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Secreted Virulence Factors: Evolution, Ecology and Therapeutic Manipulation

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Declaration

I declare that the following statements (a-c) are true.

(a) that the thesis has been composed by the candidate, and

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(c) that the work has not been submitted for any other degree or professional qualification except as specified.

Richard Allen (the candidate)

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Papers arising

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Abstract

Bacterial infections are an increasing cause for concern as resistance spreads to the majority of our front line antibiotics. To counter antibiotic resistance, new treatment regimens and drug targets are being investigated, including directly targeting bacterial virulence (pathogen-induced harm to the host), and therapies which target resistance mechanisms. The outcome of successful treatment with these compounds is not always killing or halting growth of bacteria, therefore selection for resistance to these types of therapeutics is complex. This complexity is increased by the secretion of many virulence factors, meaning their effects are shared with neighbouring individuals. In addition virulence factors show high phenotypic plasticity due to regulation by processes like quorum sensing (QS), which further complicates treatments targeting virulence, or the regulatory processes themselves.

Using the example of quorum sensing inhibitors this study shows the importance of understanding the function and ecology of targeted virulence factors, to predict the selection for resistance to anti-virulence drugs. Later chapters elaborate on this to show how quorum sensing control affects selection on secreted virulence factors. The use of anti-virulence drugs as adjuvants is discussed, with a study showing that the interaction between QS inhibition and translation inhibitors is dependent on the environment. The selection for resistance to combinations of antibiotics and adjuvants is investigated using co-amoxiclav as an example, showing that treatment with high doses of adjuvant are robust to the evolution of resistance.

Lay Summary

Antibiotics kill the pathogenic bacteria that make people sick in a variety of illnesses ranging from coughs, through to food poisoning and up to highly fatal worldwide diseases like tuberculosis. Bacteria divide very quickly and live in large populations so they quickly gain mutations in their DNA which prevent them from being killed by certain antibiotics, thus making them antibiotic resistant. When populations containing a small proportion of resistant bacteria are treated with antibiotics, only the resistant bacteria survive. The death of the susceptible bacteria allows resistant bacteria to grow better, so resistant bacteria become more common. As resistant bacteria are transferred between individuals and into the environment, the prevalence of resistance renders antibiotics ineffective. In most cases these antibiotic types will never again be of use in the fight against infection.

To counter the spread of antibiotic resistance, new (anti-virulence) drugs are being developed which aim to prevent bacteria from making people sick (preventing virulence) rather than killing the bacteria. This means that even when resistant bacteria are present in a population treatment with the drug will not kill all the susceptible bacteria, so resistant bacteria will not spread as fast. This work finds that in order to understand the long term effects of anti-virulence drugs (whether resistance will spread), we need to understand how and why bacteria make people ill. Later chapters investigate how communication between bacteria in a population allows bacteria to make us sick and the effect of anti-virulence drugs that prevent communication between bacteria. Anti-virulence drugs can also be used in combination with normal antibiotics, making antibiotics more effective at killing bacteria. This thesis investigates interactions between bacterial communication and

antibiotic killing, as well as whether bacteria can mutate to become better at growing in the presence of combinations of drugs.

1 Introduction

1.1 Infectious diseases

1.1.1 What is a parasite?

Parasites are organisms which colonise another organism (host), relying on the host for at least some of its nutritional requirement and causing some damage to this host (Price, 1980). Parasitism is common at all scales of biology and host damage is usually associated with significant fitness costs to the host (Price, 1980; Anderson and May, 1982). This has resulted in a multitude of host defences to parasitism, notably the complex and costly immune systems of multicellular organisms (Danilova, 2006), but also adaptations like CRISPR sequences to prevent phage infection of bacteria and archaea (Sorek *et al.*, 2008).

Unlike viruses and the majority of eukaryotic parasites, bacterial pathogens are usually facultative or opportunistic parasites which can exist outside the host or in a commensal lifestyle within a host (Brown *et al.*, 2012). Many of the most problematic human bacterial pathogens can be considered as opportunistic pathogens, as they spend a significant amount of time in either environmental niches or in commensal roles within a host (Brown *et al.*, 2012). These opportunistic pathogens will experience selection in environments other than the site of pathogenic exploitation (see chapter 2).

1.1.2 Virulence and virulence factors

In biomedical science virulence is defined as the ability of a pathogen to harm the host (Silverstein and Steinberg, 1990). This is the definition used throughout this thesis, but it is worth noting that other fields use distinct definitions that focus on the ability of a pathogen to kill the host (Alizon *et*

al., 2009), invade and grow within the host (Montarry *et al.*, 2010) or reduce host fitness (Anderson and May, 1982).

The role and evolution of virulence has received a lot of attention. It is usually assumed that virulence is a side effect of successful exploitation of the host. This suggests that virulence should evolve to an optimal value dictated by the trade off between the need to exploit the host and evade the immune system and the need to keep the host alive to transmit (Anderson and May, 1982). However this does depend on whether a live host is required for transmission (Richardson, 2001). Other hypotheses suggest that virulence may be an unfortunate side effect of selection outside of areas of virulent colonisation (Brown *et al.*, 2012; Levin and Eden, 1990), or that virulence is the result of within host selection for competitive ability (Lysenko *et al.*, 2010).

At a molecular level the ability to cause virulence is defined by the presence of virulence factors (VFs), the tools of the trade for bacterial pathogens. Virulence factors are genes involved in the exploitation of the host and are identified as genes that increase the ability of pathogen to cause damage to the host but do not reduce pathogen fitness in a hospitable *in vitro* environment (Silverstein and Steinberg, 1990). Virulence factors include adhesins (Nowrouzian *et al.*, 2006), siderophores (Meyer *et al.*, 1996), immune evasion genes (Lysenko *et al.*, 2010), biofilms (Costerton *et al.*, 1999), toxins to kill host cells (Raymond *et al.*, 2012) and enzymes (Rumbaugh *et al.*, 2009) to break down host tissue. Many of these products are secreted into the environment which can have important implications for selection on virulence factors in certain environments (Allen *et al.*, 2014, chapter 2). For most infections of humans the host environment is also characterised by much higher concentrations of antibiotics compared to the environment

outside the host. Therefore antibiotic resistance mechanisms are important determinants of clinical outcome but traditionally they are not included as virulence factors (Dubois *et al.*, 2009) despite being useful to survive in an antibiotic treated host.

1.1.3 Pseudomonas aeruginosa

Pseudomonas aeruginosa is an opportunistic pathogen found in many environments including soil, water, plant and animal hosts (Goldberg, 2000; Kung *et al.*, 2010). This generalist behaviour is partly facilitated by a large genome with a high proportion (approximately 9.4%) of regulatory elements (Stover *et al.*, 2000). These regulatory elements allow *P. aeruginosa* to plastically change its phenotype as a response to the environment (Adaptive phenotypic plasticity, Dotsch *et al.*, 2015; Ghalambor *et al.*, 2007; West-Eberhard, 2003). Many of the phenotypes that shift with the environment are secreted factors (Dotsch *et al.*, 2015); siderophores to scavenge iron; extracellular enzymes to access nutrients; toxins to kill host cells and inhibit the growth of competitors and quorum sensing molecules for signalling.

In a human host *P. aeruginosa* causes complications for compromised individuals, exploiting damage to external membranes to infect burn wounds (Holder, 1993), or chronically infecting of individuals with genetic disorders like cystic fibrosis patients (Döring, 1993). During chronic infection *P. aeruginosa* adapts to new conditions, acquiring mutations which lead to new phenotypes (Huse *et al.*, 2010; Smith *et al.*, 2006), such as the repression of signalling (Hoffman *et al.*, 2009; D'Argenio *et al.*, 2007) or mucoid biofilm production (Bragonzi *et al.*, 2009). Other changes that can occur are the exhibition of a mutator phenotype although this may not be adaptive (Montanari *et al.*, 2007). For treatment of chronic infections patients are routinely given large doses of antibiotics. As a gram negative organism, *P.*

aeruginosa is intrinsically resistant to many antibiotics, and has genetic resistance mechanisms to many others (Hancock and Speert, 2000). This has led to *P. aeruginosa* being classified as a pathogen of serious concern according to the CDC (Antibiotic/ Antimicrobial resistance report, CDC). As well as being an important human pathogen *P. aeruginosa* is a common study organism for laboratory studies of plasticity, infection, quorum sensing and social behaviours (Diggle *et al.*, 2007; Dotsch *et al.*, 2015; Ross-Gillespie *et al.*, 2015; Rumbaugh *et al.*, 2000).

1.1.4 Escherichia coli

Escherichia coli is a second gram negative pathogen that was used in this study. Often called the workhorse of microbiology, *E. coli* is routinely used in the laboratory, for studies of evolution (Lenski *et al.*, 1991) and molecular biology (Hermsen *et al.*, 2015). *E. coli* has many published genomes which together with the wealth of primary literature, transciptomic and proteomic work means we have a very good idea about how the metabolism of this organism functions (Hermsen *et al.*, 2015).

The high resolution genetic data that we have about *E. coli* has allowed further classification of the species into different ecotypes depending on their life history (Cohan, 2006), predominantly their infectious lifestyle. Unlike *P. aeruginosa* which has flexible phenotype resulting from a high degree of plasticity in gene expression, *E. coli* has a simpler genome (5.3 % regulatory elements) but samples show a high degree of diversity due to genetic changes (Rasko *et al.*, 2008). Different ecotypes are usually associated with the acquisition of new genes through horizontal gene transfer or gene loss (Robinson *et al.*, 2006). Sequencing has identified that *E. coli* has a relatively small core genome with a large and diverse accessory genome (Lukjancenko *et al.*, 2010).

E. coli is an important pathogen in its own right. The species is a commensal of the human gastrointestinal tract but many ecotypes are able to infect sites outside the gut where they can cause pathogenesis (Köhler and Dobrindt, 2011) and other strains will cause pathogenesis in the gut, most notably Enterohemorrhagic *Escherichia coli*, EHEC: O157 (Robinson *et al.*, 2006). Antibiotics are key to treating *E. coli* infections but antibiotic resistance is common.

1.2 Experimental evolution and antimicrobial resistance

1.2.2 Experimental evolution

Observations of extant species in the natural environment, combined with methods such as phylogenetics, fossil records or even real time sampling for phylodynamics (Hughes *et al.*, 2012) can tell us much about the selection process. For organisms with short generation times it is useful to impose stringent controlled conditions for selection in a closed system to investigate how adaptation proceeds. This approach sacrifices some realism for a greatly enhanced ability to manipulate selective forces (Buckling *et al.*, 2009).

The first experimental evolution studies were probably performed by William Dallinger, who over the course of several years in the 1870's increased the temperature used to incubate a "minute septic organism", showing an increase in thermal tolerance (Dallinger, 1878). The use of experimental evolution was little utilised for about 100 years but has since been performed with many other organisms including fruit flies and plants. In 1988 Richard Lenski started the long term evolution of *E. coli* in a closed environment (Lenski *et al.*, 1991) which is still going after 50, 000 generations (Fox and Lenski, 2015).

On the other hand experimental evolution with bacteria can be performed in the order of weeks or even days (Pena-Miller *et al.*, 2013). In order to speed up the process, or investigate specific mutants a mutation step can be performed first (Maeda *et al.*, 2012) or defined mutants can be seeded into the initial population (Kümmerli *et al.*, 2015). However, this does have the drawback of only investigating specific adaptations (Popat *et al.*, 2015b).

Experimental evolution in a variety organisms has provided insights into important biological processes including sex (Lagator *et al.*, 2014), multicellularity (Koschwanez *et al.*, 2013; Ratcliff *et al.*, 2012), host parasite interactions (Gomez and Buckling, 2011) and sociality (Ross-Gillespie *et al.*, 2015; Sandoz *et al.*, 2007) . Experimental evolution in microbes is particularly attractive because the very short generation times, ease of environmental manipulation and the possibility of freezing evolved populations which is useful for fitness assays.

1.2.3 Antibiotics

Antibiotics are compounds which have useful therapeutic activity in killing or inhibiting the growth of microbes (Clatworthy *et al.*, 2007; Yim *et al.*, 2007). Antibiotics may have bacteriostatic or bacteriocidal activity depending on whether they inhibit bacterial division or kill bacteria respectively, although many antibiotics exhibit both activities depending on concentration (Pankey and Sabath, 2004). Since the first use of antibiotics like suphonamide and penicillin, antibiotics have been vital to modern medicine enabling the easy treatment of many bacterial infections that were previously fatal, and additionally allowing modern surgery to proceed with minimal risk of infection. In the early days of antibiotics (the golden age of antibiotic discovery), many different antibiotic classes were discovered but since then antibiotic development has slowed dramatically (Coates *et al.*, 2011).

1.2.4 Antimicrobial resistance

Antibiotic resistance, the ability to grow in the presence of previously inhibitory concentrations of antibiotics, is one of the main problems currently facing modern medicine (Antibiotic/ Antimicrobial threat report, CDC). Upon exposure to inhibitory concentrations of antibiotics, bacterial populations will decline resulting in clearance. However if mutants that are resistant to the antibiotic already exist in the population, or occur *de novo* during antibiotic treatment, these mutations will spread in the population (Lipsitch and Samore, 2002). The death of susceptible bacteria due to the antibiotic leads to competitive release through the freeing up of resources, thus allowing resistant bacteria to spread quickly (de Roode *et al.*, 2004).

There has been a lot of study of how antibiotic concentration affects selection for resistance, the most common regimen is to hit bacteria with the highest reasonable concentration in the hopes of killing all bacteria quickly (Lipsitch and Samore, 2002). This stems from studies of tuberculosis which generally forms chronic infections where resistance mutations occur *de novo* during antibiotic treatment (Lipsitch and Samore, 2002). The logic of applying this to all treatments has recently been called into question (Read *et al.*, 2011). Adaptation is faster when the decline in fitness in a new environment is greater, so selection for resistance is stronger when antibiotic inhibition is greater (de Roode *et al.*, 2004; Pena-Miller *et al.*, 2013). Therefore it may be better to treat with lower doses, especially for pathogens where environmental resistance is common, because then the role of inhibition in preventing mutational supply is much less important (Read *et al.*, 2011).

Common resistance mechanisms involve mutations in the antibiotic target, the use of alternate metabolic pathways, excluding the antibiotic from the cell or destruction of the antibiotic (Nikaido and Pagès, 2012; Bush, 2013).

Resistance mechanisms will often be initially costly; mutations in the target proteins will affect the normal functioning of the protein, enzymes to deactivate antibiotics will be costly to produce and efflux pumps can affect the normal physiology of the cell. The fitness effects of resistance mutations will depend on the specific resistance mutation, the genetic background, and the environment, including antibiotic concentration (Hall, 2013; MacLean *et al.*, 2010b). Costs of resistance will have the largest effect when antibiotic concentration is low, which may lead to selection against resistance, however in many cases compensatory mutations will ameliorate this cost (Andersson and Hughes, 2010; Levin *et al.*, 2000; Schrag and Perrot, 1996).

Although resistance mechanisms are a major problem for resistance, phenotypic resistance is also an important clinical problem (Levin and Rozen, 2006). This describes situations where otherwise susceptible bacteria are resistant to antibiotics due to growth phenotypes resulting from their environment or stochastic phenotypic switches. This results in bacteria that appear susceptible to antibiotics during susceptibility testing *in vitro*, but are recalcitrant to treatment with these same antibiotics. Two particularly problematic examples of phenotypic resistance are persisters (Lewis, 2010, slow growing bacterial cells) or biofilm growth (Costerton *et al.*, 1999).

As antibiotics are often produced by bacteria, resistance genes may originate from these producing organisms (D'Costa *et al.*, 2006; Wright, 2007). Regardless of the natural function of antibiotics, bacteria have been exposed to antibiotics long before we co-opted them for clinical use. This is evident from the presence of resistance genes in frozen records dating millions of years before the clinical use of antibiotics (D'Costa *et al.*, 2011).

Of course human use of antibiotics also plays a role, not only use in a nosocomial setting but also use in agriculture and aquaculture. As well as creating selection in treated hosts, use of antibiotics in these settings may lead to contamination of the environment with antibiotics, increasing selection for resistance in soil, water and other natural habitats (Martinez, 2009). On the other hand resistance mechanisms may serve other functions in addition to resistance such as heavy metal tolerance or may be incorporated into integrons with other resistance genes (Ploy *et al.*, 2000). Efflux pumps are a common resistance mechanism which are particularly problematic because the broad range of compounds that they protect against (Nikaido and Pagès, 2012).

Once resistance mechanisms exist they may be transferred to other individuals or even species by the processes of horizontal gene transfer, through transformation, transduction or conjugation (Ochman *et al.*, 2000). In addition many bacteria are intrinsically resistant to certain antibiotics. The classic case being that gram negative organisms are resistant to the majority of β-lactam antibiotics because the outer cell membrane prevents the drug from accessing the cell wall (Hancock and Speert, 2000). Thankfully these resistance mechanisms are so much a part of the general cellular physiology that they cannot be transferred through horizontal gene transfer. This has resulted in antibiotics where resistance mechanisms cannot be transferred from the producer organism (Ling *et al.*, 2015).

1.2.6 Combating resistance

The rapid selection for resistant individuals upon exposure to antibiotics and the multitude of ways in which existing antibiotic resistance mechanisms can disseminate across bacterial strains and species has led to high levels of resistance to the majority of front line antibiotics (Davies and Davies, 2010).

Therefore combating resistance is a key problem to which several solutions have been proposed.

The simplest solution is to continue to produce more antibiotics to treat resistant pathogens regardless of the spread of resistance, this will lead to a treadmill effect where new antibiotics will continually need to be discovered (Read and Huijben, 2009). Currently we are not discovering new antibiotics at a sufficient rate for this, in part because we may have exhausted all of the easily discoverable antibiotics or even all of the possible antibiotic classes (Coates *et al.*, 2011). Although the latter is unlikely there is probably a limit to the number of different targets that can be lethally inhibited in bacteria. It is likely that there are many new antibiotic classes to be discovered given we are currently only able to culture approximately 1% of bacterial diversity (Ling *et al.*, 2015). Recently the ability to culture new bacterial species has yielded promising candidates to be developed as antibiotics (Ling *et al.*, 2015; Nichols *et al.*, 2008). Bringing a lead compound to a final product that can be used clinically is an expensive and risky process, especially given the rapid development of resistance, so pharmaceutical companies have lost interest in the development of antibiotics (Coates *et al.*, 2011). The recent governmental focus on antibiotic resistance together with new legislation will hopefully reverse this.

Rather than trying to produce new antibiotics at the current rate of resistance evolution, we can attempt to slow the treadmill down or even stop it completely. Although the possibility of resistance was proposed shortly after the use of antibiotics became prevalent (Rosenblatt-Farrell, 2009), little was done about it, possibly because so many new antibiotics were being developed at the time. Microbiology has taken huge strides in the subsequent decades, particularly with respect to the evolution of antibiotic resistance.

Improved public awareness about the use of antibiotics as well as theoretical and experimental studies investigating treatment regimens will all help to increase the lifespan of antibiotics (Bonhoeffer *et al.*, 1997; Pena-Miller *et al.*, 2012). Treatment with combinations of therapeutics is also promising. Both the treatment with combinations of antibiotics and antibiotics in combination with adjuvant therapies (which inhibit antibiotic resistance mechanisms) can make older antibiotics relevant again (Chait *et al.*, 2007; Christensen *et al.*, 2012; Pena-Miller *et al.*, 2013).

Finally completely new treatment options are a potential way to deal with antibiotic resistant bacteria. Cationic antimicrobial peptides have shown promise although resistance is still likely to develop and there is concern that this will weaken our bodies own natural defences (Perron *et al.*, 2006). Phage therapy is another promising alternative therapeutic avenue with the potential for phage treatments that can evolve to adapt to resistance mechanisms. Despite this treatments are likely to be highly species (or even strain) specific, which may prove difficult for regulation of treatment (Levin and Bull, 2004; Smith *et al.*, 1987). Lastly anti-virulence therapeutics aim to reduce a bacterium's virulence without clearing the pathogen (Allen *et al.*, 2014; Clatworthy *et al.*, 2007; Ross-Gillespie *et al.*, 2014). Although in most cases these treatments are still in early stages, they may slow the spread of resistance (see chapter 2).

1.3 Social evolution

1.3.1 Social behaviours

Social behaviours are actions that have fitness consequences for both the individual performing the action (actor) and individuals the behaviour is directed to (recipients, West *et al.*, 2006). These behaviours are generally split into a two by two matrix based on whether the behaviour is beneficial or

costly to the actor and recipient (table 1.1). Behaviours that benefit other individuals and are maintained (at least in part) due to a beneficial effect on the recipient are cooperative (West *et al.*, 2006), these include altruistic and some mutually beneficial behaviours.

Table 1.1: Classification of social behaviours based on their effect on lifetime reproductive fitness of actor and recipient.

		Fitness effect on recipient	
		Positive	Negative
Fitness effect on actor	Positive	Mutually Beneficial	Selfish
	Negative	Altruistic	Spiteful

The broad definition of social traits includes selfish behaviours like enhanced growth that will benefit the actor but are detrimental to other individuals. Although social in nature, selfish behaviours rarely need a sociobiological explanation because the direct fitness benefit is enough to explain maintenance. Benefits and costs are measured in terms of the lifetime reproductive benefits (West *et al.*, 2006). Therefore even in the case of behaviours with an initial investment cost, such as fighting over mates, the chance to mate outweighs the cost of fighting over the lifetime of the individual (and averaged over fights).

Mutually beneficial behaviours still provide a direct benefit to the actor, but also benefit other individuals. In some cases these behaviours can be simply explained by shared interest, so that the direct benefit is a sufficient explanation for the behaviour. For example cooperative breeding increases the size of the group (group augmentation), thus increasing survival of the individual (Kokko *et al.*, 2001). In other cases of mutually beneficial behaviours there is still a conflict of interest so that the mutually beneficial

behaviours must be stabilised to prevent one interaction partner from exploiting the other. A common example is cleaner fish mutualisms, small cleaner wrasse set up cleaner stations where they eat the parasites infesting the skin of client fish, the cleaner (actor) benefits because parasites are a source of food and the client (recipient) benefits because the removal of parasites prevents disease (Bshary and Grutter, 2002). However the cleaner wrasses can get better quality food by using the opportunity presented while cleaning to eat living tissue from the client, this results in a greater fitness benefit to the cleaner but a fitness reduction to the client (a selfish behaviour, Bshary and Grutter, 2002) Mutually beneficial interactions between cleaners and clients are the norm so what stops the cleaners from taking advantage of their clients (cheating)?

In 1971, Trivers suggested that these types of mutually beneficial behaviours could be stabilised by behavioural plasticity, such that individuals only perform helpful behaviours to individuals that they have previously received helping behaviours from. This behavioural rule was termed reciprocity or reciprocal altruism (and later direct reciprocity), and has been shown to be effective in small human social experiments and simulations where a popular formalisation is tit for tat (Axelrod and Hamilton, 1981) – cooperate on first move, then copy partner moves.

Although there is evidence for direct reciprocity in rhizobia-legume interactions (Kiers *et al.*, 2003, 2011) where interactions are highly repeated, direct reciprocity generally has a high cognitive burden. It requires repeated interactions to learn and remember how cooperative other individuals are especially for large groups (Boyd and Richerson, 1988). Therefore alternatives to direct reciprocity have been suggested. Indirect reciprocity bases cooperative behaviour on the reputation (observed interactions) of

others removing the need for repeated interactions (Boyd and Richerson, 1989). Generalised reciprocity states that individuals should cooperate more if they previously received cooperative interactions, this greatly reduces the cognitive burden (because only one state needs to be remembered, Milinski and Wedekind, 1998) and also the need for repeated interactions (Pfeiffer *et al.*, 2005).

1.3.2 Kin selection

Altruistic and spiteful behaviours are actions that respectively aid or hinder other individuals at a cost to the actor (over its lifetime) so how would these behaviours be maintained? In 1964 Hamilton showed that even costly behaviours could be selected for if they provide a benefit to related individuals (Hamilton, 1964). This work can be summarised as Hamilton's rule:

$$rb > c \qquad (eqn. 1.1)$$

Where c and b are the costs and benefits of the behaviour respectively. Relatedness (r) is a statistical regression coefficient which describes the similarity of recipients and actors at cooperation loci (Smith *et al.*, 2010). This rule means that a behaviour can be favoured by kin selection if the benefit to other related recipients, weighted by the relatedness at the cooperation loci, is greater than the cost to the actor. Although explanations are usually framed in terms of altruistic behaviour, it can be shown that using a negative benefit (detriment to recipient) and a negative relatedness (recipient less related to the actor than the population average) can allow this rule to explain spite (West and Gardner, 2010).

In order for kin selection to maintain costly traits there must be a mechanism to ensure that altruistic behaviour is directed towards kin. This may simply

be the result of population structuring due to population viscosity or limited dispersal so that behaviours directed randomly to the population are more likely to target relatives (Kümmerli *et al.*, 2009). Alternately kin discrimination may function so that behaviours are specifically targeted at kin, or the fitness effects of behaviours vary for kin and non kin (Brown and Buckling, 2008). As relatedness is defined at the loci involved in cooperation rather than whole genome relatedness, kin discrimination may be achieved through greenbeards, visible signals of the possession of cooperation alleles (Dawkins, 1989; Gardner and West, 2010). These signals must be the result of genes tightly linked to cooperation alleles or pleiotropic effects of cooperation alleles to prevent the occurrence of falsebeards (individuals possessing the signal but not the cooperation allele).

1.3.3 Social traits in bacteria

In the last 3 decades, with technological advances we have come to see that bacteria cannot simply be understood as isolated individuals (West *et al.*, 2006; Brown and Buckling, 2008). Bacteria interact with other individuals in a multitude of ways covering spiteful, mutually beneficial, altruistic and selfish behaviours (table 1.1). The simplicity and experimental tractability of interactions between bacteria means that identifying the specific costs and benefits of social behaviours is relatively simple. Predictions about selection based on these fitness effects can then be tested using experimental evolution. Socio-microbiology studies are making important contributions to the study of social interactions in general (Kümmerli *et al.*, 2009; Popat *et al.*, 2015b; Gardner *et al.*, 2004), but also the role of these interactions in natural settings (Foster and Bell, 2012; Kümmerli *et al.*, 2014) and importantly how we can better treat bacterial infections (Brown *et al.*, 2009; Mellbye and Schuster, 2011).

Bacteria have been shown to produce a wide spectrum of social behaviours. Cooperative spore formation in the slime mould *Dictyostelium discoideum* (Foster *et al.*, 2004) and the bacterium *Myxococcus xanthus* (Velicer *et al.*, 2000) is an altruistic act. In both species individuals that form the stalk terminally differentiate to better enable the rest of the population to disperse. It has been shown that in *Dictostelium*, this process is stabilised by the presence of a greenbeard locus (Foster *et al.*, 2004). Other behaviours such as cross feeding, a process where metabolic products of one bacterium are used by another, may be mutually beneficial if both species benefit, although exploitative interactions may also result (Estrela *et al.*, 2012). In addition the secretion of public goods, like proteases and siderophores is potentially a mutually beneficial behaviour as individuals also benefit from their own public goods, but secreted products may also cover a range of other behaviours (McNally and Brown, 2015). Although public goods production is mutually beneficial cooperative behaviours are still open to exploitation by cheats in a mixed environment as long as there is some private cost of performing the behaviour (Dumas and Kümmerli, 2012; Diggle *et al.*, 2007).

Rapid growth of bacteria would be a selfish behaviour. Rate and yield of growth have a trade off, therefore slow, efficient growth is a cooperative trait which may be selected for in highly structured populations (MacLean, 2007). On the other hand the release of bacteriocins or temperate phage into the environment by cell lysis is clearly costly (fatal) to the individual. However, possession of integrated phage or the linked bacteriocin-antidote gene complex protects against lysis. This provides a kin recognition mechanism so that spiteful behaviours are targeted to non-kin (Gardner *et al.*, 2004).

1.3.4 Signalling

A signal is any trait that elicits a response in another individual, which evolved because of the response elicited and is effective because the receiver's response evolved (Smith and Harper, 2003). Signals can range from structures such as elongated tails in sunbirds that demonstrate fitness based on a handicap (Zahavi, 1975; Evans and Hatchwell, 1992), to audio calls like alarm calls in primates (Seyfarth *et al.*, 1980). In bacteria signalling takes the form of molecules (either proteins or biosynthetic products) that are either secreted into the environment or displayed on the surface of cells (Foster *et al.*, 2004; LeRoux *et al.*, 2015; Winzer *et al.*, 2002).

There is some confusion about what constitutes a signal, particularly in microbiology literature where signalling is a newer idea (Winzer *et al.*, 2002). The need for both the signalling act and the receivers response to have evolved is key to identifying signals. If a structure provides information to another individual, but did not evolve for this reason then it is a cue, or if the response to the signal has not evolved then this is coercion (Smith and Harper, 2003) Many signalling molecules in bacteria are possibly better classified as cues (Winzer *et al.*, 2002), and theory suggests that there are opportunities for coercion with bacterial signalling (Brown and Johnstone, 2001).

1.3.5 Quorum sensing

Quorum sensing (QS) is the paradigm of bacterial cell-cell signalling. Bacteria secrete small signal molecules into the environment and then detect the concentrations of these signals, giving information about the biotic (Darch *et al.*, 2012; Fuqua *et al.*, 1994) and abioitc (Hense *et al.*, 2007; Redfield, 2002) aspects of the environment. Signal molecules take many forms including peptides and small molecules, but are usually relatively cheap to produce (Keller and Surette, 2006).

Quorum sensing often regulates the expression of a large proportion of the genome (Gilbert *et al.*, 2009; Antunes *et al.*, 2007), which includes disproportionate numbers of secreted genes (Popat *et al.*, 2015a) and is often involved in the regulation of virulence (Rumbaugh *et al.*, 2009). Furthermore in *Staphylococcus aureus,* methicillin resistance impairs quorum sensing through the *agr* locus reducing virulence (Rudkin *et al.*, 2012). For other bacteria such as *Vibrio cholerae* quorum sensing may be essential to regulate relatively complex cycle of phenotypes required for infection (Zhu and Mekalanos, 2003). This has led to suggestions that QS may be an attractive target for therapeutics (Schuster *et al.*, 2013; Rutherford and Bassler, 2012).

Since the first discovery of signalling in bacteria, the functional role of QS regulation has been a topic of speculation. Initially it was thought to be a mechanism to sense density (Fuqua *et al.*, 1994) a view that is still common today. Since then quorum sensing has been hypothesised to be involved in sensing mass transfer in the environment (Hense *et al.*, 2007; Redfield, 2002). Many bacteria possess multiple integrated quorum sensing systems, and it has been shown that multiple signals with different properties (e.g. decay rates) can allow bacteria to assess multiple aspects of the environment including density and mass transfer (Cornforth *et al.*, 2014).

Quorum sensing signalling is often highly specific to bacterial species or even strains of bacteria, to avoid coercive strategies and cheating (Eldar, 2011). On the other hand the discovery of signalling systems that are shared between many bacterial species (particularly AI-2), suggests that signals may function to allow communication at a more broad scale (Xavier and Bassler, 2003). Given the difficulty of stabilising cooperative behaviours between species these molecules are likely to be cues or coercive structures,

particularly given that AI-2 is likely to be a metabolic by product (Winzer *et al.*, 2002).

1.3.5 Quorum sensing in P. aeruginosa

P aeruginosa has 3 major interlinked quorum sensing systems (figure 1.1). Two of these systems use N-acyl homoserine lactones (HSLs) as signalling molecules a common strategy in gram negative species. The Las network uses 3-oxo-C12 N-acyl HSL (referred to as C12), the Rhl system uses C4 N-acyl HSL (referred to as C4) and the PQS system uses akyl quinalone signals (Rutherford and Bassler, 2012; Schuster *et al.*, 2013). All these signalling systems interact but they have classically been regarded as a hierarchy with the Las system at the top of the hierarchy (the master regulator). Recently this view is being called into question, with studies showing that phosphate depletion stress can lead to Las independent activation of the other QS systems (Jensen *et al.*, 2006; Lee *et al.*, 2013). In *P. aeruginosa* quorum sensing controls 6-11% of the genome (Gilbert *et al.*, 2009). Quorum sensing has been implicated in the control of siderophores, extracellular enzymes, stress responses, swarming, virulence and biofilm formation (Jimenez *et al.*, 2012).

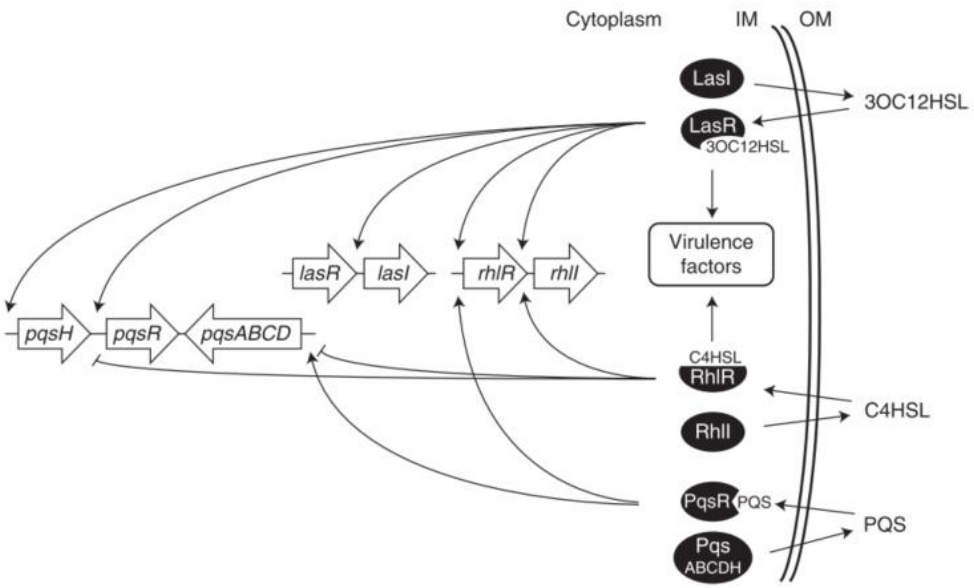

Figure 1.1: A simple schematic of the interactions between the three quorum sensing systems in *P. aeruginosa*. Arrows represent upregulation while blind arrows represent repression. IM = inner membrane, OM = outer membrane. 3OC12HSL = C12 signal, C4HSL= C4 signal. Dark circles are proteins and hollow arrows are genes. Reproduced from Rutherford and Bassler, 2012

P. aeruginosa is a popular organism for investigating quorum sensing. It has been used to look at cheating strategies and the effects of multiple gene control on cheating (Dandekar *et al.*, 2012; Diggle *et al.*, 2007; Sandoz *et al.*, 2007). Studies have looked at how quorum sensing regulates density dependent benefits (Darch *et al.*, 2012) and how information from multiple signalling networks can be integrated to control gene expression (Cornforth *et al.*, 2014). As an important opportunistic pathogen *P. aeruginosa* is an important model of the role of QS in virulence (Rumbaugh *et al.*, 2000), and a number of QS inhibitory drugs have been developed using *P. aeruginosa* (Hentzer *et al.*, 2002; Kaufmann *et al.*, 2006).

1.4 Synopsis

This thesis investigates secreted virulence factors and determinants of antibiotic resistance. Chapter 2 reviews literature on the different ways that we can inhibit bacterial virulence with anti-virulence therapeutics. Using published examples the selection for resistance to anti-virulence therapeutics is investigated. Chapter 2 argues that the fitness effects of the inhibited virulence factors are the major determinant of selection for resistance to an anti-virulence drug. The fitness effects of virulence factors will depend on the ecology of the bacterium including selection in alternate environments which may select for virulence factors even if they serve no benefit in the host. This chapter also reviews literature about how population structure affects selection for virulence factors, due to exploitation of costly virulence factors (produced by cooperators) by cheats that contribute less to virulence. The chapter shows that population structuring is particularly important for the evolution of resistance to QS inhibitors. This is because the signalling feedback that is intrinsic to QS networks can result in different selection for resistance with population structure, depending on the way that QS is inhibited.

The complexities of QS networks are shown in chapter 3. This chapter shows that positive feedback between signal production and signal reception leads to correlations between the proportion of cheat strains that do not respond to signal and signal concentration. This means that the expression of QS controlled genes will depend on the proportion of cheats in the population. For secreted QS regulated genes this is important because these public goods will not be expressed when cheats are at a high proportion in the population, reducing exploitation. This chapter uses a meta-population model to show that this can reduce the amount of population structuring (which influences r in equation 1.1) needed to maintain public goods production.Chapter 4

investigates the interaction between QS inhibitors and antibiotics. It has previously been shown that QS inhibitors act synergistically with the translation inhibitor tobramycin. However this is only in the context of preventing the development of functional biofilms which provide phenotypic resistance to antibiotics. This chapter shows that when growth is planktonic (single cells in liquid medium), quorum sensing makes *P. aeruginosa* more susceptible to translation inhibitors. As QS inhibitors weakly inhibit quorum sensing this chapter shows they act antagonistically with tobramycin, a translation inhibitor. The results of this chapter are consistent with the hypothesis that the protein synthesis burden resulting from upregulation of the majority of the QS regulon may produce this interaction.

As current QS inhibitors are only weakly effective, chapter 5 investigates the established synergy between clavulanate (β-lactamase inhibitor) and amoxicillin (β-lactam antibiotic), used together clinically as co-amoxiclav. Using theory and experimental evolution this chapter show that selection for resistance mechanisms depends on the ratio of the components of co-amoxiclav, even when the inhibitory effect is similar between ratios. Results show that resistance evolves slower when clavulanate (the adjuvant) is at higher proportions. This chapter also investigates how the drug interaction experienced by the bacterium is affected by selection, showing evidence that selection leads to a reduction in the synergy between amoxicillin and clavulanate.

Work in the appendix investigates the potential of the QS network to adapt to new environments where QS regulated traits are required to grow, but a quorum cannot be reached to upregulate them. These preliminary results are accompanied by a discussion of the problems associated with this chapter leading it to be moved to the appendix.

2 Targeting virulence: can we make evolution-proof drugs?

Published in a similar format as an opinion article in Nature Reviews Microbiology under the same name:

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Written and researched by RCA with guidance and editing from SPB. RP and SPD provided minor suggestions for edits.

2.1 Abstract

Anti-virulence drugs are a new type of therapeutic targeting virulence factors, potentially revitalising our drug development pipeline with new targets. Because anti-virulence drugs disarm rather than kill or halt pathogen growth, it has been hypothesised that they will generate much weaker selection for resistance. However, recent studies have demonstrated that mechanisms of resistance to anti-virulence drugs exist, seemingly damaging the 'evolution proof' claim. Here we highlight a critical distinction between whether resistance can emerge and whether it will spread under drug selection to a high frequency. We review recent work on the environment dependent costs and benefits of virulence factor expression that will underlie selection for resistance to anti-virulence drugs. Based on these findings we argue that selection for resistance can be reduced or even reversed with appropriate combinations of target and treatment environment, opening a path towards evolutionarily robust novel therapeutics.

2.2 Introduction

It is well established that our current practices of antibiotic use are unsustainable, due to the spread of antibiotic resistant pathogens (Davies and Davies, 2010). Resistance mechanisms are readily acquired by both *de*

novo mutation (Toprak *et al.*, 2012) and horizontal gene transfer from environmental reservoirs (D'Costa *et al.*, 2011; Wright, 2007). Viable resistance mechanisms have even been demonstrated for therapeutics like vancomycin or cationic antimicrobial peptides where resistance was once thought to be impossible (Wright, 2007; Habets and Brockhurst, 2012). If an antibiotic kills or inhibits the growth of sensitive strains, this will allow any resistant strains to grow in a competitor-free environment, creating strong selection for antibiotic resistance mechanisms (Lipsitch and Samore, 2002). Although resistance is often initially costly, secondary mutations that ameliorate this cost quickly spread so that the frequency of resistance does not decline when antibiotic use is reduced (Andersson and Hughes, 2010). For instance, mutations in *rspL* conferring Streptomycin resistance in *Escherichia coli* impose costs by slowing peptide elongation (Schrag and Perrot, 1996). However secondary mutations in *rpsD* and *rpsE* increase the rate of elongation, removing the cost of resistance (Schrag and Perrot, 1996; Levin *et al.*, 2000). The rapid spread of resistance means that the clinical lifespans of antibiotics are short, reducing profits and therefore incentives for the development of novel antibiotics, thus compounding the issue of resistance (Coates *et al.*, 2011).

So what can be done when the very action of antibiotics strongly selects for resistance? Rather than kill or halt bacterial growth, one emerging strategy is to 'disarm' pathogens (Clatworthy *et al.*, 2007; Rasko and Sperandio, 2010) by targeting virulence directly using anti-virulence (AV) drugs (Box 2.1). As AV drugs are not designed to directly harm their targets, numerous papers have argued that they will have little effect on the fitness (net growth rate) of the pathogen in the host (Rasko and Sperandio, 2010; Clatworthy *et al.*, 2007), and therefore approach the ideal of an 'evolution proof' therapeutic that does

not impose selection for resistance. Resistance to antibiotics is commonly defined and quantified as the recovery of bacterial population growth in the face of antibiotic exposure (Wright, 2007). However as we illustrate below there is often a significant disconnect between bacterial growth and the expression of virulence factors (figure 2.1). Therefore a resistance definition expressed purely in terms of growth recovery of the pathogen will not suffice for resistance to AV drugs. Here we define resistance to an anti-virulence drug as the recovery of virulence factor expression in the face of AV drug treatment

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Box 2.1- How can we target virulence

Virulence factors (VFs) are molecular determinants of virulence; pathogen genes that are non-essential to *in vitro* growth in rich media but cause increased virulence upon infection of a host (Silverstein and Steinberg, 1990). VFs are the key target of anti-virulence (AV) drugs (including antibodies and enzymes that are not small molecules). Several AV drugs and their targets are listed below (for more exhaustive lists see Rasko and Sperandio, 2010; Fernebro, 2011).

Bicyclic 2-pyridones bind to the PapC and FimH chaperones, preventing interaction of the chaperone-pilus subunit complex with the usher, inhibiting pilus formation in *Escherichia coli* (Pinkner *et al.*, 2006).

Virstatin inhibits expression of the toxin co-regulated pilus in *Vibrio cholerae* but also affects regulation of cholera toxin (Hung *et al.*, 2005).

2-imino-5-**arylidene thiazolidinone** inhibits type 2 and type 3 secretion systems in a wide variety of gram negative pathogens, likely due to an effect on the conserved secretin protein involved in both processes (Felise *et al.*, 2008).

B81-2 is a salicylidene acylhydrazide molecule which impedes VirB8 dimerisation in *Brucella abortus*, thus inhibiting type 4 secretion (Smith *et al.*, 2012). Similar salicylidene acylhydrazide compounds inhibit type 3 secretion in several other pathogens (Wang *et al.*, 2011).

Urtoxazumab is one of many anti-toxin antibodies, it is in clinical trials as an inhibitor of shiga toxin function in enterohemorrhagic *E. coli* (Yamagami *et al.*, 2001; López *et al.*, 2010).

Phosphonosulfonates inhibit CrtM, preventing biosynthesis of staphyloxanthin, a golden pigment that protects *Staphylococcus aureus* from reactive oxygen species (Liu *et al.*, 2008). Phosphonosulphonates are one of several AV drugs which are repurposed from existing drugs, reducing development time and costs.

AiiA enzyme is a lactonase isolated from *Bacillus* species it degrades the lactone bond of acyl homoserine lactone (AHL) molecules used as quorum sensing (QS, Box 3) signals (Dong *et al.*, 2001).

BuT DADMe-ImmA is a transition state analogue that inhibits the MTAN enzyme, preventing synthesis of QS signals in *V. cholerae* and *E. coli* (Schramm *et al.*, 2008).

C-30 is a derivative of natural furanone compounds that targets the LasR receptor in *Pseudomonas aeruginosa* (Hentzer *et al.*, 2002), it is one of many inhibitors that target the signal receptor complex.

We set out to dissect the hypothesis that anti-virulence drugs may be evolution proof. On first examination the hypothesis appears to be clearly false, because resistance has already been reported in several cases. Resistant strains have been isolated in clinical settings (García-Contreras *et al.*, 2013b;

Maeda *et al.*, 2012) and have been generated in laboratory systems (Maeda *et al.*, 2012; Hung *et al.*, 2005; Smith *et al.*, 2012).

For example the inhibitory impact of the salicylidene acylhydrazide drug B81-2 (Box 2.1) on type 4 secretion system expression is significantly diminished in several mutants identified by directed mutagenesis of the target protein VirB8, demonstrating that mechanisms of resistance are available to selection (Smith *et al.*, 2012). Examples like this have led to suggestions that resistance will hinder clinical efficacy (Defoirdt *et al.*, 2010; García-Contreras *et al.*, 2013a). However, the existence of mechanisms of resistance does not necessarily mean that this resistance will spread and become a clinical problem (Read *et al.*, 2011).

In this article we highlight a critical distinction between whether potential mechanisms of resistance exist (a question of mechanism) and whether potential mechanisms of resistance will spread to a high frequency in treated populations (a question of selection). The ubiquity of resistance mechanisms observed in natural populations (Maeda *et al.*, 2012; García-Contreras *et al.*, 2013b; D'Costa *et al.*, 2006; Defoirdt *et al.*, 2010) suggests that it is the question of selection that is most critical; as it is selection that governs the persistence and spread of any potential resistance mechanism. Given the inevitability of resistance mechanisms, will they spread in the face of widespread AV drug deployment? What can we do to mitigate the spread of resistance to AV drugs? To understand these questions, we must first consider the consequences of virulence factor (VF) expression for pathogen fitness. More colloquially, what is virulence for?

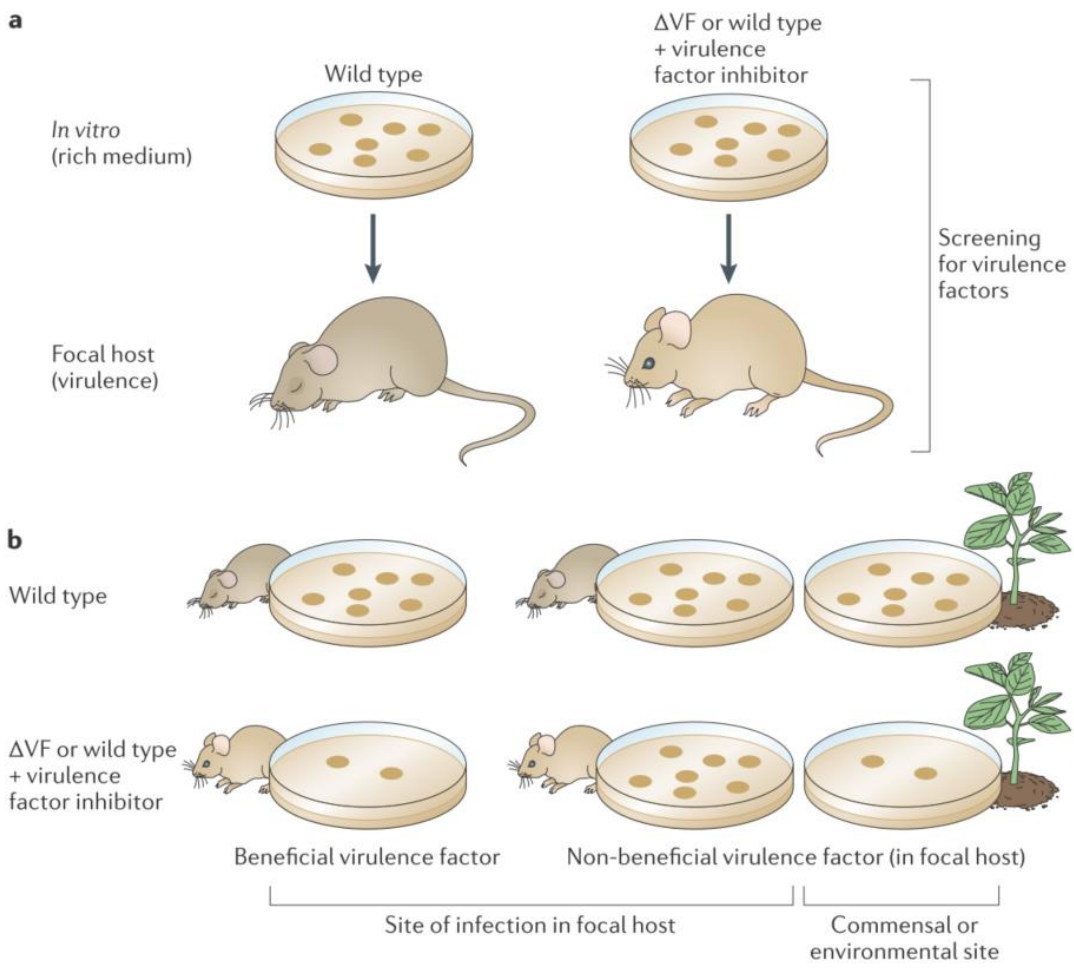

Figure 2.1: The effects of virulence factors on fitness and virulence in different environments. a) Virulence factors are commonly defined as molecular determinants of virulence; pathogen genes that are non-essential to *in vitro* growth in rich media but cause increased virulence upon infection of a host (Silverstein and Steinberg, 1990). Importantly, this definition of VFs makes no predictions about the consequences of VF expression for pathogen fitness in the host. The figure shows that a mutant strain in which the virulence factor has been deleted (ΔVF) can have identical fitness to a wild-type strain in rich medium *in vitro* but causes less pathology in the focal host. b) The effects of VF expression on pathogen fitness can be variable, particularly between sites of infection and commensal/environmental sites. The number of colonies represents pathogen fitness (net growth) in the specified environment. If an inhibitor targets a beneficial virulence factor that aids

growth at the site of infection , pathogen fitness will be reduced, causing selection for resistance to the inhibitor. If an inhibitor targets a non-beneficial virulence factor that does not aid growth at the site of infection, there will be no effect on pathogen fitness and no selection for resistance at the site of infection.

2.3 Ecological and evolutionary reasons for virulence

The evolution of virulence (pathogen-induced host damage) is a major puzzle in evolutionary biology, and has generated a range of responses to the underlying theoretical question 'why harm the source of your livelihood, your host' (Alizon *et al.*, 2009)? The dominant hypothesis states that virulence is an unavoidable cost or side effect of growing within a host and transmitting to the next host and so is maintained as the result of a trade-off between the costs of host pathology and the benefits of transmission to a new host (Anderson and May, 1982; Alizon *et al.*, 2009). Other hypotheses highlight the importance of selection in non-disease settings, where alternate functions of virulence factors can co-incidentally select for VF-induced damage to human hosts (Figure 2.1, discussed in the following section, Levin and Eden, 1990; Brown *et al.*, 2012).

We argue that understanding the selective forces maintaining the carriage and expression of a virulence factor is vital to understanding the selective pressures for resistance to an anti-virulence drug which targets that VF. The identification of virulence factors classically revolves around a simple screen for non-essential genes that are predictive of damage in a model host system (figure 2.1a, Silverstein and Steinberg, 1990; Bae *et al.*, 2004). Evidence for VFs being 'non-essential' is inevitably found in rich *in vitro* growth media, often simply the ability of a mutant to grow to a density that can be used in further assays (Bae *et al.*, 2004), far from the *in vivo* conditions governing selection for resistance to AV drugs. Here, we focus on whether the targeted virulence

factor provides a selective advantage to the target pathogen at the *in vivo* site of treatment. In figure 2.2 we outline our predictions on the evolutionary robustness of differing AV strategies, which are expanded on below.

