

Cheating and resistance to cheating in natural populations of the bacterium *Pseudomonas fluorescens*

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Bacteria perform cooperative behaviors that are exploitable by noncooperative cheats, and cheats frequently arise and coexist with cooperators in laboratory microcosms. However, evidence of competitive dynamics between cooperators and cheats in nature remains limited. Using the production of pyoverdine, an iron-scavenging molecule, and natural soil populations of *Pseudomonas fluorescens*, we found that (1) nonproducers are present in the population; (2) they co-occur (<1cm³) with pyoverdine producers; (3) they retain functional pyoverdine receptors; and (4) they can use the pyoverdine of on average 52% of producers. This suggests nonproducers can potentially act as social cheats in soil: utilizing the pyoverdine of others while producing little or none themselves. However, we found considerable variation in the extent to which nonproducers can exploit producers, as some isolates appear to produce exclusive forms of pyoverdine or kill nonproducers with toxins. We examined the consequences of this variation using theoretical modeling. We found variance in exploitability leads to some cheats gaining increased fitness benefits and others decreased benefits. However, the absolute gain in fitness from high exploitation is lower than the drop in fitness from low exploitation, decreasing the mean fitness of cheats and subsequently lowering the proportion of cheats maintained in the population. Our results suggest that although cooperator-cheat dynamics can occur in soil, a range of mechanisms can prevent nonproducers from exploiting producers.

KEY WORDS: Bacteria, Cooperation, Cheating, Pyoverdine, Social Evolution, Soil.

Cooperation is prevalent at all levels of biological organization: genes cooperate to produce cells, cells cooperate to produce organisms, and organisms cooperate to form societies (Bourke 2011). Yet cooperative behaviors are open to exploitation by noncooperative “cheats”: individuals that increase their own fitness by exploiting the cooperative behaviors of others (Ghoul et al. 2014a). Theory suggests that a number of factors allow cheats and cooperators to coexist through frequency-dependent selection. Cheats can have an advantage when rare if this lets them better exploit cooperators (Ross Gillespie et al. 2007). Conversely, cooperators have an advantage when rare if the behavior provides some direct benefit, if they gain preferential access to cooperative goods, or if populations are spatially structured (Gore et al. 2009; Frank 2010; Koschwanez et al. 2011).

However, there remains a lack of evidence that cooperators and cheats coexist in natural settings (Harrison 2013; Jones et al. 2015; Riehl & Frederickson 2016). First, many studies examining cooperator-cheat dynamics make use of artificially created cheats (Greig & Travisano 2004; Griffin et al. 2004; Diggle et al. 2007; Sandoz et al. 2007; Gore et al. 2009). The extent to which similar cheats arise in natural populations remains unclear (Sachs et al. 2010; Bozdogan & Greig 2014; Jones et al. 2015). Second, while less cooperative or noncooperative individuals are found in natural populations, this may reflect environments where there is selection for less cooperation, rather than cheating per se (Ghoul et al. 2014a,b; Jones et al. 2015). Third, there is a range of mechanisms that could prevent potential cheats from successfully exploiting the cooperative behavior of others (West et al. 2007). These

include preferentially directing cooperation toward relatives, and directing harming behaviors toward nonrelatives or noncooperators (Griffin & West 2003; Kiers et al. 2003; Sharp et al. 2005). For example, bacteria produce bacteriocins (toxins) that could potentially prevent cheats from exploiting cooperative behaviors (Riley & Gordon 1999; Wang et al. 2015).

We examine the extent to which cheating and the above complexities occur in natural populations of the soil bacterium *P. fluorescens*. The cooperative trait we examine is the production of an extracellular iron-scavenging molecule, pyoverdine (Varma & Chincholkar 2007). While iron is essential for bacterial growth, levels of soluble iron in soils are often low (Wandersmann & Delepelaire 2004, Lindsay & Schwab 1982, Colombo et al. 2014). Iron-starved cells secrete pyoverdine into the environment where it binds Fe(III) and is transported into the cell where Fe(III) is dissociated and reduced to bioavailable Fe(II) (Greenwald et al. 2007). However, after binding iron, pyoverdine molecules can in principle be taken up by any other cells with an appropriate receptor, not just the cell that produced them (West & Buckling 2003). Pyoverdine production has been studied extensively in the laboratory, where it has been demonstrated to be a cooperative trait open to exploitation by nonproducing cheats (Griffin et al. 2004; Jiricny et al. 2010; Ghoul et al. 2014b; Kummerli & Ross Gillespie 2013).

We examined isolates of *P. fluorescens* from soil at multiple sites in a local park and asked: (1) Do pyoverdine nonproducers exist in natural soil populations? (2) Can nonproducers exploit the pyoverdine produced by other cells in the population? One reason they might not be able to is if different strains produce different types of pyoverdine that require a specific receptor—this specificity could be thought of as a form of kin discrimination. (3) Do the bacteriocins produced by cells play a role in preventing pyoverdine exploitation? Bacteriocins tend to kill unrelated isolates, and so could be one mechanism of preventing unrelated lineages from exploiting cooperative behaviors (Inglis et al. 2009; Strassmann & Queller 2014). Finally, our experimental work revealed that nonproducers could exploit pyoverdine produced by some isolates and not others. Consequently, we used theoretical modeling to ask: (4) How does variation in the ability of cheats to exploit cooperators influence whether cheats and cooperators can coexist in the same population?

Material and Methods

SITE DESCRIPTION AND SAMPLING

We collected isolates of the *P. fluorescens* group from soil samples at multiple sites in University Parks, Oxford, as described previously (Bruce et al. 2017). The soil in the park is an alluvial sandy loam with a pH of ~ 7.8 . Briefly, soil samples were collected from eight sites in undisturbed regions of the park and at

each site we sampled a 1 m transect consisting of four patches: an initial patch (patch 0) and patches 1 cm (patch 1), 10 cm (patch 2), and 100 cm (patch 3) from the initial patch. We randomly selected seven isolates from each patch in each site, giving 224 isolates from 32 patches for use in the study. All isolates were sampled, cultured, isolated, and frozen in under 48 h to minimize the potential for evolution in the laboratory and all subsequent tests were performed using these freezer stocks.

