

LETTER

Cheating, trade-offs and the evolution of aggressiveness in a natural pathogen population

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Abstract

The evolutionary dynamics of pathogens are critically important for disease outcomes, prevalence and emergence. In this study we investigate ecological conditions that may promote the long-term maintenance of virulence polymorphisms in pathogen populations. Recent theory predicts that evolution towards increased virulence can be reversed if less-aggressive social ‘cheats’ exploit more aggressive ‘cooperator’ pathogens. However, there is no evidence that social exploitation operates within natural pathogen populations. We show that for the bacterium *Pseudomonas syringae*, major polymorphisms for pathogenicity are maintained at unexpectedly high frequencies in populations infecting the host *Arabidopsis thaliana*. Experiments reveal that less-aggressive strains substantially increase their growth potential in mixed infections and have a fitness advantage in non-host environments. These results suggest that niche differentiation can contribute to the maintenance of virulence polymorphisms, and that both within-host and between-host growth rates modulate cheating and cooperation in *P. syringae* populations.

Keywords

Arabidopsis thaliana, bacteria, competition, microbial ecology, *Pseudomonas syringae*, virulence.

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INTRODUCTION

The evolutionary and ecological mechanisms that maintain life history variation in pathogens are fundamental to an understanding of disease emergence, epidemiology and infection outcomes (Ochman & Moran 2001; Barrett *et al.* 2008). Aggressiveness (i.e. within-host-growth), is among the most important of pathogen life history traits, affecting the incidence and impact of pathogens in human, agricultural and natural systems. For the many pathogenic microbes that occur in genetically diverse populations (Read & Taylor 2001), classical theoretical investigations of virulence evolution predict that resource competition among competing strains favours strains with higher virulence, because more virulent strains are more aggressive and transmit at faster rates (May & Anderson 1983; Nowak & May 1994). However, results from recent empirical studies (Gower & Webster 2005; Staves & Knell 2010) suggest that evolution towards increased aggressiveness is not an inevitable outcome of within-host competition. Classical theory of pathogen evolution is therefore unlikely to be sufficient to describe evolution in mixed infections.

Pathogenicity in many microbes depends on the secretion and acquisition of extracellular molecules that enhance performance via subversion of host immune responses and facilitation of access to host nutrients (Salmond 1994; Preston *et al.* 2005). These metabolically costly (Bartra *et al.* 2001; Griffin *et al.* 2004) pathogenicity factors modify the host environment for all co-occurring pathogen strains, and thus act as common goods (Frank 2010a) that may be exploited by non-secreting ‘cheater’ strains (Chao *et al.* 2000; Brown *et al.* 2002; West & Buckling 2003). The secretion of common goods is thus a type of altruistic behaviour, the evolution of which has been shown by theory to require restrictive conditions. As a consequence, evolutionary theorists have sought to explain the maintenance of cooperative

pathogen strains (Brown *et al.* 2002; Ross-Gillespie *et al.* 2007; West *et al.* 2007; Racey *et al.* 2010).

In testing for cheating, previous studies have used strains that were experimentally evolved or artificially engineered to use extracellular molecules secreted by cooperator genotypes (Turner & Chao 1999; Griffin *et al.* 2004; Harrison *et al.* 2006). These experiments have confirmed a potential for cheating to occur under laboratory conditions, but whether or not such dynamics occur in nature is not known. Meanwhile, theory to explain the maintenance of cooperator–cheater polymorphisms has focused on mechanisms that can be observed *in vitro*, notably on kin selection (Griffin *et al.* 2004). However, polymorphisms in nature may be maintained by mechanisms that are not apparent *in vitro*, eliminating the need for the moderate to high relatedness inherent in kin-selection models (Frank 2010b). Herein we present the first evidence for a cooperator–cheater polymorphism in nature, and through a combination of data and models show that this polymorphism is likely maintained in part by a trade-off between within-host and between-host reproduction.

Studies of pathogen dynamics *in vitro* typically focus on competition in a single environment, i.e. within the host. However, pathogens may also compete outside the host. Indeed, for the many ecologically and agriculturally important pathogens that are opportunistic, or maintain free-living stages, survival and reproduction in the environment is likely to be essential. Such life-history strategies entail survival and propagation in non-host environments for long periods of time, enabling pathogens to persist when hosts are not present (Dwyer 1994; Bonhoeffer *et al.* 1996; Gandon 1998). Survival in the environment has been shown by models (Gandon 1998) to play a crucial role in pathogen competition, although the effect of reproduction outside the host has been rarely explored. One of our

goals is to incorporate this life-history strategy into models of pathogen competition.

Herein, we investigate how within-host growth (i.e. aggressiveness) and reproduction in the environment together mediate bacterial competition in nature. We studied the pathogenic bacterium *Pseudomonas syringae*, which occurs at high frequency in populations of the plant *Arabidopsis thaliana* in the Midwestern US (Kniskern *et al.* 2011). Pathogenic strains of *P. syringae* are natural parasites of *A. thaliana* (Jakob *et al.* 2002). While infections are typically sublethal, disease symptoms and pathogen densities within-host plants are negatively related to host fecundity (Roux *et al.* 2010). *Pseudomonas* is a diverse and ubiquitous bacterial genus that includes species with a range of symbiotic and free-living life histories. Pathogenicity in this group is facilitated by the type three secretion system (TTSS) (Preston 2007), a needle-like apparatus that translocates effector proteins into host cells (Buttner & Bonas 2002). Collectively, effector proteins subvert host defences and facilitate the release of nutrients from host cells (Göhre & Robatzek 2008). Because *P. syringae* inhabits extracellular environments on the leaf surface and in the leaf mesophyll, benefits conferred by the TTSS and associated effectors have the potential to benefit neighbouring individuals even if nearby strains lack mechanisms facilitating pathogenicity.

