The evolution of metabolic cooperation in bacterial communities – causes and consequences

Dissertation

To Fulfill

the Requirements for the Degree of

„doctor rerum naturalium“ (Dr. rer. nat.)

Submitted to the Council of the Faculty of Biological Sciences

of the Friedrich Schiller University Jena

by M.Sc. Daniel Preussger

born on March 7th, 1987 in Löbau (Sachsen)

Gutachter:

1) Prof. Dr. Christian Kost, Abteilung für Ökologie,
Universität Osnabrück

2) Prof. Dr. Erika Kothe, Institut für Mikrobiologie,
Friedrich-Schiller-Universität Jena

3) Kristina Hillesland PhD, Faculty of Biology,
University of Washington

Datum der Verteidigung:

28.02.2020

Table of Contents

Summary

Cooperative interactions within bacterial communities are widespread and important for their survival. In these often obligate interactions, primary metabolites are frequently exchanged. However, evolutionary theory predicts selfish individuals to have an advantage over cooperative ones by maximizing their own fitness instead of directing costly resources to others. Given that cooperation is widespread in nature despite this problem; certain mechanisms have to exist that explain the emergence and maintenance of cooperative metabolite exchange. In fact, fitness of cooperators that exchange metabolites was shown to be superior to an autonomous strategy provided that cooperators can preferentially interact with each other. However, the evolutionary factors favouring the emergence of cooperative cross-feeding as well as the consequences for the strains involved remain poorly understood. Further research therefore could add experimental evidence to ecological and evolutionary causes and consequences of cooperative cross-feeding. This thesis aims at investigating the causes and consequences of metabolic cooperation in bacteria. The findings are given as three major parts:

1. Comprehensive review of the literature regarding what is known to date about bacterial cross-feeding interactions, its origins, and the predicted consequences of metabolic cooperation in bacteria.
2. Identifying mechanisms that favour the evolution of metabolic cooperation.
3. Determining the consequences of synergistic coevolution on the genomic level.

1 Ecology and evolution of metabolic cross-feeding interactions in bacteria

A conceptual framework was developed that provides a general classification of the diverse kinds of metabolite exchange and aims at explaining evolution as well as maintenance of cooperative metabolite exchange. The framework represents a synthesis of empirical and theoretical studies covering the fields of genetics, microbiology, microbial ecology, systems biology, and evolutionary biology. From this resulted a complete evolutionary process that describes (i) the initial causes of metabolic interdependencies, (ii) the ecological and evolutionary mechanisms supporting cooperation to emerge from these interdependencies, and (iii) the theoretical consequences of synergistic coevolution that finally point towards the evolution of multicellularity. In detail reasons for adaptive gene loss are explained, which causes

metabolic interdependencies that hence could evolve towards cooperative cross-feeding provided that interdependent genotypes repeatedly interact for sufficient evolutionary time. Characteristics of bacteria were evaluated for according properties that facilitate metabolite exchange between interdependent genotypes as well as repeated interaction and thus help in evolving and maintaining metabolic cooperation. In this way certain bacterial lifestyles and molecular mechanisms facilitating association as well as competition were identified and linked to the current concepts of evolutionary theory that explain cooperation. Furthermore, the accumulated evidence on bacterial cross-feeding interactions was quantitatively analysed with regards to the identity of the interaction partners involved, the type of metabolites exchanged as well as the mode of metabolite exchange. Findings illustrate the diverse combinations of interacting partners and provide insight into the huge variety of metabolites that are exchanged.

2 Group-formation facilitates rapid evolution of cooperative cross-feeding in bacteria

Studying cross-feeding interactions within a bacterial community under natural conditions is challenging, due to a complex web of interactions between community members. In addition, the evolutionary ancestors from which the interaction evolved is required to quantify evolutionary consequences relative to it. Furthermore, it is difficult to distinguish between the varying selection- pressures that originate from complex webs of interactions within bacterial communities. Thus, it remains unclear, whether or not a genotype adapted to a cross-feeding interaction. Utilizing bacterial model systems under laboratory conditions provides a potent solution to overcome these problems. An obligate cross-feeding interaction was therefore established between Escherichia coli strains by deleting biosynthetic functions for amino acid production. The resulting strains lacked the ability to either produce tryptophan or tyrosine, and hence needed to exchange these amino acids when being cultivated together. Using cocultures of these auxotrophic mutants, an evolution experiment was performed. Additional experimental groups consisting of monocultures that lacked the obligate interaction served as control groups, representing adaptation to culture conditions and genetic manipulation (i.e. auxotrophy for amino acids). Rapid evolution of cooperative cross-feeding was observed within less than 150 generations in replicated populations of cocultured auxotrophs. Pervasive formation of multicellular clusters was identified as major strategy for amino acid exchange within cocultures and observed. Clusters of cells therefore represent the major sites of reproduction and exhibit a nascent lifecycle during which cells formed

multicellular clusters followed by disassociation into individual cells or smaller clusters that followed the experimentally imposed rhythm of serial propagation. By comparing these results to controls (i.e. amino acid-supplemented monocultures of auxotrophs), the observed cooperative exchange of amino acids as well as the strategy of growth within clusters was shown to be exclusively observed in the context of obligate cross-feeding. The success of cooperators within cocultures was linked to cooperators experiencing strong selective advantages within aggregates of cells. Positive fitness feedbacks within multicellular clusters between cooperators and non-cooperators were identified as the causative mechanism. Single cooperative cross-feeders were demonstrated to invade a population of non-cooperators (e.g. irrespective of the absence of a complementary cooperative partner) due to positive fitness feedbacks within multicellular clusters. Interestingly, when cultivated in isolation, phenotypes from cocultures showed a consistent decrease in growth relative to the ancestor. These findings indicate that mutations that increased fitness on the level of the multicellular cluster came at the cost of individual cells, thus suggesting fitness decoupling which is indicative of selection operating on the level of groups.

3 Synergistic coevolution accelerates the rate of molecular evolution

Ecological interactions are key drivers of evolutionary change. Even though it is well-documented that antagonistic coevolution can cause genetic divergence and accelerate molecular evolution, the evolutionary consequences of synergistic coevolution remain poorly understood. The observed evolution of cooperative cross-feeding from a by-product interaction raised the question of associated effects on numbers as well as spectra of accumulated mutations relative to the independent lifestyle of control groups. By utilizing comparative genomics based on sequencing whole populations and isolated clones, differences in numbers as well as spectrum of accumulated mutations between experimental groups were quantified. Strikingly, the results of this analysis indicate that also synergistic coevolution can speed up the rate of molecular evolution. Coevolution resulted in the emergence of metabolic cooperation that coincided with a significantly increased number of mutations in the genomes of coevolved auxotrophs as compared to genomes of control groups. Moreover, coevolved cooperative populations showed an increased degree of parallel evolution as well as divergent evolutionary trajectories relative to both control groups, suggesting restriction of adaptation to genetic modifications as well as abiotic environment. Together, these findings demonstrate that

similar to antagonistic interactions, also synergistic coevolution can cause rapid and divergent evolution that in the long-run may cause speciation driven by mutualistic interactions.

In summary, using experimental evolution and comparative genomics the causes and consequences of bacterial metabolic cooperation were investigated in this thesis. Presented findings potentially have far-reaching implications on the evolution of natural microbial communities that frequently exhibit obligate metabolite exchange as well as reproduction within multicellular structures. Findings therefore finally point towards multicellularity to potentially originate from metabolic interdependencies among bacteria.

Zusammenfassung

Kooperative Interaktionen innerhalb von Bakteriengemeinschaften sind weit verbreitet und entscheidend für deren Überleben. Bei diesen oft obligaten Interaktionen kommt es häufig zum Austausch von Primärmetaboliten. Die Evolutionstheorie jedoch prognostiziert, dass egoistische gegenüber kooperativen Individuen einen Vorteil haben, indem sie ihre eigene Fitness maximieren anstatt kostspielige Ressourcen an andere abzugeben. Dennoch sind vielseitige Kooperationen von Bakterien in der Natur bekannt. Folglich müssen bestimmte Mechanismen existieren, die die Entstehung und Aufrechterhaltung eines kooperativen Austausches von Metaboliten erklären.

Tatsächlich hat sich gezeigt, dass die Fitness von Kooperationspartnern, die Metabolite austauschen, einer autonomen Strategie überlegen ist, vorausgesetzt, die Kooperationspartner können bevorzugt miteinander interagieren. Die evolutionären Faktoren, die die Entstehung eines kooperativen Metabolitenaustausches (z.B. von Aminosäuren, Vitaminen oder Nukleotiden) begünstigen, sowie die Konsequenzen für die beteiligten Bakterienstämme sind bisher jedoch unzureichend aufgeklärt. Die intensive Erforschung könnte daher die ökologischen und evolutionären Ursachen und Konsequenzen des kooperativen Austausches von Metaboliten experimentell belegen.
Die vorliegende Arbeit untersucht die Ursachen und Folgen der metabolischen Kooperation bei Bakterien. Die Ergebnisse umfassen folgende Schwerpunkte:

1. Eine umfassende Literaturrecherche zu den bisher bekannten Wechselwirkungen zwischen Bakterien, ihren Ursprüngen und den prognostizierten Folgen der metabolischen Kooperation bei Bakterien.
2. Die Ermittlung von Mechanismen, die die Entwicklung von kooperativen Metabolitaustausches begünstigen.
3. Die Bestimmung der Konsequenzen einer synergistischen Koevolution auf genomischer Ebene.

1 Ökologie und Evolution metabolischer Wechselwirkungen bei Bakterien.

Um die Evolution und Erhaltung des kooperativen Austauschs von Metaboliten bei Mikroorganismen zu erklären, wurde ein Konzept entwickelt, das eine allgemeine Klassifizierung der verschiedenen Arten des Metabolitenaustausches erlaubt. Dieses Konzept stellt eine Synthese aus empirischen und theoretischen Studien dar, die die Bereiche Genetik, Mikrobiologie, Mikrobenökologie, Systembiologie und

Evolutionsbiologie einschließen. Dadurch konnte ein kompletter evolutionärer Prozess beschrieben werden (i) beginnend mit den initialen Ursachen metabolischer Abhängigkeiten die sich (ii) im Rahmen synergistischer Koevolution durch ökologische und evolutionäre Mechanismen zu einer Kooperation entwickeln, was (iii) für die beteiligten Bakterienstämme theoretische Konsequenzen hat, zum Beispiel die Evolution von Charakteristika mehrzelliger Organismen. Im Detail werden Gründe für den adaptiven Genverlust erklärt, die zu metabolischen Abhängigkeiten führen und sich daher zu einem kooperativen Austausch von Metaboliten entwickeln können. Dies setzt voneinander abhängige Bakterien voraus, welche wiederholt und ausreichend lange interagieren um den beschriebenen evolutionären Prozess zuzulassen. Zu diesem Zweck wurden alle bekannten Merkmale von Bakterien auf ihre Eignung hin untersucht, den Austausch von Metaboliten zwischen gegenseitig abhängigen Bakterien sowie deren wiederholte Interaktion zu gewährleisten und so zur Entwicklung und Aufrechterhaltung des kooperativen Austausches beizutragen. Auf diese Weise wurden entsprechende bakterielle Lebensstile und molekulare Mechanismen identifiziert und mit den aktuellen Konzepten der Evolutionstheorie verknüpft, die metabolische Zusammenarbeit erklären. Darüber hinaus wurden die gesammelten Belege für bakteriellen Metabolitenaustausch quantitativ auf die Identität der beteiligten Interaktionspartner, die Art der ausgetauschten Metaboliten, sowie die Mechanismen des Metabolitenaustausches analysiert. Die Ergebnisse veranschaulichen wie vielfältig die verschiedenen Kombinationen von Interaktionspartnern sind und geben einen Einblick in die Vielzahl der ausgetauschten Metaboliten.

2 Die Bildung von Gruppen ermöglicht die rasche Evolution von kooperativem Austausch von Metaboliten in Bakterien.

Die Untersuchung von Interaktionen in einer Bakteriengemeinschaft unter natürlichen Bedingungen stellt aufgrund von komplexen Wechselwirkungen zwischen den Beteiligten Bakterien eine Herausforderung dar. Um die Evolution einer Interaktion zu erforschen, wird darüber hinaus ein Vorfahre benötigt, der es erlaubt die Effekte eines evolutionären Prozesses auf seine Nachfahren quantifizieren zu können. Zudem können die unterschiedlichen Selektionsdrücke, die aus den komplexen Wechselwirkungen innerhalb der Bakteriengemeinschaften resultieren, nur schwer differenziert werden. Es bleibt daher unklar ob eine beobachtete Veränderung im Kontext von Metabolitenaustausch adaptiv ist oder ob diese andere Ursachen hat.

Bakterielle Modellsysteme unter Laborbedingungen bieten eine potente Lösung zur Überwindung dieser Herausforderungen. Zu diesem Zweck wurde eine beiderseitig obligate Beziehung zwischen Stämmen von Escherichia coli generiert, indem die biosynthetische Fähigkeit der Aminosäureproduktion entfernt wurde. Die modifizierten Bakterienstämme (Auxotrophe) waren nicht fähig, die Metabolite Tryptophan oder Tyrosin zu produzieren und sind folglich zu einem Austausch dieser Aminosäuren bei gemeinsamer Kultivierung gezwungen. Unter Verwendung dieser Kokulturen wurde schließlich ein Evolutionsexperiment durchgeführt. Zusätzliche experimentelle Gruppen, bestehend aus Monokulturen, denen die obligate Interaktion fehlte, dienten als Kontrollgruppen, die die Anpassung an Kulturbedingungen und genetische Manipulation (z.B. Auxotrophie für Aminosäuren) widerspiegeln. Die rasante Evolution von kooperativem Austausch von Aminosäuren wurde in weniger als 150 Generationen in den Populationen der kokultivierten Auxotrophen beobachtet. Die Bildung von mehrzelligen Clustern wurde als Hauptstrategie für den Aminosäureaustausch und damit für die Reproduktion innerhalb der Kokulturen identifiziert. Des Weiteren weisen die Kokulturen einen einfachen Lebenszyklus auf: Multizelluläre Cluster vergrößern sich durch Zellteilung gefolgt von der Auflösung in wenige kleinere Cluster und überwiegend Einzelzellen, welche sich unter günstigen Wachstumsbedingungen (frisches Medium) wieder in multizellulären Clustern organisieren. Diese Dynamik in der Populationsstruktur folgte dem Rhythmus der regelmäßigen Überimpfung während des Evolutionsexperimentes und stellt folglich einen Lebenszyklus dar.

Vergleiche mit den Kontrollgruppen (mit Aminosäuren ergänzten Monokulturen) zeigten, dass die beobachtete Wachstumsstrategie der Auxotrophen innerhalb von Clustern, sowie die Kooperation ausschließlich im Zusammenhang mit der obligaten Interaktion beobachtet wurden. Der Erfolg von Kooperationspartnern in Kokulturen konnte durch starke selektive Vorteile in den multizellulären Clustern erklärt werden. Aminosäureaustausch in diesen Clustern führte zu einer positiven Rückkopplung von Fitness zwischen interagierenden Zellen. Es konnte gezeigt werden dass dieser Mechanismus es einzelnen kooperativen Zellen erlaubte sich massiv in einer Population von normalen Zellen welche nur geringe Mengen an Aminosäure abgeben auszubreiten, was diesen in Abwesenheit der Cluster signifikant schlechter gelang. Interessanterweise zeigten solche kooperativen Phänotypen aus Kokulturen, wenn sie isoliert kultiviert wurden, ein konsistent geringeres Wachstum als deren Ursprungsstämme es vor dem Evolutionsexperiment gezeigt hatten. Diese Ergebnisse weisen darauf hin, dass

Mutationen, die die Fitness auf der Ebene des multizellulären Clusters erhöhten, zu Lasten der Fitness auf Ebene der einzelnen Zellen gingen. Diese Beobachtung kann dadurch erklärt werden das Selektion auf der Ebene der multizellulären Cluster operiert hat, also produktivere Cluster einen Vorteil hatten, was jedoch zur Folge hatte dass die Fitness einzelner Zellen irrelevant wurde.

3 Synergistische Koevolution beschleunigt die molekulare Evolution.

Ökologische Interaktionen sind wichtige Einflussfaktoren des evolutionären Wandels. Obwohl gut dokumentiert ist, dass antagonistische Koevolution genetische Divergenz verursacht und die molekulare Evolution beschleunigen kann, sind die evolutionären Konsequenzen synergistischer Koevolution nur unzureichend bekannt. Die beobachtete Evolution des kooperativen Metabolitenaustausches als direkte Folge der obligaten Interaktion wirft die Frage der daraus resultierenden Effekte auf die Anzahl, sowie auf die Diversität akkumulierter Mutationen im Verhältnis zum unabhängigen Lebensstil der Kontrollgruppen auf. Durch einen Vergleich auf Genomebene, der auf der Sequenzierung ganzer Populationen und isolierter Klone basiert, wurden Unterschiede in der Anzahl, sowie im Spektrum der akkumulierten Mutationen zwischen Versuchsgruppen quantifiziert. Die Ergebnisse dieser Analyse zeigen eindrucksvoll, dass auch die synergistische Koevolution die Geschwindigkeit der molekularen Evolution beschleunigen kann. Die Koevolution hatte eine signifikant erhöhte Anzahl von Mutationen im Genom von koevolvierten Auxotrophen im Vergleich zu Genomen von Kontrollgruppen zur Folge hatte. Darüber hinaus zeigten die kooperativen Populationen einen erhöhten Grad an paralleler Evolution, sowie divergente Evolutionsverläufe in Bezug auf beide Kontrollgruppen. Diese Unterschiede zu den Kontrollgruppen deuten auf eine Einschränkung der Anpassung an die genetischen Veränderungen (die entfernten Aminosäurebiosynthesegene) sowie an die abiotische Umgebung hin.

In dieser Arbeit wurden experimentelle Evolution und komparative Genomanalysen angewendet um die Ursachen und Konsequenzen der Evolution von metabolischer Kooperation zu erforschen. Die Resultate haben höchstwahrscheinlich weit reichende Auswirkungen auf evolutionäre Prozesse in mikrobiellen Gemeinschaften welche häufig obligaten Austausch von Stoffwechselprodukten aufweisen und zudem in multizellulären Strukturen gedeihen. Die gewonnenen Einsichten deuten schließlich darauf hin, dass sich multizelluläre Lebewesen ausgehend von metabolischen Abhängigkeiten zwischen Bakterien entwickeln können.

Introduction

Introduction

Bacteria exist virtually everywhere (1, 2), they are found aloft in the stratosphere where they resist hard radiation (3, 4), and they survive in abyssal depth within sediments or oil reservoirs where they tolerate extreme pressure and heat (5, 6). Microbial life remarkably influenced earth's history (7, 8), significantly controls the habitability of the whole biosphere (9), and is essentially important for global biogeochemical cycles (1, 10, 11) as well as multicellular organisms (12). Such evolutionary success, striking impact, and ubiquitous pervasiveness of bacteria can be basically attributed to innovative metabolic functions that allow utilizing and transforming countless compounds, of which humankind avails oneself since millennia.

Starting with the advent of microscopy, classical research gained deep insights into molecular biology, adaptive capabilities and mechanisms, impressive taxonomic diversity, and metabolic versatility of bacteria, yet has mostly focussed on single species. However, no organism is an island and therefore "… the pure culture is, with some exceptions such as certain microbes in direct cooperation with higher organisms, a laboratory artifact" (13). Indeed, the actual success and impact rarely lies in single superior species - microorganisms engage in a myriad of metabolic interactions that promote coexistence within bacterial communities (14-16) such as synergistic interactions (17). Among the diverse interactions within these communities it is thus ultimately metabolite exchange that cements the abovementioned significance of bacteria for life on earth. Due to metabolite exchange benefits are achieved within microbial communities (18, 19), for instance in the context of joint degradation of biological matter (20, 21), and awareness increased during recent years that a variety of these behaviours are indeed cooperative (14, 18, 22). These metabolic interactions are preferentially performed within taxonomically diverse communities (23-25) that are predominantly organised in cooperatively built biofilms or clusters of cells (1, 26-29). In essence microbial life is hence marked by metabolic interactions within spatially organised structures from which ultimately multicellular organisms arose in a process that was substantially driven by cooperation (30).

But what are the evolutionary consequences of living within bacterial communities and performing diverse ecological interactions that span the continuum between competition and cooperation? Indeed, there is no simple answer to that question and we

just begin to understand evolutionary effects on the level of single interactions such as the consequences originating from antagonism (31, 32) while we almost completely lack insights for metabolic cooperation albeit assumed to be an essential prerequisite for multicellularity to evolve (30, 33-35). What is more, there are in fact many open questions regarding cooperation in general: For instance under which ecological conditions does cooperation evolve and how can it be maintained despite of the presence of non-cooperators?

1.1 Cooperative interactions in the microbial world

Members of microbial communities engage in a variety of interactions that span the continuum between competition and cooperation (24, 36, 37). Due to the limitation of nutrients, space, and other resources it is intuitive that members of associations are coerced to compete against each other. Indeed, competitive interactions were found to dominate in microbial communities (38-40) and to strongly influence community structure as well as composition (22, 24, 40-43). However competition alone does not explain the given diversity of bacterial communities (37): Benefits of cooperative interactions in combination with other advantages apparently exceed costs of local competition. Since different definitions for cooperative interactions exist (13, 44), I will first define cooperation for this work as a mutually beneficial interaction between individuals that pay a cost for cooperating but generate a joint advantage. A widely acknowledged as well as well-studied kind of cooperation is for instance via public goods: metabolites that are released by a producing cell into surroundings hence are available for neighbouring cells. This type of interaction could be denominated as unidimensional cooperation, since one trait is collectively performed, which is frequently referred to as “sociality” or “social interaction”. Related individuals engage in a joint endeavour such as the production of siderophores (45, 46), biofilm-matrix (37), biosurfactants (47), and extracellular enzymes (48, 49). In the framework of kin selection theory public goods hence benefit relatives which indirectly increases the fitness of the producer (45). Motivated by Hamilton’s rule and the concept of inclusive fitness, kin selection hence provides understanding for the evolution of cooperative or altruistic behaviour among relatives or genotypes that share a certain allele (50-53).

In contrast to sociality, cooperative cross-feeding of metabolites between partners that exhibit complementary in biosynthetic functions has received much less attention. Indeed, examples for cooperation based on metabolite exchange are known (14, 18). The

difficulty to identify a certain cross-feeding interaction as “cooperation” lies within the defining measure: A costly provisioning of a certain compound is frequently challenging to verify. In addition, studying metabolite exchange does not automatically imply the aim to identify cooperation and with this to determine ecological measures such as costs for production. And even if cooperation is of interest, studying individual interactions within complex communities is not trivial (19, 54-56), which also applies to assigning metabolite flow between specific partners (19). Due to these intricacies, chapter 1 comprises a comprehensive review of literature with the aim to generate an overview of what is known about the interaction partners bacteria engage in cross-feeding with as well as the metabolites that are exchanged (see table 1 in the supplement for chapter 1 for an overview).

1.2 The problem of cooperation

Cooperation is based on the mutual exchange of goods or services to the benefit of both interacting partners. Provisioning a partner for instance with a certain metabolite however is costly, since shared resources cannot be allocated to growth and reproduction any more. These costs become particularly significant if shared resources are specifically overproduced for a cooperative partner, which intuitively implicates reciprocation for investments to pay off. However cooperators run at risk to be exploited by non-cooperative individuals or third parties. Assuming that both cooperators and non-cooperators have equal access to shared resources, the latter will generate more offspring and eventually dominate. In well-mixed surroundings exchanged metabolites for instance can also be seen as exploitable public goods that cooperators as well as non-cooperators have equal access to (38, 57). Under these conditions evolutionary theory predicts selfish individuals to be favoured by natural selection, which in the long run potentially causes the breakdown of cooperation (45, 51, 58, 59). The ultimate outcome could be the tragedy of the commons, *e.g.* extinction of cooperators (60, 61). And even by disregarding susceptibility for exploitation, complementary cooperators still face the challenge to encounter each other reliably albeit living within mixed poly-microbial communities (38). Mechanisms are thus required that help in finding and exchanging resources between interaction partners, otherwise cooperators will be selected against. And even if cooperators evolve and find common ground by chance, conflicts are likely to emerge in newly established groups (62). In consequence the benefit of newly established cooperation needs to exceed the negative effects of conflicts and increased

competition due to group formation.

1.3 Evolutionary explanations for metabolic cooperation

For cooperative cross-feeding to evolve it first requires a mutual demand that needs to arise in previously autonomous individuals. But how and why should a prototrophic (*i.e.* self-sustaining) genotype loose a vital biosynthetic function and in addition evolve a cooperative trait? To answer that question, a brief summary of theory and insights will be presented in the following. In addition, chapter one provides a more detailed overview of what is known to date.

Importantly, the majority of microbial species is expected to lack certain essential metabolic functions (22, 63), indicating dependencies on the external supply of vital metabolites to be common. This is further corroborated by unculturability of most bacterial species and we begin to understand the relevance of metabolic interdependencies as potential origin for this (64-66). The apparently prevalent lack of vital functions can be explained by natural selection favouring the fittest, yet not the most independent phenotype. In line with this, the Black Queen Hypothesis predicts adaptive gene loss due to externally supplied metabolites rendering biosynthetic functions redundant (67), which was experimentally demonstrated under laboratory conditions (68). In addition adaptive loss of genes can occur within the context of species interactions as well, thus generating the potential to intensify interactions between species, which is then termed compensated trait loss (69).

The general explanation for these processes is an increase in fitness due to utilizing costly metabolites from surroundings and in addition possibly by reducing costs of enzyme-production. Given that the preferred lifestyle within biofilms or cellular aggregates facilitates population structure and that the inhabitants of these structures exhibit a variety of dependencies, this has several far-reaching implications. Processes are enabled that potentially drive the evolution of cooperative cross-feeding from these interdependencies or alternatively from shared by-products. A large body of literature suggests several evolutionary mechanisms that facilitate the evolution of cooperation among microbes (27, 44, 45, 70). In the majority of studies focus lies on cooperation among genealogically related individuals (*i.e.* social interactions), however theory on sociality among microorganisms is based on assumptions that are not met by cooperation based on metabolite cross-feeding. Even if mediated by a single gene (71), social interactions are based on relatedness while cooperative cross-feeding is based on

complementary in certain biosynthetic functions. It is thus rather difference than similarity motivating metabolic cooperation, as predictions for the evolution of mutualisms show (72). Therefore, identified ecological and evolutionary mechanisms that explain the evolution and maintenance of cooperation in one type might not apply equally well to the other type.

This is why several theoretical predictions were tailored for the case of unrelated interaction partners (58, 73-78). However, despite fundamental differences, many conclusions and requirements can be formulated for cooperation in general. In essence, mechanisms for cooperation should ensure cooperators to interact more likely with other cooperators and less likely with non-cooperators (73), a concept that was already suggested by Hamilton for altruistic interactions (79). This strikingly simple prerequisite can be transformed into a common concept termed 'positive assortment' to which many studies essentially point towards, and which covers both intraspecies and interspecies interactions (44, 61, 73). This ultimately raises the question about supporting mechanisms available for bacteria to achieve assortment in the context of cooperative cross-feeding. Bacteria indeed possess an impressive variety of tools that are potent in facilitating positive assortment of partners as described in chapter 1 (see also chapter 1, figure 8).

Intuitively cross-feeding species benefit from spatial proximity (80) for instance by direct contact (81, 82). What is more even if cross-feeding via diffusion is not favourable under certain conditions, exchange still could be realized by direct connections between cells (*e.g.* nanotubes (83-85), or by outer membrane vesicles (86, 87). As pointed out by Tarnita 2017 (44), mechanisms for assortment can be categorized into two main features: Cooperators can simply stay together (*i.e.* partner fidelity) or alternatively find common ground (*i.e.* partner choice). While the former strategy verifies a continuing interaction of already associated partners, the latter either enables co-localization and with it association or the elimination of potential exploitive non-cooperators. Cooperative genotypes of course can employ both strategies in an alternating way, since separation can happen unintendedly or necessarily due to disturbance or dispersal, respectively. Both principles describe basically the association of partners within groups, which has several advantages as suggested for social interactions by theoretical work (88). An emergent feature of spatially organised associations is limited diffusion, which results in restriction of access for others or in other words privatization of exchanged metabolites (89). In line with this, reduced diffusion was demonstrated to constrain exploitation in

thick biofilms (90), and growth within such structures causes self-organisation and hence exclusion of non-cooperative individuals (89, 91, 92). The level to which the exchange of metabolites needs to be privatized is highly context-dependent and defined by physicochemical properties of the focal compound as well as the underlying cost-benefit ratio.

Importantly, the "aim" to cooperate is not necessarily the causative driving force to associate, and group formation therefore can be seen as a process independent of the evolution of cooperative cross-feeding as well as prior to it. Of course, a spatially structured population facilitating spatial proximity can arise by a variety of selection pressures, which were already discussed by Lyons and Kolter (2015) in the context of the evolution of multicellularity (28). This could be protection (28) from either the abiotic (oxygen, pH, and drought), or the biotic environment (predation (93, 94), competition (42), or immune system (95)), as well as resilience to environmental threads (96-98). Further advantages of living within groups or biofilms on the metabolic level comprise spatial fixation to a niche (energy- and carbon-sources), joint niche construction and exploration (synergism (13)), dealing with nutritional stress (99, 100), and co-localization of complementary genotypes (22). The logical order of events in this scenario would hence be first the evolution of mechanisms that facilitate positive assortment (44, 101) by different means and second the evolution of cooperative cross-feeding due to repeated interactions as well as the initiation of a positive feedback-loop between locally accumulating generations.

1.4 Evolutionary consequences

The transition towards obligate metabolite exchange that becomes cooperative implies several consequences for interacting genotypes or species. Since there is virtually a complete lack of experimental studies on evolutionary consequences of metabolic cooperation in bacteria, most knowledge is of hypothetical nature or adapted from other metabolic or cooperative interactions. When two partners that mutually exchange metabolites enter an evolutionary process marked by increased investment, both become dependent on reciprocity to stay competitive, and fitness and thus fate are coupled. Considering an obligate nature of the interaction, cross-feeding partners face diverse implications. By exchanging metabolites required for anabolism, growth becomes a function of received quantities hence depends on how many complementary genotypes (*i.e.* partners) are in vicinity of a focal recipient. Increased frequency in one genotype

thus triggers growth in the respective other genotype until shared resources become limited and *vice versa* - an oscillating process termed negative frequency-dependent selection (68, 102-104). During evolutionary time-scales the coupling of fitness should motivate partners to further invest into the interaction thereby causing a positive feedback-loop. Such increased investment was reported to evolve in imposed mutualisms (105, 106) and in principle resembles division of (metabolic) labour (102, 107). Classically focus in the concept of dividing labour is on social interactions (107, 108), yet principles can also be adapted to specialisation in cross-feeding interactions. In principle the defined prerequisites for division of labour are (i) phenotypes performing different tasks, (ii) cooperation, and (iii) positive selection of cooperative traits in the context of the interaction (107, 109). The advantage of dividing metabolic tasks among different cells likely lies in saving costs and therefore in increased fitness. Fitness was for instance observed to even increase by 20% over the wildtype level by synthetically engineering the division of metabolic labour between two *E. coli* genotypes (102).

Another predicted effect is that evolutionary dynamics between entities that divide labour can drive extreme specialisation that in the final stages causes the inability to reproduce independently (110, 111). In line with this metabolic interdependency was reported to intensify during experimental evolution causing loss of autonomy in one mutualistic partner (112), a phenomenon that is expected to be common in nature (112) and that might be causative for natural isolates not growing under laboratory conditions. Since frequently observed in the context of metabolite exchange between species (112-115), the loss of biosynthetic functions could be a general consequence of synergistic coevolution. Indeed, such metabolic interdependencies are a potential explanation for the widespread unculturability of bacteria (66) due to disruption of essential ecological setup, known as “the great plate count anomaly” (116). This exemplifies how risky loss of complementary partners hence interdependency can be. Besides effects supported by empirical evidence, theoretical work predicts changes in the rate of molecular evolution caused by coevolutionary dynamics, a continuous process of reciprocal adaptation. In fact changes in one partner will frequently imply effects on the other partner that will show an adaptive response, which in consequence alters the adaptive landscape for the first. One theory, the Red King Hypothesis, suggests decelerated molecular evolution for synergistic coevolution (117) however empirical evidence from analysing the life-history in an ant-plant mutualism showed the opposite phenomenon (118), which is rather predicted for antagonistic interactions. The latter is described by the Red Queen

Hypothesis and was already demonstrated to cause accelerated rates of genome evolution in a bacteria-phage antagonism (31), thereby restricting adaptation to abiotic culture conditions due to the necessity of increasing resistance (32, 119).

1.4.1 Shifts in the level of selection

Given that metabolic interactions promote coexistence within bacterial communities (14-16), and that communities are predominantly organised in biofilms or clusters of cells (1, 26-29), the question arises whether the individual cell or rather the group of cells is "seen" by selection. Put differently: Coinciding with the predominant lifestyle of bacteria, groups of cells facilitate assortment of partners hence promote metabolite exchange, which could initiate a phenomenon based on emergent properties of groups – *i.e.* selection operating on the group-level. When emergent characteristics of a group become relevant for the fitness of constituting cells, the group can become the unit of selection given that certain requirements are met (35): When looking on the level of many coexisting groups of interacting cells that exhibit variability in their composition and thus differences in fitness (35), between-group competition can favour more cooperative groups as already pointed out by Darwin in the context of competing tribes of humans (120).

Under these conditions, groups become 'evolutionary individuals' (*i.e.* a unit of biological organisation) and thus units of selection given that traits are heritable, variability between units occurs, and this variation results in fitness differences (35, 121-123). As a consequence of group-level selection, the fitness of lower level units (*i.e.* individual cells) however is subordinated, while adaptation is directed towards increasing fitness of the group-level. Selection operating on the group-level comes hence at the cost of decreasing fitness of individual cells when growing in isolation. This implication was experimentally demonstrated in a model system of *Pseudomonas fluorescens* and termed fitness decoupling (124). By further anticipating the process of fitness decoupling replication will eventually be coupled with reproduction of the higher-level entity, which is associated with complete loss of autonomy in individual cells (35, 107). In line with this, multilevel selection (MLS) theory (specifically MLS-2), states that the fitness of whole groups should indeed be decoupled from individual's fitness (125), which is also supported by modelling selection on the level of groups showing that it comes at the cost of individual fitness (74). In a nutshell it takes nothing more than regularly dividing groups and differential reproduction of cooperating

individuals therein to enable dynamics that cause a shift in selection towards the level of groups (76).

When the level of selection shifts to such a higher level unit, this is termed a transition in individuality (35). The unit natural selection operates on thus determining its frequencies, is termed an ‘evolutionary individual’ (121, 122). One important implication of such a transition is that non-cooperators may have an advantage within single affected groups, yet will locally decrease productivity while purely cooperative groups will produce most offspring and succeed (46, 126). When a transition in individuality occurs, this can have further implications such as the evolution of multicellular organisms.

1.4.2 Hierarchical evolutionary transitions and multicellularity

During earth’s history the evolution of multicellular organisms from previously simpler unicellular forms of life occurred repeatedly (127, 128). Multicellularity (*i.e.* multicellular organisms) can be broadly defined as reproductive units (*i.e.* ‘evolutionary individuals’) that (i) are formed by any kind of cell-cell adhesion, and (ii) should involve a kind of intercellular communication for coordination of activities (28), yet there is no clear definition for a multicellular organism likely due to many defining measures being located on a continuous scale thus eluding from clear categorization (129). The origin of a multicellular organism from previously unicellular simpler units involves a hierarchical evolutionary transition (HET) towards a new unit of biological organisation (30, 35, 130-132). For a HET (synonymously used to “transitions in individuality” (133)) to occur several prerequisites need to be met and the new unit of biological organisation needs to fulfil several criteria:

First of all to potentially experience a HET it requires that groups of cells (*i.e.* the new units of biological organisation to-be) are formed and that these groups undergo a life cycle. This is for instance already fulfilled by a unicellular ancestor forming groups (*i.e.* the unit) reproducibly under certain ecological conditions (*i.e.* the life cycle) (134, 135). For instance, starvation-induced aggregation illustrates such a situation (30), and fits the ecology-first scenario assuming group formation due to a pre-existing feature (136, 137). Importantly groups of cells feature emergent properties that in the first instance are immanently advantageous (*e.g.* protection or resilience) and ultimately enabled the evolution of multicellular organisms (28). One putative way towards multicellularity was hence identified in assortment within groups (also termed coming together (CT) (30,

138)) of similar genotypes, which can be facilitated by adhesion (139) or aggregation (128, 132, 140, 141), in combination with a life cycle caused by any means.

Groups that undergo a life cycle however need to fulfil further requirements as formulated by van Gestel and Tarnita 2017 (30): "…a group could be expressed as a facultative life stage only in response to certain recurrent environmental conditions…", yet needs to be "…formed sufficiently frequently for selection to potentially act on the group stage." New units of biological organisation undergoing a life cycle thus need to experience a shift in the level of selection towards the group-level as described in the previous chapter. In consequence, group-fitness of the multicellular organism becomes an emergent property and is not resembled by the fitness of constituent cells any more (125, 142, 143). Further key aspects for new units of biological organisation and with it for the evolution of multicellularity were reviewed by van Gestel and Tarnita 2017 (30) and identified in cooperation (107, 144, 145), mutual dependency (35, 146, 147), division of labour (148), resolved conflict (149, 150), and finally indivisibility of single units from the organism as well as their integration (133, 151, 152). Depending on the current stage of a HET, only few or even all of these aspects can apply to a multicellular entity. However there is still a lack of clear understanding how these processes took place and which general principles guided these transitions in a mechanistic as well as evolutionary manner (139).

Conceptually transitions in individuality can be discriminated based on the entities from which finally a multicellular organism evolves: Egalitarian transitions involve different hitherto free-living bacteria, while fraternal transitions comprise identical entities carrying out social interactions (141). Major driving forces that can initiate an evolutionary process towards multicellularity were reported for predation (94, 153), but also simple selection for faster settling and other causes were demonstrated to enable such a process (124, 154-156). The utilized model systems that resemble hallmarks of multicellular entities to date meet characteristics described for fraternal transitions (94, 124, 153-156). In contrast, multicellular aggregates of different genotypes performing cooperative cross-feeding resemble characteristics leading towards an egalitarian transition (141). Indeed, metabolic cross-feeding is well acknowledged as possible route towards such a transition (33-35), yet evolutionary approaches utilizing such an interaction to study HET were not reported to date.

1.5 Aims of the thesis

Bacteria thrive in diverse communities, where they form complex webs of metabolite exchange (19, 22, 157) that is frequently observed to be of cooperative nature (14, 18, 22). However, studying such interactions within bacterial communities remains challenging (19, 54-56) and only little is known about the origins as well as evolutionary implications of metabolic cooperation (105, 106, 112, 158). Nevertheless, evidence on bacterial cross-feeding interactions increased rapidly during the recent decade. In addition, scientific advances allowed predictions about how cross-feeding interactions can emerge, which mechanisms potentially drive the evolution of metabolic cooperation, as well as the potential implications of this evolutionary process. By adding up all these pieces to a greater picture, it has become possible to describe a complete process initially causing metabolic interdependencies (67, 68) and finally pointing towards cooperative exchange within units (*e.g.* aggregates of interacting cells) that show hallmarks of multicellularity (30, 132, 134). In detail, multicellular entities are characterized by selection operating on the level of these entities that potentially enter a lifecycle, and interdependencies of individual cells intensify until complete loss of autonomy (30, 132-134). Based on this described process, a conceptual framework was developed that resembles a synthesis of experimental evidence, empirical insights, and theory from microbiology, microbial ecology, and evolutionary biology.

Chapter 1:
Ecology and evolution of metabolic cross-feeding interactions in bacteria

Literature reporting cross-feeding interactions was reviewed and analysed with regards to the interaction partners and metabolites exchanged. A general classification of the diverse kinds of metabolite exchange is presented. In addition, different theoretical and empirical insights were combined to explain the complete evolutionary process starting with (i) the origins of cross-feeding, (ii) routes towards metabolic cooperation, (iii) maintenance, and (iv) potential evolutionary consequences that result from obligate cross-feeding of essential metabolites. Specifically, bacterial lifestyles and mechanisms of bacterial interactions that facilitate the evolution and maintenance of metabolic cooperation are considered.

In order to add experimental evidence to ecological and evolutionary causes and consequences of cooperative cross-feeding, a bacterial model system based on obligate metabolite exchange was designed, which was subjected to experimental evolution under

laboratory conditions. Furthermore, the question was whether a coevolutionary process towards metabolic cooperation would give rise to different routes of adaptation as compared to a solitary and independent lifestyle. This is why two additional control groups were integrated into the experimental design. This approach enabled us to clearly attribute observed results of adaptation either to abiotic culture conditions or rather to the obligate cross-feeding interaction. Moreover, it was of specific interested in conditions that cause continuous mixing of interaction partners, *i.e.* the absence of spatial structure facilitating assortment of complementary interaction partners. To generate a bacterial model system to address these issues, *Escherichia coli* was genetically modified. *E. coli* is a well-known bacterium that can easily be genetically manipulated (159) as well as resequenced (160, 161), and was previously successfully used in studies of experimental evolution (162-164). Derived strains lacked either the ability to produce tryptophan or tyrosine, hence needed to exchange these amino acids when cultivated together (*i.e.* as coculture) in shaken minimal medium, which did not facilitate population structure (*i.e.* assortment of complementary strains) and is therefore predicted to limit a cooperative exchange of amino acids. Monocultures of these strains and the wildtype were used as control groups.

Chapter 2: Causes for metabolic cooperation

The model system consisting of cocultures and control groups was subjected to experimental evolution for 150 generations to address the question whether and to which extent cooperative cross-feeding would evolve under shaken culture conditions. To answer these questions, the derived interaction was characterized for productivity (*i.e.* fitness on the level of populations and individuals), degree of cooperative amino acid exchange, and changes in the spatial organisation of cells. Differences relative to the ancestral interaction were quantified to determine significance of changes hence to verify increased investments in shared amino acids. By comparisons with control groups, phenotypic changes could be attributed to adaptation to the biotic interaction, thus ruling out factors resulting from adaptation to the culture conditions or genotype used. Based on these measures, the aim was to identify the mechanisms that could have promoted cooperative exchange and to experimentally demonstrate these mechanisms to indeed promote cooperation. In addition, the ecological consequences (*e.g.* additional dependencies) that may have resulted from the coevolutionary process were of interest.

What are the consequences on the genomic level? This question was addressed by comparative genomics examining the quantity of accumulated mutations, divergence in evolutionary trajectories, and extent of similarity in the spectrum of mutated genes between cocultures and control groups.

Chapter 3: Consequences of the evolution of metabolic cooperation on the molecular level

Since increased rates of molecular evolution were reported for antagonistic coevolution (31), it was asked whether the evolutionary process in cocultures (investigated in chapter two) could influence genome evolution as well. Furthermore, it was of interest whether experimental groups either have certain mutated sites in common or whether the obligate interaction is causal for divergent evolutionary directories between groups. Therefore, the genomes of whole populations and single isolates from the three experimental groups were sequenced. Subsequently, mutations that occurred in the genomes of strains derived from evolved cocultures and control groups were identified in order to determine quantitative differences in the rate of molecular evolution. The spectrum of mutations was used to generate distance trees illustrating evolutionary trajectories of all analysed samples. As a supporting measure, the extent of parallel evolution was determined within and between groups. By taking all of these measures into account, our attempt was to formulate general conclusions about the evolutionary consequences of cooperative cross-feeding relative to an independent lifestyle.

Chapter 1

Chapter 1

Ecology and evolution of metabolic cross-feeding interactions in bacteria*

Bacteria frequently exchange metabolites with other micro- and macro-organisms. In these often obligate cross-feeding interactions, primary metabolites such as vitamins, amino acids, nucleotides, or growth factors are exchanged. The widespread distribution of this type of metabolic interactions, however, is at odds with evolutionary theory: why should an organism invest costly resources to benefit other individuals rather than using these metabolites to maximize its own fitness? Recent empirical work has shown that bacterial genotypes can significantly benefit from trading metabolites with other bacteria relative to cells not engaging in such interactions. Here, we will provide a comprehensive overview over the ecological factors and evolutionary mechanisms that have been identified to explain the evolution and maintenance of metabolic mutualisms among microorganisms. Furthermore, we will highlight general principles that underlie the adaptive evolution of interconnected microbial metabolic networks as well as the evolutionary consequences that result for cells living in such communities.

*Published manuscript, shared authorship: Ecology and evolution of metabolic cross-feeding interactions in bacteria. D'Souza G, Shitut S, Preussger D, Yousif G, Waschina S, and Kost C. Natural Products Reports, Review Article, 2018, 35, 455-488, doi: 10.1039/c8np00009c.

1 Introduction

Bacteria are amongst the most ancient life forms on our planet (165, 166). Even, the last common universal ancestor (LUCA) has been suggested to strongly resemble bacteria that dwell in extreme environments (167, 168). During their evolutionary history of about 3.2 billion years, bacteria managed to colonize virtually every conceivable habitat on earth including air, soil, water, as well as other organisms such as animals and plants (2). Due to their widespread distribution and high abundance, bacteria play significant ecological roles in driving global biogeochemical cycles (10), determining homeostasis of the biosphere (9), and controlling the development, behaviour, and health of multicellular organisms (12).

In nature, bacteria usually exist within taxonomically and genotypically diverse communities (23-25). In these assemblages, bacteria compete for a wide variety of limiting resources such as favourable living spaces, nutrients, and minerals. Moreover, due to their metabolic activities, bacteria transform the environments they live in, thus drastically influencing the growth and metabolism of other co-occurring organisms (169). Strong selection pressures resulting from both of these factors have not only given rise to a plethora of ecological interactions, but also different bacterial strategies to survive and reproduce under these conditions (24). Accordingly, a large proportion of a bacterial cell's genetic material (between 17 and 42%) can encode traits that are involved in mediating ecological interactions (15).

For heuristic purposes, ecological interactions between two individuals are typically classified based on the net fitness effects that result for the organisms involved. The typological spectrum of interactions resulting from this classification scheme ranges from antagonistic (i.e. negative fitness consequences) over neutral (i.e. no interaction) to beneficial interactions (i.e. positive fitness consequences) (24). Examples of antagonistic behaviours displayed by bacteria include the active secretion of toxins such as colicins or antibiotics that kill or inhibit the growth of other bacteria (170, 171), thus providing the toxin-producing bacteria with a competitive advantage. Evolutionary theory predicts that natural selection should favour such strategies that selfishly enhance the fitness of one organism at the expense of another one (40). Indeed, a large body of work has demonstrated the prevalence of antagonistic interactions in natural microbial communities (24, 40, 41).

However, in recent years, awareness has grown that bacteria also show a range of cooperative behaviours, in which one individual helps another one at a cost to itself. A good example for this is so-called *public goods*. These are metabolites that are costly to produce, yet are released into the extracellular environment. As a consequence, these *public goods* do not only benefit the producing cell, but also other cells in the local group or population. Examples include antibiotic-degrading enzymes (48), motility-enhancing biosurfactants (47), matrix components for biofilms (172), or iron-scavenging molecules (173). Why would cells invest resources into behaviours that can be easily exploited by individuals that reap the benefits without bearing the costs for producing the public good? In most of the abovementioned cases, the individual producing the public good and the beneficiaries are genealogically related. Thus, by helping its relatives, the cooperative individual can increase the chance that its own genes are indirectly propagated. This so-called *kin-selection* can explain altruistic cooperative behaviours among closely related individuals (45).

The situation, however, is different for synergistic interactions that involve unrelated individuals or different species that reciprocally exchange metabolites such as sugars, growth factors, or amino acids with each other (18). A number of recent studies have suggested that these types of synergistic interactions might actually be common in the prokaryotic world (17, 63, 174). In many of these cases, the interactions are also obligatory for the individuals involved, meaning they can only exist when the required metabolite is externally supplied, for example by another bacterium (63, 174). This type of metabolic interactions begs an evolutionary explanation: Why should a bacterium give up its metabolic autonomy and rather rely on other organisms to provide essential metabolites? Moreover, why would a bacterial cell produce metabolites to benefit other, potentially unrelated individuals and not use these resources to maximize its own fitness?

In this article, we address these questions. By particularly focussing on metabolic interactions between two or more bacterial partners, we aim at developing a conceptual framework that allows not only to classify different types of metabolic interactions, but also to explain the evolution and maintenance of these relationships. In addition, we analyse how common metabolic cross-feeding interactions are in nature and what evolutionary consequences result for the organisms involved. The comprehensive picture that emerges from this analysis may provide an orientation to scientists that are new to this interesting field of study and identify avenues for future research.

2 Metabolic cross-feeding interactions

2.1 Historical account

A first and important step in understanding the origin of metabolic exchange in bacteria is to obtain a historical perspective on the discovery of this phenomenon. Early studies on what is now known as *cross-feeding* often discuss the phenomenon in the context of symbiosis (175, 176). These studies mainly focussed on microbial interactions that impact plant growth (e.g. root nodule bacteria (177, 178), mycorrhiza (179)) or play important roles for the fermentation of dairy products (i.e. lactic acid bacteria (180-182)). Back in 1887, Carl Garrè, a Swiss surgeon, was one of the first to mention that "*one organism prepares food for another organism by changing the medium on which it grows*" (176). Later in 1892, the British botanist Marshall Ward stumbled upon cross-feeding while trying to unravel the mystery of the Ginger-beer Plant. The substance in question is used to ferment ginger beer, a non-alcoholic, naturally sweetened beverage, from saccharine and ginger. Ward found out that this plant was, in effect, a symbiotic association between a yeast and bacteria that formed solid, semi-translucent, lumpy masses. More importantly, he found that an exchange of metabolites between both partners was an integral part of the fermentation process (183). Around this time, such mixed cultures of microbes were referred to as *microbial associations* (176). In 1897, Wilhelm Pfeffer, a German botanist and plant physiologist, introduced the terms *conjunctive* and *disjunctive* symbiosis to highlight the dependency of either partners for growth (184). Marshall Ward also proposed the use of terms like *antibiosis* and *metabiosis* to distinguish between negative and positive effects that result from an interaction for the partners involved (185).

The term *cross-feeding* was coined by Hermann Reinheimer in 1921 - a British biologist who was interested in the evolutionary significance of cooperative symbiotic interactions (186). Reinheimer suggested two terms to differentiate the source of food or metabolite, namely *in-feeding* for within-kingdom exchange and *cross-feeding* for between-kingdom exchange. This distinction, however, was not adopted by the scientific community at large. Instead, the term cross-feeding was subsequently used to describe interactions that involved an exchange of molecules and, thus, enhanced growth. Interestingly at this time, cross-feeding between auxotrophic strains was also used as a

methodological tool to elucidate biochemical pathways (187, 188). Notable work was done by Veikko Nurmikko, a Finnish microbiologist, who introduced the use of dialysis chambers to separate two auxotrophic strains of lactic acid bacteria such that they exchange metabolites via diffusion (189). In subsequent years, metabolic cross-feeding interactions were used to study the concerted degradation of herbicides (190-192) or fatty acids (193), the enhanced production of amino acids (194), and to characterize auxotrophic strains (195-197). Interestingly, until today, mixed cultures of natural bacterial isolates are employed to identify novel pathways for the degradation of complex hydrocarbons like crude oil (198-200) or toxic industrial dyes (201, 202).

Towards the end of the 1980's, microbiologists began to study bacterial interactions from an ecological and evolutionary perspective. Among them, Julian Adams and co-workers initiated long-term chemostat cultures of *Escherichia coli* in glucose-limited conditions (203, 204). An intriguing observation from their continuous cultivation experiments was that bacterial strains repeatedly evolved mutations in the acetyl CoA synthetase enzyme. This mutation allowed the uptake of exogenous acetate resulting in a stable coexistence of these mutants with wild type strains that secreted acetate as a by-product of glucose metabolism (205). Several subsequent studies analysed similar cases of diversifying selection in initially clonal populations that resulted from the evolution of metabolic cross-feeding interactions (206-209).

In recent years, the phenomenon of metabolite exchange has gained momentum with an increasing number of working groups studying this type of ecological interactions from different perspectives and using different methodological approaches. However, depending on their research focus and scientific background, a number of different terms are used to describe qualitatively similar interactions. For example, terms like syntrophy (210), synergism (17), symbiosis (175), mutualism (175), or obligately mutualistic metabolism (210) are often used interchangeably. Each of these terms describes a reciprocal exchange of molecules, yet in specific contexts. For instance, *syntrophy* denotes cases where the metabolism of two organisms are energetically coupled (210), while *synergism* simply refers to interactions from which both interacting partners benefit (17).

2.2 Classification of cross-feeding interactions

Given that a number of different terminologies are used to describe qualitatively similar ecological interactions, we begin by providing an unambiguous and

comprehensive classification scheme to name different types of metabolic cross-feeding interactions. Due to the focus of this review, we only discuss interactions that involve an exchange of primary metabolites. In reality, however, bacteria often trade metabolites against other beneficial services such as detoxification of toxic metabolites or protection from predators (24). Even though we do not treat these interactions in detail, a similar nomenclature and conceptual logic can be applied to them as well.

Our classification framework categorizes metabolic interactions along two main axes: (i) the degree of reciprocity (i.e. *unidirectional* versus *bidirectional* metabolite flow), and (ii) the investment by the involved partners (i.e. the cost to produce the exchanged metabolite) (Fig. 1). The first parameter, *degree of reciprocity*, categorizes cross-feeding interactions based on whether the metabolite exchange is unidirectional (one-way) or bidirectional (reciprocal) (Fig. 1). The second parameter, *investment*, divides cross-feeding interactions according to the cost of biosynthesis that the interacting partners bear during the interaction, resulting into two sub-categories (i) by-product cross-feeding (Fig. 1A,B) and (ii) cooperative cross-feeding (Fig. 1C,D). *By-product cross-feeding* is the exchange of metabolites that results from a selfish act of the producer (45). For example, by-products can be secreted due to the degradation of complex hydrocarbons (211), the accidental leakage of metabolites through the bacterial membrane (212), or overflow metabolism (213). In general, the production of metabolic by-products is independent of the presence of an interaction partner and positively correlated with producer's growth.

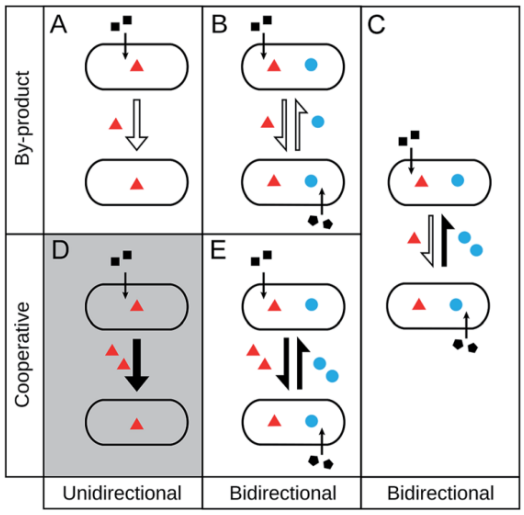

Figure 1: Types of cross-feeding interactions. Cross-feeding interactions can be classified based on the degree of reciprocity (columns) and the investment of the interacting partners (rows). (A) Unidirectional by-product cross-feeding: one partner produces a metabolic by-product that benefits the respective other. (B) Bidirectional by-product cross-feeding: reciprocal exchange of metabolic by-products between two partners. (C) Unidirectional cooperative cross-feeding: one partner bears a cost for producing a metabolite that benefits the respective other one. This box is marked in grey, because this case is hypothetical and expected to be strongly disfavoured by natural selection. (D) Bidirectional cooperative cross-feeding: reciprocal exchange of a costly metabolite that benefits both partners. (E) By-product reciprocity: One partner produces a costly metabolite to benefit another cell, which in turn supplies the producer with increased amounts of a metabolic by-product

In contrast, *cooperative cross-feeding* occurs if one partner actively invests resources to produce metabolites that benefit an interaction partner (Fig. 1C,D). In this case, the cooperating cell is producing more of the metabolites than it would require for its own growth. Enhanced levels of metabolite production can be caused by an increased expression of the corresponding biosynthetic genes (214), a greater flux through the respective metabolic pathway (215), diverting resources into the production of a given metabolite (158, 216), or harbouring a multi-copy plasmid that encodes the biosynthetic genes (217). In any case, a cell bearing this cost is significantly less fit than a cell that is not carrying the burden of increased metabolite production (158). Thus, an important difference between cooperative cross-feeding and an exchange of by-products is that cooperative cross-feeding must have been favoured by natural selection. In other words, a newly emerged mutant that produces increased amounts of a given metabolite found itself in an ecological setting, in which this cooperative trait was selectively favoured despite the concomitant fitness costs.

When cross-feeding interactions are classified in these two dimensions, it is possible to obtain five different outcomes. First, *unidirectional by-product cross-feeding* is when one cell releases a metabolic by-product that benefits another individual (Fig. 1A). Ecologically, this type of interaction is equivalent to a commensalism. A classic example is the evolution of acetate-cross-feeding in populations of *E. coli* (206), in which glucose-utilizing cells release acetate as a metabolic by-product into the growth environment. Even though acetate contains less energy than glucose, it represents an unexploited resource. Thus, mutants emerge that preferentially utilize acetate.

Bidirectional by-product cross-feeding involves the reciprocal exchange of metabolic by-products between two interacting partners (Fig. 1B). This phenomenon, which is sometimes also referred to as synergism or proto-cooperation, can, for example, be observed between ammonia oxidizing microbes (AOM) and nitrite oxidizing bacteria (NOB) (218). AOM oxidize ammonia to give nitrite, which is converted to nitrate by the NOB. However, a recent analysis shows that NOB (like *Nitrospora* sp.) convert urea to ammonia and carbon dioxide, which in turn is taken up by the AOM, thereby resulting in a bidirectional by-product cross-feeding between AOM and NOB(219).

Unidirectional cooperative cross-feeding is a possibility that only exists theoretically (Fig. 1C). In reality, however, mutants that produce metabolites without being rewarded for the increased investment are strongly selected against and thus should exist likely only transiently.

Bidirectional cooperative cross-feeding involves interactions, in which each of two partners produces a costly metabolite that benefits the respective other type (Fig. 1D). Unfortunately, due to a lack of the corresponding evolutionary ancestors from which a given interaction evolved, it is usually difficult if not impossible to infer cooperative cross-feeding in natural microbial populations: control genotypes not showing the focal interaction would be needed as a baseline, against which genotypes displaying a cooperative investment can be compared. This is why the best-studied examples come from laboratories, in which this type of interaction has been synthetically engineered. One of these synthetic cross-feeding systems has been generated by gene deletions in *E. coli* (102). The first deletion rendered the cells dependent on a certain amino acid for growth, while the second deletion increased production of the metabolite required by the respective partner for growth. Monocultures of each genotype were unable to grow and amino acid overproduction resulted in a significant fitness cost for the corresponding

mutants (102). In coculture, however, both types were significantly fitter than the corresponding WT, from which all mutants were generated.

The last type of interactions resulting from this classification scheme is a special case called *by-product reciprocity* (Fig. 1E) (220, 221). This interaction represents a mixed case, in which one partner produces a costly metabolite to benefit its corresponding partner (i.e. a cooperative act), yet receives metabolic by-product in return. In this case, the cooperative individual produces the costly metabolite to increase the amount of by-product it obtains from its partner. Such an instance of cross-feeding has been observed in experimental cocultures of *Salmonella enterica* ser. *Typhimurium* and *E. coli*, wherein *E. coli* depended on *S. enterica* for methionine, while *S. enterica* consumed metabolic by-products released from *E. coli* (158). When both strains evolved in coculture, *S. enterica* started to produce significantly higher amounts of methionine as compared to evolved monocultures of *S. enterica* (158). In other words, *S. enterica* started to actively invest in methionine production to maximize the amount of metabolic by-products it obtained from its partner.

2.3 Ways to study cross-feeding interactions

In the following two sections, we will provide an overview over different methodological approaches that have been employed to identify and characterize metabolic cross-feeding interactions in bacteria (Fig. 2).

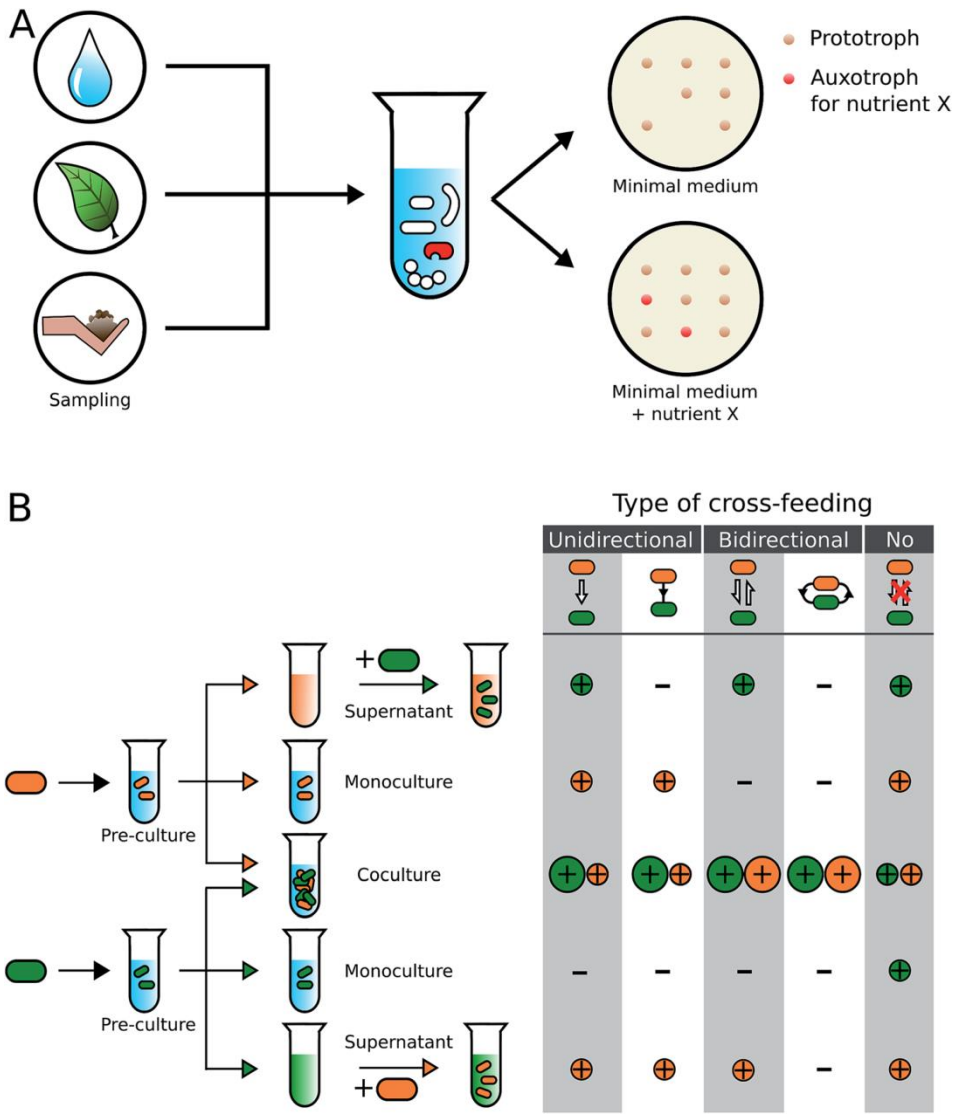

Figure 2: Experimental identification of metabolic auxotrophies and obligate cross-feeding interactions. (A) Samples obtained from natural environments are plated on selective minimal medium agar plates. Auxotrophic genotypes (shown in red), whose growth depends on an external supply of metabolites such as amino acids, vitamins, or nucleotides, can be identified by comparing their growth on metabolite-supplemented and unsupplemented medium. (B) Isolated strains (indicated in orange and green) are subjected to different diagnostic growth conditions to characterize the type of cross-feeding interaction, in which they engage. Both genotypes are first grown in a minimal medium that is supplemented with components to allow growth of a pre-culture. This culture is then exposed to three growth conditions: (i) centrifugation and filtration to obtain a cell-free supernatant, (ii) inoculation as a monoculture in unsupplemented minimal medium, and (iii) inoculation as a coculture with the second genotype in unsupplemented minimal medium. The cell-free supernatant of one genotype serves as the culture medium for the second genotype. By quantifying the growth of each genotype in each condition (+ = growth, - = no growth) and comparing the growth between conditions (size of the correspondingly coloured circles), the type of cross-feeding interaction can be identified. Besides the directionality (uni- or bidirectional), it can also be determined whether nutrients are exchanged via a transfer through the extracellular environment (white arrow between cells) or in a contact-dependent manner (black lines connecting cells).

2.3.1 Culture-dependent approaches

Isolating bacteria from environmental samples on agar plates and observing their growth patterns are classical microbiological techniques to study cross-feeding interactions. For this, environmental samples (e.g. soil, water, animal gut) are collected. Next, bacteria are isolated and purified on suitable agar plates that are often composed of a rich growth medium to also allow cultivation of strains with complex nutritional requirements. Finally, the isolated strains are either grown in monoculture or together with different partners in defined minimal growth media. Finding that some of the isolated strains can only grow in coculture yet not alone, is strongly pointing towards metabolic interactions (Fig. 2) (222). Subsequently, the isolated partners and the exchanged compounds can be identified by genomic and chemical analyses, respectively.

Sequencing, the whole genomes of the isolated strains and/ or manipulating their genome (e.g. by mutagenesis) can shed further light on the molecular basis of the observed interaction. Intrinsic problems of this type of approaches are that only a fraction of the bacteria that were actually present in an environmental sample can be isolated and cultivated under laboratory conditions (see section 4.5). Moreover, conditions that bacteria face in nature (e.g. spatial structure of soil particles, availability of specific nutrients, pH, etc.) are difficult to simulate under laboratory conditions. Moreover, mixing a certain number of different strains in all possible combinations of pairwise cocultures (222, 223) might bring together strains that would not meet in their natural habitat, thereby biasing the view on the true spectrum of existing interactions. Nevertheless, culture-dependent approaches have provided valuable insights into the rich diversity of metabolic interactions that exists within microbial communities (Fig. 2, Table S1 – see supporting information for chapter 1) (224-227) and should be seen complementary to the so-called culture-independent approaches.

2.3.2 Culture-independent approaches

The development of various meta-omics techniques revolutionized the study of microbial communities, because it allowed to also include prokaryotes that cannot be cultivated under laboratory conditions. Many studies using these approaches predicted metabolic cross-feeding interactions among community members through sequencing and annotating the metagenome of the community or the whole genome of individual clones (55, 228-230). Moreover, the combination of selective staining methods (e.g.

fluorescence *in-situ* hybridization (FISH)) with high-resolution microscopic techniques affords to analyse spatial arrangements within microbial communities, and thus use close spatial proximities as an indicator for possible metabolic interactions (54, 231, 232). On the other hand, comparing changes in the transcriptomes of cells in mono- and cocultures (using microarray, RNA-Seq; Table S1) provides a powerful tool to qualitatively analyse ecological interactions between two bacterial genotypes. The up-regulation of genes in coculture, which are involved in the production of certain metabolites, hints at a possible exchange of these compounds (224, 233, 234). The next step is usually the chemical identification of the exchanged metabolites in the cell-external environment using mass-spectrometry- or NMR-based approaches (235-237).

The major advantage of culture-independent approaches is that they provide hypotheses without the laborious and potentially biased isolation of environmental microorganisms. A downside, however, is that these techniques strongly depend on the quality of both the extraction process (i.e. DNA, RNA, or proteins) and the obtained reads. Furthermore, divergent sequences, the presence of metabolic enzyme homologs, and promiscuous enzymes with yet uncharacterized catalytic capabilities could lead to a potential overestimation of metabolic dependencies. As a consequence, the performed studies mainly provide hypotheses that need to be verified in subsequent experiments. Thus, many recent studies combine culture-dependent and independent approaches as complementary techniques to capture a more holistic picture of the microbial community (224, 233, 238).

2.4 Distribution of cross-feeding interactions in nature

How prevalent is metabolite cross-feeding in nature? To address this question, we have screened the available literature for cases, which experimentally demonstrated cross-feeding of building block metabolites using natural isolates (Table S1). In total, 77 studies were included that reported about 135 different interactions covering the period of 1952 to 2016. The metabolites identified in these studies were divided into the following six main categories: carbon source, nitrogen source, amino acids, nucleotides, vitamins, and others (i.e. phosphorus, iron, or organic compounds). Hormones, growth factors, or electron exchange were deliberately excluded from the analysis.

The results of this meta-analysis indicated that metabolite cross-feeding is indeed very common both among different bacterial species and between bacteria and members of other kingdoms including archaea, fungi, animals, protists, and plants (Fig. 3).

Moreover, cross-feeding of different molecules (Fig. 3B) was remarkably diverse with regards to the lifestyle of the involved partners and the habitats, in which the interaction occurred (215, 232, 239-242) (Table S1). Another insight that emerged from this comparative analysis was that in many cases, interacting bacterial cells tended to be localized in close spatial proximity, presumably to facilitate an exchange of metabolites (54, 231). In general, photosynthetic and nitrogen-fixing organisms commonly traded carbon and nitrogen respectively against other commodities (237, 243, 244), which represents a major input of these fundamental elements into the global biochemical cycles.

