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Inferring adaptation in social microbes from experimental evolution under relaxed selection

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by
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ABSTRACT OF THE DISSERTATION

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Microbes exist against a backdrop of other organisms, and the interactions between microbes have major consequences on their traits, their evolution, and their impact on the world. Microbial interactions and the adaptations that enable them are extremely diverse – they can unlock abilities beyond the reach of individual cells or lead to a population's destruction, they can be temporary or permanent, they can be between genetically identical cells or different species entirely. The first chapter of this dissertation reviews microbial interactions and the related concept of the evolution of conflict and cooperation.

To be certain a trait is an adaptation at all, a researcher must demonstrate both that the trait benefits an organism's fitness but also *how* it benefits fitness – that is, what the selective pressures are, if any, that resulted in the trait's evolution and maintenance. Microbes' small size

and the resulting difficulty of observing microbial interactions directly makes this an especial challenge for microbes and behooves the use of creative approaches.

This dissertation focuses upon the social amoeba *Dictyostelium discoideum*, which engages in interesting interactions both within its own species and with other microbial species. To infer the existence of adaptations in *D. discoideum* and its partners to these interactions, I employed an experimental evolution approach. Unlike many experimental evolution experiments, which involve applying an artificial selective pressure to a population and observing how it adapts, my studies instead focus on removing existing selective pressures as a way to infer what they were in the first place. I am looking for adaptations by evolving them away.

In my second chapter, I apply this concept to look for evidence of *D. discoideum* adaptations to cheating. *D. discoideum* undergoes a social life cycle wherein potentially unrelated cells aggregate to produce chimeric multicellular structures that should be especially vulnerable to exploitation by uncooperative cheater genotypes. While this phenomenon is well studied in the laboratory, some disagreement remains about its adaptive role in nature. To what extent is cheating (or resisting being cheated by others) an important selective pressure on *D. discoideum* in nature? In my study I evolved *D. discoideum* under conditions in which cheating was impossible and thus any selective pressure to cheat should be relaxed. I found evidence that under these conditions, *D. discoideum* evolves a reduced ability to cheat (or an increased susceptibility to being cheated). These results are consistent with other studies indicating an adaptive role for cheating in nature, and validate my approach as a way to study other microbial traits with difficult-to-assess adaptive values.

In my final chapter, I apply a similar approach to a more complicated interaction that occurs between *D. discoideum* and three endosymbionts in the genus *Paraburkholderia*. In nature *D. discoideum-Paraburkholderia* interactions appear to be common and past studies suggest there are positive and negative fitness consequences for both partners. In an attempt to characterize whether these interactions have a history consistent with an overall cooperative or antagonistic relationship, I experimentally evolved multiple strains of *D. discoideum* and several bacterial species without access to one another. Without partners, any selective pressures normally exerted by *D. discoideum* and its endosymbionts upon one another should be relaxed. My results provided evidence of antagonistic adaptations on the part of some symbionts, but few overall or species-level trends. Instead, different strains appeared to respond to experimental evolution differently. These results suggest that *D. discoideum* and *Paraburkholderia*'s relationship may differ considerably in character depending on the strains involved, and reinforce the need to design experiments with strain variation in mind when studying microbial interactions.

Chapter 1: Microbes: Social Evolution

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1.1 Abstract

Microbes engage in diverse interactions to which social evolution theory developed for animals can be usefully applied. In turn, studies on microbes offer insight into social evolution theory as it applies to larger organisms. Here we review key evolutionary concepts as applied to microbial interactions, then describe some prominent examples of how microbes interact to obtain resources, communicate, move, attack and defend themselves from competitors, prey, or predators, and influence multicellular hosts.

1.2 Introduction

Microbes live rich social lives. They collaborate and compete; they are predators and prey; they are friends and enemies. Microbes live in groups and have for a very long time. Some of the oldest fossils are stromatolites – 3.5 billion year old fossilized biofilms of a sort that can still be found living in modern hypersaline lagoons(Walter 1977). Microbes often live in large

populations and at high densities, often including many species in close proximity. They communicate with kin and non-kin, share and compete, sacrifice for allies, and poison their foes. Their interactions run the gamut from simple to complex, from facultative to obligate, from friendly to lethal.

Many of these interactions find easy comparison to those of more familiar organisms. The soil bacterium *Myxococcus xanthus* is a social predator, swarming across the soil in huge groups searching for bacterial prey(Velicer and Vos 2009). It does not take much imagination to see why their groups are called wolf packs. *Bacillus subtilis* clones cooperate to build towering biofilm structures that call to mind the mounds of African termites, complete with circulatory systems shaped to draw oxygen inside(Wilking, Zaburdaev et al. 2013). Other microbes interact in ways that are unique to microbes, like magnetotactic bacteria that align themselves into multicellular magnets(Keim, Martins et al. 2004).

Many of the central tools used to understand sociality in microbes are ideas developed by animal behaviorists. Microbes lack conventional nervous or endocrine systems, and yet, as in animals, the evolution and ecology of their behavior is dominated by concepts of cost and benefit, direct and indirect fitness benefits, mutualism and parasitism, and exploitation and kin recognition. Careful application of these concepts can guide our understanding of microbes' evolutionary past and future and their considerable influence on human health, agriculture, and ecosystem function.

In turn, microbes can help us understand non-microbial life. With their uniquely small size, huge populations, metabolic diversity, genetic tractability, and rapid generation times, microbes are useful models that enable experiments testing theories in behavior, evolution,

population biology, and ecology that would be impractical with larger organisms. Interactions between microbes even inform our understanding of cell-cell interactions in multicellular organisms. Eukaryotes only came about due to cooperation between microbes, a major evolutionary transition that set the stage for the immense morphological diversity of the past half billion years(Hedges, Blair et al. 2004). The line between microbial sociality and social (and non-social) function in multicellular eukaryotes is blurrier than one might expect.

In this review we first outline key evolutionary concepts as applied to microbial interactions, then describe some prominent examples of how microbes interact to obtain resources, to communicate, to move and disperse, to attack and defend, and to influence multicellular hosts. Where possible, we will relate microbial examples to conceptually similar examples in macrobes. The term "microbes" here means nothing apart from small size, and encompasses a vast diversity of unicellular and even some multicellular organisms. The examples in this review primarily feature bacteria, but conceptually analogous examples can be found in archaea, viruses, and microbial eukaryotes as well.

1.2.1 Microbial Organismality – What is a Microbial Individual?

Microbes can straddle the line between single-celled and multicellular life. Many microbes live in densely-packed groups, some with complex morphology that calls to mind the tissues of animals and other large eukaryotes. This complexity has led some researchers to liken some microbial groups not to populations or communities of interacting individuals, but rather to multicellular organisms in their own right.

When is this comparison appropriate? What is a microbial individual? Is it a single cell, or is it a group of cells? At what level of organization is the fitness of a trait expressed? In animals, the answer to these questions is often too obvious to warrant much thought, but the same ambiguities surround some clonal, colonial, or chimeric species. Understanding the evolution of a worker ant's traits is sometimes helped by considering the ant's entire colony, not just the ant, an individual. The same can be true for some microbial groups.

Degree of cooperation and conflict is the key. An individual organism need not be a single body, but its constituent units must have closely aligned fitness interests(Queller and Strassmann 2009). The balance of cooperation and conflict in a group dictates to what degree it is appropriate to assign a single fitness to it (rather than separate fitnesses for its constituent units). Importantly, 'individual' or 'organism' need not be binary terms – groups with different degrees of cooperation and conflict may be thought of as having different degrees of 'organismality'.

The degree of cooperation and conflict – and thus the organismality – of microbial groups depends upon their ecological and environmental context. Most research on microbes is performed on a single clonal lineage of a single species. Under these conditions, genetic conflicts of interest are minimized, at least until mutation generates new genetic diversity within the experimental population. In nature, however, microbes often live in close association with many competing strains, both conspecific and heterospecific. With genetic differences come potential conflicts, and so highly diverse natural bacterial groups are rarely organismal in the same way the clonal cells in an animal body are. Recent advances in our awareness of microbial sociality have sometimes inspired overzealousness in interpreting microbial traits as adaptive cooperation(Nadell, Xavier et al. 2008). For most microbes, it is most helpful to think of a clonal

lineage as an individual organism (Figure 1.1). This approach can help explain the evolution of traits and behaviors – like programmed cell death – that are impossible to justify from the perspective of a single cell without overlooking the major role played by conflict between competing lineages.
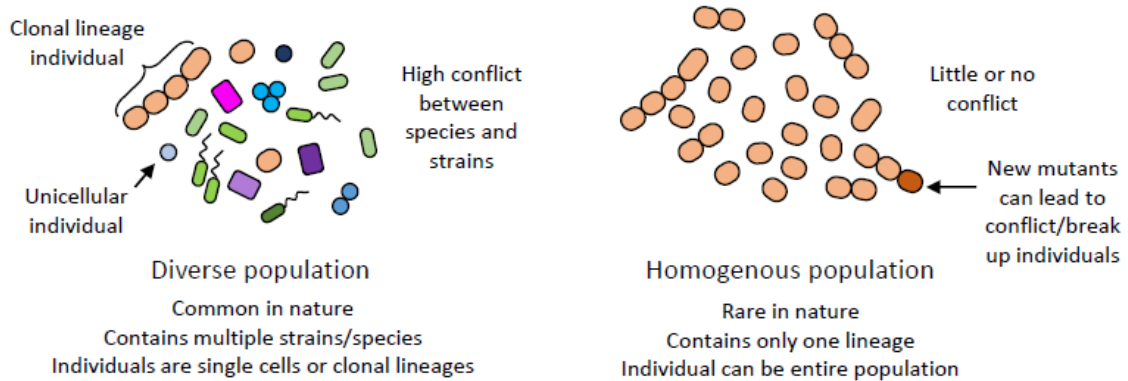
Figure 1.1 - Microbial individuals. Microbial individuals are usually single cells or clonal lineages. Genetic differences between strains or species drive conflict between the members of most natural microbial communities. In rare situations when populations are clonal, the entire population may be considered a single individual. Even these, however, break down as mutations occur and introduce new variation.

1.2.2 Microbial Cooperation

Just as lions band together in prides or wildebeest in herds, most microbes are gregarious and live in groups. Many microbes cooperate within species to gather resources, move, attack, or defend themselves in ways that would be impossible for single cells. As in animals, key to understanding the evolution of intraspecific cooperation in microbes is distinguishing between

cooperation that results in a direct fitness benefit for the cooperators and altruistic cooperation, wherein an individual pays a net fitness cost to confer a fitness benefit on a recipient.

Cooperation driven by direct fitness benefits can often be thought of as synergy achieved by performing a task cooperatively that might otherwise be performed alone. Such interactions result in all cooperators gaining fitness from the decision to cooperate, and are widespread in microbes and larger organisms alike. Often more interesting, however, are cases of altruistic cooperation, wherein at least some cooperators incur a net decrease of fitness from cooperating. Altruistic interactions within colonies of ants – wherein most individuals entirely sacrifice their own reproduction to facilitate their queen's – puzzled Charles Darwin, for how could selection result in a trait which reduced fitness? Darwin speculated that selection on family groups may hold the explanation. A century later, this idea was expanded on and formalized as the concept of kin selection on inclusive fitness(Hamilton 1964)**.** An individual's inclusive fitness incorporates not only the impact of its traits on its own reproduction, but also the impact on relatives who may share the genes underlying those traits. Hamilton's Rule mathematically describes the conditions under which costly – even suicidal – traits can be selected for, and has proven key in understanding altruistic behavior in microbes and macrobes alike.

Kin selection as formalized by Hamilton's Rule emphasizes the importance of three parameters: the cost of a trait to an actor, the benefit of the trait to the recipients, and the relatedness between actors and recipients. Estimating these parameters, especially in natural contexts, is crucial but often non-trivial because costs and benefits are not absolute measures but relative to the selfish alternatives of donor and recipient. Costs and benefits of microbial interactions can vary considerably. The most extreme examples involve – like the ants that puzzled Darwin – some participants sacrificing all fitness, either by specializing in exclusively

non-reproductive tasks or even actively destroying themselves. For such costly traits to evolve, the benefits they achieve must be substantial and directed as much as possible to close relatives. Mixed populations of multiple species or even just multiple strains of a single species should not generally evolve altruism.

High relatedness is key for most altruistic traits and there are many ways to achieve it. Mechanisms that direct benefits preferentially to relatives (or harm preferentially to non-relatives) are collectively known as kin discrimination(Strassmann, Gilbert et al. 2011)**.** Simplest and probably most important of these for microbes is limited dispersal. When dispersal is limited, local interactions are most likely to occur between close relatives. Many microbes reproduce clonally and move slowly, resulting in patches of genetically identical cells descended from a single progenitor, especially when a small propagule disperses to a previously-unoccupied patch. At high densities, limited space can drive spontaneous segregation of low-relatedness populations into high-relatedness sectors, such that cooperating strains are likely to interact with cooperating relatives and non-cooperators with non-cooperators (Nadell, Foster et al. 2010).

More complex kin discrimination mechanisms increase relatedness via differential effects on relatives and non-relatives. In animals, kin discrimination is often a function of memory or learning, such as when ants and other social insects guard their colonies against conspecifics lacking the correct cues. Without brains, microbes must take different approaches. Some microbes resist mixing with non-relatives – unrelated colonies of the bacterium *Proteus mirabilis* and many other bacteria create distinct boundaries called Dienes lines at points of contact, rather than merging into a larger group (Budding, Ingham et al. 2009). Other microbes express adhesion molecules which facilitate aggregation with other cells bearing the same adhesion

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factors(Smukalla, Caldara et al. 2008). Still other microbes benefit relatives by destroying non-relatives via the secretion of bacteriocins that kill cells lacking the correct immunity gene. Such systems destroy conspecific competitors but leave close kin unharmed, and can increase local relatedness and facilitate the evolution of cooperative traits.

When relatedness is low, exploitation can follow and destabilize altruistic traits. Even an altruistic trait that strongly benefitted a population of cooperators is vulnerable in competition with individuals who benefit from cooperation but do not pay the costs to cooperate themselves. Such individuals gain individual fitness benefits at the expense of the population as a whole. Economic and evolutionary theory calls this phenomenon the tragedy of the commons, and it has been a special focus of evolutionary biology for decades to explain why such conflicts do not preclude the evolution and maintenance of cooperation. Much empirical work suggests that in microbes, at least, they often do.

Microbes perform many biological processes by secreting chemicals into their environment. These chemicals can metabolize resources, defend against attack, facilitate microbial movement, or even communicate information, but they also render many microbes especially vulnerable to the threat of exploitation. Secreted chemicals are energetically expensive and public – their benefits can be enjoyed not just by the producer but by neighboring cells as well – and thus in microbes even many apparently non-social functions gain a social element. Combined with microbes' fast generation times and high mutation rates, this lack of privatization makes exploitation a particular obstacle to cooperation in many microbes, and in fact mutants deficient in cooperative traits are frequently isolated from laboratory, wild, and clinical microbial populations(Rainey and Rainey 2003, West and Buckling 2003, Dénervaud, TuQuoc et al. 2004).

A. Division of labor in Cyanobacteria

light

O_2

sugars

nitrogenase

CO_2

Vegetative cell photosynthesizes sugars

NH_4^+

Heterocyst fixes nitrogen

N_2

B. Bacterial quorum sensing

Unbound receptor is inactive

Bound receptor binds to promoter

Gene is expressed

Low cell density
Low autoinducer concentration

High cell density
High autoinducer concentration

C. Social development in *Dictyostelium discoideum*

Unicellular amoebae starve and aggregate

Stalk cells die during fruiting body formation

Amoebae   Mound   Mobile slug   Fruiting body

D. Biofilm development in *Pseudomonas aeruginosa*

Dispersal

Secreted matrix

Channels

Colonization   Microcolony formation   Mature biofilm

E. Symbiont exploitation in *Candidatus* Hodgkinia cicadicola

Amino acids

Symbionts provide amino acids within host crypt

Within-host competition leads to complementary gene loss

Larger symbiont populations required to maintain benefits

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Figure 1.2 - Example microbial interactions. **A. Division of labor in Cyanobacteria**. Some cells within clonal filaments differentiate into heterocysts (large, round cell, right). Heterocysts abandon oxygen-producing photosynthesis in order to fix nitrogen with the oxygen-sensitive enzyme nitrogenase. Vegetative and heterocyst cells divide labor by exchanging sugars and nitrogen. **B. Bacterial quorum sensing.** Quorum sensing cells constitutively secrete autoinducer (yellow triangles). At low cell densities, autoinducer concentration remains low and autoinducer-activated transcription factors (orange circles) are inactive. At high cell densities, autoinducer binds to and activates transcription factors, which in turn bind to and activate gene expression. **C. Social development in *Dictyostelium discoideum*.** Upon starving, vegetative amoebae aggregate into a mobile, multicellular slug, which moves through the soil. Fruiting body formation follows, during which a minority of the cells (blue) sacrifice themselves to develop into a stalk to hold the remainder of the aggregate (red) as it develops into durable spores. **D. Biofilm development in *Pseudomonas aeruginosa*.** Cells adhere to and colonize a surface through a combination of active migration and division. Secreted extracellular matrix components (dark green) accumulate into a complex structure. Cells detach and disperse from the biofilm's upper layer via autolysis of cells in the lower layer. **E. Symbiont exploitation in *Candidatus* Hodgkinia cicadicola.** Hodgkinia cells live within specialized cells in the abdomens of 17-year cicadas (*Magicicada tredecim*) and produce amino acids (yellow and orange triangles) required by their hosts. Competition between strains within a single host favors fast growth rates, selecting for loss of amino acid production. In time, strains fragment into multiple complementary lineages, each producing only a fraction of the necessary amino acids. The host must accommodate increasingly large symbiont populations to maintain sufficient amino acid production.

1.3 How Microbes Use Sociality

1.3.1 Obtaining Extracellular Resources

Not all resources can be directly drawn from the environment. Often resources exist in an unavailable form, tied up in molecules too large or unwieldy to be imported into the cell. Many microbes secrete exoproducts – often protein enzymes – to liberate these resources. For example, *Myxococcus xanthus* and its relatives are soil bacteria that actively prey on other bacteria by secreting biolytic toxins to kill and enzymes to digest their prey (Daft, Burnham et al. 1985).

Another family of exoproducts are siderophores, chemically-diverse macromolecules produced by most major bacterial lineages in order to sequester iron from their environments (Hider and Kong 2010). Though iron is one of the most abundant elements on earth, the vast majority of it exists in the biologically-unavailable ferric form (Fe^{2+}), and so for many microbes it is a crucial limiting resource. Diverse bacteria solve this limitation by producing and secreting siderophores which bind ferric iron with extremely high avidity. Once bound, siderophores are taken up again into the cells. Cytoplasmic enzymes catalyze the conversion of ferric iron into ferrous iron (Fe^{3+}), nondestructively removing it so that the siderophores can be secreted and used again. Siderophores allow microbes to survive in environments with very low iron concentrations, including inside the bodies of macrobe hosts.

Exoenzymes and siderophores are relatively energetically expensive to produce and secrete, and once secreted can diffuse away from the cell that produced them and benefit its neighbors. As such, these are well-studied model systems for the evolution of altruistic cooperation and exploitation. *Pseudomonas aeruginosa* strains that produce siderophores can grow in iron-limited media, but readily evolve non-producing mutants (West and Buckling

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2003). Non-producers lose their ability to grow without exogenous iron, in competition against producers have a higher growth rate due to not incurring the energetic costs of siderophore production. In a well-mixed liquid culture in iron-limited media, non-producers will ultimately outcompete producers to the point of causing a population crash. Such outcomes exemplify the evolutionary instability that can result from exploitable cooperative traits.

Another way microbes cooperate to obtain resources is via cross-feeding mutualisms, typically between species specializing in metabolizing different substrates. This can entail one or more participants actively metabolizing waste products from the others. Interactions like these benefit the participants either by one partner directly providing the other with substrates or by one partner consuming end products such that a metabolic pathway in the other is more energetically favorable. Waste byproduct cross-feeding interactions do not require participants to pay a cost and so generally do not risk the evolution of exploitation (Seth and Taga 2014).

