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LETTER

An evolutionary mechanism for diversity in siderophore-producing bacteria

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Abstract

Bacteria produce a great diversity of siderophores to scavenge for iron in their environment. We suggest that this diversity results from the interplay between siderophore producers (cooperators) and non-producers (cheaters): when there are many cheaters exploiting a siderophore type it is beneficial for a mutant to produce a siderophore unusable by the dominant population. We formulated and analysed a mathematical model for tagged public goods to investigate the potential for the emergence of diversity. We found that, although they are rare most of the time, cheaters play a key role in maintaining diversity by regulating the different populations of cooperators. This threshold-triggered feedback prevents any stain of cooperators from dominating the others. Our study provides a novel general mechanism for the evolution of diversity that may apply to many forms of social behaviour.

Keywords

Altruism, chromodynamics, metapopulation, *Pseudomonas aeruginosa*, pyoverdine type, regulation, siderophore specificity, tag-based cooperation, tagged public good.

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INTRODUCTION

Bacteria require iron for survival and growth and have evolved a variety of mechanisms to scavenge for the insoluble form Fe^{3+}. One such mechanism is the production of siderophores, which are molecules that bind (chelate, in chemical terms) iron with a high affinity (Guerinot 1994). Siderophores are diverse (more than 500 different siderophores are known; Ratledge & Dover 2000; Wandersman & Delepelaire 2004), but all have essentially the same structure, consisting of a functional unit that ligates with iron molecules (transferrins, lactoferrins) and a peptide backbone interacting with a receptor on the surface membrane of the bacteria. An important property of siderophores is their specificity: the siderophore receptor of a bacterium can generally only recognise the peptidic chain of a single siderophore (Hohnadel & Meyer 1988; Cornelis *et al.* 1989; de Chial *et al.* 2003; Spencer *et al.* 2003), although some strains can recognise multiple siderophore types by expressing different receptors (Barelmann *et al.* 2002; Ghysels *et al.* 2004).

How siderophore diversity has emerged and why it persists is not completely understood. Here, we will investigate this question focusing on *Pseudomonas aeruginosa*. *Pseudomonas aeruginosa* produces several types of siderophores, the main class of which are called pyoverdines (Cornelis & Matthijs 2002). Of these there exist three distinct structural types: types 1, 2 and 3. Each strain of *P. aeruginosa* can produce only one of these types, and possesses the corresponding type-specific pyoverdine receptor (Cornelis *et al.* 1989). Moreover, considerable variation has been reported within each structural type (Smith *et al.* 2005; Bodilis *et al.* 2009).

Smith *et al.* (2005) proposed an explanation of how selection could cause diversity at the pyoverdine receptor locus: they argue that siderophore diversity could be a defence against exploitation by non-siderophore-producing bacteria. If there are many non-siderophore producers, it would be beneficial for a mutant to produce a siderophore that is distinct in structure and then incompatible with the dominant population, which would create diversifying selection on the pyoverdine gene (Smith *et al.* 2005; Tummler & Cornelis 2005). However, this explanation assumes that non-siderophore-producing strains are sufficiently abundant to drive this selection.

The ecology and epidemiology of *P. aeruginosa* are not known in great detail, but although there are reports of pyoverdine-negative mutants, these are normally considered to be exceptions. Indeed, such negative strains readily appear *in vitro*, when cultures are kept for sufficiently long time (Harrison & Buckling 2009). Pyoverdine-negative strains have been reported in cystic fibrosis sufferers, but they appear normally after a patient has been infected with a pyoverdine positive clone (De Vos *et al.* 2001). Although coinfection of *P. aeruginosa* has been reported (McCallum *et al.* 2001) this suggests that infection with pyoverdine-negative strains would normally arise through mutation from pyoverdine positive strains in the same host. All these observations suggest that the natural abundance of pyoverdine-negative strains is low, which raises the question of whether the defence against cheating can actually explain the observed pyoverdine diversity.

Siderophores are secreted into the extracellular environment to bind iron and where they can be taken up by any organism that has a suitable receptor. Clones of the same strain share siderophores between them, but not with other strains. Hence, siderophores can be considered as a receptor-specific public good. Assuming that the production of siderophores is costly, this public good system favours pyoverdine-negative mutants. In the context of this public good game, not producing pyoverdine is a cheating strategy (De Vos *et al.* 2001; West & Buckling 2003; Harrison & Buckling 2009).

