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Report

The Social Biology of Quorum Sensing in a Naturalistic Host Pathogen System

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Summary

Many microorganisms cooperate by secreting products that are commonly available to neighboring cells. These "public goods" include autoinduced, quorum-sensing (QS) molecules and the virulence factors activated by these signals [1–4]. Public goods cooperation is exploitable by cheaters, cells that avoid the costs of production but gain an advantage by freeloading on the products of others [5–8]. QS signals and responses can be cooperative under artificial laboratory conditions [1–4, 9], but it remains unclear whether QS is cooperative in nature: little is known about the frequency of cheaters in natural populations [10, 11], and cheaters may do poorly because of the importance of QS in major transcriptional networks [12–14]. Here, we investigate the cooperative nature of QS in a natural system: the Gram-positive insect pathogen *Bacillus thuringiensis* and the larvae of the diamondback moth, *Plutella xylostella*. Although we find evidence of cooperation, QS null mutants are not effective cheats in vivo and cannot outcompete wild-type strains. We show that spatial structure limits mutant fitness and that well-separated microcolonies occur in vivo because of the strong population bottlenecks occurring during natural infection. We argue that spatial structure and low densities are the norm in early-stage infections, and this can explain why QS cheaters are rare in *B. thuringiensis* and its relatives [10]. These results contrast with earlier experiments describing the high fitness of Gram-negative QS cheaters and suggest that QS suppression ("quorum quenching") can be clinically effective without having negative impacts on the evolution of virulence.

Results and Discussion

Here, we investigated whether the production and response to quorum-sensing (QS) signals are cooperative in vivo and explored the factors affecting the fitness of potential social cheats of the insect pathogen *Bacillus thuringiensis*. This bacterium is a widespread specialist parasite of invertebrates and is closely related to important human pathogens such as *Bacillus anthracis*, the causative agent of anthrax, and *Bacillus cereus*, which can cause potentially lethal food poisoning [15]. This work used previously described QS null mutants that either fail to produce, but can respond to, an oligopeptide QS signal (Δ*papR*, designated signal-null mutant) or fail to respond to signal (Δ*plcR*, designated signal-blind mutant) [16, 17]. We conducted infection and competition experiments in vivo and in vitro to test the predictions of social evolution theory and examined the population structure of fluorescently labeled mutants and wild-type (WT) bacteria in early-stage infections.

We expected that responding to a QS signal would be costly because the PlcR regulon in *B. thuringiensis* and *B. cereus* is responsible for 70% of the extracellular proteome at the stationary phase [18] and is predominantly composed of virulence factors, with roles in host cell lysis (phospholipases, proteases, hemolysins) and food poisoning (enterotoxins) [19]. We found that both mutants had faster growth at the exponential phase in L broth. Competition experiments in broth over 24 hr show that Δ*plcR* mutants could consistently outcompete WT bacteria, whereas Δ*papR* mutants were fitter than WT at low frequencies (Supplemental Experimental Procedures and Figure S1 available online).

Group Level Benefits of QS In Vivo

If QS-regulated virulence factors are cooperative, the benefits of signal response should be shared between both producers and nonproducers within the host. For pathogens, these shared benefits can manifest as improved reproductive rates within the hosts [20] or improved infectivity and access to hosts [8]. This was tested in experiments with single-strain infections and mixed infections of WT and both mutants, with five mutant frequencies at three inoculum doses. Nearly 700 insects were infected in this experiment; on average, we processed 26 cadavers in each treatment. All experiments were conducted with bacteria that do not produce crystal (Cry) toxin because plasmids carrying *cry* genes tend to become lost during genetic manipulation. Cry toxin was added exogenously at a standard concentration of 0.0125 $\mu g\ \mu l^{-1}$ in all experiments because it is essential for host invasion.