One very interesting intraspecific cross-feeding interaction takes place between cells in the clonal filaments produced by some species of cyanobacteria (Figure 1.2a). Viewing one of these filaments under magnification makes it immediately obvious that the constituent cells exist in multiple differentiated forms. Strung along the filament of vegetative cells like beads are large, spherical heterocysts – terminally-differentiated, non-photosynthetic cells specializing in nitrogen fixation (Kumar, Mella-Herrera et al. 2010). Heterocysts use nitrogenase to fix biologically unavailable diatomic nitrogen (N_2) into ammonia (NH^{4+}), which is shared along the filament through channels between adjacent cells. The purpose for this specialization is unusually clear-cut – nitrogenase and its accessory proteins are extremely sensitive to the oxygen produced by photosynthesis, and so a cell can either fix carbon or fix nitrogen, but not both. Other cyanobacterial species address this strong tradeoff temporally rather than spatially,

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switching back and forth between nitrogen fixing and photosynthesis, but by delegating the tasks to separate cells, filamentous cyanobacteria achieve both processes with greater efficiency. In addition to requiring sugars from their photosynthetic neighbors, heterocysts are incapable of dividing, and so can only have evolved via indirect fitness effects. Like sterile worker ants or the non-reproductive somatic cells that make up most of a multicellular eukaryote's body, heterocysts sacrifice all future fitness to assist their relatives' reproduction.

1.3.2 Chemical Communication, Cues, and Coercion

Microbes speak a chemical language. Like animals, microbes coerce one another, respond to cues, and even engage in true communication. Explaining the evolution of communication is a difficult problem microbiologists have inherited from animal behaviorists. Why should any organism spend energy to send signals for the benefit of another, particularly between species? Why should a signal's recipient respond honestly? Kin selection can offer an explanation for some intraspecies communication, but between species the risk of exploitation should often preclude true communication.

Complicating the issue is a lack of semantic clarity. In evolutionary biology parlance, true communication is a trait or behavior that influences another organism and that has evolved primarily for this purpose in both sender and recipient(Diggle, Gardner et al. 2007). Key here is that in true communication, both parties receive a fitness benefit. Establishing this is often difficult, which has led to the word communication being misapplied to traits that are actually cues (wherein the recipient responds to a trait the sender did not send for that purpose, as when a shark hones in on the electric pulses produced by its prey's nervous system) or coercion (wherein a sender selfishly manipulates a recipient, as when a bola spider mimics moth pheromones to

attract prey into its reach). True communication, cue, and coercion are not exclusive categories, and a single trait can function as more than one in different contexts.

The most widely studied such example in microbes is quorum sensing, a potentially social regulatory mechanism that many microbes use to detect and respond to local cell density (Figure 1.2b) (Fuqua, Winans et al. 1994). Quorum sensing cells constitutively produce autoinducers – small molecules that can readily cross the cell membrane via diffusion or active transporters. As populations increase in density, local autoinducer concentrations increase commensurately. Once autoinducer concentrations rise above a threshold (a 'quorum' of cells), they bind to and activate cytoplasmic transcription factors that in turn upregulate gene expression throughout the genome. With quorum sensing, cells can maintain separate suites of genes for living in low cell density and high cell density environments.

Genes controlled by quorum sensing are many and varied (in the bacterium *Pseudomonas aeruginosa*, more than 10% of the genome is controlled by quorum sensing (Schuster and Greenberg 2006)), but often are involved in social traits. Most bacterial genes for public goods are controlled by quorum sensing, including production of exoenzymes, antibiotics, biofilm matrix components, and conjugation machinery, as are genes responsible for swarming motility, type VI secretion-mediated killing of competitors, and the formation of biofilms. Typically quorum sensing is believed to primarily benefit participants by allowing them to refrain from producing expensive exoproducts or engaging in social behaviors when there are too few cells present to realize their benefits(Darch, West et al. 2012). An exoenzyme, for instance, may only be effective enough to recoup the cost of its production if its total concentration is above a threshold. With quorum sensing, cells can suspend production until there are enough cells to reach that threshold. In pathogens, quorum sensing often controls virulence factor production,

and may help cells coordinate 'sneak attack' strategies wherein they remain avirulent and hidden from the host immune system until overwhelming numbers can be mustered(Parsek and Greenberg 2000)**.**

Quorum sensing is very widespread among bacteria, and similar mechanisms have been found in archaea, fungi, other eukaryotic microbes, viruses, and potentially even multicellular eukaryotes. Many bacteria even maintain multiple quorum sensing circuits in parallel – *P. aeruginosa,* for instance, uses no fewer than seven separate circuits arranged in a hierarchical network. The near-ubiquity of quorum sensing poses interesting questions, as quorum sensing is both a cooperative trait and a communication trait. Given that cells must incur a cost to produce and respond to autoinducer, quorum sensing should be vulnerable to exploitation (Diggle, Griffin et al. 2007). Quorum sensing-deficient mutants are common in both experimental populations and in clinical isolates of pathogens (Dénervaud, TuQuoc et al. 2004)**.** Of these, 'signal-blind' mutants which produce autoinducer (and so encourage other cells to produce quorum sensing-regulated public goods) but are incapable of responding to it themselves are especially prevalent (Diggle, Gardner et al. 2007). Within clonal or near-clonal populations, kin selection on indirect benefits may maintain quorum sensing, but the potential of exploitation to undermine quorum sensing's role for coordinating social traits has led some researchers to propose non-social explanations for its evolution (Redfield 2002). There is evidence that autoinducers may serve other functions beyond signals of cell density, which may provide sufficient direct benefits to hinder the evolution of quorum sensing-deficient strains in nature. In some bacteria, quorum sensing regulates essential genes in addition to genes for facultative social traits. Quorum sensing signal-blind mutants in *P. aeruginosa* are deficient at synthesizing adenosine, imposing a direct metabolic tradeoff (Dandekar, Chugani et al. 2012). A similar mechanism in the same species

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involves the quorum sensing-controlled production of cyanide and a protein conferring immunity to cyanide toxicity (Wang, Schaefer et al. 2015). Cooperating cells produce both cyanide and the immunity protein when in high density conditions. Signal-blind strains cannot produce the immunity protein and are killed by cyanide produced by the cooperators. Cyanide production in *P. aeruginosa* can be thought of as a sanctioning behavior used by cooperators to punish defectors, akin to similar behaviors observed in social insects and macaques.

Kin selection offers a compelling mechanism for maintaining quorum sensing in populations of relatives, but it cannot explain interspecific communication. While quorum sensing systems are widespread across bacteria, most are highly specific, involving autoinducer/receptor pairs discerning enough not to respond to quorum sensing signals produced by other species. A few, however, are more promiscuous. The autoinducer AI-2 is produced by bacteria in many taxa, which has driven speculation that it may function to coordinate actions across multispecies populations like recruitment and development in the complex polymicrobial biofilms that create dental plaques (Kolenbrander, Palmer Jr et al. 2010). Other studies have shown that *Burkholderia cepacia* growing in the lungs of patients with cystic fibrosis can upregulate virulence factors in response to the autoinducers produced by *P. aeruginosa* (Eberl and Tümmler 2004)**.** However, without evidence that these interactions have evolved due to fitness benefits in both participants, these are not compelling examples of true communication. A more likely explanation is that *B. cepacia* is using autoinducers produced by *P. aeruginosa* for its own purposes as a cue, or that *P. aeruginosa* is using its autoinducers to manipulate *B. cepacia* into producing virulence factors for its own benefit (Diggle, Gardner et al. 2007).

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1.3.3 Cooperative Movement and Dispersal

Microbes use diverse mechanisms to move through their environments, whether as part of their normal lifestyle – like a leopard that must move across its territory in search of prey – or to disperse their offspring – like marine invertebrates releasing gametes to be distributed by ocean currents. Many of these mechanisms require the cooperation of many cells working in concert.

Isolated bacteria move through liquid environments with rotating, turbine-like flagella, but across solid surfaces, surface tension makes this impossible. Many bacteria solve this limitation with a collective motion behavior called swarming motility(Kearns 2010). Swarming bacteria – like the predatory soil-bacterium *Myxococcus xanthus* – aggregate into large groups called rafts, which produce and secrete surfactants into their environment in order to disrupt surface tension. Cells within rafts link together and develop multiple flagella, which, when rotated, allow the entire raft to pull itself along the surfactant-lubricated terrain. Swarming motility allows *M. xanthus* cells to search for prey in the soil much faster than if they were working alone.

Myxococcus xanthus also depends upon cooperation to produce spores and disperse. *M. xanthus* and its relatives undergo a complicated life cycle including both unicellular and multicellular stages (Velicer and Vos 2009). *M. xanthus* normally inhabit the soil in large, diffuse groups called wolf packs, moving in distinctive rippling patterns via swarming motility and secreting exoenzymes to kill and digest other bacteria. When food resources run low, however, cells must work together to produce spores and disperse to greener pastures. Starving cells aggregate into mound-like groups – sometimes containing multiple unrelated strains – and

differentiate to produce a fruiting body. Most (more than 90 percent) of the cells in the aggregate sacrifice themselves, either to produce a stumpy structure around the base of the mound, or autolyzing themselves to liberate resources. Only the final ten percent survive, climbing atop the mound of their dead companions and developing into desiccation- and starvation-resistant spores. Other, less-well-studied myxobacteria produce more elaborate fruiting bodies with tall, branched stalks or densely clustered sori. The formation of the myxobacterial fruiting body and the requisite sacrifice of most of the colony appears to facilitate dispersal by lifting spores out of the soil. The unusual way that these multicellular structures are formed – by aggregation of potentially unrelated cells instead of clonally from the division of a single cell as seen in multicellular eukaryotes – has interesting evolutionary consequences. Myxobacteria aggregates can be chimeric, containing multiple different genotypes, and thus there is potential conflict between genotypes over which cells will make the requisite sacrifices to produce the fruiting body's stalk. Genotypes that do not contribute fairly readily evolve in laboratory studies, and can in some cases outcompete the cooperating wild type cells to the point of rendering the entire aggregate incapable of sporulating. Evidence for the importance of this threat is found in myxobacteria's elaborate kin discrimination behaviors – many genotypes of *Myxococcus* can recognize their own kin and will not aggregate with strains from a different incompatibility type.

Other microbes work together to produce mobile multicellular bodies. The social amoeba *Dictyostelium discoideum* is a eukaryote with a facultatively multicellular life cycle somewhat similar to that of *Myxococcus xanthus*, with starving cells aggregating and many of them sacrificing themselves to produce a tall fruiting body to maximize dispersal of spores (Figure 1.2c) (Strassmann and Queller 2011). Before fruiting, the multicellular aggregate assembles into a large, motile slug, which can crawl through the soil many times faster than the individual

amoebae from which it is made and find an optimal spot to fruit. The benefits of moving in a group need not be only mechanical either – some microbes can work together to gain senses. Magnetotactic bacteria found in hyper-saline lagoons form hollow sphere-shaped colonies and align their magnetic-crystal-laden inclusion bodies such that the entire colony has a net magnetic moment. Thusly assembled, the colony can sense and use magnetic fields to navigate through the water column (Keim, Martins et al. 2004).

1.3.4 Defending Against Attack

For many organisms, there is safety in numbers. Defense against attack, especially via predation by larger organisms, is believed to be one of the central selection pressures that has driven the independent evolution of multicellularity in disparate microbial taxa. Algae in the presence of a flagellate predator readily evolve a multicellular lifestyle in the laboratory, growing in clusters that help them resist predation (Boraas, Seale et al. 1998)**.**

One very prominent collective defense employed by many bacteria involves growing in dense, sessile colonies called biofilms(Hall-Stoodley, Costerton et al. 2004) (Figure 1.2d). Biofilms place bacteria of a single species or multiple species in close proximity within a secreted extracellular matrix. A biofilm matrix facilitates bacterial adhesion, but also concentrates and retains water or nutrients and forms a physical barrier that can protect the cells within from physical and chemical attack. Cells growing in a biofilm often exhibit markedly increased resistance to desiccation, predation, and toxic chemicals. Biofilm-forming pathogens are of considerable medical significance due to their role in various chronic infections, and their resistance to conventional antibiotic therapies make them difficult and expensive to treat.

We now understand that biofilm growth is the primary lifestyle for most microbes (Hall-Stoodley, Costerton et al. 2004). Biofilms can be found growing on virtually any surface, including in soil, on the surface of solid particles suspended in water or air, and on the integument and within the bodies of larger organisms. Often a biofilm's structure will be as simple as a layer or pile of cells, but some biofilms grow elaborate morphologies with tower-like structures interspersed with a network of fluid-filled channels that facilitate the movement of water and nutrients into the biofilm's interior. Far from being simple voids, some of these channels even drive active circulation via differential water loss from the biofilm surface(Wilking, Zaburdaev et al. 2013). Polymicrobial biofilms can feature defined strata of species with different metabolic roles, going from aerobic species on the biofilm exterior to strict anaerobes in the deepest levels(Okabe, Hiratia et al. 1996).

The ubiquity and complexity of some biofilms has led some researchers to liken them to multicellular organisms like plants and animals, with different cells within the biofilm coordinating tasks to altruistically benefit the population as a whole. Social evolution theory gives reason to doubt this interpretation, however, particularly for biofilms composed of multiple species. Many of the collective behaviors of biofilms are more parsimoniously explained by direct benefits to the constituent cells – that is, cooperation in a biofilm is often mutualistic rather than altruistic(Nadell, Xavier et al. 2008). Much of the structural complexity seen in biofilms need not reflect coordinated signaling between cells as it does in animal or plant tissues, but rather individual cells optimizing their behavior to suit different microniches within the biofilm. Cells growing deep in the interior of the biofilm are exposed to a different environment than that seen by cells on the periphery, with lower resource concentrations and more waste products. Further, conflict between cell lineages within a biofilm can drive structural complexity by

incentivizing competitors to grow upwards towards higher nutrient concentrations (Xavier and Foster 2007) or by specialists competitively excluding cells from microniches (Picioreanu, Kreft et al. 2004)**.** Often biofilms are more akin to a community of individual cells rather than a single multicellular organism.

Nonetheless, when immobilized within a biofilm, bacterial populations are structured, which can facilitate the evolution of cooperative traits. When cells are embedded in a matrix and unable to move, their neighbors are likely to be disproportionately clonemates. Local relatedness can thus be high, and bacteria more likely to be affected by local biotic and abiotic features of their environment than if they were free-swimming. In laboratories, some single-species bacterial biofilms show robust division of labor that may represent altruistic cooperation. In *Bacillis subtilis,* interior and peripheral cells specialize in different tasks(Liu, Prindle et al. 2015). Peripheral cells have greater access to most resources to support growth, but periodically suspend growth to allow nutrient concentrations in the interior to recover. In return, interior cells supply the peripheral cells with ammonia and act as a protected nest egg from which the biofilm can recover if attacked by antibiotics. *Pseudomonas aeruginosa* biofilms grown under nutrient-limited conditions form distinctive mushroom-like structures, with bulbous crown cell aggregates suspended atop narrow stalks(Klausen, Aaes-Jørgensen et al. 2003) (Figure 1.2d). Crown and stalk cells represent distinct subpopulations – stalk cells are non-motile and grow first, after which the motile crown cells climb atop the stalk. Stalk and crown cells have very different gene expression patterns, with stalk cells producing most of the biofilm's necessary public goods and perhaps even autolysing to support the growth and dispersal of the crown cells**.** In these examples, comparing the biofilm population to a single clonal multicellular organism may be appropriate.

1.3.5 Chemical Warfare

Microbes engage in constant chemical warfare. Competing strains deploy a diverse arsenal of chemical weapons, either secreted into the environment(Riley and Wertz 2002) or injected directly into competing cells with syringe-like secretion systems(Coulthurst 2013). Many of these mechanisms have social elements. Often genes involved in toxin production and deployment are regulated by quorum sensing, only activating when cells grow at high density and their effects are maximized. Sometimes the targets of microbial attacks are other species – predators, prey, or competitors – but some systems specialize on killing conspecifics.

A famous example of the latter are the bacteriocins, a wide category of biocidal peptides produced by many bacterial taxa(Riley and Wertz 2002). Bacteriocins specifically destroy or inhibit the growth of closely-related strains while leaving untouched any cells carrying the correct immunity protein. The most well-known bacteriocins are the colicins produced by gut bacteria like *Escherichia coli* and its relatives. Colicin systems typically involve three components – the toxin itself, which destroys DNA or tears holes in cell membranes, an immunity protein that confers specific immunity to the toxin, and a lysis protein which causes a fraction of colicin-producing cells to autolyse and deploy their deadly cargoes. The fact that cells must die to release their colicins makes clear that selection for the production of colicins must act on indirect fitness benefits rendered to the producing cells' surviving relatives. By killing conspecifics that do not carry the correct immunity protein, colicin-producing cells reduce competition on the cells that do, which are likely to be kin. While colicins are not a kin recognition system under the strictest definition (they cue onto the presence or absence of the

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immunity protein rather than kinship per se), by killing competing lineages they can create local patches of high relatedness and may thereby facilitate the evolution of other cooperative traits.

Colicins drive other social dynamics as well. Producing colicins – and the immunity proteins that prevent collateral damage – is energetically expensive. Microbes must leverage the metabolic costs of producing colicins and immunity proteins with the threat posed by competing strains' attacks. These costs likely explain why strains do not accumulate immunity proteins to all colicins simultaneously. Further, the cost of colicin production and resistance can drive a complex nontransitive relationship between colicin-producing cells, non-producing sensitive cells, and non-producing cells resistant to the colicins' effects. Under some conditions, a 'rock-paper-scissors' like dynamic is achieved, where producers outcompete sensitive cells by killing them with colicins, resistant non-producers outcompete producers by not spending energy producing colicins, and sensitive non-producers outcompete resistant non-producers by not spending energy producing immunity proteins(Kerr, Riley et al. 2002, Kirkup and Riley 2004).

1.3.6 Interacting with Macrobe Hosts

In addition to the microbe-microbe interactions that are the focus of this review, many microbes interact with eukaryotic hosts. All macrobe life evolves and lives against a dense backdrop of diverse microbes, and in recent decades there has been a surge of interest in how host-associated microbes impact host health(McFall-Ngai, Hadfield et al. 2013). Microbes and their hosts can be mutualists or antagonists, their associations can be long-lasting or transient, and they can be horizontally or vertically transmitted. The evolution of conflict and cooperation between a host and a microbial symbiont can be described by many of the same ideas as are applied to microbe-microbe or macrobe-macrobe interactions. Further, often microbes' effects on

the hosts they infect are themselves social phenomena that depend upon cooperation between microbes. In such situations interactions occur – and selection can operate – at multiple levels simultaneously.

Some microbes are pathogens that work together to parasitize macrobe hosts like humans. These interactions can be mild and chronic or brief and lethal, and the factors that influence virulence are complex. For many pathogens, virulence is a cooperative trait. Some bacterial pathogens, like *Pseudomonas aeruginosa*, band together to form biofilms which protect them from the host's immune system (as well as antibiotic treatment)(Bjarnsholt, Jensen et al. 2009). Many virulence-related functions are controlled by quorum sensing(Rutherford and Bassler 2012), which allows pathogens to coordinate their actions or hide critical antigens from the immune system until sufficient numbers have grown. Some pathogens attack their hosts with secreted toxins which are, from the pathogens' perspective, public goods (and accordingly often controlled by quorum sensing.)

