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Pyoverdin cheats fail to invade bacterial populations in stationary phase

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Abstract

Microbes engage in cooperative behaviours by producing and secreting public goods, the benefits of which are shared among cells, and are therefore susceptible to exploitation by nonproducing cheats. In nature, bacteria are not typically colonizing sterile, rich environments in contrast to laboratory experiments, which involve inoculating sterile culture with few bacterial cells that then race to fill the available niche. Here, we study the potential implications of this difference, using the production of pyoverdin, an iron-scavenging siderophore that acts as a public good in the bacteria *Pseudomonas aeruginosa*. We show that (1) nonproducers are able to invade cultures of producers when added at the start of growth or during early exponential growth phase, but not during late exponential or stationary phase; (2) the producer strain does not produce pyoverdin in the late exponential and stationary phases and so is not paying the cost of cooperating during those phases. These results suggest that whether a nonproducing mutant can invade will depend upon when the mutation arises, as well as the population structure, and raise a potential difficulty with the use of antimicrobial treatment strategies that propose to exploit the invasive abilities of cheats.

Introduction

Bacterial growth relies on the costly secretion of a range of exoproducts such as nutrient-scavenging molecules, quorum-sensing (QS) signals, biofilm polymers and toxins (Crespi, 2001; Miller & Bassler, 2001; West *et al.*, 2006; Nadell *et al.*, 2009). Cells in a local population can benefit from their neighbour's exoproduct secretion, which can act as cooperative 'public goods' (Crespi, 2001; Velicer, 2003; Griffin *et al.*, 2004; West *et al.*, 2006). Consequently, populations of cells that produce costly exoproducts (cooperators) are potentially susceptible to exploitation by 'cheats' that benefit from the cooperative production of others without contributing to the cost (Ghoul *et al.*, 2014).

Controlled experimental studies *in vitro* and *in vivo* have shown that mutants that do not produce exoproducts can invade populations of exoproduct producers (Griffin *et al.*, 2004; Diggle *et al.*, 2007; Sandoz *et al.*, 2007; Kohler *et al.*, 2009; Rumbaugh *et al.*, 2009). Abundant evidence supports the existence of mutants that do not produce certain exoproducts in natural populations. For example, siderophore mutants with reduced iron-scavenging ability of *Pseudomonas aeruginosa* have been isolated from clinical and environmental populations (Smith *et al.*, 2006; D'argenio *et al.*, 2007; Bodilis *et al.*, 2009; Jiricny *et al.*, 2014; Andersen *et al.*, 2015) and of *Vibrio* species from the ocean (Cordero *et al.*, 2012); quorum-sensing mutant strains of *Vibrio cholera*, *P. aeruginosa* and *Staphylococcus aureus* have been isolated from epidemic populations and infections of the cystic fibrosis lung, urinary tract and blood (Schaber *et al.*, 2004; Joelsson *et al.*, 2006; Traber *et al.*, 2008; Huse *et al.*, 2010; Jiricny *et al.*, 2014). Despite the presence of putative cheats and, therefore, the risk of cheat invasion, cooperation appears to be the norm in natural settings (Hibbing *et al.*, 2010; Levin, 2014). Probably the most important mechanism for maintaining cooperation in natural bacterial

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populations is population structure leading to cooperation being directed towards relatives, and hence favoured by kin selection (West & Buckling, 2003; Griffin *et al.*, 2004; Julou *et al.*, 2013).

Another challenge is how competitor–cheat dynamics have been characterized in the laboratory. Bacterial cells in natural populations live in a dynamic state, fluctuating between cycles of growth and bacteriostasis (Llorens *et al.*, 2010). When nutrients are abundant, cells are multiplying rapidly, similar to experimental cultures in exponential phase; however, nutrients are often limited in nature and cells enter a nongrowing state, similar to the stationary phase of laboratory cultures (Kolter *et al.*, 1993; Finkel, 2006; Yang *et al.*, 2008; Gefen *et al.*, 2014). In contrast, laboratory experiments involve co-inoculation of both strains from lag phase, at low density into sterile media, where cells then compete, essentially during exponential phase. We therefore lack an experimental model of invasion that more accurately captures 'real-world' processes of invasion through mutation and migration. This may be a significant omission as there are a number of reasons why potential cheats may not be able to invade a natural population because nongrowing cells generally have reduced levels of exoproduct synthesis compared to exponential phase (Kolter *et al.*, 1993; Gefen *et al.*, 2014).

