Communication, Cooperation & Conflict in Quorum Sensing Bacteria

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Abstract:

The scientific community has gathered an extremely detailed and sophisticated understanding of the genetic and molecular underpinnings of microbial communication. How these microbial communication systems arise and are maintained over evolutionary timescales however has received relatively little attention. Some major questions remain unanswered such as; what is the function of small diffusible molecules? How does population structure affect the dynamics of social communication and what is the link between the ecology of communication and the virulence of a pathogenic population? Borrowing concepts from evolutionary theory can help to unravel these fundamental questions in the context of microbial communication as it has done in other taxa displaying social behaviours. In addition microbial model organisms in which molecular and genetic tools are abundant lend enormous power to empirical tests of evolutionary theory. This work combines both of these in an attempt to understand the evolution of bacterial communication using the model organism *Pseudomonas aeruginosa* and its well characterised Quorum Sensing systems. Specifically the focus is in three areas. Firstly this study reveals that the stability of bacterial signalling is vulnerable to perturbations in cost and benefit and genetic conflict. Secondly this study finds that spatial structure (biofilm vs planktonic) influences the outcome of social competition over signalling and reduces population viability. Thirdly this study finds that interspecific and intraspecific competition over public goods impose divergent selective pressures on communication.

Publications Arising

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Liu X, Jia J, **Popat R**, Ortori CA, Li J, Diggle SP, Gao K, Camara M. (2011) Characterisation of two quorum sensing systems in the endophytic *Serratia plymuthica* strain G3: differential control of motility and biofilm formation according to life-style. BMC Microbiol. 11(1):26.

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Definitions of Key Terms

Quorum Sensing, the production and detection of small diffusible molecules produced shared and detected by cells within a population often thought to allow cells to monitor population density.

Cost & Benefit, implying a cost or benefit to the fitness of an individual.

Social Behaviour, a behaviour with fitness consequences for both actor and recipient.

Cooperation, a social behaviour with a positive fitness effect on the recipient. e.g. public goods.

Public goods, a costly behaviour that benefits individuals other than the actor, often used to describe microbial secretions that favourably modify the environment.

Communication, an act or structure produced by one organism (actor) which influences the behaviour of another organism (recipient). This can be further subdivided into:

Signal, a communication by an actor that has evolved owing to its effect on the recipient and to which the response has also evolved.

Cue, a communication to which response of the recipient has evolved.

Coercion, a communication which has evolved due to its effect on the actor.

Signals must on average benefit both actor and recipient in order to be maintained by selection over generations.

Chapter 1: Introduction

1.1 Quorum Sensing

1.1.1 What is Quorum Sensing?

Konrad Lorenz, the Austrian born founding father of ethology (the study of animal behaviour), achieved a long cherished dream of mankind, the ability to speak to animals. Lorenz listened to, recorded and learnt to emulate the calls of migrating geese in his local countryside (Bernwand Droscher 1964). Realising that these calls were issued in repeats of varying lengths he conducted field experiments in which he would issue the calls himself and observe the response of flocks of geese. In this way he was able to determine that the more repeats in each call, the faster the geese moved. The calling seemed to be a way in which to communicate to the flock the intention of moving, either slowly in a favourable grazing patch, and more quickly perhaps to move from an unfavourable grazing patch and sometimes very quickly to take off and move away altogether.

A few decades later in the laboratories of Nealson and Hastings a luminescent marine bacterium '*Photobacterium fischeri*' was being cultured. Nealson and colleagues discovered that they could induce this luminescence at lower population densities when it was grown in spent supernatant from a stationary phase culture (Nealson et al. 1970; Nealson & Markovitz 1970). This led to the purification and characterisation of *N*-(3-oxohexanoyl)-homoserine lactone (3-oxo-C6-HSL) a member of the *N*-acyl homoserine lactone (AHL) family of molecules (Eberhard et al. 1981). Using 3-oxo-C6-HSL it was possible to induce the luminescence of mutants incapable of producing the molecule and luminescing in a way analogous to the 'playback' experiments of Lorenz.

Since the times of Lorenz and Nealson, scientists have uncovered an enormous diversity of communication systems in nature. It is difficult to imagine an organism completely devoid of communication. Bacterial communication of the sort described by Nealson is often termed Quorum Sensing (QS), a term coined by Fuqua and colleagues (W. Fuqua et al. 1994). This bacterial behaviour

seemed similar to a legal quorum, a threshold number of people required to be present before a motion can be passed. Although initially thought to be a phenomenon restricted to a few marine *Vibrio* species, it was later shown that the production of the β-lactam antibiotic, 1-carbapen-2-em-3-carboxylic acid (carbapenem) by the terrestrial plant pathogen *Erwinia carotovora* was also regulated by 3-oxo-C6-HSL (Bainton, Bycroft, et al. 1992a; Bainton, Stead, et al. 1992b). Many Gram-negative species have been shown to possess AHL-signalling systems that regulate a wide variety of different phenotypes (Williams et al. 2007; Williams 2007). QS is not restricted to Gram-negative bacteria; a number of Gram-positive organisms employ small, modified oligopeptides as extracellular QS molecules. These peptides activate gene expression by interacting with two-component histidine protein kinase signal transduction systems. For example, in *Staphylococcus aureus*, the expression of a number of cell density-dependent virulence factors is regulated by the global regulatory locus *agr* (Peng et al. 1988). The fervent search for molecules involved in bacterial intercellular communication has been so productive that a lexicon of compounds have now been described as QS signal molecules in both Gram-negative and Gram-positive bacteria (Lyon & Richard P Novick 2004; Williams et al. 2007). Some authors have cautioned against labelling them all as QS molecules (Winzer et al. 2002). This raises a central and often underestimated problem in this field. What is QS and what is it's function? Closely related, how should we go about classifying and identifying communication in nature?

1.1.2 Common features of QS systems

Bacteria produce a vast array of extracellular products and conditioned medium is likely to change the behaviour of cells. Some of these exoproducts are toxins, metabolites or extracellular enzymes all of which are expected to change the behaviour and gene expression of the cells from which they originate. To distinguish these from molecules used in communication four criteria have been suggested (Winzer et al. 2002). It was suggested that a cell-to-cell signal molecule (CCSM) is one;

1. whose production occurs during specific phases of growth, under certain physiological conditions or in response to changes in the environment.

2. which accumulates extracellularly and is recognised by a specific receptor.

3. whose accumulation generates a concerted response once a threshold concentration has been reached.

4. where the response extends beyond the physiological changes required to metabolise or detoxify the compound.

The last criterion is of particular importance. It allows us to distinguish between CCSM's and other cellular responses such as the metabolism of lactose (Monod 1961; Miller 1980) or the conversion of tryptophan to indole (Baca-DeLancey & South 1999; Wang & Ding 2001) by *Escherichia coli.* The response of *E. coli* to lactose or indole does not extend beyond the handling of those particular stimuli though the rest of Winzer et. al.'s criteria are met. Even more subtle a distinction comes when we consider the production of autoinducer 2 (AI-2) in *E. coli* and its role in gene regulation. Although AI-2 meets the criteria for a CCSM in *Vibrio harveyi* (Bassler et al. 1994; Bassler 1999), it is also widely conserved among bacterial species causing speculation that it may be a interspecific communication molecule (Bassler & Greenberg 1997; Bassler 1999; Surette & B. L. Bassler 1998). For example in *E. coli,* a microarray study comparing gene expression in an O157:H7 wild type and a *luxS* mutant, unable to produce AI-2, found that more than 400 genes were up or down regulated in the mutant (Sperandio et al. 2001; DeLisa et al. 2001). The conclusion that this constitutes a global regulatory signalling system however may be unjustified. The LuxS protein also has a metabolic role in the bacterial cell. It is responsible for the conversion of *S*-ribosylhomocysteine (SRH, derived from *S*-adenosylhomoserine a byproduct of cellular methylation reactions involving S-adenosylmethionine (SAM)) into homocysteine and the ribose derivative, AI-2 thus preventing potent

inhibition of methyl transferases and recycling metabolites. It is unsurprising therefore that when *luxS* is mutated, such an important metabolic process has wide implications for genomic expression patterns. In addition the action of AI-2 in *E. coli* has not been shown to occur via a specific receptor casting further doubt as to its identity as an interspecies communication molecule (Rezzonico 2008). Later in this chapter we will revisit the definitions of communication from an evolutionary perspective, supplying further clarity on what is and what is not a signal.

In addition to Winzer et. al.'s criteria for CCSM's, it is interesting to note that many QS systems in Gram positive and Gram negative bacteria utilise autoinduction in some form. In the context of QS this means that the part of the response to the QS molecule is upregulation of synthesis of the molecule (Fig 1.1).

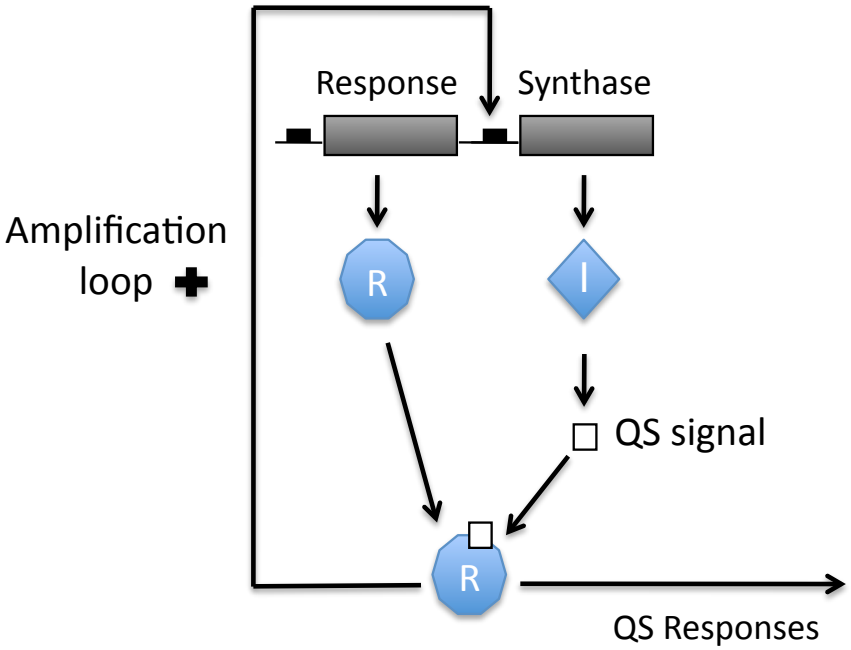


Figure 1.1: A schematic drawing of typical autoinduction in QS. Though the exact circuit may vary, this is a common feature of QS systems. The signal binds to and activates its cognate receptor forming a transcriptional regulator which among other things, induces the biosythesis of more QS molecule.

Pseudomonas aeruginosa has three QS systems which all utilise autoinduction, and these are described in greater detail below. Analogous to the AHL-based systems of *P. aeruginosa, Vibrio* species utilise the *luxIR* QS system in which AHL's activate their cognate transcriptional and upregulate the *luxI* synthase genes. Staphylococci utilise a QS system based on auto inducing peptides (AIP's). The *agr* operon encodes the synthesis of a peptide containing a thiolactone ring (AIP) via AgrD and AgrB (Novick et al. 1995). The AIP binds to AgrC on the cell surface causing signal transduction through phosphorylation of AgrA which induces the expression of the effector, a regulatory RNA, RNAIII (Novick et al. 1993; Novick et al. 2000). Streptococci utilise a QS system analogous to the *agr* system of Staphylococci revolving around the CSP peptides and sharing autoinduction as a common feature (Li et al. 2002). Not all Gram-positive bacteria with *agr* homologues show evidence of autoinduction. For example, the *agr* locus in *Listeria monocytogenes* is transcribed equally in mid exponential and early stationary phase of growth (Garmyn et al. 2009). In addition the ComX intercellular communication molecule in *Bacillus* species does not regulate its own production (Schneider & Palmer 2002; Auchtung & Lee 2006).

There is a diversity of different QS systems and wherever homologues are sought and found in other species, a diversity of function is uncovered. For example AI-2 can be considered a signal in *V. harveyi* where its autoinduction occurs via a specific receptor but its homologous system in *E. coli* does not regulate transcription through a specific receptor and does not cause autoinduction. It is possible that core metabolic processes can be exaggerated through evolution and subsequently gain new functions as intercellular signals. The diversity of QS systems that has been identified raises some interesting questions as to their design. For instance when is autoinduction found? Does this relate to the functionality of QS systems (e.g. metabolic vs. signal)? Given the vast number of studies on QS in a variety of species a comparative approach may aid us in understanding the relationship between their design (genetic and molecular architecture) and functionality (role in nature).

1.1.3 Diffusion or Quorum Sensing?

Much of the rhetoric used to describe QS alludes to a population wide coordination of physiology and behaviour, often surmounting challenges that would be impossible to achieve with a single cell. The implicit assumption is that such population wide coordination is adaptive, and that the function of QS is to sense population density. This has been challenged on the basis that a single cell could benefit by sensing diffusion rate which if sufficient could negate the benefits of costly secretion, and this has been termed Diffusion Sensing (DS, Redfield 2002). In fact it has been shown that a single cell when confined to a small compartment can become quorate (Qazi et. al. 2001, Boedicker et al. 2009; Hagen et al. 2010), though the biological relevance or adaptivity of such single celled QS remains to be empirically examined. Since QS is inherently social and DS is inherently asocial, there has been rigorous debate about which is the true function of these small diffusible signals in nature (Hense et al. 2007).

Despite the apparent conflict between these two hypotheses they can be reconciled in a number of ways. Firstly Hense et. al. suggest that bacterial cells are unable to resolve population density and diffusion rate (Hense et al. 2007). Instead they respond to conflated information about both. In either case it is adaptive to respond when signal concentrations are sufficiently high. The authors call this efficiency sensing (ES) and contextualise it by suggesting that QS and DS can be seen as extreme cases of ES. Another unification of QS and DS can be achieved via consideration of two signal molecules simultaneously. If the two signals have different stabilities in the extracellular environment and the organism is able to respond not only to concentration but to the ratio of the two, this is sufficient for the resolution of diffusion and population density independent of each other (Brown, SP. personal communication).

Though it is possible that a single cell can benefit from sensing diffusion, it has been both theoretically and empirically observed that a defecting strategy (QS

non-responding or non-producing) gains an advantage over the resident QS cooperating strategy in the population due to social exploitation of public goods regulated by QS (Brown & Johnstone 2001; Diggle et al. 2007a; Sandoz et al. 2007; Czárán & Hoekstra 2009; Wilder et al. 2011). Thus even if the primary function of QS were sensing diffusion, the inevitable social conflict requires that its maintenance be explained in terms beyond the direct benefit to a single cell. Thus another way of reconciling QS and DS is to say that in the evolutionary pathway to QS, DS is the first step. DS emerges readily as it provides a direct benefit to participant cells. As soon as two cells have adopted this strategy its benefits now depend on the strategy of others in the population as well as the need to sense diffusion. This need for a resolution to social conflict is a hallmark of a social trait. This will be elaborated later in the introduction.

Recent work in our laboratory has set out to test the central assumption of QS in that response to high population density is beneficial (Darch, West, Winzer, Diggle, unpublished). A signal negative *lasI* mutant of *P. aeruginosa* was grown to high or low population density. The subsequent addition of signal allowed the population access to QS dependent nutrients in the medium. The addition of signal resulted in a higher proportional growth increase at high than at low population densities. Experiments such as this are long overdue and will aid us in understanding the role of QS in nature.

1.1.4 QS in P. aeruginosa

Pseudomonas aeruginosa employs three interlinked QS systems that regulate more than 500 genes, and at least 6 % of the genome (Arevalo-Ferro et al. 2003; Schuster et al. 2003; Wagner et al. 2003; Schuster & Greenberg 2006). All involve the production of small diffusible molecules that elicit responses when a threshold concentration of the molecules and therefore population density, is reached (Pesci et al. 1999; C. Fuqua et al. 2001). The *las* and *rhl* QS systems each have an AHL signal synthase and an AHL binding response regulator (Fig. 1.2). The response regulator is activated by binding to the AHL signal molecule to form a transcriptional regulator whose role is both to complete the positive

feedback loop and to drive the expression of the rest of the target genes (Fuqua et al. 1994).

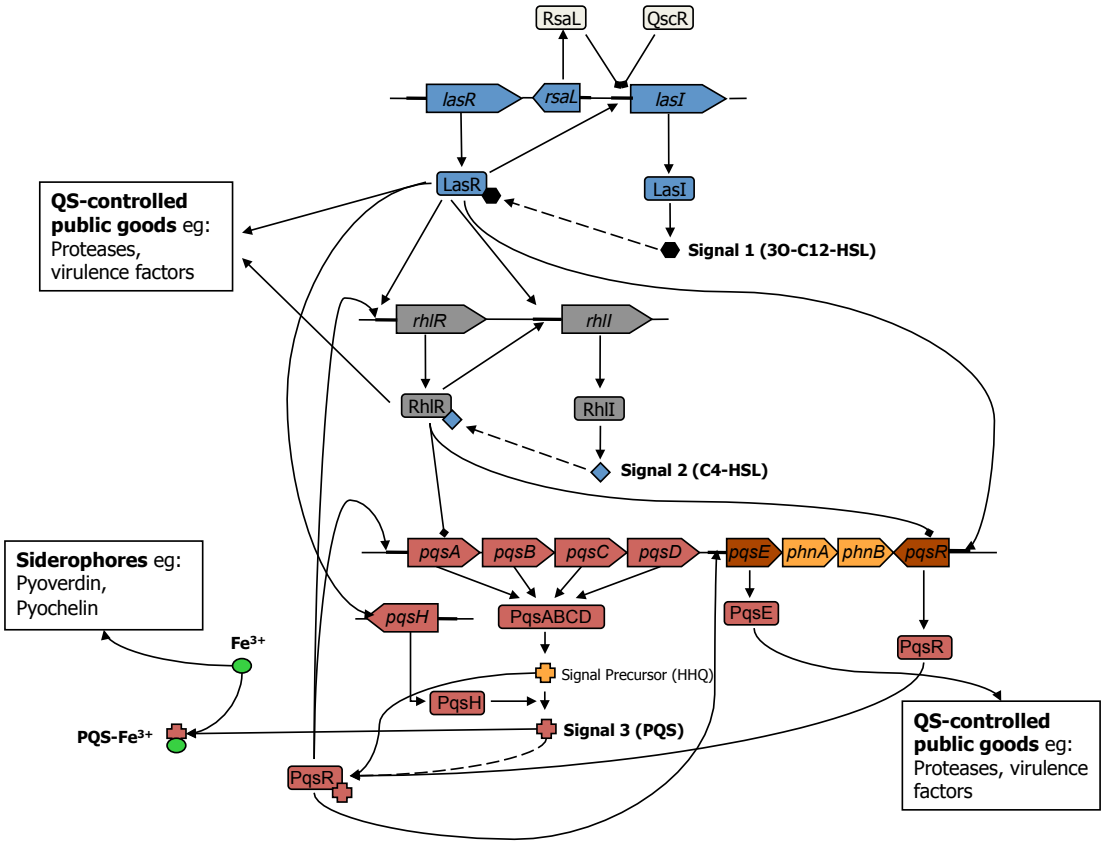


Figure 1.2: Schematic diagram of *P. aeruginosa* QS. A critical concentration of LasR/3O-C12-HSL induces expression of *rhlR* and the *rhl* quorum sensing system. AHL and AQ-dependent QS are linked since LasR/3-oxo-C12-HSL is required for expression of *pqsH*, while *pqsR* is positively regulated by LasR/3-oxo-C12-HSL. *pqsA* and *pqsR* are repressed by the action of RhlR/C4-HSL. HHQ is produced via *pqsA* itself regulated by *pqsR*. PQS and HHQ induce the expression of *pqsA* in a PqsR-dependent manner. Arrows represent positive regulation; Diamond heads represent negative regulation.

The behavioural responses to threshold concentrations of AHL include; the production of virulence factors that coordinate an attack on the host (Winzer & Williams 2001), the production of surfactants that aid social motility across a surface (Medina et al. 2003) and the production of the structural components of a cohesive biofilm community (Davies et al. 1998). The third QS system employs the Pseudomonas Quinolone Signal (PQS). The products of the *pqsABCD* operon direct the production of the signal precursor HHQ which is converted to PQS via the action of PqsH (Fig. 1.2). The PqsR-PQS and PqsR-HHQ receptor signal complexes both upregulate the expression of the *pqsABCD* operon as well as the effector PqsE which, although an enzyme has a regulatory influence on a host of genes including virulence factors (Diggle et al. 2003; Déziel et al. 2004).

Though the above depicted genetic circuit of *P. aeruginosa* QS is well established and the three systems are doubtlessly interlinked, the exact nature of the links and the hierarchy have been recently disputed (Dekimpe & Déziel 2009). There are many local variants of the PAO1 lab strain that differ dramatically at both the genotypic and phenotypic level and discordant observations of QS behaviour are often attributed to this local variation (Klockgether et al. 2010). In addition to this the QS systems do not act in isolation. There are various global transcriptional regulators that influence it and integrate other environmental stimuli into the QS regulatory network (Withers et. al. 2001, Shrout et al. 2006; Duan & Surette 2007; Venturi 2006). It is possible that QS is an evolutionarily plastic regulatory network which can be reprogrammed over generations to aid adaptation to new environments. An exciting avenue of future research would be to investigate the extent to which QS circuits can be and have been reprogrammed in different environmental and clinical isolates.

1.1.5 The Role of QS in P. aeruginosa biofilm formation

A biofilm is a highly structured and cohesive community of cells, normally attached to a surface (Costerton & Lewandowski 1995). Biofilms colonise such diverse places as the teeth of a human, the inside of medical devices, and the stones of shallow coastal sea habitats. The biofilm is normally attached to the

surface using protein or exopolysaccharide adhesins and held together using a scaffold of polymers such as alginate, the *pel* and *psl* polysaccharides (Ryder et al. 2007; Ghafoor et al. 2011), extracellular DNA (Allesen-Holm et al. 2006) and sugar binding lectins (Diggle et al. 2006). The biofilm scaffold or matrix contains membrane vesicles that may have a proteolytic or antibiotic resistance function (Beveridge 2006; Mulcahy et al. 2008; Høiby et al. 2010). The biofilm is a mode of growth observed in a wide range of bacteria including *P. aeruginosa*. Biofilm formation has been suggested to follow discrete stages (Sauer et al. 2002; Klausen, Aaes-Jørgensen, et al. 2003a; Klausen, Heydorn, et al. 2003b) characterised by a dramatic change in genome-wide expression patterns (Finelli et al. 2003; Waite et al. 2006). Bacterial biofilms are of particular importance in the clinical setting where they likely contribute to chronicity of infection and resistance to antimicrobial therapy (Moskowitz et al. 2004; Bjarnsholt et al. 2009).

In a much cited attempt to asses the role of QS in biofilm formation Davies et. al. (1998) observed that *lasI* and *lasI*/*rhlI* double mutants can attach to a surface but form flat and undifferentiated biofilms compared to the highly structured biofilms of the wild type and *rhlI* mutant. They also observed that addition of synthetic 3-oxo-C12 HSL signal restored the biofilm differentiation confirming a role for *lasIR* based signalling in biofilm differentiation. Similarly other studies have found that a class of QS inhibitor (QSI) compounds called furanones inhibit biofilm growth and that mutations in the response genes *lasR*/*rhlR* render biofilms more susceptible to the antibiotic tobramycin (Hentzer et al. 2002; Hentzer et al. 2003; Hentzer & Givskov 2003). In contrast to this Heydorn et. al. report flat and structurally indistinguishable biofilms of the wild type and QS mutants and another study using high flow conditions reports structured biofilms with no consistent variation between wild type and QS mutants (Heydorn et al. 2002; Purevdorj et al. 2002).

QS integrates information about the social and physical environment and in response, regulates a suite of genes some of which are known to be important

for biofilm formation. However since QS is not the only regulatory network responsible for regulating these genes it is unsurprising that small differences in strains or test conditions can yield drastically different observations (Shrout et al. 2006). QS regulated factors with a known influence on biofilm formation include rhamnolipids which are important for maintaining open water channels and microcolony maturation (Davey et al. 2003; Lequette & Greenberg 2004), pyoverdine (Banin et al. 2005), *lecA* and *lecB* (Tielker et al. 2005; Diggle et al. 2006) and extracellular DNA (Allesen-Holm et al. 2006).

1.2 Social Evolution

1.2.1 Hamilton's Rule

Where evolutionary theory is concerned, a social behaviour has fitness consequences for both the actor performing the behaviour and the recipient (Sachs et al. 2004; West et al. 2006; West et al. 2007). Given fitness effects on actor and recipient, social behaviours are classified into four categories: mutual benefit, selfishness, altruism and spite (Fig. 1.3). All altruistic and some mutually beneficial behaviours involve co-operation. A co-operative trait is any that increases the fitness of an individual other than the actor and that has evolved at least in part because of this effect. Distinctions between social behaviours help us identify behaviours that evolve and are maintained by different mechanisms. For example, it is easy to see how mutually beneficial and selfish behaviours evolve, they increase the fitness of the actor. However, altruism and spite are different – they decrease the fitness of the actor.

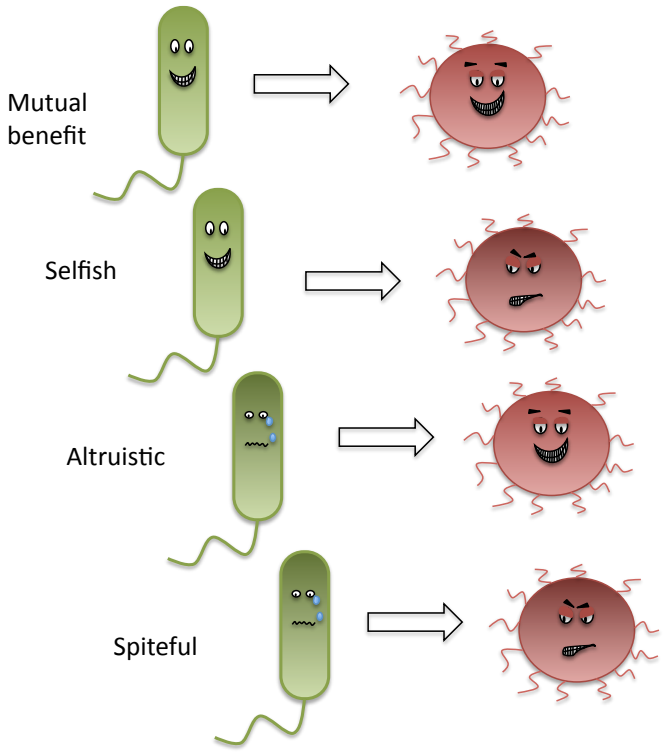


Figure 1.3: There are four classes of social behaviour defined by the fitness effect on actor (green) and recipient (red).

A classic example of altruism is the sterile worker castes of eusocial insects such as ants or bees (Bourke 2001; Wilson 2005). If the sterile workers lack the opportunity to reproduce and pass on their genes, how do successive generations still contain them? Sterile worker bees and ants commit evolutionary suicide by not reproducing and instead working to aid the reproduction of their queen. An analogous situation in the microbial world is fruiting body formation by the amoeba *Dictyostelium discoideum*. During fruiting body formation, approximately 20% of cells lyse, producing a strong cellulosaic structure that supports the spore-forming cells (Strassmann et al. 2000). Darwin himself struggled with the idea that organisms of any kind would perform a behaviour that decreased their fitness or solely increased the fitness of another. He framed the problem in the following way:

> *'If it could be proved that any part of the structure of any one species had been formed for the exclusive good of another species, it would annihilate my theory, for such could not have been produced through natural selection'*
>
> Charles Darwin, On the Origin of Species, Chapter 6: Difficulties on Theory.

In 1964 William Hamilton developed a framework (Hamilton's Rule) for understanding the evolution of social behaviours. His central prediction was that altruism can be favoured by selection when;

1 $rb - c > 0$,

where r is a coefficient of relatedness, b is the benefit of the behaviour to the recipient and c the cost to the actor (Hamilton 1964b; Hamilton 1964a). Hamilton's Rule has been successful in explaining altruism in many taxa where altruism is directed towards relatives, but is it relevant for microorganisms?

1.2.2 Social Evolution in Microbes

The generality of Hamilton's Rule is highlighted by the fact that it can be applied to microorganisms as well but the manifestation of Hamiltonian relatedness is markedly different. Furthermore there have been some important developments to the theory that are particularly potent when considering microorganisms.

Hamiltons Rule (eqn 1) includes a coefficient of relatedness r. In eukaryotes this relatedness is akin to coancestry but it is important here to make the distinction as there are far reaching implications in the world of microbes (Crespi 2001; West et al. 2006; Nadell et al. 2008a). The definition of r is given by;

2 $r = (pAR - pAX)(pAA - pAX)$,

where pAR is the probability that a randomly chosen gene from the focal locus of the actor is identical (in function) to a randomly chosen gene drawn from the focal locus of the recipient; pAA the probability that two randomly chosen genes (with replacement) from the focal locus in the actor are identical and pAX is the probability that a randomly chosen gene from the actor is identical to a randomly chosen gene from a random population member.

When considering the spread of a eukaryotic trait from rare ($pAX \rightarrow 0$, and relatedness is akin to coancestry) this gives a well known pattern of relatedness e.g. $r = 0.5$ between full siblings and 0.25 between half siblings etc. However when this definition is applied to haploid, clonal microorganisms ($pAA = 1$) a completely different pattern emerges. Individuals are on average related to their clonemates by $r = 1$ and $r = 0$ to individuals from other lineages. Since this relatedness is with respect to a certain behaviour it may change between behaviours. In addition if there is mutation in the locus controlling a behaviour, the ancestral strain will be related with $r = 0$ to its mutant offspring. Conversely if a gene associated with cooperation has a pleiotropic function this can constrain social adaptation (Foster et al. 2004). The manifestation of 'r' is a crucial contrast to diploid eukaryotes and implies that the evolutionary stability

of social behaviours in bacteria may depend heavily on mutation rates (Harrison & Buckling 2005; Harrison & Buckling 2007), horizontal gene transfer (Ellis et al. 2007) and population structuring (Ross-Gillespie et al. 2007; Kümmerli et al. 2009). In addition it implies that kin selection can influence the social interactions of genealogically distant individuals such as in greenbeards (Gardner & West 2009). A greenbeard locus is one that encodes the cooperative trait and its recognition simultaneously such that the expression of the cooperative trait can be directed exclusively to others that also express it. This can be seen as the extreme of hamiltonian relatedness at only the locus encoding the cooperative trait and can theoretically allow for stable cooperation of genealogically distinct taxa via hamiltonian relatedness.

Individual organisms are more likely to be cooperative towards related individuals (or individuals likely to share the genes for the cooperative behaviour) except when that comes at a cost to other related individuals. Put another way, viscous populations lead to neighbours that are highly related. This causes strong competition between relatives, which may negate the kin-selected benefits of cooperation. This thought led the development of theory on the spread of cooperation in the face of local competition (Griffin & West 2002) and subsequent empirical testing of these theories (Griffin et al. 2004; Kümmerli et al. 2009). Two prominent theoreticians in this field have developed methods to incorporate the scale of competition into Hamilton's Rule (Queller 1994; Frank 1998). Subsequent extensions to the theory reveal that the effect of local competition can be circumnavigated if each additional individual arising in the population increases the availability of nutrients such as through public goods production (Pfeiffer & Bonhoeffer 2003; Grafen 2007).

1.2.3 Evolution of QS

Social cheating in the microbial world is well documented. In *Pseudomonas fluorescens* cultures, clones defecting from polysaccharide production can still stick to the air surface biofilm provided by others (Rainey & Rainey 2003). Clones of *Myxococcus xanthus* defective in fruiting body formation can

nonetheless exploit fruiting body formation of others in the population (Fiegna et al. 2006). *P. aeruginosa* isolates deficient in siderophore production can exploit the iron scavenging of others in the population (Jiricny et al. 2010). QS is no exception to this, in fact the problem of social cheating in QS is two-fold. First, since QS regulates many public goods, QS response defection results in an exploitative advantage to the defector by a metabolic cost saving on public goods production (Diggle et al. 2007a). In addition to this there is the problem of stable signalling. Since a signalling interaction must on average increase the fitness of both signaller and receiver to be evolutionarily stable, deviations from this can undermine the maintenance of a signalling system (Maynard-Smith & Harper 2003; Diggle, Gardner, et al. 2007b). QS therefore represents a dual evolutionary dilemma. Hamilton's Rule allows predictions to be made about when such behaviours are evolutionarily stable. For example any influence on the behaviour which reduces its cost or increases its benefit to the fitness of the individual performing the behaviour increases its evolutionary stability. This can be extrinsic such as the availability of nutrients or substrates needed to perform the behaviour or it can be intrinsic such as the facultative regulation of costly cooperative traits. Importantly the relatedness of individuals cooperating versus the relatedness of individuals competing will also influence the selection for cooperation (Griffin et al. 2004; Diggle et al. 2007a). In chapter 4 of this thesis we will revisit the requirements for the evolutionary stability of signalling and test the central prediction that common interest can maintain honest signalling.

1.3 QS Evolution and Virulence

1.3.1 QS Regulation of Virulence

The virulence of an organism often depends on its ability to produce and release exotoxins that can damage a host. By using several animal host models, including nematodes and mice, QS-dependent control of individual virulence determinants and virulence itself has now been demonstrated in several human pathogenic organisms. These include *Burkholderia pseudomallei*, the causative agent of melioidosis (Ulrich, DeShazer, Brueggemann, et al. 2004a), *B. mallei* (Ulrich, DeShazer, Hines, et al. 2004b), *B. cenocepacia* (Eberl 2006), *V. cholerae* (Lin & Kovacikova 2007) and *S. aureus* (Fleming et al. 2006).

P. aeruginosa is a Gram-negative bacterium, capable of causing disease in plants, animals and humans (Rahme et al. 1995). It is a major source of nosocomial infections and is a leading cause of mortality in cystic fibrosis (CF) patients (Govan & Deretic 1996). As an opportunistic human pathogen, *P. aeruginosa* can colonize a wide variety of anatomical sites. This is because the organism produces an arsenal of extracellular virulence factors which are capable of causing extensive tissue damage and bloodstream invasion which consequently promotes systemic dissemination. Many of these exoproducts are regulated in a cell density-dependent manner via QS. The *lasIR* QS system regulates the production of virulence factors including elastase, the LasA exoprotease, alkaline exoprotease and exotoxin A (Williams et al. 2007). The *rhlIR* system induces the production of rhamnolipid, elastase, LasA exoprotease, hydrogen cyanide, pyocyanin, siderophores and the cytotoxic lectins LecA and LecB (Latifi et al. 1995; Winson et al. 1995; Latifi et al. 1996; Pearson et al. 1995).

While *P. aeruginosa* can cause severe infections in burn wounds and intubated patients, it is particularly problematic in the lungs of patients with CF where it causes chronic infections which result in extensive tissue damage, reduction in lung function and ultimately death (Govan & Deretic 1996). It has been demonstrated that *P. aeruginosa* QS genes are expressed in the lungs of CF

patients (Erickson et al. 2002) and that sputum from the CF lung often contains QS signal molecules suggesting that QS virulence is important during pathogenesis in CF (Middleton et al. 2002). In CF infections, *P. aeruginosa* is more resistant to antibiotic treatment than is predicted by susceptibility tests on planktonic cells and it is thought that the added resistance of *P. aeruginosa* is due, in part, to a 'biofilm' lifestyle within the lung (Singh et al. 2000).

1.3.2 Virulence as a therapeutic target

QS systems regulate bacterial pathogenesis and therefore represent novel targets for anti-infective therapies. As such there has been much interest in the discovery of QS inhibitors (QSI). The first naturally occurring agents found to possess such QS antagonistic activity were brominated furanones produced by the Australian macro-alga *Delisea pulchra* (Givskov et al. 1996). Biofilms grown in the presence of synthetic furanone derivatives were rendered susceptible to antimicrobial killing with the antibiotic tobramycin and detergent SDS in contrast to the untreated biofilms (Hentzer et al. 2003). Further reports of QSI compounds derived from food, herbal and fungal sources have been published and their potential therapeutic role investigated in biofilm and pulmonary murine models (Bjarnsholt, Jensen, Rasmussen, et al. 2005b; Rasmussen et al. 2005). A recent study identified three compounds that inhibited the synthesis of molecules required for the activation of the *P. aeruginosa* alkyl-quinolone (AQ) regulatory pathway (Lesic et al. 2007). This limited *P. aeruginosa* infection in mice, without affecting bacterial viability and such compounds provide a starting point for the design and development of novel anti-pathogenic agents targeted at important human pathogens.

Other approaches to QSI include antibiotics with QSI activity (e.g. Azithromycin), lactonolytic enzymes (e.g. AiiA) and synthetic structural mimics of QS molecules that lack QS activity (e.g. *N*-acylcyclopentylamides). Azithromycin (AZM) is a macrolide antibiotic that interferes with *P. aeruginosa* AHL QS mediated virulence below MIC. Recently Kai et. al. have shown that concentrations of AZM insufficient for the inhibition of QS related genes (e.g. *lasI*, *rhlI*) nonetheless

downregulate the production of enzymes in the biosynthetic pathway upstream of AHL biosynthesis such as glycine, methylene (by *gly*, *gcv*), homocysteine (*metF*), methionine (*metH*) and S-adenosyl methionine (SAM, *metK*) (Nalca et al. 2006; Kai et al. 2009; Köhler et al. 2010). AiiA is a lactonase enzyme isolated from *Bacillus* spp (Dong et al. 2000) which inactivates AHL mediated signalling via hydrolysis of the lactone ring (Dong et al. 2001) similar to a pH dependent reversible chemical reaction (Yates et al. 2002). *N*-acylcyclopentylamides are QS antagonistic structural analogs of AHL's found in *P. aeruginosa* and have been shown to interfere with QS via inhibition of the signal to receptor interaction (Ishida et al. 2007).

1.3.3 Lessons from the evolutionary approach

Traditional approaches to antibacterial treatment fall into two categories; bacteriocidal, killing the target organism and bacteriostatic, preventing the growth of the target organism. Generally this has been done by disrupting essential functions of the cell such as cell wall biosynthesis, protein biosynthesis or DNA replication and repair (Walsh 2000; MacLean et al. 2010). Such an aggressive kill or maim strategy presents any resistant variant with a huge selective advantage thus selecting for resistance. In addition the targeting mechanisms of bacteriocidal and bacteriostatic drugs rely on specific domains or amino acid sequences of proteins such that mutations at the target site can confer resistance to drugs. Other mechanisms of resistance include modifying the antibiotic compound to render it ineffective or pump it out using efflux pumps on the cell wall. Since many drugs originate from natural compounds of which the target species may have had experience in its evolutionary history, resistance can be acquired through the elaboration of existing efflux or modification proteins rather than completely novel mechanisms. When combined with the rapid generation times it is unsurprising that resistance to bacteriocidal or bacteriostatic antibiotics is only a matter of time.