PYOVERDINE SCREEN OF ISOLATES

We screened all isolates in the collection for the ability to produce pyoverdine, allowing us to identify low/nonproducing isolates and reveal any variation in pyoverdine production across the population. To do this, we cultured isolates from freezer stocks in 2 mL of Kings B (KB) [20 g protease peptone No3 (Beckton Dickinson Ltd., Oxford, UK), 10 mL glycerol, 1.5 g $K_2HPO_4 \cdot 3H_2O$ and 1.5 g $MgSO_4 \cdot 7H_2O$ per litre of dH_2O] media in 24-well plates at 25 °C, shaken overnight at 200 rpm. Cell density was standardized and diluted to an optical density of 0.2 (A_{600}) with M9 minimal media (6.8 g Na_2HPO_4 , 3 g KH_2PO_4 , 0.5 g NaCl and 10 g NH_4Cl per litre of dH_2O) and inoculated into 96-well plates containing iron-limited casamino acid media [5 g casamino acids, 1.18 g $K_2HPO_4 \cdot 3H_2O$, 0.25 g $MgSO_4 \cdot 7H_2O$, per litre of dH_2O supplemented with the iron chelator, human apo-transferrin 100 $\mu g mL^{-1}$ (Sigma) and 20 mM $NaHCO_3$] (six replicates per isolate, 200 μL of media, and 2 μL of bacterial culture). We then incubated cultures for 48 h at 25 °C before measuring cell density (A_{600}) and fluorescence (400/460 nm excitation/emission, cut-off at 475 nm) using a fluorimeter (SpectraMax M2, Molecular Devices, California, USA). Pyoverdine production per cell was calculated as the relative fluorescent units (RFU) of a sample corrected for cell density. Isolates that grew poorly in iron-limited media and produced very little or no pyoverdine (those isolates with average values below the fifth percentile for pyoverdine production per cell) we classified as nonproducers. We also cultured isolates that we classified as nonproducers in iron-replete CAA media under the same conditions, to confirm that iron limitation and a failure to produce sufficient pyoverdine are responsible for poor growth in iron-limited media.

MLST OF ISOLATES

We used multilocus sequence typing (MLST) of three housekeeping genes to provide a measure of genetic similarity between isolates; allowing us to explore the relationship between successful cross-feeding and genetic similarity, and also the phylogenetic origins of any pyoverdine nonproducing isolates.

DNA extraction and PCR

We extracted genomic DNA using the Wizard Genomic DNA purification kit (Promega, Wisconsin, USA) and amplified three

housekeeping genes (*gyrB*, *RpoB*, and *RpoD*; Table S1.) for each isolate. All reactions were performed in 50 μL volume containing 1 U of DreamTaq polymerase (Thermo Fisher Scientific, Massachusetts, USA), 5 μL of DreamTaq Buffer, 100 mM of each primer, 0.2 mM of each dNTP, 40.75 μL of ddH₂O, and 20 ng of DNA template. We used the following cycling conditions: 95 °C for 3 min, then 35 cycles of 95 °C for 30 sec, 58 °C/60 °C/64.5 °C for 30 sec, 72 °C for 45 sec, and a final extension of 72 °C for 5 min (annealing temperatures; Table S1).

Sequencing and analysis

We purified PCR products before they were Sanger sequenced by SourceBioscience (Nottingham, U.K.) with the respective primer pairs used for PCR amplification used as forward and reverse sequencing primers (Table S1). The quality of the resulting sequences was checked using Geneious Pro (Biomatters Ltd, Auckland, New Zealand) generating a consensus sequence for each isolate before trimming and aligning the sequences to obtain an identical length sequence for each gene in each isolate. We then constructed a concatemer of all three genes for each isolate; from which we calculated pairwise genetic distances between isolate pairs using the Jukes-Cantor model and constructed a neighbor-joining tree using MEGA6 (Tamura et al. 2013).

TEST OF RECEPTOR FUNCTION

We tested isolates that we had identified as pyoverdine nonproducers for their ability to use purified pyoverdine to sequester iron from the environment, allowing us to assess their potential to act as cheats. Pyoverdine nonproducer cells are completely incapable of growth in iron-limited media supplemented with purified pyoverdine if they lack appropriate pyoverdine receptors (Ghysels et al. 2004). To do this, we cultured nonproducers in 2 mL of KB media in a 24-well plate at 25 °C, shaken overnight at 200 rpm. Cell density was standardized and diluted to an optical density of 0.2 (A_{600}) using M9 minimal media before inoculating 2 μL into 96-well plates containing 200 μL of iron-limited media (12 replicates). Half of each isolates replicates were supplemented with purified pyoverdines from *P. fluorescens* (Sigma, Missouri, USA) to approximately 800 RFU and wells containing supplemented and nonsupplemented iron-limited media, but free of cells, served as negative controls. We incubated cultures at 25 °C for 24 h and then measured cell density (A_{600}) in supplemented and unsupplemented replicates.

PYOVERDINE CROSS-FEEDING EXPERIMENTS

We tested nonproducing isolates for their ability to use the pyoverdine-containing supernatant of other isolates to increase their growth, allowing us to determine whether nonproducers are potentially able to exploit pyoverdine producers in the population.

First, we extracted supernatant from cultures of pyoverdine producing isolates. Pyoverdine producers were cultured in 2 mL of KB media in 24-well plates at 25 °C, shaken overnight at 200 rpm, and standardized cell density to an optical density of 0.2 (A_{600}) using M9 minimal medium before inoculating 60 μL into 6 mL of iron-limited CAA in 30-mL glass vials. Cultures were incubated statically for 48 h at 25 °C before being passed through a 0.22 μm filter to remove bacterial cells. We passed the cell-free supernatants through a centrifugal unit containing a 3 kDa membrane (EMD Millipore, Massachusetts, USA) to remove cellular debris and large extracellular products, such as bacteriocins, while retaining pyoverdine molecules (Cordero et al. 2012). Purified supernatants were stored at -20 °C until required for growth enhancement experiments.

