

1 Co-occurring soil bacteria exhibit a robust competitive hierarchy and lack of non-
2 transitive interactions

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14 **Microbial communities are typically incredibly diverse, and this diversity is thought**
15 **to play a key role in community function. However, explaining how this diversity**
16 **can be maintained is a major challenge in ecology. Temporal fluctuations and**
17 **spatial structure in the environment likely play a key role, but it has also been**
18 **suggested that the structure of interactions within the community may act as a**
19 **stabilizing force for species diversity. In particular, if competitive interactions are**
20 **non-transitive as in the classic rock-paper-scissors game, they can contribute to the**
21 **maintenance of species diversity; on the other hand, if they are predominantly**
22 **hierarchical, any observed diversity must be maintained via other mechanisms.**
23 **Here, we investigate the network of pairwise competitive interactions in a model**
24 **community consisting of 20 strains of naturally co-occurring soil bacteria. We find**
25 **that the interaction network is strongly hierarchical and lacks significant non-**
26 **transitive motifs, a result that is robust across multiple environments. Moreover, in**
27 **agreement with recently proposed community assembly rules, the full 20-strain**
28 **competition resulted in extinction of all but three of the most highly competitive**
29 **strains, indicating that higher order interactions do not play a major role in**
30 **structuring this community. The lack of non-transitivity and higher order**
31 **interactions *in vitro* indicates that other factors, such as temporal or spatial**
32 **heterogeneity, must be at play in enabling these strains to coexist in nature.**

33

34 Despite their small size, microbes play outsized roles at multiple ecosystem scales, from
35 the planetary¹ to that of the human individual². Like their macroscopic counterparts,
36 microbes typically exist in diverse communities whose functions are intimately related to
37 their structure. Diversity impacts an ecological community's stability, resilience to
38 perturbations, and its ability to provide ecosystem services³. Therefore, a long-standing

39 area of interest in microbial ecology has been understanding the factors that give rise to
40 the diversity observed within microbial communities^{4,5}. A better understanding of the
41 structure of microbial communities is desirable for both managing existing microbial
42 communities⁶ and, eventually, engineering them *de novo*⁷.

43 Many factors can contribute to the generation and maintenance of diversity in ecological
44 communities. Non-transitivity⁸, bistability⁹, weak interactions¹⁰, facilitation, multiple
45 limiting factors, and spatial or temporal segregation¹¹ have all been hypothesized to play
46 a role; however, there is little empirical data regarding the relative importance of each of
47 these factors in actual natural communities. By investigating the network of underlying
48 interactions among the members of a given community, we can better understand each
49 factor's relative importance in structuring the community¹². Since interspecific
50 competition is thought to be a dominant factor in determining whether a given species
51 can persist in a community^{13,14}, the network of competitive interactions between species
52 may be particularly informative of the structure of the community within which the
53 interaction takes place. Features of competitive interaction networks that could contribute
54 to community diversity can include non-transitive motifs such as the classic rock-paper-
55 scissors triad, network modularity¹⁵, or overall trends towards weak interactions among
56 species

57 While non-transitivity in particular is often cited as a potential driver of interspecies
58 coexistence^{16,17,18}, the degree to which it occurs in natural communities remains largely
59 unknown. Indeed, Levine and colleagues recently asserted that despite the theoretical
60 potential of non-transitive interactions to stabilize community structure, there is scant
61 evidence that they are widespread in natural systems, and that further empirical studies
62 are warranted¹⁹. Recent experimental work using a field-parameterized model of
63 competition in annual plants²⁰ and naturally co-occurring *Streptomyces* bacteria⁹ suggest
64 that rock-paper-scissors type interactions may be less common in natural communities
65 than we might assume; however, further studies of competitive interaction networks in
66 diverse ecological communities are warranted, particularly among phylogenetically
67 diverse natural assemblages.

68 Here, we add to this small but growing body of research that suggests that non-transitive
69 interactions may play a less significant role in maintaining species diversity than is
70 commonly assumed. We use a model system composed of heterotrophic bacteria isolated
71 from a single soil grain. By competing in all pairwise combinations in laboratory culture,
72 we find that the overarching feature of the resulting interaction network is a strong
73 competitive hierarchy, a feature that is naturally at odds with a high incidence of non-
74 transitivity. Therefore, in the natural environment of these bacteria, other factors must be
75 at play that account for their ability to co-occur.

