Social Evolution in Natural Microbial Populations



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Declaration

I declare that this thesis was composed by myself and that the work contained herein is my own except where explicitly stated in the text. This work has not been submitted for any degree or professional qualification except as specified.

John Bruce, Michaelmas term 2017

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Publications and contributions

The following publications have arisen from this thesis and are presented in chapters 2 and 3:

- Bruce, J.B., West, S.A. and Griffin, A.S., 2017. Bacteriocins and the assembly of natural *Pseudomonas fluorescens* populations. *Journal of evolutionary biology*, *30*(2), pp.352-360.

- Bruce, J.B., Cooper, G.A., Chabas, H., West, S.A. and Griffin, A.S., 2017. Cheating and resistance to cheating in natural populations of the bacterium *Pseudomonas fluorescens*. *Evolution*, *71*(10), pp.2484-2495.

Chapters 3 and 4 are my own work, supervised by Stuart West and Ashleigh Griffin who contributed to the interpretation of the results and the manuscripts.

I contributed to two other publications that are included in the appendices:

- Ghoul, M., West, S.A., Johansen, H.K., Molin, S., Harrison, O.B., Maiden, M.C., Jelsbak, L., Bruce, J.B. and Griffin, A.S., 2015. Bacteriocin-mediated competition in cystic fibrosis lung infections. *Proceedings Royal Society B*, 282(1814) p. 20150972

- Ghoul, M., West, S.A., McCorkell, F.A., Lee, Z.B., Bruce, J.B. and Griffin, A.S., 2016. Pyoverdin cheats fail to invade bacterial populations in stationary phase. *Journal of evolutionary biology*, *29*(9), pp.1728-1736.

Abstract

To survive and thrive in their environment, bacteria engage in a multitude of cooperative and competitive behaviours. These behaviours often rely upon the secretion of extracellular public goods that are costly to produce but are essential for effective communication between cells, scavenging of iron and nutrients, protection from biotic and abiotic stresses and engaging in antagonistic competition. These cooperative and competitive behaviours have been studied extensively under controlled laboratory conditions but the extent to which social interactions can drive evolutionary change, shape population structure or promote and maintain diversity in natural bacterial populations remains unclear. In this thesis I explore the extent, form and consequences of social interactions in natural bacterial populations and also how a non-social bacterial trait, the production of functional amyloids, can shape cooperative and competitive dynamics.

Using natural soil populations of the bacterium *Pseudomonas fluorescens*, I explore (1) what drives bacteriocin-mediated competition in these populations by revealing the extent of bacteriocin production in the population and testing whether the strength of interaction is determined by spatial proximity, genetic relatedness or overlap in resource requirements. Using the same isolates I also reveal (2) the extent of potential public goods cheats in the population and demonstrate that potential cheats my be limited in their ability to proliferate by the presence of different forms of public good they cannot utilise. I then take an experimental approach to (3) investigate how a non-social bacterial trait, the production of functional amyloids, can shape cooperator-cheat dynamics in populations of *Pseudomonas aeruginosa*. I reveal that functional amyloids appear to bind public goods involved in iron acquisition and this may limit their

availability to public goods cheats. Finally, I (4) assess the contribution of functional amyloids towards defence against conspecific bacteriocins, revealing that their presence appears to contribute towards individual defence against these toxins but not collective defence.

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Chapter 1: Introduction

Social Evolution Theory

A behavior is social when it has fitness consequences both for the individual performing the behavior (the actor) and another individual (the recipient). These behaviours can have a net positive (+) or negative (-) effect on the fitness of both individuals and can be categorized accordingly (Hamilton 1964). Behaviours with positive effects on both the actor and the recipient (+/+) and those with a negative effect on the actor but a positive effect on the recipient (-/+) are termed 'mutually beneficial' and 'altruistic' respectively, and are usually discussed under the umbrella term of 'cooperative' behaviours (Sachs 2004, West et al. 2007). Selfish behaviours result in a positive outcome for the actor and a negative outcome for the recipient (+/-), while spiteful behaviours have a negative effect on both the actor and the recipient of the behaviour (-/-).

While all social behaviours can be placed into one of these categories, explaining the maintenance of certain behaviours is more challenging. In what Darwin referred to as the 'struggle for existence', an individual performing a selfish behaviour (+/-) that increases its fitness at the cost of another individual will be more likely to survive and reproduce (Darwin 1859). Mutually beneficial behaviours (+/+) are also maintained as performing the behavior has a positive fitness effect on the actor. While these behaviours can be explained through positive fitness effects for the actor, other behaviours require more detailed explanation. Both altruistic behaviours (-/+) and spiteful behaviours (-/-) have a negative effect on the fitness of the actor. In the Darwinian 'struggle for existence', how can a behavior that has a negative effect on reproductive success be maintained in a population?

W.D. Hamilton provided a means of confronting this problem (Hamilton 1963, 1964). Out of his desire to reconcile altruistic behaviours (-/+) with the Darwinian maxim that individuals should always maximize their personal reproductive success, he developed the concept of inclusive fitness theory. Hamiltons' great insight was to partition the classical measure of fitness (reproductive success) into a direct component (c) and an indirect component, weighted by the genetic relatedness (r) between the interacting individuals (rb). Hamilton's rule specifies the circumstances under which a gene for a particular social behavior will spread by natural selection, famously expressed as the inequality rb-c >0. This essentially recognizes that genes for a particular behavior can be spread directly through producing offspring (direct fitness) or through aiding reproduction of other carriers of the gene (indirect fitness).

Inclusive fitness theory thus provides a means of explaining the maintenance of behaviours that have a negative effect on the fitness of the actor. Altruistic behaviours (-/+) can be maintained when the benefit to the recipient of the behavior (b), weighted by relatedness (r) between the interactants is greater than the cost to the actor (c). Similarly, spiteful behaviours can be maintained when the cost to the actor (c), is less than the product of the negative benefit (b) and the negative relatedness (r) between individuals.

Social Microbes

Social interactions were traditionally studied in animal, bird and insect populations but microbes also engage in an incredible diversity of social behaviours: they help, protect, harm and cheat others in a multitude of different ways (West et al. 2007). Bacteria

cooperate to perform a range of functions, usually through the production of public goods. These goods are small molecules produced and secreted into the environment, where they can be utilized by all proximate cells and provide a collective benefit. These include quorum sensing molecules used to coordinate collective behaviours, siderophores and proteases used to extract iron and nutrients from the environment and the production of exopolysaccharides to form a protective matrix around cells (Diggle et al. 2007, Griffin et al. 2004, Sandoz et al. 2007, Irie et al. 2016). However, as with all cooperative behaviours, these can be exploited by 'cheats' that do not produce the public good but exploit its production by others (Ghoul et al. 2014). The vast majority of bacteria also produce bacteriocins: anti-competitor toxins that kill or inhibit the growth of other strains or species (Gordon et al. 2007). These bacteriocins often require cell lysis to be released into the surrounding environment, making bacteriocin production a rare example of a spiteful behavior in nature (Gardner et al. 2004).

The coming together of the fields of social evolution and microbiology has been an unqualified success. Bacteria have proved an incredibly tractable system with which to test evolutionary theory due to their short generation times, the ability to generate mutants and the level of control over experimental parameters such as population size and nutrient availability (Buckling et al. 2008). This has allowed successful testing of many predictions from social evolution theory: for example, how relatedness and the scale of competition influence the maintenance of cooperation in the face of social cheating and their influence upon spiteful bacteriocin production (Griffin et al. 2004, Inglis et al. 2009). Study of laboratory populations has also shed light on the mechanisms that promote the maintenance of cooperative behaviours: bacteria can tightly regulate production of public goods, public goods can be confined close to

producing cells or cells can promote spatial segregation to limit the ability of cheats to invade cooperative populations (Xavier et al. 2011, Ghoul et al. 2016, Kummerli et al. 2009, McNally et al. 2016, Mund et al. 2017). Ever greater understanding of the lives of bacteria will reveal the mechanisms, be they individual or population level traits, that contribute to the maintenance of cooperative and competitive behaviours in microbes.

As we observe the lives of bacteria in ever-greater detail, it becomes clear that social behaviours, such as the production of public goods, do not occur in isolation. Private bacterial traits, or indeed interactions between secreted public goods, can also shape cooperative and competitive dynamics between strains. For example, cells producing exopolysaccharides can prevent the diffusion of quorum sensing molecules away from producing cells (Emge et al. 2016, Yang et al. 2017). Similarly, exopolysaccharides produced by *Vibrio cholera* can limit the diffusion of digested chitin away from the digesting cells, ensuring the benefits of digestion accrue to those cells that paid the cost (Drescher et al. 2014). *Pseudomonads* also produce Fap fibrils: these are functional amyloids, needle-like protein structures that protrude from the cell surface and that contribute to biofilm stiffness and surface attachment (Dueholm et al. 2010). They also transiently bind multiple secreted public goods produced by *Pseudomonas aeruginosa*, such as quorum sensing molecules and pyocyanin (Seviour et al. 2015). This raises the possibility that a non-social trait, production of functional amyloids, may partially privatize public goods secreted by cooperators, limiting their availability to social cheats. There are a multitude of ways different social and private traits of bacteria can effect each other, but only by understanding how these apparently discrete traits interact can we fully understand how they shape cooperative and competitive dynamics.

Laboratory manipulation of bacterial populations has allowed us a window into what limits cheat invasion in bacterial populations and how spiteful behaviours can shape competitive dynamics. Yet the extent, form and consequences of social interactions in natural bacterial populations remain comparatively unexplored. Can social interactions drive evolutionary change, influence population structure, or promote and maintain diversity in natural bacterial populations? Sampling natural bacterial populations can give a snapshot of the diversity and distribution of social traits and allows inferences to be made concerning the role these behaviours have played in shaping natural communities.

Bacteriocin production is widespread in natural bacterial isolates and evidence suggests it can promote spatial structuring in sediment and marine environments (Gordon et al. 2007, Gutierrez Perez et al. 2013, Cordero et al. 2012). However, studying natural populations also allows us to ask questions about *why* bacteria engage in these behaviours that cannot be answered using defined mutants in the laboratory. For instance, what drives bacteriocin mediated conflict? Recent work suggests that genetic similarity between strains is an important predictor of the strength of interaction between bacteria (Schustra et al. 2012, Cordero et al. 2012, Hawlena et al. 2012) but other factors such space and resource requirements may also play an important role (Kinkel et al. 2014). Similarly, it is well established that artificially created public goods cheats spread in laboratory populations (Griffin et al. 2004, Diggle et al. 2007). Public goods cheats also appear, and are maintained, as long as cooperative strains are present in natural bacterial populations in the cystic fibrosis lung and on marine particulate matter (Andersen et al. 2015, Cordero et al. 2012). Yet what factors prevent cheats from spreading through natural populations remain unclear. Laboratory work suggests

mechanisms such as spatial distance between cheats and cooperators, producing different structural forms of public good that a cheat cannot use and actively antagonizing exploitative cells, can all limit cheat proliferation in laboratory settings (Weigert et al. 2017, Inglis et al. 2016, Lyons & Kolter 2017). Only by studying bacteria in natural systems can we evaluate the contribution of these mechanisms to the maintenance of cooperative and competitive behaviours in microbes.

Thesis aims and chapters

The main aims of this thesis are to explore the extent, form and consequences of social interactions in natural bacterial populations and also how a non-social bacterial trait, the production of functional amyloids, can shape cooperative and competitive dynamics.

In chapter 2, I use natural populations of *Pseudomonas fluorescens* sampled from soil to explore what drives bacteriocin-mediated competition in bacteria. The majority of bacteria appear to be capable of producing bacteriocins and these have been shown to shape the outcome of competition in laboratory settings. However, what drives these interactions in natural settings, and any consequences, remain unclear. I reveal the extent of bacteriocin production in these populations and test whether the strength of interaction is determined by spatial proximity, genetic relatedness or overlap in resource requirements.

In chapter 3 I use production of the public good pyoverdine in the same isolates of *Pseudomonas fluorescens* to explore the extent of social cheating in soil bacterial populations. Pyoverdine cheats can invade populations of cooperators in the laboratory, yet evidence for cooperators and cheats co-occurring in natural settings is somewhat

limited. I reveal the extent of pyoverdine non-producers in these populations and explore their potential to act as public goods cheats in natural settings.

In chapter 4 I explore how the production of functional amyloids by *Pseudomonas aeruginosa* can shape cooperator-cheat dynamics. It is well established that confining secreted public goods close to the cells that produced them can limit the ability of cheats to invade a population of cooperators. Functional amyloids produced by cells appear to bind multiple secreted public goods, potentially limiting their diffusion away from cells. I test the ability of functional amyloids to prevent loss of the iron-chelators pyoverdine and PQS to dilution and assess their impact on competition between cooperators and cheats over iron.

In chapter 5, I assess the contribution of functional amyloids towards individual and collective defense against conspecific bacteriocins. Functional amyloids have been shown to defend *E.coli* cells against attack from tailed-bacteriophage, which are structurally similar, and ancestral, to the R/F bacteriocins produced by *Pseudomonads*. I test whether addition of purified functional amyloid can protect cells that do not produce them from bacteriocins, whether functional amyloid overproducers are protected to a greater extent than wildtype cells and whether biofilms constructed with functional amyloids are protected to a greater degree than those without.

Chapter 2: Bacteriocins and the assembly of natural *Pseudomonas fluorescens* populations

Abstract

When competing for space and resources, bacteria produce toxins known as bacteriocins to gain an advantage over competitors. Recent studies in the laboratory have confirmed theoretical predictions that bacteriocin production can determine coexistence, by eradicating sensitive competitors or driving the emergence of resistant genotypes. However, there is currently limited evidence that bacteriocin-mediated competition influences the coexistence and distribution of genotypes in natural environments, and what factors drive interactions towards inhibition remain unclear. Using natural soil populations of *Pseudomonas fluorescens*, we assessed the ability of the isolates to inhibit one another with respect to spatial proximity in the field, genetic similarity and niche overlap. The majority of isolates were found to produce bacteriocins, however, widespread resistance between coexisting isolates meant relatively few interactions resulted in inhibition. When inhibition did occur, it occurred more frequently between ecologically similar isolates. However, in contrast with results from other natural populations, we found no relationship between the frequency of inhibition and the genetic similarity of competitors. Our results suggest that bacteriocin production plays an important role in mediating competition over resources in natural settings and, by selecting for isolates resistant to local bacteriocin production, can influence the assembly of natural populations of *Pseudomonas fluorescens*.

Introduction

The majority of bacteria produce small anti-microbial toxins, known as bacteriocins, which kill or inhibit the growth of sensitive competitors (Riley & Wertz 2002; Cascales *et al* 2007). Bacteria avoid the harmful effects of these toxins by carrying an immunity gene or lacking appropriate cell surface receptors, allowing harm to be directed towards competitors (Ghequire & De Mot 2014). The harm caused by bacteriocin production is often important for the successful invasion, or defense, of an established bacterial population: bacteriocin producers frequently invade and outcompete sensitive competitors in laboratory microcosm experiments (Riley & Gordon 1999; Wloch-Salamon *et al* 2008; Greig & Travisano 2008; Libberton *et al* 2015; Waite *et al* 2009). However; despite widespread recognition of the importance of bacteriocins in mediating competition between genotypes, we still have a limited understanding of this process in natural environments (Hibbing *et al* 2010; Whitaker 2009). In particular, it remains unclear why bacterial genotypes inhibit some competitors and not others, and whether bacteriocin-mediated interactions can influence the distribution of genotypes in free-living bacterial populations.

The abundance of bacteriocin producing genotypes in nature is often assumed to be a consequence of competition for limited resources (Gordon *et al.* 2007). Inhibitory interactions are expected to occur more frequently between ecologically similar genotypes, which require more of the same resources, and less frequently between ecologically dissimilar competitors (Frielich *et al* 2011). However, very closely related isolates, despite requiring similar resources, are often unable to inhibit each other due to shared resistance mechanisms (Hawlena *et al* 2010). Conflating predictions concerning ecological and genetic similarity suggests that inhibitory interactions should occur more

frequently between genetically 'intermediate' competitors (Schoustra *et al* 2012). While inhibitory interactions do appear to occur more frequently between genetically 'intermediate' competitors in marine and host-associated bacterial populations, direct evidence that competition over resources promotes inhibitory interactions remains scant (Cordero *et al* 2012; Hawlena *et al* 2012; Kinkel *et al* 2014). Our limited understanding of bacteriocin-mediated competition in natural settings also extends to the consequences of these behaviours. In the laboratory, bacteriocin production can influence the diversity of microcosm populations by eradicating sensitive competitors, or driving the emergence of resistant genotypes (Kerr *et al* 2002; Le Gac & Doebeli 2009; Abrudan *et al* 2012). However, it remains unclear whether bacteriocin-mediated interactions are strong enough, or occur frequently enough, to impact the distribution of genotypes in natural bacterial populations (Perez-Gutierrez *et al* 2013; Ghoul *et al* 2015).

Here, we examine bacteriocin-mediated interactions between natural isolates to reveal what factors promote inhibitory interactions in nature, and explore their consequences for the distribution of genotypes in soil. We used natural populations of the *Pseudomonas fluorescens* group, an important plant commensal, as our study system. We isolated *Pseudomonas fluorescens* from soil at multiple sites in a local park and assessed the ability of isolates to inhibit each other with respect to spatial proximity. We expect that if bacteriocin production has influenced the distribution of genotypes in the population, inhibition would occur less frequently between coexisting isolates. We also constructed nutrient use profiles for each isolate, and measured genetic distances between isolate pairs, to examine interactions in the context of both the extent to which isolates are likely to compete for resources and genetic similarity.

Material and Methods

Site description & sampling

We carried out sampling at multiple sites in University Parks, a managed parkland in Oxford consisting of sports fields, tended gardens and undisturbed parkland. We collected soil samples from eight sites in undisturbed regions of the park; the average distance between sites was approximately 250m. At each site we sampled a 1m transect consisting of four patches: a focal patch, and patches 1cm, 10cm and 100cm from the focal patch (Fig S1). At each patch we cleared surface debris and collected $1cm^3$ of soil from the surface. We carried out all sampling in a single day in June 2014 and soil samples were stored in the laboratory at 23°C and processed within two hours.

Sample processing & isolate identification

We isolated bacterial cells from soil samples as follows – 1g of sample was suspended in 6ml of minimal salts media (M9: 6.8 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl and 10 g NH_4Cl (Sigma Aldrich, UK ltd), per litre of dH_2O) in 15ml falcon tubes. We vortexed soil suspensions vigorously and plated 80µl of each suspension onto *Pseudomonas* isolation agar plates (47.4g *Pseudomonas* isolation agar and 20g glycerol per litre dH_2O). Plates were incubated at 23ºC for 48 hours before we randomly selected 30 isolates from each patch, transferred them to fresh KB agar plates (20 g protease peptone N^o3 (Difco, UK ltd), 10 mL glycerol, 1.5 g $K_2HPO_4.3H_2O$, 12g agar and 1.5 g $MgSO_4.7H_2O$ (Sigma Aldrich UK ltd), per litre of dH_2O) and incubated overnight at 23°C.

We used colony PCR to identify isolates belonging to species of the *Pseudomonas fluorescens* group. PCRs contained 12.5µl of 2x MangoMix (BioLine, US), 10.5µl of

ddH_2O, 0.5 µM of each primer (Table S1) and 1µl of DNA template (colony pick suspended in 100 µl dH_2O) up to a volume of 25 µl. The cycling conditions consisted of: 5 min at 95°C; 34 cycles of 94°C for 30s, 55°C for 10s, 72°C for 45s and a final extension of 72°C for 5 min before cooling to 4°C. All reactions were carried out with a positive (*Pseudomonas fluorescens* SBW25), negative (*Pseudomonas aeruginosa* PAOl) and blank control. We established the presence or absence of amplified PCR product using gel electrophoresis.

We randomly selected seven positive isolates from each patch, in each site (7 isolates x 4 patches x 8 sites = 224 isolates) for use in the study. These isolates were grown for 24 hours at 23ºC on an orbital shaker at 200 rpm in 6ml of KB media ((20 g protease peptone N^{o}3 (Difco, UK ltd), 10 mL glycerol, 1.5 g $K_2HPO_4.3H_2O$ and 1.5 g $MgSO_4.7H_2O$ (Sigma Aldrich UK ltd), per litre of dH_2O) and frozen in 50% glycerol at -80ºC.

Assaying inhibitory phenotypes of isolates

We assayed the ability of isolates from the focal patch to inhibit the growth of isolates from patches 1cm, 10cm and 100cm away in the same site. We also assessed their ability to inhibit isolates from the focal patch of other sites, allowing us to determine whether the frequency of inhibition caused by isolates increased with spatial distance from the focal patch locally (up to 1m away) and further afield (between sites). We determined the ability of isolates to inhibit each other's growth with pyocins by testing whether an isolates supernatant inhibits the growth of other isolates. We spotted KB agar plates with supernatants, spread a lawn of each culture over the plate and recorded

which supernatants inhibited which bacterial lawns. Assays were carried out in duplicate.

Supernatant Extraction

To extract pyocin-containing supernatants, we cultured 56 isolates (seven isolates from the focal patch of each site) for 24 hours in 6ml of KB media (23ºC at 200 rpm). We measured the cell density of cultures at A600 (SpectraMax), standardized each culture to an OD of ~0.3 and diluted this 10-fold in fresh KB media. Cultures were incubated for 12 hours before we added 100µl of 0.02mg/µl mitomycin C (Sigma, UKltd), a strong inducer of bacteriocin production, and incubated for a further four hours (Pentermann *et al* 2014). We then centrifuged cultures at 6861rpm for ten minutes, obtaining a clear, cell-free supernatant by filter sterilizing with a 0.02µm filter and storing at -20ºC until required.

Inhibition Assays

We spotted KB agar plates with 15µl of supernatant and allowed the plates to dry at room temperature. We cultured all 224 isolates from freezer stocks for 24 hours in 6ml of KB media (23ºC at 200 rpm). Cell density was standardized to an OD of ~0.5 and diluted 10-fold in M9 before 70µl of culture was spread onto the supernatant-spotted KB agar plates. We incubated plates at 23ºC for ~12 hours, or until a uniform lawn of bacterial growth was visible, and 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 (1 for inhibition, 0 for no inhibition). Plates were checked regularly after 12 hours as zones of inhibition caused by some supernatants could become overgrown. We recorded patterns of inhibition and

resistance in a 7 x 28 matrix for each set of within-site interactions and a 56 x 56 matrix for between-site interactions.

Each supernatant was spotted on a KB plate to ensure it was cell free. As a control, each lawn was also spotted with KB media treated identically to supernatant cultures; to demonstrate that residual mitomycin C was not responsible for zones of inhibition. Bacteriophage might also be responsible for inhibition on bacterial lawns, which manifest as areas of slightly reduced growth with characteristic plaques. We observed no plaque formation around the zones of inhibition on our plates and no evidence of self-inhibition, suggesting bacteriophage were not responsible.

Characterizing inhibitory molecules

We repeated the inhibition assays that resulted in one isolate inhibiting another in order to determine what mediated inhibitory interactions. *Pseudomonads* can produce an array of inhibitory molecules that vary greatly in size: large phage tail-like toxins known as tailocins, smaller proteineous toxins known as S-type pyocins and other assorted small inhibitory molecules (Ghequire & De Mot 2014). We repeated inhibition assays after passing pyocin-containing supernatants through different sized filters and determined whether isolates were capable of killing using large (>100kDa), medium (>3kDa<100kDa) or small (<3kDa) molecules.

We obtained cell-free supernatant from 35 isolates (those capable of inhibiting another isolate) as previously. To obtain supernatant containing large inhibitory molecules, we passed 500 µl of the cell free supernatant of each isolate through a 100kDa spin filter (EMD Millipore, Ltd). The flow through was discarded and the remaining pyocin-

containing supernatant was made up to 500µl using M9 solution. To obtain supernatant containing small inhibitory molecules, we passed 500 µl of the cell free supernatant of each isolate through a 3kDa spin filter and retained the flow through. The flow through contains only particles smaller than 3kDa thus all larger, potentially inhibitory molecules are removed from the supernatant. To obtain supernatant containing intermediately sized inhibitory molecules, we passed 500 µl of the cell free supernatant of each isolate through a 100kDa spin filter, the flow through was collected and passed through a 3kDa filter. This flow through was discarded and the retained supernatant was made up to 500µl using M9 solution. We then performed inhibition assays as previously and recorded which size fraction of their supernatants isolates were capable of inhibiting with.

MLST of focal isolates

We used multi-locus sequence typing (MLST) of four housekeeping genes to provide a measure of genetic similarity between isolate pairs, allowing us to explore the relationship between inhibition and genetic similarity of our isolates.

DNA Extraction and PCR

We extracted genomic DNA using the Wizard Genomic DNA purification kit (Promega, Switzerland) and amplified four housekeeping genes (gyrB, RecA, RpoB and RpoD; Table S1.) for each of the focal isolates. All reactions were performed in 50µl volume containing 1U of DreamTaq polymerase (Thermo Scientific), 5µl of DreamTaq Buffer, 100mM of each primer, 0.2mM of each dNTP, 40.75µl of ddH_2O and 20ng of DNA template. We used the following cycling conditions: 95ºC for 3 minutes, then 35 cycles of 95ºC for 30s, 58ºC/60ºC/64.5ºC for 30s, 72ºC for 45s and a final extension of

72ºC for five minutes. The annealing temperatures for each primer set are found in the Table S2.

Sequencing and Analysis.

Purified products were sent to SourceBioscience (Nottingham, UK) for Sanger sequencing with the respective primer pairs used for PCR amplification used as forward and reverse sequencing primers (Table S1). We checked the quality of the resulting sequences using Geneious Pro (Biomatters, Ltd.), 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 constructed a concatemer of all four genes for each isolate; from which we calculated pairwise genetic distances between isolate pairs using the Jukes-Cantor model (Mega6) and constructed a neighbor-joining tree showing the relationships between focal isolates.

Resource Competition

We assayed the ability of each isolate to grow on different carbon sources and calculated a measure of niche overlap between isolate pairs, allowing us to explore the relationship between inhibition and resource use in our isolates.

We measured the growth of each isolate on different carbon sources, in duplicate, using EcoPlates (Biolog Ltd). EcoPlates plates contain 31 different carbon sources commonly found in soil. We cultured all 56 isolates for 24 hours in 6ml of KB media (23ºC at 200rpm) before standardizing cultures to an OD of ~0.25 and diluting 10-fold. We inoculated each well of the plates with 150ul of culture and incubated at 23ºC for 48 hours. We measured the absorbance at A_{600} immediately after inoculation and after 48

hours. The absorbance of the control well was subtracted from the reading for each well and a measure of niche overlap was calculated (Kinkel *et al* 2014).

Niche overlap = {(Σ minimum absorbance *a,b*)/total absorbance *a* + (Σ minimum absorbance *a,b*)/total absorbance *b*)}/2

Where (minimum absorbance *a*, *b*) is the minimum absorbance value for a pair of isolates (*a* and *b*) on a given nutrient; these values are summarized over all 31 nutrients. The total absorbance for an isolate is the sum of absorbance values for that isolate over all 31 nutrients. Therefore, niche overlap between a pair of isolates is the mean proportion of total nutrient use that overlaps between the two isolates.

Statistical Analysis

We tested for significant differences in inhibition caused by isolates within their site and inhibition they caused between sites using a generalized linear mixed model (GLMM). We used inhibition between isolates as a binary response variable and distance (within-site or between-site) as the explanatory variable and included producing isolate and lawn isolate as random effects to account for the fact isolates are used in multiple comparisons. We also tested for significant differences in inhibition caused by focal isolates against isolates from other patches within sites using GLMM, with inhibition as a binary response variable and spatial distance between isolates as the explanatory variable. We included site and isolate as random effects to take into account the multiple sites used in the analysis and that isolates were used in multiple comparisons respectively.

We wish to determine if genetic similarity between isolates could explain whether isolates were more or less likely to inhibit another isolate. To determine if there was any correlation between frequency of inhibition and genetic distance between isolates, we used a generalized linear model (GLM) with inhibition as a binomial response variable and genetic distance between isolates as the explanatory variable.

Finally, to determine whether there was any correlation between frequency of inhibition and niche overlap we used a GLM with inhibition as a binomial response variable and niche overlap between isolates as the explanatory variable.

Results

Isolate collection and inhibition profiles

We selected seven isolates from a single patch at each of our eight sites (56 isolates) for further analysis, the majority of which were genetically distinct. MLST analysis of four housekeeping genes revealed that 39 of the 56 focal isolates were unique (70%) and the average pairwise genetic distance between isolates was 0.031, ranging from 0.000 to 0.047. A Maximum Likelihood (ML) tree of our focal isolates with type strains of the *P.fluorescens* complex suggest that our isolates are part of the *P.fluorescens* group within the complex and that they are comprised of multiple different species (Fig S2). Rarefaction analyses suggest that a considerable portion of diversity present in the population was sampled (Fig S3).

The majority of isolates produced a molecule capable of causing inhibition, and were inhibited by at least one of the bacteriocins produced by isolates in our collection. Approximately two-thirds (63%) of isolates produced an inhibitory molecule that could inhibit the growth of at least one other isolate and over three-quarters (79%) of isolates were inhibited by at least one other isolate (Fig 1). In only 7% of interactions did the supernatant of one isolate inhibit the growth of another isolate (Fig 2).

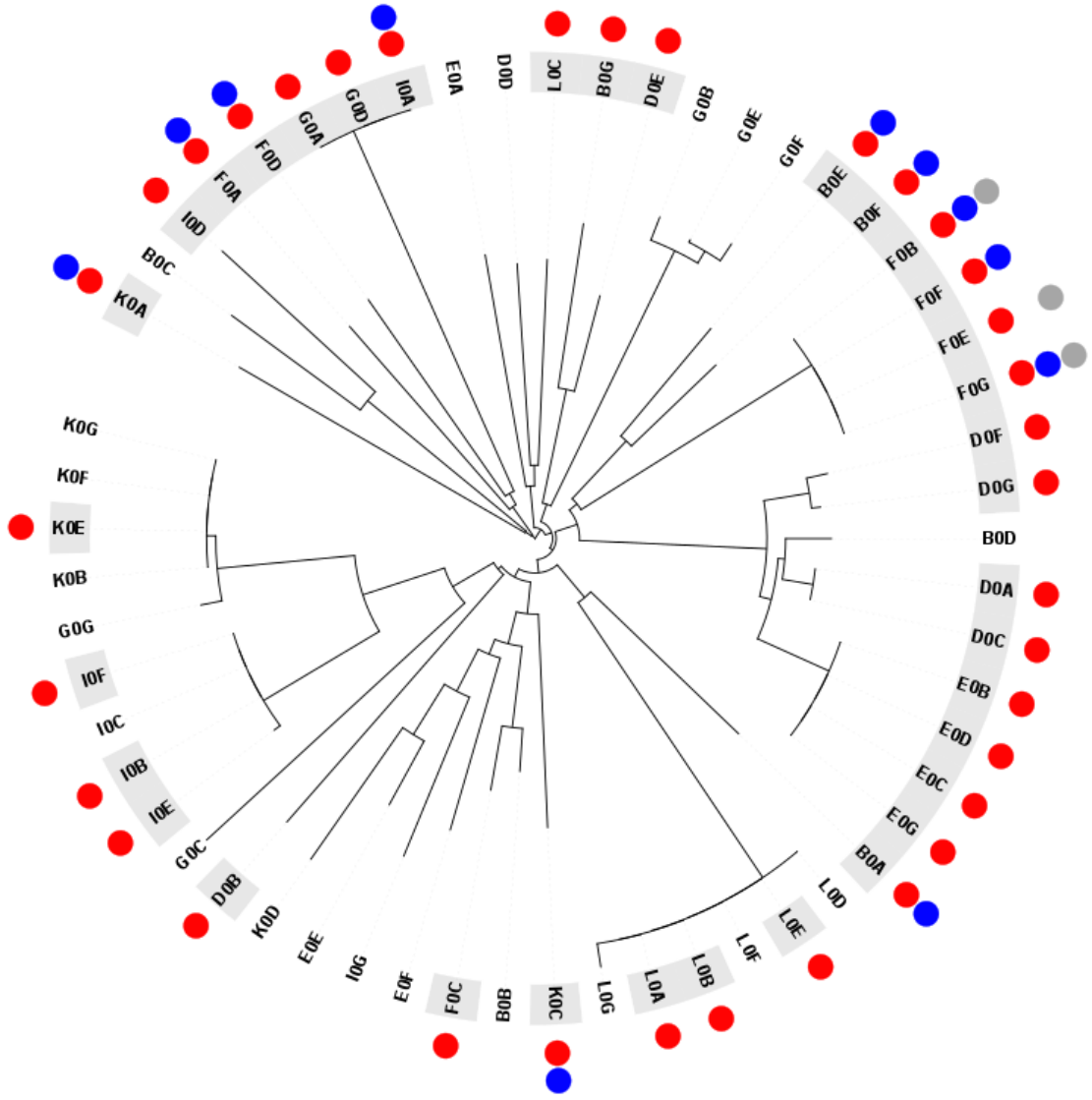


Figure 1: Inhibitory capacity and phylogeny of isolates based on four housekeeping genes. Outer ring highlights inhibitory isolates (grey) and non-inhibitory isolates (white). Coloured circles indicate the ability to inhibit another isolate with large particles (>100kDa, Red), medium particles (<100kDa, Blue) and small particles (<3kDa, Grey). The majority of isolates are genetically distinct and are capable of producing at least one type of inhibitory molecule.

The inhibitory molecules produced by the isolates in our collection varied in size, and, therefore, likely represent a range of different bacteriocin types. We found that isolates produce large (e.g. R and F types), intermediate (e.g. S types) and small (unknown) inhibitory molecules. All inhibitory isolates can inhibit using products >100kDa in size, while 20% and 9% of inhibitory isolates were capable of inhibiting with medium (<70kDa) and small (<3kDa) products respectively. We find that isolates with 100% sequence similarity at four housekeeping genes can show different patterns of inhibition and resistance (Fig 2).

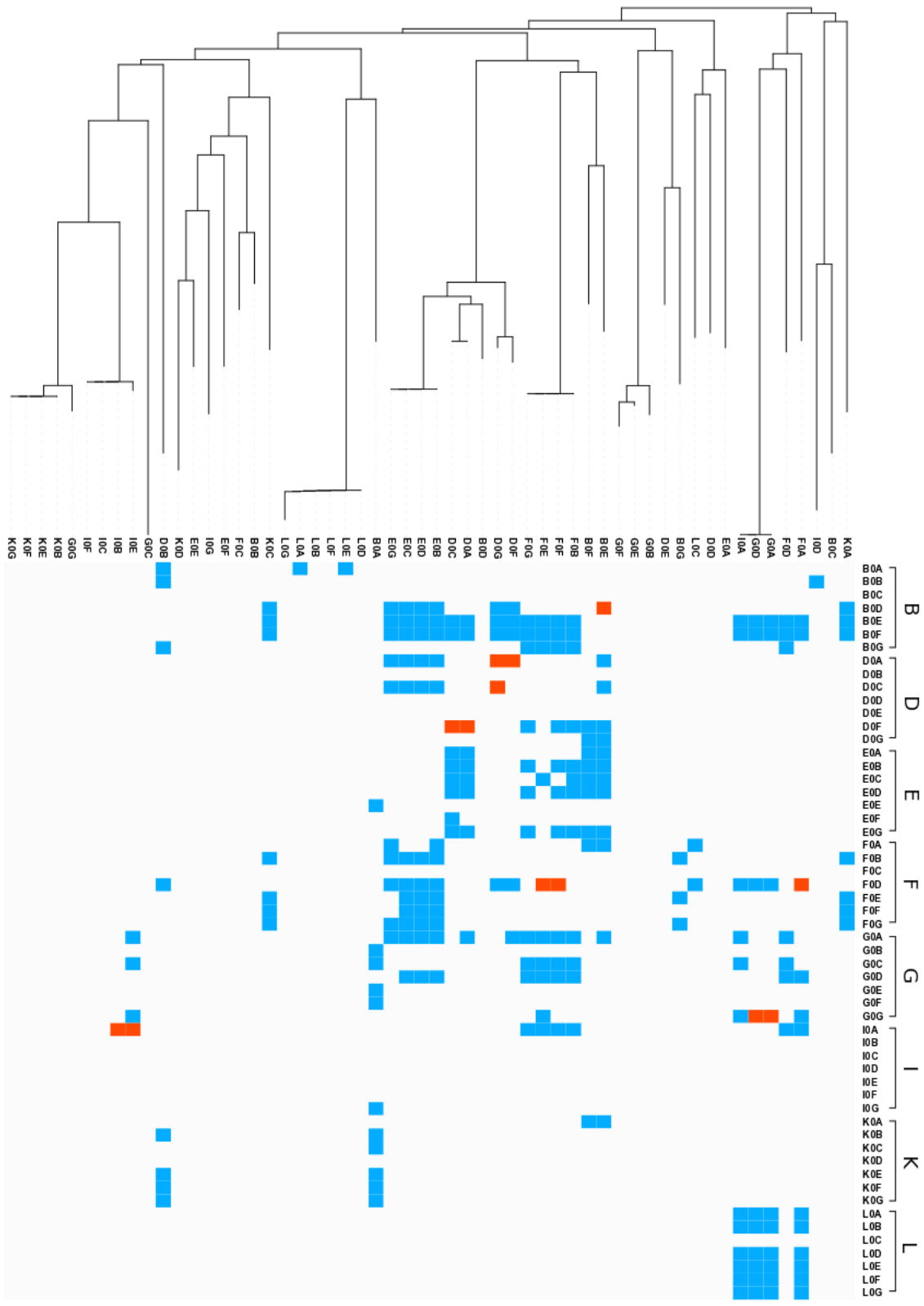


Figure 2: Phylogeny and inhibition profiles of the isolates. The left hand side shows a phylogenetic tree of all isolates, while the top shows isolates grouped by sampling site. Grey squares indicate no inhibition, blue squares indicate an inhibitory interaction between isolates from different sites (between-sites) and red squares indicate inhibitory interactions between isolates from the same site (within-sites). The majority of interactions do not result in inhibition, while genetically identical isolates can have different inhibition and resistance profiles.