If a common good can be exploited by cheaters, a feedback may arise that leads to significant diversity. The reason is that strategies may evolve recognition schemes to exclude the cheaters, as already hypothesised by Hamilton (1964). Hamilton also realised that such discrimination schemes themselves are vulnerable to new cheaters. A number of studies have shown that this can result in a type of Red

Queen evolutionary race, in which cheaters continually attempt to 'crack' the recognition scheme, while the cooperators continually devise new schemes to outrun the cheaters. A siderophore's variable part acts as a tag used in recognition (West *et al.* 2007). The dynamics that govern siderophore dynamics could thus be very similar to the Red Queen's races reported in the studies of green beard genes and other tag-based cooperation models (Riolo *et al.* 2001; Axelrod *et al.* 2004; Jansen & van Baalen 2006; Rousset & Roze 2007; Traulsen & Nowak 2007).

However, as we will see, some aspects of the bacteria metapopulation make that this system has a rather different dynamics, leading to alternating periods of random drift and episodes of intense selection. The interplay between complex population dynamics and selection that favours diversity is even richer than previously thought. The episodic nature of selection by cheaters has important consequences for the design of experimental tests of the hypothesis that diversity is generated by social dynamics, as we will discuss in some detail.

In the Methods and Results sections we will study the maintenance of siderophore diversity using a mathematical model. For those not interested in the mathematical details we will attempt to explain our results verbally in the Discussion section.

METHODS: MODEL DESCRIPTION

The model describes a set of hosts which can be colonised by bacteria. Some strains of bacteria can produce a common good in the form of a siderophore, and the production of siderophores increases the output of the colony. We assume that there are different strains of bacteria, each producing a different type of siderophore and having only the corresponding receptor for the siderophores produced: a strain cannot use siderophores produced by other strains. The production of siderophores and the expression of the receptor are controlled by different but adjacent genes (Merriman *et al.* 1995; Ghysels *et al.* 2004; Smith *et al.* 2005) (*fpvA* and *pvd* respectively in *P. aeruginosa*). We will consider them as a single locus, since a single mutation would obviously be detrimental because of a mismatch between the siderophore and its receptor. We also assume that there are siderophore-negative strains, which have lost the ability to produce siderophores, but are still capable of using the siderophore for which they possess the corresponding receptor. Therefore, we characterise a strain by two traits that evolve independently: the strategy (cooperation or cheating) and the type of siderophore it produces and/or can use. This conveniently captures the functionality of the biology of the system whilst allowing mathematical analysis.

Within a patch: local dynamics

Patches colonised by a strain of cooperators are vulnerable to compatible cheaters, because cheaters benefit from the siderophores while not paying any production cost. The appearance of compatible cheaters leads to the breakdown of cooperation, as they invade and oust the cooperators. Patches colonised by the cheaters are vulnerable to incompatible cooperators however, because these cooperators can keep the benefits of siderophores to themselves. Finally, because of positive frequency dependence, we assume that a patch dominated by one strain of cooperators cannot be invaded by a rare cooperator with a different siderophore type. Similarly, cheaters cannot invade patches of different cheaters. Assuming local dynamics are fast, the transitions are instantaneous, and patches only contain a single strain of bacteria.

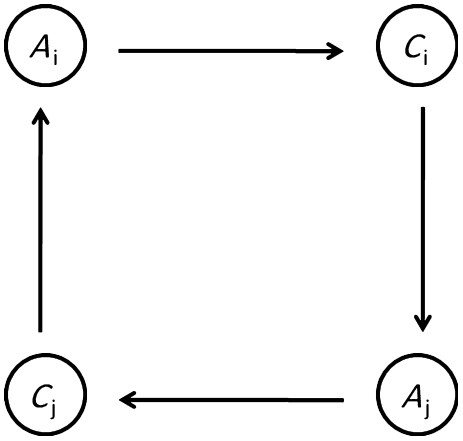

Figure 1 Outcomes of the common good game. If a patch is occupied by a cooperator, it is vulnerable to invasion by a compatible cheater (with rate φb_C). If a patch is occupied by a cheater, it is vulnerable to invasion by an incompatible cooperator (with rate b_A). In principle, the diagonal transitions ($C_i \leftrightarrow C_j$ and $A_i \leftrightarrow A_j$) are possible too through random drift, but this will be such a slow process that it can be ignored.

