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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; Schaaff *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. The 'relatedness' of two individuals can be defined as the probability of them sharing a common allele at a locus governing social interaction or cooperative action (Frank 1998). High relatedness favours cooperation via kin selection (Hamilton 1964), whereas competition between less related individuals favours the evolution of non-cooperating social cheats, which pay none of the costs of cooperation but enjoy the benefits of their neighbours' efforts. Understanding the evolution and ecology of cooperative traits is important when trying to predict the virulence of mixed (i.e. low relatedness) infections (Brown *et al.* 2002). Mutators are predicted to reduce relatedness and hence generate cheating genotypes more readily. Mutators are also predicted to have access to a greater area of the adaptive landscape, allowing them to produce 'fitter' cheats which can persist at higher relative densities (Harrison & Buckling 2005; Harrison & Buckling in preparation). While it is possible that mutators could also have access to fitter cooperating genotypes (e.g. with reduced costs of production), the reduction in relatedness generated by elevated mutation rates leads to an asymmetry in the strength of selection in favour of cheating over cooperation. We would thus expect mutators to be selected against under conditions that favour cooperation, namely, where ecological conditions result in high relatedness within demes.

The effect of relatedness on cooperation is itself mediated by the scale of competition (Hamilton 1964; West & Buckling 2003; Griffin *et al.* 2004). When competition is entirely local (i.e. due to within-patch or soft selection), selection will favour those individuals who

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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 unlikely to experience no migration (e.g. Saccheri & Hanski 2006). Any experiment designed to investigate the effect of relatedness on mutator dynamics should, therefore, incorporate a degree of global competition.

The production of iron-scavenging siderophores by *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 *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 hypermutability accelerates 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 mutator bacteria in a 1 : 1 ratio. For high relatedness, three demes were inoculated with a single wild-type clone and three with a single mutator clone. For low relatedness, each deme was inoculated with one wild-type clone and one mutator clone. Colonies from each deme were transferred to fresh growth medium every 24 h. Global competition was imposed via hard selection: all six microcosms within a population were mixed before transferring single (high-relatedness treatment) or pairs (low-relatedness treatment) of colonies to fresh microcosms as appropriate. Mixing demes represents global competition as the probability of colonies from any given deme being selected for transfer depends upon the total productivity of that deme relative to the others in the metapopulation (Saccheri & Hanski 2006). It should be noted that the treatments do not differ in relatedness at 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 PAOΔ*mutS* (Oliver *et al.* 2004), which has a deletion of the mismatch repair gene *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 PAOΔ*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 $K_2HPO_4 \cdot 3H_2O$, 0.25 g $MgSO_4 \cdot 7H_2O$, per litre), made iron limited by the addition of 70 μg 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 PAOΔ*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 $K_2HPO_4 \cdot 3H_2O$, 1.5 g $MgSO_4 \cdot 7H_2O$, 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. 4.6×10^7 versus 2.3×10^7 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 PAOΔ*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 μg 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 ddH_2O. 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

$$\frac{1-(A_i/A_{\mathrm{ref}})}{\ln(\mathrm{Density}\ i)},$$

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 bottlenecking 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: $F_{1,13}=6.46$; $p=0.025$). 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 3*a*). 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 3*b*). 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 bottlenecking 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: $F_{1,11}$ for mean

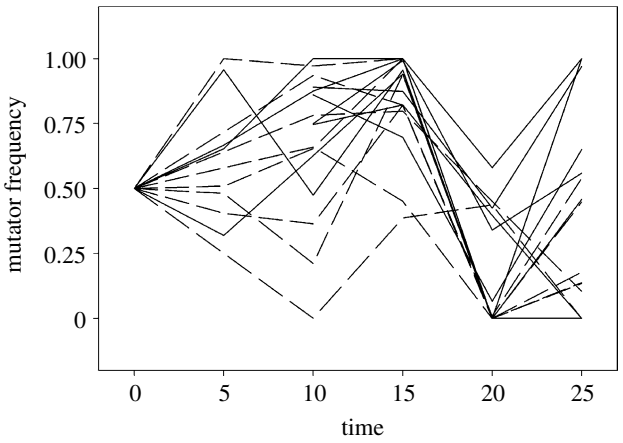

Figure 1. Mutator frequencies oscillated over time in all metapopulations. Dotted lines show metapopulations with high intra-deme relatedness, solid lines show metapopulations with low intra-deme relatedness. NB, the data for timepoint 5 are missing for one of the blocks; lines have been drawn connecting timepoints 0 and 10 in these cases.