Strikingly, the nature of the exchanged metabolites drastically depended on the corresponding partner, with which bacteria interacted (Fig. 3C). For example, plants and protists tended to mainly provide bacteria with (assimilated) carbon (245-247). In return, bacteria commonly supplied plants with nitrogen (245) and algae with vitamins, which ~50% of all algal species cannot produce autonomously (248). In general, bacteria are an important source of nitrogen for fungi, protists, plants, and animals. Animals commonly provide shelter and food to bacteria (e.g. in the gut (242)), while receiving a wide range of the metabolites including amino acids and vitamins in return. Interestingly, based on the collected data, bacteria are the only partner who cross-feed nucleotides either with other bacteria (229) or with members of other kingdoms (234).

Our literature survey also revealed that some specific types of cross-feeding interactions attracted more research attention than others. It is important to keep in mind that this pattern does not reflect an increased prevalence of these interactions in nature. For example, cross-feeding between *Streptococcus* and *Lactobacillus* has been extensively studied during the last decades, because of the biotechnological interest in these strains that are used in dairy production. On the other hand, many interactions remain likely undiscovered, because of a lack of scientific inquiry or because technical difficulties thwart the isolation and analysis of partners involved.

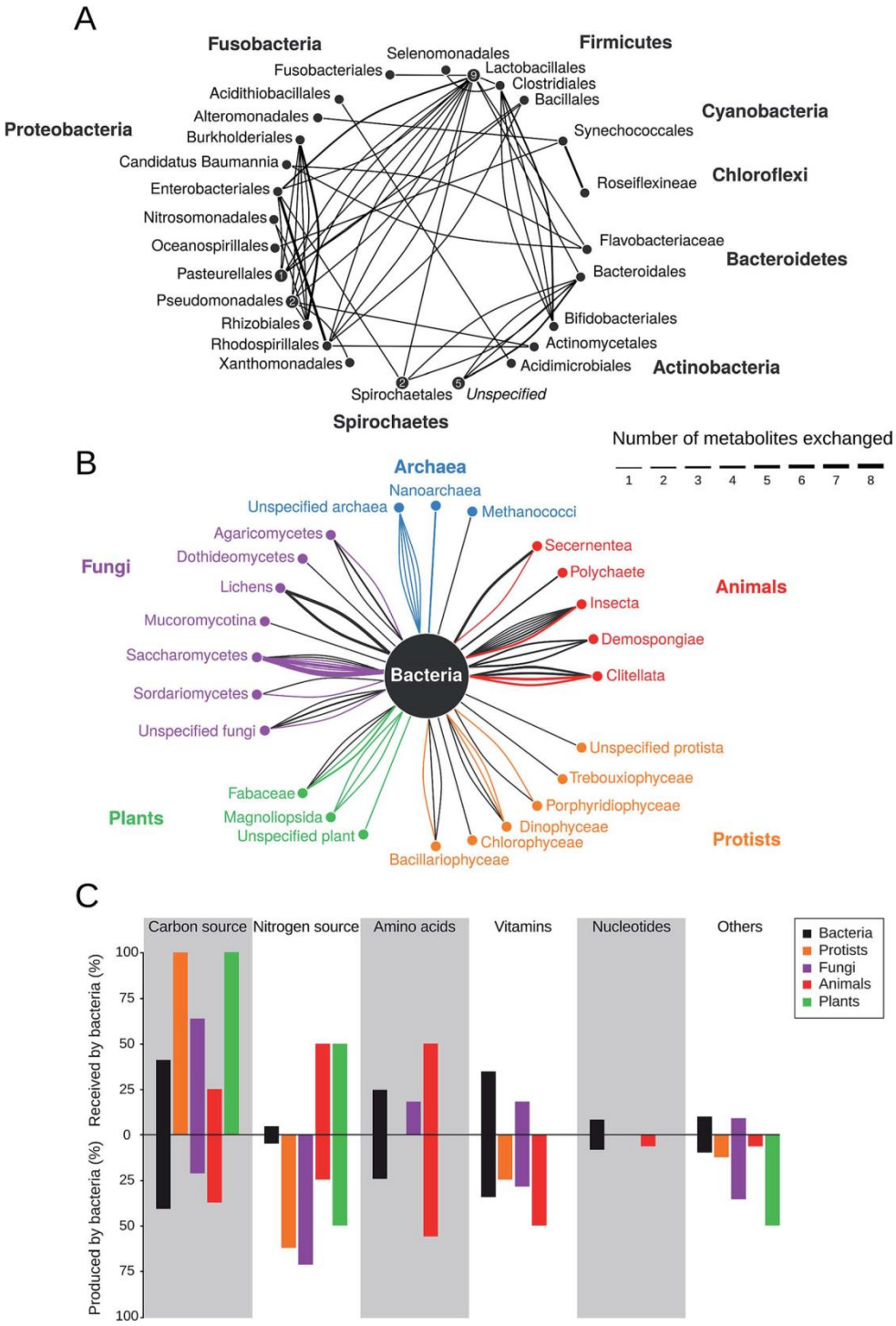

Figure 3: Prevalence of metabolic cross-feeding interactions. Data is the result of a meta-analysis of 78 studies that included 135 different cross-feeding interactions (Table S1). (A) Metabolic cross-feeding interactions (edges) between bacteria (n=68). Bacteria from the same order are summarized in nodes and nodes are grouped by the respective phylum. Numbers within nodes represent instances of within-order cross-feeding interactions. The thickness of edges indicates the number of different metabolites that are exchanged. (B) Interactions between bacteria and organisms from other kingdoms (n= 67). Edge thickness is scaled as in (A) and its colour corresponds to the partner that is producing the exchanged metabolite. (C) Percentage of specific metabolite classes that are either received (upper half) or produced (lower half) by bacteria in cross-feeding interactions relative to the total number of cases in each category (n=135).

2.5 Mechanisms of metabolite transfer

Given that cross-feeding is so common in the microbial world (Fig. 3, Table S1), the question arises how metabolites are transferred between bacterial cells. Considering the large variety of bacterial lifestyles (e.g. biofilm growth versus planktonic cells, endosymbionts versus free-living bacteria) as well as the structural diversity of metabolites that can be exchanged, it is likely that bacteria use different mechanisms to transfer material from one cell to another one. Modes of metabolite exchange can be classified into *contact-independent* and *contact-dependent*. In this context, *contact* denotes a direct physical connection between two or more interacting cells.

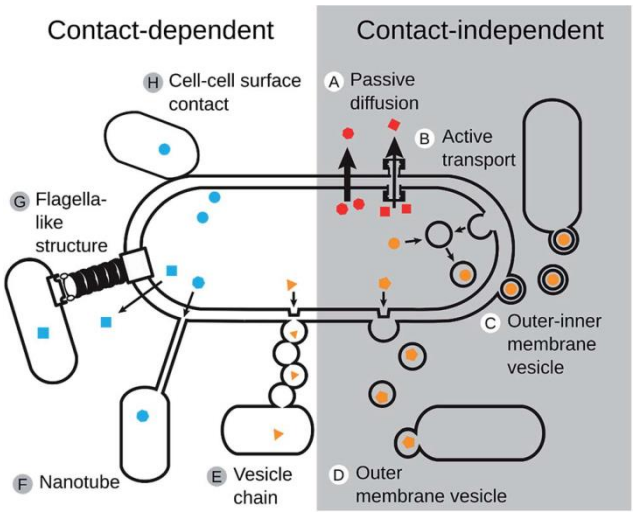

Figure 4: Mechanisms of metabolite transfer. Molecules can be transferred from one cell to another one using (A-D) contact-independent- or (E-H) contact-dependent means of cross-feeding in bacteria. Contact-independent mechanisms that are based on the diffusion through the extracellular environment require a release of the exchanged molecule by (A) a passive diffusion across the cellular membrane(249-251) or (B) an active transport of molecules via membrane-based transporters(252, 253). Alternatively, chemicals can be transferred via membrane vesicles with (C) a bilayer formed of the outer and inner membrane (254, 255) or (D) a single membrane(86). A contact-dependent exchange of metabolites between two cells can be mediated by (E) outer membrane vesicles that link to form a chain(87).or (F) intercellular nanotubes that allow an exchange of cytoplasmic contents (83, 84). Moreover, also (G) flagella-like structures (80, 214) or (H) a direct surface contact (81, 82) can facilitate the exchange of metabolites between cells.

2.5.1 Contact-independent mechanisms

Planktonic cells use various mechanisms to exchange metabolites via the extracellular environment (Fig. 4A-D). A metabolite transfer via the surrounding medium can result from an intentional or unintentional release of the focal metabolite into the environment, or alternatively, through the budding-off of vesicles that contain the exchanged good. By

secreting a metabolite into the surrounding, it is made available to all neighbouring cells. Such a so-called *public good* can, however, cannot only be used by the intended recipient (e.g. other cooperative cells), but also by other, non-cooperating genotypes in the surrounding. Another disadvantage of this mode of transfer is that the released metabolite might be chemically altered (39, 256), degraded (257, 258), or be lost by diffusion. An alternative transfer mechanism that can help to solve some of these problems is to exchange membrane vesicles that contain the traded commodity. Such vesicles not only protect the transported molecules, but potentially also allow for a more specific and targeted exchange (259).

Passive diffusion

The process of passive diffusion includes the passage of molecules through the cell membrane, often along concentration gradients and without the involvement of ATP (Fig. 4A). This type of exchange is commonly observed for small molecules like hydrogen, formate, potassium, volatile compounds like methanol (260), as well as metabolites like vitamins (246), acetate (261), amino acids, and intermediates of the TCA cycle (e.g. 2-ketoglutaric acid, gluconate) (262). Metabolites that are transferred in this way are often released as a result of overflow metabolism (213), thus giving rise to interactions, in which by-products are being exchanged.

In this type of interactions, the speed of diffusion limits the exchange of metabolites and thus the growth of both interacting partners. For instance, *Syntrophomonas wolfei* and *Methanobacterium formicium* exchange either hydrogen or formate as an electron carrier depending on the spatial distance between cells (249). Close proximity promotes electron transfer via hydrogen, because of its rapid diffusion through the medium, whereas formate is used for longer-distances (250).

Active transport

Molecules that require an active transport are usually unable to cross the bacterial membrane due to their molecular weight, charge, or polarity. Examples include some amino acids (263), siderophores (264, 265), enzymes (266), polymers (267, 268), and vitamins (18). In these cases, the exchanged molecule needs to be exported into the extracellular environment, involving energy-dependent transport systems such as the ATP-binding cassette (ABC) transporter family (269) or the phosphotransferase system (270). Also, corrinoids, a group of compounds, which consist of four pyrrole rings, fall into this category (18). Both gram positive and gram negative bacteria feature specific transporters for corrinoids (i.e. *Btu*FCD and *Btu*BFCD, respectively) (252). Cobalamin

(i.e. vitamin B_{12}) is such a corrinoid, which is actively transported through the bacterial membrane. Cobalamin and its analogues have been identified in human faeces and are likely produced by members of the gut community. However, not all prokaryotes in the human gut can synthesize cobalamin. For instance, *Bacteroides thetaiotaomicron* contains multiple transporters for the uptake of externally available cobalamin(253), suggesting corrinoid cross-feeding in the gut.

Vesicle-mediated transport

Membrane vesicles (MVs) are small, spherical encapsulations that form via protrusion of the outer membrane and subsequent pinching off from the cell (271-273) (Fig. 4 C,D). For this reason, MVs consist primarily of outer membrane material (i.e. proteins, lipopolysaccharides, phospholipids) and encapsulate periplasmic components such as proteins (274-277), enzymes (48, 278, 279), nucleic acids (280), or signalling molecules (281, 282). In addition, bacterial MVs are well-known shuttles for communication signals, which are especially common in pathogenic bacteria (272, 275, 283). As such, MVs provide an enclosed, protected environment for the exchanged molecules from the external chemical milieu.

Currently, two types of membrane vesicles are known from bacterial isolates. The first and most common type are outer membrane vesicles (OMVs). The second kind of vesicles is called outer-inner membrane vesicles (O-IMVs). These O-IMVs are formed by the protrusion of both the inner and outer membrane and contain cellular contents, especially nucleic acids (254). O-IMVs have been detected in *Shewanella vesiculosa*, *Pseudomonas aeruginosa*, *Neisseria gonorrhoeae,* and *Acinetobacter baumanii* (255).

Membrane vesicles are common and produced by a variety of different bacterial species. Until recently, research on MVs has mainly focussed on their role in transporting virulence factors from bacteria to host cells. Hence, little is known on whether MVs are also involved in transferring nutrients between bacterial cells. An exception is a study, in which the marine cyanobacterium *Prochlorococcus* was shown to release large quantities of OMVs (86). Besides DNA and RNA, OMVs also contained protein, which supported the growth of other marine bacteria such as *Altermonas* sp. and *Halomonas* sp., indicating cross-feeding of organic carbon. More work is necessary to fully evaluate the role of MVs as a means to shuttle nutrients between bacterial cells.

2.5.2 Contact-dependent mechanisms

Contact-dependent means of metabolite transfer are per definition based on a physical contact between interacting cells and in some cases, involve dedicated structures to shuttle materials from one cell to another one. Thus, these types of mechanisms require not only an increased energetic investment to establish these structures, but also a strategy to find and connect to suitable interaction partners. General advantages of this mode of transfer are that the exchanged molecules are protected from the extracellular milieu and that interactions partners can potentially be specifically chosen.

Vesicle chains

OMVs are not only used as transporting agents themselves, but also as building block materials to establish cell-cell conduits (Fig. 4E). For instance, predatory bacteria of the species *Myxococcus xanthus* link multiple individual membrane vesicles together to from so-called vesicle chains (87). The MVs within these chains contain lipids, sugars (fucose and mannose), carbohydrates (N-acetylglucosamine and N-acetyl-galactoseamine), and certain proteins that are required for coordinated movement (CglB and Tgl) (284). Vesicle chains provide an intercellular network for material transport. The molecular details, however, of how materials are transported within these interconnected vesicles remain unknown.

Nanotubes

Advancement in imaging techniques to study cocultures of interacting bacteria has led to the discovery of several structures that might be used to transfer cytoplasmic materials between bacterial cells (Fig 4F). For example, unshaken cells of *Bacillus subtilis*, for example, use nanotubes for shuttling cytoplasmic proteins and plasmid DNA to cells of the same or different bacterial species (84, 85). These tubes were observed to connect neighbouring cells (intercellular nanotubes) as well as extending from cells into the surrounding (extending nanotubes) (85). The membranous envelope of these structures was found to be constricted at certain points, giving the tube a sequential, bead-like appearance with a continuous lumen that is similar to the abovementioned vesicle chains (85). In another study, nanotubes were found to be used to transport essential amino acids between auxotrophic genotypes of *E. coli* that have been incubated under shaking conditions (83). Here, intercellular connections consisted of membrane-derived lipids, showed a continuous lumen, and were used to transport cytoplasmic materials between

bacterial cells of the same or different species. In both nanotube-forming species (i.e. *B. subtilis* and *E. coli*) it remains unclear whether interaction partners are actively chosen (e.g. by receptors on the cell surface or chemotaxis) or if cell-attachment is unspecific (e.g. mediated by non-specific adhesins or sticky polymers).

Flagella-like filaments

A contact-dependent exchange of metabolites does not always rely on dedicated structures such as membrane vesicles or nanotubes, but can also be facilitated by already existing structures that are repurposed (Fig. 4G). The fermentative bacterium *Pelotomaculum thermopropionicum* was shown to form aggregates when cocultured with the methanogen *Methanothermobacter thermautotrophicus* to facilitate the transfer of hydrogen (80). Analysis of these aggregates indicated that flagella were mediating this interaction (214). Gene expression analysis confirmed that binding of a flagellin protein (i.e. FliD) induced an up-regulation of genes for enzymes involved in methanogenesis. Thus, the flagellum is not only used to ensure physical proximity, but also to synchronize the metabolism of both interacting partners.

Cell-cell contact

The formation of extracellular appendages like nanotubes likely represents a significant cost to nutrient-limited cells, which should be avoided by cross-feeding bacteria. When cells are in a close physical contact such as within multicellular aggregates, the metabolite exchange is likely assisted by direct membrane contact (Fig. 4H). The green sulfur bacterium *Prosthecochloris aestaurii* for example is photoautotrophic, yet requires an electron donor to grow. The latter can be provided by a heterotrophic partner such as *Geobacter sulfurreducens* (81), which supports growth of *P. aestaurii* when both partners show an intimate cell contact. Additionally, a trans-outer membrane cytochrome complex in *G. sulfurreducens* was shown to be essential for cross-feeding of electrons. Another case of direct cell contact mediating an exchange of cytoplasmic materials was observed in a synthetic consortium of *Clostridium acetobutylicum* and *Desulfovibrio vulgaris* Hildenborough (82). In these cocultures, *D. vulgaris* could grow despite its inability to grow in monoculture. Differential labelling of cytoplasmic membrane and the peptidoglycan showed the absence of the peptidoglycan layer in the region of cell contact (82).

3 The evolution and maintenance of metabolic cross-feeding interactions

The reported ubiquity of metabolic cross-feeding interactions in bacteria raises a fundamental question: Why should bacterial cells start to actively invest resources to benefit other, potentially unrelated individuals? Natural selection predicts that organisms should maximize their fitness at the expense of others. How does this reconcile with an exchange of biosynthetic products that, in many cases, incurs significant fitness costs to the producing cell. For cooperative interactions, such as an exchange of costly metabolites, evolutionary theory predicts strategies should be favoured that reap cooperative benefits without reciprocating (45). These non-cooperating types, which utilize exchanged metabolites without contributing to their production, gain a significant fitness advantage over cells carrying this burden. Ultimately, the short-term advantage gained by non-cooperators can, at least theoretically, result in an extinction of cooperating genotypes (61, 285) ,thus representing a permanent threat to the existence of cooperative cross-feeding interactions. Consequently, a theory to solve this so-called *tragedy of the commons* needs to not only explain the emergence of reciprocal cross-feeding interactions, but also to provide mechanisms that can help explain the persistence of these cooperative relationships in the long-run.

The problem, however, is multi-faceted, since different levels of biological organization can affect the dynamics of cross-feeding interactions in different ways. This is because cross-feeding interactions are strongly influenced by, for example, (i) the mode of function and regulation of the individual enzymes involved in the biosynthesis of the exchanged metabolites, (ii) the cellular allocation of limited resources (e.g. nutrients, expression machinery, space) to the cellular functions that are required for the cross-feeding interaction, as well as (iii) the biotic composition of the bacterial community that determines the frequency of potential producers and consumers of exchanged metabolites. Also features of the ecological environment like i) the diffusibility of chemicals, (ii) the availability of nutrients, or (iii) the degree of spatial structuring will decide about the evolutionary fate of a metabolic cross-feeding interaction.

Hence, understanding the evolutionary trajectories that lead to obligate cross-feeding interactions requires the identification of the metabolic, physiological, and ecological factors enabling metabolite exchange as well as the evolutionary mechanism stabilising these interactions in the bacteria's natural environment.

3.1 Metabolic factors

3.1.1 Economics of microbial metabolite trade

Any biosynthetic function that consumes resources incurs a metabolic cost to the cell, because the used resources are not available anymore for other cellular processes. In general, bacterial cells face the problem to optimally allocate limited resources to different cellular functions (286). One particular resource allocation problem is the distribution of fluxes (i.e. the rate at which a metabolic substrate is converted to a specific product) through the metabolic network to optimally provide building block metabolites like amino acids, nucleotides, or lipids for cell growth (287). The biosynthesis of each of these metabolites has a metabolic cost that depends on the resource requirement of the corresponding biosynthetic pathway. As cellular resources and available nutrients in the environment are usually limited, the anabolism of a bacterium is closely linked to its fitness (169). As a consequence, a resource-efficient and tightly controlled metabolite production is vital for an organism to successfully compete with other co-occurring species for limited resources. In the case of cross-feeding interactions, each of the two interacting partners invests parts of its resources into the production of shared metabolites. A potential explanation that can account for this behaviour is a division of metabolic labour: the costs for producing increased amounts of metabolites to allow growth of interaction partners may be less than the energy saved for not having to produce other metabolites that each cell receives in return (102).

This situation strongly resembles trading interactions in human societies and there is growing appreciation in the scientific literature that the advantage of metabolic trade in bacterial communities can be assessed by applying economic models (288),(289). One particular useful concept to investigate biochemical interdependencies between microorganisms is the theoretical framework of *comparative advantages*. In 1814, David Ricardo developed this economic theory to explain how two countries could benefit from international trade (290). Comparative advantages can quantitatively explain how the resource costs to produce required goods (e.g. metabolites) can translate into mutual benefits if two parties (e.g. different bacterial species) engage in trade of the respective goods (Fig. 5). Hence, such comparative advantages are likely important preconditions for the evolution of specialisation and cooperative biological trade (289).

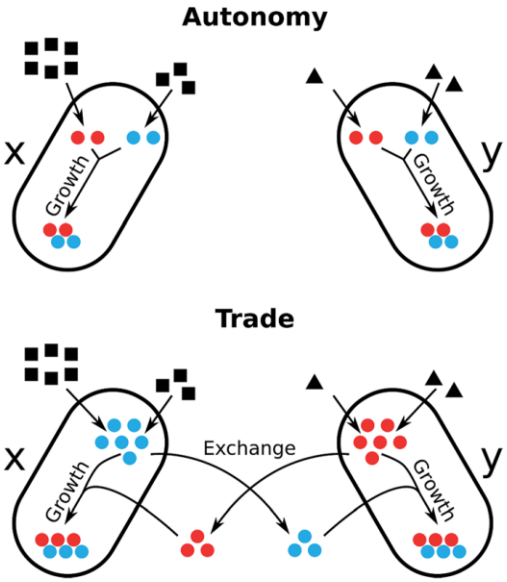

Figure 5: Economics of microbial metabolite trade and the role of comparative advantages. The scheme depicts the consequences on cell growth resulting from two opposing metabolic strategies, metabolic autonomy (above) and metabolite trade (below), in the presence of comparative advantages. Two bacteria (*x* and *y*) require two metabolites (red and blue) for cell growth. Each organism uses a different substrate from the environment and is able to produce each metabolite from the respective substrate. Both organisms differ in their metabolic costs to produce the two metabolites: bacterium *x* requires 3 units of its substrate to produce 1 unit of the red metabolite and 1.5 units substrate to produce the blue metabolite. In contrast, bacterium *y* requires 0.5 units of its substrate to produce the red metabolite and 1 unit substrate to synthesise the blue metabolite. Hence, organism *y* has an absolute advantage to produce both the red and the blue metabolite, as it requires less units of resources to synthesize them compared to bacterium *x*. However, organism *x* has a comparative advantage to produce the blue metabolite, because it can produce twice as many blue as red metabolites when it reallocates all resources from the production of red metabolites to the synthesis of blue. Analogously, organism y has a comparative advantage to produce the blue metabolite over the production of the red metabolite. Let us assume that red and blue metabolites are required in equal quantities for cell growth. In case of metabolic autonomy, bacterium *x* requires 9 units of its resource to produce 2 units each of the red and blue metabolite. *y* requires 3 units of its resource to produce the same amounts. If each organism specialises for the biosynthesis of the metabolite for which it has the comparative advantage (*x*: blue, *y*: red) and trades half of the produced metabolites with the other organisms, each organism can dedicate 50% more of each metabolite to its growth, while consuming the same amount of resources. Thus, the trade of the red and blue metabolites can be mutually beneficial to both organisms. Adapted from (288).

Also, trade-offs in the cellular metabolic networks of single organisms could explain the benefits of metabolite exchange between different cells. Metabolic trade-offs occur if improving the metabolic cost efficiency of one metabolic process or pathway (e.g. due to adaptations) is coupled with increased costs for a different process. Such biochemical conflicts are known to play a central role in the evolution of specialisation (291) and several trade-offs have been identified for a wide range of different metabolic processes in bacteria (for a recent review see Ferenci, 2010 (292)). Thus, trading metabolites may allow bacteria to increase resource efficiency by segregating conflicting metabolic pathways into separate cells (293).

3.1.2 Molecular basis for comparative advantages and biochemical conflicts in metabolite production

Several studies stress the importance of metabolic trade-offs (16, 19, 291) and comparative advantages (288, 294) for the adaptive evolution of cooperative cross-feeding interactions. However, what molecular mechanisms could cause trade-offs and/or comparative advantages in metabolite production within bacterial communities? Quantitative fitness consequences of metabolic trade in synthetic bacterial communities are often explained by the architecture of the underlying metabolic network or the topology of the corresponding biosynthetic pathway (174, 216). This is because the structure of a species' metabolic network, which determines its ability to produce a given metabolite from available substrates (Fig. 6), is known to determine metabolite production costs (295-297).

Molecular causes of comparative advantages

Differences in the architecture of metabolic networks and/ or different resource preferences between bacterial species can entail different costs for metabolite production. This can cause reciprocal comparative advantages that can promote the evolution of cross-feeding interactions. Since different species often differ in the structure of their metabolic network, it is likely that these species also differ in their biosynthetic costs to produce different metabolites (298). Hence, reciprocal comparative advantages likely exist between phylogenetically distant species, which also differ in the structures of their metabolic networks (288).

Another possibility of how the metabolic network structure can generate comparative advantages is different substrate preferences among bacteria. Coexisting bacterial species frequently utilise a distinct sub-set of carbon sources that are available in the environment (57, 299). Different carbon sources often enter the metabolic network at different locations (Fig. 6B). It has been shown that the point, at which a carbon source enters the central metabolic network, strongly affects the distribution of metabolic fluxes (300, 301) and, in this way, also the production costs of individual amino acids (296). Hence, a consequence of diverse carbon source preferences is that one species of the bacterial community can have a comparative advantage in the biosynthetic cost efficiency of a specific set of metabolites over another species, which in turn has a comparative advantage in the production of another set of metabolites (Fig. 6C).

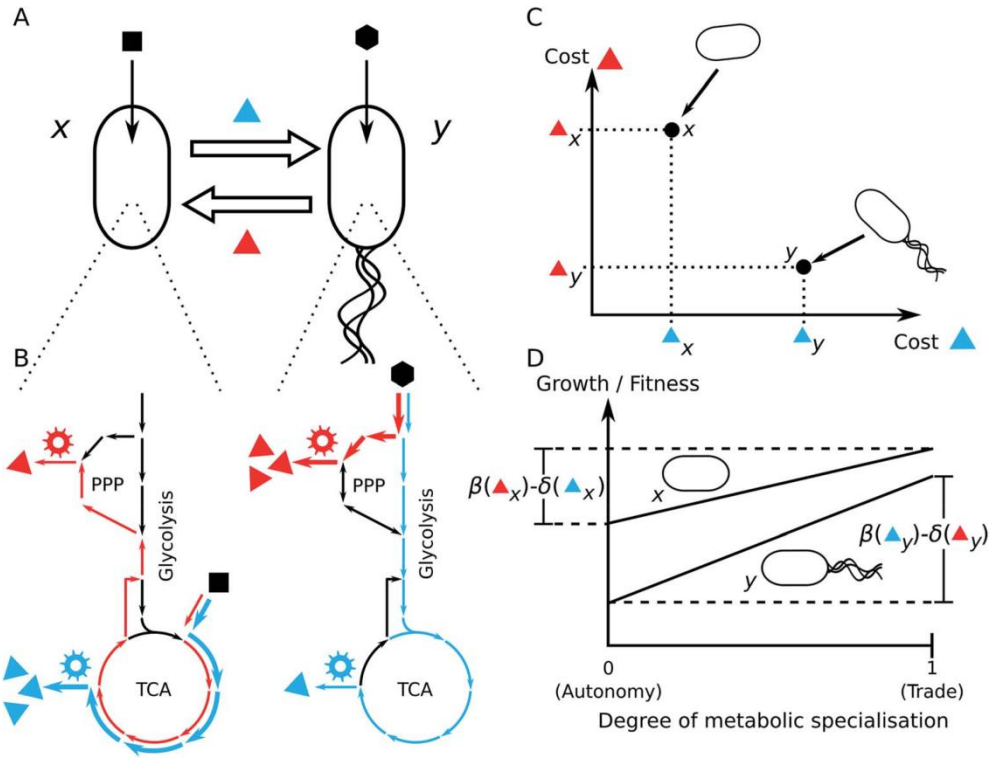

Figure 6: Possible advantages of metabolite cross feeding. (A) Cross-feeding interaction of two metabolites (blue and red triangles) between two different bacterial strains x and y. Each strain uses a different growth substrate (black square/ hexagon). (B) Scheme of the central metabolic networks including glycolysis, Pentose Phosphate Pathway (PPP), and TCA cycle. The two distinct substrates enter the metabolic networks at different positions. The reactions that are significantly involved in the chemical transformation from the substrate to the exchanged metabolites are highlighted in blue or red, respectively. Gear wheels denote the biosynthetic machineries (i.e. enzymes) that utilise precursor metabolites from central metabolic pathways for the production of the focal metabolites. (C) Schematic diagram of the differences of metabolic costs to synthesize the blue and red metabolites between the different strains. (D) Growth and fitness consequences of metabolic trade. The effect of metabolic trade for strain x is thus a function of the benefits β for not having to synthesise the red metabolite minus the costs δ that are associated with the overproduction of the metabolite that is produced to cover the demand of strain y. The fitness/growth effect of the cross-feeding interaction for strain y is determined by the benefits β that y receives by not having to synthesise the blue metabolite minus the costs δ to synthesise the red metabolite for strain x.

Comparative advantages can also arise in bacterial communities due to spatial structure. In a spatially structured bacterial population, cells may experience unequal access to different resources due to a heterogeneous distribution of chemicals on a micro-scale (302). For instance, in bacterial biofilms, nutrients are mainly accessible for cells residing close to the surface. In contrast, excreted metabolic by-products are likely enriched in the inner part of the biofilm and therefore more available to cells that dwell in the subsurface (302, 303). In fact, it has been observed for a variety of different

biofilm-forming bacteria that cells exhibit distinct metabolic phenotypes depending on their positioning within the biofilm and thus, the local environmental conditions to which cells are exposed (304). Such differences in metabolic phenotypes may cause reciprocal comparative advantages in the production of different metabolites that can promote cross-feeding interactions between different cells within the biofilm.

However, heterogeneity in metabolic phenotypes within bacterial populations is not limited to spatially structured environments or species with different genetically determined metabolic capabilities. Phenotypic heterogeneity can also arise in homogenous environments (305, 306), which in turn can give rise to reciprocal comparative advantages between different phenotypes, thereby promoting a cooperative exchange of metabolites. Bacterial populations frequently display heterogeneity, where two essential metabolic functions are partitioned between two subpopulations. Prominent examples are nitrogen fixation and photosynthesis in cyanobacteria (307) or acetate and acetoin production in *Bacillus subtilis* populations (308).

Molecular causes of biochemical conflicts

Biochemical conflicts between two metabolic functions commonly arise due to resource allocation trade-offs (292). Different metabolic functions usually compete for the same cellular resources, e.g. the same precursor metabolites, ATP, or the use of the cellular transcription-/ translation machinery (295). Importantly, resources that are consumed by one metabolic function are not available anymore to another one. Thus, the metabolic flux through one pathway might limit the activity of other metabolic processes (309, 310). Segregating such antagonistic biochemical processes into different bacterial cells can resolve the biochemical conflict between them (291).

The above-mentioned examples illustrate that comparative advantages as well as biochemical conflicts in metabolite production between co-occurring organisms are prevalent in natural bacterial communities and are thus important determinants for the evolution of cooperative metabolite exchange. While biochemical conflicts and comparative advantages can explain the mutual fitness benefit that results from metabolic cross-feeding interactions, it is important to note that in isolation they are not sufficient to explain the evolution of cooperative interactions, since they do not provide a mechanism to prevent the exploitation of exchanged metabolites by non-cooperating cells (see 3.4).

3.1.3 Metabolite leakage: the first step towards the evolution of metabolic interactions

Many metabolic functions are *leaky*, which means that the products of these biochemical transformations are released into the extracellular environment, thus making them available to other cells (311, 312) (Fig. 7A,B). Metabolite leakage can facilitate the evolution of unidirectional by-product cross-feeding interactions as well as metabolic interdependence (Fig. 7A). This is because neighbouring cells can take advantage of the released resource, thus saving the costs of producing these metabolites autonomously (see section 3.1.4). Cells, which use the metabolic by-products of other cells, adjust their metabolism by redistributing metabolic fluxes, which in-turn can cause leakage of other metabolites. The resulting mosaic of different metabolic strategies potentially provides the basis for the emergence of new metabolic dependencies (Fig. 7B,C).

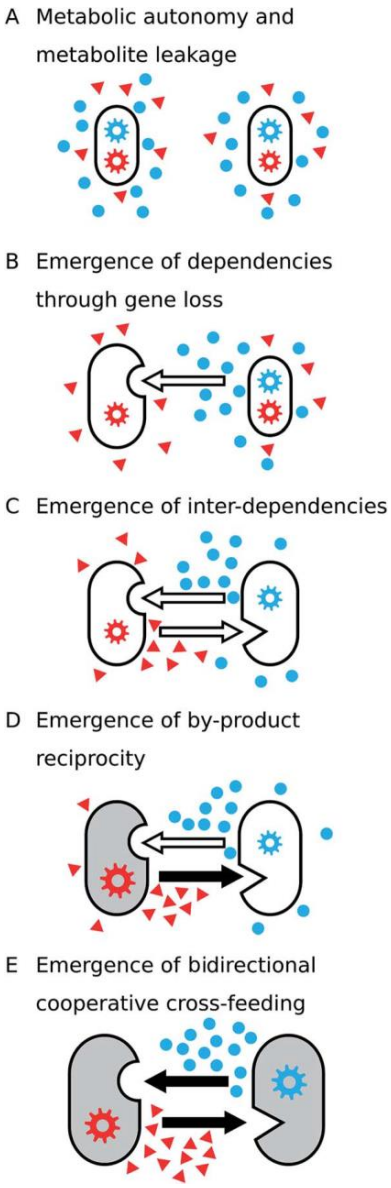

Figure 7: Hypothetical model to explain the evolution of cooperative metabolic cross-feeding. (A) Initially, each of two strains have the metabolic capacity (gear wheels) to synthesize the two growth-required metabolites (red triangles and blue circles). Besides the biosynthesis for cell growth, both strains release a fraction of the produced metabolites into the environment (i.e. metabolite leakage, see section 3.1.3). (B and C) Metabolites that are released as by-products are available to neighbouring cells. Losing the capacity to synthesize one of the focal compounds and use environmental pools instead (empty arrows) provides a growth advantage (see section 3.1.4) and thus results in the establishment of (B) unidirectional and eventually (C) bidirectional cross-feeding interactions. (D) When by-products are reciprocally exchanged, one partner can benefit from unilaterally increasing its metabolite production (filled arrow), because it automatically increases the amount of metabolic by-products (here: blue metabolite) it receives in return. (E) Cooperative cross-feeding interactions emerge if each of the involved organisms starts to actively invest resources into metabolite production to benefit the respective partner. Benefits received by the organisms in each step are indicated by increasing cell size.

3.1.4 Emergence of by-product cross-feeding through gene loss

Loss or deactivation of a metabolic gene by mutation can render the survival of the resulting auxotrophic mutant contingent on an environmental supply of the focal metabolite. Potential sources for this metabolite are besides decaying organic matter, mainly other eukaryotic (313-315) or prokaryotic organisms in the mutant's environment (316-318). Thus, the mutational loss of a conditionally-essential biosynthetic gene is a key step towards the establishment of an obligate metabolic cross-feeding interaction (Fig. 7B).

Auxotrophies are common in nature

In nature, bacterial genome sizes vary greatly (319) ranging from the largest genomes of about 14 Mb (i.e. *Sorangium cellulosum*) (320) to the smallest known genomes of 0.16 Mb (i.e. *Candiatus* Carsonella) (321). The lower end of this spectrum includes many genomes, which are significantly smaller than the estimated minimal genome size for *autonomous* bacterial growth and survival of ~400 kb (321). Interestingly, even seemingly identical members of the same bacterial species can differ greatly in their gene repertoire (322, 323). Observation of this recurring pattern in several bacterial taxa like *Salmonella*, *Escherichia*, and *Prochlorococcus* has led to the development of a concept called the *pan-genome* (324-326). In this framework, all genes that are found in all isolates/ genotypes of a given species are called the *core* genome, while genes that are only present in some genomes are referred to as the *pan-* or *auxiliary* genome (325). Systematic analyses, in which the ability of known bacterial genomes to produce all primary metabolites a bacterium required for growth was scrutinized, revealed that in fact 76% of all 949 eubacterial genomes analysed were unable to produce at least one of 25 different metabolites (63). Interestingly, the list of predicted auxotrophic taxa did not only contain endosymbiotic bacteria, but also many bacteria with a free-living lifestyle. Hence, metabolic auxotrophies are likely common in natural microbial communities.

3.2 Evolutionary mechanisms driving the loss of biosynthetic genes and functions

What drives the loss of biosynthetic functions from bacterial genomes? Two main evolutionary mechanisms have been suggested to account for these losses: adaptive advantages and genetic drift. Mutants that have lost the ability to autonomously produce a certain metabolite can be selectively favoured over metabolically autonomous genotypes, when the focal compound is sufficiently available in the cell's environment.

Alternatively, even if auxotrophic mutants suffer of a reduced fitness relative to prototrophic cells, random genetic drift in small bacterial populations (e.g. of a bacterial endosymbiont) can result in the fixation of these maladaptive mutations on a population-level. Thus, both the environmental availability of metabolites and the size of bacterial populations need to be taken into account when explaining the widespread distribution of biosynthetic loss-of-function mutants in natural microbial communities.

3.2.1 Adaptive loss of biosynthetic functions in metabolite-rich environments

In principle, two evolutionary explanations can account for an adaptive loss of genes. First, selection is expected to remove a subset of genes from a bacterial genome that might not be essential in a given environment. Retaining genes that do not contribute to a bacterial cell's fitness is costly, because of the burden resulting from the functioning of the corresponding gene products within the cellular context (327). Moreover, the expression of unneeded proteins reduces the amount of resources that are available for other cellular processes (328, 329). This is why mutants that lack these non-required functions may be adaptively favoured and thus increase in frequency relative to types that still carry these genes. This process, which is called *genome-streamlining* (330, 331), can be considered as a way to cellular economization (330, 332). This process should be particularly important in large bacterial populations, where the effect of natural selection is very strong (332-334). As a consequence, any fitness-enhancing mutation including loss-of-function mutations (e.g. deletions, frame-shifts) will be fixed in the population. Indeed, many free-living bacteria such as *Prochlorococcus* (335) or *Candiatus* Pelagibacter ubique (334), which are oligotrophic and live in aquatic ecosystems that are relatively nutrient-deficient yet stable in terms of resource turnover (336), feature genomes of reduced sizes.

The second possibility is that selection favours a loss of biosynthetic functions in bacteria, when the resulting metabolic deficiency can be compensated by an environmental uptake of the corresponding compound. Indeed, several laboratory-based studies with *Bacillus subtilis* (337), *Escherichia coli* (63, 68) , and *Pseudomonas fluorescens* (338) clearly showed that amino acid auxotrophic bacterial strains gain a significant growth advantage (i.e. up to 20% relative to their prototrophic counterpart) when the metabolite they require for growth was sufficiently available in the environment. What can explain the strong fitness advantage observed in auxotrophic genotypes? Zamenhof and Eichhorn (1967), who first described this phenomenon, suggested that when the metabolite is present in the extracellular environment, bacteria

that shut down their endogenous machinery to produce the metabolite gain a selective advantage over prototrophic cells, because they save the costs associated with producing the metabolite (337). Costs that could be saved by auxotrophic bacteria include (i) *energetic costs* that are required to drive biochemical reactions (295, 297), (ii) *ribosome costs* that accrue for building the translational machinery (328, 339), (iii) *protein costs* that stem from the need to produce the biosynthetic protein machinery (339), as well as (iv) *carbon costs* that result from the allocation of raw materials to produce the focal metabolite (296). All in all, a significant proportion of a bacterial cell's energy budget is allocated to amino acid biosynthesis (297). Given that many natural habitats of bacteria are rich in metabolites that bacterial cells require for growth (e.g. amino acids (340, 341), vitamins (342), and nucleobases (343)), it appears plausible that natural selection may favour auxotrophic mutants that save the costs of metabolite production in these environments.

Compelling evidence for the importance of natural selection for driving gene loss in bacteria comes from several evolution experiments (327, 344). Lee and Marx (2012) found that non-essential, accessory genes were frequently lost from almost 80 % of evolving *Methylobacterium extorquens* AM1 populations that adapted to minimal medium (344). In this case, gene loss was accompanied by an increase in fitness, suggesting that selection favoured the loss of unneeded genes when adapting to a specific environment (344). In another study, Koskiniemi (2012) tested the fitness consequences (327) of losing stretches of DNA from the genome of the bacterium *Salmonella enterica* and found that fitness-increasing deletions were rapidly fixed in populations that had been serially propagated in the same nutrient environment. Furthermore, *E. coli* populations that were selected in an amino acid-containing environment frequently lost the ability to autonomously biosynthesize these metabolites, with the evolved auxotrophies conferring an adaptive advantage (68).

Mutational deactivation versus transcriptional downregulation of metabolic genes

If gene loss is so beneficial, why then do bacteria not downregulate their biosynthetic machinery when the corresponding product is sufficiently available in the extracellular environment? In this way, cells could enjoy the benefits resulting from gene deactivation, yet retain the ability to grow autonomously when external metabolite pools are depleted. Two main reasons likely explain why a mutational gene loss or deactivation is likely more important in the context of metabolic cross-feeding

interactions than a regulatory inactivation of the same biosynthetic pathways. First, the ability to sense environmental conditions in order to determine whether or not it is beneficial to switch from an autonomous metabolite production to an environmental uptake requires the maintenance of extensive sensory and regulatory machinery. The production and maintenance of such a system likely requires a significant investment of resources and these costs would have to be outweighed by benefits resulting from it - even if the system remains in a certain configuration for extended periods of time. Second, a cell that is able to switch between an environmental uptake and an autonomous metabolite production has to be fitter than a cell, which specializes in just one strategy. A significant factor that works to the disadvantage of a regulation-based phenotype is the time and energy it takes to switch between both states. Indeed, a prototrophic genotype of *E. coli* that was cultivated in a minimal medium only rarely used environmentally supplied amino acids, while an auxotrophic loss-of-function mutant of the same genetic background gained a significant fitness advantage from tapping this resource (63). Even though it is not known at the moment whether the same pattern is true for other species as well, the above example clearly illustrates that auxotrophic and prototrophic cells are in different physiological states (83, 345, 346) and that at least prototrophic *E. coli* do in the presence of amino acids not transition to become functional auxotrophs (63).

3.2.2 Random genetic drift

The second main evolutionary mechanism that has been suggested to explain the loss of biosynthetic genes from bacterial genomes is random genetic drift. In populations of small size, random changes in allele frequencies can result in the fixation of maladaptive genes. Accordingly, genetic drift has been suggested to be the main cause for the extreme genome reduction that is commonly observed in endosymbiotic or endoparasitic bacteria (316, 347, 348). Several arguments seem to support this interpretation.

First, bacterial populations within host cells are usually small (10^3-10^4 cells ml^{-1}) and are subject to repeated reductions in their size (i.e. *population bottlenecks*) during transmission from parent host to its offspring (313, 348, 349). A reduction in effective population sizes (N_e) can greatly affect the impact of genetic drift (334, 349). N_e is the size of an idealized population that experiences the same magnitude of genetic drift as an existing population (334, 349, 350). However, this important parameter is difficult to estimate due to the stochasticity of genetic drift (332, 334). Accordingly, the fixation

probability of a mutant allele in a given populations depends on the product of N_e and s (i.e. the coefficient of selection) (332, 334). When $N_e s > 1$, the fate of the mutant allele is primarily determined by selection, whereas when $N_e s < 1$, genetic drift determines the fixation probability of this allele (332, 334, 349, 350). Thus, when N_e is low, mutations with deleterious effects can persist and even become fied in the populations (349), because under these conditions, natural selection is less effective in eliminating deleterious mutation (332).

Second, intracellular bacteria live in a nutrient-rich and rather constant environment. Thus, losing essential biosynthetic functions may not be penalized as strongly as is the case for bacteria living in nutrient-limiting conditions.

Third, reduced or absent levels of recombination in the intracellular environment of the host significantly restrict the opportunity to purge deleterious mutations (348, 349). As a consequence, deleterious mutations even in key biosynthetic genes accumulate in the genome - a process, which has been termed *Muller's Ratchet* (332, 348). Given that deletions of genes from bacterial genomes appear to be much more common than insertions (331, 351), *Muller's Ratchet* is likely an important evolutionary force to account for gene loss in small bacterial populations. Indeed, *Salmonella typhimurium* populations that were repeatedly subjected to single-cell bottlenecks in rich medium revealed slow-growing phenotypes and auxotrophic loss of function mutants, which the authors interpret as evidence for an accumulation of deleterious mutations (352). Overwhelming evidence for the loss of biosynthetic genes from endosymbiotic or endoparasitic genomes stems from comparative genomic studies (315, 316, 353). Unfortunately, due to difficulties to cultivate the bacterial strains involved, it is often not possible to quantify the fitness consequences resulting from gene loss for the corresponding genotypes. Moreover, it often remains unclear whether drift or selection caused the observed pattern.

3.3 Consequences of biosynthetic gene loss

3.3.1 Cellular physiology: Optimization of metabolic uptake

The mutational inactivation of a conditionally-essential biosynthetic gene can profoundly affect the physiology and behaviour of the ensuing auxotrophic genotype. Many of these changes help the nutrient-deprived cell to cope with starvation for the focal metabolite. For instance, it has been shown that amino acid auxotrophic *E. coli*

strains increase the expression levels of genes associated with cross-membrane transport of the required metabolite (63, 354)

Moreover, the loss of a biosynthetic gene that causes metabolite starvation is well-known to trigger a stringent cellular response, which globally reorganizes the metabolism to economise available resources (345, 354). Another consequence of auxotrophy-causing mutations, which has been observed in several species, is the formation of intercellular nanotubes (83, 84) (Fig. 4F). These physical intercellular connections allow the auxotrophic mutant to derive the focal metabolite from other cells that are still able to produce the required metabolite. Taken together, the abovementioned cellular responses to biosynthetic gene loss represent immediate physiological consequences that promote the evolution of cross-feeding interactions by either helping to establish unidirectional interactions or by adjusting their own metabolic processes to efficiently use the exchanged compounds.

3.3.2 Cellular interactions: The emergence of by-product reciprocity

A central step towards the evolution of cooperative cross-feeding interactions is the transition from pure by-product cross-feeding between two interacting organisms (Fig. 7C) to an interaction, in which costly metabolites are actively produced by one individual to benefit the corresponding partner (Fig. 7E). This transitional step includes the so-called *by-product reciprocity* interactions, where one organism performs a costly cooperative act such as an enhanced production of a metabolite, which is consumed by the corresponding partner. The actively overproduced metabolite benefits the recipient organism by fuelling its metabolic processes. As a consequence of the enhanced metabolic activity, the recipient organisms release higher amounts of metabolic by-products, thus benefiting the organisms that perform the cooperative act (Fig. 7D) (25, 355, 356). It is well-known that increasing the metabolic rate of a microorganism (i.e. population growth) also elevates the amount of metabolic by-products that are released (25, 203). Thus, for two organisms that reciprocally exchange metabolic by-products, any mutation that will increase the production levels of the exchanged metabolite in one of the two partners, will be immediately rewarded by increased return levels. This automatic feed-back not only stabilizes the costly cooperative investment, but also paves the way for the evolution of reciprocal cooperative cross-feeding interactions.

3.4 Evolution of cooperative cross-feeding

The challenge with explaining the evolution of cooperative cross feeding lies in the fact that cooperation, such as the overproduction of metabolites, is costly. Thus, non-cooperating free-riders that do not contribute to the production of the traded metabolite may still benefit from the cooperative interaction. Consequently, whenever both cooperators and non-cooperators have an equal probability to gain access to the cooperative public good, natural selection predicts non-cooperative genotypes to increase in frequency, which may ultimately result in a collapse of the cooperative interaction (45, 51, 58, 59). Another potential problem in complex microbial communities is that even if cooperative cross-feeding evolved, the respective genotypes need to ensure continued interaction with other, metabolically complementary genotypes (38). Hence, a key question in this context is which evolutionary mechanisms can stabilize metabolic cross-feeding within and between bacterial species.

An answer to this question needs to take both the ecophysiological intricacies of the focal interaction as well as its eco-evolutionary context into account (44). For example, metabolite exchange via diffusion through the extracellular environment intrinsically imposes several limitations on conditions under which cross feeding can exist. In well-mixed environments, these so-called *public goods* should theoretically be equally available to both producing and non-producing cells (38, 57) and are thus prone to exploitation. Alternatively, metabolites may be transferred in a contact-dependent manner (e.g. via intercellular nanotubes (83)), which restricts the access to the exchanged metabolites. The ecological conditions under which cross feeding between bacterial genotypes, showing either means of metabolite transport, can be maintained, is therefore drastically different, and will likely result in different evolutionary outcomes.

3.4.1 Positive assortment

Many different evolutionary mechanisms have been suggested to facilitate the emergence and maintenance of cooperative interactions in microbial communities (27, 44, 45, 70). Several of these have been identified for other forms of microbial cooperation than cooperative cross-feeding. However, in many cases, their logic applies equally well to interactions, in which costly metabolites are exchanged. A major conceptual advance in the field was the recognition by Fletcher & Doebeli (2009) that cooperation will be favoured by natural selection whenever mechanisms exist that either (i) increase the probability that cooperative phenotypes repeatedly interact with other

cooperators and/ or (ii) decreases the chance of cooperators to encounter non-cooperators (73). The beauty of this concept, which has been termed *positive assortment*, is that it accommodates both interactions within- and between-species. Previously, intraspecific cooperation was mainly explained by Hamilton's rule, which predicts that selection will favour cooperative behaviours, if the benefits to the recipient times the relatedness between actor and recipient outweigh the costs to the actor (51). The concept of relatedness, however, does clearly not apply to interactions between different species. This is why a range of different theoretical models and conceptual approaches had to be devised for this case (58, 72-78).

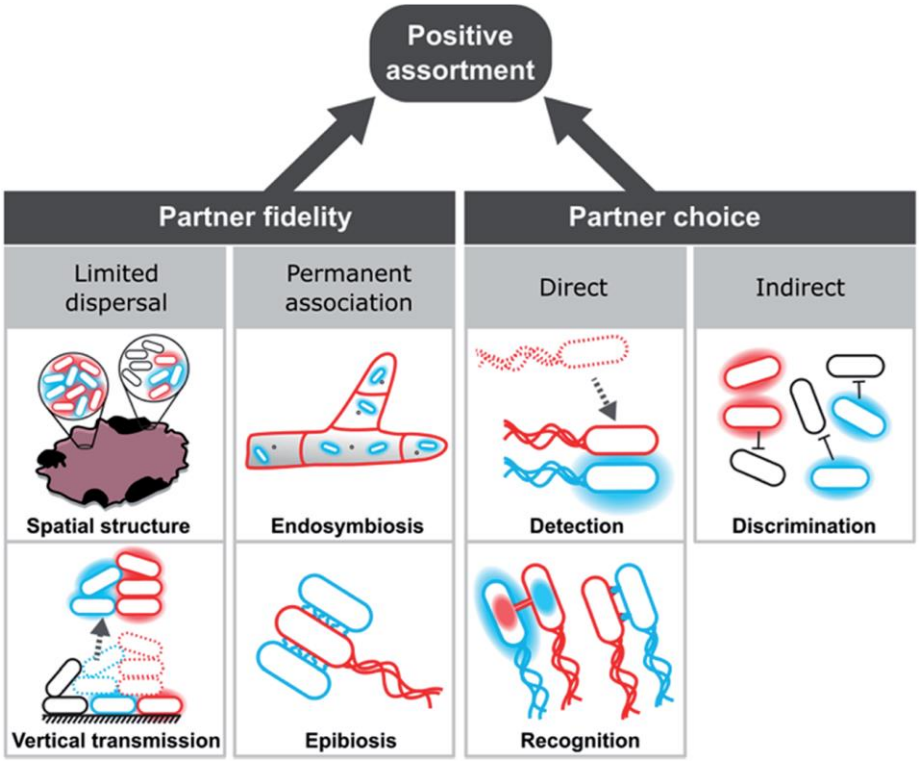

Figure 8: Evolutionary mechanisms promoting the emergence and maintenance of metabolic cooperation by positive assortment. Red and blue cells represent cooperating genotypes with halos depicting exchanged metabolites, while black cells indicate non-cooperating cells. Positive assortment of cooperating genotypes is either achieved by *partner fidelity* or *partner choice* mechanism. Partner fidelity results from *limited dispersal* in spatially structured environments or from budding/ fission events of multicellular clusters (vertical transmission). Alternatively, partner fidelity can be due to *permanent associations* between partners, as is the case for endosymbiotic or epibiotic interactions. Partner choice mechanisms can be direct or indirect, with the former being achieved by detection or recognition of potential partner cells, while the latter is due to an active discrimination against unsuitable or non-cooperative cells.

3.4.2 Mechanisms promoting positive assortment

In the following, we will classify mechanisms that have been previously suggested to facilitate positive assortment of cooperative phenotypes and which are relevant to the evolution of cooperative cross-feeding. Our framework divides mechanisms into two main categories (Fig. 8): i) *partner fidelity* (i.e. *staying together* (44))– i.e. mechanisms that ensure repeated interactions among cooperators due to a physical co-localisation, and ii) *partner choice* (i.e. *coming together (44))* – i.e. mechanisms that facilitate either the localisation and subsequent association with suitable interaction partners or the antagonizing of unsuitable interaction partners. Each of these main categories can be further subdivided. Partner fidelity can result from *limited dispersal* in spatially structured environments or be due to permanent adhesion among interacting cells when new groups of cells are formed (Fig. 8). Alternatively, *permanent associations* such as endosymbiosis or epibiosis can also facilitate repeated interactions among cooperative phenotypes.

Partner choice can be subdivided into *direct* and *indirect* mechanisms (Fig. 8). Direct partner choice can be due to *detection*, which involves the active finding and subsequent interaction with specific genotypes or *recognition* that allows cross-feeding genotypes to identify compatible genotypes. Alternatively, indirect partner choice mechanisms operate via the exclusion or inactivation of non-cooperating genotypes. The resulting local enrichment of cooperating cells can also facilitate positive assortment.

These mechanisms should not be seen as mutually exclusive, but multiple processes can operate simultaneously. The contact-dependent inhibition (CDI) system, for instance, exemplifies the combination of partner choice by specific adhesion with non-partner discrimination via inactivation of targeted cells (357). Here, a two-component secretion system facilitates binding and toxin delivery into target cells that display specific receptors. However, carriers of immunity proteins, such as close relatives of the cell expressing the CDI system, are unharmed. This system is widely distributed in alpha-, beta-, and gamma-proteobacteria (357) and can even mediate the specific assembly of multicellular biofilm communities (358, 359). Below, we will explain each of these mechanisms in more detail and highlight a number of relevant examples.

3.4.2.1 Partner fidelity

Limited dispersal

Limited dispersal refers to cases, in which groups of cells that exchange metabolites with each other, remain associated for extended periods of time. This type of increased population viscosity emerges in spatially structured communities that grow on surfaces or in multicellular aggregates.

In recent years, overwhelming evidence has accumulated that bacteria mainly grow attached to surfaces (i.e. a biofilm) or other bacteria (i.e. free-floating clusters), and thus prefer an aggregative lifestyle over a planktonic, free-living state (29, 360-362). Simulations demonstrated the formation of such groups can not only facilitate cooperative interactions (88), but also conflicts among group members (363). Hence, the benefit of a newly established cooperation needs to outweigh the negative effects of conflicts and increased competition due to group formation. Importantly, the formation of biofilms and cell clusters can be caused by a number of non-social reasons, such as the protection from the abiotic (oxygen, pH, and drought) and biotic environment (predation (93, 364), competition, and immune system), attachment to a local niche (energy- and carbon-sources), or joint niche construction and exploitation (synergism) (28).

Once established, several important consequences result for cells that grow in spatially structured communities. First, metabolites that are released into the cell-external environment potentially accumulate (303) and are thus increasingly available to resident cells. Second, local feedbacks increase the fitness of cell patches with a favourable combination of genotypes, while groups of incompatible types show weaker growth. This results in an interesting effect: the enhanced growth of cooperative groups leads to a spatial segregation of cooperating and non-cooperating cells and thus a spatial exclusion of non-cooperators from the shared goods (57, 89, 90, 92, 365). This pattern emerges due to a self-organization of initially well-mixed populations (89, 91, 365-368) and can even prevent the invasion of non-cooperative, motile cells (103). This aspect of matrix-assisted population structure can be conceptualized as a passive means of non-cooperator exclusion. In combination, these effects strongly favour the evolution and maintenance of cooperative cross-feeding interactions as evidenced by numerous theoretical (75, 366, 369) as well as experimental studies (89-92, 370-372). Empirical evidence from syntrophic bacterial communities further corroborates this interpretation: the spatial distribution of metabolically interdependent members appears to be key for functioning

of these consortia (373, 374). Taken together various insights point towards positive assortment by spatially structured populations or population viscosity to promote cooperation.

Bacterial cells that live in a spatially structured, aggregative community face the problem that with increasing cell density, competition for resources such as space and nutrients also increases. Thus, at one point, colonies need to disperse to populate new substrates. For cells that have started to engage in obligate cooperative cross-feeding, it is therefore crucial to remain associated with other, metabolically complementary cells. Since parts of biofilms are known to get detached (361), they likely function as a propagule to initiate a new biofilm. Notably, groups of cells that protrude from a biofilm or cell cluster are prone to be detached and dispersed more easily. In fact, this is the case for highly productive local groups (375). In this way, cells that are more cooperative are more likely to transmit offspring to the next generation, thus enhancing selection for cooperative phenotypes. This type of group dispersal, which represents a type of vertical transmission, ensures that complementary and potentially coevolved genotypes interact for extended periods of time (Fig. 8). This is also the case for planktonic macrostructures and multicellular magnetotactic prokaryotes (MMPs) that exhibit propagule formation and fission of whole cell groups, respectively (28, 376).

Permanent associations

Extreme cases of *staying together* are permanent associations (Fig. 8), where prokaryotic cells either live on (*epibiosis*) or in (*endosymbiosis*) another organism. Examples include associations among archaea (377) as well as interaction of bacteria with other bacteria (378-381), fungi (382-386), protists (387-391), or multicellular eukaryotes (389, 392, 393).

A characteristic feature of these associations is the enormous potential of vertical transmission over evolutionary time when new generations of host-symbiont interactions are established. The tight fitness coupling of cells living in such close associations aligns their evolutionary interests, thereby limiting conflicts among interacting partners. This is particularly promoted by the fact that the fitness of these consortia is often not a property of individual cells any more, but an emergent feature that results from the interaction among host and symbiont. Selection acting on this level is therefore expected to increase complementarity and enhance metabolic cooperation among partners. Indeed, known cases of both epibiosis and endosymbiosis are frequently based on the reciprocal

exchange of metabolites between both partners (Fig. 3B). Thus, to understand these systems it is key to identify the causal mechanisms that initiate the assembly of these associations as well as the evolutionary forces that can tip the balance in favour of cooperative cross-feeding.

The first step in the evolution of a stable *endosymbiosis* is that a bacterial cell enters the intra- or intercellular environment of its host. Even though cases exist where environmental, commensal or already mutualistic bacteria successfully established as endosymbionts (394), parasitic bacterial strains have an advantage. Their intrinsic ability to enter host cells or tissues despite the presence of anti-bacterial defence strategies (e.g. immune system) enables them to persistently colonize the host (382). This then provides the opportunity for natural selection to transform the initially antagonistic interaction into a mutually beneficial one. This is corroborated by phylogenetic studies showing that proteobacterial mutualists were more frequently derived from parasitic than from free-living ancestors (395). Even if bacterial lineages do not feature strategies to repeatedly re-infect their host or be vertically transmitted, the host might strongly benefit from evolving means to transmit beneficial symbionts to its offspring itself (394). Unfortunately, direct experimental evidence to identify the factors that complete the transition to an obligately endosymbiotic association is lacking, thus complicating the exact assignment of cause and effect. Two key requirements that likely need to be fulfilled are (i) a strict vertical transfer of bacterial symbionts across host generations and (ii) a mutual benefit arising from this interaction. Whenever these criteria are fulfilled, natural selection can act on the symbiotic interaction. Common outcomes of this coevolutionary process are a drastic reduction in genome size of the symbiotic bacteria (393, 396) as well as the emergence of a metabolic complementarity of host and symbiont, where many different metabolites, precursors, or biochemical functions are reciprocally exchanged (397).

The conditions favouring *epibiotic associations* are rather similar to the ones described previously for endosymbiotic interactions. The prospective epibiont needs to exhibit either features of a parasite or beneficial characteristics of a mutualist to allow repeated interactions with the respective host. In both cases, the ability to attach to the host is required, ultimately enabling the epibiont to exploit and adapt to the resources released by the host. This initially one-sided interaction opens the door to evolve a reciprocal exchange of metabolites, given that host and epibiont complement each other (387, 398). Benefits that are associated with this interaction for the host and/ or the

epibiont favour a permanent association. Host-epibiont coevolution as well as competition with other groups of hosts and epibionts should intensify reciprocal interactions like cooperative cross-feeding.

An example for epibiosis is the TM7 phylotype (TM7x) that is obligately associated with *Actinomyces odontolyticus* in the oral cavity (378). The epibiont TM7 features a drastically reduced genome (~700 genes) (378) and derives all of its amino acids from its host *A. odontolyticus*. In phases of extreme starvation, TM7 can even kill its host, thus classifying as a parasitic interaction. Another well-studied case is the phototrophic consortium *Chlorochromatium aggregatum*, in which a flagellated β-proteobacterium within the family Comamonadaceae is surrounded by *Chlorobium chlorochromatii*, a green sulfur bacterium (399). In these physiologically highly intertwined consortia, the central bacterium provides motility and receives photosynthetically fixed carbon in return from its epibionts.

3.4.2.2 Partner choice

Partner choice is the second main principle that facilitates positive assortment among cooperative genotypes via either selecting preferred interaction partners (*direct partner choice*) or antagonizing undesirable or unsuitable cells (*indirect partner choice*).

Important to recognize for partner choice mechanisms is that they inherently rely on a mixing of interaction partners after every round of association and disassociation. A consequence of this is that these intermittent periods of mixing reduce the chance to repeatedly encounter the exact same genotype in subsequent rounds of interaction. Under these conditions, mutations, whose effects are highly partner-specific, are less likely to spread in the global gene pool, simply because they reduce the number of potential interaction partners. On the other hand, more favourable combinations of interaction partners could result from this process than would be expected to emerge in a regime exclusively relying on limited dispersal. However, at the moment, the above-mentioned population-genetic consequences are purely hypothetical and await experimental validation.

Direct partner choice

Detection as a partner choice mechanism involves the ability of bacterial strains, which are unable to produce certain metabolites autonomously, to find and identify other cells that are capable of providing these metabolites. This can be achieved in different ways. First, other cells or groups of cells release the required metabolite into the

environment and the focal cell uses chemotaxis to trace the source of these metabolites. More cooperative cells will release more metabolites and are thus easier to find. Hence, the required metabolite is the most reliable indicator of a producing cell and should thus be the preferred cue for an auxotrophic cell. However, the focal cell can also use other chemicals to find suitable cells via positive chemotaxis. Here, in principle any metabolite, which can be sensed and followed along a concentration gradient, can serve as a cue. For example, quorum sensing, when used as a means of interspecific communication (400, 401), can reveal the species identity of the signalling cell and with it also the potential of competition (402) or metabolic compatibility (403). For interactions with higher organisms, bacteria are well-known to show a specific chemotactic response towards their hosts (404-407), while so far only few experimental examples of chemotaxis as a means of recognition among different bacterial species exist (362, 408, 409). Recently, Laganenka *et al.* (2016) reported that chemotactic accumulation followed by autoaggregation can also occur within species, which in this case, was mediated by the quorum sensing signal autoinducer-2 within populations of *E. coli* (410). In general, bacteria feature very sensitive molecular machineries to sense metabolites and quorum sensing molecules (411, 412). Even though these systems likely evolved in a different ecological context, they can dramatically enhance the ability of cells to identify possible interaction partners, even in taxonomically diverse communities.

Even though the ability to find other cells can lead to positive assortment, this mechanism alone cannot explain the evolution or maintenance of cooperative cross-feeding. Positive feedback among interactions partners is again also necessary to operate in parallel: more cooperative cells that show positive chemotaxis have a fitness advantage over non-cooperative or non-chemotactic cells, thus increasing their frequency in the local community. This means that after detection of suitable cells, metabolites that are produced and exchanged must preferentially benefit cooperative interaction partners. This can be achieved by recognition mechanisms (specific or unspecific) that allow attachment or group formation (see below). Indeed, the combination of chemical signalling with subsequent interspecies aggregation was recently observed in a mutually beneficial interaction between two bacterial species isolated from iron snow (362). More research, however, is necessary to fully evaluate the potential of this, at the moment rather theoretical possibility, to serve as a mechanism to identify suitable interaction partners.

The second main group of direct partner choice mechanisms is called *recognition.* Bacterial cells can recognize suitable interaction partners in two fundamentally different ways. First, recognition can be passive, by unspecifically attaching to other cells in the environment. In this case, growth of the focal cell will depend on the metabolic complementarity and cooperativity of the more or less randomly chosen interaction partner. Nevertheless, positive feed-back within interacting groups should increase the frequency of cooperators in a given population and thus raise the chance for a cooperative cell to encounter other cooperative cross-feeders in subsequent rounds of attachment and detachment (i.e. positive assortment).

Second, bacterial cells can be equipped with mechanisms, which allow them to specifically adhere to clonemates or members of other species that feature certain characteristics (103, 413). In the context of biofilm formation, such behaviours are commonly referred to as *co-aggregation* (413, 414). One evolutionary advantage of specific adhesion over random attachment is that specific adhesion minimizes the chance of associating with harmful, non-complementary, or non-cooperative genotypes. Moreover, adhesion can be key to the establishment of functional groups such as for the joint degradation of organic matter and the associated successful development of multispecies biofilms (413, 414). Adhesion over multiple generations finally causes progeny to locally accumulate in clusters. Such a formation of interacting groups helps in directing resources to closely-related, cooperative genotypes. Thus, both mechanisms of unspecific adhesion and specific recognition help to solve the public goods dilemma by privatizing the exchanged metabolites within interacting groups (415).

Recognition in microorganisms predominantly operates via structures on the outer membrane (413, 416, 417) and is generally based on affinity between a receptor and an identification molecule (418). Accordingly, the mostly protein-based adhesins represent receptors that facilitate recognition via binding to specific structures on the surface of another cell (419). Other specific recognition systems can be pili (419), homotypic receptors for self-recognition (420-422), the contact-dependent inhibition system (CDI) (357), and type VI secretion systems (T6SSs) (423). These systems mediate a remarkable spectrum of partner choice ranging from interactions between different kingdoms such as the host choice of microbiota (424) to specific autoaggregation with members of the same species (425).

The molecular machinery that determines whether or not two cells can interact with each other basically functions like a *greenbeard* gene (27) - i.e. a gene that allows

cooperators to recognize other cooperators and thus to preferentially direct benefits towards them. Increasing evidence demonstrates greenbeards to be substantially more common in microorganisms than originally thought (71, 416, 426, 427) For instance, the *Flo1* flocculation gene of the yeast *Saccharomyces cerevisiae* and the *csaA* self-adhesion gene in the slime mould *Dictyostelium discoideum* were identified to represent such greenbeard genes that are key for the establishment of cooperative groups (416, 428). Only carriers of the greenbeard genes are part of these groups and thus can enjoy cooperative benefits (51, 59, 71). In cooperative cross-feeding, however, recognition and cooperation are two separate functions. Hence, a non-cooperative carrier of the green beard allele could evolve and thus undermine the identification system. Nevertheless, recognition likely requires close physical contact, which automatically entails the abovementioned advantages of spatially structured metabolic interactions. Thus, a dedicated recognition system would still allow narrowing the spectrum of possible interaction partners to a subset of principally suitable cells. While research on microbial cooperation has focused more on traits like protection, reproduction, and siderophore production, cross-feeding of metabolites has received less attention so far. Thus, future work should evaluate how common such genetic recognitions systems are in natural microbial communities and whether they are also involved in stabilizing metabolic interactions.

Another attachment mechanism is the establishment of intercellular connections such as nanotubes, nanopods, or vesicle chains that are used to exchange materials between interacting cells (Fig. 4). In many of these cases, large clusters of interconnected cells emerge *(83, 87)* that should favour cooperative cross-feeding, because interactions within these clusters are highly localized. Analogous to cells growing on two-dimensional surfaces, also the self-organized growth of cells within three-dimensional networks should favour clusters of cooperative cells and penalize non-cooperators by spatial isolation.

Indirect partner choice

Indirect partner choice mechanisms lead to positive assortment of complementary cooperators by antagonizing non-complementary or non-cooperative genotypes. In general, this group of mechanisms either kills other genotypes or species or prevents them from invading a local population/ community. Consequently, all cells within the social group need to be resistant to the harmful behaviour. By excluding other,

potentially non-cooperating genotypes from cooperative benefits, indirect partner choice mechanisms can result in a local enrichment of cooperating genotypes.