Inhibition, Distance, Genetic Distance and Niche Overlap

An analysis incorporating all the factors used in the study suggests significant differences in inhibition attributable to the distance between isolates and the degree of niche overlap, but not the genetic distance between isolates (Table S3).

We found that inhibition occurred less frequently between isolates from the same site, and more frequently between isolates from different sites (Figure 3a; GLMM: $Z = 4.796$, $P = 1.62 \times 10^{-6}$). There was also a trend, within sites, for inhibition to occur more frequently between isolates separated by greater distances, but this relationship was not significant (figure 3b; GLMM: $Z=1.130$, $P = 0.259$).

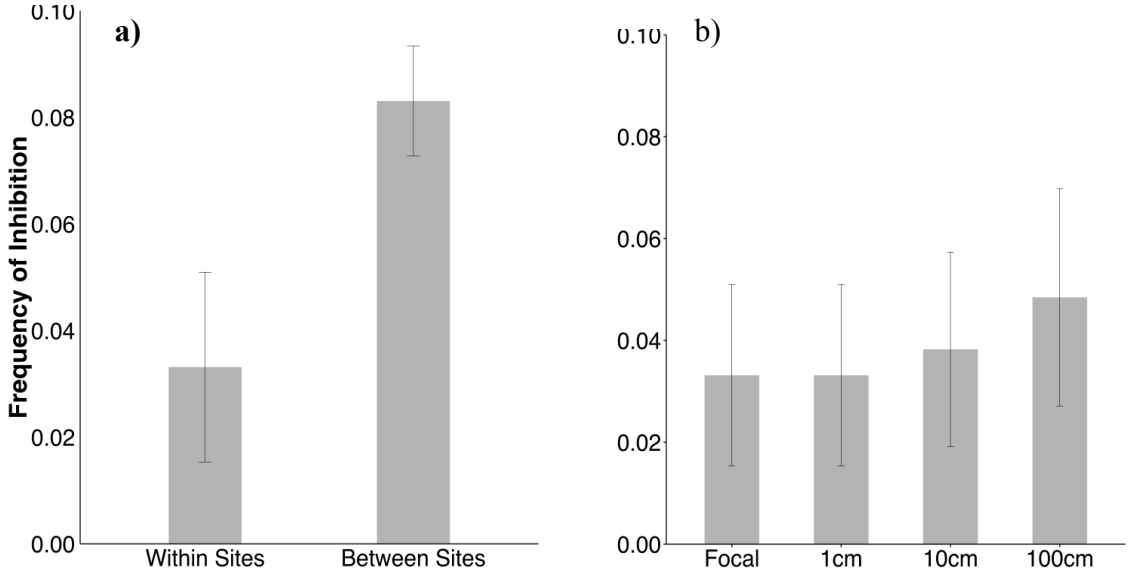


Figure 3: Frequency of inhibitory interactions between isolates. a) The frequency of inhibitory interactions between coexisting isolates (within-sites, n=372) is significantly lower than that between non-coexisting isolates (between-sites, n=2744). **b**) The frequency of inhibitory interactions observed between coexisting isolates and in interactions between isolates separated by 1cm, 10cm and 100cm within-sites (n=392 for each distance). Error bars indicate 95% confidence intervals.

We find that isolates were no more likely to inhibit genetically similar isolates than any other isolate (Fig 4; GLM: $t_{44} = -0.89$, $P = 0.37$) but that inhibition occurred more frequently between isolates with greater niche overlap (Fig 5; GLM: $t_{329} = 7.02$, $P = 1.23e^{-11}$)

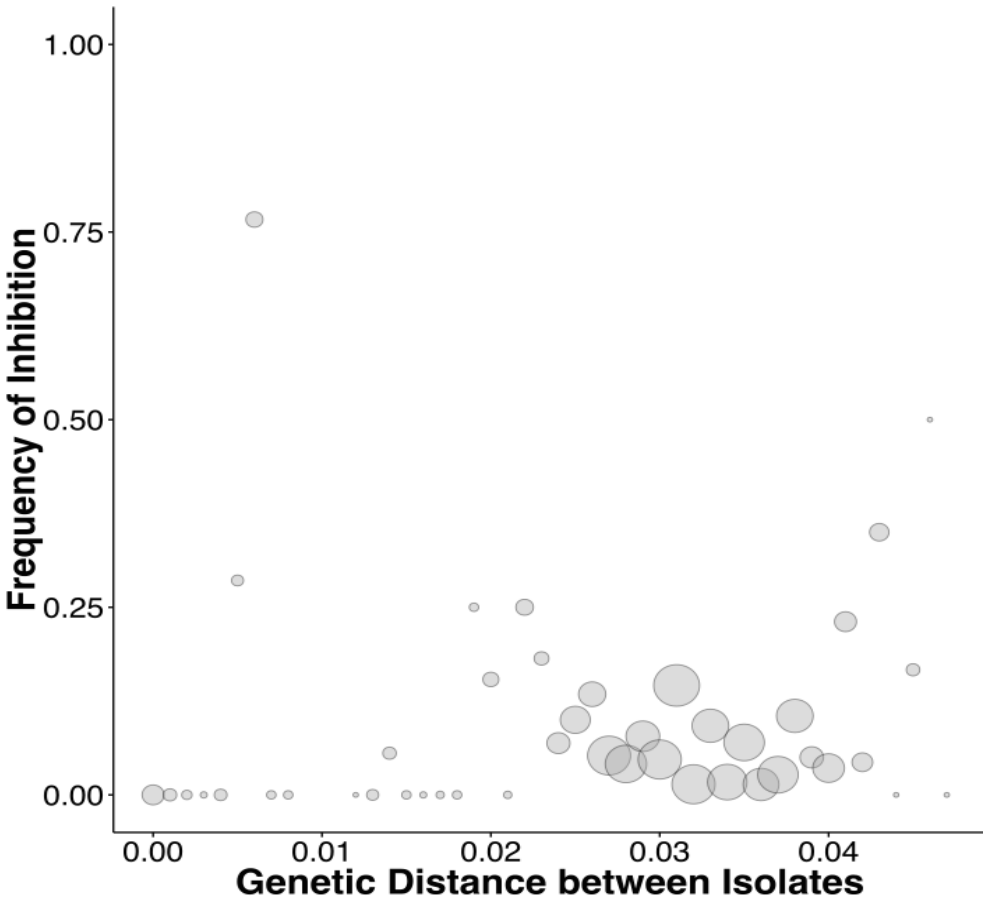


Figure 4: Inhibitory interactions and genetic distance between isolates. Inhibitory interactions occur no more frequently between genetically similar isolates than between genetically dissimilar isolates. The size of the point reflects the number of interactions: smallest points indicate <5 interactions and largest points indicate >200 interactions (n=3080).

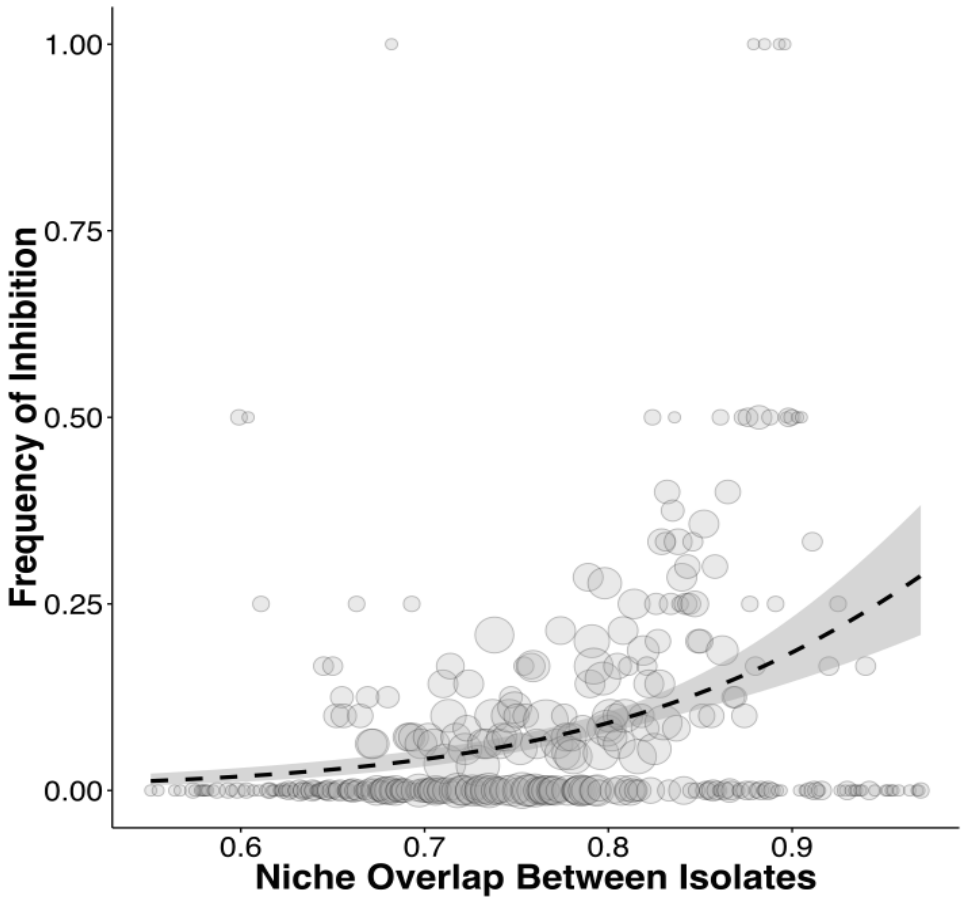


Figure 5: Inhibitory interactions and niche overlap between isolates. Inhibitory interactions occur more frequently between isolates with greater niche overlap. The size of the points reflects the number of interactions, smallest points indicate <2 interactions and largest points indicate >24 interactions (n=3080).

Discussion

Our results show that natural populations of *Pseudomonas fluorescens* are intensely competitive: the majority of isolates produce bacteriocins and some are capable of producing two or more types (Fig 1). However, resistance to bacteriocins is so widespread that relatively few interactions actually result in one isolate inhibiting another (Fig 2). In contrast with previous studies, we find no evidence that inhibition occurs more frequently between genetically more similar or more dissimilar isolates (Fig 4). However, we do show that inhibition occurs more frequently when competitors

have greater niche overlap, suggesting bacteriocins are important in mediating competition over resources in natural settings (Fig 5). Finally, we found that inhibitory interactions occur significantly less frequently between coexisting isolates: suggesting that bacteriocin production plays a role in shaping natural *Pseudomonas fluorescens* populations by selecting for isolates resistant to local bacteriocin producers (Fig 3).

Despite the potential for bacteriocin-mediated conflict, resistance appears to be so widespread that relatively few interactions actually result in one isolate inhibiting another. Resistance to the bacteriocins of conspecifics is common in other bacterial populations: in natural *Vibrio* and *Bacillus* populations less than 10% of interactions resulted in inhibition despite approximately half of isolates producing bacteriocins (Cordero *et al* 2012, Perez-Gutierrez *et al* 2013). Our results provide further evidence that resistance is prevalent and either easily acquired, or easily transferred between isolates, in natural settings. Acquiring resistance is thought to come at a significant cost to the cell, yet resistance was the most common outcome of competition even between isolates unlikely to encounter each other frequently (Inglis *et al* 2016, Opsata *et al* 2010). Why maintain costly resistance against the toxins of rarely encountered competitors? Bacteriocins, such as the phage-tail like toxins produced by our isolates, often target essential components of the cell membrane, such as siderophore or lipopolysaccharide (LPS) receptors (Kohler *et al* 2010; Cascales *et al* 2007; Elfrash *et al* 2012; Bayesse *et al* 1999). Mutations in these receptors can confer resistance to bacteriocins, and a single mutation may, by altering the structure of surface receptors, confer resistance against multiple bacteriocins (Scanlan *et al* 2015).

We found that bacteriocin-mediated inhibition occurs more frequently between competitors with similar resource requirements. Competition over limited resources is thought to be a key driver in the evolution of competitive phenotypes and selection for bacteriocin production is likely strongest when resource competition is intense (Cornforth & Foster 2013). By producing bacteriocins that harm ecologically similar competitors, isolates maximize the fitness benefits of costly toxin production, as most resources liberated are beneficial to the producer (Schoustra *et al* 2012). Our results suggest that resource competition is an important factor driving the evolution of bacteriocin production in natural populations and are consistent with the limited evidence available from other bacterial populations: inhibitory interactions between *Streptomyces* are often more intense when competitors have similar resource requirements (Kinkel *et al* 2014, Schlater *et al* 2014). Directing harm towards stronger resource competitors also allows isolates to avoid engaging in costly conflict with ecologically distinct competitors, which may partly explain the coexistence of multiple *P.fluorescens* genotypes at each site in our study.

Contrary to expectations, we found no evidence that inhibition occurs more frequently between genetically more similar or dissimilar competitors. Resource competition is thought to be strongest between genetically similar competitors (Frielich *et al* 2011, Jousset *et al* 2011). Consequently, with the exception of competition between identical or very closely related isolates, inhibition is expected to occur more frequently between genetically similar competitors. However, our results emphasize that resource competition is the key factor driving interactions towards inhibition in natural populations. While harming strong resource competitors will often equate to more frequent inhibitory interactions between non-identical, genetically similar competitors,

as observed in a recent study of clinical and laboratory *P. aeruginosa* isolates, the relationship is likely weaker in natural settings where resource use can vary significantly between even closely related isolates (Schoustra *et al* 2012, Belotte *et al* 2003, Kraemer *et al* 2015). Our results suggest that, while genetic similarity will often be a good predictor of the potential for inhibition between competitors, understanding what drives interactions towards inhibition in natural settings will also require knowledge of the resource requirements of each competitor.

Finally, we found that bacteriocin-mediated inhibition occurs less frequently between coexisting isolates, suggesting bacteriocin production selects for genotypes resistant to local bacteriocin producers. The low levels of inhibition we observe between coexisting isolates could occur if 1) isolates interact more frequently with identical or closely related competitors, who produce similar bacteriocins and are thus resistant, or 2) if unrelated isolates have evolved resistance to local bacteriocin producers (Ghoul *et al* 2015; Kerr *et al* 2002). These outcomes are not mutually exclusive: each of our sites is genotypically diverse, while many harbor a numerically dominant genotype, suggesting both processes may be important. Attenuation of inhibitory interactions between coexisting isolates is also reported in *Bacillus* populations found in marine sediment, which raises the question of why bacteriocin production is maintained in these populations when resistance is prevalent locally (Perez-Guttierez *et al* 2013). One explanation is that bacteriocin production provides a fitness advantage in competition with invading genotypes: recent empirical studies have shown that invasion of a population can be prevented by bacteriocin production and that genotypically rich populations are often less invasible than their homogenous counterparts (Libberton *et al* 2015; Jousset *et al* 2011). The genotypically diverse, locally resistant patches of

bacteriocin producers we find at each of our sites may represent structured, stable collections of genotypes resistant to invasion by conspecific competitors.

Overall, our results suggest an important role for bacteriocin production in natural free-living bacterial populations. We provide empirical evidence that suggests bacteriocin production mediates competition over resources in natural bacterial populations, and show that bacteriocin production can influence the assembly of bacterial populations by selecting for isolates resistant to local bacteriocins.

Appendix

Supplemental Material and Methods

Phylogeny

The Multi-Locus Sequence Analysis (MLSA) phylogeny was constructed using our 56 focal isolates and type strains representing each group of the *P.fluorescens* complex. Type strains are described in Andreoni (2014) and Garrido-Sanz (2016) and partial sequences for the gyrB, recA, rpoB and rpoD housekeeping genes for type strains were retrieved from GenBank (Bensen 2011). Sequences were aligned using MUSCLE (Edgar 2004), alignments cut and concatenated using MEGA software (Tamura 2013). To obtain a phylogeny we used the Maximum-Likelihood (ML) method and the Tamura-Nei model with 10000 bootstraps.

Rarefaction curves

From a concatenation of 4 housekeeping genes (gyrB, recA, rpoB and rpoD) in our focal isolates we produced a presence/absence matrix of polymorphisms at all variable sites in the alignment. We scored each polymorphism, rather than every polymorphic

site, as many sites segregated for 3 or 4 nucleotides. The matrix was used as the input to the program EstimateS 8.2 (Colwell 2013) to create rarefaction curves. For the analysis, we treated individual sequences as samples and individual polymorphisms as 'species' after Vos et al (Vos 2008).

Statistical analysis

We carried out an analysis incorporating the multiple factors used in our study (Distance, Niche Overlap and Genetic Distance). We used a generalized linear mixed effects model (GLMM) with inhibition between isolates as a binary response variable; distance, niche overlap and genetic distance between isolates as explanatory variables and included producing isolate and lawn isolate as random effects to account for the fact isolates are used in multiple comparisons.

Supplementary Figures

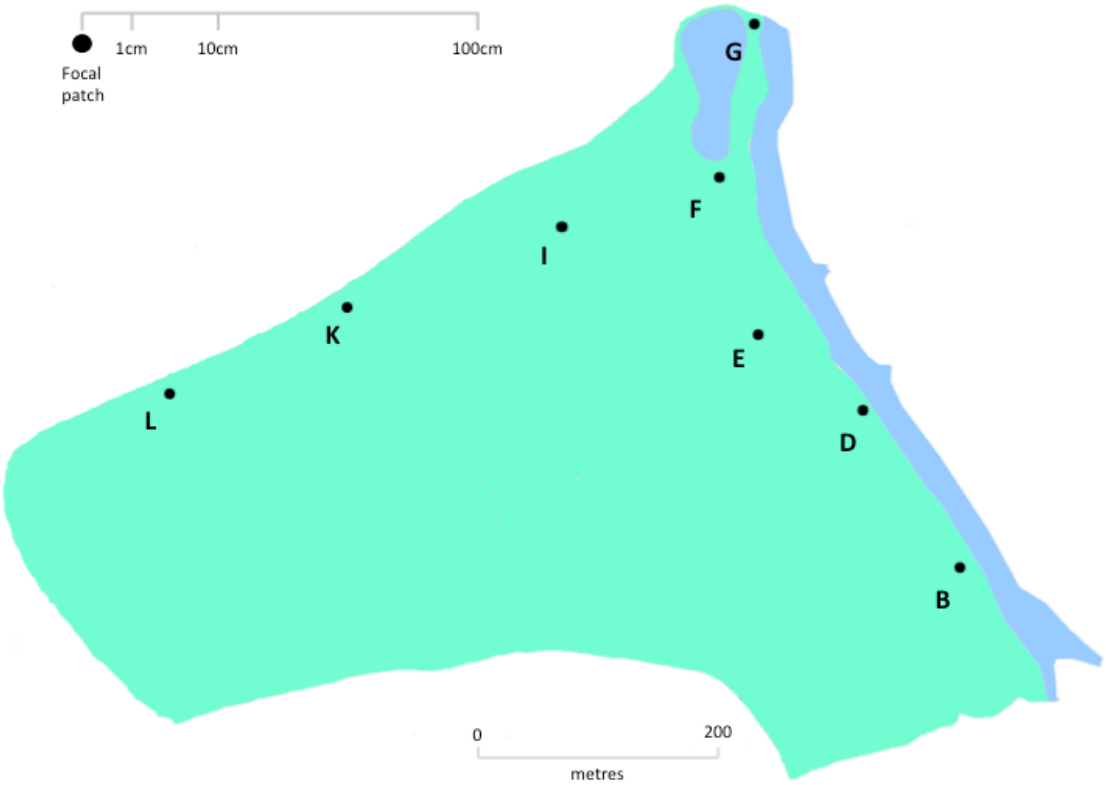


Figure S1 – Transect and sampling sites. A schematic map of University Parks, Oxford, showing sampling sites used in the study and a schematic of the transect sampled at each site.

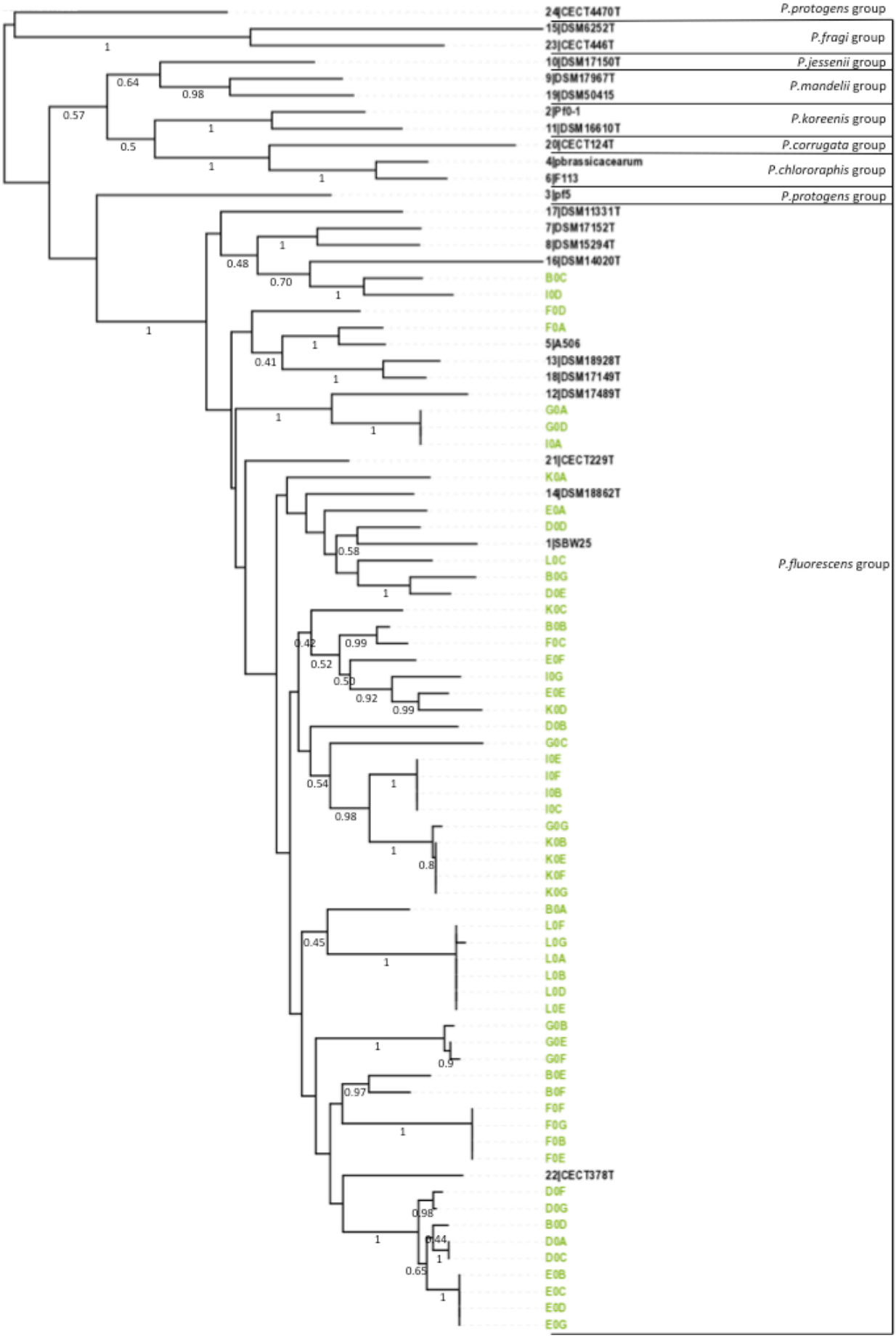

24|CECT4470T
15|DSM6252T
23|CECT446T
10|DSM17150T
9|DSM17967T
19|DSM50415
2|Pf0-1
11|DSM16610T
20|CECT124T
4|pbrassicacearum
6|F113
3|pf5
17|DSM11331T
7|DSM17152T
8|DSM15294T
16|DSM14020T
B0C
I0D
F0D
F0A
5|A506
13|DSM18928T
18|DSM17149T
12|DSM17489T
G0A
G0D
I0A
21|CECT229T
K0A
14|DSM18862T
E0A
D0D
1|SBW25
L0C
B0G
D0E
K0C
B0B
F0C
E0F
I0G
E0E
K0D
D0B
G0C
I0E
I0F
I0B
I0C
G0G
K0B
K0E
K0F
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L0F
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L0A
L0B
L0D
L0E
G0B
G0E
G0F
B0E
B0F
F0F
F0G
F0B
F0E
22|CECT378T
D0F
D0G
B0D
D0A
D0C
E0B
E0C
E0D
E0G
P.protogens group
P.fragi group
P.jessenii group
P.mandelii group
P.koreenis group
P.corrugata group
P.chlororaphis group
P.protogens group
P.fluorescens group

Figure S2 – Phylogenetic tree of focal isolates and *P.fluorescens* complex type strains. A phylogenetic tree of 56 focal isolates and type strains from each group of the *P.fluorescens* complex based on partial sequences of the gyrB, recA, rpoB and rpoD genes and constructed using the ML method and the Tamura-Nei model. Only bootstrap values above 40% (from 10000 replicates) are shown. Black text indicates type strains and green text indicates isolates from this study.

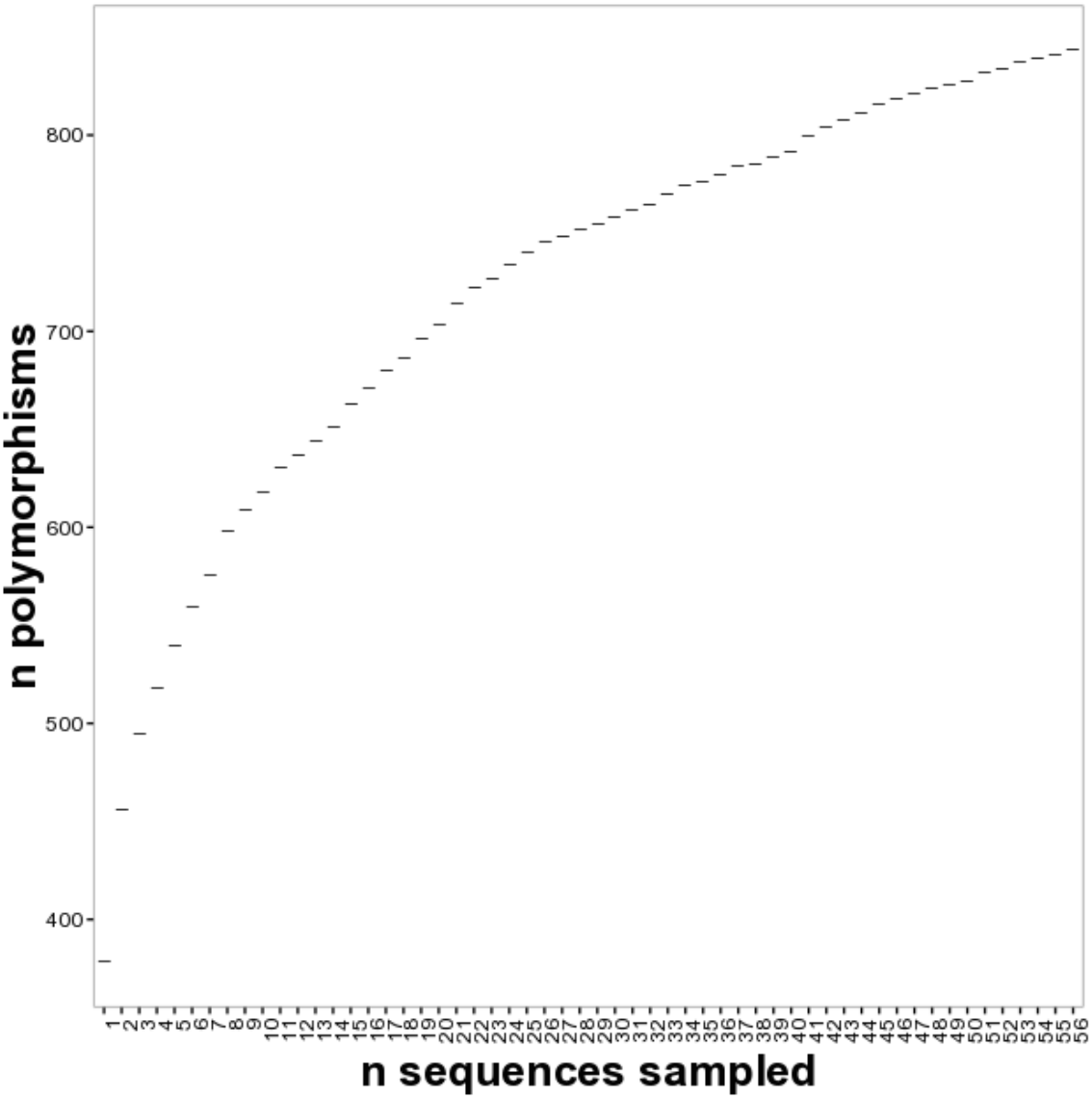


Figure S3 – Rarefaction curve of the focal isolates in this study. The number of polymorphisms in a concatemer of 4 housekeeping genes (gyrB, recA, rpoB and rpoD) as a function of sample size.

Supplementary tables

Table S1. Primer Sequences

Primer	Gene Product	Sequence	Length
16S_F	16S ribosomal RNA	AGAGTTTGATCCTGGCTCAG	922bp
16S_R		CTACGGCTACCTTGTTACGA	
gyrB_F	DNA gyrase subunit B	GGTGGTCGATAACTCCATCG	492bp
gyrB_R		CGCTGAGGAATGTTGTTGGT	
recA_F	recombinase A	TGGCTGCGGCCCTGGGTCAGATC	435bp
recA_R		ACCAGGCAGTTGGCGTTCTTGAT	
rpoB_F	RNA polymerase subunit beta	TGGCCGGTCGTCACGGTAACA	477bp
rpoB_R		CCGAAACGCTGACCACCGAAC	
rpoD_F	RNA polymerase sigma factor	CTGATCCAGGAAGGCAACATCGG	480bp
rpoD_R		ACTCGTCGAGGAAGGAGCG	

Table S2. PCR and Sequencing Primers and Annealing temperatures

Gene	Forward	ºC	Reverse	ºC
16S	16S_F	58	16S_R	58
gyrB	gyrB_F	58	gyrB_R	58
recA	recA_F	60	recA_R	60
rpoB	rpoB_F	64	rpoB_R	64
rpoD	rpoD_F	58	rpoD_R	58

Table S3. Analysis incorporating Distance, Niche Overlap and Genetic Distance

Formula: Inhibition ~ Distance + Niche Overlap + Genetic Distance + (1|Producer Isolate) + (1|Victim Isolate)
Family: Binomial

Fixed Effects:

	Estimate	Std.Error	Z Value	Pr(>|z|)
Intercept	-11.628	1.6179	-7.187	6.62E-13
Distance	2.0447	0.4039	5.063	4.14E-07
Niche Overlap	7.6231	1.7065	4.467	7.93E-06
Genetic Distance	-23.7563	12.8062	-1.855	0.0636

Random Effects:

	Name	Variance	Std. Dev
Producer Isolate	Intercept	5.751	2.398
Victim Isolate	Intercept	1.573	1.254

Chapter 3: Cheating and resistance to cheating in natural populations of the bacterium *Pseudomonas fluorescens*

Abstract

Bacteria perform cooperative behaviours that are exploitable by non-cooperative 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) non-producers are present in the population; (2) they co-occur ($<1cm^3$) with pyoverdine producers; (3) they retain functional pyoverdine receptors and (4) they can utilize the pyoverdine of on average 52% of producers. This suggests non-producers 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 non-producers can exploit cooperators, as some cooperators appear to produce exclusive forms of pyoverdine or kill non-producers with toxins. We examined the consequences of this variation using theoretical modeling. 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 non-producers from exploiting cooperators.

Introduction

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 behaviours are open to exploitation by non-cooperative 'cheats': individuals that increase their own fitness by exploiting the cooperative behaviours 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 behaviour provides some direct benefit, if they gain preferential access to cooperative goods or if populations are spatially structured (Gore et al. 2009, Koschwanez et al. 2011, Frank 2010).

However, there remains a lack of evidence that cooperators and cheats coexist in natural settings (Harrison 2013, Jones et al. 2015, Riehl & Frederickson 2016). Firstly, many studies examining cooperator-cheat dynamics make use of artificially created cheats (Griffin et al. 2004, Greig & Travisano 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, Bozdag & Greig 2015, Jones et al. 2015). Secondly, while less cooperative or non-cooperative 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). Thirdly, there is a range of mechanisms that could prevent potential cheats from successfully exploiting the cooperative behaviour of others (West et al. 2007). These include preferentially directing cooperation towards relatives, and directing harming behaviours towards non-

relatives or non-cooperators (Kiers et al. 2003, Griffin & West 2003, Sharp et al. 2005). For example, bacteria produce bacteriocins (toxins) that could potentially prevent cheats from exploiting cooperative behaviours (Riley 1999, Wang et al. 2015).

We examine the extent to which cheating and the above complexities occur in natural populations of the soil bacterium *Pseudomonas 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. 2013). 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 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 lab, where it has been demonstrated to be a cooperative trait open to exploitation by non-producing cheats (Griffin et al. 2004, Jiricny et al. 2010, Ghoul et al. 2014b, Kummerli et al. 2013).

We examined isolates of *Pseudomonas fluorescens* from soil at multiple sites in a local park and asked: (1) Do pyoverdine non-producers exist in natural soil populations? (2) Can non-producers 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 behaviours (Inglis et al. 2009, Strassmann & Queller 2014). Finally, our experimental work revealed non-producers 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 & sampling

We collected isolates of the *Pseudomonas fluorescens* group from soil samples at multiple sites in University Parks, Oxford, as described previously (Bruce et al. 2016). 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 1m transect consisting of four patches: an initial patch (patch 0) and patches 1cm (patch 1), 10cm (patch 2) and 100cm (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 hours 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/non-producing isolates and reveal any variation in pyoverdine production across the population. To do this, we cultured isolates from freezer stocks in 2ml of Kings B (KB) media in 24-well plates at 25ºC, shaken overnight at 200rpm. Cell

density was standardized and diluted to an optical density of 0.2 (A_{600}) with M9 minimal media and inoculated into 96-well plates containing iron-limited casamino acid media (6 replicates per isolate, 200µl of media and 2µl of bacterial culture). We then incubated cultures for 48hrs at 25°C before measuring cell density (A_{600}) and fluorescence (400/460nm excitation/emission, cut off at 475nm) using a fluorimeter (SpectraMax M2, Molecular Devices, UK). 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 5th percentile for pyoverdine production per cell) we classified as non-producers. We also cultured isolates we classified as non-producers 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 multi-locus 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 non-producing isolates.

DNA Extraction and PCR

We extracted genomic DNA using the Wizard Genomic DNA purification kit (Promega, Switzerland) and amplified three housekeeping genes (gyrB, RpoB and RpoD; Table S1.) for each isolate. All reactions were performed in 50µl volume containing 1U of DreamTaq polymerase (Thermo Fisher Scientific), 5µl of DreamTaq

Buffer, 100mM of each primer, 0.2mM of each dNTP, 40.75µl of ddH2O and 20ng of DNA template. We used the following cycling conditions: 95ºC for 3 minutes, then 35 cycles of 95ºC for 30s, 58ºC/60ºC/64.5ºC for 30s, 72ºC for 45s and a final extension of 72ºC for five minutes (Annealing temperatures; Table S2).

Sequencing and Analysis

We purified PCR products before they were Sanger sequenced by SourceBioscience (Nottingham, UK) 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.), 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 non-producers for their ability to use purified pyoverdine to sequester iron from the environment, allowing us to assess their potential to act as cheats. Pyoverdine non-producer cells are completely incapable of growth in iron-limited media supplemented with purified pyoverdine if they lack appropriate pyoverdine receptors (Ghysels et al. 2003). To do this, we cultured non-producers in 2ml of KB media in a 24-well plate at 25ºC, shaken overnight at 200rpm. Cell density was standardized and diluted to an optical density of 0.2 (A_{600}) using M9 minimal media before inoculating 2µl into 96-well plates containing 200µl of iron-

limited media (12 replicates). Half of each isolates replicates were supplemented with purified pyoverdines from *P. fluorescens* (Sigma) to approximately 800 RFU and wells containing supplemented and non-supplemented iron-limited media, but free of cells, served as negative controls. We incubated cultures at 25ºC for 24hrs and then measured cell density (A_{600}) in supplemented and unsupplemented replicates.

Pyoverdine cross-feeding experiments

We tested non-producing isolates for their ability to utilize the pyoverdine-containing supernatant of other isolates to increase their growth, allowing us to determine whether non-producers 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 2ml of KB media in 24-well plates at 25ºC, shaken overnight at 200rpm and standardized cell density to an optical density of 0.2 (A_{600}) using M9 minimal medium before inoculating 60µl into 6ml of iron-limited CAA in 30ml glass vials. Cultures were incubated statically for 48hrs at 25ºC before being passed through a 0.22µm filter to remove bacterial cells. We passed the cell-free supernatants through a centrifugal unit containing a 3kDa membrane (Amicon®) to remove cellular debris and large extracellular products, such as bacteriocins, while retaining pyoverdine molecules (Cordero et al. 2012). Purified supernatants were stored at -20ºC until required for growth enhancement experiments.