We then assayed for the effects of the supernatant extracted as described above, on growth of nonproducing isolates. Nonproducers were cultured in 2 mL of KB media in 24-well plates at 25 °C, shaken overnight at 200 rpm before standardizing cell density to an optical density of 0.2 (A_{600}) with M9 minimal media. Two microliters of standardized culture was inoculated into 96-well plates containing 180 μL of iron-limited media which we supplemented with 20 μL of purified supernatant from a pyoverdine-producing isolate. Each nonproducer (11 isolates) was grown in iron-limited CAA media supplemented with the supernatant of each isolate in the collection (224 growth enhancement tests per nonproducer, five replicates each) and alone in 200 μL of iron-limited CAA media. We incubated cultures for 24 h at 25 °C before measuring final cell density at A_{600} .

ASSESSING NONPRODUCER SUSCEPTIBILITY TO PRODUCERS BACTERIOCINS

Competitive behaviors that harm or kill competitors may potentially protect pyoverdine producers from exploitation. We assayed whether potential cheats in our study were more likely to be inhibited by the bacteriocins of producing strains they were able to exploit, compared to those they were unable to exploit. First, we extracted bacteriocin-containing supernatants from each isolate in the population by culturing isolates for 24 h in 6 mL of KB media (23 °C at 200 rpm), measuring cell density and standardized each culture to an optical density of ~ 0.3 (A_{600}). These cultures were then diluted 10-fold in fresh KB media and incubated shaking for 24 h at 23 °C. We then centrifuged cultures at 6861 rpm for 10 min, obtaining a clear, cell-free supernatant by filter sterilizing with a 0.22 μm filter and storing at -20 °C until required.

We then randomly selected 10 isolates that each pyoverdine nonproducer was capable of exploiting and 10 isolates each was incapable of exploiting and determined the ability of these isolates to inhibit each nonproducers growth with bacteriocins. We spotted KB agar plates with bacteriocin-containing supernatants, spread a lawn of each pyoverdine nonproducer over the plate,

and recorded which supernatants inhibited which nonproducer. Assays were carried out in triplicate. We spotted KB agar plates with 15 μL of supernatant and allowed the spots to dry at room temperature. We cultured pyoverdine nonproducers from freezer stocks for 24 h in 6 mL of KB media (23 °C at 200 rpm). Cell density was standardized to an optical density of ~ 0.1 (A_{600}) and diluted 10-fold in M9 before 70 μL of culture was spread onto the supernatant-spotted KB agar plates. We incubated plates at 23 °C for ~ 14 h, or until a uniform lawn of bacterial growth was visible, checked the plates for zones of inhibition on and around the supernatant spots and recorded whether a lawn was inhibited by a particular supernatant. Inhibition was recorded as a binary response (one for inhibition, zero for no inhibition).

STATISTICAL ANALYSIS

Of the 11 isolates we previously designated as nonproducers, we merged and averaged the data from isolates that were genetically identical at three housekeeping genes, resulting in eight independent samples. We wished to determine whether a nonproducer could use the pyoverdine of other producers in the population. To do this, we tested for significant differences between these eight nonproducers' growth in iron-limited media and in iron-limited media supplemented with the supernatants of pyoverdine producers using linear models, with final cell density as the response variable and supernatant as a categorical explanatory variable. If a nonproducer grew significantly better in supplemented iron-limited media than it did alone, we considered this successful use of the producers' pyoverdine. We recorded which supernatants nonproducers successfully used in a binary 8×224 matrix.

We carried out all statistical analyses in the R statistical environment. Except where stated, we carried out standard analyses (ANOVA, GLM, T-test etc.) assuming normal errors. All analyses using generalized linear mixed models (GLMM) included the identity of the nonproducer as a random effect, to account for the fact that we have multiple nonproducers in the population.

Results

VARIATION IN PYOVERDINE PRODUCTION

We identified 11 isolates (4.5% of the population) with values below the fifth percentile value for pyoverdine production per cell, fulfilling our criteria for consideration as nonproducers, and hence potential cheats (Fig. 1A). These isolates grow well under iron-replete conditions, indicating that the availability of iron limits the growth of nonproducers in this media (Fig. 1B). Across all isolates, growth in iron-limited media was highly correlated with pyoverdine production (LM: $t = 8.543$, $P = 2.19e^{-15}$).

Pyoverdine production per cell varied significantly both between patches (Fig. 1B, ANOVA: $F = 2.228$, $P = 0.0005$)

and between transects (ANOVA: $F = 3.015$, $P = 0.00484$) in the population. Significant differences between patches and between transects appear to be driven by transect I: patch I3 had significantly higher average levels of pyoverdine production per cell than three other patches and transect I had significantly higher levels of pyoverdine production per cell than two other transects (TukeyHSD test, Table S3). Isolates previously designated as nonproducers always co-occurred with pyoverdine producers and were found at 8 of the 32 patches sampled. The neighbor-joining tree suggests that nonproduction has arisen at least six times in the population (Fig. 1B, 2A.).

72% of isolates ($n = 224$) in the collection were genetically distinct, with the pairwise genetic distance between isolates ranging from 0.000 to 0.066 and averaging 0.04.

NONPRODUCERS HAVE RETAINED RECEPTOR FUNCTION

Nonproducers grown in iron-limited media supplemented with purified pyoverdine all grow significantly better than when cultured in iron-limited media (Fig S1, Table S3), suggesting that isolates have retained functional pyoverdine receptors despite no longer producing pyoverdine.

CROSS-FEEDING ASSAYS REVEAL DIVERSE PATTERNS OF SUCCESSFUL AND UNSUCCESSFUL SUPERNATANT USE

The growth of nonproducers in iron-limited media significantly increased when supplemented with the supernatant of on average 52% of pyoverdine-producing isolates. However, the cross-feeding assays reveal diverse patterns of successful and unsuccessful use of producers' supernatant to increase growth (Fig. 2A, C). A minority of pyoverdine producers supernatants significantly increased the growth of all nonproducers (12%), some supernatants increased the growth of none of the nonproducers (23%) and the remainder (65%) of supernatants significantly increased the growth of some nonproducers but not others (Fig. 2B).

