

# The dynamics of cooperative bacterial virulence in the field.

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**Laboratory experiments have shown that the fitness of micro-organisms can depend on cooperation between cells. While this insight has revolutionized our understanding of microbial life, results from artificial microcosms have not been validated in complex natural populations. Here, we investigate the sociality of essential virulence factors (crystal toxins) in the pathogen *Bacillus thuringiensis* using diamondback moth larvae (*Plutella xylostella*) as hosts. We show that: crystal toxins are a novel form of cooperative public good; and, in a manipulative field experiment, observed stable high relatedness and both frequency and density dependent selection on toxin production. Conditions favoring social virulence can therefore persist under natural population dynamic conditions, and social interactions (rapid cheat invasion) may account for the rarity of disease outbreaks caused by *B. thuringiensis*.**

The growth and virulence of pathogenic bacteria often depends upon cooperation between bacterial cells (1). Bacteria produce and release a range of extracellular factors, which perform a wide range of functions including nutrient acquisition, host cell lysis, biofilm formation, quorum sensing and immune evasion (2-4). The benefits derived from producing these extracellular factors are often shared with neighbouring cells, and so represent a form of cooperation (5) analogous to what economists would call the production of a 'public good'. The problem with such cooperation is that it is susceptible to 'cheating' by cells that benefit from the cooperative behavior of others, without paying the cost of cooperation themselves (2, 6, 7). Many bacterial virulence factors must be secreted to facilitate successful host invasion. As soon as these products leave the cell, they are potentially exploitable by social "cheats" (3, 8, 9). If the production of virulence factors depends on cooperation, the virulence and epidemiology of pathogenic infections will be determined by the outcome of competition between cooperative and cheating strains (10-12).

While the hypothesis that cooperation drives virulence has gained much theoretical attention, there is an absence of empirical support from natural host-parasite systems. Data from artificial microcosms has confirmed that several microbial traits are cooperative (3, 8, 9, 13, 14), and these traits can have virulence consequences (15, 16). However, the extent to which cooperation drives virulence in natural host systems, and the extent to which natural infection leads to the potential for social interactions, remains unclear. Furthermore, previous work has involved careful management of both population structure (relatedness) and population dynamics. This is a simplification, which obscures the extent to which social behaviors and population process can interact. In natural systems, levels of

cooperation are expected to influence population growth, and population-level processes (immigration, emigration, differential growth) will determine the degree of mixing of cooperators and cheats, which in turn determines the relative fitness of cooperators (14, 17). It therefore remains to be seen whether the conditions that favor bacterial cooperation persist in the face of natural population dynamics.

Here, we examine the consequences of cooperation at the individual and population levels with a mixture of laboratory and field experiments in a natural host pathogen system, involving the bacterium *Bacillus thuringiensis* and larvae of the diamondback moth, *Plutella xylostella* (Fig 1A). *B. thuringiensis* is a widespread specialist parasite of invertebrates (18). These bacteria produce diverse proteinaceous crystal toxins that form crystalline inclusions at sporulation and which determine virulence and host-range. After ingestion by an insect host, crystal (Cry) toxins are solubilised and bind to the mid-gut membrane, where they induce mid-gut cell death (19). The perforated midgut allows *B. thuringiensis* to invade the host, an essential process since access to the haemocoel and death are essential for effective replication of this pathogen (20). Cry toxin inclusions are substantial (Fig. 1b), representing around 25% of the total dry weight of *B. thuringiensis* at sporulation (21), and so their production is likely to be metabolically costly. The size of these inclusions ensures that spores of producers and non-producers of toxin can be readily distinguished with standard microscopy. Cry toxins are produced in 25-75% of clones from natural isolates of *B. thuringiensis* and related strains, suggesting that both producers and non-producers coexist stably in natural populations (22, 23). *B. thuringiensis* has been

widely studied as a source of microbial pesticides and Cry toxin genes have been incorporated into genetically modified crops.