We then assayed for the effects of the supernatant extracted as described above, on growth of non-producing isolates. Non-producers were cultured in 2ml of KB media in 24-well plates at 25ºC, shaken overnight at 200rpm before standardizing cell density to an optical density of 0.2 (A_{600}) with M9 minimal media. 2µl of standardized culture was

inoculated into 96-well plates containing 180µl of iron-limited media which we supplemented with 20µl of purified supernatant from a pyoverdine producing isolate. Each non-producer (11 isolates) was grown in iron-limited CAA media supplemented with the supernatant of each isolate in the collection (224 growth enhancement tests per non-producer, 5 replicates each) and alone in 200ul of iron-limited CAA media. We incubated cultures for 24hrs at 25ºC before measuring final cell density at A_{600}.

Assessing non-producer susceptibility to producers bacteriocins

Competitive behaviours that harm or kill competitors may potentially protect cooperators 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 hours in 6ml of KB media (23ºC 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 hours at 23ºC. We then centrifuged cultures at 6861rpm for ten minutes, obtaining a clear, cell-free supernatant by filter sterilizing with a 0.22µm filter and storing at -20ºC until required.

We then randomly selected ten isolates that each pyoverdine non-producer was capable of exploiting and ten isolates each was incapable of exploiting and determined the ability of these isolates to inhibit each cheats growth with bacteriocins. We spotted KB agar plates with bacteriocin-containing supernatants, spread a lawn of each pyoverdine non-producer over the plate and recorded which supernatants inhibited which cheats. Assays were carried out in triplicate. We spotted KB agar plates with 15µl of

supernatant and allowed the plates to dry at room temperature. We cultured pyoverdine non-producers from freezer stocks for 24 hours in 6ml of KB media (23ºC at 200 rpm). Cell density was standardized to an optical density of ~0.1 (A_{600}) and diluted 10-fold in M9 before 70µl of culture was spread onto the supernatant-spotted KB agar plates. We incubated plates at 23ºC for ~14 hours, or until a uniform lawn of bacterial growth was visible, and 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 (1 for inhibition, 0 for no inhibition).

Statistical analysis

Of the 11 isolates we designated non-producers, we merged and averaged the data from isolates that were genetically identical at three housekeeping genes resulting in 8 independent samples. We wished to determine whether a non-producer could utilize the pyoverdine of other producers in the population. To do this, we tested for significant differences between these 8 non-producers' 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 non-producer 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 non-producers successfully utilized 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 non-producer as a random effect, to account for the fact that we have multiple non-producers in the population.

Results

Variation in pyoverdine production

We identified 11 isolates (4.5% of the population) with values below the 5th percentile value for pyoverdine production per cell, fulfilling our criteria for consideration as non-producers, and hence potential cheats (Fig. 1A,C). These isolates grow well under iron-replete conditions, indicating that the availability of iron limits the growth of non-producers 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 non-producers always co-occurred with pyoverdine producers and were found at 8 of the 32 patches sampled. The neighbor-joining tree suggests that non-production has arisen at least six times in the population (Fig. 1B, 2A.).

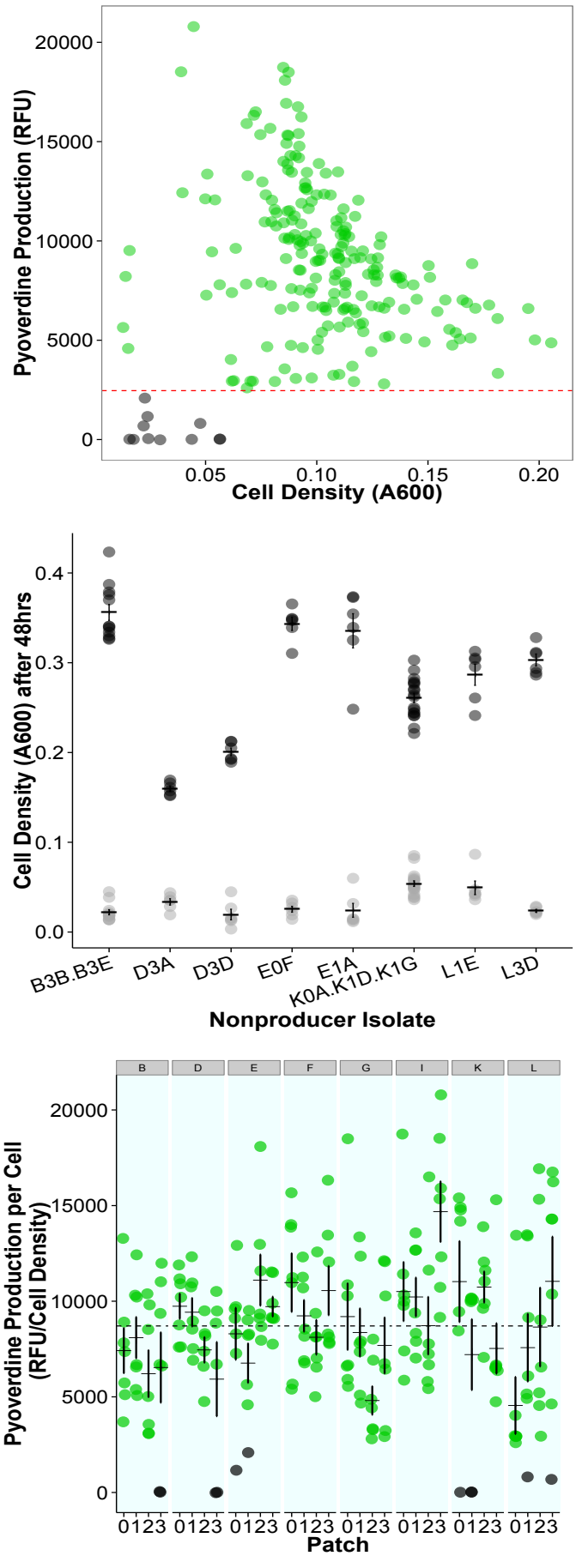


Figure 1) Pyoverdine production per cell and growth of isolates in iron-limited media. **(A)** Isolates falling below the 5th percentile for pyoverdine production per cell were considered non- producers. The horizontal red dashed line represents the 5th percentile for pyoverdine production per cell. Grey circles represent isolates classified as non-producers and green circles represent pyoverdine producers; values are the mean of 6 replicates. **(B)** Growth of non-producer isolates in CAA media and in iron-limited CAA media. All non-producer isolates grow well in CAA media but show significantly reduced growth in iron-limited CAA media. Light grey circles represent non-producers growth in iron-limited CAA media and dark grey circles represent non-producers growth in CAA media. **(C)** Pyoverdine production per cell varies significantly between patches and between transects, and non-producers co-occur with pyoverdine producers. Grey circles represent isolates classified as non-producers and green circles represent pyoverdine producers. Horizontal bars are average per cell production for each patch ± SE.

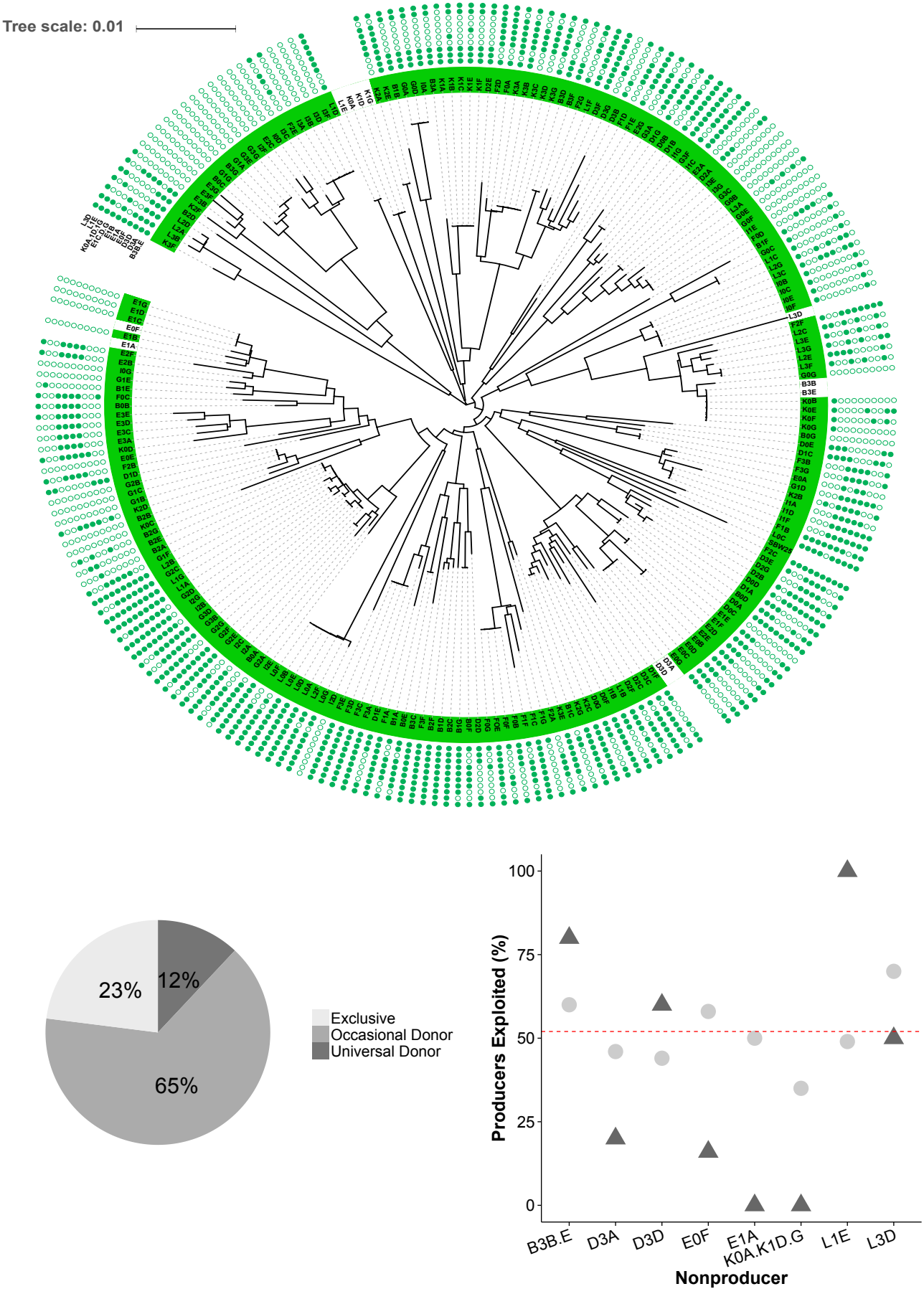

Tree scale: 0.01
23%
12%
65%
Exclusive
Occasional Donor
Universal Donor
Producers Exploited (%)
100
75
50
25
0
B3B.E
D3A
D3D
E0F
E1A
K0A.K1D.G
L1E
L3D
Nonproducer

Figure 2) (A) Phylogenetic tree of all isolates in the population. The phylogenetic tree was constructed from a concatemer of 3 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, non-producer labels are left blank. Each pyoverdine producing isolate has 8 circles associated with it, each representing the ability of a specific non-producer to use that isolates pyoverdine. Filled circles represent successful use of the isolates pyoverdine by the non-producer in the cross-feeding assays and empty circles represent unsuccessful use. **(B)** Percentage of cooperators that act as universal donors, partial donors and non-donors to pyoverdine non-producers. **(C)** The percentage of cooperators in the population that non-producers can exploit. Circles represent the percentage of isolates in the population that individual cheats can exploit, triangles represent the percentage of local, co-occuring isolates (from the same patch) that cheats can exploit.

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.

Non-producers have retained receptor function

Non-producers 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 non-producers 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, 2C). A minority of pyoverdine-producers supernatants significantly increased the growth of all non-producers (12%), some supernatants increased the growth of none of the non-producers

(23%) and the remainder (65%) of supernatants significantly increased the growth of some non-producers but not others (Fig. 2B).

There are at least three possible explanations for this observation: 1) While we have demonstrated that iron is a significant growth-limiting factor in this media (Figure 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 non-producers. It has also been suggested that siderophores may act as a trace metal buffers, increasing the availability of iron to non-producers without requiring the appropriate receptor. However, pyoverdine non-producer 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. 2003). 2) The amount of pyoverdine in producers supernatants varies and this might explain variation in exploitability i.e. 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 non-producers (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 vary in their

ability to exploit the pyoverdine produced by others (Meyer et al. 2000, Meyer et al. 2002).

Genetic similarity predicts ability of non-producers to utilize producers pyoverdines

We found that genetic similarity between non-producers and producers predicted the ability of non-producers to successfully utilize the producers' pyoverdine. Non-producer isolates were more frequently able to utilize 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 non-producers and co-occurring cooperators (from the same patch) was 0.038, ranging from 0.015 to 0.065.

Non-producers vary in their ability to use the pyoverdine of local producers

Non-producer isolates were no less likely to utilize the pyoverdine of co-occuring producers (from the same patch) than producers from the population as a whole (Fig. 3B, GLMM, z=1.529, P= 0.126). Specifically, non-producers could successfully use the pyoverdine of approximately 41.5% of co-occuring isolates and 52% of all other cooperative isolates in the population (Fig. 3B). However, the frequency of successful usage varies considerably between different non-producers when interacting with co-occuring cooperators. This ranges from a complete inability of some non-producers to use the pyoverdine of co-occuring 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 non-producers (Fig. 3B, F = 11.888, df = 7, P =0.002075).

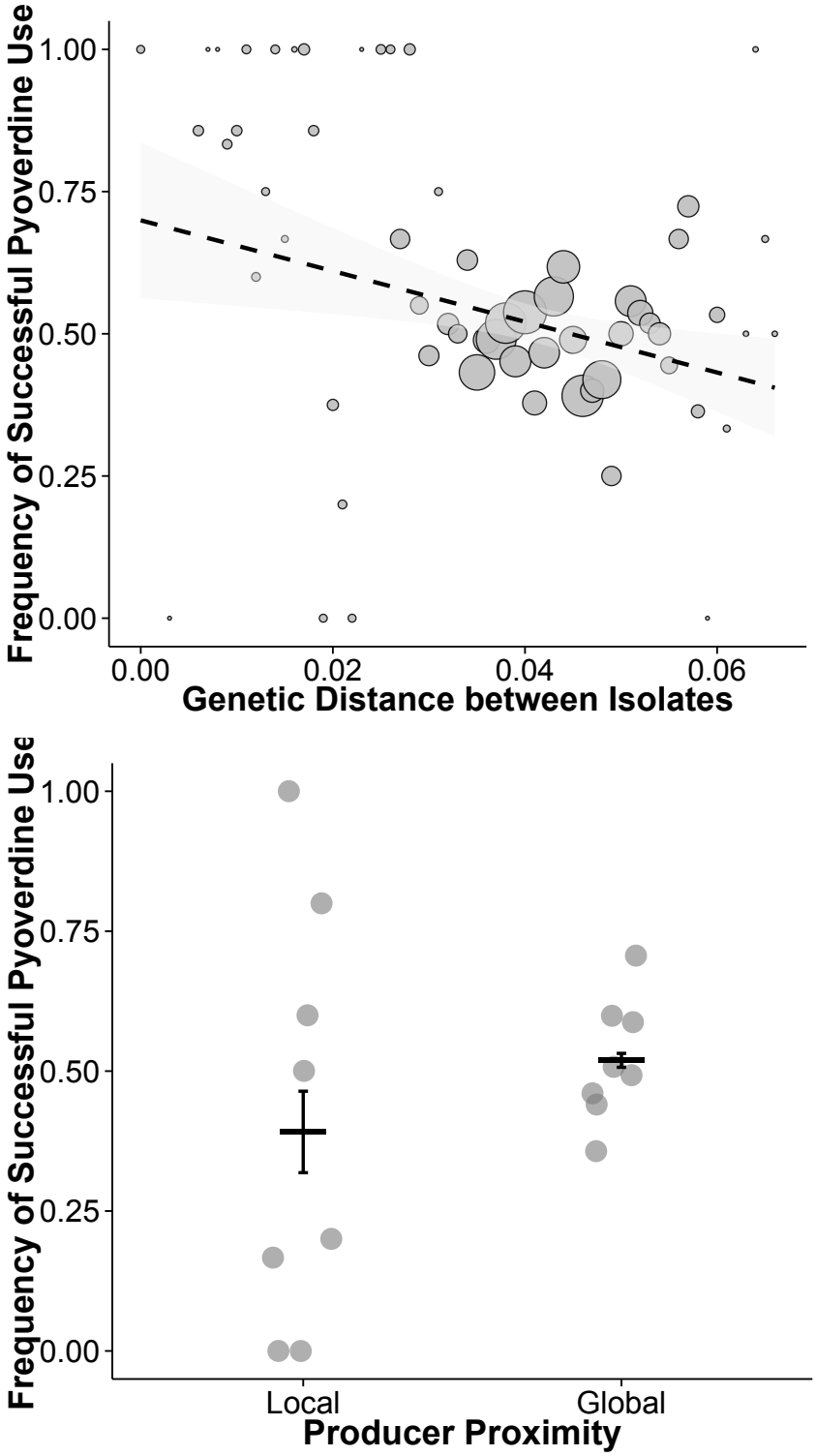


Figure 3) The effects of genetic similarity and proximity on ability to utilize pyoverdine **(A)** Non-producer isolates can successfully use the pyoverdine of genetically similar isolates more frequently than genetically dissimilar isolates. The size of the points reflects the number of interactions: smallest points indicate <5 interactions and largest points indicate >150 interactions (n=1802). **(B)** Non-producer isolates are significantly less likely to utilize the pyoverdine of co-occuring producers than from producers from the rest of the population. Points

reflect the proportion of successful pyoverdine use for each non-producer when grown with co-occuring isolates (local) or all other isolates in the population (global). Horizontal bars are average proportion of successful pyoverdine use ± SE.

Cooperators can inhibit non-producers using bacteriocins

Overall, we found that in 3.5% of the interactions we tested between pyoverdine producers and non-producers, the pyoverdine producers also released a bacteriocin that inhibited the non-producer, and hence would have prevented the non-producers from successfully cheating. The likelihood of a pyoverdine producer also releasing a bacteriocin that inhibited the non-producer did not significantly vary dependent upon whether the non-producer could utilize 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 production (Fig. 2A, 2B, 2C). 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 non-linear, 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:

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

where Q is the average production of the dispersed good by other co-operator 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:

$$W_D = p\big(x\lambda Q(E_0 - \Delta E)\big)^{\alpha} + p\big(x\lambda Q(E_0 + \Delta E)\big)^{\alpha}, \qquad (2)$$

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.

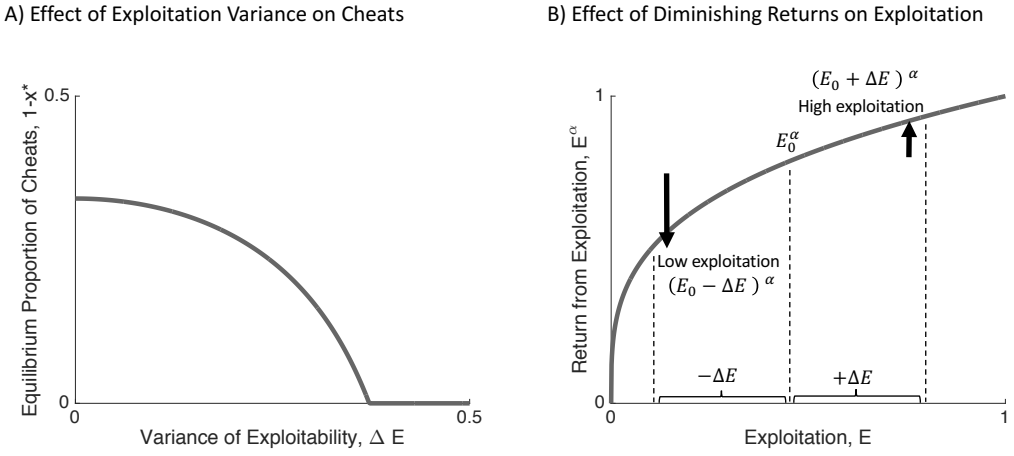


Figure 4) (A) We plot the equilibrium proportion of cheat cells (1-x*) in the population as a function of variance of exploitability (ΔE), where high variance of exploitability means that cheater cells can exploit the extracellular factor released by cooperator cells in some local

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 non-linear 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 non-linear return from the extracellular factor, which is assumed to be diminishing (α=0.3, here). If there is a non-negligible 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-ΔE)^α and a high exploitation environment with relative return (E_0+ΔE)^α. Due to the non-linear 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.

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 non-producers can potentially act as social cheats. We found that 4.5% of all isolates in the population do

not produce pyoverdine, and these non-producers were found in over a quarter (28%) of the 32 patches sampled (Fig. 1A, 1C). However, non-producers can exploit on average only 52% of pyoverdine producers in the population and only 41.5% of local, co-occuring producers (Fig. 2A, 2C). Our cross-feeding assays suggest different forms of pyoverdine are produced in the population (Fig 2A, 2B). In 3.5% of interactions, cooperators produced toxins that killed non-producers, contributing to variation in the extent to which non-producers can exploit cooperators. 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 non-producers 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 to carry multiple different receptors enabling the use of different pyoverdine structural forms (Moon et al. 2008, Ye et al. 2014, Hartney et al. 2011). These observations suggest that non-producers 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. Non-producing cheats can invade and persist in phylogenetically diverse, and spatially structured laboratory microcosms, and siderophore non-producers have been identified in *Pseudomonas aeruginosa*

populations in the CF lung and in marine populations of *Vibrio Spp.* (Joussett et al. 2013, Lujan et al. 2015, Andersen et al. 2015, Cordero et al. 2012). We do not specifically test the ability of non-producers 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 non-producers in the laboratory would imply this happening in soil, even if it wasn't 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 non-producers: pyoverdine production is a continuous trait and we find co-occurance of low and high-level producers, allowing the possibility that low-producers can exploit higher level producers (Ghoul et al. 2014b, Jiricny et al. 2010). Cheating behaviours 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, Sachs et al. 2010, Bronstein et al. 2004, Bshary & Gutter. 2002).

While non-producers 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 (Griffin et al. 2004, Greig & Travisano 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 non-producers 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 (non-linear) 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 non-linearity 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 cheats exploit cooperators? Natural *P. fluorescens* isolates are likely to produce different forms of pyoverdine, potentially preventing cheats 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 & Queller 2011). This specificity allows for uninhibited uptake of pyoverdine providing you carry the appropriate receptor, and limited or no uptake if you do not (Meyer et al. 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 aeurginosa*, with theory suggesting this may be a response to exploitation: cheats drive cooperators to produce new, exclusive structural forms of pyoverdine (Smith et al. 2006, Lee et al. 2012, Eldar 2011). This specificity allows cooperation to be directed preferentially towards 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 behaviours 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. 2003, Ghequire et al. 2014, Inglis et al. 2016b). There is little evidence that bacteriocin production has evolved as a mechanism to punish non-cooperators but susceptibility of cheats to cooperators toxins will likely

prevent successful exploitation. While this is may occur only in a small percentage of interactions, bacteriocins are only one of an arsenal of competitive mechanisms employed by bacteria to exclude non-relatives (Unterweger et al. 2014, Ruhe et al. 2013). Our results suggest that competitive mechanisms will also contribute to variability in the extent to which cheats can exploit cooperators in natural populations.

Appendix

Supplementary Figures

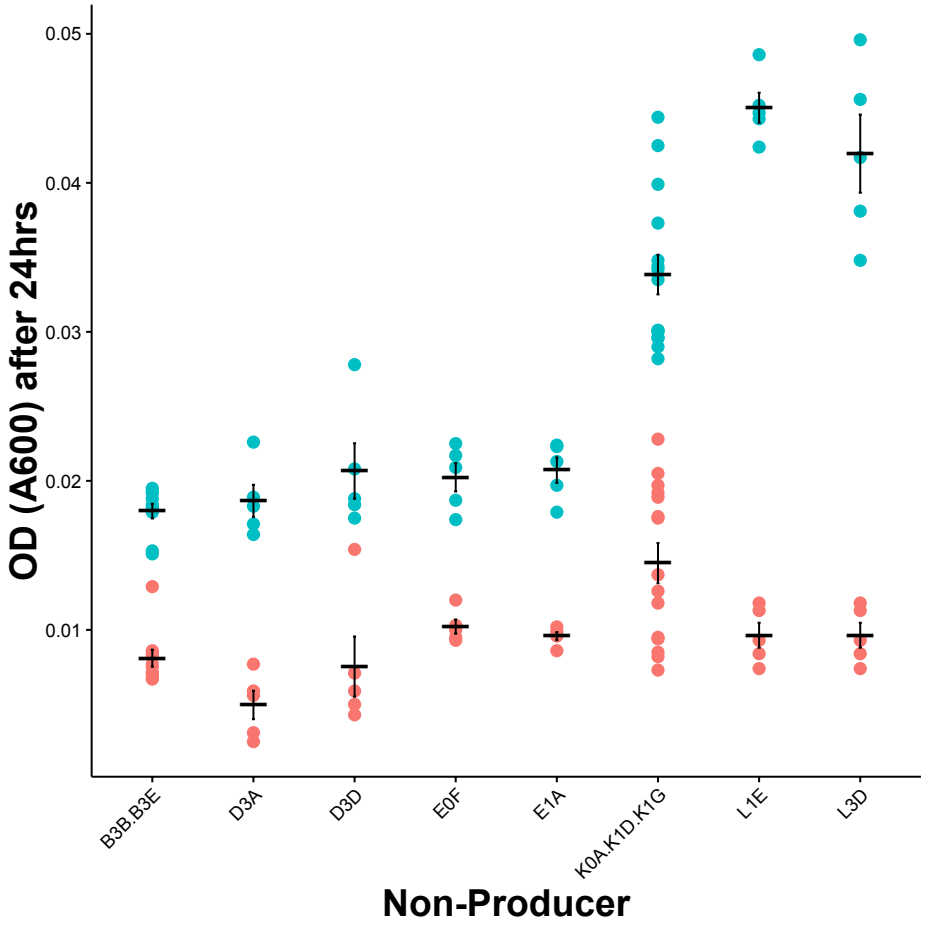


Figure S1) Growth of non-producer isolates in iron-limited CAA media and in iron-limited CAA media supplemented with purified pyoverdine (Sigma). All non-producer isolates grow poorly in iron-limited CAA media but show significantly increased growth in iron-limited media supplemented with purified pyoverdine. Red circles represent non-producers growth in iron-limited CAA media and blue circles represent non-producers growth in iron-limited CAA media supplemented with purified pyoverdine; values are the mean of 6 replicates.

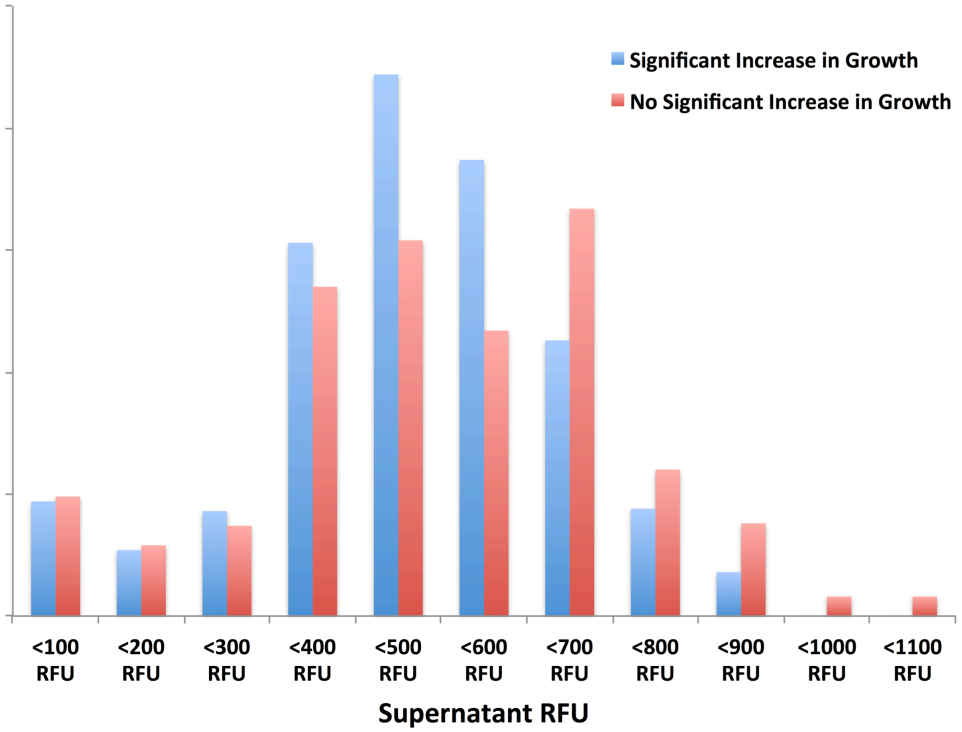


Figure S2) Histograms of levels of pyoverdine in producer supernatant for cross-feeding experiments resulting in a significant growth increase versus no significant growth increase for non-producers. N=1656 cross-feeding assays.

Supplementary Tables

Table S1. PCR and Sequencing Primers and Annealing temperatures

Gene	Primer	ºC
gyrB	gyrB_F	58
rpoB	rpoB_F	64
rpoD	rpoD_F	58
gyrB	gyrB_R	58
rpoB	rpoB_R	64
rpoD	rpoD_R	58

Primer	Gene Product	Sequence	Length
gyrB_F	DNA gyrase subunit B	GGTGGTCGATAACTCCATCG	492bp
gyrB_R		CGCTGAGGAATGTTGTTGGT	
rpoB_F	RNA polymerase subunit beta	TGGCCGGTCGTCACGGTAACA	477bp
rpoB_R		CCGAAACGCTGACCACCGAAC	
rpoD_F	RNA polymerase sigma factor	CTGATCCAGGAAGGCAACATCGG	480bp
rpoD_R		ACTCGTCGAGGAAGGAGCG	

Table S2: Tukey multiple comparisons of means
Fit: RFU.OD~Site

Site	Diff	lwr	upr	p adj
D-B	1183.1422	-2083.3682	4449.6527	0.9543748
E-B	1906.3146	-1299.1342	5111.7634	0.6068489
F-B	2656.0452	-549.4036	5861.494	0.185991
G-B	449.732	-2755.7168	3655.1808	0.9998741
I-B	3968.8546	763.4058	7174.3034	**0.0047599**
K-B	2062.4395	-1143.0093	5267.8883	0.5048824
L-B	888.4997	-2316.9491	4093.9485	0.9899869
E-D	723.1724	-2543.3381	3989.6828	0.997495
F-D	1472.9029	-1793.6075	4739.4134	0.8653528
G-D	-733.4102	-3999.9207	2533.1002	0.9972621
I-D	2785.7124	-480.7981	6052.2228	0.1578505
K-D	879.2973	-2387.2132	4145.8077	0.9916005
L-D	-294.6426	-3561.1531	2971.8679	0.9999938
F-E	749.7305	-2455.7183	3955.1793	0.99646
G-E	-1456.5826	-4662.0314	1748.8662	0.8605806
I-E	2062.54	-1142.9088	5267.9888	0.5048172
K-E	156.1249	-3049.3239	3361.5737	0.9999999
L-E	-1017.815	-4223.2638	2187.6338	0.9778872
G-F	-2206.3132	-5411.762	999.1356	0.4137782
I-F	1312.8095	-1892.6394	4518.2583	0.914466
K-F	-593.6057	-3799.0545	2611.8431	0.9992061
L-F	-1767.5455	-4972.9943	1437.9033	0.6950548
I-G	3519.1226	313.6738	6724.5714	**0.0203701**
K-G	1612.7075	-1592.7413	4818.1563	0.7847732
L-G	438.7676	-2766.6812	3644.2164	0.9998934
K-I	-1906.4151	-5111.8639	1299.0337	0.6067835
L-I	-3080.355	-6285.8038	125.0938	0.0694992
L-K	-1173.9398	-4379.3886	2031.509	0.9516211

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

Non-producer		Pyoverdine (-)	Pyoverdine (+)	t-value	Df	p-value
B3B.B3E	**Mean**	0.0081	0.01797			
	SD	0.001812304	0.001560662	13.0501	18	1.29E-10
	SE	0.000573101	0.000493525			
D3A	**Mean**	0.00496	0.01866			
	SD	0.002139626	0.002411016	9.5034	8	1.24E-05
	SE	0.00095687	0.001078239			
D3D	**Mean**	0.00754	0.02066			
	SD	0.004516968	0.004169892	4.7723	8	0.001405
	SE	0.00202005	0.001864832			
E0F	**Mean**	0.01022	0.02024			
	SD	0.001070981	0.00212791	9.4052	8	1.34E-05
	SE	0.000478957	0.00095163			
E1A	**Mean**	0.00958	0.02072			
	SD	0.000601664	0.001913635	12.729	8	1.37E-06
	SE	0.000269073	0.000855804			
K0A.K1D.K1G	**Mean**	0.01448	0.03384			
	SD	0.005224011	0.005145011	10.4547	28	3.57E-11
	SE	0.001348834	0.001328436			
L1E	**Mean**	0.00964	0.04504			
	SD	0.001876966	0.002254551	26.983	8	3.83E-09
	SE	0.000839405	0.001008266			
L3D	**Mean**	0.00964	0.04196			
	SD	0.001876966	0.00587052	11.726	8	2.56E-06
	SE	0.000839405	0.002625376			

Chapter 4: Functional amyloids promote retention of public goods involved in iron acquisition and shape cooperator-cheat dynamics

Abstract

Bacteria cooperate to perform essential functions using secreted public goods. These goods are costly produce but can be lost through diffusion away from cooperative cells, or to 'cheats' that do not produce the public good but benefit from its production in others. Limiting the diffusion of goods away from cooperative cells is therefore essential if cooperation is to be maintained. *P.aeruginosa* produces Fap fibrils, needle-like fibres on the cell surface that can transiently bind several secreted metabolites produced by cells. Here, we use strains that vary in their ability to produce Fap fibrils and in their ability to produce the iron-scavenging molecule pyoverdine to determine whether limiting the loss of public goods involved in iron-acquisition can influence cooperator-cheat dynamics during competition over iron. We show that (1) wildtype cells retain more iron-chelating metabolites than fibril non-producers; (2) purified Fap fibrils can prevent the loss of the iron-chelators PQS and pyoverdine by dilution and (3) pyoverdine non-producing cheats have higher fitness in competition with fibril non-producers than with wildtype cells. Our results suggest that by limiting the loss of costly public goods, Fap fibrils may play an important role in explaining the stability of secreted public goods in bacterial populations.

Introduction

To survive and thrive in an environment, bacteria often rely upon the secretion of extracellular public goods. These goods are metabolically costly to produce but are essential for effective communication between cells, scavenging of iron and nutrients,

protection from biotic and abiotic stresses and engaging in antagonistic competition (Popat et al. 2015, West & Buckling. 2003, Flemming et al. 2016, Ghequire & De Mot 2014). However, production of these goods can benefit both the producing cell and any neighbouring cells. Thus, public goods producers can potentially be exploited by non-producing 'cheats' that benefit from the available goods while paying none of the metabolic costs of production (Ghoul et al. 2015, Griffin et al. 2004, Diggle et al. 2007). These cheats often arise de novo under laboratory conditions and putative social cheats have been identified in a number of natural bacterial populations (Sandoz et al. 2007, Andersen et al. 2015, Cordero et al. 2012, Bruce et al. 2017).

As well as exploitation by social cheats, cells may lose return on investment in public good production simply through diffusion or advection of costly goods away from the cell in turbulent environments (Kummerli et al. 2009, Mund et al. 2016, Emge et al. 2016). These losses can be minimized through a number of different mechanisms. Cells can avoid exploitation by cheats by ensuring the benefits of public good production accrue to relatives. This can be achieved at its simplest through clonal growth but bacteria will also engage in conflict with, and kill, local unrelated cells (Nadell et al. 2013, Joulu et al. 2013, McNally et al. 2016). Forming a physical barrier can also prevent loss of public goods by slowing diffusion and/or reducing fluid flow rate around the colony: for example, thick layers of biofilm produced by *Vibrio cholera* can slow the diffusion of digested chitin, preventing its use by protease-deficient mutants (Drescher et al. 2014). However, cells may employ other mechanisms to slow or prevent the loss of secreted public goods.

One such mechanism is the production of needle-like fibrils that can bind secreted public goods. These fibrils, functional amyloids, are assembled on the cell surface and can be involved in surface attachment, provide structural rigidity to the extracellular matrix and even aid in evasion of host immune responses (Barnhart & Chapman 2006, Zeng et al. 2015, Taglialegna 2016). Recently, it was demonstrated that functional amyloids produced by *Pseudomonas aeruginosa*, Fap fibrils, are capable of binding several hydrophobic metabolites secreted by the cell (Dueholm et al. 2013, Seviour et al. 2015). Binding of the metabolites is transient: raising the possibility that serial association and dissociation with Fap fibrils may allow secreted metabolites to be locally 'bioavailable' while ensuring they are retained close to producing cells (Fig 1). If public goods producers are able to maintain preferential access to the costly goods they secrete into the environment, this could have important implications for cooperator-cheat dynamics in bacterial populations (Ross Gillespie et al. 2007).

Here, we assess whether Fap fibrils help cells retain secreted metabolites involved in iron acquisition and if this can influence cooperator-cheat dynamics. Using strains of *Pseudomonas aeruginosa* that differ in their ability to produce Fap fibrils, we assess whether production of fibrils influences growth and investment in the iron-scavenging molecule pyoverdine. We also isolate Fap fibrils from cells to determine whether fibrils can bind, and prevent the loss by dilution, of the iron-chelators pyoverdine and PQS, and subsequently whether any metabolites retained by Fap fibrils are functionally available to cells. Finally, we determine whether producing Fap fibrils influences the ability of pyoverdine producers to resist invasion by pyoverdine non-producing cheats in an iron-limited environment.

