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Report

Competition, Not Cooperation, Dominates Interactions among Culturable Microbial Species

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Summary

Microbial cells secrete numerous enzymes, scavenging molecules, and signals that can promote the growth and survival of other cells around them [1–4]. This observation is consistent with the evolution of cooperation within species [5], and there is now an increasing emphasis on the importance of cooperation between different microbial species [4, 6]. We lack, however, a systematic test of the importance of mutually positive interactions between different species, which is vital for assessing the commonness and importance of cooperative evolution in natural communities. Here, we study the extent of mutually positive interaction among bacterial strains isolated from a common aquatic environment. Using data collected from two independent experiments evaluating community productivity across diversity gradients, we show that (1) in pairwise species combinations, the great majority of interactions are net negative and (2) there is no evidence that strong higher-order positive effects arise when more than two species are mixed together. Our data do not exclude the possibility of positive effects in one direction where one species gains at the expense of another, i.e., predator-prey-like interactions. However, these do not constitute cooperation and our analysis suggests that the typical result of adaptation to other microbial species will be competitive, rather than cooperative, phenotypes.

Results

Pairwise Interactions

We are interested in the frequency of cases where microbial species help one another in a manner that is consistent with the evolution of cooperation between species [7]. Examples of cooperation between species are most familiar in the natural world from macroscopic examples like plants and pollinators where plants gain pollen dispersal and insects gain energy-rich nectar from the interaction. The critical characteristics of such evolved cooperation between species are that the presence of members of one species increases the fitness of members of the other species, and vice versa (more below) [8, 9]. We are interested then in how often fitness interactions between bacterial strains tend to be positive for both species. We assessed the fitness impacts of species interactions by comparing the productivity of single-species and multispecies cultures of 72 bacterial species where the number of cells inoculated is constant across the treatments (we relax this later). All species are collected from one environment—permanent rainwater pools in a beech tree forest known as "tree holes"—using a medium that is designed to allow as many species to grow as possible. We then study these species in spatially structured laboratory microsms containing beech leaf media that is designed to capture the natural ecology (Experimental Procedures).

If there is no interaction between microbial species, the productivity of a two-species mixture is expected to be exactly the sum of the two single species grown alone as would occur, for example, if two species occupy independent niches (Figure 1B). The sum of the productivity of each species when alone therefore forms our null for comparison with the observed productivity of the corresponding two-species mixture. If two species are mutually competitive, the mixture will be less than the null and if two species are mutually cooperative and promote each other's growth, the productivity of the mixture will be greater than this null (Figures 1C and 1D). If one species benefits from mixing while the other is harmed, the productivity can fall either above or below the null line. This class of interaction is discussed more below but we do not consider these to be examples of cooperation for the same as reason a prey species being eaten by a predator is not considered as cooperation by prey. That is, we are interested in the potential for cooperative adaptations in one species that evolved, at least in part, because of the effect that they have on the other species [7]. And it is very difficult to see how an adaptation that helps another species can evolve when it has a net fitness cost to a member of helping species as Darwin recognized: "If it could be proved that any part of the structure of any one species had been formed for the exclusive good of another species, it would annihilate my theory, for such could not have been produced through natural selection" [10]. We do not discuss commensalism independently, where one species is positively affected and another unaffected, but instead subsume this interaction within our classification of mutual benefits.

We analyzed the productivity (overall respiration from CO_2 production) of 180 two-species mixtures grown in aquatic microcosms after 7 (Figure 2A) and 28 (Figure 2B) days. In both cases, the great majority of data points lie well below the null line, which is consistent with a primary role for competition between species. The minority of the pairwise tests (7 days: 6%, 28 days: 10%) that lie above the null line are also typically only slightly above the line indicating that the gain in productivity when there are positive species interactions is typically modest. The strongest evidence for mutual cooperation is two cases out of the 180 where there is approximately a doubling of productivity by 28 days. Overall, the observed mixture productivity was 60% (±2% SE) and 63% (±3% SE) of what would be predicted under a null of no interaction after 7 and 28 days, respectively, implying a strong negative effect of mixing. Moreover, some of the net positive interactions may involve one species making gains in productivity in the mixture while the other species makes losses in productivity. In these interactions, the species that is helping is being harmed, which is analogous to exploitation rather than the evolution of cooperation between species (analogous to a predator-prey relationship).