A further critical feature of *P. syringae* is its capacity for growth and survival as a free-living saprophyte in water and soil (Morris *et al.* 2008). Hypotheses for the maintenance of cooperation in this species can thus be framed around whether or not secretion of pathogenicity factors within the host is negatively correlated with performance outside the host. Importantly, the expression of the pathogenicity-promoting TTSS can be dependent on the environmental context in which growth occurs (Higuchi *et al.* 1959). For example TTSS expression in plant-pathogenic strains of *P. syringae* is activated in oligotrophic (low nutrient) environments (Huynh *et al.* 1989). Given that *P. syringae* is found in such environments outside the host (Morris *et al.* 2008), and given that transmission among hosts may occur via environmental sources (e.g. from host contact with soil or rain) (Hollaway *et al.* 2007), the opportunity exists for energetic costs associated with the expression of the TTSS in host and non-host environments to modulate competition between strains and the maintenance of cooperation.

The utility of the *P. syringae*–*A. thaliana* interaction for investigating the maintenance of variation in pathogenicity is evidenced by the recent discovery of an apparently non-pathogenic *P. syringae* lineage carrying an aberrant TTSS (Mohr *et al.* 2008) (Supplementary Figure S1). Strains of this lineage do not cause disease, have reduced capacity for growth in plants, are limited in their capacity to deliver effectors into host cells, and do not elicit the qualitative, innate immune response that is known as a hypersensitive response or 'HR' (Clarke *et al.* 2010; Kniskern *et al.* 2011). We therefore refer to strains carrying this aberrant TTSS as 'HR–', and strains carrying the canonical TTSS as 'HR+'. Importantly, HR– strains have been found to co-occur with HR+ strains within wild *A. thaliana* populations (Kniskern *et al.* 2011). Given the established role that TTSS-secreted effectors play in pathogenesis and growth, the occurrence of a seemingly non-pathogenic clade of *P. syringae* begs the question of what ecological conditions might favour such an evolutionary transition. We thus asked two questions. First, do non-pathogenic HR– strains exhibit cheating behaviour? If so, does a trade-off between reproduction in the host and reproduction in the environment help maintain a cooperator–cheater polymorphism in this system?

Asking whether or not non-pathogenic strains cheat is equivalent to asking whether HR– strains compensate for reduced growth *in planta* via the exploitation of resources made publically available by HR+ strains (Brown 1999; Brown *et al.* 2002; Buckling & Brockhurst 2008). Asking whether or not reproduction in the environment helps to maintain the polymorphism is equivalent to asking whether HR– strains have a competitive advantage over HR+ strains when grown in the environment (Caraco & Wang 2008). To address these questions, we used a series of glasshouse and microcosm experiments in which we measured the growth of HR+ and HR– strains *in planta* (alone and in competition) and *ex planta* (in eutrophic and oligotrophic environments). In addition, we specifically investigated the role of TTSS polymorphisms in environmental fitness by performing a parallel set of experiments using genetically modified versions of HR+ and HR– strains. Finally, we developed a mathematical model to test the extent to which differences in competitive ability in within-host vs. between-host environments promote the coexistence of HR+ and HR– strains of *P. syringae*.

MATERIALS AND METHODS

Sampling of natural endophytic bacterial populations

We sampled 50 *Arabidopsis* plants from four localities (three in Michigan, one in Indiana) during spring 2008. Plants were collected in sterile 10-mL tubes and kept on ice during transport. We surface-sterilised a single non-symptomatic leaf from each plant, ground the leaf in sterile buffered 10 mM MgSO₄, and plated the homogenate onto King's medium B (KB) augmented with nitrofurantoin (50 µg mL⁻¹). After incubation at 28 °C for 2 days, colonies displaying discrete morphotypes were stored at –80 °C. To identify bacterial isolates, we amplified and sequenced part of the 16S ribosomal gene. We compared the resulting sequence against type strains in the Ribosomal Database Project. Isolates identified as *P. syringae* were then subjected to phenotypic characterisation using LOPAT tests (Lelliott *et al.* 1966) and by sequencing the *gyrB* gene to confirm species identity.

Genetic analyses and experiments

To characterise the genetic structure of *P. syringae* populations, we assembled 57 *P. syringae* isolates from the collections described above and from previous laboratory collections (Jakob *et al.* 2002; Goss *et al.* 2005). The closely related laboratory strain B728a was included as a reference. To test for the presence of alternative TTSS's within these isolates, we used PCR and phenotyping methods (Kniskern *et al.* 2011). For multi-locus sequence typing (MLST) analyses, fragments of six housekeeping genes previously used as genetic markers for this species were amplified from genomic DNA: *gyrB*, *rpoD*, *acnB*, *cts*, *gap*, *pgi*, *pfk*. Patterns of nucleotide polymorphism were assessed using DnaSP v5 (Librado & Rozas 2009). We used ClonalFrame (Didelot & Falush 2007) to estimate evolutionary relationships among the *P. syringae* isolates. We performed three separate runs on the species level dataset, executing 500 000 burn-in iterations, and 500 000 post-burn-in iterations each, always starting with a random tree, and using the default priors for all model parameters. Phylogenies were sampled every 100 iterations after the burn-in. We used the resulting 5001 sampled trees to construct 50% majority consensus phylogenies.

