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Proc. R. Soc. B 2009 **276**, 3531-3538 first published online 15 July 2009
doi: 10.1098/rspb.2009.0861

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Published online 15 July 2009

Viscous medium promotes cooperation in the pathogenic bacterium *Pseudomonas aeruginosa*

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There has been extensive theoretical debate over whether population viscosity (limited dispersal) can favour cooperation. While limited dispersal increases the probability of interactions occurring between relatives, which can favour cooperation, it can also lead to an increase in competition between relatives and this can reduce or completely negate selection for cooperation. Despite much theoretical attention, there is a lack of empirical research investigating these issues. We cultured *Pseudomonas aeruginosa* bacteria in medium with different degrees of viscosity and examined the fitness consequences for a cooperative trait—the production of iron-scavenging siderophore molecules. We found that increasing viscosity of the growth medium (i) significantly limited bacterial dispersal and the diffusion of siderophore molecules and (ii) increased the fitness of individuals that produced siderophores relative to mutants that did not. We propose that viscosity favours siderophore-producing individuals in this system, because the benefits of siderophore production are more likely to accrue to relatives (i.e. greater indirect benefits), and, at the same time, bacteria are more likely to gain direct fitness benefits by taking up siderophore molecules produced by themselves (i.e. the trait becomes less cooperative). Our results suggest that viscosity of the microbial growth environment is a crucial factor determining the dynamics of wild-type bacteria and siderophore-deficient mutants in natural habitats, such as the viscous mucus in cystic fibrosis lung.

Keywords: cooperation; kin selection; limited dispersal; population structure; public good; siderophores

1. INTRODUCTION

Why and when do individuals cooperate with one another? This is a fundamental question in evolutionary biology, as we must explain how selection can favour a trait that benefits another individual (Maynard Smith & Szathmáry 1995; Hamilton 1996; Frank 1998; Nowak 2006; West *et al.* 2007*a*). Theory shows that cooperative acts can be favoured in two ways. First, cooperation may be mutually beneficial if both actors and recipients gain direct fitness benefits through shared interests, as provided by mechanisms such as reciprocity (Sachs *et al.* 2004; Lehmann & Keller 2006; West *et al.* 2007*b*). Second, altruistic cooperation, where actors gain no direct fitness, can increase the actor's indirect fitness if cooperation is directed towards relatives that share cooperative alleles (kin selection; Hamilton 1964).

Hamilton (1964, 1972) originally suggested that limited dispersal might be a simple mechanism that generates high relatedness among interacting individuals, promoting indiscriminate altruism. However, subsequent theoretical work showed that limited dispersal also leads to increased competition between kin, which can cancel out the benefit of increased relatedness, and hence leads to no effect of the dispersal rate on selection for cooperation (Kelly 1992; Queller 1992; Taylor 1992*a*,*b*; Wilson *et al.* 1992; West *et al.* 2002*a*). Since then, a huge body of theory has shown that the relative importance of relatedness and competition, and hence whether limited dispersal favours cooperation, depends upon biological details (Goodnight 1992; Queller 1994; van Baalen & Rand 1998; Mitteldorf & Wilson 2000; Taylor & Irwin 2000; Le Galliard *et al.* 2003, 2005; Gardner & West 2006; Lehmann *et al.* 2006, 2007; Grafen 2007*b*; Alizon & Taylor 2008; El Mouden & Gardner 2008; Grafen & Archetti 2008; Johnstone 2008; Johnstone & Cant 2008; Lion & van Baalen 2008). For example, limited dispersal can promote cooperation when generations overlap (Irwin & Taylor 2000; Taylor & Irwin 2000) or when the benefit of cooperation (i.e. the extra offspring produced) can be taken up by expanding habitats (Mitteldorf & Wilson 2000; Lehmann *et al.* 2006) or exported to empty breeding sites (Alizon & Taylor 2008).