Other pathogens go to more elaborate lengths, sacrificing their own fitness for the cause. One striking example is found in infections of the enteric pathogen *Salmonella enterica* serovar Typhimurium, which infects the guts of humans. Infecting populations of *S*. Typhimurium are made up of distinct subpopulations that play different roles in the infection's overall pathogenesis(Diard, Garcia et al. 2013). Though transmission to a new host depends upon passing through the intestine, approximately one third of the cells suicidally invade gut tissue rather than remaining in the lumen. In the gut tissue the invading subpopulation secretes a cocktail of inflammatory chemicals, reducing its growth rate and triggering the ire of the host immune system. The host inflammatory response modifies the gut lumen, wreaking havoc on non-pathogenic bacteria growing within and benefitting the relatively-resistant two thirds of the

S. Typhimurium population left behind. Gut-invading *S.* Typhimurium cells sacrifice themselves to trigger the inflammatory state that the non-invasive subpopulation needs to flourish.

Not all microbial symbionts harm their hosts. Some microbes work together to provide benefits to their hosts in exchange for shelter or resources. Some of the best known host-microbe interactions are between sap-feeding insects like aphids and mutualistic bacteria like *Buchnera aphidicola*, which live inside of specialized organs within their hosts and provide amino acids not present in the hosts' nutrient-poor diets(Buchner 1965). Other hosts depend on microbes to break down molecules in their food – bacteria in the guts of termites, cows, and even humans lend their metabolic flexibility to the task. By producing or digesting molecules not usable by macrobes directly, microbes can enable their hosts to live in niches that would otherwise be impossible.

Other microbes can endow their hosts with new abilities. One famous example is the relationship between the Hawaiian bobtail squid *Euprymna scolopes* and the marine bacterium *Vibrio fischeri*(Nyholm and McFall-Ngai 2004). *V. fischeri* normally lives in ocean water but can selectively colonize crypts within the squid's mantle. When *V. fischeri* populations inside the crypt reach a sufficient density, a quorum sensing circuit activates the expression of the luciferase gene, producing light. The squid host uses *V. fischeri's* bioluminescence to counter-illuminate itself at night, matching the moonlight coming from above to obscure its silhouette to predators swimming beneath it. Because bioluminescence is energetically expensive, populations of *V. fischeri* growing in squid crypts are theoretically vulnerable to invasion by mutants that do not contribute to light production. How the mutualism is protected from exploitation is an active area of research, but appears to be in part driven by the squid regularly evicting and reacquiring

the bacteria from its environment – non-luminescent strains are pleiotropically deficient at colonizing the squid's crypts(Visick, Foster et al. 2000).

Other host/microbe interactions are more vulnerable. A striking example can be seen in the symbiosis between the long-lived cicada *Magicicada tredecim* and its intracellular bacterial symbiont *Candidatus* Hodgkinia cicadicola, which produces crucial amino acids for its host (Figure 1.2e)(Campbell, Van Leuven et al. 2015). The enzymatic pathways that synthesize these amino acids come at a cost to the bacteria, and while collectively the bacteria cannot abandon their duties without killing their host and themselves in the process, there is competition within each host that favors *Ca.* Hodgkinia strains that cut costs and produce fewer benefits. This can lead to within-host divergence of a single *Ca.* Hodgkinia lineage into as many as seventeen separate complementary lineages, each only producing one or a few amino acids and effectively defecting from production of all of the others. With no way to replace underperforming symbionts, the cicada must maintain populations of all of these lineages simultaneously. Here, conflict between bacterial symbionts within a host results in the non-adaptive evolution of reduced benefits for the host.

1.4 Conclusion

Microbes are social for many of the same reasons macrobes are. As in macrobes, interactions between microbes enable them to solve the challenges of life and impose new challenges of their own.

Microbes are worthy of study in their own right. They are ancient, ubiquitous, and of immense ecological significance. They are relevant to human health and agriculture. Most of

Earth's history has been spent dominated by microbes, and the argument can be made that they continue to dominate it now.

Microbes also have a lot to teach us about the broader concepts of biology. Many exciting and powerful experimental techniques depend upon the unique properties of microbes, and allow us to test evolutionary and ecological theory at scales that would be impossible for any other organism. The line between microbes and macrobes is fading as we learn more about the origins of eukaryotic life, the prevalence of horizontal gene transfer in our evolutionary history, and the antiquity of physiological complexity. Generalizing our understanding of social evolution in macrobes by incorporating it into a framework that includes microbes is crucial. Rather than regarding microbes as a fringe group deserving of a few special exceptions to the rules developed for macrobes, we should regard macrobes as particularly sophisticated colonies of mostly harmoniously cooperating microbes.

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Chapter 2: Reduced Social Function in Experimentally Evolved *Dictyostelium discoideum* Implies Selection for Social Conflict in Nature

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2.1 Abstract

Many microbes possess traits through which they interact with one another, but the difficulty of directly observing these interactions in nature makes interpreting their adaptive value complicated. One example is the social amoeba *Dictyostelium discoideum,* which forms aggregates wherein some cells are sacrificed for the benefit of others. Within chimeric aggregates containing multiple unrelated *D. discoideum* lineages, cheater lineages can gain an advantage over other lineages by undercontributing, but the extent to which wild *D. discoideum* has adapted to cheat is not fully clear. In this study, we experimentally evolved *D. discoideum* in an environment where it did not aggregate and where therefore any related selective pressures – including any pressures to cheat other lineages in chimeras – were relaxed. We found that *D. discoideum* lines evolved without the opportunity to cheat evolved reduced competitiveness within chimeric aggregates, suggesting that cheating adaptations were lost to drift or selection on tradeoffs when the selective pressure to cheat was relaxed. We observed a similar loss of function in average migration distance of the slug stage – a trait known to have adaptive function in nature but not under our experimental conditions – but not in spore production, which continued to be under indirect selection. The observed loss of traits that our laboratory conditions had made irrelevant suggests that these traits were adaptations driven and maintained

by selective pressures *D. discoideum* faces in its natural environment. Our results suggest that *D. discoideum* faces enough social conflict in nature to have evolved adaptations in response, and illustrate a general approach that could be applied to searching for social or non-social adaptations in other microbes.

2.2 Introduction

Microbes are capable of – and often even dependent upon – social behavior that once would have seemed beyond their abilities. Despite or perhaps because of their small size and simplicity, microbes can cooperate to sense their environment (Nealson and Hastings 1979, Fuqua, Winans et al. 1994, Abreu, Martins et al. 2007), hunt prey(Velicer and Vos 2009), kill enemies(Riley and Wertz 2002), protect friends (Vidakovic, Singh et al. 2018), move over difficult ground(Kearns 2010), collect critical nutrients(Saha, Saha et al. 2013), and more. Microbial cooperation is worthy of study in its own right for its significant consequences for human health and on the ecological services microbes provide, but also because microbes are uniquely valuable model organisms for studying major questions about evolution.

One long-standing question of interest is how cooperation evolves and is maintained despite the threat of exploitation by non-cooperating cheaters, which has been an area of active interest for evolutionary biologists for many decades (Hamilton 1963, Maynard 1964, Axelrod and Hamilton 1981, Davis 2017, Williams 2018). Many functions that larger organisms perform privately microbes must perform publicly through the production and secretion of proteins into their environment, which would seem to render them especially vulnerable to the risk of exploitation. This, combined with their short generations and high population sizes, makes social microbes especially well-suited for studying the evolution of cooperation and conflict.

Dictyostelium discoideum is an interesting eukaryotic microbe with utility as a model organism for scientists studying development, multicellularity, immunology, and cooperation and conflict (Strassmann, Zhu et al. 2000, Kessin 2001, Annesley and Fisher 2009). While it spends most of its life as a solitary unicellular hunter of bacteria, when starving, *D. discoideum* enters a multicellular life stage(Bonner 1944, Kessin 2001, Strassmann and Queller 2011). Local amoebae aggregate together and form a slug-like multicellular body that can move towards light. Upon reaching a suitable spot, the amoebae within the slug develop into a fruiting body consisting of a ball-shaped sorus of durable spores held aloft by a stalk of dead somatic cells. The spores wait dormant in the sorus to be dispersed – possibly by a passing invertebrate – to a new location with sufficient prey (Huss 1989, smith, Queller et al. 2014).

Dictyostelium discoideum aggregates form from local amoebae, which may or may not be closely related (Jahan, Larsen et al. 2021). Cells within chimeras have the opportunity for conflict that more conventional multicellular organisms – made up of the clonal descendants of a single cell – mostly avoid. In *D. discoideum* this conflict centers on the production of its fruiting body's characteristic stalk, which requires about 20% of the cells within an aggregate to die to help disperse the remainder. Such a sacrifice would not be remarkable within a clone (picture the staggering majority of human cells that toil and die just to pass on a few gametes), but in a chimeric aggregate, it creates an incentive for competing cell lineages to under-contribute to stalk production to minimize their own losses and exploit more civically-inclined lineages (Buss 1982, Armstrong 1984, Strassmann, Zhu et al. 2000).

Multiple studies have observed cheating in *D. discoideum* (Strassmann, Zhu et al. 2000, Fortunato, Queller et al. 2003, Khare and Shaulsky 2010, Santorelli, Kuspa et al. 2013). *D. discoideum* chimeras form readily in the laboratory, and careful observation of the fates of cells

within them reveal that not every cell lineage contributes equally to stalk production. Some *D. discoideum* strains appear to be consistently prone to exploiting or prone to being exploited by others (Fortunato, Queller et al. 2003, Buttery, Rozen et al. 2009) such that a simple hierarchy of cheaters and cooperators can be determined. Studies have identified mutations that cause strains to cheat (Ennis, Dao et al. 2000, Santorelli, Thompson et al. 2008, Khare and Shaulsky 2010, Santorelli, Kuspa et al. 2013), and in experimental evolution experiments where *D. discoideum* is allowed to mutate, mix, and fruit, non-fruiting obligate cheaters readily evolve and outperform cooperative strains, even to the point of causing population crashes (Kuzdzal-Fick, Fox et al. 2011). Some evidence suggests that *D. discoideum* has mechanisms for maintaining high relatedness within fruiting bodies, which may imply the existence of adaptations to reduce the risk of exploitation by maximizing the chance that cells aggregate with cooperative kin. There is evidence that *D. discoideum* actively segregates between kin and non-kin at least temporarily during aggregation (Ostrowski, Katoh et al. 2008, Benabentos, Hirose et al. 2009), though to what extent kin discrimination may protect against cheaters is unclear (Ho, Hirose et al. 2013, Strassmann 2016, Kundert and Shaulsky 2019). Nonetheless relatedness within fruiting bodies in nature is very high (Gilbert, Foster et al. 2007), probably owing to structure imposed by the way *D. discoideum* grows and disperses (Buttery, Jack et al. 2012, Smith, Strassmann et al. 2016). Signatures of the frequency-dependent selection that often accompanies evolutionary conflict have been detected in genes known to affect cheating (Ostrowski, Shen et al. 2015). *D. discoideum* was also found to recognize and respond to the presence of non-kin within a chimeric aggregate with changes in gene expression, development, and dispersal behavior relative to clonal aggregates (Noh, Christopher et al. 2020). Genes that change expression in

chimeric aggregates show signatures of increased polymorphism and rapid evolution that may indicate evolutionary conflict(Noh, Geist et al. 2018).

Despite multiple lines of evidence, however, some researchers have questioned the relevance of cheating to wild *D. discoideum*. As with any trait, directly proving that cheating is an adaptation is difficult, particularly when most studies of *D. discoideum* cheating involve laboratory-made chimeras. Non-fruiting obligate cheaters readily arise and prosper (at least over the short term) in experimental evolution experiments using *D. discoideum* under conditions imposing low relatedness (Kuzdzal-Fick, Fox et al. 2011), but zero were observed in a screen of 1039 spores isolated from 75 wild-collected fruiting bodies (Gilbert, Foster et al. 2007). This may suggest that in nature, the benefits cheaters reap by reducing stalk production in chimeras are too small or too infrequently realized to compensate for the disadvantages of being unable to produce a functioning stalk when there are no cooperative lineages to exploit. Traits previously interpreted as adaptations to mitigate the risk of exploitation (like the mechanisms driving high relatedness within *D. discoideum* fruiting bodies in nature) may instead be the result of selection on other traits that only incidentally impact social conflict (Manhes, Schaal et al. 2022). One study did not observe patterns consistent with conflict in *D. discoideum* genes expressed during the social cycle, and instead attributed high polymorphism in these genes to drift magnified by the social cycle's presumed intermittent relevance in nature(de Oliveira, Morales et al. 2019). Absent direct observation of cheating in nature, some researchers have proposed that apparent cheating is a laboratory artifact of little relevance to wild *D. discoideum*, and better explained by variation in non-social life history traits (Tarnita, Washburne et al. 2015, Wolf, Howie et al. 2015, Martínez-García and Tarnita 2016). In this, cheating in *D. discoideum* is in good company

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with other well-supported social traits in microbes that have faced similar criticisms (Redfield 2002, Jefferson 2004).

The complexity of social traits in microbes and the difficulty of observing them directly invites careful interpretation that is greatly benefitted by combining evidence from multiple different approaches. In this study, we sought to infer the presence of adaptations related to social conflict by experimentally evolving *D. discoideum* under conditions in which it never enters the social stage. Without the opportunity to aggregate, selection on social traits should be relaxed, generally leading to losses in social function normally maintained by natural selection.

Key to this approach is the assumption that when a long-standing selective pressure is removed, past adaptations driven by that pressure are likely to be lost due to drift or, more likely, pleiotropic tradeoffs with other traits. Selection upon one trait will often indirectly impact other traits, and an organism's traits often represent a compromise between mutually incompatible adaptations to different selective pressures. These compromises should tend to constrain the evolution of adaptations to overcome any particular selective pressure – an organism cannot, for example, evolve to be smaller to save energy and larger to avoid predation at the same time. If one selection pressure is relaxed, however, compromises are no longer necessary and constraints are lifted. For this reason, we should expect that relaxing a selective pressure should free an organism to lose traits that were adaptations to that pressure.
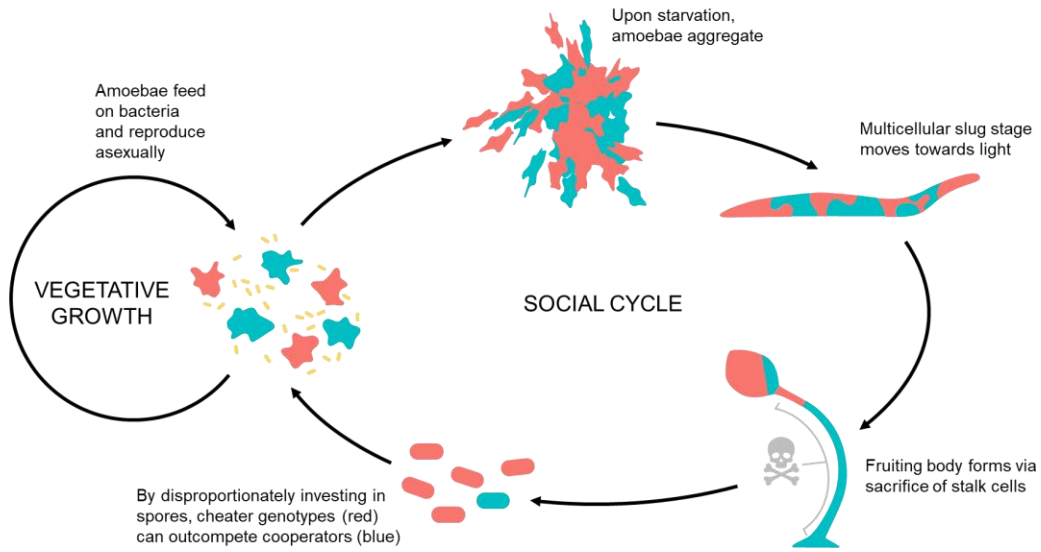
We can look for these sorts of losses and use them to infer selective pressures (and resultant adaptations) that we hypothesize are important but that are difficult to directly observe, such as the pressure that wild *D. discoideum* would experience if they were regularly exploited by cheaters. When *D. discoideum* is evolved in an environment where we know cheating is not

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relevant, it should lose adaptations related to cheating, but it cannot lose adaptations that it does not have. Therefore, loss of function when we make social conflict irrelevant in the lab is evidence that we have successfully relaxed a selective pressure that was relevant in nature. Alternatively, if the apparent cheating observed in past studies is unrelated to social conflict and is instead the result of selection on other selective pressures that we did not relax, we should see little or no change.

In this study, we measure the effects of experimental evolution under conditions where cheating cannot occur on *D. discoideum's* ability to cheat (or to resist being cheated) during the formation of chimeric fruiting bodies. In addition, we assayed the effects of experimental evolution on two other phenotypes with different connections to *D. discoideum* sociality to validate our logic. We assayed spore production, which is closely tied to cell number, a trait we expect to be selected for both in nature and under our laboratory conditions. Because it is positively correlated with fitness in the laboratory, spore production is effectively indirectly selected for and so should not decrease when *D. discoideum* is evolved without its social stage. We also assayed the distance travelled by *D. discoideum* slugs before fruiting body formation, which is likely to be adaptive in nature, as it is a complex trait and should assist *D. discoideum* in dispersal and enable it to reach the soil surface during fruiting (Castillo, Switz et al. 2005). In laboratory conditions where no slugs are formed, however, it is irrelevant, or even potentially maladaptive if there are tradeoffs between slug migration and more useful traits. Slug migration distance should be negatively correlated with fitness in the laboratory, and so we should expect to see evolved *D. discoideum* lines evolve reduced migration distance. Together spore production and slug migration make for a pair of controls flanking cheating on either side – the former a trait

likely to be positively correlated with fitness in the lab, the latter likely to be negatively correlated with fitness in the lab, and cheating sitting somewhere unknown in between.

A   *D. discoideum* social cycle allows apparent cheating

Upon starvation,
amoebae aggregate

Amoebae feed
on bacteria
and reproduce
asexually

Multicellular slug stage
moves towards light

VEGETATIVE
GROWTH

SOCIAL CYCLE

Fruiting body forms via
sacrifice of stalk cells

By disproportionately investing in
spores, cheater genotypes (red)
can outcompete cooperators (blue)

B   **Experimental evolution**

By maintaining food availability
and frequent transfers, prevent
D. discoideum from entering the
social cycle

serial passage

Evolve in vegetative cycle for
many generations. Selection on
social traits is relaxed, social
function should be lost to drift or
pleiotropy.

C   **Assaying slug stage mobility**

Ancestor   Evolved

Experimentally evolved
D. discoideum should
be less mobile

D   **Assaying cheating**

Combine *D. discoideum* and RFP-
labelled control at 50:50 ratio

Ancestor   +   Control

Evolved   +   Control

If cheating is adaptive,
experimentally evolved *D.
discoideum* may be
underrepresented in
chimeric fruiting bodies

40
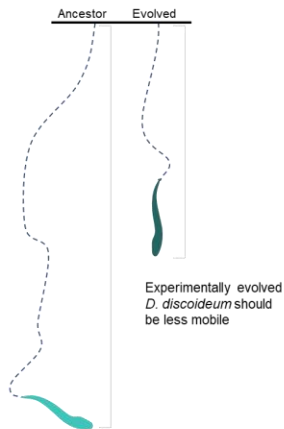
Figure 2.1 - Experiment Overview. A) simplified schematic of *D. discoideum's* social cycle, in which multiple genotypes (red and cyan) can potentially aggregate into chimeric multicellular bodies. Most of the time, amoebae are unicellular and grow vegetatively. Upon starvation, cells aggregate and develop into a multicellular slug stage, which moves towards light, and then forms a sessile fruiting body. Formation of the fruiting body requires the sacrifice of about 20% of cells to produce a stalk, and so cheater genotypes (red) can gain an advantage by under-contributing to stalk formation. **B**) Experimental evolution of *D. discoideum* under conditions which prevent it from entering the social cycle should drive the loss of traits previously maintained by selective pressures related to it. **C)** Slug mobility assays - Experimentally evolved *D. discoideum* (dark green) should travel less far during the slug stage than its ancestor (teal) due to relaxed selection. **D)** Cheating assays - Experimentally evolved *D. discoideum,* when mixed equally into a chimera with an RFP-labelled control strain (red), should be less well represented among the spores of the resulting fruiting body than its ancestor due to relaxed selection.

