Kin selection, quorum sensing and virulence in pathogenic bacteria

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Bacterial growth and virulence often depend upon the cooperative release of extracellular factors excreted in response to quorum sensing (QS). We carried out an in vivo selection experiment in mice to examine how QS evolves in response to variation in relatedness (strain diversity), and the consequences for virulence. We started our experiment with two bacterial strains: a wild-type that both produces and responds to QS signal molecules, and a lasR (signal-blind) mutant that does not release extracellular factors in response to signal. We found that: (i) QS leads to greater growth within hosts; (ii) high relatedness favours the QS wild-type; and (iii) low relatedness favours the lasR mutant. Relatedness matters in our experiment because, at relatively low relatedness, the lasR mutant is able to exploit the extracellular factors produced by the cells that respond to QS, and hence increase in frequency. Furthermore, our results suggest that because a higher relatedness favours cooperative QS, and hence leads to higher growth, this will also lead to a higher virulence, giving a relationship between relatedness and virulence that is in the opposite direction to that usually predicted by virulence theory.

Keywords: communication; inclusive fitness; signalling; cheat

1. INTRODUCTION

The growth and virulence of pathogenic bacteria often depends upon the cooperative production of extracellular factors, which are released in response to a cell-to-cell signalling process that has been termed quorum sensing (QS) [1,2]. Cells release small diffusible signal molecules, which have two effects. First, their uptake stimulates the release of extracellular factors, such as enzymes and nutrient-scavenging molecules, which facilitate growth. Second, their uptake stimulates the production of more signal molecules in a process that has been termed autoinduction. This leads to a positive feedback at high cell densities, which markedly increases production of extracellular factors themselves, but are able to benefit from those produced by others [4–6]. Genetic relatedness provides a solution to this problem, because the benefits will then be shared with individuals who share the genes for cooperatively producing extracellular factors [7]. However, while this kin selection explanation is appealing for QS and a range of other microbial social traits, there is a lack of direct empirical tests [8–10]. Furthermore, most empirical support for the application of kin selection to such microbial cooperation and signalling has come from the relatively unnatural environment of a liquid culture in a test tube [11–13], and it is not clear that cells will interact to the same extent under more natural conditions, such as during infection [14–16].

Here, we examine bacterial infections of mice, to test how relatedness influences selection for QS, with an experimental evolution approach. We study Pseudomonas aeruginosa, an opportunistic pathogen of plants and animals, including humans [17,18]. We used two bacterial strains: a wild-type that both produces and responds to QS signal molecules, and a lasR (signal-blind) mutant that does not respond to signal [16]. The lasR mutant represents a ‘free-rider’ that does not produce any QS-regulated extracellular factors. We started our experiment with a 50:50 mixture of these two strains, and then maintained them under conditions of either relatively low or relatively high relatedness. Our prediction is that the wild-type will be favoured under conditions of low relatedness, whereas the lasR mutant will be favoured under conditions of high relatedness. In addition, we test our underlying assumption that the wild-type is better able to proliferate in mice than the lasR mutant.

2. METHODS

(a) Bacterial strains and acute wound mouse model

We used two strains of P. aeruginosa: PA14 (which is a commonly used, fully virulent wild-type strain) and a PA14 lasR

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mutant strain (PA14:lasR), which does not respond to signal molecule [16]. We used female Swiss Webster mice (Charles Rivers Laboratories, Wilmington, MA) that were 8–10 weeks old and weighed approximately 20 g. Before infecting with bacteria, we anesthetized the mice and then shaved their backs before administering a dorsal, full-thickness scald burn that covered approximately 15 per cent of their total body surface. Directly after burning, we infected the wound with $10^2$ colony-forming units (CFU) of \textit{P. aeruginosa}, injected subcutaneously at the wound site. We allowed the bacteria to grow in the infected mice for 24 h, after which we euthanized the mice, excised the wound tissue and removed their livers. We then homogenized all of the tissue in sterile phosphate buffer saline (PBS) and pooled the homogenates. We then serially diluted the pooled homogenates, and plated them onto \textit{Pseudomonas} isolation agar (PIA), so that we could obtain single colonies for counting.

