The Dynamics of Cooperative Bacterial Virulence in the Field

Ben Raymond,[1]* Stuart A. West,[2] Ashleigh S. Griffin,[2] Michael B. Bonsall[2,3]

Laboratory experiments have shown that the fitness of microorganisms can depend on cooperation between cells. Although this insight has revolutionized our understanding of microbial life, results from artificial microcosms have not been validated in complex natural populations. We investigated the sociality of essential virulence factors (crystal toxins) in the pathogen *Bacillus thuringiensis* using diamondback moth larvae (*Plutella xylostella*) as hosts. We show that toxin production is cooperative, and in a manipulative field experiment, we observed persistent high relatedness and frequency- and density-dependent selection, which favor stable cooperation. Conditions favoring social virulence can therefore persist in the face of natural population processes, and social interactions (rapid cheat invasion) may account for the rarity of natural disease outbreaks caused by *B. thuringiensis.*

The growth and virulence of pathogenic bacteria often depends on cooperation between bacterial cells (*1*). Bacteria produce and release a range of extracellular factors that 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 neighboring 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 detemined by the outcome of competition between cooperative and cheating strains (*10*–*12*).

Although 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 have confirmed that several microbial traits are cooperative (*3*, *8*, *9*, *13*, *14*) and that 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 remain unclear. Furthermore, previous work has involved careful management of both population structure (relatedness) and population dynamics. This is a simplification that obscures the extent to which social behaviors and population processes can interact. In natural systems, levels of cooperation are expected to influence population growth, and population-level processes (immigration, emigration, and 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, which determine virulence and host-range. After ingestion by an insect host, crystal (Cry) toxins are solubilized and bind to the midgut membrane, where they induce midgut cell death (*19*). The perforated midgut allows *B. thuringiensis* to invade the host, an essential process because access to the hemocoel and death are essential for effective replication of this pathogen (*20*). Cry toxin inclusions are substantial (Fig. 1B), representing ~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 nonproducers of toxin can be readily distinguished with standard microscopy. Cry toxins are produced in 25 to 75% of clones from natural isolates of *B. thuringiensis* and related strains, which suggests that both producers and nonproducers 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 nonproducers, who avoid the cost of producing the toxin. We created toxin nonproducers by

Fig. 1. (**A**) Bagged plants at experimental field site on Wytham Farm, Oxfordshire. (**B**) Phase-contrast micrograph of cells from asporulated culture *B. thuringiensis kurstaki,* isolate HD-1 (ST8) just before lysis; parasporal protein crystals lie adjacent to oval spores and are marked with solid triangles. [Micrograph courtesy of B. A. Federici]

[1]School of Biological Sciences, Royal Holloway University of London, Egham, TW20 0EX, UK. [2]Department of Zoology, University of Oxford, Oxford, OX1 3PS, UK. [3]St. Peter's College, Oxford, OX1 2DL, UK.

*To whom correspondence should be addressed. E-mail: ben.raymond@rhul.ac.uk

curing them of Cry-toxin–bearing plasmids via culture at high temperature (*24*) (supplementary 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 nonproducers (*24*). Nonproducers were unable to infect the host without being coinoculated 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 ~30% producers (Fig. 2A). In these mixed infections, the proportions of nonproducers increased relative to the producers over the course of infection, which implied that their growth rates were higher because they avoided the costs of toxin production (Fig. 2B). This supports the hypothesis that toxin production is cooperative and that solubilized toxin in the larval midgut can be exploited by nonproducers to gain access to the hemolymph (*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 hemocoel, there is no further benefit from extra toxin production, and so growth will be greater when there is a higher proportion of nonproducers, 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 nonproducers grow better), which can lead to greater population growth within hosts at an intermediate proportion of producers (Fig. 2, B and 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 nonproducers will depend on the extent to which they are able to coinfect hosts with toxin producers, which will depend on 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 nonproducers and, hence, increase the extent to which nonproducers 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 (Fig. 1A). 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 (figs. S2 and S3) (*24*). We took two leaf samples per plant at time 0, and 14, 28, and 56 days after 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 nonproducers (Fig. 3). When we examined across plants, nonproducers did better when at higher cell densities (df = 9, likelihood ratio = 4.71, P = 0.030) and when producers were more common [mixed-model analysis of variance (ANOVA), df = 8, likelihood ratio = 33.8, $P < 0.0001$]. This is expected given that these factors would make nonproducers more likely to coinfect hosts with producers. Furthermore, the nature of the frequency dependence was such that both types, producer and nonproducer, were able to increase in fitness when rare. We then analyzed frequency dependence within plants, 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 nonproducers over this period was significantly negatively correlated with the starting proportion of nonproducers ($F_{1,31}$ = 32.7, $P < 0.0001$) (Fig. 3B). It was noteworthy that the relative fitness of the nonproducers was >1.0 when rare and reached <1.0 when common, which showed again that both producers and nonproducers can invade when