We found no clear evidence for shared benefits in terms of mean reproduction. Although WT bacteria tended to reach higher population densities, mutants in mixed infections did not benefit from the presence of WT (Figure 1A). These data were variable, and the distribution of spore densities between hosts was strongly bimodal, with data clustered near zero and around 10^3 cfu μl^{-1} in homogenized cadavers (Figure 1B). When bacterial counts were very low, insects died because of the effect of Cry toxin on gut paralysis, but bacteria did not successfully colonize the cadaver. QS-activated virulence genes did, however, provide a shared benefit in terms of enabling successful colonization. Infection success, defined in terms of exceeding a minimum reproductive threshold, was positively correlated with the total concentration of WT bacteria in each treatment (Figure 1D, $F_{1,25} = 8.5$, $p = 0.0076$, generalized linear model with quasibinomial errors). Neither the type of QS mutant nor the number of mutant bacteria affected infection success ($F_{1,24} = 0.3$, $p = 0.6$; $F_{1,24} = 1.0$, $p = 0.33$). This suggests that the shared benefit of QS is improved access to host resources, as has been found for other *B. thuringiensis* virulence factors [8].

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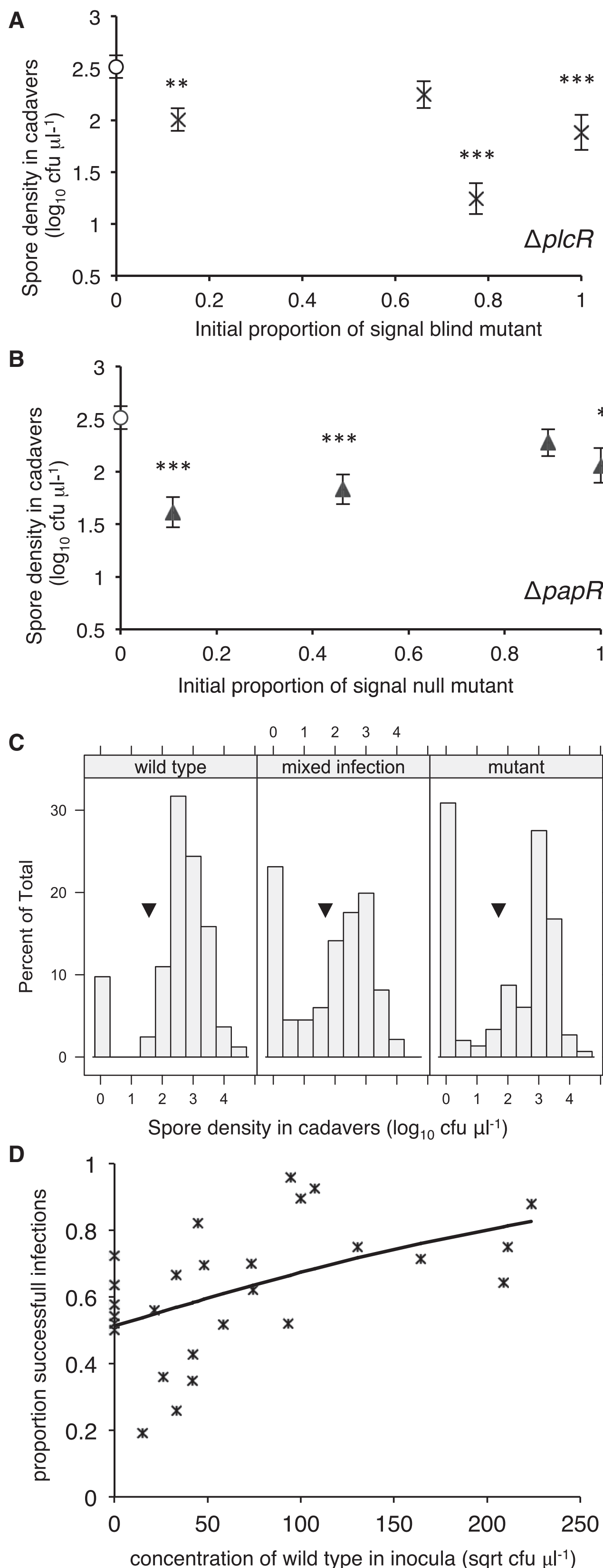

