Dynamics of Interbacterial Cooperation and Cheating

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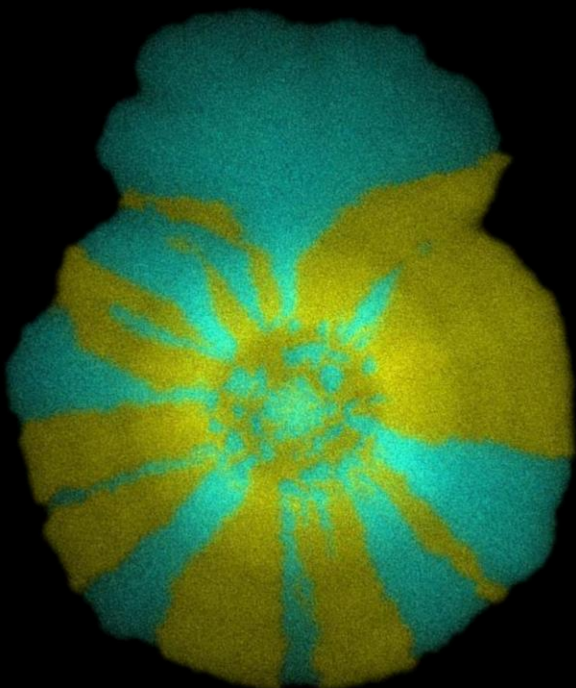

Dissertation presented to obtain the Ph.D degree in Evolutionary Biology

Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

Oeiras,
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UNIVERSIDADE NOVA DE LISBOA

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ITQB-UNL | Av. da República, 2780-157 Oeiras, Portugal
Tel (+351) 214 469 100 | Fax (+351) 214 411 277

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From left to right: Rui Oliveira (member of the jury), Rolf Kümmerli (member of the jury), Isabel Gordo (thesis co-supervisor), Özhan Özkaya, Karina B. Xavier (thesis supervisor), Adriano Henriques (member of the jury), Cecília Arraiano (president of the jury), Francisco Dionísio (member of the jury).

To my family and friends

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Bu kadar uzağa ve bu kadar uzun zamanlığına gitmeme rağmen, bu doktora için bana cesaret veren, beni hiç bir zaman yalnız bırakmayan, benim her türlü halime sabreden, hayattaki en kıymetli varlıklarımdan, Ece, senin varlığın bile benim için yeterince büyük bir şükran kaynağı; iyi ki varsın. Beni bugünlere getiren, ne kadar uzakta olsam da aslında hep yanımda, attığım her adımda, aldığım her nefeste benimle olan, anneme, babama, babaanneme, ve çok değerli, bir tanecik kardeşim Özge'ye ne kadar teşekkür etsem azdır. Uzakta olsam da beni asla yalnız bırakmayan, Ümmühan teyzeme de her şey için teşekkür ederim. Son olarak, doktora mezuniyetimi göremeden vefat eden, biricik dedem, rahmetli Kemal Arıkan'a bana geçen tüm emekleri için teşekkür ederim.

Summary

Bacterial communities face multiple environmental constraints in their environments. One of the most prevalent ways that bacteria overcome these constraints is the production of public goods. By definition, public goods are compounds that generate benefit for the entire population, producer and other individuals alike. Non-producers of public goods can avoid the cost of production of the public goods but can still benefit from them. When mixed with producers, the energy that non-producers save from not producing the public goods allows them to grow at higher rates than the producers. This can cause the non-producer of the public goods to behave as cheaters, increase in frequency in the overall population and eventually diminish the cooperation and thus the production of the public goods. The lack of public goods production can lead to the drastic reduction of the carrying capacity of the overall population. This phenomenon is defined as the tragedy of the commons in evolutionary biology.

Bacteria are a great model to investigate the cooperator/cheater dynamics. Previous studies have focused on single trait-single constraint scenarios, demonstrating that bacterial mutants can act as cheaters and showed various mechanisms to avoid cheating and maintaining cooperation. This thesis concentrates on the characterization of the cheating phenomenon by quantifying the magnitude of the relative fitness of the cheaters under various biotic and abiotic conditions. Towards this goal, a comparative approach was used to determine the similarities and differences between two different and independent social traits, production of iron-siderophore pyoverdine and quorum- sensing regulated elastase.

As important as understanding the dynamics of “single trait-single constraint” settings is, they are unlikely to occur in nature. In “multiple traits-

multiple constraint" settings, a cheater for one trait can potentially be a cooperator for another. In that scenario, the interactions among players might affect their social roles, leading to complex dynamics different from those generated in single trait-single constraint settings. In order to explore this possibility, this work presents a novel tri-partite co-culture system involving three social actors under two different environmental constraints. The actors are wild-type *Pseudomonas aeruginosa* (full cooperator) and two social mutants (cheaters/partial cooperators): *lasR* (quorum sensing mutant unable to produce and secrete the public good called elastase) and *pvdS* (unable to produce and secrete the iron-siderophore pyoverdine). These two mutants are typically used individually in countless sociomicrobiology studies and, additionally, are recurrently isolated in *P. aeruginosa* chronic infections associated with cystic fibrosis. The environmental constraints are the presence of casein as the sole carbon source (whose digestion requires elastase) and iron limitation (which requires pyoverdine production). This two traits-two constraints system allowed describing the existence of strong context dependent ecological interactions between these mutants in orthogonal social traits, which can induce or prevent a drastic collapse of the population. The findings presented here (**Chapter II**) demonstrate that by having more than one environmental constraint and more than one social mutant, cheating of one of the social mutants can be averted simply by the interaction with the other mutant. This thesis also provides experimental evidence for how quorum sensing mechanism can prevent the extinction of cooperation.

To investigate if these observations apply beyond the specific social players and constraints studied here experimentally; this thesis presents a mathematical model and determines the universal factors governing these social interactions (**Chapter III**). By using a simple model, end result of the competition (increase of cheater frequency and an eventual fixation), onset

and intensity of the tragedy of the commons (defined as the drop in the mean fitness of the population) due to cheater invasion can be predicted in a one trait-one constraint system. Furthermore, we describe a model to simulate quorum sensing regulation of a public good trait, and show how this mechanism can prevent fixation of the cheater mutants and thus full extinction of cooperation. Finally, the mathematical models in this chapter demonstrate that social dynamics of various strains in the long term composition of population in ‘multiple traits-multiple constraints’ systems are determined by the differences between the costs of the social traits involved, whereas their benefits only affect the mean fitness of the population. The model also predicts the scenarios for which coexistence of multiple social mutants is possible.

Overall, the work presented in this thesis provides experimental data and theoretical basis that support a dynamic view of cooperation and cheating that is affected by the cost spent for the cooperative behavior and the mechanisms that control the cost and benefits of the public goods. Also, this work shows that the bacterial cooperation/cheating dynamics are dependent on the genotypes and constraints present in the environment, oscillating from pure competition to a possible division of labor. Moreover, this study reveals the difference between the costs of the traits as the main factor determining social dynamics in complex populations under multiple constraints. This work provides a theoretical framework for the development of new approaches to treat infections involving social mutants, such as cystic fibrosis, by manipulating social interactions among pathogens.

Sumário

As comunidades bacterianas enfrentam múltiplas limitações ambientais nos seus habitats. Uma das estratégias mais comuns que as bactérias usam para ultrapassar estas limitações é a produção de bens comuns. Bens comuns são produtos externos que são secretados para fora das células, ficando acessíveis tanto a produtores como a não produtores. As bactérias que não produzem bens comuns conseguem evitar o seu custo de produção e ainda assim beneficiar deles. A energia economizada por não produzirem os bens comuns permite-lhes que tenham taxas de crescimento mais altas que os produtores. Isto leva a que os não produtores de bens comuns se comportem como “cheaters”, aumentando a sua frequência na população e, eventualmente, diminuindo a cooperação e consequente produção de bens comuns. A falta de produção de bens comuns pode levar à redução drástica da capacidade de suporte de toda a população. Este fenómeno é definido em biologia evolutiva como “Tragédia dos Comuns”.

As bactérias são um bom modelo de estudo de dinâmicas cooperador/”cheater”. Estudos anteriores focaram-se num cenário de limitação de um único recurso, demonstrando que bactérias mutantes podem agir como “cheaters” e mostrando vários mecanismos para evitar o “cheating” e manter a cooperação. Esta tese centra-se na caracterização do fenómeno de “cheating” focando-se na quantificação da sua magnitude quando sob diferentes condições bióticas e abióticas. Para isso foi usada uma abordagem comparativa focada nas semelhanças e diferenças entre dois recursos sociais independentes, produção de ferro-sideróferos de pioverdina e regulação de elastase por quórum sensing.

Apesar de ser importante compreender a dinâmica na limitação de um único recurso, este cenário é pouco provável de ocorrer na natureza. Num cenário de limitação de múltiplos recursos, um “cheater” para um recurso pode potencialmente ser um cooperador para outro recurso. As interações neste cenário podem afectar as funções sociais dos intervenientes, desencadeando dinâmicas complexas diferentes das geradas num cenário de limitação de um único recurso.

Para explorar esta possibilidade, este trabalho apresenta um sistema de co-cultura tripartido envolvendo três intervenientes sociais em dois ambientes com limitações. Os intervenientes são *Pseudomonas aeruginosa* tipo selvagem (cooperador completo) e dois mutantes sociais (“cheaters”/cooperadores parciais): *lasR* (mutante de quórum sensing incapaz de produzir elastase) e *pvdS* (incapaz de produzir o sideróforo de pioverdina). Estes dois mutantes são tipicamente usados individualmente em inúmeros estudos de sociomicrobiologia e, além disso, são recorrentemente isolados em infecções crónicas de *P. aeruginosa* associadas a fibrose cística. As limitações ambientais são a presença de caseína como única fonte de carbono (que requere elastase para a sua digestão) e a limitação de ferro (que requere a produção de pioverdina). Este sistema de limitação destes dois recursos permite descrever a existência de uma forte dependência de interações ecológicas entre estes mutantes com recursos sociais ortogonais, induzindo ou prevenindo um drástico colapso da população. As descobertas aqui descritas demonstram que a competição entre “cheaters” com recursos ortogonais pode prevenir o colapso da população que seria causado por um dos intervenientes (**Capítulo II**). Esta tese também providencia evidências experimentais demonstrando como o mecanismo de quórum sensing pode prevenir a extinção de cooperação.

Para investigar se estas observações se aplicam para além dos intervenientes sociais e limitações testadas experimentalmente, esta tese apresenta um modelo matemático e estabelece factores universais que determinam estas interações sociais (**Capítulo III**). Usando um modelo simples, o resultado da competição (aumento da frequência do "cheater" e eventual fixação), início e intensidade da "tragédia dos comuns" (definida como a queda na média de "fitness" da população) devido à invasão do "cheater" pode ser prognosticado num sistema de uma limitação, Além disso descrevemos um modelo que simula a regulação por quórum sensing de um bem comum, mostrando como este mecanismo pode evitar a fixação de mutantes "cheater" e, consequentemente, a total extinção da cooperação. O nosso modelo mostra que a dinâmica social em sistemas limitantes em múltiplos recursos é determinada pelas diferenças entre os custos do recurso social implicado, enquanto os seus benefícios apenas afectam a média de "fitness". Finalmente, os modelos matemáticos aqui apresentados demonstram que a dinâmica social de várias estirpes na composição populacional ao longo do tempo em cenários de limitações de múltiplos recursos é determinada pela diferença entre os custos dos factores envolvidos, enquanto que os seus benefícios apenas afectam a "fitness" média da população. Os modelos também prevêem cenários cuja coexistência de vários mutantes sociais é possível.

Em resumo, o trabalho apresentado nesta tese fornece evidências experimentais e base teorética que suportam uma teoria dinâmica de cooperação e "cheating", afectada pelo custo do comportamento cooperativo, e os mecanismos que controlam o custo e benefício dos bens comuns. Para além disso, este trabalho mostra que as dinâmicas de cooperação e "cheating" das bactérias são dependentes do genótipo e das limitações presentes no ambiente, oscilando entre a competição pura e uma possível divisão do trabalho. Este estudo revela também que a diferença dos custos de recursos é o principal factor determinante da dinâmica social em populações complexas sob múltiplas limitações. Este trabalho fornece também um enquadramento teórico do desenvolvimento de novas abordagens para tratar infecções que envolvem mutantes sociais, como a fibrose cística, através da manipulação das interacções sociais entre os patógenos.

Thesis Outline

The work presented here investigates the cooperation and cheating dynamics in *Pseudomonas aeruginosa* strain PA01, particularly upon two social traits that this bacterium possesses: production of iron-siderophore pyoverdine and quorum sensing regulated elastase. This thesis focuses specifically on the quantification of the magnitude of cheating and its relation to the onset of the tragedy of the commons, the regulatory and ecological mechanisms that affect the cooperator/cheater dynamics and the parameters that affect the competition among various social genotypes.

Chapter I contains a general introduction to sociomicrobiology, ranging from its general perspectives to more specific and recent findings in the field, lists the various ways that bacteria cooperate and cheat, and the mechanisms that are shown to prevent cheating in cooperative behaviors in microorganisms.

Chapter II focuses characterizing single cooperative traits in isolation, demonstrating the cheating behavior for each trait, identifying factors affecting the cheating magnitude. This chapter also identifies the effects of the competition among the cheaters of orthogonal social traits on one another and shows how these socio-ecological interactions or “cheating on cheaters” can help avoiding a drastic population collapse.

Chapter III investigates the main parameters that govern the cooperator/cheater dynamics in public goods competitions by using simple mathematical models and testing various single- or multiple-cheater competition scenarios including quorum sensing regulation that is experimentally shown to maintain cooperation.

Chapter IV summarizes the data shown in the previous chapters and speculates the findings achieved by analyzing them. This chapter also focuses on the directions that the future studies can take, and possible transitional work that can be done to achieve new therapeutic approaches to treat bacterial infections by exploiting bacterial cooperator/cheater dynamics.

List of Abbreviations

3-oxo-C12-HSL	N-3-oxododecanoyl-homoserine lactone
AHL	Acyl-homoserine lactone
CAA	Casamino acids
CAS	Casein
DNA	Deoxyribonucleic acid
ELA	Elastase
IQS	Integrative Quorum Sensing Signal (2-(2- hydroxyphenyl)-thiazole-4-carbaldehyde)
LB	Luria-Bertani Broth
OD	Optical density
PCR	Polymerase chain reaction
PG	Public goods
PBS	Phosphate buffer solution
PQS	Pseudomonas Quinolone Signal (2-heptyl-3-hydroxy-4-quinolone)
PVD	Pyoverdine
QS	Quorum sensing
RNA	Ribonucleic acid
Tf	Human apo-Transferrin
WT	Wild-type

List of Figures

Chapter I: General Introduction

Preliminary notes

Part of this chapter is included in the publication titled as "Maintenance of cooperation for single and multiple social traits" which is a review paper that is under revision for Journal of Bacteriology.

Introduction to Sociomicrobiology

Social Evolution Theory

Living beings reproduce and thus, multiply (Cleland and Chyba, 2002; Luisi, 1998; Oparin, 1961). By multiplication, the interactions among them become inevitable (Foster, 2009, 2010; Lehmann and Keller, 2006; West et al., 2006). During asexual reproduction, after each cell division, the daughter cells (at the least, initially) will be in physical proximity, and each division increases the chance of interactions among individual cells due to multiplicity. In sexual reproduction, the interaction between individuals is not only an outcome but also *a priori* requirement during this process of reproduction (Walker and Williams, 1976). Therefore, one can argue that to understand any biological process, interactions among individuals should always be taken into consideration, as no living being comes into existence and dies, in absolute isolation. One can even argue that life in total isolation is not possible, thus being social to a certain degree is inevitable. In this context, we can define sociality as the degree of interactions between individuals (Fisher et al., 2013; Garcia and De Monte, 2013; Walker and Williams, 1976; Wilson, 1975). Physical conditions of the environment where organisms live can dictate the level of sociality. For example, the physical proximity between individuals is one of the key factors that can change the degree of the interactions between the individuals thus, their sociality (Abbot et al., 2011; Foster and Xavier, 2007; Wilson, 1975). Physical factors in the environment such as diffusion can drift the individuals apart and limit the degree of interactions between those individuals (Dobay et al., 2014; Kümmerli et al., 2009; Taylor et al., 2013).

When studying the interactions between two individuals uni-directionally, we can define the individual who is committing the act as the ‘actor’ and the individual who is receiving the effect of the act as the ‘recipient’ (West et al., 2007b). Thus we can define this kind of interaction between individuals as a social behavior which applies fitness consequences on both the actor and the recipient (Sachs et al., 2004; West et al., 2007b). These consequences can vary depending on the fitness effects they pose on the individuals (Figure 1). When the fitness effect of the interaction is positive on both the actor and the recipient, this social behavior can be classified as mutual benefit (Waite and Shou, 2012; West et al., 2007a). When the social behavior poses a positive effect on the actor and a negative effect on the recipient, it can be sorted as selfishness (Krupp, 2013; West et al., 2007b). If the consequence of the social behavior is negative on the fitness of the actor but positive on the fitness of the recipient, these kinds of social behaviors are considered to be altruistic (Abbot et al., 2011; Van Dyken and Wade, 2012a, 2012b; Kreft, 2004; Lehmann and Keller, 2006; West and Gardner, 2010; West et al., 2007a). Finally, if the fitness of both the actor and the recipient are affected negatively by the social behavior, it can be defined as spiteful behavior (Foster et al., 2001; Gardner et al., 2004; Inglis et al., 2009; West and Gardner, 2010).

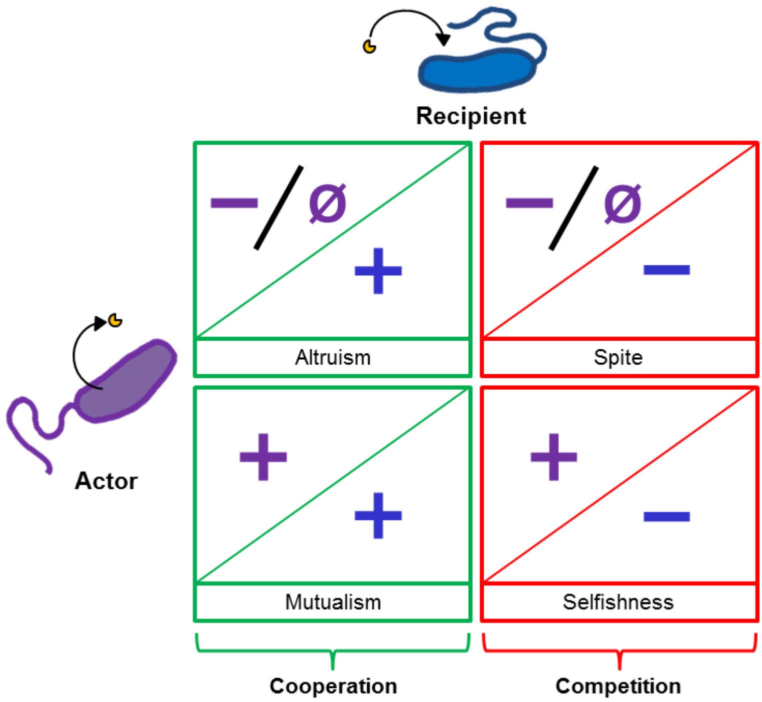


Figure 1. Classification of social behaviors depending on the fitness effects of an action on the actor and the recipient

Behaviors that have positive fitness effect on the recipient and negative or null effect on the actor is defined as 'altruism', If the behavior has positive fitness outcomes for both the actor and the recipient can be defined as 'mutualism'. Mutualism and altruism are considered to be cooperative behaviors. When a behavior has a negative fitness effect on the recipient and negative or null fitness effect on the actor it can be classified as 'spite'. Behaviors that have a positive fitness effect on the actor but a negative fitness effect on the recipient can be defined as a 'selfish behavior' or 'selfishness'. Spite and selfishness can be grouped as competitive behaviors.

Cooperation and cheating

1. Cooperation

Cooperation is a social behavior that has a positive outcome on the fitness of the recipient regardless of the fitness change of the actor (Crespi, 2001; Foster and Xavier, 2007; Griffin et al., 2004; Lehmann and Keller, 2006). Thus, both mutualistic and altruistic behaviors can be considered as cooperative. Any interaction which is costly to the actor, even if it is causing a positive effect on the recipient's fitness is difficult to explain in the context of the Darwinian evolution which predicts the survival of the individual that produces the highest numbers of progeny, which places individuals at the focus of selection (Foster, 2010; Hamilton, 1964a). Therefore, one expects that cooperative behaviors, which have a cost on the actor, should also have an indirect positive benefit on the cooperator which should overcome the cost spent; if not, altruistic behaviors should not be selected via natural selection. On the contrary, they are observed in all levels of life in nature (Allen and Nowak, 2013; Harrison, 2013; West et al., 2006, 2007a, 2007b).

Although Darwin also touched this problem in his book, Origin of Species, as the selection may be applied to the family as well as the individual (Darwin, 1859), William D. Hamilton laid the mathematical explanation of evolution and maintenance of cooperation (Hamilton, 1964a, 1964b). He argued that if the selective pressure is on the group of individuals which are related, depending on the proximity of their relatedness, the cost of the social behavior on the actor, and the benefit of it on the recipient, the altruistic behavior could be favored by natural selection (Hamilton, 1964a, 1964b). Hamilton explained that the overall fitness of an individual cannot be explained only by the behaviors that have direct fitness benefits on the individual who perform the behavior such as the number of progeny that the individual produces. The indirect fitness benefits that the actor of the altruistic behavior gets by performing this social behavior and providing fitness benefits to its relatives, the individuals to which it has a genetic similarity also have to be added to the overall fitness of that individual. Thus Hamilton argued that the direct and indirect fitness of an organism sums up to its "inclusive fitness" (Abbot et al., 2011; Foster et al., 2006; Gardner et al., 2009, 2014; Lehmann and Keller, 2006; West and Gardner, 2013) (Figure 2). He argued that natural selection operates on the inclusive fitness which is the sum of reproductive success of the individual (direct fitness) and its effects on increasing the reproductive success of its relatives (indirect fitness).

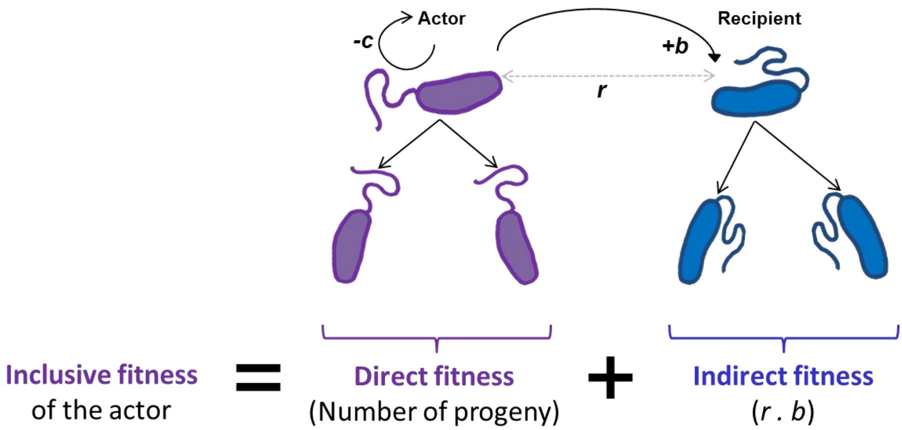


Figure 2. Inclusive fitness theory

The inclusive fitness of an individual is the sum of its direct fitness, the number of its progeny, and its indirect fitness, the fitness benefit (*b*) that the individual inflicts on the individuals that are in close genetic relation to itself (*r*) with a cost on its own direct fitness (*c*).

Hamilton proposed mathematical models to justify the conditions when a cooperative behavior can be selected. Derived from Hamilton's mathematical models and thus, called after him, 'Hamilton's rule' suggests that when $r\, b > c$, where r is the genetic relatedness between the actor and the recipient of the cooperative act and b is the benefit of the recipient from this act while *c* is the cost that actor pays to perform this act (Hamilton, 1964a, 1964b). As the rule suggests the indirect fitness component that is the term (*r b*), the benefit that the actor provides to its relative, in terms of the degree of the relatedness of the recipient to the actor, has to be greater than the cost of this act to the direct fitness of the actor for this cooperative behavior to be favored by natural selection.

In agreement with Hamilton's rule, cooperation tends to occur between close relatives (Fisher et al., 2013; Gilbert et al., 2007; Krupp et al., 2012; West et al., 2007a). Closer relatives share more recent common ancestors and thus more common genes. Genes that are expressed by an individual that increase the reproductive success of a related individual, or in other words a *kin*, can be selected. Since the related individual is likely to carry the same gene, the frequency of that gene in the gene pool can increase. This type of selection is referred as 'kin selection' (Damore et al., 2012; Gardner et al., 2009, 2011; Smith, 1964).

However, evidence of cooperation has been seen in unicellular organisms in planktonic forms where the interactions and exchange of exoproducts are relatively difficult due to diffusion, or in other occasions such as biofilms where cells get together as communities, and cooperating for certain traits, and superorganisms that are made of individuals which are highly similar in genetic background live as one united community ($0<r<1$), to multicellular organisms where cells with identical genetic code exist and operate together as one individual ($r=1$). Therefore, it is clear that cooperation is manifested in many levels of life as a spectrum.

2. Cheating

According to Hamilton's rule, cooperative behavior requires a cost to be paid by the actor of the behavior on the direct fitness of the actor (Frank, 1998; West et al., 2007b). This depends on the excludability of the benefit that is normally directed to the recipient, a close relative of the cooperative actor. However, the act itself can be vulnerable to exploitation. This exploitation can occur when individuals that are not contributing to the cooperative behavior can benefit from the cooperative action (Ghoul et al., 2014a; West et al., 2007b), especially when these individuals are not significantly related to the cooperator but still benefit from the action. In such scenario (r b) value would be smaller than the c value, therefore one would expect that such cooperative act will not be favored by natural selection. These organisms, which can benefit from a cooperative behavior without contributing to it (or contributing relatively less than the cooperators), are called cheaters (Frank, 2010; Ghoul et al., 2014a; West et al., 2006, 2007b). Cheaters can be individuals from the same species as the cooperators or members of entirely different species as long as they can benefit from the cooperative behavior.

In other words, cheaters are individuals, in an environment where cooperative behavior is selected, which can gain benefits from the cooperative behavior without cooperating themselves or are contributing to the cooperation less than the rest of the individuals (Ghoul et al., 2014a). When the cooperators are absent, the fitness of the cheaters should be lower than the situation where they are with cooperators. Not paying the cost but still benefiting from the cooperative behavior gives cheaters a relative fitness advantage over the cooperators (Figure 3). Therefore, (when the concentration of the public good correlates to the fitness of the population) cheaters have a frequency dependent fitness advantage as they have a fitness advantage when rare but a lower advantage if not enough cooperators are present to support the cooperative behavior (Ross-Gillespie et al., 2007; Wilder et al., 2011). Thus, as the fitness of the cheaters is higher when rare, it is long argued that the selective pressures for cheaters to increase in frequency should diminish the cooperative behavior (Stewart and Plotkin, 2014; Yurtsev et al., 2013). Therefore, for cooperative behaviors to be stable, it has been proposed that mechanisms to prevent cheating also exist (Bruger and Waters, 2015). To study the problems of how cooperation can be sustained in a population dominated by non-cooperators, and once established, how cooperation can be maintained against possible exploitations by cheaters various models were proposed. More recently and increasingly popularly, microorganisms are used as models to study these questions (Brown and Buckling, 2008; Foster; Parsek and Greenberg, 2005; West et al., 2007a; Xavier, 2016).

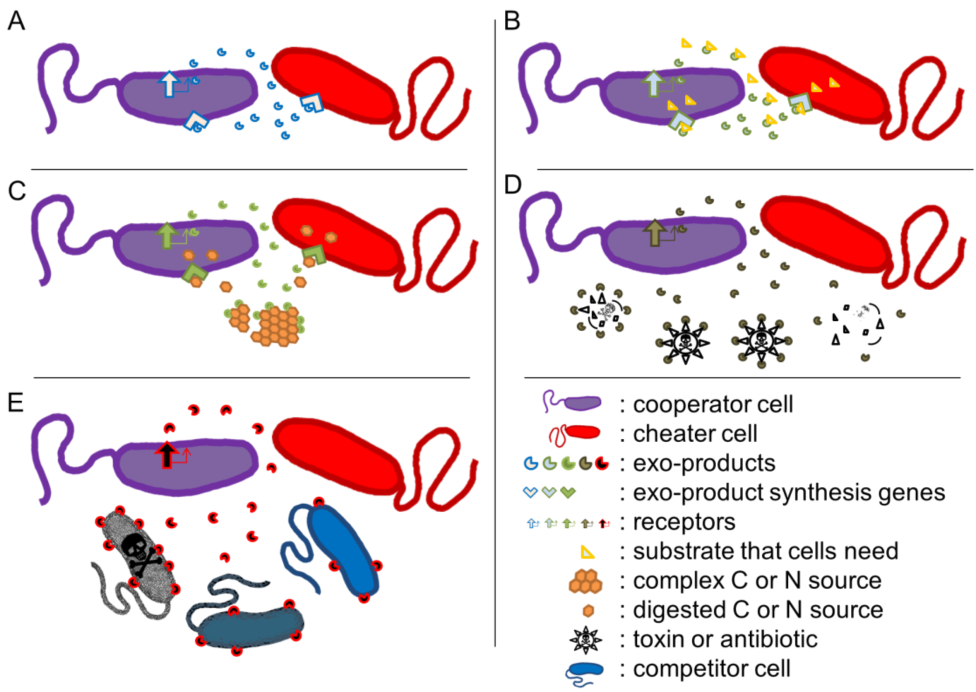


Figure 3. Bacterial cooperation and cheating

(A) Cooperators are able to synthesize and secrete public goods molecules. Both cooperator and cheater cells have access to these molecules since they both have the receptors. This direct cheating can be seen in cheating for QS signaling molecules or for rhamnolipids or in biofilms (Rumbaugh et al., 2009; Wilder et al., 2011; Xavier et al., 2011).

(B) Cooperators can synthesize and secrete public goods molecules which bind and bring other molecules to the cell. These molecules are generally called as scavenging molecules such as iron siderophores (e.g. pyoverdine, pyochelin). A mutant that avoids the cost of synthesis and secretion but still possesses the receptor can behave as a cheater in this example (Dumas et al., 2013; Kümmerli et al., 2015; Ross-Gillespie et al., 2015; Zhang and Rainey, 2013).

(C) Cooperators produce molecules which can cleave/digest other molecules. Both cooperators and cheaters can access the digested and smaller molecules which are available for intake. A good example of this system is the digestion of casein by elastase (Eldar, 2011; Stintzi et al., 1998; Wang et al., 2015; Wilder et al., 2011).

(D) Cooperators produce molecules to detoxify the environment or breakdown the antibiotics (e.g. catalase production to decompose hydrogen peroxide or beta lactamase secretion to get resistance to beta lactam antibiotics). Cheaters do not pay the cost of the production of such molecules, however, benefit from the detoxified environment or the breakdown of antibiotics simply by being there (Morris et al., 2014; Yurtsev et al., 2013).

(E) Cooperators produce toxins to kill cells of other competitor species. This makes the niche available for the cooperators and cheaters alike. Thus cheaters benefit from the toxin secretion of the cooperators, just by being there without paying the cost of production of such molecules (Cezairliyan et al., 2013; Foster, 2010; Helaine et al., 2014; Hosseinidoust et al., 2013; Inglis et al., 2009; Li et al., 2014).

Social dynamics of microorganisms

Studying social behavior in living systems, experimentally, poses various difficulties generally caused by the complexity of these systems. Establishing which behaviors are cooperative, which individuals are the cooperators, which are cheaters, which genes control which behaviors, and which environmental context favor which cooperative behavior is rather complicated in many animal, plant, or fungus models.

