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Strategies of microbial cheater control

Michael Travisano[1] and Gregory J. Velicer[2]

[1]Department of Biology and Biochemistry, University of Houston, Houston, Texas 77204, USA
[2]Department of Evolutionary Biology, Max-Planck Institute for Developmental Biology, Spemannstrasse 37, 72076 Tübingen, Germany

The potential benefits of cooperation in microorganisms can be undermined by genetic conflict within social groups, which can take the form of 'cheating'. For cooperation to succeed as an evolutionary strategy, the negative effects of such conflict must somehow be either prevented or mitigated. To generate an interpretive framework for future research in microbial behavioural ecology, here we outline a wide range of hypothetical mechanisms by which cheaters might be constrained.

CHEATING (see Glossary) arises from competition for the benefits derived from COOPERATION or ALTRUISM [1]. If benefits that result from COLLECTIVE ACTION [2,3] can be obtained without contribution, then cheaters might prosper to the detriment of social groups by reducing the value of cooperation among its members. Until recently, cheating, cooperation and sociality have not been commonly mentioned in discussions of microbes, however many of the factors affecting cheating dynamics in insect and animal societies can also be observed in simple microbial systems [4,5]. The evolution of cooperation, cheating and the restraint of cheating are fundamental issues in evolutionary theory that also have applied significance in the context of microbial pathogens with social life histories. Here we describe possible ways in which the negative group-level effects of cheating (or CHEATING LOAD [6]) in cooperative microbes might be restricted. In light of these potential strategies of cheater restraint, we also discuss how cheating might be affected by different microbial life histories, particularly in the myxobacteria and eukaryotic slime moulds that cooperatively develop into fruiting structures (Box 1).

Cheating load

Cheaters can have substantial negative consequences in the groups in which they are found. A familiar example of this can be observed in tumours and their consequent effects [7]. Tumour cells arise from normal cells as a result of mutation and as normal cells do, they benefit from access to nutrients and protection from the external environment that is provided by the body. However, in contrast to normal cells, tumour cells cheat by proliferating without restriction and without contributing to overall function. It is primarily the unrestricted growth of tumours that causes their deleterious effects, because surrounding tissues are disrupted. If cheating cells remain localized forming a benign tumour, cheating might have only modest effects. However, strongly deleterious effects are much more probable when cheating spreads throughout the body as occurs in metastatic cancer, resulting in tissue and organ failure. Therefore, the deleterious effects of cheating on the group are largely determined by the aggressiveness of growth and dispersive ability of tumour cells. The more aggressive the tumour, the greater the short-term benefit for the cheater (greater proliferation of the cheater genotype), but also greater is the likelihood of long-term costs as a result of increased mortality and reduced reproduction of the whole organism. The potential for cheating in multicellular organisms is often reduced because many have a unicellular stage (i.e. zygote) from which all cells are derived [8–11]. The clonality of somatic cells reduces the likelihood that selectively important differences among cells will arise, and promotes cooperation among cells to enhance the reproductive output of the group due to KIN SELECTION [12,13].

How do these points relate to cheating in microbes? To the extent that cooperation is important to fitness in microbial societies, the occurrence and spread of cheating individuals can be as deleterious to microbes as cancer is to a body. For example, the myxobacterium *Myxococcus xanthus* undergoes multicellular development and forms a