By targeting secondary metabolites and virulence factors using QSI and leaving the essential functions of the cell intact, we may circumvent the intense selection

for resistance. For instance theory predicts that when the organisational structure of a population of cells is attacked the emergence of resistance is several orders of magnitude slower than when the essential functions of individuals within that population are attacked (Andre & Godelle 2005). Crucially the strength of selection for resistance to QSI will depend on the requirement of QS for growth and fitness in the host (Defoirdt & Boon 2010). We must also be cautious about the effect of QSIs on the population dynamics of virulence. For example a recent study reports that the administration of the QS antagonistic antibiotic azithromycin, although reducing the bacterial load in infections, increases the prevalence of virulent clones proficient in cooperative secretions of virulence factors (Köhler et al. 2010). The authors suggest that by inhibiting QS, the selection for cheating is reduced due to reduced QS regulated public goods available to defectors.

Another exciting avenue for treatment is the use of population dynamics of social traits to deliver antimicrobials or to replace alleles at the population level with alleles more favourable to treatment (Brown et al. 2009; Saeidi et al. 2011). The logic is as follows; a public goods defective cheat is introduced into the infective population and spreads via within host selection for public goods. If the introduced cheat also contains a locus for the production of antimicrobials, triggered post inoculation via an exogenously inducible promoter, this can be used to further reduce the bacterial load. Since QS defective public goods cheats are known to invade infections and reduce virulence, this approach has a good empirical foundation (Rumbaugh et al. 2009; Köhler et al. 2009). This is similar to the gene drive mechanism proposed for the control of insect pests (Sinkins 2000; Sinkins & Godfray 2004; Sinkins 2006).

Chapter 2: Materials and Methods

2.1 *General Methods and Conditions*

2.1.1 Bacterial Strains and Plasmids

No.	Strain	Details	Phenotypic Effect	Origin
1	NPAO1	Nottingham PAO1	Null	Paul Williams
2	NPAO1 *lasB*::CTXlux	1 containing a fusion of the *lasB* promoter region to *luxCDABE* using the mini CTX lux system.	Luminesces when *lasB* promoter is active.	Karima Righetti
3	NPAO1 *lasI*::CTXlux	1 containing a fusion of the *lasI* promoter region to *luxCDABE* using the mini CTX lux system.	Luminesces when *lasI* promoter is active.	Karima Righetti
4	NPAO1 pMM::Gfp	1 containing the pMM::Gfp plasmid.	Cells fluorescent at 509nm when excited at 488nm.	Marco Messina
5	NPAO1 pMM::Mcherry	1 containing the pMM::Mcherry plasmid.	Cells fluorescent at 610nm when excited at 587nm	Marco Messina
6	NPAO1 Δ*lasR*	1 with a Gentamicin resistance cassette inserted into the *lasR* coding region.	Non responder to 3-oxo-C12-HSL.	Beatson 2002
7	NPAO1 Δ*lasR* pMM::Mcherry	6 containing pMM::Mcherry plasmid.	Cells fluorescent at 610nm when excited at 587nm	This study
8	NPAO1 Δ*lasR* CTXlux	6 with a promoterless CTX lux insertion.	Constitutively luminescent.	This study
9	NPAO1 Δ*lasIR*	1 with Gm cassette replacing *Pst*I fragment shared by *lasR* and *lasI* using the suicide vector pSB219.7A.	Non responder to and non producer of 3-oxo-C12-HSL.	This study
10	NPAO1 Δ*lasIR* *lasB*::CTXlux	9 containing a fusion of the *lasB* promoter region to *luxCDABE* using the mini CTX lux system.	Luminesces when *lasB* promoter is active.	This study
11	NPAO1 Δ*lasIR* *lasI*::CTXlux	9 containing a fusion of the *lasI* promoter region to *luxCDABE* using the mini CTX lux system.	Luminesces when *lasI* promoter is active.	This study
12	S 17-1 pSB1142	*E. coli* harbouring plasmid engineered to respond to long chain AHL's	Luminescent in the presence of 3-oxo-C12-HSL.	Winson 1998

No.	Strain	Details	Phenotypic Effect	Origin
13	S 17-1 p56536	*E. col*i harbouring plasmid engineered to respond to short chain AHL's	Luminescent in the presence of C4-HSL.	Winson 1998
14	NPAO1 Δ*pqsA*	1 containing an in frame chromosomal deletion of *pqsA*.	Non producer of Alkyl Quinolones (AQs).	Diggle 2006
15	NPAO1 Δ*pqsA* *pqsA*::lux	15 containing a plasmid borne promoter fusion to pqsA (Mini-CTX lux *pqsA*).	Luminesces when the *pqsA* promoter is active.	Diggle 2007
16	NPAO1 Δ*pqsAH*	1 containing an in frame chromosomal deletion of *pqsA* and *pqsH*.	Non producer of AQs and non converter of HHQ into PQS.	Diggle 2007
17	NPAO1 Δ*pqsR*	1 containing an in frame chromosomal deletion of *pqsR.*	Non responder to PQS.	Diggle 2003
18	NPAO1 Δ*pqsR* pMM::Gfp	1 harbouring a plasmid expressing Gfp.	Cells fluorescent at 509nm when excited at 488nm.	This study
19	NPAO1 Δ*pqsR* pMM::Mcherry	1 harbouring a plasmid expressing Mcherry.	Cells fluorescent at 610nm when excited at 587nm	This study
20	NPAO1 Δ*pqsE*	1 containing an in frame chromosomal deletion of *pqsE.*	Mutant in main PQS effector protein, attenuated for many PQS dependent phenotypes.	Diggle 2003
21	BPAO1	PAO1 WT from Belgium	Null	Pierre Cornelis
22	BPAO1 *pvdE*::*lux*	21 containing a chromosomal fusion of mini-CTX lux to the promoter region of *pqsE*.	Luminesces when *pqsE* promoter is active.	Diggle 2007
23	BPAO1 Δ*pvdDpchEF*	21 mutated in *pvdD* and *pchEF*.	Non producer of siderophores pyoverdine and pyochelin.	Pierre Cornelis
24	BPAO1 Δ*pvdDpchEF* CTXlux	23 containing a chromosomal insertion of promoterless mini-CTX lux.	Constitutively luminescent.	This study

No.	Plasmid	Details	Origin
1	pMM::Gfp	pME6032 with Gfp cloned into the MCS and lacIQ removed for constitutive expression of Gfp.	Marco Messina
2	pMM::Mcherry	pME6032 with Mcherry cloned into the MCS and *lacIQ* removed for constitutive expression of Mcherry.	Marco Messina
3	plasB::CTXlux	Mini CTX lux plasmid with *lasB* promoter region cloned into the MCS.	Karima Righetti
4	plasI::CTXlux	Mini CTX lux plasmid with *lasI* promoter region cloned into the MCS.	Karima Righetti
5	pSB219.7A	pRIC380 *P. aeruginosa suicide* vector carrying Δ*lasRI*::Gm on *Spe*I fragment.	Beatson 2002

The strain PAO1 is often referred to as wild type (WT) in this thesis. PAO1 was originally isolated in 1954 from a wound in Melbourne, Australia (Holloway 1955, 1975). It has since become the reference strain of *Pseudomonas aeruginosa* with a fully sequenced genome. Much of the molecular and genetic work undertaken on this species uses PAO1 however it's relevance to contemporary infections could be questioned and in any case it represents a minute fraction of the natural diversity of *P. aeruginosa* isolates in the environment. Therefore the label 'WT' is used here more to refer to the reference strain or ancestor as opposed to the natural state of the organism as its contemporary diversity stands.

2.1.2 Growth Media and Conditions

Antibiotics

Stock solutions of antibiotics were prepared according to Sambrook et al. (Sambrook 2001), and stored at -20 °C. Ampicillin (Amp) was used from a 50 mg/ml in 50 % (v/v) ethanol (EtOH) stock, carbenicillin (Cb) from a 50 mg/ml stock in deionised water (dH_2O) and gentamicin from a 50 mg/ml stock in dH_2O. The final concentration of antibiotics added to media for the selection and maintenance of plasmids was: Amp 100 µg/ml (*E. coli*); Cb 300 µg/ml (*P. aeruginosa*) and gentamicin 15 µg/ml (*E. coli*) and 100 µg/ml (*P. aeruginosa*). All reagents were filter sterilised before use (0.2 µm pore Minisart venting filter, Sartorius, Germany).

Synthetic AHLs and AQ's

Synthetic 3-oxo-C12-HSL and C4-HSL were made by A. Truman under the supervision of Dr. S. R. Chhabra at the Centre for Biomolecular Sciences, University of Nottingham (Chhabra et al. 1993). Stocks of 1 µg/ml 3-oxo-C12-HSL and 10 µg/ml C4-HSL in acetonitrile were used. Compounds were stored at -20 °C. Synthetic PQS and its precursor 2-heptyl-4-quinolone (HHQ) were synthesised by A. Truman under the supervision of Dr. S. R. Chhabra and stored at -20 °C. Both were dissolved in methanol and PQS kept as 10 mM stock, HHQ as 5 mM stock.

Trace metals solution

Trace metals solution was prepared according to the formulation provided by the Center of Biomedical Microbiology, Technical University of Denmark. It consisted of iron (II) sulphate 2000 mg, manganese sulphate monohydrate 200 mg, copper sulphate 200 mg, zinc sulphate heptahydrate 200 mg, cobalt sulphate heptahydrate 100 mg, sodium molybdate-6, 4-dihydrate 120 mg and boric acid 50 mg made up to 1 L in dH_2O, pH 4.5. The solution was autoclaved at 121 °C for 20 min at 15 p.s.i. (which caused the iron to precipitate).

Growth media

Media were prepared using dH_2O and autoclaved at 121 °C for 20 min at 15 p.s.i. The sterility of large volumes of media was ensured by running the autoclave cycle with 'load sensing' activated and placing the temperature probe in an equivalent volume of liquid. This often required a prolonged heating phase in excess of 90 min.

Quorum Sensing Medium

The Quorum Sensing Medium (QSM) consisted of M9 Minimal Medium Salts including Na_2HPO_4 (6.8 gl^{-1}), KH_2PO_4 (3 gl^{-1}), NaCl (0.5 gl^{-1}) which was autoclaved. Folowing that the filter sterilised supplement solutions NH_4Cl (10 mM), $CaCl_2$ (0.1 mM) and $MgSO_4$ (1 mM) were added (final concentrations stated. Lastly the carbon sources Bovine Serum Albumin (BSA 1 % w/v) and Cas Amino Acids (CAA 0.1 % w/v) were added and the medium was filter sterilised by filtration through a 0.22 μM pore filter.

QSM was developed during the course of this study, with the aim of generating a QS dependent environment. To that end protein in the form of BSA is the major carbon source. Protein hydrolysis requires protease and the *lasB* protease is tightly *lasRI* regulated. Early attempts at such a medium failed due to the fact that QS does not occur from the outset of a very dilute culture. To overcome this the medium is supplemented with a small dose of free amino acids. Hence QS is not required for growth per se but is required for maximal growth. The suitability of the medium for further experiments was determined due to a significant difference between PA01 WT and its *lasIR* mutant in final culture density.

SSD Medium

The Staphylococcal Siderophore Detection (SSD) medium consisted of KH_2PO_4 (2 mM), NaCl (7.9 mM), NH_4Cl (17.2 mM), 50 μM $MgCl_2.6H_20$, 1.2 % (vol/vol) 1.5 M Tris-HCl (pH8.8) solution, 20 mM glucose, CAA (6 gl^{-1}), 39 μM tryptophan, 32 μM

nicotinic acid, and 6 μM thiamine-HCl. SSD medium was iron depleted by treating with 10 g of Chelex 100 (Bio-Rad) per liter and stiring for 1 h at 25 °C to remove divalent and trivalent metal ions. The iron depleted medium was sterilized by filtration, which also removed the resin.

Luria Bertani medium

All bacterial strains were routinely grown in Luria Bertani (LB) broth unless otherwise stated. LB broth was prepared as described by (Sambrook 2001) and consisted of tryptone 10 g, yeast extract 5 g and sodium chloride 10 g made up to 1 L in dH_2O. LB agar was prepared by addition of 0.8 % (w/v) Technical Agar No. 3 (Oxoid) to LB broth.

Soft-top agar

LB soft-top agar consisted of tryptone 10 g, sodium chloride 5 g and Technical Agar No. 3 (Oxoid) 6.5 g made up to 1 L in dH_2O.

Pseudomonas Isolation Agar

Pseudomonas Isolation Agar (PIA) was prepared according to the manufacturer's instructions and consisted of peptone 20 g, magnesium chloride 1.4 g, potassium sulphate 10 g, irgasan 0.025 g and agar 13.6 g containing 20 ml glycerol made up to 1 L in dH_2O.

FAB medium

A-10 media was prepared according to Haagensen et al. (Haagensen 2006). It consisted of ammonium sulphate 20 g, disodium (hydrogen) phosphate dihydrate 60 g, potassium phosphate 30 g, sodium chloride 30 g made up to 1 L in deionised water, pH 6.4 ± 0.1. FB media (Haagensen 2006) consisted of 10 ml 1 M magnesium chloride, 1 ml 1 M calcium chloride, 1 ml trace metals solution made up to 9 L in Milli-Q water. FAB minimal media was prepared by aseptically mixing 1 part A-10 media with 9 parts FB media and adding glucose as the carbon source to a final concentration of 0.3 mM (Haagensen 2006).

Sucrose/agar broth

Sucrose agar and broth were used in recombinant-selective experiments. A stock concentration of 50 % (w/v) sucrose was made by adding 25 g sucrose made up to 50 ml in sterile dH_2O and filter sterilised. Sucrose broth consisted of tryptone peptone 10 g and yeast extract 5 g in 900 ml sterile dH_2O, pH 7. To this, 100 ml sucrose stock solution was added aspetically, giving a final sucrose concentration of 5 % (w/v).

Long term storage of bacterial strains

For long-term storage of bacterial strains, 0.75 ml of an overnight bacterial culture was added to 0.75 ml 50 % (v/v) glycerol and mixed thoroughly in a 2 ml Micro tube (Sarstedt, Germany) before being flash frozen in liquid nitrogen. The tubes were then stored at -80 °C.

2.1.3 Assesment of growth via optical density

Many of the growth experiments in this thesis rely on measurements of optical density (OD) defined as $OD_\lambda = \log_{10}(I_0/I)$, where I is the intensity of light at a wavelentgh (λ) once it has passed through the sample and I_0 is the intensity of light at that wavelength before it passes through the sample. The calculation is based on the Beer-Lambert law which states that transmittance (I/I_0) varies exponentially with the concentration of the absorbing species in the sample. This is a popular method for growth assays as it enables the simultaneous and automatic monitoring of culture density in microplate readers. Growth data in the form of cell counts of colony forming units (CFU) is normally presented with a logarithmic y-axis scale. Such presentation of CFU data enables the visual comparison of exponential growth rates (doubling rates). In this thesis OD data is presented on a linear y-axis scale.

Fitness in bacterial cultures can be measured in a number of different ways (see Frank 1996) but the currency of interest in the present experiments is final culture density (also called 'productivity' or 'carrying capacity'). In QSM

medium a PAO1 WT and *lasIR* mutant grow at similar initial rates but to different final densities. In a metapopulation context (between patches, see 2.2.10) the difference in final density dictates the likelihood of propagation into the next patch thus the representation of OD growth data on linear y-axes is deemed appropriate. In the current experimental system estimates of fitness based on final densities amplify the fitness difference of a WT and *lasIR* mutant when compared to estimates based on doubling rate. If however fitness estimates were based on doubling rates they would remain significant and would bear relevance to fitness of longer term propagation. In other words a small difference in doubling rate over 24 h would result in populations of dramatically different sizes over longer timescales.

2.2 *Evolution of Signalling Methods*

2.2.3 Investigation of gene transcription using lux promoter fusions

The wells of a 96 well plate were filled with 300 µl diluted culture and incubated for 24 h at 37 °C. Measurements of OD_{600} and luminescence RLU (Relative Light Units) were collected every h. Transciptional activity of the promoter region was presented as (RLU / OD_{600}) to correct for culture density.

2.2.4 Exoprotease Production

Culture density was measured as OD_{600}. Cultures were then centrifuged for 2 min at 14000 rpm and the supernatant filtered through a 0.2 µm pore filter. 100 µl of supernatant was added to 900 µl of buffer (100 mM Tris, 1 mM $CaCl_2$, pH 7.5), containing 20 mg Elastin Congo Red (ECR). The mixture was incubated for 18 h at 37 °C in a shaking incubator. Excess ECR was centrifuged and the absorbance of the supernatant determined at A_{495}. Exoprotease content of the supernatant was given as (A_{495} / OD_{600}) to correct for culture density.

2.2.5 Extraction of signal molecules

Cultures were incubated for 18 hr at 37 °C in a shaking incubator. Cultures were then centrifuged at 13000 rpm for 5 min and the supernatant was filter

sterilised to remove all cells. Equal volumes of supernatant and acidified ethyl acetate were mixed in a 50 ml universal and thoroughly vortexed. When the aqueous (bottom) and organic (top) phases are separated the top half was removed into a 30 ml glass universal and replaced with fresh acidified ethyl acetate. The supernatant was extracted three times and the resulting solvent was dried off in a vacuum centrifuge. The samples could also be dried under nitrogen gas flow. Dried samples could be stored at -20 °C. This process concentrated AHL and AQ molecules in the supernatants for later analysis. When subsequently used for analysis the dried samples were reconstituted in 30 µl methanol. For high throughput purposes, molecules could be extracted from supernatants as small as 1 ml in volume.

2.2.6 Detection of AHL molecules by TLC / Biosensor Overlay

Extracted AHLs were analysed by thin layer chromatography (TLC) based on previously developed methods (Shaw et al. 1997; McClean et al. 1997). For the detection of the long chain 3-oxo-C12-HSL, 5 µl of each sample was spotted onto a 20 x 20 cm Alugram reverse phase RP-2/UV254 plate (Macherey-Nagel) along with 1 µl of the synthetic standard. The plate was placed upright in a glass container such that the bottom of the plate was submerged in 45 % (v/v) MeOH overnight. This caused the solvent to be absorbed along the plate carrying with it the AHL molecules and spreading them out according to the physical properties of the molecules. It was then overlaid with soft top agar (100 ml) containing 5 ml of an overnight culture of *E. coli* S17 λpir pSB1142. This *E. coli* strain contains a plasmid carrying a functional *lasR* gene and a *lasI* promoter region fused to *luxCDABE*, thus it does not make any native AHL molecules but responds to long chain AHL molecules by producing light. Following incubation at 37 °C for 3 hr the light production on the plate was viewed using a Berthold Luminograph LB980 (Winson et. al. 1998).

2.2.7 High Throughput Biosensor Assay

To be able to assess the signal molecule content of several cultures simultaneously and rapidly, 15 µl of solvent extracts were added to 300 µl cultures of the bioreporters S17-1 pir pSB1142 (for the detection of long chain AHL's) on a 96 well microplate and light output was monitored over a period of 4 hr in a Tecan Infinite microplate reader.

2.2.8 LCMS detection of signal molecules

Dried samples kept at -20 °C and supplied in 2 ml eppendorfs were reconstituted in 50 µl methanol, vigorously mixed and sonicated for 5 min. They were then centrifuged for 10 min at 9447 g and the clear supernatant transferred by pipette to a glass insert within an HPLC vial. Each sample vial was sealed with a rubber/ PTFE lined screw cap. Post injection, samples were kept at -20 °C.

Liquid Chromatography

The instrument was primed and equilibrated for an hour prior to the chromatographic run . Using the Agilent 1200 series HPLC, comprising degasser, binary pump, column heater and autosampler the mobile phase consisted of formic acid 0.1% in water (A) and formic acid 0.1% in acetonitrile (B) as a gradient run over 20 min at a flow rate of 0.3 ml/min. The mobile phase was maintained at 90% A for 1 min then reduced to 50% by 4 min, 20% by 4.5 min and 0% by 13 min where it was maintained for 1 min. It was then returned to 90% by 15 min. The column used was an Ascentis Express C18 150 x 2.1 mm internal diameter, 2.7 um particle size maintained at 50 °C. After each 10 ul injection the needle was washed 3 times in methanol.

Mass Spectrometry

The instrument was cleaned and calibrated in positive ion mode for full scan, isolation and fragmentation prior to use. Using the Bruker HCT Plus ion trap in multiple reaction mode (MRM) and Hystar software, ions were generated using positive ion electrospray from the Agilent HPLC system. Using the Smartfrag

option on the software, the trap was set to isolate from full scan and then fragment ions at m/z 172.1, 260.2, 298.2, 244.2 and 316.2. The ion charge control was used to prevent charge overload in the trap. The instrument was optimised using the smart parameter setting for m/z 298. The monitored mass range was 80 - 340 m/z.

Data Analysis

Bruker DataAnalysis version 3.3 was used to interrogate the acquisitions. Extracted ion chromatograms (EIC) of m/z 102.1 were produced from the positive ion MSMS of m/z 172.1, 298.2 and 316.2 and 328.2. EIC of m/z 175.1 were produced from the positive ion MSMS of m/z 260.2. EIC of m/z 159.1 were produced from the positive ion MSMS of m/z 244.2. Retention times and peak spectra were matched to the 10 uM standard (x3) injected at intervals throughout the run of each method. Injections of sample solvent were also monitored to assess carryover. Peak areas were determined and the results transferred to PDF file for dissemination.

2.2.9 Competition Experiments

Precultures were washed and mixed in the relevant ratios according to OD_{600}. Mixtures were then a. diluted and plated onto LB agar and b. diluted into fresh medium to initiate cultures in a 96 well plate. After 24 h of incubation at 37 °C cultures were diluted and plated onto LB. Initial and final relative frequencies were determined by colony counts, the strains could be differentiated by a promoterless CTXlux insertion in a light camera (Berthold Luminograph LB980). Relative fitness of the cheating mutant was calculated as;

$$w = \frac{p_1(1 - p_0)}{p_0(1 - p_1)}$$

where p_0 is the initial proportion of cheats in the population and p_1 is their final proportion (Gillespie 2007).

During ecological time-scale competition experiments it is very unlikely that de novo mutants will arise and spread to proportions sufficient to distort the results. Such an effect is only observed after several serial passages in longer term 'evolutionary' experiments.

2.2.10 Experimental Evolution

5 replicate 5 ml QSM cultures for each treatment were initiated with a single PAO1 lasI::lux colony and incubated at 37 °C for 24 h. These cultures were then diluted and plated out to single colonies. Resulting colonies were inoculated into 300 µl LB cultures and incubated at 37 °C for 16 hr. These LB cultures were used to initiate the subsequent QSM cultures in the following way. Each replicate QSM population consisted of 10 subpopulations. Each subpopulation was initiated with a mixture of the LB cultures from its predecessor according to treatment (High Relatedness = 1 colony, Mid Relatedness = 2 colonies, Low Relatedness = 10 colonies), after correcting for OD_{600} and washing inoculating cells in fresh QSM. These subpopulations were then incubated at 37 °C for 24 h, after which they were pooled within treatments, diluted and plated to single colonies. These colonies were used to initiate the subsequent round and so the procedure continued for a total of 20 transfers. Phenotypic and genotypic analyses were performed on the populations and individuals isolated from the populations after 20 transfers of experimental evolution. 20 transfers comprises approximately 120 generations.

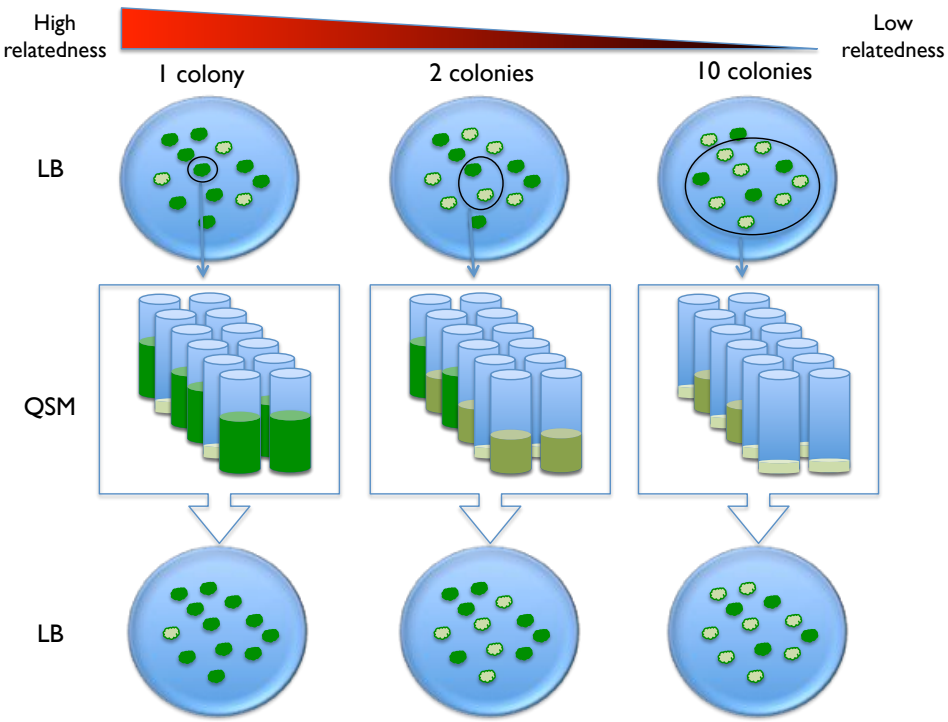


Figure 2.1: Schematic drawing of the experimental evolution procedure showing how the relatedness treatments are generated. Each subsequent subpopulation is inoculated by a mixture of colonies from the pooled subpopulations of its predecessor.

The splitting and pooling of populations into subpopulations derives from a study where it is demonstrated that such a regime is necessary to maintain selection for public goods traits (Griffin et al. 2004). This arises due to the effect of local competition. For example, high relatedness selects for costly cooperation but this can be counterbalanced if these highly related individuals are also competitors (Griffin & West 2002). In the context of the experiment; cooperation in the form of exoprotease production causes a population to grow to higher density. There is however no selective advantage to growing to high density without the splitting and pooling of subpopulations (see Fig 2.1).

The same experimental protocol was employed to study the evolution of signalling and response under manipulations of signal environment. This was created by the addition of excess signal molecule (3-oxo-C12 HSL) or signal inhibitor (C10 CPA). All of the treatments in this experiment also contained 5

replicate populations and all of the populations were maintained under conditions that tended towards high relatedness (Figure 2.2).

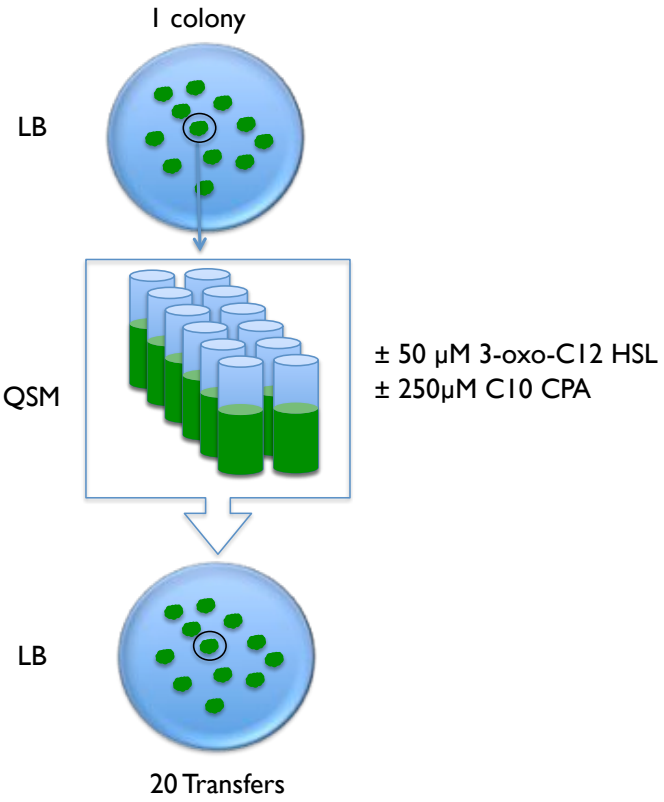


Figure 2.2: Schematic drawing of the experimental evolution procedure showing how the signal manipulation treatments are generated.

2.3 *Biofilm Methods*

2.3.1 Crystal Violet Stained Biofilms

Washed precultures were aspirated into a clear flat-bottom 96 well plate (each well containing 200 µl) and incubated for 24 h at 37 °C. The culture was then removed and microtitre plate wells were washed three times with 250 µl sterile Phosphate Buffered Saline. After washing, plates were air dried and 250 µl Methanol was added to each well in a fume hood for 15 min to fix the biofilms. The methanol was removed and plates were air dried again. Following fixation, 250 µl 0.1 % Crystal Violet (CV) solution was added to each well and left at ambient temperature for 5 min to stain the biofilms. Excess stain was removed by adding and removing tap water to and from the wells until the water was clear. The plates were air dried and the CV stain solubilized with 33 % glacial acetic acid in a fume hood. Absorbance at A_{600} was read in a plate reader and is taken to represent the extent of biofilm formation.

Where biofilm were treated with Tobramycin (60 $\mu g ml^{-1}$) or SDS (0.3 %), the liquid culture was removed from the plates and replaced with fresh medium containing either the antiobiotic or the biocide or dH_2O as a control. This was incubated for 3 hours after which the CV staining procedure was performed.

2.3.2 Flow Cell Biofilms

A detailed description of the flow cell biofilm method is available elsewhere (Haagensen 2006; Pamp et al. 2009). It was used here with a few slight modifications (see Shanika Crusz PhD Thesis University of Nottingham 2010, for detailed description of modifications). Briefly, the flow cells comprised of a perspex block with 3 flow channels with dimensions 40 x 4 x 4 mm. Silicone tubing extended from the in and outlets of each channel. A glass coverslip was attached to the flow cell with silicone glue forming a seal for the flow chambers. The entire construction (see Fig. 2.3) was washed with 1 litre 0.5 % (v/v) sodium hypochlorite solution and rinsed with 2 litres de-ionised H_2O. After overnight pre-conditioning of the system with flowing sterile medium, flow

chambers were inoculated with washed cultures diluted to OD_{600} 0.1 and incubated statically at 37 °C for 1 hr. Flow was then initiated and the whole system was incubated at 37 °C for up to 7 days.

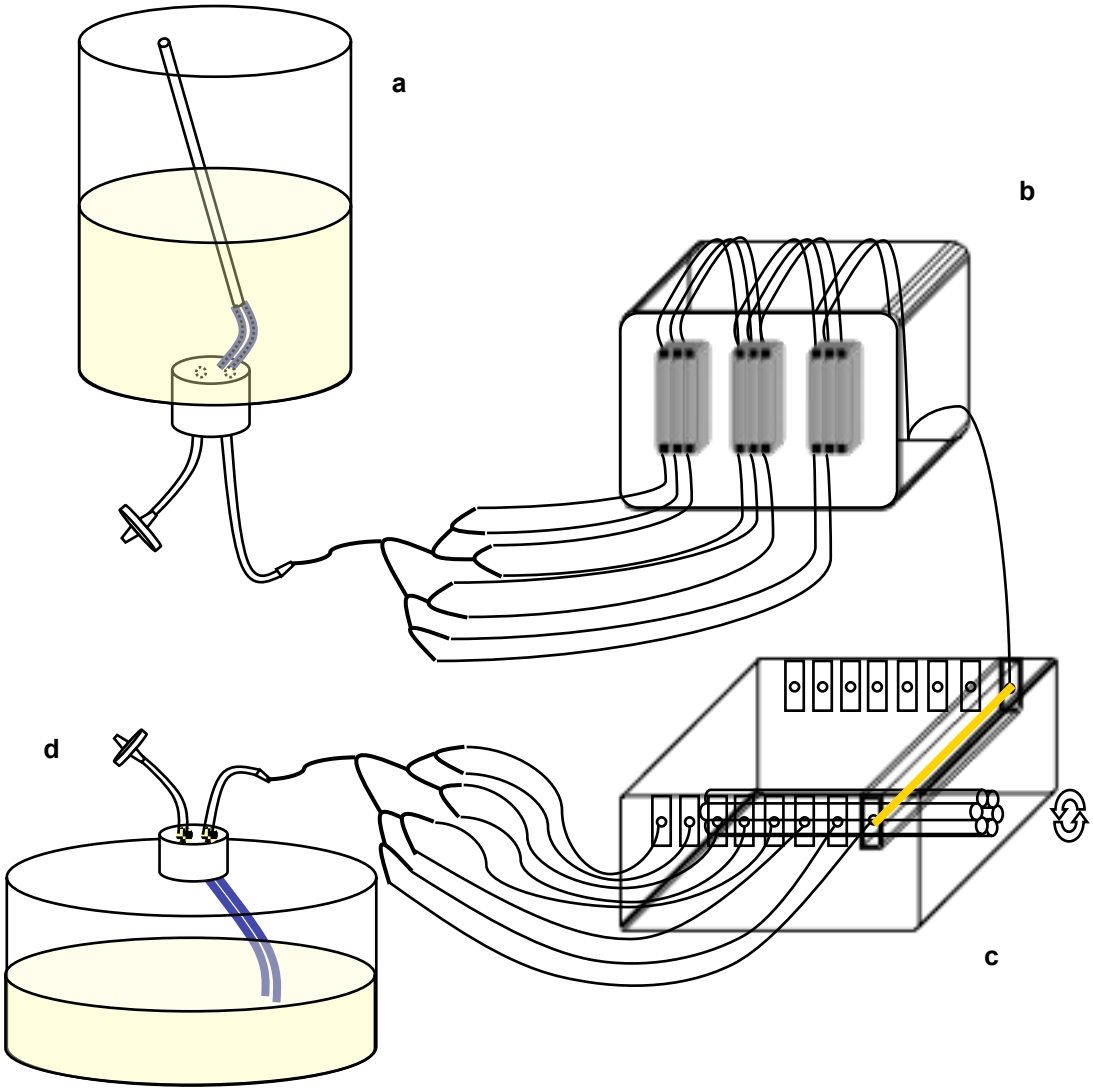


Figure 2.3: Schematic drawing of the flow cell biofilm culture system complete with modifications. Medium flows from the medium bottle (a), through the flow chambers (b) and the persitaltic pump (c) and into the waste container (d).

Confocal z-stack images were taken using a Zeiss LSM 700 with 488 nm and 563 nm metal halide laser sources to excite Gfp and Mcherry fluorescent proteins respectively and a 408 nm laser to excite DNA bound DAPI fluorescent stain.

Biofilm physical parameters were calculated using Comstat2 (Heydorn, Nielsen, et al. 2000b; Heydorn, Ersbøll, et al. 2000a), a software written for the open source imageJ platform (Abràmoff & Magalhães 2004).

2.3.3 Peg Lid Biofilms

Washed precultures were aspirated into a Nunc TSP 96 well (peg lid) plate (each well containing 200 µl) and incubated for 24 h at 37 °C. The lids of these plates have protrusions or pegs that sit in each well. Rather than removing and adding solutions or media the lid can be transferred to a plate containing fresh medium or the relevant solution. Following incubation, lids were washed three times in sterile PBS and then either stained with Crystal Violet as in 2.3.1 or placed into plates containing sterile PBS and centrifuged at 4300 rpm for 30 min to remove the cells. The resulting cell suspension was diluted and plated out to single colonies and the relative frequency of cheating mutant containing a promoterless mini CTXlux insertion was determined by counting luminescent colonies.

2.3.4 DNA Precipitation and Detection

Cultures were sampled at varying time intervals, centrifuged at 14000 rpm for 2 min. Resulting supernatants were filter sterilised using a 0.2 µM pore filter. To remove proteins from the supernatants 100 µl TE buffer was added to 150 µl supernatant followed by 100 µl phenol:chlorophorm:isoamyl alcohol (PCIA). This mixture was vortexed for 10 s and centrifuged at 13000 rpm for 5 min. The resulting mixture separated into two phases, the top phase contained the DNA so was removed and 15 µl 3M Sodium Acetate (NaOAC) was added. This mixture was vortexed and then 450 µl cold Ethanol was added. This mixture was cooled to -20 °C for 16 h to precipitate the DNA. Following precipitation the mixture was centrifuged at 13000 rpm for 10 min at 4 °C to pellet the DNA. The supernatant was removed and the pellet washed with cold Ethanol and air dried. The DNA pellet was then resuspended in 20 µl de-ionised H_2O. DNA in the concentrated DNA samples was then either detected using gel electrophoresis in

0.8 % agarose containing ethidium bromide or quantified using the NanoDrop spectrophotometer.

2.3.5 Microtitre plate assay for DNA release

This high throughput assay for the detection of DNA release was taken from a previous study (Allesen-Holm et al. 2006). 96 well plate cultures were initiated with cultures containing 0.05 μM Propidium Iodide (PI). Propidium Iodide is a nucleic acid stain which fluoresces when bound to DNA or RNA. The cultures were then incubated at 37 °C for 24 h. At 1 hr time intervals culture density was assessed by measuring OD_{600} and eDNA content was assessed by fluorescence readings at ex488 / em560.

2.3.6 Lysis assay

Logarithmic phase cultures were centrifuged at 13000 rpm for 1 min, washed and resuspended in sterile PBS to OD_{600} 1. These cell suspensions were then treated with either a methanol control or 50 μM PQS dissolved in methanol. Suspensions were then sampled at 3, 6 and 24 hr. DNA content of the samples was quantified using the Invitrogen PicoGreen DNA detection kit and CFU was determined by plating out and colony counting.

2.3.7 DAPI staining

To locate and quantify extracellular DNA (eDNA) in flow cell biofilms the DNA binding fluorescent stain 4',6-diamidino-2-phenylindole (DAPI) was used. The stain was first diluted to (0.1 $\mu g ml^{-1}$) and 1 ml of this preparation was fed into the inlet tubes of the desired flow channels under the same flow conditions used for growth of biofilms. The DAPI stain was visualised by excitation with a 408 nm laser.

