Rapid evolution of stability and productivity at the origin of a microbial mutualism

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Abstract

Mutualistic interactions are taxonomically and functionally diverse. Despite their ubiquity, the basic ecological and evolutionary processes underlying their origin and maintenance are poorly understood. A major reason for this has been the lack of an experimentally tractable model system. We examine the evolution of an experimentally imposed obligate mutualism between sulfate-reducing and methanogenic microorganisms that have no known history of prior interaction. Twenty-four independent pairings (cocultures) of the bacterium *Desulfovibrio vulgaris* and the archaeon *Methanococcus maripaludis* were established and followed for 300 community doublings in two environments, one allowing for the development of a heterogeneous distribution of resources and the other not. Evolved cocultures grew up to 80% faster and were up to 30% more productive (biomass yield per mole substrate) than the ancestors. The evolutionary process was marked by periods of significant instability leading to extinction of two of the cocultures, but resulted in more stable, efficient, and productive mutualisms for most replicated pairings. Comparisons of evolved cocultures with those assembled from one evolved and one ancestral mutualist showed that evolution of both species contributed to improved productivity. Surprisingly, however, overall improvements in growth rate and yield were less than the sum of individual contributions, suggesting antagonistic interactions between mutations from the coevolved populations. Physical constraints on the transfer of metabolites in the evolution environment affected the evolution of *M. maripaludis* but not *D. vulgaris*. Together, these results show that challenges can imperil nascent obligate mutualisms and demonstrate the evolutionary responses that enable their persistence and future evolution.

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Introduction

The existence of mutually beneficial interactions between species has often puzzled evolutionary biologists because of the benefits of avoiding costly investments in genetically unrelated populations (1, 2). Such interactions are thought to originate when species begin trading byproducts or evolve from parasitic relationships (2, 3). The persistence of newly-formed, nascent mutualisms depends on their ability to adapt to several ecological challenges. The mutualists must initially use preexisting traits, so the functional basis for the mutualism is unlikely optimal. Second, their growth may be less stable because it depends on a resource that is produced by another population. This situation can lead to extinction of the mutualism if the abundance of one or both populations gets too low, or if one population stops cooperating, especially if the mutualism is obligate (1, 4, 5). Finally, adaptation to mutualism may also be affected by properties of the environment in which the mutualism is occurring. In particular, the spatial distribution of interacting populations affects the transfer of resources between them and may be key to their stability (6-9).

The effect of these challenges on evolution of new mutualisms is difficult to study without the possibility of experimentation with the original populations. Some adaptations arising early in mutualistic associations from pre-existing traits have been identified through comparative analyses, a common approach for studying evolution (10, 11). However, empirical data on the evolutionary and ecological dynamics giving rise to these adaptations is scarce because the original populations and ecological conditions are unknown.

Here, we use experimental evolution to address this issue while avoiding the methodological limitations of past comparative approaches. We control the selective environment and examine adaptations as they occur (12) and use microorganisms so that we can establish initially identical replicate populations and therefore analyze the role of chance events in determining evolutionary outcomes (13). A similar approach has been used to rigorously examine a variety of questions involving interactions between genotypes and species, including the evolution of predator-prey interactions (14, 15), the evolution of intraspecific cooperation (16, 17) and the ecological factors that stabilize commensal relationships against competition (7, 18, 19). The evolution of mutual cooperation between distinct species has not been addressed with experimental evolution, except to explore the relationships between mutualism and parasitism (20-22).

We now use experimental evolution to study the first steps in the evolution of a new mutualism that we formed by experimentally imposing a requirement for exchange of byproducts (3). A nascent syntrophic mutualism was established by pairing the bacterium *Desulfovibrio vulgaris* Hildenborough with the archaeon *Methanococcus maripaludis* S2 (23). This mutualism is based on interspecies transfer of hydrogen, a byproduct of anaerobic metabolism that is commonly exchanged among species that inhabit anoxic environments (24). Both species can be propagated in pure culture on appropriate substrates, but in the conditions used in our experiments they can only grow through syntrophic cooperation, or 'feeding together'. In the absence of hydrogen and sulfate they feed together by cooperating to complete the following energy-yielding reaction:

$$2\ \text{Lactate} + H_2O \rightarrow 2\ \text{Acetate} + CH_4 + H^+ + HCO_3^-$$

D. vulgaris ferments lactate, producing acetate, carbon dioxide, and hydrogen, but this reaction does not produce enough energy for growth unless the concentration of one of the reaction products is kept very low. *M. maripaludis* ensures this condition is met by consuming hydrogen and using it to reduce carbon dioxide to methane. Growth of *M. maripaludis* depends on the availability of hydrogen produced by *D. vulgaris* because no other suitable substrate is available. Thus, *D. vulgaris* provides food for *M. maripaludis* as a byproduct of lactate fermentation and *M. maripaludis* provides a permissive growth environment as a byproduct of feeding. Syntrophies similar to our experimental system function in the sediments of freshwater lakes, the guts of ruminants, and anaerobic digesters used to process waste (23). *Desulfovibrio* and related species may also function in syntrophies that degrade other complex growth substrates (24), sometimes involving obligate syntrophs (25), may use carbon monoxide or formate in lieu of hydrogen (26, 27), and may have acquired syntrophy related genes through horizontal gene transfer (28).

