DISS. ETH NO. 29340

Ecology and evolution of synergy and antagonism among microbes

A thesis submitted to attain the degree of

DOCTOR OF SCIENCES

(Dr. sc ETH Zürich)

presented by

LISA FREUND

MSc, Friedrich Schiller Universität Jena

born on 30.03.1990

accepted on the recommendation of

Prof. Dr. Gregory J. Velicer, ETH Zürich (supervisor)

Prof. Dr. Alex Hall, ETH Zürich (co-examiner)

Dr. Clara Torres-Barceló, French National Institute for Agriculture, Food and Environment (INRAE) (co-examiner)

Dr. Marie Vasse, French National Center for Scientific Research (CNRS) (co-examiner)

2023

Acknowledgements

Danke!

Thank you!

Dhanyavaad!

Grazie!

Merci!

謝謝!

Gracias!

Dank je!

Completing a PhD is a journey that requires perseverance, dedication and the relentless support of many individuals. I am well aware that I could have never accomplish any of this by myself and luckily there are many individuals that encouraged, inspired and helped me along the way. To each of you, I offer my heartfelt appreciation and thanks for the invaluable role you have played in making this achievement possible. Your contributions, whether big or small, have left an indelible mark on this work and on me as a person.

Firstly, I would like to thank my PhD supervisor Greg Velicer for his support and guidance along all those years. Your insightful feedback and expertise as brilliant scientist have been invaluable. I was lucky to have such a patient, kind and understanding person as a mentor.

I also would like to thank Clara Torres-Barceló, Alex Hall and Marie Vasse for agreeing to be in my committee and for taking the time to read my thesis.

Many thanks to all my colleagues from the institute and especially to everyone from the Velicer lab. Nicco, Heike, Séb, Rita, Roli and Dani, thank you for always being willing to help and sharing you knowledge, the lab environment wouldn't be the same without you. Thank you Francesca, for becoming such a great friend over the past years, for always

being supportive and sharing all the ups and downs with me. Marco, you have made work lots more fun, thanks for being such an honest and direct person and never getting tired of helping me. Thank you Sarah, Kaitlin, Ramith, Jessica, Rebekka, Fabienne, Thomas, Jasper, Siobhan, Sabrina, Macarena, Ever, Gleb, David, Sophie, Marijn and Jos for all your help, all the fun lunches, dinners, chats and discussions. I sincerely thank Berit, Jana, Chris and Sonja for being great office mates. Hélène for helping me with phage related work and Simon for showing me the first steps in R. Thanks to every past and present member in the institute.

I owe a debt of gratitude to Samay and Marie. I have learned so much from both of you and without your help I could have never achieved any of this. Samay, thank you for all the fun times, your unwavering support, your relentless encouragement, and your faith in me. Marie, you are one of the most caring, supportive and kindhearted people I ever met, thanks for all the positive energy. Thank you both for everything, you are great mentors, each in your own way.

I am deeply grateful to my friends for being such a valuable part of my life for so many years, especially Christin and Shraddha, for always being there to listen whenever I need someone to talk to, regardless of whether we've talked recently or not for months.

Ohne die Unterstützung meiner Familie wüsste ich nicht wie weit diese Reise gegangen wäre. Ihr habt stehts versucht mir bestmöglich den Rücken zu stärken, auch wenn es manchmal schwer gefallen sein muss mich zu verstehen. Ihr habt mich ermutigt meine Ziele zu verfolgen und ich bin unendlich dankbar für eure Liebe, eure Unterstützung und eure unermüdliche Ermutigung. Und Oma, jetzt bin ich fertig mit meiner Arbeit.

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Abstract

Humans have long shown interest in understanding relationships between organisms and scientists have long investigated how organismal interactions are structured and evolve, including conspecific interactions within and between social groups and interactions between species. Biological communities are made up of a variety of interacting individuals, from large animals to tiny microorganisms, all of which are connected in one way or another. Bacteria, however, form the foundation of nearly all ecosystems, and knowing how they interact in the face of constant abiotic and biotic perturbances is crucial to understanding their population dynamics, their diversification and evolutionary history. The aim of this thesis is to better understand the importance of beneficial and antagonistic interactions and how they shape the ecology and evolution of bacterial populations. More specifically, it focuses on the effect that different levels of intergroup migration can have on within-group interactions and on how bacteria-phage interactions evolve. To address these topics, *Myxococcus xanthus* was used as a model system. The social bacterium *M. xanthus* is commonly found in soil communities and performs a variety of social behaviors, ranging from cooperative swarming and predation to the formation of multicellular fruiting bodies that harbor stress resistant spores that germinate in a cooperative manner. A unique social life cycle and interactions with many other organisms, such as prey or bacteriophages, make *M. xanthus* an attractive study organism for understanding the evolution of social behavior. First, we investigated how different levels of migration between groups of *M. xanthus* affect group-level performance and the resulting dynamics within groups (Chapter 1). Using populations that evolved under either low levels of intergroup migration or regular intergroup mixing, we show that group-level performance is higher when evolved under low levels of migration, as a result of reduced intragroup conflict. The focus of Chapter 1 is on intraspecific interactions. We see how limitation of intergroup migration during evolution can have a major influence on the evolution of cooperation in bacteria. In Chapter 2, we ask whether how evolution in populations engaging in cooperative intraspecific interactions affects subsequent interspecific interactions, using the example of antagonistic interactions between *M. xanthus* and the virulent phage Mx1. Specifically, we investigated how evolution by *M. xanthus* in the absence of phage and under high

selective pressure on intraspecific cooperation behaviors, such as cooperative motility, later affects interspecific interaction with phage (Chapter 2). We show that evolution in the absence of phage can lead to increased host resistance. Diversification in susceptibility to phage among evolved populations was not primarily caused by differences in the selective environments experienced by evolving bacteria, but rather by variation in random mutational input. The character of direct interactions between natural isolates of *M. xanthus* and phage Mx1 are the topic of Chapter 3. Bacteria-phage interactions are commonly looked at from the antagonistic perspective of the phage towards the bacterial cell. Here, we have identified apparent anti-phage defense mechanisms mainly in the form of diffusible extracellular compounds released by *M. xanthus* cells that harm free phage particles. The ability of bacterial cells to fight phage threats prior to infection may represent a previously unknown form of protection. These results highlight the complexity and importance of interaction effects for the evolution of biological diversity and social evolution, and how latent effects can play important roles alongside immediate responses to direct interactions.

Zusammenfassung

Seit dem Bestehen der Menschheit ist deren Interesse an biologische Interaktionen ausserordentlich hoch. Wissenschaftler haben stehts versucht die Interaktionsstrukturen zwischen Individuen derselben oder gar unterschiedlicher Arten nachvollziehen zu können, sie zu verstehen und zu lernen wie sich im Laufe der Zeit entwickelt haben. Lebensräume bestehen aus einer Vielzahl von Individuen, die miteinander interagieren. Angefangen bei den grössten Tierarten bis hin zu den winzigsten Mikroben, die alle auf die ein oder andere Art und Weise miteinander verbunden sind. Die Grundlage eines jeden Ökosystems bilden jedoch die aller kleinsten, eine Vielzahl von winzigen Bakterien. Nachzuvollziehen wie diese Bakterien in anbetracht von andauernden biotischen und abiotischen Störungen interagieren, bildet das Fundament um deren Populationsdynamiken, deren Vielfalt und deren Evolutionsgeschichte zu verstehen. Die vorliegende Arbeit befasst sich mit der Bedeutung von vorteilhaften und antagonistischen Interaktionen zwischen Mikroben und gibt Einblick wie diese dazu beitragen bakterielle Populationsdynamiken und deren Evolution zu formen. Konkreter beschäftigt sich diese Arbeit mit dem Einfluss von Gruppenbildung auf die daraus resultierenden Interaktionen innerhalb solcher Gruppen und auf die Auswirkungen von Bakterien-Phagen Interaktionen auf die soziale Evolution von Bakterien. Um diese Themen zu adressieren, wurde *Myxococcus xanthus* als Modelsystem verwendet. Es wird davon ausgegangen, dass das sozial lebende Bakterium *M. xanthus* Bestandteil vieler bakterieller Bodengemeinschaften ist. Heraus sticht es durch seine Vielzahl sozialer Verhaltensweisen, beginnend bei kooperativer Fortbewegung und Räuber-Beute Verhalten, bis hin zur Bildung multizellulärer Fruchtkörper, die stressresistente Sporen beherbergen, welche bei Nahrungsüberfluss auf kooperative Art und Weise wieder auskeimen. Ihr einzigartiger sozialer Lebenszyklus und die andauernden Interaktionen mit einer grossen Anzahl anderer Organsimen machen *M. xanthus* zum idealen Studienobjekt, um die Evolution sozialer Verhaltensweisen zu verstehen. Zunächst haben wir untersucht, wie Unterschiede bei Ein- und Abwanderung zwischen Gruppen von *M. xanthus* die Dynamiken innerhalb der Gruppe verändert (Kapitel 1). Mit Hilfe von Populationen, die entweder unter geringer Ein- und Abwanderung zwischen Gruppen oder aber mit häufigem Mixen der Gruppen evolviert wurden, zeigen wir, wie das

Konfliktpotential innerhalb der Gruppen, die wenig Ein- und Abwanderung ausgesetzt waren, abnimmt. Solche intraspezifischen Interaktionen innerhalb von Gruppen haben einen grossen Einfluss auf die Evolution von Kooperation und das Sozialleben der Bakterien. Im ersten Kapitel wird gezeigt, wie sich intraspezifische Interaktionen auf die Evolution intraspezifische Interaktionen auswirken können. Im zweiten Kapitel wird untersucht, ob sich solche Interaktionen auch auf interspezifische Interaktionen auswirken, und zwar am Beispiel der antagonistischen Interaktionen zwischen *M. xanthus* und dem virulenten Phagen Mx1. Wir beschäftigten uns mit dem Potenzial der „Phagen-blinden Evolution“, d. h. der Evolution von Bakterien in Abwesenheit von Phagen, auf die späteren Auswirkungen der Wirt-Parasit-Interaktionen (Kapitel 2). Wir konnten eine insgesamt höhere Resistenz der Wirtszellen nachweisen, obwohl diese zuvor ohne direkten Selektionsdruck durch Phagen evolviert wurden. Hierbei waren Unterschiede im selektiven Umfeld nicht die Haupursache für die Diversifizierung, vielmehr führten Mutationen innerhalb eines Umfelds zu einem hohen Grad an Diversifizierung. Die Auswirkungen direkter Wechselwirkungen zwischen *M. xanthus* und dem Phagen Mx1 werden in Kapitel 3 thematisier. Die Interaktionen zwischen Bakterien und Phagen werden in der Regel aus der antagonistischen Perspektive des Phagen gegenüber der Bakterienzelle betrachtet. Hier konnten wir Abwehrmechanismen gegenüber Phagen aufzeigen, die hauptsächlich durch diffusionsfähige extrazelluläre Verbindungen, welche von *M. xanthus* Zellen freigesetzt werden, freie Phagenpartikel schädigen. Bakterienzellen, die in der Lage sind, die Bedrohung durch Phagen bereits vor der Infektion zu bekämpfen, könnte eine neue Art des Schutzes vor Phagenattacken ermöglichen. Diese Ergebnisse verdeutlichen die Komplexität direkter und latenter Auswirkungen von Interaktionen auf das Sozialleben von *M. xanthus* und deren Bedeutung für die Entwicklung biologischer Vielfalt und sozialen Evolution.

General introduction

Interactions between organisms

For many years, researchers were under the impression that certain organisms, such as single-celled bacterial species, acted relatively independently of each other. The discovery of molecules produced by microbial cells with the ability to inhibit the growth of other microbes provided strong early evidence that microbes in fact interact extensively (Madigan *et al.,* 2018). Since then, many other forms of interaction such as quorum sensing and biofilm formation have revealed that single-celled organisms engage in frequent interactions both within and across species. It is now well accepted that virtually all organisms found on our planet engage in many direct and indirect exchanges with individuals of other species within the ecological communities in which they reside (Agrawal *et al.*, 2007).

The effect that organisms that share the same living space within a community have on each other's survival and reproduction is defined as biological interaction. Since almost every environment in nature is colonized by living organisms (Rothschild and Mancinelli, 2001), ranging from large animals to tiny microbes, their interactions form the basis of how ecosystems are shaped, the processes within them, and their productivity and biodiversity (Attiwill and Adams, 1993; Rooney and McCann, 2012; Latati *et al.*, 2019; Ratzke *et al.*, 2020). For example, predation can affect the sizes of interacting populations thereby impact many features of a community.

Biotic interactions can occur between individuals of the same species (intraspecific) or different species (interspecific). For both types of interactions, their effects can be viewed as direct or indirect. For example, immediate interactions between two individuals are classified as direct, with immediate effects on both interacting partners. However, because most species live in diverse communities that have complex trophic configurations, indirect interaction effects among multiple species are very frequent. Interaction effects are indirect when interactions between some individuals ultimately affect other individuals that were not involved in the original interactions

(Wootton, 1994). A wolf preying on a deer is viewed as a direct interaction, whereas effects of wolf predation on deer on plants consumed by deer (and other plants interacting with such consumed plants) are indirect interactions. Indirect interactions are much more complex because they involve multiple species and levels of interaction.

Another common way of classifying biological interactions is by distinguishing between beneficial and harmful outcomes for the partners involved. In competition, both interacting partners are harmed (Birch, 1957). In resource competition, two or more partners compete for the same resource without interacting directly. It was also long thought to be the driving force of how communities are structured (Elton, 1946), but we now know that many types of interactions, both direct and indirect, strongly influence community and ecosystems functioning (Agrawal *et al.*, 2007). Another form of competition is interference competition, where partners interact directly in a negative manner, for example in seeking to deny another partner access to a desired resource through aggressive behavior.

Antagonistic interactions take place when one partner benefits and another is harmed by an interaction, for example as in predation and parasitism. (Mills and Shenk, 1992). An interaction where organisms have a mutually beneficial relationship, for example when one partner provides food and the second provides protection, are mutualistic interactions. In the aphid-ant mutualism, aphids provide nutrient-rich sugars and ants provide protection against enemies (Stadler and Dixon, 2005). Three other types of interactions, commensalism, amensalism and neutralism, are perhaps less well known. Commensalism is when one partner benefits from interacting and the other is neither helped nor harmed (Mathis and Bronstein, 2020), whereas in amensalism one partner is harmed and the other is unaffected (Kitching and Harmsen, 2008) and in neutralism two species without niche overlap share a habitat and therefore interaction effects are neutral.

As discussed above, different types of interactions are ubiquitous in all ecosystems and they can have strong effects on communities and species dynamics. For example, beneficial group living in animals involves cooperative interactions among individuals within a group (although such cooperation within groups is often associated with antagonistic interactions between groups) (Rubenstein, 1978; Fryxell *et al.*, 2007). If individuals within a group share a common goal, such as protection from predators, interactions between these individuals can increase the likelihood of survival. For example, if an individual subject to predation is isolated, it is much less likely to survive. Imagine a single wildebeest being exposed to a lion pack on the hunt. However, through

within-group cooperation the chance of survival is much larger for everyone (Fryxell *et al.*, 2007, 2022). This way, group formation by prey helps in stabilizing predator-prey dynamics by reducing the likelihood of rapid predator population increase and the risk of extinction of one or both populations (Fryxell *et al.*, 2007). The importance of interactions for group success is not just prevalent for hunting or protection, but also for the rearing of young (Clutton-Brock *et al.*, 2000), foraging success (Galef and Giraldeau, 2001), shelter from environmental conditions (Gilbert *et al.*, 2006), and swimming efficiency (Hemelrijk *et al.*, 2015), just to name a few. Taken together, within-group interactions have a strong influence on the survival and reproduction of individuals in social species.

Interactions within the microbial world

Social interactions are not only common in higher organisms, but have been shown to be of extraordinary importance among microbes as well. Bacteria were long viewed as relatively asocial organisms, but it has become abundantly clear that microbes live in diverse communities in nature in which bacteria interact extensively with individuals from the same as well as different species; some rely on within-group interactions for their very survival. In contrast to a solitary lifestyle, many bacteria benefit from high density during quorum sensing and formation of biofilms (Darch *et al.*, 2012; Ma *et al.*, 2012), collective movement when searching for nutrient sources (Kaiser and Crosby, 1983) or production of virulence factors or antibiotics (Podbielski and Kreikemeyer, 2004). Furthermore, bacteria are involved in complex community interaction networks not only with other bacterial but also eukaryotic species.

Social interactions among bacteria are often context dependent. For example, production of siderophores by *Pseudomonas* in iron-limiting environments is a cooperative act, in which bacteria produce siderophores that are available to every individual in the neighborhood (West and Buckling, 2003). Siderophore non-producers can exploit this public good and outcompete producers when iron is limited, thus siderophore mutants are cheaters and can enjoy adaptive advantage in the presence of producers (Butaitė *et al.*, 2017). However, it was demonstrated that even in the iron-rich environment similar to lungs of cystic fibrosis patients, siderophore non-producers can emerge easily (Zhang and Rainey, 2013). In this case though, the non-producers do not

appear to enjoy adaptive advantage because they exploit siderophores produced by the cooperators/producers, but rather due to a better ability to utilize available resources. Hence, outcomes in both instances are the same, i.e. siderophores non-producers can outcompete producers, but the nature of interaction is very different.

Cooperation and communication are typical examples of positive interactions and are the foundation of pro-social behavior. Cooperative behavior often provides protection, secures access to nutrients, or helps with dispersal (West *et al.*, 2007). Bacteria can engage in different forms of cooperative interactions which include public good cooperation, in which all individuals in a group can potentially receive equal benefits. They also exhibit division of labor, in which different individual specialize in distinct tasks, which allow better survival and reproduction. Biofilm formation, for example, involves sophisticated quorum sensing mechanisms where bacteria within a group engage in collective activities that increase their survival rate (Williams *et al.*, 2007; Preda *et al.*, 2019).

However, acts of cooperation involve investment of resources. Hence the question comes as to why individuals should invest costly resources when they can potentially reap the benefits of cooperation from others without paying the same cost. Cooperation can sometimes be promoted by direct fitness benefits, where cooperative individuals obtain a direct net fitness gain from their cooperative acts. In contrast, if the direct fitness of a cooperative participant is decreased, cooperation might be explained through indirect benefits towards kin sharing alleles, promoting cooperative benefits that mediate kin selection. Hamilton argued that altruistic behavior is more likely to occur among individuals that have a high degree of genetic relatedness (Hamilton, 1964a, 1964b). Population viscosity, or the restriction of movement into and out of a group, enhances relatedness between group members. Hence, maintenance of high relatedness through limited migration is thought to play a crucial role in the stabilization of interactions that require production of public goods (Nowak *et al.*, 2010), which are highly abundant in bacterial cooperation. Another theoretical framework developed to help explain cooperation is multilevel selection, where natural selection not only acts on the individual alone, but also on groups of individuals, which can result in the evolution of traits that benefit the group as a whole (Traulsen and Nowak, 2006) .

Negative interactions like competition and predation are also ubiquitous in bacterial life cycles. For example, many bacteria produce compounds toxic for surrounding bacteria, outcompeting them for a desired resource. Alongside resource competition and predation, interactions with bacterial viruses, bacteriophages, are a common type of antagonistic interactions (Chevallereau *et al.*, 2022). Because of the high abundance of both bacteria and phage (Hendrix *et al.*, 1999), frequent encounters are inevitable and generate strong selection pressure. Antagonistic coevolution with cycles of adaptation and counter adaptation is a major driving force of evolutionary change of both bacteria and phage (Buckling and Rainey, 2002) and can play a leading role in the diversification of microbial communities and how interactions within multispecies communities are structured (Weinbauer *et al.,* 2004, Rodriguez-Valera *et al*.,2009).

Phages affect their bacterial host in many ways, selecting for increased resistance mechanisms (Meyer *et al.*, 2012) at the level of phage recognition, attachment, replication or release. For example, modification of bacterial membrane structure can hinder phage attachment (Seed, 2015). Many bacteria carry CRISPR-Cas immune systems that remember previous phage infections by insertion of phage-derived sequences in the bacterial genome (Houte *et al.*, 2016). Vice versa, phages have evolved advanced infection strategies when coevolving with the bacterial hosts (Hampton *et al.*, 2020). Phages are a leading cause of changes in bacterial mutation rates (Pal *et al.*, 2007) and of genetic innovation (Touchon *et al.*, 2016).

Depending on the infection mechanism of the phage, evolutionary outcomes might vary. Virulent phages with a lytic life cycle kill the host upon adsorption through induction of membrane lysis of the host cell (Madigan *et al.,* 2018). Temperate phages, however, either adopt the lytic or the lysogenic life cycle, where upon adsorption, the phage genome is integrated into the host genome and can lie dormant as a prophage (Echols, 1972). The prophage genome can be transmitted to the next generation of bacterial cells. Such genome integration can affect the gene expression of the host substantially and even be the cause of beneficial novel traits like virulence factors and metabolic genes in some bacterial species, enabling them to colonize new ecological niches and survive changing environments. It has been shown that a knockout event in the prophage of the pathogenic *E. coli*139 reduces their ability to colonize the animal host considerably (Li *et al.*, 2018). Lysogeny can therefore have a strong influence on the adaptation and the evolution of microbial communities and highlights the shift form antagonistic to beneficial interactions (Obeng *et al.*, 2016).

Generally, how interactions shape ecological and evolutionary patterns in microbes are strongly dependent on the type, duration and the strength of the ongoing interaction. The focus is commonly on coevolutionary processes, where two species shape each other's evolution. Such direct adaptations to local conditions are known as a primary cause of evolutionary change and biological diversity, yet species interact in very complex webs with each other, often including multiple species that can be indirectly linked, where phenotypic changes in one species caused by interactions of a second can in turn lead to changes of an additional interaction (Schmitz 2010).

Structure of the thesis

Interactions within and between species are crucial for the survival and success of bacterial populations. Their ubiquity drives the need to further study how such interactions shape population structures and how they are influenced either directly or latently, when environments change over time. To help address related questions the model organism and social bacterium *Myxococcus xanthus* was used in this thesis, which will be introduced in the section: Model system *Myxococcus xanthus.*

The first part of my thesis examines intraspecific interactions within and between groups of *M. xanthus*. Reduced intragroup conflict in many eukaryotic systems has been attributed to limited intergroup migration and the selection for group-level performance (Pruitt and Goodnight, 2014). If this is the case in microbial social groups has been little studied. We used experimental evolution to manipulate populations over multiple generations in order to study effects of migration on aspects of social evolution. Since social groups of *M. xanthus* populations in nature exhibit within-group diversity, which is lower than the between-group diversity, we initiated an evolution experiment to ask whether the standing variation within and between social groups evolves differently when populations were allowed to mix repeatedly relatively to a low-migration regimen in which it was more likely to maintain long term interaction dynamics within each social group. These experiments revealed that limited population mixing results in greater within-social group synergy over evolutionary timescales than when populations were repeatedly mixed. Further, and contrary to expectations, we observed that the populations

from the high-migration regimen were highly diverse. In this chapter I have discussed how selection might operate to result in such interesting outcomes (Chapter 1).

The second part puts the focus on intraspecific interactions between *M. xanthus* and its virulent phage Mx1. The 2nd chapter focuses on how adaption to a variety of environments involving intraspecific interactions latently affect interspecific interactions with phage. We used pre-evolved bacterial populations that were exposed to environments that varied in nutrient level and/or nutrient source. Evolved populations were then exposed to phage, which they had never encountered during experimental evolution. Here, the focus is on what impact previous adaptation involving intraspecific interactions have on subsequent interspecific interactions. Chapter 3 deals with direct interactions between a diverse panel of *M. xanthus* natural isolates and phage Mx1. When thinking about bacteria-phage interactions, the focus is commonly on antagonism of bacteria by phage; here, we investigate the side of apparent bacterial defense mechanisms that hinder phage infection already at an extracellular stage through diffusible secretions that inactivate phage particles. Such bacterial secretions can function as public goods, which might benefit entire populations.

Model system *Myxococcus xanthus*

One of the best studied bacteria, that exhibit strong social interactions is the deltaproteobacterium *M. xanthus* (Muñoz-Dorado *et al.*, 2016). As part of the Myxococcales order, which are gram-negative microbes that are predominantly isolated from soil environments, they are best known for their multicellular life cycle, where they cooperate with conspecifics during motility, predation, formation of multicellular fruiting bodies filled with stress resistant spores, and germination (Figure 1).

