

Social evolution theory for microorganisms

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Microorganisms communicate and cooperate to perform a wide range of multicellular behaviours, such as dispersal, nutrient acquisition, biofilm formation and quorum sensing. Microbiologists are rapidly gaining a greater understanding of the molecular mechanisms involved in these behaviours, and the underlying genetic regulation. Such behaviours are also interesting from the perspective of social evolution — why do microorganisms engage in these behaviours given that cooperative individuals can be exploited by selfish cheaters, who gain the benefit of cooperation without paying their share of the cost? There is great potential for interdisciplinary research in this fledgling field of sociomicrobiology, but a limiting factor is the lack of effective communication of social evolution theory to microbiologists. Here, we provide a conceptual overview of the different mechanisms through which cooperative behaviours can be stabilized, emphasizing the aspects most relevant to microorganisms, the novel problems that microorganisms pose and the new insights that can be gained from applying evolutionary theory to microorganisms.

Virulence

The damage caused to the host by a parasite or pathogen, measured as the decrease in host fitness.

Cheater

An individual who does not cooperate (or cooperates less than their fair share), but can potentially gain the benefit from others cooperating.

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This is an exciting time for researchers interested in the social behaviour of microorganisms (BOX 1). There is a growing awareness that microorganisms communicate and cooperate to perform a wide range of multicellular behaviours, such as dispersal, foraging, biofilm formation, 'chemical warfare' and quorum sensing^{1–6}. These behaviours are provoking interest both in their own right and because of the implications that follow from the fact that many of these behaviours are involved in bacterial virulence.

Microbiologists are rapidly gaining a greater understanding of the molecular mechanisms involved in these behaviours, and the underlying genetic regulation^{1–3,5–7}. In this literature it is often assumed that cooperation is favoured because it provides a benefit at the population or species level^{8,9}. However, evolutionary theory shows that this idea cannot work, because the population is at risk from invasion by selfish individuals (cheaters or free-loaders), who do not cooperate but can obtain the benefit of cooperation from others^{10–13}. More generally, explaining cooperation is one of the greatest problems for evolutionary theory^{14,15}. Microorganisms are particularly useful for addressing this problem because of the opportunities that they offer for genetic manipulation and experimental evolution¹⁶.

There is huge potential for interdisciplinary research in this area, combining both mechanistic and evolutionary

approaches (BOX 2). However, a significant limiting factor is the extent to which social evolution theory is properly communicated to microbiologists. Additionally, over the past 40 years social evolution theory has experienced numerous red herrings that have led to confusion and pointless debate^{17–21}. By making use of the debates that have already arisen and been solved in the evolutionary literature, microbiologists can avoid wasting time on red herrings and possible sources of confusion. Fortunately, during this time, evolutionary biologists have also arrived at a relatively unified body of theory that can generally be applied^{14,15,17,22–24}. Here, we summarize this theory, emphasizing the aspects most relevant to microorganisms, the novel problems that microorganisms pose and the insights that can be gained from applying evolutionary theory to microorganisms.

The problem of cooperation

Cooperation is a difficult behaviour for evolutionary biologists to explain — why should an individual carry out a costly cooperative behaviour for the benefit of other individuals or the local group^{10,25}? This seems to go completely against the Darwinian idea of 'survival of the fittest'. Consequently, cheaters who do not cooperate, but gain the benefit from others cooperating, would gain a competitive edge and be able to invade and take over the population. This problem is well known in the

Cooperation

A behaviour that benefits another individual (the recipient) and which is maintained (at least partially) because of its beneficial effect on the recipient.

Tragedy of the commons

A situation when individuals would do better to cooperate, but cooperation is unstable because each individual gains by selfishly pursuing their own short-term interests.

Public goods

A resource that is costly to produce, and provides a benefit to all the individuals in the local group or population.

Signal

Something that alters the behaviour of another individual, which evolved because of that effect, and which is effective because the receiver's response has also evolved.

Actor

A focal individual who performs a behaviour.

fields of economics and human morality, where it is called the *tragedy of the commons*²⁶. The tragedy is that as a group, individuals would benefit from cooperation, but cooperation is not stable because each individual can gain by selfishly pursuing their own short-term interests. Hardin showed this by considering a shared pasture that could be used by a number of shepherds²⁶. It is in each shepherd's interest to add additional sheep, even if this causes overgrazing, because he gains all the benefit of adding additional sheep but pays only a fraction of the cost of overgrazing as it is shared between all the shepherds. There are numerous other human examples, such as declining fish stocks due to overfishing or when there is poor public support for vaccination programmes.

The fact that cooperation can provide a benefit at the population level is not a solution to this problem, as is sometimes suggested^{8,9}. This can be demonstrated by examining cooperation from the perspective of an individual or a gene. If an individual can increase its reproductive success, and therefore increase the transmission of genes to the next generation (that is, increase its fitness) by not cooperating, then it can spread through a population of cooperators even if this reduces the productivity of the population (natural selection is not a far-sighted process). Individuals die and reproduce far more quickly than populations, and so selection that is due to the population-level consequences of behaviours will be much weaker than individual selection^{11,12}. The idea that selection favours behaviours owing to population-level benefits was the

subject of considerable debate in the evolutionary literature during the 1960s and 1970s, where it was called group selection (BOX 3). The conclusion from a large body of theoretical and empirical work was that it was not an important evolutionary force.

To demonstrate the problem of cooperation in a microbial context, consider when bacteria produce what are called *public goods*. Public goods are products that are costly for the individual to produce but that provide a benefit to the individuals in the local group or population¹⁷. The problem in this case is what stops the spread of cheats that do not produce public goods but benefit from those produced by others (FIG. 1a). The iron-scavenging molecules produced by many bacteria, called siderophores, are a good example, as demonstrated by experiments with *Pseudomonas aeruginosa*²⁷. Siderophore production is beneficial when iron is limiting, as shown by the fact that wild-type strains that produce siderophores grow faster than mutant strains that do not. However, siderophore production is also costly, as demonstrated by the fact that mutants grow faster than wild-type strains in an iron-rich environment. Consequently, in mixed populations where both wild-type and mutant bacteria are present, the mutants can gain the benefit of siderophore production without paying the cost, and therefore increase in frequency, as they out-compete the cooperative wild-type bacteria²⁷ (FIG. 1a).