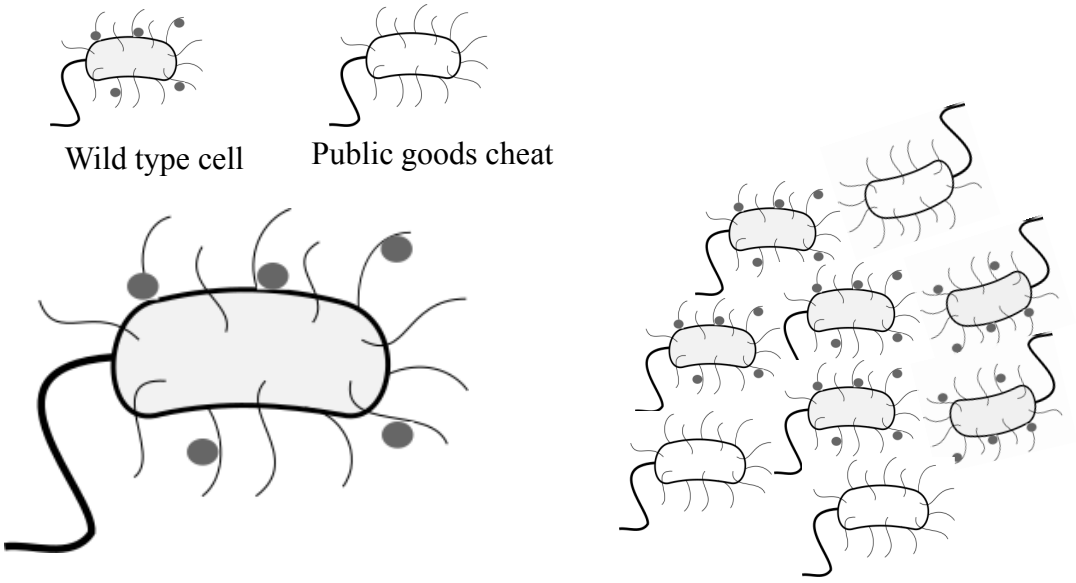


Figure 1. Hypothesized mechanism of metabolite retention. Wildtype *Pseudomonas aeruginosa* cells produce amyloid fibrils, needle-like protein fibers, which can bind secreted metabolites. Binding is transient: metabolites rapidly associate and dissociate with fibrils, keeping the metabolites close while allowing them to perform their function in the environment. In spatially structured populations, this may keep metabolites close to the cells that produce them, ensuring the benefits of public good production accrue to kin.

Material & Methods

Media and Bacterial Strains

For growth in a rich, iron-replete environment we used Kings Broth (KB) medium (20g Protease Peptone N^{o}3 (BD Biosciences), 10g glycerol, 1.5g $K_2HPO_4.3H_2O$ and 1.5g $MgSO_4.7H_20$ per litre of dH_2O) and for growth in an iron-limited environment we used Casamino Acid (CAA) medium (5g Casamino acids, 1.18g $K_2HPO_4.3H_2O$ and 0.25g $MgSO_4.7H_2O$ per litre of dH_2O) supplemented with 20mM sodium bicarbonate and 100μg ml^{-1} of the iron chelator human apo-transferrin. We wash and dilute bacterial cultures using M9 minimal salt media (6.8g Na_2HPO_4, 3g KH_2PO_4, 0.5g NaCl and 10 g NH_4Cl per litre of dH_2O). For purification of Fap fibrils, we cultured strains in colony factor antigen (CFA) medium (10g hydrolyzed casein, 50mg $MgSO_4$ and 1.5g yeast extract per litre of dH_2O). All reagents were purchased from Sigma unless otherwise stated.

We use strains that differ in their ability to produce Fap fibrils and the siderophore pyoverdine in our experiments (Table 1.). The PAO1 strain produces wildtype levels of Fap fibrils and pyoverdine. PAO1ΔFap is a mutant with the genes involved in Fap fibril production, FapA-FapF, inactivated by allelic replacement with a gentamicin resistance cassette (Dueholm et al. 2013). We also use mutants that cannot produce the iron-scavenging siderophore pyoverdine. PAO9 is UV-induced mutant derived from PAO1 that is incapable of producing pyoverdine and produces small white colonies that are easily distinguished from pyoverdine producing cells. PAO1ΔpvdD is a clean deletion mutant defective for the pyoverdine synthetase gene pvdD. For isolating Fap fibrils, we use a PAO1 strain harboring a plasmid containing the *Pseudomonas* Fap operons under the control of an isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible lacUV5 promoter (PAO1 pFap). Prior to our experiments, we cultured strains overnight for 15 hours at 37°C in 6ml of KB media, at 200rpm on an orbital shaker.

Strain	Fap Fibrils	Pyoverdine
PAO1	+	+
PAO1ΔFap	-	+
PAO1 pFap	+	+
PAO1ΔPvdD	+	-
PAO9	+	-

Table 1. Summarising phenotypic differences between strains used in the study.

Measuring Growth and Pyoverdine Production

To determine whether producing Fap fibrils influenced the growth of strains, or altered their investment in pyoverdine, we cultured fibril producers and non-producers under

iron-limited conditions. We washed overnight cell cultures in M9 media, standardized to an optical density (A_{600}) of 0.2 and diluted 100-fold before inoculating 200µl of iron-limited CAA media with 2µl of diluted culture in a 96-well plate. We measured cell density (absorbance at 600nm) and pyoverdine production (Relative Fluorescent Units, using an excitation of 400nm and emission of 460nm respectively) every 30 minutes for 24 hours using a SpectraMax i3x multi-mode platform (Molecular Devices). We calculated pyoverdine available per cell at each time point ($RFU_{400\text{-}460}$ / A_{600}) and the rate of pyoverdine production per cell per minute (($RFU_{400\text{-}460\ (2)}$–$RFU_{400\text{-}460\ (1)}$)/30 min)/$A_{600(2)}$). Each treatment was replicated 6 times per strain.

Cells retention of iron-chelating metabolites

To determine whether Fap fibrils influence cells ability to retain iron-chelating metabolites, we measured the iron-chelating activity of fibril producers and non-producers after removing unbound metabolites using a CAS assay (Schwyn & Neilands. 1987). We washed overnight cell cultures in M9 media, standardized to an optical density (A_{600}) of 0.2 and diluted 100-fold before inoculating 6ml of iron-limited CAA media with 60µl of diluted culture. We incubated vials statically for 48 hours at 37°C before vortexing and passing 2ml of culture through a 0.22µm syringe filter. We washed cells held on the membrane to remove unbound iron-chelating metabolites by passing 20ml of M9 solution through the syringe filter and re-suspended cells by passing 2ml of M9 solution through the opposite end of the filter. 100µl of CAS solution (Schwyn & Neilands 1987) was added to 100µl of re-suspended cells in a 96-well plate before we incubated the plate in darkness for 30 minutes. Iron-chelating molecules remove iron from the CAS solution, changing the colour of the solution. We quantified this change by measuring absorbance at A_{630}. We also measured the optical

density of the re-suspended cells (A_{600}) and calculated the iron-chelating activity per cell ($1-A_{630}/A_{600}$). Each treatment was replicated 22 times.

Binding of iron-chelators to Fap fibrils.

We wished to determine if Fap fibrils can bind iron-chelating metabolites, and whether bound metabolites are functionally available to cells. We exposed isolated fibrils to the iron-chelators PQS and pyoverdine and quantified iron-chelating activity of the fibrils after repeated washing to remove unbound molecules. We then assessed the ability of fibrils exposed to iron-chelators to promote the growth of a pyoverdine deficient mutant in iron-limited media.

Isolation of Fap fibrils

We inoculated PAO1 pFAP, an inducible Fap fibril overproducing strain, into KB media and incubated overnight (37°C, 200rpm) before inoculating 400ml of CFA media with 4ml of the overnight culture in a 2L flask with 40µg/ml of tetracycline. Cultures were incubated at 37°C, 200rpm to an optical density (A_{600}) of ~0.5 before inducing expression of Fap fibrils with 1mM of IPTG and incubating for a further 8 hours. We harvested cells by centrifugation (28,000g, 30 mins, 20°C), resuspended cells in 30ml of buffer (10mM Tris-HCL, pH 8.0) and homogenized manually before adding 10ml of enzyme mix (0.4mg/ml RNaseA, 0.4mg/ml DNase I, 4mg/ml lysozyme, 4mM $MgCL_2$ and 0.4% Triton X-100). This mix was homogenized by three cycles of freeze-thawing using a -80°C freezer and a 37°C water bath, followed by a final incubation of 2hrs at 37°C. We then added 5ml of 20% sodium dodecyle sulfate (SDS), boiled the sample for 20 minutes and collected insoluble material by centrifugation (28,000g, 30 mins, 20°C). The pellet was then resuspended in 27ml buffer with 3ml of 20% SDS and boiled for 20

minutes before insoluble material was collected by centrifugation. We repeated this step twice. To wash away residual SDS, we resuspended the pellet in 25ml of buffer and centrifuged (28,000g, 30 mins, 20°C), repeating this step a further two times before re-suspending the pellet in 3ml of buffer after the final centrifugation.

To confirm the presence of amyloid structures in our purified protein solution, we used a Thioflavin T fluorescence assay. We suspended 0.5mg/ml of lysozyme and 0.5mg/ml of purified protein solution in 10mM Tris-HCl (pH 8.0) and 40µM Thioflavin T. 10mM Tris-HCl (pH 8.0) and lysozyme, a protein lacking any amyloid structures, served as negative controls. Using a Spectramax i3x, we recorded the emission spectra of the samples from 470-600nm after excitation at 450nm.

Fap fibril retention of iron-chelating metabolites

We suspended purified Fap fibrils in 400µl buffer (10mM HEPES, pH 7.4) to concentrations of 5µg/ml and 50µg/ml, and included a control with no fibrils (0µg/ml). PQS or pyoverdine were added to final concentrations of 200µM and 100µM respectively and the mixtures were incubated overnight (22°C, 400rpm). Each treatment was replicated 6 times. The mixtures were centrifuged (5 minutes, 1000g), the supernatant discarded and the retentate resuspended in 1ml of buffer and vortexed. This step was repeated 3 times and following the final centrifugation, retentate was resuspended in 400µl of buffer.

To determine whether fibrils prevented the loss of PQS and pyoverdine by dilution, we quantified the iron chelating activity of the mixes after repeated washing. We added

100µl of each mixture to 100µl of CAS solution in a 96-well plate, incubated the plate in darkness for 30 minutes and measured absorbance at 630nm.

We also determined whether iron-chelators retained by fibrils were available for use by cells and could stimulate the growth of a pyoverdine non-producer strain, PAO1ΔpvdD, in iron-limited media. Overnight cultures of the pyoverdine non-producer were washed in M9 media, standardized to an OD of 0.2 and diluted 100-fold into 180µl of iron-limited CAA media in a 96-well plate. We then made up the volume in each well to 200µl with the different concentrations of fibrils (0, 5, or 50µg/ml) exposed to buffer, PQS or pyoverdine. Plates were incubated statically at 37°C and we measured the optical density of cultures (A_{600}) at 0hrs and 24hrs respectively.

Relative fitness of pyoverdine non-producers

To determine whether producing Fap fibrils influenced the ability of public goods producers to resist invasion by social cheats, we competed fibril producers and fibril non-producers (both pyoverdine producers) against the pyoverdine non-producer PAO9 under iron-limited conditions. After washing overnight cultures in M9 media, standardizing optical density (A_{600}) to 0.2 and diluting 100-fold, we mixed each combination of producer and non-producer at a ratio of 10:1 (producers: non-producers). 6ml of iron-limited CAA media was inoculated with 60µl of each mixture and incubated cultures statically at 37°C for 24 hours. Each treatment was replicated 20 times. We measured the frequency of producers and non-producers before and after 24hrs of incubation by plating onto KB agar (12g agar per litre of KB medium) and counting the number of colonies of producers and non-producers. We calculated the relative fitness of non-producers using the formula $w=(p2(1-p1))/(p1(1-p2))$ where p1

and p2 are the proportion of pyoverdine non-producers in the population before and after competition occurs respectively. A value of w >1 indicates that the non-producer has a higher fitness that the pyoverdine producer and w < 1 indicates that the producer has a higher fitness than the non-producer.

Statistical Analyses

We carried out all statistical analyses in the R statistical environment (v3.3.3, http://www.R-project.org). Except where stated, we carried out standard analyses (T-Test, Linear models, etc.) assuming normal errors. All analyses using Linear Mixed-Effect Models (LMEM) included day as a random effect, to account for the fact that replicates of the experiments were carried out on different days.

Results

Population Growth and Pyoverdine Production.

To determine whether the deletion of the Fap operon has consequences for growth or alters investment in pyoverdine, we measured growth and pyoverdine production of fibril producers and non–producers. After 24hrs of growth in iron-limited media, we found no significant difference in cell density ($t=0.519$, $df=10$, $p=0.615$), pyoverdine production ($t=-0.135$, $df=10$, $p=0.895$) and pyoverdine availability per cell ($t=-0.899$, $df=10$, $p=0.389$) (Fig 2). These results suggest that not producing fibrils does not significantly alter non-producer investment in pyoverdine production nor detrimentally affect its growth in an iron-limited environment.

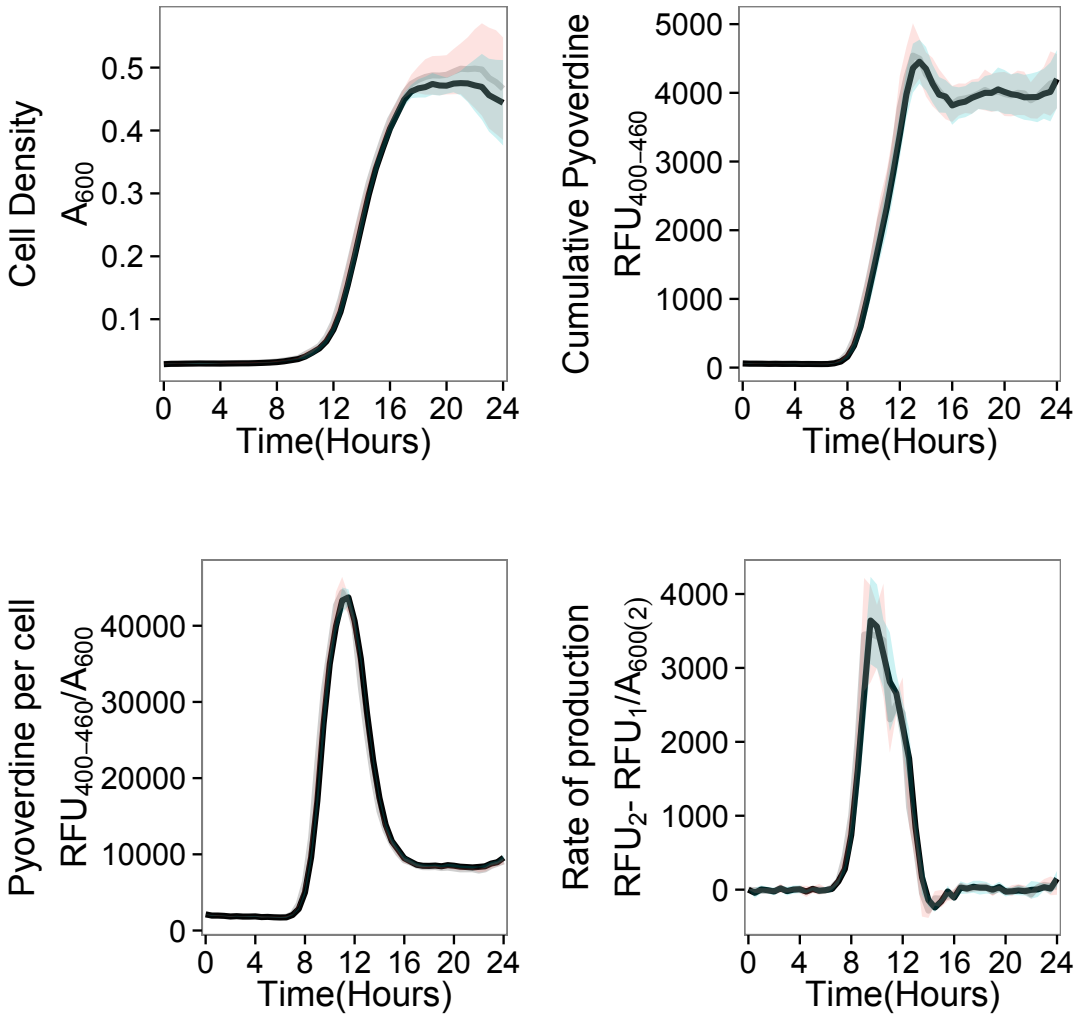


Figure 2. Growth and pyoverdine production of Fap fibril producers (black lines, grey envelope) and non-producers (grey lines, pink envelope) over 24hrs in iron-limited media. Mean and standard error envelope are plotted for each**. (a)** Growth of strains, measured by cell density at A_{600} **(b)** Cumulative pyoverdine available, measured by $RFU_{400-460}$ **(c)** Pyoverdine available per cell, measured by $RFU_{400-460}/A_{600}$ **(d)** Rate of pyoverdine production, measured per cell per 30 minutes $((RFU_2–RFU_1)/30\ min)/A_{(600(2))}$. Cell density, available pyoverdine and pyoverdine available per cell were not significantly different between fibril producers and non-producers.

Retention of iron-chelating metabolites.

We assessed the ability of fibril producers and non-producers to retain iron-chelating metabolites after washing cells. After cells had been washed to remove unattached metabolites, we found that the iron-chelating activity, standardized by cell density, of fibril producers was significantly greater than that of non-producer cells (LMEM,

t=2.256, df=41, p=0.0295) (Fig 3). This suggests that producing fibrils can promote retention of iron-chelating secreted metabolites under conditions where otherwise they would be removed.

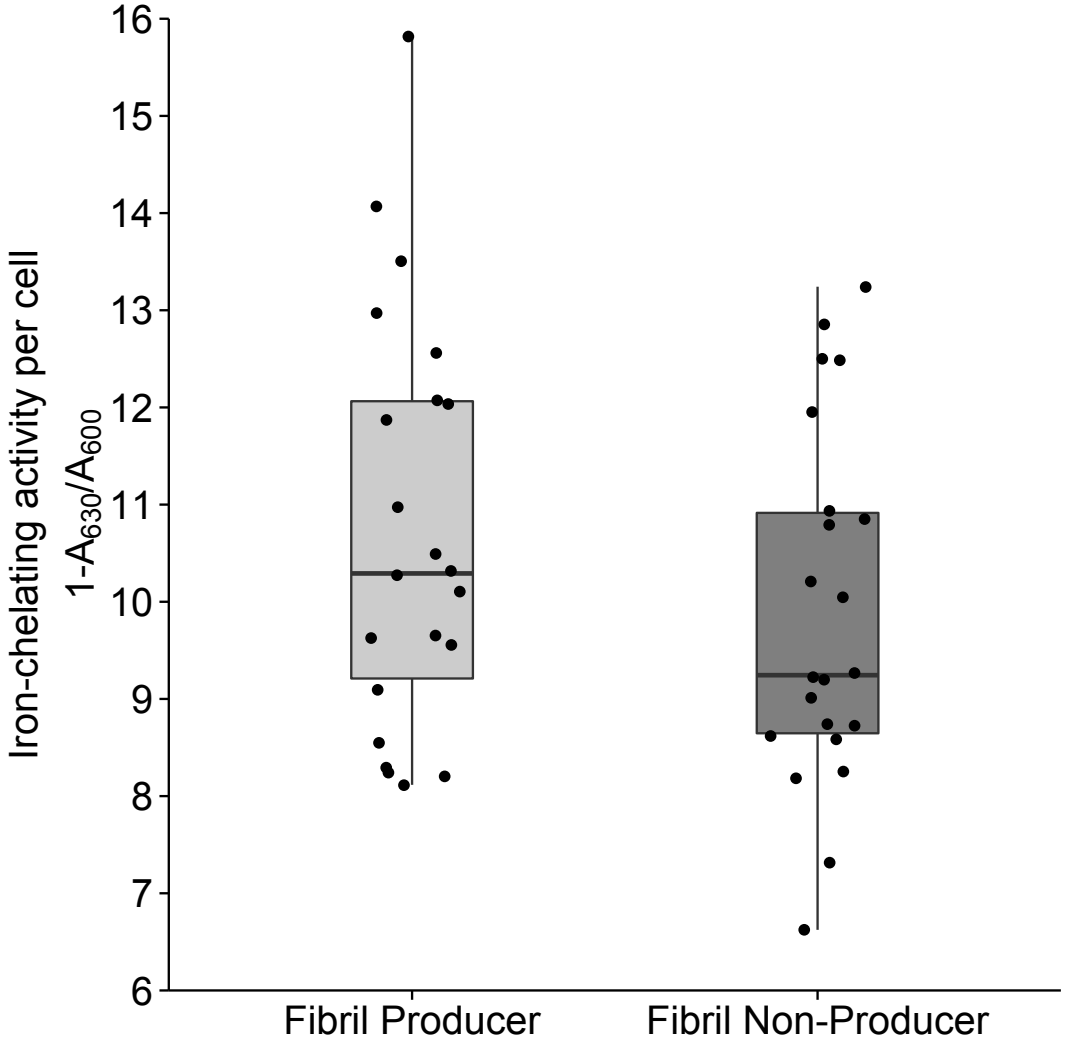


Figure 3. Retention of iron-chelating metabolites by fibril producers and non-producers. Iron chelating activity was measured as iron-chelating activity per cell ($(1-A_{630})/A_{600}$). Boxplots give the median value, boxes show the interquartile range and whiskers extend to the largest or smallest value respectively. Iron-chelating activity of fibril producers was significantly greater than that of non-producers.

We also isolated fibrils and determined whether they could prevent the loss of the known iron-chelators, pyoverdine and PQS, by dilution. A strong fluorescence

emission, with a maximum at ~490 nm when excited at 450 nm from our Thioflavin T assays confirmed the presence of amyloid structures in our purified protein solution (Fig S1). We found that isolated fibrils exposed to pyoverdine or PQS showed increased iron-chelating activity relative to fibrils exposed to buffer, as signified by a significant interaction effect between fibril concentration and pyoverdine ($F_{3,32}$= 12.984, p=0.00105) or PQS ($F_{3,32}$= 82.95, p=2.12x10^{-10}) (Fig 4a). Increasing concentrations of fibrils, without the addition of pyoverdine or PQS, did not increase levels of iron-chelation ($F_{1,16}$=0.008, p=0.931). This suggests that the increase in iron-chelating activity is due to the retention of pyoverdine and PQS by fibrils and not the fibrils themselves.

We also determined whether pyoverdine or PQS molecules retained by fibrils could be utilized by pyoverdine non-producers in an iron-limited environment. We found that fibrils exposed to pyoverdine increased the growth of a pyoverdine non-producer strain, PAO1ΔpvdD, relative to fibrils exposed to buffer, as signified by a significant interaction effect between fibril concentration and pyoverdine ($F_{3,32}$=50.43, p=4.68x10^{-8}) (Fig 4b). Fibrils alone did not increase the growth of cells ($F_{1,16}$=1.281, p=0.274), suggesting that the growth of the pyoverdine non-producer was promoted by the increasing availability of the siderophore pyoverdine in the iron-limited environment. We found that fibrils exposed to PQS actually repressed the growth of the pyoverdine non-producer relative to fibrils exposed to buffer ($F_{3,32}$=-4.983, p=0.032) (Fig 4b). This effect is likely due to the fact that PQS is an iron-chelator but not a dedicated siderophore. Thus, it will bind iron but if cells lack a means of recovering the iron from PQS molecules it only serves to further iron-limit the surrounding environment.

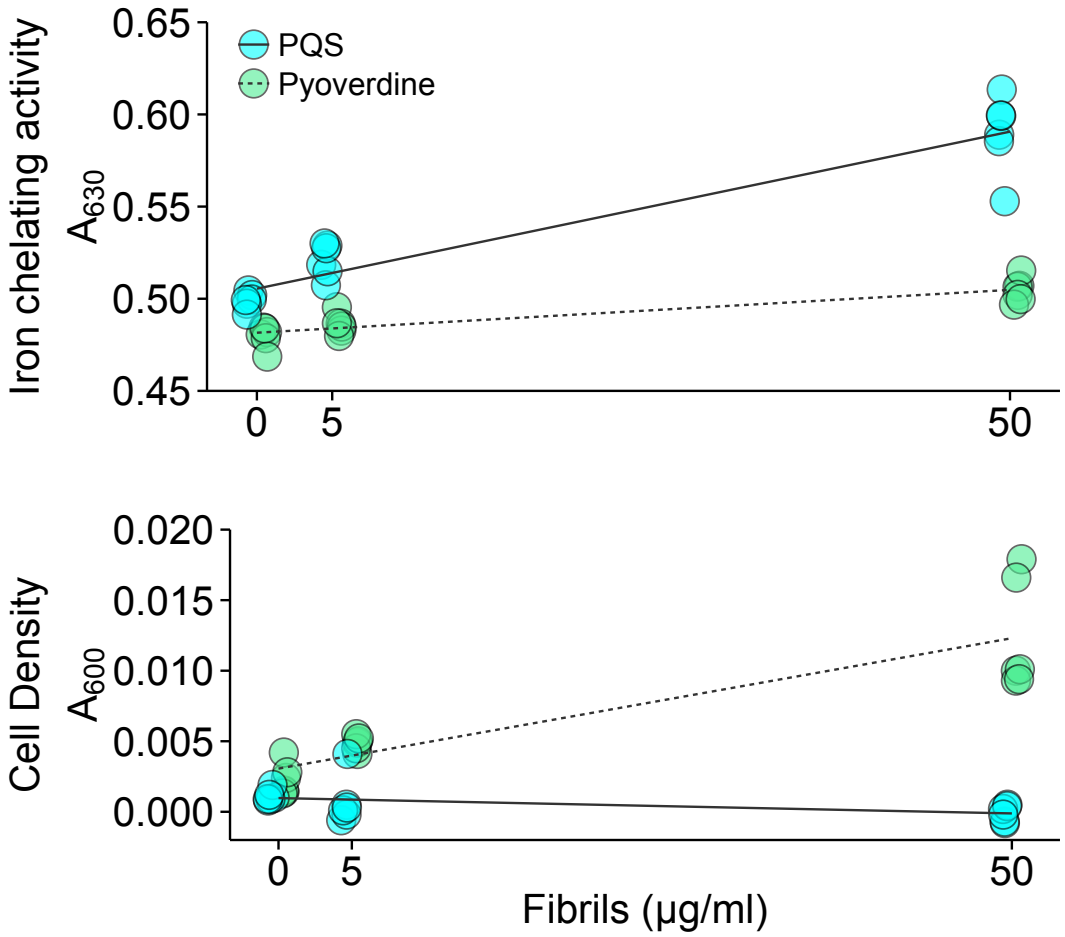


Figure 4. Retention of iron-chelating metabolites by Fap fibrils. **(a)**. Iron-chelating activity, measured as (1-A_{630}), of 0 µg/ml, 5 µg/ml and 50 µg/ml of purified Fap fibrils treated with PQS (cyan) or pyoverdine (green). Iron-chelating activity increases with increasing fibril concentration for both pyoverdine and PQS treatments. **(b)** Growth, measured as $A_{600(24hrs)}$-$A_{600(0hrs)}$, of a pyoverdine non-producer (PAO1ΔpvdD) in iron-limited media supplemented with 0 µg/ml, 5 µg/ml and 50 µg/ml of fibrils treated with PQS (pink) or pyoverdine (green). Growth of pyoverdine non-producer increases with increasing concentrations of fibrils treated with pyoverdine and decreases with increasing concentrations of fibrils treated with PQS.

Competition Assays

To determine whether producing fibrils can shape competition over iron, we competed fibril producers and non-producers with a pyoverdine non-producing strain (PAO9). We found that in mixed cultures, pyoverdine non-producers increase in frequency when in

competition with both fibril producers (w=2.70, t=6.139, df=19, p=6.737x10^{-6}) and non-producers (w=3.81, t=10.24, df=19, p=3.587 x10^{-9}) (Fig 5). However, pyoverdine non-producer relative fitness is significantly greater in competition with Fap fibril non-producers than with fibril producers (LMEM t=3.06, df=36, p=0.00416). These results suggest that producing fibrils can limit the invasion of a pyoverdine non-producer into a population of pyoverdine producers.

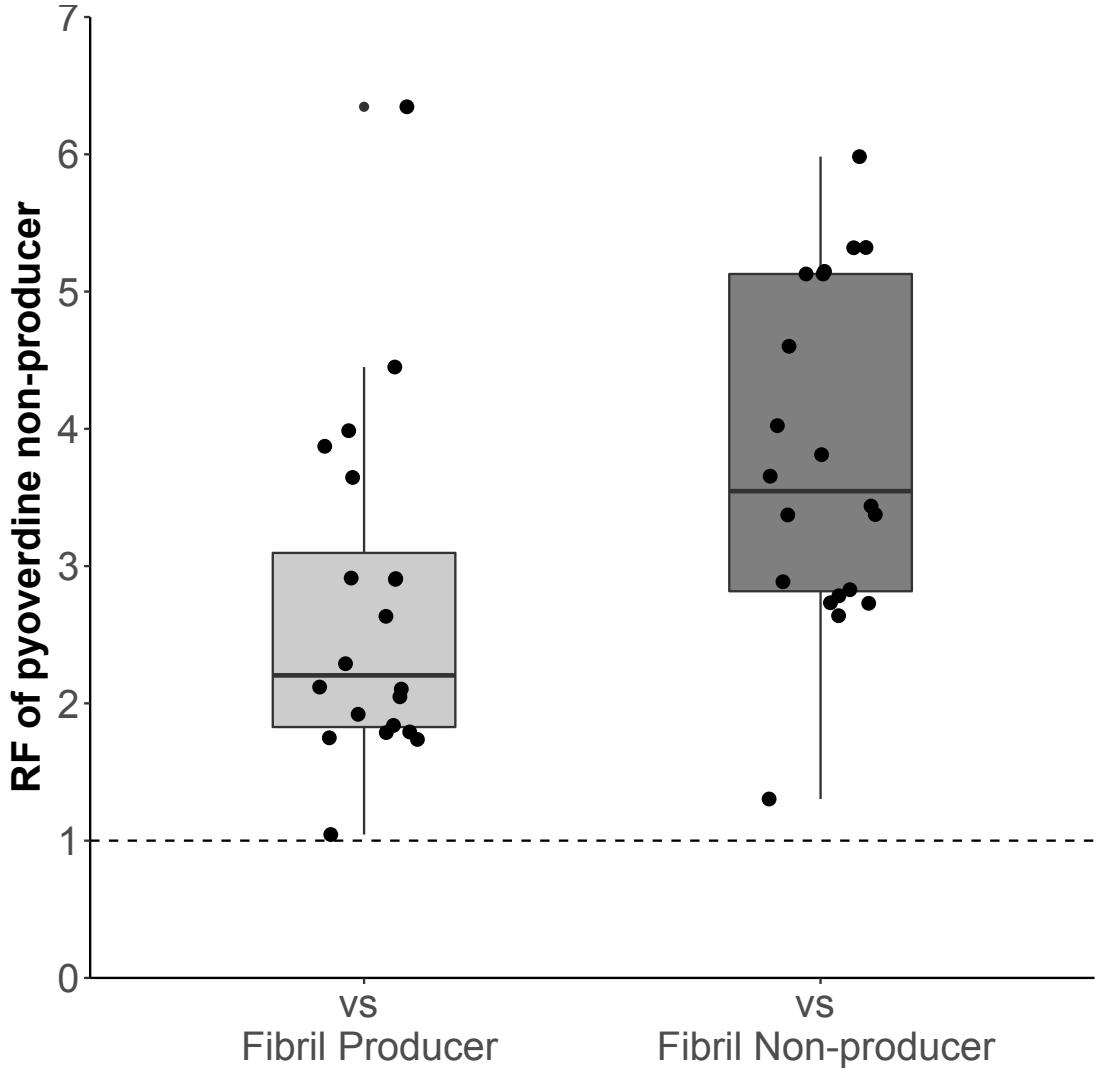


Figure 5. Relative fitness (RF) of a pyoverdine non-producer (PAO9) in competition with fibril producers and non-producers in iron-limited media. Boxplots give the median value, boxes show the interquartile range and whiskers extend to either 1.5 times the interquartile range or to the largest or smallest value respectively. Outliers are shown as small circles. Pyoverdine non-producer significantly increase in frequency over 24hrs in competition with both fibril

producers and non-producers, but pyoverdine non-producer relative fitness is significantly higher when competed against fibril non-producers than against fibril producers.

Discussion

Our results suggest that by producing Fap fibrils, *Pseudomonas aeruginosa* may limit the loss of secreted metabolites involved in iron acquisition and that this can impact competition between public goods producers and cheats over iron. We found that failing to produce Fap fibrils did not alter population growth or investment in the secreted public good pyoverdine (Fig 2), but did appear to help retain iron-chelating metabolites under conditions where otherwise they would be lost (Fig 3). Our CAS assays reveal that Fap fibrils can bind the iron-chelators pyoverdine and PQS and prevent their loss by dilution (Fig 4a) and that metabolites retained by fibrils were functionally available to cells (Fig 4b). Finally, our competition assays reveal that pyoverdine non-producers do significantly better in competition with pyoverdine producers lacking Fap fibrils than with wild-type cells (Fig 5).

Fibril producers are able to retain more iron-chelating metabolites after washing than non-producers (Fig 3) and fibrils appear to bind both pyoverdine and PQS, preventing their loss by dilution (Fig 4a). This suggests a role for fibrils in retaining costly iron chelating metabolites secreted by cells. Transient binding between fibrils and PQS occurs due to the hydrophobic nature of both Fap fibrils and PQS: potentially explaining the strong iron chelating activity of fibrils exposed to PQS despite repeated washing (Fig 4a)(Seviour et al. 2015). Pyoverdine, as a larger amphiphilic molecule, would not appear to be predisposed to binding through hydrophobic interactions (Kummerli et al. 2014). However, it may possess hydrophobic domains that allow a more limited binding to fibrils. Why is binding, and subsequently limited diffusion, of

secreted metabolites likely of importance to cells? The majority of cells will experience conditions in their natural environments that remove costly secreted metabolites by diffusion or convection, limiting their utility to producing cells (Liu & Tay. 2002, Drescher et al. 2014,). Limiting diffusion of secreted metabolites may allow cells to invest in less of a costly, recyclable public good if it is not constantly being lost to the environment (Kummerli & Brown. 2010). It may also increase the range of conditions under which producing extracellular metabolites is a viable strategy: for example, limiting diffusion of quorum sensing molecules may expand the range of conditions under which QS controlled behaviours are inducible (Emge et al. 2016, Kim et al. 2016).

How does the presence or absence of Fap fibrils influence competition over iron? Our results show that pyoverdine non-producers do significantly better in competition with fibril non-producers than with fibril producers (Fig 5). One possible reason for this is that failure to produce fibrils alters investment in pyoverdine production. This is important because non-producers gain a competitive advantage only when a cooperator is paying the cost of producing a public good (Ghoul et al. 2016). Thus, if cells that do not produce fibrils increase per cell pyoverdine production, or increase the duration of pyoverdine production, this may increase the competitive advantage to non-producers. However, we see no significant difference in per cell production of pyoverdine between fibril producers and non-producers (Fig 2), suggesting that altered investment in pyoverdine does not explain the success of pyoverdine non-producers in the population.

As fibrils appear to prevent the loss of pyoverdine by dilution (Fig 4a), it may be that during competition with social cheats, fibril producers can limit or slow the diffusion of

pyoverdine away from producing cells. This would have the effect of partially privatizing pyoverdine: ensuring the benefits of pyoverdine production accrue to producing cells and proximate cooperators, and limiting the competitive advantage of non-producers (Scholz & Greenberg 2016, Dobay et al. 2014). Another, non-mutually exclusive explanation, is that fibrils can help trap iron close to cells by binding PQS. PQS, while a strong iron chelator, is not a dedicated siderophore. Thus it has been suggested that PQS may act as an iron trap, binding iron close to cells and making it easier for dedicated siderophores such as pyoverdine to shuttle iron into cells (Diggle et al. 2007). Previous studies have demonstrated that diffusion of secreted public goods can be limited, through maintaining cell-cell contacts or simply by environmental viscosity (Julou et al. 2013, Kummerli et al. 2009). Our results suggest that binding of secreted metabolites by Fap fibrils may also limit the diffusion of secreted metabolites and consequently play a role in shaping cooperator-cheat dynamics.

Cooperative public goods production is common in bacterial populations despite the potential loss of costly public goods through diffusion or advection, or to social cheats (Hibbing et al. 2010). We have demonstrated that Fap fibrils can prevent loss of iron-chelating metabolites by dilution and limit the invasion of social cheats during competition over iron. Interestingly, other secreted metabolites that serve as public goods, such as pyochelin, pyocyanin and the QS molecules C4-HSL and C12-HSL are also small, hydrophobic molecules and likely predisposed to binding to Fap fibrils. Future work should seek to reveal if Fap fibrils can limit the loss of these secreted metabolites, particularly in turbulent environments experienced by cells in their natural environments. By helping limit the diffusion of public goods, Fap fibrils may play an

important role in explaining the stability of secreted public goods in bacterial populations.

