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Repression of competition favours cooperation: experimental evidence from bacteria

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Abstract

Repression of competition (RC) within social groups has been suggested as a key mechanism driving the evolution of cooperation, because it aligns the individual's proximate interest with the interest of the group. Despite its enormous potential for explaining cooperation across all levels of biological organization, ranging from fair meiosis, to policing in insect societies, to sanctions in mutualistic interactions between species, there has been no direct experimental test of whether RC favours the spread of cooperators in a well-mixed population with cheats. To address this, we carried out an experimental evolution study to test the effect of RC upon a cooperative trait – the production of iron-scavenging siderophore molecules – in the bacterium *Pseudomonas aeruginosa*. We found that cooperation was favoured when competition between siderophore producers and nonsiderophore-producing cheats was repressed, but not in a treatment where competition between the two strains was permitted. We further show that RC altered the cost of cooperation, but did not affect the relatedness among interacting individuals. This confirms that RC *per se*, as opposed to increased relatedness, has driven the observed increase in bacterial cooperation.

Introduction

Repression of competition (RC) within social groups has been suggested as a key mechanism driving the evolution of cooperation and the major evolutionary transitions (Leigh, 1977; Alexander, 1979, 1987; Buss, 1987; Maynard Smith, 1988; Maynard Smith & Szathmary, 1995; Szathmary & Maynard Smith, 1995; Frank, 1995, 2003, in press; Ratnieks *et al.*, 2006; Gardner & Grafen, 2009). RC unites the proximate interests of social partners, such that individuals can only increase their fitness by maximizing the reproductive output of the social group. Under such conditions, a cooperative trait can spread in a population when it increases group productivity. RC is therefore a mechanism that need not rely upon indirect (kin-selected) benefits mediated by relatedness among interacting individuals (Frank, 2003).

Competition among group members can be repressed in various ways (Frank, 2003; Sachs *et al.*, 2004; West *et al.*, 2007a). For example, it can act through randomization of reproductive success, a mechanism ensuring that individuals cannot alter their reproductive success relative to their group-mates, and hence can maximize their inclusive fitness only by maximizing the total success of their group. Randomization of reproductive success has potentially driven the evolution of a wide range of cooperative phenomena including cooperation between homologous chromosomes through fair meiosis (Leigh, 1977) and cooperation in human societies through socially imposed monogamy (Alexander, 1979, 1987). Alternatively, RC can occur through: (i) policing as observed in the social hymenoptera where workers prevent each other from reproducing or eat each others eggs (Hammond & Keller, 2004; Wenseleers & Ratnieks, 2006a,b), or at the genome level where the selfish spread

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of transposable elements and mobile repetitive DNA sequences is policed by RNA interference (Moazed, 2009); (ii) punishment as occurring in humans and other vertebrates (Clutton-Brock & Parker, 1995; Bshary & Grutter, 2002; Fehr & Gächter, 2002); (iii) host-imposed sanctions as observed in the legume–rhizobia mutualism (Kiers *et al.*, 2003); and (iv) mechanisms that reduce symbiont diversity as a measure to prevent mixing of competitive symbiotic lineages within the host (Frank, 1996, 1997) (e.g. uniparentally inherited cytoplasmic elements: Eberhard, 1980; Hurst, 1994; incompatibility of nonvertical transmitted fungus cultivars in fungus-growing ants: Poulson & Boomsma, 2005).

Despite its enormous potential for explaining cooperation across all levels of biological organization (Maynard Smith & Szathmary, 1995; Szathmary & Maynard Smith, 1995), there has been no direct experimental test of how RC mediates the evolution of cooperation. Previous work has investigated whether RC mechanisms such as policing, punishment and sanctions coerce individuals to cooperate, but a more direct test of theory, whereby RC is imposed experimentally to examine its consequences for the dynamics of cooperator evolution, is lacking. Our aim here is to carry out the first experimental evolution study to assess the effect of RC by randomization of reproductive success upon a cooperative trait – the production of iron-scavenging siderophore molecules – in the bacterium *Pseudomonas aeruginosa* (Griffin *et al.*, 2004). Wild-type bacteria release siderophores into the local environment to scavenge insoluble iron, making it available for bacterial metabolism (Guerinot, 1994; Ratledge & Dover, 2000; Wandersman & Delepelaire, 2004; Kümmerli *et al.*, 2009c). The production of siderophores is a cooperative trait subject to kin selection (West *et al.*, 2007b), because the siderophores can be shared among neighbouring cells, providing benefits to both the cell that produced them and other cells (Griffin *et al.*, 2004; Harrison *et al.*, 2006; Buckling *et al.*, 2007; Ross-Gillespie *et al.*, 2007, 2009; Kümmerli *et al.*, 2009a,b,c; Jiricny *et al.*, in press). Consequently, siderophore production can be exploited by nonsiderophore-producing individuals (cheats), which avoid the cost of siderophore production, whilst retaining the benefits by taking up iron in complex with siderophores produced by others.

