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An experimental test of whether cheating is context dependent

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Abstract

Microbial cells rely on cooperative behaviours that can breakdown as a result of exploitation by cheats. Recent work on cheating in microbes, however, has produced examples of populations benefiting from the presence of cheats and/or cooperative behaviours being maintained despite the presence of cheats. These observations have been presented as evidence for selection favouring cheating at the population level. This apparent contradiction arises when cheating is defined simply by the reduced expression of a cooperative trait and not in terms of the social costs and benefits of the trait under investigation. Here, we use two social traits, quorum sensing and iron-scavenging siderophore production in *Pseudomonas aeruginosa*, to illustrate the importance of defining cheating by the social costs and benefits. We show that whether a strain is a cheat depends on the costs and benefits associated with the social and abiotic environment and not the absolute expression of a cooperative trait.

Introduction

The growth of microbes often relies on the costly secretion of a range of exofactors such as biofilm polymers, nutrient-scavenging molecules and toxins (Crespi, 2001; West *et al.*, 2006; Xavier, 2011). The benefit of producing exofactors can be shared with other cells in the local group, in which case they can represent cooperative traits (Griffin *et al.*, 2004; West *et al.*, 2006). The problem with cooperation is that it is susceptible to exploitation by 'cheats', which do not perform the costly cooperative behaviour, but benefit by exploiting the cooperation of others (Ghoul *et al.*, 2013).

Recent work on cheating in microbes, however, has produced results that are apparently inconsistent with theoretical predictions about the outcome of competition between cooperators and cheats. For example, whereas it has been shown that the production of iron-scavenging siderophore molecules can be a cooperative trait in bacteria (Griffin *et al.*, 2004), a recent study has argued that this is not the case (Zhang & Rainey, 2013). Another example is that, while cheating, by definition, should come with an associated fitness cost to the cooperators and the group, a recent study on yeast found that group productivity is maximized by a mixed population of cooperators that produce invertase and cheats that did not (MacLean *et al.*, 2010). More generally, it is a widely held assumption that a cheat can be identified simply by reduced expression of a cooperative trait, relative to others.

We suggest that inconsistencies in the experimental literature can arise when cheating is defined as reduced production of some factor and not in terms of the relative costs and benefits. As with any social trait, the definition of cheating depends upon the social costs and benefits (Hamilton, 1964; West *et al.*, 2007). Consequently, reduced production of an exofactor will not always be cheating (Ghoul *et al.*, 2013). Put simply, whether a certain strain or genotype is a cheat is context dependent such that cheating depends on its social and abiotic environment. Here, we illustrate that cheating is context dependent using two traits commonly used in experimental studies of social behaviour in microbes: the use of quorum sensing (QS) to coordinate the production and release of exofactors, and the production of iron-scavenging pyoverdin (pvd) siderophore molecules, in the opportunistic pathogen *Pseudomonas aeruginosa*. These two systems each offer different opportunities to manipulate the abiotic environment (requirement for QS to grow) and the social environment (strains with variable pyoverdin production).

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Our first aim is to use the QS system to test the effect of the abiotic environment. We use a wild-type PAO1 strain as a cooperator and an isogenic *lasR* mutant, a signal-blind QS mutant, as a putative cheat. Strains that have a mutation in the *lasR* gene do not respond to autoinducing *N*-acyl homoserine lactone (AHL) signal molecules and therefore cannot synthesize QS-dependent exofactors to acquire nutrients (Diggle *et al.*, 2007; Mellbye & Schuster, 2011; Schuster *et al.*, 2013). We compete the putative *lasR* cheat with the cooperator in an environment that requires QS for the production of exofactors, and in an environment, where QS production of exofactors is not necessary for survival. We predict that the *lasR* mutant will act as a cheat in the nutrient-poor conditions, but not in the nutrient-rich environment.

Our second aim is to use the pyoverdin system to test the effect of the social context on cheating behaviour. We use a wild-type *P. aeruginosa* strain as a cooperator, and as putative cheats, we use mutants isolated from selective lines from a wild-type ancestor, which are defective in pyoverdin production. We compete pairs of strains that produce different quantities of pyoverdin in an iron-limited environment. We predict that whether a strain acts as a cheat depends upon its pyoverdin production relative to its competitor strain, and not its absolute level of pyoverdin production.