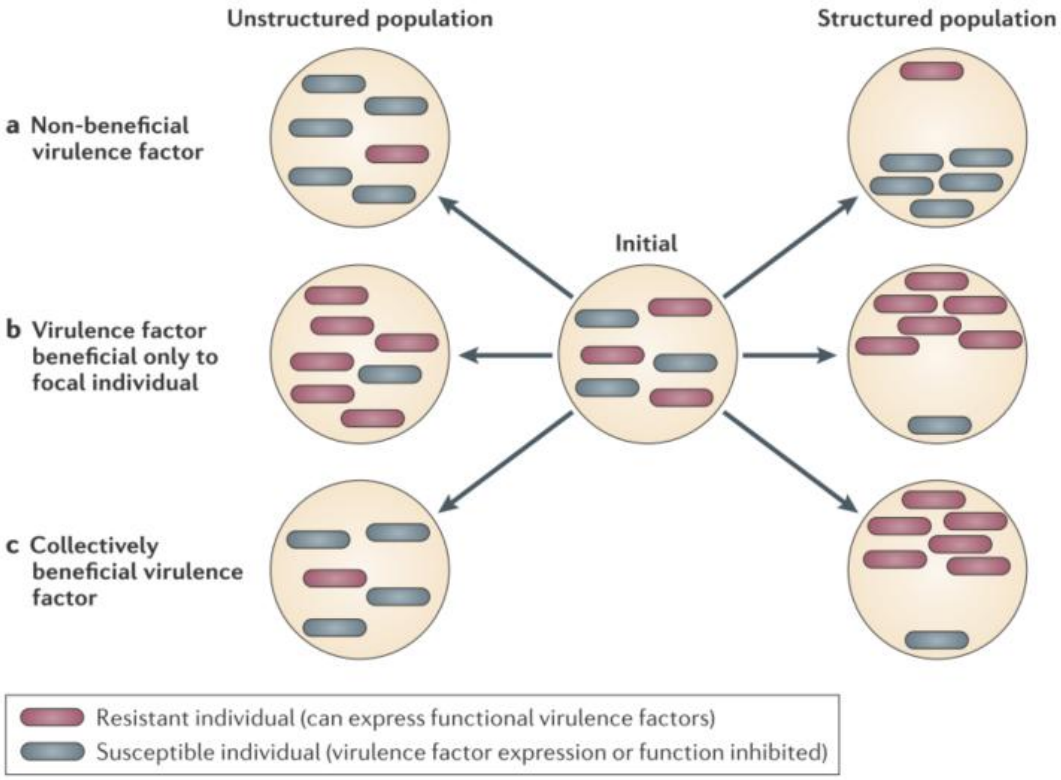

Figure 2.2: Predicted selection on resistance to antivirulence therapeutics. The direction of selection on resistance mechanisms is predicted to vary as a function of local benefits of virulence-factor expression and population structure. a) The cost of expressing non-beneficial virulence factors selects against resistance, regardless of the population structure. b) The benefits of expressing individually beneficial virulence factors select for resistance, regardless of the population structure. c) The benefits of collectively beneficial virulence-factor expression can be exploited by susceptible individuals, but only if the population is unstructured.

2.4 Evolution-proof targets: non-beneficial virulence factors

The first scenario is the simplest: the VF has no benefit at the site of infection (site of colonisation and damage in the focal host), just as they commonly show no *in vitro* benefit in rich media (figure 2.1a). If a virulence factor offers no benefits to a pathogen at the site of infection, then targeting this VF with a therapeutic at the site of infection will not impose any within-host selection for resistance. Resistance could even be selected against because treatment allows the sensitive bacteria to avoid the metabolic costs of inappropriate VF expression and potentially reduced transmission from an ill host (Figure 2.2, Box 2.2 prediction 1). While the logic is clear, the empirical question of whether there are VFs that offer no fitness benefit to a pathogen at the site of infection is more open to debate.

Box 2.2 Key predictions

1) AV drugs will select *against* resistant strains when the targeted VF confers no benefits to the pathogen at the site of treatment

2a) AV drugs will select *for* resistant strains when the targeted VF confers direct benefits to the pathogen at the site of treatment

2b) AV drugs will generate weaker selection for resistance if the target VF is conditionally beneficial and/or conditionally expressed

3a) AV drugs will select *against* resistant strains when the targeted VF confers collective benefits to a well-mixed population

3b) AV drugs will select *for* resistant strains when the targeted VF confers collective benefits to a sufficiently structured population

4) AV drugs reducing the supply of quorum-sensing signals (e.g. signal degrading enzymes) will generate weak selection on resistance in well-mixed populations

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2.4.1 Coincidental virulence factors

The best candidates for non-beneficial VFs are found in opportunistic pathogens that normally exploit distinct environments (non-human environments or commensal compartments within human hosts, Brown *et al.*, 2012), and whose VFs are the products of coincidental selection in these distinct environments for phenotypes not associated with virulence (Figure 2.1b, Levin and Eden, 1990; Brown *et al.*, 2012). Potential candidates can be found among the extra-intestinal pathogenic *E. coli* (ExPEC) strains (such as uropathogeneic and meningitis associated *E. coli*). ExPEC strains are opportunistic pathogens frequently isolated from healthy intestinal flora that also cause a variety of diseases in distinct extra-intestinal sites such as the brain or urinary tract; sites that are associated with poor transmission compared with the intestine (Köhler and Dobrindt, 2011).

ExPEC associated VFs including adhesins (P-pili) and iron acquisition factors (Yersiniabactin, Nowrouzian *et al.*, 2006; Diard *et al.*, 2010) are associated with persistence as a commensal in the intestine. OmpA and lipopolysaccharide have also been shown to be beneficial for interactions with amoeba in the environment (Alsam *et al.*, 2006). Although these VFs are associated with virulence in an extraintestinal mouse model (subcutaneous injection of bacteria into inbred mice) they conferred no measurable benefits in the presence of a variety of biological stressors (Diard *et al.*, 2010). Furthermore, phylogenetic analyses show that extraintestinal virulence is ancestral and conserved within certain *E. coli* sequence types (B2 sequence type). This suggests that coincidental selection among commensals in the

intestine is responsible for the maintenance of these VFs, and not direct selection for virulence outside the intestine (Gall *et al.*, 2007). P-pili biogenesis in ExPEC is targeted by Bicyclic 2-pyridones (Box 2.1, Pinkner *et al.*, 2006), however the selective consequences of Bicyclic 2-pyridones will depend on which environment they are deployed in. If treatment is specifically targeted at the urinary tract then we would predict that resistance would not be selected for yet pathology would be reduced. In the more likely case of systemic treatment (including both the urinary tract and the intestine), however, resistance may be selected for in the intestine where P-pili are reported to confer a selective advantage.

Although various VFs have been hypothesised to be selected for outside of the human host (Levin and Eden, 1990; Steinberg and Levin, 2007), the list of candidate 'locally non-beneficial' VFs is currently very short, largely we believe because of a lack of research focus on mapping the costs and benefits of VF expression to the pathogen, at the site of infection (for exceptions, see the plant pathogen literature, e.g. Montarry *et al.*, 2010). We suggest that pathogen fitness is an overlooked quantity; future work should seek to identify the fitness costs and benefits of VF expression in relevant host environments. Competition experiments between VF knockouts in the site of virulent colonisation is key to identify these fitness effects.

2.5 Evolution-prone targets: beneficial virulence factors

One of the key attributes of AV therapeutics is that they transform pathogenic populations into a less virulent state rather than clearing pathogens directly, meaning that long courses of AV drugs may be required to maintain a state of reduced virulence. Several AV drugs have, however, been shown to aid clearance. Furanone inhibitors of quorum sensing (Box 2.1) increase immune or antibiotic associated clearance of *P. aeruginosa*,

because of the effects of quorum sensing on immune modulation and biofilm formation respectively (Wu *et al.*, 2004; Christensen *et al.*, 2012). While this renders AV treatment potentially more attractive (particularly as a combination therapy with traditional antibiotics), it also implies that some of the VFs inhibited by furanone compounds must obviously be beneficial to a pathogen in the host, at least in the context of an intact immune system and/or concurrent antibiotic treatment.

A number of VFs have now been demonstrated to confer a range of benefits within the host in addition to protective roles described above. These benefits include providing access to limiting resources (Oogai *et al.*, 2011; Genco and Dixon, 2001) and enhancing interspecific competitive ability (Stecher *et al.*, 2007; Lysenko *et al.*, 2010). For instance, blocking expression of cholera toxin and the toxin co-regulated pilus in *Vibrio cholerae* with Virstatin (Box 2.1) has negligible effect on growth *in vitro* but markedly decreased colonisation of an infant mouse model (6 day old mice, infected using a stomach tube), indicating that these VFs increase fitness within a host (Hung *et al.*, 2005). The coupling between VF expression and pathogen fitness means that AV drug treatment will be detrimental to pathogens. In this case a resistant mutant will recover fitness leading to selection for resistance, at least on the within host scale (Levin and Bull, 1994, Figure 2.2, Box 2.2 prediction 2a). In accordance with this expectation a resistant mutant with a point mutation in the target of virstatin (*toxT*) outcompeted a susceptible strain in a treated host (Hung *et al.*, 2005).

2.5.1 Species and environment specificity

Unlike antibiotics, the targets of AV drugs are only likely to be beneficial in specific environments, which may be within, or outside of the site of infection (Diard *et al.*, 2010); or indeed outside of the host entirely (Steinberg

and Levin, 2007). In addition, the targets of AV drugs are often specific to certain pathogen species (narrow spectrum). Therefore populations outside of critical sites where the VF is expressed and is beneficial are unlikely to experience selection for resistance.

As an example of why the environmental specificity of AV drugs is beneficial consider treatment of *Staphylococcus aureus* with phosphonosulfonates (Box 2.1, Liu *et al.*, 2008). Phosphonosulfonates inhibit CrtM, an enzyme responsible for the biosynthesis of staphyloxanthin, a golden pigment that protects *S. aureus* from reactive oxygen species. Susceptible *S. aureus* cells are not directly harmed by the drug, but will be killed by reactive oxygen species (ROS) at the site of infection. Phosphonosulphonates will therefore impose strong selection for resistance at the site of infection (even though death is caused by the immune system not directly by the drug). However, in its commensal lifestyle *S. aureus* is exposed to relatively minor ROS challenge, and as a result *crtM* expression has no effect on nasal colonisation(Liu *et al.*, 2008). Therefore populations at the commensal site will not be under strong selection for resistance. This specificity can be viewed as an extension of the principle of narrow-spectrum antibiotics to include specificity of environment as well as species specificity. By restricting the population from which resistance can be selected (compared with antibiotics), environmental specificity will similarly slow the evolution of resistance (by restricting mutational supply, Hall *et al.*, 2010, and exposure to selection, Dyken and Wade, 2010), even for VFs that are tightly coupled to fitness in the site of infection (Box 2.2 prediction 2b).

Even for virulence factors that are strongly beneficial within a site of infection, note that the epidemiological spread of resistance mechanisms restoring VF expression can be largely or completely halted if the infection

site is an epidemiological 'dead end', with transmission instead coming from commensal or environmental populations (Sokurenko *et al.*, 2006; Meyers *et al.*, 2003).

2.6 Contingent targets: socially mediated virulence factors

2.6.1 Public goods

Within the broad class of beneficial VFs, an important distinction must be made between VFs that return an immediate and private benefit to the (focal) bacterium expressing the trait (e.g. adhesins), and collectively beneficial VFs that return a benefit to a group or neighbourhood of bacteria (e.g. secreted siderophores, enzymes and toxins). Many virulence factors fall into the second 'cooperative' category (Nogueira *et al.*, 2012), characterised by the secretion of costly molecules that scavenge, digest or liberate resources promoting growth (Brown *et al.*, 2002; Buckling *et al.*, 2007; Nogueira *et al.*, 2009; Rumbaugh *et al.*, 2009). From a social evolution perspective, these secretions are characterised as 'public goods', costly individual contributions to a collectively beneficial enterprise (West *et al.*, 2007). Theoretical work has shown that targeting collectively beneficial VFs can greatly reduce selection for resistance because of the general property that public goods can be exploited by neighbours (André and Godelle, 2005).

2.6.2 Exploitation of social behaviours

A widely corroborated result of social evolution theory is that cooperative behaviours are vulnerable to local exploitation by cheats that do not perform the cooperative behaviour. For instance in *P. aeruginosa* non-producers (cheats) of the secreted siderophore pyoverdin avoid paying the metabolic costs of pyoverdin production, but are still able to use siderophores produced by their cooperative neighbours (Griffin *et al.*, 2004). As a result,

cheats increase in frequency in a well-mixed environment (random interactions e.g. a shaken flask, West *et al.*, 2007; Brown *et al.*, 2009; Griffin *et al.*, 2004), and within hosts in animal infection models (Rumbaugh *et al.*, 2009; Harrison *et al.*, 2006) where this also reduces virulence (Rumbaugh *et al.*, 2009).

The local advantage of cheats (non-producers of a secreted, collective VF) relative to cooperators (producers of the VF) translates into the prediction that AV drugs targeting collectively beneficial VFs will select *against* resistance in a well-mixed environment (André and Godelle, 2005). If a drug inhibits a collectively beneficial VF, the susceptible population become phenotypic cheats (Mellbye and Schuster, 2011). These cheats will then socially exploit any resistant individual that is able to produce the collectively beneficial VF, leading to selection against resistance in mixed populations (Mellbye and Schuster, 2011, Figure 2.2, Box 2.2 prediction 3a). In *P. aeruginosa* quenching pyoverdin extracellularly with gallium is robust to the evolution of resistance in conditions where pyoverdin is required for growth (Ross-Gillespie *et al.*, 2014).

2.6.3 The effects of structure

The evolutionary robustness of targeting collectively beneficial VFs has one important caveat. Cooperative behaviours can be under positive selection if the population is sufficiently structured, because genes promoting cooperation will then preferentially help gene-copies in neighbouring cells (Hamilton, 1964; Griffin *et al.*, 2004; Diggle *et al.*, 2007; Kümmerli *et al.*, 2009). Therefore within-host structuring is likely to select for resistant mutants that maintain expression of collectively beneficial VFs, because the benefits of cooperative investments by resistant individuals will preferentially fall to clonally related (resistant) neighbours (Figure 2.2, Box 2.2 prediction 3b).

There is growing evidence for within host structuring (genetic segregation) in a number of host pathogen systems from systemic *Salmonella enterica* infections in mice (Grant *et al.*, 2008) to *P. aeruginosa* lung infections in cystic fibrosis patients (Willner *et al.*, 2012). At a more local scale biofilms are a common and problematic feature of many bacterial infections (Costerton *et al.*, 1999), and are characterised by significant genetic structuring (Kreft, 2004). Biofilms are a target for anti-virulence therapeutics (Brackman *et al.*, 2011b) which means that resistant cells could then make biofilms and exclude susceptible cells (Oliveira *et al.*, 2015), this structuring could then allow selection for resistance to collectively beneficial VFs. Other examples where expression of virulence factors could generate population structure would be where VFs allow colonisation of a new niche such as penetration of mucosal barriers (Raymond *et al.*, 2012).

2.7 Quorum sensing as a therapeutic target

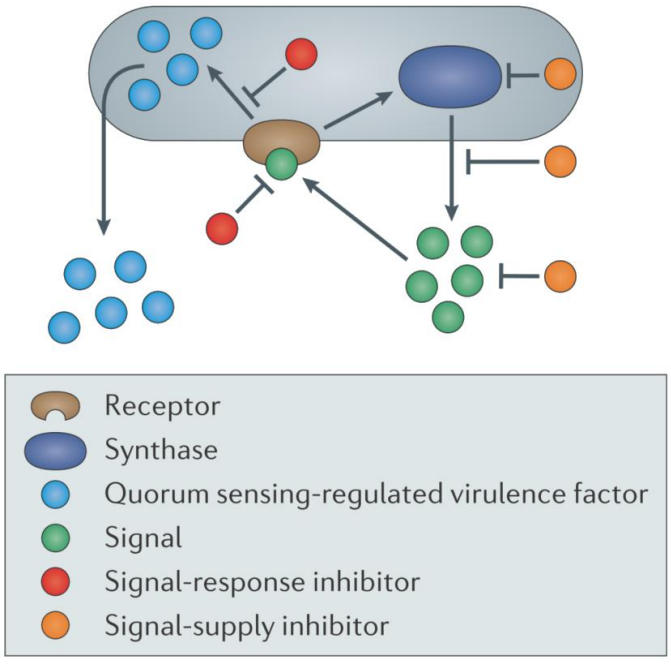
Quorum sensing (QS) is a cell-cell signalling behaviour that has received a lot of attention as a potential therapeutic target because it often controls numerous VFs (Box 2.3). We highlight the distinction between 'signal response' inhibitors (impairing the ability of individual cells to respond to signal molecules) and 'signal supply' inhibitors (impairing the production/persistence of signals in the environment), as we predict that these two approaches will present different risks of resistance evolution (Box 2.3, Figure 2.3).

Box 2.3 Quorum sensing as a regulator of virulence and how we can target it

Quorum sensing (QS) is a cell-cell communication system that controls many phenotypes, including virulence, in many bacterial pathogens including *Erwinia carotovora, Staphylococcus aureus, Pseudomonas aeruginosa* and *Vibrio*

cholerae (for relevant reviews see, Rutherford and Bassler, 2012). QS contributes to virulence by regulating a number of virulence factors, predominantly collectively beneficial VFs (Gilbert *et al.*, 2009, e.g. proteases, lectins, toxins and biofilm polymers). This has spurred interest in QS inhibitors (or quorum quenchers) as anti-virulence drugs. Several QS inhibitors have been shown to reduce virulence and aid clearance of pathogens in both animal and plant models of infection (Wu *et al.*, 2004; Dong *et al.*, 2001; Wright *et al.*, 2005a; Papaioannou *et al.*, 2009).

The specific QS network architecture varies across species, but the key steps of signal production and signal response are constant, as in any communication system (Rutherford and Bassler, 2012; Smith and Harper, 2003). We propose that this creates 2 functional classes of QS inhibitors (shown on the diagram below), signal supply inhibitors (orange) and signal response inhibitors (red), depending on whether a drug inhibits the function of the signaller or the receiver. Signal production can be chemically complemented by other individuals in the population, whereas signal reception cannot; this creates different selection pressures on resistance to signal supply inhibitors and signal reception inhibitors as we discuss later.



Signal response inhibitors are exemplified by antagonistic receptor binding drugs including signal molecule analogues like peptide inhibitors of the *agr* system in *Staphylococcus aureus*

(Wright *et al.*, 2005a) or mimics of acyl homoserine lactone (AHL) signals used by gram negatives (Ishida *et al.*, 2007). Response inhibitors include compounds that interfere with the responses to the received signal like cinnemaldehyde inhibitors of *Vibrio cholerae* QS (Brackman *et al.*, 2011a). Signal supply inhibitors can inhibit the production (But-DADMe-ImmA, Box 2.1, Schramm *et al.*, 2008) and theoretically export (Ni *et al.*, 2009) of signal, or they can inactivate signal in the environment, degrading them (AiiA, box 2.1, Dong *et al.*, 2001) or binding to them (antibodies raised against AHL, Kaufmann *et al.*, 2006, and peptide signal molecules, Park *et al.*, 2007).

2.7.1 Resistance to quorum sensing inhibitors

The relatively large research focus on QS inhibitors has led to some of the best characterised examples of resistance mechanisms to anti-virulence drugs (Defoirdt *et al.*, 2010). Resistance to furanone competitive inhibitors by overexpression of the MexAB-OprM efflux pump, can be selected for in vitro in *P. aeruginosa* and similar resistance mechanisms have been found in clinical isolates (Maeda *et al.*, 2012). The isolation of QS inhibitor resistance from clinical isolates is particularly concerning, and highlights the risks posed by cross-resistance to QS inhibitors, driven by antibiotic selection for broad activity resistance mechanisms such as efflux pumps (Maeda *et al.*, 2012).

Overexpression of the *traR* QS receptor in *Agrobacterium tumefaciens* reduced the antagonistic capabilities of compounds that inhibited signal binding in a wild type strain (Zhu *et al.*, 1998). Mutagenesis of the *luxR* QS receptor of *V. fischeri* in an *E. coli* reporter strain documented mutations that reduce antagonistic binding of several competitive inhibitors to the LuxR receptor (Koch *et al.*, 2005). This study also revealed that because competitive inhibitors have a similar structure to the signal, resistance to these

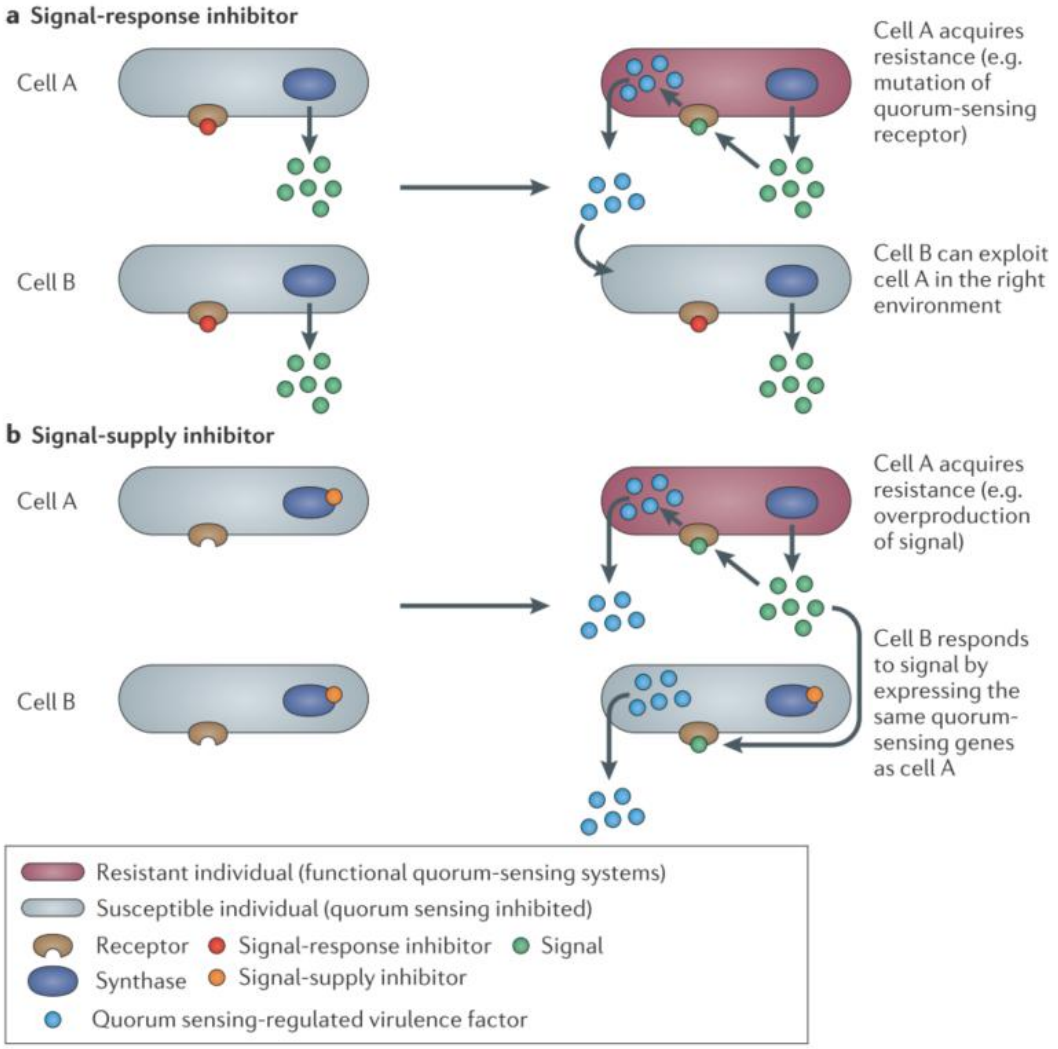


Figure 2.3: The mechanistic target of the quorum sensing inhibitor influences the direction of selection for resistance. a) If the inhibitor targets signal response (e.g. receptor blocking), only a resistant mutant can sense signal and produce VFs; collectively beneficial VFs can be exploited by neighbours so that resistance is selected against. b) If the inhibitor targets signal supply (e.g. signal cleaving enzymes), only resistant mutants will produce signal, inducing VF production in neighbours. For signal supply inhibitors the costs of VF production are shared and resistant individuals are neither favoured nor disfavoured compared with neighbouring susceptibles (assuming a negligible cost of signal production).

antagonists may also reduce sensitivity to the native signal, constraining the number of mutations that lead to effective resistance (Koch *et al.*, 2005; Eldar, 2011). A similar constraint would also occur if a change in signal structure

caused resistance (Eldar, 2011). Signal alteration has not been documented as a response to QS inhibition, but there is natural variability of peptide signals used by the *agr* locus in *S. aureus* and other staphylococci (Wright *et al.*, 2005b). There is also high variability in signal production levels in many strains with QS systems (Defoirdt *et al.*, 2010), and strains of *V. cholerae* with constitutive activation of QS regulated genes (Joelsson *et al.*, 2006) will be insensitive to QS inhibition (if it occurs upstream of the constitutive QS activation Defoirdt *et al.*, 2007). Similarly if individual VFs escape from QS regulation (Chugani *et al.*, 2012) VF expression will then be unaffected by QS inhibitors. Resistance may also occur by direct inactivation of the QS inhibitor but this has not been documented.

As before, the selective pressures acting on resistance will determine the fate of the resistance mechanisms that arise. In Table 2.1 we summarise our predictions about selection on resistance as a function of the benefits of QS controlled genes, within-host structure and the mechanism of QS inhibition.

2.7.2 Signal reception inhibitors

Signal reception inhibitors make susceptible cells signal blind, reducing the production of QS-regulated virulence factors. Therefore signal reception inhibitors will impose similar selective forces to those described for AV drugs that directly inhibit VF expression (Figure 2.2), with selection dependent on the costs and benefits of the regulated VFs in the treatment environment (Dandekar *et al.*, 2012).

If QS regulated VFs are collectively beneficial, inhibitors of signal reception render susceptible individuals phenotypic 'cheats'. For instance genetic knockouts of *P. aeruginosa* show that signal blind cheats are able to exploit protease produced by signal responsive individuals during well-mixed growth in vitro and in animal models (artificial wounds of mice infected with

high density *P. aeruginosa* culture, Rumbaugh *et al.*, 2009), when secreted protease is required for growth (Mellbye and Schuster, 2011; Diggle *et al.*, 2007, first row Table 1). As described in figure 2.2, this conclusion will change if the population is structured and resistant mutants can group together to form cooperative patches.

Table 2.1: Predicted direction of within host selection on resistance to quorum sensing (QS) inhibitors Resistance mechanisms are predicted to increase (+), decrease (-) or drift (no deterministic increase/decrease) (0) in frequency as a function of inhibition mechanism, population structuring and resource environment.

Inhibition Target		Signal response		Signal supply	
Population Structure		Unstructured	Structured	Unstructured	Structured
Benefit of QS Regulated VFs	No Benefit	**-**	**-**	**0**	**-**
	Benefit to focal individual only	**+**	**+**	**0**	**+**
	Collective Benefit	**-**	**+**	**0**	**+**

By contrast, if the QS regulated VFs only benefit the individual that expresses them, they cannot be exploited regardless of structuring and resistance will always be positively selected (Mellbye and Schuster, 2011; Maeda *et al.*, 2012). Furanone inhibitors of QS can select for resistance in *P. aeruginosa* grown on adenosine even in a well-mixed environment because QS dependent adenosine catabolism is intracellular (private) and therefore cannot be socially exploited by neighbouring cells (Maeda *et al.*, 2012).

2.7.3 Signal supply inhibitors

Inhibitors of signal supply (targeting either signal production or environmental persistence via signal degrading enzymes) will reduce signal levels in susceptible populations (Schramm *et al.*, 2008), therefore attenuating

the expression of QS-controlled virulence factors (Park *et al.*, 2007). If resistance mechanisms arise then active signal in the environment will only be produced by resistant individuals, but will be accessible to all individuals at equal (initially low) concentrations in a mixed environment. All individuals will express QS controlled VFs to an equal extent (if at all), meaning that, the benefits and costs of the virulence factors will not affect selection for resistance, so resistance may be neutral and subject to genetic drift. For example in *P. aeruginosa* genetic knockouts that are unable to synthesise signal do not outcompete strains that can synthesise signal when competed in a well-mixed environment where protease is required for growth (Wilder *et al.*, 2011). If individuals are resistant because they express more signal than susceptibles (rather than signal being insensitive to degradation), the cost of signal production in nutrient poor environments may still select against resistance. In contrast if a population is structured, signal will be preferentially observed by resistant individuals, meaning that only these cells will produce and enjoy the benefits of VFs, selecting for resistance if QS serves any benefit in the environment (Table 2.1, Box 2.2 prediction 4).

2.7.4 Multiple targets

QS influences the expression a significant proportion of the genome (approximately 5-10% for *Pseudomonas aeruginosa*, Schuster and Peter Greenberg, 2006; Chugani *et al.*, 2012), including multiple virulence factors. This broad-based influence on virulence expression is a major part of the attraction of QS inhibition, however it also raises the concern that such a large perturbation to cell functioning will drive selection for resistance (García-Contreras *et al.*, 2013a). We argue that despite the large expression footprint of QS inhibition, selection for resistance is not inevitable, and is

again environmentally determined. Given that approximately 90% of the QS regulon is activated by QS (Schuster *et al.*, 2003), resistance to QS inhibition will pose a significant cost in simple environments where the QS regulon is redundant (Diggle *et al.*, 2007), driving selection for sensitivity. When one or a few QS-controlled traits confer individual or collective advantages, these benefits must then be titrated against the simultaneously incurred costs of expression of other, redundant, traits. In defined environments conferring both individual and collective advantages to QS (protein plus adenosine media), the individual benefit of QS-mediated adenosine catabolism was generally sufficient to drive selection for QS (and by inference, resistance to QS inhibition), overcoming the costs of redundant gene expression plus the social costs of collective protein degradation (Dandekar *et al.*, 2012). The complex and highly interactive nature of QS regulation in *P. aeruginosa* also raises the prospect of more nuanced strategies of interference with QS. Recent work has demonstrated that a mix of partial receptor antagonism and agonism produces the most effective net reduction of critical virulence phenotypes (O'Loughlin *et al.*, 2013).

2.8 Changes in intrinsic virulence

We have discussed the selective fate of mutants that are resistant to AV drugs (able to express the targeted VFs in the presence of the drug), but are otherwise identical to their susceptible ancestor. Resistance is an important axis of potential evolution in response to AV drugs, but it is not the only dimension along which an evolving pathogen might respond. Here, we briefly discuss how pathogens may recover their fitness by altering their intrinsic (drug free) VF expression (Vale *et al.*, 2014), as one of the most important of these dimensions.

2.8.1 Increased virulence

Most worrying are examples where the use of AV drugs may select for higher intrinsic virulence of pathogens, as this would be particularly detrimental to untreated patients that are not protected by the therapeutic. As discussed earlier there is often a trade off between host morbidity and transmission (both of which may be the result of virulence). Therefore theory suggests that interventions that limit the virulent exploitation of a host (such as AV drugs), can select for higher intrinsic levels of virulence by relaxing the constraint of host death on transmission (Gandon *et al.*, 2001). Köhler et al found that by blocking costly collective VF expression in *P. aeruginosa*, QS inhibition (with the signal synthesis inhibitor azithromycin) reduced within-host selection for avirulent mutants (cheats), maintaining more virulent wild-type genotypes (Köhler *et al.*, 2010). Lastly it is possible that interference with regulatory processes may increase virulence by selecting for constitutive expression of virulence factors that are normally plastically expressed (Joelsson *et al.*, 2006).

2.8.2 Reduced virulence

AV drugs may also select for reduced virulence, as has been proposed for anti-toxin vaccines which inhibit the function of toxins once they have been produced (Soubeyrand and Plotkin, 2002). In the presence of the toxin inhibitor, the toxin serves no function (imposing only a metabolic cost), so loss of the virulence factor confers no cost to the pathogen in the treated host, but reduces the metabolic costs of producing a VF. Therefore VF-negative strains are under positive selection (Soubeyrand and Plotkin, 2002). In support of this, an antitoxin vaccine targeted against diphtheria toxin, (a metabolically costly phage-encoded toxin in *Corynebacterium diphtheriae)* led to a return to a commensal state, with a decrease in toxin positive strains (Pappenheimer, 1984). However, pathogens that become resistant to the anti-

toxin vaccine and re-express a beneficial VF will be more fit than VF negative strains, which may explain why this result has not been replicated (Gandon and Day, 2008). Anti-toxin therapeutics may also be overcome by overexpressing the toxin, which would also increase virulence in untreated hosts (Gandon and Day, 2008). It is imperative that these conflicting outcomes are reconciled as several anti-toxin therapeutic antibodies are already in clinical trials (Lowy *et al.*, 2010; López *et al.*, 2010).

Although phages are not AV therapeutics (as they kill pathogens) it is interesting that certain phage therapies may select for avirulence. Phages co-evolve with bacterial pathogens, so often the only long term bacterial resistance strategy is to lose the receptor for phage entry; if the receptor is a VF the outcome will be avirulence (Filippov *et al.*, 2011; Smith *et al.*, 1987). There would still be strong selection to express a modified receptor if it is beneficial but unlike antitoxin therapeutics, mutations causing over expression of the VF would increase susceptibility to the phage and would be selected against. The ability to synthetically manipulate phage specificity (Marzari *et al.*, 1997), may make this an attractive and achievable goal in the future.

2.9 Conclusions

There is real potential for the development of new and effective anti-virulence drugs, thanks to improved screening methodologies using genetically modified strains (Rasmussen *et al.*, 2005), drugs that target processes associated with virulence in several pathogenic species (Felise *et al.*, 2008; Hong *et al.*, 2012) and positive results of AV drugs in clinical trials (Lowy *et al.*, 2010; López *et al.*, 2010). But what are the potential lifetimes of anti-virulence drugs and how can we extend their effective use in the face of bacterial evolution? We have argued that while mechanisms of resistance to

these novel therapeutics are inevitable (and several already observed, Smith *et al.*, 2012; García-Contreras, Maeda, *et al.*, 2013; Maeda *et al.*, 2012; Hung *et al.*, 2005; Koch *et al.*, 2005; Zhu *et al.*, 1998), their rise in frequency under the action of drug selection is not inevitable and can even be reversed for particular combinations of VF target and treatment environment.

Unlike traditional antibiotics, where resistance is always advantageous (for an intriguing exception see, Chait *et al.*, 2007), the selective picture for anti-virulence drugs is more nuanced with resistance being potentially costly even in the presence of drugs. We outline a series of predictions (Box 2.2) about the direction of selection on resistant mutants, as a function of the match between the VF target and the pathogen's infection environment (figure 2.2, table 2.1). Our predictions suggest that a truly 'evolution proof' combination of VF target and treatment environment – where the drug treatment consistently selects against resistance – is possible (Box 2.2 predictions 1 and 3), in particular for cases where the targeted VF damages the host but serves no benefit to the pathogen. However, we caution that the benefits of VF expression are in general poorly understood and often mediated indirectly via effects on resistance to immune- or antibiotic-mediated clearance (Wu *et al.*, 2004; Christensen *et al.*, 2012). We suggest that through a careful integration of molecular and evolutionary microbiology, real progress can be made in the design and effective use of more evolutionarily robust novel therapeutics both alone and in combination with existing traditional therapeutics. Further progress in understanding and managing the evolutionary risks of anti-virulence drugs is currently limited by our lack of data on the costs and benefits of virulence-factor expression during infection. We strongly encourage more work in this direction. The effects of well studied virulence factors on growth of the pathogen within a

relevant host should be determined using competition experiments between strains with and without the VF at the site of virulent colonisation.

3 Quorum sensing protects bacterial cooperation from exploitation by cheats

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An article in a similar format has been accepted for publication by the ISME Journal and is in advance online publication.

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RCA developed the idea and performed the experiments. SPB and LM developed the theoretical model. LM designed the meta-population analysis. RCA and LM performed the statistical analysis. RCA wrote the manuscript with input from LM, SPB and RP.

3.1 Abstract

Quorum sensing is a cell-cell communication system found in many bacterial species, commonly controlling secreted cooperative traits including extracellular digestive enzymes. We show that the canonical quorum sensing regulatory architecture allows bacteria to sense the genotypic composition of high density populations, and limit cooperative investments to social environments enriched for cooperators. Using high density populations of the opportunistic pathogen *Pseudomonas aeruginosa* we map per-capita signal and cooperative enzyme investment in the wild-type as a function of the proportion of non-responder cheats. We demonstrate mathematically and experimentally that the observed response rule of 'cooperate when surrounded by cooperators' allows bacteria to match their investment in cooperation to the composition of their group, therefore allowing the maintenance of cooperation at lower levels of population structuring (i.e. lower relatedness). Similar behavioural responses have been described in

vertebrates under the banner of 'generalised reciprocity'. Our results suggest that mechanisms of reciprocity are not confined to taxa with advanced cognition, and can be implemented at the cellular level via positive feedback circuits.

3.2 Introduction

Traits that increase the fitness of other individuals are a ubiquitous feature of life, from viruses to vertebrates (Bshary and Grutter, 2002; Griffin *et al.*, 2004; Melis *et al.*, 2011; Rand *et al.*, 2009; Turner and Chao, 1999). Across biological scales, cooperative individuals face the challenge of competition with non-cooperative 'cheats' that reap the rewards of cooperation without paying the full costs (Ghoul *et al.*, 2014a; Smith, 1964). If individuals have fixed strategies (constitutively cooperative or non-cooperative), then cooperators can only outcompete cheats if the net costs of cooperation are sufficiently offset by increased rates of interaction with fellow co-operators (positive genetic assortment or relatedness, Hamilton, 1964; Frank, 1998).

While much theory has been built on the assumption of constitutive strategies, behavioural plasticity in social traits is increasingly recognised as the norm, not only in vertebrates (Rutte and Taborsky, 2007), but also in microbes (Kuemmerli and Brown, 2010; Parkinson *et al.*, 2011; Xavier *et al.*, 2011) and even in viruses interacting with conspecifics (Leggett *et al.*, 2013). The importance of behavioural control of cooperative effort has been emphasised since Trivers' (Trivers, 1971) pivotal work on reciprocity highlighted that behavioural feedbacks can allow individuals to match their investment in cooperation to others (i.e. creating phenotypic assortment), and thus protect cooperative strategies from exploitation even in well-mixed groups (Nowak and Sigmund, 1998; Pfeiffer *et al.*, 2005; Trivers, 1971; Fletcher and Zwick, 2006; Clutton-Brock, 2009). Mechanisms of reciprocity

are often suggested to be cognitively complex, because of the need to remember previous interactions with multiple individuals (Milinski and Wedekind, 1998; Stevens and Hauser, 2004). However one form of reciprocity which has been suggested to be simple enough to be achievable by most organisms is 'generalised reciprocity' (Pfeiffer *et al.*, 2005). This form of reciprocity is often stated as an individual cooperating on its next interaction if it was the recipient of a cooperative action on its previous interaction (Pfeiffer *et al.*, 2005). Importantly this behavioural rule removes the requirement for the recognition of individuals or extensive memorisation of past events, as only action of the partner (but not its identity) in the previous interaction is needed (Pfeiffer *et al.*, 2005). Generalised reciprocity has been observed in both humans (Stanca, 2009) and rats (Rutte and Taborsky, 2007).

Generalised reciprocity also referred to as upstream indirect reciprocity (Nowak and Roch, 2007), because it relies on indirect interactions with individuals not previously interacted with (Nowak and Sigmund, 1998), and because cooperative behaviour is influenced by cooperation received from others (upstream), as opposed to depending on cooperation actions observed in others (downstream) (Boyd and Richerson, 1989). For a interacting population (n-person games) generalised reciprocity means that individuals increase the probability of cooperation (Boyd and Richerson, 1988) or cooperative investment (Takezawa and Price, 2010) as the proportion of cooperators in the population increases. Generalised reciprocity has been shown to require small groups in order to invade because of the need to reach a threshold number of cooperators in order for generalised reciprocators to cooperate (Boyd and Richerson, 1988). However this problem is greatly reduced when a continuously variable cooperative

investments are allowed, especially when cooperation is efficient (Takezawa and Price, 2010)

In bacteria, cooperative interactions are commonly mediated by secreted factors that individual cells produce at a cost to provide shared benefits for a local neighbourhood of cells, for example iron-scavenging siderophores (Griffin *et al.*, 2004; Kuemmerli and Brown, 2010) or secreted digestive enzymes (Diggle *et al.*, 2007; Sandoz *et al.*, 2007). The cost of secreting proteins is considerable, and has resulted in selection for cheaper amino acid residues in secreted proteins (Nogueira *et al.*, 2009). It has been suggested that quorum sensing (QS), a form of cell-cell communication observed across diverse bacterial species (Rutherford and Bassler, 2012), has evolved in part to restrict secretion investments when they are inefficient (Cornforth *et al.*, 2014; Darch *et al.*, 2012; Hense *et al.*, 2007; Redfield, 2002). We argue that the benefits of regulating secreted proteins through quorum sensing extend to the context of genetically mixed groups.

In genetically mixed groups there may be mutants that have alternate quorum sensing strategies. To achieve quorum sensing, individuals secrete diffusible signals into the environment and regulate gene expression in response to the concentration of signal (Seed *et al.*, 1995). Secreted factors are over-represented, and predominantly upregulated, in the quorum sensing regulon both in *Pseudomonas aeruginosa* (Schuster and Peter Greenberg, 2006; Gilbert *et al.*, 2009; Popat *et al.*, 2015a) and other bacteria (Antunes *et al.*, 2007; Barnard *et al.*, 2007). This means that in genetically mixed groups mutants that do not respond to QS signals function as social cheats by not producing shared secreted proteins (Diggle *et al.*, 2007; Sandoz *et al.*, 2007).

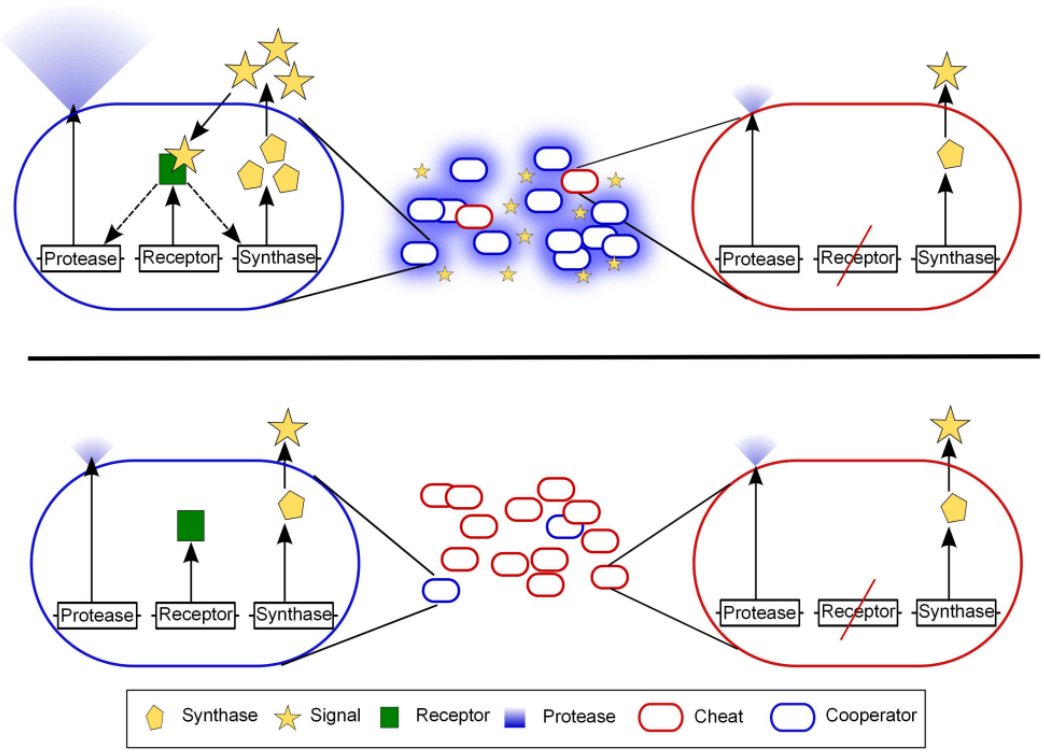

Figure 3.1: QS network architecture produces the generalised reciprocity rule of 'cooperate when surrounded by cooperators'. Top) When cooperators (blue) are common, positive signal autoregulation in cooperators leads to high environmental signal concentrations. In response to high signal concentration cooperators express more secreted enzyme, increasing population yield. Cheats (red) lack a signal receptor so they do not respond to signal, and so do not express signal or secreted enzyme above basal levels. They are able to exploit cooperators, spreading in the population. Bottom) When cooperators are rare there are few cells undergoing positive feedback in signal production (autoregulation) so signal levels remain low. Cooperators invest little in protease production (acting as phenotypic cheats), keeping costs of cooperation low and reducing exploitation by cheats.

A critical design feature in most QS regulatory networks is positive autoregulation of signal production (figure 3.1), a coupling between signal production and signal detection (Goryachev, 2009). Signal responsive wildtype (WT) individuals increase both their rate of signal (Seed *et al.*, 1995) and secreted enzyme (Pearson *et al.*, 1994) production in response to higher

environmental signal concentrations, whereas non-responsive mutants (cheats) produce minimal and invariant quantities of signal and enzyme secretions (figure 3.1). We suggest that the positive feedback loop governing signal production in the wildtype will cause correlations between signal concentrations and the proportion of WT cooperators (genetic composition) in mixed populations. This will provide a cue to the genotypic composition of the population (figure 3.1). The wildtype QS response of increasing enzyme secretions with signal concentration (Pearson *et al.*, 1994) will then lead to cooperative investment increasing with the proportion of WT cooperators in the population, the outcome of generalised reciprocity. We therefore predict that the native QS architecture will limit the ability of non-responder cheats to socially exploit the wildtype in a high density environment.

A literature review shows that non-responsive cheater mutant (*ΔLasR* mutants) proportion shows huge variation in *P. aeruginosa*, both in samples from infections and experimental evolution studies, varying from 0-100% per population (Table 3.1). As non-responsive mutants produce little to no signal high density populations composed solely of non-responsive mutants will have signal concentrations equal to that of a low density population composed entirely of responsive individuals. Therefore variation in the proportion of non-responsive individuals is common and exposes populations to the full range of signal concentrations that would be seen with density variation in a clonal population of individuals. The resulting correlation between signal concentration and cheater proportion will allow signal concentration to act as a reliable cue of cheater proportion, thus creating the potential for generalised reciprocity mediated by QS to protect cooperators from exploitation by cheaters.

ΔLasR proportion	Environment	Reference
40-95%	Cystic fibrosis patients	(Köhler *et al.*, 2010)
0-100%	Cystic fibrosis Patients	(Hoffman *et al.*, 2009)
2-35%	Mouse models of infection	(Rumbaugh *et al.*, 2009)
0-40%	Experimental evolution *in vitro*	(Sandoz *et al.*, 2007)
0-80%	Experimental evolution *in vitro*	(Dandekar *et al.*, 2012)

Table 3.1: LasR defective mutants show large variation in local proportion.

Building on existing theory (Cavaliere and Poyatos, 2013; Takezawa and Price, 2010; Taylor and Day, 2004) we present mathematical models predicting that generalised reciprocity mediated by QS signals protects public goods investments from exploitation by cheats that are un-responsive to signal. We test this hypothesis by manipulating strain mixing in the opportunistic pathogen and model QS bacterium *P. aeruginosa*. We demonstrate that the wildtype strategy of cooperative investment conditional on autoregulated signal concentration increases the range of conditions (degree of population structure and cooperator proportion) where the regulated cooperative trait can be maintained in the face of non-responsive genetic cheats.

3.3 Methods

3.3.1 Assessing the co-operative phenotype of populations

Wild-type (WT) *Pseudomonas aeruginosa* (PAO1) and an isogenic *lasR* knockout (*ΔlasR*) were used as genetic cooperators and cheats respectively (Diggle *et al.*, 2007). The WT was marked (to distinguish it from the ΔlasR mutant) with a *luxCDABE* cassette under the control of the *lasB* promoter (made using the mini CTX*lux* plasmid, Becher and Schweizer, 2000) so that luminescence could have been used to assess elastase production (although

this was not used as the elastin congo red assay for elastase was more reliable and detects elastase directly). The WT and *ΔlasR* strains were mixed at defined proportions ranging from 0 to 1 and used to seed cultures at an optical density (600nm) of 0.01. This treatment was used to manipulate initial cheat proportion to assess its effects on signal concentration and protease production in the population. Planktonic cultures were grown in 96 well plates (200ul volumes) for 6 hours in a defined quorum sensing medium (QSM, Popat *et al.*, 2012) which has protein (bovine serum albumin) as the main carbon source. As proteins are metabolised by elastase, a secreted protease upregulated by quorum sensing, QS is required for growth to high density. All *P. aeruginosa* cultures were mixed, by shaking at 250rpm with an orbit of 37mm allowing mixing of small volumes (Duetz, 2007), in order to reduce population structure. After 6 hours incubation at 37^{o}C (cultures produce most signal and response at this time), cultures were centrifuged to separate supernatant and cells. The remaining cell suspension was mixed with equal parts 1:1 LB broth:glycerol and frozen at -80^{o}C while supernatant was filter sterilised (0.22μm pore). Frozen populations were later defrosted on ice and proportions were assayed by vortexing, diluting and plating cultures on LB agar plates, and then manually counting colonies using luminescence to distinguish WT and *ΔlasR* colonies. Unfortunately the size of the experiment prevented plating of cultures once competition was complete. However any freezer effects should have affected start and end samples equally and we have no reason to believe any effects would change with WT proportion During the counting process the experimenter was blind to treatment.

For measurements of protease activity 50μl of sterile supernatant was added to 450μl of elastin congo red buffer (100mM Tris, 1mM $CaCl_2$, pH=7)

containing 20mg/ml elastin congo red (Ohman *et al.*, 1980). Tubes were incubated horizontally with shaking at 150rpm and 37°C for 18 hours. After incubation supernatant was removed and the absorbance at 495nm was read. To determine signal concentrations filtered culture supernatent was diluted 1/100 in LB broth and mixed at equal concentrations with exponentially growing bioreporter strains at OD 0.1 (600nm) in LB broth. Synthetic signal at various concentrations was treated similarly to create a calibration curve. Signal bioreporters were grown for 3 hours at 37°C taking reads of optical density and luminescence every 15 minutes. When luminescence was at a high level (1.75 and 3 hours growth for C12 and C4 respectively) a calibration curve was fitted and used to calculate signal concentrations in experimental samples. Bio-reporters were S17-1 *Escherichia. coli* containing either the p56536 or pSB1142 plasmids (Winson *et al.*, 1998), which luminesce in response to short and long chain AHLs respectively. All populations of *E. coli* showed the same growth dynamics regardless of whether supernatent, signal or control were added suggesting there was no effect of P. aeruginosa supernatent on *E. coli* growth.

3.3.2 Competition experiments

Mixed populations of WT and *ΔlasR* were set up as before, with the following modifications. The WT was marked (distinguished from the ΔLasR mutant) with the *luxCDABE* cassette in a constitutively active (promoterless) position (Popat *et al.*, 2012) and mixed cultures were grown for 40 hours in two 96 well plates (plate did not affect competition). To exclude the effect of drying during extended incubation treatments samples were randomly allocated into the central 60 wells of the 96 well plates. The outer 36 wells of the plate contained growing cultures to minimise edge effects of oxygen gradients. These outer wells were not used for analysis. Before and after

growth, cells were frozen and later defrosted to ascertain proportions and CFU (colony forming units) as before. Relative fitness of the WT (v) was calculated as: $X_1(1-X_0)/(X_0\ (1-\ X_1))$ where X_0 and X_1 are the proportions of WT cells at the start and end of competition respectively (Ross-Gillespie *et al.*, 2007). Fitness calculated with an alternate fitness measure using the ratio of number of doublings (Lenski *et al.*, 1991) is qualitatively similar as can be seen in S3.5. The change in proportion of WT over competition can be seen in figure S3.3.

3.3.3 Statistics

All statistical analyses were performed in R (R Core Team, 2013). Signal and protease phenotype data were modelled as a function of WT proportion at 6 hours (X_1) with a separate model for each of the 3 phenotypes measured (3-oxo-C12 signal, C4 signal and Elastase):

$$Phenotype(X_1) \sim c + mX_1^{(1+q)} + k.\,\text{final\_optical\_density} \qquad \text{(Eqn. 3.1)}$$

This full model was fitted using the non linear least squares (nls) function in R (Bates and Watts, 1988) which uses a least squares method of fitting non-linear curves assuming a Gaussian error structure. Where terms could be dropped F-tests were used to compare models with and without (parameter set to zero) the term to confirm that removal resulted in better predictive power. Where the exponent was significantly different from 1 ($q \neq 0$, which was all 3 phenotypes) m could not be dropped from the model because q becomes undefined. Therefore F tests could not be performed and the significance of m is reported using t tests to assess whether it is significantly different from zero. See tables 2-4 for summaries of this process. There was slight variation in the optical density of the samples used to measure phenotypes, but this was removed from all three models (k=0) due to lack of

explanatory power based on F-tests, all other terms were always significant. *p*-values reported in the results section are taken from the minimal model. The statistical model for protease production was used for the function of cooperative effort with WT proportion to convert between WT proportion and protease production for the analysis in figure 3.3.