Appendix

Supplementary Figure

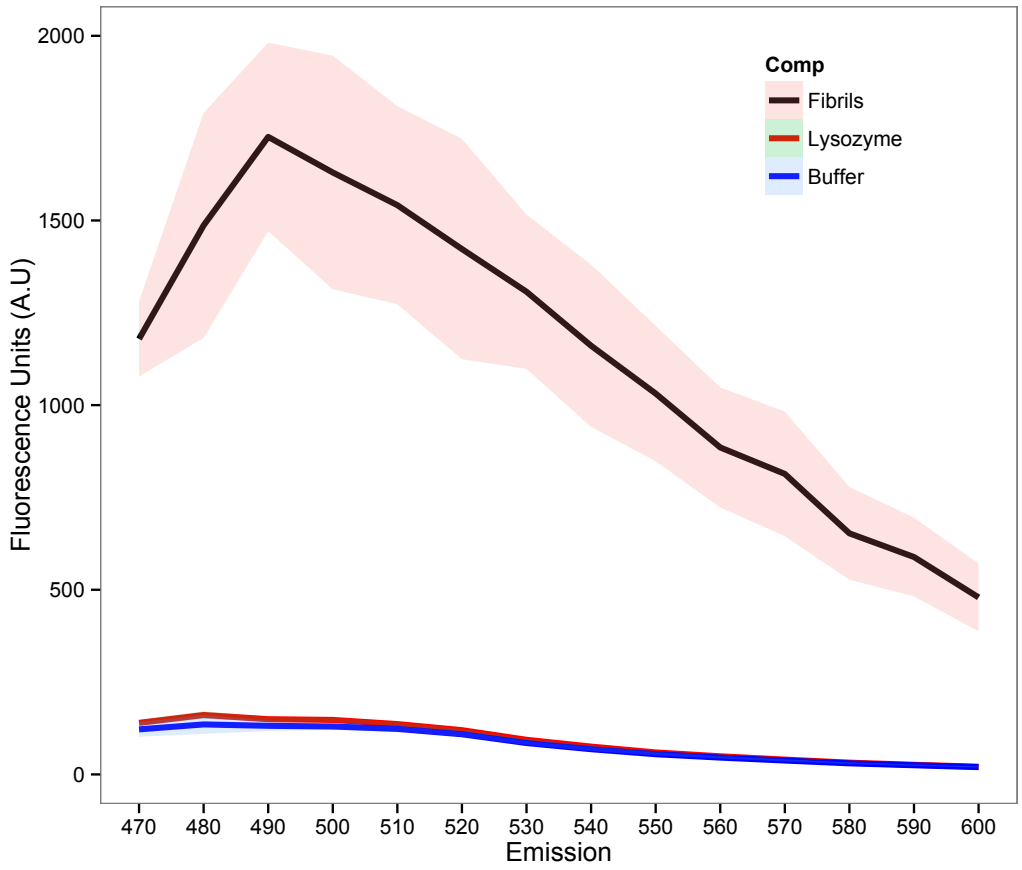


Figure S1: Fluorescence emission of 40µM Thioflavin T when mixed with 0.5mg/ml of lysozyme (red line), 0.5mg/ml of purified protein solution (black line) or 10mM Tris-HCl buffer (pH 8.0)(blue line) when excited at 450 nm.

Chapter 5: Assessing the contribution of functional amyloids towards individual and collective defence against phage-derived bacteriocins in *Pseudomonas aeruginosa*

Abstract

Bacteria are frequently under attack from other strains and species during competition for space and resources. These attacks often come in the form of secreted toxins known collectively as bacteriocins, which kill or slow the growth of susceptible cells. Protection against bacteriocins commonly occurs through possession of a corresponding immunity gene, or through mutation of the receptors bacteriocins use to enter the cell. Functional amyloids, needle-like fibres produced by bacteria and a major component of the biofilm matrix, contribute to individual and collective defenses against tailed-bacteriophage in *Escherichia coli*. Here, we assess whether the analogous Fap fibrils produced by *Pseudomonas aeruginosa* contribute to the individual and collective defense against phage-derived bacteriocins. We show that (1) purified fibrils increase survival of fibrils non-producing cells exposed to bacteriocins; (2) fibril over-producers show increased survival upon exposure to bacteriocins relative to wildtype cells and (3) upon exposure to bacteriocins, biofilms produced by fibril non-producers are no less viable than wildtype biofilms. Our results suggest that Fap fibrils may contribute to individual defense against conspecific phage-derived bacteriocins but do not contribute to collective defense in biofilms.

Introduction

Bacteria are frequently confronted with various biotic and abiotic stressors. If not under pressure from environmental stresses such as heat, desiccation or fluctuations in pH or

salinity, cells are often under attack from disgruntled hosts, or the huge range of predators and viruses that target bacterial cells (Poole et al 2012, Jousset 2011, Seed et al 2015, Jensen et al 2010). Another persistent threat comes from other bacteria: most bacteria produce an array of bacteriocins, and other small antimicrobials, to kill cells with similar space and nutrient requirements (Riley 1999, Russel et al. 2017). To survive and compete in natural microbial populations, cells need to adequately protect themselves against the harm wrought by non-kin.

Bacteria have developed a variety of individual and collective mechanisms for doing just that. Individual cells negate the effects of toxins they produce, allowing them to target competitors while avoiding harm to themselves or kin (Cascales 2007, Ghequire & De Mot 2014). However, cells can often be found carrying 'orphan' immunity genes, protecting them against bacteriocins they themselves do not produce (Ghoul et al 2015, Cordero et al 2012). Competitors' bacteriocins can also be rendered useless by preventing their access to the cell: for example, mutations in LPS receptors can prevent binding and entry of bacteriocins to *P.aeruginosa* cells (Kohler et al 2010, Penterman et al 2014). Cells also invest in collective defense: when confronted with ecological competition, cells will up-regulate biofilm production, encasing cells in a protective matrix (Oliviera et al 2015, Kaplan 2011). Commonly used antibiotics can be prevented from penetrating the biofilm matrix: for example, penetration of tobramycin and colistin into *P.aeruginosa* biofilms is limited due to electrostatic interactions with components of the biofilm matrix (Billings et al 2013, Tseng et al 2013). It remains unclear to what extent collective defenses involving biofilm formation can protect cells against bacteriocins produced by closely related competitors.

One trait that has the potential to contribute to both individual and collective defense against ecological competition in *Pseudomonads* is the production of functional amyloids known as Fap fibrils. These needle-like fibres protrude from the cell surface and contribute to surface attachment, the mechanical robustness of biofilms, and can bind metabolites secreted by cells (Dueholm et al. 2013, Zeng et al 2015, Seviour et al 2015). Recently, it was shown that the curli fibres of *E.coli*, also functional amyloids, protect cells from attack by tailed bacteriophage (Vidakovic et al 2017). Curli fibres cause cells to pack more densely in biofilm, reducing space between cells while also filling the spaces between cells, preventing bacteriophage penetrating into biofilms. They can also protect individual cells: curli fibres enmesh cells and bind bacteriophage particles, preventing them reaching the cell surface. While the Fap fibrils produced by *P.aeruginosa* are genetically distinct from curli, the amyloid forming component contains similar amyloid repeat sequences and inhibitors of curli formation also prevent Fap fibril formation (Dueholm et al 2010, Taylor et al. 2011). Given that the R/F type bacteriocins of *P.aeruginosa* are derived from tailed bacteriophage and remain structurally very similar, we hypothesized that Fap fibrils may contribute to individual and collective protection of *P.aeruginosa* from these bacteriocins (Nakayama et al. 2002, Ghequire & De Mot 2014).

We assess whether Fap fibrils can contribute to the protection of *P.aeurginosa* cells from conspecific bacteriocins. We (1) identified a *P.aeruginosa* isolate that inhibits PAO1 in a manner consistent with the use of R/F type bacteriocins; (2) assessed whether purified Fap fibrils can protect planktonic cells during exposure to these bacteriocins and whether cells that over-produce Fap fibrils are less susceptible to these bacteriocins than wildtype cells and (4) determined whether the viability of biofilms

constructed by fibrils producers and non-producers differ significantly after exposure to bacteriocins.

Material and Methods

Media and Bacterial Strains

We carried out all overnight culturing and experiments in LB media (10g Tryptone, 5g yeast extract, 5g NaCl per litre of dH_2O). When experiments required enumeration of colony forming units, we plated diluted cultures onto LB agar (10g Tryptone, 5g yeast extract, 5g NaCl and 15g agar per litre of dH_2O) We wash and dilute bacterial cultures using M9 minimal salt media (6.8g Na_2HPO_4, 3g KH_2PO_4, 0.5g NaCl and 10g NH_4Cl per litre dH_2O) For purification of Fap fibrils, we cultured strains in colony factor antigen (CFA) media (10g hydrolysed casein, 50mg $MgSO_4$ and 1.5g yeast extract per litre of dH_2O). All reagents were purchased from Sigma unless otherwise stated.

We use strains that differ in their ability to produce Fap fibrils in our experiments. Our PAO1 strain produces wildtype levels of Fap fibrils and our PAO1Δfap strain is a mutant with the genes involved in Fap fibril production (FapA-FapF) inactivated by allelic replacement with a gentamicin resistance cassette. We also use a PAO1 strain harbouring a plasmid containing the *P.aeruginosa* Fap operon under the control of an isopropyl B-D-1-thiogalactopyranosidde (IPTG) inducible lacUVS promoter (PAO1 pFap). Leaky expression of the Fap operon under the control of the lac promoter leads to higher expression even without induction. We cultured all strains overnight for 15 hours at 37°C in 6ml of LB media at 200rpm on an orbital shaker prior to all experiments.

Supernatant extraction, concentration and killing activity

To determine whether Fap fibrils can contribute to the protection of *P.aeruginosa* cells from bacteriocins, we had to identify a strain that produced phage tail-derived bacteriocin that inhibits PAO1. We identified an isolate from a cystic fibrosis (CF) lung collection that had previously been shown to inhibit PAO1, extracted and size fractioned its bacteriocin-containing supernatant and determined its ability to inhibit PAO1 (Ghoul *et al.* 2015).

Supernatant extraction and size fractioning

We grew cultures of the CF isolate 16:14 overnight in 6ml of LB media at 37°C, 200rpm on an orbital shaker. The next day we measured the density of the overnight cultures, standardized to an optical density of 3, diluted 10-fold and added 1ml of standardized culture to 100ml of LB media in a 200ml glass shake flask and incubated at 37°C, 200rpm. After 13 hours of growth, we added 100µg/ml of mitomycin C and incubated for a further 4 hours. Mitomycin C is a strong inducer of bacteriocin production in *P.aeruginosa*. We then centrifuged the cultures at 6000rpm for 10 minutes to remove cells and passed the supernatant through a 0.22um syringe filter. *P.aeruginosa* can kill using large, bacteriophage-derived bacteriocins known collectively as tailocins and also using smaller protein bacteriocins called S pyocins. We size-fractioned supernatants to separate large (>100kDa) and smaller (>3kDa but <100kDa) bacteriocins by passing supernatants sequentially through 100kDa and 3kDa spin filters and retaining the concentrated supernatants.

To determine if killing is caused by larger tailocin bacteriocins or the smaller S type pyocins, we performed spot inhibition assays. We spotted LB agar plates with 15µl of

the large or medium supernatant size fractions and allowed the plates to dry. We then spread 70µl of an overnight culture of PAO1, standardized to an OD of 2 and diluted 100-fold, onto the plates and incubated at 37°C. After 12 hours we checked the plates for zones of little or no growth around the supernatant spots. Each size-fraction was replicated four times.

Planktonic inhibition assay

To elucidate the effects of the supernatant on the viability of PAO1, we assessed the effects of two different dilutions of the concentrated supernatant on the viability of PAO1 in liquid culture. We took overnight cultures of PAO1, washed and adjusted the OD of cultures to 2 in M9 media before diluting 10-fold. We added 180ul of standardized culture to the wells of a 96-well plate containing either 20µl of concentrated supernatant diluted 1:50 in M9 solution, 20ul of supernatant diluted 1:200 in M9 solution or 20µl of M9 solution as an untreated control. Each treatment was replicated 6 times. We incubated the 96-well plate at 37°C, shaking at 150rpm for one hour before diluting cultures 10^{-4} in M9 media and plating 40ul of diluted culture onto LB agar plates. Plates were incubated at 37°C and we counted colony forming units after 20 hours.

To determine whether fibril overproducers are protected from tailocin bacteriocins in liquid culture, we repeated the planktonic killing assays described above using wildtype and fibril overproducing cells. We calculated survival after treatment with bacteriocins as the percentage CFUs relative to the average of the untreated control.

Fap fibril isolation and protection assay

To determine whether Fap fibrils can protect *P.aeruginosa* from R/F type bacteriocins, we added purified Fap fibrils to populations of cells that did not produce fibrils, and then exposed them to bacteriocins, assessing survival.

Fap fibril isolation

We inoculated PAO1 pFAP, an inducible Fap fibril overproducing strain, into KB media and incubated overnight (37°C, 200rpm) before inoculating 400ml of CFA media with 4ml of the overnight culture in a 2L flask with 40µg/ml of tetracycline. Cultures were incubated at 37°C, 200rpm to an optical density (A_{600}) of ~0.5 before inducing expression of Fap fibrils with 1mM of IPTG and incubating for a further 8 hours. We harvested cells by centrifugation (28,000g, 30 mins, 20°C), resuspended cells in 30ml of buffer (10mM Tris-HCL, pH 8.0) and homogenized manually before adding 10ml of enzyme mix (0.4mg/ml RNaseA, 0.4mg/ml DNase I, 4mg/ml lysozyme, 4mM $MgCL_2$ and 0.4% Triton X-100). This mix was homogenized by three cycles of freeze-thawing using a -80°C freezer and a 37°C water bath, followed by a final incubation of 2hrs at 37°C. We then added 5ml of 20% sodium dodecyle sulfate (SDS), boiled the sample for 20 minutes and collected insoluble material by centrifugation (28,000g, 30 mins, 20°C). The pellet was then resuspended in 27ml buffer with 3ml of 20% SDS and boiled for 20 minutes before insoluble material was collected by centrifugation. We repeated this step twice. To wash away residual SDS, we resuspended the pellet in 25ml of buffer and centrifuged (28,000g, 30 mins, 20°C), repeating this step a further two times before re-suspending the pellet in 3ml of buffer after the final centrifugation.

To confirm the presence of amyloid structures in our purified protein solution, we used a Thioflavin T fluorescence assay. We suspended 0.5mg/ml of lysozyme and 0.5mg/ml

of purified protein solution in 10mM Tris-HCl (pH 8.0) and 40µM Thioflavin T. 10mM Tris-HCl (pH 8.0) and lysozyme, a protein lacking any amyloid structures, served as negative controls. Using a Spectramax i3x, we recorded the emission spectra of the samples from 470-600nm after excitation at 450nm.

Protection assay

We grew the fibril non-producer in 6ml of LB media at 37°C, 200rpm on an orbital shaker. After 15 hours, we washed cells in M9 media, standardized to an OD of 2 and diluted 10-fold. We added 170µl of standardized culture to the wells of a 96-well plate containing 20ul of diluted supernatant (1:50) in M9 solution or 20µl of M9 media as an untreated control. We then made up each well to 200µl with solutions of either 0µg/ml, 5µg/ml, 50µg/ml or 100µg/ml of Fap fibrils. Each treatment-fibril combination was replicated 6 times. After incubating at 37°C, 150rpm for one hour we diluted cultures 10^{-4} and plated 50µl of culture onto LB agar plates. We incubated plates at 37°C and recorded CFUs after 20 hours. We calculated survival after treatment with bacteriocins as the percentage CFUs relative to the average untreated control for each concentration of fibrils.

Biofilm viability assay

We examined whether biofilms lacking Fap fibrils were less viable after treatment with bacteriocins than their wildtype counterparts. We first assessed whether Fap fibril production affects biofilm biomass and then quantified viability of 24hr biofilms of fibril producers and non-producers after treatment with bacteriocins.

Crystal violet assay

We grew wildtype, non-producer and overproducer cells overnight at 37°C, 200rpm in 6ml of LB. After standardizing to and OD of 3 and diluting 1000-fold into LB media, we add 100µl of culture to the wells of a 96-well plate, seal with a breathable membrane and incubate statically at 37°C for 24hrs. We remove planktonic cells, submerged the plate in dH_2O three times to remove unattached cells and added 150µl of 0.1% crystal violet before incubating at room temperature for 15 minutes. After removing crystal violet, we rinsed the plate three times with dH_2O, blotted dry and air-dried overnight. We then added 200µl of 30% glacial acetic acid and incubated the plate for 15 minutes at room temperature. After transferring 150µl of the mix to a new plate, we measured absorbance at 550nm, using 30% glacial acetic acid as a blank. Each strain was replicated 18 times.

Biofilm viability assay

We quantified viability of cells in biofilms after treatment with bacteriocins using a resazurin viability assay: a fluorescent assay that detects cellular metabolic activity. Resazurin is reduced to highly fluorescent resorufin by enzymes in metabolically active cells. This conversion only occurs in viable cells and thus, the amount of resorufin produced is proportional to the number of viable cells. We grew biofilms of wildtype, producer and non-producer cells for 24hrs as described above. After removing planktonic cells and washing with 200µl of M9 solution three times to remove unattached cells, we added 150µl of supernatant diluted 1:50 in M9 solution, 150µl of supernatant diluted 1:200 in M9 solution or 150µl of M9 solution as an untreated control. After incubating statically for one hour at 37°C, we removed supernatant and washed wells gently with 200µl of M9. We then added 200µl of LB media to each well and 0.5µg/well of resazurin before incubating statically at 37°C. After 4 hours, we

measured fluorescence in each well (ex 550nm, em 590nm). We calculated viability after treatment with bacteriocins as the percentage RFU relative to the average of the untreated control for each isolate. Each strain*treatment combination was replicated 5 times

Results

PAO1 is inhibited by the >100kDa size fraction of CF isolate 16:14 supernatant

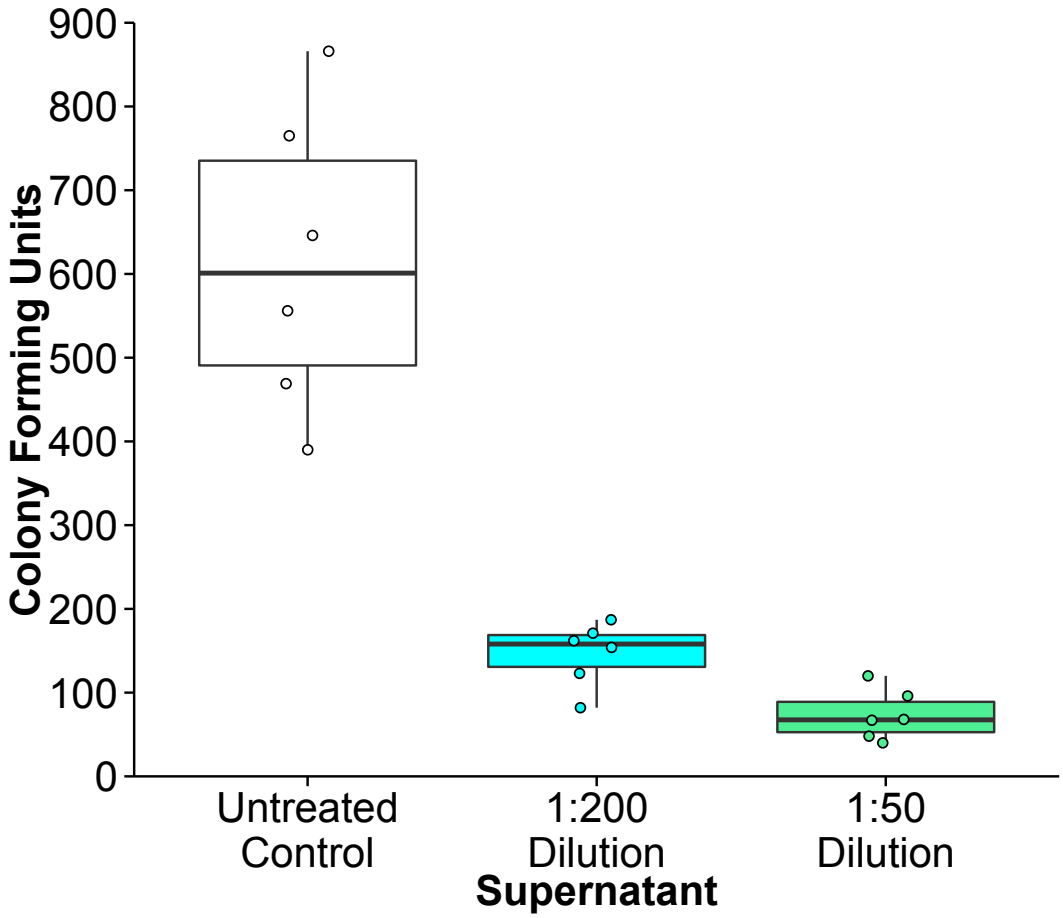


Figure 1. Inhibition of PAO1 planktonic cells by the >100kDa size fraction of isolate 16:14 supernatant. Solid lines are the median value, boxes show the interquartile range and whiskers extend up to 1.5 times the interquartile range. Both a 1:200 and a 1:50 dilution of supernatant significantly reduce the number of viable cells relative to an untreated control

To elucidate the effects of the large size fraction of isolate 16:14 supernatant on the viability of PAO1, we determined which size fraction of the supernatant caused inhibition and assessed the effects of different dilutions of the concentrated supernatant on the viability of PAO1 in liquid culture. Our plate inhibition assays show that the large (>100kDa) size fraction prevented growth of PAO1 while the smaller (>3kDa but <100kDa) size fraction does not. The spots of inhibition caused by the large size fraction of the supernatant were tight circles, not diffuse like spots caused by S type bacteriocins, consistent with inhibition caused by an R/F type bacteriocin. We observed no plaque formation around zones of inhibition, suggesting bacteriophage were not responsible for inhibition.

After one hour of exposure to the >100kDa size fraction of the supernatant, we found that the number of colony forming units was significantly reduced relative to an untreated control for both the 1:200 dilution (ANOVA, $F_{1,10}=38.99$, $p= 9.58e^{-05}$) and the 1:50 dilution of bacteriocin-containing supernatant (ANOVA, $F_{1,10}=53$, $p=2.66e^{-05}$). These results suggests that the >100kDa size fraction of the supernatant is inhibitory towards PAO1 and that this is likely due to the presence of R/F type bacteriocins.

Fap fibrils protect fibril non-producers from putative R/F bacteriocins

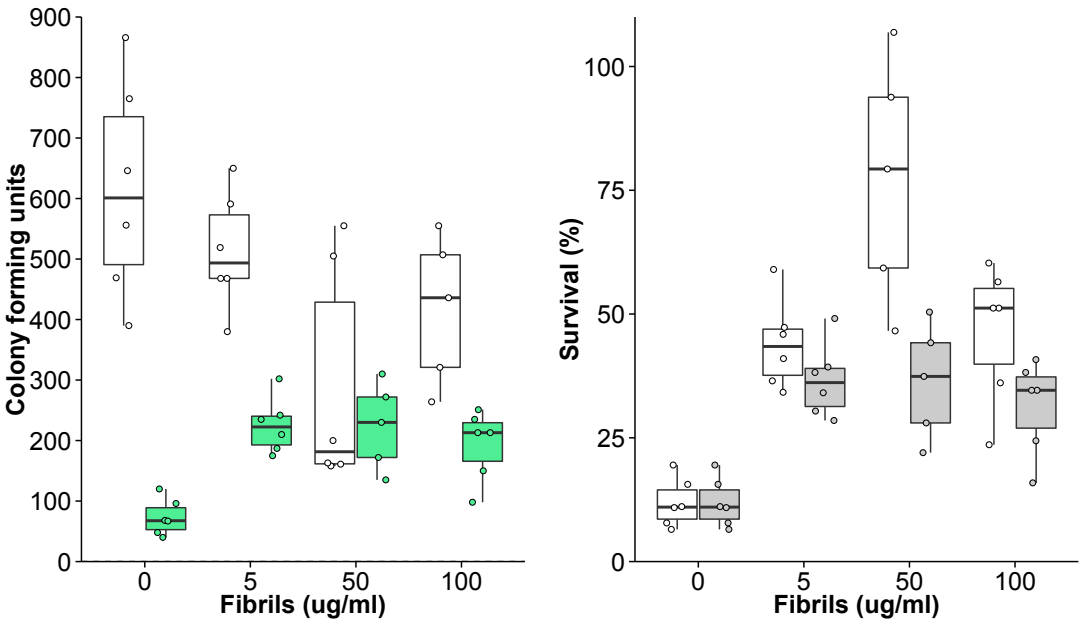


Figure 2. Survival of fibril non-producer cells treated with bacteriocins. **A**) Colony forming units after treatment with a 1:50 dilution of supernatant (green) or M9 solution control (white) for each concentration of fibrils. **B**) Percentage survival of populations treated with 1:50 dilution of supernatant relative to the average of an untreated control for each fibril concentration (white) and relative to the no fibrils-no supernatant control (grey). Solid line is the median value, boxes are the interquartile range and whiskers extend to 1.5 times the interquartile range.

We assessed the ability of Fap fibrils to protect fibril non-producing cells by exposing them to bacteriocins and various concentrations of purified Fap fibrils. After one hour of exposure to bacteriocin containing supernatant, we find that the addition of fibrils significantly increased survival of the fibril non-producer relative to untreated controls (ANOVA, $F_{3,19}$=19, p=6.06e^{-05}). Pairwise comparisons show significantly increased survival relative to an untreated control after addition of 5µg/ml (t_{10}=3.876, p= 0.001018) but addition of 50µg/ml (t_{10}=7.519, p= 4.16e-07) and 100µg/ml of fibrils did (t_{10}=4.178, p= 0.000511). However, addition of fibrils also appears to reduce the number of colony forming units (CFUs) in untreated controls (ANOVA, $F_{3,19}$=4.9, p=

0.0109). Pairwise comparisons show that the addition of 5µg/ml of fibrils did not significantly decrease the number of CFUs in untreated control (t_{10}=-1.164, p=0.25881) but addition of 50µg/ml (t_{10}=-3.685, p=0.00157) and 100µg/ml of fibrils did (t_{10}=-2.148, p=0.04478). As a more conservative estimate of the effect of Fap fibrils on survival, we also compared survival of cells to the no fibrils-no supernatant control and found that survival is still significantly improved by the addition of Fap fibrils (ANOVA, $F=11.09_{3,19}$, p=0.0002). Pairwise comparisons show significantly increased survival relative to an untreated control after addition of 5µg/ml (t_{10}=4.912, p= 7.47e-05), 50µg/ml (t_{10}=5.152, p= 0.000138) and 100µg/ml of fibrils (t_{10}=4.912, p= 0.000815).

Fibril overproducers are protected from bacteriocins in liquid culture

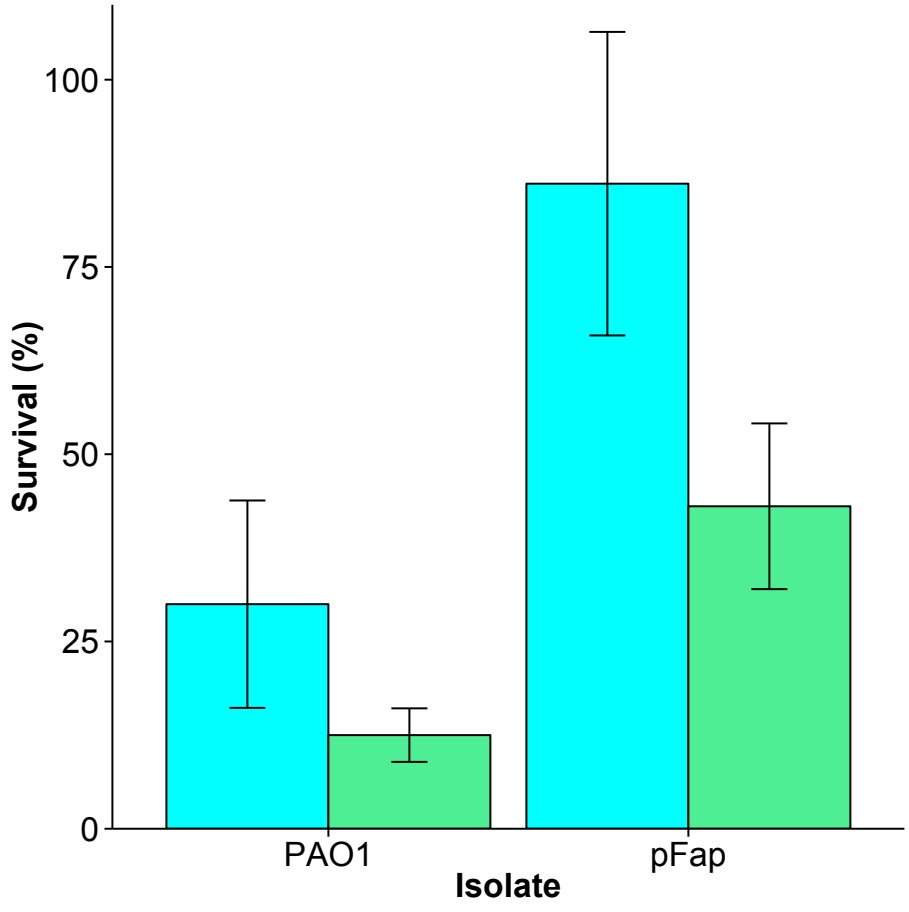


Figure 3. Survival of wildtype and fibril overproducing cells exposed to bacteriocins. The percentage of surviving cells relative to the average of an untreated control is significantly higher for fibril overproducing cells when exposed to a 1:50 supernatant dilution (green) but not for a 1:200 dilution (blue). Error bars are standard errors, n=6.

We determined whether overproducing Fap fibrils in liquid culture could protect cells from bacteriocins by exposing wildtype cells and fibril overproducing cells to bacteriocins and quantifying survival. After one hour of exposure, we find that a significantly higher percentage of overproducing cells survive relative to the wild type when exposed to a 1:50 dilution of supernatant (ANOVA, $F_{1,10}$=6.9, p=0.0253) and higher survival, but not significantly so, when exposed to a 1:200 dilution of supernatant (ANOVA, $F_{1,9}$=4.789, p=0.0564). This suggests that fibril overproducers are more protected that wildtype cells when exposed to bacteriocins in liquid culture.

Biofilms lacking Fap fibrils are no less viable than wildtype biofilms after exposure to bacteriocins

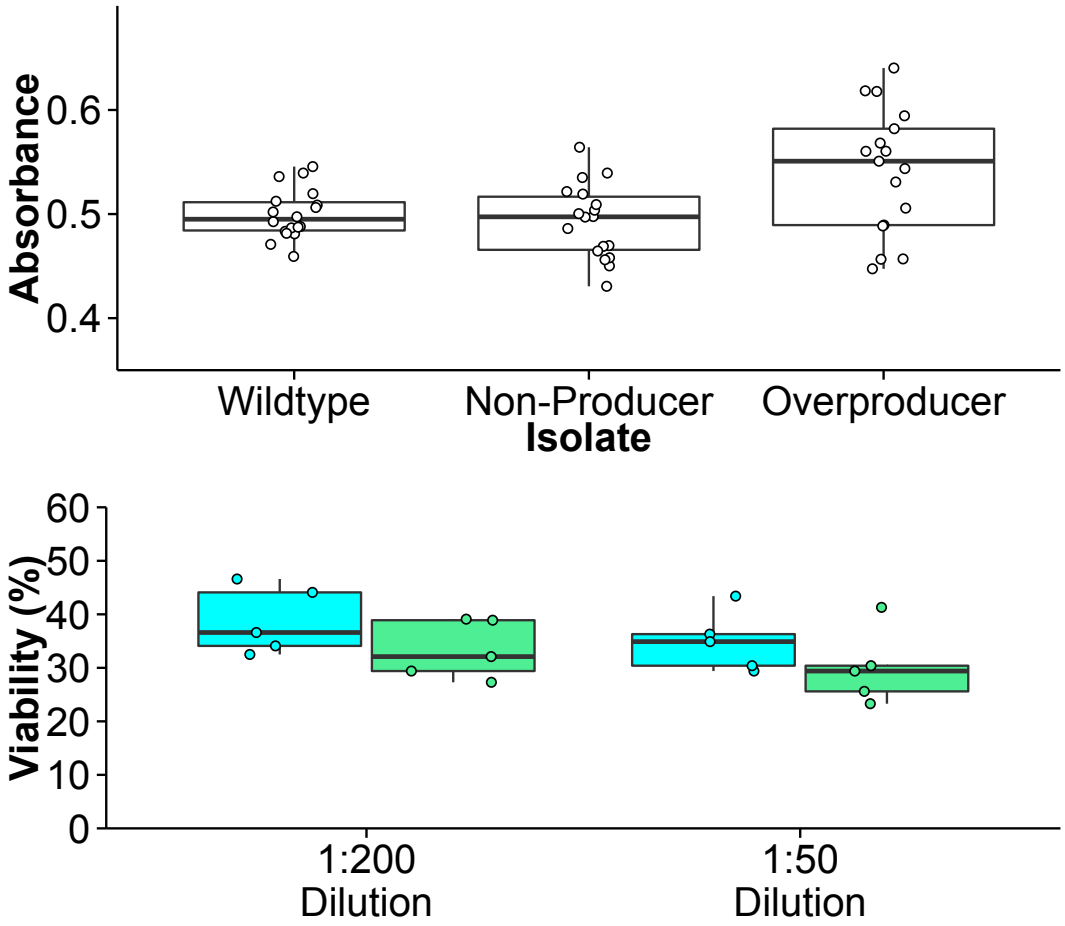


Figure 4. 24hr biofilm biomass and viability after exposure to bacteriocins. **A)** Biofilm formation by wildtype, non-producer and overproducer cells. The amount of biofilm produced by wildtype and non-producer cells does not differ significantly but overproducers produce significantly more biofilm than the wildtype. Assays were replicated 18 times per isolate. **B)** Percentage viability of 24hr biofilms relative to the average of an untreated control. Viability of wildtype biofilms (Blue) is not significantly greater than fibril non-producer biofilms (Green) after exposure to a 1:200 supernatant dilution and 1:50 supernatant dilution. Each treatment was replicated 5 times. Solid line is the median value, boxes are the interquartile range and whiskers extend up to 1.5 times the interquartile range.

To assess whether Fap fibrils contribute to the protection of cells in biofilms from bacteriocins, we quantified biofilm biomass of wildtype cells, non-producer cells and overproducer cells before assessing their viability after exposure to bacteriocins. We

found that not producing Fap fibrils does not significantly alter the amount of biofilm biomass (t_{34}=-0.447, p=0.657) but that overproducing Fap fibrils leads to significantly greater biofilm biomass relative to the wildtype (t_{34}=3.244, p=0.0021). This suggests that overproduction of Fap fibrils may also increase expression of other biofilm components such as the Pel and PSL polysaccharides or alginate. As the fibril overproducer produces significantly more biofilm biomass than the wildtype and non-producer, we excluded it from the biofilm viability assays. After exposing 24hr biofilms to bacteriocins for one hour, we find that despite fibril non-producers displaying lower percentage viability relative to the wildtype, this difference was not significantly different for either a 1:200 supernatant dilution (ANOVA, $F_{1,8}$=2.15, p=0.181) or a 1:50 dilution (ANOVA, $F_{1,8}$=1.502, p=0.255). This suggests that Fap fibrils do not contribute significantly to protecting cells in a biofilm from harm when exposed to conspecific bacteriocins.

Discussion

Our results suggest that Fap fibrils may contribute to individual protection against phage tail-derived bacteriocins. We identified a *P.aeruginosa* isolate that inhibits PAO1, and size-fractioning of its supernatant suggests inhibition is consistent with killing caused by R/F type bacteriocins (Fig 1). We found that purified Fap fibrils significantly increased the survival of fibril non-producing cells when exposed to bacteriocins in liquid culture (Fig 2); and that cells that overproduce Fap fibrils are protected to a greater extent than wildtype cells (Fig 3). Finally, our viability assays reveal that biofilms produced by fibril non-producers are not significantly less viable than wildtype biofilms after exposure to bacteriocins (Fig 4B).

While size fractioning of an inhibitory isolates supernatant suggests killing is caused by the production of R/F bacteriocins, it would be useful to confirm this directly. A plausible alternative is that inhibition is caused by bacteriophage, which are similar in size to R/F bacteriocins (Ghequire & De Mot 2014). Our spot assays never display the characteristic plaques that appear when bacterial cells are attacked by bacteriophage, suggesting this is unlikely. However, if inhibition were to be the result of attack by bacteriophage, our results would still have relevance to the protection of cells, not from intraspecific competition but from parasitism. Another possibility is that during the size fractioning and concentrating process smaller S type bacteriocins fail to pass through the filter and are also concentrated in the large size fraction. However, as our smaller size fraction does not inhibit PAO1 cells, this would require that all S type bacteriocins failed to pass through the filter, which seems unlikely. It would be useful to confirm inhibition is the result of R/F bacteriocin production and to be able to accurately quantify the amount of bacteriocins in solution rather than rely upon dilutions of concentrated supernatant. This could be achieved using a modified purification assay that can provide bacteriocin solutions that are 70-90% pure (Williams et al. 2008) and carrying out quantitative pyocin assays (Kageyama 1964) to establish the number of bacteriocins per microliter of solution.

Fibril non-producers exposed to bacteriocins and purified Fap fibrils show significantly higher survival than cells exposed to bacteriocins alone (Fig 2). This suggests there may be a role for Fap fibrils in protecting cells against R/F bacteriocins produced by conspecific competitors. However, the mechanism behind increased survival remains unclear. The curli fibres of *E.coli* enmesh cells and bind bacteriophage particles, preventing them from reaching the cell surface (Vidakovic et al 2017). It may be

possible that purified Fap fibrils are binding to R/F bacteriocins, preventing them acting upon cells. Yet, adding more fibrils does not increase survival, suggesting if binding of bacteriocins occurs its efficacy is limited in shaken liquid culture. An alternative explanation is that addition of Fap fibrils may induce aggregate formation, limiting access of R/F bacteriocins to aggregate associated cells (). Fap fibril overproducers tend to form aggregates even in shaken liquid culture and aggregation is a well-established mechanism for protection against predators, viruses and antibiotics (Zeng et al 2015, Hahn et al 2001, Darch et al 2017, Haaber et al 2012). Quantifying aggregate sizes with and without addition of Fap fibrils will give some indication of whether protection is a direct consequence of interactions between Fap fibrils and bacteriocins or the product of cells aggregating.

