Fleming Prize Lecture 2010

Correspondence
Stephen P. Diggle
steve.diggle@nottingham.ac.uk

Microbial communication and virulence: lessons from evolutionary theory

Stephen P. Diggle

School of Molecular Medical Sciences, Centre for Biomolecular Sciences, University Park, University of Nottingham, NG7 2RD, UK

At the heart of tackling the huge challenge posed by infectious micro-organisms is the overwhelming need to understand their nature. A major question is, why do some species of bacteria rapidly kill their host whilst others are relatively benign? For example, *Yersinia pestis*, the causative organism of plague, is a highly virulent human pathogen whilst the closely related *Yersinia pseudotuberculosis* causes a much less severe disease. Using molecular techniques such as mutating certain genes, microbiologists have made significant advances over recent decades in elucidating the mechanisms that govern the production of virulence factors involved in causing disease in many bacterial species. There are also evolutionary and ecological factors which will influence virulence. Many of these ideas have arisen through the development of evolutionary theory and yet there is strikingly little empirical evidence testing them. By applying both mechanistic and adaptive approaches to microbial behaviours we can begin to address questions such as, what factors influence cooperation and the evolution of virulence in microbes and can we exploit these factors to develop new antimicrobial strategies?

Introduction

It is an exciting time to be a microbiologist. The last 40 years have seen amazing advances in our understanding of the molecular mechanisms that govern social behaviours such as antibiotic production, virulence, motility and biofilm formation in a diverse range of micro-organisms. Such mechanistic insights allow us to genetically manipulate bacteria to modify such behaviours. These approaches are leading to the development of new antimicrobial strategies which is of huge importance in an era where multi-antibiotic resistance is increasingly problematic.

Whilst a huge amount of work and progress is being made in our understanding of genetic systems and mechanisms, relatively little attention is paid to the evolutionary aspects of social behaviour. Studying behaviours from an evolutionary perspective can help to explain why they exist and are maintained in nature. These studies have traditionally been undertaken by the development of evolutionary theory and comparatively little empirical data exists which tests them.

Traditionally, microbiologists and evolutionary biologists have studied social behaviours from differing perspectives. Microbiologists are primarily interested in the genetic mechanisms controlling the behaviour (“how” questions) whereas the interest of the evolutionary biologist can be found in studying the fitness consequences of a particular behaviour which helps explain why these systems are found in nature (“why” questions). However, whilst these may be different approaches, they should be viewed as complementary and not

contradictory. By combining both mechanistic and adaptive approaches we can begin to address questions such as, what factors influence cooperation and the evolution of virulence in microbes and how can we exploit these to develop new antimicrobial strategies?

This review summarizes the basic principles of social evolution theory and how it can be applied to microbes. It discusses previous experimental work that has been performed in this field and how this work can be applied to infectious disease.

Classifying social behaviours in micro-organisms

There is a huge body of work explaining social behaviours in micro-organisms from a molecular perspective and comparatively little from an evolutionary standpoint. Social behaviours in microbes have, until relatively recently, been ignored by evolutionary biologists, yet there has been much work done in this area both theoretically and empirically in higher organisms. It is therefore useful to define what a social behaviour is. Social behaviours are those which have fitness consequences for the actor performing the behaviour and for the recipient, and can be broadly categorized into four types depending on the fitness consequences for the actor performing the behaviour and for the recipient (Fig. 1). A behaviour that increases the direct fitness of the actor is mutually beneficial if the recipient also benefits, and selfish if the

Fig. 1. Classification of social behaviours.

recipient suffers a loss. A behaviour that reduces the fitness of the actor is altruistic if the recipient benefits, and spiteful if the recipient suffers a loss (West *et al.*, 2006; West & Gardner, 2010). It is important to understand the nature of the interaction between actor and recipient as then we can make very different predictions as to how such a behaviour evolves and is maintained in populations.

For example, altruism involves behaviours that increase the fitness of other individuals who share the genes for altruism. Therefore, by directly helping a relative to reproduce and ignoring non-relatives, the genes for altruistic behaviour are maintained. However, there is a more sinister side to altruism. In certain situations spiteful behaviour may evolve which directly harms recipients, which intuitively does not seem like an altruistic act. However, if you increase the fitness of your close relatives by harming competitors, then this type of behaviour may be favoured (West & Gardner, 2010). The main point here is that both types of behaviour indirectly increase the fitness of close relatives but each is likely to involve different evolutionary pressures and mechanistic processes. One of the future challenges for microbiologists is to understand the multitude of behaviours that have been described in a wide range of species from a more adaptive angle, which will increase our understanding of the nature of these behaviours. For example, it has already been shown, both theoretically and experimentally that bacteriocin production fulfils the criteria of a spiteful behaviour (Inglis *et al.*, 2009; Gardner *et al.*, 2004), whereas quorum sensing could be considered more altruistic in nature (West *et al.*, 2007b; Diggle *et al.*, 2007c). Both are likely to result in increased close relative fitness at a cost to individuals but via very different mechanisms.

The problem of cooperation

Explaining cooperative behaviours remains a significant challenge for evolutionary biologists as they reduce the fitness of the actor. The question then becomes, why help others at a cost to yourself as this appears to conflict with Charles Darwin's idea of the survival of the fittest? Natural

selection favours those individuals with the greatest reproductive success relative to the rest of the population. It is therefore difficult to see why cooperation, which reduces the fitness of the actor and increases the fitness of other individuals, can be favoured. Despite this, there are numerous examples of cooperative behaviours at all levels of biological organization from microbes to man.

Firstly, we need to define what cooperation is. A cooperative 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. Cooperation includes all altruistic behaviours and many (but not all) mutually beneficial behaviours. An example of mutual benefit that is not cooperative is when an elephant produces dung. This benefits a dung beetle but the benefit is inconsequential and has not driven the evolution of dung and is therefore not true cooperation (West *et al.*, 2007a, b).

