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# Emergent coexistence in multispecies microbial communities

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**Understanding the mechanisms that maintain microbial biodiversity is a critical aspiration in ecology. Past work on microbial coexistence has largely focused on species pairs, but it is unclear whether pairwise coexistence in isolation is required for coexistence in a multispecies community. To address this question, we conducted hundreds of pairwise competition experiments among the stably coexisting members of 12 different enrichment communities in vitro. To determine the outcomes of these experiments, we developed an automated image analysis pipeline to quantify species abundances. We found that competitive exclusion was the most common outcome, and it was strongly hierarchical and transitive. Because many species that coexist within a stable multispecies community fail to coexist in pairwise co-culture under identical conditions, we concluded that multispecies coexistence is an emergent phenomenon. This work highlights the importance of community context for understanding the origins of coexistence in complex ecosystems.**

Explaining species coexistence and the bewildering diversity of ecological communities is a major goal of ecology (*1*). Historically, this problem has been investigated through the lens of species interactions and population dynamics. This work has played a central role in theoretical ecology (*2*, *3*), establishing, for example, the importance of competitive interactions for community stability (*4*, *5*) and the criteria required for stable coexistence in species pairs and pairwise networks (*6*, *7*). An important caveat is that the ability of any model to fully capture the population dynamics of empirical populations is limited, and interactions between species are often modulated by environmental context (*8*, *9*) and by the presence of additional species (*10*, *11*). As a result, in recent years, research has started to shift to directly study coexistence networks (*12*–*14*). A central question is whether the known outcome of competition between pairs of species, i.e., their coexistence or competitive exclusion, can be leveraged to predict the composition of complex communities and the paths leading to their assembly (*12*, *13*, *15*). If this approach were fruitful, then it would circumvent the need to know the full mathematical structure of population dynamics models to predict community assembly (*14*).

There are two opposing views on species coexistence (Fig. 1A). A reductionist perspective is that multispecies coexistence is an additive affair, and all of the coexisting members of a community must also coexist as pairs when isolated from the community context (*14*). An alternative view is that coexistence in a multispecies community is a more complex, or emergent, property of the community, which is not exhibited by its most elementary units of coexistence, pairs of species in isolation (*16*). Which of these two views best reflects the reality of empirical communities (Fig. 1A)? Determining which view is more accurate requires deconstructing a community into species pairs to determine whether all possible combinations can coexist. If most can, then the reductionist view is supported. If few can, then coexistence is an emergent property of the community, as is seen in nontransitive competition [i.e., as in the rock, paper, scissors game (*17*–*24*)], which may allow multiple species to coexist even when none of them do as a pair in isolation (*17*–*24*).

Resolving this question is essential in microbial ecology given the enormous and still largely unexplained diversity of microbial ecosystems (*13*, *25*). Directly testing the two hypothesized scenarios described above is generally not feasible in natural microbial communities because of their diversity. Even if we managed to isolate most community members from a given habitat, the number of replicate environments that we would need to recreate to culture every single pair would scale quadratically with the community richness. Recent studies have taken a synthetic approach by reconstituting species pairs from natural communities in well-controlled laboratory environments (*14*, *26*, *27*). Although these studies found support for the reductionist hypothesis, their limitation lies in the small fraction of coexisting species that could be isolated and the differences between the laboratory environment and the original community habitat.