Cells that overproduce Fap fibrils show significantly increased survival relative to wildtype cells upon exposure to R/F bacteriocins (Fig 3.). Again this suggests that Fap fibrils have a role in protecting cells from R/F bacteriocins but what causes this effect is unclear. It may be that individual overproducer cells encase themselves in fibrils, as seen in *E.coli* cells, preventing bacteriocins from accessing the cell surface (Vidakovic et al 2017). Another, non-mutually exclusive, explanation is that overproduction of Fap fibrils alters the expression of other traits that have a protective effect against bacteriocins. For example, accumulation of extracellular DNA in *Salmonella enterica* culture can increase resistance to antimicrobial peptides by activating a polymixin resistance operon (Lewenza et al 2013). Given that overproducing Fap fibrils can alter the expression of many traits relative to wildtype cells, we cannot rule out that increased survival is due to differential regulation of another trait and not a direct consequence of increased amounts of Fap fibrils. Comparing survival of wildtype and fibril non-

producer cells upon exposure to bacteriocins will shed more light on whether Fap fibrils confer protection to individual cells.

We hypothesized that biofilms constructed by fibril producing cells might be better protected during exposure to bacteriocins than biofilms of fibril non-producers. However, while wildtype biofilms were not significantly more viable after exposure to bacteriocins than non-producer biofilms (Fig 4b.). The curli fibres of *E.coli* fill the spaces between cells in biofilms, physically preventing large inhibitory particles such as bacteriophage from penetrating the biofilm (Vidakovic et al 2017). Our results suggest that the ability of Fap fibrils to prevent phage-derived R/F bacteriocins from entering *P.aeruginosa* biofilms may be limited. However, it would be interesting to determine more directly whether R/F bacteriocins penetrate with equal ease into biofilms constructed with and without fibrils. This could be accomplished by determining whether killing is localized at the periphery or occurs throughout biofilms using live/dead staining, or by staining R/F bacteriocins and directly visualizing their penetration into the biofilm.

Although no information is available comparing wildtype and fibril non-producer cells, fibril overproducing cells use significantly less Pel and Psl polysaccharide and significantly more alginate when constructing biofilms (Zeng et al 2015). Changes in the ratio of matrix components may alter parameters like cell density and orientation within the biofilm, making biofilms more or less penetrable to R/F bacteriocins and other antimicrobials (Hentzer et al 2001). More specific interactions between antimicrobials and matrix components also play a part in collective defense: for example, the Psl polysaccharide can bind the antibiotic colistin and prevent its

penetration into the biofilm (Billings et al 2013). However, there is currently very little known about how bacteriocins interact with different components of the biofilm matrix. It may be that, although insignificant, the small reduction in viability in biofilms constructed by fibril non-producers is due to changes in cell density and orientation, or matrix composition, due to the lack of Fap fibrils.

To conclude, bacteria are under persistent attack from closely related strains and species during competition for space and resources and subsequently invest in both individual and collective defenses. We have demonstrated that Fap fibrils produced by *P.aeruginosa* contribute to individual defense of cells exposed to bacteriocins but their contribution towards collective defense is insignificant. Future work should seek to determine mechanistically how Fap fibrils contribute to protection against bacteriocins. This may be a direct effect where fibrils form a physical barrier between cells and bacteriocins, or electrostatically bind bacteriocins. Another, non-mutually exclusive, possibility is that protection is a secondary effect arising due to fibrils promoting aggregation of cells or differential regulation of other traits. Given the scope for using bacteriocins as a more selective method for treating bacterial infection, individual and collective defenses to these toxins will likely impact on the efficacy of such treatments.

Chapter 6: Summary and Discussion

Each chapter of this thesis contains its own extensive discussion pertinent to that particular study. My aim in this chapter is to discuss the key findings and general observations of the thesis.

My aim in this thesis was to explore social interactions in bacterial populations. I studied populations of *Pseudomonas fluorescens* isolated from soil samples to find evidence for competitive interactions, and explore the consequences of this competition, in natural bacterial populations (Ch.2). I also demonstrate the potential for social cheating in these populations and reveal factors contriving to limit the spread of cheats in natural settings (Ch.3). Finally, I have used experimental populations of *Pseudomonas aeruginosa* to reveal how a non-social bacterial trait, the production of functional amyloids, can shape cooperator-cheat dynamics during competition over iron (Ch.4), and may also play a role in defending cells during conflict involving bacteriocins (Ch.5).

Chapter 2: Bacteriocins and the assembly of natural *Pseudomonas fluorescens* populations

Competitive dynamics involving bacteriocin production have been studied extensively under laboratory conditions (Kerr et al 2002, Inglis et al 2009). Still, comparatively little work has been dedicated to understanding the extent to which conflict involving the use of bacteriocins occurs in natural bacterial populations, what drives these inhibitory interactions, and any consequences for the diversity and distribution of genotypes in natural settings (Ghoul et al 2015, Cordero et al 2012).

Sampling natural soil populations of *Pseudomonas fluorescens*, I find that the majority of isolates produce bacteriocins, and yet only 7% of interactions resulted in inhibition. Our results are similar to those found in natural populations of *Bacillus* in marine sediment and marine populations of *Vibrio* (8% and 2% of interactions respectively) (Gutierrez-Perez et al 2013, Cordero et al 2012). However, I also reveal that these inhibitory interactions appear to occur more frequently between isolates with greater metabolic overlap. This suggests that antagonism is driven by competition over resources in natural bacterial populations, also providing support for hypotheses suggesting competition between individuals will be strongest when resource requirements are similar (Kinkel, et al 2014, Russel et al 2017). We have shown a trend towards greater frequency of antagonism with greater metabolic overlap, however further dissection of the metabolic requirements of strains would shed light on why strains inhibit some competitors and not other. Are antagonistic interactions driven by competition over particular, preferred carbon sources? Or are cells that specialize on particular carbon sources more or less antagonistic than cells that utilize multiple carbon sources? Laboratory studies have previously made clear that bacteriocin production can be important for determining the outcome of competition, our results further demonstrate the utility of sampling natural bacterial populations to answer questions concerning what drives the evolution of these competitive traits.

Finally, I find that inhibitory interactions occur less frequently within than between sampled patches, suggesting bacteriocin productions plays a role in shaping bacterial population structure in soil environments (Ch 2, Fig 3). Studies have shown that bacteriocin production can promote spatial structuring in laboratory populations but the

distribution of microbes in the environment has commonly been attributed to random dispersal and environmental selection (Kerr et al 2002, De Wit et al 2006). Our results contribute to a growing number of studies demonstrating that social interactions, particularly antagonistic interactions, can contribute to spatial structure in natural bacterial populations (Cordero et al 2012, Kinkel et al 2014, Hawlena et al 2012). Finer-grain sampling, over distances at which microbes are capable of interacting, would help determine if, and at what scales, spatial structuring is the result of clonal expansion of a single genotype or collectives of diverse genotypes resistant to each others bacteriocins (Cordero et al 2016).

Chapter 3: Cheating and resistance to cheating in natural populations of the bacterium *Pseudomonas fluorescens*

It is well established that, under laboratory conditions, public goods cheats can invade populations of cooperators (Griffin et al 2004, Diggle et al 2007). However, evidence for cooperator-cheat dynamics in natural bacterial populations remains relatively limited (Cordero et al. 2012, Andersen et al 2015). Using natural soil populations of *Pseudomonas fluorescens* and the siderophore pyoverdine as a public good, I reveal the extent of, and limitations to, public goods cheating in a natural bacterial population.

I found that 4.5% of isolates in the population produce very little or no pyoverdine, but retained functional pyoverdine receptors and co-occurred with pyoverdine producers. This suggests that these isolates can potentially act as social cheats in natural settings. The frequency of potential cheats in our populations are similar to those recently determined in other soil and freshwater populations of *Pseudomonads* (8% of isolates) but low relative to those in marine populations *Vibrio* where non-producers constitute

nearly 60% of the population (Butaite et al 2017, Cordero et al 2012). This raises interesting questions concerning what determines the frequency of cheats in natural settings: are differences in frequency attributable to differences in population densities experienced by cells in marine and soil settings? Or do highly structured populations limit the ability of cheats to spread in populations?

In contrast to laboratory populations, where cheats can exploit cooperators with impunity, I find that non-producers can only 'cheat' around half the cooperators in the population. Our findings suggest that if cheating is contingent on interacting with particular cooperators, variation in the extent to which non-producers can exploit producers is likely the norm in natural settings. Another recent study concerning siderophore exploitation in soil populations of *Pseudomonads* found similar variation in exploitation and confirmed that this was due different forms of pyoverdine present in the population (Butaite et al 2017). This diversity may go some way to explaining the differences in cheat frequency between different populations discussed above: *Vibrio* populations show no diversity in siderophore molecules or receptors, allowing cells to cheat all producers (Cordero et al 2012). However, this raises further questions concerning what drives this diversification in siderophores and their receptors. One hypothesis is that escape from cheating drives diversification of pyoverdine molecules and receptors; and yet diversity appears to be absent in marine *Vibrio* where cheats reach a high frequency in the population (Lee et al 2012).

Further barriers to successful exploitation can also arise in the form of conflict: I find that a small number of cooperators produce bacteriocins that inhibit non-producers. Recent evidence suggests that in populations of *Bacillus*, antagonism is correlated with

the ability to utilize a public good, a surfactant, produced by the focal isolate (Lyons & Kolter 2017). This suggests that at least in some species that antagonistic traits may have evolved at least partly to protect against public good exploitation. I do not provide evidence here that bacteriocin production evolved, even in part, to protect against cheats. However, determining whether the ability to inhibit another strain or species is correlated with production of different public goods in naturally occurring bacterial populations would shed further light on why inhibitory interactions occur between some strains and not others.

Chapter 4: Functional amyloids promote retention of public goods involved in iron acquisition and shape cooperator-cheat dynamics

Costly, recyclable public goods are of little utility if they rapidly diffuse away from producing cells or are utilized by public goods cheats. I test whether a recently documented non-social bacterial trait, the production of functional amyloids known as Fap fibrils, can limit the loss of cooperative public goods involved in iron acquisition and influence cooperator-cheat dynamics during competition over iron.

I find that cells that do not produce functional amyloids retain less iron-chelating metabolites and that the fibrils appear to bind the iron-chelators PQS and pyoverdine, preventing their loss by dilution. Preventing the loss of recyclable goods to the environment may have important fitness implications for cells. The amount of public good a cell produces, and the rate at which it produces it, determines the ability of cheats to invade populations of cooperators (Ghoul et al 2016). Transient binding of public goods to functional amyloids may allow cells to downregulate production of a costly public goods sooner, reducing the costs of production and subsequently limiting

the ability of cheats to invade. Secreted public goods also rapidly diffuse away from cells in the environment: this work suggests a plausible mechanism by which cells can partially privatize secreted public goods, allowing the benefits of production to be directed towards cooperative cells.

Populations of cells in biofilms, or unattached aggregates are constantly subject to fluid flow (Persat et al 2015). Diffusion and advection can rapidly remove secreted metabolites from around cells, potentially limiting the ability of cells to communicate or cooperate to extract nutrients from the surrounding environment (Emge et al 2016). Transient binding of cooperative public goods to functional amyloids may allow cells to acquire resources, or communicate and coordinate behaviours using quorum sensing molecules, under conditions where it would not otherwise be possible. These hypotheses could be tested in simple flow cells: quorum sensing reporters inserted into both wildtype and fibril non-producer cells would allow quantification of the timing and duration of quorum controlled behaviours under fluid flow. Other secreted public goods, such as the quorum sensing molecule C12-HSL and pyocyanin, also appear to be predisposed to binding to Fap fibrils: this suggests functional amyloids may be quite a general mechanism for the retention of secreted public goods (Seviour et al 2015).

Overall, our results suggest that binding of iron-chelators by Fap fibrils may limit diffusion of secreted metabolites away from producer cells and subsequently limit their availability to cheats.

Chapter 5: Assessing the contribution of functional amyloids towards individual and collective defence against phage-derived bacteriocins in *Pseudomonas aeruginosa*

Bacteria are regularly under attack from other bacterial strains and species during competition for resources and subsequently posses a range of individual and collective defense mechanisms. I assessed the contribution of the Fap fibrils produced by *Pseudomonas aeruginosa* towards both individual and collective defense against phage-derived bacteriocins.

I find that addition of purified fibrils to cells exposed to bacteriocins significantly increases survival, while cells that overproduce Fap fibrils also display significantly higher survival than wildtype cells upon exposure to bacteriocins. This suggests that Fap fibrils may contribute to individual defense against bacteriocins. However, it is remains unclear mechanistically whether this is a direct effect arising from fibrils interacting with bacteriocins or a secondary effect, possibly arising due to fibrils promoting aggregation. If fibrils do indeed provide a direct protective effect against bacteriocins, this may be important when considering the potential use of bacteriocins to treat infection. The efficacy of bacteriocins against cells may be improved if used in conjunction with treatments that prevent the formation of amyloid fibrils: preventing amyloid formation would remove both a direct effect of amyloid protection, and any secondary effect by preventing or limiting aggregation.

I also find that biofilms constructed by cells that do not produce Fap fibrils are not significantly less viable after exposure to bacteriocins than wildtype biofilms. This suggests that the contribution of Fap fibrils to collective defense against phage-derived

bacteriocins is limited. While many studies have assessed the contribution of various polymers to protection of biofilms against different antibiotics, how the protein elements of biofilms contribute to collective protection has received relatively little attention (Munn & Wosniak 2012). The analogous curli fibres produced by *E.coli* organize cells in the biofilm, while simultaneously filling gaps between cells, to prevent penetration by tailed-phage (Vidakovic et al 2017). It would be interesting to see if production of Fap fibrils by *Pseudomonas aeruginosa* alters important parameters of the biofilm, such as cell density and orientation in an analogous way. While I find no evidence that Fap fibrils promote collection protection against phage-derived bacteriocins, they may contribute, directly or indirectly, to protection against other pressures such as bacteriophage or antibiotics. A good start would be to determine whether bacteriocins, and other harmful particles, penetrate with equal ease into biofilms constructed by fibril producers and non-producers, allowing a more direct evaluation of the ability of Fap fibrils to contribute to collective defense in *Pseudomonas aeruginosa*.

Insights and future work

In this section I discuss some of the themes that run throughout my thesis, highlight where my work has contributed to our understanding and suggest some interesting areas for future work.

Toxin-mediated competition in microbes

Toxin-mediated competition appears to be a salient feature of microbial life. Competition experiments carried out in laboratory settings have established that cells use toxins such as bacteriocins to gain a fitness advantage over competitors. Yet

questions concerning the ecology, evolution and regulation of these competitive traits remain to be explored in any detail: when do we expect toxin-mediated competition to occur? How common is toxin-mediated conflict in natural settings? How is toxin production regulated and why? Why is there such diversity in toxin form and function?

In the first chapter of my thesis I sample and screen microbial populations to provide a snapshot of competition in a natural environment. Competition is expected to be intense when cells have considerable metabolic overlap and resources are limiting (Ghoul & Mitri. 2015). It is anticipated that microbes will sense ecological competition, when damage to the cell or nutrient limitation trigger stress responses, and respond accordingly with the production of competitive toxins (Cornforth & Foster. 2013). Our sampling revealed that antagonistic interactions in which one strain kills another using bacteriocins occur more frequently between cells with greater metabolic overlap. These results provide evidence that toxin-production appears to be geared towards the elimination of competitors with similar resource requirements and suggest that competition over nutrients may indeed be an important driver in the evolution of toxin production and its regulation. Our results however, do not explicitly link the sensing of resource competition and the production of anti-competitor toxins. An interesting next step could be to explore whether regulation of toxin production is linked to resource overlap between ecologically congruent strains and how this impacts on the ability of strains to co-inhabit a particular niche. Do cells with greater metabolic similarity trigger aggressive responses in competitors? By looking for correlations between resource use and transcriptional responses to competition, it should be possible to demonstrate empirically whether cells regulate toxin production in response to specific resource competitors.

Individual bacterial strains sampled from natural environments are often capable of producing more than one type of bacteriocin or other anti-microbial toxins (Ghoul et al. 2015, Gordon 2007). However, it remains unclear why cells retain the capacity to produce so many toxins when carriage and production of multiple toxins is presumably costly. Our sampling of natural microbial populations also revealed diversity in the type of toxins produced by cells. While sampling and screening natural populations can provide details of the diversity at any particular moment in time, it can say little about how and why that diversity is present. Cells may produce multiple different types of bacteriocin for a number of different reasons: for example, toxins may be produced in response to different competitors or different forms of competition. Evidence that cells fine-tune responses to damage is limited but could be tested empirically by triggering different types of stress response in cells, for example by causing DNA damage or rupturing cell membranes, and using transcriptomics to monitor whether different toxins are produced in response to particular stressors. Different toxins might also be produced under different ecological conditions, varying in response to changes in for example, salinity or pH levels. Some toxins produced by *Pseudomonads*, S pyocins, use receptors involved in iron-uptake to enter cells (Baysse et al. 2003). It would be interesting to look at whether these S pyocins are produced more or less relative to other toxins when iron is limiting in the environment. Producing a cocktail of bacteriocins may also be more effective against competitors than a single bacteriocin. Again there is limited evidence for this in the literature but recent evidence suggests that T6SS effectors demonstrate conditional efficacy and can act synergistically with other effectors to harm the targets of competition (LaCourse et al. 2018). It would not be unreasonable to suspect something similar may occur with cocktails of toxins that are released

extracellularly and this could be tested empirically using strains that produce different combinations of bacteriocins.

Social interactions and microbial ecology

Microbes have proven to be an incredibly tractable system with which to test predictions of social evolution theory. However, there has also been a drive to understand how microbial cooperation and competition can shape microbial ecology. It is well established that toxin producers can kill sensitive competitors and that public goods cheats can invade populations of cooperators under laboratory conditions. Yet whether these competitive dynamics occur in natural populations and any consequences for the diversity, distribution and stability of microbial communities are only beginning to receive attention.

Toxin-mediated competition has been demonstrated to drive spatial structuring of microbial populations in simple laboratory experiments (Kerr et al. 2002). However it has been suggested that high levels of dispersal in natural populations mean that interactions between cells are transient and that competitive interactions may not contribute significantly to population structure (Cordero et al. 2012). We predicted that if this were the case, that antagonistic interactions would occur locally with the same frequency as they occur in the population as a whole. However, we found that antagonistic interactions were attenuated locally, suggesting that even in environments probably characterized by high rates of dispersal and constant environmental flux, competitive interactions appear to shape the structure of bacterial populations. Again sampling and screening natural populations can only provide a snapshot of the population at a particular moment in time. Future work should seek to answer questions

concerning *why* antagonism is attenuated locally. This could occur if less fit strains are competitively excluded from the local environment, or if competing strains split the niche, either by segregating in space or by each competitor specializing on different resources (Ghoul & Mitri. 2015). These questions could be addressed using evolution experiments in the laboratory or simple microcosms. By competing strains with similar nutrient requirements over many generations, and manipulating experimental parameters, it should be possible to determine whether certain conditions promote the competitive exclusion of resource competitors and others result in the splitting of the niche. This approach would also allow us to ask questions concerning the regulation of competitive behaviours in these environments: for example, if cells diverge in their resource requirements, do we also see less intense stress responses and concomitant a decreases in toxin production?

Our work also reveals the potential for social interactions to contribute to the generation and maintenance of microbial diversity. In the laboratory, public goods cheats can arise, invade populations of cooperators and be maintained in the population. Again, however, it is unclear to what extent, in transient environments characterized by high rates of dispersal and environmental flux, public goods cheats arise and persist in natural populations. Our work demonstrates that public goods cheats have arisen multiple times independently in a natural soil population and can utilize the siderophores produced by those around them. Public goods cheats have previously been identified in infection and in marine environments, our results demonstrate that they can also occur in complex soil environments (Ghoul et al. 2015, Cordero et al. 2012). However, we also find that contrary to results from these other systems, that cheats don't appear to be capable of cheating everyone in the soil environment. Our results, and those from a

similar study, suggest that there is diversity in the structure of the siderophores produced by cells and that they can only be utilized if a cell has the appropriate receptor (Butaite et al. 2017). It has been suggested that cheating can drive the evolution of novel forms of public good as a means of escaping pressure from cheats (Lee et al. 2012). In this scenario, antagonistic co-evolution between cheats and cooperators drive the emergence of new receptor-molecule combinations that cheats cannot utilize. Our results again give a snapshot of the diversity receptors and molecules produced by cells and cannot give insight into how this diversity emerges and is maintained. Future work could test empirically whether cheating promotes the evolution of novel receptor-molecule pairs using an experimental evolution approach. Experimental lines of siderophore producers could be exposed to cheats in environments where cooperation is necessary, the lines monitored for their ability to resist cheats and then screened for mutations that alter the structure of the siderophore molecule or the fpvA receptor. How interactions between producers of different forms of public good and their associated cheats add up at the community level in natural populations is unclear but evidence from the laboratory suggests that diversity may be maintained in these populations as through non-transitive competitive dynamics (Inglis et al. 2016).

Synergy between bacterial traits

Bacterial social traits are often studied in isolation. I have shown that the expression of a non-social trait, the production of functional amyloids, can limit the ability of siderophore cheats to invade a population of cooperators. This is believed to occur because the binding of secreted metabolites to functional amyloids limits diffusion of the costly metabolites and allows cells to benefit from the costly products they produce (Seviour et al. 2015). Our results emphasize that bacterial social traits do not occur in

isolation and suggest that superficially unrelated traits can potentially have a profound influence on the production, regulation and efficacy of bacterial social behaviours. Interactions between traits may be synergistic, where increased or decreased expression of one trait makes another trait more or less beneficial. A recent study emphasized the role of synergy in the evolution of multicellularity, demonstrating that the production of public goods can facilitate the formation of cooperative clumps of cells (Biernaskie & West 2015). Recently it was demonstrated that production of exopolysaccharides in *Vibrio cholerae* populations confines digested chitin close to the cells that produce chitinase, increasing the benefits of chitinase production (Drescher et al. 2014). Understanding the relationships between social and non-social traits will be important if we are to understand how and why cooperative behaviours are maintained in natural bacterial populations. It may also be important from an applied perspective. If the benefits to a cell of performing a behaviour are contingent on the expression of another trait, this trait may be a likely target for therapeutics. For example, functional amyloids are known to bind secreted metabolites important for iron acquisition and communication. Administering compounds that prevent the formation of functional amyloids may interfere with the ability of cells to communicate or grow in the host, reducing population density and facilitating the eradication of the population with conventional antibiotics.

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Appendix 1: Bacteriocins and the assembly of natural *Pseudomonas fluorescens* populations.

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Bacteriocins and the assembly of natural *Pseudomonas fluorescens* populations

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Keywords:

bacteriocins;
competition;
Pseudomonas fluorescens;
resource competition;
soil.

Abstract

When competing for space and resources, bacteria produce toxins known as bacteriocins to gain an advantage over competitors. Recent studies in the laboratory have confirmed theoretical predictions that bacteriocin production can determine coexistence, by eradicating sensitive competitors or driving the emergence of resistant genotypes. However, there is currently limited evidence that bacteriocin-mediated competition influences the coexistence and distribution of genotypes in natural environments, and what factors drive interactions towards inhibition remain unclear. Using natural soil populations of *Pseudomonas fluorescens*, we assessed the ability of the isolates to inhibit one another with respect to spatial proximity in the field, genetic similarity and niche overlap. The majority of isolates were found to produce bacteriocins; however, widespread resistance between coexisting isolates meant relatively few interactions resulted in inhibition. When inhibition did occur, it occurred more frequently between ecologically similar isolates. However, in contrast with results from other natural populations, we found no relationship between the frequency of inhibition and the genetic similarity of competitors. Our results suggest that bacteriocin production plays an important role in mediating competition over resources in natural settings and, by selecting for isolates resistant to local bacteriocin production, can influence the assembly of natural populations of *P. fluorescens*.

Introduction

The majority of bacteria produce small antimicrobial toxins, known as bacteriocins, which kill or inhibit the growth of sensitive competitors (Riley & Wertz, 2002; Cascales *et al.*, 2007). Bacteria avoid the harmful effects of these toxins by carrying an immunity gene or lacking appropriate cell surface receptors, allowing harm to be directed towards competitors (Ghequire & De Mot, 2014). The harm caused by bacteriocin production is often important for the successful invasion, or defence, of an established bacterial population: bacteriocin producers frequently invade and outcompete sensitive competitors in laboratory microcosm experiments (Riley & Gordon, 1999; Greig & Travisano, 2008; Wloch-Salamon *et al.*, 2008; Waite & Curtis, 2009; Libberton *et al.*, 2015). However, despite widespread recognition of the importance of bacteriocins in mediating competition between genotypes, we still have a limited understanding of this process in natural environments (Hibbing *et al.* 2010; Whitaker, 2009). In particular, it remains unclear why bacterial genotypes inhibit some competitors and not others, and whether bacteriocin-mediated interactions can influence the distribution of genotypes in free-living bacterial populations.

The abundance of bacteriocin producing genotypes in nature is often assumed to be a consequence of competition for limited resources (Gordon *et al.*, 2007). Inhibitory interactions are expected to occur more frequently between ecologically similar genotypes, which require more of the same resources, and less frequently between ecologically dissimilar competitors (Freilich

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et al., 2011). However, very closely related isolates, despite requiring similar resources, are often unable to inhibit each other due to shared resistance mechanisms (Hawlena *et al.*, 2010a,b). Conflating predictions concerning ecological and genetic similarity suggests that inhibitory interactions should occur more frequently between genetically 'intermediate' competitors (Schoustra *et al.*, 2012). Although inhibitory interactions do appear to occur more frequently between genetically 'intermediate' competitors in marine and host-associated bacterial populations, direct evidence that competition over resources promotes inhibitory interactions remains scant (Cordero *et al.*, 2012; Hawlena *et al.* 2012; Kinkel *et al.*, 2014). Our limited understanding of bacteriocin-mediated competition in natural settings also extends to the consequences of these behaviours. In the laboratory, bacteriocin production can influence the diversity of microcosm populations by eradicating sensitive competitors, or driving the emergence of resistant genotypes (Kerr *et al.*, 2002; Le Gac & Doebeli, 2009; Abrudan *et al.*, 2012). However, it remains unclear whether bacteriocin-mediated interactions are strong enough, or occur frequently enough, to impact the distribution of genotypes in natural bacterial populations (Perez-Gutierrez *et al.*, 2013; Ghoul *et al.*, 2015).

Here, we examine bacteriocin-mediated interactions between natural isolates to reveal what factors promote inhibitory interactions in nature, and explore their consequences for the distribution of genotypes in soil. We used natural populations of the *Pseudomonas fluorescens* group, an important plant commensal, as our study system. We isolated *P. fluorescens* from soil at multiple sites in a local park and assessed the ability of isolates to inhibit each other with respect to spatial proximity. We expect that if bacteriocin production has influenced the distribution of genotypes in the population, inhibition would occur less frequently between coexisting isolates. We also constructed nutrient use profiles for each isolate, and measured genetic distances between isolate pairs, to examine interactions in the context of both the extent to which isolates are likely to compete for resources and genetic similarity.

Material and methods

Site description and sampling

We carried out sampling at multiple sites in University Parks, a managed parkland in Oxford consisting of sports fields, tended gardens and undisturbed parkland. We collected soil samples from eight sites in undisturbed regions of the park; the average distance between sites was approximately 250 m. At each site, we sampled a 1-m transect consisting of four patches: a focal patch, and patches 1, 10 and 100 cm from the focal patch (Fig. S1). At each patch, we cleared surface debris and collected 1 cm^3 of soil from the surface. We carried out all sampling in a single day in June 2014 and soil samples were stored in the laboratory at 23 °C and processed within 2 h.

Sample processing and isolate identification

We isolated bacterial cells from soil samples as follows – 1 g of sample was suspended in 6 mL of minimal salts media (M9: 6.8 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl and 10 g NH_4Cl (Sigma Aldrich Company Ltd., Gillingham, UK), per litre of dH_2O) in 15-mL falcon tubes. We vortexed soil suspensions vigorously and plated 80 μL of each suspension onto *Pseudomonas* isolation agar plates (47.4 g *Pseudomonas* isolation agar and 20 g glycerol per litre dH_2O). Plates were incubated at 23 °C for 48 h before we randomly selected 30 isolates from each patch, transferred them to fresh KB agar plates (20 g protease peptone N^o3 (Becton Dickinson U.K. Ltd., Oxford, UK) 10 mL glycerol, 1.5 g $K_2HPO_4.3H_2O$, 12 g agar and 1.5 g $MgSO_4.7H_2O$ (Sigma Aldrich Company Ltd), per litre of dH_2O) and incubated overnight at 23 °C.

We used colony PCR to identify isolates belonging to species of the *P. fluorescens* group. PCRs contained 12.5 μL of 2× MangoMix (Bioline Reagents Ltd., London, UK), 10.5 μL of ddH_2O, 0.5 μM of each primer (Table S1) and 1 μL of DNA template (colony pick suspended in 100 μL dH_2O) up to a volume of 25 μL. The cycling conditions consisted of 5 min at 95 °C, 34 cycles of 94 °C for 30 s, 55 °C for 10 s, 72 °C for 45 s and a final extension of 72 °C for 5 min before cooling to 4 °C. All reactions were carried out with a positive (*P. fluorescens* SBW25), negative (*Pseudomonas aeruginosa* PAO1) and blank control. We established the presence or absence of amplified PCR product using gel electrophoresis.

We randomly selected seven positive isolates from each patch, in each site (seven isolates × four patches × eight sites = 224 isolates) for use in the study. These isolates were grown for 24 h at 23 °C on an orbital shaker at 200 rpm in 6 mL of KB media ((20 g protease peptone N^o3 (Becton Dickinson U.K. Ltd), 10 mL glycerol, 1.5 g $K_2HPO_4.3H_2O$ and 1.5 g $MgSO_4.7H_2O$ (Sigma Aldrich Company Ltd) per litre of dH_2O) and frozen in 50% glycerol at −80 °C.

Assaying inhibitory phenotypes of isolates

We assayed the ability of isolates from the focal patch to inhibit the growth of isolates from patches 1, 10 and 100 cm away in the same site. We also assessed their ability to inhibit isolates from the focal patch of other sites, allowing us to determine whether the frequency of inhibition caused by isolates increased with spatial distance from the focal patch locally (up to 1 m away) and further afield (between sites). We determined the ability of isolates to inhibit each other's growth with pyocins by testing whether an isolate supernatant

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inhibits the growth of other isolates. We spotted KB agar plates with supernatants, spread a lawn of each culture over the plate and recorded which supernatants inhibited which bacterial lawns. Assays were carried out in duplicate.

Supernatant extraction

To extract pyocin-containing supernatants, we cultured 56 isolates (seven isolates from the focal patch of each site) for 24 h in 6 mL of KB media (23 °C at 200 rpm). We measured the cell density of cultures at A600 (SpectraMax), standardized each culture to an OD of ~0.3 and diluted this 10-fold in fresh KB media. Cultures were incubated for 12 h before we added 100 μL of 0.02 mg μL^{-1} mitomycin C (Sigma, UKltd), a strong inducer of bacteriocin production, and incubated for a further 4 h (Pentermann *et al.*, 2014). We then centrifuged cultures at ~13000 *g* for 10 min, obtaining a clear, cell-free supernatant by filter sterilizing with a 0.02-μm filter and storing at −20 °C until required.

Inhibition assays

We spotted KB agar plates with 15 μL of supernatant and allowed the plates to dry at room temperature. We cultured all 224 isolates from freezer stocks for 24 h in 6 mL of KB media (23 °C at 200 rpm). Cell density was standardized to an OD of ~0.5 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 ~12 h, or until a uniform lawn of bacterial growth was visible, and 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 (1 for inhibition, 0 for no inhibition). Plates were checked regularly after 12 h as zones of inhibition caused by some supernatants could become overgrown. We recorded patterns of inhibition and resistance in a 7 × 28 matrix for each set of within-site interactions and a 56 × 56 matrix for between-site interactions.

Each supernatant was spotted on a KB plate to ensure it was cell-free. As a control, each lawn was also spotted with KB media treated identically to supernatant cultures, to demonstrate that residual mitomycin C was not responsible for zones of inhibition. Bacteriophage might also be responsible for inhibition on bacterial lawns, which manifest as areas of slightly reduced growth with characteristic plaques. We observed no plaque formation around the zones of inhibition on our plates and no evidence of self-inhibition, suggesting bacteriophage was not responsible.

Characterizing inhibitory molecules

We repeated the inhibition assays that resulted in one isolate inhibiting another in order to determine what mediated inhibitory interactions. *Pseudomonads* can produce an array of inhibitory molecules that vary greatly in size: large phage tail-like toxins known as tailocins, smaller proteineous toxins known as S-type pyocins and other assorted small inhibitory molecules (Ghequire & De Mot, 2014). We repeated inhibition assays after passing pyocin-containing supernatants through different sized filters and determined whether isolates were capable of killing using large (>100 kDa), medium (>3 kDa and <100 kDa) or small (<3 kDa) molecules.

We obtained cell-free supernatant from 35 isolates (those capable of inhibiting another isolate) as previously. To obtain supernatant containing large inhibitory molecules, we passed 500 μL of the cell-free supernatant of each isolate through a 100-kDa spin filter (EMD Millipore, Billerica, MA, USA). The flow through was discarded and the remaining pyocin-containing supernatant was made up to 500 μL using M9 solution. To obtain supernatant containing small inhibitory molecules, we passed 500 μL of the cell-free supernatant of each isolate through a 3-kDa spin filter and retained the flow through. The flow through contains only particles smaller than 3 kDa; thus, all larger, potentially inhibitory molecules are removed from the supernatant. To obtain supernatant containing intermediately sized inhibitory molecules, we passed 500 μL of the cell-free supernatant of each isolate through a 100-kDa spin filter, and the flow through was collected and passed through a 3-kDa filter. This flow through was discarded and the retained supernatant was made up to 500 μL using M9 solution. We then performed inhibition assays as previously and recorded which size fraction of their supernatants isolates were capable of inhibiting with.

MLST of focal isolates

We used multilocus sequence typing (MLST) of four housekeeping genes to provide a measure of genetic similarity between isolate pairs, allowing us to explore the relationship between inhibition and genetic similarity of our isolates.

DNA extraction and PCR

We extracted genomic DNA using the Wizard Genomic DNA purification kit (Promega, Switzerland) and amplified four housekeeping genes (gyrB, RecA, RpoB and RpoD; Table S1.) for each of the focal isolates. All reactions were performed in 50 μL volume containing 1 U of DreamTaq polymerase (Thermo Scientific), 5 μL of DreamTaq Buffer, 100 mM of each primer, 0.2 mM of each dNTP, 40.75 μL of ddH_2O 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 s, 58 °C/60 °C/64.5 °C for 30 s, 72 °C for 45 s and a final extension of 72 °C for 5 min. The annealing temperatures for each primer set are found in the Table S2.

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Sequencing and analysis

Purified products were sent to SourceBioscience (Nottingham, UK) for Sanger sequencing with the respective primer pairs used for PCR amplification used as forward and reverse sequencing primers (Table S1). We checked the quality of the resulting sequences 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 constructed a concatemer of all four genes for each isolate using Mega 6.0 software (Tamura *et al.*, 2013). From this, we calculated pairwise genetic distances between isolate pairs using the Jukes-Cantor model and constructed a neighbour-joining tree showing the relationships between focal isolates.

Resource competition

We assayed the ability of each isolate to grow on different carbon sources and calculated a measure of niche overlap between isolate pairs, allowing us to explore the relationship between inhibition and resource use in our isolates.

We measured the growth of each isolate on different carbon sources, in duplicate, using EcoPlates (Biolog, Inc., Hayward, CA, USA). EcoPlates plates contain 31 different carbon sources commonly found in soil. We cultured all 56 isolates for 24 h in 6 mL of KB media (23 °C at 200 rpm) before standardizing cultures to an OD of ~0.25 and diluting 10-fold. We inoculated each well of the plates with 150 μL of culture and incubated at 23 °C for 48 h. We measured the absorbance at A_{600} immediately after inoculation and after 48 h. The absorbance of the control well was subtracted from the reading for each well and a measure of niche overlap was calculated (Kinkel *et al.*, 2014).

$$\begin{aligned}&\text{Niche overlap}\\&=\{(\sum \text{minimum absorbance } a,b)/\text{total absorbance } a\\&+(\sum \text{minimum absorbance } a,b)/\text{total absorbance } b)\}/2\end{aligned}$$

where (minimum absorbance *a*, *b*) is the minimum absorbance value for a pair of isolates (*a* and *b*) on a given nutrient; these values are summarized over all 31 nutrients. The total absorbance for an isolate is the sum of absorbance values for that isolate over all 31 nutrients. Therefore, niche overlap between a pair of isolates is the mean proportion of total nutrient use that overlaps between the two isolates.

Statistical analysis

We tested for significant differences in inhibition caused by isolates within their site and inhibition they caused between sites using a generalized linear mixed model (GLMM). We used inhibition between isolates as a binary response variable and distance (within site or between site) as the explanatory variable and included producing isolate and lawn isolate as random effects to account for the fact isolates are used in multiple comparisons. We also tested for significant differences in inhibition caused by focal isolates against isolates from other patches within sites using GLMM, with inhibition as a binary response variable and spatial distance between isolates as the explanatory variable. We included site and isolate as random effects to take into account the multiple sites used in the analysis and that isolates were used in multiple comparisons, respectively.

We wish to determine whether genetic similarity between isolates could explain whether isolates were more or less likely to inhibit another isolate. To determine whether there was any correlation between frequency of inhibition and genetic distance between isolates, we used a generalized linear model (GLM) with inhibition as a binomial response variable and genetic distance between isolates as the explanatory variable.

