High relatedness selects against hypermutability in bacterial metapopulations

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Mutation rate and cooperation have important ecological and evolutionary consequences and, moreover, can affect pathogen virulence. While hypermutability accelerates adaptation to novel environments, hypermutable lineages (‘mutators’) are selected against in well-adapted populations. Using the model organism Pseudomonas aeruginosa, we previously demonstrated a further potential disadvantage to hypermutability, namely, that it can accelerate the breakdown of cooperation. We now investigate how this property of mutators can affect their persistence in metapopulations. Mutator and wild-type bacteria were competed for 250 generations in globally competing metapopulations, imposing conditions of high or low intra-deme relatedness. High relatedness favours cooperating groups, so we predicted that mutators should achieve lower equilibrium frequencies under high relatedness than under low relatedness. This was observed in our study. Consistent with our hypothesis, there was a positive correlation between mean mutator and cheat frequencies. We conclude that when dense population growth requires cooperation, and when cooperation is favoured (high relatedness), demes containing high frequencies of mutators are likely to be selected against because they also contain high frequencies of non-cooperating cheats. We have also identified conditions where mutator lineages are likely to dominate metapopulations; namely, when low relatedness reduces kin selection for cooperation. These results may help to explain clinical distributions of mutator bacteria.

Keywords: cooperation; mutation rate; Hamilton’s rule; metapopulations; Pseudomonas aeruginosa; microbial ecology

1. INTRODUCTION

The importance of mutation rate in evolution has been the subject of much theoretical and empirical research (Rainey 1999; Sniegowski et al. 2000; de Visser 2002). Hypermutability is also thought to be a significant risk factor in bacterial infections of animals, including humans (Oliver et al. 2000; Giraud et al. 2002; Ciofu et al. 2005). Experimental studies with hypermutable lineages of bacteria (‘mutators’) have successfully been used to expand our understanding of how and when alterations in mutation rate affect the evolution of a population. When adaptation is limited by the beneficial mutation rate, as is the case in novel or changeable environments, mutator alleles can hitch-hike with beneficial mutations to reach high frequencies (Ishii et al. 1989; Sniegowski et al. 1997; Taddei et al. 1997; de Visser et al. 1999; Tanabe et al. 1999; Giraud et al. 2001; Schaff et al. 2002). However, when a population is well adapted to its environment, the increased rate of deleterious mutations produced by mutators selects against hypermutability (Trobner & Piechocki 1984; Funchain et al. 2000; Giraud et al. 2001).

A further disadvantage may accrue to hypermutable lineages when population growth depends upon cooperative behaviours. Cooperative behaviours are common in nature, and often underpin virulence-related traits in pathogenic microbes (West & Buckling 2003; Harrison et al. 2006; West et al. 2006). Two key variables which affect the evolution of cooperation are the relatedness of interacting individuals and the scale of competition.

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are most successful within their group: the overall success of the group is unimportant. This creates a significant selective advantage for cheats, and cooperation breaks down under such conditions (West & Buckling 2003; Griffin et al. 2004). Under global competition, on the other hand, groups compete with groups and cooperating demes grow to higher densities than do cheating demes. This leads to cooperators persisting in the metapopulation and visible differences in equilibrium levels of cooperation under high versus low relatedness (West & Buckling 2003; Griffin et al. 2004). Selection for cheating under local competition can counteract kin selection due to high relatedness. This is because neighbours compete intensely with one another regardless of kinship. Under local competition, therefore, relatedness affects only the speed with which cooperation declines. Further, entirely local competition is biologically unrealistic as patches are highly interconnected, and incorporation of a degree of global competition.