Studies on microbes, on the other hand, have shown that microbes also possess a high variety of cooperative behaviors which make them behave as multicellular-like communities rather than individual cells as un-related organisms (Foster, 2010; West et al., 2006). It is now well accepted that microbes generally cooperate via secretion of extracellular products which function as public goods that are available to the whole community (Popat et al., 2016). These public goods can vary in structure and function (Figure 3). Secretion of extracellular surfactants to reach further carbon and nitrogen sources (Xavier and Kim, 2011), siderophores to scavenge iron from the environment (Brockhurst et al., 2008; Dumas and Kümmerli, 2012; Dumas et al., 2013; Imperi et al., 2009; Kümmerli et al., 2009; Visca et al., 2007), extracellular proteases that digest complex carbon sources to ingestible smaller particles (Wang et al., 2015; Wilder et al., 2011), signaling molecules to regulate community level gene regulations (Popat et al., 2008, 2012; Rumbaugh et al., 2012a), various molecules involved in the formation of biofilms (Crusz et al., 2012; Mitri et al., 2011; Xavier and Foster, 2007; Xavier et al., 2009), toxins to kill competitors (Foster, 2010; Inglis et al., 2009; Mitri et al., 2013), detoxifying agents to ameliorate the habitat (Morris et al., 2014; Yurtsev et al., 2013) and even light production to establish a mutualistic relationship with a multicellular host (Clevenger and Fast, 2012; Foster, 2010; Milton, 2006; Sachs et al., 2004) are some of the social behaviors discovered and often studied in microbes (Figure 3).

All these cooperative behaviors are studied extensively to understand how these interactions have evolved and how they are maintained despite the potential presence of cheaters. Identification of the genes controlling the production of these public goods in microbes enabled the construction of mutants in these traits (Schuster et al., 2010; Tanouchi et al., 2012; Zhang and Rainey, 2007). Mutants constructed to over-produce, to under-produce, to produce but not to benefit (receptor mutants – *losers*), or to benefit but not to produce (synthesis mutants – *cheaters*) have been used to address these questions (Table 1).

Table 1. Roles that can be seen in social behaviors in bacteria

Cooperators pay the cost of the social trait and reap the benefits. Cheaters avoid the cost of the social trait, however, can gain the benefits if there are public goods produced by others. Losers, although not seen in nature can be engineered to pay the cost of the public goods like the cooperators, however, cannot access its benefits. Loners are the strains that are independent of the social trait that the other strains are competing for (Inglis et al., 2016; Pacheco et al., 2015). Thus they either pay no cost or gain no benefit from that public good, (*) or they produce some alternative public good only they can benefit.

Social behavior	Cost	benefit
Cooperator	paid	gained
Cheater	not paid	gained
Loser	paid	not gained
Loner	not paid*	not gained*

Tragedy of the commons in microorganisms

A cheater mutant in a population of cooperator individuals, by avoiding the cost of public good production, will have a relative fitness advantage over the cooperators. This advantage is greater when the cheaters are at low frequency since there will be more cooperators to cheat upon. Presumably, cheaters can use the energy that they save from not contributing to the cooperation in the binary division and thus have a higher growth rate than the cooperators (Ghoul et al., 2014a). Given time, several cell divisions after, this difference in growth rate results in an increase in the frequencies of the cheater compared to the frequency of the cooperator (Van Dyken and Wade, 2012a). It has been frequently hypothesized that if cheaters reach frequency the threshold where the proportion of cooperators in the population is not enough to sustain the entire population, a decrease in the growth capacity of the community will be observed because of the environmental pressures which are no longer being compensated by the cooperative act. In principle, this decrease can lead to levels of growth yield similar to that of a cheater growing in a monoculture, in the absence of cooperators (Hardin, 1968; MacLean, 2008; Maclean and Gudelj, 2006; Stewart and Plotkin, 2014). This phenomenon of cheater takeover followed by the collapse of cooperation and the reduction of growth capacity is called the tragedy of the commons, a term first coined by the biologist Garret Hardin, then used heavily in economics and in the recent years in evolutionary biology (Hardin, 1968). Garret Hardin defined the tragedy of the commons as the scenario in which each individual maximize their own direct fitness in competition for a limited resource, which is accessible by all individuals, and as a result of this process, overall fitness of the population decreases due to the ultimate reduction of the resource (Hardin, 1968, 1994).

The tragedy of the commons generally refers to a fitness decrease in the overall population due to overexploitation of a common (natural) resource which is extrinsic to the individuals that exploit it. These commons are considered to be non-excludable and the access to them is rivalrous for the competing individuals (Figure 4). When there is a compound produced by certain individuals in the population that can provide benefit to whole population (and there is no rivalry for this production), these intrinsic compounds or in other words, 'social' public goods, are also non-excludable, and thus, can be exploited by the individuals that do not contribute to the production of these public goods or contribute relatively less than the others (Rankin et al., 2007) (Figure 4). This is known as the public goods dilemma (Dionisio and Gordo, 2006). In biological systems, such interactions among producers and non-producers benefiting from the same public goods can lead to eventual domination of the non-producers, and thus depletion of the social public good. With the lack of these public goods, the fitness of the whole population can decrease, and this decrease can cause the numbers of this population to shrink or completely disappear, similarly to the tragedy of the commons scenarios. However, while the fitness of the whole population decreases by the overexploitation of an extrinsic compound in the case of the tragedy of the commons; in the case of the public goods dilemma, it depends on a compound that is intrinsic to the competing individuals.

It has been shown that the public goods dilemma and the tragedy of the commons can occur simultaneously in the biological systems (as in this thesis) (Dionisio and Gordo, 2006; West and Buckling, 2003). This situation occurs when bacteria produce public goods (e.g. elastase, pyoverdine), and this production benefits the whole population non-excludably and the action of this production is non-rivalrous (as in public goods dilemma), however, the consequent effect of these public goods can behave as a non-excludable but rivalrous commons (e.g. digested carbon source via the proteolytic activity of elastase or pyoverdine bound iron molecules) (Figure 4), and thus, lead to a tragedy of the commons (Dionisio and Gordo, 2006).

In this thesis, we will refer to the population collapse (reduction in the numbers of individuals in a population) that is seen in bacterial populations due to exploitation of bacterial exoproducts as the tragedy of the commons as it was referred in studies which used similar setting to ours (Asfahl et al., 2015; Dandekar et al., 2012; Wang et al., 2015). Although similarities can be found between this definition of the tragedy of the commons and the phenomenon commonly referred as 'Darwinian extinction' or 'evolutionary suicide' in which adaptations lead to the extinction of the population (J. Rankin and López-Sepulcre, 2005; Rankin et al., 2007; Webb, 2003), we highlight that the tragedy of the commons, by addressing fitness decreases caused by the exploitation of the public goods (or the consequent effects of these public goods), better explains the loss of fitness observed in the bacterial population upon invasion of non-producers.

The tragedy of the commons is demonstrated *in vitro* bacterial cultures under specific abiotic constraints (Asfahl et al., 2015; Dandekar et al., 2012; Wang et al., 2015). Although often theorized (Dionisio and Gordo, 2006; MacLean, 2008), the tragedy of the commons, or in other words population collapse due to cheater spread, is hard to be observed in natural settings (due to difficulties in sampling). Although it is documented that cheaters can arise and spread in natural populations, population collapse due to this has not been observed (Andersen et al., 2015). This poses the question what are the mechanisms that can prevent this phenomenon from happening in nature. Cooperation and the genes controlling cooperative behaviors such as public goods production in bacteria are well documented (West et al., 2007a). The sole existence of these behaviors shows that they are favored and selected by natural selection (Hamilton, 1964a, 1964b). It also indicates that there must be mechanisms to protect cooperation from cheater invasions (Bruger and Waters, 2015).

Garret Hardin argues that there are two main mechanisms that can prevent the tragedy of the commons from happening, privatization and regulation (Hardin, 1968, 1994). As discussed, human societies generally use both solutions to deal with the problem of managing the public goods. Societies which tried either only privatization (e.g. capitalism) or only the strict regulation of public property via policing (e.g. communism) did not function optimally. Most of the modern societies use both solutions to regulate different public goods. While some public goods can be privatized, such as land by putting a fence around it, some simply cannot be privatized such as clean air since the atmosphere is not excludable, in other words, it is near impossible to put a fence around it. Thus countries police each other to restrict their pollutions (Figure 4).

The categorization of goods in Figure 4 can also be applied into microbial compounds. As humans, bacteria also compete for goods, or in other words compounds that increase their fitness in given environments. Most of the studies on bacterial social behaviors (as in this thesis) focus on the cooperation via secreted products or the outcomes of these compounds. In general, these products are not fully ‘public’ goods as the bacteria have to compete to have access to these goods as their concentrations limited to the availability of the producers. This shifts bacterial exoproducts more to the ‘common’ goods. Although some bacterial cooperative behaviors can be defined as public goods as described in Figure 4 such as light production in the light organ of *Euphrymna scolopes* via *Vibrio fischeri* (Engebrecht et al., 1983; Miyashiro and Ruby, 2012; Sachs et al., 2004), other bacterial cooperative behaviors such as pyoverdine production fits more in the common goods definition. Depending on the environment, being more or less viscous, pyoverdine can be more public or private, respectively (Kümmerli et al., 2009). Thus, these observations show that depending on the molecule (or the outcome of a behavior such as bioluminescence) or the environment that bacteria occupy can place these compounds in a continuum in the chart that can be seen in Figure 4.

Recent studies provide strong evidence that microorganisms also adopt similar strategies to avoid the tragedy of the commons (Asfahl et al., 2015; Dandekar et al., 2012; Wang et al., 2015). Some strategies directly involve the privatization of the public goods, and thus prevent a total cheater takeover (Craig Maclean and Brandon, 2008). On the other hand, as further discussed below, cooperation in bacteria can be policed either via rewarding or by punishing.

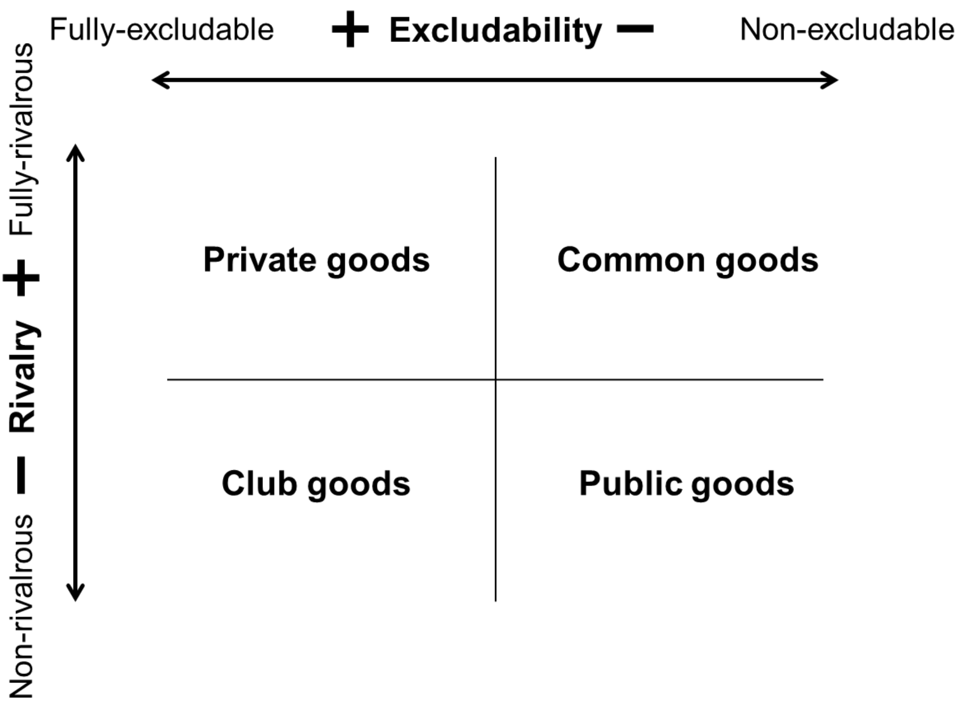


Figure 4. Classification of goods that have positive fitness effects on individuals

The goods are classified by their rivalry and their excludability. Public goods must be non-rivalrous and fully-excludable. If a molecule is privatized meaning that it is excluded from the recipients or become rivalrous then it becomes more like a private good (Dionisio and Gordo, 2006).

Mechanisms to prevent cheating in microbial populations

Studies on various public goods traits in microorganisms revealed multiple mechanisms to prevent cheating in those systems. Mainly five distinct mechanisms were proposed to explain the maintenance of cooperation and prevention of the tragedy of the commons in bacterial populations recently reviewed in Bruger and Waters, 2015:

1. Quorum Sensing

Bacteria secrete small molecules called autoinducers to the extracellular environment (Ng and Bassler, 2009; Pereira et al., 2013; Rumbaugh et al., 2009). The more cells in a given space, the higher the concentration of these molecules would get. Bacteria have specific receptors for detecting these autoinducers. When cells reach a density threshold, which is detected by a certain critical concentration of autoinducers, the bacterial cells in that given niche alter the expression of the genes regulated by the autoinducer in a synchronized manner (Li et al., 2014; West et al., 2012). This process is called quorum sensing since it enables bacteria to make ‘decisions’ depending on their ‘quorum’, or more precisely, according to their population density (Even-tov et al., 2016; Ng and Bassler, 2009) (Figure 5).

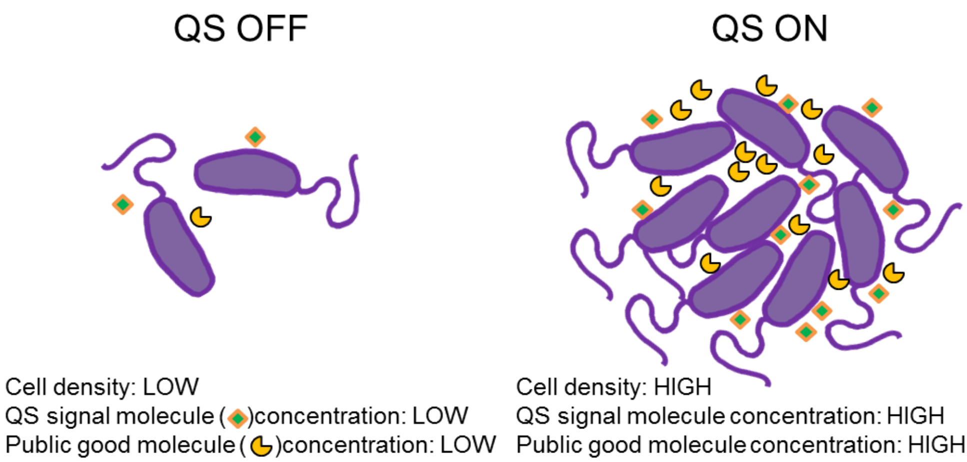


Figure 5. Quorum sensing and the regulation of public goods production

Quorum sensing is a mechanism that allows bacteria regulate certain genes via asserting their cell densities by secreting and detecting small signalling molecules called autoinducers. When the concentration of these molecules increases, the regulation of associated genes in the bacterial genome also alters. This allows bacteria to produce public goods only when the cell densities of the producers are high enough to get the benefits of this action directly

Very often the genes controlled by quorum sensing are genes involved in the production of public goods (Heilmann et al., 2015; Parsek and Greenberg, 2005; Whiteley et al., 1999). Bacteria secrete public goods to their extracellular medium as a response to environmental constraints (Bachmann et al., 2013; Van Dyken and Wade, 2012b; Foster, 2010; Frank, 2009). Often the concentration of the public goods is important for its efficacy. For example, the concentration of elastase, an enzyme required to digest complex protein sources such as casein into casamino acids, is an example of a public good where a certain concentration is required for efficient degradation of casein and the liberation of casamino acids to be used as carbon and nitrogen sources (Dandekar et al., 2012; Delden et al., 1998). Therefore, if the whole bacterial population secretes elastase, a high concertation of elastase as a pool can efficiently digest casein into casamino acids which will be available to all the cells in the environment (Heilmann et al., 2015).

Quorum sensing also makes the cooperative behavior, which it regulates, more favorable by natural selection since it increases the relatedness among the cooperative individuals by inducing the public good production when the producers are in high density and in close proximity (Rumbaugh et al., 2012b; Schluter et al., 2016). Cooperation, regulated by quorum sensing, is only induced when the cooperative individuals, which are in close proximity to each other, reach the quorum threshold. Therefore, the public goods produced by the cooperative individuals would mostly benefit themselves. Thus, it can be said that quorum sensing regulation of public goods actually can increase the excludability of these molecules by regulating the timing of the production (Gupta and Schuster, 2013; Schuster and Greenberg, 2006).

2. Privatization and metabolic constraint

Private goods are entities that the individuals produce as a response to their environment, to increase their fitness (Dandekar et al., 2012). However, in contrast to public goods, they are only accessible by the producers. Cheating is practically not possible on private goods production (Figure 4). In human societies, privatization is an ancient, well-documented and common practice to solve disputes over commons (Hardin, 1968, 1994). To prevent the tragedy of the commons from happening, people privatized commons as they simply put fences around them (Hardin, 1968). Thus, the regulation of that given commons would only give benefit to its owner. If the owners follow a detrimental policy to use their fenced land, they will be the only sufferer of the consequences and not the whole population.

Privatization of the public goods is well documented in yeast. Invertase is an enzyme of the budding yeast *Saccharomyces cerevisiae* that hydrolyses sucrose into glucose and fructose (Craig Maclean and Brandon, 2008; Gore et al., 2009; Koschwanez et al., 2013). About 99% of the fructose and glucose monosaccharides then diffuse to the environment and can be shared by the whole population (Doebeli and Hauert, 2005; Gore et al., 2009). Thus they generally behave as public goods. However, non-producers of this trait can be prevented from cheating properly on the producers since the invertase producers are able to have priority to access to these monosaccharides. About 1% of privatization of the monosaccharides is enough to avoid a cheater takeover and a possible tragedy of the commons and sustain a population which can contain both cooperators and cheaters (Naumov et al., 1996).

In bacterial populations, it was recently proposed that metabolic constraint is one of the mechanisms to avoid cheating and the tragedy of the commons by coupling the regulation of product goods production with private goods (Dandekar et al., 2012) (Figure 6). In *Pseudomonas aeruginosa*, LasIR quorum sensing circuit sits atop of several quorum sensing circuits which operate as serial systems. LasR, the master regulator of quorum sensing systems in this bacterium regulates the production of various public goods while regulating several private goods as well. One of the public goods that *lasR* regulates is LasB elastase (Dandekar et al., 2012). Elastase is secreted outside of the cell to cleave large casein molecules into smaller bits to make cells able to ingest them as carbon and nitrogen sources. When casein is the sole carbon source, *lasR* mutants behave as cheaters. However, *lasR* also controls the production of the enzyme to digest adenosine. Differently than elastase, the enzyme that digests adenosine is kept intracellularly thus; it is considered a private good (Dandekar et al., 2012). When casein and adenosine are the carbon sources in the environment, a *lasR* intact cell can digest both goods while in a monoculture of *lasR* mutants can digest neither. In mixture, *lasR* mutants can use the elastase and intake the digested casein molecules however these cannot digest adenosine, while strains with a functional LasR can digest both. The extra carbon source that the LsrR+ strain can access provides them with an extra benefit in competition with *lasR,* thus protecting the population of LasR+ cooperators from being invaded by *lasR* cheater mutants. Thus, coupling public and private goods can prevent the tragedy of the commons from happening in this scenario (Dandekar et al., 2012; Sanchez and Gore, 2013).

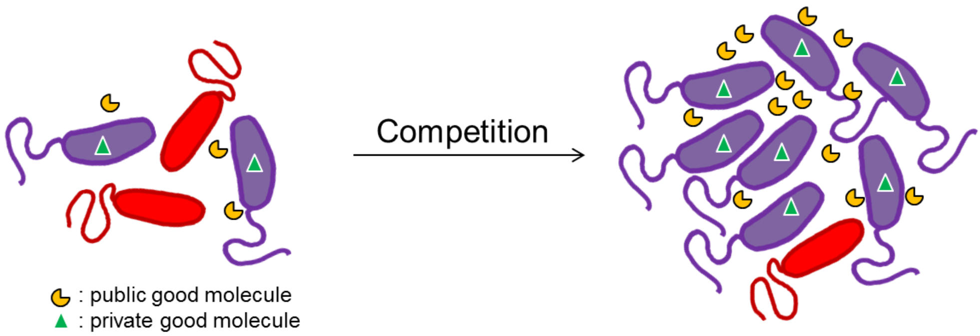


Figure 6. Metabolic constraint

When the same gene regulates a public good and an interlinked private good, the mutant of that gene loses the competition against the strain that has that gene functional in an environment where both public and private goods are needed to sustain metabolic functions.

3. Metabolic prudence

One of the mechanisms that has been proposed to prevent cheating in bacteria is called metabolic prudence (Xavier et al., 2011). To invade new niches, *Pseudomonas aeruginosa* is capable of producing rhamnolipids, a carbon-rich surface polymer, which enables bacteria to swarm into unpopulated areas. Xavier and colleagues showed that this bacterium only invests in rhamnolipid production in metabolically un-balanced conditions when carbon concentrations are higher in relation to nitrogen concentrations (Xavier et al., 2011). Bacteria require both carbon and nitrogen to divide and reproduce whereas the production of rhamnolipids requires carbon but not nitrogen. Thus, by investing in rhamnolipid production only when the C/N ratio does not favor cell division the cells minimize the cost of this public good. Additionally, under these conditions, there is no chance for nonproducers to reproduce with a higher growth rate than the producers. Therefore, when bacteria suffer from lack of nitrogen, they can induce rhamnolipid production and swarm away towards unpopulated areas which might have nitrogen and carbon at the same time. Once that happens, bacteria can start reproducing again. The way that *Pseudomonas aeruginosa* cooperates on this behavior indicates a facultative cooperative behavior (Figure 7).

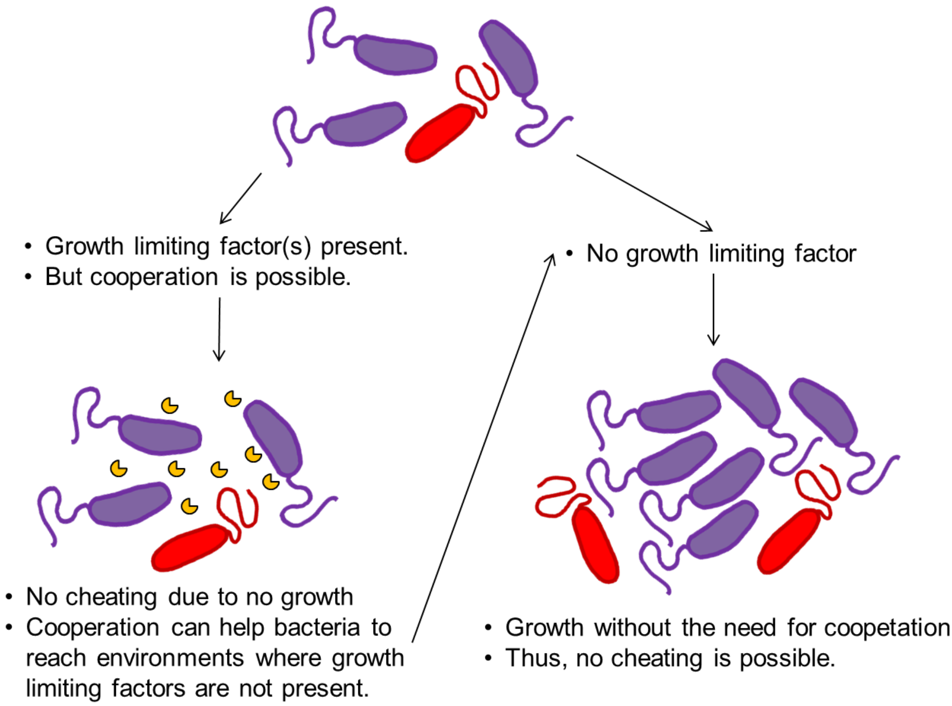


Figure 7. Metabolic prudence

Regulation of the production of public goods when there are molecules available for public good production but not for binary division

Although it is has been proposed that metabolic prudence can be ubiquitous in nature, the examples are limited to the one explained above. It is a mechanism that needs to be studied in various cooperative systems.

4. Policing

Policing is a ubiquitous mechanism among many eukaryotic organisms (Foster and Ratnieks, 2000, 2001b; Foster et al., 2002; Kümmerli, 2011; Lehmann and Keller, 2006; Rankin et al., 2007). It can be defined as an aggressive act that targets the cheating individuals to remove their fitness advantage that comes from their abuse of the cooperative behavior. It can operate either as harming the non-cooperative individual (punishing the cheaters) (Figure 8) or limiting their benefit from the cooperative behavior thus increasing the relative benefit that the cooperators gain from the cooperative behavior (rewarding the cooperator) (Figure 9). Policing is a well-documented mechanism among mammals such as in social primates, birds in their investment of the care of their offspring and the social insects for their collective scavenging behavior (Foster and Ratnieks, 2001a, 2000; Kümmerli, 2011; Manhes and Velicer, 2011).

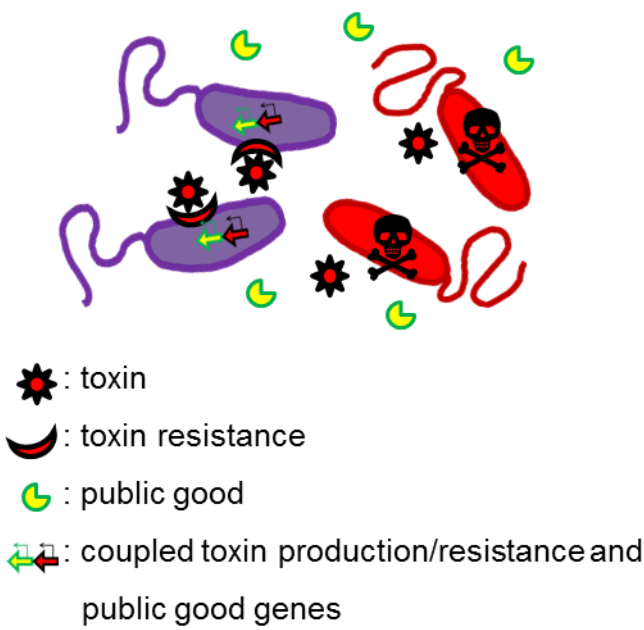


Figure 8. Policing via direct harm

When toxin secretion-toxin resistance system is coupled with a public good production, cheaters for the public good are directly harmed (and killed) by the toxins.

One common example of policing as a punishing/rewarding behavior is observed in bacteria belonging to *Rhizobia* genera which occupy the roots of plants and forms a mutual relationship (Sachs et al., 2004; Waite and Shou, 2012). Plants supply oxygen and various nutrients to the bacteria while bacteria fixe nitrogen that the plant cannot obtain by itself. Additionally, plants can sense the amount of the nitrogen from the bacteria and alter the levels of oxygen and nutrients in return. A similar policing behavior is observed between the bacterium *Vibrio fischeri* and their squid host (Milton, 2006; Sachs et al., 2004). While bacteria produce the bioluminescence that the squid uses for camouflage, the squid supplies the bacteria with nutrients.

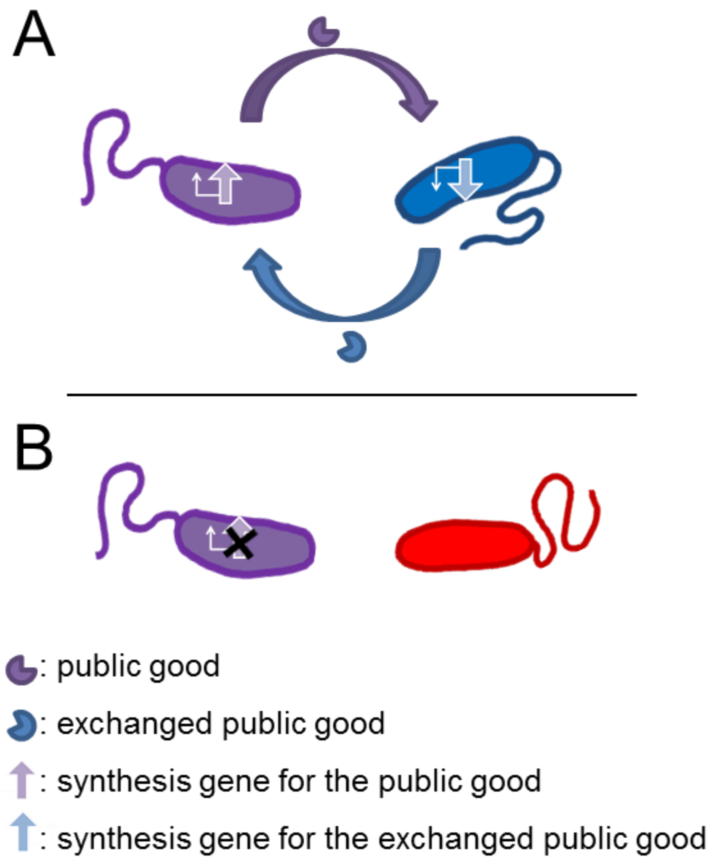


Figure 9. Policing via reciprocity

(A) When both cooperative partners (purple and blue cells) supply each other with the public goods they need, cooperation can be sustained.

(B) If one partner (red cell) does not supply the exchanged public good, the cooperator (purple cell) can avoid the public good production that it makes to reciprocate.

In *Pseudomonas aeruginosa*, cyanide production and resistance against it are coupled traits (Wang et al., 2015). And these traits are both regulated under the RhlIR system. RhlIR system is regulated by LasIR system. LasR, the master regulator of quorum sensing in *P. aeruginosa*, also controls several public goods genes, including LasB elastase production. When grown in casein medium, *lasR* mutants arise. However, by mutating in *lasR* gene, they cannot produce nor have resistance against cyanide. Thus the cooperators, *lasR* intact population, can have immunity over their own cyanide production, and also benefit from the casein digestion via elastase. By coupling cyanide immunity with a public good production polices the cooperation against cheater invasions (Wang et al., 2015).

5. Spatial structure and limited dispersal

Spatial structures such as biofilms can let related cells to stay in close proximity and thus increase relatedness in a given area. Increased relatedness, according to Hamilton’s rule, favors cooperation. Also, increased viscosity can limit the diffusion and dispersal of the related cells, and the public goods produced by them, thus increasing their benefit and in a way making the public good production more protected against possible cheaters (Krakauer and Pagel, 1995; Kreft, 2004; Kümmerli et al., 2014; Lion and Baalen, 2008; Nadell et al., 2010; Xavier et al., 2009).

The ways that bacteria can create and sustain its spatial structure can vary depending on the species. It is shown that antagonistic relations between different bacterial species can result in segregation. Segregation of different genotypes allows each genotype to interact with itself (Bucci et al., 2011). Therefore segregation via antagonistic relation can create niches with higher relatedness which favors cooperative behaviors. This can be summarized as competition paving the way of cooperation.

Biofilms are one of the most common ways that bacteria build a spatial structure (Figure 10). Extracellular lipids, polysaccharides, and nucleic acid molecules make up the biofilm matrix where cells can anchor and switch from a motile, planktonic phase to a sedentary phase (Xavier and Foster, 2007; Zhang et al., 2009). The biofilm matrix does not only influence diffusion of these static cells but also diffusion of the exoproducts secrete to the environment. Thus the molecules that are secreted generally stay around the cells that secrete them. Also, when a cell divides the sister cells tend to stay adherent rather than drifting apart (Dobay et al., 2014; Kümmerli et al., 2009; West et al., 2012). This way an exoproduct that is produced by one cell is more likely used by its closest relatives which are also more likely to be the producers of these exoproducts. Therefore, biofilms by providing a spatial structure can increase interactions among relatives and this favor maintenance of cooperation among bacteria. However, this positive effect of close relatives sticking together can be diminished with the negative effect of the increased competition among these close relatives (Taylor, 1992; West et al., 2002).