Materials and methods

Bacterial strains

We used two strains in our QS experiment: a *P. aeruginosa* wild-type PAO1 strain and an isogenic PAO1 insertion mutant *lasR*::Gm (*lasR*) (Popat *et al.*, 2012) which is a signal-blind mutant that does not respond to autoinducer signal and hence fails to induce the synthesis of exofactors even at high cell densities (Diggle *et al.*, 2007).

We used three strains in our siderophore experiment: the wild-type *P. aeruginosa* 206-12-strain, $10a^+$ (a cystic fibrosis isolate from Seattle, WA, USA (Jiricny *et al.*, 2010)) and two spontaneous pyoverdin mutants, $10a^-$ and $10c^-$, derived from the pyoverdin-producing wild types, 206-12-$10a^+$ and $10c^+$, respectively, as described in Jiricny *et al.* (2010). In this study, we refer to the wild-type strain $10a^+$ as strain A, and the pyoverdin mutants $10a^-$ and $10c^-$, as strains B and C.

Prior to experimentation, we cultured all strains from freezer stocks for 24 h at 37 °C on an orbital shaker at 180 rpm. In the QS experiment, strains were cultured in lysogeny broth [LB, 14 g broth miller, (Fisher Scientific, Loughborough, UK) per litre of distilled water (dH_2O)]. In the pyoverdin experiment, strains A, B and C were cultured in King's broth media [KB, 20 g protease peptone N^o3 (Beckton Dickinson Ltd., Oxford, UK), 10 mL glycerol, 1.5 g $K_2HPO_4.3H_2O$ and 1.5 g $MgSO_4.7H_2O$ (Sigma-Aldrich Ltd., Gillingham, UK), per litre of dH_2O]. We centrifuged the overnight cultures and discarded the supernatant. We then washed the cell pellet in minimal salts media (M9, 6.8 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl and 10 g NH_4Cl (Sigma-Aldrich Ltd.), per litre of dH_2O) to remove any residual iron and carbon resources from the KB or LB media. We assayed cell density at an absorbance of 600 nm (A_{600}) and standardized each set of cultures to the same density.

Testing the effect of the abiotic context in a QS system

Measuring lasI and lasB expression

Our QS experiment relies on the assumption that PAO1 continuously invests in exofactor synthesis in different media. We tested whether PAO1 continuously invest in exofactor synthesis by quantifying the gene expression of *lasI* [regulates *N*-(3-oxododecanoyl)-L-homoserine lactone (3O-C_{12}-HSL) production] and *lasB* (codes for elastase production) using *lasI* and *lasB* CTX*lux* reporters conjugated into PAO1 (strains PAO1 *lasI::lux* and PAO1 *lasB::lux*) that were then grown in different media (Jadhav *et al.*, 2011; Popat *et al.*, 2012). Cultures were grown for 24 h in rich casamino acid media [CAA, 1% casamino acids weight/volume (w/v) (Sigma-Aldrich Ltd.), in M9 solution] and in a minimal media containing bovine serum album [BSA, 1% BSA w/v (Sigma Aldrich, UK ltd) and 0.1% CAA w/v in M9 solution] as described by Darch *et al.* (2012). Cell growth in CAA does not require QS-controlled exofactor production, whereas growth in BSA requires QS-dependent exofactor secretions (Diggle *et al.*, 2007; Darch *et al.*, 2012). We determined bioluminescence as a function of cell density by using a combined, automated luminometer-spectrometer (Genios Pro, Tecan Group, Reading, UK). Luminescence and turbidity (A_{600}) were automatically measured every 30 min. We calculated luminescence per cell as relative light units (RLU)/A_{600} at each time point. We replicated each treatment six times.

Competition assays

We carried out competition assays in CAA and BSA media, respectively. We grew the *lasR* mutant and PAO1 strains in a 6 : 100 and 5 : 100 ratio, respectively, in vials containing 2 mL volume of either CAA or BSA media. The BSA media are supplemented with minimal amounts of CAA to initiate QS for the wild-type strain during the competitions (Darch *et al.*, 2012; Popat *et al.*, 2012). To control for social effects on relative fitness, we cultured the *lasR* mutant and wild-type strains separately as monocultures. Relative frequencies at the start of each experiment were obtained by plating 40 μL of 10^2 dilutions on LB agar [25 g LB Miller agar (Fisher Scientific) per litre of dH_2O] and then incubated them at 37 °C. We then incubated all cultures for 24 h at 37 °C. At 24 h, after the strains

had competed through the exponential growth phase and reached the stationary phase, we plated out 20 μL and 100 μL of 10^6 dilutions of the CAA and BSA mixed cultures, respectively. We then diluted the BSA monocultures two-fold and the CAA monocultures 10-fold using M9 medium prior to measuring the cell densities at A_{600}, to compare the growths of mutant and wild type in the different media environments.