For competition data, growth rate was calculated based on the CFU of each strain before and after competition as (Final CFU-Initial CFU)/Initial CFU. Linear mixed-effects models were used to model the natural logarithm of strain growth rates as a function of individual cooperation (protease production per WT cell) and average group cooperation (protease production in a whole population) with competition well included as a random effect. To do this, the model (shown in figure 3.2c) of the relationship between WT proportion and protease production in the population (with an exponent of 2.49) was used. Group cooperation was calculated as (WT proportion)$^{2.49}$ and individual cooperation in the WT as (WT proportion)$^{2.49}$/(WT proportion) or (WT Proportion)$^{1.49}$. Although there is a small amount of error on the exponent value of 2.49 we were unable to take this error into account in the later analyses. A model with effects of individual cooperation, individual cooperation squared, average group cooperation, and average group cooperation squared was fitted and simplified in a stepwise manner using F tests and likelihood ratio tests (with reporting of the χ^2 statistic). F tests taken from this full model, showed that while the quadratic term for average group cooperation did not significantly improve model fit (effect of group cooperation squared: $F_{1,52} = 2.52$, $p > 0.05$), all other terms were significant in the model (effect of group cooperation: $F_{1,52} = 134.33$, $p < 0.0001$ $\beta = 44.3$, s.e = 0.26; effect of individual cooperation: $F_{1,49} = 210.79$, $p < 0.0001$ $\beta = 1.81$, s.e = 0.54; effect of individual cooperation squared:

$F_{1,49}$ = 50.16, p < 0.0001, β = -4.91, s.e = 0.69), for more information see table 3.5. The final fitted form of the statistical model (using estimated coefficients) was

$$\ln(growth\ rate) = 3.57 + 4.43 * y + 1.81 * x - 4.91 * x^2 \qquad \text{(Eqn. 3.2)}$$

where x is individual cooperation (equal to (WT proportion)$^{1.49}$ for the WT and zero for the *ΔlasR* mutant), and y is group cooperation (equal to (WT proportion)$^{2.49}$). A limitation of this analysis is the inability to take into account the error associated with the parameter estimates for these models in later analysis, therefore errors are not propagated.

The solid curve to predict final CFU (colony forming units) in figure 3.3d is produced from estimates for WT and ΔlasR predicted by equation 3.2 using the equation:

$$final\ cfu = initial\ WT\ CFU.(1 + WT\ growth\ rate) + Initial\ \Delta lasR\ CFU.(1 + \Delta lasR\ growth\ rate \qquad \text{(Eqn. 3.3)}$$

Similarly the solid curve to predict WT fitness in 3.3e uses the growth rate estimates for WT and ΔlasR predicted by equation 3.2 using the equation:

$$WT\ relative\ fitness = \frac{(1+WT\ growth\ rate)}{(1+\Delta lasR\ growth\ rate)} \qquad \text{(Eqn. 3.4)}$$

To produce the dashed curves in figure growth rate was calculated according to constitutive cooperation, that is constant individual cooperation and group cooperation proportional to WT proportion (dashes lines in figure 3.2c).

C4 Full model and simplification				
Parameter	F stat (DF)	p-value	$t_{1,20}$	p-value
c	6.41 (1,21)	0.019	2.76	0.012
m	NA	NA	8.54	<0.001
q	10.43 (1,21)	0.004	2.31	0.031

k	3.55 (1,20)	0.074	-1.88	0.075
C4 Final Model				
Parameter	Estimate	s.e	$t_{1,21}$	p-value
c	0.265	0.098	2.70	0.013
m	1.636	0.208	7.87	<0.001
q	1.628	0.758	2.15	0.044
n=24, residual s.e=0.311 on 21 DF				

Table 3.2: Statistical table showing model simplification and final parameter estimates for the fixed effects model of C4 production. Response variable is C4 signal production measured after 6 hours of P. aeruginosa populations with WT proportion shown in figure 3.2a. This model is fitted using least squares with a Gaussian error structure.

C12 Full model and model simplification				
Parameter	F stat (DF)	p-value	$t_{1,20}$	p-value
c	5.46 (1,21)	0.029	2.54	0.019
m	NA	NA	9.62	<0.001
q	23.28 (1,21)	<0.001	3.47	0.002
k	0.035 (1,20)	0.853	-0.19	0.852
C12 Final model				
Parameter	Estimate	s.e	$t_{1,21}$	p-value
a	0.025	0.010	2.61	0.016
m	0.251	0.024	10.38	<0.001
q	2.836	0.793	3.58	0.002
n=24, Residual s.e 0.034 on 21 DF				

Table 3.3: Statistical table showing model simplification and final parameter estimates for the fixed effects model of C12 signal production. Response variable is 3-oxo-C12 signal production measured after 6 hours growth of *P. aeruginosa* populations shown in figure 3.2a. This model is fitted using least squares with a Gaussian error structure.

Protease Full model and model simplification				
Parameter	F-stat (DF)	p-value	$t_{1,20}$	p-value
c	97.92 (1,21)	<0.001	22.55	<0.001
m	NA	NA	12.00	<0.001
q	14.39 (1,21)	0.001	3.13	0.005
k	3.024 (1,20)	0.097	-1.75	0.096
Protease Final model				
Parameter	Estimate	s.e	$t_{1,21}$	p-value

c	0.091	0.004	22.24	<0.001
m	0.097	0.008	11.58	<0.001
q	1.489	0.492	3.02	0.006
n=24, Residual s.e on 21 DF = 0.013				

Table 3.4: Statistical table showing model simplification and final parameter estimates for the fixed effects model of elastase production. Response variable is elastase production measured after 6 hours growth of *P. aeruginosa* populations, measured by an elastin congo red assay, data is shown in figure 3.2c. This model is fitted using least squares with a Gaussian error structure.

Growth Rate Model simplification					
Parameter	F stat DF	F-value	p-value	χ^2_1	p value
Group_cooperation	1,53	134.33	<0.001	12.99	<0.001
Group_cooperation^2	1,52	2.52	0.140	3.27	0.071
Individual_cooperation	1,49	210.79	<0.001	13.36	<0.001
Individual_cooperation^2	1,49	50.16	<0.001	38.72	<0.001
Growth Rate minimal model					
Parameter	Estimate	s.e	t stat DF	t-statistic	p value
Intercept	3.57	0.12	1,53	29.89	<0.001
Group_cooperation	4.43	0.26	1,53	17.12	<0.001
Individual_cooperation	1.81	0.54	1,49	3.36	0.002
Individual_cooperation^2	-4.91	0.69	1,49	-7.08	<0.001
number of observations =106, number of groups =55					
Variance of random term = 0.207, residual variance = 0.138					

Table 3.5: Statistics for model simplification and final parameter estimates for the mixed effects model of growth rate for the WT and ΔlasR strain with WT proportion shown in figure 3.3a. The response variable is the natural logarithm of growth rate calculated as detailed in the methods. This model is fitted using a Gaussian error structure. The model includes a random effect of well on the model intercept to account for the fact that each well gives a data point for both the WT and the ΔlasR growth rate. χ^2_1 statistics report likelihood ratio tests.

3.3.4 Theoretical framework

We first consider a scenario where cooperators invest a constant amount in cooperation regardless of the composition of their group. For this scenario of constitutive cooperative effort the payoff is

$$w(g, G) = a + bG - cg \qquad \text{(Eqn. 3.5)}$$

where g is an individual's breeding value (1 = cooperator, 0 = cheat), G is the mean breeding value of the population, a is the intrinsic payoff, b is the benefit of cooperation and c is the cost of cooperation. From this we obtain the covariance between fitness and breeding value (i.e. the expected change in genotype frequency owing to selection) as

$$cov(w, g) = b\ cov(G, g) - c\ var(g) \qquad \text{(Eqn. 3.6)}$$

Dividing through by $var(g)$, the variance in breeding value, we get the regression of fitness on breeding value ($\beta_{w,g}$) which must be positive for the selection of cooperation

$$\beta_{w,g} = b\ \beta_{G,g} - c \qquad \text{(Eqn. 3.7)}$$

where $\beta_{G,g}$ is the regression of average group genotype on individual genotype (i.e. relatedness). We will assume that founders are binomially distributed into sub-populations with n founders from a total population which has proportion p cooperators. The regression of group genotype on individual genotype can then be simplified as follows:

$$\beta_{G,g} = E(G|g = 1) - E(G|g = 0) \qquad \text{(Eqn. 3.8a)}$$

$$\beta_{G,g} = \frac{\sum_{k=0}^{n}\left[\left(\frac{k}{n}\right)^2\binom{n}{k}p^k\,(1-p)^{n-k}\right]}{p} - \frac{\sum_{k=0}^{n}\left[\left(\frac{k}{n}\right)\left(1-\frac{k}{n}\right)\binom{n}{k}p^k\,(1-p)^{n-k}\right]}{1-p} \qquad \text{(Eqn. 3.8b)}$$

$$\beta_{G,g} = \frac{1}{n} \qquad \text{(Eqn. 3.8c)}$$

Substituting equation 3.8c into equation 3.7 and rearranging gives us equation 3.21 from the results.

We next consider a scenario where cooperators increase their relative investment linearly from 0 when no cooperators are present to 1 when their

group is entirely cooperators. For these assumptions the payoff for a plastic cooperator is

$$w(g,G) = a + bGG - cGg \qquad \text{(Eqn. 3.9)}$$

giving the following covariance between fitness and breeding value

$$cov(w,g) = b\ cov(GG,g) - c\ cov(Gg,g) \qquad \text{(Eqn. 3.10)}$$

Dividing by *var*(*g*) and rearranging.

$$\beta_{w,g} = b\ \beta_{GG,g} - c\ \beta_{Gg,g} \qquad \text{(Eqn. 3.11a)}$$

$$\beta_{w,g} = b\ \beta_{GG,g} - c[E(Gg|g=1) - E(Gg|g=0)] \qquad \text{(Eqn. 3.11b)}$$

$$\beta_{w,g} = b\ \beta_{GG,g} - c\ E(G|g=1) \qquad \text{(Eqn. 3.11c)}$$

Again assuming a binomial distribution of founders into subpopulations the regression coefficient and expectation can be simplified as

$$\beta_{GG,g} = \frac{\sum_{k=0}^{n}\left[\left(\frac{k}{n}\right)^3\binom{n}{k}p^k\,(1-p)^{n-k}\right]}{p} - \frac{\sum_{k=0}^{n}\left[\left(\frac{k}{n}\right)^2\left(1-\frac{k}{n}\right)\binom{n}{k}p^k\,(1-p)^{n-k}\right]}{1-p} \qquad \text{(Eqn. 3.12a)}$$

$$\beta_{GG,g} = \frac{1+2p(n-1)}{n^2} \qquad \text{(Eqn. 3.12b)}$$

and

$$E(G|g=1) = \frac{1+p(n-1)}{n} \qquad \text{(Eqn. 3.13)}$$

Substituting equations 3.12b and 3.13 into equation 3.11c gives equation 3.23 of the results.

3.3.5 Metapopulation framework

Metapopulations are commonly used to manipulate structure in microbiology to study social behaviours (Griffin *et al.,* 2004). We used a metapopulation framework to model the effect of signal-meditated

generalised reciprocity on the evolution of cooperation. We assume that the metapopulation is structured into an infinite number of groups, each of which is founded by n individuals. Given these assumptions, we can calculate the proportion of cooperative individuals that find themselves resident in a group with k cooperative founders as

$$Q_{coop}(k) = \frac{\frac{k}{n}\binom{n}{k}p^k(1-p)^{n-k}}{p} \qquad \text{(Eqn. 3.14)}$$

and the proportion of cheats that find themselves resident in a group with k cooperative founders as

$$Q_{cheat}(k) = \frac{\left(1-\frac{k}{n}\right)\binom{n}{k}p^k(1-p)^{n-k}}{1-p} \qquad \text{(Eqn. 3.15)}$$

We can then write the difference in fitness between cooperators and cheats as

$$w_{coop} - w_{cheat} = \sum_{k=0}^{n} Q_{coop}(k) r_{coop}\left(\frac{k}{n}\right) - Q_{cheat}(k) r_{cheat}\left(\frac{k}{n}\right) \quad \text{(Eqn. 3.16)}$$

where $r_{coop}(k/n)$ and $r_{cheat}(k/n)$ give the growth rates of cooperators and cheats in groups with proportion k/n cooperators. We compare the behaviour of this fitness difference when $r_{coop}(k/n)$ and $r_{cheat}(k/n)$ are calculated first under the natural scenario of QS control of cooperation, and secondly under the assumption of constitutive cooperation. In the scenario of QS controlled cooperation, the growth rate of cooperators is given as

$$r_{coop}\left(\frac{k}{n}\right) = e^{3.57+1.81*(k/n)^{1.49}-4.91*\left((k/n)^{1.49}\right)^2+4.43*(k/n)^{2.49}} \qquad \text{(Eqn. 3.17)}$$

and for cheaters as

$$r_{cheat}\left(\frac{k}{n}\right) = e^{3.57+4.43*(k/n)^{2.49}} \qquad \text{(Eqn. 3.18)}$$

In the scenario of constitutive cooperation, we simply replace all instances of k/n with 1, giving

$$r_{coop}\left(\frac{k}{n}\right) = e^{4.91} \qquad \text{(Eqn. 3.19)}$$

and

$$r_{cheat}\left(\frac{k}{n}\right) = e^{8.01} \qquad \text{(Eqn. 3.20)}$$

We evaluated the fitness differential for these two scenarios across varying cooperator frequency (p) and founder number (n) to assess the consequences of signal-mediated generalised reciprocity for the evolution of cooperation.

3.4 Results

3.4.1 What are the behavioural rules governing signal production and signal response?

The regulatory architecture (how the components of the system interact to regulate expression of the regulon) of QS controlled secreted enzyme production suggests that increasing the local proportion of wildtype (versus signal non-responsive cheats at a fixed population size) will increase the per capita production rate of both signal (Seed *et al.*, 1995) and secreted protease (Pearson *et al.*, 1994) by WT individuals. To test this prediction and to map the functional forms of the behavioural response, we manipulated the initial proportion of WT cooperators and measured the resulting concentration of the two primary *P. aeruginosa* signal molecules 3-oxo-C12-HSL (N-(3-oxododecanoyl)-L-homoserine lactone) and C4-HSL (N-(butanoyl)-L-homoserine lactone), plus secreted protease (elastase) enzyme (a critical *P. aeruginosa* virulence factor and bacterial public good, expressed under positive QS control). Our results are consistent with increases in per-capita C4, C12 and secreted enzyme production by WT individuals with increased WT proportion. Specifically, in figure 3.2 we see that phenotypes at the population level increase as an accelerating function of WT proportion at 6 hours, allowing us to reject the null hypothesis of constant per capita

production (exponent of 1). For C4, fitted exponent = 2.63, s.e = 0.758, t_{21} = 2.148, $p < 0.05$. For C12, fitted exponent = 3.84, s.e = 0.793, t_{21} = 3.576, $p < 0.001$. For secreted enzyme, fitted exponent = 2.49, s.e = 0.492, t_{21} = 3.024, $p < 0.01$. Inset plots show the predicted per capita WT cell phenotype (signal production or cooperative investment). Although QS is often considered to regulate genes in a binary fashion (as a threshold behaviour) our results (figure 3.2c and S3.4c) support a view that QS responses are continuous rather than binary, as has also been suggested by a previous study (Darch *et al.*, 2012).

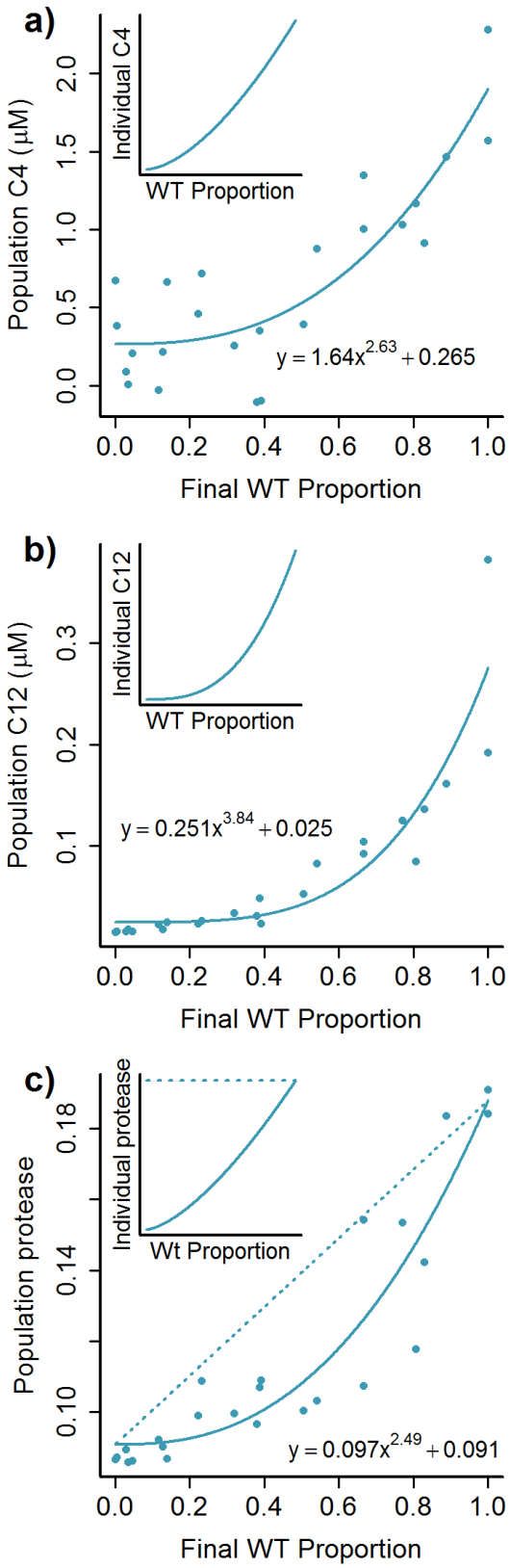

Figure 3.2: Per-capita signal and secreted enzyme investment increases with wildtype cooperator proportion. Mixed populations of cheats and cooperators were grown for 6 hours in quorum sensing media. After growth, AHL signal and secreted protease concentrations were measured for the population. Plate counts were used to determine cooperator proportion at 6 hours. Equations for fitted models are presented on the graphs. Insets show a transformation (with exponent reduced by 1) of the fitted models to show the change in per capita WT phenotype. a) Total 3-oxo-C12 N- acyl HSL concentration (inset, per WT individual). b) Total C4 N-acyl HSL concentration (inset, per WT individual). c) Total protease (elastase) concentration (inset, per WT individual), the equivalent constitutive response (constant maximal WT investment) is shown as dashed lines in c).

3.4.2 How does QS control of cooperation shape competition between cooperator and cheat genotypes?

To ask whether the native functional response "cooperate when surrounded by cooperators" (figure 3.2c) protects protease enzyme secretion from exploitation, we performed 40 hour competition experiments between WT cooperators and non-responder cheats at varying initial proportions. The growth rate of both strains increased with the initial WT proportion (figure 3.3a). In order to relate growth rate to the underlying costs and benefits of cooperative enzyme secretion, we use our estimates of individual and collective cooperative effort as a function of WT proportion at 6 hours (figure 3.2c). We fit a linear mixed-effect model of the log of growth rate (figure 3.3a) as a function of individual (figure 3.2c, inset) and collective (figure 3.2c, main) enzyme secretion phenotypes. The analysis partitions out the effects of mean group phenotype (figure 3.3b) and individual phenotype (figure 3.3c). While the growth rate of both strains increases with initial WT proportion (figure 3.3a) due to the benefits of group cooperation (figure 3.3b), the cheat strain benefits more at high initial WT proportion because secreted enzyme production is individually costly, (figure 3a,c). Fixing cooperative effort as constant (using the dashed line in figure 3.2c instead of the solid line to map WT proportion to cooperative effort) allows us to predict the growth rate of the 2 strains given constitutive WT cooperation (dashed lines in figure 3.3a).

We can use our fitted model of growth rate to summarise the yield data (measured as CFU) and relative fitness data for the competition experiments in figures 3.3d and 3.3e (data points and solid lines). These show good agreement with the data capturing the superlinear increase in yield) and increasing exploitation of the WT when common. A reduction in yield at very high initial WT frequencies is also seen which results from the way population growth is modelled; because at very high WT Proportion there

are very few fast growing cheats (MacLean *et al.*, 2010). When cooperative effort is constitutive (dashed lines) our predictions indicate that yield increases more linearly and the WT is always exploited. Experimentally fixing per-capita WT cooperative effort at a constant (but increased) level through the addition of excess signals gives qualitatively similar results (figure S3.1).

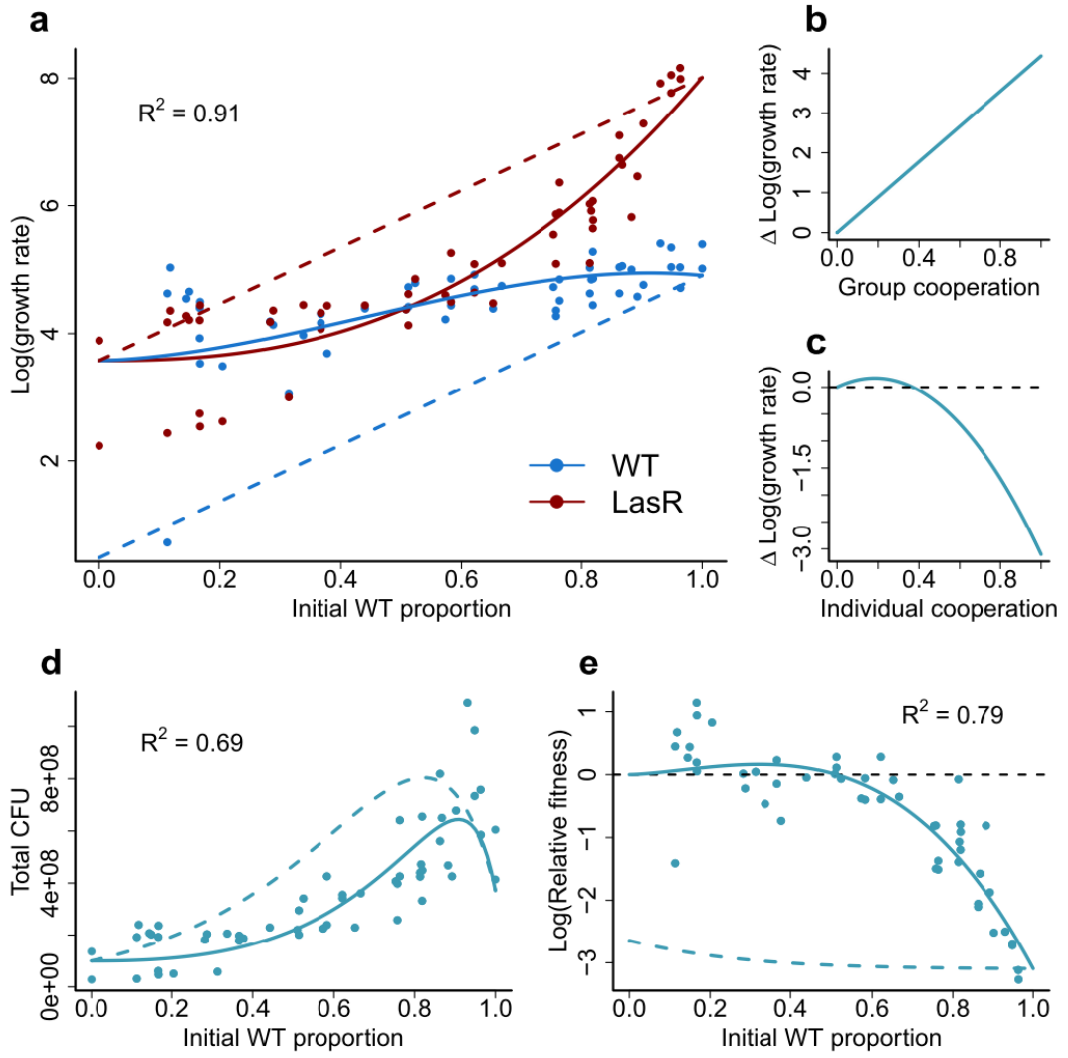

Figure 3.3: Generalised reciprocity limits social exploitation when WT cooperators are rare. Competition between WT cooperators and cheats (*ΔlasR*) over 40 hours in media requiring secreted protease enzyme for growth. a) Log growth rate (natural logarithm) of the 2 strains averaged over the length of competition. b)

The predicted linear effect of group cooperative effort shown as change in the natural logarithm of growth rate (effect of group cooperation: β = 4.43, s.e. = 0.26, $F_{1,53}$ = 134.33, p < 0.0001). c) The predicted quadratic effect of individual cooperative effort on log shown as change in the natural logarithm of growth rate (effect of individual cooperation: β = 1.81, s.e. = 0.54, $F_{1,49}$ = 210.79, p < 0.0001; effect of individual cooperation squared: β = –4.91, s.e. = 0.69, $F_{1,49}$ = 50.16, p < 0.0001). d) How population yield (CFU / 100μl) varies with WT proportion. e) How WT relative fitness varies with WT proportion. Solid fitted lines are generated using the mixed linear model for log growth rate data shown in 3.3a which assume that cooperative investment varies with WT Proportion according to the solid fits in figure 2c. Dashed lines are predictions for constitutive cooperative effort using the same model but assuming cooperative effort varies with WT Proportion according to dashed lines in 2c. Individual points are direct measures of growth rate, yield and relative fitness measured from competition experiments.

3.4.3 What are the implications of signal autoregulation for selection across a meta-population?

To explore the consequences of QS regulatory control of cooperation, we next build and analyse a simple model of bacterial fitness as a function of their social neighbourhood (Chuang *et al.*, 2009; de Vargas Roditi *et al.*, 2013), with and without quorum sensing regulatory control of cooperation. For simplicity we assume linear costs and benefits of cooperation, as has been done previously (Brown and Johnstone, 2001). Under the assumption of constitutive cooperation, we find that a cooperative strain is under positive selection whenever $b\beta_{G,g} > c$, where b and c are the benefits of cooperation and $\beta_{G,g}$ is the coefficient of relatedness (in regression coefficient form), a measure of genetic assortment between co-operators (with breeding value g) and their social group (with breeding value G). When we assume that WT and cheat strains are allocated at random (binomially) to sub-populations,

the relatedness term reduces to the inverse of the number of patch founders, n (methods), meaning cooperation will be favoured whenever

$$\frac{b}{n} > c \quad \text{(Eqn. 3.21)}$$

To introduce plastic cooperative effort, we make the conservative assumption that individual cooperative investment increases linearly with the proportion of cooperators (in practice, investment increases even faster with increasing Proportion of cooperators, figure 3.2c). This assumption implies that the costs of cooperation for an individual increase linearly with the local proportion of co-operators, whereas the per capita benefits of cooperation increase quadratically (more co-operators and more effort). As a result, the condition for cooperation to be favoured is now

$$b\beta_{GG,g} > c\beta_{Gg,g} \quad \text{(Eqn. 3.22)}$$

Using the binomial distribution to derive the regression coefficients (methods), and substituting the values for $\beta_{Gg,g}$ and $\beta_{GG,g}$ into equation 3.22 we see that the condition for quorum sensing controlled cooperation to be favoured is:

$$\frac{b}{n} > c\frac{1+p(n-1)}{1+2p(n-1)} \quad \text{(Eqn.3. 23)}$$

where p is the proportion of individuals in the metapopulation that are cooperators (g=1). Comparing the right hand sides of inequalities 3.21 and 3.23, we can see that cooperation is more easily favoured by selection under signal mediated generalised reciprocity whenever $p > 0$ and $n > 1$; that is so long as some cooperators are present and groups are not clonal. Where $p = 0$ and/or $n = 1$ the inequalities are identical. Thus signal mediated generalised

reciprocity is expected to protect cooperative individuals from exploitation by cheaters.

Our theoretical model suggests that signal mediated generalised reciprocity helps protect cooperators from exploitation by cheaters in a metapopulation. However it makes the simplifying assumptions that there is no structure in the population and the related assumption that cooperation can never have any private benefit. To assess our theoretical predictions we used our fitted fitness functions (figure 3.3) to determine the consequences of signal-mediated generalised reciprocity for the evolution of cooperation in a metapopulation. Our results confirm that generalised reciprocity reduces the level of relatedness necessary for cooperation to be favoured, allowing cooperation to be more readily maintained in non-clonal groups (figure 3.4). In figure 3.4 the final column shows a metapopulation where subpopulations have an infinite number of founders, this is equivalent to a well mixed population and thus is identical to the data in figure 3.3.

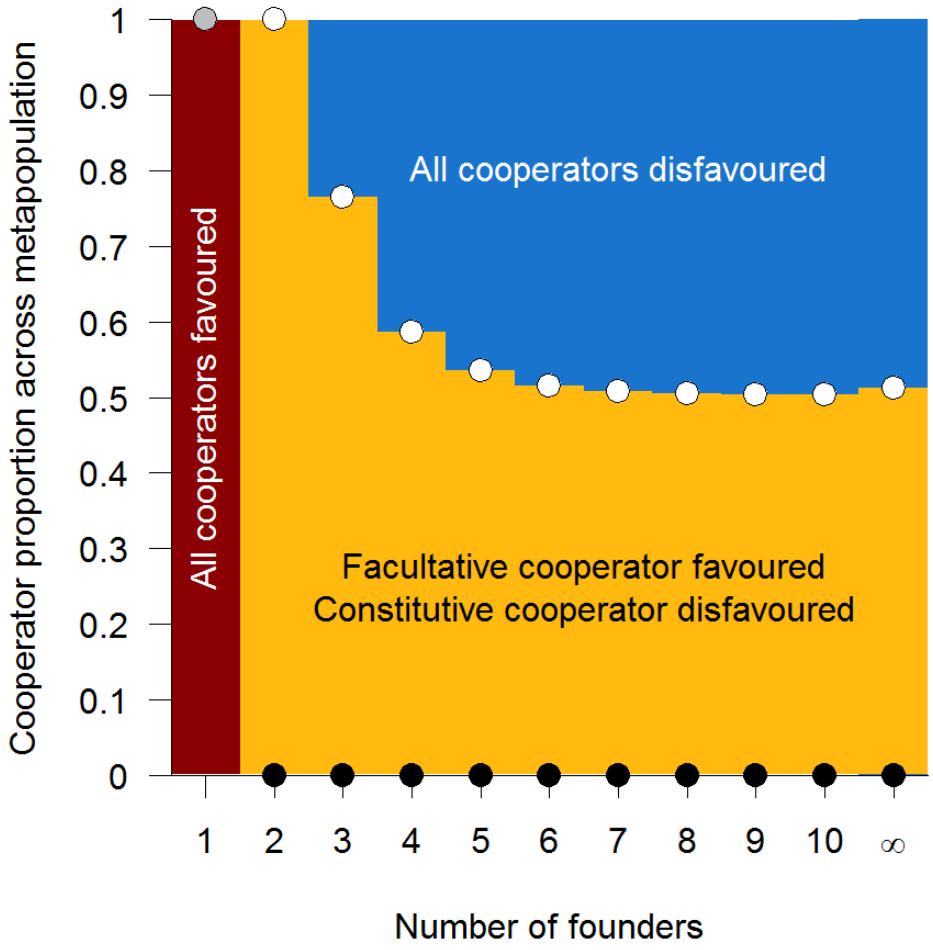

Figure 3.4: Generalised reciprocity stabilises cooperation over a larger range of conditions in a metapopulation. Selection determined using statistical model fits shown in figure 3.3 and the metapopulation framework described in the methods. Dots represent equilibria for constitutive (black), QS-regulated (white), and either form of cooperation (grey). Constitutive cooperation is only favoured when the population has a clonal structure (1 founder per subpopulation). QS regulated (facultative) cooperation is favoured for lower levels of relatedness, including a well-mixed population (infinite number of founders).

3.5 Discussion

Quorum sensing bacteria commonly place the control of both secreted signals and proteins under the positive influence of extracellular signal molecule concentration (Antunes *et al.*, 2007; Gilbert *et al.*, 2009; Rutherford

and Bassler, 2012). We illustrate that this well-studied mechanism allows bacteria to assess the extent of cooperation in the surrounding population (figure 3.2), and respond with a rule similar to generalised reciprocity that may limit exploitation by signal non-responsive cheats (figures 3.3 and 3.4). In comparison to constitutive cooperation, the rule of 'cooperate when surrounded by cooperators' moderates individual cooperative effort to be closer to the surrounding population phenotype (i.e. increasing phenotypic assortment Fletcher and Zwick, 2006) and reduces exploitation by cheats. Our results in figure 3.4 predict that facultative cooperation may lead to co-existence of cooperators and cheats in natural settings.

As can be seen in figure 3.2c and S3.4c, when the WT is rare cooperative investment is low. Our analysis in figure 3.3c indicates that although cooperation at high levels is costly to the individual, a low level of cooperation significantly benefits the individual. This is due to the significant positive effect of individual cooperation on growth. Even though there is a significant negative effect of individual cooperation squared, the net effect will always be positive in our model (as long as the effect of individual cooperation is positive) at low values of individual cooperation regardless of the size and sign of other coefficients. We experimentally measure the costs of expression in figures S3.4b and S3.4d showing that the low level of QS induction when the WT is rare results in low or negligible costs. This experiment removes the benefits of protease production (there is no protein in the environment), but given the low cost of QS induction at a low level we would see a WT relative fitness greater than 1 if there is any individual benefit of low level QS induction. Individual benefits may be due to preferential access to the low level of protease in the environment, for example due to association of protease with the membrane of the producing

cell or residual spatial structure as *P. aeruginosa* can form planktonic aggregates (Schleheck *et al.*, 2009). Quorum sensing controls many genes so other traits such as stress responses may be responsible for individual benefits (García-Contreras *et al.*, 2014; Davenport *et al.*, 2015). Private benefits are not required for our metapopulation results, if we alter our metapopulation framework so that cheats produce the level of protease that is most individually beneficial (corresponding to the maxima of the curve in figure 3.3c), we still see a benefit of QS control of public goods (figure S3.2). In addition we see an area of positive frequency dependence, producing bistability (figure S3.2).

The key to generalised reciprocity in the *P aeruginosa* system (and others sharing the key signal autoregulation design) is the pleiotropic action of the LasR signal receptor protein (Foster *et al.*, 2004; Dandekar *et al.*, 2012). As a transcription factor it determines both the level of signal production and the level of QS regulated secreted factors. The *lasR* gene (and thus signal production) resembles a greenbeard mechanism, generating a detectable signal that is mechanistically linked to investment in cooperation (Biernaskie *et al.*, 2013; Dawkins, 1989; Eldar, 2011). We do not demonstrate that the observed response rules are evolutionary stable strategies (Cavaliere and Poyatos, 2013; Takezawa and Price, 2010) and it is possible that signal production could become unlinked from signal response thus breaking the pleiotropy and the ability for generalised reciprocity to protect the WT from exploitation. In particular, the WT regulatory response appears vulnerable to a coercive cheating strategy of high signal, low response (Brown and Johnstone, 2001; Eldar, 2011). However clinical studies commonly isolate low signal, low response *LasR* mutants (table 3.1) and recent experimental evolution suggests that a coercive signalling phenotype is not readily

available to selection (Popat *et al.*, 2015b), which the authors suggest is because signal production and signal response are coupled.

A role is proposed for WT quorum sensing to sense the genetic composition of a high density population; however it is unknown to what extent this is adaptive in natural populations of *P. aeruginosa*. The functional role of quorum-sensing is the subject of significant debate, with QS implicated as a device to sense variation in density (Darch *et al.*, 2012), mass transfer (Boedicker *et al.*, 2009; Redfield, 2002) or the spatial patterning of a population (Hense *et al.*, 2007). These environmental factors, along with others, will affect the concentration of signals. Like other hypotheses for functions of quorum sensing the importance of QS in sensing genotypic composition will depend on how much variation there is in *ΔLasR* proportion (or similar signal blind mutations) in the environment and clinical setting compared with variation in other factors. Our review of current literature suggests that there are high levels of variation in the proportion of *Δ*LasR defective strains in a natural setting (table 3.1), with cheat proportion varying from 0-100% within local sub-populations. High density populations with high cheat frequencies will have similar signal concentrations to low density populations composed entirely of cooperators. Therefore populations in natural and laboratory conditions vary in cheat proportion, and can lead to the same range of signal concentrations which suggests that cheat proportion may be at least as important as population density in determining signal concentrations in nature.

The addition of genotype sensing to the list of potential QS functions should not be seen as incompatible with the hypotheses mentioned above. Recent work has argued that using multiple signals allows *P. aeruginosa* to improve resolution of both population density and mass transfer properties

(Cornforth *et al.*, 2014). It is possible that wildtype populations growing clonally can use QS to draw sophisticated inferences about the state of their social and physical environment (Darch *et al.*, 2012; Diggle *et al.*, 2007; Hense *et al.*, 2007), and in addition can use the same QS apparatus to reduce social exploitation when confronted with non-responder cheat genotypes (figures 3.2-3.4).

An understanding of QS regulated secretions takes on an additional importance given the current focus on quorum sensing as a therapeutic target (Allen *et al.*, 2014; Rasko and Sperandio, 2010; Rutherford and Bassler, 2012; Schuster *et al.*, 2013). Under the action of QS receptor blocking therapeutics, sensitive strains will behave like non-responder cheats, while resistant mutants can potentially maintain wild-type behaviour (Allen *et al.*, 2014; Mellbye and Schuster, 2011), and risk being counter-selected in well mixed populations, due to their investment in cooperation. However, our results highlight that resistant cooperators will invest little in cooperation when they arise as a low proportion of the population, expressing a similar phenotype to susceptible strains, leading to relaxed selection for or against resistance (Gerdt and Blackwell, 2014). Similarly if therapeutics targeting signal supply are used they will reduce the concentration of active signal (Dong *et al.*, 2001; Gutierrez *et al.*, 2009). The perceived proportion of cooperators will then be lower, so genetic cooperators will cooperate less, again relaxing selection for or against genetic cooperators, potentially leading to the maintenance of elevated virulence (Köhler *et al.*, 2010). Our data highlights the need to understand drug targets in detail in order to predict the evolutionary consequences of treatment with new therapeutics (Allen *et al.*, 2014; Ross-Gillespie *et al.*, 2014; Vale *et al.*, 2014).

Plasticity is commonly observed in bacterial species, and the importance of plasticity in cooperative phenotypes is increasingly becoming apparent (de Vargas Roditi *et al.*, 2013; Kuemmerli *et al.*, 2009). What then can behavioural ecology tell us about plasticity in microbial cooperation (Inglis *et al.*, 2014)? Our results suggest that a behavioural rule of 'cooperate if surrounded by cooperators' is found in bacteria. This approximates generalised reciprocity: cooperate based on previous cooperative experiences regardless of identity of partners (Pfeiffer *et al.*, 2005). Generalised reciprocity has been reported in humans (Stanca, 2009) and rats (Rutte and Taborsky, 2007), and the small cognitive demands of generalised reciprocity suggest that it may be more universally applicable. We find that bacteria implement a similar social rule, suggesting that generalised reciprocity may not be confined to taxa with advanced cognition, and can be implemented at the cellular level via simple positive feedback regulatory circuits.

4 Antagonism Between Quorum Sensing Inhibition and Antibiotics Targeting Protein Synthesis

In preparation for submission as a research article with the same name. Contributing authors are: Richard C Allen, Roman Popat, Nancy Obeng and Sam P. Brown.

RCA and SPB came up with the idea. RCA designed and performed all experiments except data shown in figure 5.2 and 5.6. Data for figure 5.2 are a re-analysis of previously published data. Data in figure 5.6 was collected by NO under the suggestion of RCA and RP. RCA analysed all data and wrote the chapter.

4.1 Abstract

Quorum sensing (QS) inhibitors have been proposed as novel therapeutics to treat bacterial infections. These compounds have been shown to act synergistically with conventional antibiotics, acting as adjuvants that increase the efficacy of antibiotic clearance. However this interaction has only been shown in the context of QS inhibitor mediated biofilm disruption. This work investigates the interaction between QS inhibitors and protein synthesis inhibitors during planktonic growth.

This study develops and tests the hypothesis that the upregulation of genes in the quorum sensing regulon places a burden on the translational machinery of the cell, reducing its ability to respond to protein synthesis inhibitors. The ability of cyclopentylamide (C10-CPA) to inhibit quorum sensing in *Pseudomonas aeruginosa* without affecting growth is demonstrated. Knockouts of both the receptor (*lasR*) and synthase (*lasI*) for the *P. aeruginosa*

QS system, and inhibition of quorum sensing via C10-CPA reduce the potency of tobramycin, an aminoglycoside antibiotic that inhibits translation. This work suggests that treatments which reduce expression of the QS regulon may act antagonistically with certain antibiotics during planktonic growth. Together with previous work documenting a synergistic effect between similar treatments during biofilm growth this suggests that interactions between drug treatments are dependent on the environment.

4.2 Introduction

In many bacteria quorum sensing (QS) controls a significant proportion of the genome (Antunes *et al.*, 2007; Gilbert *et al.*, 2009; Jimenez *et al.*, 2012), which include genes involved in stress responses, and phenotypes involved in resistance to antibiotics (García-Contreras *et al.*, 2014; Goo *et al.*, 2012; Jimenez *et al.*, 2012). Therefore it has been proposed that QS inhibitors (chapter 2) may be used in combination with traditional antibiotics (Gill *et al.*, 2014). QS inhibitors may increase the efficacy of antibiotics (Bjarnsholt *et al.*, 2005; Brackman *et al.*, 2011b; Christensen *et al.*, 2012; Roy *et al.*, 2013) serving the role of an adjuvant. Adjuvants (initially a term used primarily in vaccine literature) are molecules that do not kill the bacterium in and of themselves but potentiate the factor that kills a pathogen (either exogenous antibiotics or the immune system), increasing the efficacy of killing (Gill *et al.*, 2014). As an adjuvant increases the efficacy of an antibiotic the pair of compounds act synergistically (Gill *et al.*, 2014).

Drug interactions are important parameters for predicting selection for resistance (Michel *et al.*, 2008; Yeh *et al.*, 2009). Synergistic drug interactions will increase the action of the component drugs, and so synergy is sought for clinical use to increase the drug efficacy while keeping drug concentrations low (Ankomah *et al.*, 2013). However for synergistic drug combinations

resistance to one drug not only removes the effect of that drug but also reduces the efficacy of the drug it is combined with, producing greater selection for resistance (Hegreness *et al.*, 2008). Antagonistic interactions have lower efficacy, meaning that higher drug concentrations are needed, however resistance to one drug increases the effect of the other so antagonistic interactions will reduce selection for resistance (Chait *et al.*, 2007; Hegreness *et al.*, 2008). For strongly antagonistic interactions resistance may even be selected against (Chait *et al.*, 2007). Therefore understanding drug interactions is important to predict resistance evolution and design treatment strategies.

The furanone QS inhibitor C-30 (Hentzer *et al.*, 2003) can be used to treat growing *P. aeruginosa* cultures, where it reduces the ability to form biofilms (Bjarnsholt *et al.*, 2005; Christensen *et al.*, 2012), a QS regulated trait (Jimenez *et al.*, 2012). These cultures can then be treated with tobramycin, a protein synthesis inhibitor commonly used to treat *P. aeruginosa* infections (Hancock and Speert, 2000). Biofilm growth leads to phenotypic resistance to antibiotics (Costerton *et al.*, 1999), so the cultures treated with C-30 are more susceptible to tobramycin (Bjarnsholt *et al.*, 2005; Christensen *et al.*, 2012). In this treatment context quorum sensing inhibition (C-30) increases the activity of tobramycin, so these compounds act synergistically. However this synergy has only been shown in the context of biofilm growth. Both biofilm growth and planktonic growth are present in infections (Hall-Stoodley and Stoodley, 2009) so it is important to know how this drug interaction affects planktonic cells.

This chapter investigates whether QS inhibition may have other effects on antibiotic interactions. In the context of planktonic growth, does QS inhibition still act synergistically with antibiotics? As quorum sensing

controls such a large number of genes (6-11% of the genome, Gilbert *et al.*, 2009), it places a metabolic burden on the cell (Ghoul *et al.*, 2014b; Diggle *et al.*, 2007) which may affect susceptibility to inhibitors of the protein synthesis pathway (Greulich *et al.*, 2015; Hall *et al.*, 2011). QS mutants are often isolated in clinical environments where bacterial populations are treated with antibiotics (Hoffman *et al.*, 2009; Smith *et al.*, 2006). Based on these ideas and published observations, the hypothesis that QS inhibition acts antagonistically with protein synthesis antibiotics is tested using *P. aeruginosa.* This opportunistic pathogen is a popular model for investigating quorum sensing (Cornforth *et al.*, 2014; Diggle *et al.*, 2007; Gilbert *et al.*, 2009; Rumbaugh *et al.*, 2000) and so several compounds have been identified as possible quorum sensing inhibitors (Hentzer *et al.*, 2003; Ishida *et al.*, 2007; Kaufmann *et al.*, 2006; Schramm *et al.*, 2008). If QS inhibition does act antagonistically with protein synthesis antibiotics then these antibiotics should have a weaker effect when QS is impaired (either by genetic knockout or by inhibition from QS inhibitors).

To inhibit quorum sensing 2 QS inhibitors were initially tested; cyclopentylamide with a 10 carbon chain (C10-CPA, figure 4.1, Ishida *et al.*, 2007) and azithromycin a macrolide antibiotic (figure 4.1). C10-CPA is a structural mimic of the 3-oxo-C12 N-acyl homoserine lactone signal used by the Las QS system in *P. aeruginosa*. Azithromycin is used to treat *P. aeruginosa* infections with positive effects despite being used below the minimal inhibitory concentration (MiC). Published work suggests this is due to the inhibitory effects of azithromycin on quorum sensing in P. aeruginosa (Kai *et al.*, 2009; Tateda *et al.*, 1996), however other labs argue that it is simply due to effects on growth (Glansdorp *et al.*, 2008). Azithromycin is initially tested as a QS inhibitor, but it has effects on growth at sub-MiC concentrations.

Azithromycin is also a protein synthesis inhibiting antibiotic, and is used to treat *P. aeruginosa* so it's interaction with QS inhibition through other means is also tested.

As both quorum sensing inhibitors have only moderate effects on growth genetic knockdown of quorum sensing was used also used to manipulate expression of the QS regulon, this is a common method to mimic QS inhibition (Gerdt and Blackwell, 2014; Mellbye and Schuster, 2011). Knockouts of the synthase gene (*lasI*) of the Las QS system as well a double knockout of the QS receptor (*lasR*) and synthase (*ΔlasIR*) were used to test that effects were due to defects in the overall QS system rather than specific mutations. *lasB* (elastase) is used as reporter of QS regulon expression. To disentangle the effect of quorum sensing and growth inhibition this chapter also uses two types of media. Quorum sensing media (QSM) where QS is required for growth, because the main carbon source is protein which is metabolised by proteases (including *lasB)* regulated by the QS system. Rich media (RM) has casamino acids as the main carbon source, these are freely available without the need for quorum sensing. These media (and similar media) are often used to investigate quorum sensing (Diggle *et al.*, 2007; Mellbye and Schuster, 2011).

To assess the types of antibiotics that quorum sensing may interact with 3 antibiotics from different classes were used (figure 4.1). Tobramycin is an aminoglycoside antibiotic that inhibits protein synthesis through action on the 30S subunit of the bacterial ribosome. Azithromycin is tested as a QS inhibitor but in killing *P. aeruginosa* it also inhibits protein synthesis. Unlike tobramycin, azithromycin acts on the 50S subunit. Finally carbenicillin is a carboxypenicillin which inhibits cell wall formation, it has no documented

effects on protein synthesis so it should not interact with quorum sensing inhibition according to the hypothesis proposed here.

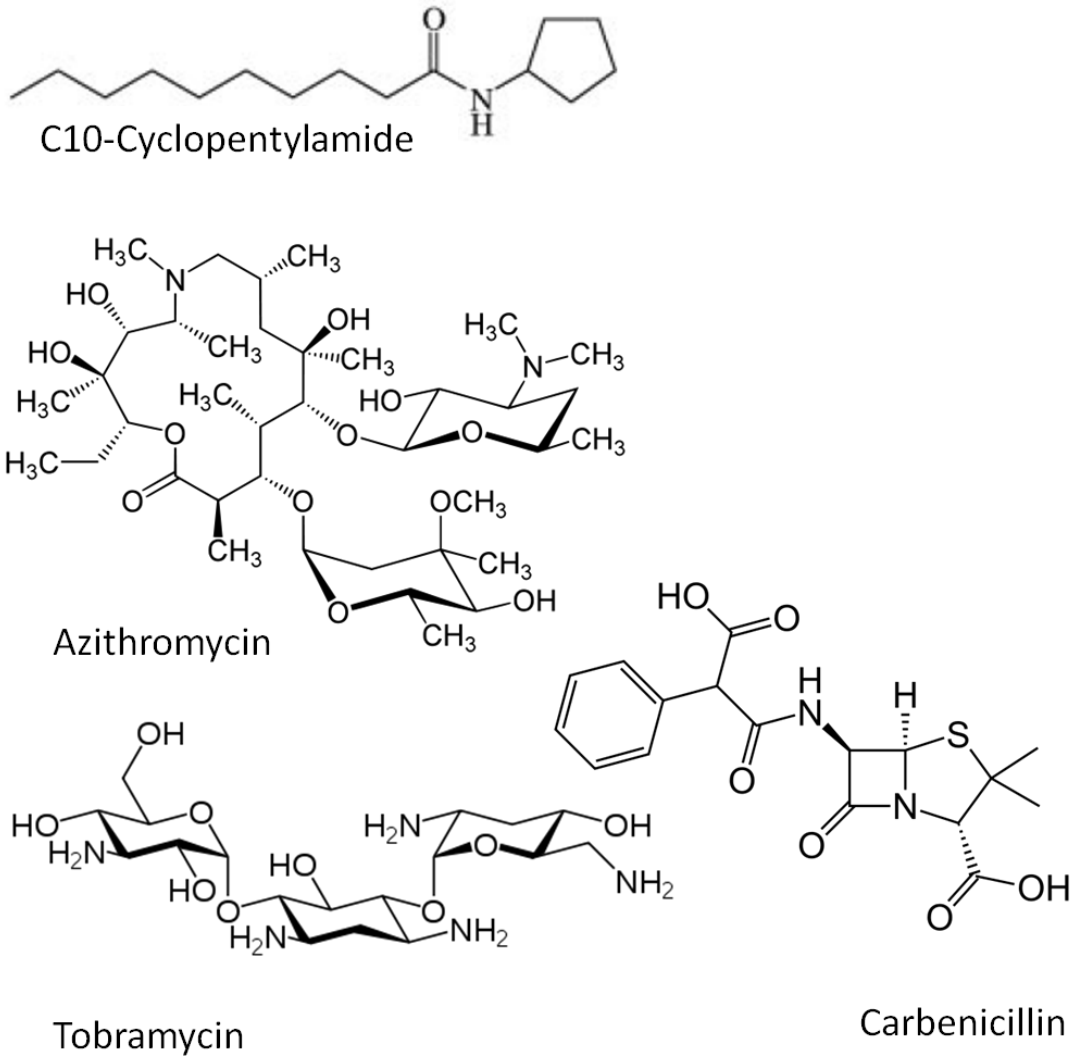

Figure 4.1: Compounds used in this chapter. C10-cyclopentylamide (C10-CPA), an inhibitor of the Las QS system, designed to mimic the Las signal molecule 3-oxo-C12 HSL. **Azithromycin**, a bacteriocidal macrolide antibiotic that acts by binding to the 50s subunit of the ribosome. This compound has published QS inhibitory activity. **Tobramycin**, a bacteriocidal aminoglycoside antibiotic that acts by binding to the 30s subunit of the ribosome. **Carbenicillin** a bactericidal β-lactam antibiotic that acts by inhibiting cell wall synthesis.

4.3 Methods

4.3.1 Strains and Media

P. aeruginosa (Nottingham PA01) was used for all experiments in the main text. All transformations were produced in the Diggle lab in Nottingham and kindly provided. *ΔlasI* and *ΔlasIR* knockouts were made by inserting a gentamycin resistance cassette into the *lasI* and *lasR* genes, which code for the synthase and receptor of the Las QS system respectively. Importantly resistance is specific to gentamycin and does not confer resistance to tobramycin or azithromycin. *lasB*::*lux* reporters were made by inserting a *luxCDABE* reporter under the control of the *lasB* promoter in a neutral position, the native *lasB* gene was not interfered with. This was used to detect the expression of the QS regulon. Insertions were produced using the mini CTXLux plasmid system (Winson *et al.*, 1998), in the Diggle lab.