Figure 1. The Shared Benefits of QS Are in Improved Host Infectivity Rather Than in Mean Pathogen Reproduction

(A and B) Mutants did not clearly benefit from the presence of WT bacteria in mixed infections in terms of mean reproductive rates. Results tended to be variable: statistical models in which mutant frequency was fitted as a factor were better than models with frequency as a covariate (Δ*plcR* signal blind: $F_{3,390} = 7.80$, $p < 0.0001$; Δ*papR* signal null: $F_{3,381} = 4.78$, $p = 0.0028$). Asterisks indicate significant differences from 100% wild-type infections (open symbols) in post hoc treatment comparisons. Total inoculum dose also affected reproduction (Δ*plcR*: $F_{1,391} = 17.1$, $p < 0.0001$; Δ*papR*: $F_{1,385} = 20.5$, $p < 0.0001$). Data shown indicate means ± SE.

(C) The bimodal distribution of bacterial reproduction in cadavers accounted for much of this variation. Mutant refers to both classes of QS mutants.

(D) The probability of establishing a successful infection increased with the square root of wild-type bacteria in inocula. Successful infections were defined as those resulting in 10^2 cfu μl^{-1} or more in homogenized cadavers (a total of 5×10^4 cfu per insect); this threshold is indicated with solid triangles in (C).

Null Mutants Show Frequency- and Density-Dependent Fitness but Are Poor Cheats

If QS behaviors were cooperative in vivo, we would also expect that QS null mutants would be able to exploit the secretions of WT producers and gain a growth advantage in mixed infections by avoiding the metabolic costs of signals and/or QS-regulated traits [5]. Theory also predicts that the fitness of cheats should increase with the abundance of public goods and therefore be negatively frequency dependent and positively density dependent as opportunities for cheating increase with the abundance of public goods [21]. Although we found that the fitness of mutants increased with frequency of WT producers and with density (i.e., inoculum dose), their fitness was never significantly greater than that of WT bacteria (Figures 2A and 2B). Only at the lowest experimental frequencies (10% mutants) and highest dose (5×10^4 cfu μl^{-1} in inocula) did the fitness of mutants approach that of WT strains, with a higher fitness for the Δ*papR* mutant compared to the Δ*plcR* mutant (Figure 2A). Density dependence and frequency dependence indicate that ecological neighbors are modulating the fitness of mutants, implying that there is some social exploitation of QS products. However, in contrast to previous results [2, 3], this level of cheating was insufficient to compensate for the low fitness of mutants.

A Stepwise Decline in Mutant Fitness with Density Suggests that Mutants Are Less Able to Transit the Midgut

What, then, is the cause of this low fitness and the ineffective level of cheating? The PlcR regulon is a major transcriptional regulator [18], so any defects could generally impair bacterial reproduction in vivo. Single genes can affect a range of phenotypic traits (pleiotropy), in which case loss of traits with direct fitness benefits could prevent cheats, which are also deficient in indirectly beneficial traits, from outcompeting cooperators [22, 23]. One pleiotropic consequence of QS null mutants is reduced motility [24], which might impair colonization of the midgut. Alternatively, the PlcR regulon encodes secreted proteins as well as proteins that are membrane bound [18].