2.4 *Genetic Modifications*

2.4.1 PCR

PCR amplifications were performed according to the protocol of Saiki et al. (Saiki et al. 1988) in a final volume of 50 µl. For general PCR reactions, 0.5 µl Taq DNA polymerase in 5 x buffer (GoTaqTM Promega, UK) or 0.5 µl Phusion high-fidelity DNA polymerase in 5 x Phusion HF buffer (New England Biolabs, Inc.) were used. Approximately 1 µg of each primer was included in the reaction mix containing 1.5 mM $MgCl_2$ and 2mM dNTPs. When amplifying P. aeruginosa DNA, 3 % (v/v) Dimethyl sulphoxide (DMSO) was added to the reaction mixture to aid DNA melting. The DNA template used was either from whole cells transferred from a fresh colony or 1 µg purified DNA from a chromosomal or plasmid preparation. Reactions were carried out for a total of 30 cycles. Briefly, the DNA template was initially denatured at 94 °C for 5 min (hot start), followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55-58 °C for 30 s and then extension at 72 °C for 1-3 min depending on the length of the DNA to be amplified (1 min per kb). The last cycle finished with a final extension stage at 72 °C for 8 min to ensure completion of all strands. The annealing temperature was varied depending on the nature of the primers used and the stringency levels required. Reaction mixes were then stored at 4 °C.

2.4.2 Generation of a lasIR *mutant*

Plasmid transfer from *E. coli* donor to *P. aeruginosa* recipient cells was carried out by bacterial conjugation. Donor and recipient cells were each grown in 5 ml of LB by overnight incubation. *P. aeruginosa* recipient strains were grown at 42 °C to inactivate a restriction enzyme system which degrades incoming foreign DNA, whilst *E. coli* donor strains were grown at 37 °C. Cells were harvested from 1.5 ml of each culture by centrifugation at 13,000 rpm for 1 min, washed with 1 ml sterile LB and then repelleted under the same conditions. The *Pseudomonas* pellet was resuspended in 1 ml LB broth and this suspension was added to the *E. coli* pellet. The pellets were resuspended well and centrifuged at 13,000 rpm for

1 min. The majority of the supernatant was removed, leaving approximately 100 µl, in which the mixed bacterial pellet was thoroughly resuspended. This was spotted as several small drops onto a LB agar plate and allowed to air dry. It was then incubated at 30 °C for at least 6 h to allow bacterial mating to occur. For each conjugation, a control containing only *P. aeruginosa* or *E. coli* was performed and treated in exactly the same way as the actual samples.

Cells were scraped from the plate using a sterile L-shaped disposable spreader and 1 ml sterile LB. The recovered cells were vortexed and aliquots of 100 µl were spread on to PIA plates containing the appropriate antibiotics to select for *P. aeruginosa* transconjugants and incubated overnight at 37 °C. No growth was expected on the control plates.

To select for double crossover events, a random sample of the resulting transconjugants were grown in 5% salt free sucrose selection broth medium for 16 hr and plated onto 5% salt free sucrose agar. The colonies growing on sucrose were the grid plated onto carbenicillin 400 $\mu g ml^{-1}$ and gentamicin 100 $\mu g ml^{-1}$. Colonies growing on gentamicin but not on carbenicillin were taken to contain the insertion but not the remainder of the plasmid thus being double crossover mutants. The insertion was detected by PCR and the phenotype of the mutants was confirmed by signal molecule extraction and detection.

2.4.4 Insertion of lasI::CTXlux *and* lasB::CTXlux *transcriptional fusion*

The promoter fusion constructs were generated using the mini CTX lux system (Winson 2002) and inserted into *P. aeruginosa* using conjugation by the *E. coli* donor strain S 17-1 as described in 2.4.2. A promoterless mini CTX lux insertion was used to label strains with constitutive lux expression for the differentiation of strains in competition experiments.

2.5 Statistical Analyses

All statistical analyses were performed using R (Ihaka 1996) and various open source packages within R. Graphical data representation was also programmed in R. The statistical work flow was to (1) identify the appropriate parametrical test, (2) check its assumptions against the data to determine the suitability of the test. If a statistical test was deemed unsuitable due to violations of its assumptions, the data was transformed using rank preserving methods such as logarithms or square roots. If the assumptions of the test were still not met after transformation a suitable non-parametric alternative was sought. (3) Once a test had been performed its result formed the basis of the acceptance or rejection of the null hypothesis.

Chapter 3: The evolution of signalling: cost to benefit ratio

3.1 Introduction and aims

3.1.1 Studying the evolution of signalling

Communication abounds in nature and yet some basic questions about the evolution of communication remain elusive. From Konrad Lorenz and his gosling entourage, to our contemporary knowledge of whales, bees, crickets, birds, frogs and the like, we have understood the communication of many different animals (Shorey 1976; Bradbury 1998; Hauser 2003). Understanding them normally comes in the form of observing behaviours associated with the communication such as putty-nosed monkey alarm calls and the rapid escape response when they are issued. Attempting to 'speak' animal languages has also aided our understanding of them. For example, playback or signal manipulation experiments have helped us to understand which kind of mating signals are attractive to females or which parts of a particular signal are required to induce a response. It is tricky however to observe the *evolution* of the charismatic signallers whose languages we have learnt, such as the honeybee, and the nightingale. It is for this reason that in the study of signal evolution, there is an abundance of theories about how signalling systems evolve and a comparative lack of empirical data. As with many other behaviours, microbial systems lend themselves to explicit tests of evolutionary theory. The next two chapters focus on observing the evolution of signalling and testing some general and some specific predictions of evolutionary theory using QS in *P. aeruginosa*.

3.1.2 Measuring the costs and benefits of signalling

Microbial systems are amenable to the study of evolution for the simple fact that populations can evolve in short spaces of time. More subtly though, microbial systems are useful to evolutionary studies because of the degree to which they can be manipulated and the accuracy with which this can be done. Thirdly it is possible to directly observe the costs and benefits of such manipulations at the population level by measuring the growth of a culture (equivalent to reproduction).

To study the evolution of signalling it is necessary to make manipulations to the signal or signaller and measure the change in response by the receiver. Many such experiments have been performed using birds, mammals, frogs or insects where the signal or signaller can be mimicked or manipulated to produce a change in the behaviour of the receiver (Cheney 1982; Levin 1996; Hauser 1998; Irvine 1998; Poole 1999). These usually take the form of playback experiments of auditory signals or direct manipulation of a visual signal and they allow us to asses various elements of the signalling interaction. Though it is possible in such systems to understand the effect of a signal manipulation on its receiver there are limitations. For example, it is not possible to provide signal manipulations over the lifetime of an organism and measure the effect on lifetime reproductive output. Nor is it possible to understand the effect of a signal manipulation at the population level and over evolutionary time scales. Uniquely, bacterial QS offers such an opportunity and it is used here for the first time to study the evolution of signalling and response under manipulations of signalling.

3.1.3 Aims of this study

This chapter sets out to construct an experimental study system in which a signalling behaviour can be manipulated such that individuals performing signalling behaviour are doing so either in excess or insufficiently for an optimal response. The aims of this study are;

1. To describe the effects of excess signal molecule and signal quencher supplementation on the QS behaviour and fitness of populations.
2. To describe the long term adaptive consequences of excess signal molecule and signal quencher supplementation.
3. To probe the molecular and mechanistic route taken by adaptation to such manipulations.

3.2 Results

3.2.1 Manipulations of signalling

Much of the work in this chapter uses a growth medium (QSM) in which QS is required for maximal growth of *P. aeruginosa*. This is a minimal medium containing two carbon sources; CAS Amino Acids (CAA) and Bovine Serum Albumin (BSA). A small amount of free amino acids in the form of CAA allows the culture to begin to grow. When this is depleted, exoprotease is required to continue growth. Since the *lasB* exoprotease and other exoproteases in *P. aeruginosa* are tightly regulated by the *lasIR* QS system (Toder et al. 1991; Toder et al. 1994; Rust et al. 1996; Pearson et al. 1997), this means QS is required for maximal growth in this environment. The following data demonstrates this relationship (Fig. 3.1).

PAO1 WT has an actively transcribed *lasI* gene and produced more exoprotease than a *lasIR* and therefore grew to a higher density in QSM (Figure 3.1). These simple experiments confirmed the importance of QS in this medium and also demonstrated that by manipulations of the *lasIR* QS system, it was possible to influence the behaviour of a bacterial population with implications for the fitness of that population.

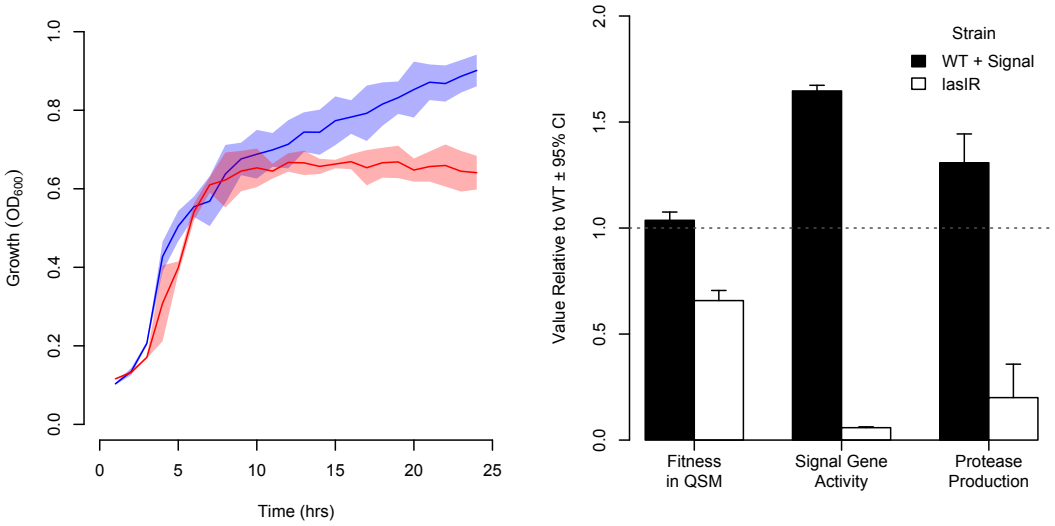


Figure 3.1: Growth curves of PAO1 (blue) and PAO1 Δ*lasIR* mutant (red) in QSM (left). Lines represent the means of 6 replicates and the polygon represents the 95% confidence interval around the mean. Three phenotypes (fitness, signal gene expression and exoprotease production) of PAO1 summarised under manipulations to QS (addition of signal and mutation of *lasIR*, right). Black bars represent relative values of PAO1 in the presence of excess exogenous 3-oxo-C12-HSL and white bars represent values of PAO1 Δ*lasIR.* To enable comparison across all three measures the values are given as a proportion of PAO1 in the absence of signal molecule. Bars represent the mean of 6 replicates. Error bars represent the 95% confidence interval around the mean.

3.2.2 Altering the cost:benefit ratio of signalling

It is possible to synthesise and add exogenous signal molecule and molecules similar in structure and which bind to the cognate receptor protein but do not activate it as a transcriptional factor thus behaving as an inhibitor or Quorum Quencher (QQ). The goal of the following experiments was to manipulate the QS behaviour of a bacterial population such that the production of public goods in response to QS was either excessive or insufficient for maximal growth in the QSM medium. This was done in two ways, firstly by the addition of excess signal molecule (3-oxo-C12-HSL) and secondly by the addition of a known inhibitor of the *lasIR* QS system, N-decanoyl cyclopentylamide (C10-CPA, Ishida et al. 2007).

Cells gain a fitness benefit from QS activity in QSM as shown above (Fig 3.1). When excess signal was added however, it was expected that the cost of the response would increase more than the benefit. This is because although the addition of excess signal induces an increase in the signalling and exoprotease production of PAO1 it does not cause an increase in growth (see figure 3.1). This may be because PAO1 already produces an optimal amount of exoprotease for growth in QSM. An increment in exoprotease production beyond such an optimum does not return an equivalent increment in growth thus the cost:benefit ratio is increased. When adding QQ on the other hand, and inhibiting QS, some metabolic cost is saved but this is likely outweighed by the inhibition of growth and vastly reduced access to overall nutrient resources.

It was therefore predicted that in the QSM environment, when sufficient concentrations of either 3-oxo-C12 HSL signal molecule or C10-CPA QQ molecule are added, QS behaviour is modified such that it is no longer suitable for maximal growth in QSM. Firstly to test whether excess 3-oxo-C12 HSL signal molecule altered QS activity and fitness cultures of PAO1Δ*lasI lasB::lux* were initiated with varying concentrations of signal molecule. A Δ*lasI* mutant was used here to remove native signal production allowing complete experimental control of signal levels in the cultures. QS activity was measured as light output and growth was measured as optical density of the culture. Growth and fitness

are equated in these monoculture experiments and in monoculture experiments throughout.

Maximal growth in LB is about twice that of QSM as may be expected between a rich and a defined medium (Figure 3.2, top). The maximal expression of *lasB* (corrected for culture density) in LB is about 5 times that of QSM indicating that resources necessary for QS activity are more abundant in a rich medium (Figure 3.2, bottom). Interestingly the expression of lasB peaks in the exponential growth phase in LB but is maintained throughout growth in QSM. To visualise the effect of signal concentration on the costs and benefits the data are plotted as a cross section of the time series as indicated by the red dashed lines in Figure 3.2.

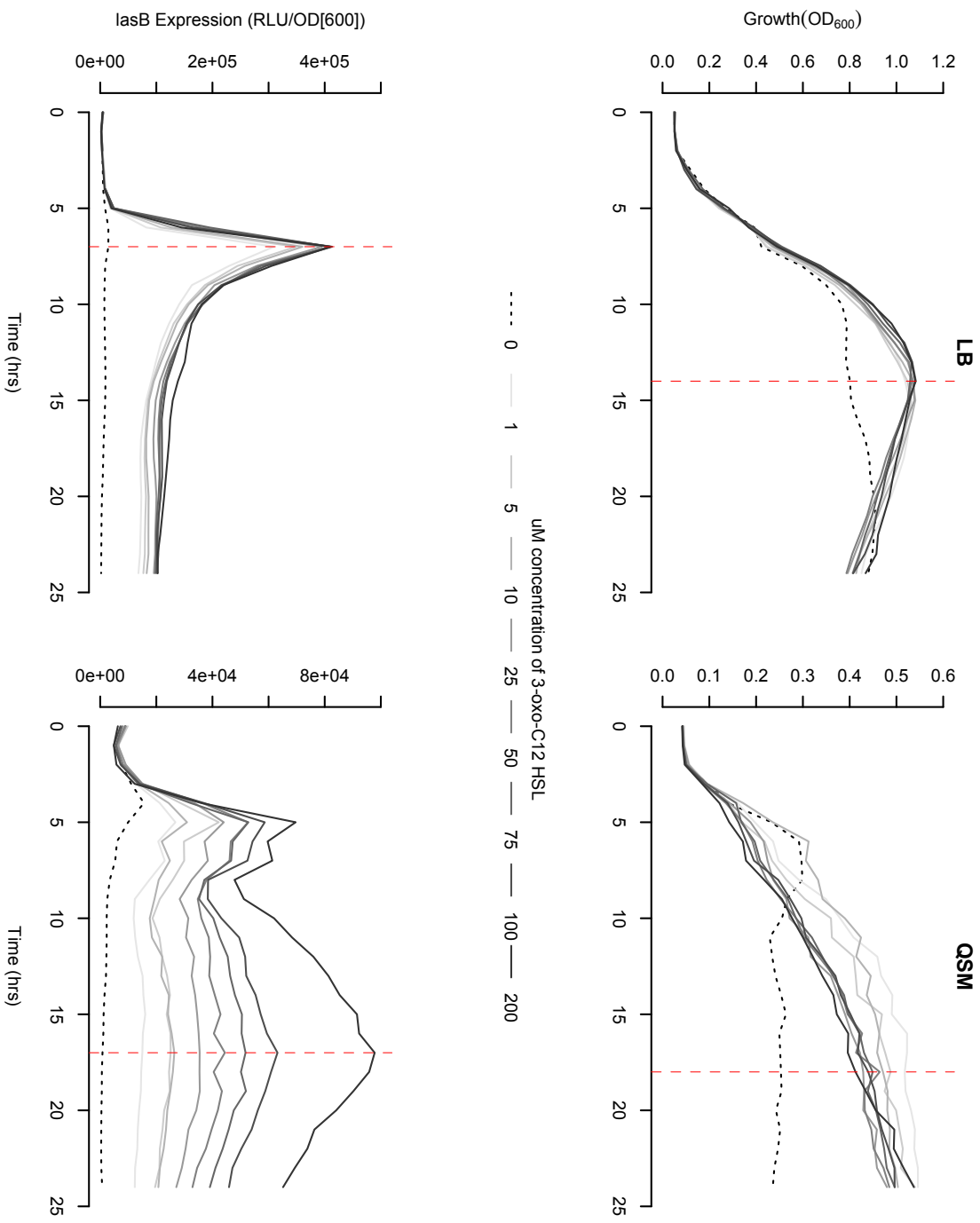


Figure 3.2: The effect of adding varying concentrations of the signal molecule 3-oxo-C12 HSL to a signal negative Δ*lasI* mutant on growth (top) and *lasB* exoprotease expression (bottom) in a rich medium (LB, left) and a QS dependent medium (QSM, right) over a 24 hr period of incubation.

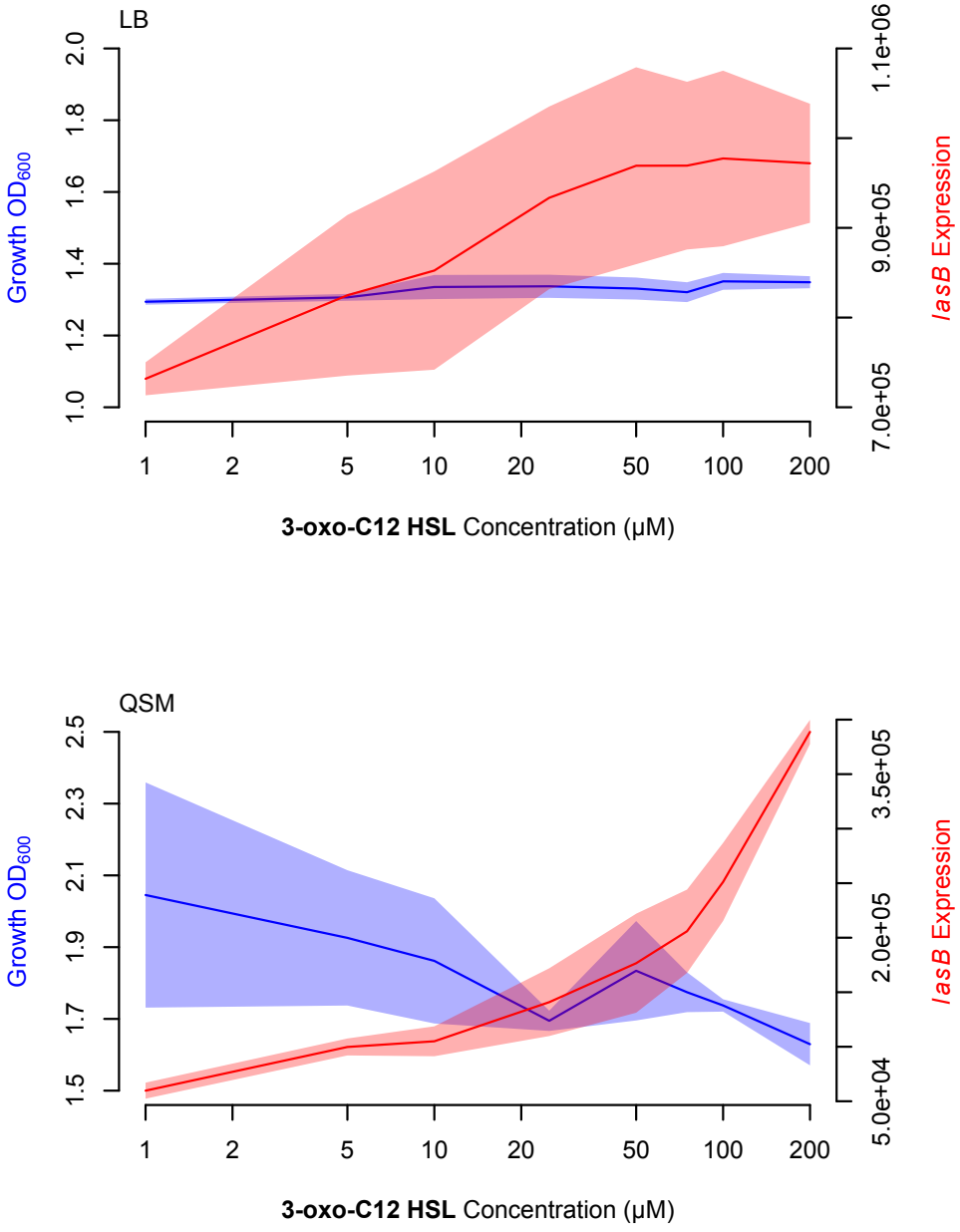


Figure 3.3: The effect of 3-oxo-C12 HSL signal concentration on maximal growth and *lasB* exoprotease expression in LB and QSM of a signal negative Δ*lasI* mutant. All values are represented as a proportion of of the corresponding value when no signal is added i.e. fold change observed upon the addition of signal. Dark lines represent the mean of 3 replicates and the shaded polygon represents the 95 % confidence interval around the mean.

Values over 1 indicate an increase in either growth or *lasB* expression upon the addition of signal and this was true for all of the measures taken (Figure 3.3). At 1 μM 3-oxo-C12 HSL there was a slight benefit to growth and a large increase in response (*lasB* expression) in LB (Figure 3.3, left). In QSM however, at 1 μM 3-oxo-C12 HSL there was a large benefit to fitness and a smaller increase in response (Figure 3.3, right). In LB there was a weak positive relationship between signal concentration and fitness ($F_{1,6} = 14.48$, $p < 0.01$, $r^2 = 0.6582$) and a strong positive relationship between signal concentration and response ($F_{1,6} = 98.66$, $p < 0.001$, $r^2 = 0.9331$). In QSM there was a strong negative relationship between signal concentration and fitness ($F_{1,6} = 27.45$, $p < 0.01$, $r^2 = 0.7907$) and a strong positive relationship between signal concentration and response ($F_{1,6} = 131.8$, $p < 0.001$, $r^2 = 0.9492$).

Let us consider first the effect of adding a small amount of signal (1 μM) to a signal negative mutant. The benefit to growth was greater in QSM than in LB (Figure 3.3, top). This confirms that dependence on QS for growth is higher in QSM than in LB. There was only a slight benefit of QS to growth in LB. This is probably because apart from the lasB exoprotease, many other extracellular and intracellular processes are regulated by the *lasIR* QS system and at least one of them is of benefit to growth, even in a rich medium. At 1 μM signal concentration there was a much greater QS response in LB than in QSM (Figure 3.3, bottom). This is possibly because in LB the constituents necessary for 3-oxo-C12 HSL biosynthesis are more readily available than in the defined medium QSM.

Let us now consider the relationships between growth, fitness and signal concentration. In LB increased response with increasing signal concentration has little effect on growth. In QSM increased response with increased signal concentration has a negative effect on growth. We can infer from this that in LB the cost:benefit ratio remains similar over a large range of signal concentrations. Crucially in QSM we were able to alter the cost:benefit ratio of signalling such that the response of a population is suboptimal. Additional exoprotease

production by the population upon excess signal addition resulted in a decrease in fitness. The next section examines the effect of adding a known Quorum Quencher in the same way. To test whether excess C10-CPA QQ molecule molecule altered QS activity and fitness, cultures of PAO1 *lasB::lux* were initiated with varying concentrations of C10-CPA QQ molecule. QS activity was measured as light output and growth was measured as optical density of the culture.

Upon the addition of a quorum quenching molecule C10-CPA, the growth of PAO1 WT was impaired slightly in LB and greatly impaired in QSM. Expression of *lasB* was decreased in both media but seems to be only delayed at lower concentrations of C10-CPA in LB. This again may be due to the variable costs of signal biosynthesis in different media. As previously and to visualise relationships between fitness, response and quencher concentration the data are plotted as a cross section of the time series as indicated by the red dashed lines in Figure 3.4.

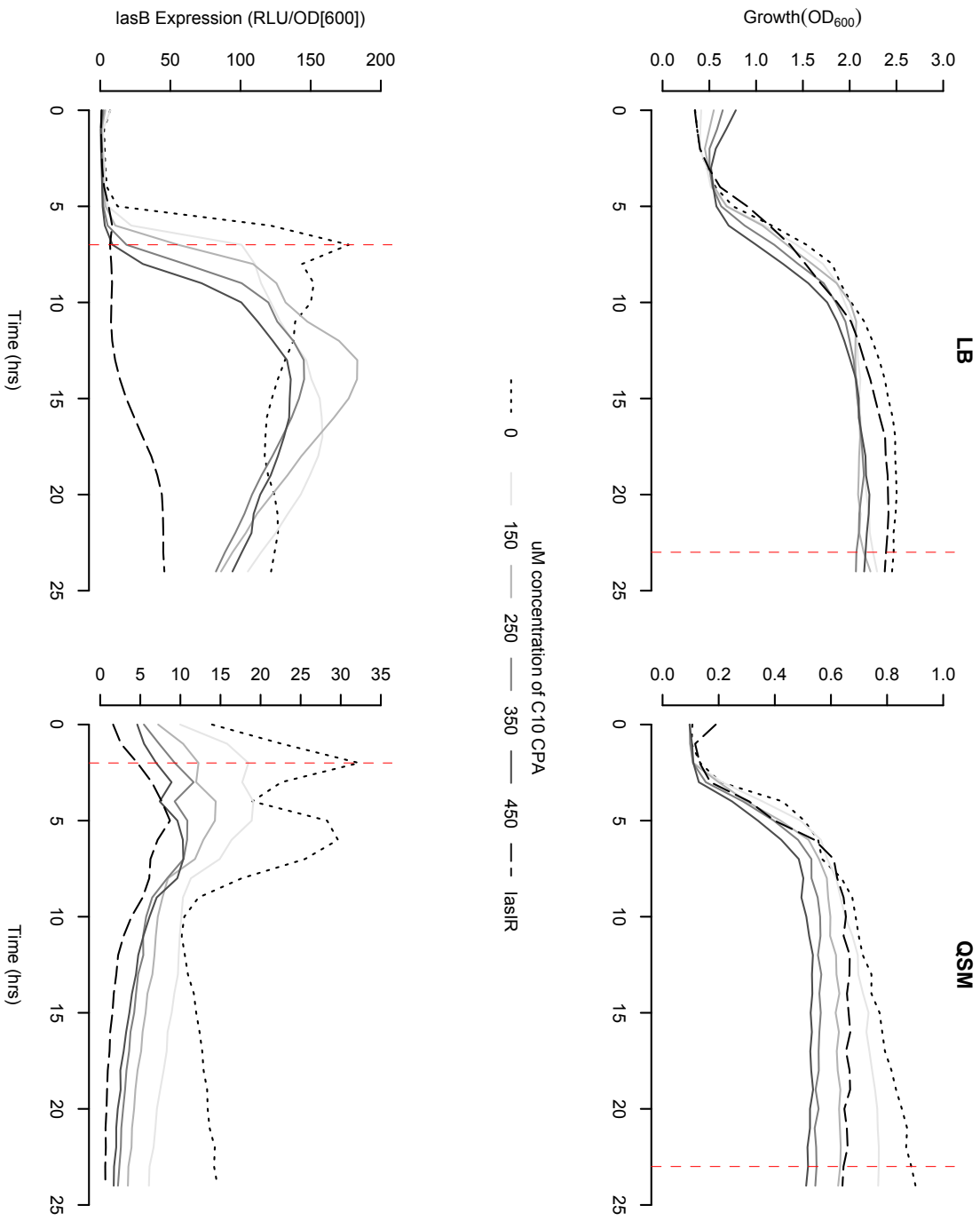


Figure 3.4: The effect of adding varying concentrations of the Quorum Quenching molecule C10-CPA to a WT on growth (top) and *lasB* exoprotease expression (bottom) in a rich medium (LB, left) and a QS dependent medium (QSM, right) over a 24 hr period of incubation.

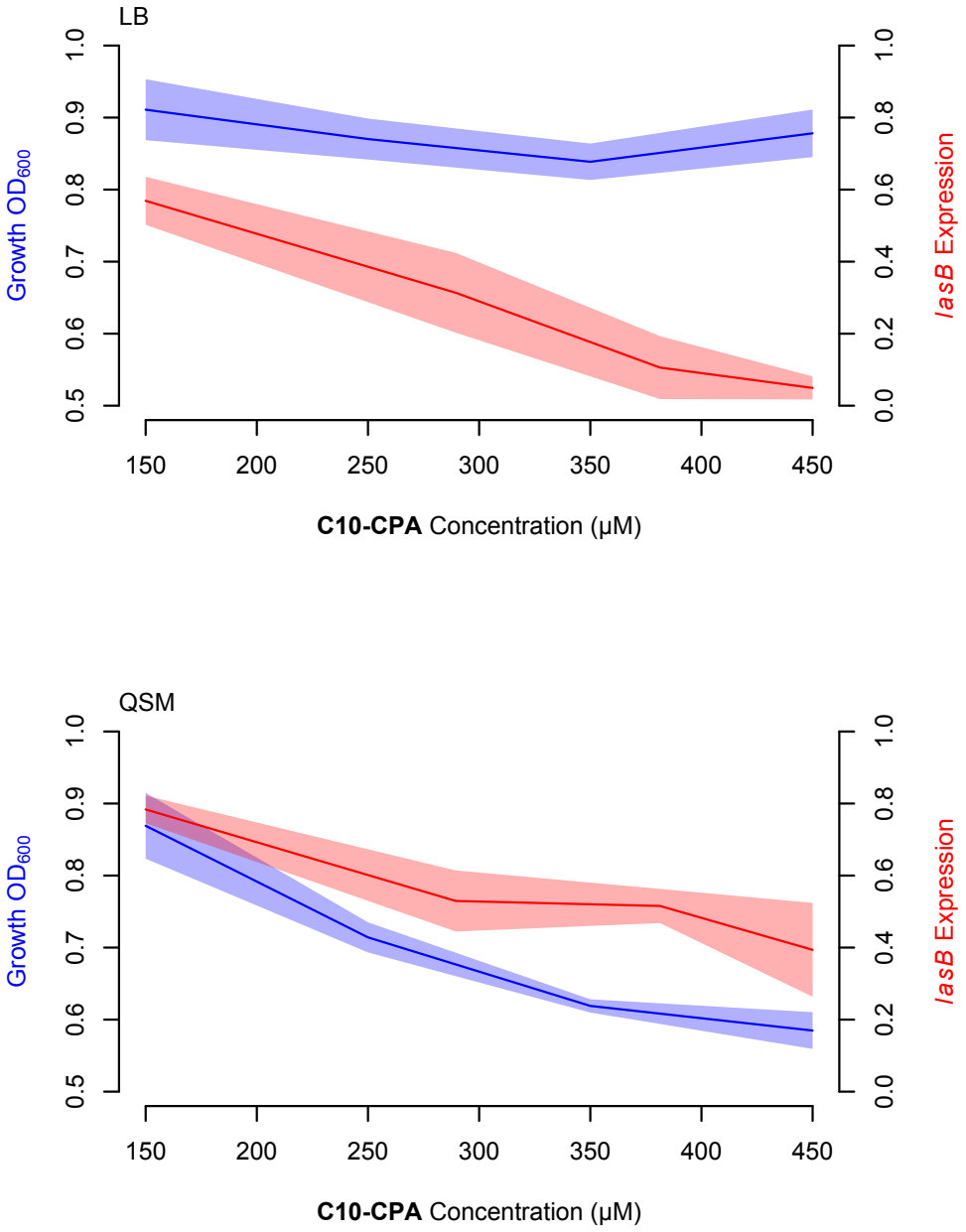


Figure 3.5: The effect of C10-CPA quencher concentration on growth and *lasB* exoprotease expression in LB and QSM of a WT. All values are represented as a proportion of the corresponding value when no quencher is added. Solid lines represent the mean for 3 replicates and shaded areas represent the 95 % Confidence Interval around the mean.

All values are below 1, indicating that there was a decrease from WT levels upon the addition of C10-CPA for all measures taken. There was no significant relationship between growth in LB and C10-CPA concentration ($F_{1,2}$ = 0.9364, p > 0.05, r^2 = -0.022) despite a significant negative relationship between response and C10-CPA concentration ($F_{1,2}$ = 29.92, p < 0.05, r^2 = 0.906). Therefore the minor cost to growth in LB upon the addition of C10-CPA is not QS dependent. There was a significant negative relationship between growth in QSM and C10-CPA concentration ($F_{1,2}$ = 24.67, p < 0.05, r^2 = 0.8875) and a corresponding negative relationship between response and C10-CPA concentration ($F_{1,2}$ = 19.64, p < 0.05, r^2 = 0.8614).

QS response could be quenched in both a rich medium and QSM however, in the rich medium this did not coincide with a reduction in fitness. In QSM it is possible to reduce the fitness by quenching QS response.

3.2.3 Adaptive response to varying cost:benefit ratio of signalling

In the previous section it was demonstrated that the addition of sufficient concentrations of either signal molecule or quorum quenching molecule altered the QS behaviour in such a way that caused a detriment to fitness. A necessary condition for this is that QS activity be positively linked to fitness. It is possible to *increase* response beyond its optimum and cause a detriment to growth and it is possible to *reduce* response beyond its optimum and cause a detriment to growth. A question that so far has never been empirically examined in the evolution of signalling is whether it is possible to overcome such alterations to signalling through adaptation and if so; what route does such adaptation take. For instance where excess signal is added, individuals may be favoured to respond at a lower rate, produce less signal or both. Or indeed, in the complex network of QS regulation some other regulatory path may be emphasised in favour of the one being manipulated. To test this, populations of PAO1 WT *lasI::lux* initiated from a single colony were serially cultured in QSM media containing either no addition, 50 μM 3-oxo-C12 HSL or 250 μM C10 CPA, for a total of 20 transfers (See Materials and Methods for full details). Following this

the populations were assayed for *lasI* expression using the *luxCDABE* reporter fusion, exoprotease production using the Elastin Congo Red assay and fitness in QSM in simple growth experiments.

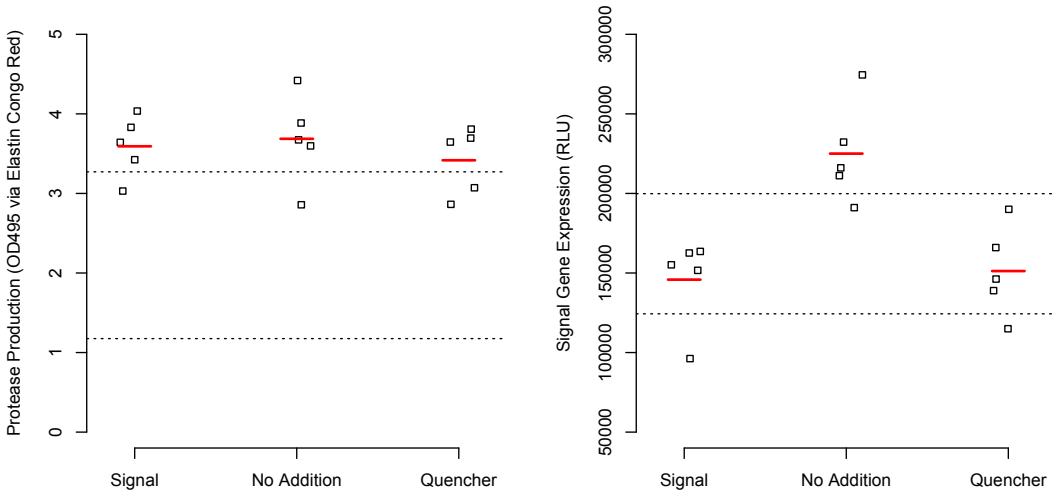


Figure 3.6: Exoprotease production and signal synthase gene expression in populations resulting from evolution in the presence and absence of either 50 µM 3-oxo-C12 HSL or 250 µM C10 CPA after 20 transfers. Each dot represents a replicate population, red bars represent the group mean and dashed lines represent the ancestral mean (top) and the mean for a Δ*lasIR* mutant (bottom).

After 20 transfers of experimental evolution, it was observed that the resulting populations did not differ in the production of LasB exoprotease (Figure 3.5, $F_{2,12} = 0.4358$, $p > 0.05$) but the addition of either signal or quencher caused a reduction in signal synthase (*lasI*) gene expression (Figure 3.5, $F_{2,12} = 11.39$, $p < 0.01$). The effects of adding either signal or quencher did not differ significantly from each other ($F = 0.084$, $p > 0.05$, by grouping factors levels). To test whether these changes in signal synthase gene expression are as a result of adaptation, fitness of the resulting populations was measured in QSM and compared with fitness in the evolved environment (i.e. in the presence or absence of signal or quencher).

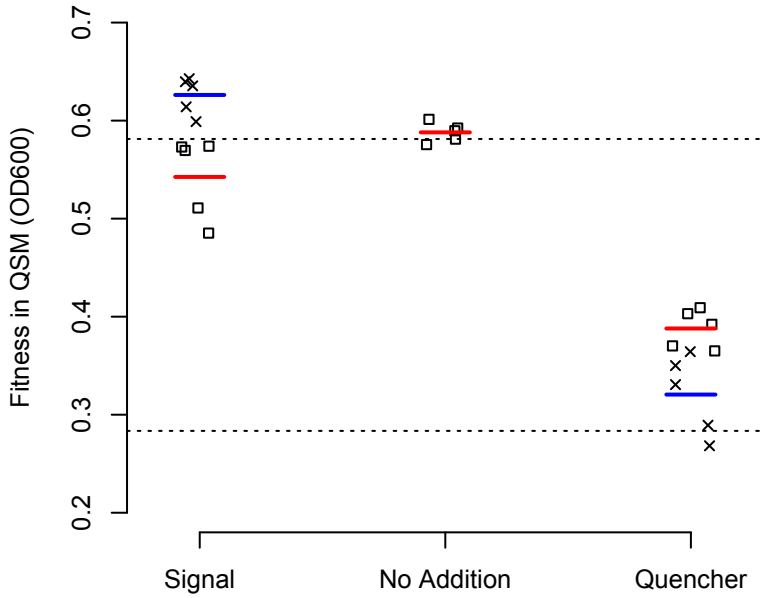


Figure 3.7: The fitness of populations resulting from evolution in the presence or absence or signal or quencher in QSM (squares, red bars), and the environments in which they evolved (crosses, blue bars) e.g. QSM + signal for the signal treatment. Dashed lines represent the ancestral the ancestral mean (top) and the mean for a Δ*lasIR* mutant (bottom).