Premixed LB broth and LB agar powder (Acros organics) was used for everyday culture of strains. Experiments used defined quorum sensing media (QSM, which needs quorum sensing for growth to high density) and rich media (RM, where growth does not require quorum sensing). These media consisted of an M9 minimal media base (containing Na_2HPO_4, KH_2PO_4 NaCl and Nh_4Cl) with the addition of 1mg/ml MgSO4, 100μg/ml CaCl2, and Hutners trace elements (Hutner *et al.*, 1950). For quorum sensing medium this was supplemented with 0.1% (w/v) casamino acids and 1% (w/v) bovine serum albumin (Popat *et al.*, 2012). For rich medium the M9 minimal medium was supplemented with 0.5% (w/v) casamino acids as the sole carbon source. Defined media was sterilised by filter sterilisation (0.22μm), LB media, and other media components which are not heat sensitive were sterilized by autoclaving at 121°C for 20 minutes.

4.3.2 Antibiotics and quorum sensing inhibitors

C10-CPA (cyclopentylamide with a 10 carbon chain) was synthesised in Nottingham and purchased as a solid through the Nottingham University website (www.nottingham.ac.uk/quorum/). This compound was dissolved in methanol. Azithromycin was purchased as a solid and dissolved in ethanol. Carbenicillin and tobramycin were purchased as a solids and dissolved in deionised water. All compounds were stored at -20°C. The level of solvent was always the same even when the concentrations of compounds was varied. Where the solvent was not water, solvent was never present above a concentration of 0.1% (v/v).

4.3.3 Growth assays

Strains to be used were thawed from -80°C storage and streaked onto sterile LB agar plates. After overnight (static, 37°C) growth, single colonies were picked and used to inoculate overnight cultures in LB (150rpm, 37°C). Overnight cultures were diluted 1/100 in the morning and re-incubated under the same conditions until mid-exponential phase. These mid-exponential cultures were washed in the assay media and the optical density (600nm) was measured. Washed cultures were corrected so that the starting optical density was 0.01 (after drug treatments added). Washed cultures were split into different treatments and drugs were added (i.e. at the start of growth). Finally cultures for each treatment were split into at least 3 replicates and were aliquoted as 200µl volumes into the central wells of a 96 well plate. All wells without treatments contained 200µl of deionised water, or media blanks.

96 well plates were incubated (static, 37°C) in a varioskan plate reader (Fisher scientific), with a protocol which takes readings of optical density (600nm) and luminescence every hour. Plates were incubated with the lid on to

prevent evaporation of cultures, a heat gradient in the plate reader prevented condensation from forming on the lids of the 96 well plates.

4.3.4 Elastin congo red assay

To measure protease activity directly an elastin congo red assay was used (Ohman *et al.*, 1980). Supernatant was extracted from cultures after the growth assay and were pooled within treatments (2 x 200µl wells for each pool, reducing 6 replicates to 3). Pooled cultures were centrifuged and supernatant was removed and filter sterilised (0.22µm). 50µl of supernatant was diluted 1/10 in 20mg/ml elastin congo red (ECR) in elastin congo red buffer (100mM Tris, 1mM $CaCl_2$, pH=7) in centrifuge tubes. These were incubated on their sides at 37°C with agitation in an orbital shaker (150rpm, 37mm orbit), blanks containing fresh media were also incubated. After 24 hours tubes were removed and centrifuged (13,000rpm, 1 minute). 200µl of supernatant was then removed and the absorbance of solubilised dye was measured at 495nm. This assay directly measures protease activity and was used to corroborate the observations seen with the lasB:luxCDABE reporter.

4.3.5 Transcriptome analysis

For QS to place a metabolic burden on the cell, protein synthesis must be increased. Therefore previously published (Cornforth *et al.*, 2014; Popat *et al.*, 2015a) transcriptomics data was used to assess whether induction of the QS system, led to overall upregulation or downregulation of gene expression To summarise the methods, double synthase knockouts (*ΔlasI*, *ΔrhlI*) had exogenous C4-HSL and 3-oxo-C12 HSL added to the cultures at a concentration of 15µM each. Cultures were grown in LB for 8 hours with agitation, before RNA was extracted and expression was measured using microarrays. QS regulated genes were those that were shown to vary significantly between the treatments. The analysis here compares the fold

change in the expression of QS regulated genes between the double synthase mutant with no signal and the double synthase mutant with both signals added simultaneously. Both signals were added because in a WT induction of the Las system generally leads to induction of the Rhl system.

4.3.5 Statistics and analysis

Measured luminescence from the *lasB* reporter was divided by the optical density of the culture to calculate relative luminescence (RLU). For summaries of growth the integral under the growth curve was used, to take into account both rate and yield of growth. Maximum values of RLU were used for summaries of the reporter activity because of the sharp peak and decline seen in time series (e.g. figure 4.8), and to reduce the feedback of growth seen at later time points.

All statistics were performed in the R computing environment (R Core Team, 2013). The statistical analysis for data in figure 5.2 used a t test to assess whether the average $\log_2$ fold change in gene expression is significantly different from zero. All other data were fitted by generalised linear models using a Gaussian error structure and an identity link function before being reduced to a minimal model to identify significant terms. Briefly, a relevant maximal model was identified and then reduced to a minimal model in a stepwise manner. At each step of model reduction all terms (or effects) that were not currently included in an interaction were tested for significance as grounds for including them in the model. Terms were dropped if the result of an F test comparison of models with and without the term of interest was not significant at α=0.05 (i.e. accepting the null hypothesis of no significant effect of the term). At each step only one term could be dropped so where several effects were non-significant the new model with the lowest AiC

(Akaike information criterion) was chosen as the best model reduction at that step.

When no terms (not included in higher order interactions) could be dropped without a significant effect, the current model was considered the minimal model. Statistical support for all terms in the minimal models was assessed as above but through comparison of the minimal model and the minimal model with the term dropped. The estimates of the coefficients for each term and its associated standard error are also reported as well as other aspects of the model. Significance is not reported for some main effects involved in significant interactions, because the model without the main effect could not be formulated with fewer parameters (an increase in degrees of freedom). Full statistical results are reported in tables near the relevant figures.

4.4 Results

4.4.1 Quorum sensing induction predominantly upregulates gene expression

Using transcriptome data collected from a previous study (Cornforth *et al.*, 2014; Popat *et al.*, 2015), shows that for all QS regulated genes the average change in expression with QS induction is approximately 2 fold, a significant upregulation (figure 4.2, $t_{1,263} = 8.51$, $p < 10^{-10}$, $\log_2$(Fold change estimate) = 1.081, s.e = 0.128). This indicates that on average genes are upregulated when quorum sensing is induced which means that QS induction could place a burden on protein synthesis.

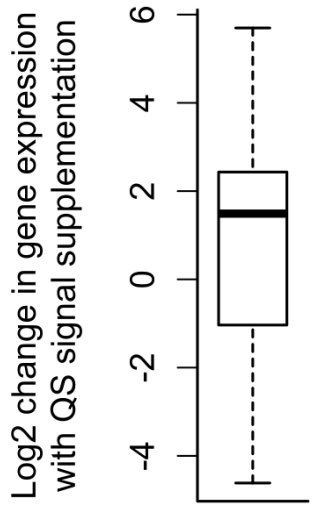

Figure 4.2: Fold change in expression of quorum sensing regulated genes with QS induction. Boxplot shows the distribution of fold changes for 264 genes which were shown to show significant regulation by the Las and Rhl quorum sensing systems. Both C4 and 3-oxo-C12 HSL were used for signal supplementation (n=264).

4.3.1 Effects of C10-CPA alone

To test whether C10-CPA was an effective QS inhibitor without direct effects on growth, WT *P. aeruginosa* was treated with increasing doses of CPA in media where QS is required for growth or media where growth is independent of QS. A QS mutant was also used to assess whether CPA had effects on a strain that doesn't use QS.

When grown in a medium requiring quorum sensing (QSM figure 4.3a), increasing doses of the QS inhibitor C10-CPA reduces the expression of QS controlled *lasB* in a dose dependent manner ($F_{1,28}$ = 36.91, p<0.001, β = -5.647x10^2, s.e = 92.950). As the medium requires quorum sensing controlled factors for growth, C10-CPA also reduces the growth of the WT ($F_{1,28}$ = 23.27, p< 0.001, β = -0.002, s.e = 3.200x10^{-4}). In a medium where QS is not required for growth (figure 4.3b) a reduction is seen in the expression of LasB with increasing C10-CPA ($F_{1,13}$ = 45.38, p<0.001, β= -7.522x10^2, s.e = 1.117x10^2), but the growth of the WT strain is not significantly reduced ($F_{1,28}$ = 4.00, p= 0.067).

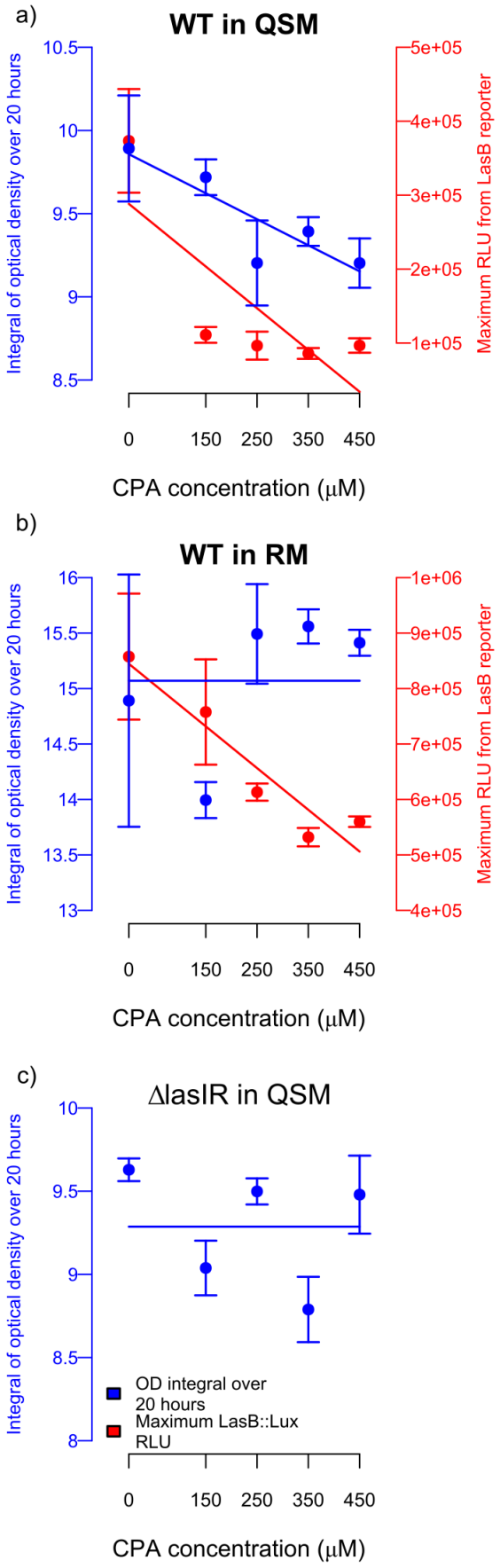

Figure 4.3: The effect of C10-CPA on growth and *lasB* expression in *P. aeruginosa* with varying requirements for quorum sensing. Each figure shows the integral of growth and the maximum expression of luminescent elastase reporter during 20 hours growth with varying concentrations of C10-CPA. Elastase expression in the WT always decreases with increasing C10-CPA and growth decreases when QS regulon expression is reduced in an environment requiring QS. Points are mean values with error bars showing confidence intervals (1.96*s.e). a) Growth and elastase expression of PAO1 wild-type in quorum sensing medium (n=6). b) Growth and elastase expression of PAO1 wild-type in rich medium (n=3). c) Growth of PAO1 *ΔlasIR* in quorum sensing medium (n=6).

a) Integral of OD over 20 hours			b) Maximum LasB:luxCDABE RLU		
Effect	$F_{1,28}$	p-value	Effect	$F_{1,28}$	p-value
CPA	23.28	<0.001	CPA	36.91	<0.001
Effect	Estimate	s.e	Effect	Estimate	s.e
Intercept	9.857	0.093	Intercept	2.882e5	2.662e4
CPA	-0.002	3.200e-4	CPA	-5.647e2	92.950
n=30, residual deviance 2.156 on 28 DF			n=30, residual deviance 1.771e11 on 28 DF		

Table 4.1: Statistical table of fixed effects model for the response of WT *P. aeruginosa* to increasing concentrations of C10-CPA in an environment where QS is required for growth (QSM). a) Response variable is the integral of optical density measures over 20 hours of growth. b) Response variable is the maximum relative luminescence (RLU) reported from a *lasB* reporter. Although models a) and b) have a similar structure and use measures taken from the same experiment they are otherwise unlinked.

a) Integral of OD over 20 hours			b) Maximum LasB:luxCDABE luminescence		
Effect	$F_{1,13}$	p-value	Effect	$F_{1,13}$	p-value
CPA	4.00	0.067	CPA	45.38	<0.001
Effect	Estimate	s.e	Effect	Estimate	s.e
Intercept	15.070	0.190	Intercept	8.44e5	3.198e4
			CPA	-7.522E+02	1.117E+02
n=15, residual deviance 7.610 on 14 DF			n=15, residual deviance 5.933e10 on 13 DF		

Table 4.2: Statistical table of fixed effects model for the response of WT *P. aeruginosa* to increasing concentrations of C10-CPA in an environment where QS is not required for growth (RM). a) Response variable is the integral of optical density measures over 20 hours of growth. b) Response variable is the maximum relative luminescence (RLU) reported from a LasB reporter. Although models a) and b) have a similar structure and use measures taken from the same experiment models are otherwise unlinked.

a) Integral of OD over 20 hours		
Effect	$F_{1,28}$	p-value
CPA	1.864	0.183
Effect	Estimate	s.e
Intercept	9.287	0.068
n=30, residual deviance 4.075 on 29 DF		

Table 4.3: Statistical table of fixed effects model for the response of P. aeruginosa *ΔlasIR* to increasing concentrations of C10-CPA in an environment

where QS is required for growth (QSM). a) Response variable is the integral of optical density measures over 20 hours of growth.

Finally if quorum sensing is abolished by genetic manipulation (*ΔlasIR*, figure 4.3c) addition of C10-CPA has no effect on growth in quorum sensing media ($F_{1,28}$ = 1.8644, p = 0.183). *ΔlasIR* does not express *lasB* so luminescence is not shown.

4.3.2 Effects of azithromycin alone

As for C10-CPA, *P. aeruginosa* was also treated with azithromycin, in media with different QS requirements for growth and against strains with and without complete QS systems. When WT *P. aeruginosa* is treated with increasing doses of azithromycin in quorum sensing medium (figure 4.4a), luminescence from the *lasB* reporter decreases with increasing azithromycin dose ($F_{1,28}$ = 6.61, p= 0.016, β = -1.054x10^4, s.e = 4.097 x10^3). Increasing doses of azithromycin also lead to decreased growth in quorum sensing medium ($F_{1,28}$ = 204.59, p < 0.001, β = -0.245, s.e = 0.017). However in a rich medium where quorum sensing is not required for growth (figure 4.4b) azithromycin still reduces growth by a comparable amount ($F_{1,28}$ = 289.51, p < 0.001, β = -1.035, s.e = 0.057). However in this medium *lasB* expression *increases* with increasing azithromycin dose ($F_{1,28}$ = 17.045, p < 0.001, β = 8.147x10^4, s.e = 1.973x10^4). Finally in a *ΔlasIR* mutant defective for QS (figure 4.4c) azithromycin has a significant negative effect on growth in quorum sensing medium ($F_{1,28}$ = 39.26, p < 0.001, β = 0.171, s.e = 0.0273).

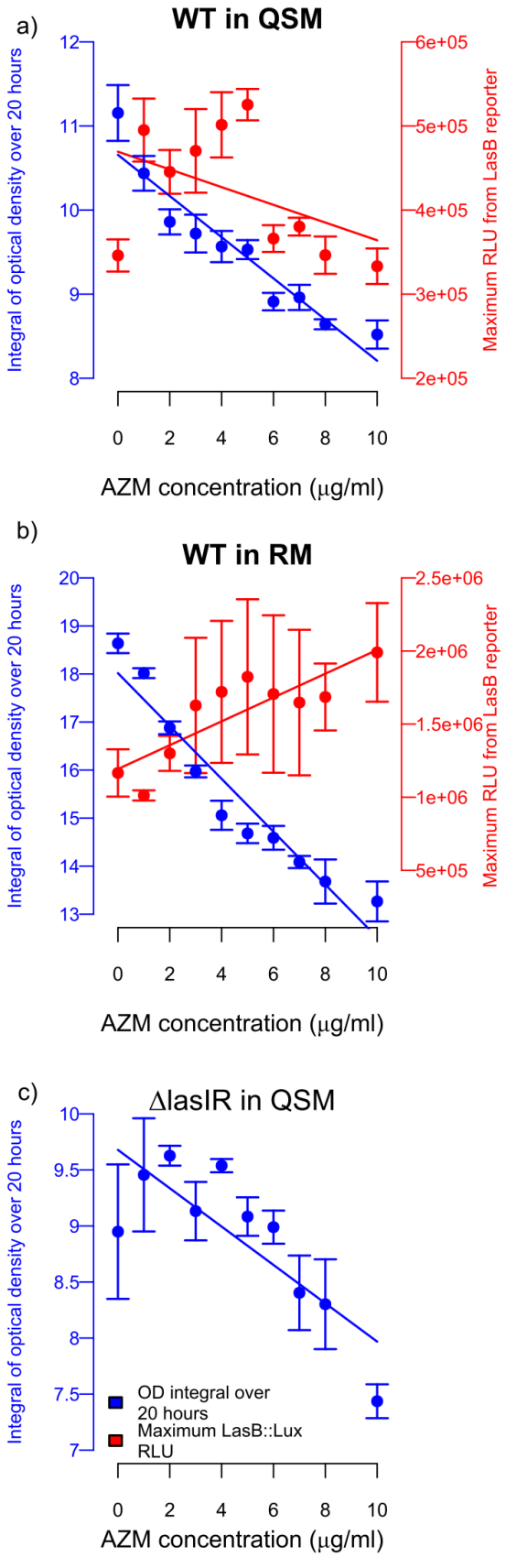

Figure 4.4: The effect of azithromycin (AZM) on growth and *lasB* expression in *P. aeruginosa* with varying requirements for quorum sensing. Each figure shows the integral of growth and the maximum expression of luminescent elastase reporter during 20 hours growth with varying concentrations of azithromycin. Growth always decreases when as azithromycin dose increases. Solid lines connect mean values while shaded regions are 95% confidence intervals of the mean. a) Growth and elastase expression of PAO1 wild-type in quorum sensing medium (n=3). b) Growth and elastase expression of PAO1 wild-type in rich medium (n=3). c) Growth of PAO1 *ΔlasIR* in quorum sensing medium (n=3).

WT growth in QSM treated with AZM		
Effect	$F_{1,28}$	p-value
AZM	204.59	<0.001
Effect	Estimate	s.e
Intercept	10.657	0.0944
AZM	-0.245	0.017
n=30, residual deviance 2.276 on 28 DF		

WT LasB expression in QSM treated with AZM		
Effect	$F_{1,28}$	p-value
AZM	6.61	0.016
Effect	Estimate	s.e
Intercept	4.694×10^{5}	2.259×10^{4}
AZM	-1.054×10^{4}	4.09710^{3}
n=30, residual deviance 9.078×10^{7} on 28 DF		

Table 4.4: Statistical table of fixed effects model for the response of WT *P. aeruginosa* to increasing concentrations of azithromycin (AZM) in an environment where QS is required for growth (QSM). a) Response variable is the integral of optical density measures over 20 hours of growth. b) Response variable is the maximum relative luminescence (RLU) reported from a LasB reporter. Although models a) and b) have a similar structure and use measures taken from the same experiment they are otherwise unlinked.

WT growth in RM treated with AZM		
Effect	$F_{1,28}$	p-value
AZM	289.51	<0.001
Effect	Estimate	s.e
Intercept	18.016	0.178
AZM	-1.035	0.057
n=30, residual deviance 8.111 on 28 DF		

WT LasB expression in RM treated with AZM		
Effect	$F_{1,28}$	p-value
AZM	17.045	<0.001
Effect	Estimate	s.e
Intercept	1.193×10^{6}	1.088×10^{5}
AZM	8.147×10^{4}	1.973×10^{4}
n=30, residual deviance 3.023×10^{12} on 28 DF		

Table 4.5: Statistical table of fixed effects model for the response of WT *P. aeruginosa* to increasing concentrations of azithromycin (AZM) in an environment where QS is not required for growth (RM). a) Response variable is the integral of optical density measures over 20 hours of growth. b) Response variable is the maximum relative luminescence (RLU) reported from a LasB reporter. Although models a) and b) have a similar structure and use measures taken from the same experiment they are otherwise unlinked.

ΔlasIR growth in QSM treated with AZM		
Effect	$F_{1,28}$	p-value
AZM	39.26	<0.001
Effect	Estimate	s.e
Intercept	9.678	0.150
AZM	-0.171	0.0273
n=30, residual deviance 5.782 on 28 DF		

Table 4.6: Statistical table of fixed effects model for the response of *P. aeruginosa ΔlasIR* to increasing concentrations of azithromycin (AZM) in an environment

where QS is not required for growth (RM). a) Response variable is the integral of optical density measures over 20 hours of growth.

4.3.3 Differential potency of antibiotics on WT and QS impaired strains

The experiments detailed in figure 4.4 suggest that azithromycin is not an effective QS inhibitor (having variable effects on QS and direct effects on growth). However comparison between figure 4.4a and 4.4c suggests that the efficacy of azithromycin may be affected by the QS system. To test this directly WT *P. aeruginosa* and the *ΔlasIR* strain were treated with azithromycin. These strains were also treated with another protein synthesis antibiotic (tobramycin) and an antibiotic that had a completely different mechanism of action (carbenicillin) to test if the effect is more general.

As is evident from an overview of figure 5.6 there is diversity in the way that the different antibiotics affect the 2 strains. In rich medium the WT strain grows slightly better when there is no antibiotic present ($F_{1,54}$ = 4.604, p < 0.036, strainWT= 0.130, s.e = 0.061). Azithromycin ($F_{1,54}$ = 186.62, p < 0.001, β = -0.144, s.e = 0.011) and carbenicillin ($F_{1,54}$ = 185.84, p <0.001, β = -0.057, s.e = 0.004) have clear main effects on the growth of the 2 strains, but neither azithromycin ($F_{1,52}$ = 0.96, p = 0.331) or carbenicillin ($F_{1,53}$ = 1.74, p = 0.193) efficacy is affected by possession of a functioning QS system. On the other hand the negative dose response curve of tobramycin (β = -11.287, s.e = 0.0761) is significantly different between the WT and *ΔlasIR* strains ($F_{1,54}$ = 6.47, p = 0.014, StrainWT:TOB = -2.505, s.e= 0.985). The *ΔlasIR* mutant is less affected at high doses so that rank order of the growth integral changes with tobramycin dose.

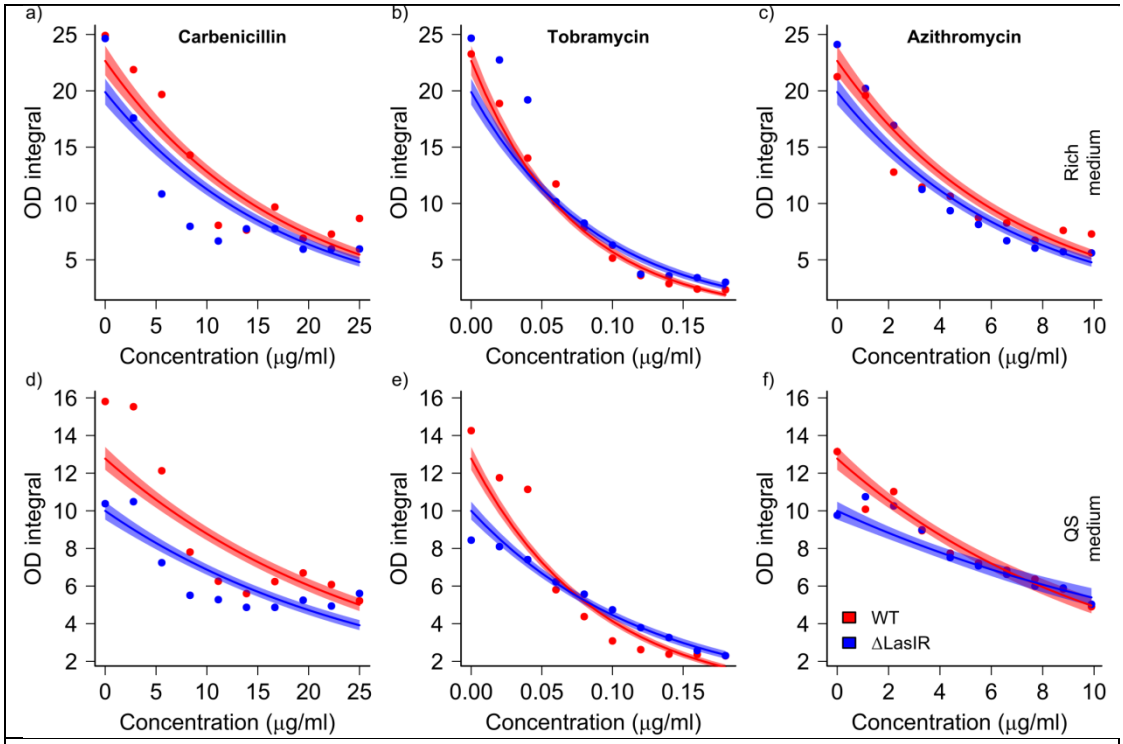

Figure 4.5 Growth of WT and *ΔlasIR* strains of *P. aeruginosa* exposed to antibiotics shows a diversity of strain interactions in dose response curves. (a, b & c) growth in rich medium. (d, e & f) growth in quorum sensing (QS) medium. (a & d) strains treated with increasing concentrations of carbenicillin. (b & e) Strains treated with increasing concentrations of tobramycin. (c & f) Strains treated with increasing concentrations of azithromycin. Data points are individual (unreplicated) integrals of growth curves over 24 hours. Solid lines are minimal linear models of the logarithm of OD integral as predicted by antibiotic concentration (tables 4.7 and 4.8), shaded areas are 95% confidence integrals of the model fit.

Effect	DF	F-Statistic	P-value
Strain WT	1,54	4.604	0.036
CB	1,54	185.84	<0.001
AZM	1,54	186.62	<0.001
TOB	NA	NA	NA
WT:CB	1,53	1.74	0.193
WT:AZM	1,52	0.96	0.331
WT:TOB	1,54	6.47	0.014
Effect	Estimate	s.e	
Intercept	2.987	0.058	
Strain WT	0.130	0.061	

CB	-0.057	0.004	
AZM	-0.144	0.011	
TOB	-11.29	0.761	
WT:TOB	-2.505	0.985	
n=60, residual deviance= 2.277 on 54 degrees of freedom			

Table 4.7 Statistical table for fixed effects model of growth of a WT *P. aeruginosa* and a QS deficient strain (*ΔlasIR*) in rich medium with the presence of increasing doses of carbenicillin, tobramycin and azithromycin. Response variable was the natural logarithm of optical density integral over 24 hours. The interaction of each antibiotic dose with strain was used to assess whether the two strains had different susceptibility to each antibiotic. F tests not performed for main effects of tobramycin concentration as it cannot be dropped from the minimal model with an increase in degrees of freedom.

Effect	F stat DF	F-Statistic	p value
Strain WT	1,53	18.70	<0.001
CB	1,53	134.21	<0.001
AZM	NA	NA	
TOB	NA	NA	
WT:CB	1,52	1.11	0.297
WT:AZM	1,53	5.19	0.027
WT:TOB	1,53	15.98	<0.001
Effect	Estimate	s.e	
Intercept	2.302	0.048	
Strain WT	0.245	0.057	
CB	-0.037	0.003	
AZM	-0.062	0.011	
TOB	-8.072	0.602	
WT:AZM	-0.033	0.015	
WT:TOB	-3.210	0.803	
n=60, residual deviance = 1.344 on 53 degrees of freedom			

Table 4.8 Statistical table for fixed effects model of growth of a WT *P. aeruginosa* and a QS deficient strain (*ΔlasIR*) in quorum sensing medium with the presence of increasing doses of carbenicillin, tobramycin and Azithromycin. Response variable was the natural logarithm of optical density integral over 24 hours. The interaction of each antibiotic dose with strain was used to assess whether the two strains had different susceptibility to each antibiotic. F tests were not performed for main effects of, tobramycin concentration or azithromycin concentration because

they could not be dropped from the minimal model with an increase in degrees of freedom.

Results are similar in QS medium although the effects are more clear. The main effect of strain is clearly significant here, because the WT grows better using BSA ($F_{1,53}$ = 18.70, p < 0.001, StrainWT = 0.245, s.e = 0.057). Carbenicillin had a significant effect on growth ($F_{1,53}$ = 134.21, p < 0.001, β = -0.037, s.e. = 0.003) but the effect was not significantly different between the two strains ($F_{1,52}$ = 1.11, p = 0.297). However the negative effects of tobramycin (β = -8.072, s.e = 0.602) and azithromycin (β = -0.062, s.e = 0.011) were greater in the QS functional WT (Tobramycin $F_{1,54}$ = 11.987, p < 0.01, StrainWT:TOB = -3.210, s.e = 0.803; Azithromycin $F_{1,53}$ = 5.1853, p < 0.05, StrainWT:AZM = 0.033, s.e = 0.015), with the *ΔlasIR* again being more fit than the WT at high concentrations of tobramycin.

4.3.4 Investigation of C10-CPA tobramycin antagonism

Results in figure 4.5 show that QS impairment has an antagonistic effect on the efficacy of certain protein synthesis inhibitors. However this data uses genetic knockout of the QS system, whereas the action of QS inhibitors like C10-CPA is much less strong. So the interaction between C10-CPA and protein synthesis antibiotics is was tested, using tobramycin, as it had the strongest interaction in the previous experiment (figure 4.5). As a positive control, and also to test an alternate QS impaired mutant *P. aeruginosa ΔlasI* was also treated with tobramycin (but not CPA).

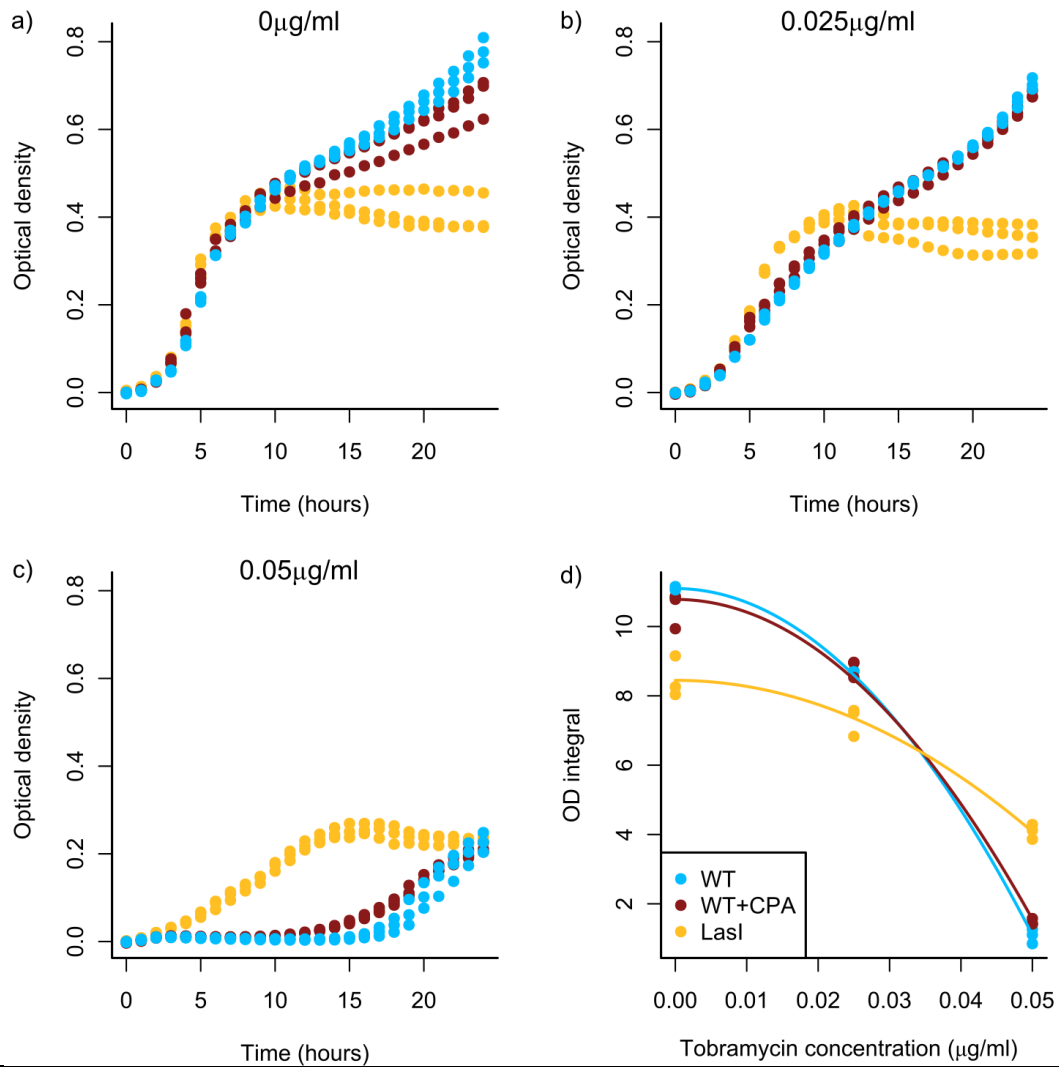

Figure 4.6 Growth of strains with impaired quorum sensing activity is less sensitive to tobramycin. Untreated PAO1 WT, C10-CPA (100μM) treated PAO1 WT and PAO1 *ΔlasI* grown in the presence of increasing concentrations of tobramycin. a) Growth without tobramycin. b) growth with 0.025μg/ml tobramycin. c) Growth with 0.05 μg/ml tobramycin. d) Comparison of OD integrals, models are minimal linear models of OD integral against tobramycin concentration squared. (n=3, all replicates are plotted).

Effect	F stat DF	F-statistic	P-value
QS impairment	2,21	78.65	<0.001
TOB^2	NA	NA	NA
QS:TOB^2	2,21	123.71	<0.001
Effect	Estimate	s.e	
Intercept	11.095	0.163	

QS_CPA	-0.313	0.231	
QS_LasI	-2.649	0.231	
TOB^2	-3996.59	109.69	
QS_CPA:TOB^2	306.940	155.125	
QS_LasI:TOB^2	2249.860	155.125	
n=27, residual deviance = 2.566 on 21 degrees of freedom.			

Table 4.9 Statistical table of the fixed effects model for growth of strains with varying QS impairment WT, CPA treated WT and a QS deficient genetic knockout (ΔlasI) in the presence of increasing concentrations of tobramycin. Response variable is the integral of growth over 24 hours. An interaction between tobramycin dose and QS impairment was used to assess whether QS status affected susceptibility to tobramycin. F tests were not performed for the main effect of, tobramycin concentration squared because the term could not be dropped from the minimal model with an increase in degrees of freedom.

Effect	F stat DF	F-statistic	P-value
QS impairment	1,14	2.21	0.160
TOB^2	NA	NA	NA
QS:TOB^2	1,14	4.702	0.048
Effect	Estimate	s.e	
Intercept	11.095	0.149	
QS_CPA	-0.313	0.211	
TOB^2	-3996.59	100.091	
QS_CPA:TOB^2	306.940	141.551	
n=18, residual deviance = 1.425 on 14 degrees of freedom			

Table 4.10 Statistical table of the fixed effects model for growth of strains with varying QS impairment, WT and CPA treated WT in the presence of increasing concentrations of tobramycin. Response variable is the integral of growth over 24 hours. An interaction between tobramycin dose and QS impairment was used to assess whether QS status affected susceptibility to tobramycin. This model was fitted to the same data as table 4.9 but with the *ΔlasI* data removed to test for a interaction between addition of CPA and tobramycin, as the interaction in table 4.9 is mostly driven by the difference between WT and *ΔlasI*. F tests were not performed for main effects of, tobramycin concentration squared because the term could not be dropped from the minimal model with an increase in degrees of freedom.

Figures 4.6a-4.6c show growth of a QS knockout *ΔlasI* and WT *P. aeruginosa* with and without C10-CPA grown in QSM with increasing concentrations of tobramycin (0, 0.025 and 0.05 μ/ml). As the concentration of tobramycin increases the growth of all strains is hindered but those that have impaired quorum sensing systems (*ΔlasI* and WT + CPA) are affected to a lesser extent (figure 4.6d). Multiple regression with the 3 QS treatments as 3 levels in a single factor shows that QS impairment has a significant negative main effect on growth ($F_{2,21}$ = 78.65, p<0.001, QS_CPA = -0.313, s.e = 0.231, QS_LasI = -2.649, s.e = 0.231). The negative main effect of tobramycin concentration squared (β = -3996.59, s.e = 109.69) varies significantly between the levels of QS impairment ($F_{2,21}$ = 123.71, p < 0.001; QS_CPA:TOB^2 = 306.940, s.e = 155.125, LasI:TOB^2 = 2249.860, s.e = 155.125).

The effect of QS treatment is predominantly driven by the difference between the WT and *ΔlasI* dose response curves (figure 4.6). To test for a real effect of CPA addition a subset of data was used (without *ΔlasI*), to show that there is a real difference in the WT response to tobramycin if treated with CPA (table 4.10). Using this subset the main effect of QS impairment (CPA addition) is not significant although this term is still included in the model to correctly interpret the interaction ($F_{1,14}$ = 2.21, p = 0.160, QS_CPA = -0.313, s.e = 0.211). However the interaction between the negative main effect of tobramycin concentration squared (β = -3996.59, s.e = 100.091) and QS impairment is still significant ($F_{1,14}$ = 4.702, p = 0.048, QS_CPA: TOB^2 = 3.06.940, s.e = 141.551). This suggests that the hypothesis of an additive effect according to bliss independence (which states that if drugs act additively then the dose response curve of one should be independent of the presence of the other, Yeh *et al.*, 2009; Bliss, 1939) can be rejected. As tobramycin is less effective in

the presence of C10-CPA these data suggest antagonism between the QS inhibitor and the antibiotic.

To understand the antagonism between QS inhibition and tobramycin luminescence from a *lasB* reporter was used to assess QS activity with increasing doses of tobramycin (figure 4.7a-4.7c). A dose dependent increase in relative luminescence from the reporter with the square root of tobramycin concentration was observed ($F_{1,14}$ =78.04, $p < 0.001$, β = 1.483 x10^6, s.e = 1.679x10^5). This was surprising but azithromycin (another translation inhibitor) was also suggested to increase *lasB* expression under some conditions (Figure 4.3). C10-CPA addition reduced the luminescence from the reporter ($F_{1,15}$ =18.70, $p < 0.01$, QS_CPA = -1.363x10^5, s.e = 3.152x10^4). However QS inhibition did not affect how elastase expression was affected by tobramycin concentration ($F_{1,14}$ =0.582, p = 0.458). Models of reporter luminescence did not include the *ΔlasI* strain because *lasB* expression is always low and is not expected to change in the complete absence of QS.

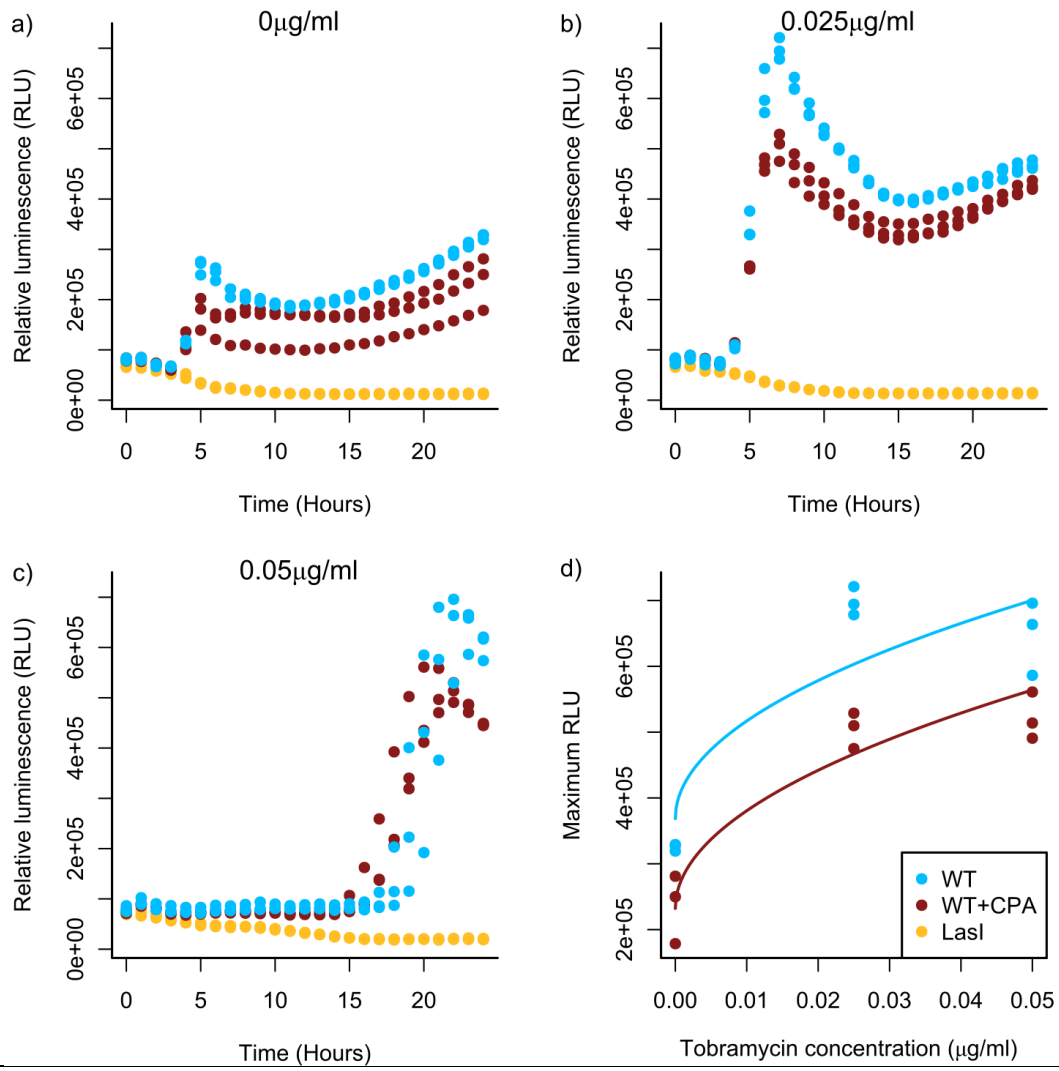

Figure 4.7 Strains exposed to tobramycin at low concentrations express elastase at higher levels Untreated PAO1 WT, C10-CPA (100μM) treated PAO1 WT and PAO1 *ΔlasI* grown in the presence of increasing concentrations of tobramycin. a) *lasB* reporter relative luminescence without tobramycin. b) *lasB* reporter relative luminescence with 0.025μg/ml tobramycin. c) *lasB* reporter relative luminescence with 0.05 μg/ml tobramycin. d) Comparison of maximum relative luminescence. Models are minimal linear models of maximum relative luminescence against the square root of tobramycin concentration. (n=3, all replicates are plotted).

Effect	F stat DF	F-statistic	P value
QS impairment	1,15	18.701	<0.001
TOB^0.5	1,15	78.04	<0.001
QS:TOB^0.5	1,14	0.582	0.458
Effect	Estimate	s.e	

Intercept	3.686×10^5	3.088×10^4	
QS_CPA	-1.363×10^5	3.152×10^4	
TOB^0.5	1.483×10^6	1.679×10^5	
n=18, residual deviance = 6.708×10^{10} on 15 degrees of freedom			

Table 4.11 Statistical table of the fixed effects model for *lasB* expression of strains with varying QS impairment WT, CPA treated WT in the presence of increasing concentrations of tobramycin. Response variable is the maximum luminescence from a lasB:luxCDABE reporter. An interaction between tobramycin dose and QS impairment was used to assess whether QS status affected the change in *lasB* expression seen with increasing tobramycin dose.

The increase in expression from the reporter could be an artefact of an effect of tobramycin on the luminescence cassette of the reporter, therefore a follow-up experiment was performed to assess the activity of elastase directly. WT *P. aeruginosa* was grown in QSM for 24 hours in the presence or absence of 0.025µg/ml tobramycin. In addition to optical density and luminescence from the *lasB* reporter, the protease activity of the supernatent was measured through the elastin congo red assay after 24 hours growth (figure 5.9c). This experiment corroborates the results of the luminescence reporter, showing that protease concentration in the media is indeed increased when tobramycin is added ($F_{1,4}$ = 72.47, p >0.001, TreatmentTOB = 0.011, s.e = 0.001).

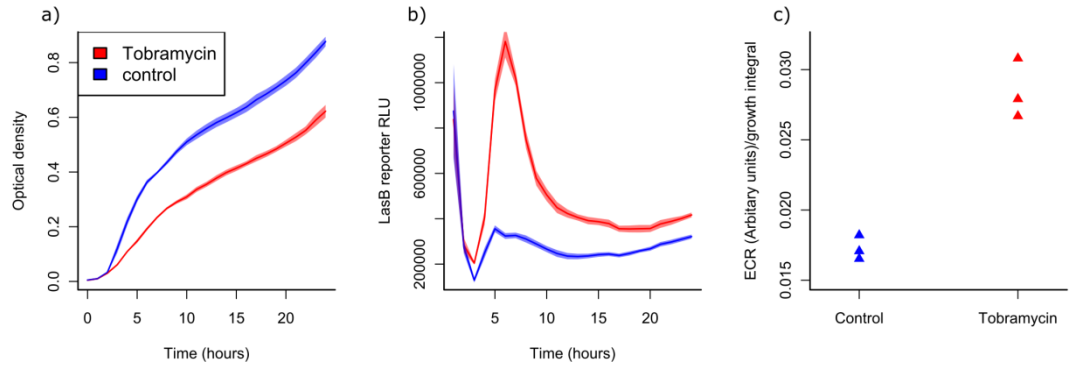

Figure 4.8: Sub MiC concentrations of tobramycin reduce growth, increase elastase reporter luminescence and increase protease production. PAO1 WT was grown with and without 0.025µg/ml tobramycin. a) Optical density of cultures over 24 hours growth, solid lines are mean values and shaded areas are 95% confidence intervals (n=6). b) Relative luminescence (RLU) of cultures over 24 hours growth, solid lines are mean values and shaded areas are 95% confidence intervals (n=6). c) Protease activity of cultures after 24 hours growth measured by an elastin congo red assay (arbitrary units), divided by the integral of growth curves (n=3)

Effect	F stat DF	F statistic	P value
Tobramycin	1,4	72.47	0.001
Effect	Estimate	s.e	
Intercept	0.017	0.001	
Tobramycin	0.011	0.001	
n=6, residual deviance = 1.038×10^{-5} on 4 degrees of freedom			

Table 4.12 Fixed effects model for the effect of tobramycin on protease expression in WT *P. aeruginosa*. Response variable is Elastin congo red dye absorbance divided by growth integral. The model assumes a Gaussian error structure and uses an identity link function.

4.5 Discussion

The majority of QS regulated genes are upregulated as quorum sensing is induced (figure 5.2 & Popat, Cornforth, *et al.*, 2015). Additionally QS impaired strains are common in antibiotic treated hosts (Hoffman *et al.*, 2009; Smith *et al.*, 2006). Based on these observations, the hypothesis that translation inhibiting antibiotics are less active against *P. aeruginosa* when

quorum sensing is impaired is tested. Put another way there is antagonism between QS inhibitors and translation inhibiting antibiotics.

This chapter investigates the effects of 2 published quorum sensing inhibitors, azithromycin and C10-CPA. Azithromycin had off target effects and failed to inhibit elastase expression so it was not further investigated as a QS inhibitor. On the other hand C10-CPA, showed modest activity as a QS inhibitor with minimal off target effects. C10-CPA reduced expression of the QS reporter (*lasB*) by 30-40% (figure 4.3) in rich media. Inhibition increased to 60-80% in QSM because the reduction in QS induction reduced density which feeds back into the QS network reducing expression further (Darch *et al.*, 2012). As QS inhibitors are in the early stage of development they often have low efficacy (Defoirdt *et al.*, 2013) as is evident here.

Figure 4.2 demonstrates that QS regulated genes are predominantly upregulated, this is likely to contribute to the cost of quorum sensing induction (Diggle *et al.*, 2007; Ghoul *et al.*, 2014b). Early growth using casamino acids (which are freely available independent of QS) is increased by a small but significant amount when QS activity is reduced (figure S4.1, table S4.1, effect of C10-CPA on early growth, $F_{1,26} = 64.3$, $p<0.001$, DrugCPA = -0.101, s.e = 0.043). So does the cost of quorum sensing affect sensitivity to protein synthesis antibiotics? The data show that both of the antibiotics that inhibit protein synthesis (azithromycin and tobramycin), have a weaker effect on the strain with an impaired QS system (*ΔlasIR*). The potency of Carbenicillin, a β-lactam used as a negative control, was not affected by the QS status of *P. aeruginosa*. Figure 4.6 shows that there is a slight but significant reduction in tobramycin efficacy with addition of C10-CPA at 100μM. Re-analysing the data in figure 4.6d (Chapter S4) using the three QS treatments as a continuous variable of QS functioning (with WT having a

value of 1 and *ΔlasI* being 0), suggests that C10-CPA is reducing QS functioning by 13.4% (SEM = 6.3%). This value is consistent with the amount of inhibition measured in figure 5.3 at 100μM C10-CPA.

Finally results suggest that addition of tobramycin increases the expression of the *lasB* reporter (figures 4.7), a result corroborated by direct measures of protease activity (figure 4.8). Although expression of *lasB* is reduced at high doses of tobramycin, the initial increase in expression is surprising given that tobramycin inhibits translation. One hypothesis for this is the inclusion of stress response genes in the QS regulon (García-Contreras *et al.*, 2014; Goo *et al.*, 2012). Addition of antibiotic at low doses may trigger greater induction of quorum sensing, and thus increased expression of QS induced genes (even if not involved in stress responses). In support of this antibiotics have been shown to induce oxidative stress (Kohanski *et al.*, 2007), which can feed into the *P. aeruginosa* QS systems through OxyR binding to *rsaL* and *pqsR* (Wei *et al.*, 2012) which may lead to greater induction of the QS system. The upregulation of the QS regulon does not detract from the stated hypothesis to explain antagonism, if the QS regulon is induced this will place a further burden on translation. However given that QS regulates stress response genes, upregulation of the QS regulon may be a beneficial response to translation inhibitors under certain conditions (García-Contreras *et al.*, 2014; Bjarnsholt *et al.*, 2005). This may contribute to the different results that are found with studies in alternate conditions (Bjarnsholt *et al.*, 2005; Christensen *et al.*, 2012).

In conclusion these experiments suggest that, under the conditions that we investigate, QS inhibition is antagonistic to the action of tobramycin (and potentially other antibiotics that target translation). These results support the hypothesis that increased protein synthesis that accompanies induction of

the QS system places an increased burden on translation so that the cell is unable to respond to translation inhibition by antibiotics. A similar conclusion (which our results are also consistent with) has been drawn to explain the increased efficacy of aminoglycosides on slow growing cells (Greulich *et al.*, 2015). This work suggests that slower growing cells have a slower ribosome production rate and so are less able to respond to irreversible ribosome binding antibiotics like tobramycin (Greulich *et al.*, 2015).

Although the antagonistic interaction of C10-CPA on tobramycin efficacy is slight, similar results are seen for genetic knockouts suggesting that antagonism is not specific to C10-CPA. Antagonism suggests that combinations of QS inhibitors and translation inhibitors will have lower efficacy but reduced selection for resistance (Hegreness *et al.*, 2008), but this hypothesis is simplistic for this instance. Comparing the results detailed here with published literature shows that QS inhibition acts both antagonistically and synergistically (Christensen *et al.*, 2012; Bjarnsholt *et al.*, 2005) with tobramycin. The growth phase (biofilm or planktonic) of *P. aeruginosa* appears to have a large effect how these therapies interact. Given that *P. aeruginosa* is exists in both states during infection (Hall-Stoodley and Stoodley, 2009) it is unclear how the change in drug interactions will shape selection. The result that QS deficient strains are less susceptible to commonly used antibiotics like tobramycin suggests that maybe this contributes to selection for QS defective strains (D'Argenio *et al.*, 2007). To test this hypothesis would require further investigation as clinical infections are complex and there are other hypotheses to account for the presence of *ΔlasR* mutants, such as social effects (Köhler *et al.*, 2010).