To investigate lateral gene transfer (LGT) of TTSS's, we targeted *Pseudomonas* strains for which sequence data for the 16S ribosomal RNA gene and the conserved TTSS gene *brtN* were available. We also selected three representative *P. syringae* isolates from the sample described above. We tested for recombination within and among gene fragments using RDP v3.4 (Martin *et al.* 2005). Phylogenetic trees were generated using MrBayes v3.1.2 (Ronquist & Huelsenbeck 2003), where we implemented a general time reversible model, with gamma distributed rate variation across sites and a proportion of invariable sites. MCMC analysis was run for five million generations and trees sampled every 1000 generations. The first 12 500 trees were discarded and the remaining 37 500 trees were summarised in a 50% majority rule consensus tree. Results were compared in multiple independent runs to ensure parameter convergence. Tree figures were constructed using MEGA v4 (Tamura *et al.* 2007). Cross-species LGT events were identified using bipartition dissimilarity (Boc *et al.* 2010) as implemented in the software T-REX (Makarenkov 2001).

To resolve the ecological costs and benefits associated with alternative TTSS's, we performed reverse genetic analysis by targeted knockout of a TTSS regulatory gene (*brpL*), and subsequent phenotypic characterisation of mutant strains. In particular, we followed the methods described in Jakob *et al.* (2007), by using marker-exchange mutagenesis to delete *brpL* from the *P. syringae* strains PNA29.1a (HR+) and LP205a (HR-). Detailed methods describing these experiments can be found in the supplementary information.

***In planta* bacterial growth**

For *in planta* growth experiments, we used the *A. thaliana* ecotype, Ga-0 as host. Ga-0 does not express major gene resistance (HR) in response to any of the bacterial strains characterised for growth. An HR+ strain (PNA29.1a) and two HR- strains (ME.Cv.B50, LP205a) were grown overnight in liquid KB, whereupon 50 mL of bacterial cell suspension was diluted in 500 mL KB, grown for 4–5 h, centrifuged at 1351 *g* for 10 min, re-suspended in sterile 10 mM MgSO₄ buffer, and diluted to OD₆₀₀ = 0.2 with 10 mM MgSO₄ buffer. For co-inoculations, we maintained the concentration of individual bacterial strains at 2.5 × 10⁸ cfu mL⁻¹. Twenty-four replicate plants were infected by spraying plants until the leaf surface was saturated. After 3 days, bacterial population size was estimated from four leaves per plant by removing a leaf disc, sterilising the disc in 70% ethanol, grinding in 200 µL of 10 mM MgSO₄, and plating using a WASP2 spiral plater (Microbiology International, Frederick, MD, USA) on KB. Three days later, we counted colonies using a PROTOCOL colony counter (Microbiology International). HR+ and HR- strains were distinguished based on previously identified differences in colony morphology. We confirmed the accuracy (100%) of identifying strains using morphology by randomly selecting 96 colonies for testing by PCR amplification of TTSS genetic variants specific to HR+ and HR- strains (Kniskern *et al.* 2011). We further confirmed that these were the correct strains by re-sequencing the *gyraseB* gene for four colonies belonging to each morphotype. Bacterial population sizes were log-transformed to normalise the data, and analysed using ANOVA.

***In vitro* bacterial growth**

We conducted microcosm experiments to assess the growth of HR+ and HR- strains. Bacteria were initially grown overnight from stocks

in liquid KB. We diluted 0.5 mL of the resultant bacterial cell suspension in 4.5 mL of fresh KB for 4–5 h; centrifuged at 3000 rpm for 10 min., re-suspended in sterile 10 mM MgSO₄ buffer, and diluted to OD₆₀₀ = 0.2 in 10 mM MgSO₄ buffer. These were then diluted 10-fold in 10 mM MgSO₄ to obtain a final titre of 2.5 × 10⁴ cell mL⁻¹. We conducted microcosm experiments in 96-well plates. For each experiment, 20 µL of the OD-adjusted cell cultures (i.e. approximately 250 cells) was added to 180 µL sterile medium. Media used were (1) Kings broth (KB); (2) minimal medium (MM) containing fructose and mannitol as sugar sources (Jakob *et al.* 2007); (3) soil wash (5 g bulk soil collected from the University of Chicago campus gardens, autoclaved in 50 mL 10 mM MgSO₄).

First, five HR+ and four HR- strains were assayed on KB and MM (six replicates of each). Growth in KB and MM was assessed by measuring OD₆₀₀ at 30 min intervals (with 5 s shaking preceding each measurement) for 60 h on a Infinite F200 microplate reader (Tecan USA, Durham, NC, USA). Overall growth was calculated as the total area under the growth curves. Mean growth of HR+ with HR- strains was compared using a one-way ANOVA. Second, growth of wild-type HR+ and HR- strains (PNA29.1a and LP205a) was compared with the growth of corresponding mutant strains where the TTSS had been rendered non-functional (see above). Growth rates were calculated as the total area under the growth curve, and growth was compared using one-way ANOVA. Finally, growth in soil of an HR+ strain (PNA29.1a) was compared with growth of two HR- strains (ME.Cv.B50, LP205a) in two separate experiments. The soil wash experiment was conducted in deep-well 96-well plates with 10-fold higher volumes (200 µL inoculum into 1800 µL medium) than microcosm experiments. In these experiments, the HR+ and HR- strains were grown either in monoculture or inoculated into the same microcosm. Growth in the soil wash was assessed after 48 h using a WASP spiral plater and PROTOCOL colony counter as above, and analysed using a two-way ANOVA (with strain and monoculture/mixture as the independent variables).

Theory

Previous theory of cheater-cooperator dynamics models *in vitro* bacterial growth by assuming that populations approach a carrying capacity (Brown 1999; Ross-Gillespie *et al.* 2007). We are interested in competition in nature, and therefore consider disease transmission and reproduction in the environment. Because disease transmission only occurs during part of the year, we constructed a separate sub-model for the period during which disease transmission occurs, which we then combined with a multi-generation model that allows for competition within the soil.