There are at least three possible explanations for this observation: (1) Although we have demonstrated that iron is a significant growth-limiting factor in this media (Fig. 1B), we have not demonstrated that iron is the *only* limiting factor: it may be possible that metabolites other than pyoverdine in the supernatant can increase or decrease the growth of nonproducers. It has also been suggested that siderophores may act as a trace metal buffers, increasing the availability of iron to nonproducers without requiring the appropriate receptor. However, pyoverdine nonproducer cells are incapable of growth in iron-limited media supplemented with purified pyoverdine if they lack appropriate pyoverdine receptors, suggesting pyoverdine does not act as a trace metal buffer (Ghysels et al. 2004). (2) The amount of pyoverdine in producers

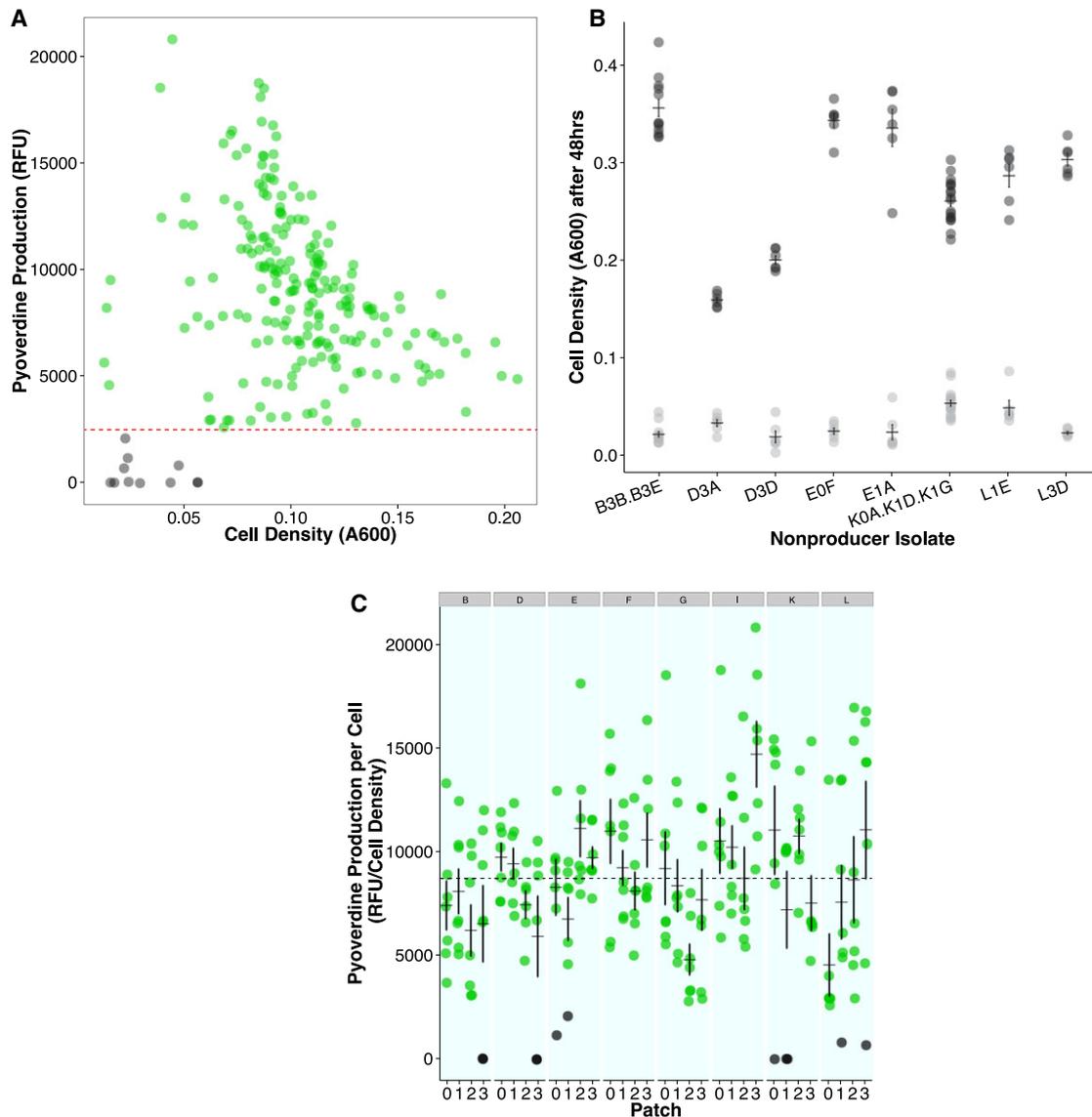


Figure 1. Pyoverdine production per cell and growth of isolates in iron-limited media. (A) Isolates falling below the fifth percentile for pyoverdine production per cell were considered non producers. The horizontal red dashed line represents the fifth percentile for pyoverdine production per cell. Gray circles represent isolates classified as nonproducers and green circles represent pyoverdine producers; values are the mean of six replicates. (B) Growth of nonproducer isolates in CAA media and in iron-limited CAA media. All nonproducer isolates grow well in CAA media but show significantly reduced growth in iron-limited CAA media. Light grey circles represent nonproducers growth in iron-limited CAA media and dark grey circles represent nonproducers growth in CAA media. (C) Pyoverdine production per cell varies significantly between patches and between transects, and nonproducers co-occur with pyoverdine producers. Gray circles represent isolates classified as nonproducers and green circles represent pyoverdine producers. Horizontal bars are average per cell production for each patch \pm SE.

supernatants varies and this might explain variation in exploitability, that is, addition of supernatants resulting in no significant increase in growth might simply contain very little pyoverdine. However, we compared the levels of pyoverdine in producer supernatant for cross-feeding experiments that resulted in a significant growth increase versus no significant growth increase for nonproducers (Fig. S2), and found that the distributions do not significantly differ from each other (Kolmogorov-Smirnov test,

$D = 0.1818$, P -value = 0.9934). This suggests that the amount of pyoverdine in the supernatant does not explain the variation in exploitability during cross-feeding assays. (3) The diverse patterns of successful and unsuccessful use of producer supernatants may occur because the population produces multiple forms of pyoverdine and these are not equally accessible to other isolates in the population. There is evidence that multiple different forms of pyoverdine are produced by *Pseudomonads* and that strains