76 **Results**

77 To probe the network of pairwise interactions in a community of diverse microbes, we
78 isolated a collection 20 strains of naturally co-occurring heterotrophic bacteria from a
79 single grain of soil. This strain collection is phylogenetically diverse and spans 16 species
80 across seven genera and five families (Fig. 1a and Methods). Similar to ref²¹, we co-

81 inoculated all pairwise combinations of the 20 strains at varying initial fractions and
82 propagated them through at least five growth-dilution cycles. During each growth cycle,
83 cells were cultured for 24 hours and then diluted by a factor of 100 into fresh media. The
84 final outcome of competition was determined by plating the cultures on solid agar and
85 counting colonies, which are morphologically distinct (Fig. 1c and Supplementary Fig.
86 1). Plating results were confirmed via next-generation sequencing for a random subset of
87 the pairs (Supplementary Fig. 6).

88 Pairwise competitions resulted in one of three qualitatively different outcomes: exclusion,
89 coexistence, or bistability (Fig. 2a-c and Methods). In 153 of the 190 pairs (81%), only
90 one strain could invade the other and drove it to extinction, an outcome we call exclusion.
91 Nineteen pairs (10%) were mutually invulnerable, and thus exhibited coexistence over the
92 time span of the experiment. Finally, 15 pairs (8%) were mutually non-invasive, an
93 outcome that we call bistability. In a small number of pairs (3; 2%), we were unable to
94 determine the outcome due to contamination. Due to the high incidence of exclusion and
95 bistable outcomes, we conclude that these strains interact in the experimental
96 environment primarily through competition.

97 To quantify the strains' overall competitive ability, we define each strain's competitive
98 score to be its mean final fraction across all pairwise competitions. The competitive
99 scores that we measured spanned nearly the entire possible range, from a low of 0.03 to a
100 high of 0.91 (Fig. 1b and Supplementary Table 1).

101 The strains exhibit a strong competitive hierarchy. Very few strains were able to exclude
102 a strain with a higher competitive score; out of 187 pairwise competitions measured, only
103 five resulted in the lower-ranked strain excluding the higher-ranked one (Fig. 2d). The
104 degree of hierarchy in this interaction network is highly significant when compared to
105 networks with randomized outcomes ($p < 10^{-19}$; Fig. 2e). To assess whether the
106 hierarchical pattern was specific to a particular environment, we repeated the
107 competitions with subsets of the full 20-strain collection in different growth media and
108 with different dilution rates (Supplementary Fig. 2). We found that the resultant
109 interaction networks in these different environments were also highly hierarchical,
110 despite changes in which strains were most competitive (Fig. 2f and Supplementary Fig.
111 3). Thus, we conclude that hierarchy in pairwise competition is a robust feature of this
112 model community.

113 Next, we asked what characteristics of a strain might best predict its performance in
114 competition. We hypothesized that strains that grow well in monoculture will have
115 competitive advantages over strains that grow more poorly. Indeed, we found that
116 exponential growth rate (r) was positively correlated with competitive score (Spearman's
117 $\rho = 0.77$; $p < 10^{-4}$; Fig. 3a) and that the typical outcome was for the strain with the
118 higher r to exclude the strain with the lower r , which occurred for 67% of pairs (Fig 3c).
119 Carrying capacity (K) in monoculture was less predictive of competitive superiority, but
120 was still significantly correlated (Spearman's $\rho = 0.55$, $p < 0.05$; Fig. 3b). In general,
121 the likelihood of outcomes other than the stronger grower outcompeting the weaker
122 grower decreases for large differences in r and K (Supplementary Fig. 4). While
123 differences in these two parameters can be indicators of the likelihood of a given

124 competitive outcome, there are many exceptions, and, indeed, some of the stronger
125 competitors do not necessarily have correspondingly strong single-species growth
126 parameters. Thus, while the each species' intrinsic growth ability correlates with
127 competitive ability, the significant number of exceptions indicates that growth ability
128 alone does not fully explain the hierarchical competitive structure that we observe.

129 An important corollary of the high degree of hierarchy we observed in the interaction
130 network is that non-transitive motifs are vanishingly rare. Non-transitive motifs are
131 instances in which a clear competitive hierarchy among members of a sub-group does not
132 exist, the classic example being a rock-paper-scissors (RPS) triad. Of the 987 triads in our
133 collection for which complete pairwise outcome data are available, only three (0.3%)
134 display the RPS topology. This number is significantly less than is found in randomized
135 networks, where on average 14% of triads were RPS ($p < 10^{15}$; Fig. 4). Furthermore, the
136 three triads that we classify as RPS each feature strains that display unusually high
137 variability from experiment to experiment, possibly due to rapid evolution, and further
138 efforts to characterize these triads failed to reproduce the non-transitive network
139 topology. As dictated by its hierarchical structure, our network is also highly enriched for
140 perfectly hierarchical feedforward loops, which were observed in over 50% of triads (Fig.
141 4). Due to the paucity and irreproducibility of observable non-transitive relationships
142 among our strains *in vitro*, we conclude that such relationships are unlikely to be a
143 significant contributor to their coexistence in a natural environment.