Here, we sought to directly test the hypotheses in an empirical system that is well suited for this purpose. Our starting point was a collection of bacterial enrichment communities that we have recently assembled in well-controlled synthetic environments containing glucose as the single externally supplied limiting nutrient (Fig. 1B) (*9*, *28*–*31*). These communities formed in a manner that is similar to the "random zoo" model in theoretical ecology (*32*). In brief, 12 soil and plant microbiomes were resuspended in separate test tubes containing M9 minimal medium (*9*) (Fig. 1B). This provided us with a diverse pool of bacterial species containing between 110 and 1290 exact sequence variants (ESVs) (fig. S1) (*9*). These 12 initial microbiota solutions were then inoculated by a 125-fold dilution into separate bioreactors containing M9-glucose growth medium (see the materials and methods), incubated for 48 hours under static conditions at 30°C, and then serially passaged 12 times each (~84 bacterial generations under our conditions) (Fig. 1B) (*9*). Community composition at various time points was determined by 16*S* ribosomal RNA (rRNA) amplicon sequencing. All communities contained multiple ($N$ < 25) coexisting ESVs belonging primarily to the families Enterobacteriaceae and Pseudomonadaceae (Fig. 1B) (*9*, *28*, *30*, *33*). It was thus possible in this system to deconstruct multiple stable communities and reconstitute and compete most pairs of species under the same starting conditions. This experimental system allowed us to evaluate whether all pairs of organisms that coexist as a part of a multispecies community also coexist in isolation (*29*), thus directly testing whether coexistence is a pairwise or an emergent phenomenon.

## RESULTS

### *Coexistence is stable in our enrichment communities*

To establish whether coexistence in these multispecies enrichment communities is stable, we set out to analyze the published community assembly dynamics data from previous studies (*9*, *34*, *35*) in which the frequencies ($x_i$) of all ESVs were quantified at the end of each transfer ($i$) for 26 representative communities (fig. S2). We determined the invasion fitness [$F = \log(x_i/x_{i-1})$] of every ESV in these communities over their full assembly dynamics ($i$ = 2,3, ..., 12; see the materials and methods) and found that a large majority of the ESVs found at the end of the experiment exhibited hallmarks of negative frequency–dependent selection. For 95/99 of these ESVs, the dependence between fitness and frequency was best fit by a negative regression slope (fig. S3), and the equilibrium frequency ($x^*$) predicted from this linear regression model [the frequency for which $F(x^*) = 0$] agreed very well with the empirically observed equilibrium

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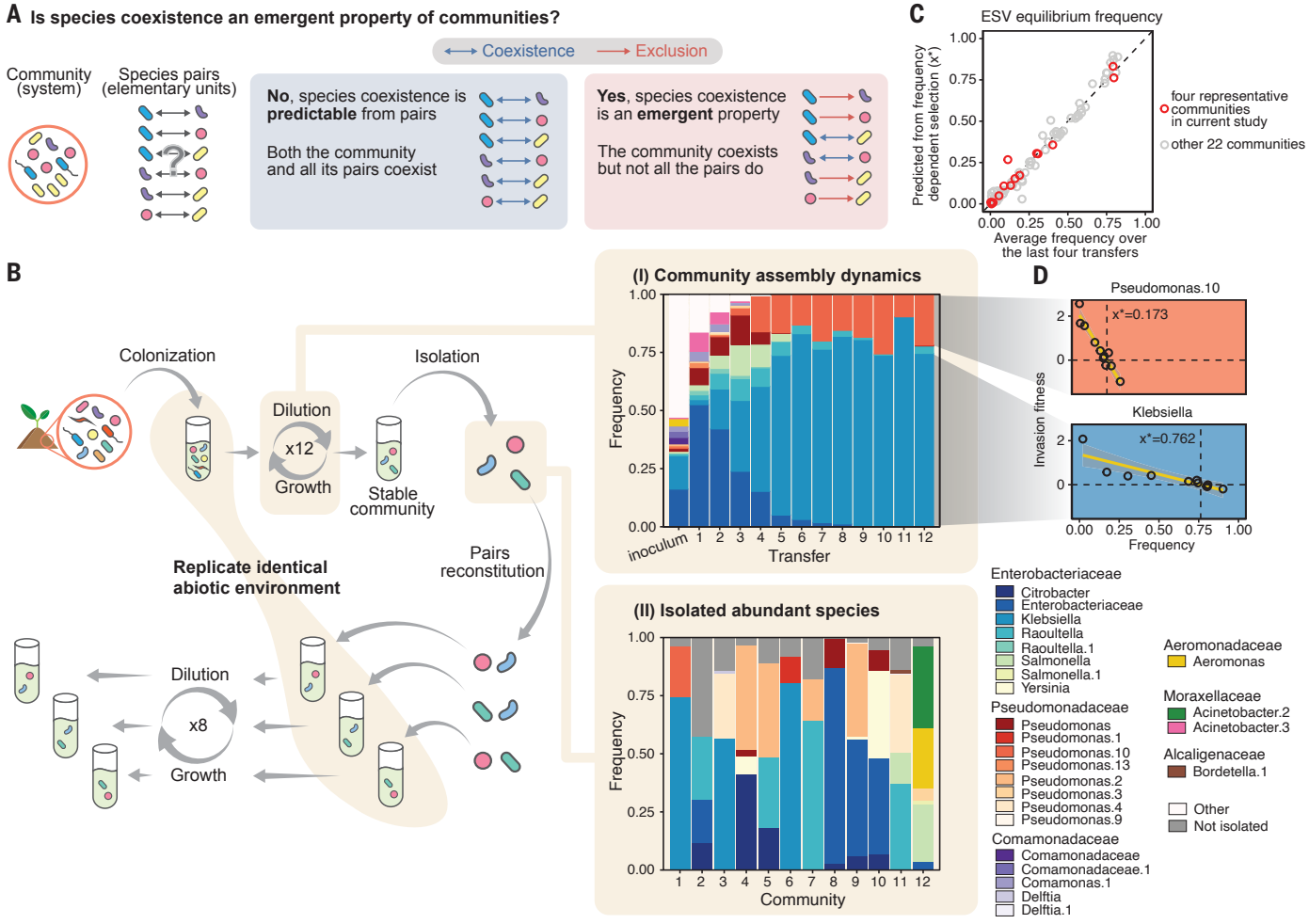