We constructed replicate populations, divided into 18 subpopulations, with six subpopulations being either seeded by cooperators alone, cheats alone, or an equal mix of the two bacterial strains. After a 24-h growth period, we spread each subpopulation on an agar plate and selected colonies from those plates according to two different selection regimes (Fig. 1). In the control treatment, we implemented complete dispersal, whereby the probability of a colony being transferred to the next generation is given by the productivity of its plate compared to other plates in the population, and the relative frequency of its type (i.e. cooperator vs. cheat) on its plate. In the RC treatment, we also implemented complete dispersal with higher productive plates being more likely to provide colonies for future generations. However, we suppressed cheating on mixed plates by giving both cooperator and cheat colonies an equal probability of being transferred to the next generation (i.e. this randomizes reproductive success between the two strains). Our previous studies (Griffin *et al.*, 2004; Kümmerli *et al.*, 2009a) have shown that complete dispersal leads to conditions where Hamilton's rule is generally not satisfied because the relatedness among interacting individuals is relatively low, which selects against cooperation. Hence, a possible increase in cooperator frequency in our RC treatment must be a direct consequence of the imposed randomization of reproductive success, which is predicted to reduce the cost of cooperation but not to alter relatedness. To verify this, we calculated relatedness among interacting individuals as well as the costs and benefits of cooperation to predict and simulate the evolution of cooperation in both treatments using a Hamiltonian approach.

Material and methods

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 PA6609 (PAO9), which is unable to produce pyoverdin (Meyer *et al.*, 1996) and pyochelin (Jiricny, N., Diggle, S., West, S. & Griffin, A. unpublished data). PAO9 was derived by UV mutagenesis from methionine auxotroph PAO6409, which in turn was derived from PAO1 (Hohnadel *et al.*, 1986). Although PAO9 was derived from PAO1, it is likely to differ from PAO1 also on other traits than siderophore production. To control for initial starting frequencies of cheats and cooperators before each experimental growth phase, we grew both strains separately for 24 h in 200 μL standard King's medium B (KB) on a 96-well plate in an orbital shaker (200 rpm) at 37 °C. Because PAO9 grows to slightly lower densities than PAO1 under these conditions, we diluted PAO1 cultures to the same optical density as PAO9 (OD at 600 nm, measured with SpectraMax M2, Molecular Devices).

Experimental design

For the control treatment and the RC treatment, we initially created populations consisting of 18 subpopulations. We seeded each subpopulation by approximately 10^6 cells from KB cultures, whereby six subpopulations were either seeded by a mix of two cooperator cultures, two cheat cultures or a 1 : 1 mix of a cooperator and a cheat culture, resulting in a 1 : 1 overall population ratio

