

Social Dynamics in Natural Populations of

Pseudomonas aeruginosa



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Declaration

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

Melanie Ghoul, Trinity 2014

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Publications

The following published papers have arisen from this thesis and are presented in chapters 2 and 3.

- ∞ Ghoul M., Griffin A. S. & West S. A. 2014. Toward an evolutionary definition of cheating. *Evolution* 68:318-331.
- ∞ Ghoul M., West S. A., Diggle S. P., & Griffin A. S. 2014. An experimental test of whether cheating is context dependent. *Journal of Evolutionary Biology* 27:551-556.

The unpublished chapters 4 and 5 are collaborative efforts though in each case the majority of the work is my own. Chapter 4 is my own work, John Bruce contributed to the laboratory work, and it was supervised by A.S Griffin and S.A West, who contributed to the experimental design, interpretation of the results and the manuscript. Chapter 5 is my own work, and was supervised by A.S. Griffin, and S.A. West who contributed to interpretation of the results and the manuscript and M. C. Maiden and his group contributed useful discussion and help with the bioinformatics BIGSdb software and data interpretation: Keith Jolley and Odile Harrison trained me to use the software and interpret the output data. James Bray uploaded and assembled the bacterial genomes and annotated them with all ribosomal and bacterial multilocus sequence typing DNA sequences.

I contributed to two other published papers that are included in the appendix:

- ∞ Jiricny N., Molin S., Foster K., Diggle S. P., Scanlan P. D, Ghoul M., Johansen H. K., Santorelli L. A., Popat R., West S. A., & Griffin A. S. 2014. Loss of Social Behaviours in Populations of *Pseudomonas aeruginosa* Infecting Lungs of Patients with Cystic Fibrosis. PLoS ONE 9, e83124
- ∞ Friman V. P., Ghoul M., Molin S., Johansen H. K., & Buckling A. 2013. *Pseudomonas aeruginosa* Adaptation to Lungs of Cystic Fibrosis Patients Leads to Lowered Resistance to Phage and Protist Enemies. PLoS ONE 8(9): e75380.

Abstract

Microbes rely on collective behaviours, such as communication and cooperation to survive and form communities. The majority of these social behaviours are mediated by the secretion of public good molecules into a shared environment such that they can be utilized by neighbouring cells. Therefore, individuals that engage in costly cooperative behaviour are susceptible to exploitation by selfish cheats that gain the benefit of cooperation without investing their share of the public good cost. Understanding such bacterial social interactions and the underlying molecular mechanisms gives insight into their complex social life in natural environments and can be used to develop alternative treatments for pathogenic bacteria that rely on such social interactions for virulence and to infect hosts. In this thesis I examine social behaviours expressed by the opportunistic pathogen, *Pseudomonas aeruginosa*. I develop an understanding of bacterial social dynamics, particularly competitive dynamics between cooperator and cheat strains and strains that engage in bacteriocin-mediated chemical warfare. I investigate bacterial cheat-cooperator systems in several ways: 1) I begin with a review describing the evolution of and response to cheating across a range of organisms and discuss the confusion that arises in identifying cheats particularly in microbial studies and therefore propose a key to identify cheating behaviour. 2) I empirically test whether cheating behaviour is context dependent in bacterial populations and reveal that the ability to cheat varies with the abiotic and social environment, which are two fluctuating conditions in natural environments. 3) I take an experimental approach to investigate why cheat invasion is not commonly observed in natural bacterial populations by testing the effect of cooperative bacterial growth dynamics on cheating ability. I find that secretion of public goods varies with bacterial growth dynamics and physiological growth stages which may explain why cheat invasion is more commonly observed in lab cultures and not in established natural populations. 4) In the final chapter I experimentally use natural isolates to examine the role of bacteriocins in mediating competition in pathogenic populations and find that contrary to empirical and theoretical work, bacteriocins do not play a significant role in strain competitive success and dominance. The thesis has laid groundwork for studying and understanding the role of social behaviours in bacterial systems and for further exploring social dynamics in natural bacterial populations.

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Foreword

I do not provide a detailed review in the introductory chapter because I provide reviews in chapters 2-5 of the specified topics.

Chapter 1. Introduction

Thesis aims and outline

In this thesis I examine the social dynamics among strains of the bacteria *Pseudomonas aeruginosa*. Until recently, social behaviours have been classically studied in animals, however, it is now known that bacterial cells rely on similar social interactions to coordinate behaviours necessary for scavenging food, invading new niches or protecting established niches from competing lineages. Learning how bacterial cells interact can impact how we understand their social dynamics in natural populations and use this knowledge for further research, such as to develop novel treatments for microbial infections.

I use pathogenic and environmental isolates of *P.aeruginosa* to investigate how and when strains express cheating behavior in cooperative systems and to determine the role of bacteriocin-induced dynamics, by studying bacteriocin production and resistance traits in shaping microbial populations.

In chapter 2, I review the evolution of, and responses to cheating across a range of organisms, which are often seen as fundamental issues in the fields of evolution. The problem of cooperation is often defined as: what stops cheats invading? However, others have argued that the term and the evolutionary implications are misleading and have led to error. The use of the term cheat has been used in many different ways to mean different things. The aims of this chapter are to: 1) provide a formal definition of cheating that conveys useful information and can be widely applied; 2) provide a conceptual framework that identifies the underlying selective forces involved in

cheating, and the response to cheating; 3) identify the major issues associated with the use of the term cheat in empirical microbial studies.

In chapter 3, I move on to experimentally test whether cheating is context dependent. This chapter is a proof of concept of the ideas discussed in chapter 2. A problem with microbial studies is that cheating is often defined by reduced expression of a cooperative trait and not in terms of the social costs and benefits of that trait. This has led to an accumulation of contradictory evidence in the literature of whether cheating occurs or not, which I seek to resolve. I experimentally show that cheating depends on the costs and benefits associated with the social and abiotic environment and not the absolute production of a cooperative trait. This demonstrates the importance of experimentally confirming cooperator-cheat dynamics before making inferences about the evolution of these social behaviours, whether in contrived laboratory or natural populations.

In chapter 4, I experimentally test the ability of cheats to invade growing and established populations of cooperative bacteria. Experimental evolution studies are often limited to co-inoculating a cheat and cooperator into sterile media at a low density, which are then allowed to compete through their growth curves. We know that reduced investment in the secretion of public goods reduces a cheat's selective advantage in exploiting cooperators. I show that the competitive dynamics of a cheat's ability to invade depends on the cooperators different growth stages, which are characterized by different rates and therefore costs of public good secretions. I demonstrate that cheating may not often be a threat in natural microbial populations that commonly exist in stationary-like phase, which is characterized by no growth and

reduced rates of public good secretions, potentially making natural cooperative populations immune to cheats.

In chapter 5, I explore bacteriocin-mediated interactions across *P.aeruginosa* isolates infecting the lungs of cystic fibrosis (CF) patients. I experimentally test bacteriocin production and resistance patterns across isolates from different stages of lung infections. I use this data to determine whether bacteriocin-induced competitive dynamics shapes bacterial populations and whether this can be used in the epidemiology of *P.aeruginosa* to predict patterns of infections across CF patients. I also show how competitive dynamics shape the evolution of the bacteriocin production and resistance traits in natural isolates.

In Chapter 6 I briefly summarise the conclusions and results of chapters 2 to 5 and discuss future directions.

In the remainder of this chapter I review social evolution theory and its application in the microbial field to introduce some of the concepts addressed in this thesis and I give a brief overview of the model organism – *Pseudomonas aeruginosa*.

Social Evolution Theory

Social traits

Social interactions are observed across all forms of life, from unicellular to the most complex organisms (Maynard Smith and Szathmary 1995). A social behaviour is categorized according to the personal fitness consequences it has on both the actor

and the recipient, which can be beneficial (+) or costly (-) (Hamilton 1964; West et al. 2007c). Hamilton's (1964) social behaviours are classified into four categories (Table 1): (1) mutual benefit (+/+), where the behavior is beneficial to both the actor and recipient; (2) altruism (-/+), where the behavior is harmful for the actor but beneficial to the recipient; (3) selfishness (+/-), where the actor benefits at the expense of the recipient; (4) spite (-/-), where the behavior is detrimental to both actor and recipient (Hamilton 1964; West et al. 2007c).

Table 1 Hamilton's classification of social behaviours

		Effect on recipient	
		+	-
Effect on actor	+	Mutual benefit	Selfishness
	-	Altruism	Spite

Table adapted from West, S. A., *et al.* (2007)

Cooperation is defined as a behaviour that benefits the recipient and is selected for because of this beneficial effect (West et al. 2007c). Cooperation is an umbrella term that includes altruistic and mutually beneficial behaviour (Sachs et al. 2004; West et al. 2007a). However, such behaviours contradict Darwin's 'survival of the fittest', which predicts the evolution of selfish behaviour that is beneficial to the actor enhancing its relative fitness. Hamilton (1964) realized that this could not explain the evolution of altruistic traits that are costly to the actor but beneficial to the recipient (-/+), such as the sterile workers of social insects. Consequently, Hamilton pointed out that reproductive success could not provide a general definition of Darwinian fitness. Instead, he showed that organisms would be selected to maximize their inclusive fitness, which is defined as the sum of an individual's own reproductive success

(direct fitness) and success through helping relatives reproduce (indirect fitness) (Hamilton 1964).

When considering whether a trait will be favoured by selection it can be useful to partition selection into direct and indirect components. This is formalised by Hamilton's (1963, 1964) rule, which states that a trait will be favoured by selection when $rb - c > 0$, where c is the fitness cost to the actor, b is the fitness benefit to the recipient, and r is their genetic relatedness. The $-c$ and rb terms represent direct and indirect fitness respectively; each of these terms may be positive or negative, and so could apply to any trait or behavior, for example those in Table 1. From a genetic perspective, a gene can increase in frequency in the next generation via direct transmission to offspring (direct fitness) or by indirectly increasing the transmission of those gene copies in other individuals. Indirect fitness benefits most commonly occur through helping close relatives and because genes are identical by descent from a common ancestor, this is referred to as 'kin selection' (Maynard-Smith 1964).

Exploiting cooperative systems

The problem with cooperation is that it is susceptible to selfish responses termed cheating. Cheats are defined as "individuals who do not cooperate (or cooperate less than their fair share), but are potentially able to gain the benefit of others cooperating" (Axelrod and Hamilton 1981; West et al. 2007c). Specifically a behaviour or trait is cheating if both: (a) it increases the reproductive success of the actor who performs the behaviour or trait and decreases the reproductive success of the recipients who are affected or 'cheated' by the behaviour (i.e. it is +/-); and (b) the positive fitness gain of the cheater (the + in +/-) arises because it is exploiting the cooperative behaviour of another individual (Ghoul et al. 2013). Cheating is therefore defined both from an individual perspective as a maximizing agent and in the context of cooperation. This

definition of cheating can be applied as widely as the definition of cooperation, both within and between species (West et al. 2007c, b).

Griffin *et al.* (2004) demonstrates that kinship plays a crucial role in the evolution of cooperation, however, relatedness is reduced with the emergence of non-cooperative individuals (either by mutation or migration) into the system. These cheats will grow at a faster rate because they reap the cooperative benefit without investing in the cost of its production, and consequently cheats will have a greater fitness than cooperators (West and Buckling 2003; Griffin et al. 2004). The evolution of cheating, the mechanisms by which cooperation is maintained despite cheating, and why cheats do not spread to fixation are fundamental issue in the field of social evolution (Bourke 2011).

Social Evolution in Microorganisms

More recently, microbial systems have opened up new opportunities for experimentally testing social evolution theory. It is now well established that microbes are not solitary creatures but live in communities and rely on multicellular-like collective behaviour to survive (Crespi 2001; Xavier 2011). Interactions among microbial cells occur via the production of costly ‘public good’ molecules secreted into the shared environment, which provide a collective benefit to neighbouring cells. For example molecules are used for cell-cell communication (quorum sensing signals), scavenging nutrients (siderophores), making biofilms (polymers), and chemical warfare with competitors (bacteriocins; (Crespi 2001; West et al. 2007a). Microbes are tractable model organisms for studying social evolution because of the

ease with which they can be cultured and manipulated for experimental studies under controlled conditions. Microbial sociality increases our evolutionary understanding of the purpose of microbial traits while elucidating the mechanistic and genetic basis of social behaviours observed in nature (West et al. 2007a).

Microbes are of particular interest because they are a major pathogen of humans and research on their social behaviours has bridged together evolutionary and medical disciplines. The concept of “Hamiltonian medicine” has emerged as a result of the awareness that cooperative bacterial behaviour is important in determining virulence of infections (Foster 2005; Crespi et al. 2014). As a result there has been a surge in the application of social evolution as a means to develop novel medical treatments for microbial infections, such as using cheats to invade an infective pathogenic population (Rumbaugh et al. 2009); or using spiteful bacteriocins as antimicrobials (Cotter et al. 2013); or combining both strategies into constructing engineered social cheats, termed “Trojan horses” that are used to invade the infectious population and attack it with toxins (Brown et al. 2009). Rumbaugh et al. (2009) have already shown that quorum sensing (QS) mutant cheats can exploit wild-type cooperator-produced virulence factors, invade the infectious wildtype cooperative population and, therefore, reduce its virulence levels *in vivo*. Brown et al. (2009) propose that Trojan horses can be designed as less virulent cheat strains that invade an infectious population by exploiting its virulence factors, while also driving medically beneficial alleles, such as antibiotic susceptibility, or allelopathic traits such as bacteriocins, or bacteriophages, into the infecting bacterial population that still expresses cooperative behaviours.

Applications of social evolution to microbial infections

Before the implementation of cheats or Trojan strategies to treat pathogenic infections, bacterial competitive dynamics need to be further examined. A key question is: can cheat strains invade established infections? Rumbaugh et al's (2009) QS mutants invade wildtype populations when the mutant and wildtype strains are co-inoculated. However, wildtype bacteria in nature – soil, water, or infections are usually found as already established populations (Kolter et al. 1993; Mulvey et al. 2001; Finkel 2006; Llorens et al. 2010; Gefen et al. 2014). Therefore it is important to determine whether cheats can invade already established populations.

Another question is: how do bacteriocins mediate competition among co-existing strains? Bacteriocins are toxins ubiquitously produced by bacteria, to eliminate competitor strains of the same species (Riley and Gordon 1999; Riley and Wertz 2002). For a cheat strain to successfully invade it must be protected from bacteriocins produced by other strains. Understanding how bacteriocin-mediated interactions shape bacterial populations can help predict the patterns of infection and what types of strains dominate. As a result this can be used to engineer cheat or Trojan strains carrying certain toxin types and protected from other strains' toxins to successfully invade different infections.

Table 1. Common bacterial social behaviours

Public good	Cost	Benefit	Cheats	References
Siderophores	Resources used for siderophore synthesis and secretion	Scavenge iron necessary for growth in iron-limited environments	Siderophore mutants	Griffin et al. (2004)
Biofilm formation	Synthesis of an extracellular matrix to form a structured population	Higher oxygen concentration and availability on the air-liquid interface, protection against environmental stressors	Non-producers of adhesive polymer	Rainey and Rainey (2003)
β lactamase enzymes	Synthesis and secretion of antibiotic resistant β lactamase enzymes	Resistant to exposure of β lactam antibiotics in the environment	β lactam susceptible cells	Dugatkin et al. (2008)
Rhamnolipid biosurfactants	Synthesis and secretion of rhamnolipid biosurfactants	Allows collective migration over surfaces to harvest nutrients	Rhl-negative mutants	Xavier et al. (2011)
QS autoinducers	Synthesis of autoinducer chemicals to sense the density of individuals in the environment	Provides information necessary for a more effective and synchronised virulent attack	LasI mutants	Diggle et al. (2007)
QS exoproducts	Exoproducts are produced and secreted in response to autoinducer signals sensed in the surrounding to induce variety of cooperative behaviours	Exoproducts are required for various uses: nutrient scavenging (siderophores) for growth, virulence factors to invade host, polymers for structured growth (biofilms), and surfactants to enhance movement (rhamnolipids)	LasR mutants	Diggle et al. (2007)
Bacteriocins	Synthesis and secretion of pyocins	Colonise a new niche or protect a niche by killing competitors	Resistant cells that do not produce bacteriocin but are resistant to it	Michel-Briand and Baysse (2002)

Study Species- *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a ubiquitous, versatile and adaptable gram-negative bacterium colonising and thriving in a diversity of ecological niches. Its social behaviours have been well characterized and documented (Table1) and its genome has been fully sequenced (Stover et al. 2000) facilitating the ability to link various social traits to the responsible genes, and construct genetically engineered strains. It has the capacity to cause disease across a range of hosts from plants to animals (Stover et al. 2000) and is classified as a superbug because it has increasingly evolved resistance to all classes of antibiotics (Gales et al. 2001). *P.aeruginosa* is an

opportunistic human pathogen, one of the most common causes of nosocomial infections and a major cause of morbidity and mortality in immuno-compromised individuals. This bacterium has gained considerable attention as a pathogen of human patients that suffer from burn wounds and particularly for causing chronic lung infections of cystic fibrosis (CF) patients. *P.aeruginosa* CF lung infections are the major cause of patient mortality with more than 95% of CF deaths caused by respiratory failure (Govan and Deretic 1996; Govan et al. 2007).

P.aeruginosa commonly colonises the CF lung in the adolescent years. Infections progress from acute, which commonly consists of multiple strains, to chronically colonized lungs, which are typically clonal (Fegan et al. 1991; Godard et al. 1993; Duport et al. 1995). Although the infection can be cleared with antibiotic treatment during early stages of colonization, it eventually persists as an established chronic infection, which is impossible to eradicate with antibiotics (Doring et al. 2011).

In contrast to wildtype or strains isolated from early-staged acute infections, *P.aeruginosa* strains isolated from patients with chronic infections of the CF lung are characterised by slow growth, lack of quorum sensing function and deficiencies in the secretion of a range of public good molecules such as virulence factors, phenazine production, biofilm formation and siderophore production (De Vos et al. 2001; Lee et al. 2005; Smith et al. 2006; D'argenio et al. 2007; Hogardt et al. 2007; Bragonzi et al. 2009; Winstanley and Fothergill 2009; Doring et al. 2011; Mowat et al. 2011; Jiricny et al. 2014). These changes are usually explained as adaptations to the lung and selective pressures such as antibiotics (Smith et al. 2006; Hogardt et al. 2007), however, it is also possible that competitive dynamics between cooperators and cheats contributes to loss of social trait expression, as observed in experimental evolution studies in the lab (Griffin et al. 2004; Diggle et al. 2007).

Chapter 2. Toward An Evolutionary Definition Of Cheating*

Abstract

The term ‘cheating’ is used in the evolutionary and ecological literature to describe a wide range of exploitative or deceitful traits. While many find this a useful short hand, others have suggested that it implies cognitive intent in a misleading way, and is used inconsistently. We provide a formal justification of the use of the term “cheat” from the perspective of an individual as a maximizing agent. We provide a definition for cheating that can be applied widely, and show that cheats can be broadly classified on the basis of four distinctions: (i) whether cooperation is an option, (ii) whether deception is involved, (iii) whether members of the same or different species are cheated, and (iv) whether the cheat is facultative or obligate. Our formal definition and classification provide a framework that allow us to resolve and clarify a number of issues, regarding the detection and evolutionary consequences of cheating, as well as illuminating common principles and similarities in the underlying selection pressures.

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1. Introduction

The problem of explaining the evolution of cooperative behavior is commonly framed in terms of the need to explain the selective forces that resist invasions by “cheats” but there is a lack of consensus about what a “cheat” actually is. The terms ‘cheat’ or ‘cheating’ are employed to describe a variety of traits and behaviors across a wide range of systems, from dishonest signalers to exploiters of mutualisms (Table 1). This suggests that many workers find the terminology a useful way to convey information about behavior. In addition, the need to explain why cheating does not lead to the breakdown of social systems is frequently posed as a major research challenge (Yu and Pierce 1998; Bronstein 2001; Kiers et al. 2003; Travisano and Velicer 2004).

In contrast it, has also been argued that the term cheat is misleading and should be avoided in the biological context. First, it has been suggested that the term ‘cheat’ is anthropomorphic and implies a cognitive intent that may not be appropriate in the majority of organisms (Bronstein 2001; Noe 2006; Douglas 2008). In some cases, alternative terms have been suggested or used such as ‘exploiter’ or ‘greedy’. Second, while other intentional terms such as altruism and spite are defined purely in terms of fitness costs and benefits (Hamilton 1964), the term ‘cheat’ also implies something about how the fitness consequences arise. Third, the term ‘cheat’ has been used inconsistently across a diversity of biological systems (Table 1). It is not always clear what exactly a cheat is, whether a cheat is always a cheat, how selection favors individuals that cheat, or how we would expect others to respond to cheating. If different researchers use the term cheat in different ways, then this will impede

attempts to unify our understanding of both the evolutionary biology of cheats, and the subsequent implications.

We aim to resolve these points of contention and the potential confusion arising from the use of ‘cheating’ in the evolutionary and ecological literature. We first examine general arguments for when intentional language can be used in biology, and provide a definition for cheating that can be applied widely. We use this definition as a framework to point out potential problems when empirically testing for cheats and for classifying the different forms of cheating, which have been observed in nature. Finally, we consider how and when selection is expected to lead to an effective counter-strategy to being cheated, and whether examining social behaviors from the perspective of cheats is always necessary or the most useful way forward.

Term	Organism
Nectar robbers ^a	Birds and insects
Non-hoarders ^b	Birds and mammals
Mutualism exploiters ^c	Insects, birds, plants, fishes
Mimics ^{c, d}	Insects, plants, arachnids
Social parasites ^{e, f}	Microbes
Hyperparasites ^g	–
Brood parasites ⁿ	Birds and insects
Aprovechados ^{c, i, j}	Plants and Insects
Kleptoparasites ^k	Insects, birds, plants, mammals, reptiles, amphibians, fishes
Scroungers ^k	Insects, birds, plants, mammals, reptiles, amphibians, fishes
Free loaders ^{l, m}	Insects and microbes
Defectors ^{i, n, o}	Fishes, insects, microbes, humans
Non-cooperators ^{n, p}	Microbes and humans
Non-producers ^p	Microbes
Cancer cells ^q	–
Illegitimate emitters ^d	Arachnids, insects, and plants
Dishonest signalers ^r	Insects
Somatic parasites ^s	Microbes
Beneficiary mutant ^t	Microbes

Table 1. Examples of traits that have been classified as cheating.

^a (Maloof and Inouye 2000). ^b (Andersson and Krebs 1978). ^c (Bronstein 2001). ^d (Dicke and Sabelis 1992). ^e (Strassmann and Queller 2011). ^f (Fiegna and Velicer 2003). ^g (Hochberg et al. 2000). ^h (Davies 1992, 2000; Kilner and Langmore 2011). ⁱ (Tyre and Addicott 1993; Addicott and Tyre 1995). ^j (Yu and Pierce 1998). ^k (Barnard 1984). ^l (West et al. 2006). ^m (Bronstein et al. 2006). ⁿ (West et al. 2007c). ^o (Bshary 2002). ^p (Xavier 2011). ^q (Nunney 1999). ^r (Tibbetts and Dale 2004). ^s (Matapurkar and Watve 1997). ^t (Morris et al. 2012).

2. Can the use of intentional terms like cheat be justified?

Biologists often borrow terms from everyday speech to describe phenomena. The advantage of this is that it can provide a shorthand that conveys meaning in an efficient way. One of the clearest examples of this is how the term ‘selfish’ was used to help explain the “gene’s eye” view by Dawkin’s (1976) in *The Selfish Gene*. Another example is how the term “altruism” is used to describe helping behaviors that are costly to perform, but provide a benefit to another individual (Hamilton 1964).

Confusion can arise when terms are poorly defined or used inconsistently, as previously shown in relation to terms such as altruism and eusociality (Crespi and Yanega 1995; West et al. 2007c).

Cheat and cheating are usually used in ways that suggest an intention or purpose to deceive or exploit others, in line with dictionary definitions. The Oxford dictionary (2010) definition of a 'cheat' is: "a person who behaves dishonestly in order to gain an advantage". The definition of the verb to "cheat" is: "gain an advantage over or deprive of something by using unfair or deceitful methods". A potential problem with the term cheat is that it is anthropomorphic and implies cognitive intent where there is not (Bronstein 2001; Bshary and Bronstein 2004). In particular, individuals are often referred to as cheats, in cases where they lack the cognitive ability to intentionally cheat and gain fitness, such as bacteria, slime molds, plants and many animals.

However, evolutionary theory provides a formal justification for the use of intentional language. Natural selection will lead to organisms that appear to be designed to maximize their fitness, with our most general definition of fitness being inclusive fitness (Fisher 1930; Hamilton 1964; Grafen 2006; West et al. 2007c; West and Gardner 2013). Another way of saying this is that natural selection will lead to organisms that act with the appearance of intention, as if they are trying to maximize their fitness. This does not mean that any or all intentional language is justified, but rather that intentional language is justified when it can be linked to an individual acting as a maximizing agent analogy, in terms of its fitness or reproductive success (Grafen 1999).

The advantage of using intentional language in this way is that it provides an useful shorthand that describes classes of traits, in a way that provides information about fitness consequences and gene dynamics (West et al. 2007c). For example, if we

know that a behavior is ‘altruistic’, then this tells us that both: (a) the behavior is costly to perform, but benefits others; and (b) natural selection will have favored this behavior because it is directed towards relatives (Hamilton 1964, 1970). We would want the term cheat to provide analogously useful information. Other examples of justified and useful intentional language include the terms cooperation, selfish, signal and deception (Maynard Smith and Harper 2003; West et al. 2007c).

3. What is a Cheat?

We define cheating as: (i) a trait that is beneficial to a cheat and costly to a cooperator in terms of inclusive fitness (ii) when these benefits and costs arise from the actor directing a cooperative behavior towards the cheat, rather than the intended recipient.

The distinction between “cheat” and “recipient” is key to our definition. Confusion can arise because both are individuals that benefit from a cooperative act but they differ in the fitness consequences of that benefit to the actor that performs the cooperative act, and, therefore, in their effect on selection for cooperation. This follows from the formal definition of cooperation as a behavior that is favored by selection, at least in part, from its beneficial effect on the recipient (West et al. 2007c). If the benefit of cooperation goes to the intended recipient, then this provides a direct or indirect benefit to the actor that can favor cooperation. If the benefit goes to the cheat, then it decreases the fitness of the cooperator, potentially selecting against cooperation.

It is important to note that not every individual that benefits from a cooperative act is either a recipient or a cheat: a fringe-lipped bat (*Trachops cirrhosis*) that uses the

mating calls of male frogs to locate and prey on them, or a parasitoid fly (*Euphasiopteryx ochracea*) that uses the songs of male crickets to locate a host on which to lay eggs (Cade 1975; Zuk and Kolluru 1998; Page and Ryan 2008) are not the “recipient” of the cooperative act of signaling. Other examples include nectar robbers, which steal a flower’s nectar, but are not the recipients that fulfill the flower’s pollination services (Richardson 1995; Maloof and Inouye 2000), or a man who raids a beehive is not the “recipient” of the cooperative act of producing honey. In all of these cases, the cooperative act is only favored by selection as a result of a benefit provided to the intended recipients – female frogs and crickets, pollinators and colony mates. We suggest that it is not useful to include examples of eavesdropping or stealing in a definition of cheating, because the fitness consequences for cooperation in these examples are not driven by the fact that the behaviors being exploited are social.

Our requirement that the benefit and cost result from cooperation is required to distinguish cheating from other selfish behaviors. For example, consider when anglerfishes lure prey with the bioluminescent flesh growth on their head, or when chameleons camouflage themselves to hide from predators. In both cases a cooperative interaction is not being exploited, and so we wouldn’t want to classify this as cheating - real prey don’t want to be eaten, and un-camouflaged prey do not want to be found respectively. This emphasizes that the problem of cooperation is to explain why cheating does not lead to the breakdown of cooperation (Sachs et al. 2004; West et al. 2007b; Bourke 2011).