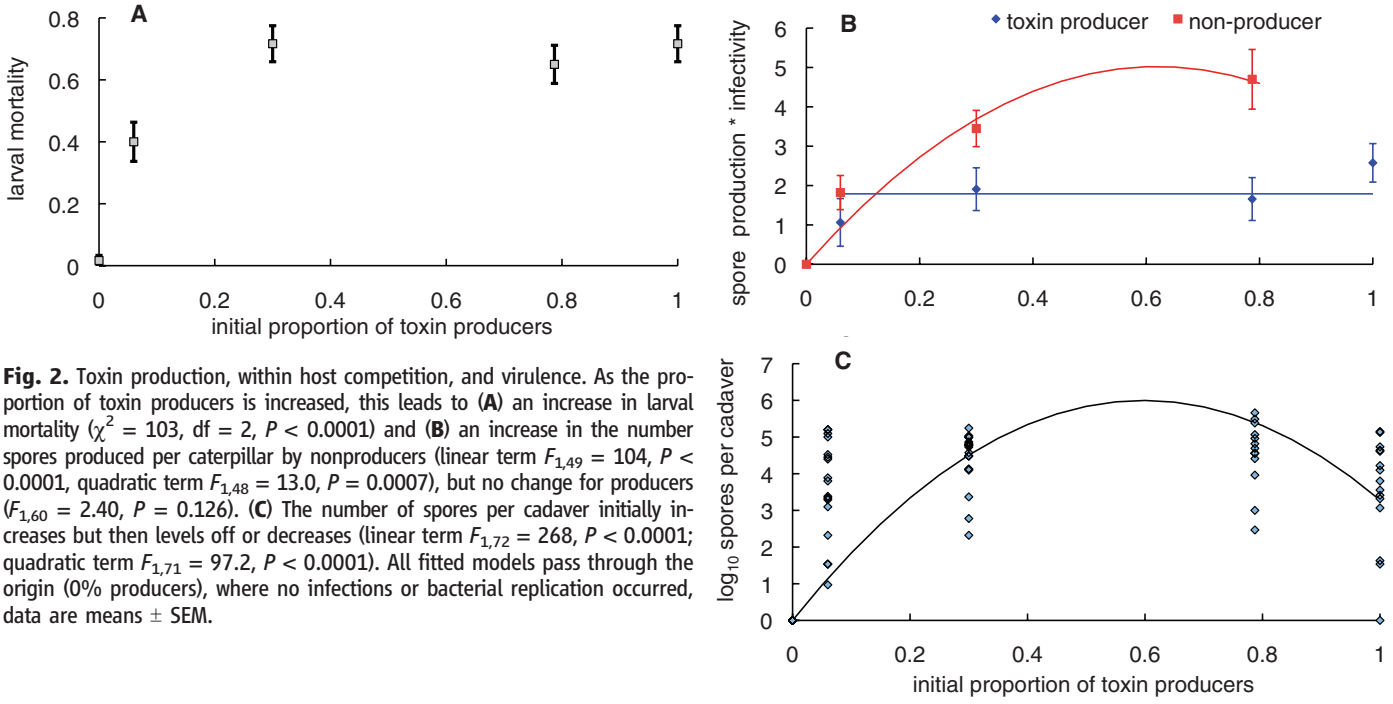

Fig. 2. Toxin production, within host competition, and virulence. As the proportion of toxin producers is increased, this leads to (**A**) an increase in larval mortality (χ^2 = 103, df = 2, $P < 0.0001$) and (**B**) an increase in the number spores produced per caterpillar by nonproducers (linear term $F_{1,49}$ = 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 ± SEM.