To allow for maximum generality, we assumed that both the cooperators and cheaters are able to reproduce outside the host. We further assumed that host plants infected by a cooperator strain might be secondarily infected by a non-pathogenic (cheater) strain, but that cheaters cannot infect a host plant alone. Meanwhile, we assume facultative pathogenicity, meaning that both strains are capable of reproducing outside the host. Infection of host plants is expected to depend on the density of both hosts and pathogens, so we use a simple epidemic model (Keeling and Rohani 2007). Because *Arabidopsis* infected with *P. syringae* do not recover, we modified the standard model to eliminate recovery. The resulting model allows for reproduction both in soil and in plants. Although the model is based on the *P. syringae*–*A. thaliana* system, we note that the basic model

assumptions are valid for a wide range of fungal and bacterial pathogens.

For simplicity, we begin by considering soil growth only. We then define x_n and y_n to be the densities of the two microbes in generation n . Our model is then:

$$x_{n+1} = \frac{\rho x_n}{1 + \hat{a}x_n + \hat{b}y_n}, \quad (1)$$

$$y_{n+1} = \frac{\lambda y_n}{1 + \hat{c}x_n + \hat{d}y_n}. \quad (2)$$

Here ρ and λ are the reproductive rates of the two microbes, while \hat{a} , \hat{b} , \hat{c} , \hat{d} are the competition coefficients.

Arabidopsis thaliana typically has only one generation per year, and so it is reasonable to assume that transmission only occurs when leaf tissue is available to be infected. In allowing for within-host growth and infections, we therefore model transmission separately from the dynamics of competition outside the host. We use a standard disease model (Daley & Gani 2001), except that infected host plants do not die during the period of transmission, so we eliminate losses of infected hosts:

$$\frac{dH}{dt} = -\beta V_0 H, \quad (3)$$

$$\frac{dI_V}{dt} = \beta V_0 H - \alpha A_0 I_V, \quad (4)$$

$$\frac{dM}{dt} = \alpha A_0 I_V. \quad (5)$$

Here H is uninfected hosts, I_V is hosts infected with the cooperator, and M is hosts infected with both the cooperator and the cheater. Also, V_0 and A_0 are the respective initial numbers of the cooperators and cheaters, and β and α are the transmission rates of the cooperators and cheaters respectively. We are thus assuming that the non-pathogenic strain can only infect hosts that are already infected with the pathogenic strain.

In the Supplementary Information, we solve equations (3)–(5) to calculate the number of progeny of each strain at the end of the transmission period. As part of this calculation, we define K to be the total host population during transmission. To add pathogen progeny from the infection model to the environment model, we next identify the number of pathogenic progeny V_0 at the beginning of transmission with the number of pathogenic progeny in generation n , $V_0 \equiv x_n$. Likewise, we identify $A_0 \equiv y_n$. Our long-term model is then:

$$x_{n+1} = \frac{\rho x_n}{1 + \hat{a}x_n + \hat{b}y_n} + K\phi \frac{\beta K x_n}{\beta x_n - \alpha y_n} (e^{-\alpha y_n t} - e^{-\beta x_n t}) + K\gamma \left[1 - e^{-\beta x_n t} - \frac{\beta x_n}{\beta x_n - \alpha y_n} (e^{-\alpha y_n t} - e^{-\beta x_n t}) \right], \quad (6)$$

$$y_{n+1} = \frac{\lambda y_n}{1 + \hat{c}x_n + \hat{d}y_n} + K\omega \left[1 - e^{-\beta x_n t} - \frac{\beta x_n}{\beta x_n - \alpha y_n} (e^{-\alpha y_n t} - e^{-\beta x_n t}) \right]. \quad (7)$$

For the pathogenic strain, the term $K\phi \frac{\beta K V_0}{\beta V_0 - \alpha A_0} (e^{-\alpha A_0 t} - e^{-\beta V_0 t})$ represents the net number of progeny produced by singly infected hosts, while $K\gamma \left[1 - e^{-\beta V_0 t} - \frac{\beta V_0}{\beta V_0 - \alpha A_0} (e^{-\alpha A_0 t} - e^{-\beta V_0 t}) \right]$ represents the net number produced by doubly infected hosts. The parameters ϕ

and γ are thus the net number of progeny per host that are produced by each type of host. By ‘net’, we mean that each parameter takes into account over-winter survival of progeny. Meanwhile, progeny of the non-pathogenic strain are produced only by doubly infected hosts, and so the net number of progeny of this strain is $K\omega \left[1 - e^{-\beta V_0 t} - \frac{\beta V_0}{\beta V_0 - \alpha A_0} (e^{-\alpha A_0 t} - e^{-\beta V_0 t}) \right]$. The parameter ω is thus the net number produced per host.

RESULTS AND DISCUSSION

Within-host dynamics

We first examined the genetic diversity of *P. syringae* isolated from naturally occurring populations of *A. thaliana*. The survey showed that both HR+ and HR– clades are present in nature (Fig. 1). Evolutionary diversification in clade 2c suggests that HR– strains have been maintained over long periods (Fig. 1a). We then examined the frequency at which HR+ and HR– strains co-occur in a natural setting. Given the established role that TTSS-secreted effectors play in pathogenesis (Preston *et al.* 2005), we predicted that HR+ strains should greatly outnumber HR– strains in the plant environment. In surveys of natural populations, however, we found that HR+ and HR– strains occur at similar frequencies within populations of wild *A. thaliana* (Fig. 1b).