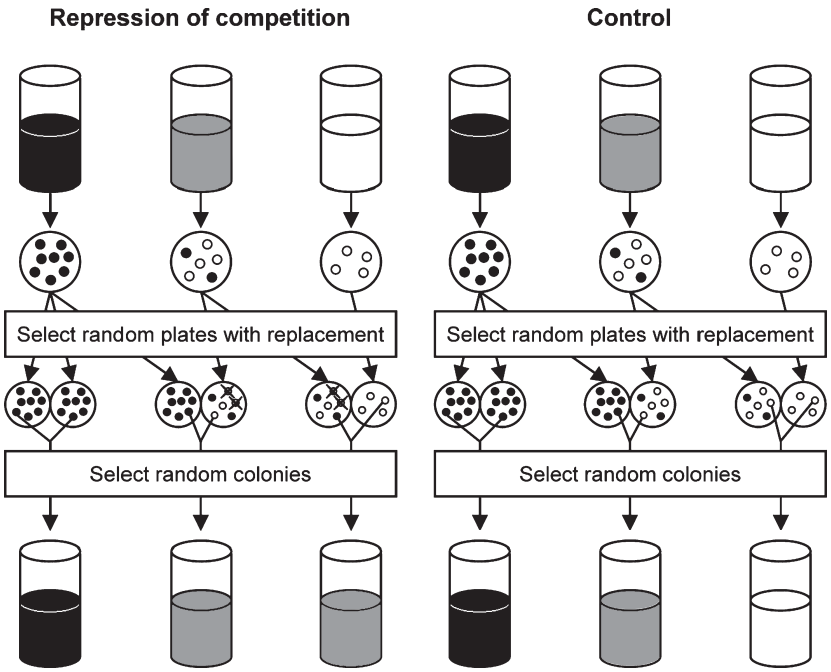

Fig. 1 Experimental design repressing or permitting competition between a siderophore-producing cooperator and nonsiderophore-producing cheat strain of the bacterium *Pseudomonas aeruginosa*. Populations consisted of 18 subpopulations (for simplicity only three subpopulations per treatment are shown), which we seeded with either two cooperator colonies (black tubes), two cheat colonies (white tubes) or a 1 : 1 mix of the two strains (grey tubes). After a 24-h growth period, we individually plated cultures onto agar. We then seeded each subpopulation of the next growth generation with two colonies, each of which we derived from a randomly chosen (with replacement) agar plate, with the probability of choosing any plate being proportional to its productivity. We repressed competition between the two bacterial strains in mixed cultures by setting the probability of selecting a cooperator vs. a cheat colony to 1 : 1 (indicated by the crosses on mixed plates), which represses the advantage cheats normally have in mixed cultures. In the control treatment, competition between the two strains was permitted with the probability of selecting either a cooperator or cheat colony being directly proportional to their relative frequencies on that plate. The whole experiment entailed 12 selection steps for both treatments in fourfold replication.

of cooperators and cheats (Fig. 1). We grew each subpopulation in a 30-mL glass universal containing 6 mL of casamino acids (CAA; 5 g casamino acids, 1.18 g K_2HPO_4*$3H_2O$, 0.25 g $MgSO_4$*$7H_2O$, per litre) supplemented with 20 mM NaHCO3 (sodium bicarbonate) and 100 μg mL^{-1} human apo-transferrin (Sigma, Gillingham, UK) (Meyer *et al.*, 1996). Apo-transferrin, combined with bicarbonate is a powerful natural iron chelator and was used to bind the free Fe(III) in the CAA media, preventing nonsiderophore-mediated uptake of iron by bacteria. Subpopulations were then grown for 24 h in a static incubator at 37 °C.

After 24 h, we plated equal volumes from all subpopulations individually onto KB agar. We incubated the plates overnight at 37 °C and then quantified the number of PAO1 and PAO9 colony forming units (CFU) on each plate. Colonies of PAO1 and PAO9 differ in their morphology and can easily be distinguished based on colour differences: PAO1 colonies are green because of the presence of fluorescent pyoverdin molecules, whereas colonies of PAO9 are smaller and white because of the absence of pyoverdin molecules. Consistent with our previous findings (Griffin *et al.*, 2004; Harrison *et al.*, 2006; Ross-Gillespie *et al.*, 2007, 2009; Kümmerli *et al.*, 2009a,b,c; Jiricny *et al.*, in press), mutant monocultures grew to significantly lower densities than wild-type monocultures under these iron-limited conditions (reduction in growth = 37.2% ± 1.8%, $t_{695} = 17.41$, $P < 0.0001$, Table 1). In contrast, the mutants increased in relative frequency in mixed cultures where they acted as cheats (relative cheater fitness $v = 1.57 \pm 0.04$, a value that is significantly > 1: $t_{488} = 15.32$, $P < 0.0001$, Table 1). Note that the relative fitness of cheats in mixed cultures is given as $v = x_2(1 - x_1)/x_1(1 - x_2)$, where x_1 is the initial proportion of cheats and x_2 is their final proportion. Because $x_1 = 0.5$ in our experiment, $v = x_2/(1 - x_2)$.

We then seeded each subpopulation of the next growth generation with two colonies (Fig. 1), each of which we derived from a randomly chosen (with replacement) agar plate, with the probability of choosing any plate being proportional to its productivity (i.e. CFU). Hence, more productive plates were more likely to transmit higher numbers of colonies to the next generation. Monoculture plates delivered a random colony of their respective type (cooperator or cheat). If we chose a

Table 1 Simulating the evolution of cooperation using our experimental design with fitness parameters from various experimental evolution studies on *Pseudomonas aeruginosa*. Simulations are based on 20 000 replicates each.

	This study	Griffin *et al.*, 2004	Kümmerli *et al.*, 2009a
Standardized growth (w)			
$W_{cooperator}$	1.000	1.000	1.000
W_{mix}	0.762	0.994	0.893
W_{cheat}	0.628	0.631	0.729
Relative fitness in mix (v)			
V_{cheat}	1.571	1.776	1.996
Hamilton's parameters			
r	0.486	0.486	0.486
$b = W_{cooperator} - W_{cheat}$	0.372	0.369	0.271
$c = W_{mix}(V_{cheat} - 1)/(V_{cheat} + 1)$	0.169	0.278	0.297
Simulations for control treatment			
rb-c	0.012	−0.099	−0.165
Proportion of cooperators	0.600	0.008	0.002
Transfers to equilibrium	211	397	68
Simulations for RC treatment			
rb (note that here $c = 0$)	0.181	0.179	0.132
Proportion of cooperators	1.000	1.000	0.998
Transfers to equilibrium	55	199	154

