



Defining Division of Labor in Microbial Communities

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Abstract

In order to survive and reproduce, organisms must perform a multitude of tasks. However, trade-offs limit their ability to allocate energy and resources to all of these different processes. One strategy to solve this problem is to specialize in some traits and team up with other organisms that can help by providing additional, complementary functions. By reciprocally exchanging metabolites and/or services in this way, both parties benefit from the interaction. This phenomenon, which has been termed *functional specialization* or *division of labor*, is very common in nature and exists on all levels of biological organization. Also, microorganisms have evolved different types of synergistic interactions. However, very often, it remains unclear whether or not a given example represents a true case of division of labor. Here we aim at filling this gap by providing a list of criteria that clearly define division of labor in microbial communities. Furthermore, we propose a set of diagnostic experiments to verify whether a given interaction fulfills these conditions. In contrast to the common use of the term, our analysis reveals that both intraspecific and interspecific interactions meet the criteria defining division of labor. Moreover, our analysis identified non-cooperators of intraspecific public goods interactions as growth specialists that divide labor with conspecific producers, rather than being social parasites. By providing a conceptual toolkit, our work will help to unambiguously identify cases of division of labor and stimulate more detailed investigations of this important and widespread type of inter-microbial interaction.

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Introduction

In order to survive and reproduce, organisms need to accomplish many different tasks such as the acquisition of food, defense against enemies, growth, repair, and so on. In modular organisms that consist of multiple, lower-level units, different tasks are executed by specific modules that are specialized for their respective task, a phenomenon that is called *division of labor* (DOL) [1–3] or *functional specialization* [4,5]. Breaking down complex processes into simpler steps eliminates unnecessary constraints that stem from the need to perform several tasks simultaneously or switch between them, thus significantly enhancing the efficiency, with which the whole process can be

executed. Due to the tremendous advantages that can result from dividing tasks among several, lower level units, the principle of DOL can be found at all levels of biological organization [6–8].

Enzymes that can catalyze two biochemical reactions with different efficiencies can—after a duplication event of the underlying gene—be selected to perform both functions with an increased specificity [9,10]. Also, the evolutionary success of multicellular organisms is likely due to the differentiation into several cell types or tissues that all serve specific functions and synergistically interact with each other to enhance the performance of the whole organism. The prime example for DOL, however, is *eusociality*, which evolved multiple times independently in the animal kingdom [11]. Analogous to the

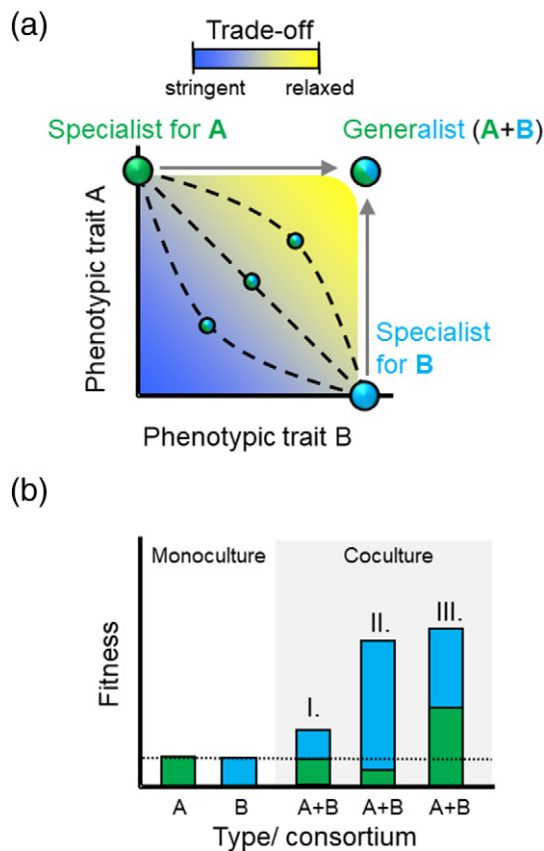


Fig. 1. Trade-offs can cause DOL. (a) Consider a situation in which an organism needs to be able to perform two different tasks (A + B) in order to maximize its fitness. If the organisms can perform both tasks simultaneously equally well, it is considered to be a *generalist*. A trade-off occurs when an improved performance of one task comes at the expense of a reduced ability to perform the respective other task. Under these conditions, organisms need to specialize and are thus only able to perform either one of the two tasks (A or B) or display reduced abilities to perform both tasks simultaneously. The shape of the dashed lines connecting the two specialist strategies (i.e., pareto fronts) dictates how stringent (blue background) or relaxed (yellow background) a certain trade-off is. (b) A synergistic interaction between two specialists (A and B) can enhance fitness of the entire consortium. The graph shows the fitness both types reach under mono- (left) and coculture conditions (right). When interacting with each other, both types can display several different consortium-level growth phenotypes: (I) fitness of both partners combines additively, (II) one partner benefits, while the other displays a reduced fitness relative to monoculture conditions, and (III) both partners benefit (i.e., their fitness is enhanced compared to monoculture conditions). DOL can be seen in consortia of type III and intraspecific consortia of type II (see main text for further explanation).

previous examples, also in this case, DOL provides benefits at the level of the social colony and is thought to be one of the main factors responsible for the tremendous ecological success of eusocial ants

or termites [12,13]. These insect societies divide labor at two levels of organization: first, reproductive DOL in the form of a single (or multiple) fertile queen (s) that exclusively focus on the reproduction of a given colony, while sterile workers perform all tasks related to colony maintenance and growth. This dichotomy is functionally equivalent to the distinction of germline and soma [14], as it is commonly observed in multicellular eukaryotes [15,16]. Second, sterile workers within a eusocial colony are differentiated into castes that are defined by different sizes and ages, and which fulfill special functions within the colony. This type of DOL is analogous to the abovementioned cells and tissues of multicellular organisms that fulfill certain tasks that all contribute to the growth and maintenance of the whole organisms [13,17].

Finally, also bacteria commonly show DOL, such as, for example, the development of fruiting bodies in *Myxococcus xanthus* [18]. Formation of this complex structure requires the contribution of multiple cells performing different tasks, including cell lysis, the formation of peripheral rods, or the development into spore cells. Spores and lysed cells mainly exist inside of fruiting bodies, whereas peripheral rods remain outside. Peripheral rod cells have been proposed to enhance the survival of *Myxococcus* in its natural habitat, because they do not undergo cell division, but can probably respond to sudden change in environmental conditions [19]. Furthermore, lysed cells provide nutrients that allow spore cells to differentiate during development.

Another prominent examples of DOL within the bacterial kingdom are filamentous cyanobacteria such as *Anabaena* or *Nostoc* species that differentiate into specialized cell types to segregate incompatible biochemical reactions such as photosynthesis and nitrogen fixation. Photosynthesis occurs in vegetative cells and the adjacent heterocysts fix nitrogen, which requires a highly regulated system to ensure efficient distribution of resources along the filament [20,21].

Besides the abovementioned cases of bacterial DOL, there are many other examples of behaviors that appear to be cooperative or beneficial at the level of a bacterial consortium. However, given the paradigmatic cases mentioned above, it is frequently not clear whether or not these other cases constitute real examples of DOL, or rather some different type of ecological interaction. One example for such an ambiguous case is the biodegradation of a complex substrate such as a polysaccharide (e.g., hemicellulose [22–24]) or xenobiotics (e.g., organophosphate esters and pentachlorophenol [25–29]). In these cases, degradation of a single molecule requires many sequential biochemical reactions. However, most commonly, it is not a single species that breaks down the entire substrate all alone, but multiple species that work together to achieve this

goal. In these cases, every species catalyzes an individual step of the degradation pathway and releases an intermediate metabolite, which is then passed on and used by the next species in the chain. Such sequential interactions help to not only efficiently utilize the available resources but also to remove toxic intermediates that often inhibit the growth of other community members. In this process, different bacterial species come together and collectively degrade a substrate much more efficiently than any of the participating species could achieve in isolation [26,30,31]. Moreover, such interaction chains probably emerge, because downstream species benefit from utilizing metabolites that are liberated from preceding species. However, does this example describe a true case of DOL? And if not, why not?