Here, we experimentally assessed how the growth stage of an exoproduct producer bacterial strain affects its susceptibility to invasion by a nonproducer strain. We use the model system of pyoverdin production in *Pseudomonas aeruginosa*. Pyoverdin is an iron-scavenging siderophore molecule, which has been shown in a number of detailed studies to act as a public good in iron-limited conditions (Griffin *et al.*, 2004; Harrison & Buckling, 2009; Kümmerli & Ross-Gillespie, 2014). In previous work with this system, it has been demonstrated that under iron-limited conditions, the relative fitness of cheats is positively correlated with pyoverdin production of cooperative competitors (Jiricny *et al.*, 2010). We use a wild-type *P. aeruginosa* strain as a cooperator and a mutant strain defective for pyoverdin production as a putative cheat. We test whether the invasive potential of nonproducers varies with the rate of pyoverdin production per cell through the growth curve of a cooperative population and, therefore, whether producer *P. aeruginosa* populations in variable growth phases are more or less susceptible to exploitation and invasion.

Materials and methods

Model system

We use pyoverdin production in the opportunistic gram-negative pathogen, *P. aeruginosa* as a model cooperative trait. Pyoverdin is an iron-scavenging siderophore molecule that is secreted by cells under iron-limited conditions (Guerinot, 1994; West & Buckling, 2003). Iron is essential for bacterial growth, but is generally a major limiting nutrient because it is found in an insoluble Fe (III) form in the environment and is actively withheld by hosts during infection (Ratledge & Dover, 2000). Once pyoverdin is bound to iron, it becomes available for metabolism and can be shared by neighbouring cells, acting as a public good (Griffin *et al.*, 2004). Pyoverdin molecules are highly durable and can be recycled and reused multiple times (Faraldo-Gomez & Sansom, 2003; Imperi *et al.*, 2009). Therefore, when sufficient levels accumulate, bacterial cells down-regulate production (Faraldo-Gomez & Sansom, 2003; Imperi *et al.*, 2009; Kummerli & Brown, 2010).

Bacterial strains

We used two *P. aeruginosa* strains in our experiments: the wild-type PAO1 strain (referred to throughout as producer), which produces pyoverdin, and a UV-induced, pyoverdin-mutant PAO9 strain (referred to throughout as nonproducer) derived from PAO6049, a mutant of PAO1 (Rella *et al.*, 1985; Hohnadel *et al.*, 1986), which has been shown to exploit pyoverdin producers under specified conditions (Griffin *et al.*, 2004; Ross-Gillespie *et al.*, 2007). PAO1 and PAO9 are morphologically distinct and have different colours. This pair, therefore, provides reliable colony counts, even if the production of pyoverdin decreases over the extended time in culture (> 24 h) involved in this experiment. Previous competition assays with multiple strain pairs find no significant difference in how the PAO1/PAO9 pair behaves when compared with an isogenic knockout, a deletion mutant of PAO1 defective for the pyoverdin synthetase gene *pvdD* (PAO1*ΔpvdD*) (Ross-Gillespie *et al.*, 2007, 2009; Jiricny *et al.*, 2010). In addition, because PAO9 is derived from PAO1, the strains share the same bacteriocin profile and we phenotypically confirmed that they do no inhibit each other using bacteriocins based on the protocol described by Ghoul *et al.* (2015) (See Appendix S1).