However, despite the large amount of theoretical work in this area, there is a severe lack of experimental studies testing the predictions of theory and determining the extent to which competition reduces selection for cooperation (West *et al.* 2001; Griffin *et al.* 2004; Kümmerli *et al.* 2009*a*). This is despite the fact that population viscosity has been proposed as a key factor in numerous systems, including the evolution of multicellularity (Michod & Roze 2001; Pfeiffer & Bonhoeffer 2003), microbial cooperation (Crespi 2001; Velicer 2003; West *et al.* 2006, 2007*c*), eusociality in insects

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Electronic supplementary material is available at http://dx.doi.org/10.1098/rspb.2009.0861 or via http://rspb.royalsocietypublishing.org.

Received 19 May 2009
Accepted 24 June 2009

(Bourke & Franks 1995; Lehmann *et al.* 2008), cooperative breeding in vertebrates (Griffin & West 2002; Johnstone & Cant 2008) and even interspecific mutualism (Doebeli & Knowlton 1998; Bever & Simms 2000; West *et al.* 2002*b*).

Here, we study the effect of viscous medium on cooperative behaviour in the context of public good production—the production of iron-scavenging siderophore molecules by the pathogenic bacterium *Pseudomonas aeruginosa*. Iron is a major limiting factor for bacterial growth, because most iron in the environment is in the insoluble Fe(III) form and is actively withheld by hosts during opportunistic infections (Guerinot 1994; Ratledge & Dover 2000; Budzikiewicz 2001; Wandersman & Delepelaire 2004; Miethke & Marahiel 2007; Visca *et al.* 2007). In response to iron deficiency, *P. aeruginosa* releases siderophore molecules into the local environment to scavenge insoluble iron, making it available for bacterial metabolism. It has previously been shown that, in planktonic culture and in an acute infection model, the production of siderophores fits the definition of a cooperative social trait (West *et al.* 2007*b*), which provides direct and indirect fitness benefits (Griffin *et al.* 2004; Harrison *et al.* 2006, 2008; Buckling *et al.* 2007; Ross-Gillespie *et al.* 2007, 2009; Harrison & Buckling 2009; Kümmerli *et al.* 2009*a*,*b*). Specifically, siderophore production provides a fitness benefit to both the producing cell and neighbouring cells. Thus, individuals that avoid the cost of siderophore production but exploit the siderophores produced by others can be considered as cheats (West *et al.* 2007*b*).

Here, we aim to determine how altering the viscosity of the environment affects the relative success of cooperative siderophore producers and siderophore-defective mutants in mixed cultures. We inoculated growth medium with cells of a wild-type siderophore-producing clone and cells of a mutant that lacks the primary (pyoverdin) and the secondary (pyochelin) siderophore. Viscosity was manipulated by varying the agar concentration of the growth medium. We tested whether viscosity limited the dispersal of bacteria and/or the diffusion of the public good (siderophores) and then measured the relative fitness of the two strains after a competition period. We predicted that increased viscosity would limit dispersal of both cells and siderophores. We hypothesized that increasing the viscosity of the medium would increase the relative fitness of cooperative siderophore producers for two reasons. First, if dispersal is reduced, sharing of siderophores is more likely to occur among cooperators (i.e. relatives; see Allison 2005 for microbial public goods in general). Second, as siderophore diffusion becomes limited, public goods will not diffuse far from the individuals that produced them, hence limiting the number of individuals contributing to the local public good pool, which increases the probability that siderophores in complex with iron are taken up by the producer itself. In other words, we predict that the importance of both the direct and indirect fitness benefits of siderophores will vary as a function of medium viscosity.

2. MATERIAL AND METHODS

(a) *Strains*

We used *P. aeruginosa* strain ATCC 15692 (PAO1) as the siderophore-producing wild-type, which produces both the primary siderophore, pyoverdin, and the secondary siderophore, pyochelin (Ankenbauer *et al.* 1985; Budzikiewicz 2001). As the siderophore-negative mutant, we used strain PA06609 (PAO9), which is unable to produce pyoverdin (Meyer *et al.* 1996) and pyochelin (N. Jiricny, S. Diggle, S. West & A. Griffin 2007, unpublished data). PAO9 is a mutant derived by UV mutagenesis from methionine auxotroph PAO6409 (Hohnadel *et al.* 1986), which in turn was generated by transposon mutagenesis from PAO1 (Rella *et al.* 1985).