By grouping the levels of a factor variable it is possible to test whether each of the groupings in Figure 3.7, explains a significant amount of the variation in fitness. Populations evolved in either the presence of signal or quencher had reduced fitness in QSM ($F = 6.117$, $p < 0.05$). Populations evolved in the presence of signal had greater fitness in the presence of the signal than when it was removed ($F = 20.70$, $p < 0.001$). This suggests that those populations adapted to the presence of signal molecule. In the case where quencher was added to evolving populations, despite having had time to adapt, they were less fit in the presence of the quencher than in its absence ($F = 13.45$, $p < 0.01$). It may in fact be that some minor adaptation has occurred but populations have not been able to overcome the fitness cost imposed by quorum quenching. To determine whether within group variation is concealed by population level assessments of exoprotease and signal production these experiments were

repeated on individual clones isolated from the populations (5 clones from each replicate population (5) in each treatment (3) = 5 x 3 x 5 = 75 clones).

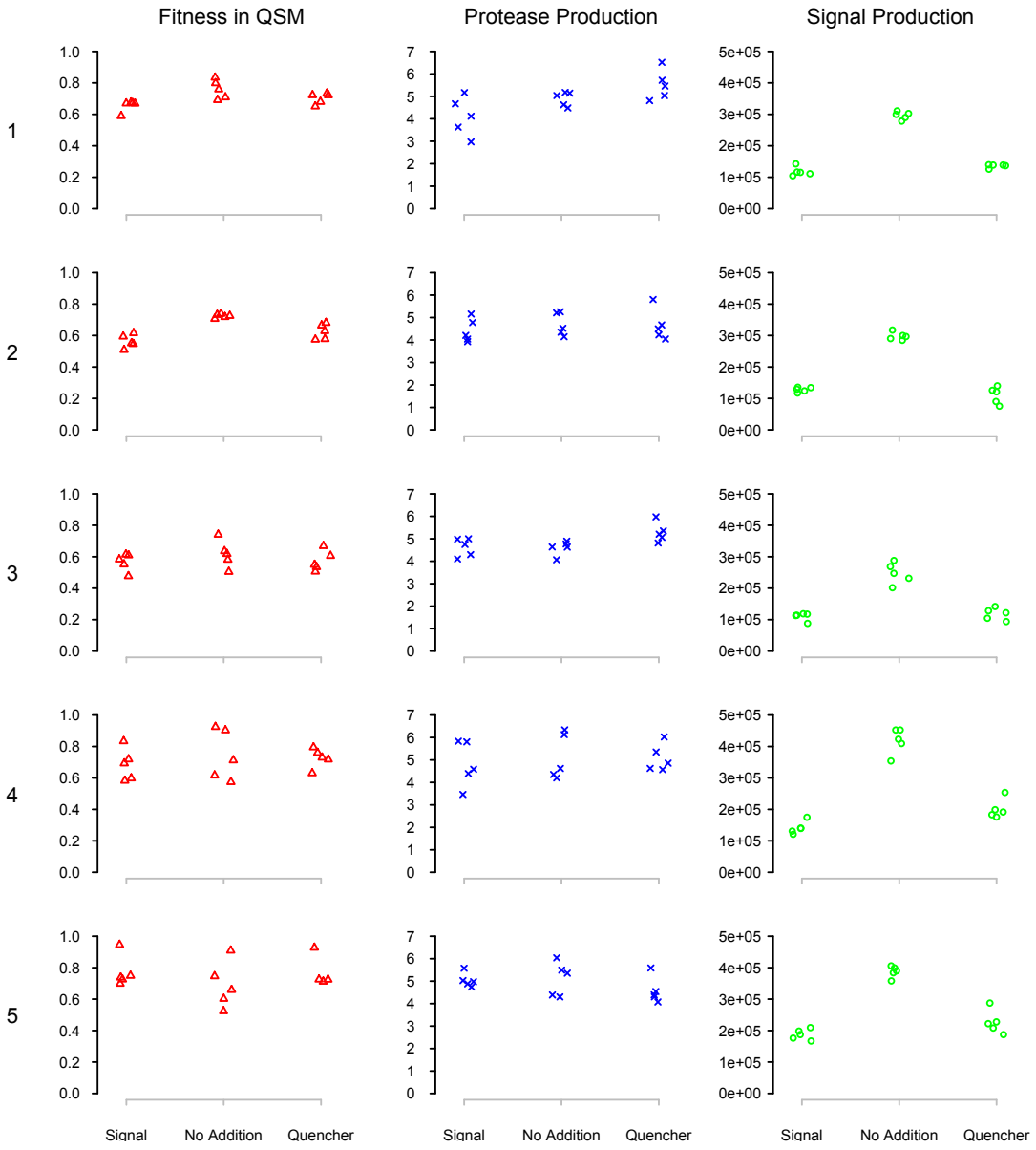


Figure 3.8: Fitness in QSM (OD 600), LasB exoprotease production (OD 495) and signal production (RLU/OD600) of individual clones from populations evolved in the presence or absence of signal quencher. Each horizontal triplet (1-5) represents a replicate of the experiment.

Taking into account all 5 replicates of the experiment (Fig 3.8), the addition of signal or quencher treatments did not explain significant levels of variation in Fitness in QSM ($F_{2,68}$ = 2.462, p > 0.05) or LasB exoprotease Production ($F_{2,68}$ = 2.462, p > 0.05) but did explain a significant amount of the variation in Signal gene expression ($F_{2,68}$ = 297.9, p < 0.05). It seems that some of the variation in fitness in QSM, within replicate populations is concealed by assays at the population level.

Both populations evolved with the addition of either signal or quencher show a reduced signal output. Despite this, those same populations continue to make exoprotease (Figure 3.8). If the ratio of the LasB exoprotease production : signal production was taken to be the responsiveness of the populations, we would conclude that these populations have become exquisitely sensitive to signal. This would be counterintuitive as an adaptation to suboptimal signal levels and resulted in two hypotheses. First; the responsiveness of populations evolved in the presence of either signal or quencher molecules indeed became more sensitive to signal or second; another of the QS signals that influences *lasB* exoprotease expression was emphasised in place of the *lasIR* QS system. To assess responsiveness directly, an aliquot of each replicate population, was used to inoculate cultures in rich medium (LB) in the presence and absence of 3-oxo-C12 HSL signal molecule. Since the ancestor of the experiment contained the *lasI::lux* promoter fusion and expression of this requires a functional signal-receptor complex, responsiveness was taken to be the light output in the presence of signal / the light output in the absence of the signal.

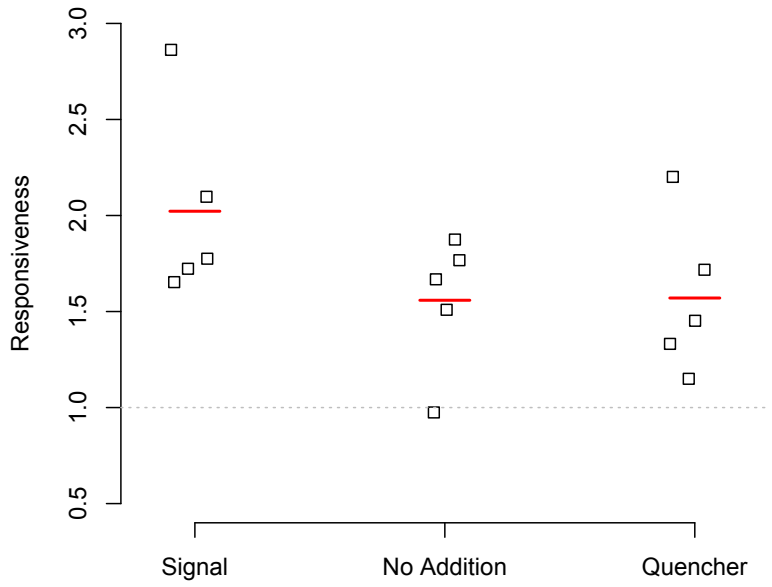


Figure 3.9: Responsiveness of populations evolved under the presence or absence of 3-oxo-C12 HSL or C10-CPA. Values given are a ratio between *lasI::lux* activity in the presence and absence of 50 µM 3-oxo-C12 HSL. Each data point represents a replicate population and the red lines represent the mean in that group. The dashed line at 1 represents an equal *lasI::lux* activity in the presence and absence of 50 µM 3-oxo-C12 HSL.

Evolution in the presence of either 3-oxo-C12 or C10-CPA did not result in a change in responsiveness to 3-oxo-C12 HSL (Fig 3.9, $F_{2,12} = 1.94$, $p > 0.05$). This leads to the rejection of the hypothesis that those populations retain exoprotease production in spite of decreased signal production due to increased sensitivity to signal. To test whether populations had instead emphasised another signal molecule to regulate the production of *lasB* exoprotease, cultures were initiated and signal molecule extractions performed on sterile cell free supernatants. A signal molecule profile was generated by adding signal molecule extracts to three reporter strains designed to detect either long or short chain AHL's or AQ's.

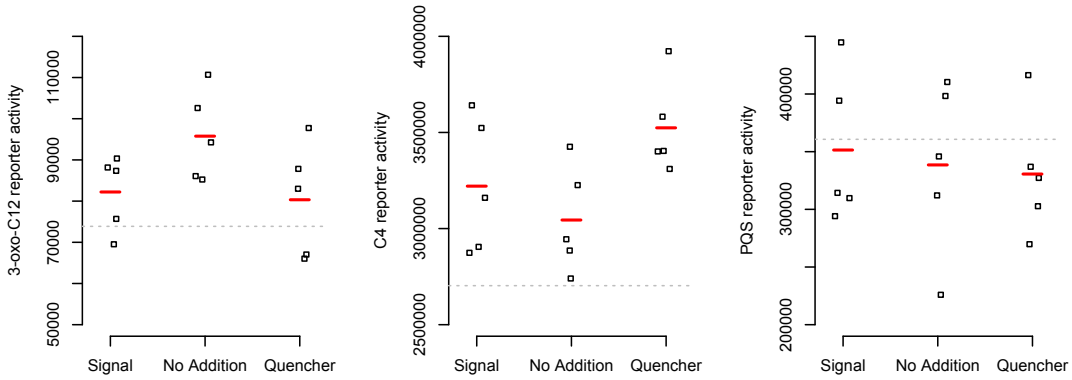


Figure 3.10: Signal molecule extraction and profiling of populations evolved under the presence or absence of 3-oxo-C12 HSL or C10-CPA. Values are Relative Light Units (RLU) of biosensor strains designed to detect long chain AHL's (pSB1142, left), short chain AHL's (p56536, middle) and AQ's (PAO1 Δ*pqsA pqsA::lux*, right). Each point represents a replicate population, the red lines represent the mean for each group. The dashed grey lines represent the ancestral mean.

The mean reporter activity was lower for long chain AHL's and higher for short chain AHL's in populations evolved in the presence of signal or quencher compared to the control though there was no significant difference between the groups ($F_{2,12}$ = 2.74 and 3.41 respectively, $p > 0.05$). Evolution in either the presence or absence or signal or quencher had no effect on the production of AQ's ($F_{2,12}$ = 0.13, $p > 0.05$). These results suggest the possibility that C4-HSL is emphasised in populations where cost:benefit ratio is distorted over generations. In the absence of statistically significant differences between groups however, it cannot be stated as a firm conclusion. These results will be discussed further in the discussion of this chapter.

3.5 Discussion

The power with which one can manipulate microbial study systems is unparalleled. In this investigation for example two chemically synthesised molecules were used, a signal molecule identical in chemical structure to the native 3O-C12-HSL signal molecule and a signal molecule mimic (C10-CPA) used to quench the native receptor (LasR) of the signal. These can be added in varying concentrations and at different times if necessary. Combined with the use of knock-out mutants and promoter fusions to the *luxCDABE* operon, it is possible to study the effects of manipulations to signal not only on the response but also the consequence of the altered response. This study is an example of when precise and powerful manipulations to a microbial study system unlock the potential to answer questions in an untapped area of evolutionary theory.

The present study system uses a medium in which the *lasIR* QS system is necessary for maximal growth. A Δ*lasI* mutant does not grow well in this system and a small amount of 3-oxo-C12 supplementation restores QS response and maximal growth. Any increase beyond this optimal concentration reduces the growth restoration despite a continually increasing QS response. This leads to the conclusion that if sufficient excess signal molecule is provided, the resulting response to signal is above and beyond that necessary for growth. Once the hydrolysis of protein by QS regulated exoproteases is saturated (cannot be increased by further production of exoprotease) a further increase in exoprotease production provides no further benefit to the population and incurs only a cost. The provision of a signal quencher to a QS positive PAO1 WT strain however, reduces QS response to the native signal and therefore impedes maximal growth in QSM. It is perhaps surprising that PAO1 QS is tuned to growth in the QSM medium such that any perturbation results in a fitness decline. This is due to the medium composition having been designed for maximal growth of PAO1 WT.

The QSM medium imposes the condition that QS is necessary for maximal growth. To demonstrate the importance of this environment, signal

manipulation experiments were also performed in a rich medium (LB) where the benefit of QS is relaxed. Although the QS response could be induced or impeded, there were no fitness consequences in a nutrient rich growth environment. The reasons for this are likely twofold; firstly there is no benefit to QS activity, nutrients are freely available and secondly, the cost of QS activity is reduced due to a diverse nutrient base from which to supply AHL biosynthesis. QS inhibition manifests predominantly as a delay in QS response in a rich environment compared to an overall decrease in a defined medium. This is also consistent with reduced cost of QS in a rich medium.

This study observed the long term evolutionary responses to such manipulations. When signal molecule or signal quencher is continually provided over generations (approx. 120), the expression of the native signal synthase was significantly reduced compared to a control or the ancestor. Despite this the production of exoprotease remained the same as in the control and the ancestor. This is a striking and initially counterintuitive result. A decrease in signal synthase expression should be accompanied by a decrease in behaviours regulated by that signal. This led to several hypotheses and data was obtained to distinguish between a few of them. Firstly it is possible given a low signal synthase expression and normal exoprotease production that the populations in question had evolved an acute sensitivity to signal. This possibility was excluded via a direct assessment of responsiveness of those populations. Secondly it is possible that another AHL signal was emphasised instead of 3-oxo-C12 HSL and that this was responsible for the maintained exoprotease production.

When the signal molecule profile for populations evolved in the presence or absence of either signal or quencher is examined it emerges that there is no significant difference in long chain AHL's, short chain AHL's and Alkyl-Quinolones between any of the groups. Though the mean long chain AHL production is lower in populations evolved in the presence of either signal or quencher there is no significant difference between the groups. This is counter to expectation given the drastic reduction in *lasI::lux* expression. This may be

due to the fact that the biosensor is sensitive to long chain AHL's such as 3-oxo-C10 HSL as well as 3-oxo-C12 HSL, though to a lesser degree (Winson et al. 1998).

The main novel finding of this study is that populations evolved in the presence of excess signal molecule or quencher undergo a regulatory change in the production of LasB exoprotease. It is possible that this is still under QS control and that the QS circuit has been reprogrammed with respect to the production of LasB exoprotease. For instance a change in the regulatory region of QS controlled exoprotease genes could result in greater sensitivity to transcriptional induction by the signal-receptor complex. Alternatively it is also possible that exoprotease is produced constitutively. Crucially, the fitness effects of evolutionary change to such manipulations was assessed. In the case of signal addition, evolved populations were less fit that the ancestor when the signal was subsequently removed. In the presence of signal however (in the evolved environment), this fitness was higher than the control. This is clear evidence of adaptation. Populations evolved in the presence of signal suffer a fitness cost when the signal is subsequently removed but gain a fitness advantage (free signal) in their evolved environment. In the case of quencher addition, the fitness of evolved populations was reduced when the quencher was subsequently removed, but decreased further when in the presence of quencher. Though there may have been some adaptation, the effect of quenching on populations evolved in the presence and absence of quencher is needed to determine this. It may be of note however to clinical microbiologists with an interest in therapeutic QS quenching, that the fitness of populations evolved in the presence of quencher was reduced compared to the control.

Finally and to draw the obvious comparisons with the animal world, the power of microbial systems should be made clear. As explained previously one could issue the recorded alarm call of a putty-nosed monkey. One could then asses the response of the individuals. Much harder would be to evaluate the effect of their altered response. For example does the continual artificially induced flight

response reduce the likelihood of predator evasion. It would then be all but impossible to issue such false alarm calls to an entire population over generations and observe the change in frequency of different response strategies over evolutionary time scales. Such is the power of the test tube and petri dish.

Chapter 4: The evolution of signalling: common interest

4.1 Introduction and aims

4.1.1 QS as Biological Communication

Communication is an act or structure produced by one individual that influences the behaviour of another. As such, communication is inherently social and we can categorise types of communication based on the conditions that are required for them to evolve. There is a large body of literature dedicated to definitions of biological communication but those proposed by Maynard-Smith and Harper are used in this chapter (Maynard-Smith & Harper 2003). Based on two criteria, their classification aids us in understanding how different types of communication evolve. Firstly, 'did the act or structure evolve due to a particular response?' and secondly, 'is the act or structure effective because the response has evolved accordingly?'. Answering these questions leads us to determine whether an act or structure may be a true signal, an environmental cue that guides an action, or a coercion (Maynard-Smith & Harper 2003; Diggle, Gardner, et al. 2007b). Such distinctions are important because understanding the precise biological nature of an interaction between individuals allows us to make predictions about how such interactions evolve and are maintained in natural populations.

It is useful to explore a few examples to illustrate this. Consider a cow grazing on a field. It respires releasing carbon dioxide which is detected by a mosquito. The mosquito follows the carbon dioxide gradient to find the cow and feeds on it. The carbon dioxide is effective due to an evolved response on the part of the mosquito but its production by the cow did not evolve for that reason so in this scenario we should consider carbon dioxide to be a cue and not a signal. Now consider two rhinoceros beetles. In a territorial dispute they fight on a branch and one pushes the other over the side. This act evolved owing to its effect on the receiver, but the receiver does not benefit from an accordingly evolved response. This scenario is therefore a coercion. Finally, consider an alarm call

from a putty-nosed monkey. When a predator is detected, the alarm call is sounded and all in earshot rush to safety. The alarm has evolved due to the response from its receivers and the receivers benefit from an evolved response. In this case the alarm call can be termed a signal. These examples are used here simply to illustrate the differences between types of communication. It is however possible that a putty-nosed monkey issues an alarm call in the absence of a predator simply to move his competitors from a favourable feeding patch. To properly categorise such communication, Maynard-Smith and Harper's two criteria (see above) must be measured. With respect to bacterial QS, there is a tendency in the literature to term all the molecules and interactions as 'signals' even if the above-mentioned criteria are unknown.

Let us turn our attention to a QS bacterial population. When a QS-regulated exoenzyme is important for growth, many deviations from the normal strategy could confer a benefit (Sandoz et al. 2007; Diggle et al. 2007a; Czárán & Hoekstra 2009). A mutant that does not respond to QS by producing the enzyme would benefit nonetheless from the enzyme production of others. A mutant that does not signal benefits from sensing population density as signalled by other cells in the population. A mutant that overproduces the signal, yet does not respond to it, benefits from overproduction of the costly enzyme by its neighbours. Experimentally it has now been demonstrated that QS is subject to invasion by cheats and that QS cheating can be detrimental to the population (Diggle et al. 2007a; Sandoz et al. 2007).

4.1.2 QS evolution and common interest

Since signalling is vulnerable to various types of social exploitation, we may ask what forces maintain it in nature? Three broad mechanisms can maintain reliable signalling. Firstly a signal can be a reliable index of quality. For example, the pitch of a deer roar is strongly correlated with the size of its larynx and linked to the body size of the male (Reby 2003). Therefore a roar provides reliable information about the male. Secondly if a signal is very costly, this reduces the likelihood of cheating. For instance, a nightingale sings for most of

the night and loses up to 10% of its body weight in doing so (Thomas 2002; Kipper et al. 2006). An inferior male could also sing but is less likely to do so if the metabolic cost is unaffordable. Thirdly a reliable signal can be maintained by common interest. Common interests in bacterial populations are likely to arise through Hamiltonian relatedness, as described elsewhere (see Chapter 1). For instance if on average, a cooperative cell interacts with other cells that share the gene for cooperation then the shared interest in reproduction removes the advantage of cheating.

With an evolutionary model of QS, Brown and Johnstone explore how signalling and response to the signal evolves with varying relatedness (Brown & Johnstone 2001). Their model assumes that QS signals and response are (1) costly to the individual cells producing them; (2) beneficial to the group of interacting cells (cooperative) and (3) that cooperation is more beneficial at high population density. First they find that selection for public goods production in response to the signal is reduced with decreasing relatedness. When on average a cooperative individual encounters individuals that do not share the genes for cooperation, it does not pay to cooperate. Second the authors find that selection for signalling to elicit cooperation peaks at intermediate relatedness. The cells need information on population density in order to reap the rewards of cooperation which are density dependent in this model. To gain this information via a signal, there needs to be an agreed signalling convention - so long as every cell follows this convention, the resulting signal density can be reliably decoded. Importantly, this 'agreed convention' could be high or low, and work equally well. Under high relatedness, the model predicts that selection on signal cost will drive signal level to the lowest level that is consistent with reliable signal functioning, i.e. sufficient to overcome background noise. Selection on signalling under genetic conflict can favour increased signal production to coerce cooperation from competitors. In return this will select for 'devaluation' of the signal currency and less cooperation for a given input. This ratchet continues until a stable equilibrium is reached, and the prediction is that this stable equilibrium will peak under intermediate genetic conflict.

In a spatially explicit simulation model Czaran et. al. explore the social evolution of different QS strategies (Czárán & Hoekstra 2009). They allow for variation in three hypothesised loci, C, S and R standing for Cooperation, Signalling and Response which can be either functional or inactive. This creates 8 possible genotypes. When set into motion the authors observed a stable coexistence of two of these, "Honest" denoted CSR which cooperates, signals and responds and "Ignorant" denoted csr which does none of these. The equilibrium shifts in the favour of "Ignorant" when diffusion rate is increased as this mixes interactions between non-related individuals (i.e. decreases relatedness). As the threshold number of cells resulting in a quorum increases, the system becomes highly sensitive to changes in relatedness. For example, when a large number of cells is required for a quorum, small amounts of diffusion will disrupt interactions between related cells and prevent a quorum from being reached. Most interestingly, across much of the parameter space and particularly at intermediate levels of effective relatedness, "Liar" genotypes which do no cooperate or respond but nonetheless produce signal are present. For example when diffusion is sufficient to allow exploitation of cooperating genotypes, selection for signal production allows "Liar" genotypes to prosper more than silent exploitative genotypes.

The two theoretical studies, though very different in their approach produce similar conclusions. First that exploitation of cooperative signalling is commonplace and can be stabilised by high relatedness. Second that when QS regulates cooperation, a plethora of social interactions are possible and can coexist. In particular, cooperative, exploitative and coercive strategies should be relatively common in nature. This chapter sets out to test the predictions of such theoretical analyses.

4.1.3 Aims of this study

Specific theoretical analyses of QS evolution predict that common interest in signalling can be generated through high Hamiltonian relatedness and that such conditions favour the maintenance of QS. Czaran et. al. (2009) also predict that different QS genotypes/phenotypes producing different strategies can stably coexist under conditions of lower Hamiltonian relatedness. This chapter aims to test these predictions using experimentally evolved populations of *P. aeruginosa* in the following way.

1. Evolving populations under conditions favouring either high, intermediate or low relatedness.

2. Measuring the public goods and signalling behaviours of populations and individual clones resulting from such evolution.

4.2 Results

4.2.1 Signal Strategies and Cheating

Changes in the signalling strategy of either the resident cooperator or the invading cheat can affect the outcome of conflict over public goods investment. To study QS cheating under purely experimental manipulations of the signal environment it was necessary to create a cheating strategy that has no native signal production. A Δ*lasIR* double mutant was created to this end, using an existing suicide construct (see Methods 2.4.4 and Appendix A1). Competition experiments using this mutant highlight the importance of signal strategy on the outcome of social conflict. Mixed cultures of WT and *lasIR* mutant were inoculated first at varying frequency of the mutant and then at a constant frequency in the presence and absence of either 3-oxo-C12 HSL signal or C10-CPA quencher (Fig. 4.1).

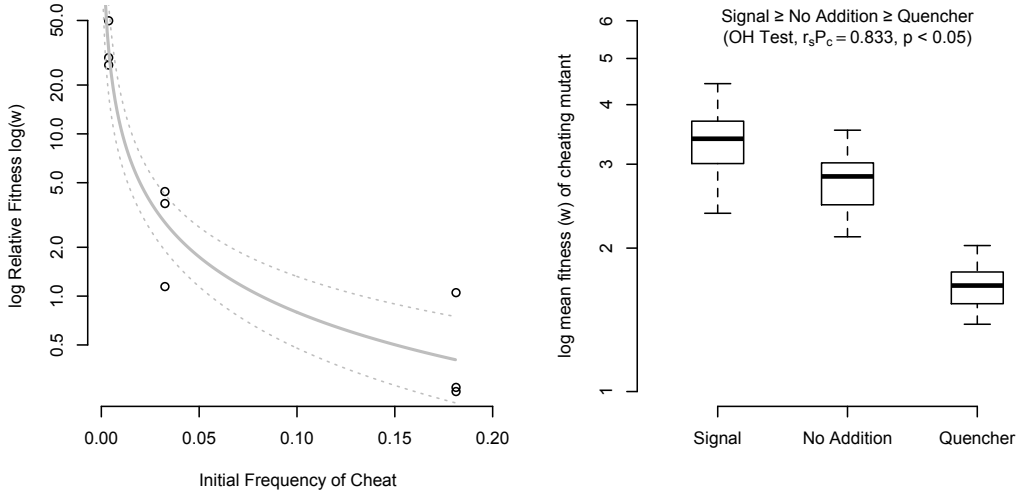


Figure 4.1: Fitness in QSM of a Δ*lasIR* cheating mutant at varying initial frequencies in the population (left). The line is a log regression and the dashed lines are 95% confidence intervals of the regression. The relative fitness of a Δ*lasIR* mutant (right) inoculated at a initial frequency of 0.05 in coculture with a WT with the addition of either 50 μM 3-oxo-C12 HSL signal or 250 μM C10-CPA Quorum Quencher.

A cheating *lasIR* mutant gained a fitness advantage in a WT population ($F_{1,7}$ = -5.69, $p < 0.001$) subject to negative frequency dependent selection (Fig 4.1 left, $F_{1,7}$ = -8.88, $p< 0.001$). To investigate the effect of signal strategy on the fitness of a cheating mutant competition experiments were performed in the presence or absence of either additional signal molecule or a known quorum quencher (Ishida et al. 2007). This mimics a scenario where the sum of signal output of the two competing strains is either reduced or increased. The fitness of a rare cheating mutant is modulated by the signal environment with the highest fitness achieved at high levels of signal (Fig 4.1 right, $r_S P_C$ = 0.833, $p < 0.05$). The Ordered Heterogeneity test was implemented here as this enables the testing of a directional alternative hypothesis i.e. Signal ≥ No Addition ≥ Quencher with at least one inequality strict (Gaines 1994; Rice 1994).

4.2.2 Adaptive response to varying relatedness

As seen in the previous chapter, perturbations to the cost : benefit ratio of signalling cause an adaptive response to the signalling phenotype. Theory also predicts that varying Hamiltonian relatedness over evolutionary timescales varies the selection for signalling and response. Specifically, a previous theoretical model predicts that whilst response in the form of public goods production decreases with decreasing relatedness, signalling peaks at intermediate relatedness (Brown & Johnstone 2001).

To test this further, experimental evolution was performed using the global competition design (A. S. Griffin et al. 2004) and varying relatedness by passaging 1 (High Relatedness), 2 (Mid Relatedness) and 10 (Low Relatedness) clones for the next round of selection (Fig. 4.2). With conflict over public goods this is an experimental design which should tend towards relatedness values of 1, 0.5 and 0.1. After 20 passages the populations were assayed for fitness in QSM, exoprotease production and *lasI* signal gene expression.

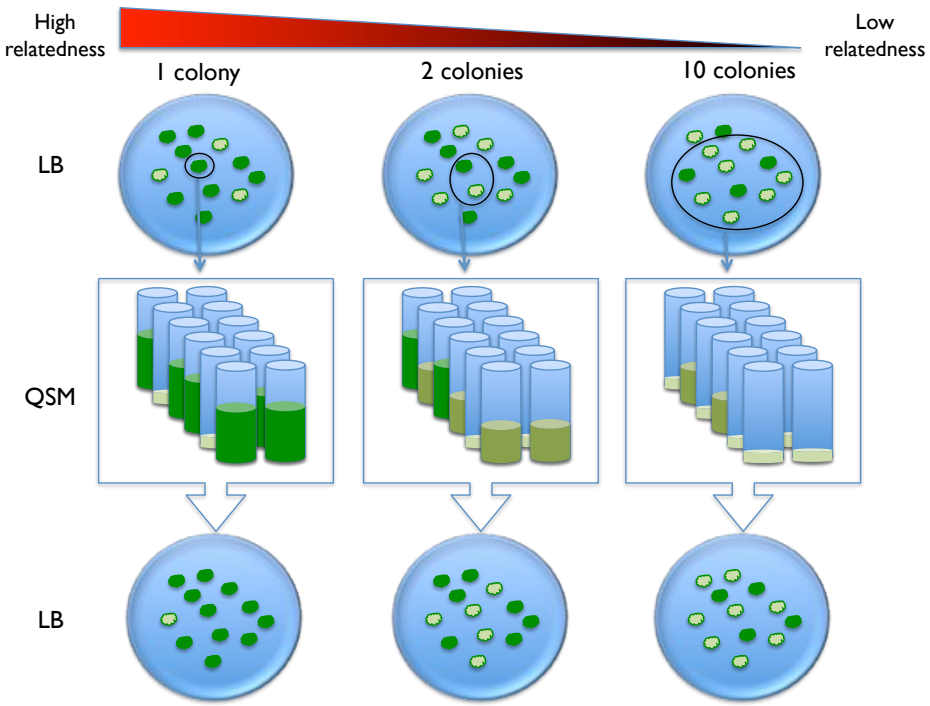


Figure 4.2: Schematic drawing of the experimental evolution procedure showing how the relatedness treatments are generated. Each subsequent subpopulation is inoculated by a mixture of colonies from the pooled subpopulations of its predecessor.

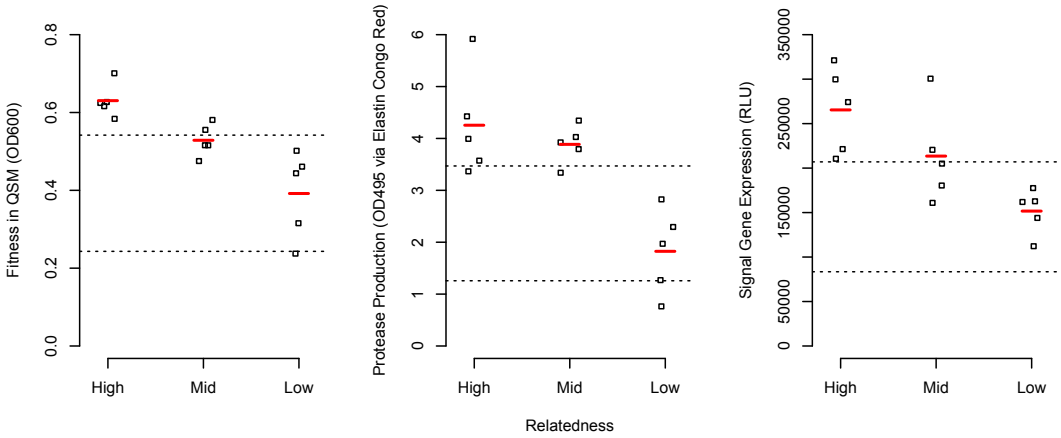


Figure 4.3: Fitness in QSM (left), exoprotease production (middle) and *lasI* signal gene expression (right) at the population level for populations evolved under varying relatedness regimes. Each point represent a replicate population. Red bars represent the mean for each group, dashed lines represent the ancestral mean (top) and the mean for a *lasIR* mutant.

Upon the manipulation of relatedness, populations suffered a decrease in fitness ($F_{2,12}$ = 13.61, p < 0.001), exoprotease production ($F_{2,12}$ = 14.02, p < 0.001) and *lasI* gene expression ($F_{2,12}$ = 8.315, p < 0.01) with decreasing relatedness (Fig 4.3). In the case of exoprotease production this is in accordance with expectation, as high relatedness selects for cooperation. In the case of signalling the result is counter to a previous theoretical prediction. There are several possible reasons for this that will be discussed in detail at the end of the chapter. First to reveal within population variation, the analysis was repeated using single clones isolated from each population (Fig. 4.4, Total = 75 clones).

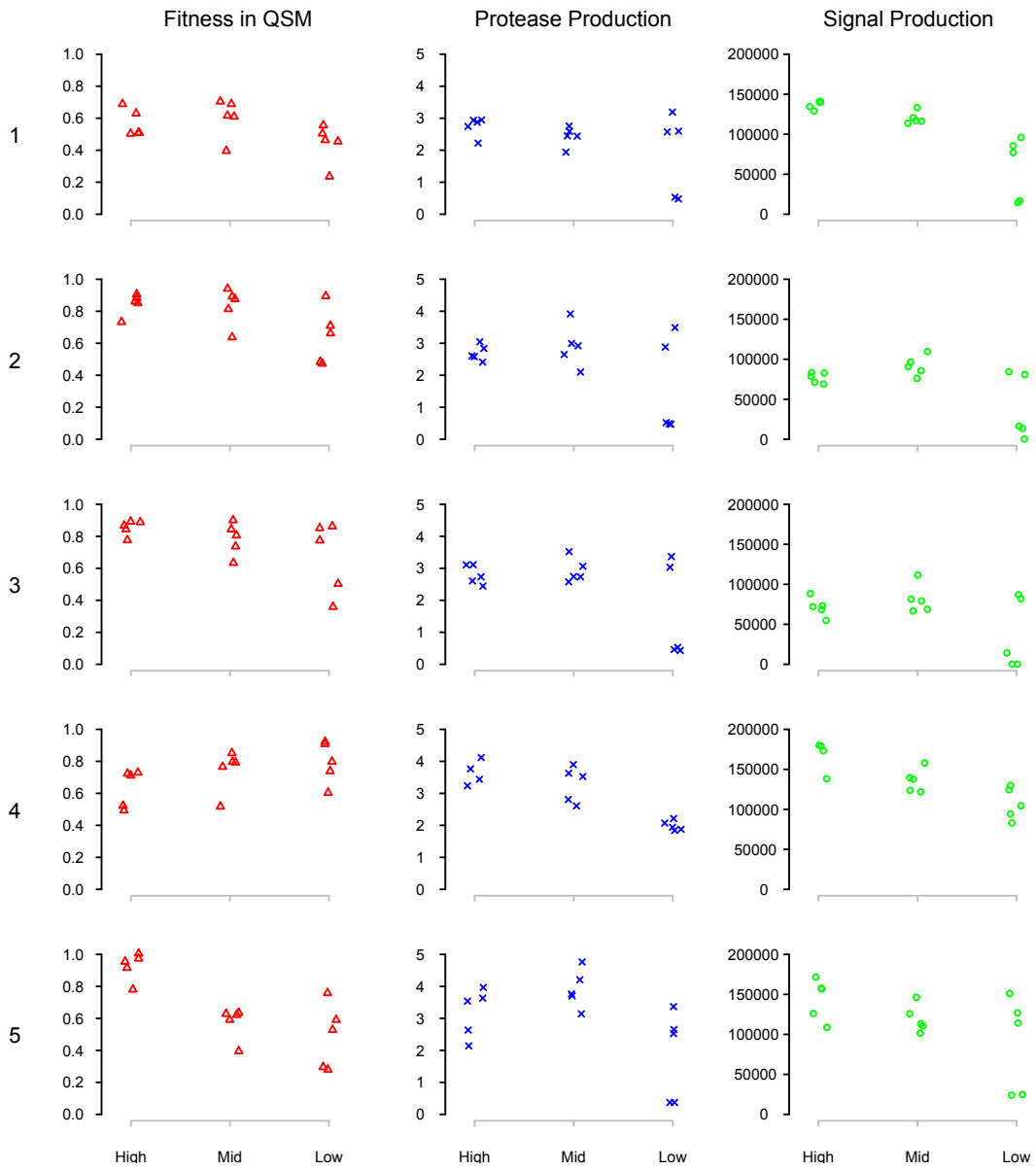
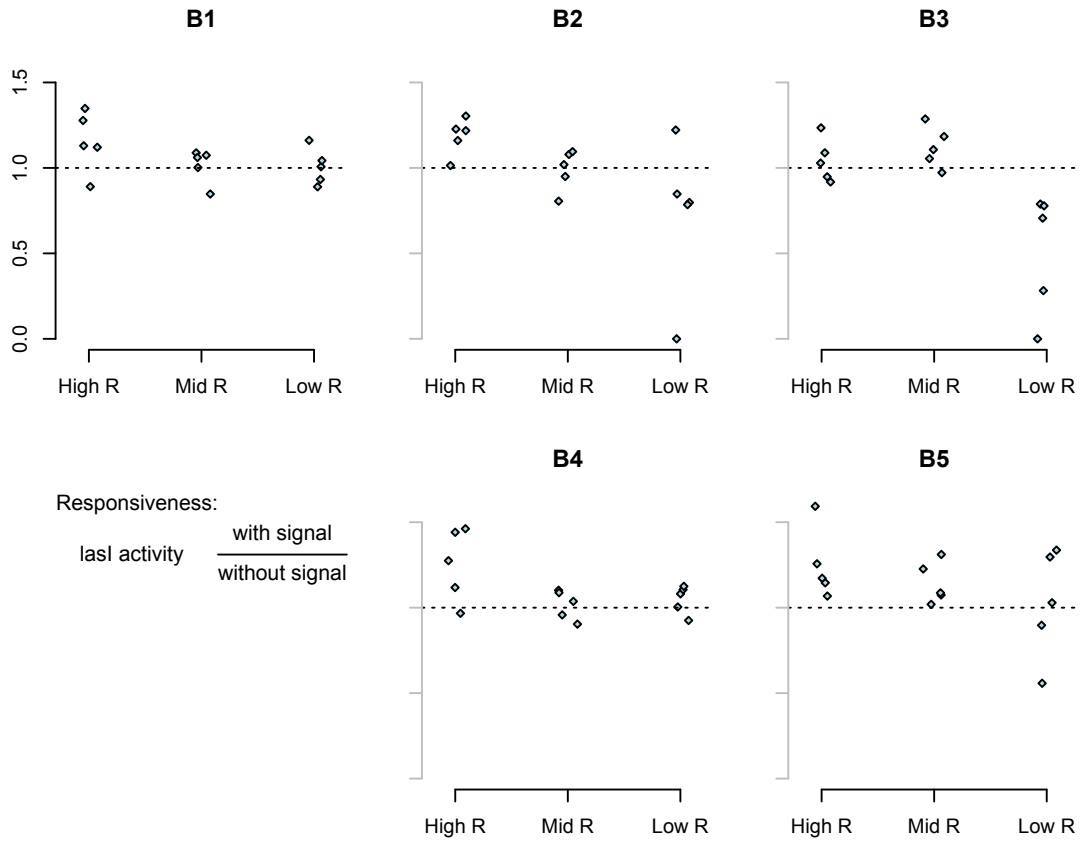


Figure 4.4: Fitness in QSM (left), Las B exoprotease production (middle) and *lasI* signal gene expression (right) of individuals clones from populations evolved under varying relatedness regimes. Each horizontal triplet represents a replicate population of the experiment. Each data point represents a single clone isolated from that population.