The strains of *D. vulgaris* and *M. maripaludis* used here have been propagated in pure culture in the laboratory for years and were also isolated from very different environments (29, 30)**,** so the selective environment during adaptation to syntrophy is expected to be similar to that of a nascent mutualism. Both strains must rely on traits that have been adapted to pure culture growth. Their continued association is also dependent upon the individual success of both syntrophic partners. Finally, efficient interspecies transfer of hydrogen, and possibly other materials, depends upon the spatial distribution of each species and the resources they produce. Thus, our experimental design also incorporated spatial heterogeneity as an environmental factor so we could test how the efficiency of byproduct transfer affected mutualist evolution.

To explore the evolution of this model mutualism, 24 nearly identical cocultures were evolved independently for 300 coculture doublings in two environments that promoted different distributions of populations, substrates, and metabolites. Throughout the experiment, nearly all of the cells were free-living and not aggregated. Twelve cultures were evolved in an environment where cells and substrates were uniformly distributed and metabolic byproducts were transferred by rapid shaking of the culture. The remaining twelve replicates were evolved in cultures that remained static during incubation (but mixed weekly for propagation), creating an environment in which substrates and metabolites could be transferred only by diffusion. To test how the heterogeneity of the environment affected the evolution of each species in mutualism, we isolated the species populations from each coculture and used them to produce cocultures of mixed ancestry. These cocultures were compared to cocultures with only ancestral or only evolved populations.

Results

Evolutionary changes in stability of syntrophic communities. The early stage of mutualist evolution was characterized by erratic growth (Fig 1). Cocultures typically consumed all of the resources within a week and achieved stationary-phase densities between 0.25 and 0.35 $OD_{600\ nm}$. However, after the first four transfers, a few cocultures did not show appreciable growth (0.0-0.06 OD_{600}) and were not transferred until they reached stationary-phase densities one or more weeks later. Every coculture entered one of these slow-growth phases at least once during the first six months of propagation. The final densities achieved by slow-growing cocultures varied from 0.15 to 0.35 OD_{600}. The timing, frequency, and duration of slow growth varied

considerably among cocultures, suggesting the involvement of a stochastic process. This stochastic process resulted in the extinction of two cocultures in the heterogeneous evolution environment. After about 30 transfers, erratic growth became infrequent and coculture growth cycles stabilized (Fig. 1).

Increases in growth rate and yield of evolved communities. As the cocultures became stable, they consistently achieved higher densities in stationary phase than at the beginning of the evolution experiment (Fig. 1). To test whether each coculture was also growing faster, we used freezer stocks of each evolved and ancestral coculture to inoculate media selective for *D. vulgaris* and *M. maripaludis*, thereby separating the populations in each coculture. These populations were acclimated in pure culture conditions before being used to inoculate new cocultures to minimize differences in previous acclimation to syntrophic growth. We could therefore more accurately measure differences caused by genetic changes accumulated in evolved cocultures. Only twenty cocultures were tested in these experiments because two cocultures went extinct and two others did not reach their 45th transfer for several weeks after these assays were completed.

Nineteen of the twenty cocultures tested exhibited a significant improvement in growth rate relative to the ancestor, indicating that one or both species had adapted to some aspect of the syntrophic environment. The average doubling time of the ancestral cocultures was 20 h (± 1.3, 95 % confidence interval) in the uniform (U) environment and 23 h (± 1.3) in the heterogeneous (H) environment. By contrast, evolved cocultures doubled every 13 hours on average when grown in the treatment in which they evolved (± 1.5, H-evolved; ± 1.7, U-evolved; when the U-

evolved coculture that did not improve was removed the average for U-evolved cocultures was 12 ± 0.2 h). Thus U-evolved cocultures improved roughly 1.6-fold, while H-evolved cocultures improved 1.8-fold on average (Fig 2a).