After incubating the agar plates for 17 h at 37 °C, we recorded the colony counts of each strain which are distinguishable by colony phenotypes. *lasR* mutant colonies have smooth edges surrounded by a halo, whereas PAO1 colonies have rugged edges.

Testing the effect of the pyoverdin social context

Measuring pyoverdin production

We estimated the relative pyoverdin production per cell by the ratio of relative fluorescent units (RFU), at excitation and emission wavelengths of 400 nm and 460 nm, respectively, to absorbance (A_{600}, cell density), as described by Jiricny *et al.* (2010). We replicated each assay six times per culture and calculated the mean pyoverdin production per cell by RFU/A_{600} (Kummerli *et al.*, 2009b).

Competition assays

We compared the relative fitness of strain C to that of strain B, and relative fitness of strain B to that of strain A (wild type), by competing C : B and B : A in a 1 : 10 ratio, in vials containing 6 mL of iron-limited CAA media. In each case, the lower-producing pyoverdin strain (putative cheat) is introduced at a lower frequency than the higher-producing strain (putative cooperator) (see results). We also cultured each strain separately in a monoculture. Relative frequencies at the start of each experiment were obtained by plating 10 μL of 10^1 dilution of mixed cultures on KB agar (12 g agar per litre of KB medium), which we then incubated at 37 °C. We then incubated all cultures for 24 h at 37 °C. At 24 h after the strains had competed through the exponential growth phase and reached the stationary phase, we plated 10 μL of 10^4 dilution on KB agar plates and incubated them at 37 °C. We then diluted the monocultures 10-fold with M9 medium and assayed the cell density of each monoculture at A_{600} to compare the growths of each strain.

After incubating the agar plates for 17 h at 37 °C, we recorded the colony counts of each strain. We phenotypically distinguished colonies of each strain based on colony shape and pigmentation. The strains that produce more pyoverdin are greener in colour (Jiricny *et al.*, 2010). We ensured that the plates are not incubated longer than 17 h because siderophores diffuse into the agar and are taken up by neighbouring colonies adding to their pigmentation and making them less distinguishable.

Statistical analysis

We calculated the relative fitness (ω) of the putative cheat, which is the change in frequency over time relative to the wild type. This is given by $\omega = x_2(1\text{-}x_1)/x_1(1\text{-}x_2)$ where x_1 is the mean initial proportion of mutants at 0 h from the sample population and x_2 is the final proportion of each sample at 24 h (Otto & Day, 2007; Ross-Gillespie *et al.*, 2007). A value of $\omega > 1$ indicates that the cheat has a higher fitness than the cooperator, and a value $\omega < 1$ indicates that the cheats have a lower fitness than cooperators. We replicated each competition assay six or twelve times and logged ($\log_{10}$) the ω values before calculating the mean across replicates to eliminate any bias from pseudo-replication variation (Jiricny *et al.*, 2010). We carried out all analysis in R statistical software (http://www.R-project.org).

Results

Varying the abiotic context in a QS system

Competition and monoculture assays

The *lasR* mutant acts as a cheat in BSA media, where QS is required for growth, but not in CAA media, where exofactor synthesis is not required for growth. In mixed cultures, containing PAO1 and the *lasR* mutant, the *lasR* mutant increases in frequency, in both BSA media (Fig. 1; $\omega_{lasR} = 1.7 \pm 0.4$, $T = 3.33$, $P = 0.007$) and CAA media (Fig. 1; $\omega_{lasR} = 2.2 \pm 0.6$, $T = 4.04$, $P = 0.002$). In monocultures, the *lasR* mutant grows significantly less well than PAO1 in BSA media (Fig. 2a; $T = 9.66$, $P = 0.0001$), but significantly better than PAO1 in CAA media (Fig. 2b; $T = 11.55$, $P = 1.6 \times 10^{-6}$).