Whilst controlling for variation between different blocks or replicates of the experiment (1 - 5, Figure 4.4), individuals displayed a reduced fitness ($F_{2,71}$ = 5.340, $p < 0.01$), reduced exoprotease production ($F_{2,71}$, = 19.23, $p < 0.001$) and reduced signal production ($F_{2,71}$, = 14.33, $p < 0.001$). These results confirm the previous observations at the population level. It is visible from plotting values from individual clones that in some populations, there is within population variation in traits that is concealed either by calculating the mean or by performing assays on crude samples of the entire population (Figure 4.4). It is known that different strategies such as signal blind and signal negative can invade a WT QS strategy (Diggle et al. 2007a). To test whether the deviant QS strategies which have invaded the populations were signal negative or signal blind, they were cultured in the presence and absence of exogenous 3-oxo-C12 HSL. Absorbance and light production of the cultures was measured. Although the *lux* reporter in these cells was fused to the promoter of *lasI*, in the ancestor, the increased expression of *lasI* in the presence of signal would indicate a functioning signal-receptor complex and therefore QS response. The ratio of light production in the presence : in the absence of signal molecule was taken to represent responsiveness.



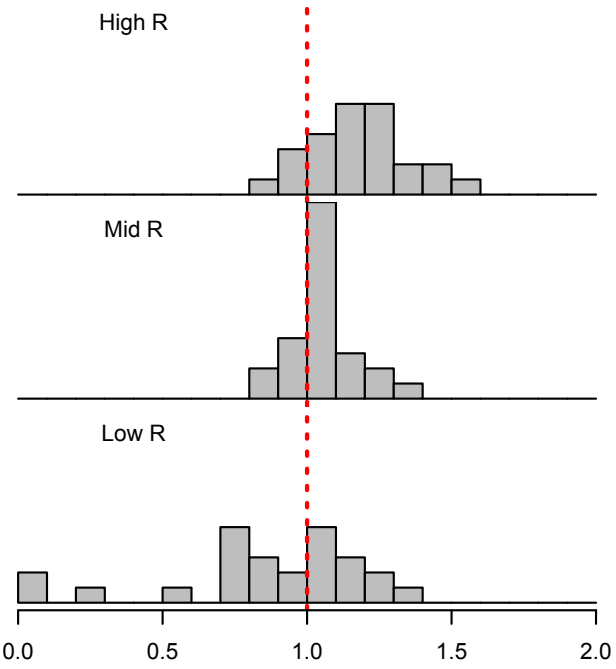


Figure 4.5: The responsiveness of individual clones resulting from evolution under varying relatedness regimes plotted for each repeat of the experiment (top, B1 - 5) and all together as a frequency histogram (right).

Relatedness treatment explained a significant amount of variation in responsiveness with a trend towards lower responsiveness with decreasing relatedness ($F_{2,68}$ = 12.679, $p < 0.001$). The resulting inference is that high relatedness maintains selection for response. These results demonstrate within population variation in LasB exoprotease production, signal gene expression and responsiveness in the lower relatedness groups. Such variation may represent adaptations in the overall QS circuit and may influence production of other signal molecules which regulate diverse phenotypes. To characterise such variation with greater resolution one of the replicate populations (1) was resampled and isolated clones were assayed for several QS signal molecules. LasB exoprotease production and *lasI* signal gene expression of these clones was measured. Then a liquid biosensor assay was performed using a reporter for long chain AHL's. Then an organic solvent extraction was performed and extracts analysed by LCMS for 3-oxo-C12 HSL in its native and open-ring form, C4-HSL, PQS and HHQ. The results are summarised in the table below.

	protease	signal gene expression	Bio sensor	C4-HSL	open ring C12	PQS	3-oxo-C12 HSL	HHQ
PAO1	1	1	1	1	1	1	1	1
Δ*lasIR*	0.15	0.25	0.77	0.99	1.36	0.83	0.63	1.22
3a	0.12	0	0.52	0.65	0.73	2.02	0.35	3.1
3b	0.13	0	0.48	0.62	0.81	2.55	0.4	3.37
3c	0.14	0.28	0.46	0.32	0.83	1.48	0.52	1.86
3d	0.15	0.3	0.41	0.34	0.94	1.54	0.59	1.93
3e	0.14	0.28	0.36	0.31	1.02	1.43	0.53	1.69
3f	0.14	0.27	0.43	0.4	1.24	1.46	0.47	1.86
3g	0.13	0	0.39	0.72	1	1.8	0.42	2.44
3h	1.17	0.81	0.53	0.57	0.88	0.6	0.8	0.88
3i	0.14	0	0.49	0.05	1.12	0.2	0.67	0.12
3j	1.14	0.92	0.55	0.43	1.03	0.7	0.63	0.85

Table 4.1: Summary of several QS phenotypes of clones isolated from a single replicate population evolved under conditions that would favour low relatedness. The mean values for a PAO1 WT and its isogenic Δ*lasIR* mutant are included for comparison. All values are given as a proportion of the PAO1 ancestral WT.

From the phenotypes summarised in Table 4.1, we can see that two of ten clones isolated produced WT levels of exoprotease and the rest were similar to a Δ*lasIR* mutant. Some of the phenotypes summarised in Table 4.1 correlate strongly. Isolates can be grouped according to similarities and differences in these 8 phenotypes simultaneously using a Principal Components Analysis (PCA). This type of analysis reduces the dimensionality in a dataset with several explanatory variables such as this one, according to the correlations between variables. The result is a table where only a few explanatory variables explain the majority of the variation in the original dataset. Put another way, PCA combines the similarities found across all samples and reveals the differences between them

with respect to all of the (in this case 8) explanatory variables included. Such an analysis is useful for instance in determining whether the phenotypes place clones into discrete groups and which phenotypes are most different between those groups. PCA was performed on this data and the results are summarised below.

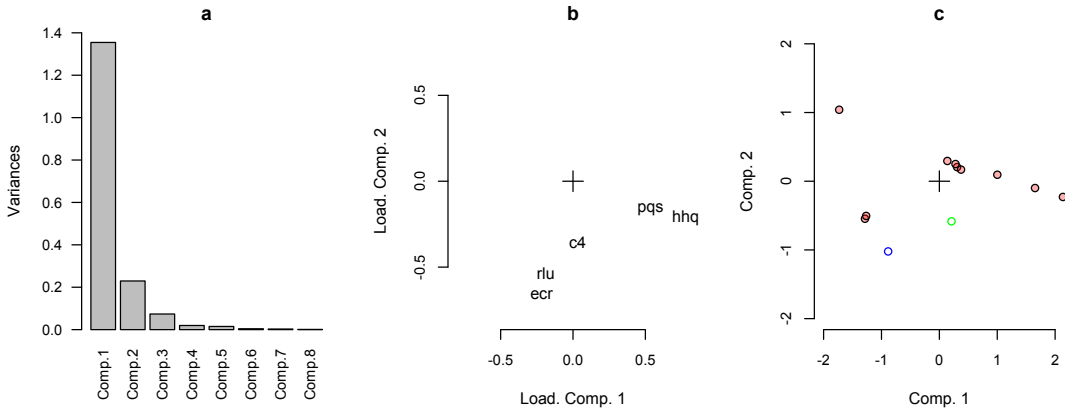


Figure 4.6: PCA analysis of 10 clones isolated from a single population evolved under condition favouring low relatedness. The residual variance after inclusion of successive principal components (a), The loadings of phenotypes on principal components 1 and 2 (b) and the scores of individual clones on principal components 1 and 2 (c). Phenotypes with low loading scores on principal components 1 and 2 are omitted from (b) for clarity of the diagram. Each data point in (c) represents a single clone (red) and the PAO1 WT (blue) and Δ*lasIR* mutant (green) are included for comparison.

After PCA, principal components 1 and 2 explained the majority of the variation in the original dataset (Figure 4.6a). This means that there is a high degree of correlation between some of the original explanatory variables, the 8 phenotypes measured here. The loadings of each phenotype, that is the effect of each phenotype on the principal components 1 and 2 are represented in figure 4.6b. For example the bottom left hand corner of the plot represents high values of exoprotease production (ecr) and signal gene expression (rlu). The bottom

right hand corner of the plot represents high values of PQS (pqs) and HHQ (hhq). The phenotypes with low loading scores are omitted on figure 4.6b for clarity. From this plot it is visible that exoprotease production, signal gene expression, PQS, HHQ and C4-HSL all have large effects on the relative positions of clones on the plot. Finally figure 4.6c places each sample onto a plot between principal components 1 and 2 including the WT and Δ*lasIR* controls. From this plot we can see that two of the clones have relatively high values of exoprotease production and signal gene expression, confirming our observation of table 4.1. The rest of the clones have relatively low values of exoprotease production and signal gene expression and a spread of values of PQS and HHQ production. Interestingly, exoprotease non-producers do not always have the same phenotypic profile as the cheating Δ*lasIR* knockout mutant which was generated for this study. This may give us a hint as to what kind of mechanistic reshuffling has occurred during adaptation to low relatedness. Some insights to this will be made in the discussion.

4.1 Discussion

In the previous chapter, manipulations of the *P. aeruginosa lasIR* QS system by addition of excess signal or quencher molecules were described in detail. When signal or quencher was added to PAO1 WT in sufficient quantities, it was possible to modify the behaviour of cells such that a fitness cost was incurred. The cost:benefit ratio was displaced resulting in a decrease in fitness. In this chapter using mixed cultures of PAO1 WT and an isogenic Δ*lasIR* mutant it was observed that the mutant had a relative fitness advantage over the WT when rare (cheating). This advantage decreased as the frequency of the Δ*lasIR* mutant increased, termed 'negative frequency dependent selection'. It was then observed that upon the addition of excess signal or quencher molecule that the rare fitness advantage of a cheating mutant could be modified. Addition of signal increases the cost of QS to a WT whilst increasing the benefit of QS to all. Addition of quencher decreased the cost of QS to the WT whilst decreasing the benefit of QS to all. As such, varying the level of QS activity only affects the cost of QS for WT whereas the benefits are always shared. This means that under natural conditions, signalling strategy, that is the decision of how much to invest in signalling, could influence the outcome of competition between competing genotypes. This is also supported by two explicit theoretical examinations of QS evolution, described in the introduction to this chapter (Brown & Johnstone 2001; Czárán & Hoekstra 2009). This chapter set out to test the predictions of these two theoretical models.

It was observed that after 20 transfers of experimental evolution, populations displayed a reduction in LasB exoprotease production, QS *lasI* gene expression, response to QS signal and therefore fitness in QSM with decreasing relatedness. The reduction in exoprotease production confirms a central prediction of social evolution theory; that cooperative traits can be maintained by high relatedness (Hamilton 1964b; Hamilton 1964a; Brown & Johnstone 2001). This was true both for population level phenotypic analysis and individual clones isolated from those populations. With regards to the production of signal, the results

contradict the specific theoretical prediction that signal output peaks at intermediate relatedness (Brown & Johnstone 2001). There may be several reasons for this. Firstly it may be that following a reduction in public goods output (reducing effective relatedness), a consequent increase in signal output does not compensate sufficiently for reduced public goods output and so is not favoured by selection. Brown and Johnstone's theoretical model assumes that both public good output and signal production vary in a continuous manner. If this assumption is violated, selection for increased signalling at intermediate relatedness may be reduced. For example, following the emergence of a cheat strategy which completely abolishes response or exoprotease production, an increase in signal output would be ineffective. Interestingly the order of events during adaptation to the social environment is likely to influence the outcome and diversity of emergent strategies (Eldar 2011). In addition it is possible that where more than one signal can be used to regulate a response trait with redundancy, a devaluation in one signal could be compensated for by the emphasis of another. This may also undermine the prediction of Brown & Johnstone (2001) however this study does not find any strong evidence that such 'switching' between signals occurred.

The present results demonstrate a lack of emergence of 'super-signallers' where theory predicts them. Maynard-Smith and Harper discuss other mechanisms which could constrain the evolution of 'super-signalling'. Excluding 'common interest' which is explicitly examined in this study, the two remaining classes of mechanism include 'indices' and 'costly signalling'. These will be discussed in turn as possible mechanisms which constrain the evolution of 'super-signalling'.

The autoinduction feedback loop, a common feature of QS circuits in both Gram-positive and Gram-negative bacteria entails that signal overproduction requires a functional response to signal (Williams et al. 2007; Popat et al. 2008). Therefore although selection at intermediate relatedness may favour a 'super-signalling' strategy, its emergence may be constrained by pleiotropic function of the response regulator. The effects of pleiotropic costs on the constraints to

adaptations to the social environment has been described in *Dictyostelium discoideum*. The loss of function in a gene *dimA* confers a lack of response to the stalk formation signalling factor DIF-1 thus preventing altruistic death. This could potentially allow cells to cheat however, since *dimA* is also required for inclusion into the spore forming section of the multicellular slug such cheating is prevented. A similar constraint may be present in *P. aeruginosa*. For example a loss of function in *lasR* allows cells to save energy on the production of a plethora of enzymes and virulence factors whilst gaining the advantage of their production by others. Since *lasR* is required for the overproduction of signal molecule however, the subsequent evolution of 'super-signalling' may be constrained. In a recent study it was suggested that although *rhlR* mutants grow worse than a WT in a QS dependent medium, they do not behave as cheats in a co-culture possibly due to the increased cost of PQS production (Wilder et al. 2011). In this case the pleiotropic activity of *rhlR* prevent social cheating.

Constraints to deviant signalling can also occur via 'costly signalling' such that the production of signal is so costly that producing a false signal is strongly disfavoured. The biosynthetic pathways to AHL production in *P. aeruginosa* are inextricably linked to other processes (Heurlier et al. 2006) such as fatty acid metabolism (Moré et al. 1996; Parsek et al. 1999) and the utilisation of adenosine/inosine (Schuster et al. 2004) as well as the amino acids glycine and serine (Schuster et al. 2003). Thus the over-activation of AHL biosynthesis whilst favoured by selection in the social environment may incur hidden opportunity costs with respect to other metabolic processes. This could also explain the lack of 'super-signalling' at intermediate relatedness in the data presented here.

Lastly this study found that under conditions favouring low relatedness, a diversity of phenotypes evolved within populations. This prompted further investigation and a QS profiling of a subset of clones isolated from a low relatedness treatment. It was observed that clones could be categorised into producers and non-producers of exoprotease and signal molecule. Notably there

were no non-producers of exoprotease that produced WT levels of signal molecule. Amongst the non-producers, there was a spread of phenotypes regarding other known QS molecules. Specifically some showed high levels of PQS and HHQ production where others did not. The three QS systems in *P. aeruginosa* are highly interconnected. The LasR-3-oxo-C12 HSL complex upregulates *pqsA-E*, *pqsH* and *pqsR*. Therefore a mutation in the *lasIR* system should result in a reduced amount of PQS. The variety of levels of HHQ and PQS production in the LasB exoprotease and signal non-producers in the present experiment may point to the unpredictable effects of perturbations of one QS system on the functioning of another (Wilder et al. 2011), or indeed it may be an indication that the non-producing phenotype is arrived at via more than one regulatory route.

Chapter 5: Communication, cooperation and conflict in biofilms

5.1 Introduction and aims

5.1.1 The Significance of biofilms

A biofilm is a highly structured and cohesive community of cells, normally attached to a surface (Costerton & Lewandowski 1995). Biofilms colonise such diverse places as the teeth of a human, the inside of medical devices, and the stones of shallow coastal sea habitats as well as water pipes or drinking sources (Jass 1997; Verran 2000; Rittmann 2004; Frank 2000). Biofilms are also extremely important in a clinical context, where surfaces vulnerable to colonisation include; indwelling medical devices and prostheses, dental unit water lines, endoscope tubing and wounds (Lindsay & Vonholy 2006). It is now widely accepted that biofilms confer a large increase in the resistance to traditional hospital antibiotics and biocides (Drenkard 2003; Smith 2008). A striking recent study has found that antibiotic treatment regimes based on the latest biofilm antibiotic susceptibility testing fare no better in terms of patient or microbiological outcome than randomised susceptibility testing (Moskowitz et al. 2011). Such work has cast doubt upon contemporary clinical susceptibility testing and its relevance to the efficacy of the drugs subsequently administered to patients.

5.1.2 The role of QS in P. aeruginosa *biofilm formation*

In a much cited study assessing the role of QS in biofilm formation, Davies et. al. observed that *lasI* and *lasI/rhlI* mutants attached to a surface but formed flat and undifferentiated biofilms compared to the highly structured biofilms of the wild type and *rhlI* mutant (Davies et al. 1998). They also observed that addition of 3-oxo-C12 HSL signal product restored biofilm differentiation confirming a role for *lasIR* based signalling in biofilm differentiation. Similarly other studies have found that a class of QSI compounds called furanones inhibit biofilm growth and that mutations in the response genes *lasR* and *rhlR* render biofilms more susceptible to the antibiotic tobramycin. In contrast to this, Heydorn et. al. reported flat and structurally indistinguishable biofilms of the wild type and QS mutants and a further study using high flow conditions reported structured biofilms with no consistent variation between wild type and QS mutants (Arne Heydorn et al. 2002; Purevdorj et al. 2002). QS integrates information about the social and physical environment and in response, regulates a suite of genes some of which are known to be important for biofilm formation. However since QS is not the only regulatory input for these genes, it is not surprising that small differences in strains or test conditions can yield drastically different observations. QS regulated factors with a known influence on biofilm formation include rhamnolipids which are important for maintaining open water channels and microcolony maturation (Davey et al. 2003; Lequette & Greenberg 2004), pyoverdine (Banin 2005), *lecA* and *lecB* (Tielker et al. 2005; Diggle et al. 2006) and extracellular DNA (Allesen-Holm et al. 2006).

Though we do not know the exact contribution of QS to biofilm development a recent article explores the possibility of quite divergent roles of QS in biofilms. Nadell et. al. (2008) use an individual based simulation model, taking into account the activity of several individual cells, to investigate why some bacteria upregulate polymer secretion at high density and some downregulate it. They find that downregulation of polymer secretion at high population density can be beneficial when it coincides with dispersal events. They suggest that the link

between QS and polymer secretion during biofilm formation at high cell density can be linked to the requirements of acute vs. chronic infections. Also their results confirm that the relative strength of competition within and between biofilm populations can be pivotal to the evolution of cooperative signalling.

5.1.3 Biofilms as a social trait

There are many extracellular products that contribute to a biofilm which could be considered public goods but there are few explicit tests of this idea. One system in which public goods cheating in biofilms has been studied is *Pseudomonas fluorescens*. When grown in static microcosms, an ancestral planktonic smooth morph (SM) *P. fluorescens* diversifies into the bottom dwelling fuzzy spreader (FS) and the surface dwelling wrinkly spreader (WS). All three spatial niche specialists are maintained by negative frequency-dependent selection (Rainey & Travisano 1998). By constitutive over-expression of a cellulosaic exopolymer, the WS forms a surface mat at the air-broth interface, the most productive part of the microcosm due to the abundance of oxygen. It has been shown that mutations arising *de novo* in a population of WS create cheating SM phenotypes that do not produce the polymer but still inhabit the biofilm, decreasing the overall buoyancy of the mat (Rainey & Rainey 2003). This might lead to the prediction that strain mixing, mutation and genetic diversity would lead to a decrease in Hamiltonian relatedness and therefore an increase in biofilm cheating over generations. However it has been documented that local competition for resources generates diversifying selection on resource use and resultant diverse biofilms are more productive and more resistant to invasion by cheats (Brockhurst et al. 2006). Additionally it has been shown that the rate of physical disturbances can affect the level of cooperation in such biofilms. Catastrophic disturbances could manifest in many forms such as flooding, fire, antimicrobial treatment or dispersal into new and inhospitable patches. Such disturbances may be commonly experienced by bacterial populations. When disturbances are frequent, the population does not reach sufficient density for biofilms to form. When disturbances are infrequent,

competition within the biofilm leads to selection for cheating. Cooperation in the biofilm therefore peaks at intermediate frequency of physical disturbance (Brockhurst et al. 2007).

5.1.4 Cheating and the role of spatial structure

It is theoretically well established that selection for cheating on secreted public goods is moderated by the spatial structure of a population, how far the public good are shared and the rate at which cells migrate (all things which alter relatedness within a population). For instance, a sessile biofilm community reproducing clonally, would be expected to have a higher relatedness than a well-mixed planktonic population (Tolker-Nielsen et al. 2000; Kreft 2004; Nadell et al. 2010, Figure 5.1). Although this would increase cooperative activity between clone-mates and therefore increase selection for cooperation, it would also increase local competition between clone-mates. These two could cancel each other out and so have no effect on selection for cooperation (Queller 1994; Frank 1998). The effect of local competition between relatives can be overcome if the production of the public good in question increases the patch size or local availability of nutrients i.e. a hydrolytic enzyme (Pfeiffer & Bonhoeffer 2003; Kreft 2004; Grafen 2007; Kümmerli et al. 2009). Therefore the effect of population spatial structure on selection for cooperation depends on the nature of the cooperative behaviour. It could be argued however that in many cases, public goods increase local capacity and therefore selection for those public goods increases with spatial structuring in spite of local competition.

A recent study investigated the selection for cooperation whilst varying viscosity of the environment (Kümmerli et al. 2009). The researchers found that both cell dispersal and diffusion of public goods (siderophores) is limited by increasing viscosity. They then observed that the fitness of a cheating mutant is reduced with increasing viscosity. The authors suggest that this is due to the restricted diffusion of siderophores away from producers making the trait less cooperative.

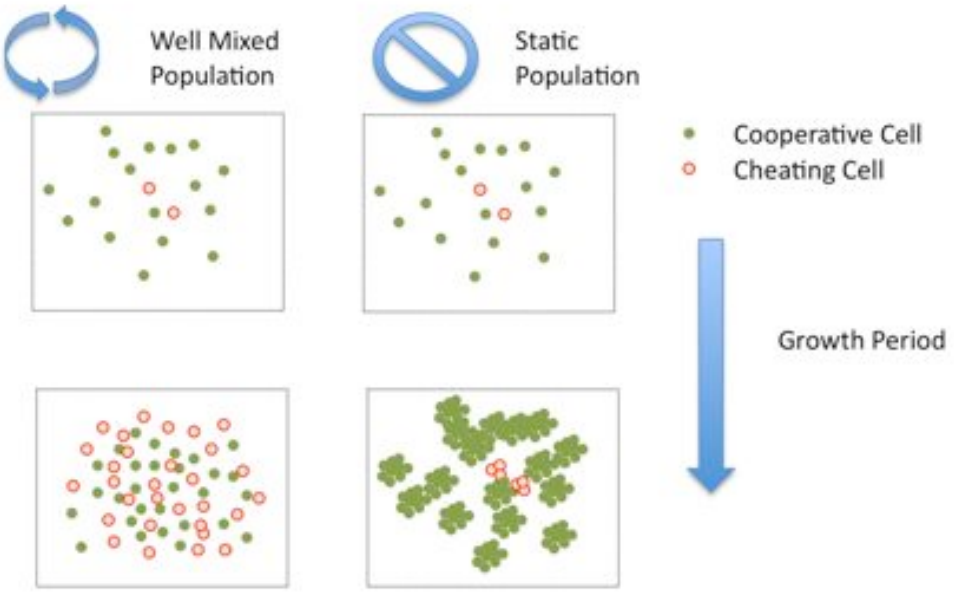


Figure 5.1: Schematic drawing of bacterial cells growing in well mixed or static populations. In well mixed populations, the public good is shared widely so public goods cheats can proliferate. In poorly mixed populations, the public good may not be shared so widely and therefore cheating cells may not be able to proliferate.

5.1.5 Aims of this study

Much work has been undertaken on the role of QS in biofilm development. Despite this, no universal pattern can be observed and culture conditions are likely to be important. The biofilm spatial structure is also likely to modify selection for public goods cheating. This chapter aims to combine some of these ideas with the following aims:

1. To investigate the role of QS in biofilm development under the current study system (i.e. QS dependent QSM culture conditions).
    a. Is QS important in biofilm development?
    b. Does QS cheating undermine biofilm development and robustness?
2. To investigate the role of spatial structuring of biofilms on the selection for cheating.
    a. What is the effect of growth mode (biofilm vs. planktonic) on the selection for cheating?

5.2 Quorum sensing and biofilm development

Quorum Sensing regulates a host of diverse behaviours, many of which enable *P. aeruginosa* to obtain nutrients from its environment or to modify the environment in a favourable way. The exact role of QS in biofilm development is much debated (Parsek & Greenberg 2005) and likely dependent on culture conditions. To test whether QS is important for biofilm development, cultures of WT and *lasR* mutant monocultures were grown in both rich medium (LB) and medium requiring QS for maximal growth (QSM). The *a priori* prediction is; if QS is important for biofilm development, a *lasR* mutant should demonstrate reduced biofilm development independently of any reductions in planktonic growth. Two biofilm culture methods were employed 1; microtitre plates with Crystal Violet (CV) staining and 2; flow cells with confocal laser scanning microscopy (CLSM).

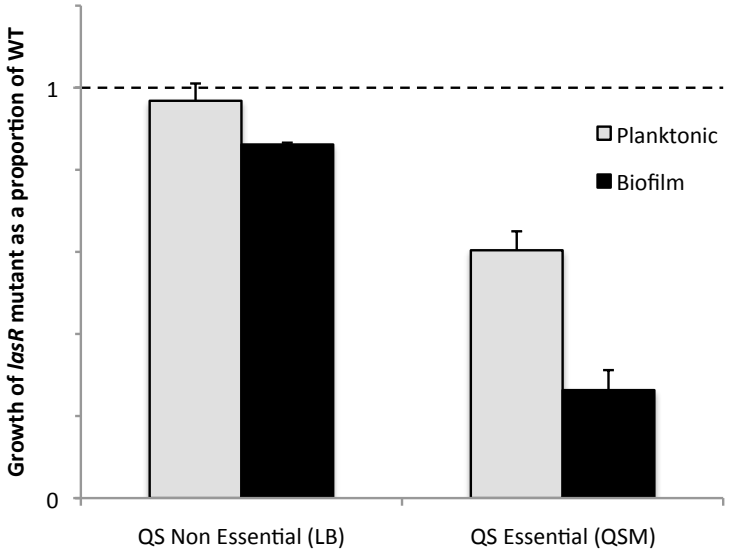


Figure 5.2: Growth of *lasR* mutant in planktonic (grey bars) and biofilm (black bars) mode in rich medium (LB) and QS essential medium (QSM). Values are shown as a proportion of the WT under identical conditions for each treatment. Values represent the means of three replicates and error bars represent the standard error of the mean.

In CV stained microtitre plates it was possible to compare planktonic and biofilm growth in the same experiment (Figure 5.2). The *lasR* mutant grew to a lesser extent in biofilm compared to planktonic mode and in a QS essential compared to QS non essential environment ($F_{1,8}$ = 64.44 and 298.65 respectively, $p<0.001$) with a significant interaction between the two ($F_{1,8}$ = 17.68, $p < 0.01$). Thus the lack of QS activity caused the greatest reduction in growth in biofilm mode where QS was essential for maximal growth. This result suggests that QS activity is important for biofilm formation beyond the need for nutrient acquisition. To examine more closely the effect of QS on biofilm formation and structure, biofilms were cultured in a flow cell system and quantified using Confocal Laser Scanning Microscopy (CLSM) and image analysis.

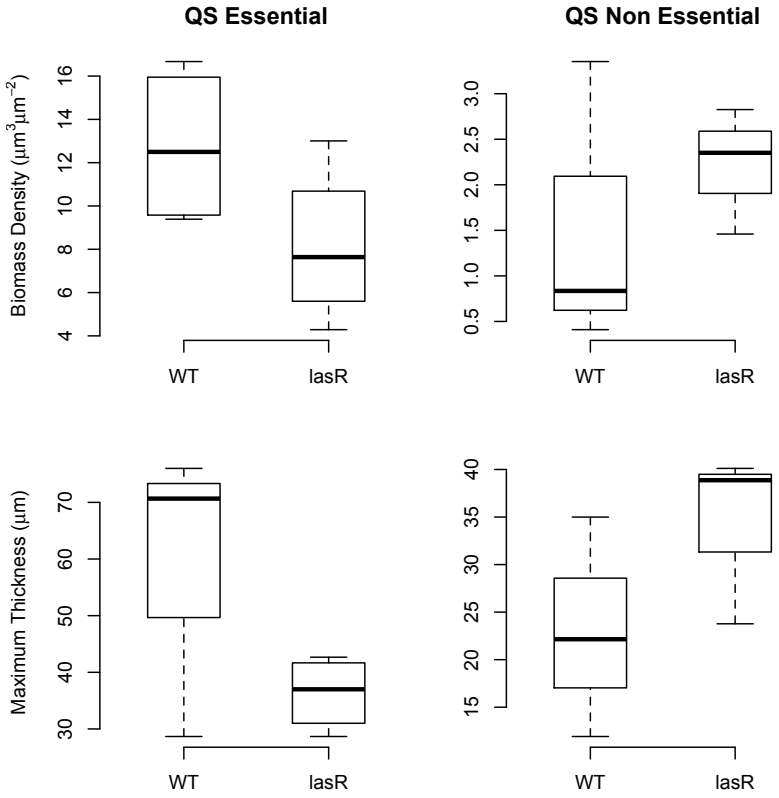


Figure 5.3: Biomass and thickness calculations of biofilms in flow cell chambers of PAO1 and *lasR* mutant in QS Essential (+ PAO1 LB supernatant, n = 8) and QS Non-Essential Media (FAB + glucose, n = 6), calculated from images obtained by CLSM. The thick black lines represent the median values, the boxes represent the interquartile range and the whiskers represent the extreme values.

The goal of the following experiment was to parameterise a QS dependent flow cell medium such that a WT grew larger biofilms than its QS mutant counterpart. However due to the diffusibility of QS molecules and excreted public goods and their constant removal in a flow system, growth in QS dependent media was difficult to achieve. A condition had to be created where sufficient growth was possible. These experiments were performed in a number of different ways (see Appendix A2) but best results were achieved by adding a 1:10 dilution of sterilised PAO1 LB culture supernatant to the medium. The results are summarised in Figures 5.3 and 5.4. There was no significant difference in biomass of PAO1 and *lasR* biofilms under both QS essential and QS non-essential

conditions (Wilcoxon Rank Sum Tests, p=0.7 and 0.7, Figs. 5.3, 5.4). The thickness of biofilms showed a trend towards thicker PAO1 biofilms under QS essential conditions but thicker *lasR* mutant biofilm under QS non-essential conditions (Wilcoxon Rank Sum Tests, p=0.2 and 0.2, Figure 5.3, 5.4). In addition the difficulty in culturing biofilm under flow conditions strongly indicate that diffusible factors are important for growth per se in QSM. The following figure displays representative images from that experiment (Fig. 5.4)

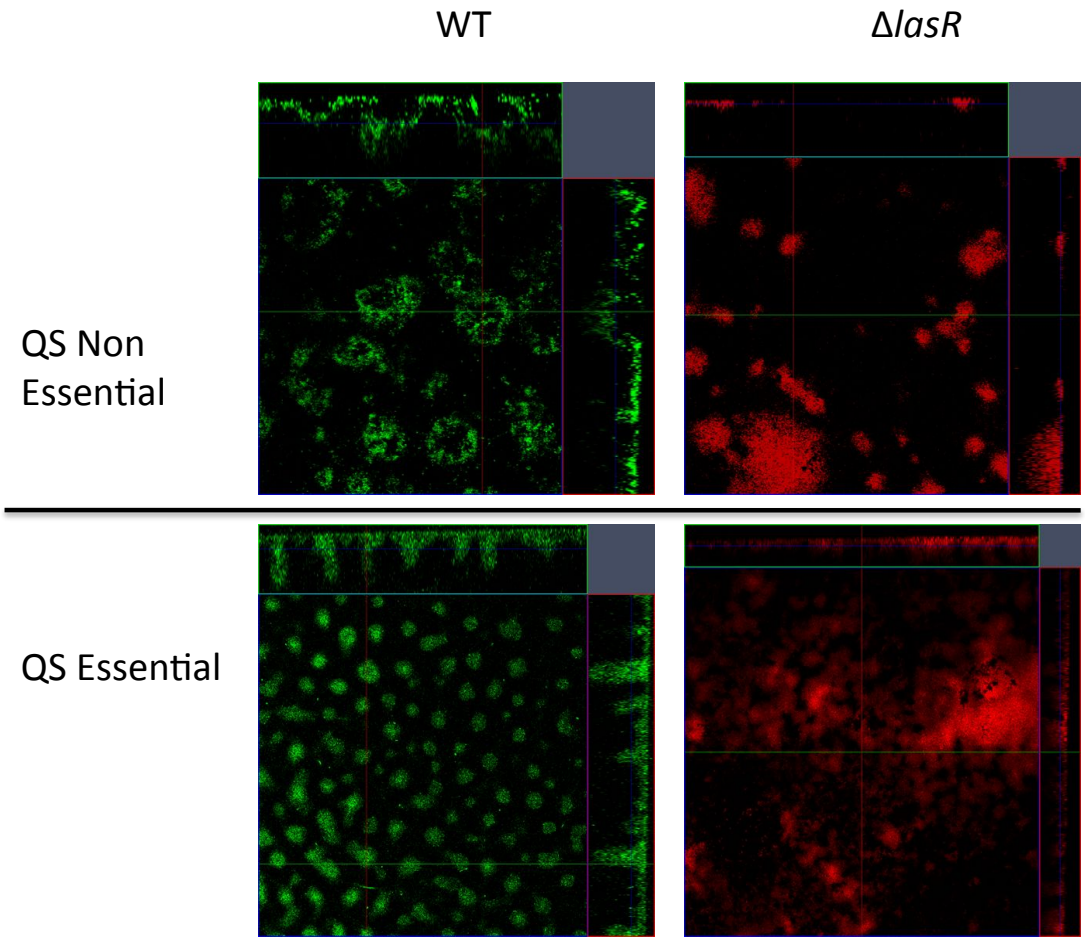


Figure 5.4: Confocal Image Stacks of biofilms grown under QS Non-Essential conditions (glucose supplemented) and QS Essential conditions (QSM + PAO1 LB supernatant) of PAO1 WT labelled with GFP and isogenic Δ*lasR* mutant labelled with MCherry. Central panels are cross sections in XY and top/right panels are cross sections in XZ/ YZ respectively.

Where public goods are important for growth or survival it is known that the presence of cheats causes a reduction in the overall fitness of the population in both biofilm and planktonic modes (Rainey & Rainey 2003), which is termed here as cheat load. Therefore another way to test the importance of QS for population fitness is to measure the fitness of a planktonic population in the presence and absence of cheating mutants that cause a detriment to the population by decreasing QS activity. Since QS-regulated LasB exoprotease production is important for growth in QSM (Diggle et al. 2007a; Sandoz et al. 2007) a reduction of population fitness by cheat load is expected.

In the next section of this chapter the application of cheat load to cultures is used to investigate the important of QS on the fitness of first biofilm and then planktonic cultures with the hypothesis that QS is important for biofilm development above and beyond nutrient acquisition. First, if QS is important for population fitness, the presence of QS cheats should cause a detriment to growth and it is predicted that this detriment can be negated by the addition of excess signal molecule to the culture. To test this, cultures were initiated with varying ratios of PAO1 WT and isogenic PAO1 Δ*lasR*. To test whether addition of exogenous signal molecule could negate the detrimental effect of cheat load, this was also done in the presence and absence of 50 μM 3-oxo-C12 HSL.

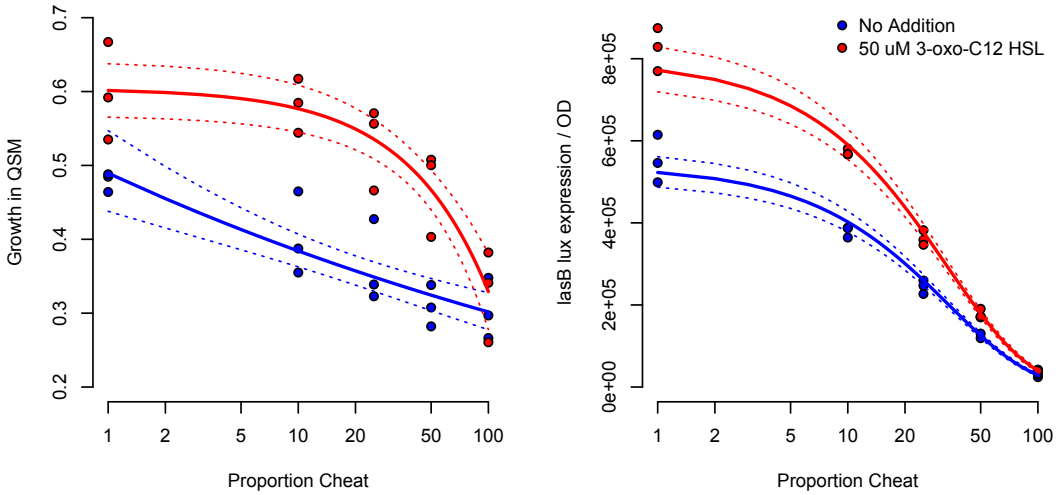


Figure 5.5: The effect of cheat load on growth in QSM (fitness, left) and lasB::lux expression (response, right) in the presence (red) and absence (blue) of 50 μM 3-oxo-C12 HSL. The points represent the data collected and the lines represent predictions made using linear modelling and the 95% confidence intervals of those predictions.

