Advances in the social evolution and ecology of bacterial public goods

Submitted by

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signature: ……..

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Publications

The following published papers have arisen from this thesis and are presented in chapters 3, 4 and 6:

- S O'Brien, AMM Rodrigues, A Buckling. 2013. The evolution of bacterial mutation rates under simultaneous selection by interspecific and social parasitism. Proceedings of the Royal Society B: Biological Sciences 280 (1773), 20131913

- S O'Brien, D Hodgson, A Buckling. 2014. Social evolution of toxic metal bioremediation in *Pseudomonas aeruginos*a. Proceedings of the Royal Society B: Biological Sciences 281 (1787), 20140858

- S O'Brien, D Hodgson, A Buckling.2013. The interplay between microevolution and community structure in microbial populations. Current opinion in biotechnology 24: 821-825

Chapters 2, 3 and 5 are collaborative efforts, although in each case the majority of work is my own. The motivation for Chapter 2 arose from discussion with Professor Michael Cant, who contributed to interpretation of results and provided comments on the manuscript. In chapter 3, Rodrigues carried out the theoretical component of the study and provided comments on the manuscript. In chapter 5, Dr Adela Luján contributed to experimental design and interpretation of results.

Chapters 4 and 6 were my own work, supervised by Hodgson (who provided comments on both manuscripts) and Buckling. All chapters in this thesis were primarily supervised by Buckling, who, in every case, provided input on experimental design, analysis of results and comments on the manuscript.

Contents

List of figures

Chapter 5

Chapter 6:

Foreword

I do not provide a detailed review of the literature in chapter 1, because I provide reviews of each relevant topic in chapters 2-6.

Abstract

The altruistic production of public goods is one of most popular puzzles in evolutionary biology, and is most commonly explained by the indirect fitness benefit accrued by producers. I develop our understanding of the ecology and evolution of public good production by considering how inter- and intraspecific interactions can affect indirect fitness benefits, and ultimately, the evolutionary trajectory of public good cooperation in a bacterial public good system: 1) I demonstrate the ability of public good cooperators to adapt to the presence of cheats by reducing their own cooperative output, constraining cheat fitness as a consequence. 2) I examine the relative contributions of inter- (bacteriophage) and intraspecific (social cheats) parasites on shaping bacterial mutation rates, and demonstrate that social cheats can gain a fitness advantage in the presence compared with the absence of interspecific parasites. 3) I formally show for the first time, that siderophore-mediated detoxification can be an altruistic trait, rapidly selecting for the evolution of *de novo* cheats, and discuss the implications this process may have for community structure and function. 4) I extend (3) to assess the impact the natural microbial community has on the fitness consequences of siderophore-mediated detoxification in a natural soil environment. 5) I discuss the interplay between rapid microbial evolution and community context, and propose the impacts such interplay may have for biotechnological applications.

1. Introduction

Social evolution theory

Social evolution theory is concerned with the lifetime fitness consequences of interactions between individuals. Interactions can be classified into one of four variants of social behaviour depending on its impact on the reproductive success of the 'actor' and 'recipient' (Hamilton 1964, 1970; West et al. 2006, 2007a) (figure 1.1). These categories are 1) **mutualism**, where both actor and recipient incur a direct benefit, 2) **altruism**, where a cost is incurred to the actor but the recipient benefits, 3) **selfishness/parasitism**, where the actor gains a benefit at the detriment of the recipient and 4) **spite**, where the behaviour is harmful for both actor and recipient. There is plasticity in these social interactions: for example, cleaner fish, who readily eat ectoparasites found on the skin of the fish they service, prefer to eat the mucous and tissue of their clients (Grutter & Bshary 2003). The extent to which the behaviour is mutualistic or parasitic depends on ectoparasite load; increased ectoparasites reduce the benefit accrued to 'selfish' cleaner fish (Cheney & Cote 2005). However, as I will demonstrate in this thesis, mechanisms evolve to constrain the effect selfish individuals can have on the productivity of cooperative genotypes.

While the evolution of behaviours incurring direct fitness benefits require little explanation, more problematic is the explanation for why an individual would act to its own detriment. While spite has been generally neglected because it requires problematic negative relationships between interactors (Hamilton 1970; Foster et al. 2001; Gardner & West 2004a,b), the most general theory that predicts the conditions under which altruistic behaviours will be favoured is inclusive fitness theory. This theory is based on statistical associations between other individuals with the same cooperating 'gene', or as a proxy, close relatives. The generality of inclusive fitness stems from its ability to partition natural selection into direct and indirect effects. This models the effect of the altruistic behaviour on the altruistic gene's reproductive success in both the actor (direct) and recipient (indirect), weighted by the probability that the altruistic gene is shared between social partners (Hamilton 1964; 1970). Simplifying this leads to Hamilton's rule which states that natural selection will favour an altruistic trait when $rb > c$, {where b is the benefit to recipient, c is the

cost to the actor and *r* is the probability that the gene is shared between actor and recipient, i.e. genetic relatedness} (Hamilton 1964). Overall, inclusive fitness theory has proven itself as a general, versatile model for explaining patterns in nature as well as predicting the evolutionary trajectory of social interactions (Trivers 1985; Bourke & Franks 1995; Lehmann & Keller 2006; West et al. 2007b; Bourke 2011; West & Gardner 2013), and so it will underpin the conceptual reasoning used throughout this thesis.

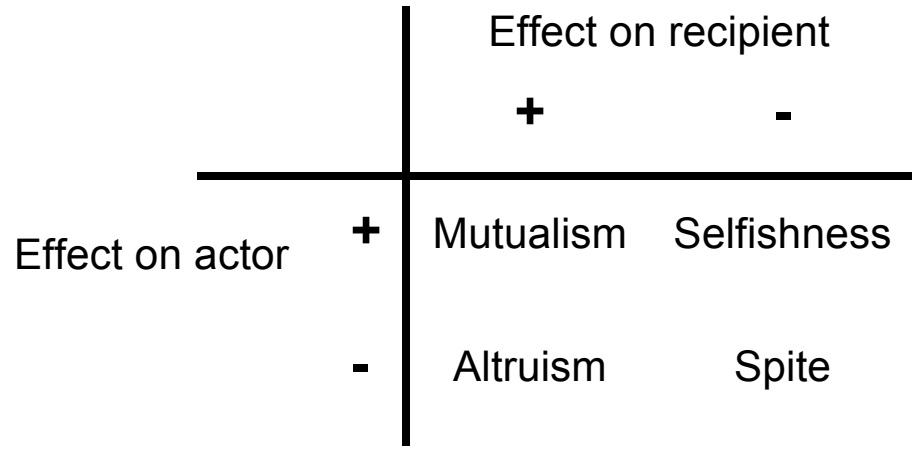

Figure 1: A classification of social behaviours (from West et al. 2006)

Social interactions in microbes

Microbes exhibit a wealth of social interactions, ranging from altruistic production of indole by antibiotic-tolerant bacteria, helping susceptible bacteria resist antibiotics (Lee et al. 2010), spiteful chemical (bacteriocin) warfare (Gardner et al. 2004; Inglis et al. 2009, 2011, 2012) and the evolution of selfish siderophore-negative cheats (Griffin et al. 2004). Recently, there has been an upsurge in research aiming to understand these interactions because i) they are a tractable model system for studying the evolution of social behaviour on ecological timescales (West et al. 2006, 2007c), ii) social behaviours can be important predictors of virulence in bacterial infections (e.g. van Baalen & Sabelis 1995; Frank 1996; Gandon et al. 2002; Day & Burns 2003, Buckling & Brockhurst 2008; Leggett et al. 2013) and iii) developing stable bacterial communities relevant for biotechnology is limited by the effect social

interactions can have in shaping the structure and function of these communities (e.g. Norman et al. 2004). In this thesis I examine the ecology and evolution of bacterial public goods, focusing on the production of siderophores, best known for their ability to chelate unavailable iron. As with any cooperative system, siderophore producers can be invaded by anti-social 'cheats' who pay none or a reduced cost[1] of the behaviour but reap the rewards. Siderophore production can be classified as altruistic or 'weakly' altruistic (West et al. 2006) depending on whether the benefit of production accrues predominantly to the producer or related individuals. One determinant of this is the extent of spatial structure or viscosity; at extreme levels of structure, producers and their siderophores will be kept together, at intermediate structure the benefits of siderophores will accrue to other, closely related individuals, and in the absence of structure, the probability of the beneficiary sharing the same altruistic gene is greatly reduced (Kummerli et al. 2009, 2014). In the latter scenario, altruism is not supported by an individual's ability to maximise their inclusive fitness, and so cooperation is not sustainable. Accordingly, in well-mixed iron-limited environments, *de novo* siderophore cheats evolve rapidly and can invade populations when inoculated in a 1:1 mixture with cooperators (Griffin et al. 2004), a result not observed in spatially structured environments (Luján et al. 2015).

The mechanism of siderophore-chelation

Iron is crucial for bacterial growth, and siderophores enable bacteria to access iron when present in the unavailable ferric (Fe^{3+}) form. Siderophores function by chelating Fe^{3+}, binding to a species-specific cell surface receptor, and transporting the Fe^{3+} into the cell where is reduced to Fe^{2+} and available for cellular metabolism (figure 1.2). While the primary role of siderophores is to access ferric iron, they can in fact bind to a wide array of toxic heavy metals, albeit with less affinity than Fe^{3+}. Many of these metals, which can diffuse into cells, are toxic to bacteria. However, on binding to siderophores the ability of these metals to diffuse into the cell is lost. Moreover, unlike iron-loaded

[1] Labelling an individual as a 'cheat' is context-dependent, relative to the levels of siderophore produced by wildtype, in addition to the extent of iron-limitation (Ghoul et al. 2014). Down-regulating mutants should only be labelled as 'cheats' when they exhibit a fitness cost grown as monoculture relative to wildtype (benefit to recipient), and outcompete wildtype in co-culture (cost of production).

siderophores, these complexes cannot be taken into the cell efficiently, and so, in the presence of many heavy metals, siderophore producers exhibit higher growth than non-producers (Braud et al. 2010) (figure 1.2).

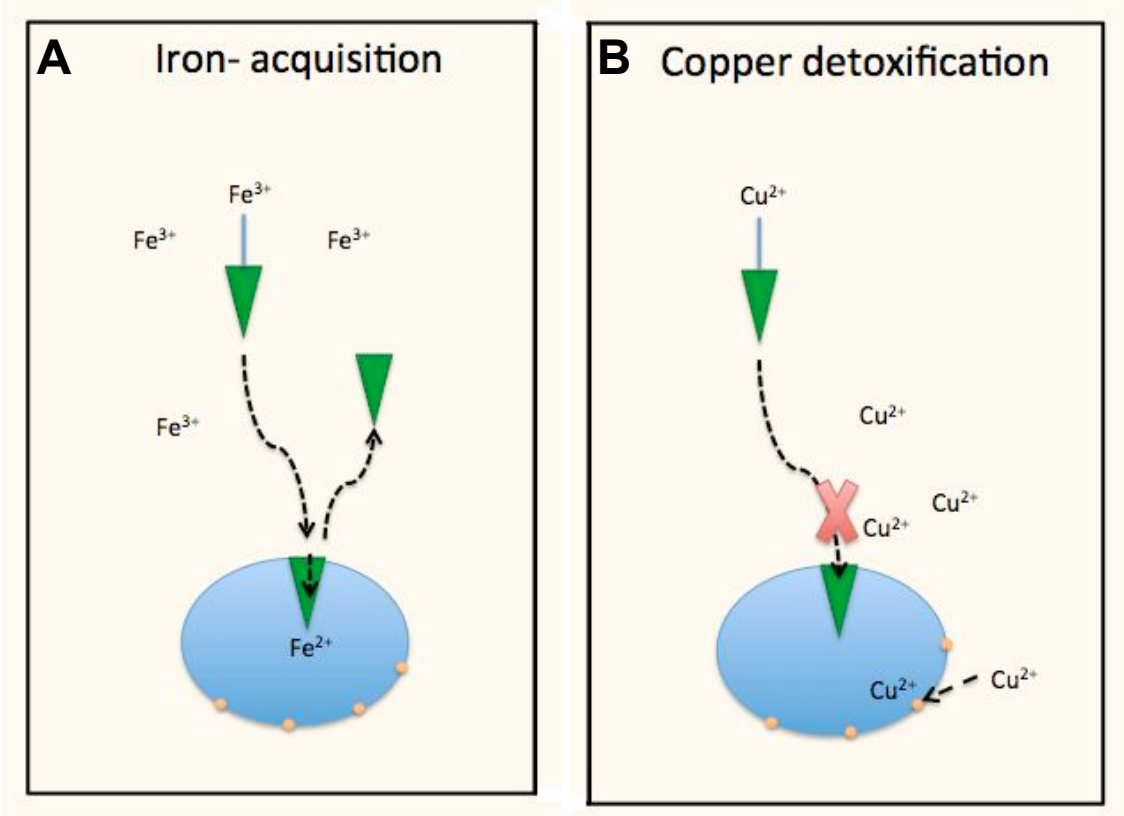

Figure 2: Mechanism of siderophore-mediated iron-acquisition and copper detoxification. Green inverted triangles: siderophores; Blue oval: bacterial cell; Orange circle: cell membrane porin (facilitating diffusion of Cu^{2+} into cell). **A**) Siderophores chelate Fe^{3+} (which cannot diffuse into cells), the complex binds to a siderophore-receptor and is taken into the cell. The siderophore is then recycled back into the environment. **B)** Siderophores bind toxic copper (which would otherwise diffuse into cells), however since the complex is not efficiently transported into the cell, this binding reduces extracellular toxicity.

Advances in the social evolution and ecology of bacterial public goods

In this thesis I will touch on and develop the above themes. Specifically:

Chapter 2:

Populations of siderophore producers are, under certain environmental conditions, vulnerable to invasion by siderophore-negative 'cheats' (Griffin et al.

2004). Such cheats impose a fitness cost on cooperators such that mutations will be favoured that confer resistance to cheating. I investigate whether populations of cooperators, under selection for high productivity, can adapt to the presence of cheats. I show that cooperators reduce their own cooperative output, and upregulate an alternative method of iron-acquisition which cheats also perform, thus reducing the fitness advantage of cheats. More generally, this highlights how cooperators can adapt to the presence of public goods cheats in a system where cooperators and cheats are indistinguishable.

Chapter 3:

I experimentally and theoretically investigate the conflicting impact of intraspecific parasites (social cheats) and interspecific parasites (bacteriophage) on shaping bacterial mutation rate. I also demonstrate that siderophore cheats can have a selective advantage in the presence of parasites; parasite fitness is limited by bacterial growth, and so cooperators cannot benefit from the enhanced productivity their cooperation brings. This work highlights that 1) interspecific interactions may be important in determining whether cooperative interactions matter and 2) the negative impact cheats can have on population fitness is likely to be constantly fluctuating, manipulated by slight changes in the environment.

Chapter 4:

Stemming from work by Braud et al. (2010), I show, for the first time, that siderophore production in the context of detoxification is an altruistic trait, vulnerable to invasion by non–siderophore producing cheats. Furthermore, social interactions in this context are likely to have a much greater impact than those occurring in classical experiments performed in iron-limited media; *de novo* cheats evolve more rapidly and reach higher frequencies than those in iron-limited KB. This work highlights that siderophores are much more pliable than simple iron-chelators, and their ecology and evolution may be subject to more complex selection pressures than assumed in simple iron-limited experiments. Finally, I discuss the potential role of interspecific interactions in shaping the outcome of cooperation in this context.

Chapter 5:

I extended chapter 4 to investigate whether siderophore-based cooperation actually matters in a semi-natural soil microcosm, in the presence and absence of the natural microbial community. I show how the outcome of cooperation is affected by spatial structure in soil, compared with homogenous conditions used in chapter 4. Moreover, I demonstrate the importance of interspecific interactions for the ecology of siderophores. I also make some general predictions about how community-level public good production can be maintained in natural environments, with the ultimate aim of manipulating robust bacterial communities of “super cooperators” with enhanced bioremediation capabilities.

Chapter 6:

The interplay between species interactions and their effect on shaping community context is explored in more general terms. I discuss the relevance of the natural microbial community in determining the fitness and hence evolutionary trajectory of a focal trait, and in turn, discuss the effect of rapid evolution within a species on community composition. I expose the surprising lack of empirical investigation into the impact of the community in shaping the outcome of social interactions and vice versa, and highlight the importance of filling this gap in order for social evolution theory to have robust applications in biotechnology and for the development of novel therapeutics.

2. Cooperative adaptation in the presence of public goods cheats

Abstract

Cooperation in nature is ubiquitous, but is often susceptible to social cheats who pay little or no cost of cooperation but reap the benefits. The effect such cheats have on reducing population productivity suggests that there is selection on cooperators to mitigate the adverse fitness effects of cheats. While mechanisms have been elucidated for scenarios where there is a direct association between the producer and cooperative product, such as kin recognition and policing/punishing, it is unclear whether cooperators can act to suppress cheating in a diffusive public good scenario, whereby attributing a public good to a particular individual is likely to be problematic. Here, we investigate the real time evolutionary response of cooperators to cheats when cooperation is mediated by a diffusible public good; the production of iron-scavenging siderophores in *Pseudomonas aeruginosa*. By evolving cooperators under conditions where there is selection for high productivity in the presence and absence of cheats, we found that cooperators adapted to the presence of cheats by downregulating siderophore production at no obvious cost to population productivity. This reduction in siderophore output and thus iron-availability was compensated for via the upregulation of pyocyanin; an extracellular secondary metabolite and virulence factor with strong redox activity that can promote soluble ferric iron. We find that when evolving in the presence of a high frequency of cheats, cooperators mitigate the negative effect of cheats by finding an alternative route to achieving a similar outcome. Moreover, that the addition of exogenous pyocyanin had a stronger effect in enhancing growth in siderophore-negative cheats compared with cooperators, further supports this interpretation.

1. Introduction

Cooperative behaviour (any action selected at least partly because of its beneficial effect on another individual (West et al. 2007a)) owes its ubiquity to incurring a net fitness benefit to individuals in cooperating groups. However, when cooperation carries a cost it is associated with social cheats who pay little

or no cost of cooperation but reap the rewards. This fitness advantage facilitates their invasion of cooperating groups, which can impose a large cost on the population as a whole, described as a 'Tragedy of the Commons' (Hardin 1988; Velicer et al. 2000; West & Buckling 2003; Harrison & Buckling 2005; Harrison et al. 2006). As a result, a number of mechanisms have evolved that impede the negative impact of cheats. Many organisms, including long-tailed tits (Russell & Hatchwell 2001), bumble bees (Shykoff & Schmid-Hempel 1991) and toads (Waldman & Adler 1979, Waldman 1982), preferentially direct help toward kin, while tactics such as punishing/policing cheats have been well documented, for example, in queenless ants (Monnin et al. 2002), honeybees (Ratnieks & Visscher 1989) and humans (Fehr & Gachter 2000, 2002).

Our understanding of how cooperators adapt to the presence of cheats has been greatly enhanced by studies of microbes, due to their suitability for carrying out real-time evolution experiments as well as identifying the genetic basis of behaviours. While kin recognition *per se* has not been reported for microbes, there are cases of microbial 'green beards' (genes that code a phenotypic effect that enables carriers to recognize one another, permitting the bearer to direct cooperative behaviour to other individuals possessing that phenotypic trait (Hamilton 1964; Dawkins 1976; Keller & Ross 1998)). For instance, in *Saccharomyces cerevisiae,* a green beard gene can inhibit cheat exploitation during flocculation. The green beard in question is the FLO1 gene, and only carriers form strong bonds with one another; non-carriers (cheats) remain outside the floc and although they avoid the cost of flocculation, they are paradoxically more vulnerable to infection (Smukalla et al. 2008). Note that such green beards are not limited to microbes: heterozygous *Bb* fire ant workers are able to isolate and kill homozygous *BB* queens on the basis of a transferable odour cue (Keller & Ross 1998). Furthermore, antagonistic pleiotropy has been reported to contribute to a policing/punishment- style mechanism for constraining the effect of cheating in microbes. For instance, in *Dictyostelium discoideum,* lack of the *dimA* gene results in cells that cheat by avoiding differentiation into non-reproducing prestalk cells, but consequently are excluded from reproducing spores; hence pleiotropy contributes to restricting the spread of cheats (Foster et al. 2004). Similarly, cells of *Vibrio fischeri* that avoid the cost of luciferase production used for bio-illumination by the squid

Euprymna scolopes, also exhibit reduced growth in the squid light organ (Visick et al. 2000). Finally, several studies have demonstrated real-time evolution of cheat resistance in cooperator populations, e.g. *Pseudomonas fluorescens* biofilm coevolution between cheats and cooperators, whereby cooperators become better at resisting cheats who are themselves becoming more efficient invaders (Zhang et al. 2009), and the evolution of cooperating mutants that oppose cheat productivity when they are under-represented in dead end stalk cells in *Myxococcus xanthus* (Manhes & Velicer 2011), and *Dictyostelium discoideum* (Khare et al. 2009), although the mechanisms of cheater resistance in these cases is unclear.

While the above examples relate to cheat-resistance when cooperation involves direct physical association between producer and the trait itself, it is less clear if and how adaptation may occur in a diffusive public good scenario, where there is usually no clear physical link between producer and product. Hence, directly targeting cheats for punishment/policing, or directing benefits of cooperation toward kin is problematic. Moreover, simple point mutations can lead to the rapid production of cheats, making opportunities for pleiotropy unlikely (Stern 2000). Here, we investigate the real time evolutionary response of cooperators to cheats when cooperation is mediated by a diffusible public good that is individually costly and carries a group-level benefit: the production of iron-scavenging siderophores (namely pyoverdine and pyochelin), by *Pseudomonas aeruginosa*. Cheats evolve rapidly in this system, avoiding the cost of siderophore production while still availing of increased iron (Harrison & Buckling 2005; O'Brien et al. 2013) and are commonly encountered in natural populations (de Vos et al. 2001; Smith et al. 2006; Jiricny et al. 2014). By evolving cooperators under conditions where there is selection for high productivity in the presence and absence of cheats, we find that cooperators adapt to the presence of cheats by downregulating siderophore production at no obvious cost to population productivity. This reduction in siderophore and thus iron-availability was compensated for via the upregulation of a secondary metabolite and virulence factor with iron-reducing properties, pyocyanin.