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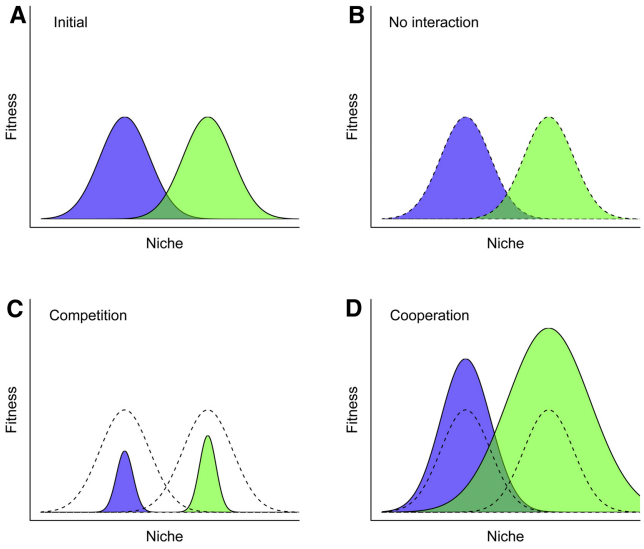

Figure 1. Models of Species Interactions

Productivity (fitness) of two species is shown across a niche axis.
(A) Initial niche space occupied by the two species.
(B) If there are no interactions, species productivity is unaffected by the presence of the other species.
(C) If there are net negative interactions, such as interference competition, the niches of some species might be altered and the overall productivity of the community is lowered.
(D) If there are net positive interactions, such as facilitation, the species benefit from the presence of other species and the overall productivity of the community increases. If the effect of interaction is to benefit one species and harm the other, the overall community productivity may increase or decrease and these cases are discussed more in the text. Dashed curves are the initial niche (A) plotted for comparison.
See also Figure S1.

Some of the net negative interactions may also include cases where one species is helped and the other is harmed. Although there is a positive component to these interactions, they again are inconsistent with the evolution of cooperation. Moreover, in these cases it is clear anyway that negative effects are dominant. In sum, even using the most conservative estimate that all net positive interactions are mutually beneficial the pairwise data do not support the importance of strong cooperation between the species studied. Only a small minority of interactions are mutually beneficial.

We considered whether the patterns of interaction could be understood in terms of phylogenetic similarity. For example, one might expect increased potential for cooperation among species that are from different phylogenetic groups, owing to decreased metabolic similarity. We, therefore, reanalyzed the data based upon whether the interacting bacteria in the experiment were from the same or different phylogenetic division. This analysis, however, revealed no differences as a function of phylogeny. In both cases, there were similarly negative effects of one species on another irrespective of whether the species were from the same division (Figure S1 available online).

Finally, one might consider that the typical lack of cooperation that we observe is because our experiment puts together species that do not typically interact in natural environments. For example, this might be because they have different niches and thus occupy different microhabitats within the tree hole. Although it is near impossible to assess the exact niches overlap of microbes in nature, our experiments are intended to mimic the tree-hole environment (Experimental Procedures). If such microhabitat selection does occur, therefore, the species are also not expected to interact in the microcosms and the majority of pairwise interactions will fall on the line of no interaction. Instead, species typically fall below the line, consistent with competition and niche overlap. In combination with our species coming from one very specific environment, we conclude that these species are likely to meet and interact under natural conditions.

Multispecies Interactions

A focus on pairwise interactions is an effective way to establish the potential for cooperation between different microbial species [11]. However, this approach neglects the potential for higher order interactions that occur when sets of three, four or more species engage in loops of mutually beneficial interaction, which are central to many properties of ecological networks [12–16]. We therefore also compared the productivity of a range of mixtures, which contain up to 72 species, against our null line that assumes there is no interaction among the constituent species. If higher-order positive interactions are more important than pairwise interactions for multispecies cooperation, the productivity of these more diverse species mixtures will more often lie above the null line than the pairwise mixtures. We assayed 615 multispecies mixtures chosen at random as described in [17]. Figures 2D and 2E compare single-species productivity to productivity observed in multispecies mixtures.