Growth and QS response in QSM both decreased with increasing presence of cheats in the culture (t_{13} = -5.98, $p < 0.001$ and t_{26} = -7.52, $p < 0.001$ respectively). This decline in growth and response could be counteracted by the addition of excess signal molecule (Figure 5.5, red). At high proportions of cheat the addition of signal had little effect due to the decreased QS response of the population. At lower levels of the cheat, response (*lasB* expression) increased greatly with addition of signal but growth did not. Growth increase with the addition of signal was greatest at intermediate proportions of cheat in the population. The reasons for this will be discussed fully at the end of this chapter. The lack of increase in fitness of a pure WT culture upon the addition of excess signal molecule is initially counterintuitive (Fig 5.5, left). Despite this, the reduction in QS activity in the presence of cheat and the associated reduction in fitness (growth in QSM) confirm the previous finding that QS is important for fitness in this environment.

If QS is additionally important in biofilm development, the reduction in fitness should be even greater than the effect on planktonic culture. To test the effect of cheat load on the fitness of biofilm cultures, microtitre plate cultures of PAO1 WT in QSM medium were initiated with varying proportions of Δ*lasR* mutant in the inoculum. Both planktonic and biofilm growth were measured. Since the occupant cells of a biofilm tend to be less metabolically active and more slowly growing there is a possibility that differences observed in growth at any one point are due to the difference in growth rate alone. To eliminate this possibility planktonic and biofilm cultures were grown to their peak or to stationary phase (24 h for planktonic cultures and up to 4 days for biofilm cultures, see Appendix A3).

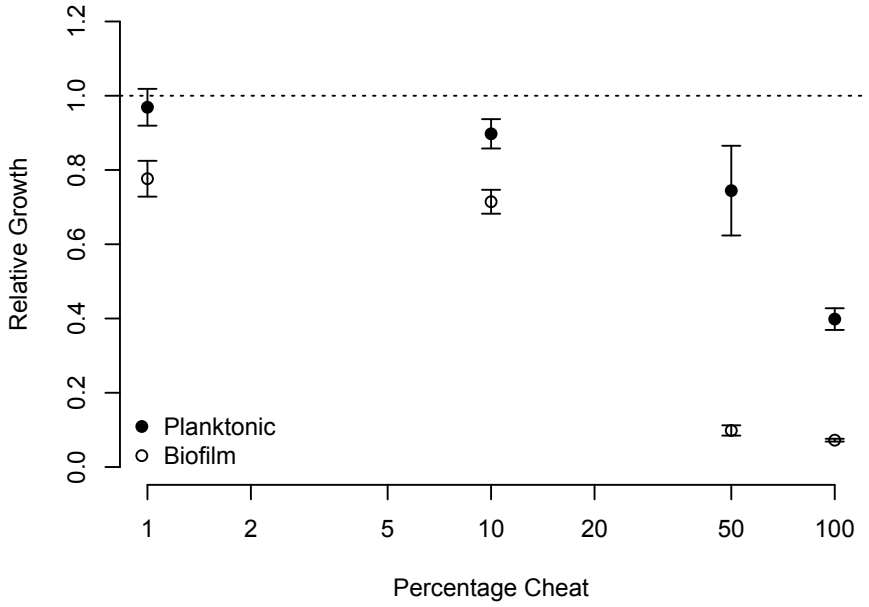


Figure 5.6: Peak growth of WT populations containing various proportions of Δ*lasR* in the innoculum in both Planktonic (blue) and Biofilm (red) mode. Values are given as a proportion of growth of a pure WT culture. Values represent the mean of 6 replicates and the error bars represent the standard error of the mean.

Increasing cheat load decreased growth in both planktonic and biofilm modes but the reduction in biofilm growth was greater (Figure 5.6, paired t-test, t =

-3.117, $p < 0.05$). This is consistent with the hypothesis that QS plays a role in biofilm formation that goes beyond the acquisition of nutrients.

It is known that biofilms provide a protection from environmental insults such as antibiotics, and biocides and foraging (Davies et al. 1998; Bjarnsholt, Jensen, Burmølle, et al. 2005a; Bjarnsholt et al. 2007; Matz et al. 2005; Matz & Kjelleberg 2005; Colvin et al. 2011). Since QS cheat load decreases biofilm formation, it was predicted that the resulting biofilms would be more susceptible to environmental challenge. To test this biofilms were grown under varying degrees of cheat load and established biofilms were challenged with either the antibiotic Tobramycin or the biocide Sodium Dodecyl Sulphate (SDS), both known to disrupt the biofilms of *P. aeruginosa* (Davies et al. 1998; Bjarnsholt, Jensen, Burmølle, et al. 2005a) and then stained with CV to measure the extent of biofilm remaining. Biofilm remaining after challenge was calculated as CV staining after treatment divided by the mean CV staining with control treatment for each cheat load category.

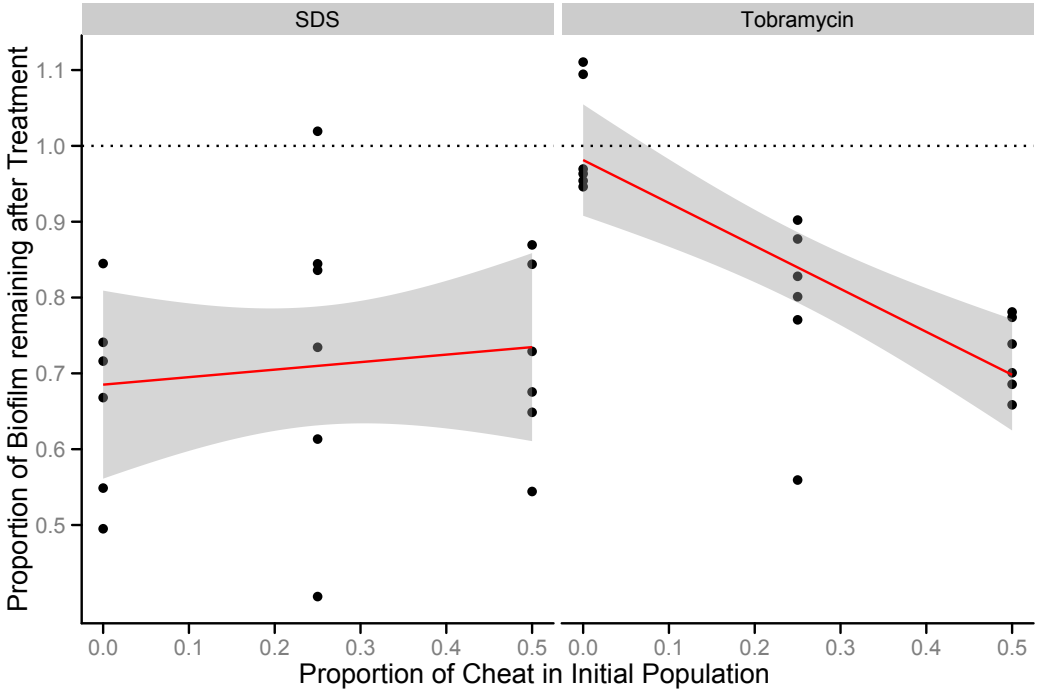


Figure 5.7: Proportion of biofilm remaining after treatment with either 0.2 % SDS or 60 μgml^{-1} Tobramycin. The dashed line at 1 represents the level of biofilm formation of a pure WT population treated with H_20 as a control. Red lines and grey shaded areas represent the relationships described by a linear regression.

The addition of SDS to established biofilms decreased the biofilm mass ($t = -5.38$, $p < 0.001$) but the damage done by SDS was not dependent on the cheat load in the population ($t = 0.55$, $p > 0.05$). Addition of Tobramycin to established biofilms had no effect on pure WT biofilms ($t = -0.52$, $p > 0.05$) but did significantly damage biofilms with increasing cheat load ($t = -5.27$, $p < 0.001$). These results are consistent with the hypothesis that cheat load increases the vulnerability of biofilms to environmental insult in the case of tobramycin but not SDS.

5.3 Spatial Structure and QS cheating

There have been many theoretical analyses of the effect of spatial structure on public goods cheating. Diverse approaches such as game theory and individual based modelling have been employed to attempt to answer this question (Frank 1996; Boots & Sasaki 1999; Pfeiffer & Bonhoeffer 2003; Grafen 2007; Mouden & Gardner 2008). The main prediction of this body of theory is that spatial structure, diffusion rate, migration and any non-random ordering of the population can influence the dynamics of public goods exploitation if like genotypes are grouped. Put another way, any phenomenon which alters Hamiltonian relatedness in the population modifies the selection for cheating. Biofilms are considerably structured in space with restricted diffusion and a greater degree of population viscosity when compared to planktonic growth (Nichols et al. 1989; Lawrence et al. 1994).

To test whether biofilm growth altered the dynamics of public goods exploitation a pegged lid microtitre plate assay was used, in which a polyethylene peg was suspended into each well of a microtitre plate, providing a surface for attachment. Following a period of incubation, the biofilm attached to the peg could be separated from the rest of the population and live cells recovered from pegs by centrifugation. Using this method it was possible to assess the relative fitness of a cheating mutant in biofilm and planktonic growth modes from the same population. To confirm that any differences in fitness were due to social cheating the frequency of cheat was also varied with the prediction that a social cheating strategy should be subject to negative frequency dependant selection.

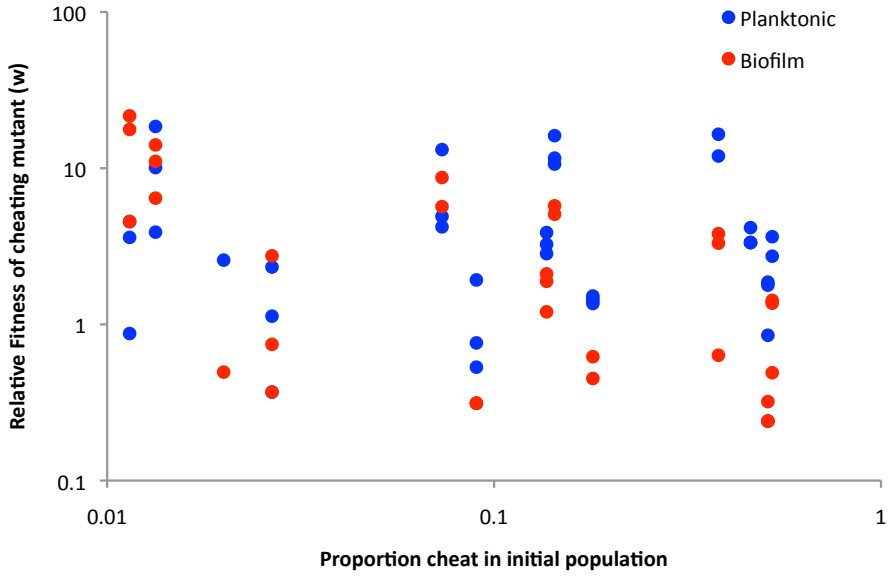


Figure 5.8: Relative fitness of cheating mutant in WT populations with varying proportions of cheat in the initial population in both Planktonic (blue) and biofilm (red) growth modes. Each data point represents a replicate of the experiment.

In the pegged lid microtitre plate experiments relative fitness did not vary with proportion cheat in the initial population (Figure 5.8). Relative fitness of a cheating mutant (w, see methods 2.2.9) took values above and below one indicating that it was sometimes inferior in fitness to the WT. In addition the lack of negative frequency dependent selection suggests that there was no social exploitation of the public good. Only a small amount of cells were recoverable from the surface of the pegs (1.21E+07 CFUml-1 compared to 2.53E+09 CFUml-1 obtained from the planktonic culture). To confirm that the biofilm was in fact removed upon centrifugation, pegs were stained with Crystal Violet with and without prior centrifugation. Centrifugation did not significantly decrease biofilm staining (Wilcoxon test, $p > 0.05$) suggesting that little biofilm was removed in the procedure (Appendix A4).

Bacterial cells are possibly able to move freely from the surface attached biofilm to the liquid medium and back in the pegged lid culture system. This may distort

any effect that spatial structure has on the fitness of a cheat. To circumvent that problem, separate competition experiments on the surface of agar (colony) and in liquid cultures of otherwise identical media (QSM) were run in parallel and compared (Fig. 5.9). Agar colonies were created by spotting the liquid innoculum onto the surface of the agar. It was confirmed that the *lasR* cheating mutant formed thin and insubstantial colonies compared to the WT, representing poor growth (Appendix A5).

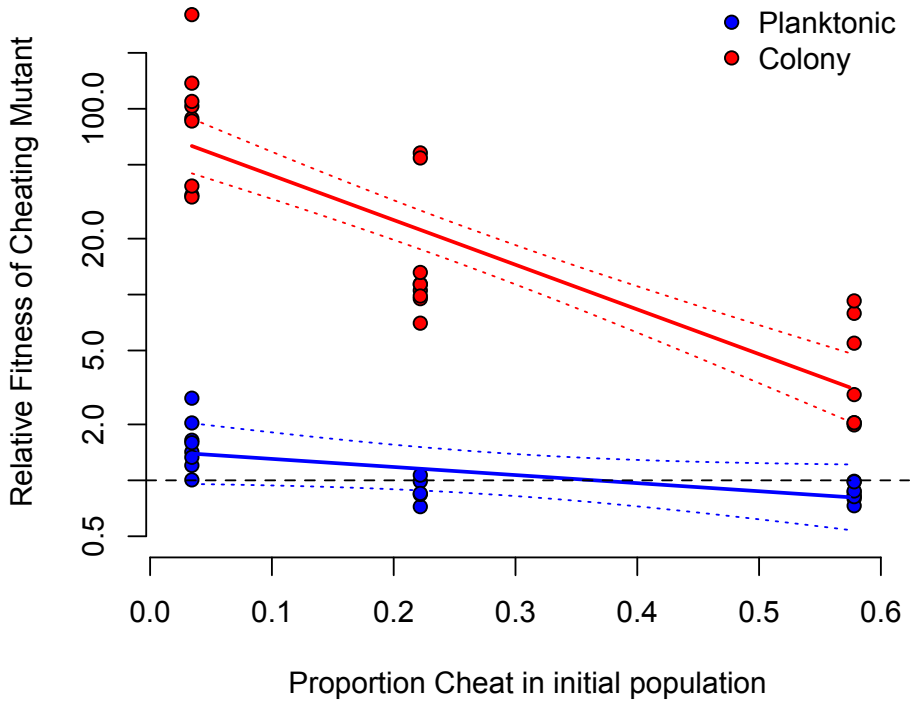


Figure 5.9: Relative fitness of cheating mutant in WT cultures with varying proportions of cheat in the initial population in both planktonic (QSM broth) and colony (QSM agar) growth modes. The points represent data collected and lines represent predictions generated from linear models with 95% Confidence Intervals. The dashed line at 1 represents the point at which the fitness of WT and mutant are equal.

The fitness of a cheating mutant was higher and declined with frequency at a greater rate in colony mode compared to planktonic growth (Fig. 5.9, $t_{14} = 14.25$, $p < 0.001$ and $t_{14} = -5.77$, $p < 0.001$). This result is counter to the prediction that spatial structuring reduces the selection for cheating. Since cheating in this system is dependent on QS activity of the WT it is possible that QS is initiated much earlier in colonies with the close aggregation of cells and signal molecules, thus enabling cheats to spread even more rapidly than in liquid.

5.4 Discussion

5.4.1 QS is important in biofilm development

This study confirms that QS can be important in biofilm development given certain conditions. A central feature of this study system is a growth medium where QS is required for maximal growth in batch culture. Therefore it was expected that a lack of QS activity incurs a cost to growth. This was confirmed and additionally it was observed that QS activity is also necessary for biofilm development under the same conditions. This is also expected as biofilm development is a form of growth.

This study then attempted to transfer these conditions to a flow cell biofilm system with which detailed parameters of monoculture and mixed strain biofilms could be measured such as relative frequency of the two strains and total biomass and thickness. Biofilms grown under flow conditions however, did not behave in quite the same way. It was troublesome to implement the QSM medium in a flow culture system. Specifically it was observed that under conditions likely to favour the WT public good producer or cooperator, no growth or very poor growth was obtained. Conversely under conditions that would relax the advantage of the WT, growth of both the WT and its isogenic *lasR* mutant were not significantly different (See Appendix A2). This led to the hypothesis that diffusion of either signal molecules or public goods was too great under flow conditions for biofilms to establish. Further work would be needed to explicitly test this hypothesis but such a mechanism may have important implications. For example where troublesome biofilms form in pipes or medical catheters under constant flow, if the conditions could be adjusted such that growth is dependent on public goods production or signalling, then the relative abundance of public goods producers and cheats could be controlled.

5.4.2 Cheat load, biofilms and frequency dependent survival.

This study then utilised batch culture biofilms specifically to test the effect of the presence of a portion of the population not contributing to public goods

production. It has been previously shown that such a 'cheat load' can cause a detriment to the population as a whole (Rainey & Rainey 2003; Griffin et al. 2004). It was observed that with increasing cheat load, populations suffered a reduction in overall growth and biofilm development. Previous biofilm studies have shown that a surface attached community of cells is often characterised by slow growth when compared to a well mixed batch culture (Heydorn, Ersbøll, et al. 2000a). Therefore to directly compare these two modes of growth an initial experiment was carried out to determine when the peak of biofilm development was observed. The extent of biofilm development in batch culture peaks after some period of incubation and then decreases again, perhaps at the onset of starvation. This may come about if the biofilm architecture is actively maintained or dispersal and consumption of the biofilm extracellular matrix may be a specific response to starvation. Interestingly as cheat load in the population increases, so peak biofilm development is not only reduced but also delayed (see Appendix A3). This may occur because a population under cheat load is not able to use nutrients as quickly and so starvation occurs later. This is interesting as it means that the most robust and largest biofilms are sometimes founded by populations under cheat load, depending on the time at which they were observed. No such effect was found in planktonic growth.

The present study found that the QS response of a population decreases with increasing cheat load. Also noteworthy is that the largest increase in growth upon the addition of exogenous signal molecule was found at intermediate levels of cheat load. This is probably because the medium was designed for optimal growth of a PAO1 WT. Thus upon the addition of exogenous signal molecule a pure WT culture did not gain an extra growth advantage, despite a large increase in expression of the LasB exoprotease. Perhaps the conversion of BSA to its amino constituents was already saturated at the native endogenous signal level. Conversely at high cheat load, when few cells in the population are responding to signal, no extra growth advantage was derived due to a corresponding lack of QS response. When compared directly, the reduction in biofilm development

due to cheat load was greater than would be expected given the reduction in overall planktonic growth.

This is strong evidence that either the *lasIR* QS system is particularly important in biofilm development under the present conditions or indeed that the *lasIR* QS system regulates another factor important for biofilm development. In either case the result indicates a positive relationship between biofilm development and survival and frequency of QS cooperators. A similar scenario has been described in a bacterium (*Myxococcus xanthus*) where intense cheat load of individuals not contributing to spore formation decrease the overall sporulation efficiency of populations leading to increased risk of local extinction (Fiegna & Velicer 2003). The present study also identified such an effect with respect to QS cheat load and biofilm development. Such effects may be widespread in microbial populations since all that is required is a positive relationship between survival and frequency of cooperators.

The present study then went on to investigate the effect of cheat load on biofilm survival when challenged with a biocide and an antibiotic. Previous reports found that the killing by both tobramycin and SDS was dependent on QS activity with a WT monoculture better able to survive such challenges when compared to a *las* and *rhl* defective mutant, though neither investigated the effect of killing on mixed culture biofilms (Davies et al. 1998; Bjarnsholt, Jensen, Burmølle, et al. 2005a). This study found found that cheat load increased susceptibility of populations to the antibiotic tobramycin but not the biocide SDS. Therefore, an increase in nonresponding QS cheat load has at least three detrimental effects on population survival. Firstly cheats decrease overall population growth, secondly they decrease biofilm development and thirdly this makes the population more susceptible to antimicrobial intervention. The results suggest that such a double cost occurs here due to the decrease in QS response of the population as a whole, once it is diluted with non responding cheaters. This observation adds to our growing arsenal of means with which to control troublesome bacteria populations using an understanding of population level phenomena. For

instance it has recently been proposed that a cheating mutant able to invade an established infection could serve as an efficient means of delivering toxins or as a gene replacement technique (Sinkins 2006; Brown et al. 2009).

5.4.3 Spatial Structure and QS cooperation

The present study set out to test a central prediction of social evolution theory; spatial structure influences the evolutionary dynamics of cooperation. When like genotypes interact individuals can maximise their inclusive fitness by increasing the reproduction of their neighbours. A pegged lid biofilm culture system was employed to test this. In this system it was possible to harvest viable cells from both the planktonic and biofilm phase of the culture. It was expected that the relative fitness of a cheating mutant would be negatively frequency dependent. There was no significant relationship between frequency and relative fitness in either the planktonic or biofilm phase in this culture system. This was surprising but may have arisen if cells were able to move freely between the biofilm and planktonic phase throughout growth. The lack of frequency dependence predicted by the spatial structure in a biofilm may have obscured the negative frequency dependence predicted in a well-mixed population. It was then observed that relatively little of the biofilm could be removed from the pegs via centrifugation (See Appendix A4). Removing relatively small amounts of a poorly formed biofilm due to cheat load, may have further decreased the ability to detect an effect of growth mode. To circumnavigate this experimental difficulty another culture method was used. Growth on agar plates represents an environment more spatially restricted than liquid culture with no possibility of interactions between the two.

On agar plates a cheating mutant had a greater relative fitness and a greater negative frequency dependence when compared with liquid cultures. This is contrary to the *a priori* prediction that spatial structure selects for cooperation due to increased interactions between clone-mates. There are two compelling explanations for this; either (a) local competition between relatives negates the effect of kin selection or (b) the spatial restriction of movement of both cells and

signal molecules increases the QS activity of resident cooperators thus increasing the total available resource. Such an increase in activity would incur a cost to the resident cooperator but its benefits would be shared by all in the population. Contrary to the present findings, another similar study conducted on siderophores finds that the viscosity of the agar increases the selection for cooperation (Kümmerli et al. 2009). Since the effect of local competition must also occur in this system, the increase in cheater fitness observed in the QS cheating system points towards increased QS activity. These results when considered in combination highlight the importance of interactions between the genetic regulation of public goods production and the spatial ecology of a population and their effect on the selection for cooperation.

Chapter 6: eDNA as a structural public good in biofilms

6.1 Introduction and aims

6.1.1 Cheating and the nature of public goods

Bacteria are the ultimate saprophytes, they export a vast range of secreted factors which modify the environment for their benefit. Some are small diffusible molecules, such as QS signals, and some are large polymeric substances and protein complexes of all sizes. In addition the way that such secreted factors interact with the biotic and abiotic environment may differ widely. For instance secreted factors may aid in motility to escape a hostile environment or promote adhesion to a favourable environment. Secreted factors may be involved in nutrient acquisition or toxin production. Secreted factors often play multiple roles in modifying the environment to suit the producing cell. The effect of the nature of public goods on their evolutionary dynamics has received little attention. The nature of public goods may be the direct consequence of adaptation or they may impose constraints upon adaptation. For example a recent theoretical and experimental study found that the durability of public goods influences the selection for cheating (Kümmerli & Brown 2010). Specifically the study showed that when a public good is durable (i.e. can be used by successive generations), that the selection for cheating is reduced. Once the initial cost of production has been paid facultative down-regulation of public goods production makes a WT and cheating strategy equivocal, a cheating strategy is no longer at an advantage. Their theoretical model predicts that this should occur only if the trait is facultatively regulated. This minimises the cost of production to the cooperator whilst maximising the benefit.

Similarly another study proposed a molecular mechanism which can constrain the evolution of cheating (Xavier & Kim 2010). The authors found that a *Pseudomonas aeruginosa* Δ*rhlA* mutant, unable to produce rhamnolipids essential for swarming motility, can hitchike on the rhamnolipid rich swarms of

the wild type (WT) parent strain. Although the Δ*rhlA* mutant was swept along by the WT swarm it did not gain an advantage for cost saving defection. This was found to be due to a regulatory control of rhamnolipid production by the WT, such that production was only performed when carbon was plentiful and when growth rate was slowing. Such regulation ensures that rhamnolipid production occurs only when metabolic cost is minimised. When the researchers placed rhamnolipid production under constitutive control, a defecting Δ*rhlA* mutant quickly outcompeted the resident WT. These studies demonstrate that public goods are diverse and will not all have the same evolutionary dynamics. This will depend on the nature of the public good, the growth environment, and how its production is regulated by the producer.

6.1.2 eDNA in P. aeruginosa biofilms

It is now the prevailing opinion in the microbial literature that biofilm growth represents a highly relevant phenomenon often found in nature as opposed to the the more traditional, well mixed planktonic experiments. This mechanism of growth results in a complex structure of numerous cells embedded in an extracellular matrix (ECM) and with extremely variable micro-niches. It is known that the biofilm ECM is a complex mixture of secreted, polymers, proteins and small molecules (Flemming & Wingender 2010). It is also known that without the production of such structural factors, a biofilm is either unable to form, or suffers a reduction in differentiation or stability (Branda et al. 2005; Ryder et al. 2007; Ma et al. 2009; Colvin et al. 2011). What is not known to the same extent, is whether such architectural factors are subject to social cheating. This might be expected as structural components of the biofilm are likely to be costly to produce and in addition they often provide a benefit to the population. It has been increasingly reported that biofilms of many microbial taxa contain extracellular DNA (Flemming & Wingender 2010). A few studies in particular have focused on the eDNA content of *P. aeruginosa* (Whitchurch et al. 2002; Yang et al. 2007; Bass & Russo 2010). PCR amplification of specific genes and the banding patterns generated by random amplification of DNA using short

primers (RAPD) and restriction enzyme digestion confirmed the intracellular origin of this DNA (Allesen-Holm et al. 2006). Treatment with DNA degrading enzymes demonstrated that the DNA contributes to the structural integrity of biofilms (Nemoto et al. 2003). The exact mechanism of release is as yet unclear although QS has been implicated (Allesen-Holm et al. 2006; Häussler & Becker 2008).

Allesen-Holm et. al. (2006) assayed the amount of recombinant β-galactosidase released into the supernatant as a proportion of the total β-galactosidase in the culture to determine the amount of cellular material being released into the supernatant. The release of this cytoplasmic enzyme should be increased by lysis of cells. The authors observed that a *P. aeruginosa* PAO1 WT released more β-galactosidase into the supernatant than a Δ*lasIrhlI* mutant. They then developed a high throughput method, which is adopted later in this chapter, using propidium iodide (PI) as a non-permeating nucleic acid stain in cultures to measure their eDNA content. Using this method they observed that the eDNA deficiency of the Δ*lasIrhlI* mutant can be complemented using AHL signal molecules. Additionally they observed that a PQS deficient Δ*pqsA* mutant was impaired in eDNA production whilst a strain Δ*psqL,* a mutant which is no longer able to produce the alkyl quinolone N-oxides and which produces more of the others AQs than the WT, also overproduced eDNA. The implication is that either AQs such as PQS/HHQ play a role in promoting eDNA release or that the AQ N-oxides inhibit eDNA release. Another study found that the PQS molecule has both anti and pro-oxidant activities (Häussler & Becker 2008). The researchers corroborated the finding that PQS enhances DNA release and they suggested that this is due to a PQS-mediated response to oxidative stress.

The conclusion that can be derived from the studies described above is that eDNA is beneficial for biofilm structural integrity, but the exact mechanism of its production will influence its evolutionary dynamics, and these remain to be explored.

6.1.3 Aims of this study

The social dynamics of diffusible public goods have been well studied in the literature. The polymeric structural elements of biofilm architecture may also be public goods, but the exact mechanisms of production are predicted to influence the evolutionary dynamics. eDNA release has been linked to PQS production but its exact role has not yet been elucidated. This chapter aims to contribute to these findings in the following way;

1. To investigate the QS regulation of eDNA in biofilms, and the possibility of public goods cheating on such structural elements of a biofilm.
    a. What is the role of the PQS system in the release of eDNA in biofilms?
    b. Is there potential for mutant strategies to emerge that can cheat on eDNA production?

6.2 Results

6.2.1 Is eDNA export due to lysis of P. aeruginosa cells?

Several studies have shown that eDNA is released by bacterial cells and in particular some of these confirm the cellular origin of the extracellular DNA (Nemoto et al. 2003; Allesen-Holm et al. 2006). Either an active cellular eDNA export mechanism is employed or DNA is released by lysis of the cells. To distinguish between these two, PAO1 cultures were grown to mid log phase in LB at 37°C in a shaking incubator, washed and incubated in phosphate buffered saline solution (PBS, non-growth medium) in the presence and absence of PQS. Both CFU and eDNA content was then monitored to determine whether PQS could directly cause lysis of the cells.

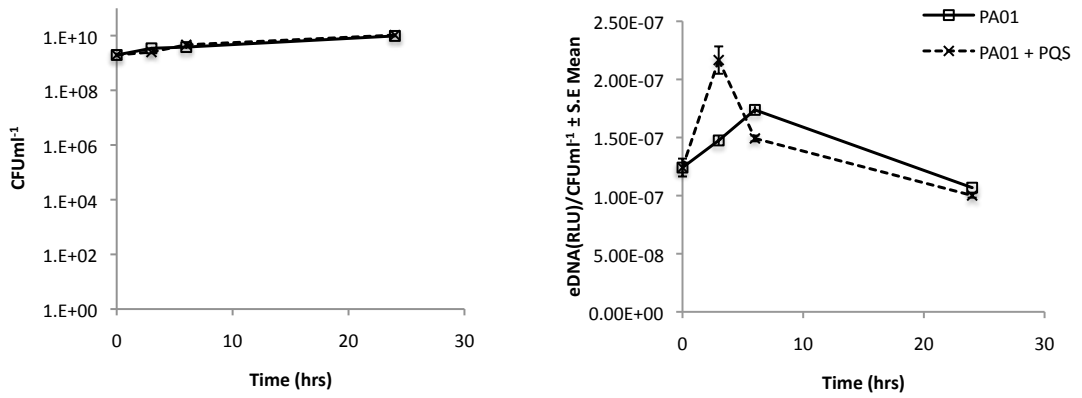


Figure 6.1: $CFUml^{-1}$ (left) and eDNA release, measured by fluorescence of PicoGreen (right), of WT PAO1 in a non growth medium, across a 24 h incubation period at 37°C in the presence and absence of 50 μM PQS. Points represent the mean of three replicates and error bars represent the standard error of the mean.

$CFUml^{-1}$ approximately doubled over the course of 24 h though there was no significant difference in the presence or absence of PQS ($t_{1,6}$ = 1.48, p > 0.05, Figure 6.1). eDNA content of the supernatant peaked at intermediate time intervals but was greater in the presence of PQS. This experiment demonstrates

that the PQS molecule, though stimulating increased eDNA release, does not itself cause lysis of PAO1 cells.

6.2.2 The role of PQS in eDNA release

PQS mutants have previously been shown to produce less eDNA (Allesen-Holm et al. 2006; Häussler & Becker 2008). In these previous studies the chemical complementation of eDNA production with synthetic PQS was not tested. If these same mutants respond to PQS molecules produced by the WT, they would not be able to exploit production of eDNA in a mixed culture. Therefore it was first determined whether it was possible to complement eDNA deficiency in PQS mutants upon the addition of exogenous PQS and its precursor molecule HHQ, since this can also act as a signal molecule (Déziel et al. 2004). The concentration of eDNA was measured in the presence and absence of 50 μM PQS or HHQ both by phenol:chloroform:isoamyl alcohol extraction from supernatants followed by gel electrophoresis, and by the addition of Propidium Iodide (PI) to cultures and measurement of fluorescence. All assays were performed in a defined medium (FAB + glucose) containing no DNA.

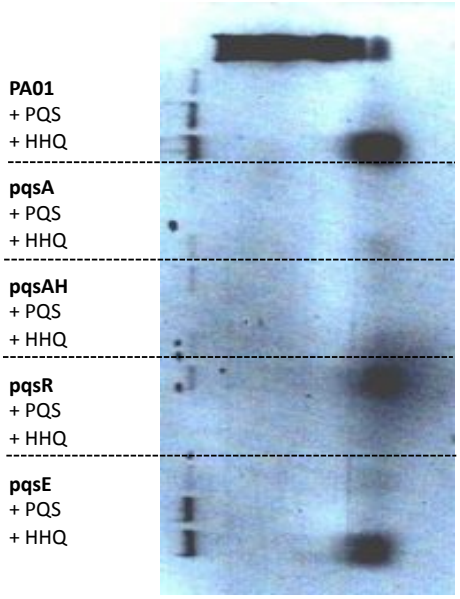


Figure 6.2: Phenol Chlorophorm Isoamyl Alcohol (PCIA) extraction of eDNA from supernatants of WT and mutants grown in the presence and absence of 50 μM PQS or HHQ, followed by visualisation via EthBr. eDNA is seen both as chromosomal DNA (left) and fragments (right). The top lane is the ladder.

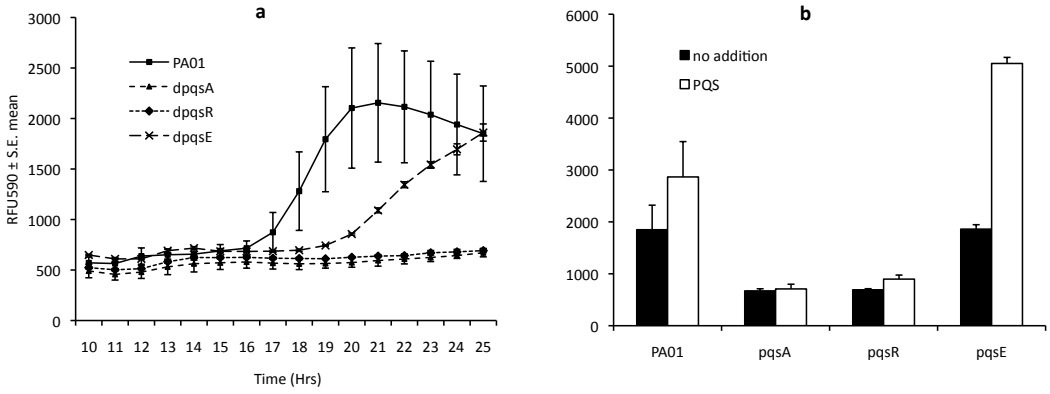


Figure 6.3: eDNA release, fluorescence of Propidium Iodide, of PQS mutants during a period of incubation (a) and at the last time point (25 h) in the presence and absence of 50 μM PQS (b). Points/bars represent the mean of three replicates and error bars represent the standard error of the mean.

Both DNA extractions and PI staining revealed that *pqsA* and *pqsR* mutants were significantly reduced in eDNA release and this could not be complemented by the addition of either PQS or HHQ (Figure 6.2, 6.3). The *pqsE* mutant was delayed in eDNA release but responded to PQS and HHQ with a dramatic increase in eDNA release. Growth measured by OD_{600nm} did not vary significantly between any of the treatments (Kruskall Wallis Test, $\chi^2 = 13.89$, $p > 0.05$).

The lack of response to PQS or HHQ makes the *pqsA* and *pqsR* mutants possible candidates for eDNA cheating. Interestingly both the WT and *pqsE* mutants increased DNA release upon addition of PQS and HHQ. These data lead us to ask; if PQS is important in eDNA release, why does the addition of PQS to cultures not stimulate eDNA release. One possible answer is that a secondary molecule, the production of which is directed by *pqsA,* is the stimulant for eDNA release.

6.2.3 Does eDNA increase biofilm development?

The results of this chapter have shown that PQS stimulates the production of eDNA, although the mechanism for its release has not been determined. Previously it has been shown that eDNA helps to stabilise biofilms (Allesen-Holm et al. 2006), suggesting that it could function as a public good within biofilms. However, in order for eDNA to be considered a public good, there must be some measurable fitness benefit of its production. Therefore it was necessary to test whether a deficiency in eDNA production reduces biofilm development. To test this, the same mutants were grown in 20 ml universals in the presence and absence of 50 μM PQS or HHQ. After 24 h of incubation, planktonic growth (OD), biofilm growth (Crystal Violet Staining) and eDNA content (Nanodrop™ Spectrometer) were measured. 20 ml universals were used in order to obtain sufficient volume for DNA extraction from supernatant.

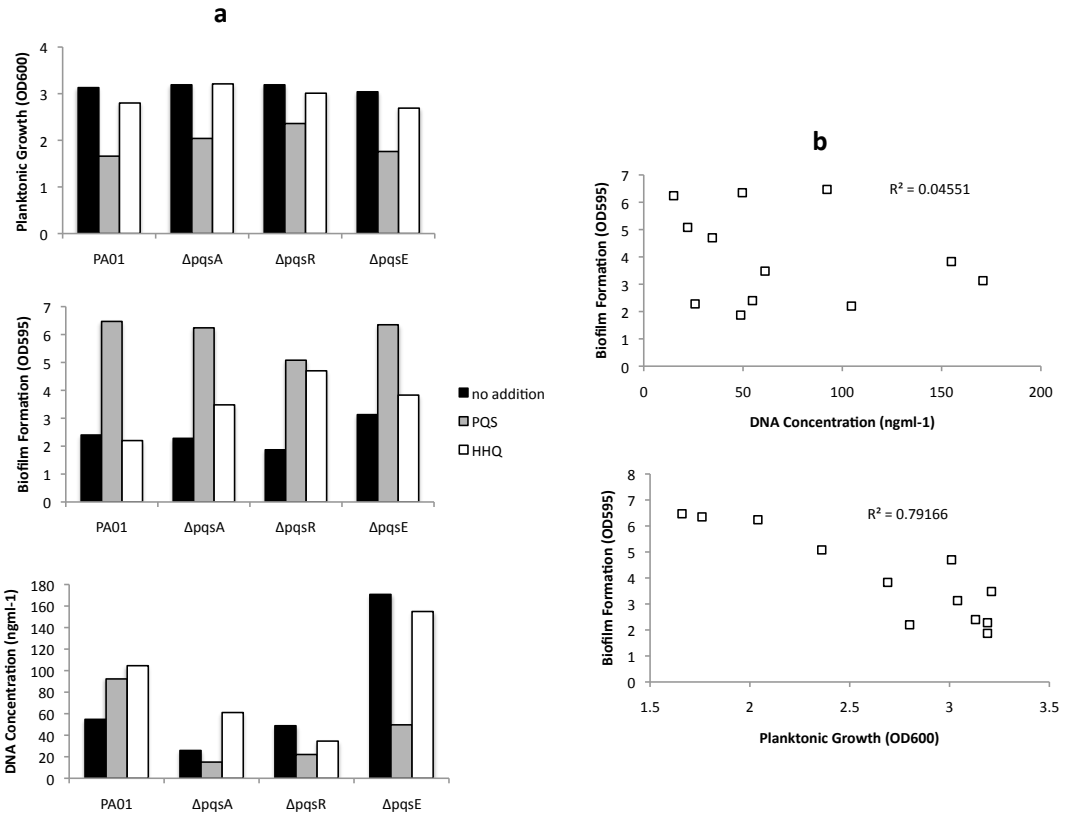


Figure 6.3: Planktonic Growth, Biofilm development and eDNA content of supernatants from cultures grown over 24 h (a) and the correlation between eDNA content and biofilm formation and planktonic growth and biofilm development (b) of several mutants in the PQS biosynthetic pathway in the presence and absence of 50 µM PQS or HHQ.

