doi: 10.1111/j.1420-9101.2010.01939.x

Fitness correlates with the extent of cheating in a bacterium

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Keywords:

cheating;
cooperation;
Pseudomonas aeruginosa;
public goods;
pyoverdin;
siderophores.

Abstract

There is growing awareness of the importance of cooperative behaviours in microbial communities. Empirical support for this insight comes from experiments using mutant strains, termed 'cheats', which exploit the cooperative behaviour of wild-type strains. However, little detailed work has gone into characterising the competitive dynamics of cooperative and cheating strains. We test three specific predictions about the fitness consequences of cheating to different extents by examining the production of the iron-scavenging siderophore molecule, pyoverdin, in the bacterium *Pseudomonas aeruginosa*. We create a collection of mutants that differ in the amount of pyoverdin that they produce (from 1% to 96% of the production of paired wild types) and demonstrate that these production levels correlate with both gene activity and the ability to bind iron. Across these mutants, we found that (1) when grown in a mixed culture with a cooperative wild-type strain, the relative fitness of a mutant is negatively correlated with the amount of pyoverdin that it produces; (2) the absolute and relative fitness of the wild-type strain in the mixed culture is positively correlated with the amount of pyoverdin that the mutant produces; and (3) when grown in a monoculture, the absolute fitness of the mutant is positively correlated with the amount of pyoverdin that it produces. Overall, we demonstrate that cooperative pyoverdin production is exploitable and illustrate how variation in a social behaviour determines fitness differently, depending on the social environment.

Introduction

Explaining cooperation is fundamental to understand the major evolutionary transitions from individually replicating organisms to complex, structured animal societies (Leigh, 1991; Maynard Smith & Szathmáry, 1995). A behaviour is cooperative if it provides a benefit to another individual as well as to the performer and has evolved, at least partially, because of this benefit to another (West *et al.*, 2007b). Recently, there has been much interest in the primary role cooperative behaviours play in the lives of microorganisms (Crespi, 2001; Velicer, 2003; West *et al.*, 2006; Foster *et al.*, 2007; West *et al.*, 2007a). Individual microbial cells routinely communicate and cooperate to perform a range of activities, such as biofilm formation, chemical defence, spore dispersal and foraging for iron using siderophores.

In microorganisms, the social nature of a trait is usually investigated by comparing the performance of cooperative strains to mutant strains that do not perform, or invest less in, that trait. If these mutant strains increase in relative fitness by exploiting cooperative strains, they are termed 'cheats'. Cheats increase fitness by avoiding or incurring less of the costs of cooperation, while continuing to benefit from the cooperative behaviour of others (Strassmann *et al.*, 2000; Velicer *et al.*, 2000; Foster

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et al., 2004; Greig & Travisano, 2004; Griffin *et al.*, 2004; Fiegna & Velicer, 2005; Fiegna *et al.*, 2006; MacLean & Gudelj, 2006; Diggle *et al.*, 2007a; Sandoz *et al.*, 2007; Rumbaugh *et al.*, 2009). The extent to which traits are social is commonly tested by competing mutant strains against wild-type strains and examining both the extent to which the mutants are able to exploit the wild type and the group level consequences. However, experiments performed to date have utilized mutant phenotypes that either carry large fitness effects, which can lead to complications such as frequency-dependence (Ross-Gillespie *et al.*, 2007), or exhibit pleiotropic effects on other traits (Foster *et al.*, 2004; Diggle *et al.*, 2007c). In addition, many studies focus on genetic knockout mutants that do not perform the putatively cooperative trait and so have not tested the fitness consequences of cheating to a greater or lesser extent (Foster *et al.*, 2004; Greig & Travisano, 2004; Fiegna *et al.*, 2006; MacLean & Gudelj, 2006; Diggle *et al.*, 2007a; Ross-Gillespie *et al.*, 2007, 2009; Rumbaugh *et al.*, 2009).

Here, we make and test specific predictions of the consequences of cheating behaviour by using a collection of mutants that differ in the extent to which they express a potentially social trait. The trait that we examine is the production of pyoverdin by the bacterium *Pseudomonas aeruginosa*. Pyoverdin is a siderophore – an iron-scavenging molecule that is released extracellularly in response to iron-limited conditions (Visca *et al.*, 2007). Iron is an essential nutrient but often a major limiting factor for bacterial growth in both environmental and parasitic strains as its commonest form, Fe(III), is insoluble under normal conditions, and host organisms actively withhold iron during bacterial infections (Ankenbauer *et al.*, 1985; Schwyn & Nielands, 1987; Ratledge & Dover, 2000; Griffin *et al.*, 2004). The production of pyoverdin is metabolically costly to the individual; and as these molecules can be utilized by other cells following release, they represent a potential cooperative public good (West & Buckling, 2003). We and others have previously demonstrated that mutant *P. aeruginosa* strains that do not produce siderophores are able to exploit the iron-chelator production of other cells and that the presence of such strains reduces population growth (Griffin *et al.*, 2004; Harrison & Buckling, 2005; Harrison *et al.*, 2006; Ross-Gillespie *et al.*, 2007; Harrison *et al.*, 2008; Kümmerli *et al.*, 2009a–c; Ross-Gillespie *et al.*, 2009). The knockout strains and PAO6609 mutant (Hohnadel *et al.*, 1986) used in these studies did not provide sufficient variation in pyoverdin expression for our purposes. We therefore generated and analysed a collection of novel, spontaneously evolving pyoverdin mutants to investigate the effects of varying investment in cheating behaviour.