The production of iron-scavenging siderophores by \textit{Pseudomonas aeruginosa} is a classic example of cooperation via production of a ‘public good’ (West & Buckling 2003; Griffin et al. 2004) and a useful model system for studying cooperation (Griffin et al. 2004; Harrison & Buckling 2005). Siderophores enhance population growth under iron-limited conditions and are necessary for virulence in acute \textit{P. aeruginosa} infections (Meyer et al. 1996; Harrison et al. 2006), meaning that useful inferences regarding cooperation, mutation and pathogen virulence can be made. We have previously used this system to show that hypermutable alleles accelerate the breakdown of cooperation when competition is entirely local (Harrison & Buckling 2005). We now use the same system to examine the fate of hypermutable lineages in globally competing metapopulations (within- and between-patch competition) under conditions of high or low intra-deme relatedness.

Our experimental design was based on that of Griffin et al. (2004) and comprised two treatments in which relatedness was either high or low. Metapopulations comprising six demes (iron-limited broth microcosms) were initially inoculated with wild-type and mutant bacteria in a 1:1 ratio. For high relatedness, three demes were inoculated with a single wild-type clone and three with a single mutant clone. For low relatedness, each deme was inoculated with one wild-type clone and one mutant clone. Colonies from each deme were transferred to fresh growth medium every 24 h. Global competition was imposed via hard selection: all six demes in a single microcosm were incu-bated at 37°C and were allowed to grow from time zero, as this is defined with respect only to loci governing siderophore production. After a few transfers, most demes in the low-relatedness treatment will be inoculated with one cooperator and one cheat clone and the relatedness will be low. Iron-limited CAA represents a novel environment for the bacteria, and as such we expect mutator lineages to adapt more rapidly to the growth in these microcosms.

2. EXPERIMENTAL PROCEDURES

(a) Bacterial strains

The tetracycline-resistant strain PAO985 (University of Washington) was used as the wild type, and the strain PAO\textDelta mutS (Oliver et al. 2004), which has a deletion of the mismatch repair gene \textit{mutS}, was used as the mutator. This strain has a spontaneous mutation rate more than two orders of magnitude higher (Oliver et al. 2004) than that of strain ATC 15692 (PAO1), from which both PAO\textDelta mutS and PAO985 are derived.

(b) Growth conditions

Our study comprised 16 metapopulations of bacteria, divided into two treatment groups of eight metapopulations each; the experiment was carried out in two equal blocks. Each metapopulation comprised six demes (glass microcosms). Microcosms contained 6 ml casamino acids medium (CAA: 5 g casamino acids, 1.18 g K$_2$HPO$_4$, 0.25 g MgSO$_4$ \cdot 7H$_2$O, per litre), made iron limited by the addition of 70 \mu g ml$^{-1}$ human apotransferrin (a natural iron chelator) and 20 mM sodium bicarbonate (necessary for iron chelator activity; Meyer et al. 1996). High-relatedness metapopulations initially comprised three microcosms inoculated with a single colony of PAO985, and three microcosms inoculated with a single colony of PAO\textDelta mutS. In low-relatedness metapopulations, each of the six microcosms was inoculated with a single colony of both strains.

Microcosms were incubated for 24 h at 37°C on an orbital shaker. Individual microcosms were then homogenized using a vortex mixer. Aliquots of culture from each of the six microcosms within a metapopulation were mixed and the mixture plated onto King’s B agar (KB: 10 g glycerol, 20 g proteose peptone no. 3, 1.5 g K$_2$HPO$_4$, 3H$_2$O, 0.25 g MgSO$_4$ \cdot 7H$_2$O, per litre). Single colonies (high relatedness) or pairs of colonies (low relatedness) were picked at random and used to inoculate six fresh microcosms. While our design meant that low-relatedness demes were always inoculated with twice as many cells as were high-relatedness treatments (approx. 4.6 \times 10^7 versus 2.3 \times 10^7 cells), the total number of cell divisions occurring within demes should not differ between the two treatments as stationary phase will be reached long before transfers take place.