2. Materials and methods

2.1 Strains and growth media

We used one the following 3 variants of *Pseudomonas aeruginosa* strains as the siderophore producing wildtype 1) PAO1 (Stover et al. 2000) 2) gentamicin-resistant PAO1 (PAO1^{R}) or 3) gentamicin-resistant PAO1 with a *lacZ* reporter gene insertion (PAO1 ^{R}*lacZ).* These strains were generated by integrating a gentamicin resistance cassete (Tn7-gm) and a *lacZ* gene (with a gentamicin resistance cassette; Tn7-gm-*lacZ*), respectively at the *att*::Tn7 locus in *P. aeruginosa* PAO1 using the methods of Choi and Schweizer (2006). PAO1*ΔPvdDΔPchEF* is a gentamicin-susceptible isogenic mutant strain of PAO1 with genes encoding both primary and secondary siderophores, pyoverdine and pyochelin knocked out (Ghysels et al. 2004), which we used as an obligate siderophore cheat. All of our experiments were carried out in Kings Medium B (KB) (King et al. 1954): (10 g glycerol, 20 g proteose peptone no. 3, 1.5 g $K_2HPO_4.3H_2O$, 1.5 g $MgSO_4.7H_2O$ per litre). Where stated, KB medium was made iron-limited by the addition of freshly made filter-sterilised 100μg/ml human apotransferrin and 20mM $NaHCO_3$ to KB medium immediately before use. Since siderophore production is repressed when there is an excess of Fe^{2+} (Leoni et al. 2000, Ratledge & Dover 2000), iron-limitation ensures that siderophores are essential for growth and stimulates their production. Gentamicin was used at a concentration of 30 μg/ml and 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was added at a concentration of 90 μg/ml. Cultures were incubated at 37°C for 24h, shaken at 180rpm unless stated otherwise.

2.2 Costs and benefits of siderophore production

We firstly wished to confirm that siderophore production is an altruistic behaviour in iron-limited KB medium (individually costly yet carries a group-level benefit). We conducted an ecological experiment co-culturing PAO1 (cooperator) and PAO1*ΔPvdDΔPchEF* (siderophore-negative cheat) in monoculture and in a 1:1 co-culture, quantifying population growth after 24 hours. Eighteen starting cultures were propagated in 30ml universal containers with loose lids (microcosms), each containing 6ml iron-limited KB medium. Microcosms were divided into 3 treatments (0%, 50% and 100% cheats), with 6

replicate populations for each treatment, and proportions of bacteria inoculated so that final density of bacteria added to each microcosm was ~ 10^6 CFU's (colony forming units). Densities were assessed by diluting and plating the contents of each microcosm on KB hard agar (prepared by the addition of 12g agar to KB broth before sterilising) and incubating for 24h, after which individual CFU's could be counted. Colony types were categorised by colour into either siderophore producers (yellow/green) or non-producers (white) based on the fact that the primary siderophore of *P. aeruginosa*, pyoverdine, is yellow/green (see Harrison & Buckling 2005). To verify this distinction we grew monocultures of siderophore producer and non-producer static in 200μl iron-limited KB medium (siderophore-stimulating conditions), with 6 replicates for each strain. After 24 hours, pyoverdine production (RFU) for each strain was quantified using a pyoverdine-specific excitation-emission assay (Ankenbauer et al. 1985; Cox & Adams 1985; Prince et al. 1993), measuring fluorescence of each culture at 460nm, following excitation at 400nm, using a Biotek Synergy 2 spectrophotometer. Optical density (OD) was also measured at 600nm, and the ratio RFU/OD was employed as a quantitative measure of per capita pyoverdine production (Jiricny et al. 2010; Harrison 2013). White colonies produced pyoverdine at significantly lower levels than yellow/green cooperators (Welch t-test, $t_{5.211}$=34.5358, p<0.0001), and so, colony colour could be used as a proxy for distinguishing pyoverdine cheats from cooperators.

2.3 Evolution Experiment

We evolved cooperators (PAO1^R) for ~ 200 generations, in the presence and absence of a high frequency (90%) of siderophore-negative cheats (PAO1*ΔPvdDΔPchEF*), under conditions where there was selection for high productivity in cooperator populations (i.e. global competition and high cooperator relatedness). PAO1^R was cultured in 6ml KB medium, after which it was diluted and cultured on KB hard agar. A single colony was then selected and cultured once more in 6ml KB medium. This single-strain culture was used to establish populations.

Seventy-two microcosms containing 6ml iron-limited KB medium were divided into 36 treatment microcosms (1:9 mixture of cooperator and cheat), and 36 control microcosms (100% cooperator). We set up 6 replicate populations for each

treatment and control group, with each population comprising 6 ‘patches’. Each patch was inoculated with either ~10^6 CFU’s cooperator (control), or in a 1:9 mixture of cooperator and cheat, so that the final density was also ~10^6 CFU’s. After 24h of growth, 100μl was removed from each patch within a population and combined, resulting in a 600μl mixture for each population. This design selected for patches with high productivity, since genotypes from the most productive patches were overrepresented in the mixture, and any phenotype that reduced patch productivity was selected against.

Each mixture was diluted and grown on KB hard agar supplemented with 30μg/ml gentamicin at 37°C for 24h. The use of gentamicin eliminated cheats from our treatment group at this stage, minimising any effect of coevolution between cheats and cooperators on siderophore production. Six colonies from each agar plate were randomly selected, grown independently static in 180μl KB medium and subsequently used to inoculate a new patch with ~10^6 CFU’s. This ensured high cooperator relatedness within each patch and hence the potential for selection for cooperation was continually re-established. In treatment populations, these cooperator colonies were inoculated with the ancestral cheat in a 1:9 ratio as before. A total of 18 transfers were carried out (~200 generations), but cultures were allowed to grow for 4 days (rather than 24h) between transfers for the last 4 transfers to allow beneficial mutations more opportunities to increase in frequency, and thus reach detectable levels.

2.4 Assays

After ~200 generations, each population (consisting of 6 patches each) was diluted and cultured on KB hard agar to i) note any morphotypic variation among colonies ii) assess per capita siderophore production using both a pyoverdine-specific excitation-emission assay (Ankenbauer et al. 1985; Cox & Adams 1985; Prince et al. 1993) and chrome azurol S assay (CAS) (Schwyn & Neilands 1987) for total siderophore production and iii) determine levels of pyocyanin, an alternative iron-acquisition agent. Since some siderophores are more costly than others, we began by specifically measuring the most costly and efficient chelator, pyoverdine. Thirty colonies from each population were randomly selected, grown independently static in 200μl iron-limited KB medium (siderophore-stimulating conditions) and analysed for pyoverdine production

(RFU) as described in section 2.2. Additionally, we performed a chrome azurol S (CAS) assay on the same cultures to assess levels of total siderophore production (not solely pyoverdine). The CAS assay method was taken from Schwyn & Neilands (1987), with the modification that one volume of double distilled H_2O was added to the CAS solution prior to use (see Harrison & Buckling 2005; Harrison et al. 2008). 50μl from each of 30 single colony cultures prepared perviously per population was combined, centrifuged to pellet cells and a 50% CAS assay performed on the supernatant, measuring A_{630} of cultures and cell-free supernatant (reference culture). A measure of iron chelator activity relative to the reference culture in each population was given by $[1-(A_{pop}/A_{ref})]$ (Harrison & Buckling 2005; Harrison et al. 2008), standardised by the optical density (OD_{600}) of the relevant culture. Ancestral wildtype, cheat and uninoculated KB broth were included in all of our assays as controls. Finally, pyocyanin production was measured in each of our evolved populations by measuring A_{691} of each population, and then standardised by OD_{600} (Reszka et al. 2004).

2.5 Quantifying fitness of evolved populations: competition experiments

Cheats were eliminated from each population prior to competition experiments by growing cultures on 30μg/ml gentamicin- supplemented KB agar. A sample was taken of the resulting cheat-free colony growth with a sterile 200μl pipette tip, inoculated in 6ml KB medium, and grown for 24h. Despite the control populations being already cheat-free, this process was carried out on all populations to control for any plating effects. Ancestral and cheat cultures were also inoculated in 6ml KB medium and grown for 24h.

We investigated whether evolved populations had a competitive advantage over their ancestor, and whether this could be affected by the presence/absence of a high frequency of cheats. We established 2 groups of 72 microcosms (6 replicates per evolved population, in the presence and absence of cheats), with each microcosm containing 6ml iron-limited KB media. The first group of microcosms were inoculated with ~$5x10^5$ CFU's ancestral $PAO1^R$*lacZ* (gentamicin-resistant and *lacZ* insertion strain), ~$5x10^5$ CFU's of the appropriate evolved population and ~10^6 CFU's of PAO1*ΔPvdDΔPchEF* (siderophore-negative cheat), so that cheat strains represented a high percentage (~90%) of

the total bacterial density in each microcosm. The second group of microcosms (cheat-free competitions) were inoculated with 10^6 CFU's ancestral PAO1^R*lacZ* and ~10^6 CFU's of the appropriate evolved population. Microcosms were incubated for 24h after which populations were diluted, plated on KB agar supplemented with 30μg/ml gentamicin and 90μg/ml Xgal, and colonies were counted as before. Gentamicin enabled the removal of cheats at the counting stage, which otherwise would have dominated the plates and resulted in very low cooperator counts. Evolved and ancestral cooperator strains were distinguished by a dark blue appearance of the ancestral strain on Xgal-supplemented agar. Finally, to confirm the neutrality of the *lacZ* insertion in the ancestral strain, it was competed in a 1:1 co-culture against the original ancestor (both strains were gentamicin-resistant).

2.6 Addition of exogenous pyocyanin

In order to ascertain whether exogenous pyocyanin could 'rescue' siderophore cheats under iron-limited conditions, we established 48 microcosms, each containing 6ml iron-limited KB. Twenty-four of these microcosms were inoculated with ~10^6 CFU's wildtype PAO1 (cooperator), and the remainder with ~10^6 CFU's siderophore negative cheat PAO1*ΔPvdDΔPchEF.* The 24 microcosms per strain were subdivided into 4 groups of 6 microcosms to allow 4 treatments per strain: control (no pyocyanin), 10μM pyocyanin, 30μM pyocyanin and 50μM pyocyanin. Pyocyanin (purified from *P. aeruginosa*) was purchased as a 5mg/ml ready-made solution in DMSO from Sigma-Aldrich. Following the addition of pyocyanin, microcosms were incubated, and after 24h diluted in M9 minimal salt solution, plated on KB agar and CFU's counted to assess density, as before.

2.7 Evolved population growth rate in monoculture under static and shaken conditions

We wished to determine whether the impact on fitness of producing predominantly siderophore or pyocyanin to access iron is dependent on the degree of environmental spatial structure, as has been previously observed for siderophores (Kummerli et al. 2009). Twelve microcosms were established containing 6ml iron-limited KB medium, and another 12 containing 6ml iron-limited soft KB agar, created by the addition of 1% agar to standard broth before

sterilising, and adding 100μg/ml human apotransferrin and 20mM sodium hydrogen carbonate ($NaHCO_3$) prior to cooling. 10^7 CFU's of each evolved line were propagated in one microcosm containing KB medium and another containing KB soft agar, and tubes were incubated shaken (liquid medium) and static (soft agar). After 24h sterile glass beads were added to each tube and microcosms were vortexed for 20s to allow the agar to break up and the contents to homogenize. Microcosms were then diluted in M9 minimal salt solution, plated on KB hard agar and CFU's counted to assess cell density.

2.8 Statistical analyses

All data were analysed using R version 2.15.1 (R Core Team, 2012). We determined population Malthusian growth rate (*m*) as ln(final density/start density) (Lenski et al. 1991). Relative fitness of strain *x* compared with strain *y* (W(*x*)) was calculated in co-culture as *m*(strain *x*)/*m*(strain *y*), and in monoculture as *m*(strain *x*)/mean(*m*(strain *y*)). When W(*x*) = 1, fitness of strain *x* = strain *y.* Following an F-test to compare variances, and a Shapiro-Wilk normality test, we used Student's t –tests and Wilcoxon rank-sum tests to compare *m* values in monoculture, or *W* values between treatments. To assess whether (W(*x*)) was significantly different from 1, we used 1-sample t-tests and Wilcoxon signed-rank tests.

1 and 2-sample t-tests were used to compare per capita total siderophore production between evolved populations and ancestor (using mean ancestral siderophore production as the alternative value in 1-sample tests), and a Kolmogorov-Smirnov test for non-parametric data with unequal variances was employed to compare the frequency of a novel morphotype in control and treatment populations. A 1-sample t-test, and Wilcoxin signed-rank test enabled the comparison of pyoverdine levels in evolved control and treatment populations with ancestral pyoverdine production, respectively. To compare per capita pyoverdine between wildtype and control populations, a linear mixed effects revised (LMER) model using maximum likelihood was performed, assigning condition (treatment/control) as a fixed factor and population as a random factor, controlling for the presence of 30 datapoints for each of 12 populations.

To determine fitness of evolved populations relative to ancestor in the presence/absence of cheats, an LMER (using maximum likelihood) was employed to account for non-independent datapoints (6 replicates per population), assigning 'population' as a random factor and both condition (treatment/control) and cheats (present/absent) as fixed explanatory factors (including interaction).

A general linear model (GLM) was used to investigate whether pyocyanin production is affected by evolution condition (control/treatment lines) and frequency of a novel morphotype present in each population (including interaction). To investigate any relationship between pyocyanin and pyoverdine investment we pooled data from both growth conditions, and analysed using an LMER, assigning condition (control/treatment population) as a random factor, pyoverdine (including linear and squared terms) as a fixed explanatory numeric variable and the natural log of pyocyanin as a numeric response variable:

Finally, the effect of exogenous pyocyanin on growth rate was calculated as the change in growth relative to the control at each pyocyanin concentration using selection coefficient (r): *m*(strain *x*) - mean(*m*(strain *y*)). Using a GLM, the effect of pyocyanin concentration (continuous numeric variable) and strain identity (cooperator or cheat) on promoting growth was investigated (including the interaction). To ensure the effect of adding 10μM pyocyanin was accounted for, the control treatment relative selection coefficient was included and standardized to zero.

3. Results

3.1 Costs and benefits of siderophore production

Cooperator populations exhibited a higher growth rate compared with cheats when grown in isolation (Wilcoxon rank-sum test: W=34, $p<0.05$), however this effect was reversed in 1:1 co-culture, whereby cheats had a growth rate advantage over cooperators (1-sample t-test of relative fitness against 1: $t_5=2.743$, $p<0.05$, figure 1). Thus, while siderophore production carries a group-level fitness benefit, is individually costly in this context (consistent with Griffin et al. 2004).

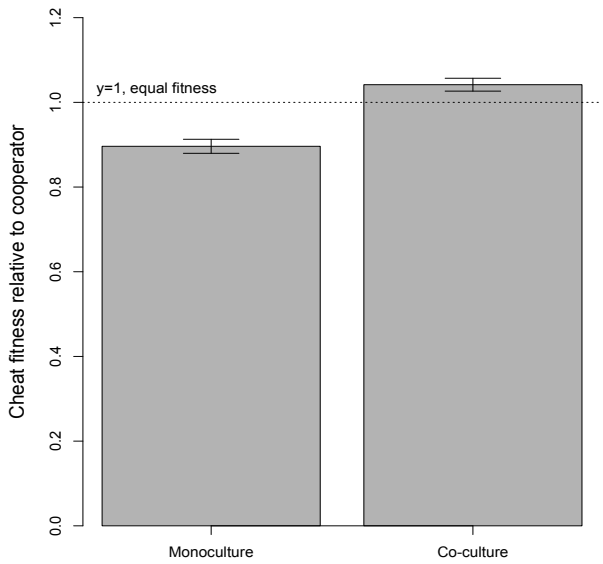

Figure 1: Relative fitness (*W*) of cheat in monoculture and co-culture with cooperator. In monoculture, *W*(cheat) < 1 (Wilcoxon signed rank test: *V*=0, *p*<0.05), whereas in co-culture *W*(cheat) > 1 (1-sample t-test: t_5=2.743, *p*<0.05). Each *W*(cheat) datapoint in monoculture is calculated as *m*(cheat)/mean(*m*(cooperators)), and in co-culture as *m*(cheat)/(*m*(cooperator). Data are means (n=6) ± SEM

3.2 Siderophore production following evolution

Following ~ 200 generations of growth, populations grown in the presence of cheats exhibited reduced per capita total siderophore production compared with ancestor (1-sample t-test (alt=0.70672), t_5=3.4056, *p*<0.05, figure 2A) and cheat-free control populations (Student's t-test, t_{10}=2.7662, *p*<0.05, figure 2A). Testing for pyoverdine specifically, treatment populations showed decreased pyoverdine output compared with ancestor (1-sided Wilcoxon signed-rank test, alternative = 7876.512, *V*=4, *p*<0.0001, figure 2B) and control populations (LMER: $X^2_{1,3}$=9.7914, *p* <0.01, figure 2B). Control populations did not differ from ancestral total per capita siderophore production (1-sample t-test (alt=0.70672) t_5*=0.6999*, *p* = 0.51, figure 2A), but per capita pyoverdine output was reduced over the course of the experiment (1-sample t-test, alternative = 7876.512, t_{179}=18.6277, *p*<0.0001, figure 2B). We also recorded the appearance of a novel morphotype with a slightly raised surface and reduced surface area in evolved populations. After ~200 generations, not only was the frequency of this

morphotype significantly higher in our treatment than control populations (Kolmogorov- Smirnov test, D=0.8333, p<0.05, figure 3), it went to fixation in 3 out of 6 treatment populations.

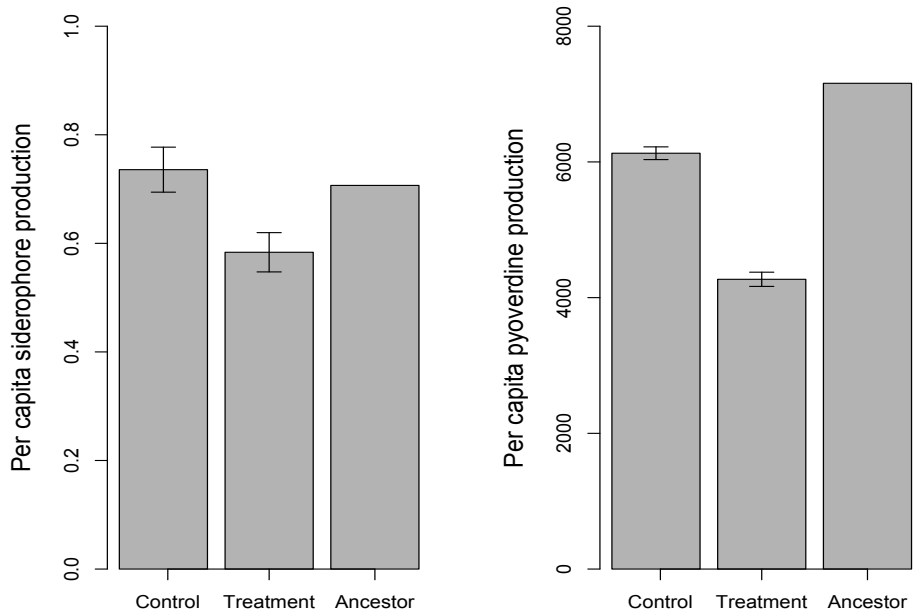

Figure 2: Siderophore **(A)** and pyoverdine **(B)** production by control (- cheats), treatment populations (+ cheats) and ancestor. After ~200 generations, treatment populations exhibited reduced per capita total siderophore production (1-sample t-test (alt=0.70672), t_5=3.4056, p <0.05) and pyoverdine production (1-sided Wilcoxin signed-rank test, alternative = 7876.512, V=4, p<0.0001), relative to the ancestor. Data are means (n=6) ± SEM.

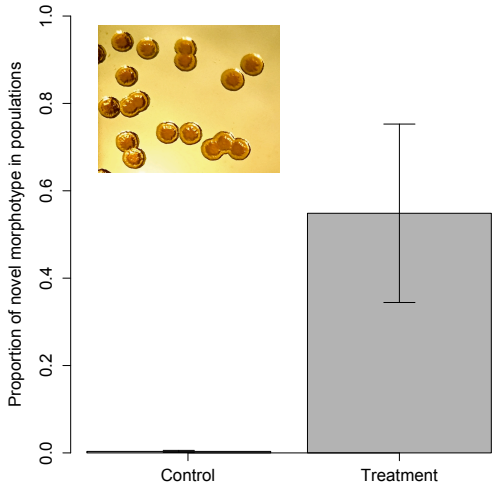

Figure 3: After 200 generations of growth, the proportion of a novel morphotype in our treatment and control populations (Kolmogorov-Smirnov test, D=0.8333, p<0.05). Data are means (n=6) ± SEM. *Inset*: image of novel morphotype with raised surface and reduced surface area.

3.3 Quantifying fitness of evolved populations

To determine adaptation to cheats, we competed evolved lines against the ancestor in the presence and absence of 90% cheats: conditions mimicking the evolutionary conditions. We found an interaction between the presence or absence of cheats, and evolution condition (control/treatment), so that treatment populations were better at preventing the invasion of the ancestor only when cheats were present at high frequencies (LMER: $X^2_{1,5}$=24.044, p<0.0001, figure 4).

Given that our experimental design also selected for high within-population yields, we measured the mean growth rate of evolved populations as monocultures under the selective (iron-limited) conditions, finding no difference in mean growth rate between control and treatment lines (Student's t-test, t_{10}= 0.0268, p=0.98, figure 5). Finally, we verified that the use of an ancestor possessing a *lacZ* genetic marker in this experiment did not alter relative fitness: in direct 1:1 competition with non-*lacZ* ancestor, W(*lacZ*) did not differ from 1 (1-sample t-test (alt=1), t_5=-1.5659, p= *0.18*).

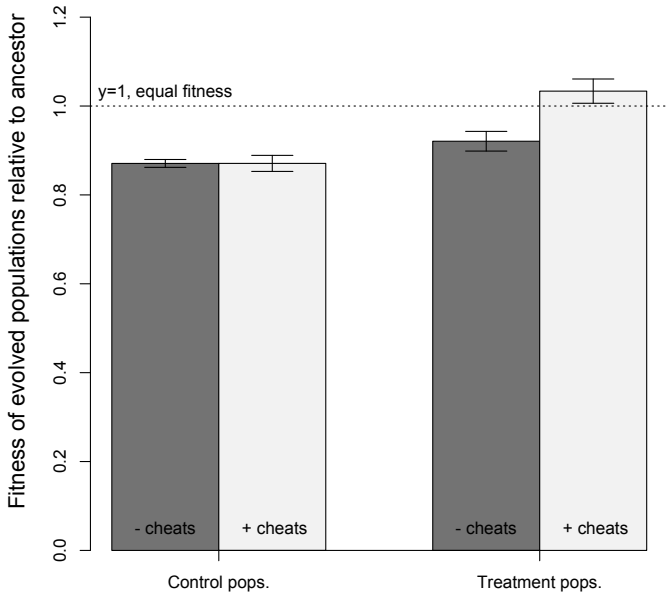

Figure 4: Relative fitness of evolved control and treatment populations in a 50/50 co-culture with ancestral wildtype, in both the presence and absence of a high frequency of cheats. We found a significant interaction between the presence or absence of cheats, and evolution treatment (control/treatment lines), so that treatment lines were only better at preventing the invasion of the ancestral wildtype when cheats were present at high frequencies (LMER: $X^2_{1,5}$=24.044, p<0.0001). Data are means (n=6) ± SEM.