In the first part of our article, we describe the isolation of pyoverdin-deficient mutants, and characterize them at both the phenotypic and genotypic level. We generated artificial selection lines from 11 strains of *P. aeruginosa*, representing different genetic backgrounds (see Materials and methods section). We measured the pyoverdin-producing ability of mutants isolated from these lines in three ways: (1) measurement of expression of the pyoverdin biosynthesis gene *pvdE* and the sigma factor *pvdS*, which is involved in the regulation of pyoverdin biosynthesis genes; (2) estimation of pyoverdin production per cell by taking optical density and fluorescence readings of cultures; and (3) measurement of the depletion of iron from culture media. This enabled us to test for correlations between genetic and phenotypic measurements of pyoverdin production.

In the second part of our article, we describe the use of mutant strains to test three more specific predictions of the consequences of cheating. First, if a mutant is a cheat, its relative fitness (measured by the increase or decrease in proportion of cheats over a set time in a competitive mixed culture) should be negatively correlated with the quantity of public good that it produces when competing in a mixed culture with a wild-type, cooperative strain (Brown, 1999; West & Buckling, 2003). Mutants that produce fewer public goods will avoid greater costs of production, while retaining the ability to exploit pyoverdin production of cooperators. Second, when grown in a mixed culture with a mutant, the absolute fitness of a cooperative strain should be positively correlated with the quantity of public good that the mutant produces. If mutants produce any public good, then this will provide a benefit to the wild-type individuals as well. Third, when grown in monoculture, the absolute fitness of the mutant (i.e. carrying capacity in the stationary phase) should be positively correlated with the quantity of public good that it produces. This prediction arises because the public good provides a benefit, and emphasizes that producing less public good can be either beneficial (prediction 1) or costly (prediction 3), depending upon the social environment.

Materials and methods

Creation of putative pyoverdin mutant–wild type pairs

We isolated 26 spontaneous mutants of putative reduced pyoverdin production from selective lines initiated with 11 parental strains (Table 1). We grew the 11 parent strains in monoculture for up to 25 days in 30-mL glass universal vials containing 6 mL volumes of minimal medium: CAA: 5 g casamino acids, 1.18 g $K_2HPO_4.3H_2O$, 0.25 g $MgSO_4.7H_2O$, per litre. Cultures were incubated at 37 °C statically or in an orbital shaker at 1.34 *g*. Some monocultures were also made iron-limiting [100 μg mL^{-1} of the iron-chelator human apo-transferrin (Sigma); and 20 mM sodium bicarbonate (Schwyn & Nielands, 1987; Meyer *et al.*, 1996)] to further encourage the emergence of siderophore mutants. At 24-h intervals, 60 μL of each culture was transferred into 6 mL fresh medium. Every 3–4 days, we spread 20 μL of a 10^{-6} dilution of each

Table 1 Description of ancestral *Pseudomonas aeruginosa* strains.

Strain N°	Strain name	Strain description	Laboratory source	Reference
1	PAO1 (ATC 15692)	Laboratory isolate	Europe	(Ghysels *et al.*, 2004; Rumbaugh *et al.*, 2009)
2	PAO6049	Methionine auxotroph derived from PAO1 by transposon-mutagenesis	Zürich, Switzerland	(Rella *et al.*, 1985)
3	Holloway isolate I	Diverged PAO1 laboratory isolate	Holloway collection, Nottingham, UK	(Holloway *et al.*, 1979)
4	Holloway isolate II	Diverged PAO1 laboratory isolate	Holloway collection, USA	(Holloway *et al.*, 1979)
5	Holloway isolate III	Diverged PAO1 laboratory isolate	Holloway collection, Italy	(Holloway *et al.*, 1979)
6	UCBPP-PA14	Human clinical isolate	USA	(Rahme *et al.*, 1995)
7	ATCC 013	–	American Type Culture Collection, Manassas, VA, USA	
8	1–60	Cystic fibrosis (CF) isolate	Seattle, WA, USA	
9	2–164	CF isolate	Seattle, WA, USA	
10	206–12	CF isolate	Seattle, WA, USA	
11	MSH	Environmental Isolate	Mount St. Helens, WA, USA	

culture onto King's B (KB)-agar plates (1 L KB medium: 20 g proteose peptone N°3, 10 mL glycerol, 1.5 g $K_2HPO_4.3H_2O$, and 1.5 g $MgSO_4.7H_2O$ per litre; with 12 g agar) and incubated these overnight at 37 °C. As pyoverdin is a bright green pigment, we examined mature colonies by eye and isolated those that appeared paler than colonies of the ancestral wild type. A maximum of three colonies were isolated per line, each potential pyoverdin mutant paired with a colony of wild-type phenotype isolated from the same plate, i.e. of the same line and cultured in identical conditions (see Table S1).