We will therefore characterise the state of a patch by the type of strain that is currently inhabiting it: we denote A_i and C_i the fraction patches that contain cooperators of type i and cheaters of type i, respectively. Figure 1 summarises the transitions in the states that the model allows.

Between patches : The metapopulation model

Patches are colonised by dispersing bacteria with rates b_A and b_C for cooperators and cheaters respectively. We assume that cooperators are more efficient in exploiting their hosts than cheaters (on their own), therefore cooperator patches will produce more dispersers, and we have $b_A > b_C$. We differentiate the invasion by cheaters in an empty patch and in a patch already occupied by a compatible cooperator: as cheaters benefit from siderophores already produced by compatible cooperators in a cooperator patch, we could assume that colonisation of a cooperator patch by compatible cheaters is facilitated by a factor $\varphi > 1$. For simplicity however, we will assume that cheaters will invade cooperator patches as easily as empty patches, so that $\varphi = 1$ and will thus not appear in the model (see Data S1 for results taking $\varphi > 1$ into account).

New strains appear in the metapopulation through mutation. We describe two types of mutation affecting the local dynamics: with rate ε, the mutation from siderophore-producing to non-siderophore-producing strategy leads to the replacement of cooperators by compatible cheaters inside a patch, and with rate δ, the mutation of siderophore type leads to the replacement of cheaters by incompatible cooperators inside a patch. Since the mutation of siderophore type needs a double mutation (on the siderophore and the receptor genes), we impose $\varepsilon > \delta$. We ignore all the other mutation possibilities, as they are irrelevant for the local dynamics: for instance, the mutation from non-siderophore-producing strategy to producing would render the mutant (now cooperator) vulnerable to the all-cheater resident population, and thus it could not invade. Thus, an empty patch can change status when it is colonised, a colonised patch can change status either by local extinction, by competition from other strains, or as a consequence of the fixation of a mutant. The dynamics of a

metapopulation with n types of siderophore producers are governed by the following differential equations

$$\frac{dA_i}{dt} = (1 - \Sigma A - \Sigma C)b_A A_i - mA_i - b_C A_i C_i + b_A A_i(\Sigma C - C_i) - \varepsilon A_i + \frac{\delta}{n-1}(\Sigma C - C_i)$$

$$\frac{dC_i}{dt} = (1 - \Sigma A - \Sigma C)b_C C_i - mC_i + b_C A_i C_i - b_A C_i(\Sigma A - A_i) + \varepsilon A_i - \delta C_i$$

with $\Sigma A = \sum_{j=1}^{n} A_j$ and $\Sigma C = \sum_{j=1}^{n} C_j$.

Here, $(1-\Sigma A-\Sigma C)b_A A_i$ represents the colonisation of empty patches by A_i, mA_i the extinction of A_i patches, $b_C A_i C_i$ the colonisation of A_i patches by C_i, $b_A A_i(\Sigma C-C_i)$ the colonisation of C_j ($j \neq i$) patches by A_i, εA_i the conversion from A_i to C_i, $\frac{\delta}{n-1}(\Sigma C - C_i)$ the conversion from patches C_j ($j \neq i$) to A_i. Similarly, $(1-\Sigma A-\Sigma C)b_C C_i$ represents the colonisation of empty patches by C_i, mC_i the extinction of C_i patches, $b_A C_i(\Sigma A-A_i)$ the colonisation of C_i patches by A_j ($j \neq i$) patches, δC_i the conversion from patches C_i to A_j.