3.4 Growth in spatially structured environments

We speculated that reduced siderophore production of evolved populations may be disadvantageous in spatially structured environments, where the persistence of siderophores may be crucial. Indeed, in static iron-limited KB soft agar, populations which had evolved in the presence of cheats manifested lower growth rates compared with non-cheat adapted populations (Student's t-test, t_{10}= 3.2821, p<0.01, figure 5).

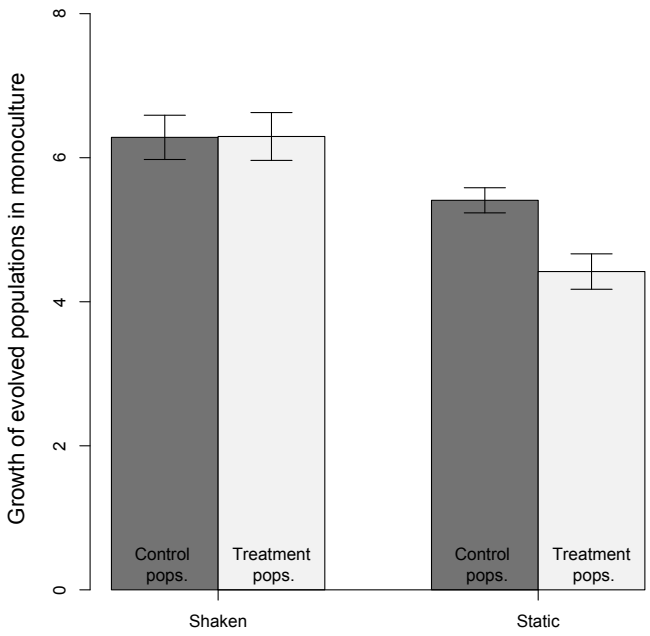

Figure 5: Evolved populations grown as monoculture in shaken iron-limited KB broth and static iron-limited KB soft agar. Population growth (*m*) calculated as ln(final density/start density). In shaken treatments, there is no difference in population growth between control and treatment lines (Student's t-test: t_{10}= 0.0268, p=0.98). However, in static conditions control lines exhibited a marked fitness advantage compared with treatment lines (Student's t-test, t_{10}= 3.2821, p<0.01). Data are means (n=6) ± SEM.

3.5 Pyocyanin and novel morphotypes

Our competition experiments exposed colour differences among our populations, ranging from yellow/green to blue/green, which we found to be instigated by increased pyocyanin levels in some populations. Although treatment populations showed elevated pyocyanin levels compared with control populations, variation between populations meant that this increase was insignificant (GLM, $F_{1,9}$=0.2445, p=0.63). Crucially, there was a generally negative relationship between pyocyanin and pyoverdine; however, this took the form of a positive parabolic function so that pyocyanin levels decreased with increasing pyoverdine levels, slightly increasing once more as pyoverdine values became extremely high (LMER, $X^2_{1,4}$= 9.6716, p<0.01, figure 6).

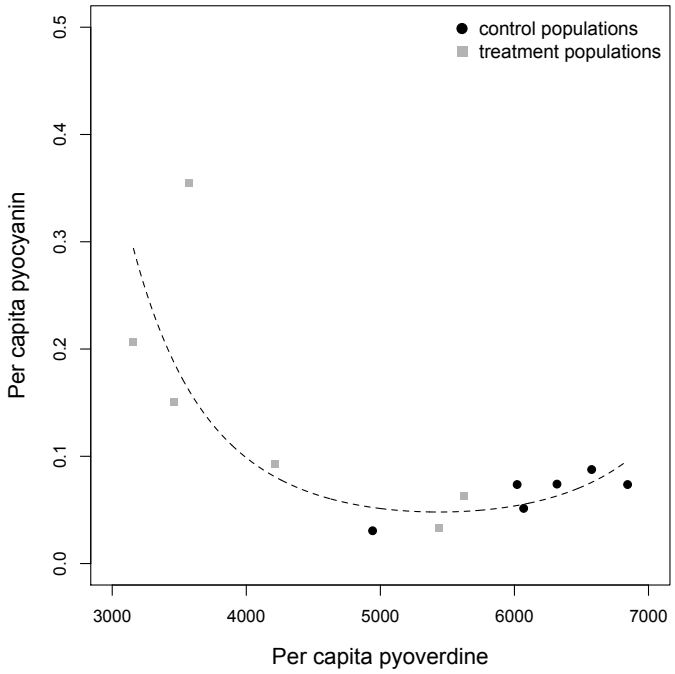

Figure 6: Elevated levels of pyocyanin is associated with downregulated pyoverdine production (LMER, $X^2_{1,4}$=9.6716, p<0.01), n=12.

3.6 Addition of exogenous pyocyanin

The addition of exogenous pyocyanin was beneficial for both siderophore-producing and non-producing strains; however, the effect of increasing pyocyanin production on growth rate was greatest for non-producing strains (GLM, strain identity x pyocyanin concentration interaction, $F_{1, 44}$=12.018, p=0.001, figure 7).

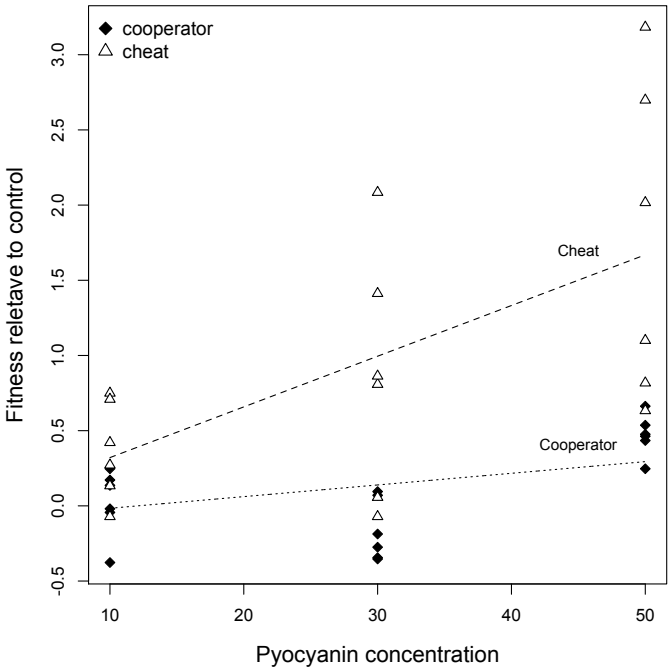

Figure 7: The effect increasing pyocyanin has on fitness (r) is greatest in cheat populations (GLM, strain identity x pyocyanin concentration, $F_{1,44}$ =12.018, p=0.001). Fitness (r) relative to control population (no pyocyanin added) was calculated as *m*(strain *x*) – mean(*m*(strain *y*)). Lines are plotted based on predictions from minimal GLM model.

4. Discussion

Here, we investigated how populations of the bacterium *P. aeruginosa* selected for high productivity adapted to the presence of public good cheats, where public goods in this case are the primary and secondary iron-scavenging siderophores pyoverdine and pyochelin. We found that after ~200 generations cheat-adapted populations manifested increased fitness in the presence of cheats, relative to control populations, while displaying no apparent growth rate cost as monocultures. Cheat-adapted populations evolved greatly reduced siderophore production, but with a concomitant increase in pyocyanin: an extracellular secondary metabolite with strong redox activity that can promote soluble ferric iron. These results suggest that public good producers compensate for the presence of cheats by finding an alternative way to obtain iron, i.e. through the upregulation of iron-reducing pyocyanin. That the addition of exogenous pyocyanin had a stronger effect in enhancing growth of

siderophore-negative cheats compared with cooperators, further supports this interpretation.

Unlike siderophores, pyocyanin is not an iron-chelator, but a reducing agent that converts ferric (Fe^{3+}) to ferrous iron (Fe^{2+}) (Cox 1986). The resulting ferrous ions can diffuse into cells via cell-surface porins (eliminating the requirement of a siderophore-specific receptor), while ferric ions are insoluble. The importance of pyocyanin as an alternative mechanism to obtain iron is apparent from recent studies investigating adaptation of *P. aeruginosa* to toxic levels of the antimicrobial gallium nitrate (García-Contreras et al. 2014; Ross-Gillespie et al. 2014). Pyoverdine binds to Fe^{3+} and Ga^{3+} and these complexes enter the periplasm via the FpvA transporter. However, since Ga^{3+} cannot be reduced it remains bound to pyoverdine in the periplasmic space, where it is toxic to cells. Resistant mutants downregulate pyoverdine to reduce the rate at which toxic gallium enters the cell, having the knock-on effect of reducing pyoverdine-mediated iron uptake. Cells compensate for this by producing pyocyanin at higher concentrations, consequently increasing access to ferrous iron without exposing themselves to gallium toxicity (García-Contreras et al. 2014; Ross-Gillespie et al. 2014).

Our data suggest that, like siderophores, pyocyanin may also act as a public good. Specifically, we show that the growth rate of siderophore cheats in iron-limited media can be rescued to some extent by the addition of exogenous pyocyanin. Moreover, studies in mouse models demonstrate reduced growth and virulence of pyocyanin negative mutants compared with wildtype (Mahajan-Miklos et al. 1999; Cao et al. 2001; Lau et al. 2004a), but that mutant growth is enhanced by the presence of wildtype producers in mixed infections, or the addition of exogenous pyocyanin (Lau et al. 2004a). Note that in this case, pyocyanin is probably not linked to iron scavenging, as non-producers had intact siderophores, and pyocyanin has a range of additional *in vivo* activities such as apoptosis of neutrophils that could enhance growth. This then begs the question: why weren't pyocyanin overproducers in our evolved cheat-adapted populations exploited by individuals making less pyocyanin? The most likely explanation is that pyocyanin over-production imposes a small metabolic cost (relative to siderophores), at least under these experimental conditions; hence

pyocyanin non-producers would have little, if any, fitness advantage. However, our results do not rule out the possibility that pyocyanin producers could ultimately be exploited by the evolution of pyocyanin cheats in this or other environments.

It could be reasoned that if pyocyanin production facilitates iron acquisition with a reduced cost, then why produce diffusible siderophores at all? Our growth rate experiment in a viscous environment suggests that the increased ability of siderophores to persist and 'scout' for iron makes them necessary in increasingly static, viscous environments such as sputum and soil: populations of primarily pyoverdine producers (control populations) outcompeted populations of primarily pyocyanin producers (treatment populations) in static but not shaken environments. Differences in oxygen availability (and consequently the predominant form of iron) between these conditions fail to explain our results, as iron would be in the oxidized, unavailable Fe^{3+} form in shaken tubes and the opposite outcome would be predicted. Consistent with this interpretation, a study of the diffusive properties of 189 iron-scavenging siderophores from environments that vary in their degree of structure, demonstrated that while highly diffusible far-reaching siderophores are maintained in spatially structured and viscous environments, local-acting siderophores are favoured in unstructured, liquid environments, because they are less likely to diffuse unused into the environment while also increasing direct and indirect fitness benefits for the producer (Kummerli et al. 2014).

Competing ancestral against evolved cooperator populations in the presence and absence of cheats demonstrated that evolved control populations were consistently outcompeted by the ancestor, while evolved treatment populations managed to negate this only in the presence of cheats. The inherent disadvantage of evolved populations could not be attributed to differences in growth between $PAO1^{R}$LacZ and $PAO1^{R}$, and was potentially a consequence of population bottlenecking resulting from transfer of single clones, which could have resulted in the fixation of deleterious mutations.

It is always debatable whether *in vitro* results are relevant to the real world. While siderophore cheats are present in natural pops (De Vos et al. 2001;

Cordero et al. 2012) and can have a selective advantage when rare (Luján et al. 2015), it is unclear if selection has acted in ways that mitigate their exploitation, as observed here. It has been speculated that diversity in the pyoverdine locus and Fpv1 receptor is driven by the presence of a high frequency of cheats, who are unable to bind novel pyoverdine structures (Smith et al. 2005; Lee et al. 2012). However, it is not clear whether cheats drive this pyoverdine diversification *per se*, or if this diversity evolved for other reasons and the presence of cheats help to conserve this diversity. Indeed, it is notable that generalist receptors exist that can recognise multiple siderophore types, suggesting that unique siderophore types are not likely to be a particularly effective strategy against exploiting non-producers (Barelmann et al. 2002; Ghysels et al. 2004). Alternatively, our finding that pyoverdine production evolved to lower levels in populations grown with and without cheats, and that populations that compensated by increasing pyoverdine exhibited no difference in fitness compared with non-compensating populations (in the absence of cheats), suggests that pyoverdine is produced at higher concentrations than is required to sustain growth. While such robustness to cheating may be an effective temporary strategy, an alternative explanation is that overproduction is a result of the diffusion of pyoverdine away from producing organisms. Finally, in contexts where siderophore non-producers are at high frequency, such as in chronic infections (De Vos et al. 2001) strong selection may be imposed to obtain iron in different ways, such as via pyocyanin as observed here. Indeed, pyocyanin over-producing genotypes are associated with exacerbated CF infections (Fothergill et al. 2007; Mowat et al. 2011), although these correlational data can be open to different interpretations, and not all exacerbations exhibited high numbers of pyocyanin overproducing isolates. Moreover, the viscous environment commonly encountered in CF lung sputum suggests that pyocyanin-mediated iron acquisition is likely to be inefficient in this context. However, any factors promoting escalation in pyocyanin levels warrants more investigation since pyocyanin directly harms host cells, kills competitors, and results in more virulent infections (Baron & Rowe 1981; Sorensen & Klinger 1987; Lau et al.2004a, b; Norman et al. 2004; Mowat et al. 2011; Mc Dermott et al. 2012). While these studies provide anecdotal evidence that mechanisms are in place to mitigate the cost of siderophore cheats, the necessity and route of such adaptation is likely to vary depending on spatial

structure, environmental viscosity, cheat frequency, relatedness and the extent of iron-limitation.

More generally, little is known about mechanisms in place to mitigate the effect of such 'secret cheats', whereby it is difficult or impossible to identify and hence punish cheating individuals. In *P. aeruginosa* the expression of a diffusible surfactant rhamnolipid only when the cost of doing so is minimal, has been interpreted as a mechanism to cope with a high cheat load (Xavier et al. 2011), effectively creating a cost-free public good. Similarly, in cooperatively breeding vertebrates, helping can be accentuated by supplementary feeding (Russell et al. 2007; Clutton-Brock et al. 1999). In human societies, the infiltration of secret cheats may be mitigated by evoking feelings of guilt, shame or immorality in cheats, or alternatively, by providing a direct or indirect benefit to cooperating individuals (Hamilton 1964; Wang et al. 2012; Hodgson 2014). For example, in developing countries, $285 billion is lost per year due to tax evasion in the domestic shadow economy or the 'black market' (Cobham 2005). While tax compliance is generally enforced via punishment of tax debtors either directly or indirectly, in a shadow economy this proves to be a far more difficult route. Governments in Zimbabwe, Gambia and Uganda, to name a few, have adopted instead a strategy of publicly recognising the highest taxpayers on 'Taxpayers Appreciation Day', hence, individuals gain a social benefit from paying tax. This ability to associate cooperators with an added fitness benefits, which excludes cheats, is comparable to increased resistance to infection experienced by individuals who pay the cost of flocculation in *Saccharomyces cerevisiae* (Smukalla et al. 2008). However, in these cases, the ability to directly identify cooperators (but not necessarily cheats) is essential. Here, we report the evolved adaptive response of cooperators to the presence of a high frequency of cheats, in perhaps a more rare cooperative scenario whereby neither cooperators nor cheats can be directly identified.

3. Public goods, parasites and mutation rate[2].

Abstract

Many bacterial populations harbour substantial numbers of hypermutable bacteria, in spite of hypermutation being associated with deleterious mutations. One reason for the persistence of hypermutators is the provision of novel mutations, enabling rapid adaptation to continually changing environments, such as coevolving virulent parasites. However, hypermutation also increases the rate at which intra-specific parasites (social cheats) are generated. Inter-specific and intra-specific parasitism are therefore likely to impose conflicting selection pressure on mutation rate. Here, we combine theory and experiments to investigate how simultaneous selection from inter- and intra-specific parasitism affects the evolution of bacterial mutation rates in the plant-colonizing bacterium *Pseudomonas fluorescens.* Both our theoretical and experimental results suggest that phage presence increases selection for mutator bacteria. Moreover, phages imposed a much greater growth cost than social cheating, and when both selection pressures were imposed simultaneously, selection for cooperation did not affect mutation rate evolution. Given the ubiquity of infectious phages in the natural environment and clinical infections, our results suggest that phages are likely to be more important than social interactions in determining mutation rate evolution.

1. Introduction

One hypothesis for the evolution of sexual reproduction is an increased ability to evade parasites: the “Parasite Red Queen” hypothesis (Van Valen 1973; Jaenike 1978; Hamilton 1980, 1990; Lively & Dybdahl 2000; Brockhurst 2011; Morran et al. 2011; Hartfield & Keightley 2012). Parasites become adapted to the most frequent host genotypes, and host sex can result in novel genotypes that escape infection by the most common parasites. However, parasites can

[2] Published as: O’Brien S, Rodrigues AMM & Buckling A. 2013. The evolution of bacterial mutation rates under simultaneous selection by inter-specific and social parasitism. Proceedings of the Royal Society B: Biological Sciences 280 (1773), 20131913 (see Appendix)

quickly adapt, and so there is continual selection for genotypic diversity in the presence of parasites. However, another form of parasitism, social parasitism, may itself be a cost of sexual reproduction. Specifically, the genetic diversity produced by sex means that interacting individuals are less likely to share the same alleles for cooperation. This reduction in relatedness reduces the indirect benefits required to select for cooperative behaviour. This idea that "sex is an antisocial force in evolution" was first proposed by Wilson (1975), and has been explored both theoretically and comparatively (Boomsma 2007, 2009; Hughes et al. 2008; Cornwallis et al. 2010; Lukas & Clutton-Brock 2012) demonstrating that highly cooperative societies such as those of the eusocial insects or cooperative breeders are likely to be preceded by strict lifetime monogamy (rather than promiscuity), whereby relatedness between interacting individuals is predictably high. Sexual reproduction can therefore create a trade-off between resistance to inter- and intra-specific parasitism (Hamilton 1987; Sherman et al. 1988).

The evolution of mutation rates in obligatory asexual species may be affected by similar conflicting selection pressures from inter- and intra-specific parasitism. Many bacterial populations harbour hypermutable bacterial strains (10-1000 fold increase in genomic mutation rate (Sniegowski et al. 1997, 2000; Taddei et al. 1997; Jayaraman 2009)) with the frequency of hypermutators sometimes in excess of 30% (Oliver et al. 2000) in clinical infections. Hypermutators increase in frequency through hitch-hiking with beneficial mutations, hence continual changing selection pressures tend to result in increases in mutator frequencies (Taddei et al. 1997; Giraud et al. 2001; Desai & Fisher 2011). Antagonistic coevolution with viruses creates continual selection for novel resistant phenotypes, and has been shown to confer a large selective advantage on hypermutator bacteria *in vitro* (Pal et al. 2007; Morgan et al. 2010). However, hypermutators have been shown to be selected against when cooperative behaviours are advantageous (specifically, the production of siderophores: extracellular iron-scavenging public goods). This is because hypermutators generate non-producing cheats more readily, reducing relatedness in populations founded by mutators (Harrison & Buckling 2005, 2007; Racey et al. 2010). Beyond bacteria, selection for cooperation can keep relatedness high through processes such as kin recognition (Hamilton 1964;

Griffin & West 2003) reproductive skew (Heinze 1995) and within-group segregation (Queller et al. 1992).

Here, we combine theory and experiments to investigate how simultaneous selection from inter- and intra-specific parasitism affects the evolution of bacterial mutation rates. We use the well-studied plant-colonizing bacterium *Pseudomonas fluorescens* SBW25, which undergoes extensive reciprocal evolution of defense and counter-defense with an obligate killing bacteriophage, φ2, in both nutrient media (Buckling & Rainey 2002a) and soil (Gómez & Buckling 2011). For the cooperative trait, we focus on the extracellular production of iron-scavenging siderophores, which are individually costly to produce but can be used by neighbouring conspecifics. As a result, non-producing cheats can readily invade but reduce iron-dependent population growth rate in iron-limited environments in both *P. fluorescens* SBW25 (Morgan et al. 2012) and the closely related bacterium, *P. aeruginosa* (Griffin et al. 2004). We experimentally manipulated these opposing selection pressures by competing *P. fluorescens* SBW25 and an isogenic mutator in iron-limited or iron-rich media (only the former conditions require siderophore production), and in the presence or absence of phages, in experimental metapopulations. Our experimental design simulated global competition, whereby all patches within a metapopulation were mixed and clones from this mixture were used to inoculate new patches. In doing so, genotypes from the more successful patches were overrepresented in subsequent generations, while those from less successful patches were underrepresented. Consequently, any phenotype that reduced group productivity was selected against. To ensure selection for cooperation, we regularly established new patches in the metapopulations with single clones (high relatedness) (Griffin et al. 2004; Harrison & Buckling 2007). This design allowed the benefits of diversity to be realised by hypermutators, but also kept selection for cooperation high.

2. Theoretical model and analysis

We develop a simple mathematical model to make qualitative predictions about the evolution of bacterial mutation rates μ in the presence of social cheats and parasites. We assume that higher mutation rates both increases bacterial resistance to parasites and the local abundance of social cheats. We consider a

metapopulation composed of a large number of patches, each of which is colonised by a single bacterial strain with mutation rate μ, with $0 \leq \mu \leq 1$. All strains are assumed to be functional for the cooperative trait, and cheats arise by loss-of-function mutations. While cheats impose a growth cost on cooperative lineages, we assume that cheats are poor colonisers of new patches, and therefore evolutionarily unviable. We assume that the fitness w of a bacterial strain with mutation rate μ is a function of its survival S and its growth G, such that $w(\mu) = SG$.