Competition for space and other resources is common-place in microbial communities (22, 39, 42, 429, 430). Under these conditions, genotypes that can exclude or inactivate competitors are clearly at an advantage, thus explaining the widespread distribution of antagonistic behaviours among microorganisms (40, 430, 431). Whenever bacteria antagonize others, an almost automatic consequence is that these behaviours decrease the genetic diversity in the local community, thereby increasing the cohesiveness of the group displaying the harmful behaviour or being immune to its consequences. Antagonistic behaviours can facilitate the emergence and spread of cooperative behaviours within such groups, because metabolites that are produced as a cooperative act are more likely to benefit other group members. Discrimination against others can be achieved passively by generating a restrictive chemical environment, for instance by resource-depletion or pH-modification (432-434). The resulting competitive exclusion will reduce the number of genotypes present and possibly enrich close relatives of the strains causing the environmental modification. A property emerging from this process is often that already established bacterial communities, such as the ones colonizing corals (435), plant roots (425, 436), or the intestine of animals (434, 437, 438), cannot be invaded by other bacteria. This phenomenon has been termed *bacterial interference* or *colonization resistance* (437, 439, 440). In the context of metabolic cooperation within a spatially structured bacterial community, the ability to prevent the establishment of foreign bacterial genotypes may help to promote the exclusion of exploitative individuals and thus to stabilize metabolic cooperative interactions.

Alternatively, bacteria can actively express phenotypes that inhibit or kill other bacteria in the vicinity. Besides the production of antimicrobial compounds such as antibiotics or bacteriocins (434), bacteria use a range of other strategies to eliminate competitors including harmful extracellular vesicles (441), contact-dependent inhibition systems (CDI) (42), or type VI secretion systems (T6SS) (42, 442). Importantly, by killing genotypes that do not carry the gene that confers resistance against the antagonistic trait, cells that express the antagonistic trait and are resistant against it qualify as *harming greenbeards* (71, 427). In such a scenario, the local elimination of susceptible genotypes generally increases genetic homogeneity for the greenbeard allele and promotes positive assortment. For example, the CDI-system of *B. thailandensis* was demonstrated to be capable of antagonizing carriers of different BcpA-CT and BcpI

proteins, which were hence successfully excluded from a biofilm (427). Mutual antagonism between two bacterial species via the T6SS was demonstrated to cause segregation of different genotypes as well (43). Also in *Bacillus* species that were isolated from fresh water sediments, antagonistic interactions that were based on bacteriocin-like substances facilitated the assembly of cohesive communities even in a homogeneous aquatic environment (431). Under these conditions, positive assortment can be facilitated when multiple cooperative genotypes display the same antagonistic behaviour. This can even be orchestrated in a synchronized release of the antagonizing molecule in many individuals by quorum sensing (443-445).

Both passive and active discrimination mechanisms differ drastically in the specificity, with which they affect other genotypes. For example, antibiotics usually kill or inhibit a broad range of bacterial species (446, 447). In contrast, bacteriocins typically exhibit a narrow killing spectrum (446), enabling producers to specifically target other coexisting species or genotypes of the same species (434, 448). Increasing evidence suggests that microorganisms are able to discriminate relatives with high selectivity: antagonistic interactions were identified to facilitate positive assortment among conspecific genotypes (27, 37, 43, 430, 449, 450). *Proteus mirabilis*, *Myxococcus xanthus*, and *Bacillus subtilis* are examples for bacterial species that can identify and antagonize other strains (451-453). When different populations of these species grow on two-dimensional surfaces, the formation of physical boundaries, so-called demarcation- or Dienes lines, indicate the presence of discrimination mechanisms (451-453). Strikingly, the underlying recognition mechanisms are usually highly selective and allow discrimination even within a species (418, 451). Differences in a few loci already cause the formation of Dienes lines between swarming colonies of these bacteria.

The often very high specificity of antagonistic discrimination mechanisms combined with the fact that individual bacteria commonly use multiple discrimination mechanisms simultaneously (454) suggests that bacteria aim at maximizing the chance to interact with desired genotypes of the same or different species. Here, the presence of antagonistic functions and the corresponding resistance genes serves as a system to discriminate bearers of these alleles (kin) from non-bearers (non-kin) (454). Since these alleles can also be transferred via horizontal gene transfer(455), kinship does not necessarily imply affiliation to the same species. For this reason, the term *kind discrimination* is more applicable under these conditions (27). If members of the same kin/kind group engage in a mutualistic interaction, any kin/kind discrimination

mechanism will help to protect cooperation from exploitation, as recently demonstrated for swarms of *Bacillus subtilis* (454). Moreover, positive assortment of genotypes in populations of *Vibrio cholerae* that was mediated via the T6SS correlated with increased cooperation (456). Accordingly, a phylogenetic comparison revealed that the extent of released public goods, in this case proteins, correlated with the total number of T6SS per strain, suggesting that positive assortment via killing promotes the evolution of public goods cooperation (456). Unfortunately, research in this field is just beginning to appreciate the potential significance of indirect partner choice mechanisms for the evolution of cooperative interactions. Thus, further research is necessary to fully evaluate to which extent positive assortment by specific discrimination mechanisms can favour cooperative cross-feeding among different genotypes or species.

4 Consequences of obligate metabolite cross-feeding

The evolutionary transition from a metabolically autonomous, free-living life-style to a state, in which the fitness of a bacterial cell hinges on the obligate cross-feeding of metabolites with other organisms has a number of significant evolutionary consequences for the focal genotype. Some of these effects are well-documented, while others are rather based on theoretical considerations. In the following, we will provide an overview over the manifold ramifications arising from obligate cooperative cross-feeding.

4.1 Negative frequency-dependent selection

As outlined above evolutionary theory predicts obligate cooperative interactions that are based on an exchange of public goods to be notoriously unstable (45, 51, 58, 59). Particularly when cooperating and non-cooperating individuals have equal access to the cooperative public good, non-cooperators are expected to gain a significant fitness advantage relative to cooperating types, which ultimately should result in a collapse of the cooperative interaction. Surprisingly, this outcome is rarely observed in cases where essential metabolites are cooperatively exchanged (but see (457)). Instead, obligate cooperative cross-feeding interactions are commonly stabilized by *negative frequency-dependent selection*. *Frequency dependence* describes an evolutionary process, in which the fitness of a given genotype depends on the relative frequency of other genotypes in the total population. In the case of negative frequency-dependent selection, the fitness of a certain genotype decreases as it becomes more common in a given population. From

this, results a stabilizing force that maintains interacting genotypes in the long-run (458-461).

Negative frequency-dependence has been shown to operate in both one-way by-product (68, 462) and two-way cooperative cross-feeding interactions (102). In both cases, frequencies of the two interacting cell types oscillated around a stable equilibrium point that most likely was determined by rates of metabolite production and consumption. Interestingly, the same pattern prevails when non-cooperators are included: also consortia consisting of amino acid cross-feeding *E. coli* cells and non-cooperating auxotrophs (102) or cocultures between producers of a public good and the corresponding non-cooperators (90, 104, 459, 463-465) were stabilized by negative frequency-dependent selection. Observing negative frequency-dependent selection for different types of metabolic interactions in both spatially structured and unstructured environments suggests this pattern is a common principle emerging in synergistic microbial communities.

However, cooperators and non-cooperating individuals of the abovementioned examples are unlikely to have equal access to the cooperatively produced metabolite. Instead, mechanisms of positive assortment, which have either evolved or result as a by-product from other features of the interaction, ensure the cooperatively produced metabolite is predominantly benefitting other producing genotypes. Examples of such mechanisms include a privatization of metabolites or biosynthetic functions due to contact-dependent exchange mechanisms (83, 102) or the localization of public goods in spatially structured environments (89-92, 370-372).

As a consequence, no genotype can take over in these obligate interactions. Instead, negative frequency-dependence maintains genotypic diversity (57, 366, 466). A prediction that follows from this is that the evolution of cooperative cross-feeding should promote a metabolic and genotypic diversification within microbial communities. Indeed, this pattern has been repeatedly observed in theoretical models (57) and experimentally evolved microbial communities (68, 203).

4.2. Coevolutionary dynamics

Unfortunately, very little is known on the coevolutionary consequences resulting from cooperative cross-feeding (467). An important aspect is certainly the positive feed-back loop that result when positive assortment assures repeated interactions among complementary partners across generations (i.e. partner fidelity feedback (221): In

reciprocal cross-feeding interactions, an increased metabolic investment on one side automatically enhances growth and thus also metabolite production on the other side (158). Thus, continued coevolution is expected to increase productivity in cross-feeding communities, which is corroborated by experimental evidence (105).

For antagonistic interactions such as host-parasite interactions, both empirical and theoretical work suggest increased rates of molecular evolution (i.e. red-queen effect) (31), mainly affecting loci involved in virulence and resistance. In contrast, much less research has been devoted to the question how mutualistic interactions - such as cooperative cross-feeding - affect rates of evolution. Theoretical work implies that in mutualistic interactions, species generally evolve more slowly to increase their share of the benefits (i.e. red-king effect (117)). Experimental tests whether horizontally or vertically transmitted bacterial symbionts indeed evolve to some point of evolutionary stasis (468), however, revealed inconclusive patterns (469). Thus, further work should examine in more detail how synergistic coevolution affects the rate of evolution.

Finally, also the genomic signature that results from synergistic coevolution of cross-feeding genotypes is not very well understood. Given the transient nature of cross-feeding interactions, it is for example unclear, whether a pattern of reciprocal coevolutionary change, in which mutations in one interaction partner favour a specific set of mutations in the other partner, can be expected. Coevolution experiments with syntrophic consortia consisting of a sulfate reducer (*Desulfovibrio vulgaris*) and a hydrogenotrophic methanogen (*Methanococcus maripaludis*) for 1,000 generations documented an extremely rapid loss of functional independence of *Desulfovibrio vulgaris*, which was driven by a mutational inactivation of genes involved in sulfate respiration (112). Also pairs of lactic acid bacteria (i.e. *Streptococcus thermophiles* and *Lactobacillus delbrueckii* subsp. *bulgaricus*) that have been serially propagated in coculture to ferment yoghurt reciprocally exchange a larger number of metabolites in a mutualistic manner (115). This striking metabolic complementarity, which likely evolved in response to the ecological interaction between both species, is largely due to gene loss (113, 114). Given that consortia of co-occurring bacterial endosymbionts (54, 353, 470) display a similar pattern on a genomic level, it is tempting to speculate that metabolic complementarity is a common outcome of a synergistic metabolic coevolution.

4.3 Formation of intercellular metabolic networks

The frequent loss of metabolic genes from microbial genomes along with intricate patterns of cross-feeding, where other coexisting genotypes compensate the functional deficiency suggests that within bacterial consortia, metabolism is often a community-level property and not a feature of an individual cell any more. If true, this implies that a bacterial communities' metabolism is in essence a *super-metabolic network*, where each member of the community performs specialized biosynthetic or catabolic tasks. Indeed, recent metagenomic and empirical surveys of environmental bacterial communities have revealed that individual genotypes in a community can have distinct patterns of amino acid auxotrophies, such that some members lack multiple biosynthesis pathways, at the same time specializing in the production of another set of amino acids (18, 114, 471, 472). Also specialized genotypes have been found, which provide biosynthetically expensive amino acids to other community members (471). Moreover, coevolved symbiotic interactions between bacteria and higher organisms often show signatures of a *functional fusion*, in which interacting parties operate as one integrated metabolic unit. For instance, the partitioning of biosynthetic pathways between host and endosymbionts (349, 473) or between multiple, co-occurring endosymbionts (318, 474) illustrates that only the sum of the organismal metabolic pathways can satisfy the nutritional needs of all interacting parties (318).

Metabolic cross-feeding interactions can strongly determine community *structure* and *function* (471). As discussed above, the establishment of such interactions is driven by a complex interplay between fitness-advantages of individual mutants and the eco-evolutionary dynamics between multiple genotypes. Ultimately, the highly-conserved structure of core metabolic pathways in otherwise rather divergent bacterial lineages could guide the evolutionary self-organization of metabolic exchange even between very different bacterial species (475). Whether and to which extent microbial communities act as one integrated *metabolic unit* that maximizes both productivity and stability of the entire consortium, however, needs to be addressed in future studies. Here, targeted analyses of the metabolic abilities and activities of individual strains living in natural bacterial communities are necessary to identify whether the distribution of metabolic functions within these communities is indeed determined by global constraints that are dictated by the structure of the underlying metabolic network (476, 477).

4.4 Bacterial unculturability

In 1932, Razumov was isolating freshwater bacteria and noticed that the majority of bacteria, which he observed under the microscope, resisted cultivation on agar plates (478). Since then, microbiologists have repeatedly corroborated that less than 1% of all archaea and bacteria can be grown *in vitro* (479) - a problem that has been popularized as *the great plate count anomaly* (116). Accordingly, recent advances in metagenome sequencing of environmental samples revealed not only the presence of a tremendous microbial diversity, but also discovered completely novel genera and even phyla, which have not been isolated yet (480-482).

Many potential explanations have been proposed to account for the unculturability of most bacterial species. These can be grouped into four main categories:

(1) Niche mismatch: Mismatch between the physiological and nutritional requirements of a bacterial strain and the conditions provided (e.g. pH, temperature, salts, minerals, and nutrient levels) (483, 484).

(2) Dormancy: Bacterial cells might be viable but unculturable (485, 486).

(3) Antagonistic effects: Competitively superior strains outcompete others and/ or produce toxic compounds (e.g. antibiotics) to kill or inhibit other strains (487, 488).

(4) Obligate metabolic interactions: Bacterial strains have evolved obligate metabolic relationships with other neighbours in their environment. Thus, attempts to isolate a single species must fail, because of the lacking nutrients or biochemical functions (66, 489).

Although all of the above-mentioned reasons likely contribute to explaining the unculturability of many bacterial isolates, accumulating experimental evidence suggests that an obligate exchange of metabolites or biochemical functions among bacterial strains is particularly important in this context. For example, attempts to preserve ecological interaction within microbial communities have significantly increased bacterial recovery. In 2002, Kaeberlein *et al*. designed a diffusion chamber, which allowed an exchange of metabolites between cells and their environment, but prevented a mixing of cells (490). Using this system, the authors managed to isolate novel cultures, which increased bacterial culturability to up to 50% (491). Interestingly, some of the isolated strains did not grow on synthetic media afterwards, indicating that a direct contact with the native microbial community was essentially required for growth (490). Other related isolation techniques are *enrichment cultures*, in which environmental conditions are tailored to favour certain genotypes (492), or *dilution cultures*, where

environmental samples are diluted to low, but known cell numbers (493). Both approaches frequently do not result in the isolation of individual strains, but mixtures of strains that can only grow together but not in isolation (55). Thus, this method provides the opportunity to isolate coexisting genotypes that cross-feed essential metabolites and study their interaction in more detail. Taken together, the available evidence suggests that metabolic interdependencies within natural microbial communities are an important determinant of the commonly observed unculturability of natural bacterial isolates.

4.5 Levels of selection and transitions in individuality

When auxotrophic bacteria engage in cooperative cross-feeding with other bacterial cells, the question arises whether natural selection acts on individual bacterial cells or if groups of cells are the *unit of selection*. Before addressing this question, some relevant key terms need to be defined: An *evolutionary individual* is the unit, whose frequencies are adjusted by natural selection (121, 122). Traits that determine the Darwinian fitness of such an individual must be heritable, variable, and give rise to differential reproduction among competing individuals that differ in the respective trait (121, 123). As a consequence, natural selection does not only act on, for example, populations of bacterial cells, but can simultaneously operate on lower (e.g. plasmids inside of bacterial cells) or higher levels (e.g. multicellular prokaryotes such as cyanobacteria). A *transition in individuality* is now observed when the level of selection is shifted from lower level units that are units of selection themselves, to a higher-level entity, which is composed of several lower-level units (123). The fitness of the collective that emerges during this transition is not proportional to the average fitness of the assembled lower-level units, but an emergent property of the higher-level entity (125, 142, 143). Moreover, after the transition "*genetic information is transmitted between generations*" such that "*entities that were capable of independent replication before the transition can replicate only as part of a larger whole of it*" (35) . Major leaps in biological complexity have resulted from evolutionary transitions, during which previously independent organisms were functionally integrated to form a new, higher-level entity (150, 494, 495). Eminent examples of such transformative events include the origin of the eukaryotic cell (496, 497) or the emergence of plastids from a cyanobacterial progenitor (498).

How does natural selection now act in bacterial communities that engage in obligate cooperative cross-feeding of metabolites? First, it is important to recognize that under these conditions, fitness is not only determined by the traits of the individual cells, but as a property that emerges from interactions among cells (102, 499). Even if cells that do not invest in the cooperative production of shared metabolites may save the cost of metabolite production (104, 500), their lack of investment in cooperative cross-feeding likely curtails their own fitness. This is largely due to the fact that multi-level selection acts on both the level of individual cells and on groups of cells (e.g. sub-populations, different parts of the same biofilm, cell clusters, and so on). Auxotrophic cells of a local community assemble in groups to facilitate the exchange of metabolites (83, 501). Growth of cells within such a group depends on the genotypic composition of the whole

group: clusters that contain more cooperative cells grow more than clusters consisting mainly of non-cooperators (Fig. 9). Even if non-cooperators gain an advantage in their local group, they are selected against on a global level, if more cooperative groups export their productivity in subsequent rounds of assembly and disassembly. Different mechanisms of positive assortment (Fig. 8) and principles of self-organization (89, 367), ensure that cooperative cells remain associated with other cooperators over time.

A second important consequence of living in a close metabolic entanglement with other bacterial cells is that the mutational landscape, which is available to a cell to improve its fitness, will likely dependent on the current interaction partner. Thus, the spectrum of mutations that is expected to be favoured within cross feeding interactions should be radically different from those that might be beneficial in a metabolically autonomous bacterium. Hence, mutations that arise from within these interactions and which improve the performance of the cell group (e.g. mutations increasing among-cell adhesion or levels of metabolite production) are likely maladaptive outside these interactions and thus, should be interpreted as group-level adaptations.

Finally, the question remains: Are groups of bacteria, whose survival depends on obligate cross-feeding of metabolites, evolutionary individuals? Put differently: What is the unit that is most relevant to evolution – the individual cell that is unable to survive in isolation, or the group of cells, in which auxotrophic bacteria can thrive? If groups were the relevant evolutionary individual, cell groups would have undergone a transition in individuality. To answer this question, it is useful to consider cases, in which a new individual has been formed by natural selection upon the merging of previously independent lower-level units and identify hallmarks that characterize these cases (Table 1).

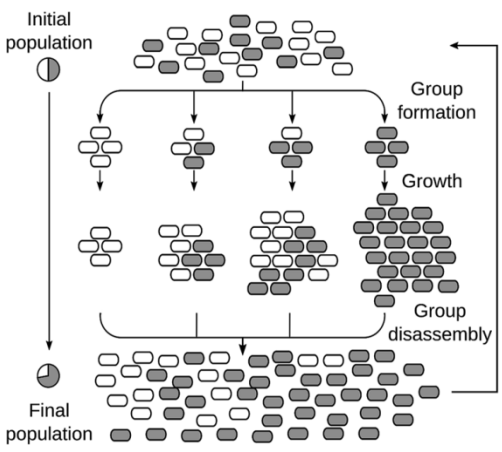

Figure 9: Multilevel selection favours cooperative cross-feeding. Cells in a global pool assemble into clusters consisting of non-cooperative (white) and cooperative (grey) cells. On a cluster-level, non-cooperating cells gain a selective advantage over cooperators, because they save the costs of metabolite production. However, the productivity of clusters depends on the relative proportion of cooperative cells within a cluster. As a consequence, differential growth of clusters selects against non-cooperation and favours cooperation on a global level. Modified after (46) and (126).

Evaluating whether consortia of cross-feeding bacteria fulfil these criteria indicates that even though important features such as mutual dependence, functional specialisation, cooperation, and cell-attachment result almost automatically from cooperative cross-feeding, a striking difference is that these interactions are often transient (Table 1). Due to the often non-permanent nature of association between cells, heritability of group-traits is likely low. Moreover, cross-feeding consortia do not form a cohesive unit that is clearly delimitable from other cells in the environment, but rather a delicate network of transiently interacting cells. Nevertheless, selection is expected to favour extended associations between compatible genotypes. Moreover, frequency-dependent selection and spatial self-organization within clusters should adjust the mixture and the positioning of cells within clusters, thus maximizing the supply of limiting metabolites for cooperative cells (502). Mutations that were favourable in the context of one group, might work equally well when the focal mutant is combined with other genotypes, thus compensating for the lack of a strict vertical transmission. Finally, repeated bouts of association and disassociation allow to purge detrimental mutations on a cluster-level, thus accelerating molecular evolution. Taken together, consortia of bacteria that engage in obligate cooperative cross-feeding do not form a coherent, multicellular organism. Still, their performance results from complex metabolic interactions among the constituent cells, which is more than the sum of its parts. Future work is necessary to determine how durable cross-feeding interactions are and how this affects coevolution of interacting cells.

Table 1: Consortia of bacteria that engage in obligate cooperative cross-feeding of metabolites show hallmarks of a transition in individuality.

Characteristic	Cooperative cross-feeding bacteria	Example
Mutual dependence[1]	yes	(503)
Functional specialisation of cells/ division of labour[2]	yes	(36, 504)
Cooperation	yes	(61, 505)
Group-level reproduction[3]	likely yes, to some extent	(506)
Cell-attachment	yes, but likely transient	(83, 501)
Strict vertical transmission	likely not	
Conflict resolution strategies	yes	(89, 507)
Coordination of cellular activities	yes	(251, 499)

[1] Reproduction only as part of a multicellular consortium
[2] Synergistic fitness benefits arise upon combination of functions
[3] Groups of cross-feeding bacteria beget new groups

5 Concluding remarks

Our comprehensive analysis of the available literature revealed how commonly bacteria are involved in metabolic cross-feeding interactions with other bacteria, archaea, or eukaryotic organisms (Fig. 3). Particularly striking was the tremendous diversity in terms of mode of metabolite exchange that characterized the analysed interactions (Fig. 4). What also became obvious from screening the available literature, however, is that despite intensive efforts to study the molecular details of numerous metabolic cross-feeding interactions during the past decades (18, 115, 210), ecological or evolutionary aspects of these interactions are still notoriously understudied. Moreover, research attention so far has been mainly directed towards a relatively small set of model systems that have been studied in more detail (18, 115, 210). Thus, more empirical work is required to systematically compare cross-feeding interactions of different environmental origins and taxonomic compositions. In addition, the prevalence and ecological significance of metabolite cross-feeding should be increasingly analysed in different natural microbial communities, especially focussing on its importance for structuring these communities. Finally, the analysis of isolated consortia should be complemented by studies of synthetically engineered or experimentally evolved interactions, in which the causal molecular and evolutionary factors can be identified much easier.

With the growing realization that metabolic interactions within microbial communities and populations are key for determining human health (12), global biogeochemical cycles (10) or the yield in biotechnological production processes (508), the need to understand rules that govern the emergence and evolution of these interactions is becoming particularly urgent.

Undoubtedly, the development of new technologies to chemically identify and characterize exchanged metabolites, to derive transcriptional and proteomic information of individual genotypes in a coculture context, as well as to differentially label and image interacting cells under controlled conditions will significantly advance the study of microbial metabolite exchange (12). Especially the possibility to combine different methods will provide exciting opportunities, such as to image living cocultures on a single-cell level and simultaneously visualize the distribution of metabolites with a high spatial resolution. Moreover, current computational advances in simulating metabolic processes of cells that are embedded in complex communities hold the potential to predict bacterial metabolite exchange interactions based on the genome sequence of the organisms present in the community (509, 510).

A wealth of exciting research opportunities is waiting in this rapidly emerging field. Interesting questions that should be addressed in the future include (i) Which ecological factors determine the assembly of metabolically interacting consortia in natural microbial communities (e.g. motility, chemotaxis, antagonistic interactions)? (ii) How stable/ transient are obligate metabolic interactions in natural environments? (iii) Which evolutionary consequences result for genotypes that transition into an obligate metabolic dependency with other genotypes (e.g. local adaptation, genome streamlining, improved efficiency)? (iv) Which rules govern the division of metabolic functions within microbial communities? or (v) Are clusters of metabolite cross-feeding cells evolutionary individuals?

Evolution does not only proceed by giving rise to new species, but also by merging previously independent organisms into new life-forms (511). Consequently, intricate metabolic interdependencies between two or more individuals are a general feature of life. Answering the abovementioned questions using metabolite cross-feeding within microbial communities as a tractable model therefore holds the potential to help resolve the fundamental evolutionary problem of how biological complexity can emerge from the establishment of cooperative interactions among simpler units.

Chapter 2

Chapter 2

Group formation promotes evolution of metabolic cooperation in bacteria

Interdependency based on biosynthetic products is common in nature and the associated exchange of metabolites is frequently cooperative. Due to ecological importance and proposed relevance for the evolution of multicellularity, cooperative cross-feeding interactions receive increasing attention. However there are still few empirical insights into how a cross-feeding interaction can become cooperative and what such an evolutionary process entails on involved genotypes. Here we show rapid evolution of cooperative cross-feeding within less than 150 generations between two genotypes of *Escherichia coli* performing obligate amino acid exchange under conditions that are predicted to hamper cooperation: Well-mixed liquid culture that lacks abiotic spatial structure. We identify pervasive formation of multicellular clusters as major strategy for efficient amino acid exchange thus place of reproduction and report dynamics of alternating association and disassociation of these clusters. By comparing results with negative controls (*i.e.* independently growing monocultures), we show observed strategy association as well as cooperation to be exclusively present in the context of obligate cross-feeding. Observed pervasive success of cocultures was linked to cooperators experiencing strong selective advantages due to positive fitness feedbacks within clusters.

1 Introduction

Metabolic interactions are a widespread phenomenon and play a critical role for biogeochemical cycles (10) and whole ecosystems to function (512). The accompanied metabolic conversions are predominantly performed by microorganisms that rarely act as single species, but rather within diverse communities. Analysis of such communities by modern sequencing techniques revealed a remarkable lack of vital biosynthetic functions (18, 63, 114, 471, 472), which strongly indicates metabolite exchange and interdependencies to be a major feature of bacterial communities (22). Involved partners frequently benefit by the cooperative exchange of metabolites (250, 417, 467). However, theory suggests selection on the level of individuals to promote selfish over cooperative phenotypes due to fitness benefits, ultimately causing the interaction to break down (45, 61, 513). Moreover cooperators are unlikely to meet other cooperators when rare, which adds another layer of complexity in explaining the origin and maintenance of cooperation.

Solutions to this problem are suggested by numerous theoretical work as well as *in silico* studies that address the evolution of cooperation: Multiple mechanisms for assortment (44, 61) are proposed to facilitate cooperating partners to predominantly interact with each other, which allows avoiding non-cooperators and hence invested goods to pay off (89). In line with these insights recent work demonstrates cooperative interactions to easily evolve under laboratory conditions confirming abiotic spatial structure to promote cooperation by enforcing positive assortment of partners (92, 106). In contrast to these experimentally imposed conditions, the environment frequently either lacks spatial structure, or experiences disturbance. Microbes hence need to attach to each other, or enforce the formation of biofilms on substrates, or both. In this context improved biofilm formation for instance supported a mutualistic interaction within a static liquid environment (514). However, there are only few empirical studies that investigate metabolic cooperation and its evolution aside experimental conditions that mimic proposed mechanisms for assortment (105, 112). In addition, conducted studies rarely focus on effects associated with the evolution of cooperation relative to an independent lifestyle, albeit insights are important to understand the complex as well as potentially conflicting routes of adaptation to abiotic as well as biotic factors. Such a comparison is especially helpful in clearly attributing evolved cooperative traits to be adaptive within the context of the interaction or to rather be mere by-products of adaptation to abiotic culture conditions. We therefore asked which mechanisms (i.e. eco-

evolutionary driving forces) could reward a cooperative phenotype within theoretically unfavourable surroundings and what differences this implicates compared to a solitary lifestyle.

Here we investigate the evolution of cooperation within a well-mixed environment. Therefore two *E. coli* genotypes, unable to either synthesize tyrosine or tryptophan, performed reciprocal cross-feeding of these amino acids. We report cooperative metabolite exchange to rapidly emerge from the initial by-product interaction during serial propagation of these cocultures during ~150 generations. Interestingly, we consistently observed a nascent lifecycle of association and disassociation of cellular aggregates that followed the experimentally imposed rhythm of serial transfers. By utilizing control groups with an independent lifestyle as reference to cocultures we disentangle adaptive responses to obligate cross-feeding from adaptations to culture conditions as well as genotypic background. In detail, we identified cluster formation within cocultures throughout the conducted evolution experiment as (i) specific to cross-feeding populations and (ii) predominant hot-spots of growth. This observation of obligate cross-feeding partners forming their own spatial structure by aggregation is in line with a previously reported study (501). Moreover our results show cooperative phenotypes to (i) exclusively evolve within cocultures, and (ii) specifically experience a selection advantage within multicellular clusters even under the absence of a complementary cooperative partner. Preventing cluster formation significantly decreased gained advantages, which indicates local feedback loops within clusters to reward cooperation. Finally, in contrast to significantly increased fitness in cocultures, members of these populations consistently showed reduced growth-performance in isolation suggesting a shift in selection to the level of whole groups at the cost of individual fitness.

Taken together our results demonstrate multicellular clusters as an emergent property of obligate metabolite exchange to follow a nascent lifecycle, and to facilitate assortment and thus the evolution of cooperation within an environment that lacks spatial structure. Together with suggested selection on the group-level the evolved model system shows hallmarks of a multicellular entity. What is more we report the first empirical evidence of cooperators experiencing strong positive selection albeit exclusively interacting with non-cooperators and link this selection advantage with emergent properties of interacting within a group., i.e. a multicellular cluster.

2 Results

To determine whether a cooperative metabolic interaction can evolve from an interaction that initially depends on a reciprocal exchange of by-products an evolution experiment was conducted using three different treatment groups. First, prototrophic wildtype cells of *Escherichia coli* were serially propagated in minimal medium. Second, two genotypes of *E. coli* that each lacked the ability to autonomously produce one amino acid (i.e. tyrosine or tryptophan) were cultivated in monoculture in a minimal medium that contained the amino acid each strain required for growth in non-saturating concentrations (i.e. 50 µM). Third, the two auxotrophic genotypes were grown in coculture in a minimal medium without the supplementation of any amino acid. These two auxotrophic mutants were chosen, because they showed a marginal growth when cocultured, which was likely due to an exchange of the amino acids each strain essentially required for growth.

The two monoculture treatments were included to assess the potential of metabolically autonomous or auxotrophic genotypes to adapt to their abiotic environment. In addition, because amino acids have been supplemented in growth-limiting concentrations to monocultures of auxotrophs, these strains should experience starvation for amino acids. In contrast, auxotrophs evolving in coculture should adapt to both their abiotic environment and their interaction partner. Specifically, under these conditions, auxotrophic strains could only improve in fitness when their partner increased the amount of amino acid they required for growth.

Twelve replicate populations of each experimental group were serially propagated for a total of 20 cycles of growth and subsequent dilution into fresh minimal medium (Fig. 1a). Populations were initially transferred every seven days. However, since cocultures of auxotrophs rapidly increased in growth, the transfer interval was reduced after the fifth cycle to three days.

2.1 Cocultures of auxotrophs rapidly improved in fitness

Given that compensatory mutations can occasionally enable growth-deficient genotypes to revert towards prototrophic phenotypes (515). One of the first tests after the evolution experiment has been terminated was to verify whether auxotrophic genotypes had retained their loss of function phenotype over the course of the evolution experiment. Plating all derived populations on agar plates that did not contain any amino acids revealed that in ten populations of cocultured auxotrophs, both cell types were still

present at the end of the evolution experiment. However, two populations completely consisted of reverted phenotypes that had re-evolved the ability for prototrophic growth. These replicates as well as their cognate controls (i.e. auxotrophic monocultures) were excluded from further analysis.

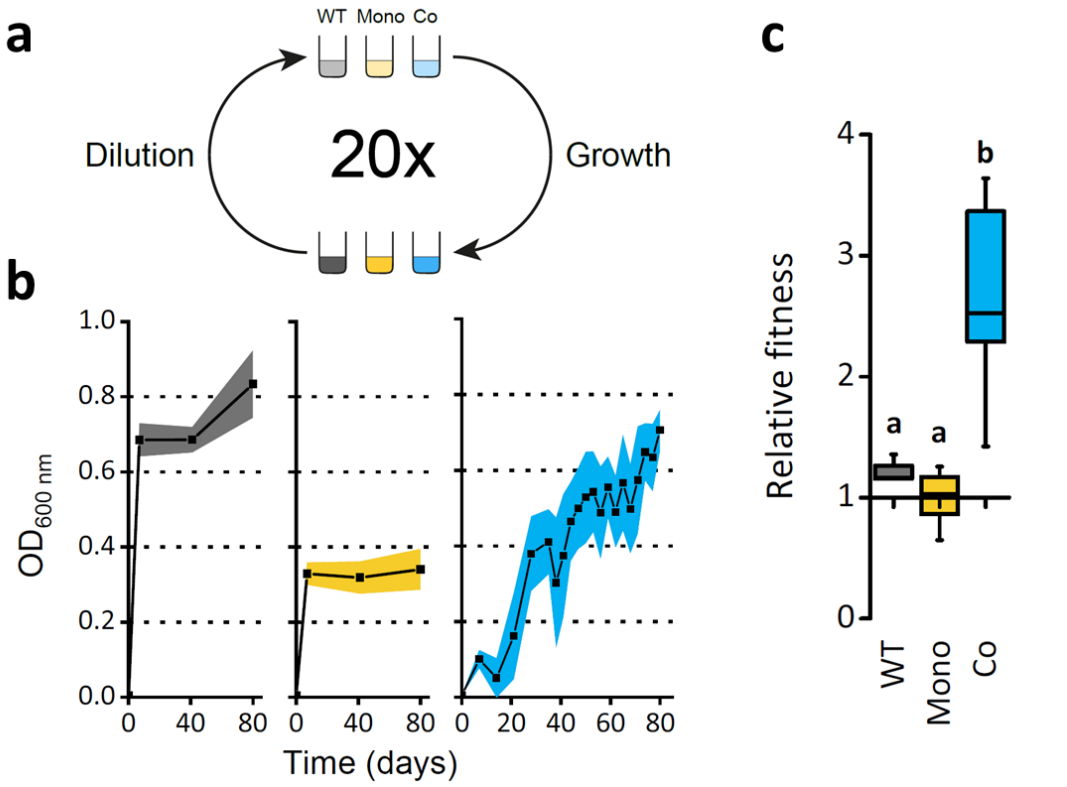

Figure 1: Productivity and fitness significantly improved in cross-feeding cultures. (a) Experimental setup including two control groups lacking obligate interactions (WT: Prototrophic wild type, grey; Mono: Monocultures of amino acid auxotrophic genotypes, yellow), and one group performing obligate amino acid exchange (Co: Mixed populations of two genotypes auxotrophic for tyrosine or tryptophan, blue). (b) Averaged optical density (OD600 nm) during serial propagation in minimal medium in three groups of *Escherichia coli* cultures (WT: n=12; Mono: n=19; Co: n=10). Errors are given as 95% confidence intervals. Data points at 0 days show initial optical densities of 0.005 after inoculation. For control groups only time points after 7, 41, and 80 days are given. (c) Observations in final culture densities were confirmed by determining fitness relative to the corresponding ancestor in WT, Mono, and Co. Different letters indicate significant difference in relative fitness (ANOVA with LSD *post hoc*-test: $P<0.001$, n=12 for WT, n=19 for Mono, and n=10 for Co). Relative fitness for WT and Co is significantly different to 1 (One-sample t-test, difference to 1: $P<0.05$ for WT and $P<0.001$ for Co)

Next, we asked whether or not the three different treatment groups increased in growth over the course of the evolution experiments. For this, growth of all experimental populations was measured by quantifying the optical density they reached after a growth cycle during different time points of the evolution experiment. Comparing the growth

each of the three groups reached at the beginning and at the end of the experiment, revealed a marginal (WT: 1.2-fold, Auxmono = 1.04-fold) increase in the growth of both wild type and auxotroph control groups (Fig. 1b). In contrast, cocultures of auxotrophs showed a much stronger (7-fold) increase in growth. Interestingly, even though cocultures of auxotrophs showed only marginal growth at the beginning of the evolution experiment, their growth at the end was statistically indistinguishable from the levels reached by the ancestral wild type (Independent samples t-test: $P=0.6$; $n_{WT}=12$, $n_{Co}=10$).

A similar pattern emerged when the fitness of all derived cultures, quantified as the improvement in growth, was compared to the fitness of their respective evolutionary ancestors: Both the wild type and cocultures of auxotrophs significantly increased in fitness (One-sample t-test, difference to 1: $P<0.05$ for WT and $P<0.001$ for Co), while the fitness of monocultures of auxotrophs remained unchanged (Fig. 1c). Strikingly, the experienced increase in fitness was markedly higher in cocultures (2.5-fold) than in prototrophic wild type cultures (1.2-fold). Further comparison of absolute fitness measures (*i.e.* Malthusian growth parameters) revealed that derived populations achieved similar levels (Fig. S1) albeit showing differences in optical densities (Fig. 1b). Cocultures hence finally reached fitness-levels of control groups.

Thus, taken together, results suggest that the synergistic coevolution experienced by auxotrophic types in coculture enhanced their rate of adaptation relative to the two control groups that were capable of independent growth.

2.2 Cocultures of auxotrophs evolved overproduction of exchanged amino acids

The increased growth observed in derived cocultures of auxotrophic genotypes suggested that within these cultures, both parties must have increased the amount of amino acids they produced to support the growth of their corresponding partner. In contrast, derived populations of both control groups are not expected to have changed their production level of the two focal amino acids. To verify this possibility, different clones were isolated from both ancestral and derived populations of all three treatment groups to determine changes in their production levels of tyrosine and tryptophan over the course of the evolution experiment.

Unexpectedly, when plated on indicator agar plates that were used to identify individual genotypes, noticeable changes in colony morphology were observed: eight out of ten cocultures of derived auxotrophs showed colony morphologies that clearly differed from the one of the corresponding ancestral type in terms of colony size and

colour (see methods) for at least one partner, while only three out of 19 auxotrophic monocultures analysed showed more than one morphotype. None of the derived wild type populations showed an apparent change in their colony morphology. To consider this variation in subsequent experiments, four representatives of each identified morphotype were isolated. In principle, amino acids could be transferred between bacterial cells in two different ways. First, cells could release the metabolites and exchange them via diffusion through the extracellular environment. Second, bacteria may use contact-dependent structures to derive cytoplasmic metabolites from other bacterial cells (i.e. so-called nanotubes, (83)) in which case metabolites do not leave the intracellular environment. In these cases, a nanotube-mediated removal of amino acids from the cytoplasm of donor cells can even increase the donor cell's production levels of the corresponding metabolite (516).

Thus, to simultaneously quantify the amount of amino acids that is being transferred via diffusion through the extracellular environment as well as potentially by cytoplasmic exchange, isolated clones as well as their corresponding evolutionary ancestors were individually supplemented with the amino acid they essentially required for growth and cocultured together with a second genotype of *E. coli* that was auxotrophic for the focal amino acid. Therefore the former strains functioned as donors while the ladder were recipients hence were used as amino acid biosensors, whose growth correlates with the amount of amino acids shared by the donor strain (517). Comparing the levels of supported biosensor growth between derived clones and their evolutionary ancestors in this way indicated a significantly increased production of the two focal amino acids tyrosine and tryptophan in coevolved auxotrophs (Mann-Whitney Test: $P=0.002$; $n=140$ for evolved auxotrophs; $n=20$ for ancestral auxotrophs). In contrast, auxotrophic monocultures showed the opposite trend of supporting significantly less biosensor growth than their evolutionary ancestors (Mann-Whitney Test: $P<0.001$; $n=88$ for evolved auxotrophs; $n=19$ for ancestral auxotrophs), while these measures did not change over the course of the evolution experiment in the prototrophic wild type for tryptophan (Mann-Whitney Test: $p=0.315$; $n=6$), yet decreased for tyrosine (Mann-Whitney Test: $P<0.05$; $n=6$) (Fig. 2a). Additional comparisons in absolute measures of biosensor to donor ratios confirm these differences and clearly demonstrate coevolved auxotrophs to share significantly more amino acids than derived control groups (Fig. S3).

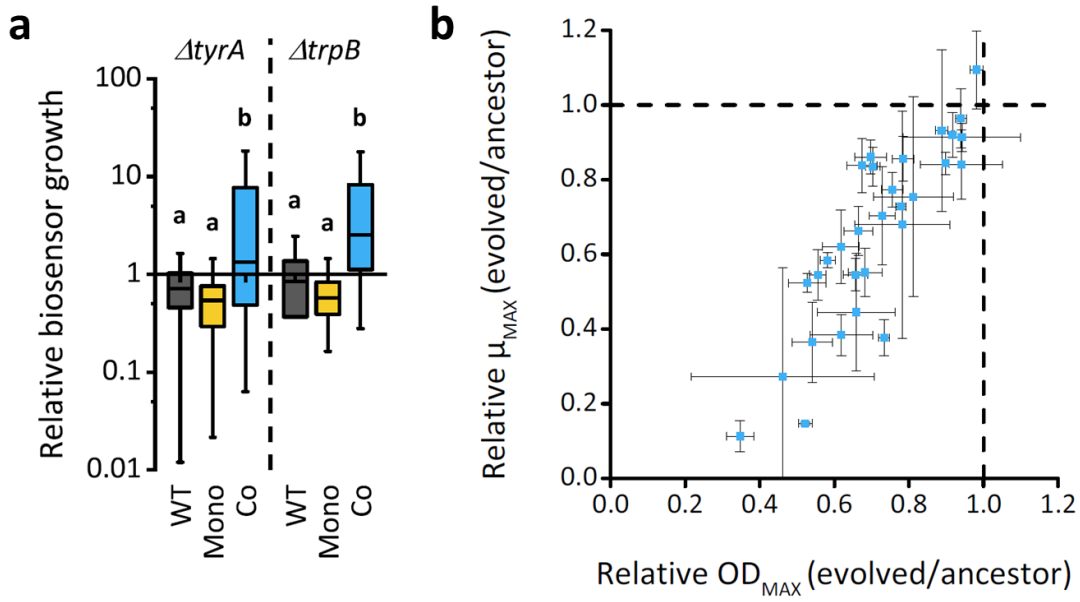

Figure 2: A costly cooperative trait exclusively evolved in cocultures. (a) Increased levels of shared amino acids were detected in derived strains from cocultures. Isolates from evolved populations (wild type: WT, auxotrophic monocultures: Mono, auxotrophic cocultures: Co) as well as corresponding ancestors were utilized as amino acid donors for *E. coli* biosensor strains (recipients) auxotrophic for tyrosine (*ΔtyrA*) or tryptophan (*ΔtrpB*). The culture medium contained amino acid essential for the respective donor, but lacked the amino acid essential for the recipient to grow. Thus, biosensor growth reflects levels of shared amino acid by the donor. Results are given as fold change in biosensor to donor ratios achieved by evolved isolates relative to corresponding ancestors. Isolates of morphotypes (nWT=12; nMono=88; nCo=140) and ancestors (nWT=12; nMono=19, nCo=20) were replicated three times. The change in the potential to supplement another amino acid auxotrophic strain with tyrosine or tryptophan is significantly different between evolved auxotrophs from cocultures and controls (one-way ANOVA followed by Dunnett T3 *post hoc* test: $P<0.01$ for both comparisons). (b) Decrease in growth performance reveals fitness costs in isolates from cocultures. Growth kinetics under rich amino acid supplementation were used to calculate maximum optical densities and maximum growth rates for 30 morphotypes from cocultures and corresponding ancestors. Values are given as mean with standard errors from ratios of evolved isolate relative to the ancestor (n=4). Dotted lines indicate ancestral levels.

As a supplementary measure of investment we analysed levels of tyrosine and tryptophan in culture supernatants of individual clones. For this, isolated clones as well as their corresponding ancestors were individually supplemented with the amino acid they essentially required for growth and cultured in minimal medium until they had reached late exponential growth phase or early stationary phase. In these stages of the growth curve highest levels of amino acid were expected. After growth the cell-free supernatant of these populations was harvested and the concentration of tyrosine and tryptophan was quantified using tools of analytical chemistry (LC-MS/MS). Thus, the

experimental setup in regards to culture conditions was equivalent to the biosensor experiment, except the presence of a biosensor that continuously removed the required amino acid from the system. Hence we ultimately tested for constitutive overproduction even under the absence of a coevolved partner or any alternative recipient. Comparison with ancestral strains revealed evolved phenotypes from cocultures to release increased amounts of amino acid albeit growing in isolation (Fig. S2). Results of the biosensor experiment were confirmed for tyrosine auxotrophs, which released significantly more tryptophan into culture supernatants than ancestors (Fig. S2). Determined amounts of tryptophan furthermore show a weak positive correlation with promoted growth in the corresponding biosensor (Pearson correlation: $P=0.01$; $R=0.340$, $n=168$). In addition, decreased amino acid concentrations in control groups are in line with observed reduction of growth in biosensors. Isolates from auxotrophic monocultures even showed a drastic decrease in tryptophan towards levels found in blank culture medium (Fig. S2). Analysis of tyrosine concentrations supported observed biosensor growth within the wild type control group, which showed relative concentrations in median to be below ancestral levels. However, results are inconsistent between both experiments for evolved tryptophan auxotrophs. Detected tyrosine concentrations appeared to be significantly increased in isolates from auxotrophic monocultures (Fig. S2), however did not translate in biosensor growth which was significantly decreased (Fig. 2a, Wilcoxon signed ranks test: $P<0.001$, $n=10$ for ancestors and $n=44$ for isolates). This reverse trend is less pronounced in isolates from cocultures, however there was no significance as well as linear relationship found between amino acid release and biosensor growth (Pearson correlation: $P=0.091$; $R=-0.107$, $n=252$). In comparison, results in evolved isolates from cocultures show that auxotrophy causes major differences in the frequency as well as required conditions to detect cooperative cross-feeding. Evolved tryptophan auxotrophs from cocultures repeatedly require the presence of a recipient or partner to initiate cooperative cross-feeding, while tyrosine auxotrophs more frequently performed the evolved cooperative trait even in isolation. Out of the 21 isolated tryptophan auxotrophic mutants, 12 morphotypes (57%) in median displayed increased production levels of tyrosine in biosensor experiments and 8 (38%) in the supernatant analysis (Fig. S4). In contrast, 12 (86%) out of 14 tyrosine auxotrophic isolates shared more tryptophan with a biosensor, while 9 (64%) released more tryptophan (Fig. S4). The observed discrepancies between biosensor supplementation on the one hand and detected amounts in culture supernatants on the other hand importantly revealed phenotypes that produced

increased amounts of amino acid that in contrast did not promote respective biosensor-frequencies, suggesting secretion of additional metabolites hampering growth. Discrepancies furthermore show that quantification of shared resources in isolation from interaction partners hence by significantly altering ecological context is less suitable to detect cooperative phenotypes, yet helps in identifying permanent overproduction mutants.

Together, these results show that auxotrophic genotypes that evolved in coculture started to produce increased amounts of amino acids, presumably to support the growth of their respective partner. Finding that this was not observed in the two control groups suggests that the obligate metabolic interaction was driving this pattern.

2.3 Adaptation to coevolved partner is costly

Given that the coevolved auxotrophic genotypes have significantly increased their production level of tryptophan and tyrosine in response to the selection regime, we wondered whether this raised investment into the corresponding interaction partners translated into fitness costs to the overproducing cells. To test this, the growth performance of isolates from auxotrophic cocultures was compared with the one of their corresponding ancestors using minimal medium to which the required amino acid has been supplemented in sufficient amounts. Strikingly, despite the supplementation with high levels of amino acids (150 µM for both tryptophan and tyrosine), the growth performance of all virtually all derived auxotrophs from cocultures was consistently below the level of the ancestral auxotrophs (Fig. 2b). The only exception to this was one isolate, whose maximum growth rate improved in the course of the evolution experiment by in average ~10% (Fig. 2b). This result shows that adaptation to the coevolved partner, which included the increased production of the exchanged amino acid (Fig. 2a), incurred a significant cost.

2.4 What explains the increased growth of coevolved auxotrophs?

Releasing increased amounts of a costly metabolite into a spatially unstructured environment is at odds with evolutionary theory, which predicts natural selection should operate against such producers: If the amino acid is openly presented as a public good, it will be equally available to both mutants that produce it in increased amounts and all other cells that still show unchanged production levels. Since the mutant has to carry the burden of metabolite overproduction, but does not receive a benefit in return, it should

be disfavoured by natural selection and thus be readily lost from the population. However, it is well-known that stressed bacterial cells including *E. coli* commonly attach to other bacterial cells (29, 82, 83, 303, 410, 501, 518). By decreasing distance between cells the exchange of metabolites via diffusion in this way is enhanced (519). The resulting multicellular aggregates would create a spatial structure, in which an increased investment in form of increased amino acid production levels will likely be immediately rewarded: by locally enhancing the growth of the respective partner, producers automatically receive more amino acids in return. If this mechanism is true, three main conditions should be met: First, auxotrophic cells that interact in coculture should form aggregates consisting of multiple cells, while this behaviour should not be seen in both control groups. Second, growth of auxotrophic genotypes should depend on a physical contact between cells. Third, a positive fitness feedback should operate for cells that are part of a multicellular cluster, but not when they exist in a unicellular, planktonic state.

2.5 Cocultures of auxotrophs predominantly interact within multicellular clusters

To test the propensity of the derived populations of the three different experimental groups to form multicellular clusters, the size distribution of cellular aggregates within populations was analysed during their exponential growth phase by laser diffraction spectroscopy. This experiment revealed that in derived auxotrophic cocultures, the majority of cells (i.e. between 68 - 97% of all cells) existed within clusters of an average diameter of 45 µm. In contrast, populations of both derived WT and monocultures of auxotrophs were almost exclusively present in a unicellular form (Fig. 3a). This pattern was corroborated, when cultures of derived populations were analysed by fluorescence microscopy. Pairs of evolved auxotrophs that were labelled with green or red fluorescent proteins indeed formed multicellular clusters under coculture conditions (Fig. 3b). Interestingly, the observation that individual cells frequently displayed two fluorescent colours within the same cell (see Fig. 3b) suggests an intercellular transport of cytoplasmic materials such as proteins and amino acids via for example intercellular nanotubes (83).

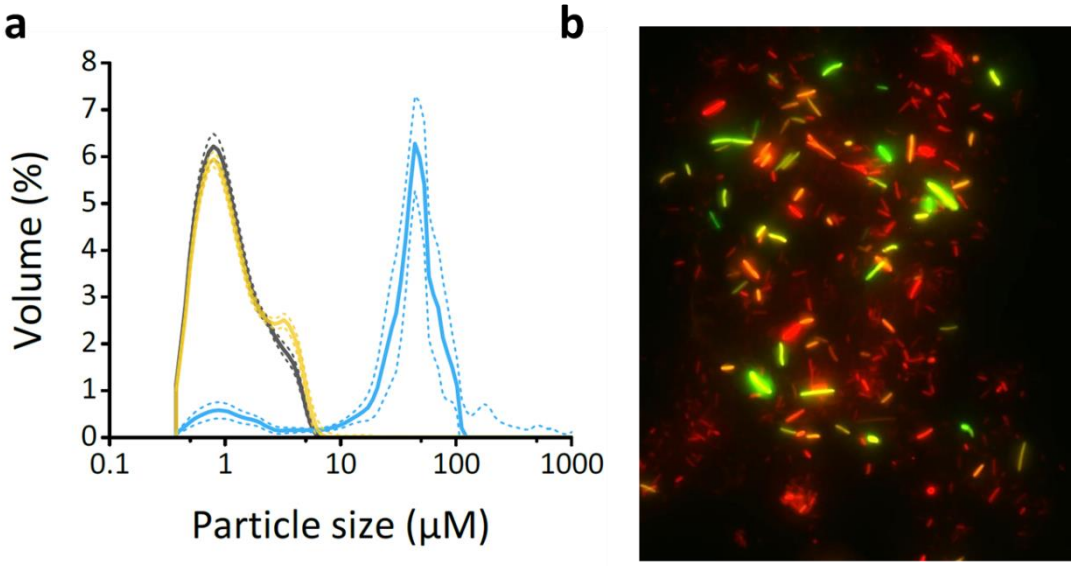

Figure 3: Multicellular clusters are exclusively formed within cocultures and harbour the majority of populations. (a) Size distribution in volume-percent of single cells and clusters of cells in terminal populations of wildtype (grey, n=12), monoculture (yellow, n=24), and coculture (blue, n_{Total}=30, n=10). Populations were analysed during exponential growth phase by laser diffraction spectroscopy. Lines are given as median with errors (95% confidence intervals) as dotted lines. The threshold to count particles as cell clusters was set at 10 µM, which is the maximum approximate length of two *E.coli* daughter cells that still stick together after division. To quantify differences in the extent of cluster formation, ratios of total volumes of particles >10 µM and <10 µM were calculated for all samples and compared between experimental groups. Cocultures exhibit significantly higher ratios than wildtype or monocultures (Dunnett T3 *post hoc* test, P<0.001; n_{WT}=12; n_{Mono}=19, n_{Co}=10). (b) Fluorescence microscopy z-stack image of a cell cluster harbouring two isolated genotypes from terminal cocultures that were labelled with either eGFP (green) or mCherry (red). Yellow or orange cells potentially contain both markers indicating exchange of cytoplasmic material as reported in Pande *et al.* 2015 (83).

In order to clarify, whether cluster formation is a derived trait that emerged during the evolution experiment or a property that always characterizes auxotrophic genotypes, the degree of cluster formation was compared between ancestral and derived consortia of auxotrophic genotypes. In addition, multiple time-points (i.e. early, intermediate, and late) of a growth cycle were analysed in both cases, to unravel whether auxotrophs persistently aggregate or undergo cycles of cluster formation and dissociation. Extensive cluster formation was consistently observed within cross-feeding populations during all time points analysed (Fig. 3a, Fig. S5) including the ancestral condition (Fig. S6), suggesting that it is not a derived trait. We hence conclude that aggregation did not evolve de novo in the course of the evolution experiment, but is a feature that characterizes auxotrophic genotypes in general. Analysing the progression of

cluster formation over the course of a growth cycle revealed that at the beginning, the majority of cells (~ 90%) were part of clusters, while an increased proportion of single cells within cocultures of auxotrophs was observed at the end of the growth cycle (i.e. in median 35-50%, Fig. S6). The disassembly of clusters into single cells was more pronounced and occurred much earlier in evolved cocultures than in ancestral cocultures (Fig. S6).

Given that the cultivation conditions of the evolution experiment included continuous shaking at high speed, it was unfortunately not possible to trace the fate of individual clusters for extended periods of time. Nevertheless, the results of this experiment indicate that the formation of clusters is likely a dynamic process of aggregation and disaggregation into either smaller clusters or individual cells that form a new group of cells as nutrients become available again.

2.6 Growth of auxotrophic cocultures is contact-dependent

The prevalence of multicellular aggregates in all cocultures suggested this behaviour is advantageous for auxotrophic mutants. One likely possibility is that the spatial proximity per se allows auxotrophic mutants to exchange amino acids more efficiently. To test this hypothesis, both ancestral and derived pairs of auxotrophic genotypes were grown in a device that allows to cultivate both populations either together in the same compartment or separated by filter membrane, which permits passage of free amino acids in the culture medium, yet prevents a physical contact between bacterial cells. Indeed, separating interaction partners in this way significantly reduced growth in both ancestral and evolved cocultures, thus confirming that physical contact between cells was key for an efficient transfer of amino acids between cells (Fig. S7). The fact that introducing the filter membrane affected the net growth of derived consortia less strongly than the ancestral consortium implies derived clones feature adaptations that make them less dependent on a very close physical contact with their partner strain. This could include, for example, an increased liberation of free amino acids into the extracellular environment in derived, but not the ancestral consortia. Together, this experiment confirmed that a close proximity among aggregated cells was necessary – particularly during early stages of the evolution experiment – to enhance growth by facilitating the exchange of amino acid between cross-feeding cells.

2.7 Positive feedback loops favour cooperation within multicellular clusters

We next asked which evolutionary mechanism facilitated the observed evolution and maintenance of metabolic cooperation in replicated populations. Given that in all replicates analysed, auxotrophic cells assembled into multicellular clusters (Fig. 3) and that these cluster were key to efficient growth of auxotrophs (Fig. S7), one conceivable explanation could be that cooperation was favoured on a cluster-level. Specifically, cells that increased their investment into the amino acid that they produce to support the growth of other cells, might be rewarded for this investment when they are part of a multicellular cluster, yet penalized when they are alone. By enhancing the growth of the respective other cells, amino acid overproducers might receive more of the amino acid they require for growth in return, thus enhancing their own fitness. The resulting positive feedback-loop should favour cooperative cells.

To test this hypothesis, we designed and performed an invasion-from-rare experiment that mimicked the emergence of a cooperative phenotype within a coculture of otherwise non-cooperative auxotrophs during early phases of the evolution experiment. Under these conditions, a newly evolved cooperator (i.e. the invader) is initially rare in frequency and competes with its evolutionary ancestor, which is common and shares the same auxotrophy (i.e. the competitor), for the amino acids that are produced by the respective other auxotrophic strain (i.e. the partner). Importantly, both the competitor as well as the partner feature ancestral levels of amino acid production. To evaluate the advantage that is gained by interacting within multicellular clusters, cooperators and their respective competitors were either cocultured together with the partner in the same environment or, alternatively, separated with a filter membrane, thus inhibiting the formation of joint clusters (Fig. 4). If positive fitness feedbacks operate on cooperative cells when being part of a cluster, the invasion success of cooperative auxotrophs should be high in the absence, but low in the presence of the filter membrane. For this experiment, six cooperative phenotypes, which have been isolated from evolved cocultures, were used as invaders. As a prerequisite only cooperative phenotypes were used that facilitated high levels of biosensor growth in the previous experiment hence indeed acted cooperatively (see methods and Fig. S4). In parallel, also the invasion success of the corresponding ancestors of selected phenotypes was tested, to control for effects that could emanate from the genetic background used (i.e. auxotrophy and phenotypic markers). When contact is allowed, these non-evolved auxotrophs should be

significantly less able to invade an ancestral consortium of auxotrophs than the derived cooperators.

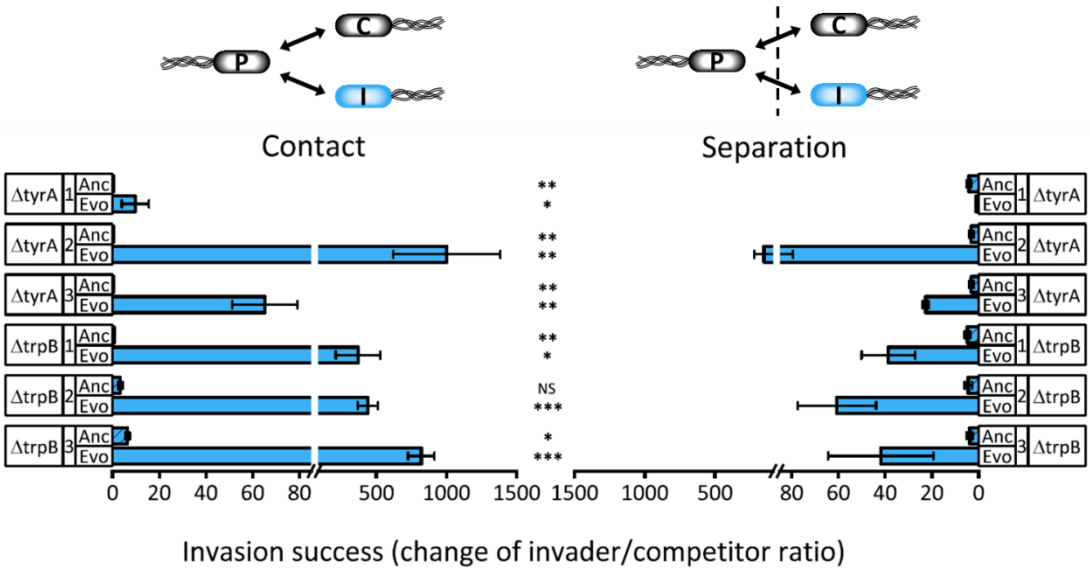

Figure 4: Spatial proximity promotes positive selection of cooperative phenotypes. The potential to invade into a population consisting of non-evolved tyrosine and tryptophan auxotrophs was tested for six cooperative phenotypes isolated from evolved cocultures as well as their respective ancestors (*ΔtyrA* 1-3, and *ΔtrpB* 1-3; Evo or Anc, respectively). Invaders (blue cell: "I") and competitor genotypes of similar auxotrophy (black cell: "C") competed for the focal amino acid shared by partner genotypes of complementary auxotrophy (black cell: "P"). Competition either took place in mixed cultures or in populations that were separated on the level of auxotrophy by a membrane filter. Initial frequencies of partner, competitor, and invader were ~50%, ~50%, and 0.05%, respectively. Ratios of invaders and their competitors were determined via plating before and after 72 h incubation. Invasion success is given as the average change of invader to competitor ratio during the experiment (errors are given as 95% CI). Treatments were directly compared for each tested cooperator (*i.e.* invader). Invasion success was significantly higher for all cooperative isolates when contact was allowed, while results were inconsistent for tested ancestors (paired samples t-test: *P<0.05, **P<0.01, ***P<0.001, NS: not significant; n=5).

These invasion-from-rare experiments revealed for all tested cases that in the absence of the filter, evolved cooperating types strongly increased in frequency and that this invasion success was significantly reduced when the two competing auxotrophs were separated from their respective partner by introducing a filter membrane (Fig. 4). Some invaders achieved a 1,000-fold increase in frequency and finally reached similar frequencies as their interaction partner, thus pointing to a tremendous selective advantage resulting for derived cooperators. To allow further conclusions we confirmed formation of multicellular clusters in three-partite consortia during cultivation without membrane filter by determining the size distribution via laser diffraction spectroscopy (Fig. S8). This supports the interpretation that the advantage, experienced by cooperative

cells, was likely due to a positive fitness feedback, which operated within aggregates of interacting cells. The fact that none of the control genotypes was able to invade the ancestral consortium and reach an increased frequency corroborated that neither the auxotrophy-causing mutation nor the phenotypic marker was involved in improving the invasion success of evolved isolates. In line with this ancestors consistently increased significantly less in frequency than respective cooperative phenotypes when contact was enabled (paired samples t-test: $P<0.001$; $n=30$). Taken together, these results provide strong experimental evidence that cooperative types gained a strong fitness advantage over non-cooperative auxotrophs when being part of a multicellular cluster.

3 Discussion

The evolution of cooperation within populations of well-mixed bacterial cells poses a major problem for evolutionary biology: why should individuals start to invest costly resources to benefit other bacteria, rather than utilizing these resources to maximize their own fitness? For these situations, evolutionary theory predicts that newly emerged cooperative genotypes that pay a cost for performing a cooperative behaviour, yet are not receiving any additional benefits for this investment in return, should be rapidly selected against and thus be lost from a given population (45, 51, 58, 59).

Here we show that cooperative cross-feeding of essential metabolites can rapidly evolve in populations of bacteria, whose growth requires a reciprocal exchange of essential metabolites among two bacterial genotypes. Cocultures rapidly increased fitness, while individual isolates decreased in fitness when cultivated alone indicating reduced independency, which was reported for an evolved mutualism as well (112). The transition from the initial by-product interaction into a costly cooperation was due to the formation of multicellular clusters of bacteria. These structures not only enhanced the exchange of metabolites between cells even in a well-mixed environment, but also resulted in positive fitness feedbacks that benefitted cooperative mutants when being part of a cluster. It is well-known that spatially structured environments facilitate the evolution and maintenance of cooperative interactions (83, 158, 294, 520-524). Several causal reasons can account for this phenomenon. First, random mixing of cooperative and non-cooperative genotypes before a given surface is colonized results in local patches that differ in their genotypic composition. In areas were multiple cooperative genotypes co-localize by chance, cells can grow more than in patches, which are

dominated by non-cooperating types. In addition, if the cooperation is based on an exchange of metabolites, released compounds may locally accumulate and preferentially benefit resident cells (89, 303). In case the interaction lasts long enough, this gives non-cooperating cells the chance to reciprocate, for example by acquiring additional mutations that close the cooperative loop (106). Second, as cells grow, self-organization within expanding bacterial populations can lead to a spatial segregation of cooperative and non-cooperative cells, thus resulting in an exclusion of non-cooperators from cooperative benefits (89, 92). However, the problem with this is that the colonization of a spatially structured surface is usually a dead end. How can the increased productivity of more cooperative patches be exported to the next generation of bacteria despite of dispersal or disturbance? Our work resolves this issue by showing that even in spatially unstructured environments, cooperation can evolve. The key criterion for this to happen is that bacteria generate a spatially structured population by themselves that is independent of a surface-attached growth. Within these free-floating, multicellular aggregates, similar principles as outlined above are likely to operate. In particular, the invasion-from-rare experiment conducted strongly suggests local fitness feedbacks within multicellular clusters to explain the observed evolution of cooperation. Moreover, in our experiment cells apparently underwent dynamic cycles of aggregation and disaggregation or budding (Fig. S6). Consequently, clusters with a higher proportion of cooperative cells likely leave more offspring than less cooperative clusters, which allows them to export their enhanced productivity to the next generation. In the following interaction round, cells aggregate again and the cycle repeats. These observed dynamics in population structure are reminiscent of a nascent life-cycle

Interestingly, the formation of multicellular aggregates was not a derived trait, but characterized already ancestral cocultures of auxotrophs. Under our experimental conditions, auxotrophic cells could only grow when they derived amino acids from other cells in their environment. By physically attaching to other cells, the spatial distance between donor and recipient is reduced, which likely facilitates an exchange of metabolites between cells (501). In this way, the loss of metabolites by diffusion into the extracellular environment is reduced (303). However, what triggered the formation of multicellular aggregates?

One likely explanation is a physiological stress response that resulted from the starvation of auxotrophs for the two amino acids tyrosine and tryptophan. In our experimental set-up, auxotrophs that were grown in cocultures most likely experienced

phases of severe amino acid deprivation, while this was not (or to a lesser extent) the case for monocultures of amino acid-supplemented auxotrophs. Starvation for amino acids is known to trigger the so-called stringent response in auxotrophic bacteria (525). Under these conditions, expression of the leucine-responsive regulator protein (Lrp) is up-regulated (526), which in turn stimulates phase variation of fimbriae (527) hence increases the likelihood of activating their production (528) and in addition activates production of extracellular polymeric substances (529). Altogether this leads to autoaggregation (530). An alternative mechanism could be the formation of intercellular nanotubes that is also induced by amino acid starvation in auxotrophic bacteria (83). The detection of double-labelled cells in auxotrophic cocultures (Fig. 3b) corroborates that these cells exchanged cytoplasmic materials – most likely via nanotubes.

By linking starvation to the formation of multicellular aggregates, this regulatory pathway may have been the key molecular driver orchestrating the cellular behaviours that gave rise to the evolutionary dynamics observed in this study. Reportedly the stringent response is highly conserved among bacteria (531). Strikingly, also in the social bacterium *Mxococcus xanthus* the transition from a unicellular to a multicellular lifestyle is sensitive to changes in the availability of nutrients and controlled by the stringent response (531). In unicellular eukaryotes that aggregate to generate a multicellular form during some parts of their life cycle, starvation is the stimulus triggering these behaviours as well (532). Together, this accordance suggests that starvation could be a potent driver for emergence of an aggregative multicellularity in a broad range of phylogenetically different organisms.

Taken together, the results presented in this work show how simple changes in the genomes of bacteria, in this case the loss of two biosynthetic genes, can set off an evolutionary dynamic that drastically reconfigures the ecology and evolution in an entire microbial community. The fact that these mutations forced the two resulting strains to interact with each other in order to grow, paved the way for the initial by-product interaction to evolve into a cooperative metabolic interaction. Key factors driving this change were (i) the assortment of auxotrophic bacteria into multicellular clusters, (ii) a dynamic aggregation and disaggregation of these clusters, and (iii) positive fitness feedbacks that operated on cells within these clusters and favoured cooperative mutants. Given the prevalence of auxotrophic bacteria in natural microbial communities (56, 63), the well-known propensity of bacteria to form multicellular aggregates in order to facilitate an exchange of metabolites (377, 378, 533-535), and the ease with which

cooperative interactions evolve between those auxotrophs (this study) it is likely that cooperative metabolic interactions may be much more common than previously thought. To date however we still lack insights about consequences of such a process relative to a solitary lifestyle. Therefore future work utilizing next generation sequencing in evolved cocultures and control groups may reveal the trajectory and extent to which synergistic coevolution influences genome evolution.

4. Material and methods

4.1 Strains and plasmids

To synthetically design an obligate cross-feeding interaction, *Escherichia coli* BW25113 (159) was used as the wild type, and genetically modified by P1 transduction (536) to generate in-frame knockout mutants by replacement of target genes with a kanamycin resistance cassette (159, 537). These mutants lacked the genes *trpB* or *tyrA* that encode for enzymes responsible for the terminal amino acid biosynthesis step of tryptophan or tyrosine, respectively. Generated auxotrophic genotypes hence contained an in-frame replacement of the targeted amino acid biosynthesis gene (*trpB* or *tyrA*) with a kanamycin cassette. Both amino acid auxotrophic genotypes were combined in cocultures that resembled the starting point of the evolution experiment. To allow phenotypic discrimination of these two auxotrophic genotypes on agar plate, the marker genes *araDAB* (derived from *E. coli* REL607 (538)) and the functional *lacZ*-gene (derived from E. coli MG1655 (539)) were additionally introduced by P1 transduction. As a result, strains carrying the functional alleles for arabinose utilization and β-galactosidase appear blue on modified TA-agar (540) that additionally contained 0.1 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) and 50 µg ml^{-1} x-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), while the WT phenotype appears red. The presence of the resistance cassette as well as the marker genes, and amino acid auxotrophy was first confirmed on respective selective agar plates and further verified in the course of whole genome resequencing.