We made stock cultures of each putative mutant clone from overnight cultures grown at 37 °C in 30-mL glass universal vials containing 6 mL volumes of standard KB medium and frozen at −80 °C in a 3 : 2 mix of KB glycerol: 50/50 KB medium/glycerol. Our isolates were labelled using a three-character notation. For example: $1a^+$ was isolated from ancestral strain 1 (see Table 1) and has a wild-type pyoverdin phenotype (indicated by $^+$); $1a^-$ has a mutant pyoverdin phenotype and was isolated from the same selective line, at the same timepoint, as $1a^+$ (see Table S1).

Of the 26 putative pyoverdin mutants we generated by this method, 19 proved to be true mutants following pyoverdin production assay. This indicates that characterisation of pyoverdin phenotype by eye is a reasonable method for detecting mutants, but requires additional measurements for full accuracy. The final mutant–wild type pairs are listed in Table 1. In two instances (in lines 5 and 6), two phenotypically distinct putative siderophore mutants were isolated from a single agar plate on the same day ($5a^-$ & $5b^-$, and $6a^-$ & $6b^-$) and so were paired with a single wild-type colony from that plate ($5a^+$ and $6a^+$, respectively). Therefore, the 19 pyoverdin mutants isolated across the collection were only paired with 17 wild-type strains, making, on the inclusion of the previously analysed PAO6609 and its paired wild type, PAO1 (Griffin *et al.*, 2004; Ross-Gillespie *et al.*, 2007; Kümmerli *et al.*, 2009a), a collection of 20 mutant–wild type pairs.

Engineered pyoverdin mutants

P. aeruginosa secretes two siderophores, pyoverdin and pyochelin. We concentrated on the former, as its iron-binding capacity is five-fold that of pyochelin (Ankenbauer *et al.*, 1985; Budzikiewicz, 2001). Although our study did not measure the production of the secondary siderophore pyochelin, we included three siderophore-deficient single-gene knockout strains in all experiments to ensure that, if present, pyochelin was not interfering with any pyoverdin readings. These strains were pyoverdin- and/or pyochelin-negative unmarked deletion mutants engineered from PAO1 by knocking-out the genes for the pyoverdin synthetase *pvdD* and/or the pyochelin synthetases *pchE* and *pchF* (Ghysels *et al.*, 2004). These three strains were PAO1*ΔpvdD* (*pvd*-), PAO1*ΔpchEF* (*pch*-) and PAO1*ΔpvdD pchEF* (*pvd*-/*pch*-). The *pvd*- strain, as a well-established pyoverdin-deficient mutant, was included as a control in some analyses.

Construction of *pvdE* and *pvdS* reporter fusions

The genes *pvdE* and *pvdS* are involved in the production and release of pyoverdin. PvdS is an alternative sigma factor that, among other functions, directs gene expression for and during pyoverdin synthesis (Leoni *et al.*, 2000). It is also known to regulate the expression of *pvdE*, which plays an uncharacterized role in the biosynthesis of pyoverdin (Visca *et al.*, 2007).

To verify that our observations of pyoverdin production in the mutant–wild type pairs were supported at the genetic level, we conjugated *pvdE* lux-based reporter fusions into 11 of the 19 mutant–wild type pairs ($1b^+/1b^-$; $1c^+/1c^-$; $2a^+/2a^-$; $3a^+/3a^-$; $4a^+/4a^-$; $5a^+/5a^-$; $5a^+/5b^-$;

$7a^+/7a^-$; $9a^+/9a^-$; $10b^+/10b^-$; and $10c^+/10c^-$) and *pvdS* lux-based reporter fusions into nine of the pairs ($1b^+/1b^-$; $1c^+/1c^-$; $2a^+/2a^-$; $3a^+/3a^-$; $4a^+/4a^-$; $7a^+/7a^-$; $9a^+/9a^-$; $10b^+/10b^-$; and $10c^+/10c^-$), as previously described (Diggle *et al.*, 2007b).

Measuring pyoverdin production

We prepared experimental cultures from freezer stock in 200 μL volumes of KB medium in a 96-well microtitre plate incubated at 37 °C, 1.34 *g* for 24 h. The 36 outer wells of these plates, being more susceptible to evaporation, were not inoculated with culture. 1 μL of each culture was then transferred to a 200 μL volume of iron-limited CAA in a 96-well microtitre plate. Plates were incubated statically at 37 °C. The apo-transferrin in the CAA binds iron, triggering siderophore production in iron-starved bacteria. Siderophores compete with the apo-transferrin for the iron and facilitate siderophore-mediated iron uptake into cells.