The problem of cooperative behaviour is that due to the fitness costs on the actor, there is the potential of exploitation by cheats or freeloaders who do not cooperate (or cooperate less) and who therefore gain a fitness advantage in the population. A famous example of this can be found in the 'tragedy of the commons' which describes human economics and morality. The classic example given is that of a number of shepherds sharing a pasture to graze their sheep. To an individual shepherd, it is in their interests to add more sheep to the land because they gain more benefit but at a fraction of the cost. However, by individuals pursuing this line of action, the field is at risk of overgrazing. The tragedy is that if all shepherds cooperated, everyone would ultimately benefit (Hardin, 1968).

In microbes, cooperative behaviours might seem simpler than those that have been described in higher organisms but they share a similar fundamental property: a shared investment in a group resource. Many microbial social behaviours come in the form of public goods which are released into the surrounding environment and are costly for individuals to make but provide a benefit for all other individuals in the population (Fig. 2) (West *et al.*, 2007b). Microbes make a wide variety of public goods including siderophores for iron scavenging, β -lactamases, exopolysaccharides, toxins, proteases and signal molecules (West *et al.*, 2007b).

How can cooperation be maintained?

Given the problems of cooperation described above, how can cooperative behaviours be maintained in nature? If there is a direct fitness benefit to the individual performing the behaviour, cooperation is mutually beneficial. An example of direct fitness benefits can be found in group size. For example, in many cooperative breeding species, larger group size may provide a benefit to all the members of the group through factors such as greater survival or higher foraging success. In this case, individuals can be

Fig. 2. Exploiting public goods production. (a) Cheating cells do not pay the cost of public goods production and (b) can exploit the benefits of public goods produced by co-operating cells and gain a fitness benefit within the population.

selected to help rear offspring that are not their own, in order to increase group size (West *et al.*, 2007a). There are a number of ways that cooperation involving direct fitness benefits can be maintained, including punishment, sanctions, reciprocity and policing (West *et al.*, 2007a).

However, direct fitness benefits do not adequately explain the existence of altruistic behaviours. Darwin himself understood that explaining altruistic behaviours was a major problem for his theory and suggested that certain characteristics could be favoured because they improve the reproductive success of relatives. This idea was formalised in 1964 by the evolutionary biologist William Hamilton. By developing Hamilton's Rule, which describes mathematically whether a gene for altruistic behaviour will spread in a population, Hamilton recognized that genes can be favoured by natural selection if they increase the reproductive success of its bearer (direct fitness) and also increase the reproductive success of other individuals that carry the same gene (indirect fitness). The most common reason for two individuals to share genes in common is for them to be genealogical relatives (kin) and so this process is often termed 'kin selection' although Hamilton himself used the term 'inclusive fitness'. Simply put, by helping a close relative reproduce, an individual transmits genes to the next generation, albeit indirectly (Hamilton, 1964a, b).

There are two main ways that kin selection can work. One is termed kin discrimination and this is where an individual can distinguish relatives from non-relatives and preferentially direct aid towards them. An example of this can be found in the long tailed tit (*Aegithalos caudatus*) where kin and non-kin breed within each social unit and helpers are failed breeders. It was shown that potential helpers do not become helpers in the absence of close kin and when given a choice between helping broods belonging to kin and non-kin within the same social unit, virtually all helped at the nest of kin (Hatchwell *et al.*, 2001). Kin discrimination has also been demonstrated in malaria parasites. *Plasmodium chabaudi* parasites use kin discrimination to evaluate the genetic diversity of their infections and they adjust their behaviour in response to environmental cues (Reece *et al.*, 2008).

A second way that kin selection can work is via limited dispersal. Here, relatives are kept close together, increasing

the probability of interactions occurring amongst relatives, which favours indiscriminate altruism towards neighbours. This does not require complex recognition systems and therefore is likely to be important in a broad range of organisms, including microbes. Limited dispersal favours cooperation in the opportunistic pathogen *Pseudomonas aeruginosa*, where it has been shown that by increasing viscosity in the growth medium this (i) limited bacterial dispersal and the diffusion of siderophore public good molecules and (ii) increased the fitness of individuals that produced siderophores relative to mutants that did not. Therefore, in this example, viscosity favours siderophore-producing individuals because the benefits of siderophore production are more likely to accrue to relatives (i.e. greater indirect benefits) (Kümmerli *et al.*, 2009a, b).

A contentious issue for some microbiologists is what defines relatedness within a population of micro-organisms? For example, if an infection is initiated by a founding clone and this accumulates mutations over time, then can you still consider these mutants to be related to the initial infecting strain? Relatedness (r) is a measure of genetic similarity and in eukaryotes this can be relatively easily determined using molecular markers (Queller & Goodnight, 1989). However, r is more difficult to determine in micro-organisms both in single species and mixed species populations. Consider a bacterial species that is unable to produce a public good due to a mutation in a gene important in its biosynthesis. Even if this mutation involved a single nucleotide point mutation, then from a social evolution perspective, this strain is unrelated to strains containing an intact locus even if the rest of the genome is identical. This mutation would produce a cheat that can exploit cooperating strains which still produced the public good, resulting in a breakdown of the social behaviour in question. Therefore r must be measured at the locus (or loci) that controls the social behaviour of interest. In the example given above, those cooperative individuals carrying an intact locus are related ($r=1$) whilst those carrying a mutation are unrelated to cooperators ($r=0$). Public good loci can also be carried on mobile genetic elements such as plasmids (Nogueira *et al.*, 2009). In this case, genetic identity ($r=1$) at particular loci encoding social behaviours can potentially be shared between species.

Advantages of using microbes to study cooperation

Most of the experimental work performed to date on social evolution theory has involved work on higher organisms. Microbes constitute the majority of the diversity of life seen on our planet and so the previous focus of research performed on other animals may be a misleading representation of the factors that govern social evolution. The variety of social behaviours described in microbes provides us with unique opportunities to test some of the fundamental principles of social evolution and how they apply to other organisms.