2.3 Methods

Culture conditions – We performed experimental evolution using SM/5 media (Loomis and Sussman 1966) (2 g glucose (Fisher Scientific), 2 g BactoPeptone (Oxoid), 2 g yeast extract (Oxoid)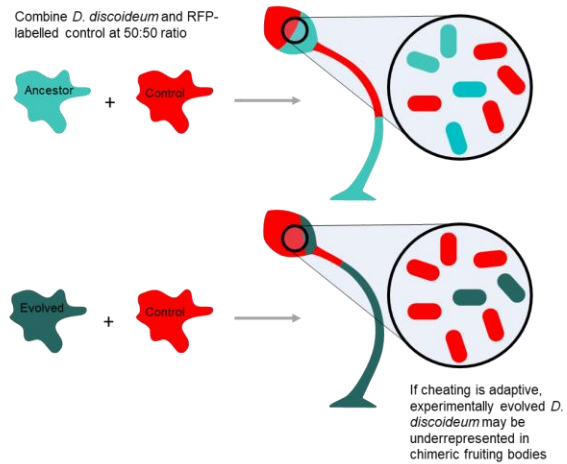, 0.2 g $MgCl_2$ (Fisher Scientific), 1.9 g $KHPO_4$ (Sigma-Aldrich), 1 g K_2HPO_5 (Fisher Scientific), and for solid media 15 g agar (Fisher Scientific) per liter deionized water). To start a fresh culture of *D. discoideum*, we diluted spores from -80°C glycerol frozen stocks in KK2 buffer (2.25g KH_2PO_4 (Sigma-Aldrich) and 0.67g K_2HPO_4 (Fisher Scientific) per liter deionized water). We plated 1.0×10^5 total spores onto an SM/5 plate along with 200µl *K. pneumoniae* food bacteria resuspended in KK2 to an OD_{600} of 1.5. To start a fresh culture of any bacterial strain, we streaked stocks from the -80°C freezer for isolation on SM/5 plates. We performed slug migration and spore production assays on nutrient-free starving agar plates.

Antibiotic curing of *D. discoideum* – Many wild *D. discoideum* isolates are infected by *Paraburkholderia* symbionts which affect their fitness and behaviors (DiSalvo, Haselkorn et al. 2015). In order to remove symbionts, 1.0×10^5 *D. discoideum* spores were plated on SM/5 agar medium containing 30ug/mL tetracycline and 10ug/mL ciprofloxacin with 200µl of *K. pneumoniae* resuspended in KK2 to an OD_{600} of 1.5. We allowed plates to grow at room temperature under ambient light until formation of fruiting bodies (3-5 days). We collected spores as above, then diluted and plated again as above. We then collected spores and performed spot test assays (described in (Brock, Douglas et al. 2011)) and PCR using *Paraburkholderia*-specific primers (Garcia, Larsen et al. 2019) to verify successful curing.

Experimental evolution – Experimental evolution was performed as part of a larger experiment involving *D. discoideum* and various bacterial endosymbionts. Three replicate lines each of ten strains were plated on SM/5 plates. We incubated all lines at room temperature under ambient light and transferred 0.5% of the population to fresh plates every 48 hours. We performed transfers by first harvesting all cells into 10mL KK2 buffer using gentle pipetting and scraping of the agar surface. We then thoroughly vortexed the resulting suspensions, diluted them 20-fold, and plated 100uL onto fresh plates with 200uL of an $OD_{600} = 1.5$ *K. pneumoniae* suspension to serve as food. The 48-hour transfer interval was selected to preempt *D. discoideum's* fruiting stage and prevent direct selection on social traits. Every fifth transfer, we additionally froze 1mL of the undiluted suspension of harvested cells at -80°C with 60% glycerol.

Following experimental evolution (after the 30th transfer) *D. discoideum* lines were checked for cross-contamination using fragment analysis. We extracted DNA from 100uL of the undiluted suspension of harvested cells using CHELEX resin beads and amplified using fluorescently-tagged PCR primers specific to highly variable microsatellite loci known to differ in length

between *D. discoideum* strains (Smith 2004)**.** Fragment analysis of resulting amplicons was performed by Genewiz and evolved strains were compared to ancestors. No cross-contamination was detected.

Slug migration assays – In order to compare how experimental evolution affected slug migration distance, we performed assays on ancestral and evolved lines. Each assay was performed in triplicate on non-nutrient agar plates (13 cm diameter). We marked a 10 cm secant line on the back of each plate. We plated 50μL of an OD_{600}=50.0 suspension of *K. pneumoniae* in KK2 buffer containing 10^7 *D. discoideum* spores along the secant line. We allowed the loaded sample to dry and wrapped the plates individually in aluminum foil. On each wrapped plate, we made a small pinhole opposite the starting line through which light could enter. We placed the wrapped plates on the laboratory bench under a light source and left them undisturbed for 8 days. At the end of the 8 days, we unwrapped the plates and photographed using a Canon EOS 5D Mark III camera.

We used Fiji (Schindelin, Arganda-Carreras et al. 2012) to perform image processing and obtain slug migration distances. First, we scaled images and overlayed a 1cm x 1cm grid. We marked fruiting bodies on each image and measured their distance from the starting line. For the final analysis, we did not include fruiting bodies that developed directly on the starting line (distance=0).

Spore production assays – 1 day after plates from the slug migration assays were imaged, we collected the fruiting bodies from each plate to assay spore production. In order to compare the fraction of *D. discoideum* aggregates that had formed slugs and migrated, we collected fruiting bodies that formed on the starting line and fruiting bodies that had travelled towards the pinhole

into two separate tubes of KK2 buffer for each plate. We then gently mixed the suspensions and counted the spores under a light microscope using a hemocytometer. We calculated a total number of spores for each plate by combining the spore counts from the starting zone and the movement zone.

Cheating assays - In order to assay cheating, we determined the proportion of fluorescent spores in fruiting bodies developing from an initially 50:50 mix of a strain of interest and the RFP-labelled control strain RFP-NC28.1. We plated $2x10^5$ spores for each strain onto SM/5 agar plates. During mid-log stage (approximately 34-36 hours), we collected vegetative cells and washed three times with cold KK2 buffer, counted using a hemacytometer, and diluted each suspension to 10^8 cells/mL. We combined equal volumes of the focal strain and the labelled control strain and gently mixed to get 50:50 mix suspensions. We prepared UV-sterilized $13mm^2$ AABP 04700 (Millipore) filter squares. We pipetted 15uL of the 50:50 mix suspension into the center of each of 3 filter squares pre-dampened with KK2. We transferred filters onto KK2 (non-nutrient) agar plates to initiate immediate aggregation and development and incubated plates for 5 days at room temperature.

After incubation, we examined and selected 2 filters from each plate to assay. We prioritized filters that showed no evidence of slugs having escaped onto the surrounding agar – otherwise selection was random. We collected each filter and any fruiting bodies that had grown atop them with sterile forceps into 500uL KK2 buffer, vortexed thoroughly, and took photographs with bright-field and fluorescence microscopy. We captured at least 5 fields for each sample (representing around 1000-2000 spores). Each assay was performed on three separate days.

We counted total spores and fluorescent spores using Fiji (Schindelin, Arganda-Carreras et al. 2012). We first manually converted micrographs into binary images using a brightness threshold set individually for each image to account for minor differences in contrast and brightness between samples. We then used Fiji's Count Particles function, filtering for particles between 15-200 um^2 and between 0.5 and 1.0 circularity (settings which consistently resulted in very similar results to manual counting).

We determined proportion of fluorescent spores by dividing the number of fluorescent spores (count from fluorescent image) by the total spores (count from brightfield image). We also determined a percentage fluorescence for the RFP-NC28.1 control for each assay and divided this proportion out of the results for the 50:50 mix samples in order to compensate for incomplete labelling.

Statistical analysis - We performed analyses using R version 4.0.4 (R Core Team, 2015) with the *lme4* package (Bates et al., 2015) and the *emmeans* package (Lenth, 2022).

To analyze changes in spore production and slug migration distance, we used linear mixed effects models with treatment (ancestor vs evolved) as a fixed effect and strain as a random effect. The model used to analyze spore production data was Average Spore Production ~ Treatment + (1|Strain/Line). The model used to analyze slug migration was Average Migration Distance ~ Treatment + (1|Strain/Line). In the slug migration assays, in order to account for fruiting bodies that developed at the starting line (migration distance = 0), we weighted each plate's contribution to the model by the fraction of fruiting bodies with nonzero migration distances.

To analyze changes in representation among spores in chimeric fruiting bodies, we used a two-tailed, two-proportion Z test.

In order to convey the most biologically meaningful insight, we have reported our results in terms of compatibility intervals rather than single p-values (Berner and Amrhein 2022).

2.4   Results

Spore production assays –

We measured the effects of experimental evolution on spore production by comparing the spores produced by ancestor and evolved lines. While spore production itself should not have been under direct selection in our experimental evolution experiment, we expected that it would be indirectly selected for by virtue of its close connection with the total number of cells on the plate (due to growth rate, efficient use of available resources, etc).

Ancestral *D. discoideum* produced an average of 6.9×10^6 spores per plate. As expected, experimentally evolved *D. discoideum* produced an average of 1.5×10^6 (+21.9%) more total spores than their ancestors. Possible values for the true increase most compatible with our data ranged from 2.5×10^5 to 2.7×10^6 (+3.7% to +40.1%) (95% CI).

significantly more total spores than their ancestors ($\beta = 1.51 \times 10^6$, SE=6.34×10^5, t=2.392, p<0.05) **(Figure 2.2)**.
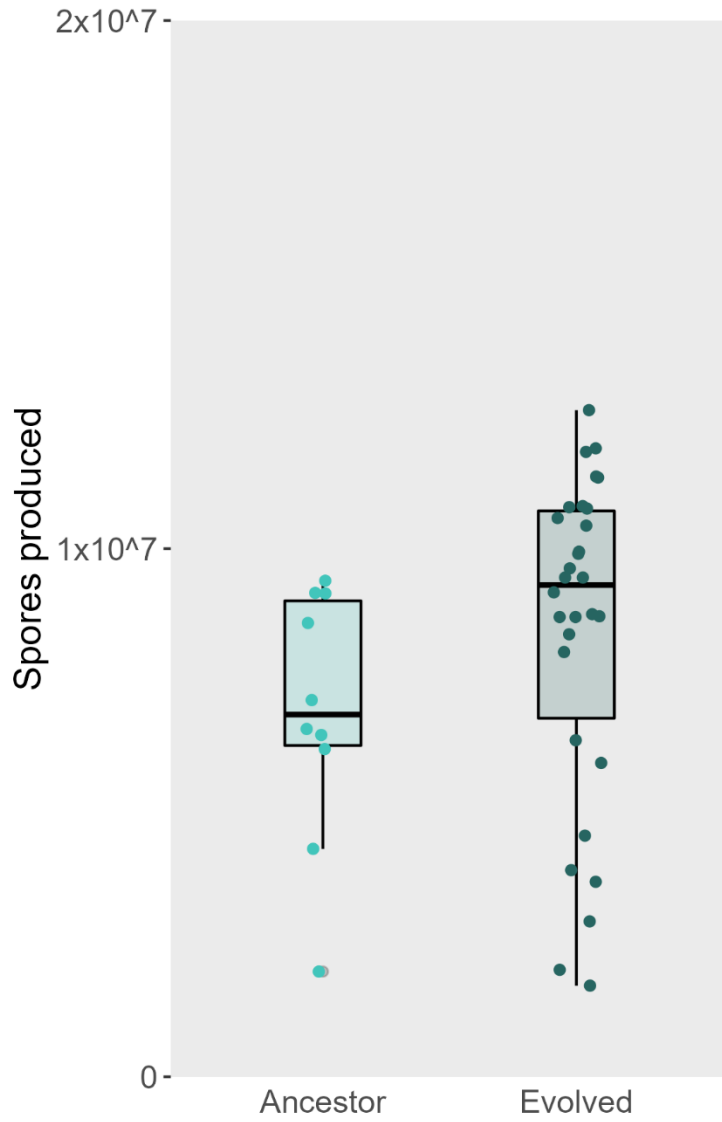
Figure 2.2 - Experimentally evolved *D. discoideum* strains evolve increased spore production. A) *D.
discoideum* strains evolved without the opportunity to aggregate produce significantly more spores than
their ancestors.

Slug mobility assays –

We measured the effects of experimental evolution on slug migration by assaying the average distance travelled by slugs produced by ancestral and evolved lines from a starting position. As migration distance should be selected for in nature but not in the laboratory, we expected to see reductions in migration distance. These reductions would suggest the loss of adaptations that facilitated slug migration in nature but were negatively correlated with fitness in the laboratory.

Ancestral *D. discoideum* slugs migrated an average of 4.01cm from the starting line. As expected, experimentally evolved *D. discoideum* slugs migrated an average of 0.49cm (-12.2%) less far than their ancestors. Possible values for the true decrease most compatible with our data ranged from 0.50cm to 0.48cm (-12.3% to -11.9%) (95% CI) **(Figure 2.3B)**.
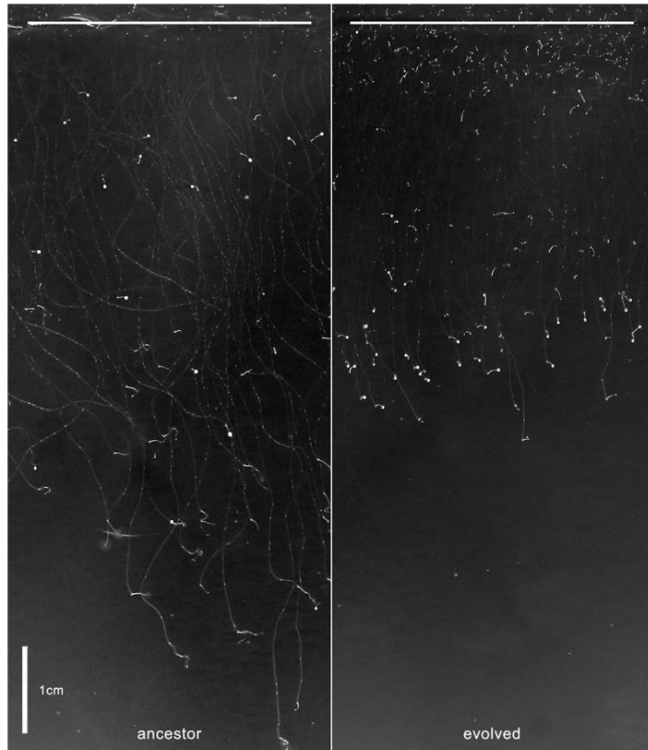
Figure 2.3 - Experimentally evolved *D. discoideum* strains evolve reduced slug mobility. A)
Representative image of slug mobility assays. *D. discoideum* suspensions were plated along the starting
line (top of the figure). Slugs migrated towards a light source (bottom of figure). B) *D. discoideum* strains
evolved without the opportunity to aggregate migrate shorter distances during the slug stage than their
ancestors.

Cheating assays –

We measured the effects of experimental evolution on cheating by assaying ancestral and
evolved lines' representation among the spores of chimeric fruiting bodies. We performed

cheating assays by combining ancestral or evolved lines with an RFP-labelled control strain (NC28.1) at a 50:50 ratio. The resulting fruiting bodies were collected, suspended in buffer, and photographed using brightfield and fluorescence microscopy to determine counts of total spores and fluorescently labeled spores. Deviation from the starting 50:50 ratio is interpreted as cheating.

The selective importance of cheating in nature is disputed, but cheating is certainly not selected for under our laboratory environment. If wild isolates' ability to cheat or avoid being cheated by other lineages in nature is the result of adaptations driven by selective pressures on cheating, then relaxing those selective pressures should result in loss of those adaptations and reduced cheating function.

Ancestral *D. discoideum* strains contributed an average of 54.27% of spores in chimeric fruiting bodies with RFP-NC28.1, suggesting that overall the wild strains could cheat NC28.1. Evolved *D. discoideum* strains contributed an average of 3.10% fewer spores in competition with NC28.1 compared to their ancestors. Possible values for the true decrease most compatible with our data ranged from -2.65% to -3.56% (95% CI) **(Figure 2.4)**.

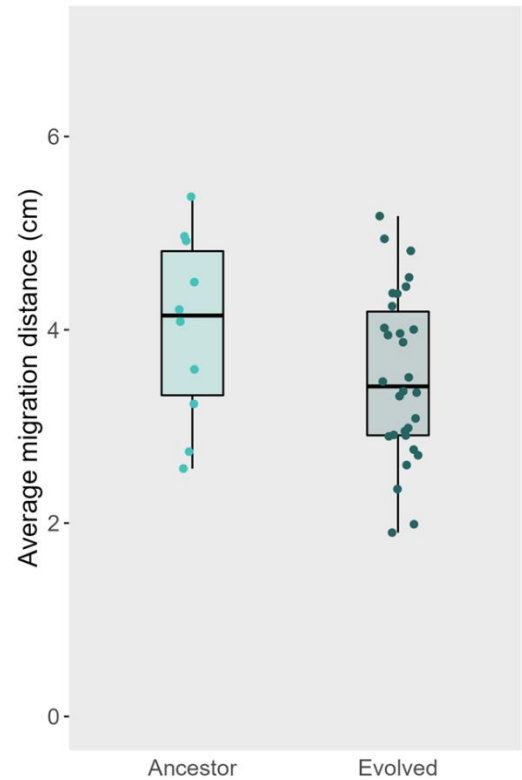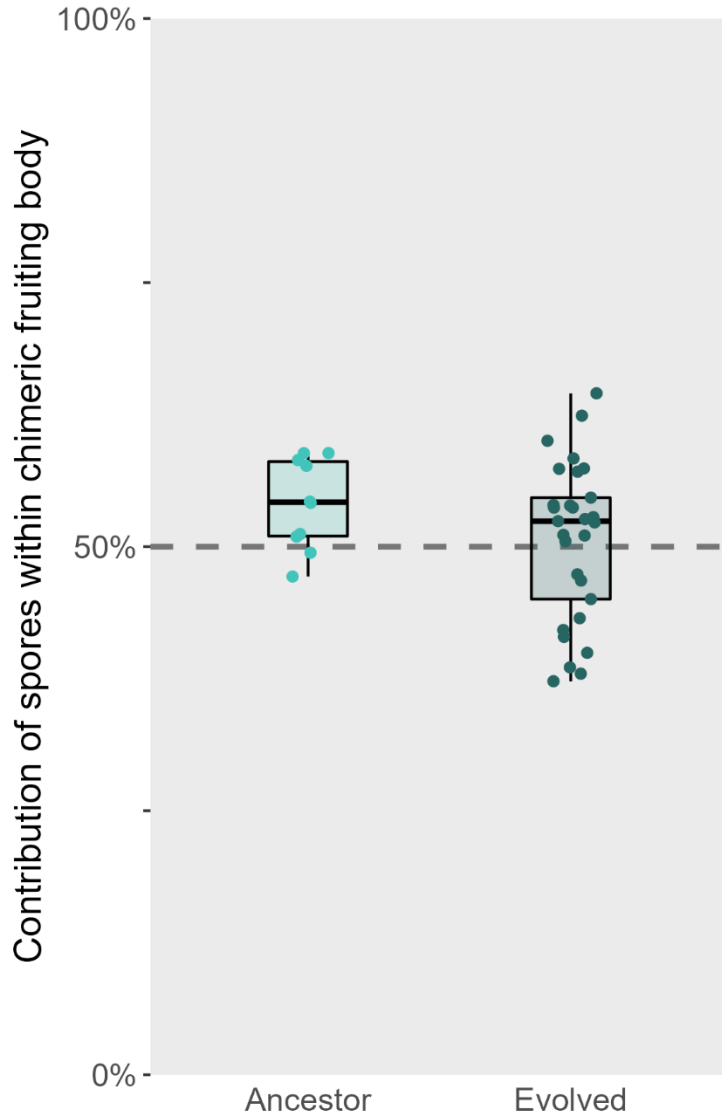**Figure 2.4 - Experimentally evolved *D. discoideum* strains evolve reduced ability to cheat.** Spores of ancestral *D. discoideum* strains are slightly overrepresented within chimeric fruiting bodies made by combining equal numbers of the focal strain and a labelled control strain. When competed against the same control strain, evolved *D. discoideum* strains contribute a lower percentage of spores within fruiting bodies compared to their ancestors, suggesting that experimental evolution resulted in reduced cheating ability (or reduced ability to resist being cheated upon).