Cheats require a mechanism for cheating others. Our definition of cheat includes individuals that intercept cooperation meant for someone else, and individuals that manipulate others to direct cooperation towards them. An example of interception,

would be an individual that did not perform, or performed less of an indiscriminate cooperative behavior, but still benefited from the cooperation of others, such as a bacterial cell that did not produce iron scavenging siderophore molecules (Griffin et al. 2004). In this case the mechanism of cheating is just to cooperate less, but specific exploitative traits are also possible.

An example of cheating by manipulation is when fork-tailed drongos (*Dicrurus adsimilis*) give false predator-warning alarm calls, to make pied babblers or meerkats flee, allowing the drongos to steal their food (Ridley et al. 2007; Flower 2011). In this case, the drongos are manipulating the babblers and the meerkats to perform what would normally be a cooperative response to an alarm call. A second example is when cleaner fish (*Labroides dimidiatus*) take a bite from the fish client, rather than eating/cleaning the parasites off them (Bshary and Grutter 2002). In this case, the cleaner fish are exploiting the cooperative behavior of the client fish to stay there and have its parasites eaten. Another example is when some firefly females of the genus *Photuris* mimic the light flash pattern of *Photinus* females of another species, to lure in males to what he perceives to be a receptive female, only to be preyed on by the *Photuris* female (Lloyd 1983).

Cooperation occurs at all levels of biological organization, and so we should also expect to see cheating at all levels of biological organization (Bourke 2011). Meiotic drivers, such as sex chromosome drivers, cheat other alleles at that locus (Burt and Trivers 2006). Cells within multicellular organisms can cheat other cells. For example, in the fruiting bodies of *Dictyostelium discoideum* slime molds, or *Myxooccus xanthus* bacteria, cells could increase their likelihood of being in the reproductive spore head, rather than the stalk that holds the spore head up (Strassmann et al. 2000; Velicer et al. 2000). Similarly, cancers can spread as cheats

within a multicellular organism (Bourke 2011).

We distinguish between putative or potential cheats, that might not be favored by natural selection, and cheats that are maintained in populations as adaptations. Individuals can arise by mutation, or be experimentally created by genetic and phenotypic manipulations, that cooperate to a lesser extent and are therefore potential cheats (Foster et al. 2007; West et al. 2012b). For example, bacteria mutants that produce less of an extracellular public good, such as iron scavenging siderophore molecules, or rhizobia bacteria that are prevented from fixing nitrogen for their host plants (Kiers et al. 2003; Griffin et al. 2004). Whether this provides a fitness benefit, allowing them to be ‘successful’ cheats that can spread and be maintained in the population is another matter. The success of such mutants will depend upon factors such as whether the population structure will allow them to exploit cooperators, or if their social partners have mechanisms to reduce cooperation with non-cooperators. This distinction allows us to have a formal definition for cheating adaptations that are maintained in the population, while also talking about how cooperation can be stable against potential cheats.

It is necessary to distinguish between the fitness consequences of being cheated and the fitness consequences of interacting with a cheat. While the fitness effect of being cheated is costly (negative) to the recipient, this does not mean that interacting with a cheat will necessarily result in a net reduction of fitness. For example, consider the cooperative interaction between legume plants (*Glycine max*) and their rhizobia bacteria (*Bradyrhizobium japonicum*), where rhizobia provide their plant hosts with nitrogen, and are given carbon in exchange. If a rhizobia strain were able to benefit by providing relatively less nitrogen to their host plant, while still receiving carbon, then it would be a successful cheat, at a (relative) cost to the fitness of their host plant

(Kiers et al. 2003). Nonetheless, it could still be better for the plant to interact with this cheater strain than to not interact with it, when the smaller amount of nitrogen that it provides is better than getting no nitrogen at all.

4. How do we find cheats?

Consider a species of bacteria where individuals produce and release a molecule to acquire a resource from the environment. For example, the siderophore molecules produced by many bacteria to scavenge iron from the environment (West and Buckling 2003). If we detect individual variation in levels of production of this molecule, could we say that low producers are cheating? We suggest that there are three key questions that need to be considered when trying to determine whether individuals are cheats.

(i) Is a cooperative trait being exploited?

In the context of our bacterial example, is the production and release of the molecule a cooperative behavior? The first step in testing this is to grow two strains that differ in the amount of the molecule that they produce, either alone or in a mixed population. If the molecule is cooperative and individuals that produce less are acting as cheats, then we predict, when grown in an environment where the molecule is required for growth, that: (a) populations of cells that produce the molecule (the putative cooperators) should grow better than populations of cells that produce less of the molecule (the putative cheats); (b) when grown in mixed populations, the cells that produce less of the molecule (the putative cheat) should increase in frequency (Griffin et al. 2004). These results would show that the trait provides a benefit at the

group level that can be exploited by individuals who do less. Put simply, the putative cheats are acting as cheats. A series of finer predictions can also be made, regarding how the fitness of putative cheats will also depend upon their frequency in the population, population density and the relative extent to which they produce the molecule (West et al. 2006; Ross-Gillespie et al. 2007; Ross-Gillespie et al. 2009; Jiricny et al. 2010; West et al. 2012b).

(ii) Does variation in behavior evolve from selection to cheat?

The experiments described above can identify cheats, but this does not mean that they evolved or are being maintained in the population as cheats. For example, strains that produce less of the molecule may have been isolated from environments where the molecule was not required to scavenge resources, and so they have just evolved a lower production for this reason. This can be a difficult problem to address. One option is to obtain multiple strains from an environment where you know that the strains can interact. Another option is to take a phylogenetic approach and examine the order in which different traits evolved. For example, Cordero et al. (2012), surveyed siderophore production across a large number of *Vibrio* populations, and showed that, while siderophore production is often lost, the ability to uptake siderophores is always retained. This suggests that there was selection to retain the ability to utilize siderophores produced by other strains, rather than just selection to lose the trait.

(iii) Are the studies carried out in an appropriate environment?

Consider the scenario where we have a cooperator and a cheat strain, that evolved in a certain environment, but we carry out the growth assays in a different environment where there is an excess of resources such that the molecule provides no benefit. In

this case, cheats avoids the cost of producing the molecule and grow better in both the mixed and monocultures. This could lead us to concluding that the trait was not social (e.g. Zhang & Rainey 2013), which would be an error, as it is social, just not in that particular environment. To give an analogy, this would be like testing whether lions foraged cooperatively, but doing the study in a zoo, where there was *ad lib* food and they didn't do any actual foraging. While it can be difficult to know the appropriate environment, it is very unlikely to be one where the molecule is not needed, because if that were the case, there would not have been selection to produce the molecule.

To give another possible scenario, imagine that we had two strains, neither of which were cheats. One strain produces a relatively large amount of the molecule (it came from a harsh environment where the molecule was required) and the other produces a relatively low amount of the molecule (it came from a rich environment where the molecule was less required). Now imagine that we carried out our growth assays in an intermediate environment, where only an intermediate amount of molecule was optimal. In this case, a mixed culture would produce the molecule at a rate closer to the optimal amount, and hence grow at a faster rate than the two monocultures. While we might be tempted to conclude that this means 'cheats' aid population growth, this would be misleading. Instead, what we have shown is that when some individuals are over-cooperating, this can be partially compensated for at the level of the population, by other individuals cooperating at lower levels (MacLean et al. 2010). Furthermore, the result in our thought experiment is an artifact of the conditions under which the experiment has been carried out in the laboratory. If we had used a strain that had evolved in the intermediate environment, we would expect it to produce an intermediate level of the molecule, such that the addition of cheats would always decrease population growth.

It is important to distinguish cases of true cheating from cases of differential trait expression, because this distinction is key to understanding how cooperative groups resist the invasion of cheats and, therefore, ultimate questions about the evolution of cooperation. For example, if we accepted the low producers from the previous paragraph as “cheats”, we would get the wrong answers to these ultimate questions. Tolerance of being cheated is not favored as a result of population level benefits and it is not favored because cheats may also cooperate to some extent. Cheating is bad for cooperators, by definition, and we can only understand how and why cooperation persists by understanding how cheating is resisted by direct or indirect (kin selected) benefits (Sachs et al. 2004; West et al. 2007b; Bourke 2011).

Another example of the need to recognize that cheating is bad for cooperation is provided by Rainey’s (2007; Rainey and Kerr 2010) suggestion that the germ line could have evolved by cheating in early multicellular organisms. If germ cells were cheats, then they would have imposed a cost on the soma cells, which would have led to a germ-soma conflict, and selection for the soma to suppress the germ (Bourke 2011). Instead, the germ line has evolved as a form of cooperation, that provided an inclusive fitness benefit to both germ and soma cells (Queller 2000; Bourke 2011), as supported by the fact that sterile cells are more likely to occur in species where the multicellular groups are clonal (Fisher et al. 2013).

We focused on a bacterial example in this section to illustrate general problems. We did this because, as illustrated above, we think that the term cheat is sometimes misapplied within the microbial literature, in a way that leads to the implications being over-interpreted. We provide other examples below, when discussing why cheats don’t take over. This problem has arisen, in part, because of the extent to which both environmental condition and strain behavior can be experimentally

manipulated in studies of microbes. In animals, such extreme manipulations are not possible, and the social fitness consequences of traits can be clearer, making such over-interpretation harder. For example, consider the parental behavior in a fictional species of bird where males and females form monogamous pairs, and raise one chick per breeding attempt. Now suppose a mother feeds at the optimal rate, but that the male feeds at a much higher rate, such that there is more food than the chick can eat. In this situation, we would conclude that the male is feeding at too high a rate, possibly due to an experimental manipulation. It is clearly incorrect to claim that the female is a cheat. However, analogous claims have been made in some microbial studies, by carrying out studies in environments where some strains are over-performing behaviors.

5. A key to cheating

Ideally, our definition of cheating can be applied to a diverse range of biological phenomena, ranging from molecular nutrient scavenging by bacteria to raising false alarm calls by drongos (Fig. 1). These behaviors have ostensibly little in common, with little overlap in the research that goes on between them. Our aim in this section is to categorize cheating behaviors in a way that illuminates common principles and similarities in the underlying selection pressures. We categorize cheats on the basis of four distinctions:

1. Is cooperation in the strategy set of the cheat?
2. Is deception involved?
3. Is the cheat of the same species as the cooperator?

4. Is the cheat facultative or obligate?

We chose these four distinctions because they divide cheats up in way that provides information about the evolutionary interaction between cooperators and cheats. Distinction (1) determines whether cheats could be selected to cooperate under certain conditions, and whether cheating has evolved from cooperation. Distinction (2) determines whether or not cheating occurs by exploiting a signaling system. Distinction (3) determines whether the cheats can replace cooperators, or just reduce the relative benefit of cooperation, potentially leading to its loss. Distinction (4) determines the consequences of the spread of cheating for cooperation – cooperation can be maintained with facultative cheats, but will be lost if obligate cheats spread to fixation.

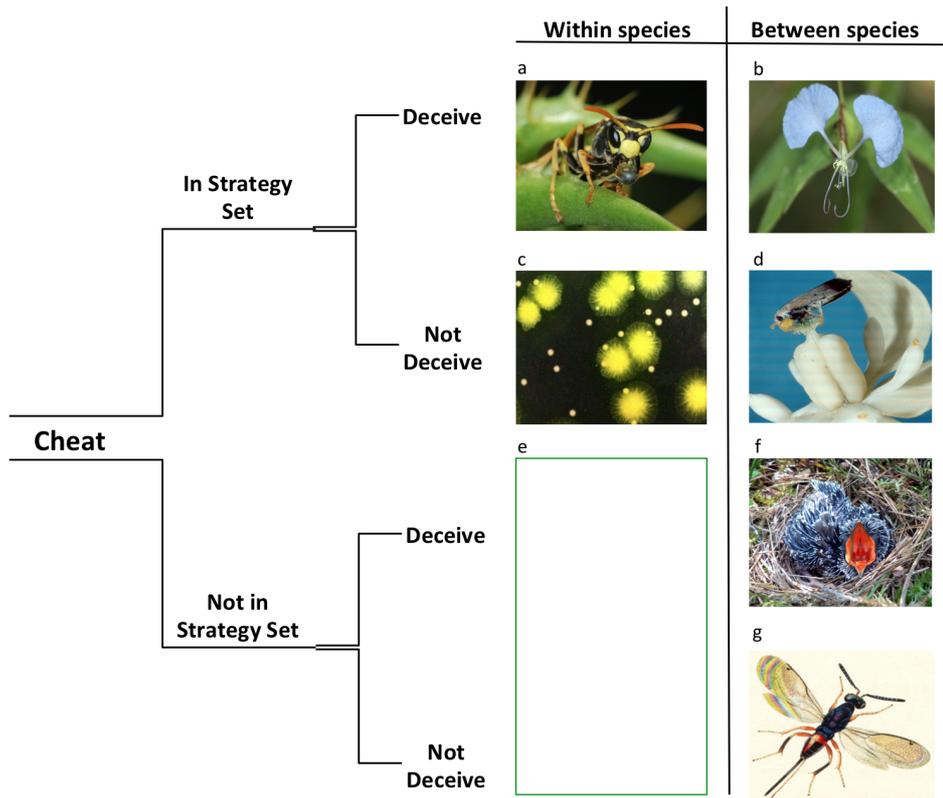


Figure 1. A key to cheating. The figure shows how different cheating behaviors can be categorized based on three distinctions: (i) whether or not cooperation is in the strategy of the cheat; (ii) whether deception is involved; and (iii) whether the cheat is of the same species as the cooperator. The examples given are: (a) *Polistes dominulus* wasp; (b) *Commelina mascarenica* flower with infertile stamens and false anthers (image courtesy of Rosie Treveylan); (c) *Pseudomonas aeruginosa* siderophore producers (yellow colonies) and non-producers (white colonies); (d) *Tegeticula* moth on a *Yucca* plant; (e) we do not know of any examples – see main text; (f) a *Cuculus canorus* cuckoo in an *Acrocephalus scirpaceus* reed warbler nest; (g) *Torymidae* fig wasp.

5.1 Is cooperation in the strategy set of the cheat?

Our first distinction is whether the cooperative trait being exploited is in the potential strategy set of the cheat, in such a way that the cheat could potentially cooperate, or was a cooperator in its evolutionary past. Examples of when cooperation is in the

strategy set include: (1) Non-pollinating fig wasps in the family Agaonidae, which enter the fruit to lay their eggs, and which have evolved from pollinator species (van Noort and Compton 1996; Herre et al. 2008). (2) *P. aeruginosa* strains that produce fewer siderophores (Griffin et al. 2004; Jiricny et al. 2010). (3) Fork-tailed drongos that give false predator-warning alarm calls, to make pied babblers or meerkats flee, allowing the drongos to steal their food (Ridley et al. 2007; Flower 2011). (4) Rhizobia strains that fix less nitrogen for their legume host, but still obtain the plant's carbon resource in return (Kiers et al. 2003).

Examples of when cooperation is not in the strategy set, include: (1) Common cuckoos (*Cuculus canorus*), which are obligate brood parasites, that lay their eggs in warbler's nests, exploiting the warbler's parent-offspring interaction (Davies 1992). (2) Non-pollinating fig wasps in the family Torymidae, which lay their eggs from outside the fruit, and compete with the pollinators for flowers in which to develop, but which have not evolved from pollinators (West and Herre 1994). (3) Bolas spiders (*Mastophora cornigera*) that mimic the sex pheromones of moths to attract and then prey on them, exploiting the cooperative communication between moths (Eberhard 1977; Stowe et al. 1987; Dicke and Sabelis 1992). (4) *Photuris* fireflies that make false signals of another species to lure in the males as prey (Lloyd 1983).

The above examples make clear that the definition of cheating we present includes cases where cheats have not evolved from cooperators. In contrast, within the mutualism literature, it is sometimes suggested that if an individual was a mutualist, it can then evolve to be a cheat, but if it was never a mutualist, its selfish behavior is simply parasitic (Kautz et al. 2009). We argue that it is useful to allow both these scenarios to be cheats because they both involve exploiting the cooperative behavior

of others and, from an evolutionary perspective, both of these types of cheats could potentially lead to a breakdown in cooperation.

5.2 Is deception involved?

Our second distinction is whether deception is involved. Deception occurs when a signal is made under circumstances where the response is beneficial to the signaler and costly to the recipient. Recipients would only be expected to respond to deceptive signals if, under other circumstances, they gain a benefit from responding to the signal (Maynard Smith and Harper 2003).

Examples of cheats that use deception (deceitful signalers) include: (1) Subordinate *Polistes dominulus* wasps with facial patterns that alter their badge of status resembling that of a more dominant wasp (Tibbetts and Dale 2004). (2) The flowers of some plants, such as *Chilogottis* or *Ophrys* orchids, that mimic the morphology and pheromone odor of female wasps/bees, to lure in male wasps/bees, that then act as pollinators (Borgkarlson and Tengo 1986; Schiestl et al. 2003). (3) Fork-tailed drongos that make false alarm calls (Ridley et al. 2007; Flower 2011). (4) *Photuris* fireflies that make false signals to lure in males as prey (Lloyd 1983). (5) Male blennioid fish (*Salaria pavo*) that mimic the color patterns and morphology of females, so that they can sneak into a harem nest to fertilize the spawned eggs (Goncalves et al. 1996). (6) Bolas spiders that mimic moth sex pheromones to lure them in as prey (Eberhard 1977; Stowe et al. 1987; Dicke and Sabelis 1992).

Examples of cheats that do not use deception include all the cases where individuals cheat by cooperating less. For example, *P. aeruginosa* LasR mutant strains, that do not respond to quorum sensing signal, but are able to exploit the cooperative public

goods produced by other strains in response to quorum sensing (Diggle et al. 2007; Sandoz et al. 2007; Rumbaugh et al. 2009; Yang et al. 2010; Popat et al. 2012).

This distinction over whether deception is involved emphasizes that many of our cheating examples involve signaling systems. Signaling is open to cheating because all stable signaling systems are cooperative (Scott-Phillips et al. 2012). The reason for this is that signaling is only evolutionarily stable when it is, on average, beneficial to both the sender and the receiver of the signal (Maynard Smith and Harper 2003). If this were not the case, then the partner, that did not benefit would be selected to either not signal or not respond, leading to the signaling system breaking down. Deception can also occur outside of signaling systems, and so not all cases of deception are cheats (Box 1). It remains to be seen whether signaling systems that are evolutionarily stable for different reasons, such as the cost (handicaps) or feasibility (indices) of dishonest signaling, or common interest, vary in their susceptibility to different types of cheating (Davies et al. 2012).

5.3 Is the cheat of the same species as the cooperator?

Our third distinction is whether cheats exploit individuals of their own species, or individuals of another species. Examples of cheating a member of the same species include: (1) Male blennid fish that mimic the color patterns and morphology of females, to sneak fertilize their eggs (Goncalves et al. 1996). (2) Fork-tailed drongos that make false alarm calls to their conspecifics (Ridley et al. 2007; Flower 2011). (3) LasR mutant strains of *P. aeruginosa*, that do not respond to quorum sensing signal (Diggle et al. 2007; Rumbaugh et al. 2009; Popat et al. 2012).

Examples of cheating a member of another species include: (1) Yucca moths that lay eggs in the developing yucca fruit that have already been pollinated, but do not

pollinate the plant in return (Tyre and Addicott 1993; Pellmyr et al. 1996). (2) Arbuscular mycorrhizal fungi, of the subgenus *Glomus*, that supply less phosphorus to their host plant (Kiers et al. 2011). (3) Rhizobia bacteria that fix a low or negligible amount of nitrogen for their legume host (Kiers et al. 2003). (4) Plants, such as those of the Orchidacea or honey mesquites (*Prosopis glandulosa*), that produce flowers without nectar, but which mimic the scent, morphology and spectral color reflectance of flowers that do provide nectar to pollinators (Lopez-portillo et al. 1993; Thakar et al. 2003; Anderson et al. 2005; Anand et al. 2007; Burkle et al. 2007; Brandenburg and Bshary 2011). (5) *Commelina mascarenica* flowers that have three false stamens that carry no pollen but are used to lure in pollinators. (6) Cleaner fishes that take a bite from the fish client, rather than eating/cleaning the parasites off them (Bshary 2002; Bshary and Grutter 2006).

Our first and third distinctions are not completely independent. While cheating between species can occur when cooperation is in the strategy set or when cooperation is not in the strategy set, we do not know any examples of cheating within a species when cooperation is not in the strategy set (Fig. 1). We would expect such cheats to be rare or non-existent, as it would require a cooperative behavior that could not be performed by a subset of the species.

5.4 Is the cheat facultative or obligate?

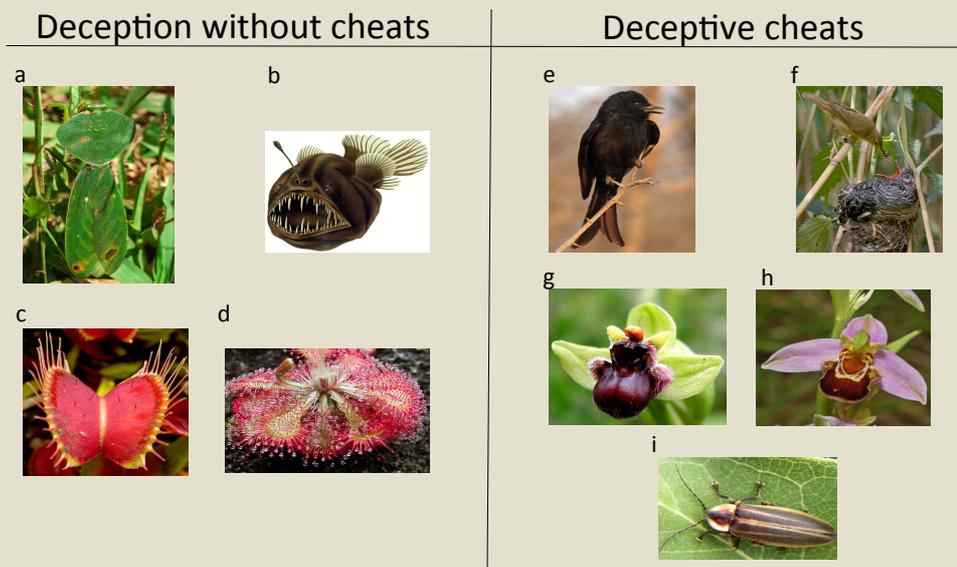
Another biologically important distinction is whether the cheat is facultative (conditional) or obligate (Travisano and Velicer 2004; Santorelli et al. 2008; Buttery et al. 2009). A facultative strategy would be one in which the relevant trait is adjusted conditionally, dependent on the cheater's social partners. For example, cooperate if with clone-mates, but cheat if with non-relatives. An obligate strategy would be one in

which cheating is expressed unconditionally. For example, never perform a cooperative behavior.

Few studies have successfully determined whether cheats are facultative or obligate, and so we have not included it in our classification figure (Fig. 1). An important exception is studies of fruiting body production in the slime mold *Dictyostelium discoideum*. When starved of food, individuals of this species aggregate together to form a motile slug that differentiates into a fruiting body with sterile stalk cells holding a viable spore head. Some strains have been shown to be cheats in that, when there are mixed genotype fruiting bodies, they contribute less than their proportional share to the dead stalk, and more to the reproductive spore head (Strassmann et al. 2000). It has also been shown that a number of strains are facultative cheats, that cheat when in mixed genotype fruiting bodies, but are able to produce a normal fruiting body when in single genotype (clonal) fruiting bodies (Fortunato et al. 2003; Santorelli et al. 2008; Buttery et al. 2009; Khare and Shaulsky 2010). Another important exception is the analogous fruiting body formation in the bacteria *Myxococcus xanthus*, where facultative cheating has also been observed (Velicer et al. 2000; Fiegna and Velicer 2005; Fiegna et al. 2006).

Box 1. Signaling systems, deception and cheating

While we have used deception as a characteristic to distinguish cheats, not all instances of deception will be cheating (see figure below). Deception is cheating when it manipulates a cooperative signaling system. In contrast, deception will not be cheating when it is exploiting a feature of the world that can be used as a guide to action, termed a ‘cue’ (Maynard Smith and Harper 2003). For example, when anglerfish lure in prey with bioluminescent ‘bulbs’, they are being deceptive, but they are not exploiting a cooperative signaling system, and so this is not cheating. An illuminating example here is given by the carnivorous venus flytrap (*Dionaea muscipula*) and sundew plants (*Drosera anglica*). If these plants were mimicking flowers to attract in and eat pollinators, then they would be exploiting the cooperative plant-pollinator signaling, and so would be cheats. However, if they were just mimicking a cue of a food source, to attract in a range of insects, including non-pollinators, then they would not be exploiting a cooperative interaction, and so would not be cheats. Empirical data supports the latter (Ellison and Gotelli 2001; Murza et al. 2006; Ellison and Gotelli 2009; Gibson and Waller 2009).



Deception without cheating and deceptive cheats. Not all deceptive behavior implies cheating. Deception can be divided into those that manipulate cooperative behaviors (cheats) and those that don't (deception without cheating). A common form of deception without cheating is deceptive predators such as: (a) *Choeradobis rhombicollis*, leaf-mimicking praying mantis; (b) anglerfish; (c) *Dionaea muscipula*, venus flytrap; (d) *Drosera* sundew plant. Examples of deceptive cheats include (e) *Dicrurus adsimilis*, fork-tailed drongo; (f) *Acrocephalus scirpaceus* reed warbler feeding a *Cuculus canorus* cuckoo chick; (g) *Ophrys bombyliflora*, bumblebee orchid; (h) *Ophrys apifera*, bee orchid; (i) *Photuris* firefly.

6. What is the response to cheating?

The presence of cheats imposes a fitness cost on cooperators such that this will favour mutations that confer resistance to cheating. The strategies available to cooperators will depend upon what kind of cooperation is being cheated, and how it is being cheated. Cooperators could respond to cheats in at least four ways.

First, cooperators could adjust their behavior facultatively in response to cheats. Lindstrom et al. (1997) found that great tits were more likely to eat worms that honestly signaled distastefulness, as the frequency of dishonest cheats (Batesian mimics) increased (Box 2). Ridley and Raihani (2007) showed that cooperatively breeding pied babblers respond more strongly to kleptoparasitic drongos alarm calls when in a small group size, where there are fewer guards to defend the group (more beneficial to respond), but rarely respond to drongo alarm calls when in larger groups where more individuals participate in predator vigilance.

Second, cooperators could be selected to reduce the cooperative behavior that is being exploited, even leading to the loss of cooperation. For example, in the presence of bacterial cheats, who did not produce and release a cooperative nutrient-acquiring molecule (public good), there could be selection for the cooperative individuals to evolve to produce less of the molecule. An alternative could be to having phenotypic switching, with some proportion of individuals producing no public good (Diard et al. 2013; Frank 2013).