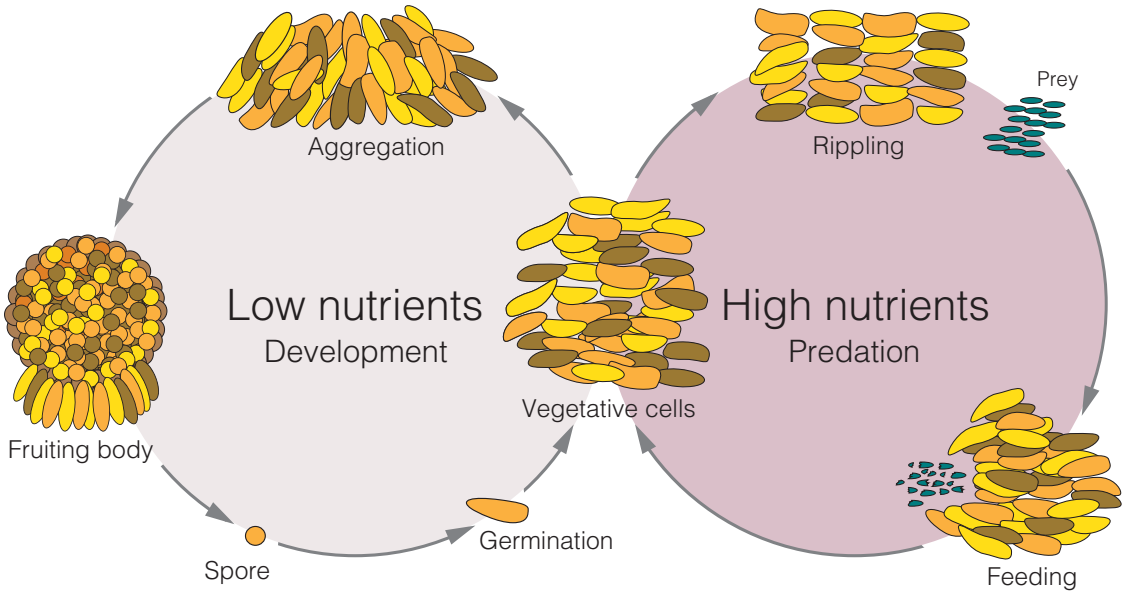

Figure 1. The life cycle of *M. xanthus*. Vegetative cells travel in swarms in search for nutrients. They prey on other bacterial cells and utilize surrounding nutrients to feed on. Once nutrients are scarce, aggregative mounds from, that later on build a structure called a fruiting body. This fruiting body contains a multitude of stress resistant spores, that start to germinate upon nutrient availability.

Within-species interactions

M. xanthus has a life cycle that consist of two major parts, the vegetative growth phase, where motile cells hunt for nutrients and the developmental phase, where, upon nutrient depletion, cells come together to form a multicellular fruiting body containing up to several thousands of stress-resistant spores (Muñoz-Dorado *et al.*, 2016). Interestingly, both of these lifecycle stages are highly social.

During the vegetative growth phase cells need to take up nutrients from their environment. The growth of *M. xanthus* populations is thought to often be density dependent. This might primarily be because of the need for extracellular digestion of complex nutrients (such polypeptides) which form a major component of *M. xanthus* diet including prey bacteria. Recently, Fiegna et. al. have demonstrated that higher density not only allows *M. xanthus* to grow better, it also results in greater survival under pH stress (Fiegna *et al.*, 2021). Interestingly, this stress tolerance mechanism is mediated by diffusible compounds, suggesting that public goods play a key role in both growth and survival of *M. xanthus* populations. Additionally, *M. xanthus* is a generalist bacterial

predator which kills and lyses cells of other species but can also absorb other organic matter (Shimkets, 1984). During predation, *M. xanthus* cells communicate to use both contact dependent and independent mechanisms of killing (Thiery and Kaimer, 2020). Importantly, contact independent mechanisms include production of antibiotics, lytic enzymes and toxins. The diffusible killing factors thus serve as public good that might benefit both producers as well as non-producing bacteria.

To be able to reach nutrient-rich spots *M. xanthus* utilizes two distinct motility systems (Hodgkin and Kaiser, 1977). The so called adventurous A-motility is utilized both in groups and by single isolated cells that scout for nutrient patches or to colonize new territories. When A-motile cells move, they secrete extracellular material that might be used as slime trails by other cells within a swarm. The second motility system, S-motility, stands for social motility and requires social interactions between cells. Cell movement is initiated through type IV pili that connect cells by extending from the leading edge of a cell pole and attaching to the surface of neighboring cells. After retraction of the pilus, the cell is pulled forward (Kaiser *et al.*, 2010). Another important factor for successful S-motility are exopolysaccharides, to which the pilus attaches and which trigger a retraction response (Li *et al.*, 2003).

Once nutrient availability is limited, a complex signaling machinery is enabled that triggers cells to come together and form mounds of cells that later develop into multicellular fruiting bodies harboring stress resistant spores. Successful fruiting body formation requires, alongside functioning signaling and nutrient-level-detection machinery, a working motility mechanism and a minimum threshold population density (Kuspa *et al.*, 1992; Kaiser, 2004; Diodati *et al.*, 2007). Upon nutrient availability, spores within a fruiting body start to germinate, which is another social process of *M. xanthus* involving public good molecules (Pande *et al.*, 2020).

Between species interactions

Myxobacteria engage in a wide variety of interactions with other groups of organisms and in general are widely studied for their large array of secretions including many antimicrobial compounds (Arakal *et al.*, 2023) that are presumably released to inhibit the growth of other bacterial species, including other Myxobacteria. *M. xanthus* is a well known predator of a large variety of other bacterial species ranging from gram negative and gram-positive bacteria, but also certain fungal species (Rosenberg and Varon, 1984; Bull *et al.*, 2002; Morgan *et al.*, 2010; Mendes-Soares and Velicer, 2013).

One of the fascinating aspects of *M. xanthus* predation is group or wolfpack predation strategy used by *M. xanthus* populations in which clusters of predator cells excrete large amounts of secondary metabolites, enzymes (Hart and Zahler, 1966), and antibiotic compounds (Xiao *et al.*, 2011) that lyse prey cells (Arend *et al.*, 2020). Additionally, research starts to focus on the potential of *M. xanthus* to serve as prey of larger eukaryotes like nematodes (Mayrhofer *et al.*, 2021).

During their life cycle *M. xanthus* is expected to engage frequently in antagonistic interactions with bacteriophages (Vasse and Wielgoss, 2018), the intracellular parasites of bacteria, since phages are the most abundant entity in the environment. Studies on myxophages are very scarce and have mainly focused on their utilization as molecular tool to study myxobacterial biology (Murphy and Garza, 2020). To date there are four distinct serological groups of myxophages (Mx1, Mx4, Mx8 and Mx9) and one prophage (Mx alpha) that have been studied (Vasse and Wielgoss, 2018). Among those are phages with a virulent as well as temperate life cycles, all being linear double-stranded DNA phages. Generally, there is very little detailed knowledge on the host range of those phages and the molecular mechanisms of such myxo-phage attacks and how they enter bacterial cells, but it is likely that they interact with extracellular and cell-surface molecules, like it is common in other types of bacteria (Silva *et al.*, 2016). Since such molecules play an important role in the social behaviors of *M. xanthus* (Wu and Kaiser, 1995), the coevolution with phage may be an important driving force of social evolution in Myxobacteria and generally play a role on how such systems interact.

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PART I

EVOLUTION AND MAINTENANCE OF SYNERGISTIC INTERACTIONS IN *MYXOCOCCUS XANTHUS* SOCIAL GROUPS

CHAPTER 1

Repeated intergroup mixing results in the loss of within-group synergy

Lisa Freund, Samay Pande, Marie Vasse & Gregory J. Velicer, in preparation.
Authors are listed in alphabetical order, which will be determined for publication.

Abstract

Group-level selection on performance at cooperative traits facilitated by limited intergroup migration appears responsible for promoting the evolution of positive interactions and reduced conflict within social groups in many macrobial systems. However, the validity of this hypothesis has received little direct experimental attention. Natural populations of the bacterium *Myxococcus xanthus* live as social kin groups in highly spatially structured soil environments, where cells develop into spore-bearing multicellular fruiting bodies upon nutrient deprivation. Interestingly, genetic variation in *M. xanthus* populations in highly structured spatially; isolates from the same natural fruiting body are often genetically distinct but are more closely related to each other than to isolates from different fruiting bodies, and the degree of genetic differentiation between fruiting-body groups increases with distance. Here we show that for populations derived from reconstituted natural fruiting-body groups, experimental evolution under conditions that select on two important social components of the *M. xanthus* life cycle – swarming and sporulation – results in better performance at those traits when intergroup migration is limited compared to evolution under a high-migration regimen. After experimental evolution, the average pure-culture swarming rate of clonal isolates from the limited-migration treatment was faster than that from the high-migration treatment. Moreover, the overall effect of pairwise mixing of clones isolated from the same fruiting body at the end of experimental evolution was neutral among strains from limited-migration treatment but negative for clones from the high-migration treatment. Interestingly, negative effects of mixing at the population level were scarcer after evolution under

limited migration than under high migration not only during swarming but also during sporulation. Together, these results suggest evolution of diversity in small spatial patches results in reduced intragroup conflict and in the diversity patterns that qualitatively resemble natural populations of *M. xanthus*. Suggesting that limited migration and selection for better intergroup performance are important drivers of diversity in the natural populations of myxobacteria.

Introduction

In many species, group living enhances population growth (1, 2) and survival (3, 4). The spectrum of within group interactions between genetically and behaviourally distinct individuals within a group can range from highly competitive (5) to cooperative (6) and altruistic (7). For example, grouping of Eurasian badgers induces strong reproductive competition that makes group living costly (8). In contrast, behavioural diversity within groups of highly related eusocial insects often has overall positive effects on group productivity (9). In such groups, the evolution of reduced conflict and positive effects of group living are prevalently associated with high relatedness between individuals (10, 11).

Many species that form genetically diverse social groups have evolved intricate mechanisms that limit intermixing of individuals from different social groups (12, 13). Barriers to limit intergroup dispersal are important because they are expected to allow selection to more readily favour synergistic within-group interactions and reduced conflict than when intergroup mixing is more extensive (14, 15). Limited intergroup migration could occur in the simplest of organisms and hence it was hypothesised that it might have played an important role in the evolution of multicellularity from unicellular ancestors (16), eusociality (17) and evolution of interspecies mutualistic interactions (18). Instances of chimeric synergy within social groups – i.e. positive effects of diversity on group-level performance – are plenty among animal species and have also been documented in microbes (19, 20). While it is highly plausible that group-level selection made possible by limitation of inter-group migration promotes the evolution of such synergies, this has received little attention experimentally, whether with animals or microbes.

Myxococcus xanthus is a soil bacterium that cooperatively forms multicellular fruiting bodies upon starvation (21). Interestingly, individual natural fruiting body groups pervasively harbour variation in social phenotypes and genetic loci among their constituent cells (22). Given that a high percentage of fruiting-body groups observed in nature are internally diverse, it seems unlikely that this within-group diversity is transient. Such within-fruiting-body diversity might be maintained by non-transitive fitness relationships (23), negative frequency dependence (24), or indirectly by trade-offs not related to social traits (25). Alternatively, such diversity might be favoured by selection

favouring co-transmission across generations of multiple genotypes because of mutualistic interactions.

Previous studies have demonstrated that *M. xanthus* populations in nature are highly structured at all geographical distances beyond individual fruiting bodies (26). Moreover, endemic within-fruiting-body diversity is lower than diversity between fruiting bodies, which increases with distance (22). Coupled with the observation that *M. xanthus* natural isolates isolated at millimetre or greater spatial scales pervasively exhibit colony-merger incompatibilities (27), the biogeographic character of natural *M. xanthus* variation indicates that diversity in this species evolves in clustered patches. Additionally, cells of most natural *M. xanthus* strains are highly adherent to one another, a feature expected to promote spatio-temporal clustering of genetic diversity.

Studies of natural *M. xanthus* populations suggest that if group-level performance during swarming and sporulation are important for the success of individual strains, then spatial clustering of endemic diversity in *M. xanthus* populations during evolution should result in lower conflict among any distinct strains co-residing within social groups, on average, than if inter-group migration were high. However, this hypothesis has not been directly tested experimentally. In light of the observation that distinct genotypes from the same natural fruiting body tend to interact neutrally or positively while genotypes from different natural fruiting bodies generally interact antagonistically when mixed (19), we performed an evolution experiment to compare the effects of high vs. low inter-group migration when selection acts on both the swarming and developmental components of the *M. xanthus* lifecycle. Specifically, we examined effects of different degrees of migration on group-level phenotypes and on within-group interactions during swarming and development.

We evolved four replicate metapopulations in each of two selection regimes (Fig S1). Each metapopulation was initiated by experimentally reconstituting three natural *M. xanthus* fruiting-body groups and inoculating three samples of each of those groups on a square agar plate in a 3 x 3 grid. Each of these natural groups was partially reconstituted by mixing equal proportions of eight distinctly clones that had previously been isolated from a single fruiting body harvested from one of three natural soil samples; those three soil samples had been collected from three forested sites separated pairwise by ~10 kilometres (22) (MC3.5.9, KF4.3.9 and GH3.5.6) (Fig S1).

Using experimentally reconstituted natural fruiting-body groups allowed us to initiate experiments with strains that are likely to have co-existed and co-evolved for extended periods under natural conditions (20). Importantly, each of the three natural groups used as sources for ancestors in this study harboured within-group genetic diversity that is likely to have evolved under the influence of substantial kin-group-level selection. A previous genomic analysis inferred that diversity within each of the three fruiting bodies evolved from a single common ancestor unique to each group and that such within-group variation had persisted for around 100 generations (20). Among the three natural fruiting bodies used here, MC 3.5.9 harbours the most diversity. Interestingly, it was also observed that genes involved in social traits such as fruiting body formation and swarming are genomic "hotspots" of selection among these clones, suggesting that the relevant social traits were under selection during the process of within-group diversification in the wild. Importantly, when mixed, distinct strains derived from the same natural fruiting body tend to show synergistic interactions or neutrality, whereas mixed strains derived from different fruiting bodies exhibit much more antagonism, suggesting the operation of kin-group level selection in nature (19). Together these data suggest that fruiting-body groups in nature compete with each other and that performance at the social traits plays a crucial role in the success of individuals and the kin groups they comprise (19).

The evolution experiment was performed with a level of nutrients high enough to fuel growth and swarm expansion from the inoculation points outward into unoccupied territory but low enough so that starvation-induced fruiting-body formation and sporulation nonetheless occurred within the eight days of each transfer cycle. The experiment was initiated by inoculating nine spots of *M. xanthus* culture samples on each of eight agar-plate grids. Each culture spot was composed of eight clones that had been isolated from one of the three natural fruiting bodies previously described (see Methods and Fig S1 for details) and we refer to each of the initially inoculated spots as a "reconstituted natural fruiting-body group". For each of the three fruiting bodies three spots were inoculated per plate. The nine resulting colony swarms were then allowed to compete for space on the plate over eight days, after which spore-bearing fruiting bodies were harvested from nine areas near lines where swarms encountered one another in a manner intended to reward greater group-level swarming with greater representation in the next growth cycle (see Methods, Fig S1).

Two treatments were initiated, with four replicate plates (or "metapopulations") initiated per treatment. In one treatment – the high-migration treatment – all spores harvested from a given metapopulation plate were mixed together at the time of spore selection before samples were inoculated in a 9-point grid on a new plate to initiate the next cycle. In the other, low-migration, treatment, the fruiting bodies from each of the nine harvest points were kept separate during the spore-selection treatment before each sample was placed on a new inoculation point to initiate the next cycle (Fig S1). This weekly regime of swarming and fruiting-body formation, fruiting-body harvest and spore selection, followed by either experimental mixing or non-mixing of sub-populations within each metapopulation and inoculation onto fresh plates was carried out successfully for 15 cycles with no population losses due to contamination.

In the high-migration treatment of this evolutionary culture regime design, mixing of endemic diversity from different natural fruiting bodies *M. xanthus* populations at the end of the first cycle of swarming and development should clearly initially result in increased conflict among co-residing strains during expression of these traits. (This is because interactions between strains from different natural fruiting bodies tend to be antagonistic.) From such a presumed initial state of increased conflict immediately after the first high-migration mixing event, we sought to understand how both group-level phenotypes and within-group interactions would evolve over time when inter-group mixing remains high, compared to the treatment in which harvested groups were not globally mixed.

Regarding group-level phenotypes, these might evolve at higher levels under low migration if either i) synergistic interactions among spatially clustered, co-evolved genotypes are important for maximising group-level performance under our experimental conditions and/or ii) negative interactions occurring between genotypes brought together by high levels of mixing tended to cause lower group-level performance throughout the experiment. However, it was also plausible *a priori* that group-level phenotypes might not differ after extended evolution under high vs. low migration, for example if inter-strain synergy were not a significant contributor to group-level performance, whether due to lack of synergy despite the presence of within-group genetic diversity or because single genotypes tended to dominate most harvested sub-populations.

Regarding the evolution of interactions, it was unclear *a priori* how much diversity might evolve within groups of either the low- or high-migration treatments in the first place. For example, even in the low-migration treatment, diversity among founding genotypes within groups that had co-existed in nature might be rapidly lost under the very

different ecological conditions of our laboratory experiment. Alternatively, such natural within-group diversity might be beneficial to group-level performance even under lab conditions and thus maintained by group-level selection in the low-migration treatment. In the high-migration treatment, regular and extensive mixing might greatly reduce within-group diversity, with a single lineage that is competitively superior across both swarming and sporulation rising to high frequency while driving other lineages to extinction. Alternatively, multiple genotypes might also be found within high-migration groups due to multiple possible causes, for example if one genotype has highest fitness during swarming while another has highest fitness during development. Despite the difficulties of predicting how much within-group diversity might evolve in our treatments, however, we hypothesized that among whatever distinct genotypes might be present within groups of either treatment at the end of our experiment, interactions among group members would tend to be more positive (or less negative) in the low-migration treatment.

Materials and Methods

Semantics:

1. Here we use the terms 'migration' and 'mixing' interchangeably. Though, migration is often described as an active process in which individuals move across locations that provide more favourable conditions for survival and/or reproduction, it can also be brought about by both biotic and abiotic factors. For example, wind, waves, anthropogenic activities have been known to mix microbial communities.

Strains: The *M. xanthus* clones used to initiate each evolving sub-population were previously isolated from three distinct natural fruiting bodies, MC3.5.9, GH3.5.6 and KF4.3.9, as described by Kraemer and Velicer (2011) (22). Each fruiting body was originally collected from an undisturbed soil site at one of three sampling areas near Bloomington, Indiana, USA, [Moores Creek (MC), Old Meyers Road (GH) and Kent Farm (KF)], each of which is ~10 km from both of the other two areas. Upon clone

isolation and characterization, Kraemer and Velicer found considerable within-fruiting-body-diversity in social swarming behaviour and sporulation abilities.

For this study, these three natural fruiting-body groups were partially reconstituted by mixing eight clones that had been isolated separately from one of the three source natural fruiting bodies and remixing them in equal proportions. In each case, the eight selected clones represent much of the phenotypic diversity previously found among clones from the respective natural fruiting body. Our experimentally reconstituted natural groups were composed of the following clones: MC3.5.9, clones C2, C5, C11, C18, C25, C29, C33, and C36; GH3.5.6, clones C3, C5, C7, C21, C26, C27, and C33; KF4.3.9, clones C6, C9, C13, C23, C32, C43, C47, C48.

Growth conditions: Evolution and post-evolution experiments performed on agar plates were incubated at 32 °C, 90% rH and liquid cultures were incubated under shaking conditions at 32 °C, 300 rpm. Prior to the start of each experiment cultures were revived from frozen stocks by inoculation on CTT hard (1.5%) agar plates followed by incubation for 3-5 days until colony growth was visible. A sample from each colony edge was transferred to 8 ml CTT liquid and incubated for 24 h, avoiding entrance into stationary phase by dilution as necessary. To initiate experiments, cultures were centrifuged at 5000 rpm for 15 min, supernatant was discarded and pellets were resuspended with liquid CTT 0.1% casitone or TPM, depending on the subsequent assay, to a density of ~5 x 10^9 cells/ml.

Experimental evolution: See Figure S1. Experimental evolution was performed under two distinct migration regimes with four replicate metapopulations each, one exhibiting limited intergroup migration and a second where migration between groups was high due to an experimental manipulation. Each replicate plate was initiated with three replicates of each of the three reconstituted fruiting-body groups (MC3.5.9, GH3.5.6 and KF4.3.9), generating nine distinct sub-populations, each comprised of eight distinct clones, as described above.

All clones were grown in CTT liquid prior to experiments. On the day evolution was initiated and every transfer day, 30 ml of heated, liquid phase (50 °C) CTT 0.1% casitone hard (1.5%) agar was poured into 10 x 10 cm square petri dishes and kept uncovered in a sterile laminar flow hood to cool until solid. On the same day, exponential-phase growth

cultures were centrifuged and resuspended with CTT 0.1% casitone CTT liquid. Eight clones were mixed in equal proportion to reconstitute each fruiting-body group (MC3.5.9, GH3.5.6 and KF4.3.9), and 10 μl of each group was then spotted three times in a grid-shape orientation on the square agar plate (~1.3 cm apart). The sub-populations in each replicate were named according to the position at which each was spotted to initiate the first cycle of evolution, as shown in Fig S1. In the first cycle of evolution, sub-populations founded from the same reconstituted natural fruiting-body group were never adjacent neighbours. Once the spots dried, plates were incubated for eight days.

Colony fronts swarmed outwards until they encountered the front of a swarm approaching from the opposite direction, after which they might have ceased progression or might have swarmed to some degree into territory already occupied by cells of a spatially opposing swarm. The interfaces of opposing fronts of genetically distinct swarms are characterized by visible demarcation lines (28). Within the eight days of each cycle, fruiting bodies formed most commonly along either side of, but not directly on, the swarm-interface line, although they sometimes also formed directly at the apparent interface.

At the end of each cycle of growth and development, fruiting bodies were harvested from near or at the swarm-interface lines between the swarms that met between the following cycle-1 inoculation points: 1|2, 2|3, 3|6, 6|5, 5|4, 4|7, 7|8, 8|9 and 9|6 (Fig S1). In all cases in which fruiting bodies were not present exactly on the demarcation line, fruiting bodies were harvested from the side of the first-listed of paired inoculation points, close to the demarcation line. The number of fruiting bodies picked from near (or at) each demarcation line was equivalent to the distance in millimeters from the first-listed inoculation point of each inoculation-point pair to the encounter line. The intent of this protocol was to reward i) faster occupation of open agar prior to swarm-front encounters and/or ii) higher levels of within-fruiting-body spore formation by transferring more fruiting bodies (and hence presumably more spores) to the next cycle from swarm fronts that had swarmed farther before encountering another swarm than those that had swarmed less.

All fruiting bodies collected from near a given interface line were placed together into a microcentrifuge tube containing 100 μl of 0.1%-casitone CTT liquid and subsequently heated for 2 h at 55 °C to ensure that only heat-resistant spores were transferred to the next cycle. At the next step, the difference between the two distinct selection regimes was implemented. In the low-migration regime, the spores harvested from each the nine

focal swarm-interface lines were not mixed with spores harvested from the other interface lines. In the high-migration treatment, however, the spores from all nine interface lines from the same replicate plate were mixed in a single liquid sample.

For the low-migration regime, cultures were then centrifuged for 10 min at 12000 rpm after heat inactivation of all vegetative cells, then resuspended with 20 μl CTT 0.1% casitone and spotted on a fresh CTT 0.1 % casitone hard (1.5%) agar plate. Each harvested sub-population culture was then spotted on one of the nine inoculation spots on a new plate at positions that were randomized except for one criterion; previously neighbouring swarms were never placed in the same order as in the previous cycle. 20-μl spore samples were spotted in two steps in which first 10 μl were spotted and then allowed to dry before a second 10 μl was aliquoted on the same spot. This was done to reduce the inoculum area relative to spotting one 20-μl aliquot. The starting positions on the new plate of each sub-population harvested from the previous plate were recorded. Here, the name of each harvested sub-population stayed the same for each cycle and was traced based on the corresponding original reconstituted fruiting-body group.