We can compare these competing hypotheses by exploring how the fitness of different mutants varies with overall in vivo bacterial reproduction, measured as the final pathogen population size in cadavers. When we compared different insect cadavers with varying reproductive rates, mutant fitness showed a stepped decline with increasing reproductive rates (Figure 2C). This pattern is not consistent with a general pleiotropic deficiency in mutant growth because this would predict that fitness would decline smoothly with the number of generations in vivo. Furthermore, the fitness of mutant and WT bacteria were indistinguishable when bacterial reproduction was low (Figure 2C). Thus, when inocula had killed hosts but bacteria

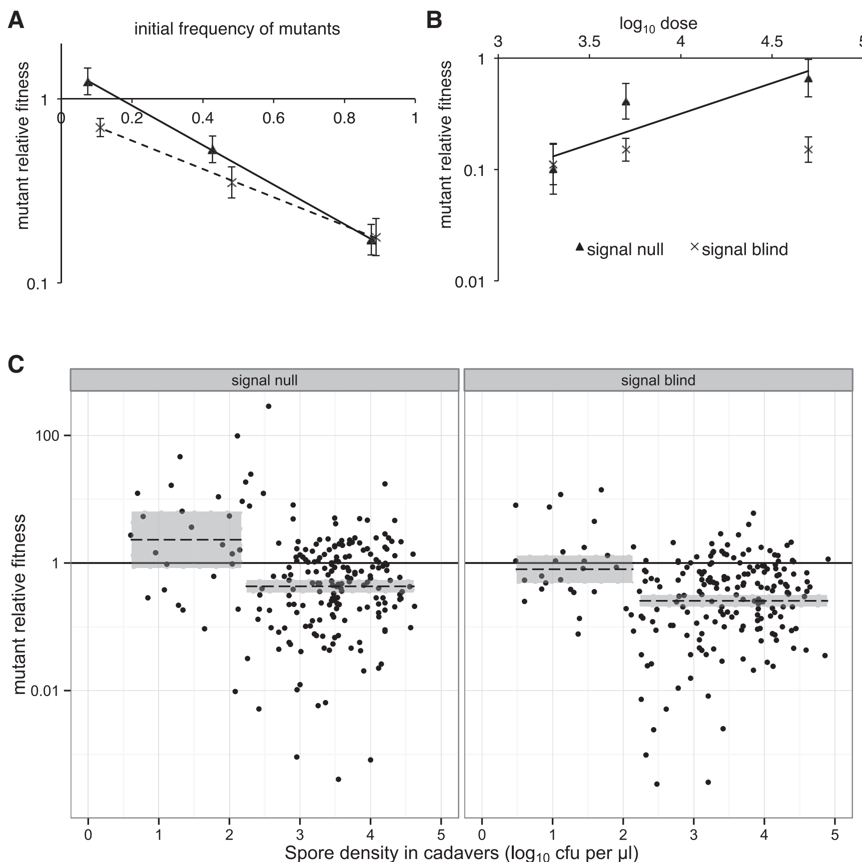

Figure 2. Relative Fitness of Mutants Is Density Dependent and Frequency Dependent

(A) Relative fitness of mutants decreased significantly with initial frequency (for Δ*papR*: $F_{1,151}$ = 65.1, p < 0.001; for Δ*papR*: $F_{1,141}$ = 35.7, p < 0.001). Note that when fitness is equal to 1, mutants have the same fitness as wild-type producers. Triangles and solid lines denote Δ*papR* signal-null mutants, whereas crosses and dashed lines denote Δ*plcR* signal-blind mutants. Data shown indicate means ± SE.

(B) Relative fitness of Δ*papR* signal-null mutants (filled symbols) was positively density dependent and increased with dose regardless of initial frequency ($F_{1,136}$ = 8.1, p < 0.005, n = 138), whereas the fitness of Δ*plcR* signal-blind mutants was independent of dose.