We first tested the extent to which toxin production is a social trait within insect larval hosts under controlled laboratory conditions. If Cry toxin production is a social trait, then the benefit of toxin production will be shared with non-producers, who avoid the cost of producing the toxin. We created toxin non-producers by curing them of Cry-toxin bearing plasmids via culture at high temperature (SOM, text), as competition between near-isogenic strains with and without these plasmids is likely to occur in natural populations (22). We inoculated *P. xylostella* with a controlled dose of spores within 24 hours, using a range of frequencies of toxin producers and non-producers (24). Non-producers were unable to infect the host without being co-inoculated with producers (Fig. 2). As caterpillars were exposed to an increasing proportion of toxin producers, the proportion of successful infections increased rapidly, reaching an asymptote after approximately 30% producers (Fig. 2A). In these mixed infections, the proportions of non-producers increased relative to the producers over the course of infection, implying that their growth rates were higher due to avoiding the costs of toxin production (Fig 2B). This supports the hypothesis that toxin production is cooperative, and that solubilised toxin in the larval midgut can be exploited by non-producers to gain access to the haemolymph (21).

Our results show that selection on toxin production differs from that in other known social traits in bacteria. Increased production of bacterial extracellular factors such as siderophores and elastases (3, 8, 9), leads to increased growth. In contrast, with Cry toxin

production, once a bacterium has successfully invaded the haemocoel, there is no further benefit from extra toxin production, and so growth will be greater when there is a higher proportion of non-producers, which avoid the cost of producing the toxin. This leads to a trade-off at the population level between the ability to infect caterpillars (which requires producers) and growth in infected caterpillars (where non-producers grow better), which can lead to greater population growth within hosts at an intermediate proportion of producers (Fig. 2B,C). However, the key cost of toxin cheats in this pathogen system is in reduced transmission. The presence of cheats may not diminish population size within hosts, but they will reduce the overall toxin production in a cadaver and lower the probability of ongoing infections (Fig. 2A).

We examined how the social costs and benefits of toxin production play out under field conditions. Theory predicts that the relative fitness of non-producers will depend upon the extent to which they are able to co-infect hosts with toxin producers, which will depend upon ecological factors such as the density of bacteria, frequency of producers, and the extent to which producers are aggregated (14, 17). For example, higher population densities, a greater frequency of producers and less aggregation between patches, will all make hosts more likely to ingest both producers and non-producers, and hence increase the extent to which non-producers can exploit cooperators. We planted 204 six-week old cabbages at Wytham Farm, near Oxford, UK and bagged them with a fine net to exclude herbivores and their natural enemies (Fig1A). One week later, we added 35 third instar diamondback moth larvae to each plant. After a further 48 hours we inoculated each plant with *B. thuringiensis* spores, with a factorial design that involved three density treatments

and five frequency treatments of toxin producers (Fig S2, Fig S3) (24). We took two leaf samples per plant at 0, 14 days, 28 and 56 days post-spraying and recovered *B. thuringiensis* spores from saline leaf washes. This regime allowed larvae that have ingested spores to die and release new inoculum onto host plants and soil between sample dates (22). Insect populations crashed after day 28 and additional larvae (N=30 per plant) were released at day 45.

We found that cooperation and virulence were determined by the way in which relative frequency and density affected the competitive dynamics of toxin producers and non-producers (Fig 3). Examining across plants, non-producers did better when at higher cell densities ( $df = 9$ , Likelihood ratio = 4.71,  $P = 0.030$ ), and when producers were more common (mixed model ANOVA,  $df = 8$ , Likelihood ratio = 33.8,  $P < 0.0001$ ). This is expected given that these factors would make non-producers more likely to co-infect hosts with producers. Furthermore, the nature of the frequency dependence was such that both types, producer and non-producer, were able to increase in fitness when rare. We then analysed frequency dependence at the within plant level, examining plants for which we had reasonable abundance of *B. thuringiensis* over two consecutive time-points, and found the same pattern. Considering changes in producer frequency from both 0 to 14 days and 14 to 28 days, we found that the relative fitness of non-producers over this period was significantly negatively correlated with the starting proportion of non-producers (Fig 3B;  $F_{1,31} = 32.7$ ,  $P < 0.0001$ ). Importantly, the relative fitness of the non-producers was  $>1.0$  when rare, and reached  $<1.0$  when common, showing again that both producers and non-

producers can invade when rare, explaining why both types are observed in natural populations.