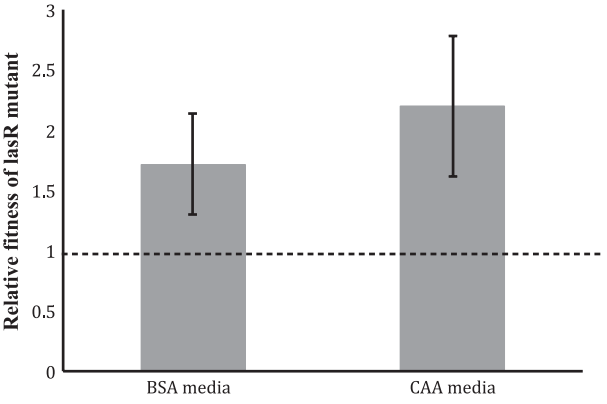

Fig. 1 Relative fitness of the *lasR* mutant in competition with PAO1 under conditions that requires quorum sensing (QS) (BSA) and that does not require QS (CAA). The *lasR* mutant increases in frequency in competition with wild type in both BSA media (where QS is required for growth) and in CAA media (where QS is not required for growth). The error bars indicate 95% confidence intervals around the means of twelve independent competition replicates.

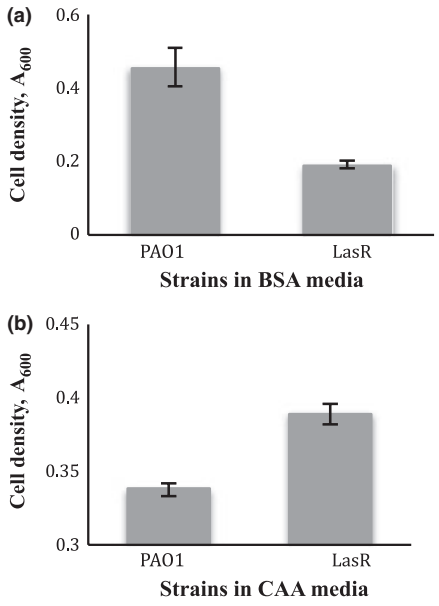

Fig. 2 Monoculture cell density (A_{600}) in media that requires quorum sensing (QS) (BSA) and media that does not require QS (CAA). In monocultures of the *lasR* and PAO1 strain: (a) PAO1 grows to a significantly higher density than *lasR* in BSA medium; (b) PAO1 grows to a significantly lower density than *lasR* in CAA medium. The error bars indicate 95% confidence intervals around the means of six independent replicates.

lasI and lasB expression

Even in CAA media, which does not require exofactors for cell growth, PAO1 continues to express QS-dependent genes and invest in exofactor synthesis over a period of 24 h of growth (Fig. S1 a and b). In CAA media, PAO1 expresses *lasB* at a significantly higher peak per cell than *lasI* (Fig. S1a; $T = 16.44$, $P = 7.544 \times 10^{-7}$). In BSA media, PAO1 expresses *lasI* at a significantly higher peak per cell than *lasB* (Fig. S1b; $T = -6.18$, $P = 0.0008$). Comparing gene expression in both media shows that PAO1 expresses *lasB* and *lasI* at significantly higher levels in CAA than in BSA media (Fig. S1 a and b, $T = -26.85$, $P = 8.962 \times 10^{-7}$ and $T = -9.91$, $P = 1.761 \times 10^{-6}$, respectively). In the BSA environment, *lasB* expression is sustained, indicating that the production of protease exofactors, such as elastase, is important for growth in this environment and is therefore maintained, whereas in the CAA medium, there is a peak of gene expression when QS induces followed by a sharp drop in expression.

Varying the social context in a public good (pyoverdin) system

Competition and monoculture assays

The outcome of competition depends on the relative and not the absolute expression of the public good, pyoverdin: strain B either cheats or is cheated, depending on its social partner. Strain A produces significantly higher levels of pyoverdin per cell than strain B, and strain B produces significantly higher levels of pyoverdin per cell than strain C (Fig. 3a; $F_{(2,15)} = 72.05$, $P = 2.03 \times 10^{-8}$). The growth rate, in monoculture, correlates with pyoverdin production, with strain A growing to higher density than strain B, which grows to higher density than strain C (Fig. 3b; $F_{(2,15)} = 272.4$, $P = 1.62 \times 10^{-12}$). We found that, in mixed cultures, when grown in an iron-limited environment, where cooperative pyoverdin production is required for growth, strain B increases in frequency when in competition with strain A (Fig. 4; $\omega_B = 1.96 \pm 0.45$, $T = 4.20$, $P = 0.008$), but decreases in frequency when grown in competition with strain C (Fig. 4; $\omega_B = 0.50 \pm 0.16$, $T = -6.32$, $P = 0.001$).