(C) Mutant fitness within cadavers varied with bacterial reproduction in vivo. Here, we analyzed the data from two fitness experiments in (A) and (B) that used an inocula concentration of 5 × 10^4 cfu/μl at a range of initial mutant frequencies. Statistical models in which fitness changed in a two-step fashion in response to bacterial reproduction in vivo (AIC = 1,619; residual deviance = 1,128; threshold effect: $F_{1,418}$ = 34.0, p < 0.0001) were more parsimonious and explained more of the variation than models fitting bacterial reproduction as a continuous variable (AIC = 1,644; residual deviance = 1,197; reproduction covariate: $F_{1,418}$ = 8.84, p = 0.003). The reproductive threshold separating infections into those with high and low mutant fitness was $10^{2.2}$ cfu μl^{-1}; this matches the bimodal pattern of bacterial reproduction shown in Figure 1C. Mutant genotype, initial proportion of mutants, and experimental replicate were fitted as main effects ($F_{1,416}$ = 16.3, p < 0.001; $F_{1,417}$ = 43.0, p < 0.001; $F_{1,415}$ = 18.7, p < 0.001). Dashed lines indicate fitted means, whereas the gray shading indicates 95% confidence intervals for the means.
See also Figure S1.

had not successfully invaded the cadaver, mutants and WT bacteria had equal fitness, indicating that mutants and WT were equally able to colonize the midgut. Both signal-blind and signal-null mutants showed the same qualitative patterns (Figure 2C). Our interpretation of this stepwise change in bacterial fitness is that these data reflect a two-step infection process in which QS mutants suffer a disadvantage in transiting from a site of low growth (the midgut) to a site of high growth (the bulk of the cadaver). It also indicates that mutants are not readily able to exploit the virulence factors of WT strains, which are producing the QS-regulated enzymes that enable them to cross the midgut epithelia. This is in perfect agreement with previous results showing that PlcR is essential for *B. thuringiensis* to gain access to the hemocoel [16], whereas a different suite of genes regulated by a second peptide signal is involved in late-stage reproduction in the cadaver [25].

Spatial Structure Limits Mutant Fitness and Occurs in Early-Stage Infections when QS Is Important for Host Invasion

Spatial structure has an important impact on the ability of mutants to exploit the products of their neighbors. If producers are aggregated in clonal microsites within the host, then QS null mutants may not be able to exploit their signals or their virulence factors in vivo. Attachment to host cells is an important early step in infection for many enteric pathogens [26]. *Bacillus* spores must also germinate before being voided from the host midgut. Population bottlenecks caused by host defenses, a shortage of suitable attachment sites, or the need for timely germination could generate significant spatial structure. We therefore tested how spatial structure affected the fitness of QS mutants. First, we examined the frequency dependence of both signal-blind and signal-null mutants in cadavers that were homogenized 3–4 days after death. When bacteria were allowed to grow in physically disrupted cadavers, mutant fitness was negatively frequency dependent, and mutant fitness exceeded WT fitness at frequencies below 10% (Figure 3A). This indicates that there is the potential for effective cheating when spatial structure is disrupted. In single-strain controls, reproduction was similar for all bacterial genotypes (Figure S2). Second, we assessed the fitness of signal-blind and signal-null mutants under conditions of varying spatial structure by conducting competition experiments in shaken and unshaken insect homogenates. Shaken insect homogenates were further disrupted by the addition of sterile glass beads. As predicted, when spatial structure was reduced, both signal-blind and signal-null mutants had significantly higher relative fitness, and signal-null mutants were capable of outcompeting WT bacteria (Figure 3B).

In order for spatial structure to be important during competition between QS mutants and WT strains, it has to occur when QS is active and important, in other words, when