There are some obvious advantages to using microbes as experimental systems. (i) Evolution can be studied in 'real-time'; (ii) clonality enhances experimental replication; (iii) environmental conditions can be easily manipulated; (iv) they can be easily stored and revived creating a rich 'fossil record' over time; (v) fast reproduction allows experiments to be run over many generations. In addition to these advantages, the recent rapid progress made in genome sequencing provides the researcher with access to a huge amount of genetic information in hundreds of bacterial species. For the first time, we are able to genetically manipulate genes known to be involved in social behaviours.

The use of micro-organisms as experimental evolution systems really began in earnest in the 1990s when *Escherichia coli* was adapted in a long-term (10 000 generation) selection experiment to a novel laboratory environment. It was demonstrated that evolution was rapid for the first 2000 generations but it was almost static for the following 5000 generations (Lenski & Travisano, 1994). Prokaryote cheating was first described in *Myxococcus xanthus*, where it was shown that cheats defective in fruiting body development were overrepresented in overall spore formation (Velicer *et al.*, 2000). Since then, a number of key empirical studies have unravelled some of the complexities and molecular mechanisms of social behaviour in the myxobacteria, which provides an excellent example of how theory and empirical work of a molecular nature can combine to further understand the behaviour of an organism (Velicer & Vos, 2009).

There is also a significant body of work using *Pseudomonas* spp. to test aspects of social evolution theory. Rainey and Rainey showed that populations of *Pseudomonas fluorescens* cooperate by producing an adhesive polymer which allows the population to colonize the air/liquid interface in liquid cultures. Defective genotypes (cheats) sabotaged this phenotype resulting in population collapse (Rainey & Rainey, 2003). *P. aeruginosa* has also been widely used as an experimental organism for evolutionary studies. Previous work has revealed that production of the iron scavenging siderophore pyoverdine is costly to produce and is subject to exploitation by non-producing cheats (Griffin *et al.*, 2004). Furthermore, it was demonstrated that the scale of competition is important in the evolution of cooperation in microbes. When competition is local (within

populations) cooperation is more vulnerable to cheating than when competition is global (between populations), demonstrating that cooperative behaviours are more likely to be maintained when populations are competing with each other (Griffin *et al.*, 2004). Put simply, an individual is more likely to cooperate with its local group if it is competing against a rival group for resources, whereas in the absence of competitors, it is competing for resources within its group and it may be in its own self interests to cooperate less. Effectively, it becomes a cheat. This may have particular relevance during infections which may involve multiple species or single species infections that diversify over time, thus reducing the relatedness between cells.

It is clear then that the production of public goods can result in social dilemmas where cheats can exploit cooperating cells. There are also ecological factors that need to be taken into account. For example, consider two different public good molecules. One is relatively unstable and is quickly broken down and the second is more durable and can persist in the environment. A theoretical approach has revealed that a group of cooperators can fare worse than a group of cheats if they inherit fewer public goods. In other words, cheats can prosper in the absence of cooperators if they find themselves in an environment containing a durable public good due to a previous cooperative venture (Brown & Taddei, 2007). This neatly demonstrates that for a full understanding of a microbial behaviour, we need to consider evolutionary, as well as ecological and molecular aspects. This provides us with huge potential for empirical studies in the future.

What is quorum sensing (QS)?

Many social behaviours performed by bacteria such as biofilm formation, swarming motility and virulence are regulated by cell-to-cell signals in a cell density-dependent manner known as QS (Williams, 2007; Diggle *et al.*, 2007a; Ng & Bassler, 2009). QS describes the phenomenon whereby the accumulation of 'signalling' molecules in the surrounding environment enables a single cell to sense the number of bacteria (cell density), and therefore the population as a whole can make a coordinated response. The signal produced regulates its own production (auto-induction) and so this leads to a positive-feedback response and greatly increased signal production. At critical cell densities, the binding of a regulator protein to the signal leads to the switch on of genes controlled by QS and a coordinated population response.

One of the earliest classic descriptions of a cell density-dependent phenotype was by Nealson *et al.* who showed that the addition of spent culture supernatants of the marine luminescent bacterium *Vibrio fischeri* to low-density cultures of the same organism induced the production of bioluminescence due to the presence of an autoinducing substance in the surrounding medium (Nealson *et al.*, 1970). In its natural environment during symbiosis in a light organ

found in certain species of squid, the autoinducer molecules accumulate to a critical concentration (usually at high bacterial cell densities) which, in turn, induces expression of the genes responsible for bioluminescence. The autoinducing molecule was later identified as an *N*-acylhomoserine lactone [AHL, specifically *N*-(3-oxohexanoyl) homoserine lactone (3O-C6-HSL)].

A further crucial breakthrough for the field came when 3O-C6-HSL was identified in organisms other than *V. fischeri* including *P. aeruginosa* and *Erwinia carotovora*, demonstrating that this type of signalling was not limited to a few species of marine symbiotic bacteria (Bainton *et al.*, 1992). In 1994, the term QS was first used (Fuqua *et al.*, 1994) and the QS research field is now vast, covering a wide range of bacterial species and a number of diverse chemical signalling molecules and systems (Williams, 2007). A simple PubMed search performed at the time of writing this article revealed that since 1994, over 2600 articles containing the term 'quorum sensing' have been published.

What defines a QS signal?

It is useful to point out that there is a tendency in the literature to describe any QS molecule that alters the behaviour of other individuals as a 'signal'. Broad use of this term can obscure the true nature of the interaction between cells either within the same species or between species, sometimes referred to as bacterial 'cross-talk' (Diggle *et al.*, 2007b; Keller & Surette, 2006). This is a lesson that has been well learnt in fields studying cooperation and signalling in animals which has led to much confusion where the same term has been used to mean different things (Maynard-Smith & Harper, 2003).

If we are to be accurate about the nature and the true role of a QS 'signal' we need to differentiate a true signal from a cue or a coercive molecule. In general, a signal is defined as 'any act or structure that alters the behaviour of other organisms, which evolved owing to that effect, and which is effective because the receiver's response has also evolved' (Maynard-Smith & Harper, 2003). In contrast, a cue involves the production of a substance by individual A which has 'not evolved' because of its effect on individual B (Table 1).