rare and explains 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

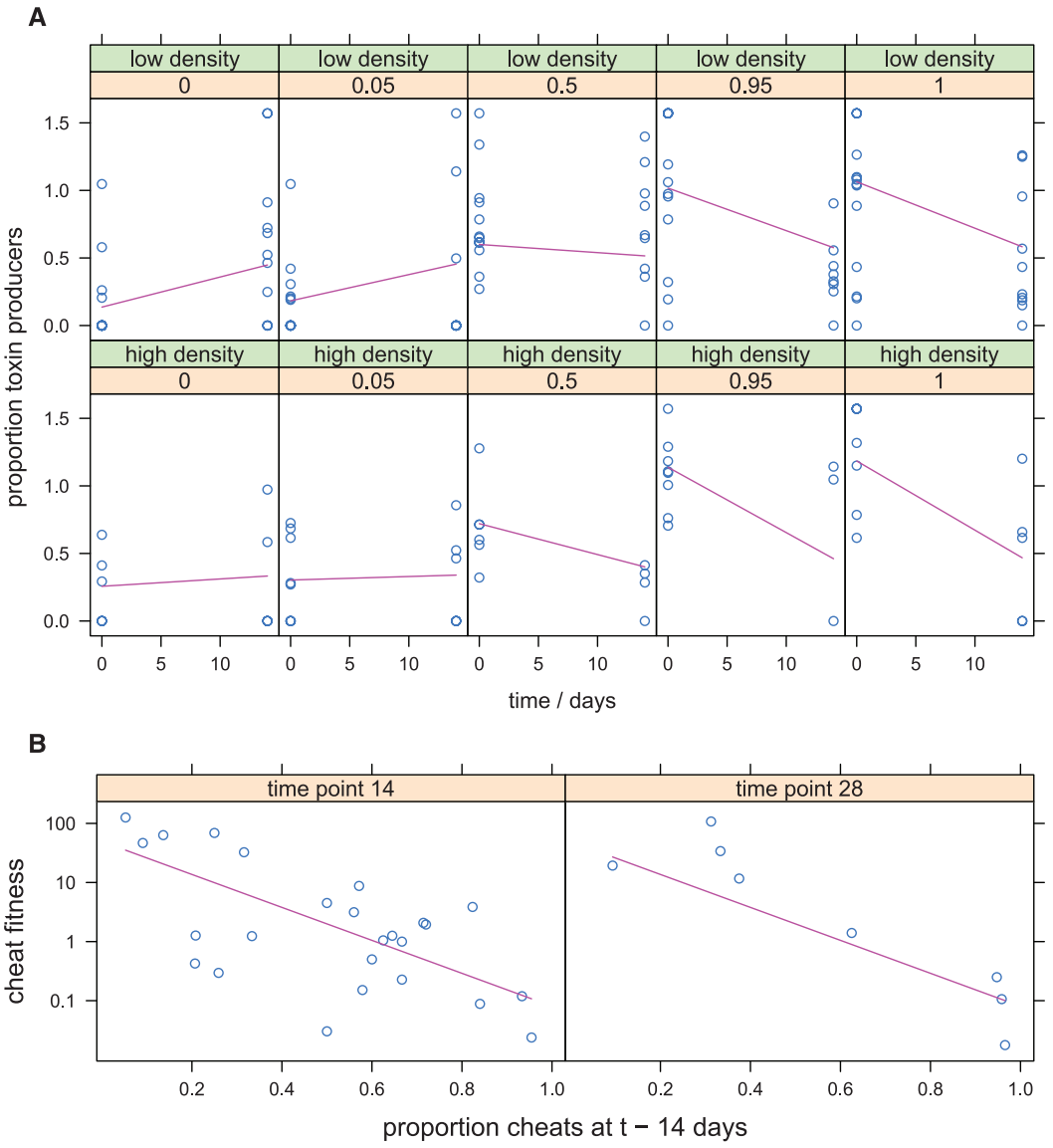

Fig. 3. Frequency- and density-dependence in the field. (**A**) The proportion of toxin producers (arc-sine square root transformed) plotted against time over the first 14 days of the experiment. The data are divided according to the initial proportion of producers in experimentally applied *B. thuringiensis* (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; producers have higher relative fitness (positive or near zero slopes) when rare and at lower population densities. (**B**) Examining changes in the frequency of nonproducers over time, within plants, the relative fitness of nonproducers was lower when they were more common, considering changes from both 0 to 14 and 14 to 28 days. Cheats have equal fitness with producers when their fitness = 1. Each data point represents a single independent plant.