144 Given the hierarchical structure of the pairwise interaction network, we wondered about
145 the potential of higher-order interactions and indirect effects among our strains to give
146 rise to a diverse community. To address this, we inoculated three replicate cultures with
147 equal proportions of all 20 strains and propagated them through five growth-dilution
148 cycles (Fig. 5b). The resulting assemblages were highly replicable, and consisted of three
149 strains representing some of the strongest competitors in pairwise experiments (Fig. 5a,c),
150 all of which were found to coexist with each other in pairwise competition. Notably, this
151 combination of survivors was consistent with the simple community assembly rule we
152 recently developed²¹: namely, that a strain is expected to survive in multispecies
153 competition if and only if it is not excluded by any other surviving species. Since
154 pairwise outcomes alone are sufficient to predict the outcome of multispecies competition
155 in this environment, we conclude that higher-order interactions are unlikely to play a
156 major role in structuring this community.

157 **Discussion**

158 Many factors can contribute to the generation and maintenance of diversity in ecological
159 communities. Non-transitivity, facilitation, bistability, weak interactions, multiple
160 limiting factors, and spatial or temporal segregation have all been hypothesized to play a
161 role²²; however, there is little empirical data regarding the relative importance of each of
162 these factors in actual natural communities. Here, we explored one such community. In
163 this work, we explored the network of pairwise interactions for a community of naturally
164 co-occurring bacteria. Our results indicate that diversity in this community is likely
165 maintained primarily due to factors including and spatial or temporal segregation or
166 multiple limiting factors, rather than frequent bistability, non-transitivity, or higher order

167 interactions, all of which have been hypothesized to play a role in generating and
168 maintaining diversity. Nonetheless, we still do not completely understand the processes
169 that give rise to the diversity we observe in nature.

170 Given that soil is a heterogeneous mixture with a multitude of microhabitats, microbial
171 co-occurrence in soil may be facilitated by niche separation and spatial de-mixing. This
172 would allow the coexistence of strains that display strong inhibitory interactions in well-
173 mixed environments. Microbes in soil also experience a strongly fluctuating environment,
174 which can lead to coexistence of multiple strains over time via the soil spore bank.
175 Members of the genus *Bacillus* are particularly well known for their spore-forming
176 ability, which may allow them to persist in a non-vegetative, and therefore non-
177 competitive state, until conditions favor their growth²³. Finally, our experimental
178 approach clearly requires that the strains to be competed be culturable in the laboratory,
179 so it is impossible for us to exclude the possibility that other strains present within the
180 soil might behave very differently.

181 Simulations of our experimental system using the generalized Lotka-Volterra model
182 (gLV) predicted that, if the underlying ecological interactions among species are assigned
183 at random, the pairwise interaction network should become less hierarchical at lower
184 death rates, corresponding to a lower daily dilution rate in our experimental setup
185 (Supplementary Fig. 5). In order to test this hypothesis, we competed a subset of pairs
186 while experimentally reducing the dilution rate from 1:100 to 1:10 (Fig. 2f). The
187 hierarchical network structure was robust to this manipulation, and remained highly
188 correlated with growth rates in monoculture. While it is possible that reducing the death
189 rate further could weaken the hierarchy by reducing the importance of a growth rate
190 advantage in determining survival, the most straightforward interpretation of our data is
191 that the hierarchy is not simply due to differences in growth rates.

192 This experimental system also gives us the opportunity to test the importance of higher
193 order interactions in shaping communities. Higher order interactions are said to take
194 place when the presence of an additional species changes the interaction between two
195 existing species²⁴, and have the potential to contribute to the maintenance of species
196 diversity²⁵. In bacterial systems, this can be driven by complex networks of selective
197 antibiotic production and sensitivity²⁶. Despite the potential for higher order interactions
198 in our model community, our simple assembly rule²¹, which disregards higher order
199 interactions entirely, accurately predicted the survivors in all-versus-all competition *in*
200 *vitro*, suggesting that higher order interactions are not a major driver of community
201 structure in this instance.

202 The observation of high levels of diversity in communities of competing organisms is a
203 long-standing paradox in community ecology²⁷. In this work, we showed that a bottom-
204 up approach to studying community assembly can be useful in narrowing down the range
205 of possible explanations for the diversity we observe in nature. However, this approach
206 necessitates removing the organisms from their natural environment, including the larger
207 community in which the species of interest are embedded. Future work combining *in*
208 *vitro* competition experiments with a more mechanistic understanding of the influence of

209 environment on species survival would help to further explain the persistence of diversity
210 in nature.