2.5 Discussion

D. discoideum is a useful model organism for studying cooperation and conflict. Its social cycle requires potentially unrelated cells to cooperate, exploit one another, and resist being exploited. While multiple lines of evidence attest to *D. discoideum's* ability to cheat in the laboratory, interpreting their relevance to *D. discoideum* in nature is not trivial. In this respect, cheating in *D. discoideum* echoes other prominent examples of microbial sociality (Redfield 2002, Jefferson 2004) and illustrates a general challenge in studying adaptations in any organism. It is easier to prove that an organism has a particular trait than it is to be certain what selective pressures (if any) drove the trait's evolution, and doubly so if the organism is too small to directly observe in its natural habitat.

This study attempts to shed light on the adaptive value of cheating in *D. discoideum* in nature by experimentally evolving wild strains of *D. discoideum* under laboratory conditions in which the social cycle – and thus cheating – is prevented. This very simple experiment hinges on the idea that relaxing selective pressures an organism is presumed to face in nature (in this case selection for social function in the form of slug migration and of cheating) will tend to result in loss of adaptations maintained by those pressures.

We expect any adaptations to relaxed selective pressures to tend to be lost due to drift or selection on other traits with which they are antagonistically pleiotropic. Adaptations are not free, and enhancing one trait will tend to result in reductions in another. While positive pleiotropic interactions exist, they are much less common than negative interactions for the same reason that beneficial mutations are less common than detrimental mutations – there are more

ways to break a system than there are to improve it (Johnson, Lahti et al. 2012). We maintained our *D. discoideum* lines under conditions where, as in nature, they were presumably selected to produce lots of offspring, but were no longer selected to engage with one another to produce slugs or fruiting bodies. Without the opportunity to form slugs, why maintain adaptations that support slug function, especially if they tradeoff with other traits that may still be useful? Without the opportunity to cheat or the risk of being cheated, why maintain adaptations for cheating? Traits not being maintained by selection should tend to atrophy – organisms 'use it or lose it' (Wcislo and Danforth 1997, Hall and Colegrave 2008, Darwin 2012, Sadier, Sears et al. 2022). By contrast, if cheating is not selected for in nature, *D. discoideum* should not have adaptations supporting it, and moving cells to an environment where cheating continues not to be adaptive should not change anything.

We looked for changes in experimentally evolved *D. discoideum's* ability to cheat that would suggest it had experienced selective pressure to cheat (or resist cheating) in its natural environment. In addition, we assayed spore production and slug migration distance, two phenotypes for which we had clear predictions. We found that evolved *D. discoideum* produced more spores than their ancestors (Figure 2.2). At first glance, spore production might be expected to decrease when *D. discoideum* is evolved under conditions where it never forms spores. We do not consider this apparent contradiction to refute our general logic, however, because we expect spore production to be tightly interrelated with the fitness of vegetative cells (which were still under selection in our experiment). We expected this indirect selection would tend to maintain adaptations that facilitated higher spore production and that we would therefore not see reduced spore production in evolved lines. The increase we observed is likely the result of *greater* selection on cell number in the laboratory relative to in nature, reflecting that the laboratory

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environment – where food is plentiful and conditions are mild – behooves cells to invest in outcompeting neighbors rather than protect themselves from no-longer-relevant hazards. We speculate that if we had broken fitness into more granular components distinguishing the efficiency of different stages in *D. discoideum's* life cycle, we would have seen reduction in the efficiency of spore production, but that in our assays it was masked behind the large fitness gains our lines realized through improvements in other traits that remained relevant. Given long enough, we expect that our experimentally evolving *D. discoideum* lines would lose the ability to produce spores entirely. In the meantime, spore production is a trait that might itself not be selected for, but that is positively pleiotropically linked with a trait (vegetative cell fitness) that is.

By contrast, our slug mobility results illustrate an example of a trait that was not associated with fitness under the conditions of our experimental evolution experiment. Slug migration, in addition to being a trait only expressed during the social stage of *D. discoideum's* life cycle, has previously been linked to the consequences of cheating over stalk production (see below) (Castillo, Switz et al. 2005)**.** In our experiment, *D. discoideum* lines were transferred to new plates with fresh food sources before starvation motivated them to aggregate, and so they never had the opportunity or need to form slugs or migrate. Any adaptations that ancestral *D. discoideum* had that improved slug migration distance should no longer have been maintained in the laboratory, and so accordingly, evolved *D. discoideum* migrated less far than their ancestors (Figure 2.3). Unlike spore production, slug mobility has no expected positive pleiotropic relationship with vegetative cell fitness and so the reduction we observed is best explained by the relaxation of selection for slug migration in nature.

By the same logic, the results of our cheating assays suggest that wild *D. discoideum* has adaptations related to social conflict. When analyzed across all strains, there was an overall reduction in the contribution to spores within chimeras for experimentally evolved lines relative to their ancestors (Figure 2.4), consistent with the idea that on average, *D. discoideum* evolved without the opportunity to cheat became either less capable of cheating or more susceptible to being cheated by others. We interpret this lost social function as a result of selection in the non-social laboratory environment on traits that have pleiotropic tradeoffs with social traits. In nature, these tradeoffs oblige wild *D. discoideum* strains to come to some compromise between being effective cheaters (or effective at resisting cheating) and being effective during the non-social stages of *D. discoideum's* life cycle. The removal of any selective pressure on the social stage freed experimentally evolved lines to evolve based upon non-social selective pressures alone. Our results are consistent with past genomic studies implying *D. discoideum* genes affecting cheating have an evolutionary history fraught with conflict (Ostrowski, Shen et al. 2015, Noh, Geist et al. 2018).

On average, evolved *D. discoideum* strains were less well represented within chimeric fruiting bodies with a labelled social competitor than their ancestors were against the same competitor. We interpreted this as evidence that evolved strains had become worse cheaters (or more susceptible to being cheated upon). However, some researchers have questioned the validity of measuring cheating based on spore representation, and have pointed out that underrepresentation within chimeric fruiting bodies could also result from variation between strains in life history traits impacting spore production but not necessarily related to social conflict (Tarnita, Washburne et al. 2015, Wolf, Howie et al. 2015, Martínez-García and Tarnita 2016). For instance, strains can vary in how they allocate cells between spores and stalk, in

whether they produce fewer, higher quality spores or more, lower quality spores, and in what proportion of cells aggregate at all rather than remain asocial vegetative cells. A strain that inherently produces fewer spores – for whatever reason - would appear to be underrepresented among the spores of a chimeric fruiting body even with no actual selection on social conflict. These alternative explanations doubtless have a place in a holistic understanding of cheating in *D. discoideum*, but are not consistent with the results of this study. *D. discoideum* evolving in our experiment were consistently transferred before they had the opportunity to aggregate, and so we expect there should have been little or no selection on traits expressed only during the fruiting process like spore/stalk allocation. To the extent to which cells' fates as spore or stalk may be determined prior to aggregation, it is possible that some selection on spore/stalk allocation might have remained in our experiment. However, because in our experimental conditions there was no benefit to be gained by forming stalks at all, we would expect any such selection to drive *D. discoideum* towards greater spore allocation, which would have resulted in apparent *gains* of cheating ability, rather than the losses we observed. Finally, while as with spore/stalk allocation it is possible that our *D. discoideum* experienced some selection to abstain from aggregation and instead act as loner cells, we did not observe the reduction in fruiting body production that we would expect from reducing spore production in this way.

Microbes lead complicated lives obscured from us by alien tininess. Understanding even some apparently central aspects of their biology is a complex task demanding multiple approaches and careful interpretation. *D. discoideum's* social cycle has been the subject of interest for decades, and yet how exactly it fits into this microbe's life in nature continues to inspire debate. The results of this study support the idea that some wild *D. discoideum* strains experience enough social conflict to have evolved adaptations to it. Further, the approach we

have employed here – looking for otherwise inscrutable adaptations by evolving them away in lab – should be applicable to a wide variety of traits in a wide variety of organisms. This approach can usefully supplement other approaches in researchers' pursuit of a more complete understanding of adaptation.

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Chapter 3: Experimental Evolution of Symbiotic Microbes Without Their Partners

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2.1 Abstract

Microbes adapt to the presence of other species, but the fitness consequences of specific interactions are difficult to study in their natural context. We experimentally evolved symbiotic microbes in an artificial environment without access to the partners with whom they interact in nature. As organisms will tend to lose adaptations that they do not need due to drift or pleiotropic tradeoffs, we expect normally symbiotic microbes evolved in isolation to lose adaptations to help or harm their natural partners. The direction and magnitude of such changes can suggest whether the microbes had historically been selected to help or harm one another. We apply this method to the symbiosis between the social amoeba *Dictyostelium discoideum* and three intracellular bacterial endosymbionts, *Paraburkholderia agricolaris, P. hayleyella,* and *P. bonniea.* We found that experimentally evolved *P. agricolaris* and *P. hayleyella* tended to become less antagonistic towards – and more susceptible to antagonism from – *D. discoideum*, suggesting that they had lost adaptations to harm and to resist harm from *D. discoideum* in nature. We also found evidence that the degree to which *D. discoideum* and *Paraburkholderia* have adapted to help or harm one another differs between strains within each species. Our results underscore the complexity of microbial interactions in nature and suggest experimental evolution under relaxed selection is a potentially useful approach for studying adaptation in microbes.

2.2 Introduction

Microbial interactions with other species take place in a world that is difficult to observe for humans quadrillions of times larger(Young and Crawford 2004, Vos, Wolf et al. 2013, Widder, Allen et al. 2016). Questions with answers that seem almost self-evident for larger organisms – such as 'who interacts with whom?', 'is interacting costly or beneficial?', and 'are

organisms adapted to help or harm other species?' – can be very difficult to answer for microbes, and particularly for microbes within their natural habitats. It is easy for a zoologist to see when a cheetah and a gazelle interact and what they are trying to do with respect to each other – they are enemies; their interactions are costly; they have adaptations to maximize their chances of success. A microbiologist wishing to develop the same kind of understanding of the relationship between two species of soil bacteria isolated from the same plot – who might equally be inseparable friends, dire enemies, or neutral bystanders who never interact at all – often needs to take a more indirect approach.

One approach involves the use of experimental evolution of populations in a laboratory environment. Experimental evolution studies have provided key insights into major evolutionary concepts (Lenski and Travisano 1994, Sniegowski, Gerrish et al. 1997, Kawecki, Lenski et al. 2012, Hoang, Morran et al. 2016), but can also be a useful tool for exploring microbial adaptation and behavior(Cooper 2018, Cooper, Honsa et al. 2020). In this study, in lieu of the typical scheme of imposing an artificial selection pressure and observing how a population evolves in response to it, we instead focus on how populations evolve in response to the removal of selective pressures they normally face. We take microbes likely to interact in nature and experimentally evolve them in the laboratory with and without access to their erstwhile partners. We then ask what potential adaptations these microbes lose when their partners (and thus any selective pressures normally imposed by their partners) are removed.

When a preexisting selective pressure is removed, adaptations driven by selection on that pressure are likely to atrophy – organisms seem to 'use it or lose it' and become less well-adapted to environments in which they do not live(Lahti, Johnson et al. 2009, Johnson, Lahti et al. 2012). For example, vestigial or transient traits like the hindlimbs of cetaceans, reduced digits

in birds and ungulate mammals, and the eyes of cave fish appear to serve little function but may be the remnants of adaptations to past environments where selective pressures were different (Darwin 1888, Darwin 2012, Sadier, Sears et al. 2022). Bacteria that live inside larger organisms, and in particular intracellular endosymbionts, experience high rates of decay and loss of genes that are essential for free-living bacteria but not necessary inside of a host (Smith, Buckley et al. 2006, McCutcheon and Moran 2012). Similarly, organisms adapt to selective pressures imposed by other organisms with which they interact and lose these adaptations when their biotic context changes. Viruses that infect an unfamiliar host tend to lose their ability to infect their original host, allowing for the creation of attenuated vaccines (Badgett, Auer et al. 2002). Island animals freed from the threat of predation lose anti-predator behaviors maintained by those on the mainland(Atkinson 2006, Berger, Wikelski et al. 2007, Carthey and Banks 2014). Further, trait losses have frequently been observed in experimental evolution experiments, wherein animal or microbial populations are often passaged in much simpler environments than those in which they evolved (Jaenike 1993, Velicer, Kroos et al. 1998, Holland and Rice 1999, Hoffmann, Hallas et al. 2001, Nilsson, Koskiniemi et al. 2005, Behe 2010, Lee and Marx 2012, Renda, Dasgupta et al. 2015).

Given enough time, mutations accumulating under relaxed selection can cause formerly adaptive traits to disappear via drift. Further, if a trait comes at some cost to the organism – whether in terms of energy, resources, or pleiotropic trade-offs with other traits – selection should actively drive its loss in an environment where it is no longer necessary. We expect this latter mechanism to be especially relevant in experimental evolution. Natural environments pose manifold challenges to organisms that require many separate tools to solve(Fraebel, Mickalide et al. 2017). Adaptations to different pressures are likely to be antagonistically pleiotropic (Rose

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1982), and so an organism's traits are often the result of a balance between the demands of different pressures. Microbes growing in simplified laboratory environments are likely to face very few, very strong selective pressures mostly relating to competition, and any tradeoffs between these traits and adaptations to their original environment will lead to selection against the latter.

In the same way that adaptations should tend to be lost when there is no longer a selective pressure to maintain them, we expect adaptations that evolved to help or harm an interspecific partner to tend to be lost when there is no longer a partner to help or to harm. By looking for such losses, we should be able to infer the manner of relationship the partners may have had. If evolution in isolation makes an organism more destructive to a partner, it suggests that the ancestral organism began with adaptations that benefitted (or at least reduced harm to) its partner's fitness that were lost when they were no longer relevant. Conversely, if evolved organisms become significantly less damaging to their partner's fitness, it suggests that the ancestral organism began with adaptations that harmed their partner in the first place. The traits that an organism loses when evolved under relaxed selection can thus provide evidence for adaptation to a cooperative or antagonistic interaction. Helpful or harmful, an organism cannot lose adaptations it does not have.

In this study we apply this logic to the ambivalent relationship between the social amoeba *Dictyostelium discoideum* and its bacterial symbionts *Paraburkholderia agricolaris, P. hayleyella,* and *P. bonniea* (Haselkorn, DiSalvo et al. 2019). *D. discoideum* is a soil-dwelling amoeba that upon starvation aggregates into a multicellular fruiting body of durable spores that sit dormant and await dispersal atop a stalk of dead cells(Bonner 1944, Kessin 2001, Strassmann and Queller 2011). Several bacteria in the genus *Paraburkholderia* are capable of intracellularly

infing *D. discoideum* and co-dispersing inside of and upon the surface of their hosts' spores throughout multiple rounds of fruiting body formation (smith, Queller et al. 2014, DiSalvo, Haselkorn et al. 2015, Haselkorn, DiSalvo et al. 2019).

Laboratory experiments suggest that the interaction between *D. discoideum* and *Paraburkholderia* can have both positive and negative fitness consequences for each partner. *Paraburkholderia* infection usually reduces the fitness of *D. discoideum* hosts (DiSalvo, Haselkorn et al. 2015), potentially because of the production of toxic compounds, the exploitation of intracellular host resources, or the disruption of normal host digestion. Despite this, *Paraburkholderia* infection also imbues *D. discoideum* with the ability to carry a simple microbiome of other bacteria – including suitable prey bacteria – as passengers through its social stage (Brock, Douglas et al. 2011, DiSalvo, Haselkorn et al. 2015, Dinh, Farinholt et al. 2018), which can facilitate *D. discoideum's* colonization of environments impoverished in food bacteria (Brock, Douglas et al. 2011). *Paraburkholderia* infection may also bolster its host's resistance to toxins (Brock, Callison et al. 2016) and serve to defend against exploitation by uninfected *D. discoideum* strains (Brock, Read et al. 2013). In turn, *Paraburkholderia* living within a host presumably enjoys a resource-rich environment where the costs of competition are minimized, as well as a dispersal advantage from riding along within its much larger and more mobile host. Direct comparisons of *Paraburkholderia* fitness with and without *D. discoideum* in the laboratory yield mixed results (Garcia, Larsen et al. 2019). While *Paraburkholderia* can survive and divide inside of *D. discoideum* cells, the presence of *D. discoideum* in a liquid coculture has been shown to significantly suppress extracellular *Paraburkholderia* growth rate.

It is not clear whether *D. discoideum* and its symbionts should be considered friends, foes, both, or neither. In at least some circumstances *D. discoideum* and *Paraburkholderia* may

benefit from their association with one another, while in others, they may suffer. To what extent *D. discoideum* and *Paraburkholderia* are likely to have evolved adaptations to help or harm one another should depend upon the specific circumstances under which they typically interact – for instance how many *Paraburkholderia* infect each *D. discoideum*, how capable *Paraburkholderia* is of switching hosts, how often *D. discoideum* can benefit from the ability to carry food, and which *D. discoideum* strains interact with which strains of which species of *Paraburkholderia*. Evolving *D. discoideum* and *Paraburkholderia* under conditions in which they cannot access one another (and thus conditions in which any unknown selective pressures they ordinarily exert upon one another in nature are relaxed) we can gain useful insight about the balance of cooperation and antagonism between these microbes in nature.

We previously published a study that outlined these ideas and attempted to demonstrate them using *D. discoideum, P. agricolaris,* and *P. hayleyella* (Larsen, Jefferson et al. 2021). Our later sequence analysis revealed cross-contamination issues that called our specific results into question. This study is intended to replicate its predecessor while incorporating numerous improvements. We employed species-specific PCR screens and fragment analyses throughout and after experimental evolution to verify that lines had not been contaminated. In addition, we expanded the original experiment with the inclusion of a third symbiont species (*P. bonniea*) as well as non-symbiotic bacteria and non-host strains of *D. discoideum*. We also added a control treatment wherein *D. discoideum* and *Paraburkholderia* were evolved in coculture to distinguish between adaptation to isolation from symbiotic partners *per se* and adaptation to other elements of the laboratory environment.

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A. Natural environment
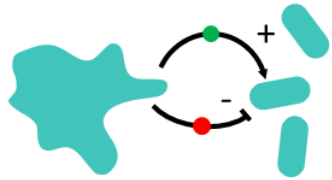
In its natural environment, *D. discoideum* may have unknown adaptations (● and ●) which help or harm *Paraburkholderia*.