Relatedness, or the spatial separation of producers and cheaters, is critical to the persistence of cooperative traits (25). Typically, relatedness is inferred from neutral population genetic markers (26). Here, we were able to score relatedness directly at the *cry* toxin gene, because strains expressing toxins are identifiable with light microscopy. Spatially aggregated populations of cooperators will emerge if plant colonization by *B. thuringiensis* is rare and there is fine-scale competition between patches of microbes based on investment in toxin production. Considering the first 14 days of the experiment, changes in relatedness tracked the changes in frequency of toxin producers, as might be expected from standard definitions of relatedness (SOM), so that treatments with initially low frequencies of toxin producers had higher relatedness at time point 14 (treatments 0 and 0.05, Fig 4, frequency\*time interaction, Likelihood ratio = 22.6,  $P < 0.0001$ ). However, despite the rapid turnover in microbial populations at the plant level, and wide fluctuation in the frequency of toxin producers between time points, relatedness at the level of the entire field plot remained high throughout the experiment (Fig 4B). The highly aggregated distribution of bacteria on growing leaf tissue (SOM, text) provides indirect support for our hypothesis of rare colonization. Evidence of the benefits of toxin production for local bacterial populations was found in the correlation between toxin production and bacterial density in the final time point of the field experiment (Fig S5; SOM, text).

These experiments illustrate how cooperative, virulent bacteria and avirulent cheats compete and co-exist in a natural environment. As social evolutionary theory predicts, non-producing cheats can outcompete producers both within hosts and within local populations. Here, we have shown that cooperation can still be stable, because cheats drive down population density, and because co-operators do better when rare (negative frequency dependence) and at low population densities (negative density dependence). The rapidity with which toxin cheats can invade patches of cooperators is likely to have consequences for bacterial population dynamics. *B. thuringiensis*, unlike most insect pathogens, very rarely causes epidemics in the field (27, 28). The increased fitness of cheats at high densities, and their ready availability in the soil, means that invasion of non-infectious cheats could rapidly curtail the direct host-host transmission required for an epidemic. In the field, *B. thuringiensis* killed cadavers tend to fall into the soil soon after death (22), suggesting that most natural infections will occur indirectly after dispersal from a soil reservoir rather than from cadaver to larva during epidemics.

The more general implications of this work will depend upon the prevalence of cooperative virulence among bacterial pathogens. However, a large number of essential virulence factors in important human pathogens are likely to be cooperative because they are exotoxins, which are necessarily secreted outside of the bacterial cell, and are therefore potentially exploitable by cheats. These include anthrax toxins, diphtheria toxin, cholera toxin, Shiga toxin, *Clostridium* spp. exotoxins, pneumolysin, Botulinum toxin, pertussis toxin, *Staphylococcus* alpha toxin, and tetanus toxin (29). In some instances (*e.g.* in *Vibrio cholerae*) the ecological similarities with *B. thuringiensis* are striking: natural populations are composed of both toxin producers and non-producers, toxin binding occurs on intestinal

receptors and activates the release of host resources, and secreted toxins are encoded on mobile elements (30). Our results suggest that social interactions between toxin producers and non-producers can explain the co-existence of virulent and avirulent bacteria, and that sociality will influence the dynamics of virulence in natural populations.

## References and Notes

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### **Supporting Online Material**

Materials and Methods

SOM Text

Figs. S1-S6

References (31-48)

**Fig. 1.** (a) Bagged plants at experimental field site on Wytham Farm, Oxfordshire. (b). Phase-contrast micrograph of cells from a sporulated culture *B. thuringiensis kurstaki*, isolate HD-1 (ST 8) just prior to lysis, parasporal protein crystals lie adjacent to oval spores, micrograph courtesy of Prof B.A. Federici.

**Fig. 2.** Toxin production, within host competition and virulence. As the proportion of toxin producers is increased, this leads to: (a) an increase in larval mortality ( $\chi^2=103$ ,  $df = 2$ ,  $P < 0.0001$ ); (b) an increase in the number spores produced per caterpillar by non-producers (linear term  $F_{1,49} = 104$ ,  $P < 0.0001$ , quadratic term  $F_{1,48} = 13.0$ ,  $P = 0.0007$ ), but no change for producers ( $F_{1,60} = 2.40$ ,  $P = 0.126$ ); (c) the number of spores per cadaver initially increases, but then levels off or decreases (linear term  $F_{1,72} = 268$ ,  $P < 0.0001$ ; quadratic term  $F_{1,71} = 97.2$ ,  $P < 0.0001$ ). All fitted models pass through the origin (0% producers) where no infections or bacterial replication occurred, data are means  $\pm$ SEM.