(b) *Cell dispersal and siderophore diffusion*

We assessed cell dispersal and siderophore diffusion in increasingly viscous medium on agar plates. The medium used was Casamino acids (CAA; 5 g CAA, 1.18 g $K_2HPO_4^{\ddagger}3H_2O$, 0.25 g $MgSO_4^{\ddagger}7H_2O$, per litre). Viscosity was manipulated by supplementing CAA with 0.1 per cent (almost liquid), 0.25 per cent (semi-liquid), 0.5 per cent (semi-solid) or 1 per cent (solid) agar. Prior to the experiment, samples of PAO1 and PAO9 were streaked onto King's medium B (KB) agar plates and inoculated overnight in a static incubator at 37°C. We then stab-inoculated one PAO1 or PAO9 colony from the KB plate into the centre of the CAA agar plate. The number of cells was approximately 10^6 and similar for the two strains. This experiment was carried out in 10-fold replication. Cell dispersal was measured as the mean number of millimetres travelled from the centre of the plate in four directions after a 24 h incubation period at 37°C in a static incubator.

To assess siderophore diffusion, we first separated siderophores from cells by centrifugation of PAO1 KB cultures at 13 000 r.p.m. for 10 min. Five microlitres of supernatant, which contains siderophores, was dropped onto the centre of a CAA agar plate supplemented with chromeazurol sulphate (CAS) in 10-fold replication. Siderophores binding to iron cause a colour change from blue to orange in the CAS reagent (Schwyn & Neilands 1987), which allows colorimetric visualization of total siderophore diffusion. Siderophore diffusion was measured as the mean number of millimetres travelled from the centre of the plate in four directions after a 24 h incubation period at 37°C in a static incubator.

(c) *Competition assays*

Prior to experimentation, PAO1 and PAO9 strains were grown for 24 h in 30 ml glass universals containing 6 ml KB in an orbital shaker (200 r.p.m.) at 37°C. We then measured optical density of these cultures at 600 nm using a spectrophotometer (SpectraMax M2, Molecular Devices). Competition between these two strains took place in a 30 ml tube containing 6 ml of CAA medium supplemented with 0 per cent (liquid), 0.25 per cent (semi-liquid), 0.5 per cent (semi-solid) or 1 per cent (solid) agar. Prior to the experiment, all CAA tubes were heated to allow the medium to liquefy. Once the medium had sufficiently cooled down but prior to solidification, we added 20 mM $NaHCO_3$ (sodium bicarbonate) and 100 $\mu g\,ml^{-1}$ human apo-transferrin (Sigma; Meyer *et al.* 1996; Griffin *et al.* 2004). Apo-transferrin, combined with bicarbonate, is a powerful natural iron chelator and was used to bind the free Fe(III) in the CAA medium, which prevents non-siderophore-mediated uptake of iron by bacteria. Before solidification of the agar, we also added approximately 10^6 cells of a 2 : 1 volume mix of siderophore-producing

wild-type (PAO1) to sidorophore-defective mutants (PAO9). This mixing resulted in a starting proportion of mutants of 0.306 (estimated on the basis of a calibration curve relating optical density of bacteria cultures to cell number). Although we started with a relatively low proportion of mutants to increase experimental power (i.e. mutant fitness is density dependent; Ross-Gillespie *et al.* 2007), additional experiments revealed that the qualitative relationship between mutant fitness and viscosity remained unaltered by mutant starting frequency (see electronic supplementary material).