For example, a human exhales carbon dioxide (CO₂) which attracts the attention of mosquitoes. The mosquito is responding to the CO₂ but we cannot call CO₂ a signal in this case, because the production of CO₂ has not evolved because of this effect (Maynard-Smith & Harper, 2003). More accurately, we can say that the mosquito is responding to a cue. Perhaps more controversially but not necessarily incorrectly, we can introduce this idea to QS in bacteria. In a bacterium such as *Erwinia carotovora* which regulates exoenzyme production via 3O-C6-HSL production (Barnard *et al.*, 2007), it is likely that 3O-C6-HSL is a true signal because it has evolved to regulate

Table 1. Types of communication are distinguished depending upon their fitness consequences to the sender and the responder

Consequences are either beneficial (+) or costly (−).

	Evolved owing to the effect on sender	Benefits the receiver to respond
Signal	+	+
Cue	−	+
Coercion	+	−

exoenzymes and importantly the receiver's response has also evolved. However, when you look closely at other microbial interactions, particularly interspecies interactions, the picture becomes less clear. For example, *Burkholderia cenocepacia* can utilize AHLs produced by *P. aeruginosa* to upregulate exoenzyme production, yet *P. aeruginosa* does not appear to be able to use AHLs produced by *B. cenocepacia* (Eberl & Tümmeler, 2004). There appears to be little benefit to *P. aeruginosa* in this interaction so it seems unlikely that molecule production by *P. aeruginosa* and response by *B. cenocepacia* have co-evolved to produce a true signalling system. In this example, it seems likely that *B. cenocepacia* uses 3O-C12-HSL produced by *P. aeruginosa* as a cue to guide future actions.

Similar problems are found in the literature on auto-inducer-2 (AI-2) signalling. AI-2 is produced by many genera of both Gram-negative and Gram-positive bacteria and the production of AI-2 by one species can conceivably regulate AI-2-dependent gene expression in another. However, we cannot broadly use the term 'signal' for all these interactions. In distantly related bacteria, the sensing of AI-2 is unlikely to have evolved because it is produced by another species (Diggle *et al.*, 2007b).

Another potential interaction exists. If the production of a substance by cell A forces a costly response from cell B, we can differentiate this from signalling and term it as coercion or chemical manipulation. In this case, the molecule producer benefits from this interaction due to the effects on the receiver (Diggle *et al.*, 2007b; Maynard-Smith & Harper, 2003; Keller & Surette, 2006). Let us return to the *B. cenocepacia* and *P. aeruginosa* example given above. The effect of 3O-C12-HSL on *B. cenocepacia* exoenzyme production may in fact benefit *P. aeruginosa*. Consider an infection involving both species. Production of a metabolically cheap molecule that increases costly exoenzyme production of another species may provide fitness benefits to *P. aeruginosa* in a co-infection as in this case, *B. cenocepacia* produces all the tissue-degrading enzymes necessary to provide nutrients that *P. aeruginosa* can also utilize. Therefore, this interaction could be considered a coercive action by *P. aeruginosa*.

Is all of this important? Yes, because understanding the nature of interactions is crucial if we are to further

understand how single and mixed species infections coordinate virulence and cause disease. This has been discussed in greater detail elsewhere (Maynard-Smith & Harper, 2003; Diggle *et al.*, 2007b; Keller & Surette, 2006).

QS and social evolution

The QS literature generally assumes that QS involves cell-density-dependent cooperation between cells and is performed for the 'good of the population'. The concept of diffusion sensing (DS) was the first major challenge to this idea. Here, it was suggested that autoinducers function chiefly to enable individual cells to sense how rapidly secreted molecules diffuse away (Redfield, 2002). Therefore, DS could allow individuals to minimize the loss of costly public goods by extracellular diffusion by first producing a metabolically cheap molecule such as an AHL. More recently, the term efficiency sensing (ES) has been introduced (Hense *et al.*, 2007). ES assumes that low cost autoinducers are released to test the efficiency of producing costlier exoenzymes, a concept that is similar to DS. However, ES includes the potential for cooperation because microcolonies (clusters) may help protect against interference by other species and cheaters. In this way, ES unifies both QS and DS, as it enables cells to sense cell density, diffusion limitation and cell distribution (clustering). While it is important to be thinking about QS in a broader evolutionary and ecological context, it is also important for the field that we do not introduce too many new terms as many of these have similar meanings and have the potential to spread confusion, which, given the huge body of previous and ongoing work on QS, is undesirable. As it stands, there is little wrong with the term 'quorum sensing'. However, there needs to be a more general understanding that QS is not just about cell density, but that many evolutionary and ecological factors also affect how a population of cells behave. This has been discussed in greater detail elsewhere (Platt & Fuqua, 2010).

Because QS involves the production of costly signals and public goods, then it is potentially exploitable by asocial cheaters; this idea has been experimentally tested using *P. aeruginosa* (Diggle *et al.*, 2007c; Sandoz *et al.*, 2007). 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 that are capable of causing extensive tissue damage and bloodstream invasion which consequently promotes systemic dissemination. Many of these exoproducts are regulated by QS (Williams & Cámara, 2009; Popat *et al.*, 2008). *P. aeruginosa* possesses two AHL-dependent QS systems. These are termed the *las* and *rhl* systems, comprising the LuxRI homologues, LasRI and RhlRI, respectively. LasI directs the synthesis of primarily *N*-(3-oxododecanoyl)-L-homoserine lactone (3O-C12-HSL) and together with the transcriptional regulator LasR regulates the production of virulence factors including elastase, the LasA protease, alkaline protease and exotoxin A (Gambello