This work presents the interesting result that activation of the Las QS system in *P. aeruginosa* increases the efficacy of tobramycin. The results collected here are consistent with the hypothesis that the increased efficacy of translation inhibitors like tobramycin is because of the burden that the QS regulon places on transcription. Further work should thoroughly test the mechanism and specificity of antagonism using other antibiotic classes particularly other classes that inhibit translation. This work also highlights that the environment will play a part in determining drug interactions. Therefore it would be interesting to assess the drug interactions across, different abiotic environments, which may also help to identify the specific reasons for antagonism between QS inhibition and translation inhibitors. Finally studies of how drug interactions affect selection for resistance, should start to look at selection in the case where the drug interactions are not static, so bacteria experience both antagonism and synergy as the environment changes.

5 Modified antibiotic, adjuvant ratios can slow the evolution of resistance: co-amoxiclav as a case study

In preparation for submission as a research article with the same name. Contributing authors: Richard C. Allen, and Sam P. Brown.

RCA came up with the idea following discussions with SPB. RCA designed experiments, modelling approaches and analysis with guidance from SPB. RCA performed all experiments and analysis. RCA wrote the chapter with edits suggested by SPB.

5.1 Abstract

Combination therapy shows promise for extending the lifetime and efficacy of existing therapeutics. An understanding of how selection for resistance to drug combinations is influenced by drug interactions and the dosages of the component drugs will help to better optimise combination therapies. This chapter investigates selection to the synergistic drug combination of amoxicillin (β-lactam) and clavulanate (β-lactamase inhibitor) experienced by *Escherichia coli* expressing the CTX-M14 extended spectrum β-lactamase. A simple theoretical model suggests that the ratio of clavulanate to amoxicillin should influence the types of resistance mutations that are most favoured by selection, independent of inhibitory activity of the drug combination. This prediction is tested using experimental evolution demonstrating that high clavulanate ratios slow the evolution of resistance. By testing selected lines in alternate drug environments the ability of drug environments to select for ratio specific resistance phenotypes is investigated. Results show that there is only partial cross resistance across drug ratios. These results also indicate

that selected lines experience reduced synergy of amoxicillin and clavulanate.

5.2 Introduction

The QS regulon, which is inhibited by C10-CPA in chapter 4 includes disparate traits which produce general resistance phenotypes to a number of antibiotics (Bjarnsholt *et al.*, 2005; Jimenez *et al.*, 2012). On the other hand a number of adjuvant compounds are targeted at specific resistance mechanisms like efflux pumps and β-lactamase enzymes, disrupting metabolic functions to restore the action of traditional antibiotics (Gill *et al.*, 2014). Examples, such as β-lactamase inhibitors are well developed and have been in clinical use for several decades (White *et al.*, 2004).

In the context of a pathogen that has an adjuvant targeted resistance mechanism, the combination of antibiotic and adjuvant are synergistically acting therapeutics. Synergy is when a combination of compounds work better when used together than the sum of the efficacy of the two compounds when used separately (i.e. greater than the additive effect). This is commonly formalised using the reference model of Loewe additivity (Ankomah *et al.*, 2013; Loewe and Muischnek, 1926): the killing effect of multiple drugs is proportional to the sum of the of the effects of the two drugs separately when the interaction is additive. In the context of bacterial killing this is often summarised as the combination index (CI) through isobologram analysis (Fraser, 1872; Zhao *et al.*, 2010):

$$CI = \frac{C_{A,x}}{IC_{x,A}} + \frac{C_{B,x}}{IC_{x,B}} \qquad (Eqn. 5.1)$$

Where x is the amount of inhibition, $IC_{x,A}$ is the inhibitory concentration of drug A on its own and ($C_{A,x}$, $C_{B,x}$) is a pair of drug concentrations where the two drugs together inhibit bacterial growth by x. For additivity, synergy and

antagonism the CI is one, less than one or greater than one respectively (Fraser, 1872; Zhao *et al.*, 2010). This can be easily visualised on a plot of a checkerboard assay, to see the effects of varying combinations of 2 drugs used together (see later).

Synergistic drug treatments are sought in clinical use of antibiotics because lower doses of antibiotics can be used, while still controlling the bacterial infection (Hegreness *et al.*, 2008). However elegant work has shown that selection for resistance to combinations of two drugs is dependent on the interactions between the two drugs, with synergistic interactions increasing selection for resistance (Chait *et al.*, 2007; Hegreness *et al.*, 2008; Yeh *et al.*, 2009).

Treatment dose plays an important role in the selection for antibiotic resistance. Clinical studies of antibiotic resistance often discuss the optimal treatment dose using the minimal inhibitory concentration (MiC); the minimal concentration of an antibiotic that inhibits overnight growth of bacteria (Mouton *et al.*, 2012). Although this measure is useful for measuring efficacy in a treatment context, it is of little use in an evolutionary context because treatment below the MiC will still impose some inhibition on growth and select for resistance (Gullberg *et al.*, 2011). An alternate measure is the minimum selective concentration (MSC) the lowest concentration at which treatment inhibits growth of the bacterium by a significant amount. The upper limit of selection for resistance is usually identified as the mutation prevention concentration (MPC), the drug concentration at which all accessible mutations are unable to confer resistance to therapeutic treatment (Andersson and Hughes, 2014). Resistance can only be selected for in the window between the MSC and the MPC, the mutation selection window (Andersson and Hughes, 2014; Michel *et al.*, 2008).

Work has shown that for interaction between two drugs the size of the mutation selection window can vary with the ratio of drugs (Michel *et al.*, 2008). Furthermore there is evidence that the evolution of resistance proceeds at a faster rate when the synergistic drugs are used at equal concentrations relative to the MiC, (Pena-Miller *et al.*, 2013). For these types of experiments it is important to control for the strength of antibiotic inhibition as this has a large effect on selection, with greater inhibition imposing stronger selection (Pena-Miller *et al.*, 2013; Oz *et al.*, 2014).

This chapter investigates drug synergy using the antibiotic amoxicillin and the adjuvant clavulanate (clavulanic acid). Amoxicillin is a bacteriocidal β-lactam antibiotic that inhibits synthesis of the bacterial cell wall (Babic *et al.*, 2006). Particularly for gram negative organisms a common mechanism of resistance to amoxicillin (and other β-lactams) is the production of β-lactamase enzymes (Babic *et al.*, 2006; King *et al.*, 2014). Clavulanic acid (clavulanate) is a β-lactamase inhibitor (Drawz and Bonomo, 2010) with competitive and suicidal action (it competes with the normal substrate for binding and is inactivated in the process of inhibition). Therefore clavulanate prevents bacterial produced β-lactamases from cleaving amoxicillin, thus making amoxicillin effective again. The combination of amoxicillin and Clavulanic acid (marketed as augmentin and co-amoxiclav) has been used clinically since 1981 (White *et al.*, 2004) and is listed on the WHO list of essential medicines (WHO Model Lists of Essential Medicines, 19th edition).

As for the majority of antibiotics, resistance mechanisms to co-amoxiclav exist. Since its clinical introduction the dosage of amoxicillin has increased from 3.3μg/ml to 17 μg/ml, to counter the increasing resistance of bacteria to amoxicillin (White *et al.*, 2004). On the other hand the dosage of clavulanic acid has not changed staying at 2.6 μg/ml (White *et al.*, 2004), so the ratio of

clavulanate to amoxicillin has decreased. Once bacteria overcome the effects of clavulanic acid, alternative treatments are used with either new antibiotics that are not cleaved by β-lactamase enzymes or new inhibitors. Bacteria overcome the β-lactamse inhibition of clavulanate by mutations in β-lactamases or acquisition of new β-lactamses through horizontal gene transfer (Ripoll *et al.*, 2011; Cantón *et al.*, 2008). Neither Clavulanic acid or amoxicillin have significant toxicity even at the highest does of these drugs used (Neu *et al.*, 1993). However clavulanic acid is able to act as a weak β-lactam as the majority of β-lactamase inhibitors mimic the shape of β-lactam antibiotics (Drawz and Bonomo, 2010).

The β-lactamase enzymes of gram positive enzymes are generally secreted into the environment, while the enzymes of gram negative species are periplasmic (remaining between the inner and outer membrane, Livermore, 1995). β-lactamases have been shown to confer a protective effect on susceptible species (Dugatkin *et al.*, 2005; Medaney *et al.*, 2015; Perlin *et al.*, 2009; Yurtsev *et al.*, 2013), acting as a public good. The extent to which resistance is shared is dependent on the localisation of the enzyme (Dugatkin *et al.*, 2005; Perlin *et al.*, 2009), but even when the enzyme is localised to the producing cell it can confer a benefit to others due to detoxification of the environment (Medaney *et al.*, 2015; Perlin *et al.*, 2009; Yurtsev *et al.*, 2013). Recently it has been shown that the ability of non-secreted enzymes to confer a protective effect is dependent on very stringent environmental conditions (Medaney *et al.*, 2015). In particular susceptible cells need to be persisters (Lewis, 2010) surviving in a less metabolically active phenotype during the period when the β-lactam antibiotics make the environment toxic (Medaney *et al.*, 2015; Perlin *et al.*, 2009).

The *E. coli* strain used here encodes CTX-M-14 on the large naturally occurring plasmid (pCT, Cottell *et al.*, 2011). This is an extended spectrum β-lactamase (ESBL) which indicates that it is active against a wider range of β-lactam antibiotics (notably cephalosporins, King *et al.*, 2014). Whether a β-lactamase enzyme is an ESBL does not affect the ability of β-lactamase inhibitors to inhibit its action. ESBLs are a pressing issue severely limiting the β-lactam antibiotics that can be used. This forces the use of carbapenems and therefore leads to more rapid development of carbapenem resistance (King *et al.*, 2014). The spread of ESBLs on plasmids is an important factor contributing to the spread of resistance (Cantón *et al.*, 2008).

This chapter tests the hypothesis that the fitness effects of mutations conferring resistance to combinations of drugs will depend on the ratio of the two drugs. In this example does the fitness effect of resistance mechanisms to amoxicillin vary with the ratio of amoxicillin in drug treatments? Second this chapter investigates the related hypothesis, that the rate of resistance evolution is dependent on drug ratio. Importantly these hypotheses are tested in the case when the overall inhibitory effect of the combination of drugs is independent of the drug ratio that strains are treated with (i.e. all ratios affect growth to approximately the same extent).

5.3 Methods

5.3.1 Strains and media

Escherichia coli strain MG1655 containing a naturally occurring pCT plasmid (Cottell *et al.*, 2011) and defective for horizontal transfer due to a knockout of the *trbA* gene (Medaney *et al.*, 2015) was used as the ancestor of all selection lines and is henceforth referred to as the ancestor. The strain was produced in the lab of Dr Ben Raymond (Medaney *et al.*, 2015) and kindly provided. The pCT plasmid is a large naturally occurring plasmid containing the CTX-

M-14 extended spectrum β-lactamse. This lactamase cleaves amoxicillin and is inhibited by clavulanate.

Media was specifically designed for these experiments to allow growth of MG1655 to a high density but not reaching carrying capacity until close to 22 hours. The media recipe was as follows: M9 media base (containing 6.78 mg/ml Na_2HPO_4, 3 mg/ml KH_2PO_4, 0.5 mg/ml NaCl and 1 mg/ml NH_4Cl) supplemented with 1mM MgSO4, 0.1mM CaCl2, 0.4% (v/v) glycerol, 0.02% casamino acids, 0.5μg/ml thiamine and Hutners trace elements (Hutner *et al.*, 1950) at 1X concentration. Throughout this text this will be referred to as *E. coli* minimal medium (EMM).

The pCT plasmid is stable, however prior to incubation for experimental evolution and growth dynamics assays the ancestor was grown in the presence of 100μg/ml ampicillin to maintain the pCT plasmid. For phenotyping of experimentally evolved strains pre-culture was without antibiotics to reduce any non genetic effects of exposure to antibiotic ratios.

5.3.2 Testing sensitivity of ancestor to clavulanate and amoxicillin

To test antibiotic sensitivity of the ancestral strain (MG1655 *ΔtrbA* with pCT plasmid) the ancestor was growth for 22 hours in Luria-Bertani (LB) broth in the presence of increasing clavulanate and amoxicillin, at all possible combinations of the two drug concentrations (checkerboard assay). This was done firstly to assess whether there was synergy between the two compounds and secondly, to identify drug ratio's and drug concentrations that inhibited growth to a similar extent (maximum concentrations in figure 5.1). Once these concentrations were identified the ancestral strain was grown in *E. coli* minimal medium at these drug concentrations (and fractions

of the maximum value) to confirm that growth was not significantly affected by drug ratio.

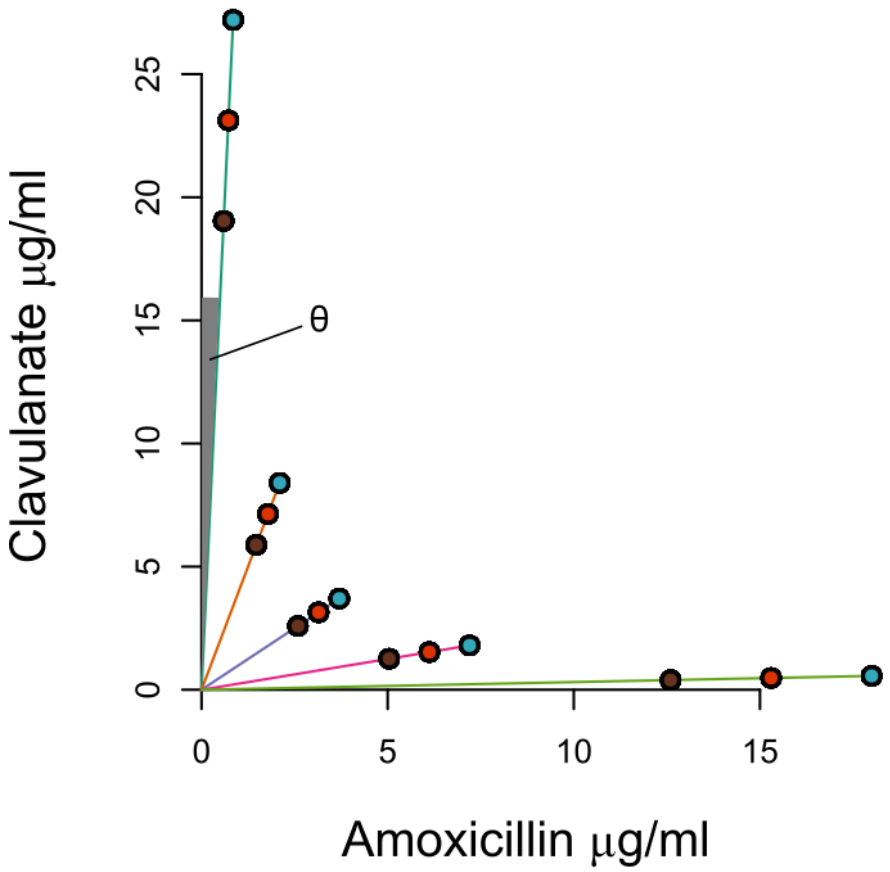

Figure 5.1: Allocating drug environments with equal inhibitory activity. Each point represents one of the drug environments used for selection. To allocate drug concentrations polar coordinates were used, defined by the angle and length of a vector from the origin to the drug concentration. Each drug combination was designated by the angle of the vector (θ) and length of the vector relative to the length of the vector for the maximum dose (V). Blue, red and brown points are dose strength (V) with values of 1, 0.85 and 0.7 respectively. All points on the same line (vector) have the same drug ratio (θ).

5.3.3 Drug ratios

Clavulanic acid (in the form of potassium clavulanate, Fluca analytical) and amoxicillin (LKT laboratories) were supplied in powdered forms, stored at

4°C and used to make stocks in deionised water. These stocks were stored at 4°C according to manufacturer's instructions, liquid stocks were not kept for longer than 14 days to minimise degradation of the compounds.

Due to the synergy between clavulanate and amoxicillin treatment regimes for selection experiments were allocated using polar coordinates as has been used previously (figure 5.1 Michel *et al.*, 2008; Pena-Miller *et al.*, 2013). Selection regimes were chosen so that the 5 different ratios of amoxicillin and clavulanic acid would have similar effects on growth . In addition to the full strength regime medium and low strength regimens were used corresponding to 85% and 70% of the maximum strength.

5.3.4 Experimental evolution

To test its ability to adapt to different drug doses *E coli* was evolved against varying drug regimens defined by drug ratio (θ) and dose strength (V). MG1655 *ΔtrbA* containing the pCT plasmid (ancestor) was grown overnight in LB (100mg/ml amoxicillin) and then diluted 1/100 into the same media and grown to mid exponential phase. The mid-exponential phase culture was washed twice in sterile EMM and diluted to an OD (600nm) of 0.0125 in sterile EMM. This culture was aliquotted into wells in 160µl volumes and made up to 200µl using drug ratios. Final volumes were 200µl and starting densities were OD = 0.01. Experimental evolution lines were set up corresponding to 5 drug ratios at 3 different strengths, plus one line which was not exposed to drugs. These 16 treatments were replicated 3 times (giving 48 lines total) and were positioned in the central wells of a 96 well plate. Plates were incubated statically at 37°C for 22 hours for each passage.

To passage experimental evolution lines, wells were mixed using a pipette to re-suspend any clumps of bacteria. The optical density of the wells was then measured and used to transfer cells to a fresh plate so that each line started at

an OD (600nm) of 0.01. Another selection experiment was performed where the dilution per passage was fixed (at 1/20), however preliminary results were the same (data not shown). All results reported here are from selection experiments with a fixed initial density. Experimental evolution was performed for 12 passages (corresponding to approximately 80 generations). Lines were frozen every 3 passages by adding 100μl of a 1:1 LB: glycerol mixture to the remaining culture after the transfer had been performed, these were then frozen at -80^{o}C.

5.3.5 Measuring cross resistance between drug environments

Although final density was measured at the end of each passage, measuring growth from stored samples is better for comparing genetic changes in resistance, because it eliminates long term effects from exposure to antibiotics. This gives a better estimate of whether there is variation in the potential for adaptation to different drug environments. In addition to measuring the growth in the presence of the drug regimen the evolved line was exposed to, evolved lines could be tested for their ability to grow in other environments. By doing this the hypothesis that certain adatations are more fit in certain drug regimens can be tested.

To assay evolutionary change in response to drug combinations, for each line the population after 6 passages was revived, chosen because this is when there was most diversity in how lines had adapted to their environment. Each line of selection was assayed for growth in its selection drug environment and drug environments (θ_A, V_A) that differed from the drug environment they were selected in (θ_S, V_S). The differing drug environments that selection lines were assayed against either kept drug ratio the same ($\theta_A = \theta_S$) and varied dose strength (V_A) or kept dose strength the same ($V_A = V_S$)

and varied drug ratio (θ_S). For varying dose strength an increased dose of 1.15 times the maximum dose was also used (V_A=1.15). Otherwise all conditions were the same, strains were grown in EMM for 22 hours statically at 37°C and mixed prior to measuring optical density. This was a large experiment, 15 selection treatments, with 3 replicates tested against 9 conditions each plus controls so treatments were semi randomly blocked across the central wells of nine 96 well plates. Each plate had three blank wells and one well containing each of the 3 control lines selected in the absence of drugs and assayed in the absence of drugs. There was small but significant variation in the growth of control lines across plates so OD values were for each plate were corrected using the growth of controls. θ_S is undefined for the control lines (selected without drugs) so these were tested against every different drug environment.

5.3.6 Statistics

All statistics were performed in R (R Core Team, 2013). Full models were produced using relevant main effects and interactions (detailed in individual tables). Fixed effects models were fitted using the glm function using a Gaussian error distribution and identity link function. For data sets where multiple measures were taken from each strain, a mixed effects model was used to take into account the effect of strain as a random effect, this was fitted using the lme function in the nlme package (Pinheiro *et al.*, 2015). For resistance profiling the large data set containing multiple factors was split into two subsets for ease of model interpretation. One data set includes all data where strains are tested for resistance to the same dose strength they are selected against (at varying drug ratios), while the other includes all data where strains are tested for resistance to the same ratio they were selected against (at different dose strengths). Both these data sets include the 45 data

points (one per evolved line) where both ratio and strength of assay are the same as those for selection.

The maximal model was reduced to a minimal model in a stepwise manner. At each step of model reduction all terms that were not currently included in an interaction were tested for significance as grounds for including them in a model. Terms (or effects) were dropped if the result of an F test (for fixed effects models) or likelihood ratio test (for mixed effects models) comparison of models with and without the term of interest was not significant at α=0.05 (i.e. accepting the null hypothesis of no significant effect of the term). At each step only one term could be dropped so where several effects were non-significant the new model with the lowest AiC (Akaike information criterion) was chosen as the best model reduction at that step. When no terms (not included in higher order interactions) could be dropped without a significant effect this was considered the minimal model. Statistical support for all terms in the minimal models was assessed as above but through comparison of the minimal model and the minimal model with the term dropped. The estimates of the coefficients for each term and its associated standard error are also reported in addition to other aspects of the model. Full statistical results are reported in tables near the first instance of model use.

5.4 Model analysis

5.4.1 Modelling β-lactam and β-lactamase inhibitor action

To increase understanding of selection for different types of resistance in varied drug environments a theoretical approach was used. A mathematical model was produced for the interaction between a β-lactamase producing strain (*E. coli*), a β-lactam antibiotic (amoxicillin) and a β-lactamase inhibitor (clavulanic acid). As a start point a simple differential equation for logistic growth was used (Brown *et al.*, 2009), modified by a cost (c_z) incurred by

lactamase production (figure 5.2a). Where B is bacterial density and z is per cell lactamase production.

$$\frac{dB}{dt} = (1-B)B - c_z\, z\, B \quad Eqn.\,5.2$$

The action of an antibiotic (A, amoxicillin) that inhibits bacterial growth in a density independent manner with rate k_1 is added. The antibiotic amoxicillin is cleaved by β-lactamase, so in the model the action of A is inhibited proportionally to z. For simplicity antibiotic is fixed at a constant concentration which represents the drug dose.

$$\frac{dB}{dt} = (1-B)B - c_z\, z\, B - k_1 A\, B(1-z) \quad Eqn.\,5.3$$

Lastly the action of an inhibitor (C, clavulanic acid) is added to the system, again for simplicity this is a fixed parameter. Clavulanic acid inhibits β-lactamase, which reduces the inhibitory effect of lactamase on amoxicillin.

$$\frac{dB}{dt} = (1-B)B - c_z\, z\, B - k_1 A\, B\big(1 - z(1-C)\big)$$
$$\frac{dB}{dt} = Logistic\ growth - cost\ of\ lactamase - modulated\ killing \quad Eqn.\,5.4$$

In this model logistic growth is density dependent, so that growth is bounded. On the other hand the costs of lactamase production and the killing effect of amoxicillin are density independent (the per individual costs are not dependent on B).

The differential equation (Eqn.5. 4) has two equilibria one trivial equilibrium at B=0 and a non-zero equilibrium at:

$$B^* = 1 - c_z z - Ak_1\big(1 - z(1-C)\big) \quad Eqn.\,5.5$$

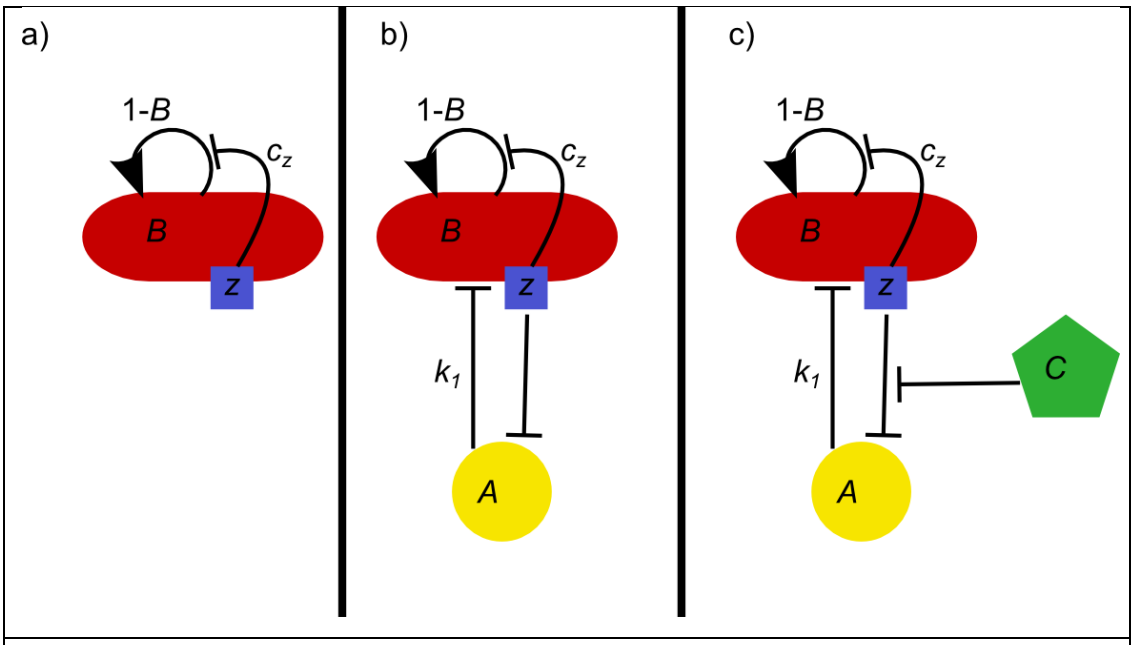

Figure 5.2: Schematic diagrams for models of β-lactamase producing *E. coli* in the presence of antibiotic and β-lactamase inhibitors. Coloured shapes show the key components of the model. Bacterial density ($B[t]$) is modelled with a differential equation of logistic growth. Growth is reduced by the action of antibiotic (A), which is in turn reduced by the action of bacterially produced β-lactamase (z), which incurs a cost to growth (c_z). The ability of β-lactamase to prevent antibiotic mediated killing is reduced by the β-lactamase inhibitor (C). Parameters associated with processes are shown by the interaction lines. a) A model of bacterial growth without any drug treatment. b) Bacterial growth in the presence of a β-lactam antibiotic that is cleaved by the β-lactamase. c) Addition of a β-lactamase inhibitor which inhibits the action of the β-lactamase.

The stability of the two equilibria for this system can be assessed by determining conditions when the derivative of the differential equation is less than zero (Otto and Day, 2007). The two stability conditions are mutually exclusive so that B^* is stable (and B=0 is unstable) when:

$$1 - c_z z - Ak_1\big(1 - z(1 - C)\big) = B^* > 0 \quad Eqn.\,5.6$$

Therefore whenever the non-zero equilibrium (B^*) is accessible it is stable.

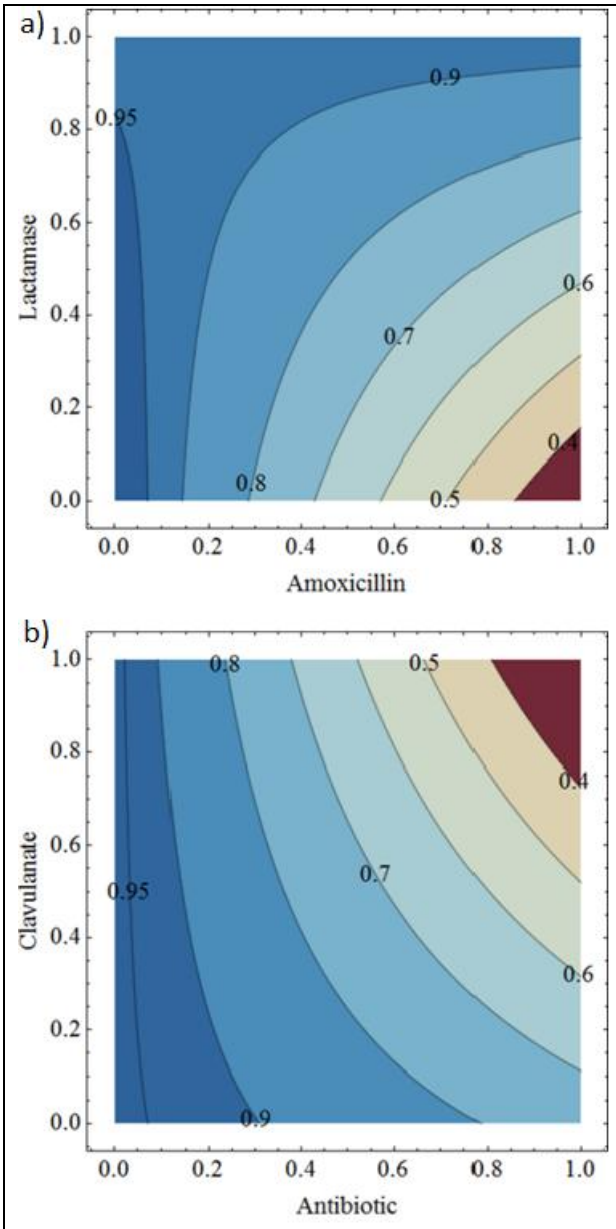

Figure 5.3: Equilibrium values of bacterial density. Contours show the value of the non-zero equilibrium (equation 6.5) of bacterial density. a) Density of a bacterium producing β-lactamase at a varying level, in the presence of varying concentrations of amoxicillin, and absence of clavulanate. b) Density of a β-lactamase producing bacterium in the presence of varying concentrations of amoxicillin and clavulanate. Parameters are: c_z = 0.05, k_1 = 0.7, z = 0.7 unless otherwise stated.

The numerical value of B^* can be plotted after setting parameters to numerical values as shown in figure 5.3. When inhibitor concentration is zero, and lactamase production and amoxicillin concentration are varied the system behaves as in figure 5.3a. When antibiotic concentration is low lactamase production reduces the final yield because lactamase is costly without any benefit in terms of protection. As antibiotic concentration increases lactamase production shows a strong benefit. In the presence of

clavulanate (figure 5.3b) the two compounds show strong synergy as is seen in our experimental results (figure 5.4).

5.4.2 Evolutionary analysis

Adaptation to the drug environment is the primary interest of this study, so per capita growth rate was used as a proxy for fitness and analysis was performed to see how the fitness of different resistance mechanisms varies with drug environment. Two drug resistance phenotypes were considered, varying lactamase production (z) and direct resistance to killing by amoxicillin (d). Lactamase production already incurs a cost (c_z) and a similar cost (c_d)was implemented for direct resistance (d is set to zero in earlier analysis). The modified form of equation 5.4 (with direct resistance parameter included) can be divided though by B to obtain the per capita growth rate $f(d,z)$, a function of direct resistance and lactamase production.

$$f(d,z) = (1-B) - c_z\, z\, - c_d d\, - k_1 A\,(1-d)\big(1 - z(1-C)\big) \quad Eqn.\,5.7$$

To assess the whether a rare mutant with altered lactamase production or direct resistance can spread, equation 5.7 was partially differentiated with respect to either d or z. This derivative then shows the change in individual growth rate associated with a small change in either d or z, indicating whether these phenotypic changes are beneficial.

$$\frac{\partial f(d,z)}{\partial z} = Ak_1(1-C)(1-d) - c_z \; Eqn.\,5.8a$$
$$\frac{\partial f(d,z)}{\partial d} = Ak_1\big(1 - z(1-C)\big) - c_d \; Eqn.\;5.8b$$

With the linear relationship between costs and benefits the second derivative of these both these functions is zero showing that the selection for these resistance phenotypes depends on the environment but not the native resistance phenotype. Both resistance phenotypes (increased d or z) are

favoured unless costs of resistance are high, as antibiotic killing increases and both types will be more beneficial. However increased lactamase production is more favoured when inhibitor is low, while increased intrinsic resistance is more favoured when the effect of β-lactamase is low, and therefore when inhibitor concentration is high (if lactamase is present i.e. $z>0$). This analysis supports the hypothesis that different drug ratios present different strengths of selection for different resistance mutations.

5.5 Results

5.5.1 Growth of ancestor with drug treatment

Clavulanate and amoxicillin act synergistically on the ancestral strain (figure 5.4). Final optical density of the ancestor is negatively affected by both amoxicillin ($F_{1,44} = 4.77$, $p = 0.035$, $\beta = -0.029$, s.e = 0.013) and clavulanate ($F_{1,44} = 25.16$ $p < 0.001$, $\beta = -0.118$, s.e = 0.023). In addition there is a negative interaction between the two compounds, their inhibitory effect is greater than additive ($F_{1,44} = 8.31$, $p = 0.006$, Clavulanate: Amoxicillin = -0.017, s.e = 0.006). This can be clearly seen in figure 5.4b, the contours of equal inhibition are significantly different than linear, they are concave so lower doses of the drugs are needed when both are used together. Values can be taken from the OD=0.6 contour to give a CI of approximately 0.5. As neither drug has potent activity on its own IC_x values are likely an underestimate so the real CI is probably lower.

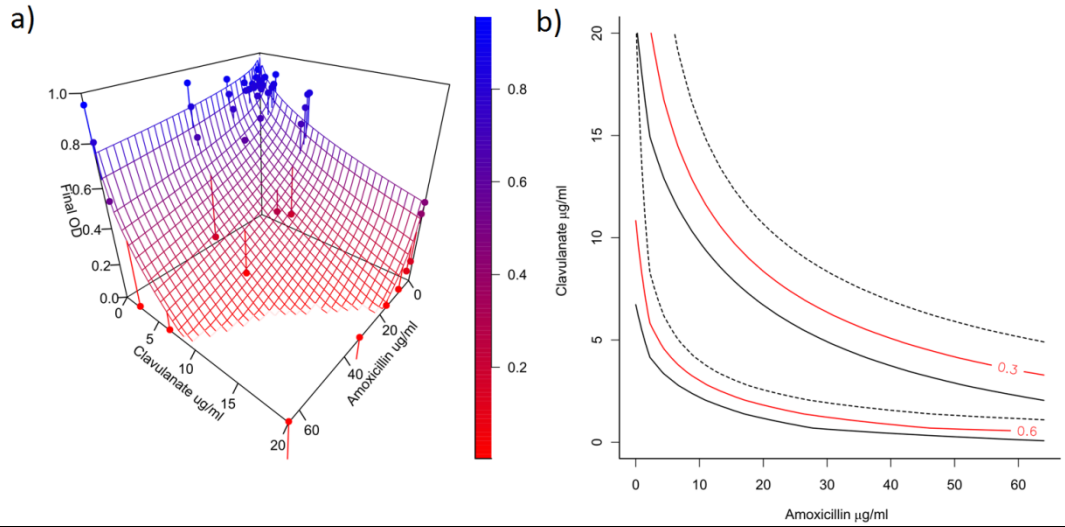

Figure 5.4: Synergy between amoxicillin and clavulanate. a) Final density of ancestral *E. coli* grown for 22 hours in LB broth, points are unreplicated data points, surface is a prediction from a linear model. b) Contours of equal bacterial density taken from predicted values of the fitted model, black lines are upper and lower (dashed) confidence intervals.

Effect	F Stat DF	F Statistic	P value
Clavulanate$^{0.5}$	1,44	25.16	<0.001
Amoxicillin$^{0.5}$	1,44	4.77	0.035
Clavulanate$^{0.5}$:Amoxicillin$^{0.5}$	1,44	8.31	0.006
Effect	Estimate	s.e	
Intercept	0.987	0.052	
Clavulanate$^{0.5}$	-0.118	0.023	
Amoxicillin$^{0.5}$	-0.029	0.013	
Clavulanate$^{0.5}$:Amoxicillin$^{0.5}$	-0.017	0.006	
n=48, residual deviance = 1.0322 on 44 DF			

Table 5.1: Statistical table of interaction between amoxicillin and clavulanate. Response variable is optical density after 22 hours growth. The square root of concentration of the two drugs was used to make the drug concentrations more linear and a statistical interaction was used to test for the interaction between the two drugs.

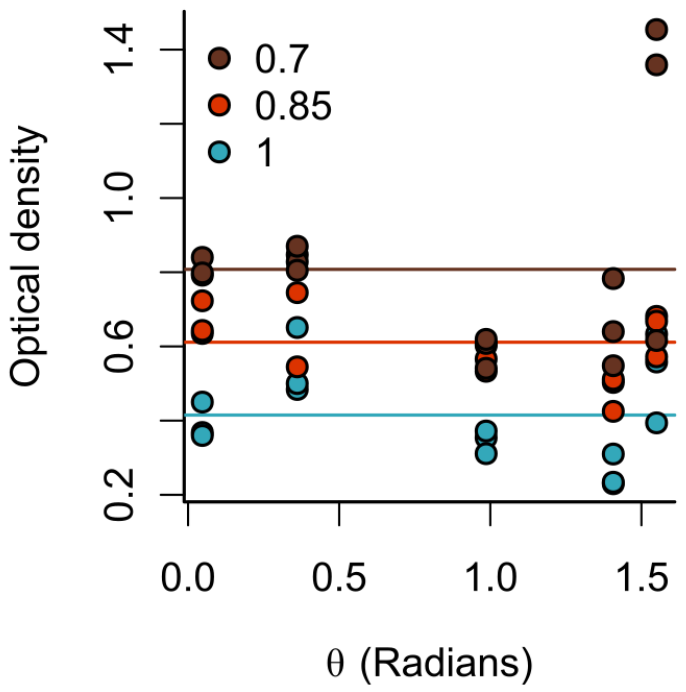

Figure 5.5: Drug ratio (θ) has no significant effect on optical density of the ancestor after 22 hours. The ancestor was grown in drug environments defined in figure 6.4, with colours representing the different does strengths (V). Lines are predictions from a linear model where dose strength (over the doses shown here) has a significant effect on final growth, but drug ratio doesn't have a statistically supported effect on final growth.

Effect	F stat DF	F statistic	P value
θ	1,42	0.213	0.647
V	1,43	35.29	<0.001
θ:V	1,41	0.753	0.391
Effect	Estimate	s.e	
Intercept	1.724	0.189	
V	-1.309	0.220	
n=45, residual deviance on 43 DF= 1.4100			

Table 5.2 Statistics for the fixed effects model of the 5 drug ratios and 3 dose strengths chosen for the experimental evolution experiment. Response variable is optical density after 23 hours growth. The interaction between strength and θ was tested to see if some drug ratios were more inhibitory at high or low doses

Using this synergistic plot the maximum drug concentrations for each of 5 drug ratios used in the selection experiments were defined. It is difficult to visualise the relation between these drug concentrations chosen using Cartesian coordinates however using polar coordinates a simple relationship can be seen (figure 5.1). Each drug environment can be defined by the drug ratio, stated as θ the angle in figure 5.1, and the dose strength V which is the length of the vector from the origin to the drug dose in figure 5.1, relative to the length of the same vector for the maximum dose strength. Each of these drug ratios (at maximal, 0.85 and 0.7 strength) was tested for its ability to inhibit the ancestral strain. The chosen drug concentrations act so that after 22 hours growth there is no difference in final density (figure 5.5) across all of the drug ratios (effect of θ, $F_{1,42} = 0.213$, $p = 0.647$), but there is a significant negative linear effect of drug ratio (effect of V, $F_{1,43} = 35.289$, $p < 0.001$) over the 3 dose strengths shown. This is important as it means that strength of inhibition (of the ancestral strain) is statistically independent of drug ratio.

Figure 5.6 shows growth curves of the ancestor compared to control lines for low amoxicillin : high clavulanate (figure 5.6a, $\theta = 0.05$) to high amoxicillin : low clavulanate (figure 5.6e, $\theta = 1.55$). However as is visible there is some variation in the shape of the growth curves between the panels. During growth in the *E. coli* minimal medium the ancestor formed clumps or weak biofilms in many of the drug treatments, which may account for the variation

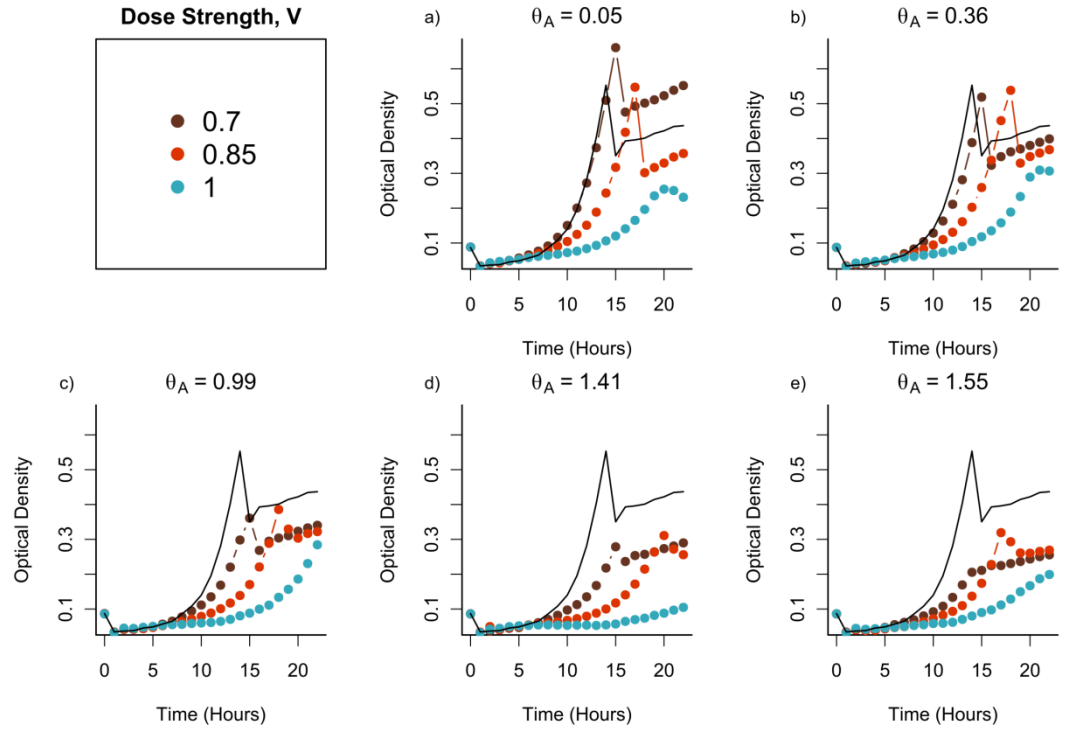

Figure 5.6: Growth of ancestral E. coli in the presence of drug regimes. Growth over 22 hours in a defined E. coli minimal medium. Panels a-e) show the drug dose regime from high clavulanate : low amoxicillin to low clavulanate : high amoxicillin. Colours indicate dosage strength as a fraction of the maximum dose as shown in figure 5.1. Black lines are the control, with no drug treatment. All points are the mean of 3 replicates.

seen. When measuring optical density for end points of growth cultures were mixed before optical density was measured.

5.5.2 Adaptation of E. coli to dosage regimes

After each of the growth cycles, cultures were mixed and optical density was measured so that a time course of adaptation can be produced (figure 5.7). Black lines are the minimal mixed linear model of adaptation in the control lines (separate from the experimental line model), in which the effect of passage (χ^2_1 = 3.50, p > 0.062) had no effect. Brown, orange and blue lines show the predicted values of minimal linear mixed models for experimental lines. Values of each co-efficient in the minimal model as well as statistics for

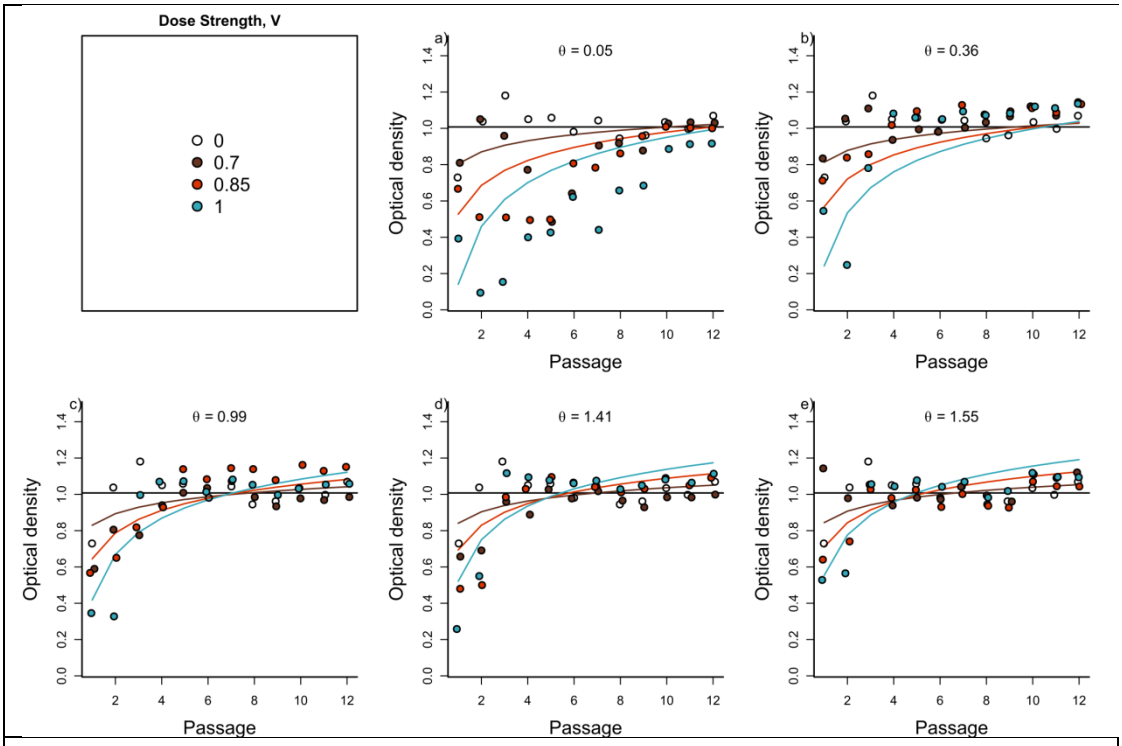

Figure 5.7: Adaptation of E. coli to the synergistic drug regime primarily depends the strength of selection imposed. Optical density of experimental evolution passages after a 22 hour growth cycle and mixing to re-suspend biofilms. Panels a-e) show the drug selection regime from high clavulanic acid : low amoxicillin (θ = 0.047 radians) to low clavulanic acid : high amoxicillin (θ = 1.550 radians). Colours indicate drug selection strength as a fraction of the maximum dose as shown in figure 5.1. Hollow points are control lines, with no drug treatment, and are the same for each pane. All points are the mean of 3 replicates. Brown, orange and blue curves are the predicted values of the fixed effects from a minimal linear mixed effects model (see methods). Black lines are the are the fitted curve of adaptation in control lines (slope =0).

Effect	χ^2_1	p-value	Model Reduction step
log(Passage)	28.57	<0.001	NA
V	29.27	<0.001	NA
θ	2.55	0.110	NA
log(Passage):θ	0.20	0.659	1
log(Passage):V	69.41	<0.001	NA
V: θ	4.42	0.036	NA
Effect	Coefficient	s.e	
Intercept	4.805	0.517	

log(Passage)	-0.731	0.136	
V	-3.672	0.602	
θ	-0.716	0.444	
log(Passage):V	1.355	0.158	
V: θ	1.108	0.517	
n=540, number of groups =45			
Variance in random term =0.052, residual variance =0.104			
Degrees of freedom = 493, 41			

Table 5.3 Mixed effects model of adaptation in lines selected against varying drug ratios (θ) and dose strengths (V). Response variable is the exponential of optical density after 23 hours at the end of each growth cycle (before lines were passaged. Selection line is included as a mixed effect, affecting the intercept of the model. Interactions between passage and drug treatment variables are tested to assess whether drug treatment affects adaptation. Interaction between dose strength and drug ratio is tested to assess whether certain drug ratios are more effective at different doses.

Effect	χ^2_1	p-value	Model reduction step
Passage	3.50	0.062	1
Effect	Estimate	s.e	
Intercept	2.754	0.047	
n=36, number of groups =3			
Variance in random term = 6.982e12, residual variance =0.079			
Degrees of freedom = 33			

Table 5.4: Mixed effects model of adaptation in control lines (not treated with any drug. Response variable was the exponential of optical density after 23 hours at the end of each growth cycle (before lines were passaged. Selection line is included as a mixed effect, affecting the intercept of the model.

each term are shown in table 5.3. Over time lines adapt and become better at growing in the drug environment. Although the base regression coefficient for log(Passage) is negative ($\chi^2_1 = 28.57$, $p<0.001$, $\beta = -0.731$, s.e = 0.136) there is a significant interaction with growth so that lines adapt to the drug environment, and high strength treatments adapt faster ($\chi^2_1 = 69.41$, $p<0.001$, log(Passage):V = 1.355, s.e = 0.158). As shown previously (figure 5.5) the ancestor is inhibited more by high strength drug environments so dose

strength has a negative effect on growth of strains at the start of evolution (χ^2_1 = 29.27, p < 0.001, V main effect = -3.672, s.e = 0.602). The effect of strength also interacts with negative main effect of drug ratio (χ^2_1 = 2.55, p=0.110, θ main effect = -0.716, s.e = 0.444) so that high clavulanate drug ratios are more greatly affected by drug dose (χ^2_1 = 4.442, p = 0.036, V:θ = 1.108, s.e = 0.517). Control lines (without drug treatment) did not show significant adaptation to experimental evolution conditions (effect of passage: χ^2_1 = 3.50, p=0.062).

The interaction between V and θ may result from the effects of drugs on growth of the ancestral strains (early in experimental evolution) or the growth of evolved strains, late in experimental evolution. An interaction of V:θ:log(passage) was not considered due to the difficulty of fitting and interpreting three way interactions. To more clearly interpret the growth of evolved strains adapted to different drug environments (and remove effects like accumulated stress from antibiotic treatment) the strains at the end of passage 6 were revived from frozen stocks (in antibiotic free media) and tested for growth in the drug environment they were selected in (figure 5.8). Here we can see that the evolved strains adapt to their environment better in low clavulanate (high θ) environments (effect of θ, χ^2_1 =4.16, p = 0.041, β = 0.192, s.e = 0.095; effect of θ^2, χ^2_1 = 74.30, p<0.001, β = -0.297, s.e = 0.032). This data also supports the results of the adaptation time course in that the difference in adaptation with θ is greater at high drug doses (θ:V interaction, χ^2_1 = 20.95, p <0.001, estimate = 0.425, s.e = 0.092). The same data is plotted in figure 5.8b to highlight that the interaction between θ and dose strength so that in populations evolved to low θ, higher dose strengths adapted more poorly, while at high θ increasing V increases adaptation.

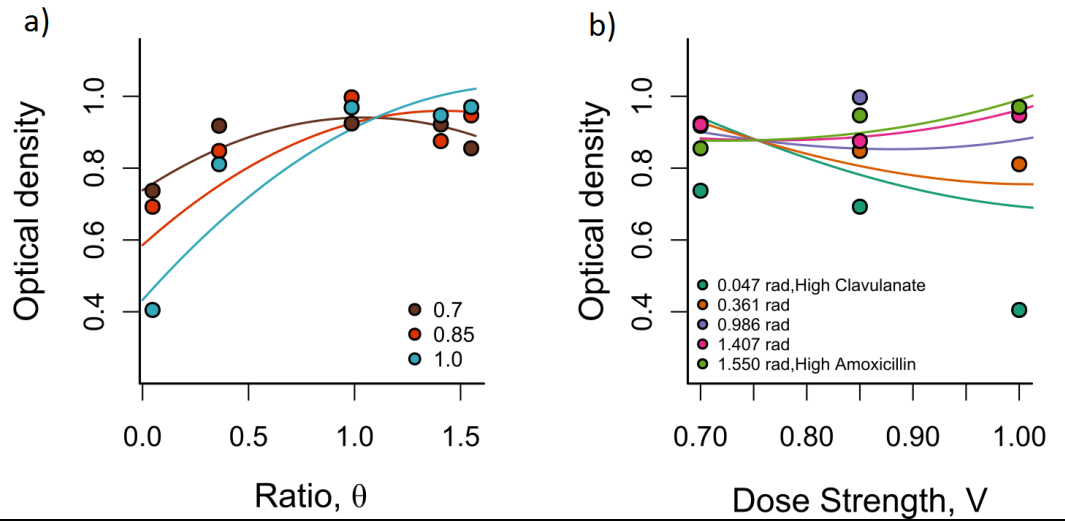

Figure 5.8: Adaptation to the selection environment after 6 passage of experimental evolution. Points only show data from assays where evolved lines were tested for growth at the same drug concentrations that they were evolved against. a) The relationship between final density and drug ratio (θ). b) The relationship between final density and dose strength. The data in the 2 panels is the same but displayed to highlight the 2 axes of treatment variation. Points are the mean values for 3 replicate lines. Lines are predictions from the fixed effects of minimal mixed effects linear models of a) varying drug ratio (table 5.5) and dose strength (table 5.6), where $\theta_A = \theta_S$ and $V_A = V_S$

Effect	χ^2_1	p-value	Model reduction step
θ_A	4.16	0.041	NA
$\theta_A{}^2$	74.30	<0.001	NA
θ_S	6.04	0.014	NA
V_S	16.68	<0.001	NA
θ_A: θ_S	31.80	<0.001	NA
$\theta_A{}^2$: θ_S	1.49	0.222	2
θ_A: V_S	20.95	<0.001	NA
$\theta_A{}^2$: V_S	1.03	0.310	1
θ_S: V_S	5.52	0.019	NA
Effect	Estimate	s.e	
Intercept	1.451	0.202	
θ_A	0.192	0.095	
$\theta_A{}^2$	-0.297	0.032	
θ_S	-0.454	0.181	
V_S	-1.019	0.235	

θ_A: θ_S	0.112	0.019	
θ_A: V_S	0.425	0.092	
θ_S: V_S	0.501	0.211	
n=225, number of groups =45			
Variance in random term = 0.0080, residual variance =0.0093			
Degrees of freedom = 176, 41			

Table 5.5 Mixed effects model for resistance of evolved lines tested against drug treatments where dose strength is the same as selection conditions but drug ratio is varied. Response variable is optical density after 22 hours growth. Selection line is included as a mixed effect, affecting the intercept of the model. Theta squared is included to allow non-linear effects of assay drug ratio (such as increased synergy). Interactions are included which allow the effect of drug ratio to vary with the environment the line was selected in and also the strength of the assay (and selection) dose.