This leads to the fundamental problem of what makes cooperative behaviours, such as siderophore production, evolutionarily stable in response to the possible invasion by cheats that arises through migration or mutation²⁸? Clearly, there must be a solution because siderophore production occurs, and in the following section of this paper we will discuss the possible solutions to this problem. Furthermore, there are numerous other examples of public goods that are produced by individual bacterial cells and used by neighbouring cells. These include extracellular products for nutrient acquisition^{29,30}, quorum sensing (QS) signalling molecules for cell–cell communication^{3,6,31,32}, antibiotics³³, immune-modulation molecules^{34–36}, antibiotic-degradation compounds (for example, β -lactamases)^{37,38} and biosurfactants (for example, rhamnolipids) for motility^{39,40}. In addition, biofilm development and maintenance can be influenced by public goods secreted into the bacterial extracellular matrix, including exopolysaccharides, such as alginate⁴¹, and adhesive polymers⁴².

Before discussing solutions to the problem of cooperation, it is useful to distinguish whether the benefits of a behaviour accrue to all the local individuals, including the actor (whole-group traits), or only local individuals other than the actor (other-only traits)⁴³. This distinction is important because the two situations can demand a different set of explanations, as described in the next section. The production of public goods is a classic example of a whole-group trait. Another example would be when the economical use of common resources leads to more efficient resource use, such as when high growth yield is coupled with low growth rate^{44,45}. Examples of other-only traits would be when cells sacrifice themselves to the benefit of the cells that are around them. In this case, the

Box 1 | A classification of social behaviours

A behaviour is social if it has consequences for both the actor and the recipient. Social behaviours can be categorized according to the fitness consequences they entail for the actor and recipient^{10,54,68} (see table). A behaviour that increases the direct fitness of the actor is mutually beneficial if the recipient also benefits, and selfish if the 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. The term 'cooperation' refers to a behaviour that increases the fitness of the recipient — cooperation can therefore be mutually beneficial or altruistic, depending on the effect on the actor. It is easy to see how natural selection favours mutually beneficial or selfish behaviour, whereas altruism and spite are more difficult to explain.

Behaviours should be classified according to their impact on total lifetime reproductive success. So, if a cooperative behaviour was costly to perform but provided a direct benefit that outweighed this cost in the long term, such as by avoiding punishment, it would be classified as mutually beneficial. Classifying specific behaviours can be complicated because of the difficulties in measuring the long-term consequences for fitness, and because behaviours can have multiple consequences — for example, public goods can have both direct and indirect fitness benefits. This means that in some situations there can be an advantage to describing behaviours according to their immediate effects, as the long-term effects might not be certain, and we are often not in a position to view them¹⁵. So, rather than classify behaviour as altruistic or mutually beneficial, it can be easier to show, for example, that the production of public goods is costly to the individual but provides a local benefit²⁷. Another problem is that different authors can use terminology differently. However, these issues are simply semantic, and confusion is best avoided by clear and specific statements that minimize jargon.

		Effect on recipient	
		Positive	Negative
Effect on actor	Positive	Mutual benefit	Selfishness
	Negative	Altruism	Spite

Box 2 | Complementary approaches to studying behaviour

Evolutionary biologists and microbiologists typically study behaviours or traits from different perspectives. Evolutionary biologists are primarily concerned with the fitness consequences or survival value of a behaviour, which are called ultimate explanations: why has this behaviour been selected for by natural selection? Microbiologists are primarily concerned with proximate explanations: what molecular and genetic mechanisms govern the particular trait or behaviour?

The crucial point here is that these methods are complementary and not alternatives. The Nobel Prize winner Niko Tinbergen famously clarified this point in the most influential paper of his career¹¹⁶. One of his classic studies to show the distinction between ultimate and proximate explanations was on how black-headed gulls remove eggshells from their nests. The mechanistic (proximate) explanation for this is that individuals are more likely to remove objects from their nest if they are white or egg-coloured, have frilly edges and if they are feather-light. The evolutionary (ultimate) explanation for this is that it makes aerial predators such as herring gulls less likely to find their brood. These explanations are clearly not competing, and both are required for a full understanding of the behaviour.

Combining evolutionary and mechanistic approaches opens many avenues of research. Mechanistic studies stimulate evolutionary research because they demonstrate behaviours that pose problems from an evolutionary perspective. Indeed, evolutionary biologists have begun to marvel at the possibilities microorganisms offer¹⁶. The evolutionary approach stimulates mechanistic research because it suggests problems for which there must be a mechanistic solution, and the kind of mechanisms involved. For example, is there a mechanism by which individuals can direct cooperation towards closer relatives? Are individuals who do not cooperate punished in some way? Does cooperation provide some longer term feedback benefit? If a molecule produced by cell A affects a response in cell B, is that a cue, a signal or a manipulation^{6,32,117}?

Direct fitness

The component of fitness gained through reproduction.

Repression of competition

When the selfish advantage of cheats is removed.

Altruistic

A behaviour that increases another individual's fitness at a cost to one's own.

Kin selection

A process by which traits are favoured because of their beneficial effects on the fitness of relatives.

Indirect fitness

The component of fitness gained from aiding the reproduction of non-descendant relatives.

Hamilton's Rule

A condition ($rb - c > 0$) that predicts when a trait is favoured by kin selection, where c is the cost to the actor of performing the behaviour, b is the benefit to the individual who the behaviour is directed towards, and r is the genetic relatedness between those individuals.

Relatedness

A measure of genetic similarity.

problem is why should an individual cell pay the cost of this sacrifice when it could just exploit the sacrifice of other cells? One of the clearest cases of this is in the formation of fruiting bodies by cellular slime moulds such as *Dictyostelium discoideum*, where some cells become spores, whereas others sacrifice themselves and become non-viable stalk cells^{46,47} (FIG. 1b). Similar differentiation occurs in *Myxococcus xanthus* bacteria, where cells become either spores or non-spores⁴⁸. Another example is when cells undergo autolysis (cell death), if this is to provide nutritional benefits to the population, such as providing DNA to help structure biofilms, or to release antimicrobial toxins (for example, bacteriocins) that kill competitors^{24,49}.