**Fig. 1. Enrichment microbial communities allowed us to test the complexity of species coexistence.** (**A**) The two hypotheses about species coexistence tested in our study. (**B**) To discriminate between the two hypotheses, we used an empirical system constructed from previously assembled enrichment in vitro bacterial communities under serial growth and dilution cycles (*9*). In inset I, we present the full assembly dynamics for a representative community, showing the frequency of each ESV at the end of every growth period (transfers). We only show ESVs >2% in frequency, each in a different color. We chose 12 representative communities with richness ranging between $N$ = 5 and $N$ = 13 ESVs at transfer 12 (inset II) and isolated most community members (colored bars) covering an average of 89.4% of the abundance. Gray bars represent ESVs that we were not able to isolate (see the materials and methods). Raw data were obtained from previous studies (*9*, *34*, *35*). (**C**) Frequency-dependent dynamics predicted the empirically observed equilibrium frequencies. Empirical equilibrium frequencies (horizontal axis) were quantified as the average frequency of an ESV in the last four transfers of the community assembly process (transfers nine to 12). To determine the predicted equilibrium frequency $x^*$ ($x$ axis), we first quantified the invasion fitness $F_i = \log(x_i/x_{i-1})$ for each ESV at each transfer and then regressed this $F_i$ against ESV frequency. This regression yielded a negative slope for 95/99 ESVs found near the equilibrium in their respective community (fig. S3), indicating that these ESVs are subject to negative frequency–dependent selection. In these cases, we estimated the equilibrium frequency $x^*$ as the $x$-intercept of the regression line (figs. S3 and S4). (**D**) Two examples of invasion fitness analysis from the community in inset I showing negative frequency–dependent selection. The yellow line represents the linear fit as determined by least-squares regression ($N$ = 11, $R^2$ = 0.92 and $N$ = 11, $R^2$ = 0.70 for the top and bottom panels, respectively). The $x$-intercept was used to estimate the equilibrium frequency $x^*$, which is shown as a vertical dashed line.

frequencies, which we determined as the average frequency of the ESV over the last four transfers (Fig. 1C, fig. S3, and materials and methods). By contrast, ESVs that were only transiently present during community assembly but were not part of the final stable community generally exhibited either negative average fitness values or equilibrium frequencies close to 0 (figs. S4 and S5). Overall, our quantitative analyses indicated that the ESVs that were present in the final transfer of our multispecies enrichment communities could invade from low frequency, fulfilling the mutual invisibility criterion of stable coexistence (*36*).