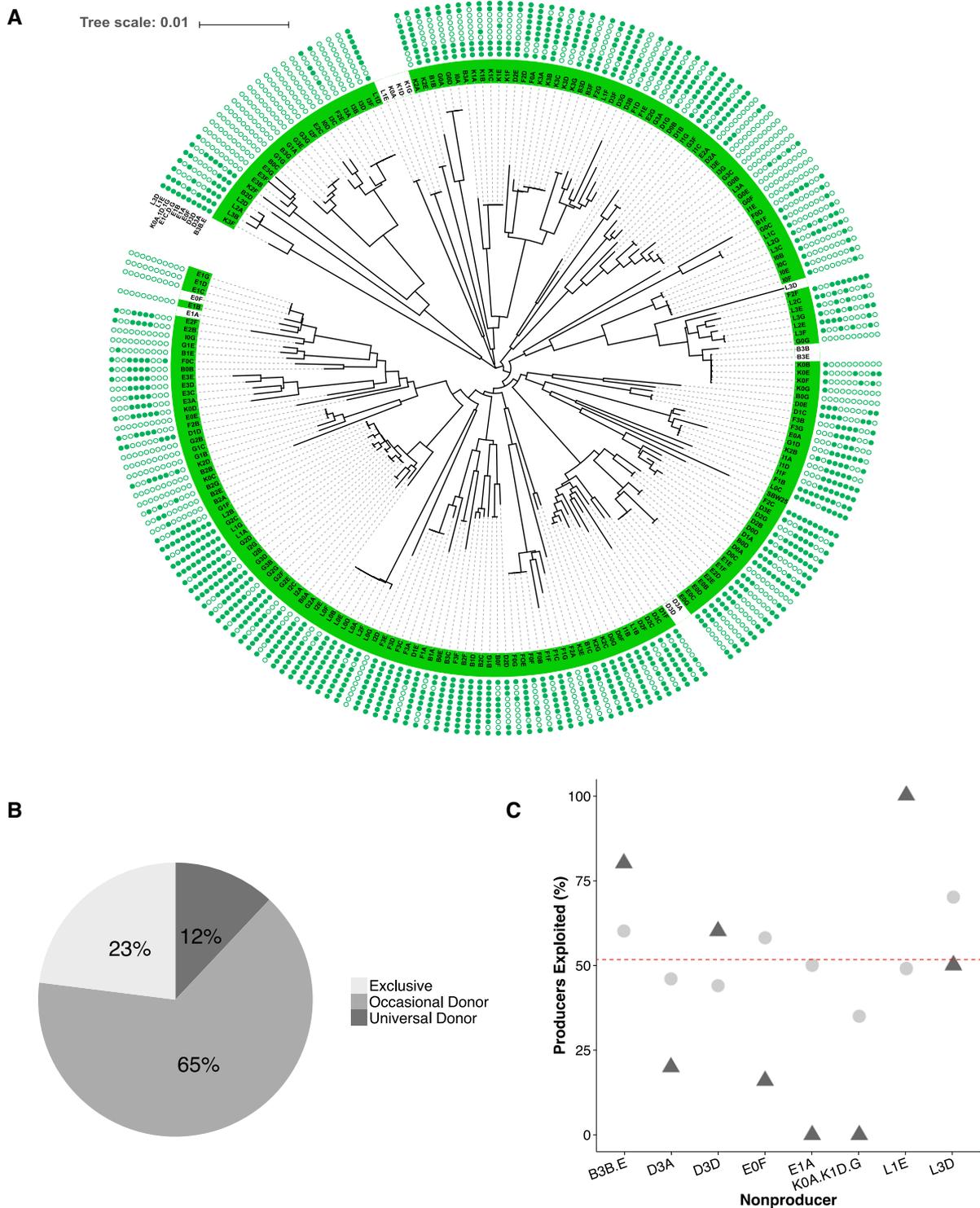


Figure 2. (A) Phylogenetic tree of all isolates in the population. The phylogenetic tree was constructed from a concatenation of three housekeeping genes (*gyrB*, *recA*, and *rpoB*) using the Neighbor-joining method and the Jukes-Cantor model of nucleotide substitution with 1000 bootstraps. Pyoverdine-producing isolates labels are highlighted in green, nonproducer labels are left blank. Each pyoverdine producing isolate has eight circles associated with it, each representing the ability of a specific nonproducer to use that isolates pyoverdine. Filled circles represent successful use of the isolates pyoverdine by the nonproducer in the cross-feeding assays and empty circles represent unsuccessful use. (B) Percentage of pyoverdine producers that act as universal donors, partial donors, and nondonors to pyoverdine nonproducers. (C) The percentage of producers in the population that nonproducers can exploit. Circles represent the percentage of isolates in the population that individual nonproducers can exploit, triangles represent the percentage of local, co-occurring isolates (from the same patch) that nonproducers can exploit.

vary in their ability to exploit the pyoverdine produced by others (Meyer 2000; Meyer et al. 2002).

GENETIC SIMILARITY PREDICTS ABILITY OF NONPRODUCERS TO USE PRODUCERS PYOVERDINES

We found that genetic similarity between nonproducers and producers predicted the ability of nonproducers to successfully use the producers' pyoverdine. Nonproducer isolates were more frequently able to use the pyoverdine of genetically similar producers than those of genetically more dissimilar isolates (Fig. 3A, GLM: $t = -2.846$, $P = 0.006$). The average genetic distance between nonproducers and co-occurring producers (from the same patch) was 0.038, ranging from 0.015 to 0.065.

NONPRODUCERS VARY IN THEIR ABILITY TO USE THE PYOVERDINE OF LOCAL PRODUCERS

Nonproducer isolates were no less likely to use the pyoverdine of co-occurring producers (from the same patch) than producers from the population as a whole (Fig. 3B, GLMM, $z = 1.529$, $P = 0.126$). Specifically, nonproducers could successfully use the pyoverdine of approximately 41.5% of co-occurring isolates and 52% of all other isolates in the population (Fig. 3B). However, the frequency of successful usage varies considerably between different nonproducers when interacting with co-occurring pyoverdine producers. This ranges from a complete inability of some nonproducers to use the pyoverdine of co-occurring producers, through to successful use of all surrounding producers pyoverdine by others. The variance in the frequency of exploitation was significantly greater when interactions occur between local, co-occurring producers and nonproducers (Fig. 3B, $F = 11.888$, $df = 7$, $P = 0.002075$).