Bacterial survival decreases as parasite density π increases, while it increases as bacterial hosts evolve resistance to parasites. For simplicity of analysis we assume that the probability that bacterial hosts acquire resistance alleles is equal to their mutation rate μ, and that parasite-induced mortality decreases linearly with resistance. As a result, the survival cost imposed by parasites on their bacterial hosts is $(1-\mu)\pi$, such that the survival of a strain with mutation rate μ is $S = 1-(1-\mu)\pi$. Note that in absence of parasites ($\pi = 0$), or when there is full resistance ($\mu = 1$), survival is assumed to be 1.

We assume that the costs associated with social cheats depend on three factors: i) the local abundance of cheats; ii) the intrinsic cost of cheats to cooperators; and iii) parasite negative density-dependent regulation of hosts' cooperative growth. We assume that the local abundance of cheats is equal to the mutation rate μ of the local bacterial strain. Cheats are assumed to impose an intrinsic cost c on cooperative growth. In addition, cooperative growth may be subject to density-dependent regulation by parasites, which reduces the impact of cheats on cooperative growth. We assume that parasite density-dependent regulation of hosts' cooperative growth is given by $\alpha\pi$, where $0 \leq \alpha \leq 1$ is the degree of parasite density-dependent regulation. Putting all these factors together, we define the overall growth cost of cheats to cooperative growth as $(1-\alpha\pi)c\mu$, and the growth of a focal lineage as $G = 1-(1-\alpha\pi)c\mu$. Note that if: i) cheats are absent ($\mu = 0$); or ii) cheats do not impose an intrinsic cost on cooperators ($c = 0$); or iii) there is full density dependent regulation ($\alpha = \pi = 1$), growth is one, $G = 1$. The fitness of a focal strain with mutation rate μ is then given by

$$w(\mu) = \overbrace{(1 - \underbrace{(1-\mu)\pi}_{\text{Cost of parasites}})}^{\text{Survival}} \overbrace{(1 - \underbrace{(1-\alpha\pi)c\mu}_{\text{Cost of social cheats}})}^{\text{Growth}}. \quad (1)$$

Our objective is to determine the evolutionary stable (ES) mutation rate (μ^*; (Maynard-Smith & Price 1973; Maynard-Smith 1982). We find that the marginal fitness benefit of a slight increase in the mutation rate is given by $B(\mu) = \pi$ (1-(1-$\alpha\pi$)$c\mu$) = πG. Thus, the marginal benefit increases with increasing parasite density π, as long as there is some growth. The marginal fitness cost of a slight increase in the mutation rate is given by $C(\mu)$ = (1-(1-μ) π)(1-$\alpha\pi$)$c\mu$ = S(1-$\alpha\pi$)c. Thus, as long as there is some survival, the growth cost is low if: i) there is strong parasite density-dependent regulation (high $\alpha\pi$); and ii) the intrinsic cost of cheats is low (low c). The ES mutation rate is the value μ^* for which the marginal cost exactly cancels the marginal benefit of a slight increase in the mutation rate, i.e. $B(\mu^*) = C(\mu^*)$. This is given by

$$\mu^* = \frac{1}{2}\left(\frac{1}{c(1-\alpha\pi)} - \frac{1-\pi}{\pi}\right). \quad (2)$$

We find that the ES mutation rate μ^* decreases with increasing intrinsic cost of cheats ($\partial\mu^*/\partial c < 0$). By contrast, the ES mutation rate μ^* increases with increasing degree of parasite negative density-dependent regulation of hosts ($\partial\mu^*/\partial\alpha > 0$), and with increasing parasite density ($\partial\mu^*/\partial\pi > 0$; see Figure 1).

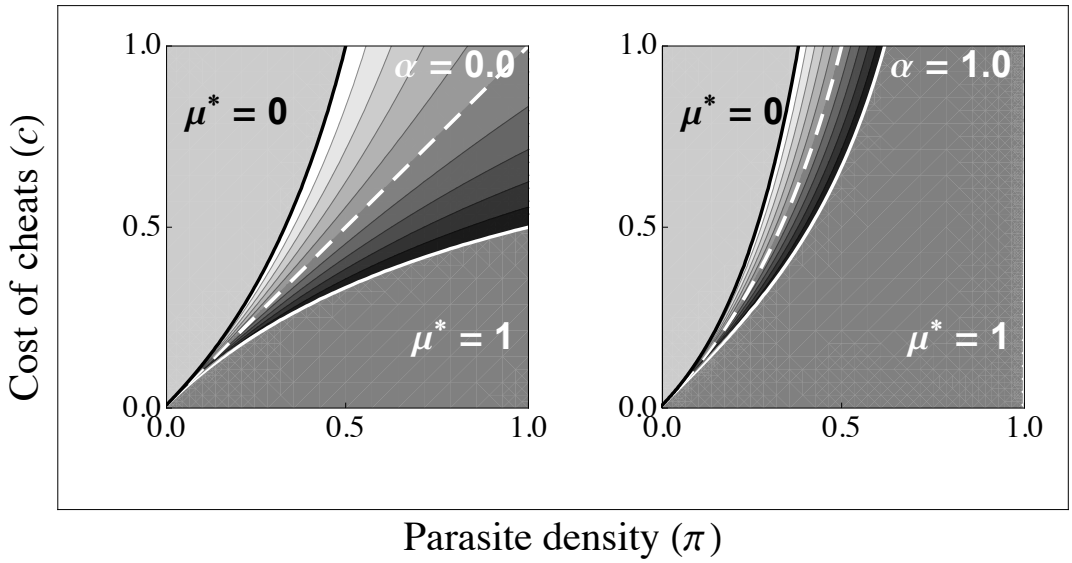

Figure 1: The ES mutation rate (μ^*) as a function of parasite density (π) and the intrinsic cost of cheats (c), for various levels of parasite density-dependent regulation of hosts (α). If there is no parasite density-dependent regulation (α = 0), then when parasite density is low and the cost of cheats is high, selection favours low mutation rates ($c \geq \pi/(1+\pi)$; $\mu^* = 0$). If parasite density is equal to the intrinsic cost of cheats, then intermediate levels of mutation rates are favoured ($\pi = c$; $\mu^* = 0.5$; dashed white line). If parasite density is high relative to the cost of cheats, then selection favours high mutation rates ($c \leq \pi/(1-\pi)$; $\mu^* = 1$). If there is strong parasite density-dependent regulation ($\alpha = 1$), the ES mutation rate becomes less sensitive to changes in the cost of cheats (c).

3. Experimental materials and methods

3.1 Strains

The *P. fluorescens* strain SBW25 (Rainey & Bailey 1996) was used as a wildtype, and the strain SBW25Δ*mutL* which has a mutation in the mismatch repair gene *mutL,* was used as a hypermutator. SBW25Δ*mutL* exhibits a spontaneous mutation rate 100-fold times higher than SBW25, and is resistant to the antibiotic gentamicin (Pal et al. 2007), allowing it to be distinguished from the wildtype. We used the lytic phage phi 2 (φ2) (Buckling & Rainey 2002a).

3.2 Experimental design

To ensure that each population was colonised by a single strain (i.e relatedness = 1) at the first transfer into medium, each bacterial culture was grown in 6ml

King's Medium B (KB; (10 g glycerol, 20 g proteose peptone no. 3, 1.5 g $K_2HPO_4.3H_2O$, 1.5 g $MgSO_4.7H_2O$, per litre), for 24 hours at 27°C, when it was diluted with M9 minimal salt solution and grown for 36 hours on KB agar at 27°C. A single colony could then be selected and further grown in KB medium at 27°C for 24 hours. This single-strain culture could then be used to establish populations.

We manipulated phage and iron availability in a fully factorial experiment, setting up each of our 4 treatments (a: iron + phage, b: iron only, c: phage only, d: no iron, no phage) in 96 well plates. We used 6 replicates for each treatment, with each replicate consisting of a metapopulation made up of 6 patches (wells). Each well was inoculated with a total of 190µl KB broth, with 3 wells of each replicate initially inoculated with approximately 10^7 colony forming units (CFUs) of clonal wildtype bacteria, and 3 wells inoculated with approximately 10^7 CFUs of clonal mutator bacteria. Iron limitation was created by the addition of 100µg/ml human apotransferrin and 20mM sodium hydrogen carbonate ($NaHCO_3$) to standard KB medium immediately before use (see Morgan et al. 2012). $NaHCO_3$ was also added to non-iron limited treatments. Approximately 10^5 phages were added to phage treatments in 10µl KB, and the same volume of KB media added to non-phage wells. Plates were incubated at 27°C under static conditions.

Every second day, aliquots (2µl) from all 4 treatments were transferred to new KB medium (+/- Fe) using a sterile pin replicator. Every 4 transfers (8 days), 100µl from each of the 6 wells within a replicate was mixed, simulating global competition (Griffin et al. 2004) and plated out on KB and KB + gentamicin agar to assess frequencies of mutator and wildtype bacteria (see below). Phage was isolated from this mixture each time, by adding 10% chloroform and centrifuging at 13000rpm, which lysed and pelleted bacteria. Six colonies from each KB agar plate were randomly selected, grown independently for 24 hours in 180 µl KB medium at 27°C, and each was subsequently used to inoculate a new well within a treatment (10^7 CFUs), to ensure high relatedness, and hence the potential for selection for cooperation was continually re-established. For phage treatments, both sympatric (10µl) phages and ancestral (10µl) phages were added (to ensure continual selection for phage resistance where sympatric

phages had reached low densities); equivalent volumes of phage-free KB media were added to no-phage treatments. Mixing and plating were performed 5 times in total, for approximately 250 generations of bacterial growth (carrying out 6 transfers instead of 4 between mixing after the 3rd assay).

3.3 Assays

The relative fitness of mutator and wildtype strains was assessed by plating a mixture of all patches within a replicate onto both KB agar (for an estimate of total density) and KB agar supplemented with 10μg/ml gentamicin (on which only gentamicin resistant mutator bacteria can grow) over 36 hours, resulting in a KB and KB+gentamicin plate for each replicate, and 6 plates of each type per treatment. Colony types were also categorised by colour into either siderophore producers (yellow/green) or non-producers (white), based on the fact that the primary siderophore of *P. fluorescens*, pyoverdine, is yellow/green. As the amount of colony pigmentation varied considerably, we classified a colony showing any yellow/green pigmentation after 36 hours as a producer (see Harrison & Buckling 2005). We carried out an additional experiment to verify that the appearance of milky white colonies indeed represented the evolution of siderophore negative cheats. We selected 19 wildtype and 19 milky colonies at random at the final timepoint, and inoculated each colony into iron-limited KB media. Populations were grown at 27°C for 24 hours, after which, fluorescence of 180μl of culture was measured at 460nm, following excitation at 400nm using a Biotek Synergy 2 spectrophotometer (standard protocol for measuring pyoverdine production (Jiricny et al. 2010; Harrison 2013). Milky colonies produced pyoverdine at significantly lower levels than wildtype (student's t test, $T_{18.017} = 15.7631$, $p < 0.0001$) and so any milky colonies that emerged during the experiment were labelled siderophore cheats.

3.4 Costs of cheating and phage parasitism

To directly assess the relative cost of cheats and parasites on the growth of wildtype bacteria, wildtype SBW25 and an isogenic cheat strain (SBW25Δ*pvdL*, with the primary siderophore, pyoverdine, knocked out, (Moon et al. 2008)) were grown in isolation in the presence and absence of phages (phi 2 (φ2)), with 8 replicates per treatment. All treatments were carried out in iron-limited KB broth to facilitate selection for cooperation under starting conditions described

above. After 24 hours at 27°C, the abundance of cheat and wildtype genotypes was assessed by plating each treatment on KB agar, incubating for 36 hours, and counting the colonies on each plate as before. We also confirmed previous work (Morgan et al. 2012) that cheats have a growth rate advantage in the presence of wildtype bacteria under these conditions by competing 50:50 mixtures of cheats and wildtype.

3.5 Statistical analyses

R software (R development core team, 2010) was employed for all statistical analyses. Maximum likelihood analysis was carried out using Linear Mixed Effects Revised models with arcsine square root (proportion of mutators/cheats) as a response variable, and iron, phage and time, including 2-way interactions, as explanatory variables. A third model with cheat frequency as the response variable was employed to investigate any affect mutator frequency might have on the appearance of cheats, and the extent to which this was affected by iron limitation. Iron and phage presence/absence were fitted as factors and time as a continuous variable, assigning 'population' as a random factor as follows:

*mutator frequency ~ iron*phage +iron*time + phage*time*+ 1|*population*
*cheat frequency ~ iron*phage +iron*time + phage*time*+ 1|*population*
*cheat frequency~ mutator frequency*iron* + 1|*population*,

where * represents an interaction and separate terms. To assess the relative costs of cheating and parasitism, student's t-tests were used to compare fitness of wildtype and cheat strains after 24 hours growth in the presence and absence of phage.

4. Experimental results

4.1 Costs of cheats and phages

To establish the relative magnitude of costs of cheating and phage parasitism on bacterial growth, we measured the densities of siderophore cheat and wildtype strains grown as monocultures in the presence and absence of ancestral bacteriophages after 24h growth under iron-limited conditions. Phages caused a significant reduction in the density of wildtype (student's t test, $T_{7.515}$ =

5.6787, $p < 0.001$), and cheat phenotypes (student's t test, $T_{11.542} = 2.4959$, $p < 0.05$). Consistent with previous work (Moon et al. 2008; Morgan et al. 2012) cheats reached significantly lower densities than wildtype bacteria in the absence of phages (student's t test, $T_{9.696} = 4.6885$, $p < 0.001$), however, in the presence of phages, this relationship was reversed and cheats grew significantly more than wildtype (student's t test, $T_{11.124} = 3.3046$, $p < 0.01$). The reduction in the density of wildtype bacteria brought about by cheats was significantly smaller than the reduction caused by the presence of phages (student's t test, $T_{6.872} = 4.5904$, $p < 0.01$). Based on our simple model predictions, these ecological results suggest that phage-imposed selection for mutators is likely to be greater than that imposed by cheats. A competition experiment between wildtype SBW25 and SBW25Δ*pvdL* verified that cheats can invade cooperators under iron-limited conditions (student's t test (alt=1), $T_5 = 2.5039$, $p = 0.05$), despite the poorer performance of cheats compared with cooperators as monocultures; i.e. pyoverdin production is an altruistic trait.

4.2 Competition between mutators and wildtype over evolutionary time scales.
We predicted that phages should select for higher mutation rates, while selection for cooperation should select against mutators. Phage-imposed selection was manipulated by the presence versus absence of phages, whereas selection for siderophore-mediated cooperation was manipulated by changing iron availability (siderophores are required when iron is limited) in experimental metapopulations. While the frequency of mutators increased through time in the presence of phages and decreased in the absence of phages (LMER: timepoint x phage interaction, $Chisq_{1,5} = 9.3343$, $p < 0.01$; Figure 2*)* iron availability had no impact on the frequency of mutators (LMER: $Chisq_{1,6} = 0.3097$, $p = 0.58$). Moreover, there was no interaction between the presence of phage and iron availability (LMER: non significant phage x iron interaction, $Chisq_{1,8} = 0.187$, $p = 0.67$). Selection for mutators via the presence of external viruses therefore was not affected by any conflicting selection for cooperation by iron limitation.

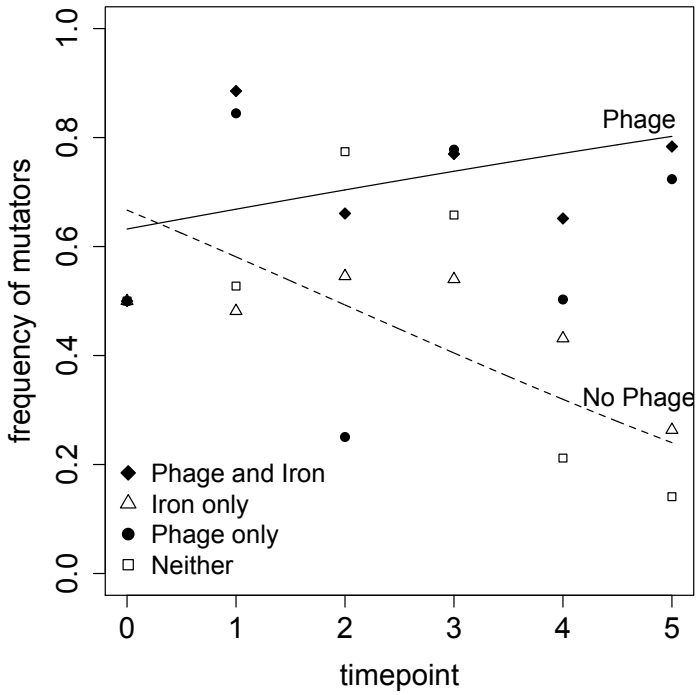

Figure 2: Changes in mutator frequency in a population through time is affected by the presence/absence of phage ($Chisq_{1,5}$ =9.3343, $p < 0.01$). Data are plotted using predictions from minimal LMER model (*mutator frequency ~ time:phage* + 1|*population).*

4.3 Evolution of siderophore cheats

The frequency of siderophore cheats increased through time in all treatments but significantly more so when there was selection for cooperation by limiting iron availability (LMER: timepoint x iron interaction $Chisq_{1,5} = 3.9498$, $p < 0.05$; Figure 3). Phage presence did promote the production of cheats through time, but this trend was not significant (LMER: non-significant phage x time interaction, $Chisq_{1,7} = 2.1579$, $p = 0.14$). Furthermore, no interaction was observed between iron availability and the presence of phages in determining cheat frequency (LMER: non-significant phage x iron interaction $Chisq_{1,8} = 0.6231$, $p = 0.43$). Cheat frequency increased with the frequency of mutators, but only in iron-rich populations (LMER: mutator frequency x iron interaction: $chisq_{1,5} = 8.1479$, $p < 0.01$).

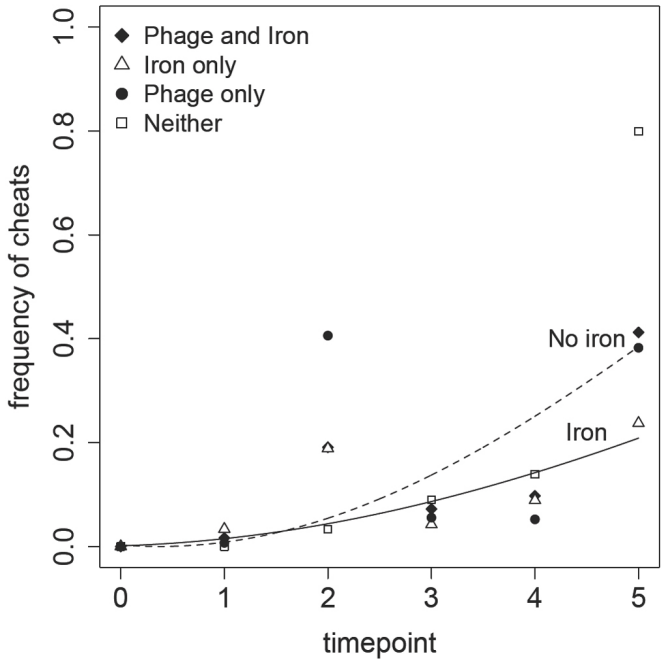

Figure 3: When Iron is limited, cheats reach higher frequencies through time than when iron is present ($Chisq_{1,5} = 3.9498$, $p < 0.05$). Data are plotted using predictions from minimal LMER model (*cheat frequency ~ time:iron + 1|population).*

5. Discussion

Here, we investigate both theoretically and empirically how selection imposed by inter-specific parasites (bacteriophages) and intra-specific parasites (social cheats) affects the evolution of mutation rates in bacteria. Theoretically, selection for mutator bacteria decreases when there is selection for cooperation because mutators are more likely to generate social cheats, and increases in the presence of phage because mutators are more likely to generate phage resistance mutations. Our short-term growth rate experiments suggest that phages impose a much greater growth rate cost than social cheating in this experimental context. Moreover, the impact of phages on density was less for cheat than wildtype populations. Feeding this back into our simple model suggests that the strength of phage-imposed positive selection on mutators will be greater than cooperation-imposed negative selection on mutators. Our evolution experiment was consistent with this prediction: mutator frequency

increased in the presence of phages but was not influenced by whether or not there was selection for cooperation.

That coevolution with phages favours mutator bacteria is consistent with previous work using this system (Giraud et al 2001; Pal et al. 2007; Morgan et al 2010). Pal *et al.* (2007) found that after approximately 170 bacterial generations mutation rates had increased 10- to 100- fold in 9 out of 36 populations in the presence of coevolving phages, whereas no significant change in mutation rate was observed in populations evolving in the absence of phages. Qualitatively identical results were observed when the wildtype and an isogenic mutator were competed at equal ratios in the presence and absence of phages. However, in these experiments bacteria were cultured as single populations. Our results reveal similar phage-imposed selection for mutation rates in high-relatedness metapopulations. Arguably, such metapopulation structure presents a more ecologically relevant scenario for bacteria, where microcolonies are founded by single bacteria, which then go extinct or go on to colonise new patches (Buckling et al. 2007).

The lack of association between selection for siderophore-mediated cooperation and mutator frequency is initially surprising, as a previous study showed such a relationship using the related bacterium, *Pseudomonas aeruginosa* (Harrison & Buckling 2007). Specifically, in an evolution experiment of a similar duration to the current study (approximately 250 generations), mutator frequency was significantly lower in iron-limited metapopulations when patches were established with single colonies (high relatedness, hence strong selection for cooperation) compared with multiple colonies. While cheats of both species do show reduced growth under iron-limited conditions (this study, Griffin et al. 2004; Moon et al. 2008; Meyer et al. 1996), the discrepancy is likely to have arisen because i) different species and media were used, and hence the cost of cheating may be different, ii) selection for cooperation in the current study was manipulated by iron-availability as opposed to number of colonies founding a patch and iii) we used the mutL mutator strain rather than mutS as in previous studies (Harrison & Buckling 2007; Pal et al. 2007), and differences in genetic effects of the specific gene may account for discrepancies. However, studies in

Escherichia coli suggest these mutations have very similar effects (Schaaperr & Dunn 1987).