To begin each new cycle of the high-migration regime, the experimentally imposed migration step was to pool all nine sets of harvested fruiting bodies from each replicate plate into a single liquid sample directly after the spore-selection step, eliminating all previous spatial population structure across harvested sub-populations and across fruiting bodies within harvested sub-populations. Centrifugation was followed by resuspension, in this case in 180 μl CTT 0.1% casitone. Two successive 10-μl aliquots were placed on each of the nine inoculation points of each new plate in the same manner as in the low-migration treatment. Since tracing each reconstituted fruiting-body group for each cycle was not possible as a result of sub-population mixing, naming of each sub-population was done according to position on the plate. The overall evolutionary cycle was repeated 15 times.

Each sub-population was stored frozen every second cycle. To do so, culture was harvested in the center of each inoculation spot two days after each transfer. This allowed an initial increase in cell density sufficient for colony expansion. The harvested culture was transferred to liquid CTT, incubated until sufficiently grown and freezer stocks were prepared with 20% glycerol.

Clone isolation: After 15 cycles of evolution, 24 clones were isolated from single fruiting bodies that developed from harvested sub-populations 1, 7 and 9 from each replicate

metapopulation, which originally were founded by reconstituted fruiting-body groups MC3.5.9, KF4.3.9 and GH3.5.6, respectively. Although ancestral fruiting-body groups and subsequent evolving sub-populations cannot be distinguished after mixing in the high migration treatment, groups used for clone isolation were chosen according to the original location of the respective sub-populations in the first cycle prior to the first instance of global mixing at the end of that cycle. The fruiting bodies from which clones were isolated at the end of cycle 15 were picked within the same areas from which fruiting bodies would have been sampled for another transfer had the experiment continued further. Single fruiting bodies were transferred to a separate microcentrifuge tube containing 500 μl ddH_2O. Tubes were heated at 50 °C for 2 h and sonicated two times 10 s with a 10 s break in between. This protocol ensured killing of vegetative cells as well as spore dispersion. Subsequently, spores were dilution-plated and allowed to germinate embedded in CTT soft (0.5%) agar; colonies were picked and grown in liquid CTT to be stored frozen until further use.

Swarming assay: To test for differences in motility, samples of whole harvested sub-populations or single clones were grown up and cell density was adjusted with CTT 0.1% casitone to 5 x 10^9 cells/ml. 10- μl culture samples containing the same number of cells were spotted in the centre of one day old CTT 0.1% casitone hard (1.5%) agar plates. The day prior, 5-cm diameter plates were prepared with 10 ml agar solidified in the sterile laminar-flow hood, covered, and stored overnight at room temperature. After the culture spot dried, plates were incubated until the next day to allow initiation of swarming. The outer rim of the swarm was then marked and plates were incubated for an additional 48 h, when the colony edge was marked again. The average swarming distance that was travelled within a day was calculated by measuring the distance between the two marked areas on four different axes. For experiments that required the combination of more than one clone, cultures were mixed in a 1:1 ratio prior to spotting on the agar plate.

Developmental competition assay: To test whether chimerism affects spore production during development in pairwise mixes, we chose the same clones that already were tested for swarming. After cultures were grown and density was adjusted to 5 x 10^9 cells/ml with TPM liquid, a 1:1-ratio aliquot of the paired clones was spotted on one day-old starvation plates. Plate preparation was same as for swarming assays, except TPM hard

(1.5%) agar instead of CTT 0.1 % casitone hard (1.5%) agar was used. Like in the swarming assays, clones were mixed pairwise, with clones paired either within or across harvested sub-populations, but here we tested for their sporulation efficiency. All paired clones were also assayed in pure clonal cultures as well. For each developmental plate, 100 μl of culture was spotted in the centre of the agar plate and incubated for five days. After mature fruiting bodies formed, they were harvested with a sterile scalpel into 1 ml of ddH_2O, heated at 50 °C for 2 h and sonicated for two times 10 s with a 10 s break in between. Cultures were diluted using ddH_2O and several dilutions were plated embedded in CTT soft (0.5 %) agar. After about four days of incubation, colonies emerged inside the agar layer and were counted. The bidirectional-mixing effect parameter B_{ij} was calculated for each mixed according to the equation from Fiegna and Velicer 2005 (29), which calculates the number of viable spores of the chimera in relation to spore counts that would be expected from monoculture results.

Results

Evolution under limited migration results in faster motility

Group swarming and fruiting-body formation are social traits of *M. xanthus* (30) that were both under selection in our evolution experiment. Mixing all nine harvested sub-populations from each plate in the high-migration treatment before re-inoculating as nine distinct spots on fresh plates was expected to result in phenotypic homogenization among inoculated reconstituted fruiting-body groups at these traits within each evolving metapopulation over time, relative to the low-migration treatment in which such experimental mixing did not occur. We first examined swarming rates of evolved harvested sub-populations to compare degrees of within-treatment phenotypic diversity and test for treatment effects on average swarming rates at the end of the evolution experiment. Overall, we could show that sub-populations evolved to have reduced their swarming ability compared to ancestral cultures when sub-populations were mixed in high-migration treatment (Fig S2, Type III ANOVA $F_{1,179} = 336.2104$, $p < 0.001$, with Tukey posthoc contrasts, $t_{179} = 20.102$, $p < 0.001$, one-sample t tests against 1 with Benjamini & Hochberg correction, $t_{179} = -17.806$, $p < 0.001$), whereas low migration between populations led to the evolution of faster swarming during evolutionary time

(Fig S2, Type III ANOVA $F_{1,179} = 336.2104$, $p < 0.001$, with Tukey posthoc contrasts, $t_{179} = 20.102$, $p < 0.001$, one-sample t tests against 1 with Benjamini & Hochberg correction, $t_{179} = 8.125$, $p < 0.001$). A strong homogenizing effect of experimental mixing on swarming rates across sub-populations within metapopulations could be observed throughout the evolution experiment (Fig S3). This difference remained evident at the end of the final cycle, when low-migration metapopulations varied more in the swarming rates of their respective harvested sub-populations than did high-migration metapopulations (Fig 1, linear model on coefficient of variation for each metapopulation $F_{3,16} = 41.772$, $p < 0.001$, with Tukey posthoc contrasts between migration treatments for each metapopulation $p < 0.001$).

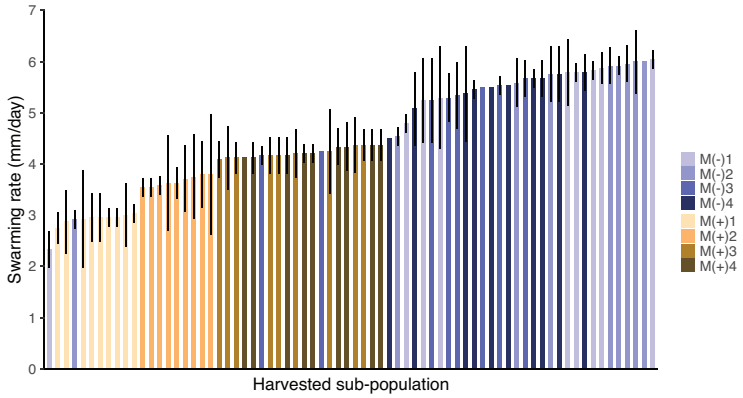

Figure 1. High migration among harvested sub-populations reduces phenotypic variation within metapopulations and causes slower swarming to evolve. Average swarming rates after the terminal evolution cycle of all harvested sub-populations evolved under low (M(-), light-to-dark purple shades) or high (M(+), tan-to-brown shades) migration are shown. Each data bar represents the mean collective swarming-rate estimate for an entire harvested sub-population (i.e. not for just a single clone isolated from a sub-population). Different shading levels of the two colour ranges distinguish the harvested sub-populations from distinct metapopulations (i.e. the nine sub-population from the same metapopulation share the same color shade). Error bars represent 95% confidence intervals of three independent biological replicates.

The terminal harvested sub-populations from the low-migration treatment were found to swarm ~41% faster, on average, than high-migration sub-populations (Fig 1, Type III ANOVA $F_{1,179} = 404.0893$, $p < 0.001$, with Tukey posthoc contrasts, $t_{179} = 20.102$, $p < 0.001$). Indeed, only two (of 36) low-migration sub-populations swarmed significantly slower than the fastest high-migration population (Fig 1, Type III ANOVA $F_{32,72} =$

46.219, $p < 0.001$, 36 posthoc one-sample t tests on estimated marginal means with Benjamini & Hochberg correction, $p < 0.001$) while 32 were significantly faster (36 posthoc one sample t tests on estimated marginal means with Benjamini & Hochberg correction, $p < 0.005$). Together, these results demonstrate a strong effect of repeated mixing of sub-populations within metapopulations on the evolution of *M. xanthus* social traits, both with respect to the degree of variation among harvested sub-populations within metapopulations and with respect to treatment-level differences in average swarming rates.

Evolution under high migration selects for negative interactions effects on swarming rates

Low intergroup migration should result in spatial clustering of genotypes over time and is thus expected to evolutionarily promote more synergy (or less antagonism) among distinct genotypes within harvested sub-populations than is high migration. However, the tendency of harvested sub-populations from the low-migration treatment to swarm faster than high-migration evolved populations might be due to either or both of two possible causes. First, the group-level difference in average swarming rate across the two treatments might be due to differences in how fast the fastest strains in those groups can swarm in separate clonal groups. Alternatively, interactions between genotypes within harvested sub-populations might contribute to or even be solely responsible for the overall treatment-level difference.

To test for contributions from either or both of these mechanisms to the observed difference between treatments in sub-population-level swarming, we first randomly selected three sub-population numbers from the low-migration treatment (HSP1, HSP 7 and HSP 9) and analysed the three harvested sub-populations within each low-migration replicate metapopulation corresponding to those numbers. For the high-migration treatment, because forced intergroup migration brought all harvested sub-populations into a single well-mixed population between each growth cycle, we randomly selected one sub-population number (HSP1) for further analysis. We then randomly isolated 24 clones from each of the 16 focal sub-populations groups (12 sub-populations from the low-migration treatment, 4 from the high-migration treatment). Using these clones, we tested for an overall difference between the high- *vs.* low-migration treatments in the average swarming rates of individual clones swarming in clonal isolation. Clones evolved

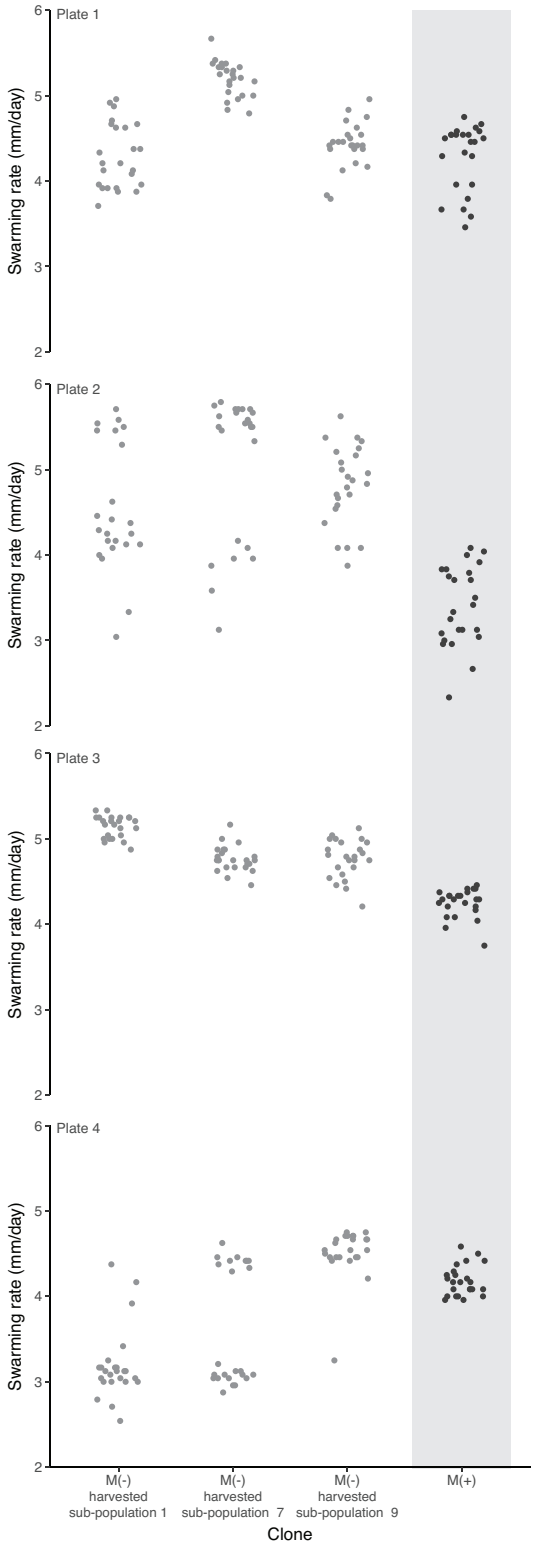

Figure 2. Limited migration results in faster average swarming among clones and greater within-sub-population variation among clone swarming rates than high migration. Average swarming rates of 24 clones isolated from harvested sub-populations 1, 7 and 9 of each metapopulation evolved in the low-migration regime (light grey dots) and harvested sub-population 1 of the high-migration regime (dark grey dots) after 15 cycles of evolution. Because the mixing manipulation of the high-migration regime homogenised harvested sub-populations within a metapopulation, just one harvested sub-population was selected for clone isolation. Dots represent the mean of three independent biological replicates.

under the low-migration regime swarmed on average significantly faster than clones evolved under the high-migration regime (4.53 mm/day 95% CI [4.48, 4.58] vs 4.03 mm/day 95% CI [3.97, 4.09], respectively; Type III ANOVA $F_{1,858} = 39.61$, $p < 0.001$, post hoc contrasts on marginal means, $t_{581} = 6.29$, $p < 0.001$) (Fig 2).

Importantly, significant swarming-rate differences between clones isolated from the same harvested sub-population were found within sub-populations from both the high- and low-migration regimens (Fig 2, Type III ANOVA, metapopulation:sub-population:isolate interaction $F_{138,858} = 2.39$, $p < 0.001$). This result indicates that multiple phenotypically distinct genotypes co-existed in at least some sub-populations at the end of 15 cycles at frequencies sufficiently high to be readily sampled by our random clone selection. However, such within- sub-population-group variation among clones was greater in magnitude among the low-migration sub-populations than high-migration populations (Type III ANOVA on coefficient of variation across clones for each sub-population, $F_{6,35} = 10.29$, $p < 0.001$, post hoc contrasts on marginal means, $t_{35} = 2.67$, $p < 0.05$). This suggests that our high migration mixing treatment resulted in the evolution of lower within-sub-population genetic diversity than the low-migration treatment, despite the experimental mixing of genotypes from all harvested sub-populations with each replicate metapopulation into a single pooled population in every cycle of the high-migration treatment.

That low-migration evolved clones swarm faster in isolation, on average, than high migration clones might suggest that the observed faster swarming by whole harvested sub-populations from the low-migration treatment might be explained more by differences in intrinsic clonal performance than by treatment-level differences in the character of genotype-genotype interactions within harvested sub-populations. Nonetheless, such interactions might also contribute to the observed difference in sub-population-level swarming rates.

To test this hypothesis, interaction effects among four distinct clonal isolates from each of the harvested sub-population HSP1, HSP7 and HSP9 within each replicate metapopulation of both migration treatments were examined. For clones evolved under high migration, the mean effect of pairwise clone mixing on swarming rate was significantly negative relative to expectations from pure-culture swarming rates of the paired clones (Fig 3A, Type III ANOVA $F_{1,131} = 104.85$, $p < 0.001$, one-sample t test against 1 with Benjamini & Hochberg correction, $t_{131} = -15.996$, $p < 0.001$). In other

words, mixed groups of two high-migration evolved clones tended to swarm more slowly than the faster of the two clones in single clone assays. In contrast, no such overall negative effect of clone mixing was observed among the low-migration evolved clones; *i.e.* the swarming rates of clone-pair groups were similar overall to swarming rates of the faster of paired clones in isolation (Fig 3A, one sample *t* test against 1 with Benjamini & Hochberg correction, $p > 0.05$).

Moreover, in the high-migration treatment, mixing generated negative mixing-effect estimates for all but one of 72 possible pairings (irrespective of the significance of individual estimates, a result extremely unlikely to have occurred by chance (binomial test, probability of 0.014, 95% CI [0.0004, 0.07], $p < 0.001$). In the low-migration treatment, only 35 of the 72 possible pair estimates were negative, an outcome close to the null-hypothesis frequency of 0.5.

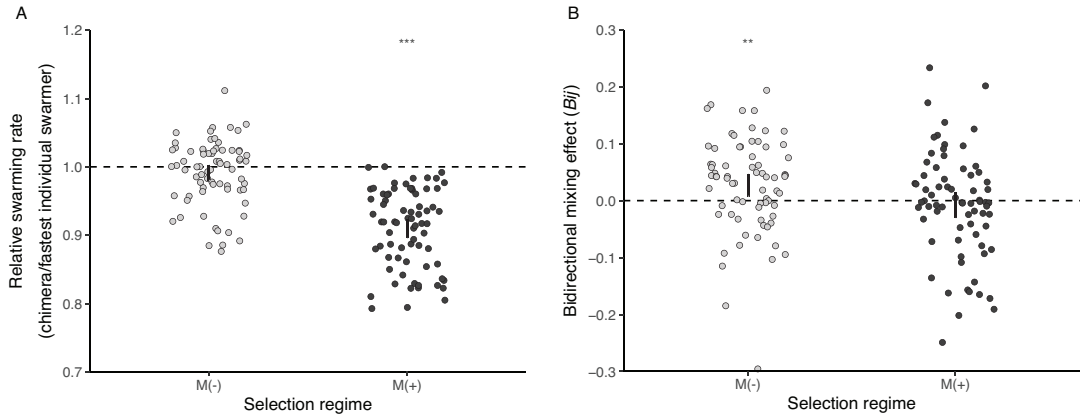

Figure 3. Average values of interaction effects between clones are higher after evolution under low migration than under high migration. Effects of mixing evolved clones in pair groups relative to performance in monoculture for swarming rates (A) and sporulation efficiency (B). Four clones of a selected number of harvested sub-populations (HSP 1, 7 and 9) were mixed in all possible pairwise combinations and tested for swarming and developmental efficiency in comparison to monoculture performance. Each dot represents one of 72 possible pairwise interactions that were independently replicated three times. Black lines represent 95% confidence intervals. Dotted lines indicate the absence of a difference between the swarming rate of a chimeric pair and that of the pair's faster clone in swarming vs. monoculture performance. Asterisks show significant average differences from monoculture performance (ANOVA and subsequent and one-sample *t* tests with Benjamini & Hochberg correction, *** $p < 0.001$, ** $p < 0.01$).

The above analyses partitioned interaction-effect data only by treatment within each replicate. In a subsequent analysis, we additionally partitioned values by harvested sub-populations within treatments, allowing us to examine the degree of (un)evenness in how much individual sub-populations contributed to the overall trend of greater negative interactions among the high-migration clones. Mixing effects of all six possible pairwise combinations of the four clones derived from each sub-population were averaged to calculate within-replicate sub-population averages, which were in turn used to calculate cross-replicate averages. For the low-migration regime, ten sub-population-level interaction effects were neutral and two were negative (Fig 4A, Type III ANOVA $F_{6,120} = 6.5473$, $p < 0.001$; one-sample t tests against 1 with Benjamini & Hochberg correction, $p < 0.05$), while for the high-migration regime only three were neutral and nine were significantly negative (Fig 4A, one-sample t tests against 1 with Benjamini & Hochberg correction, $p < 0.05$).

Together, these observations demonstrate that repeated mixing of all harvested sub-populations within each metapopulation during evolution in the high-migration regime resulted in internally diverse groups composed of genotypes that, when paired, tend to interact negatively with respect to group-level swarming rates. Thus, the difference in group-sub-population-level swarming rates between the evolutionary treatments appears to be explained by both by faster intrinsic swarming of low-migration clones and more negative interactions among high-migration clones.

Evolution under limited migration selects for positive interaction effects on spore productivity

In addition to swarming, proficiency at fruiting body formation and sporulation were selected for during the evolution experiment. We therefore also tested whether interactions among clones during development evolved similarly to how pair-wise clonal interactions during swarming evolved, at least with respect to broad directional trends. To do so, we analysed the same clones as previously. Consistent with the results from the swarming experiments, among clones evolved in the low-migration treatment, positive effects of pairwise mixing on overall spore productivity greatly outnumbered neutral or negative effects (47 positive effects, 25 negative or neutral effects) (Fig 3B, Type III ANOVA $F_{1,131} = 7.46$, $p < 0.05$, one-sample t test against 0 with Benjamini & Hochberg correction, $t_{131} = 3.010$, $p < 0.01$). In contrast, no trend toward positive mixing

effects was observed among the high-migration evolved clones; indeed a majority of mixing effects were neutral or negative (33 positive effects, 39 negative / neutral effects) (Fig 3B, Type III ANOVA $F_{1,131}$ = 7.46, p < 0.05, one-sample t test against 0 with Benjamini & Hochberg correction, p > 0.05). In line with these observations, the grand mean of mixing effects was significantly positive for low-migration evolved metapopulations (Fig 4B, grand mean (B_{ij}) = 0.027, Type III ANOVA $F_{6,120}$ = 8.4277, p < 0.001, one-sample t test against 0 with Benjamini & Hochberg correction, t_{120} = 3.346, p < 0.01) but neutral for low-migration metapopulations (grand mean (B_{ij}) = -0.007, Type III ANOVA $F_{6,120}$ = 8.4277, one-sample t test against 0 with Benjamini & Hochberg correction, t_{120} = -0.950, p > 0.05).

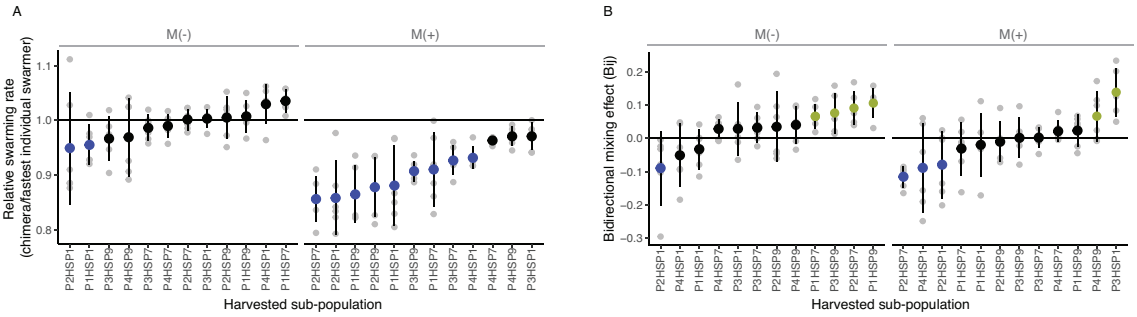

Figure 4. Within-sub-population mixing effects on overall swarming rate and sporulation efficiency. Pairwise mixing effects on swarming (A) and sporulation efficiency (B) for all six possible pairwise interactions among the four clones isolated from each harvested sub-population (HSP 1, 7 and 9) isolated from all four replicate metapopulations (P). Each large dot is the mean mixing effect of pairwise interactions (light grey dots) for a given harvested sub-population with 95% confidence intervals. Pairwise interactions were independently replicated three times. Colours show outcomes of tests for differences to 1 (A) or 0 (B) (black: no significant difference, purple: significantly decreased performance in mixture compared to monoculture, green: significantly increased performance in mixture compared to monoculture; ANOVA and subsequent and one-sample t tests with Benjamini & Hochberg correction, p < 0.05).

As with the swarming results, we also partitioned the sporulation data by harvested sub-populations, averaging the six pairwise interactions among the four clones from each sub-population for all sub-populations. In the low-migration treatment, only one sub-population mean was significantly negative, seven were neutral and four were

significantly positive (Fig 4B, Type III ANOVA $F_{6,120} = 8.4277$, $p < 0.001$, one-sample t tests against 0 with Benjamini & Hochberg correction, $p < 0.05$). For the high-migration regimen, the distribution of negative, neutral and positive interaction effects was three, seven and two respectively (Fig 4B, one-sample t tests against 0 with Benjamini & Hochberg correction, $p < 0.05$).