This evolution was continued for 25 transfers (approx. 250 bacterial generations). Every fifth day, aliquots from all six microcosms within a metapopulation were mixed together in equal proportions, and the relative densities of PAO985 and PAO\textDelta mutS, the frequency of siderophore-negative cheats and the total siderophore production were measured in each metapopulation as described below.

(c) Assays

(i) Aliquots of diluted culture were replica plated on King’s B (KB) agar (for a measure of total density), KB agar + 60 \mu g ml$^{-1}$ tetracycline (to select for
growth of PAO985 only) and CAA agar (to score siderophore producers and non-producers visually).

(ii) An aliquot of the whole-population mix was centrifuged to pellet the cells, and the supernatant (containing siderophores) was stored at −20°C. The total siderophore content of these supernatants was later determined using the chrome azurol S (CAS) method described by Schwyn & Neilands (1987), with the modification that we diluted Schwyn & Neilands’ CAS recipe 1:1 with ddH2O. The relative absorbency at 630 nm of a mixture of 50 μl supernatant, and 100 μl CAS solution (all chemicals from Sigma) decreases linearly as siderophore concentration rises. Thus, a measure of mean siderophore production per colony-forming unit (CFU) in the ith microcosm is given by

\[
1 - \left( \frac{A_i}{A_{ref}} \right) \ln(\text{Density}_i),
\]

where \(A_i\) is the absorbency of the ith sample; \(A_{ref}\) is the absorbency of a reference solution comprising 50 μl sterile growth medium plus 100 μl CAS; and density is the CFU in 50 μl of the population sample.

(d) Statistical analyses
All data were analysed using Minicab v. 13. All frequency data were arcsine square root transformed prior to analysis, except in the case of the analysis of mean mutator versus mean cheat frequencies (figure 3), where a log transformation was used. These transformations provided the best fit to the assumptions of general linear modelling in each case. For the calculation of mean cheat frequencies, only data from timepoints 5–25 were used to avoid the initial frequency of zero influencing the results.

3. RESULTS
(a) Mutators are more able to dominate metapopulations when intra-deme relatedness is low
Mutator frequencies oscillated over time in all populations (figure 1). This was unsurprising, given the stochasticity expected to result from bottlenecks at each transfer, sampling effects and the nature of mutators themselves. We therefore calculated the mean mutator frequency over time for each population and used these data for our analyses, and we stress that our results should be viewed as demonstrating how relatedness affects the range of possible outcomes of mutator/wild-type competition.

As shown in figure 2, the mean equilibrium mutator frequency was significantly higher in low-relatedness metapopulations (ANOVA with block and treatment fitted as factors: \(F_{1,11} = 6.46; p = 0.025\)). Among high-relatedness metapopulations, the mean equilibrium mutator frequency was not significantly different from 0.5 (\(T_7 = 1.26, p = 0.247\); range 0.26–0.60). Among low-relatedness metapopulations, however, mutators were more often able to rise to dominance (range 0.47–0.77). The mean equilibrium mutator frequency for this treatment was significantly greater than 0.5 (\(T_7 = 2.45, p = 0.021\)).

(b) Mutators have an increased propensity to generate siderophore cheats
Pyoverdin-negative cheats arose in all metapopulations (figure 3a). Pyoverdin is not the only siderophore produced by this species, so we also used a chemical assay to measure total siderophore production in our microcosms. Consistent with the above, total siderophore production per CFU decreased over time in all metapopulations (figure 3b). As cheat frequency and total siderophore production were subject to severe oscillations and thus showed nonlinear dynamics over time (presumably as a result of the severe bottlenecks imposed), we calculated the mean cheat frequencies over time for all metapopulations and used these data in subsequent analyses.