Our model and ecological experiment suggests a simple but potentially general interaction between social cheating and parasitism: parasites have less of an impact on reducing cheat population density, and hence the relative impact of cheating on population growth is reduced in the presence compared with the absence of parasites. This is a simple extension of the more general finding that enemies typically reduce high density populations relatively more than low density populations e.g. (Hochberg & van Baalen 1998; Lopez-Pascua & Buckling 2008). In addition to weakening selection against mutators, this reduced costs of cheats also suggests that cheats may be relatively favoured in the presence versus absence of parasites. By contrast, recent theory and experiments suggest cheating may be less favoured in the presence of parasites, because cheats are likely to be initially rare in newly colonized patches, and hence less likely to evolve resistance mutations (Morgan et al. 2012; Quigley et al. 2012). Both mechanisms are not mutually exclusive, with the former relevant when cheats are at sufficiently high frequency to affect growth and the latter when cheats are initially rare. The simultaneous operation of both mechanisms may explain our finding that phages had no net effect on the presence of cheats.

High mutation rates can result in rapid resistance to antibiotics and bacteriophage, and clinical studies have shown hypermutators to be associated with poorer lung function in CF patients (Waine et al. 2008). Therefore it is crucial to develop an understanding of how different selection pressures involved in their persistence interact with each other. Given the ubiquity of infectious phages in the natural environment (e.g. Vos et al. 2009) and clinical infections (Ojeniyi et al. 1991; Harrison 2007) our results suggest that phages are likely to be more important than social interactions in determining mutation rate evolution in natural environments.

4. Public goods cooperation and detoxification[3]

Abstract

Bacteria are often iron-limited, hence produce extracellular iron-scavenging siderophores. A crucial feature of siderophore production is that it can be an altruistic behaviour (individually costly but benefitting neighbouring cells), thus siderophore producers can be invaded by non-producing social "cheats". Recent studies have shown that siderophores can also bind other heavy metals (such as Cu and Zn), but in this case siderophore chelation actually reduces metal uptake by bacteria. These complexes reduce heavy metal toxicity; hence siderophore production may contribute to toxic metal bioremediation. Here, we show that siderophore production in the context of bioremediation is also an altruistic trait and can be exploited by cheating phenotypes in the opportunistic pathogen *Pseudomonas aeruginosa*. Specifically, we show that in toxic copper concentrations: 1) siderophore non-producers evolve *de novo* and reach high frequencies; and 2) that producing strains are fitter than isogenic non-producing strains in monoculture, and vice versa in co-culture. Moreover, we show that the evolutionary effect copper has on reducing siderophore production is greater than the reduction observed under iron-limited conditions. We discuss the relevance of these results to the evolution of siderophore production in natural communities and heavy metal bioremediation.

1. Introduction

Social behaviours in bacteria are commonplace, and are important both as model systems for understanding the evolution of cooperation, and for playing a key role in determining virulence (Crespi 2001). One well-studied example of such behaviour is the production of iron-scavenging public goods called siderophores. Siderophore production can be an altruistic behaviour (individually costly but benefitting neighbouring cells), hence can be invaded by

[3] Published as: O'Brien S., Hodgson D. & Buckling A. 2014. Social evolution of toxic metal bioremediation in *Pseudomonas aeruginosa*. Proceedings of the Royal Society B: Biological Sciences 281 (1787), 20140858 (see Appendix).

non-producing social “cheats” (West & Buckling 2003). The evolutionary maintenance of siderophore production can be explained by kin selection (Hamilton 1964; Maynard-Smith 1964): most neighbouring cells that benefit from siderophores are themselves siderophore producers (Griffin et al. 2004). While the primary role of siderophores is to chelate ferric iron, they can also play an important role in detoxifying heavy metal contaminated environments (Nair et al. 2007). Here, we empirically investigate the importance of siderophore production as an altruistic trait in the context of bioremediation relative to iron uptake.

Siderophores can bind to a wide array of toxic metals (e.g. V^{4+}, Cr^{3+}, Al^{3+}, Cu^{2+}, Eu^{3+}, Pb^{2+}, Sn^{2+} and Tb^{3+}), albeit with far less affinity than Fe^{3+} (Baysse et al. 2000; Braud et al. 2009). Many of these toxic metals are required in trace amounts for efficient metabolism, but are lethal at higher concentrations for bacteria (Heldal et al. 1985). For example, high concentrations of copper can cause oxidative stress, which can damage cellular DNA (Gaetke & Chow 2003; Valco et al. 2005). Unlike iron/siderophore complexes, which bind to species-specific siderophore receptors allowing uptake of iron (Braud et al. 2009; Miethke & Marahiel 2007; Noinaj et al. 2010), siderophores bound to other heavy metals do not enter the cell efficiently (Braud et al. 2009). As a result, siderophores can actually prevent heavy metals, which normally enter cells by diffusion, from being taken up, effectively detoxifying the environment for the microbial community. Moreover, detoxification may not just be a simple by-product of siderophore production in response to iron-limitation, as recent work shows siderophore production in *P. aeruginosa* is upregulated in toxic environments (Höfte et al. 1993; Teitzel et al. 2006; Braud et al. 2010).

As with iron chelation, siderophore production in the context of heavy metal detoxification is likely to be an altruistic trait. Previous work has shown that siderophore-producing *P. aeruginosa* has greatly enhanced growth in metal-contaminated media relative to isogenic mutants which do not produce the primary *P. aeruginosa* siderophore, pyoverdine (Braud et al. 2010). However, it has not been determined whether non-producers can invade populations of producers in this context. We confirmed this prediction using *P. aeruginosa* by: (i) following the evolution and invasion of spontaneous non-producing mutants

in initially clonal wildtype populations, and (ii) carrying out competition experiments between wildtype and an isogenic siderophore knockout mutant. We also carried out comparable experiments under non-toxic but iron-limited conditions to assess the relative importance of detoxification to iron uptake for the social evolution of siderophore production in heavy metal contaminated environments.

2. Materials and Methods

2.1 Strains and growth media

We used the *P. aeruginosa* strain PAO1 as a wildtype siderophore-producing strain, and an isogenic mutant strain with both primary and secondary siderophores, pyoverdine and pyochelin, knocked out (Ghysels et al. 2004).

Bacteria were cultured in Kings Medium B (KB) (King et al. 1954); (10 g glycerol, 20 g proteose peptone no. 3, 1.5 g $K_2HPO_4.3H_2O$, 1.5 g $MgSO_4.7H_2O$, per litre) with the addition of either copper or iron sulphate. Iron sulphate acted as a control for the effect of sulphate and copper ions *per se*. Moreover, while Cu^{2+} stimulates the production of pyoverdine (Braud et al. 2010), Fe^{2+} inhibits it (Leoni et al. 2000; Ratledge & Dover 2000). Consequently siderophore production, and hence its cost, varies among our experimental treatments. Note that both metals repress siderophore fluorescence on chelation (Braud et al 2009) meaning that bacterial fluorescence is a good proxy for free siderophore density in the medium. We identified a mildly toxic (624μM/low strength) and highly toxic (6.17mM/high strength) level of copper sulphate for wildtype, and prepared the same final concentrations of iron sulphate as a control. High copper treatments reduced population growth by 63.5% compared with high iron, while low copper reduced mean population growth by 29.25% compared with low iron. Note that the low iron treatment was not iron-limited, but a lower concentration of *supplemented* iron was added to the growth medium.

2.2 Evolution experiment and assay

We first carried out an evolution experiment to investigate whether toxicity is likely to accelerate the evolution of cheats. A single wildtype PAO1 colony was

grown in KB medium at 37°C for 24 hours, and approximately 10^5 colony forming units (CFU's) inoculated into 48 microcentrifuge tubes containing 900 µl of KB medium supplemented with copper or iron sulphate to a final molarity of 624µM or 6.17mM (12 replicates within each of 4 treatments). The tubes were shaken horizontally at 180 rpm at 37 °C, aliquoting 1% (approx. 10^6 CFU's) of each culture to new iron/copper treated medium every 24 hours for a total of 15 transfers (approximately 100 bacterial generations).

At transfer 15 the appearance of *de novo* mutants was monitored by aliquoting each population into standard KB medium (diluting by 10^{-2}), grown for 24 hours at 37 °C, after which they were diluted and plated onto KB agar to assess total density and phenotype. Growing populations in KB medium (which does not require siderophore production) ensures any mutants that have evolved are obligate cheats rather than phenotypically plastic. As the amount of colony pigmentation varied considerably, we classified a colony showing any yellow/green pigmentation after 36 hours as a producer; the primary siderophore of *P. aeruginosa*, pyoverdine, is yellow/green (see Harrison & Buckling 2005). To compliment this assay, the overall level of pyoverdine production in each population was assessed by a pyoverdine-specific excitation-emission assay (Jiricny et al. 2010; Harrison 2013). 1% of each population was transferred to iron-limited KB medium in a 96 well plate. Since siderophore production is repressed when there is an excess of Fe^{2+} (Leoni et al. 2000; Ratledge & Dover 2000) iron-limitation ensures that siderophores are essential for growth and stimulates their production. Iron limitation was created by the addition of 100µg/ml human apotransferrin and 20mM sodium hydrogen carbonate ($NaHCO_3$) to standard KB medium immediately before use. Populations were grown at 37°C for 24 hours, after which, fluorescence of 200µl of culture at 460nm, following excitation at 400nm was measured using a Biotek Synergy 2 spectrophotometer (Jiricny et al. 2010; Harrison 2013).

We carried out an additional experiment to ascertain how relevant toxic metals are in promoting cheat evolution, compared to the well-established evolution of cheats in iron-limited environments (Griffin et al. 2004). We repeated the evolution experiment as described above in non-toxic but iron-limited conditions (KB + 100µg/ml human apotransferrin and 20mM $NaHCO_3$) and in standard KB

media (nutrient-rich control), aliquoting 1% (approx. 10^6 CFU's) of each culture to new medium every 24 hours for a total of 33 transfers (approximately 200 bacterial generations). A pyoverdine-specific excitation-emission assay was then used to quantify the level of pyoverdine production in each population, controlling for cell density as described above.

2.3 Competition experiment and assay

Our short-term growth rate experiment consisted of 144 populations of bacteria (36 populations for each of the 4 treatments described above) grown in horizontally shaken 2ml microcentrifuge tubes at 180rpm, in 900µl KB medium. Within each treatment, 12 populations were inoculated with approximately 10^5 CFU's of wildtype, 12 with 10^5 CFU's of mutant, and a further 12 with equal amounts of wildtype and mutant, so that the total number of CFU's in this group was also approximately 10^5. Populations were incubated at 37 °C for 24 hours.

The intrinsic growth rate of wildtype and mutant strains was assessed by diluting and plating all populations onto KB agar, leaving for 24 hours at 37 °C after which individual CFU's could be counted. Colony types were categorised by colour into either siderophore producers (yellow/green) or mutants (white) as before. We determined the Malthusian growth rate (*m*) for both wildtype and mutant strains as ln(final density/start density) (Lenski et al. 1991). Relative mutant fitness compared to wildtype (*W*.mutant) in co-culture was calculated as *m*(mutant)/*m*(wildtype), and in monoculture as *m*(mutant)/global mean(*m*(wildtype)).

2.4 Statistical analyses

R 2.15.1 (R Development Core Team 2010) was employed for all statistical analyses. We treated metal type and strength as fixed explanatory factors and relative mutant fitness (*W*) as our continuous response variable.

a) Evolution experiment

We used a Kruskal Wallis nonparametric test, with multiple comparisons among treatment groups, to investigate whether cheat frequency was affected by metal treatment after 100 generations. This test ensured our zero-inflated data did not violate the assumptions of a parametric test. A 1-way analysis of variance and

Tukey test were employed to assess whether pyoverdine production was affected by metal treatment. We used a non-parametric Spearman rank order correlation to confirm the relationship between pyoverdine production and cheat frequency. Finally, we used a Student's t-test to investigate whether iron-limitation altered pyoverdine production, and a Welsh t-test (to tolerate unequal variances) to assess how this change compared with any toxicity-mediated reduction in pyoverdine. All measures of pyoverdine were corrected for optical cell density to obtain per capita pyoverdine production.

b) Competition experiment

We used a factorial analysis of variance to investigate whether relative mutant fitness (*W*) was affected by metal type, growth conditions (mono-versus co-culture), and metal strength, including all 2-way interactions. Additionally, a Student's t-test was employed to assess the extent of mutant invasion in each treatment condition.

3. Results

3.1 Evolution experiment

Based on the fact that copper toxicity greatly upregulates siderophore production (making it more costly), we predicted toxic copper conditions would promote the evolution of non-siderophore producing cheats, even when iron is abundant. We first carried out an evolution experiment to investigate whether heavy metal toxicity can accelerate the evolution of cheats compared to non – toxic environments. After approximately 100 generations, the proportion of mutant phenotypes was significantly higher in highly toxic (6.17mM) copper populations than all other populations (Kruskal-Wallis & Kruskal mc: H_3=29.4762, p<0.001, figure 1a). Our assay for per capita pyoverdine production was consistent with this result: production was lower in highly toxic copper treatments, and higher in low iron treatments than all other treatments. (1-way ANOVA & Tukey HSD, $F_{3,44}$=45.952, p<0.001, figure 1b). A Spearman's rank correlation revealed a strong negative correlation between pyoverdine production and cheat frequency after 100 generations (ρ = *-0.515*, n=48, p<0.001). To ensure that the increased proportion of mutant phenotypes was not a result of a higher population density in the high copper treatment, we

compared final population densities among our 4 treatments. Populations from high copper treatments in fact had lower densities than both iron treatments (1-way ANOVA, $F_{1,46}$=38.495, p<0.001), so density could not explain the increased proportion of mutants in high copper treatments.

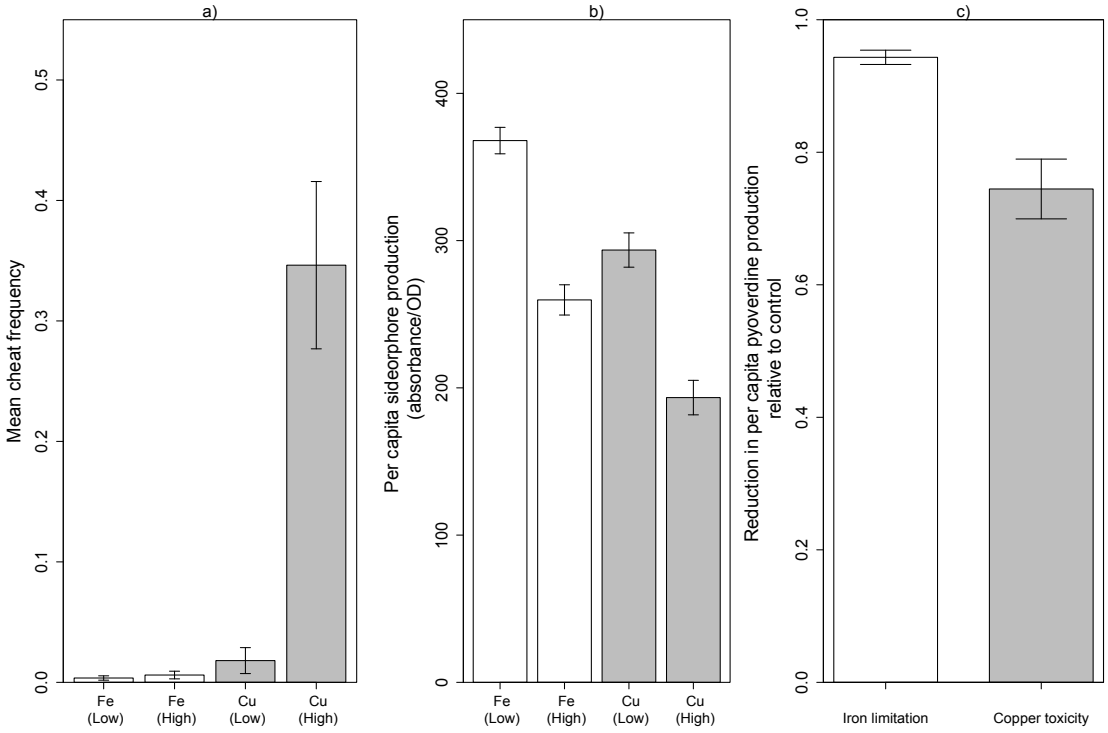

Figure 1: **a)** After 100 generations, *de novo* siderophore non-producing mutants reach far higher frequencies in highly toxic copper populations than all other populations (Kruskal-Wallis & Kruskal mc: H_3=29.4762, p<0.001 **b)** Per capita pyoverdine production is reduced after evolving in highly toxic copper for ~100 bacterial generations, compared to other metal treatments (1-way ANOVA & Tukey HSD, $F_{3,44}$=45.952, p<0.001) c) The reduction in per capita pyoverdine production instigated by high copper toxicity (relative to high iron control) is stronger than the reduction caused by iron-limitation (relative to standard KB broth). When y = 1, treatment has no effect of reducing pyoverdine relative to control. Data are means (n=12) ± SEM.

To ascertain the relative importance of copper-mediated pyoverdine reduction in bacterial populations, we repeated our evolution experiment using non-toxic iron-limited and standard KB broth. After 33 daily transfers (~200 bacterial generations) per capita pyoverdine production was lower in iron-limited populations than KB broth (Student's t-test, t_{22}=3.6208, p<0.005). Moreover, this effect of iron-limitation on pyoverdine production after 200 generations was significantly weaker than the high copper mediated decrease in pyoverdine production after just 100 generations (Welch t-test, $t_{12.254}$=4.2841, p<0.005).

3.2 Competition experiment

We next carried out an ecological growth rate experiment to assess whether it is beneficial to produce siderophores in the presence of toxic copper, but costly in competition with non-producers. To do this, we measured the densities of wildtype and mutant strains grown for 24 hours in monoculture and co-culture under low (624μM) and high (6.17mM) copper and iron conditions.

We find that in the high iron treatment, mutant relative fitness is equivalent between monoculture and co-culture conditions, and relative mutant fitness is not affected by growth conditions (relative fitness = 1; Figure 2). In low iron treatments however, mutants seem to obtain a small fitness advantage in co-culture compared to monoculture. This might be explained by the cost of producing siderophores when they are not required. Moreover, when grown at both copper concentrations, mutants in monoculture have greatly reduced fitness but recoup these costs in co-culture, greatly exceeding fitness of the wildtype in high copper (Figure 2; evidenced by 2-way interaction between metal and growth conditions: $F_{1,88}$ = 30.583, p<0.001).

We also found differential impacts of metal concentrations on mutant fitness, between the two metals (Figure 2; evidenced by 2-way interaction between metal and concentration, $F_{1, 88}$ = 11.612, p<0.001). Whereas high copper concentration greatly increased the extent of mutant invasion compared with low copper (relative fitness >1) (1-sample t-test on relative fitness, alternative=1, high copper: t_{11}= 7.3307, p<0.001, low copper: t_{11}= 2.6615, p<0.05), high iron actually prevented mutants from invading, compared with low iron (1-sample t-test on relative fitness, alternative=1, high iron: t_{11}= 1.6955,

p=0.118, low iron: t_{10}= 3.2794, p<0.01). These differential impacts of metal concentration were not affected by growth conditions (evidenced by non-significance of 2-way interaction between growth conditions and concentration, $F_{1,87}$ = 0.0229, p=0.88).

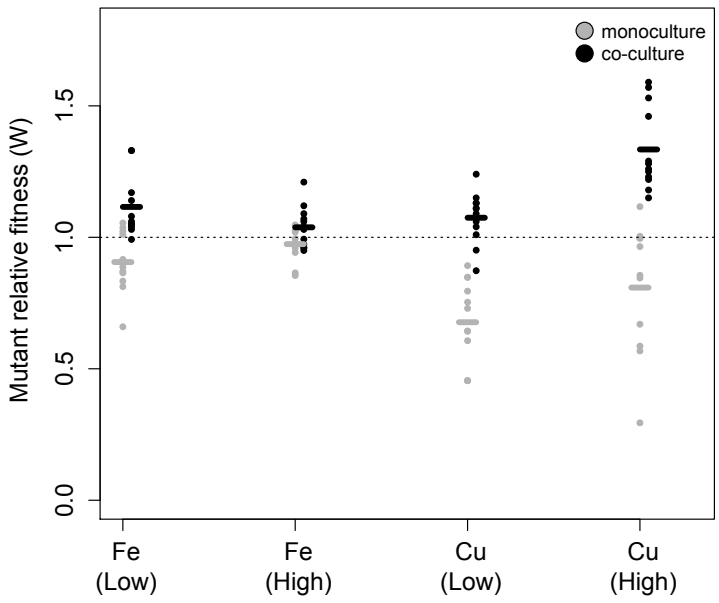

Figure 2: The relative fitness of mutants compared to wildtype (W) depends on an interaction between metal species (copper or iron) and growth conditions (mono/co-culture) (2-way interaction between metal and growth conditions: $F_{1, 88}$ = 30.583, p<0.001), and an interaction between metal species and metal concentration (high= 6.17mM, low = 624μM) (2-way interaction between metal species and concentration, $F_{1, 88}$ = 11.612, p<0.001). In co-culture (black circles), W > 1 (i.e. mutants invade) for highly toxic copper concentrations only (1-sample t-test on relative fitness, alternative=1, t_{11}= 3.338, p<0.01).

4. Discussion

In this study we demonstrate that siderophore-mediated detoxification of heavy metals by bacteria is an altruistic trait, open to invasion by non-producing social cheats. We first show that spontaneous non-producing mutants in initially clonal wildtype *P. aeruginosa* populations reach much higher frequencies in highly toxic compared with non-toxic environments. This is based on the premise that siderophore production is most costly when it is needed most (Brockhurst et al.

2008), and consequently there is stronger selection for siderophore-negative mutants. We next confirm the fitness advantage of non-producing mutants in toxic (and refute it in non-toxic) environments by competing wildtype bacteria and an isogenic siderophore-negative mutant, and also demonstrate a large growth rate benefit of siderophores in toxic environments when grown as monoculture (the latter result previously shown by Braud et al. (2010)). Finally, we validate the relative importance of toxicity-mediated social cheating by showing that the effect of toxicity in reducing pyoverdine levels is stronger than the effect of iron-limitation.

A key implication of our results is that heavy metal contamination is likely to influence the evolution of siderophore production, and hence the extent of detoxification in natural populations. As for many microbial cooperative traits, precisely how will depend on population structure and the scale of decontamination (West et al. 2007c). If populations are highly structured, such that immediate neighbours are likely to be clonal and detoxification effects are localised, contamination may favour upregulation of siderophores through kin selection (Hamilton 1964; Maynard Smith 1964) with siderophore production carrying both direct and indirect fitness benefits. However, if populations are mixed (e.g. aquatic environments, as in this study) then there is likely to be selection for reduced siderophore production as benefits are less likely to be received by clone mates (reduced indirect benefits).