Together, comparing the direction of interaction effects between the swarming and sporulation assays, in both cases low-migration mixes yielded higher values than high migration mixes, on average (Figs. 3, 4). In the swarming assays, low-migration mixes were neutral while high-migration mixes negative on average, whereas in the sporulation assays the high-migration mixes were neutral on average while the low-migration mixes were positive overall.

Discussion

In both higher animals (31) and microbes (19, 20) in which group-level performance at one or more social traits is important for fitness, limited migration between groups seems more likely to promote the evolution of within-group synergy than is high inter-group migration. However, evolutionary-scale experiments that manipulate the degree of migration between social groups are difficult to perform with animals. Here we used a model social bacterium to test the effects of population mixing on the evolution of group-level performance at one trait important to fitness – swarming rate – and on the evolution of within-group interactions for both swarming rate and sporulation, a second trait important to fitness in our experiment.

Since *M. xanthus* populations in nature live in genetically distinct social groups, we used natural social groups of *M. xanthus* to test the relative effects of different degrees of between-group migration on the evolution of within group social interactions. Our results demonstrate that limitation of migration among groups has positive effects on how within-group interactions evolve. For one group-level phenotype – swarming rate – limiting migration prevented the evolution of negative interactions that evolved in the high-migration treatment, while for another cooperation dependent phenotype – spore production – positive within-group interactions were present in evolved groups that were absent from groups evolved under high migration. These results suggest that group-level

performance at these traits was important for the fitness of individual genotypes in our evolution experiments.

While imposing selection on performance during two distinct *M. xanthus* social traits (swarming and development), in our experiments we tested the importance of an inferred aspect of *M. xanthus* population biology in nature – low intergroup migration – for shaping how within-group social interactions evolve. In nature, in addition to the high degree of physical structure in soil environments, low intergroup migration is likely promoted by multiple aspects of *M. xanthus* biology. For example, natural *M. xanthus* social groups isolated at mm-scale distances often exhibit colony-merger incompatibilities (27) and interference competition that are likely to reduce inter-group mixing that might occur due to motility (32) Additionally, the high-degree of cell-cell adhesion exhibited by many *M. xanthus* natural isolates is also likely to limit between-group migration. Our results suggest that, at least in our laboratory-evolution regime, limitation of between-group migration during evolution allows group-level selection to favor group compositions within which interactions among distinct genotypes are more positive (and or less negative) with respective to group-level performance at social traits important to fitness.

We expected that the two distinct selection regimes used during the evolution experiment would result in different relative degrees of within vs between social group competition. We hypothesised two distinct outcomes. First, a "superior" genotype that can emerge in each social group/sub-population group and thus individual sub-populations exhibit limited within group diversity. However, our results demonstrate that in a low migration regimen not only the diversity is maintained within social groups but is also beneficial for group level performance.

Similarly, mixing of social groups in the control treatment should result in multiple selective sweeps and hence loss in diversity at the metapopulation level. Interestingly we observed that the high migration regime has resulted in the evolution of diversity in which individuals compete with each other which generally results in the negative population mixing effects. Surprisingly we observed that clones from high migration treatment can have slow migration.This is surprising given that the selection was for swarming and being at the leading edge. These results can be explained by multiple hypotheses that are not mutually exclusive. First, interference competition ability of clones matters more than intrinsic swarming rate for fitness during swarming and hence strains that dominate at

interference can either be slow or fast swarmers. Second, formation and sporulation ability matter more to overall fitness than intrinsic swarming rate through the entire cycle and strains more fit at these developmental traits tend to be slower at swarming. If this was solely correct, we would not expect to see a difference between treatments. Finally, multiple antagonistic genotypes can be maintained in the population because of non-transitive fitness relationships.

Expansion of bacterial swarms and colonies create repeated population bottlenecks at the colony edge (33). This is because cells at the leading edge of the colony have greater access to the resources at the colony edge, which results in the only handful of the cells at the colony edge growing more rapidly than the rest. Such growth differences reduce the available genepool at the colony edge resulting in the population bottlenecks. Such bottlenecks can result in genetic drift and hence evolution of non-adaptive genotypes. In this context, our results are interesting and demonstrate that a greater degree of competition between sub-populations/social groups allowed by limited migration will result in the evolution of diversity that does not have negative effects on group performance. However, we did observe reduced performance in the high-migration regimen. These results suggest that the drift might operate at the colony edge when group level performance is not under selection. This hypothesis can be tested by comparing the competitive fitness of the evolved variants relative to the ancestors when both are competing for common resources. Alternatively, mixing will result in more competition among individuals. And the absence of strong group level selection component (especially in the high migration regimen) will result in the evolution of negative micing effects and competitive genotypes that have significantly higher fitness as an independent clone relative to their ancestors. Once again, this hypothesis can be tested in future by performing competition experiments between the evolved and the ancestor genotypes.

Taken together we demonstrate that limitation of intergroup migration can maintain extant within-group diversity and/or allow the evolution of novel within-group diversity that has positive, or less negative, interaction effects on group-level performance than does extensive intergroup migration over evolutionary timescales. This appears to happen because group-level performance at multiple social life-history traits across the life cycle is important in competing for common resources and low migration allows selection to favor group compositions with higher group-level performance than do conditions with high intergroup migration. Our results thus contribute to explaining the

relationship between patterns of genetic diversity and patterns of social interaction within and across natural social groups. *M. xanthus* populations in nature are highly structured and show synergy unique to within-group interactions, synergy which our results suggest would be reduced or lost if intergroup migration were higher. Finally, given that many organisms live in social groups within which genetically diverse individuals interact to positive effect, it will be of interest to similarly test for effects of variable degrees on migration on how within-group social interactions evolve in additional systems.

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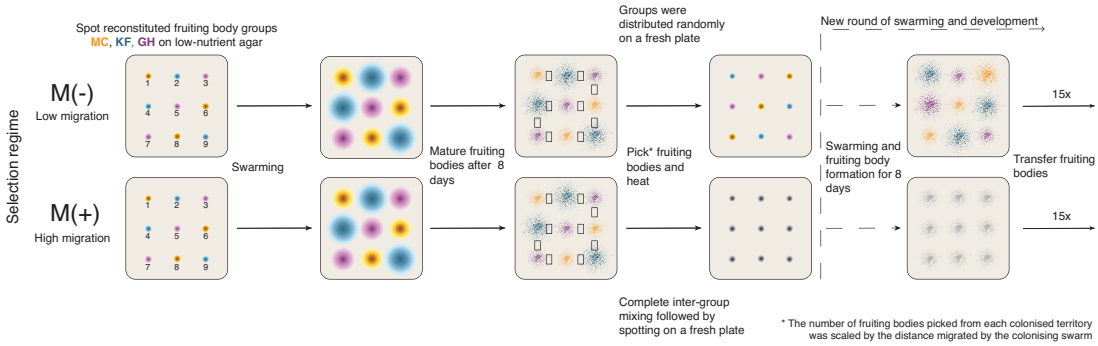
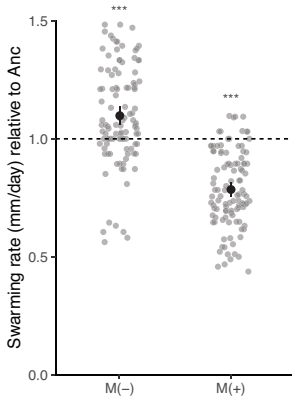
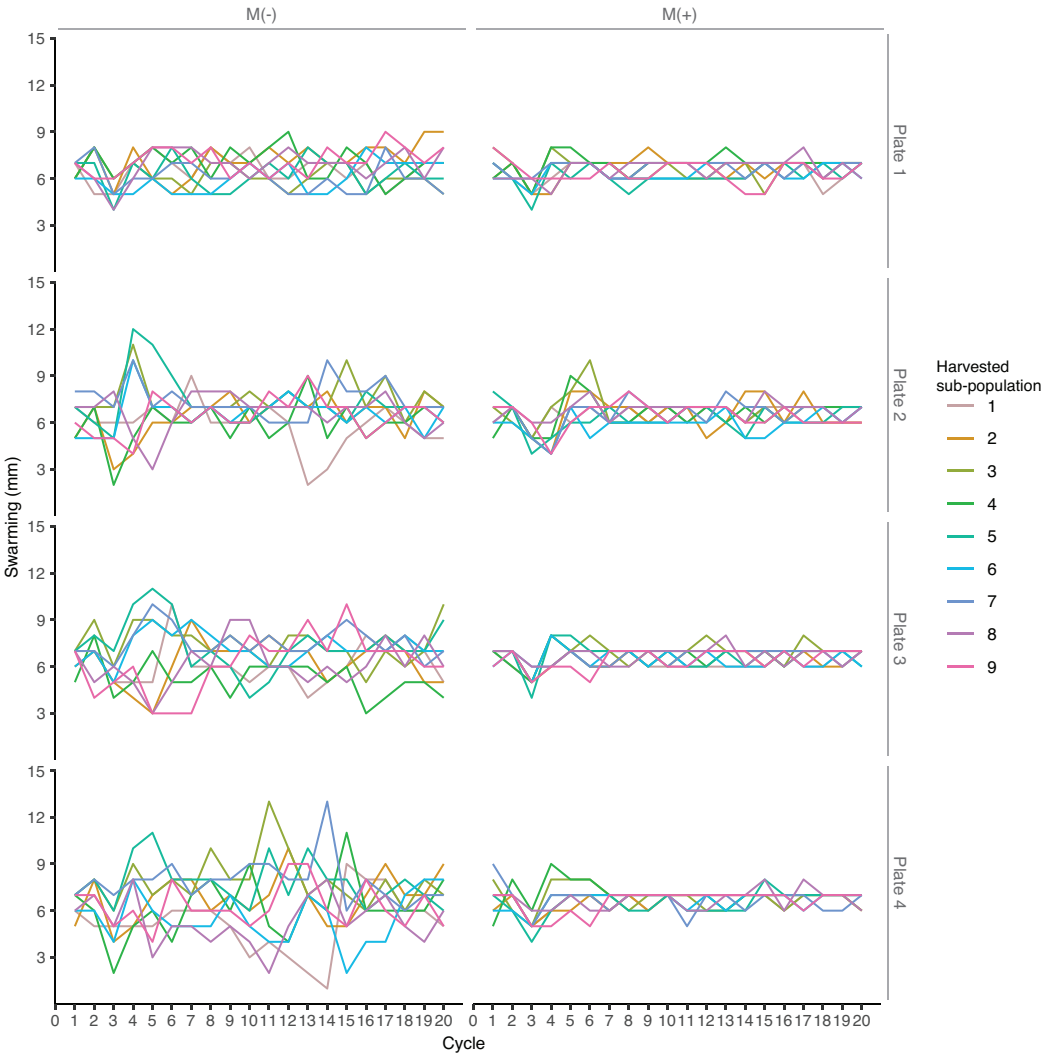
Supplementary Figures



Supplementary figure 1. Experimental design of the evolution experiment. For both selection regimes (M(-) and M(+)), three natural *M. xanthus* fruiting-body groups (depicted by different colours) were reconstituted by mixing equal ratios of eight clones that were originally isolated from these groups. Each reconstituted group was spotted three times on a low nutrient, square-shaped agar plate, representing the start of each sub-population. Mixing and spotting was replicated four times, to generate four replicate metapopulation plates for each selection regime. Plates were incubated for eight days until nutrients were depleted and mature fruiting bodies emerged. Fruiting bodies of each sub-population were picked at the merging area of two neighbouring colonies (black square area). The number of picked fruiting bodies was proportional to the distance each sub-population managed to swarm until meeting the neighbouring population. Picked fruiting bodies were heated to select for spores and subsequently transferred to a fresh agar plate. Under low migration, harvested sub-populations were not allowed to have the same direct neighbour as in the previous cycle but were otherwise spotted randomly. Lack of mixing in this treatment maintained any group-level differences between harvested sub-populations at the time of harvest. In the high-migration treatment, all picked fruiting bodies were first mixed before spotting on a fresh agar plate, homogenising all harvested sub-populations within a plate. The eight-day cycle of swarming and development was repeated 15 times.



Supplementary figure 2. Faster swarming evolved under low migration than under high migration. Swarming rates of all harvested sub-populations relative to their respective ancestor when evolved under low migration (M(-)) and high migration (M(+)). Black dots are means of relative swarming rate and error bars represent 95% confidence intervals. Asterisks show significant difference from 1 (ANOVA and subsequent and one-sample *t* tests with Benjamini & Hochberg correction, *** $p < 0.001$).



Supplementary figure 3. Higher variation of swarming distances per cycle across harvested sub-populations when evolved under low migration. Colony expansion of each harvested sub-population per evolution cycle until encounter of neighbouring sub-population. Coloured lines represent distinct sub-population within all four metapopulations (Plate 1-4) and selection treatments (low migration M(-) and high migration M(+)).

PART 2

THE ECOLOGY AND CHARARTERIZATION OF ANTAGONISTIC BACTERIA – PHAGE INTERACTIONS

CHAPTER 2

Hidden paths to endless forms most wonderful: Parasite-blind diversification of host quality

Lisa Freund, Marie Vasse & Gregory J. Velicer, 2021, *Proceedings of the Royal Society B: Biological Sciences*

Abstract

Evolutionary diversification can occur in allopatry or sympatry, can be driven by selection or unselected, and can be phenotypically manifested immediately or remain latent until realised in a newly encountered environment. Diversification of host-parasite interactions is frequently studied in the context of intrinsically selective coevolution, but the potential for host-parasite interaction phenotypes to diversify latently during parasite-blind host evolution is rarely considered. Here we use a social bacterium experimentally adapted to several environments in the absence of phage to analyse allopatric diversification of host quality - the degree to which a host population supports a viral epidemic. Phage-blind evolution reduced host quality overall, with some bacteria becoming completely resistant to growth suppression by phage. Selective-environment differences generated only mild divergence in host-quality. However, selective environments nonetheless played a major role in shaping evolution by determining the degree of stochastic diversification among replicate populations within treatments. Ancestral motility genotype was also found to strongly shape patterns of latent host-quality evolution and diversification. These outcomes show that i) adaptive landscapes can differ in how they constrain stochastic diversification of a latent phenotype and ii) major effects of selection on biological diversification can be missed by focusing on trait means. Collectively, our findings suggest that latent-phenotype evolution (LPE) should inform host-parasite evolution theory and that diversification should be conceived broadly to include latent phenotypes.

Introduction

Wonderful "endless forms" of phenotypes [1–3] often first evolve non-adaptively [4–7], even if they later prove beneficial in a new context as "exaptations" [8]. Temporally, a non-adaptively evolved phenotype might be generated immediately when its causal genotype first evolves, or only later, upon that genotype's exposure to a novel or changed environment. Here we refer to a phenotype that is potentiated by an existing genotype but not initially manifested due to environmental specificity as a 'latent phenotype'. We additionally refer to the evolution of such a genotype prior to manifestation of its initially latent phenotype as 'latent-phenotype evolution' (or 'LPE', [7]), a label independent of the causes or consequences of LPE (see Methods). LPE is intrinsically non-adaptive because the focal phenotype is not yet generated when its genetic basis first evolves. However, the genetic basis of the latent phenotype might evolve by any evolutionary mechanism (Table 1). For example, the causal genotype might arise adaptively due to one phenotypic effect beneficial in a first environment while pleiotropically potentiating a second phenotypic effect only be manifested in a distinct environment encountered later [9,10]. Alternatively, genotypes underlying LPE may evolve non-adaptively by hitchhiking with an adaptive genetic element [11] or by stochastic forces [6,12]. Variation among initially neutral alleles underlying latent phenotypes [12,13] has long been recognised as potential fuel for later adaptation to new and changing environments [14–16].

Latently evolved phenotypes can be features of individual organisms. For example, bacteria have latently evolved altered antibiotic resistance [10,17], metabolic-profile shifts [15] and changes in nutrient-uptake abilities [18]. However, outcomes of interactions between organisms can also be considered phenotypes. Examples of such "interaction phenotypes" [19,20] include reproductive incompatibility resulting from allopatric speciation, which remains latent until allopatrically diverged lineages make secondary contact [21–23]. Similarly, host-parasite interaction phenotypes [24–28] and bacterial social-interaction phenotypes [29,30] can also evolve and diversify latently in allopatry. Given that i) pleiotropy, hitchhiking and genetic drift are common, ii) manifestation of phenotypes is often context-specific (e.g. due to phenotypic plasticity [31] or, for interaction phenotypes, limitations on interaction opportunities), iii) exposure to changing or new environments is inevitable for most biological lineages, and iv)

latently evolved phenotypes often have selective significance upon their manifestation, LPE is likely to strongly contribute to long-term patterns of phenotypic evolution and diversification.

Table 1. Categories of latent-phenotype evolution (LPE). The genetic basis of a focal latent phenotype first evolves in a temporally prior environment E_P. We distinguish three mechanisms responsible for evolution of the causal genotype: adaptation, hitchhiking and stochasticity (corresponding to alleles *a*, *h* and *s*, respectively). By definition, each allele potentiates a latent phenotype LP_y (right superscript) in the prior environment E_P that is then manifested in the later-encountered environment E_L (MP_y). In addition, this allele may cause a non-advantageous manifested phenotype MP_x (left superscript) in the prior environment E_P, that might or might not also be manifested in E_L (as shown by the use of the brackets). All phenotypes manifested in E_L can have positive, negative, or no fitness effect. The first (non-header) row of the table describes a form of pleiotropy - a delayed, environment-contingent pleiotropic effect of an adaptive allele. The rows below describe scenarios consistent with the common meaning of 'cryptic genetic variation' (see Methods).

Prior environment E_P	**Later environment E_L**	**Cause of allele's increase in E_P**	**Allele's adaptive status in E_P**
$^{MPx}a^{LPy}$	$^{(MPx)}a^{MPy}$	adaptation (direct selection)	adaptive
h^{LPy}	h^{MPy}	hitchhiking (indirect selection)	non-adaptive
$^{MPx}h^{LPy}$	$^{(MPx)}h^{MPy}$	hitchhiking (indirect selection)	non-adaptive
s^{LPy}	s^{MPy}	stochasticity (mutation/drift)	non-adaptive
$^{MPx}s^{LPy}$	$^{(MPx)}s^{MPy}$	stochasticity (mutation/drift)	non-adaptive

Bacteria engage in a vast array of interactions, including with their own viral parasites, bacteriophages. Bacteria-phage interactions are determined by the match between phage-infectivity and bacterial-resistance mechanisms, which can result in narrow to broad host ranges [32–34]. Diverse mechanisms to resist phage have evolved at all major stages of

the infection cycle: including preventing phage adsorption, impeding post-entry reproduction and assembly, and stopping virion release through abortive systems that kill both phage and host [32,35]. Selection to resist infection can lead to host-phage incompatibility, as antagonistic coevolution between phage and their hosts leads to rapid local adaptation [36,37] and diversification [27,38,39]. However, how much host quality - the degree to which a host genotype or population facilitates parasite growth - is shaped directly by parasite-imposed selection versus indirectly from byproducts of other selective forces (e.g. resource competition [26] or other predators [40]) or stochastic forces has been little investigated.

Perhaps all microbes express some social traits [41], but some have evolved extraordinarily complex suites of cooperative behaviours [42]. Myxobacteria, including the model species *Myxococcus xanthus,* engage in cooperative swarming [43] during group predation [44] and in multicellular fruiting-body development [45]. As predators of many microbes, myxobacteria are predicted to strongly shape the structure and evolution of soil microbial communities [46,47]. Myxobacteria are themselves subject to selective pressure by myxophage [48], which in turn are likely to strongly shape myxobacterial social evolution. For example, cell-surface molecules such as type-IV pili and O-antigen serve as phage receptors in many bacteria [49,50] and also function in *M. xanthus* social behaviours [51,52]. Thus, just as bacteria-phage coevolution can indirectly shape bacterial social interactions [53–55], social evolution in the absence of phage is likely to latently alter the character and diversity of future host-parasite interactions. Analyses of experimentally-evolved lineages [29,30] suggest that intra-specific social interactions between natural *M. xanthus* lineages [56] often evolve latently. But how LPE shapes future antagonistic interactions of myxobacteria with other species, including with phage and their own prey, remains unexplored.

In this study, we test for LPE - including diversification - of host-virus interactions using the virulent myxophage Mx1 [57] and bacterial populations from an evolution experiment named MyxoEE-3 (see Methods). In MyxoEE-3, populations of *M. xanthus* underwent selection for increasing fitness at the leading edge of colonies expanding spatially by growth and motility, with cells near the leading edge transferred to a fresh plate at the end of each evolutionary cycle [29,30,58]. Importantly, evolving populations never encountered phage during MyxoEE-3, thereby allowing us to test whether and how

phage-blind adaptation to multiple environments indirectly shapes the character of interactions with obligate parasites.

We first analyse host-phage LPE in eight MyxoEE-3 treatments that share a common ancestor but differed in selective environment with respect to surface structure (hard or soft agar), nutrient availability (high or low nutrient levels) and/or nutrient source (nutrient medium alone or with prey lawns). We examine effects of environment and chance on the direction and degree of average phage-blind host-quality evolution and also on the degree of within-treatment diversification. If LPE is mediated predominantly by alleles that increased due to selection during MyxoEE-3, adaptive landscape structure [59,60] may shape LPE outcomes, including the degree to which latent phenotypes diversify stochastically. We further analyse how ancestral motility genotype shapes LPE by testing for effects of debilitating each of the two *M. xanthus* motility systems on latent host-quality evolution and diversification.

Materials and Methods

Semantics and nomenclature.

On use of 'latent-phenotype evolution'. Large bodies of literature examine modes by which the genetic basis of latent phenotypes evolves, for example as initially adaptive alleles that potentiate initially unrealised pleiotropic phenotypes [9,61,62] or as initially neutral alleles, variation for which is commonly referred to as "cryptic genetic variation" (CGV) [12,13]. However, there does not appear to be a well-established generic label for the evolution of the genetic basis of latent phenotypes that is impartial with regard to causes or consequences of such evolution and that applies equally to the phenotypes of individuals and organismal-interaction phenotypes.

We adopt 'latent' [63] over 'cryptic' due to the frequent association of the latter with selectively neutral alleles [12,13,16] (despite exceptional applications to initially adaptive alleles [64]) and the evocation of future manifestation by 'latent'. We use 'latent-phenotype evolution' to focus primary attention on the process of evolution over time, which may result in loss of variation due to fixation of an allele underlying a latent phenotype, rather than primarily on within-population variation at loci encoding such alleles, which is the focus of CGV. We also note that LPE in our sense is distinct from

(but may nonetheless be related to) the latent evolvability of a genotype, population or species, i.e. the potential for future evolution of novel forms, functions or diversity [65–68].

On use of 'manifestation'. Here we use 'manifestation' (and variations thereof) rather than 'expression' to refer to the actualization of a genetically caused phenotype. This is because we conceive expression to be actualization of a phenotype by an individual organism, but we desire a term that also applies generically to organism-interaction phenotypes. Actualization of the latter may be prevented simply by lack of spatial proximity between the relevant organisms rather than by lack of phenotypic expression by individuals.

MyxoEE-3. To facilitate reference to the broader evolution experiment of which the treatments examined were a part, we refer to the overall experiment as MyxoEE-3 [7] (Myxobacteria Evolution Experiment, with '3' indicating the temporal rank of the first publication from MyxoEE-3 [69] relative to first publications from other MyxoEEs). Shared features of MyxoEE-3 treatments have been described previously [29,30,58]. Treatments examined here are summarised in Table S1.

Strains and procedures

Strains. In MyxoEE-3, ancestral strains differing in motility genotype and antibiotic-resistance marker were selected for increased fitness at the leading edge of expanding colonies. Multiple treatment sets of replicate populations adapted to different environmental conditions that varied in nutrient level, nutrient type or agar concentration (Table S1).

The ancestral reference strains GJV1 and GJV2 have two functional motility systems: adventurous (A) and social (S) motility (hereafter referred to as the A+S+ motility genotype) [70]. Deletion of one gene essential for either motility system led to strains that were defective in A-motility (A-S+; deletion of *cglB*; strains GJV3 and GJV5) or S-motility (A+S-; deletion of *pilA*; strains GJV4 and GJV6). GJV1, GJV3 and GJV4 are rifampicin-sensitive, whereas GJV2, GJV5 and GJV6 are rifampicin-resistant variants of the corresponding motility type (Table S1). Two distinct sub-clone genotypes of GJV1 (represented by GJV1.1 and GJV1.2) previously found to differ by one mutation were used to establish the rifampicin-sensitive A+S+ MyxoEE-3 populations and thus were examined here also [29]. No phenotypic differences between these clones were found in

our assays (see *Statistical analysis* in Supplementary Material*)*. A single sub-clone was used for each of the other five ancestral strains since there are no known mutational differences between the ancestral sub-clones used to found the respective MyxoEE-3 populations.