Competition took place over a period of 12 h (culture in the mid-exponential phase), 24 h (end of the exponential phase) or 36 h (stationary phase) in a static incubator at 37°C (see fig. S1 in the electronic supplementary material for growth curves). The different competition periods were chosen to test the generality of our predictions in a system where the relative success of siderophore producers and non-producers is density-dependent (Ross-Gillespie *et al.* in press). Each treatment combination (agar concentration versus competition time) was carried out in 12-fold replication, resulting in 144 competition assays. After the competition period, 6 ml of M9 solution (12.8 g Na_2HPO_4, 3 g $KHPO_4$, 0.5 g NaCl, 1 g NH_4Cl, per litre) was added to the tubes, and cultures were vortexed until the agar broke up and bacteria were washed out into the solution. To determine the final ratio of wild-type and mutants, dilutions of the washed-out cultures were plated onto KB agar and the number of colony-forming units (CFUs) was counted. The two strains were distinguished by their colour difference: wild-type (PAO1) colonies are green, whereas mutant (PAO9) colonies are white. We then calculated the relative fitness (v) of mutants as $v = [x_2(1-x_1)]/[x_1(1-x_2)]$, where x_1 is the initial proportion of mutants and x_2 is their final proportion (Otto & Day 2007). The fitness value of v therefore signifies whether mutants increased in frequency ($v > 1$), decreased in frequency ($v < 1$) or remained at the same frequency ($v = 1$) over the competitive period. Note that v is a measure for the cumulative amount of selection occurring over the growth period and can therefore be regarded as a proxy for the exact value of relative fitness, which is difficult to estimate as the selection coefficient presumably varies between the exponential growth phase and the stationary phase owing to density and frequency effects (Ross-Gillespie *et al.* 2007, in press). However, because the growth pattern is similar for all agar treatments (fig. S1*b*, electronic supplementary material), v correctly represents observed fitness differences between treatments.

We further carried out a control experiment to check whether monoculture growth of PAO1 and PAO9 was differentially affected by agar supplementation *per se*. We subjected monocultures of both strains to the four different agar treatments in the CAA medium, as described earlier. Tubes were arranged in pairs (six PAO1–PAO9 tube pairs for each agar treatment). After a growth period of 24 h, dilutions of monocultures were plated onto KB agar and the number of CFUs was counted. Within paired samples, we then divided CFU (PAO9) by CFU (PAO1) and tested whether this value differs between agar supplementation treatments. Such a paired design was necessary to account for the fact that absolute cell densities after 24 h growth differed between strains (i.e. mutants grow to lower densities because of their deficient production of siderophores) and increased with more agar added (because agar represents an additional carbon source, see fig. S1*b*, electronic supplementary material).

(d) *Statistical analysis*

To test whether cell dispersal and siderophore diffusion decrease with increasing agar supplementation, we performed generalized linear model (GLM) analyses. We implemented travelled versus non-travelled millimetres (strictly bounded and discrete values from 0 to 43 mm) on the agar plate as our variable with quasi-binomially distributed errors, the agar concentration (log transformed) as a covariate and the strain as a fixed factor. A quasi-binomial error distribution was used to eliminate the significant overdispersion observed with a binomial error distribution. Interaction terms were removed from the model when non-significant (Crawley 2007).

We built linear models to test whether relative mutant fitness (dependent variable) varied as a function of agar concentration (covariate) and/or competition time (fixed factor). Values of relative mutant fitness were logarithmically transformed prior to analysis to achieve normally distributed errors. Because culture growth significantly increased with agar supplementation ($F_{1,142} = 101.4$, $p < 0.001$; probably because agar represents an additional food source) and was significantly negatively associated with relative mutant fitness ($F_{1,137} = 26.6$, $p < 0.001$), we included log-transformed values of number of cells per microlitre after growth as a covariate in our model. For *post hoc* pairwise comparisons, we used the false discovery rate control method (Benjamini & Hochberg 1995) to adjust the nominal $\alpha = 0.05$. All statistical computations were carried out with R 2.8.0 (http://www.r-project.org).

3. RESULTS

(a) *Increased viscosity impedes cell dispersal and siderophore diffusion*

Cell dispersal significantly decreased with increased viscosity (figure 1*a*; GLM: $t_{77} = -8.13$, $p < 0.001$). There was no significant difference in the dispersal distances between the siderophore-producing wild-type and the siderophore-defective mutant strain, and no interaction between the strains and the degree of viscosity (strain: $t_{77} = -0.06$, $p = 0.95$; interaction: $t_{76} = -0.33$, $p = 0.75$).