After 24 h, we retrieved cultures (which had reached carrying capacity in stationary phase) and assayed for cell density at an absorbance of 600 nm (A_{600}) (Ankenbauer *et al.*, 1985; Schwyn & Nielands, 1987) and for pyoverdin production, measured in relative fluorescence units (RFU), at an excitation wavelength of 400 nm and an emission wavelength of 460 nm (Ankenbauer *et al.*, 1985; Cox & Adams, 1985; Prince *et al.*, 1993) using a fluorimeter (SpectraMax M2; Molecular Devices, Wokingham, UK). We replicated each assay seven times per culture and calculated the mean pyoverdin production per cell by RFU/A_{600} (Kümmerli *et al.*, 2009c).

Time and cell-density dependent measurement of bioluminescence

To monitor expression of *pvdE* and *pvdS*, we diluted 6 mL 24-h KB cultures of the 20 lux-tagged strains (1 : 100 in 200 μL volumes of fresh, iron-limited medium) and cultured these statically in 96-well microtitre plates at 37 °C. We determined bioluminescence as a function of cell density by using a combined, automated luminometer-spectrometer (Genios Pro, Tecan UK Ltd, 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 10 h.

Measuring siderophore iron-binding capacity

The iron-binding potency of the pyoverdin produced by each strain was determined as a percentage of siderophore units (SU) using the functional chrome azurol S (CAS) liquid assay (Schwyn & Nielands, 1987; Payne, 1994). We cultured the 20 mutant–wild type pairs and an additional three replicates of PAO1 from freezer stock in 2 mL volumes of KB medium in 24-well microtitre plates and incubated these in an orbital shaker (37 °C, 1.34 *g*).

After 24 h, we used 2 μL of each culture to inoculate 200 μL volumes of iron-limiting medium. Following 24-h incubation at 37 °C, we measured the cell densities of the cultures, diluted each to 0.1 A_{600} with double-distilled (dd) H_2O and centrifuged 1 mL at 10,400 *g* for 6 min. We then removed 25 μL of supernatant from each culture and diluted these 1 : 4 with 75 μL ddH_2O in a 96-well microtitre plate.

We then added 100 μL of CAS assay solution and 2 μL of a shuttle solution of 0.2 M 5-sulfosalicylic acid (Payne, 1994) to each well and left the plate to stand in the dark for 30 m. We then measured the colour change-associated removal of iron from the CAS reagent at A_{630} using the Spectramax M2. The mean A_{630} of five wells of 100 μL ddH_2O with 100 μL CAS assay solution and 2 μL shuttle solution was taken as the reference (*r*). We calculated the iron-binding capacity of the supernatants (*s*) in SU as

$$\mathrm{SU} = \left(\frac{\mathrm{mean}A_r - A_s}{\mathrm{mean}A_r}\right) \times 100$$

We replicated each CAS assay four times and corrected all measurements: dividing readings by the mean SU value of the four PAO1 supernatants assayed per replicate.

Measuring relative mutant fitness

To determine the fitness of each pyoverdin mutant relative to that of its paired wild type, we grew mutant strains in competition with a paired wild type at a 1 : 10 ratio in 6 mL volumes of iron-limited medium. On KB-agar plates, 20 μL of 10^4–10^6 dilutions of these mixed cultures were plated at 0 h and again after 24-h incubation at 37 °C. This period covered the exponential growth phase and the start of stationary phase.

Following 18-h incubation of the plates at 37 °C, we recorded the number of mutant and wild-type colonies on each. Colonies were scored by phenotype as well as pigmentation. Counts were not performed after 18 h as siderophores begin to diffuse into the agar, where they can be taken up by neighbouring colonies and add to their pigmentation. We calculated the relative fitness (*v*) of each putative mutant by

$$v = \frac{x_2(1-x_1)}{x_1(1-x_2)}$$

where x_1 is the initial proportion of mutants in the sample population and x_2 is their final proportion (Otto & Day, 2007; Ross-Gillespie *et al.*, 2007, 2009). The value of *v* therefore signifies whether cheats increase in frequency ($v > 1$), decrease in frequency ($v < 1$) or remain at the same frequency ($v = 1$). We replicated each competition assay six times and took the mean fitness value to indicate how efficiently the mutant could exploit its corresponding wild-type strain when cultured in

competitive conditions, increasing or decreasing in frequency to the disadvantage or benefit of the wild type. We repeated all fitness analyses, logging the raw values of *v* before calculating the mean across the replicates, to test for any bias resulting from within-replicate variation in *v*. These analyses gave the same results and, no bias being found, are not shown.

Statistical analyses

We used paired *T*-tests to test for significant differences in bioluminescence, pyoverdin production and iron-binding capacity per cell between the pyoverdin mutants and the wild-type strains. We used standard general linear model methods implemented in MINITAB 15 (http://www.minitab.com), and when required, logarithmically transformed variables to normalize the residuals.

Results

Do the isolated mutants produce less pyoverdin than their paired wild type?