Glossary

ALTRUISM: Behaviour that has an individual fitness cost but provides a fitness benefit to others. Cooperative behaviour in the presence of cheaters constitutes altruism toward the cheaters.
CHEATING: Obtaining benefits from a collectively produced public good (see below) that are disproportionately large relative to a cheater's own contribution to that good.
CHEATING LOAD: The degree to which obligately defecting cheaters decrease the group-level benefits of cooperation in chimeric social groups.
COLLECTIVE ACTION: The combined effect of individual behaviours within a group.
COOPERATION: Proportional contribution by individuals to a collectively produced public good. (See [5] for distinctions between types of cooperation.)
DEFECTION: Disproportionately small contribution by individuals to a collectively produced public good. Biologically, defection does not necessarily entail cheating (i.e. gaining an advantage from defection). Some mechanisms of defection might not enhance the relative ability of defectors to exploit a relevant public good.
KIN SELECTION: Natural selection for alleles shared by close relatives that might cause individuals to behave in a manner that is detrimental to their own individual fitness but beneficial for the spread of the alleles under selection.
PUBLIC GOOD: Any fitness-enhancing resource that is accessible to multiple individuals within a local group. A pre-existing public good (such as rainwater) originates independently of the group that benefits from it. Alternatively, a collectively produced public good (such as an intercellular signal) is generated by members of the group that use it.

Corresponding authors: Michael Travisano (mtrav@mac.com), Gregory J. Velicer (gregory.velicer@tuebingen.mpg.de).

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Box 1. Lifestyles of *Myxococcus* and *Dictyostelium*

A common feature of myxobacteria and eukaryotic slime moulds is their cooperative mode of development [20,66]. In both cases, nutrient starvation causes masses of individual cells to gather and form distinct multicellular structures, which are stationary in *Myxococcus* and motile in *Dictyostelium*. When starvation is prolonged, aggregated groups from both genera form fruiting bodies in which only a portion of the population develops into stress-resistant spores. The spores remain dormant until they germinate when they encounter new resources. From this brief outline, it would appear that myxobacteria and slime moulds have converged to have remarkably similar life histories, despite their vast genetic and physiological differences [67,68]. However, this view is only partially correct because of behavioural differences before development.

Myxobacteria appear to be cooperative throughout most stages of their life cycle. After sporulation, they are thought to primarily forage in groups, collectively releasing antibiotics and enzymes into the environment that digest organic materials, including other microorganisms, which are then ingested for growth. Although social cohesion in *M. xanthus* is not absolutely obligate before development, it does move primarily in groups on most laboratory surfaces. Moreover, survival under stress and vegetative growth [69] can be enhanced by social interactions, at least under some laboratory conditions.

By contrast, *Dictyostelium* slime moulds are largely independent before development. Similar to myxobacteria, slime moulds are predatory, but importantly they attack their prey as individuals, chasing them down by chemotaxis and engulfing them by phagocytosis. Assuming that this difference in pre-development sociality (observed primarily in the laboratory) also occurs in nature, it might have broad implications for sociality during development. *Dictyostelium* slugs are less likely to be composed primarily of a few closely related genotypes [70], because its greater degree of individual cell movement allows more opportunity for distinct genotypes to mix. The lower degree of relatedness within fruiting bodies predicted for *Dictyostelium* by this reasoning would encourage greater social conflict [18,39] because there would be less opportunity for kin selection to occur relative to the myxobacteria, which appear to have little genetic diversity within individual fruiting bodies (M. Vos and G. Velicer, unpublished).