211 **Methods**

212 *Strain isolation and identification*

213 Bacterial strains were isolated from a single grain of soil collected in September, 2015 in
214 Cambridge, Mass., U.S.A. The grain weighed ~1 mg and was handled using sterile
215 technique. The grain was washed in phosphate-buffered saline (PBS) and serial dilutions
216 of the supernatant were plated on nutrient agar (0.3% yeast extract, 0.5% peptone, 1.5%
217 bacto agar) and incubated for 48 hr at room temperature. Isolated colonies were sampled
218 and cultured at room temperature in 5 mL nutrient broth (0.3% yeast extract, 0.5%
219 peptone) for 48 hr. To ensure purity, the liquid cultures of the isolates were diluted in
220 PBS and plated on nutrient agar. Single colonies picked from these plates were once
221 again grown in nutrient broth for 48 hr at room temperature and the resulting stocks were
222 stored in 20% glycerol at -80 °C.

223 The 16S rRNA gene was sequenced via Sanger sequencing of DNA extracted from
224 glycerol stocks carried out at GENEWIZ (South Plainfield, New Jersey, U.S.A.).
225 Sequencing was performed in both directions using the company's proprietary universal
226 16S rRNA primers, yielding assembled sequences ~1100 nt in usable length. Species
227 names were assigned using the Ribosomal Database Project's Seqmatch module²⁸ based
228 on the type strain with the highest seqmatch score relative to the query strain. Three
229 strains (*B. toyonensis* 1, 2, and 3) had identical 16S rRNA sequences, and were therefore
230 differentiated using a 404-bp fragment of the *pyrE* gene amplified using the primers 5'-
231 TCGCATCGCATTATTAGAA-3' and 5'-CCTGCTTCAAGCTCGTATG-3' following
232 protocols described in ref²⁹. A list of the strains used, their GenBank accession numbers,
233 competitive scores, and inferred growth parameters is given in Supplementary Table 1.
234 For phylogenetic analysis, sequences were aligned using MUSCLE³⁰ and a tree was
235 constructed using PhyML 3.0^{31,32}.

236 *Estimation of single-species growth parameters*

237 The carrying capacity of each individual strain was estimated to be its optical density at
238 600 nm (OD₆₀₀) in 0.2X nutrient broth after five repeated growth-dilution cycles, starting
239 from an initial OD₆₀₀ of 3×10^{-3} . Growth curves at OD₆₀₀ were measured in flat-bottomed
240 96-well microtiter plates (BD Biosciences) with lids sealed with Parafilm in a Tecan
241 Infinite M200 Pro plate reader over 48 hr at 25 °C with maximum shaking. An
242 approximation of the exponential growth rate of each individual strain was extracted from
243 the growth curves using the time each strain took to reach a threshold optical density. The
244 time-to-threshold method was chosen over other estimates of growth rate due to wide
245 variations in growth patterns across the strains, which led to difficulties in fitting
246 parameters to other population growth models.

247 *Competition experiments*

248 Prior to competition experiments, cells were streaked out on nutrient agar plates, grown
249 for 48 hr at room temperature, and then stored at 4 °C for up to two weeks. Single
250 colonies were picked from these plates and grown for 24 hr at room temperature in 0.2X
251 nutrient broth.

252 The competitions were initiated by diluting each individual strain in 0.2X nutrient broth
253 to an OD₆₀₀ of 3×10^{-3} . The diluted cultures were then mixed by volume to the desired
254 starting ratios of 0.05/0.95 and 0.95/0.05 (Strain A/Strain B). The competitions were
255 performed in 200 μ L volumes in flat-bottomed 96-well microtiter plates sealed with Titer
256 Tops® polyethylene sealing films (Diversified Biotech). For each growth-dilution cycle,
257 the cultures were incubated at 25 °C and shaken at 900 rpm for 24 hr. At the end of each
258 cycle, the cultures were thoroughly mixed and then diluted by a factor of 100 into fresh
259 medium. OD₆₀₀ was measured at the end of each cycle, and final species fractions were
260 estimated after five (or, in the case of initially low plating density, seven) cycles.

261 To measure the final species fractions, the co-cultures were diluted by a factor of 10^4 - 10^6
262 (depending on OD₆₀₀) in PBS. Seventy-five μ L of the diluent was plated onto 10 cm
263 Petri dishes containing 25 mL of nutrient agar and incubated at room temperature for 48
264 hr. All but a small fraction of the strain pairs have distinct colony morphologies, so
265 species fractions were estimated by counting colonies of each type (median: 51 colonies
266 per plate). Next-generation sequencing of a subset of the co-cultures affirmed the overall
267 accuracy of the plating technique (Supplementary Fig. 6).

268 *Determining the outcome of competition*

269 The result of competition was classified as one of three outcomes: exclusion of a single
270 strain, coexistence of both strains, or bistability. A strain was said to exclude its
271 competitor if it was the sole strain observed from both starting frequencies after 5 cycles,
272 or if it excluded its competitor when starting from an initial frequency of 0.95 and
273 achieved a frequency of 0.85 or greater when starting from an initial frequency of 0.05.
274 Pairs were considered bistable if the strain that started out at a frequency of 0.95 excluded
275 the competitor. All other outcomes were classified as coexistence.