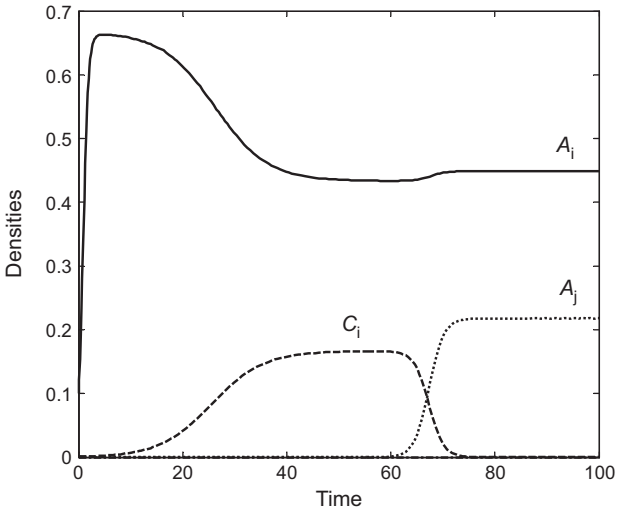

Figure 2 How cheaters disappear from patches when there is more than one type of siderophore. First both A_i and C_i are going to equilibria $\bar{A}$ and $\bar{C}$. The appearance of a competitor (A_j) drives the population of C_i to extinction and A_j settles in an all-cooperator system (neutrally stable). Parameters : $b_A = 3$, $b_C = 1.2$, $M = 1$.

RESULTS

We will start by assessing under what conditions new types can invade in the population and first analyse the existence and invasibility of equilibria without explicit mutation, so that $\varepsilon = \delta = 0$. We first determine the equilibria of the system. Then, we explore under which conditions a metapopulation using a single siderophore type can be invaded by a strain using a new type of siderophore.

Equilibria

We denote $\bar{A}$ and $\bar{C}$ the equilibria for the total populations of altruists and cheaters, respectively. We will only consider the simultaneous non-zero equilibrium for both strategies ($\bar{A} > 0$ and $\bar{C} > 0$). Solving the equilibrium for arbitrary n yields

$$\bar{A} = n\left(\frac{M}{b_A + b_C - b_A n} - \frac{b_C}{b_A + b_C - b_C n}\right)$$

$$\bar{C} = n\left(-\frac{M}{b_A + b_C - b_A n} + \frac{b_A}{b_A + b_C - b_C n}\right)$$

As $\bar{A}$ and $\bar{C}$ are proportions, feasible equilibria constrain between 0 and 1. For a metapopulation with only one type of siderophore produced ($n = 1$), a feasible equilibrium can exist, with cooperators and cheaters present. If we increase the number of siderophore types ($n \geqslant 2$) however, there is no solution with $0 < \bar{C} < 1$ anymore: the cheaters cannot stably persist in the metapopulation if there is more than one siderophore type (Fig. 2).

The fact that no diverse equilibrium is possible raises the question what will happen in the long run when siderophore diversity increases.

Invasibility

Even if cheaters cannot persist in a diverse metapopulation this does not mean they do not play a role in determining siderophore diversity. In order to gain an intuitive understanding of their role, we now investigate the invasion by mutant types using a novel siderophore into a system which has a resident cooperator and cheater who share a single siderophore ($n = 1$). We consider first what happens if a mutant cheater appears in the metapopulation. This mutant cheater has no advantage because it cannot use the siderophores produced by the resident cooperator, and therefore, it cannot invade (see Data S2 for a mathematical description).

Then, we consider what happens if a mutant cooperator that uses a new type of siderophore arises in a population. This mutant is initially not encumbered by any cheaters, so it has an advantage over the resident cooperator which is being exploited by its cheater, and will invade the system. When this mutant invades, the resident cooperator will decrease in density. Eventually, the resident density will drop below a threshold density, denoted A_T, which is the density value above which a cooperator can support the presence of a corresponding cheater (see Data S3 for a mathematical derivation). As a result, the resident cheater decreases and once it has gone extinct, the two strains of cooperators will coexist in a neutral equilibrium (Fig. 3). However, a cheater can reappear whenever one particular corresponding strain of cooperators exceeds the A_T threshold, triggering the same mechanism to regulate the cooperators, protecting the emerged diversity. Thus, although they cannot persist in the metapopulation, cheaters have an ephemeral but important role in regulating siderophore diversity. The overall effect will depend on the level of the threshold density A_T, the processes (such as drift) that allow some cooperator strains to exceed this threshold, and the processes (such as mutations) that reintroduce cheaters into the population. We will explore these effects in the next section using stochastic simulations.