In this review, we aim at clarifying this issue by (a) exploring conditions that favor DOL, (b) defining key criteria that have to be fulfilled for an interaction to classify as DOL, (c) deriving a list of diagnostic experiments to unambiguously identify true cases of DOL that allow distinguishing it from other kinds of synergistic bacterial interactions, and (d) analyzing examples for structurally different synergistic bacterial interactions in light of our classification framework.

Specialization and Trade-offs

Many of the tasks organisms need to fulfill in order to maximize their fitness are not independent of each other, but are genetically, biochemically, or physiologically interconnected [32]. For example, the amount of resources that can be allocated to two different processes is limited. As a consequence, investing more resources in one task comes at the expense of having to invest less in another one. Alternatively, the optimal conditions to perform one biochemical reaction can be suboptimal for catalyzing another one, thus preventing the simultaneous execution of both biochemical reactions. Such relationships, where one trait can only be improved or performed at the expense of another process, is called a *trade-off* [33,34] (Fig. 1a).

Trade-offs are common and are very important determinants of an organism's fitness. As a consequence, organisms that adapt to certain environmental conditions need to specialize and, in this process, improve in performing some tasks, while becoming worse at others. In bacteria, trade-offs affect several physiological processes including nutrient utilization, metabolism, antibiotic resistance, tolerance to abiotic stress, and virulence behaviour. From this result different evolutionary strategies along a continuum of possible combinations of phenotypic traits. Interestingly, these relationships

can manifest not only among members of the same species, but also, in cases where the trade-off is evolutionarily conserved, between different species [35–37]. In any case, the ability of individuals to perform both functions simultaneously is determined by the shape of the trade-off function (Fig. 1a). Specifically, when the trade-off is rather stringent, the shape of the trade-off is concave, while convex relationships point to more relaxed trade-offs (Fig. 1a). Three extreme ends of this distribution are (i) the point where the trade-off is absent and individuals can display both phenotypes in an unconstrained manner (i.e., “*generalist*”) or (ii) the two positions, at which only one of the two traits is expressed (i.e., “*specialist*”). In general, trade-offs can have different mechanistic causes, some of the most important ones will be discussed in turn.

Allocation of resources

Optimal resource allocation is key for maximizing Darwinian fitness [38]. As cellular resources are usually limited, any biosynthetic function that requires certain resources inevitably also incurs a metabolic cost to the cell, because fewer resources are available to perform other cellular processes [39]. One particular resource allocation problem is the distribution of metabolic fluxes by the metabolic network for cell growth. When cellular resources are abundant, bacterial cells often switch from respiration to fermentation to achieve faster growth, thus causing a redistribution of their protein machinery [40]. During this process, cellular protein pools undergo a dramatic rearrangement. Specifically, respiratory enzymes are substituted by glycolytic enzymes, which enhances the speed of growth at the cost of a reduced yield [41]. Besides bacterial growth, resource allocation trade-offs are also commonly observed in pathogenic bacteria infecting their host. For example, the plant pathogen *Ralstonia solanacearum* faces a trade-off in resource allocation between proliferation and the costly production/secretion of virulence factors (e.g., exopolysaccharides, or EPS) that are necessary to infect the host plant [42].

Biochemical trade-off

The fact that many different biochemical reactions are usually performed in the same intracellular environment, can lead to incompatibilities between reactions at the molecular level. This type of trade-off can be resolved by segregating antagonistic processes into different cells (compartmentalization) or by performing incompatible reactions at different times (temporal differentiation) [4,43].

One example for a biochemical trade-off is the incompatibility between photosynthesis and nitrogen fixation due to the irreversible inhibition of the

nitrogen-fixing enzymes (i.e., *nitrogenase* complex) by oxygen that is produced during photosynthesis [20,44]. Multicellular cyanobacteria solve this problem by spatial differentiation of cells that either perform photosynthesis (i.e., vegetative cells) or fix nitrogen (i.e., heterocysts) [21]. Another solution to the same problem has been found by non-heterocystous cyanobacterial species, such as *Plectonoma boryanum*, which differentiate temporally into photosynthetically active or nitrogen-fixing cells. The switch between both states is either regulated by an endogenous circadian rhythm or triggered by external signals [45].

Evolutionary trade-off

Evolutionary trade-offs emerge when adaptation to one environment results in a loss of fitness in another environment [46]. Such a phenotypic specialization can be due to two distinct genetic processes: *mutation accumulation* and *antagonistic pleiotropy*. Mutation accumulation is the mutational deactivation or loss of genes that are not needed in the current environment, and are thus not maintained by selection. Loss of the corresponding biochemical function can impair the mutant's ability to grow and survive in another environment [47–50]. In contrast, antagonistic pleiotropy is when one gene or operon has multiple effects that opposingly affect fitness. This linkage can cause trade-offs, where a mutation that enhances fitness in one environment is maladaptive in another one [47]. The rapid adaptation of *Pseudomonas fluorescens* bacteria to unshaken environmental conditions represents a compelling example for antagonistic pleiotropy. In this case, diversification, which is driven by mutation and selection, leads to the emergence and stable coexistence of three niche specialists with distinct colony morphologies [51,52]. The ability of one of these types (i.e., so-called *wrinkly spreader*) to form a biofilm at the air–liquid interface resulted in the concomitant loss of other phenotypic features such as the capacity to utilize different carbon sources [53]. Other studies, where catabolic defects have been observed in *Escherichia coli* cells adapting to a minimum glucose-environment for more than 50,000 generations, attributed the observed cost of specialization to the stochastic accumulation of mutations in currently unused genes [54].

Together, the abovementioned processes result in a mosaic of bacterial strains that have adapted to very specific environmental conditions and, as a consequence, have adopted a certain evolutionary strategy. When trade-offs prevent the evolution of generalism, an ecological interaction between two or more individuals that occupy different ends of the same trade-off function can significantly enhance the fitness of all individuals relative to their performance

in isolation [33]. This phenomenon is called DOL [3,55].

Defining DOL

Comparing many qualitatively different interactions to identify similarities and differences to unambiguous cases representing DOL revealed four main conditions that have to be fulfilled for an ecological interaction to be classified as DOL. These are (1) functional complementarity, (2) synergistic advantage (+/+), (3) negative frequency-dependent selection, and (4) positive assortment.

Functional complementarity

All partners participating in the interaction trade a commodity (i.e., a product of a biochemical reaction such as a metabolite or an enzymatic function such as the detoxification of an environmental chemical) that they can produce more efficiently (i.e., in larger amounts, more energy-efficient, etc.) than the other interaction partner(s). The requisite functional differentiation can be due to trade-offs that resulted in a phenotypic specialization of individuals along a trade-off function (Fig. 1a). Alternatively, two or more genotypes may also have experienced a divergent evolutionary history in the past, yet display a functional complementarity that is mutually beneficial when they encounter each other.

Synergistic advantage (+/+)

A synergistic advantage has to result from the interaction (i.e., it has to be mutually beneficial) for each of the parties involved, in order to classify as DOL. For this, the sum of lifetime fitness payoffs that results for each participating individual from the interaction has to be more than the sum of individual fitnesses, when the interaction partners exist in isolation (Fig. 1b). To fulfill this criterion, it is not essential that the interaction has evolved, because it has been favored by natural selection in the past. As a consequence, cases of DOL do not only include cooperative interactions, in which the interacting individuals invest costly resources to benefit their respective interaction partners and which has evolved for this purpose, but also interactions, in which partners trade by-products, whose production is independent of the presence of the corresponding partner.

The only exceptions to this rule are interactions within species, where it can happen that one of the interaction partners involved is less fit when being part of the interaction as compared to its performance outside the interaction. In these cases, the focal individual shows a cooperative behavior that is

costly to itself, yet beneficial to its closely related interaction partner(s) [56–58]. Both *direct* and *indirect* fitness consequences result from these *altruistic* behaviors: by helping to increase the reproductive success of close relatives, the altruistic individual can indirectly pass on its genes to the next generation [59,60]. Thus, by interacting with each other, all partners can reach a higher consortium-level fitness than any of the parties could achieve in isolation. This effect should not be seen in interspecific interactions, where interaction partners are per definition less closely related, thus minimizing indirect fitness benefits [61].