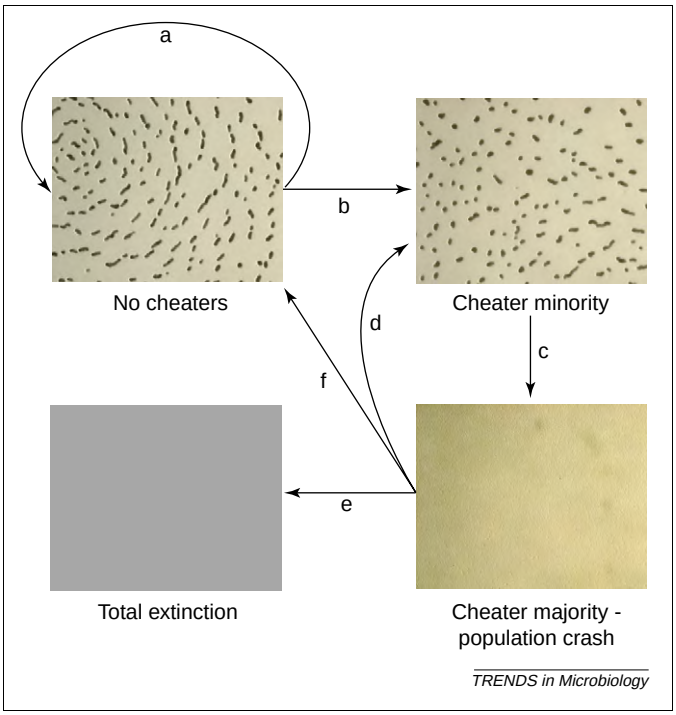

Figure 1. Cheater-induced extinction events during competitions between wild-type and cheater genotypes of *Myxococcus xanthus*. **(a)** In the absence of cheaters, the wild-type forms healthy fruiting bodies (upper left) over repeated cycles of starvation-induced development. **(b)** Cheaters were mixed as a small minority (1%) with wild-type and the mixed populations allowed to undergo repeated cycles of development and vegetative growth. The cheaters used in these experiments are themselves severely defective at development in pure culture, but sporulate more efficiently than the wild-type when present as a minority in mixed populations. In the initial round of development after cheater introduction, cheaters had little effect on fruiting body morphology (upper right) or total spore production. **(c)** Cheaters rapidly rose to high frequency, however, causing failure of normal development (lower right) and population crashes after reaching majority status. After such crashes, three distinct population fates were observed. **(d)** In some cases, both the cheater and wild-type survived the crash, but with the wild-type having regained the majority. **(e)** In other competitions, the crash was so severe that either the entire population went extinct or **(f)** only the wild-type survived while the cheater faced extinction. The quantitative population dynamics for these experiments can be found in [6]. Images are shown at 3× magnification.

spore-containing fruiting body when nutrients become scarce (Box 1) [14]. When cheaters are common, however, they can severely reduce population size by disrupting fruiting body formation and sporulation [15], even to the extent of driving populations to extinction (Figure 1) [6]. In *Pseudomonas flourescens*, cooperatively formed biofilm mats are disrupted by genotypes that fail to contribute to mat coherence and stability but that benefit by growing within the biofilm [16]. Even in *Saccharomyces cerevisiae*, cheaters can benefit from the activity of cooperator genotypes, as cheater genotypes do not produce a diffusable, extracellularly excreted enzyme that aids in nutrient uptake [17]. Therefore cheaters can be evolutionarily successful (at least in the short-term), and cooperating genotypes, such as the cells that make up a multicellular organism, face the problem of how to prevent or alleviate the costs of cheating. However, the potential for cheating is much higher in populations of social microbes, such as myxobacteria and slime moulds, because in contrast to the cell populations in most multicellular organisms, these microbes might arise from mixtures of distinct genetic lineages [15,18] (F. Fiegna and G. Velicer, unpublished). In social microbes, the potential for kin selection to enhance the fitness of other individuals in the society is muted with respect to cells in a multicellular organism, because many individuals might be unrelated.

Societal control in microbes

An understanding of cheating in social microbes can be obtained by testing specific evolutionary and mechanistic hypotheses. To facilitate the testing of species-specific hypotheses, several mechanisms that might be used to restrain the spread of cheaters are described. Some readers might argue that one or more of the proposed scenarios require an implausible degree of sophistication on the part of microbes. Nonetheless, explicit consideration of a broad range of theoretical possibilities could help to prevent premature dismissals. Moreover, these hypotheses are intended to be provocative and to encourage new avenues of research on quorum-sensing, biofilms, virulence factor production and other forms of microbial cooperation. Although the manifestation of each category would differ across biological systems, the underlying concepts are equally relevant to microbial, insect, animal and human societies. For illustrative purposes, we focus on the well-known developmental

processes of *Myxococcus* [14,19] and *Dictyostelium* [20], in which individuals cooperate to build spore-bearing fruiting bodies but not all participants gain the social benefit of becoming a spore.