Stochastic simulations

Mutations have a number of important effects. The first is that it may lead to genetic drift, which has the potential to favour (at random) one of the cooperator strains. The second is the occasional appearance of cheater mutants which, as we have seen, protects diversity. The diversity in such a system thus depends on a balance between

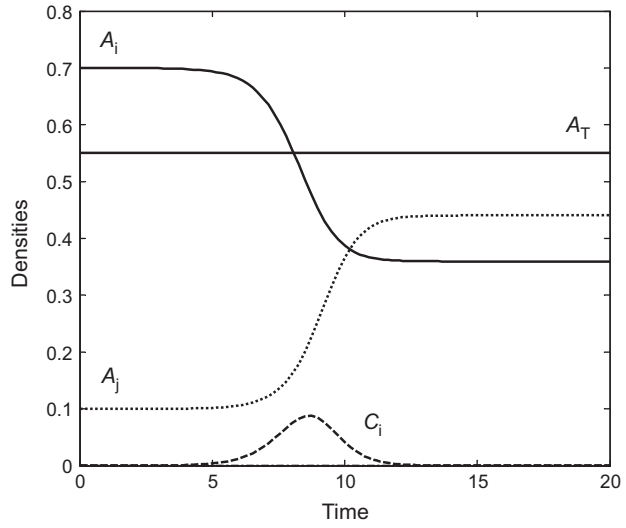

Figure 3 The role of the cheaters. Cheaters can appear in the population if one of the cooperating strains exceeds a threshold density A_T. The cheater of the abundant cooperator increases and regulates its population, then disappears again. The amplitude between high and low cooperators is reduced, and cheaters cannot re-invade until the same process perturbs the relative frequencies of the cooperators. Parameters : $b_A = 5$, $b_C = 3$, $M = 1$.

generation of new types through mutation, loss through drift but also regulation by cheaters. To study the dynamics of this diversity, we used a simulation model. This simulation model is a stochastic version of the deterministic metapopulation model, in which the number of patches is finite. Otherwise, the patch state transitions follow the same rules as in the deterministic model (colonisation, extinction, competition between strains, mutations), in discrete time.

We first study the effect of stochasticity in a polymorphic metapopulation of cooperators without cheaters. Because all strains of cooperators are competitively neutral with respect to each other, demographic stochasticity makes that types eventually disappear. Unsurprisingly, in the absence of cheaters, the metapopulation eventually loses all its initial diversity and becomes monomorphic (Fig. 4a) because of genetic drift.

The presence of cheaters in the metapopulation has a protective effect on the population diversity: diversity is invariably maintained in all the simulations we carried out (Figs 4b and 6a,b). This is in stark contrast with the results of our simulations without cheaters and shows how cheaters can act as a force against the loss of diversity in siderophore types through genetic drift. Moreover, in agreement with our theoretical analysis, while being critical to diversity, cheaters remain at very low frequencies in the population, and increase only episodically. Figure 5 shows clearly the mechanism maintaining diversity: through genetic drift, one type is starting to increase in frequency, reducing the frequency of the other type present. At some point, when the frequency of this cooperator crosses the threshold A_T, we observe some time later a short appearance of the corresponding cheater, reducing the frequency of the cooperator below A_T, before disappearing again. The genetic drift is thus counteracted by the cheaters, which act as a regulating force which is absent most of the time but appears when one of the cooperating strains becomes dominant (i.e. crosses the threshold), thus protecting diversity.

Figure 5 demonstrates how diversity is promoted and maintained by cheaters in the metapopulation. But it leaves unanswered the