For this study, we used MyxoEE-3 populations that evolved for 40 two-week cycles under high-nutrient conditions (CTT growth medium, see recipe below), low-nutrient conditions (0.1% Casitone-CTT) or with prey (*Escherichia coli* or *Bacillus subtilis* and CTT). Additionally, the agar concentration in each environment was either high (1.5% hard agar, HA) or low (0.5% soft agar, SA) (Table S1). During evolution, replicate populations derived from each of the six ancestors (GJV1-GJV6) grew and swarmed (to their ability) outwards on the surface of each selection environment for two weeks, after which a patch of defined size was collected from the leading edge of each colony and transferred to a fresh plate. Importantly, these populations never interacted with phage during MyxoEE-3.

The virulent myxophage Mx1 [57] is a double-stranded DNA Myoviridae, morphologically similar to coliphages T2 and T4 [48,71]. A single source stock of Mx1 was generated by infecting a growing liquid culture of GJV1. We isolated phage particles with 10% chloroform and filtration (0.22 μm) and titered the resulting Mx1 stock using double agar overlay plaque assays on the highly susceptible *M. xanthus* strain DZ1 [72].

Assays of Mx1 growth on MyxoEE-3 ancestors and evolved populations in liquid culture. All host-quality experiments were performed in liquid CTT medium (10 mM Tris pH 8.0, 8 mM $MgSO_2$, 10g/l casitone, 1 mM KPO_4, pH 7.6) supplemented with 0.5 mM $CaCl_2$ in case of phage infection and incubated at 32 °C and 300 rpm. Prior to each experiment, bacteria were inoculated onto CTT 1.5% agar from frozen stocks and incubated at 32 °C and 90% rH until sufficiently grown. Colony-edge samples were transferred to 8 ml CTT liquid and incubated shaken at 300 rpm. When the bacterial cultures reached mid-log phase, cells were centrifuged (15 min, 5000 rpm) and resuspended with CTT liquid to ~2 x 10^8 cells/ml. To initiate the host-quality assays in liquid culture, aliquots of the same Mx1 phage stock at ~2 x 10^6 particles/ml were added to the density-standardised bacterial populations, resulting in a multiplicity of infection (MOI) of ~0.01. Phages were allowed to infect both ancestral and evolved MyxoEE-3 bacterial populations for 24 h and viable phage population size after 24 h in liquid was

our measure of host quality. We performed four replicates of this experiment, each divided into three randomised blocks.

To quantify and compare Mx1 population sizes at the end of the host-quality assay in liquid, we performed plaque assays on the indicator strain DZ1. DZ1 is highly susceptible to Mx1 [72] and thus maximises the detection of viable phage particles. To isolate the phage from the bacteria, 100 μl of chloroform were added to 1 ml of liquid culture and this mixture was incubated for 5 min under constant shaking/vortexing to disrupt bacterial cells. Subsequently, dead cells and phage were separated by centrifugation (3 min, 12000 rpm). Supernatant containing the phage was stored at 4 °C. Phage numbers were then estimated by serial dilution followed by mixing 10 μl of each dilution with 10 μl of the indicator strain DZ1 (~10^8 cells/ml) and 1 ml CTT 0.5% agar. This mixture was poured onto 5 ml CTT 1.5% hard agar and plates were then closed immediately and incubated until Mx1 plaques within the soft-agar-embedded lawns of DZ1 became visible and could be counted.

M. xanthus growth in the presence of phage. To assess effects of phage on growth of populations P65-P72 (Table S1), we measured optical density (OD 595 nm) of liquid cultures growing with and without phage. Overnight cultures of each ancestor and evolved population were diluted in equal volumes into two 15 ml cultures in 100 ml flasks, one of which was infected with phage (MOI of 0.01), and incubated shaken as described above. Measurements were taken at 0, 14, 16, 18, 20, 22 and 24 h.

Statistical analysis. Details of statistical analysis can be found in the Supplementary Material.

Results

Phage-blind evolution lowered host quality overall while environment mildly shaped treatment means

Host quality depends on a combination of host features, including extracellular components phage must bypass or penetrate to reach the cell surface [73], surface and membrane components that phage utilise for invasion [49], harmful intracellular components phage must avoid or neutralise [74] and beneficial intracellular components that phage exploit for growth. Any of these components might be altered during adaptation in the absence of phage. If selective pressures imposed by distinct MyxoEE-3 environments often differ in their effects on traits important to phage invasion and growth, MyxoEE-3 treatments might often vary in host quality. If not, more variation in host quality should be found among replicate populations within treatments than between treatment means.

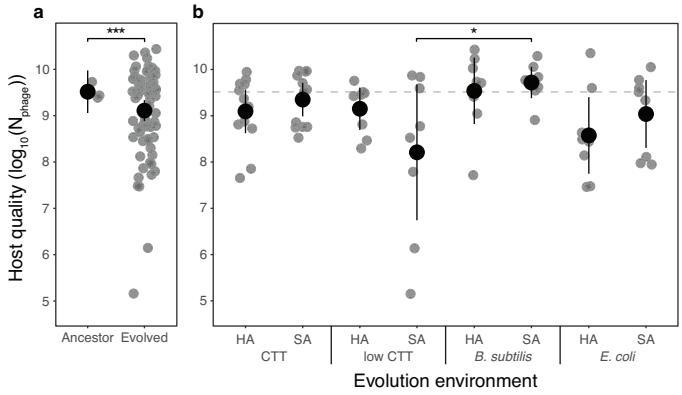


Figure 1. Diversification of latent host quality during phage-blind evolution occurred predominantly within rather than between selective environments. Cross-replicate means of host-quality measurements (grey circles) for ancestors and all evolved populations (a) and evolved populations categorised by evolution environment (b) with corresponding overall category means (black circles) and 95% confidence intervals. HA and SA indicate hard and soft agar environments, respectively. Host quality is measured as Mx1 phage population sizes 24 h after initial infection of bacterial populations (log-transformed data). The dashed line corresponds to average phage population size after growth on the experimental ancestors GJV1 and GJV2. The asterisks indicate statistically significant differences: two-sample two-sided *t*-test (a, *** $p < 0.001$) and the one pairwise comparison in which treatment means differ significantly (b, post-hoc Tukey test, mixed linear model, * $p < 0.05$).

Evolved populations and their ancestors were exposed to phage epidemics in shaken liquid culture for 24 h before phage population sizes were determined to quantify host quality. On average across all 72 populations descended from the A+S+ ancestor, evolved populations supported less phage growth than their ancestors (Figs. 1a, S1; $t_{54} = -4.78$, $p < 0.001$). The observed trend of decreased host quality suggests that adaptation to laboratory conditions generally increased resistance to a major natural stress (in this case phage). This is an intriguing outcome, as laboratory "domestication" of natural isolates usually relaxes selection for natural stresses [26,75], often resulting in corresponding trait losses [9,76,77]. Nonetheless, evolved bacterial populations might have become lower-quality hosts by two very different mechanisms - either by becoming more rapidly killed by phage and thereby supporting less phage growth overall or by individual bacteria becoming more resistant to phage. We investigate these hypotheses for one MyxoEE-3 treatment below. However, because absolute fitness is what matters from the phage perspective, our primary emphasis is on host quality *per se*, irrespective of what specific traits underlie its unselected evolution.

Despite the overall decrease in host quality across all evolved populations, no individual treatment considered separately changed significantly from the ancestor (Dunnett contrasts, all $p > 0.1$), reflecting variable outcomes among replicate populations within treatments (Fig. 1). However, selection environment did nonetheless have a small but significant effect on the structure of evolved host-quality outcomes (Fig. 1b; mixed linear model, $F_{7,64} = 2.4$, $p = 0.03$). This effect was driven predominantly by a difference between two environments - populations that evolved with *B. subtilis* as prey on soft agar were higher-quality hosts, on average, than populations evolved on low-nutrient soft agar (Fig. 1b; post-hoc multiple comparisons with Tukey method for p-value adjustment $t_{66} = -3.24$, $p = 0.04$).

We further tested whether environmental features shared across subsets of treatments affected average host-quality evolution and thus grouped treatments by nutrient type (high- and low-casitone CTT, *B. subtilis* or *E. coli*) and agar type (hard or soft agar), irrespective of other factors. Agar type did not influence mean host-quality evolution (Fig. S2a; $F_{1,71} = 0.028$, $p = 0.87$) but nutrient type did (Fig. S2b; $F_{3,69} = 3.73$, $p = 0.015$). The latter outcome is caused predominantly by the low-nutrient and *B. subtilis* subsets, with populations having grown at low-nutrient levels evolving lower host quality than

populations that evolved with *B. subtilis* as prey (post-hoc multiple comparison adjusted with the Tukey method, $t_{71} = 2.9, p = 0.02$).

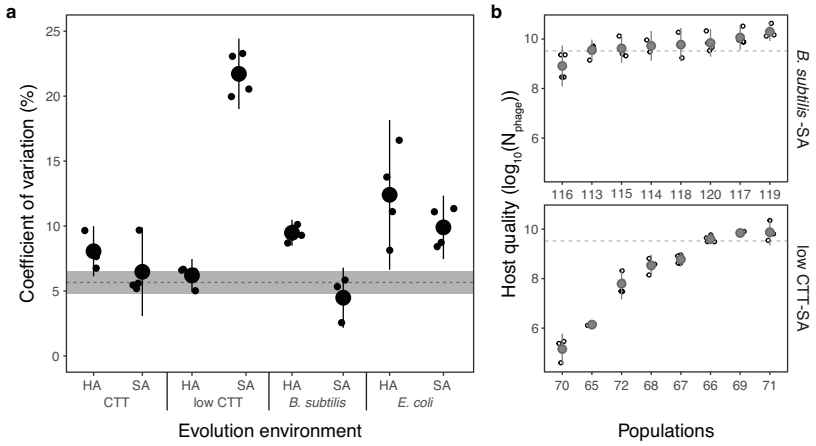


Figure 2. Selective environments differentially constrained stochastic diversification of host quality within treatments. (a) Within-treatment coefficients of variation (CVs) of phage population size 24 h post infection. Small and large circles represent within-replicate-assay CV estimates across evolved populations and cross-replicate-assay means for each treatment, respectively. HA and SA differentiate hard and soft agar environments, respectively. Error bars show 95% confidence intervals. For comparison, the dashed line indicates the between-treatment CV (i.e. the cross-replicate-assay average of the CV among host quality mean for each treatment). Grey shaded area is the corresponding 95% confidence interval. (b) Host quality of evolved populations from the least (upper panel) and most (lower panel) evolutionarily diversified treatments. Grey circles are the means across four biological replicates (open circles) and error bars represent 95% confidence intervals.

Degrees of within-treatment diversification varied greatly across selective environments

Although divergence among treatment means due to environmental differences was limited, we noted a high degree of diversification among populations overall that was not evenly distributed across treatments. To visualise host-quality diversification at multiple levels, we compared the coefficient of variation within vs. between selective environments for all eight treatments with A+S+ ancestors. Variation among populations within environments (on average 9.9%, ranging from 4.5 to 21.8%, Fig. 2a) greatly exceeded variation across environments (5.7%, calculated among within-treatment means, $t_{38} = 4.11, p < 0.001$), further confirming that chance differences in the mutational trajectories of replicate populations contributed more to overall diversification than did systematic differences between treatments.

Environments might differ in the degree to which they allow latent phenotypes to diverge stochastically among replicate populations if i) LPE is caused largely by mutations that evolved due to selection rather than drift and ii) the adaptive landscapes of distinct environments differ in their ranges of accessible adaptive pathways with regard to their indirect effects on host quality. We found that the degree of host-quality diversification among replicate populations adapted to the same environment varied greatly across treatments (Figs. 2a, S1; $F_{7,21} = 48.83$, $p < 0.001$). At one extreme, populations evolved on soft agar with *B. subtilis* diverged very little in host quality (Fig. 2). At the other extreme, populations evolved on low-nutrient soft agar diversified much more than populations in any other treatment (Fig. 2). Such variation in diversification across environments indicates that many of the mutations underlying LPE evolved due to selection and that distinct adaptive landscapes differ in how much they constrain latent-phenotype divergence.

Some populations latently evolved complete resistance to phage antagonism

Post-infection phage population sizes varied by more than ten-fold across replicate evolved host populations within all treatments, more than 100-fold in five treatments and nearly five orders of magnitude in one treatment (low-nutrient soft agar, Figs. 1, S1). Given such diversity, we tested whether bacterial growth would be suppressed to a degree inversely correlated with phage population growth. It is not obvious that such a correlation will occur, because, as noted above, phage growth might be low on both highly susceptible and highly resistant bacterial genotypes and thereby prevent a correlation. On highly susceptible hosts, phage growth may be low because phage strongly suppress the increase of their only growth substrate. In contrast, phage will not grow much from even large populations of highly resistant hosts. In this scenario, phage productivity could be maximal on bacterial populations exhibiting intermediate growth in the presence of phage.

During experimental epidemics with evolved populations from the most diversified evolutionary treatment (P65-P72, low-nutrient soft agar), we tracked both phage growth and *M. xanthus* population dynamics, the latter in comparison to bacterial growth in the absence of phage. None of these evolved populations grew less in the presence of phage than their ancestor, indicating that increased susceptibility to phage killing was not a general mechanism by which host quality often decreased during MyxoEE-3 (Fig. 3). As expected, these populations varied greatly in the degree to which Mx1 hindered their

growth relative to their growth in the absence of phage (Fig. 3). Three evolved populations (P67, P68 and P72), like the ancestors, grew very little over the 24 h epidemics, both relative to the phage-free controls and in absolute numbers. The other five populations all grew significantly more than their ancestors, again both relative to the phage-free controls and in absolute numbers (Dunnett test against the ancestor, all p values < 0.005). The two evolved populations that supported the least phage growth (P65 and P70, Fig. 2b) exhibited the highest bacterial growth, which was not significantly lower than phage-free growth after 24 h. Thus, complete (or nearly complete) resistance to viral load is found to evolve indirectly.

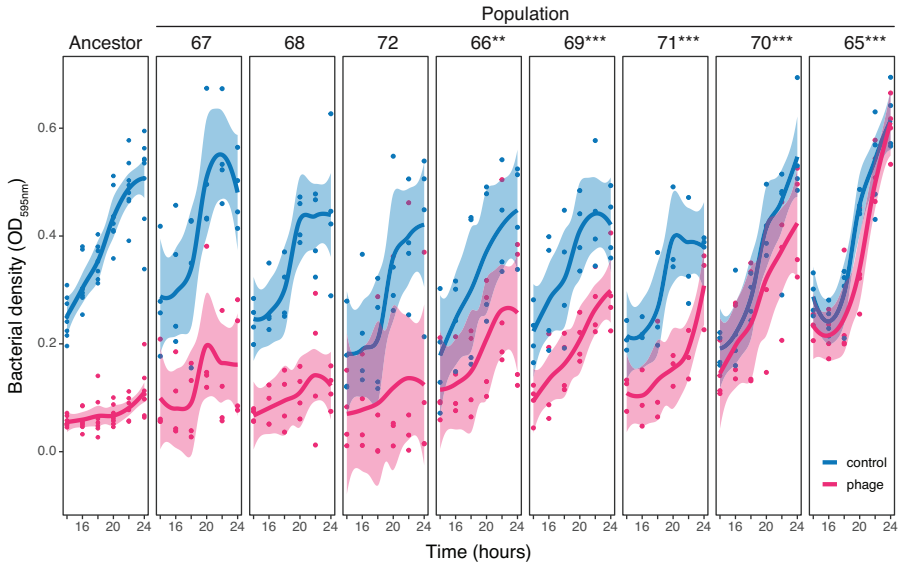


Figure 3. Diversity of indirect evolution of bacterial resistance to growth suppression by phage. Growth of the ancestors and of MyxoEE-3 populations evolved on low-nutrient soft agar (P65-P72) in the presence (red) and absence (blue) of phage. Data points show optical density (OD_{595nm}) measurements over time for four temporally separate biological replicates, trendlines track conditional mean values of locally weighted regressions and grey areas represent 95% confidence intervals of the fit. The asterisks indicate significant differences to the ancestors (Dunnett test, mixed linear model, ** $p < 0.01$ and *** $p < 0.001$).

In contrast to the hypothetical scenario presented above, total phage productivity was found to weakly correlate with bacterial growth reduction (Spearman's rho correlation $r_S = 0.31$, $n = 40$, $p = 0.058$, Figs. 3, S3). However, large numbers of phage were able to grow from bacterial populations that exhibited very different degrees of growth

suppression by phage. For example, Mx1 consistently grew to large population sizes on the ancestors, P66-P69 and P71, yet these populations varied greatly in the degree to which their growth was suppressed by phage. These results reveal idiosyncrasy in relationships between host growth and phage growth and thus point to those relationships evolving by diverse molecular mechanisms.

Intriguingly, we also noted substantial evolution and diversification of growth dynamics among the evolved populations in this treatment in the absence of phage, with several (e.g. P67, P68, and P71) slowing or ceasing growth earlier than the ancestors and other evolved populations (e.g. P65 and P70). Thus, variation of mutational input across replicate populations generated substantial diversification of growth dynamics in liquid media in the absence of phage.

Ancestral motility genotype determines ancestral host quality, mean host-quality evolution and within-treatment host-quality diversification

Motility not only allows organisms to search for new resources but also allows active flight from biotic and abiotic danger. In bacteria, motility can allow cells to escape from non-motile phage particles [78]. On the other hand, motility-related surface structures such as type-IV pili can also make bacteria susceptible to phage attack by acting as phage receptors [79]. To our knowledge, the relationship between motility and host-phage interactions, either behaviourally or evolutionarily, has yet to be examined for bacteria with multiple motility systems. We exploited the design of MyxoEE-3 - which included not only an ancestor with both *M. xanthus* motility systems intact (A+S+), but also ancestors lacking a gene essential for either motility system (A-S+, *ΔcglB* and A+S-, *ΔpilA*, Table S1), to test for motility-genotype effects on ancestral host quality and subsequent host-quality evolution.

We quantified total phage productivity after 24 h of growth in liquid on all motility-genotype ancestors and all descendant populations that evolved on CTT hard or soft agar during MyxoEE-3. The absence of *cglB* in the A-S+ ancestors had no effect on phage growth (motility effect in mixed linear model $F_{2,4} = 12.47$, $p = 0.019$, posthoc contrasts $t_4 = -0.66$, $p = 0.8$), whereas the absence of *pilA* in the A+S- ancestors increased phage productivity nearly ten fold (posthoc contrasts $t_4 = 4.79$, $p = 0.019$, Fig. 4). Thus, production of pilin, but not of CglB, greatly reduces a phage epidemic.

Ancestral motility genotype also affected the character of host-quality evolution. Mean host quality of A-S+ populations decreased significantly from their ancestral values

during evolution ($t_{55} = 2.83, p = 0.007$) and decreased significantly more than did the host quality of A+S+ or A+S- populations, while A+S+ populations decreased more than did A+S- populations ($F_{2,54} = 8.34$, $p < 0.001$, all post-hoc contrast p values < 0.001). Moreover, ancestral motility genotype also affected degrees of diversification within treatments, with evolved host quality spanning over four orders of magnitude among the A-S+ populations but less than a factor of ten among the A+S- populations (Fig. 4). The large differences in both ancestral phenotypes and evolutionary patterns between the A+S- category of populations versus the two categories with intact S-motility indicate that pilin production potentiates latent evolutionary reduction and diversification of host quality.

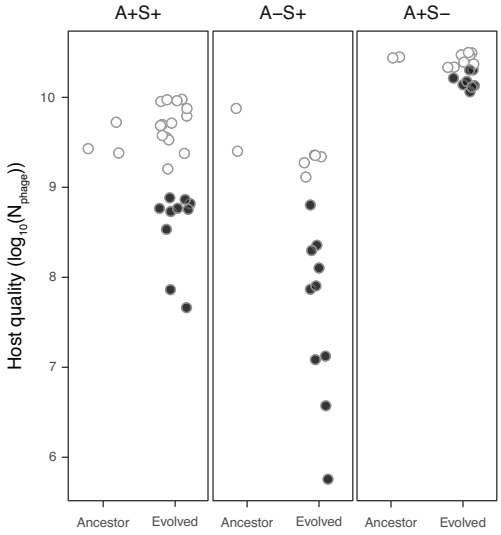


Figure 4. Ancestral motility genotype determines both degree of trait-mean evolution and degree of stochastic diversification for host quality. Phage population size 24 h after infection of ancestors and evolved populations with both motility systems intact (A+S+) or lacking either system (A-S+ or A+S-). Each data point represents the mean of four biological replicates. Colours show the difference between evolved populations and their respective ancestors (open circles: non-significant difference, black circles: significant difference; Dunnett test, mixed linear model).

Discussion

Previously, Meyer *et al*. found that the *Escherichia coli* Long-Term Evolution Experiment (LTEE) populations adapting to one selective environment in the absence of phage evolved changes in their interactions with multiple phage types [26]. Replicate LTEE populations evolved increased susceptibility toward phage T6* and increased resistance toward bacteriophage lambda with some degree of parallelism. Here we asked whether multiple distinct phage-free selective environments might differentially shape how bacterial populations descended from a common ancestor would diverge from each other in quality as phage hosts. We tested for such inter-treatment divergence with respect to both mean host quality and the degree of stochastic within-treatment diversification among replicate populations.

Overall, MyxoEE-3 populations tended to support less phage growth than their ancestors and diversified greatly, including some lineages that evolved nearly complete resistance to negative phage effects on bacterial population growth. We found high degrees of diversification in host quality among replicate populations within treatments. Indeed, among A+S+ populations, distinct selective environments drove only a small degree of divergence between selective-environment treatments in mean host quality; the primary diversifying force was chance variation in mutational input across replicate populations (Figs. 1 and 2).

Yet despite the limited effect of selection on divergence of treatment means, selection nonetheless strongly shaped the character of intrinsically non-adaptive diversification of latent phenotypes. Specifically, selective environments determined the degree to which replicate populations diversified in latent host quality, thus indicating that many of the mutations driving such divergence first evolved due to selection. Further, this result indicates that distinct adaptive landscapes can differ not only in the number of adaptive mutational pathways replicate populations might follow [60,80,81], but concomitantly can also differ in the range of latent phenotypic effects generated by those adaptive pathways [7]. Thus, distinct natural environments may often differ in the character of latent phenotypic diversity they allow to evolve.

Determination of latent stochastic diversification of host-parasite interactions by environment-specific features of fitness landscapes might apply not only to host evolution but to parasite evolution also. For example, consider a scenario with animal viruses in

which different initial host species for a given viral type differ in fitness-landscape structure and thereby allow different ranges of adaptive mutational pathways to be followed by evolving viral populations. Such differences might in turn generate differences in latent virus-host interaction phenotypes, including potential for jumps to novel host species (e.g. zoonosis).

We further investigated whether ancestral motility genotype is important to ancestral host-parasite interactions and/or their subsequent evolution, including extent of diversification (Fig. 4). While type-IV pili are the very means of cell invasion by phage in some host species [49,50], we found that production of pilin, the building-block of type-IV pili [51], both greatly reduces phage population growth in our ancestral genetic background and promotes greater latent evolutionary reduction and diversification of host quality than occurs in populations lacking pilin (Fig. 4). These immediate and evolutionary effects, respectively, of pilin production may be mediated not by pilin *per se* but by the *M. xanthus* exopolysaccharide (EPS) matrix, which is positively regulated by pilin production [69,82]. The EPS matrix is necessary for effective S-motility [83] and mediates cell-cell adhesion [84]. We hypothesise that the EPS matrix hinders Mx1 access to its adsorption receptor (which remains unknown), thereby explaining the nearly ten-fold increase in phage growth resulting from deletion of *pilA* to generate the A+S- genotype. This hypothesis would suggest that in A+S+ and A-S+ ancestors, evolution of the EPS matrix may have often provided greater protection against phage compared to ancestral EPS, thereby promoting greater diversification than among populations lacking ancestral EPS.