Discussion

We have demonstrated that whether or not a strain, with reduced expression of a potentially cooperative

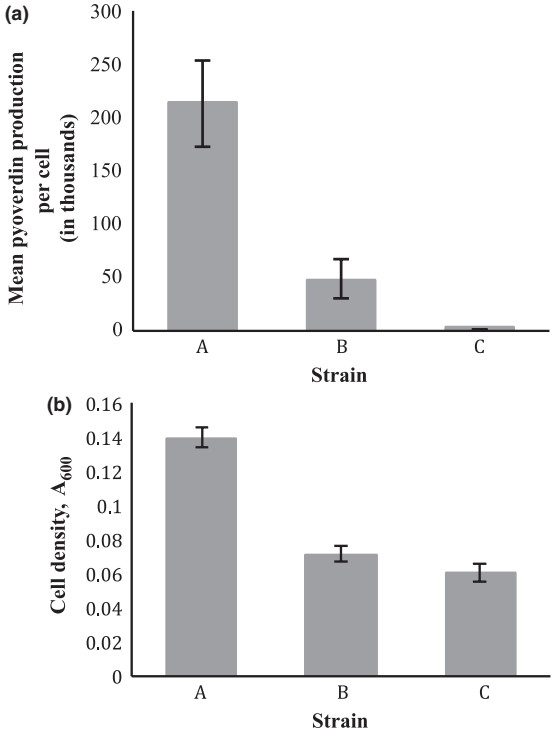

Fig. 3 (a) Mean pyoverdin production per cell for each strain, and (b) monoculture cell density under iron-limited conditions. In iron-limited CAA medium where pyoverdin is required for growth: (a) wild-type strain A produces significantly more pyoverdin ($ppc_A = 2.13 \times 10^5 \pm 2.07 \times 10^4$) than mutant strain B, which produces significantly more ($ppc_B = 4.87 \times 10^4 \pm 9.47 \times 10$) than mutant strain C ($ppc_C = 3.73 \times 10^2 \pm 75.51$); (b) pyoverdin production is correlated with cell density (A_{600}), where strain A grows to a significantly higher density than strain B, and strain B grows significantly better than strain C. The error bars indicate 95% confidence intervals around the means of six independent replicates.

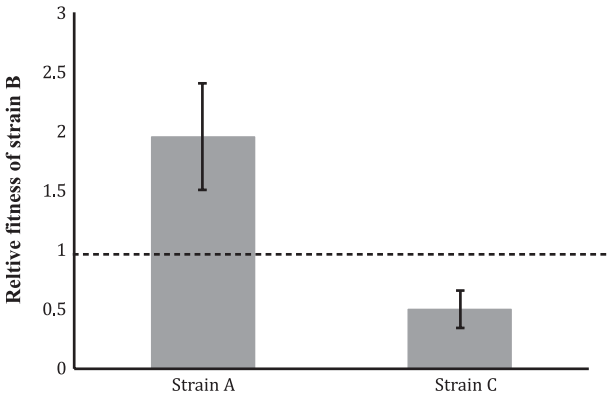

Fig. 4 Relative fitness of strain B in competition with strain A and with strain C under iron-limited conditions. In mixed cultures of iron-limited CAA media, strain B increases in frequency when in competition with strain A, but strain B decreases in frequency when competing with strain C. The error bars indicate 95% confidence intervals around the means of six independent competition replicates.

trait, acts as a cheat depends upon the abiotic and biotic environment. Cheats can increase in frequency at the cost of another strain by exploiting cooperation (Ghoul *et al.*, 2013). Using the QS system, we found that a *lasR* mutant, which does not respond to QS signal, acts as a cheat to PAO1's cooperative production of exofactors in a QS-dependent environment, but not in an environment where QS is not required for growth (Figs 1 and 2). Examining pyoverdin iron-scavenging molecules, we found that whether a strain acted as a cheat depends upon its pyoverdin production relative to the strain it is interacting with, and not its absolute level of pyoverdin production (Figs 3 and 4).