Prior to experimentation, we cultured both strains from freezer stocks for 12 h at 37 °C on an orbital shaker at 200 rpm in KB media (20 g protease peptone N°3 (Beckton Dickinson, UK Ltd, Oxford, UK), 10 mL glycerol, 1.5 g $K_2HPO_4.3H_2O$ and 1.5 g $MgSO_4.7H_2O$ (Sigma Aldrich UK Ltd, Gillingham, UK), per litre of dH_2O). We centrifuged the overnight cultures and discarded the supernatant and then washed the cell pellet in minimal salt media (M9: 6.8 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl and 10 g NH_4Cl (Sigma Aldrich, UK Ltd), per litre of dH_2O) to remove any residual iron and carbon resources from the KB media. Cell density was assayed at an absorbance of 600 nm (A_{600}), standardized to the same starting density and diluted 100-fold before inoculation.

Measuring growth and pyoverdin production

We first measured pyoverdin production through different stages of growth from point of inoculation. We constructed 48-h growth curves for both producer and nonproducer strains in iron-limited casamino acid media (CAA 5 g casamino acids, 1.18 g $K_2HPO_4.3H_2O$, 0.25 g $MgSO_4.7H_2O$, per litre of dH_2O) supplemented with the iron chelator, human apo-transferrin (100 μg mL^{-1}, Sigma Aldrich, UK), and 20 mM sodium bicarbonate (Schwyn & Neilands, 1987; Meyer *et al.*, 1996) to induce siderophore production (Griffin *et al.*, 2004). Turbidity at A_{600} and pyoverdin production in relative fluorescent units (RFU) at excitation and emission wavelengths of 400 and 460 nm, respectively, were automatically measured every 30 min for 48 h using a combined, automated fluorescence spectrometer (Synergy2 BioteK, BioTek, Swindon, UK as described by Jiricny *et al.* (2010)). We calculated the relative pyoverdin availability per cell by the ratio of RFU to A_{600} at each time point (Kummerli *et al.*, 2009) and measured the rate of pyoverdin production per cell per minute by $((RFU_2–RFU_1)/30\ min)/A_{600(2)})$. Each treatment was replicated 12 times per strain.

Competition assays to assess the ability of nonproducers to invade

In order to test for the effect of growth stage on the ability of the nonproducer to invade, we inoculated cultures of the producer strain with the nonproducer at time point zero, and at 4-h intervals over a period of 24 h, then once more at 48 h (Table 1). Each culture of the producer strain was initiated with 60 μL of inoculum (10^2 dilution) into 6 mL of CAA media. At each of the eight time points, the nonproducer culture was introduced at appropriate dilutions to ensure that we controlled for the differences in frequency across treatments (see Table 1). We did this by tracking density of the producer along its growth curve and adjusting the nonproducer dilution accordingly. All nonproducer cells were in exponential growth phase at the time they were added to the producer population. Competition cultures were then grown statically for 48 h at 37 °C. We recorded the relative frequencies of producer and nonproducer cells at the start of competition and then again at 24 and 48 h of competition by counting colonies grown on KB agar plates (12 g agar per litre of KB medium; the plates were incubated at 37 °C overnight; Table 1). Producer and nonproducer colony-forming units (CFU) are distinguishable from each other by phenotype. Producer colonies are green in colour with rugged edges, whereas nonproducer colonies are smaller, white in colour with smooth edges. See Table 1 for details of replication.

Controlling for ability of producer cultures in stationary phase to inhibit cell growth

To determine whether an impaired ability of nonproducer cells to invade at stationary phase was due to the accumulation of toxin metabolites or the lack of nutrients, we tested the ability of nonproducer and producer cells to grow in sterile spent media of 48-h producer cultures. First, we grew monocultures of the producer strain, using the same conditions as above to obtain a supply of stationary-phase spent media. After 48 h of growth, we centrifuged the cultures for 10 min at 5000 *g* and filter-sterilized the supernatant using 0.2-μm filter tips before storage in fresh glass vials.

We constructed growth curves to determine whether freshly extracted vs. aged spent media had more inhibitory effect on the absolute growth of strains (inducing a longer lag phase and overall lower growth densities) and whether inhibitory molecules degrade with time and allow producer and nonproducer cells to grow better. For this, we inoculated five replicates of each freshly extracted, 1- and 3-day-old spent media with producer and nonproducer exponential-phase cells and grew the monocultures for 24 h taking A_{600} reads every 15 min using the spectrometer (Synergy2 BioteK; Fig. S2).