The 19 pyoverdin mutants, PAO6609 and the *pvd*- and *pvd-/pch*-strains produced significantly less pyoverdin than their paired wild types, as measured by RFU/A_{600} (Fig. 1, paired *T*-test: $T = 7.72$, $P < 0.0001$). The mutants also exhibited significantly lower levels of *pvdE* and *pvdS* gene expression and iron-binding capacity (SU) than the wild type (paired *T*-test: $T = 4.97$, $P < 0.0001$; $T = 2.87$, $P = 0.019$; and $T = 6.17$, $P < 0.0001$, respectively).

Is there a correlation between gene activity and phenotype?

We tested whether our quantification of pyoverdin production correlated with gene expression and the ability to collate iron, across the spontaneous mutants. In the mutant lines with lux-based reporter insertions, the relative pyoverdin production per cell was positively correlated with both relative mutant *pvdE* gene expression (Fig. 2a, adjusted $r^2 = 0.39$, $F_{(1,9)} = 7.26$, $P = 0.025$) and relative mutant *pvdS* gene expression (Fig. 2b, adjusted $r^2 = 0.56$, $F_{(1,7)} = 11.05$, $P = 0.013$). Our measurements of pyoverdin production in mutants estimated from fluorescence and absorbance readings therefore corresponded with the expression of both a synthesising gene, *pvdE*, and a sigma factor that regulates pyoverdin biosynthesis, *pvdS*. Furthermore, the pyoverdin production per cell of the mutants was positively correlated with iron-binding capacity (SU) (Fig. 2c, adjusted $r^2 = 0.42$, $F_{(1,17)} = 14.03$, $P = 0.002$). Thus, the amount of pyoverdin released by each mutant was a significant indicator of the capacity of that mutant to harvest iron from its environment.

Is mutant growth in monoculture positively correlated with pyoverdin production?

We first tested whether pyoverdin production is beneficial to mutant growth in an iron-limited environment. As predicted, in iron-limited media, the carrying capacity of mutant strains at stationary phase was positively correlated with their pyoverdin production. Mutants that produced less pyoverdin per cell grew to lower cell densities than monocultures of mutants expressing higher levels of pyoverdin production (Fig. 3, all strains: adjusted $r^2 = 0.45$, $F_{(1,19)} = 17.24$, $P = 0.001$ and spontaneous mutants only: adjusted $r^2 = 0.43$, $F_{(1,17)} = 14.7$, $P = 0.001$). Wild-type strains exhibited an identical trend (data not shown, all strains: adjusted $r^2 = 0.46$, $F_{(1,17)} = 16.31$, $P = 0.001$ and paired with spontaneous mutants only: adjusted $r^2 = 0.48$, $F_{(1,15)} = 16.0$, $P = 0.001$).

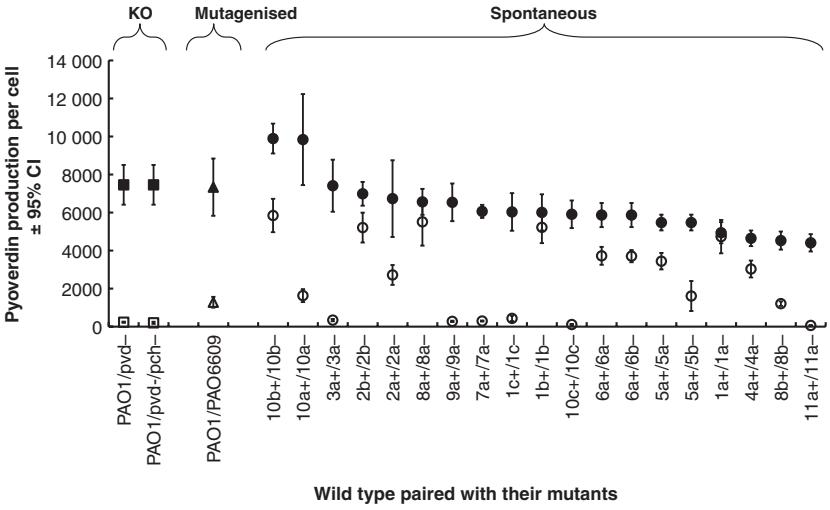

Fig. 1 The range of reduction in pyoverdin production per cell (RFU/A_{600}) in spontaneous mutants (circles), the mutagenized PAO6609 mutant (triangle) and two genetic knockout (KO) strains (squares) compared to wild type (filled symbols). Values are means ± 95% confidence intervals (CI) across seven replicates.