For the quantification of shared amino acids as a measure for cooperativeness, biosensors were generated for detection of tyrosine and tryptophan levels released by a donor strain (517). The corresponding genotypes carrying the functional *lacZ*-gene and the knockout of *trpB* or *tyrA* were generated as described above. The derived genotypes *E. coli ΔtrpB::kan lacZ* and *E. coli ΔtyrA::kan lacZ* were further modified to enable

quantification of colony forming units (CFU) on agar plates irrespective of exhibiting low frequencies within a population of other *E. coli genotypes*. For this, the kanamycin resistance cassette was replaced with a chloramphenicol resistance cassette using the method described by Datsenko and Wanner 2000 (537). In detail, the chloramphenicol cassette (the *camR* gene) from pKD3 was amplified by PCR using the reported primers for both frt sites that direct site-specific recombination. After transforming the Red helper plasmid pKD46 into both genotypes, electroporation with the PCR-product was performed (537). Generated constructs containing *camR* were selected on LB agar with 30 µg ml^{-1} chloramphenicol and restored sensitivity for kanamycin was confirmed.

Plasmids pJBA24-*egfp* (517) or pJBA24-*mCherry* (83), that constitutively express the respective fluorescent protein as well as a β-lactamase, were transformed into the WT, the auxotrophic genotypes, as well as selected evolved genotypes that were isolated from derived populations of the long-term evolution experiment.

4.2 Culture conditions and general procedures

In all experiments, cells were grown using minimal medium for *Azospirillium brasilense* (MMAB) (541) without biotin using 0.5 % glucose instead of malate as a carbon source. To obtain MMAB agar, two-fold concentrated Kobe-agar (30 g l^{-1}) was added to 2-fold concentrated MMAB medium in a 1:1 ratio. Unless otherwise noted, culture conditions were kept constant between experiments (30 °C, 225 rpm) and precultures of auxotrophic genotypes were supplemented with amino acid (150 µM tryptophan or tyrosine, respectively). If not indicated differently these amino acid concentrations were generally used for supplementation. Bacterial strains were freshly streaked on LB agar and incubated for 24 h or until single colonies showed sufficient size for inoculation of liquid cultures. Individual colonies were used as biological replicates to inoculate 1 ml overnight precultures in 96 deep-well plates (max. volume: 2 ml, Thermo Scientific Nunc), which were diluted to an optical density 600 nm (OD_{600nm}) of 0.1 the next day. Unless otherwise specified, these precultures were subsequently used to inoculate 1 ml MMAB medium with a final OD_{600nm} of 0.001. To enable blue-white staining agar contained 0.1 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) and 50 µg ml^{-1} x-Gal (5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside). Antibiotics were used at the following concentrations: kanamycin 50 µg ml^{-1} and chloramphenicol 30 µg ml^{-1}.

4.3 Evolution experiment

The evolution experiment comprised three major experimental groups: The main group of interest, *i.e.* populations performing the obligate cross-feeding interaction, and two control groups, *i.e.* monocultures of prototrophic wildtype as well as generated auxotrophic genotypes. Aim of the control groups was to directly compare observations in derived cocultures with those made in derived monocultures hence to clearly attribute observations to be causative for the obligate interaction, the genetic modifications, or the abiotic culture conditions. To generate the synthetically designed obligate by-product interaction, complementary phenotypes (*i.e. E. coli* $Tyr^{-}Ara^{+}$ Lac^{+} & *E. coli* Trp^{-} Ara^{-} Lac^{-}, or the reverse combination of phenotypic labelling and auxotrophy) were combined in cocultures in an initial ratio of 50:50. Two major control groups contained either monocultures of the different generated auxotrophic strains to determine effects of adaptation to genotypic background (prototrophy or auxotrophy, and presence or absence of phenotypic marker genes) or monocultures of the wildtype to determine effects of adaptation to abiotic culture conditions and phenotypic labelling (presence or absence of phenotypic marker genes). Each six biological replicates of each genotype (*i.e.* the wildtype, the Δ*trpB* knockout, and the Δ*tyrA* knockout either without or with both phenotypic markers) were used to start the evolution experiment, adding up to twelve WT monocultures, twelve cocultures, and 24 monocultures of auxotrophs. Auxotrophic monocultures were incubated under identical conditions as cocultures and wildtype aside from required amino acid supplementation of 50 µM tyrosine or tryptophan. Amino acids were by purpose kept at limiting concentrations to mimic conditions and starvation stress of cocultures. To start the evolution experiment 4 ml of minimal medium in 20 ml scintillation vials (Wheaton Industries Inc., USA) were inoculated with an initial OD_{600nm} of 0.005. Populations were initially transferred every seven days for a total of five transfers, following 15 transfers every three days, adding up to a total of 80 days or approximately ~150 generations of bacterial growth. At the end of each cycle, optical densities were determined at 600 nm via spectrophotometry in a plate reader (Spectramax M5, Applied Biosystems; United States) and 20 µl of culture were transferred into 4 ml of fresh MMAB-medium. Depending on the length of the cycle (*i.e.* three or seven days), glycerol stocks (20% glycerol) were prepared each six or seven days and stored at -80 °C. Cocultures were regularly tested for revertant phenotypes that showed prototrophic growth (i.e. that were capable to grow on MMAB agar without amino acid supplementation). Out of twelve cocultures, two were excluded

from further analysis due to prototrophic phenotypes. Accordingly, the two cognate replicates of auxotrophic monocultures were excluded from further analysis as well.

In addition, one replicate of *E. coli* Δ*tyrA* Δ*araDAB* Δ*lacZ* from monocultures was excluded from further analysis due to a contamination of the culture. Terminal populations were spread on modified TA agar plates to isolate evolved clones based on differences in colour and colony morphology. For each detected morphotype (i.e. colony morphology) in auxotrophic populations, four individual colonies were isolated as biological replicates, which were used in subsequent experiments. Phenotypic diversity was observed in eight out of ten derived cocultures of auxotrophic genotypes resulting in a total of 35 isolated phenotypes, and three out of 19 auxotrophic monocultures yielding a total 22 isolated phenotypes. All monocultures of the prototrophic wild type remained phenotypically homogeneous. Isolates were stored at -80 °C until further analysis.

4.4 Relative fitness of ancestral vs. evolved populations

To characterize the achieved improvements in growth of evolved populations, fitness relative to the corresponding ancestor was determined. Therefore, cultures of evolved populations as well as the respective ancestors were directly inoculated from cryo-stocks and incubated for 72 h in 4 ml of MMAB medium. The number of colony-forming units (CFUs) was estimated at 0 h and 72 h by plating on modified TA agar. The Malthusian parameter M (538) describes the reproductive capacity of a given number of individuals during a given period of time and was calculated as a measure for fitness of evolved populations (denoted as “Evo”) relative to their respective ancestor (denoted as “Anc”) as

$$M_{\mathrm{Evo}} \,/\, M_{\mathrm{Anc}} \;=\; \ln(N_{\mathrm{f,Evo}}/N_{\mathrm{i,Evo}}) \,/\, ln(N_{\mathrm{f,Anc}}/N_{\mathrm{i,Anc}})$$

with N_i as the initial number of CFU and N_f as the final CFU-count after 72 h. Each evolutionary lineage was analysed using six replicates.

4.5 Growth performance of isolates from cocultures in single cultivation

After determining fitness on the level of whole cross-feeding populations, the ability to grow autonomously (except amino acid supplementation) was tested in isolated phenotypes from cocultures to evaluate side-effects of adapting to cooperative cross-feeding on independence. Ancestral auxotrophs were analysed as well to finally quantify these effects.

Therefore growth kinetics were monitored by measuring optical density at 600nm every 30 min for a total of 72 hours in an Tecan Infinite F200 Pro plate reader (Tecan Group Ltd., Switzerland). Strains were cultivated in a 384 well plate containing 50 µl minimal medium per well, which was supplemented with the required amino acid. During each cycle (*i.e.* 30 min), the microtiter plate was shaken trice for 3 min. Determined growth curves were used to calculate relevant growth parameters. Magellan software (Tecan Group Ltd., Switzerland) was utilized to calculate μ_{MAX} using six time points and OD_{MAX} using eight time points. Readouts for evolved phenotypes (n=4) were divided by averaged values of ancestors (n=6) with the respective genotypic background.

4.6 Quantification of amino acid production levels using biosensors

To compare the amount of tyrosine or tryptophan that was produced by ancestral and evolved strains, both types were used as amino acid donor in coculture with auxotrophic biosensor strains. Auxotrophic donors were supplemented the required amino acid, while biosensors growth was depended on the shared amount of amino acid by the donor. Growth of a biosensor will hence correlate with the released amount of amino acid by a given donor (517). For this, the generated strains *E. coli* Δ*trpB* Δ*araDAB lacZ camR* and *E.coli* Δ*tyrA* Δ*araDAB lacZ camR* were utilized as biosensors, since their numbers could be determined irrespective of frequency when in coculture with a donor. Auxotrophic donor strains were supplemented with 150 µM of the respective amino acid, while WT cells were cultivated in the absence of externally supplied amino acids. Cocultures of donors and recipients (i.e. amino acid biosensors) were inoculated in a 1:1 ratio in 1 ml MMAB medium and incubated for 72 h. The number of CFUs (colony forming units) of donor and biosensor was determined at the beginning as well as after the coculture experiment (i.e. after 3d) by plating. To enable phenotypic discrimination between both types, populations were plated either on either LB agar plates containing xGal as well as IPTG (resulting in white and blue colonies, respectively) or TA agar plates (resulting in white and red colonies, respectively). In addition, cocultures were spread on LB agar containing chloramphenicol to determine cell numbers of biosensors in a very low abundance. In parallel to cocultures, monocultures of biosensors were incubated in MMAB medium without amino acid supplementation to determine basal growth, which was subtracted from absolute CFU in cocultures to calculate net-growth in the presence of a donor strain. The experiment was replicated three times for each ancestral population as well as each isolated clone from evolved cocultures.

4.7 Amino acid quantification in culture supernatants

Released amounts of tyrosine and tryptophan were quantified as described for tryptophan analysis in Pande *et al.* 2015 (83) that adapted the protocol from Jander *et al.* 2004 (542). Precultures for all replicates of isolated phenotypes were incubated until stationary phase and supernatants were harvested by centrifugation (3,800g for 20 min) in a 96 filter plate (0.2 µM AcroPrep^{TM} 96 filter plate, Pall Corporation, USA). Culture supernatants were directly used for analysis after adding 10 µg/ml of ^{13}C, ^{15}N-labelled amino acid mix (Isotec, Miamisburg, OH, USA).

4.8 Contact-dependent growth

To determine whether physical contact between cells is necessary to facilitate the exchange of amino acids and thus growth, interacting genotypes were cultivated in a device that allows to grow two cell populations either in the same compartment, or separated by a filter membrane (0.2 mm, polyethersulfone, Pall GmbH, Germany), which prevents a physical contact between cells, but a transfer of amino acids through the extracellular environment (i.e. Nurmikko cells, (83, 543)). In this experiment, both pairs of ancestral auxotrophic genotypes and derived genotypes were analysed that have been cocultured together. In cases were multiple morphotypes have been isolated from evolved auxotrophs, all isolated clones were mixed in equal ratios according to auxotrophy-causing mutations. Each combination was replicated four times. The initial OD_{600nm} was set to 0.001 with each auxotrophy representing 50% of the initial population. In cases were multiple isolates have been isolated from the same derived population of cocultured auxotrophs, their initial density was adjusted such that their combined density reached an OD_{600nm} of 0.001, as well. Each Nurmikko cell contained 4 ml MMAB minimal medium and was incubated under shaking conditions (i.e. 150 rpm, 30 °C). Total numbers of CFUs were determined after 0 h and 72 h incubation on modified TA agar.

4.9 Cluster formation

In order to determine the propensity of the different experimental treatment groups to form multicellular clusters, cultures that have been isolated from different time points of the evolution experiment were subjected to a laser diffraction spectroscopy. This technique utilizes diffraction patterns of a laser beam that is passed through a solution

and thereafter through a Fourier lens on a detector array to measure particle size distributions. To this end, ancestral or derived populations of the three experimental groups (i.e. prototrophic WT, auxotrophic monocultures, auxotrophic cocultures) were directly inoculated from cryo-stocks into glass bottles containing MMAB medium. Monocultures of auxotrophs were additionally supplemented with 50 µM of the respective amino acid. The total culture volume was adjusted to the optical density reached during the exponential growth phase (i.e. WT: 20 ml, auxotrophic monocultures: 50 ml, coculture: 100 ml). Due to the increased variation observed in test experiments, each coculture population (n=10) was replicated three times, while each population of control groups was only replicated once (n=12 for WT, and n=24 for auxotrophic monocultures). Cluster formation was verified for ancestral (0 days) and evolved populations (80 days). Additional time points that were checked for cluster formation from the evolution experiment were t4 (28 days), t11 for cocultures and wildtype (53 days), t12 for auxotrophic monocultures (56 days). Analysis of particle size distribution was performed utilizing a Beckman Coulter LS 13 320 laser-diffractometer with universal liquid module using the Fraunhofer optical model. If necessary, precultures were either diluted until a sufficient obscuration (~5%) was reached or were used undiluted in case of low optical density. Analysis was performed with precultures in exponential growth phase with pump speed set to 6% to minimize shear forces that degrade cell clusters over time. Each sample was measured three times for one minute. Averaged output files of these individual measurements were used for further analysis.

4.10 Invasion-from-rare experiment

To determine whether or not physical contact and local interactions favour evolved cooperative phenotypes in multicellular clusters consisting of ancestral, non-cooperating genotypes, an invasion from rare experiment was conducted. For this experiment, the invasion success of cooperative phenotypes within a population of less cooperative auxotrophs was quantified using Nurmikko cells and non-evolved ancestral auxotrophs were used as controls. Tripartite populations (i.e. two ancestral auxotrophs plus one invader – an evolved cooperator or ancestral auxotroph) were either grown separated on the level of the auxotrophy by a filter membrane or under conditions that allowed mixing among genotypes. Suitable cooperative phenotypes were selected among evolved isolates from cocultures based on their cooperative supplementation of an auxotrophic biosensor strain as indicated by the results of the biosensor experiment (Figure S4). In

this way, a total six isolates (Av1b, Av6lb, Bv1r, Av1rw, Bv5bb, and Bv6b) were chosen with at least one representative for each of the four genotypic combinations of auxotrophy and phenotypic marker genes, which have been used in the evolution experiment. Two ancestral auxotrophs were used to found a coculture in a 1:1 ratio and an initial OD_{600nm} of 0.005 to which an invader (i.e. evolved or ancestral auxotroph) was added with a 0.05 % initial frequency. Each combination including a particular invader was replicated five times and conditions were identical to the Nurmikko cell experiment mentioned above. CFUs were determined by plating during the onset of the experiment and after three days of incubation. To discriminate biosensor strains in established tripartite consortia, cultures were plated on MMAB agar plates containing one of the required amino acids. Further discrimination of the respective auxotroph (i.e. competitor) and the invader sharing the same auxotrophy required the use of indicator dyes in the respective MMAB agar plates as described above for the biosensor experiment. To determine low frequencies of invaders, plating was additionally performed on LB agar containing 50 µM kanamycin. The invasion success is given as the change in invader-to-competitor ratio, which in principle was adapted from calculations for the selection coefficient (544). The reason behind adapting calculations was that mathematical operations for the selection coefficient do not consider negative growth. This was however observed in few competitor frequencies likely due to the evolved phenotype resembling a strong competitor itself. Ratios were determined at the initial time point (inoculation) and at the end of cultivation after three days. In detail invasion success was calculated by dividing the final ratio of invader to competitor CFU counts with the initial ratio of invader to competitor CFU counts.

Supporting information

The following supporting information is available for this chapter starting with page 194

Chapter 3

Chapter 3

Synergistic coevolution accelerates genome evolution

Ecological interactions are key drivers of evolutionary change. Even though it is well-documented that antagonistic coevolution can cause genetic divergence and accelerate molecular evolution, the evolutionary consequences of synergistic coevolution remain poorly understood. Here we show experimentally that also synergistic coevolution can speed up the rate of molecular evolution. Pairs of auxotrophic genotypes of the bacterium *Escherichia coli*, whose growth depended on a reciprocal exchange of amino acids, were experimentally coevolved and compared to amino acid-supplemented monocultures of auxotrophs or metabolically autonomous wild type cells as a control. Coevolution resulted in the emergence of a strong metabolic cooperation that coincided with a significantly increased number of mutations in the genomes of coevolved auxotrophs as compared to genomes of control groups. Moreover, coevolved cooperative populations showed an increased degree of parallel evolution as well as divergent evolutionary trajectories relative to both control groups suggesting constrained adaptation to the abiotic environment. Together, these results demonstrate that similar to antagonistic interactions, also synergistic coevolution can cause rapid and divergent evolution that in the long-run may drive speciation driven by mutualistic interactions.

1. Introduction

The evolution of microorganisms is fundamentally driven by ecological interactions (545), but the extent to which positive as well as negative relationships contribute to this ongoing process remains puzzling. It is hence crucial to specifically unravel the consequences each ecological interaction has on its own to finally understand the complex interplay and the resulting outcome of diverse selection pressures acting simultaneously on microbial species. Recent work on antagonistic interactions uncovered significant consequences on coevolving species: In accordance to the red queen hypothesis (546), coevolution with their bacterial host increased the rate of molecular evolution in phages, which in return increased genetic variation in the host (32, 547). What is more, adaptation to the abiotic environment was demonstrated to be constraint by adaptation to ecological interactions and vice versa (32, 119, 548, 549). Taken together antagonistic interactions were shown to significantly influence rate as well as trajectory of evolution and are likely a "major driver of evolutionary change within species" (31).

In contrast, only little is known about consequences cooperative interactions impose on coevolving partners in general. One theory, termed the Red King effect (117), suggests that decelerated evolution can be advantageous for one mutualistic partner under relaxed selection (550) and when unilateral increase of received benefits is favoured (75, 117). However, this outcome requires both partners to differ in their rates of evolutionary change (117), a scenario intuitively met by species from different kingdoms that frequently engage in mutualisms, yet rather not by cooperating bacterial species. While fulfilling these conditions, Rubin and Moreau (2016) show in a seminal study of ant-plant mutualisms that cooperative plant-ants in contrast to predictions exhibit higher rates of genome evolution than respective non-symbiotic species (118). Aside from these insights only a few studies actually addressed synergistic coevolution by utilizing microbial model systems (105, 106, 112, 158), reporting first insights on imposed consequences on genome evolution (112), yet conflicts in adaptation between the biotic and the abiotic environment remain virtually unclear.

In previous work we demonstrated synergistic coevolution to rapidly and repeatedly result in metabolic cooperation within replicated bacterial populations. Initially, cocultures of two *Escherichia coli* strains that either lacked the ability to synthesize tyrosine or tryptophan barely grew in minimal medium by the enforced exchange of these amino acids. Hence, as a consequence of the interactions obligate nature, fitness

and thus fate of the cocultured genotypes (i.e. cross-feeding partners) was coupled. Importantly the experimental design invoked control groups which comprised monocultures of the prototrophic wild type as well as auxotrophic genotypes from cocultures. Monocultures lacked the obligate interaction but experienced identical treatment as cocultures, hence functioned as reference for adaptation to abiotic culture conditions as well as genotypic background. Cooperative cross-feeding independently evolved within ten replicated cocultures in less than 150 generations, which was accompanied by a significant improvement in fitness towards levels of evolved monocultures. This demonstrates an astonishing capability of an auxotrophic consortium to rapidly compensate the loss of vital biosynthetic functions. We were next interested in associated implications of the observed de-novo evolution of metabolic cooperation relative to control groups on the genomic level. By utilizing modern sequencing techniques we successfully attributed observations to either resemble a consequence of synergistic coevolution or rather adaptation to abiotic culture conditions as well as genotypic background (i.e. auxotrophy).

We present the first empirical evidence of synergistic coevolution to (i) accelerate molecular evolution, (ii) drive parallel evolution among replicated populations while partners do not share mutated sites, and (iii) cause evolutionary trajectories that are highly divergent to an independent lifestyle.

2. Results

We previously demonstrated cooperation to rapidly evolve from an obligate by-product interaction within short evolutionary time of less than 150 generations. Cooperative cross-feeding exclusively evolved within replicated cocultures of auxotrophic *E. coli* strains, while adaptation to the abiotic environment rather decreased levels of shared amino acids in control groups, which suggests strong positive selection for cooperation within cocultures. Moreover, in contrast to independent control groups, cocultures consistently showed dramatic changes in population structure by the formation of multicellular clusters, which were demonstrated to promote cooperators to increase in frequencies. These results clearly unravel a divergent development between auxotrophic cocultures and control groups, which indicates that evolution in cocultures is driven by adaptation to the obligate interaction, yet not to abiotic culture conditions. We were hence interested in quantifying associated consequences of the evolution of cooperation on the genomic level, especially relative to a lifestyle that lacks the obligate

interaction, by direct comparisons of evolutionary trajectories of the three experimental groups. Therefore resequencing of 63 evolved clonal isolates (*i.e.* isolated morphotypes, as reported in previous work) and 24 whole-population samples was performed in the three experimental groups (auxotrophic cocultures, auxotrophic monocultures, and wildtype monocultures). Analysis revealed in total 325 and 205 mutations for clonal samples and whole-population samples, respectively. Sequenced isolates and whole populations hence exhibited mean numbers of 5.2 and 8.5 mutations per analysed sample. Based on the detected spectrum of mutations within and between groups, differences were visualised on the gene-level utilizing distance trees (Fig. 1a, and Fig. 2a).

2.1 Parallel evolution in cocultures is divergent to prototrophic and auxotrophic monocultures

As suggested by previous insights, cocultures indeed cluster as one consistent group sharing a common branch that is distinct to control groups (Fig. 1a). Except two wildtype populations that root early into the branch of cocultures close to the coalescence, control groups in general share two separate main branches. In detail both of these branches comprise populations of both control groups independent of genotype. Control groups hence show a low preference of replicated populations being mapped to similar branches. Taken together the observed topology indicates levels of parallel evolution to be highest within auxotrophic cocultures. To quantitatively evaluate the extent of parallel evolution, we determined the Jaccard Index (J) as a measure of parallelism for all possible comparisons within and between experimental groups (Fig. 1b). The extent of similarity in accumulated mutations was computed on the gene level. Comparisons confirm degree of parallelism to be significantly higher in cocultures than in both control groups (Fig. 1b). Mean J values range between 0.11 for wild type and 0.09 for auxotrophic monocultures, while reaching 0.2 in cocultures. Between-group comparisons furthermore reveal low degree of parallelism among experimental groups in general with a mean J value of 0.04, which is illustrated by few overlaps in the spectrum of mutated genes (Fig. S1a). Accumulated mutations hence not only concerned different genes due to the presence or lack of the obligate interaction, yet amino acid auxotrophy as well influenced genome evolution (Fig. S1b). Furthermore, experimental groups only have one altered gene in common (Fig. S1a). In conclusion, significant differences in evolutionary trajectories were identified on the population level. Given that minimal

overlaps in the spectrum of mutated genes were observed (Fig. S1a), we conclude that the obligate interaction as well as the genotypic background of auxotrophic strains completely altered adaptation relative to the prototrophic wildtype.

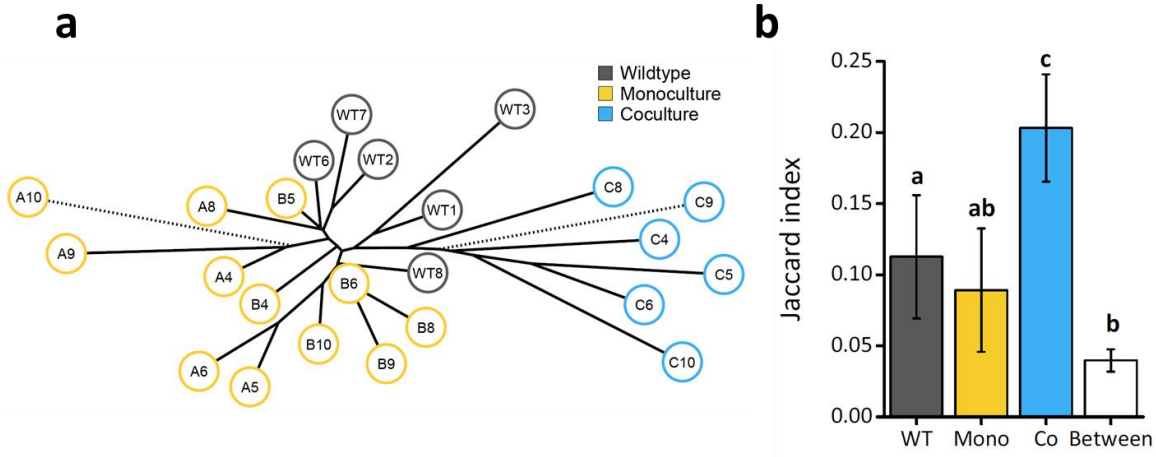

Figure 1: Evolution of cooperation drives parallel evolution that is highly divergent from an independent lifestyle. (**a**) Distance tree derived from a population level genotyping matrix utilizing standard neighbour joining. Nodes are colour-coded by experimental group. Evolutionary trajectories differ between control groups (WT = wildtype; A = tyrosine,- and B = tryptophan auxotrophic monocultures) and cross-feeding populations (C = cocultures). Cocultures cluster as one group (*e.g.* share a branch) distinct to control groups that lack the cross-feeding interaction. Labels containing similar numbers indicate a common ancestor among auxotrophic populations. Dotted lines indicate shortened branches that exceeded a certain threshold (see methods). (**b**) Degree of parallel evolution within and between experimental groups on the level of mutated genes given as mean Jaccard indices with 95% confidence intervals. Letters indicate significant differences (Dunett T3 *post hoc* test: $P < 0.05$, WT: n=15; Mono: n=30; Co: n=15, Between (all between-group comparisons): n=216).

2.2 Partner-specific evolutionary trajectories

Population-level analysis did not allow mutations to be assigned to a specific auxotrophic partner in cocultures. Therefore we analysed the spectrum of mutations in clonal samples, *i.e.* isolates from evolved wildtype monocultures (n=10), auxotrophic monocultures (n=22), and cocultures (n=31). Remarkably, cross-feeding partners are mapped to completely distinct branches depending on auxotrophy (Fig. 2a), which reveals partner-specific evolutionary trajectories. While isolates from cocultures auxotrophic for tryptophan share one main branch, tyrosine auxotrophic isolates are distributed over three branches that also comprise individual isolates from tyrosine auxotrophic monocultures. Interestingly, these isolates from monocultures share a common ancestor with respective isolates from cocultures and root early into the same

branch in all four observed cases (Fig. S2a). This either indicates a predisposition for certain mutations which is unlikely or could be a result of coincidence by picking biological replicates that already carried the shared mutation before the evolution experiment was initiated. Re-sequencing of respective replicates to confirm the presence of mutations within initial cocultures is therefore recommended and will be part of further work.

As described above, we isolated and resequenced all morphotypes of each population (*i.e.* optically discriminable phenotypes on agar-plate). Morphotypes of similar auxotrophy that were isolated from the same population expectedly mapped to similar side-branches, since these clones likely originated from a common lineage hence shared several mutations (Fig. S2a). In detail, isolates that shared similar auxotrophy and originated from the same auxotrophic coculture or monoculture approximately shared 75% and 33% of mutations, respectively. The remaining isolates from control groups are mapped on separate branches with no consistent grouping preference of identical genotypes, which is in line with observed topology in mapped population samples. We quantitatively evaluated the extent of parallel evolution on the gene-level in clonal samples as well. Determined J values for all possible comparisons within and between experimental groups show similar patterns of observed parallelism as analysed populations (Fig. S2b). However, when groups are separated by auxotrophy, tyrosine auxotrophic genotypes show significantly reduced within-group parallelism (Fig. 2b). Levels of similarity are indeed significantly higher in cocultures than in monocultures, yet tyrosine auxotrophic isolates from both experimental groups show significantly lower degree of parallelism than respective tryptophan auxotrophic isolates (Fig. 2b). J values among tyrosine auxotrophs from monocultures do not even differ with between-group comparisons. These comparisons furthermore confirm low degree of parallelism among experimental groups, which is underpinned by few overlaps in the spectrum of mutated genes between groups of auxotrophic isolates (Fig. S1b). However, there is one exception: comparisons between the wildtype and tryptophan auxotrophs from monocultures resulted in mean J values of 0.1 (data not shown), which is at the level of three within group comparisons (Fig. 2b). Taken together resequencing of clonal samples confirmed observed patterns in analysed population samples and revealed a general trend of highly divergent evolutionary trajectories between auxotrophic partners within cocultures.

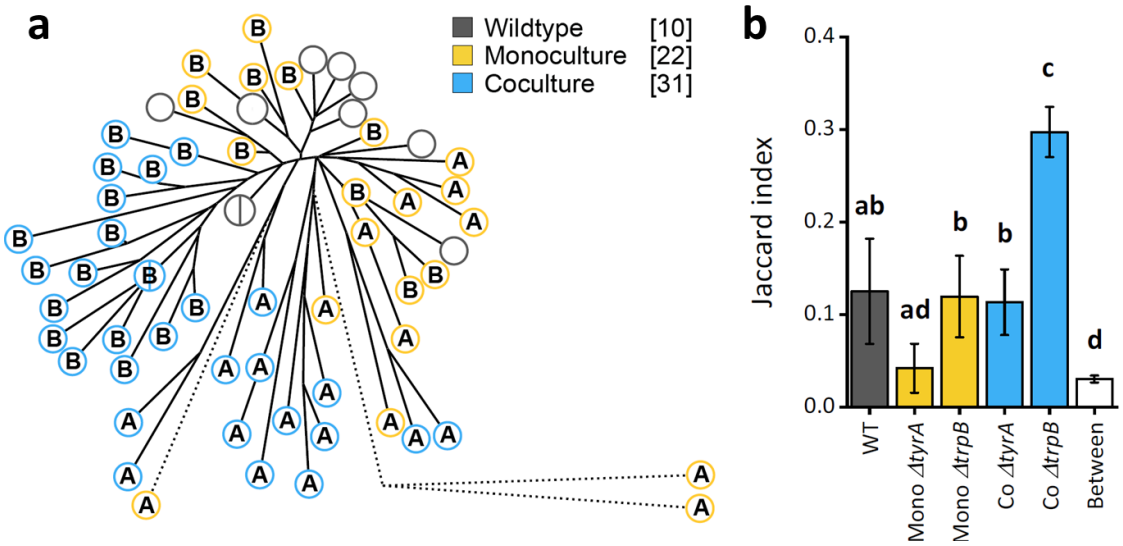

Figure 2: Coevolved auxotrophs show higher levels of divergence as well as degree of parallel evolution than control groups. (**a**) Distance tree of clonal samples (A = tyrosine auxotrophic,- and B = tryptophan auxotrophic isolate) utilizing standard neighbour-joining based on a genotyping matrix. Tryptophan auxotrophic isolates from cocultures (n=18) cluster as one distinct group, while respective tyrosine auxotrophs (n=13) are split into three branches and share in certain cases mutated genes with tyrosine auxotrophs from monocultures (n=11). Dotted lines indicate shortened branches that exceeded a certain threshold (see methods). (**b**) Degree of parallel evolution within and between experimental groups given as mean Jaccard indices with 95% confidence intervals. Letters indicate significant differences between groups (Dunett T3 *post hoc* test: $P < 0.01$, WT: n=45; Mono: n=55 for both auxotrophies; Co: n=78 for Δ*tyrA* and n=153 for Δ*trpB*; Between (all between-group comparisons): n=1567). The degree of parallel evolution was significantly higher in cocultures for both auxotrophies.

2.3 Between-group divergence on the functional level

We next analysed the spectrum of detected mutations between experimental groups on the functional level. Genes carrying a mutation were therefore counted in clonal samples and duplicates (*i.e.* due to morphotypes from the same population carrying an identical mutation) were removed before further analysis. Consistent for both auxotrophies is that genes involved in stress response as well as resistance are predominantly mutated in isolates from cocultures, indicating that the native reaction to stress was unfavourable in cross-feeding cocultures. Regulation appears to be affected more frequently in tryptophan auxotrophs from cocultures than in auxotrophic monocultures, while the reverse trend could be observed for energy production and conversion, where not a single isolate from cocultures carried a respective mutated gene. In contrast, isolates with tyrosine auxotrophy more frequently carried mutations in genes associated with metabolism and transport than their counterparts in monocultures. Functional analysis

hence revealed that auxotrophic partners from cocultures on the one hand show patterns that likely resulted from adapting to the interaction, on the other hand analogies specific to auxotrophy were observed as well. Mutations in groups of auxotrophic isolates in general appear more diverse on a functional level, while the prototrophic wildtype predominantly carries mutations in genes involved in metabolism and transport.

2.4 Loss-of-function mutations are most abundant in cocultures

Comparative analysis showed major between-group differences in the spectrum of mutated genes, yet no genomic region that commonly accumulated more mutations in all three experimental groups was observed, which indicates elevated local mutation rates not being relevant in our system (Fig. S3). Identified mutations have different effects on the function of the respective gene, ranging from neutral synonymous SNPs to loss of function mutations that can be caused by various modifications such as nonsense SNPs, frameshifts, and larger deletions or insertions frequently mediated by mobile elements. When comparing the abundance of these different types of mutations on the population-level, cocultures indeed show the highest counts for single nucleotide polymorphisms (SNPs), insertions and deletions (INDEL), and mobile elements (MOB), yet there are no obvious differences between groups in the general distribution of mutations (Fig. S5). Since associated implications are potentially highest when a function is deleted, we next compared frequencies of loss of function mutations on the clonal level to allow discrimination of auxotrophic partners. Therefore genes carrying a mutation were counted in clonal samples, and duplicates (*i.e.* due to morphotypes from the same population carrying an identical mutation) were removed on the population-level to avoid pseudo-replication. In addition, mutator genotypes were not considered. Highest frequencies of loss of function mutations were identified in cocultures while lowest frequencies were identified in auxotrophic monocultures: Percentages ranged between ~33% and ~27% in cocultures, and ~15% and ~18% in monocultures for tyrosine and tryptophan auxotrophs, respectively. Wildtype isolates showed intermediate frequencies of ~23%.

2.5 Evolution of metabolic cooperation accelerated molecular evolution

We next quantified the extent rapid evolution of cooperative metabolite exchange influenced genome evolution in cross-feeding populations. Therefore each detected mutation was counted as a single event to compare absolute numbers of accumulated

mutations between experimental groups. Re-sequencing revealed a population consisting of an established genotype carrying a non-sense mutation in the gene *mutT*. Inactivation of *mutT* causes a hypermutator phenotype that rapidly accumulates predominantly single nucleotide polymorphisms (SNPs) (551). Since mutations are rather deleterious than beneficial (552, 553) and therefore rarely adaptive in hypermutators, we excluded the focal population A10 and respective isolates from further analysis (see Fig. 1a & Fig. S2a for shortened branches that indicate high genetic load, and Fig. S3 for numerous individual mutations in isolates).

Comparative analysis showed absolute numbers of mutations to be significantly higher in cocultures than in control groups on both the clonal as well as the population-level, while wildtype and auxotrophic monocultures showed similar levels of accumulated mutations in both comparisons (Fig. 3a and b). Considering polymorphisms in population samples expectedly resulted in higher numbers of detected mutations than in clonal samples, however numbers within population samples of cocultures appeared to be higher than the sum of mutations detected within individual clones. If mutations are rare, they indeed might not be detected by analysing single clones. However, mutations with higher frequencies of >20% were exclusively detected within population samples yet not clonal samples (a complete list of mutations can be found in supplementary tables 2 and 3 for clonal samples and population samples, respectively). One potential explanation lies in sample preparation of whole populations. Analysing derived cocultures on the population-level from the fossil record required another cycle of cultivation, which likely caused additional mutations to accumulate whereas sub-cultivation of individual isolates did not cause this effect. In average wildtype monocultures accumulated 6 and 2.3, auxotrophic monocultures 4.7 and 3.1, and cocultures 13.7 and 5.6 mutations in analysed populations and clones, respectively. In conclusion, our analysis shows that cocultures accumulated significantly more mutations than control groups within ~150 generations (Fig. 3). Our results hence suggest accelerated molecular evolution in cross-feeding populations as a consequence of the evolution of metabolic cooperation.

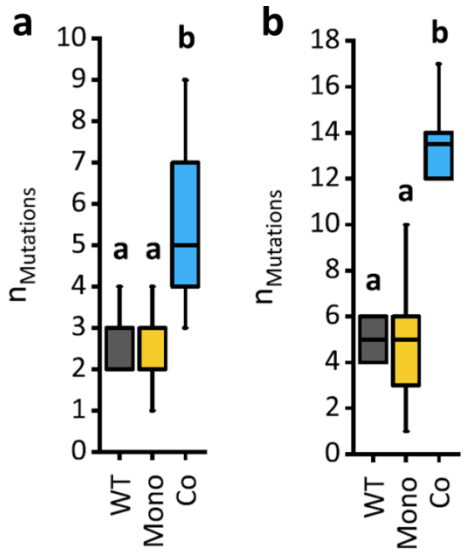

Figure 3: Cocultures accumulated more mutations than controls. Comparison of absolute numbers of mutations between all analysed samples demonstrates significantly faster accumulation in populations of cross-feeding auxotrophs (Co) relative to the wildtype (WT) and auxotrophic monocultures (Mono). Mutator genotypes were excluded from analysis. (**a**) Statistical analysis of clonal resequencing data showed that the obligate interaction had a significant effect on the accumulated number of mutations (Univariate linear model: $P<0.05$, WT: n=10, Mono: n=20, Co: n=31) and that isolates from cocultures accumulated significantly more mutations than isolates from both control groups (Bonferroni *post hoc* test: $P < 0.05$, WT: n=10, Mono: n=20, Co: n=31). (**b**) Numbers of detected mutations on the population level are significantly higher in cocultures as well (Bonferroni *post hoc* test: $P < 0.05$, WT: n=6, Mono: n=11, Co: n=6). Differences in mean values are more pronounced between groups than in clonal samples due to the accumulative effect of counting mutations of both partners within cocultures.

3. Discussion

Driven by adaptation and counter-adaptation, antagonistic coevolution was demonstrated to increase rates of molecular evolution (31) by following the principles of an evolutionary arms race as described by the red queen hypothesis (546). These insights were feasible due to experimental coevolution in replicated populations under laboratory conditions, an approach that was already utilized to study a variety of antagonistic interactions (545). For mutualistic interactions evolution experiments were conducted as well, yet with the focus on evolvability (105) as well as consequences of adaptation to the interaction (112), and verification of empirical predictions on the evolution of cooperation (106, 158). Hence these studies successfully addressed *i.a.* effects of ecological setup and adaptive processes on the evolution and maintenance of mutually beneficial interactions, while consequences in genome evolution on a quantitative level especially relative to an independent lifestyle remain unclear.

By utilizing comparative genomics we quantified the extent of molecular evolution during adaptation to an obligate metabolic interaction on the one hand and possible adaptive routes to abiotic culture conditions on the other hand. Analysis revealed high levels of parallelism among cocultures, yet clear divergence in evolutionary trajectories as well as low extent of similarity to control groups. Highest levels of parallel evolution were identified in cross-feeding populations (Fig. 1b) and tryptophan auxotrophic isolates from these populations (Fig. 2b). Taking into account the lower extent of similarity within tyrosine auxotrophic partners (Fig. 2b) we conclude that tryptophan auxotrophs predominantly determined elevated J values at the population level.

Since cultivated under limiting amino acid supplementation, we expected monocultures of auxotrophs to have several mutated genes in common with cocultures that experienced limiting conditions especially during the early stages of evolution as well. However, results indicate no such overlaps in both groups albeit assumed to experience similar starvation stress. The observed divergence between control groups and cocultures suggests hence conflicts in selection: When considering detected mutations within control groups to possibly resemble adaptation to abiotic culture conditions or deletion of a vital biosynthetic function, adaptation to both seems to be strongly restricted by the evolution of metabolic cooperation. Albeit evolving under almost identical culture conditions, experimental groups shared not more than one mutated site on the basis of each six analysed populations (Fig. S1a). In detail, either *rph* itself or the intergenic region of *pyrE/rph* exhibited mutations which are known to restore a deficiency in pyrimidine-biosynthesis (554, 555).

Moreover we observed striking differences in cocultures depending on auxotrophy. Partners almost completely differed in the spectrum of mutated sites (Fig. S1b). Together with differences to control groups this suggests adaptation to the specific function (*i.e.* provisioning of an amino acid) and to the changing requirements during the course of synergistic coevolution. This interpretation is supported by the spectrum of mutated genes showing major differences on the functional level (Fig. S4). Surprisingly we observed similar patterns in auxotrophic monocultures that highly differed in the spectrum of mutated sites as well (Fig. S1b), which illustrates how a single loss-of-function mutation can completely alter the adaptive landscape and therefore the spectrum of beneficial mutations a certain mutant has access to. Besides individual spectra of mutations cocultures more frequently accumulated loss of function mutations than control groups. In line with observations reported by Hillesland *et al.* 2014 (112)

we conclude as well that the early development of an obligate metabolic interaction caused additional interdependencies, which is corroborated by reduced growth performance of cooperative phenotypes that were cultivated in isolation as reported in previous work.

By quantifying total numbers of mutated sites we show for the first time rates of genome evolution to be significantly increased in both interaction partners due to the evolution of metabolic cooperation (Fig. 3). Taking into account high levels of similarity in cross-feeding populations, observations made in this study are likely a result of adaptive evolution. Our observations appear to be reminiscent to insights in antagonistic coevolution were molecular evolution appears to be accelerated as well (31), while adaptation to the abiotic environment is restricted (32, 119). Despite these analogies we do not interpret our results to be in contradiction to predictions made by the Red King hypothesis, which in contrast to our intra-species model system assumes unequal evolutionary rates of differing partners (117). Additionally the initial stages of developing metabolic cooperation were marked by rapid improvement in fitness indicating strong selection, which furthermore is in contrast to another identified premise for the Red King effect: weak or relaxed selection (550). The question however remains about which mechanism caused elevated evolutionary rates as well as parallel evolution in both partners during synergistic coevolution in replicated populations. This could be either explained by few available routes of adaptation or strong/intense positive selection of most beneficial mutations. One major difference between experimental groups was the potential of fitness gain during the evolution experiment, since ancestral cocultures showed very limited growth. As previously reported fitness in average increased by 290% in cocultures, 20% in wildtype populations, and only 5% in auxotrophic monocultures. Given these differences, strong selective pressure operating on most beneficial mutations likely was the underlying driving force causative of extensive parallelisms in the evolution of metabolic cooperation, an interpretation shared with another study that investigated the evolution of cooperation in microbes finding rapidity and repeatability as well (556). Strong selection pressure during serial passages potentially facilitated multiple selective sweeps that rapidly established cooperative mutants and finally could have caused accelerated molecular evolution. Importantly we reported in previous work (chapter 2) such sweeps to indeed take place in our experimental setup. Invasion success of cooperators was demonstrated to be facilitated by positive feedback-loops in multicellular aggregates. Further work utilizing

comparative genomics in the fossil record of the conducted evolution experiment could confirm these rapid selective sweeps of cooperators as well as coevolutionary responses of interacting partners.

Taken together, our results provide first experimental evidence that synergistic coevolution can increase the rate of molecular evolution. Our study demonstrates the advantages of combining resequencing of single isolates with whole-population analysis when studying interactions in bacterial populations, especially when these consist of different genotypes of the same species. This complementary approach allowed detailed analysis of accumulated mutations for parallelism, evolutionary rate, spectrum of affected functions, and partner-specific responses. We show that the evolution of metabolic cooperation caused highly divergent spectra of mutations between interacting partners as well as analysed lifestyles. Our insights have fundamental implications for microbial populations that carry out metabolite exchange and eventually might engage in cooperatively sharing resources.

4. Material and Methods

4.1 Bacterial strains.

We used *Escherichia coli* BW25113 (159) as the wild type (WT), which was genetically modified by P1 transduction (536). Derived auxotrophic genotypes contained an in-frame replacement of the targeted amino acid biosynthesis gene (i.e. *trpB* or *tyrA*) with a kanamycin cassette. To allow discrimination of different genotypes on agar plates, the phenotypic marker genes *araDAB* (derived from *E. coli* REL607 (538)) and *lacZ* (derived from *E. coli* MG1655 (539)) were additionally introduced into WT and auxotrophic strains by P1 transduction. As a result, one set of strains carried the functional alleles for arabinose utilization and β-galactosidase, which appears blue on TA-Agar (540) supplemented with 0.1 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) and 50 µg mL^{-1} X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), while the other set of WT phenotypes appears red.

4.2 Culture conditions.

In all experiments, minimal medium for *Azospirillium brasilense* (MMAB) (541) with 0.5 % glucose instead of malate and without biotin was used as culture medium. Cultures were incubated under shaking conditions at 30 °C and 225 rpm. Only monocultures of auxotrophic genotypes were supplemented with tryptophan or tyrosine (150 µM for precultures and 50 µM for the evolution experiment). To start an experiment, bacterial strains were freshly streaked from cryo-stocks on LB agar plates and incubated for 18-24 h at 30 °C. Individual colonies were used as biological replicates to inoculate each 1 ml MMAB of overnight preculture, which were set to an optical density (OD_{600nm}) of 2 the next day. Respective aliquots of these were subsequently used to inoculate 4 ml MMAB medium with an initial OD_{600nm} of 0.005. In the case of auxotrophic cocultures each genotype was inoculated with an initial OD_{600nm} of 0.0025.

4.3 Evolution experiment.

Complementary auxotrophic strains (*E. coli ΔtrpB::kan araDAB lacZ, and E. coli ΔtyrA::kan ara-* Δ*lacZ* or the reverse combination of phenotypic labelling) were combined in cocultures to generate a synthetically designed obligate by-product interaction. To determine effects of genotypic background (prototrophy or auxotrophy, and presence or absence of phenotypic marker genes) on the accumulation of mutations, two control groups contained monocultures of utilized genotypes. Six biological

replicates of each generated genotype were used to start the evolution experiment, adding up to twelve WT monocultures, twelve cocultures of auxotrophs, and 24 monocultures of auxotrophic genotypes (i.e. 12 of each type). Populations were initially transferred every seven days for a total of five transfers, which was followed by 15 transfers every three days, adding up to a total of 80 days or approximately 153 generations. At the end of each cycle, optical densities were determined in 200 µl culture in microtiter plates by spectrophotometry in a plate reader (Spectramax M5; Applied Biosystems; United States) and 20 µl of culture were transferred into 4 ml of fresh MMAB-medium. Depending on the cycle-length, glycerol stocks (20% glycerol) were prepared each six or seven days and stored at -80 °C. Cocultures were regularly tested for revertant phenotypes that showed prototrophic growth, thus that were capable to grow on MMAB-Agar without amino acid supplementation. Out of twelve cocultures, two cocultures were excluded from further analysis due to the evolution of prototrophic phenotypes. Accordingly, also the matching biological replicates in auxotrophic monocultures were excluded from further analysis. In addition, one replicate of *E. coli ΔtyrA::kan ara-* Δ*lacZ* from monocultures was excluded due to contamination. Terminal populations were spread on modified TA agar plates to isolate evolved clones based on colour and colony morphology for whole-genome resequencing. Phenotypic diversity was observed in eight out of ten cocultures, and three out of 19 monocultures of auxotrophs, while all WT monocultures remained phenotypically homogeneous. Isolates were stored at -80°C until further analysis.

4.4 Genome resequencing and analysis.

Evolved populations were sequenced on the level of isolated clones and on the metagenome-level. For this, isolates from terminal populations were incubated in LB medium and whole populations in the respective native minimal medium until maximum optical density was reached. Genomic DNA was extracted using the Epicentre MasterPure^{TM} Complete DNA & RNA purification kit (MC85200, Biozym Scientific, Germany). Further steps were performed by the Max Planck-Genome-centre Cologne, Germany (https://mpgc.mpipz.mpg.de/home/): Quality control of samples was performed on Genomic DNA ScreenTape Analysis® using TapeStation Analysis Software A.02.01 (Agilent Technologies, United states), followed by TruSeq compatible library preparation. Clonal samples were sequenced on the Illumina HiSeq2500 platform in 100-bp paired-end mode for all WT and coculture samples, and in 150-bp paired-end mode for samples from auxotrophic monocultures. Observed coverage was

approximately 75-fold or above at quality scores above 30. Sequencing was successfully performed for 65 clonal samples in total. Numbers of sequenced clones depended on the number of observed morphotypes on agar plates (see above). Further analysis of mutations revealed two cases of clones from the same population of cocultures to exhibit identical mutations. To avoid pseudoreplication, the corresponding pairs were hence treated as one. Besides genotypes isolated from derived populations of WT (n=10), cocultures (n=31; with *ΔtrpB*: n=18 and *ΔtyrA*: n=13), and monoculture (n=22; with *ΔtrpB*: n=11 and *ΔtyrA*: n=11) also the six ancestral genotypes were sequenced, to identify mutations that were already present at the beginning of the evolution experiment. Metagenomes were sequenced on the Illumina HiSeq3000 platform using 150-bp single-end mode. To allow quantitative comparison of accumulated mutations without sampling bias, whole populations were analysed from WT- (n=6) and coculture populations (n=6) as well as from the corresponding populations of auxotrophic monocultures (n=12, with *ΔtrpB*: n=6 and *ΔtyrA*: n=6). Observed coverage was approximately 1,250-fold at quality scores above 30. Mapping of reads on the published reference genome of *Escherichia coli* BW25113 (CP009273_1) (557) and identification of mutations was performed using the BRESEQ-pipeline (160, 161). For population samples, the polymorphism mode with the "Polymorphic Read Alignment (RA) Evidence" option "--polymorphism-minimum-coverage-each-strand" was set to 40. Identified mutations and evidence for new junctions ("Unassigned new junction evidence") were rechecked by verifying individual reads to sufficiently indicate the presence of the mutations and to be only mapped to one dedicated site in the genome, especially when using the polymorphism mode. If reads, indicating a certain mutation, mapped elsewhere with 100% homology (determined by using NCBI nucleotide BLAST (558)), for instance as in the case of highly homologous tRNA encoding genes, respective mutations were rated as false-positive and excluded from further analysis. Absolute numbers of mutations were determined by counting each mutation regardless of size or structure as a single event. Evidence for complex mutations was first resolved in clonal samples as described for BRESEQ (161) and further used to successfully resolve all detected complex mutations in population samples by confirming identical architecture (for instance reads to be tiled at identical positions). All identified mutations are listed in supplementary tables 2 and 3.

4.5 Quantification of parallel evolution.

The Jaccard Index (*J*) was calculated to estimate parallel evolution at the level of shared mutated genes between samples as described in (559). J-values range between zero and one with lower values indicating fewer mutations occurred simultaneously in the compared samples and larger numbers pointing to an increased similarity between samples. *J* was calculated for all possible combinations within individually sequenced isolates and within whole population samples, excluding comparisons with the same sample.

4.6 Distance trees.

Divergent evolution between experimental groups was analysed with a standard neighbour-joining method and GrapeTree (560) was used for visualisation. All identified mutations were summarized in a genotyping matrix that indicated whether a given gene within each sample was either mutated or showed the WT allele. Complex mutations that affected more than one gene were treated as single alleles as well, since these mutations, as single evolutionary events, separate a mutant from another lineage. Branches within distance trees were shortened when exceeding a certain threshold and displayed as dotted lines for clonal samples as well as metagenomes.

Supporting information

The following supporting information is available for this chapter starting with page 201

Figure S1: Experimental groups share few mutated genes.

Figure S2: Degree of parallel evolution based on mutated genes in clonal samples.

Figure S3: Mutated sites in evolved isolates show the extent of within-group parallelism and between-group divergence.

Figure S4: Proportion of mutated genes in clonal samples categorized by function.

Figure S5: Spectrum of detected mutations in population samples.

Table S1: Nomenclature of evolved populations and isolates.

Table S2: List of detected mutations in clonal samples.

Table S3: List of detected mutations in population samples.

General Discussion

General Discussion

Life is marked by consistent change on evolutionary timescales and ecological interactions are major drivers of this process (31, 69). Interactions among microorganisms in fact are frequently based on the exchange of metabolites, but little is known about the mechanistic causes favouring the evolution of a cooperative exchange of metabolites from the mere exchange of metabolic by-products as well as the consequences resulting from it.

In this work, the first step was to summarize current theory on cooperation in the context of metabolic cross-feeding. Therefore, a synthesis of theory, computational predictions, and empirical results was composed to describe a complete evolutionary process starting with simple by-product exchange, emerging metabolic dependencies, and eventually the evolution of cooperative cross-feeding. One major conclusion for the evolution of cooperative metabolite exchange was the requirement for assortment of partners in spatial proximity, for instance due to generating population structure within groups of cells (37, 44). In an evolutionary approach presented in chapter 2, this prediction was empirically tested by synthetically engineering obligate metabolite exchange between planktonic bacterial cells under well-mixed culture conditions that do not facilitate population structure (*i.e.* assortment of partners (44) (Tarnita 2017)). The established obligate interaction comprised two *Escherichia coli* genotypes lacking the biosynthetic ability to produce either tryptophan or tyrosine. During serial propagation, cooperative cross-feeding evolved remarkably rapidly in replicated populations. Auxotrophic consortia even reached fitness levels of the wild type by the cooperative exchange of increased levels of required amino acids. The formation of multicellular clusters consisting of both auxotrophic genotypes was identified as the ecological mechanism causing this pattern. Despite the well-mixed culture conditions that according to evolutionary theory should limit the evolution of cooperation, spatial proximity within groups of cells was demonstrated to selectively favour cooperative phenotypes. Thus, the results of this experiment represent the first empirical evidence for a single cooperative phenotype to experience strong positive selection within a group of non-cooperative individuals despite the lack of a complementary cooperator. Imaging of multicellular clusters containing different fluorescently labelled genotypes showed double-labelled cells, which strongly suggests the exchange of cytoplasmic material as

reported previously (83) (Pande et al 2015). Furthermore cross-feeding populations were observed to undergo dynamics in the organisation of cells: At each transfer, cocultures were predominantly unicellular, yet aggregated to groups that resembled the major places of growth to again disassemble into single cells during the later growth phase until the next cycle was started. Isolated members from cooperating populations showed a consistent decrease in growth performance when compared to ancestors, suggesting that adaptation to cooperative cross-feeding overruled selection to maximize fitness in individuals. Due to the utilization of control groups consisting of wild type and auxotrophic monocultures, measures indicating cooperation and observed cluster formation could be clearly attributed to the obligate cross-feeding interaction hence were neither a side-effect of culture-conditions nor due to the genotypes used.

The observed pervasive success in evolved cooperative cross-feeding hence raised the question of associated effects on numbers as well as spectra of accumulated mutations relative to the independent lifestyle in control groups. Therefore, the genomic consequences resulting from the evolution of metabolic cooperation were analysed (chapter 3). In particular, sequencing individually isolated clones and whole populations revealed accelerated rates of molecular evolution as well as increased levels of parallel evolution in cocultures of auxotrophic genotypes relative to the two control groups. Consistent to this observed extent in similarity cocultures share a common branch on a distance tree, yet control groups mapped to different branches indicating divergent evolutionary trajectories. In addition, auxotrophic genotypes shared only few mutated sites hence showed extreme partner-specific spectra in mutated genes. Marginal overlaps in targets of selection between cocultures and control groups in addition revealed conflicts in adaptation to biotic and abiotic environment. Altogether, causes for cooperative cross feeding were identified in self-assembly properties of *E. coli* via aggregation of auxotrophic bacterial cells of *E. coli* and self-organization during repeated bouts of assembly and disassembly of multicellular clusters were identified to facilitate the observed evolution of cooperation. Consequences imposed by the evolution of cooperation were identified in reduced individual fitness, an altered lifestyle within multicellular clusters, completely altered trajectory of evolution, and completely altered adaption relative to control groups. These results are indicative of metabolic division of labour, a shift in selection to the level of groups, and dynamics towards a hierarchical evolutionary transition (HET) (30) (van Gestel & Tarnita 2017) that will be evaluated in sections three, four, and five of the discussion, respectively.

1. Experimental evolution in bacterial model systems as an effective tool to study species interactions

The bacterium *Escherichia coli* is the most studied microorganism. It is easily to genetically modify, exhibits short generation times, and large populations, which provides opportunity for selection to rapidly become manifest in genomic change (538). It is hence no surprise that *E .coli* has been subject of a variety of evolution experiments (68, 561, 562), some of which have been conducted for decades (538, 563), and a few even comprise coevolutionary approaches (545). As the enforced interdependency between two amino acid auxotrophic genotypes was integral to address the question of how cooperative cross-feeding can evolve from a by-product interaction, the strain *E. coli* BW25113 was selected since it lacks the ability to exchange genetic material with other bacterial cells such as conjugation or natural competence (537, 557). The absence of these mechanisms was a prerequisite for stable auxotrophy even during serial passages of cultivation, since the probability for gene transfer between genotypes was minimized. Since little was known about the coevolution of interdependent bacterial genotypes under laboratory conditions (105, 158), the model system was designed as simple as possible to minimize variables.

Thus, an obligate intraspecific interaction was established, which may not resemble the typical type of cross-feeding, *i.e.* metabolite exchange between different species, yet exhibits similar dependencies for growth (*i.e.* growth is coupled to the amount of metabolite exchanged) as a mutualistic interaction. The interaction was based on amino acid exchange since amino acid auxotrophy is widespread in microorganisms (63). These genotypes hence need to compensate their deficiency by the uptake from the environment. Such external sources include decaying organic matter or secretions by micro- or macroorganisms. This could either happen unintendedly, by passive leakage of certain metabolites through a bacteria's cellular membrane, or by active secretion as part of a cross-feeding interaction. Due to these different sources, amino acids are likely publicly available to bacterial cells (16, 564). Importantly, amino acids as a public good were recently demonstrated to enable adaptive gene loss in an evolutionary approach: Prototrophic genotypes lost vital biosynthetic functions and hence became auxotrophic for supplemented amino acids (68). These insights show that the loss of biosynthetic functions can be adaptive. Considering these insights, the model system utilized in this work resembles the consequent next step towards coexisting genotypes that lack different vital biosynthetic functions: A situation in which auxotrophic genotypes

become interdependent. Indeed, a number of other studies used *E. coli* to investigate metabolic interactions between genotypes or species as well (83, 89, 102, 106, 158, 174, 501, 516, 565), with a subset including an evolutionary approach (106, 158). Evolution experiments exploring cross-feeding interactions frequently lack control groups to determine the effect of adaptation to the abiotic culture conditions used. This is likely because this specific information was not required for the main questions addressed in the previously mentioned studies. However, including these controls into the experimental design allows drawing conclusions pertaining to adaptation. Our model system illustrates the power of experimental evolution utilizing interdependent cocultures and independent control groups. The system illustrates how bacterial cells can immediately form interacting groups that intrinsically promote cooperative phenotypes via the emergent property of reciprocal feedback-loops. It furthermore demonstrates that control groups enable clear attribution of observations to the obligate interaction rather than to the abiotic environment. Cocultures and control groups were exposed to serial propagation during 80 days corresponding to ~150 generations, which revealed increased levels of evolvability in replicated cross-feeding populations relative to control groups. The previously inefficiently growing consortium acquired the ability to cooperatively exchange elevated amounts tyrosine and tryptophan. Such dramatic increase in productivity finally resulting in fitness levels that were statistically indistinguishable from wild type levels was unexpected and observed for the first time. The intricacy of terming an evolved interaction “cooperation” however lies in providing evidence that evolutionary changes were adaptive in the context of the interaction and not a mere by-product of adapting to genomic modifications or culture conditions. This for instance concerned the increased release of amino acids and associated costs in evolved cocultures. The solution to this challenge reveals the strength of the experimental design, which lies in the integrated control groups. These allowed the causal linkage between observation and aspired attribution to cooperation since they functioned as negative control.

2. Analysis of mutated sites showing parallel evolution within cocultures

Since analysis presented in chapter three aimed at quantifying general consequences, the focus was not on single genes and the putative role as well as implication of the detected mutation. Genes that were noticeable frequent targets of selection in replicated cocultures will therefore be discussed in section two of the general discussion. Genome analysis of members from ten evolved cocultures of auxotrophic genotypes revealed highly auxotrophy-specific spectra of mutated sites with nearly no overlaps in affected genes between partners. In either several or in a few cases even all ten independently evolved populations, genes appeared to be consistently affected, hence indicating important targets of selection. This raises the question whether there are any conclusions possible when looking at these genes and the potential effect of detected mutations. In tryptophan-auxotrophic isolates, *lrp*, *ompF*, *rpoS*, and *trpD* or *trpE*, and in tyrosine-auxotrophic isolates *cyoC* or *cyoE*, *rpe*, *hemL*, and *sspA* were repeatedly found to exhibit changes that comprised missense, nonsense, or frameshift mutations, or even complete deletions and, in one case, even a five-fold amplification. In the following, these genes will be discussed in more detail. The putative impact of detected mutations, which frequently caused a loss-of-function phenotype, in the context of amino acid cross-feeding will be evaluated and finally conclusions regarding general consequences will be drawn. If one of these genes is found to be mutated in one of the control groups as well, this will be considered for discussion. Importantly, nonsense, frameshift, and deletion mutations will all be considered as putatively causing loss-of-function to simplify the discussion. When applicable, missense mutations causing an amino acid substitution were additionally predicted for causing loss-of-function. Since mutations cannot be connected to fitness without additional analysis, the phrases “loss of function” mutation and “deleterious” mutation will be used synonymously.

Four out of ten populations showed a frameshift within *lrp*, and four other populations showed missense mutations within a site spanning only 62 base pairs. The observed substitutions likely rendered Lrp non-functional, because the observed frameshift mutations point towards a beneficial effect of loss of function. This assumption is supported by supplementary PROVEAN-analysis (566, 567), which predicts observed amino acid substitutions in three out of four populations to be deleterious as well (Supplementary table 1)). The leucine-responsive regulatory protein Lrp is encoded by *lrp* and is involved in the regulation of in total 283 genes in *E. coli*, many of which are involved in the transport or biosynthesis of amino acids (568). In addition, Lrp is

involved in the regulation of pili biosynthesis, where it influences phase variation (527). A knockout of *lrp* influences the directionality of the fim-switch (527, 528) and dramatically reduces the ON-to-OFF inversion (527), thus causing the switch to remain in the ON-position, which allows prolonged pili formation. This makes perfect sense in the context of metabolite exchange within liquid shaken culture that lacks spatial structure. As demonstrated in this work (chapter II), cell-cell aggregation and as a consequence cluster formation is likely key for an efficient transfer of amino acids between cells. In this context it appears reasonable to assume that by increasing the ability to form aggregates is advantageous, since such phenotypes are more likely to become part or remain part of a multicellular cluster. Importantly, Lrp represses biosynthesis of all amino acids except threonine, yet activates catabolic pathways of amino acids, including these for aromatic amino acids (568). In addition, Lrp controls almost 75% of genes associated with stationary phase (569, 570) and was shown to provide a selective advantage over the wild type during stationary phase when non-functional (571). Increased viability during stationary phase was likely beneficial during the evolution experiment, especially when populations reached maximum cell densities early during experimental cycles and needed to persevere until the next transfer. A possible conclusion hence would be that by deregulating amino acid biosynthesis, specifically reducing catabolism and releasing anabolism, more tyrosine is produced for the partner, while increased survival under stationary phase additionally favoured positive selection of deleterious mutations in *lrp*. However, this interpretation overlooks the downside of deactivating Lrp: The cellular regulon in consequence lacks an important transcriptional activator for aromatic amino acid transporters (568). Disabling that control reduces the flux of tyrosine and tryptophan in and out of the cell. As a consequence, the vital exchange of amino acids between cells is limited, which also should slow down growth. A solution to this dilemma would be to increase the permeability of the cellular membrane, ideally specifically for aromatic amino acids.

Interestingly, mutations that comprise the outer membrane porin F appear to be strongly associated with mutations in *lrp*: Out of 14 isolates carrying mutations in *lrp*, 13 carry mutations in *ompF* as well. In addition, amino acid substitutions in OmpF are consistently found in all cross-feeding populations. OmpF belongs to the group of general outer membrane porins and is involved in a variety of transport processes (260). It is one of the most abundant proteins in the outer membrane (260) and strong experimental evidence exist showing that porins transport aromatic compounds as well

(260). However, it remains unclear whether also tyrosine and/ or tryptophan is transported in this way. Missence mutations within *ompF* were detected in all ten populations, and additionally these substitutions comprise only three amino acid residues. Since the amino acids tyrosine (Y62), glutamic acid (E139), and aspartic acid (D135) were predominantly replaced by amino acids with a shorter side chain (*e.g.* alanine, glycine, serine, and cysteine), the pore size was possibly increased, which could have enhanced permeability of these pores. This interpretation is supported by experimental evidence showing that exactly these amino acids are facing into the channel and are therefore in direct contact with the aqueous phase (572). In half of the analysed populations, additional base substitutions were observed in the intergenic region of *ompF* and *asnS*. With a distance of -115, and -122 base pairs to *ompF*, these mutations are located exactly within a predicted binding site for the transcriptional regulator CRP (**c**AMP **r**eceptor **p**rotein) (573), which suggests modification of transcription. However, it remains unclear whether these mutations increase or decrease binding affinity for CRP-cAMP. One isolate that exhibits a five-fold amplification of a 32 kBp region comprising *ompF* additionally suggests positive selection of increased permeability. Taken together, the repeated occurrence of OmpF-associated changes in the context of both amino acid-cross-feeding in general and frequently negative side effects by *lrp*-deficiency in addition suggest strong selection for increased membrane permeability. However, future work is necessary to experimentally verify this.

Another interesting gene that has been found to frequently carry mutations is *rpoS*, which encodes the alternative sigma factor σ^S – the master regulator of the general stress response in *E. coli*. At least four of the analysed populations contain *rpoS*-mutants, of which only one exhibits a missense mutation and the remaining ones a frameshift mutation. Supplementary PROVEAN-analysis (566, 567) suggests the missense mutation to be deleterious as well (Supplementary table 1), hence all four mutations are considered to be loss-of-function mutations. The latter thereby are reported to be responsible for a competitive advantage during stationary phase termed “growth advantage in stationary phase (GASP) phenotype” similar to *lrp* mutants (571, 574). Interestingly, deleterious mutations in *lrp* or *rpoS* were to 100% mutually exclusive, suggesting that either loss of function in one of the two gene products conferred a sufficient advantage during stationary phase, or epistatic effects are deleterious when losing both. Taking into account that lack of σ^S substantially affects metabolism during exponential growth phase as well (575), the question arises whether there are additional

side effects promoting positive selection of *rpoS* mutants. Loss of RpoS for instance stimulates resource acquisition from the surrounding, including amino acids and peptides (576, 577), which consequently increases the flux of tyrosine into the cell. Furthermore, tryptophanase expression is strongly upregulated by σ^S when stationary phase is initiated (578), which almost completely transforms cell internal tryptophan into indole (579, 580). As this is of course critical for a genotype, whose growth is predominantly limited by tryptophan, preventing positive regulation of tryptophanase could additionally favour mutants with deactivated σ^S. In line with this interpretation and by taking into account that no deleterious mutation was detected in *lrp* or *rpoS* in any other experimental group than the tyrosine auxotrophic partner strongly suggests these mutations to be specifically beneficial in a tryptophan auxotrophic background when carrying out obligate amino acid exchange. The same reasoning applies to nonsense- or frameshift mutations within *trpD* and *trpE* that were exclusively and frequently (nine out of ten) detected in Δ*trpB* genotypes that evolved as part of a coculture. Importantly, both of these genes represent the initial part of the *trp* operon. Observed mutations thus have polar effects on the genes located downstream and therefore almost completely shut down the whole pathway except low constitutive expression of *trpCBA* by a weak promoter (581). One potential explanation for the observed prevalence of mutated *trpD* or *trpE* is compensation of side effects due to the lack of trpB: indole is not utilized for the biosynthesis of tryptophan anymore and hence likely accumulates within the cell and the culture supernatant. Considering that high levels of indole are known to block cell division (582) and are associated with the transition from late exponential growth phase to stationary phase (580, 583), a reduction of indole biosynthesis makes perfectly sense. Furthermore, saving the costs for the biosynthesis for several enzymes of a whole pathway additionally could have favoured selection of observed mutations (63). Taken together, the frequently observed genomic changes in Δ*trpB* genotypes most likely had more than one advantage and were positively selected in the context of cooperative cross-feeding, viability during prolonged stationary phase, and compensation of side effects due to the *trpB*-knockout. In tyrosine auxotrophic isolates from auxotrophic cocultures, *rpe* was found to carry a loss of function mutation in six out of ten populations. Ribulose-5-phosphate 3-epimerase (Rpe) is part of the pentose phosphate pathway. Loss of enzymatic activity reportedly causes reduced growth on glucose minimal medium (584).

The finding that supplementation of *rpe* deletion mutants with casamino acids enhanced growth, suggests certain auxotrophy (585). These detrimental effects raise the

question of how loss of *rpe* could be advantageous in the context of amino acid cross-feeding. One potential explanation for the frequent emergence of *rpe* knockout mutations is given by *in-silico* predictions utilizing flux-balance analysis that predicts these genotypes to be overproduction mutants for tryptophan (Silvio Waschina, personal communication). Quantification of tryptophan concentrations in the culture supernatants of the respective genotypes consistently confirm these predictions (Supplementary figure S4; chapter 2). Another set of loss of function mutations affects another gene (*hemL*) that codes for an enzyme involved in tetrapyrrole-biosynthesis of *E. coli* (586). Interestingly, mutations in *hemL* and *rpe* appear to be mutually exclusive, yet it is unclear why. The gene product of *hemL* is glutamate-1-semialdehyde aminotransferase (GSA-T), which is required for biosynthesis of δ-aminolevulinate (ALA). Knockouts of GSA-T exhibit a leaky auxotrophy for ALA that allows for basal growth (587) yet were reported to be unable to grow in rich medium (159, 588). However, these mutants showed reduced growth under isolation in the work presented here, yet it remains unclear, which positive effect loss of GSA-T has in cocultures. Together with the overall reduction in growth of isolated clones (Fig. 2b; chapter 2), deleterious mutations in *rpe* and *hemL* may point to an increased interdependency among cocultures.