### *Quantification of pairwise competition assays*

To empirically test whether stable multispecies coexistence was a pairwise phenomenon in our enrichment communities, we chose 12 representative communities containing between five and 13 ESVs in stable equilibrium, plated them on their final transfer, and then selected at least three morphologically distinct isolates from each community (fig. S6 and materials and methods). Using Sanger sequencing, we obtained the full-length sequence of the 16*S* rRNA gene of these isolates, aligned it with the ESVs that were found in their communities of origin, and retained all isolates with at least 200–base pair consensus sequence and four or fewer mismatches. This resulted in a total of 62 isolates, 40 with fully matching alignments and 22 with one to four mismatches

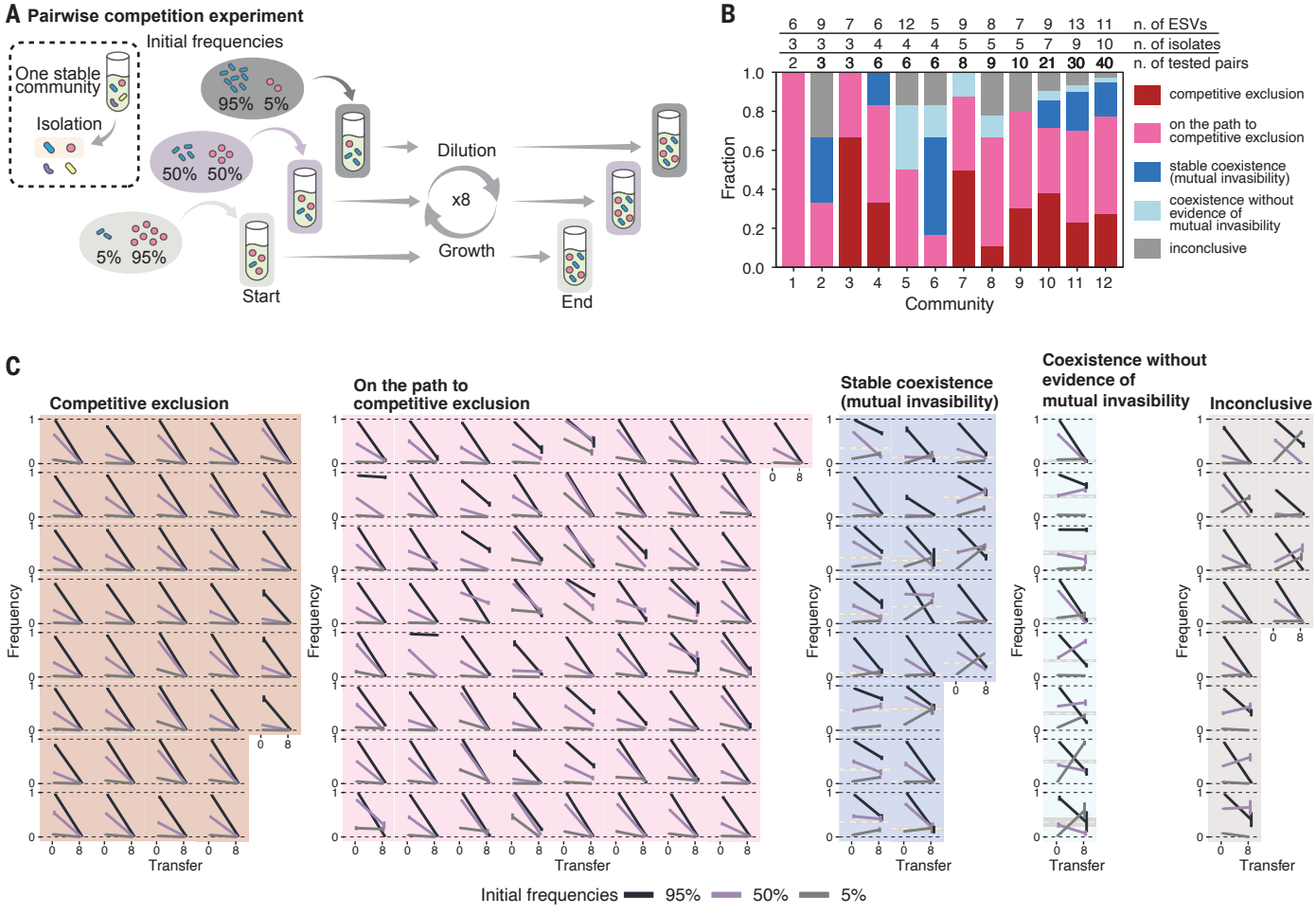


**Fig. 2. Multispecies coexistence is an emergent property of the community.** (**A**) To determine whether isolated species pairs coexist or outcompete one another, we cultured each pair at three different initial frequencies. Pairs were propagated in the same culture conditions as their community of origin for eight consecutive passages. The pairwise competition outcomes of all 12 enrichment communities are shown in (**B**), and communities are ordered by the number of strains in each community from the smallest (three taxa) to the largest (10 taxa). The numbers above in each bar show the number of ESVs, the number of isolated strains, and the number of tested pairs, respectively. Note that some communities have missing pairs because these pairs either did not have any colonies in co-culture or had low classification model accuracy. (**C**) Competition outcomes of 144 pairwise co-cultures. Mean frequencies and 95% confidence intervals were determined by Poisson sampling ($N$ = 1000; see the materials and methods). For clarity, we plotted in all cases the frequency of the isolate ending with a lower average frequency in time point 8 ($T_8$). In coexisting pairs, the mean equilibrium frequency on the final transfer is represented by a horizontal dashed line, and the 95% confidence interval (computed from Poisson sampling, $N$ = 1000) as a shaded area around it. Each of the inset grids