The common feature of cheating in *Myxococcus* and *Dictyostelium* development is that cheaters are over-represented in the spore population at the end of development relative to their initial frequency at the beginning [15,18,21]. Such an exploitative advantage in developmental mixtures of cheaters with non-cheaters might be accomplished in at least two distinct ways. First, DEFECTION from the production of an intercellular signal that is essential for fruiting body formation might give defectors an unfair advantage in the process of spore differentiation, resulting in a greater percentage of defector genotypes becoming spores than non-defectors. Alternatively, cheaters might evolve mechanisms of avoiding the fate of developmental cell death, which takes the form of cell autolysis in *Myxococcus* [14,22–25] and stalk construction in *Dictyostelium* [20]. Sophisticated cheaters might use a combination of these strategies.

Here possible anti-cheating mechanisms are discussed primarily in the context of developmental signalling, although the concepts are also broadly relevant to other cooperative microbial behaviours. Specific intercellular signals are required for successful development and are also focal points for the detection and control of cheating. Generally, signals are either 'intimate' or 'diffusive'. Intimate signals are physically associated with cooperating individuals, as in the cell-surface-associated C-signal of *M. xanthus*, which induces fruiting body morphogenesis [26–29]. Intimate signals can serve as cooperator identification tags, referred to as C-IDs and encoded by 'greenbeard' alleles [30,31]. These alleles can recognize copies of themselves in other individuals by a trait (a C-ID) that they encode. In principle, the presence or absence of C-IDs might allow cooperators to behave differently toward fellow cooperators and cheaters, respectively. For example, the *csA* gene (a greenbeard allele) in *D. discoidium* [32,33] encodes a cell membrane protein (a C-ID) that causes adhesion of cooperator genotypes and promotes their migration into the fruiting body. Diffusive signals are released into the common environment and do not remain tightly associated with their source cells, resulting in anonymity of signal contribution. Hence, diffusive signals cannot serve as direct C-IDs, but could potentially bind to C-IDs on other cells and therefore serve to identify cooperators. In *M. xanthus*, the A-signal that serves as a quorum-sensing initiator of development is a subset of amino acids [34] that appears to be diffusive.

Two strategies

Cooperative genotypes have two primary strategies by which they might reduce or eliminate cheaters and their effects within social groups: targeted benefit and targeted punishment. In the benefit strategy, individuals that contribute to the group (cooperators) gain access to group-associated benefits that are not available to cheaters. Therefore, cooperators acquire some benefits that might vary with their social contribution. In the punishment strategy, individuals not contributing to the beneficial group phenotype are actively punished by cooperators. In both strategies, cheaters are at a disadvantage to cooperators. However, the differences between the two strategies arise in their implementation and modes of circumvention. The benefit strategy can be implemented by tight integration of the mechanisms of cooperation to the modes by which social benefits are conferred. Cheaters arise by somehow breaking that connection, accruing benefits without cooperation.

The punishment strategy is more complicated both for cheaters to overcome and for cooperators to implement. To succeed within a punishment system, cheaters must not only overcome integration of altruistic behaviour (cooperation) to gain access to benefits, but must do so while punishing behaviours are directed toward them by cooperators. For punishment to occur, at least a portion of cooperators must possess specialized cheater-inhibition or killer traits that are analogous to the targeted killing of cancer cells by specialized cytolytic T cells of the mammalian immune system [35]. Punishment, by our definition, involves more than simply the absence of benefits that are conferred by social interactions.

Benefit-based strategies

Intrinsic defector inferiority

The most direct way for social genes to spread and persist would be to make their loss inherently costly. This could be accomplished by the evolution of a tight coupling of access to group benefits to mechanisms of developmental signal expression, as seen with the *csA* gene and its product in *D. discoidium*. Only those genotypes expressing correct developmental signals would promote development or suffer any individual cost to development. However, these would also be the only genotypes to have full access to group benefits. Because there is a strong selective pressure for cheating by defection to avoid the individual costs of cooperation, the social systems in which such cheating is mechanistically difficult to achieve would be advantageous over those where the selfish benefits of cheating are easily obtained by simple defection mutations. The tighter the connection between expression of developmental signals and exclusivity in development, the more difficult it is for the evolution of mutant alleles that allow access to benefits of development without the cost (Box 2).