DNA content of cultures was comparable with previous data obtained with the *pqsA* and *pqsR* mutants (Fig 6.1, 6.2, 6.3) which released less DNA and did not respond to PQS (Figure 6.3a, bottom), although the *pqsA* mutant showed an increase in eDNA release with the addition of HHQ. Counter to expectation, biofilm development increased in all mutants tested upon the addition of PQS and in some upon the addition of HHQ (Figure 6.3a, middle). There was a concomitant decrease in planktonic growth leading to the conclusion that the addition of PQS caused some general increase in surface adhesion regardless of mutations in the PQS pathway and independently of eDNA release into the

medium. In support of this there was no correlation between DNA release and biofilm formation (Figure 6.3b, top) but a strong negative correlation between planktonic growth and biofilm growth (Figure 6.3b, bottom).

This result lead to the hypothesis that although the PQS molecule appeared to aid adhesion to the surface, PQS-mediated eDNA release played no role in surface adhesion but was perhaps important in the latter stages of biofilm development. To test this, the flow cell system was used to obtain biofilms grown in FAB medium supplemented with glucose at 37°C for 4 days. Biofilms were then stained with DAPI to locate the eDNA. When used in sufficiently low concentrations, DAPI does not enter the cell, making it suitable for an eDNA stain. Using such experiments it was possible to simultaneously measure the biofilm biomass and the mass of biofilm containing eDNA.

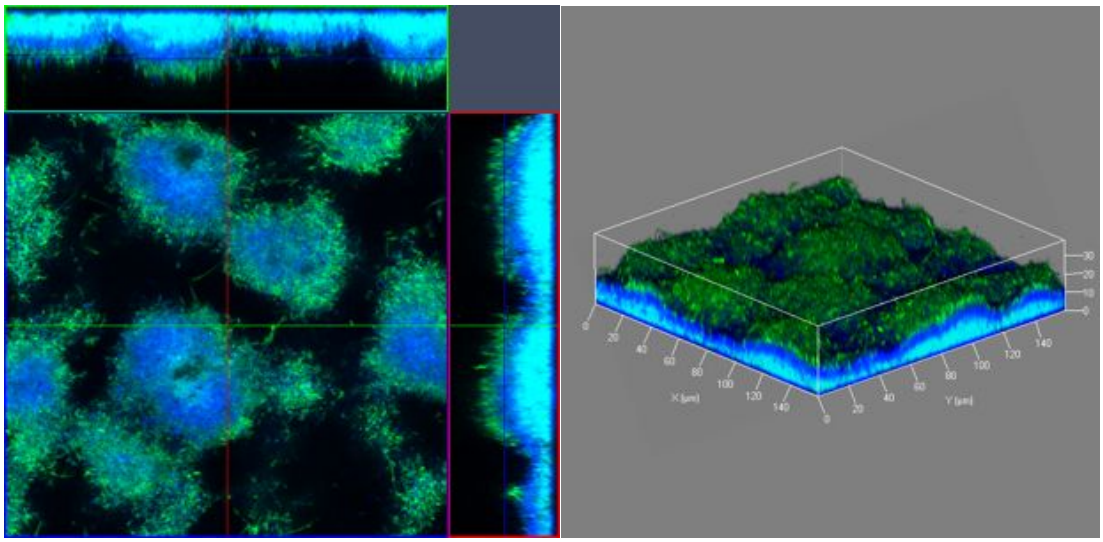


Figure 6.4: WT Biofilm grown in FAB medium supplemented with glucose. Cells expressing Gfp (green) and stained with DAPI (blue) to visualise DNA. Images were taken after 4 days. The bottom layer appears blue green as it contains both signals.

Dual channel imaging of DAPI stained, Gfp expressing biofilms appeared as three layers (Figure 6.4). The bottom layer contained both cells and DNA (bright blue), the middle layer contained only DNA (blue) and the top layer contained only cells (green). It is plausible therefore that eDNA provides a general adhesive in the biofilm particularly enabling the top layer of cells to adhere to the lower layers. Given that the DNA content of biofilms is substantial and widespread, it was predicted that the *pqsA* and *pqsR* mutants would form less substantial biofilms than the WT. For the following experiments the *pqsR* mutant was grown alongside the PAO1 WT in the flow cell system. The *pqsR* mutant was chosen as it showed no increase in eDNA production upon the addition of PQS or HHQ in any of the previous experiments. Biofilms were grown in FAB medium supplemented with glucose at 37°C for 4 days. Biofilm biomass, thickness and eDNA content was measured by CLSM and image analysis.

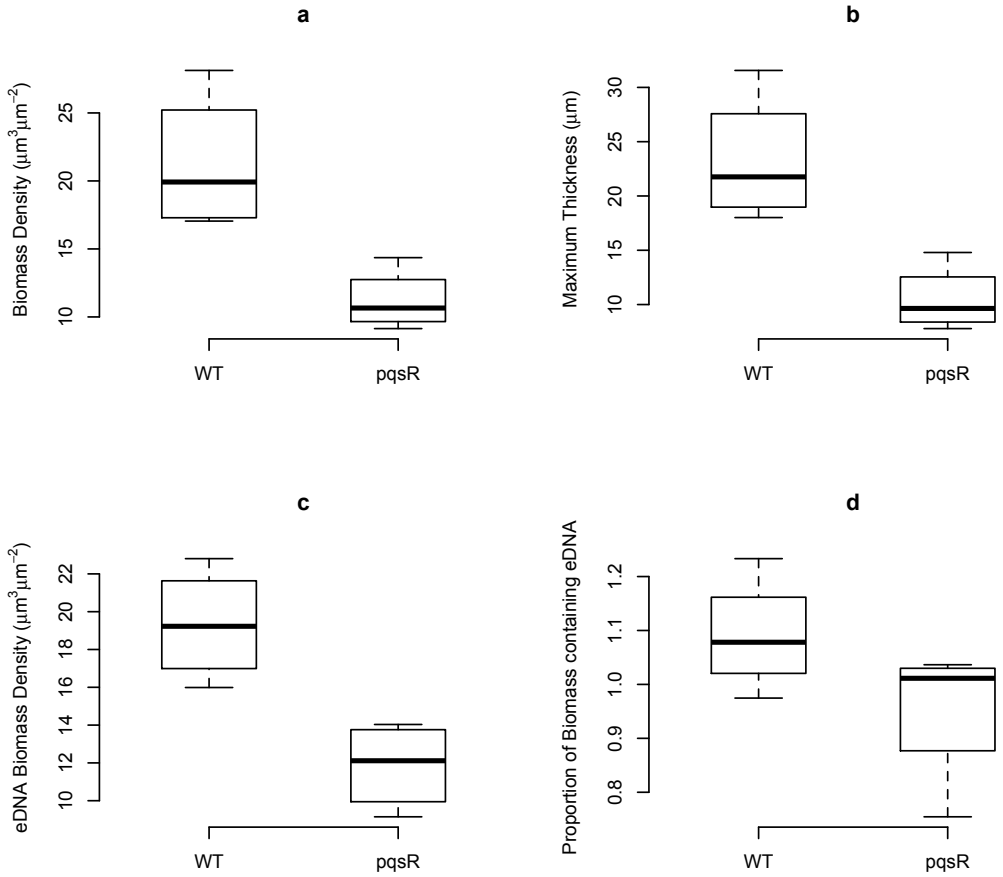


Figure 6.5: Biomass Density and thickness (a, b), absolute eDNA biomass (c) and eDNA as a proportion of total biomass (d) of biofilms formed by the WT and isogenic Δ*pqsR* mutant.

Biofilms of the Δ*pqsR* mutant were of lower biomass ($F_{1,6}$ = 12.75, $p < 0.05$), lower thickness ($F_{1,6}$ = 14.50, $p < 0.01$) and contained less eDNA ($F_{1,6}$ = 15.80, $p < 0.01$) than the WT counterpart. The proportion of biomass containing eDNA however did not significantly differ between the two strains ($F_{1,6}$ = 2.59, $p > 0.05$).

6.2.4 Can a mutant producing less eDNA cheat on a WT?

An eDNA deficient Δ*pqsR* mutant was considerably impaired in biofilm development thus making it a suitable candidate as a public goods cheating strategy. To test whether the Δ*pqsR* mutant was able to exploit the eDNA production of the WT, flow cells were inoculated with either a WT, Δ*pqsR* or a mixed culture containing WT and 10% Δ*pqsR*. The two strains could be distinguished by the use of different fluorescent protein labels. If exploitation occurred the Δ*pqsR* mutant should have shown an increase in frequency, over a period of incubation. Biofilms were grown in FAB medium supplemented with glucose at 37°C for 4 days. The physical parameters of biofilms were measured as above and in addition the relative abundance of each strain was measured for the mixed channels.

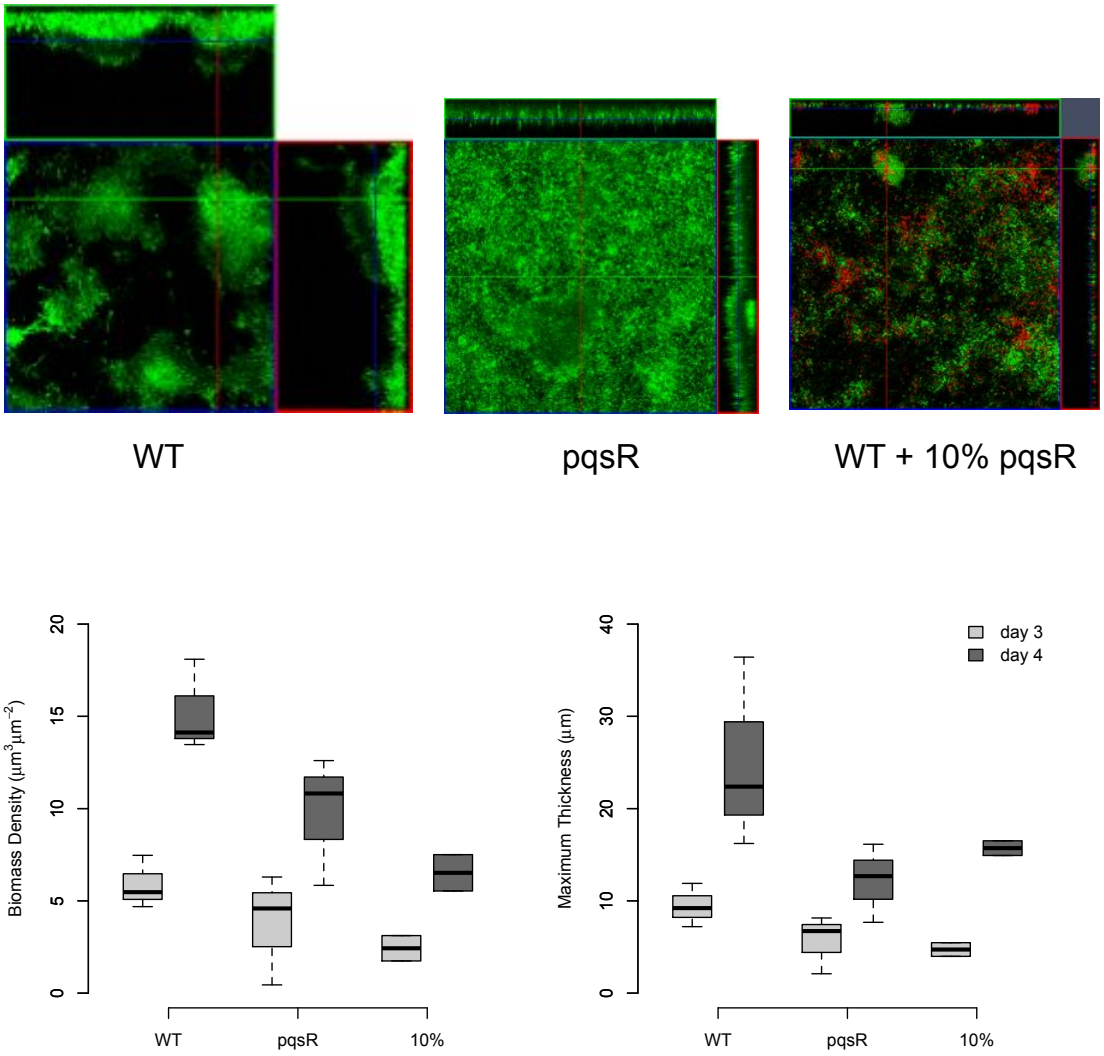


Figure 6.6: Confocal Micrographs (top) and physical parameters estimated from them (bottom) of biofilms formed by the WT (gfp labelled), Δ*pqsR* mutant (mcherry labelled) and a WT containing 10 % Δ*pqsR*.

Microscopic observations and calculated physical parameters indicated that biofilms of the WT were greater in biomass density ($F_{2,12} = 7.14$, $p < 0.01$) and thickness ($F_{2,12} = 4.62$, $p < 0.05$) than the Δ*pqsR* mutant and a mixed culture. The greatest differences were observed on day 4, later in the incubation period. This is consistent with the hypothesis that eDNA release enhances the latter stages of biofilm development as opposed to attachment and colonisation. The smaller

biofilms of a mixed culture indicate some cost to the presence of a cheating mutant. To test explicitly whether exploitation occurred, the relative fitness of the cheating mutant was calculated. Relative fitness diverged between the two replicates of mixed culture such that in one iteration the Δ*pqsR* mutant enjoyed a fitness advantage and in the other iteration it's fitness declined by day 4 ($F_{1,10}$ = 15.11, $p < 0.01$).

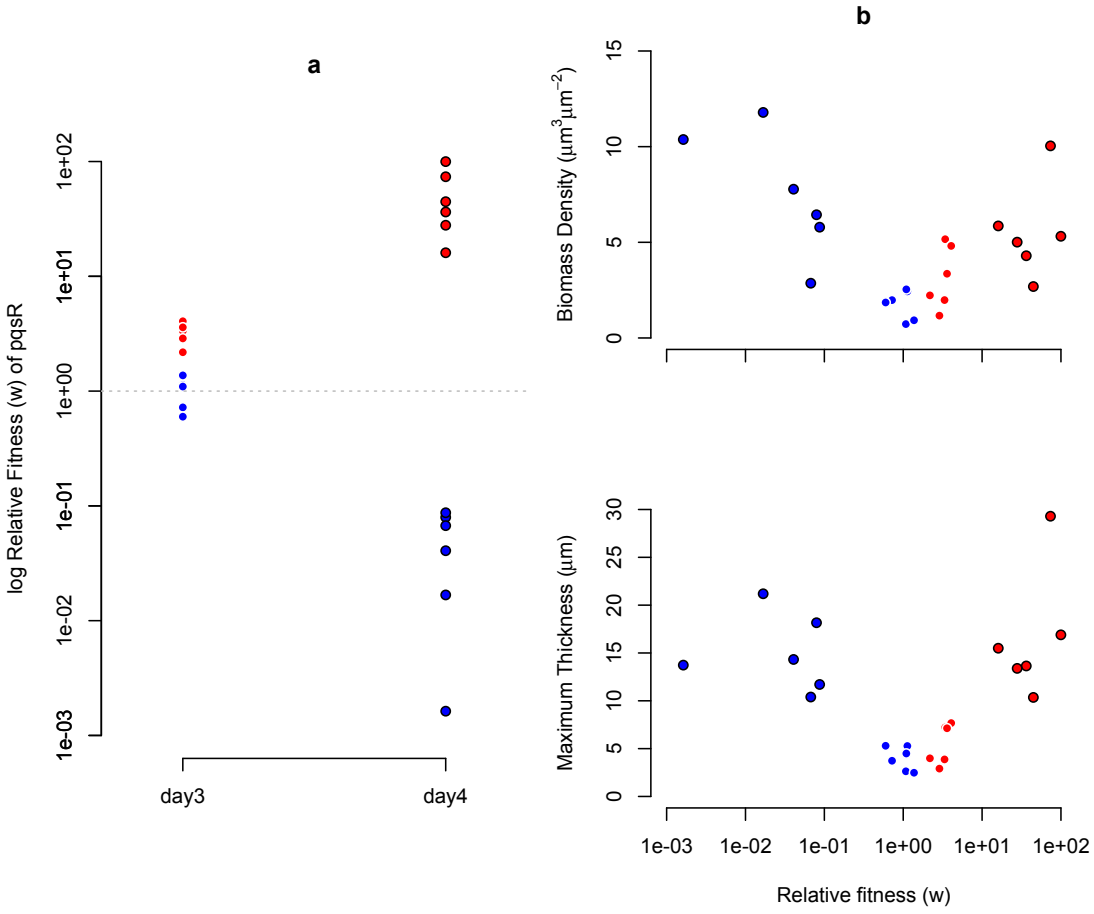


Figure 6.7: (a) Relative fitness of a Δ*pqsR* mutant. (b) Correlation of relative fitness vs. biomass and relative fitness vs. thickness. The red and blue dots represent individual confocal z-stacks taken from 2 replicates (blue/red) of the experiment. Small dots represent z-stacks from day 3 and large dots represent z-stacks from day 4.

If the presence of a Δ*pqsR* mutant incurs a cost to the biofilm, a negative relationship is expected with the extent of biofilm formation and the fitness of a mutant. This was tested using 'patches' or confocal z-stacks and comparing the fitness and the biofilm size in each (Figure 6.7). There was no negative correlation between biomass and fitness ($t_{22} = 1.07$, $p > 0.05$) or thickness and fitness ($t_{22} = 3.12$, $p > 0.05$). This indicates that although in one case the Δ*pqsR* mutant spread in the population, this was not costly to the population in terms of biofilm formation. Taken together these results suggest that although there is potential for eDNA cheating within biofilms, the prevalence of a *pqsR* mutant in the population relies on other factors than simply its eDNA output.

6.3 Discussion

6.3.1 The PQS biosynthetic pathway and eDNA release

PQS/AQ signalling has been implicated in the release of structurally important extracellular DNA in the biofilm matrix (Allesen-Holm et. al. 2006, Haussler & Becker 2008). This chapter considered whether a strategy that does not contribute to such a structural component could nonetheless benefit from its production by others in the population. Firstly, in order for such a strategy to prevail, its existence must be possible. Two criteria are important here; firstly the inability to produce eDNA and secondly the inability to respond to PQS by producing eDNA. It was observed that a growing culture of WT PAO1 increases its release of DNA upon the addition of exogenous PQS and HHQ. It was then observed that mutants in the PQS biosynthesis and response genes *pqsA* and *pqsR* are impaired in the release of eDNA when compared to their WT PAO1 parent strain and that this deficiency could not be overcome by the addition of synthetic PQS. Lastly it was observed that a mutant in the PQS effector protein *pqsE* was unaffected with respect to eDNA production. A number of basic insights stem from these observations.

The lack of eDNA production in response to PQS and HHQ by both the Δ*pqsA* and Δ*pqsR* mutants indicates that the effect of PQS on eDNA release is not a signal-receptor complex dependent phenotype. That is, the effect of PQS is not due to signal transduction via the receptor protein PqsR. If this was the case the *pqsA* mutant would have responded when the signal molecule was added. In line with this, the Δ*pqsE* mutant, does produce WT levels of eDNA. The exact function of the *pqsE* gene has remained elusive to the field but it is considered to be the main effector in traits regulated by the PQS-PqsR signal-receptor complex such as pyocyanin production. For example it is possible to complement phenotypes such as pyocyanin production, *lecA* expression, swarming and biofilm formation of knockout mutant in PQS biosynthesis by the addition of an inducible *pqsE* gene under induction (Rampioni et al. 2010). It is therefore likely

that eDNA release is not dependent upon formation of the PQS-PqsR complex and the expression of *pqsE* but caused by the presence of the PQS molecule itself.

6.3.2 The mechanism of eDNA release

The costs and benefits of performing a trait heavily influence its evolutionary dynamics. Therefore the mechanism of eDNA release is important to determine the cost of such a release. It has been suggested that PQS mediated eDNA release is caused by lysis of cells (Häussler & Becker 2008) in which case the trait would be very costly. To test the effect of PQS on cell lysis, high density cell suspensions were incubated in a medium in which cell division and metabolism was not possible (PBS) in the presence and absence of PQS. Firstly it was observed that log $CFUml^{-1}$ increased slightly in PBS over a 24 h period. This increase represented approximately 1 cell division over that incubation period confirming that cells in this solution were able to survive but not divide. No decrease in log $CFUml^{-1}$ was observed upon the addition of PQS. Despite this the addition of PQS stimulated an increase in eDNA content of the supernatant. This suggests that the PQS mediated release of eDNA is at least in part independent of cell lysis. Though previous studies elsewhere conclude that the source of eDNA is the genomic DNA of the originating organism, it has not been demonstrated unequivocally that this is due to lysis (Nemoto et al. 2003; Häussler & Becker 2008). It may be possible to determine the amount of DNA released by a single cell upon lysis e.g. by sonication and therefore deduce wether PQS mediated DNA release may indeed be due to lysis or not. There may be several simultaneously acting routes to eDNA release. For example the lysis of a cell is sure to release eDNA but at a much greater cost than the specific export of a smaller amount of DNA. Which mechanism is acting will determine in part the cost of the trait.

6.3.3 The benefit of eDNA in biofilm growth

In order for eDNA to be considered a public good, its production should confer some benefit to the population. To confirm the findings of other studies that

demonstrate the importance of eDNA in biofilm development, cultures of several *pqs* mutants in the presence and absence of PQS and HHQ were grown and the resulting biofilms stained with crystal violet. DNA content of the supernatant was also measured using DNA precipitation and detection by spectrometric analysis (Nanodrop™). These mutants and conditions were selected to obtain a range of different levels of eDNA production to test the correlation between eDNA production and biofilm formation. It was found that the addition of PQS greatly enhanced crystal violet staining in all treatments and HHQ enhanced crystal violet staining mainly in the Δ*pqsR* mutant. With respect to eDNA release, the previous findings were largely confirmed apart from a few exceptions. The Δ*pqsA* mutant responded to HHQ by producing eDNA and the Δ*pqsE* mutant showed greater eDNA release apart from in the presence of PQS. With respect to individual differences in strains, no strong conclusions can be drawn from this experiment due to each treatment combination containing only 1 replicate.

The effect of DNA concentration on biofilm formation however can be assessed by correlation. There was no correlation between DNA content and biofilm development. This led to the hypothesis that attachment and initial colonisation of the attached community, though enhanced by the presence of PQS, was not dependent upon PQS mediated DNA release but rather that DNA release played a role in the latter stages of biofilm development. In addition there was a significant negative correlation between planktonic growth and biofilm formation indicating that experimental treatments caused bacterial cells to switch between planktonic and attached modes of growth. It has been previously shown that PQS stimulates the formation and release of micro-vesicles via a direct interaction with the outer membrane lipopolysaccharide (Mashburn & Whiteley 2005). Such an interaction could condition the cells to be more adhesive to abiotic surfaces.

To investigate the effect of PQS and eDNA release on biofilm maturation biofilms were cultured in the flow cell system and stained with DAPI, a nucleic acid stain.

This enabled a simultaneous assessment of live cells using Gfp and eDNA using DAPI. It was observed that a WT PAO1 strain formed more substantial biofilms, containing a larger amount of eDNA when compared with the Δ*pqsR* mutant. DNA content as a proportion of total biomass however did not vary significantly between the two strains. There are two plausible explanations for this. Firstly it is possible that the *ΔpqsR* mutant produces the same amount of eDNA as the WT and simply grows more slowly. Secondly the WT makes more eDNA which enables it to grow to higher densities in turn causing the constant eDNA proportions. Biofilms of the WT and Δ*pqsR* mutant differ more on day 4 of incubation than on day 3. This is consistent with the hypothesis that PQS-mediated eDNA release confers a benefit to a biofilm population at the latter stages of biofilm development.

6.3.4 eDNA as an exploitable public good within biofilms

The ability of a rare Δ*pqsR* mutant to exploit a WT in a mixed culture biofilm was assessed by monitoring the relative abundance of each over a period of incubation. Relative fitness of the rare mutant diverged in the two replicates of this experiment. In one case the relative fitness increased from the initial point and in another the relative fitness drastically decreased. In addition, there was no negative relationship between fitness and biomass between patches of co-culture biofilms. Taken together, these two observations indicate that the prevalence of the Δ*pqsR* mutant is not dependent upon exploitation of the WT eDNA production. It may be that eDNA release in a biofilm is due to a different cause than in liquid culture. It is known for example that mature biofilm micro-colonies undergo lysis of the interior (Webb et al. 2003). It is also important to note that PQS influences biofilm formation through regulation of the galactose-binding lectin LecA (Diggle et al. 2006). The combinatorial effects of PQS on eDNA and LecA may make the prevalence of a Δ*pqsR* mutant unpredictable. For instance, a Δ*pqsR* mutant cell may gain a benefit from reduced eDNA production but may not be able to physically stick to the biofilm due to a reduced production of LecA.

These data should be treated as preliminary pilot data due the lack of sufficient replicates to achieve statistical power. Although it is possible to analyse the outcome of one such experiment, from the present data, it is not possible to make generalisations about WT and Δ*pqsR* biofilms. The flow cell biofilm system is excellent for obtaining detailed images and 3 dimensional reconstructions of biofilms but less useful when many replicative experiments are necessary. Future work could utilise one of the new high-throughput flow cell technologies which will likely, in time, replace more traditional flow cell systems.

Chapter 7: Intraspecific cooperation, interspecific competition

7.1 Introduction and aims

7.1.1 Multiple Social Traits and Species

A public goods dilemma can undermine population productivity and threaten the prevalence of cooperation. The exact way in which this unfolds depends upon the costs and benefits of cooperation (in part determined by the environment), and the social structuring in a population, factors which shape selection for cooperation. Bacteria engage in many such cooperative ventures simultaneously in polymicrobial communities, and so the interactions between cooperative traits and species also influence the outcome of social conflicts. This is a matter which has received relatively little attention but a few recent studies set the scene.

Harrison et. al. (2009) report that whilst pyoverdine defection in isolates of *P. aeruginosa* confers an advantage in iron limited co-culture with a WT, it incurs a pleiotropic cost to biofilm formation. This is because pyoverdine cheats form poor biofilms and cannot exploit the biofilm production of a WT. The authors suggest that this may reduce selection for pyoverdine cheating in nature as resulting biofilms would risk extinction. Brown & Taylor (2010) formulate a general framework for the joint evolution of multiple social traits. They demonstrate that in the presence of 'evolutionary synergy', i.e. when the fitness implications of one trait affect those of another in a non-additive way, the equilibrium level of such a trait could not be predicted by single trait models. This highlights the importance of considering the interaction of multiple traits particularly where the fitness effect of one is a function of the other.

7.1.2 Interspecific vs. Intraspecific competition

We have seen that the outcome of social conflict over a trait is modulated by environmental factors that affect the costs and benefits of that trait, and the environment consists of many biotic and abiotic elements. In addition, microbes

interact with many different species in the environment and within hosts. The effect of interactions between species on the outcome of social conflict within species has not been explored in depth. *P. aeruginosa* interacts with many other microbes in the CF lung (Harrison 2007a). A seminal study by the Cystic Fibrosis Foundation showed the longitudinal prevalence of microbial species present in the airways of a CF patient. *P. aeruginosa* was shown to be the most prevalent species in adults, displacing *Staphylococcus aureus* as chronic infection progressed (Cystic Fibrosis Foundation, 2004).

This prompted research into the interactions between *P. aeruginosa* and *S. aureus*. It was found that a secondary product of the PQS biosynthesis pathway in *P. aeruginosa*, the N-oxide derivative of HHQ (HHQNO), causes growth inhibition of *S. aureus* and protects it against killing by tobramycin (Hoffman et al. 2006). This is probably due to interruption of the electron transport system in *S. aureus* as the same effect can be observed using pyocyanin, a *P. aeruginosa* PQS-regulated exoproduct (Biswas et al. 2009). This may, in part, explain the lower detection of *S. aureus* in clinical samples where both species are found for example in the Cystic Fibrosis Foundation longitudinal study. In addition it has been reported that *P. aeruginosa* down regulates iron acquisition genes in the presence of *S. aureus* in a mouse model (Mashburn et al. 2005). This observation initially led to the hypothesis that *S. aureus* acts as a iron source for *P. aeruginosa*. A recent study however reports that the presence of *S. aureus* causes *P. aeruginosa* to produce more pyoverdine and increases the selection for cheating by pyoverdine non-producers. In either case these studies provide hints that the PQS system confers an advantage to *P. aeruginosa* when in co-culture with *S. aureus*. This likely involves competition for iron and may influence the intraspecific competition over iron and therefore the selection for intraspecific siderophore cheating.

7.1.3 PQS is a multifunctional molecule

The PQS signalling system has been shown to function through several routes (Diggle, Matthijs, et al. 2007c). It acts on its cognate receptor protein, PqsR, to

form a transcription factor but it can also bind iron in the environment, causing an increase in the expression of iron uptake genes in a similar way to iron starvation. The PQS precursor molecule HHQ, acts as a signal molecule but does not bind iron, and therefore does not cause induction of iron acquisition genes. It is therefore likely that the induction of iron acquisition genes by PQS is through iron starvation. In this capacity, PQS is not only a signal molecule, it influences iron levels in the environment which then induces an appropriate response. It has also been reported that PQS interacts with the lipo-polysaccharides of gram negative bacterial outer membranes, forming vesicles (Mashburn & Whiteley 2005; Mashburn-Warren et al. 2008). This leads to the hypothesis that PQS is an iron trap, closely associated with the outer membrane and trapping iron close to the cell (Diggle et. al. 2007). This could potentially affect competition for iron, both inter and intra-specifically. Firstly, an increase in public good (siderophore) output would increase the benefit and cost for producers but would only increase the benefit for non-producers, thus skewing the outcome of competition in favour of non-producers. On the other hand, trapping iron enabling its use by *P. aeruginosa,* to the exclusion of other species such as *S. aureus,* may aid in interspecific competition.

7.1.4 Aims of this study

PQS is a multi-functional molecule. As well as being a classical QS signal molecule it may play an important part in mediating inter- and intra-specific competition for iron. This chapter investigated this in the following way;

1. To investigate the effects of iron and PQS in the expression of *P. aeruginosa* iron acquisition genes in both iron deplete and iron rich environments.

2. To investigate the effect of PQS on selection for siderophore cheating in *P. aeruginosa*.

3. To investigate whether the PQS-Fe complex, once formed, is more beneficial to *P. aeruginosa* than *S. aureus*.

4. To investigate whether *P. aeruginosa* up-regulates PQS production in direct response to the presence of *S. aureus*.

7.2 Results

7.2.1 Facultative siderophore production in response to iron and PQS

To study competition for iron under iron limiting conditions, an iron limited medium Casamino Acids (CAA) was used. Supplementation of this medium with ferrous iron sulphate ($FeSO_4$) should increase growth and repress the expression of iron acquisition genes coding for siderophore transport systems (*pvd* and *pch* genes). To test this cultures of *P. aeruginosa* PAO1 WT, containing a *lux* fusion to the pyoverdine biosynthesis gene *pvdE,* were grown in CAA for 24 h at 37°C over a gradient of iron concentrations. Growth and light production were measured and represented here as a proportion of the level for cultures with no addition.

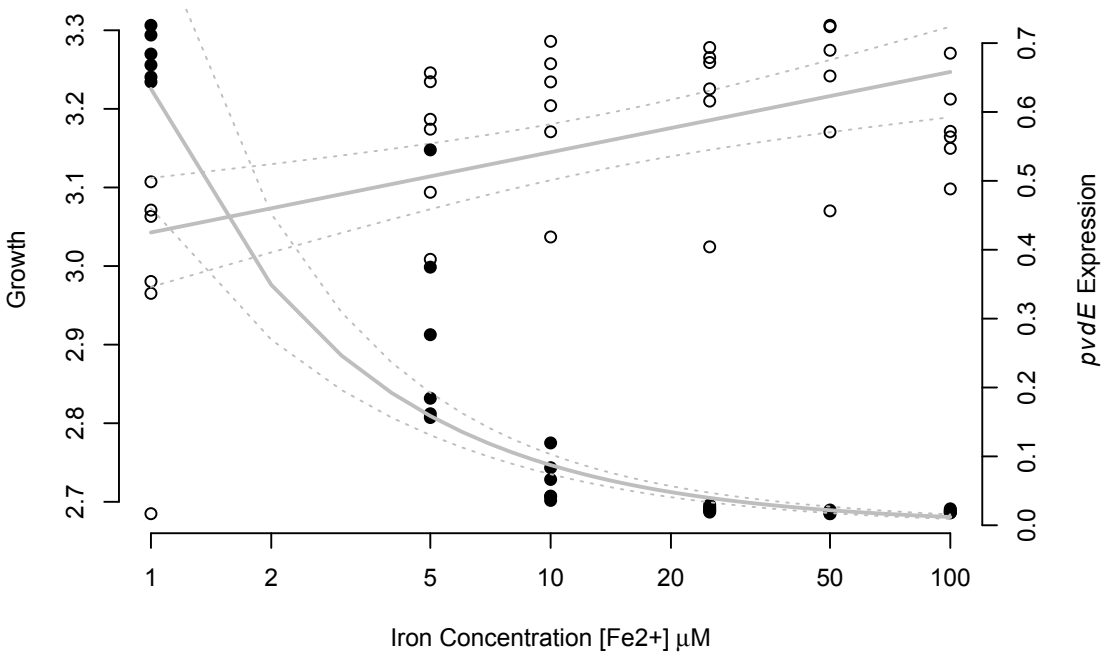


Figure 7.1: The effect of iron supplementation on growth and *pvdE* expression if PAO1 WT in an iron limited medium. Values are proportions of either growth or *pvdE* expression in the absence of iron supplementation. Open circles represent growth (left y-axis) and closed circles represent *pvdE* expression (right y-axis). Grey solid and dashed lines represent the predictions of linear models fitted to the data.

Starting with an iron deplete medium and supplementing with iron has two important implications. Firstly growth is increased with iron concentration ($t = 86.07$, $p < 0.001$, Figure 7.1) and *pvdE* expression decreased with iron concentration ($t = -2.80$, $p < 0.01$, Figure 7.1). This is in agreement with a previous study that reports the repression of *pvd* genes in response to increasing iron availability (Mashburn et al. 2005).

If PQS causes iron starvation it could be expected that the addition of PQS to iron rich cultures both increases *pvdE* expression and decreases growth. To test this, iron rich (LB medium) cultures were initiated with PAO1 *pqsA* mutant containing a *lux* fusion to the pyoverdine biosynthesis gene *pvdE* were grown in LB over a range of PQS concentrations. Growth and light production were measured and and represented here as a proportion of the level for cultures with no addition. As expected, the addition of PQS to iron rich cultures decreased growth ($t = -3.42$, $p < 0.01$, Figure 7.2) and increased *pvdE* expression ($t = 2.24$, $p < 0.05$, figure 7.2). It can be inferred from this data that *P. aeruginosa* experiences iron starvation in the presence of excess PQS.

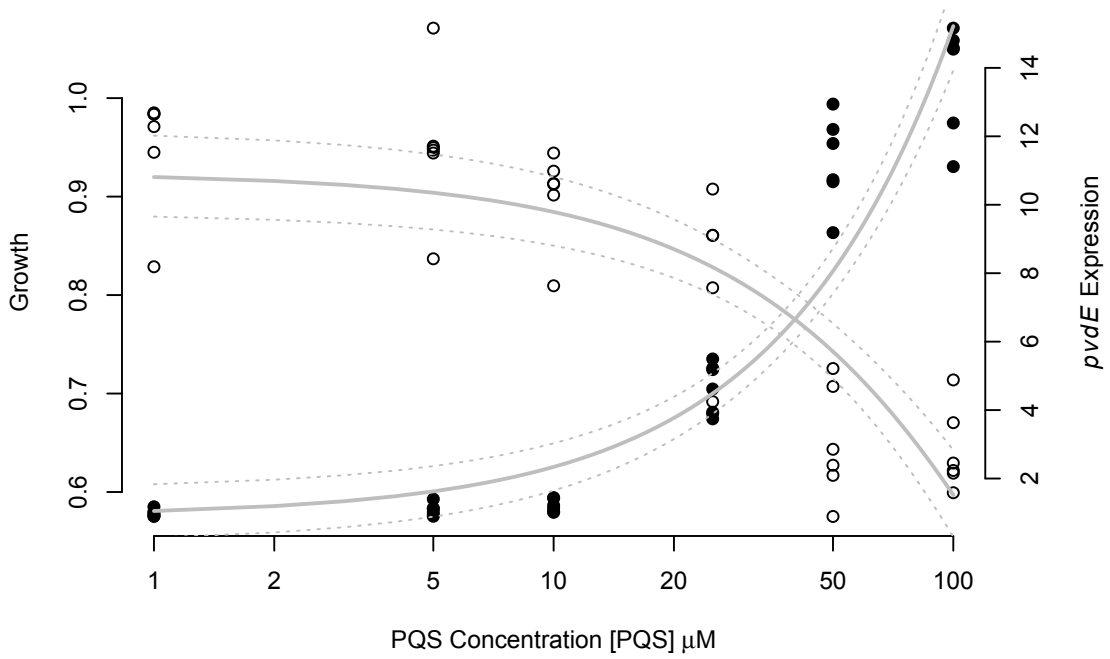


Figure 7.2: The effect of PQS on growth and *pvdE* of a PAO1 *pqsA* mutant expression in an iron rich medium (LB). Values are proportions of either growth or *pvdE* expression in the absence of PQS addition. Open circles represent growth (left y-axis) and closed circles represent *pvdE* expression (right y-axis). Grey solid and dashed lines represent the predictions of linear models fitted to the data.

7.2.2 Pyoverdine cheating and PQS

It is well known that a siderophore non-producer can exploit the production of others in the population and therefore under iron limitation enjoys a relative fitness advantage over producers subject to negative frequency dependence. When iron availability is varied, the relative cost:benefit ratio of iron acquisition is altered. When iron is plentiful, the cost of pyoverdine production is reduced by down regulation of genes involved in pyoverdine biosynthesis. When iron is scarce, the cost of pyoverdine production is paid by producers and the benefit is shared between all individuals within the population. Therefore it was predicted that during PQS-induced iron starvation, a non-producer of pyoverdine would enjoy an increased relative fitness benefit over a producer. To test this, competition experiments were performed, starting with a rare (starting frequency 1 %) non-producing Δ*pvdDpchEF* double mutant in a WT background. The Δ*pvdDpchEF* double mutant is defective in both pyoverdine (*pvd*) and pyochelin (*pch*) production, two important siderophores in *P. aeruginosa* (Serino et al. 1997; De Vos et al. 2001). This mutant was used because preliminary data indicated that the absence of one siderophore could be compensated for by up regulation of the other. Relative abundance was measured before and after a 24 hr period of incubation and relative fitness was calculated.