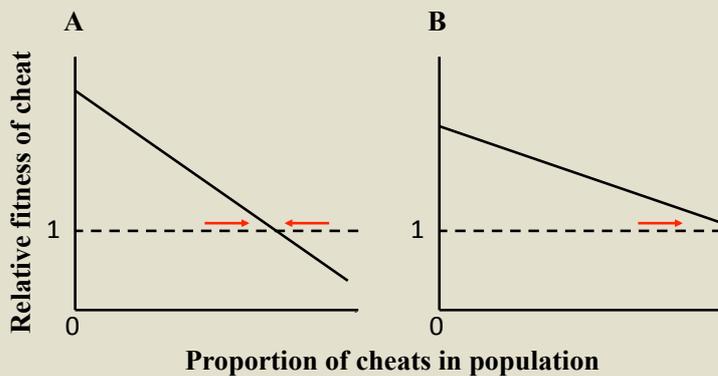
Third, cooperators could be selected to change the cooperative trait such that it is harder to exploit. For example, in the presence of bacterial cheats, who do not produce and release a cooperative nutrient-acquiring molecule (public good), there

could be selection for the cooperative individuals to evolve a receptor-specific molecule that is harder for cheats to exploit (West et al. 2007a). Empirical evidence for the evolution of cheater resistance comes from studies on both *D. discoideum* and *M. xanthus*. In both species it has been shown that the presence of cheater strains, which over contribute to the spores in fruiting bodies, selects for other strains to be less exploitable by these cheats (Fiegna et al. 2006; Khare et al. 2009). *M. xanthus*, cheats re-evolve the ability to cooperate but in a way that they are immune to exploitation by the ancestral cheats from which they evolved (Fiegna et al. 2006). While the presence of *D. discoideum* cheats selects for mutants that resist cheating but maintain cooperation (Khare et al. 2009).

If cooperators evolve to become harder to exploit, this would in turn, select on cheats to improve their ability to exploit the cooperators. Consequently, not only does the presence of cheats impose a selection pressure on cooperators, but this can lead to a coevolutionary arms race between cooperators and cheats. Possible examples of such coevolution include: (a) the conflict between brood parasitic cuckoos and their hosts, where the hosts are selected to reject cuckoo eggs, and the cuckoos are selected to circumvent this (Davies 2000; Spottiswoode and Stevens 2010; Langmore et al. 2011; Stoddard and Stevens 2011); (b) both cheats and cooperators performing better against their opponents from the past, but less well against future phenotypes, considering a laboratory selection experiment on biofilm production in *Pseudomonas fluorescens* (Zhang et al. 2009).

Box 2. Frequency dependence

Our definition of cheating suggests that because cheats can exploit the cooperation of others, they should be able to invade and spread into populations. Consequently, when we observe cheating, we might be looking at a stable equilibrium of cooperators and cheats, or at a form of cooperation that is breaking down and on its way out. A key factor here is whether the fitness of cheats is frequency dependent, such that the relative fitness of cheats decreases as they become more common in the system (Velicer et al. 2000; Fiegna and Velicer 2003; Ross-Gillespie et al. 2007). If such negative frequency dependence drives the relative fitness of the cheat below that of the cooperator, then a stable equilibrium is expected, where both cooperators and cheats will be maintained in the population (MacLean and Gudelj 2006; Gore et al. 2009; MacLean et al. 2010) (A). In contrast, if the relative fitness of the cheat does not go below that of the cooperators, then cooperation will be lost from the population (B).



Figures A and B. The relative fitness of cheats as a function of the initial proportion of cheats in a mixed population with cooperators. A relative fitness > 1.0 means that cheats have a higher fitness than cooperators, while a relative fitness < 1.0 , means that cheats have a lower fitness than cooperators. Part (A) shows when the relative fitness of cheats drops < 1.0 , in which case we would expect a stable equilibrium with both cooperators and cheats in the population. Part (B) shows when the fitness of cheats is always greater than that of cooperators, in which case we would expect the cheats to go to fixation.

At least three factors can lead to the fitness of cheats being negatively frequency dependent. First, if cheats are discriminated against, but less detected at low frequencies. This appears to be the case in cleaner fish, where the client's have an image scoring ability allowing them to interact more frequently with cleaners that mainly cooperate while avoiding cleaners that they observed cheated frequently (Bshary 2002). Second, if the presence of cheats impacts negatively on population growth, then the more cheats there are in a population, the less opportunity there will be for cheats to exploit cooperators, as has been found in bacteria (Ross-Gillespie et al. 2007; Frank 2010). Third, if cooperators are able to preferentially benefit from cooperative behaviors, such as when cooperators are clustered together, or if cooperation provides a direct as well as an indirect benefit, then this will lead to the fitness of cheats being negatively frequency dependent, as has been found in bacteria and yeast (Ross-Gillespie et al. 2007; Gore et al. 2009).

7. Do we need to talk about cheating?

We emphasize that examining social behaviors from the perspective of cheats will not always be necessary or the most useful way forward. For example, the term cheat is used very little when discussing cooperation and conflict within the social insects, such as over the sex ratio or who produces male eggs (Ratnieks et al. 2006; West 2009). In these cases, it is often more useful to think about a conflict between partners with different interests, rather than one individual, or one class of individual, cheating another.

The analogous point that terminology and concepts can be extremely useful in some cases, but not others, has already been made for other intentional language such as altruism and cooperation (West et al. 2007c). For example, terms such as altruism or spite, and theoretical tools such as Hamilton's rule, are rarely used in studies of some social traits, such as sex allocation in response to population structure or relatedness asymmetries (West 2009). This does not alter the usefulness of either the term altruism, or Hamilton's rule, when studying other issues. The general point here is that while clear and unambiguous terminology can facilitate the development of a conceptual overview, terms need only be used when useful.

Appendix

Cheat/cheating definitions	Reference
“cells that gain additional advantage from the cooperating type over and above that gained by avoiding the cost of cooperation”	(Rainey and Rainey 2003)
“individuals that have access to group benefits without contributing their fair share”	(Gilbert et al. 2007)
“We partition facultative cheating into two forms: (1) promotion of individual fitness through selfish behavior (“self-promotion”) and (2) coercion of other genotypes to act cooperatively.”	(Buttery et al. 2009)
<i>S. cerevisiae</i> “cheat by stealing the sugar digested by their neighbors without contributing the enzyme themselves”.	(Greig and Travisano 2004)
“cheaters-strains that make more than their fair share of spores in chimerae.”	(Khare and Shaulsky 2010)
“Genetic mutants that fail to perform a group-beneficial function but that reap the benefits of belonging to the group should have a withing-group selective advantage, provided that the mutants are not too common.”	(Velicer et al. 2000)
“As with any system depending on coordination, this transition is vulnerable to cheaters that do not perform a typical response (e.g., cells that fail to respond to cell-cell signals or cells that fail to halt cell division) but profit in some way from the fact that other members of the population still respond.”	(Vulic and Kolter 2001)
“Those strains that secrete invertase are considered “co-operators”, while non-producers are regarded as selfish “cheats”.”	(MacLean et al. 2010)
“In the case of multicellularity, these cheats are cancer cells.”	(Nunney 1999)
“There is strong variation between cleaners with respect to cheating of clients (i.e. feeding on client tissue instead of parasites)”	(Bshary 2002)
“Cheaters are illegitimate emitters that mimic infochemicals of other organisms to exploit a communicative system.”	(Roitberg and Isman 1992)
““producers”- who secrete a substance that breaks down antibiotic- and nonproducers (“cheats”) who do not secrete, or carry the machinery associated with secretion.”	(Dugatkin et al. 2008)
“subdivisions of signal interception includes: “spies”, “stowaways”, “boasters”; we do not go into the semantic distinctions here. The metaphors virtually always involve dishonesty and cheating”	(Zuk and McKean 2000)
“We use the term “cheater” only for individuals that belong to the mutualist species under investigation. They may either be individuals who always cheat (designated by Bronstein, 2001, as “pure exploiters”), or individuals that cooperate under some defined range of conditions (“conditional exploiters”).”	(Bshary and Bronstein 2004)
“A Proportion of nectarless flowers are known to occur within rewarding species and these are thought to be cheater flowers.”	(Thakar et al. 2003)
“caching systems based on reciprocal pilfering can be stable and are not necessarily susceptible to “cheaters”, animals that pilfer food but do not scatter hoard food themselves”	(Vander Wall and Jenkins 2003)
“When hoarders live in a group, an additional cost may arise from ‘cheaters’, who do not store food themselves but parasitize the caches made by other individuals.”	(Andersson and Krebs 1978)
“ “Aprovechados” are non-mutualistic species that take advantage of a mutualistic association. For example, some bees and birds utilize floral nectar without transferring pollen. “Cheaters” are non-mutualistic individuals within a mutualistic species. For example, individuals of some	(Tyre and Addicott 1993)

<p>“ "Aprovechados" are non-mutualistic species that take advantage of a mutualistic association. For example, some bees and birds utilize floral nectar without transferring pollen. "Cheaters" are non-mutualistic individuals within a mutualistic species. For example, individuals of some plants produce little or no nectar but are still visited by pollinators.”</p>	(Tyre and Addicott 1993)
<p>“In general, signals of quality require high and differential costs to remain honest (that is prevent low-quality cheaters from exploiting any fitness benefits associated with communicating high quality).”</p>	(Tibbetts and Dale 2004)
<p>“Cheating (<i>sensu lato</i>) occurs when individuals use mutualistic resources or services without providing mutualistic resources or services in return. <i>Aprovechados</i> (<i>sensu</i> Soberon and Martinez 1985) are individuals of non-mutualistic species, which exploit a mutualism, whereas cheaters are individuals that exploit a mutualism even though most other conspecifics act mutualistically. Both cheaters and <i>aprovechados</i> decrease the net benefit to the provider of the mutualistic services or resources.”</p>	(Addicott and Tyre 1995)
<p>“One should avoid ‘greedy’ individuals that may try to reap short-term benefit by not returning favours”</p>	(Noe 2006)
<p>“In a badge-of-status system, an obvious question is why individuals do not use their signal in an inappropriate manner or why cheating does not happen.”</p>	(Nakagawa et al. 2008)
<p>“selfish individuals derive an advantage from exploitation which is greater than the average advantage that accrues to unselfish individuals. Secondly, exploitation has no intrinsic fitness value except in the presence of the “cooperative behavior" practiced by the other members of the group”</p>	(Koeslag and Terblanche 2003)
<p>Intruding aphids cheat on the host clone by not helping in gall defence but instead invest in their development growth and reproduction.</p>	(Abbot et al. 2001; Foster 2002)
<p>“a cheater or a somatic parasite that does not participate in stalk formation but always forms spores can gain a relative fitness advantage.”</p>	(Matapurkar and Watve 1997)
<p>“ ‘Cheaters’ are individuals that hunt if they are the first to spot the prey, but stop if others join in and do not hunt if others are hunting.”</p>	(Whitehouse and Lubin 1999)
<p>“Cheating is defined as benefiting from an exaggerated advertisement that results from producing an inaccurately high level of the signal.”</p>	(Briffa 2006)
<p>“Anarchy is a counterstrategy against worker policing and an example of a ‘cheating’ strategy invading a cooperative system.”</p>	(Barron et al. 2001)
<p>In gift-giving species, males that provide worthless nuptial gifts (cheats) could potentially invade a group that provide genuine gifts.</p>	(LeBas and Hockham 2005)
<p>“ ‘cheaters’ who signal a dominant status when they are actually subordinate (or true dominants who signal a subordinate status)”</p>	(Molles and Vehrencamp 2001)
<p>“Upon receiving a clutch of eggs to fertilize, a cheater should offer no eggs in return; in other words, it will try to play the male role instead of the female role.”</p>	(Sella and Lorenzi 2000)
<p>“Why not ‘cheat’, by displaying more intensely than normal, and increase the odds of driving off a rival?”</p>	(Halperin et al. 1998)
<p>“brood parasites—cheats that procure costly care for their dependent offspring by leaving them in another species' nursery.”</p>	(Kilner and Langmore 2011)
<p>“a queen is prevented from ‘cheating’ (for example, by eating all the eggs of the other queen and replacing them with her own).”</p>	(Reeve and Nonacs 1992)

Table A1. Examples of how cheating has been defined in various studies.

Chapter 3. An Experimental Test of Whether Cheating is Context Dependent[†]

Abstract

Microbial cells rely on cooperative behaviours that can breakdown as a result of exploitation by cheats. Recent work on cheating in microbes, however, has produced examples of populations benefiting from the presence of cheats and/or cooperative behaviours being maintained despite the presence of cheats. These observations have been presented as evidence for selection favouring cheating at the population level. This apparent contradiction arises when cheating is defined simply by the reduced expression of a cooperative trait and not in terms of the social costs and benefits of the trait under investigation. Here we use two social traits, quorum sensing and iron scavenging siderophore production in *Pseudomonas aeruginosa*, to illustrate the importance of defining cheating by the social costs and benefits. We show that whether a strain is a cheat depends on the costs and benefits associated with the social and abiotic environment and not the absolute expression of a cooperative trait.

Introduction

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The growth of microbes often relies on the costly secretion of a range of exofactors such as biofilm polymers, nutrient-scavenging molecules and toxins (Crespi 2001; West *et al.* 2006; Xavier 2011). The benefit of producing exofactors can be shared with other cells in the local group, in which case they can represent cooperative traits (Griffin *et al.* 2004; West *et al.* 2006). The problem with cooperation is that it is susceptible to exploitation by ‘cheats’, which do not perform the costly cooperative behavior, but benefit by exploiting the cooperation of others (Ghoul *et al.* 2013).

Recent work on cheating in microbes however, has produced results that are apparently inconsistent with theoretical predictions about the outcome of competition between cooperators and cheats. For example, while it has been shown that the production of iron scavenging siderophore molecules can be a cooperative trait in bacteria (Griffin *et al.* 2004), a recent study has argued this is not the case (Zhang and Rainey 2013). Another example is that, while cheating, by definition, should come with an associated fitness cost to the cooperators and the group, a recent study on yeast found that group productivity is maximized by a mixed population of cooperators that produce invertase and cheats that did not (MacLean *et al.* 2010). More generally, it is a widely held assumption that a cheat can be identified simply by reduced expression of a cooperative trait, relative to others.

We suggest that inconsistencies in the experimental literature can arise when cheating is defined as reduced production of some factor and not in terms of the relative costs and benefits. As with any social trait, the definition of cheating depends upon the social costs and benefits (Hamilton 1964; West *et al.* 2007c). Consequently, reduced production of an exofactor will not always be cheating (Ghoul *et al.* 2013). Put simply, whether a certain strain or genotype is a cheat, is context dependent such that

cheating depends on its social and abiotic environment. Here we illustrate that cheating is context dependent using two traits commonly used in experimental studies of social behaviour in microbes: the use of quorum sensing (QS) to coordinate the production and release of exofactors, and the production of iron scavenging pyoverdinin (pvd) siderophore molecules, in the opportunistic pathogen *Pseudomonas aeruginosa*. These two systems, each offer different opportunities to manipulate the abiotic environment (requirement for QS to grow), and the social environment (strains with variable pyoverdinin production).

Our first aim is to use the QS system to test the effect of the abiotic environment. We use a wildtype PAO1 strain as a cooperator and an isogenic *lasR* mutant, a signal blind QS mutant, as a putative cheat. Strains that have a mutation in the *lasR* gene do not respond to autoinducing *N*-acyl homoserine lactone (AHL) signal molecules and therefore cannot synthesize QS-dependent exofactors to acquire nutrients (Diggle *et al.* 2007; Mellbye and Schuster 2011; Schuster *et al.* 2013). We compete the putative *lasR* cheat with the cooperator in an environment that requires QS for the production of exofactors, and in an environment, where QS production of exofactors is not necessary for survival. We predict that the *lasR* mutant will act as a cheat in the nutrient-poor conditions, but not in the nutrient-rich environment.

Our second aim is to use the pyoverdinin system to test the effect of the social context on cheating behavior. We use a wildtype *P. aeruginosa* strain as a cooperator and as putative cheats we use mutants isolated from selective lines from a wildtype ancestor, which are defective in pyoverdinin production. We compete pairs of strains that produce different quantities of pyoverdinin in an iron-limited environment. We predict that whether a strain acts as a cheat depends upon its pyoverdinin production relative to its competitor strain, and not its absolute level of pyoverdinin production.

Materials and Methods

Bacterial strains

We used two strains in our QS experiment: a *P. aeruginosa* wildtype PAO1 strain and an isogenic PAO1 insertion mutant *lasR::Gm (lasR)* (Popat *et al.* 2012) which is a signal-blind mutant that does not respond to autoinducer signal, and hence fails to induce the synthesis of exofactors even at high cell densities (Diggle *et al.* 2007).

We used three strains in our siderophore experiment: the wild type *P. aeruginosa* 206-12-strain, 10a⁺ (a cystic fibrosis isolate from Seattle, WA, USA (Jiricny *et al.* 2010)) and two spontaneous pyoverdinin mutants, 10a⁻ and 10c⁻, derived from the pyoverdinin-producing wildtypes, 206-12-10a⁺ and 10c⁺, respectively as described in Jiricny *et al.* (2010). In this study we refer to the wildtype strain 10a⁺ as strain A, and the pyoverdinin mutants 10a⁻ and 10c⁻, as strains B and C.

Prior to experimentation, we cultured all strains from freezer stocks for 24 hours at 37°C on an orbital shaker at 180 rpm. In the QS experiment, strains were cultured in Lysogeny Broth (LB, 14 g broth miller, (Fisher Scientific) per liter of distilled water (dH₂O)). In the pyoverdinin experiment, strains A, B, and C were cultured in King's broth media (KB, 20 g protease peptone N°3 (Beckton Dickinson, UK ltd), 10 mL glycerol, 1.5 g K₂HPO₄·3H₂O and 1.5 g MgSO₄·7H₂O (Sigma Aldrich UK ltd), per liter of dH₂O). We centrifuged the overnight cultures and discarded the supernatant. We then washed the cell pellet in minimal salts media (M9, 6.8 g Na₂HPO₄, 3 g KH₂PO₄, 0.5 g NaCl and 10 g NH₄Cl (sigma Aldrich, UK ltd), per liter of dH₂O) to remove any residual iron and carbon resources from the KB or LB media. We assayed cell density at an absorbance of 600 nm (A₆₀₀) and standardized each set of cultures to

the same density.

Testing the effect of the abiotic context in a QS system

Measuring lasI and lasB expression

Our QS experiment relies on the assumption that PAO1 continuously invests in exofactor synthesis in different media. We tested whether PAO1 continuously invests in exofactor synthesis by quantifying the gene expression of *lasI* (regulates *N*-(3-oxododecanoyl)-L-homoserine lactone (3O-C₁₂-HSL) production) and *lasB* (codes for elastase production) using *lasI* and *lasB* CTX*lux* reporters conjugated into PAO1 (strains PAO1 *lasI::lux* and PAO1 *lasB::lux*) that were then grown in different media (Jadhav et al. 2011; Popat et al. 2012). Cultures were grown for 24 hours in rich casamino acid media (CAA, 1% casamino acids weight/volume (w/v) (Sigma Aldrich, UK Ltd), in M9 solution) and in a minimal media containing bovine serum album (BSA, 1% BSA w/v (Sigma Aldrich, UK Ltd) and 0.1% CAA w/v in M9 solution) as described by Darch *et al.* (2012). Cell growth in CAA does not require QS-controlled exofactor production, while growth in BSA requires QS-dependent exofactor secretions (Diggle et al. 2007; Darch et al. 2012). We determined bioluminescence as a function of cell density by using a combined, automated luminometer-spectrometer (Genios Pro, Tecan Group, UK). Luminescence and turbidity (A_{600}) were automatically measured every 30 minutes. We calculated luminescence per cell as relative light units (RLU)/ A_{600} at each time point. We replicated each treatment six times.

Competition assays

We carried out competition assays in CAA and BSA media respectively. We grew the

lasR mutant and PAO1 strains in a 6:100 and 5:100 ratio respectively in vials containing 2 ml volume of either CAA or BSA media. The BSA media is supplemented with minimal amounts of CAA in order to initiate QS for the wildtype strain during the competitions (Darch *et al.* 2012; Popat *et al.* 2012). In order to control for social effects on relative fitness, we cultured the *lasR* mutant and wildtype strains separately as monocultures. Relative frequencies at the start of each experiment were obtained by plating 40 μL of 10^2 dilutions on LB agar (25 g LB Miller agar (Fisher Scientific) per liter of dH_2O), and then incubated them at 37°C . We then incubated all cultures for 24 hours at 37°C . At 24 hours, after the strains had competed through the exponential growth phase and reached the stationary phase, we plated out 20 μL and 100 μL of 10^6 dilutions of the CAA and BSA mixed cultures respectively. We then diluted the BSA monocultures 2-fold and the CAA monocultures 10-fold using M9 medium prior to measuring the cell densities at A_{600} , to compare the growths of mutant and wildtype in the different media environments.

After incubating the agar plates for 17 hours at 37°C , we recorded the colony counts of each strain which are distinguishable by colony phenotypes. *lasR* mutant colonies have smooth edges surrounded by a halo while PAO1 colonies have rugged edges.

Testing the effect of the pyoverdinin social context

Measuring pyoverdinin production

We estimated the relative pyoverdinin production per cell by the ratio of relative fluorescent units (RFU), at excitation and emission wavelengths of 400 nm and 460 nm respectively, to absorbance (A_{600} , cell density), as described by Jiricny *et al.* (2010). We replicated each assay six times per culture and calculated the mean

pyoverdinin production per cell by RFU/ A_{600} (Kummerli et al. 2009b).

Competition assays

We compared the relative fitness of strain C to that of B, and strain B to strain A (wild type), by competing C:B and B:A in a 1:10 ratio, in vials containing 6 ml of iron-limited CAA media. In each case, the lower-producing pyoverdinin strain (putative cheat) is introduced at a lower frequency than the higher-producing strain (putative cooperator) - see results. We also cultured each strain separately in a monoculture. Relative frequencies at the start of each experiment were obtained by plating 10 μ L of 10^1 dilution of mixed cultures on KB agar (12 g agar per liter of KB medium) which we then incubated at 37°C. We then incubated all cultures for 24 hours at 37°C. At 24 hours after the strains had competed through the exponential growth phase and reached the stationary phase, we plated 10 μ L of 10^4 dilution on KB agar plates and incubated them at 37°C. We then diluted the monocultures 10-fold with M9 medium and assayed the cell density of each monoculture at A_{600} to compare the growths of each strain.

After incubating the agar plates for 17 hours at 37°C, we recorded the colony counts of each strain. We phenotypically distinguished colonies of each strain based on colony shape and pigmentation. The strains that produce more pyoverdinin are greener in colour (Jiricny *et al.* 2010). We ensured the plates are not incubated longer than 17 hours because siderophores diffuse into the agar and are taken up by neighbouring colonies adding to their pigmentation and making them less distinguishable.

Statistical analysis

We calculated the relative fitness (ω) of the putative cheat, which is the change in frequency over time relative to the wildtype. This is given by $\omega = x_2(1-x_1)/x_1(1-x_2)$ where x_1 is the mean initial proportion of mutants at 0 hour from the sample population and x_2 is the final proportion of each sample at 24 hours (Otto and Day 2007; Ross-Gillespie *et al.* 2007). A value of $\omega > 1$ indicates that the cheat has a higher fitness than the cooperator, and a value $\omega < 1$ indicates that the cheats have a lower fitness than cooperators. We replicated each competition assay six or twelve times, and logged (\log_{10}) the ω values before calculating the mean across replicates in order to eliminate any bias from pseudo-replication variation (Jiricny *et al.* 2010). We carried out all analysis in R statistical software (<http://www.R-project.org>).

Results

Varying the abiotic context in a QS system

Competition and monoculture assays

The *lasR* mutant acts as a cheat in BSA media, where QS is required for growth, but not in CAA media, where exofactor synthesis is not required for growth. In mixed cultures, containing PAO1 and the *lasR* mutant, the *lasR* mutant increases in frequency, in both BSA media (Fig.1; $\omega_{lasR} = 1.7 \pm 0.4$, $T = 3.33$, $P = 0.007$), and in CAA media (Fig.1; $\omega_{lasR} = 2.2 \pm 0.6$, $T = 4.04$, $P = 0.002$). In monocultures, the *lasR* mutant grows significantly less well than PAO1 in BSA media (Fig.2a; $T = 9.66$, $P = 0.0001$), but significantly better than PAO1 in CAA media (Fig.2b; $T = 11.55$, $P = 1.6 \times 10^{-6}$). To ensure that the difference in cell density, as measured by A_{600} reads, is not an artifact of optical density reads, we also plated out colonies of each monoculture, counted the colony forming units per ml (CFU/ml) and confirmed that *lasR* mutant grows significantly less well than PAO1 in BSA media (CFU \pm 95%CI: CFU_{PAO1} = $1.1 \times 10^{10} \pm 4.06 \times 10^9$, CFU_{*lasR*} = $3.8 \times 10^9 \pm 1.2 \times 10^9$; $T = -3.18$, $P = 0.0193$) but significantly better than PAO1 in CAA media (CFU_{PAO1} = $9.3 \times 10^9 \pm 1.3 \times 10^9$, CFU_{*lasR*} = $2.0 \times 10^{10} \pm 6.3 \times 10^9$; $T = 3.23$, $P = 0.0204$).

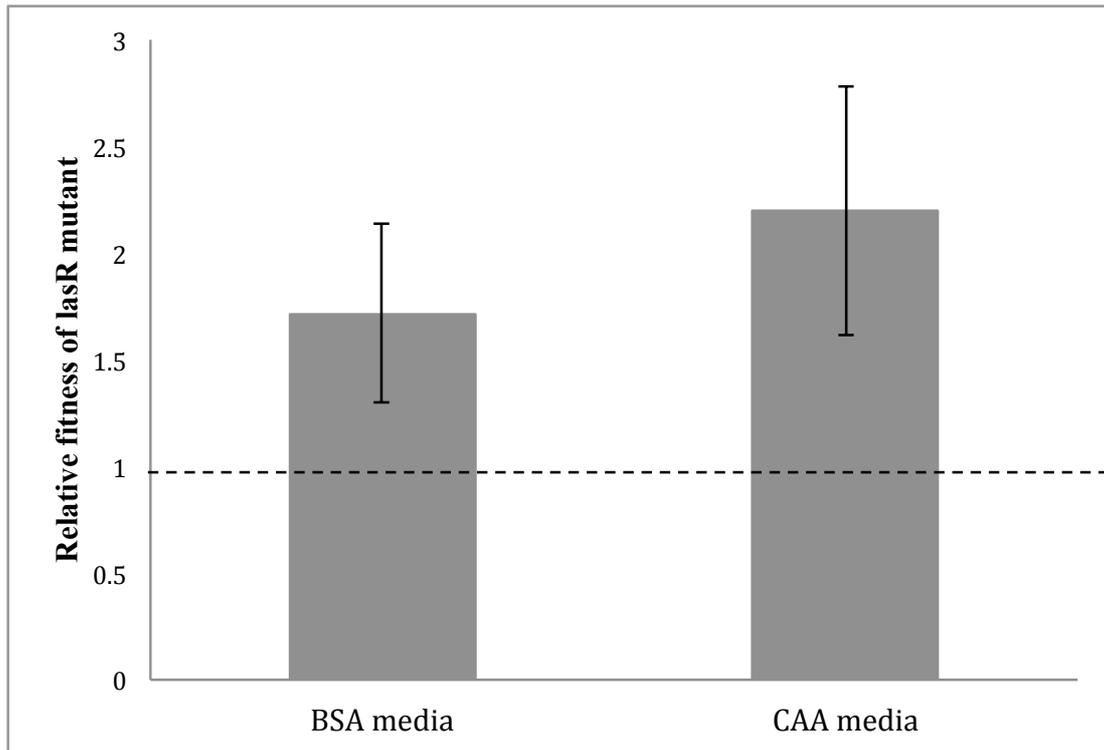


Fig. 1 Relative fitness of the *lasR* mutant in competition with PAO1 under conditions that requires QS (BSA) and that does not require QS (CAA). The *lasR* mutant increases in frequency in competition with wildtype in both BSA media (where QS is required for growth) and in CAA media (where QS is not required for growth). The error bars indicate 95% confidence intervals around the means of twelve independent competition replicates.