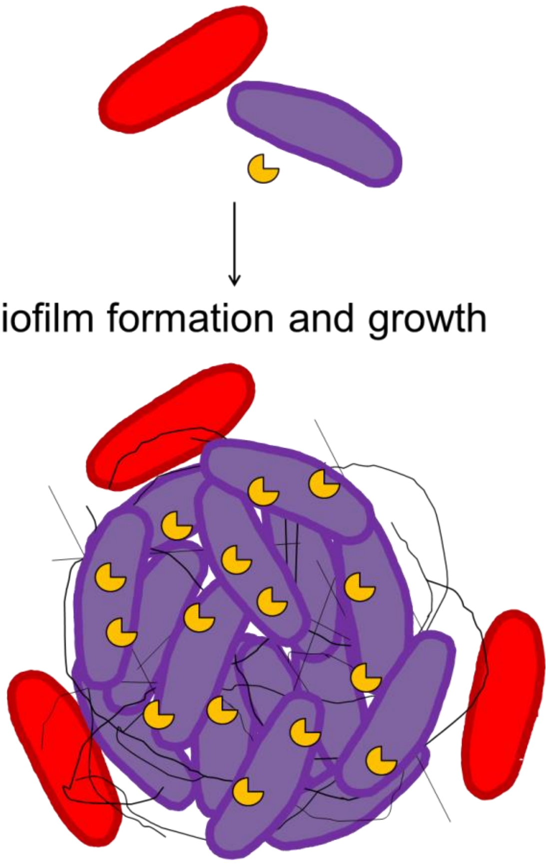


Figure 10. Biofilm formation and growth in a spatial structure

When the cooperators (purple cells) produce a biofilm and grow next to their closest kin, the polymers (black lines and curves) they secrete and bind them together and allow them to grow in close proximity also reduces the diffusion of not only the cells but also the other public good molecules (yellow pies). This way, the benefit of the public good production is mainly gained by its producers and not by the non-producer cells (red cells).

If a mutation appears in a gene that is responsible for the synthesis of an exoproduct of a cell in the biofilm, that cell can have an advantage when it is surrounded by the cooperator cells. However, the more they grow the less they can benefit from the exoproducts that are produced by the others since the cheater cells that are in the center of a cheater patch will receive less and less benefit from the exoproducts (Kreft, 2004; Li and Tian, 2012; Popat et al., 2012). Thus the spatial structure gives more benefit from the exoproduct to the producers than the cheaters. This asymmetry can reduce the fitness advantage of the cheaters that is gained by avoiding the cost to synthesize and secrete this exoproduct to a level that not producing the public good becomes non-advantageous and perhaps deleterious.

Pseudomonas aeruginosa as a model organism to study cooperation and cheating in bacterial populations

As microbes, and more specifically bacteria, have been proven to be useful model systems to study cooperation and cheating, many cooperative behaviors in bacteria have been documented (Damore et al., 2012; West et al., 2006, 2007a). *Pseudomonas aeruginosa* is a gram-negative bacterium that possesses many of the documented bacterial cooperative traits. *P. aeruginosa* can be found in various habitats such as soil and water as well as causing deadly infections in immunocompromised patients. This bacterium species has been shown to be a valuable model organism to study bacterial social evolution, cooperation/cheating dynamics, and the relation between bacterial cooperation and infections (Lutter et al., 2008; Stover et al., 2000; Tümmler et al., 2014).

P. aeruginosa can live on various surfaces and environments that overlap with human habitats. Culturing and maintaining *P. aeruginosa* populations in laboratory environments are easy and sustainable. *P. aeruginosa* is a fast growing strain which makes it a favorable species for the evolutionary adaptation experiments (Asfahl et al., 2015; Kümmerli et al., 2015; Marvig et al., 2014; Wang et al., 2015). Its genome is fully sequenced and this makes it possible to link certain social behaviors to certain genes and their regulation machineries (Balasubramanian and Mathee, 2009; Feinbaum et al., 2012; Stover et al., 2000; Wagner et al., 2003; Whiteley et al., 1999). Also, diagnosing the gene-behavior link leads the way to engineer mutant strains in this species, to conduct fully factorial studies.

P. aeruginosa possesses many of the documented bacterial social behaviors (Balasubramanian and Mathee, 2009; Feinbaum et al., 2012; Tümmler et al., 2014). It can secrete several iron-siderophore molecules such as pyoverdine and pyochelin which help bacteria scavenge iron when soluble iron concentrations are low in the environment (Imperi et al., 2009; Moon et al., 2008; Takase et al., 2000). *P. aeruginosa* has a very complex quorum sensing system which regulates many of its cooperative traits, one of which is the secretion of elastase which helps bacteria to cleave complex carbon structures into smaller pieces for the cellular intake (Jimenez et al., 2012; Wagner et al., 2003). *P. aeruginosa* is known for its ability to form robust biofilms which provide protection to the bacteria from antibiotics in the environment. It is shown that *P. aeruginosa* can secrete rhamnolipids to swim or swarm on the surfaces (Xavier et al., 2011). These secretions are also proven to be cooperative and prone to be cheated. *P. aeruginosa* can also secrete bacteriocins which allow the mechanisms of reciprocity and policing to be studied in this bacterial system (Inglis et al., 2009).

P. aeruginosa is also an opportunistic human pathogen that is the major cause of the nosocomial infections (Abdul Wahab et al., 2014; Hogardt and Heesemann, 2010; Lakshmana Gowda et al., 2013; Lutter et al., 2008; Takase et al., 2000; Wilder et al., 2009). It can lead to a high rate of mortality by causing infections in immunocompromised patients. It can commonly infect the wounds of the burn victims, and the lungs of cystic fibrosis patients (Haas et al., 1991; Mulcahy et al., 2014). *P. aeruginosa* being the most common bacterial species during the chronic phase of the cystic fibrosis infections became the focus of attention in microbiology (Hogardt and Heesemann, 2010; Marvig et al., 2013; Winstanley and Fothergill, 2009).

Commonly, in the adolescent ages of a CF patient, an environmental *P. aeruginosa* strain colonizes is the lungs of the CF patient. It was shown that the cooperative traits that *P. aeruginosa* possesses are vital for the colonization (Andersen et al., 2015; Rau et al., 2010; Smith et al., 2006a) (Figure 11). *P. aeruginosa* uses siderophores, proteases, surfactants, bacteriocins to carve itself a niche in the host tissue by competing with other invasive strains and also the host immune system elements. In this acute phase, the symptoms that are caused by the bacterial virulence factors are severe. If the patient survives with the help of antibiotic treatments, the infection proceeds to a chronic phase. Interestingly, *P. aeruginosa* samples that are isolated during this phase show a low virulence profile (Andersen et al., 2015; Jiricny et al., 2014). Various mutants of the cooperative traits are also prevalent in the isolate populations (Ciofu et al., 2010; Friman et al., 2013a; Lutter et al., 2008; Sommer et al., 2015). Mutations in quorum sensing and iron-scavenging mechanisms are especially common in this phase (Jiricny et al., 2014; Smith et al., 2006a; Wilder et al., 2009). These two systems, quorum sensing and iron-scavenging, are considered to be social traits (Jiricny et al., 2010; Kümmerli and Ross-Gillespie, 2013; Rumbaugh et al., 2009; Wang et al., 2015; Zhou et al., 2014). Quorum sensing controls various cooperative behaviors in *P. aeruginosa* and the mutation in its master regulator, *lasR*, is prevalent in most patients (Smith et al., 2006a). Similarly, *pvdS* mutants, mutants in the sigma factor of production of pyoverdine, the primary iron-siderophore of *P. aeruginosa* that are unable to induce pyoverdine production are also frequently isolated during this phase (Andersen et al., 2015; Kümmerli, 2015; Smith et al., 2006a). The mutants isolated from this phase have the characteristics of cheating behavior *in vitro*. Also, other studies showed that synthesis mutants of pyoverdine production, *pvdS*, of *P. aeruginosa* appear first. Then, the receptor mutants appear only after the pyoverdine molecule diminishes. Thus this order of appearance of synthesis and receptor mutations indicates that the selective force in CF for selecting these mutations is likely to be social (Andersen et al., 2015; Kümmerli, 2015).

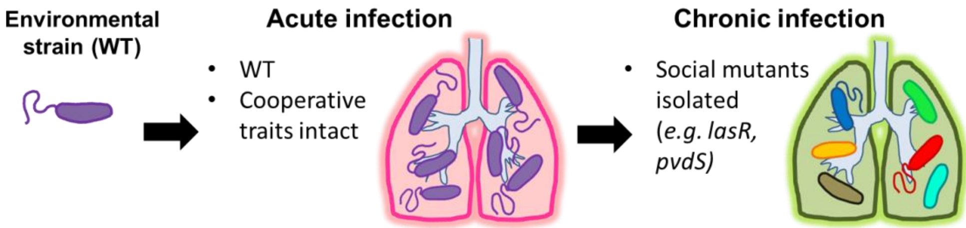


Figure 11. *P. aeruginosa* infection progression in CF lungs

Colonization initiated by a WT *P. aeruginosa* loses many of its cooperative traits throughout its adaptation in CF lungs. *lasR* and *pvdS* mutants are two of the mutants that are frequently isolated from the samples taken during the chronic phase of the infections.

Due to this link of infection progression and the bacterial social dynamics in this species, *P. aeruginosa* became one of the most well studied bacteria in sociomicrobiology. Currently, there are treatment approaches such as using engineered, cheating and antibiotic sensitive strains named 'Trojan strains' are being discussed (Brown et al., 2009). The expectation is that by using the competitive advantage of a cheating strain and after its fixation using the antibiotic therapy could one day be a viable treatment option. However, understanding the complex dynamics of the different mutants and strains during this bacterial infection is extremely important to develop safer and more effective treatments.

Chapter II: Cooperation, Cheating, and the Tragedy of the Commons

Preliminary notes

The author of the thesis participated in the planning and analysis of all the experiments presented in this chapter. The author executed all the experiments shown in this chapter. Part of this chapter is included in the publication titled as “Cheating on orthogonal social traits prevents the tragedy of the commons in *Pseudomonas aeruginosa*”. (Manuscript submitted to bioRxiv: http://biorxiv.org/content/early/2017/03/19/118240).

Abstract

Bacterial cooperation can be disrupted by non-producers, which can access public goods without paying their production cost. These cheaters can increase in frequency, exhausting the public goods and causing a population collapse. We investigated how interactions among cheaters in orthogonal social traits influence such collapse. We characterized the dynamics of *Pseudomonas aeruginosa* polymorphic populations under conditions where two social traits, production of iron-scavenging pyoverdine and quorum sensing regulated elastase, are necessary. We demonstrate that cheaters for either trait compete with both the wild type and each other and, since production of pyoverdine is costlier than elastase production, pyoverdine cheaters impair invasion by quorum sensing mutants, preventing the collapse caused by the latter.

Introduction

Although bacteria are unicellular organisms, they can engage in many group behaviors including biofilm formation, swarming motility, production and secretion of extracellular proteases and iron-chelating siderophores (Bruger and Waters, 2015; Foster, 2010; Parsek and Greenberg, 2005; Xavier, 2016). The collective production of costly, secreted compounds provides fitness benefit to the entire population and can be considered as cooperative behaviors. Cooperation is frequently under the threat of exploitation by cheaters: individuals that benefit from the cooperative action but contribute little or nothing at all to the production of the public goods. When mixed with cooperators, cheaters can increase in frequency and cause loss of cooperation by exhaustion of the public goods, leading to a collapse of the entire population, characterized by a strong decrease in the growth yield of the entire population (Rankin et al., 2007). This phenomenon, defined as the 'tragedy of the commons', was coined in economics (Hardin, 1968), but has been explored in ecology (Waite and Shou, 2012) and has also become a focus of attention in microbiology in the last decade (Asfahl et al., 2015; Dandekar et al., 2012; MacLean, 2008; Wang et al., 2015; West et al., 2006).

Several mechanisms have been proposed to explain how cooperative behaviors are still observed and maintained in microbial populations despite the emergence of cheaters. For instance, spatial structure and diffusion (Dobay et al., 2014; Drescher et al., 2014; Foster et al., 2004; Krakauer and Pagel, 1995; Kreft, 2004; Kümmerli et al., 2009; Lion and Baalen, 2008; Nadell et al., 2010; Persat et al., 2015; West et al., 2012), pleiotropy (Bachmann et al., 2013; Banin et al., 2005; Dandekar et al., 2012; Friman et al., 2013b; Harrison and Buckling, 2009; Ross-Gillespie et al., 2015; Sandoz et al., 2007; Wagner et al., 2003; Wilder et al., 2011), restricted migration (Kerr et al., 2006), social and non-social adaptations (Asfahl et al., 2015; Hammarlund et al., 2016; Kümmerli et al., 2015; Waite and Shou, 2012), policing mechanisms (Wang et al., 2015), molecular properties of public goods (Kümmerli and Brown, 2010), and metabolic strategies (Xavier et al., 2011) play significant roles in maintaining cooperation and avoiding the tragedy of the commons (Bruger and Waters, 2015). Importantly, despite all these mechanisms to inhibit cheaters' invasion, cheating behavior is still observed *in vitro* (Asfahl et al., 2015; Dandekar et al., 2012; Sandoz et al., 2007; Wang et al., 2015), *in vivo* (Czechowska et al., 2014; Rumbaugh et al., 2009), and in natural populations (Cordero and Polz, 2014; Katzianer et al., 2015; Winstanley et al., 2016).

Certain cheaters are also clinically relevant and are repeatedly isolated from the sputum samples of cystic fibrosis (CF) patients chronically infected with *Pseudomonas aeruginosa* (Andersen et al., 2015; Smith et al., 2006a; Sommer et al., 2015; Winstanley et al., 2016). CF is a genetic disorder which causes thickening of mucus in the lungs. Although initial acute infections are normally associated with colonization of the lungs by wild type (WT) *P. aeruginosa*, subsequent chronic infections consist of polymorphic populations which include mutants affected in social traits (Nguyen et al., 2014; Smith et al., 2006a; Sommer et al., 2015; Zhang and Rainey, 2013). Importantly, *in vitro* studies, which focused on one trait and one constraint at a time, demonstrated that invasion by a cheater leads to a tragedy of the commons (Asfahl et al., 2015; Dandekar et al., 2012; Wang et al., 2015). However, despite the prevalence of social cheaters in the CF lung population, population collapse due to the invasion of cheaters has not been described. Therefore, we reasoned that studying interactions among multiple social cheaters, simultaneously, under conditions where more than one social trait is required could provide new insights into social dynamics of *P. aeruginosa* populations in CF lungs and other environments. When more than one environmental constraint is present, the roles among different social mutants are likely to be more complex, since a cheater for one trait could potentially be a cooperator for another, making ‘cheater’ and ‘cooperator’ relative terms (Ghoul et al., 2014b; Zhang and Rainey, 2013). We hypothesize that in environments where multiple constraints require bacteria to express multiple cooperative traits simultaneously, competition among mutants in orthogonal social traits (traits that are not known to be functionally linked), could influence their co-existence and the magnitude of the collapse of the population. This possibility is further supported by recent theoretical and experimental studies showing that interactions between interlinked cooperative traits significantly affect the course of their evolution (Brown and Taylor, 2010; Mellbye and Schuster, 2014; Popat et al., 2017; Ross-Gillespie et al., 2015).

Here we examine the consequences of ecological interactions among social cheaters and the full cooperator in *P. aeruginosa* populations under conditions where two orthogonal cooperative traits are required.

Both *lasR* and *pvdS* mutants are used individually in a large number of sociomicrobiology studies (Cox and Adams, 1985; Dandekar et al., 2012; Griffin et al., 2004; Kümmerli and Brown, 2010; Lamont et al., 2002; Sandoz et al., 2007; Visca et al., 2007; De Vos et al., 2001) and are among the most common mutants recurrently isolated from the sputum samples of CF patients (Andersen et al., 2015; Smith et al., 2006a; Winstanley et al., 2016).

LasR is the master regulator of quorum sensing (QS) and controls the production of elastase (Figure 12). Production and extracellular secretion of elastase is essential for *P. aeruginosa* to digest complex sources of amino acids, such as casein, which serves as carbon and nitrogen source (Dandekar et al., 2012). Previous studies showed that *lasR* mutants grow poorly in media containing casein as the only carbon source, but increase in frequency when mixed with WT bacteria. This invasion of the mutant eventually leads to a collapse where the total cell numbers of the population are drastically reduced due to the depletion of producers of the essential public good (Asfahl et al., 2015; Dandekar et al., 2012; Wang et al., 2015).

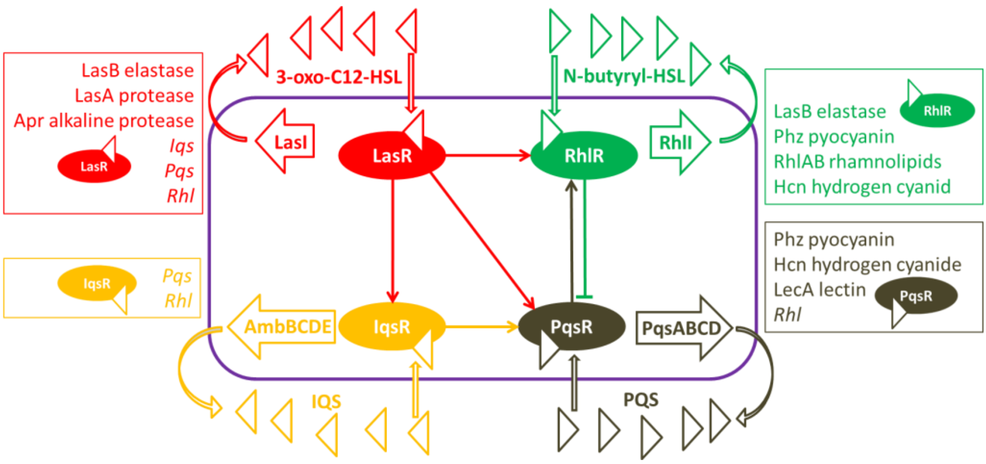


Figure 12. *Pseudomonas aeruginosa* quorum sensing circuits

There are four quorum sensing circuits identified in *P. aeruginosa*. They function in series, in which LasIR circuit sits atop. LasI synthesizes the signal molecule 3-oxo-C12-HSL which binds to LasR. This complex then regulates several genes including *lasB* (which codes for elastase) and the other three quorum sensing circuits in *P. aeruginosa,* RhlIR, PqsR, and IqsR. (Figure adapted from Lee and Zhang, 2014).

Similarly, production of pyoverdine is one of the most studied cooperative traits in bacteria (Cox and Adams, 1985; Griffin et al., 2004; Kümmerli and Brown, 2010; Lamont et al., 2002; Visca et al., 2007; De Vos et al., 2001). Although iron is one of the most abundant elements in nature, it is generally found in its insoluble ferric form (Fe^{3+}) in the aerobic environments. Since iron is vitally important in various reactions in the cell, the competition for iron is also severe in nature. In an infection, host would use their own mechanisms to limit the access to iron for bacteria by using transferrin and bacteria compete with that by using siderophores. In such iron-limited environments, pyoverdine is secreted by the *P. aeruginosa*, chelates iron from the environment and is subsequently retrieved, providing iron to the cell (Visca et al., 2007) (Figure 13). Mutants in pyoverdine synthesis (e.g. *pvdS*) do not pay the cost of its production but are still able to retrieve the iron-bound pyoverdine produced by others, gaining a fitness advantage and increasing in frequency in the population (Dumas and Kümmerli, 2012; Dumas et al., 2013; Kümmerli and Brown, 2010).

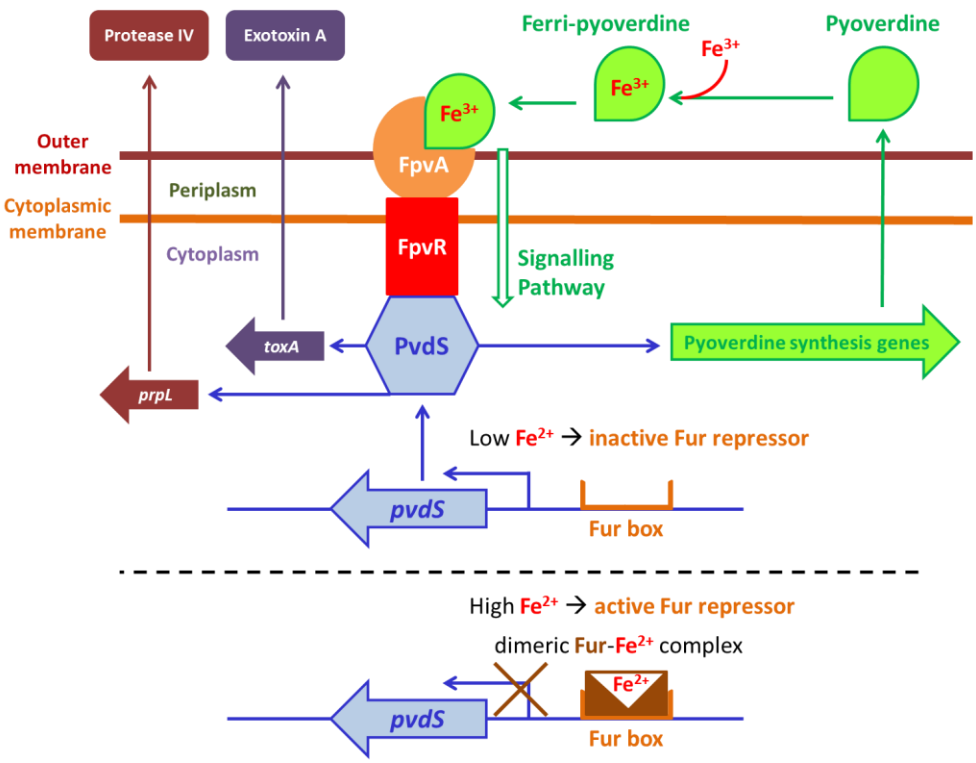


Figure 13. Pyoverdine synthesis and signalling pathway

When in high-iron conditions (when cells have enough soluble ferrous form of iron, Fe^{2+} generally stored as bacterioferritin protein), dimeric Fur-Fe^{2+} complex binds to Fur box on the promoter of *pvdS*, which represses σ-factor PvdS expression. When in low-iron condition (when bacteria lack ferrous iron), Fur repressor becomes inactive and *pvdS* is transcribed. Then σ-factor PvdS induces pyoverdine synthesis genes and thus pyoverdine molecule is produced and secreted. Pyoverdine molecules then scavenge the insoluble ferric form of iron, Fe^{3+} and form Ferri-pyoverdine complexes. Ferri-pyoverdine complexes bind to FpvA receptor and thus represses the anti-σ-factor FpvR, which would otherwise repress σ-factor PvdS. (Figure adapted from Lamont et al., 2002 and Leoni et al., 2000).

Regulation of elastase production via LasR and pyoverdine production via PvdS are not directly linked, and they are considered as orthogonal traits. However, LasR induces PQS production which has iron trapping ability that can decrease the available iron concentrations in the environment and indirectly induce the activity of PvdS (Popat et al., 2017). On the other hand, PvdS controls the production of protease IV which might serve a similar function as elastase, as both enzymes are known to digest elastin (Caballero et al., 2001; Smith et al., 2006b; Wilderman et al., 2001).

We followed the cheating behavior of a *lasR* knock-out (KO) mutant in environments where casein is the sole carbon source, and thus production of elastase is required. In addition to this ‘one constraint - one trait’ setting, we added another constraint (iron depletion) and another social player (a *pvdS* KO mutant) and studied the behavior of the population in a ‘two constraints - two traits’ setting. We quantified the cheating behavior of a *lasR* mutant in short and long-term competitions, in iron-supplied or iron-depleted casein media with or without the presence of a *pvdS* mutant. We found that the relative fitness of the *lasR* mutant is altered when the *pvdS* mutant is in the culture, but only when the *lasR* mutant produces pyoverdine. We next determined the long-term consequences of the interactions among the two mutants and the WT for the onset of the tragedy of the commons. Our results show that in the environment where the two cooperative traits are required, competition between the two mutants affects their dynamics, preventing the drastic population collapse otherwise caused by the domination of the *lasR* mutant.

Results

Effects of environmental constraints and population composition on cheating

We investigated the fitness of *lasR* and *pvdS* mutants, alone and in competition under different environmental conditions, to determine the effect of the interactions between different cooperative traits on the dynamics of the cheater frequency and on the overall fitness of the population. Both mutants grow as well as WT in media where neither elastase nor pyoverdine is required (i.e., an iron-supplied medium where casamino acids (CAA) serve as simple carbon and nitrogen source) (Figure 14A). However, when casein is the sole carbon source, *lasR* mutant has a lower growth than WT (Figure 14B) and in iron-depleted CAA medium, the growth yield of *pvdS* mutant is lower than that of WT and *lasR* mutant (Figure 14C).

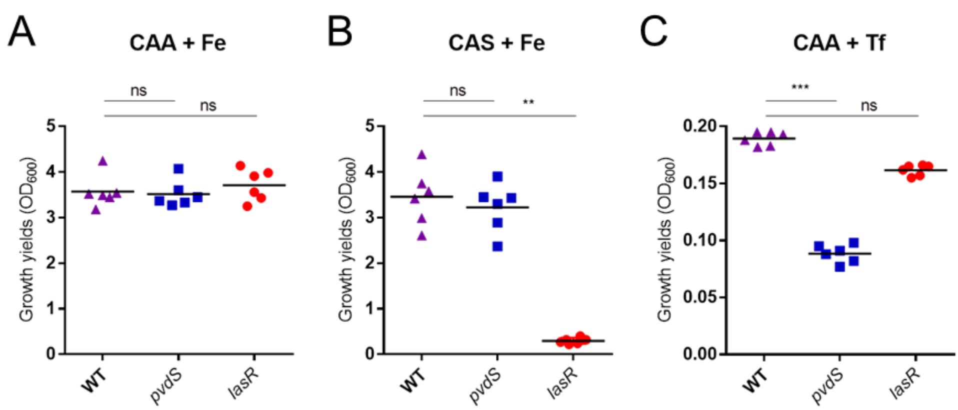


Figure 14. Growth yields (OD_{600}) of WT (purple triangles), *pvdS* (blue squares) and *lasR* (red circles) strains of *P. aeruginosa* monocultures after 48 hours of incubation in various media

(A) WT, *pvdS*, and *lasR* growth yields in iron-supplied casamino acids (CAA) medium (Kruskal-Wallis test with Dunn's correction, WT-*pvdS* ns=not significant P>0.9999, WT-*lasR* ns=not significant P>0.9999, N=6). **(B)** WT, *pvdS,* and *lasR* growth yields in iron-supplied casein medium (Kruskal-Wallis test with Dunn's correction, WT-*pvdS* ns=not significant P>0.9999, WT-*lasR* **=P=0.0034, N=6). **(C)** WT, *pvdS,* and *lasR* growth yields in iron-depleted CAA medium. (Kruskal-Wallis test with Dunn's correction, WT-*pvdS* ***=P=0.0002, WT-*lasR* ns=not significant P=0.1027, N=6).

Importantly, even though LasR regulates most of the quorum sensing genes in *P. aeruginosa*, the growth yield of *lasR* mutant was only affected significantly in media where elastase is required (and Figure 15). These data corroborate that there is no direct functional link between *lasR* and *pvdS* under the conditions tested (Figure 14A and B) (Lee and Zhang, 2014; Popat et al., 2017). Next, to obtain a condition where both constraints were present, we cultured these mutants in a medium with casein as the sole carbon source supplemented with transferrin to deplete iron (iron-depleted casein medium). Monocultures of both *lasR* and *pvdS* mutants have a lower growth yield than WT in this medium (Figure 15) because, under these conditions, elastase and pyoverdine are both required for growth. Importantly, the growth yield of *lasR* mutant is smaller than that of *pvdS* mutant.

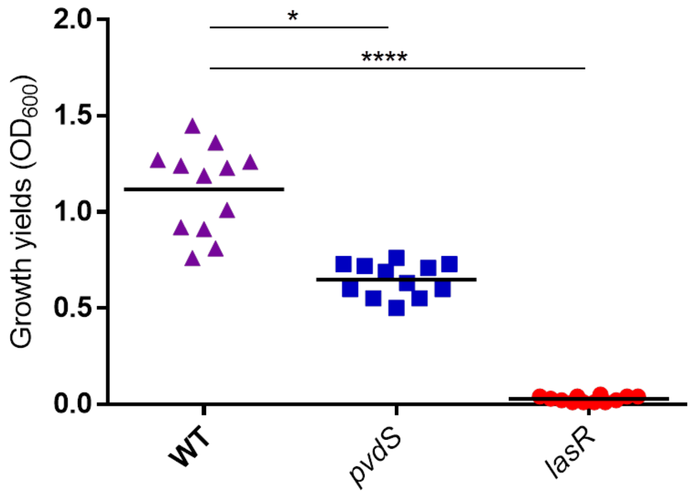


Figure 15. Growth yields of *P. aeruginosa* monocultures in iron-depleted casein medium

WT (purple triangles), *pvdS* (blue squares) and *lasR* (red circles) mutant strains grown alone (horizontal lines show means of each group, Kruskal-Wallis test with Dunn's correction, WT-*pvdS* *=P=0.011, WT-*lasR* ****=$P<10^{-3}$, N=12.)

We next determined the relative fitness of these two mutants in competition with WT. When there is no environmental constraint present, neither of the mutants shows any significant increase in frequency (Figure 16 A). However, when co-cultured with WT in iron-supplied casein medium, *lasR* mutant increases in frequency, demonstrating that it can act as cheater under these conditions (Figure 17A-left and Figure 16B). The introduction of the *pvdS* mutant in the WT:*lasR* co-cultures does not affect the cheating behavior of *lasR* mutant, since *lasR* can also increase in frequency in the triple co-culture (Figure 17A-right, and Figure 18A).

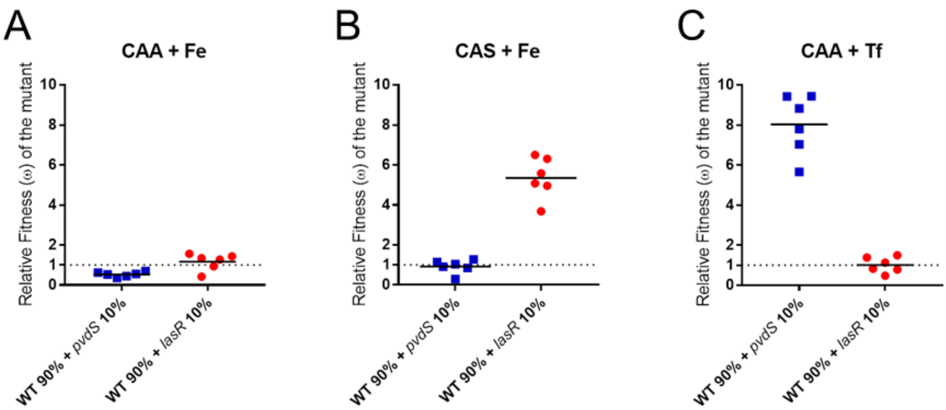


Figure 16. Relative fitness of the mutants in co-cultures with WT

Competitions with initial frequencies of 9:1 WT:*pvdS* (blue squares) or 9:1 WT:*lasR* (red circles) after 48 hours of incubation in various media. **(A)** Relative fitness of *pvdS* and *lasR* in iron-supplied CAA medium (N=6). **(B)** Relative fitness of *pvdS* and *lasR* in iron-supplied casein medium (N=6). **(C)** Relative fitness of *pvdS* and *lasR* in iron-depleted CAA medium (N=6). Dotted lines indicate no change in relative fitness (relative fitness=1).

The fact that *pvdS* mutant does not change the behavior of *lasR* mutant in the iron-supplied casein media is consistent with the fact that *pvdS* mutant does not increase in frequency, and thus it does not act as a cheater under these conditions (Figure 17B and Figure 18B). Then, we studied the behavior of these mutants in the medium with two constraints (iron-depleted casein medium). In this medium, *lasR* mutant again increases in frequency in the co-cultures with WT (Figure 17C-left). Importantly, the relative fitness of *lasR* in iron-depleted casein medium is smaller than the observed in the iron-supplied casein medium (Figure 17A-left). This is not due to a differential production of pyoverdine in *lasR* (Figure 19) but because, in iron-depleted casein medium, WT reaches a much smaller growth yield than in iron-supplied casein medium (Figure 15 and Figure 14B). In fact, when measured in units of cumulative numbers of cell divisions (CCD = final cell number – initial cell number; for more information see the Appendix) (Lee et al., 2011; Luria and Delbrück, 1943) the relative fitness per cell division of *lasR* is not significantly different in the two media ($7.27 \times 10^{-10} \pm 2.19 \times 10^{-10}$ versus $7.53 \times 10^{-10} \pm 1.20 \times 10^{-10}$ in iron-supplied and iron-depleted medium, respectively) and thus the relative fitness of the mutants in 48 hours (Figure 17) is higher in the iron-supplied medium, where the growth yield is also higher (Figure 15 and Figure 14B).