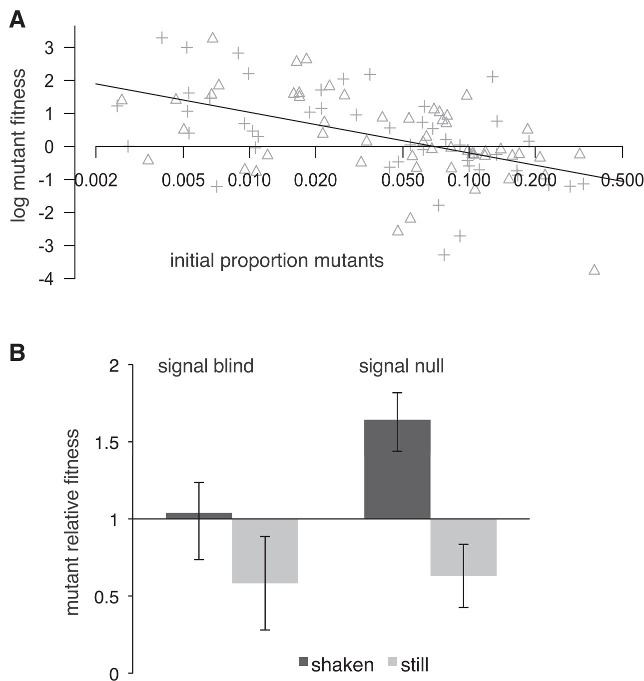

Figure 3. Spatial Heterogeneity Limited the Relative Fitness of QS Mutants in Homogenized Cadavers

(A) In homogenized insect cadavers, mutants had higher fitness than WT producers when rare (ln fitness = $-1.43 - (0.535 \times \ln p)$, where p is the initial proportion of mutants; $F_{1,98} = 36.0$, $p < 0.001$). Signal-null strains are represented by crosses, signal-blind mutants are represented by triangles, and mutant genotype had no effect on fitness in this experiment ($F_{1,97} = 0.03$, $p = 0.87$).

(B) Relative fitness of mutants was measured in mixed inocula (nine WT, one mutant) incubated under shaken and still (unshaken) conditions for 24 hr. Both mutants had significantly higher relative fitness in shaken insect homogenates than that in unshaken insect homogenates ($F_{1,62} = 9.9$, $p < 0.003$, $n = 64$).

Data shown indicate means ± SE. See also Figure S2.

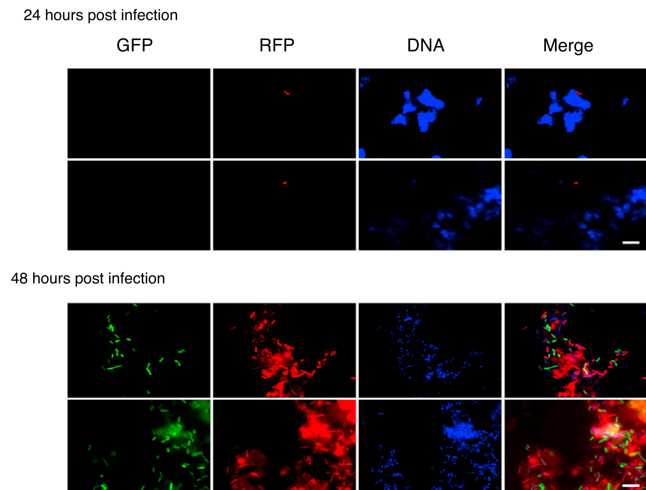

Figure 4. Dynamic Spatial Structure of Mixed Infections of WT and QS Mutants In Vivo

Third instar diamondback moth larvae ingested QS WT bacteria with a green fluorescence marker (WT GFP) and an isogenic signal-blind mutant with a red fluorescence marker (MT RFP) in a single droplet. Larvae were frozen 24 hr and 48 hr postinfection. Foci of infection were identified visually and photographed with a fluorescence microscope. The experiment was repeated with reciprocal markers, i.e., red fluorescent WT (WT RFP) and green fluorescent isogenic signal-blind mutant (MT GFP), shown in Figure S3. Nuclear DNA (visualized using DAPI) is shown in blue. Scale bars represent 15 μm.

