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

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Short title: *Bacterial cooperation in a viscous environment*

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SUMMARY

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 1) significantly limited bacterial dispersal and the diffusion of siderophore molecules and 2) increased the fitness of individuals that produced siderophores relative to mutants that did not. We propose that viscosity promotes siderophore production 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 wildtype bacteria and siderophore-deficient mutants in natural habitats, such as the viscous mucus in cystic fibrosis lung.

Key words: 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 & Szathmary 1995; Hamilton 1996; Frank 1998; Nowak 2006; West *et al.* 2007a). Theory shows that cooperative acts can be favoured in two ways. First, cooperation may be mutually beneficial if both actor and recipient gain direct fitness benefits through shared interests, as provided by mechanisms such as reciprocity (Sachs *et al.* 2004; Lehmann & Keller 2006; West *et al.* 2007b). 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 lead to no effect of the dispersal rate on selection for cooperation (Kelly 1992; Queller 1992; Taylor 1992a, b; Wilson *et al.* 1992; West *et al.* 2002a). 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; Grafen 2007b; Lehmann *et al.* 2007; 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.

76 the extra offspring produced) can be taken up by expanding habitats (Mitteldorf & Wilson
77 2000; Lehmann *et al.* 2006) or exported to empty breeding sites (Alizon & Taylor 2008).
78
79 However, despite the large amount of theoretical work in this area, there is a severe
80 lack of experimental studies, testing the predictions of theory and determining the extent to
81 which competition reduces selection for cooperation (West *et al.* 2001; Griffin *et al.* 2004;
82 Kümmerli *et al.* 2009a). This is despite the fact that population viscosity has been proposed
83 as a key factor in numerous systems, including the evolution of multicellularity (Michod &
84 Roze 2001; Pfeiffer & Bonhoeffer 2003), microbial cooperation (Crespi 2001; Velicer 2003;
85 West *et al.* 2006; West *et al.* 2007c), eusociality in insects (Bourke & Franks 1995; Lehmann
86 *et al.* 2008), cooperative breeding in vertebrates (Griffin & West 2002; Johnstone & Cant
87 2008), and even interspecific mutualism (Doebeli & Knowlton 1998; Bever & Simms 2000;
88 West *et al.* 2002b).
89
90 Here, we study the effect of viscous medium on cooperative behaviour in the context
91 of public good production – the production of iron-scavenging siderophore molecules by the
92 pathogenic bacterium *Pseudomonas aeruginosa.* Iron is a major limiting factor for bacterial
93 growth because most iron in the environment is in the insoluble Fe(III) form and is actively
94 withheld by hosts during opportunistic infections (Guerinot 1994; Ratledge & Dover 2000;
95 Budzikiewicz 2001; Wandersman & Delepelaire 2004; Miethke & Marahiel 2007; Visca *et al.*
96 2007). In response to iron deficiency, *P. aeruginosa* releases siderophore molecules into the
97 local environment to scavenge insoluble iron, making it available for bacterial metabolism.
98 We have previously shown that, in planktonic culture and in an acute infection model, the
99 production of siderophores fits the definition of a cooperative social trait (West *et al.* 2007b),
100 which provides direct and indirect fitness benefits (Griffin *et al.* 2004; Buckling *et al.* 2007;

Ross-Gillespie *et al.* 2007, 2009; Harrison *et al.* 2006, 2008; Harrison & Buckling 2009; Kümmerli *et al.* 2009a, 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.* 2007b).

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 hypothesised that increasing the viscosity of the medium would therefore 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 ATC 15692 (PAO1) as the siderophore-producing wildtype, 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 (Jiricny, N., Diggle, S., West, S. & Griffin, A. unpublished data). PAO9 is a mutant derived by UV-mutagenesis from methionine auxothroph 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; 5g casamino acids, 1.18g $K_2HPO_4*3H_2O$, 0.25g $MgSO_4*7H_2O$, per litre). Viscosity was manipulated by supplementing CAA with 0.1% (almost liquid), 0.25% (semi-liquid), 0.5% (semi-solid) or 1% (solid) agar. Prior to the experiment, samples of PAO1 and PAO9 were streaked onto 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 hour incubation period at 37°C in a static incubator.

To assess siderophore diffusion, we first separated siderophores from cells by centrifugation of PAO1 KB (King's medium B)-cultures at 13,000 rpm for 10 minutes. 5 µl 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 visualisation 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 hour incubation period at 37°C in a static incubator.