The stringent response is triggered by a stalled ribosome under amino acid limitation and appears to be subject to modification as well. Derived tyrosine auxotrophs show loss of function mutations affecting *sspA* in six out of 10 populations, of which five additionally lost *sspB* as part of a ~10 kB deletion. The stringent starvation protein A (SspA) is important for activation of stress response during stationary phase (589), since it indirectly increases expression of *rpoS* (590) by negatively regulating H-NS (589). In contrast to genotypes with non-functional Lrp or σ^S, strains that lack functional SspA have a reduced viability during prolonged stationary phase (591). Furthermore, increased sensitivity to acidification was reported in SspA mutants (589), which, however, is at odds with elevated acetate production by the coevolved partner due to the abovementioned lack of functional σ^S (575). Both detrimental effects need to be compensated by a yet unknown positive effect of these mutations to explain positive selection. One advantage could have originated from induced hypermotility due to released H-NS expression (589, 592), which potentially increased the likelihood to encounter a compatible partner for cross-feeding. This would have been particularly beneficial at the initial stage of each cycle, after cocultures were transferred from stationary phase cultures into fresh medium and shear forces due to pipetting likely

disrupted the cellular aggregates in addition to most cells being already unicellular (Supplementary figure S6; chapter 2). However, this is speculative and requires experimental verification under coculture conditions under which the respective mutations arose and were selected for. For instance, due to the coevolutionary process, a given knockout might only be plausible in a certain genetic background of the respective partner due to epistatic interactions among mutations. Another set of mutations was identified in Δ*tyrA* genotypes isolated from cocultures as well as monocultures, which suggests these may be mutations that compensate for the lack of TyrA.

Exhibiting a loss-of-function mutation, two genes are affected (*cyoC* and *cyoE*), which are both essential for functioning cytochrome *bo* oxidase complex. Hence, these are important for the respiratory system and generation of the proton motive force (593-595). One of the two genes is mutated in six out of ten cocultures and six out of ten respective monocultures. However, the putative reason why a part of the respiratory system has been deactivated in the context of tyrosine auxotrophy remains unclear, especially since expression of the whole complex (i.e. *cyoABCDE*) is lowest under growth on glucose (596), which was the sole carbon source used in this work. Taken together, in the majority of cases where parallel evolution was observed between replicates, the detected mutations caused a loss of function rather than modifying a given process. These comprised genes with large pleiotropic effects associated with global regulation (*lrp*, *rpoS*, and *sspA*) and metabolism (*trpDE*, *cyoCE*, and *rpe*). The only exceptions to this are apparent modifications of the outer membrane porin F. By further excluding identified mutations that likely compensated for genetic modifications (*i.e.* for the knockout of *trpB* or *tyrA*), it becomes obvious that genes encoding for central regulatory function were repeatedly disabled in both auxotrophic partners within replicated populations, yet not in control groups. The phenomenon of loss of function mutations in global regulators with high impact was already reported in complex environments and the underlying adaptive strategy was termed “higher-order ‘metabolic selection’” (577). By globally manipulating regulation of the whole metabolism in only one step due to pleiotropic effects, the potential for significant benefits was given in an adaptive landscape that otherwise only allows marginal gain in fitness by a single mutation (577). However, why was this observed in cocultures as well, even though the corresponding populations have been cultivated in a well-mixed environment containing only one carbon source? The obligate interaction triggering cluster formation likely generated complexity in an otherwise simplistic environment, which points towards

emergent features that arise by nothing more than two interdependent bacterial genotypes forming multicellular aggregates. In addition and in contrast to Saxer *et al.* 2014 (577), rapid improvement in fitness points towards strong selection favouring cooperative cross-feeding, allowing the conclusion that potentially both the advantage of pleiotropic effects (*i.e.* significant modifications in the metabolism) and a large benefit by a single change (*e.g.* improved viability during stationary phase) operated simultaneously. For instance, simple modification of phase variation towards elongated fimbriae expression in one partner is a side effect of a *lrp* knockout mutation and may have been an appropriate adaptation to fine-tune aggregation of compatible genotypes within multicellular clusters. What the genomic analysis did not reveal is any signature of a modified signalling pathway (*e.g.* communication) or sensing of partners being involved in the evolved cooperative interaction (e.g. quorum sensing), which may suggest compatible genotypes aggregate by chance after each transfer. These chance-events were likely sufficient for many aggregates containing both genotypes given that high numbers of cells were transferred. The evolved preference for aggregation in the Δ*trpB* genotype in combination with putative hypermotility in the Δ*tyrA* genotype can be seen as complementary adaptations that increase the probability for both coming together and staying together (44). Taken together, functional analysis of the most frequently mutated genes suggests that both auxotrophic partners showed specialisation on their specific role in the interaction. As a genetic signature of evolved metabolic cooperation, evidence for adaptation to increased amino acid production as well as flux between cross-feeding partners was identified. This, however, likely came at the cost of reduced resistance in stressful situations (576).

3. Division of labour and specialisation

An obligate interaction that is based on the cooperative exchange of amino acids resembles a strategy that is integral for animal's life: Division of labour is a well-known principle found within many hierarchical levels of organization in higher organisms, which is reflected by more than 200 differentiated cell-types fulfilling specific tasks (597) or different phenotypes in insect states (598). Classically describe for multicellular eukaryotes, division of labour has also become subject of research in microorganisms, which suggests this strategy to be relevant for simpler life forms as well (107, 599). One plausible explanation is that a given individual (cell) can only fulfil a limited number of tasks simultaneously, for instance due to tasks being in conflict to each other (291) such

as in the case of oxygen-sensitive nitrogen-fixation that requires anoxic conditions provided by a specialized cell-type (*i.e.* a heterocyst) within filamentous cyanobacteria (600). In consequence, increased efficiency is achieved by dividing tasks between different entities thus reducing costs (102, 372). The former argument is for instance represented by cyanobacteria of the genus *Anabaena* as mentioned above. Dealing with nitrogen-limiting conditions causes a differentiated cell type (*i.e.* the heterocyst) that stops photosynthesis to allow anoxic conditions for nitrogen-fixation (600, 601). The latter was theoretically predicted for a scenario in which the segregation of two vital functions into two specialised phenotypes is less costly than both functions being executed by the same individual (110). It was previously demonstrated under laboratory conditions that coupled metabolism between compatible genotypes indeed has advantages (102, 602). For instance, in a synthetically designed interaction, two interdependent genotypes gained up to 20 % fitness advantage relative to a single independent genotype (the wild type) by the mutual overproduction and exchange of vital amino acids (102). In comparison to that study, the model system utilized in the work presented here basically resembles a more primitive stage of interaction that lacks genetic modifications for amino acid overproduction. Indeed, evolved metabolic cooperation propelled fitness of auxotrophic consortia towards wild type levels. Remarkably, it required only 150 generations to allow accumulation of mutations that enabled compensation for the loss of vital biosynthetic functions. Since fitness is a function of the exchanged amino acid quantities in cocultures, I conclude that generated benefits by cooperative cross-feeding explain the remarkable increase in fitness. However, besides cooperation, fitness can also increase by adaptation to the abiotic culture conditions and an increased efficiency in resource utilization as well. However, comparisons with control groups support the conclusion of cooperative exchange causing increased fitness: First, auxotrophic monocultures that were limited in growth by the supplemented amount of amino acid only showed marginal improvements in fitness, which strongly suggests that no major innovation arouse enabling more efficient utilization of the supplemented quantities of tyrosine or tryptophan. Second, also the sequencing results suggest that derived auxotrophic cocultures neither improved in fitness due to adapting to abiotic culture conditions in general nor by using limiting resources (i.e. the carbon source glucose or the exchanged amino acids) more efficiently. Determining the individual effect of each of these mutations on fitness in the context of cocultures as well as culture conditions could confirm made conclusions.

Division of metabolic labour and with it saving costs likely accounts for the observed success despite evident deleterious effects due to deregulation and decreased stress resistance. Due to the obligate nature of the focal interaction analysed, cross-feeding of amino acids likely resulted in negative frequency-dependent selection of partners (102). An increased frequency of one partner results in depletion of the metabolite it requires for growth and an excess supply of the metabolite the corresponding other partner needs, which causes an oscillation of frequencies in both. In evolved consortia, this resulted in frequencies between 20-80% in auxotrophic partners as indicated by analysed polymorphisms in whole populations (Supplementary table 3; chapter 3). In detail, the polymorphisms are given as percentage, thus reflecting the detected frequency of a given mutation within a population sample, which hence indicates the frequency of the respective genotype (*i.e.* auxotroph).

For representing division of labour, cooperative phenotypes need to be both positively selected due to mutual fitness benefits and specialised on the given task (30, 603), which is connected to certain requirements defined by West and Cooper 2016 (107): "… individuals carry out different tasks (phenotypic variation); some individuals carry out cooperative tasks that benefit other individuals (cooperation); the division of tasks provides an inclusive fitness benefit to all of the individuals involved (adaptation)." Exactly these preconditions are met by evolved consortia that consist of two phenotypes showing specific dependencies: Characterization of evolved cocultures revealed metabolic cooperation that evolved due to mutual fitness benefits demonstrated evolved specialisation on mutually providing increased amounts of tryptophan or tyrosine. The bacterial model system studied here is hence one of few experimentally evolved examples for the division of metabolic labour among bacteria. It was further hypothesized that a consequence of dividing labour could be extreme specialisation (111). In analogy, increased efficiency by specialization (*i.e.* elevated exchange of amino acids) was observed to result in intensified interdependency (*i.e.* reduced growth under isolation from the partner). However, predictions were made in the context of dividing labour between a helping phenotype and a reproductive phenotype (*i.e.* kin selection theory) (111). Albeit carrying the same label, in this case "division of labour", there are fundamental differences between reproductive division of labour and bidirectional cooperative cross-feeding interactions. For instance fitness of individual cells during growth is coupled in the case of bidirectional cross-feeding and individuals are units of selection (direct fitness), while reproductive division of labour implies a unit of

biological organisation that consists of “helpers” and “reproductives”) (111) that experience selection on the group level (indirect fitness). Put differently success in cooperative cross-feeding interactions is not bound to relatedness in contrast to the case of “helpers” and “reproductives”. Care should be therefore taken when adapting insights between both systems. What could be compared is for instance the insight that an essential trait will increase chances for division of labour to evolve (111), which is certainly the case in this work due to the obligate nature of the interaction. Besides, extreme specialization and with that strong interdependency was predicted to be supported by limited dispersal (111), which increases the chance of repeated interactions among individuals of aligned interests (*i.e.* those with increased relatedness according to the model of Cooper and West 2018 (111). When adapted to metabolic cooperation between bacterial cells (aside from relatedness), this can be translated into cross-feeding within aggregates, as discussed in Chapter 1. Taken together, cocultures evolved specialisation towards metabolic cooperation at the cost of decreased levels of autonomy (see Chapter 2), which was besides aggregation reported for another experimentally evolved metabolic interaction between two mutualistic bacteria as well (112). Further conceiving the process of specialisation illustrates potential evolutionary dynamics towards unculturability as a result of obligate dependencies.

4. Group formation and selection on the group-level

As indicated above, aggregates of cells were identified as the dominant location of growth within replicated cocultures from the initial stage until the end of the evolution experiment. Since reportedly present in non-evolved auxotrophic cocultures (501), aggregation indeed cannot be a result of adaption in the context of cross-feeding, yet is well-known to be triggered under stress (518). Hence, cluster formation is rather a stress-response related to starvation for amino acids, which could also be observed to a marginal extent in monocultures of auxotrophic genotypes (Chapter 2). In general, the formation of groups can (i) have a variety of reasons aside cross-feeding of metabolites, and (ii) enable a variety of processes, which I will discuss in the following with focus on the evolved metabolic cooperation. First of all, group formation was identified as the feature enabling evolution of cooperation in an otherwise homogeneous environment that does not *per se* facilitate population structure hence positive assortment (44) (Chapter 2). In line with their ubiquitous presence, these clusters were demonstrated to facilitate a general growth advantage. Aggregation is a commonly observed phenomenon

when bacterial species engage in metabolite exchange (29, 80-82, 362, 376, 399, 533). Thus, it is important to understand the implications of metabolite cross-feeding, which was shown to require such persistent formation of aggregates during growth (Supplementary figure S7; chapter 2). Therefore a general chain of causality for group formation could be helpful that is based on efficiency of exchange as the general premise: By logic, the fate of an interaction that generates mutual benefits via cross-feeding of resources highly depends on the efficiency of exchange that can be either realized via simple diffusion though the extracellular environment or physical connections between cells (chapter 1). In the case of diffusion, metabolites are unequally distributed between producers and consumers due to generated gradients. Under these conditions, it is likely that individuals within a given microbial community experience an unequal availability of the shared resources, which is reminiscent of a semi-public goods situation (44). The grade of diffusion was demonstrated by modelling to decide whether cooperators can exist in the presence of non-cooperators (465). Consequently, recipients likely benefit from increasing the proximity to producers. In fact, the upper limits of distance for efficient exchange between cells without significant loss due to diffusion or uptake by others were shown to be in the micrometer-range (29, 303, 519, 521). On the microscale, even ocean water exhibits a highly-structured distribution of metabolites (604, 605) and hence fulfils the assumption of unequal availability. Given that also direct connections between cells such as nanotubes are inherently limited in length (83-85) the limitations in distance between cross-feeding partners apply to nanotubes and related structures as well. Given the inherent limitations associated with metabolite exchange, spatial proximity between cells is the only conceivable strategy for cross-feeding interactions to exchange metabolites without significant loss hence to be competitive. Any alternative scenario, in which individuals of a microbial population have equal access to a required metabolite is in consequence a rare condition and hence a rather hypothetical construct, yet circumstances might be different when gaseous compounds are exchanged due to high levels of diffusion (105). Taken together, cooperating genotypes strongly depend on spatial proximity to enable efficient exchange of shared metabolites as confirmed by pervasive group formation as well as loss in fitness due to separation in chapter 2. The formation of a group can have remarkable implications for individuals interacting therein. On the level of a single group, positive feed-back loops can operate due to reciprocal metabolite exchange. A cooperative phenotype increases growth of the respective partner that in turn increases in numbers

hence releases more metabolites required by the cooperator (158). The relevance of these dynamics was demonstrated with cooperators experiencing strong positive selection, which was specifically promoted by groups consisting of ancestral, non-cooperative phenotypes (chapter 2). When looking on the level of many coexisting groups that exhibit variability in their composition and thus differences in fitness, competition can favour more cooperative groups as already pointed out by Darwin in the context of competing tribes (120). A hierarchical shift in the level on which selection operates is the mechanistic logic behind this idea (35). It is therefore the emergent property of a group that is under selection, and, in consequence, these groups represent units of selection (chapter 1). Theory predicts showed that than regularly dividing groups and differential reproduction of cooperating individuals therein are sufficient to enable dynamics that lead to selection operating on the level of groups (76). When more productive groups and not individuals experience a selective advantage, this should have two verifiable implications for evolved consortia that can be scrutinised on the basis of presented results: First, if group selection actually operated, increased fitness is a property of the group (*i.e.* the multicellular cluster), not the individual cell, which is confirmed by determined growth in whole populations forming clusters as well as individual isolates. Evolved cross-feeding consortia show dramatically increased fitness relative to the levels achieved by the evolved wild type (chapter 2). In contrast, individual isolates consistently showed decreased growth performance in comparison to ancestral auxotrophs despite the supplementation of sufficient amounts of amino acid, indicating that fitness on the levels of individuals indeed was not subjected to selection. Second, if non-cooperators (*i.e.* phenotypes that decreased their share relative to the corresponding ancestor) evolved and subsequently were selected against due to decreased group-level fitness, evolved consortia should ideally lack such phenotypes. This holds true for conducted analysis on the level of individuals as well, in which the degree of cooperativity has been quantified by determining either amino acid accumulation in the culture supernatant or by quantifying the growth of amino acid auxotrophic biosensor whose growth depends on the amount of amino acids they derived from the cocultured test strain (chapter 2).

Out of 35 isolated phenotypes, 34 exhibited at least ancestral levels for one of the two measures (Supplementary figure 4 of chapter 2), hence did not decrease the amount of amino acids they produced over the course of the experiment. This strongly suggests that under the experimental condition of the evolution experiment, selection operated against

non-cooperating phenotypes even though both control groups reduced the amounts of amino acids they produced (chapter 2). Of course, ancestral levels do not indicate cooperative cross-feeding on the other hand. However, since these measures required significant modification of ecological context, such as removal of the coevolved partner as well as amino acid supplementation, observed levels of cooperativity are likely underestimated as discussed in chapter 2. Confirmed implications of multi-level selection thereby have a similar outcome as division of labour driving extreme specialisation: Due to increasing interdependency, individuals become fitter in the context of the group (*e.g.* the interaction), however, eventually are unable to reproduce in isolation any more (107). The haystack-model as described by Maynard Smith (see chapter 1, figure 9) is strikingly reminiscent of the population dynamics as well as clusters as units of selection as reported in this study (58). In conclusion, the presented results strongly suggest selection has operated on the level of groups of cells at the cost of individual fitness (74). This phenomenon is currently under being further investigated by utilizing a microscopy-based analysis of multicellular clusters (Christian Kost, University of Osnabrück, personal communication).

5. Hierarchical evolutionary transitions

Beforehand identified features of the derived cooperation will next be discussed in the higher-order theoretical framework of hierarchical evolutionary transitions (HET) (30). HET, similarly to "transitions in individuality" (133), describes the evolution of multicellular organisms from previously simpler unicellular forms of life – a phenomenon which has occurred repeatedly during the history of life on earth (127, 128). However, there is still a lack of clear understanding how these processes took place mechanistically as well as which evolutionary factors facilitated these transitions (62). Conceptually, transitions in individuality can be classified according to the entities from which a multicellular organism evolved. Egalitarian transitions involve different hitherto free-living units, while fraternal transitions comprise identical entities carrying out social interactions (141). A major driving forces that can trigger the evolution of multicellularity can be predation (94, 153), but also simple selection for faster settling and other causes were demonstrated to facilitate such a process (154-156, 289). However, in all of the abovementioned model systems where authors reported the emergence of features that resemble the evolution of multicellular entities meet characteristics of fraternal transitions. In contrast, multicellular aggregates of different

genotypes performing cooperative cross-feeding rather display characteristics leading towards an egalitarian transition (141). Indeed, metabolic cross-feeding is well acknowledged as possible route towards such a transition (33-35), yet evolutionary approaches to study HET were not reported to date.

Next I will discuss to which extent the evolved bacterial model system resembles hallmarks of multicellularity and with this of a new unit of biological organisation, which is central to a HET. First, evolution from a well-known unicellular ancestor is required to study the initial stages towards a HET (30) and logically the very first general prerequisite for a multicellular entity to arise is the formation of a group. One putative way multicellularity by assortment of genotypes within groups can be facilitated from previously unicellular entities is by adhesion (139) or aggregation (128, 132, 140, 141), also termed coming together (CT) (30, 138), which is indeed given in *E. coli* cocultures. Next, for groups of *E. coli* cells to undergo a HET, it is required that they develop a life cycle, which is for instance fulfilled by a unicellular ancestor reproducibly forming groups (134, 135). Such reliable cluster formation and dispersal from clusters during each experimental cycle (assembly and disassembly) as described for evolved auxotrophic consortia (chapter 2) fits these assumptions and is remarkably reminiscent of the dynamics proposed to occur during the initial steps towards multicellularity (138, 140). A similar dynamic during experimental evolution was observed in the unicellular green alga *Chlamydomonas reinhardtii* that formed multicellular clusters as a response to predation (155). Importantly, groups that undergo a life cycle need to fulfil further requirements as formulated by van Gestel & Tarnita 2017, "…a group could be expressed as a facultative life stage only in response to certain recurrent environmental conditions…", yet needs to be "…formed sufficiently frequently for selection to potentially act on the group stage." (30). In line with these requirements, metabolic interdependency was shown to cause aggregation, likely as a stress response to amino acid limitation after each transfer (the recurrent environmental condition). Furthermore, groups were the units of reproduction as well as selection as indicated by decoupled fitness, followed by disassembly into single cells at the end of each cycle likely due to saturation (*e.g.* depleted resources). Related dynamics were reported for a social interaction that was subjected to an experimentally imposed life cycle, showing that fitness became decoupled as well (124).

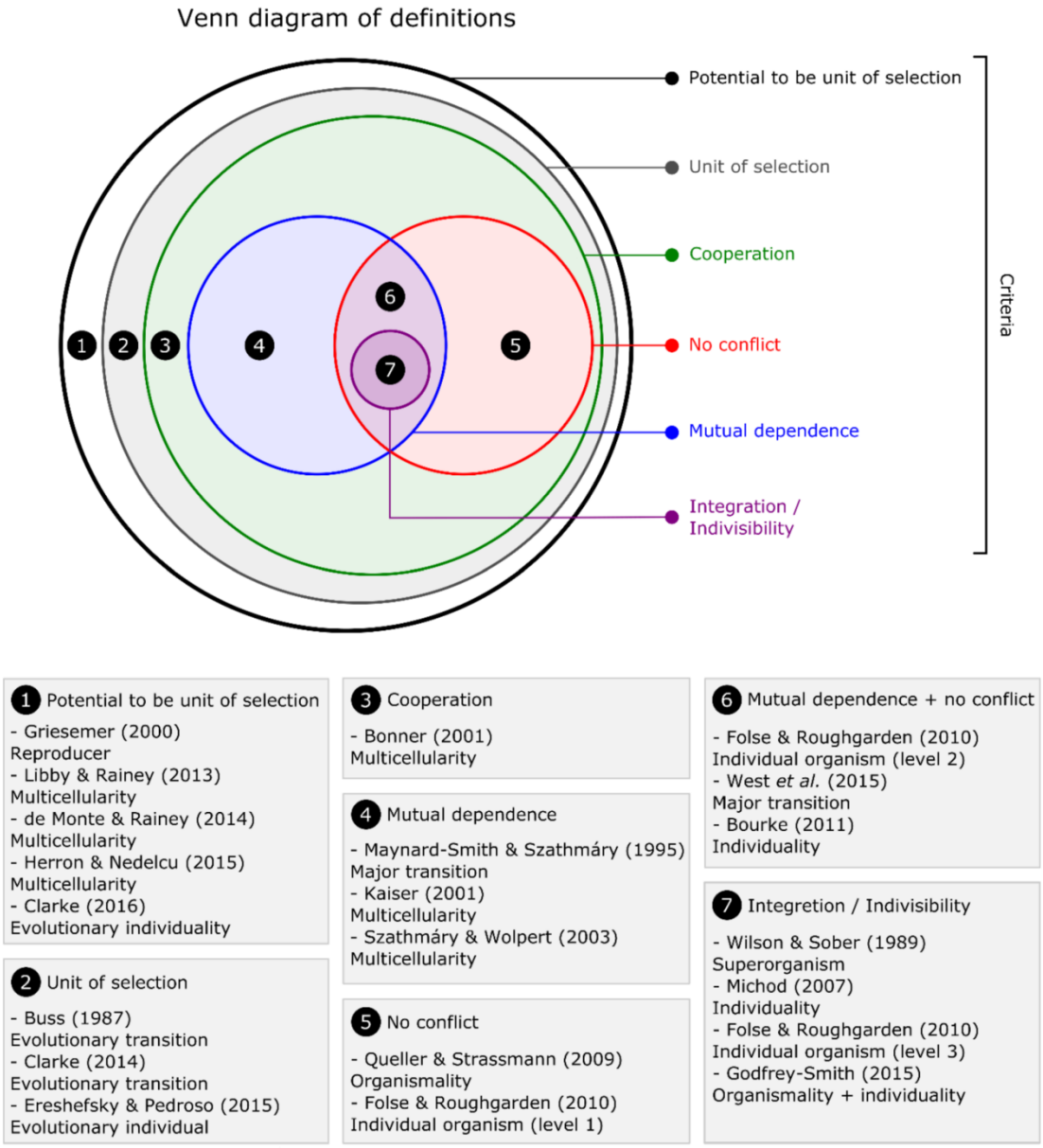

Figure 1: Comparison of definitions and requirements for a group to resemble a unit of biological organisation. The overlaps between existing definitions for the unit of biological organisation are illustrated by a Venn diagram. Figure from van Gestel and Tarnita (2017) (30).

These dynamics fit to the ecology-first scenario, which assumes group formation due to a preexisting feature (136, 137), for instance via starvation-induced aggregation as previously suggested (30) and suggested by observations in this work (chapter 2). Given all these characteristics, evolved multicellular clusters meet the minimum requirements to resemble a unit of biological organisation and thus exhibit the potential to be unit of selection (Figure 1). Further key aspects for the evolution of multicellularity were (i)

cooperation (107, 144, 145), (ii) mutual dependency (35, 146, 147), and (iii) division of labour (148). Albeit not subject to empirical tests, performance in growth and fitness measures suggests conflicts of interest play, if at all, a minor role. Since characteristics of evolved auxotrophic cocultures are in line with most of the abovementioned aspects, the requirements to resemble a unit of biological organisation are indeed fulfilled (Fig. 1) (30). Not identified was a new organizing principle (*e.g.* communication) or complexity (Fig. 1), which is unlikely to emerge within only 150 generations in the utilized model system. Even though the result presented in this work fit definitions, one has to be careful with stating that a HET occurred in the focal bacterial model system or that a new unit of biological organisation has evolved. Hence, I recommend further evolution of cross-feeding populations to pave the way towards evolutionary dynamics that in the ultimate consequence point towards the development of multicellularity. Further empirical tests should be performed to evaluate (i) the evolution of group-level adaptations (i.e. traits that can only be explained as adaptations to a group-living lifestyle yet not individual cells), (ii) the potential for complete loss of the capacity to reproduce independently, and (iii) the modification of the life cycle. Taken together the sum of insights gained in this work demonstrates cooperation within groups of cells to enable emergent higher-level evolutionary dynamics that are strikingly reminiscent of proposed development towards a higher unit of biological organisation within remarkably short evolutionary time.

6. Concluding remarks & outlook

Gained insights presented in this dissertation demonstrate for the first time how metabolic cooperation can evolve despite the absence of abiotic spatial structure, and which implications this process could have. Reported consequences resemble red-queen-like effects of accelerated molecular evolution as well as restricted adaptation to the abiotic environment. Further, detected effects on the level of fitness as well as the genome are indicative of groups as the units of selection. In addition, positive fitness-feedbacks within groups were shown to enable strong positive selection of single cooperating phenotypes. Strikingly, findings represent the first empirical evidence of cooperators succeeding despite of exclusively interacting with non-cooperative phenotypes. Observed population dynamics of assembly and disassembly showed that it takes as less as an obligate metabolic interaction causing aggregation to initiate a life cycle that followed the experimentally imposed rhythm of serial propagation. Taking

into account both the prevalence of metabolic interdependencies in bacteria and their preferred life style in multicellular structures, evolution of metabolic cooperation may be much more common than previously thought.

Furthermore, the observed evolutionary dynamics within groups of different partners that engage in metabolic division of labour fulfil important characteristics of a unit of biological organisation. These characteristics ultimately enabled evolutionary processes towards a transition in individuality, and demonstrate the ease with which division of metabolic labour in an evolutionary context drives groups to show hallmarks of nascent multicellularity. Obligate cross-feeding as a mechanism for generating groups was not empirically demonstrated before as a potential driver for a HET. Given these insights, a series of events point towards complexity can be formulated that are driven by the interplay of evolution and microbial ecology:

1. Numerous ecological driving forces, thereunder protection and competition, coerce bacteria to organize in groups that automatically provide population structure (28, 42, 606, 607).
2. Species eventually arrange as a function of metabolic complementarity (22) or dependency (16) as well as minimized potential for conflict or warfare (42, 454, 606, 607).
3. Adaptive gene loss (Morris et al 2012) and compensated trait loss not only be associated with a loss of metabolic autonomy, but further drive interdependencies, thus generating ecological interactions (69).
4. Most efficient groups produce most offspring (and push less-efficient ones aside within biofilms (89)), with the effect that (i) selection potentially shifts to the group-level, and (ii) metabolic division of labour and with it extreme specialisation is favoured.
5. If favourable ecological conditions support a continuing life cycle of these cooperators, further evolution of group properties enables a HET and with it a multicellular entity (30).

Since the ability to form aggregates is widespread in bacteria (414, 518), and metabolic dependencies seem to be common (63), I argue that the described process is not restricted to laboratory conditions. Indeed, under natural conditions selection pressures and adaptive processes are extraordinarily more multifaceted than in the experimental setup utilized in this study. Nevertheless, further investigations may focus on more complex environments, on the level of medium complexity, as well as on the

level of involved genotypes and species, ultimately including antagonistic interactions as well. Eventually, empirical studies can approach the diversity of natural microbial communities with the vision of finally designing and utilizing fine-tuned microbial associations in multiple applications that help in preserving ecosystems.

Coevolutionary processes in bacteria in addition could be utilized to generate overproduction mutants for varying compounds. The strength of bacterial populations lies in the sheer number of individuals on which selection can operate within relatively short periods of time. By synthetically designing obligate metabolic interactions that are based on the compound(s) of interest, large amounts of replicated populations can be monitored. When evolving populations fulfil defined criteria under specifically defined selection pressures, such as a minimal increase in production levels of desired metabolites, they are permitted to replace less productive ones. In this way, a powerful system is generated that ultimately selects for the desired outcome, namely overproduction. A welcome side-effect of this approach is the identification of targets for overproduction of primary metabolites or even secondary metabolites depending on a fine-tuned selection system that exclusively rewards more productive phenotypes in the framework of targeted evolution. As a consequence of this, previously unknown overproduction mutants that might not even resemble genetically modified organisms (GMO) can evolve within weeks by simply utilizing the power of selection in an evolutionary approach.

Another application lies in the optimization of synthetic bacterial communities (508, 608, 609). These might be designed for either the degradation of pollutants such as xenobiotics or the biosynthesis of desired compounds that are otherwise difficult to realize by a single genotype. Unforeseen competitive interactions, for instance stress-mediated release of growth-inhibiting compounds, potentially decrease productivity and hence efficiency and/or yield. By integrating obligate interdependencies, synthetic communities can first be stabilized and in the next step subjected to experimental evolution that selects for cooperation and against competition. Another aspect is that observed initial stages towards a HET could have significant implications for the evolution of multicellular eukaryotes. What if both the origin of multicellularity and the endosymbiotic origin of mitochondria occurred as a consequence of the same process? If two metabolically complementary species perform division of labour and hence aim at spatial proximity within groups, the coevolutionary dynamics are predicted to drive further specialisation, reduce potential of conflict due to alignment of interest, ultimately

paving the way for obligate endosymbiosis to emerge. This idea has several aspects in common with current theory about the evolution of eukaryotes, for instance conflict mediation in the context of group-level selection (610) as well as metabolic complementarity (611, 612). At the end, positive fitness effects at the group-level will determine whether endosymbiosis will be rewarded, which in the case of eukaryotes most likely happened due to a boost in energy metabolism (611). In fact, partners would significantly benefit from endosymbiosis when the life cycle still comprises a unicellular stage, which would eliminate the risk that partners do not encounter each other after dispersal from a group. This mechanistic perspective of coupled dynamics towards multicellularity as well as acquirement of mitochondria could motivate further evolution experiments that provoke positive selection of cooperating groups.

References

1. Flemming HC, Wuertz S. Bacteria and archaea on Earth and their abundance in biofilms. Nat Rev Microbiol. 2019;17(4):247-60.
2. Battistuzzi FU, Feijao A, Hedges SB. A genomic timescale of prokaryote evolution: insights into the origin of methanogenesis, phototrophy, and the colonization of land. BMC Evolutionary Biology. 2004;4(1):44.
3. Wainwright M, Wickramasinghe NC, Narlikar JV, Rajaratnam P. Microorganisms cultured from stratospheric air samples obtained at 41 km. FEMS Microbiol Lett. 2003;218(1):161-5.
4. Smith DJ, Ravichandar JD, Jain S, Griffin DW, Yu H, Tan Q, et al. Airborne Bacteria in Earth's Lower Stratosphere Resemble Taxa Detected in the Troposphere: Results From a New NASA Aircraft Bioaerosol Collector (ABC). Frontiers in microbiology. 2018;9:1752-.
5. Inagaki F, Hinrichs KU, Kubo Y, Bowles MW, Heuer VB, Hong WL, et al. DEEP BIOSPHERE. Exploring deep microbial life in coal-bearing sediment down to ~2.5 km below the ocean floor. Science. 2015;349(6246):420-4.
6. Wentzel A, Lewin A, Cervantes FJ, Valla S, Kotlar HK. Deep Subsurface Oil Reservoirs as Poly-extreme Habitats for Microbial Life. A Current Review. In: Seckbach J, Oren A, Stan-Lotter H, editors. Polyextremophiles: Life Under Multiple Forms of Stress. Dordrecht: Springer Netherlands; 2013. p. 439-66.
7. Johnston DT, Wolfe-Simon F, Pearson A, Knoll AH. Anoxygenic photosynthesis modulated Proterozoic oxygen and sustained Earth's middle age. Proc Natl Acad Sci U S A. 2009;106(40):16925-9.
8. Hazen RM, Papineau D, Leeker WB, Downs RT, Ferry JM, McCoy TJ, et al. Mineral evolution. Am Miner. 2008;93(11-12):1693-720.
9. Lovelock JE, Margulis L. Atmospheric homeostasis by and for the biosphere: the Gaia hypothesis. Tellus. 1974;26(1-2):2-10.
10. Falkowski PG, Fenchel T, Delong EF. The Microbial Engines That Drive Earth's Biogeochemical Cycles. Science. 2008;320(5879):1034-9.
11. Kieft T. Microbiology of the Deep Continental Biosphere. 2016. p. 225-49.
12. McFall-Ngai M, Hadfield MG, Bosch TC, Carey HV, Domazet-Loso T, Douglas AE, et al. Animals in a bacterial world, a new imperative for the life sciences. Proc Natl Acad Sci U S A. 2013;110(9):3229-36.
13. Schink B, Stams AJM. Syntrophism among Prokaryotes. Dworkin M, Falkow S, Rosenberg E, Schleifer KH, Stackebrandt E, editors. New York: Springer; 2006. 309-35 p.
14. Duan K, Sibley CD, Davidson CJ, Surette MG. Chemical interactions between organisms in microbial communities. Contributions to microbiology. 2009;16:1-17.
15. Phelan VV, Liu WT, Pogliano K, Dorrestein PC. Microbial metabolic exchange--the chemotype-to-phenotype link. Nat Chem Biol. 2011;8(1):26-35.
16. Zelezniak A, Andrejev S, Ponomarova O, Mende DR, Bork P, Patil KR. Metabolic dependencies drive species co-occurrence in diverse microbial communities. Proc Natl Acad Sci U S A. 2015;112(20):6449-54.
17. Schink B. Synergistic interactions in the microbial world. Antonie van Leeuwenhoek. 2002;81(1):257-61.
18. Seth EC, Taga ME. Nutrient cross-feeding in the microbial world. Frontiers in Microbiology. 2014;5.
19. Ponomarova O, Patil KR. Metabolic interactions in microbial communities: untangling the Gordian knot. Curr Opin Microbiol. 2015;27:37-44.
20. Lykidis A, Chen CL, Tringe SG, McHardy AC, Copeland A, Kyrpides NC, et al. Multiple syntrophic interactions in a terephthalate-degrading methanogenic consortium. Isme j. 2011;5(1):122-30.
21. Fowler SJ, Gutierrez-Zamora ML, Manefield M, Gieg LM. Identification of toluene degraders in a methanogenic enrichment culture. FEMS Microbiol Ecol. 2014;89(3):625-36.

22. Freilich S, Zarecki R, Eilam O, Segal ES, Henry CS, Kupiec M, et al. Competitive and cooperative metabolic interactions in bacterial communities. Nature Communications. 2011;2:589.
23. Mitri S, Foster KR. The Genotypic View of Social Interactions in Microbial Communities. In: Bassler BL, Lichten M, Schupbach G, editors. Annual Review of Genetics, Vol 47. Annual Review of Genetics. 472013. p. 247-73.
24. Little AE, Robinson CJ, Peterson SB, Raffa KF, Handelsman J. Rules of engagement: interspecies interactions that regulate microbial communities. Annu Rev Microbiol. 2008;62:375-401.
25. Sachs JL, Hollowell AC. The Origins of Cooperative Bacterial Communities. Mbio. 2012;3(3).
26. O'Toole G, Kaplan HB, Kolter R. Biofilm formation as microbial development. Annu Rev Microbiol. 2000;54:49-79.
27. Strassmann JE, Gilbert OM, Queller DC. Kin Discrimination and Cooperation in Microbes. In: Gottesman S, Harwood CS, editors. Annual Review of Microbiology, Vol 65. Annual Review of Microbiology. 65. Palo Alto: Annual Reviews; 2011. p. 349-67.
28. Lyons NA, Kolter R. On the evolution of bacterial multicellularity. Current Opinion in Microbiology. 2015;24:21-8.
29. Cordero OX, Datta MS. Microbial interactions and community assembly at microscales. Current Opinion in Microbiology. 2016;31:227-34.
30. van Gestel J, Tarnita CE. On the origin of biological construction, with a focus on multicellularity. Proceedings of the National Academy of Sciences. 2017:201704631.
31. Paterson S, Vogwill T, Buckling A, Benmayor R, Spiers AJ, Thomson NR, et al. Antagonistic coevolution accelerates molecular evolution. Nature. 2010;464(7286):275-8.
32. Scanlan PD, Hall AR, Blackshields G, Friman V-P, Davis MR, Jr., Goldberg JB, et al. Coevolution with Bacteriophages Drives Genome-Wide Host Evolution and Constrains the Acquisition of Abiotic-Beneficial Mutations. Molecular Biology and Evolution. 2015;32(6):1425-35.
33. Aravind L, Anantharaman V, Zhang D, de Souza RF, Iyer LM. Gene flow and biological conflict systems in the origin and evolution of eukaryotes. Front Cell Infect Microbiol. 2012;2:89-.
34. Martin W, Muller M. The hydrogen hypothesis for the first eukaryote. Nature. 1998;392(6671):37-41.
35. Szathmary E, Smith JM. The major evolutionary transitions. Nature. 1995;374(6519):227-32.
36. Estrela S, Trisos CH, Brown SP. From metabolism to ecology: cross-feeding interactions shape the balance between polymicrobial conflict and mutualism. The American naturalist. 2012;180(5):566-76.
37. Nadell CD, Drescher K, Foster KR. Spatial structure, cooperation and competition in biofilms. Nat Rev Micro. 2016;14(9):589-600.
38. Oliveira NM, Niehus R, Foster KR. Evolutionary limits to cooperation in microbial communities. Proc Natl Acad Sci U S A. 2014;111(50):17941-6.
39. Hibbing ME, Fuqua C, Parsek MR, Peterson SB. Bacterial competition: surviving and thriving in the microbial jungle. Nat Rev Micro. 2010;8(1):15-25.
40. Foster Kevin R, Bell T. Competition, Not Cooperation, Dominates Interactions among Culturable Microbial Species. Current Biology. 2012;22(19):1845-50.
41. Hibbing ME, Fuqua C, Parsek MR, Peterson SB. Bacterial competition: surviving and thriving in the microbial jungle. Nature Reviews Microbiology. 2009;8(1):nrmicro2259.
42. Stubbendieck RM, Straight PD. Multifaceted Interfaces of Bacterial Competition. J Bacteriol. 2016;198(16):2145-55.
43. Wong MJQ, Liang X, Smart M, Tang L, Moore R, Ingalls B, et al. Microbial Herd Protection Mediated by Antagonistic Interaction in Polymicrobial Communities. Applied and Environmental Microbiology. 2016;82(23):6881-8.
44. Tarnita CE. The ecology and evolution of social behavior in microbes. The Journal of Experimental Biology. 2017;220(1):18-24.
45. West SA, Griffin AS, Gardner A, Diggle SP. Social evolution theory for microorganisms. Nature Reviews Microbiology. 2006;4(8):597-607.
46. Xavier JB. Social interaction in synthetic and natural microbial communities. Mol Syst Biol. 2011;7:11.
47. Daniels R, Vanderleyden J, Michiels J. Quorum sensing and swarming migration in bacteria. Fems Microbiol Rev. 2004;28(3):261-89.
48. Ciofu O, Beveridge TJ, Kadurugamuwa J, Walther-Rasmussen J, Hoiby N. Chromosomal beta-lactamase is packaged into membrane vesicles and secreted from Pseudomonas aeruginosa. J Antimicrob Chemother. 2000;45(1):9-13.

49. Craig Maclean R, Brandon C. Stable public goods cooperation and dynamic social interactions in yeast. J Evol Biol. 2008;21(6):1836-43.
50. Frank SA. Foundations of social evolution. Krebs JR, Clutton–Brock T, editors. Princeton, New Jersey: Princeton University Press; 1998.
51. Hamilton WD. The genetical evolution of social behaviour, I & II. J Theor Biol. 1964;7(1):1-52.
52. Queller DC. A GENERAL MODEL FOR KIN SELECTION. Evolution. 1992;46(2):376-80.
53. West SA, Diggle SP, Buckling A, Gardner A, Griffins AS. The social lives of microbes. Annual Review of Ecology Evolution and Systematics. Annual Review of Ecology Evolution and Systematics. 382007. p. 53-77.
54. Wu D, Daugherty SC, Van Aken SE, Pai GH, Watkins KL, Khouri H, et al. Metabolic Complementarity and Genomics of the Dual Bacterial Symbiosis of Sharpshooters. PLoS Biol. 2006;4(6):e188.
55. Garcia SL, Buck M, McMahon KD, Grossart H-P, Eiler A, Warnecke F. Auxotrophy and intrapopulation complementary in the 'interactome' of a cultivated freshwater model community. Molecular Ecology. 2015;24(17):4449-59.
56. Zengler K, Zaramela LS. The social network of microorganisms - how auxotrophies shape complex communities. Nat Rev Microbiol. 2018;16(6):383-90.
57. Germerodt S, Bohl K, Lück A, Pande S, Schröter A, Kaleta C, et al. Pervasive Selection for Cooperative Cross-Feeding in Bacterial Communities. PLOS Computational Biology. 2016;12(6):e1004986.
58. Smith JM. Group Selection and Kin Selection. Nature. 1964;201(4924):1145-7.
59. Dawkins R. The selfish gene / Richard Dawkins. New York: Oxford University Press; 1976.
60. Hardin G. The Tragedy of the Commons. Science. 1968;162(3859):1243-8.
61. Nowak MA. Five rules for the evolution of cooperation. Science. 2006;314(5805):1560-3.
62. Rainey PB, Monte SD. Resolving Conflicts During the Evolutionary Transition to Multicellular Life. Annual Review of Ecology, Evolution, and Systematics. 2014;45(1):599-620.
63. D'Souza G, Waschina S, Pande S, Bohl K, Kaleta C, Kost C. Less is more: selective advantages can explain the prevalent loss of biosynthetic genes in bacteria. Evolution. 2014;68(9):2559-70.
64. Leadbetter JR. Cultivation of recalcitrant microbes: cells are alive, well and revealing their secrets in the 21st century laboratory. Curr Opin Microbiol. 2003;6(3):274-81.
65. Tyson GW, Banfield JF. Cultivating the uncultivated: a community genomics perspective. Trends in Microbiology. 2005;13(9):411-5.
66. Pande S, Kost C. Bacterial Unculturability and the Formation of Intercellular Metabolic Networks. Trends in Microbiology. 2017;25(5):349-61.
67. Morris JJ, Lenski RE, Zinser ER. The Black Queen Hypothesis: Evolution of Dependencies through Adaptive Gene Loss. mBio. 2012;3(2).
68. D'Souza G, Kost C. Experimental Evolution of Metabolic Dependency in Bacteria. PLoS Genet. 2016;12(11):e1006364.
69. Ellers J, Kiers ET, Currie CR, McDonald BR, Visser B. Ecological interactions drive evolutionary loss of traits. Ecol Lett. 2012;15(10):1071-82.
70. MacLean RC. The tragedy of the commons in microbial populations: insights from theoretical, comparative and experimental studies. Heredity. 2008;100(3):233-9.
71. Gardner A, West SA. GREENBEARDS. Evolution. 2010;64(1):25-38.
72. Foster KR, Wenseleers T. A general model for the evolution of mutualisms. J Evol Biol. 2006;19(4):1283-93.
73. Fletcher JA, Doebeli M. A simple and general explanation for the evolution of altruism. Proceedings of the Royal Society B: Biological Sciences. 2009;276(1654):13-9.
74. Wilson DS. A theory of group selection. Proceedings of the National Academy of Sciences. 1975;72(1):143-6.
75. Doebeli M, Knowlton N. The evolution of interspecific mutualisms. Proc Natl Acad Sci U S A. 1998;95(15):8676-80.
76. Traulsen A, Nowak MA. Evolution of cooperation by multilevel selection. Proceedings of the National Academy of Sciences. 2006;103(29):10952-5.
77. Antal T, Ohtsuki H, Wakeley J, Taylor PD, Nowak MA. Evolution of cooperation by phenotypic similarity. Proceedings of the National Academy of Sciences. 2009;106(21):8597-600.
78. Nadell CD, Foster KR, Xavier JB. Emergence of Spatial Structure in Cell Groups and the Evolution of Cooperation. PLOS Computational Biology. 2010;6(3):e1000716.

79. Hamilton WD. INNATE SOCIAL APTITUDES OF MAN AN APPROACH FROM EVOLUTIONARY GENETICS1975. 133-55 p.
80. Ishii S, Kosaka T, Hori K, Hotta Y, Watanabe K. Coaggregation facilitates interspecies hydrogen transfer between Pelotomaculum thermopropionicum and Methanothermobacter thermautotrophicus. Appl Environ Microbiol. 2005;71(12):7838-45.
81. Ha PT, Lindemann SR, Shi L, Dohnalkova AC, Fredrickson JK, Madigan MT, et al. Syntrophic anaerobic photosynthesis via direct interspecies electron transfer. Nat Commun. 2017;8:13924.
82. Benomar S, Ranava D, Cardenas ML, Trably E, Rafrafi Y, Ducret A, et al. Nutritional stress induces exchange of cell material and energetic coupling between bacterial species. Nat Commun. 2015;6:6283.
83. Pande S, Shitut S, Freund L, Westermann M, Bertels F, Colesie C, et al. Metabolic cross-feeding via intercellular nanotubes among bacteria. Nature Communications. 2015;6:6238.
84. Dubey GP, Ben-Yehuda S. Intercellular nanotubes mediate bacterial communication. Cell. 2011;144(4):590-600.
85. Dubey Gyanendra P, Malli Mohan Ganesh B, Dubrovsky A, Amen T, Tsipshtein S, Rouvinski A, et al. Architecture and Characteristics of Bacterial Nanotubes. Developmental Cell. 2016;36(4):453-61.
86. Biller SJ, Schubotz F, Roggensack SE, Thompson AW, Summons RE, Chisholm SW. Bacterial Vesicles in Marine Ecosystems. Science. 2014;343(6167):183-6.
87. Remis JP, Wei D, Gorur A, Zemla M, Haraga J, Allen S, et al. Bacterial social networks: structure and composition of Myxococcus xanthus outer membrane vesicle chains. Environ Microbiol. 2014;16(2):598-610.
88. van Veelen M, Garcia J, Aviles L. It takes grouping and cooperation to get sociality. J Theor Biol. 2010;264(4):1240-53.
89. Pande S, Kaftan F, Lang S, Svatos A, Germerodt S, Kost C. Privatization of cooperative benefits stabilizes mutualistic cross-feeding interactions in spatially structured environments. Isme Journal. 2016;10(6):1413-23.
90. Drescher K, Nadell Carey D, Stone Howard A, Wingreen Ned S, Bassler Bonnie L. Solutions to the Public Goods Dilemma in Bacterial Biofilms. Current Biology. 2014;24(1):50-5.
91. Momeni B, Waite AJ, Shou W. Spatial self-organization favors heterotypic cooperation over cheating. eLife. 2013;2:e00960.
92. Van Dyken JD, Müller Melanie JI, Mack Keenan ML, Desai Michael M. Spatial Population Expansion Promotes the Evolution of Cooperation in an Experimental Prisoner's Dilemma. Current Biology. 2013;23(10):919-23.
93. Corno G, Jürgens K. Direct and Indirect Effects of Protist Predation on Population Size Structure of a Bacterial Strain with High Phenotypic Plasticity. Applied and Environmental Microbiology. 2006;72(1):78-86.
94. Herron MD, Borin JM, Boswell JC, Walker J, Chen ICK, Knox CA, et al. De novo origins of multicellularity in response to predation. Sci Rep. 2019;9(1):2328-.
95. Hall-Stoodley L, Stoodley P. Biofilm formation and dispersal and the transmission of human pathogens. Trends in Microbiology. 2005;13(1):7-10.
96. Griffiths BS, Philippot L. Insights into the resistance and resilience of the soil microbial community. Fems Microbiol Rev. 2013;37(2):112-29.
97. Baho DL, Peter H, Tranvik LJ. Resistance and resilience of microbial communities – temporal and spatial insurance against perturbations. Environ Microbiol. 2012;14(9):2283-92.
98. Hiltunen T, Virta M, Laine AL. Antibiotic resistance in the wild: an eco-evolutionary perspective. Philosophical transactions of the Royal Society of London Series B, Biological sciences. 2017;372(1712).
99. Iwasa K, Murakami S. Palmelloid Formation of Chlamydomonas. Physiologia Plantarum. 1968;21(6):1224-33.
100. Iwasa K, Murakami S. Palmelloid Formation of Chlamydomonas II. Mechanism of Palmelloid Formation by Organic Acids. Physiologia Plantarum. 1969;22(1):43-50.
101. Ackermann M, Stecher B, Freed NE, Songhet P, Hardt WD, Doebeli M. Self-destructive cooperation mediated by phenotypic noise. Nature. 2008;454(7207):987-90.
102. Pande S, Merker H, Bohl K, Reichelt M, Schuster S, de Figueiredo LF, et al. Fitness and stability of obligate cross-feeding interactions that emerge upon gene loss in bacteria. ISME J. 2014;8(5):953-62.

103. Nadell CD, Drescher K, Wingreen NS, Bassler BL. Extracellular matrix structure governs invasion resistance in bacterial biofilms. ISME J. 2015;9(8):1700-9.
104. Ross-Gillespie A, Gardner A, West SA, Griffin AS. Frequency dependence and cooperation: theory and a test with bacteria. Am Nat. 2007;170(3):331-42.
105. Hillesland KL, Stahl DA. Rapid evolution of stability and productivity at the origin of a microbial mutualism. Proc Natl Acad Sci U S A. 2010;107(5):2124-9.
106. Harcombe WR, Chacón JM, Adamowicz EM, Chubiz LM, Marx CJ. Evolution of bidirectional costly mutualism from byproduct consumption. Proceedings of the National Academy of Sciences. 2018.
107. West SA, Cooper GA. Division of labour in microorganisms: an evolutionary perspective. Nat Rev Microbiol. 2016;14(11):716-23.
108. Zhang Z, Claessen D, Rozen DE. Understanding Microbial Divisions of Labor. Frontiers in microbiology. 2016;7:2070-.
109. van Gestel J, Vlamakis H, Kolter R. Division of Labor in Biofilms: the Ecology of Cell Differentiation. Microbiology spectrum. 2015;3(2):Mb-0002-2014.
110. Wahl LM. Evolving the division of labour: generalists, specialists and task allocation. J Theor Biol. 2002;219(3):371-88.
111. Cooper GA, West SA. Division of labour and the evolution of extreme specialization. Nature Ecology & Evolution. 2018;2(7):1161-7.
112. Hillesland KL, Lim S, Flowers JJ, Turkarslan S, Pinel N, Zane GM, et al. Erosion of functional independence early in the evolution of a microbial mutualism. Proc Natl Acad Sci U S A. 2014;111(41):14822-7.
113. Bolotin A, Quinquis B, Renault P, Sorokin A, Ehrlich SD, Kulakauskas S, et al. Complete sequence and comparative genome analysis of the dairy bacterium Streptococcus thermophilus. Nature Biotechnology. 2004;22(12):1554-8.
114. van de Guchte M, Penaud S, Grimaldi C, Barbe V, Bryson K, Nicolas P, et al. The complete genome sequence of *Lactobacillus bulgaricus* reveals extensive and ongoing reductive evolution. Proc Natl Acad Sci U S A. 2006;103(24):9274-9.
115. Sieuwerts S, de Bok FAM, Hugenholtz J, van Hylckama Vlieg JET. Unraveling microbial interactions in food fermentations; from classical to genomics approaches. Applied and Environmental Microbiology. 2008;74(16):4997-5007.
116. Staley JT, Konopka A. Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. Annu Rev Microbiol. 1985;39:321-46.
117. Bergstrom CT, Lachmann M. The Red King effect: When the slowest runner wins the coevolutionary race. Proc Natl Acad Sci U S A. 2003;100(2):593-8.
118. Rubin BER, Moreau CS. Comparative genomics reveals convergent rates of evolution in ant–plant mutualisms. Nature Communications. 2016;7:12679.
119. Collins S. Competition limits adaptation and productivity in a photosynthetic alga at elevated CO₂. Proceedings of the Royal Society B: Biological Sciences. 2010.
120. Darwin C. The descent of man : and selection in relation to sex. London: J. Murray; 1871.
121. Lewontin RC. The units of selection. Annual Review of Ecology and Systematics. 1970;1:1-18.
122. Hull DL. Individuality and selection. Annual Review of Ecology and Systematics. 1980;11:311-32.
123. Michod RE. Evolution of the individual. American Naturalist. 1997;150:S5-S21.
124. Hammerschmidt K, Rose CJ, Kerr B, Rainey PB. Life cycles, fitness decoupling and the evolution of multicellularity. Nature. 2014;515:75.
125. Damuth J, Heisler IL. Alternative formulations of multilevel selection. Biology and Philosophy. 1988;3(4):407-30.
126. Sober E, Wilson DS. Unto others: The evolution and psychology of unselfish behavior: Harvard University Press; 1999.
127. Bonner JT. First Signals: The Evolution of Multicellular Development2009.
128. Grosberg RK, Strathmann RR. The Evolution of Multicellularity: A Minor Major Transition? Annual Review of Ecology, Evolution, and Systematics. 2007;38(1):621-54.
129. Pepper JW, Herron MD. Does biology need an organism concept? Biol Rev Camb Philos Soc. 2008;83(4):621-7.
130. Bonner JT. On development: The biology of form.: Harvard University Press: Cambridge; 1974. 305–6 p.
131. Buss LW. The Evolution of Individuality: Princeton University Press; 1987.

132. Szathmáry E. Toward major evolutionary transitions theory 2.0. Proc Natl Acad Sci U S A. 2015;112(33):10104-11.
133. Michod RE. Evolution of individuality during the transition from unicellular to multicellular life. Proc Natl Acad Sci U S A. 2007;104 Suppl 1:8613-8.
134. Libby E, P BR. A conceptual framework for the evolutionary origins of multicellularity. Physical biology. 2013;10(3):035001.
135. Herron MD, Nedelcu AM. Volvocine Algae: From Simple to Complex Multicellularity. In: Ruiz-Trillo I, Nedelcu AM, editors. Evolutionary Transitions to Multicellular Life: Principles and mechanisms. Dordrecht: Springer Netherlands; 2015. p. 129-52.
136. Wolpert L. The evolutionary origin of development: cycles, patterning, privilege and continuity. Development. 1994;1994(Supplement):79-84.
137. West-Eberhard MJ. Phenotypic Plasticity and the Origins of Diversity. Annual Review of Ecology and Systematics. 1989;20(1):249-78.
138. Bonner JT. The origins of multicellularity. Integrative Biology: Issues, News, and Reviews. 1998;1(1):27-36.
139. De Monte S, Rainey PB. Nascent multicellular life and the emergence of individuality. Journal of biosciences. 2014;39(2):237-48.
140. Tarnita CE, Taubes CH, Nowak MA. Evolutionary construction by staying together and coming together. J Theor Biol. 2013;320:10-22.
141. Queller DC. Relatedness and the fraternal major transitions. Philosophical transactions of the Royal Society of London Series B, Biological sciences. 2000;355(1403):1647-55.
142. Okasha S. Evolution and the levels of selection. Oxford: Oxford University Press; 2006. 288 p.
143. Michod RE. On the transfer of fitness from the cell to the multicellular organism. Biology & Philosophy. 2005;20(5):967-87.
144. Buss LW. Slime molds, ascidians, and the utility of evolutionary theory. Proc Natl Acad Sci U S A. 1999;96(16):8801-3.
145. Michod RE. Darwinian dynamics : evolutionary transitions in fitness and individuality. 1999.
146. Kaiser D. Building a multicellular organism. Annu Rev Genet. 2001;35:103-23.
147. Szathmáry E, Wolpert L. The Transition from Single Cells to Multicellularity. In: Hammerstein P, editor. Genetic and Cultural Evolution of Cooperation. MIT Press: The MIT Press; 2003. p. 271-90.
148. Ispolatov I, Ackermann M, Doebeli M. Division of labour and the evolution of multicellularity. Proceedings Biological sciences. 2012;279(1734):1768-76.
149. Bourke AFG. Principles of social evolution. Oxford [England]; New York: Oxford University Press; 2011.
150. West SA, Fisher RM, Gardner A, Kiers ET. Major evolutionary transitions in individuality. Proc Natl Acad Sci U S A. 2015;112(33):10112-9.
151. Wilson DS, Sober E. Reviving the superorganism. J Theor Biol. 1989;136(3):337-56.
152. Godfrey-Smith P. Individuality and life cycles. Individuals across the Sciences. 2015:85-102.
153. Boraas ME, Seale DB, Boxhorn JE. Phagotrophy by a flagellate selects for colonial prey: A possible origin of multicellularity. Evolutionary Ecology. 1998;12(2):153-64.
154. Ratcliff WC, Denison RF, Borrello M, Travisano M. Experimental evolution of multicellularity. Proc Natl Acad Sci U S A. 2012;109(5):1595-600.
155. Ratcliff WC, Herron MD, Howell K, Pentz JT, Rosenzweig F, Travisano M. Experimental evolution of an alternating uni- and multicellular life cycle in Chlamydomonas reinhardtii. Nat Commun. 2013;4:2742.
156. Koschwanez JH, Foster KR, Murray AW. Improved use of a public good selects for the evolution of undifferentiated multicellularity. eLife. 2013;2:e00367.
157. Faust K, Raes J. Microbial interactions: from networks to models. Nat Rev Microbiol. 2012;10(8):538-50.
158. Harcombe W. Novel cooperation experimentally evolved between species. Evolution. 2010;64(7):2166-72.
159. Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, et al. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol. 2006;2:2006.0008.
160. Barrick JE, Colburn G, Deatherage DE, Traverse CC, Strand MD, Borges JJ, et al. Identifying structural variation in haploid microbial genomes from short-read resequencing data using breseq. BMC Genomics. 2014;15:17.

161. Deatherage DE, Barrick JE. Identification of Mutations in Laboratory-Evolved Microbes from Next-Generation Sequencing Data Using breseq. In: Sun L, Shou W, editors. Engineering and Analyzing Multicellular Systems: Methods and Protocols. Methods in Molecular Biology. 11512014. p. 165-88.
162. Elena SF, Lenski RE. Evolution experiments with microorganisms: The dynamics and genetic bases of adaptation. Nat Rev Genet. 2003;4(6):457-69.
163. Lenski RE. Phenotypic and genomic evolution during a 20,000-generation experiment with the bacterium *Escherichia coli*. Plant Breeding Reviews. 2004;24:225-65.
164. Barrick JE, Yu DS, Yoon SH, Jeong H, Oh TK, Schneider D, et al. Genome evolution and adaptation in a long-term experiment with Escherichia coli. Nature. 2009;461(7268):1243-U74.
165. Whitman WB, Coleman DC, Wiebe WJ. Prokaryotes: the unseen majority. Proceedings of the National Academy of Sciences. 1998;95(12):6578-83.
166. Horner-Devine MC, Carney KM, Bohannan BJ. An ecological perspective on bacterial biodiversity. Proceedings of the Royal Society of London B: Biological Sciences. 2004;271(1535):113-22.
167. McInerney JO. Evolution: a four billion year old metabolism. Nature microbiology. 2016;1:16139.
168. Weiss MC, Sousa FL, Mrnjavac N, Neukirchen S, Roettger M, Nelson-Sathi S, et al. The physiology and habitat of the last universal common ancestor. Nature Microbiology. 2016;1:16116.
169. González-Cabaleiro R, Ofiţeru ID, Lema JM, Rodríguez J. Microbial catabolic activities are naturally selected by metabolic energy harvest rate. The ISME journal. 2015;9(12):2630-41.
170. Kerr B, Riley MA, Feldman MW, Bohannan BJ. Local dispersal promotes biodiversity in a real-life game of rock–paper–scissors. Nature. 2002;418(6894):171-4.
171. Kirkup BC, Riley MA. Antibiotic-mediated antagonism leads to a bacterial game of rock–paper–scissors in vivo. Nature. 2004;428(6981):412-4.
172. Davies D, Geesey G. Regulation of the alginate biosynthesis gene algC in Pseudomonas aeruginosa during biofilm development in continuous culture. Applied and environmental microbiology. 1995;61(3):860-7.
173. Griffin AS, West SA, Buckling A. Cooperation and competition in pathogenic bacteria. Nature. 2004;430(7003):1024-7.
174. Mee MT, Collins JJ, Church GM, Wang HH. Syntrophic exchange in synthetic microbial communities. Proc Natl Acad Sci U S A. 2014;111(20):E2149-56.
175. Pound R. Symbiosis and Mutualism. The American Naturalist. 1893;27(318):509-20.
176. Marshall CE. Microbial associations. Science. 1915:306-12.
177. Dawson M. 'Nitragin'and the nodules of leguminous plants. Proceedings of the Royal Society of London. 1899;64(402-411):167-8.
178. MacDougal DT. Symbiosis and saprophytism. Bulletin of the Torrey Botanical Club. 1899;26(10):511-30.
179. Ferguson TP, Bond G. SYMBIOSIS OF LEGUMINOUS PLANTS AND NODULE BACTERIA .5. THE GROWTH OF RED CLOVER AT DIFFERENT OXYGEN TENSIONS. Annals of Botany. 1954;18(72):385-96.
180. Marshall MS. An associative study of Strept. lacticus and B. subtilis in milk. Journal of dairy science. 1920;3(5):406-13.
181. Graham V. The maintenance of viability in Lactobacillus bulgaricus cultures by growth in association with certain yeasts. J Bacteriol. 1943;45:51.
182. Soulides DA. A SYNERGISM BETWEEN YOGHURT BACTERIA AND YEASTS AND THE EFFECT OF THEIR ASSOCIATION UPON THE VIABILITY OF THE BACTERIA. Applied Microbiology. 1955;3(3):129-31.
183. Ward HM. The Ginger-Beer Plant, and the Organisms Composing it: A Contribution to the Study of Fermentation-Yeasts and Bacteria. Philosophical Transactions of the Royal Society of London B. 1892;183:125-97.
184. Pfeffer W. Pflanzenphysiologie. Ein Handbuch der Lehre vom Stoffwechsel und Kraftwechsel in der Pflanze. 2nd revised and amended ed. Engelmann, Leipzig. 1897.
185. WARD D Sc F, H MARSHALL. Symbiosis1. Annals of Botany. 1899(4):549-62.
186. REINHEIMER H. SYMBIOSIS AND THE BIOLOGY OF FOOD. Science Progress in the Twentieth Century (1919-1933). 1921;16(62):258-74.
187. Gibson F, Jones M. Tests for" cross-feeding" among bacteria. Aust. J Sci. 1954;17:33-4.
188. Nurmikko V. BIOCHEMICAL FACTORS AFFECTING SYMBIOSIS AMONG BACTERIA. Experientia. 1956;12(7):245-9.

189. Nurmikko V. The dialysis technique in the study of the vitamins and amino acids affecting associations of micro-organisms. Acta chem scand. 1955;9:1317-22.
190. Sørensen SR, Ronen Z, Aamand J. Growth in coculture stimulates metabolism of the phenylurea herbicide isoproturon by Sphingomonas sp. strain SRS2. Applied and environmental microbiology. 2002;68(7):3478-85.
191. de Souza ML, Newcombe D, Alvey S, Crowley DE, Hay A, Sadowsky MJ, et al. Molecular basis of a bacterial consortium: interspecies catabolism of atrazine. Applied and Environmental Microbiology. 1998;64(1):178-84.
192. Roberts SJ, Walker A, Parekh NR, Welch SJ, Waddington MJ. Studies on a mixed bacterial culture from soil which degrades the herbicide linuron. Pest Management Science. 1993;39(1):71-8.
193. McInerney MJ, Bryant MP, Pfennig N. ANAEROBIC BACTERIUM THAT DEGRADES FATTY-ACIDS IN SYNTROPHIC ASSOCIATION WITH METHANOGENS. Arch Microbiol. 1979;122(2):129-35.
194. Fildes P. Production of tryptophan by Salmonella typhi and Escherichia coli. Microbiology-(UK). 1956;15(3):636-42.
195. Dempsey WB, Pachler PF. Isolation and Characterization of Pyridoxine Auxotrophs of Escherichia coli. Journal of Bacteriology. 1966;91(2):642-5.
196. Pomper S. Purine-requiring and pyrimidine-requiring mutants of Saccharomyces cerevisiae. Journal of bacteriology. 1952;63(6):707.
197. Glanville E, Demerec M. Threonine, isoleucine, and isoleucine-valine mutants of Salmonella typhimurium. Genetics. 1960;45(10):1359.
198. Rahman K, Thahira-Rahman J, Lakshmanaperumalsamy P, Banat I. Towards efficient crude oil degradation by a mixed bacterial consortium. Bioresource technology. 2002;85(3):257-61.
199. Haritash A, Kaushik C. Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): a review. Journal of hazardous materials. 2009;169(1):1-15.
200. Das N, Chandran P. Microbial degradation of petroleum hydrocarbon contaminants: an overview. Biotechnology research international. 2011;2011.
201. Ali H. Biodegradation of synthetic dyes—a review. Water, Air, & Soil Pollution. 2010;213(1-4):251-73.
202. Saratale RG, Saratale GD, Chang J-S, Govindwar S. Bacterial decolorization and degradation of azo dyes: a review. Journal of the Taiwan Institute of Chemical Engineers. 2011;42(1):138-57.
203. Treves DS, Manning S, Adams J. Repeated evolution of an acetate-crossfeeding polymorphism in long-term populations of Escherichia coli. Molecular biology and evolution. 1998;15(7):789-97.
204. HELLING RB, KINNEY T, ADAMS J. The maintenance of plasmid-containing organisms in populations of Escherichia coli. Microbiology-(UK). 1981;123(1):129-41.
205. Helling RB, Vargas CN, Adams J. Evolution of Escherichia coli during growth in a constant environment. Genetics. 1987;116(3):349-58.
206. Rosenzweig RF, Sharp RR, Treves DS, Adams J. Microbial evolution in a simple unstructured environment - Genetic differentiation in *Escherichia coli*. Genetics. 1994;137(4):903-17.
207. Turner PE, Souza V, Lenski RE. Tests of ecological mechanisms promoting the stable coexistence of two bacterial genotypes. Ecology. 1996;77(7):2119-29.
208. Friesen ML, Saxer G, Travisano M, Doebeli M. Experimental evidence for sympatric ecological diversification due to frequency-dependent competition in Escherichia coli. Evolution. 2004;58(2):245-60.
209. Pfeiffer T, Bonhoeffer S. Evolution of cross-feeding in microbial populations. American Naturalist. 2004;163(6):E126-E35.
210. Morris BE, Henneberger R, Huber H, Moissl-Eichinger C. Microbial syntrophy: interaction for the common good. Fems Microbiol Rev. 2013;37(3):384-406.
211. Doyle E, Muckian L, Hickey AM, Clipson N. Microbial PAH degradation. Adv Appl Microbiol. 2008;65:27-66.
212. Shiio I, ÔTSUKA S-I, TAKAHASHI M. Effect of biotin on the bacterial formation of glutamic acid: I. Glutamate formation and cellular permeability of amino acids. The Journal of Biochemistry. 1962;51(1):56-62.
213. Paczia N, Nilgen A, Lehmann T, Gätgens J, Wiechert W, Noack S. Extensive exometabolome analysis reveals extended overflow metabolism in various microorganisms. Microbial cell factories. 2012;11(1):122.
214. Shimoyama T, Kato S, Ishii Si, Watanabe K. Flagellum mediates symbiosis. Science. 2009;323(5921):1574-.

