

1 **Pyoverdinin cheats fail to invade bacterial populations in stationary phase**

2 Melanie Ghoul*, Stuart A. West, Fergus A. McCorkell, Zhuo-Bin Lee, John B. Bruce,

3 Ashleigh S. Griffin

4 Department of Zoology, University of Oxford, Oxford, UK

5 Running title: Cheating in growing bacterial populations

6 *Address correspondence to Melanie Ghoul, melanie.ghoul@zoo.ox.ac.uk

7 Telephone: +44(0)1865271254

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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 non-producing 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 pyoverdinin, an iron scavenging siderophore that acts as a public good in the bacteria *Pseudomonas aeruginosa*. We show that: (1) non-producers 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 pyoverdinin 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 non-producing 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.

Keywords: cooperation, cheating, bacteria, growth phase

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.* 2013).

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

69 natural bacterial populations is population structure leading to cooperation being
70 directed towards relatives, and hence favoured by kin selection (West & Buckling
71 2003; Griffin *et al.* 2004; Julou *et al.* 2013).

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

86 Here, we experimentally assessed how the growth stage of an exoproduct producer
87 bacterial strain affects its susceptibility to invasion by a non-producer strain. We use
88 the model system of pyoverdine production in *Pseudomonas aeruginosa*. Pyoverdine is
89 an iron-scavenging siderophore molecule, which has been shown in a number of
90 detailed studies to act as a public good in iron-limited conditions (Griffin *et al.* 2004;
91 Harrison & Buckling 2009; Kümmerli & Ross-Gillespie 2013). In previous work with
92 this system, it has been demonstrated that under iron-limited conditions, the relative
93 fitness of cheats is positively correlated with pyoverdine production of cooperative

94 competitors (Jiricny *et al.* 2010). We use a wildtype *P. aeruginosa* strain as a
95 cooperator and a mutant strain defective for pyoverdine production as a putative cheat.
96 We test whether the invasive potential of non-producers varies with the rate of
97 pyoverdine production per cell through the growth curve of a cooperative population
98 and, therefore, whether producer *P. aeruginosa* populations in variable growth phases
99 are more or less susceptible to exploitation and invasion.

100 **Materials and Methods**

101 *Model system*

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

114 *Bacterial strains*

115 We used two *P. aeruginosa* strains in our experiments: the wildtype PAO1 strain
116 (referred to throughout as producer) which produces pyoverdine, and, a UV-induced,

pyoverdin mutant PAO9 strain (referred to throughout as non-producer) 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/PAO9 are morphologically distinct, and have different colours. This pair, therefore, provides reliable colony counts, even if production of pyoverdine decreases over the extended time in culture (>24 hours) 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 Δ *pvdD*) (Ross-Gillespie *et al.* 2007; Ross-Gillespie *et al.* 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 not inhibit each other using bacteriocins based on the protocol described by Ghoul *et al.* (2015) (See supplementary info).

Prior to experimentation, we cultured both strains from freezer stocks for 12 hours at 37°C on an orbital shaker at 200 rpm in KB media (20 g protease peptone N°3 (Beckton Dickinson, UK ltd), 10 mL glycerol, 1.5 g K₂HPO₄·3H₂O and 1.5 g MgSO₄·7H₂O (Sigma Aldrich UK ltd), per liter of dH₂O). We centrifuged the overnight cultures and discarded the supernatant and then washed the cell pellet in minimal salts media (M9: 6.8 g Na₂HPO₄, 3 g KH₂PO₄, 0.5 g NaCl and 10 g NH₄Cl (Sigma Aldrich, UK ltd), per liter of dH₂O) to remove any residual iron and carbon resources from the KB media. Cell density was assayed at an absorbance of 600 nm (A₆₀₀), standardized to the same starting density and diluted 100 fold before inoculation.

Measuring growth and pyoverdine production

We first measured pyoverdine production through different stages of growth from point of inoculation. We constructed 48 hour growth curves for both producer and non-producer strains in iron-limited casamino acid media (CAA 5 g casamino acids, 1.18 g $K_2HPO_4 \cdot 3H_2O$, 0.25 g $MgSO_4 \cdot 7H_2O$, per liter of dH_2O) supplemented with the iron-chelator, human apo-transferrin (100 $\mu g \cdot mL^{-1}$ - 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 pyoverdine production in relative fluorescent units (RFU) at excitation and emission wavelengths of 400 nm and 460 nm respectively, were automatically measured every 30 minutes for 48 hours using a combined, automated fluorescence-spectrometer (Synergy2 BioteK, as described by Jiricny *et al.* (2010)). We calculated the relative pyoverdine availability per cell by the ratio of RFU/A_{600} at each time point (Kummerli *et al.* 2009) and measured the rate of pyoverdine production per cell per minute by $((RFU_2 - RFU_1)/30 \text{ minutes}) / A_{600(2)}$. Each treatment was replicated 12 times per strain.