The comparison provides an even more stark illustration of the relative importance of competition and cooperation in multispecies mixtures. Net productivity of the multispecies mixtures lies below the null line (red line) and for the more diverse species groups the null line for no interaction is many times greater than the realized productivity. We can also compare the productivity of mixtures to the average productivity of the constituent strains in monoculture (Figures 2D and 2E, blue lines). This reveals that the mixtures do perform better than the average of the monocultures, which suggests that interspecies competition is not as strong as intraspecific competition and may indicate some degree of niche separation among the strains. However, this productivity gain is very modest and is far from that required to indicate that cooperation among species is the norm (red line). Allowing for higher order interactions among species then suggests little or no role for positive feedback loops involving three or more species in the potential for cooperation among species.

The Results Are Robust to Substitutive and Additive Experimental Design

The above data come from experiments where total cell number was held constant across treatments at the initiation of the experiment. This "substitutive experiment design" controls for total initial cell density across the diversity gradients [18]. However, a consequence of this design is that there will be fewer cells of a particular strain inoculated in mixtures of species as compared to monocultures. Under some conditions, this might unfairly disadvantage mixed-genotype treatments in our no-interaction comparison, which assumes that a two-species mixture without interaction will produce double the number of cells as compared to monocultures. Specifically, if cell growth is purely exponential then a mixed pair of genotypes that do not interact will not reach double the cell numbers of single genotypes, simply because each strain started with half the number of cells.