These initial data suggested that both pathogenic (HR+) and non-pathogenic (HR–) strains persist in nature, but did not identify the mechanisms maintaining the polymorphism. To test for the possibility of cheating by the HR– strain within hosts, we first determined the fitness benefits associated with alternative TTSS’s in HR+ and HR– *P. syringae* strains by experimentally disabling the respective TTSS’s using marker-exchange mutagenesis. As predicted, disabling the TTSS of the HR+ strain PNA29.1a resulted in a 10-fold reduction in growth *in planta*, whereas disabling the xenologous TTSS in the HR– strain LP205a had no significant effect on growth (supplementary Figure S2). Our system thus meets the predicted requirements for competitive exploitation (Brown 1999; Brown *et al.* 2002; Buckling & Brockhurst 2008), in that only HR+ strains use the effector-secreting TTSS to improve their ability to exploit hosts. In *Pseudomonas*–*Arabidopsis* interactions, there is thus an opportunity for effector-secreting pathogenic strains to be exploited by non-secreting cheaters.

To determine if cheating occurs, we tested whether or not the presence of an HR+ strain enhances the growth of less-aggressive HR– strains. In a series of greenhouse experiments, *A. thaliana* plants were surface-inoculated with equal mixtures of HR+ and HR– strains or with one strain alone. We found that HR– strains were significantly outperformed by the HR+ strain when grown alone (Fig. 2, Tukey $P < 0.01$ for both comparisons), thus satisfying initial requirements for exploitation to occur. Parallel competition experiments, however, demonstrated that this hierarchy is context dependent. As hypothesised, the presence of HR+ strains significantly enhanced the growth of HR– strains in co-inoculations (Tukey $P < 0.05$ for both comparisons), to the point that there were no statistical differences in their growth in mixed infections (Tukey $P > 0.05$ for both comparisons). These results demonstrate that TTSS-secreted pathogenicity factors can favourably modify the host environment for non-secreting bacterial strains, and are consistent with the hypothesis that non-secreting bacteria ‘cheat’ by making use of common goods produced by secretors. Nevertheless, it was not known whether the benefits of cheating were sufficient to explain the success and diversification of the HR– lineage (Fig. 1a). Moreover, under existing models

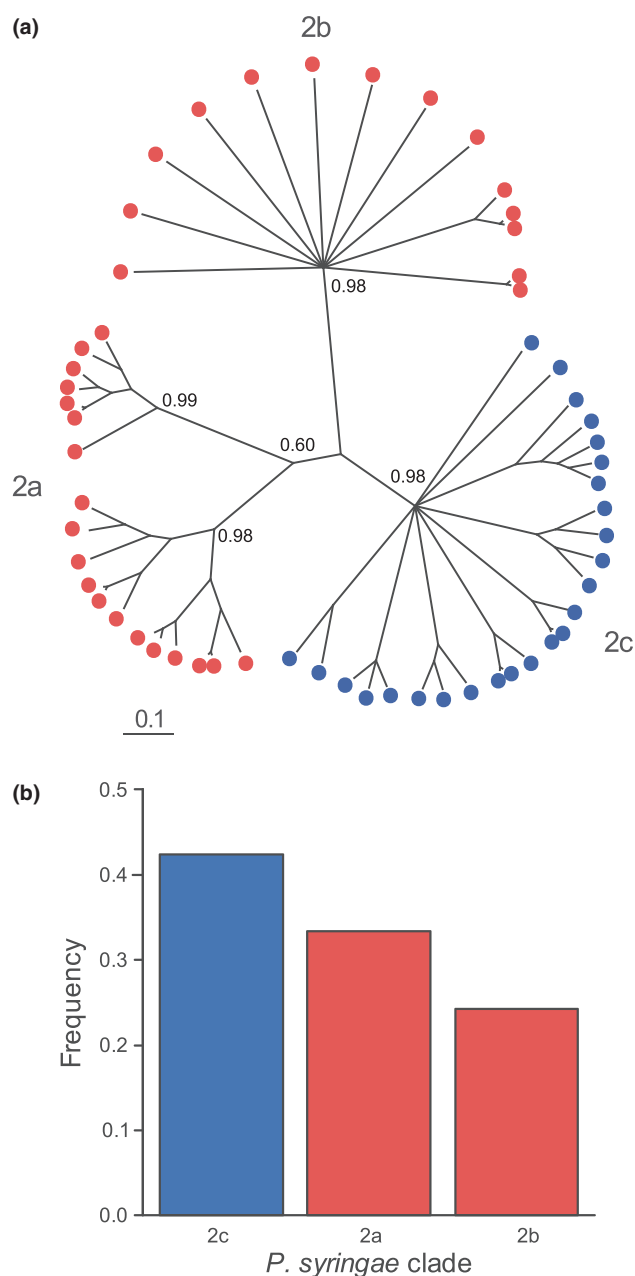


Figure 1 Diversity and frequency of *Pseudomonas syringae* strains sampled from *Arabidopsis thaliana* leaves. Cooperator HR+ strains are shown in red; cheater HR- strains in blue. (a) A 50% majority rule consensus tree generated from ClonalFrame phylogenetic analysis of five housekeeping genes demonstrating the cladistic diversification among 58 isolates of *P. syringae*. Confidence intervals are shown for the main cladistic groups only. Evolutionary diversification in clade 2c suggests that less virulent cheaters have been maintained over long periods. (b) Frequency of HR+ and HR- phenotypes collected from natural populations of *A. thaliana*. HR- phenotypes are maintained within *P. syringae* populations at relatively high frequency, despite reduced virulence of these strains.

of virulence evolution, maintenance of less fecund-cheating strains requires that cheaters have higher fitness than cooperators when they co-occur (Brown 1999; Buckling & Brockhurst 2008). In our system, the increased performance of HR- strains in competition did not translate into significant competitive superiority (Fig. 2a,b; Tukey $P > 0.05$), thus raising the question, is a cheating strategy alone sufficient to maintain the polymorphism?

