensis* strains to grow and sporulate effectively within insect cadavers is highly variable (Prasertphon et al., 1973; Suzuki et al., 2004). Effective transmission of *Bt* between larvae has been difficult to demonstrate experimentally (Takatsuka and Kunimi, 1998) and can require a high density of hosts and/or cannibalism (Knell et al., 1998). These low rates of transmission and reproduction may, in part, explain the lack of secondary infection from *Bt* microbial sprays (van Frankenhuyzen, 1993).

The variable success of *Bt* as a pathogen has led to much speculation regarding possible alternative reproductive strategies. This includes the suggestion that *Bt* is a soil micro-organism with incidental insecticidal activity (Martin and Travers, 1989); that *Bt* is part of the phylloplane microflora and has evolved to provide protection against plants (Elliot et al., 2000; Smith and Couche, 1991) and that *Bt* may be part of the commensal gut flora of many insects without causing overt disease (Jensen et al., 2003). All of these hypotheses remain largely untested, leaving the ecological niche of *Bt* a matter of continuing contention. *Bt* spores can germinate and grow vegetatively in the insect midgut in the process of lethal infections (Chiang et al., 1986) and vegetative cells of *Bt* have been isolated from the guts of soil dwelling insects (Hendriksen and Hansen, 2002). *Bacillus cereus sensu stricto* has also been described as a gut commensal in Lepidoptera and other

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invertebrates (Jung and Kim, 2006; Margulis et al., 1998). Nevertheless, it is not currently known whether *Bt* can proliferate in non-lethal infections in the larval gut and this question is the main focus of this work.

The high pH of the Lepidopteran midgut is a potential physiological barrier to the proliferation of *Bt* in the larval intestine. Early reports of *Bt* (Kreig, 1964)—cited in Burges and Hurst (1977) and *B. cereus* (Raevuori and Genigeorgis, 1975) have claimed that bacteria in this group are not capable of growth at high pH. *Bacillus thuringiensis israelensis* cannot grow at high pH in buffered media, however, pathogenesis in the midgut lowers midgut pH readily (Walther et al., 1986) and vegetative growth of *Bt* can occur 40 min after ingestion of inoculum (Chiang et al., 1986). In non-lethal infections, a functioning gut may retain a high pH and it is not clear, in general, to what extent Lepidopteran adapted strains of *Bt* can tolerate these conditions.

If sub-lethal reproduction does occur and commensal reproduction is a primary or major niche for *Bt* this begs the question of what it is the purpose of investing in a large quantity of protein-based toxin? Jensen et al. (2003) speculate that toxin receptors provide germination cues and increased germination improves the ability of *Bt* to proliferate in the gut. Toxin may of course lower pH by reducing gut function. An additional possibility is that sub-lethal pore-formation caused by low doses of endotoxin and increases the flow of haemolymph and nutrients into the midgut.

Using the diamondback moth, *Plutella xylostella,* as a host we compared the extent to which *Bt* could replicate in lethal infections, and in live hosts before death or sub-lethally infected hosts. We also tested whether a range of *B. thuringiensis* isolates were capable of growth at high pH. We hypothesized that if *Bt* can propagate infections sub-lethally in the gut then spores should either be released in the frass or that non-overt infection should be transmitted from mother to offspring. If spores are released in frass, reproduction should be indicated by the ratio of spores released to those ingested by the host. Finally, if toxin binding or pore-formation assists growth in the intestine we hypothesize that insects which are resistant to or tolerant of infection should be less suitable for sub-lethal proliferation. It follows that if toxin binding is important for sub-lethal growth the proportion of spores in frass to spores ingested should increase in insects that are more susceptible to toxins.

2. Materials and methods

2.1. Bacterial strains, preparation of inocula and enumeration

In order to be able to conclusively identify experimental inoculum from commensal *B. cereus* and other contaminants we used an antibiotic resistant strains of in all experiments. *Bacillus thuringiensis* var. *kurstaki* HD1 (*Btk*) was isolated from a commercial biopesticide preparation, DiPel ($32{,}000\ \mathrm{IU\ mg^{-1}}$; supplied by Biowise, UK) by culturing spores on *B. cereus* specific agar (BcSA; Oxoid, UK). A spontaneous rifampicin resistant mutant, *Btk* rif^R, was produced by repeated sub-culture with this antibiotic on BcSA. This was repeated until the strain could grow at $100\ \mu\mathrm{g\ ml^{-1}}$ rifampicin. The stability of this resistance was ensured by sub-culturing the strain without selection a further 10 times in Luria broth (LB; Sigma–Aldrich, UK). *Bacillus thuringiensis* var. *tenebrionis* (*Btt*) was a gift from DJI Thomas (HRI, Wellesbourne, UK). A spontaneous spectinomycin derivative, designated *Btt* spec^R, was selected using the methods described above. Additional strains of *B. t. kurstaki* were sourced from the *Bacillus* Genetic Stock Centre (BGSC 4A4, 4D4); one strain was isolated from wild *Brassica oleracea* in the UK (Dorset 7.1.o) and has been identified as *B. t. kurstaki* by the presence of parasporal inclusions and multilocus sequence typing (ST8, sequence data available at http://pubmlst.org/bcereus).

Inocula were prepared by streaking glycerol stocks onto BcSA plates. After incubation at 30 °C for 5 days bacterial biomass was removed and resuspended in sterile saline (0.85% NaCl). The cell/spore/toxin suspension was washed by 3 rounds of centrifugation and resuspension in fresh sterile saline. Spore density was then assessed using a haemocytometer and appropriate dilutions made in sterile saline containing 0.005% Triton X-100.

The total number of bacteria in experimental samples (cadavers, frass, eggs, or adult abdomens) was calculated by selective plating procedures. Samples were homogenized by grinding in sterile saline. Tenfold serial dilutions were performed and triplicate 20 μl aliquots of each dilution were dropped onto BcSA containing the appropriate selective antibiotic ($100\ \mu\mathrm{g\ ml^{-1}}$ rifampicin for *Btk* rif^R, or $100\ \mu\mathrm{g\ ml^{-1}}$ spectinomycin for *Btt* spec^R) together with $20\ \mu\mathrm{g\ ml^{-1}}$ cycloheximide to suppress fungal growth. All dilutions were then pasteurized (70 °C for 45 min) before plating out as above to generate spore counts. Plates were incubated at 30 °C for 48 h and the number of colonies counted.

2.2. Insect strains

A *Bt* susceptible population (Lab-UK) of *P. xylostella* was obtained from the Institute of Arable Crops Research, Rothamsted (Harpenden, Hertfordshire, United Kingdom). A second population of *P. xylostella* (Karak) with high levels of resistance to *Btk* was also used. Mechanisms of resistance, maintenance of resistance and insect culture methods have been described previously (Sayyed et al., 2004).

2.3. Growth at neutral and high pH

Autoclaved Luria-Bertani (LB) broth (Sigma) was adjusted to pH 10 using concentrated sterile NaOH in a laminar flow cabinet. The pH of standard LB broth and

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