In many cases, synergistically advantageous ecological interactions result in strong fitness feedbacks between individuals when they participate in the interaction, yet not when they occur in isolation. This means that an individual participating in the synergistic interaction can enhance its fitness by increasing the production of the traded function, while reducing the investment in the interaction would curtail its own fitness and thus be maladaptive. Such positive feedbacks align the interests of all interaction partners involved, which should result in more cooperative interactions in the long-run. However, for this to work, it is important that interaction partners remain associated for a sufficiently long time so that fitness feedbacks, which result from the interaction, can operate.

Negative frequency-dependent selection

Another key criterion defining DOL-based interactions is that all partners involved can coexist over extended periods of time. The prime ecological mechanism permitting stable coexistence among multiple interacting genotypes is negative frequency-dependent selection. Here, the fitness of one genotype depends of the frequency of other genotypes in the local population/community. Specifically, the fitness of the focal individual is highest when its frequency relative to other interactors in the resident population/community is low, yet decreases, as its relative frequency increases. With increasing abundance, the fitness of the focal population drops below values of the other interaction partner(s). If this pattern is observed among all individuals participating in the synergistic interaction, the numbers of all cell types will oscillate around a stable equilibrium that is most likely determined by the interplay between the costs and benefits of resulting from the interaction. In addition, epigenetic or regulatory mechanism may operate that constantly generate new copies of the two or more interacting partners (see below). Although these mechanisms contribute to further stabilizing the different types in a given population or community, negative frequency dependence is likely still important to optimally adjust their frequencies to the

current environmental conditions. Thus, negative frequency-dependent selection is a powerful mechanism to stabilize ecological interactions in the long-run.

Positive assortment

Another key requisite for an interaction to classify as DOL is that it is favored by natural selection. Even if participation in the interaction is advantageous (criterion 1) and it is ecologically stabilized by negative frequency-dependent selection (criterion 3), the interaction might still be unstable, when interaction partners participating in DOL are not repeatedly encountering each other in subsequent rounds of interaction. This can be the case when individuals within a community are mixed with other organisms not participating in the DOL, for example, when the community is disturbed. Under these conditions, mechanisms are required that increase the probability that an individual participating in the DOL (or its offspring) will encounter the required interaction partner relative to meeting other individuals not participating in the interaction. This so-called *positive assortment* can be achieved by a multitude of different evolutionary mechanisms (summarized in [62]) including the active choice or physical co-localization of suitable interaction partners. Independent of how this is achieved, positive assortment ensures that fitness feedbacks operate among interaction partners, meaning that any unilateral increase of the investment into the interaction that benefits the other partner(s) is likely immediately rewarded due to the reciprocity of the DOL.

Facultative and Non-permanent Interactions

Recent work has shown that obligate interdependencies [63–65] as well as permanent interactions [66,67] promote the evolution of cooperative behaviors, because both mechanisms help to align the evolutionary interests of all interaction partners involved. However, both parameters are not defining features of interactions qualifying as DOL. Ecological interactions are always context-dependent [68,69] and it is thus conceivable that also facultative or non-permanent interactions can meet the four defining criteria under some conditions. Imagine, for example, the case of two bacterial genotypes that are each auxotrophic for a different amino acid and can only grow, when they reciprocally exchange the amino acid their partner requires for growth. As long as both metabolites can only be derived from the other interaction partner, the reciprocal cross-feeding interaction qualifies as DOL, because all

four key criteria are fulfilled [63]. However, when amino acids become environmentally available, both strains will grow independently. Nevertheless, as soon as amino acids are used up again, auxotrophic strains will start to interact again with each other. Even if this interaction is facultative and non-permanent in the presence of environmental amino acids, it fulfills all four key criteria in their absence. Thus, also non-permanent and facultative interactions can qualify as DOL under some conditions.

Ultimately, when the advantages that result from the interaction are sufficiently strong and the interaction persists long enough, facultative interactions may also become obligate as interactions partners adapt to each other and, in this process, lose their functional independence [70].

Sources of Variation

In order to classify as a case of DOL, the interacting individuals need to show some degree of functional specialization (i.e., see criterion 1 above). In general, three main ways, of how this is achieved, can be distinguished: (1) epigenetic mechanisms, (2) regulatory mechanisms, and (3) genetic mechanisms.

Epigenetic mechanisms

Epigenetic mechanisms include all cases where members of an isogenic bacterial population (i.e., cells having the same genotype) simultaneously display two or more phenotypes that are not due to a response to microenvironmental stimuli. This phenomenon has been termed *phenotypic heterogeneity*, *bistability*, or *bet-hedging*, and is caused by mechanisms such as (i) stochastic/heterogeneous gene expression [71,72], (ii) error-prone protein synthesis [73–75], or (iii) epigenetic modifications [76,77], which simultaneously give rise to several different phenotypes that are adaptive under certain environmental conditions [78]. Epigenetic phenotypic heterogeneity has been predicted to evolve in environments that change rapidly and unpredictably, with these changes strongly affecting the fitness of the resident population [79]. Since phenotypic heterogeneity arises only in isogenic populations, this mechanism is per definition only relevant for mediating interactions between members of the same species. Phenotypic heterogeneity classifies as DOL, because the interaction involves two functionally different types (criterion 1), the clonal group benefits from displaying phenotypic heterogeneity (criterion 2), both (all) phenotypes coexist stably (criterion 3), and both (all) types show positive assortment (criterion 4).

An example for an epigenetic mechanism causing phenotypic heterogeneity has been described in *Salmonella typhimurium* expressing the virulence

locus type III secretion system I (ttss-1) inside the human gut lumen [80]. Here, isogenic subpopulations stochastically express different phenotypes that result in the simultaneous formation of an avirulent (ttss-1 OFF) and a virulent subpopulation (ttss-1 ON). One subpopulation (ttss-1 ON) grows slowly and causes gut inflammation, while the other (ttss-1 OFF) receives the benefits that result from the inflammation of the gut. As a consequence, the ttss-1 OFF cell type can grow faster, which results in the competitive exclusion of the entire resident gut microbiota. Thus, by dividing labor in this way, *S. typhimurium* can enhance the survival of the whole consortium inside a new host [78,80].

Regulatory mechanisms

A second mechanism causing phenotypic differentiation within isogenic bacterial populations is regulatory changes in gene expression in response to environmental stimuli. By responding to microenvironmental differences, cells can attain a physiological state that is optimal for the given condition without the need to change the underlying genome sequence. Besides the composition of the nutritional environment, bacteria can, for example, sense and respond to changes in oxygen availability [81], pH [82], or the presence of surfaces for attachment [83]. This process not only generates physiological and metabolic heterogeneity within microbial populations, but can also lead to ecological interactions among different strategists that can enhance survival and growth of the population as a whole. Given that positive assortment (criterion 4) is an automatic consequence of this type of phenotypic differentiation, these interactions qualify as DOL when the corresponding interaction partners display complementary phenotypes (criterion 1) that are mutually synergistic (criterion 2) and stabilized by negative frequency-dependent selection (criterion 3).

An example for regulatory differentiation within the same multicellular individual can be found in filamentous cyanobacteria such as *Anabaena* and *Nostoc*. While most of the cells within a filament are photosynthetically active, a proportion of vegetative cells differentiate into nitrogen-fixing heterocysts [44]. In this way, the biochemical incompatibility of nitrogen fixation and photosynthesis is reconciled by segregating these processes into separate cells. Although all cells share the same genetic information, photosynthetic and nitrogen-fixing cells show very distinct expression patterns that correspond to the tasks that are divided among both subpopulations [84,85]. Differentiation of this type is induced by nitrogen deprivation as well as changes in the availability of nutrients or quantity/quality of light [86].

A second example for a regulation-based DOL between different members of the same population is the collective colony expansion of *Bacillus subtilis* [87].