In addition, we used CFU counts to determine whether the freshly extracted spent media have a higher inhibitory effect on growth of the nonproducer than on the growth of producer strain. We obtained the initial number of producer and nonproducer cells by plating out 100 μL of 10^3 dilutions on KB agar. We then allowed the monocultures to grow in the fresh

Table 1 cc

Producer growth phase	Lag		Exponential					Stationary
Time in producer growth when non-producer added (hours)	0	4	8	12	16	20	24	48
Initial ratio of non-producer:producer	1 : 10	7 : 100	3 : 100	2 : 100	2 : 100	2 : 100	4 : 100	6 : 100
Plating volumes and dilutions at (hours)								
0	100 μL, 10^1	70 μL, 10^2	20 μL, 10^2	50 μL, 10^3	70 μL, 10^4	70 μL, 10^4	70 μL, 10^4	70 μL, 10^4
24	50 μL, 10^4	50 μL, 10^4	50 μL, 10^4	50 μL, 10^4	50 μL, 10^4	50 μL, 10^4	50 μL, 10^4	50 μL, 10^4
48	50 μL, 10^4	50 μL, 10^4	50 μL, 10^4	50 μL, 10^4	50 μL, 10^4	50 μL, 10^4	50 μL, 10^4	50 μL, 10^4
Replicates	28	14	14	14	14	14	22	16

spent media for 48 h static at 37 °C and then obtained cell numbers after 48 h by plating out 50 μL of 10^4 dilutions on KB agar. Each monoculture was replicated eight times per treatment. We incubated all KB agar plates at 37 °C overnight after which we recorded the colony-forming unit counts for each to determine whether the cells were dividing and increasing.

Statistical analysis

We calculated the relative fitness of the nonproducer in two ways. We first measured relative fitness, (ω), as the change in frequency over time relative to the producer, given by $\omega = x_2(1–x_1)/x_1(1–x_2)$, where x_1 is the mean initial proportion of the nonproducer from the sample population and x_2 is the final proportion in each sample (Otto & Day, 2007; Ross-Gillespie *et al.*, 2007). A value of $\omega > 1$ indicates that the nonproducer has a higher fitness than the producer, and a value $\omega < 1$ indicates that the nonproducer has a lower fitness than the producer. We calculated the mean fitness value across the competition assay replicates to indicate how efficiently the nonproducer can exploit the producer population.

We then calculated the Malthusian growth rates (R) of the producer and nonproducer strains over the course of the 48-h competition experiments. This is given by $R = \ln [N(t)/N(0)]/(\text{day})$, where $N(0)$ is the number of cells at time when nonproducers are first added into the producer culture and $N(t)$ is the number of cells at time t, the end of 48 h of the competition. We compared the growth rates of producers and nonproducers to determine (1) the change in each strain's growth along consecutive competition assays with a growing producer culture; and (2) the differences in strains' growths relative to each other. For these data, we used a GLM using log ($y + (1 - \min(y))$)-transformed rate values (see Table S1). Malthusian growth rates assume that the population is growing – if a population is declining, it would give negative and misleading estimates, and so we only compared Malthusian growth rates of both strains to each other and not relative Malthusian fitness values. All analyses were carried out in R statistical software v 2.15.2 (http://www.R-project.org).