Finally, to determine whether there was any correlation between frequency of inhibition and niche overlap, we used a GLM with inhibition as a binomial response variable and niche overlap between isolates as the explanatory variable.

Results

Isolate collection and inhibition profiles

We selected seven isolates from a single patch at each of our eight sites (56 isolates) for further analysis, the majority of which were genetically distinct. MLST analysis of four housekeeping genes revealed that 39 of the 56 focal isolates were unique (70%) and the average pairwise genetic distance between isolates was 0.031, ranging from 0.000 to 0.047. A maximum-likelihood (ML) tree of our focal isolates with type strains of the *P. fluorescens* complex suggests that our isolates are part of the *P. fluorescens* group within the complex and that they are comprised of multiple different species (Fig. S2). Rarefaction analyses suggest that a considerable portion of diversity present in the population was sampled (Fig. S3).

The majority of isolates produced a molecule capable of causing inhibition, and were inhibited by at least one of the bacteriocins produced by isolates in our collection. Approximately two-thirds (63%) of isolates produced an inhibitory molecule that could inhibit the growth of at least one other isolate and over three quarters (79%) of isolates were inhibited by at least one other isolate (Fig. 1). In only 7% of interactions did the supernatant of one isolate inhibit the growth of another isolate (Fig. 2).

The inhibitory molecules produced by the isolates in our collection varied in size, and, therefore, likely

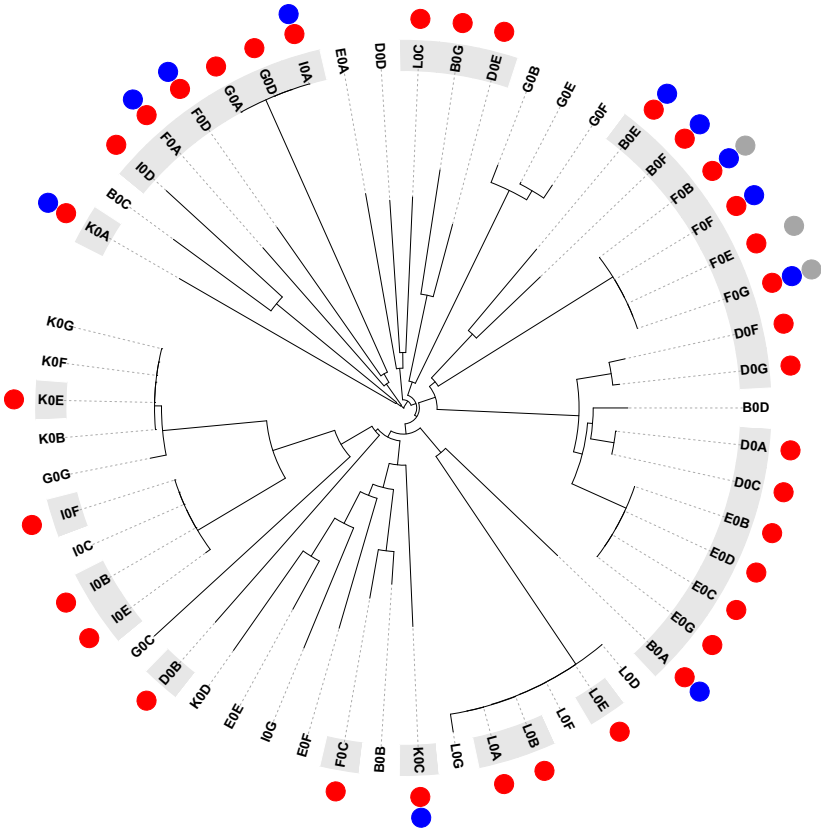


Fig. 1 Inhibitory capacity and phylogeny of isolates based on four housekeeping genes. Outer ring highlights inhibitory isolates (grey) and noninhibitory isolates (white). Coloured circles indicate the ability to inhibit another isolate with large particles (>100 kDa, red), medium particles (<100 kDa, blue) and small particles (<3 kDa, grey). The majority of isolates are genetically distinct and are capable of producing at least one type of inhibitory molecule.

represent a range of different bacteriocin types. We found that isolates produce large (e.g. R and F types), intermediate (e.g. S types) and small (unknown) inhibitory molecules. All inhibitory isolates can inhibit using products >100 kDa in size, whereas 20% and 9% of inhibitory isolates were capable of inhibiting with medium (<70 kDa) and small (<3 kDa) products, respectively. We find that isolates with 100% sequence similarity at four housekeeping genes can show different patterns of inhibition and resistance (Fig. 2).

Inhibition, distance, genetic distance and niche overlap

An analysis incorporating all the factors used in the study suggests significant differences in inhibition attributable to the distance between isolates and the degree of niche overlap, but not the genetic distance between isolates (Table S3).

We found that inhibition occurred less frequently between isolates from the same site, and more frequently between isolates from different sites (Fig. 3a; GLMM: $Z = 4.796$, $P = 1.62 \times 10^{-6}$). There was also a trend, within sites, for inhibition to occur more frequently between isolates separated by greater distances, but this relationship was not significant (Fig. 3b; GLMM: $Z = 1.130$, $P = 0.259$).

We find that isolates were no more likely to inhibit genetically similar isolates than any other isolate (Fig. 4; GLM: $t_{44} = -0.89$, $P = 0.37$) but that inhibition occurred more frequently between isolates with greater niche overlap (Fig. 5; GLM: $t_{329} = 7.02$, $P = 1.23e^{-11}$).

Discussion

Our results show that natural populations of *P. fluorescens* are intensely competitive: the majority of isolates produce bacteriocins and some are capable of producing two or more types (Fig. 1). However, resistance to bacteriocins is so widespread that relatively few interactions actually result in one isolate inhibiting another (Fig. 2). In contrast with previous studies, we find no evidence that inhibition occurs more frequently between genetically more similar or more dissimilar isolates (Fig. 4). However, we do show that inhibition occurs more frequently when competitors have greater niche overlap, suggesting bacteriocins are important in mediating competition over resources in natural settings (Fig. 5). Finally, we found that inhibitory interactions occur significantly less frequently between coexisting isolates, suggesting that bacteriocin production plays a role in shaping natural *P. fluorescens* populations by selecting for isolates resistant to local bacteriocin producers (Fig. 3).

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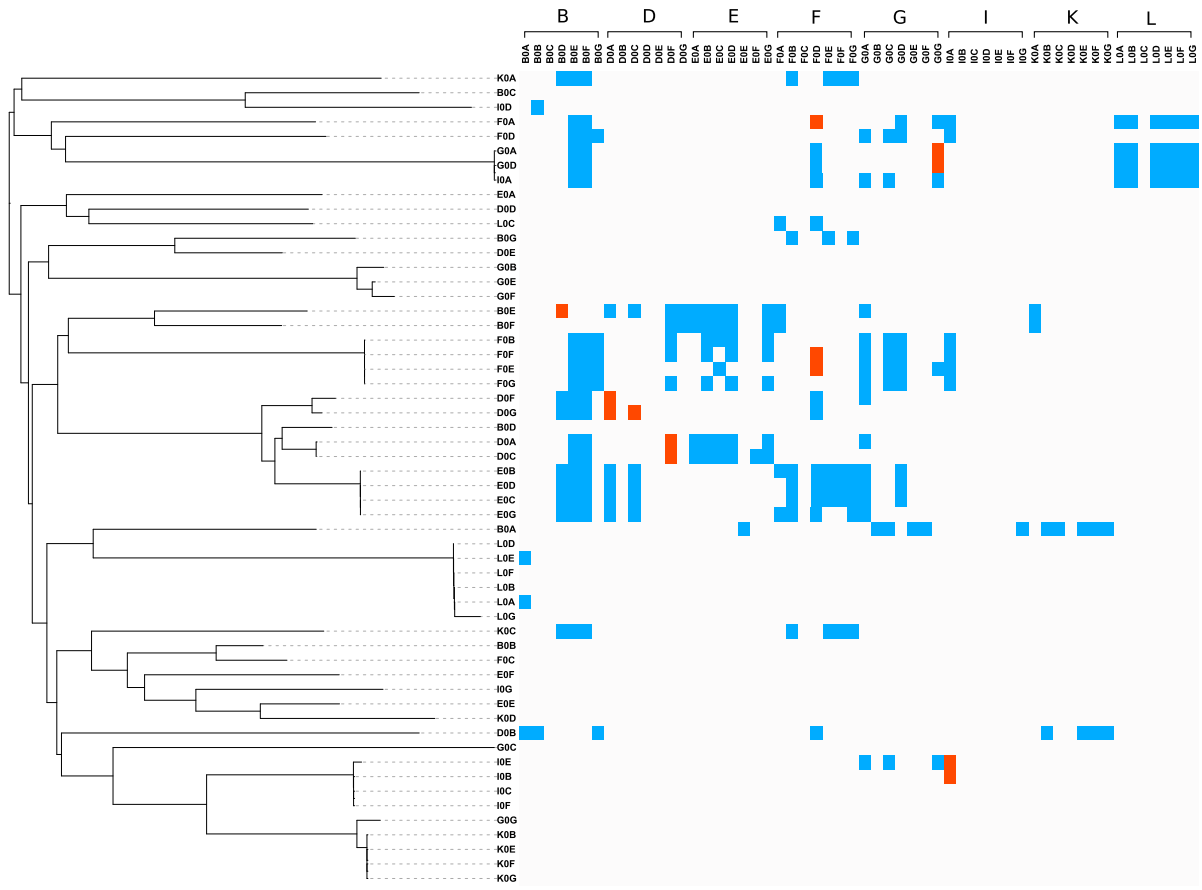


Fig. 2 Phylogeny and inhibition profiles of the isolates. The left-hand side shows a phylogenetic tree of all isolates, whereas the top shows isolates grouped by sampling site. Grey squares indicate no inhibition, blue squares indicate an inhibitory interaction between isolates from different sites (between sites) and red squares indicate inhibitory interactions between isolates from the same site (within sites). The majority of interactions do not result in inhibition, whereas genetically identical isolates can have different inhibition and resistance profiles.

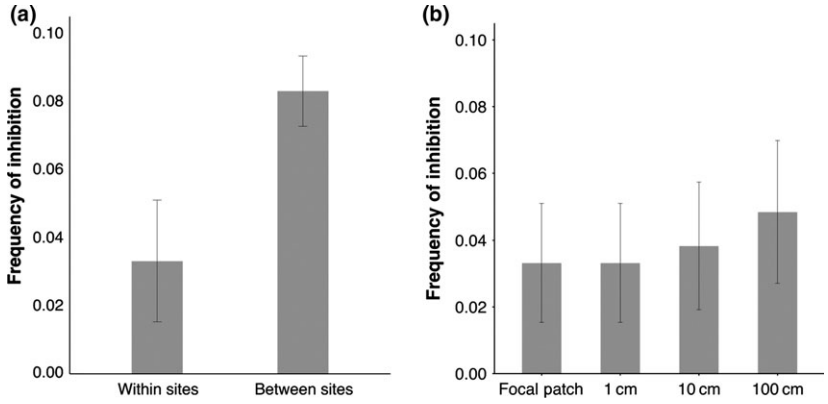


Fig. 3 Frequency of inhibitory interactions between isolates. (a) The frequency of inhibitory interactions between coexisting isolates (within sites, $n = 372$) is significantly lower than that between non-coexisting isolates (between sites, $n = 2744$). (b) The frequency of inhibitory interactions observed between coexisting isolates and in interactions between isolates separated by 1, 10 and 100 cm within sites ($n = 392$ for each distance). Error bars indicate 95% confidence intervals.

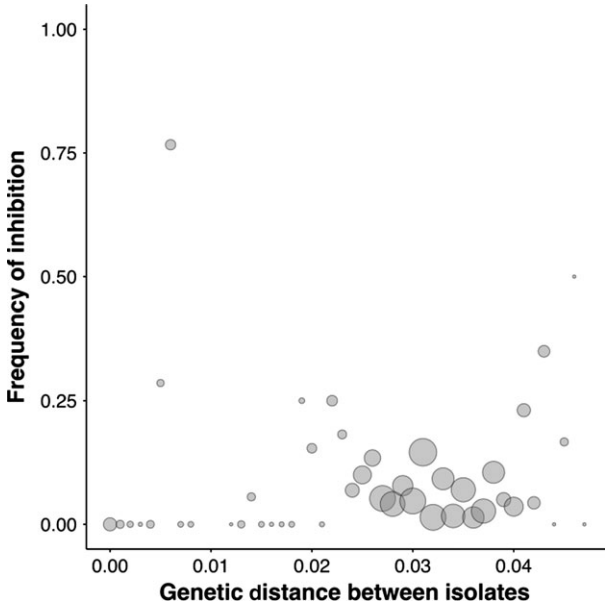


Fig. 4 Inhibitory interactions and genetic distance between isolates. Inhibitory interactions occur no more frequently between genetically similar isolates than between genetically dissimilar isolates. The size of the point reflects the number of interactions: smallest points indicate <5 interactions and largest points indicate >200 interactions (n = 3080).

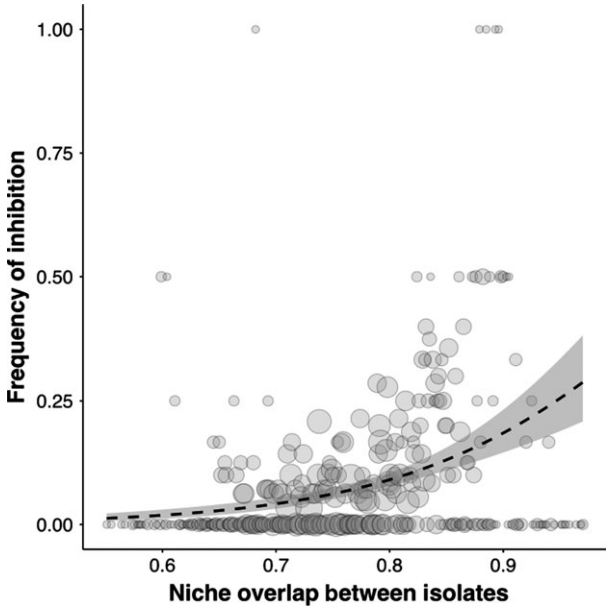


Fig. 5 Inhibitory interactions and niche overlap between isolates. Inhibitory interactions occur more frequently between isolates with greater niche overlap. The size of the points reflects the number of interactions, smallest points indicate <2 interactions and largest points indicate >24 interactions (n = 3080).

Despite the potential for bacteriocin-mediated conflict, resistance appears to be so widespread that relatively few interactions actually result in one isolate inhibiting another. Resistance to the bacteriocins of conspecifics is common in other bacterial populations: in natural *Vibrio* and *Bacillus* populations, <10% of interactions resulted in inhibition despite approximately half of isolates producing bacteriocins (Cordero *et al.*, 2012; Perez-Gutierrez *et al.*, 2013). Our results provide further evidence that resistance is prevalent and either easily acquired, or easily transferred between isolates, in natural settings. Acquiring resistance is thought to come at a significant cost to the cell, yet resistance was the most common outcome of competition even between isolates unlikely to encounter each other frequently (Opsata *et al.*, 2010; Inglis *et al.*, 2016). Why maintain costly resistance against the toxins of rarely encountered competitors? Bacteriocins, such as the phage tail-like toxins produced by our isolates, often target essential components of the cell membrane, such as siderophore or lipopolysaccharide (LPS) receptors (Baysse *et al.*, 1999; Cascales *et al.*, 2007; Kohler *et al.*, 2010; Elfarash *et al.*, 2012) . Mutations in these receptors can confer resistance to bacteriocins, and a single mutation may, by altering the structure of surface receptors, confer resistance against multiple bacteriocins (Scanlan *et al.*, 2015).

We found that bacteriocin-mediated inhibition occurs more frequently between competitors with similar resource requirements. Competition over limited resources is thought to be a key driver in the evolution of competitive phenotypes and selection for bacteriocin production is likely strongest when resource competition is intense (Cornforth & Foster, 2013). By producing bacteriocins that harm ecologically similar competitors, isolates maximize the fitness benefits of costly toxin production, as most resources liberated are beneficial to the producer (Schoustra *et al.*, 2012). Our results suggest that resource competition is an important factor driving the evolution of bacteriocin production in natural populations and are consistent with the limited evidence available from other bacterial populations: inhibitory interactions between *Streptomyces* are often more intense when competitors have similar resource requirements (Kinkel *et al.*, 2014; Schlatter & Kinkel, 2014). Directing harm towards stronger resource competitors also allows isolates to avoid engaging in costly conflict with ecologically distinct competitors, which may partly explain the coexistence of multiple *P. fluorescens* genotypes at each site in our study.

Contrary to expectations, we found no evidence that inhibition occurs more frequently between genetically more similar or dissimilar competitors. Resource competition is thought to be strongest between genetically similar competitors (Freilich *et al.*, 2011; Jousset *et al.*, 2011). Consequently, with the exception of competition between identical or very closely related isolates,

inhibition is expected to occur more frequently between genetically similar competitors. However, our results emphasize that resource competition is the key factor driving interactions towards inhibition in natural populations. While harming strong resource competitors will often equate to more frequent inhibitory interactions between nonidentical, genetically similar competitors, as observed in a recent study of clinical and laboratory *P. aeruginosa* isolates, the relationship is likely weaker in natural settings where resource use can vary significantly between even closely related isolates (Belotte *et al.*, 2003; Schoustra *et al.*, 2012; Kraemer & Kassen, 2015). Our results suggest that although genetic similarity will often be a good predictor of the potential for inhibition between competitors, understanding what drives interactions towards inhibition in natural settings will also require knowledge of the resource requirements of each competitor.

Finally, we found that bacteriocin-mediated inhibition occurs less frequently between coexisting isolates, suggesting bacteriocin production selects for genotypes resistant to local bacteriocin producers. The low levels of inhibition we observe between coexisting isolates could occur if (i) isolates interact more frequently with identical or closely related competitors, who produce similar bacteriocins and are thus resistant, or (ii) if unrelated isolates have evolved resistance to local bacteriocin producers (Kerr *et al.*, 2002; Ghoul *et al.*, 2015). These outcomes are not mutually exclusive: each of our sites is genotypically diverse, whereas many harbour a numerically dominant genotype, suggesting both processes may be important. Attenuation of inhibitory interactions between coexisting isolates is also reported in *Bacillus* populations found in marine sediment, which raises the question of why bacteriocin production is maintained in these populations when resistance is prevalent locally (Perez-Gutierrez *et al.*, 2013). One explanation is that bacteriocin production provides a fitness advantage in competition with invading genotypes: recent empirical studies have shown that invasion of a population can be prevented by bacteriocin production and that genotypically rich populations are often less invasible than their homogenous counterparts (Jousset *et al.*, 2011; Libberton *et al.*, 2015). The genotypically diverse, locally resistant patches of bacteriocin producers we find at each of our sites may represent structured, stable collections of genotypes resistant to invasion by conspecific competitors.

Overall, our results suggest an important role for bacteriocin production in natural free-living bacterial populations. We provide empirical evidence that suggests bacteriocin production mediates competition over resources in natural bacterial populations, and show that bacteriocin production can influence the assembly of bacterial populations by selecting for isolates resistant to local bacteriocins.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:
Data S1 Supplemental Material and Methods.
Figure S1 Transect and sampling sites.
Figure S2 Phylogenetic tree of focal isolates and *Pseudomonas fluorescens* complex type strains.
Figure S3 Rarefaction curve of the focal isolates in this study.
Table S1 Primer sequences.
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Appendix 2: Cheating and resistance to cheating in natural populations of the bacterium *Pseudomonas fluorescens*

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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 (<1cm^3) 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; Bozdag & 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

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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 $K_2HPO_4.3H_2O$ and 1.5 g $MgSO_4.7H_2O$ 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 $K_2HPO_4.3H_2O$, 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) 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, cutoff 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 ddH_2O, 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

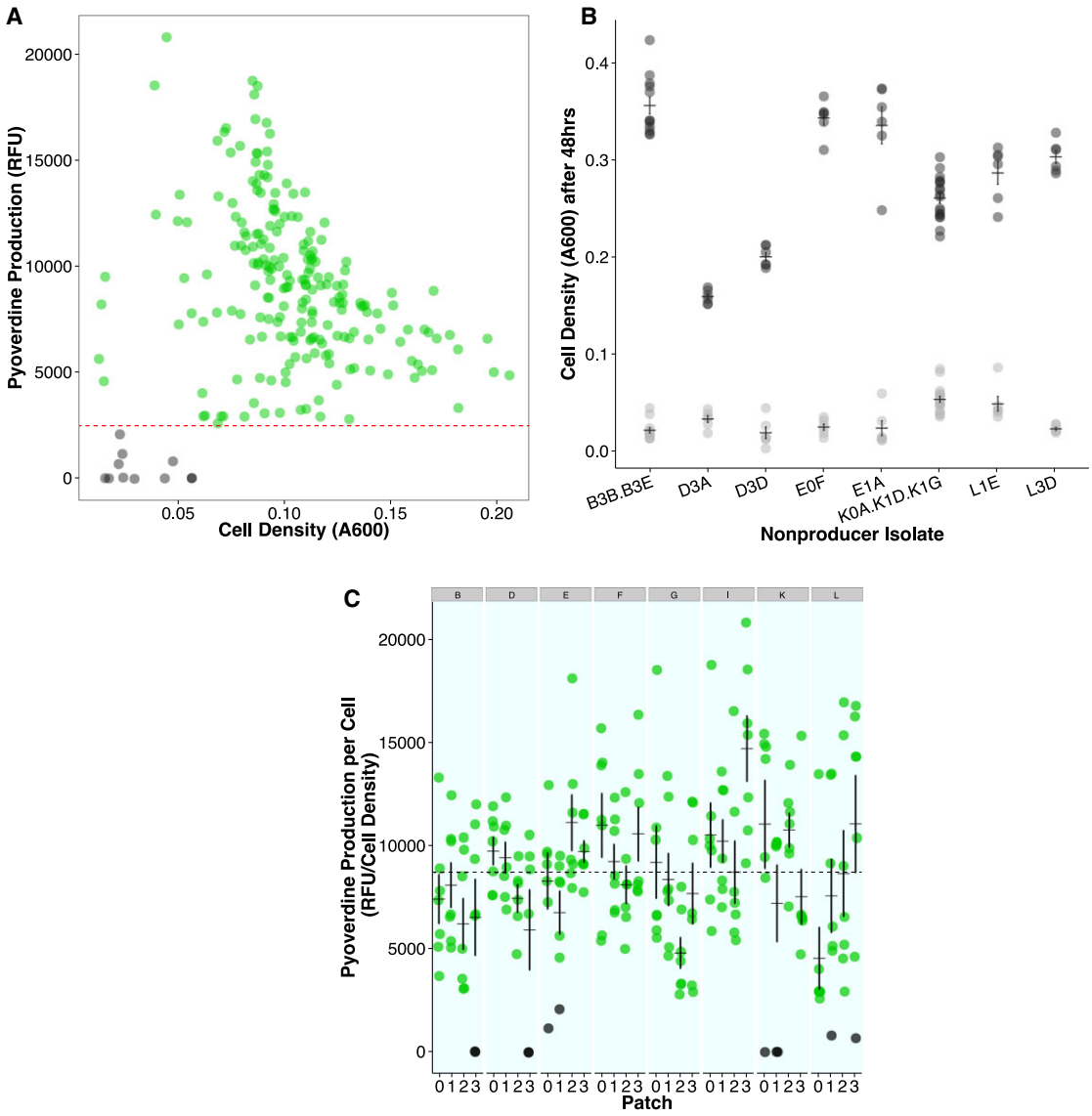


Figure 1. Pyoverdine production per cell and growth of isolates in iron-limited media. (A) Isolates falling below the fifth percentile for pyoverdine production per cell were considered non producers. The horizontal red dashed line represents the fifth percentile for pyoverdine production per cell. Gray 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 ± 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

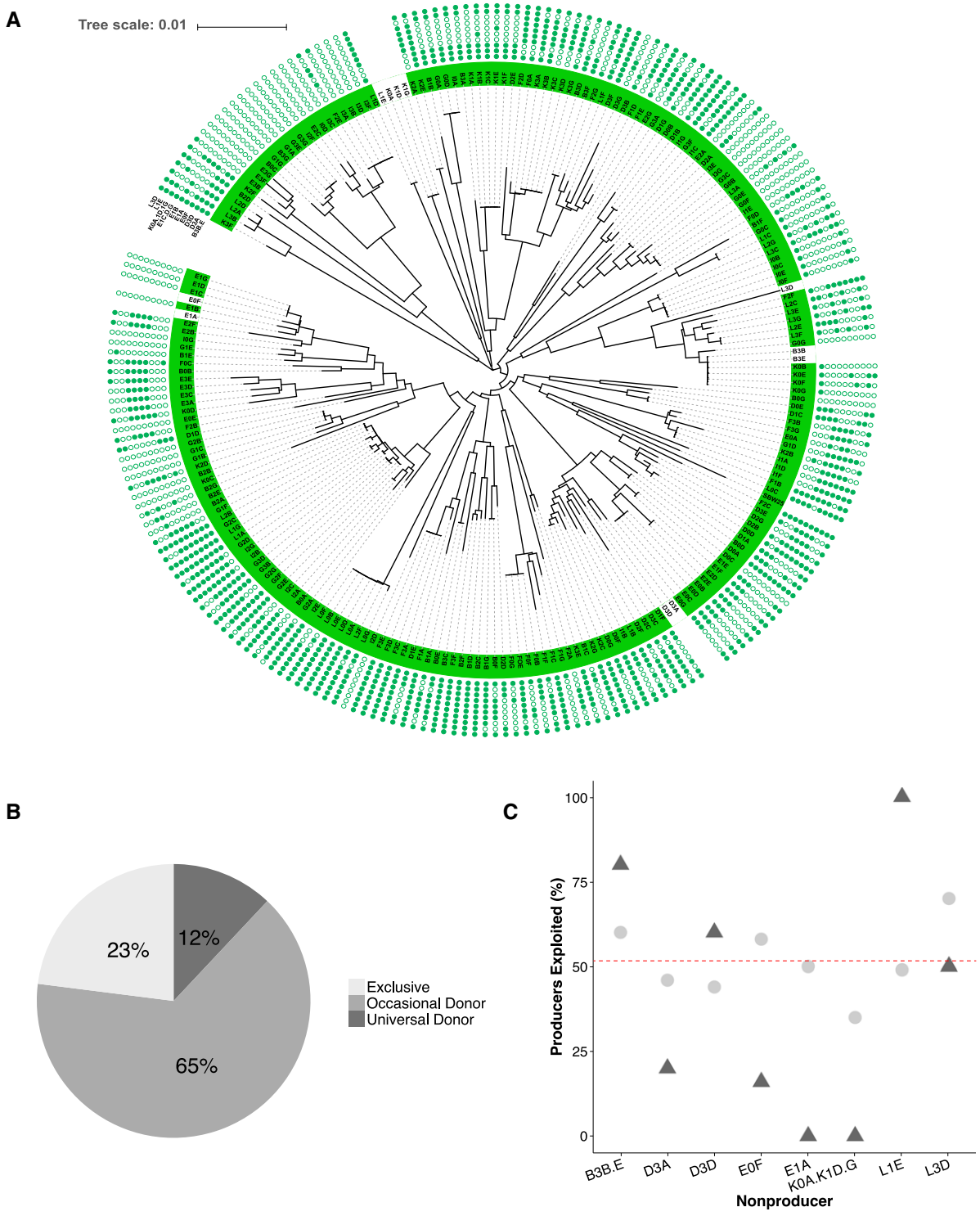


Figure 2. (A) Phylogenetic tree of all isolates in the population. The phylogenetic tree was constructed from a concatemer of three housekeeping genes (gyrB, recA, and rpoB) using the Neighbor-joining method and the Jukes-Cantor model of nucleotide substitution with 1000 bootstraps. Pyoverdine-producing 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-occuring 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-occuring 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-occuring 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-occuring pyoverdine producers. This ranges from a complete inability of some nonproducers to use the pyoverdine of co-occuring 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, $z = 0.452$, $P = 0.651$).

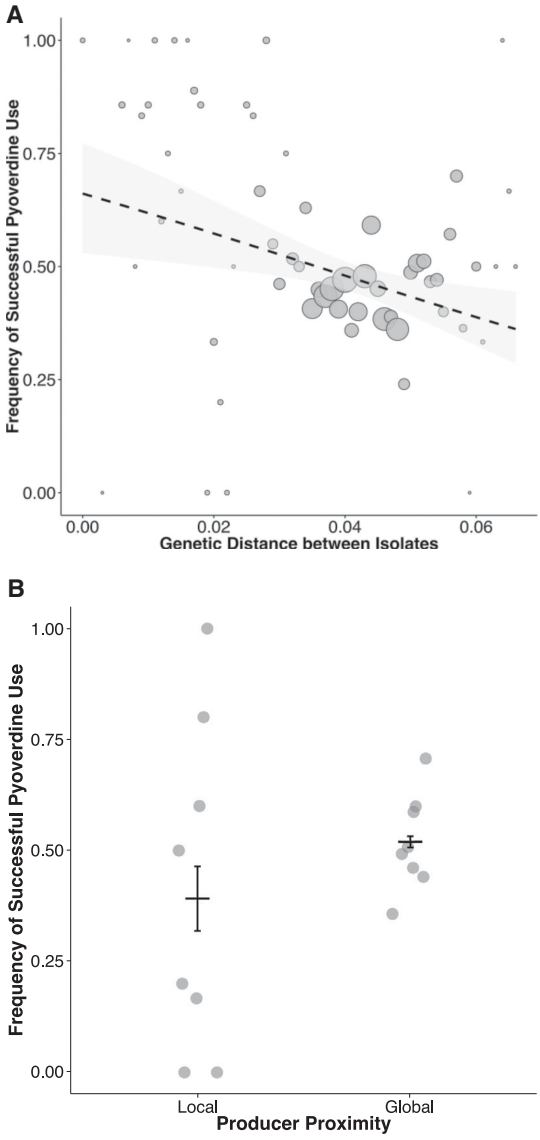


Figure 3. **The effects of genetic similarity and proximity on ability to use pyoverdine. (A) Nonproducer isolates can successfully use the pyoverdine of genetically similar isolates more frequently than genetically dissimilar isolates. The size of the points reflects the number of interactions: smallest 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-occuring 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-occuring isolates (local) or all other isolates in the population (global). Horizontal bars are average proportion of successful pyoverdine use ± SE.**

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 production (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:

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

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-occuring 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

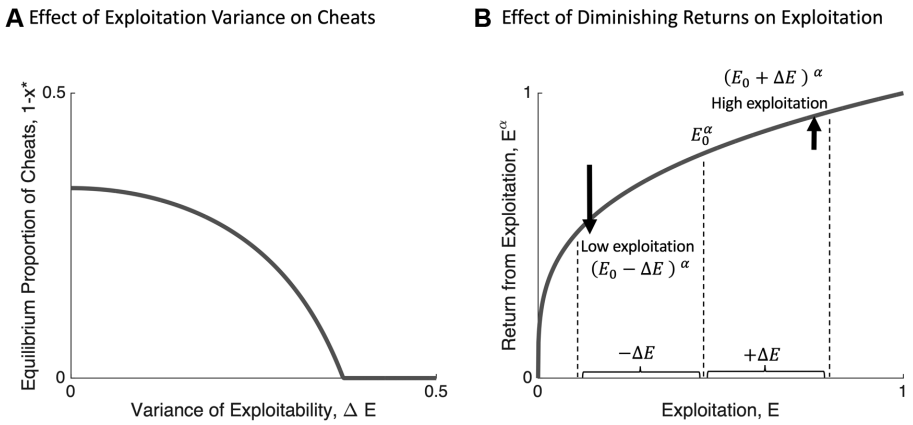


Figure 4. **(A) We plot the equilibrium proportion of cheat cells ($1 - x^*$) in the population as a function of variance of exploitability (ΔE), where high variance of exploitability means that cheater cells can exploit the extracellular factor released by cooperator cells in some local environments 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 aeurginosa*, 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

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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.

Appendix 3: Bacteriocin-mediated competition in cystic fibrosis lung infections.

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Bacteriocin-mediated competition in cystic fibrosis lung infections

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Bacteriocins are toxins produced by bacteria to kill competitors of the same species. Theory and laboratory experiments suggest that bacteriocin production and immunity play a key role in the competitive dynamics of bacterial strains. The extent to which this is the case in natural populations, especially human pathogens, remains to be tested. We examined the role of bacteriocins in competition using *Pseudomonas aeruginosa* strains infecting lungs of humans with cystic fibrosis (CF). We assessed the ability of different strains to kill each other using phenotypic assays, and sequenced their genomes to determine what bacteriocins (pyocins) they carry. We found that (i) isolates from later infection stages inhibited earlier infecting strains less, but were more inhibited by pyocins produced by earlier infecting strains and carried fewer pyocin types; (ii) this difference between early and late infections appears to be caused by a difference in pyocin diversity between competing genotypes and not by loss of pyocin genes within a lineage over time; (iii) pyocin inhibition does not explain why certain strains outcompete others within lung infections; (iv) strains frequently carry the pyocin-killing gene, but not the immunity gene, suggesting resistance occurs via other unknown mechanisms. Our results show that, in contrast to patterns observed in experimental studies, pyocin production does not appear to have a major influence on strain competition during CF lung infections.

1. Introduction

Bacteria produce antimicrobial toxins, termed bacteriocins, to kill competitors [1]. A number of experimental studies have shown that bacteriocins can play a key role in competitive dynamics among closely related bacterial strains [2,3]. Bacteriocin 'producers' can invade and outcompete 'sensitive' strains that are killed by that toxin. These producers can, in turn, be outcompeted by 'resistant' strains that are immune to the bacteriocin, but do not produce it, and hence do not pay the metabolic cost of producing it. While the 'resistant' strains can be outcompeted by 'sensitive' strains because sensitive strains do not invest in the metabolic production of expressing the immunity protein that neutralizes the toxin. In spatially structured populations this can lead to population dynamic cycling with all three strains coexisting, analogous to the rock–paper–scissors (RPS) game, because each strain can be invaded by another strain—producers outcompete sensitive, resistant outcompete producers and sensitive outcompete resistant [1,4,5].

The extent to which bacteriocins determine the dynamics of competing *Pseudomonas aeruginosa* strains in nature, or lead to RPS dynamics, is not known. In addition, previous work has focused on *Escherichia coli*, which carry bacteriocins with relatively simple toxin–antitoxin systems, where one protein is responsible for the killing action (toxin) and another immunity protein for neutralizing the killing protein [4,6]. However, in natural populations of bacteria like

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P. aeruginosa one strain usually carries and releases multiple types of bacteriocins known as pyocins, which have a diversity of killing and resistance mechanisms in addition to the toxin–antitoxin systems [2,7]. For example, three pyocin types exist (S, R and F), with each of these having multiple subtypes with varying toxicity levels [2,7–10]. The soluble S-type is similar to colicins of *E. coli* and is composed of killing and immunity genes. While the R and F type are phage-derived and have different killing and resistance mechanisms, where both consist of only a killing component with no specified immunity that neutralizes the toxin but instead resist via alteration or absence of cell-surface receptors [2,9–15].

We examine bacteriocin-mediated population dynamics in natural populations using cystic fibrosis (CF) lung isolates of *P. aeruginosa*, an opportunistic human pathogen. Early colonization of the lung is characterized by a diversity of strains that intermittently co-infect from environmental sources or other patients, while later stages are characterized by chronic infections usually dominated by one strain [13,16–19]. Our first aim was to test whether the competitive success of different strains, within hosts, can be explained by variation in pyocin production and resistance. In particular, whether the production of bacteriocins has allowed certain strains to eliminate competitors and become dominant. We examined the extent to which different strains where able to produce pyocins that inhibited the growth of other strains, comparing: (i) isolates sampled from non-lung environments and 13 CF Danish patients at different stages of infection and (ii) isolates sampled from a longitudinal study following the infection dynamics of strains within eight CF Danish patients. We sequenced the genomes of all these strains to identify the pyocin genes, examine how pyocin diversity changes during lung infections and examine the genetic basis of the inhibition patterns that we observed phenotypically. We tested the generality of our results by comparing our sequence data with that from previously sequenced epidemic strains, and from 55 isolates of a transmissible Danish isolate that has been sampled for over 38 years [20].

Our second aim was to test whether RPS dynamics occur in natural *P. aeruginosa* populations. We used all the isolates to assess the frequency of occurrence of the different types of producer, resistant and sensitive strains. Theory predicts that structured populations allow all these types to be maintained at appreciable frequencies, whereas sensitives will dominate in unstructured (panmictic) populations [4,21–23]. We do not know the extent to which human hosts represent relatively structured or unstructured populations. We focus on S pyocins because they are composed of a killing and immunity gene, and in particular, the five S pyocin types for which both killing and immunity genes are known. Our third aim was to assess how often the S pyocin immunity gene was responsible for cell resistance to the killing gene. While it has been observed that some strains appear to not be killed by S pyocins, despite lacking the immunity gene for that pyocin, the relative occurrence and importance of this in nature remains unknown [24].

2. Material and methods

We obtained three collections of lung isolates from the Department of Clinical Microbiology, Rigshospitalet, Copenhagen, where they had been isolated from CF patients treated at the Copenhagen CF clinic (collections 1, 2 and 3 below).

(a) *Pseudomonas aeruginosa* isolates

Collection 1 consisted of 41 isolates, which we grouped into categories according to their source of origin: non-lung, or from infected lungs and the stage of lung infection. Specifically, we divided these isolates into non-lung strains from the natural environment or from patient burn wounds (laboratory PAO1), acute isolates that cause a single intermittent colonization in a child, chronic isolates that cause a single one-off chronic infection for less than six months in an adult, and multiply chronic isolates that are highly transmissible and cause multiple chronic infections for longer than six months in adults (electronic supplementary material, table A1). We sequenced all isolates (see the electronic supplementary material) and based on the bacterial genome multilocus sequence typing (MLST) profiles the 41 isolates belong to 29 distinct independent genotypes. We used collection 1 to determine whether the ability to produce, inhibit and resist pyocins, measured both phenotypically and genotypically, correlates with how long a strain has been in the lung.