mixed culture plate, we selected a colony following one of two different selection regimes (Fig. 1). In the control treatment, the probability of selecting either a cooperator or cheat colony was directly proportional to their relative frequencies on that plate after the growth period. In the RC treatment, we set the probability of selecting a cooperator vs. a cheat colony to 1 : 1, which represses the advantage cheats normally have in mixed cultures (i.e. relative cheater fitness $v = 1$), and thereby randomizes the reproductive success between the two strains. Note that the cost of cooperation – given by $c = w_{mix}(v - 1)/(v + 1)$ with w_{mix} being the relative growth of mixed cultures compared to cooperator monocultures – is completely removed in our RC treatment because $v = 1$ (Frank, 1995, 2003). Furthermore, note that competition is only repressed between the cooperator and the cheat strain, but not between any new mutants within each strain. For instance, our experiment allows for better-adapted cooperating and cheating mutants to emerge, which could potentially displace the ancestral cooperators and cheats, respectively.

For stochastic sampling of plates and type of bacteria (cooperator or cheat), we used a random number generator implemented in *Mathematica* (Wolfram Research). Growth in CAA followed by colony selection was repeated 12 times in fourfold replication, allowing between 60 and 84 bacterial generations under experimental conditions. We took the proportion of cooperator colonies that were selected to seed the next generation of subpopulations as our explanatory variable. We tested whether the proportion of cooperators significantly increased or decreased across the 12 selection steps (linear regression analysis) or differed between the two treatments after the twelfth selection step (randomization two-sample t-test, as data followed a non-normal distribution).

Relatedness calculations

We used the number of cooperators and cheats after each selection step to calculate the relatedness within subpopulations. Note that the relevant measure of relatedness in this context is with respect to the cooperative trait being examined (i.e. relatedness at the siderophore loci) and not relatedness across the whole genome (Hamilton, 1964; Grafen, 1985). Relatedness with respect to any trait is formally defined as the ratio of the covariance of genetic breeding value for the trait between social partners and the variance in genetic breeding values: $r = \mathrm{cov}(X,Y)/\mathrm{cov}(X,X)$, where X is the genetic breeding value of the focal individual, Y is the genetic breeding value of its social partners and cov is the statistical covariance (i.e. $\mathrm{cov}(X,Y) = \mathrm{E}(X \times Y) - \mathrm{E}(X)\mathrm{E}(Y)$ and $\mathrm{cov}(X,X) = \mathrm{E}(X^2) - \mathrm{E}(X)^2$, where E denotes an expectation or arithmetic mean: Frank, 1998). We may arbitrarily assign cooperators a breeding value of $X = 1$ and cheats $X = 0$; hence, $\mathrm{E}(X^2) = \mathrm{E}(X) = \mathrm{E}(Y)$, and $r = (\mathrm{E}(X \times Y) - \mathrm{E}(X)^2)/(\mathrm{E}(X) - \mathrm{E}(X)^2)$. Noting that subpopulations can exist in three different states according to whether they have been founded by zero (n_0), one (n_1) or two (n_2) cooperator colonies, $\mathrm{E}(X)$ is equivalent to the proportion of cooperators in the population: $p = (n_1 + 2n_2)/[2(n_0 + n_1 + n_2)]$; and $\mathrm{E}(X \times Y)$ is equivalent to the probability of sampling two individuals from the same patch and these both having value 1: $\mathrm{E}(X \times Y) = (n_1/4 + n_2)/(n_0 + n_1 + n_2)$. Thus, we can re-express relatedness as

$$r = \frac{\dfrac{n_1 + 4n_2}{4(n_0 + n_1 + n_2)} - p^2}{p - p^2}.$$

Because relatedness is undefined ($r = 0/0$) if either cooperators ($p = 1$) or cheats ($p = 0$) succeed in going to fixation, we discarded those replicates in which fixation had occurred when calculating the average of relatedness (in a particular generation) over a number of replicates. We tested whether relatedness significantly increased or decreased across the 12 selection steps (linear regression analysis) or differed between the two treatments (randomization two-sample t-test). For the latter analysis, we used relatedness values from the fourth selection step, a time point before any of the replicates went to fixation.