Results

Population growth and pyoverdin availability

We begin by reporting data on growth and pyoverdin availability in monocultures of both strains, justifying two key assumptions underlying our experimental design: pyoverdin production is required for strain growth in iron-limited conditions (Figs 1a and S1), and producers produce more pyoverdin than nonproducers, which secrete a negligible residual amount (Fig. 1b). Pyoverdin production rate by the producer strain peaks at 9.5 h of growth, after which the rate drops and remains low (Fig. 1a,b). Peak cumulative pyoverdin availability per producer cell occurs during the exponential phase at 17 h and is significantly higher than the residual pyoverdin available per nonproducers, which peaks at 48 h at the end of the growth assay (Fig. 1c; $T = 18.24$, $P = 1.107 \times 10^{-9}$). Producer pyoverdin availability per cell decreases after 17 h and is significantly lower after 24 h of growth (Fig. 1c; $T = 10.50$, $P = 4.53 \times 10^{-7}$) after which pyoverdin levels are sustained and only gradually decline. By 48 h of growth, pyoverdin per producer cell is significantly lower than at 24 h (Fig. 1c; $T = 6.46$, $P = 4.69 \times 10^{-5}$). Nonproducer pyoverdin production is negligible throughout a 48-h growth period (Fig. 1b).

Effect of producer growth stage on the ability of nonproducers to invade

The nonproducer strain was able to invade the producer strain in lag phase (0 and 4 h of growth) and early exponential phase (8, 12 and 16 h of growth; Fig. 2a,b; stats: Table S1). This corresponded with periods of relatively high pyoverdin production by producers (Figs 1b and 2c). The nonproducer persists, but does not invade in producer cultures passed mid-exponential phase at 20 and 24 h, and the producer declines and cannot invade producer cultures in stationary phase at 48 h (Fig. 2a,c; stats: Table S1). The cost of pyoverdin production decreases after mid-exponential phase, and is negligible at 48 h in stationary phase, reducing the nonproducer's competitive advantage (Fig. 1a–c).

These same results are obtained when looking at growth rates. The producer and nonproducer growth rates are lower when in competition with producers from later growth phases than in competitions with producers from early growth stages (Fig. 2b and Tables S2 and S3; $F_{3,\ 266} = 88.13$, $P < 2.2 \times 10^{-16}$). Overall, the producer strain grows significantly slower than the nonproducer during competitions (Table S2; $T = -3.88$, $P = 1.32 \times 10^{-4}$), but this difference is reduced in competitions with producer populations of later growth stages (Fig. 2b). Eventually, the nonproducer growth rate significantly drops below the producer's when in competition with a stationary-phase producer culture (Tables S2 and S3).

Assessing for the ability of producer cells in stationary phase to inhibit cell growth

Growth of both strains was significantly reduced in freshly extracted producer-spent media relative to 1-day- and 3-day-old spent media (Fig. S2; producer: $F_{2,\ 12} = 176.2$, $P = 1.27 \times 10^{-9}$; nonproducer: $F_{2,\ 12} = 102.8$, $P = 2.81 \times 10^{-8}$). Within the freshly extracted spent media treatment, nonproducer monocultures