& Iglewski, 1991; Gambello *et al.*, 1993). RhlI directs the synthesis of *N*-butanoyl-L-homoserine lactone (C4-HSL) which activates RhlR and in turn RhlR/C4-HSL induces the production of rhamnolipid, elastase, LasA protease, hydrogen cyanide, pyocyanin, siderophores and the cytotoxic lectins LecA and LecB (Gambello & Iglewski, 1991; Gambello *et al.*, 1993; Brint & Ohman, 1995; Winzer *et al.*, 2000; Winson *et al.*, 1995; Pearson *et al.*, 1994, 1995, 1997). The *las* and *rhl* systems are organized in a hierarchical manner such that the *las* system exerts transcriptional control over both *rhlR* and *rhlI* (Williams & Cámara, 2009; Popat *et al.*, 2008; Pesci *et al.*, 1997; Winson *et al.*, 1995). In addition to AHL-dependent QS, *P. aeruginosa* also produces over 50 2-alkyl-4(1*H*)-quinolones (AQs), some of which were originally identified from their antibacterial properties, although the biological function of many of these is not known (Dubern & Diggle, 2008). One of these compounds, 2-heptyl-3-hydroxy-4(1*H*)-quinolone was discovered to function as a diffusible signal molecule and termed the *Pseudomonas* quinolone signal (Dubern & Diggle, 2008; Pesci *et al.*, 1999).

Empirical studies have shown that QS in *P. aeruginosa* is both costly and exploitable by cheaters. Using a synthetic growth medium where QS is important for bacterial growth due to the production of costly exoproteases (public goods), it has been demonstrated that wild-type (PAO1) populations grow well whereas populations of QS cheat (*lasR* mutants) do not. However, crucially, in mixed culture, cheaters have a fitness advantage because they exploit the exoprotease production of wild-type cells (Diggle *et al.*, 2007c; Sandoz *et al.*, 2007). These cheaters showed a trend of negative frequency-dependent fitness (Diggle *et al.*, 2007c), which means that they show highest fitness when rare in a population and a decreasing fitness as they increase in the population (Fig. 3a). Simply put, when there are less cheaters in the population, there are more cooperators to exploit, resulting in an increased cheat fitness. Furthermore, *lasR* mutants evolve *de novo* in such a growth environment and can then spread in the population (Sandoz *et al.*, 2007).

Given the problem of cheating, how then can QS be maintained? The most likely explanation is kin selection (Hamilton, 1964a, b), because if neighbouring cells tend to be close relatives they will have a shared interest in communicating honestly and cooperating (Brown & Johnstone, 2001; Diggle *et al.*, 2007b). Kin selection is likely to be highly important in microbial social behaviours such as QS because of clonal reproduction and relatively local interactions (West *et al.*, 2006). An empirical test of the kin selection hypothesis revealed that in populations maintained under conditions of relatively high relatedness, QS was favoured. In contrast, in populations maintained under low related conditions which generally contained mixtures of QS cooperators and cheaters, QS was not favoured due to constant exploitation by cheaters (Diggle *et al.*, 2007c) (Fig. 3b). The studies show that QS is beneficial for growth in certain environments and in such cases it is obvious that QS cooperating populations will do

Fig. 3. QS and social cheating *in vitro*. (a) Invasion and relative fitness of QS *lasR*⁻ cheats are frequency-dependent in an environment requiring QS for growth. Cheats have a higher fitness when they are less common. (b) Kin selection and QS. The proportion of QS individuals (wild-type) is plotted against time (rounds of growth). QS is favoured by higher relatedness (□, low relatedness; ◆, high relatedness). Data are shown as mean ± SEM. Adapted by permission from Macmillan Publishers Ltd: *Nature*, Diggle *et al.* (2007c), copyright 2007.

better than populations of cheats. However, given that QS is important in such situations, why do cooperating cells therefore not completely outcompete mutants in mixed populations? It demonstrates that QS is social in nature, that it is subject to exploitation by cheats and that relatedness matters.

Social evolution and infection

So far, the majority of published work studying social evolution in microbes has focused primarily on planktonic cultures in the laboratory. There is less work describing how social cheating impacts populations of bacteria in more 'natural' environments such as during infections or in biofilms. Intriguingly, QS negative mutants are commonly isolated from *P. aeruginosa* lung infections (Smith *et al.*, 2006) and QS mutants, when tested in animal models of infection, are frequently less virulent than their parental strain (Pearson *et al.*, 2000; Rumbaugh *et al.*, 1999; Wu *et al.*, 2001). Empirical evidence demonstrating that these QS mutants are able to successfully 'spread' in natural populations is strikingly sparse and data showing this would imply that these mutants are social cheaters and are able to exploit the cooperation of others during infections. There is also a dramatic lack of empirical data describing the effects on virulence by multiple strains. Two theories have been suggested: (1) a greater strain diversity (low related population) will favour greater competition for resources which will lead to higher growth rates and promote those strains with the highest virulence (Frank, 1996); (2) a high relatedness will result in higher levels of cooperation and therefore virulence (West & Buckling, 2003). This 'cooperative virulence theory' also predicts that cheats can exploit cooperators. Depending upon conditions and the strains involved in the infection, both situations are possible. However, recent studies looking at the role of QS during infections support the second hypothesis and show that QS cheaters can significantly

invade a QS cooperating population, resulting in a decrease in overall virulence. In a mouse model of infection it was demonstrated that QS cheats invade a burn wound within days (Rumbaugh *et al.*, 2009). Fig. 4 demonstrates that in both mouse burn (a) and chronic wound (b) models, a QS cheat (*lasR* mutant) can invade *P. aeruginosa* populations both on the skin and systemically, and cheat fitness is frequency-dependent. It is interesting that in monoculture infections, the *lasR* mutant is significantly less virulent and is more readily cleared from the mouse. However, during a mixed infection with a cooperating strain, the cheat has a fitness that exceeds that of the cooperating strain. This suggests that during *in vivo* infections, QS cheats can exploit public goods and resources produced by a cooperating population in a manner similar to that seen in previous studies using planktonic cultures. Perhaps even more exciting is that a 50:50 inoculum of cooperator:cheat results in an attenuated infection which is as comparable in virulence to that of a *lasR* mutant alone (Rumbaugh *et al.*, 2009) (Fig. 4c). Another recent study has demonstrated that during infections in human intubated patients, a mix of QS cooperating cells and QS cheats resulted in a milder infection (Köhler *et al.*, 2009). The overall implication is that the spread of cheats within a population can significantly reduce virulence due to a breakdown in cooperation and hence in addition to the infection being less virulent, these mixed infections may be easier to treat with conventional antimicrobial therapies.