Simulation analysis

We developed a population genetic model and performed numerical simulations for the two treatments, parameterized with the fitness values obtained

from our experiment. The model provides a complete description of the experimental population in terms of the frequency and distribution of cooperators and cheats over subpopulations and over time, and it allowed us to compare the experimental data with the predictions of the model. We used our CFU counts to calculate the fitness of cheating and cooperating strains in subpopulations founded by 0, 1 or 2 cooperative colonies, which yielded the values of four fitness parameters (see Table 1). We standardized fitness values by setting the absolute fitness of pure cooperator cultures to 1. Stochastic sampling of clones to form each new subpopulation was implemented using a *Mathematica* random number generator, with the probability of success of the clone being determined by the treatment-specific criteria as in our empirical experiment (Fig. 1). We performed 20 000 replicates for both treatments and calculated the average values for the proportion of cooperators and relatedness as well as the range containing 95% of all simulated outcomes.

Results

We found that cooperation was favoured in the RC but not in the control treatment (Fig. 2a). The end proportion of cooperators was significantly higher in the RC (0.958) than in the control treatment (0.361; randomization two-sample *t*-test: $N = 7$, $P = 0.027$), and the proportion of cooperators significantly increased over time in the RC treatment (linear regression: $R^2 = 0.237$, $F_{1,46} = 15.60$ $P = 0.0003$), but not in the control treatment ($R^2 = 0.043$, $F_{1,34} = 2.58$, $P = 0.12$). In the RC treatment, three of four replicates became fixed for cooperators and the fourth replicate was also close to fixation (proportion of cooperators = 0.83; Fig. 2b). This pattern contrasts with that found in the control treatment replicates (Fig. 2c), where cooperators became extinct in one replicate, and were maintained at intermediate frequencies in two other replicates. In the fourth replicate of the control treatment, a clear phenotypic change occurred in the cooperator strain, presumably as a result of mutation, which provided this strain with a fitness advantage over cheats in mixed cultures (see Supporting Information, Fig. S1). Whilst this means that this replicate does not represent a fair comparison of cooperation and cheating, and had therefore to be excluded from our analysis, it raises a number of questions about cooperator/cheat coevolution (see Supporting Information, Appendix S1). The simulations based on our population genetic model confirmed our empirical findings that the proportion of cooperators increases in the RC treatment, while remaining at an intermediate frequency in the control treatment (Fig. 2b,c). Furthermore, the simulations show that all experimental end points lie within the 95% range of the simulated replicates.

As expected because of the same dispersal regime in both treatments, we found that relatedness did not significantly differ between the two treatments (random-