It has long been recognised that forces other than direct selection on focal traits play important roles in shaping evolutionary diversification [5,13,23,85], but latently evolved diversification is only rarely quantified [7,15,26]. We have shown that host-parasite interactions diversify greatly during parasite-blind evolution, highlighting the need to more deeply integrate LPE into our overall conception of biological diversification. The total long-term diversification of populations evolved in an original focal context can be conceived to include both divergence already actualised in that original context and the sum of all latent diversification revealed only later in new contexts. This expansive view of diversification can, in turn, inform how conservation efforts are conceived [86] to include conservation of latent phenotypes and corresponding evolutionary potential.

Data accessibility

Raw data and representative code are available from the Dryad Digital Repository: doi:10.5061/dryad.rn8pk0p86 [87].

Author contributions

L.F. designed experiments, carried out experiments and co-wrote the manuscript. M.V. designed experiments, performed statistical analysis and co-wrote the manuscript. G.J.V. designed experiments and co-wrote the manuscript.

Acknowledgements

This work was funded in part by Swiss National Science Foundation (SNSF) grants 31003A/B_16005 to GJV and an ETH Fellowship 16-2 FEL-59 to MV. The authors thank Peter Ashcroft, Marco La Fortezza, Samay Pande, Joshua Payne, Sébastien Wielgoss and all members of the ETH Zürich Evolutionary Biology group for helpful discussions and support.

Conflict of interest

The authors declare no conflict of interest.

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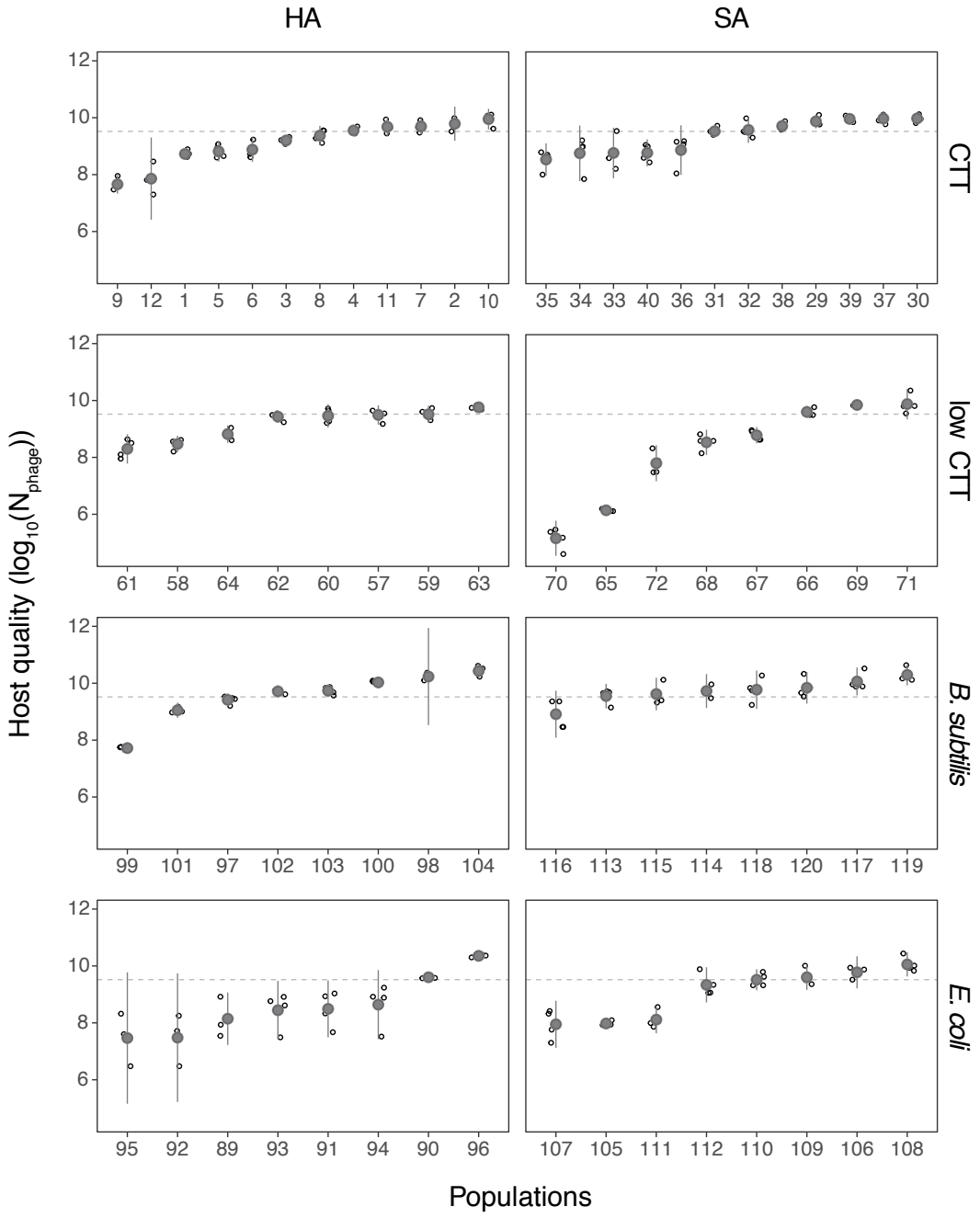
Supplementary Tables and Figures

Table S1. MyxoEE-3 treatments examined in this study.

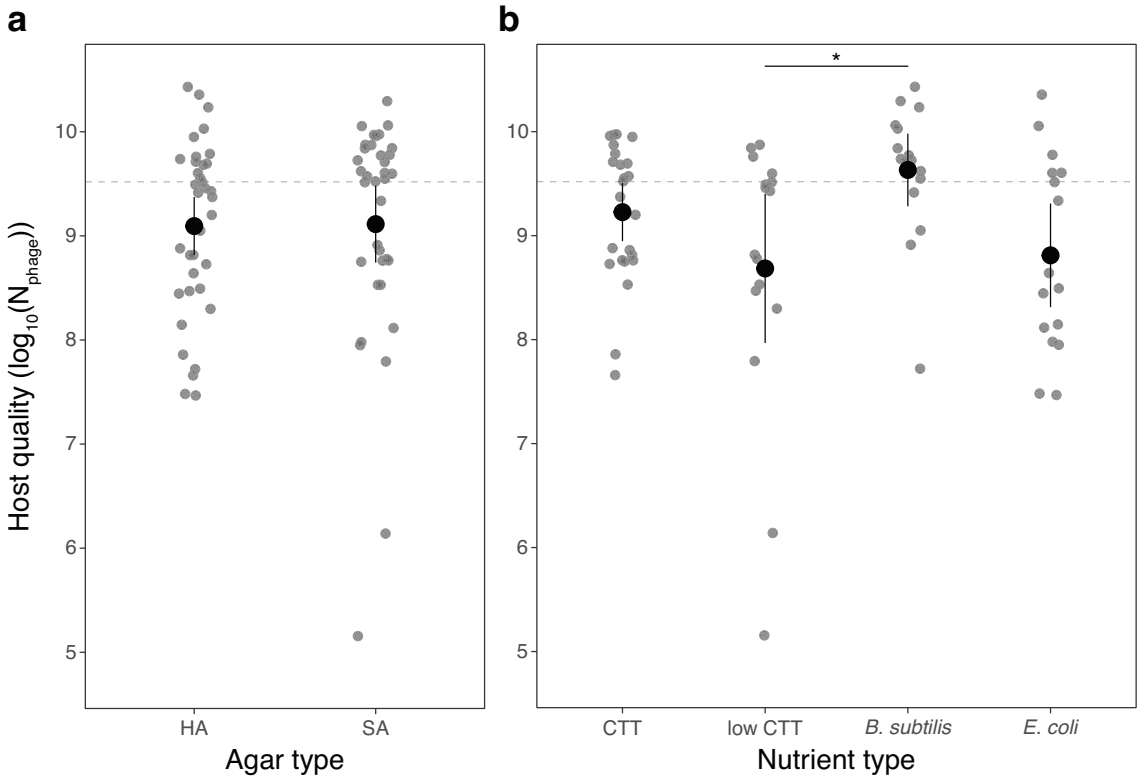
Evolved population*	Evolution environment	Ancestral motility genotype	Ancestral variants	
			Rif S	Rif R
1 -12	CTT (High-nutrient 1% Casitone CTT), HA (1.5% hard agar)	A+S+	GJV1**	GJV2
13 - 20	CTT, HA	A-S+ *ΔcglB*	GJV3	GJV5
21 - 28	CTT, HA	A+S- *ΔpilA*	GJV4	GJV6
29 - 40	CTT, SA (0.5% soft agar)	A+S+	GJV1	GJV2
41 - 48	CTT, SA	A-S+ *ΔcglB*	GJV3	GJV5
49 - 56	CTT, SA	A+S- *ΔpilA*	GJV4	GJV6
57 - 64	Low CTT (Low-nutrient 0.1% Casitone CTT), HA	A+S+	GJV1	GJV2
65 - 72	Low CTT, SA	A+S+	GJV1	GJV2
89 - 96	*E. coli* lawn grown on CTT HA	A+S+	GJV1	GJV2
97 - 104	*B. subtilis* lawn grown on CTT HA	A+S+	GJV1	GJV2
105 - 112	*E. coli* lawn grown on CTT SA	A+S+	GJV1	GJV2
113 - 120	*B. subtilis* lawn grown on CTT SA	A+S+	GJV1	GJV2

*Odd-numbered evolved populations descend from rifampicin sensitive ancestors and even-numbered populations descend from rifampicin resistant ancestors.

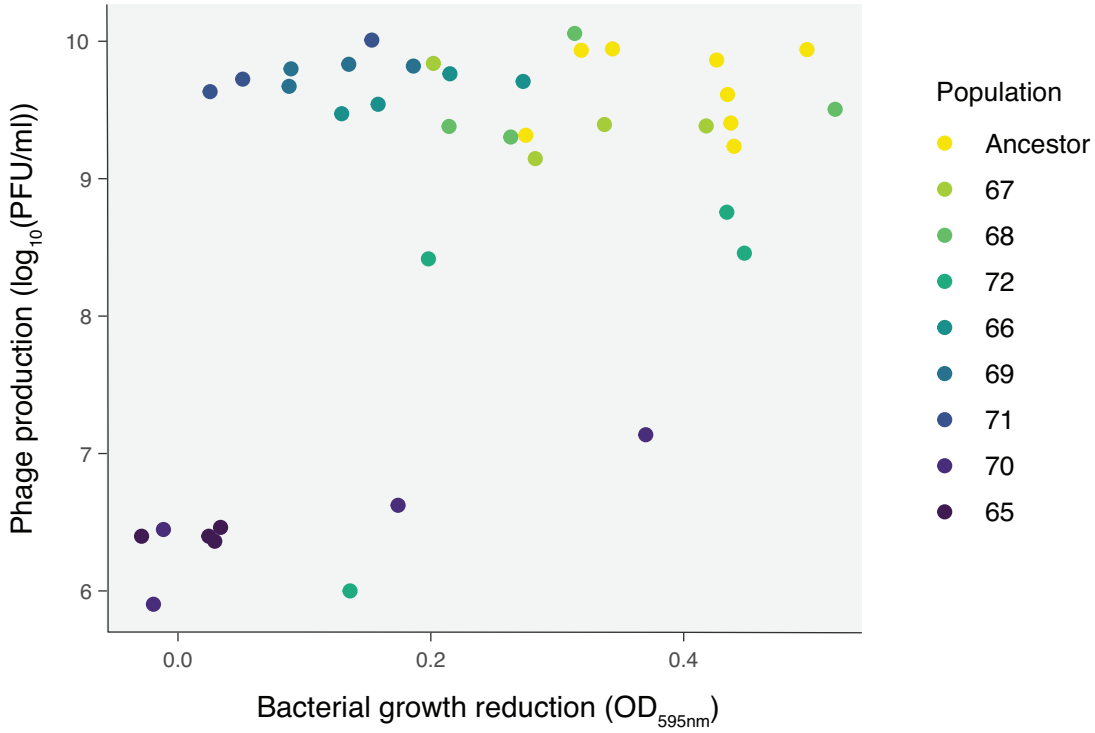
**Ancestral strain GJV1 is represented by two subclones, GJV1.1 and GJV1.2 (see Methods)



Supplementary Figure 1. Host quality of all evolved A+S+ bacterial populations. Host quality is measured as Mx1 phage population sizes 24 h after initial infection of bacterial populations (log-transformed data). Evolutionary treatments are categorized vertically by agar type (hard agar (HA) or soft agar (SA)) and horizontally by nutrient type (CTT, 0.1%-Casitone CTT, *B. subtilis* and *E. coli*). Open circles are original estimates per replicate, filled grey circles represent the mean, error bars represent 95% confidence intervals and dashed lines show average phage population size after growth on the ancestors.



Supplementary Figure 2. Host quality of evolved A+S+ populations grouped by shared selective-environment features. Mx1 phage population sizes 24 h after initial infection of bacterial populations evolved on different agar (a) and nutrient (b) types. HA and SA indicate hard and soft agar environments, respectively. Grey circles are population means over four biological replicates. Category means (over all population means within a category) are represented by black circles and error bars are 95% confidence intervals. Dashed lines correspond to average phage population size after growth on the experimental ancestors GJV1 and GJV2. The asterisk indicates the one pairwise comparison in which category means differ significantly (post-hoc Tukey tests, mixed linear model).



Supplementary Figure 3. Reduction of bacterial growth by phage correlates only weakly with phage fitness. Bacterial growth reduction is calculated as the difference in optical density (OD595nm) in the presence and absence of phage for the ancestors and the MyxoEE-3 populations evolved on low-nutrient soft agar (P65-P72). Phage fitness is the population size 24 h after initial infection of bacterial populations (log-transformed data). Colours correspond to the different populations (n = 3-8) and the colour gradient ranges from high (yellow) to low (dark purple) bacterial growth reduction.

Supplementary methods

Statistical analysis. We tested the effect of latent diversification on the final number of phage particles (log-transformed to meet normality assumptions) among evolved A+S+ populations using a mixed linear model (function lmer from the package lmerTest [1]) with the evolution environment as a fixed effect and the experimental block nested into replicates as a random factor (intercept). In addition, those populations evolved from two ancestral sub-clone genotypes of GJV1 (GJV1.1 and GJV1.2) and one sub-clone genotype of GJV2 (the rifampicin-resistant variant of GJV1). Therefore, we included the population ID nested into their ancestral background as a second random factor (intercept). However, as there was no significant difference between the rifampicin resistant and sensitive populations in final phage numbers ($F_{1,100} = 2.44$, $p = 0.12$), nor between the two clones of GJV1 ($t_{40} = 0.57$, $p = 0.57$), we combined them in the figures. To test for specific effects in the evolution environments, we used a variant of the previous model decomposing the agar type and/or nutrient types. We further measured the diversification among populations within each treatment with coefficients of variation (ratio of standard deviation to mean, multiplied by 100) and evaluated the importance of the evolution environment in shaping such divergence using the previously described mixed linear model. Bacterial growth data were analysed by i) comparing the final OD (24 h) in the presence of phage between evolved and ancestral populations (lmer model with Dunnett test, function glht from the package multicomp [2]) and ii) testing for a correlation between the phage-associated growth reduction and the phage number after 24 hours with a two-sided Spearman correlation test for paired data (function cor.test with Spearman method).

Finally, we investigated roles of both *M. xanthus* motility systems in determining host quality *per se* and its evolutionary diversification. We started by assessing the effect of motility on host quality (i.e. log-transformed final numbers of phage particles) in the ancestors using a mixed linear model with the motility system as a fixed effect and the population ID and the blocks nested into replicates as random factors. We then included the evolved populations and modified the model to account for the evolutionary state (either evolved or ancestor) as a second fixed effect and its interaction with the motility system. We tested whether the evolved populations differed from their relative ancestors within each motility subset. All multiple comparisons were performed using the emmeans

package (version 1.4.3 [3]) with *p* values adjusted with the Tukey method. All statistical analyses were performed using R version 3.6.2 and RStudio version 1.2.5033 [4,5]. We used the packages ggplot2 [6], ggpubr (version 0.4.0) [7] and ggsignif (version 0.6.0) [8] to generate the figures.

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CHAPTER 3

Remote extracellular attacks on bacteriophage

Lisa Freund[1], Marie Vasse[1] & Gregory J. Velicer

[1] Authors contributed equally

Abstract

Bacterial viruses – bacteriophages – and their hosts co-evolve to more effectively exploit and defend against the other, respectively. Known anti-phage defenses prevent attachment to the bacterial membrane or target phage nucleic acids after entry into the cell and variation in such defenses across bacteria shapes phage host range. We initially investigated the host range of the virulent myxophage Mx1 among natural isolates of the social bacterium *Myxococcus xanthus*. Mx1 was found not only to have a very restricted host range, but to itself be antagonized by most *M. xanthus* isolates. These antagonisms inactivate large fractions of viable phage populations and appear mediated by both cell-bound compounds and secreted, diffusible polypeptides. By harming phage before infection, anti-phage public goods represent a previously unknown category of defense against phage. Some isolates secrete anti-phage compounds facultatively in response to Mx1, while others appear to produce them only constitutively. The facultative responses suggest adaptation specifically to harm phage. *M. xanthus* isolates also alter the heat-stress tolerance of phage that initially survive interacting with them, with such effects being highly variable across bacterial genotypes. Indeed, some genotypes that fail to antagonize Mx1 in direct encounters nonetheless greatly reduce phage tolerance of later heat stress. Our results suggest that some bacteria have adaptations to secrete polypeptides as extracellular anti-phage agents, agents that, at least in *M. xanthus,* can both inactivate many phage immediately and weaken surviving phage. Secreted anti-phage agents raise intriguing questions regarding the costs and benefits of extracellular versus cell-associated phage-defense mechanisms.

Introduction

Bacteriophages are perhaps the most abundant type of viruses on our planet (1). They pervasively threaten but also often benefit their bacterial hosts (2–5). Phage-bacteria interactions and their evolution over billions of years cascade outward to impact bacterial social interactions (6), bacterial diversification (7), microbial-community interactions (8), many microbe-macrobe interactions (9), and global nutrient cycling (10). The range of bacterial species and strains that can support replication by a given phage type – its host range – determines its ecological impact and evolutionary potential. Some phage types can broadly infect multiple bacterial species, whereas others can replicate only within very closely related sublineages of a single species, and phage host ranges often overlap (11). Host range is likely to evolve in concert with other traits such as maximum reproductive rate (12), but the evolutionary causes and effects of host-range evolution remain under extensive investigation.

A major form of coevolution between bacteria and phage is often described as an arms race (13). On one side, phages are selected to become more efficient at invading and/or replicating within bacterial cells. On the other, bacterial cells evolve diverse defense mechanisms to survive phage attacks, targeting multiple stages in the phage life cycle.

Focusing on virulent DNA phage, successful lytic life cycles involve several stages; bacteria have evolved defenses targeting each. Upon encountering a bacterial host, a phage particle attaches to a cell-surface receptor and inserts its genetic material into the cell (14, 15). Some bacterial defenses prevent phage adsorption by modifying (16) or masking (17) potential receptors. If phage genetic material is successfully inserted, it hijacks host replication machinery to proliferate. However, phage DNA is often recognized and destroyed by a bacterial restriction-modification system (RM system) (18) or a CRISPR-Cas immune system (19). If such systems that directly target phage DNA are absent or fail to stop phage replication, phage proteins are synthesized and new particles are formed, up to hundreds of which per cell can be released when an infected cell bursts. Some bacteria can inhibit phage propagation through self-induced death using abortive infection systems (Abi systems) or by inhibiting DNA synthesis with nucleotide depletion mechanisms (20–23).

While the above-mentioned defense mechanisms against phage, by preventing replication, all have group-level consequences, they operate on or within the individual

defending host cell. Other mechanisms, however, such as the secretion of defensive diffusible secondary metabolites, have greater potential to generate protection against phage at broader community levels. Such secreted metabolites were first described in the 1950s and have recently regained attention but their molecular modes of action remain unclear (24). Such metabolites are mostly anthracycline (25) and aminoglycoside (26) antibiotics, both commonly produced by Streptomycetes, that inhibit replication by diverse DNA phages after introduction of phage genetic material into a prospective host cell. That such metabolites might have the potential to benefit cells other than their producers, and therefore function as anti-phage public goods (hereafter APPGs), raises many fascinating questions regarding the potential costs and benefits of shared *vs* private anti-phage mechanisms at individual, population, and community levels.

Like Streptomycetes, myxobacteria produce a vast array of secondary metabolites (27), some of which might, like anthracyclines and aminoglycosides, have anti-phage properties. However, soil-dwelling myxobacteria are perhaps best known for their highly social life cycles, which involve many forms of intercellular communication and coordination, including during social motility (28), group predation (29), aggregative multicellular fruiting body development (30) and social spore germination (31). Because phage adhere to cell-surface molecules that may be involved in bacterial sociality, interactions with phage are expected to have played important roles in shaping the complex cooperative behaviors of myxobacteria (32).

Although phage capable of infecting *M. xanthus* were first isolated many decades ago (33), relatively few have been isolated since and very little is known about their host ranges (34). We initially sought to characterize the host range of a virulent myxophage, specifically the range of *M. xanthus* genotypes susceptible to being exploited by the virulent myxophage Mx1 to fuel phage population growth. To this end, we introduced phage Mx1 into cultures of *M. xanthus* strains isolated worldwide and of known phylogenetic relatedness. Upon finding that many of the bacterial genotypes actually antagonize the phage rather than *vice versa*, we tested whether the antagonizing compounds produced by *M. xanthus* are diffusible or cell-bound, and whether they are produced constitutively or facultatively in response to interaction with phage. As a function of *M. xanthus* genotype, we then characterized the heat sensitivity of both diffusible antagonistic compounds produced by *M. xanthus* and of Mx1 phage that had interacted with *M. xanthus*.

Methods

Strains and culture conditions

M. xanthus natural isolates examined in Rajagopalan *et al*. 2015 were selected to include, in most cases, two independent isolates from each of eight globally distributed sampling locations/regions, thus encompassing a wide range of phylogenetic distances among strains (35). Strain GJV1, a derivative of the reference strain DK1622 (36), was included also. Frozen stocks of bacterial isolates were inoculated on CTT hard-agar plates (CTT HA; 10 mM Tris pH 8.0, 8 mM $MgSO_2$, 10 $g.l^{-1}$ casitone, 1 mM KPO_2, pH 7.6; 1.5% agar (37)) and incubated at 32 °C with 90% rH until sufficiently grown. Samples of growing plate cultures were transferred to CTT liquid medium (same as CTT-HA without agar) and incubated at 32 °C, 300 rpm until mid-log-phase. To initiate each experiment, cultures were centrifuged (3 min at 12000 rpm) and resuspended to ~2 x 10^8 cells ml^{-1} in CTT liquid.

Here we studied the virulent myxophage Mx1, a double-stranded DNA phage (33). The Mx1 stock was generated by infecting the lab strain GJV1 growing in liquid CTT. Phage particles were isolated using 10% chloroform followed by filtration (0.22 μm).

Infection assays

Phage infection was initiated with density-adjusted bacterial cultures (~2 x 10^8 cells ml^{-1}) and diluted phage (MOI ~0.01) in 4 ml CTT liquid supplemented with $CaCl_2$ (0.5 mM). Cocultures were incubated shaken at 300 rpm, 32 °C for 24 h. To determine final phage numbers, 10% chloroform was added to each sample. Samples were incubated shaking and vortexed to ensure the release of phage particles from bacterial cells. After centrifugation (3 min at 12000 rpm) and removal of the supernatant containing all phage particles, viable phage particles were determined in double-agar-overlay plaque assays. To do so, we used *M. xanthus* strain DZ1 as an indicator strain, as it is highly susceptible to infection by Mx1 and allows clear plaque formation (38). 10 μl of DZ1 suspension at 10^{10} cells ml^{-1} and 10 μl of serial diluted phage were mixed with 1 ml CTT soft agar (CTT SA; CTT liquid with 0.5% agar) in the liquid state cooled to 50 °C and mixture was then poured on top of solidified CTT HA plates. After 24 h of incubation at 32 °C, we counted PFU numbers.