215. Belenguer A, Duncan SH, Calder AG, Holtrop G, Louis P, Lobley GE, et al. Two routes of metabolic cross-feeding between Bifidobacterium adolescentis and butyrate-producing anaerobes from the human gut. Applied and environmental microbiology. 2006;72(5):3593-9.
216. Wintermute EH, Silver PA. Emergent cooperation in microbial metabolism. Mol Syst Biol. 2010;6.
217. Baumann L, Baumann P, Moran NA, Sandström J, Thao ML. Genetic characterization of plasmids containing genes encoding enzymes of leucine biosynthesis in endosymbionts (Buchnera) of aphids. Journal of molecular evolution. 1999;48(1):77-85.
218. Lipschultz F, Zafiriou O, Wofsy S, McElroy M, Valois F, Watson S. Production of NO and N2O by soil nitrifying bacteria. Nature. 1981;294(5842):641-3.
219. Koch H, Lücker S, Albertsen M, Kitzinger K, Herbold C, Spieck E, et al. Expanded metabolic versatility of ubiquitous nitrite-oxidizing bacteria from the genus Nitrospira. Proceedings of the National Academy of Sciences. 2015;112(36):11371-6.
220. Leimar O, Connor RC. By-product benefits, reciprocity, and pseudoreciprocity in mutualism. Genetic and cultural evolution of cooperation. 2003:203-22.
221. Sachs JL, Mueller UG, Wilcox TP, Bull JJ. The evolution of cooperation. Q Rev Biol. 2004;79(2):135-60.
222. Russel J, Røder HL, Madsen JS, Burmølle M, Sørensen SJ. Antagonism correlates with metabolic similarity in diverse bacteria. Proceedings of the National Academy of Sciences. 2017;114(40):10684-8.
223. Madsen JS, Røder HL, Russel J, Sørensen H, Burmølle M, Sørensen SJ. Coexistence facilitates interspecific biofilm formation in complex microbial communities. Environ Microbiol. 2016;18(8):2565-74.
224. Rosenthal AZ, Matson EG, Eldar A, Leadbetter JR. RNA-seq reveals cooperative metabolic interactions between two termite-gut spirochete species in co-culture. Isme Journal. 2011;5(7):1133-42.
225. Graber JR, Breznak JA. Folate cross-feeding supports symbiotic homoacetogenic spirochetes. Applied and environmental microbiology. 2005;71(4):1883-9.
226. Kaiser P. Diazotrophic Mixed Cultures of Azospirillum Brasilense and Enterobacter Cloacae. In: Fendrik I, del Gallo M, Vanderleyden J, de Zamaroczy M, editors. Azospirillum VI and Related Microorganisms: Genetics — Physiology — Ecology. Berlin, Heidelberg: Springer Berlin Heidelberg; 1995. p. 207-12.
227. Kives J, Guadarrama D, Orgaz B, Rivera-Sen A, Vazquez J, SanJose C. Interactions in Biofilms of Lactococcus lactis ssp. cremoris and Pseudomonas fluorescens Cultured in Cold UHT Milk. Journal of dairy science. 2005;88(12):4165-71.
228. Woyke T, Teeling H, Ivanova NN, Huntemann M, Richter M, Gloeckner FO, et al. Symbiosis insights through metagenomic analysis of a microbial consortium. Nature. 2006;443(7114):950-5.
229. Foster J, Ganatra M, Kamal I, Ware J, Makarova K, Ivanova N, et al. The Wolbachia genome of Brugia malayi: endosymbiont evolution within a human pathogenic nematode. PLoS Biol. 2005;3.
230. Ghignone S, Salvioli A, Anca I, Lumini E, Ortu G, Petiti L, et al. The genome of the obligate endobacterium of an AM fungus reveals an interphylum network of nutritional interactions. ISME J. 2012;6(1):136-45.
231. Mark Welch JL, Rossetti BJ, Rieken CW, Dewhirst FE, Borisy GG. Biogeography of a human oral microbiome at the micron scale. Proceedings of the National Academy of Sciences. 2016;113(6):E791-E800.
232. Dekas AE, Poretsky RS, Orphan VJ. Deep-Sea Archaea Fix and Share Nitrogen in Methane-Consuming Microbial Consortia. Science. 2009;326(5951):422-6.
233. Walker CB, Redding-Johanson AM, Baidoo EE, Rajeev L, He ZL, Hendrickson EL, et al. Functional responses of methanogenic archaea to syntrophic growth. Isme Journal. 2012;6(11):2045-55.
234. Sieuwerts S, Molenaar D, van Hijum S, Beerthuyzen M, Stevens MJA, Janssen PWM, et al. Mixed-Culture Transcriptome Analysis Reveals the Molecular Basis of Mixed-Culture Growth in Streptococcus thermophilus and Lactobacillus bulgaricus. Applied and Environmental Microbiology. 2010;76(23):7775-84.
235. Jahn U, Summons R, Sturt H, Grosjean E, Huber H. Composition of the lipids of Nanoarchaeum equitans and their origin from its host Ignicoccus sp. strain KIN4/I. Arch Microbiol. 2004;182(5):404-13.

236. Sawanon S, Kobayashi Y. Synergistic fibrolysis in the rumen by cellulolytic Ruminococcus flavefaciens and non-cellulolytic Selenomonas ruminantium: Evidence in defined cultures. Anim Sci J. 2006;77(2):208-14.
237. Foster RA, Kuypers MMM, Vagner T, Paerl RW, Musat N, Zehr JP. Nitrogen fixation and transfer in open ocean diatom-cyanobacterial symbioses. ISME J. 2011;5(9):1484-93.
238. Rivière A, Gagnon M, Weckx S, Roy D, De Vuyst L. Mutual Cross-Feeding Interactions between Bifidobacterium longum subsp. longum NCC2705 and Eubacterium rectale ATCC 33656 Explain the Bifidogenic and Butyrogenic Effects of Arabinoxylan Oligosaccharides. Applied and Environmental Microbiology. 2015;81(22):7767-81.
239. Deveau A, Brulé C, Palin B, Champmartin D, Rubini P, Garbaye J, et al. Role of fungal trehalose and bacterial thiamine in the improved survival and growth of the ectomycorrhizal fungus Laccaria bicolor S238N and the helper bacterium Pseudomonas fluorescens BBc6R8. Environmental Microbiology Reports. 2010;2(4):560-8.
240. Gobbetti M, Corsetti A, Rossi J. The sourdough microflora. Interactions between lactic acid bacteria and yeasts: metabolism of carbohydrates. Appl Microbiol Biotechnol. 1994;41(4):456-60.
241. Ramsey MM, Rumbaugh KP, Whiteley M. Metabolite Cross-Feeding Enhances Virulence in a Model Polymicrobial Infection. PLoS Pathog. 2011;7(3):e1002012.
242. Engel P, Moran NA. The gut microbiota of insects – diversity in structure and function. Fems Microbiol Rev. 2013;37(5):699-735.
243. Kaiser C, Kilburn MR, Clode PL, Fuchslueger L, Koranda M, Cliff JB, et al. Exploring the transfer of recent plant photosynthates to soil microbes: mycorrhizal pathway vs direct root exudation. New Phytologist. 2015;205(4):1537-51.
244. Chapman RL. Algae: the world's most important "plants"—an introduction. Mitigation and Adaptation Strategies for Global Change. 2013;18(1):5-12.
245. Bach MK, Magee WE, Burris RH. Translocation of Photosynthetic Products to Soybean Nodules and Their Role in Nitrogen Fixation. Plant Physiology. 1958;33(2):118-24.
246. Croft MT, Lawrence AD, Raux-Deery E, Warren MJ, Smith AG. Algae acquire vitamin B12 through a symbiotic relationship with bacteria. Nature. 2005;438(7064):90-3.
247. Amin SA, Green DH, Hart MC, Küpper FC, Sunda WG, Carrano CJ. Photolysis of iron–siderophore chelates promotes bacterial–algal mutualism. Proc Natl Acad Sci U S A. 2009;106(40):17071-6.
248. Croft MT, Warren MJ, Smith AG. Algae Need Their Vitamins. Eukaryotic Cell. 2006;5(8):1175-83.
249. Boone DR, Johnson RL, Liu Y. Diffusion of the interspecies electron carriers H2 and formate in methanogenic ecosystems and its implications in the measurement of Km for H2 or formate uptake. Applied and Environmental Microbiology. 1989;55(7):1735-41.
250. Schink B, Stams AJM. Syntrophism Among Prokaryotes. In: Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F, editors. The Prokaryotes: Prokaryotic Communities and Ecophysiology. Berlin, Heidelberg: Springer Berlin Heidelberg; 2013. p. 471-93.
251. Krause SMB, Johnson T, Samadhi Karunaratne Y, Fu Y, Beck DAC, Chistoserdova L, et al. Lanthanide-dependent cross-feeding of methane-derived carbon is linked by microbial community interactions. Proceedings of the National Academy of Sciences. 2017;114(2):358-63.
252. Zhang Y, Rodionov DA, Gelfand MS, Gladyshev VN. Comparative genomic analyses of nickel, cobalt and vitamin B12 utilization. BMC Genomics. 2009;10(1):78.
253. Degnan PH, Barry NA, Mok KC, Taga ME, Goodman AL. Human gut microbes use multiple transporters to distinguish vitamin B 12 analogs and compete in the gut. Cell host & microbe. 2014;15(1):47-57.
254. Pérez-Cruz C, Carrión O, Delgado L, Martinez G, López-Iglesias C, Mercade E. New Type of Outer Membrane Vesicle Produced by the Gram-Negative Bacterium Shewanella vesiculosa M7T: Implications for DNA Content. Applied and Environmental Microbiology. 2013;79(6):1874-81.
255. Pérez-Cruz C, Delgado L, López-Iglesias C, Mercade E. Outer-inner membrane vesicles naturally secreted by gram-negative pathogenic bacteria. PLoS One. 2015;10(1):e0116896.
256. Xavier KB, Bassler BL. Interference with AI-2-mediated bacterial cell–cell communication. Nature. 2005;437(7059):750-3.
257. Uroz S, Chhabra SR, Camara M, Williams P, Oger P, Dessaux Y. N-Acylhomoserine lactone quorum-sensing molecules are modified and degraded by Rhodococcus erythropolis W2 by both amidolytic and novel oxidoreductase activities. Microbiology-(UK). 2005;151(10):3313-22.

258. Wang B-Y, Kuramitsu HK. Interactions between oral bacteria: inhibition of Streptococcus mutans bacteriocin production by Streptococcus gordonii. Applied and environmental microbiology. 2005;71(1):354-62.
259. Mashburn LM, Whiteley M. Membrane vesicles traffic signals and facilitate group activities in a prokaryote. Nature. 2005;437:422.
260. Nikaido H. Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev. 2003;67(4):593-656.
261. Ren Z, Ward TE, Regan JM. Electricity Production from Cellulose in a Microbial Fuel Cell Using a Defined Binary Culture. Environmental Science & Technology. 2007;41(13):4781-6.
262. Zhou J, Ma Q, Yi H, Wang L, Song H, Yuan Y-J. Metabolome Profiling Reveals Metabolic Cooperation between Bacillus megaterium and Ketogulonicigenium vulgare during Induced Swarm Motility. Applied and Environmental Microbiology. 2011;77(19):7023-30.
263. Krämer R. Secretion of amino acids by bacteria: physiology and mechanism. Fems Microbiol Rev. 1994;13(1):75-93.
264. Crosa JH, Walsh CT. Genetics and assembly line enzymology of siderophore biosynthesis in bacteria. Microbiology and molecular biology reviews. 2002;66(2):223-49.
265. Furrer JL, Sanders DN, Hook-Barnard IG, McIntosh MA. Export of the siderophore enterobactin in Escherichia coli: involvement of a 43 kDa membrane exporter. Mol Microbiol. 2002;44(5):1225-34.
266. Arnosti C. Microbial extracellular enzymes and the marine carbon cycle. Annual review of marine science. 2011;3:401-25.
267. Flemming H-C, Wingender J. The biofilm matrix. Nature Reviews Microbiology. 2010;8(9):623-33.
268. Wingender J, Neu TR, Flemming H-C. What are bacterial extracellular polymeric substances? Microbial extracellular polymeric substances: Springer; 1999. p. 1-19.
269. Rees DC, Johnson E, Lewinson O. ABC transporters: the power to change. Nature reviews Molecular cell biology. 2009;10(3):218-27.
270. Postma P, Lengeler J, Jacobson G. Phosphoenolpyruvate: carbohydrate phosphotransferase systems of bacteria. Microbiological reviews. 1993;57(3):543-94.
271. Nasarabadi A, Berleman JE, Auer M. Outer Membrane Vesicles of Bacteria: Structure, Biogenesis, and Function. Biogenesis of Fatty Acids, Lipids and Membranes. 2017:1-15.
272. Kulp A, Kuehn MJ. Biological functions and biogenesis of secreted bacterial outer membrane vesicles. Annual review of microbiology. 2010;64:163-84.
273. Beveridge TJ. Structures of gram-negative cell walls and their derived membrane vesicles. Journal of bacteriology. 1999;181(16):4725-33.
274. Fiocca R, Necchi V, Sommi P, Ricci V, Telford J, Cover TL, et al. Release of Helicobacter pylori vacuolating cytotoxin by both a specific secretion pathway and budding of outer membrane vesicles. Uptake of released toxin and vesicles by gastric epithelium. The Journal of pathology. 1999;188(2):220-6.
275. Kato S, Kowashi Y, Demuth DR. Outer membrane-like vesicles secreted by Actinobacillus actinomycetemcomitans are enriched in leukotoxin. Microbial pathogenesis. 2002;32(1):1-13.
276. Wai SN, Lindmark B, Söderblom T, Takade A, Westermark M, Oscarsson J, et al. Vesicle-mediated export and assembly of pore-forming oligomers of the enterobacterial ClyA cytotoxin. Cell. 2003;115(1):25-35.
277. Yokoyama K, Horii T, Yamashino T, Hashikawa S, Barua S, Hasegawa T, et al. Production of Shiga toxin by Escherichia coli measured with reference to the membrane vesicle-associated toxins. FEMS microbiology letters. 2000;192(1):139-44.
278. Patrick S, McKenna JP, Seamus O, Dermott E. A comparison of the haemagglutinating and enzymic activities ofBacteroides fragiliswhole cells and outer membrane vesicles. Microbial pathogenesis. 1996;20(4):191-202.
279. Kadurugamuwa JL, Beveridge TJ. Virulence factors are released from Pseudomonas aeruginosa in association with membrane vesicles during normal growth and exposure to gentamicin: a novel mechanism of enzyme secretion. Journal of Bacteriology. 1995;177(14):3998-4008.
280. Dorward DW, Garon CF. DNA is packaged within membrane-derived vesicles of gram-negative but not gram-positive bacteria. Applied and environmental microbiology. 1990;56(6):1960-2.
281. Mashburn-Warren L, Mclean R, Whiteley M. Gram-negative outer membrane vesicles: beyond the cell surface. Geobiology. 2008;6(3):214-9.
282. Remis JP, Costerton JW, Auer M. Biofilms: structures that may facilitate cell–cell interactions. The ISME journal. 2010;4(9):1085-7.

283. Yonezawa H, Osaki T, Kurata S, Fukuda M, Kawakami H, Ochiai K, et al. Outer membrane vesicles of Helicobacter pylori TK1402 are involved in biofilm formation. BMC microbiology. 2009;9(1):197.
284. Berleman JE, Allen S, Danielewicz MA, Remis JP, Gorur A, Cunha J, et al. The lethal cargo of Myxococcus xanthus outer membrane vesicles. Frontiers in microbiology. 2014;5.
285. Hardin G. The Tragedy of the Commons∗. Journal of Natural Resources Policy Research. 2009;1(3):243-53.
286. Goelzer A, Fromion V. Bacterial growth rate reflects a bottleneck in resource allocation. Biochimica et Biophysica Acta (BBA)-General Subjects. 2011;1810(10):978-88.
287. Varma A, Palsson BO. Stoichiometric flux balance models quantitatively predict growth and metabolic by-product secretion in wild-type Escherichia coli W3110. Applied and environmental microbiology. 1994;60(10):3724-31.
288. Tasoff J, Mee MT, Wang HH. An economic framework of microbial trade. PLoS One. 2015;10(7):e0132907.
289. Hammerstein P, Noë R. Biological trade and markets. Phil Trans R Soc B. 2016;371(1687):20150101.
290. Ricardo D. The works and correspondence of David Ricardo Vol. 1: On the principles of political economy and taxation1817.
291. Johnson DR, Goldschmidt F, Lilja EE, Ackermann M. Metabolic specialization and the assembly of microbial communities. Isme j. 2012;6(11):1985-91.
292. Ferenci T. Trade-off mechanisms shaping the diversity of bacteria. Trends in microbiology. 2016;24(3):209-23.
293. Lilja EE, Johnson DR. Segregating metabolic processes into different microbial cells accelerates the consumption of inhibitory substrates. The ISME journal. 2016;10(7):1568-78.
294. Werner GDA, Strassmann JE, Ivens ABF, Engelmoer DJP, Verbruggen E, Queller DC, et al. Evolution of microbial markets. Proceedings of the National Academy of Sciences. 2014;111(4):1237-44.
295. Kaleta C, Schäuble S, Rinas U, Schuster S. Metabolic costs of amino acid and protein production in Escherichia coli. Biotechnology Journal. 2013:n/a-n/a.
296. Waschina S, D'souza G, Kost C, Kaleta C. Metabolic network architecture and carbon source determine metabolite production costs. The FEBS journal. 2016;283(11):2149-63.
297. Akashi H, Gojobori T. Metabolic efficiency and amino acid composition in the proteomes of Escherichia coli and Bacillus subtilis. Proceedings of the National Academy of Sciences. 2002;99(6):3695-700.
298. Heizer Jr EM, Raiford DW, Raymer ML, Doom TE, Miller RV, Krane DE. Amino acid cost and codon-usage biases in 6 prokaryotic genomes: a whole-genome analysis. Molecular biology and evolution. 2006;23(9):1670-80.
299. Hu P, Dubinsky EA, Probst AJ, Wang J, Sieber CM, Tom LM, et al. Simulation of Deepwater Horizon oil plume reveals substrate specialization within a complex community of hydrocarbon degraders. Proceedings of the National Academy of Sciences. 2017;114(28):7432-7.
300. Zhao J, Shimizu K. Metabolic flux analysis of Escherichia coli K12 grown on 13 C-labeled acetate and glucose using GC-MS and powerful flux calculation method. Journal of biotechnology. 2003;101(2):101-17.
301. Sabarly V, Aubron C, Glodt J, Balliau T, Langella O, Chevret D, et al. Interactions between genotype and environment drive the metabolic phenotype within Escherichia coli isolates. Environ Microbiol. 2016;18(1):100-17.
302. Welch JLM, Rossetti BJ, Rieken CW, Dewhirst FE, Borisy GG. Biogeography of a human oral microbiome at the micron scale. Proceedings of the National Academy of Sciences. 2016;113(6):E791-E800.
303. Flemming H-C, Wingender J, Szewzyk U, Steinberg P, Rice SA, Kjelleberg S. Biofilms: an emergent form of bacterial life. Nat Rev Micro. 2016;14(9):563-75.
304. Stewart PS, Franklin MJ. Physiological heterogeneity in biofilms. Nature Reviews Microbiology. 2008;6(3):199-210.
305. Ackermann M. A functional perspective on phenotypic heterogeneity in microorganisms. Nature Reviews Microbiology. 2015;13(8):497-508.
306. Healey D, Axelrod K, Gore J. Negative frequency-dependent interactions can underlie phenotypic heterogeneity in a clonal microbial population. Mol Syst Biol. 2016;12(8):877.
307. Kumar K, Mella-Herrera RA, Golden JW. Cyanobacterial heterocysts. Cold Spring Harbor Perspectives in Biology. 2010;2(4):a000315.

308. Rosenthal AZ, Qi Y, Hormoz S, Park J, Li SH-J, Elowitz M. Metabolic Interactions Between Dynamic Bacterial Subpopulations. bioRxiv. 2017:208686.
309. Wortel MT, Noor E, Ferris M, Bruggeman FJ, Liebermeister W. Profiling metabolic flux modes by enzyme cost reveals variable trade-offs between growth and yield in Escherichia coli. bioRxiv. 2017:111161.
310. Carroll SM, Lee M-C, Marx CJ. SIGN EPISTASIS LIMITS EVOLUTIONARY TRADE-OFFS AT THE CONFLUENCE OF SINGLE- AND MULTI-CARBON METABOLISM IN METHYLOBACTERIUM EXTORQUENS AM1. Evolution. 2014;68(3):760-71.
311. Morris JJ. Black Queen evolution: the role of leakiness in structuring microbial communities. Trends in Genetics. 2015;31(8):475-82.
312. Kallus Y, Miller JH, Libby E. Paradoxes in leaky microbial trade. Nature Communications. 2017;8(1):1361.
313. Wernegreen JJ. Genome evolution in bacterial endosymbionts of insects. Nat Rev Genet. 2002;3(11):850-61.
314. Thomas GH, Zucker J, Macdonald SJ, Sorokin A, Goryanin I, Douglas AE. A fragile metabolic network adapted for cooperation in the symbiotic bacterium Buchnera aphidicola. BMC systems biology. 2009;3:24.
315. Moran NA, McCutcheon JP, Nakabachi A. Genomics and evolution of heritable bacterial symbionts. Annual Review of Genetics. Annual Review of Genetics. 422008. p. 165-90.
316. McCutcheon JP, Moran NA. Extreme genome reduction in symbiotic bacteria. Nature Reviews Microbiology. 2012;10(1):13-26.
317. McCutcheon JP, Moran NA. Functional convergence in reduced genomes of bacterial symbionts spanning 200 My of evolution. Genome Biology and Evolution. 2010;2:708-18.
318. McCutcheon JP, Moran NA. Parallel genomic evolution and metabolic interdependence in an ancient symbiosis. Proc Natl Acad Sci U S A. 2007;104(49):19392-7.
319. Mira A, Klasson L, Andersson SG. Microbial genome evolution: sources of variability. Current opinion in microbiology. 2002;5(5):506-12.
320. Han K, Li Z-f, Peng R, Zhu L-p, Zhou T, Wang L-g, et al. Extraordinary expansion of a Sorangium cellulosum genome from an alkaline milieu. Sci Rep. 2013;3.
321. Nakabachi A, Yamashita A, Toh H, Ishikawa H, Dunbar HE, Moran NA, et al. The 160-kilobase genome of the bacterial endosymbiont Carsonella. Science. 2006;314(5797):267-.
322. Puigbò P, Lobkovsky A, Kristensen D, Wolf Y, Koonin E. Genomes in turmoil: quantification of genome dynamics in prokaryote supergenomes. BMC Biology. 2014;12(1):1-19.
323. Gordienko EN, Kazanov MD, Gelfand MS. Evolution of Pan-Genomes of Escherichia coli, Shigella spp., and Salmonella enterica. Journal of Bacteriology. 2013;195(12):2786-92.
324. Tettelin H, Riley D, Cattuto C, Medini D. Comparative genomics: the bacterial pan-genome. Current opinion in microbiology. 2008;11(5):472-7.
325. Medini D, Donati C, Tettelin H, Masignani V, Rappuoli R. The microbial pan-genome. Current opinion in genetics & development. 2005;15(6):589-94.
326. Biller SJ, Berube PM, Lindell D, Chisholm SW. Prochlorococcus: the structure and function of collective diversity. Nat Rev Microbiol. 2015;13(1):13-27.
327. Koskiniemi S, Sun S, Berg OG, Andersson DI. Selection-driven gene loss in bacteria. PLoS Genet. 2012;8(6).
328. Shachrai I, Zaslaver A, Alon U, Dekel E. Cost of unneeded proteins in E. coli is reduced after several generations in exponential growth. Molecular cell. 2010;38(5):758-67.
329. Dekel E, Alon U. Optimality and evolutionary tuning of the expression level of a protein. Nature. 2005;436(7050):588-92.
330. Dufresne A, Garczarek L, Partensky F. Accelerated evolution associated with genome reduction in a free-living prokaryote. Genome Biology. 2005;6(2).
331. Mira A, Ochman H, Moran NA. Deletional bias and the evolution of bacterial genomes. Trends in Genetics. 2001;17(10):589-96.
332. Martínez-Cano DJ, Reyes-Prieto M, Martínez-Romero E, Partida-Martínez LP, Latorre A, Moya A, et al. Evolution of small prokaryotic genomes. Frontiers in Microbiology. 2014;5:742.
333. Swan BK, Tupper B, Sczyrba A, Lauro FM, Martinez-Garcia M, Gonzalez JM, et al. Prevalent genome streamlining and latitudinal divergence of planktonic bacteria in the surface ocean. Proc Natl Acad Sci U S A. 2013;110(28):11463-8.
334. Batut B, Knibbe C, Marais G, Daubin V. Reductive genome evolution at both ends of the bacterial population size spectrum. Nat Rev Microbiol. 2014;12(12):841-50.

335. Grote J, Thrash JC, Huggett MJ, Landry ZC, Carini P, Giovannoni SJ, et al. Streamlining and Core Genome Conservation among Highly Divergent Members of the SAR11 Clade. Mbio. 2012;3(5).
336. Bristow LA, Mohr W, Ahmerkamp S, Kuypers MM. Nutrients that limit growth in the ocean. Current Biology. 2017;27(11):R474-R8.
337. Zamenhof S, Eichhorn HH. Study of microbial evolution through loss of biosynthetic functions- Establishment of defective mutants. Nature. 1967;216(5114):456-8

338. Kim W, Levy SB. Increased fitness of Pseudomonas fluorescens Pf0-1 leucine auxotrophs in soil. Applied and environmental microbiology. 2008;74(12):3644-51.
339. Wessely F, Bartl M, Guthke R, Li P, Schuster S, Kaleta C. Optimal regulatory strategies for metabolic pathways in *Escherichia coli* depending on protein costs. Mol Syst Biol. 2011;7.
340. Lochhead AG. Soil bacteria and growth-promoting substances. Bacteriological Reviews. 1958;22(3):145-53.
341. Moura A, Savageau MA, Alves R. Relative amino acid composition signatures of organisms and environments. PLoS One. 2013;8(10):e77319.
342. Schmidt E, STARKEY RL. SOIL MICROORGANISMS AND PLANT GROWTH SUBSTANCES: II. TRANSFORMATIONS OF CERTAIN B-VITAMINS IN SOIL. Soil Science. 1951;71(3):221-32.
343. Levy-Booth DJ, Campbell RG, Gulden RH, Hart MM, Powell JR, Klironomos JN, et al. Cycling of extracellular DNA in the soil environment. Soil Biology and Biochemistry. 2007;39(12):2977-91.
344. Lee MC, Marx CJ. Repeated, selection-driven genome reduction of accessory genes in experimental populations. PLoS Genet. 2012;8(5):e1002651.
345. Paul BJ, Berkmen MB, Gourse RL. DksA potentiates direct activation of amino acid promoters by ppGpp. Proceedings of the National Academy of Sciences. 2005;102(22):7823-8.
346. Santos V, Hirshfield I. The Physiological and Molecular Characterization of a Small Colony Variant of Escherichia coli and Its Phenotypic Rescue. PLoS One. 2016;11(6):e0157578.
347. Martínez-Cano DJ, Reyes-Prieto M, Martinez-Romero E, Partida-Martinez LP, Latorre A, Moya A, et al. Evolution of small prokaryotic genomes. Frontiers in Microbiology. 2015;5.
348. Moran NA. Accelerated evolution and Muller's rachet in endosymbiotic bacteria. Proceedings of the National Academy of Sciences. 1996;93(7):2873-8.
349. Wernegreen JJ. Endosymbiont evolution: predictions from theory and surprises from genomes. Ann NY Acad Sci. 2015:n/a-n/a.
350. Gillespie JH. Population genetics: a concise guide: JHU Press; 2010.
351. Kuo C-H, Ochman H. Deletional Bias across the Three Domains of Life. Genome Biology and Evolution. 2009;1:145-52.
352. Nilsson AI, Koskiniemi S, Eriksson S, Kugelberg E, Hinton JCD, Andersson DI. Bacterial genome size reduction by experimental evolution. Proc Natl Acad Sci U S A. 2005;102(34):12112-6.
353. Łukasik P, Nazario K, Van Leuven JT, Campbell MA, Meyer M, Michalik A, et al. Multiple origins of interdependent endosymbiotic complexes in a genus of cicadas. Proceedings of the National Academy of Sciences. 2017.
354. Traxler MF, Summers SM, Nguyen HT, Zacharia VM, Hightower GA, Smith JT, et al. The global, ppGpp-mediated stringent response to amino acid starvation in Escherichia coli. Mol Microbiol. 2008;68(5):1128-48.
355. Aanen DK, Henrik H, Debets AJ, Kerstes NA, Hoekstra RF, Boomsma JJ. High symbiont relatedness stabilizes mutualistic cooperation in fungus-growing termites. Science. 2009;326(5956):1103-6.
356. Connor RC. Pseudo-reciprocity: Investing in mutualism. Animal Behaviour. 1986;34(5):1562-6.
357. Aoki SK, Diner EJ, de Roodenbeke CT, Burgess BR, Poole SJ, Braaten BA, et al. A widespread family of polymorphic contact-dependent toxin delivery systems in bacteria. Nature. 2010;468(7322):439-42.
358. Garcia EC, Anderson MS, Hagar JA, Cotter PA. Burkholderia BcpA mediates biofilm formation independently of interbacterial contact-dependent growth inhibition. Mol Microbiol. 2013;89(6):1213-25.
359. Anderson MS, Garcia EC, Cotter PA. Kind Discrimination and Competitive Exclusion Mediated by Contact-Dependent Growth Inhibition Systems Shape Biofilm Community Structure. PLoS Pathog. 2014;10(4):14.

360. Zobell CE. The Effect of Solid Surfaces upon Bacterial Activity. Journal of Bacteriology. 1943;46(1):39-56.
361. Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: from the Natural environment to infectious diseases. Nat Rev Micro. 2004;2(2):95-108.
362. Mori JF, Ueberschaar N, Lu S, Cooper RE, Pohnert G, Kusel K. Sticking together: inter-species aggregation of bacteria isolated from iron snow is controlled by chemical signaling. ISME J. 2017;11(5):1075-86.
363. Rainey PB, De Monte S. Resolving Conflicts During the Evolutionary Transition to Multicellular Life. In: Futuyma DJ, editor. Annual Review of Ecology, Evolution, and Systematics, Vol 45. Annual Review of Ecology Evolution and Systematics. 452014. p. 599-620.
364. Liébana R, Arregui L, Santos A, Murciano A, Marquina D, Serrano S. Unravelling the interactions among microbial populations found in activated sludge during biofilm formation. FEMS Microbiology Ecology. 2016;92(9):fiw134-fiw.
365. Datta MS, Korolev KS, Cvijovic I, Dudley C, Gore J. Range expansion promotes cooperation in an experimental microbial metapopulation. Proc Natl Acad Sci U S A. 2013;110(18):7354-9.
366. Estrela S, Brown SP. Metabolic and Demographic Feedbacks Shape the Emergent Spatial Structure and Function of Microbial Communities. PLOS Computational Biology. 2013;9(12):e1003398.
367. van Gestel J, Weissing FJ, Kuipers OP, Kovacs AT. Density of founder cells affects spatial pattern formation and cooperation in Bacillus subtilis biofilms. ISME J. 2014;8(10):2069-79.
368. Tecon R, Or D. Cooperation in carbon source degradation shapes spatial self-organization of microbial consortia on hydrated surfaces. 2017;7:43726.
369. Nowak MA, Bonhoeffer S, May RM. Spatial games and the maintenance of cooperation. Proc Natl Acad Sci U S A. 1994;91(11):4877-81.
370. Burmølle M, Webb JS, Rao D, Hansen LH, Sørensen SJ, Kjelleberg S. Enhanced Biofilm Formation and Increased Resistance to Antimicrobial Agents and Bacterial Invasion Are Caused by Synergistic Interactions in Multispecies Biofilms. Applied and Environmental Microbiology. 2006;72(6):3916-23.
371. Strassmann JE, Queller DC. Fine-scale spatial ecology drives kin selection relatedness among cooperating amoebae. Evolution. 2016;70(4):848-59.
372. Wahl LM, Associate Editor: Michael PM, x, Gibbons. The Division of Labor: Genotypic versus Phenotypic Specialization. The American Naturalist. 2002;160(1):135-45.
373. Pernthaler A, Dekas AE, Brown CT, Goffredi SK, Embaye T, Orphan VJ. Diverse syntrophic partnerships from deep-sea methane vents revealed by direct cell capture and metagenomics. Proceedings of the National Academy of Sciences. 2008;105(19):7052-7.
374. Haroon MF, Hu S, Shi Y, Imelfort M, Keller J, Hugenholtz P, et al. Anaerobic oxidation of methane coupled to nitrate reduction in a novel archaeal lineage. Nature. 2013;500(7464):567-70.
375. Kovács ÁT. Impact of spatial distribution on the development of mutualism in microbes. Frontiers in Microbiology. 2014;5(649).
376. Norlund KLI, Southam G, Tyliszczak T, Hu Y, Karunakaran C, Obst M, et al. Microbial Architecture of Environmental Sulfur Processes: A Novel Syntrophic Sulfur-Metabolizing Consortia. Environmental Science & Technology. 2009;43(23):8781-6.
377. Jahn U, Gallenberger M, Paper W, Junglas B, Eisenreich W, Stetter KO, et al. Nanoarchaeum equitans and Ignicoccus hospitalis: New Insights into a Unique, Intimate Association of Two Archaea. Journal of Bacteriology. 2008;190(5):1743-50.
378. He XS, McLean JS, Edlund A, Yooseph S, Hall AP, Liu SY, et al. Cultivation of a human-associated TM7 phylotype reveals a reduced genome and epibiotic parasitic lifestyle. Proc Natl Acad Sci U S A. 2015;112(1):244-9.
379. Husnik F, McCutcheon JP. Repeated replacement of an intrabacterial symbiont in the tripartite nested mealybug symbiosis. Proceedings of the National Academy of Sciences. 2016;113(37):E5416-E24.
380. Overmann J. Phototrophic Consortia: A Tight Cooperation Between Non-Related Eubacteria. In: Seckbach J, editor. Symbiosis: Mechanisms and Model Systems. Dordrecht: Springer Netherlands; 2002. p. 239-55.
381. Xia Y, Kong Y, Thomsen TR, Halkjær Nielsen P. Identification and Ecophysiological Characterization of Epiphytic Protein-Hydrolyzing Saprospiraceae ("Candidatus Epiflobacter" spp.) in Activated Sludge. Applied and Environmental Microbiology. 2008;74(7):2229-38.

382. Kobayashi DY, Crouch JA. Bacterial/Fungal Interactions: From Pathogens to Mutualistic Endosymbionts. Annual Review of Phytopathology. 2009;47(1):63-82.
383. Honegger R. 15 The Symbiotic Phenotype of Lichen-Forming Ascomycetes and Their Endo- and Epibionts. In: Hock B, editor. Fungal Associations. Berlin, Heidelberg: Springer Berlin Heidelberg; 2012. p. 287-339.
384. Partida-Martinez LP, Monajembashi S, Greulich K-O, Hertweck C. Endosymbiont-Dependent Host Reproduction Maintains Bacterial-Fungal Mutualism. Current Biology. 2007;17(9):773-7.
385. Li Z, Yao Q, Dearth SP, Entler MR, Castro Gonzalez HF, Uehling JK, et al. Integrated proteomics and metabolomics suggests symbiotic metabolism and multimodal regulation in a fungal-endobacterial system. Environ Microbiol. 2017;19(3):1041-53.
386. Guo H, Glaeser SP, Alabid I, Imani J, Haghighi H, Kämpfer P, et al. The Abundance of Endofungal Bacterium Rhizobium radiobacter (syn. Agrobacterium tumefaciens) Increases in Its Fungal Host Piriformospora indica during the Tripartite Sebacinalean Symbiosis with Higher Plants. Frontiers in Microbiology. 2017;8(629).
387. Edgcomb VP, Breglia SA, Yubuki N, Beaudoin D, Patterson DJ, Leander BS, et al. Identity of epibiotic bacteria on symbiontid euglenozoans in O2-depleted marine sediments: evidence for symbiont and host co-evolution. ISME J. 2011;5(2):231-43.
388. Epstein SS, Bazylinski DA, Fowle WH. Epibiotic Bacteria on Several Ciliates from Marine Sediments. Journal of Eukaryotic Microbiology. 1998;45(1):64-70.
389. Bernhard JM, Buck KR, Farmer MA, Bowser SS. The Santa Barbara Basin is a symbiosis oasis. Nature. 2000;403(6765):77-80.
390. Nowack ECM, Melkonian M. Endosymbiotic associations within protists. Philosophical Transactions of the Royal Society B: Biological Sciences. 2010;365(1541):699-712.
391. Ramanan R, Kim B-H, Cho D-H, Oh H-M, Kim H-S. Algae–bacteria interactions: Evolution, ecology and emerging applications. Biotechnology Advances. 2016;34(1):14-29.
392. Cai L, Zhou G, Tian R-M, Tong H, Zhang W, Sun J, et al. Metagenomic analysis reveals a green sulfur bacterium as a potential coral symbiont. Sci Rep. 2017;7(1):9320.
393. Moran NA, McCutcheon JP, Nakabachi A. Genomics and Evolution of Heritable Bacterial Symbionts. Annual Review of Genetics. 2008;42(1):165-90.
394. Sachs JL, Skophammer RG, Regus JU. Evolutionary transitions in bacterial symbiosis. Proceedings of the National Academy of Sciences. 2011;108(Supplement 2):10800-7.
395. Sachs JL, Skophammer RG, Bansal N, Stajich JE. Evolutionary origins and diversification of proteobacterial mutualists. Proc R Soc B-Biol Sci. 2014;281(1775).
396. Toft C, Andersson SGE. Evolutionary microbial genomics: insights into bacterial host adaptation. Nat Rev Genet. 2010;11(7):465-75.
397. Zientz E, Dandekar T, Gross R. Metabolic Interdependence of Obligate Intracellular Bacteria and Their Insect Hosts. Microbiology and Molecular Biology Reviews. 2004;68(4):745-70.
398. Liu Z, Müller J, Li T, Alvey RM, Vogl K, Frigaard N-U, et al. Genomic analysis reveals key aspects of prokaryotic symbiosis in the phototrophic consortium “Chlorochromatium aggregatum”. Genome Biology. 2013;14(11):R127.
399. Wanner G, Vogl K, Overmann J. Ultrastructural characterization of the prokaryotic symbiosis in "Chlorochromatium aggregatum". Journal of Bacteriology. 2008;190(10):3721-30.
400. Zohar B-A, Kolodkin-Gal I. Quorum Sensing in Escherichia coli: Interkingdom, Inter- and Intraspecies Dialogues, and a Suicide-Inducing Peptide. In: Kalia VC, editor. Quorum Sensing vs Quorum Quenching: A Battle with No End in Sight. New Delhi: Springer India; 2015. p. 85-99.
401. Clinton A, Rumbaugh KP. Interspecies and Interkingdom Signaling via Quorum Signals. Israel Journal of Chemistry. 2016;56(5):265-72.
402. Levy R, Borenstein E. Metabolic modeling of species interaction in the human microbiome elucidates community-level assembly rules. Proc Natl Acad Sci U S A. 2013;110(31):12804-9.
403. Bernhardsson S, Gerlee P, Lizana L. Structural correlations in bacterial metabolic networks. BMC Evolutionary Biology. 2011;11(1):20.
404. de Weert S, Vermeiren H, Mulders IHM, Kuiper I, Hendrickx N, Bloemberg GV, et al. Flagella-Driven Chemotaxis Towards Exudate Components Is an Important Trait for Tomato Root Colonization by Pseudomonas fluorescens. Molecular Plant-Microbe Interactions. 2002;15(11):1173-80.
405. Wadhams GH, Armitage JP. Making sense of it all: bacterial chemotaxis. Nat Rev Mol Cell Biol. 2004;5(12):1024-37.

406. Bay G, Nahar N, Oubre M, Whitehouse MJ, Wardle DA, Zackrisson O, et al. Boreal feather mosses secrete chemical signals to gain nitrogen. New Phytologist. 2013;200(1):54-60.
407. Tout J, Jeffries TC, Petrou K, Tyson GW, Webster NS, Garren M, et al. Chemotaxis by natural populations of coral reef bacteria. ISME J. 2015;9(8):1764-77.
408. Miller TR, Hnilicka K, Dziedzic A, Desplats P, Belas R. Chemotaxis of Silicibacter sp. Strain TM1040 toward Dinoflagellate Products. Applied and Environmental Microbiology. 2004;70(8):4692-701.
409. Sieg RD, Poulson-Ellestad KL, Kubanek J. Chemical ecology of the marine plankton. Natural Product Reports. 2011;28(2):388-99.
410. Laganenka L, Colin R, Sourjik V. Chemotaxis towards autoinducer 2 mediates autoaggregation in Escherichia coli. Nature Communications. 2016;7:12984.
411. Sourjik V, Berg HC. Receptor sensitivity in bacterial chemotaxis. Proceedings of the National Academy of Sciences. 2002;99(1):123-7.
412. Müller J, Kuttler C, Hense BA. Sensitivity of the quorum sensing system is achieved by low pass filtering. Biosystems. 2008;92(1):76-81.
413. Rickard AH, Gilbert P, High NJ, Kolenbrander PE, Handley PS. Bacterial coaggregation: an integral process in the development of multi-species biofilms. Trends in Microbiology. 2003;11(2):94-100.
414. Katharios-Lanwermeyer S, Xi C, Jakubovics NS, Rickard AH. Mini-review: Microbial coaggregation: ubiquity and implications for biofilm development. Biofouling. 2014;30(10):1235-51.
415. Julou T, Mora T, Guillon L, Croquette V, Schalk IJ, Bensimon D, et al. Cell–cell contacts confine public goods diffusion inside Pseudomonas aeruginosa clonal microcolonies. Proceedings of the National Academy of Sciences. 2013;110(31):12577-82.
416. Smukalla S, Caldara M, Pochet N, Beauvais A, Guadagnini S, Yan C, et al. FLO1 Is a Variable Green Beard Gene that Drives Biofilm-like Cooperation in Budding Yeast. Cell. 2008;135(4):726-37.
417. Kouzuma A, Kato S, Watanabe K. Microbial interspecies interactions: recent findings in syntrophic consortia. Frontiers in Microbiology. 2015;6(477).
418. Wall D. Kin Recognition in Bacteria. In: Gottesman S, editor. Annual Review of Microbiology, Vol 70. Annual Review of Microbiology. 702016. p. 143-60.
419. Vengadesan K, Narayana SVL. Structural biology of gram-positive bacterial adhesins. Protein Science. 2011;20(5):759-72.
420. Dautin N, Bernstein HD. Protein Secretion in Gram-Negative Bacteria via the Autotransporter Pathway. Annual Review of Microbiology. 2007;61(1):89-112.
421. Heras B, Totsika M, Peters KM, Paxman JJ, Gee CL, Jarrott RJ, et al. The antigen 43 structure reveals a molecular Velcro-like mechanism of autotransporter-mediated bacterial clumping. Proceedings of the National Academy of Sciences. 2014;111(1):457-62.
422. Sherlock O, Vejborg RM, Klemm P. The TibA Adhesin/Invasin from Enterotoxigenic Escherichia coli Is Self Recognizing and Induces Bacterial Aggregation and Biofilm Formation. Infection and Immunity. 2005;73(4):1954-63.
423. Liu L, Hao S, Lan R, Wang G, Xiao D, Sun H, et al. The Type VI Secretion System Modulates Flagellar Gene Expression and Secretion in Citrobacter freundii and Contributes to Adhesion and Cytotoxicity to Host Cells. Infection and Immunity. 2015;83(7):2596-604.
424. McLoughlin K, Schluter J, Rakoff-Nahoum S, Smith Adrian L, Foster Kevin R. Host Selection of Microbiota via Differential Adhesion. Cell Host & Microbe. 2016;19(4):550-9.
425. Bogino PC, de las Mercedes Oliva M, Sorroche FG, Giordano W. The Role of Bacterial Biofilms and Surface Components in Plant-Bacterial Associations. International Journal of Molecular Sciences. 2013;14(8):15838-59.
426. Heller J, Zhao J, Rosenfield G, Kowbel DJ, Gladieux P, Glass NL. Characterization of Greenbeard Genes Involved in Long-Distance Kind Discrimination in a Microbial Eukaryote. PLoS Biol. 2016;14(4):e1002431.
427. Danka ES, Garcia EC, Cotter PA. Are CDI Systems Multicolored, Facultative, Helping Greenbeards? Trends in Microbiology. 2017;25(5):391-401.
428. Queller DC, Ponte E, Bozzaro S, Strassmann JE. Single-Gene Greenbeard Effects in the Social Amoeba Dictyostelium discoideum. Science. 2003;299(5603):105-6.
429. Liu X, Ramsey MM, Chen X, Koley D, Whiteley M, Bard AJ. Real-time mapping of a hydrogen peroxide concentration profile across a polymicrobial bacterial biofilm using scanning electrochemical microscopy. Proceedings of the National Academy of Sciences. 2011;108(7):2668-73.

430. Ghoul M, Mitri S. The Ecology and Evolution of Microbial Competition. Trends Microbiol. 2016;24(10):833-45.
431. Pérez-Gutiérrez R-A, López-Ramírez V, Islas Á, Alcaraz LD, Hernández-González I, Olivera BCL, et al. Antagonism influences assembly of a Bacillus guild in a local community and is depicted as a food-chain network. The ISME Journal. 2013;7(3):487-97.
432. Sipiczki M. Metschnikowia Strains Isolated from Botrytized Grapes Antagonize Fungal and Bacterial Growth by Iron Depletion. Applied and Environmental Microbiology. 2006;72(10):6716-24.
433. Lindgren SE, Dobrogosz WJ. Antagonistic activities of lactic acid bacteria in food and feed fermentations. FEMS Microbiology Letters. 1990;87(1-2):149-64.
434. Fons AGTKM. Mechanisms of Colonisation and Colonisation Resistance of the Digestive Tract Part 2: Bacteria/Bacteria Interactions. Microbial Ecology in Health and Disease. 2000;12(2):240-6.
435. Peixoto RS, Rosado PM, Leite DCdA, Rosado AS, Bourne DG. Beneficial Microorganisms for Corals (BMC): Proposed Mechanisms for Coral Health and Resilience. Frontiers in Microbiology. 2017;8(341).
436. Rudrappa T, Biedrzycki ML, Bais HP. Causes and consequences of plant-associated biofilms. FEMS Microbiology Ecology. 2008;64(2):153-66.
437. Buffie CG, Pamer EG. Microbiota-mediated colonization resistance against intestinal pathogens. Nat Rev Immunol. 2013;13(11):790-801.
438. Stecher B, Berry D, Loy A. Colonization resistance and microbial ecophysiology: using gnotobiotic mouse models and single-cell technology to explore the intestinal jungle. Fems Microbiol Rev. 2013;37(5):793-829.
439. He X, McLean JS, Guo L, Lux R, Shi W. The social structure of microbial community involved in colonization resistance. ISME J. 2014;8(3):564-74.
440. Caballero S, Kim S, Carter RA, Leiner IM, Susac B, Miller L, et al. Cooperating Commensals Restore Colonization Resistance to Vancomycin-Resistant Enterococcus faecium. Cell Host & Microbe. 2017;21(5):592-+.
441. Li Z, Clarke AJ, Beveridge TJ. Gram-Negative Bacteria Produce Membrane Vesicles Which Are Capable of Killing Other Bacteria. Journal of Bacteriology. 1998;180(20):5478-83.
442. Russell AB, Peterson SB, Mougous JD. Type VI secretion system effectors: poisons with a purpose. Nature Reviews Microbiology. 2014;12:137.
443. Quadri LEN. Regulation of antimicrobial peptide production by autoinducer-mediated quorum sensing in lactic acid bacteria. Antonie van Leeuwenhoek. 2002;82(1):133-45.
444. Shanker E, Federle MJ. Quorum Sensing Regulation of Competence and Bacteriocins in Streptococcus pneumoniae and mutans. Genes. 2017;8(1):15.
445. van der Ploeg JR. Regulation of Bacteriocin Production in Streptococcus mutans by the Quorum-Sensing System Required for Development of Genetic Competence. Journal of Bacteriology. 2005;187(12):3980-9.
446. Cotter PD, Ross RP, Hill C. Bacteriocins [mdash] a viable alternative to antibiotics? Nat Rev Micro. 2013;11(2):95-105.
447. Bassetti M, Merelli M, Temperoni C, Astilean A. New antibiotics for bad bugs: where are we? Annals of Clinical Microbiology and Antimicrobials. 2013;12(1):22.
448. Zacharof MP, Lovitt RW. Bacteriocins Produced by Lactic Acid Bacteria a Review Article. APCBEE Procedia. 2012;2(Supplement C):50-6.
449. Chao L, Levin BR. Structured habitats and the evolution of anticompetitor toxins in bacteria. Proceedings of the National Academy of Sciences. 1981;78(10):6324-8.
450. Rendueles O, Zee PC, Dinkelacker I, Amherd M, Wielgoss S, Velicer GJ. Rapid and widespread de novo evolution of kin discrimination. Proceedings of the National Academy of Sciences. 2015;112(29):9076-81.
451. Gibbs KA, Greenberg EP. Territoriality in Proteus: Advertisement and Aggression. Chemical Reviews. 2011;111(1):188-94.
452. Vos M, Velicer GJ. Social Conflict in Centimeter-and Global-Scale Populations of the Bacterium Myxococcus xanthus. Current Biology. 2009;19(20):1763-7.
453. Stefanic P, Kraigher B, Lyons NA, Kolter R, Mandic-Mulec I. Kin discrimination between sympatric Bacillus subtilis isolates. Proceedings of the National Academy of Sciences. 2015;112(45):14042-7.
454. Lyons Nicholas A, Kraigher B, Stefanic P, Mandic-Mulec I, Kolter R. A Combinatorial Kin Discrimination System in *Bacillus subtilis*. Current Biology. 2016;26(6):733-42.

455. Borgeaud S, Metzger LC, Scrignari T, Blokesch M. The type VI secretion system of Vibrio cholerae fosters horizontal gene transfer. Science. 2015;347(6217):63-7.
456. McNally L, Bernardy E, Thomas J, Kalziqi A, Pentz J, Brown SP, et al. Killing by Type VI secretion drives genetic phase separation and correlates with increased cooperation. Nature Communications. 2017;8:11.
457. Waite AJ, Shou W. Adaptation to a new environment allows cooperators to purge cheaters stochastically. Proceedings of the National Academy of Sciences. 2012;109(47):19079-86.
458. Xavier JB, Foster KR. Cooperation and conflict in microbial biofilms. Proceedings of the National Academy of Sciences. 2007;104(3):876-81.
459. Gore J, Youk H, van Oudenaarden A. Snowdrift game dynamics and facultative cheating in yeast. Nature. 2009;459(7244):253-6.
460. Sanchez A, Gore J. Feedback between Population and Evolutionary Dynamics Determines the Fate of Social Microbial Populations. PLoS Biol. 2013;11(4):e1001547.
461. Cordero OX, Polz MF. Explaining microbial genomic diversity in light of evolutionary ecology. Nat Rev Micro. 2014;12(4):263-73.
462. Ribeck N, Lenski RE. Modeling and quantifying frequency-dependent fitness in microbial populations with cross-feeding interactions. Evolution. 2015;69(5):1313-20.
463. Greig D, Travisano M. The Prisoner's Dilemma and polymorphism in yeast SUC genes. Proceedings of the Royal Society of London Series B: Biological Sciences. 2004;271(Suppl 3):S25-S6.
464. MacLean RC, Fuentes-Hernandez A, Greig D, Hurst LD, Gudelj I. A Mixture of "Cheats" and "Co-Operators" Can Enable Maximal Group Benefit. PLoS Biol. 2010;8(9):e1000486.
465. Borenstein DB, Meir Y, Shaevitz JW, Wingreen NS. Non-Local Interaction via Diffusible Resource Prevents Coexistence of Cooperators and Cheaters in a Lattice Model. PLoS One. 2013;8(5):e63304.
466. Doebeli M, Ispolatov I. Complexity and Diversity. Science. 2010;328(5977):494-7.
467. Hillesland KL. Evolution on the bright side of life: microorganisms and the evolution of mutualism. Ann N Y Acad Sci. 2018;1422(1):88-103.
468. Law R. Evolution in a mutualistic environment. The biology of mutualism: Ecology and evolution. 1985:145-70.
469. Sachs JL, Essenberg CJ, Turcotte MM. New paradigms for the evolution of beneficial infections. Trends Ecol Evol. 2011;26(4):202-9.
470. Ankrah NY, Luan J, Douglas AE. Cooperative metabolism in a three-partner insect-bacterial symbiosis revealed by metabolic modeling. J Bacteriol. 2017.
471. Embree M, Liu JK, Al-Bassam MM, Zengler K. Networks of energetic and metabolic interactions define dynamics in microbial communities. Proc Natl Acad Sci U S A. 2015;112(50):15450-5.
472. Garcia SL, McMahon KD, Martinez-Garcia M, Srivastava A, Sczyrba A, Stepanauskas R, et al. Metabolic potential of a single cell belonging to one of the most abundant lineages in freshwater bacterioplankton. Isme j. 2013;7(1):137-47.
473. Shigenobu S, Watanabe H, Hattori M, Sakaki Y, Ishikawa H. Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera sp* APS. Nature. 2000;407(6800):81-6.
474. McCutcheon JP, von Dohlen CD. An interdependent metabolic patchwork in the nested symbiosis of mealybugs. Current Biology. 2011;21(16):1366-72.
475. Braakman R, Follows MJ, Chisholm SW. Metabolic evolution and the self-organization of ecosystems. Proceedings of the National Academy of Sciences. 2017;114(15):E3091-E100.
476. Schloss PD, Handelsman J. Metagenomics for studying unculturable microorganisms: cutting the Gordian knot. Genome biology. 2005;6(8):229.
477. Chiu H-C, Levy R, Borenstein E. Emergent biosynthetic capacity in simple microbial communities. 2014.
478. Razumov A. The direct method of calculation of bacteria in
water. Comparison with the koch method. Mikrobiologiya. 1932;1:131–46.
479. Amann RI, Ludwig W, Schleifer KH. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiological Reviews. 1995;59(1):143-69.
480. Hugenholtz P, Goebel BM, Pace NR. Impact of Culture-Independent Studies on the Emerging Phylogenetic View of Bacterial Diversity. Journal of Bacteriology. 1998;180(18):4765-74.
481. Wrighton KC, Thomas BC, Sharon I, Miller CS, Castelle CJ, VerBerkmoes NC, et al. Fermentation, Hydrogen, and Sulfur Metabolism in Multiple Uncultivated Bacterial Phyla. Science. 2012;337(6102):1661-5.

482. Michael S. Rappé , Giovannoni SJ. The Uncultured Microbial Majority. Annual Review of Microbiology. 2003;57(1):369-94.
483. Tamaki H, Sekiguchi Y, Hanada S, Nakamura K, Nomura N, Matsumura M, et al. Comparative Analysis of Bacterial Diversity in Freshwater Sediment of a Shallow Eutrophic Lake by Molecular and Improved Cultivation-Based Techniques. Applied and Environmental Microbiology. 2005;71(4):2162-9.
484. Biosca EG, Flores R, Santander RD, Díez-Gil JL, Barreno E. Innovative Approaches Using Lichen Enriched Media to Improve Isolation and Culturability of Lichen Associated Bacteria. PLoS One. 2016;11(8):e0160328.
485. Huai-Shu X, Roberts N, Singleton FL, Attwell RW, Grimes DJ, Colwell RR. Survival and Viability of Nonculturable Escherichia coli and Vibrio cholerae in the Estuarine and Marine Environment. Microbial ecology. 1982;8(4):313-23.
486. Lennon JT, Jones SE. Microbial seed banks: the ecological and evolutionary implications of dormancy. Nat Rev Micro. 2011;9(2):119-30.
487. Velicer GJ, Mendes-Soares H. Bacterial predators. Current Biology. 2009;19(2):R55-R6.
488. Kerr B, Margaret A R, Marcus W F, Brendan J. M. B. Local dispersal promotes biodiversity in a real-life game of rock–paper–scissors. Nature Communications. 2002;418: 171–4.
489. D'Onofrio A, Crawford JM, Stewart EJ, Witt K, Gavrish E, Epstein S, et al. Siderophores from Neighboring Organisms Promote the Growth of Uncultured Bacteria. Chemistry & Biology. 2010;17(3):254-64.
490. Kaeberlein T, Lewis K, Epstein SS. Isolating "Uncultivable" Microorganisms in Pure Culture in a Simulated Natural Environment. Science. 2002;296(5570):1127-9.
491. Nichols D, Cahoon N, Trakhtenberg EM, Pham L, Mehta A, Belanger A, et al. Use of Ichip for High-Throughput In Situ Cultivation of "Uncultivable" Microbial Species. Applied and Environmental Microbiology. 2010;76(8):2445-50.
492. Garcia SL, McMahon KD, Grossart H-P, Warnecke F. Successful enrichment of the ubiquitous freshwater acI Actinobacteria. Environmental Microbiology Reports. 2014;6(1):21-7.
493. Button DK, Schut F, Quang P, Martin R, Robertson BR. Viability and Isolation of Marine Bacteria by Dilution Culture: Theory, Procedures, and Initial Results. Applied and Environmental Microbiology. 1993;59(3):881-91.
494. Clarke E. Origins of evolutionary transitions. Journal of biosciences. 2014;39(2):303-17.
495. Fraser CM, Gocayne JD, White O, Adams MD, Clayton RA, Fleischmann RD, et al. The minimal gene complement of *Mycoplasma genitalium*. Science. 1995;270(5235):397-403.
496. Douglas AE. Symbiosis as a General Principle in Eukaryotic Evolution. Cold Spring Harbor Perspectives in Biology. 2014;6(2):a016113.
497. Estrela S, Kerr B, Morris JJ. Transitions in individuality through symbiosis. Current Opinion in Microbiology. 2016;31:191-8.
498. Bhattacharya D, Archibald JM, Weber APM, Reyes-Prieto A. How do endosymbionts become organelles? Understanding early events in plastid evolution. BioEssays : news and reviews in molecular, cellular and developmental biology. 2007;29(12):1239-46.
499. Shitut S, Ahsendorf T, Pande S, Egbert M, Kost C. Metabolic coupling in bacteria. bioRxiv. 2017:114462.
500. Dunny GM, Brickman TJ, Dworkin M. Multicellular behavior in bacteria: communication, cooperation, competition and cheating. BioEssays : news and reviews in molecular, cellular and developmental biology. 2008;30(4):296-8.
501. Marchal M, Goldschmidt F, Derksen-Müller SN, Panke S, Ackermann M, Johnson DR. A passive mutualistic interaction promotes the evolution of spatial structure within microbial populations. BMC Evolutionary Biology. 2017;17(1):106.
502. Shou W, Ram S, Vilar JM. Synthetic cooperation in engineered yeast populations. Proceedings of the National Academy of Sciences. 2007;104(6):1877-82.
503. Overmann J. The phototrophic consortium "Chlorochromatium aggregatum"–a model for bacterial heterologous multicellularity. Recent Advances in Phototrophic Prokaryotes: Springer; 2010. p. 15-29.
504. Doebeli M. A model for the evolutionary dynamics of cross-feeding polymorphisms in microorganisms. Popul Ecol. 2002;44(2):59-70.
505. Biernaskie JM, West SA. Cooperation, clumping and the evolution of multicellularity. Proc R Soc B-Biol Sci. 2015;282(1813).
506. Brenner K, Arnold FH. Self-Organization, Layered Structure, and Aggregation Enhance Persistence of a Synthetic Biofilm Consortium. PLoS One. 2011;6(2):e16791.

507. Bruce JB, Cooper GA, Chabas H, West SA, Griffin AS. Cheating and resistance to cheating in natural populations of the bacterium Pseudomonas fluorescens. Evolution. 2017;71(10):2484-95.
508. Cavaliere M, Feng S, Soyer OS, Jimenez JI. Cooperation in microbial communities and their biotechnological applications. Environ Microbiol. 2017;19(8):2949-63.
509. Bauer E, Zimmermann J, Baldini F, Thiele I, Kaleta C. BacArena: Individual-based metabolic modeling of heterogeneous microbes in complex communities. PLOS Computational Biology. 2017;13(5):e1005544.
510. Harcombe WR, Riehl WJ, Dukovski I, Granger BR, Betts A, Lang AH, et al. Metabolic resource allocation in individual microbes determines ecosystem interactions and spatial dynamics. Cell reports. 2014;7(4):1104-15.
511. Margulis L. Origin of eukaryotic cells: Evidence and research implications for a theory of the origin and evolution of microbial, plant and animal cells on the precambrian Earth: Yale University Press; 1970.
512. Newman DK, Banfield JF. Geomicrobiology: how molecular-scale interactions underpin biogeochemical systems. Science. 2002;296(5570):1071-7.
513. Hofbauer J, Sigmund K. Evolutionary Game Dynamics2003.
514. Røder HL, Herschend J, Russel J, Andersen MF, Madsen JS, Sørensen SJ, et al. Enhanced bacterial mutualism through an evolved biofilm phenotype. The ISME Journal. 2018;12(11):2608-18.
515. Blank D, Wolf L, Ackermann M, Silander OK. The predictability of molecular evolution during functional innovation. Proc Natl Acad Sci U S A. 2014;111(8):3044-9.
516. Shitut S, Ahsendorf T, Pande S, Egbert M, Kost C. Nanotube-mediated cross-feeding couples the metabolism of interacting bacterial cells. bioRxiv. 2018:114462.
517. Bertels F, Merker H, Kost C. Design and Characterization of Auxotrophy-Based Amino Acid Biosensors. PLoS One. 2012;7(7).
518. Trunk T, Khalil HS, Leo JC. Bacterial autoaggregation. AIMS Microbiology. 2018;4(microbiol-04-01-140):140.
519. Weigert M, Kümmerli R. The physical boundaries of public goods cooperation between surface-attached bacterial cells. Proceedings of the Royal Society B: Biological Sciences. 2017;284(1858).
520. Bever JD, Richardson SC, Lawrence BM, Holmes J, Watson M. Preferential allocation to beneficial symbiont with spatial structure maintains mycorrhizal mutualism. Ecol Lett. 2009;12(1):13-21.
521. Kim HJ, Boedicker JQ, Choi JW, Ismagilov RF. Defined spatial structure stabilizes a synthetic multispecies bacterial community. Proceedings of the National Academy of Sciences. 2008;105(47):18188-93.
522. Yamamura N, Higashi M, Behera N, Yuichiro Wakano J. Evolution of mutualism through spatial effects. J Theor Biol. 2004;226(4):421-8.
523. Wakano JY, Nowak MA, Hauert C. Spatial dynamics of ecological public goods. Proceedings of the National Academy of Sciences. 2009;106(19):7910-4.
524. Allen B, Gore J, Nowak MA. Spatial dilemmas of diffusible public goods. eLife. 2013;2:e01169.
525. Traxler MF, Zacharia VM, Marquardt S, Summers SM, Nguyen H-T, Stark SE, et al. Discretely calibrated regulatory loops controlled by ppGpp partition gene induction across the 'feast to famine' gradient in Escherichia coli. Mol Microbiol. 2011;79(4):830-45.
526. Chen CF, Lan J, Korovine M, Shao ZQ, Tao L, Zhang J, et al. Metabolic regulation of lrp gene expression in Escherichia coli K-12. Microbiology-(UK). 1997;143 (Pt 6):2079-84.
527. Blomfield IC, Calie PJ, Eberhardt KJ, McClain MS, Eisenstein BI. Lrp stimulates phase variation of type 1 fimbriation in Escherichia coli K-12. J Bacteriol. 1993;175(1):27-36.
528. Kelly A, Conway C, T OC, Smith SG, Dorman CJ. DNA supercoiling and the Lrp protein determine the directionality of fim switch DNA inversion in Escherichia coli K-12. J Bacteriol. 2006;188(15):5356-63.
529. Costa OYA, Raaijmakers JM, Kuramae EE. Microbial Extracellular Polymeric Substances: Ecological Function and Impact on Soil Aggregation. Frontiers in Microbiology. 2018;9(1636).
530. Schembri MA, Christiansen G, Klemm P. FimH-mediated autoaggregation of Escherichia coli. Mol Microbiol. 2001;41(6):1419-30.
531. Boutte CC, Crosson S. Bacterial lifestyle shapes stringent response activation. Trends Microbiol. 2013;21(4):174-80.

532. Du Q, Kawabe Y, Schilde C, Chen ZH, Schaap P. The Evolution of Aggregative Multicellularity and Cell-Cell Communication in the Dictyostelia. Journal of molecular biology. 2015;427(23):3722-33.
533. Summers ZM, Fogarty HE, Leang C, Franks AE, Malvankar NS, Lovley DR. Direct exchange of electrons within aggregates of an evolved syntrophic coculture of anaerobic bacteria. Science. 2010;330(6009):1413-5.
534. Cerqueda-Garcia D, Martinez-Castilla LP, Falcon LI, Delaye L. Metabolic analysis of Chlorobium chlorochromatii CaD3 reveals clues of the symbiosis in /`Chlorochromatium aggregatum/'. ISME J. 2014;8(5):991-8.
535. Kim SR, Matsui K, Yamada M, Gruz P, Nohmi T. Roles of chromosomal and episomal dinB genes encoding DNA pol IV in targeted and untargeted mutagenesis in Escherichia coli. Molecular Genetics and Genomics. 2001;266(2):207-15.
536. Thomason LC, Costantino N, Court DL. E. coli Genome Manipulation by P1 Transduction. Current Protocols in Molecular Biology: John Wiley & Sons, Inc.; 2001.
537. Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc Natl Acad Sci U S A. 2000;97(12):6640-5.
538. Lenski RE, Rose MR, Simpson SC, Tadler SC. Long-term experimental evolution in *Escherichia coli*. I. Adaptation and divergence during 2,000 generations. The American Naturalist. 1991;138:1315-41.
539. Blattner FR, Plunkett G, Bloch CA, Perna NT, Burland V, Riley M, et al. The complete genome sequence of Escherichia coli K-12. Science. 1997;277(5331):1453-&.
540. Levin BR, Stewart FM, Chao L. Resource-Limited Growth, Competition, and Predation: A Model and Experimental Studies with Bacteria and Bacteriophage. The American Naturalist. 1977;111(977):3-24.
541. Vanstockem M, Michiels K, Vanderleyden J, Van Gool AP. Transposon Mutagenesis of Azospirillum brasilense and Azospirillum lipoferum: Physical Analysis of Tn5 and Tn5-Mob Insertion Mutants. Applied and Environmental Microbiology. 1987;53(2):410-5.
542. Jander G, Norris SR, Joshi V, Fraga M, Rugg A, Yu SX, et al. Application of a high-throughput HPLC-MS/MS assay to Arabidopsis mutant screening; evidence that threonine aldolase plays a role in seed nutritional quality. Plant J. 2004;39(3):465-75.
543. Nurmikko V. Microbiological determination of vitamins and amino acids produced by microorganisms, using the dialysis cell. Applied microbiology. 1957;5(3):160-5.
544. Dykhuizen DE. EXPERIMENTAL STUDIES OF NATURAL-SELECTION IN BACTERIA. Annual Review of Ecology and Systematics. 1990;21:373-98.
545. Brockhurst MA, Koskella B. Experimental coevolution of species interactions. Trends Ecol Evol. 2013;28(6):367-75.
546. Van Valen L. Molecular evolution as predicted by natural selection. Journal of Molecular Evolution. 1974;3(2):89-101.
547. Pal C, Macia MD, Oliver A, Schachar I, Buckling A. Coevolution with viruses drives the evolution of bacterial mutation rates. Nature. 2007;450(7172):1079-81.
548. Gorter FA, Hall AR, Buckling A, Scanlan PD. Parasite host range and the evolution of host resistance. J Evol Biol. 2015;28(5):1119-30.
549. Lawrence D, Fiegna F, Behrends V, Bundy JG, Phillimore AB, Bell T, et al. Species Interactions Alter Evolutionary Responses to a Novel Environment. PLoS Biol. 2012;10(5).
550. Veller C, Hayward LK, Hilbe C, Nowak MA. The red queen and king in finite populations. Proc Natl Acad Sci U S A. 2017;114(27):E5396-E405.
551. Miller JH. Spontaneous mutators in bacteria: insights into pathways of mutagenesis and repair. Annu Rev Microbiol. 1996;50:625-43.
552. Fisher RA. The genetical theory of natural selection. [2d rev. ed.] ed: New York Dover Publications; 1958. 291 p.
553. Eyre-Walker A, Keightley PD. The distribution of fitness effects of new mutations. Nat Rev Genet. 2007;8(8):610-8.
554. Conrad TM, Joyce AR, Applebee MK, Barrett CL, Xie B, Gao Y, et al. Whole-genome resequencing of Escherichia coli K-12 MG1655 undergoing short-term laboratory evolution in lactate minimal media reveals flexible selection of adaptive mutations. Genome Biology. 2009;10(10):1-12.
555. Herring CD, Raghunathan A, Honisch C, Patel T, Applebee MK, Joyce AR, et al. Comparative genome sequencing of Escherichia coli allows observation of bacterial evolution on a laboratory timescale. Nat Genet. 2006;38(12):1406-12.