This type of motility depends critically on the synergy between two specialized cells: surfactin-producing cells that reduce the friction between cells and their substrate and matrix-producing cells that form multicellular clumps. Both cell types emerge from regulatory differences between two subpopulations up-regulating either *srfA* (surfactin production) or *tapA* (matrix production). Differentiation into these two subpopulations is regulated by a complex interplay between quorum sensing, responses to microenvironmental differences, and a temporal dynamics of gene expression. In combination, these regulatory links allow cells to divide labor during colony expansion [87].

Genetic mechanisms

The final category includes all cases, in which phenotypic differences that give rise to synergistic interactions between members of the same or different bacterial species are due to genetic differences between the interacting individuals. The most important causal processes in this category are (i) loss-of-function mutations, (ii) gain of function mutations, (iii) horizontal gene transfer, and (iv) antagonistic pleiotropy.

First, mutations that inactivate or down-regulate a gene can lead to synergistic interactions. Mutants that have lost the ability to autonomously produce a certain metabolite can be favored by natural selection, when the loss is compensated by another member of the community [88,89]. Recent empirical studies suggest that bacteria can divide labor if they trade certain cellular building blocks such as amino acids [90,91], vitamins [92,93], or extracellular matrix components [94,95] with members of the same or another bacterial species, rather than producing these compounds on their own. Thus, loss-of-function mutations can provide an advantage to a group of interacting individuals and enhance the productivity of the whole consortium even in nutrient-limiting environments [63].

Strikingly, loss of function mutations can also result in new phenotypes that enhance synergistic interactions with other organisms. For instance, a recent study observed a novel phenotype that arose in a cross-feeding system between *E. coli* and *Salmonella enterica*, when they were experimentally coevolved together for more than 200 generations in a spatially structured environment. In this study, the authors report the emergence of a mutated *E. coli* strain that secreted a costly sugar (galactose), which in turn could be used by *Salmonella* as a resource. These galactose-secreting *E. coli* mutants evolved in multiple replicate lineages and were in all cases due to a frameshift mutation in an existing enzyme (i.e., galactokinase, *galK*) that blocked galactose metabolism [96]. Although these mutations were costly when *E. coli* was grown on lactose alone, they were beneficial in coculture with *Salmonella*, thus con-

tributing to the maintenance of this mutualistic interaction.

Second, also the acquisition of a novel phenotypic capability via a gain-of-function mutation or horizontal gene transfer can enhance the production of a metabolite or an enzyme that plays a role in a synergistic interaction between two or more partners. An example of both of these genetic mechanisms is the acquisition of antibiotic resistance [97], which can benefit other strains in the vicinity that are sensitive to the corresponding antibiotic [98–100].

Mutations that affect more than one phenotypic trait are said to have a pleiotropic effect. *Antagonistic pleiotropy* describes cases, in which a mutation increases the expression of one trait, while the development of another one is decreased. Thus, antagonistic pleiotropy has the potential to create trade-offs between two phenotypes that are exclusively due to the underlying genetics and not caused by, for example, a differential allocation of resources. Consequently, mutations causing antagonistic pleiotropy could also lead to DOL, when different types that do or do not carry the focal mutation interact with each other synergistically and fulfill the abovementioned criteria for DOL. However, to the best of our knowledge, no case has been reported yet where a mutation causing antagonistic pleiotropy gave rise to a DOL-type synergistic interaction. Hence, future work should explore this theoretical possibility and identify the role of antagonistic pleiotropy as a genetic cause for DOL in bacteria.

Classification of Interactions

In the following, we will provide an overview over different ecological interactions among members of the same or different bacterial species and classify them according to whether or not they fulfill the abovementioned defining criteria for DOL. For this, we categorize ecological interactions among bacteria along two main axes. First, we distinguish cases of intra- *versus* interspecific interactions. Second, we differentiate unidirectional (linear) interactions and bidirectional (reciprocal) interactions. All interactions considered are synergistic in the sense that at least one partner benefits from the interaction. This can, for example, be due to the release of a certain metabolite into the environment or the detoxification of an environmental chemical that enhances the growth of another cell in the same environment. For heuristic reasons, we only analyze interactions consisting of two or three members. These should be seen as the simplest units, from which more complex interaction networks are constructed. The same logic, with regard to whether or not the focal interaction represents a case of DOL, should apply to more complex cases containing more interaction partners as well.

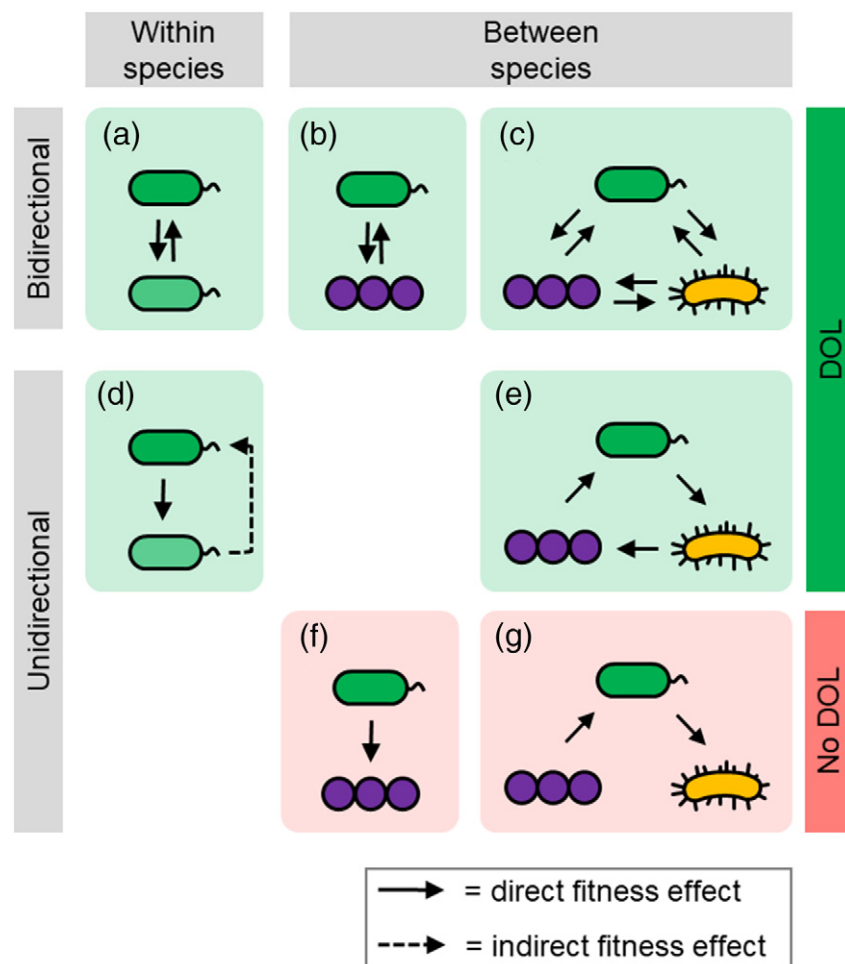


Fig. 2. Classification of pairwise and three-way interactions as DOL. Interactions can be uni- or bidirectional as well as occur within one or between two microbial species. Arrows indicate fitness benefits that are exchanged between interaction partners that can be direct (straight line) or indirect (dashed line). For an interaction to classify as DOL, four main criteria need to be fulfilled, which is only the case in closed (reciprocal) interactions (green), yet not in open (linear) interactions (red). (a) Mutually beneficial (reciprocal) interaction within two conspecific genotypes. (b) Mutually beneficial (reciprocal) interaction within two heterospecific genotypes. (c) Mutually beneficial (reciprocal) interaction between three different species. (d) Unidirectional interaction between two members of the same species. (e) Unidirectional interaction between three members of three different species. (f) Unidirectional interaction between two members of two different species. (g) Unidirectional (linear) interaction chain between members of three different species.