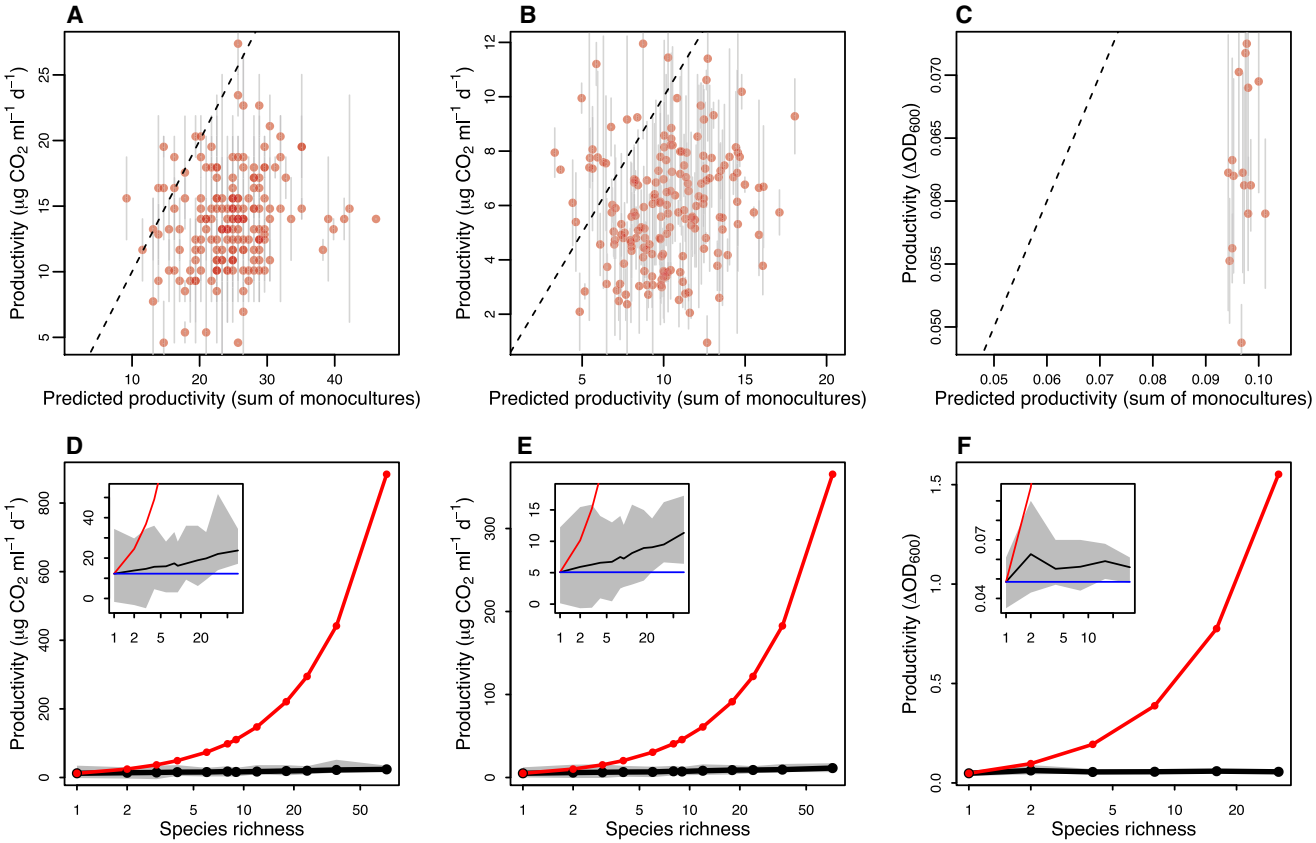

Figure 2. Interactions among bacterial isolates

(A–C) Pairwise interactions between 72 bacterial strains over (A) 7 days, (B) 28 days, and (C) between 32 strains over 7 days. The observed data were collected by measuring total productivity in randomly chosen pairwise mixtures. Predicted values (y axis) are the sum of the monoculture productivity. The dashed line is where predicted values equal observed values, which will occur if there are no interactions between species (Figure 1B). Grey vertical lines are SEs.

(D–F) These data are extended across multiple levels of species richness up to 72-species at (D) 7 days, (E) 28 days, and (F) for the 32-species experiment at 7 days. The y axis is total community productivity measured as total respiration measured as change of pH in an indicator as described in Experimental Procedures. The black line is mean observed values with the full data range (min to max) shown in gray, which is only just visible in the main figures. The red line is mean sum of the monoculture productivities, which will occur if there is no interaction among species (Figure 1B). The blue line is the average of the monoculture productivities of the constituent species. The same data are plotted in the inset figures but different y axis values are used to see all data at the lower end of the data range.

We do not expect the described inoculation effects to be important in our data because the experiments were run until cells were close to stationary phase where initial densities become unimportant for final yield. We nevertheless wanted to make sure that our conclusions were not affected by inoculation conditions. We therefore ran a second independent experiment involving 32 species from the same tree-hole population in which the number of cells per species were constant across all treatments (an "additive" design [19]). In this case, for example, mixtures of two species had double the density of single species. These data showed the same patterns as the original data, with none of the two-species mixtures exceeding what would be expected if species productivity was additive (Figure 2C). Furthermore, there was again no evidence for the importance of higher-order positive interactions, which involve more than two species (Figure 2F).

Discussion

There is a substantial literature that documents positive interactions among microbial species [1, 4, 6, 20], leading some to conclude that positive interactions predominate in microbial communities: "Given that mixed biofilms are ubiquitous and found in both ecological and clinical environments, one can assume that synergistic interactions between species predominate over antagonistic ones, particularly synergies that facilitate a robust coexistence" [4]. While such statements may yet prove correct for some ecologies, our data are consistent with a view of microbial species interactions where competition is by far the dominant outcome [21], with cooperation among species absent from most interactions. Moreover, even in the set of pairwise interactions that are consistent with cooperation, the net benefits to the species involved remain relatively modest (Figure 2).

Our primary analysis is limited to one ecosystem. However, data from other environments provide consistent findings. In the supplemental materials, we analyze data from a study of marine bacteria that show a very similar pattern to those seen for our rainwater-pool species [22] (Figure S2). How do our findings relate to the frequent emphasis on positive interactions in the literature? There are a number of points where our study can depart from previous work on cooperation

among bacterial species. It is not our intention to criticize the studies we discuss below as each stands on its own merits. However, these studies tend to have different goals to ours and it is important to look at the similarities and differences in order that we evaluate the generality of our conclusions.