indicates the change in frequency from the initial time point ($T_0$) to the final one ($T_8$). The background color represents the competition outcomes, and the line color indicates the three initial frequencies. To establish significant changes in frequency between $T_0$ and $T_8$ in each experiment, we used Wilcoxon–Mann-Whitney tests with $N$ = 2000 and a significance threshold of $P < 0.05$ (see the materials and methods).

(fig. S7 and materials and methods), covering on average 89.4% of the ESV composition of the original communities (Fig. 1B).

We then performed every possible pairwise competition experiment among the isolates of each community by mixing inocula of pairs of isolates and passaging each mixture for eight growth-dilution cycles in the same glucose minimal medium at the same temperature (30°C) used in the original community enrichment experiments (Fig. 2A). All pairwise competition experiments were performed three times, each at a different starting count proportion of ~5:95, ~50:50, and ~95:5 (Fig. 2A and materials and methods). During each growth cycle, the cells were incubated for 48 hours, after which the resulting culture was diluted 125-fold into fresh medium, as was done in the original community assembly experiment (*9*). At the end of the last dilution cycle, we measured the composition of our pairwise co-cultures by plating them on Petri dishes and counting the colonies belonging to each isolate.

To avoid human bias in colony morphology identification, we adopted an automated image-processing pipeline (fig. S8) combined with a machine-learning approach for classification using 159 × 3 = 477 co-culture images on the basis of 40 colony morphology features (figs. S9 and S10, table S1, and supplementary materials). The pipeline started by extracting color channels and correcting for uneven backgrounds, followed by segmenting colony objects and extracting the morphological features from these. These colony features were analyzed using random forest classification to determine whether each colony present in the co-culture image belonged to one morphotype or another (fig. S10). This approach allowed us to quantify the number of colony-forming units of each of the two competitors in pairwise co-culture. Of