Interactions qualifying as DOL

Bidirectional interactions within species

When two tasks are divided among two members of the same species and the fitness benefit that both parties gain when interacting with each other is significantly higher than the fitness they could achieve in isolation, bidirectional interactions qualify as DOL (Fig. 2a). Helping other members of the same species does not only benefit the acting individual directly, but, depending on the relatedness between both individuals, possibly also indirectly. Thus, an individual can increase its own fitness by enhancing the growth and survival of close relatives [57].

This type of interactions, where two conspecific partners engage in a reciprocal exchange of benefits, is known to occur in different bacterial species [95,101,102]. A recent example has been described for the soil bacterium *B. subtilis* that forms biofilms, for example, at the air–liquid interface or on plant roots [95]. These biofilms benefit the cells that are involved in their production by providing a tighter adhesion to surfaces [103], resistance against harsh environmental conditions [104], or a better access to oxygen [51]. To establish a biofilm, several costly compounds need to be secreted that make up the biofilm matrix. These involve, besides extracellular DNA and certain structural proteins such as TasaA, mainly EPS. Cells within these biofilms can divide labor by only producing one of these matrix

components. This is achieved by displaying phenotypic heterogeneity, which results in matrix non-producers, EPS producers, and types that produce both EPSs and TasA [95]. Strikingly, mixtures of genetically engineered specialists that produced either only TasA or EPS showed negative frequency dependent selection and were more productive in terms of cell numbers produced than wild-type cells or monocultures of both types [95]. Thus, this system qualifies as DOL, because it fulfills all defining criteria mentioned above. The reasons why we still find phenotypic heterogeneity in the wild-type cells and not the seemingly superior strategy of a genetically determined DOL may be that, in the absence of positive assortment, a genetically differentiated population may lose essential components when dispersing to a new environment, while this is unproblematic when the differentiation is controlled epigenetically.

Bidirectional interactions between species

Bidirectional interactions between two species are functionally equivalent to the intraspecific case (Fig. 2b). However, one important difference is that the genetic relatedness between both interactors is low. As a consequence, only direct and not indirect fitness benefits result from the interaction. Nevertheless, because heterospecific strains are likely to differ in their genetic repertoire [105,106], they are also more likely to display a functional complementarity than two strains belonging to the same species. Since both parties benefit from the interaction, these direct fitness benefits can be sufficient to stabilize bidirectional interactions between species [107–110].

One example of an interaction involving two partner species is the obligate metabolic mutualism that has been experimentally evolved between a sulfate-reducing bacterium and a methanogenic archaeon. In this interaction, *Methanococcus maripaludis* consumes hydrogen, which is liberated by *Desulfovibrio vulgaris* as a by-product that results from reducing carbon dioxide to methane. By consuming hydrogen, *M. maripaludis* creates permissive environmental conditions that allow *D. vulgaris* to grow. The fact that serial propagation was sufficient to transform the interaction that initially exclusively relied on an exchange of by-product benefits into an ecologically stable and mutually beneficial cooperative interaction points to very strong fitness feedbacks that operate among both interaction partners [108,111].

Another kind of bidirectional interaction between two species can be observed in natural communities of nitrifying bacteria. In a process called nitrification, ammonia is first oxidized to nitrite by ammonia-oxidizing microorganisms (i.e., bacteria and/or archaea) and subsequently to nitrate by nitrite-oxidizing bacteria [112,113]. This DOL between two functional groups has been attributed to the

length of the underlying metabolic pathways, which maximizes ATP production and hence growth rate when they are shorter [112]. The fact that the bidirectional interaction between ammonia-oxidizing microorganisms and nitrite-oxidizing bacteria is complementary, synergistic, ecologically stable, and shows signs of positive assortment suggests nitrification fulfills all criteria to classify as DOL [113,114].

Empirical evidence for a three-way interaction comes from synthetic communities consisting of the three species of soil bacteria *Azotobacter vinelandii*, *Bacillus licheniformis*, and *Paenibacillus curdlanolyticus*. When coexisting in close spatial proximity, all members of this community can survive in a complex environment containing nitrogen gas, penicillin G, and carboxymethyl-cellulose, in which none of the three species could persist in isolation. This was possible because every species contributed a function that was essential to ensure the survival of the respective other strains and hence the entire consortium: *A. vinelandii* utilized gaseous nitrogen and converted it into amino acids that served as a nitrogen source for the other species. *B. licheniformis* degraded the available antibiotic with the help of β -lactamases, thus allowing the other two penicillin-sensitive species to grow. Finally, *P. curdlanolyticus* provided a carbon source to the whole community by degrading carboxymethyl-cellulose into glucose using cellulases. This synthetic example clearly demonstrates that the presence of different, functionally complementary species can allow a multipartite community to grow in a complex environment [115] (Fig. 2c). Unfortunately, it has not been tested in the cited work whether the interaction is stabilized by negative frequency-dependent selection and positive assortment. Given that the survival of each species hinged on its synergistic interaction with the other community members, it would be interesting to test experimentally whether this three-way interaction is stabilized by natural selection in the long run.

Unidirectional interactions within species

A huge body of literature exists on so-called *public goods cooperation*, where one genotype releases a cooperative function into the environment that is equally accessible to all members of the resident community [116]. Examples involve the production of a siderophore to chelate iron [117,118], invertase to cleave sucrose [119], a protease to degrade protein [120], or polymeric substances to produce biofilms [51]. Given that the production of these public goods is costly to the producing individual, evolutionary theory predicts that mutants, which do not produce the cooperative function anymore, should be favored by natural selection, because

they save the costs for producing the public good, yet still benefit from the production of other cells in the vicinity [121–123]. These mutants are commonly referred to as *free-riders* [57,121,124] or *cheaters* [124,125], because they appear to have abandoned production of the cooperative public good for seemingly selfish reasons.

However, viewed through the lens of DOL, these mutants may not be selfish non-cooperators, but specialists that grow faster than the public good-producing cells. Given that both producing and non-producing cells are genealogically related [116], non-producers might be interpreted as altruists that help close relatives to grow [116].

Although it has been frequently predicted that the emergence of non-cooperating mutants should result in a collapse of the focal interaction [121–123,126,127], experiments finding evidence for this prediction are generally rare (but see [128–130]). In addition, all of these studies assume a perfect mixing of cooperator, non-cooperator, and the public good—a condition that might be hardly ever met in the environments, in which bacteria occur naturally. In contrast, rather than breaking down, it has been shown in many natural and laboratory-based systems that populations containing both producing and non-producing cells display frequency-dependent selection that maintains both types in the long-run [63,119,131–137]. Moreover, in some cases, the simultaneous presence of producing and non-producing types even enhanced the productivity of the whole consortium [138] and permitted coexistence among species [139]. In other words, growth of the entire population would be reduced if every cell invested in the production of the public good. Instead, when only a subset of all (otherwise isogenic) cells produces the public good, the whole population can grow faster and is thus fitter than a population without non-producing cells. This type of interaction can hence be viewed as a case of DOL between producing and non-producing cells that trade a direct fitness advantage (i.e., the benefit resulting from the public good) against an indirect fitness advantage (i.e., slow-growing producers help fast-growing non-producers that are very close relatives to maximize their fitness) (Fig. 2d). The selective reason for this could be that producers of the public good serve as a dispersal stage that colonizes new environments and then begets non-producers to maximize population-level growth and potentially enhance competition against other species.

This principle is nicely illustrated by the filamentous soil bacterium *Streptomyces coelicolor*. Like many other actinomycetes, this species has the capacity to produce a vast range of different secondary metabolites that function as antibiotics [140]. Interestingly, wild-type cells of *S. coelicolor* do not produce large amount of antibiotics. However,

their genome shows an enormous degree of instability that gives rise to subpopulations of cells with deletions or amplifications of large genomic sequences. These mutated cells produce increased amounts of antibiotics at the expense of a drastically reduced growth. Although it has not been explicitly tested in this system, growth of the entire colony likely depends on the need for antibiotics. When no competitor is present, wild-type cells can grow unrestrictedly, while competitive situations should limit their growth and favor the antibiotic-producing mutants that in turn creates new opportunity for the wild type to grow. This study demonstrates a trade-off between growth and antibiotic production that gives rise to a DOL between two types specializing in either function. Although in this case the mutant is the producer of the public good, a similar logic applies to cases where the mutant is the non-producing, fast-growing variant.