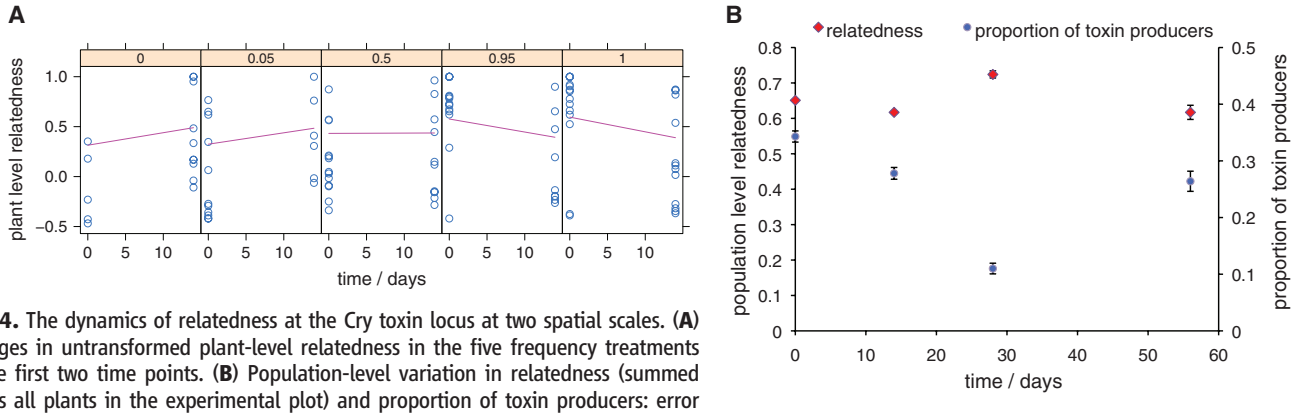

Fig. 4. The dynamics of relatedness at the Cry toxin locus at two spatial scales. (**A**) Changes in untransformed plant-level relatedness in the five frequency treatments at the first two time points. (**B**) Population-level variation in relatedness (summed across all plants in the experimental plot)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

competition between patches of microbes based on investment in toxin production. When we considered 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 (supplementary materials), 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 (supplementary materials and supplementary 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; supplementary materials and supplementary text).

These experiments illustrate how cooperative, virulent bacteria and avirulent cheats compete and coexist in a natural environment. As social evolutionary theory predicts, nonproducing 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 cooperators 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 mean that invasion of noninfectious 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*), which suggests 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 on 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, diptheria 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 nonproducers, 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 nonproducers can explain the coexistence of virulent and avirulent bacteria and that sociality will influence the dynamics of virulence in natural populations.