bacteria need to cross the midgut barrier [16]. We therefore examined the distribution of fluorescently labeled WT and Δ*plcR* bacteria within hosts in the early stages of infection. Consistent with our hypothesis, we observed a strong population bottleneck. Very few *Bt* spores or vegetative cells remained within the midgut 24 hr postingestion (Figure 4). From the 6 larvae and 60 sections that were examined, we only observed a total of 25–30 WT cells and 2 mutant cells. Spatial structure was also evident: the bacterial population was composed of isolated microcolonies (mean patch size = 1.8 cells, SE = 0.34, mode = 1) (Figures 4 and S3). This result was independent of the markers used to identify WT and Δ*plcR* bacteria (Figure S3). Forty-eight hours postingestion, the host midgut had largely disintegrated, and hosts contained large numbers of vegetative cells of both genotypes.

It is therefore likely that spatial structure and population bottlenecks limit the fitness of QS mutants in this system because mutants are likely to be spatially separated from WT cells at the point when QS-regulated virulence factors assist host invasion. The subtly different behavior of signal-blind and signal-null cheats also indicates that spatial structure is important. We expected signal-blind mutants to be better cheats than signal-null mutants because the many large secreted proteins of the PlcR regulon should be more costly than the small amounts of signal peptide (Figure S1). However, in mixed infections with WT bacteria, the fitness of signal-blind mutants was consistently lower than that of signal-null bacteria (Figure 2, combined ANOVA for both experiments, $F_{1,415} = 40.7$, $p < 0.00013$). A structured environment in which small signal peptides diffuse more rapidly than the larger enzymes would explain why signal-null mutants are better able to exploit producers in vivo.

This ecology is different from the established social model of QS-regulated virulence, based on *P. aeruginosa* in vitro or on mice with burn wounds [2, 3]. In contrast to the results here, QS mutants of *P. aeruginosa* are very effective cheaters and outcompete WT bacteria over a wide range of frequencies [2, 3]. *P. aeruginosa* is an opportunistic pathogen in humans and typically infects immune suppressed hosts. In mice, additional barriers to infection are bypassed by injecting bacteria [27]. Although QS-regulated virulence facilitates the spread of infection, these virulence factors were not required to establish the initial infection. We argue that the ecology of infections in a burn wound model might be unusual. Hosts generally put up significant resistance to infection, and bacteria may have to survive a number of severe bottlenecks as successive barriers to infection are overcome [28]. We know little of the evolutionary ecology of QS in other bacterial systems. The low frequency (1%) of spontaneous QS null mutants isolated from *B. thuringiensis* and *B. cereus* culture collections [10] provides circumstantial support that QS cheats have low fitness in this group. In *Staphylococcus aureus*, signal-deficient QS mutants are good cheaters in an insect model and readily appear in persistent human infections but may persist poorly outside the host [9, 11].

Cooperative behavior and social conflict can potentially be exploited in direct clinical intervention [29, 30]. Cooperative signaling in particular may be a viable clinical target of

antivirulence drugs via "quorum quenching," in which chemicals that suppress the virulence of pathogens in vivo facilitate immune- or antibiotic-mediated clearance of infection [31]. Understanding the fitness implications of virulence expression and how virulent and avirulent bacteria compete in vivo can help us predict the efficacy of antivirulence drugs and how they might select for resistance or altered virulence [32]. For instance, a clinical assessment of *P. aeruginosa* cystic fibrosis patients showed that quorum quenching can, counterintuitively, maintain virulent cooperative QS populations because it prevents the invasion of cheats [33]. We found that spatial structure and host defenses prevent the proliferation of cheats and that virulence was linked to host invasion and pathogen fitness. Thus, with strong spatial structure, cheat invasion is already impeded, and quorum quenching is not expected to provide additional selection pressure for the maintenance of collectively beneficial virulence traits. In fact, with spatial structure, quorum quenching may select for the downregulation of costly and now-ineffective virulence traits [32]. However, spatial structure may make the evolution of resistance to quorum-quenching drugs more likely because resistant individuals would not be competing locally with cheats and would have a selective advantage when rare [32].

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2014.08.049.

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