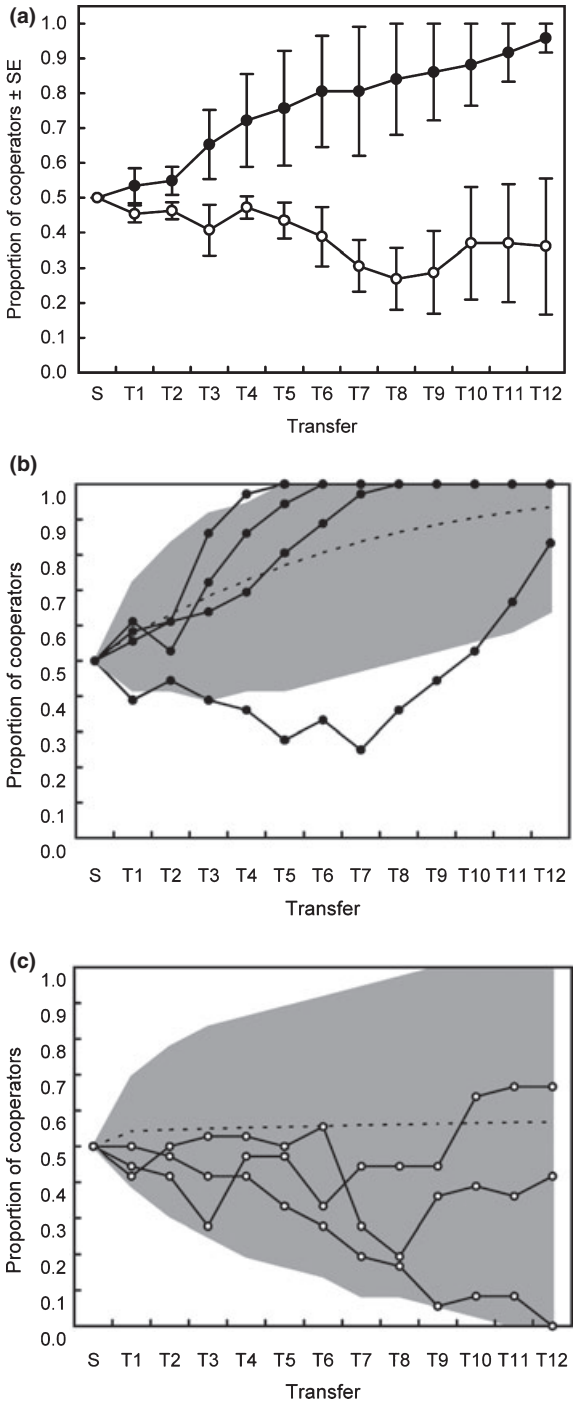

Fig. 2 Repression of competition (RC) and the evolution of cooperation. (a) The mean proportion of cooperators in mixed populations with cheats significantly increased when competition between the two strains was repressed (black circles), but did not significantly change when competition between the two strains was allowed (open circles; control treatment). (b) & (c) Individual replicates in the RC and the control treatments, respectively. Dotted lines and shaded areas indicate simulated means, and the range containing 95% of all simulated outcomes based on 20 000 replicates.

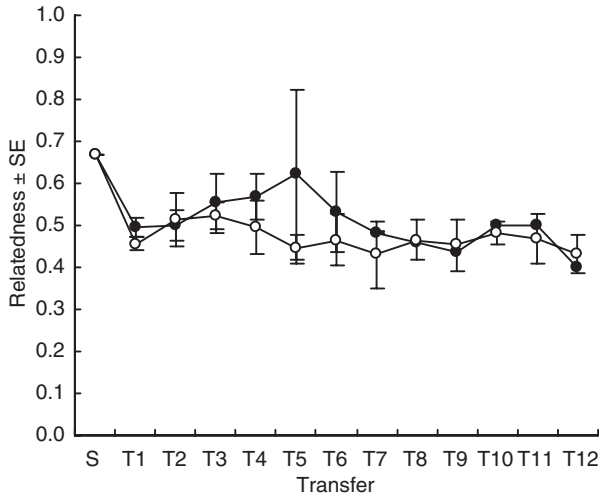

Fig. 3 Repression of competition (RC) and the role of relatedness. Within-subpopulation relatedness in mixed populations of cooperators and cheats did not significantly differ between the two experimental treatments; i.e. in situations where competition between the two strains was repressed (black circles) or not repressed (open circles). Note that after the fourth transfer, some of the replicates had to be discarded because relatedness became undefined ($r = 0/0$) because of the fixation of either cooperators or cheats in the population (see text for details).

ization two-sample *t*-test: $N = 7$, $P = 0.44$) and remained stable over time (Fig. 3; linear regression for RC treatment: $R^2 < 0.001$, $F_{1,26} = 0.68$, $P = 0.42$; for control treatment: $R^2 < 0.001$, $F_{1,33} = 0.92$, $P = 0.35$). This shows that increased cooperation in the RC treatment was not due to a change in relatedness, but was the effect of randomization of reproductive success, which altered the cost of cooperation in such a way as to promote cooperation. Note that relatedness is undefined ($r = 0/0$) if either cooperators or cheats fix in the population. Consequently, we had to discard the replicates in which fixation had occurred and compared relatedness values from the fourth selection step, a time point before any of the replicates went to fixation. Data from our simulations confirmed that relatedness was not different between the two treatments (RC: $r = 0.4858 \pm 0.0010$; control treatment: $r = 0.4861 \pm 0.0007$, mean across the twelve transfers: $t_{22} = 0.68$, $P = 0.50$) and showed that relatedness should approximate $r = 0.5$, which is the expected baseline relatedness given the experimentally imposed regime of complete dispersal and two clones per patch.

To evaluate the range of possible outcomes of cooperator evolution under our experimental conditions, we performed numerical simulations with different sets of fitness parameters obtained from our previous experimental evolution work on *P. aeruginosa* (Griffin *et al.*, 2004; Kümmerli *et al.*, 2009a). We found that the evolution of cooperation greatly varied between the different sets of fitness parameters in the control but not in the RC treatment (Table 1). In the control treatment, our simulations show that the dynamics of cooperation depends upon the balance between the fitness benefit (b) that cooperator strains experience over cheater strains in monocultures, and the fitness cost (c) that cooperators infer from being exploited by cheats in mixed cultures (Table 1). Consistent with Hamilton's rule (Hamilton, 1964), we show that the frequency of cooperators remains fairly stable when $rb\text{-}c = 0$ as found in this experiment. In contrast, if $rb\text{-}c < 0$, then cooperators are disfavoured, as observed when fitness values from our previous studies were used to parameterize our model (Table 1). In the RC treatment, the cost of cooperation is removed ($c = 0$) such that Hamilton's rule reduces to $rb > 0$. Because this condition is satisfied for all sets of fitness parameters, cooperation similarly increased in all simulations (Table 1). This shows that RC is a powerful mechanism that promotes cooperation under conditions that would otherwise be unfavourable for cooperation (e.g. because of low relatedness).