Siderophore diffusion was also significantly limited by increased viscosity (figure 1*b*; GLM: $t_{35} = -7.32$, $p < 0.001$). Across the entire viscosity range, cell dispersal was not significantly different from siderophore diffusion (GLM: $t_{73} = 0.80$, $p = 0.43$). However, there was a significant interaction between viscosity and the type of dispersing agent (cell versus siderophore: $t_{73} = 3.58$, $p < 0.001$), with cells dispersing significantly further than siderophores in the 0.25 per cent ($t_{18} = -10.30$, $p < 0.001$) but not in the 0.5 per cent ($t_{16} = -2.03$, $p = 0.059$) agar supplementation treatment, whereas siderophores diffused significantly further than cells in the 1 per cent agar supplementation treatment ($t_{17} = 6.88$, $p < 0.001$).

(b) *Relative fitness of mutants varies with viscosity and competition time*

As predicted, under iron limitation, mutant monocultures grew to significantly lower densities than wild-type monocultures in all agar supplementation treatments (mean ratio mutant CFU : wild-type CFU = 0.47 ± 0.04 (0% agar), 0.39 ± 0.09 (0.25% agar), 0.33 ± 0.07 (0.5%

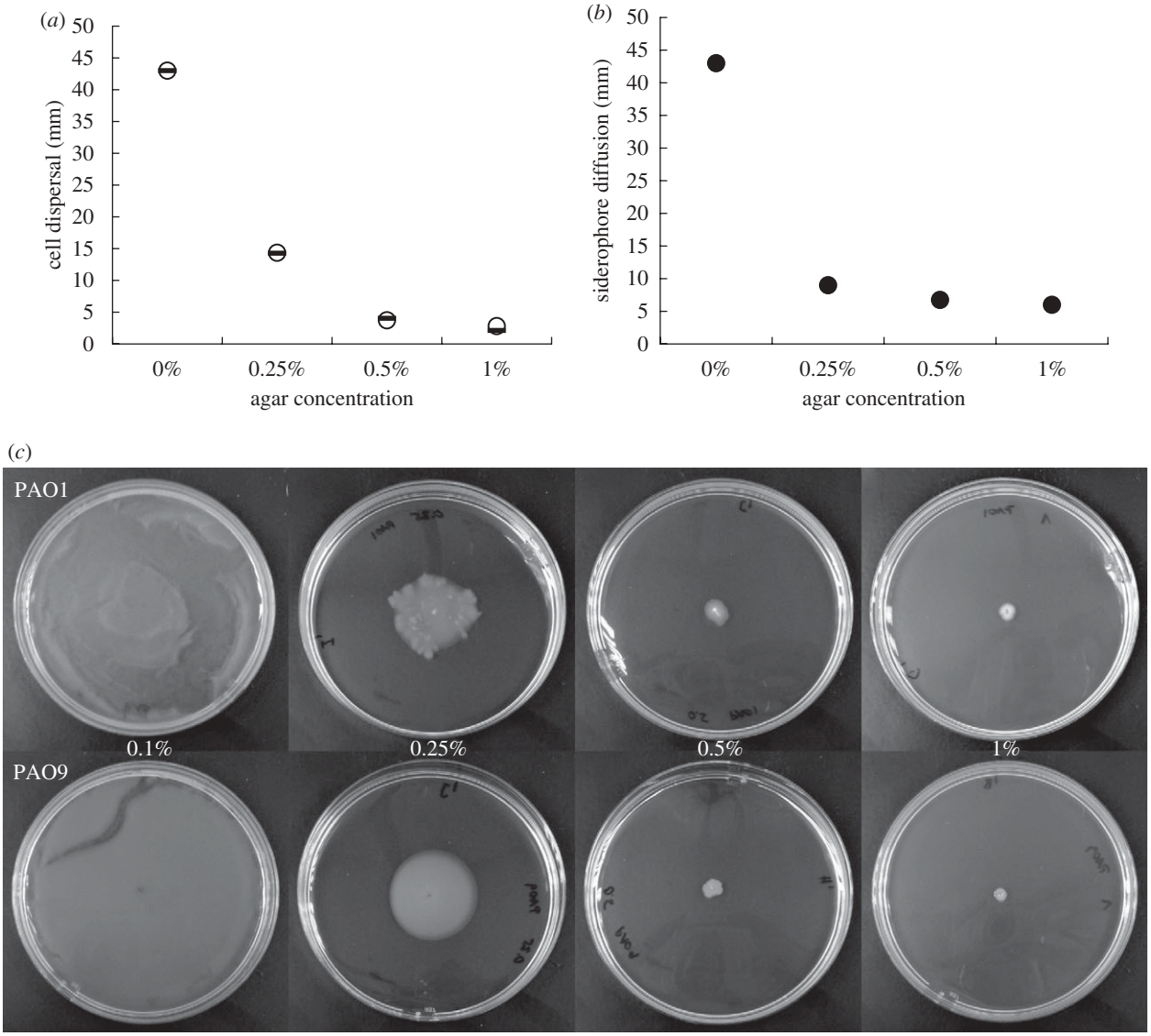

Figure 1. Increasing agar concentration in growth medium (i.e. increasing viscosity) significantly impedes (*a*) cell dispersal (open circles, wild-type siderophore-producing strain PAO1; black bars, siderophore-defective mutant PAO9) and (*b*) the diffusion of siderophores of PAO1 (filled circles). The 95 per cent confidence intervals are smaller than the circle symbols and are therefore not shown. The photographs in (*c*) show examples of bacterial populations used to generate the data graphed in (*a*).

agar) and 0.45 ± 0.10 (1% agar); one-sample *t*-tests for differences from unity: $5.5 \leq t_5 \leq 14.4$, all $p < 0.005$). Relative mutant monoculture growth did not differ significantly between agar supplementation treatments (ANOVA: $F_{3,20} = 0.68$, $p = 0.57$).