References and Notes

1. S. A. West, A. S. Griffin, A. Gardner, S. P. Diggle, *Nat. Rev. Microbiol.* **4**, 597 (2006).
2. M. A. Brockhurst, M. E. Hochberg, T. Bell, A. Buckling, *Curr. Biol.* **16**, 2030 (2006).
3. S. P. Diggle, A. S. Griffin, G. S. Campbell, S. A. West, *Nature* **450**, 411 (2007).
4. S. A. West, A. Buckling, *Proc. R. Soc. Lond. B Biol. Sci.* **270**, 37 (2003).
5. J. L. Sachs, U. G. Mueller, T. P. Wilcox, J. J. Bull, *Q. Rev. Biol.* **79**, 135 (2004).
6. S. A. Frank, *Foundations of Social Evolution* (Princeton Univ. Press, Princeton, NJ, 1998).
7. S. P. Brown, *Proc. R. Soc. Lond. B Biol. Sci.* **266**, 1899 (1999).
8. A. S. Griffin, S. A. West, A. Buckling, *Nature* **430**, 1024 (2004).
9. K. M. Sandoz, S. M. Mitzimberg, M. Schuster, *Proc. Natl. Acad. Sci. U.S.A.* **104**, 15876 (2007).
10. S. P. Brown, M. E. Hochberg, B. T. Grenfell, *Trends Microbiol.* **10**, 401 (2002).
11. S. A. Frank, *Q. Rev. Biol.* **71**, 37 (1996).
12. A. Buckling, M. A. Brockhurst, *Heredity* **100**, 484 (2008).
13. J. E. Strassmann, Y. Zhu, D. C. Queller, *Nature* **408**, 965 (2000).
14. R. C. MacLean, I. Gudelj, *Nature* **441**, 498 (2006).
15. K. P. Rumbaugh *et al.*, *Curr. Biol.* **19**, 341 (2009).
16. T. Köhler, A. Buckling, C. van Delden, *Proc. Natl. Acad. Sci. U.S.A.* **106**, 6339 (2009).
17. A. Ross-Gillespie, A. Gardner, S. A. West, A. S. Griffin, *Am. Nat.* **170**, 331 (2007).
18. B. Raymond, P. R. Johnston, C. Nielsen-LeRoux, D. Lereclus, N. Crickmore, *Trends Microbiol.* **18**, 189 (2010).
19. M. Soberón *et al.*, *Science* **318**, 1640 (2007).
20. B. Raymond, S. L. Elliot, R. J. Ellis, *J. Invertebr. Pathol.* **98**, 307 (2008).
21. H. Agaisse, D. Lereclus, *J. Bacteriol.* **177**, 6027 (1995).
22. B. Raymond, K. L. Wyres, S. K. Sheppard, R. J. Ellis, M. B. Bonsall, *PLoS Pathog.* **6**, e1000905 (2010).
23. F. A. Collier, S. L. Elliot, R. J. Ellis, *FEMS Microbiol. Ecol.* **54**, 417 (2005).
24. Materials and methods are available as supplementary materials on *Science* Online.
25. W. D. Hamilton, *J. Theor. Biol.* **7**, 1 (1964).
26. O. M. Gilbert, K. R. Foster, N. J. Mehdiabadi, J. E. Strassmann, D. C. Queller, *Proc. Natl. Acad. Sci. U.S.A.* **104**, 8913 (2007).
27. M. Porcar, P. Caballero, *J. Appl. Microbiol.* **89**, 309 (2000).
28. J. Myers, *Adv. Ecol. Res* **18**, 179 (1988).
29. L. M. Prescott, J. P. Harley, D. A. Klein, *Microbiology* (McGraw-Hill, New York, ed. 5, 2002).
30. J. Reidl, K. E. Klose, *FEMS Microbiol. Rev.* **26**, 125 (2002).

Acknowledgments: Raw field data are available in the supplementary materials. This work was supported by a Natural Environment Research Fellowship award (to B.R.), the Biotechnology and Biological Sciences Research Council (grant no. BBC 5127021), the European Research Council, and the Royal Society. The authors declare no conflicts of interest.

Supplementary Materials

www.sciencemag.org/cgi/content/full/337/6090/85/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S6
References (*31*–*45*)

20 December 2011; accepted 28 May 2012
10.1126/science.1218196

A Single Promoter Inversion Switches *Photorhabdus* Between Pathogenic and Mutualistic States

Vishal S. Somvanshi,[1]* Rudolph E. Sloup,[1] Jason M. Crawford,[2]† Alexander R. Martin,[1] Anthony J. Heidt,[1] Kwi-suk Kim,[1] Jon Clardy,[2] Todd A. Ciche[1]‡

Microbial populations stochastically generate variants with strikingly different properties, such as virulence or avirulence and antibiotic tolerance or sensitivity. *Photorhabdus luminescens* bacteria have a variable life history in which they alternate between pathogens to a wide variety of insects and mutualists to their specific host nematodes. Here, we show that the *P. luminescens* pathogenic variant (P form) switches to a smaller-cell variant (M form) to initiate mutualism in host nematode intestines. A stochastic promoter inversion causes the switch between the two distinct forms. M-form cells are much smaller (one-seventh the volume), slower growing, and less bioluminescent than P-form cells; they are also avirulent and produce fewer secondary metabolites. Observations of form switching by individual cells in nematodes revealed that the M form persisted in maternal nematode intestines, were the first cells to colonize infective juvenile (IJ) offspring, and then switched to P form in the IJ intestine, which armed these nematodes for the next cycle of insect infection.

Pathogenic and mutualistic bacteria can exist in different states in their host to survive sudden environmental shifts such as antibiotic exposure or host immune activation (*1*, *2*). *Photorhabdus luminescens* bacteria are bioluminescent symbionts of *Heterorhabditis bacteriophora* nematodes, and the two organisms (as a mutualistic pair) infect, kill, and reproduce inside insects. Nematodes in the infective juvenile (IJ) stage penetrate an insect host and