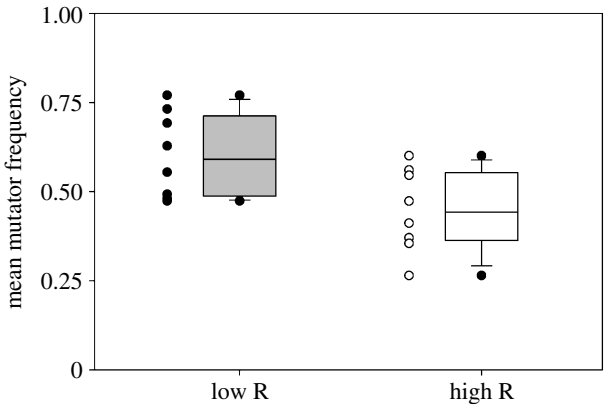

Figure 2. Box plots and individual data points showing the distributions of mean mutator frequencies. Open symbols correspond to metapopulations with high intra-deme relatedness, closed symbols to metapopulations with low intra-deme relatedness.

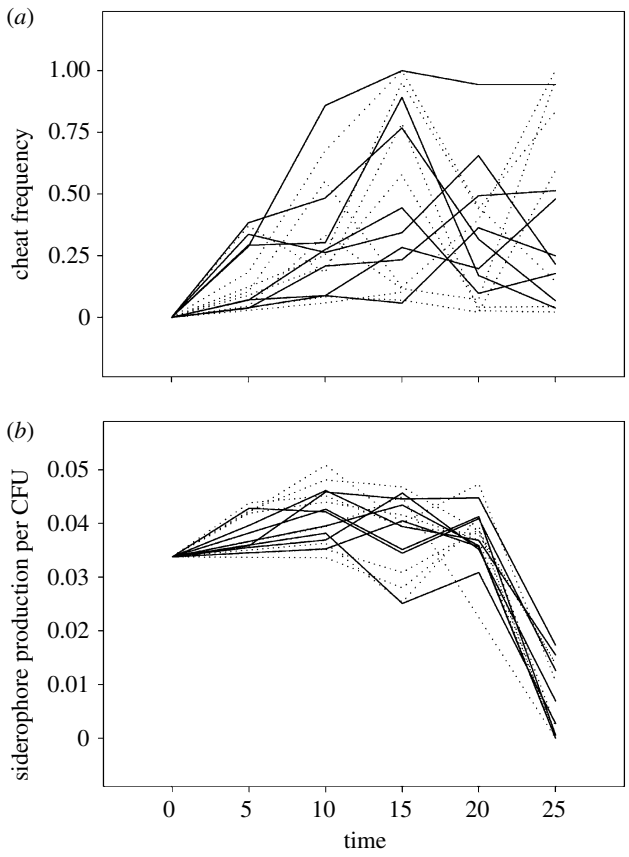

Figure 3. Siderophore cheats appeared in all metapopulations and their frequency oscillated over time. (*a*) Pyoverdin-negative cheat frequencies; (*b*) total siderophore production per CFU. Dotted lines show metapopulations with high intra-deme relatedness, solid lines show metapopulations with low intra-deme relatedness.

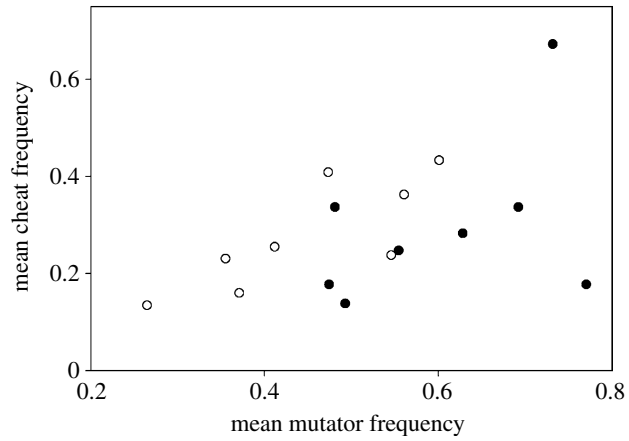

Figure 4. Mean mutator frequency and mean cheat frequency are positively correlated ($p=0.042$). Open circles show high intra-deme relatedness data, closed circles show low intra-deme relatedness data.

mutator frequency = 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 co-operation 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 (LeClerc *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; Schaaff *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 intraspecific 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 kinesiotherapy. 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 kinesiotherapy, 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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