Selection for siderophore production in contaminated environments will of course also be influenced by the primary selection pressure acting on this trait: iron availability. Arguably, the ubiquity of siderophores suggests bacteria are generally iron-limited, and there is both direct and indirect evidence of siderophore exploitation by cheats in natural populations (Meyer et al. 1997; De Vos et al. 2001; Pirnay et al. 2002; Pirnay et al. 2005; Cordero et al. 2012; Lee et al. 2012). Toxic environments may then simply further increase selection for the production and exploitation of siderophores, in a similar response to reduced iron availability. However, there are reasons to believe that selection on siderophores for detoxification may be stronger than for iron-scavenging in some contexts. First, heavy metal contamination might logically be associated with an excess of bioavailable iron. Indeed, lower pH conditions increase iron

solubility (reducing the advantage of siderophores), while potentially increasing the bioavailability of toxic heavy metals. In this context, siderophore production is likely to be driven by the need for detoxification rather than iron-scavenging. Second, the spatial scale of the effect of siderophores on iron acquisition and detoxification may differ, altering indirect fitness benefits. We would speculate that the spatial scale of benefit for iron acquisition is likely to be more local, simply because the binding of siderophore-iron complexes to the correct receptor facilitates the transport of iron into the cell, and hence these complexes have a shorter half-life. Third, while siderophore-mediated iron acquisition is relatively species specific (although there is some evidence that species can exploit each other's iron-siderophore complexes - see Meyer et al. (1999) and D'onofrio et al. (2010)), decontamination can potentially benefit the entire community. This means that siderophore exploitation may not only occur within species but also between species. Again it is unclear how siderophore production will be affected in this context, but it is likely that this will make exploitation relatively easier, hence increasing the frequency of non-producing individuals in the populations. This multi-species interaction has recently been coined as the Black Queen hypothesis (Morris et al. 2012), and predicts that public goods will be lost first in the more abundant species and continue to be lost until any further loss is offset by the cost. Notably, our finding that toxicity-mediated pyoverdine reduction is much stronger than the reduction caused by iron-limitation suggests that the social responses of *P.aeruginosa* to toxic environments may play a key role in environmental processes.

Our results may also be relevant in trying to improve the effectiveness of applying siderophore indirectly or directly for decontamination purposes. This approach has proved successful. For example, siderophores isolated from *Pseudomonas azotoformans* removed 92.8% of arsenic from the environment, much higher than EDTA (76.4%) and tap water (65.8%) (Nair et al. 2007), while the addition of *Alcaligenes eutrophus* has been demonstrated to decrease solubility of Cd, Zn and Pb in sandy soils (Diels et al 1999). Moreover, siderophore-producing bacteria have also been employed as a mechanism to enhance phytoremediation, whereby bacteria are added to soil containing metal-accumulating plants (Wu et al. 2006; Li & Ramakrishna 2011; Rojas-Tapias et al. 2012), although not always successfully (see Kuffner et al. 2008).

Siderophore containing filtrates have also been shown to enhance fungal growth with 0.8-32.4% and 0.7-20.8% biomass increase for Cd and Zn, respectively (Cao et al. 2012).

A key point highlighted by our study is that bioremediation of any kind that relies on a steady production of siderophores, may be impeded by strong selection for siderophore-negative cheats, reducing remediation efficiency. If this is the case, the implications for bioremediation are notable, whereby a high level of bacterial cooperation is essential for efficient removal of toxic materials. Notably, in natural soils, we have found copper concentrations as high as 600ppm (unpublished data), far higher than even our 392ppm high copper treatment, therefore our results represent realistic levels of copper toxicity experienced by microbial communities. Our results predict that bioremediation may be made more efficient by establishing conditions that make cheats less viable; for example, by promoting the oxidation of Fe^{2+} ions to Fe^{3+}, through the addition of lime-containing materials, or an alternative alkaline substance. Since Fe^{3+} is not readily bioavailable to bacteria, siderophores must chelate iron as well as detoxify, ensuring only populations with a high level of siderophore production are sustainable.

5. Public goods cooperation in a community context

Abstract

Microbial social interactions are ubiquitous, and are important for both environmental functioning and as determinants of virulence. Recent work has indicated that within-species social interactions may be strongly affected by community context. Despite this, empirical studies investigating the ecology and evolution of social interactions have only been carried in single-species systems or *in vitro* 'communities' consisting of a few species. Here, we investigate how the cooperative production of detoxifying public goods (siderophores) by the opportunistic pathogen *Pseudomonas aeruginosa* is shaped by the presence of the natural microbial community (NMC), in a soil environment. Siderophores are predominantly used by bacteria for iron-acquisition but have also been shown to play a role in bacterial detoxification of heavy metals. The crucial difference between these two contexts it that while only conspecifics can benefit from siderophore-mediated iron-acquisition, the entire community can benefit from detoxification. We find an overall cost to not producing siderophores, but that the presence of both siderophore-producing conspecifics and the natural microbial community negates this costs in copper-contaminated environments only. These results suggest that siderophores act as a community-wide public good in heavy metal -polluted environments. Our results may have implications for industrial siderophore-based bioremediation approaches, which rely on a consistently high production of siderophores to maintain high bioremediation efficiency.

1. Introduction

Bacteria do not live in isolation; they exist within a complex network of social, competitive, predator-prey and host-parasite interactions. Such interactions can have a marked effect on a species' evolutionary trajectory, which in turn may influence community context itself. There is growing evidence that the direction and strength of evolution may be fundamentally altered by community context (Stinchcombe & Rausher 2001; Buckling & Rainey 2002a,b; Strauss et al. 2006; Meyer & Kassen 2007; Pal et al. 2007; De Mazancourt et al. 2008; Friman et al.

2008; Harrison et al. 2008; Johansson 2008; Paterson et al. 2010; Collins 2011; Zhang & Buckling 2011; Lawrence et al. 2012; Morgan et al. 2012; Friman & Buckling 2013; Gómez & Buckling 2013a; Celiker & Gore 2014; Fiegna et al. 2014). Despite this, the vast majority of microbial studies investigating the evolution and ecology of a focal trait occur in well-mixed environments comprising of a single species. Although such studies are crucial for investigating fundamental factors that can influence a focal trait's fitness, they tell us little about whether they actually matter in nature. Here, we examine the effect of the natural microbial community on the evolution of public goods production in a focal species of bacteria, *Pseudomonas aeruginosa*. Specifically, we investigate how the community can affect cooperative siderophore-mediated heavy metal detoxification: a system that is vulnerable to non-producing 'cheats' who pay little or no cost of production, but can benefit from siderophores produced by the sideorphore-producing community (Braud et al. 2010; O'Brien et al. 2014).

Despite that bacterial public goods are crucial determinants of virulence (West & Buckling 2003; Buckling & Brockhurst 2008) and environmental functioning (Ahmed & Holmstrom 2014) there are currently only a handful of studies exploring the impact of the community on the evolution of bacterial public goods. Harrison et al. (2008) demonstrated the impact of multispecies competition on shaping the evolutionary trajectory of a focal trait: siderophore production. When in direct competition with *Staphylococcus aureus*, siderophore production in *P. aeruginosa* was upregulated and consequently non-producing 'cheats' evolved more rapidly compared to when in the absence of *S. aureus.* Predation or parasitism may also affect evolutionary trajectories of social interactions; Morgan et al. (2012) found that in the presence of the lytic DNA bacteriophage, SBW25Φ2, *Pseudomonas fluorescens* siderophore cooperators are less vulnerable to invasion by cheats (versus populations grown in the absence of phage) because resistant mutations are more likely to arise in the numerically dominant cooperator population. While 'communities' in such studies consist of just two species, they nevertheless indicate that the natural microbial community is an important determinant of the outcome of public goods cooperation.

Reinforcing this idea are simulations demonstrating that the effect of the community on the evolution of cooperative secretions depends on the degree of nutrient competition; when competition is high, the community inhibits cooperation as cooperators are eradicated before they become established, whereas under low competition cooperators benefit from the community as they are less vulnerable to cheats of their own species (Mitri et al. 2011).

Recently, the role of the community in mediating reductive gene loss has been documented by the Black Queen hypothesis (BQH) (Morris et al. 2012). One species may benefit by losing an essential biosynthetic 'leaky' function, such as a vital public good, provided the function is retained by at least one other species in the community. For instance, in the presence of hydrogen peroxide (H_2O_2), a toxic by-product of marine photosynthesis, *Escherichia coli* carriers of the catalase-peroxidase gene KatG and susceptible cells of *E. coli* are able to coexist for 1200 generations as the carrier reduces cost and increases efficiency of KatG production and the susceptible remains dependant on the detoxification capabilities of the carrier. In contrast, in the absence of H_2O_2, the susceptible drives the carrier to extinction (Morris et al. 2014). While the exact evolutionary explanations have not been elucidated, there is evidence of Black-Queen mediated gene loss operating in nature. For example, in natural marine environments, most *Prochlorococcus sp.* lack the KatG enzyme while it is retained in *Synechococcus sp*. (Scanlan et al. 2009; Morris et al. 2011). Endosymbionts may take advantage of host's public goods, for example *Buchnera sp.* (endosymbionts of *Acyrthosiphon pisum),* have undergone substantial genome reduction, losing the ability to synthesise many factors supplied by the host, such as non-essential amino acids, fatty acid biosynthesis, genes involved in transport and secretion, DNA repair and cell defence (Shigenobu et al. 2000; Moran et al. 2009). Moreover, growth of unculturable bacteria can be enhanced through the addition of siderophores isolated from neighbouring species, such as the ability of acyl-desferrioxamine siderophores isolated from *Micrococcus luteus* culture supernatant to induce growth of the otherwise unculturable *Maribacter polysiphoniae* (D'onofrio et al. 2010), indicating that the community is driving the loss of this costly biosynthetic trait (D'onofrio et al. 2010). In general, these examples suggest that a good strategy is to be part of a lineage that avoids paying the cost of a specific public good,

but live in close proximity to a lineage that does. Moreover, the extent to which the community affects the evolution of cooperative secretions is likely to be stronger when the entire community benefits from the public good, and not solely conspecifics, such as in the context of sideorphore-mediated detoxification.

Despite such studies indicating that the community can have an important impact on evolution, there has been no experimental investigation into how the relative costs and benefits of public good production is affected by the natural microbial community. Here, we examine whether cooperative siderophore-mediated heavy metal detoxification is affected by the microbial community in a natural soil environment. While siderophores are predominantly iron-scavenging molecules produced by bacteria when iron is limited, they also chelate an array of other toxic heavy metals. Bound toxic metals are unable to enter cells efficiently by diffusion nor enter cells via the species-specific siderophore receptor used for iron-uptake. Hence, in toxic metal environments, siderophore production can be advantageous to cells: as evidenced by i) decreased sensitivity of siderophore producers compared with non-producers in toxic metal environments (Braud et al. 2009, 2010; O'Brien et al. 2014) and ii) the ability of 100 mM Cu^{2+} and Ni^{2+} to upregulate the primary siderophore, pyoverdine by 290% and 380%, respectively (Braud et al. 2010).

2. Materials and Methods

2.1) Bacterial Strains

Pseudomonas aeruginosa strain PAO1 was used as a siderophore producing wildtype strain, and an isogenic mutant strain PAO1*ΔPvDΔPch* (Ghysels et al. 2004) with both primary and secondary siderophores pyoverdine and pyochelin knocked out, was used as a siderophore negative mutant. To facilitate isolation of these strains from the natural microbial community following inoculation, we inserted a gentamicin-resistant cassette into both strains prior to use. Furthermore, we inserted a neutral Lac Z reporter gene into PAO1 (producer), so that this strain appeared blue/green on xgal-supplemented media, enabling producers and non-producers to be distinguished with ease. To isolate the natural microbial community (NMC), 40g of fresh 50% peat-free soil was added

to 200mls M9 minimal salt solution and incubated shaken at 150rpm at 28 °C for 24h. Following this period, the supernatant was plated out neat on LB hard agar to verify the presence of a microbial community, and also on LB supplemented with 30µg/ml gentamicin to ensure the community was susceptible to this antibiotic concentration.

2.2) The effect of copper concentration on wildtype growth

We first established a concentration of $CuSO_4$ that hindered growth of *P. aeruginosa,* without resulting in population extinction. Eighteen microcosms were filled with 30g twice-autoclaved 50% peat-free soil in round 90mm petri dishes. Luján et al. (2015) demonstrated that *P. fluorescens* non-producers experience a fitness cost in soil that they recoup in the presence of producers, in 30% and 70% peat-free soil. Hence, we can assume that our 50% peat-free soil is iron-limited and will incur a fitness cost of not producing siderophores. Each microcosm was inoculated with ~10^7 CFU's/ml PAO1, and placed in an environmental chamber at 26 °C and 75 % RH. After 48 hours of growth, we i) assayed population density of each microcosm (see part *2.4* below) and ii) supplemented each microcosm with 2ml filter sterilised 0.1M $CuSO_4$, 0.5M $CuSO_4$ or ddH_2O, with 6 replicates for each treatment. Microcosms were returned to the environmental chamber for 6 days, after which density was assessed once again. Population growth per day was >0 for control (no $CuSO_4$) (1-sample t-test, alt=0, t_5= 10.4949, p<0.001, figure 1), <0 for .5M $CuSO_4$ (1-sample t-test, alt=0, t_5= 3.5099, p<0.05, figure 1) and was not different from 0 in 0.1M $CuSO_4$ treatments (1-sample t-test, alt=0, t_5= 1.0526, p=0.34, figure 1). Consequently, 0.1M $CuSO_4$ was the chosen copper concentration for the subsequent experiments.

2.3) Competition experiment

We wished to assess the impact of the NMC on siderophore-mediated detoxification, by quantifying the relative fitness of producers and non-producers in each treatment in a fully factorial experimental design (+/- Copper, +/- NMC, figure 2). We established 72 microcosms in 90mm round petri dishes containing 30g of twice-autoclaved 50% peat-free soil, and inoculated ~10^7 CFU's/ml PAO1, 10^7 CFU's/ml PAO1*ΔPvDΔPch* or both strains in a 1:1 mixture (so that the total inoculum was 10^7 CFU's/ml), into to 24 microcosms each.

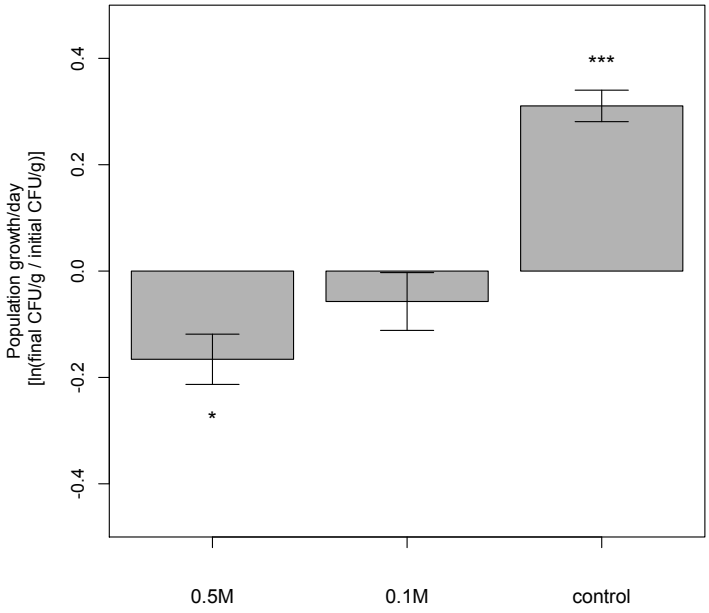

Figure 1: In order to first establish a concentration of $CuSO_4$ that hindered wildtype population growth (without causing extinction), wildtype population growth was assessed in the presence of .1M and .5M $CuSO_4$. Population growth per day was >0 for control (no $CuSO_4$) (1-sample t-test, alt=0, t_5= 10.4949, p<0.001) <0 for .5M $CuSO_4$ (1-sample t-test, alt=0, t_5= 3.5099, p<0.05) and was not different from 0 in 0.1M $CuSO_4$ treatments (1-sample t-test, alt=0, t_5= 1.0526, p=0.34). 0.1M $CuSO_4$ was selected as the concentration for the remaining experiments. Data are means (n=6) ± SEM.

Finally, the isolated NMC was inoculated into 36 microcosms (12 each from PAO1, PAO1*ΔPvDΔPch* and 1:1 mixture) and 2ml M9 minimal salt solution was added to remaining microcosms. Microcosms were placed in an environmental chamber at 26 °C and 75 % RH for 48 hours. Following this period we i) assessed population density of *P. aeruginosa* strains in each microcosm (see part 2.4 below) and ii) added 2mls filter-sterilised 0.1M $CuSO_4$ to 36 plates (12 each from PAO1, PAO1*ΔPvDΔPch* and 1:1 mixture), and 2ml sterile ddH_2O to the remaining microcosms, in a fully factorial experimental design (figure 2). Microcosms were returned to the environmental chamber at 26°C and 75 % RH for 5 days, after which, the population density of *P. aeruginosa* in each microcosm was assayed once more.

	+ NMC	- NMC
+ Copper	Wildtype [6]	Wildtype [6]
	Mutant [6]	Mutant [6]
	1:1 co-culture [6]	1:1 co-culture [6]
- Copper	Wildtype [6]	Wildtype [6]
	Mutant [6]	Mutant [6]
	1:1 co-culture [6]	1:1 co-culture [6]

Figure 2: Experimental design for competition experiment examining the effects of copper and the natural microbial community (NMC) on siderophore production in *P. aeruginosa.* (wildtype: PAO1 siderophore producer, mutant: PAO1ΔPvDΔPch siderophore non-producer)

2.4) Assaying for P. aeruginosa population density

Initial density was taken 2 days post inoculation (enabling bacteria to reach modest densities in order to withstand the addition of copper and/or NMC) and again 5 days after the addition of copper and/or NMC (final density). 1g of soil was transferred from each microcosm to 6mls M9 minimal salt solution in 30ml glass vials. Vials were shaken for 2 hours at 28°C at 180rpm, after which the supernatant was diluted in M9, and plated on LB agar plates supplemented with 90µg/ml Xgal and 30µg/ml gentamicin. Plates were incubated at 37°C for 18 hours, when individual colonies could be identified and counted.

2.5) Statistical Analyses

All data were analysed using R version 2.15.1 (R Core Team, 2012). We determined population Malthusian growth rate per day (*m*) as ln(final density/start density) / growth duration (days) [24]. Relative fitness of strain *x* compared with strain *y* (W(*x*)) was calculated in co-culture as *m*(strain *x*)/*m*(strain *y*), and in monoculture as *m*(strain *x*)/mean(*m*(strain *y*)). When W(*x*) = 1, relative fitness of strain *x* = strain *y.*

Population growth rates (*m*) were compared between strains using 2-sample t-tests or Wilcoxon rank-sum tests if they failed to comply with assumptions of

normality. We used general linear models (GLM's) and F-tests to investigate 1) whether copper (presence/absence) and the NMC (presence /absence) impacted growth (*m*) of producing and non-producing strains, including the interaction and 2) whether copper (presence/absence), NMC (presence /absence) and condition (mono / co-culture), including a 3-way interaction, impacted the relative fitness of mutant compared to wildtype (*W*.mutant). In this case, the natural log of *W*.mutant was assigned as the numeric response variable to facilitate a Gaussian error structure. Finally, treatments in the presence and absence of copper were analysed independently using two separate GLM's to investigate the effects of growth condition and the NMC on *W*(mutant).

To assess whether relative fitness (W(*x*)) was different from 1, 1-sample t-tests or Wilcoxon signed-rank tests were employed. Our dataset contained a mutant population which had an unusually high starting density (and hence reduced growth rate) and was consequently not included in our analysis. However removing this datapoint did not affect the outcome of any statistical significance.

3. Results

3.1) Analysis of growth rates

We examined the relative impact of copper toxicity, and the NMC on growth rate (*m*) of producer and non-producer populations. Based on the premise that bacteria can utilise siderophores produced by the NMC in the context of copper detoxification, but not for iron-acquisition, we predicted that copper would reduce growth rate of both producers and non-producers, but that the impact of copper on growth rate would be ameliorated in the presence of the NMC. Indeed, in both producer and non-producer populations, the impact of copper on reducing growth was only evident in the absence of the NMC (GLM, copper*NMC interaction, producers: $F_{1,20}$=25.616, p <0.0001, non-producers: $F_{1,19}$=32.355, p<0.0001, figure 3).