Relative mutant fitness declined significantly as viscosity increased (ANOVA: $F_{1,137} = 9.65$, $p = 0.002$; figure 2) and did so across all competition time periods (linear regression after 12 h: $R^2 = 0.198$, $F_{1,46} = 12.86$, $p = 0.001$; after 24 h: $R^2 = 0.396$, $F_{1,46} = 31.82$, $p < 0.001$; after 36 h: $R^2 = 0.342$, $F_{1,46} = 25.43$, $p < 0.001$). Relative mutant fitness also varied across competition times, with a significant interaction between viscosity and competition time (ANOVA, competition time: $F_{2,137} = 17.0$, $p < 0.001$; interaction: $F_{1,137} = 4.68$, $p = 0.011$). Pair-wise comparisons revealed that relative mutant fitness was significantly greater after 24 h ($t_{137} = 4.22$, $p < 0.001$) and after 36 h ($t_{137} = 2.70$, $p = 0.008$) than after 12 h; there was no significant difference in relative mutant fitness between the 24 and 36 h competition periods ($t_{137} = 1.13$, $p = 0.26$).

Consistent with previous findings (Griffin *et al.* 2004; Ross-Gillespie *et al.* 2007; Kümmerli *et al.* 2009*a*, *b*), relative fitness of mutants was significantly greater than 1 after the 24 h competition period in the 0 per cent agar treatment (one-sample *t*-test: $t_{11} = 3.43$, $p = 0.006$; figure 2). Thus, under these conditions, siderophore-defective mutants are successful cheats. In contrast, relative mutant fitness in this treatment was not significantly different from 1 after 12 h ($t_{11} = -0.74$, $p = 0.47$) nor after 36 h ($t_{11} = 0.81$, $p = 0.44$). In the treatments with 0.25, 0.5 and 1 per cent agar supplementation, relative mutant fitness was significantly lower than 1 ($-21.5 < t_{11} < -4.03$, all $p < 0.002$) for all time periods except for the 0.25 per cent agar treatment after 24 h (value not significantly different from 1: $t_{11} = -0.77$, $p = 0.46$).