Mutators may affect a variety of traits, including numerous social behaviours. We have hypothesized, however, that mutators are selected against in this system because they break down siderophore-mediated cooperation. We therefore tested for a positive correlation between the mean mutator and mean cheat frequencies in each metapopulation. Consistent with our hypothesis and with our previous work (Harrison & Buckling 2005), a positive correlation was found (general linear model with block and treatment fitted as factors: \(F_{1,11} = 7.45\) for mean
mutator frequency ($Z_{mut}=5.30$, $p=0.042$). The strength of this relationship did not differ between high- and low-relatedness treatments ($F_{1,11}$ for interaction term $=0.04$, $p=0.842$). These results are shown in figure 4 (note the right shift of low-relatedness data relative to high-relatedness data, showing higher mutator and cheat frequencies in these metapopulations).

4. DISCUSSION

(a) High relatedness selects against mutators in bacterial metapopulations

Here, we tested the hypothesis that kin selection for cooperation can affect the success of mutator alleles in bacterial metapopulations. Global competition favoured groups which contained higher total densities, and hence higher proportions of cooperators. Selection for cooperation was augmented by imposing high intra-deme relatedness or attenuated by imposing low intra-deme relatedness.

We conclude that the increased ability of mutators to respond to individual-level selection for cheating means that demes containing high mutator frequencies are more likely to selected against under high, as opposed to low, intra-deme relatedness. Under such conditions, mutators were unlikely to dominate metapopulations. We have also identified conditions where mutators can readily dominate metapopulations; namely, when relatedness is low. These results are consistent with our previous predictions (Harrison & Buckling 2005) and with work by other authors (Giraud et al. 2001). Previously published observations that migration in a metapopulation can select against mutator lineages (Giraud et al. 2001; Le Chat et al. 2006) may be partially explicable by the effect of hypermutability on cooperation if migration leads to global competition. It should be noted, however, that increases in diversity (i.e. decreases in relatedness) can sometimes reduce the success of cheats if diversification promotes character displacement among cooperators (Brockhurst et al. 2006; see also Smith et al. 2005). It would be very useful to synthesize models of diversification enhancing cooperation with kin selection models in order to obtain a more realistic picture of how the effects of population structure on selective forces interact to influence cooperation.

It is interesting that mutators were able to persist in all metapopulations, despite global competition favouring cooperation. Even under high relatedness, mutators represented 26–60% of the metapopulation. As our experimental microcosms represented a novel environment for the ancestral bacteria, this may be the result of a trade-off between the increased adaptive potential of mutator bacteria, and their increased propensity to break down cooperation. Further, mutators are expected to decrease relatedness and to be able to access cheating genotypes with increased ability to persist at high relative frequencies (Harrison & Buckling 2005; Harrison & Buckling in preparation).

(b) Mutators, siderophores and virulence in pathogen metapopulations

The results presented here suggest that further theoretical and empirical work in this area could help to explain environmental and clinical distributions of mutator bacteria, and contribute to our understanding of how mutator variants of pathogenic bacteria affect virulence.

Mutators are much more common in clinical isolates than in conspecific environmental populations (Le Clerc et al. 1996). Further, P. aeruginosa mutators are more commonly observed in long-term chronic infections than in acute infections (Oliver et al. 2000). These observations have previously been attributed to the fluctuating environment experienced during long-term in vivo growth.
as a result of host immune responses and medical intervention (Oliver et al. 2000, 2004; de Visser 2002; Giraud et al. 2002; Schaff et al. 2002). The appearance of hypermutable variants of pathogenic bacteria is associated with increased antibiotic resistance (Oliver et al. 2000; Ciofu et al. 2005) and as such mutators represent a significant risk factor for chronically colonized patients. Understanding the evolutionary ecology of mutators may thus benefit our understanding of chronic infection progression; indeed, understanding the evolution and ecology of pathogen communities may be vital in the development of new and more effective prophylaxis.