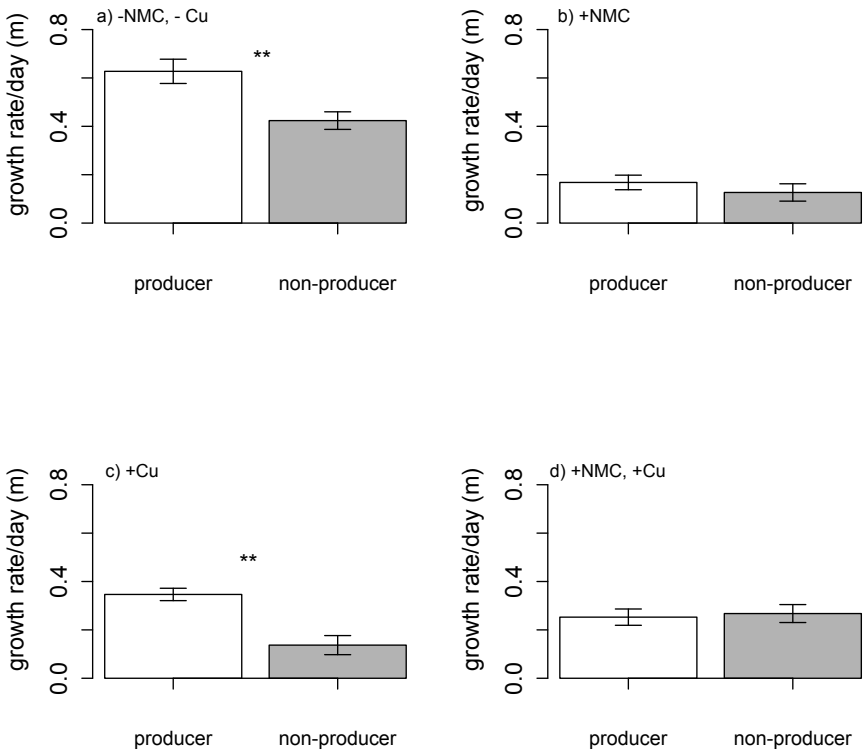

Figure 3: Growth rates (m) of independently grown producers and non-producers. While copper has a negative impact on growth of both producers and non-producers, this effect is negated in the presence of the NMC. (GLM, copper*NMC interaction, producers: $F_{1,20}$=25.616, p <0.0001, non-producers: $F_{1,19}$=32.355, p<0.0001). Data are means (n=6) ± SEM

3.2) Relative fitness measures

We next explored how the impact of the NMC and copper toxicity on absolute growth is manifested differently in producer and non-producer populations, quantifying fitness of the non-producing mutant relative to producers, *W*.mutant, under each treatment condition. We found that under monoculture conditions, the NMC can work to negate the negative impact of copper on non-producer fitness (i.e. non-producers can use detoxifying siderophores produced by the NMC), whereas in co-culture, the NMC cannot ameliorate the fitness costs of toxicity (3-way interaction between the presence of copper, NMC and growth conditions (mono or co-culture) (GLM, copper*NMC*condition: $F_{1,39}$=4.3829, p<0.05, figure 4))

In the absence of copper (solely iron-limited; where siderophores are generally species specific), non-producers are predicted to be able to exploit conspecific siderophores for iron-acquisition but not that of the majority of the NMC. However, neither the presence of producing conspecifics (GLM: $F_{1,21}$=1.1011, p=0.31, figure 4a), nor the NMC (GLM: $F_{1,20}$=0.4079, p=0.53, figure 4a) increased non-producer fitness relative to producers, suggesting non-producers were not able to exploit conspecific producers sufficiently well enough to significantly increase their growth. Furthermore, we could not detect any interaction between the presence of producing conspecifics and the NMC on shaping non-producer relative fitness (GLM: $F_{1,19}$=0.5183, p=0.48, figure 4a). Finally, while non-producers do incur a cost of not producing siderophores in iron-limited conditions (1-sample Wilcoxon signed rank test, alt=1: V=9, p<0.05, figure 4a), we were unable to find any evidence that non-producers could invade populations of producers when in initiated at a 1:1 mixture, and non-producers remained less fit than producers (1-sample Wilcoxon signed rank test alt=1, V=12, p<0.05, figure 4a).

Focusing now on toxic copper treatments, where siderophores can act as a community-public good; non-producers are predicted to suffer a growth cost of not producing siderophores, which can be negated in the presence of producing conspecifics or producing members of the NMC. Accordingly, we found a positive effect of the NMC on non-producer relative fitness under monoculture growth conditions, which was less evident when non-producers were grown in co-culture with producers (GLM, condition*NMC interaction: $F_{1,20}$=4.2963, p=0.05, figure 4b). However, both the NMC (GLM: $F_{1,21}$=6.6906, p<0.05, figure 4b) and presence of conspecific producers (GLM: $F_{1,21}$=10.748, p<0.01, figure 4b) independently enhanced relative fitness of non-producers.

Given that the NMC differentially affected growth rates in monoculture and co-culture, we determined the fitness of non-producers relative to producers for each trait combination separately. Non-producers incurred a fitness cost of not producing siderophores in toxic copper (1-sample t-test, alt=1: t_5=5.2973, p<0.01, figure 4b), and that this cost was negated in the presence of producers (Wilcoxon signed rank test, alt=1: V=12: p=0.84, figure 4b). The effect of copper

on reducing *W*.mutant (relative to *W*.mutant=1, where producer and non-producer fitness is equal) was greater than the reduction brought about by iron-limitation (Student's t-test: t_{10}= 2.1854, p=0.05, figure 4), so that this effect could not be solely explained by iron-limitation. Based on the premise that siderophore-mediated detoxification is not species-specific, the NMC is predicted to negate the cost of being a non-producer since our focal *P. aeruginosa* non-producer can utilise siderophores produced by the entire community for detoxification. Accordingly, there was no obvious cost to the non-producer in the presence of the NMC, both in the presence and absence of producers (1-sample t-test, alt=1: -producers: t_5=0.3957, p=0.71, +producers: t_5=1.6486, p=0.16, figure 4b).

4. Discussion

Here, we investigate how cooperative siderophore-mediated detoxification is shaped by the presence of the natural microbial community (NMC), in a natural soil environment. In iron-limited soil environments, we find a cost to not producing siderophores, which cannot be rescued by conspecific producers or the NMC. In toxic copper soils, we find a higher cost to not producing siderophores, compared with the cost observed in solely iron-limited soils, however this cost is negated in the presence of conspecific producers and/or the NMC. This work demonstrates the importance of the NMC in shaping the outcome of cooperative traits, when the cooperative trait is not-species specific. Moreover, our study suggests that while non-producers cannot efficiently exploit conspecific siderophores produced in iron-limited spatially structured environments, they are able to do so in heavy metal contaminated environments.

Despite our finding that in the absence of the NMC, non-producers are more efficient in exploiting producers for detoxification rather than iron-acquisition, non-producers fail to fully invade populations of producers in either context. Luján et al. (2015) demonstrated that *P. fluorescens* non-producers exhibit a cost of not producing siderophores in iron-limited soil microcosms, but can negate this cost in a 1:1 co-culture with producers, and furthermore, can invade populations of producers when inoculated from rare at 1:100. This discrepancy

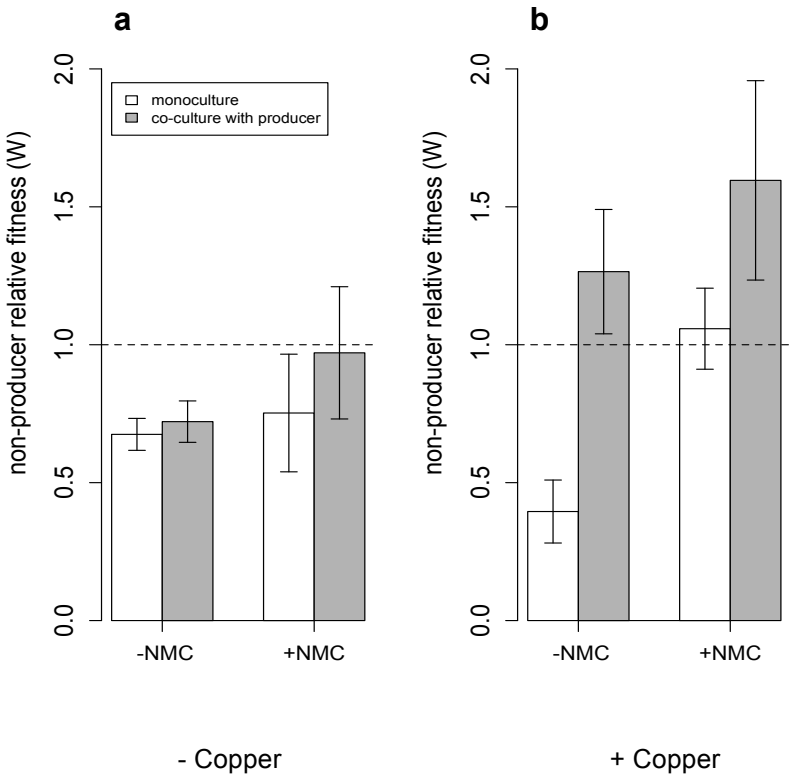

Figure 4: Non-producer relative fitness (*W.* mutant) in (a) the absence of copper (solely iron-limited) and (b) in the presence of copper. In the absence of copper **(a)** neither the presence of producing conspecifics (GLM: $F_{1,21}$=1.1011, p=0.31), nor the NMC (GLM: $F_{1,20}$=0.4079, p=0.53) increase *W.*mutant. In the presence of copper **(b)** *W.*mutant is enhanced in both the presence of producing conspecifics and the NMC, and is best described by a 2-way interaction between social condition and NMC, so that the positive effect of the NMC on *W.*mutant is strongest in the absence of producers (GLM, condition*NMC interaction: $F_{1,20}$=4.2963, p=0.05).When y=1, fitness of producer and non-producer is equal. Data are means (n=6) ± SEM.

may be explained by enhanced growth of *P. fluorescens* (K = ~10^6) in soil relative to *P. aeruginosa* (K = ~10^4). Higher densities can increase relative fitness of non-producers because non-producers are better able to exploit the cooperative siderophore production of other cells when they are physically closer to them (Greig & Travisano 2004; Ross-Gillespie et al. 2009). However, the finding that non-producers cannot invade producers when initiated at 1:1 is

in contrast to studies carried out in iron-limited (Griffin et al. 2004) and toxic copper (O'Brien et al. 2014) liquid media. This then begs the question: why are non-producers able to fully exploit siderophores in liquid media but not in soil? Soil spatial structure tends to keep producers and their products together, meaning less siderophore is lost to the environment and siderophore that is normally exploitable by cheats is less accessible (Kummerli et al. 2014). Moreover, such bacterial clusters are generally clonal; the beneficiaries of cooperative products are highly related to the producer so that the system is less vulnerable to cheats (Kummerli et al. 2009). While this is bad news for cheaters of iron-scavenging, it may be less of a concern for detoxification. Since siderophore-heavy metal complexes do not enter the cell, they are likely to have a more far-reaching effect than siderophore-Fe^{3+} complexes, meaning that non-producers on the exterior of these clusters may eventually benefit from a reduction in toxicity. Spatial structure is ubiquitous in the microbial world and there is increasing evidence that it can shape the outcome of cooperation (Cordero et al. 2012; Luján et al. 2015). Our work demonstrates that while spatial structure can constrain the evolution of cheating in siderophore mediated iron acquisition, it may not be as prevalent in curtailing detoxification cheats.

Our findings unequivocally demonstrate that the presence of the NMC can have a marked effect on the fitness of a focal strain, specifically by negating the fitness cost of not producing siderophores in the context of detoxification. When non-producers were cultured in the presence of siderophore producers, there was no appreciable effect of the NMC on enhancing non-producer fitness, presumably because producers were at ample frequency for non-producers to exploit. One caveat is that the effect of the community may be a result of other environmental changes associated with the addition of the community (e.g. pH), and not strictly siderophores. Nonetheless, out results give an indication that the community *per se* may play a crucial role in shaping the fitness of these traits, in particular by enhancing the fitness of non-producers. Furthermore, while in single-species systems the relative fitness of non-producers is negatively frequency dependent (Ross-Gillespie et al. 2007), our results indicate that in nature, a lineage of non-producers may be able to reach fixation in a population (providing that adequate cooperative product is produced by the community), as predicted by the Black Queen hypothesis (BQH) (Morris et al. 2012).

Microbes exhibit a wealth of such 'leaky' public goods, which could be reasoned to have beneficial effects for the entire community. For example, β -lactamase production by Gram-negative bacteria benefits the surrounding community by inactivating β -lactam antibiotics, preventing them binding to penicillin binding proteins in the cell wall (Ciofu et al. 2000; Dugatkin et al. 2005). Dugatkin et al. (2005) demonstrated that β –lactamase enzymes are indeed public goods; an ampicillin sensitive strain of *E.coli* was able to gain a fitness benefit from TEM-1 β lactamase produced by a second strain of *E.coli.* Immune-modulation molecules, which supress the function of the host immune system, are also likely to indirectly benefit the microbial pathogenic community. This includes the production of quorum-sensing signal molecules, which can have negative pleiotropic effects on eukaryotic cells, particularly those involved in host immunity. Two of these signal molecules 3-Oxo-C12-HSL and PQS exhibit an arsenal of defences against the host immune system, including the acceleration of apoptosis in macrophages and neutrophils (Tateda et al. 2003), inhibition of cell proliferation and IL-2 release and inhibition of tumour necrosis factor alpha release from human monocytes (Hooi et al. 2004). Such sharing of public goods among species is not limited to bacteria; parasitic helminths have developed strategies that dampen their host's immune response (Allen & MacDonald 1998; Hartgers & Yazdanbakhsh 2006; Kamal & Khalifa 2006; Correale & Farez 2007; Cooke 2008; Hartmann et al. 2013). For example, *Onchocerca volvulus* interferes with effector T cell function and surpresses antigen-specific stimulation *in vitro* (Hartmann et al. 2013). Similarly, *Brugia malayi, Nippostrongylus braziliensis and Toxocara canis* implanted into the lungs of mice, resulted in immunosuppression by a reduction in T cell proliferations (Allen & MacDonald 1998). Moreover, the dampening of the immune response initiated by parasitic helminths has been demonstrated to increase virulence of viral infections such as Human Immunodeficiency virus (HIV), Hepatitis B virus (HBV), and Hepatitis C virus (HCV) (Kamal & Khalifa 2006). Despite the prevalence of such leaky public goods *in vivo*, we have little idea as to how their fitness and hence evolutionary trajectory (and consequently their impact on virulence) is affected by the presence of the entire community. Moreover, the role of the community in shaping trait fitness and *vice versa* is not limited to cooperative traits; for example, production of reuterin (a broad-spectrum

antimicrobial) by lactobacillus inhibits growth of species representing an array of bacterial genera including *eschericheria, salmonella, shingella, proteus, pseudomonas, clostridium, and staphylococcus* (Axelsson et al.1989). Such antagonistic traits are potentially more likely to speed to up the rate of evolutionary change and hence strengthen the impact of the community.

It is difficult to predict how such interspecific interactions, whereby relatedness between producer and recipient is low, can be maintained in complex ecological networks. The Black Queen hypothesis (BQH) suggests that one species within a community may benefit by losing an essential biosynthetic 'leaky' function, such as a vital public good, provided the function is retained by at least one other species in the community (Morris et al. 2012). This reduces the requirement for high relatedness; sequential loss of a cooperative trait will proceed from species to species, until any further cheating will be offset by the cost to the individual of not having enough public goods. However, recent studies have challenged the idea that interspecific cooperation can be maintained, because i) they render a cell dependant on a lineage which may not be nearby (Oliviera et al. 2014) ii) the evolution of cooperative exchanges reduces community productivity relative to an autonomous strain that produces everything it needs (Oliviera et al. 2014) and iii) competitive interactions are likely to predominate over cooperative ones (Foster & Bell 2012). This leads to a specific set of conditions under which interspecific cooperation will only be maintained when satisfied: namely that there are intermediate levels of mixing (Oliviera et al. 2014), the overall effect of a second species on a focal species is beneficial and there is high intraspecific relatedness (Mitri & Foster 2013). The ability of the community to sustain a population of non-producers (i.e. interspecific cooperation) will have a marked impact on the relative frequency of producers or non-producers in a focal population, which may be critical when designing synthetic communities of producers for biotechnological applications.

There is overwhelming evidence that siderophores are capable of reducing heavy-metal toxicity, both in the lab and in natural soils (Nair et al. 2007; Braud et al. 2009; 2010, Cao et al. 2012; O'Brien et al. 2014). Despite this, more permanent adaptations such as augmented efflux systems, compartmentalisation, and plasmid-based *cop* systems are commonly observed

in nature to cope with the toxic effects of heavy metals. Lin & Olson (1995) isolated copper resistant bacteria from a copper corroded water distribution system and observed that 62% of the total isolates exhibited substantial resistance to copper, among which 49% of isolates had *cop* or *cop*-like gene systems as well as both compartmentalization and efflux systems. Moreover, activation of 4 *cop* genes in the plant pathogen *Pseudomonas syringe* enhanced copper resistance by accumulating and compartmentalizing copper in the cells periplasm (Cooksey et al.1994). Since the mechanisms of siderophore upregulation are already established, what might promote the evolution of such novel resistance mechanisms? The most intuitive explanation is that siderophores are effective enough to be advantageous but are not powerful enough to completely mitigate the effect of toxicity. Here, we report that while siderophore producers gain an advantage in toxic conditions, they fail to entirely recoup fitness compared with growth in non-toxic environments. The cost of siderophore production suggests that there is a limit to upregulation, and perhaps the most efficient route to resistance is to take advantage of siderophores in conjunction with the evolution of a novel mechanism of resistance. Second, a “fingers in pies” approach to resistance may reduce the negative effect siderophore cheats can have on a population. Siderophore cheats that evolve from a population carrying a novel mechanism of resistance will continue to detoxify via this novel mechanism; thus, the negative effect of cheating on population fitness is reduced. Third, the tendency of siderophores to chelate an array of heavy metals reduces the number of free siderophores available to chelate ferric iron. For example, a low pyoverdine-iron uptake was observed when grown with 500 equivalents of pyoverdine loaded with Ag^{+}, Ga^{3+}, Hg^{2+}, Al^{3+}, Zn^{2+}, Mn^{2+}, Cd^{2+} or Co^{2+} (Braud et al. 2009), suggesting that in the presence of high concentrations of these metals, selection should act to promote alternative mechanisms of resistance. While the addition of Ga^{3+} to iron-starved *P. aeruginosa* is detrimental, depriving cells from essential siderophore-bound Fe^{3+}, Ross-Gillespie et al. (2014), found no evidence of evolved novel mechanisms of gallium resistance. However, as the damaging effects were mediated via iron inhibition and not because gallium is toxic *per se,* this is unsurprising. Finally, the effect of metal species in promoting alternative mechanisms of resistance should be considered. While pyoverdine produced by *P. aeruginosa* can inhibit the uptake of Al^{3+}, Cu^{2+} and Ni^{2+} by 79%, 85% and

40% respectively, it does not affect uptake of Ga^{3+} (Braud et al. 2010), and there is no evidence for pyoverdine-bound Tl^{+}, Tb^{3+}, Sn^{2+}, Pb^{2+}, Ni^{2+}, Eu^{3+}, Cu^{2+} and Cr^{2} inhibiting pyoverdine-iron uptake (Braud et al. 2009). In general, while siderophore upregulation may be a good short-term strategy in toxic conditions, selection for increased novel mechanisms of resistance will depend on the extent and nature of heavy metal toxicity and iron-limitation. However environmental contamination resulting from ore-containing minerals is associated with an arsenal of toxic heavy metals, so novel mechanisms of resistance should be the ultimately favoured approach.

Our results may have implications for improving the effectiveness of applying siderophore either directly or indirectly for decontamination purposes. While directly adding siderophores has proved successful (e.g. Nair et al. 2007; Cao et al. 2012) the addition of siderophore producing bacteria to soil (the effectiveness of which relies on consistently high siderophore levels) has had mixed results (Wu et al. 2006; Kuffner et al. 2008; Li & Ramakrishna 2011; Rojas-Tapias et al. 2012). The ability of bacteria to adapt and evolve on ecological timescales, combined with the fitness benefit non-producers experience in co-culture suggests that populations may be vulnerable to the rapid evolution of *de novo* cheats in toxic soils. One potential way to restrain the evolution of cheats in natural systems is to artificially create conditions whereby siderophore-production is essential for growth. Cheats cannot invade in co-culture under iron-limited soil environments, because they cannot gain access to siderophores produced by clusters of siderophore-producing bacteria. Consequently, any cheats that arise in response to detoxification will be subjected to reduced personal iron uptake and will go extinct. Hence, by ensuring iron is predominantly present in the unavailable ferric form Fe^{3+} (through the addition of $CaOH_2$), it may be possible to constrain any negative effect siderophore cheats may have on reducing bioremediation efficiency. More generally, our results demonstrate the importance of community context for understanding the evolution of social traits, which, in addition to ecosystem processes such as remediation, can have a major impact on disease virulence.

6. The interplay between rapid evolution and community context[4]

Abstract

The structure of microbial communities is key to their functionality. However, this structure is likely to be influenced by adaptive genetic change in members of the community, which can occur over a matter of days. Changes in community structure can in turn influence the evolutionary trajectories of species within the community, further altering community structure. Microbial communities provide evidence for this interplay between rapid evolution and community structure. To date, studies are primarily limited to simple in vitro systems, but we suggest similar processes are inevitably operating in both natural and derived communities important for industry.

Introduction

Microbial communities are key to a range of natural processes, ranging from health of their eukaryotic hosts (Huttenhower et al. 2012) to global nutrient cycling (Treseder et al. 2012). This functionality has been exploited by industry for the production of food, biofuels and waste treatment (Faust & Raes 2012). Recent years has seen a growing interest in both understanding the functionality of these communities and ultimately designing communities with optimal functionality (Purnick & Weiss 2009; Klitgord & Segre 2011; Chakraborty et al. 2012; Faust & Raes 2012). Surprisingly, there has been little attention on the importance of rapid evolution in determining community structure and function in these applied contexts.

There is now unequivocal evidence that microbes undergo rapid evolutionary change, such that evolutionary and ecological (i.e. changes in species composition within communities) time scales are often blurred (Buckling et al.

[4] Published as: O'Brien S, Hodgson, DJ & Buckling A. 2013. The interplay between microevolution and community structure in microbial populations. Current Opinion in Biotechnology 24:821-5 (see Appendix)

2009). As a result, rapid evolution (or microevolution) can potentially play a major role in the structure and function of microbial communities (Johnson & Stinchcombe 2007; Schoener 2011). Community context can in turn affect the rate and trajectory of evolution of focal species within the community, which in turn is likely to feedback into determining community structure (Johnson & Stinchcombe 2007; Schoener 2011) (figure 1). In this brief review, we discuss evidence for this interplay between microevolution and community structure in microbial communities. Although we are unaware of any work that explicitly addresses this interaction, evidence from experimental evolution studies in the lab provides a good foundation for considering how this potentially crucial issue is important in contexts directly relevant to biotechnology.

Rapid evolution on ecological timescales

Experimental evolution studies have shown real-time adaptive evolution in bacteria, viruses, fungi and algae (Lenski et al. 1991; Dettman et al. 2007; Cowen 2008; Buckling et al. 2009; Lachapelle & Bell 2012). Crucially, rapid evolution also occurs in more natural environments. For example, Köhler et al. (2009) found that clonal infections of *Pseudomonas aeruginosa* rapidly evolved changes in their social behaviour in intensive care patients. Specifically, in a number of patients Quorum-Sensing (*lasR*) mutants arose from initially wildtype populations, and dominated infections over a matter of days. Another clinical study documented the evolution of rapid genetic diversity from initially clonal populations of *Escherichia coli* during relatively short-term infections (Levert et al. 2010). Finally, a controlled evolution study in a more natural setting (soil, including the resident community) demonstrated that bacteria and viruses undergo reciprocal evolution of resistance and infectivity (antagonistic coevolution) over a matter of weeks; a similar time scale to that observed *in vitro* (Buckling & Rainey 2002a; Gómez & Buckling 2011).