B. Experimental evolution

Evolve *D. discoideum* in isolation

serial passage

In the laboratory in the absence of *Paraburkholderia*, adaptations to help or hurt it are no longer useful and should be lost

Evolve *D. discoideum* and *Paraburkholderia* together

serial passage

When evolved with *Paraburkholderia* present, adaptations to help or hurt it may remain relevant and so be retained.
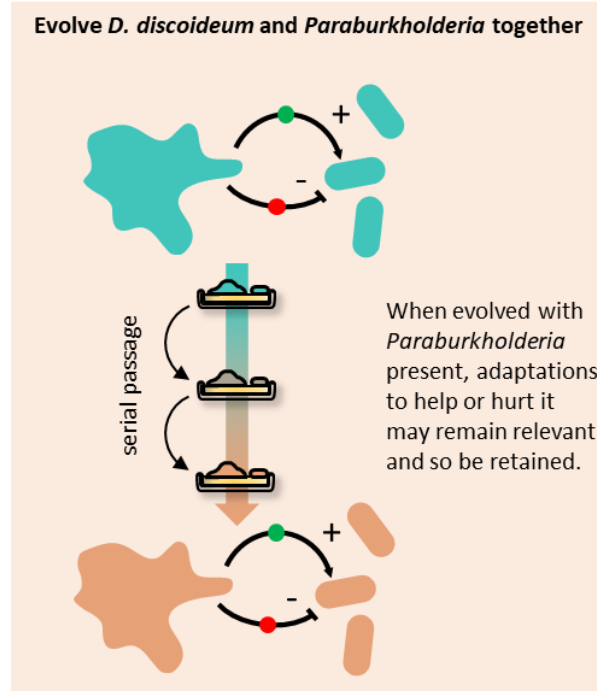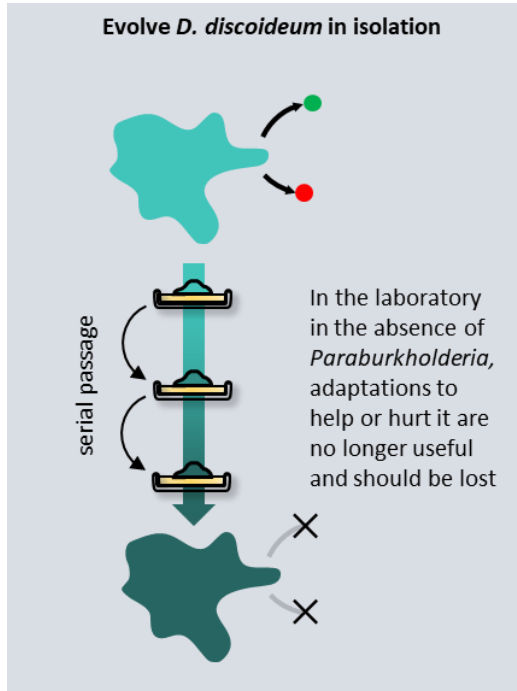
C. Reinfect with *Paraburkholderia* and measure changes in its fitness in coculture

or

If evolved *D. discoideum* harms *Paraburkholderia* more, **ancestral *D. discoideum* was helpful**

If evolved *D. discoideum* helps *Paraburkholderia* more, **ancestral *D. discoideum* was harmful**

Presence of *Paraburkholderia* during evolution means no relaxed selection. Changes in *D. discoideum's* effects may have nothing to do with *Paraburkholderia*.

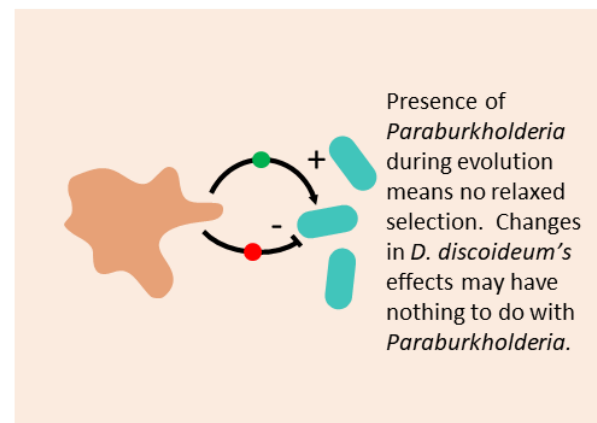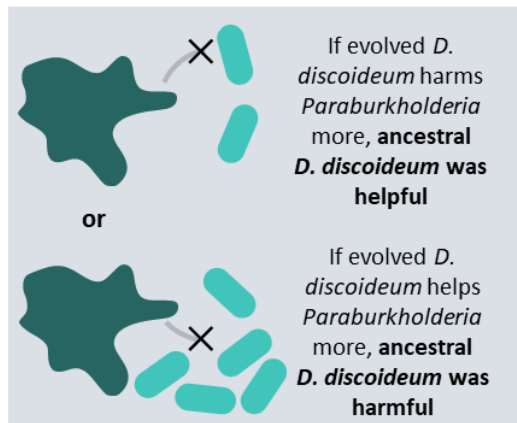**Figure 3.1 – using trait loss during experimental evolution to infer whether *D. discoideum* has adaptations which help or harm *Paraburkholderia* in nature.** Example logic for finding adaptations in *D. discoideum* which affect *Paraburkholderia* fitness.

A. In nature*, D. discoideum* potentially has adaptations which increase or decrease *Paraburkholderia* fitness. Here we represent these adaptations as secreted products (green or red circles) but any trait with an associated cost could be expected to follow the same logic. **B.** these adaptations are costly or irrelevant in *Paraburkholderia's* absence (left), but potentially still useful in its presence (right). **C.** Loss of beneficial or antagonistic adaptations can be inferred by reintroducing ancestral *Paraburkholderia* and assessing its fitness in the presence of *D. discoideum* evolved in isolation.

This figure depicts the scheme for finding adaptations in *D. discoideum* which affect *Paraburkholderia*. We applied exactly parallel logic to look for adaptations affecting *D. discoideum's* susceptibility to *Paraburkholderia's* effects and to look for adaptations in *Paraburkholderia*.

2.3   Methods

Strain selection – We selected three pairs each of naturally-occurring *D. discoideum/P. agricolaris* partners (QS70/bQS70, QS159/bQS159, QS161/bQS161), *D. discoideum/P. hayleyella* partners (QS11/bQS11, QS21/bQS21, QS69/bQS69), and *D. discoideum/P. bonniea* partners (QS395/bQS395, QS481/bQS481, QS859/bQS859).

Additionally, we selected three *D. discoideum* isolates with no associated *Paraburkholderia* infection (QS6, QS9, QS18 – hereafter called 'non-hosts') and three edible non-symbiont bacteria collected from the field together with *D. discoideum*(Brock, Haselkorn et al. 2018) to represent potential prey species (*Achromobacter aegrifaciens (*AaegC*), Comamonas kerstersii (*CkerE*), Pseudomonas fluorescens* (Pf)) (hereafter 'prey'). As we have reason to believe our non-host and prey strains are on average less intimate partners in nature than *D.*

discoideum/Paraburkholderia symbiont pairs, we expected that inclusion of these strains would help validate our prediction that loss of symbiotic partners should drive trait loss.

In all cases, we cured *D. discoideum* of *Paraburkholderia* infection using antibiotics (see below) before use, then reinfected as needed for phenotypic assays to ensure a consistent infective dose.

Culture conditions – We conducted all experiments using SM/5 media(Loomis and Sussman 1966) (2 g glucose (Fisher Scientific), 2 g BactoPeptone (Oxoid), 2 g yeast extract (Oxoid), 0.2 g $MgCl_2$ (Fisher Scientific), 1.9 g $KHPO_4$ (Sigma-Aldrich), 1 g K_2HPO_5 (Fisher Scientific), and for solid media 15 g agar (Fisher Scientific) per liter deionized water). To start a fresh culture of *D. discoideum*, we diluted spores from -80°C glycerol frozen stocks in KK2 buffer (2.25g KH_2PO_4 (Sigma-Aldrich) and 0.67g K_2HPO_4 (Fisher Scientific) per liter deionized water). We plated $1.0x10^5$ total spores onto an SM/5 plate along with 200µl *K. pneumoniae* food bacteria resuspended in KK2 to an OD_{600} of 1.5. To start a fresh culture of any bacterial strain, we streaked stocks from the -80°C freezer for isolation on SM/5 plates.

Antibiotic curing of *D. discoideum* – In order to remove associated bacteria, $1.0x10^5$ *D. discoideum* spores were plated on SM/5 agar medium containing 30ug/mL tetracycline and 10ug/mL ciprofloxacin with 200µl of *K. pneumoniae* resuspended in KK2 to an OD_{600} of 1.5. We allowed plates to grow at room temperature under ambient light until formation of fruiting bodies (3-5 days). We collected spores as above, then diluted and plated again as above. We then collected spores and performed spot test assays (described in (Brock, Douglas et al. 2011)) and PCR using *Paraburkholderia*-specific primers to verify successful curing.

Experimental evolution – We separated natural pairs of *D. discoideum/P. agricolaris, D. discoideum/P. hayleyella,* and *D. discoideum/P. bonniea* from their partners as described above

and experimentally evolved them either isolated from or reinfected and cocultured with their partners. The coculture treatment was intended to serve as a control to distinguish effects of the absence of symbiotic partners *per se* and effects of other elements of the laboratory environment. In addition, we evolved non-host *D. discoideum* strains not known to harbor *Paraburkholderia* and potential prey bacteria not known to infect *D. discoideum* (see strain selection for details). Absent natural partners, non-host and prey strains lacked coculture treatments.

All lines were evolved in triplicate on SM/5 plates. Lines including *D. discoideum* were supplemented with 200µl of *K. pneumoniae* food bacteria resuspended in KK2 to an OD_{600} of 1.5 on SM/5 plates. We incubated all lines at room temperature under ambient light and transferred 0.5% of the population to fresh plates every 48 hours by first harvesting all spores and cells into 10mL KK2 buffer using gentle pipetting and scraping of the agar surface, thoroughly vortexing the resulting suspensions, diluting them 200-fold, and plating 100µl onto fresh plates. Plates containing *D. discoideum* were additionally supplemented with 200uL of *K. pneumoniae* suspension as a food source.

The 48-hour transfer interval was selected to preempt *D. discoideum's* fruiting stage and avoid selection for non-fruiting cheaters observed in previous studies (Kuzdzal-Fick, Fox et al. 2011). Every fifth transfer, we froze 1mL of the undiluted suspension of harvested cells at -80°C with 60% glycerol to act as a frozen archive.

During the experimental evolution, routine checks were done for contamination of bacterial lines. We extracted DNA from 100uL of the undiluted suspension of harvested cells by boiling and ran PCR using primers specific for *P. agricolaris* and *P. hayleyella*(Garcia, Larsen et al. 2019). In a small number of cases, tested lines showed amplification of the incorrect species or

did not show amplification of the correct species, suggesting contamination had occurred. Affected lines were discarded and resumed from the most recent frozen ancestors using the process described above. Following experimental evolution (after the 30th transfer) *D. discoideum* lines were checked for cross-contamination using fragment analysis. We extracted DNA from 100µl of the undiluted suspension of harvested cells using CHELEX resin beads and amplified using fluorescently tagged PCR primers specific to highly variable microsatellite loci known to differ in length between *D. discoideum* strains (Smith 2004). Fragment analysis of resulting amplicons was performed by Genewiz and evolved strains were compared to ancestors. In order to maximize the usefulness of both tests for contamination, transfers were completed in a predefined order such that no two lines of the same species were transferred sequentially, and replicate lines were transferred at different times with reagents replaced and the workstation cleaned in between each replicated set.

At the conclusion of the experimental evolution, lines of cocultured *D. discoideum* and *Paraburkholderia spp.* were separated in order to separately assay their evolved changes. To collect only *D. discoideum* without *Paraburkholderia*, we followed the antibiotic curing process outlined above. To collect only *Paraburkholderia* without *D. discoideum*, we centrifuged collected suspensions at 300xG for 10 minutes, discarded the resultant pellets, and plated the supernatants on fresh SM/5 plates. We inspected plates after 5 days of growth to verify that we had successfully eliminated any *D. discoideum*.

After all lines had evolved in isolation for 30 transfers (representing approximately 130 generations), we reintroduced evolved strains to their partners and used two assays to test how evolution in isolation had changed the fitness effects that strains imposed upon their partners and the susceptibility of strains to their partners reciprocal effects. In all assays, *D. discoideum* and

Paraburkholderia were paired with their naturally occurring partners, which had originally been co-isolated. All *Dictyostelium* lines were cured before use (see above) and reinfected for each assay to ensure consistency. For strains that did not have naturally occurring partners (the three non-host *D. discoideum* strains and the three prey bacteria strains), we used the *Paraburkholderia agricolaris* strain bQS70 and the *D. discoideum* strain QS6 respectively as partners in our experiments.

Assays of *D. discoideum* spore production - To observe how experimental evolution of bacteria affected their impact on *D. discoideum* fitness, we compared total spore production of ancestral *D. discoideum* when uninfected, and when infected by ancestral bacteria, bacteria evolved in isolation, or bacteria coevolved in the presence of *D. discoideum.*

In order to observe how experimental evolution of *D. discoideum* affected its susceptibility to the effects of bacterial infection, we compared total spore production of ancestral *D. discoideum, D. discoideum* evolved in isolation, and *D. discoideum* coevolved in the presence of bacteria when uninfected and when infected by ancestral bacteria.

For each spore production assay, we first suspended spores of each *D. discoideum* line of interest in KK2 buffer to a concentration of 10^6 spores/mL. We suspended *K. pneumoniae* food bacteria and each bacterial symbiont line of interest in KK2 buffer at an OD_{600} of 1.5. We inoculated SM/5 plates with 100µl of the appropriate *D. discoideum* suspension (~10^5 spores) and 200µl of either 100% *K. pneumoniae* suspension or a 95:5 (vol:vol) mixture of *K. pneumoniae* and the appropriate bacterial symbiont suspension. We incubated plates at room temperature under ambient light for 5 days, after which we harvested all spores by washing plates with 10mL of a detergent solution of 0.1% NP40 in KK2. We diluted the resulting spore suspensions and

counted on a hemocytometer to estimate total spore production across the entire plate. We performed each assay three times.

Assays of bacterial growth rate – In order to observe how experimental evolution of *D. discoideum* affected its impact on bacterial symbiont fitness, we compared growth rates of ancestral bacteria when cultured alone and when cocultured with ancestral *D. discoideum, D. discoideum* evolved in isolation, or *D. discoideum* coevolved in the presence of bacterial symbionts.

In order to observe how experimental evolution of bacterial symbionts affected their susceptibility to the presence of *D. discoideum,* we compared growth rates of ancestral bacteria, bacteria evolved in isolation, and bacteria coevolved in the presence of *D. discoideum* when cultured alone and when cocultured with ancestral *D. discoideum.*

For each growth rate assay, we inoculated each bacterial line of interest from freezer stocks into 2mL liquid SM/5 media and grew them to stationary phase overnight at 30°C in a shaking incubator. We quantified bacterial suspensions using a spectrophotometer and diluted them to an OD_{600} of 0.1. We prepared *D. discoideum* amoeba suspensions by inoculating SM/5 plates with *D. discoideum* spores from freezer stocks and *K. pneumoniae* food bacteria, incubating for ~48 hours at room temperature, and harvesting amoebae by washing plates with KK2. To remove residual *K. pneumoniae,* we washed the resulting *D. discoideum* suspensions 3 times by centrifuging at 300xG for 3 minutes then resuspending the pellet in KK2. After the third wash, we resuspended pellets in KK2 buffer containing 30ug/mL tetracycline and 10ug/mL ciprofloxacin for 1 hour. We then centrifuged and washed the antibiotic-treated suspensions 2 more times with fresh KK2, counted amoebae using a hemocytometer, and diluted them to 10^7

amoebae/mL. We obtained growth curves from a Tecan Spark microplate reader. We prepared wells of a 96 well plate by combining 100µl SM/5 broth with either 10µl OD_{600} 0.1 *Paraburkholderia* suspension or 10µl KK2 and either 10µl 10^7 amoebae/mL *D. discoideum* suspension or 10µl KK2. We took OD_{600} measurements every 15 minutes for 48 hours to produce a growth curve for each well. We fit growth curves and calculated maximum specific growth rates using the fitr script (https://github.com/dcangst) in R.

We used an identical process to measure how experimental evolution of bacteria affected their susceptibility to the effects of *D. discoideum* by comparing growth rates of ancestral and evolved bacteria in the presence and absence of ancestral *D. discoideum*.

Statistical analyses - We analyzed results using random effect negative binomial models with replicate line as a random effect. Models were evaluated primarily using ANOVA to compare AIC values.

For data comparing the effects of ancestral and evolved bacteria on ancestral *D. discoideum* spore production, the final, AIC-minimized model was Sporeproduction ~ Treatment*Strain + (1|Line).

For data comparing the susceptibility of ancestral and evolved bacterial growth rates to the presence of ancestral *D. discoideum,* the final, AIC-minimized model was Growthrate ~ Treatment+Strain + (1|Line).

For data comparing the effects of ancestral and evolved *D. discoideum* on ancestral bacterial growth rates, the final, AIC-minimized model was Growthrate ~ Strain + (1|Line).

For data comparing the susceptibility of ancestral and evolved *D. discoideum* spore production to the presence of ancestral bacteria, the final, AIC-minimized model was Sporeproduction ~ Strain + (1|Line).

We performed analysis using R version 4.0.4 (Team 2013), the *lme4* package(Bates, Maechler et al. 2015), the *MASS* package (Ripley, Venables et al. 2013), and the *emmeans* package(Lenth 2022).

2.4 Results

Experimentally evolved *Paraburkholderia* have reduced antagonistic effects on ancestral *D. discoideum* hosts

To assay how experimental evolution of *Paraburkholderia* affected its fitness effects on its *D. discoideum* host, we compared *D. discoideum* spore production when uninfected and when infected by (1) ancestral bacteria, (2) bacteria experimentally evolved in the absence of *D. discoideum,* and (3) bacteria experimentally evolved in coculture with *D. discoideum* (Figure 3.2A). We analyzed spore count data using a negative binomial mixed effects model incorporating line as a random effect.

Most tested ancestral bacteria had a negative effect on *D. discoideum* spore production under the conditions of our assay (Figure 3.2B). Our final model indicated a significant interaction between *Paraburkholderia* strain and experimental evolution treatment (χ^2 (16)=103.674, p<0.0001) but not between bacterial species and treatment. Overall, experimentally evolved *Paraburkholderia* – whether evolved in isolation or in coculture with *D. discoideum* – tended to

become less toxic to their hosts. Estimated marginal means indicate that different strains

responded differently to evolution treatment (Figure 3.2C) (see Supp. Table 1 for estimates).
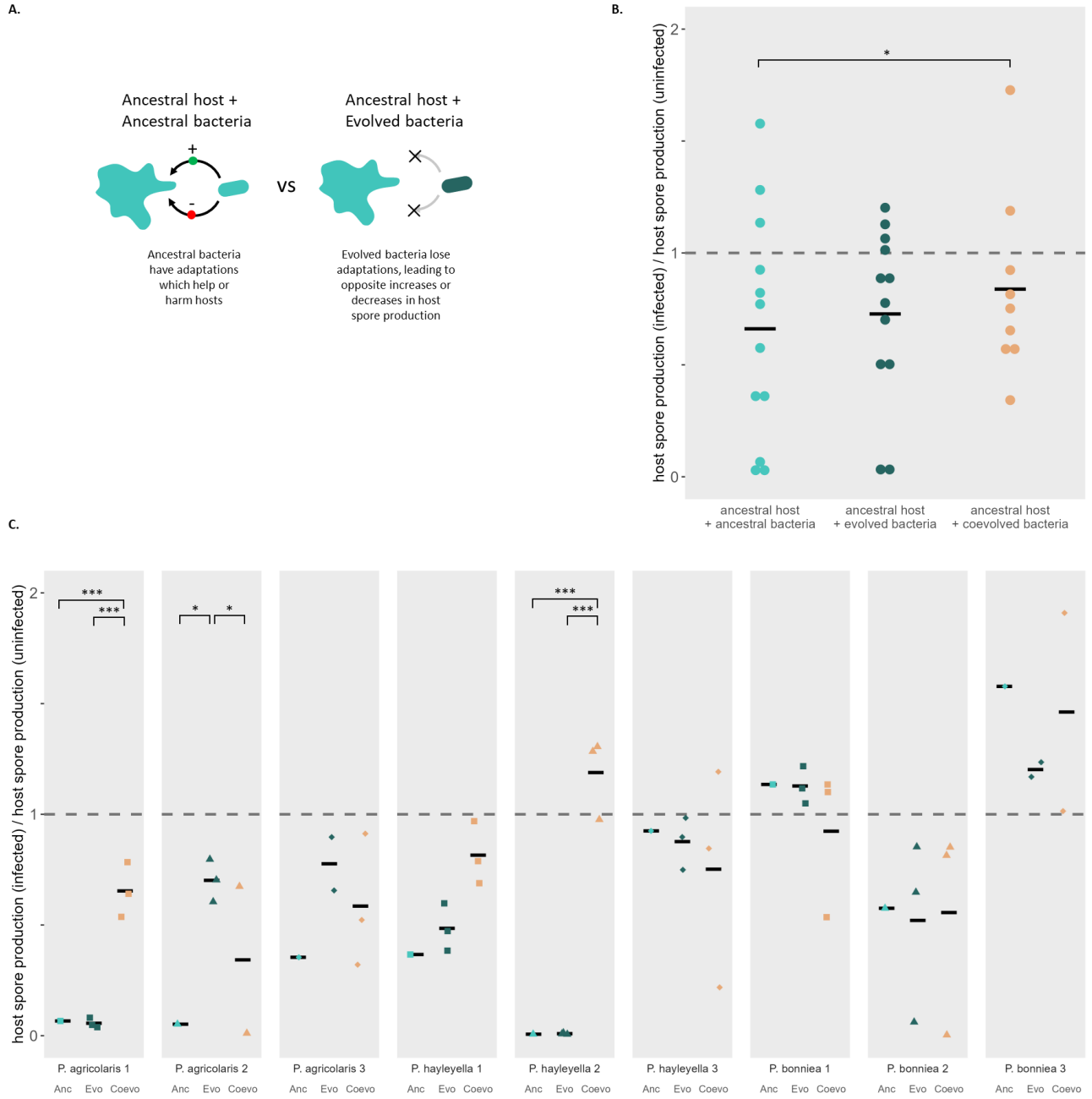
Figure 3.2 – Experimental evolution reduces *Paraburkholderia* antagonism towards *D. discoideum* hosts. **A.** Comparing the effects of ancestral and evolved bacteria on ancestral *D. discoideum spore production* to look for bacterial adaptation to help or harm hosts. **B.** Infected *D. discoideum* spore production (as a proportion of uninfected *D. discoideum).* Colors denote ancestral bacteria (teal), bacteria evolved in isolation (dark green), and bacteria coevolved with *D. discoideum* (orange). Each point represents an average of three independent lines. **C.** Data from panel B separated by strain and line. Each point represents an independent line.