Much of the published work in this area has focused on bacterial populations within the respiratory tracts of cystic fibrosis (CF) patients. Individuals with CF are colonized by bacterial pathogens in early infancy, leading to chronic infection by diverse communities of microbes and, usually, eventual death from respiratory failure (Lyczak et al. 2002). Pseudomonas aeruginosa is one of the commonest CF pathogens, and is associated with a significant increase in patient morbidity (Nixon et al. 2001). Over the course of chronic P. aeruginosa infection, both increases in mutation rate (Oliver et al. 2000; Ciofu et al. 2005) and decreases in siderophore production (De Vos et al. 2001; Smith et al. 2006) are observed. Further, recurrent colonization and genetic diversification of founder clones lead to increases in both inter- and infraspecific diversity within the lung (Bergan & Hoiby 1975; Kresse et al. 2003; Saiman 2004; Moore et al. 2005; Smith et al. 2006). Thus, we expect that relatedness will decrease over the course of chronic infections. The CF lung microflora exists in spatially distinct communities (Armstrong et al. 1996; Gutierrez et al. 2001) which may be expected to undergo periodic mixing as a result of kinesitherapy. Thus, it is possible that the CF microflora represents a globally competing metapopulation with low relatedness (if inter-deme mixing is common) or numerous distinct locally competing patches with low relatedness (if mixing is less common). Gaining a fuller picture of the structure of this ecosystem could help greatly in ascertaining the role of cooperation, and its effects on virulence, in chronic CF infections (Brown et al. 2002).

This leaves us with several possible explanations for the observed frequencies of P. aeruginosa mutators and siderophore cheats in such communities. Siderophore production is an excellent example of a cooperative trait necessary for virulence in acute infections (Meyer et al. 1996; Harrison et al. 2006), and low relatedness has been shown to lead to decreased virulence in such infections (Harrison et al. 2006). It has been assumed that, as siderophore production declines over time in CF isolates of P. aeruginosa, and as free iron levels can be elevated in the CF respiratory tract (Stites et al. 1998, 1999), siderophores are not necessary in chronic infections. However, this has not been explicitly tested. Declines in siderophore production could also be the result of local competition and/or low relatedness reducing the extent to which cooperative production of siderophores is favoured. This effect could be exacerbated by the appearance of mutator genotypes. As mutators have not been recovered from non-CF patients with acute P. aeruginosa infections (Oliver et al. 2000), it seems that simple environmental novelty is not sufficient to explain the presence of mutators in the CF airways (although the constantly fluctuating conditions experienced during chronic infection may still afford some advantage to mutators). Examining P. aeruginosa populations from different sites within patients could help to narrow down these possibilities and to develop models of ecosystem structure in vivo. Comparing microbial communities from patients with differing treatment histories, or from patients before and after kinesitherapy, would reveal the effect of these interventions and help to uncover the interplay between adaptation and cooperation in the lung.

Moving on from the distribution of mutators within an infected patient, groups of patients in periodic close contact in CF centres or hospitals may themselves represent a metapopulation, as patient-to-patient transmission of pathogens is known to occur (Cheng et al. 1996; Ledson et al. 1998; Geddes 2001; Salunkhe et al. 2005). Studying groups of cross-infected patients as a metapopulation may help us to understand which strains or clones transmit readily through large numbers of patients. Taking an ecological view of cross-infection could also allow the identification of patients who are likely to be a ‘hub’ of infection. As transmissible clones can have high virulence or be antibiotic resistant (Cheng et al. 1996; Salunkhe et al. 2005), minimizing the contact between such patients and their peers could be a useful addition to preventive treatment.

5. CONCLUDING REMARKS

In summary, we have shown that high relatedness can select against mutator lineages of bacteria in experimental metapopulations. This is because high relatedness favours cooperation via kin selection. On the other hand, when kin selection is tempered by decreases in relatedness, mutators are more likely to dominate metapopulations. It is clear that simple in vitro studies of microbes can make a valuable contribution to our understanding of pathogen ecology and evolution, and how these affect virulence-related traits. This could aid in targeting the existing and future therapies more effectively.

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