Community context affects evolutionary change

Species within a community are not distinct entities and interact in many different ways. Many of the clearest examples of interspecific interactions dramatically altering microevolution are where there are strong antagonistic

interactions between species; one species has a very negative impact on the fitness of the other, such as the effect of virulent parasites on their hosts. This is because highly damaging parasites impose strong selection for resistance, and if the parasite subsequently evolves to overcome this resistance (i.e. hosts and parasite coevolve), then parasite-imposed selection will be relatively continual (Hamilton 1980). For example, the bacterium *P. fluorescens* and a phi2 (a 40KB ds DNA phage) have been shown to undergo continual cycles of resistance and infectivity (Buckling & Rainey 2002a) and this can dramatically alter the evolution of *P. fluorescens* traits not directly associated with phage resistance, including ecological diversity (Buckling & Rainey 2002b), mutation rates (Pal et al. 2007) and intraspecific cooperative behaviours (Morgan et al. 2012). Other studies have shown that predators (Meyer & Kassen 2007; Friman et al. 2008; Friman & Buckling 2013) and competitors (Harrison et al. 2008; Collins 2011; Lawrence et al. 2012) can also have a major impact on the short-term evolutionary trajectories of experimental bacterial populations.

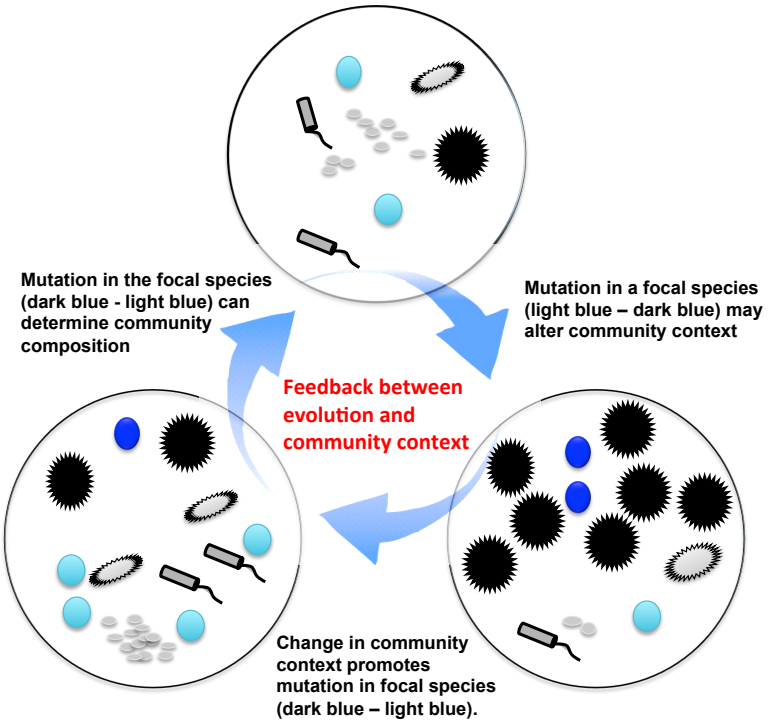

Figure 1: A mutation in a focal species (light blue-dark blue) can alter the frequency of the various interacting species (greyscale). Such change in community context further promotes evolutionary change in the focal species, so that there is feedback between evolution and community context.

Can we make any generalisations about the impact of community complexity on microevolution of focal species? A long-standing prediction is that interactions with other species should speed up evolutionary change, because other species create strong and continually changing selection pressures (Van Valen 1973; Stenseth & Smith 1984). A number of studies support this prediction, including recent work using *P. fluorescens* and phi2 that demonstrate more rapid (adaptive) molecular evolution in genes involved in phage attachment when coevolving with *P. fluorescens*, compared to when they evolved with a non-evolving host (Paterson et al. 2010). However, there is currently no empirical work to determine if this prediction holds in the context of interactions within whole communities as opposed to just one (or a few) enemy species. On the one hand, increasing the number of interacting species may result in even more rapid evolution, as selection acts on increasing numbers of traits. On the other, interactions with large numbers of species may instead constrain the rate of evolutionary change if adaptations to one species trade off with adaptations to other species.

While species interactions may (or may not) speed up evolution *per se*, perhaps a more relevant consideration for industrial processes such as waste treatment is how species interactions affect adaptation to the physical environment. The physical conditions within bioreactors change through time as a result of variation in the input of organic matter, and genetic adaptation to these changing conditions is likely to increase the rate at which substrates are broken down. Recent theory suggests that community complexity constrains genetic adaption to the physical environment, because species sorting in response to environmental change (i.e. the most successful species increase in frequency) reduces the effectiveness of natural selection (i.e. sorting of genes within species) (de Mazancourt et al. 2008) Experimental results are consistent with this general prediction, but not the specific mechanisms. For example, Lawrence et al. (2012) found that bacteria propagated within 5 species communities evolved to feed on each other's excreted metabolites, but also grew less well in the absence of the rest of the community than did species evolved as monocultures. This suggests a trade-off between adaptation to biotic and abiotic components of the environment. A similar trade-off between biotic

and abiotic adaptation has been demonstrated in viruses: adaptation to a thermally deteriorating environment was constrained when viruses antagonistically coevolved with their bacterial hosts compared to when hosts populations were held evolutionary constant (Zhang & Buckling 2011). In this example, reduced population size in the coevolving virus populations also contributed to poorer abiotic adaptation. It is however possible that community complexity may in fact enhance abiotic adaptation where horizontal gene transfer brings together beneficial mutations from different species into the same genome (Ochman et al. 2000). This is simple speculation, however, and demonstrates the need for research into how interactions among the natural microbial community may be altering the rate of evolution and consequently influencing the effectiveness of industrial processes that principally rely on microbial communities.

Microevolution affects community structure and function

Microevolutionary changes in individual species are likely to have little consequence for the functioning of entire communities, assuming the structure of the community remains relatively constant. However, it is well known from ecological studies of plants and animals that some species have disproportionately large effects on community structure as a whole, with their removal resulting in radical changes in community composition (Paine 1966). Moreover, not one, but all species are likely to be evolving within microbial communities. Rapid evolution is therefore likely to play a major role in structuring microbial communities. For example, rapid evolution in human gut microbiota might promote genetic diversity and alter mutualistic interactions between microbiota and host (Schluter & Foster 2012; Ursell et al. 2012) When bacterial diversity is high, there is selection for the most competitive bacterial genotypes rather than the ones most beneficial to the host, destabilising the mutualism (Schluter & Foster 2012). Furthermore, disturbances in microbiota diversity underlie diseases such as Crohn disease, and research is now exploring the use of manipulation of gut microbial communities to treat these conditions, using probiotics and prebiotics (Preidis & Versalovic 2009; Chow et al. 2010).

Unfortunately, our understanding of the role of real-time evolution on community structure and function is very limited. *In vitro* experiments using simple predator-prey and host-parasite species convincingly show that rapid evolution can significantly alter population dynamics of the interacting species (Buckling & Hodgson 2007; Yoshida et al. 2003, 2007). For example, by using a combination of mathematical models and experiments, the evolution of *E. coli* resistance to T4 phages was shown to be responsible for significant alterations in bacteria-virus population cycles and densities (Yoshida et al. 2007). An even more obvious outcome of the evolution of host resistance to viruses is phage extinction (Lenski & Levin 1985). The likely relevance of these in vitro studies has recently received support from a study of bacteria and virus dynamics in a wastewater treatment plant (Shapiro et al. 2010). Specifically, the relative abundance of operational taxonomic units (OTUs; based on 99% similarity of 16S rDNA) through time correlated with infective phage density, such that increases in density of a bacterial group subsequently resulted in an increase in the density of phage infecting that particular group. Crucially, different host clones within a single OTU showed very different resistance profiles to phages isolated from different time points, which is consistent with rapid coevolution between bacterial resistance and phage infectivity altering population densities.

The importance of rapid evolution altering community structure is not limited to interactions between trophic levels, but also operates within trophic levels. A recent study Hansen et al. (2007) showed how a 2-species cross-feeding interaction between *Acinetobacter* Sp. and *Pseudomonas putida* (*P. putida* fed on a waste product of the *Acinetobacter*) evolved to be more exploitative after a few days growth as biofilms. Evolved *P. putida* started to grow in closer proximity to the *Acinetobacter*, resulting in a negative impact on *Acinetobacter* growth, presumably as a result of increased competition for oxygen. Crucially, this evolved interaction, while having a negative impact on the *Acinetobacter*, increased total community biomass. Similarly, the mutualistic coevolution between a sulphate reducing bacterium, *Desulfovibrio vulgaris* and the methanogenic Archaeon, *Methanococcus maripaludis*, evolved to be 30% more productive (per mole of substrate) after a few hundred generations of co-culture (Hillesland & Stahl 2010)

While demonstrating that rapid evolution can affect community structure, the above studies only demonstrate the impact of evolution on the interaction between two species within a community. We are aware of one in vitro study (Lennon & Martiny 2008) that shows that changes in 2 species interactions (specifically bacteria and viruses, again) resulting from evolution can affect nutrient cycling. Surprisingly, studies that investigate the consequences of rapid evolution for "real" communities are dominated by studies of large organisms. For example, a recent study has shown how past adaptive diversification of sticklebacks into distinct morphotypes can affect the structure and function of natural communities (Harmon et al. 2009). Specifically, distinct stickleback morphotypes (limnetic and benthic forms), that have repeatedly diversified in Canadian lakes around 10000 years ago, were independently introduced into experimental mesocosms and the consequences for the density of prey species and a range of ecosystem traits were measured. While extremely elegant, this (Harmon et al. 2009), and other related studies (e.g. Schoener 2011) do not demonstrate that evolution *in situ* can affect community structure on what are normally considered ecological timescales. Nonetheless, they provide a good justification for careful consideration of how rapid evolution may be disturbing microbial community structure, particularly in industrial processes where microbe community stability may be crucial.

Perspectives

The interplay between microevolution and community context is intricate, but imperative. Despite this, there have been no thorough studies investigating the interplay between real-time evolution and community structure in either natural or synthetic communities relevant to industry. Such studies are inevitably difficult, but much could be learnt from experiments and observations that compare real-time phenotypic and genomic evolution of a focal species and changes in community structure. Theoretical models could determine if generalisations about microevolution and community structure can be made, although the disadvantage is that they trade off generality for specificity. For example, is microevolution more important in changing the structure of relatively simple versus highly diverse communities? This question is crucial for determining the benefits of developing simplified synthetic communities to carry out defined functions, as opposed to using existing natural communities. The

implications of rapid evolution in biotechnology are unclear, but could explain the change in function of industrial processes. While this review provides very few explicit answers as to the real importance of microevolution-community structure feedback, we hope it highlights its relevance to natural ecosystems, the importance of considering the issue in microbial biotechnology and finally, emphasises the need for novel approaches for further research in this area.

7. Discussion

Each of the chapters in this thesis contained their own extensive discussion. The aim of this chapter is to briefly review what has been achieved in each of the preceding chapters, and to highlight some emerging general points.

Chapter 2: Cooperative adaptation in the presence of public good cheats

- It is difficult to appreciate how cooperators can adapt to the presence of a high frequency of cheats in a diffuse public good system, whereby individuals cannot be directly identified as a cooperator or cheat.
- Siderophore producers can adapt to the presence of a high frequency of non-evolving pyoverdine and pyochelin-negative cheats, by downregulating their investment into siderophores, without any obvious fitness cost. They compensate for the ensuing reduction in iron availability by upregulating their production of pyocyanin; an extracellular secondary metabolite with strong redox activity that can promote soluble ferric iron. Crucially, since cheats also produce pyocyanin, the fitness disparity between cheats and cooperators is reduced.
- More generally, cooperators may adapt to the presence of a high frequency of cheats in a diffusible system by switching to an alternate mechanism to achieve the same outcome.

Chapter 3: Public goods, parasites and mutation rate.

- While the literature has suggested that bacterial mutation rate is susceptible to conflicting selection pressure from interspecific (bacteriophage) and intraspecific (social cheats), this work experimentally and theoretically demonstrated that interspecific parasites are more important in driving bacterial mutation rate than selection for siderophore cooperation in *P. fluorescens.*
- There is a simple but general interaction between social cheating and parasitism: parasites have less of an impact in reducing cheat versus cooperator population density, and so, the impact of cheating on

population growth is reduced in the presence versus absence of parasites.

Chapter 4: Public goods cooperation and detoxification

- Siderophore production in toxic copper growth media is an altruistic trait that can be exploited by non-siderophore producing cheats.
- In monoculture, siderophore producers out-competed non-producers as siderophores are required for detoxification. However, non-producers gained the advantage of siderophores produced by producers in co-culture, without paying the cost of production, and thus invaded the producing population. Hence, siderophore production in this context carries a cost to the producer and benefit to the recipient.
- While *de novo* cheats evolved in toxic copper and iron-limited growth media, the effect of copper in driving the evolution of these cheats was greater than the effect of iron-limitation.
- In natural populations, the influence of detoxification on shaping cooperation may be more relevant than iron-limitation under low pH, spatially structured, and multi-species environments.
- These results may have important consequences for the efficient use of bacterial siderophores in bioremediation.

Chapter 5: Public goods cooperation in a community context

- While siderophore production in iron-limited and toxic copper soil microcosms carries a benefit to the recipient, no cost of production could be detected, i.e. non-producers could not invade populations of producers in co-culture.
- Populations of non-siderophore producing mutants benefit by being in the presence of the natural microbial community, presumably because non-producers can use siderophores produced by the community in the context of iron-limitation, and to a greater extent, copper detoxification.
- Soil spatial structure may have the effect of keeping siderophore costs low because it keeps relatives together and reduces the amount of siderophore lost to the environment. Siderophore-loaded copper is likely

to be more far reaching than siderophore-loaded iron; hence the beneficiaries of detoxifying siderophores may be over larger spatial scales. Thus, the effect of spatial structure on constraining the cost of siderophores and thus cheating may be more prominent in the context of iron-acquisition than detoxification.

- This suggests implications for improving the effectiveness of applying siderophore either directly or indirectly for decontamination of soils, which relies on a consistently high production of siderophores by bacteria.

Chapter 6: The interplay between rapid evolution and community context

- The functionality of microbial communities is likely to be influenced by rapid evolution among members of the community; however, studies beyond simple 2-species communities are extremely limited.
- In turn, community context can affect the rate and trajectory of evolution of a focal species or trait. While community complexity may accelerate evolution because a multitude of co-evolving species create strong and changing selection pressures, complexity resulting in a large number of species interactions can impede the rate of evolution if adaptation to one species trades off with adaptation to another.
- Community complexity may result in a trade-off between adaptation to the abiotic and biotic components of the environment, so that the rate of adaptation to a changing environment is constrained.
- Incorporating the natural community into microbial evolution experiments may lead to better predictions of evolutionary trajectories.
- This may be directly relevant in the context of biotechnology, e.g. for the efficient production of food, biofuels and waste treatment.

General remarks

Some general themes have emerged from these studies:

The role of the natural microbial community in shaping cooperative traits

While experimental studies of a single bacterial species in a homogenous liquid media environment are crucial in their own right for testing social evolution

theory, they tell us little about whether such traits occur in nature and more importantly if they actually *matter.* This thesis has discussed in detail the ability of such interspecific interactions to shape the evolution of a focal trait (chapter 6); evidenced in chapter 3 by the presence of a lytic bacteriophage parasite increasing the frequency of hypermutators, and hence *de novo* cheat frequency in a bacterial population. Despite that these two-species 'communities' present another level of complexity in our systems, they are poor indicators of how the community is likely to alter the outcome of social interactions in nature. Isolating the natural microbial community and carrying out experiments in the presence and absence of this community (chapter 5) can gauge how all community interactions act collectively to influence the fitness costs and benefits of a focal trait. Where this approach has been taken, the microbial community i) inhibited the adaptive radiation of bacteria into specialists (Gómez & Buckling 2013a), ii) did not affect the ability of a bacteriophage to select for increased mutation rate (Gómez & Buckling 2013b) and iii) negated the cost of siderophore cheating when grown as monocultures in toxic and iron-limited soils (Chapter 5).

"Secret cheats" in societies

Social evolution theory has enjoyed astonishing success in terms of explaining the evolution and maintenance of cooperative behaviour, a challenge once described by Lord Robert May as 'the most important unanswered question in evolutionary biology, and more generally in social sciences". In principal, we now have abundant knowledge about why organisms cooperate with one another, inclusive fitness and global competition being the main drivers of such behaviour. However, we know less about the mechanisms by which cooperators themselves can constrain the spread of cheating once it is established. While mechanisms such as kin recognition and policing/punishing have been postulated to maintain cooperation, in public good scenarios the inability to associate the producer with the public good renders these approaches unlikely. Extensive research of the literature failed to discover evidence of a single adaptation by cooperators to public good cheats, which is remarkable given the ubiquity of cheats in nature and ease of *de novo* cheat evolution in the lab. Given the success of social evolution theory it is probable that poor empirical support is not due to a misunderstanding of how selection

operates but rather that some crucial details of the systems biology has been overlooked.

Perhaps a good strategy is to pinpoint mechanisms used by cooperators to constrain cheats in non-microbial public good systems. In human societies, income tax is a public good, susceptible to non-paying cheats; however, in most systems, restraining cheats relies on punishment/policing and hence the ability to distinguish cheats from cooperators. A punishment approach is arguably less efficient when cheats are at high frequency, where a better strategy might be to avoid the cost of punishment and change from a public based system to a private one. When tax collection systems are inefficient and subsequently associated with a high abundance of cheats, many services such as health, transport and household waste collection become privatised. Perhaps this is what we observe in chapter 2; although the observation that bacteria can benefit from exogenous pyocyanin indicates that it may not represent a private but simply a less exploitable public good.

Potentially more relevant to our microbial system is an individual's contribution to their carbon output or personal recycling; such systems are more vulnerable to non-identifiable 'secret cheats', whereby an individual incurs negligible cost of cheating, provided the proportion of cooperators remains high. Constraining cheats in this context may be a result of education and campaigning, whereby a cheating is associated with guilt and cooperating with sense of satisfaction, ultimately playing on a human's sense of morality. While this strategy is not applicable to microbes, it indicates that the same issue of public good cheats is encountered in all walks of life; some mechanisms of adaptation are perhaps easier to identify than others.

Social interactions matter, sometimes

In this thesis I have explored how social interactions can shape ecosystems and communities within. The most obvious example is perhaps the ability of cooperative siderophore production to aid in the detoxification of heavy-metal contaminated environments (chapters 4 and 5). This work, combined with evidence from studies involving the addition of siderophore-producing bacteria to heavy-metal contaminated soils (Wu et al. 2006; Li & Ramakrishna 2011;

Rojas-Tapias et al. 2012), clearly demonstrates the effect siderophore production can have in controlling important aspects of the abiotic environment. When the benefits of social interactions extend beyond conspecifics, their effects are likely to have consequences for community diversity and stability. Although this idea is manifested in chapter 5, it is also relevant to chapter 2, whereby social interactions result in the evolution of enhanced levels of pyocyanin. Pyocyanin has an antimicrobial effect on the community, killing non-pseudomonads and destroying community diversity, reducing the efficiency of microbial communities designed for biotechnology-based applications such as the degradation of crude-oil (Norman et al. 2004). A key point here is that social interactions are likely to matter most when the benefits (or indeed, costs) of the social trait is realised by the community and not solely conspecifics.

Social interactions don't matter, sometimes

Studies such as Harrison & Buckling (2005, 2007) and Racey et al. (2010), have indicated that social interactions, specifically, selection for siderophore cooperation, can restrain the evolution of a high bacterial mutation rate as it reduces the average relatedness between actor and recipient. In chapter 2, I discuss how this is irrelevant in the presence of a co-evolving lytic bacteriophage (which selects for a high supply of mutations in the host population). In nature, the ubiquity of phage suggests that selection for cooperation may be irrelevant in constraining mutation rate; the cost of being killed is higher than a reduced rate of growth.

The sociobiology of pyoverdine is context-dependent

This is perhaps an obvious statement, yet debate over the cooperative nature of pyoverdine production still rumbles on (Zhang & Rainey 2013; Kummerli & Ross-Gillespie 2014). Arguments made by Zhang & Rainey (2013) have interpreted the finding that pyoverdine is maladaptive in KB media, as evidence that pyoverdine cannot be labelled as cooperative, yet KB media is iron-replete and so this finding fits perfectly with social evolution theory. It is clear that any environment that results in a trait being costly and maladaptive will select against that trait. This however, does not deter from the potential cooperative nature of a trait in an environment where the trait is required. I have avoided being drawn into such arguments by quantifying the costs and benefits of

pyoverdine production in each environmental condition used in this thesis (iron-limited KB medium (chapter 2 and 3), iron-rich KB medium (chapter 4), toxic copper KB medium (chapter 4), iron limited soil (chapter 5) and toxic copper (& iron-limited) soil (chapter 5).

It is wise to be prudent in making assumptions about the role of cooperation in motivating pyoverdine production nature, and not to be overzealous in labelling pyoverdine mutants isolated from natural populations as social cheats, as it is difficult to discern whether the loss of the trait follows from cheating or another driver. However, the importance of the role of cooperation in controlling pyoverdine production in natural systems depends on the motivation for the experiment in question, namely as a model system for studying the stability of cooperation *per se*, or predicting microbial social interactions in infections, for example.

General conclusions

Social evolutionary biology owes much of its success to its firm, conceptually simple, theoretical underpinnings. While such work has been crucial in determining environmental mechanisms driving the evolution and maintenance of cooperation, it remains difficult to assess its ability to predict the outcome of cooperation in more complex microbial environments. Research into the evolution and maintenance of cooperation has begun to recognise the importance of examining trait evolution in increasingly complex scenarios. My research has aimed to highlight the importance of such complexity, demonstrating the effect of inter and interspecific interactions in shaping the outcome of public good cooperation.

There are many potentially useful directions for future research. While our understanding of the role of environmental factors such as viscosity, spatial structure and resource limitation in influencing siderophore production is robust, we know little of the mechanisms employed by bacteria to deal with public good cheats once they have evolved. My research has exposed one possible mechanism, but different routes may be taken in spatially structured natural environments. Moreover, our knowledge of how the natural community can affect the outcome of cooperation is still poor, despite progress made in recent

years. Resolution of these questions is paramount, particularly in light of research applying social evolution theory to developing novel therapeutic approaches that focus on interfering with microbial pyoverdine production (Imperi et al. 2013; de Carvalho & Fernandez 2014), and the 'hacking' of siderophores for use as drug-delivery agents (Mollmann et al. 2009). Finally, the application of social evolution theory to enhancing the efficiency of heavy metal bioremediation is a novel and exciting idea that has stemmed from this thesis. Combining this with further investigation into siderophore-based community interactions could ultimately lead to artificially designing stable, cooperative communities with highly efficient detoxification rates.

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