(b) In vivo selection experiment

Our selection experiment contained two treatments: relatively high and relatively low relatedness (figure 1). The variation in relatedness in our experiment is with respect to QS, which is the trait in whose evolution we are interested. We are able to focus on QS because the wild-type and \textit{lasR} mutant are initially identical at other parts of the genome, and so there is no genetic variation for other traits to influence selection. We varied relatedness by initiating each mouse infection with either one clone (relatively high relatedness) or 10 (relatively low relatedness) clones. We used black to symbolize mice infected with the normal QS wild-type (PA14), white to symbolize mice infected with the mutant that does not respond to signal (PA14::lasR), and grey to symbolize mice infected with a mixture of these two types.

![Figure 1. Experimental design. We varied relatedness by infecting each mouse (subpopulation) with either one clone (relatively high relatedness) or 10 (relatively low relatedness) clones. We use black to symbolize mice infected with the normal QS wild-type (PA14), white to symbolize mice infected with the mutant that does not respond to signal (PA14::lasR), and grey to symbolize mice infected with a mixture of these two types.](http://rspb.royalsocietypublishing.org/Downloaded from)
(10^2 each) of 10 clones. In order to calculate the relative frequency of lasR mutants after each round of infectivity, we also plated wound homogenates onto PIA containing gentamicin (100 \( \mu \)g ml\(^{-1}\)). We were able to distinguish lasR mutants from PA14 on this media, because the mutants harbour a gentamicin resistance cassette. The key point here is that in our low relatedness treatment, each mouse is infected by a mixture of clones, which can be a mixture of both the wild-type and the lasR mutant, and so these two different types are able to interact within mice. This allows competition within hosts, where the lasR mutant could potentially exploit the extracellular factors produced by the wild-type, as well as between the bacteria from different mice.

We repeated this experiment three times, giving a total of six replicate selection lines (three at high relatedness and three at low). We repeated the selective regime for up to six passages through novel mice, with some selection lines being terminated earlier because either the wild-type or the lasR mutant had gone to fixation. Overall, our experiment involved the passaging of bacteria through a total of 191 mice. Our experimental design is analogous to previous in vitro experimental evolution studies examining how population structure and evolution influences the evolution of social traits such as QS [11–13,20,21], except that the subpopulations were grown in mice, not test tubes.

3. RESULTS
We found that the QS wild-type was favoured under conditions of high relatedness, whereas the lasR (no response) mutant was favoured under conditions of low relatedness (figure 2; \( F_{1,4} = 284.9, p < 0.0001 \)). Comparing mice that had been infected with only the QS wild-type or the lasR mutant, we found that the QS wild-type grew to significantly higher densities (figure 3; \( F_{1,60} = 4.3; p = 0.043 \)). This result held irrespective of whether we analysed only the wound tissue, or the combined samples from both wound tissue and liver.

We then estimated the virulence consequences of variation in relatedness, by combining the results of this selection experiment with data that we have already collected on the mortality rate in infections of mice. Specifically, we have previously found that the percentage of mice that had died 5 days after infection was 100 per cent (9/9), 67 per cent (6/9) and 56 per cent (5/9) for infections initiated with the QS PA14 wild-type, the lasR mutant and a 50 : 50 mixture of the two, respectively [16]. Consequently, we estimate that high relatedness would lead to a greater virulence (100% mortality after 5 days) than low relatedness (67% mortality after 5 days; figure 4).

4. DISCUSSION
We found that QS was favoured by a relatively high relatedness (figure 2). In the high relatedness treatment, the QS wild-type and lasR mutant occurred in different
mice, and so the greater growth of the QS wild-type (figure 3) led to the wild-type increasing in frequency until it was fixed at 100 per cent. In contrast, in the low relatedness treatment, the QS wild-type and lasR mutant were able to co-exist in the same mice. When this happens, lasR mutants are able to exploit the extracellular factors produced by the QS wild-type, without paying the cost of producing them, and so the lasR mutants increase in frequency [16]. Put simply, conditions of high relatedness mean that cooperators interact with cooperators, and so cannot be exploited, whereas conditions of low relatedness mean that cooperators and cheats can interact, allowing cheats to exploit cooperators. Overall, these results provide clear support for kin selection theory, both as applied to QS and more generally [4,7,10,22]. Furthermore, by showing how relatedness influences selection on a specific molecular mechanism of pathogenesis, we have demonstrated how social evolution can shape selection on pathogen virulence (figure 4).