Supernatant assays

We investigated whether the observed antagonisms were mediated by secreted diffusible compounds. To recover cell-free supernatants (Fig. S1), cultures with bacteria and phage were centrifuged (5 min at 12000 rpm) 24 h after infection and the phage-containing supernatant was carefully removed and titered. 3 ml of the supernatant was transferred to a new flask, to which ~3.7 x 10^6 phage particles ml^{-1} were added, followed by incubation for another 24 h. The viable phage population size was determined using the soft-agar-overlay assay. Control assays in which bacteria were grown in the absence of phage were performed as above, except phage particles were not added to the bacterial culture for the first 24 h of incubation, only to the supernatant for the second 24 h incubation period.

Temperature assays

We tested whether high temperature eliminates the negative effect on phage of exposure to *M. xanthus* supernatant (Fig. S2). To do so, we co-cultured Mx1 with all bacterial isolates and titered phages in the resulting supernatants. Supernatant aliquots were then heated at 45, 55, 65 or 75 °C for 30 min and cooled down 10 min on ice. The number of viable phages was determined a second time for each heat-treated supernatant. We then exposed phages to the heat-treated supernatants and samples were handled as noted previously (stage 2 of Fig. S2). For control treatments without phage interaction, phages were not added to bacterial cultures, only to the purified supernatant.

Test for supernatant effects on phages T4 and DMS3vir

To test whether *M. xanthus* compounds that inactivate Mx1 phage also harm bacteriophages that infect non-myxobacterial species, we exposed phage T4 (39), a virulent phage of *Escherichia coli* and DMS3vir (40), a virulent phage of *Pseudomonas aeruginosa*, to supernatants from co-cultures of *M. xanthus* isolates and Mx1 phage. We co-cultured Mx1 with each of the five bacterial isolates that previously showed the biggest supernatant effect on Mx1 and extracted the supernatant as described previously. Subsequently, ~2 x 10^6 phage particles ml^{-1} of T4 and DMS3vir were added to separate supernatants of each bacterial genotype. After 24 h of incubation, PFUs were quantified using the double-agar-overlay assay with the respective host strain of either phage. We used *E. coli* strain MG1655 as host for T4 and *P. aeruginosa* strain UCBPP-PA14

csy3::LacZ (40) as host for DMS3vir. To be sure that Mx1 particles remaining in the supernatant have no lytic effect on *E. coli* or *P. aeruginosa,* a control treatment was included that tested for possible plaques of Mx1 on both bacterial strains in absence of T4 or DMS3vir. No such plaques were observed at any plating dilution.

Statistical analysis

All experiments were performed in three or four temporally separate, independent biological replicates. Analyses were performed using R version 4.1.2 and Rstudio version 2021.09.0.351 (41, 42). Strain-specific effects were tested for with one-way ANOVA followed by two-tailed Dunnett tests (package multcomp (43)) to compare against single treatments. For supernatant experiments, we first calculated ratios of observed *vs* expected phage numbers and pre *vs* post heat-treatment values and tested for between-strain differences with one-way ANOVA. Upon detection of significant effects of the strain identity, differences between individual observed and expected values were tested for with multiple one- or two-sample paired *t* tests with correction for multiple testing (Benjamini & Hochberg). Alternatively, a single *t* test was performed combining the data for all strains.

Results

Mixtures of Mx1 phage with liquid cultures of 16 *M. xanthus* natural isolates and the reference strain GJV1 were incubated for 24 h before the number of phage particles capable of forming plaques on the *M. xanthus* indicator strain DZ1 (hereafter referred as 'viable phage') from each co-culture was determined. The *M. xanthus* genotypes varied greatly in their effects on viable phage numbers (Fig. 1; linear model, $F_{17,36} = 854.33$, $p < 0.001$). However, our results indicated that Mx1 has a very narrow host range, as only two of the 17 bacterial strains allowed Mx1 to increase in viable-population size, namely GJV1 and Sulawesi 05 (posthoc Dunnett contrasts, both $p < 0.001$). These strains are positioned in different subclades of a whole-genome based phylogeny (Fig. 1).

Surprisingly, among the remaining 15 strains in which Mx1 is unable to complete its lytic life cycle, ten caused large decreases in viable-phage population sizes (reductions of 97.6-99.9%), while five had no significant effect on phage numbers. This is an intriguing

observation, since phage numbers were expected to remain constant over short periods when bacterial cells cannot be utilized as a suitable host. No clear relationship between phylogenetic position and the three categories of effects on phage number - neutral, successful phage replication, and antagonism of phage - is evident.

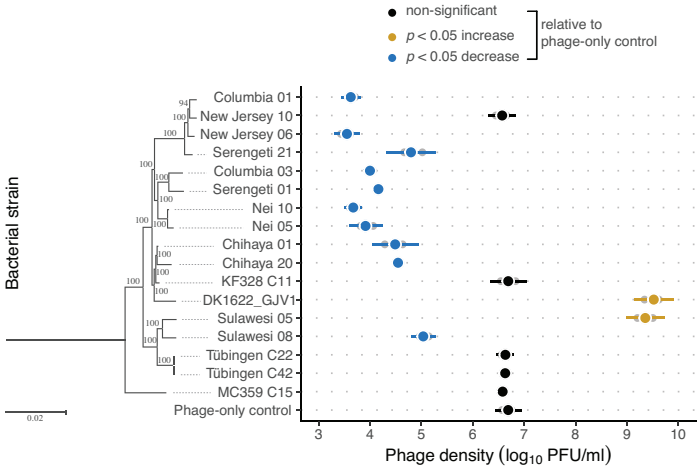

Figure 1. Most *M. xanthus* natural isolates antagonize bacteriophage Mx1, which has a very narrow host range. Phage density 24 h after infection of bacterial strains and in the absence of bacteria (control, black circle). Circles are mean $\log_{10}$-transformed PFU values across three biological replicates (light gray dots) and error bars represent 95% confidence intervals. Colors show outcomes of tests for differences between phage numbers for phage populations that interacted with *M. xanthus vs* control populations that did not (black: no significant difference, yellow: significant increase in phage number due to interaction with *M. xanthus*, blue: significant reduction; ANOVA and subsequent Dunnett contrasts, $p < 0.05$). The phylogeny is a maximum-likelihood tree reproduced from Rajagopalan *et al*. 2015 inferred from ~4.5 Mbp of orthologous genome sequence using *M. xanthus* strain *M. fulvus* HW-1 (64) as an outgroup. Bootstrap values supporting branch inferences are shown.

Phage inactivation is partially caused by diffusible public goods

Reductions of viable phage numbers upon interaction with bacteria might result from diverse mechanisms. These include intracellular mechanisms such as integration into host DNA to form prophage, prevention of phage genome replication or particle assembly, as well as extracellular mechanisms such as diffusible or cell-surface-bound compounds that alter the phage in a manner preventing successful growth on strain DZ1. We began investigating these alternatives by testing whether diffusible compounds secreted by the phage-antagonistic *M. xanthus* strains might be partly or fully responsible for the observed reductions in phage numbers.

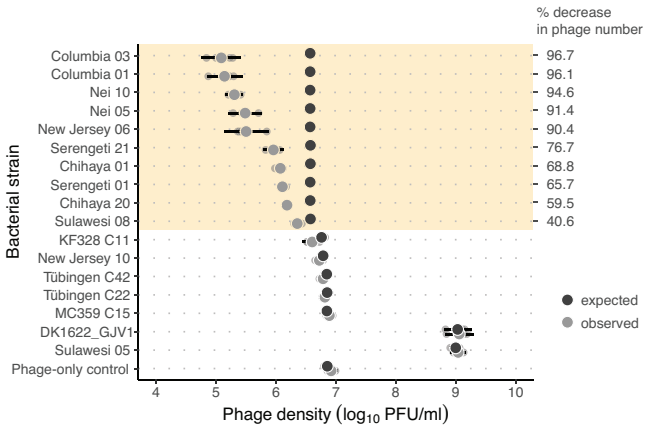

Figure 2. Antagonism of phage is mediated predominantly by diffusible bacterial secretions. Phage density after 24 h exposure to supernatant extracted from cultures in which bacteria and phage interacted over the previous 24 h. Expected numbers represent the sum of PFU counts immediately after 24 h of stage 1 bacteria-phage interaction (Fig. S1) and the number of phage particles added at the start of experiment stage 2 (to determine the effect of supernatant on newly added phage, Fig. S1). Observed numbers are PFU counts after incubation with supernatant at the end of stage 2. Means of $\log_{10}$-transformed PFU values and 95% confidence intervals are shown. Light gray dots represent biological replicates ($n = 4$). Percentage values indicate what proportion of decreases in phage number caused by *M. xanthus* shown in Fig. 1 can be attributed to diffusible supernatant compounds based on this assay. Significant differences between observed and expected phage numbers are indicated by colored background (one-way ANOVA followed by two-sample paired *t* tests with Benjamini & Hochberg correction, $p < 0.05$).

After exposing bacterial cells to phage (or to media only in no-phage controls) in stage 1 of this experiment (Fig. S1), cell-free supernatant (or media only in no-bacteria controls) was harvested and tested for potential effects on phage viability after new phage were added at the start of stage 2. We calculated observed and expected phage numbers for each bacterial interaction treatment. The expected number in each case was the sum of the number of viable phages remaining in the supernatant at the end of stage 1 and the number of new phages added at the start of stage 2. The observed number was the count of viable phages remaining after exposure to the harvested supernatant during stage 2. The difference between observed and expected phage counts varied as a function of bacterial-strain identity (ANOVA, $F_{17,54} = 101.63$, $p < 0.001$ followed by posthoc contrasts).

Supernatants from the same ten strains that antagonized phage when they could interact directly with bacterial cells (Fig. 1) were found to reduce viable phage counts below expected values (Fig. 2, one-sided paired *t*-tests with Benjamini-Hochberg

correction, $p < 0.05$). This outcome indicates that diffusible secreted compounds are at least partially responsible for the anti-phage antagonisms observed in our original experiments. Among those ten strains, the diffusible phage-antagonistic compounds present in supernatant accounted for 40-96% of the total antagonistic effects of these strains observed in our original experiment (Fig. 2).

Anti-phage compounds are expressed both constitutively and facultatively

Secretion of phage-inactivating compounds might be either constitutive or rather facultatively expressed upon interaction with phages. To examine these hypotheses, we compared effects of supernatants of bacteria that either had or had not previously interacted with Mx1 phage (experiment stage 1, Fig. S1) on the survival of phage freshly exposed to the supernatants (experiment stage 2, Fig. S1).

In one treatment of this experiment, the ten bacterial strains that significantly reduced viable phage numbers in the Fig. 2 experiment were infected with phage during stage 1 and culture supernatant was harvested after bacteria-phage interaction. As in the Fig. 2 experiment, a known number of new phage particles was then exposed to the harvested supernatant in stage 2. Nine of the ten strains again significantly reduced viable phage counts (Fig. 3, one-sided paired *t*-tests with Benjamini and Hochberg correction, $p < 0.05$), the sole exception being Sulawesi 08, which was therefore excluded from additional analysis in this experiment.

In a second treatment, the bacteria were not exposed to Mx1 phage in stage 1; harvested supernatant therefore contained only diffusible compounds secreted in the absence of phage. Of the nine strains still under consideration, supernatant from all but one (Chihaya 20) caused a decline in phage number (one-sided paired *t*-test with Benjamini and Hochberg correction, $p < 0.05$), indicating that they produce compounds harmful to phage even in the absence of the viruses.

However, for several bacterial strains, the degree of phage inactivation by supernatant was found to differ between supernatant from phage-exposed *vs* non-phage-exposed bacteria. Specifically, the supernatants of strains Columbia 01, Columbia 03, Nei 05 and Nei 10 showed greater negative effects on phage when the bacteria had been previously exposed to phage than when they had not (Fig. 3, one-sided paired *t*-tests with Benjamini and Hochberg correction, $p < 0.05$); a difference for strain Chihaya 20 was nearly

significant as well (p = 0.056). No effect of bacteria-phage interaction on supernatant potency was detected for the remaining four strains.

These results indicate that some *M. xanthus* isolates secrete some anti-phage compounds constitutively while secreting other compounds only facultatively in response to interacting with phage, whereas other phage-antagonistic isolates appear to secrete their antagonistic compounds only constitutively. Thus, distinct *M. xanthus* strains appear to differ qualitatively in the behavioral modes by which they neutralize phage threats.

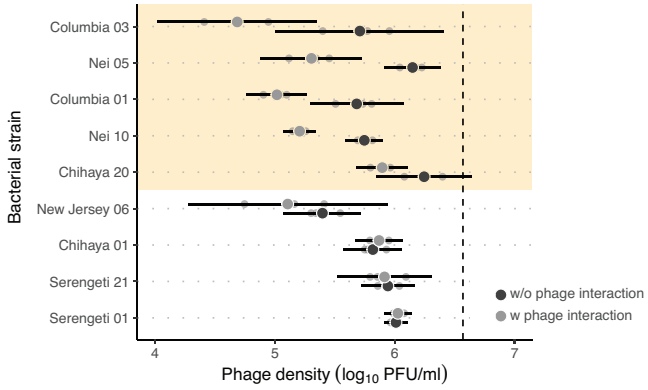

Figure 3. Secretion of phage-inactivating compounds by *M. xanthus* is often a facultative response to interaction with phage. Mean values of $\log_{10}$-transformed PFU counts for Mx1 phage exposed to supernatant from cultures containing both bacteria and phage (gray) or from cultures with bacteria only (dark gray). Error bars represent 95% confidence intervals. Light gray dots indicate biological replicates (n = 3). The dashed line indicates the number of phages initially added to the supernatant. Colored shading indicates significant differences between treatments with bacteria+phage supernatant and bacteria-only supernatant (one-way ANOVA followed by two-sample paired t tests with Benjamini & Hochberg correction, $p < 0.05$).

Diffusible anti-phage compounds are heat sensitive

If the diffusible antagonistic compounds revealed by our experiments are proteinaceous (or require polypeptides to function), heat treatment should inactivate those compounds and eliminate the antagonistic effects. To test this hypothesis, we heated supernatants at 45, 55, 65 and 75 °C before exposing a known number of added phage particles (Fig S2). Exposure to 55, 65 and 75 °C eliminated negative effects of supernatant on phage (Fig. S3, one-sided paired t-tests, $p > 0.05$), whereas in most cases exposure to 45 °C did not, suggesting that proteinaceous factors are necessary for the observed phage inactivation.

The robustness of phage surviving antagonistic supernatant depends on the source bacterial genotype

Our experiment testing for heat inactivation of phage-antagonistic compounds in supernatant revealed an unexpected result from the 65 °C treatment, in which antagonistic activity of supernatant compounds was eliminated by the heat treatment. Comparison of plaque forming unit (PFU) values immediately before and after heat treatment of supernatant from cultures that included both bacteria and phage revealed a significant decline in phage number in supernatants from 13 strains (Fig. 4, one-sided paired *t*-tests with Benjamini-Hochberg correction, $p < 0.05$). The observation that phage-antagonistic compounds in supernatant are fully deactivated by 65 °C heat (Fig. S3) suggests that these decreases in viable phage counts were due to the heat treatment. In contrast, in the phage-only control in which liquid was harvested from phage suspensions lacking bacteria, viable phage counts did not decrease upon exposure to 65 °C, indicating that Mx1-particle viability is not intrinsically sensitive to this level of heat stress. These results thus suggest that for phage that survived interacting with bacterial cells and diffusible compounds produced by cells prior to heat treatment, some of those interactions reduced the tolerance of the surviving phage particles to subsequent heat stress.

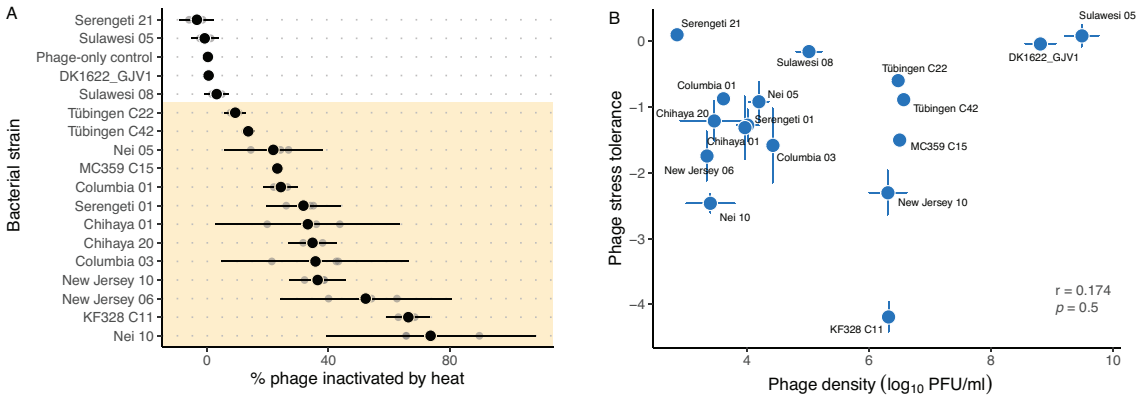

Figure 4. Interaction with bacteria reduces the heat-tolerance of phage to varying degrees as a function of bacterial genotype. A) Percentage of previously bacteria-exposed phage inactivated by exposure to 65 °C heat stress. Mean values and 95% confidence intervals are shown. Light gray dots indicate replicate values ($n = 3$). Colored shading highlights significant differences between phage PFU before *vs* after heat exposure (one-way ANOVA followed by two-sample paired *t* tests with Benjamini & Hochberg correction, $p < 0.05$). B) Heat-stress tolerance does not correlate with phage density after interaction with bacteria and prior to the onset of heat stress. Mean values ± SME of $\log_{10}$-transformed PFU counts (Spearman correlation).

Such effects were not identical across bacterial strains. Four bacterial strains had no effect on post-interaction phage tolerance of heat stress (GJV1, Serengeti 21, Sulawesi 05 and Sulawesi 08). However, only two of those four strains (GJV1 and Sulawesi 05) were among the seven strains that failed to reduce viable phage population size in the Fig. 1 experiment. In contrast, Serengeti 21 and Sulawesi 08 both antagonized Mx1 directly by reducing particle counts in the Fig. 1 experiment but failed to negatively impact the heat-stress tolerance of those phage that did survive interacting with them.

Intriguingly, five *M. xanthus* strains that did not reduce viable Mx1 counts in our first experiment nonetheless reduced the heat tolerance of phage particles that survived interacting with them (Fig. 1; New Jersey 10, KF328c11, Tübingen C22, Tübingen C24 and MC359c15). For example, strain KF328c11 had no detectable effect on phage numbers immediately after Mx1 had been exposed to this strain, but phage populations that had interacted with this strain were reduced by more than 70% upon exposure to 65 °C heat stress. Thus, some *M. xanthus* strains harm Mx1 latently, reducing their future resilience under stress without immediately inactivating them.

Because viable phage counts varied at the start of our heat treatment (due to variable effects of different bacterial genotypes), we tested whether viable-phage density at the start of the 65 °C heat treatment (the last step of stage 1 of this experiment, Fig. S2) might have impacted the results shown in Fig. 4A. However, no correlation between stress tolerance and phage number immediately prior to heat treatment was detected (Pearson's correlation $r_s = 0.174$, $n = 17$, $p = 0.518$). Thus, the observed variation in heat-stress tolerance of Mx1 phage is caused by different effects of the distinct *M. xanthus* genotypes to which Mx1 was exposed. Bacteria had no effect on phage survival at lower temperatures of 45 and 55 °C (Fig. S4, one-sided paired *t*-test with Benjamini-Hochberg correction, $p > 0.05$).

We further tested whether bacterial-strain effects on Mx1 heat-stress tolerance depend on direct interaction between bacterial cells and phage or might rather be caused by diffusible compounds in supernatant derived from bacterial cultures that had not been exposed to phage (Fig. S2). We found that for almost all strains, supernatant from phage-free cultures reduced phage stress tolerance as much as did exposing phage to cultures still containing bacterial cells (Fig S5, two-sided paired *t*-tests with Benjamini-Hochberg correction, $p > 0.055$). This indicates both that i) the compounds that reduce phage heat-stress tolerance are generally diffusible secretions and ii) these compounds are generally

secreted constitutively in the absence of phage. This result further suggests that the compounds that inactivate phage upon bacteria-phage interaction, which in several cases are produced by bacteria facultatively in response to interaction with phage (Fig. 3), are often distinct from the compounds that reduce the heat-stress tolerance of phage. For example, interaction with Mx1 causes several strains (Colombia 01, Colombia 03, Nei 05, Nei 10, and Chihaya 20) to facultatively secrete (at least) one compound that directly inactivates many phage particles (Fig. 3), but in the absence of interaction with Mx1, these same strains also constitutively secrete at least one different compound that reduces Mx1 heat-stress tolerance (Fig. S5). Thus, individual *M. xanthus* strains appear to often produce multiple distinct compounds that exert different antagonistic effects on phage.

Anti-myxophage compounds do not exert generic anti-phage effects

Finally, we began exploring what range of phage the anti-myxophage compounds secreted by several *M. xanthus* strains might antagonize. To do so, we tested whether supernatants from five *M. xanthus* genotypes co-cultivated with Mx1 significantly reduce viable populations of the *Escherichia coli* phage T4 (39) (another tailed, myoviridae phage) or the *Pseudomonas aeruginosa* phage DMS3vir (40) capable of infecting host strains of those species. In only one supernatant-phage pairing (DMS3vir with Nei 10 supernatant) were phage counts significantly reduced by exposure to *M. xanthus* supernatant (Fig. S6, posthoc Dunnett contrasts, $p < 0.001$). Given the structural similarities of T4 and Mx1, both of which are tailed myoviridae, our results indicate that *M. xanthus* APPGs have narrow target ranges; their activities even might be largely specific to phage that can infect myxobacteria (or a subset thereof).

Discussion

The persistent threat of death from virulent phage has inevitably selected for bacterial defenses (13). Previously known mechanisms that hinder phage infection exert their anti-phage effects within or on the bacterial cell (17, 44–46). We have shown that many *M. xanthus* isolates secrete diffusible public goods that inactivate, and in some cases latently injure, lytic phage extracellularly, before entry into the bacterial cell. In doing so, these myxobacteria greatly reduce extracellular viable phage population sizes. Strains that

inactivated phage did so most effectively when direct cell-phage contact was possible, implicating cell-associated factors, but most of these strains also secreted APPGs. *M. xanthus* strains varied greatly in their anti-phage effects, including in the magnitude, production mode and character of those effects, implicating differences in the detailed mechanisms employed. APPGs were produced only constitutively by some strains, but in others were secreted facultatively in response to bacterial interaction with phage. Some strains only produced APPGs that immediately inactivate phage, while others only produced APPGs that latently injure the phage, compromising their ability to survive future environmental stress; yet other strains produced both categories of compounds.

The APPGs produced by *M. xanthus* appear to be polypeptides (or require polypeptides to function) because exposure to 55 °C heat eliminated all anti-phage activity of bacterial supernatants. These APPGs thus differ in molecular character from non-proteinaceous APPGs produced by some Streptomycetes, namely aminoglycoside and anthracyclines antibiotics (24). Such APPG polypeptides might be secreted directly into extracellular space from the cell membrane. Alternatively, secreted outer-membrane vesicles (OMVs) might be involved (47). OMVs can mediate interspecific killing (48) and have been hypothesized to play roles in cell-cell communication (49) and predation (50). In some species, OMVs have been found to have act as phage traps, with phage attaching to receptor proteins on the OMV surface and introducing their DNA into OMVs rather than cells and thus failing to replicate (51, 52). OMVs secreted by myxobacteria might serve a similar role and/or might bear polypeptides that directly inactivate phage particles upon contact.

Multifunctionality. Diffusible bacterial secretions serve highly diverse functions; they can mediate cooperative interactions such as communicating information about cell density or nutritional status (53) or mediate antagonisms such as killing competitors (54) or prey (55). We have found that bacterial secretions can also act extracellularly as anti-phage agents. One possible benefit of using public goods as anti-phage agents is multi-functionality; some diffusible secretions might mitigate phage threats while also serving some other function. For example, aminoglycoside and anthracycline antibiotics produced by some Streptomycetes have the potential to both kill competitor bacteria extracellularly and, as DNA-intercalating agents, intracellularly prevent replication of injected phage DNA. Once future work has defined the molecular identity of

myxobacterial APPGs, it will be of interest to test whether they serve additional functions, for example in predation or conspecific interference competition.