276 *Calculating competitive score and network hierarchy score*

277 The competitive score s_i of each strain i was defined as its mean fraction f_{ij} after co-
278 culture with each of the $n - 1$ competitor strains:

$$s_i = \left(\sum_{i \neq j} f_{ij} \right) / (n - 1)$$

279 The hierarchy score (h) for an n -member network is calculated as:

$$h = \sum_{s_i > s_j} f_{ij}$$

280 The network hierarchy score for the observed set of competitive outcomes was then
281 compared against the distribution of scores for 10,000 simulated networks in which each
282 pair was randomly assigned an outcome of exclusion, coexistence, or bistability with
283 probability proportional to the incidence of each outcome in the empirical dataset. The
284 resulting distribution of hierarchy scores was approximated using the normal distribution
285 to determine p -values.

286 *Identifying network motifs*

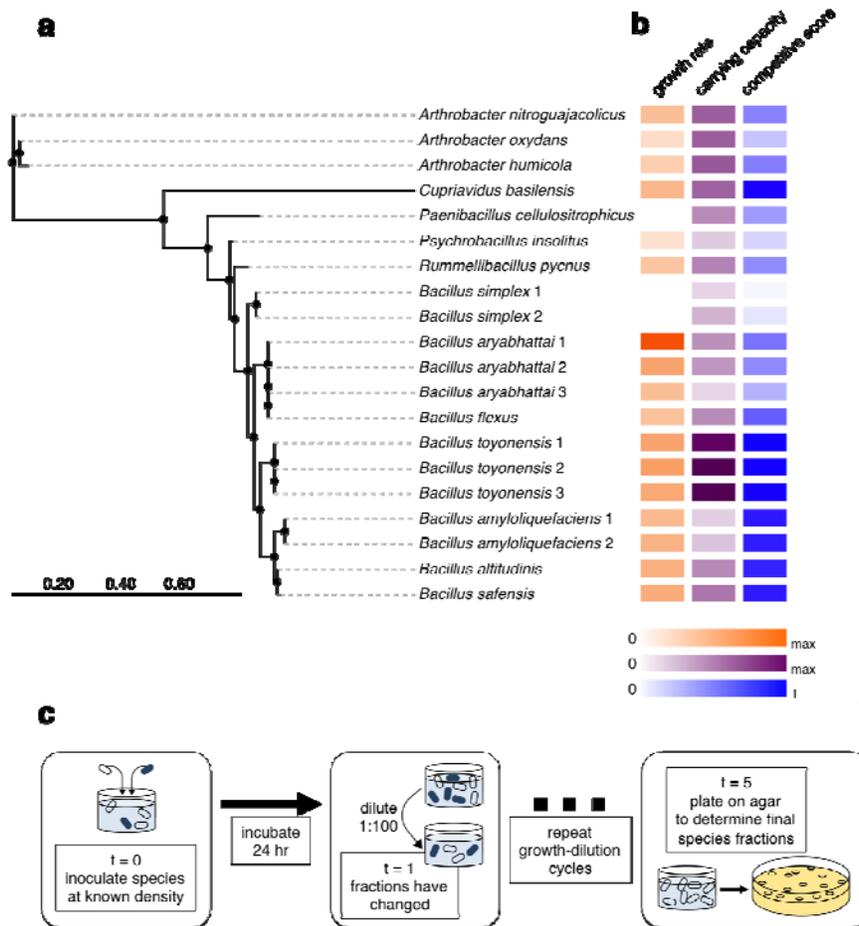
287 The frequencies of distinct topologies among the $\binom{20}{3} = 1140$ three-strain networks were
288 enumerated using the FANMOD software package³³. Random networks were simulated
289 by assigning the outcome of exclusion to each pair of strains within the simulated
290 network with the probability 0.818, which is equal to the fraction of pairs in the empirical
291 dataset that exhibited exclusion. The occurrences of rock-paper-scissors and feedforward
292 loop motifs were enumerated for 1000 simulated networks and approximated by a normal
293 distribution to determine two-sided p -values.

294 *Data and code availability*

295 The data that support the findings of this study are available from the corresponding
296 authors upon reasonable request. An implementation of the routine for estimating the
297 distribution of hierarchy scores and motifs in randomized networks is also available upon
298 reasonable request.