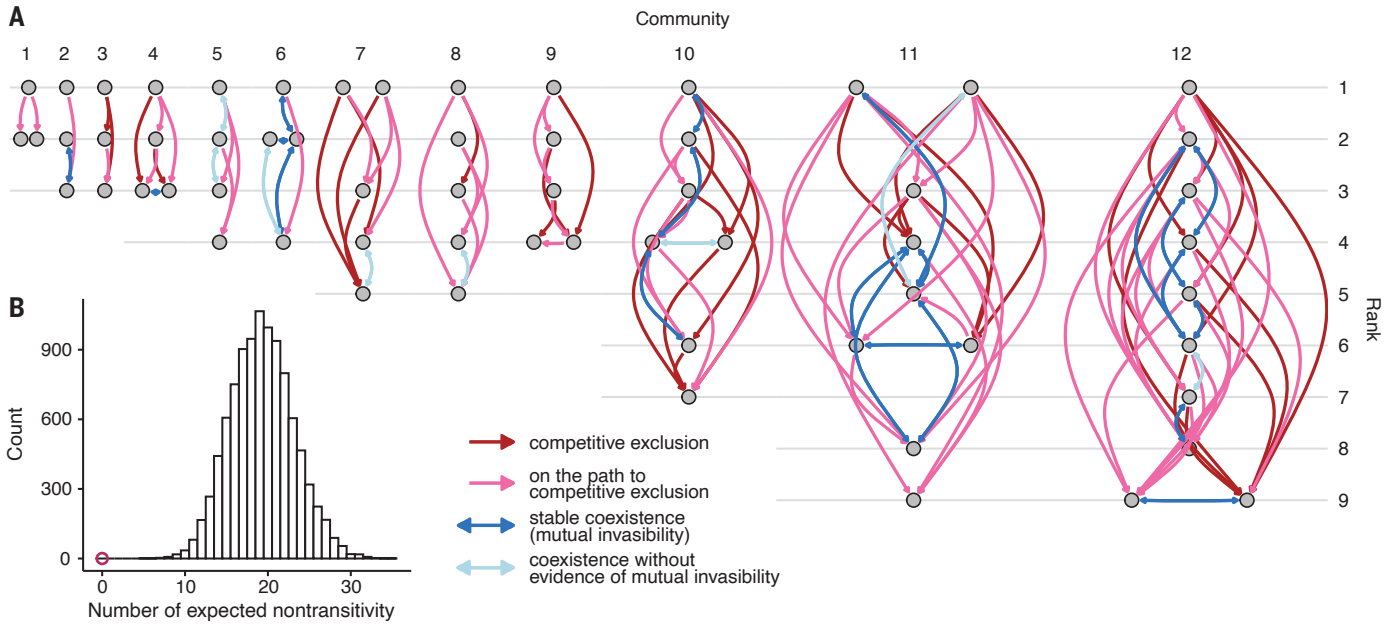


**Fig. 3. Competitive hierarchy prevails among species pairs in stably coexisting communities.** (**A**) All isolates in our 12 communities were rank-ordered from top to bottom on the basis of the number of other isolates that they excluded in pairwise competition, using data from the experiments shown in Fig. 2. The gray nodes in the network represent each individual isolate. Red arrows point from the winning isolate of a pairwise competition to the losing one. Blue lines connect isolates that coexist. (**B**) Binomial distribution with $N = 77$, $P = 0.25$ showing the expected number of pairs that exhibit transitivity if we randomly swapped the coexistence and exclusion links in (A). The red open circle marks the experimentally determined number of trios that broke transitivity (in our case, zero).

the 159 competing pairs, six did not yield a measurable optical density in either of the three competition assays regardless of their inoculation frequencies, and no colonies were detected. Because we could assign neither coexistence nor competitive exclusion to these pairs, which were also formed by pairs of isolates that were not present at the final transfer in monoculture, we excluded them from further analysis. We removed nine additional pairs for which the trained model performed poorly on the validation datasets (accuracy score <0.9; fig. S11 and materials and methods). We therefore used $N = 144$ pairs in our analysis. The automated pipeline approach agreed well with visual colony identification, yielding comparable results for both the total colony count on a plate [$R^2 = 0.85$; root-mean-square deviation (RMSD) = 17.67; $N = 381$] and the relative frequency of different colony morphotypes ($R^2 = 0.87$; RMSD = 0.17; $N = 381$) (fig. S12) for the 127 pairs with an accuracy score > 0.9 that could be discriminated by eye.