Experimentally evolved *Paraburkholderia* have increased susceptibility to growth suppression by *D. discoideum* in coculture

To assay how experimental evolution of *Paraburkholderia* affected its susceptibility to the growth suppression of cocultured *D. discoideum,* we compared the growth rate with and without the presence of ancestral *D. discoideum* of (1) ancestral bacteria, (2) bacteria evolved in the absence of *D. discoideum* hosts, and (3) bacteria evolved in coculture with *D. discoideum* (Figure 3.3A). We analyzed growth rate data using a negative binomial mixed effects model incorporating line as a random effect.

Most tested ancestral *D. discoideum* had a negative effect on bacterial growth rates. Our final model indicated a significant effect of treatment (χ^2 (16)=32.80, p<0.05) and strain (χ^2

(16)=32.80, p<0.05). There was no significant interaction between strain and treatment, indicating that different bacteria responded similarly to the presence of *D. discoideum* (Figure 3.3C).
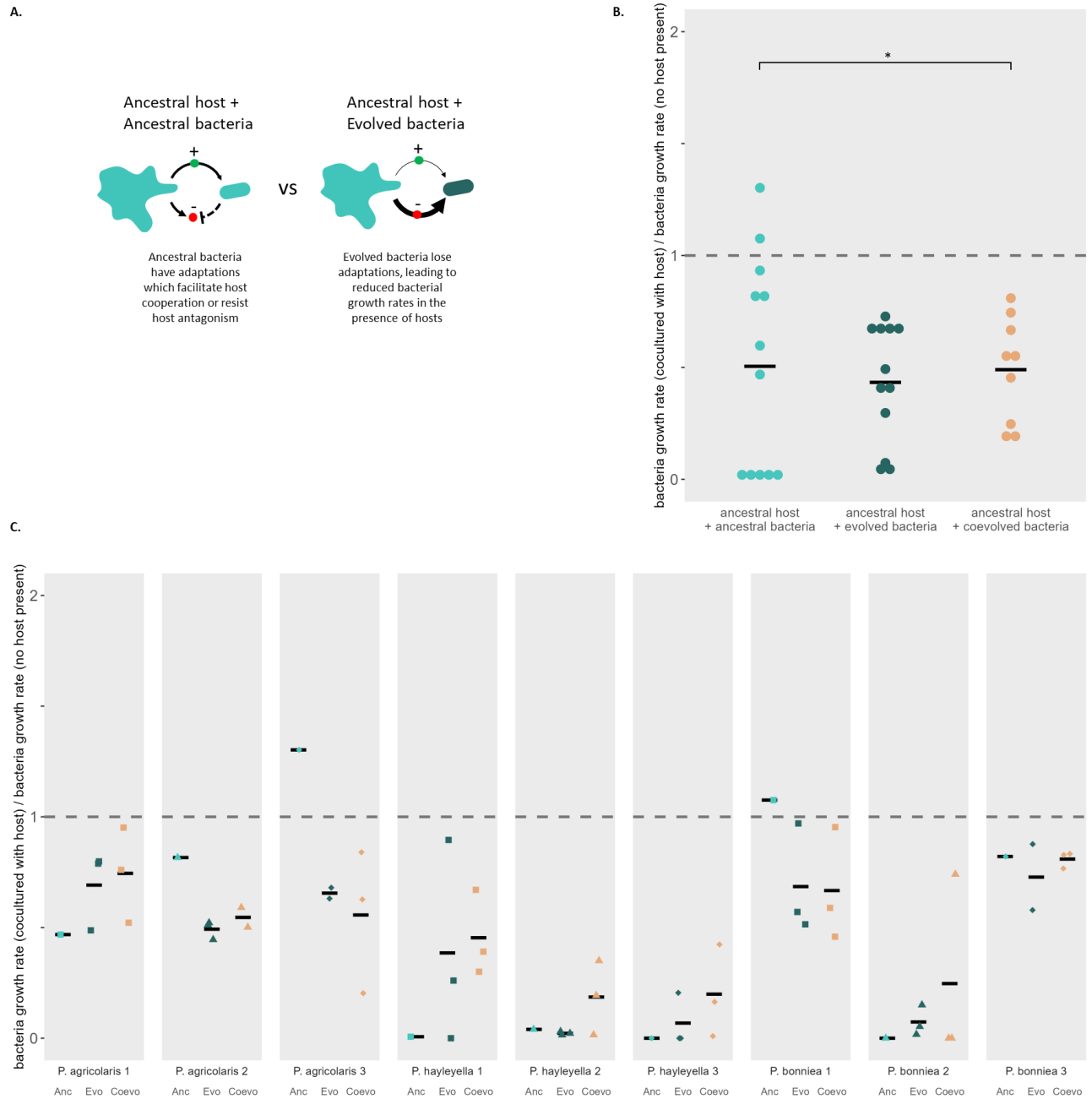
Figure 3.3 – Experimental evolution increases *Paraburkholderia* susceptibility to *D. discoideum* antagonism. A. Comparing the effects of ancestral *D. discoideum* on the growth rate of ancestral and evolved bacteria to look for bacterial adaptation to resist host antagonism or facilitate host cooperation. **B.** Bacteria growth rate in the presence of *D. discoideum* (as a proportion of growth rate with no *D. discoideum* present). Colors denote ancestral bacteria (teal), bacteria evolved in isolation (dark green),

and bacteria coevolved with *D. discoideum* (orange). Each point represents an average of three

independent lines. **C.** Data from panel B separated by strain and line. Each point represents an

independent line.

Experimentally evolved *D. discoideum* did not change in their ability to suppress bacterial growth in coculture.

To assay how experimental evolution of *D. discoideum* affected its ability to suppress the growth

rate of bacteria in its environment, we compared bacterial growth rates in the absence of *D.*

discoideum and when cocultured with (1) ancestral *D. discoideum,* (2) *D. discoideum*

experimentally evolved in the absence of bacterial symbionts, and (3) *D. discoideum*

experimentally evolved in coculture with bacterial symbionts (Figure 3.4A).

Most tested ancestral *D. discoideum* had a negative effect on bacterial growth rates under the

conditions of our assay (Figure 3.4B). We did not find significant effects of experimental

evolution *on D.* discoideum's effect on growth rates, nor significant differences between strains.
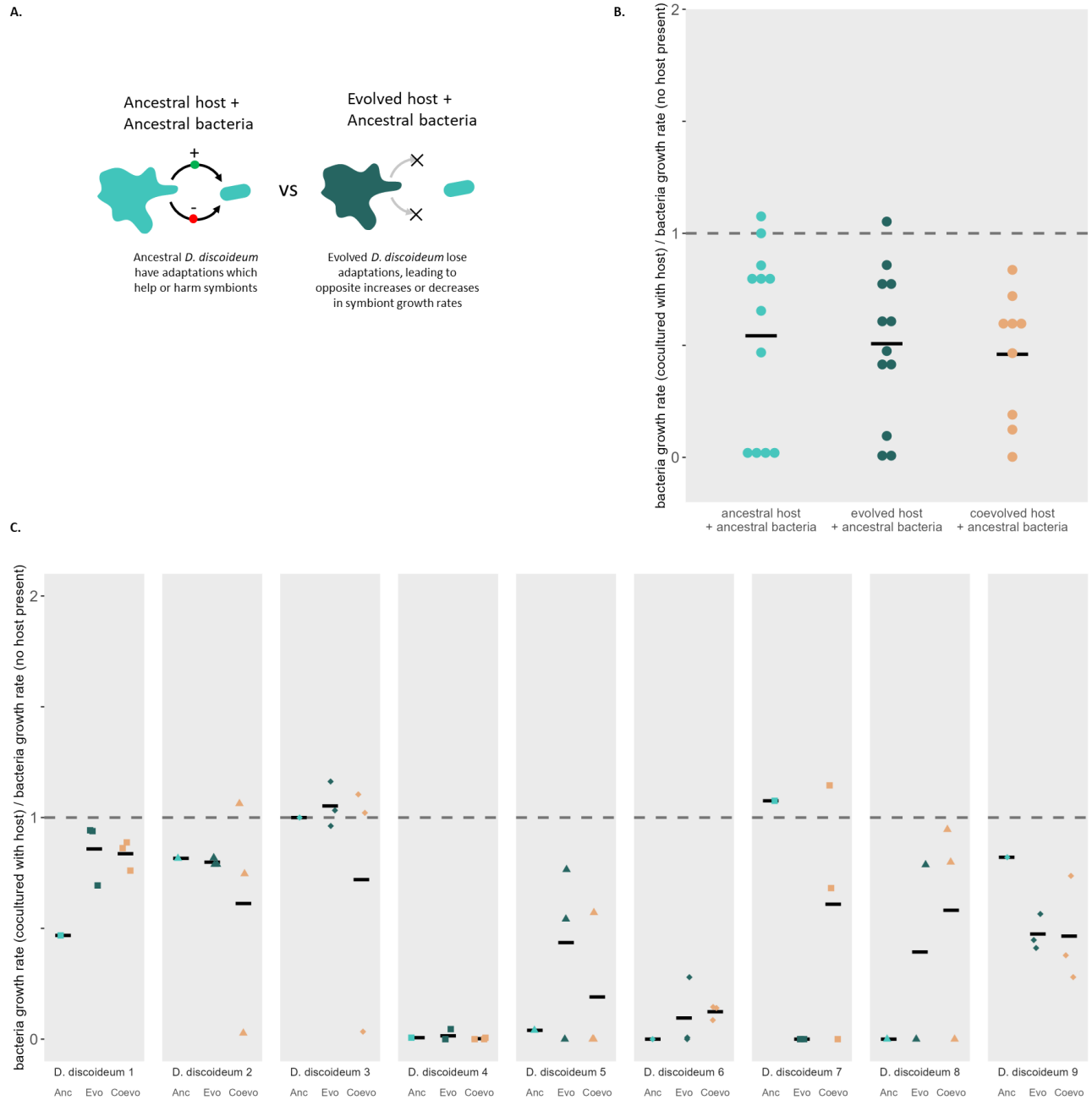
(Figure 3.4C)

Figure 3.4 – Experimental evolution did not significantly impact *D. discoideum* ability to suppress bacterial growth. **A.** Comparing the effects of ancestral and evolved *D. discoideum* on the growth rate of ancestral bacteria to look for *D. discoideum* adaptation to help or harm bacteria. **B.** Bacteria growth

rate in the presence of *D. discoideum* (as a proportion of growth rate with no *D. discoideum* present*).* Colors denote ancestral *D. discoideum* (teal), *D. discoideum* evolved in isolation (dark green), and *D. discoideum* coevolved with *bacteria* (orange)*.* Each point represents an average of three independent lines. **C.** Data from panel B separated by strain and line. Each point represents an independent line.

Experimentally evolved *D. discoideum* did not gain or lose susceptibility to the antagonistic effects of *Paraburkholderia* infection.

In order to assay how experimental evolution of *D. discoideum* affected its susceptibility to the negative fitness consequences of bacterial infection, we compared the spore production of infected and uninfected (1) ancestral *D. discoideum*, (2) *D. discoideum* evolved in the absence of bacterial symbionts, and (3) *D. discoideum* evolved in coculture with bacterial symbionts (Figure 3.5A).

Most *D. discoideum* produced fewer spores when infected by ancestral bacteria under the conditions of our assay (Figure 3.5B). We did not find significant effects of experimental evolution *on D.* discoideum's susceptibility, nor significant differences between strains. (Figure 3.5C)

A.

Ancestral host + Ancestral bacteria

Ancestral *D. discoideum* have adaptations which facilitate symbiont cooperation or resist symbiont antagonism

VS

Evolved host + Ancestral bacteria

Evolved *D. discoideum* lose adaptations, leading to reduced host spore production in the presence of symbionts
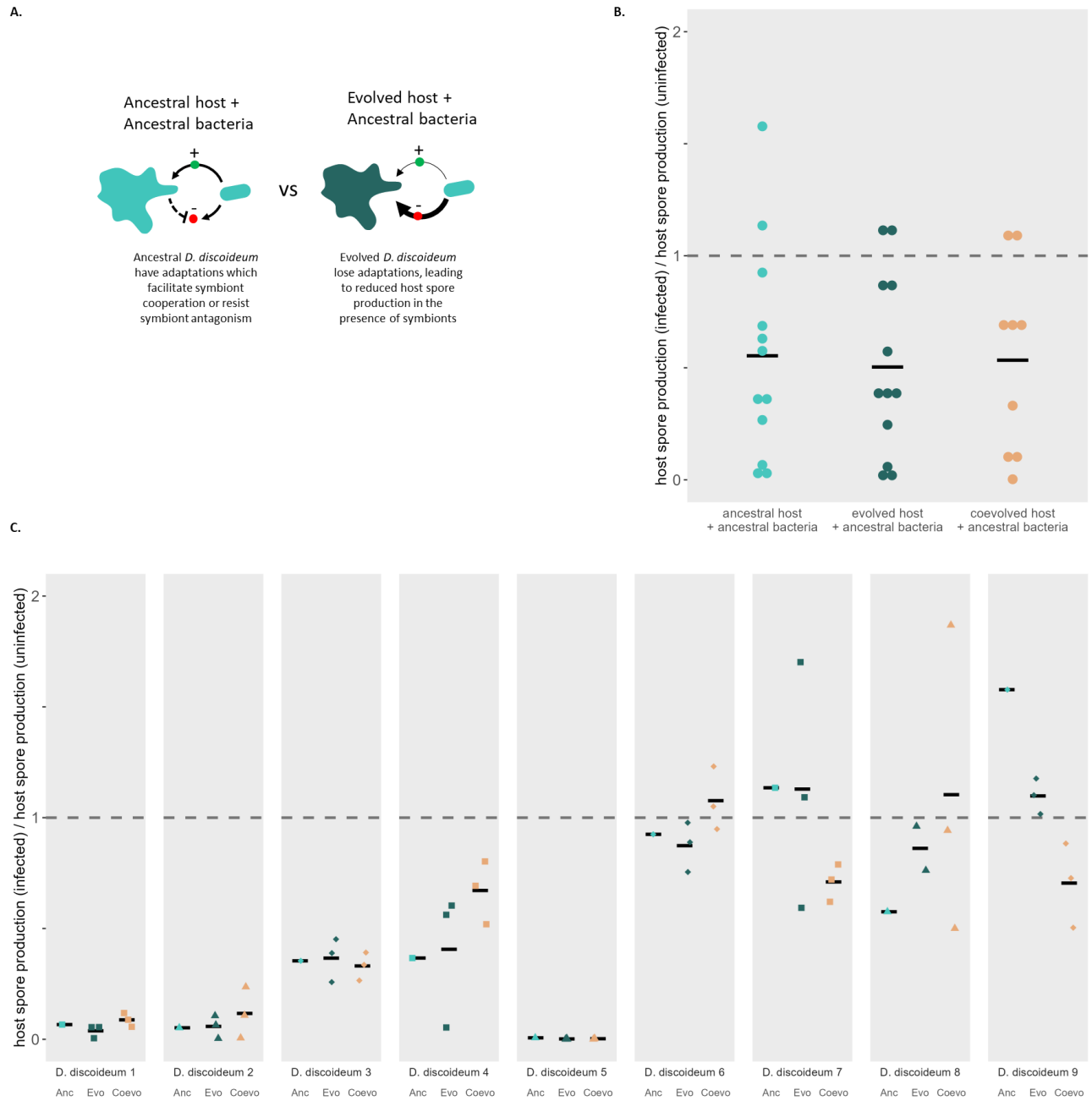
B.

C.

Figure 3.5 – Experimental evolution did not significantly impact *D. discoideum* susceptibility to bacterial infection. A. Comparing the effects of ancestral bacteria on the spore production of ancestral

and evolved *D. discoideum* to look for *D. discoideum* adaptation to resist symbiont antagonism or facilitate symbiont cooperation. **B.** Infected *D. discoideum* spore production (as a proportion of uninfected *D. discoideum).* Colors denote ancestral *D. discoideum* (teal), *D. discoideum* evolved in isolation (dark green), and *D. discoideum* coevolved with *bacteria* (orange). Each point represents an average of three independent lines. **C.** Data from panel B separated by strain and line. Each point represents an independent line.

2.5 Discussion

In a laboratory environment where they have no access to one another, *D. discoideum* and *Paraburkholderia* should tend to lose adaptations they have to help or harm one another in nature. In this study, we sought to use this prediction to infer the selective pressures *D. discoideum* and *Paraburkholderia* impose upon one another in nature. Rather than ask how a microbe adapts to novel artificial selective pressures as in many experimental evolution studies, we study how microbes lose adaptations when the selective pressures they experience in nature are taken away.

Experimentally evolved *Paraburkholderia* were less toxic towards their ancestral *D. discoideum* hosts, although the effect was only statistically significant in the coevolution treatment (Figure 3.2B). This trend was most clear in *P. agricolaris* and *P. hayleyella*, but not significant in *P. bonniea* (Figure 3.2C) or the prey bacteria**.** *Paraburkholderia* becoming less toxic in the laboratory could be interpreted as evidence that the wild *Paraburkholderia* strains with which we started the experiment had adaptations that made them toxic in the first place. When moved to the laboratory where selective pressures are relaxed, these adaptations may have

become neutral or even maladaptive and thus evolved away over time. However, the fact that reduced toxicity was more evident in the coevolution treatment suggests that it was not necessarily driven by the availability of symbiotic partners *per se*, and may have involved changes in selective pressures related to abiotic aspects of the environment.

Experimentally evolved *Paraburkholderia* evolved changes in their susceptibility to growth suppression, consistent with wild ancestor strains having possessed adaptations to resist *D. discoideum* antagonism that were rendered irrelevant when no *D. discoideum* was present (Figure 3.3A). This effect was clearest in *P. agricolaris* (Figure 3.3B), which had also evolved the clearest changes in its own toxicity. Taken together, these two observations suggest that *P. agricolaris*' relationship with *D. discoideum* in nature might be especially important, and be the driving force behind multiple adaptations.