Explaining cooperation

Given the problem of cooperation, how can cooperative behaviours be maintained? There is a huge amount of literature on this topic, and it is possible to clarify the different possibilities in several ways^{15,17,23,24,50–52}. Our aim here is to provide a classification that is useful for microorganisms and that can be understood by microbiologists who are not specialists in evolutionary theory.

Cooperation is when a behaviour benefits another individual (BOX 1). We divide possible explanations for cooperation into two broad groups. First, the cooperative behaviour can provide a direct fitness benefit to the reproductive performance of the individual performing the behaviour that outweighs the cost of performing the behaviour. In this case, cooperation is mutually beneficial (BOX 1). We divide this into situations where individuals have a shared interest in cooperation and situations where there is a mechanism for enforcing cooperation

or removing the advantage of cheating (this is called repression of competition).

Second, and harder to explain from an evolutionary perspective, are cooperative behaviours that decrease the direct fitness of the individual that performs them. In this case, cooperation is altruistic (BOX 1) and can only be explained if cooperation is directed towards individuals who share cooperative genes¹⁰. This is called kin selection¹¹, or an indirect fitness benefit⁵³. A complication here is that a cooperative behaviour can provide both a direct and indirect fitness benefit. In particular, whole-group traits such as siderophore production provide a benefit to both the individual that produces them and to nearby relatives. In this case, whether a behaviour is mutually beneficial or altruistic will depend on the relative importance of the direct and indirect fitness benefits. This is in contrast to other-only traits, which can only be explained by indirect fitness benefits.

Kin selection

Kin selection theory provides an explanation for altruistic cooperation between relatives¹⁰. By helping a close relative to reproduce, an individual is still passing on its own genes to the next generation, albeit indirectly. This theory is encapsulated in a pleasingly simple form by Hamilton's rule^{10,25}, which states that altruism is favoured when $rb - c > 0$; where c is the fitness cost to the altruist, b is the fitness benefit to the beneficiary and r is their genetic relatedness (see **Supplementary information S1** (box)). This predicts that greater levels of altruistic cooperation are expected when r or b is high and c is low (FIG. 2a).

Kin selection and limited dispersal. Kin selection requires a sufficiently high degree of relatedness between cooperating individuals. Hamilton suggested two possible mechanisms for this¹⁰. First, limited dispersal (population viscosity) would tend to keep relatives together^{10,54}. In this case, altruism directed indiscriminately towards all neighbours will be favoured as neighbours tend to be relatives. This type of mechanism is likely to be of huge importance in microorganisms as asexual reproduction means that single cells colonize and grow in a local area. In this case, the individuals interacting over a small area will be clonal, corresponding to $r = 1$, which can be conducive to the evolution of cooperation.

This form of kin selection, involving limited dispersal, has been suggested to be important for the production of public goods^{28,34,55}. It applies to any public good that is dispersed on a scale where bacteria tend to be close relatives. Experimental support for this idea has been obtained from the multiple-generation selection experiment on the production of siderophores in *P. aeruginosa*²⁷. It was observed that a wild-type strain that produced siderophores out-competed a selfish mutant strain when cultured under conditions of high relatedness, but not when relatedness was lower. Relatedness was manipulated by allowing the bacteria to grow and interact in groups derived from a single clone (relatively high relatedness) or from two clones (relatively low relatedness)²⁷.

Box 3 | Group selection

It is useful to distinguish between two types of group selection²⁰.

Old group selection. During the 1960s, Wynne-Edwards¹¹⁸ argued for the importance of group selection in its original or 'old' form, considering relatively cooperative behaviours such as reproductive constraint. He realized that populations of cooperators could be invaded easily by a selfish cheat that reproduced at the maximum rate, and suggested that the differential survival of groups was the solution to this problem. In groups consisting of selfish individuals, resources would be over-exploited, and the group would become extinct. By contrast, groups consisting of altruistic individuals who restricted their birth rate would not over-exploit their resources, and not become extinct. Therefore, by a process of differential survival of groups, behaviour evolved that was for the good of the group.

During the 1960s and 1970s however, a large body of theoretical and empirical work accumulated against this idea. In practice, groups do not become extinct fast enough and individuals move too frequently between groups for this to be an important process¹¹⁹. It is now generally accepted that this form of group selection is unimportant, and that this kind of thinking leads people to the false conclusion that individuals will do something because it is 'for the good of the species'.

New group selection. In the 1970s and 1980s a new form of group selection was developed, based on the idea that at certain stages of an organism's life cycle interactions take place between only a small number of individuals¹²⁰. Under these conditions, cooperative behaviour can be favoured. This kind of new group selection is sometimes referred to as trait-group or demic selection. The new group selection models therefore rely on within-population group selection whereas the old group selection theory focuses on between-population group selection¹²¹.

However, it has since been shown that kin selection and the new group selection are just different ways of conceptualizing the same thing. They are mathematically identical, and are both valid^{18,20,122–124}. Both approaches tell us that increasing the group benefits and reducing the individual cost favours cooperation. Similarly, group selection tells us that cooperation is favoured if we increase the proportion of genetic variance that is between-group as opposed to within-group, but this is equivalent to saying that relatedness is increased. In all cases where both methods have been used to look at the same problem, they give identical results (see TABLE 1).

Avoiding confusion. Although the equivalence of the kin selection and new group selection approaches has long been appreciated^{20,122,123}, there has been a huge amount of fruitless debate in this area, mainly owing to semantics^{17,18,20,121}. This was partly due to the group selection literature redefining several terms, such as altruism, that already had valuable and clear meanings²⁰. Our worry here is that although this debate was solved decisively during the 1960s–1980s by evolutionary biologists, it seems to reoccur and lead to confusion as new fields embrace the relevant aspects of social evolution¹²¹, and this is starting to occur within the microbiology literature^{4,8,9}.