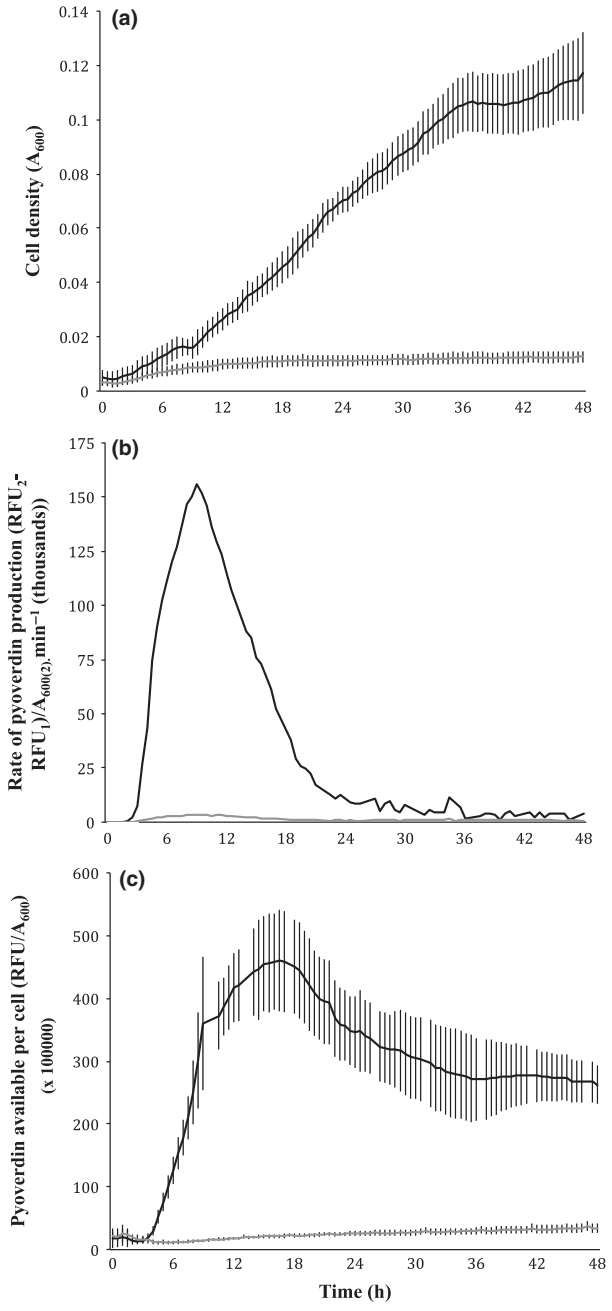

Fig. 1 Producer (black) and nonproducer (grey) (a) growth curves, (b) rate of pyoverdin production and (c) cumulative pyoverdin available per cell, over a duration of 48 h in iron-limited media: (a) growth measured by cell density at A_{600} of the producer is significantly better in iron-limited conditions than that of the nonproducer; (b) rate of pyoverdin production, measured per cell per minute $((RFU_2–RFU_1)/30\ min)/A_{600(2)})$, is negligible through the nonproducer's growth relative to the producer, and the producer's production rate peaks at 9.5 h during exponential growth; (c) peak pyoverdin levels available per cell, measured by $(RFU_{400,460}/A_{600})$, are significantly higher for producers than for nonproducers. The error bars indicate standard deviation around the mean values.

have significantly higher CFU counts than producers after 48 h of growth (Fig. 3; $T = -3.02$, $P = 0.0126$).

Discussion

We found that in competition assays between pyoverdin producers and nonproducers, the nonproducer was able to invade producer populations in lag and early exponential phase, persists but does not invade in late exponential phase and declines and does not invade in stationary phase (Fig. 2). Our data are consistent with the explanation that when the producer's pyoverdin production rates are highest, from lag to early exponential phase, the nonproducer strain has a clear fitness advantage (Figs 1 and 2). As the producer growth slows, its rates of pyoverdin production drop and the ability of nonproducers to invade is reduced (Fig. 2; Table S1). Finally, as the population of producers reaches stationary phase, when pyoverdin production rates are negligible, nonproducers are unable to invade and even decline in relative frequency (Fig. 2).

The durability of pyoverdin is key to interpreting our results: once pyoverdin accumulates, it is recycled, allowing the cells to down-regulate production as a cost-saving strategy (Imperi *et al.*, 2009; Kummerli & Brown, 2010; Dumas & Kummerli, 2012). Nonproducers introduced into a population of producers in late exponential phase may benefit from the exploitation of iron made available by high levels of pyoverdin, but its competitive advantage starts to decline because producer cells are no longer bearing the cost of pyoverdin production. This does not appear to be a direct cooperative resistance mechanism selected in response to cheat invasion, but is likely a result of the intrinsic properties of the pyoverdin public good molecule, and the subsequent effect on the molecule's regulation.

One possible explanation for the inability of nonproducers to invade in late exponential phase is that the nonproducer strain experiences stationary-phase cultures as a hostile environment either due to the presence of inhibitory molecules or due to the lack of nutrients. Although our experiments show that in the absence of cell competition, pyoverdin-rich supernatant extracted from the stationary-phase cultures of producers is more enriching for the nonproducer cells than it is for itself (Fig. 3), this is perhaps because the producer continues to bear some cost of pyoverdin production. However, inhibitory molecules secreted by producers seem to also act on the nonproducer cells: freshly extracted spent media from producer culture at stationary phase inhibits producer and nonproducer cell growth significantly more than aged spent media. This is consistent with the hypothesis that inhibitory molecules degrade over time and that inhibitory molecules produced in stationary phase also prevent both producers and nonproducers from growing during competition.