The QS experiment showed that whether or not a strain that produces less of some exofactor is a cheat depends upon the abiotic environment. In both the CAA and BSA conditions, the *lasR* mutant (putative cheat) increased in frequency in mixed cultures with the PAO1 wild type (putative cooperator; Fig. 1.) Although these mixed culture results are consistent with the mutant being a cheat, they could also be explained by some nonsocial fitness advantage to the *lasR* mutant, such as avoiding the cost of producing unneeded exofactors (i.e. the mutants are better adapted to the medium). The possibility for this alternative explanation is why we also needed to compare the growth of the strains in monocultures. In monocultures, PAO1 grew better in BSA, but the *lasR* mutant grew better in CAA (Fig. 2). These results suggest that QS provides a benefit in BSA, but not in CAA. Taken together, the monoculture and mixed culture results suggest that in BSA conditions, QS is a cooperative behaviour (PAO1 does better in monoculture) that can be exploited by *lasR* cheats (*lasR* mutants do better in mixed culture). In contrast, in a CAA environment, QS is a costly trait that provides no benefit at the population level and is not cooperative (*lasR* mutants do better in mixed culture and monocultures).

These results illustrate that the *lasR* mutant increases in frequency in the mixed cultures for different reasons – because it is a cheat in conditions requiring QS (BSA), and because it is avoiding the cost of producing not needed exofactors in conditions where QS is not required for survival (CAA). The latter is not cheating, highlighting that it is essential to both carry out monoculture controls (Fig. 2) and test for cheats in an appropriate (natural) environment (West *et al.*, 2012; Ghoul *et al.*, 2013; Kümmerli & Ross-Gillespie, 2013). Although determining the appropriate environment can be hard with bacteria, we would only expect bacteria to produce exofactors that aid growth – natural selection would quickly weed out anything not needed (Fisher, 1930; Morris *et al.*, 2012). Consequently, the first step should be to test for cheating in an environment where the potentially cheatable (cooperative) trait provides a benefit at the population level (Kümmerli & Ross-Gillespie, 2013), such as the BSA, QS-dependent environment.

The pyoverdin experiment showed that whether or not a strain that produces a certain amount of some exofactor is a cheat depends upon the biotic or social environment. Taking strain B as our focal strain, we found that strain B acted as a cheat when in competition with Strain A, which produced more siderophores (Figs 3 and 4). In contrast, we found that strain B was cheated by strain C, which produced less siderophores (Figs 3 and 4). The point here is that cheating is relative to the individuals being interacted with. What matters is how much exofactor you produce relative to the cells that you interact with, and not the absolute level produced (Ghoul *et al.*, 2013).

How do our results help us understand the possible contradictions in the existing literature? Zhang & Rainey (2013) concluded that siderophore production was not a cheatable cooperative trait, but is 'unnecessary and maladaptive' because they carried out their experiments in an environment where siderophore production was not needed (Kümmerli & Ross-Gillespie, 2013). More specifically, their results are analogous to our QS experiments in CAA. The general point here is that examining the social costs and benefits of a trait will give misleading results if experiments are carried out in environments where the trait has no function. MacLean *et al.* (2010) found that a mixed culture of cells that produce invertase, and cells that did not, was able to grow to higher population densities than a culture of cells that just produce invertase. This occurs because the producers keep producing invertase, even when all the sucrose has been used up and hence when invertase is not needed (MacLean *et al.*, 2010). At this time, invertase production is not cooperative anymore, and so the nonproducers increase group productivity not because they are cheating *per se*, but because they are not producing a

molecule when it is not needed. This is also analogous to our QS experiments in CAA.

Our results clarify some of the necessary steps to take when designing microbial experiments to study the evolution of cooperation, cheating and the persistence of these behaviours with changing biotic and abiotic conditions. It is useful here to distinguish between two broad types of microbial study. If the aim is to use a microbial system to test theoretical predictions, it is necessary to experimentally set up the costs and benefits for cooperation to occur in that environment, such that cheats can exploit that cooperation (Griffin *et al.*, 2004; Kümmerli *et al.*, 2009a; Kümmerli & Ross-Gillespie, 2013). In contrast, if the aim is to determine the nature of selection on a trait under natural environments, where the social and abiotic conditions are expected to be much more variable than highly controlled experimental systems, it should not be assumed that putative cheats and cooperators are always social behaviours (Rumbaugh *et al.*, 2009; West *et al.*, 2012). In this case, it is essential to first determine the social costs and benefits associated with putative cooperators and cheats, so that the occurrence of cooperation and cheating can be confirmed, before making inferences about their evolution.

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Supporting information

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

Figure S1 PAO1 expression (RLU/A_{600}) of lasI and lasB genes per cell (a-b) and strain growth curves in (c-d) in CAA and BSA media.

Data deposited at Dryad: doi:10.5061/dryad.3v0n3

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