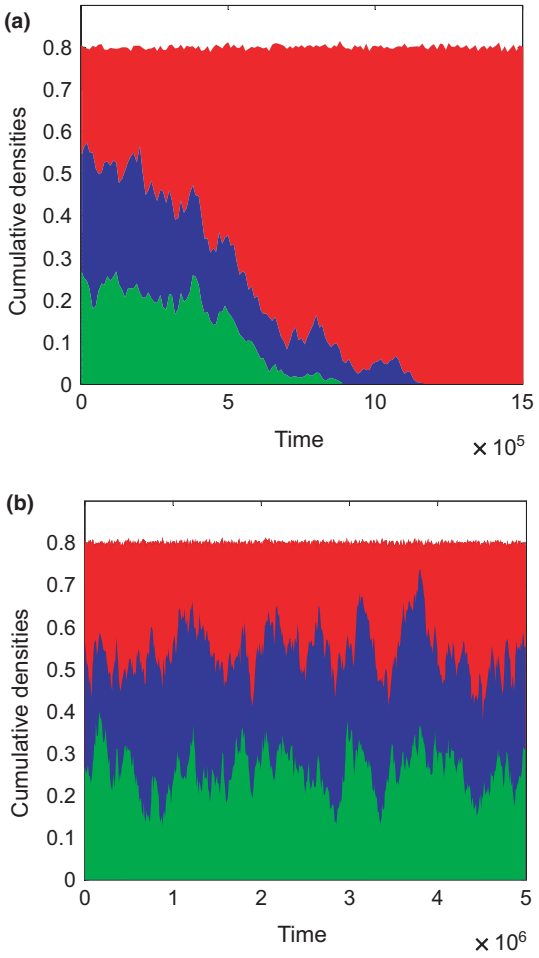

Figure 4 Stochastic simulations for an initially diverse system of three different cooperators. Graphs show the cumulative densities of cooperators: A_1 in green, A_2 in blue, A_3 in red. (a) Dynamics in absence of cheating strains. Over time, some strains disappear (A_1 and A_2) and eventually the system reverse to a monomorphic population. (b) Dynamics in presence of cheating strains. Strains vary in densities, but do not disappear even for a longer period of time. Parameters: $b_A = 5$, $b_C = 3$, M=1.

question as to whether this diversity is bounded, and if so, to what level it is regulated. Figure 6a,b shows the effect of mutation rates on the resulting level of diversity. High mutation rates of the cooperating to cheating strategy (ε) lead to high diversity in the metapopulation (Fig. 6a). This is because the cheating strains appear quicker in the population, preventing the disappearance of types through the genetic drift: if a strain of cooperator increases in frequency, it is regulated when the corresponding cheaters appear (Fig. 5), but the delay between the increase of cooperators and the appearance of the corresponding cheaters can lead to the extinction of other less abundant types. The level of diversity also depends on the rate of appearance of new siderophore types δ. If the new strains appear quickly, diversity will be high, and on the contrary if new strains appears rarely, the diversity is lower (Fig. 6b).

DISCUSSION

We used a mathematical model to investigate how siderophore diversity can be maintained in populations of siderophore-producing