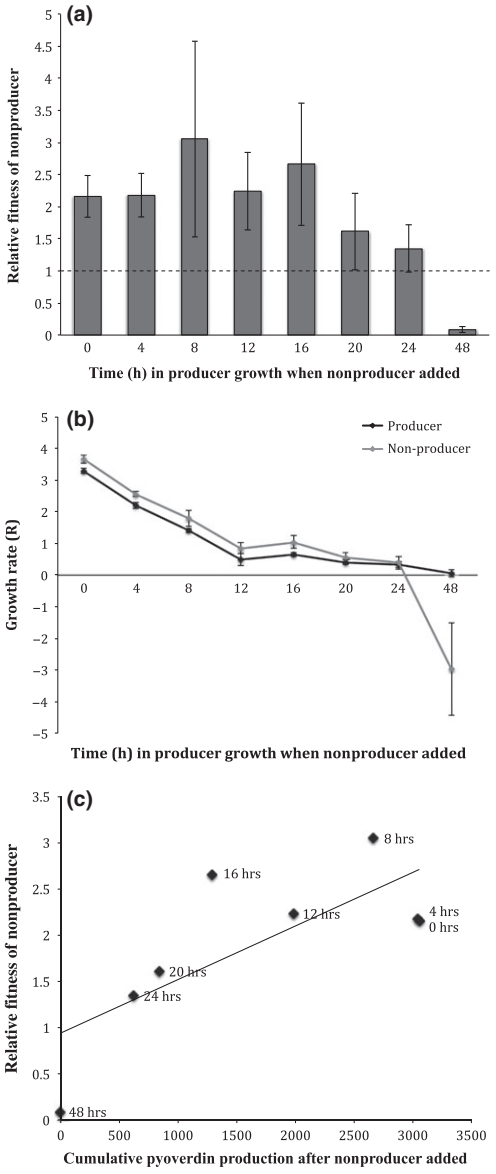

Fig. 2 (a) Relative fitness of nonproducer in competition with producers at different stages of growth, (b) growth rates of producer and nonproducer strains during competition, (c) relative fitness of nonproducer correlated with the producer's cumulative pyoverdin production after the nonproducer is introduced (RFU (48 h)- RFU(introduction time of nonproducer): (a) nonproducers significantly increase in frequency after 48 h of competition with producers when introduced at 0, 4, 8, 12 and 16 h of producer growth. (b) The producer has significantly slower growth rates than the nonproducer, but this difference decreases and switches over consecutive competitions and at 48 h. The error bars for (a) and (b) indicate 95% confidence intervals around the mean values; (c) the nonproducer's relative fitness value is positively correlated with the producer's pyoverdin production levels at the different time points in the producer's growth.

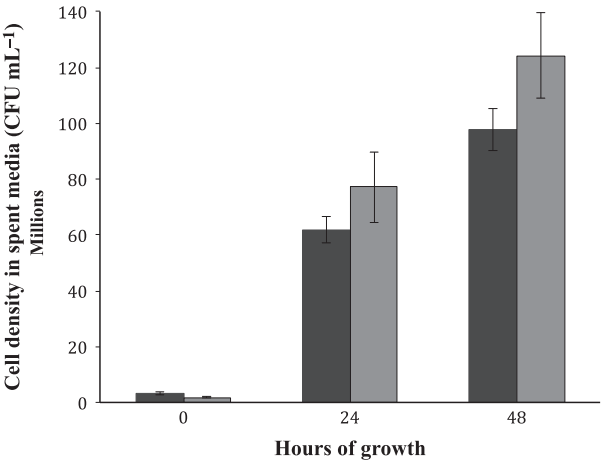

Fig. 3 Monoculture cell growth in freshly extracted producer-spent media. Cell density is measured as colony-forming units per mL (CFU mL^{-1}) in the spent media of 48-h producer monocultures. Over a period of 48 h, nonproducers (grey) grow to a significantly higher density than producers (black) in the producer's freshly extracted spent media. The errors bars indicate 95% confidence intervals around the mean values.