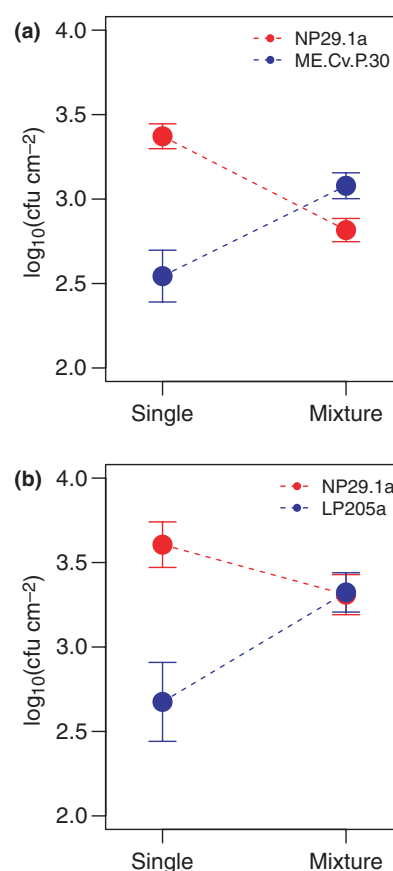


Figure 2 Host-associated benefits of alternative type three secretion system (TTSS's). Data points show mean (\pm standard error) *in planta* growth of HR+ (red) and HR- (blue) strains when inoculated singly, or in mixtures, across two experiments. Strains are (a) NP29.1a and ME.Cv.B50 and (b) NP29.1a and LP205a. For both experiments, inoculation treatment significantly affected growth of *Pseudomonas syringae* genotypes (a: $F_{1,88} = 8.51$, $P < 0.01$; b: $F_{1,90} = 29.76$, $P < 0.0001$). In single inoculation experiments, the HR- strain was outperformed by the HR+ strain (Tukey $P < 0.01$ for both comparisons). When co-inoculated, growth of HR+ and HR- strains were indistinguishable (Tukey $P > 0.05$ for both comparisons), due to an increase in growth of HR- strains (Tukey $P < 0.05$ for both comparisons).

This question is difficult to answer definitively because precise outcomes will depend on the genetic and environmental contexts within which interactions take place. For example, in *P. syringae*, the relative aggressiveness of different HR+ strains can be highly variable depending on the genotypes of the interacting host strains, and the environmental conditions over the course of the infection. The resources made available to HR- strains in co infection scenarios may, in turn, vary depending on the fecundity of the HR+ strain in that host background. Indeed, it is reasonable to hypothesise that the fitness of cheating strains should be positively correlated with the fecundity of cooperating strains, so that increasing aggressiveness of cooperators would allow the maintenance of cheaters with decreased aggressiveness. Finally, the demographic contexts within which interactions occur are also likely to be important in determining the relative fitness of cheaters and cooperators. In particular, previous studies suggest that the costs and benefits of co infection should vary depending on the initial relative frequencies of competing strains (50 : 50 in our experiments), with

the fitness of cheaters negatively related to their initial frequency (Ross-Gillespie *et al.* 2007). These many complexities leave open the possibility that coexistence can be maintained depending only on variation in dynamics occurring *in planta*. Rather than attempting to

define those hypothetical conditions, we instead focused on a more general, and less explored, coexistence mechanism: a trade-off in competitive ability between within-host and between-host environments.