Unidirectional interaction circles between species

This type of interaction involves an interaction chain of more than two partners, where the actions of the last member in the chain positively affect the fitness of the first member (Fig. 2e). By closing the loop in this way, the fitness of each community member is inevitably linked to one of its respective interaction partners. As long as these interactions are complementary and synergistic, the resulting fitness coupling is expected to select for increased cooperation among its community members. In addition, selection should favor mechanisms that allow types to repeatedly interact with each other or remain associated for extended periods of time [62]. Failing to cooperate within such a hypercycle-like arrangement [141] will curtail the fitness not only of the less cooperative individual but, as a consequence, also of the entire community. Hence, consortia that differ in the degree, to which their members invest resources into the interaction (i.e., their cooperativeness), will also differ in their consortium-level fitness. As a consequence, groups of cells that cooperate more efficiently should in the long run outcompete less cooperative groups.

One example for such an interaction between multiple species has been shown to occur in a synthetically constructed community of cellulose-degrading bacteria [142]. In this case, five bacterial strains (*Clostridium straminisolvans* CSK1, *Clostridium* sp. strain FG4, *Pseudoxanthomonas* sp. strain M1–3, *Brevibacillus* sp. strain M1–5, and *Bordetella* sp. strain M1–6) coexisted stably for 20 serial transfers. In this complex community, synergistic interactions are fuelled by the degradation of cellulose as the primary substrate by one community member. The resulting degradation products are liberated and consumed by two other strains that in turn produce by-products that serve as the

primary food source for two other community members. In this example, the circle of interactions is closed, because (i) removal of two different degradation products enhances the growth of the primary cellulose degrader, which otherwise would inhibit its growth, and (ii) three other strains consume oxygen and neutralize the pH that creates more optimal conditions for the primary cellulose degrader [142].

Interactions not qualifying as DOL

All interactions that do not meet one or more of the four defining criteria (i.e., (1) functional complementarity, (2) synergistic advantage (+/+), (3) negative frequency-dependent selection, and (4) positive assortment) do not qualify as DOL. This includes all linear or unidirectional interactions, where the fitness of the helping individual is independent of the fitness or behavior of the beneficiary. Although both partners might reciprocally adapt to each other, due to the lack of reciprocity, natural selection will not favor an increased investment of the exchanged trait in the donor. This situation is even aggravated when the two interacting individuals belong to different species, in which case also no indirect fitness advantages can compensate for potential costs that might accrue to the producing individual.

Unidirectional interactions between species

Any unidirectional by-product interaction, in which one individual releases a metabolite as a waste product into the extracellular environment that in turn is consumed by a member of a different species, falls into this category (Fig. 2f).

An example for a unidirectional interaction between two species is the exploitation of public goods, which are produced to support the growth of close relatives of the producing cells, by members of other species. For instance, an extracellular enzyme that inactivates an antibiotic (e.g., β lactamases that hydrolyse β -lactam antibiotics [131,143]) can protect both the bacterium that produces it and its neighbors [98]. The detoxifying enzyme is a public good, because it is costly to produce and equally available to all cells in the vicinity [99,100,144]. Since the producing individual carries the burden of the public good but does not benefit, when the released function provides a growth advantage to members of other species, this type of interactions does not qualify as a case of DOL. In contrast, when the producer of the beneficial function and the beneficiary are closely related, the interaction would qualify as DOL (see above). Interestingly, however, is the observation that a unidirectional public good interaction between two species, where one species produces the

public good and the other one consumes it, can be ecologically stabilized by intraspecific competition between producing and non-producing individuals for the public good [139,145].

Sequential interactions between species

Sequential interaction chains involving more than two interaction partners do not qualify as examples for DOL when no fitness feedbacks operate between the different links in the chain (Fig. 2g). Consider, for example a sequential reaction, in which a complex biopolymer is microbially degraded (i.e., a food chain). What is commonly observed is that not a single bacterial species will break down the substrate all alone, but rather consortia consisting of multiple different species accomplish this task. It frequently works like this that one species attacks the substrate, and, in this process, liberates by-products that in turn are catabolized by other species in the local environment [146,147]. The consortia involved in breaking down complex substrates include very often several different species that degrade the focal substrate in a stepwise manner [2,26,148,149]. Although the first species in the chain liberates by-products that benefit other members of the consortium, the fact that the interaction is unidirectional prohibits fitness feedbacks to operate (Fig. 2g), disqualifying it as DOL according to the definition mentioned above. The groups of bacteria that assemble to jointly degrade a certain substrate are likely also not dispersed together, but increase in frequency as a consequence of their incidental local occurrence as the substrate becomes available [150].

An example of such a sequential interaction without fitness feedbacks is the metabolic interactions between multiple bacterial strains of the human gut microbiota. Recently, it has been shown in a gnotobiotic mouse model that gut symbionts of the species *Bacteroides ovatus* that digest complex polysaccharide like inulin and xylan liberate monosaccharides such as glucose and fructose, which benefit other species in the gut including *Bacteroides vulgatus* and *Lactobacillus paracasei* [120]. These monosaccharides are then further catabolized by *B. vulgatus* and *Faecalibacterium prausnitzii*, which in turn liberate short-chain fatty acids like acetate and butyrate [151] that might be used by *Desulfovibrio piger* and other gut bacteria [152]. In this case, the intermediate and last members of the chain benefit from the action of their respective anterior partner that provides the growth-limiting substrate. However, no fitness feedback is known to operate between different members of the chain, which precludes classification of this interaction as a case of DOL (Fig. 2g).