Quorum-sensing as cheater control

When social defectors are physiologically capable of exploiting contributor signals (i.e. they cheat), quorum-sensing can provide cooperators with an advantage. Consider a signal cascade of at least two signals, for example, 'X' and 'Y', that act at distinct but consecutive stages (X first) of a cooperative process and that X-defectors can advantageously exploit Y. Suppose also that contribution of Y is only advantageous when X is present above a minimum threshold concentration. Under these conditions, it would be advantageous for potential Y-contributors to make Y production dependent on the concentration of X, which is simply a mechanism of quorum-sensing. This not only prevents costly Y production when the total population density is too low, but

Box 2. Killer immunity

Production of diffusive bacteriocins by some strains of *Escherichia coli* and other bacteria [45] is a well-known form of microbial altruism [71,72] with multiple facets that relate to cheater control. Colicins, which are bacteriocins carried by *E. coli*, are encoded by tightly linked three-gene clusters that also encode immunity and lysis genes and that typically reside on plasmids [45]. Colicin production kills unprotected competitors, thereby reserving resources for colicin-producing cells. But it is also costly because occasional cell lysis is the general mechanism for release of colicins into the environment. In this system, the three-gene cluster can be viewed as a greenbeard allele, the immunity protein as a C-ID (cooperator identification tag), and the toxin serves as a diffusible signal that discriminates between immune cooperators and sensitive individuals without the C-ID. Sensitive genotypes include those that have defected from cooperation, by loss of the cluster or silencing of the genes, as well as unrelated cells.

Colicin production exemplifies three cheater control categories: intrinsic defector inferiority (IDI), xenophobia and policing. Colicinogenic strains are protected from at least one form of defection within their own ranks by IDI. Defection from costly colicin production by loss of the entire three-gene cluster is prevented by the need to retain colicin immunity in the presence of colicin-producing individuals. To bypass this built-in cheater control, defectors would need to eliminate colicin production or lysis gene expression while retaining expression of the immunity gene [73]. Moreover, the system provides inherent xenophobic protection from cheating by simply killing all neighbouring unrelated genotypes that are sensitive to colicins. Finally, because this mode of IDI results from susceptibility to a harmful diffusive compound produced by altruists, it can also be viewed as a form of policing in which a costly compound provides a general group benefit (the killing of all proximate non-immune competitors), which inherently includes the killing of at least some defectors as well. In this instance, however, the policing function did not evolve to protect the fruits of a cooperative behaviour that is distinct from the policing itself. Rather, policing is built into the very structure of an altruistic behaviour that evolved as a generalized anti-competitor strategy. Any defectors that lose the entire three-gene cluster are inherently inferior because their defection puts them into the class of 'strangers' that are the objects of xenophobia. The automatic classification of such defectors as strangers can be viewed as built-in policing.

also when the frequency of X-defectors is too high, even if the total density is also high. Therefore, cooperators might 'assess' the probable cheating load and adjust their developmental strategy accordingly. In this scenario, rare X-defectors can invade a population consisting entirely or primarily of cooperators. However, upon reaching sufficiently high frequency the X-defectors cause cooperative production of Y to cease and thereby lose their advantage from social defection. Under conditions in which the social interactions being cheated upon are very important to fitness, such a 'shutdown' strategy would be counter-productive, and quorum-sensing would be unlikely to specifically evolve as a mechanism to prevent cheating. Cheater control through quorum-sensing, if it occurs, is probably an indirect by-product of positive selection for other social benefits that are conferred by quorum-sensing.