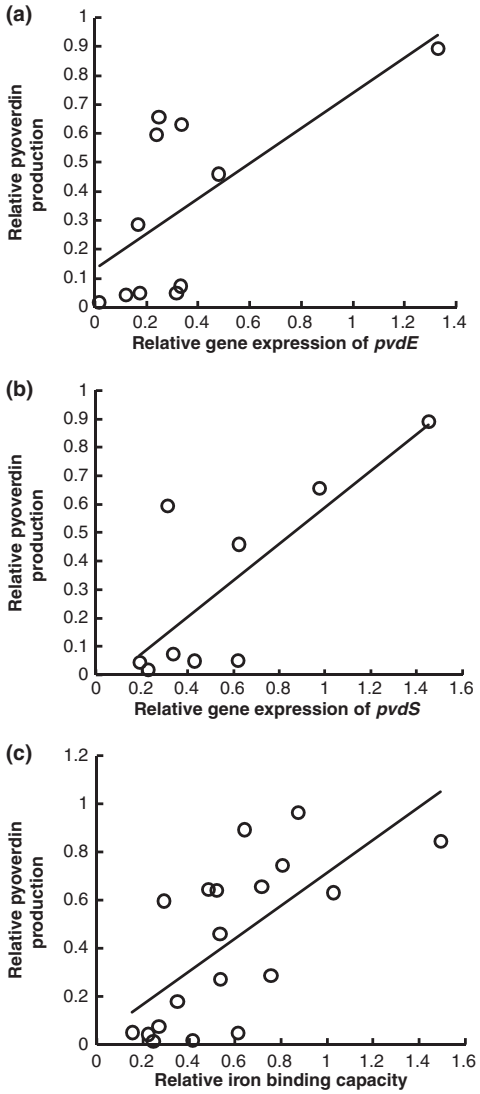

Fig. 2 Pyoverdin gene synthesis activity correlates with fluorescence and iron-binding activity. After 24-h growth in iron-limited conditions, spontaneous mutant pyoverdin production relative to that of the paired wild types was positively correlated with the following: (a) relative mutant *pvdE* gene expression at 10 h; (b) relative mutant *pvdS* gene expression at 10 h; and (c) relative mutant iron-binding capacity at 24 h. Values are means across seven replicates for relative pyoverdin production, one replicate for relative bioluminescence, and across six replicates for relative iron-binding capacity.

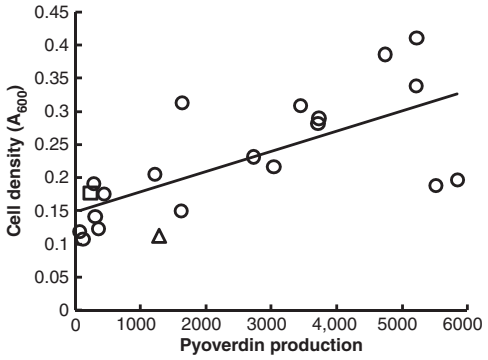

Fig. 3 Pyoverdin production is beneficial in iron-limited conditions. The cell density of mutants was positively correlated with the amount of pyoverdin produced per cell (RFU/A_{600}) when grown in monoculture for 24 h. Symbols: spontaneous mutants (circles); mutagenized PAO6609 (triangle); and genetic knockout *pvd-* (square). Values are means across six replicates for cell density and across seven replicates for pyoverdin production.

Does pyoverdin production by mutants provide a benefit to wild-type cells in mixed culture?

We examined how the growth of wild types was influenced by the presence of mutants. Wild-type strains grew less well in mixed culture with their paired mutant than they did in monoculture (paired *T*-test, all strains: $T = 3.06$, $P = 0.006$; spontaneous mutants only, $T = 2.68$, $P = 0.015$) suggesting a cost of exploitation by mutants. Both the wild-type cell density and relative fitness were positively correlated with the relative pyoverdin production of their paired mutant (Fig. 4, cell density, all lines: adjusted $r^2 = 0.61$, $F_{(1,19)} = 32.39$, $P < 0.0001$; cell density, paired with spontaneous mutants only: adjusted $r^2 = 0.58$, $F_{(1,17)} = 25.33$, $P < 0.0001$; relative fitness, all lines: adjusted $r^2 = 0.39$, $F_{(1,19)} = 13.55$, $P = 0.002$; and relative fitness, paired with spontaneous mutants only: adjusted $r^2 = 0.38$, $F_{(1,17)} = 11.83$, $P = 0.003$). This shows that pyoverdin produced by the mutants can be utilized by the wild-type cells and demonstrates that although mutants have relatively reduced investment in pyoverdin synthesis, they may still provide some benefit to neighbours.

In multiple regression, unlike in monoculture, the quantity of pyoverdin produced by the wild type did not significantly influence wild-type cell density at carrying capacity in mixture (all lines: adjusted $F_{(1,19)} = 3.2$, $P = 0.09$ and spontaneous mutants only: $F_{(1,17)} = 2.16$, $P = 0.16$).

Is mutant fitness in mixture negatively correlated with relative mutant pyoverdin production?