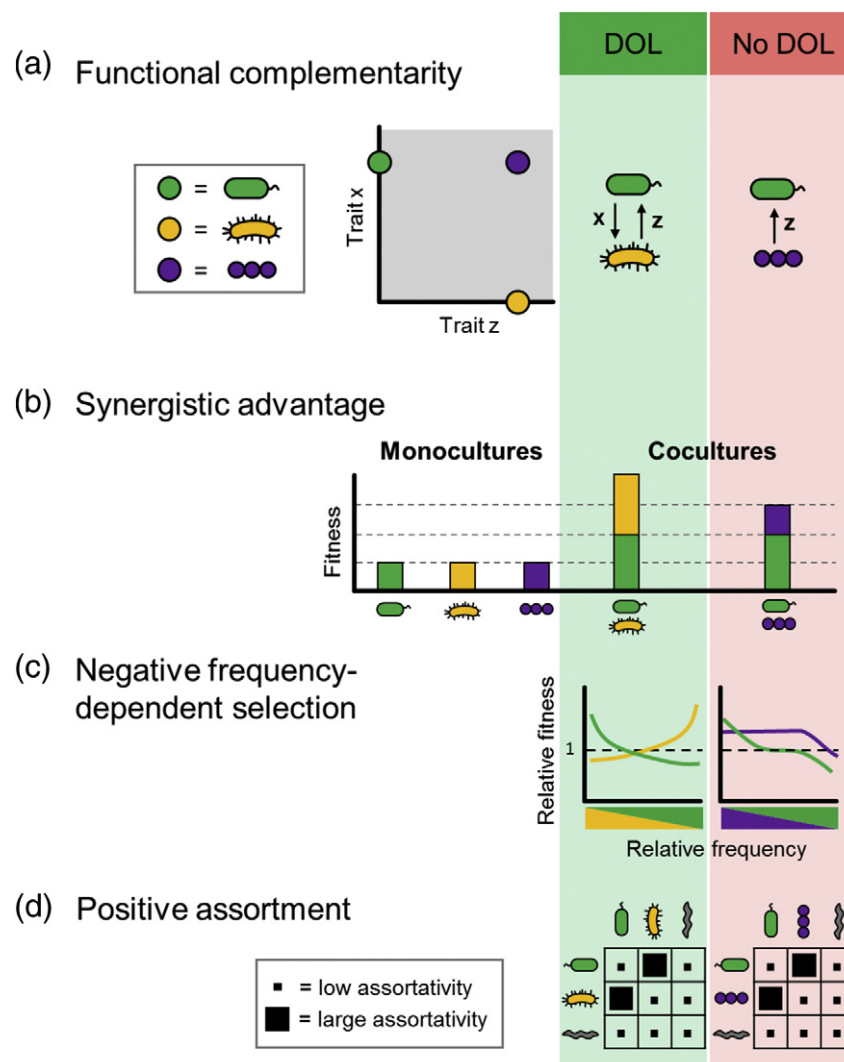


Fig. 3. Diagnostic experiments to identify DOL. (a) Phenotypic complementarity between two or more individuals can be elucidated by identifying and quantifying traits (x and z) that affect the fitness of the respective other interaction partner. Such an exchanged function could for instance be the production of metabolites (e.g., siderophores or nutritional compounds) or the detoxification of certain compounds in the environment. In this example, the green and orange bacteria specialize in traits x and z , respectively, while the purple bacterium is a generalist that can perform both functions equally well. Based on these constellations, a possible bidirectional interaction emerges for the green and orange bacterium, while a possible interaction between the green and purple individual that is based on traits x and z is unidirectional. (b) Synergistic advantages can be tested by comparing the individual's fitness when growing in mono- and cocultures. Interactions are mutually beneficial when the fitness of all interactions partners is significantly increased in coculture relative to monoculture conditions. The only exception to this is interactions within species, where the cost of altruistic behaviors can lower the fitness of strains in coculture relative to monoculture conditions. Thus, in the example, the interaction between the green and orange bacterium does fulfill this criterion, while the unidirectional interaction between the green and the purple strain does not. (c) Negative frequency-dependent selection can be experimentally addressed in reciprocal invasion-from-rare experiments, where the initial ratio of the interacting organisms is altered and the resulting relative fitness for each organism is quantified. Negative frequency-dependent selection occurs when the relative fitness of each species is highest, when its relative frequency is low and decreases with an increasing initial frequency. In the example, the orange and green bacteria show negative frequency dependence, while the purple individual does not. (d) In mixed cultures, which include besides the focal individuals also types, which do not participate in the DOL interaction, positive assortment can be quantified as an increased probability to interact with other types engaging in DOL, relative to non-participating individuals. In the example, both interactions (green + orange, green + purple) show positive assortment, because in both cases, the green individual requires the respective other one for providing trait z . Together, although the interaction between the green and the purple bacterium shows positive assortment and thus fulfills the fourth criterion, it does not qualify as DOL, because the first three defining criteria are not met.

Diagnostic Experiments to Identify DOL

In the following, a set of diagnostic laboratory experiments is suggested that can be used to evaluate whether a given interaction fulfills the abovementioned four criteria defining DOL (Fig. 3). First, metabolic complementarity (Fig. 3a) can be investigated by means of comparative genomics, as the individuals' genomes can provide evidence for the physiological capabilities of the organisms. For instance, an analysis of the genomes of the two bacterial symbionts (*Baumannia cicadellinicola* and *Sulcia muelleri*) of the insect (*Homalodisca coagulata*) suggested striking complementarity of biosynthetic capabilities for the production of essential metabolites between both symbionts, suggesting selection favored this type of division of metabolic labor [153]. However, if possible, sequencing data should be complemented by experiments to confirm the assumed functional relationship. This can be achieved, for example, by analyzing profiles of nutrient utilization and quantifying the release of metabolic by-products or other functions into the environment. Such experiments can identify differences in niche specialization [154] and point to potential cross-feeding interactions [155].

Second, synergistic advantages resulting from species interactions can be determined in experiments, which compare the growth (fitness) of all interaction partners under mono- and coculture conditions (Fig. 3b). Such experiments provide evidence for synergistic advantages when the fitness of all individuals/species in coculture exceeds the fitness they can achieve in monocultures under the same environmental conditions. As soon as one species does not benefit from the interaction or even has a reduced fitness in coculture, the interaction is not mutually beneficial and does therefore not represent a case of DOL. As discussed above, an exception to this is interactions within species, where one individual can altruistically invest resources that benefit conspecific interaction partners at a cost to itself. Finding that strains are unable to grow in monoculture, but only under coculture conditions, would point to an obligate nature of the focal interaction under the given environmental conditions [156–159].

Third, the question whether a given interaction is ecologically stabilized by negative frequency-dependent interaction can be addressed in reciprocal invasion-from-rare experiments (Fig. 3c). The rationale of these experiments is the assumption that in a given community of $n \geq 2$ members, all types can stably coexist, when they can invade (i.e., increase in frequency) a resident community consisting of the respective other type(s). If this is the case, the frequency of each type should reach a stable equilibrium point, around which abundances of each subpopulation fluctuate. By initiating the

experiment with different ratios between the invader and the resident community and subsequently monitoring their fitness (i.e., change in the number of colony-forming units between two time points), a graph can be produced that allows to plot the ratio of the invader to the resident community (x-axis) over the relative fitness of the invader depending on this ratio (y-axis). If the fitness of the invader is highest when it is rare, yet decreases as its relative frequency increases, the experiment would point to negative frequency-dependent selection. The point at which the fitness of both types (i.e., the invader and the resident community) is equal, is the equilibrium point, around which frequencies should fluctuate. Negative frequency-dependent interaction is a powerful ecological mechanism that stabilizes multiple different genotypes, and which is commonly observed in a broad range of qualitatively different ecological interactions [63,95,160,161].

Fourth, for DOL to persist in the long-run, the participating individuals need to display an increased probability to interact with each other in subsequent rounds of interaction, relative to other, co-occurring types that take advantage of the interaction, but do not reciprocate (Fig. 3d). This mechanism, which is called positive assortment, can manifest in different ways, including (i) one or both phenotypes are continuously created from the respective other type (e.g., phenotypic heterogeneity or regulatory mechanisms), (ii) both types remain physically associated with each other for extended periods of time (permanent association, vertical transmission), (iii) both types show positive assortment in spatially structured environments, (iv) one or both individuals can identify and actively choose their preferred interaction partner, or (v) one or both individuals antagonize non-preferred interaction partners. Independent of how this is achieved, the diagnostic criterion to assess positive assortment is to quantify interaction probabilities of all types involved. Since this is often difficult to achieve experimentally, spatial proximity between cells can be used as a proxy. Assuming that two cells with a close distance are more likely to interact with each other than two cells that are further apart, the degree of assortment can be quantitatively determined by analyzing microscopic images. One possibility to determine the degree of assortment among cell populations is to measure the local neighborhood of one cell type relative to its frequency in the global community [162]. The higher the degree of positive assortment, the more effective is the reestablishment of the ecological interaction in subsequent rounds of interaction and the more stable is the interaction in the long-run. This approach has been previously applied to analyze colonies grown on agar plates [163] or cryo-sectioned biofilms [164–166].

Discussion

The main aim of this paper was to conceptually reconsider DOL in microbial communities in order to develop a framework that allows to unambiguously identify cases falling in this category. In this way, we did not only identify four main criteria that an interaction among two or more microorganisms has to fulfill in order to qualify as a true case for DOL (i.e., (1) functional complementarity, (2) synergistic advantage (+/+), (3) negative frequency-dependent selection, and (4) positive assortment), but also suggested a set of diagnostic experiments that can be applied to verify whether a given interaction qualifies as DOL. Systematically analyzing different interactions in light of this framework revealed several interesting insights.