(a) Relatedness and quorum sensing

Our experiment examined the consequences of variation in genetical relatedness for a single trait (whether or not to respond to QS), and not across the whole genome. We did this because we were interested in how that trait evolves, and so wished to remove noise due to selection on other traits. If we are interested in how a single trait evolves in response to relatedness, then the relatedness that matters, as defined in Hamilton’s rule, is the genetical relatedness of that trait [7,19,23–25]. More specifically, relatedness is defined statistically as the genetical similarity between social partners relative to the rest of the population [19,26]. Consequently, in our experiment, a high relatedness means that hosts are infected with either the wild-type or the lasR mutant, such that cells will interact with identical cells, such that the lasR cells are not able to exploit the extracellular factors produced by the wild-type. In contrast, a low relatedness means that hosts can be infected by both the wild-type and the lasR mutant, such that the lasR cells are able to exploit the extracellular factors produced by the wild-type.

It is important to distinguish here between experiments with genetic manipulations at a single locus (in this case lasR) and more natural scenarios. In experiments such as we have carried out here, there is only genetic variation and hence the potential for relatedness to vary at the loci of interest, with no variation, and hence $r = 0$ at all other loci (relatedness is defined relative to the population, and so if you are as genetically similar to your social partner as to the whole population, then $r = 0$, even if you are genetically identical—this is analogous to why humans should not be expected to be especially altruistic to chimpanzees just because of the high similarity across our genomes [26]). While such manipulations are a bit artificial, they are experimentally useful, because it allows us to focus on selection on a single trait, such as QS.

In contrast, in natural scenarios, relatedness will be more similar across the whole genome [19]. A key point here is that while it is genetical relatedness at that locus that determines selection on a locus [7,23], as emphasized by selection on greenbeard genes [7,27,28], there is something special about when common ancestry causes genetic relatedness across the genome [19]. Common ancestry causes approximately the same relatedness across all alleles, and hence allows different parts of the genome to pull in the same direction to produce adaptations [19]. A potentially important complicating factor here, which does not arise in organisms typically used to study social evolution (such as insects and vertebrates), is the potential for horizontal gene transfer, and how it can cause relatedness to vary across the genome [8,29,30]. Measuring relatedness in natural populations of microbes, let alone if and how it varies across different social traits, remains a major task [15].

(b) Relatedness, strain diversity and virulence

The prediction from our results—that higher relatedness will lead to higher virulence (figure 4)—is in the direction opposite to that from classical virulence theory. Numerous theoretical models have predicted that a lower relatedness between the parasites infecting a host (higher strain diversity) will lead to greater competition for host resources, and hence selects for a higher growth rate that leads to greater virulence [31–33]. We obtained the opposite result in our experiment, because a higher relatedness favoured greater cooperation between bacterial cells, which facilitates bacterial growth and hence leads to host mortality. This supports the predictions of theoretical models that have allowed for such cooperation [5,34,35], and is in agreement with previous empirical work on a bacterial phage [36,37].

A key difference here is that classical models assume that growth rate can be varied in response to the level of competition, whereas our results flow from the growth rate, depending upon the level of cooperation [5,38,39]. It is not that one group of models is wrong and the other correct, but rather that they focus on different types of social interaction, with different mechanisms of pathogenesis (public goods and cooperation versus restraint and a tragedy of the commons). In natural infections, there could be an interaction between these factors, with host mortality placing a cap on the growth rate that would be selected for at high relatedness [5], or even different relationships from other forms of social trait [39–41]. Within the specific context of our experiment, bacteria were passaged before host death, and so host death per se did not influence selection on QS, although theory suggests that the effects of host death on virulence can be relatively negligible compared with the effect of cooperation [5]. More generally, the majority of ‘virulence factors’, whose production are associated with virulence in bacterial infections, appear to be extracellular factors [9], and so we would expect that cooperation will be a driving factor in the virulence of bacterial infections.

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