Effect	Likelihood ratio	P-value	Model reduction step
V_S	7.97	0.005	NA
V_A	0.84	0.359	4
V_A ²	58.86	<0.001	NA
θ_S	6.80	0.009	NA
V_S: θ_S	8.66	0.003	NA
V_A: θ_S	3.80	0.051	1
V_S: V_A	6.39	0.094	3
V_A ²: θ_S	0.03	0.861	2
V_A²: V_S	31.03	<0.001	NA
Effect	Estimate	s.e	
Intercept	1.931	0.285	
V_S	-0.945	0.332	
V_A ²	-1.516	0.180	
θ_S	-0.604	0.227	
V_S: θ_S	0.801	0.264	
V_A²: V_S	1.214	0.209	
n=180, number of groups =45			
Variance in random term = 0.0127, residual variance =0.0110			
Degrees of freedom = 133, 41			

Table 5.6 Mixed effects model for resistance of evolved lines tested against drug treatments where drug ratio is the same as selection conditions but dose strength is varied. Response variable is optical density after 22 hours growth. Selection line is included as a mixed effect, affecting the intercept of the model. Dose strength

squared is included to allow non-linear effects of assay dose strength. Interactions are included which allow the effect of dose strength to vary with the environment the line was selected in and also the drug ratio in the assay (and selection).

In summary the data suggests that dose strength has a strong effect on the speed of adaptation to amoxicillin and clavulanate, with high doses being more inhibitory initially and then causing faster and greater adaptation. High clavulanate environments (low θ) are suggested to reduce adaptation, an effect that is increased at high dose strengths.

5.5.3 Correlation of resistance between drug environments

The theoretical results of this chapter suggest that high and low clavulanate environments should select for different resistance mutations, this would predict that mutants selected in low clavulanate would fare relatively poorly in high clavulanate environments and vice versa. Therefore the growth of evolved strains were tested in alternate drug environments to the ones they were selected against. Due to experimental constraints it was not possible to test all selected lines against every alternate drug environment, therefore alternate treatments were considered along the two axes of drug ratio and dose strength. The response to changes in dose strength was assessed (figure 5.9) by keeping drug ratio fixed ($\theta_A = \theta_S$) and changing the strength of the assay dose (V_A). The response to changing drug ratio was assessed (figure 5.10) by keeping dose strength fixed ($V_A = V_S$) and varying assay drug ratio (θ_A).

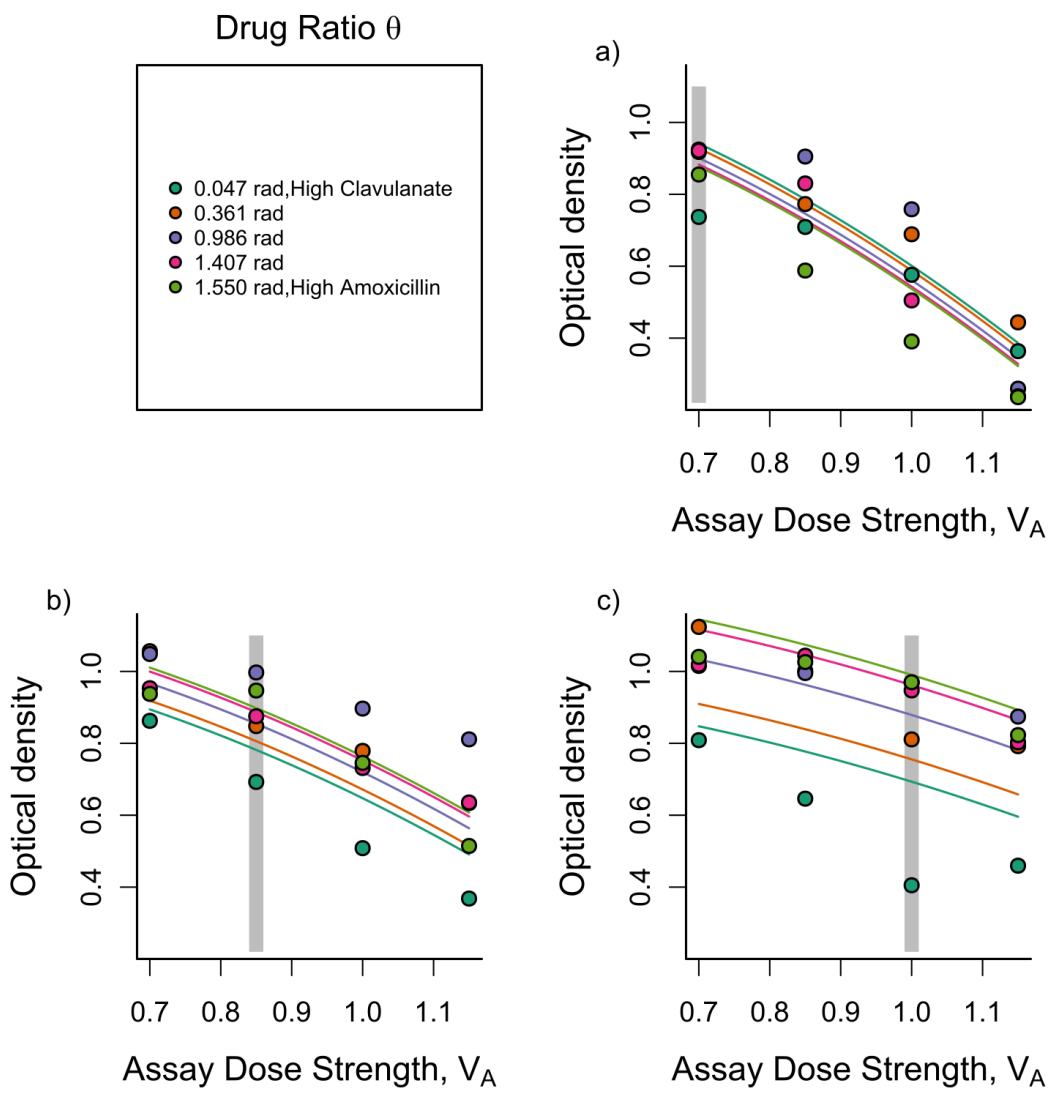

Figure 5.9: High dose strengths predispose bacteria to growth at higher drug doses of the same ratio of drugs. Each panel shows lines evolved to dose strengths (V_S) of (a) 0.7, (b) 0.85 and (c) 1, assayed for growth at alternate assay dose strengths (V_A). Colours differentiate drug ratios for both selection history and assay ($\theta_S = \theta_A$). Solid lines show predicted values from the fixed effects of a minimal linear mixed effects model detailed in table 5.6. The grey bars indicate the dose strength (V_S) that lines were selected against. Points are the mean of three replicate lines.

A linear mixed effects model was fitted to the data in figure 5.9, significance and coefficient values are shown in table 5.6 (and not repeated in the text, to aid text flow). The model indicates that strains evolved to higher drug doses

have increased growth (higher final density after 22 hours) in the presence of all drug doses, shown by the increase in intercept of plots with selection drug dose (V_S). In addition strains evolved to higher drug doses are better able to deal with increases in drug dose, as shown by the reduced magnitude of the negative slope in lines selected at high dose strengths (V_S:V_A^2). The lines selected to different drug ratios behave the same way when exposed to varying dose strengths, as shown by the non significant interactions of θ_S:V_A and θ_S:V_A^2. However, the strains evolved to low θ_S do poorly against all drug environments, as shown by the significant effects of θ_S and V_S:θ_S interaction (lower intercept of lines with low θ).

A separate linear model is fitted to the data in figure 5.10, with significance and coefficient values shown in table 5.5 (and not repeated in the text). Strains evolved to low clavulanate drug ratios (high θ_S) are significantly better at growing in these environments than other lines, as shown by the positive θ_S:θ_A. All strains grow poorly in the high clavulanate (low θ_A) environments although at low dose strengths the lines evolved at low θ_S grow better in high clavulanate than lines evolved at drug ratios with low clavulanate. On the other hand the strains evolved in environments defined by high clavulanate drug ratios and high dose strengths grow poorly in all environments (including the environment they were selected in) compared to the growth of other lines in these drug environments. Additionally specificity of resistance is more prevalent in lines selected at high dose strengths, as seen by the significant positive estimate of V_S:θ_A (greater slope of curves at high dose strengths).

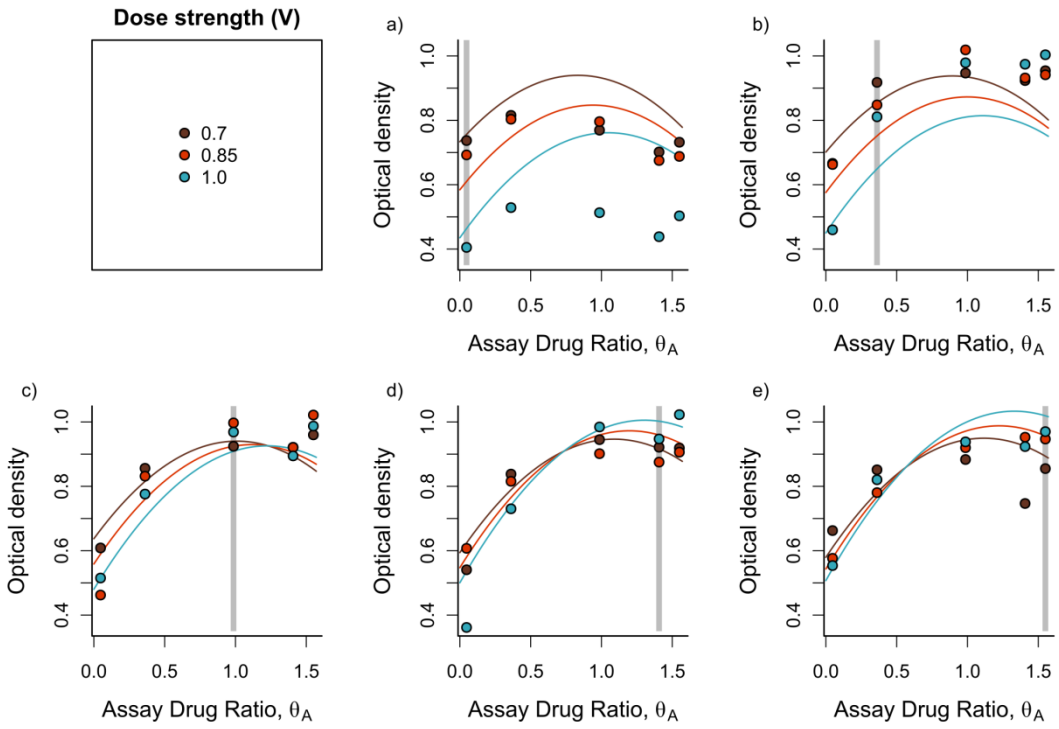

Figure 5.10: The evolution of synergy in selection lines evolved to specific drug ratios (θ). Each panel shows lines evolved to drug ratios (θ_S) of (a) low θ_S to (e) high θ_S, assayed for growth at alternate drug ratios (θ_A). Colours differentiate drug strengths for both selection history and assay ($V_S = V_A$). Solid lines show predicted values from the fixed effects of a minimal linear mixed effects model detailed in table 5.5. The grey bars indicate the drug ratio (θ_S) that lines were selected against. Points are the mean of three replicate lines.

To summarise, figure 5.9 indicates that not only do high dose strengths more efficiently select for resistance, the resistance mechanisms that evolve are better at dealing with further increases in dose. Figure 5.10 provides support for the hypothesis that resistance mechanisms that are selected in one environment do not confer resistance in other environments. Our model suggests this effect is greatest when dose strength is highest. A clear result of figure 5.10 is also that strains evolved to high clavulanate ratios are not only

poorly adapted to their own selection environment (although sometimes better than other lines), but also to other drug environments.

5.6 Discussion

Combinations of drugs have been suggested as a solution to the spread of antibiotic resistance (Yeh *et al.*, 2009; Gill *et al.*, 2014; Bonhoeffer *et al.*, 1997; Christensen *et al.*, 2012) and for some pathogens combination therapy has been in use for decades (Ankomah and Levin, 2012; White *et al.*, 2004). Recent work has suggested that the ratio of drugs used in combination therapies may affect selection for resistance (Pena-Miller *et al.*, 2013; Michel *et al.*, 2008), however for the most part these studies do not keep the strength of selection constant which has a well established effect on the evolution of drug resistance (Oz *et al.*, 2014). This work varies the drug ratio and dose strength (figure 5.1) to create different drug environments (of the same two drugs) where the inhibitory effect is independent of drug ratio. This allows testing of the effect that drug ratio has on selection for resistance, while controlling for the effect of selection strength. The results show that drug ratios with a high concentration of the adjuvant clavulanate (β-lactamse inhibitor) may present less selection for resistance (figure 5.7 and 5.8), even when the inhibitory effect of the drug combination on the ancestor is similar. The results also corroborate that resistance generally evolves faster at when the inhibitory effect of the drug combination is greater.

A simple model shows that different ratios of β-lactam antibiotics and β-lactamase inhibitors can differentially select for certain types of resistance mechanisms, which may account for the difference in the rate of adaption in the selection experiments. The result that additional β-lactamase inhibitor reduces selection for β-lactamase expression is counter to recent theory and experimental observations (Yurtsev *et al.*, 2013), however the two approaches

cover different situations. Yurtsev and colleagues investigate the ability of a resistant strain (producing a fixed amount of β-lactamase) to protect a sensitive strain from concentrations of antibiotic that have no effect on growth of the resistant strain. In this situation addition of β-lactamase inhibitor reduces the amount of detoxification done by a single resistant cell, increasing the density of the resistant strain required to detoxify the environment enough to allow susceptible strains to grow. The model presented here investigates the per capita growth rate in a situation where antibiotic affects growth of all individuals. It shows that because clavulanate reduces the benefits of β-lactamase (inhibiting its activity) but not the costs (incurred during production) increased lactamase production should be favoured less as clavulanate concentration increases.

The hypothesis that different environments select for different resistance mutations raises an important question, namely how does evolution in one drug environment select for resistance to other environments? Studies have shown that there is correlated selection for resistance to different antibiotics (Oz *et al.*, 2014; Toprak *et al.*, 2012; Schenk *et al.*, 2014), but studies investigating evolution to drug ratios often do not assay evolved resistance at altered ratios (Pena-Miller *et al.*, 2013). Treatment regimens are not fixed for combination therapies, the guideline doses for co-amoxiclav change with history, geography and pathology as well as over the course of infection due to different pharmacokinetics and pharmacodynamics (Ankomah *et al.*, 2013). Does resistance to one drug environment (drug ratio and dose strength), confer resistance to other drug environments?

Figures 5.9 and 5.10 show that selection in one drug environment also confers resistance to other drug environments, but the effects of resistance are not the same for all new drug environments, (either with changing dose

strength or drug ratio). Figure 5.9 supports the idea that dose strength is a major factor in determining resistance but also suggests that resistance to high doses is also beneficial at low doses. Several interesting patterns are apparent in figure 5.10, firstly selection in treatments with high θ_S does not confer resistance when assayed by treatment at low θ_A. Second there is cross resistance to different drug environments to the extent where selected lines do not grow best when exposed to the drug ratio they evolved to (see figure 5.10b). Lastly strains evolved to low values of θ_S do not grow well at any drug ratio. These evolved lines also show reduced growth in the absence of drugs (figure S5.2) suggesting that they may have become poorly adapted, tentatively this could suggest an evolutionary trap (Ross-Gillespie *et al.*, 2014).

The interaction between two drugs is predominantly dependent on the drugs used for combination therapy (Yeh *et al.*, 2006). Interactions also depend on how the bacterium responds to these drugs, and is thus dependent on the environment and physiology of the bacterium (chapter 4). In addition consider this example, if the target bacterium does not possess a β-lactamase, or the β-lactamase mutates to become inhibitor resistant, the clavulanate won't act synergistically with amoxicillin. Therefore the drug interaction depends on the genetics and metabolism of the bacterium, these traits are heritable and can therefore evolve. Do we see evolution of *E. coli* to modulate drug interactions?

Given that all drug ratios inhibit growth of the ancestor (and evolved control lines) by a similar amount (figure 5.5 and S5.1), a departure from equal growth at each ratio indicates a change in the drug interaction experienced by the evolved bacteria. The significant negative θ_A^2 term in the model suggests, that there is a move towards a less synergistic relationship between

the two drugs, although a full checkerboard analysis of selected lines (as for figure 5.4) is needed to get the exact shape of the drug interaction. This measurement of evolutionary change in the drug interaction experienced by a bacterium has interesting consequences for treatment with combinations of drugs. Previous reporting of synergy to antagonism change during experimental evolution (Pena-Miller *et al.*, 2013) is misleading in reporting antagonism because multiple divergent populations were used to measure the response the drug combination.

Why is adaptation to high clavulanate environments slower than adaptation to low clavulanate environments? The theoretical model detailed here suggests that high clavulanate environments should select for direct resistance to amoxicillin rather than over-expression of β-lactamase. At high concentrations of clavulanate β-lactamases will be strongly inhibited (or fully inhibited) even if produced at increased levels. On the other hand direct resistance to amoxicillin will be more effective with high concentrations of clavulanate because the effective level of amoxicillin experienced will be higher (as it will not be broken down by β-lactamases). It may be that there are less available mutations which confer direct resistance to amoxicillin compared with ways of increasing CTX-M-14 production. For example amplification of a gene copy number is more easy to achieve than evolution of new or altered functions needed for amoxicillin resistance. The β-lactamase is on a plasmid so the plasmid copy number can increase or gene expression from the single gene could be increased. Direct resistance to amoxicillin can occur by point mutations in penicillin binding proteins, reduced expression of outer membrane porins, or drug efflux (Drawz and Bonomo, 2010), these resistance mechanisms should be available to selection but may be less so than increased β-latamase expression.

β-lactamases have been shown to act as public goods under specific conditions (Medaney *et al.*, 2015; Perlin *et al.*, 2009) so it was expected that adaptation to co-amoxiclav may be impaired because of exploitation of the public good (West *et al.*, 2006). This does not seem to be the case as the environment where β-lactamase was expected to be most beneficial was where resistance developed fastest, this is not that surprising as the conditions used here do not facilitate persister formation or secretion of β-lactamases.

Further work on this project will sequence end points of selection to identify mutations that are responsible for resistance in different selection environments, to further test the predictions detailed here. A full checkerboard analysis will also be performed on selected lines to measure the evolved drug interaction more thoroughly and robustly test for changing drug interactions. In the case where resistance to the drug combination can be attributed to only one of the drugs in the combination, the drug combination could be modified to replace that component with another version (e.g. tiarcillin to replace amoxicillin and sulbactam to replace clavulanate, Gill *et al.*, 2014). This study suggests that using ratios with high clavulanate (or other β-lactamase inhibitor) would slow resistance and reduce selection for increased β-lactamase expression, when resistance to the β-lactam antibiotic arises it could be replaced with another β-lactam.

6 Discussion

6.1 Summary

It has been suggested that antibiotic resistance is conceptually uninteresting to evolutionary biologists, because the outcome is always the same, the appearance of resistance mutations and their spread through the population (Antonovics *et al.*, 2007; Read and Huijben, 2009). As antibiotic resistance becomes a more pressing and widely studied topic the subtleties of antibiotic resistance become apparent (Greulich *et al.*, 2015; Hall, 2013; Michel *et al.*, 2008). As the supply of antibiotics diminishes we now turn to new ways of treating bacterial infections, including new ways of using antibiotics (Gill *et al.*, 2014; Yeh *et al.*, 2009) and new therapeutic targets (Allen *et al.*, 2014; Rasko and Sperandio, 2010).

In this thesis I have shown examples where new ways of treating bacterial infections lead to more complex selection for resistance. Targeting virulence factors and other non-essential bacterial components will mean the effect of therapeutics and therefore selection for resistance will be dependent on the environment (Chapters 2 and 4). Regulation may play a key role in adaption when therapeutics disrupt non-essential genes which are often regulated in a plastic manner (Chapter 2 and 3). This is particularly true for quorum sensing (QS) where different ways of inhibiting QS regulation can lead to different selection for resistance (Chapter 2) and where resistance is dependent on the proportion of resistant individuals in the population (Chapter 3). Similarly combination therapies add complexities compared with single drug therapy. It is established that the interaction between therapeutics is a key parameter determining efficacy and evolutionary robustness (Hegreness *et al.*, 2008), but here I show that interactions between

therapeutics can depend on the environment (chapter 4) and that the drug interaction experienced by a bacterium can change with selection (chapter 5).

More complex selection dynamics bring both further risks and rewards. By taking advantage of the complex selection it may be possible to create therapeutic strategies that slow (Ross-Gillespie *et al.*, 2014) or even reverse (Chait *et al.*, 2007; Mellbye and Schuster, 2011) selection for resistance, as is shown in chapter 5. On the other hand more the more complex processes involved in selection create more risks for therapeutic treatments if used in the wrong context. There is still room for improvement in the way we prescribe conventional antibiotics (Read *et al.*, 2011) and the complexities when using new treatments uncover pitfalls which may lead to faster evolution of resistance (Michel *et al.*, 2008) or even other changes in the life history of pathogens, most worryingly increases in virulence (Allen *et al.*, 2014; Gandon *et al.*, 2001; Köhler *et al.*, 2010). I would argue that not only has it never been more interesting for ecologists and evolutionary biologists to investigate drug resistance, it has also never been more important.

6.2 Effects of combination therapies on selection for resistance

Combination therapies show a lot of promise for extending the lifespan of antibiotics, these include combinations of antibiotics (Ankomah *et al.*, 2013; Michel *et al.*, 2008; Pena-Miller *et al.*, 2013) or antibiotics combined with adjuvants (compounds that do not kill but increase the efficacy of antibiotics, Christensen *et al.*, 2012; Gill *et al.*, 2014). However, Using two compounds means more targets for selection, resistance to one drug can spread if the pharmacokinetics and pharmacodynamics allow refuges where only one drug is at high enough doses (Ankomah *et al.*, 2013). The efficacy and evolutionary robustness drug combinations depends on the interaction

between them, with synergistic interactions being more effective but less robust and antagonistic interactions being less effective and more robust (Hegreness *et al.*, 2008). Furthermore the drug ratio that drugs are used at has important consequences for selection for resistance (Michel *et al.*, 2008; Pena-Miller *et al.*, 2013).

Part of this thesis looks at the potential for evolution of resistance to combinations of antibiotics with adjuvants. As in previous studies, this work shows that selection for resistance increases with antibiotic inhibition (Chapter 5,Oz *et al.*, 2014; de Roode *et al.*, 2004), even when resistance needs to occur *de novo*. More interesting however is the finding that the rate of evolution of resistance depends on the ratio of the adjuvant and antibiotic compound. This indicates that even for accepted drug combinations there may be room for dosing regimens to be optimised, particularly given that the results show that high levels of adjuvant slow resistance whereas the ratio of adjuvant to antibiotic has been decreasing for co-amoxiclav clinical use (White *et al.*, 2004). The theoretical and experimental work in chapter 5 also suggests that different ratios of clavulanate and amoxicillin may also select for resistance to different components of the drug combination. It has been suggested that as we may be close to exhausting viable targets for antibiotics so that antibiotics should be preserved over adjuvants where possible (Gill *et al.*, 2014). Based on this suggestion we could begin to tailor combination treatments to select for resistance to one of the two compounds (a sacrificial adjuvant component of the treatment).

Through the studies in chapter 4 and chapter 5 I stress the idea that drug interactions are not static. In addition to being determined by the drugs chosen for treatments (Yeh *et al.*, 2006), interactions are also dependent on the bacterium and so will vary with treatment environment (chapter 4) and

bacterial genetics (chapter 5). While the selection of bacteria to combinations of therapeutics has received a lot of interest in recent years (Ankomah *et al.*, 2013; Christensen *et al.*, 2012; Michel *et al.*, 2008; Ocampo *et al.*, 2014; Pena-Miller *et al.*, 2013), selection in the context of changing drug interactions has not been studied. Studies that look at how drug interactions change as bacterial metabolism evolves (under drug selection) and how selection proceeds when drug interactions are variable would be of great interest to both basic microbiology and medical interventions. Another axis of treatment variation where study is greatly needed is the time when therapeutics are given. In the lab compounds are usually given prophylactically (at the start of growth, to undeveloped low density cultures). Prophylactic treatment is rarely needed in practice and it usually results in higher potency (Christensen *et al.*, 2012) compared with treating developed cultures.

6.3 Effects of targeting virulence factors on selection for resistance

There has been huge interest in alternate methods of treating bacterial infections (Allen *et al.*, 2014; Clatworthy *et al.*, 2007; Rasko and Sperandio, 2010). Anti-virulence therapeutics (including QS inhibitors) have been investigated here (chapter 2 and chapter 4), but as for other avenues like treatment with bacterial competitors (Brown *et al.*, 2009), these treatments will be heavily dependent on the ecology of the pathogen. Although bacterial physiology (e.g. growth rate, Greulich *et al.*, 2015), can subtly affect antibiotic susceptibility, antibiotic killing (or halting of growth) is predominantly affected by drug concentration (Andersson and Hughes, 2014). Thus selection for resistance is then determined by the antibiotic concentration and the costs of resistance (MacLean *et al.*, 2010b). As for antibiotics the fitness of strains resistant to antivirulence drugs will depend on the dose of the drug

and the costs of resistance, but also on the fitness effects of the targeted virulence factor (Chapter 2), which will depend on the environment and the pathogen strain.

Many virulence factors are secreted molecules or traits that can otherwise be considered as public goods (Nogueira *et al.*, 2009). Public goods production (by co-operators) can be exploited by cheats (West *et al.*, 2007; Diggle *et al.*, 2007). Therefore resistance to therapeutics that inhibit public goods can be exploited by susceptible individuals (which act as cheats), selecting against resistance (Mellbye and Schuster, 2011). Again, selection on resistance will depend on the environment, whether or not the public good is required for growth or whether the population is structured (Mellbye and Schuster, 2011; Dandekar *et al.*, 2012; Griffin *et al.*, 2004).

Quorum sensing controls a large suite of genes in many pathogens so even if quorum sensing is inhibited the effects will be variable with the pathogen and the environment (Mellbye and Schuster, 2011). In many cases the overall effect of treatment will depend on the net effect of all the genes in the QS regulon which may include significant effects from different traits (Dandekar *et al.*, 2012, chapter 2). Preliminary work in the appendix suggests that for QS controlled traits the potential for adaptation is highly dependent on the selection environment. Further investigation will perform more thorough models of the mutation process to quantify the contribution of repeated *de novo* cheat emergence (amateur cheats) to the frequency of cheats.

6.4 Limitations

The sequencing techniques used in the appendix are a reflection of financial limitations (Schlötterer *et al.*, 2014) where the cost of library preparation meant that only a preliminary test with a single line was possible. Further

work may look to sequence more genotypes at the individual level (rather than pooled) to ascertain the genomes of individuals rather than SNP frequencies. However sequencing pools of clones reduces library costs significantly and would allow more samples (and more thus more environments) to be investigated. Sequencing of several more lines would be needed to ensure that conclusions are general. However before making this investment, further investigation into methods of analysing this data needs to be done, to make best use of the data, explicitly genetic methods need to be used, but these need to be applicable to SNP data where associations between SNPs (i.e. whether they are in the same genome) is not possible.

Chapter 3 presents an in depth study of how phenotypic plasticity can affect selection for social traits. This study greatly benefits from the integration of mathematical models with experimental data, however the complexity of these models makes it difficult to account for the error in parameter estimates by propagation of errors. Error in the model prediction of protease expression with WT proportion will then lead to errors in the model of growth rate, which uses the protease expression prediction to estimate cooperative effort. Future work of this type must be designed from the outset to either minimise the potential for errors to be propagated, or to allow propagation of error to be done using relatively simple approaches.

Spectrophotometers allow quick reads of optical density to assess bacterial population density. This approach is invaluable for high throughput experiments where counting colony forming units is too time consuming, or time course experiments which rely on non-destructive sampling. However in the presence of certain antibiotics bacteria have been shown to filament so that bacterial cells become larger (Gottfredsson *et al.*, 1998). As optical density effectively measures cell mass rather than cell number an increase in

cell size that occurs with certain antibiotic treatments will cause underestimates of cell number (Domínguez *et al.*, 2001). The estimation of bacterial biomass (rather than cell number) is still relevant for measures of antibiotic efficacy in most cases it is important to know exactly what growth parameter antibiotics are affecting. Therefore in future experiments plate counts and optical density measures will be used to produce calibration curves for relevant combinations of strains and antibiotics (at varied concentrations).

6.5 Further work

To extend the work performed during my PhD it would be useful to investigate new QS inhibitors which have stronger effects (O'Loughlin *et al.*, 2013) and to see if the antagonism between QS inhibition and translation inhibiting antibiotics is more general. In addition the hypothesis detailed here suggests that any trait that places a burden on translation will increase the efficacy of translation inhibitors. This hypothesis should be investigated further to differentiate the effects of increased protein synthesis from changes in growth. It would be particularly useful to test whether this could select against toxin positive strains, as toxins may confer an enormous burden on translation (e.g. diptheria toxin represents 5% of total protein synthesis in *Corynebacterium diphtheriae*, Pappenheimer and Gill, 1973).

Ongoing work aims to identify the mutations that confer resistance to co-amoxiclav in the selection experiment by whole genome sequencing all replicate populations of the high dose evolved lines after 6 passages of evolution. This will further test the hypothesis that varying drug ratios select for different mutations. This will also aid in identifying the reason why selection for resistance to high clavulanate treatment is so slow to evolve. It will be useful to investigate whether similar observations hold for other

synergistic drug combinations including combinations of 2 antibiotics (i.e. both compounds kill), but also interesting to perform similar experiments for antagonistic interactions. For antagonistic interactions would selection to the drug environment lead to reduced antagonism, reducing drug interactions; or would antagonism increase, reducing the overall effect of the combination of drugs.

Having already separated selection populations for the sequencing approach used in work in the appendix it will be of interest to see the fitness of non-producer fractions compared with the preceding and subsequent producer fractions. This has recently been done for host parasite co-evolution (Gomez and Buckling, 2011) studies and very recently for cheat cooperator dynamics of siderophore production in *P. aeruginosa* (Kümmerli *et al.*, 2015). Not only would performing similar studies on QS cheats be of interest to understand co-evolution, but it would be of interest to compare the diverse populations of cheats and co-operators, which has not been done previously.

Antibiotic resistance is a problem of vital social and economic importance, but is also a problem that is interesting to us as scientists. Evolutionary biology has made progress in understanding the processes that affect the evolution of resistance both to conventional antibiotics ad new therapeutic strategies. Despite this, examples where evolutionary biology has made contributions to healthcare policy are rare. Evolutionary biology must continue to play to its strengths, understanding how the environment and ecology affects selection for resistance. However there needs to be greater integration with medical microbiology if we are to have a lasting impact on the antibiotic resistance crisis.

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Appendix

Chapter S3

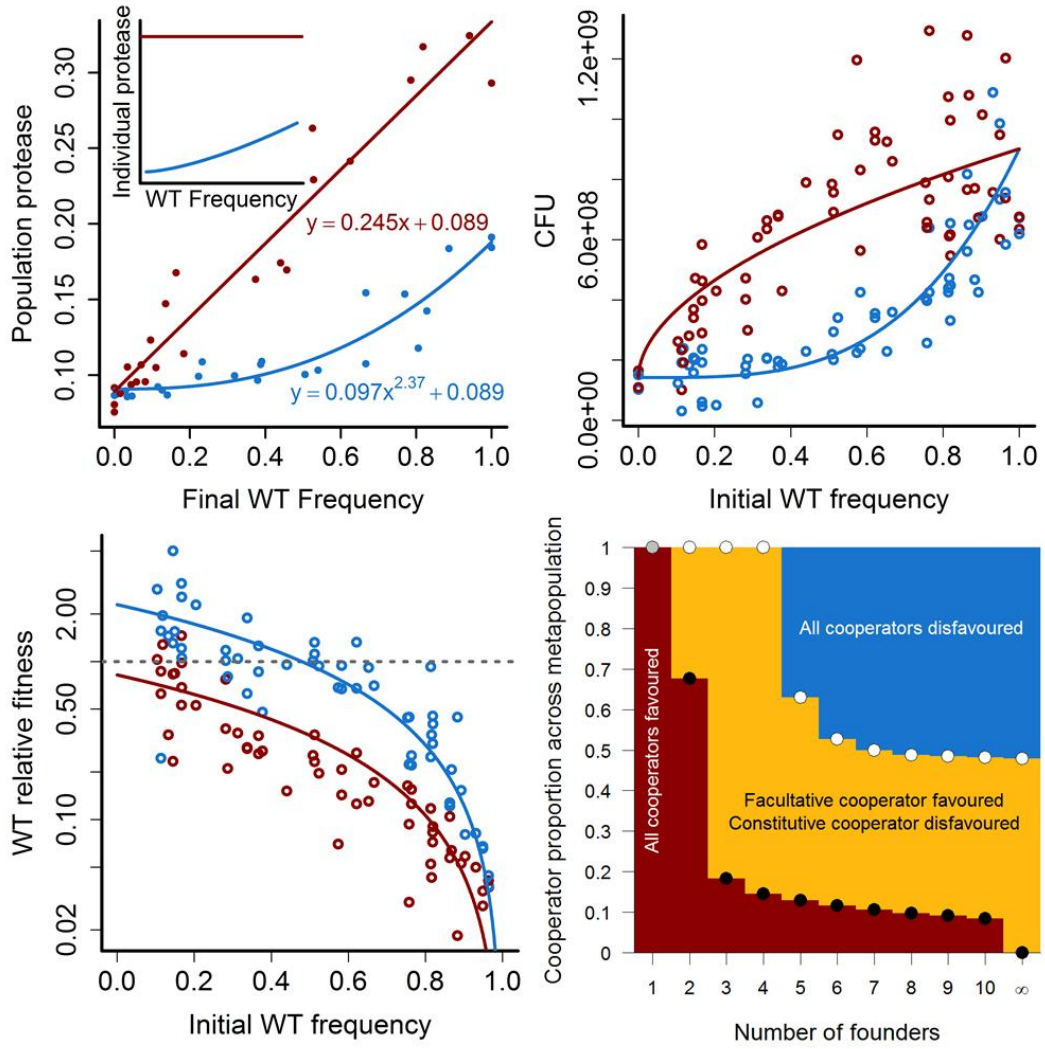

Figure S3.1: Protease production and competitive ability of wildtype cooperators when cooperation is experimentally fixed by adding QS signals in excess. Signals (3-oxo-C12 and C4) were both added at a concentration of 45μM to generate data in red. Native response and competitive ability, without signal addition, are included in blue for comparison. Solid lines are fitted non-linear models. Topleft) Total protease production, as measured by an elastin congo red assay. Inset shows protease per WT individual. Topright) Population yield, measured as CFU/ 100μl. Bottomleft) WT relative fitness, plotted on log scale. Bottomright) Selection for

cooperation in the metapopulation framework with varying numbers of founders, calculated using the solid curves for yield and relative fitness. QS regulated (facultative) cooperation is favoured over a much larger parameter space, including a well-mixed population (infinite number of founders). Dots represent equilibria for constitutive (black), QS-regulated (white), and either form of cooperation (grey).

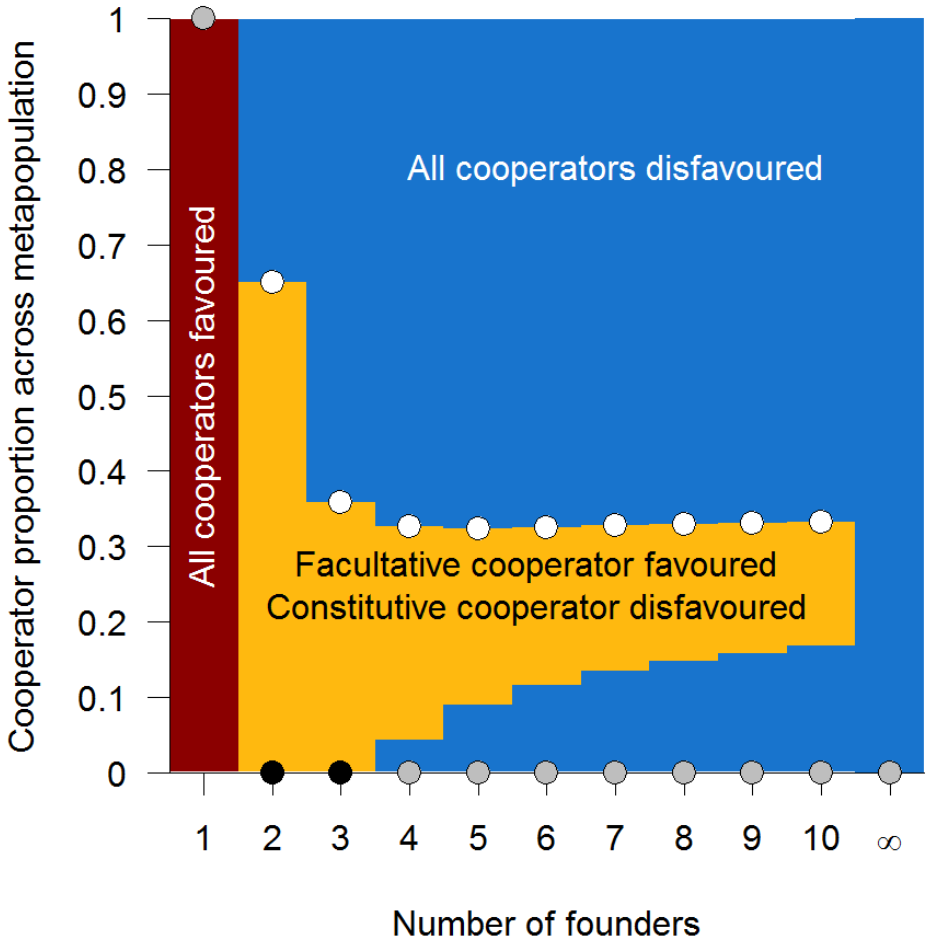

Figure S3.2: Selection for cooperation in a metapopulation using our experimental data to allow cheats to cooperate at the optimal level (maximising their individual benefit).Selection determined using statistical model fits shown in figure 3.3 and the metapopulation framework described in the methods. The cheat is considered to cooperate the amount that maximises their direct fitness (the maximum of the curve in Fig. 3.3c). Dots represent equilibria for constitutive (black), QS-regulated (white), and either form of cooperation (grey). Constitutive cooperation is only favoured when the population is completely structured (1 founder per subpopulation). QS regulated (facultative) cooperation is favoured over a much larger parameter space. For founder numbers of four and above bistability occurs in the system. An equilibrium of coexistence between cheats and facultative cooperators can be

reached once facultative cooperators are above a threshold frequency, while below this frequency an equilibrium of only cheaters is favoured

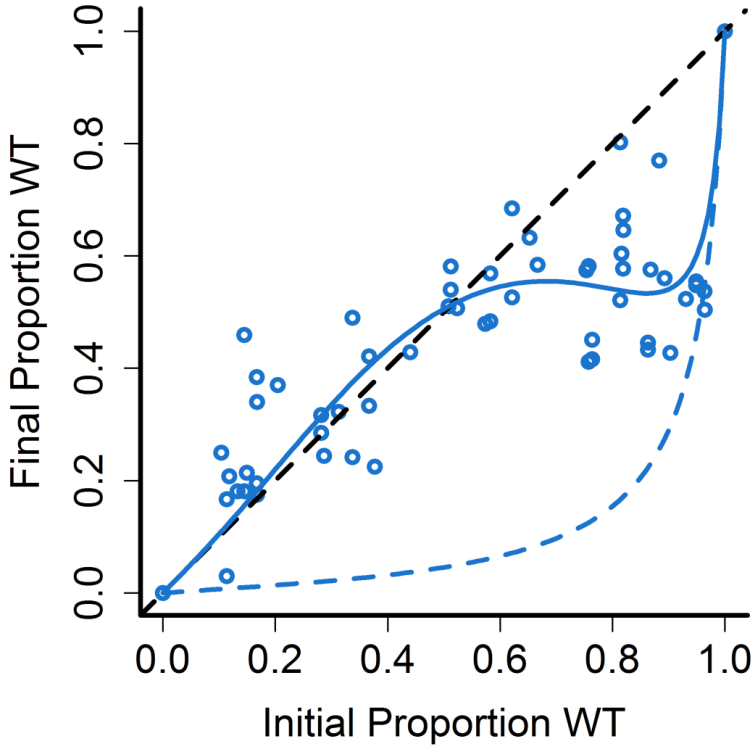

Figure S3.3: Change in frequency of the WT strain over the course of 40 hours competition. WT proportion was measured by plate counts at the start and end of competition, data in blue show competition under native control of cooperation. Sold curves show fits for the model fitted to the data in figure 3.3a, dashed lines show the predictions of the same model for constitutive cooperation.

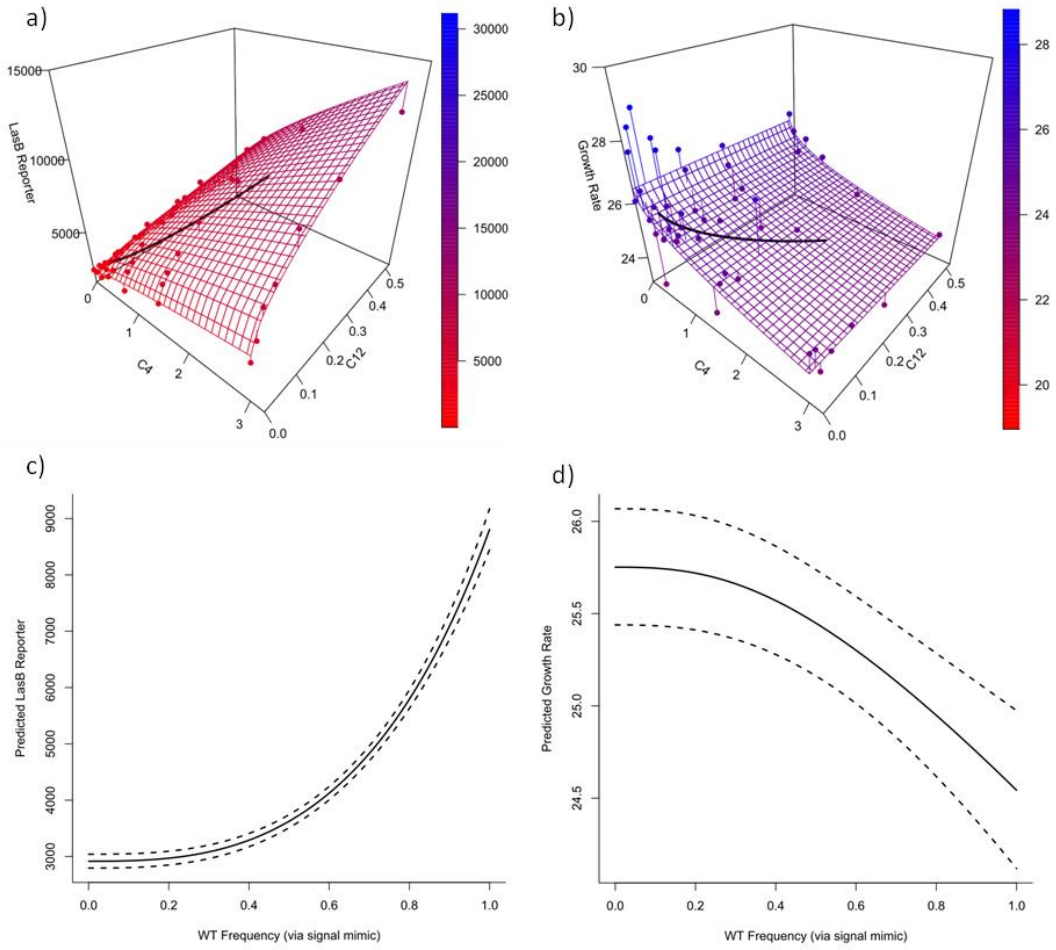

Figure S3.4: The effect of QS activation on growth in an environment where protease is not group beneficial. Pure cultures of double synthase (*ΔlasI ΔrhlI*) mutant were grown for 4 hours in QSM lacking the protein component. Quorum sensing was induced by the addition of C4 and 3-oxo-C12 signals to cultures at the start of growth. a) Luminescence measured from a LasB reporter after 4 hours growth, showing elastase expression increases in a diminishing manner with both C4 and 3-oxo-C12 signals. b) Growth rate of the synthase mutant over 4 hours growth, showing a diminishing cost with increasing concentration of C4 AHL. Black lines on surfaces show the path of signal concentrations measured and shown in figures 3.2a and 3.2b. The trajectory of these lines is shown in more detail in figure c and d. dashed lines are the 95% confidence intervals of the surface predictions.

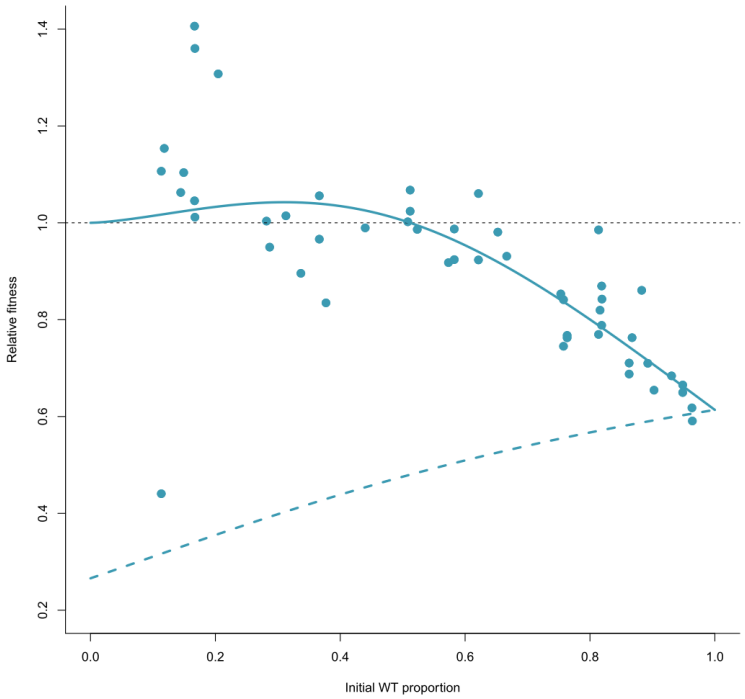

Figure S3.5: Alternate calculation of fitness of the WT in the style of figure 3.3e. Fitness was calculated as $\log[N_{WT}(1)/ N_{WT}(0)]/\log[N_{lasR}(1)/N_{lasR}(0)]$ where $N_{WT}(0)$ and $N_{WT}(1)$ are the nuber of WT individuals at the start and end of competition respectively. This equation is equivalent to that published by Lenski and colleagues (Lenski *et al.*, 1991)

Quorum sensing media recipe

M9 Minimal media supplemented with: 1M $CaCl_2$, 1M $MgSO_4$, Hunters trace metals (Hutner *et al.*, 1950), 1% (w/v) bovine serum albumin and 0.1% (w/v) casamino acids. Filter sterilise (0.22µm filter).

Experimentally enforcing constitutive cooperation

For both the experiments measuring protease phenotype and competitive ability a second treatment was included which was treated identically except that 3-oxo-C12 HSL and C4 HSL were added at 45µM each (excess). Models were fitted to protease activity and yield using power law equations similar to the main text but models allowed all parameters to vary between the 2

treatments. For fitness data, power law models were fitted to cheat frequency (1-WT frequency), but otherwise model specification was the same. All models were then reduced to minimal models as in the main text. Relative fitness of the WT (v) was calculated as: $X_1(1-X_0)/(X_0\ (1-\ X_1))$ where X_0 and X_1 are the proportions of WT cells at the start and end of competition respectively (Ross-Gillespie *et al.*, 2007).

With signal addition the exponent of the protease production curve was reduced compared to the native signal treatment (H_0 : Exponents the same between treatments, fitted difference = -1.31, s.e = 0.544, t_{43} = -2.398 , $p < 0.05$), and was no different from 1 (H_0 : Signal treatment exponent=1, fitted exponent = 0.95, s.e = 0.093, t_{43} = 0.580, $p > 0.5$). However although the amount of secreted enzyme produced by each individual WT was constant, secreted enzyme was produced at an increased rate compared with the native treatment (H_0 : Effective slope the same between treatments, fitted difference = 0.15, s.e = 0.014, t_{44} = 10.582, $p < 0.001$). For yield (as measured by CFU), the exponent of the curve for the signal addition treatment was reduced (H_0 : Exponents the same between treatments, fitted difference = -2.93, s.e = 0.568. t_{111} = -5.152, $p < 0.001$) but was significantly different from 1 (H_0 : Exponent =1, fitted exponent = 0.53, s.e = 0.089, t_{111} = -5.30, $p < 0.001$). The maximal yield of the 2 treatments was not significantly different (H_0 : Effective slope is the same between treatments, fitted difference = 1.45x10^8, s.e = 7.408x10^7, t_{110} = 1.961, $p > 0.05$).

For fitness data, the intercept (at a WT frequency of 1) was not significantly different between treatments (H_0 : intercept is the same between treatments, fitted difference = -0.19, s.e = 0.151, t_{107} = -1.307, $p > 0.1$) or different from zero (H_0 : intercept = 0, fitted intercept = 0.01, s.e = 0.115, t_{108} = 0.115, $p>0.5$). However the effective slope (fitness at a WT frequency of 0) was significantly

reduced with signal addition (H_0 : Effective slope the same between treatments, fitted difference = -1.47, s.e = 0.199, t_{109} = -7.397, p< 0.001), and for both treatments the slope was significantly different from zero (H_0 : Effective slope for native treatment = 0, fitted slope = 2.29, s.e = 0.184, t_{109} = 12.490 p < 0.01; H_0 Effective slope for signal addition treatment = 0, fitted slope = 0.82, s.e = 0.136, t_{109} = 6.064, p < 0.001).

Inducing variable levels of competition to measure costs of quorum sensing

To measure costs of signalling a double synthase mutant (ΔLasI, ΔRhlI) was grown axenically in the QSM medium lacking the protein component, under otherwise identical conditions to the mixed populations. Both C4 and 3-oxo-C12 AHL signals were added to the cultures at the start of growth at a range of concentrations observed in figure 2a and 2b. After 4 hours the optical density (600nm) was measured and used to calculate growth rate (final density – initial density)/initial density. Elastase expression was measured using *LuxCDABE* cassette under the control of the *LasB* promoter (made using the mini CTXLux plasmid (Becher and Schweizer, 2000).

Maximal statistical models were fitted to log(growth rate) and squareroot (reporter luminescence) as multiple regressions on the squareroot of signal concentrations with an interaction term. Reported values are taken from minimal models. We see that protease expression (measured by a luminescent reporter, figure S4a) increases in a diminishing manner with concentration of both C4 (effect = 14.51, s.e = 1.36, t_{45} = 10.68, p < 0.001) and 3-oxo-C12 (effect = 23.00, s.e = 3.33, t_{45} = 6.91, p <0.001) AHL's. In addition there is a positive interaction between the two (effect = 29.27, s.e = 3.62, t_{45} = 8.08, p < 0.001). Quorum sensing activation has a cost in the growth rate (figure S4.4b) which is also diminishing with C4 concentration (effect = -0.056, s.e. =

0.010, t_{47} = -5.39,$p < 0.001$), although the effect of increasing C12 has no significant effect on growth. Using the signal environments at different proportions (taken from figure 2a and 2b) we can predict elastase expression (figure S4.4c) and growth rate (figure S4.4d) of the WT at different WT proportions in mixed populations. Dashed lines show the upper and lower confidence intervals on these predictions based on errors in the predictions in the surface from S4.4a and S4.4b.