(c) Competition essays

Prior to experimentation, PAO1 and PAO9 strains were grown for 24 hours in 30 ml glass universals containing 6 ml KB in an orbital shaker (200 r.p.m.) at 37º C. We then measured optical density of these cultures at 600nm using a spectrophotometer (SpectraMax M2, Molecular Devices). Competition between these two strains took place in 30 ml tube containing 6 ml of CAA medium supplemented with 0% (liquid), 0.25% (semi-liquid), 0.5% (semi-solid) or 1% (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 20mM $NaHCO_3$ (sodium bicarbonate) and 100µgml^{-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 media, 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 wildtype (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

174 started with a relatively low proportion of mutants to increase experimental power (i.e. mutant
175 fitness is density-dependent, see Ross-Gillespie *et al.* 2007), additional experiments revealed
176 that the qualitative relationship between mutant fitness and viscosity remained unaltered by
177 mutant starting frequency (see electronic supplementary material (ESM).
178
179 Competition took place over a period of 12h (culture in mid exponential phase), 24h
180 (end of exponential phase) or 36h (stationary phase) in a static incubator at 37º C (see ESM
181 figure S1 for growth curves). The different competition periods were chosen to test the
182 generality of our predictions in a system where the relative success of siderophore producers
183 and non-producers is density dependent (Ross-Gillespie *et al.* 2009). Each treatment
184 combination (agar concentration versus competition time) was carried out in 12-fold
185 replication resulting in 144 competition assays. After the competition period, 6 ml of M9
186 solution (12.8g Na_2HPO_4, 3g $KHPO_4$, 0.5g NaCl, 1g NH_4Cl, per litre) were added to the
187 tubes, and cultures were vortexed until the agar broke up and bacteria were washed out into
188 the solution. To determine the final ratio of wildtype and mutants, dilutions of the washed-out
189 cultures were plated onto KB agar and the number of colony forming units (CFUs) was
190 counted. The two strains were distinguished by their colour difference: wildtype (PAO1)
191 colonies are green, while mutant (PAO9) colonies are white. We then calculated the relative
192 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
193 and x_2 is their final proportion (Otto & Day 2007). The fitness value of v therefore signifies
194 whether mutants increased in frequency ($v > 1$), decreased in frequency ($v < 1$), or remained
195 at the same frequency ($v = 1$) over the competitive period. Note that v is a measure for the
196 cumulative amount of selection occurring over the growth period and can therefore be
197 regarded as a proxy for the exact value of relative fitness, which is difficult to estimate as the
198 selection coefficient presumably varies between the exponential growth phase and the

199 stationary phase due to density and frequency effects (Ross-Gillespie *et al.* 2007, 2009).
200 However, because the growth pattern is similar for all agar treatments (ESM figure S1b), v
201 correctly represents observed fitness differences between treatments.
202
203 We further carried out a control experiment to check whether monoculture growth of
204 PAO1 and PAO9 was differentially affected by agar supplementation *per se*. We subjected
205 monocultures of both strains to the four different agar treatments in CAA medium as
206 described above. Tubes were arranged in pairs (six PAO1-PAO9 tube pairs for each agar
207 treatment). After a growth period of 24 hours, dilutions of monocultures were plated onto KB
208 agar and the number of CFUs was counted. Within paired samples, we then divided CFU
209 (PAO9) by CFU (PAO1) and tested whether this value differs among agar supplementation
210 treatments. Such a paired design was necessary to account for the fact that absolute cell
211 densities after 24-hour growth differed between strains (i.e. mutants grow to lower densities
212 because of their deficiency to produce siderophores) and increased with more agar added
213 (because agar represents an additional carbon source, see figure S1b).
214
215 *(d) Statistical analysis*
216 To test whether cell dispersal and siderophore diffusion decrease with increasing agar
217 supplementation, we performed generalized linear model (GLM) analyses. We implemented
218 travelled versus non-travelled millimetres (strictly bounded and discrete values from zero to
219 43 mm) on the agar plate as our variable with quasibinomially distributed errors, the agar
220 concentration (log-transformed) as a covariate and the strain as a fixed factor. A
221 quasibinomial error distribution was used to eliminate the significant overdispersion observed
222 with a binomial error distribution. Interaction terms were removed from the model when non-
223 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; most likely 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/µl after growth as a covariate in our model. For *post-hoc* pairwise comparisons, we used the false discovery rate (FDR) control method (Benjamini & Hochberg 1995) to adjust the nominal α=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 1a+b; GLM: t_{77}=-8.13, p<0.001). There was no significant difference in the dispersal distances between the siderophore-producing wildtype 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 1c; 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