The hypothesis of phage-targeted adaptation. In evolutionary biology, high degrees of trait-function specificity and trait complexity are often considered to strengthen arguments that a given trait evolved as a particular adaptation (56). For example, the high complexity and specificity of CRISPR-Cas systems strongly suggest that they evolved primarily as adaptations for defense against phage. But there are often reasons why a given trait effect may not have evolved as an adaptation *per se*. For example, the multiple antagonistic effects of aminoglycoside and anthracycline antibiotics hinder clarity regarding their primary adaptive benefits. Their antagonism on some phage may have been a major selective contributor to their initial evolution and subsequent maintenance, but also might be largely a mechanistic byproduct of selection for their anti-bacterial effects. Contributing to this ambiguity, production of anthracycline or aminoglycoside antibiotics has not been shown (to our knowledge) to protect producing strains from phage capable of introducing DNA into producer cells or their conspecifics. Effects against phage with host ranges relevant to antibiotic-producing strains is a prerequisite for inferring that the anti-phage effects of these antibiotics are adaptations in their own right.

Our demonstration that many *M. xanthus* genotypes secrete public goods that inactivate a phage type – Mx1 – capable of killing producer conspecifics promotes the basic plausibility of the hypothesis that the anti-phage effects of these APPGs are adaptations *per se*. The additional demonstration of facultative specificity, namely that many of these strains secrete some APPGs only after direct exposure to Mx1, further strengthens the argument for this hypothesis. In turn, this also increases the plausibility of the hypothesis that the anti-phage effects of cell-associated (*i.e.* non-diffused) compounds (Figs. 1 and 2) and of APPGs produced constitutively (Fig. 3) are adaptive *per se*.

Susceptible-phage ranges. Once the molecular identities of *M. xanthus* APPGs are known, defining the ranges of phage genotypes that they each can inactivate or latently injure – their susceptible-phage ranges – will also be of interest. These ranges, together with the host-ranges of targeted phage, have large implications for understanding the costs and benefits of APPG production across variable community compositions. For example, the sociobiological and community-ecology implications of APPG production

will differ greatly between broad (potentially including inter-species effects (24)) *vs* narrow susceptible-phage ranges. We have shown that the anti-myxophage compounds secreted by several *M. xanthus* strains do not generically harm all bacteriophages, *e.g.* phages of *E. coli* and *P. aeruginosa* (Fig. S6); defining their susceptible-phage ranges more precisely requires further study. Investigation of the mechanistic bases of variation in susceptibility to APPGs should improve understanding of functionally important differences in phage-particle composition and form across phage types.

Conspecific diversity in anti-phage mechanisms. As noted previously, *M. xanthus* strains vary greatly in their anti-phage effects, including in the magnitude (Figs. 1-3), production mode (Fig. 3) and character (Fig. 1 *vs* Fig. 4) of those effects. Differences in the magnitude of phage inactivation might be explained by either differences in the molecular character of anti-phage compounds produced by different strains (including possibly cocktails of multiple compounds by individual strains) or differences in production levels of the same compound(s). Similarly, facultative inactivation of phage in response to exposure to Mx1 might result from either increased production of anti-phage compounds already produced constitutively at a lower level or from production of compounds only secreted at all upon interaction with the phage.

Fitness implications of latent injury. Animal injury from physical violence between conspecifics or between predators and prey is common and can reduce the future fitness of injured parties (57). Our experiments reveal that, in addition to extracellularly inactivating phage with public goods, some bacteria can latently injure phage particles without immediately inactivating them. Supernatants from several *M. xanthus* strains reduced the physical robustness of Mx1 phage that survived supernatant exposure, reducing their ability to survive future heat stress. This suggests that phage in natural populations that survive encounters with myxobacterial cells or their secretions may often be compromised in their ability to survive a variety of future environmental stresses.

Our results reveal a previously unknown category of bacterial defense against phage, namely compounds that actively harm phage particles prior to introduction of genetic material into the cell, rather than prevention of absorption or prevention of phage-DNA/RNA replication after successful entry. While UV radiation (58) and other abiotic factors (59) are known to physically weaken phage particles, less is known about compounds of biotic origin that do so. Future characterization of how *M. xanthus* APPGs physically debilitate and/or latently injure phage is thus of interest. The observations that

some strains only inactivate Mx1, others only latently injure the phage, and yet others do both suggest that, at least in some cases, inactivation and latent injury are mediated by distinct compounds.

Social and community implications. Our findings raise intriguing questions regarding the relative costs and benefits of distinct anti-phage defense mechanisms, played out over multiple biological scales, as well as their relative contributions to shaping phage host ranges. All effective defense mechanisms deployed by any cell confer social benefits to other cells simply by reducing phage reproduction and thus the likelihood that others will be infected. Other cost/benefit features, however, differ across defense categories. Bacteria that undergo apoptosis in response to phage infection gain no self-benefit at the cellular level (even if there is a self-benefit at the genetic level) (60, 61). Cell-internal defenses such as CRISPR-Cas systems that allow potential survival of infected cells can provide a cellular-level self-benefit, but at the risk of death if phage circumvent the internal defense (62). Prevention of phage absorption without other harm to phage particles confers the self-benefit of protecting potential victim cells from infection, but leaves unabsorbed phage at large to potentially infect other cells.

By reducing external viable-phage population sizes, however, extracellular inactivation of phage particles potentially benefits both APPG producers and other cells within relevant proximity that might otherwise be susceptible. Future research might investigate the relative effectiveness of extracellularly-acting APPGs *vs* other defense strategies at limiting phage epidemics across distinct ecological conditions. The sociobiological effects and evolutionary fate of any given form of APPG production will be determined by the relative degrees to which producer cells protect themselves and fellow producers *vs* non-producers from being harmed by phage. Production of APPGs is thus a social behavior that should be incorporated into models of multi-trait social evolution (63), as well as models of microbial-community interaction networks.

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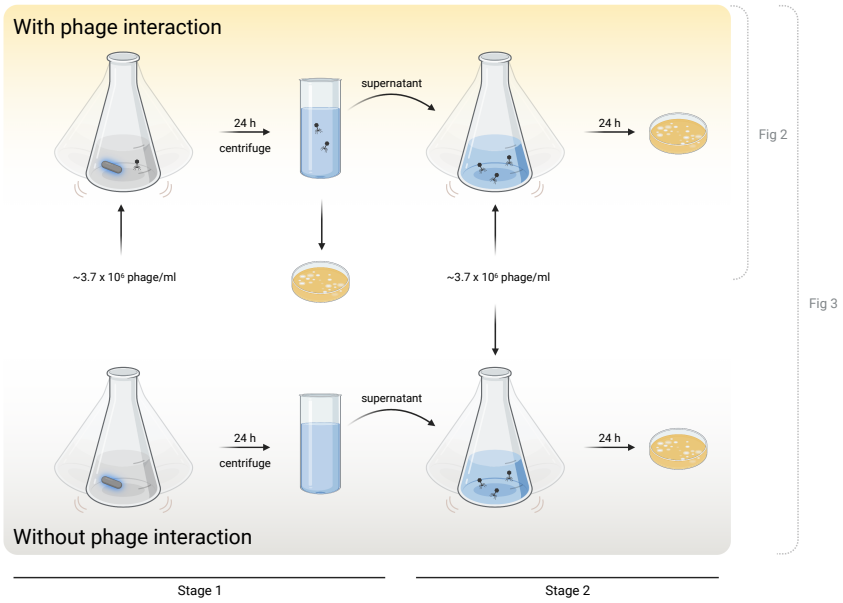
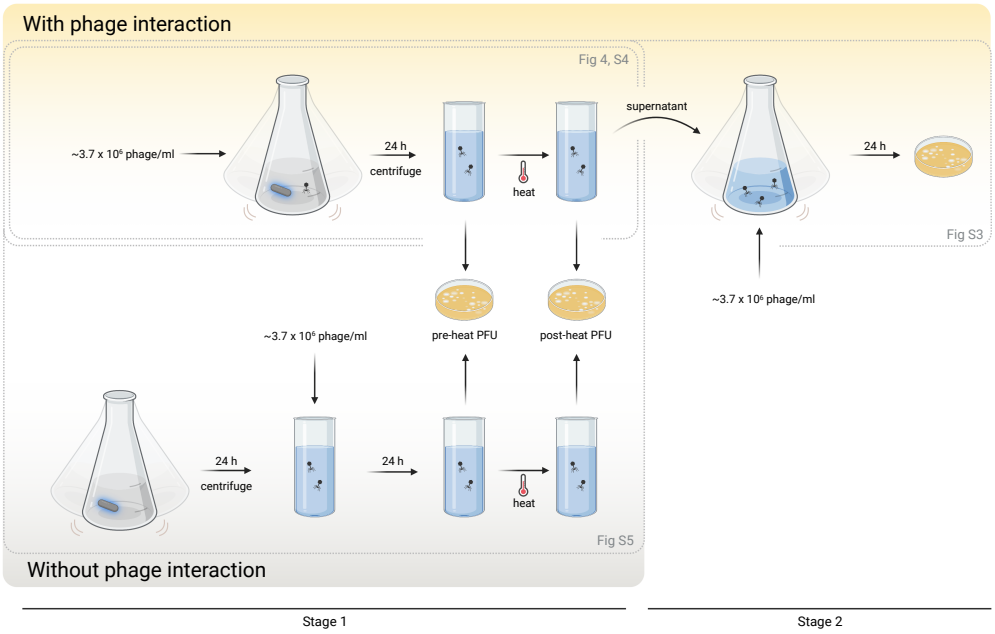
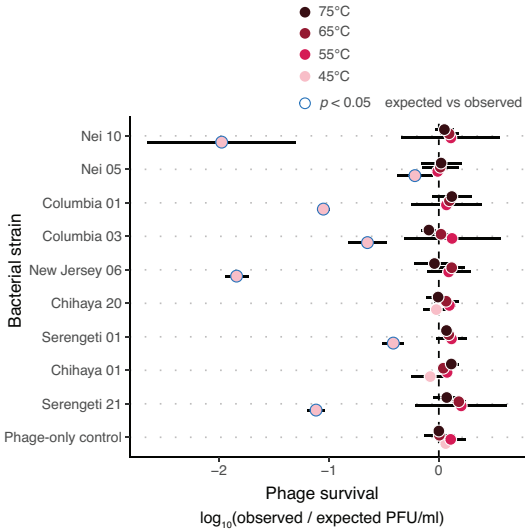
Supplementary Figures



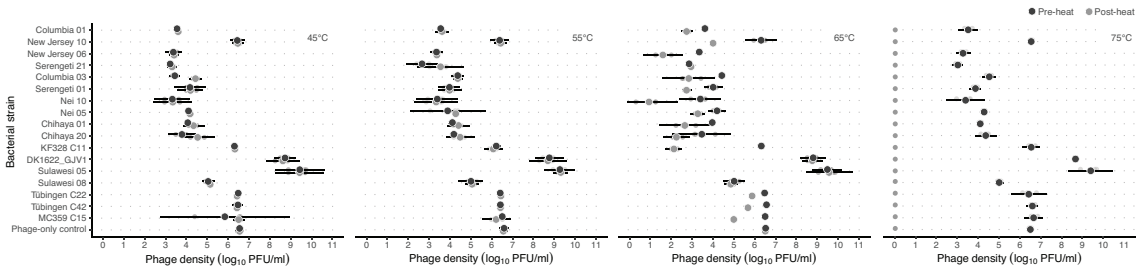
Supplementary figure 1. Design of experiments for Figs. 2 and 3. The top part of the diagram depicts the treatment in which direct contact between bacteria and phage was possible. Mx1 and *M. xanthus* natural isolates were mixed in co-culture to initiate stage 1 of this treatment. After 24 h of incubation, cultures were centrifuged and viable-phage population sizes in supernatants were determined by dilution plating. To initiate stage 2, new phage particles were exposed to supernatant, effects of which on viable-phage population size were determined by dilution plating after another 24 h of incubation. The bottom part of the diagram shows the treatment in which direct contact between bacteria and phage was prevented. In this treatment, Mx1 was exposed to supernatants from phage-free bacterial cultures. Phage-only controls were also run for both the top and bottom protocols. Created with BioRender.com.



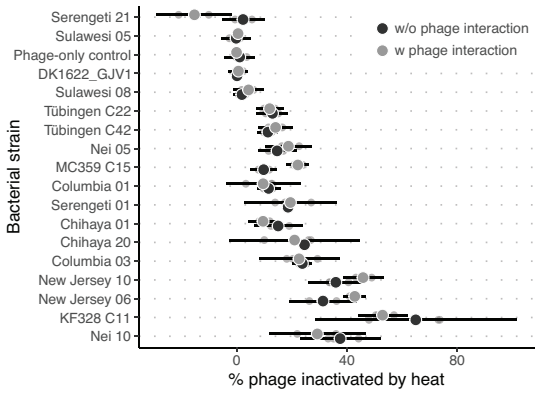
Supplementary figure 2. Design of experiments for Fig. 4 and supplementary figures 3-5. The top part of the diagram depicts the treatment in which direct contact between bacteria and phage was possible; Mx1 and *M. xanthus* natural isolates were mixed in co-culture to initiate stage 1 of this treatment. After 24 h of incubation, cultures were centrifuged and viable-phage population sizes (pre-heat PFU counts) were determined by dilution plating, after which the phage suspensions were heated at 45, 55, 65 or 75 °C and viable-phage population counts were again assessed (post-heat PFU counts). To initiate stage 2, new phage particles were added to supernatant, effects of which on viable-phage population size were determined by dilution plating after another 24 h of incubation. The bottom part of the diagram shows the treatment in which direct contact between bacteria and phage was prevented. This part of the experiment was performed for stage 1 only. The supernatant of bacterial cells was harvested after 24 h of bacterial growth, after which phage were added and the suspensions then underwent the same protocol as in the top panel. Phage-only controls were also run for both the top and bottom protocols. Created with BioRender.com.



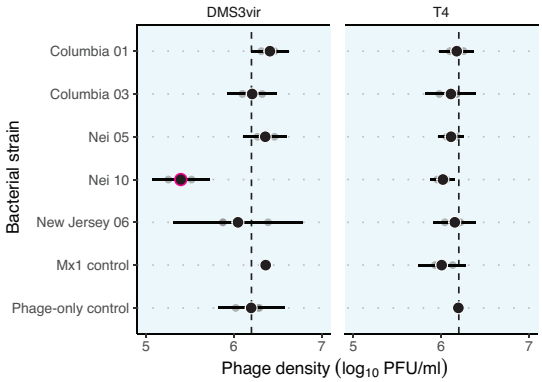
Supplementary figure 3. Heat deactivates diffusible phage-deactivating compounds produced by *M. xanthus*. 24h after phage exposure to nine bacterial strains that reduce viable phage population size, cell-free supernatant was treated at different temperatures and subsequently new phage particles were added to test for and quantify effects of heat-treated supernatant on viable phage population size. Plotted are mean values of the difference between expected and observed $\log_{10}$-transformed PFU counts. Expected numbers represent the sum of PFU counts in cell-free supernatant at the end of stage 1 of the experiment (see Fig. S2) and the number of phage particles added at the start of stage 2. Observed numbers represent PFU counts after incubation with heat-treated supernatants at the end of stage 2. Colored dots indicate means at different temperatures. Error bars represent 95% confidence intervals. Significant differences between expected and observed phage numbers are highlighted by blue circles around dots (one-way ANOVA followed by two-sample paired *t* tests with Benjamini & Hochberg correction, $p < 0.05$). Observed numbers of each phage control replicate were used as expected numbers in the 75 °C treatment, since the number of phage that were added in stage 2 were lower than 3.7×10^6 phage particles ml^{-1}.



Supplementary figure 4. Differential stress tolerance of Mx1 as a function of bacterial-genotype interaction history is evident only under 65°C stress. Log_{10}-transformed PFU counts pre- and post-heat exposure (light gray and black, respectively) after coculture with different *M. xanthus* strains. Pre- and post-heat PFU counts do not significantly differ for phage treated with 45 or 55 °C (one-way ANOVA, $p > 0.05$) but do differ after 65 °C treatment (one-way ANOVA, $p < 0.05$). When the temperature is raised to 75 °C, all phage particles are inactivated irrespective of their interaction history. Light gray circles represent single replicate values ($n = 3$) and error bars are 95% confidence intervals. The lower error bar of Nei 10 in the 65°C treatment graph expands into the negative range and was shortened for visual purposes.



Supplementary figure 5. *M. xanthus* secretion of diffusible compounds that reduce Mx1 heat-stress tolerance is independent of exposure to phage. Percentage-means of phage particles inactivated at 65 °C after exposure to supernatant derived from bacteria previously exposed to phage (gray dots) or not (dark gray dots). 95% confidence intervals are shown. Small light gray circles indicate individual replicate values (n = 3). No significant effects of prior exposure to phage were detected (one-way ANOVA followed by two-sample paired t tests with Benjamini & Hochberg correction, $p > 0.05$).



Supplementary figure 6. Supernatants from *M. xanthus* isolates co-cultured with Mx1 generally fail to harm the *E. coli* phage T4 or the *P. aeruginosa* phage DMS3vir. Right and left panels show PFU counts for the phages T4 and DMS3vir, respectively, on their respective indicator strains after exposure to supernatant from five *M. xanthus* strains co-cultured with Mx1 or from an Mx1-only control. The phage control shows the respective number of T4 or DMS3vir after 24 h incubation with no exposure to supernatant. The dashed vertical line shows the number of T4 or DMS3vir particles added. Red circle indicates treatment where phage count was significantly reduced by exposure to Mx1 treated supernatant (Dunnett contrasts, $p < 0.001$). Error bars are 95% confidence intervals.

Outlook

Adaptation as a response to changing environments and alternating biological interactions lays the foundation for success and survival of all organisms. Understanding the path that a species follows over time as it evolves and adapts to its environment is of extraordinary importance to understand the history, diversity and functioning of life on earth. Such evolutionary trajectory can be viewed as patterns of change in traits over time and can be influenced by internal as well as external factors. For example, organisms may develop towards increased size or complexity (Szathmáry and Smith, 1995), or towards greater specialization (Grant and Grant, 1996) as a response to a particular environment. Gaining more and more knowledge to understand evolution in an ecological context will help us getting insight into the past and allow us to make predictions about the future of species and ecosystems (Thompson, 1999), it will advance our understanding in the spread of diseases or the development of antibiotic resistance (Davies and Davies, 2010; Funk *et al.*, 2010) and will help us understand the impacts of human activities on the ecosystems (Karl and Trenberth, 2003).

Experimental evolution is a widely used tool for evolutionary biologists to simplify complex biological systems and study the mechanisms of evolution and how organisms respond to changes in the environment in a controlled setting. Especially bacterial systems are frequently used to study evolutionary questions because of their fast reproductive cycle, the ease with which they can be handled, and asexual reproduction (McDonald, 2019). Experimental evolution with bacterial model systems have given us insight in the emergence of multicellular structures from single cells (Ratcliff *et al.*, 2012), showed differences in adaptation processes when evolving in one environment during long term evolution (Lenski, 2017; Lenski *et al.*, 1991), or coevolution experiments between bacteria and phage that showed how both partners evolve in response to each other over time (Brockhurst and Koskella, 2013; Buckling and Rainey, 2002; Scanlan *et al.*, 2015).

The first chapter of this thesis focused on the question **if limited migration can contribute to a reduction in within-group conflict** among genetically diverse microbial populations, like we see it in higher organisms like animal species. Using experimental evolution, we could show that groups of socially living bacteria like *M. xanthus* benefit

from group structures where individuals stay together, even if the groups are internally diverse. A lower overall group performance and negative within-group interactions were mainly observed when groups of individuals were randomly mixed. Maintenance of within-group diversity through limited between group migration resulting in reduced levels of competition between clones of a group, is shedding new light on the drivers on the evolution of diversity in bacteria. Bacterial model systems with a social lifecycle like *M. xanthus* allows us to further address questions like ecological factors required for the emergence of diversity within groups, the requirements for the evolution of division of labor in microbial species or task specialization of groups of cells.

The second chapter of the thesis dealt with the **importance of latent effects of evolution on evolutionary diversification**. Through *M. xanthus* populations that were previously evolved in different environments, we showed that irrespective of no phage encounter during evolution, the subsequent phage interaction was highly diverse in terms of being a good quality host for the phage. Latent phenotypic diversification highlights an additional level of complexity then thinking about diversification of host-parasite interactions, that are frequently studied with focus on direct selection pressure (Brockhurst *et al.*, 2003; Buckling and Rainey, 2002). Incorporating the concept of latent phenotype evolution more broadly, for example when thinking about antibiotic resistance evolution or effects of climate change, which can help broaden our understanding and the wide-ranging consequences of other mechanisms than direct selection.

The third chapter circles around the phenomenon of **extracellular mechanisms of phage defense**. We could show that *M. xanthus* does not only fight the threat of phage attack through intracellular mechanisms after the phage genome has already entered the cell, but that a large variety of tested strains utilized anti-phage defense that decreased the number of viable phage particles in the surrounding of the bacterial cells through secretions. Such extracellular phage antagonism results in group-level consequences that potentially act at a much larger community scale by reducing the external viable phage number and therefore protecting from phage attack.

Biological systems are extraordinarily complex. We are just beginning to fully understand their complexity and further researching the interplay between evolution and the ecology of such systems will provide us with a more comprehensive understanding.

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CURRICULUM VITAE

Lisa Freund, born March 30th, 1990

EDUCATION

2017 – 2023	**PhD in Evolutionary Biology** with Prof. Dr. Gregory J. Velicer ETH Zürich, Switzerland
2014 – 2017	**MSc in Microbiology** Friedrich-Schiller University Jena, Germany
2010 – 2014	**BSc in Biology** area of specialization: Microbiology Friedrich-Schiller University Jena, Germany

RESEARCH / TEACHING EXPERINCE

2020 – 2022	**Supervisor Bachelor/Master students**, ETH Zürich Supervised one bachelor thesis and one semester project (master level) Supervised two term paper students (master level, environmental systems science) Tutor for environmental biology seminar students (bachelor level)
2019	**Course development** for Life Science Zürich Learning Center Zürich Course development for hands-on life science course for school classes *"Antibiotic resistance and its development"*
2019 and 2022	**Teaching assistant**, ETH Zürich Lab assistant and supervisor (master level course *Antibiotic resistance in soil microbial communities*)
06/2014 – 10/2015	**Research assistant**, Max Planck Institute for Chemical Ecology Jena/Germany Experimental Ecology and Evolution research group Prof. Dr. Christian Kost
03/2013 – 08/2013	**Internship**, Max Planck Institute for Chemical Ecology Jena/Germany Experimental Ecology and Evolution research group Prof. Dr. Christian Kost

PUBLICATIONS

L. Freund, S. Pande, M. Vasse and G.J. Velicer. Repeated intergroup mixing results in the loss of within-group synergy *[in prep]*

L. Freund*, M. Vasse* and G.J. Velicer. Remote extracellular attacks on bacteriophage. *co-first authors, *[in prep]*

L. Freund[#], M. Vasse and G.J. Velicer (2021) Hidden paths to endless forms most wonderful: Parasite-blind diversification of host quality. *Proceedings of the Royal Society B* 288, 20210456 [#]corresponding author

S. Pande*, S. Shitut*, **L. Freund***, M. Westermann, F. Bertels, C. Colesie, I. B. Bischofs and C. Kost. (2015) Metabolic cross-feeding via inter-cellular nanotubes among bacteria. *Nature communications* 6:6238 *co-first authors

CONFERENCE CONTRIBUTIONS

2022 18th International Symposium on Microbial Ecology (ISME 2022)
Poster presentation: *Phage wars the bacteria strike back. Anti-phage activities of M. xanthus*.

2021 47th International Meeting on the Biology of the Myxobacteria (virtual)
Oral presentation: *Phage wars the bacteria strike back. Anti-phage activities of M. xanthus*.

2019 25th European Meeting for PhD Students in Evolutionary Biology (EMPSEB 2019, Portugal)
Oral presentation: *Limited migration selects for social synergy in a cooperative microbe*.

2018 2nd Joint Congress on Evolutionary Biology (Montpellier, France)
Poster presentation: *Limited migration among bacterial social groups reduces within-group antagonism*.

2016 43rd International Meeting on the Biology of the Myxobacteria (Interlaken, Switzerland)
Oral presentation: *Effect of inter-group migration on within and between group variation in Myxococcus xanthus*.