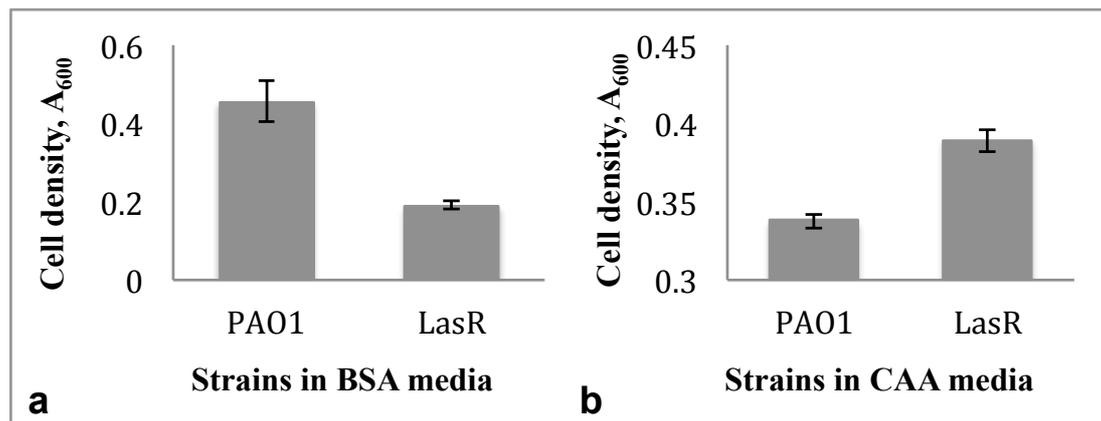


Fig. 2 Monoculture cell density (A_{600}) in media that requires QS (BSA) and media that does not require QS (CAA). In monocultures of the *lasR* and PAO1 strain: (a) PAO1 grows to a significantly higher density than *lasR* in BSA medium; (b) PAO1 grows to a significantly lower density than *lasR* in CAA medium. The error bars indicate 95% confidence intervals around the means of six independent replicates.

lasI and lasB expression

Even in CAA media, which does not require exofactors for cell growth, PAO1 continues to express QS-dependent genes and invest in exofactor synthesis over a period of 24 hours of growth (Fig. 3 a and b). In CAA media, PAO1 expresses *lasB* at a significantly higher peak per cell than *lasI* (Fig. 3a; $T= 16.44$, $P= 7.544 \times 10^{-7}$). In BSA media PAO1 expresses *lasI* at a significantly higher peak per cell than *lasB* (Fig. 3b; $T= -6.18$, $P= 0.0008$). Comparing gene expression in both media shows that PAO1 expresses *lasB* and *lasI* at significantly higher levels in CAA than in BSA media (Fig. 3 a and b, $T= -26.85$, $P= 8.962 \times 10^{-7}$ and $T= -9.91$, $P= 1.761 \times 10^{-6}$, respectively). In the BSA environment *lasB* expression is sustained indicating that the production of protease exofactors, such as elastase, is important for growth in this environment and is therefore maintained. Whereas in the CAA medium, there is a peak of gene expression when QS induces followed by a sharp drop in expression.

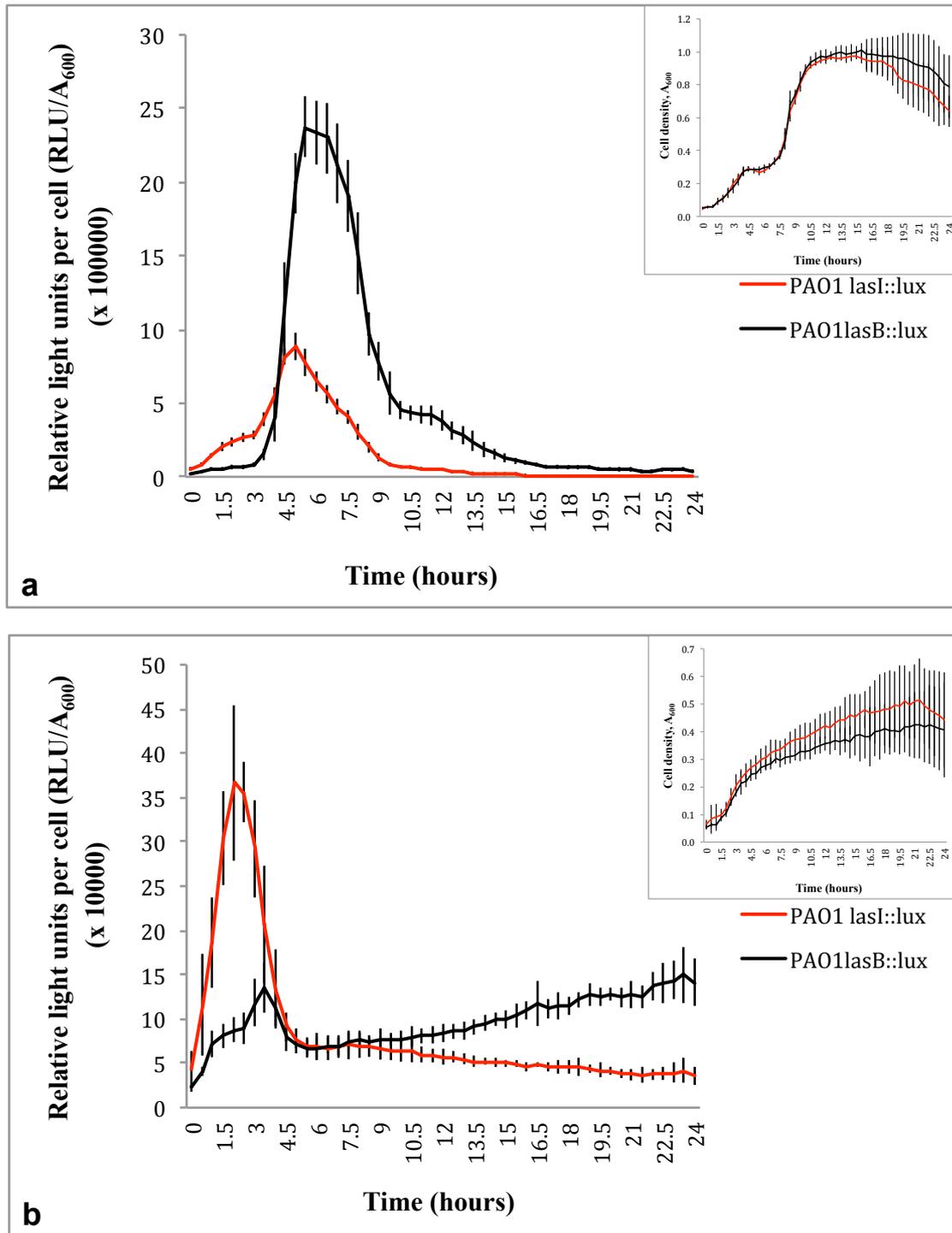


Fig. 3 a-b PAO1 expression (RLU/ A_{600}) of *lasI* and *lasB* genes per cell and strain growth curves in (a) CAA and (b) BSA media. The *lasI* gene directs the synthesis of the autoinducer N-(3-oxododecanoyl) homoserine lactone (3O- C_{12} -HSL), and *lasB* codes for elastase in the PAO1 wildtype. Cultures of each lux-tagged PAO1 were grown in each medium and every half hour relative light unit (RLU) and cell density (A_{600}) measurements were taken for a period of 24 hours of growth. a) In CAA media, PAO1 expresses *lasB* significantly more than *lasI*. b) In BSA media PAO1 expresses *lasI* significantly more than *lasB*. The error bars indicate standard deviation around the means of six independent replicates.

Varying the social context in a public good (pyoverdinin) system

Competition and monoculture assays

The outcome of competition depends on the relative and not the absolute expression of the public good, pyoverdinin: strain B either cheats or is cheated, depending on its social partner. Strain A produces significantly higher levels of pyoverdinin per cell than strain B, and strain B produces significantly higher levels of pyoverdinin per cell than strain C (Fig.4a; $F_{(2,15)} = 72.05$, $P = 2.03 \times 10^{-8}$). The growth rate, in monoculture, correlates with pyoverdinin production, with strain A growing to a higher density than strain B, which grows to a higher density than strain C (Fig.4b; $F_{(2,15)} = 272.4$, $P = 1.62 \times 10^{-12}$). To confirm that the difference in cell density, as measured by A_{600} reads, is not an artifact of optical density reads, we also plated out colonies of each monoculture, counted the colony forming units per ml (CFU/ml) and confirmed that strain A grows significantly better than strain B, which grows better than strain C (CFU \pm 95%CI: $CFU_A = 7.22 \times 10^7 \pm 1.66 \times 10^7$, $CFU_B = 4.05 \times 10^7 \pm 6.05 \times 10^6$, $CFU_C = 3.25 \times 10^7 \pm 9.38 \times 10^6$; $F_{(2,15)} = 12.64$, $P = 0.000605$). We found that, in mixed cultures, when grown in an iron-limited environment, where cooperative pyoverdinin production is required for growth, strain B increases in frequency when in competition with strain A (Fig.5; $\omega_B = 1.96 \pm 0.45$, $T = 4.20$, $P = 0.008$), but decreases in frequency when grown in competition with strain C (Fig.5; $\omega_B = 0.50 \pm 0.16$, $T = -6.32$, $P = 0.001$).

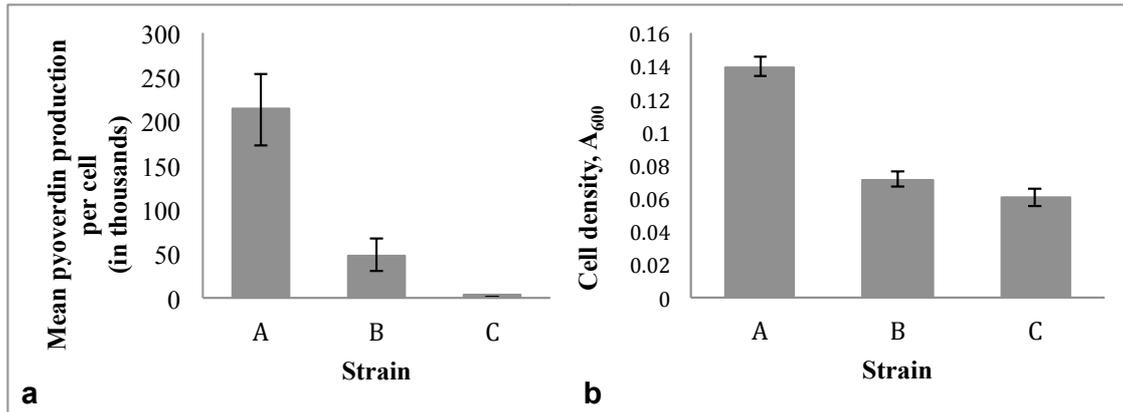


Fig. 4 (a) Mean pyoverdinin production per cell, and (b) monoculture cell density. In iron-limited CAA medium where pyoverdinin is required for growth: (a) wildtype strain A produces significantly more pyoverdinin ($ppc_A = 2.13 \times 10^5 \pm 2.07 \times 10^4$) than mutant strain B, which produces significantly more ($ppc_B = 4.87 \times 10^4 \pm 9.47 \times 10^3$) than mutant strain C ($ppc_C = 3.73 \times 10^2 \pm 75.51$); (b) pyoverdinin production is correlated with cell density (A_{600}), where strain A grows to a significantly higher density than strain B, and strain B grows significantly better than strain C. The error bars indicate 95% confidence intervals around the means of six independent replicates.

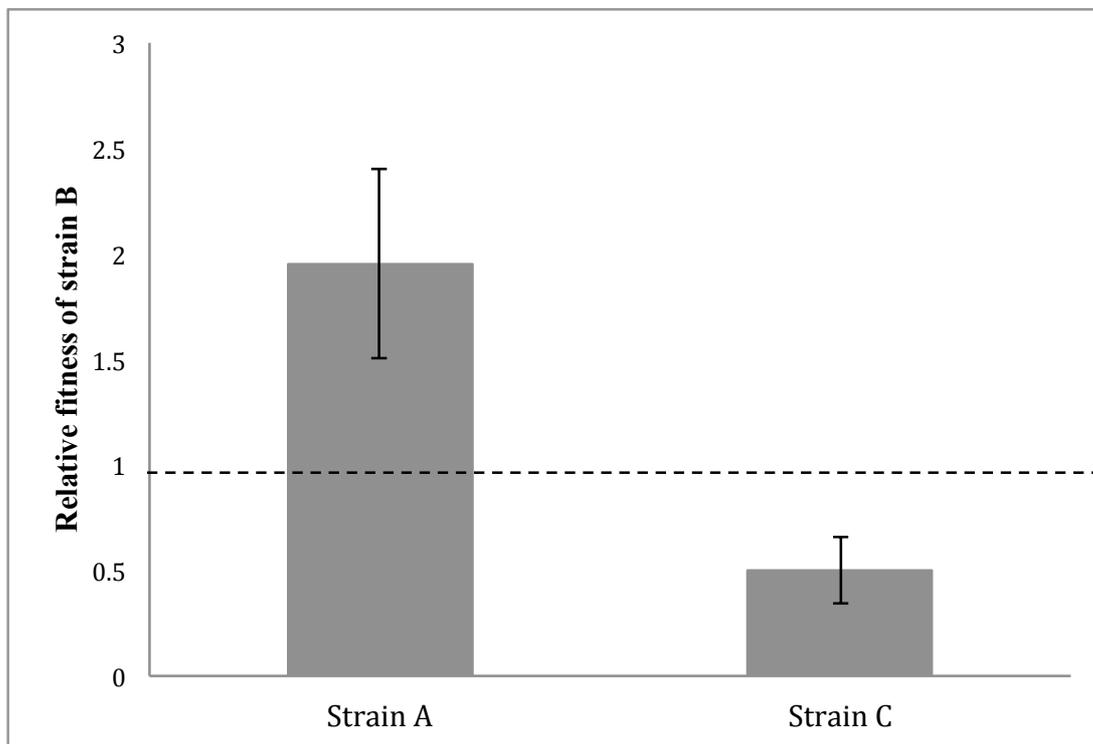


Fig. 5 Relative fitness of strain B in competition with strain A and with strain C under iron-limited conditions. In mixed cultures of iron-limited CAA media, strain B increases in frequency when in competition with strain A, but strain B decreases in frequency when competing with strain C. The error bars indicate 95% confidence intervals around the means of six independent competition replicates.

Discussion

We have demonstrated that whether or not a strain, with reduced expression of a potentially cooperative trait, acts as a cheat depends upon the abiotic and biotic environment. Cheats can increase in frequency at the cost of another strain by exploiting cooperation (Ghoul *et al.* 2013). Using the QS system, we found that a *lasR* mutant, which does not respond to QS signal, acts as a cheat to PAO1's cooperative production of exofactors in a QS-dependent environment, but not in an environment where QS is not required for growth (Figs. 1 and 2). Examining pyoverdinin iron scavenging molecules, we found that whether a strain acted as a cheat depends upon its pyoverdinin production relative to the strain it is interacting with, and not its absolute level of pyoverdinin production (Figs. 4 and 5).

The QS experiment showed that whether or not a strain that produces less of some exofactor is a cheat depends upon the abiotic environment. In both the CAA and BSA conditions, the *lasR* mutant (putative cheat) increased in frequency in mixed cultures with the PAO1 wildtype (putative cooperator; Fig 1.) Whilst these mixed culture results are consistent with the mutant being a cheat, they could also be explained by some non-social fitness advantage to the *lasR* mutant, such as avoiding the cost of producing unneeded exofactors (i.e. the mutants are better adapted to the medium). The possibility for this alternative explanation is why we also needed to compare the growth of the strains in monocultures. In monocultures, PAO1 grew better in BSA, but the *lasR* mutant grew better in CAA (Fig. 2). These results suggest QS provides a benefit in BSA, but not in CAA. Taken together, the monoculture and mixed culture results suggest that in BSA conditions, QS is a cooperative behavior (PAO1 does better in monoculture) that can be exploited by *lasR* cheats (*lasR* mutants do better in

mixed culture). In contrast, in a CAA environment, QS is a costly trait that provides no benefit at the population level and is not cooperative (*lasR* mutants do better in mixed culture and monocultures).

These results illustrate that the *lasR* mutant increases in frequency in the mixed cultures for different reasons – because it is a cheat in conditions requiring QS (BSA), and because it is avoiding the cost of producing not needed exofactors in conditions where QS is not required for survival (CAA). The latter is not cheating; highlighting that it is essential to both carry out monoculture controls (Fig. 2), and test for cheats in an appropriate (natural) environment (West et al. 2012b; Ghoul et al. 2013; Kümmerli and Ross-Gillespie 2013). While determining the appropriate environment can be hard with bacteria, we would only expect bacteria to produce exofactors that aid growth – natural selection would quickly weed out anything not needed (Fisher 1930; Morris et al. 2012). Consequently, the first step should be to test for cheating in an environment where the potentially cheatable (cooperative) trait provides a benefit at the population level (Kümmerli and Ross-Gillespie 2013), such as the BSA, QS-dependent environment.

The pyoverdinin experiment showed that whether or not a strain that produces a certain amount of some exofactor is a cheat depends upon the biotic or social environment. Taking strain B as our focal strain, we found that strain B acted as a cheat when in competition with Strain A, which produced more siderophores (Figs. 3 & 4). In contrast, we found that strain B was cheated by strain C, which produced less siderophores (Figs. 3 & 4). The point here is that cheating is relative to the individuals being interacted with. What matters is how much exofactor you produce relative to the cells that you interact with, and not the absolute level produced (Ghoul et al. 2013).

How do our results help us understand the possible contradictions in the existing literature? Zhang and Rainey (2013) concluded that siderophore production was not a cheatable cooperative trait, but is “unnecessary and maladaptive” because they carried out their experiments in an environment where siderophore production was not needed (Kümmerli and Ross-Gillespie 2013). More specifically, their results are analogous to our QS experiments in CAA. The general point here is that examining the social costs and benefits of a trait will give misleading results if experiments are carried out in environments where the trait has no function. MacLean *et al.* (2010) found that a mixed culture of cells that produce invertase, and cells that did not, was able to grow to higher population densities than a culture of cells that just produce invertase. This occurs because the producers keep producing invertase, even when all the sucrose has been used up, and hence when invertase is not needed (MacLean *et al.* 2010). At this time, invertase production is not cooperative anymore, and so the non-producers increase group productivity not because they are cheating *per se*, but because they are not producing a molecule when it is not needed. This is also analogous to our QS experiments in CAA

Conclusion

Our results clarify some of the necessary steps to take when designing microbial experiments to study the evolution of cooperation, cheating, and the persistence of these behaviours with changing biotic and abiotic conditions. It is useful here to distinguish between two broad types of microbial study. If the aim is to use a microbial system to test theoretical predictions, it is necessary to experimentally set up the costs and benefits for cooperation to occur in that environment, such that cheats can exploit that cooperation (Griffin et al. 2004; Kummerli et al. 2009a; Kümmerli and Ross-Gillespie 2013). In contrast, if the aim is to determine the nature of selection on a trait under natural environments, where the social and abiotic conditions are expected to be much more variable than highly controlled experimental systems, it should not be assumed that putative cheats and cooperators are always social behaviours (Rumbaugh et al. 2009; West et al. 2012b). In this case, it is essential to first determine the social costs and benefits associated with putative cooperators and cheats, so that the occurrence of cooperation and cheating can be confirmed, before making inferences about their evolution.

Chapter 4. Cooperative Bacteria in Stationary Phase are Immune to Cheating

Abstract

Microbes engage in cooperative behaviours by producing and secreting public goods that are shared among cells and are therefore susceptible to exploitation by non-producing cheats. Despite the risk of cheats invading, cooperation dominates natural microbial life. A continuing challenge is to determine how natural cooperative populations resist cheating and to what extent experimental studies can give insight into bacterial life outside of a test tube culture. In nature bacteria exist as established populations in a non-growing state similar to the stationary phase of laboratory cultures. A limitation with experimental bacterial studies is that cheating is often tested for in competitions initiated with cooperator populations that are at a low density at the start of their growth - in lag phase. Here, we use the production of pyoverdine, an iron scavenging siderophore, as a public good in the bacteria *Pseudomonas aeruginosa*, to test how the growth stage of a cooperative population affects the ability of cheats to invade. We show that a strain's ability to cheat correlates with the rates of pyoverdine production, which varies with the bacterial growth stage.

Introduction

Bacterial growth relies on the costly secretion of a range of exoproducts such as nutrient scavenging molecules, quorum sensing (QS) signals, biofilm polymers and toxins (Crespi 2001; West et al. 2006). Cells in a local population can share the benefit of exoproduct secretion, and exoproducts can, therefore, act as cooperative “public goods” (Crespi 2001; Griffin et al. 2004; West et al. 2006). Consequently, populations of cells that produce exoproducts (cooperators) are potentially susceptible to exploitation and invasion by ‘cheats’ that do not produce the costly exoproduct but benefit from the cooperative production of others (Ghoul et al. 2013).

Controlled experimental studies *in vitro* and *in vivo* have shown that mutants that do not produce exoproducts can invade a population of exoproduct producers (Diggle et al. 2007; Rumbaugh et al. 2009). Furthermore, evidence exists that this process can occur in natural populations: *Pseudomonas* and *Vibrio* strains that do not produce iron scavenging siderophores have been isolated from lung infections in patients with cystic fibrosis and the ocean respectively (Smith et al. 2006; D'argenio et al. 2007; Bodilis et al. 2009; Cordero et al. 2012; Jiricny et al. 2014) and *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Vibrio cholera* and *Bacillus cereus* quorum sensing mutants have been isolated from infections of the cystic fibrosis lung, urinary tract, or blood, and epidemic, environmental and spontaneous lab mutant strains (West et al. 2012a; Jiricny et al. 2014). However, despite the risk of cheat invasion, cooperation is evidently the norm in natural bacterial populations (Hibbing et al. 2010; Levin 2014). The challenge remains, therefore, to determine the mechanism that potentially limits cheating in natural microbial populations and to assess the

extent to which the findings of experimental evolution studies can be applied to natural populations of bacteria.

One notable difference between bacterial cells in experimental settings and those living in nature is likely to be the physiological growth state. In nature, populations of bacteria are more likely to compete with one another in established populations that are in a non-growing state, similar to the stationary growth phase of laboratory cultures (Kolter et al. 1993; Wallis et al. 1999; Mulvey et al. 2001; Finkel 2006; Llorens et al. 2010; Gefen et al. 2014). The typical procedure for introducing cheats to cooperative populations in the lab involves co-inoculation of both strains at low density into sterile media, where cells then compete through all physiological growth phases. We have a poor understanding, therefore, of the ability of cheats, that emerge by mutation and/or migration, to invade established populations of cooperative cells. This may be a significant omission as there are a number of reasons why potential cheats may not be able to invade an established population: in stationary phase, cells generally have reduced levels of exoproduct synthesis compared to exponential cells (Kolter et al. 1993; Gefen et al. 2014); and if exoproducts are durable, the level of production may be downregulated and negligible or low when the cheats are introduced, such that they would have little or no fitness advantage (Brown and Taddei 2007; Kummerli et al. 2009b; Kummerli and Brown 2010; Xavier et al. 2011). Here, we describe the results of an experimental test of how growth stage of cooperative bacteria affects their ability to resist invasion by cheats. We use the model system of pyoverdinin production in *Pseudomonas aeruginosa* –pyoverdinin is an iron-scavenging siderophore molecule, which has been shown in a number of detailed studies to act as a public good in iron-limited conditions (Griffin et al. 2004). In previous work with this system, it has been demonstrated that under iron-limited

conditions, the relative fitness of cheats is positively correlated with pyoverdinin production of cooperative competitors (Jiricny et al. 2010). We use a wildtype *P.aeruginosa* strain as a cooperator and as a putative cheat we use a mutant, defective for pyoverdinin production. We aim to determine how the invasive potential of cheats varies with the rate of pyoverdinin production per cell though the growth curve of a cooperative population and, therefore, whether cooperative *P.aeruginosa* populations in stationary phase cultures- the state bacteria commonly occurs at in nature- have increased resistance to invasion.

Materials and Methods

Model system

We examine pyoverdinin production in the opportunistic gram-negative pathogen, *Pseudomonas aeruginosa*. Pyoverdinin is an iron-scavenging siderophore molecule that is secreted by cells in iron-limited conditions (Kummerli et al. 2009b). Iron is essential for bacterial growth but is generally a major limiting nutrient because it is found in an insoluble Fe(III) form in the environment and is actively withheld by hosts during infection (Ratledge and Dover 2000). Therefore, bacterial cells almost always invest in pyoverdinin production to chelate iron and once the molecules are secreted they can be shared by neighbouring cells, acting as a public good (Griffin et al. 2004). Pyoverdinin molecules are highly durable and can be recycled and reused multiple times such that bacterial cells can downregulate production when sufficient levels accumulate (Faraldo-Gomez and Sansom 2003; Imperi et al. 2009; Kummerli and Brown 2010)

Bacterial strains

We used two *Pseudomonas aeruginosa* strains in our experiments: the wildtype PAO1 strain which produces pyoverdinin, and the pyoverdinin UV-induced mutant PAO9 strain derived from PAO6094, a mutant of PAO1 (Harrison et al. 2006).

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

Growth curves and measuring pyoverdinin production

In order to determine how pyoverdinin production varied during the bacterial growth stage from point of inoculation we constructed 48 hour growth curves for both strains PAO1 and PAO9 in iron-limited casamino acid media (CAA 5 g casamino acids, 1.18 g K₂HPO₄·3H₂O, 0.25 g MgSO₄·7H₂O, per liter of dH₂O) supplemented with the iron-chelator, human apo-transferrin (100 µg·mL⁻¹- Sigma Aldrich, UK), and 20 mM sodium bicarbonate (Schwyn & Nielsens, 1987; Meyer et al.1996) to induce siderophore production (Griffin et al 2004). We determined fluorescence as a function of cell density by using a combined, automated fluorescence-spectrometer (Synergy2

BioteK). Turbidity at A_{600} and pyoverdinin production in relative fluorescent units (RFU) at excitation and emission wavelengths of 400 nm and 460 nm respectively, as described by Jiricny *et al.* (2010), were automatically measured every 30 minutes for 48 hours. We calculated the relative pyoverdinin availability per cell by the ratio of RFU/A_{600} at each time point (Kummerli *et al.* 2009b). We measure the rate of pyoverdinin production per cell per minute by $((RFU_2 - RFU_1)/30 \text{ minutes}) / A_{600(2)}$. We replicated each treatment 12 times per strain.

Competition assays

In order to test for effect of growth stage on the ability of cheats to invade we inoculated cheats (PAO9) into a cooperative (PAO1) culture at different points of its growth curve (Table 1). We competed the putative cheat, PAO9, with a growing population of PAO1 starting at time point zero and then at 4 hour intervals over a period of 24 hours and then at 48 hours of PAO1 growth in 6ml of iron limited CAA media. Initially we coinoculated 60 μ L of a mixed culture of PAO9 and PAO1 which competed for a total of 48 hours. We also inoculated 6ml of iron limited CAA media with 60 μ L of PAO1 that we grew static at 37°C for 4, 8, 12, 16, 20, 24 and 48 hours as monocultures after which we introduced PAO9 putative cheats at a lower frequency into the growing population of PAO1 and allowed them to compete for 48 hours (Table 1). We obtained the relative frequencies prior to competition, 24 hours into competition and 48 hours at the end of competition by plating out dilutions of mixed cultures onto KB agar (12 g agar per liter of KB medium; Table 1). We incubated all KB agar plates at 37°C overnight after which we recorded the colony counts for each strain. PAO1 and PAO9 colony forming units are distinguishable from each other by phenotype. PAO1 colonies are green in colour with rugged edges

while PAO9 colonies are smaller, white in colour with smooth edges.