### *Multispecies coexistence is an emergent property*

In 26.4% of the pairs (38/144), one of the two competitors had become competitively excluded by the end of the last dilution cycle in all three competition experiments (i.e., no colonies were detected on the plates) regardless of its starting inoculation proportion (Fig. 2, B and C, dark red). We marked these outcomes as competitive exclusion. For 45.1% of the pairs (65/144), the frequency of the losing species declined ($\Delta F < 0$) in all three competition experiments regardless of its initial proportion (Fig. 2, B and C, light red box, and materials and methods). This indicates that its trajectory was on the path to competitive exclusion. Adding these outcomes to the competitive exclusion category, we found that 71.6% of the pairs (103/144) failed to coexist in the absence of the other community members. These results were not driven by the poor competitive ability of the least-abundant ESVs, because eliminating from the analysis those isolates with ESVs with <0.05 frequency in the stable multispecies communities still produced a majority of competitive exclusion outcomes (61/84 = 72.6%) (fig. S13).

All 12 communities contained at least one pair, but generally more, that could not coexist in isolation (Fig. 2B). The fraction of pairs exhibiting competitive exclusion was similar across communities regardless of their richness (Fig. 2B). These results are not consistent with the additive assembly rule proposed previously (*14*), which would have predicted less-diverse communities composed only of those taxa that can coexist in isolated pairs. Therefore, complex multispecies coexistence could not be reduced to pairwise relationships in our communities, and it is thus likely an emergent property of the whole community.

A substantial fraction of pairwise competitions (28.5% of the pairs, 41/144) did not result in competitive exclusion, indicating that pairwise coexistence may still be common among members of a stable multispecies community. Among these 41 pairs, 29 were still coexisting in all three competition experiments after eight transfers (Fig. 2, B and C, blue). To identify those pairs that coexist stably, we apply the mutual invasibility criterion, which requires that both species must be able to invade each other from low frequency (*36*) (Fig. 2, B and C, dark blue). Methodologically, this requires that $\text{sign}(\Delta x) = \text{sign}[x^* - x(T_0)]$ for both species in all three pairwise competition experiments (see the materials and methods). Here, $\Delta x$ denotes the change in a species frequency between the final and initial transfers, $x^*$ is the equilibrium frequency for that species (which we determined by averaging the final transfer frequencies of the three experiments; see the materials and methods), and $x(T_0)$ is the species' inoculation frequency on the first day of the experiment. This condition was met in 21 of the 29 coexisting pairs. The criteria for mutual invasibility were not met in the remaining eight coexisting pairs, so we classified these as

coexisting without evidence of mutual invasibility (Fig. 2, B and C, light blue). The remaining fraction of our pairs (12/144, 8.3%) did not offer conclusive results because the outcome of the competition was not consistent in the three experiments. We left these as inconclusive (Fig. 2, B and C, gray).

### *Competitive exclusion is hierarchical and transitive*

In an effort to better explore the structure of our pairwise competition network, we used the competition outcomes shown in Fig. 2 to rank all isolates in each community by the number of competitors that each of them excluded (see the materials and methods). We found that competitive exclusion was almost fully hierarchical: In all but one of the 103 pairs in which one of the two isolates was excluded, the lower-rank species was the one that was excluded (Fig. 3A). The ranks in the competitive hierarchy were positively correlated with the frequency rank of the corresponding ESV in the parent community (Spearman's $\rho = 0.42$, $P < 0.001$, $N = 62$; fig. S14), but this pattern was mostly driven by less-diverse communities (fig. S14), which recapitulates previous findings from plant communities (*37*). We also found that competitive hierarchy was positively correlated with the strain's growth rate in glucose medium (Pearson's $r = -0.314$, $P = 0.0129$, $N = 62$; fig. S15). Regarding the type of metabolism, respiro-fermenters had a higher average competitive rank (mean = 2.54) than obligate respirers (mean = 4.72) (Wilcoxon–Mann-Whitney test $P < 0.001$, $N = 62$; fig. S16), a pattern consistent with our previous work (*9*, *28*).