We did not detect overall or symbiont species-level changes in experimentally evolved *D. discoideum's* ability to suppress *Paraburkholderia's* growth rate (Figure 3.4A). While there was initial variation between ancestral *D. discoideum* strains (Figure 3.4B), we did not observe reductions in growth suppression that we would expect if it was an adaptation to antagonizing *Paraburkholderia* in nature.

We did not detect any overall or symbiont species-level changes in experimentally evolved *D. discoideum's* susceptibility to *Paraburkholderia* toxicity (Figure 3.5A). While hosts of different *Paraburkholderia* species varied in their susceptibility (particularly apparent comparing hosts of *P. agricolaris* and *P. bonniea*) (Figure 3.5B), experimental evolution did not result in the increases in susceptibility we would have predicted if *D. discoideum* had lost no-

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longer-relevant resistance mechanisms in the laboratory. At the strain level, all three *P. bonniea* strains showed significant changes, but in inconsistent directions (Figure 3.5C).

We included the bacteria *Achromobacter aegrifaciens, Comamonas kerstersii,* and *Pseudomonas fluorescens* to represent plausible prey of *D. discoideum* that did not infect amoebae intracellularly. We assumed these strains would have weaker relationships (if any) with *D. discoideum* than *Paraburkholderia* did. Consistent with these expectations, we did not detect statistically significant changes in toxicity or susceptibility to growth suppression in these prey species at any level. The single exception was *A. aegrifaciens,* which evolved significantly increased susceptibility to *D. discoideum* growth suppression. This suggests *A. aegrifaciens* may have had adaptations granting them some resistance in the first place.

Our results suggest that whether the relationship between *D. discoideum* and its *Paraburkholderia* symbionts is cooperative, antagonistic, or neutral in nature may vary considerably even among closely related strains. We observed relatively few statistically significant overall or species-level trends. Instead, different strains of the same species sometimes showed markedly different degrees of initial antagonism (for example, see Figure 3.2C) and sometimes opposite responses to experimental evolution**.** Though the strains we employed in this study were isolated at roughly the same site at roughly the same time, the apparent differences in the degree to which they have adapted to cooperate or antagonize their partners may reflect adaptation to conditions varying over smaller scales of space and time. The relationship between *D. discoideum* and *Paraburkholderia* is sensitive to environmental conditions, and if different strains experienced slightly different conditions, they might well adapt in different directions or to different degrees. Importantly, the high strain level variation we detected underscores the importance of designing experiments using multiple strains when

possible, and of not carelessly extrapolating observations based upon observations of a single strain.

In some cases replicate lines started from the same ancestral population arrived at different outcomes by the end of the experiment. This non-parallelism reflects the effect of drift and contingency on evolutionary outcomes. Past experimental evolution experiments(Kuzdzal-Fick, Fox et al. 2011, Larsen, Jefferson et al. 2021) had led us to believe the timeframe of this study was long enough to see changes, but we expect that a longer experimental evolution regime would likely have led to more parallel outcomes.

Though our chief interest was in how being isolated from one's symbiotic partner would drive evolutionary change, of course the environment in which we evolved our lines is likely to have differed from their natural environment in other ways that have nothing to do with interspecific interactions. Our purpose in including a treatment wherein bacteria and *D. discoideum* partners were plated and transferred in coculture and were both free to evolve (hereafter the 'coevolution treatment') was to provide a control to distinguish between trait losses driven by isolation from other species *per se* and trait losses driven by some other abiotic aspect of the laboratory environment. Our expectation was that if *D. discoideum* or *Paraburkholderia* had adaptations specifically to help or harm one another, we would detect changes in the evolution in isolation treatment where such adaptations had been rendered irrelevant but *not* in the coevolution treatment where the partners could still affect one another. Surprisingly, however, we also found multiple examples where we detected changes in the coevolution treatment but not in the evolution in isolation treatment. These instances seem to indicate that while the presence or absence of partners did have an effect on *D. discoideum* and

Paraburkholderia's evolution, it sometimes did so through selective pressures that existed in the coevolution treatment in the laboratory but not in nature.

For example, the *P. agricolaris* strain bQS70 evolved significantly reduced toxicity when experimentally coevolved with *D. discoideum* but not when evolved alone. This result seems intuitive, except that it suggests that it only became in bQS70's best interest to limit the harm it did to its *D. discoideum* cohabitants after we had moved it to the laboratory. It is not obvious why *D. discoideum* and *Paraburkholderia's* fates should be more tightly linked in the apparently simple laboratory environment of our experiment than they were in nature. One interesting possibility is that perhaps living *D. discoideum* somehow assisted bQS70 cells in being transferred to the next plate, similar to how *D. discoideum* may aid bacterial dispersal in nature. While we designed our experiment such that, to the extent that it was possible, the evolution in isolation and coevolution treatments only differed in the presence or absence of interspecific partners, there were unavoidable differences that may explain different evolutionary outcomes in the two treatments. Most importantly, wild *D. discoideum* strains require the presence of prey bacteria (*K. pneumoniae*, in our experiment) which compete with *Paraburkholderia.* This could explain why *Paraburkholderia* might have an especial interest in limiting the damage it did to *D. discoideum* in the laboratory – because *D. discoideum* preys upon its chief competitor. Another plausible difference between the isolation and coevolution treatments besides the presence of symbiotic partners *per se* is in effective population size of one or both species, which could have impacted the strength of selection and drift.

Even traits with clear adaptive function come with associated costs, whether in the form of energetic costs of the traits themselves or more complicated tradeoffs between traits(Guillaume and Otto 2012, Rodríguez-Verdugo, Carrillo-Cisneros et al. 2014). A microbe

living in nature must make compromises between mutually incompatible traits impacted by multiple selective pressures. When the microbe is moved to a simpler laboratory environment with fewer selective pressures, some compromises will no longer be necessary and traits should be lost to reflect that. In principle, it is possible that pleiotropic effects could instead result in gains of functionality(Meyer, Agrawal et al. 2010), but these situations should be the exception. Most pleiotropy will be negative rather than positive for the same reason that most mutational effects are negative – there are more ways to break complex adaptations than there are to enhance them(Johnson, Lahti et al. 2012). Given time, geographically isolated populations tend to lose reproductive compatibility – effectively a loss of function – rather than increase it, and symbionts living in a simplified host environment tend to lose adaptations to their prior environments rather than enhancing them(Dodd 1989, McCutcheon and Moran 2012, Campbell, Van Leuven et al. 2015).

Past studies suggest that the interactions between *D. discoideum* and its *Paraburkholderia* symbionts are highly context-specific(Scott, Queller et al. 2022), helping or harming either partner's fitness depending on for instance the relative abundances of host, symbiont, and other prey bacteria(Brock, Douglas et al. 2011, DiSalvo, Brock et al. 2014, DiSalvo, Haselkorn et al. 2015, Scott, Queller et al. 2022). These parameters are not trivial to measure in nature and are likely to vary in time and space. Accordingly, it is valuable to look not only at how *D. discoideum* and *Paraburkholderia* behave in the lab, but also to look for evidence of an evolutionary history consistent with cooperation or antagonism. In this study we focus primarily upon changes in – but not on the absolute values of – the phenotypes measured before and after experimental evolution. Observing, for example, high toxicity in one *Paraburkholderia* strain and low toxicity in another is not necessarily sufficient to conclude that the former is a

parasite in nature and the latter is not. More useful to us is whether our strains became *more* toxic (or less toxic) over the course of our experiment. We can be more confident drawing conclusions about changes in phenotypes than we can about the phenotypes themselves. For an evolved change in phenotype to be misleading there would need to be not just an environmental effect due to the difference between laboratory and natural environments, but a more complex genotype-by-environment interaction. Even if, for example, the density of *Paraburkholderia* we used in our experiments was unrealistically high, observing a shift towards reduced toxicity would lead us to the wrong conclusions only if the relative harmfulness of the ancestral and evolved genotypes changes in sign between the laboratory and field environments.

Our results provide new insights into *D. discoideum* and *Paraburkholderia's* relationship in nature. Most of all, they suggest that no one-size-fits-all description can be made, and that just as the relationship between *D. discoideum* and *Paraburkholderia* can be very different under different environmental contexts, it can also be very different depending on the specific strain (s) being considered. The different outcomes we observed even between closely related strains emphasize the need to account for variation within species when studying wild microbes and the risk of overattributing results derived from observations of only one or a few strains.

Our results underscore the complexity and contingency of biotic interactions in microbes, and the need for overlapping lines of evidence when trying to interpret them. Studies directly observing interactions in the laboratory are powerful (Lenski and Travisano 1994, Sniegowski, Gerrish et al. 1997, Elena and Lenski 2003, Jessup, Kassen et al. 2004, Denef, Mueller et al. 2010, Kawecki, Lenski et al. 2012, Hoang, Morran et al. 2016) but carry crucial limitations when their results must be applied to microbes in nature (Carpenter 1996). The approach we have taken in this study takes advantage of the differences between natural and laboratory

environments by looking for traits lost when selective pressures are relaxed. Similar approaches could usefully supplement other studies of adaptation, particularly those performed using microbes that do not have long histories of use in the laboratory and for which the natural context may be especially unclear or hard to recreate.

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<u>Chapter 4: Conclusions</u>

The apparent ability of natural selection to optimize organisms' traits to the challenges they face is arguably the central aspect of life from which nearly all other aspects derive. Broadly speaking, adaptation is the reason living things look the way they do and act the way they act. However central its role, however, adaptation is not the only way evolution occurs. It is tempting for the incautious evolutionary biologist to observe a trait, ponder how it may increase an organism's fitness, and construct a narrative to support it, but in fact not every trait of every organism is necessarily adaptive. To rigorously establish that a trait is an adaptation, it must be shown that it increases the organism's fitness, for example by experimentally removing the trait in a mutant organism and competing it against its wild-type ancestor.

This gold standard is, of course, not practical for every organism, or even hardly any organisms at all, and so most of the time evolutionary biologists wishing to study adaptation must employ other approaches which address it more indirectly. The presented studies outline one approach involving experimental evolution and apply it to intraspecific social traits in the social amoeba *Dictyostelium discoideum* and interspecific social traits in *D. discoideum* and the endosymbiotic bacteria *Paraburkholderia agricolaris, P. hayleyella,* and *P. bonniea.*

Though the complexity of the social behavior in *D. discoideum* and *Paraburkholderia* would seem to make an adaptive explanation more likely than not, direct demonstration of the fitness consequences of these traits on *D. discoideum* and *Paraburkholderia* in nature is not trivial. While both microbes grow readily in the laboratory, they are difficult and labor-intensive to isolate in their natural environment, and observations of how they behave in the laboratory do not necessarily reflect how they behave in nature. Infecting 10^5 amoebae of the *D. discoideum* strain QS159 with 10^5 cells of *P. agricolaris* strain bQS159 on a flat SM/5 agar plate at 25°C under fluorescent lighting results in drastically reduced fitness. But what if you infect with less? Or do it in the dark? Or more, but at a different temperature? We have an incomplete understanding of under what real life conditions *D. discoideum* and *P. agricolaris* normally interact, and we know that at least some of these conditions can strongly affect the outcome of their interactions.

The way around this is to combine observations made in the laboratory with other techniques that interrogate the two species' evolutionary history. One common way to do this is using sequence analysis – past studies have done just that to explore the question of *D. discoideum* cheating I described in my second chapter. My study approaches the same question from a slightly different angle.

My approach can be summarized as 'inferring adaptations by evolving them away'. In its execution, this is almost identical to a traditional experimental evolution experiment in which a population of interest is evolved under some selective pressure imposed by the researcher. In my case, I evolved wild microbes likely to engage in intra- and interspecific interactions in nature under conditions in which those interactions were no longer possible. As expected, the microbes adapted to the laboratory conditions. What I believe to be a new (or at least underappreciated) insight is that if the selective pressures experienced by the microbes during the experiment are relaxed relative to those they normally experienced in nature, adaptations to the latter should tend to be lost. Rather than focus upon how the subjects adapt to their new artificial environment, I focus on how they lose adaptations to their original environment. We do not know exactly what selective pressures *D. discoideum* and *Paraburkholderia* experience in nature, but organisms cannot lose adaptations they do not have. If we relax a selective pressure that we believe to be important in nature (in this case any selective pressures exerted by other microbes), and we then see losses in related traits, we can take this as indirect evidence that those pressures had driven the evolution of those traits in the first place.

The main reason this approach works is because of pleiotropic tradeoffs between traits. Whether in the laboratory or in nature, natural selection will drive evolution of increased fitness, but how different traits impact fitness depends upon the environment. In nature, organisms must face many different challenges over space and time, and the tools to solve those challenges are likely to conflict with one another. These conflicts can derive from incompatibilities intrinsic to the challenges themselves – for instance, a cell could not become larger to resist predation and smaller to facilitate dispersal at the same time – or simply a matter of costs. Ultimately an organism's traits will represent some compromise between competing selective pressures. When

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some of those selective pressures are relaxed, some compromises are no longer necessary, and organisms should evolve accordingly.

Laboratory environments are often much simpler than natural environments. In my studies, I evolved microbes on agar plates under constant lighting, humidity, pH, and temperature, with abundant food, with minimal structure, and most importantly with a radically simplified biotic context in which organisms with which *D. discoideum* and *Paraburkholderia* would ordinarily have to contend were no longer relevant. Evolving microbes under these conditions almost certainly relaxes many selective pressures and opens opportunities to stop compromising between traits. I expected to see big changes as natural selection drove the microbes towards a new balance, optimizing for traits that continued to matter in the lab environment (e.g. growth rate) at the expense of traits that no longer mattered (e.g. ability to resist the antagonistic effects of other microbes no longer present).

In my first study, I was chiefly interested in if my experimentally evolved *D. discoideum* strains would become worse at cheating (or worse at resisting being cheated upon). *D. discoideum's* social cycle is a well-studied model for cooperation and conflict, a perennial subject of interest within evolutionary biology. *D. discoideum* can form chimeric multicellular aggregates and so would seem to be especially vulnerable, as multicellular organisms go, to conflict between cell lineages. *D. discoideum* lineages can exploit other lineages within a chimeric aggregate by undercontributing to the production of the fruiting body stalk. While there are multiple lines of evidence that suggest cheating of this kind to be important, nonetheless its relevance has been questioned by some researchers, in part because most research on cheating has taken place in the laboratory. My *D. discoideum* lines – evolved without the need or opportunity to make fruiting bodies at all – evolved a small but significant reduction in their

ability to cheat a labelled control strain. In addition, evolved lines were less functional during the slug stage that precedes fruiting body formation, as judged by the distance they were capable of travelling. These results were consistent with my expectations, as any selection pressures favoring adaptations related to cheating and slug migration should have been mostly or entirely relaxed in my experiment where fruiting bodies or slugs were never formed. My results reinforce earlier studies indicating that cheating is important enough to *D. discoideum* in nature that at least some strains have adaptations supporting it.

The results of my second study were more difficult to interpret. In this study, I focused on interspecific interactions presumed to take place between *D. discoideum* and three species of endosymbiotic bacteria in the genus *Paraburkholderia.* Past work in our laboratory suggests that *D. discoideum* and *Paraburkholderia* have a complex relationship with both cooperative and antagonistic elements. Given that the fitness consequences *D. discoideum* and *Paraburkholderia* inflict upon one another appear to be highly context specific, I sought to use *D. discoideum* and *Paraburkholderia* evolved without access to one another to infer whether either microbe had adaptations related to their interaction. I primarily observed losses in antagonistic effects and resistance to antagonistic effects in *P. agricolaris*, suggesting that it may have an adversarial interaction with *D. discoideum* in nature important enough to drive adaptations. However, I observed an unexpected degree of variation between strains.

The results of both of my studies indicate an importance for variation between strains of the same species. In my cheating study, while there was an overall reduction in cheating ability among experimentally evolved *D. discoideum*, some strains appeared to lose more ground than others. This suggests that different *D. discoideum* strains may have adapted to social conflict to different degrees. This trend was even more apparent in my second study involving *D.*

discoideum's interaction with *Paraburkholderia*, where sometimes strains of the same species evolved in opposite directions. While this outcome arguably opens more questions than it answers – why do some strains behave one way and other strains another? – it makes for a general lesson about interpreting the results of experiments on microbes. It suggests that studies of microbes isolated from nature should whenever possible compare results from multiple strains in an attempt not to overdraw conclusions that may not apply to a system in general.

Further research is necessary to determine how and why different strains of *D. discoideum* and *Paraburkholderia* might have adapted to different degrees of intra- and interspecific conflict. I speculate that the variation I observe reflects, once again, that microbes living in nature are likely to be forced to balance tradeoffs. Some strains, I suspect, are akin to 'social specialists', adapting to improve outcomes of interactions with other microbes at the expense of other functions. I suspect that opportunities for wild *D. discoideum* to cheat or be cheated upon are common enough that there is some impetus to evolve strategies to capitalize on them, but not so common that such adaptations are mandatory for survival. Strains investing differently in improving the outcome of social conflict will presumably rise or fall with the frequency of such conflict, which will depend upon the environment. For example, in environments where *D. discoideum* is more mobile – perhaps due to the amount of moisture or the abundance of invertebrate vectors – its populations will tend to be less viscous and the relatedness between neighboring cells lower. These conditions would increase the chance that unrelated cells will aggregate, increasing the opportunities for social conflict and thus the potential benefits of investing in adaptations related to cheating. Similarly environments with consistently abundant prey populations will – somewhat like the conditions in which I evolved my lines in the lab – decrease the need for *D. discoideum* to enter its social cycle at all, and thus

presumably decrease the benefits of cheating. Similar arguments can be constructed to speculate about the source of variation between strains in apparent antagonism between *D. discoideum* and *Paraburkholderia.* The value of adapting to intra- or interspecific conflict depends upon environmental parameters that are likely to vary over space and time, leading to different strains adapting to different degrees. Future research should attempt to achieve a finer grained accounting of how microbial strains with different social traits are distributed over variable environments.

The approach I took in my studies has strengths but also attendant challenges. First of all, it is potentially applicable to a wide variety of systems. The chief assumption that negative pleotropic tradeoffs will tend to drive trait loss when selective pressures are relaxed should hold true for any organism, microbe or macrobe. Ideal subjects will be organisms that are amenable to experimental evolution and that are difficult to study via more direct methods. The approach can be applied to organisms for which researchers' understanding of mechanistic and/or genetic details are very limited. It is also versatile with respect to the potential adaptations being studied – my studies focused on social traits because they are complex and especially difficult to observe in nature, but in theory any selective pressure that can be experimentally relaxed in the laboratory could be used to look for adaptations driven by that selective pressure.

The key limitation of this approach, however, lay in careful selection of laboratory conditions that will, as much as possible, isolate and relax only and exactly the putative selective pressure the researcher wants to study. The differences between laboratory and natural environments are helpful to this approach in that they should maximize the chances that the trait of interest will change due to selection on pleiotropically linked traits. At the same time, since a simplified laboratory environment will tend to relax many different selective pressures

simultaneously, results may be difficult to attribute to a specific selective pressure. One option, as I attempted to demonstrate in my study of *D. discoideum* and *Paraburkholderia*, is to employ multiple different treatments with different test environments. In my study, for example, I attempted to distinguish between the effects of isolation from symbiotic partners *per se* from other aspects of the laboratory environment by including an isolation and a coevolution treatment. These two treatments were designed only to differ in the presence or absence of symbionts, but as discussed in Chapter 3, nonetheless carried some other, unintended differences that may have complicated interpretation of their results. Future studies of this sort may benefit from more granular treatments designed to relax selective pressures more surgically. Such treatments could also be used to study more complex systems – for instance, in a system involving three or more species, there could be treatments removing each species individually.

Microbes enable powerful techniques that would not be practical for larger organisms, but even those living in the soil in our backyards inhabit a world that is alien to us. Understanding them demands researchers apply many different approaches. The approach I took in my studies emphasizes the importance of the environment in understanding microbes and offers a way to infer how they evolved in nature by watching how they evolve in a laboratory.