The effect of relatedness can be shown by considering how relatedness is determined by the number of clones in an area or patch. At one extreme, if only one clone colonizes each patch then relatedness will be high, and each patch will be colonized by a cooperator or a cheater. Cooperation leads to greater growth rates, and so cooperators will out-compete cheaters²⁷. Consequently, at high relatedness cooperation is evolutionarily stable²⁸. At the other extreme, if multiple colonies colonize each patch then relatedness will be low, and each patch can contain a mixture of cooperators and cheaters. In this case, cheaters can exploit and out-compete cooperators²⁷ and so cooperation will not be evolutionarily stable²⁸. This stresses the fact that population benefits alone cannot explain cooperation.

As mentioned earlier, the production of public goods can potentially provide a direct and an indirect (kin selected) fitness benefit. This means that some production of public goods, such as siderophores, could still be favoured when interacting with non-relatives if the benefits outweigh the costs to the cell that produces them²⁸. The relative cost and benefit of siderophore production will vary with factors such as environmental conditions and population density. Therefore, it is possible that the production of public goods, such as siderophores, can be altruistic under some conditions and mutually beneficial under others¹⁸. However, we suspect that the dominating factor will usually be kin selection, which favours altruism, as is supported by the siderophore selection experiment²⁷.

Kin selection and kin discrimination. The second mechanism for generating sufficiently high relatedness to make kin selection important is kin discrimination, when an individual can distinguish relatives from non-relatives and preferentially direct aid towards them¹⁰. This has been demonstrated in numerous cooperatively breeding vertebrate species, where the feeding of young is preferentially directed towards close relatives⁵⁶. By contrast, there has been little research on the importance of kin discrimination in microorganisms. An exception is in malaria parasites, where kin discrimination has consequences for the evolution of the sex ratio (the proportion of male gametocytes) in the transmission stages, and there is evidence both for and against these parasites assessing relatedness⁵⁷.

One form that kin discrimination could take in microorganisms is through specificity. With the production of public goods, selection would favour the production of highly specific molecules that other lineages (clones) could not utilize. Consistent with this, in *P. aeruginosa*, there is variation across strains in the form of the pyoverdine siderophore produced and in the ability to take up iron that has been chelated by pyoverdines produced by other strains⁵⁸. Furthermore, sequence data suggest that the genes involved in pyoverdine production are under selection for novelty and specificity (diversifying selection)⁵⁹. This form of behaviour is unlikely to be restricted to iron acquisition in microorganisms. *Staphylococcus aureus* strains utilize an autoinducing signal peptide (AIP), which

Kin discrimination

When behaviours are directed towards individuals depending on their relatedness to the actor.

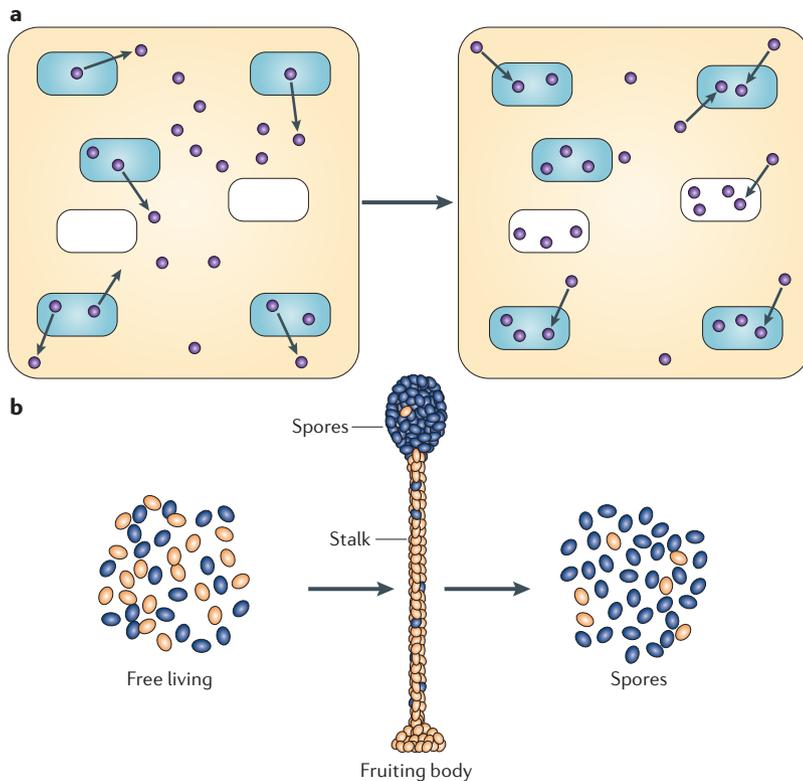


Figure 1 | The problem of cooperation. **a** | The tragedy of the commons with public goods. Cheats (white oblongs) who do not pay the cost of producing public goods (purple circles) can still exploit the benefits of public goods produced by other cells (green oblongs). **b** | Altruistic sacrifice. When two lineages come together to form a fruiting body, a cheat (blue lineage) would increase its reproductive success by contributing less towards stalk formation, and more towards spore production, compared with the other (orange) lineage.

controls the expression of several virulence determinants, including the exotoxins used to convert host tissue into nutrients for bacterial growth²⁹. Four variants of this peptide have been reported to be produced by *S. aureus*, which has allowed *S. aureus* strains to be divided into four AIP-producing groups (AIP1–AIP4) (REF. 60,61). Experimentally, AIP1 has been shown to induce virulence in AIP1-producing strains but to inhibit virulence in AIP2-, AIP3- and AIP4-producing strains⁶². Other

examples of within-species diversity and specificity include the identification of >25 bacteriocins (colicins) in *Escherichia coli*⁴⁹, and the diverse 2-alkyl-4(1H)-quinolone (AHQ) secondary metabolites produced by *P. aeruginosa*, which possess antibacterial, immunomodulatory and signalling properties^{63,64}.

The extent to which diversity and specificity lead to kin discrimination is unclear, as their importance can be limited by conflicting selection on individuals to be able to take up all forms of molecules produced. Indeed, this conflict could prevent kin discrimination from being evolutionarily stable⁶⁵. A potential solution to this problem is if the variation in a relatedness cue is maintained by another selective pressure, such as host immune evasion⁶⁶. Overall, this is an area where there is an urgent need for further theoretical and empirical research to address even the most basic questions, let alone generalizations such as in what environments we should and should not find kin discrimination.