The first point of difference is that we attempt an unbiased assessment of the frequency of positive interactions among strains. Other studies have a different goal and perform screens for a focal phenotype in order to rapidly focus in on the phenotype for further study. Many of these studies actively seek positive interactions, and are therefore intentionally unrepresentative. The emphasis of these studies then is that there can be positive interactions among genetically different strains and species, not that they are the norm. This includes studies of synergistic biofilm formation [23, 24] and the observation of improved growth of some species in coculture [25].

A second difference is that we apply the evolutionarily appropriate definition of between-species cooperation that both species must increase their productivity in coculture, which is reflected in our minimal cutoff: two strains must produce more than the sum of the biomass of either alone. This may appear stringent, but note that this cutoff is simply the line of zero interaction among species as would occur with complete niche separation (Figure 1B). Below this cutoff, one of the two strains is being harmed by the interaction, which is not consistent with the evolution of cooperation between species (it does not pay to help a species that harms you, see results) [8, 26]. Other studies, which find evidence of positive interactions, do not estimate total productivity in direct competition and focus primarily upon one-way positive interactions [11, 25, 27, 28]. Despite these differences, one can attempt estimates of the maximum frequency of positive mutual interactions in some previous studies. These are likely to be overestimates for the reasons discussed above. Nevertheless, these all suggest a minority of mutual positive interactions: synergism among strains in biofilm formation, 14% [24]; growth promotion through sideorphore secretion, 10% [25]; and growth promotion among *Streptomyces* strains, 4% [11] (K. Vetsigian, personal communication).

The third difference is that we do not select for, or engineer, cooperative interactions in the laboratory. Theoretical modeling shows that stable cooperation can occur among microbial species, but also emphasizes that it requires specific conditions that will limit its emergence [9, 12, 29, 30]. This is reflected in a series of empirical studies that use engineering or evolution to study cooperation. For example, one can find metabolic cooperation among sets of auxotrophic mutants in *Escherichia coli* [31], and promote cooperation among species by introducing artificial spatial structure that limits competition [32]. In addition, experiments have used engineering or natural selection to show that mutually beneficial interactions can be generated [33–35]. However, laboratory evolution does not necessarily lead to mutually beneficial interactions or coexistence and the other outcomes include competition [36] or the weakening of interactions following character displacement [37].

Our study only considers interactions among species that will actually grow in the laboratory. This limitation is a necessary condition of any direct assessment of species interactions, but it could bias our estimates toward sets of species that are more competitive. This would be the case if the difficulties in culturing bacteria are due to mutually beneficial interactions among species that are needed to get bacteria to grow. For example, anaerobic "syntrophic" interactions occur when consortia of several species act together to break down complex substances, which we would not detect in our respiration assays, e.g. [38]. There is some evidence that positive interactions among species do increase the yield of culturable bacteria [25, 27, 28]. However, as discussed above, these appear to be positive in one direction only whereby of a "helper" strain that produces a factor that enables a recipient species to grow, something recently termed "black queen" evolution [39]. More work is needed to evaluate the effects of the recipients on the helpers but, to the extent that this effect is negative, these interactions would not constitute mutualism that would lead us to underestimate the frequency of cooperation among species. Moreover, an estimate of the frequency of this class of one-way positive interactions is modest at 10% [25]. In sum, the studies that have looked specifically at promoting the culturability of bacteria also do not suggest that large-scale mutual cooperation is common. Nevertheless, it will be important to reevaluate our conclusions as bacteria culturability improves.

Our statistical estimate that cooperation among species is relatively rare does not, of course, exclude the fact that there are examples of cooperation among microbial species [1, 4, 6, 20]. These include dental species [40] and the bacterium *Pelotomaculum thermopropionicum*, which uses its flagella to physically attach itself to the methanogenic archaeon *Methanothermobacter thermautotrophicus*, where flagella attachment induces the latter to exchange metabolic services [41]. Such examples notwithstanding, our analyses suggest that competition rather than cooperation dominates interactions among microbial species. This conclusion has implications for our understanding of microbial communities. In particular, it warns against the untested assumption that bacterial communities can be viewed as superorganisms, which function as a single cohesive adaptive unit [42–46].