First, the term DOL is traditionally associated with multicellular organisms [6,167–169] or colonies of eusocial insects [11–13,17], where several different lower-level units take over specialized functions, which, in combination, enhance the performance of the higher-level entity (i.e., multicellular organisms or eusocial colony). In our view, these paradigmatic cases of intra-specific or intra-individual interactions represent extreme manifestations along a continuum of interactions that otherwise also includes associations that are looser and less highly evolved. This is why we also included interactions between different species in our scheme—a view that is shared by other authors as well [112,148,170]. Although in these interactions the degree of genealogical relatedness between interaction partners is naturally low, synergistic partnerships between species share many characteristics with intraspecific interactions, such as the functional complementarity, the synergistic advantage that results from the interaction, and a coupling of evolutionary interests. The main difference between intra- and interspecific interactions is the mode of how individuals repeatedly re-establish associations with their complementary partner(s): DOL within the same species relies on a phenotypic differentiation of already closely neighboring cells via epigenetic, regulatory, or genetic mechanisms. The resulting positive assortment among close relatives increases the chance that any investment in the interaction is also benefitting the desired interaction partners. In contrast, the challenge faced by interspecific interactions is that two or more cells that are already phenotypically differentiated need to develop mechanisms that allow them to reliably re-associate with their desired partner to maintain the interaction in the long-run. This can be achieved by various mechanisms (for a recent review, see [62]). In any case, shared habitat preferences and significant fitness advantages resulting from the interaction likely exert strong selection pressures that favor mechanisms of positive assortment.

Based on these considerations, it appears arbitrary to us to distinguish interactions within and between species in the context of DOL.

Second, when thinking about criteria that define DOL, we adopted a definition that on first sight might appear rather permissive and less stringent than previous uses of the term [171]. However, the reasons for this were as follows. Criteria like, for example, “the interaction has to be evolved for this purpose,” are rather restrictive and often difficult to test experimentally. Even if an interaction, in which two bacterial genotypes share complementary functions to the benefit of both interacting partners has not evolved for this purpose, it can still have a significant impact on the evolution of both strains involved. Moreover, if sharing two complementary functions aligns the evolutionary interests of both interacting partners, very strong selection pressures should operate that not only maintain the interaction in the long-run but also lead to reciprocal adaptations and enhanced investments of all participating individuals. For example, bacterial genotypes commonly lose the ability to perform certain metabolic functions, when the corresponding metabolites are sufficiently available in the extracellular environment [160,172]. When environmental levels of the required metabolites drop below certain thresholds that are critical for the survival of the newly evolved auxotrophic mutants, other strains in the vicinity that are still able to produce the focal metabolite can serve as a source for this essential nutrient [63,158,173]. In this way, interaction networks emerge by self-organizing principles that are governed by the production and consumption levels of the different metabolites [174,175] or the dynamics of metabolite diffusion in the environment [65,163,176]. In the long-run, these interactions should intensify [96,108] and become more obligate [177].

Moreover, by including negative frequency-dependent selection and positive assortment as defining criteria, we excluded transient and ephemeral interactions that lack the potential to evolve into longer-lived and tighter synergistic relationship. However, our definition does not require the interaction to be essential: also facultative interactions can qualify as DOL, if they fulfill our four main criteria. Ultimately, ecological interactions are always context-dependent. As a consequence, the same pair of genotypes may display a clear DOL in one environment, yet live independent from each other in another one. Embracing these cases is essential, because they can help to understand the factors that drive the evolution of obligate and non-reversible DOL. The criteria and diagnostic experiments defined here provide a heuristic tool to clearly differentiate between these different cases.

Third, one of the main insights that emerged from our analysis is the reinterpretation of non-cooperators in intraspecific public good interactions as energy-saving growth specialists that divide labor with their public good-producing conspecifics. Given that in nature, a population of a bacterial species has to solve more than one evolutionary problems [178], it is likely more efficient to segregate these tasks into different cells that each specialize in performing one or few of these functions, rather than every cell investing resources into all of them. A DOL of this type essentially requires that each individual shares the benefits resulting from its investment with other clonemates—a condition that is easily met in public good-based interactions. Exploitation of these openly presented resources by other species is often hampered by specificity-enforcing mechanisms such as a requirement for dedicated uptake systems as is the case for siderophores [179] or the need to be resistant to a toxin that is produced as a public good [140].

Hence, focusing on just one of these traits might be misleading and paint a wrong picture that does not adequately reflect the conditions experienced by bacteria under natural conditions. This new view can help to explain the widespread occurrence of public goods cooperation in nature, despite the ubiquity of non-cooperators within these systems [121,123]. A mathematical model developed to test the plausibility of this idea revealed that intraspecific DOL in public good interactions is promoted when (i) compound sharing is high and (ii) production costs can be shared among a sufficient number of producing individuals [180]. Interestingly, analyzing the distribution of several social traits simultaneously (i.e., production of biosurfactants, biofilm matrix components, proteases, siderophores, and toxic compounds) within natural communities of *Pseudomonas* revealed the existence of individuals that carried the burden of producing multiple public goods, while other types only showed a reduced investment in these traits [178]. More work is definitely necessary to identify the rules that govern the distribution of biochemical functions within microbial communities. Nevertheless, adopting this new perspective when considering non-producers of public goods can help to better understand the evolutionary causes for their emergence as well as inspire new experiments to identify their role in natural microbial communities.

Taken together, our comprehensive analysis revealed many examples from the microbial world that fulfilled hallmarks of DOL. Thus, it can be concluded that DOL is a ubiquitous principle that is key to many biological systems for enhancing the efficiency of a certain process or to solve a given evolutionary problem. By providing a conceptual toolkit, our work should help to unambiguously identify and classify cases of DOL and stimulate a

more detailed investigation of this important and widespread type of intermicrobial interaction.

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DOL, division of labor; *Salmonella enterica*, *Salmonella enterica* serovar Typhimurium; EPS, exopolysaccharides.

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Glossary

Altruism: A behavior or trait that increases the fitness of the recipient, but decreases the fitness of the actor

Antagonistic pleiotropy: Phenomenon where a mutation in a gene has a positive effect on one and a negative effect on another phenotypic trait

Bet-hedging: Survival strategy, in which a clonal population produces phenotypic variants in stochastic or unpredictably chan-

ging environments. This type of phenotypic heterogeneity ensures that a subpopulation is well-prepared for environmental changes, even though its fitness in the current environment is reduced.

Cooperation: A behavior that is costly to the producer and beneficial to the recipient, and which evolved because of this beneficial effect

Darwinian fitness: Heritable ability of a genotype of organisms to contribute offspring to the next generation

Direct fitness: Fitness component that results from reproduction of the focal individual

Epigenetic mechanism: Phenotypic change in isogenic bacteria that is due to non-genetic mechanisms affecting gene expression, protein levels, or other ways to alter the cell's phenotype. The resulting phenotypes may or may not be heritable depending on the rate of phenotypic change. The ultimate cause for the epigenetic mechanism may still be a mutation in the underlying genetic sequence.

Eusociality: This evolutionarily highest level of colonial organization is defined by (i) cooperation in caring for the young, (ii) reproductive division of labor, where some adult individuals (i.e., sterile workers) reduce their own lifetime reproductive potential to raise the offspring of others (i.e., fertile queens), and (iii) overlap of at least two generations that can contribute to colony labor. Eusociality has evolved for example in ants, bees, wasps, and termites.

Functional complementarity: Phenomenon, where two or more interacting organisms perform different essential biochemical functions in a complementary way

Generalist: An individual that is able to thrive under a broader range of environmental conditions or has a broader range of phenotypic capabilities than a specialist.

Indirect fitness: Fitness component that results from aiding the reproduction of close relatives.

Metabolic flux: The rate at which a metabolic substrate is converted to a specific product through a metabolic pathway

Metabolic by-product: A metabolite that is actively or passively released from a cell, because it is toxic or currently not used as a resource. However, other cells in the vicinity can benefit from a consumption of these metabolites

Mutualism: Cooperation between two species

Niche specialization: Evolutionary process, during which a population or species becomes better adapted to a particular set of biotic and abiotic conditions

Pleiotropy: Phenomenon, where one locus affects more than one phenotypic traits

Public good: A resource that is costly to produce and released into the extracellular environment. All individuals in the vicinity can potentially benefit from utilizing the public good.

Relatedness: Measure of genetic similarity between two individuals

Specialist: An individual that has less phenotypic capabilities than a generalist or is specialized to survive in a subset of environmental conditions a generalist can survive in

Streamlined genome: Reduction in genome size that is favored by natural selection

Synergistic interaction: Interaction between two species that benefits all individuals involved

Trade-off: Relationship between two fitness-related traits such that an increase in the performance of one trait comes at the expense of a reduced ability to perform the other one