Competition assays to assess ability of non-producers to invade

In order to test for the effect of growth stage on the ability of the non-producer to invade we inoculated cultures of the producer strain with the non-producer at time point zero, and at four hour intervals over a period of 24 hours, then once more at 48 hours (Table 1). Each culture of the producer strain was initiated with 60 μL of inoculum (10^2 dilution) into 6ml of CAA media. At each of the eight time-points, the non-producer culture was introduced at appropriate dilutions to ensure we controlled for differences in frequency across treatments (see Table 1). We did this by tracking density of the producer along its growth curve and adjusting the non-producer dilution

165 accordingly. All non-producers cells were in exponential growth phase at the time
166 they were added to the producer population. Competition cultures were then grown
167 statically for 48 hours at 37°C. We recorded the relative frequencies of producer and
168 non-producer cells at the start of competition, and then again at 24 hours and 48 hours
169 of competition by counting colonies grown on KB agar plates (12 g agar per liter of
170 KB medium; plates incubated at 37°C overnight; Table 1). Producer and non-
171 producer colony forming units (CFU) are distinguishable from each other by
172 phenotype. Producer colonies are green in colour with rugged edges while non-
173 producer colonies are smaller, white in colour with smooth edges. See Table 1 for
174 details of replication.

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

176 To determine whether impaired ability of non-producer cells to invade at stationary
177 phase was due to accumulation of toxin metabolites or lack of nutrients, we tested the
178 ability of non-producer and producer cells to grow in sterile spent media of 48-hour
179 producer cultures. First we grew monocultures of the producer strain, using the same
180 conditions as above to obtain a supply of stationary phase spent media. After 48 hours
181 of growth we centrifuged the cultures for ten minutes at 5000 rcf, and filter sterilized
182 the supernatant using 0.2 µm filter tips before storage in fresh glass vials.

183 We constructed growth curves using the spectrophotometer to determine whether
184 freshly extracted versus aged spent media had more inhibitory effect on the absolute
185 growth of strains (inducing a longer lag-phase and overall lower growth densities) and
186 whether inhibitory molecules degrade with time and allow producer and non-producer
187 cells to grow better. For this we inoculated five replicates of each freshly extracted,
188 one and three day-old spent media with producer and non-producer exponential phase

cells and grew the monocultures for 24 hours taking A_{600} reads every 15 minutes using the spectrometer (Synergy2 BioteK; Fig. S2).

In addition we used CFU counts to determine whether the freshly extracted spent media has a higher inhibitory effect on growth of the non-producer than the producer strain. We obtained the initial number of producer and non-producer cells by plating out 100 μ L of 10^3 dilutions on KB agar. We then allowed the monocultures to grow in the fresh spent media for 48 hours static at 37°C and then obtained cell numbers after 48 hours by plating out 50 μ L of 10^4 dilutions on KB agar. Each monoculture was replicated 8 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 non-producer in two ways. We first measured relative fitness, (ω), as the change in frequency over time relative to the producer, given by $\omega = x_2(I-x_1)/x_1(I-x_2)$, where x_1 is the mean initial proportion of the non-producer 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 non-producer has a higher fitness than the producer, and a value $\omega < 1$ indicates that the non-producer has a lower fitness than the producer. We calculated the mean fitness value across the competition assay replicates to indicate how efficiently the non-producer can exploit the producer population.

We then calculated the Malthusian growth rates (R) of the producer and non-producer strains over the course of the 48-hour 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 non-producers

are first added into the producer culture and $N(t)$ is the number of cells at time t , the end of 48 hours of the competition. We compared the growth rates of producers and non-producers to determine: (1) change in each strain's growth along consecutive competition assays with a growing producer culture, and (2) differences in strains' growths relative to each other. For this data we used a GLM using $\text{Log}(y + (1 - \min(y)))$ transformed rate values (see table S1). Malthusian growth rates assume 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 analysis was carried out in R statistical software v 2.15.2 (<http://www.R-project.org>).