PAO1 growth phase	Lag		Exponential					Stationary	
Time in PAO1 growth when PAO9 added (hours)	0	4	8	12	16	20	24	48	
Initial ratio of PAO9:PAO1	1:10	7:100	3:100	2:100	2:100	2:100	4:100	6:100	
Plating volumes & dilutions at (hours)	0	100 μL , 10^1	70 μL , 10^2	20 μL , 10^2	50 μL , 10^3	70 μL , 10^4			
	24	50 μL , 10^4	50 μL , 10^4	50 μL , 10^4	50 μL , 10^4	50 μL , 10^4	50 μL , 10^4	50 μL , 10^4	50 μL , 10^4
	48	50 μL , 10^4	50 μL , 10^4	50 μL , 10^4	50 μL , 10^4	50 μL , 10^4	50 μL , 10^4	50 μL , 10^4	50 μL , 10^4

Table. 1 Experimental protocol. In the eight sets of competition assays PAO9 is co-inoculated with PAO1 cooperator populations and PAO9 is added to independent growing population of PAO1 at 4 hourly intervals up to 24 hours through the lag and exponential growth phase and again at 48 hours during the stationary growth phase of PAO1. Both strains are then allowed to compete for up to 48 hours. Mixed cultures were plated out prior to competition at the specified dilutions and volumes, at 0 hours, when PAO9 is first added to a PAO1 population, then 24 hours into competition and again 48 hours after competition.

We also cultured each strain separately as a monoculture in 6ml of iron-limited CAA which were incubated at 37°C for a total of 72 hours and assayed for cell density at A_{600} at 24 hours, 48 hours and 72 hours. Each monoculture and mixed competition per time point was replicated 14 times except for the co-inoculated competitions that were replicated 28 times, competitions with 24 hour old PAO1 populations were replicated 22 times, and with 48-hour old PAO1 populations were replicated 16 times due to overlapping repetition with different lines.

Control assays: growth in sterile PAO1 supernatant

To determine whether the abiotic environment of the PAO1 cultures after 24 and 48 hours of growth inhibits strain growth, due to metabolic toxin accumulation or a

limiting growth nutrient, we grew PAO1 and PAO9 as monocultures in spent media of 24 and 48-hour PAO1 cultures. First we inoculated 60 μL of PAO1 in 6ml of iron-limited CAA media and allowed them to grow as monocultures for 24 hours and 48 hours static at 37°C. After 24 and 48 hours we centrifuged the cultures for 10 minutes at 5000 rcf, filter sterilized the supernatant, using 0.2 μm filter tips, into fresh glass vials. We then inoculated the supernatant (spent media) of the 24-hour and 48-hour PAO1 cultures with PAO1 and PAO9 strains. We obtained the initial number of PAO1 and PAO9 cells by plating out 100 μL of 10^3 dilutions on KB agar. We allowed the monocultures to grow in the spent media for 48 hours static at 37°C and then obtained cell numbers after 24 and 48 hours by plating out 50 μL of 10^4 dilutions on KB agar. Each monoculture was replicated 8 times per treatment.

We incubated all KB agar plates at 37°C for 15 hours after which we recorded the colony-forming unit (CFU) counts for each monoculture to estimate the initial and final number of cells in the cultures to determine whether the cells were dividing and increasing.

Statistical analysis

We calculated the relative fitness (ω) of the PAO9 putative cheat, which is the change in frequency over time relative to the PAO1 wildtype. This is given by $\omega = x_2(1-x_1)/x_1(1-x_2)$ where x_1 is the mean initial proportion of the PAO9 cheat from the sample population and x_2 is the final proportion in each sample (Otto and Day 2007; Ross-Gillespie *et al.* 2007). A value of $\omega > 1$ indicates that the cheat has a higher fitness than the cooperator, and a value $\omega < 1$ indicates that the cheats have a lower fitness than cooperators. We calculated the mean fitness value across the competition assay

replicates to indicate how efficiently the putative cheat can exploit the cooperative population. In order to eliminate any bias from pseudo-replication variation we logged (\log_{10}) the ω values before calculating the mean across replicates (Jiricny *et al.* 2010), this analysis gave the same results and therefore is not shown. We carried out all analysis in R statistical software v 2.15.2 (<http://www.R-project.org>).

Results

Population growth and pyoverdinin availability

Monocultures of PAO1 grew to a significantly higher cell density than PAO9 during all stages of growth in iron-limited CAA media indicating the importance of pyoverdinin for growth in this environment (Fig. 1a). Pyoverdinin production per cell per min for PAO1 increases during early exponential phase and peaks at 9.5 hours of growth, after which the rate drops and remains low fluctuating around lower production rates and rates almost equal to zero (Fig.1). PAO9 pyoverdinin production is negligible throughout a 48-hour growth period, compared to PAO1 (Fig. 1b). Peak cumulative PAO1 pyoverdinin availability per cell (ppc) occurs during the exponential phase at 17 hours and is significantly higher than peak ppc of PAO9, which occurs at 48 hours (Fig.1c; $T = 18.24$, $P = 1.107 \times 10^{-9}$). PAO1 ppc decreases after 17 hours and is significantly lower after 24 hours of growth (Fig.1c; $T = 10.50$, $P = 4.53 \times 10^{-7}$) after which pyoverdinin levels are sustained and only gradually decline. By 48 hours of PAO1 growth pyoverdinin per cell is significantly lower than at 24 hours (Fig.1c; $T = 6.46$, $P = 4.69 \times 10^{-5}$).

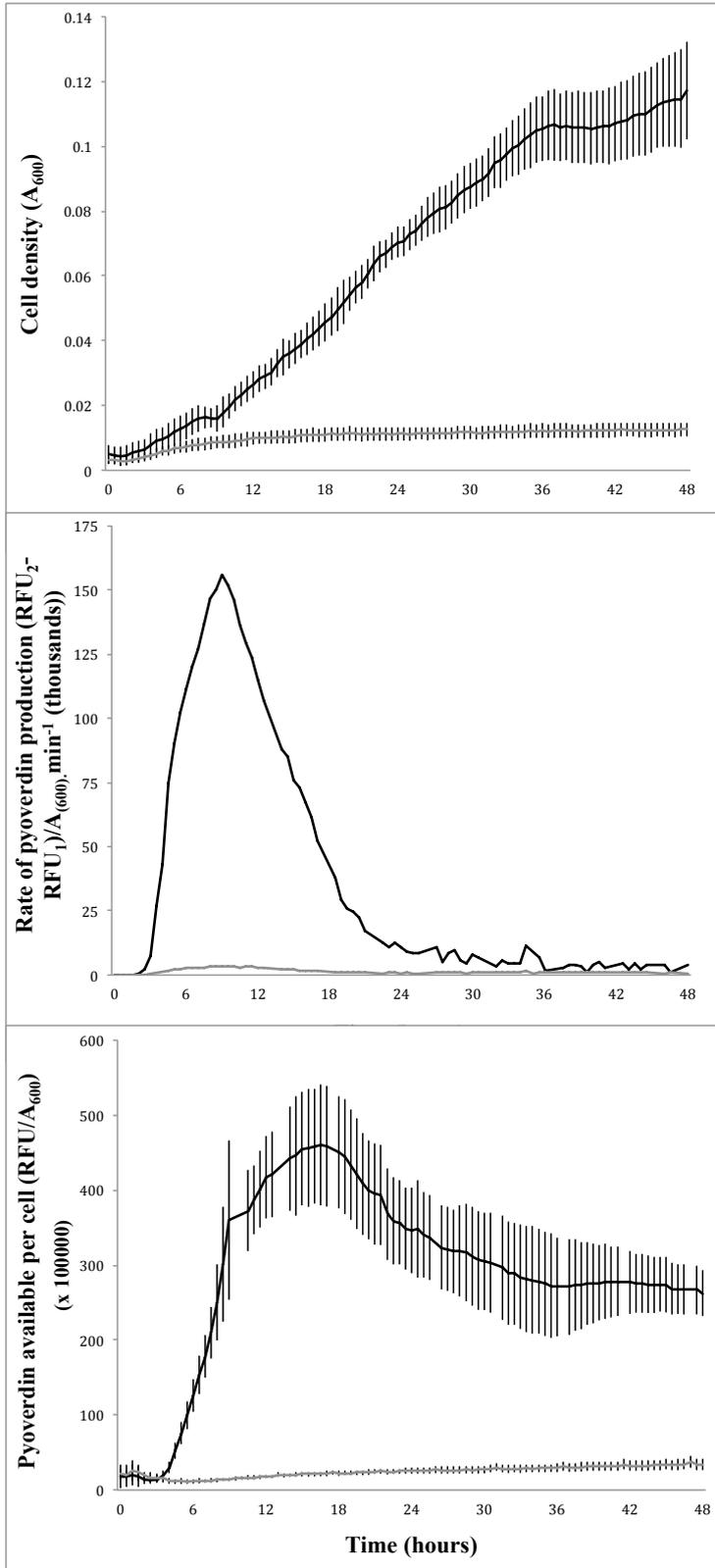


Fig. 1 Cooperator (black) and cheat (grey) (a) growth curves, (b) Rate of pyoverdinin production and (c) cumulative pyoverdinin available per cell, over a duration of 48 hours in iron-limited media: (a) Growth, measured by cell density at A_{600} , of the cooperator is significantly better in iron-limited conditions than the putative cheat; (b) Rate of pyoverdinin production, measured per cell per minute ($(\text{RFU}_{400,460}/A_{600} \cdot \text{min}^{-1})$), is negligible throughout the cheat's growth relative to the cooperator, and cooperator production rate peaks at 9.5 hours during exponential growth; (c) Peak pyoverdinin levels available per cell, measured by $(\text{RFU}_{400,460}/A_{600})$ are significantly higher for cooperators than cheats. The error bars indicate standard deviation around the mean values.

Effect of growth stage on the ability of cheats to invade

Once PAO9 is introduced into a PAO1 population, they are grown together to compete for 48 hours. After 48 hours of competition, PAO9 is able to cheat when introduced in PAO1 cultures during its lag phase at 0 and 4 hours and early exponential phase at 8, 12 and 16 hours of growth (Fig. 2 a & b; Table S1). PAO9 was not able to invade when introduced in PAO1 cultures passed mid-exponential phase at 20 and 24 hours or stationary phase at 48 hours, when pyoverdinin per cell levels and rate of pyoverdinin production have dropped (Fig. 2 a & b).

The monoculture controls confirm that PAO9 grows significantly less well than PAO1 in iron-limited CAA media after 24, 48 and 72 hours of growth (Fig. A1; T = -8.23, P = 9.07×10^{-9}).

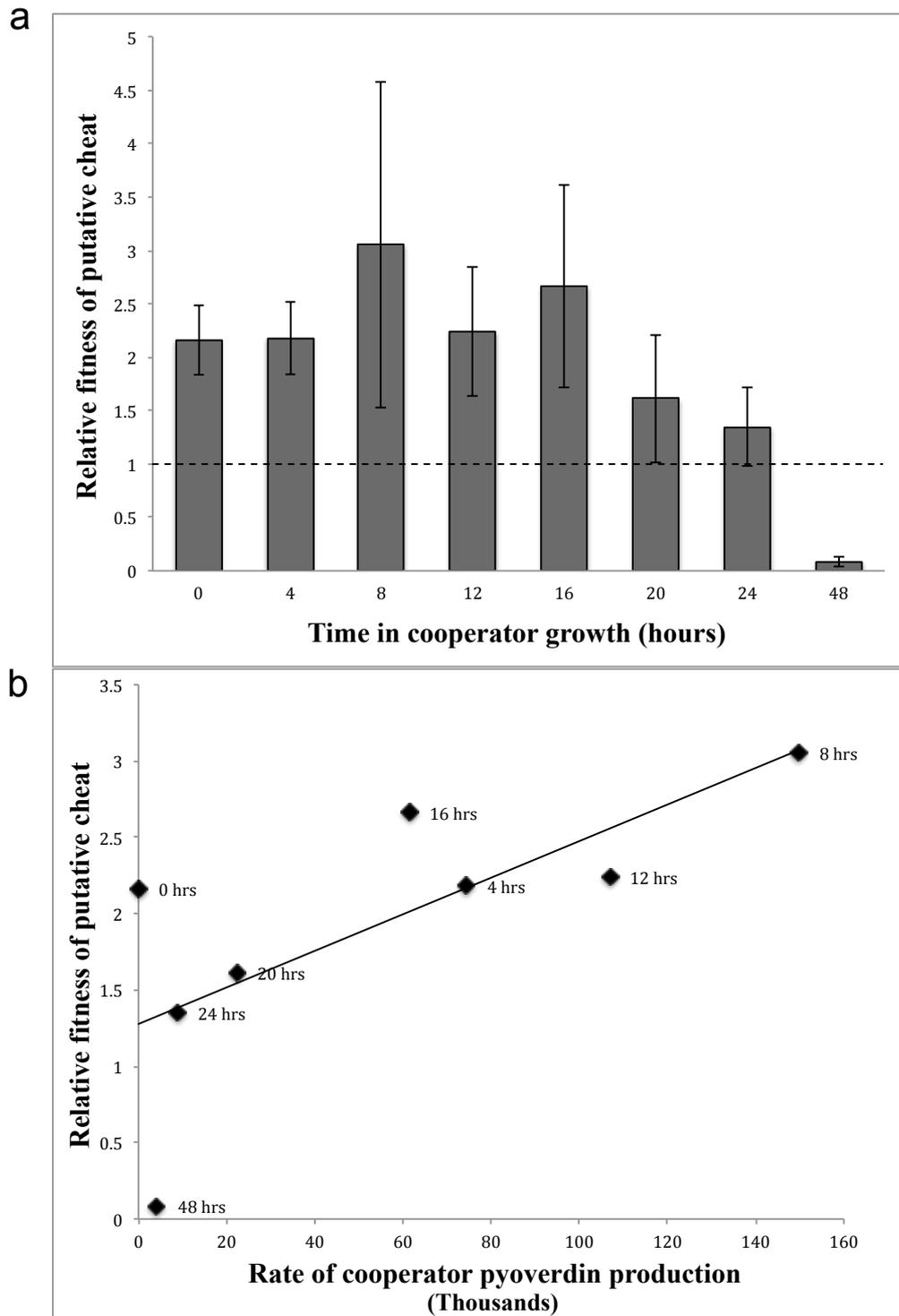


Fig. 2 (a) Relative fitness of putative cheat in competition with cooperators at different stages of growth and (b) Relative fitness of cheat correlated with the rate of cooperator's pyoverdine production: (a) cheats significantly increase in frequency after 48 hours of competition with cooperators when introduced at 0, 4, 8, 12, and 16 hours of cooperator growth. The error bars indicate 95% confidence intervals around the mean values; (b) the cheat's relative fitness value is positively correlated with the rate of the cooperator's pyoverdine production at the different time points in its growth (hours).

Assay controlling for enhanced ability of cooperative cells in stationary phase to inhibit growth of cheats

To determine whether the abiotic environment of the PAO1 cultures after 24 and 48 hours of growth inhibits growth of either strain we determined if each of the strains grew as monocultures in PAO1 spent media. PAO1 and PAO9 increase in cell density over 48 hours when grown in the spent media of 24 and 48 hour old PAO1 cultures (Fig. 3). After 48 hours of growth in the pyoverdinin-rich spent media, PAO9 monocultures have significantly higher colony counts than PAO1 in 24 and 48-hour spent media treatments (Fig. 3; $T = -2.63$, $P = 0.021$ and $T = -3.02$, $P = 0.0126$ respectively).

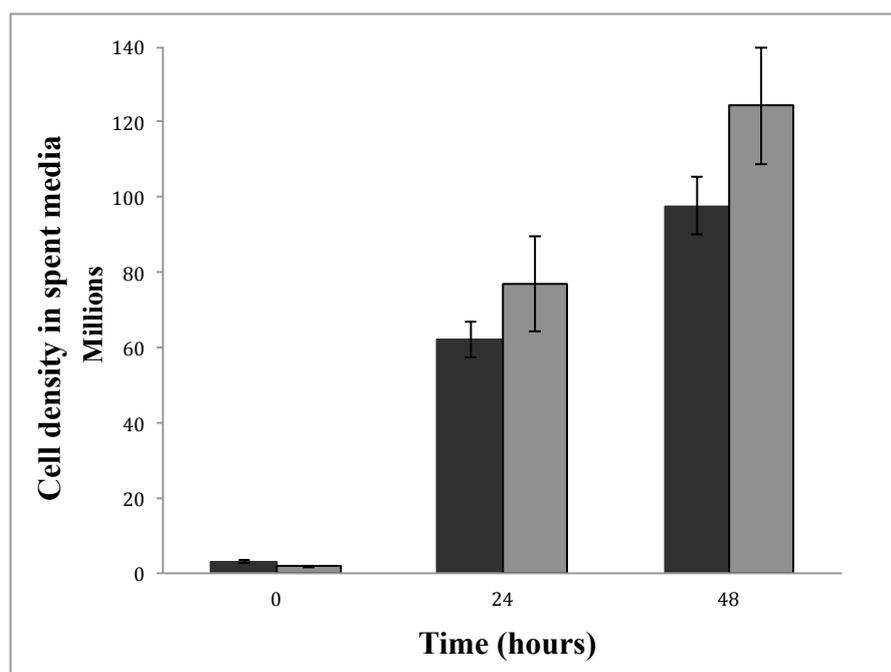


Fig. 3 Monoculture cell growth in cooperator spent media. Cell density is measured as colony-forming units per ml (CFU/ml) in the spent media of 48-hour cooperator monocultures. Cooperator (black) and cheat (grey) growth is not inhibited in the cooperator's spent media over a period of 48 hours. Cheats grow to a significantly higher density than cooperators. The errors bars indicate 95% confidence intervals around the mean values.

Discussion

This study demonstrates the importance of growth dynamics to evolutionary dynamics of social behaviour: specifically, populations of cooperators in stationary phase are immune to cheat invasion. More specifically, our results suggest that the ability of cheats to invade is due to pyoverdine production rates of cooperative cells (Fig. 1 & 2). During the early stages of growth (lag and early exponential phase) pyoverdine production rates are highest among cooperative cells and the cheating strain has a clear fitness advantage (Fig. 1 & 2). As growth continues (late exponential phase), cheats cannot invade but are still able to persist (Fig. 2; Table A1). Finally, as population size of cooperators reaches stationary phase, cheats are unable to invade and in fact, are seen to decline in competition with cooperators (Fig.2) The durability of pyoverdine is key to this interpretation: once pyoverdine accumulates, it is recycled, allowing cells to downregulate production as a cost-saving strategy (Imperi et al. 2009; Kummerli and Brown 2010). A cheat introduced into a population of cooperators in late exponential phase may benefit from the exploitation of iron made available by high levels of pyoverdine, but its competitive advantage has been removed. In stationary phase, cooperative cells are also avoiding the cost of pyoverdine production.

Alternatively, stationary phase cooperative cultures may be relatively hostile environments in which to be introduced. The cheat strain in our experiments may have been prevented from invading by accumulation of metabolic toxins, or depletion of growth nutrients as the cooperative cultures age. We controlled for this by growing our cheat strain in the sterile supernatant of the cooperative culture from late exponential and stationary phase. In fact, when grown as monocultures in these

environments, the cheat strain grew significantly better than the cooperative strain (Fig. 3) demonstrating that the reduced ability of the cheat to invade is not due to an inhibitory environment. Not only is cooperator supernatant enriching for the cheat, it was more enriching for the cheat than it is for itself (Fig. 3), most likely because the cooperator continues to invest in pyoverdine production.

It remains to be shown whether the changing cooperative-cheat dynamics through population growth we describe here are representative of other cooperative public good traits in bacteria. The fitness benefits of many exoproducts are density dependent and controlled by quorum sensing (Darch et al. 2012; West et al. 2012a) or produced only at certain stages of population growth. Xavier et al. (2011) show that cooperators regulate expression of biosurfactants delaying its production to times when it is least costly to produce: during stationary phase when cell division rate is lowest and when the environment is limited in nutrients except for carbon sources that are in excess of what is needed for growth and, therefore, used for biosurfactant production. However, when engineered to constitutively produce biosurfactant during exponential phase, when it is more costly, a non-producing cheat invades the cooperative population (Xavier et al. 2011). Exoproducts potentially acting as public goods are also known to vary in durability and ability to be re-used and this could have a significant impact on the fitness differences between cooperators and cheats (Brown and Taddei 2007; Kummerli and Brown 2010).

Conclusion

In conclusion, our results demonstrate that a cooperative strain of bacteria was immune to invasion by putative cheats in a stationary physiological state, characteristic of established, stable populations in nature. These results have important ecological implications because although cheating may play an important role in the competitive dynamics of bacteria when growth from low density is required, such as colonizing a new niche, our results suggest that the threat to established bacterial populations is potentially minimal. This is significant, as bacteria exist in nature in a non-growing stationary-like phase in soil, water, biofilms and as established chronic infections (Kolter et al. 1993; Wallis et al. 1999; Mulvey et al. 2001; Finkel 2006; Llorens et al. 2010; Gefen et al. 2014). While experimental evolution studies have made a significant contribution to our understanding of cooperative behaviour in bacteria, we can usually only guess which of the parameters we have shown to be important in the lab are likely to be driving social dynamics in natural populations of bacteria. The complexity of microbial ecosystems can make this a daunting task for biologists. For example, it has been increasingly observed that non-growing bacteria in nature may be in a different stage than the commonly studied early stationary phase lab cultures, but may exist in a later physiological stage of their lab culture life cycle referred to as a long-term stationary phase (Kolter et al. 1993; Finkel 2006). Bacteria in long-term stationary phase rely on various stress-response genes to survive, which can explain how bacteria persist the sporadic and fluctuating environmental conditions in nature. These stressors select for cells that are better adapted to the environmental conditions of long-term stationary phase and are known as growth advantage stationary phase (GASP) cells (Finkel 2006; Gefen et al. 2014).

Therefore, the selection for GASP cells in established natural populations can have big implications on bacterial competitive dynamics that may completely mask the effects of cooperator-cheat social interactions. Perhaps the appeal of the results of this experiment is the suggestion of a relatively simple and ubiquitous scenario for the maintenance of cooperative traits that is likely to hold in a range of parameter space in nature.

Appendix

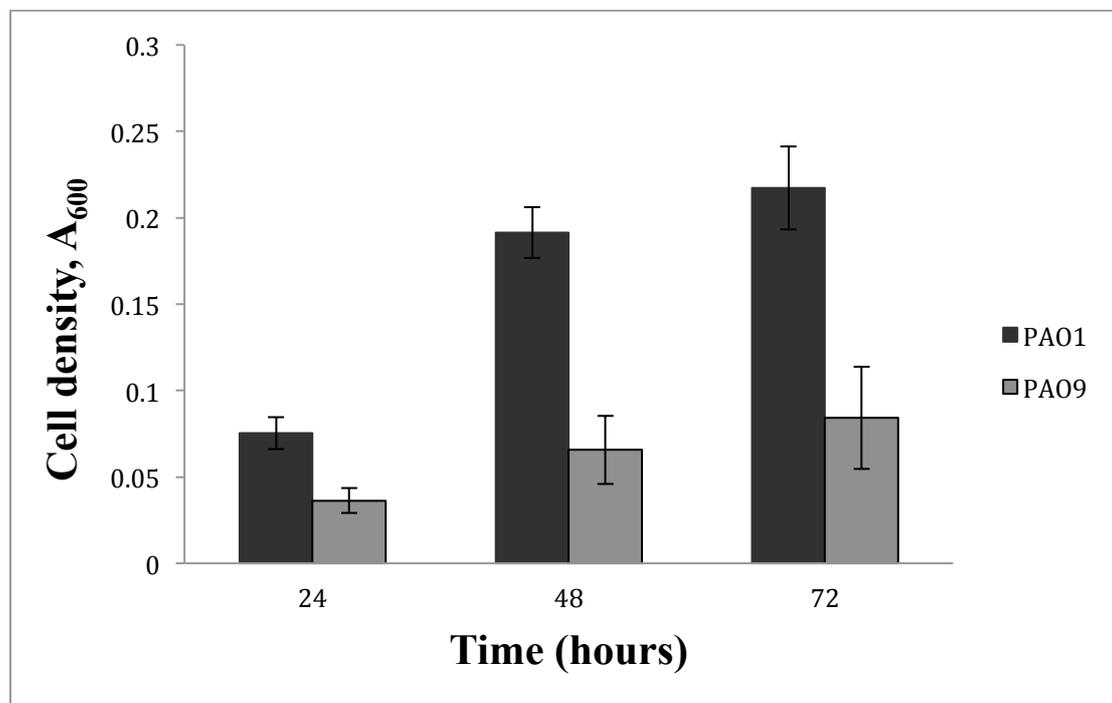


Fig. A1 Monoculture cell density (A₆₀₀) in iron-limited CAA media that requires pyoverdinin production for growth. PAO1 grows to a significantly higher density than the PAO9 after 24, 48 and 72 hours of growth. The error bars indicate 95% confidence intervals around the means of fourteen independent replicates. To account for non-independence of the repeated measures of the replicates over time I analysed the data as a mixed model in the R software using the lmer-Test package with replicates per strain as a random effect and nested time as a fixed effect.

PAO9 added to PAO1 population grown for	Relative PAO9 fitness \pm 95% CI (after 1 & 2 days)	Test-statistic (t)	df	P- value
0 hours	$\omega_1 = 1.8 \pm 0.32$	4.88	27	4.157×10^{-5}
	$\omega_2 = 2.2 \pm 0.33$	6.99	27	1.617×10^{-7}
	$\omega_3 = 1.3 \pm 0.21$	2.67	27	0.013
4 hours	$\omega_1 = 1.3 \pm 0.15$	3.97	13	0.002
	$\omega_2 = 2.2 \pm 0.34$	6.81	13	1.24×10^{-5}
	$\omega_3 = 1.8 \pm 0.33$	4.45	13	0.0007
8 hours	$\omega_1 = 1.6 \pm 0.75$	1.5	13	0.159
	$\omega_2 = 3.1 \pm 1.52$	2.64	13	0.02
	$\omega_3 = 2.2 \pm 0.71$	3.18	13	0.007
12 hours	$\omega_1 = 1.6 \pm 0.28$	3.93	13	0.002
	$\omega_2 = 2.2 \pm 0.60$	4.04	13	0.002
	$\omega_3 = 1.6 \pm 0.30$	3.56	13	0.004
16 hours	$\omega_1 = 2.4 \pm 0.77$	3.53	13	0.004
	$\omega_2 = 2.7 \pm 0.95$	3.42	13	0.005
	$\omega_3 = 1.2 \pm 0.26$	1.59	13	0.136
20 hours	$\omega_1 = 2.0 \pm 0.80$	2.5	13	0.027
	$\omega_2 = 1.6 \pm 0.60$	2.00	13	0.067
	$\omega_3 = 0.9 \pm 0.24$	-0.80	13	0.437
24 hours	$\omega_1 = 1.6 \pm 0.53$	2.08	21	0.05
	$\omega_2 = 1.3 \pm 0.40$	1.85	21	0.079
	$\omega_3 = 1.0 \pm 0.24$	0.03	21	0.978
48 hours	$\omega_1 = 0.2 \pm 0.09$	-19.29	15	5.32×10^{-12}
	$\omega_2 = 0.1 \pm 0.05$	-41.73	15	2.2×10^{-16}
	$\omega_3 = 0.2 \pm 0.12$	-13.59	15	7.789×10^{-10}

Table A1. Relative fitness values of PAO9 in competition with PAO1 at different stages of its growth. The relative fitness of PAO9 is measured between the start of competition and 24 hours later (ω_1), between the start of competition and 48 hours later (ω_2), and to check if PAO9 continues to increase or decrease in frequency during course of competition we measure the relative fitness between 24 and 48 hours of competition (ω_3). In the lag and early exponential stage of PAO1 growth (0-16 hours) when pvd production rates are highest, PAO9 shows a higher relative fitness after 2 days of competition than after 1 day, indicating that cheating is sustained throughout PAO1's growth until it establishes and reaches stationary. However, in later stages of PAO1 exponential growth (20-24 hours), PAO9 has a lower relative fitness after 2 days of competition than after 1 day indicating that PAO9 couldn't invade. In 48-hour stationary phase PAO1 populations, PAO9 significantly decreases in frequency and in some cases go extinct ($\omega < 1$, $\omega = 0$) because the rate of PAO1 pvd production is almost negligible, and pyoverdinin per cell is significantly lower at 48 than at 24 hours.