An extreme case of emergent coexistence may occur when coexistence networks are nontransitive (*17*, *24*). However, we found that nontransitive cycles were unlikely to stabilize coexistence in our communities. Of 77 triplets of species that could be connected by competitive exclusion links in our 12 communities, we did not find a single violation of transitivity (Fig. 3B). Because the expected fraction of nontransitive triplets in a random network is $P = 1/4$, the probability of observing this outcome by chance is given by $P(0) = (1/4)^{77} = 4.4 \times 10^{-47}$ (Fig. 3B).

## Discussion

The aim of this study was to empirically test whether coexistence in microbial communities is a pairwise phenomenon or if it is an emergent property of the community. To address this question, we isolated most members of 12 stable enrichment communities and determined whether each possible pair could coexist in the absence of the other members of their communities under the same culture conditions as in the enrichment. Although a substantial fraction of pairs did coexist (29/144, 20.1%), a majority (103/144, 71.5%) of them ended up in competitive exclusion, with one of the two members becoming excluded or on the path to it. This indicates that coexistence could not be reduced to a pairwise phenomenon in our enrichment communities and that the community context is generally required for species pairs to coexist. Our finding contrasts with the outcome of a recent empirical study supporting the reductionist hypothesis, which concluded that the coexistence of multiple species in bottom-up assembled communities requires every pair to coexist in isolation (*14*).

Given that both hypotheses can be correct in different communities (*14*, *16*), our results prompt the question of under which conditions each is most likely to occur. We have not yet determined whether the complex nature of multispecies coexistence in our enrichment communities derives from higher-order interactions, or if it can be explained by a complex network of pairwise interactions. Another possible factor that may stabilize coexistence, but which our study has not addressed, is the rapid emergence of intra-strain diversity through evolutionary processes. Evolution of new species interactions, such as the appearance of a new mutualism, may mediate the emergent coexistence of pairs of strains that would otherwise end in competitive exclusion (*16*). As for broader evolutionary patterns, we did not find a correlation between pairwise coexistence and sequence similarity (fig. S17), although our analysis was limited to the 16*S* marker gene. Finally, spatial structure is also known to affect microbial coexistence [e.g., (*38*)], but the number and nature of spatial niches could not be identified in a straightforward manner in our experiments.

Theoretical studies have suggested that nontransitivity can stabilize the coexistence of multiple competing species in the presence of spatial heterogeneity (*18*) or when competitors have differential competitive abilities on multiple limiting resources (*17*, *21*). Although the idea of nontransitivity is well established in theory, empirical studies on its prevalence are sparse. Our mutual invasion experiments with 144 species pairs from each of the 12 communities did not find a single nontransitive trio, suggesting strongly hierarchical competition among our species. This discrepancy between theory and our findings may be caused by the underlying ecological interactions among competing species. In our communities, exploitation of the single externally supplied limiting nutrient and cross-feeding appeared to be the dominant ecological interactions determining the community structure (*9*, *28*), whereas nontransitivity may emerge through interference competition (*39*) or through changes in species' competitiveness across resources (*40*).

Our experiments suggest that pairwise coexistence is not necessarily required for the stable assembly of multispecies communities. However, more complex assembly rules might still be found to predict and explain multispecies coexistence. Future empirical work with communities assembled under growing environmental complexity will be necessary to establish how factors such as spatial structure, the number of supplied resources, the existence of higher-order interactions, and fluctuating conditions may influence the complexity of coexistence in multispecies communities.

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## SUPPLEMENTARY MATERIALS

science.org/doi/10.1126/science.adg0727
Materials and Methods
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Tables S1 to S3
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MDAR Reproducibility Checklist

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**Editor's summary**
The question of how organisms coexist in communities is a pivotal issue for ecologists. To answer this question in a natural ecosystem would require the imposing task of isolating and competing all coexisting members. To render the question experimentally tractable, Chang *et al*. isolated organisms from stable synthetic bacterial communities and competed all possible combination of pairs of organisms to test their ability to live together. Competitive exclusion occurred in a majority of pairs, whereas a minority of pairs coexisted. Therefore, species coexistence is in part the result of networks of interactions and is an emergent property of community assembly, pointing to the importance of sustaining biodiversity. —Caroline Ash