Future perspectives

It is now increasingly recognized that microbes engage in a wide range of social behaviours. Our understanding of the molecular mechanisms of such actions are now being complemented by theoretical and empirical studies examining the evolutionary and ecological factors governing these. In an era of increasing multi-antibiotic resistance and with a dwindling supply of antibiotics useful to

Fig. 4. QS and social cheating *in vivo*. Invasion and relative fitness of QS *lasR*⁻ cheats are frequency-dependent in (a) acute burn and (b) chronic wound mouse models. In all cases, mutants have a higher fitness when they are less common. (c) The survival curves for mice (burn model) infected with either the PA14 wild-type, a QS mutant (*lasR*⁻), or a 50:50 mixture of the two, plotted against time (days after burn/infection). The survival rate of mice infected with the 50:50 mixture of wild-type and mutant is significantly greater than that of the wild-type. Reprinted from *Curr Biol* **19**, Rumbaugh *et al.*, Quorum sensing and the social evolution of bacterial virulence, pp. 341–345, Copyright 2009, with permission from the Elsevier.

clinicians, can an understanding of social evolution in microbes help the design and development of novel and effective antimicrobial strategies? Studies demonstrating that mixed infections containing both QS cooperators and cheats in both mice (Rumbaugh *et al.*, 2009) and humans (Köhler *et al.*, 2009) are less virulent than infections of pure cooperators, suggest that asocial cheats could potentially be used as part of a therapeutic regime. There are a number of potential benefits in using cheats to treat infections: (1) they reduce the virulence of the infecting bacterium; (2) they spread less readily in a host; (3) they can be engineered to be antibiotic sensitive and so can be effectively cleared from a host; (4) reduction of virulence ‘buys time’ to allow the host to mount an effective immune response; and (5) targeting a group behaviour leads to less chance of resistance developing. In addition to this, cheats could be engineered to contain ‘Trojan Horse’ genes which are beneficial alleles that could be introduced into an antibiotic resistant infection due to the cheat’s ability to exploit

cooperators (Brown *et al.*, 2009). There is a precedent for the idea of driving alleles through a population in an attempt to replace a harmful population with a benign one. Work in this area has been pioneered in populations of insect pathogens and vectors (Alphey *et al.*, 2002; Burt, 2003). For example, work is currently under way to develop selfish genetic elements that will infect populations of mosquito malaria vectors. Millions of pounds worth of funding has been poured into this enterprise and yet there has been no suggestion of attempting the same approach in populations of microbes, probably because of ethical and regulatory considerations. This is despite the fact that driving genes into microbe populations encounters nothing like the scale of obstacles facing projects on insect pathogen control (Sinkins & Gould, 2006).

The idea of ‘quorum quenching’ (QQ) has often been discussed to have the potential to downregulate virulence in pathogenic organisms such as *P. aeruginosa* and involves

the degradation of QS signals (Dong *et al.*, 2007). Much work has been performed in this area and a number of laboratories are actively working on QQ compounds as antimicrobial strategies (Bjarnsholt & Givskov, 2007). However, social evolution provides us with a cautionary tale. A recent study in human intubated patients has shown that quenching QS in *P. aeruginosa* infections led to a reduction in the fitness of *lasR* mutants which spread in the absence but not in the presence of QQ treatment. Effectively, this led to the selection of more virulent strains within the infection and diminished natural selection towards reduced virulence (Köhler *et al.*, 2010).

Whilst there is clearly huge potential in developing strategies to tackle microbial gene regulatory systems such as QS, we should also be aware of the evolutionary implications of doing so. After all, how many scientists had the foresight to predict the rise and impact of multi-antibiotic resistance 50 years ago?

Acknowledgements

Firstly, I would like to thank the Society for General Microbiology for the award of the 2010 Fleming Prize Lecture on which this paper is based. I gratefully acknowledge the many excellent collaborators and colleagues I have worked with over the years, specifically Paul Williams, Miguel Camara, Klaus Winzer, Shanika Crusz, Steve Atkinson, Stephan Heeb, Stuart West, Ashleigh Griffin, Andy Gardner, Kendra Rumbaugh, Sam Brown, Roman Popat, Sophie Darch and Eric Pollitt. This work has been supported by funding from the Royal Society.