Results

Population growth and pyoverdine availability

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

gradually decline. By 48 hours of growth pyoverdine per producer cell is significantly lower than at 24 hours (Fig.1c; $T = 6.46$, $P = 4.69 \times 10^{-5}$). Non-producer pyoverdine production is negligible throughout a 48-hour growth period (Fig. 1b).

Effect of producer growth stage on the ability of non-producers to invade

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

These same results are obtained when looking at growth rates. The producer and non-producer growth rates are lower when in competition with producers from later growth phases than competitions with producers from early growth stages (Fig. 2b & Table S2 & S3; $F_{3, 266} = 88.13$, $P < 2.2 \times 10^{-16}$). Overall the producer strain grows significantly slower than the non-producer 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 non-producer growth rate significantly drops below the producer's when in competition with a stationary phase producer culture (Table S2 & S3).

Assessing for 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: $F_{2,12} = 176.2$, $P = 1.27 \times 10^{-9}$; non-producer: $F_{2,12} = 102.8$, $P = 2.81 \times 10^{-8}$). Within the freshly extracted spent media treatment, non-producer monocultures have significantly higher CFU counts than producers after 48 hours of growth (Fig. 3; $T = -3.02$, $P = 0.0126$).

Discussion

We found that in competition assays between pyoverdin producers and non-producers, the non-producer 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 is consistent with the explanation that when the producer's pyoverdin production rates are highest, from lag to early exponential phase, the non-producer strain has a clear fitness advantage (Fig. 1 & 2). As the producer growth slows, its rates of pyoverdin production drop and the ability of non-producers to invade is reduced (Fig. 2; Table S1). Finally, as the population of producers reaches stationary phase, when pyoverdin production rates are negligible, non-producers 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 cells to downregulate production as a cost-saving strategy (Imperi *et al.* 2009; Kummerli & Brown 2010; Dumas & Kummerli 2012). Non-producers 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

284 cooperative resistance mechanism selected in response to cheat invasion, but is likely
285 a result of the intrinsic properties of the pyoverdine public good molecule, and the
286 subsequent effect on the molecule's regulation.

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

300 Our study demonstrates the importance of the bacterial growth phase to evolutionary
301 dynamics of social behavior, with producers of a public good immune to invasion in
302 late exponential and stationary phase. Our data is consistent with the explanation that
303 the ability to invade is correlated with the costs incurred by competitors when
304 producing a public good. It remains to be shown whether the changing cooperative-
305 cheat dynamics through population growth we describe here are representative of
306 other cooperative public good traits in bacteria. The fitness benefits of many
307 exoproducts are density dependent and controlled by quorum sensing (Bassler 1999;
308 Williams *et al.* 2007) or produced only at certain stages of population growth (Xavier

309 *et al.* 2011). Xavier *et al.* (2011) show that cooperators regulate expression of
310 biosurfactants in a way that reduces cost of production; in contrast to wildtype,
311 cooperators engineered to constitutively produce biosurfactant were vulnerable to
312 invasion. This study and our results emphasise that cooperators can only be exploited
313 when they are actually cooperating, and hence paying the cost of cooperation. Sharing
314 the benefits from past cooperation is not enough for cheat invasion.

315 Our results may also have implications for antimicrobial treatment strategies that rely
316 on the invasive ability of cheats (Brown *et al.* 2009). Experimental studies *in vitro*
317 and *in vivo* have shown that quorum sensing cheats can invade, leading to a reduction
318 in virulence (Diggle *et al.* 2007; Rumbaugh *et al.* 2009). It has also been argued that
319 ‘trojan horse’ cheats could be used as a mechanism to drive medically beneficial
320 alleles such as antibiotic susceptibility into the resistant infective population (Brown
321 *et al.* 2009). A limitation with this previous work is that it has focused on co-
322 inoculating potential cheats into a wildtype population at the onset of its growth.
323 However, these strategies may not be effective against a population of slowly growing
324 infecting cells producing low levels of exoproducts.