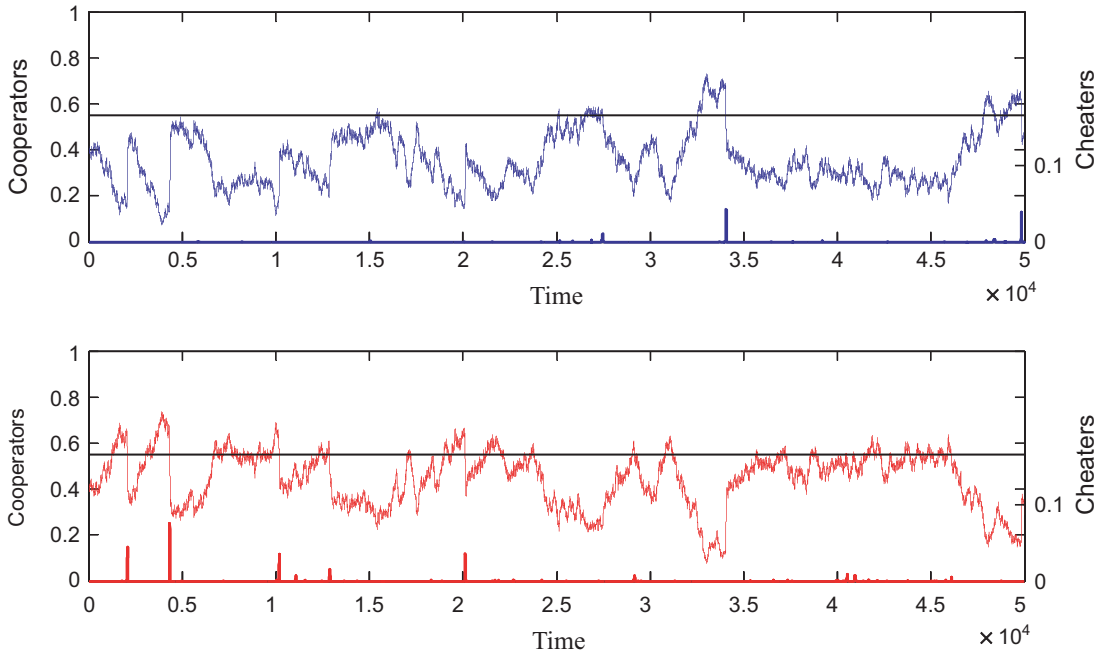

Figure 5 Dynamics for a two-type system. In blue, cooperator and cheater 1, and in red, cooperator and cheater 2. The upper fluctuating lines correspond to the densities of cooperators, and the lines below consisting of isolated peaks to the densities of cheaters. When one of the cooperating strains increases in frequency, the other decreases. If one of the cooperators goes above the threshold A_T (black horizontal line), the corresponding cheating strain invades, which regulates the cooperators, and then disappears. Parameters: $b_A = 5$, $b_C = 3$, $M = 1$, $N = 5000$.

bacteria. We found that cheaters can play a crucial role in the maintenance and regulation of siderophore diversity. Because siderophore-negative cheaters can act as a regulating agent, the cheating strains increase in density if the specific siderophore they can use is produced in sufficient abundance, which results in a decrease of the cooperators that produce this siderophore. The cheaters then disappear. This frequency-dependent mechanism selects against all siderophore-producing strains that becomes sufficiently dominant. Our analysis suggests that this mechanism favours diversity even if the cheaters occur infrequently in the metapopulation and at a very low density.

Our results thus support the hypothesis that siderophore diversity results from cheating, as suggested by Smith *et al.* (2005). However, this hypothesis appears to rely on the selection pressure caused by cheaters, which often seem to be absent. Although cheating strains arise readily in laboratory experiments (Harrison & Buckling 2009), they seem rare in natural populations (West & Buckling 2003). Is it then possible that siderophore diversity has nevertheless arisen as an evolutionary response to non-siderophore-producing strains? Our model shows that this can indeed be the case: fluctuations from drift in the relative densities of siderophore can make certain strains temporarily abundant. If this happens, non-siderophore-producing strains will be selected for and rapidly increase, and will reduce the density of these abundant strains, increasing the densities of other siderophore producers in the process. This mechanism prevents producing strains from reaching very high or very low densities and thus maintains diversity through episodic outbreaks of non-siderophore-producing strains.

Siderophore diversity is not regulated as a dynamic equilibrium, in which the forces that increase and decrease the diversity counteract each other and balance out, but as a dynamic process in which the drift in the population keeps changing the densities of the different siderophore-producing strains in the population. Only once a strain crosses the threshold do the cheating strains emerge and does the regulation kick in. This implies that stochastic processes play a key role: they govern both the drift in a strain's frequency and the appearance of new mutants. The dynamics are thus characterised by an alternation of periods of steady genetic drift with episodes of rapid and intense evolution.

This is also found in other tag-based cooperation models (Riolo *et al.* 2001; Axelrod *et al.* 2004; Jansen & van Baalen 2006; Rousset & Roze 2007) in which diversity results from non-equilibrium dynamics. If there were no incentives to cheat it would pay all cooperators to produce the same type of siderophore, that is to equally share the benefits of their altruistic acts. Only when there are cheaters around it pays for cooperators to deviate from the dominant type and share benefits with members using the same receptor. This results in (locally) unstable dynamics governed by rarity advantage and commonness penalty. In Jansen & van Baalen's (2006) beard chromodynamics model different types occur in amorphous clusters, leading to a dominance of cheaters. In the model presented here types appear in homogeneous clusters, leading to dynamics in which altruists prevail. Whether a similar relationship between siderophore production costs and tag diversity exists as in the Jansen & van Baalen (2006) model remains to be tested.

In our model, the final levels of siderophore diversity depend on the mutation rates. Moderate diversity can evolve when mutation rates are low, but high mutation rates lead to a high diversity. This result is consistent with the discovery of hypermutant strains of *P. aeruginosa*, which have unusually high mutation rates (Oliver *et al.* 2000; Buckling *et al.* 2007), particularly for the *mutS* gene, involved in the DNA-