REFERENCES

- Alphey, L., Beard, C. B., Billingsley, P., Coetzee, M., Crisanti, A., Curtis, C., Eggleston, P., Godfray, C., Hemingway, J. & other authors (2002). Malaria control with genetically manipulated insect vectors. *Science* **298**, 119–121.
- Bainton, N. J., Bycroft, B. W., Chhabra, S. R., Stead, P., Gledhill, L., Hill, P. J., Rees, C. E., Winson, M. K., Salmond, G. P. & other authors (1992). A general role for the *lux* autoinducer in bacterial cell signalling: control of antibiotic biosynthesis in *Erwinia*. *Gene* **116**, 87–91.
- Barnard, A. M., Bowden, S. D., Burr, T., Coulthurst, S. J., Monson, R. E. & Salmond, G. P. (2007). Quorum sensing, virulence and secondary metabolite production in plant soft-rotting bacteria. *Philos Trans R Soc Lond B Biol Sci* **362**, 1165–1183.
- Bjarnsholt, T. & Givskov, M. (2007). Quorum-sensing blockade as a strategy for enhancing host defences against bacterial pathogens. *Philos Trans R Soc Lond B Biol Sci* **362**, 1213–1222.
- Brint, J. M. & Ohman, D. E. (1995). Synthesis of multiple exoproducts in *Pseudomonas aeruginosa* is under the control of RhlR–RhlI, another set of regulators in strain PAO1 with homology to the autoinducer-responsive LuxR–LuxI family. *J Bacteriol* **177**, 7155–7163.
- Brown, S. P. & Johnstone, R. A. (2001). Cooperation in the dark: signalling and collective action in quorum-sensing bacteria. *Proc Biol Sci* **268**, 961–965.
- Brown, S. P. & Taddei, F. (2007). The durability of public goods changes the dynamics and nature of social dilemmas. *PLoS ONE* **2**, e593.
- Brown, S. P., West, S. A., Diggle, S. P. & Griffin, A. S. (2009). Social evolution in micro-organisms and a Trojan horse approach to medical intervention strategies. *Philos Trans R Soc Lond B Biol Sci* **364**, 3157–3168.
- Burt, A. (2003). Site-specific selfish genes as tools for the control and genetic engineering of natural populations. *Proc Biol Sci* **270**, 921–928.
- Diggle, S. P., Crusz, S. A. & Cámara, M. (2007a). Quorum sensing. *Curr Biol* **17**, R907–R910.
- Diggle, S. P., Gardner, A., West, S. A. & Griffin, A. S. (2007b). Evolutionary theory of bacterial quorum sensing: when is a signal not a signal? *Philos Trans R Soc Lond B Biol Sci* **362**, 1241–1249.
- Diggle, S. P., Griffin, A. S., Campbell, G. S. & West, S. A. (2007c). Cooperation and conflict in quorum-sensing bacterial populations. *Nature* **450**, 411–414.
- Dong, Y. H., Wang, L. H. & Zhang, L. H. (2007). Quorum-quenching microbial infections: mechanisms and implications. *Philos Trans R Soc Lond B Biol Sci* **362**, 1201–1211.
- Dubern, J. F. & Diggle, S. P. (2008). Quorum sensing by 2-alkyl-4-quinolones in *Pseudomonas aeruginosa* and other bacterial species. *Mol Biosyst* **4**, 882–888.
- Eberl, L. & Tümmler, B. (2004). *Pseudomonas aeruginosa* and *Burkholderia cepacia* in cystic fibrosis: genome evolution, interactions and adaptation. *Int J Med Microbiol* **294**, 123–131.
- Frank, S. A. (1996). Models of parasite virulence. *Q Rev Biol* **71**, 37–78.
- Fuqua, W. C., Winans, S. & Greenberg, E. (1994). Quorum sensing in bacteria: the LuxR–LuxI family of cell density-responsive transcriptional regulators. *J Bacteriol* **176**, 269–275.
- Gambello, M. J. & Iglewski, B. H. (1991). Cloning and characterization of the *Pseudomonas aeruginosa lasR* gene, a transcriptional activator of elastase expression. *J Bacteriol* **173**, 3000–3009.
- Gambello, M. J., Kaye, S. & Iglewski, B. H. (1993). LasR of *Pseudomonas aeruginosa* is a transcriptional activator of the alkaline protease gene (*apr*) and an enhancer of exotoxin A expression. *Infect Immun* **61**, 1180–1184.
- Gardner, A., West, S. A. & Buckling, A. (2004). Bacteriocins, spite and virulence. *Proc Biol Sci* **271**, 1529–1535.
- Griffin, A. S., West, S. A. & Buckling, A. (2004). Cooperation and competition in pathogenic bacteria. *Nature* **430**, 1024–1027.
- Hamilton, W. D. (1964a). The genetical evolution of social behaviour. I. *J Theor Biol* **7**, 1–16.
- Hamilton, W. D. (1964b). The genetical evolution of social behaviour. II. *J Theor Biol* **7**, 17–52.
- Hardin, G. (1968). The tragedy of the commons. The population problem has no technical solution; it requires a fundamental extension in morality. *Science* **162**, 1243–1248.
- Hatchwell, B. J., Ross, D. J., Fowlie, M. K. & McGowan, A. (2001). Kin discrimination in cooperatively breeding long-tailed tits. *Proc Biol Sci* **268**, 885–890.
- Hense, B. A., Kuttler, C., Müller, J., Rothballer, M., Hartmann, A. & Kreft, J. U. (2007). Does efficiency sensing unify diffusion and quorum sensing? *Nat Rev Microbiol* **5**, 230–239.
- Inglis, R. F., Gardner, A., Cornelis, P. & Buckling, A. (2009). Spite and virulence in the bacterium *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **106**, 5703–5707.
- Keller, L. & Surette, M. G. (2006). Communication in bacteria: an ecological and evolutionary perspective. *Nat Rev Microbiol* **4**, 249–258.
- Köhler, T., Buckling, A. & van Delden, C. (2009). Cooperation and virulence of clinical *Pseudomonas aeruginosa* populations. *Proc Natl Acad Sci U S A* **106**, 6339–6344.