PYOVERDINE PRODUCERS CAN INHIBIT NONPRODUCERS USING BACTERIOCINS

Overall, we found that in 3.5% of the interactions we tested between pyoverdine producers and nonproducers, the pyoverdine producers also released a bacteriocin that inhibited the nonproducer, and hence would have prevented the nonproducers from successfully cheating. The likelihood of a pyoverdine producer also releasing a bacteriocin that inhibited the nonproducer did not significantly vary dependent upon whether the nonproducer could use the pyoverdine from that producer (GLMM, $z = 0.452$, $P = 0.651$).

EXPLOITABILITY AND CHEAT-COOPERATOR COEXISTENCE

We found that there is significant variation in the extent to which potential cheats can exploit cooperators, which is caused by variation in both pyoverdine exploitability and bacteriocin pro-

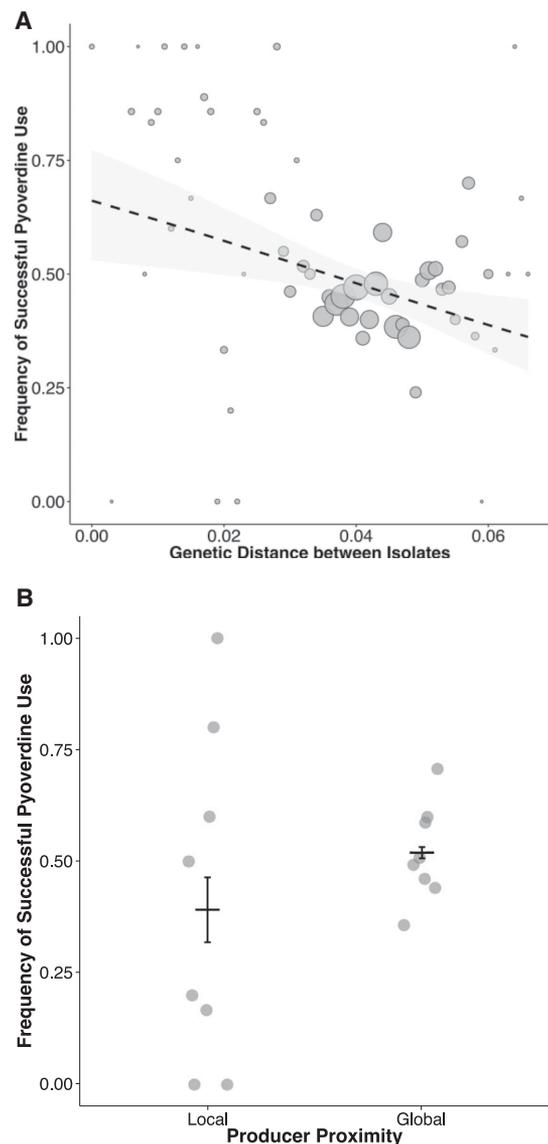


Figure 3. The effects of genetic similarity and proximity on ability to use pyoverdine. (A) Nonproducer isolates can successfully use the pyoverdine of genetically similar isolates more frequently than genetically dissimilar isolates. The size of the points reflects the number of interactions: smallest points indicate <5 interactions and largest points indicate >150 interactions ($n = 1802$). (B) Nonproducer isolates are no less likely to use the pyoverdine of co-occurring producers than from producers from the rest of the population. Points reflect the proportion of successful pyoverdine use for each nonproducer when grown with co-occurring isolates (local) or all other isolates in the population (global). Horizontal bars are average proportion of successful pyoverdine use \pm SE.

duction (Fig. 2A–C). In this section, we examined theoretically the influence of such variation for whether cheats and cooperators will coexist. Our hypothesis is that, because the benefits from cooperation are often diminishing (Ross-Gillespie et al. 2007; Gore et al. 2009; Cornforth et al. 2012, Frank 2011), variance

in exploitability will decrease the relative fitness of cheats, and hence make it harder for them to coexist with cooperators.

We model a simple public goods scenario that could be applicable to a range of microbial traits (West et al. 2007). We assume a large population of cells composed of cooperators and cheats. The cooperators invest a fraction $q \in [0, 1]$ of their resources into the production of an extracellular factor that provides a benefit to the local population of cells (public good). We assume that the benefit from a fraction $1 - \lambda$ of the dispersed good is returned to the cell that produces it, and that the remaining fraction λ goes to other cells. The parameter λ determines how well the good disperses and is shared—a lower value of λ implies less shared and, in the extreme of $\lambda = 0$, we have a private good. If bacteria gain a greater than random share of any extracellular factor that they produce, this can lead to the fitness of cheats being frequency dependent (Ross-Gillespie et al. 2007; Gore et al. 2009). In contrast, the cheats do not produce the extracellular factor. We assume that the population is composed of a fraction x of cooperators and a fraction $1 - x$ of cheats.

We allow the benefit obtained from the extracellular factor to be nonlinear, as determined by the shape parameter α (>0) (Fig. 4A). We assume $\alpha < 1$, such that the synergistic effect is diminishing, as is thought to be the case for extracellular factors such as iron scavenging siderophore molecules (Ross-Gillespie et al. 2007). This gives the following personal fitness for a focal cooperator cell:

$$W_C = (1 - q) \times ((1 - \lambda)q + x\lambda Q)^\alpha, \quad (1)$$

where Q is the average production of the dispersed good by other cooperator cells (others-only; Pepper 2000). The first term quantifies the private cost due to production of the good ($1 - q$) and the second term captures the benefit from the dispersed good (produced by the focal cell, and other cells).