249 in the 0.25% (t_{18}=-10.30, p<0.001) and but not in the 0.5% (t_{16}=-2.03, p=0.059) agar
250 supplementation treatment, whereas siderophores diffused significantly further than cells in
251 the 1% agar supplementation treatment (t_{17}=6.88, p<0.001).
252
253 *(b) Relative fitness of mutants varies with viscosity and competition time*
254 As predicted, under iron limitation mutant monocultures grew to significantly lower densities
255 than wildtype monocultures in all agar supplementation treatments (mean ratio [mutant
256 CFU]:[wildtype CFU] = 0.47±0.04 (0% agar), 0.39±0.09 (0.25% agar), 0.33±0.07 (0.5%
257 agar), 0.45±0.10 (1% agar); one-sample t-tests for differences from unity: $5.5 \leq t_5 \leq 14.4$, all
258 p<0.005). Relative mutant monoculture growth did not differ significantly between agar
259 supplementation treatments (ANOVA: $F_{3,20}$=0.68, p=0.57).
260
261 Relative mutant fitness declined significantly as viscosity increased (ANOVA:
262 $F_{1,137}$=9.65, p=0.002, figure 2) and did so across all competition time periods (linear
263 regression after 12 hours: R^2=0.198, $F_{1,46}$=12.86, p=0.001; after 24 hours: R^2=0.396,
264 $F_{1,46}$=31.82, p<0.001; after 36 hours: R^2=0.342, $F_{1,46}$=25.43, p<0.001). Relative mutant
265 fitness also varied across competition times, with a significant interaction between viscosity
266 and competition time (ANOVA, competition time: $F_{2,137}$=17.0, p<0.001; interaction:
267 $F_{1,137}$=4.68, p=0.011). Pair-wise comparisons revealed that relative mutant fitness was
268 significantly greater after 24 hours (t_{137}=4.22, p<0.001) and after 36 hours (t_{137}=2.70,
269 p=0.008) than after 12 hours; there was no significant difference in relative mutant fitness
270 between the 24- and 36-hour competition periods (t_{137}=1.13, p=0.26).
271
272 Consistent with previous findings (Griffin *et al.* 2004; Ross-Gillespie *et al.* 2007;
273 Kümmerli *et al.* 2009a, b), relative fitness of mutants was significantly greater than one after

the 24-hour competition period in the 0% 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 one after 12 hours (t_{11}=-0.74, p=0.47) or after 36 hours (t_{11}=0.81, p=0.44). In the treatments with 0.25%, 0.5% and 1% agar supplementation, relative mutant fitness was significantly lower than one ($-21.5<t_{11}<-4.03$, all $p<0.002$) for all time periods except for the 0.25% agar treatment after 24 hours (value not significantly different from one: t_{11}=-0.77, p=0.46).

4. DISCUSSION

Our findings demonstrate that population viscosity can favour public good producers under conditions where both the dispersal of individuals and the public good is limited. There are two reasons for this. Firstly, 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). Secondly, 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 emphasises 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.* 2007a).

How does the biological system that we have studied compare with the assumptions of related theoretical models of cooperation in structured environment? One difference is that

299 general theoretical models usually assume an island-structured population consisting of
300 patches (groups or subpopulations), and variable migration rate between patches (Taylor
301 1992a, b). In contrast, we have investigated the consequences of structuring within patches,
302 where individuals may be more likely to interact with closer individuals (i.e. variable
303 viscosity within patches). This is more analogous to general models that assume stepping
304 stone dispersal, or interactions on a graph (Comins *et al.* 1980; Hauert *et al.* 2004; Ohtsuki *et*
305 *al.* 2006; Grafen 2007a; Lehmann *et al.* 2007; Taylor *et al.* 2007), or models which have been
306 specifically developed with bacteria in mind (Ross-Gillespie *et al.* 2007, 2009). Another
307 difference is that the range over which the potentially cooperative behaviour is expressed
308 (siderophore diffusion) also varies with the viscosity of the media in our experiment. This
309 would be analogous to the spatial size of the group (i.e. number of social interactions) co-
310 varying with the dispersal rate in a theoretical model (i.e. lower dispersal leads to smaller
311 group sizes or to interactions being more likely with closer individuals on a graph – in the
312 extreme case, n=1 and trait would not be social). One way of conceptualising this with an
313 existing public good model (Ross-Gillespie *et al.* 2009), is that the size of the group,
314 containing interacting individuals, is negatively correlated with the relatedness between
315 individuals within a group (i.e. n would be negatively correlated with r in the public goods
316 model of Ross-Gillespie *et al.* 2009). The effect of viscosity on both individual dispersal and
317 the range over which the potentially cooperative trait is expressed is likely to be extremely
318 common in microorganisms such as bacteria, where the secretion of extracellular public
319 goods appears to be the most common social trait (Allison 2005; West *et al.* 2006, 2007c).
320
321 Our results show that the relative decrease of mutant fitness in viscous medium is a
322 general pattern found for different cell densities (i.e. competition times, figures 2) and starting
323 frequencies of mutants (figure S2). Crucially, medium viscosity and competition time