Discussion

We have provided the first direct experimental support – by means of a selection experiment – for the general prediction that RC favours cooperation because it aligns individual interests with the interest of the social group (Leigh, 1977; Alexander, 1979, 1987; Buss, 1987; Maynard Smith & Szathmary, 1995; Frank, 2003). More specifically, our results provide support for the prediction that if reproductive success is randomized within a group, the benefits of cheating are repressed, whereas the relatedness among interacting individuals and benefits of cooperation remain unchanged – resulting in a net benefit of cooperation (Frank, 1995). This demonstrates that randomization of reproductive success *per se*, as opposed to increased relatedness, is responsible for the selective advantage of cooperation. Such randomization has been suggested as a key force in the evolution of cooperation, with examples including Mendelian segregation of chromosomes (Leigh, 1977), and socially imposed monogamy within human societies (Alexander, 1979, 1987). For example, Leigh (1977) suggested that because fair meiosis (Mendelian segregation) ensures that paired homologues of chromosome have equal chances of ending up in a gamete, it acts as a mechanism that prevents costly conflicts at the group (genome) level such that each part of the genome can increase its own success only by enhancing the total number of progeny. Alexander (1987) applied this idea to human social groups where he argued that socially imposed monogamy equalizes and therefore randomizes reproductive success among men, thereby reducing within-group sexual competition. Consequently, an individual can increase its reproductive success only when enhancing the success of the entire group, which makes groups with resolved conflicts more successful than groups with unresolved conflicts.

In our experiment, competition between the two bacterial strains was externally imposed by the experimenter, which is similar to hosts controlling endosymbiont diversity (Frank, 1996, 1997), and was not based on a specific biological mechanism inherent to *P. aeruginosa*. However, RC seems of general importance in maintaining cooperative systems in microbes (Travisano & Velicer, 2004). For instance, the evolution of character displacement among *Pseudomonas fluorescens* lineages in biofilms was found to serve as a mechanism that represses competition among lineages, increased group productivity and made diverse biofilms less susceptible to invasion by cheats (Brockhurst *et al.*, 2006). Furthermore, the evolution of cheating-resistant mutants has been observed in the fruiting body-forming species *Dictyostelium discoideum* (Khare *et al.*, 2009) and *Myxococcus xanthus* (Fiegna *et al.*, 2006), which repress competition between strains in chimaeric aggregations to guarantee a fair allocation of cells to spore formation.

Our experimental (Fig. 2c) and simulation (Table 1) data show that cooperators are either maintained or decreased in frequency with complete dispersal in our control treatment. These findings contrast with the results from an engineered microbial cooperative system (Chuang *et al.*, 2009), in which cooperators increased in frequency under complete dispersal despite being selected against in mixed cultures with cheats (i.e. conforming to the 'Simpson's paradox; Simpson, 1951; Blyth, 1972). Such a paradoxical result could emerge because there was a large covariance between the absolute growth rate of subpopulation types and their proportion of cooperators. Indeed, when parameterizing the control treatment of our model with fitness values from Chuang *et al.* (2009), we also found that cooperators rapidly went to fixation because here $rb\text{-}c >> 0$ ($r = 0.486$, $b = 0.916$, $c = 0.066$). However, with complete dispersal, $rb\text{-}c >> 0$ is unlikely to occur under natural conditions because c tends to be positively correlated with b (Kümmerli *et al.*, 2009c; Jiricny *et al.*, in press). In other words, producing more of the public good increases growth (higher b) of cooperator relative to cheat monocultures, but also makes cooperators more vulnerable to exploitation (higher c) in mixed cultures, such that a large covariance is not expected. This correlation appears to prevent the occurrence of the Simpson's paradox in most systems, so that cooperators are expected to either coexist with cheats or else go extinct under complete dispersal as found for our cooperative trait.

In conclusion, our simple experimental design has captured the essence of the problem of whether RC favours cooperation and has allowed a rigorous test of basic theory. Although such an approach had to be undertaken in quite an artificial environment, it is extremely useful in dissecting the essential components needed for a biological mechanism – such as randomization of reproductive success – to function as a promoter of cooperation. Furthermore, our findings demonstrate that the observed results were not a trivial consequence of the experimental design and that the underlying biology of the study organism played an important role. We observed *de novo* within-strain evolution in our bacterial populations, as demonstrated by the phenotypic change in the cooperator strain in one of the control replicates (see Supporting Information, Appendix S1). Hence, evolution was 'open ended', which allowed within-strain and between-strain evolution to occur, and which in turn influenced the fitness values of cooperators and cheats and therefore their relative success in our experiment. This is best demonstrated in our complete dispersal control treatments where the evolutionary outcomes differ across our various experiments (Table 1), which is most likely attributed to differences in the within-strain evolutionary paths taken by bacteria during the course of the experiment.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 A new cooperator mutant that evades exploitation?