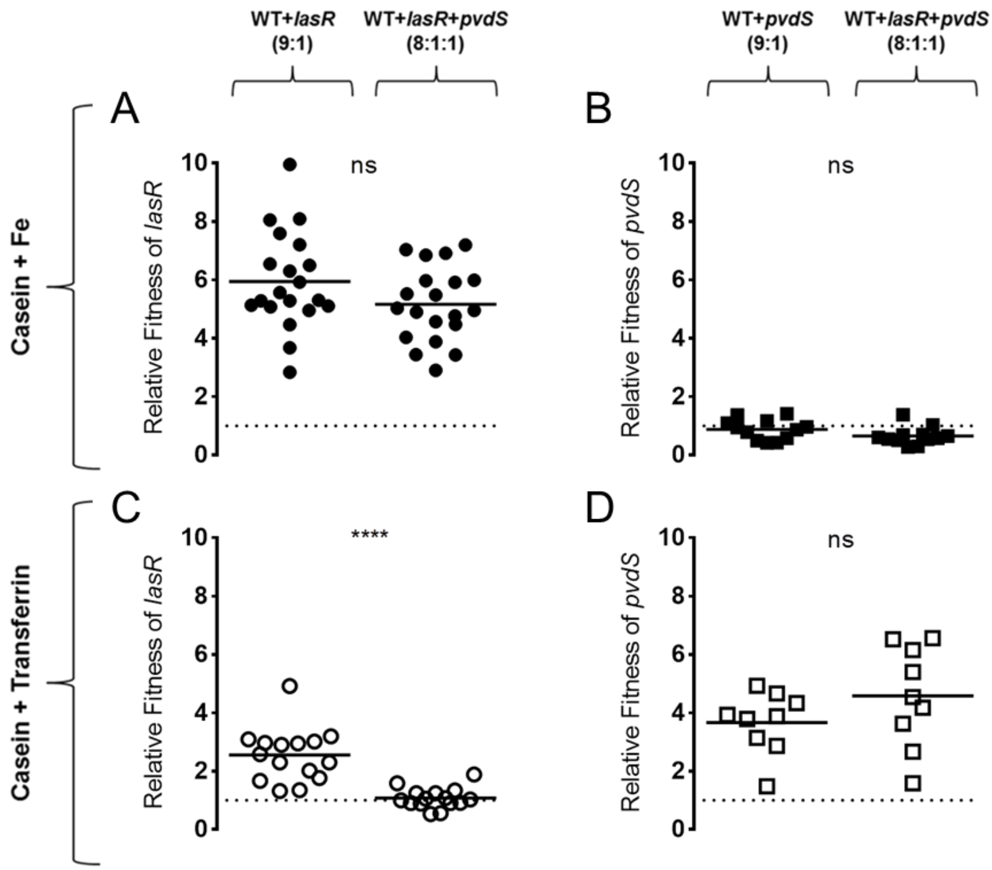


Figure 17. **Relative fitness of *lasR* or *pvdS* in iron-supplied or iron-depleted casein media in double or triple co-cultures**

(A) Relative fitness of *lasR* (circles) in co-culture with WT, or with WT and *pvdS* in iron-supplied casein media (ns=not significant, P=0.1207, N=20). **(B)**

Relative fitness of *pvdS* (squares) in co-culture with WT, or with WT and *lasR* in iron-supplied casein media (ns, P=0.1600, N=12). **(C)** and **(D)** same as in (A) and (B), respectively, but in iron-depleted casein media (****=$P<10^{-3}$, N=15 and ns, P=0.2581, N=9, for (C) and (D), respectively). Relative fitness of *lasR* or *pvdS* mutants is calculated in respect to all the other strains in the population. Relative fitness > 1 (above the dotted lines) indicate conditions where the frequency of the mutant increased in relation to the rest of the strains in the population during the competition. Initial ratios of the strains in each co-culture are 9:1 for WT:*lasR* and WT:*pvdS*, and 8:1:1 for WT:*pvdS*:*lasR*. Symbols indicate individual replicates and horizontal lines indicate the means of each group.

Interestingly, when *pvdS* mutant added to the competition in iron-depleted casein medium, it acts as a cheater in co-cultures with WT (Figure 17D-left), and in triple cultures with *lasR* and WT (Figure 17D-right and Figure 18D).

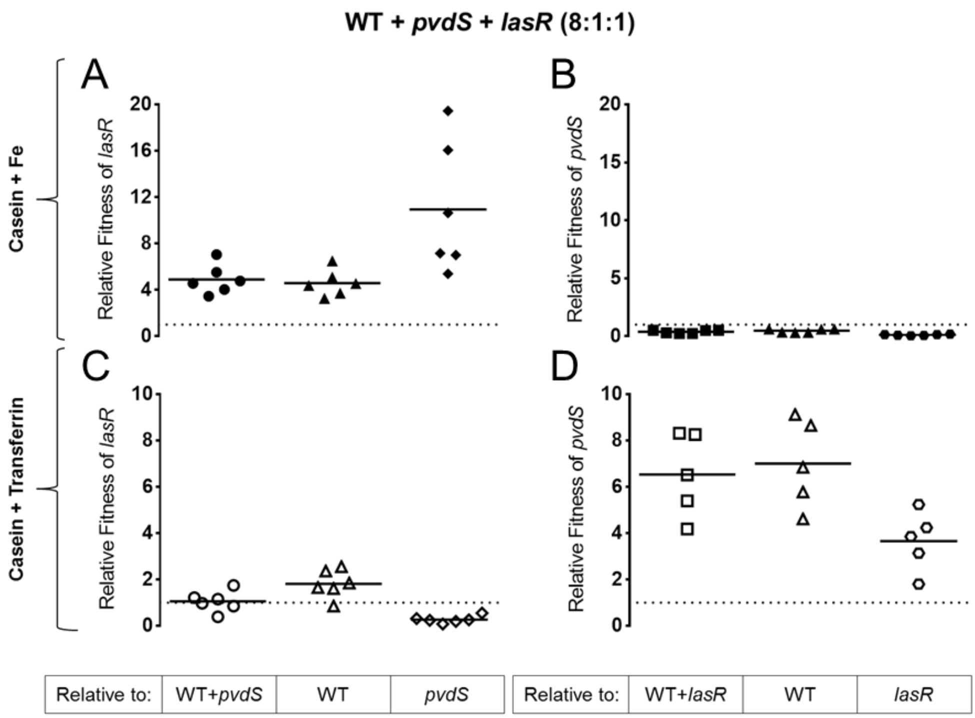
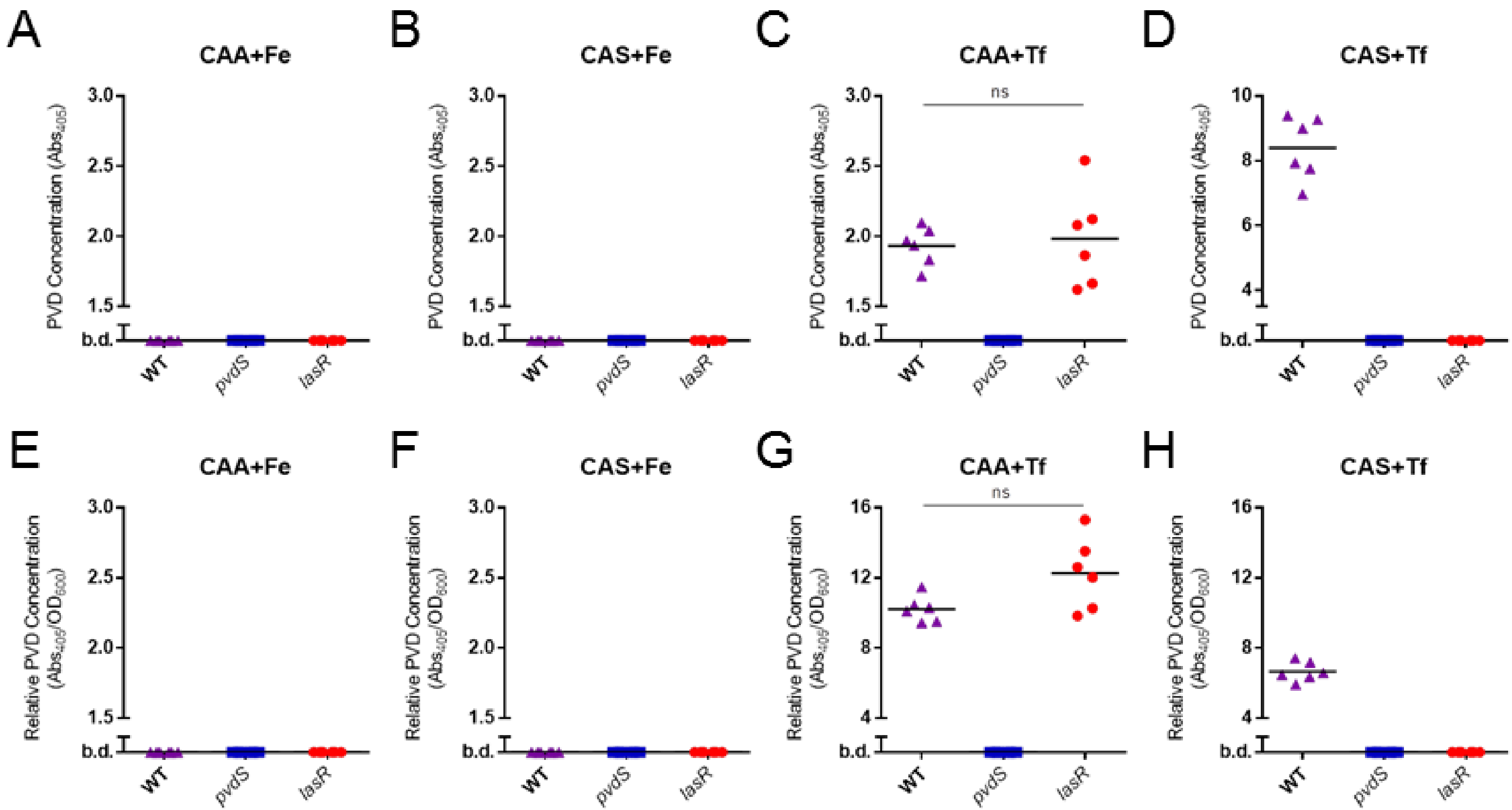


Figure 18. Fitnesses of *lasR* and *pvdS* relative to the rest of the population, or to WT, or the other mutant

Competitions in iron-supplied or iron-depleted casein media with WT:*pvdS*:*lasR* triple co-culture with the initial frequencies 8:1:1 (Data from Figure 17). **(A)** Fitness of *lasR*, in iron-supplied casein media, relative to WT:*pvdS* (circles), WT (triangles) and *pvdS* (diamonds). **(B)** Fitness of *pvdS*, in iron-supplied casein media, relative to WT:*lasR* (squares), WT (filled) and *lasR* (hexagons). **(C)** and **(D)** same as in (A) and (B) (but with empty symbols), respectively, but in iron-depleted casein media. Symbols indicate individual replicates and horizontal lines indicate the means of each group. Dotted lines indicate no change in relative fitness (relative fitness=1).

Strikingly, in the triple cultures under the condition where both traits are required, the presence of *pvdS* mutant results in a significant decrease in the ability of *lasR* mutant to act as a cheater (Figure 17C-right, and Figure 18C). These results show that the costs and benefits of the two social traits studied here are context dependent and support the conclusion that the behaviors of the social mutants vary not only with the environment, but also with the level of polymorphism in the population.



A
CAA+Fe
PVD Concentration (Abs405)
3.0
2.5
2.0
1.5
b.d.
WT
pvdS
lasR
B
CAS+Fe
C
CAA+Tf
ns
D
CAS+Tf
10
8
6
4
E
Relative PVD Concentration (Abs405/OD600)
F
G
16
12
H

Figure 19. Total (Abs_{405}) and relative (Abs_{405}/OD_{600}) pyoverdine (PVD) concentrations of WT, *pvdS*, and *lasR* monocultures after 48 hours of incubation in various media

Total PVD concentrations (Abs_{405}), **(A)** in iron-supplied CAA medium, **(B)** in iron-supplied casein medium, **(C)** in iron-depleted CAA medium, **(D)** in iron-depleted casein medium**.** Relative PVD concentrations (Abs_{405}/OD_{600}), **(E)** in iron-supplied CAA medium, **(F)** in iron-supplied casein medium, **(G)** in iron-depleted CAA medium, **(H)** in iron-depleted casein medium. (Comparisons are done via Mann-Whitney test; ns=not significant, $P>0.05$; for all experiments N=6; b.d.: below detection)**. Methodology:** PVD concentration measurements are done after 48 hours of growth in 37^{o}C shaker by centrifuging the cells at 14000 r.p.m. for 4 minutes (Eppendorf Centrifuge 5418) and collecting the supernatant, measuring their absorbance at 405nm (Abs_{405}) in optical cuvettes as 1:10 dilutions with PBS solutions in a Thermo Spectronic Helios δ spectrophotometer.

Cheating capacity and the onset of the tragedy of the commons

We next asked what would be the long-term consequences of these differences in cheating capacities for the overall fitness of the population by performing long-term propagations (Figure 20).

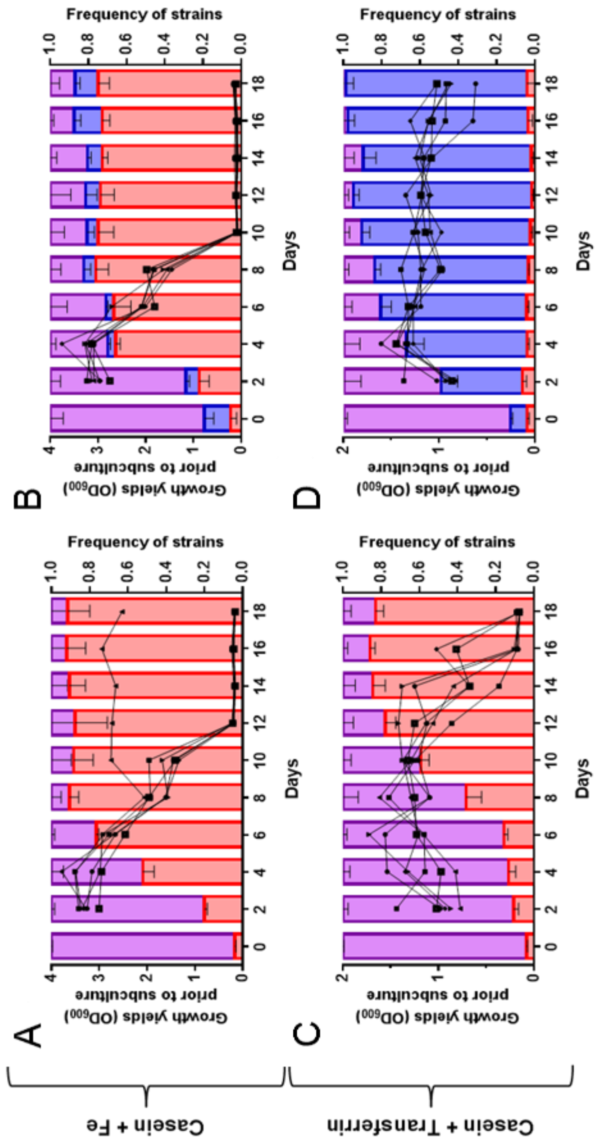


Figure 20. **Effects of abiotic and biotic factors on growth yields and strain composition of the population in long-term propagations**

Left Y-axes show the OD_{600} values prior to subculture; black symbols are the OD_{600} values of 6 biological replicates tested for each condition. Right Y-axes show the frequencies of WT (purple), *lasR* (red), and *pvdS* (blue) mutants 48 hours after subculturing; data are shown as bars and represent the means of 6 biological replicates, error bars indicate SD. X-axes show the days of propagations to fresh media. **(A)** WT and *lasR* mutant co-cultures mixed at an initial frequency of 9:1 in iron-supplied casein media. **(B)** WT, *lasR* and *pvdS* mutants triple co-cultures mixed at initial an initial frequency of 8:1:1 in iron-supplied casein media. **(C)** and **(D)** same as in (A) and (B) but in iron-depleted casein media.

We started co-cultures with WT:*lasR* or WT:*lasR*:*pvdS* (at 9:1 and 8:1:1 initial ratios, respectively), in either iron-supplied casein medium (Figure 20 A and B), or in iron-depleted casein medium (Figure 20 C and D). The illustrations of the four experimental conditions are shown in Figure 21.

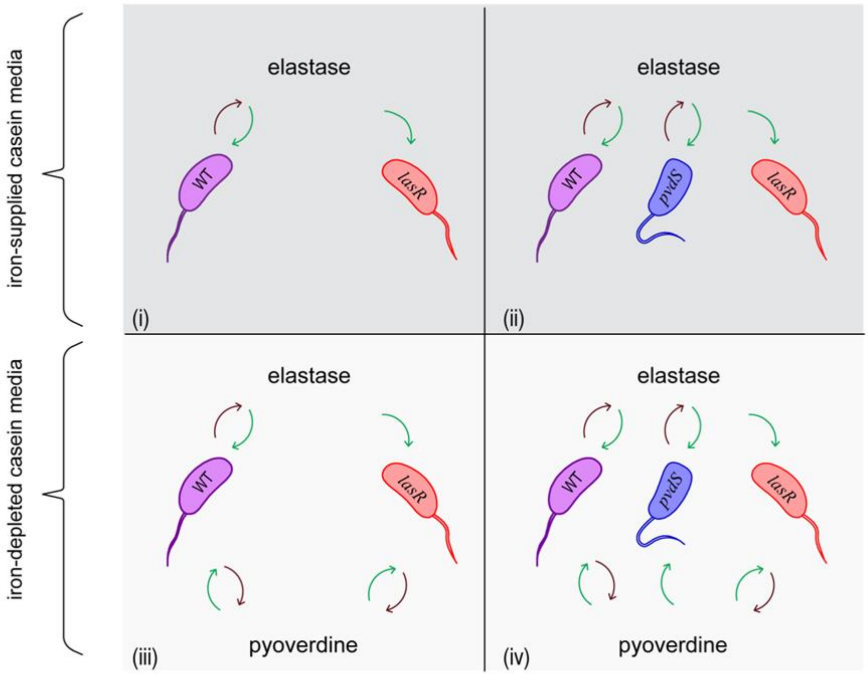


Figure 21. **Four scenarios representing the environmental constraints and population compositions tested**

(i) double co-culture in iron-supplied casein medium (WT and one mutant, under one environmental constraint); (**ii)** triple co-culture in iron-supplied casein medium (WT and two mutants, under one environmental constraint); **(iii)** double co-culture in iron-depleted casein medium (WT and one mutant, under two environmental constraints); **(iv)** triple co-culture in iron-depleted casein medium (WT and two mutants, under two environmental constraints). Dark-red arrows indicate a paid cost for the production of elastase and/or pyoverdine, and the green arrows indicate a benefit from these behaviors. Dark-grey backgrounds indicate optimal iron concentrations; light-grey backgrounds indicate depleted iron concentrations in the media.

Propagations were performed by culture transfer to fresh media every 48 hours. Before each passage, cell density and frequencies of WT, *pvdS,* and *lasR* cells were determined. Growth yields in OD_{600} and colony forming units (CFUs) are shown in Figure 20 and Figure 22, respectively.

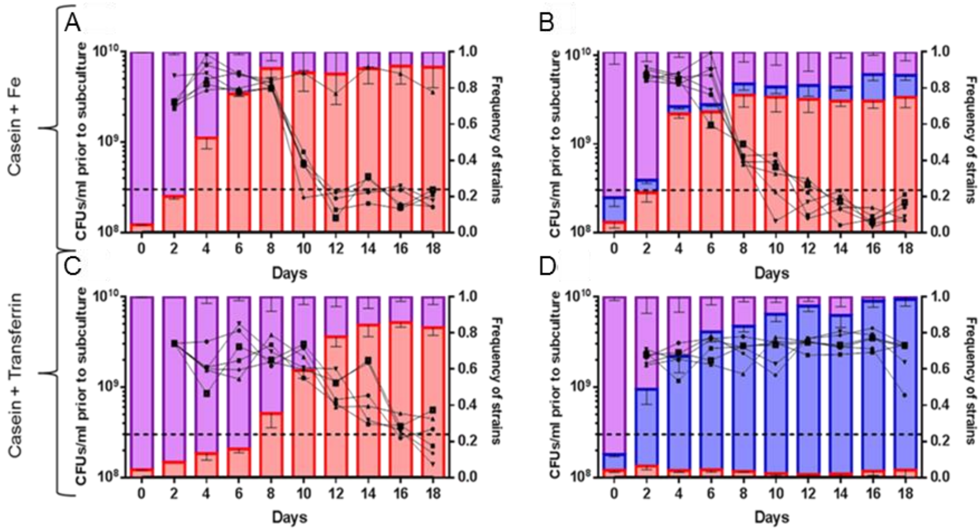


Figure 22. **Data from the experiments shown in Figure 20 with the growth yields prior to subculture, shown as CFUs/ml**

Dash lines indicate the approximate monoculture growth yields of *lasR* monoculture ($3x10^8$ CFUs/ml).

We observed that, in the long-term propagations, in five out of six replicates of WT:*lasR* co-cultures in iron-supplied casein medium, *lasR* mutant quickly increased in frequency throughout the first 8 days (4 passages), reaching up to 90% of the population (red bars in Figure 20A). The total cell densities of the populations (black lines) rapidly decreased to levels similar to that of *lasR* monocultures (OD_{600} = 0.03) by day 12, and no recovery was observed in subsequent passages (Figure 20A, and Figure 23).

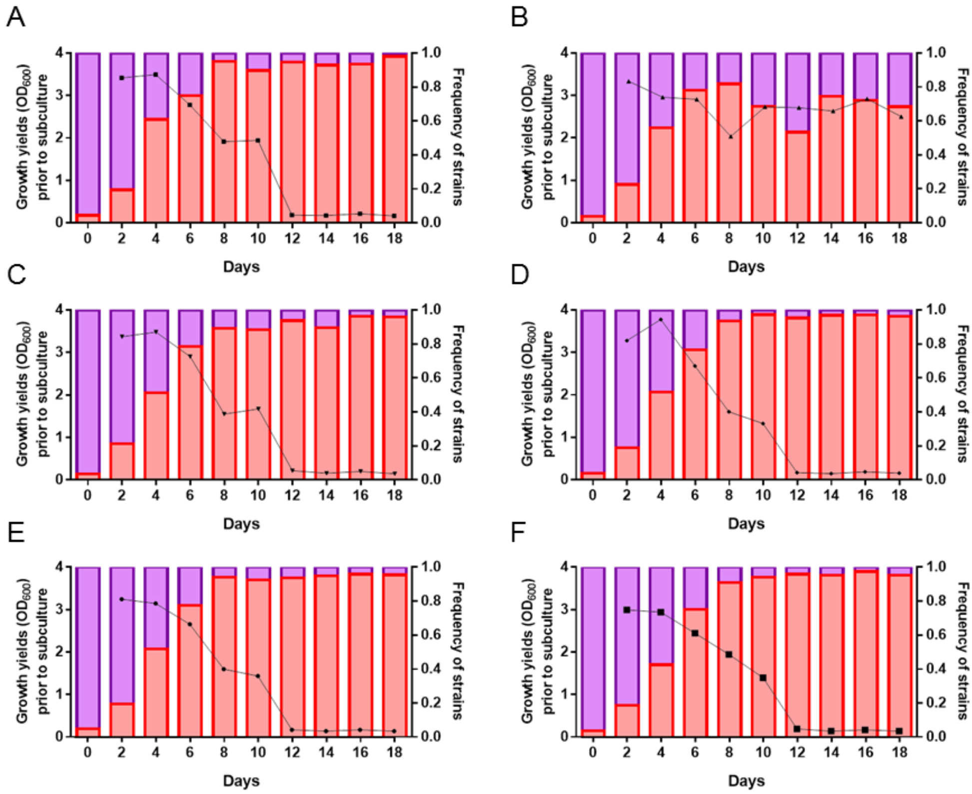


Figure 23. Individual biological replicates from the Figure 20A

WT:*lasR* populations, which are co-cultured with initial frequencies of 9:1, in iron-supplied casein media. 'X' axes show the days of propagations to fresh media. Left 'Y' axes show the growth yields (OD_{600}), prior to subculture; data shown as black lines are the growth yields (OD_{600}) of the cultures measured at the late stationary phase (48 hours after the inoculation) values. Right 'Y' axes show the frequencies of WT (purple) and *lasR* (red).

We defined this density, which was reached by day 12 of the propagation (OD_{600} = 0.03), as the 'collapse threshold' caused by the domination of *lasR* mutant. One replicate out of six did not follow this trend; in this case, no population collapse was observed, and the total cell numbers remained high throughout the experiment (Figure 23B). The cause of this difference is currently under investigation, but the fact that it only occurred in one of the six replicates suggests that the WT in this particular replicate may have acquired *de novo* beneficial mutation(s), that could prevent invasion of *lasR* mutant, and these are likely to be non-social mutation(s).

Next, we analyzed long-term competitions in triple co-culture (WT, *pvdS*, and *lasR*; respectively 8:1:1) in iron-supplied casein medium (Figure 20B, and Figure 24).

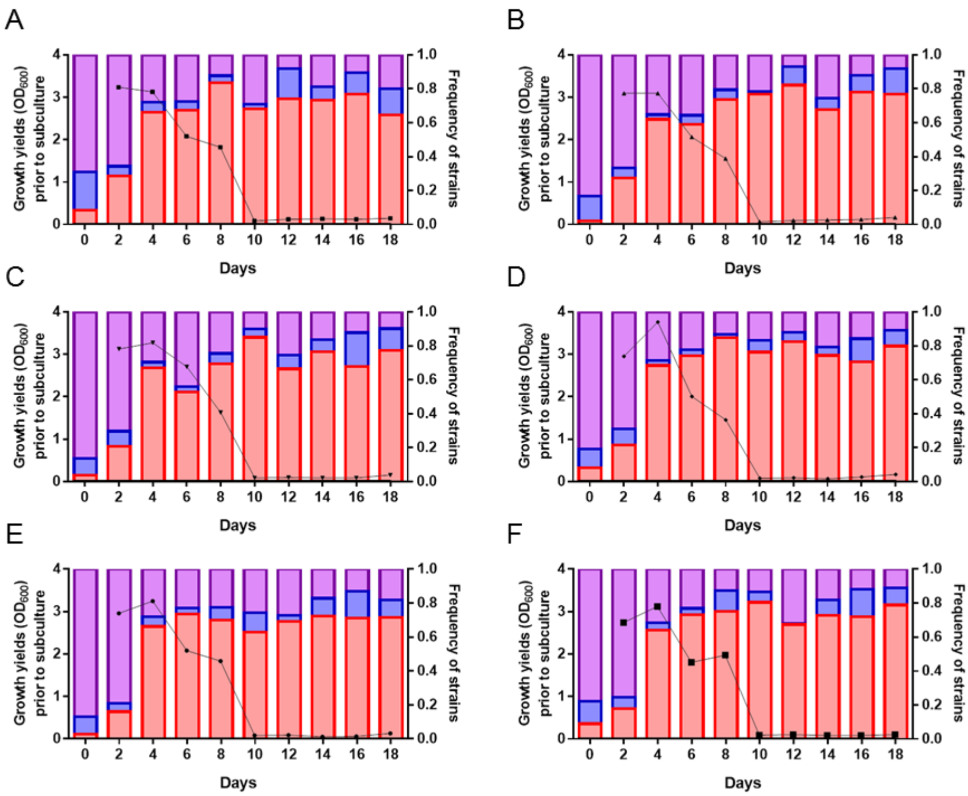


Figure 24. Individual biological replicates from the Figure 20B

WT:*lasR*:*pvdS* populations, which are co-cultured with initial frequencies of 8:1:1, in iron-supplied casein media. ‘X’ axes show the days of propagations to fresh media. Left ‘Y’ axes show the growth yields (OD_{600}) prior to subculture; data shown as black lines are the growth yields (OD_{600}) of the cultures measured at the late stationary phase (48 hours after the inoculation) values. Right 'Y' axes show the frequencies of WT (purple), *pvdS* (blue), and *lasR* (red).

In this case, we observed an increase in *lasR* frequency, similar to that of WT and *lasR* co-cultures seen in Figure 20A, which was also accompanied by a drastic decrease in the overall population density. At day 10 of the propagation, the six populations reached the collapse threshold. The frequencies of *pvdS* mutant varied between 4% and 15% throughout the duration of the experiment with no indication of any sustained increase (blue bars, Figure 20B). This result is consistent with the predictions from the relative fitness measurements (Figure 17B).

Then we propagated WT:*lasR* co-cultures in the medium with two constraints (Figure 20C, and Figure 25).

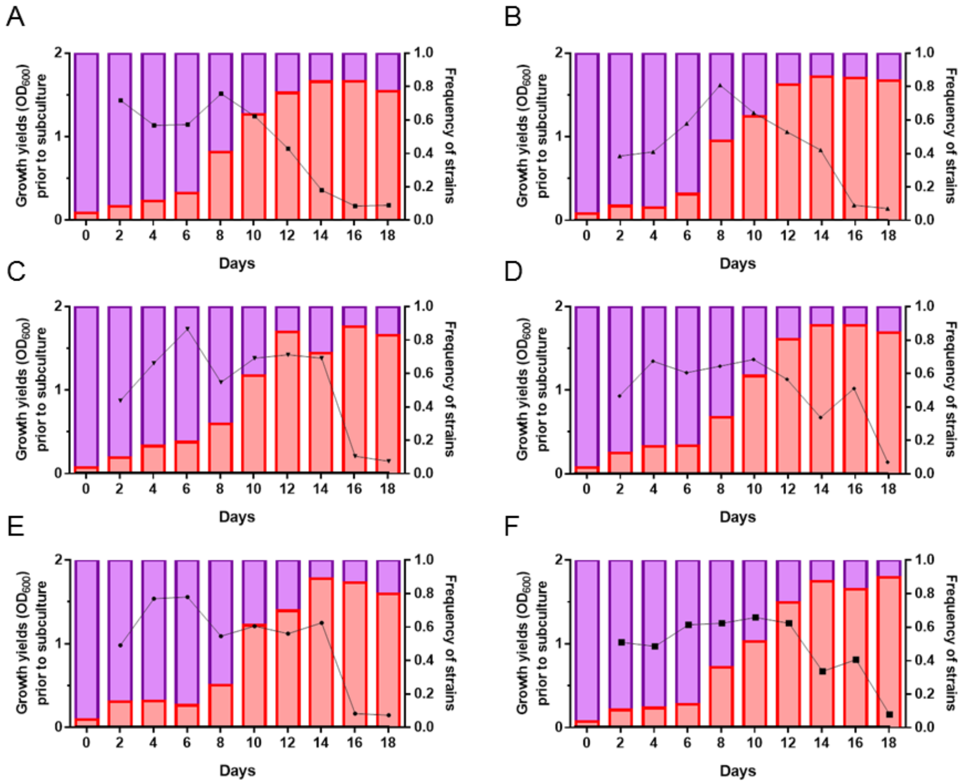


Figure 25. Individual biological replicates from the Figure 20C

WT:*lasR* populations, which are co-cultured with initial frequencies of 9:1, in iron-depleted casein media. 'X' axes show the days of propagations to fresh media. Left 'Y' axes show the growth yields (OD_{600}) prior to subculture; data shown as black lines are the growth yields (OD_{600}) of the cultures measured at the late stationary phase (48 hours after the inoculation) values. Right 'Y' axes show the frequencies of WT (purple) and *lasR* (red).

In these propagations, *lasR* mutant also increases in frequency throughout the first days, but at a slower pace than in iron-supplied medium. The total cell numbers remain high until days 10-12, but, as the *lasR* frequencies increase to about 80%, the density of the population decreases, reaching the collapse threshold by day 18.

Hence, in all the three scenarios described here, the dominance of *lasR* mutant is followed by a drastic population collapse due to the tragedy of the commons (Figure 20 A – C).