Chapter S4

Testing cross resistance between C30 and C10-CPA

As QS inhibitors are relatively new, few studies have characterised resistance, but two strains resistant to the quorum sensing inhibiting furanone compound C30 were obtained with kind permission from the Wood lab (Maeda *et al.*, 2012). These strains were *Pseudomonas aeruginosa* PA14 WT and isogenic transposon mutants in *ΔmexA* and *ΔnalC* as produced in a previous study (Maeda *et al.*, 2012). These strains both overexpress efflux pumps.

The C-30 resistant strains grew more slowly in QSM, compared to the WT (Fig 5.5, Main effect of strain at 10 hours, $F_{2,27} = 392.96$, $p < 10^{-10}$; Main effect of strain at 36 hours, $F_{2,27} = 47.003$ $p < 10^{-5}$). This difference was predominantly driven by the reduced growth of the *ΔmexR* mutant.After 10 hours growth addition of C10-CPA had a positive effect on density (Main effect of C10-CPA, $F_{1,26} = 64.30$, $p < 10^{-5}$), indicating that QS was costly for initial growth and inhibition through C10-CPA reduced this cost. After 36 hours, addition of the QS inhibitor had a negative effect on the growth of strains (Main effect of C10-CPA, $F_{1,26} = 31.596$, $p < 10^{-5}$), indicating that QS inhibition reduced growth on the protein carbon source.

After 10 hours growth there was no significant interaction between strain and CPA addition (C10-CPA:Strain interaction , $F_{2,24} = 0.912$, $p > 0.05$), indicating that all strains were affected by CPA in the same way at this point in growth. On the other hand after 36 hours growth there was a significant interaction between strain and drug inhibition (C10-CPA:Strain interaction , $F_{2,24} = 3.981$, $p < 0.05$), indicating that addition of the drug affected final growth to differing extents. Looking at individual components of the fitted linear model the effect of the C10-CPA (compared to the WT) was

significantly *stronger* in for the *ΔnalC* mutant ($t_{1,24}$ = -2.27, $p < 0.05$) but not significantly different for the *ΔmexR* mutant ($t_{1,24}$ = 0.321 ,$p > 0.5$).

Strains with knockouts conferring resistance to an alternate QS inhibitor C-30 showed no detectable cross resistance to C10-CPA, despite the normal broad effects of efflux pumps (Nikaido and Pagès, 2012). On the other hand it is clear that these mutations impaired growth in QSM (figure 5.5), which is consistent with these efflux pumps reducing intracellular AHL concentration (Minagawa *et al.*, 2012).

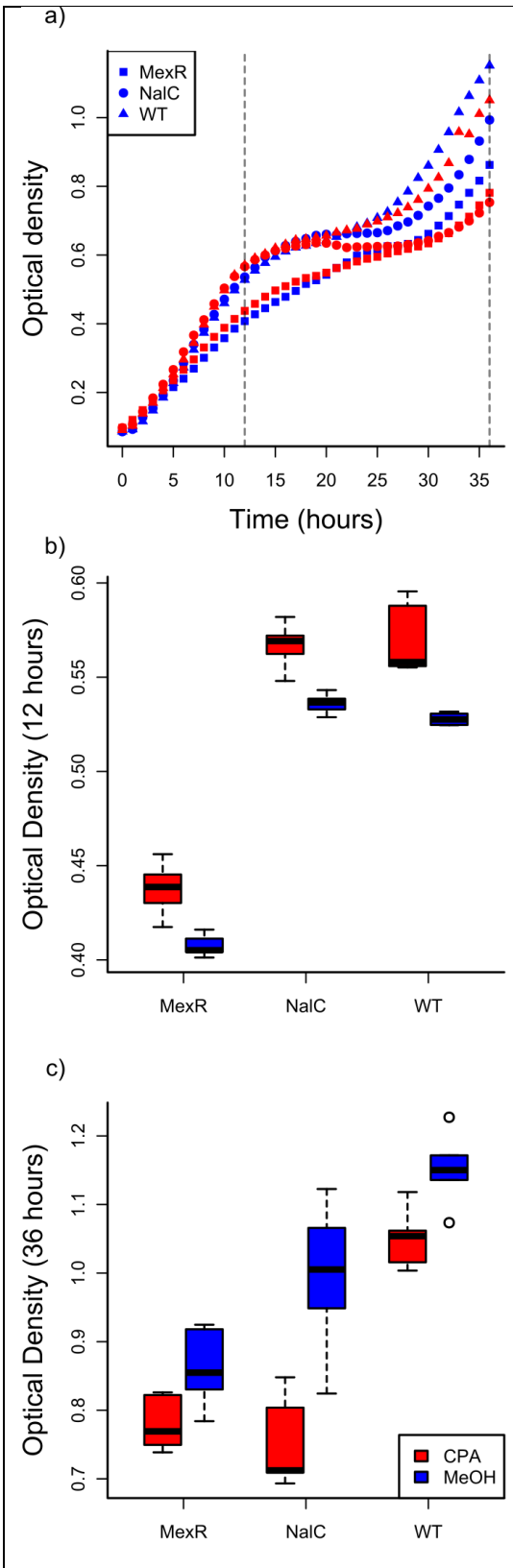

Figure S4.1: Growth of PA14 mutants resistant to the QS inhibitory furanone C30 are impaired for growth in QS media and are not resistant to C10-CPA. a) Growth of PA14 strains in quorum sensing media. Blue points are treated with 400µM C10-CPA, Red points are untreated. Points are mean values, dahed lines are at 12 and 36 hours. b) Density of populations after 12 hours growth. c) Density of cultures after 36 hours growth. All treatments were replicated 5 times.

Parameter	F stat DF	F Stat	p value
Strain	2,26	392.96	<0.001
Drug	1,26	64.3	<0.001

Strain:Drug	2,24	0.91	0.415
Parameter	Estimate	s.e	
Intercept	0.440	0.004	
StrainNalC	0.129	0.005	
StrainWT	0.127	0.005	
DrugMeOH	-0.034	0.004	
n=30, residual deviance= 0.0036 on 26 DF			

Table S4.1 Fixed effects model of the effect of C10-CPA on growth of PA14 mutants after 12 hours growth.

Parameter	F stat DF	F Stat	p value
Strain	NA	NA	NA
Drug	NA	NA	NA
Strain:Drug	2,24	3.98	0.032
Parameter	Estimate	s.e	
Intercept	1.152	0.031	
StrainNalC	-0.158	0.043	
StrainMexR	-0.289	0.043	
DrugCPA	-0.101	0.043	
StrainNalC:DrugCPA	-0.139	0.061	
StrainMexR:DrugCPA	0.0197	0.061	
n=30, residual deviance= 0.1129 on 24 DF			

Table S4.2 Fixed effects model of the effect of C10-CPA on growth of PA14 mutants after 36 hours growth.

Estimating the effect of C10-CPA on azithromycin sensitivity relative to genetic knockouts of lasI gene

To estimate the effect of C10-CPA at 100μM on tobramycin antagonism relative to a genetic knockout a model similar to the one in figure 5.7 was produced.

$$OD = C + c\,(B + x\,A) + \big(M + m\,(B + x\,A)\big)Concentration^2 \qquad Eqn.S4.1$$

A is zero for all treatments except WT treated with C10-CPA and B is zero for all treatments except the *ΔlasR*. Therefore C and M are the intercept and slope of the WT on tobramycin concentration squared. The difference between the intercept and slope for the WT and *ΔlasR* strains is then c and m respectively. The fractional activity of 100μM C10-CPA is the x. This results

of this model are very similar to the model in figure 5.7 except that the effect of C10-CPA on intercept and slope are not independent. Model output is shown below.

```
Parameters:
    Estimate Std. Error t value Pr(>|t|)
C  1.112e+01  1.465e-01  75.866  < 2e-16 ***
c -2.666e+00  2.195e-01 -12.146 3.15e-11 ***
M -3.994e+03  1.070e+02 -37.323  < 2e-16 ***
m  2.247e+03  1.517e+02  14.814 6.31e-13 ***
x  1.337e-01  6.298e-02   2.123   0.0452 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 0.3423 on 22 degrees of freedom

Number of iterations to convergence: 3
Achieved convergence tolerance: 2.356e-06
```

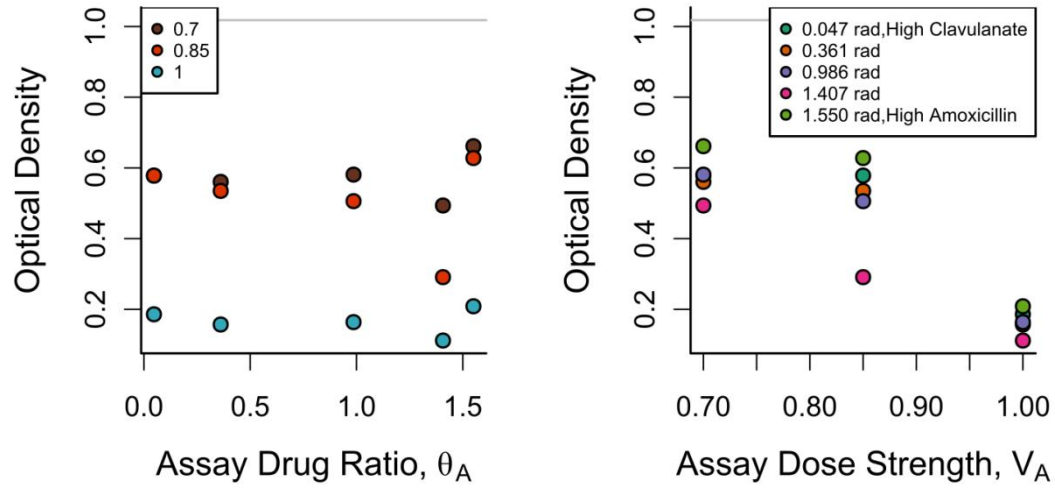

Figure S5.1: The growth of control lines of experimental evolution grown in varying drug environments. Control lines evolved in the absence of drugs for 6 passages were grown for 22 hours in varying drug environments. a) Effect of assay drug ratio on growth, the colours represent varying assay dose strengths. b) Effect of assay dose strength on growth, colours represent varying assay drug ratios. The same data is used for (a) and (b). Points are the mean of the growth for 3 replicate lines. grey lines are the growth of control lines in the absence of drugs.

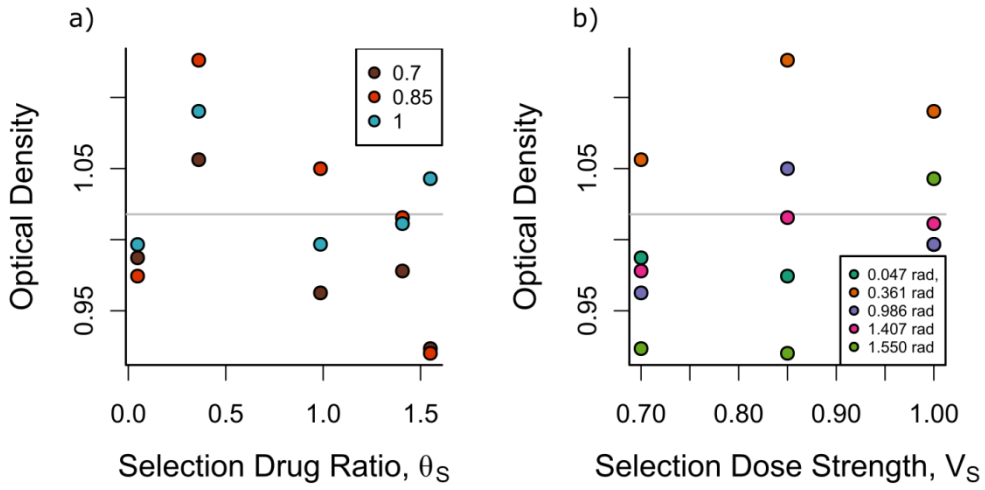

Figure S5.2: The growth of experimental lines grown in the absence of drugs. Experimental lines evolved in drug environments defined by θ_S and V_S for 6 passages were grown for 22 hours in the absence of drugs. a) Effect of selection drug ratio on growth, the colours represent varying selection dose strengths. b) Effect of selection dose strength on growth, colours represent varying selection drug ratios. The same data is used for (a) and (b). Points are the mean of the growth for 3 replicate lines. grey lines are the growth of control lines in the absence of drugs.

Appended chapter: Selecting For Early Expression of Quorum Sensing

Contributing authors: Richard C. Allen, Nick Lowery, Katsiaryna Usachova, Sam P. Brown.

Contributions: RCA, NL and SPB came up with the idea and designed the experiments. RCA and NL performed the experimental evolution. KU performed pilot experiments for phenotyping of evolved lines. RCA performed the phenotyping experiments and analysis described here.

Note in correction

During the defence of this thesis both examiners raised serious concerns about this chapter, and questioned the need to include it in the thesis. As a substantial body of work that has been written up, but does not meet the standard of the rest of the thesis, this chapter has been entirely moved to the appendix.

The problem with this project was that there were too many hypotheses being investigated, rather than a simpler clearly set out hypothesis. This led to an experimental design with many factors in a full factorial design leading to 8 treatments but only 3 replicate lines per treatment. The process of transferring from one passage to another was labour intensive and the experimental evolution process lasted for approximately 3 months. Evolution, phenotyping and analysis could have been greatly improved by focussing on one or two factors, and having more replicates.

The mixing treatment was achieved by either having tubes shaken or static, this was intended to manipulate population structure and the ability of cells to clump together but it had large effects on growth speed due to better

aeration of mixed cultures, this project was redone use of shaken cultures only is recomended as static cultures grew very poorly. Most of the interesting results suggestsed here stem from the change in media so it may have been sensible to additionally drop the genetic background treatment, using only the WT strain. With fewer variables each treatment could be better replicated simpler models could be used to explain data, putting more focus on correlation between measured phenotypes. To test a more general hypothesis of evolution with public and private nutrient sources it would also be of benefit to have at least two media with both social and private QS dependent nutrient sources, however it should be noted that the comparison between adenosine and protein has been used to make a social vs private comparison multiple times in high impact papers in the field (Dandekar *et al.*, 2012; Mellbye and Schuster, 2011).

Finally the sequencing analysis presented at the end of this chapter suffers from a lack of time and resources. Although a significant amount of money was used to pay for sequencing, the depth of sequencing required and the cost of library preparation meant that only one experimentally evolved line could be sequenced. This prevents general conclusions from being drawn. Unfortunately read depth and multiple time points are required to answer the specific question so the only option would be to spend more money for more sequences to do this. The coverage we have of the one experimentally evolved line should allow robust predictions to be made for this line, however a more detailed analysis must be done using genetic methods rather than analysing SNPs independently.

The following is the chapter as it appeared in the examined version of the thesis and has not been altered. All figure or chapter numbers (4.X or S4.X) refer to this chapter not to chapter 4 in the main text.

4.1 Abstract

Quorum sensing allows bacteria to alter gene expression once sufficient population density is reached (quorum). However many studies investigate quorum sensing in environments when the quorum is easy to reach. This chapter investigates the dilemma produced in *Pseudomonas aeruginosa* when quorum sensing upregulated enzymes are required for growth from the outset, but starting density is too low for the quorum sensing system to turn on. Results suggest that selection for early expression of private traits (nucleoside hydrolase) leads to upregulation of these traits with uncoupling from quorum sensing. In contrast selection in an environment requiring early expression of public goods (elastase) leads to changes in the quorum sensing network. To further investigate social dynamics, the spread of cheat strains is investigated. High resolution sequencing of a line selected to express elastase early, identifies mutations in motility and biofilm formation as possible adaptations to the environment. This sequencing also indicates that the dynamics of cheat lineages are a balance between persistence and adaptation to a cheat lifestyle and repeated *de novo* cheat emergence by mutation.

4.2 Introduction

Quorum sensing (QS) is a means for bacteria to gain information about the biotic and abiotic factors of their environment (Cornforth *et al.*, 2014; Darch *et al.*, 2012; Hense *et al.*, 2007), in order to coordinate gene expression changes. The canonical role for quorum sensing is to sense density (Fuqua *et al.*, 1994); bacteria need to reach a quorum before the quorum sensing regulon is induced. However the density of the quorum varies with the environment (Cornforth *et al.*, 2014) and may be as low as a single cell in confined spaces (Redfield, 2002).

Experiments often allow *Pseudomonas aeruginosa* to reach quorum by including a free carbon component in the media (Popat *et al.*, 2015b), inoculating cultures at a high density (Sandoz *et al.*, 2007) or transferring signals over from overnight cultures upon inoculating experiments (Mellbye and Schuster, 2011). This work performs experimental evolution of *P. aeruginosa* in environments where quorum sensing is required for growth from the outset, but no means of reaching a quorum is provided. These experiments test whether *P. aeruginosa* is able to adapt to the dilemma of needing QS regulated genes to grow to the density required for QS induction.

Increased or early expression of the QS regulon may be required for growth but it also presents problems. The secreted proteins regulated by QS can act as public goods, meaning that cheats that do not express the QS regulon can spread by exploitation of public goods. As wild-type (WT) *P. aeruginosa* does not induce the QS regulon at low densities, it could exploit mutants that have evolved to express the QS regulon at low density (Ghoul *et al.*, 2014b). The potential for exploitation may be greater at low density, secreted factors (and the breakdown products of secreted enzymes) can move away from the cell (due to diffusion or flow), so the benefits of secretions will be small at low density (Cornforth *et al.*, 2014; Darch *et al.*, 2012; Koschwanez *et al.*, 2013). To investigate the effect of exploitation, two selection media were used contrasting between exploitable and private QS regulated traits. Media contained as the sole carbon source, either protein (Diggle *et al.*, 2007; Mellbye and Schuster, 2011) utilised by elastase - a secreted protease (*lasB*), or adenosine (Dandekar *et al.*, 2012; Heurlier *et al.*, 2005; Mellbye and Schuster, 2011) utilised by the intracellular enzyme *nuh* (nucleoside hydrolase).

Many experimental evolution studies have shown that cheats occur by mutation and spread due to the exploitation of cooperative behaviours in their neighbours, especially when the environment is unstructured (Dandekar *et al.*, 2012; Griffin *et al.*, 2004; Sandoz *et al.*, 2007). Do cheats consistently arise *de novo,* or once they arise do they persist and adapt to a cheater lifestyle, gaining mutations that make them better at exploiting cooperators? If cheats persist as a subpopulation, it would allow the co-evolution of cooperators and cheats (Kümmerli *et al.*, 2015; Gomez and Buckling, 2011). In addition clinical samples often contain large numbers of cheats (Hoffman *et al.*, 2009; Smith *et al.*, 2006), which are able to gain adaptations to the CF lung environments (D'Argenio *et al.*, 2007).

Several hypotheses can be made about how *P. aeruginosa* could adapt to express QS regulated genes early. The quorum density may be reduced by increased basal signal expression, or increased sensitivity of the receptor (Popat *et al.*, 2015b). As detailed in chapter 2, this would have different effects on neighbouring cells; for example increased signal production would alter the expression of neighbours while increased signal reception would only affect expression in the mutant (Allen *et al.*, 2014; Wilder *et al.*, 2011). It has been recently shown that the ratio of signals can affect expression of subsets of the QS regulon (Cornforth *et al.*, 2014; Popat *et al.*, 2015a) so *P. aeruginosa* may alter only one of the QS systems; for example there is evidence that the Rhl system can compensate for Las system deficiency (Delden *et al.*, 1998) in *ΔlasR*. Half the selection lines have *ΔlasR* mutants as the ancestor, which allows investigation of the effect of genetic background on the types of adaptations available. When the QS system is damaged (by the knockout of *lasR*), it may be more likely that adaptations occur

downstream by uncoupling genes from QS regulation and leading to QS expression.

Not all potential adaptations are reliant on changes in the QS regulation of genes; yeast cells have been shown to adapt to low density growth by evolving imperfect separation leading to clumping (Koschwanez *et al.*, 2013). *P. aeruginosa* is a prolific biofilm former (Costerton *et al.*, 1999) and has been shown to produce planktonic biofilms (Schleheck *et al.*, 2009), which could serve the role of clumps. In this selection experiment clumps would serve several beneficial roles. Clumping would lead to higher local signal densities when overall population density is lower. Public goods (and their products) would be more readily used by the clumps before they diffuse away (Koschwanez *et al.*, 2013; Cornforth *et al.*, 2014). Lastly clumping would increase the structure of the population. To investigate clumping, selection lines contrast static environments with shaken environments, intended to make it harder for clumps to persist.

These experiments show that the potential to adapt to environments where QS induction is required at low density, is variable with the genetic background, nutrient environment and mixing of the population (table 4.1). Investigating within-population diversity suggests that cheating organisms are able to spread and hinder adaptation at the population level. Finally, detailed sequencing of a single line shows that mutations involved in virulence and motility are selected for. In addition this data suggests that while some cheat lineages persist, most cheats arise repeatedly over the course of selection.

Agitation	Genotype	Media	Numeric ID	Symbol	Final sample

Yes	WT	AdM	1	Circle	21
			2	Triangle	21
			3	Square	21
		PrM	4	Circle	21
			5	Triangle	21
			6	Square	21
	ΔLasR	AdM	7	Circle	21
			8	Triangle	21
			9	Square	21
		PrM	10	Circle	21
			11	Triangle	21
			12	Square	6
No	WT	AdM	13	Circle	21
			14	Triangle	21
			15	Square	15
		PrM	16	Circle	21
			17	Triangle	21
			18	Square	15
	ΔLasR	AdM	19	Circle	21
			20	Triangle	21
			21	Square	21
		PrM	22	Circle	6
			23	Triangle	6
			24	Square	6

Table 4.1 Treatments for each selection line.

4.3 Results

4.3.1 Ancestral growth

Media without casamino acids are inhospitable for *P. aeruginosa*. As can be seen in figures 4.1a and 4.1c, WT PAO1 and a *ΔlasR* knockout have similar growth dynamics in PrM. Other strains taken from a clinical isolate library have varying growth dynamics. There is similar variation for growth in AdM, however the WT strain clearly has higher absolute fitness than the QS mutant over 48 hours growth (figure 4.1b and 4.1d).

Despite removing all the casamino acid free carbon from PrM there is still rapid growth up to approximately OD=0.2; this indicates that some of the carbon from the bovine serum albumin added to the medium is freely accessible even to strains that are impaired for quorum sensing (figure 4.1a).

4.3.2 Adaptation

Over the course of experimental evolution, the time taken (in days) for each line to reach the threshold was recorded as a real time record of adaptation. This data can be seen in table 4.2 and is plotted (as reciprocal of time to threshold) in figures 4.2a and 4.2b. Although the data is noisy, loess fits to the data show that in PrM both shaken cultures reach the threshold quicker after selection. On the other hand the static *ΔlasR* line does not appear to adapt to the selection environment and all three lines were stopped after 6 passages due to time constraints. In addition the WT line selected in a static tubes, does not adapt well to the selection environment, particularly between passages 10 and 15 where the approximate growth rate of these lines slows dramatically.

In the AdM environment the WT already grows quite well (figure 4.1b) and as a result there is no strong trend of adaptation. On the other hand the *ΔlasR* mutant lines show an increase in growth rate over the course of the selection experiment. Unfortunately this data is not balanced and is unsuitable for statistical analysis.

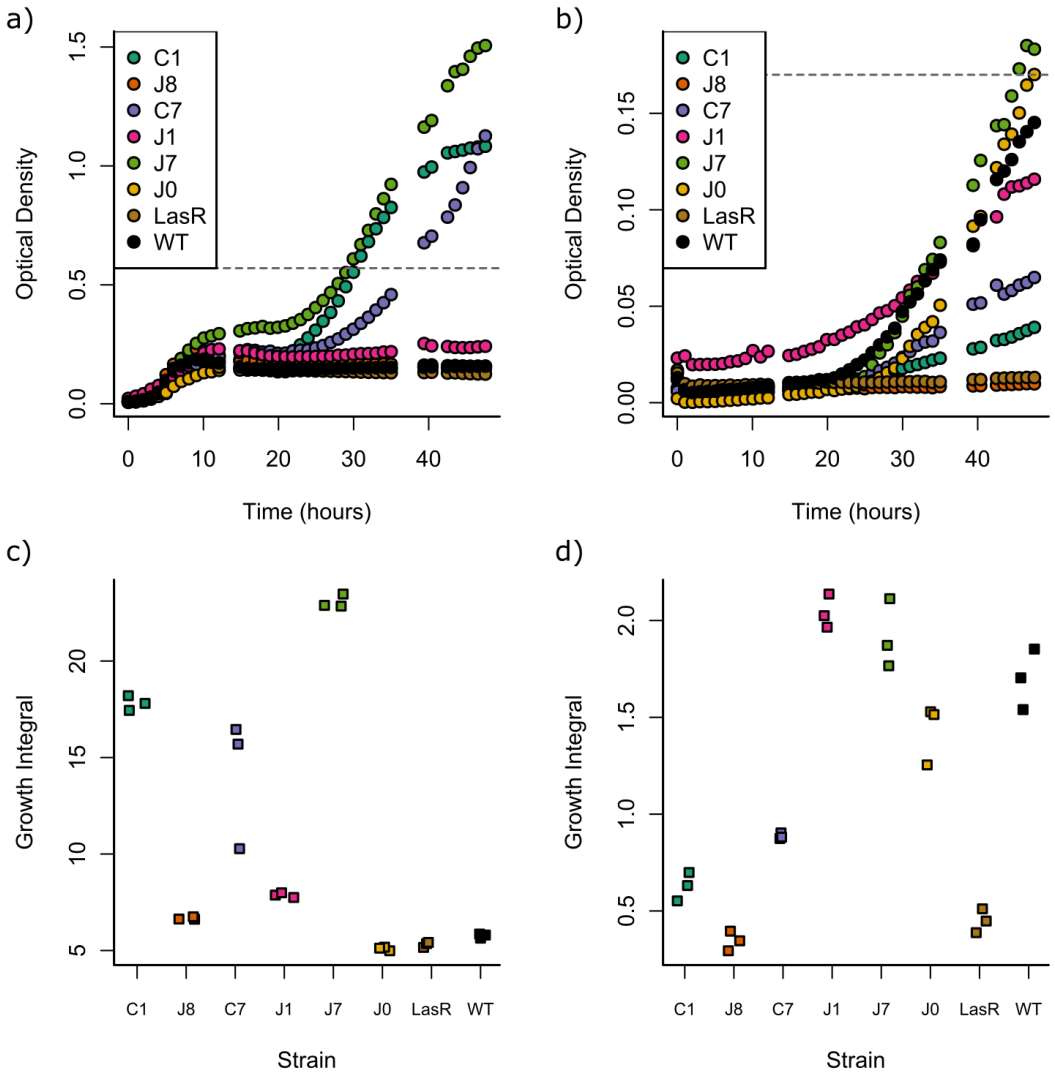

Figure 4.1: Diverse growth dynamics of strains in media with protein and adenosine as the sole carbon source. Growth of clinical isolates and PAO1 controls over 48 hours in a medium with protein (PrM, a & c) and adenosine (AdM, b & d) as the sole carbon source. Lower panels show the integral of 3 replicate growth curves for each strain in each medium. Dashed lines in (a) and (b) are the optical density thresholds for passage during experimental evolution.

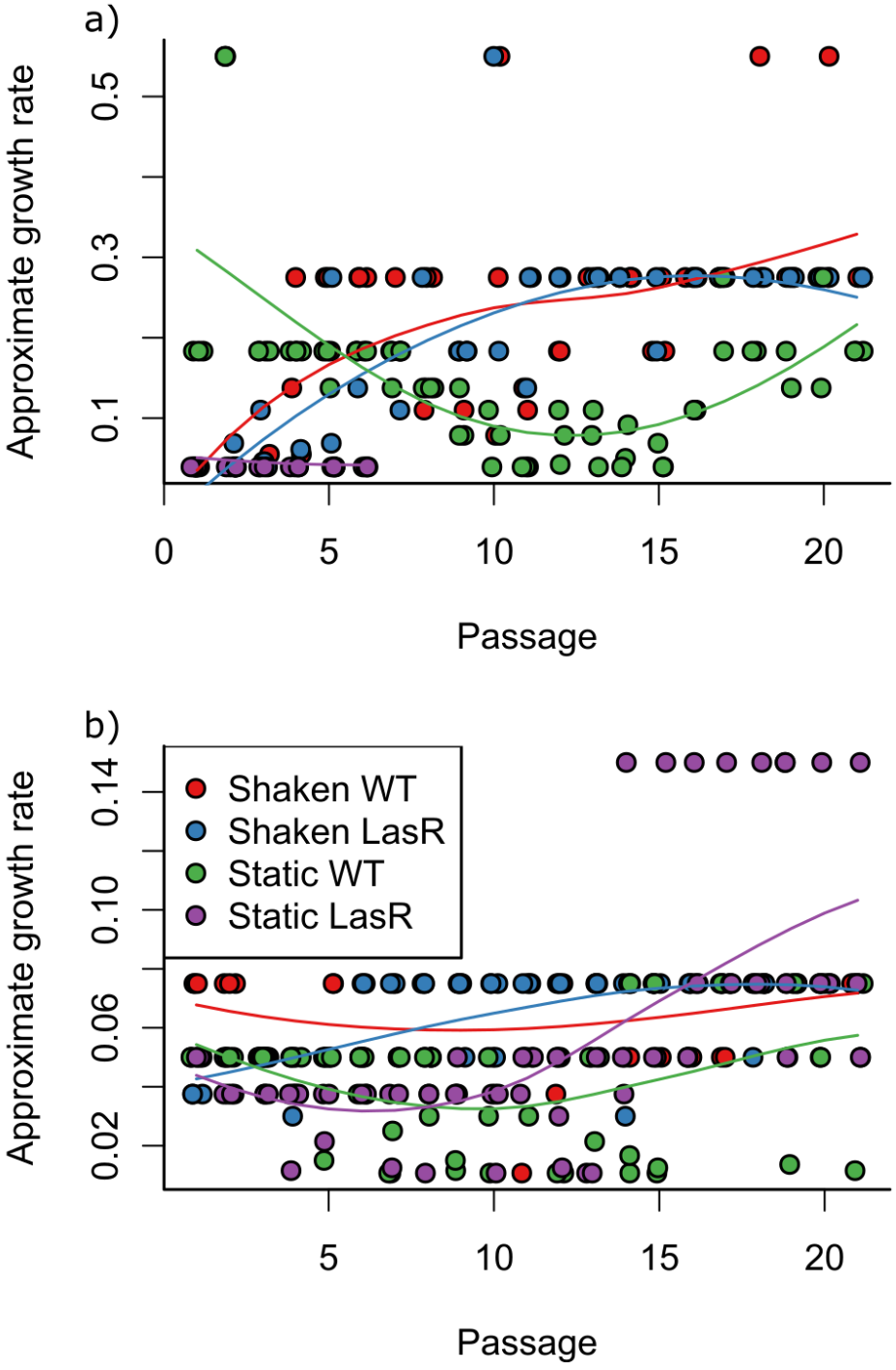

Figure 4.2: Adaptation of lines over time. Approximate values of growth rate of selection lines over 21 passages in a) PrM and b) AdM. Growth rate is approximated as he reciprocal of time to threshold. Curves are loess fits implemented to better show the trend, not for statistical analysis.

		Passage																				
Treatment	Line #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Shaken Adenosine WT	1	2	2	3	4	2	3	3	2	2	4	3	2	2	2	2	3	2	2	2	3	2
	2	2	2	3	3	3	2	3	3	2	3	14	4	2	3	3	2	3	2	2	2	2
	3	2	2	3	3	2	2	2	3	2	2	2	2	3	3	2	2	3	2	2	2	2
Shaken Protein WT	4	14	14	10	10	3	2	3	5	5	7	5	3	2	2	3	2	2	2	2	2	2
	5	14	14	3	2	2	2	3	2	3	1	2	2	2	2	2	2	2	2	2	1	2
	6	14	14	12	4	2	2	2	2	3	2	4	3	2	2	3	2	2	1	2	2	2
Shaken adenosine LasR	7	4	3	3	3	3	3	2	2	2	2	2	2	2	2	2	2	2	3	3	2	3
	8	4	3	3	5	3	2	14	3	3	3	2	3	2	2	2	2	2	2	1	2	2
	9	4	3	3	4	3	3	2	2	2	2	2	2	2	5	2	2	2	2	2	2	2
Shaken Protein LasR	10	14	8	5	3	2	3	5	2	3	1	2	2	2	2	2	2	2	2	2	2	2
	11	14	14	12	9	8	4	3	4	3	3	4	2	2	2	3	2	2	2	2	2	3
	12	14	14	14	14	14	14	14														
Nonshaken Adenosine WT	13	3	3	3	3	3	3	3	3	13	5	3	14	7	2	14	3	2	2	2	2	2
	14	3	3	3	3	3	3	6	3	4	3	3	14	3	14	2	3	2	2	11	3	13
	15	3	4	3	4	10	4	14	5	10	15	5	3	3	9	12						
Non-shaken Protein WT	16	3	1	3	3	3	3	4	4	4	7	14	7	5	11	14	5	2	3	4	4	3
	17	3	1	3	3	4	3	3	4	7	5	14	5	7	6	8	5	3	3	3	2	3
	18	3	1	3	3	3	3	3	4	7	14	14	13	14	14	14						
Non-shaken Adenosine LasR	19	3	4	4	4	4	4	4	4	4	20	4	12	14	1	1	1	1	1	1	1	1
	20	3	4	4	4	4	4	4	4	4	4	3	5	14	3	3	2	2	2	2	2	3
	21	3	4	4	13	7	4	12	14	3	4	3	3	3	4	3	3	2	2	3	2	2
Non-Shaken Protein LasR	22	14	14	14	14	14	14															
	23	14	14	14	14	14	14															
	24	14	14	14	14	14	14															

Table 4.2: The time taken (days) to grow to threshold for all 24 lines of experimental evolution. Empty boxes are lines that did not

4.3.3 Growth of selection lines

The end points of each selection line (generally after 21 passages, see table 4.1 for more details) were assayed for growth in both PrM and AdM with the integral of the growth curve used as a summary statistic, to account for changes in rate and yield of growth (fig 4.3). For growth in PrM, the minimal model of the data includes a term for the selection media which causes a significant reduction in model fit if dropped (H_0: no effect of selection medium, effect of selection in AdM = -2.92, $F_{1,20} = 8.1725$, $p < 0.01$). The model also includes a positive effect of selection in a shaken environment, in relation to a static environment ($F_{1,22} = 14.388$, $p < 0.01$). Finally the minimal model includes a small positive effect of passage on growth integral ($F_{1,21} = 4.34$, $p = 0.0502$); this term is included to account for different selection lengths and it is hard to interpret given that the lines with few passages were stopped because they did not grow well.

The minimal model for the integral of growth in AdM includes a significant positive effect of selection in AdM compared with selection in PrM ($F_{1,22} = 21.08$, $p < 0.001$). Again the minimal model includes a marginally significant positive effect of passage number on growth in AdM ($F_{1,21} = 4.17$, $p = 0.054$) but no other effects are significant. There is no significant overall correlation between growth PrM and AdM ($F_{1,22} = 3.60$, $p > 0.05$). However, there is a significant negative main effect of selection in PrM ($F_{1,21} = 16.21$, $p < 0.001$) and a significant interaction between the two ($F_{1,20} = 15.98$, $p < 0.001$). Therefore there is a positive relationship between growth in PrM and AdM only for strains evolved in PrM (Fig 4.4).

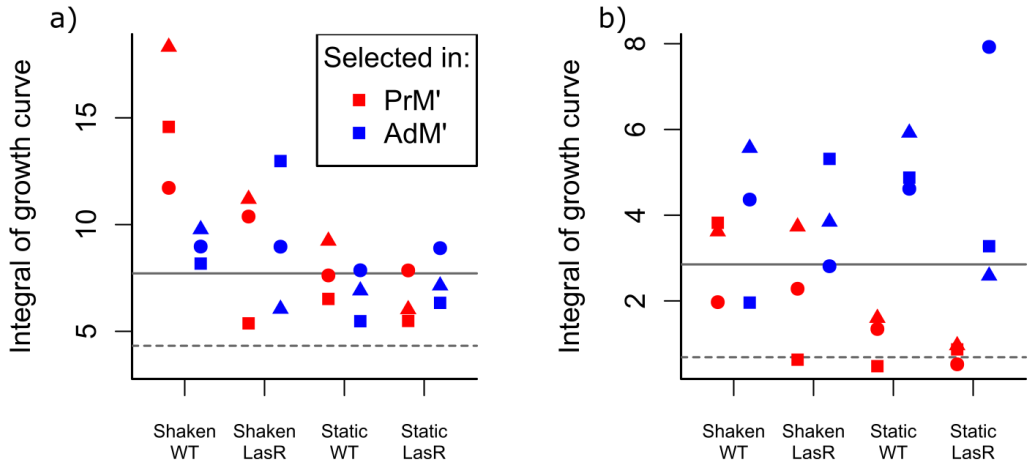

Figure 4.3 Integral of the growth curves for end points of selection, in PrM and AdM. Points show the integral of growth curves over 48 hours for the end point of each selection line. These are assayed in either (a) PrM or (b) AdM. Different shapes are used to allow easy comparison across graphs in this chapter; first, second and third replicates use circles, triangles and squares respectively. Sold lines are the mean of 3 replicates for growth of the WT ancestor, dashed lines are the mean of three replicates for the ΔLasR ancestor.

Lines seeded with the WT ancestor and grown statically in PrM were chosen to be investigated further. Figure 4.5 shows the integral of growth of these lines (16, 17 and 18) for each of frozen time-point of these samples(collected after 3, 6, 9, 12, 15, 18 and 21 passages). Overall we see that there is a general decrease in the integral of growth in both PrM (figure 4.5a) and AdM (figure 4.5b). It is notable however that in line 16 we see a recovery in growth of the line in PrM for later passages (regression of growth on passage = -3.16, $F_{1,18} = 10.03$ $p < 0.01$; regression of growth on passage^2 = 0.13, $F_{1,18} = 9.91$, $p < 0.01$) In figure 4.5a all the time-points for line 18 grow poorly, this line responded badly to selection but the different lines were tested on different days so the large anomaly is probably at least in part attributable to experimental discrepancies.

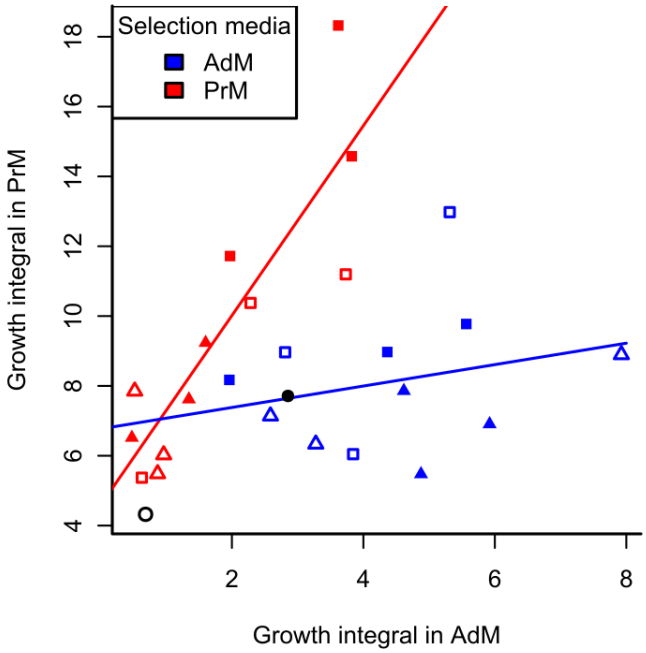

Figure 4.4: Correlation of growth in the two selection media. Growth integrals of end points of selection in PrM and AdM. Points are coloured by the environment they were selected in. Squares and triangles are shaken and static selection environments respectively. Filled and Hollow points are selection lines with WT and *ΔlasR* ancestors respectively. Black circles are ancestors. Lines are predictions from minimal linear models.

4.3.4 Aggregation of cells

The variation in repeat optical density readings briefly separated by shaking were used to assess whether cells were liable to clump during growth (methods). There is a significant relationship between whether cells clump together during growth and clumping during growth for PrM (figure 4.6a, $F_{1,22} = 6.63$, $p < 0.05$.) and AdM (figure 4.6b, $F_{1,22} = 26.89$, $p < 0.001$) but the correlation is greater for AdM (Pearson correlation 0.49 vs 0.75). The causal nature of these relationships is not known and may in part be due to the fact that more dense cultures form biofilms more easily, rather than an adaptive effect of clumping. There is no significant relationship between clumping in the two media (figure S4.1, $F_{1,22} = 2.49$, $p > 0.1$) suggesting that clumping

adaptation is at least media specific, but it may also indicate that it is simply a product of growth.

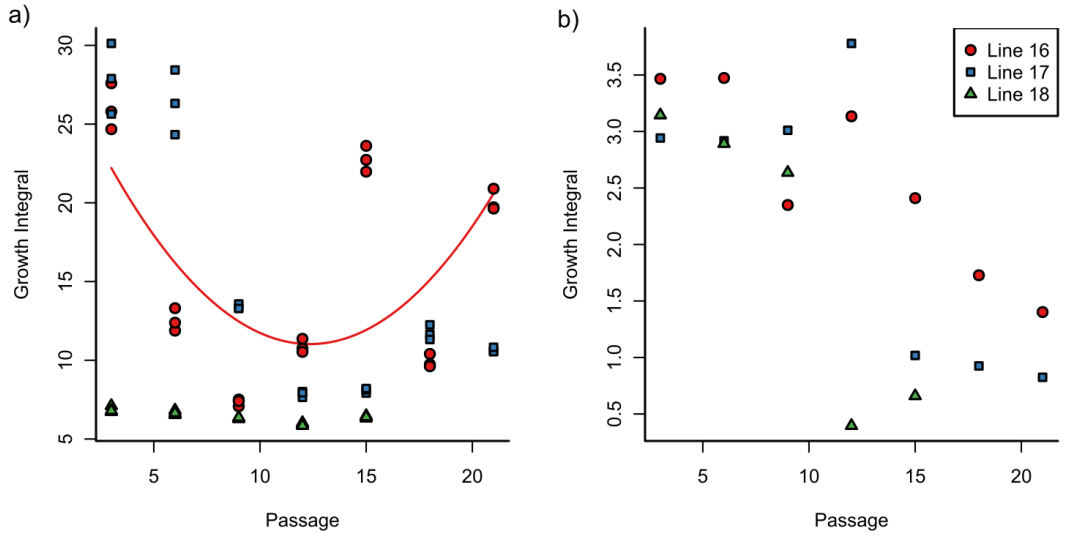

Fig 4.5: Change in growth dynamics over time in lines 16, 17, and 18. Each point is the mean of the integral of 3 replicate growth curves in either (a) PrM' or (b) AdM' over 48 hours. The curve in (a) is a linear model fitted to the line 16 data, with a significant effect of passage^2.Data for the 3 different lines was collected on 3 different days.

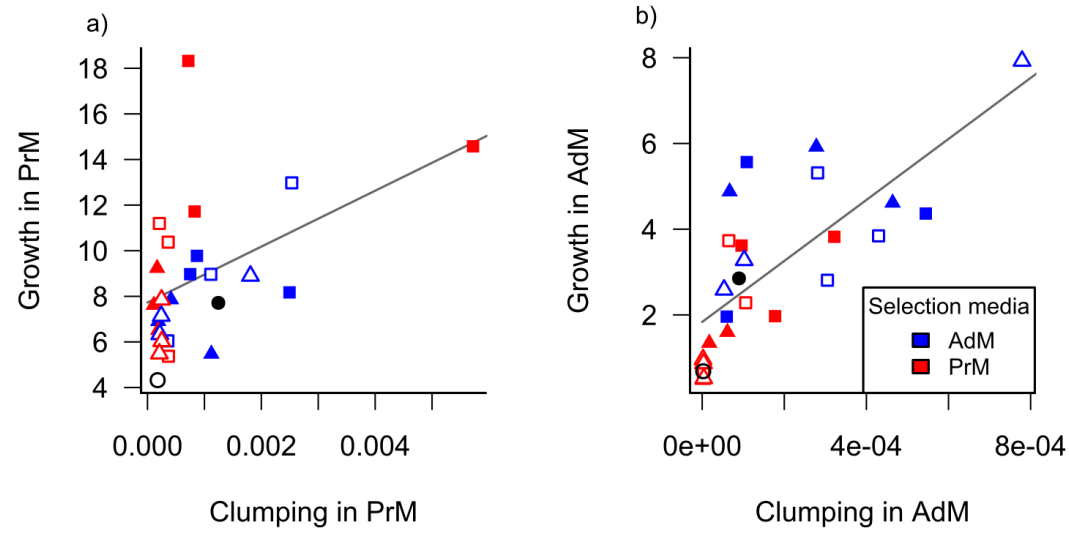

Figure 4.6: Correlation of growth with clumping. The integral of growth shows a significant relationship with measures of clumping in a) PrM and b) AdM. Points are coloured by the environment they were selected in. Squares and triangles are

shaken and static selection environments respectively. Filled and Hollow points are selection lines with WT and *ΔlasR* ancestors respectively. Black circles are ancestors. The grey lines are predictions from linear models.

4.3.5 Elastase production by end points of selection

To get an idea of evolved phenotypes, all final time-points were grown for 6 hours in PrM, before supernatent was tested for protease activity. For measures of protease production (and signal concentration) it is important to note that the density of the samples was not the same when supernatant was taken to assay protease culture density after 6 hours is shown in the appendix (figure S4.2). The different lines have varying levels of protease activity however model selection shows that there is no statistical difference between the selection treatments in terms of protease activity (figure S4.3). Only a positive effect of both passage ($F_{1,22} = 10.24$, $p<0.01$) and optical density at 6 hours ($F_{1,21} = 4.76$, $p<0.05$) are significant. Despite this many of the evolved samples appear to be producing protease at an increased level compared with the ancestral level. ECR activity has a significant effect on growth in PrM ($F_{1,22} = 4.34$, $p < 0.05$) but not in AdM ($F_{1,22} = 2.25$, $p > 0.1$), as is shown in figure 4.7.

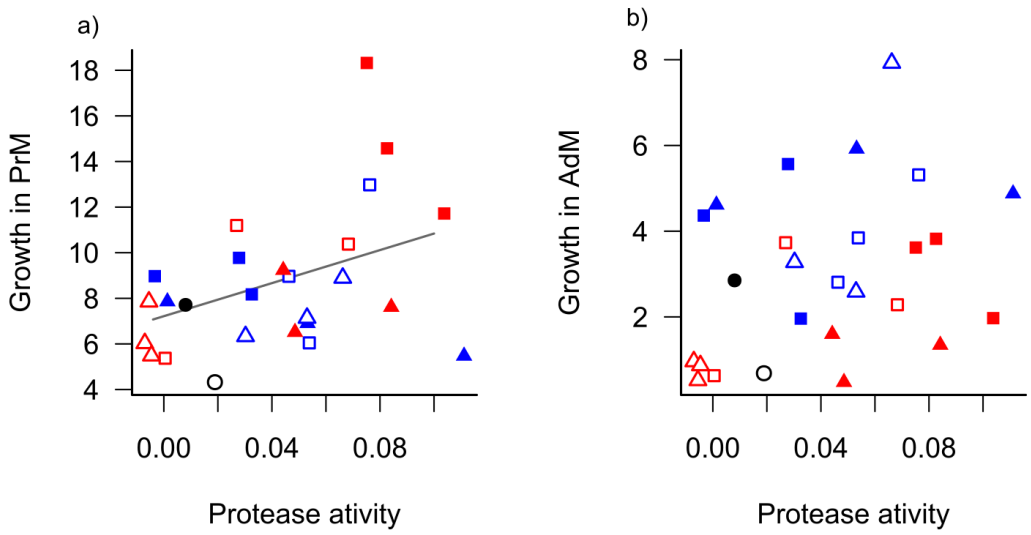

Figure 4.7: Correlation of growth with protease activity. The integral of growth shows a significant relationship with protease activity in a) PrM but not in b) AdM. Points are coloured by the environment they were selected in. Squares and triangles are shaken and static selection environments respectively. Filled and Hollow points are selection lines with WT and *ΔlasR* ancestors respectively. Black circles are ancestors. The grey lines are predictions from linear models.

4.3.5 Signalling production of end points of selection

The concentration of 3-oxo-C12 and C4 AHL signals in supernatent after 6 hours growth in PrM were measured using bioreporters (figure 4.8). In general levels of C12 production are reduced compared to the levels of signal in the ancestor. Generalised linear models show that there is still a significant effect of ancestor, with WT selection lines producing more C12 signal ($F_{1,22}$ = 14.03, p<0.01) *ΔlasR* selection lines, no other factors had a significant effect on C12 production. As can be seen in figure 4.8a all of the evolved strains had a reduced level of C12 signal production. On the other hand, some of the lines seeded with the *ΔlasR* ancestor produce signal at levels similar to the *ΔlasR* ancestor, and some have evolved greatly increased levels of signal production.

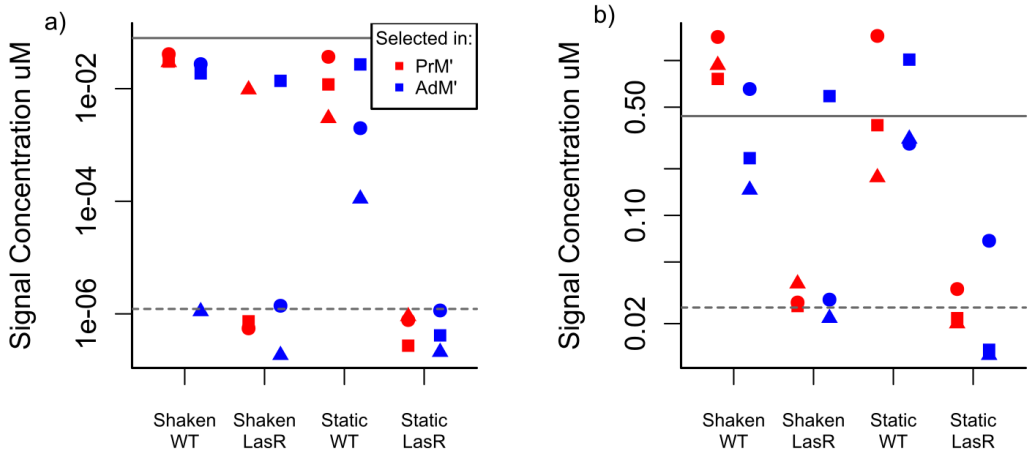

Figure 4.8: Signal concentrations after 6 hours growth in PrM' for the end points of selection. a) The concentration of 3-oxo-C12 after 6 hours growth. b) The concentration of C4 after 6 hours growth. Different points are used to allow easy comparison across graphs in this chapter; first, second and third replicates use circles, triangles and squares respectively. Solid lines are the mean of 3 replicates for growth of the WT ancestor, dashed lines are the mean of three replicates for the ΔLasR ancestor. Note the log scales of signal concentration.

For levels of C4 signal, again the ancestor was the only predictor that had a significant effect on signal production ($F_{1,22}$ = 16.00, p<0.001). For C4 production several lines produce signal at a level higher than the ancestors, and in the case of line 9 a *ΔlasR* ancestor evolved to produce signal at a level greater than the WT. The concentrations for the two signals in the evolved lines had a highly significant linear relationship ($F_{1,22}$ = 155.23, $p < 10^{-10}$) and compared to the concentration of signals in the WT ancestor there is a shift towards greater production of C4 signal (figure 4.9a). Despite this C4 had no significant predictive effect on growth in either PrM (figure S4.4b, $F_{1,22}$ = 3.99, p = 0.058) or AdM (figure S4.4d, $F_{1,22}$ = 0.04, p>0.1). On the other hand C12 signal concentration was predictive of growth in PrM (figure S4.4a, $F_{1,22}$ = 6.42, p <0.05) but not AdM (figure S4.4b, $F_{1,22}$ = 0.01, p > 0.1). These

relationships are likely driven by the effect of C12 (figure 4.9b, $F_{1,22} = 10.64$, $p < 0.01$) and C4 (Figure 4.9c, $F_{1,22} = 16.01$, $p < 0.001$) on protease production.