Our study demonstrates the importance of the bacterial growth phase to evolutionary dynamics of social behaviour, with producers of a public good immune to invasion in late exponential and stationary phase. Our data are consistent with the explanation that the ability to invade is correlated with the costs incurred by competitors when producing a public good. It remains to be shown whether the changing cooperative–cheat dynamics through population growth we describe here are representative of other cooperative public good traits in bacteria. The fitness benefits of many exoproducts are density dependent and controlled by quorum sensing (Bassler, 1999; Williams *et al.*, 2007) or produced only at certain stages of population growth (Xavier *et al.*, 2011). Xavier *et al.* (2011) show that cooperators regulate the expression of biosurfactants in a way that reduces the cost of production; in contrast to wild type, cooperators engineered to constitutively produce biosurfactant were vulnerable to invasion. This study and our results emphasize that cooperators can only be exploited when they are actually cooperating and hence paying the cost of cooperation. Sharing the benefits from past cooperation is not enough for cheat invasion.

Our results may also have implications for antimicrobial treatment strategies that rely on the invasive ability of cheats (Brown *et al.*, 2009). Experimental studies *in vitro* and *in vivo* have shown that quorum-sensing cheats can invade, leading to a reduction in virulence (Diggle *et al.*, 2007; Rumbaugh *et al.*, 2009). It has also

been argued that 'trojan horse' cheats could be used as a mechanism to drive medically beneficial alleles such as antibiotic susceptibility into the resistant infective population (Brown *et al.*, 2009). A limitation with this previous work is that it has focused on co-inoculating potential cheats into a wild-type population at the onset of its growth. However, these strategies may not be effective against a population of slowly growing infecting cells producing low levels of exoproducts.

Experimental evolution studies have provided some unique opportunities to experimentally test the prediction of social evolution theory and have made a significant contribution to our understanding of cooperative behaviour in bacteria. However, the parameters that have been shown to be important for driving social dynamics in the laboratory are likely to have more complex interactive effects in natural populations of bacteria. This study emphasizes the necessity to test the effect of bacterial growth rate on the production of exoproducts and therefore the ability of putative cheats to invade as we have done here. Cheating in natural populations may play an important role in the competitive dynamics of bacteria when growth from low density and secretion of exoproducts is required, such as colonizing a new niche. However, susceptibility to cheating is potentially minimal in populations with prolonged slowly or nongrowing bacterial populations with reduced exoproduct secretions. If one considers the fluctuations between growing and nongrowing conditions, then cheats might still be maintained. Our results demonstrate that a cooperative strain of bacteria was immune to invasion by putative cheats in a stationary physiological state, characteristic of nutrient-starved or established natural populations such as biofilms (Mulvey *et al.*, 2001; Finkel, 2006; Garrett *et al.*, 2008; Llorens *et al.*, 2010; Gefen *et al.*, 2014). This has significant ecological and medical implications, as bacteria likely exist in nature in continuously fluctuating growth phases in soil, water, biofilms and as established chronic infections (Kolter *et al.*, 1993; Mulvey *et al.*, 2001; Finkel, 2006; Yang *et al.*, 2008; Llorens *et al.*, 2010; Gefen *et al.*, 2014).

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1 Monoculture cell density (A_{600}) in iron-limited CAA media that requires pyoverdin production for growth.

Figure S2 24-h growth curves of producer (black) and nonproducer (grey) in producer-spent media.

Table S1 Relative fitness values of the nonproducer in competition with the producer strain at different stages of its growth.

Table S2 Model results for the change in growth rates of each strain over time with strain type as an interaction effect.
Table S3 Growth rates of the producer and nonproducer strain during competition.
Appendix S1 Testing the inhibitory effect of strains PAO1 and PAO9 on each other.

Data deposited at Dryad: doi: 10.5061/dryad.n2s14

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