Chapter 5. Bacteriocin-mediated competition in Cystic Fibrosis Lung Infections

Abstract

Bacteriocins are antimicrobial toxins produced by bacteria to kill competitors of the same species. Both theory and laboratory experiments have suggested that bacteriocin production and immunity can play a key role in the competitive dynamics of different bacterial strains. The extent to which this is the case in natural populations, especially pathogens of humans, remains to be tested. We examined the role of bacteriocins in the competitive dynamics of the pathogen *Pseudomonas aeruginosa*, infecting the lungs of humans with cystic fibrosis. We found that: (1) isolates from later infection stages caused less inhibition of other strains, were significantly more inhibited by the pyocins produced by other strains and carried a significantly lower diversity of pyocin types; (2) this difference between earlier and later infections appears to be caused by the difference in pyocin diversity between the competing genotypes and not by pyocin genes being lost by strains over time; (3) pyocin inhibition profiles do not significantly explain why certain strains are better at outcompeting other strains within lung infections; (4) strains frequently carry the gene to produce a killing pyocin, but not the immunity gene, because they can acquire resistance via other unknown mechanisms.

Introduction

Bacteria produce antimicrobial toxins, termed bacteriocins, to kill competitors (Riley and Gordon 1999). A number of experimental studies have shown that bacteriocins can play a key role in the competitive dynamics among closely related bacterial strains (Michel-Briand and Baysse 2002; Riley and Wertz 2002). Bacteriocin ‘producers’ can invade and outcompete ‘sensitive’ strains that are killed by that toxin. These producers can, in turn, be outcompeted by ‘resistant’ strains that are resistant to the bacteriocin, but do not produce it, and hence do not pay the cost of producing it. In spatially structured populations this can lead to population dynamic cycling with all three strains coexisting, analogous to the a rock-paper-scissors game, because each strain can be invaded by another strain - producers outcompete sensitives, resistants outcompete producers, and sensitives outcompete resistants (Durrett and Levin 1997; Riley and Gordon 1999; Kerr et al. 2002).

The extent to which bacteriocins determine the dynamics of competing strains in nature, or lead to rock-paper-scissor dynamics, is not known. In addition, previous work has focused on *E.coli*, which carry bacteriocins with relatively simple toxin anti-toxin systems, where one protein is responsible for the killing action and another immunity protein for neutralizing the killing protein (Kerr et al. 2002). However, in natural populations of bacteria like *Pseudomonas aeruginosa* one strain usually carries and releases multiple types of bacteriocins known as pyocins, which have a diversity of killing and resistance mechanisms in addition to the toxin-antitoxin systems (Michel-Briand and Baysse 2002; Parret and De Mot 2002). For example, three pyocin types exist (S, R and F), with each of these having multiple subtypes with varying toxicity levels (Uratani and Hoshino 1984; Michel-Briand and Baysse

2002; Parret and De Mot 2002; Heo et al. 2007; Kohler et al. 2010). The soluble S-type is similar to colicins of *E.coli*, and is composed of killing and immunity genes while the R and F type are phage-derived, and have different killing and resistance mechanisms, where both consist of only a killing component (Fyfe et al. 1984; Uratani and Hoshino 1984; Sano and Kageyama 1993; Sano et al. 1993; Duport et al. 1995; Michel-Briand and Baysse 2002; Scholl et al. 2009)

We examine bacteriocin-mediated population dynamics in natural populations using cystic fibrosis (CF) lung isolates of *P.aeruginosa*, an opportunistic human pathogen. Early colonization of the lung is characterized by a diversity of strains that intermittently co-infect from environmental sources or other patients, while later stages are characterized by chronic infections usually dominated by one strain (Fegan et al. 1991; Godard et al. 1993; Duport et al. 1995). Our first aim is to test whether the competitive success of different strains, within hosts, can be explained by variation in pyocin production and resistance. In particular, whether the dominant strains outcompete other strains by producing bacteriocins. We examined the extent to which different strains were able to produce pyocins that inhibited the growth of other strains, comparing: (a) isolates sampled from non-lung environments and thirteen Danish patients at different stages of infection; (b) isolates sampled from a longitudinal study following the infection dynamics of strains within eight Danish patients. We sequenced the genomes of all these strains to identify the pyocin genes, examine how pyocin diversity changes during lung infections, and examine the genetic basis of the inhibition patterns that we observed phenotypically. We tested the generality of our results by comparing our sequence data with that from previously sequenced epidemic strains, and from 55 isolates of an epidemic Danish isolate that has been sampled for over 38 years.

Our second aim is to use all the isolates to assess the frequency of occurrence of the different types of producer, resistant and sensitive strains in natural *P.aeruginosa* populations. Theory predicts that structured populations allow all these types to be maintained at appreciable frequencies, whereas sensitives will dominate in unstructured (panmictic) populations (Chao and Levin 1981; Czaran et al. 2002; Kerr et al. 2002; Biernaskie et al. 2013). We do not know the extent to which human hosts represent relatively structured or unstructured populations. We focus on S pyocins because they are composed of a killing and immunity gene, and in particular the five S pyocin types for which both killing and immunity genes are annotated. Our third aim is to assess how often the S pyocin immunity gene is responsible for cell resistance to the killing gene. While it has been observed that some strains appear to not be killed by S pyocins, despite lacking the immunity gene for that pyocin, the relative occurrence and importance of this in nature remains unknown (Denayer et al. (2007).

Materials and Methods

Materials

For all the experiments we used Kings broth (KB) media and agar to culture bacteria (20 g peptone, 1.5 g $K_2HPO_4 \cdot 3H_2O$ and 1.5 g $MgSO_4 \cdot 7H_2O$ for the media and an additional 12 g agar powder for KB agar, per liter of distilled water (dH_2O)). We used M9 minimal salts solution to dilute cell cultures (6.8 g Na_2HPO_4 , 3 g KH_2PO_4 , 0.5 g NaCl and 10 g NH_4Cl pre liter of dH_2O). We used the Magellan plate reader v.7.0 and the fluorimeter SpectraMax M2, Molecular Devices, to take endpoint OD reads for

constructing optical density growth curves. The camera box (Syngene Ltd, UK) G:box EF², was used for all pictures of bacterial agar plates.

***P. aeruginosa* isolates**

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

Collection 1 consists of 41 isolates, which we grouped into categories according to their source of origin: non-lung, or from infected lungs, and the stage of lung infection. Specifically, we divided these isolates into non-lung strains from the natural environment or from patient burn wounds (lab PAO1), acute isolates that cause a single intermittent infection in a child, chronic isolates that cause a single one-off chronic infection for less than six months in an adult, and multiply chronic isolates that are highly transmissible and cause multiple chronic infections for longer than six months in adults (Table A1). Based on bacterial MLST profiles the 41 isolates belong to 29 distinct independent genotypes. We used collection 1 to determine whether the ability to produce, inhibit and resist pyocins, measured both phenotypically and genetically, correlates with how long a strain has been in the lung. We included a category of non-lung strains because all CF patients are initially colonized by isolates from an environmental reservoir and can be re-infected multiple times with either new genotypes from the environment or other patients (Folkesson et al. 2012). Therefore we compare genotypes that have never adapted to the lung and those that have progressively adapted for longer to determine whether competitive colonization of the lung is mediated by bacteriocins. Although the non-lung isolates are not from Danish

environmental reservoirs and the lab domesticated PAO1 strain is used in the non-lung category, a previous study (Ernst et al. 2003) and our study shows (bacterial phylogeny Fig. A3) that environmental and clinical isolates do not cluster separately but together, and the non-lung isolates of this study are more closely related to lung isolates than other non-lung isolates sampled from the similar environmental sources.

Collection 2 consists of the genome sequences of 55 *P.aeruginosa* clinical CF lung isolates, previously sequenced and obtained from Lars Jelsbak's lab at the Department of Systems Biology at the Technical University of Denmark. The 55 isolates are of the same genotype and belong to the DK2 clone type, a transmissible lineage of *P.aeruginosa* in Denmark, and were sampled from 21 patients with multiply chronic CF infections over 38 years between 1972 and 2008 (Marvig et al. 2013). We used these genome sequences to screen for the bacteriocin genes and analyse whether they are lost or mutated over time during infections.

Collection 3 consists of longitudinal isolates that had been sampled from the onset of infection and over many years from 8 different patients, three of which suffer from chronic infections and all others from acute infections (Table A3). Altogether there are 122 isolates that, based on bacterial MLST profiles, belong to 17 different genotypes. Three of the patients are currently in the chronic stage of infection and five in the acute stages. We used this collection to track changes in bacteriocin production over time in isolates of the same genotype and to track competitive dynamics of co-infecting genotypes within the same lung environment.

Collection 4 consists of *P. aeruginosa* isolates, which have been previously sequenced and we could find in GenBank. These isolates include CF lung isolates (the Liverpool strain LESB58, an isolate from chronically infected patient in Boston PA2192, the Manchester epidemic strain C3719 and PACS2; accession numbers:

NC_011770, NZ_CH482384, NZ_CH482383, NZ_AAQW01000001) and non-lung isolates (one from a burn wound patient PA14 and a human clinical non-respiratory isolate from Argentina PA7; accession numbers: NC_008463 and NC_009656 respectively). We used these genome sequences to compare the pyocin gene profiles of other epidemic strains to the Danish epidemic strains and non-lung strains of our collection.

Experimental protocols

Bacterial growth curves

We grew each isolate for 24 hours at 37°C at 200 rpm in 6 ml of KB media inoculated from a frozen stock in 20 ml glass vials. We measured optical density (OD) at 600 nm (A_{600}), and standardized all cultures to an OD of 1.0 using M9 solution to dilute. We used 1 μ L of standardized culture to inoculate 200 μ L of KB media in a 96 well plate. To construct growth curves we set the plate to incubate at 37°C for 44 hours with automatic periodic shaking every half hour for fifteen seconds prior to OD measurements totaling 89 OD reads. We then plotted the growth curves to determine when each isolate reached stationary phase.

Phenotypic characterisation of pyocin production/resistance

Preparation of supernatant

We cultured all isolates for 24 hours at 37°C at 200 rpm in 6 ml of KB media inoculated from a frozen stock in 20 ml glass vials. We measure cell density at A_{600} and standardised all cultures to an OD of 1. We then inoculated 30 μ L of each

standardised culture into 6 ml of fresh KB media in 20 ml glass vials and allowed them to grow at 37°C at 200 rpm until they reach stationary phase. We then centrifuged the cultures for 10 minutes at 5000 rcf (6861 rpm) to obtain a clear supernatant, which we filter sterilized (0.2 µm pore size) into sterile tubes and placed in the freezer at -20°C. We spotted a control KB agar plate with 20 µl of each culture's supernatant and incubated them overnight to ensure they are free of bacterial growth.

Preparation of bacterial lawns

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

Experimental design

We measured the ability of different strains to inhibit each other by testing whether a strain's pyocin-carrying supernatant inhibits a bacterial culture of another strain. We spotted KB agar plates with the supernatants, and then spread a lawn of each culture (Fig. 1). We placed a 20µl spot of each supernatant onto KB agar plates and allowed the spots to dry into the agar before spreading a lawn of 50µl of each diluted culture. We then set the plates to incubate at 37°C for 12 hours till a uniform lawn had appeared. We replicated the same lawn with each set of spots three times. Each plate consisted of control sterile KB media spots and a spot of self-supernatant for each isolate's lawn.

We examined the lawns for zones of inhibition, or reduced growth on all supernatant spots. We took pictures of the plates using a camera with contrasting colour images in

order to better visualize and detect reduced growth areas when the inhibition areas were not clear zones (Fig. 1). As a control, we confirmed that sterile KB media and self-supernatant spots did not cause any inhibition of the lawns. We then recorded which strains inhibited each other and compiled a 41 x 41 matrix organized into 4 isolate categories of non-lung and three different stages of a CF infection across the patients. We also compiled a separate matrix for within patient isolate interactions for the 8 different patients.

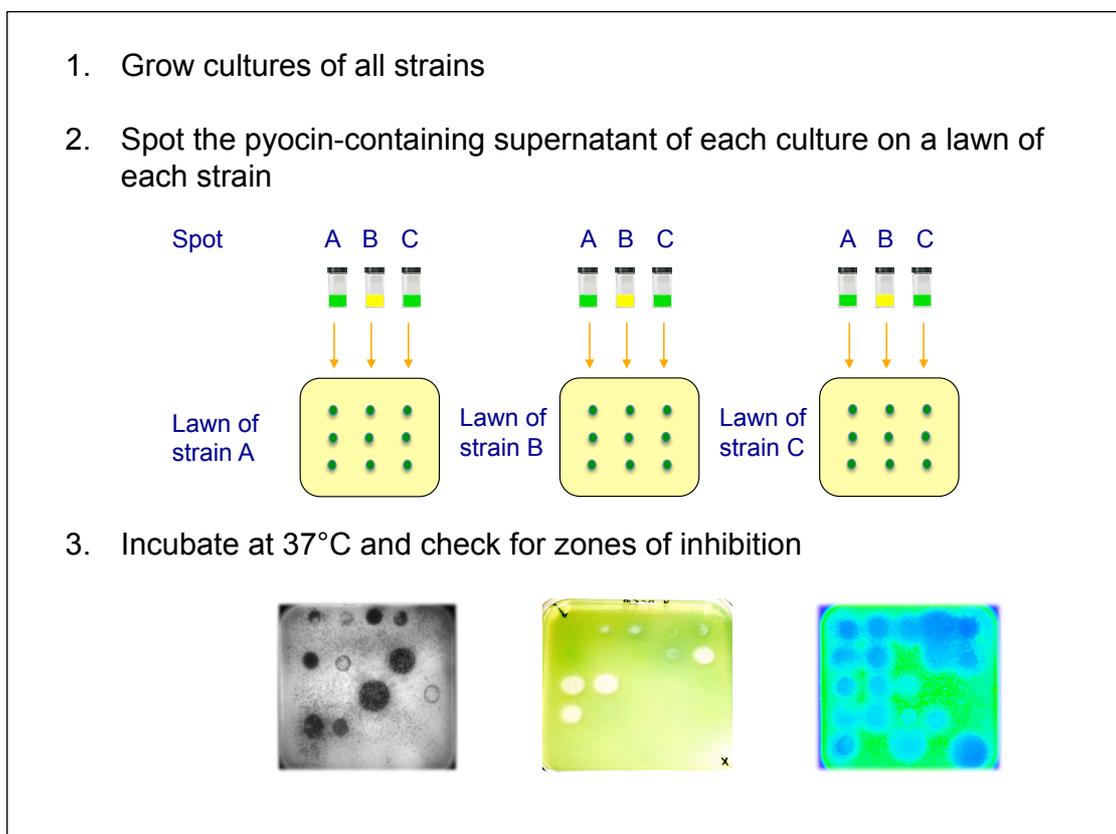


Fig. 1 Experimental Design. Larger inhibition zones are typical of S pyocins that diffuse, while zones of the same size as the initial supernatant spot are typically a result of cell death by R/F pyocin.

Genome sequences

We extracted genomic DNA and had them sequenced for the 41 non-lung and CF lung isolates from different stages of infection (collection 1). We used the Promega Wizard® genomic purification kit and protocol. We quantified the final DNA

concentration using the Promega QuantiFluor™ Dye Systems kit and protocol. We assessed the genomic DNA to meet the quality control criteria: 1) a nanodrop 260/280 ratio between 1.8-2 and the 260/230 ratio between 2-2.2; 2) run the samples on 0.7% agarose gel and ensure the DNA gives a distinct band with no smearing. We sent the samples to the Wellcome Trust Centre for Human Genetics. Multiplexed genomic DNA libraries were prepared prior to paired end sequencing over one lane of a flow cell. Sequencing conditions: Illumina Hi-Seq with 100 base pair paired end reads and on average 116 times coverage. The Isolates from the 8 additional patients (collection 3) had already been sequenced at the Department of Clinical Microbiology, Rigshospitalet, Copenhagen.

Data analysis

Genome sequence analysis - screening for pyocin genes

All isolate genome sequences were FastQ files with paired-end short sequence reads that were assembled *de novo* using the velvet assembly which were then uploaded to the PubMLST BiGSDB database (Jolley and Maiden 2010). We assessed the diversity of isolates using multilocus sequence typing (MLST) where the genomes were screened for alleles of the 7 housekeeping loci, *acs*, *aro*, *mut*, *nuo*, *pps*, *trp* and *gua* of *P.aeruginosa* using pubmlst.org. The MLST concept is extended to the whole genome scale screening for allele variants of a diversity of other loci and we analysed this data in combination with the key housekeeping genes to identify distinct genotypes among the isolates (Jolley and Maiden 2010). For further data analysis of collection 1, we grouped isolates with identical MLST profiles together as one independent sample to avoid any pseudoreplication (Fig. A1).

We screened all isolate genomes for the soluble S pyocin (S1, S2, S3, S4, S5 and AP41) loci and their allele variants for the killing and corresponding immunity gene using a known sequence as a reference available from previously sequenced genomes in NCBI Genbank (Table A4). We also screened the genomes for regulatory (prtN and prtR) and lysis genes (hol and lys) as well as the R and F pyocin structural genes (Fig. 2 & Table A4). We used pyocin loci R2 and F2 as reference genes to screen for other potential alleles that belong to other R and F pyocin subtypes. The R pyocin is characterized by the presence of the whole suite of genes between hol-lys and the F pyocin by the presence of the toxin loci between lys-trpG (Fig. 2). We tagged the genomes of all isolates and annotated each of the loci and its identified alleles.

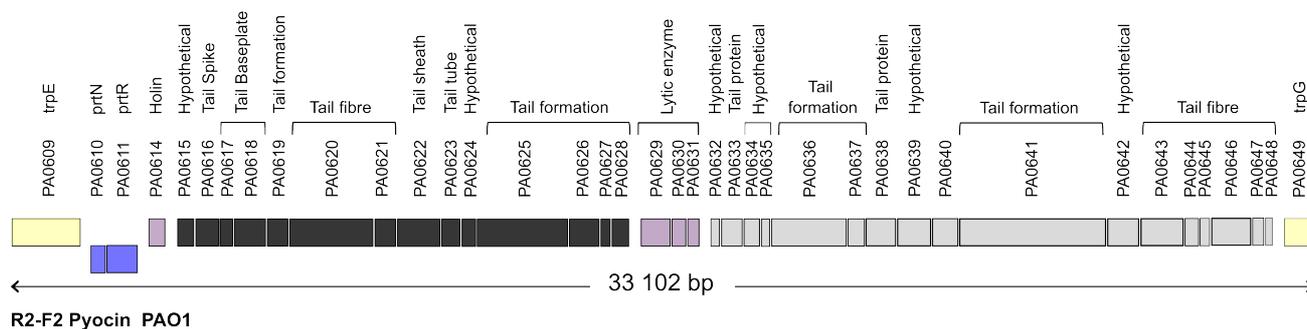


Fig. 2 Genetic organization of R and F pyocin components. The R and F pyocins occur in two gene clusters in tandem lying between trpE and trpG and ranging from the open reading frames (ORFs) PA0610-PA0648, which consists of the regulatory genes in blue (are transcribed in the opposite direction), a lysis gene cassette in purple is common to R, F and S type pyocins and R2 and F2 specific regions in black and grey respectively, encoding the structural toxin proteins Individual protein functions are labeled. (Figure is an adaptation from (Nakayama et al. 2000; Michel-Briand and Baysse 2002)).

For some of the F2 toxin loci, alleles could not be identified due to high sequence dissimilarity. In such cases we checked for the presence of open reading frames (ORF) in the expected location of the F –specific region and if present we marked as

present but with an unidentified allele. In all isolates the genes for R and F pyocins are found between *trpE* and *trpG* in one region lying on one contig, facilitating the search for ORFs in the missing R or F regions. Pyocin subtypes F2 and F3 genes responsible for tail fibre formation, consist of three ORFs, PA0643, PA0644 and PA0645 that compose a gene cassette, that is duplicated as seen by the high sequence homology with ORFs PA0646, PA0647 and PA0648 (Nakayama et al. 2000). Whereas in the F1 subtype, the duplicated tail formation region is absent (Kuroda and Kageyama 1981; Nakayama et al. 2000).

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

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

Statistical analysis of phenotypic inhibition profiles

For the 41 isolates taken from different stages of infection (collection 1), we merged and averaged the data of all isolates with identical MLST genotypic profiles in each isolate category resulting in a total of 29 independent samples (Fig. A1). To avoid pseudoreplication, we estimated the number of times isolates of identical genotypes are inhibited or inhibiting and used the averaged value for the analysis. We tested for significant differences in inhibition with time spent in the lung (as estimated by type of CF lung infection) using a generalized linear (GLM) analysis with level of inhibition as a response variable and two explanatory variables: time spent in lung and type of *P.aeruginosa* isolate category. We tested for changes in the proportion of inhibition caused by isolates' supernatant as a function of time isolates spent in lung and the proportion of lawns inhibited by supernatants as a function of time bacterial lawn isolates spent in lung. We also specifically tested for differences in the level of inhibition caused by isolates' supernatants of each category on the isolate lawns within each category and differences in the level of inhibition in the isolate lawns of each category caused by the isolates' supernatants of each category.

For the longitudinal samples from the 8 CF patients (collection 3), we did not merge isolates of the same genotype as one independent sample because the aim of these experiments is to test for significant differences in inhibition with time of infection across isolates infecting the same lung. We used a GLM analysis to test for significant differences in inhibition levels with time (in years) each isolate has spent infecting the lung. In the analysis we modeled the proportion of killing caused by isolates' supernatant as a response variable and time of infection as a continuous variable with genotype as a fixed effect. This analysis was done on isolates per patient and isolates across all patients for more general patterns. The data from both isolate collections is

proportion data and so we normalized for residuals prior to analysis using an arcsine square-root transformation.

Analysis of S pyocins

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

To assess whether the S immunity gene is responsible for cell protection from S pyocins we used the pyocin gene profiles in all isolates to predict how often one cell carrying the S pyocin will kill a cell that does not carry that S pyocin (Fig. 3). Then we determine how often killing is observed from the phenotypic experimental data. We then record how often resistance of the target cell is due to presence of the immunity gene. When the same genotype showed variation in its S pyocin profile, we also considered the isolates as independent samples. We recorded resistance or susceptibility from the experimental competitive interactions when more than 50% of the isolates of each independent genotype are susceptible or resistant. If exactly 50% of the isolates of a genotype show resistance and the other 50% susceptibility, we record that as data that cannot be interpreted which occurred in 29 out of 635 cases. In the majority of cases, there was no discrepancy among isolates within a genotype, and so the criteria we use above do not significantly change the results if not applied.

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

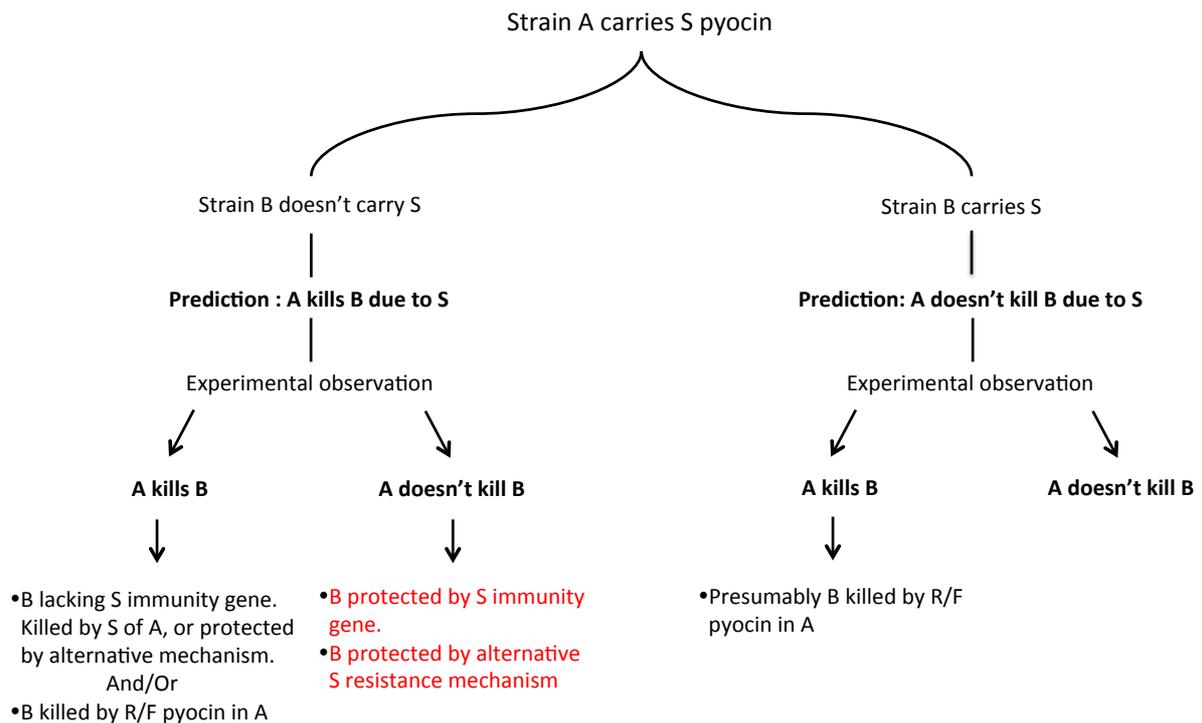


Fig. 3 Flowchart representing S-pyocin mediated killing/resistance. Predicted strain killing is compared with experimentally observed killing and we genetically determine how often observed strain resistance is due to presence of the immunity gene or an alternative unknown mechanism.

Results

Pyocin production and resistance

Comparison across patients sampled at different stages of infection

Considering the 41 isolates taken from different stages of infections (collection 1), we found that strains that were isolated from later stage infections were both less likely to inhibit and more likely to be inhibited by other strains (Fig. 4 & A1; Inhibiting: $F_{7, 108} = 10.63$, $P = 4.03 \times 10^{-10}$; Inhibited: $F_{7, 108} = 11.14$, $P = 1.53 \times 10^{-10}$; $N=29$ distinct genotypes). The interaction terms between the type of lawn inhibited, and the type of supernatant causing inhibition were not significant for either the supernatants inhibiting ($F_{4, 111} = 16.91$, $P = 7.43 \times 10^{-11}$) or the lawns being inhibited ($F_{4, 111} = 18.9$, $P = 7.10 \times 10^{-12}$).

We found that strains that were isolated from later infection stages had a lower diversity of pyocins (Fig.5; GLM, $F_{1, 27} = 5.003$, $P = 0.034$). Lower pyocin diversity in transmissible strains can be either due to gene loss over infection time, or the strains never having had them in the genomes. To determine whether the pyocin genes are lost from the genomes when a strain infects for long periods we examined the 55 isolates taken over 38 years from one of the Danish multiply chronic transmissible genotypes (DK2; collection 2). These isolates do not show loss of or mutation accumulation in the pyocin genes over time. This data indicates that pyocin genes of transmissible isolates are not lost with increasing infection time but were never present such that transmissible strains are characterized by a lower pyocin diversity relative to non-chronic isolates.