Effects of orthogonal cheating on preventing the tragedy of the commons

Our short-term competitions revealed that the cheating ability of *lasR* is influenced by both abiotic and biotic conditions, as the presence of *pvdS* in the low iron conditions reduces the relative fitness of *lasR* mutant (Figure 17C). Therefore, we investigated if, under low iron conditions, *pvdS* could protect a polymorphic population from the drastic population collapse caused by the *lasR* invasion. Figure 20D (and the individual replicates in Figure 26) shows that in the propagation of triple co-cultures in iron-depleted casein medium; *lasR* cannot increase in frequency (it stays at approximately 3% throughout the experiment).

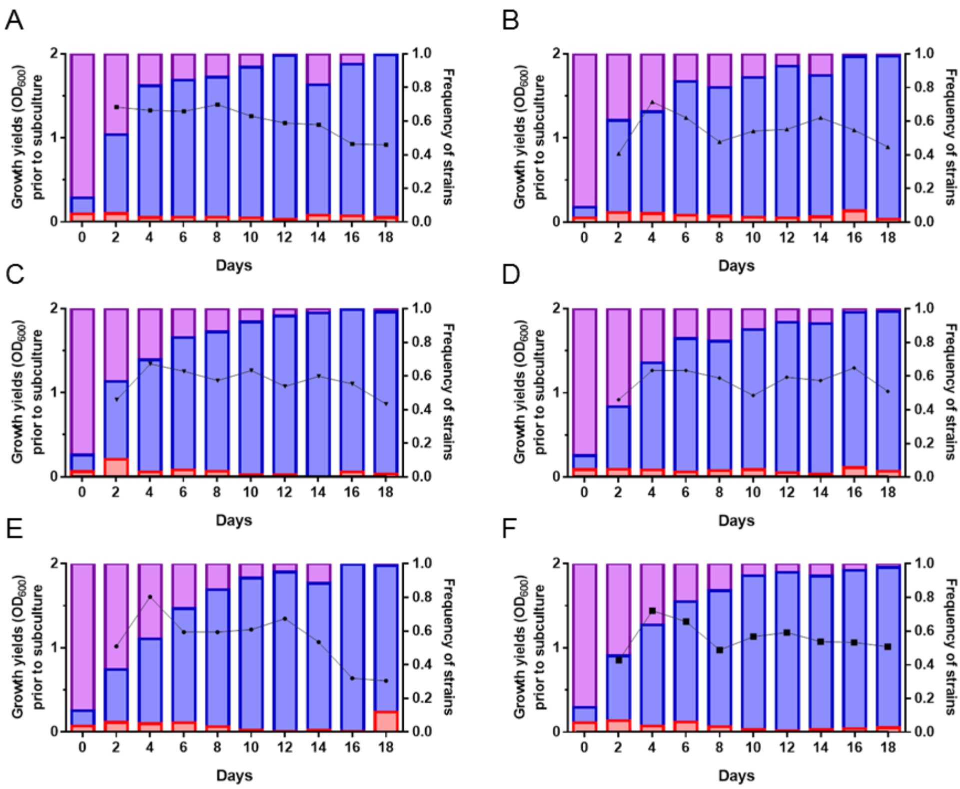


Figure 26. Individual biological replicates from the Figure 20D

WT:*lasR*:*pvdS* populations, which are co-cultured with initial frequencies of 8:1:1, in iron-depleted casein media. 'X' axes show the days of propagations to fresh media. Left 'Y' axes show the growth yields (OD_{600}) prior to subculture; data shown as black lines are the growth yields (OD_{600}) of the cultures measured at the late stationary phase (48 hours after the inoculation) values. Right 'Y' axes show the frequencies of WT (purple), *pvdS* (blue), and *lasR* (red).

In contrast, *pvdS* rapidly spreads during the first 12 days to an average frequency of 96% at day 18. Despite the *pvdS* domination, cell densities of the overall populations stay high. These indicate that, under these conditions, the presence of only 4% of pyoverdine producers in the population is enough to sustain the growth of the entire populations to levels similar to the WT mono-cultures. This interpretation is supported by the results shown in Figure 27, representing growth yields of mixed cultures with different starting frequencies of *pvdS*.

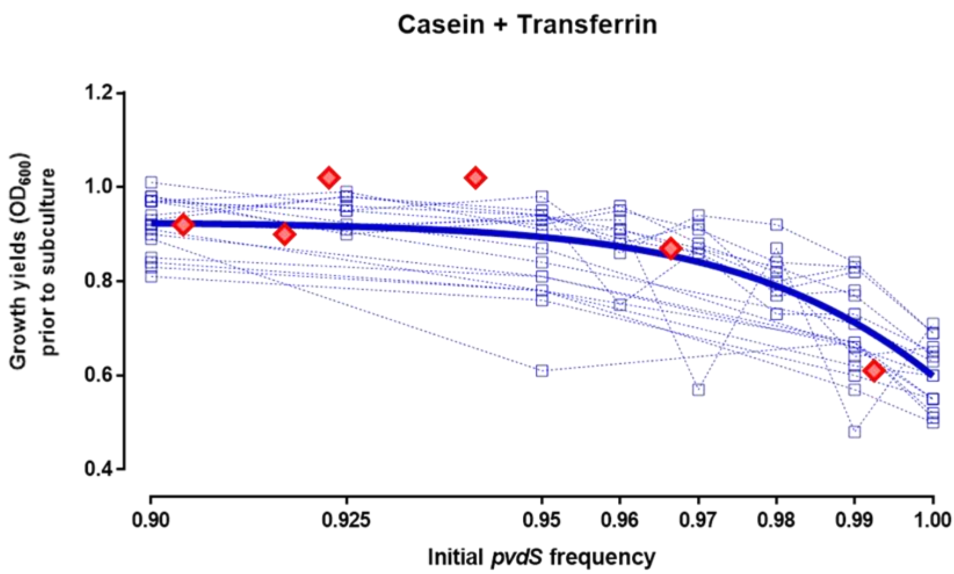


Figure 27. Effect of the initial frequencies of *pvdS* mutant in the co-cultures with WT, on the overall growth yields of the population

Each blue square represents one short term competition (48 hours) in iron-depleted casein media. Initial frequencies of *pvdS* are shown in the 'X' axis (curve indicates the log regression of these short term competitions). Red diamonds show the OD_{600} measurements and matching inoculum frequencies of *pvdS* mutant from different co-cultures of the 18th day of the experiment shown in Figure 20D (OD_{600} of the cultures with 3% WT and 97% *pvdS* vs. 100% WT, $P=0.1316$; and OD_{600} of the cultures with 2% WT and 98% *pvdS* vs 100% WT, $P<0.05$).

Overall, the domination of *pvdS* mutant in the triple cultures with the two constraints has a remarkable effect on the outcome of the propagations in terms of the growth yields; *pvdS* domination prevents expansion of *lasR* and thus the drastic population collapse of the population, which occurs when *lasR* mutant dominates (OD_{600}=0.03, Figure 20 A – C). This occurs because, in this environment where both *lasR* mutant and WT are induced to produce pyoverdine*,* even though *lasR* mutant still increases in frequency in relation to the WT (Figure 18C), it loses against *pvdS,* given the high relative fitness of *pvdS* against both WT and *lasR* in this medium (Figure 17).

In Figure 20D, although there is no *pvdS* fixation, Figure 27 shows that this could be because of *pvdS* not reaching high enough frequencies (~97%) of the entire population. We experimentally tested whether this would happen if the propagation were prolonged in competitions with initial *pvdS* frequencies similar to those at the 18th day of the competitions in Figure 20D. The results in Figure 28, below show that when the competitions are initiated with *pvdS* frequencies similar to those at day 18 in Figure 20D, *pvdS* always reaches fixation when co-cultured either with WT(Figure 28 A, B, and C) or with WT and *lasR* mutant (Figure 28 D, E, and F).

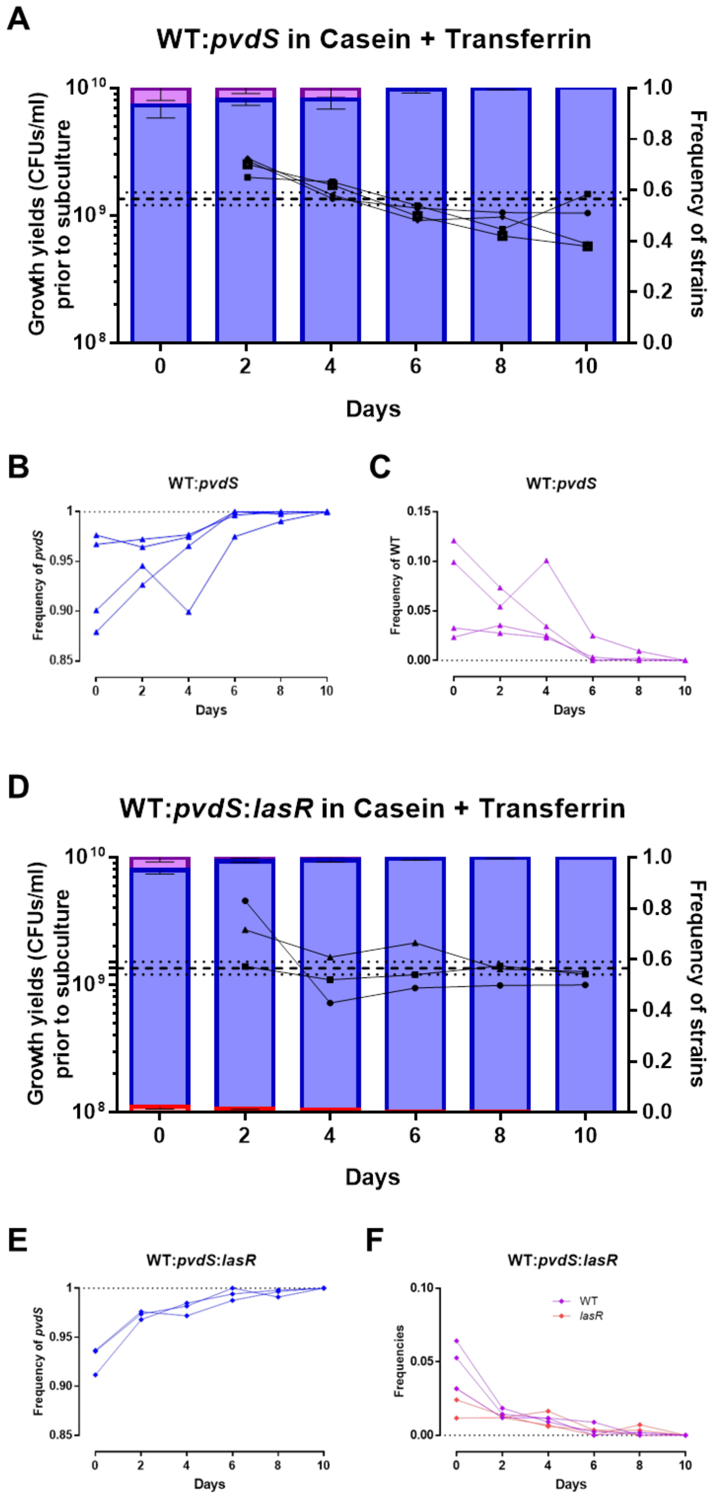

A
WT:pvdS in Casein + Transferrin
Growth yields (CFUs/ml) prior to subculture
Frequency of strains
Days
B
WT:pvdS
Frequency of pvdS
Days
C
WT:pvdS
Frequency of WT
Days
D
WT:pvdS:lasR in Casein + Transferrin
Growth yields (CFUs/ml) prior to subculture
Frequency of strains
Days
E
WT:pvdS:lasR
Frequency of pvdS
Days
F
WT:pvdS:lasR
WT
lasR
Frequencies
Days

Figure 28. *pvdS* reaches fixation.

Propagations of WT:*pvdS* (**(A)**, **(B)**, and **(C)**) and WT:*lasR*:*pvdS* cultures (**(D)**, **(E)** and **(F)**) in iron-depleted casein media throughout 10 days by passing the cultures to fresh media with a 1/1000 dilution after each 48 hours of growth ('X' axes show the days of propagation and the initial frequencies are shown at day 0). (A) Frequency changes of WT (purple) and *pvdS* (blue) in WT:*pvdS* co-cultures shown as stacked bars (right 'Y' axes) as the growth yields (CFUs/ml) of 4 biological replicates are shown as black lines (left 'Y' axes). (B) A detailed presentation of the frequency changes of *pvdS* in WT:*pvdS* co-cultures (blue). (C) A detailed presentation of the frequency changes of WT in WT:*pvdS* co-cultures (purple). (D) Frequency changes of WT (purple), *pvdS* (blue), and *lasR* (red) in WT:*pvdS*:*lasR* co-cultures shown as stacked bars (right 'Y' axes) as the growth yields (CFUs/ml) of 3 biological replicates are shown as black lines (left 'Y' axes). (E) A detailed presentation of the frequency changes of *pvdS* in WT:*pvdS* co-cultures (blue). (F) A detailed presentation of the frequency changes of WT (purple), and *lasR* (red), in WT:*pvdS*:*lasR* co-cultures. Dash lines indicate the mean monoculture *pvdS* growth yields in the same media and the dotted lines indicate SD in (A) and (D) (mean: 1.35×10^9 CFUs/ml; ±SD=1.12×10^0 CFUs/ml). Dotted lines indicate full fixation of *pvdS* to 100% of the population in (B), (C), (E) and (F). For (A), (B), and (C) N=4; and for (D), (E), and (F) N=3.

As a control, we also performed the long-term propagation experiments in media with no constraints. As expected, we did not observe any significant change in the population densities (Figure 29).

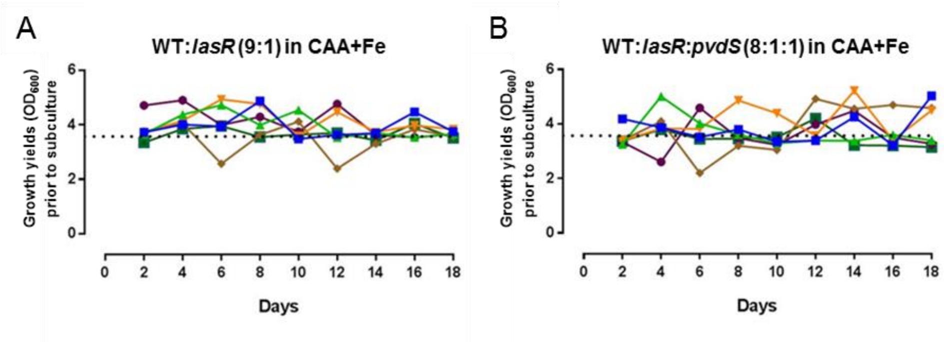


Figure 29. **Propagations of *P. aeruginosa* cultures in a medium with no constraints**

(A) WT and *lasR* co-culture initially mixed (9:1) in iron-supplied CAA media. **(B)** WT, *pvdS* and *lasR* co-culture initially mixed (8:1:1) in iron-supplied CAA media. 'X' axes show the days of propagations to fresh media. 'Y' Axes show the growth yields as OD_{600} prior to subculture, each colored line indicates one propagated culture (N=6), dash lines indicate the monoculture WT growth yields in the same medium (mean=3.57, ±SD=0.357, N=6).

Effects of quorum sensing regulation in preventing the extinction of cooperation upon emergence of cheaters

As Figure 20 A, B, and C show, *lasR* does not reach fixation at the 18th day of the competitions. To test if *lasR* would eventually reach fixation given enough time, we then propagated WT:*lasR* co-cultures, starting with similar frequencies to the co-cultures at the end of the propagations in Figure 20A. Figure 30A shows the results of the propagations of WT:*lasR* cocultures with initial lasR frequencies around 85%, does not change in frequencies throughout the propagations for 6 days. This shows that *lasR* cannot reach fixation under these circumstances.

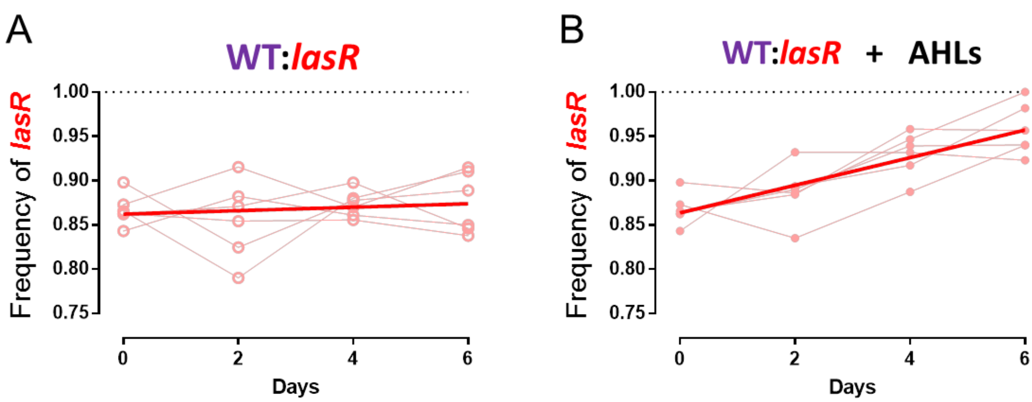


Figure 30. Frequencies of *lasR* in propagations of WT:*lasR* co-cultures (with initial *lasR* frequencies similar to the 18th day of Figure 20A) in iron-supplied casein media in the absence or presence of exogenously added AHLs (3OC$_{12}$-HSL)

Initial frequency of 80-90% of *lasR* were used. Cultures were propagated throughout 6 days by passing the fresh media each 48 hours. **(A)** Frequency changes of *lasR* in WT:*lasR* co-cultures (red). **(B)** is the same as (A) but with 5µM AHLs (3OC$_{12}$-HSL) added to the media. Red lines indicate linear regressions. Dotted lines represent 100% domination of *lasR*.

Given that the *lasR* gene and elastase production are regulated via quorum sensing, we hypothesized that QS could be responsible for the lack of fixation of *lasR* mutant. We repeated the propagation experiment, shown in Figure 30A, with the addition of the QS autoinducer AHLs ($3OC_{12}$-HSL) to the culture medium. The addition of AHLs abolishes the QS-dependent regulation of elastase by locking the LasR regulator on its ON state. With the addition of AHLs, the frequency of *lasR* mutant increases throughout the competitions until fixation (Figure 30B).

We then tested whether the onset the tragedy of the commons would be altered if AHLs are added from the beginning of a competition with WT and *lasR* when *lasR* starts from relatively low frequencies. Towards this end, we conduct two parallel propagation experiments, one similar to Figure 20A, and the other is the same but with the addition of exogenously added AHLs. We measured the changes in the frequencies (Figure 31 A and B) and in growth yields (Figure 30 C and D). We find that the addition of AHLs do not affect the onset of the tragedy of the commons but similarly to the Figure 30, addition of AHLs let *lasR* reach fixation. Without the additional AHLs, similarly to Figure 30, *lasR* frequencies get stabilized around 90%. Interestingly, and similar to Figure 20A, one biological replicate out of six in each set of the experiment did not follow the same trend as the other replicates.

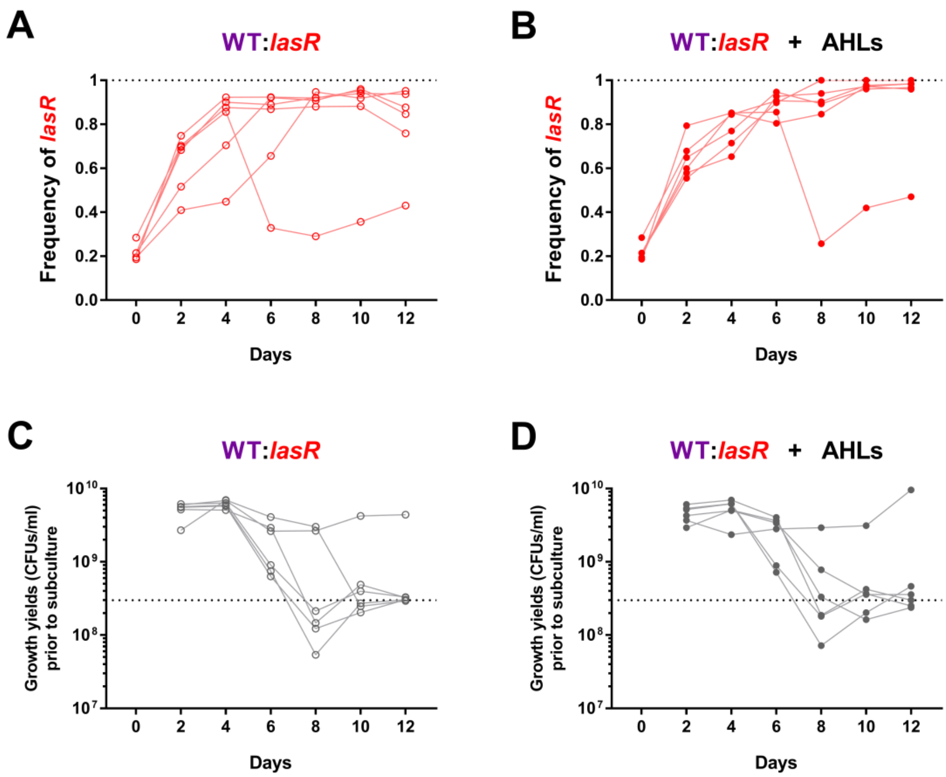


Figure 31. Frequencies and growth yields of *lasR* in propagations of WT:*lasR* co-cultures in iron-supplied casein media in the absence or presence of exogenously added AHLs (3OC$_{12}$-HSL)

Initial frequencies of 20% of *lasR* were used. Cultures were propagated throughout 12 days by passing the fresh media each 48 hours. **(A)** Frequency changes of *lasR* in WT:*lasR* co-cultures (red). **(B)** is the same as (A) but with 5µM AHLs (3OC$_{12}$-HSL) added to the media. **(C)** Growth yields (CFUs/ml) prior to subculture) of the WT:*lasR* populations (grey lines). **(D)** is the same as (C) but with 5µM AHLs (3OC$_{12}$-HSL) added to the media. Dotted lines in (A) and (B) represent 100% domination of *lasR*. Dotted lines in (C) and (D) indicate the monoculture growth levels of *lasR*.

We conclude that quorum sensing regulation of production of a public good prevents full domination of the QS cheater, maintaining cooperation in populations, however, does not affect the onset of the tragedy of the commons. On the other hand, if the cheater which wins is affected in the production of a public good that is not regulated via QS (e.g. *pvdS*) this mutant can dominate the entire population (Figure 28).

Manipulation of the environmental constraints to induce or prevent the tragedy of the commons

We reasoned that if strong ecological interactions dominate in long-term dynamics over *de novo* adaptive mutations, alterations of the abiotic factors in the triple cultures should modify the role of each mutant by changing the costs and benefits of the cooperative traits. Indeed, changing the carbon source from casein to CAA during the course of the propagation eliminated the behavior of *lasR* mutant as a cheater, and this environmental change was sufficient to protect the WT:*lasR* co-cultures from population collapse (Figure 32A).

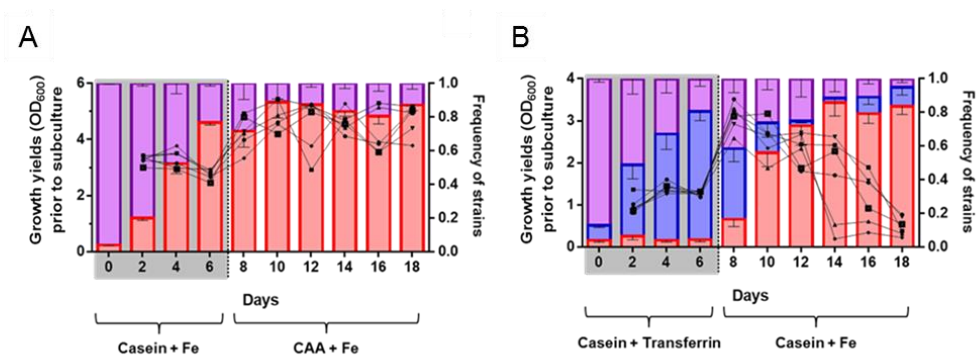


Figure 32. Results of manipulations of abiotic conditions in long-term propagations

Aliquots of either the WT:*lasR* co-cultures propagated in iron-supplied casein media (Figure 20A) or the WT:*lasR*:*pvdS* triple co-cultures propagated in iron-depleted casein media (Figure 20D) for 6 days were transferred into either iron-supplied CAA medium or into iron-supplied casein medium, respectively. **(A)** Relief of complex carbon constraint by changing casein in iron-supplied casein medium to CAA, thus making it a medium with no constraints after the 6th day of the competitions (N=6, data from the first 6 days are from Figure 20A). **(B)** Relief of low iron constraint by adding iron instead of iron depleting transferrin and changing iron-depleted casein medium into iron-supplied casein medium after the 6th day of the competitions (N=6, data from the first 6 days are from Figure 20D). Legends as in Figure 20

Conversely, the addition of iron to the iron-depleted casein medium (thus making it iron-supplied) reverts the expansion of *pvdS* mutant, favoring a consequent increase in *lasR* cheating capacity, ultimately causing the collapse of all the populations at day 18 (Figure 32B). We confirmed that changes in final frequencies observed in Figure 32B were not due to the high starting frequencies of *pvdS*; even though the selective advantage of *pvdS* is frequency dependent, this mutant is capable of cheating even at frequencies higher than 90% (Figure 33).

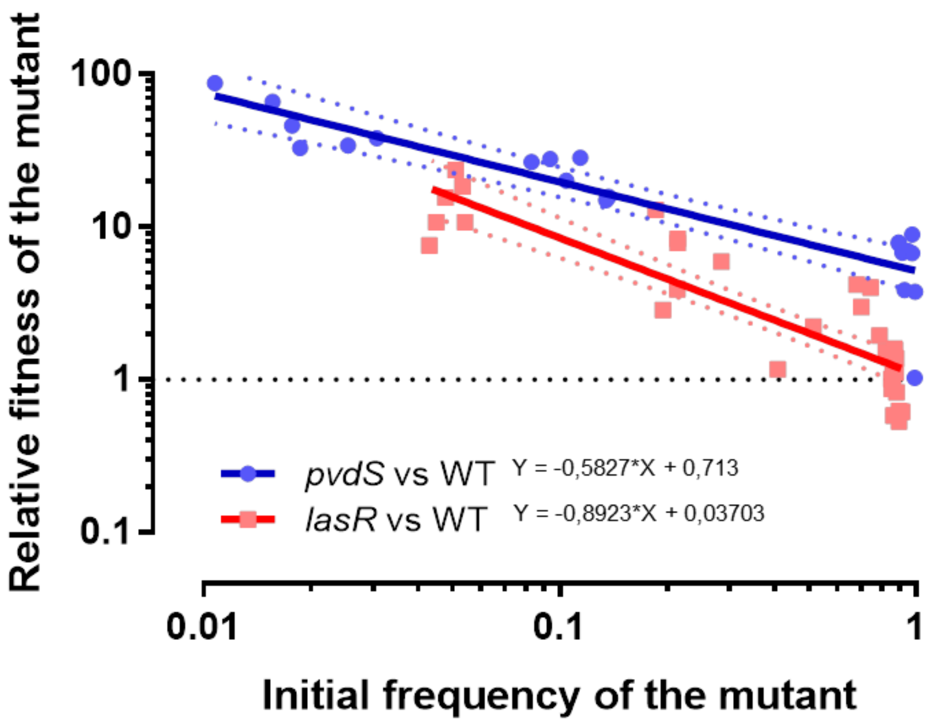


Figure 33. Frequency-dependent selection for *pvdS* and *lasR*

The change in relative fitness of either mutant in relation to their initial frequencies in co-culture with WT in iron-depleted or iron-supplied casein media for *pvdS* (blue circles) or *lasR* (red squares), respectively. ‘X’ axis shows the frequencies of *pvdS* or *lasR* in the beginning of the competition with WT. ‘Y’ axis shows the relative fitness values of *pvdS* or *lasR* over WT after 48h of incubation. Lines indicate linear regressions; slopes of the lines are shown on the figure (Comparison of the lines: F=8,525, DFn=1, DFd=52, **=P=0,0052; the lines are significantly different). Red and blue dots indicate the 95% confidence intervals of the corresponding lines. The gray dotted line indicates no change in relative fitness (no cheating, relative fitness=1).

Overall, these results show that by changing the roles of *lasR* and *pvdS* mutants, it was possible to revert the social and ecological dynamics of the populations in a very predictable and reproducible manner. The different consequences of these abiotic manipulations are related to the distinct characteristics of the two mutants studied here, *i. e.*, the tragedy of the commons caused by the invasion of *pvdS* causes a small drop in cell density while the invasion of *lasR* leads to a much greater decrease in density.

Discussion

The classical experimental approach in sociomicrobiology has been to study one trait and one constraint at a time. The simplicity of such an approach has substantially increased our understanding of the dynamics of cooperative and non-cooperative clones and revealed several mechanisms involved in the maintenance of cooperation (Bruger and Waters, 2015; Parsek and Greenberg, 2005; Xavier, 2016). The ability of *lasR* or *pvdS* mutants to behave as cheaters is well documented under these 'one constraint-one trait' laboratory settings. However, even though *lasR* and *pvdS* mutants are commonly isolated from bacterial populations colonizing CF lungs, population collapse due to the invasion by these mutants has not been seen in patients (Cordero and Polz, 2014; Katzianer et al., 2015; Smith et al., 2006a; Winstanley et al., 2016).

Here, we established an experimental setup where WT cooperates in more than one trait: production of elastase and pyoverdine. Under this environment with two constraints, the *lasR* mutant is a cheater for elastase but, a cooperator for pyoverdine, whereas *pvdS* mutant does the opposite. In this environment, the advantage of *pvdS* mutant for not producing pyoverdine is higher than that of *lasR* for not producing elastase (Figure 17C and Figure 17D). As a consequence of the different costs associated with the different traits, in 3-way competitions, *pvdS* causes a reduction in the relative fitness of *lasR* mutant and dominates the population. This domination of *pvdS* prevents the population from a potential drastic collapse caused by the invasion of *lasR* mutants (compare Figure 20D with Figure 20 A – C). Although the tragedy of the commons due to the domination of *pvdS* mutant can also occur (Figure 27), the consequent decrease in cell density, is much less drastic than the decrease in growth yields observed upon the domination of *lasR* mutant (Figure 15 and Figure

20 A – C). This happens because the difference between the benefit and the cost of pyoverdine production is much smaller than that of elastase production.

The results from the 3-way competition demonstrate that by having more than one environmental constraint and more than one social mutant, a scenario likely to be closer to the conditions in nature (such as in the lungs of CF patients), the cheater for the trait with the highest cost is expected to dominate. The consequence of that domination for the population collapse will depend on the benefit-cost that such trait entails. Importantly, the degree of the population collapse caused by the tragedy of the commons can eventually have very different consequences for the host. In case of a trait whose difference in benefit to cost is high, a drastic collapse on the density of the population caused by the cheater in that trait is expected. If a drastic collapse in density takes place, clearance of the pathogen is more likely to occur, resulting in a higher benefit to the host. In contrast, if the mutant for the trait with a low benefit to cost difference (as for *pvdS*) wins, a weak collapse occurs, to the detriment of the host.

Our results highlight the importance of the difference between costs of the different traits for the population dynamics, and the difference between benefits and costs of each trait for the mean fitness of the population. By studying two different traits, we were able to compare the cost-benefit payoffs of each trait, which we show, that are not always linear or symmetrical. Whereas a high costly trait, such as pyoverdine production, can give a relatively smaller benefit; a trait that costs little to produce can give relatively larger benefits as in quorum sensing regulated elastase production. Given the relationship between the costs, in a fluctuating environment, polymorphism of various mutants of cooperative traits, as it occurs in CF infections, is possible. Additionally, as shown here for *lasR* mutant, quorum sensing regulation can also favor the maintenance of polymorphism; as such regulation alters the values of the cost and benefits of the traits.

Altogether, our results provide support for a dynamic view of cooperation and cheating that is dependent on the genotypes and constraints present in the environment. We demonstrate how changes in the abiotic environment can make a social mutant to stop cheating on one trait while still cooperating on other traits also susceptible to cheating. Studying such multidimensional relations in interbacterial cooperation and cheating dynamics could provide a better understanding for providing new therapeutically approaches for the treatments of infections such as those in the lungs of CF patients.