To determine the cost of pyoverdin production, we examined how the fitness of mutants correlates with relative pyoverdin production when grown in competition with paired wild-type strains in iron-limited conditions. In mixed cultures, the fitness of mutants, relative to the wild type, was negatively correlated with the mutants' relative pyoverdin production (Fig. 5, all strains: adjusted $r^2 = 0.49$, $F_{(1,19)} = 20.21$, $P < 0.0001$ and spontaneous mutants only: adjusted $r^2 = 0.6$, $F_{(1,17)} = 27.63$, $P < 0.0001$. Relative pyoverdin

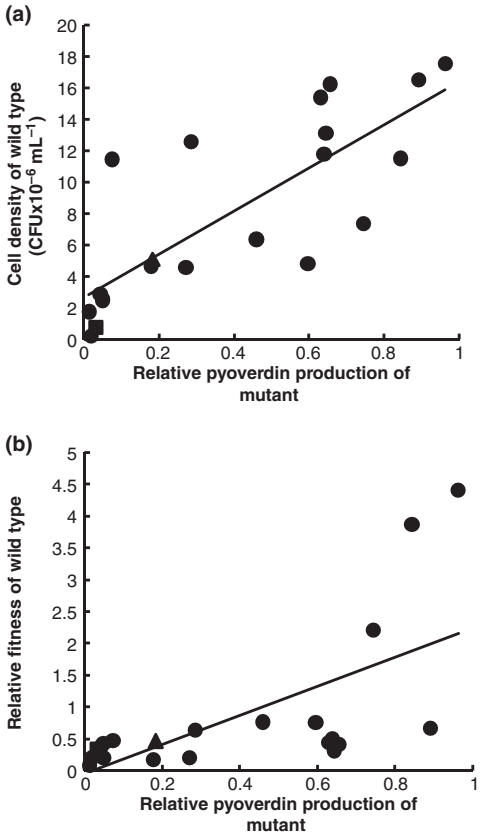

Fig. 4 Pyoverdin production determines fitness of cooperators and cheats in iron-limited conditions. Cell density (a) and relative fitness (b) of the wild type were positively correlated with the relative pyoverdin production of the paired mutants when grown in iron-limited conditions for 24 h in a competitive 10 : 1 wild type: mutant mix. Symbols: paired wild type of spontaneous mutants (circles); PAO1 paired with mutagenized PAO6609 (triangle); and PAO1 paired with *pvd*- (square). Values are means across six replicates for cell density and fitness and across seven replicates for relative pyoverdin production.

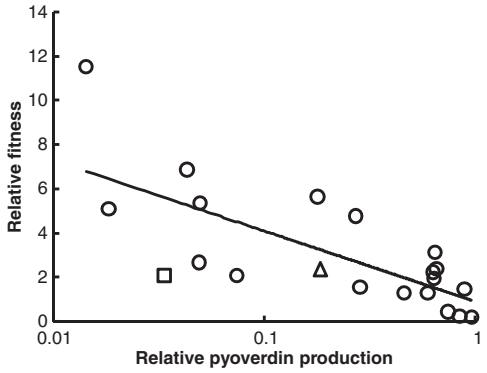

Fig. 5 The fitness consequences of reduced pyoverdin production. When a mutant was grown in a mixture with its wild-type strain in iron-limited conditions, the relative fitness of the mutant was negatively correlated with the relative amount of pyoverdin that it produced. Symbols: spontaneous mutants (circles); mutagenized PAO6609 (triangle); and *pvd*- (square). Values are means across seven replicates for relative pyoverdin production and across six replicates for relative fitness.

production logarithmically transformed in both datasets), demonstrating the benefit the mutants accrue by reducing investment in, and therefore the cost of, pyoverdin synthesis.

Discussion

Our results demonstrate the social nature of pyoverdin production *in vitro* and support all three of our theoretical predictions on cheating behaviour. Specifically (1) in mixed culture with a cooperative wild-type strain, mutant relative fitness is negatively correlated with mutant pyoverdin production; (2) in mixed culture, the absolute and relative fitness of the wild-type strain is positively correlated with mutant pyoverdin production; and (3) in monoculture, absolute mutant fitness is positively correlated with mutant pyoverdin production. In addition, our results show strong correlations between our three measurements of pyoverdin production: pyoverdin biosynthesis gene expression, secreted pyoverdin fluorescence and pyoverdin iron-binding efficacy (Fig. 2), indicating that any single method gives an accurate representation of pyoverdin activity in *P. aeruginosa*. Furthermore, as the spontaneous pyoverdin mutants we selected from a diverse range of parental strains all produced significantly lower levels of pyoverdin than their ancestral wild type (Fig. 1), it is probable that the spontaneous emergence of cheats is a common challenge to pyoverdin production in *P. aeruginosa* populations.

In support of our first prediction, we confirmed that the relative fitness of mutants is negatively correlated with the relative amount of pyoverdin they produce when grown in mixed culture with wild type (Fig. 5). Mutants avoid the metabolic cost of production, but retain the ability to benefit from pyoverdin produced by a cooperator. The greater the reduction in pyoverdin production by the cheat, the greater its fitness advantage when grown with a cooperator. Furthermore, previous work has shown that cooperators compensate for the deficit in soluble iron created by cheat consumption by upregulating pyoverdin production (Kümmerli *et al.*, 2009c). In support of our second prediction, we observed that absolute growth (cell density) and relative fitness of cooperative wild types were positively correlated with relative pyoverdin production of mutants in mixed culture (Fig. 4), confirming that pyoverdin produced by the mutants can be utilized by the wild type. We did not see, or expect to see, a corresponding positive relationship

between wild-type fitness and pyoverdin production by the wild type in mixture. This is because the benefit to wild type will be obscured by increased relative fitness of a cheat paired with a more cooperative wild type: the more pyoverdin the wild type produced, the fitter the cheat.