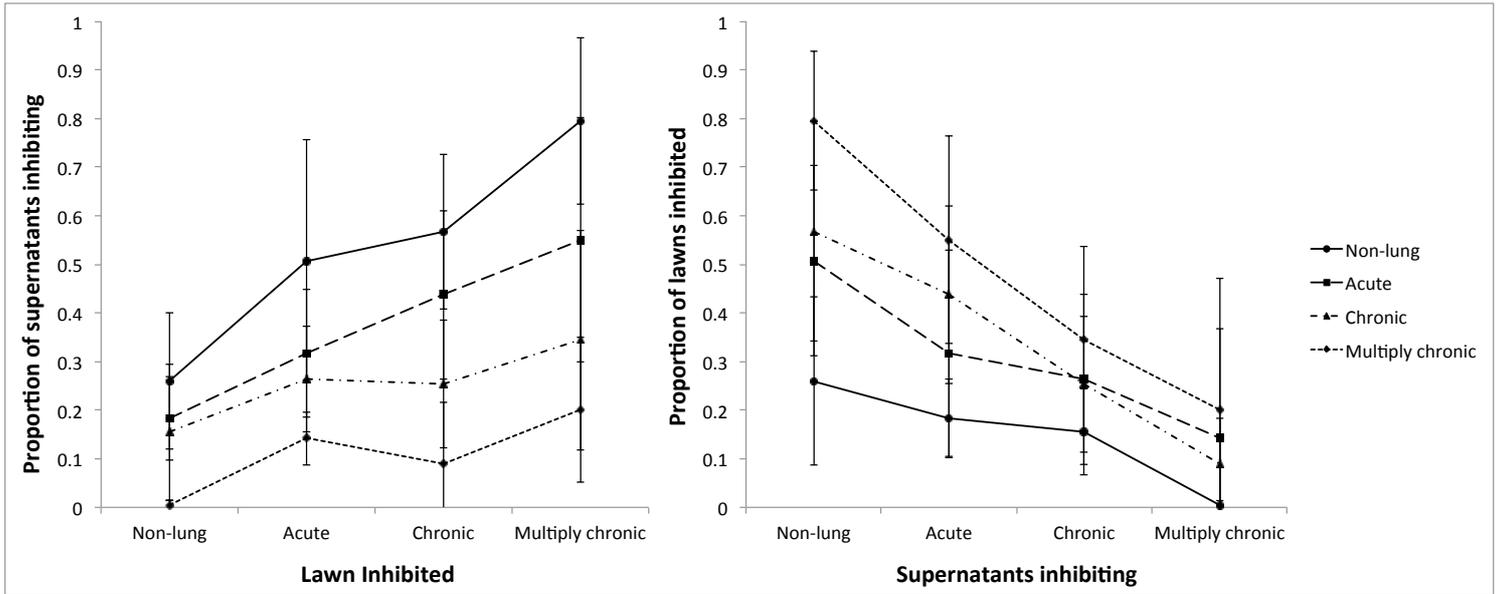


Fig. 4 Proportion of supernatants inhibiting and lawns inhibited. Strains that were isolated from later stage infections were both (a) less likely to inhibit and (b) more likely to be inhibited by other strains. The error bars indicate \pm 95% confidence interval bars around mean values.

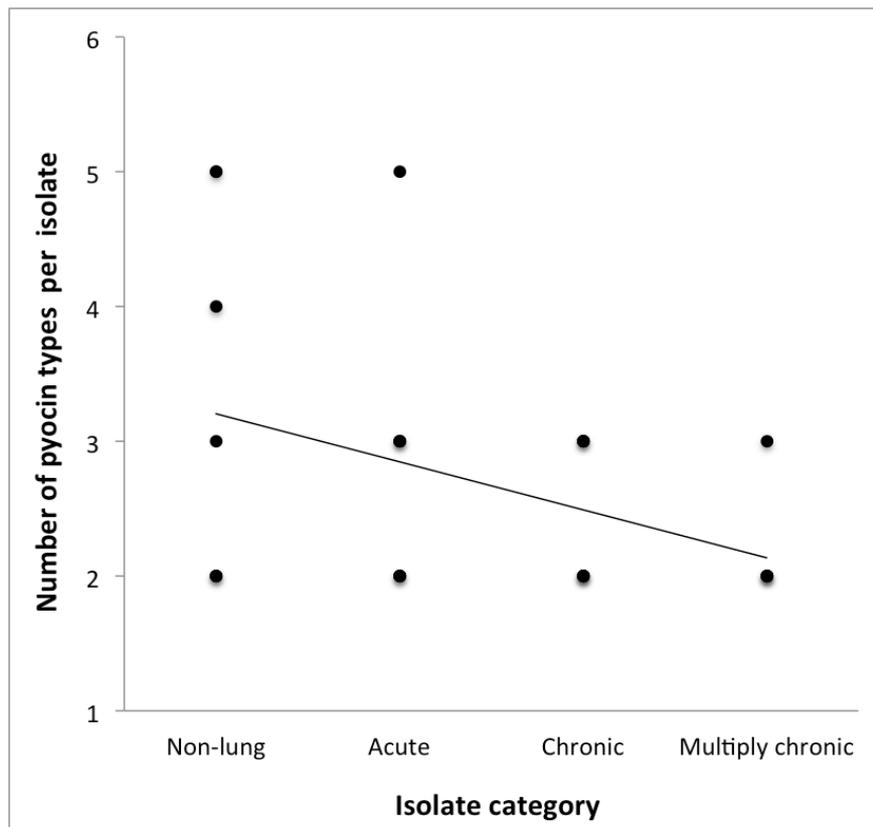


Fig. 5 Pyocin diversity across different stages of infection. Isolates from later stage infections carry fewer pyocin types than isolates from early infections and non-lung environments.

To test the generality of our results, particularly that Danish multiply chronic strains carry a reduced diversity of pyocins, we analysed the genomic pyocin data on six *P. aeruginosa* isolates from GenBank, four chronic or epidemic CF strains from the UK, Boston and an unknown source, and two clinical non-lung isolates (collection 3). We found the same pattern in these isolates that we had found in our Danish isolates. Similar to the multiply chronic isolates of our collection the CF isolates (except for the Liverpool strain) also show absence of all the R pyocin genes but retain the F genes, while the clinical non-lung isolates carry both the R and F pyocin genes, similar to the non-lung isolate category of our collection (Fig. A2).

Comparison over time within patients

We then analysed longitudinal isolates, taken over a 6-10 year period, from 8 patients. We found that, within patients, isolates' inhibition levels did not change over time infecting (Table A5; $T_7 = -0.70$, $P=0.505$, $N=8$) – inhibition increased in 2 patients, decreased in 4, and there was no inhibition in 2 patients. Examining the genomic data of isolates in patients with multiple infecting genotypes, pyocin diversity does not vary significantly with the likelihood of persisting in the lung ($T_{7.73} = -0.43$, $P = 0.676$, $N=5$).

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

competition (Fig. 6). In contrast, in the third patient, the inhibited genotype persisted longer in the infection.

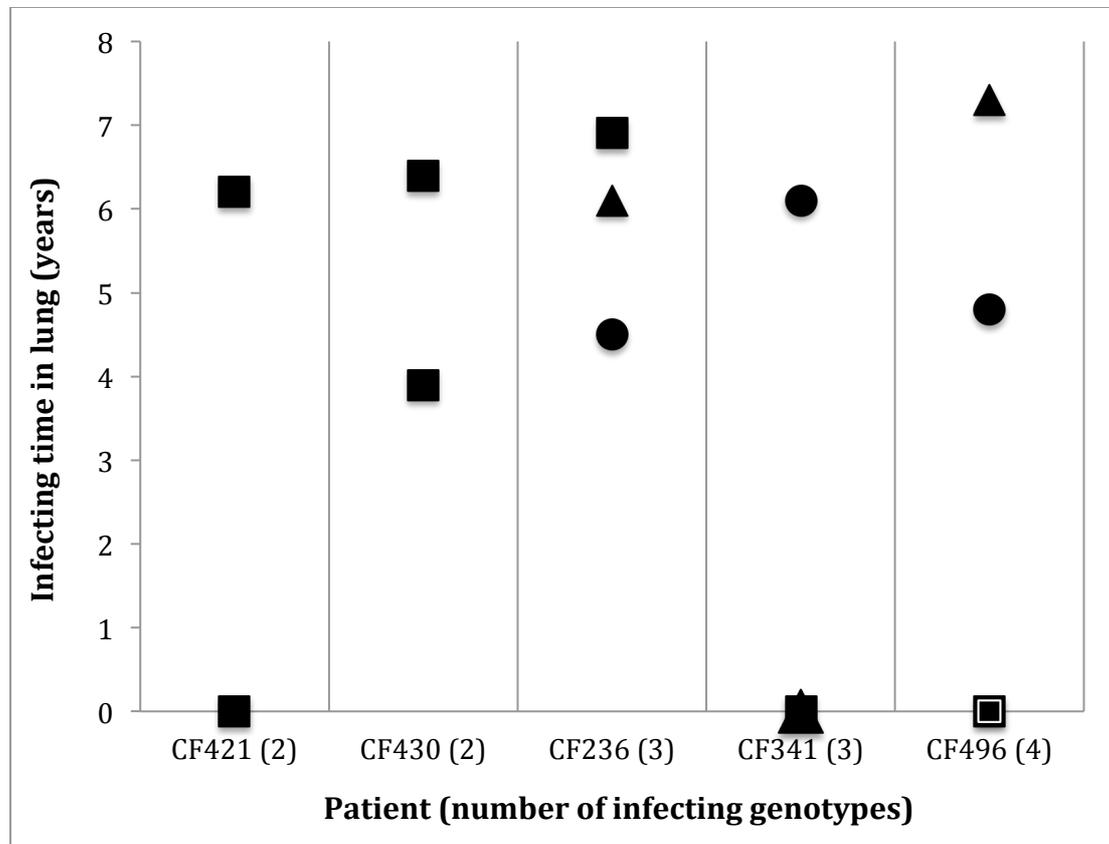


Fig. 6 Within patient competitive dynamics. Considering the eight patients where we were able to follow strain dynamics over time, five were infected by multiple strains. These patients contained 14 distinct genotypes, and none of the patients were infected with the same genotype. In only three of these five patients at least one of the strains was able to inhibit another strain in the infection. Squares (■) represent genotypes that are not inhibited nor cause any inhibition; (▲) triangles are genotypes that inhibit the susceptible circle genotypes (●). In two out of these three patients (CF236 & CF496), the inhibiting genotype (▲) persisted longer in the infection, consistent with a role of bacteriocin-mediated competition. In the other patient (CF341), the inhibited genotype (●) persisted longer in the infection, contrary to the prediction from bacteriocin dynamics.

Linkage between S pyocin killing and immunity

We then examined the extent to which killing and immunity co-occurred for the five S-type pyocins. We focused on S-type pyocins because they are composed of two

genes, one responsible for the killing activity and an immunity gene that protects against the killing. Natural populations have been observed to contain strains that carry both, the toxin and immunity genes (toxin producers), just the immunity gene (resistants), or neither (sensitives).

Overall, 98% (53/54) of all independent isolates in all collections contained at least one S-pyocin gene. The average number of pyocin types per isolate was 1.5, giving 80 (53 x 1.5) occurrences of pyocin genes. Amongst those, 51% (41/80) contained both the toxin and the immunity genes, 35% (28/80) only the killing gene and 14% (11/80) only the immunity gene, (Fig.7; $\chi^2_{2,80} = 25.46$, $P = 2.96 \times 10^{-6}$). The relatively high occurrence of only the killing gene is surprising, because this would lead to the isolate killing itself.

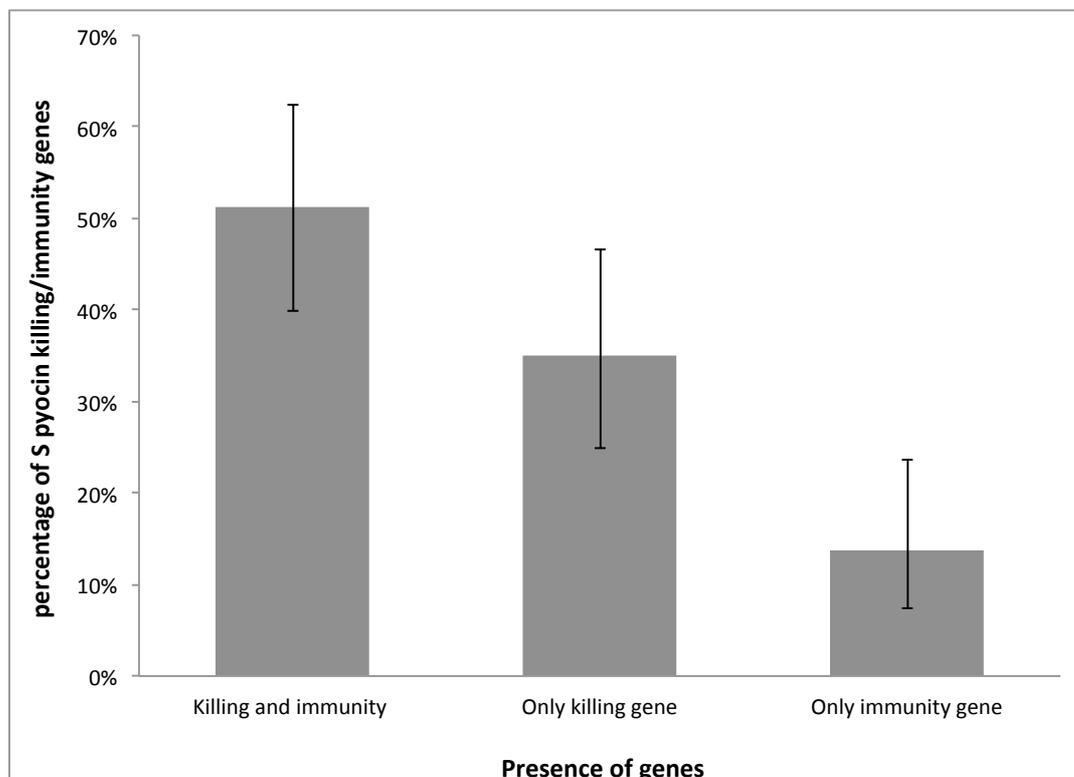


Fig. 7 Soluble pyocin killing and immunity genes. Killing and immunity genes are found together (51%), or either the killing gene (35%) or the immunity gene (14%) alone. The error bars indicate \pm 95% confidence intervals.

We examined the extent to which strains might have resistance mechanisms other than the specific immunity genes (Fig. 3). We considered 877 strain interactions (competitions), including the isolates from the different stages of infection and the longitudinal isolates from the 8 patients (collection 1 and 4). Examining our genetic data, we found that in 635 of these competitions, one strain carried at least one S-type pyocin that the other strain did not. In 33% (207/635) of these cases, we found that the toxin carrying strain was able to inhibit the growth of the other strain. In the 403 interactions where growth was not inhibited, we found that in 19% (77/403) of cases the strain not inhibited contained the immunity gene to the pyocin(s) being produced by the killing strain. In the remaining 326 cases (81%, 326/403), the strain did not have the immunity gene(s) and so must be using some alternative mechanism to resist the pyocin. Alternative resistance mechanisms protect strains significantly more frequently than the immunity gene (Fig. 8; $\chi^2_{1,403} = 305.23$, $P < 2.2 \times 10^{-16}$).

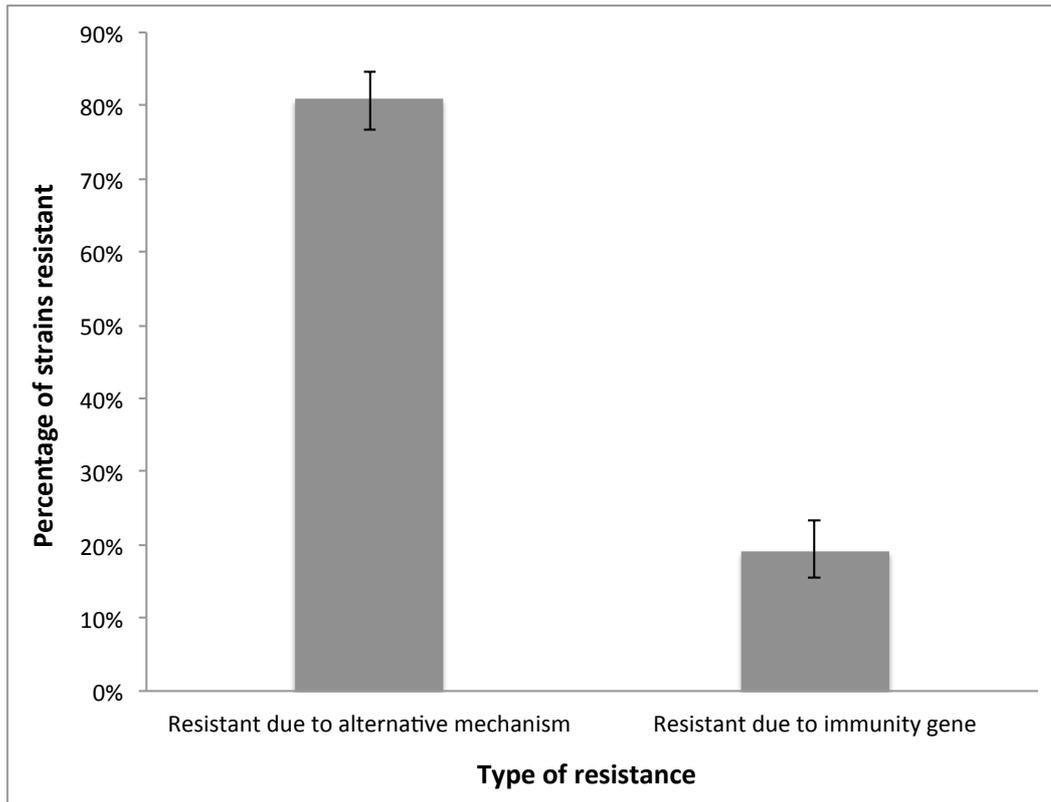


Fig. 8 Protection from *S* pyocins by unknown resistance mechanisms. Considering cases where one strain does not carry a pyocin type carried by another (403 strain pairs), resistance via some other mechanism is much more common (81%) than resistance via the immunity gene (19%). The error bars indicate \pm 95% confidence intervals with correction for continuity around proportion values.

Discussion

We found that, in *P. aeruginosa* infections of the cystic fibrosis lung: (1) isolates from later infection stages caused less inhibition of other strains, were significantly more inhibited by the pyocins produced by other strains (Fig.4), and carried a significantly lower diversity of pyocin types (Fig. 5 & A3); (2) pyocin genes were not lost from a chronic transmissible strain over time; (3) within infections, over time, there is no change in inhibition / inhibiting levels or pyocin diversity; (4) pyocin inhibition profiles do not significantly explain which strains persist longer within lung

infections (Fig. 6); (5) 35% of S pyocins occurred only as the killing gene, without the immunity gene to that pyocin (Fig. 7); (6) in cases where one strain does not carry an S pyocin type carried by another, resistance via other mechanisms is much more common (81%) than resistance via the immunity gene (19%; Fig. 8).

Bacteriocins and strain dominance

We found no support for the hypothesis that certain strains are able to achieve dominance in the CF lung through bacteriocins (Fig. 4 & 6). In fact, we found the opposite pattern, that more dominant / persistent strains, taken from later infection stages are less able to kill and more easily killed by other strains (Fig. 4). Our genetic analysis suggests that this pattern arises because isolates from later infections carry fewer pyocin genes (Fig. 5). This pattern could occur through strains losing pyocin genes over time, or through strain competition, with more competitive strains producing less pyocins (Tredgett et al. 1990; Romling et al. 1994; Tummler and Kiewitz 1999; Ernst et al. 2003; Eberl and Tummler 2004). Our results suggest the latter with more competitive strains producing fewer pyocins (Fig. 5) rather than pyocin genes being lost from strains during infection.

Why do more dominant strains which cause chronic infections carry a lower diversity of pyocins (Fig. 5; Tredgett et al. (1990)? A number of possible selective forces, which are not mutually exclusive, may be involved. A higher strain diversity, both within and across species, may favour a higher pyocin diversity during early stages of infection (Morse et al. 1980; Blackwell et al. 1982; Campagnari et al. 1994; Jelsbak et al. 2007; Williams et al. 2008; Waite and Curtis 2009; Bakkal et al. 2010; Yang et al. 2011). Another possibility is that higher pyocin diversity may be more costly in chronic infections if it leads to a higher antibiotic susceptibility – for example, expression of the R/F region increases strain susceptibility to fluoroquinolone

antibiotics due to induction of the lysis system (Brazas and Hancock 2005). However, this does not explain maintenance of the F pyocin in the multiply chronic isolates of our collection, unless carrying either R or F rather than both, also makes the cell less inducible and therefore more resistant to antibiotics.

Killing and immunity genes

We found that 35% of S pyocins are found as the killing gene without the corresponding immunity gene (Fig. 7). This is surprising because it suggests that these strains would kill themselves. However, we also found that strain resistance often occurs without presence of the immunity gene. Considering cases where one strain does not carry a pyocin type carried by another, resistance via some other mechanism is much more common (81%) than resistance via the immunity gene (19%; Fig. 8). Possible alternative resistance mechanisms could be due to production of another immunity protein, that a secondary receptor is needed in addition to the primary receptor to allow entry of the pyocin into the cell or that cells have mutations in or are lacking the primary S pyocin receptors (Baysse et al. 1999; Denayer et al. 2007; Elfarash et al. 2014).

We didn't find evidence for bacteriocin-mediated competition explaining strain dynamics and/or producing rock-paper-scissor dynamics. Such dynamics are still expected even when there are multiple bacteriocin types in a population (Biernaskie et al. 2013). One possible explanation is that *P. aeruginosa* bacteriocins do not appear to involve single production / immunity genes, as we have shown immunity can occur via alternative mechanisms. This contrasts with *E.coli* where bacteriocins are only neutralized by the immunity proteins (Cascales et al. 2007). Our results suggest that the outcome of strain competition is being driven primarily by other factors, which

influence the ability to survive and grow in the CF lung. These could include susceptibility to antibiotics or attack from the immune system.

Conclusion

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

Appendix

Isolate category	Isolate name	Isolate source
Non-lung	PAO6049*	Lab PAO1
	ATCC15692*	Lab PAO1
	P3*	Lab PAO1
	P76*	Lab PAO1
	P100*	Lab PAO1
	P93*	Lab PAO1
	MSH	Mt St Helen's
	R4 [§]	Sink
	R5 [§]	Sink
	F-2 ⁺	Water isolate
	F-3 ⁺	Water isolate
	N3	Columbia, polluted water
	N7	Columbia, soil
	N18	Columbia, soil
Acute (Intermittent)	B3-0	Child with CF #3
	B6-0•	Child with CF #6
	B6-2•	Child with CF #6
	B6-3	Child with CF #6
	B7-1 [⊙]	Child with CF #7
	B7-3 [⊙]	Child with CF #7
	B11-1	Child with CF #11
	B12-0	Child with CF #12
	B13-2	Child with CF #13
	B28-1	Child with CF #28
Chronic (1-off chronic)	2-1	Adult with CF #2
	2-3	Adult with CF #2
	6-2	Adult with CF #6
	11-1	Adult with CF #11
	11-6♦	Adult with CF #11
	11-7	Adult with CF #11
	16-1	Adult with CF #16
	16-2	Adult with CF #16
	16-10♦	Adult with CF #16
	16-14	Adult with CF #16
Multiply Chronic (multiple chronic)	2-4	Adult with CF #2
	2-5*	Adult with CF #2
	6-1	Adult with CF #6
	7-1*	Adult with CF #7
	10-1	Adult with CF #10
	11-10*	Adult with CF #11
	16-3	Adult with CF #16

Table A1. Four categories of *P.aeruginosa* isolates and time to grow to stationary phase. *§+•⊙♦ Isolates with same symbols are genetically identical based on bacterial MLST profiling

Patient origin	Year of sample
CF84	1972
CF66	1973
CF66	2008
CF66	2002
CF66	1992
CF510	2006
CF43	1973
CF333	2003b
CF333	1997
CF333	2007b
CF333	2007a
CF333	1992a
CF333	2004a
CF333	2004b
CF333	1992b
CF333	2005
CF333	1994
CF333	2003c
CF333	2004c
CF333	2007c
CF333	2003a
CF333	1991
CF311	2002
CF30	1979
CF248	2002
CF243	1986
CF243	2002
CF240	2002
CF224	2002a
CF224	1987
CF224	2002b
CF223	2002
CF222	2001
CF211	1997a
CF211	2006a
CF211	1997b
CF211	2006b
CF206	2002
CF180	2002a
CF180	2002b
CF177	2002
CF173	1984a
CF173	1997
CF173	2005
CF173	1984b
CF173	1999
CF173	1991
CF173	1984c
CF173	1992
CF173	2002
CF173	1994
CF173	1995
CF119	1979
CF114	1973
CF105	1973

Table A2. 55 DK2 isolates from 21 patients sampled over 38 years.