determined whether or not mutants can exploit siderophore producers, and hence the extent to which siderophore production is a social (cooperative) behaviour or not (for a detailed discussion of terminology, see West *et al.* 2007b). 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 becomes less social (cooperative). However, even in very viscous media, siderophore production remained a social trait, because: (a) siderophores diffused away from its producers (figure 1c); (b) cell division leads to bacteria being very close to each other (and also highly related); with both (a) and (b) 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.* 2007b).

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 due to differences in measurement techniques, it is clear that mucus 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 in press). 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

349 an important virulence factor (virulence factor expression assays: Lamont *et al.* 2002; insect
350 acute infections: Harrison *et al.* 2006), this could be an additional benefit of mucolysis.
351
352 More generally, the consequences of population viscosity for the virulence of
353 pathogenic species are predicted to depend upon the mechanism of virulence (Frank 1996,
354 1998; Boots & Sasaki 1999; Brown *et al.* 2002, West & Buckling 2003, Wild *et al.* 2009). If
355 the ability of a pathogen to grow within a host is limited by the production of public goods
356 such as siderophores, then a lower viscosity (i.e. greater dispersal) between and within hosts
357 can lead to a lower relatedness, a lower production of public goods and hence a lower
358 virulence (e.g. Harrison *et al.* 2006; Rumbaugh *et al.* 2009). In contrast, if parasites are
359 prudently reducing growth to avoid overexploiting their hosts, then a decreased relatedness
360 due to lower viscosity would favour a higher virulence, as a consequence of trying to obtain a
361 greater proportion of the host resources (e.g. Kerr *et al.* 2006; Boots & Mealor 2007).
362

363 **Author contributions**

364 RK, ASG, SAW, AB & FH conceived the study. RK & FH carried out the experimental work
365 and analysed the data. RK, ASG, SAW, AB & FH wrote the paper.

366

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375

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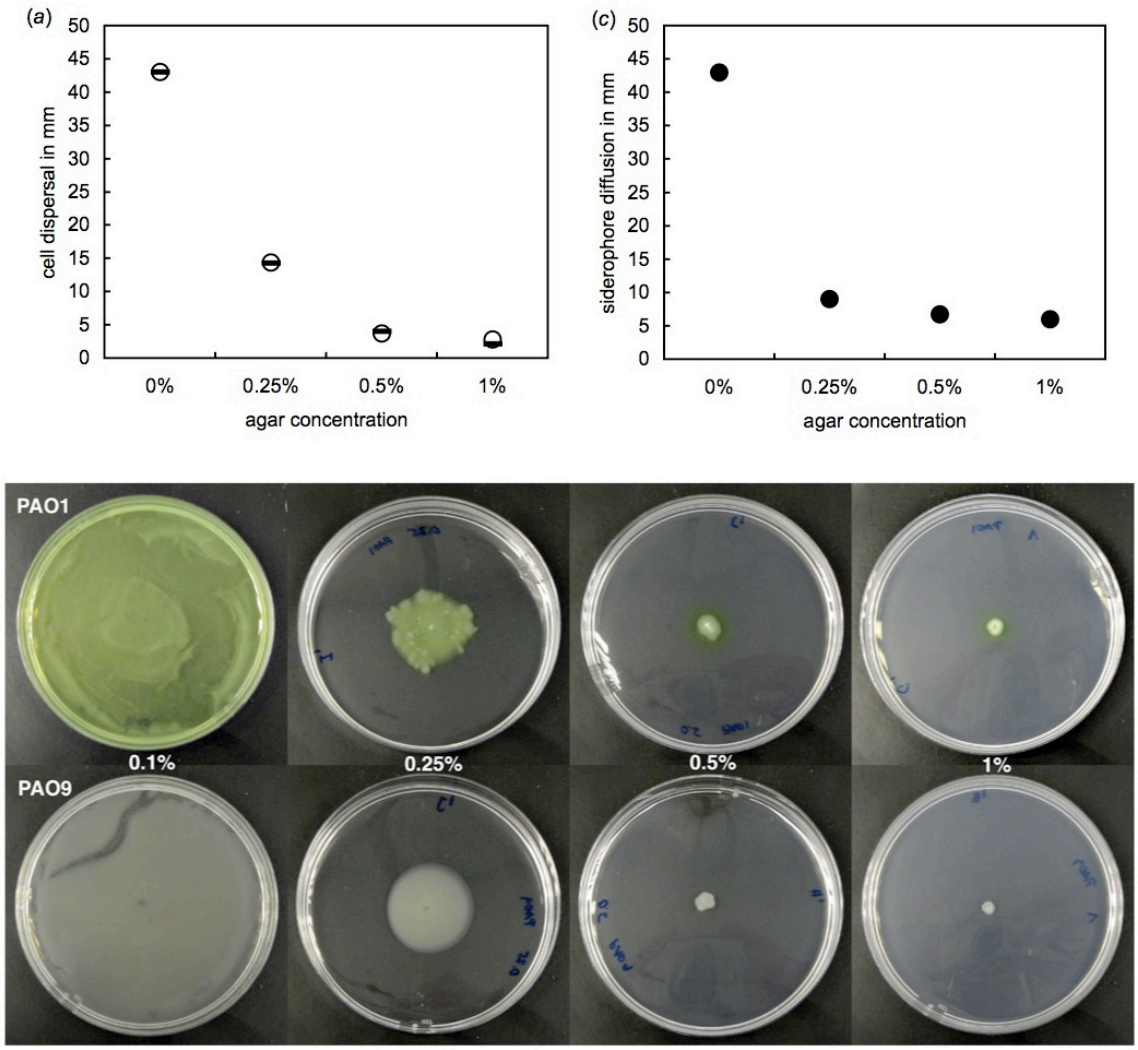