299

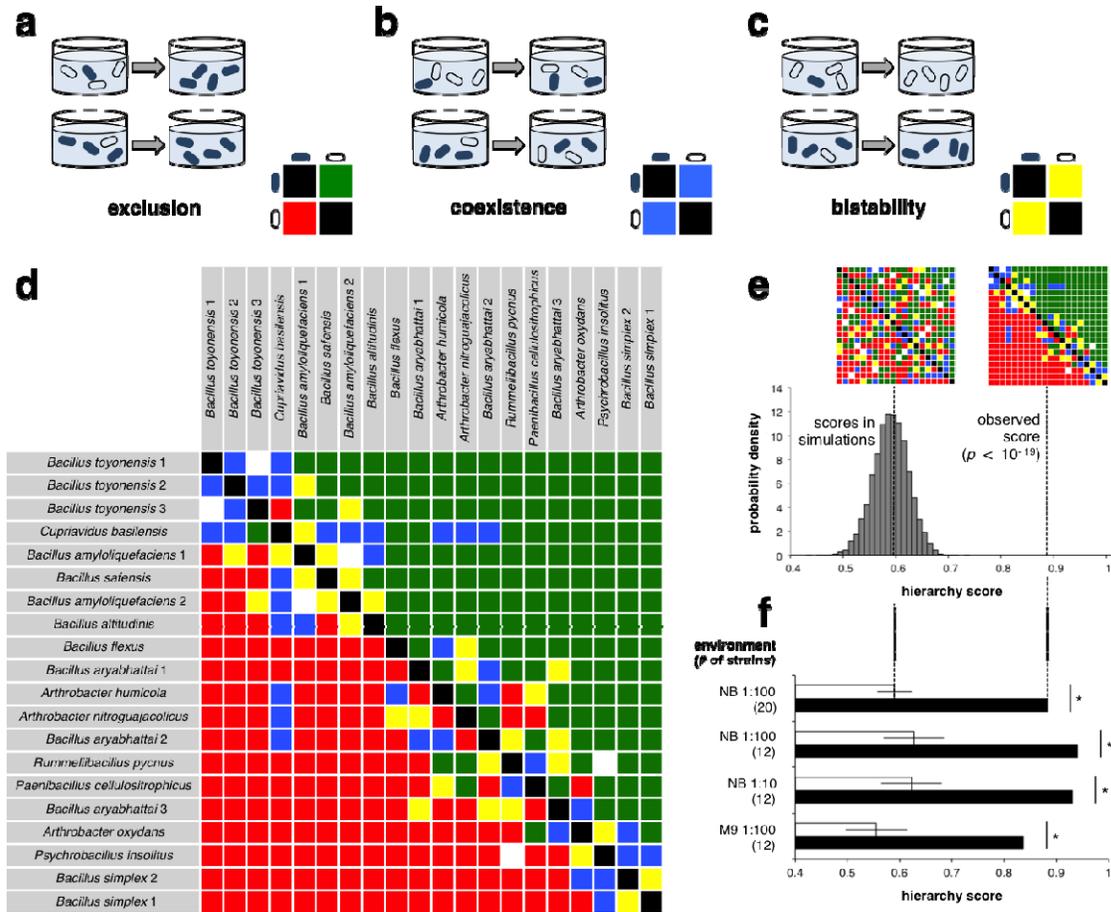
300 **Figures**



301

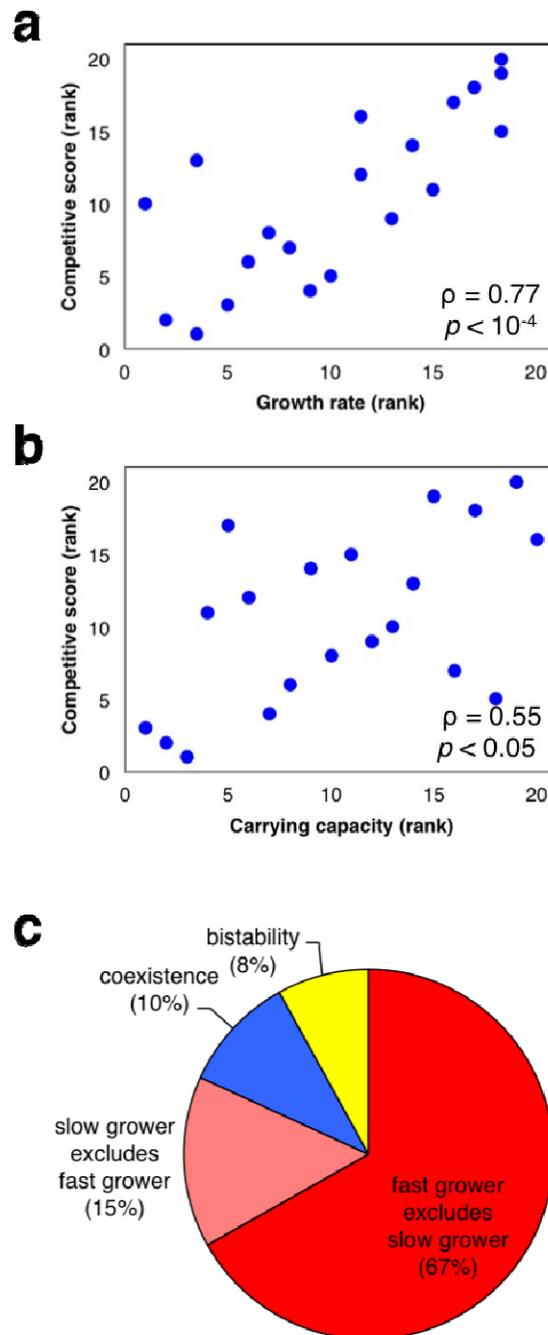
302 **Figure 1. Twenty strains of bacteria isolated from a single grain of soil were**
303 **competed against each other in all pairwise combinations. a,** Phylogenetic tree of the
304 20 strains used in this study. Tree was constructed using the full 16S gene. **b,** Growth rate
305 (orange) and carrying capacity (purple) of each strain in monoculture, as well as
306 competitive score against other strains (blue). Lighter shades correspond to lower values,
307 while darker shades correspond to higher values. **c,** All 190 pairwise combinations of the
308 soil isolates were competed in the laboratory. Colonies of different strains were visually
309 distinct, allowing determination of final species fractions at the end of competition.