**Fig. 3.** Frequency- and density-dependence in the field. (A) The proportion of toxin producers (arc-sine square root transformed) plotted against time over the first 14 days of the experiment. The data is divided according to the initial proportion of producers in experimentally applied *B. thuringiensis* (i.e. 0, 0.05, 0.5, 0.95 and 1.0) and the density of this inoculum (low or high). The relative fitness of producers can be inferred from the slopes of the fitted model; producer have higher relative fitness (positive or near zero slopes) when rare and at lower population densities. (B) Examining changes in the frequency of non-producers over time, within plants, the relative fitness of non-producers

was lower when they were more common, considering changes from both 0-14 and 14-28 days. Cheats have equal fitness with producers when their fitness = 1.

**Fig. 4.** The dynamics of relatedness at the Cry toxin locus at two spatial scales. **A.** Changes in untransformed plant level relatedness in the five frequency treatments in the first two time points. **B.** Population level variation in relatedness (summed across all plants in the experimental plot) and proportion of toxin producers: error bars for relatedness are 99% jackknifed CLs, calculated with  $n = 125, 81, 84,$  and  $50$  plants, for time points  $0, 14, 28$  and  $56$  respectively. Proportional data are means  $\pm$  SEM for  $n = 2335, 1863, 1097$  and  $614$  stains for time points  $0, 14, 28$  and  $56$ .