4. DISCUSSION

Our findings demonstrate that population viscosity can favour public good producers under conditions where

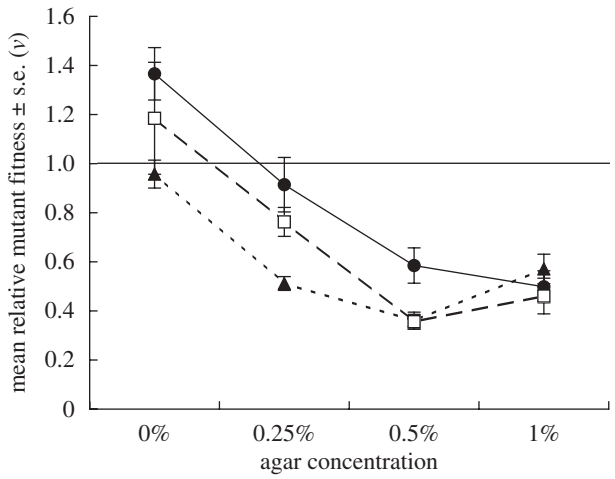

Figure 2. Increasing agar concentration in growth medium (i.e. increased viscosity) significantly reduces relative fitness of a siderophore-defective mutant (PAO9) compared with a siderophore-producing wild-type strain (PAO1) in competition assays of 12 h (triangles), 24 h (circles) and 36 h (squares). Value of $v > 1$ signifies that mutants increased in frequency and therefore represents conditions under which they can be considered as successful cheats.

both the dispersal of individuals and the public good are limited. There are two reasons for this. First, limited dispersal is likely to increase the probability of public good sharing occurring among relatives (i.e. the recipients of siderophores are likely to share alleles for siderophore production). Second, limited public good diffusion is likely to result in increased direct fitness benefits because fewer individuals contribute to the local public good pool, which results in individuals being more likely to take up iron-loaded siderophores that they themselves produced (i.e. the trait becomes less social and cooperative and more selfish). This emphasizes that the extent to which traits such as siderophore production are mutually beneficial (direct benefits outweigh the production costs) or altruistic (direct benefits do not outweigh production costs), or even not social, will depend upon factors such as population structure and dispersal (Rousset 2004; West *et al.* 2007*a*).

How does the biological system that we have studied compare with the assumptions of related theoretical models of cooperation in a structured environment? One difference is that general theoretical models usually assume an island-structured population consisting of patches (groups or subpopulations) and variable migration rate between patches (Taylor 1992*a*,*b*). In contrast, we have investigated the consequences of structuring within patches, where individuals may be more likely to interact with closer individuals (i.e. variable viscosity within patches). This is more analogous to general models that assume stepping-stone dispersal, or interactions on a graph (Comins *et al.* 1980; Hauert & Doebeli 2004; Ohtsuki *et al.* 2006; Grafen 2007*a*; Lehmann *et al.* 2007; Taylor *et al.* 2007), or models that have been specifically developed with bacteria in mind (Ross-Gillespie *et al.* 2007, 2009). Another difference is that the range over which the potentially cooperative behaviour is expressed (siderophore diffusion) also varies with the viscosity of the medium in our experiment. This would be analogous to the spatial size of the group (i.e. number of social interactions) covarying with the dispersal rate in a theoretical model (i.e. lower dispersal leads to smaller group sizes or to interactions being more likely with closer individuals on a graph—in the extreme case, $n = 1$ and trait would not be social). One way of conceptualizing this with an existing public good model (Ross-Gillespie *et al.* in press) is that the size of a group containing interacting individuals, is negatively correlated with the relatedness between individuals within that group (i.e. n would be negatively correlated with r in the public good model of Ross-Gillespie *et al.* in press). The effect of viscosity on both individual dispersal and the range over which the potentially cooperative trait is expressed is likely to be extremely common in micro-organisms such as bacteria, where the secretion of extracellular public goods appears to be the most common social trait (Allison 2005; West *et al.* 2006, 2007*c*).