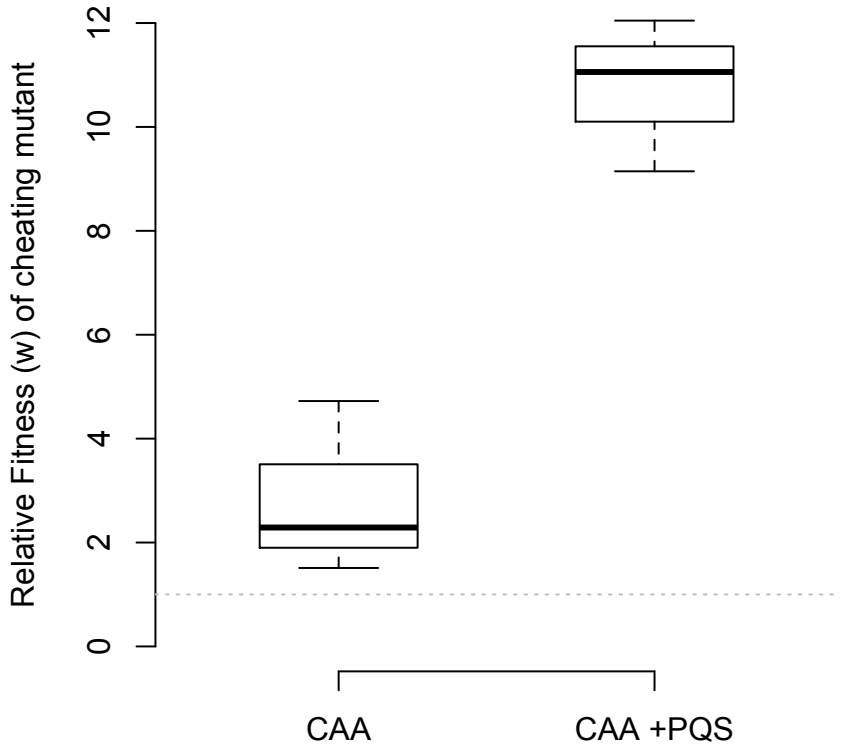


Figure 7.3: The relative fitness of a rare (1%) Δ*pvdDpchEF* mutant in the presence and absence of 20 μM PQS after a 24 hr period of incubation. The dashed horizontal line at w = 1 represents the point at which neither producer or non-producer has a relative fitness advantage over the other.

In accordance with the prediction, relative fitness of a cheating mutant increased upon the addition of PQS to the culture ($F_{1,4} = 37.66$, $p < 0.01$, Figure 7.3). This is consistent with the previous data showing that a WT experiences iron starvation and increased siderophore output with increasing PQS concentration.

7.2.3 Utilisation of the PQS-Fe complex

It has previously been suggested that, since PQS has a high affinity for the LPS of the cell membrane, it may act as an iron trap (Diggle et. al. 2007, Mashburn-Warren et al. 2008). The 3-hydroxyl moiety of the PQS molecule enables it to bind ferric iron and the resulting complex can be scavenged by *P. aeruginosa pvd* and *pch* siderophores. The PQS-iron complex can restore the growth deficiency of *P. aeruginosa* under iron deplete conditions (Diggle, Matthijs, et al. 2007c). However it may also be possible that *S. aureus* is able to utilise this complex as an iron source which would reduce any competitive advantage to *P. aeruginosa*. To test whether *S. aureus* was able to utilise the PQS-Fe complex, iron limited cultures of *P. aeruginosa* PAO1 and *S. aureus* RN6390R were initiated in the presence and absence of PQS, Fe_2SO_4 and the PQS-Fe complex. A defined medium in which both species could be cultured was used here (SSD medium) and Chelex-100 was used to remove iron. Growth was measured and is represented here as a proportion of growth in CAA with no addition in order to compare the effects on proportional growth across the two species.

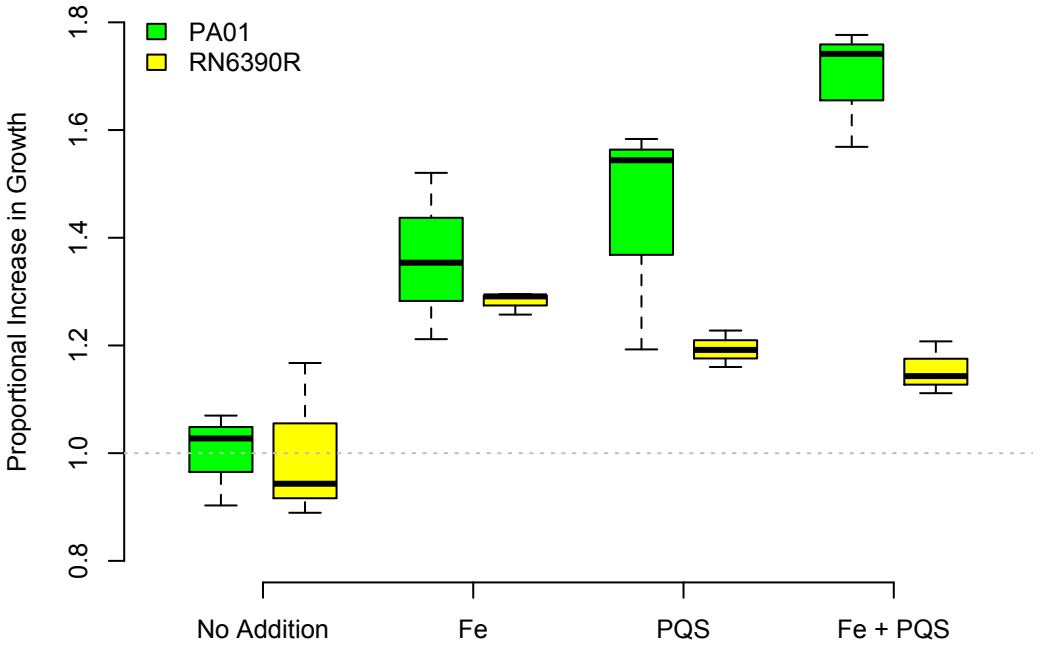


Figure 7.4: The effects of adding 20 µM PQS, 7 µM Fe_2SO_4 and 20 µM PQS-Fe complex to iron limited cultures of on *P. aeruginosa* PAO1 and *S. aureus* RN6309R. Values are given as a proportion of growth with no addition (horizontal line at 1). There are three replicates in each treatment-strain combination. The thick horizontal bars represent the median value, the boxes represent the interquartile range and the whiskers represent the extreme values.

In a fully factorial ANOVA analysis it was possible to distinguish the effects of Fe, PQS and the interaction of their effects when they were added together as a complex. ANOVA, used throughout this thesis can enable the disentanglement of several interacting factors influencing a single response. For example, using ANOVA we can determine the effects of adding PQS and iron on the growth of bacterial cultures and whether or not those effects interact (i.e. is the combined effect different from the sum of the individual effects). This requires that we collect data on each individual treatment and the combined treatments as has

been done in this experiment (Figure 7.4, above). The results of this analysis are summarised in the table below (Table 7.1).

Factor	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
strain	1	0.28	0.28	19.36	0.00039	***
PQS	1	0.26	0.26	18.07	0.00054	***
Fe	1	0.28	0.28	18.94	0.00043	***
strain:PQS	1	0.19	0.19	12.84	0.00229	**
strain:Fe	1	0.05	0.05	3.61	0.07447	.
PQS:Fe	1	0.07	0.07	4.67	0.04531	*
Residuals	17	0.25	0.01			

Table 7.1: The output of an ANOVA analysis on the effects of strain, PQS and Fe on relative growth. Factors are listed in the left hand column where interactions are denoted with a colon symbol. All except the interaction between strain and iron (strain:Fe) explain a significant amount of the variation in proportional growth.

From the analysis it can be inferred that the addition of PQS and Fe increase growth ($F_{1,16} = 18.26$, $p < 0.001$) and ($F_{1,16} = 19.15$, $p < 0.001$) respectively. The effect of PQS but not iron is dependent upon the strain ($F_{1,16} = 12.96$, $p < 0.01$) and ($F_{1,16} = 3.65$, $p > 0.05$) respectively. The effect of adding Fe depends upon the presence of PQS ($F_{1,16} = 4.75$, $p < 0.05$).

To explain this in a more biological context; addition of iron benefits both strains, the addition of PQS benefits *P. aeruginosa* more than *S. aureus* and this effect is enhanced when Fe is already present (i.e. when the PQS-Fe complex is added). Therefore, although the addition of PQS alone favours *P. aeruginosa*, the difference is greater when PQS-Fe is added. This results suggests that the PQS-Fe complex is more beneficial to *P. aeruginosa* than *S. aureus*.

7.2.4 PQS production in response to the presence of S. aureus

It has previously been reported that *P. aeruginosa* up-regulates PQS biosynthesis in a medium designed to mimic CF sputum compared with a standard rich growth medium (Palmer et al. 2005). Sputum is one environment where both *P. aeruginosa* and *S. aureus* co-exist as both are important pathogens in the CF lung (Harrison 2007a). Since these species are often found together in such an environment, it was hypothesised that *P. aeruginosa* can detect the presence of other species and produce extracellular factors that aid in interspecific competition. Due to the down-regulation of siderophores in the presence of *S. aureus*, it has also been suggested that *S. aureus* provides an iron source to *P. aeruginosa* via lysis of the cells (Mashburn et al. 2005). To test whether *P. aeruginosa* upregulates PQS biosynthesis in response to the presence of *S. aureus,* and whether *S .aureus* provides an iron source to *P. aeruginosa,* iron limited cultures of PAO1 Δ*pqsA pqsA*::lux were supplemented with either sterile *S. aureus* LB supernatant, washed *S. aureus* cells suspended in LB and sterile LB and Fe as controls. Growth and expression of *psqA* were monitored. A mutant in *pqsA* was used to avoid HHQNO mediated growth inhibition of the *S. aureus* cells (Hoffman et al. 2006).

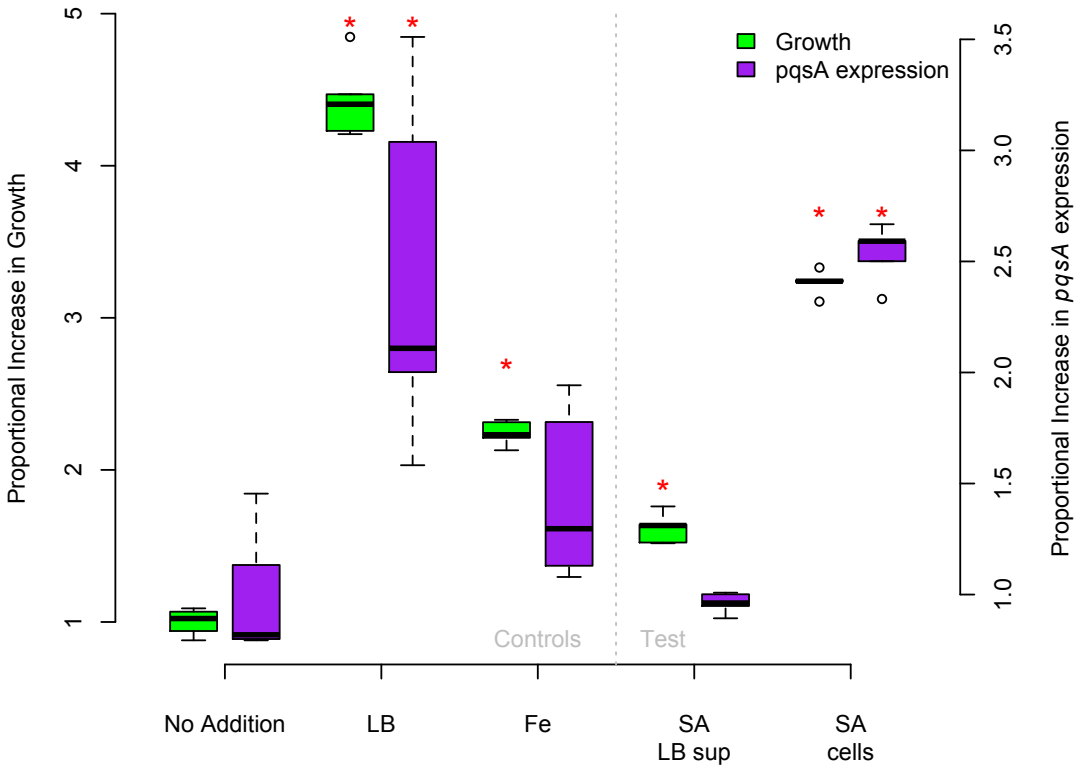


Figure 7.5: The effect of adding *S. aureus* supernatant or washed cells on the growth (green boxes, left axis) and *pqsA* expression (purple boxes, right axis) of *P. aeruginosa*. Data are shown as a proportion of the No Addition treatment and the red asterisks indicate that those groups are significantly different from their corresponding No Addition treatment.

By grouping factor levels within an ANOVA and comparing resulting models, the pairwise differences between No Addition and subsequent treatments was tested. Growth was significantly increased by the addition of LB, Fe and both the supernatant and cells from a *S. aureus* culture. Expression of *pqsA* was increased by the addition of LB and *S.aureus* cells but not by Fe or *S. aureus* supernatant (Figure 7.5, red asterisks).

7.3 Discussion

7.3.1 Facultative cooperation and cheating

The data presented here, together with several previous studies corroborate the observation that *P. aeruginosa* produces iron scavenging siderophores facultatively, tuning down their costly production when iron is plentiful. This study additionally finds that when excess PQS is present, its iron binding capacity can reduce the free iron in the environment and induce siderophore production in *P. aeruginosa.* This in turn increases the selection for cheating as a siderophore cheat does not pay the cost of additional siderophore production but gains the benefit of their presence.

7.3.2 Utilisation of the PQS-Fe complex

Previous studies implicate that the *P. aeruginosa* PQS biosynthetic pathway provides an enhancement of competitive ability against *S. aureus*. A product of the PQS biosynthetic pathway, HHQNO interferes with electron transport and selects for highly tolerant small colony variants (SCV's) in *S. aureus* as does the PQS-regulated pigment pyocyanin (Lépine et al. 2004; Hoffman & Déziel 2006). In addition, this study found that once the PQS-Fe complex is formed, it is of greater benefit to *P. aeruginosa* than to *S. aureus*, although it does benefit the iron-limited growth of both species. The presence of PQS alone gives *P. aeruginosa* a larger growth benefit than *S. aureus* but this is enhanced when both PQS and Fe are present in the PQS-Fe complex form. The simplest explanation for this is that *P. aeruginosa* siderophores have a higher affinity for PQS bound iron than do the siderophores of *S. aureus*. In addition, the interactions of PQS with the *P. aeruginosa* outer membrane may further trap the iron close to the *P. aeruginosa* cell walls removing it from the vicinity of *S. aureus* cells. The combinatorial effects of HHQNO, pyocyanin and PQS iron binding make the PQS system of *P. aeruginosa* a formidable tool against *S. aureus*. This however comes at a cost. The extra investment in siderophore production leaves

the population vulnerable to increased cheating by con-specific defectors in siderophore production.

7.3.3 Staphylococcus aureus as a nutrient source

Since PQS effectively aids in *P. aeruginosa* in competition against *S. aureus*, an experiment was designed to test whether PQS biosynthesis increased in a specific response to the presence of *S. aureus*. It was found that whilst the expression of *pqsA* was increased by the presence of *S. aureus* cells, this was also true of the addition of LB, a simple increase in nutrient availability. However in the case of adding LB, all of the culture consisted of *P. aeruginosa* whereas upon the addition of *S. aureus* cells the culture contained both species. Therefore the per cell bioluminescence detected when *S. aureus* cells were also present in the population was likely underestimated. Spent supernatant of *S. aureus* cultures and iron increased the growth but not the *pqsA* expression. The increase in *pqsA* expression was only observed in treatments that added fresh LB to the medium. The present study unfortunately does not rule out a specific response to *S. aureus* and this may merit further study. Overall this study describes how a multifunctional signal molecule can affect the balance between inter- and intra-specific competition. The forces of inter-specific competition are likely to occur in most natural microbial consortia and so this example may be one of many that may be found in the microbial world.

Chapter 8: Conclusions and Future Directions

8.1 The Evolution of Signalling

Evolutionary theory must always precede its empirical appraisal. There can be instances however when empirical assessments of theory follow very slowly on from theoretical analyses. Often this is due to limitations of knowledge and technology with which we handle empirical systems but sometimes limitations are imposed by the organisms themselves. Genetically tractable microbial experimental systems with fast generation times have boosted the speed with which we can examine evolutionary theory (Buckling et. al. 2009). In addition to this, microbial systems can be cultured under an enormous variety of conditions, consist of one or many species, infect many hosts, display social behaviours, become subject to predation and communicate. There are many behaviours in nature that are paralleled in the microbial world. This means that often its is possible to use microbial systems to examine more general questions in the evolution of behaviour. The crucial caveat however is that the specific nuances and biological details of every organism and study system can dramatically influence the manifestation of otherwise general biological phenomena. This thesis attempted to some general and some specific questions with regard to the evolutionary theory of communication.

Quorum sensing in *P. aeruginosa* is a thriving area of research in molecular and medical microbiology due to the chronic and aggressive infections it causes and the potential to interfere with pathogenicity through QSI (Bjarnsholt & Givskov 2008). It is owing to the rich history of molecular and genetic advances in the study of *P. aeruginosa* QS that this study was able to manipulate QS behaviour and observe the consequences. Both the addition of synthetic signal molecule and the addition of signal quenching molecule were able to alter QS response in a dramatic manner. When combined with the condition that QS response is needed for growth, these alterations to QS response caused a detriment to fitness. Bacterial populations responded to such selection over evolutionary timescales by reducing the native production of signal, primarily by reducing

expression of the *lasI* signal synthase gene. Surprisingly however the same populations maintained production of a QS regulated exoprotease, in spite of a reduction in signalling effort. This has two important implications. First the regulation of exoprotease may have been altered in some way. Second the linkage between signal and response may have been broken. Either of these changes would constitute a deviation from the well researched and described QS system of PAO1, the ancestral strain used in this study. If these observations are genuine and can be corroborated by other researchers, the implication would be that *P. aeruginosa* QS is evolutionarily plastic. The ability of populations to reorganise the genomic architecture of traits could be a formidable source of potential for adaptation and contribute to the ability of *P. aeruginosa* to colonise multiple and diverse habitats.

Signalling behaviours are vulnerable to defection or coercion, if on average the production of signal or the response elicited does not benefit both the signaller and the receiver (Maynard-Smith & Harper 2003). Since participant individuals in signalling and response are rarely genetically identical, their evolutionary interests will not always overlap. Though microbial populations reproduce clonally, the fast generation times allow mutations to arise and spread rapidly including at the loci encoding communication, leading to genetic conflicts. Three classes of mechanisms which overcome this dilemma have been suggested. Firstly the cost of signalling if sufficient reduces the opportunity to provide a false signal when it only benefits the signaller. Secondly if the production of signal and the benefit of response are so closely linked that a discrepancy is impossible. For example when a tiger scratches a tree to mark his territory, the height of those scratches dictates the benefit of response. Since there cannot be a discrepancy between the height of the scratches and the size of the tiger, response is always beneficial. This type of mechanism has been termed a handicap (Zahavi 1975, Grafen 1990). Thirdly, common interest between signaller and receiver removes the competitive advantage of defection or coercion by either party such that stable signalling results (Johnstone & Grafen 1992, Brown & Johnstone 2001). Common interest can arise through several

routes. Repeated interactions between pairs of individuals creates a covariance in fitness between them (Sachs & Bull 2004). Repression of competition can reduce the advantage of selfishness (Frank 2003). Common interest can also arise through relatedness at the relevant loci as in Hamilton's rule. For example honey bees share a common interest in waggle dance signalling as all female workers are related and sterile, thus their success depends on that of the queen.

This study provided evidence that common interest through relatedness maintains signalling in bacterial QS. The results suggest that signalling as well as response decrease with decreasing relatedness, though it is difficult to completely disentangle the two without a more detailed analysis. For example, when a reduction in exoprotease and signal production is observed simultaneously, and given the tight co-regulation of both by autoinduction, its cause could be loss of function in signalling, response or both. Nonetheless phenotypically, clones evolved in low relatedness treatments produced less signal, exoprotease and responded to a lesser degree to exogenously added signal molecule.

An Evolutionary Stable Strategy (ESS) model of QS under varying relatedness predicts that signalling should peak with intermediate relatedness (Brown & Johnstone 2001). The model predicts that the extremes of relatedness select for lower signal effort. At extreme high relatedness where a response is guaranteed, selection favours the minimum amount of signal needed to elicit a response. At extreme low relatedness where response is rare, selection favours an abolishment of signalling. Thus signal effort is maximised at intermediate relatedness. The empirical data herein does not support this prediction and there may be several reasons for this. Firstly it may be that once a reduction in public goods effort has occurred, a subsequent increase in signal effort does not sufficiently counteract this. Secondly if a reduction in public goods effort occurs through reduced response, this would also entail a reduced signal effort, therefore constraining adaptation by increased signalling. This would be similar to the index type of mechanism to maintain honest signalling. Lastly there may

be an opportunity cost to signalling such that AHL biosynthesis reduces the efficacy of other metabolic processes. Since the experiments herein were carried out in a defined minimal medium this cost may be accentuated and reduce the opportunity for excessive signalling.

Given the lack of emergence of super-signalling strategies where theory predicts them, a future avenue for research would be to examine the costs of signalling and possible constraints on super-signalling. For example preliminary data to this study revealed that a knockout mutant in *lasR* negated signal production in PAO1 but not in PA14. Therefore it is possible that if response to selection on public good production occurs at the *lasR* locus, its pleiotropy constrains the evolution of super-signalling in PAO1 but perhaps not in PA14. Closely related to this is the possibility that such autoinduction systems, in which the pleiotropic response mechanisms which governs response as well as signalling, themselves are a mechanism to constrain coercion. It has already been documented in the amoeba *D. discoideum* that pleiotropy can constrain the evolution of social defection (Foster et. al. 2004). The QS system of *P. aeruginosa* may be another example of this. Furthermore this could be experimentally tested by placing signalling and response under constitutive expression thus breaking the genetic linkage and allowing adaptation at these loci to occur independently. The *a priori* prediction would be that super-signalling strategies would emerge in such population. Another approach to answering the same question would be to employ the comparative method to probe the design and function of QS systems. In this case that would involve the comparison of phylogenetic data and phenotypic data to determine whether autoinduction contributes to the maintenance of honest in signalling.

8.2 Quorum Sensing in Biofilms

Many studies have attempted to determine the role of QS in biofilm formation and in some species it has been elucidated successfully (Vuong et. al. 2000, Vuong et. al. 2003). In *P. aeruginosa* there have been conflicting reports and it is likely that the influence of QS on biofilm formation is indirect (Parsek & Greenberg 2005). This effect is likely compounded by the involvement of many factors en route to biofilm formation such that there is redundancy of function, for example the formation of the extracellular matrix of biofilms. In the present study system using the QSM medium, QS plays a role in biofilm formation that goes beyond the simple requirement for QS-dependent nutrient acquisition. Whilst investigating the role of QS in biofilm formation, this study revealed a relationship between QS, population density and survival. In environments where QS is important such a relationship may be common and may be important in understanding the evolutionary dynamics of QS cooperation. If for instance the emergence of a cheating subpopulation limited the population density, this may increase the threat of local extinction. Such a mechanism may increase the strength of population level selection for cooperation. In addition, understanding the relationship between population density and survival may help to conceptualise and engineer new antimicrobial strategies where the target is a population level phenomenon such as population density. For example the data presented in this thesis suggests that a limitation to population density imposed by social competition increases the efficacy of the antibiotic tobramycin. To build upon this, it would be exciting to investigate whether such a strategy is feasible in an infection model and whether pathogen eradication is possible through the increased extinction threat imposed by limitation to population density.

This thesis investigated the effect of spatial structure on the outcome of conflict over QS public goods production. Experiments comparing the fitness of a rare mutant revealed a drastically increased fitness in colony vs. liquid culture. This was counter to the prediction that reduced spatial mobility decreases selection

for cheating in clonally reproducing microorganisms by grouping like genotypes. There are two likely explanations for this. Firstly it is possible that where diffusible public goods are necessary for growth, the restricted diffusion resulting in a concentration of public goods results in increased opportunity for cheating. Secondly it is possible that where QS regulated public goods are required for growth the induction of QS regulated public goods production may be induced earlier or to a greater degree by restricted diffusion of signal molecules. Both of these mechanisms would increase the fitness of a cheating mutant. Interestingly this result is counter to another study which finds that viscosity decreases the selection for cheating on siderophores (Kummerli 2009). Since siderophores have not been shown to be QS regulated it is likely that the increased selection for cheating with increased viscosity in this study is due to the concentration of QS molecules leading to early or amplified QS public goods production by the resident wild type. Taken together these results suggest that the effect of spatial structure on the selection for cheating may depend on the exact nature of regulation of the public good in question. Future experiments could determine the selection for public goods cheating in QS v.s non-QS regulated public goods across a range of viscosities with the hypothesis that reduced diffusion increases selection for cheating when QS is required for public goods production.

This study investigated the possibility that PQS mediated DNA release acts as a biofilm public good. Whilst the mechanism and regulation of DNA release remains obscure, it is difficult to make predictions about the fate of various eDNA deficient mutants when grown in co-culture with a wild type. The results herein suggest that the PQS molecule itself does not cause lysis of the cells and that DNA release can occur independently of cell lysis. Lysis however may be influenced via PQS mediated interactions with other cellular processes and remains a candidate mechanism for DNA release observed in other studies. Indeed it may be the major source of DNA observed in flow cell biofilms in this study. Although the results herein suggest that PQS can induce DNA release in the absence of lysis, this passive mechanism may incur a minimal cost since

these experiments were carried out in media containing no carbon source. If this PQS mediated mechanism of DNA release requires no energetic input it is not likely to result in a social dilemma in the same way as *lasIR* regulated exoprotease. Therefore unless PQS influences DNA release through cell lysis in an energy dependent manner, PQS deficient mutants are unlikely to behave as cheats. It remains possible that PQS is not the main effector of DNA release but that another product of the PQS biosynthetic pathway is more directly involved. For instance it was observed that HHQ when added to cultures induced the DNA release of PAO1 and its Δ*pqsE* mutant counterpart. Future work on this topic must determine the exact role of the PQS biosynthetic pathway in DNA release before the evolutionary dynamics of such a trait can be predicted. In addition PQS has already been shown to influence other factors important in biofilm formation such as lectins (Diggle 2003, 2006). Therefore the role of PQS in biofilm formation may be multifactorial and further conflate findings on DNA release.

8.3 Intra and Interspecific competition for Iron

Microbial populations are likely to consist of several species and competition for resources must be resolved between as well as within species. This study described how competition within species can interact with competition between species generating opposing outcomes for social competition. It is known that *P. aeruginosa* coexists with *S. aureus* in the CF lung where *P. aeruginosa* eventually displaces *S. aureus* possibly through production of PQS-dependent virulence factors (Harrison 2007, Mashburn et. al. 2005, Palmer et. al. 2005). This study investigated an additional role of PQS in iron competition. It was demonstrated that the presence of PQS and its iron binding capacity reduced the available iron causing production of siderophores to overcome iron starvation. This then increased the opportunity for siderophore defection and increases the fitness of a rare siderophore cheat. On the other hand, when formed, the PQS-Fe complex causes greater growth enhancement of *P. aeruginosa* than *S. aureus* under iron limiting conditions. PQS production therefore may be under divergent selection imposed by inter and intraspecific competition for iron. Further work could examine this more specifically with experimental evolution under purely interspecific or purely intraspecific competition for iron. PQS is a multifunctional molecule but in that fact it is not alone. AHL's have been shown to be potent inhibitors of the immune system and selection on AHL production is likely to be influenced by several factors. The extent to which interspecies and inter taxonomic interactions influence selection for multifunctional signal molecules is an exciting avenue of future research.

Quorum Sensing in *P. aeruginosa* is very well characterised with respect to its genetic and molecular mechanisms and lends itself very well to the study of previously intractable evolutionary questions. In addition the population dynamics of cooperative and communicative social traits determine in part the virulence of the population and contribute to a full understanding of disease. Therefore a two way interaction between microbiology and evolutionary biology places a ladder on the tree so that its highest hanging fruit may be picked.

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Appendix

A1 Mutagenesis of lasIR region

A mutation was introduced into the PAO1 WT (Nottingham) strain which replaced the *lasIR* region with a gentamicin resistance cassette. This was verified by PCR and detection of an increase in band size, indicating successful insertion. In addition the extraction and detection of 3-oxo-C12 HSL showed that mutants no longer produced long chain AHL's also indicating successful mutagenesis.

Figure AΩ: An insertion of a gentamicin resistance cassette into the *P. aeruginosa lasIR* region increases the size of a PCR product amplifying the *lasIR* region (gel electrophoresis, left). The pSB1142 bioreporter for long chain AHL's no longer responds to clones that have undergone mutagenesis of *lasIR* (thin layer chromatography with bioreporter overlay, right). Lane 1 contains a PAO1 control, lanes 2-5 contain candidate clones for successful mutagenesis (both figures).

A2 Summary of QSM flow cell experiments

Experiment	Medium	Innocula	48 hr static incubation	Supernatant	Results
RPFC3	1 in 10 QSM	RPFC3*	No	No	Single ells on coverslip
RPFC4	1 in 10 QSM	RPFC3	No	No	No growth
RPFC5	1 in 10 QSM	RPFC3	No	No	No growth
RPFC6	1 in 10 QSM	RPFC3	No	No	Slight growth in 1 channel
RPFC7	1 in 10 QSM	RPFC3	Yes	Yes (2b)	Growth in 3 channels. WT formed biofilms, -lasR did not.
RPFC8	1 in 10 QSM	RPFC3	Yes	Yes (2b)	Growth in all channels. WT grew in chains possibly due to nutrient stress. -lasR formed biofilms.
RPFC9	1 in 10 QSM	RPFC3	Yes	Yes (2b)	This experiment did not reach conclusion.
RPFC10	1 in 10 QSM	RPFC3	Yes	Yes (2b)	consistant difference between WT and *lasR*
RPFC11	1 in 10 QSM	RPFC3	Yes	1st day	?
RPFC12	1 in 10 QSM	RPFC3	Yes	1st day	?
RPFC13	1 in 10 QSM	RPFC3	No pump speed halved	no	Monolayer Biofilms observed
RPFC14	QSM (0.1% BSA)	LB o/n	No pump speed halved	no	No Growth observed

Table A1: Summary of flow cell experiments using the QSM environment detailing

A3 Growth in QSM under cheat load

Cheat load slows the rate and reduces the extent of growth in QSM. In the case of biofilms this means that the peak growth is later than 24 h (Figure A1b)

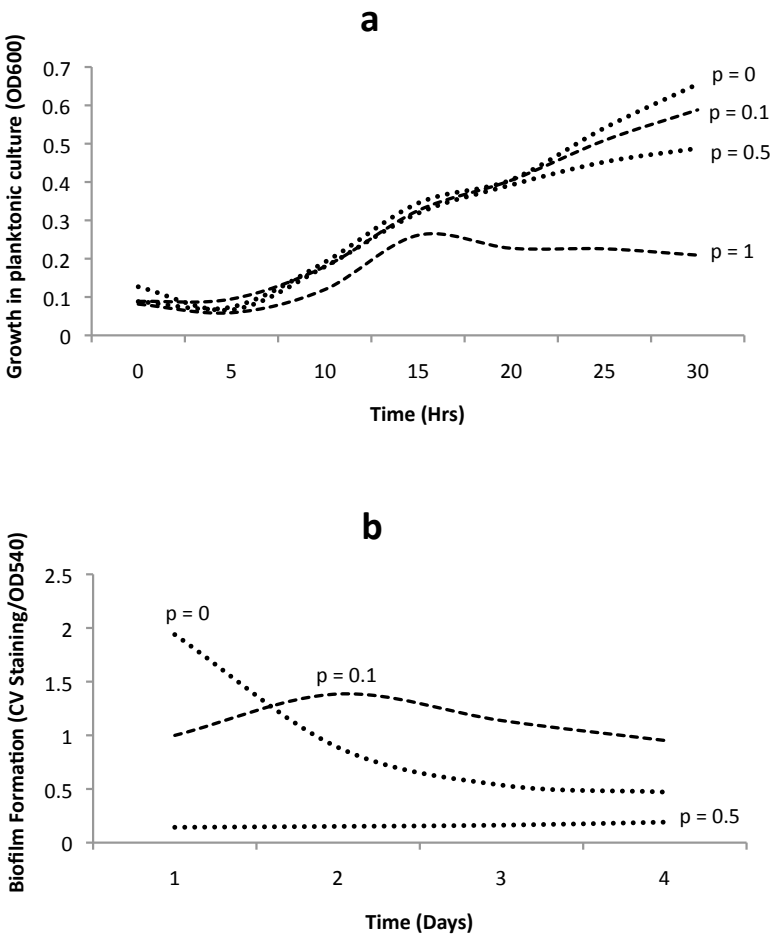

Figure A1: Growth of PAO1 cultures in planktonic (a) and biofilm (b) growth modes in the presence of varying proportions of cheating mutant (p) in the initial population.

A4 CV Staining of Peg-Lid Biofilms

There is no significant difference in Crystal Violet staining of the pegs before and after centrifugation (Figure AΩa). This indicates that little of the biofilm is removed by this procedure. In addition very little biofilm had formed where the initial proportion of cheat was 0.5 or greater (Figure AΩb).

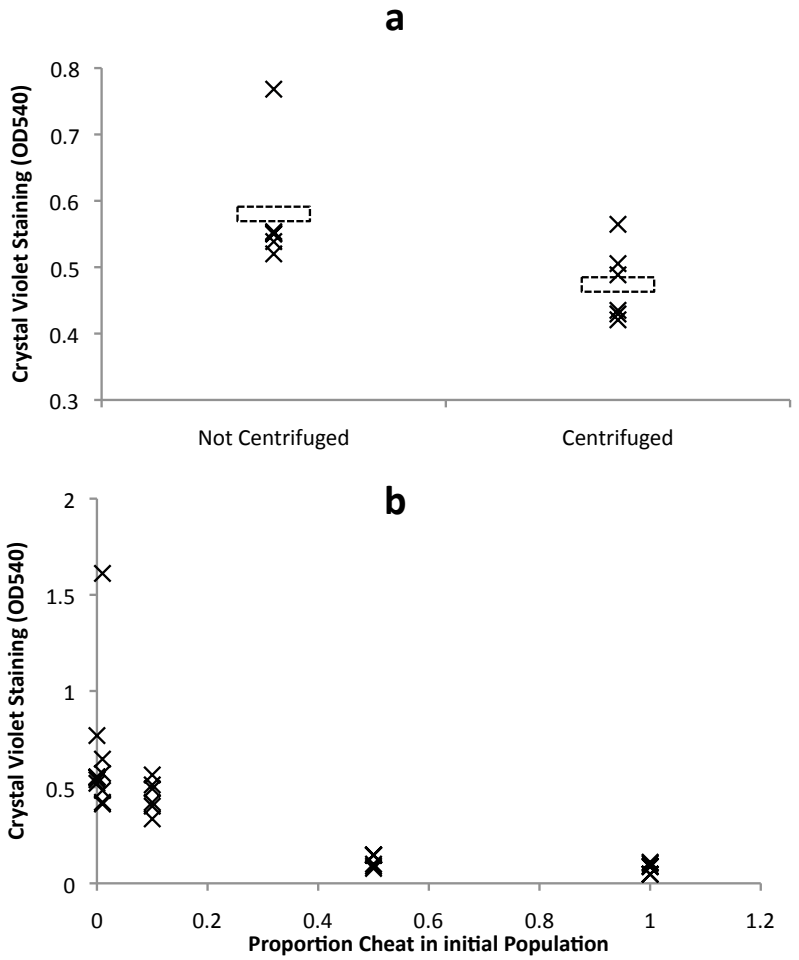

Figure A2: Crystal Violet staining of pegged lid biofilms at 24hr before and after centrifugation (a) and in the presence of varying proportion of cheating

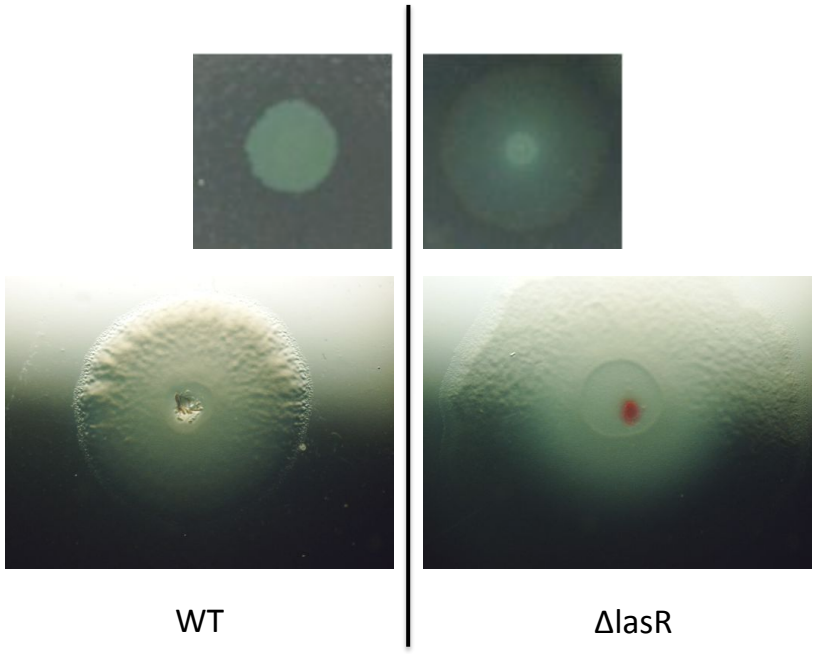

Figure A3: Photographs of *P. aeruginosa* growing on QSM agar. The WT forms thick substantial colonies when spotted onto the plate, the *lasR* mutant forms diffuse, thin and insubstantial colonies.