An extreme form of kin discrimination occurs in so-called ‘green-beard’ genes, which specifically direct cooperation towards other individuals that carry the same gene^{10,13,67}. This requires a gene that causes a phenotypic effect that can be recognized by other individuals who have this gene and who can adjust their behaviour accordingly. Green beards are likely to be rare, because cheats that display the green beard without also performing the cooperative behaviour could invade and overrun the population. However, one of the few examples is in a microorganism — the *csa* cell-adhesion gene in *D. discoideum*. Individuals with this gene adhere to each other in aggregation streams, excluding mutants who do not, and cooperatively form fruiting bodies⁶⁷.

Kin discrimination can also be used to direct harmful or spiteful behaviours at non-relatives^{68,69} (BOX 1). This is advantageous when it releases resources or reduces competition for closer relatives. Bacteriocin production and other forms of ‘chemical warfare’ provide one of the clearest examples of spite⁷⁰. Discrimination against non-relatives can occur with bacteriocins because the bacteriocin gene and an immunity gene that encodes a factor that deactivates the bacteriocin are linked genetically³³. If this linkage is broken, cheats can arise that produce the antidote but save on the cost of producing

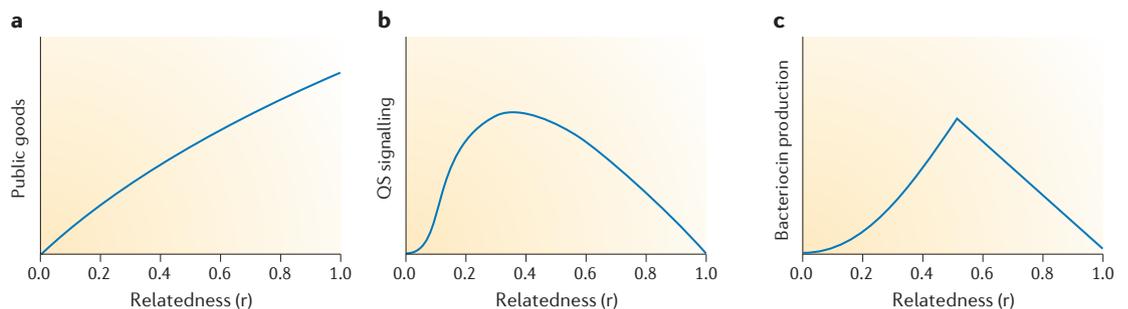


Figure 2 | Some relationships between genetic relatedness (*r*) and social behaviours. The production of public goods, such as siderophores, increases monotonically as relatedness between social partners increases²⁸ (**a**). Quorum sensing (QS) signalling (**b**) and bacteriocin production (**c**) are both predicted to show a dome-shaped function of relatedness, with signalling⁹¹ and bacteriocin production⁷⁰ maximized at an intermediate relatedness.

Cue
Something that can be used by an individual as a guide for future action.

Spiteful
A behaviour that decreases another individual's fitness at a cost to one's own.

Table 1 | **Misconceptions and red herrings in social evolution theory**

Misconception or red herring	Reality
Kin selection will not be important in microorganisms because it requires complex sensory systems that can recognize kin.	Kin selection can work without kin discrimination, if processes such as limited dispersal lead to interacting individuals being highly related ¹⁰ . Also, there is the potential for kin discrimination in microorganisms.
Kin selection requires clonality ($r = 1$).	Kin selection can work when $r < 1$, depending on the relative benefit:cost ratio. However, higher levels of cooperation are expected as relatedness increases.
There is a 'new' form of group selection, which can be invoked to explain cooperation in cases where kin selection cannot.	Kin selection and the new group selection are just different ways of conceptualizing the same process — they are mathematically identical and both valid ^{17,20,124} (BOX 3). However, most workers focus on kin selection methodology because it is usually easier to construct models, interpret the predictions of theory, and apply the models to real biological cases, and it reduces the potential for confusion.
The Prisoner's Dilemma (PD).	The PD provided a useful way of conceptualizing the problem of cooperation in the 1970s and 1980s ^{15,115} . However, it makes specific assumptions and has been surpassed by more powerful and general methods for examining the problem of cooperation ¹⁷ . The PD focuses attention on reciprocal altruism, which is a small subclass of the mechanisms for repressing competition within groups, and is unlikely to be of general importance outside humans ^{125–127} . In microorganism examples where the PD has been invoked ^{4,30,128} , it is easier, more appropriate and more general to conceptualize with the tragedy of the commons and kin selection ^{28,129} .

r, relatedness.

the toxin⁷¹. The degree of kin discrimination will depend on the diversity and specificity of bacteriocins, and the mechanisms by which these are generated⁴⁹.

Direct fitness benefits to cooperation

In the above section we have discussed how kin selection can explain altruistic cooperation. The alternative explanation for cooperation is that it provides a direct fitness benefit to the individual that performs the cooperative behaviour. In this case, cooperation is mutually beneficial and not altruistic (BOX 1). In this section we consider how direct benefits to cooperation can occur: first, if individuals have a shared interest in cooperation, and second, when there is a mechanism to enforce cooperation or remove the advantage of cheating (repression of competition).

Shared interest (no enforcement). Cooperation will be favoured by selection when individuals have a shared selfish interest in cooperating^{52,54}. The simplest possibility that can lead to the evolution of cooperation is when an otherwise selfish behaviour benefits another individual (one-way by-product benefit), such as when the waste product of one individual provides a nutrient for another. In this case, the behaviour is mutually beneficial, providing a direct benefit to the actor who performs the behaviour, irrespective of what other individuals do, but also providing a benefit to another individual. Benefits can flow in both directions, as is found when two interacting individuals each feed on a by-product of the other (known as cross-feeding or two-way by-product benefit). These by-products can occur between different phenotypes (variants or morphs) of the same species or between different species.

We would not class these behaviours as cooperation because the benefit to neighbouring individuals is simply the by-product of selfish behaviour. We suggest that a behaviour should only be classed as cooperative if it is maintained, at least partly, because of its beneficial

effect on the recipient. However, cooperation can evolve in response to by-product benefits. For example, if individuals A and B feed on by-products of each other, then selection can favour increased by-product production by individual A, because it would lead to individual B doing better and producing more by-product that can then be used by individual A. In this case, individual A would be performing a cooperative behaviour (by-product reciprocity).