Conditional misers

The most sophisticated benefit strategy requires the individualized response of microbes to the presence of C-IDs on other cells. If cells defect from producing an intimate signal that also serves as a C-ID, they might be actively and specifically excluded from social benefits. For example, cooperation might require a signal cascade with an initial signal (Q) followed by a subsequent signal (R) that is tied directly to the social benefit. Cells that fail to produce the first signal (Q) might not be included in the subsequent signalling (R) that is essential for the social benefit. In this scenario, fellow Q-contributors recognize one another and proceed to share a subsequent signal (R), but automatically withhold R from any cell that lacks the C-ID that tags Q-contribution.

Punishment-based strategies

Policing

Cooperative genotypes might take more aggressive action against defectors by targeted 'policing', where a pro-active punishment harms defectors but not cooperators. This is similar to the way that major defections from tax contribution are sometimes punished by fines or jail-time in human societies. In our definition, such punishment decreases defector fitness more than mere exclusion from access to benefits. For example, a toxin to which only defectors are susceptible might be generated by contributors, as occurs in bacterial colicin production (Box 2) and is thought to occur in k1 killer toxin expression and immunity in *S. cerevisiae* [36].

Anti-defector policing [37,38] that is costly to enact would be an instance of second-order cooperation and might be performed by all signal-cooperators (as above). Policing itself would then be open to cheating by defection. However, policing might also be accomplished by only a subset of the signal-cooperators and the subsequent benefits of such policing could arise in two ways. Policing might evolve as an altruistic trait, in which police suffer a fitness decrease on behalf of their non-policing, signal-contributing kin. This type of policing would require some form of group-level selection (e.g. kin selection [39]) to evolve and be maintained by natural selection [40]. Alternatively, the fitness costs of policing might be offset by benefits that are only associated with a particular policing function. For example, the resources of cheaters (signal-defectors) could be sequestered by those cells that undertake policing, such as would occur in cannibalism [41]. In this case, policing would be open to cheating by 'corrupt' policers that 'punish' non-cheaters as well as cheaters to acquire even more resources for themselves [42].

Xenophobia

Are all cheaters enemies from within or are there also foreign invaders? In social insects, many known examples of cheating involve queens that usurp a colony of a distinct species and use its workers to raise their own offspring [43]. In at least some cases, this is accomplished by host-odour imitation as a rejection-avoidance strategy [44]. If cheating across kin boundaries is common in microbes, although these boundaries remain obscure, an effective strategy of cheater control might be a form of generalized xenophobia. Possible mechanisms of 'stranger' exclusion include the production of compounds that kill, hinder, repel or physically exclude alien genotypes, whether they are cheaters or not. Xenophobia is common among bacteria

in the form of toxin secretion [45]. Such interference competition might serve not only to protect resources from competitors, or turn competitors themselves into resources, but might also serve to exclude potential cheaters (Box 2).

However, what if a society of close relatives suffers more from cheating by their own kin than from resident foreigners? Several documented cases of microbial cheating suggest that this might often be the case among microbes [5,15,46,47]. Many of the cheaters in these studies were closely related to the exploited cooperative strain, in some cases possibly differing by only a single mutation. If most cheating occurs via simple non-contribution mutations in previously cooperative lineages of a social group, then generalized interference competition will be ineffective against cheaters. Moreover, anti-cheater detection and punishment (i.e. policing) among close microbial relatives could be mechanistically difficult to evolve owing to the overall similarity of cheaters to non-cheaters.

Pure colonization

If the effects of cheating are particularly severe, then selection against cheaters can potentially occur as a function of population structure rather than direct selection either for cooperators or against cheaters within populations [48,49]. This mode of selection is somewhat akin to 'opting out' or leaving one social group to start or join another, and is a frequently invoked strategy to prevent cheating in human societies [50,51]. However, to be an evolutionarily successful strategy for microbes it would require conditions in which natural selection acts simultaneously at multiple levels of biological organization [52–56]. If cooperative behaviour provides a substantial benefit to individual fitness, then the impact of cheating on cooperative groups and their non-cheating members can be large (Figure 1) [6]. In the absence of an effective method to contain the effects of cheating within populations, selection against cheating might occur between populations. Populations founded with few or no cheaters, or those populations founded with genotypes that do not readily evolve cheating genotypes, will have a selective advantage to those suffering from high cheating loads. However, this mode of evolution can be limited because it requires that differences between groups rather than between individuals be the primary source of fitness variation [57–59].