We assume that there is variance in how well the cheats can exploit the extracellular factor produced by different strains of cooperators. For simplicity, we assume that cheats can find themselves in two scenarios: with probability p , they are relatively good at exploiting the extracellular factor in their local environment ($E_0 + \Delta E$), and with probability $1 - p$, they are relatively bad at exploiting the extracellular factor in their local environment ($E_0 - \Delta E$). We assume that each environment is equally likely ($p = 1/2$). Thus, the parameter E_0 is the expected exploitation of a cheat and ΔE quantifies the variance in cheater exploitation, which will be high if there is a large difference in how well cheats can exploit some cooperative strains over others. This leads to the following personal fitness of a focal cheater cell:

$$W_D = p(x\lambda Q(E_0 - \Delta E))^\alpha + p(x\lambda Q(E_0 + \Delta E))^\alpha, \quad (2)$$

where the first and second terms are the realized fitness in the low and high exploitation environments, respectively. We assume that cooperators throughout the population are monomorphic with respect to cooperation and thus that $Q = q$, which we hold fixed in this analysis.

As the proportion of cheats in the population increases, the proportion of cooperators necessarily decreases and, as a result, the background density of the dispersed good drops for both cheats and cooperators (Fig. 4B). Cheater fitness therefore increases as cheaters become more rare and decreases as cheats become more common in the population (frequency dependence; Ross-Gillespie et al. 2007; Gore et al. 2009).

We ask how the variation in the extent to which cheats can exploit cooperators (ΔE) influences the equilibrium proportion of cheats ($1 - x^*$). We set cooperator and cheat fitness as equal and solve for x^* , giving:

$$1 - x^* = \max\left(\frac{\lambda\theta p^{1/\alpha} - (1 - q)^{1/\alpha}}{\lambda(\theta p^{1/\alpha} - (1 - q)^{1/\alpha})}, 0\right), \quad (3)$$

where $\theta = ((E_0 - \Delta E)^\alpha + (E_0 + \Delta E)^\alpha)^{1/\alpha}$.

Discussion

Our results suggest that in natural bacterial populations, pyoverdine nonproducers can potentially act as social cheats. We found that 4.5% of all isolates in the population do not produce pyoverdine, and these nonproducers were found in over a quarter (28%) of the 32 patches sampled (Fig. 1A, C). However, nonproducers can exploit on average only 52% of pyoverdine producers in the population and only 41.5% of local, co-occurring producers (Fig. 2A, C). Our cross-feeding assays suggest different forms of pyoverdine are produced in the population (Fig. 2A, B). In 3.5% of interactions, pyoverdine producers produced toxins that killed nonproducers, contributing to variation in the extent to which nonproducers can exploit producers. Furthermore, our model suggests that this variability in exploitability may reduce the mean fitness of cheats; leading to a lower proportion of cheats being maintained in the population (Fig. 4A).

Pyoverdine production is energetically costly and its ubiquity suggests isolates are iron limited in their natural environment (Dumas et al. 2013). We did not specifically measure iron levels in soil samples, as our intent here was to test whether social interactions can potentially explain variation in pyoverdine production, not to assess isolates responses to environmental iron availability. Pyoverdine production can be lost if alternative iron sources are available, but these nonproducers have retained functional pyoverdine receptors which are costly even when expressed at low levels (Marvig et al. 2014; Nguyen et al. 2014; Andersen et al. 2015). *Pseudomonas fluorescens* isolates are also known

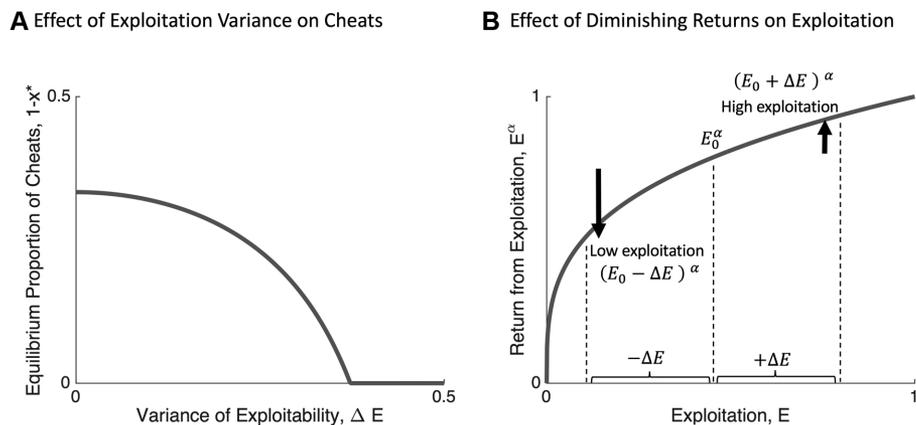


Figure 4. (A) We plot the equilibrium proportion of cheat cells ($1 - x^*$) in the population as a function of variance of exploitability (ΔE), where high variance of exploitability means that cheater cells can exploit the extracellular factor released by cooperator cells in some local environments much better than in others. Here, we assume that the cooperators invest a fraction 0.5 of their private resources toward production of the extracellular factor, that the nonlinear return from the extracellular factor is diminishing (with shape term, $\alpha = 0.5$) and that mean exploitation is 0.5. (B) We show the relative return to cheater cells from exploitation in different local environments. If there is no cheat exploitation variance, then cheats receive a relative return of E_0^α , where E_0 is the expected exploitation and α quantifies the nonlinear return from the extracellular factor, which is assumed to be diminishing ($\alpha = 0.3$, here). If there is a nonnegligible variance in cheat exploitation, then we quantify this (symmetric) variance with the term ΔE . Cheats then experience two local environments with equal probability: a low exploitation environment where the relative return is $(E_0 - \Delta E)^\alpha$ and a high exploitation environment with relative return $(E_0 + \Delta E)^\alpha$. Due to the nonlinear fitness return, the low exploitation environment leads to a larger absolute change in fitness than the high exploitation environment (compare size of the arrows), and so an increased variance leads to a drop in the relative fitness of cheats.