Figure S1 Competition assays between evolved (ev) and ancestral (anc) cooperator (PAO1) and siderophore-defective (PAO9) strains.

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Supporting Information

A new cooperator mutant that evades exploitation?

We observed that the phenotype of the cooperator strain changed in one of the replicates of the control treatment. Specifically, the new phenotype had a doubled growth in monocultures, and outcompeted the siderophore-defective strain in mixed cultures. We hypothesized and tested, whether this phenotypic change, presumably due to mutation, provides a fitness advantage because it reduces the extent to which the cooperators can be exploited by the cheats. We isolated evolved PAO1 and PAO9 colonies from the tenth selection step (i.e., the time point when cooperators went to fixation). We put the isolated strains into competition with each other and with the respective ancestral strains from the freezer stock in six-fold replication under iron limited conditions (6 mL of Casamino acids supplemented with sodium bicarbonate and human apo-transferrin). After a 24h-competition period at 37°C in a static incubator, we plated diluted cultures onto KB plates and assessed the CFU of the two strains following overnight incubation of the plates at 37°C. We then calculated the relative fitness of cheats as $v = x_2(1 - x_1)/x_1(1 - x_2)$, where x_1 is the initial proportion of cheats and x_2 is their final proportion. Because x_1=0.5 in our experiment, $v = x_2/(1 - x_2)$. We then tested whether cheats increased in frequency $v > 1$, decreased in frequency $v < 1$, or remained at the same frequency $v = 1$ using one-sample *t*-tests. We further tested whether v differs between competitions involving evolved or ancestral cooperators using two-sample *t*-tests.

These experiments confirmed that the evolved cooperator strain grew to significantly higher densities than the ancestral cooperator strain (increase in growth = 108%±3%: t_{10}=19.29, P<0.0001) and significantly outcompeted both the ancestral (t_5=-7.58, P=0.0006) and the evolved (t_5=-67.33, P<0.0001) siderophore-defective strain (Fig. S1). Moreover, we found evidence for co-evolution, as the evolved cooperator strain exploited the co-evolved siderophore-defective strain more efficiently than the ancestral siderophore-defective mutant (t_{10}=3.88, P=0.0031). This pattern was not found in the competition assays with the ancestral cooperator strain, which was outcompeted by both the ancestral and the evolved siderophore-defective strain in the same order of magnitude (no significant difference: t_{10}=0.74, P=0.48, Fig. S1). These experiments suggest that cooperators can adapt to their social environment due to beneficial

mutations that prevent exploitation by siderophore-defective strains (see Fiegna *et al.*, 2006, for an analogues example in *Myxococcus xanthus*). Although beneficial, such mutations might be relatively rare as no similar incident has been observed in our previous selection experiments (Griffin *et al.*, 2004; Kümmerli *et al.*, 2009).

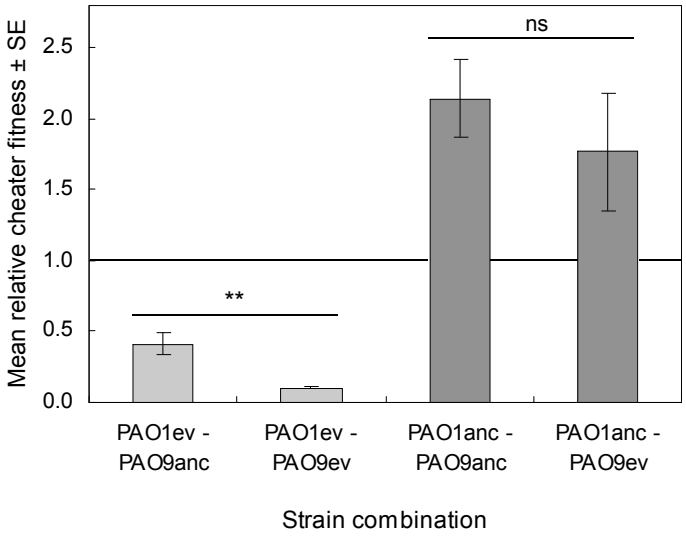

Figure S1. Competition assays between evolved (ev) and ancestral (anc) cooperator (PAO1) and siderophore-defective (PAO9) strains show that the ancestral cooperator is significantly outcompeted by cheat strains (mean relative cheater fitness > 1: t_{11}=3.92, P=0.002), whereas the evolved cooperator is no longer susceptible to cheating and has a significant advantage over cheating strains (mean relative cheater fitness < 1: t_{11}=12.40, P<0.0001). This evolved cooperator mutant was isolated from one of the control treatment replicates.

Supplemental references

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