The manner in which new social groups are established is an important evolutionary parameter [43,60] and might play a significant role in determining the fates of microbial cheaters. If new groups are often founded by single individuals (or small groups of identical clones), within-group genetic diversity in general, and cheaters in particular, can be regularly purged by new acts of colonization. Resulting groups of pure cooperators will have an advantage over groups with high cheating loads. Interestingly, *M. xanthus* has two distinct motility systems, one of which ('S-motility') is social in nature and therefore requires high cell density to function efficiently [61]. During both S-motility and sporulation within fruiting bodies, cells are bound together by a complex web of extracellular fibrils [62] that hinder single cells from independently establishing new distinct colonies. The second motility system ('A-motility'), however, allows cells to move as isolated individuals and might therefore provide a means of shedding cheaters by clonal colonization [63]. The possibility that the motility behaviour of *M. xanthus* (group versus individualistic) might be affected by the presence of cheaters (or genetic diversity *per se*) within local groups is intriguing, even though the relative roles of A- and S-motility in the natural life history of *M. xanthus* remain unclear.

Perspective

Just how sophisticated can microbial cheating and anti-cheating strategies be? In principle, there are two modes of defection by which cheating might occur: obligate and facultative. Obligate defectors are unable to alter their defector status as a function of group composition. In other words, obligate defectors do not cooperate any more with fellow defectors than they do with non-defectors, and pure groups of obligate defectors are at a disadvantage to pure cooperator groups (at least when cooperation is important for fitness). Alternatively, facultative defectors can modify their behaviour to be exploitative toward unrelated genotypes but cooperative to their own kin, and are therefore not socially defective in pure groups. For simplicity, our discussion here has been limited to cheating via obligate defection, but facultative cheating might be common. One study [18] suggests that at least *Dictyostelium* might be capable of evolving such complex strategies of facultative defection [5].

Any major transition in evolution [11,40] requires mechanisms to mitigate the negative effects of competition among individuals within the new cooperative social unit. Even simple evolutionary transitions to primitive forms of cooperation [16,64] can, in principle, be undermined by the appearance of cheaters [16]. Failure to mediate conflict at one level leads to disintegration of cooperative functions at higher levels [16]. In the transition from fully independent single cells to populations of relatively loose association (such as myxobacteria and slime moulds) and to cells with strong associations (such as those within a metazoan organism), increasingly complex strategies of cheater control will possibly occur. One mode of increasing sophistication is to simultaneously incorporate multiple strategies. For example, many metazoans use both of the penalization strategies (i.e. policing and xenophobia) and the pure-colonization strategy (by passage through a single-cell bottleneck).

Have any behaviours of cooperative microbes evolved specifically to constrain the negative effects of cheaters on cooperative endeavours? If so, have cheaters subsequently evolved constraint-avoidance strategies? More broadly, are there long-term co-evolutionary 'arms races' between cheating and anti-cheating strategies [65], and are such strategies widespread throughout microbial interactions as might be observed in a biofilm? As the mechanistic details and population dynamics of cooperative microbial societies become better understood (Box 3), these intriguing questions are likely to become important for understanding fundamental aspects of microbial social behaviour.

Box 3. Goals for microbial behavioural ecology and social evolution

Long-term goals of these disciplines are to understand how cooperation evolves in microorganisms and to assess its importance in nature. To achieve these numerous intermediate goals need to be met:

i. Rigorously demonstrate cooperative behaviour in microbial systems.
ii. Demonstrate that cheating is at least theoretically possible.
iii. Identify the genes involved in sociality and cheating.
iv. Make evolutionary predictions and test them directly.
v. Test hypotheses about potential mechanisms of cheater control.
vi. Examine the generality of the genetic results by 'bioinformatic' surveys.

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