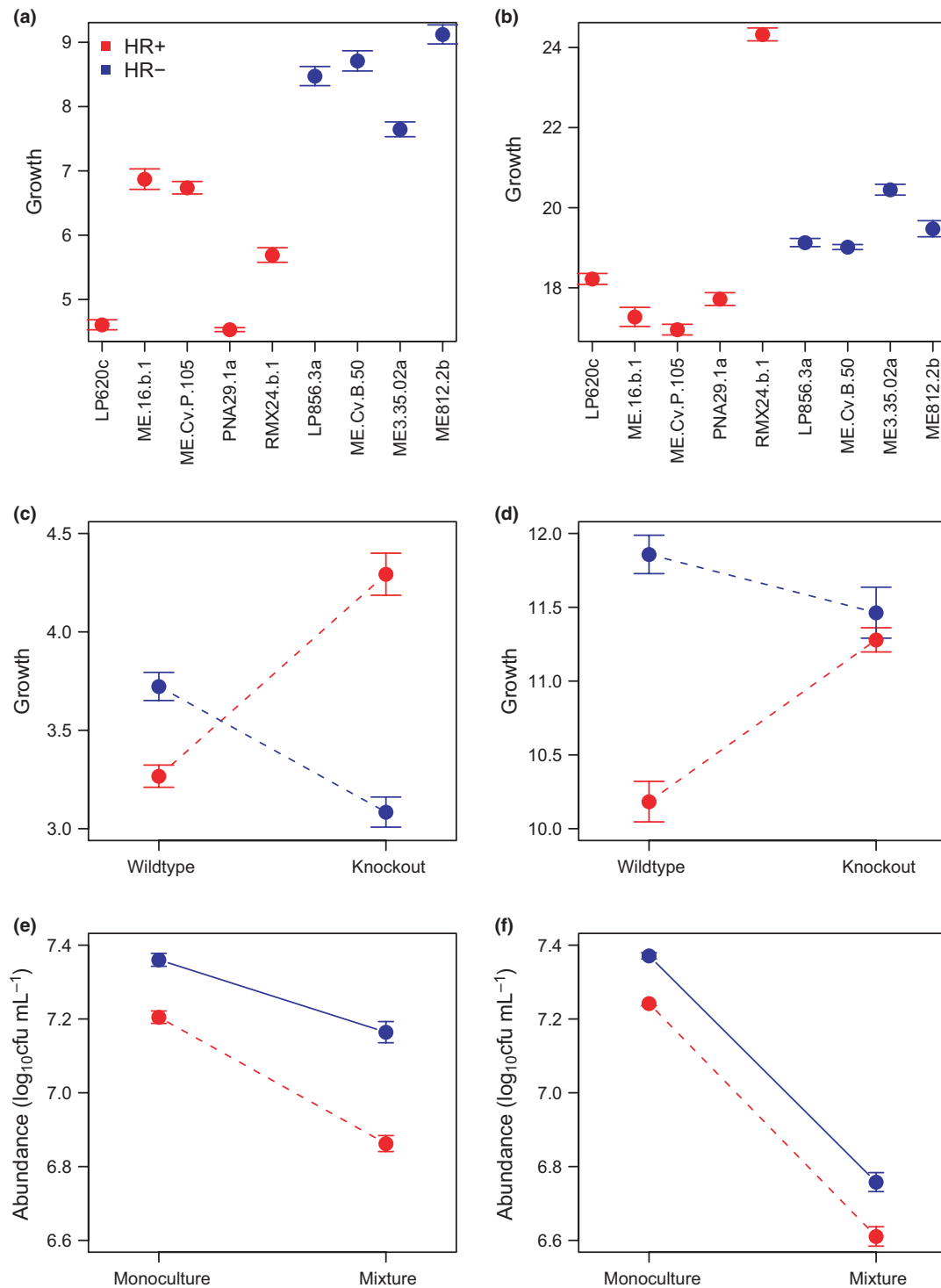


Figure 3 Saprophytic growth of HR+ and HR- strains under different environmental conditions. Five HR+ and four HR- strains were grown in (a) nutrient-poor environment and (b) nutrient-rich environment. Data points are mean (\pm standard error) growth of HR+ (red) and HR- (blue) strains. HR+ growth increased, whereas HR- growth declined, when a key TTSS gene (*hrpL*) was knocked out. Growth of the HR+ knockout was significantly higher than growth of the HR- strain in (c) oligotrophic but not in (d) nutrient-rich environments. In soil, HR- growth for (e) LP205a and (f) ME.Cv.B50 was significantly higher than growth of HR+ strain NP29.1a whether alone or in competition. Growth was estimated as the area under the growth curve over 60 h (a-d) or as the density of colony-forming units after 48 h (e,f) (see Materials and Methods).

Dynamics in non-host environments

P. syringae is capable of growth as a free-living saprophyte in water and soil (Morris *et al.* 2008), and this survival in the environment not only allows for persistence in the absence of a host but also provides a potential transmission pathway between annual plant generations (Hollaway *et al.* 2007). The potential for trade-offs between performance within and between hosts to contribute to the maintenance of polymorphism in *P. syringae* populations is evidenced by experiments showing that for HR+ strains, activation of TTSS expression is triggered in *ex planta* oligotrophic environments (Huynh *et al.* 1989). This led us to hypothesise that the presence of the canonical TTSS may lead to lower fitness for the HR+ strain compared with the HR- strain in oligotrophic conditions outside the host. To test this hypothesis, we measured the *ex planta* growth of HR+ and HR- strains using a series of microcosm experiments in low- and high-nutrient environments. Consistent with our hypothesis, HR- isolates consistently and significantly outperformed HR+ isolates in an oligotrophic environment (Fig. 3a; $F_{1,7} = 17.5$, $P = 0.003$). In contrast, in a nutrient-rich environment (in which TTSS expression is not triggered), there was no statistical difference in growth of HR+ and HR- strains (Fig. 3b; $F_{1,7} = 0.2$, $P = 0.7$).

We next directly tested whether physiological costs associated with the expression of the ancestral TTSS underlie differences in the fitness of HR+ and HR- strains. To do this, we performed a parallel set of experiments using genetically modified HR+ and HR- strains in which the expression of the TTSS was prevented. Consistent with results obtained for naturally occurring variants, disabling the TTSS of the HR+ strain resulted in significantly higher growth of the knockout strain compared with the wild type strain in both the oligotrophic (Fig. 3c, 31% higher growth, Tukey $P < 10^{-7}$) and nutrient rich (Fig. 3d, 11% higher growth, Tukey $P = 0.0003$) environments. There is thus a large direct cost associated with the expression of the canonical TTSS, particularly in oligotrophic environments where growth of the knockout HR+ strain significantly outperformed the wild type HR- strain (Tukey $P = 0.0016$). In contrast, disabling the xenologous TTSS in the HR- strain LP205a resulted in a statistically significant decline in performance in oligotrophic environments (Fig. 3e, 18% lower growth Tukey $P = 0.0004$), while growth was not significantly altered in a nutrient-rich medium (Fig. 3f, Tukey $P = 0.345$). Although we have been unable to identify the reason for the lower performance of HR- knockouts in oligotrophic environment, these results provide further evidence that those strains expressing the xenologous TTSS have the potential to perform well when resources are scarce.

To verify the ecological relevance of these results, we performed a second set of experiments examining the growth of experimental strains in soil, either alone or in competition. Again, HR- strains significantly outperformed HR+ strains (Fig. 3e: $F_{1,20} = 111.2$, $P < 10^{-8}$; Fig. 3f: $F_{1,20} = 52.5$, $P < 10^{-6}$), clearly demonstrating that HR- strains have higher fitness under commonly encountered conditions outside the host. Importantly, given that transmission of *P. syringae* between hosts may occur indirectly via environmental sources (Hollaway *et al.* 2007), our results reveal that TTSS polymorphisms in *P. syringae* populations also have the potential to influence rates at which hosts are infected by different pathogen lineages.