310



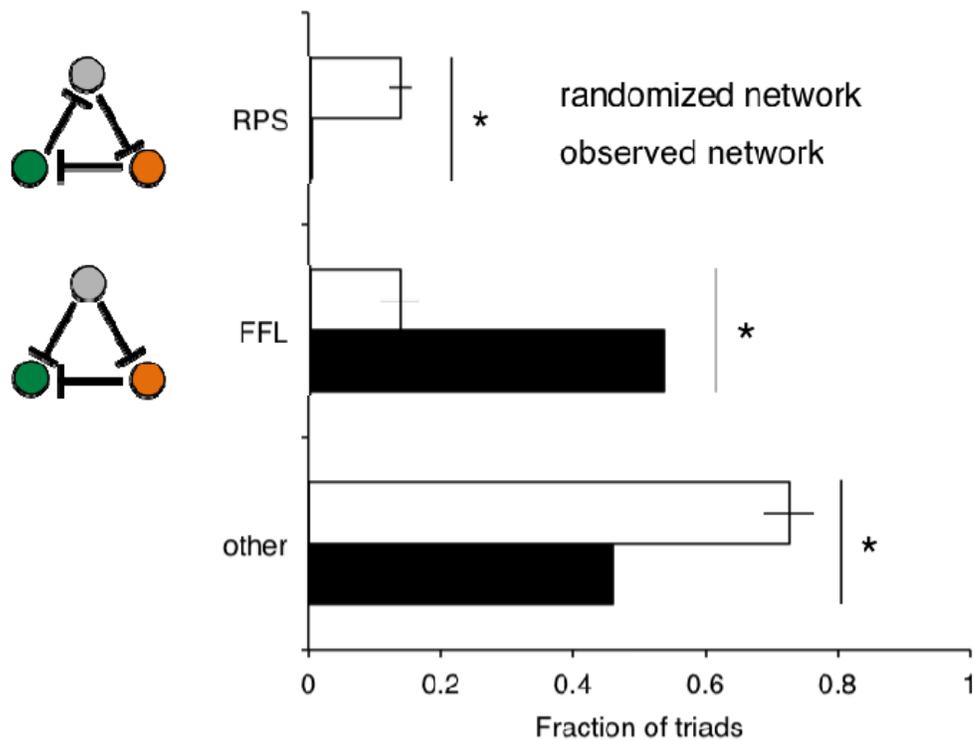
311

312 **Figure 2. The network of pairwise interactions among strains is strongly**
 313 **hierarchical.** **a-c**, Changes in relative abundance over time in three hypothetical pairs:
 314 one in which the outcome was competitive exclusion; one in which the outcome was
 315 stable coexistence; and one in which the outcome was bistability. The color-coded
 316 matrices inset into each diagram indicate the qualitative outcome for the row species in
 317 competition with the column species. **d**, Pairwise outcome matrix for the entire 20-strain
 318 collection. Outcomes are color coded as for **a-c**, with white indicating an indeterminate
 319 outcome. Rows and columns are sorted in decreasing order of each strain's competitive
 320 score. **e**, Histogram of hierarchy scores for randomized outcome matrices. The hierarchy
 321 score for a given matrix is calculated by summing the final fractions of the row strain in
 322 competition with the column strain across all row-column pairs in the upper triangle of
 323 the matrix. The difference is highly significant ($p < 10^{-20}$). **f**, Hierarchy scores for
 324 pairwise interaction networks associated with varying environmental conditions and the
 325 corresponding randomized networks. NB: 0.2X nutrient broth. M9: 1X M9 minimal
 326 medium supplemented with 0.2% casamino acids, 0.4% glycerol, and 1 mM thiamine
 327 HCl. Dilution rates were either 1:100 or 1:10 per 24 hr, and experiments consisted of
 328 either the full complement of 20 bacterial strains or subsets of 12, as indicated in
 329 parentheses. Error bars represent ± 1 s.d. Differences in observed versus randomized
 330 scores were highly significant in all environments ($p < 10^{-7}$).
 331