A famous example of by-product reciprocity is the greater honeyguide, an African bird that guides humans to beehives, where it feeds on the honey left behind after human foraging⁵². Another example is in cooperative breeding vertebrates, where helping behaviours can lead to a larger group size, and therefore higher survival rates⁷². As helping is often directed towards relatives in vertebrates, this provides a clear example of how cooperation can lead to both direct and indirect (kin selected) benefits. Selection for cooperation is increased if there are synergistic benefits to cooperation, or if the underlying ecology allows cooperators to become associated across generations^{23,50,73}.

Direct benefits to cooperation could occur in multi-species biofilms — all that is required is that there is a net benefit due to the benefit received as a by-product outweighing the cost of performing the cooperative behaviour. Many biofilms are composed of multiple species. For example, colonization of human teeth and the oral mucosa can involve up to 500 species of bacteria, with huge potential for cooperation or conflict between species^{4,74,75}. Studies on the early colonizers *Streptococcus oralis* and *Actinomyces naeslundii* suggest that cooperation between these species allows them to grow where neither can survive alone⁷⁶. Another example is the mixed-species biofilm between *P. aeruginosa* and *Burkholderia cepacia* that can occur in the lungs of people with cystic fibrosis, where it is associated with morbidity and mortality^{77,78}. *B. cepacia* has been shown to upregulate the production of virulence determinants using QS

Recipient

An individual who is affected by the behaviour of the actor.

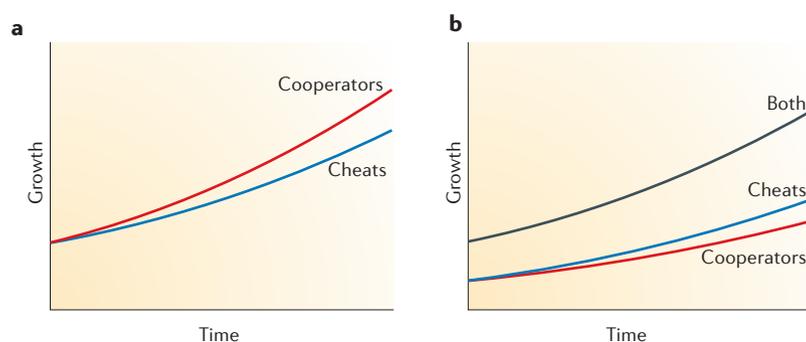


Figure 3 | The costs and benefits of producing public goods. Shown are the growth rates of (a) populations consisting of only cooperators and only cheats and (b) cheats and cooperators in a population started with a 50 :50 mixture of both. For illustration, exponential growth is assumed in conditions where the production of the public goods is favoured. The graphs show that the population as a whole grows faster when it consists of cooperators (a), but that cheats spread and out-compete cooperators in a mixed population (b). Also note that the total productivity of the mixed population (line marked Both in b) is lower than that of a pure cooperator population (in a).

molecules produced by *P. aeruginosa*. However, it has yet to be shown whether this represents signalling and cooperation or one species manipulating and exploiting the behaviour of another³².

Many examples of cooperation between microbial symbionts and their hosts can be explained by shared interests. If a single symbiont lineage infects each host then they will have a shared interest that can favour cooperation, especially if the symbiont is transmitted vertically, from parent to offspring. However, if multiple symbiont lineages infect each host, then this will break the shared interest and the tragedy of the commons arises. Specifically, each lineage would be expected to behave selfishly, at the expense of the host and other lineages — gaining the benefit from others cooperating without paying the cost of helping the host^{79,80}. In this case, cooperation would require some mechanism to repress this conflict, as we discuss below. A consequence of this is that hosts can be selected to minimize symbiont diversity, in order to minimize conflict between symbionts^{79,81}.

Repression of competition (enforcement). The problem of cooperation is that individuals can be selected to pursue their own selfish interests (cheat) to the detriment of the overall productivity of the local group. However, if the opportunities for successful cheating are removed, then individuals can only increase their reproductive success by enhancing the productivity of the group^{51,82}. This unites the interests of neighbours, and selects for cooperation. Different mechanisms have been suggested for this, including policing, punishment, sanctions and partner choice^{51,80,82–85}. Frank⁵¹ has grouped these as mechanisms for the repression of competition within groups. A classic example of this is in the social insects, ants, bees and wasps, where each worker would benefit from rearing its own sons, rather than the queen's sons. However, other workers are selected to prevent them doing this because it has a cost to colony productivity,

Manipulate

When one behaviour alters the behaviour of another individual, usually to the benefit of the actor and to the cost of the other individual.

and because the policing workers can be more related to the sons of the queen than the sons of other workers⁸⁶. This has led to selection for policing by workers, where they destroy the eggs laid by other workers. This provides a clear example of how conflicts can occur even among highly cooperative social groups. To our knowledge, such a mechanism has yet to be found (or tested for) in a microorganism.

Repression of competition can also allow cooperation across species^{23,80}. An example of this is the interaction between leguminous plants and the rhizobial bacteria that fix N_2 within the root nodules of the host plant. N_2 fixation benefits the plant because it supplies nitrogen needed for growth and photosynthesis, but it is energetically costly to the bacteria, reducing the resources that can be allocated to bacterial growth and reproduction⁸⁰. Experiments have shown that cooperation is favoured, because if the rhizobia in a nodule do not provide nitrogen to their host, the plant punishes them by decreasing the O_2 supply to that nodule, which severely reduces the growth rate of the bacteria⁸⁵. We suspect that similar processes could explain many cases of cooperation between species, such as why the bacteria *Vibrio fischeri* expensively luminesce in the light organ of their host squid; this is supported by the fact that mutants that cannot luminesce are unable to colonize light organs⁸⁷. Another possibility is cooperation in multiple-species biofilms.

A general point when considering the explanations of cooperation given above is that the different mechanisms are not mutually exclusive. We have already discussed how behaviours such as the production of public goods can provide a direct and indirect (kin selected) benefit. Another example would be that if by-product benefits occur between relatives, then this will lead to a direct and indirect benefit of cooperation. Similarly, although cooperation is favoured between relatives, if there is a mechanism for the repression of competition between relatives then this can lead to even higher levels of cooperation.