Materials and Methods

Bacterial strains, media and culture conditions

We used *Pseudomonas aeruginosa* WT strain PA01 as the full cooperator, PA01 *lasR* mutant harboring a gentamycin resistant gene inserted in *lasR* (*lasR::Gm*), and PA01 *pvdS* mutant harboring a gentamycin resistance gene inserted in *pvdS* (*pvdS::Gm*) (All three strains were provided by Kevin Foster). Iron-supplied casein medium contains casein (Sigma, Ref: C8654) (1% w/v) as the sole carbon and nitrogen sources salts (1.18 g $K_2HPO_4.3H_2O$ and 0.25 g $MgSO_4.7H_2O$ per liter of dH_2O) and 50 μM of $FeCl_3$. Iron-depleted casein medium is identical to the iron-supplied casein medium but instead of $FeCl_3$ supplementation, this medium contains 100 μg/ml of human apo-transferrin (Sigma, T2036) and 20 mM sodium bicarbonate to deplete available iron and induce pyoverdine production. The medium with no constraints contains the same salt solutions as the other media, low iron CAA (BD, Ref: 223050) (1% w/v) as the sole carbon source and 50 μM of $FeCl_3$. All cultures were incubated at 37^oC with aeration (240 rpm, New Brunswick E25/E25R Shaker) for the incubation times indicated. Cell densities were estimated by measuring absorbance (Abs) at 600 nm (OD_{600}) in a Thermo Spectronic Helios δ spectrophotometer.

Determination of genotypic frequencies

Estimation of the frequencies of each strain in the co-cultures was performed by scoring fluorescence and colony morphology of colonies obtained from plating serial PBS dilutions of the cultures. For each individual sample, three aliquots (of 50μl - 200μl, as appropriated) were plated into LB agar plates, which were used as technical replicates. Then, CFUs/ml were calculated by scoring different colony morphologies on each plate. A stereoscope (Zeiss Stereo Lumar V12) with a CFP filter was used to distinguish pyoverdine producers, which are fluorescent, from the non-fluorescent *pvdS* mutants (Ghoul et al., 2014b; Jiricny et al., 2010). *lasR* mutant colonies have distinct colony morphology: smaller with smooth edges whereas elastase producers are larger with rugged edges (Ghoul et al., 2014b). To validate the phenotypic scoring all colonies used to determine the frequency from day 18 of the propagation experiments (Figure 20D) were tested by PCR with primers for the *lasR* and *pvdS* genes. The PCR data confirmed the phenotypic scoring with 100% accuracy.

Competition experiments

We propagated six replicates under four different conditions (Figure 20). Prior to start the competition experiments, all strains were inoculated, from frozen stocks, in medium containing 1% (w/v) casein and 1% (w/v) CAA in salts solution (same as in iron-supplied casein medium, described above) for 36 hours at 37^{o}C temperature with shaking (240 rpm). Cells were then washed with PBS four times, to remove any residual extracellular factor. Next after measuring cell densities (OD_{600}), cultures were normalized to OD_{600} = 1.0 and used to inoculate the various media as described in the text and figures. The different strains were diluted into fresh media, at different ratios as specified, to a starting initial OD_{600} = 0.05. For short term competitions the relative frequencies were determined by plating an aliquot of each culture at the beginning of the experiment (t = 0), and after 48 hours of incubation. For long-term competitions, the relative frequencies were determined at t = 0, and thereafter every 48 hours before each passage. At the end of every 48 hours, 1.5 µl of each culture was transferred to 1.5ml of fresh medium (bottle-neck of 1/1000).

Statistical analysis

Independent biological replicates were separately grown from the frozen stocks of each strain. Each experiment was performed at least twice, with three biological replicates, except in Figure 17A where one experiment has only two biological replicates. Each figure (or figure panel) includes data from the biological replicates of at least two experiments. The sample size (N), corresponds to the total numbers of independent biological replicates in each figure panel and is provided in the corresponding figure legends. Relative fitness was used to determine the cheating capacity of each mutant. For both *lasR* and *pvdS* strains, the relative fitness (ω) was calculated as the frequency change over 48 hours relative to the rest of the strains in the mixture, using the following formula $\omega = f_{final}(1 - f_{initial}) / f_{initial}(1 - f_{final})$ where $f_{initial}$ is the mean of the initial proportion measured (as described above) at the beginning of the competitions while f_{final} is the mean of the final proportions of the mutant after 48 hours of competition (Ghoul et al., 2014b; Jiricny et al., 2010). We used Mann-Whitney test which is a non-parametric test that does not account for normality and it is more suitable for the sample size used in each experiment ($5<N<20$). For multiple corrections, we used Kruskal-Wallis test with Dunn's correction. For all statistical analyses, we used GraphPad Prism 6 software (http://www.graphpad.com/scientific-software/prism).

Chapter III: Modelling Cheating and the Tragedy of the Commons

Preliminary notes

The author of the thesis participated in the planning and analysis of all the experiments presented in this chapter. The author executed all the experiments shown in this chapter. Part of this chapter is included in the publication titled as "Cheating on orthogonal social traits prevents the tragedy of the commons in *Pseudomonas aeruginosa*". (Manuscript submitted to bioRxiv: http://biorxiv.org/content/early/2017/03/19/118240)

Abstract

Public goods (PG) cooperation is ubiquitous in many levels of life. The dynamics of cooperation and the dilemma that the cheating poses are the focus of many studies in fields ranging from economics to biology. Thus, understanding the dynamics of the PG cooperation and competition has a tremendous importance. Here, we build a simple mathematical model to simulate various PG games to understand the main parameters that govern these dynamics. We, first explore a minimal model, what would happen in a simple PG game with two players, cooperators and cheaters for a single trait. We show that only the cost of the cooperative trait determines the speed of the cheater invasion, and the difference between the benefit and the cost of a cooperative trait determines the intensity of the tragedy of the commons. We then model quorum sensing (QS) regulation of a PG trait implements, and show how it prevents cooperation from getting extinct. Then, as a novel approach, we test a scenario in which multiple games are being played by multiple players, where each player has a different role in each game. This scenario is more similar to that individuals often face in natural settings. Overall, the model simulates the experimental observations successfully by using only a few parameters, the cost and benefit parameters of each social trait, and the QS regulation of a trait which affects the cost and benefit parameters.

Introduction

Bacteria, being prokaryotic minuscule organisms, heavily depend on molecules that operate outside of their cells (Bachmann et al., 2013; Popat et al., 2008; Taylor et al., 2013; West et al., 2007a; Zhou et al., 2014). Many of these exo-products behave as public goods up to a degree of their accessibility (Bachmann et al., 2013; Jiricny et al., 2014). The more the public goods are accessible by all the organisms in the population, higher the selective pressure for cheating (Bachmann et al., 2013; Kümmerli and Brown, 2010). Cheating, via benefiting from public goods with no or little contribution, is considered as one of the obstacles needed to be defined and analyzed to be able to understand the dynamics of interbacterial cooperation (Ghoul et al., 2014a).

Studies showed that cheating as a phenomenon can be observed *in vitro* (Asfahl et al., 2015; Dandekar et al., 2012; Sandoz et al., 2007; Wang et al., 2015), *in vivo* (Czechowska et al., 2014; Rumbaugh et al., 2009) and in natural populations (Cordero and Polz, 2014; Katzianer et al., 2015; Winstanley et al., 2016). Other studies also confirmed that cheating mutants can arise spontaneously in populations where cooperative behaviors are necessary for survival, and can drive the whole population to extinction by increasing in frequency (Asfahl et al., 2015; Dandekar et al., 2012; Wang et al., 2015). Laboratory experiments demonstrated that engineered cheating mutants can increase in frequency in populations where cooperation is needed (Diggle et al., 2007). This creates the paradoxical outcome of the tragedy of the commons (Hardin, 1968; MacLean, 2008; Rankin et al., 2007). The tragedy of the commons happens when individuals increase their individual fitness over the competition for a public good and cause a decrease in the fitness of the overall population by exhausting that public good (Hardin, 1968; Rankin et al., 2007). In bacterial cooperation, the tragedy of the commons is generally observed when cheating organisms increase in fitness by avoiding the cost of public good production and using the public good produced by the cooperator organisms (Asfahl et al., 2015; Dandekar et al., 2012; Wang et al., 2015). This frequency increase of the mutant can only reach to a level where the public good concentration cannot provide the fitness benefit that it otherwise would in a cooperative population. When that happens, the numbers of all the individuals would go down to the levels of the cheating mutants when they grow alone (Chapter II).

Various mechanisms that prevent cheating and eventually avoid a possible tragedy of the commons are described in literature, such as quorum sensing (Allen et al., 2016), privatization (Dandekar et al., 2012; Estrela et al., 2016), spatial structure (Kreft, 2004), metabolic prudence (Xavier et al., 2011). It is tremendously important to dissect the conditions under which mechanisms operate to prevent cheater invasions and maintain cooperation. Although there is a large body of literature on interbacterial cooperation and cheating, experimentally and theoretically, modelling cheating to predict the tragedy of the commons, or the lack thereof which would be the maintenance of the population is yet to be focused on.

In this study, we explore simple mathematical models with continuous replicator equations. We show that costs and benefits of the cooperative traits are the main determinants of the outcome of the public goods competitions between cooperators and cheaters in multiple scenarios. This simple model allows us to explore the dynamics of multiple constraint-multiple trait scenarios and also the effects of quorum sensing to cheating behavior and the tragedy of the commons. Our results demonstrate with simple models of cooperator and cheater dynamics in single or multiple public good games, how the onset and the intensity of the tragedy of the commons can be predicted, and how QS regulation can affect that.

Results

Predicting the tragedy of the commons for one trait-one constraint scenarios

To understand the main factors determining the dynamics of competition among cooperators and cheaters in a simple ‘one trait-one constraint’ scenario, we built a simple mathematical model assuming that the fate of cooperators and cheaters is governed by the costs and the benefits of the cooperative traits. The model assumes that the cost (*c*) of a cooperative trait is lower than the benefit (*b*) associated with this trait ($b>c>0$). The model also assumes that the benefit provided by the cooperative trait is equal for the entire population, as it would be in the case of an equally accessible public good in a well-mixed environment. Spatial structure, diffusion, or privatization, which would alter the benefit gained from the public good for cooperators and cheaters asymmetrically, were not considered in the model. The parameters used are described in Table 2.

Table 2. Parameters for single cooperative trait public goods game

Symbols	Descriptions
coop	Cooperator
ch	Cheater
c	Cost of the cooperative trait
b	Benefit gained from the cooperative trait
ω_0	Fitness without the additional fitness effects of the cooperative traits (basal fitness)
ω_{coop}	Fitness of the cooperator of the both cooperative traits
ω_{ch}	Fitness of the cheater of the cooperative trait
$\overline{\omega}$	Mean fitness of the entire population (A proxy for OD_{600} or CFUs/ml)
p_{coop}	Frequency of the cooperator of the both cooperative traits in the entire population
p_{ch}	Frequency of the cheater of the cooperative trait in the entire population

We define the fitness of a cooperator and a cheater mixed in an environment where the public goods trait that this mutant cheats on as:

$$\omega_{coop} = \omega_0 + b\,(1 - p_{ch}) - c \qquad [1]$$

$$\omega_{ch} = \omega_0 + b\,(1 - p_{ch}) \qquad [2]$$

As it can be seen from the fitness equations above, the cost (c) that cheater avoids is the only difference between the fitness of the cooperator and the fitness of the cheater.

The change in the mean fitness is given by:

$$\overline{\omega} = \Sigma_i\, p_i(t)\, \omega_i = \omega_0 + p_{coop}(t)\, \omega_{coop} + p_{ch}(t)\, \omega_{ch}$$

$$= \omega_0 + (b - c)\,(1 - p_{ch}(t)) \qquad [3]$$

Mean fitness ($\overline{\omega}$) is used as a proxy for the biomass (OD_{600} or CFUs/ml values) as the value c indicates the energy spent for the cooperative action instead of increasing the biomass, and the value b indicates the increase in biomass due to the benefit gained from the cooperative action, and the value ω_0 is the biomass of the population without the effect of any cooperative action.

We assume that the frequencies of cooperators and cheaters change by a replicator equation system in a homogeneous environment, and ignoring stochastic effects:

$$dp_{coop} / dt = p_{coop}(t)\,(\omega_{coop} - \overline{\omega}) = p_{coop}(t)\,(- c\, p_{ch}(t)) \qquad [4]$$

$$dp_{ch} / dt = p_{ch}(t)\,(\omega_{ch} - \overline{\omega}) = p_{ch}(t)\,(c\,(1 - p_{ch}(t))) \qquad [5]$$

As it can be seen from the equations above, the change in frequency of the strains, when they are mixed, only depends on the c value and not the b value. This is because the model assumes that the public good is fully and equally accessible by each player (both *coop* and *ch*) in the game.

In particular, the change in frequency of the cheater is given by:

$$p_{ch}(t) = (p_{ch}(0)\ e^{ct}) / ((1 - p_{ch}(0)) + p_{ch}(0)\ e^{ct}) \qquad [6]$$

The equation above describes that by knowing the initial frequency of the cheater ($p_{ch}(0)$), the cost of the cooperative trait (c), and the time duration (t) that the competition takes (cumulative numbers of cell divisions are used as arbitrary units of time in our model, see Appendix), the frequency of the cheater at that given time ($p_{ch}(t)$) can be predicted.

By combining [3] and [6], we can express $\overline{\omega}$ as:

$$\overline{\omega} = \omega_0 + (b - c)\ (1 - (p_{ch}(0)\ e^{ct}) / ((1 - p_{ch}(0)) + p_{ch}(0)\ e^{ct})) \qquad [7]$$

As can be seen by the equation above (Equation [7]), the mean fitness is affected both by the benefit gained from the cooperative trait (b) and the cost spent in the cooperative trait (c), as well as the basal fitness (ω_0), and the initial frequency of the cheater ($p_{ch}(0)$).

When we use this mathematical model to simulate a competition between a cooperator and a cheater for a public good that is equally accessible by both of the players, we obtain the simulation in Figure 34.

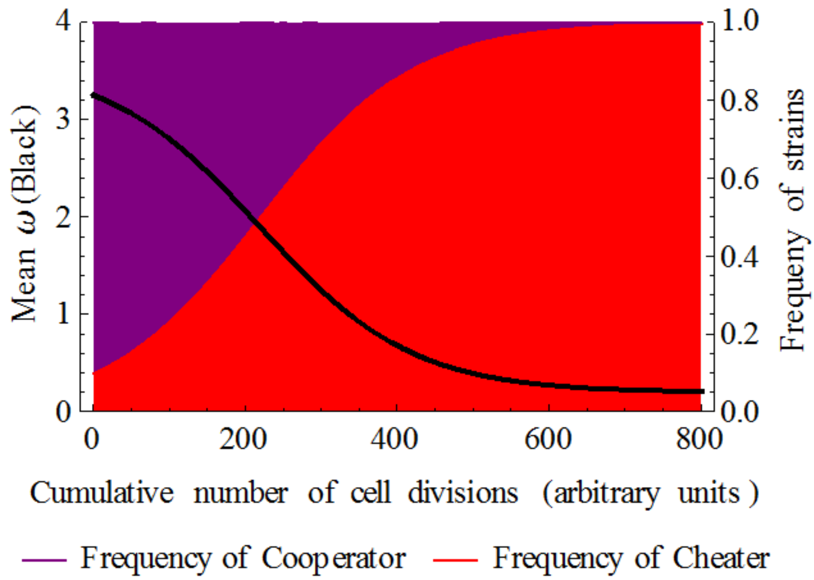


Figure 34. Dynamics of 'one trait-one constraint' cooperator - cheater competition

Left Y-axes show $\bar{\omega}$, the mean fitness of the entire population, which is a proxy of OD_{600} or CFUs/ml values prior to subculture (black line) (Equation [7]). Right Y-axes show the frequencies of p_{coop} (e.g. WT, purple), p_{ch} (e.g. *lasR*, red) (Equation [5]). X-axes show the time, represented as the cumulative number of cell divisions as arbitrary units to relate the dynamics in Chapter II (see Appendix). Values given to the parameters: $p_{coop}(0)$= 0.9, $p_{ch}(0)$=0.1, c=0.01, b=3.4, ω_0=0.2.

As it can be seen in the figure above, the mutant (*ch*, red), reaches fixation given enough time (numbers of cumulative cell divisions, see Appendix). The increase in the frequency of the mutant also causes a drastic decrease in the mean fitness. This can be interpreted as the tragedy of the commons. Here the magnitude of the cost spent, or the *c* value, is the determinant of the onset of the tragedy of the commons (Figure 35).

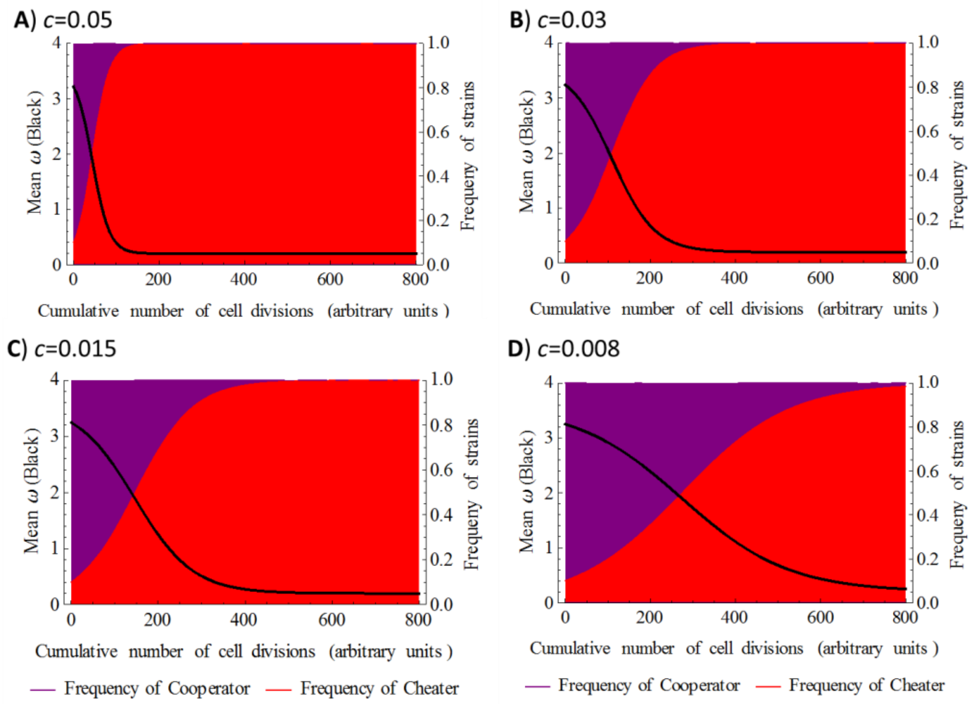


Figure 35. The cost of public good production determines the onset of the tragedy of the commons.

Axes and the rest of the parameters are as in Figure 34 except for the *c* values which are shown on the figure and noted for each panel.

The fitness equations show that the benefit gained from the public goods (*b*) can only affect the magnitude of the decrease in the mean fitness once the cheater reaches a frequency near fixation. To simulate this we give various *b* values to our model (Figure 36).

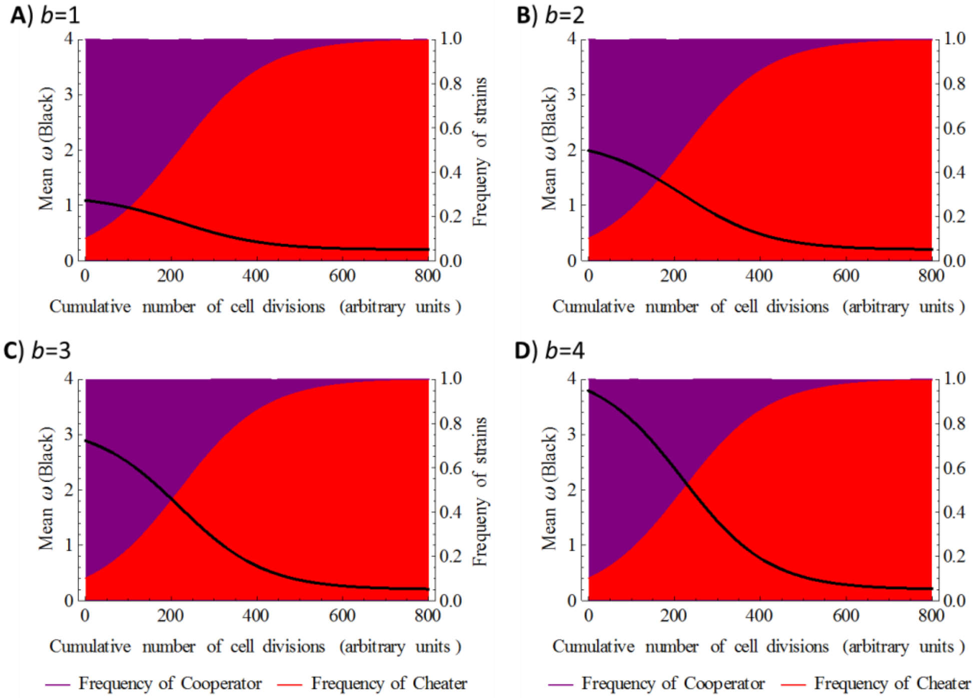


Figure 36. The benefit gained from the public good production determines the severity of the tragedy of the commons.

Axes and the rest of the parameters are as in Figure 34 except for the *b* values which are shown on the figure and noted for each panel.

Simulations of quorum sensing regulation for preventing cooperation from getting extinct

Quorum sensing is a mechanism that allows bacteria to regulate certain genes via asserting their cell densities by secreting and detecting small signalling molecules called autoinducers. When the concentration of these molecules increases, the regulation of associated genes in the bacterial genome also alters. This allows bacteria to control the expression of these genes depending on the numbers of cells in a given space (Figure 5). QS is known to control various public good synthesis genes. This allows bacteria to produce public goods only when the cell densities of the producers are high enough to get the benefits of this action directly (Allen et al., 2016; Pollitt et al., 2014; Popat et al., 2012; Wagner et al., 2003). Therefore, QS is hypothesized to be one of the mechanisms to prevent cheater invasions. We tested this in an experimental setting demonstrated in Chapter II indeed QS can prevent the cooperators from an extinction (Figure 30).

Table 3. Parameters for the QS regulation

Symbols	Descriptions
th	Quorum threshold (as a function of the non-QS strain frequency)
n	Hill coefficient for the slope of the inhibition of the QS-regulated public good

We, therefore, want to show with a simple mathematical model which parameters affect the stabilization of cooperation in a cooperative system with QS regulation (Table 3). To do that we chose to use a Hill function to model QS regulation of a PG trait as shown below:

$$f(p_{ch}) = 1 / (1 + (p_{ch} / th)^n) \qquad [8]$$

Then we introduced this Hill function (Equation [8]) to our simple model (Equations [1] and [2]), where the cost (c) and benefit (b) of the cooperative trait are reduced with a slope, n, when the frequency of the cheater (p_{ch}) reaches a given threshold value, th.

$$\omega_{coop} = \omega_0 + b\,(1 - p_{ch})\,(1 / (1 + (p_{ch} / th)^n)) - c\,(1 / (1 + (p_{ch} / th)^n)) \quad [9]$$

$$\omega_{ch} = \omega_0 + b\,(1 - p_{ch})\,(1 / (1 + (\mathrm{p_{ch}} / th)^n) \quad [10]$$

According to these fitness equations, the frequencies can be expressed as:

$$dp_{coop} / dt = p_{coop}(t)\,(\omega_{coop} - \overline{\omega})$$

$$= p_{coop}(t)\,((-c\,p_{ch}(t)) / ((p_{ch}(t) / th)^n + 1)) \quad [11]$$

$$dp_{ch}/ dt = p_{ch}(t)\,(\omega_{ch} - \overline{\omega})$$

$$= p_{ch}(t)\,((c\,(1 - p_{ch}(t))) / ((p_{ch}(t) / th)^n + 1)) \quad [12]$$

In this scenario, the mean fitness would be expressed as:

$$\overline{\omega} = \Sigma_{\mathrm{i}}\, p_i(t)\,\omega_i = \omega_0 + p_{coop}(t)\,\omega_{coop} + p_{ch}(t)\,\omega_{ch}$$

$$= \omega_0 + ((b - c)\,(1 - p_{ch}(t))) / ((p_{ch}(t) / th)^n + 1) \quad [13]$$

When we use this mathematical model to simulate a competition between a cooperator and a cheater for a public good where there is QS regulation for the cooperative trait, we obtain the numerical solutions below (Figure 37 and Figure 38).

As it can be seen in Figure 37, the rate of inhibition of PG trait, which decreases the cost and the benefit of the trait gradually, given a fixed threshold value (th), can affect the outcome of the PG competition between the cooperator (which has an intact QS system) and the cheater (which is a mutant in QS regulation and the production of the PG that is regulated via that QS system). The smaller the n value is, the longer it takes for QS regulation to diminish the cost and benefit of the PG trait (or in biological scenarios, it takes longer for the genes responsible for PG production to turn off). Thus smaller *n* values are more likely to fail to preserve cooperation from getting extinct (as in blue and orange lines). The mean fitness of the populations, however, decreases to the monoculture cheater levels in all cases, as the benefit is diminished via QS when the frequency of the cheater increases. This decrease happens earlier when the *n* value is higher.

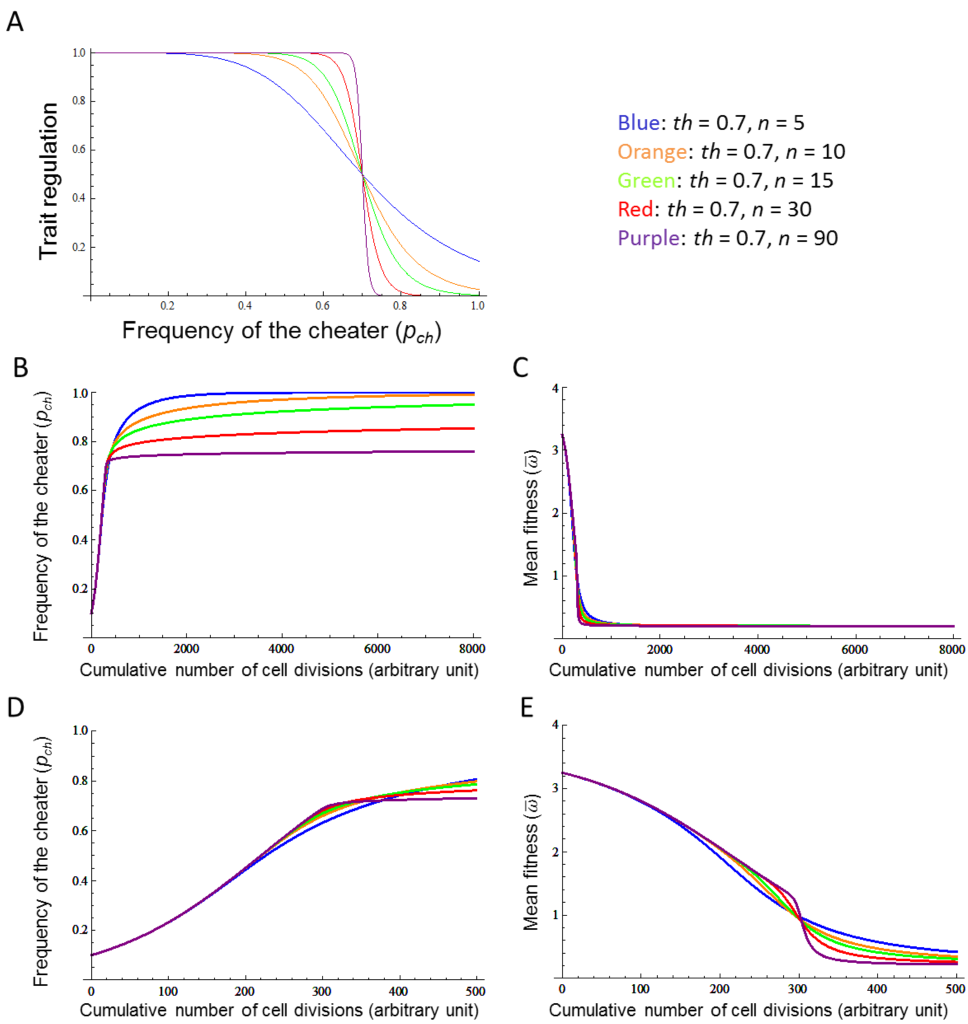


Figure 37. Simulations demonstrating the effects of the QS regulation via gradual inhibition with a rate *n*

(A) shows how various values of *n*, can affect the regulation of the PG trait via QS regulation, X-axis shows the frequency of the cheater (p_{ch}), Y-axis shows the level of trait regulation (1 means gene is fully ON, or cost and benefit are at maximum, and 0 means gene is fully OFF, or cost and benefit are 0). **(B)** shows the effects of different rates of PG inhibition via QS regulation on the frequency of the cheater during a competition with a cooperator. **(C)** shows the effects of different rates of PG inhibition via QS regulation on the mean fitness of the population. **(D)** and **(E)** are the same as (B) and (C) but show a smaller window of time during the competition to assess the differences on the frequency of the cheater and the mean fitness that different values of *n* causes. Y-axes in (B) and (D) show the frequency of the cheater (p_{ch}). Y-axes in (C) and (E) show the mean fitness ($\overline{\omega}$). X-axes in (B), (C), (D), and (E) show the time as cumulative number of cell divisions as an arbitrary unit. The rest of the parameters are as in Figure 34.

Figure 38 shows that the threshold of inhibition of the PG trait, which decreases the cost and the benefit of the trait when a certain level of frequency of the cheater is reached, given a fixed rate of decrease (*n*), can affect the outcome of the PG competition between the cooperator (which has an intact QS system) and the cheater (which is a mutant in QS regulation and the production of the PG that is regulated via that QS system). The higher the *th* value is, the more likely is for the cheater to reach fixation (p_{ch} = 1). Thus higher *th* values are more likely to fail to preserve cooperation from getting extinct (as in red and purple lines). The mean fitness of the populations, however, decreases to the monoculture cheater levels in all cases, as the benefit is diminished via QS when the frequency of the cheater increases. This decrease happens earlier when the *th* value is smaller.

As it can be seen in our simulations, a quorum sensing mechanism that diminishes the cost and the benefit of the public good production can prevent the total fixation of the cheater by removing its competitive advantage that comes from the cost paid by the cooperator. Thus the cooperators can avoid extinction.