Figure 1



Figure 2

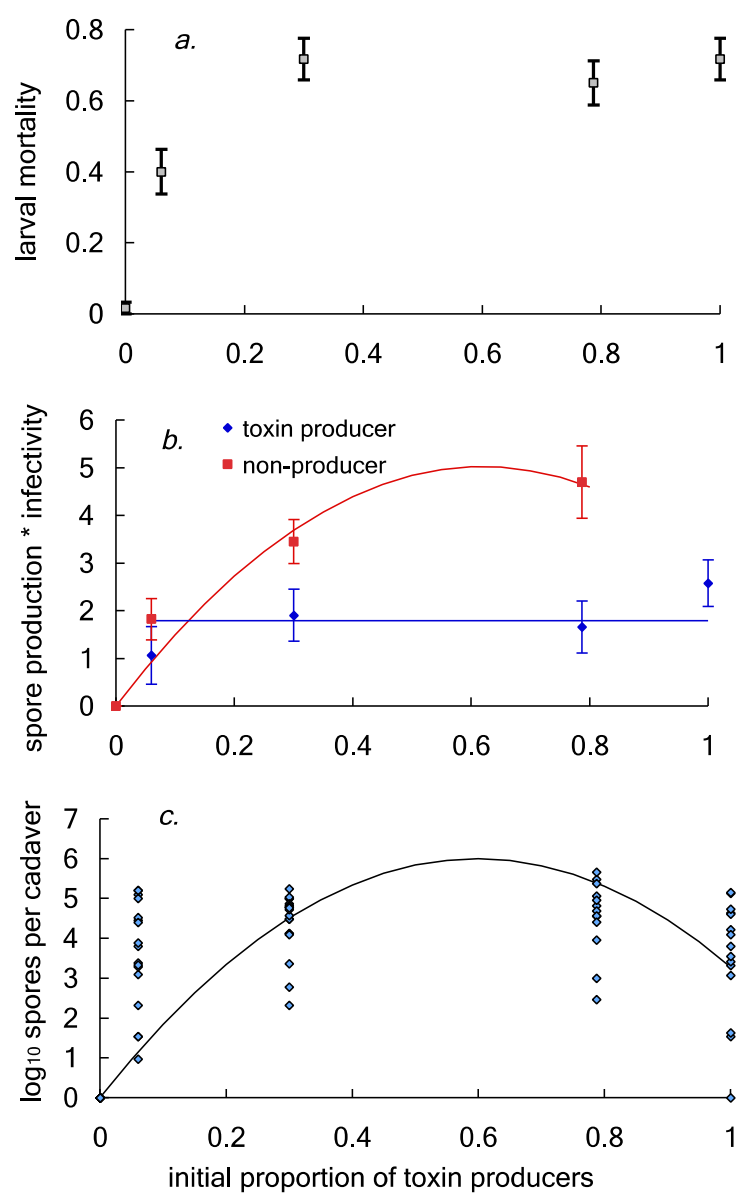


Figure 3

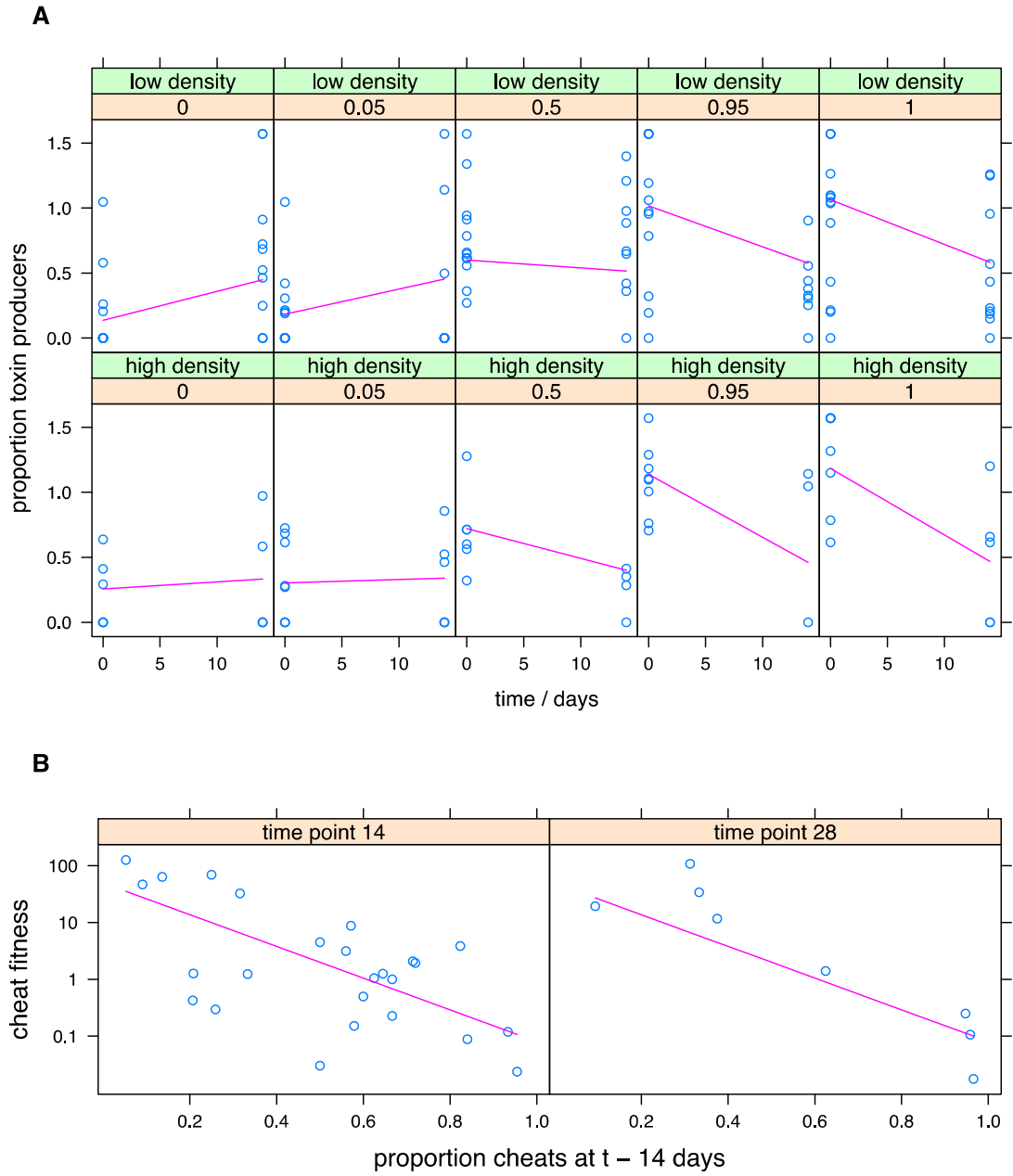


Figure 4