4.3.6 Within population diversity

To ascertain whether cheats reduced adaptation at the population level during selection, populations were spread on skimmed milk plates, and zones of clearance, and colony morphology were used to identify different protease production phenotypes. Colonies were identified as either non-producers, low producers, medium producers, high producers, and fuzzy producers (the WT ancestor was a high producer and the ΔLasR ancestor was a low producer). Fuzzy producers had a large zone of skimmed milk clearance (like high producers) but were characterised by large colonies with a fuzzy edge rather than the smooth edge seen for other colonies. All colony types were grown on PIA (*Pseudomonas* isolation agar) to assess that they were not contaminants.

Several lines had small colonies with no clearance halo, these were initially thought to be non-producers however these colonies showed no discernible growth on PIA. PCR amplification of 3 conserved genes (Lavenir *et al.*, 2007) identified that these colonies were not *P. aeruginosa*. Sequencing later identified these colonies as *Stenotrophomonas maltophilia,* a gram negative opportunistic pathogen that is sometimes mistaken for *Pseudomonas,* and

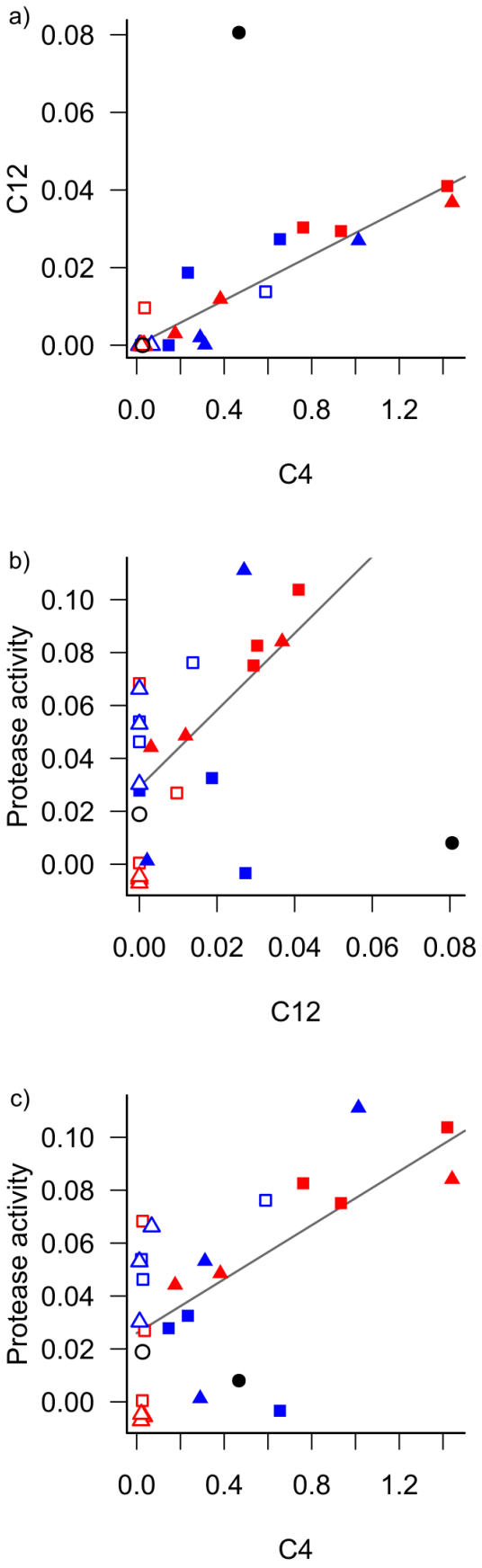

Figure 4.9: Correlation of signals and protease activity. Significant relationships are seen between a) C12 and C4 concentration. b) C12 concentration and Protease activity c) C4 concentration and protease activity. Points are coloured by the environment they were selected in. Squares and triangles are shaken and static selection environments respectively. Filled and Hollow points are WT and *ΔlasR* ancestors respectively. Black circles are ancestors which are excluded from models. The grey lines are predictions from linear models.

often co-colonises with *P. aeruginosa* (Brooke, 2012). This bacterium must have been introduced though contamination of some lines during passaging. Colonies that showed this morphology and were not able to grow on PIA were scored as SM (*S. maltophila*) like.

For end points of selection there is high within population diversity of phenotypes found in the populations of selected strains (figure 4.10) but there is some commonality within selection lines. Strains evolved in AdM often have high proportions of non producer strains. Strains evolved in static media appear to have higher proportions of fuzzy producers which would be consistent with them having a role in motility. Obviously the low producer phenotype (analogous to *ΔlasR*) is more common in the selection lines initiated with *ΔlasR* and there is only one case where the high producer line (analogous to WT) arises in these lines. Although non-producer phenotypes arise in WT initiated lines low producers only appear in a few cases. The diversity of the different treatments combined with the binomial error structure prevented adequate modelling of the data.

For lines 16, 17 and 18 the change in frequency of the different colony morphologies was tracked over the course of the selection experiment as can be seen in figure 4.11. Data shows that additional colony morphologies invade so that the frequency of the protease producers declines over time (H_0: sum of high producers and fuzzy producer frequency does not change as a fraction of *P. aeruginosa* morphologies, $F_{1,15} = 8.48$, $p < 0.05$). In particular spreading morphologies invade the population as well as morphologies that produce less protease. Selection line also has a significant effect on the frequency of protease producers ($F_{1,16} = 5.04$, $p < 0.05$) with line 16 having significantly higher proportions of producers than the other 2 lines.

Unfortunately lines 16 and 17 have a contamination by *S. maltophila,* these are shown in figure 4.11 but these colonies are excluded from statistical analysis.

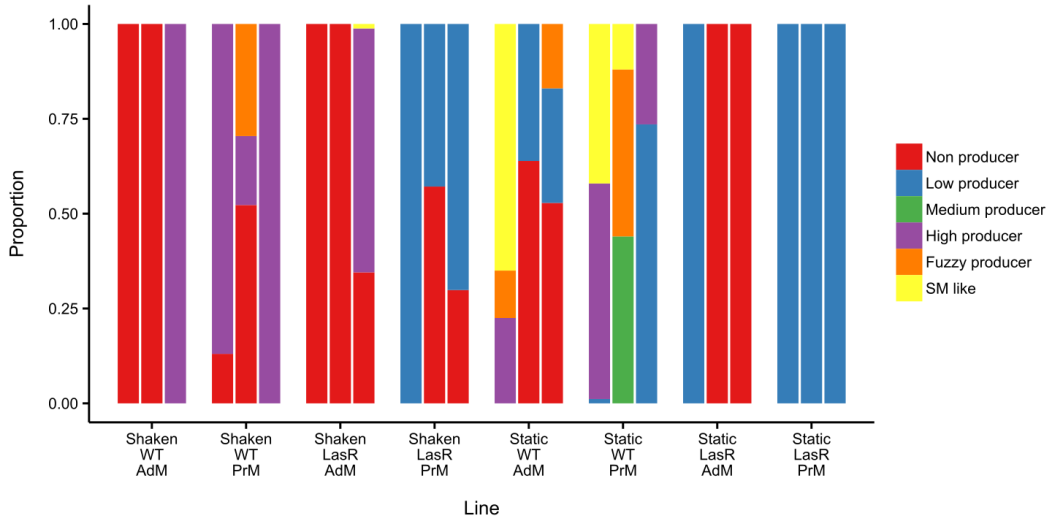

Figure 4.10: The frequency of colony morphologies for the end points of selection. Colony morphologies were determined based on colony morphology as well as the size and transparency of the halo produced by colonies on skimmed milk plates. SM like colonies were colonies with morphology similar to a contaminant identified as *S. maltophilia,* lack of growth on PIA also picked these out as contaminants.

4.3.7 Growth of morphologies

Individual clones of the morphologies were tested for their growth in PrM and AdM as monocultures (axenically). Figure 4.12a shows that in general the high producers (which resemble the WT ancestor) generally have the highest absolute fitness. Despite this one of the fuzzy producer clones from line 17 has greatly increased growth. It is difficult to make out in figure 4.12a but the fuzzy producers grow to a higher initial density at approximately 6 hours compared to the other clones, this is consistent with the larger colony size on the skimmed milk agar. In the AdM' media the high producer clones have a consistently high absolute fitness, however the fuzzy producer clone from line 17 grows to a density comparable to the high producer clones.

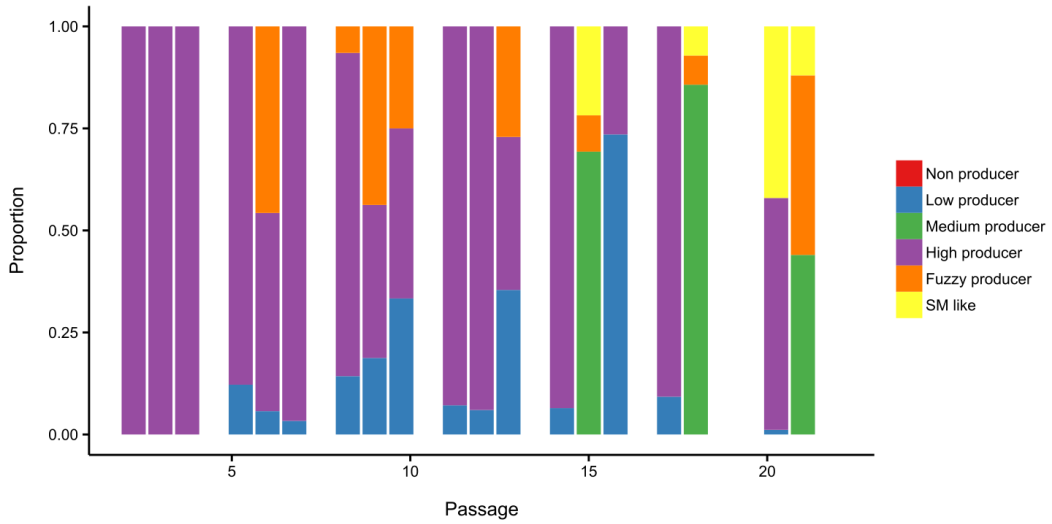

Figure 4.11: Change in frequency of colony morphologies in WT lines grown statically in PrM. Colony morphologies were determined based on colony morphology as well as the size and transparency of the halo produced by colonies on skimmed milk plates. SM like colonies were colonies with morphology similar to a contaminant identified as *S. maltophilia,* lack of growth on PIA also picked these out as contaminants. Note the x axis is not continuous; first, second and third columns for each tick mark shows the frequencies in line 16,17 and 18 respectively. Line 18 was not continued past 15 passages so the columns for 18 and 21 passages.

4.3.8 Sequencing data

To ascertain the genetic basis for adaptation, line 16 was selected for sequencing. For all populations except for passage 3 a producer and non-producer population were submitted as separate samples for sequencing. The final time point (after 21 passages) is excluded from analysis as it is contaminated. As can be seen in figure 4.13 a several SNPs spread over the

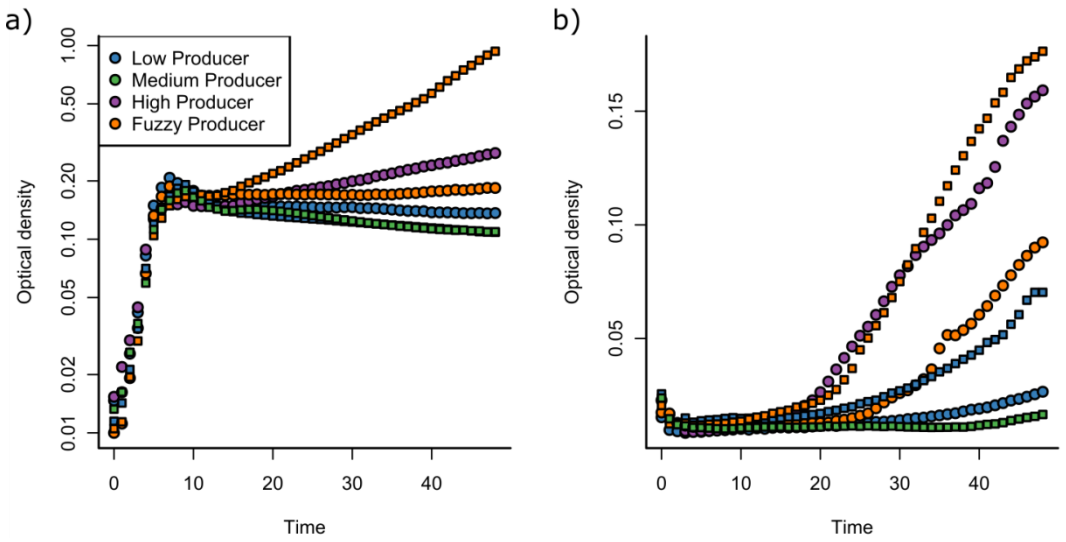

Figure 4.12: Growth of colony morphologies taken from line 16 and 17. Individual colonies were picked from populations of line 16 (circles) and 17 (squares) and tested for growth in (a) PrM (log scale) and (b) AdM. Each series represents the mean of at least 2 replicates spread across at least 2 separate clones. All line 16 clones are taken from the population after 9 passages, the line 17 low producer was taken from the population after 9 passages while the fuzzy producer and medium producer were taken from the population after 18 passages.

course of selection, and in some several cases these SNPs spread specifically in either the cheat or cooperator fraction of the selection experiment. For SNPs that are that rose to high frequency (greater than 20%) more information can be found in table 4.3 and figure 4.14.

To test whether cheater strains arise repeatedly or arise and then co-evolve with cooperators the pattern of SNP spread (figure 4.15) in the cooperator and defector populations was analysed. A mixed model with an interaction between passage and fraction was fitted to the data. The model included a random effect of SNP on the magnitude of the passage effect to account for

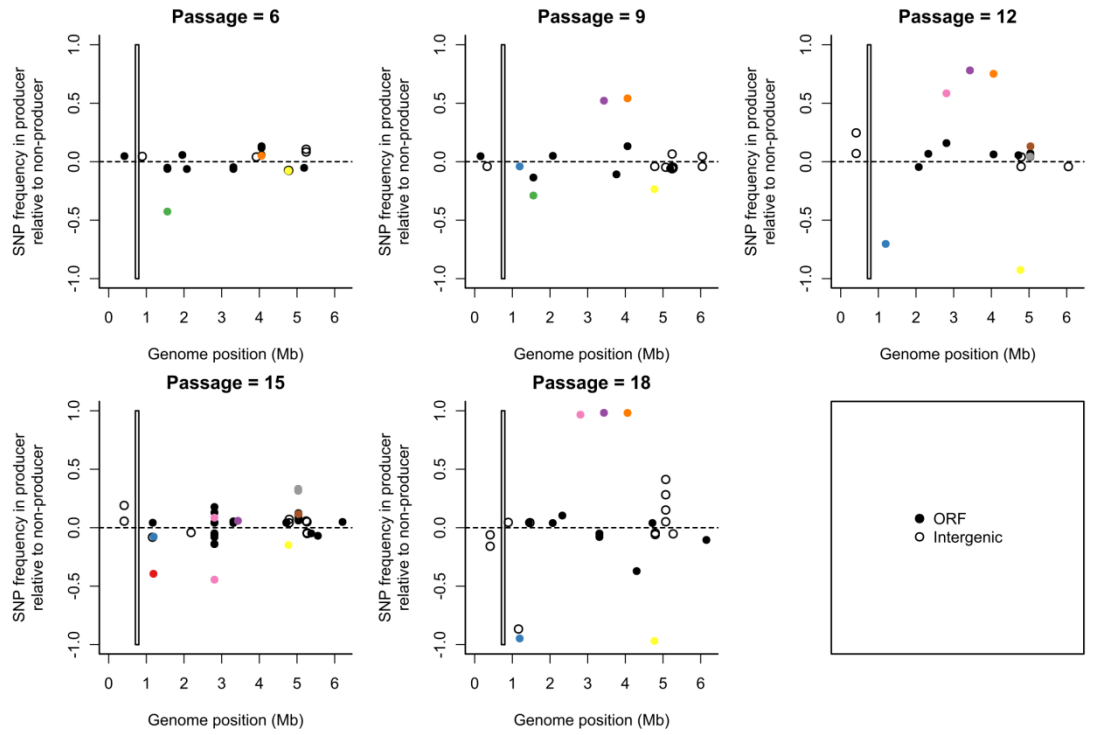

Figure 4.13: Difference in SNP frequency between producer and non-producer fractions at each timepoint. Filled points are those that are assigned to open reading frames (including putative proteins) of the PAO1 annotated genome. Only SNPs that were has a difference in frequency of magnitude greater than 0.04 (2 clones of 50) are shown for clarity. The majority of the large number of SNPs found in the shaded region were artefacts of alignment, and are not plotted. Difference is calculated as Producer frequency minus non-producer frequency. Coloured points have more detail shown in figure 4.14.

the different selective effects of SNPs. Neither the interaction between passage and fraction (Likelihood ratio = 0.143, p >0.5) or the main effect of fraction (Likelihood ratio = 2.022, p >0.1) were significant. However passage has a significant positive effect on SNP frequency ($F_{1,2411} = 16.464$, $p < 0.01$). This indicates that SNPs each on average each SNP is spreading at a similar frequency in the producer and non-producer fractions of the selection line.

	Maximum frequency

PA number	Gene	Genome coordinate	Nucleotide change	Producer	Non-Producer
No gene	No gene	411126	C→A	0.247	0.159
No gene	No gene	1163079	C→T	0.002	0.867
PA1097:	*fleQ* Transcriptional regulator	1188336	G→T	0.174	0.569
PA1102:	*fliG* motor switch protein	1195026	G→T	0.002	0.947
PA1430:	*lasR* QS receptor	1558556	G→T	0.000	0.427
PA2492:	*mexT* Transcriptional regulator	2807871	G→T	1.000	0.210
PA2492:	*mexT* Transcriptional regulator	2808022	C→T	0.175	0.619
PA3064:	*pelA* Cell wall LPS	3431725	C→A	1.000	0.236
PA3622:	*rpoS* sigma factor	4057995	C→A	1.000	0.207
PA3839:	Probable $NaSO_4$ symporter	4300264	G→A	0.004	0.371
PA4269:	*rpoC* RNA Pol β' subunit	4772400	C→A	0.053	0.969
PA4496:	Probable transporter	5031740	G→A	0.333	0.011
PA4496:	Probable transporter	5031743	A→G	0.343	0.010
PA4496:	Probable transporter	5031763	T→C	0.330	0.015
No gene	No gene	5036902	C→A	1.000	1.000
PA4500:	Probable transporter	5038674	G→T	0.708	0.592
No gene	No gene	5071546	T→G	0.282	0.037
No gene	No gene	5071547	G→A	0.413	0.048

Table 4.3: Information about the 18 SNPs that were found rose to a frequency greater than 20%. Shaded genes are shown in figure 4.13, lighter shading inicates multiple SNPs in a single gene.

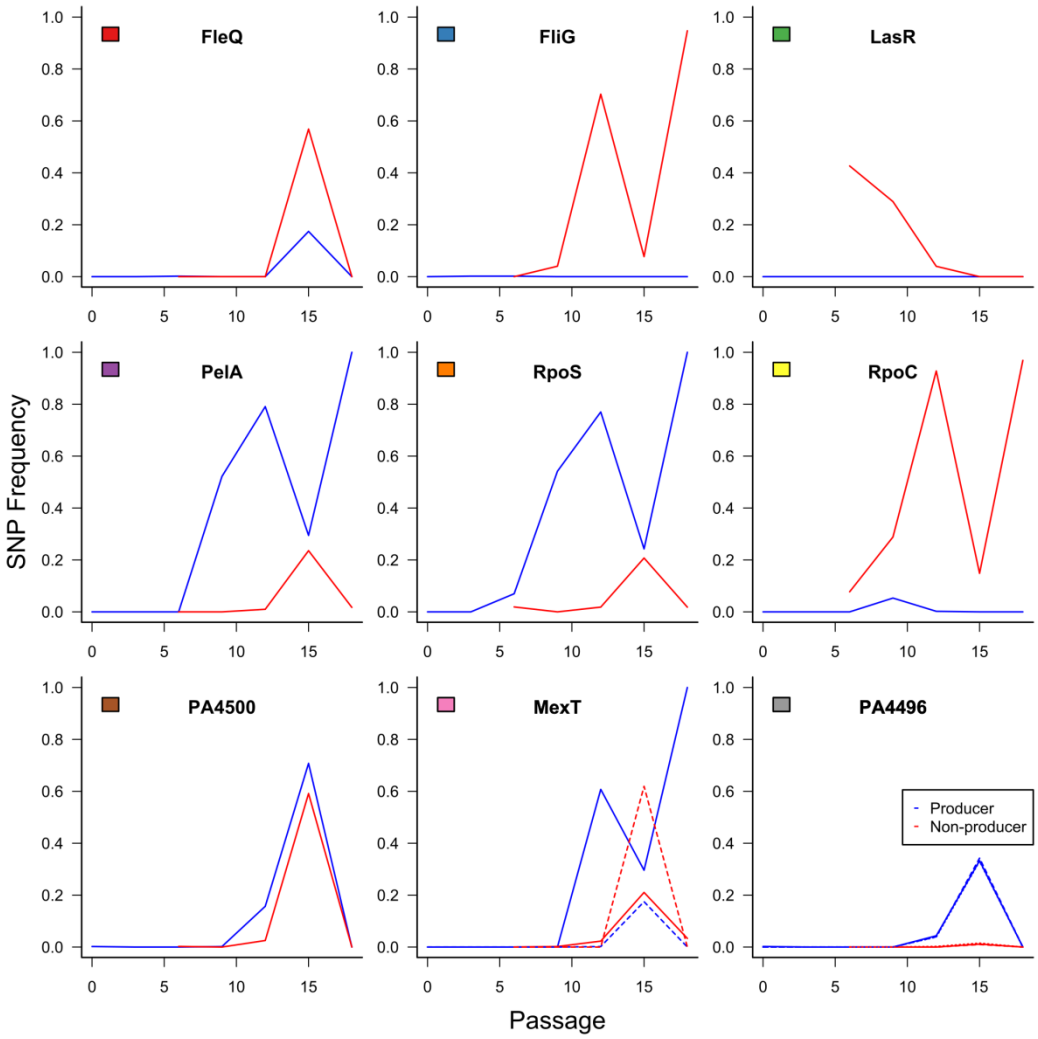

Figure 4.14: Change in frequency of SNPs in open reading frames and rose to high frequency. SNP frequency in producer and non-producer fractions is shown as blue and red lines respectively, colours in the top left are the colouring of points in figure 4.11. *mexT* and PA4496 were had several SNPs that rose to high frequency (two and three respectively) which are shown by different dash styles.

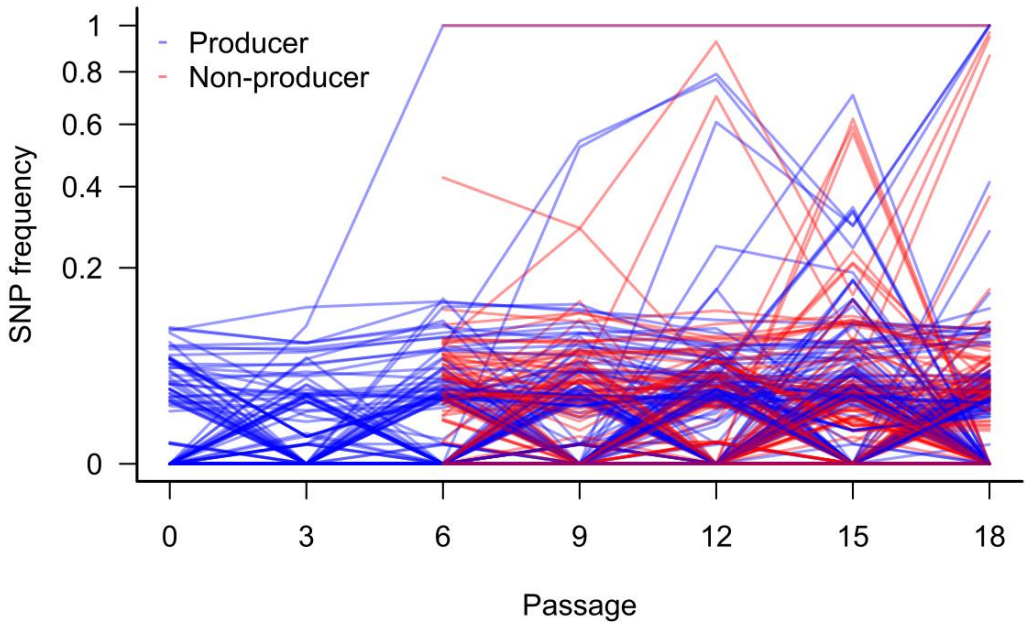

Figure 4.15: The frequencies of 293 SNPs in the producer and non-producer fractions of line 16 during selection. SNP frequency in each fraction is relative to the total proportion of that fraction rather than the total frequency in the population as a whole. SNP frequency is on a log scale.

4.4 Discussion

Using experimental evolution, this chapter shows that the quorum sensing network can adapt to environments where it is required to switch on early. Overall the data suggest that adaptations conferring early QS expression are dependent on the nutrient environment. Increased growth in PrM is reliant on greater protease production, driven by increased C12 (and probably C4) production at low density. In contrast although there is adaptation to growth in AdM, this is likely to be the result of increased expression of *nuh* independent of signal. Sequencing of selection lines shows that many of the mutations that were selected for under these conditions have roles in motility, biofilm formation and virulence regulation. As has been shown before emergence and spread of cheats hinders adaptation at the population level in environments where public goods are required (Sandoz *et al.*, 2007).

The pattern of SNPs found in cheats and cooperators suggests that the emergence of cheat lineages occurs repeatedly by de novo mutations throughout the selection experiment.

The 24 selection lines show a lot of diversity in their ability to adapt to their selection environments. For lines evolved in PrM, selection in a shaken environment is the factor that has the biggest positive effect on growth (figure 4.3). It is noticeable however that shaken environments were initially the most hostile to growth of the WT (figure 4.2). The shaking contrast was intended to manipulate population structure and break up biofilms. However it may also increase signal movement away from cells reducing signal concentrations and thus QS induction. Shaking of cultures also probably increased aeration which may have increased signal degradation, and also allowed cultures to grow faster once carbon was available (Duetz, 2007). In adenosine media lines were more consistent in their ability to adapt to the environment. Although static environments led to slow growth initially (figure 4.2), by the end of the selection experiments most strains had evolved to grow better in adenosine media than the WT. Interestingly *ΔlasR* lines did not adapt well to PrM (4 out of 6 never grew to threshold), but the *ΔlasR* lines selected in AdM grew comparably or better than their WT counterparts by the end of selection (figure 4.3).

The ability of the selected lines to grow in the alternate media environment can be studied, to give an idea of how far downstream in QS regulation adaptations occur. Figure 4.5 shows that a significant positive relationship between growth of evolved strains in PrM and AdM only exists for populations selected in PrM. This indicates that whatever adaptations are being selected to increase growth in AdM, they are not affecting PrM growth. To elaborate on this, the data show that although C12 concentration is

predictive of growth in PrM (figure S4.4a), probably through signal effects on protease production (figure 4.9). There is no relationship between the concentration of either AHL molecule and growth in AdM. The effect of signal on PrM growth suggests that adaptations are in the QS system, in comparison adaptations to growth in AdM probably occur downstream of QS regulation.

Why are different mechanisms for adaptation occurring in these different environments. One possibility is that it has to do with the scale of interactions in the two media (Dandekar *et al.*, 2012; Mellbye and Schuster, 2011). As can be seen (figures 4.10 and 4.11) protease non-producing organisms emerge during selection and exploit producers, particularly overproducers (Ghoul *et al.*, 2014b; Jiricny *et al.*, 2010). Therefore for adaptation in PrM it may be beneficial to retain plasticity through QS control, in order to restrain protease production to when benefits are greatest (Darch *et al.*, 2012; Xavier *et al.*, 2011). For AdM, maximal use of adenosine is most beneficial because it cannot be exploited (Mellbye and Schuster, 2011; Dandekar *et al.*, 2012). However it would be particularly costly to upregulate the entire QS regulon so *nuh* may be upregulated independently of the rest of the QS regulon. In many cases lines selected in AdM still produce protease so the QS system is still functional but not altered to activate earlier.

Although cheats spread over time reducing growth in PrM and AdM (figure 4.5 and 4.11),. line 16 keeps cheats at a low frequency and growth in PrM (but not AdM) recovers. This recovery occurs around passage 15 and looking at sequencing data shows this is associated with several dramatic changes in SNP frequency, these include a dramatic increase in the frequency of 2 SNPs in the producer fraction and a decrease in the frequency of many SNPs that were at high frequency. The 2 SNPs were PA4500 and PA4496 which are

annotated as probable transporters (Winsor *et al.*, 2011), as little is known about these genes it is unknown at this point if they are the cause of the recovery in growth. However it is notable that early in selection a single SNP rapidly fixed in the population (which had not split into producers and non-producers), although this SNP is intergenic it is found 173 bp from the ATG codon of PA4500 (Winsor *et al.*, 2011) well within the limits for a probable promoter region.

Other genes that have been mutated in this line are shown in table 4.3. *fleQ*, the transcriptional upregulator of flagellar machinery (Dasgupta *et al.*, 2002) as well as *fliG*, an important component of the flagellum (Chevance and Hughes, 2008), rose to high frequency in the non-producer fraction of the selected line. Motility genes have been previously found to be mutated during selection for public goods, and it is hypothesised that reduced motility increases structure and therefore reduces exploitation (Kümmerli *et al.*, 2015). That being said it is strange that mutants appear in the non-producer fraction so it would be useful to know the exact effect of these mutations. A mutation in *pelA*, an important gene in expolysaccaride synthesis (Colvin *et al.*, 2013), fixed in the producer fraction of the selection line. At this point it is unknown if this mutation results in reduction or increase in PelA function but an increase in PelA activity leading to increased biofilm production is consistent with clumping as an adaptation to the environment.

One of the first genes mutated in the non-producer fraction was *lasR*, as has been previously observed (Sandoz *et al.*, 2007), however this allele declined over time suggesting that other mutations may better adapted to a cheat lifestyle in this environment. This may be because a *lasR* mutant will still produce some protease, but also deactivating the whole QS system will

reduce expression of other genes which may be beneficial (García-Contreras *et al.*, 2014; Goo *et al.*, 2012). A mutation in the *rpoC* gene (encoding the RNA polymerase β' subunit) spread and almost fixes within the non-producer fraction of line 16, mutants in this gene have been found during adaptation to the cystic fibrosis lung (Cramer *et al.*, 2011). It may be that this mutation affects transcription of certain genes but a dramatic change in function is unlikely as RpoC is an essential protein (Cramer *et al.*, 2011). Finaly new alleles of several transcriptional regulators reached high frequency. RpoS is an RNA polymerase sigma factor involved in regulation of stress responses, virulence and QS regulation,(Dong and Schellhorn, 2010; Schuster *et al.*, 2004). MexT is a transcription factor that negatively regulates *lasB* and *rhlA* expression through its upregulation of the MexEF-oprN efflux pump, as well as negatively affecting pyocyanin and type 3 secretion independent of efflux pumps (Tian *et al.*, 2009), notably the *mexT* gene has two mutant alleles that rose to high frequency one predominantly in the producer background and one in the non-producer background.

This work show that non-producer cheats are able to spread in the selection experiment. Sequencing highlights that several alleles are specific to either the producer or non-producer background (albeit in a single line), but what are the dynamics of cheat emergence and spread. Two extremes of mutation dynamics can be predicted, here referred to as professional cheat and amateur cheat hypotheses. Professional cheats arise and persist in the population gaining more adaptations to a cheating lifestyle and preventing other cheat lineages from spreading. This will lead to co-evolution of cheat and cooperator lineages as has recently been investigated (Kümmerli *et al.*, 2015). At the other extreme, amateur cheat lineages arise repeatedly from cooperator lineages throughout selection through *de novo* mutations

conferring a cheat phenotype. These lineages are short lived, being replaced by new amateur cheat lineages over time. Looking at high frequency SNPs shows several SNPs that are specific to cheat lineages supporting the professional cheat hypothesis but these are only the most common SNPs. Across all SNPs there is no difference in the spread of a SNP between producers and non-producers. This supports the amateur cheat hypothesis, with a process where cheats arise repeatedly by mutation moving cooperator SNPs into a cheat background at a frequency similar to the cooperator fraction. Overall these observations suggest that neither the professional or amateur cheat hypothesis is wholly correct, but that the reality is somewhere in between. Some mutations can spread in cheats to high frequency but there are still new cheat lineages that arise and spread.

A recent paper elegantly shows co-evolution of cheat and co-operator lines over the course of selection (Kümmerli *et al.*, 2015), but Kümmerli and colleagues used marked cheats inoculated at the start and so had poor ability to detect de novo cheats that arose from the cooperators during selection. Also the line chosen here for sequencing held cheats at very low proportions, which may represent a specific case of cooperators keeping preventing the further adaptation in cheats (Khare *et al.*, 2009).

4.5 Methods

4.5.1 Strains and Media

Preliminary analysis of the two media (figure 4.1) used 6 strains from a strain library provided by John Govan (Edinburgh), previous analysis (not included) of these strains has determined signal production and protease production of this library and strains were chosen based on the diversity of QS strategies. This data also includes the WT and *ΔlasR* strains that were used as ancestors for experimental evolution.

Selection lines were either inoculated with Nottingham strain of PA01 (referred to as WT) or with an isogenic *ΔlasR* knockout created by inserting a gentamycin resistance cassette in the *lasR* gene, neither strain had any other markers. These strains were produced by the Diggle lab in Nottingham and kindly provided (Popat *et al.*, 2012). For signal detection bio-reporters were used, *Escherichia coli* S17-1 with either p1142B or p56536 plasmids to detect short chain (C4) and long chain (3-oxo-C12) AHLs respectively (Winson *et al.*, 1998).

Specific media was designed for the selection experiment consisting of an M9 minimal media base (containing Na_2HPO_4, KH_2PO_4 NaCl and Nh_4Cl) with the addition of 1mg/ml MgSO4, 100μg/ml CaCl2, and Hutners trace elements (Hutner *et al.*, 1950) and adding 0.1% (w/v) BSA to make casamino acid free protein media (PrM). adding 0.2% (w/v) adenosine (Heurlier *et al.*, 2005) produced casamino acid free adenosine medium (AdM). These media thus consist of environments where all carbon requires QS controlled metabolism to utilise. Due to the slow bacterial growth and repeated use of these media they were kept incubated at 37°C to detect contamination.

4.5.2 Experimental evolution

Colonies of WT and *ΔlasR* was grown overnight in LB broth and then diluted 1 in 100 in fresh media to grow to mid exponential phase. Mid-exponential cultures of both genotypes were each washed in either AdM or PrM, then optical density (600nm) was measured and the four cultures were diluted to an optical density of 0.02. These cultures were aliquoted into six replicate tubes each at a volume of 3000μl each. Three tubes were incubated statically at 37°C while the other three were incubated with agitation (150rpm, 37mm orbit) at the same temperature in the same incubator.

Optical density thresholds were set for the 2 media, based on measured growth curves in figure 4.1 and corresponding to utilisation of the carbon source and significant detectable growth. These thresholds were 0.17 and 0.57 (600nm) for growth in AdM and PrM respectively (once blanks were taken into account). Each day all tubes were assessed by eye to see if the density was close to the threshold, these tubes were removed from the incubator and the optical density was measured to assure that it was above the threshold, and therefore ready to be passaged. If a line was not passaged for 14 days it was passaged after the 14th day of growth, this was done to prevent cultures from drying out and going extinct.

To passage lines, cultures were pelleted and resuspended twice in sterile media (either AdM or PrM), the optical density of the cultures was measured again and used to dilute lines to an optical density of 0.02 in 3000μl for the next passage. Every three passages, remaining culture was mixed in a 1:1 ratio with a mixture of LB and glycerol in a 1:1 ratio (i.e. 25% glycerol final) and archived at -80C.

4.5.3 Growth dynamics

To assay growth of both ancestors and evolved strains, growth curves were produced. Pure strains (i.e stocks of ancestors) were grown overnight in LB. Overnight cultures were then washed twice separately in either AdM and PrM. Cultures were not diluted and grown to mid exponential phase to minimise change in frequency in preparing cultures and to better mimic growth during passages.

The optical density (600nm) of the washed cultures was measured and used to dilute cultures to OD = 0.02. These culture were aliquoted in 200μl volumes in the central 60 wells a 96 well plate. These plates were incubated statically in a Varioskan™ plate reader (thermo scientific) and optical density

(600nm) was automatically measured every hour for 48 hours. For assays of the growth of the final evolutionary time points, a similar protocol was used however at each hour 5 reads were taken separated by agitation this agitation was minor and would not disrupt biofilms, but was intended to move large clumps. Mean values of the 5 reads were used to produce growth curves but the variance of the 5 reads gives a crude measurement of the amount of clumping.

4.5.4 Signal concentration measures

Cultures were set up as in 4.5.4 but were incubated for six hours in PrM' at which point they were removed from the plate reader. Pilot experiments also tried using AdM, LB and defined rich medium as the initial growth medium but levels of signal were too low or growth was not sufficiently similar enough across samples (data not shown). Culture from 2 replicate wells was pooled (400µl) and filtered through a 0.22µm syringe filter to remove bacterial cells. Cell free supernatants were diluted 1/10 in LB. Signal stocks at known concentrations were used to make a dilution series for calibration.

In parallel, bio-reporter cultures were grown overnight, diluted and grown to mid-exponential phase. Plasmids p1142B or p56536 were maintained during pre-culture by addition of tobramycin or Ampicillin respectively. Diluted cell free supernatants and signal dilution series were mixed 1:1 with cultures of bioreporters at an optical density (600nm) of 0.1. These were then incubated at 37^{o}C in a Varioskan^{TM} plate reader for 3 hours, taking readings of luminescence and optical density (600nm) every 15 minutes.

4.5.5 Protease concentration measures

Supernatent was extracted from the same cultures as signal measures (4.2.5). 50µl of supernatent was diluted 1/10 in 20mg/ml elastin congo red (ECR) in elastin congo red buffer (100mM Tris, 1mM $CaCl_2$, pH=7) in centrifuge tubes.

These were incubated on their sides at 37°C with agitation in an orbital shaker (150rpm, 37mm orbit). Blanks containing fresh media were also incubated. Protease will cleave the elastin congo red so that the congo red dye becomes soluble. After 24 hours tubes were removed and centrifuged (13,000rpm, 1 minute). 200µl of supernatant was then removed and absorbance was measured at 495nm (Ohman *et al.*, 1980).

4.5.6 Colony morphology assays

To assay within population diversity evolved populations were defrosted and inoculated into 4ml of LB broth which was incubated with shaking (150rpm, 37mm orbit) for 8-10 hours. The optical density (600nm) of cultures was measured and used to dilute cultures to an optical density of $1x10^{-6}$, and then 50µl was plated onto replicate skimmed milk plates, and left to grow for 18-24 hours. The presence of a completely translucent halo was used to identify protease producing strains (Markussen *et al.*, 2014).

Skimmed milk agar was made by making 2x strength LB agar and autoclaving it under standard conditions (121°C, 15 minutes). Skimmed milk was made up from powdered milk (10% w/v) and autoclaved under milder settings (115°C, 10 minutes). Agar was melted in a microwave and then both agar and milk were brought to 55°C and thoroughly mixed at a 1:1 ratio before being poured into Petri dishes. Plates were checked for sterility by incubating a test plate overnight t 37°C.

4.5.7 Separating protease producer and non-producer phenotypes

Multiple pates with clearly visible single colonies for each sample were produced as above. These were visualised by eye and colonies were picked into a high and low producer groups based on the size of halos. This

corresponded to WT halo size (completely translucent halo clearly extending beyond the edge of the colony) and *ΔlasR* halo size (faint halo not extending beyond the edge of the colony). 60 colonies of each type were picked into 60 wells of a 96 well plate filled with 200µl of 1/3 strength LB broth using toothpicks. After inoculating liquid culture the toothpick was stabbed into a second agar plate so that the 60 colonies were equally spaced in a grid. Liquid cultures were grown overnight with agitation (250rpm, 37mm, 37°C) while agar plates were incubated statically at 37°C overnight.

After incubation, agar plates were observed to verify that the colony morphology phenotype (presence of halo) was correct for the 60 colonies on the grid, showing both accuracy of colony selection and heritability of phenotype. Fifty colonies were selected based on accurate morphology. Where more than fifty colonies were available, colonies at the edge of the agar plate were dismissed. The optical density (600nm) was measured and the 50 wells corresponding to the selected colonies were pooled, correcting for changes in growth. Distinct colony morphologies were tested on *Pseudomonas* isolation agar to confirm they were *P. aeruginosa*.

4.5.8 PCR

To further test potential contaminants PCR diagnostics were performed. Checking for the amplification and size of the *ecfX*, *gyrA* genes and the rRNA internal transcribed spacer according to the protocol in (Lavenir *et al.*, 2007). Briefly the 3 pairs of upstream and downstream primers used by lavenir and colleagues were ordered (from Integrated DNA Technologies) and were used with GoTaq® flexi according to manufacturer's instructions and using annealing temperatures and $MgCl_2$ concentrations from (Lavenir *et al.*, 2007). PCR products were visualised using gel electrophoresis stained with ethidium bromide.

4.5.9 DNA extraction

DNA was extracted using a QIAmp DNA extraction kit (Quiagen), following the manufacturers protocol for bacterial samples. DNA samples to be sent for sequencing were checked for quality by gel electrophoresis and measuring the 230/260nm infra-red ratio using a nano-drop (fischer scientific). DNA concentration was estimated using a nano-drop but verified using fluorescence measurements from a Qubit (Invitrogen).

4.5.10 Sequencing and Bioinformatics

Further preparation of DNA samples and sequencing was performed by Edinburgh Genomics. Briefly, bar-coded libraries were produced using PCR-free library preparation (550bp) and run together in a single lane of a HiSeq v4 sequencer using 125 base paired-end sequencing. Basic bioinformatics was performed by Edinburgh Genomics. Briefly, reads were aligned to the *P. aeruginosa* genome (Stover *et al.*, 2000). Variants were then called using Popoolation 2 software (Kofler *et al.*, 2011). This generated a table with the details of each SNP that differed from the reference genome, associated with the nucleotide change, as well as the frequency and coverage of the SNP in each sample, this data was returned to me. Where SNPs were in open reading frames the PA number of the relevant gene was also given

With the table the number of SNPs was reduced according to 2 criteria. Firstly coverage of the SNP had to exceed 10 reads in each sample, second all SNPs within the region of 710,000 and 800,000bp were excluded because of a known deletion in this region of the NPA01 genome which made alignment difficult and generated false SNPs. This left a total of 293 SNPs for analysis.

4.5.11 Data analysis

Maximal models for the effects of evolutionary treatments (as well as their second order interactions) on phenotypes of growth and signalling were

reduced to minimal models using the dredge function in the MuMIn package (Barton, 2011). Models of the correlation between measured phenotypes were produced by starting with sensible maximal models based on plots of the data. These simpler models were reduced manually. The frequency of phenotypes within the population was modelled using a quasibinomal model (as dispersion was high). Where data included large numbers of zero's binomial models were not possible. To assess the pattern of SNPs the subset of 268 SNPs that were polymorphic in at least one of the two fractions between 6 passages and 18 passages was used (where both producer and non-producer fractions were available). A mixed effects model was fitted (using lme in the nlme package, Pinheiro *et al.*, 2015) that assessed SNP frequency between passage 6 and passage 18 as predicted by passage, fraction and an interaction between the two. A random effect of SNP on passage was included to account for the different selective effects of SNPs. In all cases statistics are taken from analyses of variance of minimal models. Binomial models failed to converge so a model with a Gaussian distribution was used with the square root of SNP frequency to account for large number of low frequencies.

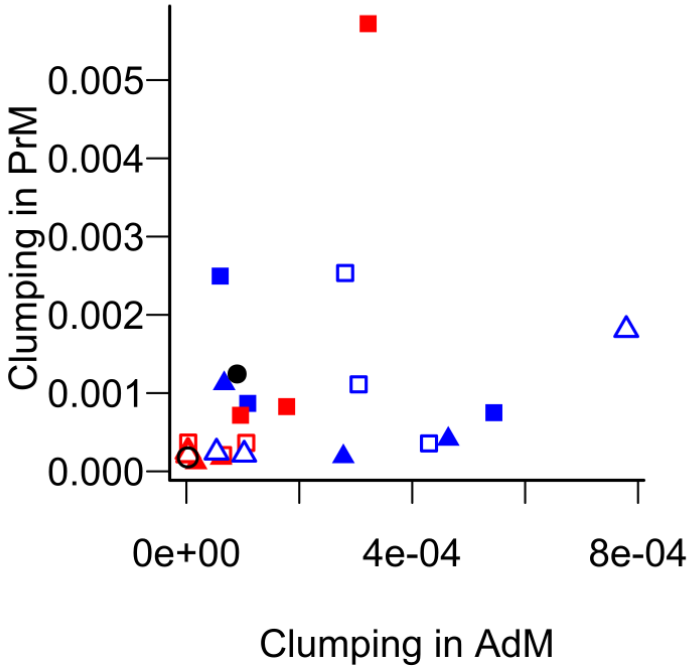

Figure S4.1: Correlation of clumping in the two selection media. Growth integrals of end points of selection in PrM and AdM. Points are coloured by the environment they were selected in. Squares and triangles are shaken and static selection environments respectively. Filled and Hollow points are WT and *ΔlasR* ancestors respectively. There is no significant relationship between the two.

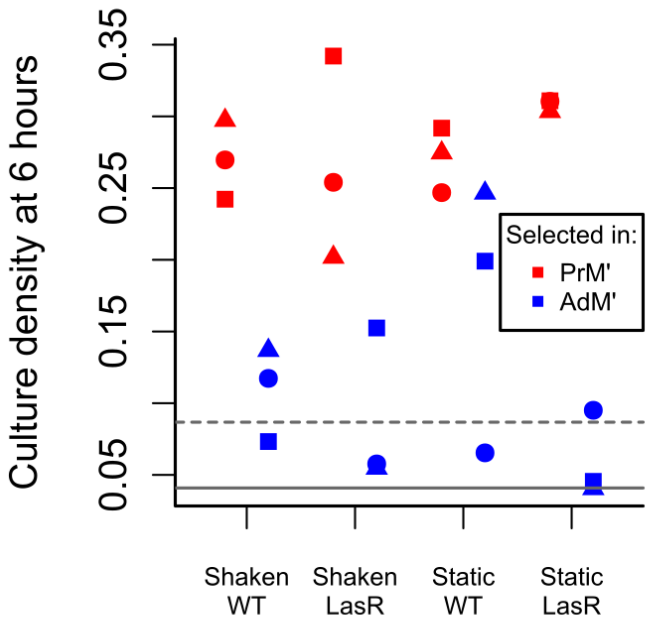

Figure S4.2: Density of cultures used to measure protease activity and signal concentrations. Cultures were grown for 6 hours in PrM, points are the mean optical density of the two 200µl wells pooled to get supernatent. First, second and third replicates use circles, triangles and squares respectively. Solid lines are the mean of 3 replicates for growth of the WT ancestor, dashed lines are the mean of three replicates for the ΔLasR ancestor.

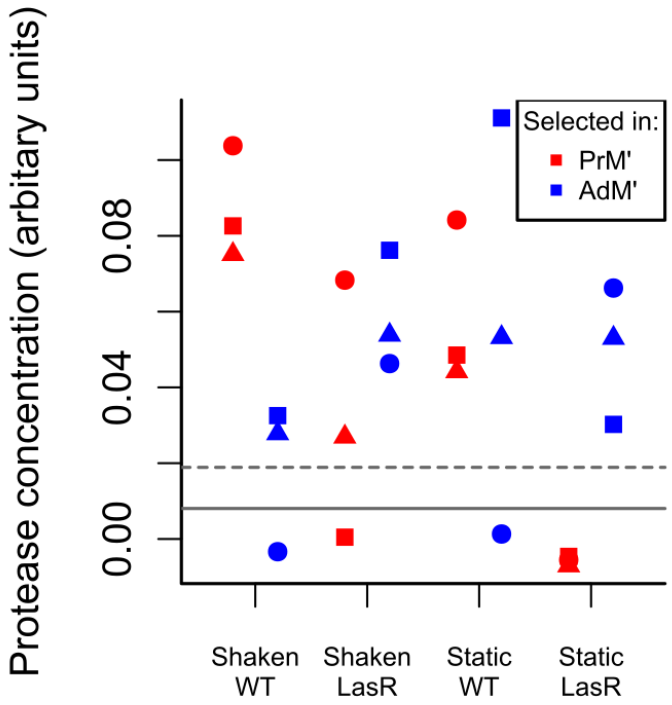

Figure S4.3: Protease activity of the end points of selection. Protease activity of cultures grown for 6 hours in PrM was measured using and elastin congo red assay. First, second and third replicates use circles, triangles and squares respectively. Solid lines are the mean of 3 replicates of the WT ancestor, dashed lines are the mean of three replicates of the ΔLasR ancestor.

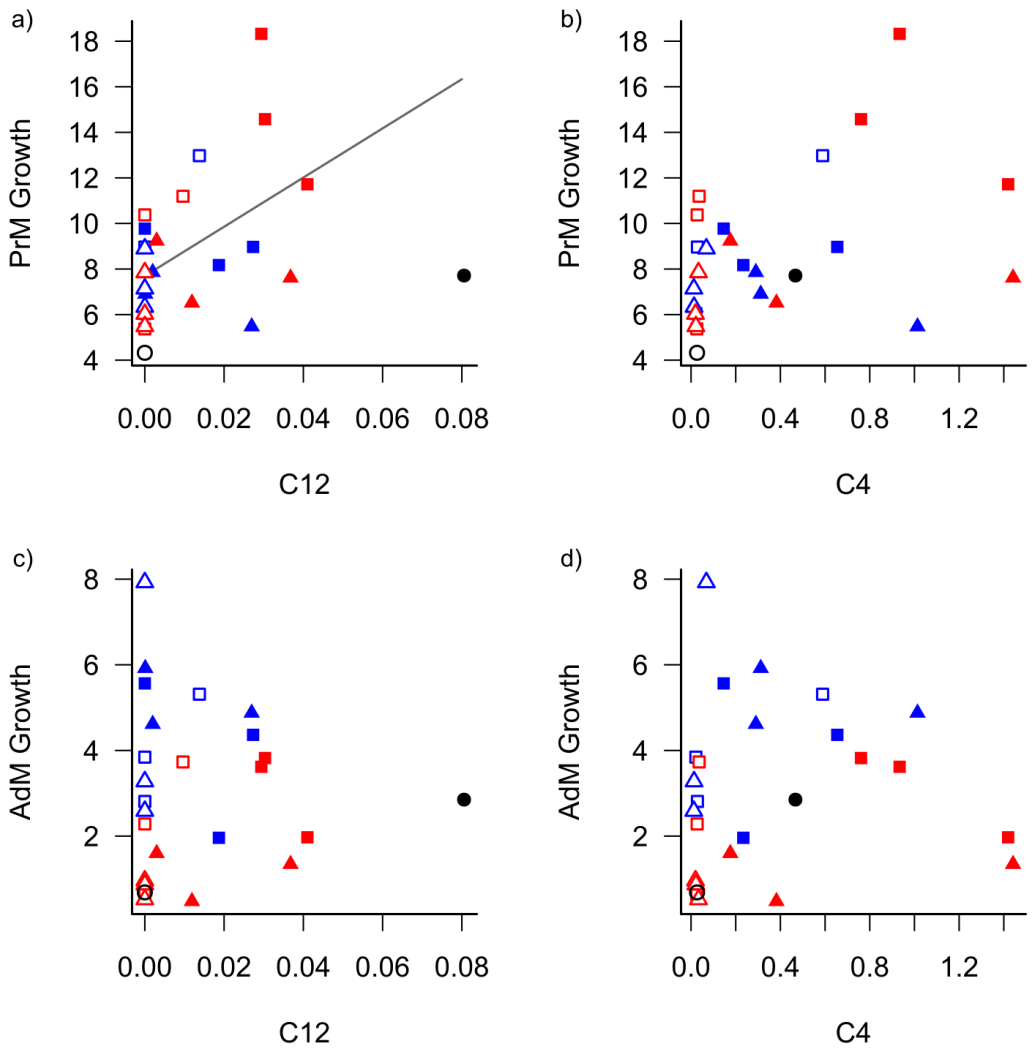

Figure S4.4: Correlation of signal concentration on growth. Growth integrals of end points of selection in PrM (a & b) and AdM (c & d) against concentration of C12 (a & c) and C4 (b & d). Points are coloured by the environment they were selected in. Squares and triangles are shaken and static selection environments respectively. Filled and Hollow points are WT and *ΔlasR* ancestors respectively. There is only a significant relationship between C12 concentration and growth in PrM, shown by the grey line in (a).