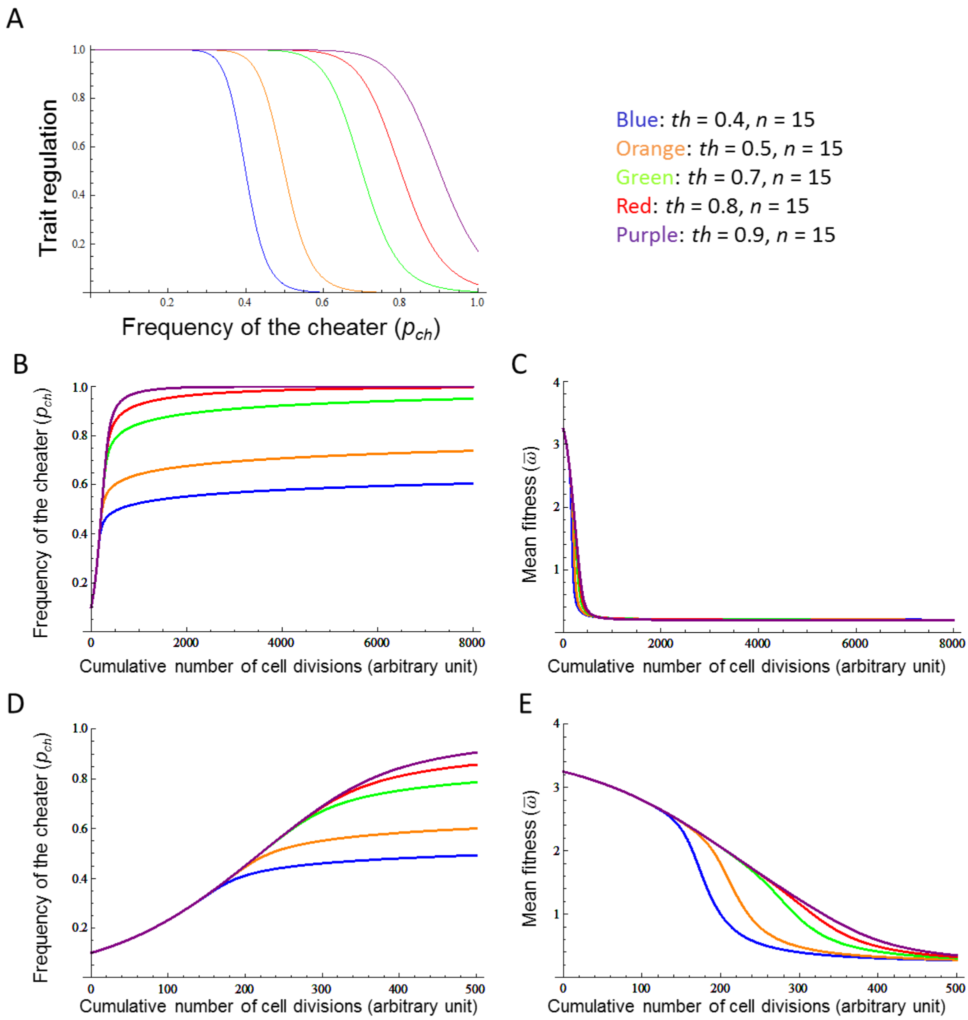


Figure 38. Simulations demonstrating the effects of the QS regulation via inhibition at a threshold *th*

(A) shows how various values of *th*, can affect the regulation of the PG trait via QS regulation, X-axis shows the frequency of the cheater (p_{ch}), Y-axis shows the level of trait regulation (1 means gene is fully ON, or cost and benefit are at maximum, and 0 means gene is fully OFF, or cost and benefit are 0). **(B)** shows the effects of different thresholds of PG inhibition via QS regulation on the frequency of the cheater during a competition with a cooperator. **(C)** shows the effects of different thresholds of PG inhibition via QS regulation on the mean fitness of the population. **(D)** and **(E)** are the same as (B) and (C) but show a smaller window of time during the competition to assess the differences in the frequency of the cheater and the mean fitness that different values of *th* causes. Y-axes in (B) and (D) show the frequency of the cheater (p_{ch}). Y-axes in (C) and (E) show the mean fitness ($\overline{\omega}$). X-axes in (B), (C), (D), and (E) show the time as cumulative number of cell divisions as an arbitrary unit. The rest of the parameters are as in Figure 34.

Changing roles in a simultaneously played multiple public goods games

After studying a single cooperative trait and its associated cooperator-cheater dynamics on public goods games, we focused on the multiple public goods games played by a full cooperator (*coop.*) and the cheaters of each public goods production (ch_1 and ch_2). We modified the model in the previous section. This model also assumes that the costs (c_1 and c_2) of the cooperative traits are lower than the benefits (b_1 and b_2) of their associated traits ($b_1>c_1>0$ and $b_2>c_2>0$). The model also assumes that the benefits provided by the cooperative traits are equally accessible by the entire population, as it would be in the case of an equally accessible public good in a well-mixed environment. Spatial structure, diffusion, or privatization, which would alter the benefit gained from the public good for cooperators and cheaters asymmetrically, were not considered in this model either. The parameters used are described in Table 4.

Table 4. Parameters for the mathematical model for the 3-way public goods game

Symbols	Descriptions
c_1	Cost of the 1st cooperative trait
c_2	Cost of the 2nd cooperative trait
b_1	Benefit gained from the 1st cooperative trait
b_2	Benefit gained from the 2nd cooperative trait
ω_0	Fitness without the additional fitness effects of the cooperative traits (basal fitness)
ω_{coop}	Fitness of the cooperator of the both cooperative traits
ω_{ch1}	Fitness of the cheater of the 1st cooperative trait
ω_{ch2}	Fitness of the cheater of the 2nd cooperative trait
$\bar{\omega}$	Mean fitness of the entire population (A proxy for OD_{600} or CFUs/ml)
p_{coop}	Frequency of the cooperator of the both cooperative traits in the entire population
p_{ch1}	Frequency of the cheater of the 1st cooperative trait in the entire population
p_{ch2}	Frequency of the cheater of the 2nd cooperative trait in the entire population

1. Simple 3-way public goods model

We define the fitness of a cooperator and two cheaters mixed in an environment where both traits that these mutants cheat on as:

$$\omega_{coop} = \omega_0 + b_1 (1 - p_{ch1}) + b_2 (1 - p_{ch2}) - c_1 - c_2 \quad [14]$$

$$\omega_{ch1} = \omega_0 + b_1 (1 - p_{ch1}) + b_2 (1 - p_{ch2}) - c_2 \quad [15]$$

$$\omega_{ch2} = \omega_0 + b_1 (1 - p_{ch1}) + b_2 (1 - p_{ch2}) - c_1 \quad [16]$$

As can be seen from the fitness definitions of these three players, the cheaters always have a higher fitness than the cooperator due to the costs (c_1 or c_2) saved. Assuming a homogeneous environment, and ignoring stochastic effects, the population changes according to the replicator equation system:

$$dp_{coop} / dt = p_{coop}(t) (\omega_{coop} - \bar{\omega}) = p_{coop}(t) (- c_1 p_{ch1}(t) - c_2 p_{ch2}(t)) \quad [17]$$

$$dp_{ch1} / dt = p_{ch1}(t) (\omega_{ch1} - \bar{\omega}) = p_{ch1}(t) (c_1 (1 - p_{ch1}(t)) - c_2 p_{ch2}(t)) \quad [18]$$

$$dp_{ch2} / dt = p_{ch2}(t) (\omega_{ch2} - \bar{\omega}) = p_{ch2}(t) (c_2 (1 - p_{ch2}(t)) - c_1 p_{ch1}(t)) \quad [19]$$

The change in the mean fitness is given by:

$$\bar{\omega} = \Sigma_i p_i(t) \omega_i = \omega_0 + p_{coop}(t) \omega_{coop} + p_{ch1}(t) \omega_{ch1} + p_{ch2}(t) \omega_{ch2}$$

$$= \omega_0 + (b_1 - c_1) (1 - p_{ch1}(t)) + (b_2 - c_2) (1 - p_{ch2}(t)) \quad [20]$$

Figure 39 shows the predicted mean fitness and final frequencies of the different strains in the population assuming different c_1/c_2 ratios. It can be easily seen that cooperators will always go extinct and that the two cheaters can only co-exist when $c_1 = c_2$.

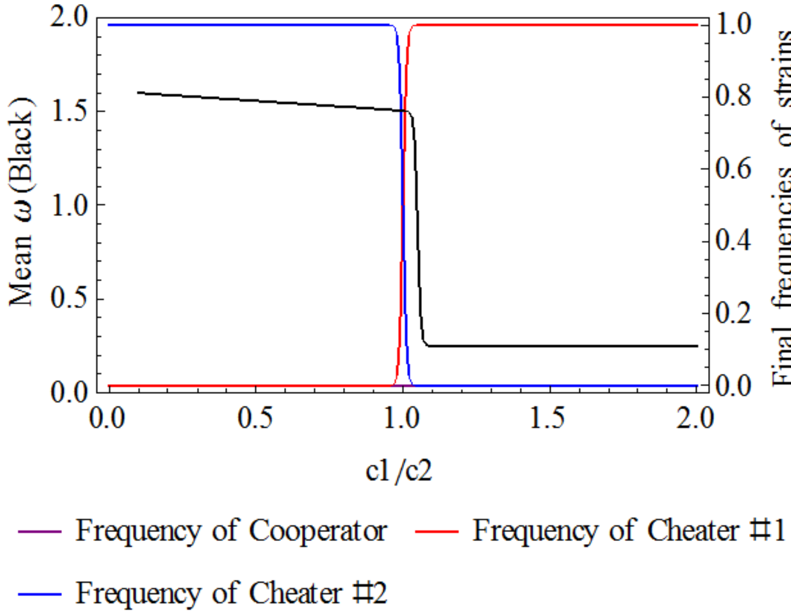


Figure 39. Mathematical model for the final frequencies of the three strains in relation to the ratio of c_1/c_2

In Right-Y axes, frequencies of cooperator of both cooperative traits (**purple**), cheater of the 1st cooperative trait (**red**), and cheater of the 2nd cooperative trait (**blue**) in relation to the ratio of c_1/c_2 either. In Left-Y axes, the mean fitness, $\bar{\omega}$, is shown in **black**. The values given to the parameters of the simulations are: $p_{coop}(0)$=0.8, $p_{ch1}(0)$=0.1, $p_{ch2}(0)$=0.1, 0.001≤c_1<0.199, b_1=1.5, c_2=0.1, b_2=0.25, ω_0=0.1, time (as arbitrary CCD)=1800.

Whenever $c_1 \neq c_2$, then the cheater that produces the more costly trait will lose. Therefore, the relation between c_1 and c_2 determines which cheater will dominate the population, independently of the benefits of these cooperative traits. On the other hand, the yield of the population will depend on, $\bar{\omega}$, which is affected by the difference between b and c values of each trait.

We simulated the four scenarios (Figure 40) corresponding to the conditions in Figure 20. In Figure 40A and Figure 40C, the cooperator for both traits (WT) and the cheater of the 1st cooperative trait compete ($p_{coop}(0)$ = 0.9 and $p_{ch1}(0)$ = 0.1), while the cheater of the 2nd cooperative trait is absent (hence $p_{ch2}(0)=0$). Whereas, in Figure 40B and Figure 40D all three strains compete ($p_{coop}(0)$ = 0.8 and $p_{ch1}(0) = p_{ch2}(0)$ = 0.1). In Figure 40A and Figure 40B, only the 1st cooperative trait is produced ($b_1 > c_1 > 0$, whereas $b_2 = c_2 = 0$) while in Figure 40C and Figure 40D both traits are expressed ($c_2 > c_1 > 0$ and $b_1 > b_2 > 0$). The c_1/c_2 ratio reflects the ratios of the relative fitnesses in Figure 17C and Figure 17D. The time scale of the simulations reflects the CCD as a more meaningful scale than days or numbers of generations (Lee et al., 2011; Luria and Delbrück, 1943). CCD values were chosen according to the final number of cells measured in the competition in Figure 17, where the CCD were significantly higher in iron-supplied casein medium than in iron-depleted casein medium for the same time period. ω_0, b_1, and b_2 are chosen to reflect the growth yields in Figure 15 and Figure 14B.

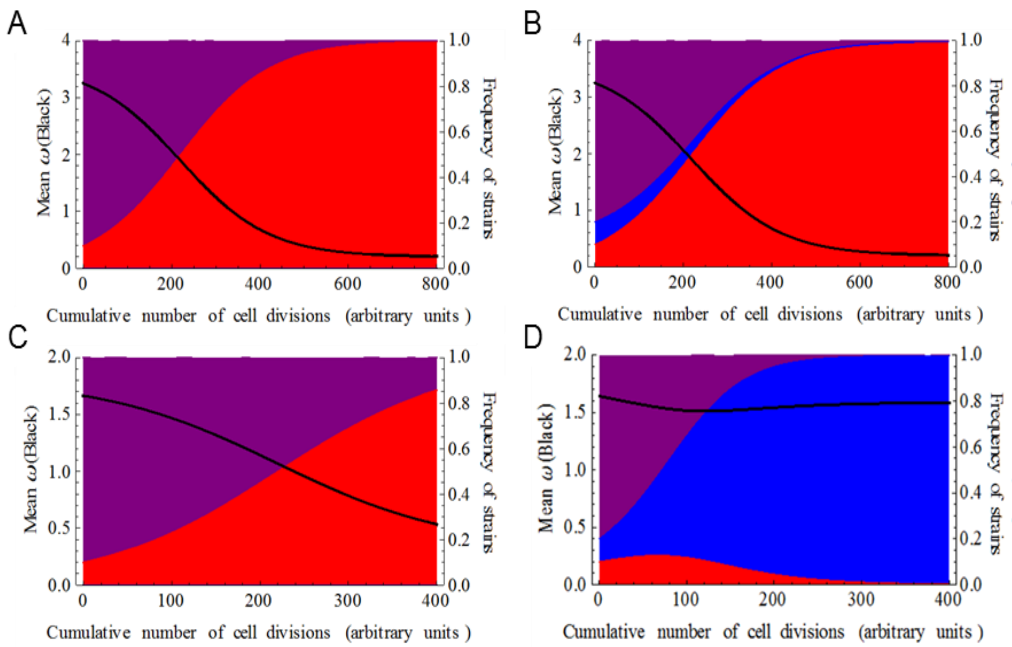


Figure 40. Simulation of four main scenarios similar to Figure 20

(A) one mutant-one constraint, **(B)** two mutants-one constraint. **(C)** one mutant-two constraints, **(D)** two mutants-two constraints. Left Y-axes show $\bar{\omega}$, the mean fitness of the entire population, which is a proxy of OD600 or CFUs/ml values prior to subculture (black lines). Right Y-axes show the frequencies of pcoop (e.g. WT, purple), p_{ch1} (e.g. *lasR*, red) and p_{ch2} (e.g. *pvdS*, blue). X-axes show the cumulative number of cell divisions as arbitrary units. The values that are given to the parameters of the simulations are: (A) $p_{coop}(0)$=0.9, $p_{ch1}(0)$=0.1, pch2(0)=0, c_1=0.01, b_1=3.4, c_2=0, b_2=0, ω_0=0.2; (B) $p_{coop}(0)$=0.8, $p_{ch1}(0)$=0.1, $p_{ch2}(0)$=0.1, c_1=0.01, b_1=3.4, c_2=0, b_2=0, ω_0=0.2; (C) $p_{coop}(0)$=0.9, $p_{ch1}(0)$=0.1, $p_{ch2}(0)$=0, c_1=0.01, b_1=1.5, c_2=0.025, b_2=0.25, ω_0=0.1; (D) $p_{coop}(0)$=0.8, $p_{ch1}(0)$=0.1, $p_{ch2}(0)$=0.1, c_1=0.01, b_1=1.5, c_2=0.025, b_2=0.25, ω_0=0.1. Note that the values of parameters used in these simulations are chosen to reflect approximately the relation between the values observed in Figure 15, Figure 14, Figure 17, Figure 20.

The results of the model for the four scenarios (Figure 40) resemble the experimental data from Figure 20, explaining changes in frequencies and the mean fitness observed reasonably well. However, this simple model always predicts complete fixation of the winning mutant (Figure 40), and cannot explain the lack of fixation of the mutants observed experimentally with *lasR* (Figure 20 A, B, and C) and *pvdS* (Figure 20D).

2. Simple 3-way public goods model including quorum sensing

Given that the *lasR* gene and elastase production are regulated via quorum sensing, we hypothesized that QS could be responsible for the lack of fixation of *lasR* mutant. QS regulation should reduce both the cost and the benefit of elastase production when the cooperators are below the QS threshold as the cells will not be producing it. We therefore modelled the effect of QS on fitness equations by assuming a Hill function where the cost (c_1) and benefit (b_1) of the 1st cooperative trait are sharply reduced when the frequency the cheater of the1st cooperative trait (p_{ch1}) reaches a given threshold value (th) (Equation [8])

Fitness equations of the cooperator and the cheaters are given as:

$$\omega_{coop} = \omega_0 + b_1 (1 - p_{ch1}) (1 / (1 + (p_{ch1} / th)^n)) + b_2 (1 - p_{ch2}) - c_1 (1 / (1 + (p_{ch1} / th)^n)) - c_2 \quad [21]$$

$$\omega_{ch1} = \omega_0 + b_1 (1 - p_{ch1}) (1 / (1 + (p_{ch1} / th)^n)) + b_2 (1 - p_{ch2}) - c_2 \quad [22]$$

$$\omega_{ch2} = \omega_0 + b_1 (1 - p_{ch1}) (1 / (1 + (p_{ch1} / th)^n)) + b_2 (1 - p_{ch2}) - c_1 (1 / (1 + (p_{ch1} / th)^n)) \quad [23]$$

The change in the mean fitness is given by:

$$\overline{\omega} = \omega_{0\,+} ((b_1 - c_1) (1 - p_{ch1}(t)) / (1 / (1 + (p_{ch1} / th)^n))) + (b_2 - c_2) (1 - p_{ch2}(t)) \quad [24]$$

In this case, fixation of one mutant can only happen if $c_1 < c_2$. When $c_1 \geq c_2$, both cheaters can co-exist in the population (Figure 41).

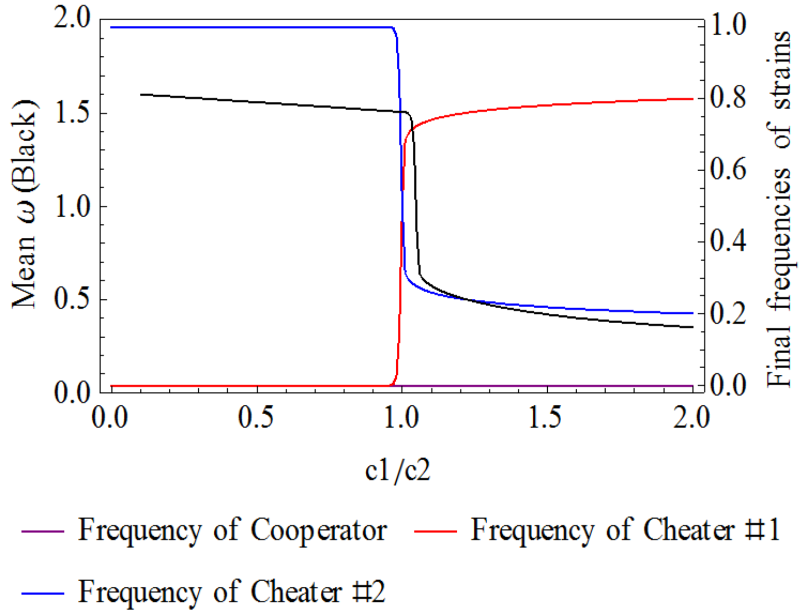


Figure 41. Mathematical model for the final frequencies of the three strains in relation to the ratio of c_1/c_2, with the influence of quorum sensing (QS) regulation on the 1st cooperative trait

Axes are as in Figure 39. The values given to the parameters of the simulations are: $p_{coop}(0)$=0.8, $p_{ch1}(0)$=0.1, $p_{ch2}(0)$=0.1, 0.001≤c_1<0.199, b_1=1.5, c_2=0.1, b_2=0.25, ω_0=0.1, time (as arbitrary CCD)=1800, *n*=30 and *th*=0.8.

As shown in Figure 42, the results of the simulations of the modified model including QS for the four experimental conditions predict accurately both the frequency dynamics and the reduction in population size (assumed to be related to the mean fitness) as the cheaters spread. It is also now clear that by adding the QS regulation to the model the simulation predicts that *lasR* will not reach fixation.

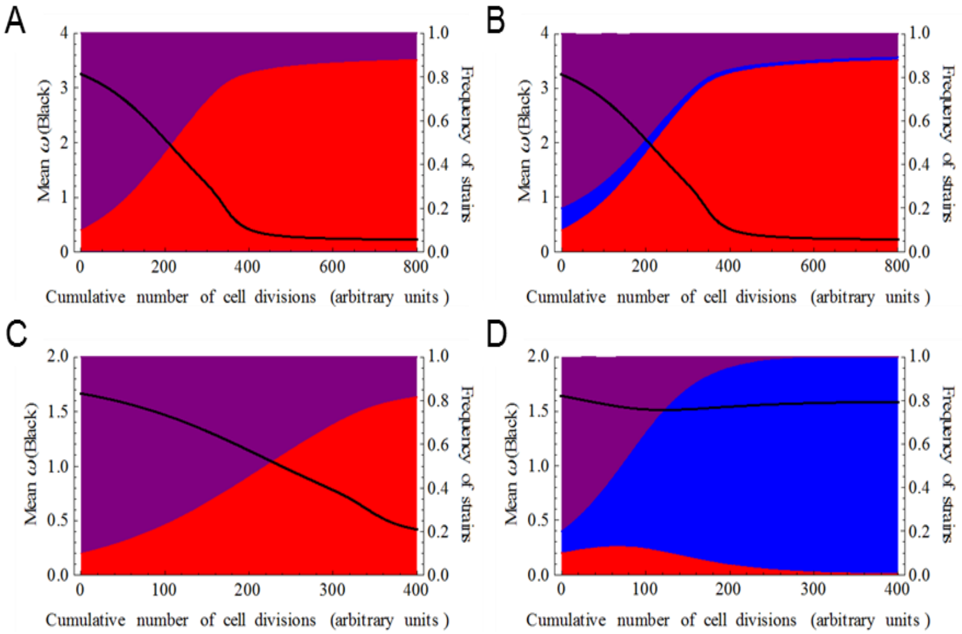


Figure 42. Results of the mathematical model simulating the four scenarios in Figure 20

Model includes quorum sensing regulation of the 1st cooperative trait (b_1 and c_1 are negatively regulated via a Hill equation as a function of the frequency of the mutant of this trait, p_{ch1}). Left Y-axes show $\bar{\omega}$, the mean fitness of the entire population which is a proxy of OD_{600} or CFUs/ml values prior to subculture (black lines). Right Y-axes show the frequencies of p_{coop} (e.g. WT, purple), p_{ch1} (e.g. *lasR*, red) and p_{ch2} (e.g. *pvdS,* blue). X-axes show the number of cell divisions as arbitrary units. The values that are given to the parameters of the simulations are: (A) $p_{coop}(0)$= 0.9, $p_{ch1}(0)$=0.1, $p_{ch2}(0)$=0, c_1=0.01, b_1=3.4, c_2=0, b_2=0, ω_0=0.2, *th*=0.8, *n*=30; (B) $p_{coop}(0)$= 0.8, $p_{ch1}(0)$=0.1, $p_{ch2}(0)$=0.1, c_1=0.01, b_1=3.4, c_2=0, b_2=0, ω_0=0.2, *th*=0.8, *n*=30; (C) p_{coop}(0)= 0.9, p_{ch1}(0)=0.1, p_{ch2}(0)=0, c_1=0.01, b_1=1. 5, c_2=0.025, b_2=0.25, ω_0=0.1, *th*=0.8, *n*=30; (D) p_{coop}(0)= 0.8, p_{ch1}(0)=0.1, p_{ch2}(0)=0.1, c_1=0.01, b_1=1.5, c_2=0.025, b_2=0.25, ω_0=0.1, *th*=0.8, *n*=30.

We also simulated alternative scenarios in an environment where both traits are needed without QS regulation or with QS regulation (only the 1st cooperative trait is regulated by QS):

When only the cooperator of both cooperative traits (e.g. WT) and the cheater of the 2nd cooperative trait (which is not regulated by quorum sensing, e.g. *pvdS*) are in competition, the cheater wins and reaches fixation as in the triple co-culture scenario, regardless of QS regulation of the 1st cooperative trait (Figure 43), while the mean fitness becomes:

$$\bar{\omega} = \omega_0 + b_1 - c_1 \quad [25]$$

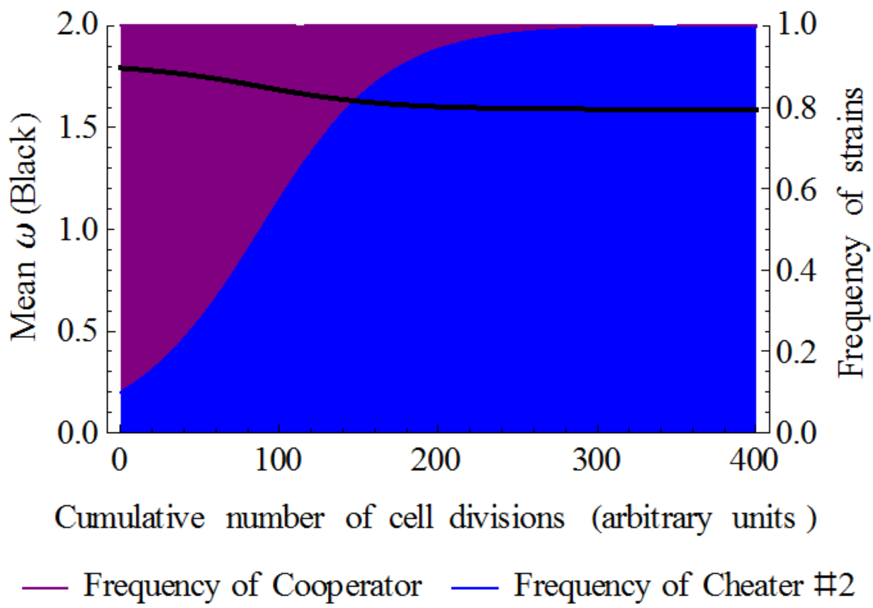


Figure 43. Simulation with two strains, full cooperator (WT) + cheater for the 2nd cooperative trait, under conditions where both public goods are produced and $c_2 > c_1$

Left Y-axis show $\bar{\omega}$, the mean fitness of the entire population which is a proxy of OD_{600} or CFUs/ml values prior to subculture (black lines). Right Y-axis show the frequencies of p_{coop} (purple), and p_{ch2} (blue). X-axis shows the cumulative number of cell divisions as arbitrary units. The values given to the parameters of the simulations were: $p_{coop}(0)$=0.9, $p_{ch1}(0)$=0, $p_{ch2}(0)$=0.1, c_1=0.01, b_1=1.5, c_2=0.025, b_2=0.25, ω_0=0.1. The results were the same regardless if the 1st was considered to be regulated by QS (n=30, th=0.8) or not (n=0, th=0).

When only two cheaters are in 1:1 competition, the cheater that saves the greater cost (here, the cheater of the 2nd cooperative trait since $c_2>c_1$) wins the competition and reaches fixation regardless of QS regulation of the 1st cooperative trait (Figure 44), while the mean fitness becomes:

$$\bar{\omega} = \omega_0 + b_1 - c_1 \quad [26]$$

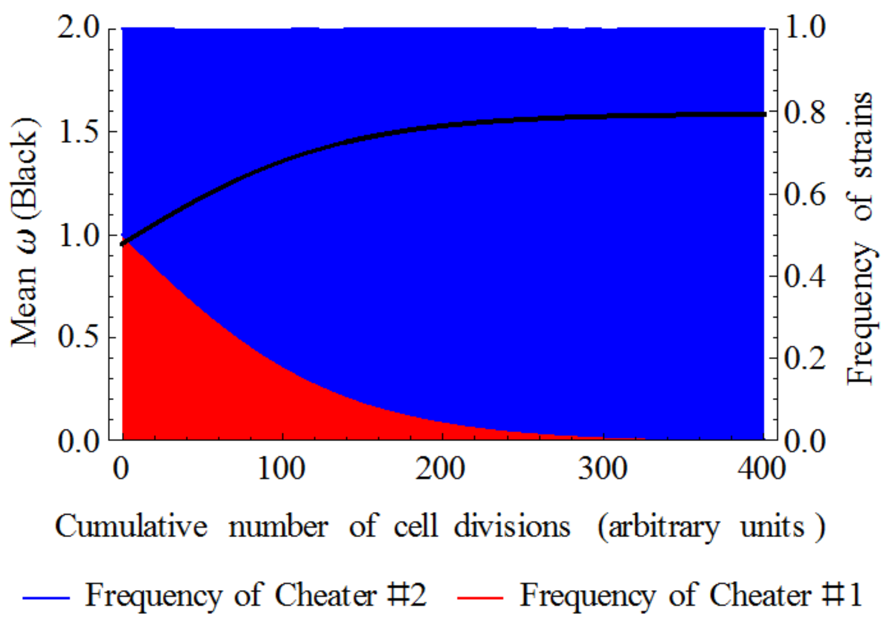


Figure 44. Simulation with the two cheaters competing with each other, under conditions where both public goods are produced and $c_2>c_1$
Frequencies of p_{ch1} (red), and p_{ch2} (blue) are shown on the Right Y-axis. The other axes are as described in Figure 43. The values given to the parameters of the simulations were: $p_{coop}(0)$=0, $p_{ch1}(0)$=0.5, $p_{ch2}(0)$=0.5, c_1=0.01, b_1=1.5, c_2=0.025, b_2=0.25, ω_0=0.1. The results were the same

regardless if QS regulation for the 1st cooperative trait was considered (n=30, th=0.8) or not (n=0, th=0).

When the cooperator of the both cooperative traits (e.g. WT) is competing with two mutants under conditions where the costs of both traits are equal (***c_1=c_2***), both cheaters increase in frequency until both of them reach 50% of the population (Figure 45), similarly with or without QS regulation of the 1st cooperative trait, while the mean fitness becomes:

$$\bar{\omega} = \omega_0 + ½\,(b_1 + b_2 - c_1 - c_2) \quad [27]$$

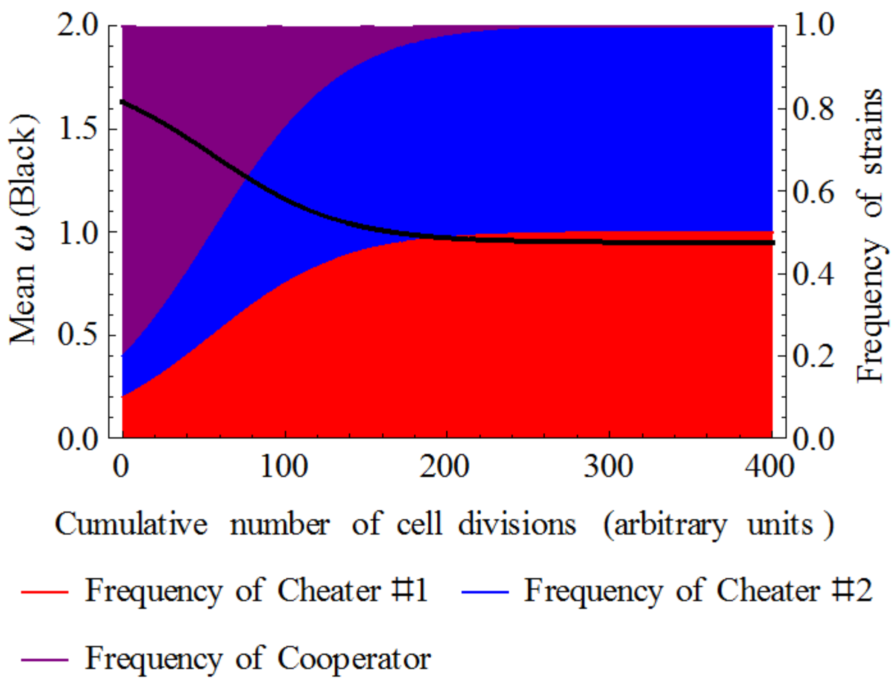


Figure 45. Simulation for a 3-way competition with the cooperator of the both cooperative traits competing with two cheaters, under conditions where both public goods are produced and c_2=c_1

Frequencies of p_{coop} (purple), p_{ch1} (red), and p_{ch2} (blue) are shown on the Right Y-axis. The other axes are as described in Figure 43. The values given to the parameters of the simulations were: $p_{coop}(0)$=0.8, $p_{ch1}(0)$=0.1,

$p_{ch2}(0)$=0.1, c_1=0.025, b_1=1.5, c_2=0.025, b_2=0.25, ω_0=0.1.The results were the same regardless if QS regulation for the 1st cooperative trait was considered (n=30, th=0.8) or not (n=0, th=0).

When the cooperator of the both cooperative traits (e.g. WT) is competing with two mutants under conditions where the $c_1 > c_2$, then the more drastic tragedy-inducing cheater becomes the winner of the 3-way competition. In this case, when the 1st cooperative is not regulated by QS, the cheater of the 1st cooperative trait wins the 3-way competition and causes a drastic collapse, with the mean fitness (Figure 46A):

$$\bar{\omega} = \omega_0 + b_2 - c_2 \qquad [28]$$

However, when the 1st cooperative trait is regulated by QS, the cheater of the 1st cooperative, while it still wins the competition, it can only increase in frequency until the QS threshold (th=0.8) and thus, cannot reach fixation (Figure 46B); and the mean fitness becomes:

$$\bar{\omega} = \omega_0 + (b_1 - c_1)\,(0.4) + (b_2 - c_2)\,(0.8) \qquad [29]$$

As a result, the cooperator of the both cooperative traits (e.g. WT) persists in the population. Therefore, presumably, the population has a greater chance to recover if the environmental conditions change. In conclusion, the QS regulation becomes relevant only under conditions where the mutant for the QS-regulated trait (here the cheater of the 1st cooperative trait) is not completely outcompeted.