Collection 2 consisted of the genome sequences of 55 *P. aeruginosa* clinical CF lung isolates [20]. The 55 isolates were of the same genotype and belonged to the DK2 clone type, a transmissible Danish lineage of *P. aeruginosa* and were sampled from 21 Danish patients with multiply chronic CF infections over 38 years between 1972 and 2008 (electronic supplementary material, table A2; [20]). We used these genome sequences to screen for the bacteriocin genes and analyse whether they are lost or mutated over time during infections.

Collection 3 consisted of longitudinal isolates that had been sampled from the onset of infection and over 6–10 years from eight different patients, three of which suffer from chronic infections and five from acute infections as defined by Marvig *et al.* [18] (electronic supplementary material, table A3). The isolates had already been sequenced at the Department of Clinical Microbiology, Rigshospitalet, Copenhagen [18]. Altogether there were 122 isolates that, based on genome MLST profiles, belonged to 17 different genotypes. We used this collection to track changes in bacteriocin production over time in isolates of the same genotype and to track competitive dynamics of co-infecting genotypes within the same lung environment.

Collection 4 consisted of *P. aeruginosa* isolates, which had been previously sequenced and we found in GenBank. These isolates include CF lung isolates (the Liverpool strain LESB58, an isolate from a chronically infected patient in Boston PA2192, the Manchester epidemic strain C3719 and PACS2; accession numbers: NC_011770, NZ_CH482384, NZ_CH482383, NZ_AAQW01000001, respectively) and non-lung isolates (PA14: from a burn wound patient and PA7: a human clinical non-respiratory isolate from Argentina; accession numbers: NC_008463 and NC_009656, respectively). We used these genome sequences to compare the pyocin gene profiles of other epidemic strains to the Danish strains and non-lung strains of our collection.

(b) Materials

For all the experiments we used King's broth (KB) media and agar to culture bacteria (20 g peptone, 1.5 g $K_2HPO_4{\cdot}3H_2O$ and 1.5 g $MgSO_4{\cdot}7H_2O$ for the media and an additional 12 g agar powder for KB agar, per litre of distilled water (dH_2O)). We used M9 minimal salts solution to dilute cell cultures (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). We used the Magellan plate reader v. 7.0 and the fluorimeter SpectraMax M2, Molecular Devices, to take endpoint OD reads for constructing optical density growth curves. The camera box (Syngene Ltd, UK) G:box EF^2 was used for all pictures of bacterial agar plates.

(c) Experimental protocols

(i) Bacterial growth curves

We grew each isolate for 24 h at 37°C at 200 r.p.m. in 6 ml of KB media inoculated from a frozen stock in 20 ml glass vials. We

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measured optical density (OD) at 600 nm (A600), and standardized all cultures to an OD of 1.0 using M9 solution to dilute. We used 1 µl of standardized culture to inoculate 200 µl of KB media in a 96-well plate. To construct growth curves we set the plate to incubate at 37°C for 44 h with automatic periodic shaking every half hour for 15 s prior to OD measurements totalling 89 OD reads. We then plotted the growth curves to determine when each isolate reached stationary phase.

(d) Phenotypic characterization of pyocin production/resistance

(i) Preparation of supernatant

We cultured all isolates for 24 h at 37°C at 200 r.p.m. in 6 ml of KB media inoculated from a frozen stock in 20 ml glass vials. We measured cell density at A600 and standardized all cultures to an OD of 1.0. We then inoculated 30 µl of each standardized culture into 6 ml of fresh KB media in 20 ml glass vials and allowed them to grow at 37°C at 200 r.p.m. until they reach stationary phase. We then centrifuged the cultures for 10 min at 5000*g* to obtain a clear supernatant, which we filter sterilized (0.2 µm pore size) into sterile tubes and placed in the freezer at −20°C. We spotted a control KB agar plate with 20 µl of each culture's supernatant and incubated them overnight to ensure they were free of bacterial growth. Although inhibition of strains by supernatants could be because of small peptides secreted into the supernatant, the broad killing zones that we observe will primarily be because of pyocins. We also excluded inhibition potentially caused by phage lysis (see the electronic supplementary material).

(ii) Preparation of bacterial lawns

We cultured isolates for 20 or 30 h (for slower growing strains) at 37°C at 200 r.p.m. in 6 ml of KB media inoculated from a frozen stock in 20 ml glass vials. We standardized cultures to OD of 1.0, and diluted them 100-fold. We then spread 50 µl of each diluted culture on the supernatant-spotted KB plates.

(iii) Experimental design—using the agar spot assay

We measured the ability of different strains to inhibit each other by testing whether a strain's pyocin-carrying supernatant inhibits a bacterial culture of another strain. We spotted KB agar plates with the supernatants, and then spread a lawn of each culture (electronic supplementary material, figure A1). We placed a 20 µl spot of each supernatant onto KB agar plates. We allowed the spots to dry into the agar before spreading a lawn of 50 µl of each diluted culture. We then set the plates to incubate at 37°C for 12 h until a uniform lawn had appeared. We replicated the same lawn with each set of spots three times. Each plate consisted of control sterile KB media spots and a spot of self-supernatant for each isolate's lawn.

We used semiquantitative assays to examine the lawns for zones of inhibition, or reduced growth on all supernatant spots for collections 1 and 3. We took pictures of the plates using a camera with contrasting colour images in order to better visualize and detect reduced growth when the inhibition areas were not clear zones (electronic supplementary material, figure A1). As a control, we confirmed that sterile KB media and self-supernatant spots did not cause any inhibition of the lawns. We then recorded which strains inhibited each other and compiled a 41 × 41 matrix organized into four isolate categories of non-lung and three different stages of a CF infection (acute, chronic one-off and multiply chronic) across the patients. We also compiled a separate matrix for within-patient isolate interactions for the eight different patients of collection 3.

(e) Data analysis

(i) Genome sequence analysis—screening for pyocin genes

All isolate genome sequences were assembled *de novo* using the assembly software Velvet, the contigs from which were uploaded to a database running the BIGSdb platform [25]. We assessed the diversity of isolates using multilocus sequence typing (MLST) where the genomes were screened for alleles of the seven key housekeeping loci, *acs*, *aro*, *mut*, *nuo*, *pps*, *trp* and *gua*, of *P. aeruginosa* available at the www.pubmlst.org/paeruginosa website. The MLST concept is extended to the whole genome scale screening for allele variants of a diversity of other loci and we analysed this data using a genome comparator tool to identify distinct genotypes among the isolates [25]. For further data analysis, we grouped isolates with identical genome MLST profiles together as one independent sample to minimize pseudoreplication.

The BIGSdb software includes 'autogagger' and 'autodefiner' tools that scan deposited whole genome sequences against loci identifying exact matches or allele variants with 98% sequence identity for which new alleles are generated. Isolates' records are updated with specified allele numbers marking the regions on assembled contiguous sequences (contigs) for defined loci. We blasted the genomes for pyocin genes and tagged the resulting allelic variants before proceeding with further analysis.

We screened all isolate genomes for the soluble S pyocin (S1, S2, S3, S4, S5 and AP41) loci and their allele variants for the killing and corresponding immunity gene using a known sequence as a reference available from previously sequenced genomes in NCBI GenBank (electronic supplementary material, table A4). We also screened the genomes for regulatory (prtN and prtR) and lysis genes (hol and lys) as well as the R and F pyocin structural genes (electronic supplementary material, table A4, figure A2). We used pyocin loci R2 and F2 as reference genes to screen for other potential alleles that belong to other R and F pyocin subtypes. The R pyocin is characterized by the presence of the whole suite of genes between the hol-lys and the F pyocin by the presence of the toxin loci between lys and trpG (electronic supplementary material, figure A2). We tagged the genomes of all isolates and annotated each of the loci and its identified alleles (electronic supplementary material, figure A3).

For some of the F2 toxin loci, alleles could not be identified because of high sequence dissimilarity. In such cases, we checked for the presence of open reading frames (ORF) in the expected location of the F-specific region and, if present, we marked them as present but with an unidentified allele. In all isolates the genes for R and F pyocins are found between *trpE* and *trpG* in one region lying on one contig, facilitating the search for ORFs in the missing R or F regions.

For our analysis a gene was considered to be present in the isolate when it is present in its fully functional coding sequence. When a sequence is truncated and partly found in the genome because it lies at the edge of a contig it is impossible to determine whether it exists in the genome as a fully functional coding sequence or a mutated non-functional gene. We considered genes lying at the edge of a contig as present and functional for further analysis. Sequences with premature stop codon mutations are considered non-functional. If one of the genes in the cassette composing the R- or F-pyocins is missing in an isolate we consider the pyocin to be non-functional and as absent. However, this was never the case, they were either all present together or none at all.

To ensure that the R- and F-pyocin loci are in fact absent from the genomes and the results are not due to a sequencing error, we checked whether any other DNA sequence lies in the R- and F-specific region of the genome. The R-pyocin genes are in a lysis cassette, between the holin and lys genes, and the F-specific region is between lys and trpG genes. We found that for all isolates not caryring R and F pyocins, there are no ORFs between the holin-lys and lys-trpG loci respectively, confirming the absence of R- and F-pyocin genes from the isolates.

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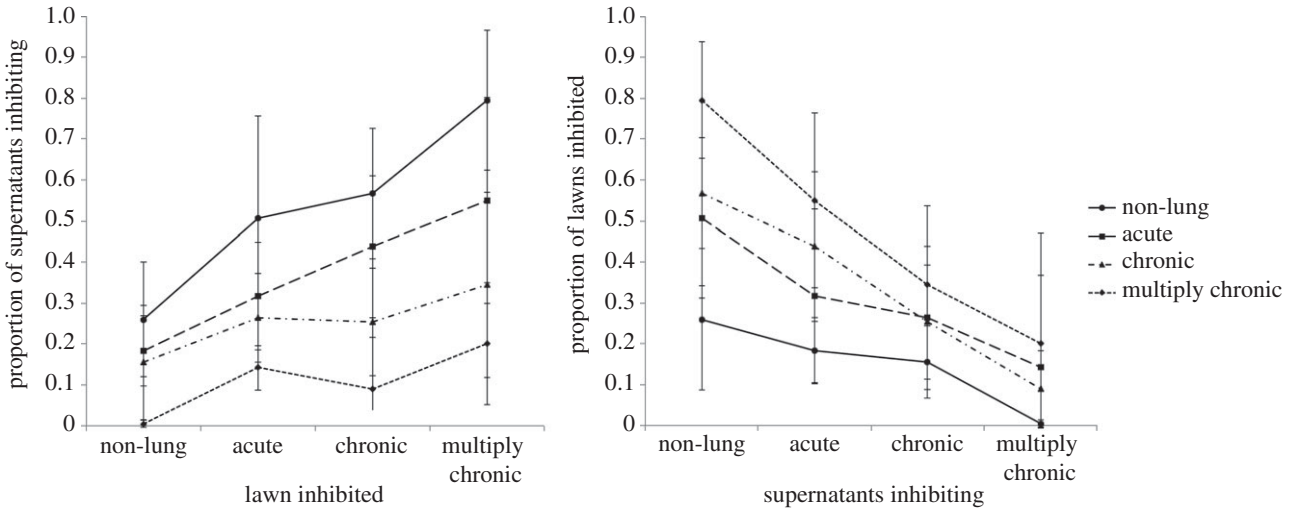


Figure 1. Proportion of supernatants inhibiting and lawns inhibited. Strains that were isolated from later-stage infections were both (*a*) less likely to inhibit and (*b*) more likely to be inhibited by other strains. The error bars indicate $\pm$95% CI bars around mean values.

(ii) Statistical analysis of phenotypic inhibition profiles

For the 41 isolates taken from different stages of infection (collection 1), we merged and averaged the data of all isolates with identical MLST genotypic profiles in each isolate category resulting in a total of 29 independent samples. To avoid pseudoreplication, we estimated the number of times isolates of identical genotypes are inhibited or inhibiting and used the averaged value for the analysis (electronic supplementary material, figure A4). We tested for significant differences in inhibition with time spent in the lung (as estimated by type of CF lung infection) using a generalized linear model (GLM) with level of inhibition as a response variable and two explanatory variables: time spent in the lung and type of *P. aeruginosa* isolate category. We tested for changes in the proportion of inhibition caused by the isolate's supernatant as a function of time isolates spent in the lung and the proportion of lawns inhibited by supernatants as a function of time bacterial lawn isolates spent in the lung. We also specifically tested for: (i) differences in the level of inhibition caused by isolates' supernatants of each category on the isolate lawns within each category and (ii) differences in the level of inhibition in the isolate lawns of each category caused by the isolates' supernatants of each category.

For the longitudinal samples from the eight CF patients (collection 3), we did not merge isolates of the same genotype as one independent sample because the aim of these experiments is to test for significant differences in inhibition with time of infection across isolates infecting the same lung. We used GLM analysis to test for significant differences in inhibition levels with time (in years) each isolate has spent infecting the lung. In the analysis we modelled the proportion of killing caused by isolates' supernatant as a response variable with time of infection as a continuous variable and genotype as a discrete variable. This analysis was done on isolates per patient and isolates across all patients for more general patterns. The data from both isolate collections are proportion data and so we normalized for residuals prior to analysis using an arcsine square-root transformation.

(iii) Analysis of S pyocins

To assess the frequency in which producer, resistant and sensitive S pyocin isolates occur we used the genetic data to assess how often an S pyocin occurred with both its killing and immunity gene, and how often either gene was found alone across all independent genotypes. When the isolates that share the same genome MLST type showed variation in their S pyocin profile, we considered the isolates as independent samples. This resulted in a total of 53 independent samples, with a total of 80 occurrences of S pyocin genes found across all.

To assess whether the S immunity gene is responsible for cell protection from S pyocins we used the pyocin gene profiles in all isolates to predict how often one cell carrying the S pyocin will kill a cell that does not carry that S pyocin (electronic supplementary material, figure A5). Then we determine how often killing is observed from the phenotypic experimental data. We then record how often resistance of the target cell occurs in the presence of the immunity gene. When the same genotype showed variation in its S pyocin profile, we also considered the isolates as independent samples here. We recorded resistance or susceptibility from the experimental competitive interactions when more than 50% of the isolates of each independent genotype are susceptible or resistant. If exactly 50% of the isolates of a genotype show resistance and the other 50% susceptibility, we record that as data that cannot be interpreted. This occurred in 25 of 635 competitive interactions that were therefore excluded from further analysis. In the majority of cases, there was no discrepancy among isolates within a genotype, and so the criteria we use above do not significantly change the results when not applied.

For the above analyses we analysed data of S1, S2, S3, S5 and AP41 pyocins, but we excluded the S4 pyocin because it is still unknown whether there is an immunity gene for it, and if there is, it has not been identified or annotated yet. We used proportion χ^2-squared to test for significant differences in the frequency of genes occurring together or alone and to test for significant differences in the frequency of strains that resist via alternative resistance mechanisms and strains that resist using the immunity gene. We implemented all analyses in R statistical software (http://www.R-project.org).

3. Results

(a) Pyocin production and resistance

(i) Comparison across patients sampled at different stages of infection

Considering the 41 isolates taken from different stages of infections (collection 1), we found that strains that were isolated from later stage infections were both significantly less likely to inhibit and more likely to be inhibited by other strains (figure 1; electronic supplementary material, figure A4; inhibiting: $F_{7,108} = 10.63$, $p = 4.03 \times 10^{-10}$; inhibited: $F_{7,108} = 11.14$, $p = 1.53 \times 10^{-10}$; $N = 29$ distinct genotypes). The interaction terms between the type of lawn inhibited, and the type of

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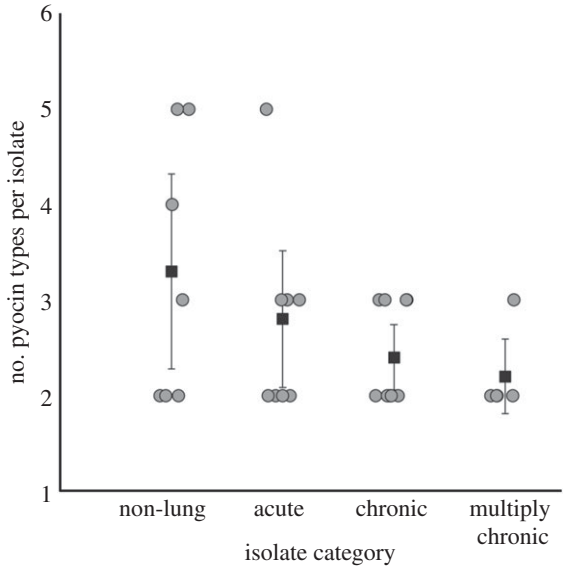


Figure 2. Pyocin diversity across different stages of infection. Isolates from later-stage infections carry fewer pyocin types than isolates from early infections and non-lung environments. Circles represent isolates and squares represent the means of number of pyocin types for each isolate category. The error bars indicate ±95% CI bars around mean values.

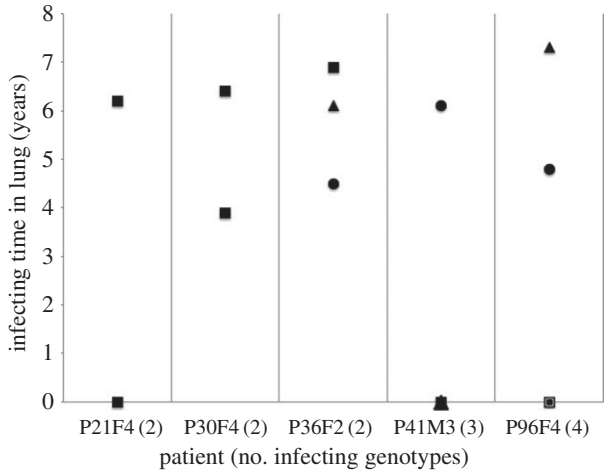


Figure 3. Within patient competitive dynamics. Considering the eight patients where we were able to follow strain dynamics over time, five were infected by multiple strains. These patients contained 14 distinct genotypes, and none of the patients were infected with the same genotype. In only three of these five patients at least one of the strains was able to inhibit another strain in the infection. Squares represent genotypes that are not inhibited nor cause any inhibition; triangles are genotypes that inhibit the susceptible circle genotypes. In two out of these three patients (P36F2 and P96F4), the inhibiting genotype (triangle) persisted longer in the infection, consistent with a role of bacteriocin-mediated competition. In the other patient (P41M3), the inhibited genotype (circle) persisted longer in the infection, contrary to the prediction from bacteriocin dynamics.

supernatant causing inhibition were not significant. Therefore, excluding the effect of interaction, there is a significant decrease in the supernatants inhibiting ($F_{4,111} = 16.91$, $p = 7.43 \times 10^{-11}$) and a significant increase in the lawns being inhibited ($F_{4,111} = 18.9$, $p = 7.10 \times 10^{-12}$) with increasing infection time in the lung.

We found that strains had a significantly lower diversity of pyocins as infections progress to chronic stages (figure 2; GLM, $F_{1,27} = 5.003$, $p = 0.034$). Lower pyocin diversity in multiply chronic transmissible strains can be either because of gene loss over infection time within a lineage, or that strain lineage never having had them in the genomes. To determine whether the pyocin genes are lost from the genomes when a strain infects for long periods we examined the 55 isolates taken over 38 years from one of the Danish multiply chronic transmissible genotypes (DK2; collection 2). These isolates do not show loss of or mutation accumulation in the pyocin genes over time. These data indicate that pyocin genes of transmissible isolates are not lost with increasing infection time but were initially never present in the genomes; such that chronic transmissible strains are characterized by a lower pyocin diversity relative to non-chronic isolates.

To compare our results to previously published strains, particularly that Danish multiply chronic strains carry a reduced diversity of pyocins, we analysed the genomic pyocin data on six *P. aeruginosa* isolates from GenBank, four chronic or epidemic CF strains from the UK, Boston and an unknown source, and two clinical non-lung isolates (collection 3). We found the same pattern in these isolates that we had found in our Danish isolates. Similar to the multiply chronic isolates of our collection, the CF isolates (except for the Liverpool strain) also show absence of all the R pyocin genes but retain the F genes, while the clinical non-lung isolates carry both the R and F pyocin genes, similar to the non-lung isolate category of our collection (electronic supplementary material, figure A3).

(ii) Comparison over time within patients

We then analysed longitudinal isolates, taken over a 6–10 year period, from eight patients. We found that, within patients, isolates' inhibition levels did not change over time infected ($T_7 = -0.70$, $p = 0.505$, $N = 8$)—inhibition increased in two patients, decreased in four, and there was no inhibition in two patients. Examining the genomic data of isolates in patients with multiple infecting genotypes, pyocin diversity does not vary significantly with the likelihood of persisting in the lung ($T_{7.73} = -0.43$, $p = 0.676$, $N = 5$).

Considering the eight patients, five had two or more genotypes infecting the lung. In two of these patients there was no inhibition between genotypes. In the remaining three patients, at least one of the genotypes was able to inhibit the growth of another genotype in that patient. In two out of these three patients, the inhibiting genotype persisted longer in the infection, consistent with a role of bacteriocin-mediated competition (figure 3). In contrast, in the third patient, the inhibited genotype persisted longer in the infection.

(b) Linkage between S pyocin killing and immunity

We then examined the extent to which killing and immunity co-occurred for the five S-type pyocins. We focused on S-type pyocins because they are composed of two genes, one responsible for the killing activity and an immunity gene that protects against the killing. Natural populations have been observed to contain strains that carry both, the toxin and immunity genes (toxin producers), just the immunity gene (resistant) or neither (sensitive).

Overall, 98% (53/54) of all independent isolates in all collections contained at least one S-pyocin gene. The average number of pyocin types per isolate was 1.5, giving 80 (53 × 1.5) occurrences of pyocin genes. Amongst those, 51% (41/80) contained both the toxin and the immunity genes, 35% (28/80) only the killing gene and 14% (11/80) only the immunity gene, (figure 4; $\chi^2_{2,80} = 25.46$, $p = 2.96 \times 10^{-6}$). The relatively

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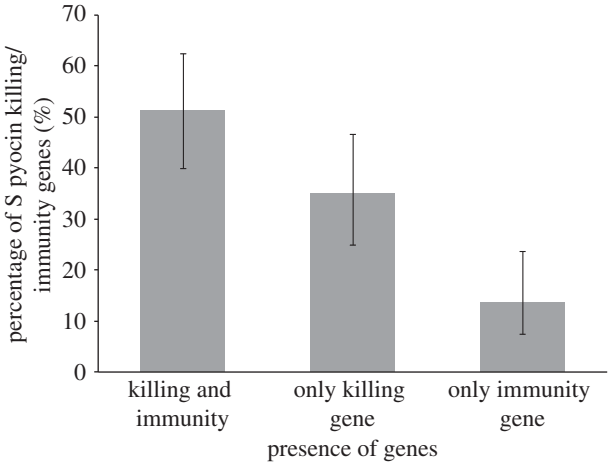


Figure 4. Soluble pyocin killing and immunity genes. Killing and immunity genes are found together (51%), or either the killing gene (35%) or the immunity gene (14%) alone. The error bars indicate ±95% CIs.

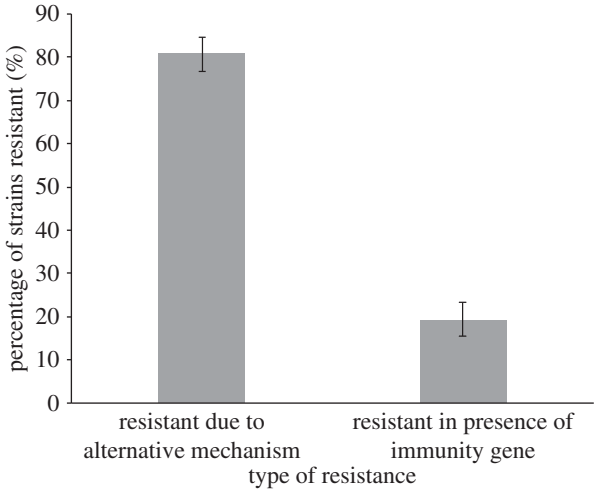


Figure 5. Protection from S pyocins by unknown resistance mechanisms. Considering cases where one strain does not carry a pyocin type carried by another (403 strain pairs), resistance via some other mechanism is much more common (81%) than resistance likely due to the immunity gene (19%). The error bars indicate ±95% CIs with correction for continuity around proportion values.

high occurrence of only the killing gene occurs only in the nuclease S pyocins (S1, S2, S3 and AP41). This is surprising, because this would lead to the isolate killing itself, however, this is not phenotypically observed for any of the strains tested in the inhibition assays. Whereas the S5 pyocin, the only known pore-forming S toxin, which is therefore not lethal when expressed, occurs a total of 13 times across genotypes but is always present with the killing and immunity gene together.

We examined the extent to which strains might have resistance mechanisms other than the specific immunity genes (electronic supplementary material, figure A5). We considered 877 strain interactions (competitions), including the isolates from the different stages of infection and the longitudinal isolates from the eight patients (collections 1 and 3). Examining our genetic data, we found that in 635 of these competitions, one strain carried at least one S-type pyocin that the other strain did not. In 33% (207/635) of these cases, we found that the toxin carrying strain was able to inhibit the growth of the other strain. In the 403 interactions where growth was not inhibited, we found that in 19% (77/403) of cases the strain not inhibited contained the immunity gene to the pyocin(s) being produced by the killing strain. In the remaining 326 cases (81%, 326/403), the strain did not have the immunity gene(s) and so must be using some alternative mechanism to resist the pyocin. Alternative resistance mechanisms protect strains significantly more frequently than the immunity gene (figure 5; $\chi^2_{1,403} = 305.23$, $p < 2.2 \times 10^{-16}$).

4. Discussion

We found that, in *P. aeruginosa* infections of the CF lung: (i) isolates from later infection stages caused less inhibition of other strains, were significantly more inhibited by the pyocins produced by other strains (figure 1) and carried a significantly lower diversity of pyocin types (figure 2); (ii) pyocin genes were not lost from a chronic transmissible strain over time; (iii) within infections, over time, there is no change in inhibition/inhibiting levels or pyocin diversity; (iv) pyocin inhibition profiles do not significantly explain which strains persist longer within lung infections (figure 3); (v) 35% of S pyocins occurred only as the killing gene, without the immunity gene to that pyocin (figure 4); (vi) in cases where one strain does not carry an S pyocin type carried by another, resistance via other mechanisms is much more common (81%) than resistance via the immunity gene (19%; figure 5).

(a) Bacteriocins and strain dominance

We found no support for the hypothesis that certain strains are able to achieve dominance in the CF lung through bacteriocins (figures 1 and 3). In fact, we found the opposite pattern, that more dominant/persistent strains, taken from later infection stages are less able to kill and more easily killed by other strains (figure 1). Our genetic analysis suggests that this pattern arises because isolates from later infections carry fewer pyocin genes (figure 2). This pattern could occur through strains losing pyocin genes overtime, or through strain competition, with more competitive strains producing less pyocins [26–30]. Our results suggest the latter with more competitive strains producing fewer pyocins (figure 2) rather than pyocin genes being lost from strains during infection.

Why do more dominant strains which cause chronic infections carry a lower diversity of pyocins (figure 2; [29])? A number of possible selective forces, which are not mutually exclusive, may be involved. Higher strain diversity, both within and across species, may favour a higher pyocin diversity required for competition during early stages of infection [19,31–37]. Another possibility is that higher pyocin diversity may be more costly in chronic infections if it leads to a higher antibiotic susceptibility—for example, expression of the R/F region increases strain susceptibility to fluoroquinolone antibiotics due to induction of the lysis system [38]. However, this does not explain maintenance of the F pyocin in the multiply chronic isolates of our collection, unless carrying either R or F rather than both, also makes the cell less inducible and therefore more resistant to antibiotics.

Within patient infections, strains show no competitive dominance as a result of bacteriocins and this is because any given cell is interacting more often with clonemates that share the same genotype. In which case the bacteriocins are useless. However, even when interacting with strains of

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different genotypes that are co-infecting, bacteriocins do not always explain strain persistence and dominance (figure 3). More often it is the strains that do no inhibit nor are inhibited by others that persist longest, implying that toxins are not useful in the CF lung environment. Despite this, strains retain a diversity of toxins, most probably as a result of adaptation to a range of environments they previously colonized.

It is important to note that it is possible that pyocin production can vary with the conditions in the CF lung that would show inhibition profiles different from our experiments. For example, stress-inducing conditions, such as anaerobic or oxidative stress, usually associated with pathogenesis in the lung, as well as mutagenic agents inducing production of all pyocin types but repressing expression of the S immunity [37–40]. It has also been speculated that the CF lung is an iron-limited environment [41], which can make cells more susceptible to S pyocin killing due to upregulated expression of ferri-pyoverdine and pyochelin receptors, which are required for iron uptake but also serve as receptors for S pyocins [2,42,43]. However, we note that our genetic analysis supports the general trends observed in the phenotype inhibition assays.

(b) Killing and immunity genes

We found that 35% of S pyocins are found as the killing gene without the corresponding immunity gene (figure 4). This is surprising because these are nuclease toxins, which suggests that these strains would kill themselves when expressed. However, we also found that strain resistance often occurs without the presence of the immunity gene, suggesting that whether or not you produce the specified immunity gene in *P. aeruginosa* is irrelevant. Considering cases where one strain does not carry a pyocin type carried by another, resistance via some other mechanism is much more common (81%) than resistance via the immunity gene (19%; figure 5). Possible alternative resistance mechanisms could be production of another 'orphan' immunity protein—as found in other pseudomonad species [44], that a secondary receptor is needed in addition to the primary receptor to allow entry of the pyocin into the cell or cells have mutations in or are lacking the primary S pyocin receptors [24,42,43].

We did not find evidence for bacteriocin-mediated competition explaining strain dynamics and/or producing RPS dynamics. Such dynamics are still expected even when there are multiple bacteriocin types in a population [21]. One possible explanation is that *P. aeruginosa* bacteriocins do not appear to involve single production/immunity genes, as we have shown immunity can occur via alternative mechanisms. This contrasts with *E. coli* where bacteriocins are neutralized by the immunity proteins [45]. Our results suggest that the outcome of strain competition is being driven primarily by other factors, which influence the ability to survive and grow in the CF lung. These could include susceptibility to antibiotics or attack from the immune system. Another explanation is that bacteriocins may be useless in the CF environment because they may not penetrate through the mucus layers and reach target cells, therefore, selecting against high-producing bacteriocin strains.

To conclude, we have found no evidence that killing by bacteriocins allows strains to outcompete other strains in the CF lung. Instead, we found the opposite result that multiply chronic Danish strains, which are better at surviving and persisting, as well as other epidemic CF strains carry fewer bactericions. Screening of bacteriocin profiles may therefore offer a method for predicting which infections are more likely to persist as chronic. A major task is to determine why lower bacteriocin diversity is correlated with the ability to persist in infections of the CF lung.

Ethics. The use of samples was approved by the local ethics committee at the Capital Region of Denmark Region Hovedstaden: registration number H-1-2013-032. All patients have given informed consent. In patients under 18 years of age, informed consent was obtained from their parents. All patient samples were anonymized prior to any experimental use or analysis.

Competing interests. We declare we have no competing interests.

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Appendix 4: Pyoverdin cheats fail to invade bacterial populations in stationary phase

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Pyoverdin cheats fail to invade bacterial populations in stationary phase

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bacteria;
cheating;
cooperation;
growth phase.

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

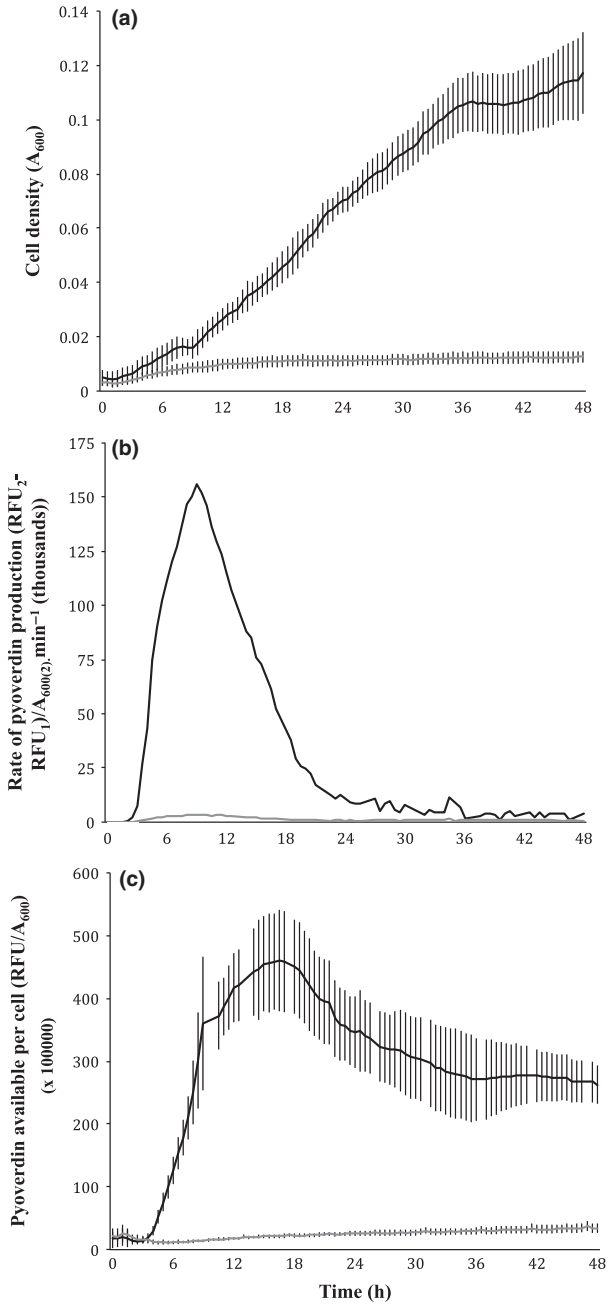


Fig. 1 Producer (black) and nonproducer (grey) (a) growth curves, (b) rate of pyoverdin production and (c) cumulative pyoverdin available per cell, over a duration of 48 h in iron-limited media: (a) growth measured by cell density at A_{600} of the producer is significantly better in iron-limited conditions 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.

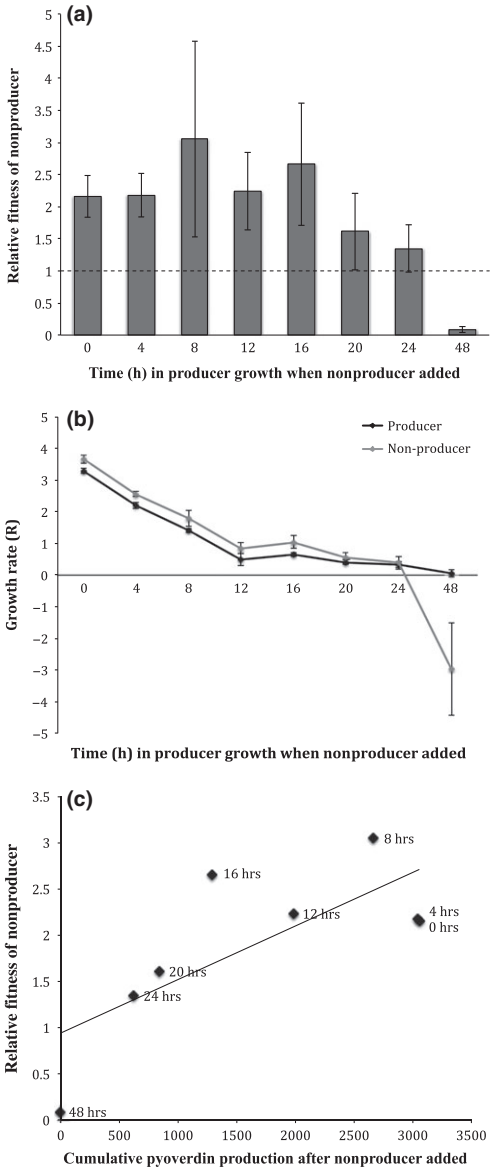


Fig. 2 (a) Relative fitness of nonproducer in competition with producers at different stages of growth, (b) growth rates of producer and nonproducer strains during competition, (c) relative fitness of nonproducer correlated with the producer's cumulative pyoverdin production after the nonproducer is 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.

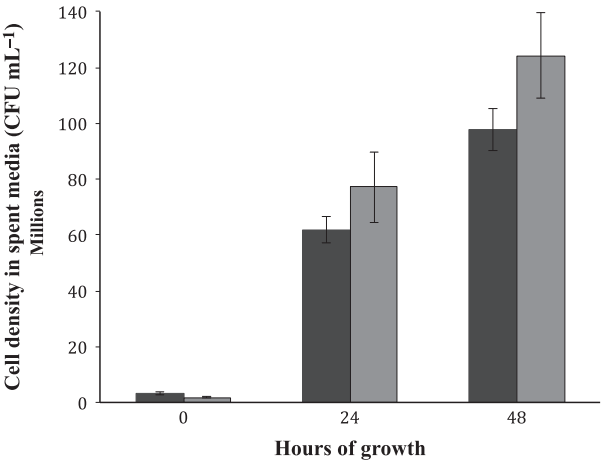


Fig. 3 Monoculture cell growth in freshly extracted producer-spent media. Cell density is measured as colony-forming units per mL (CFU mL^{-1}) in the spent media of 48-h producer monocultures. Over a period of 48 h, nonproducers (grey) grow to a significantly higher density than producers (black) in 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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The authors declare no conflict of interest.

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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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