601

602 Figure 1. Increasing agar concentration in growth medium (i.e. increased viscosity)
603 significantly impedes: a)+b) cell dispersal of a wildtype siderophore-producing strain (PAO1;
604 open circles) and a siderophore-defective mutant (PAO9; black bars) as well as; (c) the
605 diffusion of siderophores of PAO1 (filled circles). The 95% confidence intervals are smaller
606 than the size of the circle symbols and are therefore not shown.

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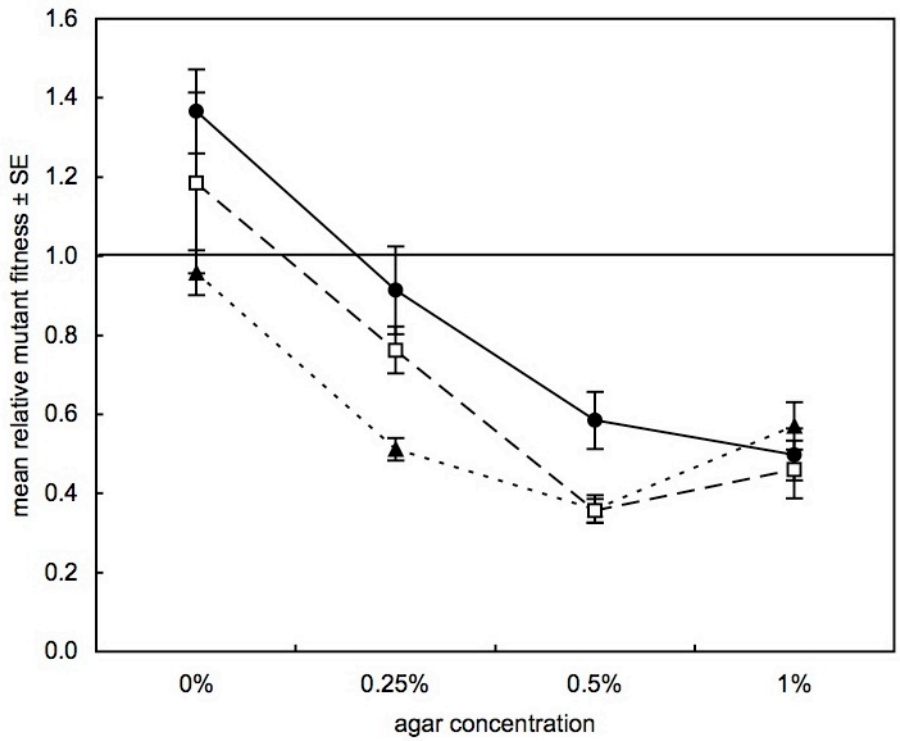

609

610 Figure 2. Increasing agar concentration in growth medium (i.e. increased viscosity)
611 significantly reduces relative fitness of a siderophore-defective mutant (PAO9) compared to a
612 siderophore-producing wildtype strain (PAO1) in competition assays of 12 hours (triangle),
613 24 hours (circles), and 36 hours (open squares). Value of $v > 1$ signifies that mutants
614 increased in frequency und therefore represent conditions under which they can be considered
615 as successful cheats.