Linking disease transmission and reproduction outside the host

Our results show that naturally occurring strains of *P. syringae* can indeed interact via cooperator–cheater dynamics, and that reproduction outside hosts contributes to the maintenance of the cooperator–cheater polymorphism. Existing theory of cooperator–cheater interactions has focused either on optimal strategies within a pathogen strain (Brown 1999; Brown *et al.* 2002), or on the effects of relatedness across strains (Griffin *et al.* 2004; Ross-Gillespie *et al.* 2007), without considering the effects on coexistence of alternative strategies. We instead consider the conditions permitting coexistence of cooperator and cheater strains within a pathogen population when we allow for competition both inside and outside hosts. Consistent with our empirical observations, coexistence is only possible if the non-pathogenic strain can reproduce outside the host, although otherwise coexistence occurs for a wide range of parameter values (Fig. 4, blue lines). Our model results therefore support our empirical observations, suggesting that exploitation of effector-secreting pathogens, in combination with a fitness trade-off between alternative environments, can explain the diversification of the HR- lineage and the maintenance of pathogenicity polymorphisms in *P. syringae* populations (Fig. 1).

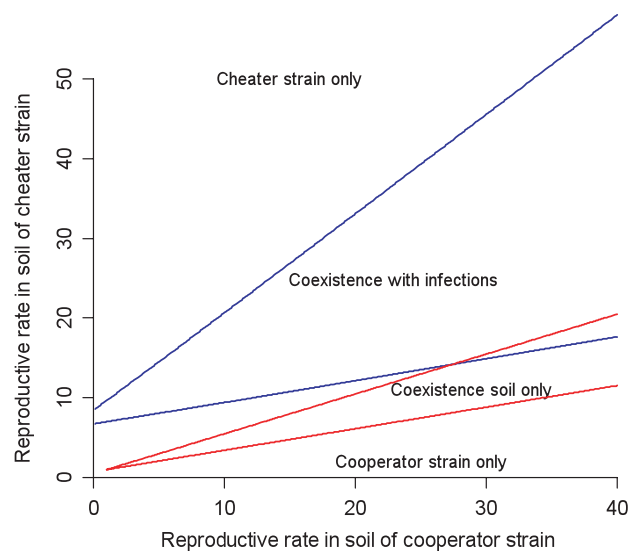


Figure 4 Values of parameters for which cooperators and cheaters coexist, as predicted by a mechanistic model of cooperator–cheater interactions (model details in supplementary material). We compared results for the full model, which allows for infections of host plants and growth outside the host, and a simpler model that allows only for growth in the environment. Consistent with experimental data (Fig. 2), we assumed that the fitness of cooperator and cheater strains in doubly infected hosts is approximately equal. Coexistence occurs when reproductive rates fall between the blue lines for the full model, and between the red lines for the non-host-only model. The model shows that coexistence is possible even if the cooperator strain has zero fitness outside the host, as evidenced by the gap between the blue lines when they meet the vertical axis. Coexistence, however, occurs for a much broader range of parameters, and is thus more likely, if the cooperator has non-zero fitness outside the host. This broadening in the region of coexistence occurs even if the cooperator's reproductive rate outside the host is less than one, which means that reproduction in the soil increases the chances of coexistence even if the cooperator cannot survive through soil-reproduction only. The figure also shows that infection of the host also improves the chances of coexistence, because the range of parameters for which coexistence occurs is lower for the soil-only model.

A basic feature of our model is that the cheater's growth rate must increase if the cooperator's growth rate in the environment increases, otherwise it will go extinct. A less intuitive result is that, as the cooperator's *ex planta* growth rate increases, coexistence becomes more likely because the *range* of cheater growth rates allowing coexistence increases. This effect occurs in a small way even for the no-infection model (red lines in Fig. 4), because here coexistence depends on the ratio of the cooperator and cheater growth rates (see Supporting Information). For the full model, the effect is much stronger because a larger number of cooperators means that there are more hosts infected with cooperating strains, and hence more opportunities for cheaters to exploit cooperators. Increases in the abundance of cooperators in non-host environments can thus be beneficial for the cheater.

Classical theories of the evolution of pathogen virulence have focused on hierarchical competition within hosts (Nowak & May 1994), to which recent observations of cooperator–cheater dynamics stand in stark contrast (West *et al.* 2007). The restriction of previous studies to *in vitro* environments, however, has limited the range of cooperator–cheater coexistence mechanisms under consideration. By instead considering bacterial growth in both host and non-host environments, we have shown that the combination of within-host and between-host reproduction can help maintain cooperator–cheater polymorphisms. By combining theory and data to show how cooperator and cheater strains coexist in nature, our work provides crucial support for a paradigm shift in which theories of pathogen evolution must account for both competition and cooperation (Chao *et al.* 2000; Brown *et al.* 2002; Buckling & Brockhurst 2008).

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AUTHORSHIP

The study was conceived and designed by LB, TB and JB. The experiments were conducted and analysed by TB and LB. The theoretical modelling was conceived by GD and JB, and conducted and written by GD. LB, TB, GD and JB wrote the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Supplementary methods.

Appendix S2 Model description and results.

Figure S1 Comparison of 16S and hrcN gene trees. *P. syringae* strains sampled from *Arabidopsis* are shown in red (HR+) and blue (HR-). Inferred LGT events are indicated by grey or blue shading of isolate names.

Figure S2 Mean (\pm standard error) growth of wildtype and hrpL mutants of the HR+ strains PNA29.1a and the HR+ strain LP205a in the *A. thaliana* ecotype Col-0. Disabling the TTSS strains resulted in a significant reduction in growth in planta of HR+ strains but not of HR- strains.

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