to carry multiple different receptors enabling the use of different structural forms of pyoverdine (Moon et al. 2008; Hartney et al. 2011; Ye et al. 2014). These observations suggest that nonproducers have lost production not because the trait is redundant in natural settings, but because they can extract sufficient iron from the pyoverdine of local producers. Nonproducing cheats can invade and persist in phylogenetically diverse, and spatially structured laboratory microcosms, and siderophore nonproducers have been identified in *Pseudomonas aeruginosa* populations in the CF lung and in marine populations of *Vibrio Spp.* (Joussett et al. 2013; Andersen et al. 2015; Lujan et al. 2015; Cordero et al. 2012). We do not specifically test the ability of nonproducers to invade populations of pyoverdine producers under laboratory conditions, as (1) these conditions are far removed from those in natural soil environments and (2) extrapolating the results of laboratory fitness assays to “real world” scenarios is problematic: demonstrating invasion of nonproducers in the laboratory would imply this happening in soil, even if it was not and vice versa. However, we do provide evidence that potential cheats can arise and persist in natural soil populations of bacteria, suggesting they may be a pervasive feature of natural bacterial populations. Cooperator-cheat dynamics are unlikely to be confined to production of siderophores, as bacteria and other microbes perform a range of potentially exploitable social traits (West et al. 2006). Cheating may not even be restricted to nonproducers: pyoverdine

production is a continuous trait and we find co-occurrence of low- and high-level producers, allowing the possibility that low producers can exploit higher level producers (Jiricny et al. 2010; Ghoul et al. 2014b). Cheating behaviors have also been observed in a number of other natural systems including fruiting body formation in social amoeba, and rhizobia plant, plant pollinator, and cleaner fish mutualisms (Strassmann et al. 2000; Bronstein et al. 2004; Sachs et al. 2010; Bshary & Gutter. 2002).

While nonproducers can still extract iron from pyoverdine, we find that they can exploit on average only 52% of pyoverdine producers in the population. This variability contrasts sharply with most previous studies, where cheats are competed against isogenic cooperators they can exploit freely (Greig & Travisano 2004; Griffin et al. 2004; Diggle et al. 2007; Sandoz et al. 2007; Gore et al. 2009). In natural populations, social interactions occur between phylogenetically diverse isolates that may differ considerably in the trait of interest, and many others (Stefanic et al. 2012; Stefanic et al. 2015; Kraemer et al. 2016). In our populations, we find that nonproducers are more likely to exploit closely related isolates than they are genetically dissimilar isolates (Fig. 3A). This suggests that potential cheats will be at an initial advantage when they arise in the population, as they will be surrounded by exploitable close relatives, but may fare less well as they encounter genetically different strains. Our results suggest that, if the ability to exploit is contingent on interacting with genetically

similar isolates, in genetically diverse natural populations, variation in the extent to which cheats can exploit cooperators is likely the norm.

We examined theoretically the consequences of this variation in exploitability for cheat-cooperator dynamics. We found that variation in exploitability reduced the mean fitness of cheats, and so led to a lower proportion of cheats being maintained in the population (Fig. 4A). In some cases, these can even lead to cheats being excluded from the population. The reason for this influence of exploitability stems from increased levels of cooperation leading to diminishing (nonlinear) benefits (Fig. 4B). Variance in exploitability leads to some cheats gaining increased benefits, and some cheats gaining decreased benefits. With traits such as siderophores, the benefits from increased cooperation are diminishing (Ross-Gillespie et al. 2007). This nonlinearity means that the absolute gain in fitness from high exploitation is lower than the absolute drop in fitness from low exploitation, a manifestation of Jensen's inequality for concave functions (Fig. 4B). Consequently, an increase in the variability of exploitation leads to a decrease in the mean fitness of cheats.

What factors underlie this variation in the extent to which nonproducers exploit producers? Natural *P. fluorescens* isolates are likely to produce different forms of pyoverdine, potentially preventing nonproducers from exploiting some producers. Pyoverdine is a structurally diverse molecule, with over 50 types identified in *Pseudomonads*, and is considered a "lock-key" or "gift-password" system involving receptor-molecule binding specificity (Meyer et al. 2007; Strassmann et al. 2011). This specificity allows for uninhibited uptake of pyoverdine provided isolates carry the appropriate receptor, and limited or no uptake if they do not (Meyer 2000; Meyer et al. 2002). This may explain why exploitation occurs more often between closely related isolates: they are more likely to harbor complementary receptors. The pyoverdine locus is under selection for diversification in *Pseudomonas aeruginosa*, with theory suggesting this may be a response to exploitation: cheats drive cooperators to produce new, exclusive structural forms of pyoverdine (Smith et al. 2005; Eldar 2011; Lee et al. 2012). This specificity allows cooperation to be directed preferentially toward close relatives, preventing cheats from exploiting the cooperative behavior (Inglis et al. 2016a).

We have focused on variation in the cooperative trait and its consequences for the extent to which potential cheats can exploit cooperators. However, we find that some cooperators produce bacteriocins that kill potential cheats. This suggests that other bacterial traits may influence the extent to which exploitation of cooperative behaviors can occur (Lyons et al. 2016). Most bacteria produce toxins that kill or inhibit the growth of closely related strains or species, and in *P. aeruginosa*, some of these toxins even target siderophore receptors (Baysse et al. 1999; Ghequire & De Mot 2014; Inglis et al. 2016b). There is little evidence

that bacteriocin production has evolved as a mechanism to punish noncooperators but susceptibility of cheats to cooperators toxins will likely prevent successful exploitation. While this may occur only in a small percentage of interactions, bacteriocins are only one of an arsenal of competitive mechanisms employed by bacteria to exclude nonrelatives (Ruhe et al. 2013; Unterweger et al. 2014). Our results suggest that competitive mechanisms will also contribute to variability in the extent to which cheats can exploit cooperators in natural populations.

AUTHOR CONTRIBUTIONS

JBB, SAW, ASG and HC designed the study, JBB carried out data collection, JBB, SAW and ASG analyzed the data, GAC and SAW conceived the model, JBB, GAC, SAW and ASG wrote the paper.

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DATA ARCHIVING

The doi for our data is <https://doi.org/10.5061/dryad.36g6r>

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Growth of non-producer isolates in iron-limited CAA media and in iron-limited CAA media supplemented with purified pyoverdine (Sigma).

Figure S2. Histograms of levels of pyoverdine in producer supernatant for cross-feeding experiments resulting in a significant growth increase versus no significant growth increase for non-producers. N=1656 cross-feeding assays.

Table S1. PCR and Sequencing Primers and Annealing temperatures.

Table S2. Tukey multiple comparisons of means.

Table S3. Comparison of non-producer growth in iron-limited media and iron limited media supplemented with purified pyoverdine.