556. Rainey PB, Travisano M. Adaptive radiation in a heterogeneous environment. Nature. 1998;394(6688):69-72.
557. Grenier F, Matteau D, Baby V, Rodrigue S. Complete Genome Sequence of Escherichia coli BW25113. Genome Announcements. 2014;2(5):e01038-14.
558. Johnson M, Zaretskaya I, Raytselis Y, Merezhuk Y, McGinnis S, Madden TL. NCBI BLAST: a better web interface. Nucleic Acids Res. 2008;36(Web Server issue):W5-9.
559. Bailey SF, Rodrigue N, Kassen R. The Effect of Selection Environment on the Probability of Parallel Evolution. Molecular Biology and Evolution. 2015;32(6):1436-48.
560. Zhou Z, Alikhan N-F, Sergeant MJ, Luhmann N, Vaz C, Francisco AP, et al. GrapeTree: Visualization of core genomic relationships among 100,000 bacterial pathogens. Genome Res. 2018.
561. Long A, Liti G, Luptak A, Tenaillon O. Elucidating the molecular architecture of adaptation via evolve and resequence experiments. Nat Rev Genet. 2015;16(10):567-82.
562. Van den Bergh B, Swings T, Fauvart M, Michiels J. Experimental Design, Population Dynamics, and Diversity in Microbial Experimental Evolution. Microbiology and Molecular Biology Reviews. 2018;82(3):e00008-18.
563. Blount ZD, Lenski RE, Losos JB. Contingency and determinism in evolution: Replaying life's tape. Science. 2018;362(6415).
564. Moe LA. Amino acids in the rhizosphere: from plants to microbes. American journal of botany. 2013;100(9):1692-705.
565. Hosoda K, Suzuki S, Yamauchi Y, Shiroguchi Y, Kashiwagi A, Ono N, et al. Cooperative Adaptation to Establishment of a Synthetic Bacterial Mutualism. PLoS One. 2011;6(2).
566. Choi Y, Sims GE, Murphy S, Miller JR, Chan AP. Predicting the functional effect of amino acid substitutions and indels. PLoS One. 2012;7(10):e46688.
567. Choi Y, Chan AP. PROVEAN web server: a tool to predict the functional effect of amino acid substitutions and indels. Bioinformatics. 2015;31(16):2745-7.
568. Cho BK, Federowicz S, Park YS, Zengler K, Palsson BO. Deciphering the transcriptional regulatory logic of amino acid metabolism. Nat Chem Biol. 2011;8(1):65-71.
569. Tani TH, Khodursky A, Blumenthal RM, Brown PO, Matthews RG. Adaptation to famine: a family of stationary-phase genes revealed by microarray analysis. Proc Natl Acad Sci U S A. 2002;99(21):13471-6.
570. Cho BK, Barrett CL, Knight EM, Park YS, Palsson BO. Genome-scale reconstruction of the Lrp regulatory network in Escherichia coli. Proc Natl Acad Sci U S A. 2008;105(49):19462-7.
571. Zinser ER, Kolter R. Prolonged stationary-phase incubation selects for lrp mutations in Escherichia coli K-12. J Bacteriol. 2000;182(15):4361-5.
572. Zhu Y, Guo T, Park JE, Li X, Meng W, Datta A, et al. Elucidating in vivo structural dynamics in integral membrane protein by hydroxyl radical footprinting. Molecular & cellular proteomics : MCP. 2009;8(8):1999-2010.
573. Zhang Z, Gosset G, Barabote R, Gonzalez CS, Cuevas WA, Saier MH, Jr. Functional interactions between the carbon and iron utilization regulators, Crp and Fur, in Escherichia coli. J Bacteriol. 2005;187(3):980-90.
574. Zambrano MM, Siegele DA, Almiron M, Tormo A, Kolter R. Microbial competition: Escherichia coli mutants that take over stationary phase cultures. Science. 1993;259(5102):1757-60.
575. Rahman M, Hasan MR, Oba T, Shimizu K. Effect of rpoS gene knockout on the metabolism of Escherichia coli during exponential growth phase and early stationary phase based on gene expressions, enzyme activities and intracellular metabolite concentrations. Biotechnol Bioeng. 2006;94(3):585-95.
576. Ferenci T. Maintaining a healthy SPANC balance through regulatory and mutational adaptation. Mol Microbiol. 2005;57(1):1-8.
577. Saxer G, Krepps MD, Merkley ED, Ansong C, Deatherage Kaiser BL, Valovska M-T, et al. Mutations in Global Regulators Lead to Metabolic Selection during Adaptation to Complex Environments. PLoS Genet. 2014;10(12):e1004872.
578. Lacour S, Landini P. SigmaS-dependent gene expression at the onset of stationary phase in Escherichia coli: function of sigmaS-dependent genes and identification of their promoter sequences. Journal of bacteriology. 2004;186(21):7186-95.
579. Newton WA, Snell EE. An inducible tryptophan synthetase in tryptophan auxotrophs of Escherichia coli. Proc Natl Acad Sci U S A. 1962;48:1431-9.
580. Gaimster H, Cama J, Hernandez-Ainsa S, Keyser UF, Summers DK. The indole pulse: a new perspective on indole signalling in Escherichia coli. PLoS One. 2014;9(4):e93168.

581. Horowitz H, Platt T. Initiation in vivo at the internal trp p2 promoter of Escherichia coli. J Biol Chem. 1983;258(13):7890-3.
582. Chimerel C, Field CM, Pinero-Fernandez S, Keyser UF, Summers DK. Indole prevents Escherichia coli cell division by modulating membrane potential. Biochim Biophys Acta. 2012;1818(7):1590-4.
583. Lelong C, Aguiluz K, Luche S, Kuhn L, Garin J, Rabilloud T, et al. The Crl-RpoS regulon of Escherichia coli. Molecular & cellular proteomics : MCP. 2007;6(4):648-59.
584. Lyngstadaas A, Sprenger GA, Boye E. Impaired growth of an Escherichia coli rpe mutant lacking ribulose-5-phosphate epimerase activity. Biochim Biophys Acta. 1998;1381(3):319-30.
585. Sakakibara Y. Involvement of the ribulosephosphate epimerase gene in the dnaA and dnaR functions for initiation of chromosome replication in Escherichia coli. Mol Microbiol. 1997;24(4):793-801.
586. Ilag LL, Jahn D, Eggertsson G, Soll D. The Escherichia coli hemL gene encodes glutamate 1-semialdehyde aminotransferase. J Bacteriol. 1991;173(11):3408-13.
587. Ilag LL, Jahn D. Activity and spectroscopic properties of the Escherichia coli glutamate 1-semialdehyde aminotransferase and the putative active site mutant K265R. Biochemistry. 1992;31(31):7143-51.
588. Goodall ECA, Robinson A, Johnston IG, Jabbari S, Turner KA, Cunningham AF, et al. The Essential Genome of Escherichia coli K-12. MBio. 2018;9(1).
589. Hansen AM, Qiu Y, Yeh N, Blattner FR, Durfee T, Jin DJ. SspA is required for acid resistance in stationary phase by downregulation of H-NS in Escherichia coli. Mol Microbiol. 2005;56(3):719-34.
590. Gentry DR, Hernandez VJ, Nguyen LH, Jensen DB, Cashel M. Synthesis of the stationary-phase sigma factor sigma s is positively regulated by ppGpp. J Bacteriol. 1993;175(24):7982-9.
591. Williams MD, Ouyang TX, Flickinger MC. Starvation-induced expression of SspA and SspB: the effects of a null mutation in sspA on Escherichia coli protein synthesis and survival during growth and prolonged starvation. Mol Microbiol. 1994;11(6):1029-43.
592. Bertin P, Terao E, Lee EH, Lejeune P, Colson C, Danchin A, et al. The H-NS protein is involved in the biogenesis of flagella in Escherichia coli. J Bacteriol. 1994;176(17):5537-40.
593. Puustinen A, Finel M, Haltia T, Gennis RB, Wikstrom M. Properties of the two terminal oxidases of Escherichia coli. Biochemistry. 1991;30(16):3936-42.
594. Saiki K, Mogi T, Ogura K, Anraku Y. In vitro heme O synthesis by the cyoE gene product from Escherichia coli. J Biol Chem. 1993;268(35):26041-4.
595. Nakamura H, Saiki K, Mogi T, Anraku Y. Assignment and functional roles of the cyoABCDE gene products required for the Escherichia coli bo-type quinol oxidase. J Biochem. 1997;122(2):415-21.
596. Cotter PA, Chepuri V, Gennis RB, Gunsalus RP. Cytochrome o (cyoABCDE) and d (cydAB) oxidase gene expression in Escherichia coli is regulated by oxygen, pH, and the fnr gene product. J Bacteriol. 1990;172(11):6333-8.
597. Heintzman ND, Hon GC, Hawkins RD, Kheradpour P, Stark A, Harp LF, et al. Histone modifications at human enhancers reflect global cell-type-specific gene expression. Nature. 2009;459(7243):108-12.
598. Smith CR, Toth AL, Suarez AV, Robinson GE. Genetic and genomic analyses of the division of labour in insect societies. Nat Rev Genet. 2008;9(10):735-48.
599. Dal Co A, Brannon C, Ackermann M. Division of labor in bacteria. eLife. 2018;7:e38578.
600. Flores E, Herrero A. Compartmentalized function through cell differentiation in filamentous cyanobacteria. Nat Rev Microbiol. 2010;8(1):39-50.
601. Golden JW, Robinson SJ, Haselkorn R. Rearrangement of nitrogen fixation genes during heterocyst differentiation in the cyanobacterium Anabaena. Nature. 1985;314(6010):419-23.
602. Dragos A, Kiesewalter H, Martin M, Hsu CY, Hartmann R, Wechsler T, et al. Division of Labor during Biofilm Matrix Production. Curr Biol. 2018;28(12):1903-13.e5.
603. West SA, Griffin AS, Gardner A. Social semantics: altruism, cooperation, mutualism, strong reciprocity and group selection. J Evol Biol. 2007;20(2):415-32.
604. Verdugo P, Alldredge AL, Azam F, Kirchman DL, Passow U, Santschi PH. The oceanic gel phase: a bridge in the DOM–POM continuum. Marine Chemistry. 2004;92(1–4):67-85.
605. Stocker R, Seymour JR. Ecology and Physics of Bacterial Chemotaxis in the Ocean. Microbiology and Molecular Biology Reviews : MMBR. 2012;76(4):792-812.
606. Oliveira NM, Martinez-Garcia E, Xavier J, Durham WM, Kolter R, Kim W, et al. Biofilm Formation As a Response to Ecological Competition. PLoS Biol. 2015;13(7):e1002191.

607. Deschaine BM, Heysel AR, Lenhart BA, Murphy HA. Biofilm formation and toxin production provide a fitness advantage in mixed colonies of environmental yeast isolates. Ecology and evolution. 2018;8(11):5541-50.
608. Jagmann N, Philipp B. Design of synthetic microbial communities for biotechnological production processes. Journal of Biotechnology. 2014;184:209-18.
609. Großkopf T, Soyer OS. Synthetic microbial communities. Current Opinion in Microbiology. 2014;18:72-7.
610. Blackstone NW. Why did eukaryotes evolve only once? Genetic and energetic aspects of conflict and conflict mediation. Philosophical transactions of the Royal Society of London Series B, Biological sciences. 2013;368(1622):20120266.
611. Lane N, Martin W. The energetics of genome complexity. Nature. 2010;467(7318):929-34.
612. Embley TM, Martin W. Eukaryotic evolution, changes and challenges. Nature. 2006;440(7084):623-30.
613. Keseler IM, Mackie A, Peralta-Gil M, Santos-Zavaleta A, Gama-Castro S, Bonavides-Martinez C, et al. EcoCyc: fusing model organism databases with systems biology. Nucleic Acids Res. 2013;41(D1):D605-D12.

Supplemental Information

Supplemental information

Supporting information for chapter 1

Table S1: Reported microbial species exchanging different metabolites.

Species A		Kingdom		Metabolite transfer						
A	B	A	B	from A to B	from B to A	Type of exchange[1]	Environment	Life-style	CD/CI[2]	Ref.
Bifidobacterium adolescentis	*Eubacterium hallii*	Bacteria	Bacteria	Lactate		By-product	Human colon	Endo-symbiont	CD	1
Bifidobacterium adolescentis	*Roseburia* sp.	Bacteria	Bacteria	Oligo-saccharides		By-product	Human colon	Endo-symbiont	CD	
Bifidobacterium longum	*Eubacterium rectale*	Bacteria	Bacteria	Acetate	Xylose, arabinose	By-product	Human colon	Endo-symbiont	CD	2
Streptococci sp.	*Aggregatibacter* sp.	Bacteria	Bacteria	Lactate		By-product	Dental plaque	Free-living	CI	3
	Capnocytophaga sp.	Bacteria	Bacteria	CO_2		By-product				
Acinetobacter sp.	*Pseudomonas putida*	Bacteria	Bacteria	Benzoate		By-product	Not mentioned	Free living	CD	4
Burkholderia sp.	*Pseudomonas* sp.	Bacteria	Bacteria	Chloro-benzoate		By-product	Not mentioned	Free living	CD	5
Staphylococcus sp.	*Azospirillum brasilense*	Bacteria	Bacteria	Aspartic acid		By-product	Soil	Free living	CD	6
Cellulomonas sp.	*Diazotrophs* sp.	Bacteria	Bacteria	Carbon source		By-product	Soil	Free living	CD	7
Streptococcus gordonii	*Fusobacterium nucleatum*	Bacteria	Bacteria	Ornithine		By-product	Oral cavity	Pathogenic biofilm	CD	8
Streptococcus gordonii	*Aggregatibacter actinomycetemcomitans*	Bacteria	Bacteria	Lactate		By-product	Oral cavity	Pathogenic biofilm	CD	9
Streptococcus suis	*Actinobacillus* sp.	Bacteria	Bacteria	Pyridine compounds		By-product	Swine pathogens	Pathogenic biofilm	CD	10
Bordetella bronchiseptica		Bacteria	Bacteria							
Pasteurella multocida		Bacteria	Bacteria							
Pleuropneumoniae sp.		Bacteria	Bacteria							
Staphylococcus aureus		Bacteria	Bacteria							
Escherichia coli		Bacteria	Bacteria							
Escherichia coli	*Lactobacilluts arabinosuts*	Bacteria	Bacteria	Phenylalanine, nicotinic acid		By-product	Not mentioned	Free living	CD	11
Escherichia coli	*Streptococcuts faecalis*	Bacteria	Bacteria	Folic acid						
Oospora lactis	*Neurospora sitophila*	Bacteria	Bacteria	Vitamin B6						
Acidithiobacillus caldus	*Acidimicrobium ferrooxidans*	Bacteria	Bacteria	Organic carbon		By-product	Pyrite leaching experiment	Free living	CD	12
Soil bacteria	Amino acid auxotrophic bacteria	Bacteria	Bacteria	Amino acids		By-product	Soil	Free living	CD	13
Pseudomonas sp.	*Hyphomicrobium sp.*	Bacteria	Bacteria	Methanol		By-product	Methane-utilizing consortia	Free living	CD	14
Prochlorococcus sp.	*Alteromonas sp.*	Bacteria	Bacteria	Carbon source		By-product	Ocean	Free living	CD	15
	Halomonas sp.	Bacteria	Bacteria							

Bacteroidetes	acI-B2	Bacteria	Bacteria	Vitamins, amino acids		By-product	Freshwater	Free living	CI	16
Bacteroidetes	acIII	Bacteria	Bacteria	Aspartic acid						
PnecC	Bacteroidetes	Bacteria	Bacteria	Thiamine						
PnecC	acI-B2	Bacteria	Bacteria	Vitamins, amino acids						
PnecC	acIII	Bacteria	Bacteria	Biotin, thiamine	Histidine					
acIII	acI-B2	Bacteria	Bacteria	Vitamins, amino acids						
Archaea	Bacteroidetes	Archaea	Bacteria	Vitamin B12						
Archaea	acIII	Archaea	Bacteria	Vitamin B12						
Archaea	acI-B2	Archaea	Bacteria	Vitamin B12						
Archaea	PnecC	Archaea	Bacteria	Vitamin B12						
Desulfovibrio vulgaris	*Methanococcus maripaludis*	Bacteria	Archaea	Alanine		By-product	Lab coculture experiment	Free living	CD	17
Ignicoccus hospitalis	*Nanoarchaeum equitans*	Archaea	Archaea	Amino acids, lipids		By-product	Not mentioned	Free living	CD	18
Archaea	*Desulfosarcin* sp.	Archaea	Bacteria	Nitrogen source		By-product	Marine sediment	Free living	CI	19
Treponema azotonutricium	*Treponema primitia*	Bacteria	Bacteria	Amino acids, H_2	Vitamins	Cooperative	Termite gut	Endo-symbiont	CD	20
Lactococcus lactis	*Treponema primitia*	Bacteria	*Bacteria*	Folate		By-product	Termite gut	Endo-symbiont	CD	21
Serratia grimesii		Bacteria								
Beijerinckia lacticogenes	*Thiobocillus ferrooxidans*	Bacteria	Bacteria	Nitrogen source	Carbon source	By-product	Not mentioned	Free living	CD	22
Streptococcus lactis	Bacteroides	Bacteria	Bacteria	Lactate		By-product	Hindguts of termites	Wihtin gut	CD	23
Ruminococcus flavefacien	*Selenomonas ruminantium*	Bacteria	Bacteria	Fiber hydrolysis product		By-product	Rumen	Gut inhabitants	CD	24
Porphyromonas gingivalis	*Treponema denticola*	Bacteria	Bacteria	Isobutyric acid	Succinic acid	By-product	Oral cavity	Free living	CD	25
Enterobacter cloacae	*Azospirillum brasilense*	Bacteria	Bacteria	Acetic & succinic acids, acetoin, 2,3-butanediol		By-product	Soil	Free living	CD	26
Bacillus sp.	*Azospirillum* sp.	Bacteria	Bacteria	Catabolic products of pectin		By-product	Soil	Free living	CD	27
Streptococcus rnutans	*Streptococcus rnutans*	Bacteria	Bacteria	Lactate		By-product	Human oral cavity	Free living	CD	28
Acetobacter suboxydans	*Lactobacillus plantarum*	Bacteria	Bacteria	Growth factor related to folic acid pathway		By-product	Not mentioned	Free living	CD	29
Acetobacter suboxydans	*Streptococcus faecalis*	Bacteria	Bacteria							
Lactobacillus plantarum	*Streptococcus faecalis*	Bacteria	Bacteria							
Thermophilic lactobacilli	*Streptococcus thermophilus*	Bacteria	Bacteria	Amino acids		By-product	Cheese	Free living	CD	30
Lacti streptococci	*Streptococcus thermophilus*	Bacteria	Bacteria							

Leuconostoc mesenteroides	*Streptococcus faecalis*	Bacteria	Bacteria	Folic acid	Amino acids	By-product	Milk and dairy product	Free living	CD	31
Lactococcus lactis	*Pseudomonas fluorescens*	Bacteria	Bacteria	Lactic acid		By-product	Milk and dairy product	Free living	CD	32
Hydrogenophaga palleronii	*Agrobacterium radiobacter*	Bacteria	Bacteria	Catechol-4-sulfonate	4-aminobenzoate, biotin, vitamin B12	By-product	4-aminobenzene--sulfonate	Free living	CD	33
Primary consumers (mainly *Pseudomonads*)	*Stenotrophomonads* sp.	Bacteria	Bacteria	Unknown		By-product	Contaminated sediment	Free living	CD and CI	34
	Acidovorax defluvi	Bacteria	Bacteria							
Streptococcus thermophilus	*Lactobacillus bulgaricus*	Bacteria	Bacteria	Purine (precursor)		Cooperative	Yogurt	Free living	CD	35
Lactobacillus bulgaricus	*Streptococcus thermophil*	Bacteria	Bacteria	Glycine, histidine		By-product	Yogurt	Free living	CD	36
Streptococcus thermophilus	*Lactobacillus bulgaricus*	Bacteria	Bacteria	Formic acid, CO_2	Amino acids, peptides	Cooperative	Yogurt	Free living	CD	37
Streptococcus sp.	*Pseudomonas aeruginosa*	Bacteria	Bacteria	2,3-butanedione		By-product	Lung of cystic fibrosis (CF) patients	Patho-genic	CI	38
Thermosynechococcus sp.	*Roseiflexus castenholzii*	Bacteria	Bacteria	Vitamins (B1, B2, B7)		By-product	Hot spring	Free living	CI	39
Baumannia cicadellinicola	*Sulcia muelleri*	Bacteria	Bacteria	Histidine	Homoserine	Cooperative	Sharpshooter (insect)	Endo-symbiont	CI	40
Baumannia cicadellinicola	*Homalodisca coagulate*	Bacteria	Animal	Histidine, vitamins, cofactors	Sugar					
Sulcia muelleri				Amino acids						
Donghicola sp.	*Phaeodactylum tricornutu*	Bacteria	Algae	Ammonium	Organic compound	By-product	Marine	Free living	CD	41
Marinobacter sp.	*Scrippsiella trochoidea*	Bacteria	Algae	Siderophores	Organic compound	By-product	Marine	Free living	CD	42
Halomonas sp.	*Amphidinium operculatum*	Bacteria	Algae	Vitamin B12	Organic carbon	Cooperative	Marine	Free living	CD	43
	Porphyridium purpureum									
Richelia intracellularis	*Hemiaulus* sp.	Bacteria	Algae	Fixed nitrogen		Cooperative	Marine	Free living	CD	44
Phytoplankton	Crenarchaea	Algae	Bacteria	Carbon or nitrogen sources		By-product	Marine and freshwater	Free-living	IN	45
Burkholderia rhizoxinic	*Rhizopus microspores*	Bacteria	Fungi	Unknown sporulation substance		By-product	Fungus mycelium	Endo-symbiont	CD	46
Pseudomonas fluorescens	*Laccaria bicolor*	Bacteria	Fungi	Thiamine, unknown metabolities	Trehalose	By-product	Soil	Free-living	CD	47
Saccharomyces cerevisiae	*Acinetobacter* sp.	Fungi	Bacteria	Ethanol		By-product	Host, soil, water	Pathogenic free living	CD	48
Saccharomyces cerevisiae	*Proteus vulgaris*	Fungi	Bacteria	Niacin like factor		By-product	Not mentioned		CD	49
Saccharomyces cerevisiae	*Lactobacillus brevis* subsp. *lindneri*	Fungi	Bacteria	Amino acids		By-product	Milk, diary product	Free-living	CD	50
Saccharornyces exiguus	*Lactobacillus brevis* subsp. *lindneri*	Fungi	Bacteria	Carbon source		By-product	Sourdough	Free-living	CD	51
Azospirillum sp.	*Crcthus srercoreus*	Bacteria	Fungi	Nitrogen	Carbon	Cooperative	Soil	Free living	CD	52
	Cellulose degrader fungi									

Trichoderm sp.	*Clostridium butyricum*	Fungi	Bacteria	Carbon source	Nitrogen compound	By-product	Soil	Free-living	CD	53
Acetobacter diazotrophicus	*Lipomyces kononenkoae*	Bacteria	Fungi	Nitrogen	Carbon	Cooperative	Not mentioned	Free-living	CD	54
Bacillus sp.	*Debaryomyces vanriji*	Bacteria	Fungi	Thiamine		By-product	Hot spring	Free-living	CD	55
Zygotorulaspora florentina	*Lactobacillus nagelii*	Fungi	Bacteria	Several amino acids		Cooperative	Water kefir	Free-living	CD	56
Cyanobacteria	Fungi	Bacteria	Fungi	Fixed Carbon, nitrogen		By-product	Not mentioned	Free-living	CD	57 & 58
Arbuscular mycorrhizal fungi	*Candidatus Glomeribacter gigasporarum*	Fungi: obligate plant biotroph	Bacteria: endo-fungal	Phosphate, amino acids, Zn		By-product	Inside AMF mycelia	Endo-symbiont	CI	59
Lichen-associat. bacteria	Lichens	Bacteria	Fungi	Fix N_2, vitamin B12, IAA, thiamine, carbon		By-product	Thallus of lichen	Free-living	CI	60
Nostoc sp.	Mycobiont	Bacteria	Fungi	Fixed N_2						
Nostoc sp.	Cholorbiont	Bacteria	Algae	Fixed N_2						
Rhizobiales	*Lobaria pulmonaria L.*	Bacteria	Fungi: lichen	Vitamins, auxins, fixed carbon & N_2		By-product	Thallus of lichen	Free-living	CI	61
Nostoc sp.	Ascomycete fungus	Bacteria	Algae	Fixed N_2						
Nostoc sp.	*Dictyochloropsis reticulat*	Bacteria	Algae	Fixed N_2						
Azotobacter sp.	*Alternaria* sp.	Bacteria	Fungi	Fixed N_2		By-product	Synthetic tripartite symbiosis	Free-living	CD	62
	Chlamydomonas sp.	Bacteria	Algae	Fixed N_2	Fixed carbon					
Cyanobacteria	*Aplysina cauliformis*	Bacteria	Animal: Sponge	Fixed Carbon, nitrogen		By-product	Marine	Free-living	not tested	63
	Neopetrosia subtriangularis									
	Aplysina fulva									
Buchnera sp.	Aphids	Bacteria	Animal	Essential amino acids		By-product	Mycetocyte symbiosis	Endo-symbiont	CI	64
Symbiotic bacteria	*Riftia pachyptila*	Bacteria	Animal	Glutamate, succinate		By-product	Trophosome	Endo-symbiont	CI	65
Wolbachia sp.	*Cimex lectularius*	Bacteria	Animal: Bedbug	Vitamin B		By-product	Bacteriome	Endo-symbiont	CI	66
Wigglesworthia glossinidia	Tsetse flies	Bacteria	Animal	Vitamins		By-product	Bacteriocytes	Endo-symbiont	CI	67
Wolbachia sp.	*Brugia malayi*	Bacteria	Animal	Ribo,-flavin, adenine dinucleotide, heme, nucleotides	Amino acids	By-product	Nematode	Endo-symbiont	CI	68
Streptococcus sp.	*Reticulitermes flavipes* (Termites)	Bacteria	Animal	Nitrogen source		By-product	Termite gut	Endo-symbiont	CD	69
Bacteroides termitidis										
Gamma-proteobacteria	*Olavius algarvensis*	Bacteria	Animal	Carbon, amino acids, vitamins	Succinate, acetate, propionate, ammonia, taurine, urea	By-product	Below the worm cuticle (*Olavius algarvensis*)	Endo-symbiont	CI	70
Deltaproteobacterial										
Blattabacterium sp.	*Blattella germanica*	Bacteria	Animal	Proline, glutamine, asparagine	Amino acids, coenzymes	By-product	Abdominal fat body	Endo-symbiont	CI	71

Buchnera aphidicola	*Acyrthosiphon pisum*	Bacteria	Animal	Amino acids		Cooperative	Bacteriocytes in the aphid	Endo-symbiont	CI	72
Rhizopium sp.	Legume plants	Bacteria	Plants	Fixed N_2	Fixed carbon	Cooperative	Soil	Free-living	CD	73
Rhizoglomus irregularis		AM fungus	Bacteria	Phosphorus		Cooperative	Dutch dune, perennial grassland ecosystem	Free-living	CD	74
	Legume plants	Bacteria	Plants	Fixed N_2						
Calystegia sepium	*Rhizobium meliloti*	Bacteria	Plants	Calystegin		By-product	Soil	Free-living	CD	75
Convolvulus arvensis										
Atropa belladonna										
Mesorhizobium mediterraneum	Chickpea and barley	Bacteria	Plants	Phosphorous		By-product	Soil	Free-living	CD	76
Plant	Soil microbiota			Sugars		By-product	Soil	Free-living	not tested	77

[1] Any cross-feeding interaction was by default considered as a 'by-product' interaction. Only in those cases, in which it was demonstrated that the exchanged metabolite incurred costs to the producer and that it was produced to benefit the interaction partner was the interaction classified as 'cooperative cross-feeding' interaction.

[2] Techniques that have been used in the reported studies: CD = culture-dependent techniques, CI = culture-independent techniques.

References:

1. A. Belenguer, S. H. Duncan, A. G. Calder, G. Holtrop, P. Louis, G. E. Lobley and H. J. Flint, Applied and Environmental Microbiology, 2006, 72, 3593-3599.
2. A. Rivière, M. Gagnon, S. Weckx, D. Roy and L. De Vuyst, Applied and Environmental Microbiology, 2015, 81, 7767-7781.
3. J. L. Mark Welch, B. J. Rossetti, C. W. Rieken, F. E. Dewhirst and G. G. Borisy, Proceedings of the National Academy of Sciences, 2016, 113, E791-E800.
4. B. B. Christensen, J. A. J. Haagensen, A. Heydorn and S. Molin, Applied and Environmental Microbiology, 2002, 68, 2495-2502.
5. A. T. Nielsen, T. Tolker-Nielsen, K. B. Barken and S. Molin, Environmental Microbiology, 2000, 2, 59-68.
6. G. Holguin and Y. Bashan, Soil Biology and Biochemistry, 1996, 28, 1651-1660.
7. D. M. Halsall and A. H. Gibson, Soil Biology and Biochemistry, 1989, 21, 291-298.
8. A. Sakanaka, M. Kuboniwa, H. Takeuchi, E. Hashino and A. Amano, The Journal of Biological Chemistry, 2015, 290, 21185-21198.
9. M. M. Ramsey, K. P. Rumbaugh and M. Whiteley, PLoS Pathogens, 2011, 7, e1002012.
10. A. Loera-Muro, M. Jacques, F. J. Avelar-Gonzalez, J. Labrie, Y. D. N. Tremblay, R. Oropeza-Navarro and A. L. Guerrero-Barrera, BMC Microbiology, 2016, 16, 128.
11. V. Nurmikko, Applied Microbiology, 1957, 5, 160-165.
12. N. Okibe and D. B. Johnson, Biotechnology and Bioengineering, 2004, 87, 574-583.
13. T. M. B. Payne, J. W. Rouatt and A. G. Lochhead, Canadian Journal of Microbiology, 1957, 3, 73-80.
14. T. G. Wilkinson, H. H. Topiwala and G. Hamer, Biotechnology and Bioengineering, 1974, 16, 41-59.
15. S. J. Biller, F. Schubotz, S. E. Roggensack, A. W. Thompson, R. E. Summons and S. W. Chisholm, Science, 2014, 343, 183-186.
16. S. L. Garcia, M. Buck, K. D. McMahon, H.-P. Grossart, A. Eiler and F. Warnecke, Molecular Ecology, 2015, 24, 4449-4459.
17. C. B. Walker, A. M. Redding-Johanson, E. E. Baidoo, L. Rajeev, Z. L. He, E. L. Hendrickson, M. P. Joachimiak, S. Stolyar, A. P. Arkin, J. A. Leigh, J. Z. Zhou, J. D. Keasling, A. Mukhopadhyay and D. A. Stahl, The ISME Journal, 2012, 6, 2045-2055.
18. U. Jahn, R. Summons, H. Sturt, E. Grosjean and H. Huber, Archives of Microbiology, 2004, 182, 404-

413.
19. A. E. Dekas, R. S. Poretsky and V. J. Orphan, Science, 2009, 326, 422-426.
20. A. Z. Rosenthal, E. G. Matson, A. Eldar and J. R. Leadbetter, The ISME Journal, 2011, 5, 1133-1142.
21. J. R. Graber and J. A. Breznak, Applied and Environmental Microbiology, 2005, 71, 1883-1889.
22. N. C. Trivedi and H. M. Tsuchiya, International Journal of Mineral Processing, 1975, 2, 1-14.
23. J. E. Schultz and J. A. Breznak, Applied and Environmental Microbiology, 1979, 37, 1206-1210.
24. S. Sawanon and Y. Kobayashi, Animal Science Journal, 2006, 77, 208-214.
25. D. Grenier, Infection and Immunity, 1992, 60, 5298-5301.
26. P. Kaiser, in Azospirillum VI and Related Microorganisms: Genetics — Physiology — Ecology, eds. I. Fendrik, M. del Gallo, J. Vanderleyden and M. de Zamaroczy, Springer Berlin Heidelberg, Berlin, Heidelberg, 1995, DOI: 10.1007/978-3-642-79906-8_21, pp. 207-212.
27. K. M. Khammas and P. Kaiser, Canadian Journal of Microbiology, 1992 38, 794-797.
28 F. H. M. Mikx and J. S. van der Hoeven, Archives of Oral Biology, 1975, 20, 407-410.
29. B. W. Koft and J. H. Morrison, Journal of Bacteriology, 1956, 72, 705-707.
30. J. ACCOLAS, P., M. VEAUX and J. AUCLAIR, Lait, 1971, 51, 249-272.
31. V. Nurmikko, Acta Chemica Scandinavica, 1952, 6, 1258-1264.
32. J. Kives, D. Guadarrama, B. Orgaz, A. Rivera-Sen, J. Vazquez and C. SanJose, Journal of Dairy Science, 2005, 88, 4165-4171.
33. E. Dangmann, A. Stolz, A. E. Kuhm, A. Hammer, B. Feigel, N. NoisommitRizzi, M. Rizzi, M. Reuss and H. J. Knackmuss, Biodegradation, 1996, 7, 223-229.
34. J. Wald, M. Hroudova, J. Jansa, B. Vrchotova, T. Macek and O. Uhlik, Frontiers in Microbiology, 2015, 6, 1268.
35. S. Sieuwerts, D. Molenaar, S. van Hijum, M. Beerthuyzen, M. J. A. Stevens, P. W. M. Janssen, C. J. Ingham, F. A. M. de Bok, W. M. de Vos and J. Vlieg, Applied and Environmental Microbiology, 2010, 76, 7775-7784.
36. E. S. Bautista, R. S. Dahiya and M. L. Speck, Journal of Dairy Research, 2009, 33, 299-307.
37. F. M. Driessen, F. Kingma and J. Stadhouders, Netherlands Milk and Dairy Journal, 1982, 36, 135-144.
38. K. L. Whiteson, S. Meinardi, Y. W. Lim, R. Schmieder, H. Maughan, R. Quinn, D. R. Blake, D. Conrad and F. Rohwer, The ISME Journal, 2014, 8, 1247-1258.
39. I. A. Rodionova, X. Q. Li, A. E. Plymale, K. Motamedchaboki, A. E. Konopka, M. F. Romine, J. K. Fredrickson, A. L. Osterman and D. A. Rodionov, Environmental Microbiology Reports, 2015, 7, 204-210.
40. D. Wu, S. C. Daugherty, S. E. Van Aken, G. H. Pai, K. L. Watkins, H. Khouri, L. J. Tallon, J. M. Zaborsky, H. E. Dunbar, P. L. Tran, N. A. Moran and J. A. Eisen, PLoS Biology, 2006, 4, e188.
41. M. Suleiman, K. Zecher, O. Yücel, N. Jagmann and B. Philipp, Applied and Environmental Microbiology, 2016, 82, 7113-7122.
42. S. A. Amin, D. H. Green, M. C. Hart, F. C. Küpper, W. G. Sunda and C. J. Carrano, Proceedings of the National Academy of Sciences of the United States of America, 2009, 106, 17071-17076.
43. M. T. Croft, A. D. Lawrence, E. Raux-Deery, M. J. Warren and A. G. Smith, Nature, 2005, 438, 90-93.
44. R. A. Foster, M. M. M. Kuypers, T. Vagner, R. W. Paerl, N. Musat and J. P. Zehr, The ISME Journal, 2011, 5, 1484-1493.
45. S. Fietz, A. Martínez-Garcia, G. Rueda, V. L. Peck, C. Huguet, M. Escala and A. Rosell-Melé, Limnology and Oceanography, 2011, 56, 1907-1916.
46. G. Lackner, N. Moebius and C. Hertweck, The ISME Journal, 2011, 5, 252-261.
47. A. Deveau, C. Brulé, B. Palin, D. Champmartin, P. Rubini, J. Garbaye, A. Sarniguet and P. Frey-Klett, Environmental Microbiology Reports, 2010, 2, 560-568.
48. M. G. Smith, S. G. Des Etages and M. Snyder, Molecular and Cellular Biology, 2004, 24, 3874-3884.
49. A. Shindala, H. R. Bungay, N. R. Krieg and K. Culbert, Journal of Bacteriology, 1965, 89, 693-696.
50. M. Gobbetti, A. Corsetti and J. Rossi, World Journal of Microbiology and Biotechnology, 1994, 10, 275-279.
51. M. Gobbetti, A. Corsetti and J. Rossi, Applied Microbiology and Biotechnology, 1994, 41, 456-460.
52. D. M. Halsall, Soil Biology and Biochemistry, 1993, 25, 419-429.
53. J. M. Lynch and S. H. T. Harper, Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences, 1985, 310, 221-226.

54. E. H. Cojho, V. M. Reis, A. C. G. Schenberg and J. Döbereiner, FEMS Microbiology Letters, 1993, 106, 341-346.
55. E. G. Rikhvanov, N. N. Varakina, D. Y. Sozinov and V. K. Voinikov, Applied and Environmental Microbiology, 1999, 65, 4292-4293.
56. J. Stadie, A. Gulitz, M. A. Ehrmann and R. F. Vogel, Food Microbiology, 2013, 35, 92-98.
57. D. C. Smith and E. A. Drew, New Phytologist, 1965, 64, 195-200.
58. G. D. Scott, New Phytologist, 1956, 55, 111-116.
59. S. Ghignone, A. Salvioli, I. Anca, E. Lumini, G. Ortu, L. Petiti, S. Cruveiller, V. Bianciotto, P. Piffanelli, L. Lanfranco and P. Bonfante, The ISME Journal, 2012, 6, 136-145.
60. M. Grube, T. Cernava, J. Soh, S. Fuchs, I. Aschenbrenner, C. Lassek, U. Wegner, D. Becher, K. Riedel, C. W. Sensen and G. Berg, The ISME Journal, 2015, 9, 412-424.
61. A. Erlacher, T. Cernava, M. Cardinale, J. Soh, C. W. Sensen, M. Grube and G. Berg, Frontiers in Microbiology, 2015, 6, 53.
62. Z. Lőrincz, É. Preininger, A. Kósa, T. Pónyi, P. Nyitrai, L. Sarkadi, G. M. Kovács, B. Böddi and I. Gyurján, Folia Microbiologica, 2010, 55, 393-400.
63. C. J. Freeman and R. W. Thacker, Limnology and Oceanography, 2011, 56, 1577-1586.
64. A. E. Douglas, Annual Review of Entomology, 1998, 43, 17-37.
65. H. Felbeck and J. Jarchow, Physiological Zoology, 1998, 71, 294-302.
66. T. Hosokawa, R. Koga, Y. Kikuchi, X.-Y. Meng and T. Fukatsu, Proceedings of the National Academy of Sciences, 2010, 107, 769-774.
67. L. Akman, A. Yamashita, H. Watanabe, K. Oshima, T. Shiba, M. Hattori and S. Aksoy, Nature Genetics, 2002, 32, 402-407.
68. J. Foster, M. Ganatra, I. Kamal, J. Ware, K. Makarova, N. Ivanova, A. Bhattacharyya, V. Kapatral, S. Kumar, J. Posfai, T. Vincze, J. Ingram, L. Moran, A. Lapidus, M. Omelchenko, N. Kyrpides, E. Ghedin, S. Wang, E. Goltsman, V. Joukov, O. Ostrovskaya, K. Tsukerman, M. Mazur, D. Comb, E. Koonin and B. Slatko, PLOS Biology, 2005, 3, e121.
69. C. J. Potrikus and J. A. Breznak, Proceedings of the National Academy of Sciences of the United States of America, 1981, 78, 4601-4605.
70. T. Woyke, H. Teeling, N. N. Ivanova, M. Huntemann, M. Richter, F. O. Gloeckner, D. Boffelli, I. J. Anderson, K. W. Barry, H. J. Shapiro, E. Szeto, N. C. Kyrpides, M. Mussmann, R. Amann, C. Bergin, C. Ruehland, E. M. Rubin and N. Dubilier, Nature, 2006, 443, 950-955.
71. M. J. López-Sánchez, A. Neef, J. Peretó, R. Patiño-Navarrete, M. Pignatelli, A. Latorre and A. Moya, PLOS Genetics, 2009, 5, e1000721.
72. C. W. Russell, A. Poliakov, M. Haribal, G. Jander, K. J. van Wijk and A. E. Douglas, Proceedings of the Royal Society B: Biological Sciences, 2014, 281, 20141163.
73. M. K. Bach, W. E. Magee and R. H. Burris, Plant Physiology, 1958, 33, 118-124.
74. M. G. A. van der Heijden, S. d. Bruin, L. Luckerhoff, R. S. P. van Logtestijn and K. Schlaeppi, The ISME Journal, 2016, 10, 389-399.
75. D. Tepfer, A. Goldmann, N. Pamboukdjian, M. Maille, A. Lepingle, D. Chevalier, J. Dénarié and C. Rosenberg, Journal of Bacteriology, 1988, 170, 1153-1161.
76. A. Peix, A. A. Rivas-Boyero, P. F. Mateos, C. Rodriguez-Barrueco, E. Martínez-Molina and E. Velazquez, Soil Biology and Biochemistry, 2001, 33, 103-110.
77. I. Kraffczyk, G. Trolldenier and H. Beringer, Soil Biology and Biochemistry, 1984, 16, 315-322.

Supporting information for chapter 2

Supporting figures

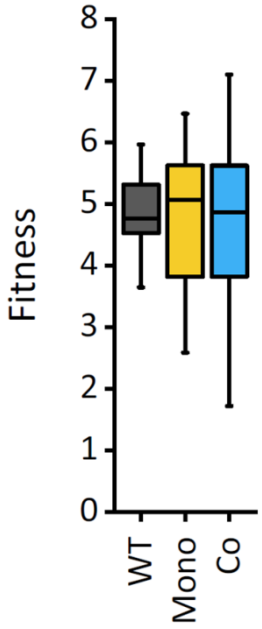

Figure S1: Absolute fitness does not differ between groups of evolved populations. Fitness in evolved populations (Wildtype: WT, auxotrophic monocultures: Mono, and cocultures: Co) given as the Malthusian parameter. Populations do not differ in fitness (ANOVA: P=0.582, n=36 for WT, n=114 for Mono, and n=60 for Co).

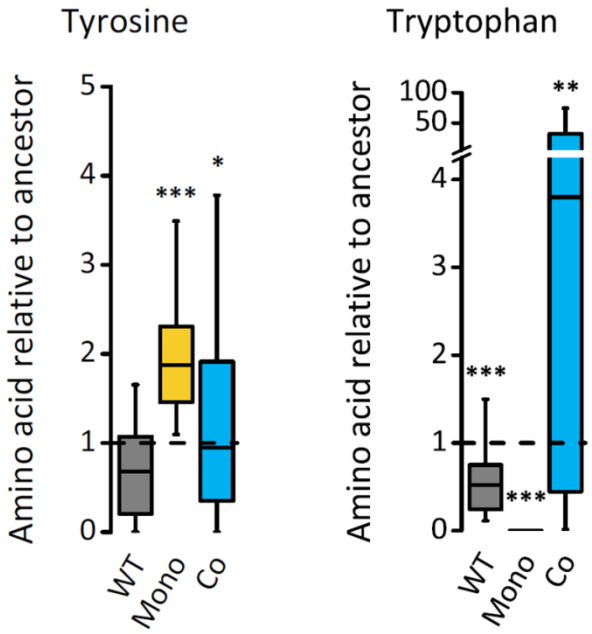

Figure S2: Quantification of released tyrosine and tryptophan levels reveals permanent overproducers. Levels of amino acids were quantified in culture supernatants of evolved phenotypes from wild type cultures (WT), auxotrophic monocultures (Mono), cocultures of auxotrophs (Co) and ancestors. Detected amounts were first set to determined cfu. Resulting measures for tyrosine and tryptophan are given as ratios of evolved isolate relative to corresponding ancestor. Significant differences to 1 were determined using One-Sample t-test: WT: n=48; Mono: n=44; Co tyrosine: n=56; Co tryptophan: n=84; ***P<0.001, **P<0.01, *P<0.05.

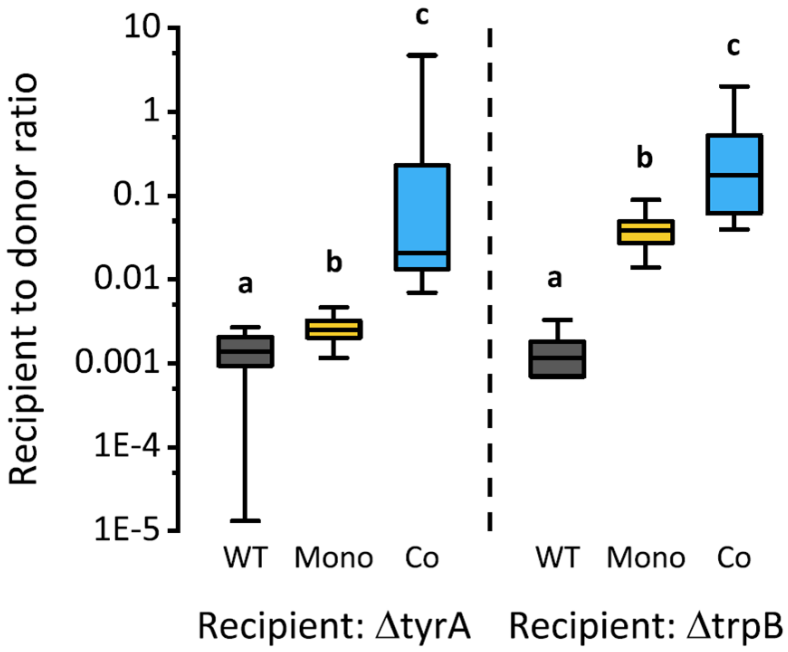

Figure S3: Superior biosensor supplementation by evolved isolates from cocultures. Highest levels of shared amino acids were detected in derived strains from cocultures. Isolates from evolved populations (wild type: WT, auxotrophic monocultures: Mono, auxotrophic cocultures: Co) were utilized as amino acid donors for E. coli biosensor strains (recipients) auxotrophic for tyrosine (*ΔtyrA*) or tryptophan (*ΔtrpB*). The culture medium contained amino acid essential for the respective donor, but lacked the amino acid essential for the recipient to grow. Thus, biosensor growth reflects levels of shared amino acid by the donor. Results are given as biosensor to donor ratios achieved by evolved isolates. Isolates from derived populations (n_{WT}=12; n_{Mono}=88; n_{Co}=140) were replicated three times. The potential to supplement another amino acid auxotrophic strain with tyrosine or tryptophan is significantly higher in evolved auxotrophs from cocultures than in controls (One-way ANOVA followed by Dunnett T3 *post hoc* test: $P<0.05$ for recipient *ΔtyrA* and $P<0.001$ for recipient *ΔtrpB*).

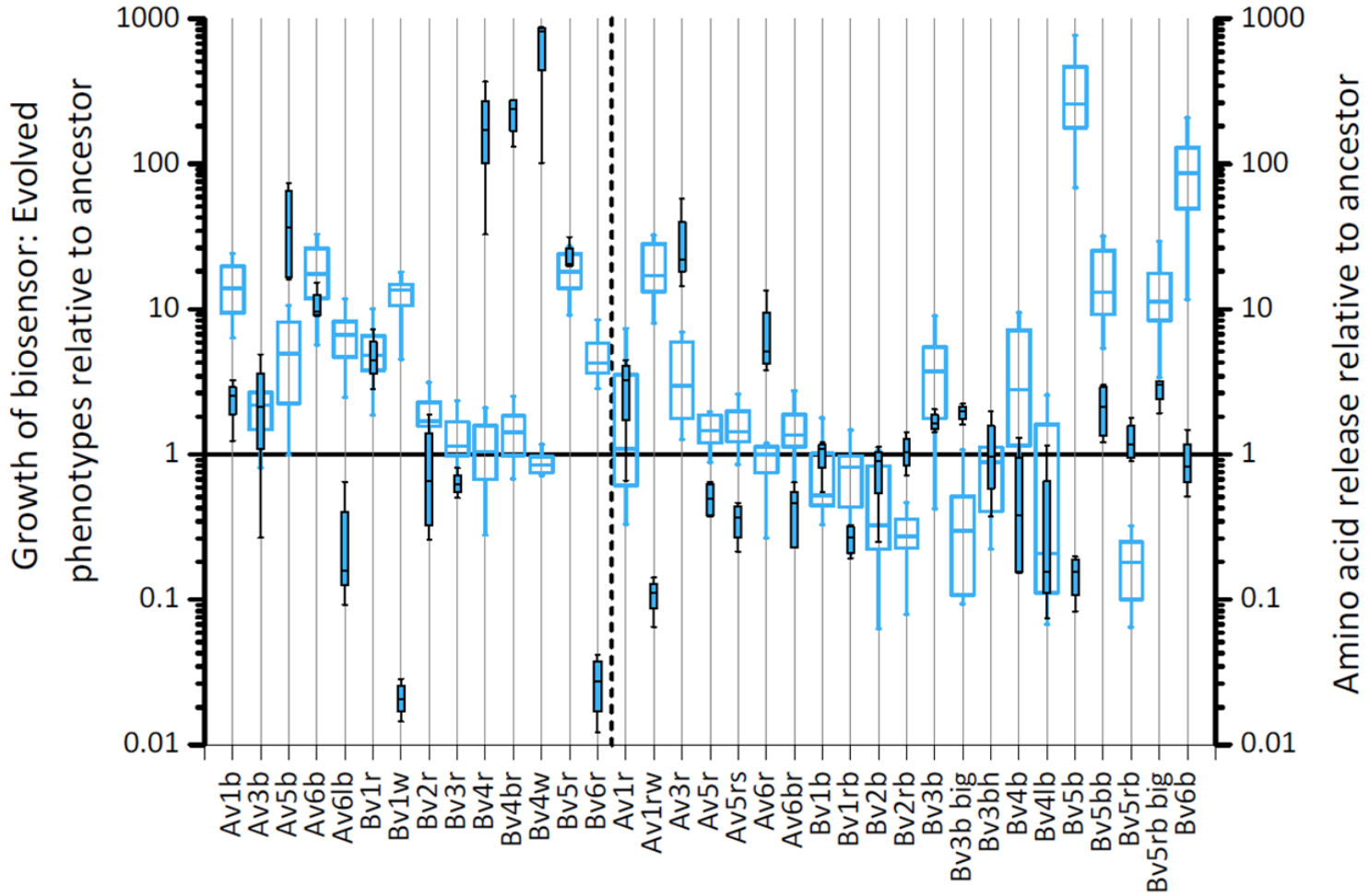

Figure S4: Individual measures of the changes in amino acid release in isolates from cocultures. Biosensor supplementation (empty boxes) and analytically determined amino acid concentrations in culture supernatant (filled boxes) are given for all 35 isolated phenotypes from cocultures. Values are given relative to the ancestor. The dotted horizontal line divides isolated phenotypes into tyrosine auxotrophs (left) and tryptophan auxotrophs (right). Isolates are labelled in regards to the population they originated from and their phenotypic appearance on modified TA-Agar plates. "Av" and "Bv" determine the initial genotypic composition of cocultures, which was either *E. coli ΔtyrA ara+ lacZ* and *E. coli ΔtrpB ara- ΔlacZ* or the reverse combination, respectively. The following digit indicates the number of replicated population, while letters are abbreviations for colony colour and/or size (e.g. "b" = blue, "r" = red, "lb" = large-blue, "rb" = red-blue, "rs" = red-small, "br" = bright red, "w" = white). Please see table S1 in the supplement for chapter 3 for an overview of analysed populations and attribution of individual isolates.

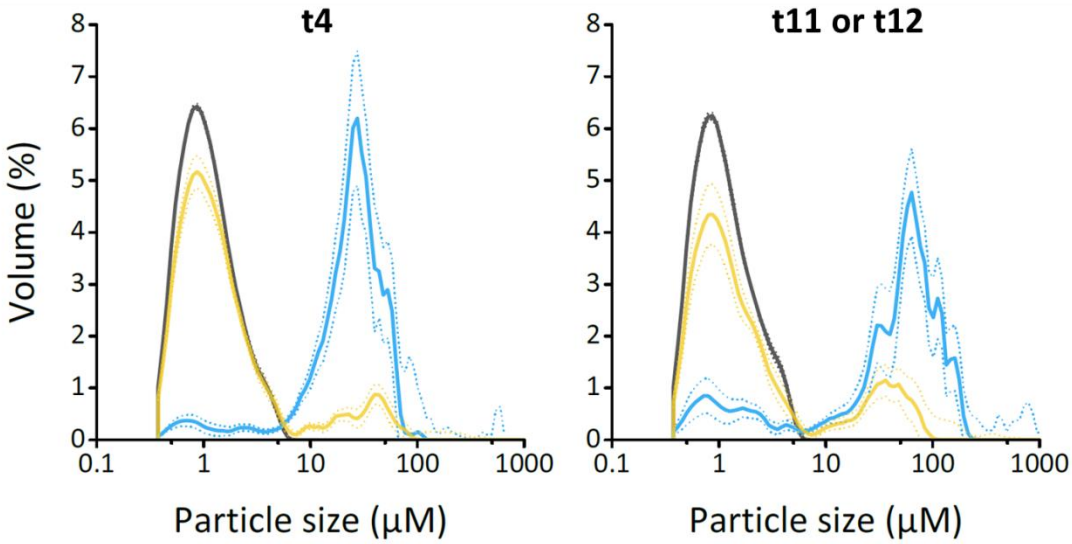

Figure S5: Analysis of size distribution in two time points of the evolution experiment confirms predominant cluster formation in cocultures. Size distribution in volume-percent of single cells and clusters of cells in wild type populations (grey, n=12), auxotrophic monoculture (yellow, n=24), and coculture (blue, n=10 - replicated three times). Populations from two time points of the evolution experiment (28 (t4) and 53 (t11) days for the wild type and cocultures; 28 (t4) and 56 (t12) days for auxotrophic monocultures) were analysed during exponential growth phase via laser diffraction spectroscopy. Lines are given as median with errors (95% confidence intervals) as dotted lines. The threshold to count particles as cell clusters was set at 10 µM, which is the maximum approximate length of two E.coli daughter cells that still stick together after division. To quantify differences in the extent of cluster formation, ratios of total volumes of particles >10 µM and <10 µM were calculated for all samples and compared between experimental groups. Cocultures exhibit significantly higher ratios than wildtype or monocultures in both analysed time points (Dunnett T3 *post hoc* test: $P<0.001$).

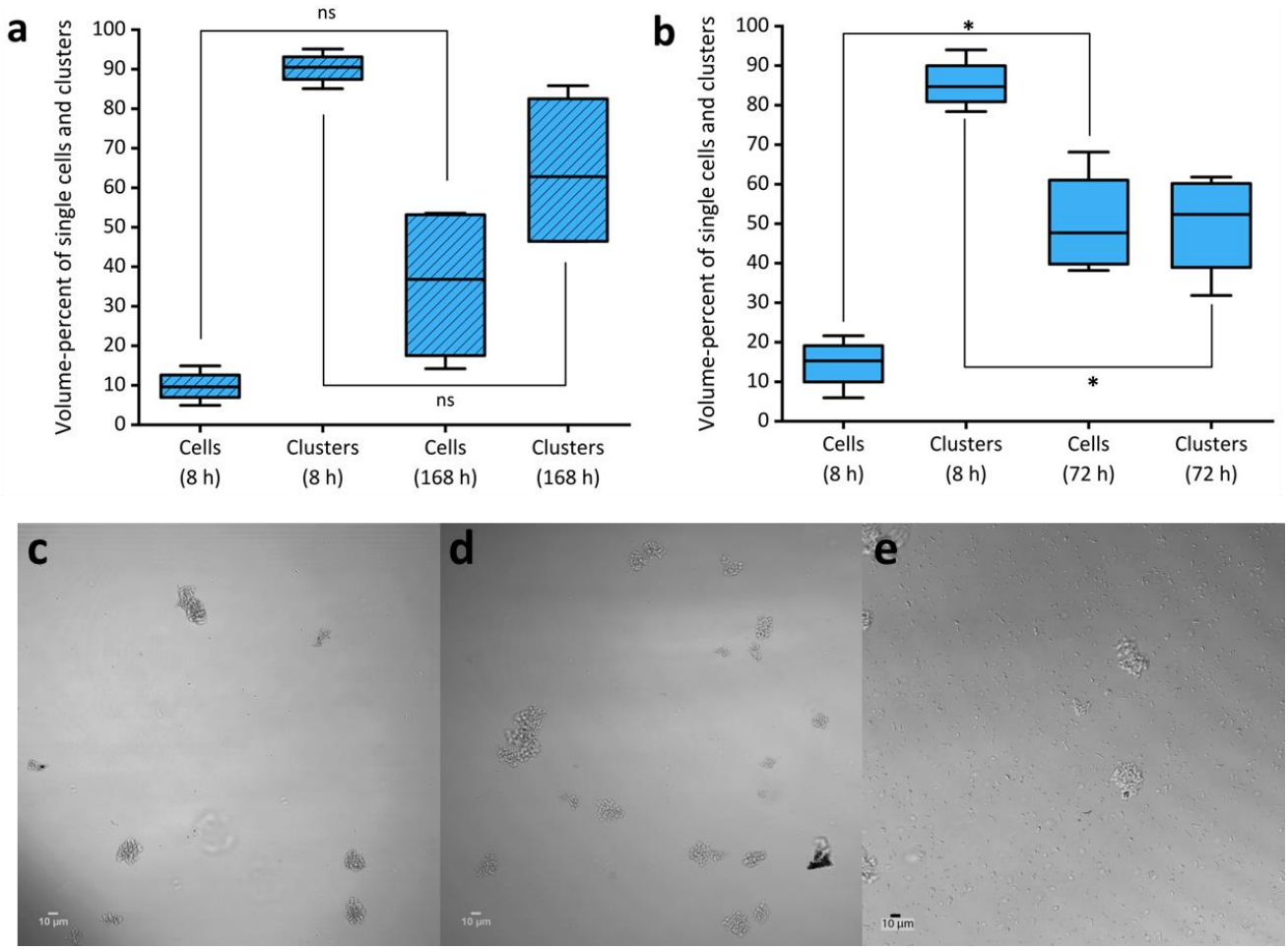

Figure S6: Nascent lifecycle of association and disassociation in cross-feeding populations. Populations of ancestral (a) and evolved (b) cocultures predominantly grow within multicellular clusters, yet start to disassociate into single cells when reaching late exponential growth phase. Transfer of saturated cocultures into fresh medium results in reassembly into clusters (data not shown). Cocultures were analysed for particle size distribution during the early (cultivation for 8 hours) and the terminal growth phase (168 hours for ancestral and 72 hours for evolved populations) utilizing laser diffraction spectroscopy. The threshold for counting particles as single cells was set conservatively to maximum 10 µM. Culture conditions were identical to the evolution experiment, yet culture volume was increased to 100 mL to meet requirements of analysis. Differences in dynamics of population structure are more pronounced in evolved cocultures (Paired samples t-test: $P<0.05$ and $n=4$ for both comparisons; significance indicated by "*") than ancestors (Wilcoxon signed ranks test: $P=0.068$ and $n=4$ for both comparisons; indicated by "ns"). (Courtesy of Samir Giri, University Osnabrück)

Microscopy pictures show clusters and single cells in an evolved coculture over time. Pictures were taken after 10 hours (c), 15 hours (d), and 22 hours (e). The earlier time points show few single cells and predominantly clusters (c ,and d), while disassociation of clusters into single cells is shown in the later time point (e). Pictures were taken with a Zeiss LSM 880 microscope at a magnification of 200x. Scale bars show 10 µm.

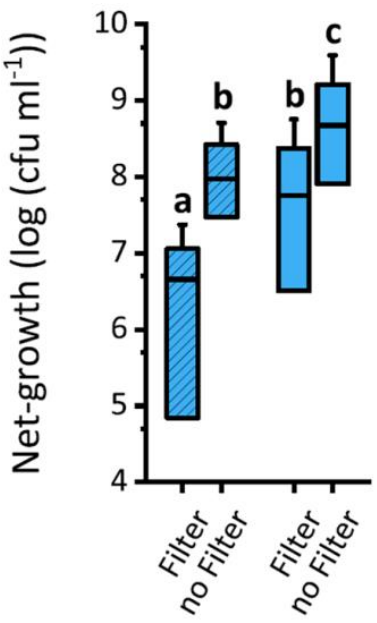

Figure S7: Cross-feeding populations show contact-dependent growth. Separation of interacting auxotrophs by a filter reduces growth in both, ancestral as well as evolved cocultures. Ancestral strains were combined to cocultures as described for initiating the evolution experiment, while isolated morphotypes were recombined pairwise on the population level and whole populations were reassembled by recombining all corresponding morphotypes in addition. Differences are more pronounced in ancestral cocultures. Letters above box-plots indicate significant differences between groups (Dunnett T3 *post hoc* test: P<0.01, n=40 for ancestral and n=144 for evolved cocultures).

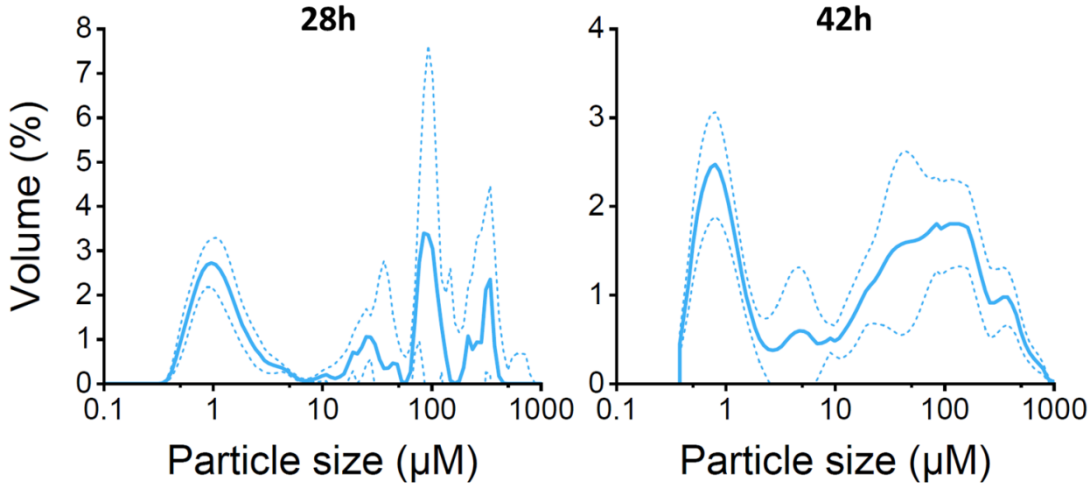

Figure S8: Analysis of size distribution within Nurmikko cells reveals prevalence of multicellular aggregates. Size distribution in volume-percent of single cells and clusters of cells in tripartite populations consisting of both *E. coli* biosensor strains and six isolates from evolved cocultures was analyzed via laser diffraction spectroscopy. Therefore ten Nurmikko cells were inoculated with technical replicates of tripartite cultures and the content (ten milliliters per cell) was pooled for further analysis. Pooled populations were analyzed after 28h and 42h incubation. Lines are given as median with errors (95% confidence intervals) as dotted lines. The threshold to count particles as cell clusters was set at 10 µM, which is the maximum approximate length of two *E.coli* daughter cells that still stick together after division. To quantify the extent of cluster formation, volumes of particles smaller than 10 µM and larger than 10 µM were calculated for all tested combinations of strains. Cell clusters in average represent ~60% of measured volume for both analyzed time points.

Supporting information for chapter 3

Supporting figures

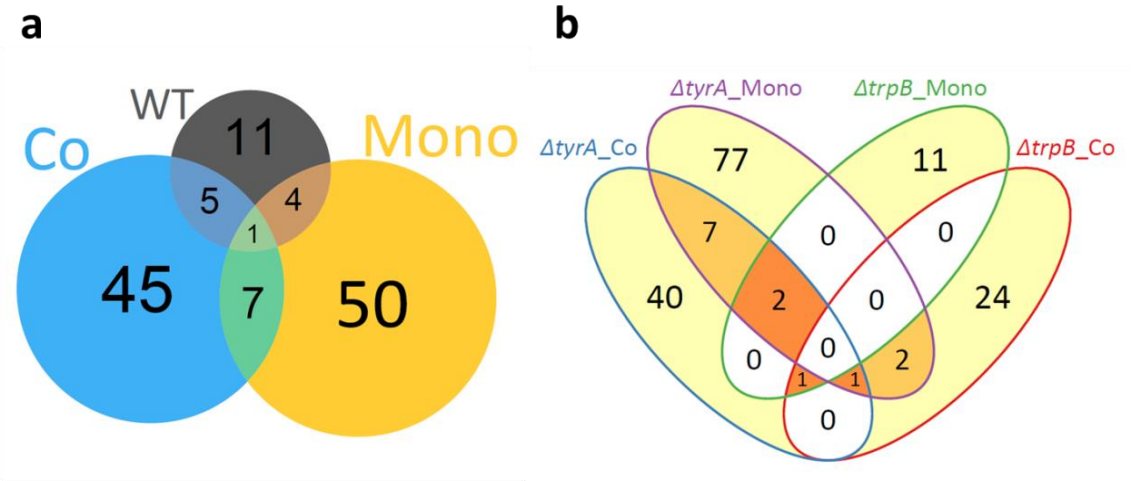

Figure S1: Experimental groups share few mutated genes. Qualitative comparisons comprise genes carrying a mutation or being subject of a deletion. Numbers in Venn-diagrams represent total counts of different genes. (**a**) Venn-diagram showing the overlap in mutated genes on the population-level between cocultures (Co), wild type (WT), and auxotrophic monocultures (Mono). Datasets were curated from population A10 (see Fig. 1a) that harbours an established mutator genotype as well as from constituents of amplifications beforehand. Due to high extent of similarity cocultures exhibit less individual genes that exhibit a mutation than monocultures of auxotrophs. (**b**) Venn-diagram showing the overlap in mutated genes between auxotrophic isolates (*i.e.* on the clonal level) from cocultures (Δ*tyrA*_Co, Δ*trpB*_Co) and monocultures (Δ*tyrA*_Mono, Δ*trpB*_Mono). Datasets were curated from constituents of amplifications beforehand, but contain mutated genes identified in mutator genotypes.

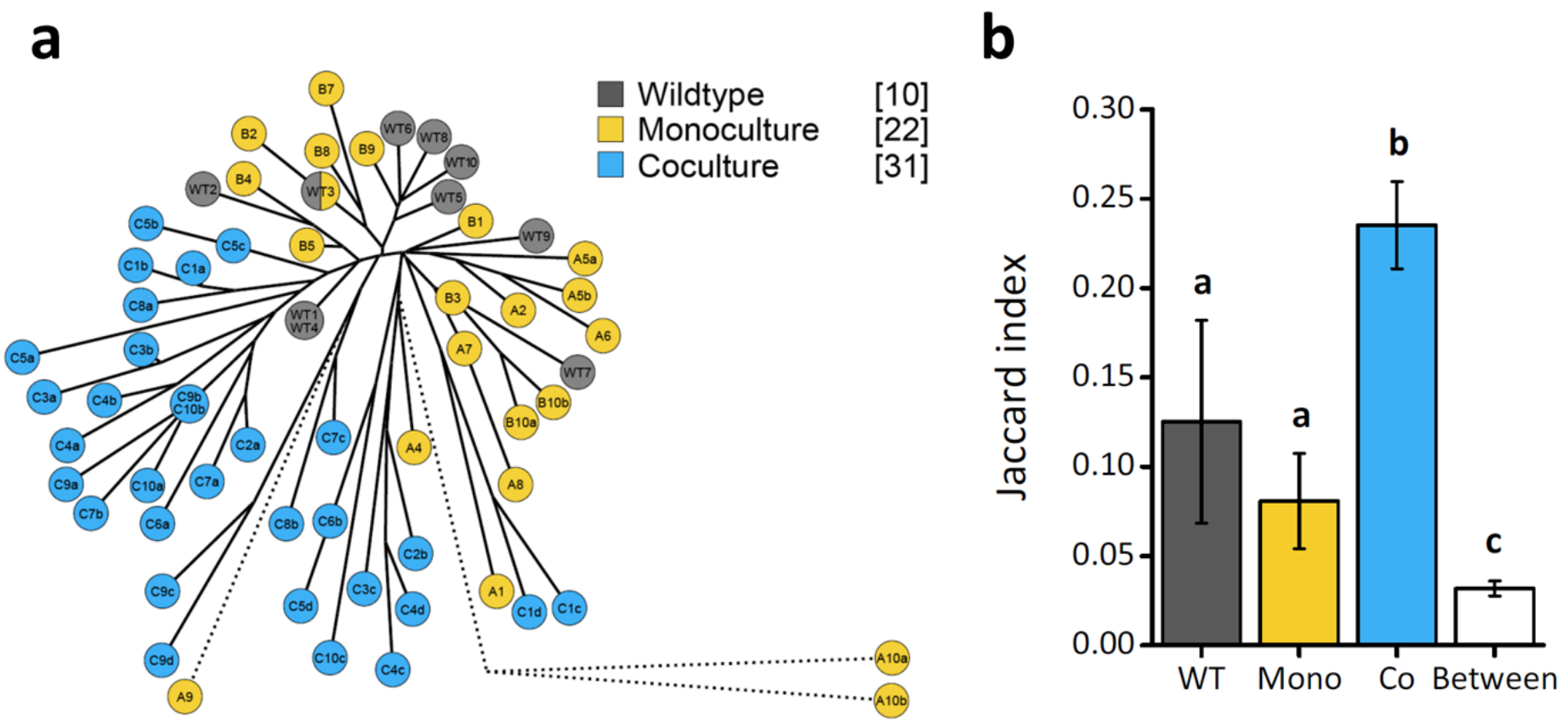

Figure S2: Degree of parallel evolution based on mutated genes in clonal samples. (**a**) Distance tree of clonal samples (A = tyrosine auxotrophic ,- and B = tryptophan auxotrophic isolate) utilizing standard neighbour-joining based on a genotyping matrix. Tryptophan auxotrophic isolates from cocultures cluster as one distinct group, while tyrosine auxotrophs from cocultures are split into three branches and share in certain cases mutated genes with tyrosine auxotrophs from monocultures. Dotted lines indicate shortened branches that exceeded a certain threshold (see methods). Similar numbers of auxotrophic isolates indicate a common biological replicate, *i.e*. the same ancestral preculture that was used to initiate the evolution experiment (See also table S1 for an overview). (**b**) Degree of parallel evolution based on mutated genes in clonal samples given as mean Jaccard indices with 95% confidence intervals. Values were calculated using Letters indicate significant differences (Dunett T3 *post hoc* test: $P < 0.05$, WT: n=45; Mono: n=110; Co: n=231, Between (all between-group comparisons): n=1567).