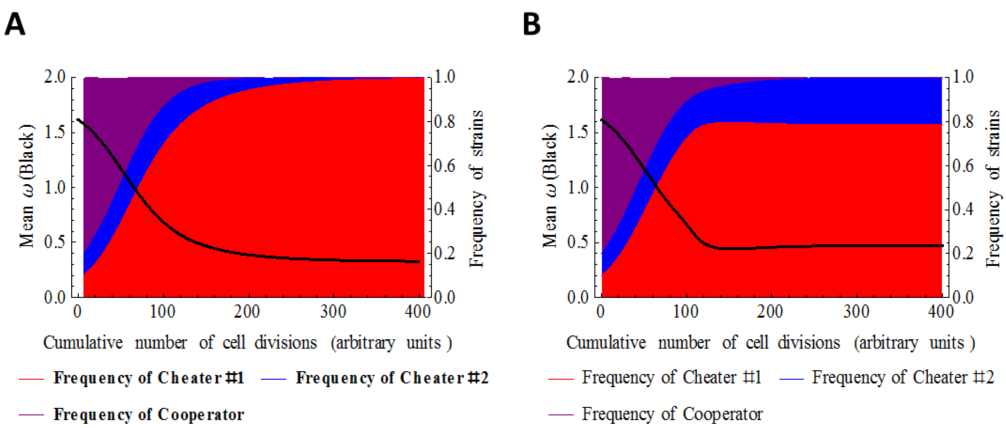


Figure 46. Simulation for a 3-way competition with the cooperator of the both cooperative traits competing with two cheaters, under conditions where both public goods are produced and c_1>c_2

Frequencies of p_{coop} (purple), p_{ch1} (red) and p_{ch2} (blue) are shown on the Right Y-axes. The other axes are as described in Figure 43. The values given to the parameters of the simulations were: **(A)** $p_{coop}(0)$=0.8, $p_{ch1}(0)$=0.1, $p_{ch2}(0)$=0.1, c_1=0.04, b_1=1.5, c_2=0.025, b_2=0.25, ω_0=0.1, n = 0, th = 0; **(B)** same as in (A) except n=30 and th=0.8, as QS regulation was considered for the 1st cooperative trait.

We conclude that quorum sensing regulation of production of a public good prevents full domination of the QS cheater, maintaining cooperation in populations. However, if the cheater which wins is affected in the production of a public good that is not regulated via QS (e.g. *pvdS*) this mutant can dominate the entire population. In summary, the results obtained from our mathematical model (Figure 42) show that the dynamics observed in our propagation experiments in Figure 20 can be explained by the relationship between the cost values of two orthogonal cooperative traits and a quorum threshold that regulates both costs and benefits of one of these traits.

Discussion

We showed that when there are no mechanisms to regulate the cost spent on producing the public good or the accessibility of the public good thus the benefit gained by each individual, the tragedy of the commons is inevitable in a continuously growing population in an unchanging environment. We further showed that the cost of the public good production determines the magnitude of the cheating or in other words the relative fitness advantage of the cheater. The difference between the cost and the benefit of the public goods, however, determines the severity of the population collapse when the cheater approaches fixation and causes a tragedy of the commons.

Importantly, the model presented here considers that the costs and the benefits of different PG traits can be different. As a consequence, the difference between the costs avoided by each cheater mutant determines which cheater wins the competition and the relation between the benefit and cost difference determines the level of collapse after that mutant wins the competition and reaches fixation.

In this chapter, we modelled QS regulation of a PG trait by using a Hill function. We assumed that an increase in cheater frequency would cause a decrease in the mean fitness (a proxy for cell densities). This assumption is supported by our experimental data in Chapter II. Thus, via a Hill function, our QS model decreases the cost and benefit of the PG trait that QS regulates when the frequency of the cheater increases. Using a Hill function allowed us to address the two aspects of quorum sensing regulation of PG traits, a threshold at which point gene regulation starts to be turned off, and a given rate for this alteration. We assume that QS regulation starts turning the genes for PG production off with a given rate (not instantaneously), and only after a threshold is reached by the cheater frequency. The predictions of our model for QS regulation are strikingly coherent to our experimental findings in Chapter II (Figure 30, and Figure 31).

Furthermore our model shows that when multiple players play multiple PG games, by changing their roles in each game, or in other words a tri-partite PG games with a full cooperator, a cheater for one trait, and another cheater for the second trait; the cheaters cease to be full cheaters since they cooperate on the trait that they are not cheating. This changes the classic dynamics of one trait-one constraint (uni-directional) PG games. Importantly, the predictions that the model supports also explain the experimental findings of tri-partite PG game dynamics in the frequency changes of the strains, the change in the growth yields (mean fitness) and the fixation or the lack of fixation of the non-producers of the public goods (Figure 20).

Overall, the simple mathematical model presented in this chapter sheds light on the understanding of which parameters govern the dynamics of the PG games in various scenarios. Knowing that a greater and unregulated cost could give a fitness advantage to one mutant strain to reach fixation in the population, or being able to determine the conditions that promote fixation of mutants of a high benefit – low cost trait that has the potential to cause a more drastic population collapse compared to a low benefit – high cost trait, can change the possible strategies for new experimental designs and therapeutic approaches to treat infections such as the ones affect CF patients. New cheater strains can be engineered to avoid a high costly trait with a minimum benefit which also cannot survive on its own (such as a mutant that can benefit the detoxification done by others but cannot survive in a toxic environment, e.g. a mutant in catalase or beta-lactamase production) to rapidly dominate the already existing virulent bacterial population to drive the entire population to an ultimate collapse.

Chapter IV: General Discussion

Preliminary notes

Part of this chapter is included in the publication titled as "Maintenance of cooperation for single and multiple social traits" which is a review paper that is under revision for Journal of Bacteriology.

Although there are a numerous and still growing number of well-designed studies to understand the conditions that enable maintenance of cooperative behaviors in bacterial communities, there are also challenges that limit the understanding of microbial social dynamics. Most previous studies have focused on one cooperative trait and study how that trait is maintained against possible cheater invasions. Each study characterizes mechanisms (See Chapter I – Mechanisms to prevent cheating in microbial populations) that play a role for the cooperative trait that is studied. This method is valid and certainly there are specific mechanisms for maintaining bacterial cooperation yet to be discovered. However, the methods for quantifying bacterial cheating have to be assessed and discussed carefully. Cheating behavior has to be defined precisely and should be quantified. Parameters that affect the magnitude of the cheating behavior have to be evaluated. Only after these challenges are faced, sociomicrobiology can move its frontiers further and help developing new therapeutic approaches for complex infections such as in the chronic infections in the lungs of CF patients.

First, it is important to determine if the cooperative trait of interest can be regulated by the cooperator as a response to external constraints. If the cooperative behavior is performed by the obligate cooperators, changing external factors can easily make cooperation vulnerable. When the cooperative trait is obligatory, meaning that it will be produced even when the product, the public good is costly but not giving any benefits, the non-producers would be vastly advantageous. This is considered as the most suitable explanation for why most cooperative traits are facultative (Allen et al., 2016; Bruger and Waters, 2015; Dumas et al., 2013; Ghoul et al., 2014a).

The fact that most cooperative traits are facultative brings the requirement of studying that particular trait in the condition that it is needed to increase the fitness of the cooperator or avoid a fitness decrease. An example of a well-documented facultative cooperation is the production of iron-siderophore molecule pyoverdine in *P. aeruginosa* studied here. Pyoverdine production is induced by the depletion of the iron concentration (Schalk and Guillon, 2013). To study pyoverdine production as a cooperative trait, first, iron concentration has to be depleted to the levels that the pyoverdine producing strain produces it in significant amounts that result in a fitness advantage in that environment (Ghoul et al., 2014b; Kümmerli and Ross-Gillespie, 2013). Otherwise, neither a cost nor a benefit due to this trait should be expected (Ghoul et al., 2014b). The advantage of the cheaters comes from avoiding the cost and reaping the benefits. When there are no costs to avoid or benefits to gain, cheating cannot be expected.

The work presented in Chapter II shows that the mutants that are avoiding the cost of producing pyoverdine (*pvdS*) or elastase (*lasR*) can only behave as cheaters when the cost of producing pyoverdine or elastase is significantly great for the producers (Figure 17). We show that the change in cheating indicates the change in cost spent by the producers and this change can be controlled by altering the environment via adding or removing abiotic constraints (Figure 32).

Also to study cheating, the mutant has to be carefully selected. If the strain is isolated after spontaneous evolution, the genome of the mutant is better to be sequenced to rule out any other mutations which could affect the conclusion of the experiment. Asfahl et al. showed that when WT *P. aeruginosa* evolved in casein media, *lasR* mutations spontaneously arose. However before these mutations arose, an earlier mutation in *psdR* gene which gave cells a non-social advantage in the intake of dipeptides, the products of the digestion of casein by elastase, swept through the population, diminishing the cheating advantage of *lasR* mutants and deferring a tragedy of the commons which could otherwise be caused by *lasR* cheating (Asfahl et al., 2015).

If the mutant is genetically engineered, the genes to be manipulated have to be accurately chosen. A mutation in the genetic machinery of the cooperative trait can create a cheater mutant, as well as an obligate cooperator, a loner, or a loser (Table 1).

Although the term “cheat” is ubiquitously used in various fields in biology, the meaning of it can vary (Ghoul et al., 2014a; West et al., 2007b). Most commonly sociomicrobiology studies define cheating as avoiding the cost of a cooperative trait, which increases the fitness or avoids decrease of the fitness of the recipients of this trait while gaining the benefit of it. Thus the conditions in which the cooperative trait is induced and to which intensity are extremely important.

To be able to call a strain a cheater, it has to be shown first that the strain of interest has a growth defect compared to the cooperative strain in monocultures. Then, in mixture with the cooperator strain, at low frequency (since cheating is frequency dependent) the mutant strain of interest should increase in frequency while the population grows. The monoculture growth shows that although the mutant strain avoids the cost, not gaining the benefit of the cooperative trait gives its growth defect. However in the mixture, assuming that the public good is fully accessible to all the individuals, then the mutant should have the same benefits as the cooperator while avoiding the cost.

Another important point while studying the emergence of cheaters is that avoiding the energy cost spent in the cooperative trait behaves as the selection coefficient to the cheater mutant only when the density of population increases. If there is no cell division, cheating has no effect on fitness and it is not observed since the measurement of cheating is calculated via calculating the relative fitness, the relative change in the frequencies of each strain. A study done by Ghoul et al. demonstrated experimentally that pyoverdine cheats cannot invade WT populations when they are introduced during the stationary phase. However, it is hard to distinguish whether this is because the cells are not growing or the cost of pyoverdine is diminished at this stage of growth (Ghoul et al., 2016). If in a culture, no death or growth of the population is expected, then cheating behavior should not be expected either. Given this definition of cheating, this thesis discusses how cheating magnitude increases with the accumulated number of cell divisions rather than the real time units (seconds, minutes, hours, days etc.), or numbers of generations which overlap (Lee et al., 2011). We argue (in Chapter II and Chapter III) that the cost avoided by the cheater is similar to a selection coefficient in a competition between a WT strain and another strain which has a beneficial mutation. Therefore, we concluded that in these scenarios cumulative numbers of cell divisions must be used as it is a more meaningful unit of time since the frequency changes are caused by the differences in the growth rates which result in different numbers of cell divisions in a given time window. Numbers of generations could be misleading in this case because populations can have the same number of generations but a different number of cell divisions if the starting yields of the populations are different (see Appendix, Figure 48 and Figure 49). In the propagation experiments shown in this thesis, the magnitude of cheating is different under different conditions even when the number of generations is the same. This occurs due to differences in growth rates which result in a different number of cumulative cell divisions for the same period of time (For more discussion see Appendix).

A better understanding of the interactions in polymorphic bacterial populations in complex environments not only provides insights into key aspects of sociomicrobiology, but also can offer a theoretical framework for the development of new therapeutic strategies against bacterial populations where social mutants can invade. *P. aeruginosa* has been the focus of many clinical CF studies because social mutants such as *lasR* and *pvdS* are repeatedly observed in the lungs of chronically infected patients (Smith et al., 2006a; Winstanley et al., 2016). Recently, it was suggested that controlled introduction of engineered *lasR* or *pvdS* cheaters into the lungs of CF patients infected by *P. aeruginosa* might decrease the bacterial population by inducing a tragedy of the commons (Brown et al., 2009; Kümmerli, 2015; Rumbaugh et al., 2009). However, in the lungs, *P. aeruginosa* faces multiple constraints similar to the ones studied here: complex carbon sources and low iron concentrations. Thus, the order of the introduction of the cheaters, the composition of the bacterial population at the onset of the intervention, and the abiotic environmental conditions in the CF lungs are determinant for the ecological outcome of the population and the success of the intervention. For example, according to our results, introducing a *pvdS* mutant into a population containing *lasR* mutants in iron-limiting conditions might avert a drastic population collapse rather than triggering it. A study on the evolution of *P. aeruginosa* strains in the CF lungs showed that appearance of *lasR* mutations is followed by that of *pvdS* mutations (and other mutation affecting iron metabolism) (Smith et al., 2006a). This might explain why a drastic population collapse does not take place in the CF lungs. Our results suggest that a successful clearance of a *P. aeruginosa* infection in CF patients via triggering a tragedy of the commons can be achieved if the cheaters are introduced when the environmental constraints are limited to the specific trait that the cheater strain cheats on. Importantly, modifications of the environmental conditions can contribute to this effect. For instance, our results predict that when a *lasR* mutant is introduced, the supply of extra iron could accelerate the drastic collapse caused by the expansion of *lasR* mutant (Figure 32B) and presumably addition of AHLs, by forcing WT to constitutively cooperate, can promote complete fixation of *lasR* mutants (Figure 30B), therefore increasing the efficacy of this potential treatment. Our studies, do not take into account the effect of *pvdS, lasR* double mutant (we found no evidence for the emergence of double mutants within the period of our experiments), which have the potential to occur *in vivo* (Smith et al., 2006a). The effect of such double mutants should be investigated in the future. However, based on our results, we can speculate that double mutants should accelerate the collapse of the population. Indeed, we have preliminary data (data not shown) that in longer propagation experiment (in Figure 20D), *de novo* mutations can occur, which include *pvdS*, *lasR* double mutants. We further note that, while our *pvdS* mutant does not show any advantage in iron-supplied medium (Figure 17B), some pyoverdine mutants in *Pseudomonas fluorescens* have been reported to be better adapted even in environments where iron concentration is not low and thus can be considered non-social mutations (Zhang and Rainey, 2013). The observation of these potentially non-social mutants indicates that iron-supplementation may sometimes fail as a potential intervention for clearance, highlighting the need for understanding the nature of these mutations and changes in dynamics caused by them.

Finally, also as this thesis shows in detail in Chapter II, the biotic components of the population of interest should be assessed. To understand how cheating affects the dynamics of complex biological systems such as the multi-species and multi-strain infections of CF lungs, the complex web of inter-species and inter-strain interactions must be addressed. There has been a growing literature focusing the inter-species and inter-strain interactions in these infections. (Bassler, 1999; Brown and Taylor, 2010; Harrison and Buckling, 2009; Leinweber et al., 2017; Oliveira et al., 2014; Popat et al., 2017; Ross-Gillespie et al., 2015). Brown and

Taylor first established a framework for the evolution of joint social traits. They showed in their theoretical study that interestingly, selfish exploitation increased with increasing relatedness while maintaining social and asocial strategies stable (Brown and Taylor, 2010). Oliveira et al. showed in their theoretical study that for the evolution of cooperation among the producers of different public goods to be stable they must be in an intermediate genetic mixing. However, even this stable state poses a constraint that the strains that rely on the exchange of the public goods as a population are less fit than a population of individuals that can produce all the public goods independently (Oliveira et al., 2014). Ross-Gillespie et al. showed that the interactions between the two iron-siderophore production traits, production of pyoverdine and pyochelin, of *P. aeruginosa* can affect the evolution of these traits in long term where pyoverdine uni-directionally suppresses pyochelin production. They showed experimentally that under strong iron limitation (where pyoverdine is produced) pyoverdine cheaters arose and thus the loss of pyoverdine resulted in an increase in pyochelin production. However under moderate iron limitation (where pyochelin is produced), although pyochelin cheaters arose and the loss of pyochelin is observed, there was no increase in pyoverdine production due to the unidirectional link between these two social traits (Ross-Gillespie et al., 2015). Popat et al. demonstrated how two genetically independent social traits, production of iron-siderophore pyoverdine, and *pseudomonas* quorum sensing signal PQS can affect each other environmentally, by demonstrating that externally added PQS can increase the cheating magnitude of the pyoverdine mutants, as it alters the iron availability in the environment (Popat et al., 2017). Leinweber et al. showed a complex social web of interactions between *P. aeruginosa*, *Burkholderia cenocepacia* and a mutant strain of *P. aeruginosa* that acts as an iron-siderophore cheat can provide co-existence and a higher biodiversity. The roles in social behaviors such as cooperators, cheaters, and more recently loners are described to have similar interaction systems to a rock-paper-scissor game (Inglis et al., 2016). Our experimental results demonstrated in Chapter II and mathematical simulations expressed in Chapter III grow on this literature and shed more light on the interbacterial cooperator and cheater dynamics of multiple social traits. We show that the cost values that the cheaters of different traits avoid vary, and the relationship among these cost values is what determines which cheater wins the competition. Additionally, we also demonstrate that mechanisms such as QS can sustain co-existence between mutants of different social traits by regulating the cost and benefit values of these traits.

This growing literature and our results demonstrated in this thesis point out to challenges in the current understanding of cheating. When the multitude of the interactions among the players with different social roles increases, the social dynamics of the system would be more like a division of labor than a simple cooperator-cheater interaction. If a complex system such as the infection of CF lungs is taken into account, the social role of each individual becomes relative to each other and in the sum of the population.

To have a simple analogy, a hunter-gatherer human population system could be imagined where the population needs both meat and vegetation consumption to grow and be maintained (Figure 47). In such population, both cooperation for meat scavenging, or hunting; and cooperation for vegetation collecting, or gathering are observed. It can be imagined that half of the population participates only in hunting and the other half participates only in gathering. If an anthropologist who studies this population only focuses on hunting as a cooperative trait, then they can misclassify the gatherers in the population as cheaters since from that point of view, the gatherers would be not hunting but yet still eating meat gathered by the hunters. And vice versa, if another anthropologist focuses only on gathering behavior, they can assess the hunters as cheating individuals who are not contributing in gathering but still benefiting from the access to the vegetation brought by the gatherers. We define this as ‘the multiple traits hypothesis’, and argue that the roles of each group, both hunters and gatherers in this analogy, can only be correctly assessed if both traits are accounted simultaneously. Only then, it can be seen that the components of this populations were not cheaters but mutual partners (Figure 47).

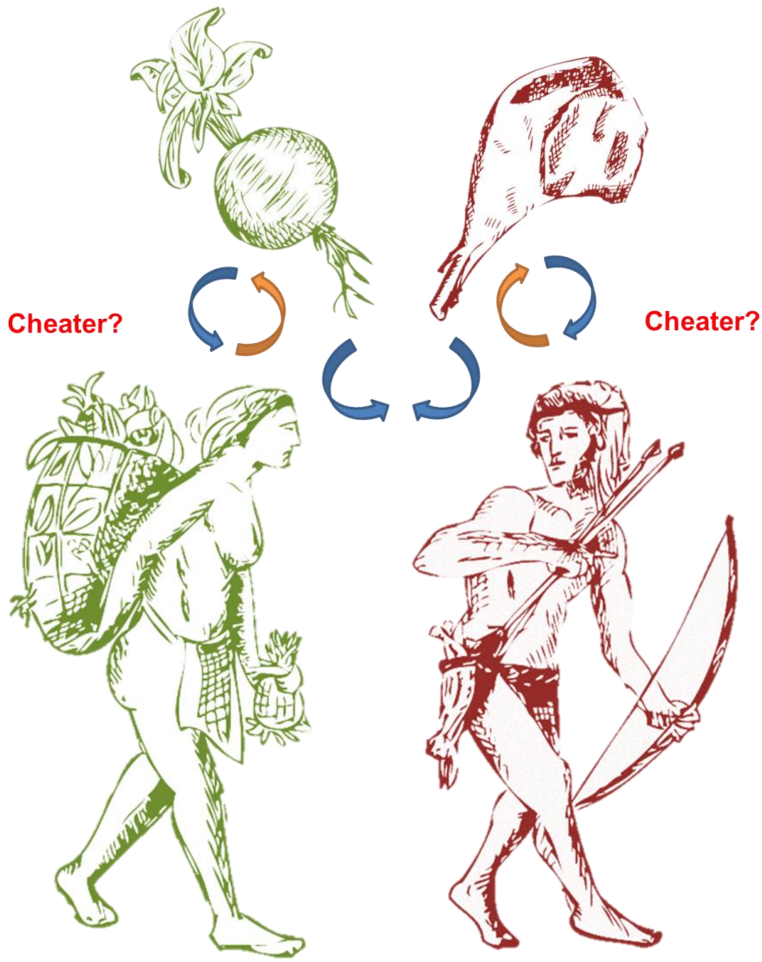


Figure 47. Multiple traits hypothesis

A gatherer collects roots and vegetables but consumes not only the resources that they collect but also meat that is collected by hunters. A hunter, on the other hand, hunts for prey but consumes not only the meat that they provided but also the vegetables and roots scavenged by the gatherers. A uni-directional approach to this di-partite system can lead to the mislabeling of either company as cheaters for the actions that they are not participating but still gaining benefit from.

(Image credit: Image hand-drawn by Özhan Özkaya and digitally edited by Rômulo Areal, inspired from the illustration "*A Kali'na hunter with a woman gatherer*" from the scanned version of "*Na'na Kali'na: Une histoire des Kali'na en Guyane"*

More parallels can be drawn between more complex organisms and bacteria to this extent. Such as a single human that is stranded in a deserted island has to overcome all the external forces by themselves, thus possess all the traits for their survival ranging from hunting and gathering to cloth making, cooking, farming, herding etc. This life style can be similar to a planktonic bacterium in a liquid environment like an ocean. This bacterium is unlikely to find another kin to interact due to high dispersal and diffusion, thus has to overcome the constraints of its environment by possessing all necessary traits. Therefore, it is not surprising that in what we define as wild type planktonic bacteria populations, each cell will generally have more cooperative traits, than the cells in more complex situations where the probability of exchanging goods is higher, such as in biofilms in infections of CF lungs where various subpopulations lose or maintain different traits, making each cell a partial cooperator in a division of labor scenario. When more humans live in close proximity to exchange goods and services, specifications to certain tasks and division of labor are generally observed. In fact, each individual will lose the ability to perform more traits, when more frequent interactions would be available with more individuals (Wood, 1991). This progression of trait loss and population growth can be observed starting from the hypothetical human on a deserted island to a hunter-gatherer society to a farming society and eventually to an industrial society in the modern times where each individual performs only one task and depends on all the other tasks to the society while the overall society becomes more robust to external changes. Certain theories in economics, such as ‘comparative advantage’, which describes how two individuals, companies, or countries can save time and energy by specializing in the production of one trait while exchanging for the others, have already been the subject of interdisciplinary studies in microbiology (Enyeart et al., 2015; Tasoff et al., 2015). These analogies and interdisciplinary studies can help to understand the complex and dense biofilms in CF infections where bacteria lose certain traits during the course of the infection however the whole population becomes more robust to external changes, antibiotics, inter-species competition, and to the immune system elements.

Conclusion

Collectively, the experimental and theoretical results presented in this thesis underline the main factors that govern the social behaviors, and the mechanisms, such as quorum sensing that can regulate them in favor of preserving cooperation. Furthermore, the novel tri-partite approach underlines the need for including polymorphism and multiple constraints in pertaining to cooperation in microbial populations. Our results demonstrate that using conditions that include more than one social trait can reveal complex and dynamic social roles in bacterial populations as well as their dependence on the environment. Understanding the dynamics of polymorphic populations in these complex environments provides insights into social interaction processes, expanding their relevance beyond sociomicrobiology, in addition to providing knowledge important for the development of new therapeutic tools.

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Appendix

Cumulative numbers of cell divisions as the timescale for cheating

To further analyze what factors affect the relative fitness of *lasR* in iron-supplemented casein and iron-depleted casein media we focused on the data demonstrated in Chapter II. The first observation that is significant is the twice as much higher yields in iron-supplemented casein media compared to iron-depleted casein (Figure 14B and Figure 15). We argue that this difference affects the relative fitness in two ways.

First, as it can be seen in Figure 17A and Figure 17C, the relative fitness of *lasR* in iron-depleted casein media is half of the one in iron-supplemented casein media. Here, cells in both media start growing from the same initial numbers (10^7 CFUs/ml, OD_{600}=0.05) but grow to different final yields (approximately $4x10^9$ CFUs/ml or OD_{600}=6 in iron-supplemented casein and $2x10^9$ CFUs/ml or OD_{600}=3 in iron-depleted casein media) which means that the cells go through different numbers of cell divisions since they experience a higher numbers of generations in iron-supplemented casein ($\log_2(4x10^9/10^7)$) than in iron-depleted casein media ($\log_2(2x10^9/10^7)$). This could be one of the effects on the final yields on the relative fitness (Figure 48).

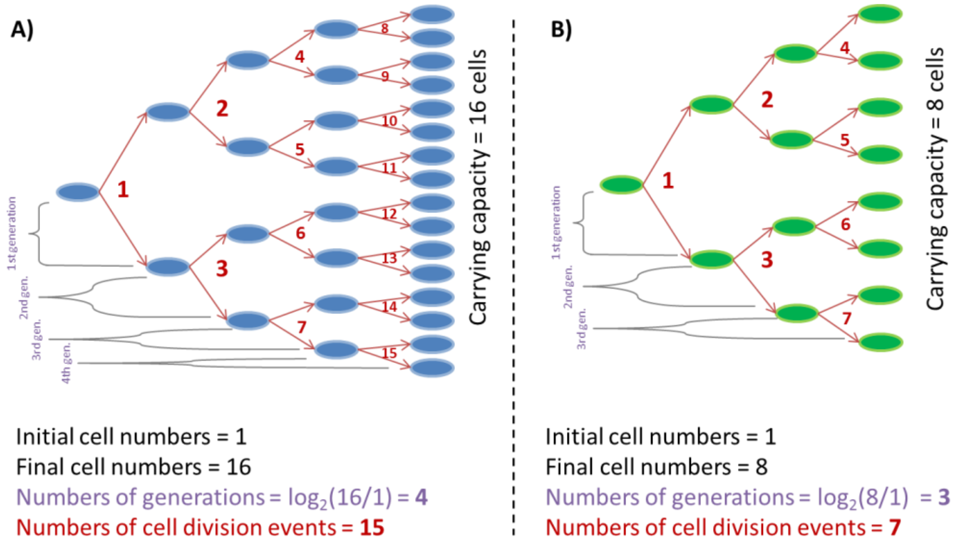

Figure 48. Cumulative numbers of cell divisions and generations in populations with different carrying capacities but same inocula.
Given that the cost is the same in both scenarios **(A)** and **(B)**, the relative fitnesses of the mutants in these conditions should be proportional to the number of cell divisions that those populations experience:

Relative fitness of A / Relative fitness of B $\cong$ 15/7

Here, the difference between the carrying capacities is important because it changes the number of generations, thus the number of cell division events.

Secondly, in Figure 20, we apply 1/1000 bottleneck to the cultures from the 2nd day and onwards. This causes the cultures to experience same numbers of generations in each media ($\log_2 10^3$). However the relative fitness of *lasR* in iron-supplemented casein media is again greater than in iron-depleted casein media (from day 2 to 4, mean ω=3,08 SD=0,71 N= 6 and mean ω=1,33 SD=0,51 N=6, respectively). Thus, the tragedy of the commons is achieved much earlier (day 12 and day 18, respectively). The role of the different yields here is different than the one in Figure 17. Here, since we propagate cells from the stationary phase to inoculate a fresh culture by using a bottleneck (1/1000 for both media), the initial numbers of cells would be proportional to the yields in the previous culture. For example, at the end of the 2nd day cultures grew to approximately $4x10^9$ CFUs/ml in iron-supplemented casein media and $2x10^9$ CFUs/ml in iron-depleted casein media. After the bottleneck, there are approximately $4x10^6$ CFUs/ml in iron-supplemented casein media and $2x10^6$ CFUs/ml in iron-depleted casein media. Given the cells will experience the same numbers of generations, they will go through different numbers of cell division events due to different initial cell numbers (Figure 49).

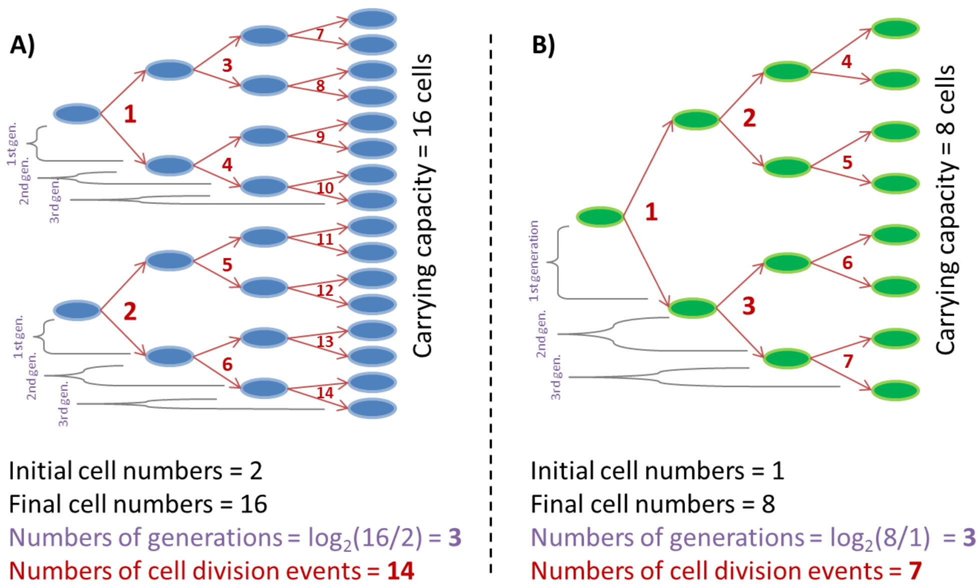

Figure 49. Cumulative numbers of cell divisions and generations in populations with different carrying capacities and different inocula. Given that the cost is the same in both scenarios **(A)** and **(B)**, the relative fitnesses of the mutants in these conditions should be proportional to the number of cell divisions that those populations experience:

Relative fitness of A / Relative fitness of B $\cong$ 14/7= 2

Here, the difference between the carrying capacities is important (considering that final population of one culture will be used to inoculate the next culture after a dilution), because it changes the number of initial cell numbers, thus the number of cell division events.

To conclude, we argue that the relative fitness advantage of *lasR* would be expressed in each cell division event and the numbers of cell divisions are not only proportional to the numbers of generations but also to the initial cell numbers (Lee et al., 2011). And this would be expressed as:

Cumulative number of cell divisions = Final number of cells – initial number of cells

$$CCD = N_{final} - N_{initial}$$

Whereas generation numbers (G) are expressed as:

$$G = \log_2 (N_{final} / N_{initial})$$

We can also express CCD as a function of N_G as below:

$$CCD = N_{initial} (2^G - 1)$$

In this thesis, we used relative fitness per cell division as the ultimate cheating magnitude unit (see Chapter II / Results / Section: "Effects of environmental constraints and population composition on cheating", pages: 52 - 53). This calculation is an approximation of the cost value that is spent in the production of the public good which gives the competitive advantage to the cheater as it avoids this cost.

As can be seen in Chapter III, when we modelled PG competition simulations as continuous replicator functions, we used CCD as the arbitrary time unit on the x-axes of the simulation plots.

“Só de aceitar tenhamos a ciência,
E, enquanto bate o sangue em nossas fontes,
Nem se engelha connosco
O mesmo amor, duremos,

Como vidros, às luzes transparentes
E deixando escorrer a chuva triste,
Só mornos ao sol quente,
E refletindo um pouco.”

Ricardo Reis, 17-VII-1914