Our results show that the relative decrease in mutant fitness in viscous medium is a general pattern found for different cell densities (i.e. competition times; figure 2) and starting frequencies of mutants (fig. S2, electronic supplementary material). Crucially, medium viscosity and competition time determine whether or not mutants can exploit siderophore producers, and hence these same variables determine the extent to which siderophore production is a social (cooperative) behaviour or not (for a detailed discussion of terminology, see West *et al.* 2007*b*). As the medium became more viscous, this impeded the ability of mutants to exploit the siderophore production of others, and hence the direct fitness consequences of siderophore production became relatively more important, and the trait became less social (cooperative). However, even in very viscous medium, siderophore production remained a social trait, because (i) siderophores diffused away from their producers (figure 1*b*) and (ii) cell division leads to bacteria being very close to each other (and also highly related); with both (i) and (ii) providing opportunities for siderophores in complex with iron being taken up by neighbouring cells. A key point here is that terms such as social and cooperation are defined at the level of individual cells and not at the colony or strain level (Hamilton 1964; West *et al.* 2007*b*).

The environmental conditions in this experiment relate to one of the environments naturally inhabited by *P. aeruginosa*: the lungs of humans with cystic fibrosis (CF). CF lungs become filled with viscous mucus (Nixon *et al.* 2001; Nielsen *et al.* 2004; Harrison 2007; Rubin 2007). A number of studies have explored the biomechanical properties of CF mucus (Charman & Reid 1972; King 1981; Nielsen *et al.* 2004; Perez-Vilar & Boucher 2004; Rubin 2007). While it is difficult to compare the results of these studies owing to differences in measurement techniques, it is clear that mucous viscosity varies among patients and over time within patients (App *et al.* 1998; Rubin 2007; Schulz *et al.* 2007). Furthermore, mucolytic substances are commonly prescribed to aid mucociliary clearance (Shak *et al.* 1990; Shak 1995; Donaldson *et al.* 2006; Evans & Koo 2009). Our *in vitro* findings suggest that siderophore-deficient mutants might increasingly be favoured in less viscous mucus and therefore that mucolysis could select for siderophore-deficient mutants. As siderophores are an important virulence factor (virulence factor expression assays: Lamont *et al.* 2002; insect acute infections: Harrison *et al.* 2006), this could be an additional benefit of mucolysis.

More generally, the consequences of population viscosity for the virulence of pathogenic species are predicted to depend on the mechanism of virulence (Frank 1996, 1998; Boots & Sasaki 1999; Brown *et al.* 2002; West & Buckling 2003; Wild *et al.* 2009). If the ability of a pathogen to grow within a host is limited by the production of public goods such as siderophores, then a lower viscosity (i.e. greater dispersal) between and within hosts can lead to a lower relatedness, a lower production of public goods and hence a lower virulence (e.g. Harrison *et al.* 2006; Rumbaugh *et al.* 2009). In contrast, if parasites are prudently reducing growth to avoid overexploiting their hosts, then a decreased relatedness owing to lower viscosity would favour a higher virulence, as a consequence of trying to obtain a greater proportion of the host resources (e.g. Kerr *et al.* 2006; Boots & Mealor 2007).

We thank Suzanne Clarke, Fredrik Inglis and Helen Morgan for their help in the laboratory and Sam Brown, Gabriel Garçía Peña, Chris Holland, Troy Day and two anonymous referees for helpful comments. This work was supported by the Royal Society, the Leverhulme Trust, a Marie Curie Intra-European Fellowship (to R.K.) and the University of Oxford (Newton-Abraham studentship awarded to F.H.). F.H. is currently funded by the European Community Framework 6 Coordination Action 'Integrating Cooperation Research Across Europe' (NEST 043318).

Author contributions. R.K., A.S.G., S.A.W., A.B. and F.H. conceived the study. R.K. and F.H. carried out the experimental work and analysed the data. R.K., A.S.G., S.A.W., A.B. and F.H. wrote the paper.

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