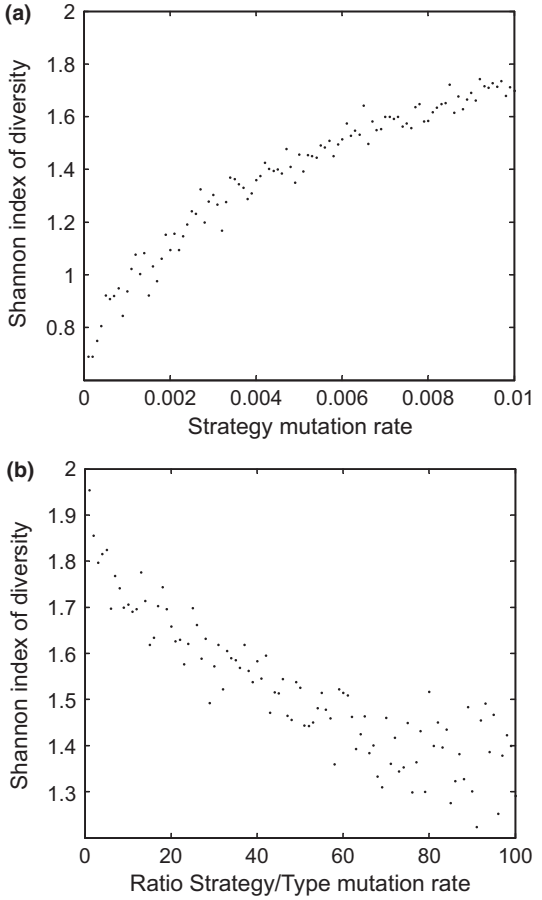

Figure 6 The effect of mutations on diversity. Simulations start with an initial monomorphic metapopulation. (a) The effect of the cooperation to cheat strategy mutation rate (ε) on the diversity. Diversity is higher if the rate ε is high. Parameters : $N = 10\,000$, $\delta = 0.1\varepsilon$. (b) The effect of the type-mutation (δ) rate on diversity. Diversity decreases and is low when the ratio ε/δ is high. Parameters: $N = 10\,000$, $\varepsilon = 0.01$.

mismatch repair mechanism (Oliver *et al.* 2002). As in the classical Red Queen, the mutation rate has the general effect of speeding up the dynamics: cooperative strains can escape their corresponding cheaters quicker, by producing new types of siderophores, and vice versa, the cheating strains can acquire the compatible siderophore receptor quicker.

Our results show that the diversity in the siderophore receptor locus can result from the selection imposed by the episodic presence of cheating strains. This finding has relevance beyond explaining siderophore diversity: a number of other organisms have been suggested as having tag-based recognition, for instance *Saccharomyces cerevisiae* has genes (*FLO*) that cause flocculation which is associated with social behaviour (Smukalla *et al.* 2008). It has also been suggested that there is considerable diversity in these genes, with closely related strains of *S. cerevisiae* often displaying very distinct phenotypes, because of an unstable tandem repeat sequence in the *FLO1* gene (Verstrepen *et al.* 2004, 2005; Smukalla *et al.* 2008). Moreover, mutation rates of the *FLO1* gene have been found to be at least 100-fold greater than the average mutation rates in the genome, suggesting that the same mechanism, as the one we highlight here, is operating in this system. We thus suggest that diversity in the *FLO* genes could also have emerged as an evolutionary response to the presence of cheaters. A further example are side-blotched lizards where the males form cooperative mate-guarding dyads based on throat colouration (Sinervo & Clobert 2003; Sinervo *et al.* 2006). Different throat colours have been observed together with the unstable dynamics in which altruistic and selfish traits alternate in dominance.

Our results suggest that to better understand diversity in siderophores, or tag-based cooperation in general, one needs to go beyond studying strains in isolation or even snapshots from a population. One needs to investigate the change in tag composition over sufficient time within such populations, and in sufficient detail. Our analysis suggests that siderophore producers, such as *P. aeruginosa*, can serve as an experimental model system to demonstrate how altruistic behaviour can go together with the dynamically unstable behaviour known as chromodynamics.

The experiment is simple in essence: create a metapopulation of bacteria and allow some movement between the patches. What we predict is that the diversity in siderophores is more persistent if cheating strains are occasionally introduced in this population. A further prediction is that the densities of cheaters will be negatively correlated to the siderophore diversity.

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SUPPORTING INFORMATION

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

Data S1 Inclusion of φ.
Data S2 Invasion of a second cooperator.
Data S3 Reinvasion by cheaters.

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