Our findings raise a conundrum. The evolution of cooperative phenotypes within a single microbial species appears to be a common occurrence [5]. Moreover, laboratory studies suggest that cooperative interactions can readily evolve between species over a few hundred generations for a variety of bacteria [34, 47]. The current study indicates that, although cooperation can readily evolve, in general, it does not evolve among natural microbial species. Why aren't there more mutually positive interactions among microbial species? One key factor may be the potential for resource competition among microbes. Indeed, there is evidence that tree-hole species tend to use similar resources as different species have similar effects on the chemical profile of media as they grow [47]. Another important factor is the extreme diversity of natural microbial ecosystems, which far outstrips that in experimental systems. If interactions with other species or strains are fleeting and unreliable, there will be little evolutionary benefit from investing in other species over short or long timescales [9, 26, 30], which will lead to the dominance of negative interactions that we observe here. Finally, it is important to re-emphasize that our data do not exclude the possibility of positive effects where one microbial species gains at the expense of another, analogous to predator-prey interactions. In this sense, it may be often true that one species is positively affected by another. This is not consistent with cooperation, however, and the study of this class of interaction will benefit from a paradigm based upon competitive, not cooperative, evolution.

Experimental Procedures

Study System

We isolated bacteria from an aquatic ecosystem. Rainwater frequently accumulates at the base of beech trees to form permanent pools of rainwater. There is no true soil but beech leaf litter supports an array of heterotrophic organisms. We use the pools here as small natural ecosystems that contain microbial strains that are likely to have been interacting over many generations. These miniature ecosystems have been used extensively as replicate, miniature ecosystems to understand the ecology of bacteria [17, 48]. We conducted two independent experiments where we collected 72 and 32 species from tree-hole environments and mixed random combinations of strains in the lab while measuring overall mixture productivity.

72-Species Experiment

The data from the 72-species experiment were previously used in an analysis of ecosystem functioning and the methodology has previously been reported in [17]. Briefly, 72 bacterial strains were collected from water-filled tree holes, identified using fatty-acid profiles, and inoculated into sterile aquatic microcosms (30 ml vials). The phylogenetic distribution of the different species is listed in Table S1. Each microcosm contained 10 ml sterile PBS (pH 7) and 50 8 mm leaf discs from freshly fallen leaves. The microcosms are not shaken but static so the leaves, along with the sides of the microcosm, allow spatial structure and biofilms to form as they would in nature. Each microcosm was inoculated with a total of 100 μl of culture from stocks that had been grown to stationary phase over at least 1 week. Because the quantity of the inoculum was held constant for all microcosms, the inoculum per species decreased with increased species richness (e.g., eight species communities were inoculated with 12.5 μl of each constituent species). The experiment consisted of a total of 683 replicated mixtures. This included all 72 monocultures and random mixtures consisting of the following: 180 2-species, 120 3-species, 90 4-species, 60 6-species, 45 8-species, 40 9-species, 30 12-species, 20 18-species, 15 24-species, 10 36-species, and 1 72-species combinations. There were two replicates of each mixture.

Cumulative community productivity was measured at intervals throughout the experiment. Here, we present the results from day 0 to day 7 (Figures 2A and 2D) and from day 0 to day 28 (Figures 2B and 2E). Community productivity was measured by inserting sterile vials containing known concentrations of NaOH into each microcosm. The CO_2 respired by the bacterial communities was estimated as the change in pH of the NaOH over each time period after subtracting appropriate negative controls. The results (Figures 2A, 2B, 2D, and 2E) are presented as μg CO_2 ml^{-1} day^{-1}.