Testing for social traits in microorganisms

In evolutionary biology, a fundamental first step in the study of any trait is to demonstrate its fitness consequences in a social context (BOX 1). This means determining the cost and benefit of a behaviour for the actor and the recipient. An advantage when addressing such problems in microorganisms is that mutant strains that do not perform the potentially social trait are often available to facilitate such work^{27,38,40,48,88,89}. Given this, the first experiment is to examine the relative fitness of the wild type (that possesses the supposedly social trait) and the mutant, alone and in mixed cultures (FIG. 3). It is particularly informative if this can be done under conditions when the behaviour is and is not favoured. For example, the benefit of siderophore production could be altered by varying iron availability.

Analogous experiments are required for other potentially social traits. For example, are mutants that do not produce adhesive factors such as alginate, which are involved in biofilm formation, able to invade and spread, reducing the overall productivity of the biofilm⁴²? What

is the relative fitness of QS cheats that do not signal, or do not respond to signals, under conditions where QS is and is not favoured? Is the production of persister cells, which survive antibiotic treatment⁹⁰, vulnerable to cheats that maximize their short-term growth? What is the relative fitness of individuals that do and do not produce bacteriocins, when grown alone or with competitors? In all of these cases, the first step forward will be simple experiments that examine the basic costs and benefits of the behaviours in a social context. This can be achieved by examining something that is expected to correlate with fitness, such as the growth rate, in cooperators and cheats when grown alone and in mixed culture.

More subtle predictions can also be made and tested. The relative advantage of cheating can be altered by varying the population density or the relative frequency of cheats — kin selection theory predicts that cheats will do better at lower frequencies and in denser populations^{30,34,38,48,91,92}. An alternative possibility is to test for conflicts of interest between non-relatives. For example, when multiple lineages combine to form a fruiting body, can one lineage increase its success at a cost to the other^{47,93,94}, and does this lead to reduced total spore production^{95,96}? If so, how and why is this variation maintained? These kinds of experiments will even be required for cases where the possible nature of social interactions is not clear. For example, in *Streptococcus pneumoniae*⁹⁷ when competent cells trigger pneumolysin release from non-competent cells (autolysis), does this represent coercion of non-competent cells against their wishes, or a signal between relatives with a shared interest³²?

There are pros and cons to the different possible ways of conducting the experiments discussed above. One issue is whether it is better to do experiments *in vitro* or *in vivo*. By doing experiments *in vitro*, conditions can be more controlled and the outcome closely monitored. However, we will often ultimately be interested in what happens *in vivo*, because these are the conditions in which the trait we are interested in has evolved. Furthermore, although things can be more complex *in vivo*, these complexities are interesting. For example, spatial structuring within a host might keep the relatedness between interacting bacteria high, even when multiple clones infect a host, increasing the advantage of cooperation²⁸. This clearly demonstrates the possible interplay between the different methodologies, as *in vitro* studies could be designed to investigate the importance of factors, such as spatial structuring, that are thought to be important *in vivo*.

Another issue is what kind of cheating mutants should be used. Strains in which the gene for a cooperative behaviour has been artificially knocked out have the advantage of well-defined, clear and large effects^{27,48,88}. By contrast, spontaneous mutations from the laboratory or isolates from the field might be more natural and give a better indication of the kind of variation that natural selection could act on⁴⁸. Clearly, in all these cases, the greatest explanatory power and demonstration of generally applicable phenomena will be obtained by combining different methods.

Comparative studies across species are another useful methodology to investigate the benefit and cost of social traits⁹⁸. Theory can make predictions about when a trait is most useful, and therefore how the occurrence of that trait should correlate with factors such as ecological variables. For example, Hamilton's rule predicts that there is greater selection for the preferential helping of relatives when the benefit of helping relatives (b) is greater. This prediction is supported in cooperative breeding vertebrates, where there is greater kin discrimination when the benefits of helping are higher, providing evidence for the importance of kin selection in these species⁵⁶. There are many possibilities for such studies in microorganisms, such as variation across species or populations in the production of public goods, or the amazing diversity in how different slime-mould species make fruiting bodies⁴⁶. For example, kin selection theory predicts greater levels of cooperation in species or populations in which interacting individuals tend to be more related. It is possible that relatedness will be influenced by dispersal rates and population structuring, which can be determined by the physical nature of the environment (for example, liquid versus soil versus different locations in a vertebrate host) or by the behaviour of the microorganisms themselves (for example, biofilm formation or budding).

Analogy and complications

Analogy with invertebrates and vertebrates can be useful for developing hypotheses to test in microorganisms¹. Compare a group of bacteria growing in a biofilm (slime city) or a small area, with a colony of social insects, such as ants. Castes are enormously important in ant societies, where individuals can develop different morphologies that enable them to specialize in varied behaviours, such as foraging, brood care or defence⁹⁹. A recent paper suggests that similar phenomena might be happening in biofilms, where variants exhibit different behaviours, such as an increased ability to disseminate or a tendency to form biofilms in an accelerated manner¹⁰⁰. An important difference here, however, is that caste determination is generally environmental in social insects¹⁰¹, whereas genetic variation was suggested in biofilms. Environmental castes might be more likely to favour altruistic cooperation because with genetic altruists, the benefits would be less likely to go to individuals who share the altruistic gene. Even if the individuals are genetically identical at all other loci, it is the genetic similarity (relatedness) at the altruism locus that is crucial here. Consequently, the pattern in biofilms might merely represent the spread of selfish mutants, although there could be the possibility for kin selection to influence the mutation rate, producing altruists that are less likely to be transmitted to the next patch.

Another recurring theme in insect and vertebrate societies is that although individuals live in cooperative groups there is still plenty of possibility for conflict within the group⁸⁶. The fundamental point here is that when $r > 0$ kin selection can favour cooperation, but when $r < 1$ individuals will have selfish interests, so when $0 < r < 1$ there will be potential for both cooperation and conflict. It is perhaps ironic that some of the best support for kin

selection theory actually comes from its ability to predict conflicts in cooperative insect colonies⁸⁶. An analogous example of simultaneous conflict and cooperation from a microorganism is that even when genetically distinct slime-mould clones cooperate to form a fruiting body, each is selected to maximize its contribution to the spores and minimize its contribution to the stalk, to the detriment of the other lineages⁴⁷. It is for this reason that thinking about bacterial populations as multicellular organisms^{8,9} can be misleading. In multicellular organisms, $r = 1$ and so there is no conflict (unless there is mutation), whereas in bacterial populations r can be < 1 , in which case there will be conflict.