332

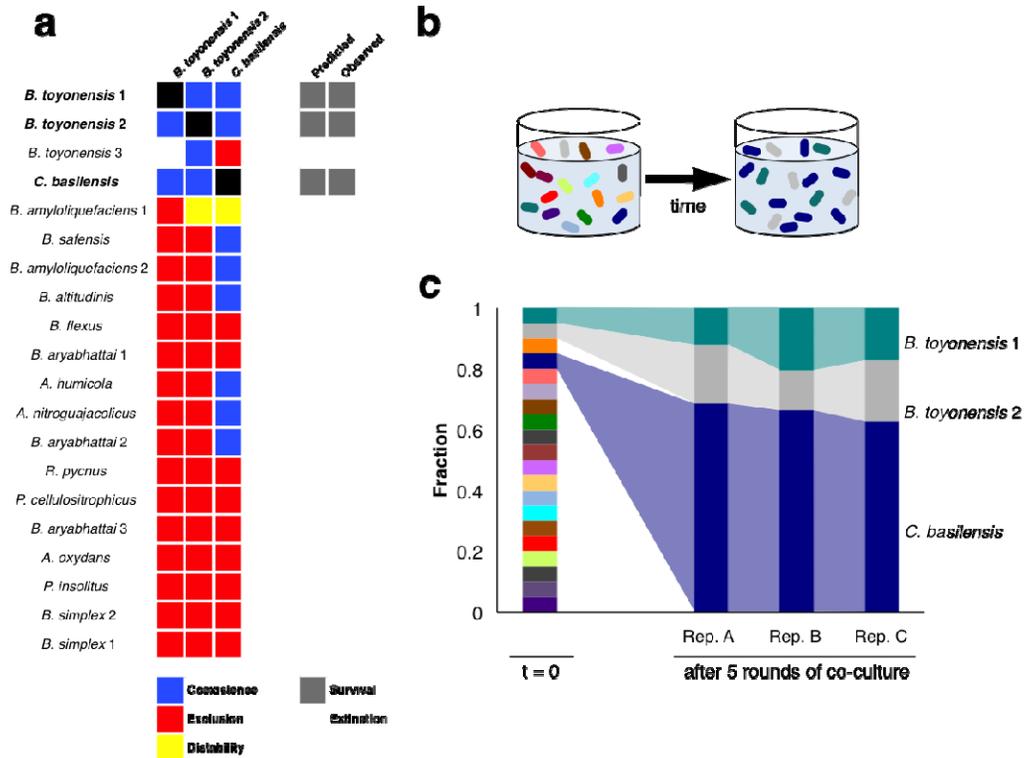
333 **Figure 3. Differences in growth parameters frequently predict the outcome of**
334 **competition. a, b,** Correlation between rank in growth rate (as estimated using a time-to-
335 threshold method) or rank in carrying capacity (as measured using OD₆₀₀) and rank in
336 competitive score. Figures reported are Spearman correlation coefficients (ρ) with two-
337 sided p -values. **c,** Distribution of competitive outcomes for all pairs, with pairs that
338 exhibit exclusion differentiated according to whether the faster or slower grower excludes
339 the other.



340

341 **Figure 4. The observed interaction network contains very few cycles.** There were
342 significantly fewer rock-paper-scissors triads and significantly more feedforward loops in
343 the network of observed outcomes as compared to 1000 randomized networks. Error bars
344 represent ± 1 s.d.. Differences in the observed versus randomized incidences were
345 highly significant for all motif categories ($p < 10^{-7}$).

346



347

348 **Figure 5. Only three species survive in all-versus-all competition, as predicted by**
 349 **pairwise outcomes.** **a**, Predictions and observed outcomes of multispecies competition
 350 (grey squares, right) based on community assembly rules incorporating the outcomes of
 351 pairwise interactions (colored squares, left). **b**, All strains were mixed in equal proportion
 352 by optical density and allowed to reach equilibrium. **c**, In three replicate cultures, only the
 353 same three strains survived, each of which was found to coexist with the other two strains
 354 in pairwise experiments.

355

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364 **Author Contributions**

365 L. M. H., J. F., and J. G. designed the study. L. M. H. performed the experiments. L. M.
366 H., J. F., and H. S. performed the analyses. L. M. H., J. F., H. S., and J. G. wrote the
367 manuscript.

368 **Competing Interests**

369 The authors declare no competing financial interests.

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