32-Species Experiment

We conducted a second experiment in order to test the effects of changing the inoculation regimen. For these experiments, we employed methods that allow the experiments to be performed using microtiter plates. Here, 32 bacterial strains were collected from a single tree-hole environment in 2011. Dilution cultures of tree-hole water were plated on R2A agar and incubated at 20°C until there were visible colonies (4–7 days). Colonies were picked into Luria broth and grown overnight at 30°C, after which they were pelleted, resuspended in 30% glycerol, and stored at −80°C. We identified 225 isolates by sequencing part of the 16S rDNA locus. Briefly, frozen stocks of each strain were streaked onto R2A agar plates and allowed to grow at 28°C until visible colonies developed. An individual colony was then picked into 10 μl molecular grade water, of which 1 μl was used in a PCR. Each PCR contained 1 μl of the bacterial suspension, 0.1 μl Taq polymerase (5 units/μl; Sigma), 1 pmol each of forward primer 63f and reverse primer 1389r, 0.2 mM dNTPs (Sigma), 0.2 μl BSA (Sigma), 2.5 μl 10× PCR buffer, and 20.8 μl molecular grade water. The PCR consisted of an initial step at 94°C to lyse the cells, followed by 30 cycles of: 94°C for 60 s, 54°C for 60 s, 72°C for 120 s, followed by a final elongation step at 72°C for 10 min. Amplicons were sequenced using the BigDye v.3.1 chemistry. Sequences were aligned trimmed using Geneious 5.5.4, and aligned using MUSCLE. Divergence between sequences was calculated as the proportion of nucleotide sites that differed between sequences. Sequences with >1% divergence were classified as belonging to different operational taxonomic units (OTUs). Thirty-two distinct OTUs were sampled at random from the pool of isolates, and these were identified using nucleotide BLAST (Table S1). Named OTUs are the top named hit from the BLAST search. We refer to these OTUs as "species" for convenience, while recognizing the substantial difficulties in defining bacterial species. It is at any rate clear that the strains used in the experiment represent widely divergent clades (Table S1).

The species were combined in aquatic microcosms that simulated the tree-hole environment. An aquatic media was created using freshly fallen beech leaves collected in October 2010 and subsequently stored at −20°C. Beech leaves (500 g) were autoclaved in 500 ml mineral water (Volvic) as described [49]. This undiluted media was diluted 32-fold in mineral water and reautoclaved. Beech leaf media was added to each well of a deep-well (1.2 ml) 96-well plate, with the volume of media dependent on the species richness treatment. Species richness treatments of 1, 2, 4, 8, 16, and 32 species were prepared from strains grown in Luria broth, centrifuged, and resuspended in sterile beech leaf media. Ten microliters of these cultures was then added to the appropriate wells, and topped up with sterile media to a final volume of 1 ml per well.

Total community productivity was measured using the MicroResp system (http://www.microresp.com) as suggested by the manufacturer (Macauley Scientific Consulting Ltd). The procedure involves using a rubber-sealing mat to isolate each well in the deep-well plate. The sealing mat has holes pierced for each well, allowing passage of gas evolved from each of the cultures. An inverted 96-well microplate containing a detection gel is clamped to the sealing mat such that the evolved gas from the cultures reacts with the detection gel. The detection gel contains a pH indicator (cresol red), which changes color as CO_2 is evolved from the cultures. The detection gel was prepared according to the manufacturer instructions. Briefly, 18.75 mg cresol red (Sigma), 16.77 g KCl, and 0.315 g $NaHCO_3$ were dissolved in 1,000 ml deionized water. This indicator solution was amended with melted 3% purified agar (2:1 indicator:agar), of which 150 μl was pipetted into a 96-well microplate. Plates were then stored in a desiccator containing soda lime for a week prior to the initiation of the experiment before being clamped onto the cultures. The experiment was run for 7 days, and the optical density of the indicator plates was measured at 600 nm at the initiation and conclusion of the experiment using a microplate reader (Biotek μQuant). Community respiration was taken as the difference between the initial and final optical density (ΔOD_{600}). The results (Figures 2C and 2F) are presented as the change in optical density over the course of the experiment after subtracting a negative control that contained no bacteria. Each mixture was replicated five times, to yield a total of 480 microcosms. This included all 32 monocultures, 16 2-species, 8 4-species, 4 8-species, 2 16-species, and 1 32-species mixtures.

Experiment Design

Both experiments were designed according to the method described [50]. Using this design, species mixtures are chosen at random within the constraint that each species is represented equally at each level of species richness. The design creates random mixtures while preventing any single species from unduly influencing the results.

Supplemental Information

Supplemental Information includes two figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2012.08.005.

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