Mechanisms for enforcing cooperation, such as punishment or sanctions, will lead to individuals who cooperate having greater direct fitness, but measuring the costs and benefits of cooperation can be more complicated in these situations^{51,80,82,85}. The trick is to untangle the cost of cooperation from the benefit of avoiding punishment. Consider the example given earlier of rhizobia in their legume hosts, where it was shown that cheating (not fixing N_2) has a direct fitness cost⁸⁵. This cost occurs at the level of the nodule, which is usually colonized by a single clone, and so there will be strong kin selection between these bacteria to avoid punishment⁸⁵. The difficulty is showing that this occurs through a punishment mechanism. Kiers *et al.* overcame this problem by elucidating the mechanism involved, showing that plants cut off the O_2 supply⁸⁵. Another possibility would be if this punishment could be removed (for example, supply O_2 experimentally), in which case we would expect cheaters to do better than cooperators. In such cases, the interplay between examining behaviour from an evolutionary and a mechanistic perspective is crucial.

A general point regarding all the experiments discussed above is that they assume huge simplifications. We know that the situation is actually more complex. For example, there is facultative variation in response to environmental conditions, such as in the amount of siderophore production¹⁰². Bacteria have multiple hierarchical QS systems, with each affecting numerous traits^{3,31}. In addition, spatial structuring through limited dispersal is not always good for cooperation as it leads to increased competition between relatives, which can reduce or remove kin selection for altruism^{17,27,54,103,104}. This last point could be of particular relevance to biofilms, where we might expect mechanisms that keep relatives together so they can cooperate, but also minimize the potential competition between them¹⁰⁵.

Pathogenic species offer a greater layer of complexity. One reason for this, as mentioned above, is that the environment will be more complex *in vivo*, especially when the host responds to the presence of the pathogen. Another factor is that any behaviour that influences virulence will also be subject to the selective pressures we have discussed in this article^{28,70,106}. For example, if multiple clones infect a host (low r), then this can lead to a tragedy of the commons over the host resources, which will select for rapid and aggressive exploitation of the host, with greater virulence¹⁰⁶. These factors

also have applied implications, as different pathogen-management strategies could select for more or less virulent parasites^{27,107}, or be designed to exploit social behaviours¹⁰⁸. However, it is crucial to gradually work up to investigating these complications in small steps that elucidate the basics first, based on a solid understanding of the underlying mechanisms.

Microbial peculiarities

Although the fundamentals of evolutionary theory are well understood, there is still a need to develop the theory to apply to specific microbiological cases. An example of this is provided by the suggestion that bacteria use QS systems to coordinate cooperative behaviours. It has been suggested that such QS systems will be favoured under conditions of high relatedness, because then kin selection would favour cooperation and signalling to coordinate this cooperation^{6,109}. However, when this was modelled explicitly, a slightly different prediction emerged — the highest level of signalling is predicted to occur at intermediate levels of relatedness⁹¹ (FIG. 2). At low relatedness there is no selection for cooperation, and so no signalling, whereas at high relatedness there is no conflict and so a minimal, cheap signal is required to coordinate cooperation. At intermediate relatedness, individuals are selected to signal at higher levels to manipulate competitors into greater levels of cooperation.

Microorganisms present their own practical problems, despite their attractions as model systems for research into social evolution. In diploid eukaryotes, relatedness can be measured relatively easily with molecular markers¹¹⁰. This has led to the problem that there has been too little focus on the costs (c) and benefits (b) of behaviours (which can be relatively difficult to measure)¹¹¹. By contrast, in microorganisms, b and c can often be relatively easy to manipulate, whereas determining r in natural populations can pose problems. The relevant relatedness is that between individuals interacting through a social behaviour, which will depend on the population structure¹⁰³. For example, even when multiple lineages infect an area, such as a human host, spatial structuring could still lead to interactants being predominantly 'clone mates' and $r \approx 1$ (see [Supplementary Information S1](#) (box)).

Another complication is that the concept of relatedness can be harder to apply in microorganisms (see [Supplementary Information S1](#) (box)). One reason for this is because genes for cooperative behaviours can be transmitted horizontally between different bacterial lineages by mobile genetic elements, such as conjugative plasmids or lysogenic phages⁵⁵. It has been suggested that this can be a way of stopping the spread of cheats in a population by 'reinfected' them with cooperative behaviour⁵⁵. This fascinating possibility requires further theoretical and empirical exploration. For example, the extent to which this could be counteracted or aided by selection to avoid or take up such genetic elements is not clear. Another reason is that, even when an area is colonized by a single cooperative clone, mutation can lead to the loss of social behaviours. The appropriate measure of relatedness for a social trait is with respect to the locus controlling that

trait^{10,19}. This means that an individual who performs a cooperative behaviour is related by $r = 1$ to another individual who performs that behaviour, and by $r = 0$ to another individual (cheater or mutant) who does not¹⁷ (see **Supplementary Information S1** (box)). Consequently, mutation can produce ‘unrelated’ cheater lineages, which could spread through a population, especially long-term populations with limited dispersal¹¹², as seems to happen in long-term *P. aeruginosa* infections¹¹³.

Conclusions

Interdisciplinary research on the social behaviour of microorganisms is at the early stages of an exponential explosion. In most cases the social nature of traits is merely speculation that needs to be verified experimentally. Is QS really communication¹⁰⁹? Are biofilms really cooperative slime cities^{4,114}? Social evolution theory provides a conceptual framework for addressing these questions.

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Acknowledgements

We thank M. Camara, B. Evans, K. Foster, N. Mehdiabadi, S. Molin, A. Ross-Gillespie, J. Strassman and P. Williams for useful discussion and comments on the manuscript, and the Royal Society, the Biotechnology and Biological Sciences Research Council and the Natural Environment Research Council (UK) for funding.

Competing interests statement

The authors declare no competing financial interests.

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