Patient	Genotype
CF236 (23 isolates)	DK01 DK15 DK53
CF341 (22 isolates)	DK02 DK19 DK20
CF382 (15 isolates)	DK32 Unidentified
CF405 (7 isolates)	DK13
CF414 (9 isolates)	DK12
CF421 (15 isolates)	DK06 DK37
CF430 (11 isolates)	DK34 DK35
CF496 (19 isolates)	DK27 DK28 DK29 DK30

Table A3. Longitudinal isolates sampled from patients over the course of infection

Pyocin	Gene function	Gene name/ Locus tag	Locus ID in BIGSDB	Alias in NCBI
S5	Killing protein	pyoS5 (PA0985)	PSEU0985	AE004091.2
S5	Immunity protein	PA0984	PSEU0984	AE004091.2
S2	Killing protein	pys2 (PA1150)	PSEU1150	AE004091.2
S1 and S2	Immunity protein	imm2 (PA1151)	PSEU1151	AE004091.2
S1	Killing protein	-	PSEU5571	D12707.1
S4	Killing protein	PA3866	PSEU3866	AE004091.2
S3	Killing protein	pyoS3A	PSEU5575	PA14_49520
S3	Immunity protein	pyoS3I	PSEU5576	X77996.1
AP41	Killing protein	-	PSEU5577	D12705.1
AP41	Immunity protein	-	PSEU5578	D12705.1
trpE	anthranilate synthetase I	trpE (PA0609)	PSEU0609	AE004091.2
R and F	Transcriptional activator	prtN (PA0610)	PSEU5581	AB030825.1 (AE004091.2)
R and F	Transcriptional repressor	prtR (PA0611)	PSEU5582	AB030825.1 (AE004091.2)
R and F	Holin	Hol (PA0614)	PSEU5583	AB030825.1 (AE004091.2)
R	Tail spike	VR2 (PA0616)	PSEU5584	AB030825.1 (AE004091.2)
R	Tail baseplate	WR2 (PA0617)	PSEU5585	AB030825.1 (AE004091.2)
R	Tail baseplate	JR2 (PA0618)	PSEU5586	AB030825.1 (AE004091.2)
R	Tail formation	IR2 (PA0619)	PSEU5587	AB030825.1 (AE004091.2)
R	Tail fibre	HR2 (PA0620)	PSEU5588	AB030825.1 (AE004091.2)
R	Tail fibre	PRF16 (PA0621)	PSEU5589	AB030825.1 (AE004091.2)
R	Tail sheath	FIR2 (PA0622)	PSEU5590	AB030825.1 (AE004091.2)
R	Tail tube	FIIR2 (PA0623)	PSEU5591	AB030825.1 (AE004091.2)
R	Hypothetical protein	PRF19 (PA0624)	PSEU5592	AB030825.1 (AE004091.2)
R	Tail formation	PRF20 (PA0625)	PSEU5593	AB030825.1 (AE004091.2)
R	Tail formation	UR2 (PA0626)	PSEU5594	AB030825.1 (AE004091.2)
R	Tail formation	XR2 (PA0627)	PSEU5595	AB030825.1 (AE004091.2)
R	Tail formation	DR2 (PA0628)	PSEU5596	AB030825.1 (AE004091.2)
R and F	Lytic enzyme	Lys (PA0629)	PSEU5597	AB030825.1 (AE004091.2)
F	Tail protein	VF2 (PA0633)	PSEU5598	AB030825.1 (AE004091.2)
F	Tail formation	HF2 (PA0636)	PSEU5599	AB030825.1 (AE004091.2)
F	Tail formation	MF2 (PA0637)	PSEU5600	AB030825.1 (AE004091.2)
F	Tail protein	PA0638	PSEU0638	AE004091.2
F	Hypothetical protein	PA0639	PSEU0639	AE004091.2
F	Tail protein	PA0640	PSEU0640	AE004091.2
F	Tail protein	PA0641	PSEU0641	AE004091.2
F	Hypothetical protein	PA0642	PSEU0642	AE004091.2
F	Hypothetical protein	PA0643	PSEU0643	AE004091.2
F	Hypothetical protein	PA0644	PSEU0644	AE004091.2
F	Hypothetical protein	PA0645	PSEU0645	AE004091.2
F	Hypothetical protein	PA0646	PSEU0646	AE004091.2
F	Hypothetical protein	PA0647	PSEU0647	AE004091.2
F	Hypothetical protein	PA0648	PSEU0648	AE004091.2
trpG	anthranilate synthetase II	trpG (PA0649)	PSEU0649	AE004091.2

Table A4. Genes screened in all isolate genomes

Model	Patient	Parameter	Estimate	SE	t	df	p
Proportion isolates killing ~ Infection time & Genotype	CF236 23 isolates, 3 genotypes-DK01, DK15 & DK53	Intercept (genotype DK01)	0.354	0.024	14.94	19	5.94x10 ⁻¹²
		Infection time	0.008	0.005	1.55		0.138
		Genotype DK15	-0.260	0.038	-6.86		1.52x10 ⁻⁶
	CF341 22 isolates, 3 genotypes- DK02, DK19 & DK20	Genotype DK53	-0.387	0.026	-14.83		6.75x10 ⁻¹²
		Intercept (genotype DK02)	1.420x10 ⁻¹⁶	2.370x10 ⁻¹	0.00	18	1.000
		Infection time	2.153x10 ⁻²	2.558x10 ⁻²	0.84		0.411
	CF382 15 isolates, 1 genotype- DK32 (and 3 isolates of unknown genotype)	Genotype DK19	2.608x10 ⁻²	2.559x10 ⁻¹	0.10		0.920
		Genotype DK20	9.231x10 ⁻¹	3.352x10 ⁻¹	2.75		0.013
		-	-	-	-	-	-
	CF405 7 isolates, 1 genotype- DK13	-	-	-	-	-	-
	CF414 9 isolates, 1 genotype- DK12	Intercept	0.093	0.071	1.310	7	0.232
		Infection time	-0.020	0.022	-0.923		0.387
	CF421 15 isolates, 2 genotypes-DK06 & DK37	Intercept (genotype DK06)	0.041	0.046	0.888	12	0.392
		Infection time	-0.001	0.012	-0.100		0.922
		Genotype DK37	-0.041	0.109	-0.377		0.712
	CF430 11 isolates, 2 genotypes-DK34 & DK35	Intercept (genotype DK34)	0.092	0.181	0.510	8	0.624
		Infection time	-0.024	0.022	-1.091		0.307
		Genotype DK35	0.132	0.169	0.785		0.455
	CF496 19 isolates, 4 genotypes- DK27, DK28, DK29 & DK30	Intercept (genotype DK27)	0.589	0.092	6.390	14	1.68x10 ⁻⁵
		Infection time	0.0115	0.0250	0.459		0.653
Genotype DK28		-0.589	0.224	-2.635		0.0196	
Genotype DK29		-0.481	0.099	-4.856		0.000255	
Genotype DK30		-0.589	0.224	-2.635		0.0196	

Table A5. Model results for the proportion of killing by each isolate of a given genotype on all other isolates infecting a patient. The proportion data is arcsine square root transformed to correct for overdispersion. In all cases genotype is added as a fixed factor. Models are all Gaussian GLMs.



Fig. A1 Phenotypic screening of inhibition/resistance profiles of isolates tested against each other. Isolates that infect the CF lung longer have the most inhibited lawns and supernatants that inhibit the least. Boxes from left to right, and top to bottom represent increasing colonization time in the lung and are divided into 1) non-lung, 2) acute, 3) chronic and 4) multiply chronic isolates. Filled squares represent lawns inhibited by pyocins. Empty squares represent no inhibition. * Isolates that are merged.

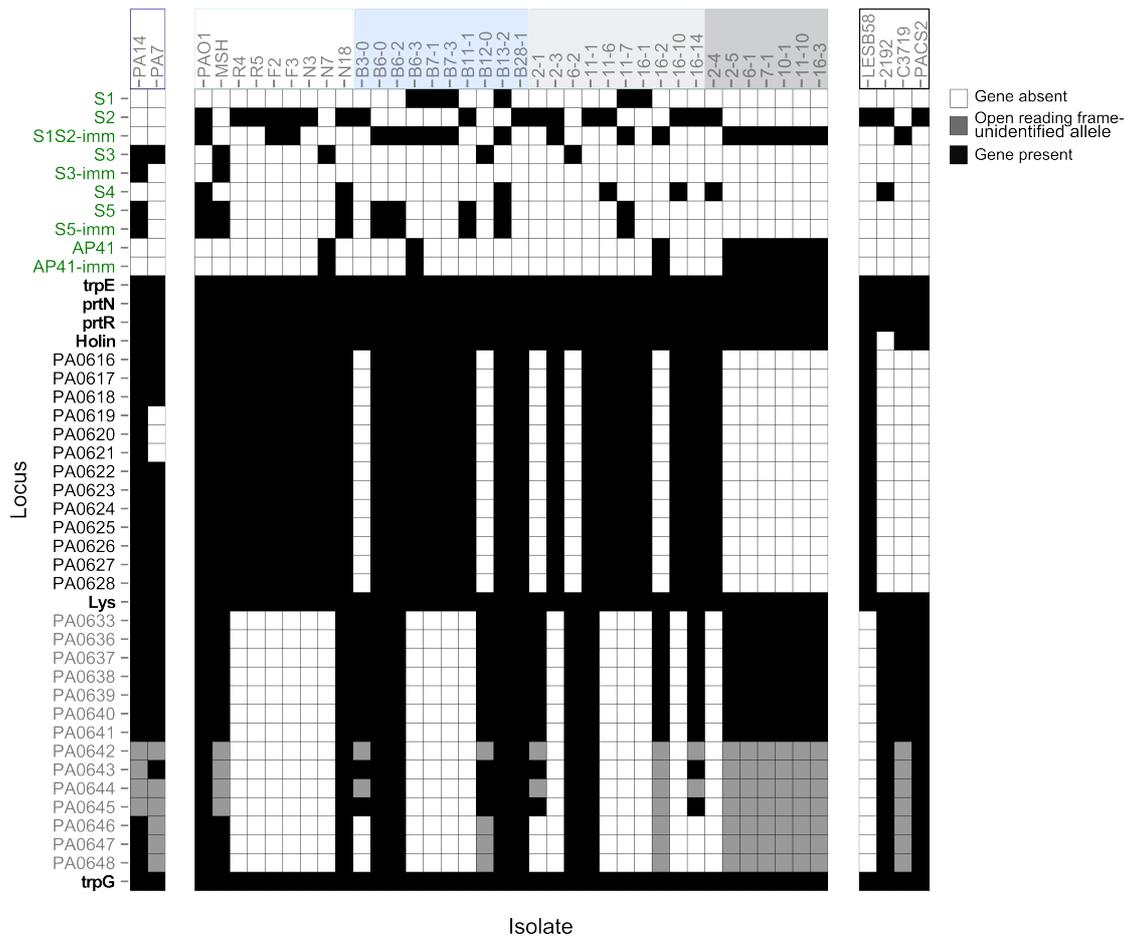


Fig. A2 Presence and absence of pyocin loci. Left to right shows the isolates in categories: 1) non-lung isolates from GenBank (accession numbers: NC_008463 and NC_009656 respectively); 2) Non-lung isolates; 3) Acute; 4) Chronic; 5) Multiply chronic; 6) Lung isolates from genbank (accession numbers: NC_011770, NZ_CH482384, NZ_CH482383, NZ_AAQW01000001). Top to bottom are the pyocin loci. Loci in green represent the soluble pyocins. In black are the loci for constructing the R-type and in grey are the loci specific to the F-type pyocin, loci in bold are common to S, R and F-types. Filled squares represent presence of open reading frame (grey) or annotated gene (black) and empty squares represent absence of gene in each isolate. Isolates B3-0, 2-1 and 16-14 are missing the F pyocin tail formation loci PA0646, PA0647 and PA0648 of subtype F2 because they carry the F1 subtype.

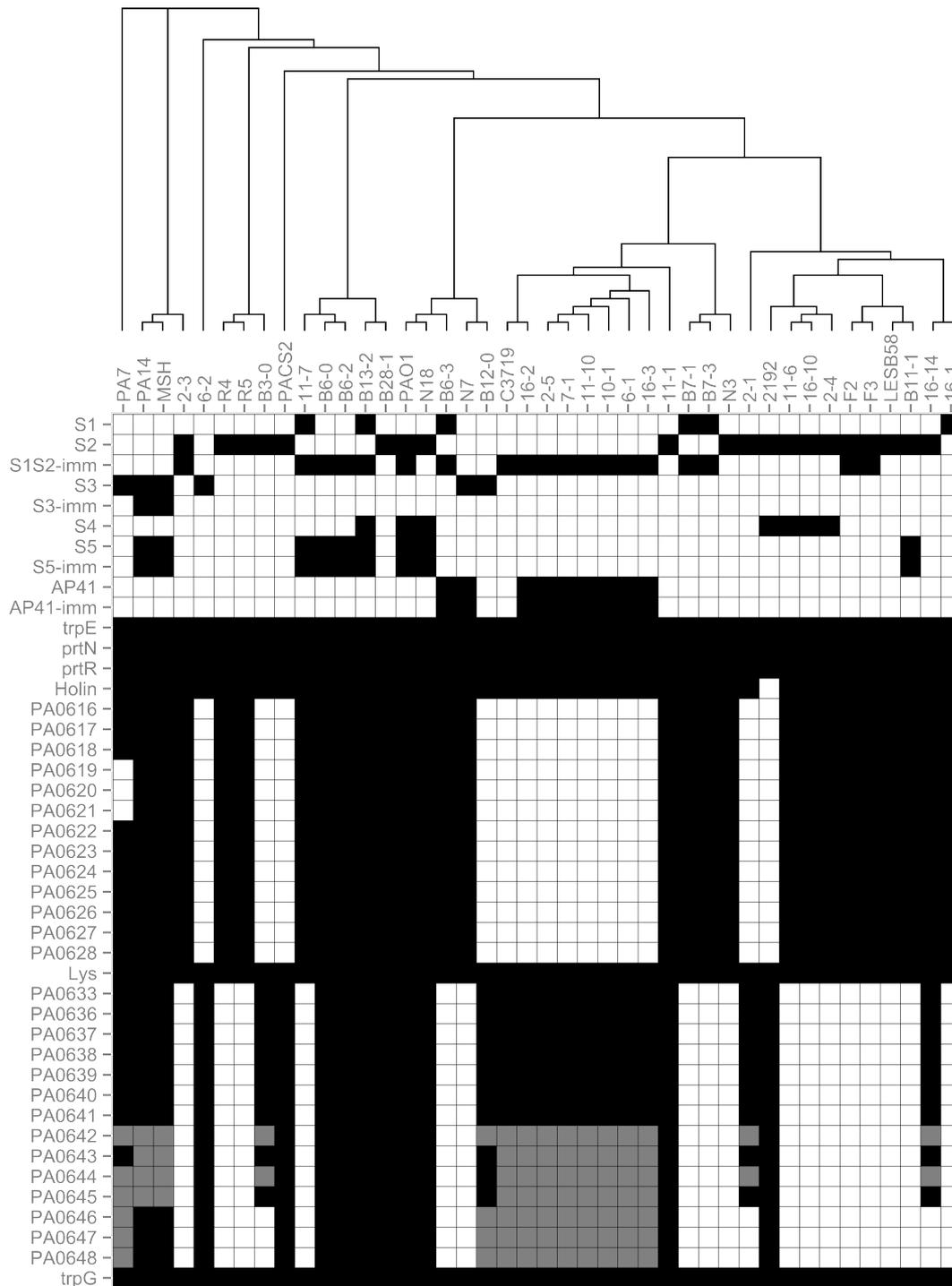


Fig A3. Isolate phylogeny tree mapped onto pyocin profiles. We constructed a phylogenetic tree from the concatenated and aligned bacterial MLST sequences using a Tamura Nei genetic distance model and the neighbour joining tree build method with no specified outgroup in Geneious software version (6.0.5) Biomatters (<http://www.geneious.com>). Isolates are not clustered based on type of infection or environmental source they have been sampled from. Similarly, Ernst et al. (2003) show in their study that environmental and clinical strains tend to cluster together. Closely related isolates do not necessarily share the same pyocin profiles. Black and grey squares represent presence of an annotated gene or an open reading frame respectively. Empty squares represent gene absence.

Chapter 6. Discussion

Each of the chapters in this thesis contains a relevant extensive discussion. The aim of this chapter is to highlight the key findings and general observations, and to discuss some general conclusions and future directions that have emerged from my work.

The aim of my thesis has been to understand the role social behaviours play in mediating competitive dynamics in *Pseudomonas aeruginosa* populations and to apply our understanding of such behaviours to natural bacterial populations. However, in natural environments (e.g. cystic fibrosis lung infections) the social and abiotic conditions are much more variable than controlled experimental systems. Therefore, it is essential to identify what the various selective factors in natural settings are, how they may affect expression of the social trait and therefore influence population dynamics (Ch. 2 & 3). Bacteria's physiological state of growth also plays a key role in determining social behaviour dynamics (Ch. 4). The use of cheats to develop novel antibacterial treatments carries less potential given that I found that established populations of bacteria are more resistant to cheat invasion. This result has important medical implications because if cheats are to be used as a medical intervention strategy, to reduce the virulence of clinical infections, this may be more difficult given that infectious symptoms manifest after cooperative pathogenic populations are already established in the host. However, the use of bacteriocins as antimicrobial agents may be more promising. I found that *P.aeruginosa* isolates that dominate and persist as chronic infections in the cystic fibrosis lung do not use bacteriocins to outcompete other strains. In fact the persisting strains are more

susceptible to other strains' bacteriocin production and carry a low diversity of bacteriocins (Ch. 5). Therefore, engineering strains to carry broad-spectrum and potent bacteriocins that target chronically infecting strains may prove to be effective.

Chapter 2. Cheats and cheating

The evolution of cheating and response of cooperators to cheating are fundamental issues in the field of evolution. A key question in social evolution is, how is cooperative behavior maintained when it is vulnerable to cheats invading? Despite arguments over the use of intentional language being misleading in the biological context such as with the term 'cheating', a formal definition for cheating in the context of social evolution, can be very useful to convey information about the evolutionary biology of the behavior, and its applications across a range of biological systems. Just as cooperation occurs at all levels of biological organisation, so does cheating. Cheats require a mechanism to exploit cooperators. Cheats either intercept cooperation meant for another cooperator or manipulate cooperators to direct the cooperative behavior toward them.

Social evolution is classically studied in animals whose behaviours are easily observable in a known environment allowing for straight forward application and interpretation of the results. However, testing for cheats is subtler when studying microbes, and therefore some empirical issues arise. Three key questions to ask when testing for cheating to avoid any experimental misinterpretation are: *i)* Is a cooperative trait being exploited? *ii)* Does variation in behavior evolve from selection to cheating? and *iii)* Are the studies carried out in an appropriate environment?

Chapter 3. Cheating is context dependent

A problem with microbial social evolution studies is that cheating is often defined as reduced expression of a cooperative trait rather than in terms of the social costs and benefits of that trait. We experimentally show that cheating depends on the costs and benefits associated with the interacting social partners, as well as the abiotic environment, and not the absolute production of a cooperative trait.

It is essential to test for cheats in an appropriate environment that requires expression of cooperative behaviours for survival, such that a monoculture of cheats cannot survive as well in the absence of cooperators in that environment. A putative cheat in mixed cultures with cooperators can increase in frequency for different reasons: *i*) because it is a cheat in an environment that requires cooperation; or *ii*) because it is avoiding the cost of producing unnecessary public goods in an environment that does not require cooperation— in which case it does not cheat. Cheating is relative to the interacting individuals, because what matters is how much public good an individual produces relative to the cells that it interacts with and not the absolute level produced.

It should not be assumed that putative cheating and cooperation are always social behaviours unless the costs and benefits have been experimentally set up for cooperation to occur in that environment. Our results demonstrate the importance of experimentally confirming the occurrence of cooperator-cheat dynamics before making inferences about the evolution of these social behaviours, whether in contrived laboratory or natural populations.

Chapter 4. Stationary phase cooperators resist cheating

A limitation with bacterial *in vivo* and *in vitro* experimental studies is that cheating and cooperation competition experiments are conducted by co-inoculating strains into sterile media or a host at a low density. Cheat and cooperator cells then compete through the stages of bacterial growth beginning with a lag phase. However, these experiments may not be representative of what happens in natural bacterial populations, which exist as established populations similar to the stationary growth phase of lab cultures. Therefore, the growth dynamics of the strains are not taken into consideration when testing for bacterial social behaviours.

The various stages of the cooperative bacteria's growth curve are characterized by changing levels and rates of public good production, which affect a cheat's ability to invade a cooperative population. Bacterial populations generally secrete public goods at a higher rate during the exponential phase, when cells divide fastest. Therefore cheats capable of exploiting this stage of cooperator growth would benefit the most. In contrast, exoproduct production and secretion are downregulated in stationary phase cultures, thereby reducing the cost of producing public goods and any fitness advantage for cheats. The type of public good produced by the cooperator also plays a key role in a cheat's invasive ability.

We examined the iron-scavenging public good pyoverdine, which is a durable molecule that is reused and recycled. As a result, once pyoverdine accumulates to sufficient levels at later stages of population growth, cooperators downregulate production as a cost-saving strategy, reducing the selective advantage to cheating. This result is similar to experiments with other public goods, such as the molecules

required for swarming, which are only produced when it is least metabolically costly for the bacteria to invest in: this is referred to as ‘metabolic prudence’, a cooperative mechanism that resists cheating (Xavier et al. 2011). However, social behaviours that require constitutive production of a public good, are potentially more susceptible to cheat invasion regardless of the cooperators’ growth stage.

The results have important implications for understanding natural bacterial populations, which likely exist in a permanent stationary-like phase of growth such that secretion of most exofactors is minimal. This may explain why cheating is not so commonly observed in nature, and cooperation dominates despite the ability of cheats to emerge and invade (Hibbing et al. 2010; Levin 2014).

Chapter 5. Bacteriocin-mediated competition

Bacteriocins are thought to play a key role in competitive dynamics among closely related strains. We test the competitive ability of natural bacterial isolates infecting the CF lung to determine whether bacteriocins do play a role in strain dominance in natural populations. Testing strains across patients from different stages of infection, we find that strains persisting as chronic infections are in fact the least likely to kill with bacteriocins and the most susceptible to other strains’ bacteriocins. In addition, these persisting chronic strains also carry a significantly lower diversity of bacteriocin types relative to environmental and infectious strains from early stages of infection. Similarly examining strains sampled over years from infection within patients, we also find little evidence that the strain dominating the infection is a result of bacteriocin competition.

Empirical and theoretical studies to date have mostly focused on relatively simple toxin-antitoxin bacteriocin systems to predict how bacteriocins shape population diversity— particularly resulting in rock-paper-scissor dynamics in structured populations. In such cases resistant strains that only carry the immunity gene and do not invest in production outcompete producer and sensitive strains. However, microbes like *P.aeruginosa* carry a diversity of bacteriocins with complex killing and resistance mechanisms. In fact, we find that among the pyocins with toxin-antitoxin systems, only in 51% of cases do both toxin and antitoxin genes occur together, while the killing gene occurs alone (35%) more frequently than the immunity gene (14%). In addition, we find that *P.aeruginosa* cells can use multiple resistance mechanisms for one pyocin type and this may explain: *i*) why the immunity gene is frequently lost and; *ii*) why it is difficult to identify the different players of the rock-paper-scissor dynamics among our strains.

Bacteriocins do not appear to be used to outcompete other strains. The results suggest that strain competition is being driven primarily by other factors, which influence the ability of some isolates to survive and grow in the CF lung. These factors could include susceptibility to antibiotics or attack from the immune system. We also do not know to what extent the CF lung is spatially structured or unstructured, which is also likely to influence the outcome of strain competition. However, a consistent result is that strains persisting as chronic infections are characterized by a lower diversity of pyocins and share similar pyocin profiles. This knowledge could potentially be used as a method for predicting infection or even designing engineered bacteriocins with which to treat such chronic infections (Gillor et al. 2005; Williams et al. 2008; Scholl et al. 2009; Kohler et al. 2010).

General Remarks and Future Direction

In this thesis I aimed to better explain the social behaviors of bacteria and gain a more detailed understanding of how their social dynamics in contrived experimental settings can give insight to their behavior in more natural environments. This opens potential research avenues to apply social behaviors to studies in natural populations and to extend such research to pathogenic bacteria as a means of developing novel treatments for bacterial infections.

Unlike environmental wildtype isolates, *P.aeruginosa* pathogens that chronically infect the CF lung are characterized by a loss of social traits or reduced secretion of public goods such as quorum sensing molecules, virulence factors, flagellar motility, toxins, biofilm formation and siderophore production (De Vos et al. 2001; Lee et al. 2005; Smith et al. 2006; D'argenio et al. 2007; Hogardt et al. 2007; Bragonzi et al. 2009; Winstanley and Fothergill 2009; Doring et al. 2011; Mowat et al. 2011; Jiricny et al. 2014). Loss of these traits is usually explained by adaptation to the CF lung environment and the selective pressures of antibiotics (Smith et al. 2006; Hogardt et al. 2007). However, as observed in many experimental studies, the emergence of cheats and competition between cooperators and cheats can explain loss of social traits (Griffin et al. 2004; Jiricny et al. 2014). More research is required to test the emergence of cheats in the CF lung; however it is difficult to determine the selection pressures *in vivo* and, therefore, to mimic the lung environment in the lab.

With more thorough and longitudinal sampling of *P.aeruginosa* isolates from patients it will be easier to observe and track evolutionary changes in the bacteria's social behaviours and long-term strain dynamics over the course of infection. Ideally, to test

for the emergence of cheats in the CF lung, it would be best to focus on a public good that is known to be under positive selection, such that expression of that social trait is required for bacterial survival. One such trait is antibiotic resistance. Patients with *P.aeruginosa* infections undergo rigorous antibiotic treatments, which inevitably select for antibiotic resistance. However, various antibiotic resistance mechanisms exist for different classes of drugs— some can protect neighbouring cells while others are strictly private, protecting only the focal cell (Fig.1). For example, resistance using an efflux pump protects only the focal cell, transporting the antibiotic into and out of the cell intact. However, resistance by production of intracellular enzymes (aminoglycosides) or secretion of enzymes (β -lactamases) (Ciofu et al. 2000) protect the focal cell but also reduce the concentration of antibiotics in the vicinity, extending the benefit of the enzyme production to neighbouring cells in a cooperative manner. Therefore, the latter resistance mechanism could be susceptible to exploitation by cheats that do not invest in enzyme production but are protected by the producer cells (Dugatkin et al. 2005a; Dugatkin et al. 2005b; Dugatkin et al. 2008). The relative indirect fitness effects conferred by the three different resistance mechanism have never been tested.

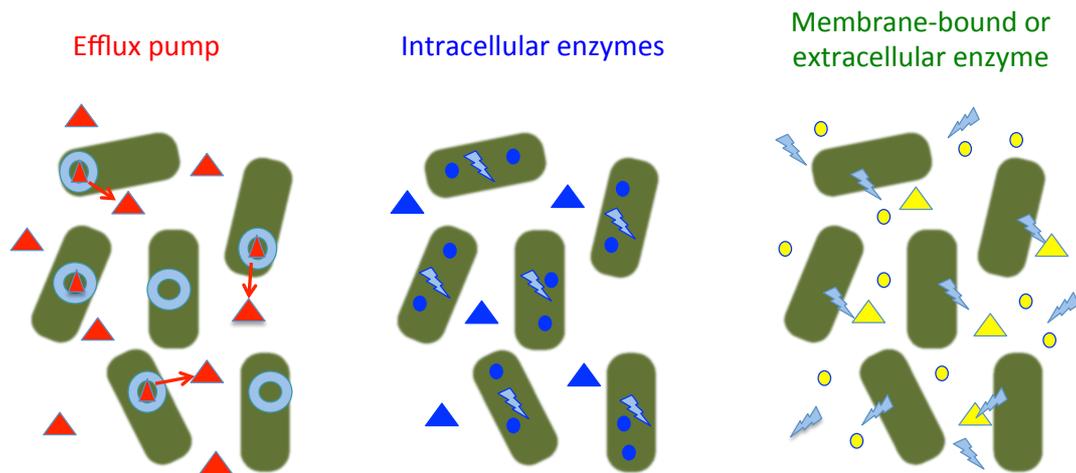


Fig. 1 Antibiotic resistance mechanism. Bacterial cells resist antibiotics like fluoroquinolones using efflux pumps (left panel). Here, only the focal cell is protected, and the antibiotic remains intact in the environment. Resistance to antibiotics like aminoglycosides requires the production of intracellular enzymes (centre): the focal cell degrades the antibiotic internally to protect itself, and in doing so reduces the concentration in the external environment to the benefit of neighbouring cells. Resistance to β -lactam antibiotics (right) relies on membrane-bound or secreted enzymes that externally degrade the antibiotic, and can therefore act as a public good in *P.aeruginosa*.

To test whether β -lactamase production is cooperative and to what extent its indirect fitness consequences affect resistance patterns in infections, I would use a collection of clinical CF isolates longitudinally sampled from long-term infections of multiple patients. First I would use nitrocefin to assay all isolates for β -lactamase production and compare patterns within and among patients. Then I would confirm whether increased resistance to β -lactam antibiotics is correlated with higher β -lactamase production to control for any effect of resistance by efflux pumps. I would also quantify β -lactamase production in all isolates when induced by antibiotic pressure, because production is facultative and is adjusted in response to the presence of antibiotics. I would finally test to what extent resistance by β -lactamase production is

cooperative and therefore exploitable by putative cheats by competing clinical isolates that show low and high productions.

To determine whether indirect fitness effects of resistance mechanisms affect strain resistance over the long-term infections I would experimentally test how resistance responds to selection. I would test the indirect fitness effects based on the type of resistance mechanisms and the costs associated with them. Information on patient treatment regimes and genome sequence of the isolates can be used to predict the response to antibiotic selection and analyse the genetic basis for resistance over the course of infection.

To conclude, this thesis highlights the impact social behaviours have in determining the fitness of microbes, particular *P.aeruginosa*, and the key role such behaviours could play in driving the evolution of strains in natural settings. However, a better understanding of what the selective pressures are *in vivo*, such as the CF lung, is necessary to: *i)* experimentally set up those conditions in the lab to determine what forces are driving strain behaviour; and *ii)* to determine to what extent the observed behavior can be attributed to competitive strain dynamics.

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Appendix

Chapters from this thesis and other research studies I contributed to that have been published are presented in their corresponding journal paper format.