- Köhler, T., Perron, G. G., Buckling, A. & van Delden, C. (2010). Quorum sensing inhibition selects for virulence and cooperation in *Pseudomonas aeruginosa*. *PLoS Pathog* **6**, e1000883.
- Kümmerli, R., Gardner, A., West, S. A. & Griffin, A. S. (2009a). Limited dispersal, budding dispersal and cooperation: an experimental study. *Evolution* **63**, 939–949.
- Kümmerli, R., Griffin, A. S., West, S. A., Buckling, A. & Harrison, F. (2009b). Viscous medium promotes cooperation in the pathogenic bacterium *Pseudomonas aeruginosa*. *Proc Biol Sci* **276**, 3531–3538.
- Lenski, R. E. & Travisano, M. (1994). Dynamics of adaptation and diversification: a 10,000-generation experiment with bacterial populations. *Proc Natl Acad Sci U S A* **91**, 6808–6814.
- Maynard-Smith, J. & Harper, D. (2003). *Animal Signals*. Oxford: Oxford University Press.
- Nealson, K. H., Platt, T. & Hastings, J. W. (1970). Cellular control of the synthesis and activity of the bacterial luminescent system. *J Bacteriol* **104**, 313–322.
- Ng, W. L. & Bassler, B. L. (2009). Bacterial quorum-sensing network architectures. *Annu Rev Genet* **43**, 197–222.
- Nogueira, T., Rankin, D. J., Touchon, M., Taddei, F., Brown, S. P. & Rocha, E. P. (2009). Horizontal gene transfer of the secretome drives the evolution of bacterial cooperation and virulence. *Curr Biol* **19**, 1683–1691.
- Pearson, J. P., Gray, K. M., Passador, L., Tucker, K. D., Eberhard, A., Iglewski, B. H. & Greenberg, E. P. (1994). Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. *Proc Natl Acad Sci U S A* **91**, 197–201.
- Pearson, J. P., Passador, L., Iglewski, B. H. & Greenberg, E. P. (1995). A second *N*-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **92**, 1490–1494.
- Pearson, J. P., Pesci, E. C. & Iglewski, B. H. (1997). Roles of *Pseudomonas aeruginosa* *las* and *rhl* quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. *J Bacteriol* **179**, 5756–5767.
- Pearson, J. P., Feldman, M., Iglewski, B. H. & Prince, A. (2000). *Pseudomonas aeruginosa* cell-to-cell signaling is required for virulence in a model of acute pulmonary infection. *Infect Immun* **68**, 4331–4334.
- Pesci, E. C., Pearson, J. P., Seed, P. C. & Iglewski, B. H. (1997). Regulation of *las* and *rhl* quorum sensing in *Pseudomonas aeruginosa*. *J Bacteriol* **179**, 3127–3132.
- Pesci, E. C., Milbank, J. B., Pearson, J. P., McKnight, S., Kende, A. S., Greenberg, E. P. & Iglewski, B. H. (1999). Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **96**, 11229–11234.
- Platt, T. G. & Fuqua, C. (2010). What's in a name? The semantics of quorum sensing. *Trends Microbiol* **18**, 383–387.
- Popat, R., Crusz, S. A. & Diggle, S. P. (2008). The social behaviours of bacterial pathogens. *Br Med Bull* **87**, 63–75.
- Queller, D. C. & Goodnight, K. F. (1989). Estimating relatedness using genetic markers. *Evolution* **43**, 258–275.
- Rainey, P. B. & Rainey, K. (2003). Evolution of cooperation and conflict in experimental bacterial populations. *Nature* **425**, 72–74.
- Redfield, R. J. (2002). Is quorum sensing a side effect of diffusion sensing? *Trends Microbiol* **10**, 365–370.
- Reece, S. E., Drew, D. R. & Gardner, A. (2008). Sex ratio adjustment and kin discrimination in malaria parasites. *Nature* **453**, 609–614.
- Rumbaugh, K. P., Griswold, J. A., Iglewski, B. H. & Hamood, A. N. (1999). Contribution of quorum sensing to the virulence of *Pseudomonas aeruginosa* in burn wound infections. *Infect Immun* **67**, 5854–5862.
- Rumbaugh, K. P., Diggle, S. P., Watters, C. M., Ross-Gillespie, A., Griffin, A. S. & West, S. A. (2009). Quorum sensing and the social evolution of bacterial virulence. *Curr Biol* **19**, 341–345.
- Sandoz, K. M., Mitzimberg, S. M. & Schuster, M. (2007). Social cheating in *Pseudomonas aeruginosa* quorum sensing. *Proc Natl Acad Sci U S A* **104**, 15876–15881.
- Sinkins, S. P. & Gould, F. (2006). Gene drive systems for insect disease vectors. *Nat Rev Genet* **7**, 427–435.
- Smith, E. E., Buckley, D. G., Wu, Z., Saenphimmachak, C., Hoffman, L. R., D'Argenio, D. A., Miller, S. I., Ramsey, B. W., Speert, D. P. & other authors (2006). Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. *Proc Natl Acad Sci U S A* **103**, 8487–8492.
- Velicer, G. J. & Vos, M. (2009). Sociobiology of the myxobacteria. *Annu Rev Microbiol* **63**, 599–623.
- Velicer, G. J., Kroos, L. & Lenski, R. E. (2000). Developmental cheating in the social bacterium *Myxococcus xanthus*. *Nature* **404**, 598–601.
- West, S. A. & Buckling, A. (2003). Cooperation, virulence and siderophore production in bacterial parasites. *Proc Biol Sci* **270**, 37–44.
- West, S. A. & Gardner, A. (2010). Altruism, spite, and greenbeards. *Science* **327**, 1341–1344.
- West, S. A., Griffin, A. S., Gardner, A. & Diggle, S. P. (2006). Social evolution theory for microorganisms. *Nat Rev Microbiol* **4**, 597–607.
- West, S. A., Griffin, A. S. & Gardner, A. (2007a). Evolutionary explanations for cooperation. *Curr Biol* **17**, R661–R672.
- West, S. A., Diggle, S. P., Buckling, A., Gardner, A. & Griffin, A. S. (2007b). The social lives of microbes. *Annu Rev Ecol Evol Syst* **38**, 53–77.
- Williams, P. (2007). Quorum sensing, communication and cross-kingdom signalling in the bacterial world. *Microbiology* **153**, 3923–3938.
- Williams, P. & Cámara, M. (2009). Quorum sensing and environmental adaptation in *Pseudomonas aeruginosa*: a tale of regulatory networks and multifunctional signal molecules. *Curr Opin Microbiol* **12**, 182–191.
- Winson, M. K., Cámara, M., Latifi, A., Foglino, M., Chhabra, S. R., Daykin, M., Bally, M., Chapon, V., Salmond, G. P. & other authors (1995). Multiple *N*-acyl-L-homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **92**, 9427–9431.
- Winzer, K., Falconer, C., Garber, N. C., Diggle, S. P., Cámara, M. & Williams, P. (2000). The *Pseudomonas aeruginosa* lectins PA-IL and PA-III are controlled by quorum sensing and by RpoS. *J Bacteriol* **182**, 6401–6411.
- Wu, H., Song, Z., Givskov, M., Doring, G., Worlitzsch, D., Mathee, K., Rygaard, J. & Høiby, N. (2001). *Pseudomonas aeruginosa* mutations in *lasI* and *rhlI* quorum sensing systems result in milder chronic lung infection. *Microbiology* **147**, 1105–1113.