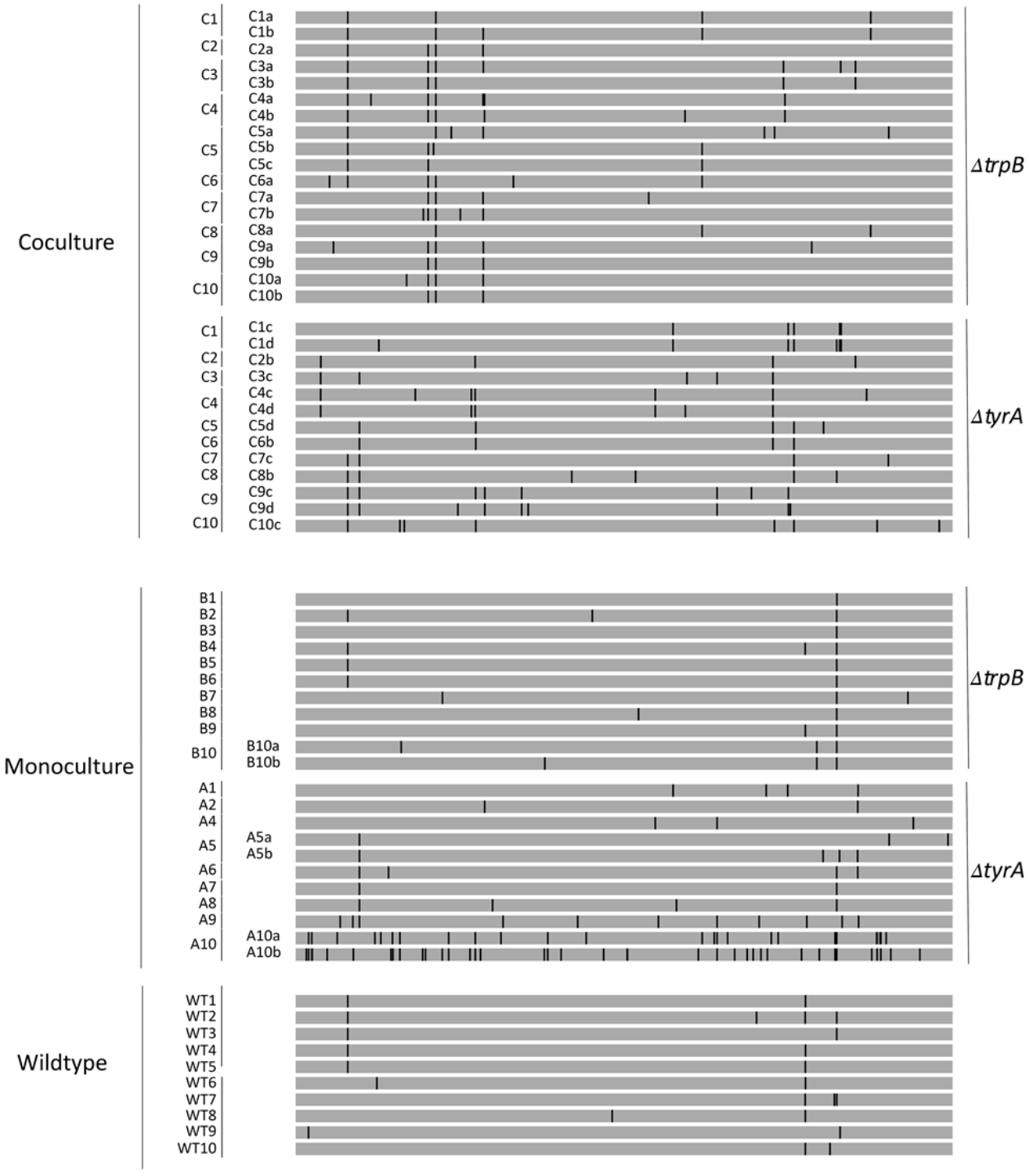

Figure S3: Mutated sites in evolved isolates show the extent of within-group parallelism and between-group divergence. Linear genomes of sequenced isolates from three experimental groups show accumulated mutations during the evolution experiment. Isolates are sorted by auxotrophy (Δ*trpB*: tryptophan auxotrophic genotype; Δ*tyrA*: tyrosine auxotropic genotype). Size and architecture of each mutation were not considered.

(Provided by Leonardo Ona Bubach, University of Osnabrück)

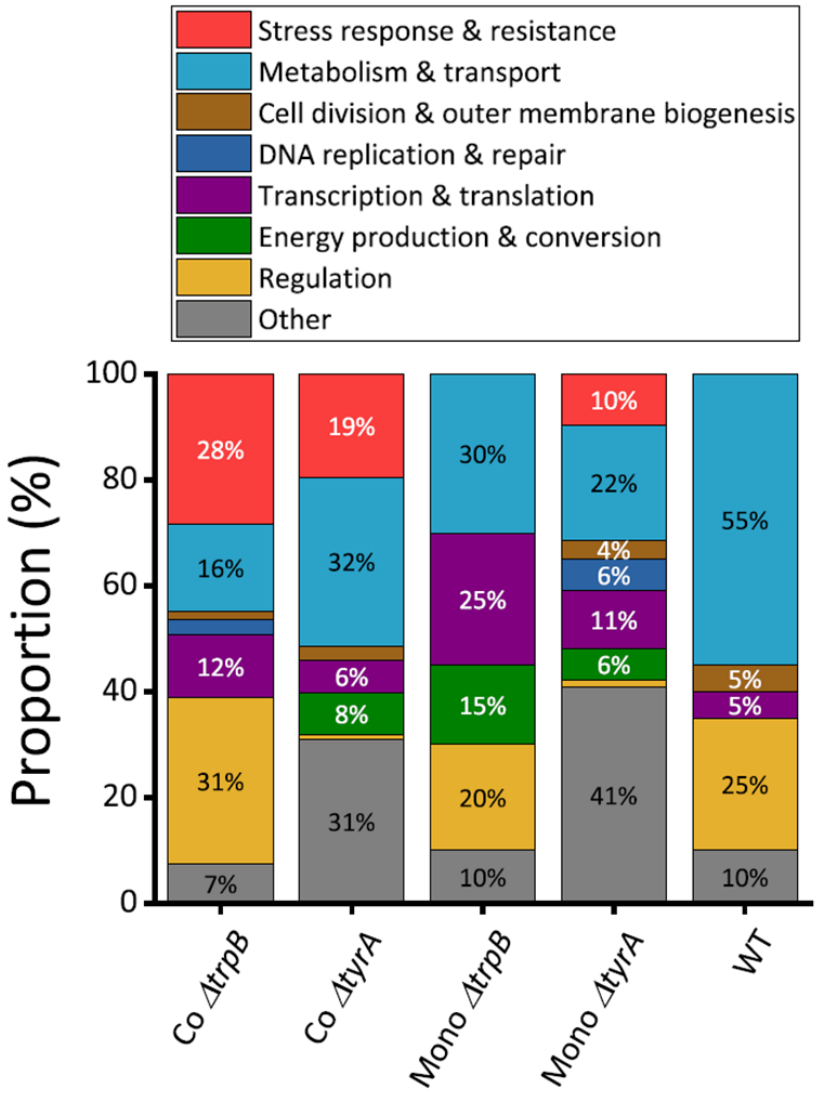

Figure S4: Proportion of mutated genes in clonal samples categorized by function. Functions of mutated genes were determined by using the EcoCyc-database (613). Mutator genotypes were included in this analysis, while genes that were part of amplifications were not considered. Datasets were corrected for pseudo-replication of genes that were identified multiple times within each population due to analysing related clones. The category "Other" predominantly comprises genes of unknown function.

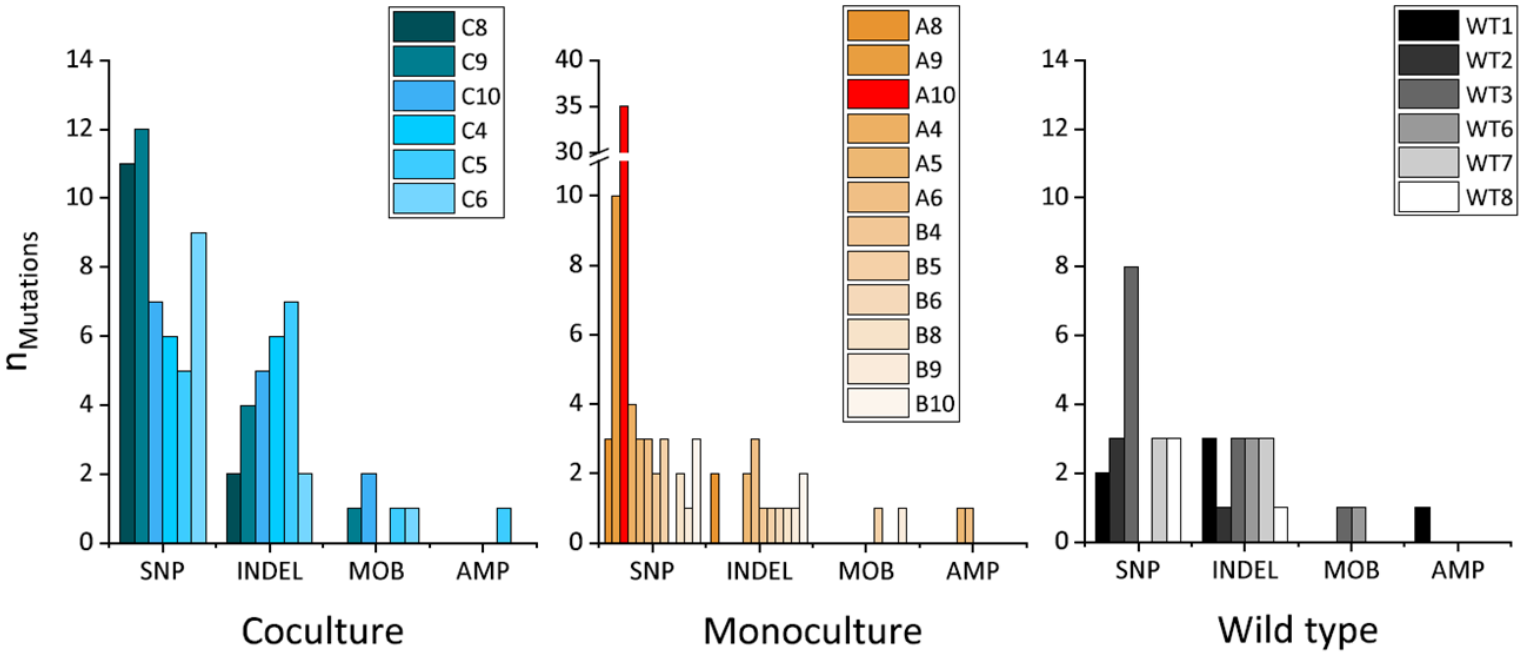

Figure S5: Spectrum of detected mutations in population samples. Depending on architecture, mutations were categorised in single nucleotide polymorphisms (SNP), insertions and deletions (INDEL), disruptions of genes by mobile elements (MOB), and amplifications of regions exceeding the size of a single gene (AMP). Population A10 (red column) contains the established mutator phenotype. Labelling of samples is explained in table S1.

Supporting tables

Table S1: Nomenclature of evolved populations and isolates. Denotations of samples from the three experimental groups (Cocultures consisting of two auxotrophic genotypes; monocultures of these genotypes, and the wildtype) are listed and if applicable sorted by auxotrophy. Denotations of population samples consisting of amino acid auxotrophs are given below "Pop". "Original" denotations are derived from experimental work and were later replaced by "New" denotations. In the case of wildtype samples denotations for whole populations and clones are similar (see "New"). If respective samples are labelled red, either sequencing was not successful (Bv2b, Bv3b) or a cross-contamination was detected (A3r) which entailed exclusion from further analysis. Yellow fields indicate samples containing a mutator phenotype. Continuous lines between samples indicate identical biological replicates used for initiating the evolution experiment.

Coculture						**Monoculture**						**Wildtype**	
	Δ trpB		*Δ tyrA*			*Δ trpB*			*Δ tyrA*				
Pop	New	Original	New	Original		Pop	New	Original	Pop	New	Original	New	Original
C1	**C1a** **C1b**	Bv1b Bv1rb	**C1c** **C1d**	Bv1r Bv1w		**B1**	**B1**	B1b	**A1**	**A1**	A1r	**WT1**	C1
C2	 **C2a**	Bv2b Bv2rb	**C2b**	Bv2r		**B2**	**B2**	B2b	**A2**	**A2**	A2r	**WT2**	C2
C3	 **C3a** **C3b**	Bv3b Bv3big Bv3bh	**C3c**	Bv3r		**B3**	**B3**	B3b			A3r	**WT3**	C3
C4	**C4a** **C4b**	Bv4b Bv4lb	**C4c** **C4d**	Bv4br Bv4r		**B4**	**B4**	B4b	**A4**	**A4**	A4r	**WT4**	C5
C5	**C5a** **C5b** **C5c**	Bv5b Bv5bb Bv5rb	**C5d**	Bv5r		**B5**	**B5**	B5b	**A5**	**A5a** **A5b**	A5r A5rs	**WT5**	C6
C6	**C6a**	Bv6b	**C6b**	Bv6r		**B6**	**B6**	B6b	**A6**	**A6**	A6r	**WT6**	D1
C7	**C7a** **C7b**	Av1r Av1rw	**C7c**	Av1b		**B7**	**B7**	B1r	**A7**	**A7**	A1b	**WT7**	D2
C8	**C8a**	Av3r	**C8b**	Av3b		**B8**	**B8**	B3r	**A8**	**A8**	A3b	**WT8**	D4
C9	**C9a** **C9b**	Av6br Av6r	**C9c** **C9d**	Av6b Av6lb		**B9**	**B9**	B6r	**A9**	**A9**	A6b	**WT9**	D5
C10	**C10a** **C10b**	Av5r Av5rs	**C10c**	Av5b		**B10**	**B10a** **B10b**	B5r B5rs	**A10**	**A10a** **A10b**	A5b A5w	**WT10**	D6

Table S2: List of detected mutations in clonal samples.
Samples from cocultures:

Predicted mutations									
position	**mutation**	**C7c**	**C7a**	**C7b**	**C8b**	**C8a**	**C10c**	**annotation**	**gene**
360,752	A→G	100%			100%		100%	V331A (GTG→GCG)	*lacI* ←
443,007	G→T	100%			100%			S52* (TCG→TAG)	*cyoE* ←
728,407	C→G						100%	G1123G (GGC→GGG)	*rhsC* →
760,381	G→T						100%	G249V (GGT→GTT)	*sucD* →
894,763	G→T			100%				A264S (GCA→TCA)	*rlmC* →
928,197	Δ1 bp			100%				coding (147/495 nt)	*lrp* →
928,488	Δ1 bp		100%					coding (438/495 nt)	*lrp* →
982,023	T→G					100%		E139A (GAA→GCA)	*ompF* ←
982,254	T→G		100%	100%				Y62S (TAT→TCT)	*ompF* ←
982,553	G→A		100%	100%				intergenic (-115/+488)	*ompF / asnS*
1,154,652	+AACT			100%				coding (1328/1434 nt)	*ptsG* →
1,263,811	C→T						100%	S64F (TCC→TTC)	*kdsA* →
1,315,284	Δ1 bp		100%					coding (358/1596 nt)	*trpD* ←
1,316,270	G→A			100%				L312F (CTC→TTC)	*trpE* ←
1,940,383	IS*1* +9 bp				100%			coding (47-55/66 nt)	*yobI* ←
2,391,144	C→T				100%			R834H (CGT→CAT)	*nuoG* ←
2,483,414	G→T		100%					F84L (TTC→TTA)	*yfdV* ←
2,860,476	G→T					100%		Y145* (TAC→TAA)	*rpoS* ←
3,370,422	Δ1 bp						100%	coding (358/639 nt)	*sspA* ←
3,508,077	IS*4* (–) +1						100%	coding (330-342/678 nt)	*rpe* ←
3,508,086	A→T	100%						C111* (TGT→TGA)	*rpe* ←
3,508,366	A→T				100%			L18Q (CTG→CAG)	*rpe* ←
3,809,219	Δ82 bp				100%				*[rph]–[rph]*
4,048,148	A→C					100%		V164G (GTG→GGG)	*glnG* ←
4,049,329	T→G					100%		H124P (CAC→CCC)	*glnL* ←
4,094,585	T→A						100%	N107Y (AAC→TAC)	*cpxA* ←
4,172,860	C→T	100%						T563I (ACC→ATC)	*rpoB* →
4,532,356	IS*1* (+) +9						100%	coding (503-511/597 nt)	*fimE* →

Samples from cocultures (continued):

Predicted mutations									
position	**mutation**	**C10a**	**C10b**	**C9a**	**C9c**	**C9d**	**C9b**	**annotation**	**gene**
259,769	A→C			100%				Y64D (TAT→GAT)	*ykfH* ←
360,752	A→G				100%	100%		V331A (GTG→GCG)	*lacI* ←
443,007	G→T				100%	100%		S52* (TCG→TAG)	*cyoE* ←
775,607	G→A		100%					W185* (TGG→TAG)	*ybgF* →
928,201	Δ1 bp			100%			100%	coding (151/495 nt)	*lrp* →
928,488	Δ1 bp	100%	100%					coding (438/495 nt)	*lrp* →
982,036	C→T	100%	100%	100%			100%	D135N (GAT→AAT)	*ompF* ←
982,56	T→A	100%	100%	100%			100%	intergenic (-122/+481)	*ompF / asnS*
1,137,696	C→A					100%		E710* (GAA→TAA)	*rne* ←
1,262,785	IS*5* (–) +4				100%			coding (10-13/810 nt)	*ychA* →
1,316,187	31 bp x 2	100%	100%					coding (1017/1563 nt)	*trpE* ←
1,316,866	Δ1 bp			100%			100%	coding (338/1563 nt)	*trpE* ←
1,327,816	C→T				100%	100%		Q838* (CAA→TAA)	*topA* →
1,586,828	C→T				100%	100%		intergenic (-129/+94)	*hipB / yneO*
1,634,074	C→T					100%		E81K (GAG→AAG)	*rrrQ* ←
2,964,912	C→T				100%	100%		intergenic (+64/-44)	*ygdR / tas*
3,207,549	G→A				100%			A382T (GCG→ACG)	*rpoD* →
3,467,928	IS*5* (–) +4				100%	100%		intergenic (-17/+106)	*rpsL / tusB*
3,482,817	Δ13 bp					100%		coding (711-723/1221 nt)	*argD* ←
3,632,942	$(C)_{5\rightarrow4}$			100%				coding (139/336 nt)	*uspB* ←

position	**mutation**	**C3a**	**C3b**	**C3c**	**C4c**	**C4a**	**C4b**	**annotation**	**gene**
170,28	Δ1 bp				100%			coding (1090/1281 nt)	*hemL* ←
170,326	IS*3* (+) +3			100%				coding (1042-1044/1281	*hemL* ←
360,752	A→G	100%	100%			100%	100%	V331A (GTG→GCG)	*lacI* ←
443,679	Δ13 bp			100%				coding (426-438/615 nt)	*cyoC* ←
524,284	$(T)_{7\rightarrow6}$					100%		pseudogene (189/491 nt)	*ybbD* →
837,145	C→T				100%			intergenic (-158/+107)	*fiu / mcbA*
928,124	+A					100%	100%	coding (74/495 nt)	*lrp* →
928,462	C→A	100%	100%					L138M (CTG→ATG)	*lrp* →
982,254	T→C	100%	100%					Y62C (TAT→TGT)	*ompF* ←
982,254	T→G					100%	100%	Y62S (TAT→TCT)	*ompF* ←
1,232,458	C→A				100%			E80D (GAG→GAT)	*ycgB* ←
1,259,153	T→A				100%			intergenic (-197/-17)	*lolB / hemA*
1,315,113	(CCAGCA					100%		coding (529/1596 nt)	*trpD* ←
1,317,092	Δ1 bp	100%						coding (112/1563 nt)	*trpE* ←
1,325,807	G→A					100%	100%	R168H (CGT→CAT)	*topA* →
2,529,193	A→G				100%			M1M (ATG→GTG) †	*crr* →
2,739,312	Δ7 bp						100%	coding (227-233/249 nt)	*rpsP* ←
2,753,751	$(T)_{6\rightarrow7}$			100%				coding (1408/1410 nt)	*yfjI* →
2,964,908	C→A			100%				intergenic (+60/-48)	*ygdR / tas*
2,965,688	T→A			100%				Y245N (TAT→AAT)	*tas* →
3,360,110	Δ10,078 b				100%			IS*5* -mediated	*[yhcE] –[sspA]*
3,360,110	Δ11,001 b			100%				IS*5* -mediated	*[yhcE] –sspA*
3,433,542	G→T	100%	100%					Q283K (CAG→AAG)	*rpoA* ←
3,443,617	Δ11 bp					100%	100%	coding (259-269/279 nt)	*rpsS* ←
3,837,085	A→G	100%						L22P (CTA→CCA)	*yicO* ←
3,941,547	Δ1 bp	100%	100%					coding (102/339 nt)	*yifE* →
4,019,893	Δ1 bp				100%			coding (7/702 nt)	*fre* →

Samples from cocultures (continued):

Predicted mutations									
position	**mutation**	**C1a**	**C1b**	**C1c**	**C1d**	**C2a**	**C2b**	**annotation**	**gene**
170,788	G→C						100%	Y194* (TAC→TAG)	*hemL* ←
360,752	A→G	100%	100%			100%		V331A (GTG→GCG)	*lacI* ←
580,519	IS*3* (+) +5				100%			coding (567-571/954 nt)	*ompT* ←
580,522	Δ2 bp				100%			coding (567-568/954 nt)	*ompT* ←
928,427	C→T					100%		A126V (GCC→GTC)	*lrp* →
982,023	T→C	100%	100%			100%		E139G (GAA→GGA)	*ompF* ←
982,56	T→A					100%		intergenic (-122/+481)	*ompF / asnS*
1,259,125	G→A						100%	intergenic (-169/-45)	*lolB / hemA*
1,315,235	Δ1 bp					100%		coding (407/1596 nt)	*trpD* ←
1,316,141	2 bp→A		100%					coding (1062-1063/1563	*trpE* ←
2,655,030	IS*1* (–) +9			100%	100%			coding (453-461/489 nt)	*iscR* ←
2,860,769	G→A	100%	100%					Q48* (CAG→TAG)	*rpoS* ←
3,360,110	Δ10,887 b						100%	IS*5* -mediated	*[yhcE] –sspA*
3,463,484	IS*1* (+) +9			100%				intergenic (-272/+12)	*chiA / tufA*
3,463,619	A→C			100%				I357S (ATC→AGC)	*tufA* ←
3,464,196	C→T			100%				G165S (GGC→AGC)	*tufA* ←
3,467,845	C→T			100%	100%			A23T (GCG→ACG)	*rpsL* ←
3,507,896	C→A			100%	100%			E175* (GAA→TAA)	*rpe* ←
3,809,170	Δ1 bp				100%			intergenic (-42/+24)	*pyrE / rph*
3,830,184	C→A			100%	100%			intergenic (+268/-129)	*yicT / setC*
3,840,246	G→A			100%	100%			T94T (ACC→ACT)	*uhpT* ←
3,941,236	G→T						100%	A31E (GCA→GAA)	*hdfR* ←
4,049,242	T→C	100%	100%					Q153R (CAG→CGG)	*glnL* ←

Samples from cocultures (continued):

Predicted mutations										
position	**mutation**	**C4d**	**C5b**	**C5a**	**C5c**	**C5d**	**C6a**	**C6b**	**annotation**	**gene**
170,28	Δ1 bp	100%							coding (1090/1281 nt)	*hemL* ←
232,44	G→A						100%		P17L (CCA→CTA)	*rnhA* ←
360,752	A→G		100%	100%	100%		100%		V331A (GTG→GCG)	*lacI* ←
443,679	Δ13 bp					100%		100%	coding (426-438/615 nt)	*cyoC* ←
928,427	C→T						100%		A126V (GCC→GTC)	*lrp* →
928,489	T→C		100%		100%				Y147H (TAC→CAC)	*lrp* →
966,495	31,651 bp		100%						amplification	*kdsB –elfG*
982,035	T→G			100%					D135A (GAT→GCT)	*ompF* ←
982,248	C→T						100%		R64H (CGT→CAT)	*ompF* ←
982,683	IS*2* (+) +5 bp						100%		intergenic (-245/+354)	*ompF / asnS*
1,091,894	IS*5* (–) +4 bp			100%					coding (596-599/987 nt)	*ycdU* →
1,232,458	C→A	100%							E80D (GAG→GAT)	*ycgB* ←
1,259,153	T→A	100%							intergenic(-197/-17)	*lolB / hemA*
1,263,967	C→T							100%	A116V (GCG→GTG)	*kdsA* →
1,264,228	C→T					100%			A203V (GCA→GTA)	*kdsA* →
1,316,015	Δ1 bp			100%					coding(1189/1563 nt)	*trpE* ←
1,529,286	$(T)_{5\rightarrow6}$						100%		coding (830/894 nt)	*yddE* ←
1,529,287	C→T						100%		E277K (GAA→AAA)	*yddE* ←
2,529,193	A→G	100%							M1M (ATG→GTG) †	*crr* →
2,741,712	C→A	100%							S131* (TCG→TAG)	*ypjD* →
2,860,039	+A		100%		100%				coding (872/993 nt)	*rpoS* ←
2,860,539	G→T						100%		N124K (AAC→AAA)	*rpoS* ←
3,299,592	Δ11 bp			100%					coding (1618-1628/1890 nt)	*deaD* ←
3,360,110	Δ10,078 b	100%							IS*5* -mediated	*yhcE –sspA*
3,360,110	Δ10,892 b					100%			IS*5* -mediated	*yhcE –sspA*
3,360,110	Δ10,906 b							100%	IS*5* -mediated	*yhcE –sspA*
3,371,313	Δ6 bp			100%					coding (249-254/393 nt)	*rpsI* ←
3,508,262	Δ2 bp					100%			coding (156-157/678 nt)	*rpe* ←
3,508,366	A→T							100%	L18Q (CTG→CAG)	*rpe* ←
3,716,165	G→A					100%			R531R (CGC→CGT)	*glyS* ←
4,176,533	A→C			100%					H419P (CAC→CCC)	*rpoC* →

Samples from monocultures of auxotrophs:

Predicted mutations									
position	**mutation**	**A7**	**A1**	**A2**	**A8**	**A4**	**A5b**	**annotation**	**gene**
443,007	G→T	100%			100%			S52* (TCG→TAG)	*cyoE* ←
443,679	Δ13 bp						100%	coding (426-438/615 nt)	*cyoC* ←
1,326,386	C→T			100%				S361L (TCA→TTA)	*topA* →
1,381,880	G→A				100%			E302K (GAA→AAA)	*tyrR* →
2,529,193	A→G					100%		M1M (ATG→GTG) †	*crr* →
2,655,030	IS*1* (–) +9		100%					coding (453-461/489 nt)	*iscR* ←
2,655,103	C→A		100%					V130F (GTT→TTT)	*iscR* ←
2,655,680	G→G		100%					intergenic (-190/+262)	*iscR / trmJ*
2,679,434	$(A)_{6\rightarrow5}$				100%			coding (241/1191 nt)	*hmp* →
2,965,688	T→G					100%		Y245D (TAT→GAT)	*tas* →
3,311,913	G→T		100%					intergenic (-265/-83)	*metY / argG*
3,463,484	IS*1* (+) +9		100%					intergenic (-272/+12)	*chiA / tufA*
3,463,619	A→C		100%					I357S (ATC→AGC)	*tufA* ←
3,464,196	C→T		100%					G165S (GGC→AGC)	*tufA* ←
3,713,593	114,908 b						100%	amplification	*[cspA] –[yicJ]*
3,809,170	Δ1 bp	100%			100%			intergenic (-42/+24)	*pyrE / rph*
3,956,890	C→A						100%	E234* (GAA→TAA)	*gpp* ←
3,957,345	Δ11 bp			100%				coding (235-245/1485 nt)	*gpp* ←
3,959,912	G→A		100%					G46S (GGC→AGC)	*rho* →
4,349,672	T→C					100%		R59R (CGA→CGG)	*cadB* ←

Samples from monocultures of auxotrophs (continued):

Predicted mutations									
position	**mutation**	**A5a**	**A10a**	**A10b**	**A9**	**A6**	**B1**	**annotation**	**gene**
68,521	C→A			100%				G228G (GGC→GGA)	*yabI* →
85,062	T→G		100%	100%				L183R (CTT→CGT)	*cra* →
107,669	G→T		100%	100%				E47* (GAA→TAA)	*mutT* →
110,351	T→G			100%				F141V (TTC→GTC)	*guaC* →
215,883	T→G			100%				M67L (ATG→CTG)	*tsaA* ←
286,949	T→G		100%					K37Q (AAA→CAA)	*insA* ←
307,295	G→T				100%			intergenic (-248/-528)	*ecpR / ykgL*
396,685	G→T				100%			intergenic (-306/-157)	*ddlA / iraP*
400,961	T→G			100%				D47A (GAC→GCC)	*proC* ←
443,007	G→T				100%			S52* (TCG→TAG)	*cyoE* ←
443,679	Δ13 bp	100%				100%		coding (426-438/615 nt)	*cyoC* ←
552,583	T→G		100%					L205L (CTA→CTC)	*folD* ←
594,186	T→G		100%					I6S (ATT→AGT)	*cusA* →
648,088	G→A					100%		S133N (AGC→AAC)	*citA* →
666,309	T→G			100%				Q251H (CAA→CAC)	*holA* ←
676,291	T→G		100%					G208G (GGT→GGG)	*djlC* →
679,856	T→G			100%				I5L (ATT→CTT)	*rihA* ←
728,407	C→G		100%	100%				G1123G (GGC→GGG)	*rhsC* →
890,099	A→C			100%				K287T (AAA→ACA)	*potF* →
909,163	A→C			100%				D36E (GAT→GAG)	*hcp* ←
909,323	T→G			100%				intergenic (-53/+91)	*hcp / ybjE*
1,027,245	A→C			100%				intergenic (-77/-350)	*serT / hyaA*
1,072,807	T→G		100%	100%				Q511P (CAG→CCG)	*putA* ←
1,222,912	A→C			100%				I6S (ATC→AGC)	*pliG* ←
1,260,081	A→C		100%	100%				Q304H (CAA→CAC)	*hemA* →
1,297,346	T→G			100%				F64C (TTC→TGC)	*oppB* →
1,441,605	A→C		100%					intergenic (-65/-171)	*feaR / feaB*
1,456,010	C→A				100%			T287T (ACC→ACA)	*paaJ* →
1,747,319	T→G			100%				K257Q (AAA→CAA)	*ydhV* ←
1,771,018	T→G			100%				F392C (TTC→TGC)	*ydiF* →
1,771,247	T→G		100%	100%				T468T (ACT→ACG)	*ydiF* →
1,865,867	T→G			100%				F409C (TTT→TGT)	*yeaI* →
1,983,151	C→G				100%			intergenic (-180/-11)	*yecH / tyrP*
2,043,291	A→C		100%					K1625Q (AAG→CAG)	*yeeJ* →
2,165,985	T→G			100%				K124Q (AAG→CAG)	*gatD* ←
2,331,566	T→G			100%				D445A (GAT→GCT)	*gyrA* ←
2,334,315	A→C			100%				S1112A (TCG→GCG)	*yfaL* ←
2,552,058	Δ6,790 bp				100%				*intZ–[eutA]*
2,833,790	T→G			100%				L303R (CTG→CGG)	*ascF* →
2,860,529	T→G		100%					I128L (ATC→CTC)	*rpoS* ←

Samples from monocultures of auxotrophs (continued):

Predicted mutations									
position	**mutation**	**A5r**	**A5b**	**A5w**	**A6b**	**A6r**	**B1b**	**annotation**	**gene**
2,861,047	A→C		100%					S356A (TCA→GCA)	*nlpD* ←
2,945,404	T→G		100%					Q139H (CAA→CAC)	*recD* ←
2,964,891	T→G		100%	100%				intergenic (+43/-65)	*ygdR / tas*
2,964,912	C→T				100%			intergenic (+64/-44)	*ygdR / tas*
2,965,686	A→C		100%	100%				K244T (AAA→ACA)	*tas* →
3,039,528	T→G		100%					*958S (TAA→TCA)	*gcvP* ←
3,089,987	T→G			100%				I82M (ATT→ATG)	*yggU* →
3,178,086	T→G			100%				intergenic (-261/-113)	*ribB / yqiC*
3,220,607	A→C			100%				I339L (ATC→CTC)	*ygjI* →
3,262,186	G→T				100%			R138M (AGG→ATG)	*yhaC* →
3,274,406	T→G			100%				pseudogene (347/375 nt)	*agaW* →
3,320,032	A→C			100%				V88G (GTT→GGT)	*ftsH* ←
3,348,437	T→G		100%					D118E (GAT→GAG)	*gltB* →
3,396,245	A→C		100%					V149V (GTT→GTG)	*csrD* ←
3,561,521	T→G			100%				I390L (ATT→CTT)	*glgC* ←
3,597,923	G→T				100%			L57L (CTG→CTT)	*rsmD* →
3,686,520	T→G			100%				K693Q (AAA→CAA)	*bcsA* ←
3,797,270	T→G		100%	100%				L76L (CTA→CTC)	*waaB* ←
3,809,167	A→C		100%	100%				intergenic (-39/+27)	*pyrE / rph*
3,809,219	Δ82 bp					100%			*[rph] –[rph]*
3,809,971	C→T						100%	intergenic (-62/-65)	*rph / yicC*
3,847,231	G→T				100%			intergenic (+229/-51)	*tisB / emrD*
3,957,279	Δ14 bp					100%		coding (298-311/1485 nt)	*gpp* ←
3,963,666	C→A				100%			L58L (CTC→CTA)	*wecB* →
4,056,312	T→G			100%				C236G (TGT→GGT)	*yihN* →
4,091,222	T→G		100%					F162C (TTC→TGC)	*sodA* →
4,094,585	T→A		100%	100%				N107Y (AAC→TAC)	*cpxA* ←
4,114,904	T→G		100%					K612T (AAA→ACA)	*priA* ←
4,119,848	T→G		100%	100%				L29W (TTG→TGG)	*metL* →
4,158,566	T→G		100%					intergenic (+191/-3)	*gltT / rrlB*
4,178,879	Δ9 bp	100%						coding (3602-3610/4224	*rpoC* →
4,191,132	A→C			100%				L213F (TTA→TTC)	*yjaH* →
4,395,715	A→C			100%				E404D (GAA→GAC)	*purA* →
4,593,453	IS*5* (–) +4	100%						coding (160-163/726 nt)	*yjjQ* →

Samples from monocultures of auxotrophs (continued):

Predicted mutations									
position	**mutation**	**B2**	**B3**	**B4**	**B5**	**B7**	**B8**	**annotation**	**gene**
360,752	A→G	100%		100%	100%			V331A (GTG→GCG)	*lacI* ←
1,028,484	G→A					100%		C297Y (TGT→TAT)	*hyaA* →
2,085,908	G→A	100%						A10A (GCG→GCA)	*hisC* →
2,411,824	C→T						100%	A422V (GCG→GTG)	*yfcC* →
3,586,809	C→T			100%				G253D (GGT→GAT)	*livG* ←
3,809,111	G→C			100%				R6R (CGC→CGG)	*pyrE* ←
3,809,170	Δ1 bp		100%					intergenic (-42/+24)	*pyrE / rph*
3,809,185	C→T				100%			intergenic (-57/+9)	*pyrE / rph*
3,809,219	Δ82 bp	100%				100%			*[rph] –[rph]*
3,809,243	Δ1 bp						100%	pseudogene (667/669 nt)	*rph* ←
4,310,911	G→T					100%		L52M (CTG→ATG)	*phnH* ←
position	**mutation**	**B6**	**B9**	**B10a**	**B10b**			**annotation**	**gene**
360,752	A→G	100%						V331A (GTG→GCG)	*lacI* ←
737,74	C→A			100%				V91V (GTG→GTT)	*dtpD* ←
1,750,759	C→T				100%			R269C (CGT→TGT)	*pykF* →
3,587,712	C→A		100%					E377* (GAG→TAG)	*livM* ←
3,669,232	T→G			100%	100%			F363V (TTC→GTC)	*yhjE* →
3,809,170	Δ1 bp				100%			intergenic (-42/+24)	*pyrE / rph*
3,809,184	G→T			100%				intergenic (-56/+10)	*pyrE / rph*
3,809,219	Δ82 bp	100%	100%						*[rph] –[rph]*

Samples from wildtype monocultures:

Predicted mutations								
position	**mutation**	**C1**	**C2**	**C3**	**C5**	**C6**	**annotation**	**gene**
360,752	A→G	100%	100%	100%	100%	100%	V331A (GTG→GCG)	*lacI* ←
3,243,770	C→A		100%				intergenic (+35/-151)	*yqjk / yqjF*
3,587,271	+T		100%				coding (296/768 nt)	*livG* ←
3,588,245	A→C					100%	L199R (CTC→CGC)	*livM* ←
3,588,985	C→A	100%					G260V (GGC→GTC)	*livH* ←
3,589,279	+A				100%		coding (485/927 nt)	*livH* ←
3,809,165	A→G		100%				intergenic (-37/+29)	*pyrE / rph*
3,809,219	Δ82 bp			100%				*[rph] –[rph]*
position	**mutation**	**D1**	**D2**	**D4**	**D5**	**D6**	**annotation**	**gene**
83,95	C→T				100%		A36V (GCG→GTG)	*ilvH* →
566,524	IS*3* (+) +4	100%					coding (176-179/552 nt)	*ybcL* →
566,528	Δ1 bp	100%					coding (180/552 nt)	*ybcL* →
2,225,507	C→T			100%			A62V (GCC→GTC)	*cdd* →
3,586,409	C→T		100%				R130H (CGC→CAC)	*livF* ←
3,588,171	G→A			100%			Q224* (CAG→TAG)	*livM* ←
3,588,201	C→A					100%	E214* (GAA→TAA)	*livM* ←
3,588,703	Δ8 bp	100%					coding (131-138/1278 nt)	*livM* ←
3,762,154	G→T					100%	pseudogene (156/252 nt)	*yibS* →
3,793,310	T→C		100%				K83E (AAA→GAA)	*waaZ* ←
3,809,170	Δ1 bp		100%				intergenic (-42/+24)	*pyrE / rph*
3,832,624	IS*1* (+) +8				100%		coding (723-730/819 nt)	*nlpA* ←
3,832,633	+G				100%		coding (721/819 nt)	*nlpA* ←
3,833,335	G→T				100%		H7N (CAT→AAT)	*nlpA* ←

Table S3: List of detected mutations in population samples.
Population samples of cocultures.

Predicted mutations									
position	**mutation**	**C8**	**C10**	**C9**	**C4**	**C5**	**C6**	**annotation**	**gene**
170,28	Δ1 bp				50.7%			coding (1090/1281 nt)	*hemL* ←
360,752	A→G	24.8%	62.3%	48.8%	29.4%	27.8%	30.2%	V331A (GTG→GCG)	*lacI* ←
443,007	G→T	41.4%		66.7%				S52* (TCG→TAG)	*cyoE* ←
443,679	Δ13 bp					50.6%	50.0%	coding (426-438/615 nt)	*cyoC* ←
728,407	C→G		76.7%					G1123G (GGC→GGG)	*rhsC* →
927,772	G→A						39.5%	intergenic (-266/-279)	*trxB / lrp*
928,201	Δ1 bp			30.9%				coding (151/495 nt)	*lrp* →
928,488	Δ1 bp		19.3%					coding (438/495 nt)	*lrp* →
966,495	31,651 bp x 5					100%		amplification	*[kdsB] –[elfG]*
982,023	T→G	60.8%						E139A (GAA→GCA)	*ompF* ←
982,035	T→G					35.7%		D135A (GAT→GCT)	*ompF* ←
982,036	C→T		20.8%	33.1%				D135N (GAT→AAT)	*ompF* ←
982,248	C→T						48.5%	R64H (CGT→CAT)	*ompF* ←
982,254	T→G				40.2%			Y62S (TAT→TCT)	*ompF* ←
982,56	T→A		21.1%	34.2%				intergenic (-122/+481)	*ompF / asnS*
982,683	IS*2* (+) +5 bp						50.6%	intergenic (-245/+354)	*ompF / asnS*
1,091,894	IS*5* (–) +4 bp					100%		coding (596-599/987 nt)	*ycdU* →
1,137,696	C→A			25.6%				E710* (GAA→TAA)	*rne* ←
1,154,225	G→A			11.4%				E301K (GAG→AAG)	*ptsG* →
1,232,458	C→A				19.6%			E80D (GAG→GAT)	*ycgB* ←
1,259,153	T→A				22.8%			intergenic (-197/-17)	*lolB / hemA*
1,263,811	C→T		75.0%					S64F (TCC→TTC)	*kdsA* →
1,263,967	C→T						44.4%	A116V (GCG→GTG)	*kdsA* →
1,264,228	C→T					49.3%		A203V (GCA→GTA)	*kdsA* →
1,282,249	A→G					6.3%	8.3%	intergenic (+267/+294)	*narI / tpr*
1,315,113	(CCAGCAGGCGAGCGCCCTGGGT)$_{1\to2}$				16.1%			coding (529/1596 nt)	*trpD* ←
1,315,951	(CATACAGGCGC)$_{1\to2}$				7.9%			coding (1253/1563 nt)	*trpE* ←
1,316,015	Δ1 bp					38.9%		coding (1189/1563 nt)	*trpE* ←
1,316,187	(TTCAATACGGCTGTCGAGATCTCTGTCCAGT)$_{1\to2}$		10.7%					coding (1017/1563 nt)	*trpE* ←
1,316,426	C→A			9.2%				E260* (GAA→TAA)	*trpE* ←

Population samples of cocultures (continued):

Predicted mutations									
position	**mutation**	**C8**	**C10**	**C9**	**C4**	**C5**	**C6**	**annotation**	**gene**
170,28	Δ1 bp				50.7%			coding (1090/1281 nt)	*hemL* ←
360,752	A→G	24.8%	62.3%	48.8%	29.4%	27.8%	30.2%	V331A (GTG→GCG)	*lacI* ←
443,007	G→T	41.4%		66.7%				S52* (TCG→TAG)	*cyoE* ←
443,679	Δ13 bp					50.6%	50.0%	coding (426-438/615 nt)	*cyoC* ←
728,407	C→G		76.7%					G1123G (GGC→GGG)	*rhsC* →
927,772	G→A						39.5%	intergenic (-266/-279)	*trxB / lrp*
928,201	Δ1 bp			30.9%				coding (151/495 nt)	*lrp* →
928,488	Δ1 bp		19.3%					coding (438/495 nt)	*lrp* →
966,495	31,651 bp x 5					18.6%		amplification	*[kdsB]–[elfG]*
982,023	T→G	60.8%						E139A (GAA→GCA)	*ompF* ←
982,035	T→G					35.7%		D135A (GAT→GCT)	*ompF* ←
982,036	C→T		20.8%	33.1%				D135N (GAT→AAT)	*ompF* ←
982,248	C→T						48.5%	R64H (CGT→CAT)	*ompF* ←
982,254	T→G				40.2%			Y62S (TAT→TCT)	*ompF* ←
982,56	T→A		21.1%	34.2%				intergenic (-122/+481)	*ompF / asnS*
982,683	IS*2* (+) +5 bp						50.6%	intergenic (-245/+354)	*ompF / asnS*
1,091,894	IS*5* (–) +4 bp					41.7%		coding (596-599/987 nt)	*ycdU* →
1,137,696	C→A			25.6%				E710* (GAA→TAA)	*rne* ←
1,154,225	G→A			11.4%				E301K (GAG→AAG)	*ptsG* →
1,232,458	C→A				19.6%			E80D (GAG→GAT)	*ycgB* ←
1,259,153	T→A				22.8%			intergenic (-197/-17)	*lolB / hemA*
1,263,811	C→T		75.0%					S64F (TCC→TTC)	*kdsA* →
1,263,967	C→T						44.4%	A116V (GCG→GTG)	*kdsA* →
1,264,228	C→T					49.3%		A203V (GCA→GTA)	*kdsA* →
1,282,249	A→G					6.3%	8.3%	intergenic (+267/+294)	*narI / tpr*
1,315,113	(CCAGCAGGCGAGCGCCCTGGGT)$_{1\rightarrow2}$				16.1%			coding (529/1596 nt)	*trpD* ←
1,315,951	(CATACAGGCGC)$_{1\rightarrow2}$				7.9%			coding (1253/1563 nt)	*trpE* ←
1,316,015	Δ1 bp					38.9%		coding (1189/1563 nt)	*trpE* ←
1,316,187	(TTCAATACGGCTGTCGAGATCTCTGTCCAGT)$_{1\rightarrow2}$		10.7%					coding (1017/1563 nt)	*trpE* ←
1,316,426	C→A			9.2%				E260* (GAA→TAA)	*trpE* ←

Population samples of cocultures (continued):

position	mutation	G	H	I	J	K	L	annotation	gene
1,316,866	Δ1 bp			13.8%				coding (338/1563 nt)	*trpE* ←
1,317,165	G→T						38.7%	C13* (TGC→TGA)	*trpE* ←
1,325,807	G→A				44.1%			R168H (CGT→CAT)	*topA* →
1,327,816	C→T			62.7%				Q838* (CAA→TAA)	*topA* →
1,530,289	Δ3 bp	11.1%						coding (584-586/681 nt)	*narV* ←
1,586,828	C→T			60.5%				intergenic (-129/+94)	*hipB / yneO*
1,634,074	C→T			24.5%				E81K (GAG→AAG)	*rrrQ* ←
1,703,101	C→G	6.3%						A642A (GCC→GCG)	*rsxC* →
2,298,492	A→G					6.2%		intergenic (+620/+95)	*eco / mqo*
2,391,144	C→T	57.0%						R834H (CGT→CAT)	*nuoG* ←
2,529,193	A→G				62.5%			M1M (ATG→GTG) †	*crr* →
2,860,476	G→T	44.1%						Y145* (TAC→TAA)	*rpoS* ←
2,860,539	G→T						45.1%	N124K (AAC→AAA)	*rpoS* ←
2,964,278	(AGGCGT)$_{1\rightarrow2}$		5.4%					coding (500/714 nt)	*ygdQ* →
2,964,912	C→T	29.6%		67.5%				intergenic (+64/-44)	*ygdR / tas*
3,207,549	G→A			32.1%				A382T (GCG→ACG)	*rpoD* →
3,270,632	(TTCAACA)$_{2\rightarrow3}$			14.6%		16.1%		coding (272/336 nt)	*prlF* →
3,299,592	Δ11 bp					37.9%		coding (1618-1628 nt)	*deaD* ←
3,360,110	Δ10,078 bp				61.4%			IS*5* -mediated	*[yhcE]–[sspA]*
3,360,110	Δ10,892 bp					56.6%		IS*5* -mediated	*[yhcE]–sspA*
3,360,110	Δ10,906 bp						49.4%	IS*5* -mediated	*[yhcE]–sspA*
3,370,422	Δ1 bp		72.7%					coding (358/639 nt)	*sspA* ←
3,371,313	Δ6 bp					39.6%		coding (249-254/393 nt)	*rpsI* ←
3,443,617	Δ11 bp				39.9%			coding (259-269/279 nt)	*rpsS* ←
3,467,928	IS*5* (–) +4 bp			68.7%				intergenic (-17/+106)	*rpsL / tusB*
3,482,817	Δ13 bp			30.8%				coding (711-723/1221 nt)	*argD* ←
3,507,954	Δ1 bp				22.8%			coding (465/678 nt)	*rpe* ←
3,508,077	IS*4* (–) +13 bp		80.1%					coding (330-342/678 nt)	*rpe* ←
3,508,262	Δ1 bp					48.7%		coding (157/678 nt)	*rpe* ←
3,508,263	Δ1 bp					49.0%		coding (156/678 nt)	*rpe* ←
3,508,366	A→T	46.8%					50.1%	L18Q (CTG→CAG)	*rpe* ←
3,803,449	G→A		16.6%					G89S (GGC→AGC)	*coaD* →
3,807,209	(TTCTGG)$_{1\rightarrow2}$		30.0%					coding (1119/1221 nt)	*dfp* →
3,809,219	Δ82 bp	32.9%							*[rph]–[rph]*
4,048,289	C→A	9.3%						R117L (CGC→CTC)	*glnG* ←
4,049,261	C→T	9.5%						G147S (GGT→AGT)	*glnL* ←
4,049,329	T→G	54.1%						H124P (CAC→CCC)	*glnL* ←
4,094,585	T→A		74.1%					N107Y (AAC→TAC)	*cpxA* ←
4,353,277	C→T						40.2%	G528D (GGC→GAC)	*dsbD* ←
4,416,066	G→T					12.7%		E48* (GAA→TAA)	*rplI* →
4,532,356	IS*1* (+) +9 bp		32.5%					coding (503-511/597 nt)	*fimE* →

Population samples of monocultures auxotrophic for tyrosine:

Predicted mutations									
position	**mutation**	**A4**	**A5**	**A6**	**A8**	**A10**	**A9**	**annotation**	**gene**
70,482	A→C					29.3%		L176R (CTT→CGT)	*thiP* ←
85,062	T→G					30.0%		L183R (CTT→CGT)	*cra* →
107,669	G→T					100%		E47* (GAA→TAA)	*mutT* →
185,655	A→C					29.2%		F113L (TTT→TTG)	*map* ←
307,295	G→T						100%	intergenic (-248/-528)	*ecpR / ykgL*
396,685	G→T						100%	intergenic (-306/-157)	*ddlA / iraP*
443,007	G→T				100%		100%	S52* (TCG→TAG)	*cyoE* ←
443,679	Δ13 bp		100%	100%				coding (426-438/615 nt)	*cyoC* ←
648,088	G→A			85.7%				S133N (AGC→AAC)	*citA* →
707,99	A→C					26.1%		M90R (ATG→AGG)	*ybfF* ←
728,407	C→G					100%		G1123G (GGC→GGG)	*rhsC* →
1,011,182	A→C					25.5%		Q4H (CAA→CAC)	*rmf* →
1,072,807	T→G					81.7%		Q511P (CAG→CCG)	*putA* ←
1,115,594	A→C					26.1%		L150R (CTG→CGG)	*solA* ←
1,230,695	T→G					28.2%		L101W (TTG→TGG)	*fadR* →
1,260,081	A→C					100%		Q304H (CAA→CAC)	*hemA* →
1,327,593	C→A			12.7%				C763* (TGC→TGA)	*topA* →
1,381,880	G→A				100%			E302K (GAA→AAA)	*tyrR* →
1,456,010	C→A						100%	T287T (ACC→ACA)	*paaJ* →
1,574,012	C→A		69.6%					E346* (GAA→TAA)	*ydeM* ←
1,594,169	8,367 bp x 2			18.0%				duplication	*[lsrK] –[yneE]*
1,763,960	T→G					15.3%		A210A (GCT→GCG)	*ydiK* →
1,771,247	T→G					35.1%		T468T (ACT→ACG)	*ydiF* →
1,983,151	C→G						100%	intergenic (-180/-11)	*yecH / tyrP*
2,119,797	T→G					27.1%		Q292P (CAG→CCG)	*wcaG* ←
2,420,808	T→G					25.7%		K155Q (AAA→CAA)	*argT* ←
2,529,193	A→G	100%						M1M (ATG→GTG) †	*crr* →
2,679,434	$(A)_{6\to5}$				100%			coding (241/1191 nt)	*hmp* →
2,838,350	T→G					26.5%		Y494S (TAC→TCC)	*hycE* ←
2,861,047	A→C					18.6%		S356A (TCA→GCA)	*nlpD* ←
2,964,891	T→G					100%		intergenic (+43/-65)	*ygdR / tas*
2,964,912	C→T						100%	intergenic (+64/-44)	*ygdR / tas*
2,964,952	G→A	31.0%						intergenic (+104/-4)	*ygdR / tas*
2,965,686	A→C					100%		K244T (AAA→ACA)	*tas* →
2,965,688	T→G	88.3%						Y245D (TAT→GAT)	*tas* →
2,965,716	C→A						84.9%	A254E (GCA→GAA)	*tas* →
3,039,528	T→G					13.8%		*958S (TAA→TCA)	*gcvP* ←
3,076,086	A→C					27.4%		intergenic (+56/+150)	*loiP / speB*
3,127,447	A→C					26.7%		intergenic (-131/-43)	*yghS / yghT*
3,262,186	G→T						100%	R138M (AGG→ATG)	*yhaC* →

Population samples of monocultures auxotrophic for tyrosine (continued):

position	mutation	A4	A5	A6	A8	A10	A9	annotation	gene
3,272,019	A→C					9.6%		S2R (AGT→AGG)	*agaR* ←
3,396,245	A→C					17.6%		V149V (GTT→GTG)	*csrD* ←
3,448,546	T→G					26.3%		E71A (GAA→GCA)	*gspA* ←
3,597,923	G→T						100%	L57L (CTG→CTT)	*rsmD* →
3,607,617	C→G					26.7%		Y197* (TAC→TAG)	*nikA* →
3,616,121	G→C				9.9%			R1190R (CGG→CGC)	*rhsB* →
3,648,862	T→G					25.7%		H134P (CAT→CCT)	*yhiD* ←
3,669,222	G→T	9.0%						M359I (ATG→ATT)	*yhjE* →
3,669,232	T→G		82.9%	24.7%				F363V (TTC→GTC)	*yhjE* →
3,713,593	114,908 bp x 3		23.2%					amplification	*[cspA] –[yicJ]*
3,797,270	T→G					35.2%		L76L (CTA→CTC)	*waaB* ←
3,809,167	A→C					84.4%		intergenic (-39/+27)	*pyrE / rph*
3,809,170	Δ1 bp				100%			intergenic (-42/+24)	*pyrE / rph*
3,809,182	T→G					7.8%		intergenic (-54/+12)	*pyrE / rph*
3,809,219	Δ82 bp		60.5%	83.5%					*[rph] –[rph]*
3,847,231	G→T						100%	intergenic (+229/-51)	*tisB / emrD*
3,956,890	C→A		16.3%					E234* (GAA→TAA)	*gpp* ←
3,957,279	Δ14 bp			84.7%				coding (298-311/1485 nt)	*gpp* ←
4,094,585	T→A					100%		N107Y (AAC→TAC)	*cpxA* ←
4,095,182	T→G					27.3%		L139F (TTA→TTC)	*cpxR* ←
4,114,904	T→G					19.7%		K612T (AAA→ACA)	*priA* ←
4,119,848	T→G					83.2%		L29W (TTG→TGG)	*metL* →
4,360,586	A→C					25.5%		T28P (ACC→CCC)	*groS* →

Population samples of monocultures auxotrophic for tryptophan:

Predicted mutations									
position	mutatio	B4	B5	B6	B8	B10	B9	annotation	gene
845,572	IS*5* (+) + 4 bp					19.7%		intergenic (-19/-331)	*rhtA / ompX*
1,427,227	G→T	100%						W187L (TGG→TTG)	*tfaR* →
1,750,759	C→T		9.5%					R269C (CGT→TGT)	*pykF* →
2,411,824	C→T	30.2						A422V (GCG→GTG)	*yfcC* →
2,758,822	$(TATGGCAC)_{6\to7}$				39.3 %			intergenic (-310/+50)	*yfjL / yfjM*
3,199,939	IS*5* (–) + 4 bp			9.7%				coding (118-121/912 nt)	*ttdA* →
3,586,809	C→T				68.7			G253D (GGT→GAT)	*livG* ←
3,669,232	T→G		100%					F363V (TTC→GTC)	*yhjE* →
3,809,111	G→C				100%			R6R (CGC→CGG)	*pyrE* ←
3,809,170	Δ1 bp		30.7			6.8%		intergenic (-42/+24)	*pyrE / rph*
3,809,171	C→T					19.4%		intergenic (-43/+23)	*pyrE / rph*
3,809,183	C→T					14.9%		intergenic (-55/+11)	*pyrE / rph*
3,809,184	G→T		15.6					intergenic (-56/+10)	*pyrE / rph*
3,809,185	C→T					41.0%		intergenic (-57/+9)	*pyrE / rph*
3,809,219	Δ82 bp	56.9	39.8	70.1			82.8		*[rph] –[rph]*
4,174,714	C→T			27.6				P1181L (CCA→CTA)	*rpoB* →

Population samples of wildtype monocultures:

Predicted mutations									
position	**mutatio**	**WT1**	**WT2**	**WT3**	**WT6**	**WT7**	**WT8**	**annotation**	**gene**
360,752	A→G	100%	100%	100%				V331A (GTG→GCG)	*lacI* ←
566,524	IS*3* (+) + 4 bp				54.1%			coding (176-179/552 nt)	*ybcL* →
566,528	Δ1 bp				54.1%			coding (180/552 nt)	*ybcL* →
1,282,421	A→G						5.5%	intergenic (+439/+122)	*narI / tpr*
1,703,101	C→G			6.6%				A642A (GCC→GCG)	*rsxC* →
1,983,404	G→C	12.5%						W81C (TGG→TGC)	*tyrP* →
2,098,857	C→A			100%				V228F (GTC→TTC)	*wbbI* ←
2,298,492	A→G					5.3%	5.5%	intergenic (+620/+95)	*eco / mqo*
2,464,622	IS*2* (+) + 5 bp			7.2%				coding (371-375/441 nt)	*yfdK* ←
2,758,822	$(TATGGCAC)_{6\to7}$	15.0%						intergenic (-310/+50)	*yfjL / yfjM*
3,243,770	C→A		18.1					intergenic (+35/-151)	*yqjK / yqjF*
3,299,638	G→T					6.1%		H528N (CAC→AAC)	*deaD* ←
3,586,394	Δ11 bp					7.5%		coding (394-404/714 nt)	*livF* ←
3,586,409	C→T					46.0%		R130H (CGC→CAC)	*livF* ←
3,586,583	Δ11 bp		32.2%					coding (205-215/714 nt)	*livF* ←
3,587,010	$(TCTCTTTCG)_{1\to2}$			13.9%				coding (557/768 nt)	*livG* ←
3,587,647	C→T			72.1%				M398I (ATG→ATA)	*livM* ←
3,587,655	+G	8.8%						coding (1186/1278 nt)	*livM* ←
3,587,676	102 bp x 2	14.0%						duplication	*livM* ←
3,588,319	$(GCTGCC)_{1\to2}$				5.9%			coding (522/1278 nt)	*livM* ←
3,588,703	Δ8 bp				51.8			coding (131-138/1278 nt)	*livM* ←
3,588,896	$(CAGCACCAG)_{1\to2}$					9.8%		coding (868/927 nt)	*livH* ←
3,612,569	G→A			14.3%				A6A (GCG→GCA)	*rhsB* →
3,650,976	C→A			10.0%				intergenic (+49/-750)	*hdeD / gadE*
3,755,572	C→T			40.6%				G10G (GGC→GGT)	*rhsA* →
3,755,620	G→A			27.1%			21.4%	V26V (GTG→GTA)	*rhsA* →
3,755,623	C→T			27.1%			21.3%	R27R (CGC→CGT)	*rhsA* →
3,809,165	A→G		100%					intergenic (-37/+29)	*pyrE / rph*
3,809,168	$(A)_{8\to7}$				100%			intergenic (-40/+26)	*pyrE / rph*
3,809,170	Δ1 bp					45.0%		intergenic (-42/+24)	*pyrE / rph*
3,809,219	Δ82 bp			10.8%			72.1%		*[rph] –[rph]*
3,809,307	+C			100%				pseudogene (603/669 nt)	*rph* ←
3,809,332	Δ1 bp	12.3%						pseudogene (578/669 nt)	*rph* ←
3,809,333	Δ1 bp	12.2%						pseudogene (577/669 nt)	*rph* ←

Supporting information for discussion

Supporting table

Table S1: Analysis for loss of function prediction of selected mutations, *i.e.* missense mutations causing an amino acid substitution, utilizing PROVEAN (566, 567).

Mutated protein:	Amino acid substitution:	Number of supporting sequences used:	PROVEAN score:	Predicted effect:
Lrp	A126V	318	-3.070	**Deleterious**
Lrp	L138M	318	-1.550	**Neutral**
Lrp	Y147H	318	-4.902	**Deleterious**
Rpe	L18Q	646	-5.613	**Deleterious**
RpoS	N124K	620	-5.620	**Deleterious**

Danksagung

Zu allererst möchte ich Lisa, Sophia und Simon danken. Der Gedanke an euch drei hat mir dazu verholfen ungeahnte Kräfte zu mobilisieren. Ihr habt besonders während der letzten Monate tapfer meine regelmäßige Abwesenheit durchgestanden (selbst wenn ich anwesend war).

Danke Lisa für deine Geduld, und für dein Verständnis. Du hast mir den nötigen Rückhalt gegeben der es mir ermöglicht hat weit über meine persönlichen Grenzen der Belastbarkeit hinausgehen zu können. Diese Dissertation ist unsere Arbeit.

Meinen Eltern und Schwiegereltern gilt ganz besonderer Dank für die Unterstützung in allen Bereichen. Ohne euch wäre die Unvereinbarkeit zwischen einer anspruchsvollen Promotion und Familie nicht zu bewältigen gewesen! Hartwin und Christina, ihr habt mir in der finalen Phase den Rücken frei gehalten und hoffe die Gelegenheit zu bekommen mich bei euch angemessen zu revanchieren. Auch Felix und Marie danke ich für die Zeit die verbrachte Zeit mit Sophia. Ganz besonders danken möchte ich auch meinen Eltern. Ihr beiden habt mich bedingungslos unterstützt egal wohin mich mein Weg geführt hat.

Christian, vielen Dank für das entgegengebrachte Vertrauen und die Freiheit selbständig Projekte zu planen und durchzuführen, für die Förderung und Forderung, für die Inspiration aus ökologischer und evolutionärer Perspektive, für den grenzenlosen Optimismus und die Hilfe beim Verfassen der Manuskripte – dort insbesondere für die Geduld beim Korrigieren so manchen denglischen Satzes.

Danke Anne (Kathrin), für die unzähligen guten Gespräche, wissenschaftlich und unwissenschaftlich. In meinem zukünftigen Berufsleben verzichte ich nur ungern auf dich als Büro,- sowie Leidensgefährtin.

Für die unermüdliche Unterstützung bei jedem erdenklichen bürokratischen Akt und darüber hinaus danke ich Grit. Du bist die gute Seele der Abteilung.

Mein besonderer Dank gilt Glen, Shraddha, Ghada, Silvio & Christian für den gemeinsamen Kraftakt den Review aller Reviews zu verfassen.

Alexander Herbig danke ich für die entscheidenden bioinformatischen Tipps zum Generieren der Distance-trees und für die absolut konstruktive, wertschätzende sowie problemlose Kooperation und den Input zum Manuskript.

Lisa Freund, Bianca Altmann und Marita Hermann danke ich für die zuverlässige Hilfe im Labor. Danke Bianca, für dein Engagement wenn es mal wieder etwas länger gedauert hat.

Danke Silvio, für bioinformatische Hilfestellungen und für die Informationen zu den vorhergesagten Überproduktionsmutanten.

Samir und Linea danke ich für die weiterführenden Analysen der Assoziation und Disassoziation von Zellclustern.

Glen und Silvio danke ich für das gelegentliche Überimpfen des Evolutionsexperiments.

Hendrik Tilger danke ich für wichtige Tipps beim Kampf zwischen Windows und Linux.

Mein Dank gilt auch Michael Reichelt für die Hilfe bei der Aminosäureanalytik.

Professor Boland möchte ich für vorrübergehende Finanzierung danken.

Special thanks to the former group of the EEEs: Holger, Samay, Anne, Glen, Shraddha, Silvio, and Lisa, for all that fun we had in the lab, the office, and during retreats. Special thanks to Holger for introducing me to the lab and explaining me a lot. Thank you all for the great time, great Indian food, and for teaching me little Hindi.

CURRICULUM VITAE

Name	**Daniel Preußger**
Address	Hufelandweg 16 07743 Jena, GERMANY
Telephone	+49 173 9643480
E-Mail	daniel.preussger@gmail.com
Birth	07.03.1987, Löbau (Sachsen)

EDUCATION

05/2013 – present — DISSERTATION PROJECT
The evolution of metabolic cooperation within bacterial communities - causes and consequences

Experimental Ecology and Evolution Group
Max Planck Institute for Chemical Ecology, Jena

Parental leave: 01/2015 – 11/2015
Parental leave: 06/2019 – 12/2019

10/2010 – 02/2013 — STUDIES OF MICROBIOLOGY; M.SC.
Thesis title: The effect of spatial structures on the evolution of cooperative amino acid cross-feeding within bacterial consortia

Friedrich-Schiller University, Jena
Master thesis: 04/2012 – 02/2013
Max Planck Institute for Chemical Ecology, Jena
Internship: 11/2011 – 03/2012
Max Planck Institute for Chemical Ecology, Jena

10/2006 – 09/2009 — STUDIES OF BIOTECHNOLOGY; B.SC.
Thesis title: Quantifizierung mikrobiellen Benzolabbaus in horizontal durchflossenen Pflanzenkläranlagen

University of Applied Science, Zittau-Görlitz
Bachelor thesis: 05/2009 – 09/2009
Helmholtz-Zentrum für Umweltforschung, Leipzig
Internship: 07/2008 – 10/2008
Biotecon Diagnostics GmbH, Potsdam

2005 — Abitur, Geschwister-Scholl-Gymnasium, Löbau (Sachsen)
General qualification for university entrance (17.07.2005)

CAREER

03/2013 – 05/2013 — Research assistant: Acetate quantification (HPLC) in supernatants of genetically modified *Escherichia coli* strains
Max Planck Institute for Chemical Ecology, Jena

12/2009 – 08/2010 — Employment: Efficacy verification of disinfectants according to DVG and DGHM regulations
Biotecon Diagnostics GmbH, Potsdam

SCIENTIFIC OUTPUT

Publications	05/2018	**Ecology and evolution of metabolic cross-feeding interactions in bacteria.** D'Souza G, Shitut S, Preussger D, Yousif G, Waschina S, and Kost C Natural Products Reports, Review Article, 2018, 35, 455-488 doi: 10.1039/c8np00009c
	in prep.	**Synergistic coevolution accelerates genome evolution.** Preussger D, Herbig A, Kost C (To be submitted to Nature Ecology & Evolution)
	in prep.	**Group formation promotes evolution of metabolic cooperation in bacteria.** Preussger D, Giri S, Muhsal L, Oña L, Kost C (To be submitted to Nature Ecology & Evolution)
Oral presentations	04/2018	**Synergistic coevolution speeds up molecular evolution.** **Mini Symposium Keynote Lecture,** VAAM – Annual Conference for General and Applied Microbiology, Wolfsburg
	08/2017	**Synergistic coevolution accelerates molecular evolution.** Meeting of the European Society of Evolutionary Biology Groningen, Netherlands
	09/2016	**Synergistic interactions speed up evolution.** MPI-CE Institute Symposium Max Planck Institute for Chemical Ecology, Jena
	12/2016	**Evolution of cooperative cross-feeding: Consequences and conclusions.** Annual JSMC-symposium Jena School for Microbial Communication, Jena
	09/2014	**Rapid evolution of obligate cooperative cross-feeding.** Annual JSMC-symposium Jena School for Microbial Communication, Jena
	10/2013	**The evolution of metabolic cooperation within bacterial communities - causes and consequences.** Annual JSMC-symposium Jena School for Microbial Communication, Jena
Poster presentations	03/2016	**Synergistic interactions speed up evolution.** VAAM – Annual Conference for General and Applied Microbiology, Jena
	09/2014	**Evolution of cooperative cross-feeding independent of spatial structure.** MPI-CE Institute Symposium Max Planck Institute for Chemical Ecology, Jena
	04/2014	**Evolution of cooperative cross-feeding independent of spatial structure.** 4th International Student Conference on Microbial Communication - MiCom 2014, Jena
Workshop	06/2014	Guarda Workshop on Evolutionary Biology Guarda, Switzerland Organized by the University of Basel

AWARDS

Best poster presentation	03/2016	VAAM – Annual Conference for General and Applied Microbiology, Jena
PhD funding (3 years)	05/2013	Excellence Graduate School Jena School for Microbial Communication - JSMC German Excellence Initiative

Selbständigkeitserklärung

Ich erkläre hiermit, dass mir die geltende Promotionsordnung (§ 5 PromO) der Fakultät für Biowissenschaften der Friedrich Schiller Universität Jena bekannt ist.

Die vorliegende Arbeit wurde von mir selbständig angefertigt. Alle Hilfsmittel, übernommene Inhalte aus anderen Quellen und von anderen Personen, inklusive persönlicher Mitteilungen, welche in dieser Arbeit Verwendung fanden oder auf welche direkt Bezug genommen wird, sind als solche eindeutig kenntlich gemacht. Insbesondere wurden alle Personen in der Danksagung genannt, die direkt an der Entstehung der vorliegenden Arbeit und den verfassten Manuskripten beteiligt waren.

Ich versichere, dass kein Promotionsberater in Anspruch genommen wurde, noch das Dritte von mir geldwerte Leistungen für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.

Ich habe die Dissertation bisher nicht in gleicher oder ähnlicher Form an einer anderen Prüfungsbehörde zum Zweck einer Promotion oder eines anderen Prüfungsverfahrens eingereicht.

Jena, den 27.06.2019

Daniel Preussger