325 Experimental evolution studies have provided some unique opportunities to
326 experimentally test prediction of social evolution theory and have made a significant
327 contribution to our understanding of cooperative behaviour in bacteria. However, the
328 parameters that have been shown to be important for driving social dynamics in the
329 lab are likely to have more complex interactive effects in natural populations of
330 bacteria. This study emphasises the necessity to test the effect of bacterial growth rate
331 on the production of exoproducts and, therefore, the ability of putative cheats to
332 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 non-growing bacterial populations with reduced exoproduct secretions. If one considers fluctuations between growing and non-growing 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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Figure legends

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

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

Fig. 3 Monoculture cell growth in freshly extracted producer spent media. Cell density is measured as colony-forming units per ml (CFU/ml) in the spent media of 48-hour cooperator monocultures. Over a period of 48 hours, non-producers (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.

Table

Table. 1 Experimental protocol. We added the non-producer strain to cultures of the producer strain at zero, and 4 hour intervals over a period of 24 hours, and then once more at 48 hours into the producers growth. Both strains are then allowed to compete for up to 48 hours. Mixed cultures were plated out prior to competition at the specified dilutions and volumes, at 0 hours, then 24 hours into competition and again at 48 hours after competition. Each set of competitions was replicated as shown in the table.

Appendix

Fig. S1 Monoculture cell density (A_{600}) in iron-limited CAA media that requires pyoverdinin production for growth. As a standard control in parallel to the competition assays, we cultured each strain separately as a monoculture to confirm that the pyoverdinin producer strain (black) grows better as a monoculture than the non-producer strain (grey). The cultures were then incubated at 37°C and assayed for cell density at A_{600} at 24 hours, 48 hours and 72 hours. The producer grows to a significantly higher density than the non-producer after 24, 48 and 72 hours of growth ($T = -8.23$, $P = 9.07 \times 10^{-9}$). The error bars indicate 95% confidence intervals around the means of fourteen independent replicates. To account for non-independence of the

repeated measures of the replicates over time we analysed the data as a mixed model in the R software using the lmer-Test package with replicates per strain as a random effect and nested time as a fixed effect.

Fig.S2. 24-hour growth curves of producer (black) and non-producer (grey) in producer spent media. Both producer and non-producer grow significantly better in aged spent media than freshly extracted spent media (producer: $F_{2,12} = 176.2$, $P = 1.27 \times 10^{-9}$; non-producer: $F_{2,12} = 102.8$, $P = 2.81 \times 10^{-8}$). Non-producer and producer growth rates are equally inhibited in 0 day and 1-day aged spent media, but the non-producer grows significantly better than the producer in 3-day aged spent media ($T_{\text{fresh}} = 1.543$, $P_{\text{fresh}} = 0.164$; $T_{1\text{day}} = 0.648$, $P_{1\text{day}} = 0.551$; $T_{3\text{day}} = -2.958$, $P_{3\text{day}} = 0.036$).

Table S1. Relative fitness values of the non-producer in competition with the producer strain at different stages of its growth. The relative fitness of the non-producer is measured between the start of competition and 24 hours later (ω_1), between the start of competition and 48 hours later (ω_2), and to check if the non-producer continues to increase or decrease in frequency during course of competition we measure the relative fitness between 24 and 48 hours of competition (ω_3). In the lag and early exponential stage of the producer's growth (0-16 hours) when pvd production rates are highest, the non-producer shows a higher relative fitness after two days of competition than after one day, indicating that cheating is sustained throughout the producer's growth until it establishes and reaches stationary. However, in later stages of producer exponential growth (20-24 hours), the non-producer has a lower relative fitness after two days of competition than after one day indicating that non-producer couldn't invade. In 48-hour stationary phase producer populations, the non-producer significantly decreases in frequency and in some cases go extinct ($\omega < 1$,

607 $\omega = 0$) because the rate of producer pvd production is almost negligible, and pyoverdine
608 per cell is significantly lower at 48 than at 24 hours.

609 **Table S2.** Model results for the change in growth rates of each strain over time with
610 strain type as an interaction effect. The data is Log (y + (1-min(y))) transformed. The
611 producer and non-producer growth rates are lower when in competition with
612 producers from later growth phases than competitions with producers from early
613 growth stages ($F_{3, 266} = 88.13$, $P < 2.2 \times 10^{-16}$). The producer growth rates decrease
614 less rapidly than the non-producer rate along the competitions with a growing
615 producer population.

616 **Table S3. Growth rates of the producer and non-producer strain during**
617 **competition.** R_1 is the growth rate between the start of competition and 24 hours later.
618 R_2 is the rate from the start of competition and 48 hours later and R_3 is the rate
619 between 24 and 48 hours of competition.

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