In support of our third prediction, we found that mutants reducing investment in pyoverdin production relative to the wild type to a greater degree were less successful when grown in isolation in iron-limiting conditions, achieving lower cell densities than the mutants expressing higher levels of relative pyoverdin production (Fig. 3). The mutants therefore increased fitness by cheating in mixture but suffered reduced fitness when grown in the absence of cooperators, demonstrating that reducing investment in pyoverdin production can be advantageous or debilitating, depending on the social environment. Overall, we demonstrate the fitness consequences of varying investment in cooperative behaviours, the vulnerability of cooperative behaviours to exploitation and how the fitness outcomes of reduced pyoverdin production lie in opposing directions dependant upon whether cheats exist in isolation or mixed culture.

Our results compare with recent investigations into cheating behaviour in other microbes: slime moulds and myxococci. Mutant *Dictyostelium discoideum* slime mould strains have, for example, been observed to produce more spores when forming fruiting bodies in mixed populations than they do in clonal populations – exploiting their neighbours to bias spore dispersal in their favour (Strassmann *et al.*, 2000; Khare *et al.*, 2009). In similar studies, *Myxococcus xanthus* mutants reduced investment in fruiting body formation while increasing relative spore production (Velicer *et al.*, 2000; Fiegna & Velicer, 2005; Fiegna *et al.*, 2006). However, unlike pyoverdin cheats, several of the *M. xanthus* mutant strains did not suffer fitness consequences when grown in isolation and some appeared to be facultative rather than obligative cheaters (Fiegna & Velicer, 2005). Furthermore, in the *D. discoideum* studies, over-representation of some strains in spores of mixed fruiting bodies did not appear to be determined by competition over spore cell allocation alone (Fortunato *et al.*, 2003). This suggests that whereas the relative fitness of the *P. aeruginosa* mutants in our study is primarily determined by the production of a single public good (pyoverdin), the consequences of cheating over cooperative fruiting body formation in slime moulds and myxococci are more complex (Santorelli *et al.*, 2008; Khare *et al.*, 2009).

In addition to demonstrating the social fitness consequences of variation in cheating within cooperative systems, our results provide a potential explanation for a well-documented clinical pattern. *P. aeruginosa* is routinely isolated from the immunocompromised lungs of cystic fibrosis (CF) patients, where it accelerates morbidity and mortality (Govan & Deretic, 1996; Hutchison & Govan, 1999; Budzikiewicz, 2001; De Vos *et al.*, 2001). It has been repeatedly observed that *P. aeruginosa* isolates that colonise the CF lung lose numerous cellular functions over time, including social behaviours such as siderophore production (De Vos *et al.*, 2001; Smith *et al.*, 2006; West *et al.*, 2006; Jelsbak *et al.*, 2007). One explanation is that such social traits are not necessary for survival in the CF lung and are selected against as the bacterial strain adapts to this environment, thereby increasing isolate fitness (De Vos *et al.*, 2001; Lee *et al.*, 2005; Smith *et al.*, 2006; Jelsbak *et al.*, 2007). However, an alternative possibility is that social cheats emerge, exploit and outcompete wild-type strains, resulting in a reduction in cooperative activity and therefore social trait expression (Griffin *et al.*, 2004; West *et al.*, 2006; Harrison, 2007; West *et al.*, 2007a; Brown *et al.*, 2009). In the latter case, studies of social behaviour in *P. aeruginosa* could inform antipseudomonal treatments for CF patients: many antibiotics are designed to target actively growing bacterial cells and so may be less effective in treating social mutant strains that grow slowly (if at all) in environments lacking locally produced public goods (West & Buckling, 2003; Andre & Godelle, 2005; Harrison *et al.*, 2006). Key tasks for the future are to repeat our experiments *in vivo* as previous work has shown that social behaviours can have significant effect on virulence (Harrison *et al.*, 2006; Rumbaugh *et al.*, 2009) and to conduct analyses of CF lung isolates that have reduced social trait expression to determine whether this loss has resulted in an increase or decrease in fitness, i.e. whether *P. aeruginosa* CF isolates causing chronic illness are outcompeted or out-compete clonal isolates harvested at a later stage of the same chronic infection.

Acknowledgments

We would like to thank Angus Buckling, Pierre Cornelis, Gary Loake and Eric Smith for kindly providing *P. aeruginosa* strains; Alan Cockayne for help with the CAS assays; Andy Gardner and Rolf Kümmerli for useful discussion; and the BBSRC, Leverhulme Trust, and Royal Society for funding.

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Supporting information

Additional Supporting Information may be found in the online version of this article:
Table S1 Isolation conditions of the mutant–wild type pairs.

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Received 13 October 2009; revised 18 December 2009; accepted 28 December 2009