These evolutionary improvements in coculture growth rate could represent general adaptations to conditions that are the same in both treatments, such as the challenge of growing on lactate without an electron acceptor. In this case, evolved cocultures would perform similarly whether they were examined in the heterogeneous environment or the uniform environment. Alternatively, the populations may have adapted to ecological conditions that are different in the two environments. In this case, cocultures that evolved in the uniform environment would perform poorly in the heterogeneous environment, and vice versa. This was tested by measuring the growth rate of all of the evolved cocultures in their alternate evolution environment (Fig. 2a). The magnitude of improvement in coculture growth rate was not affected by environment ($F_{1,18}$ = 0.41, p=0.531) or the interaction between the evolution and assay environment ($F_{1,136}$ =2.03, p=0.157). Thus, it appears that the populations have mostly adapted to general aspects of syntrophic growth and not specifically to the heterogeneity of the environment in which they evolved. Regardless of how they evolved, the relative improvement was greater when examined in the heterogeneous environment (Fig. 2a, $F_{1,136}$ =5.35, p=0.022), slightly more so if they also evolved in the heterogeneous environment (t-test, t_{136} = -2.42, p=0.0169). This latter difference was due to poorer growth of U-evolved lines in the heterogeneous environment (15 ± 1.0 h average doubling time) compared to H-evolved lines.

All evolved cocultures exhibited significant increases in yield. The magnitude of the increase was independent of the evolution environment (Fig 2b; $F_{1,18}$ =0.0, p=0.969) but was relatively higher when yield was measured in the heterogeneous environment compared to the uniform environment (Fig 2b; $F_{1,136}$ =228.9, p<0.0001). This difference in relative magnitude can be attributed to the performance of the ancestor. The average yield was similar for U-evolved (OD_{600} nm = 0.488 ±0.01, 95% confidence interval) and H-evolved (OD_{600} nm = 0.492 ±0.03,) cocultures, but the ancestor reached a yield of 0.339 ± 0.01 and 0.278 ± 0.01 when growing in the uniform and heterogeneous environments, respectively. Increased optical density corresponded with increases in average stationary-phase cell densities of both species. An average 3.9-fold increase in *D. vulgaris* cell density (to 4 x 10^8 cells/ml) and a 1.6-fold increase in *M. maripaludis* density (to 3 x 10^8 cells/ml) was determined in evolved cocultures. Although *M. maripaludis* predominated in ancestral cocultures, after 300 generations *D. vulgaris* became the predominant species in almost all U-evolved cocultures and half of the H-evolved cocultures (Supplementary material, Fig S1).

Contributions of *D. vulgaris* and *M. maripaludis* populations to improved coculture growth.

The evolutionary improvements in coculture growth rate and yield could be caused by adaptations in *D. vulgaris*, *M. maripaludis*, or both species. To identify the population responsible for these community-level changes, we compared the growth rate and yield improvements of cocultures with only one evolved species (D_EM_A, evolved *D. vulgaris* in coculture with ancestral *M. maripaludis*; D_AM_E, ancestral *D. vulgaris* and evolved *M. maripaludis*) with improvements of fully evolved cocultures (D_EM_E) in their evolution treatment only. For these comparisons, cocultures were formed from potentially genetically diverse

populations of *D. vulgaris* and *M. maripaludis* that had been isolated by enrichment with pure culture growth conditions and antibiotics. Each of the evolved *D. vulgaris* and *M. maripaludis* populations could improve syntrophic growth by themselves (Figs 3 and 4). Independently evolved *D. vulgaris* populations were able to improve coculture growth rate and yield by similar magnitudes (except coculture U12) but the capacity of *M. maripaludis* populations to affect syntrophy was more variable.

M. maripaludis populations that evolved in the heterogeneous environment had a greater and more consistent effect on coculture growth than those evolved in the uniform environment (Fig 3, Table S2). The interaction between coculture composition (D_EM_E vs D_EM_A vs D_AM_E) and evolution environment had a significant effect on coculture growth rate ($F_{2, 214} = 15.2$, $p<0.0001$). The mean growth rate improvement of U-evolved D_AM_E cocultures was lower than cocultures with H-evolved D_AM_E cocultures because only some U-evolved *M. maripaludis* populations could improve coculture growth rates while every H-evolved *M. maripaludis* population caused faster growth (Fig. 3a and b). When evolved in a uniform environment, almost all D_EM_E and D_EM_A cocultures had similar growth rate improvements, suggesting that *D. vulgaris* may be the primary determinant of this feature (Fig. 3a). In contrast, H-evolved D_EM_A and D_AM_E cocultures both yielded similar improvements as D_EM_E cocultures (Fig. 3b). Thus, either or both species in combination may have contributed to growth rate improvements of H-evolved cocultures.

The effects of *D. vulgaris* and *M. maripaludis* on coculture yield were similar, and appear unaffected by the heterogeneity of their evolution environment ($F_{2, 36} = 0.2$, $p=0.819$). Fully evolved cocultures (D_EM_E) tended to obtain higher yields than cocultures where only one of the

species had evolved in syntrophy (D_EM_A or D_AM_E) (Fig. 4a and b; $F_{2,36}=19.8$, $p<0.0001$), suggesting that both species contributed to the yield improvements of the fully evolved cocultures.

Interactions between evolved *D. vulgaris* and evolved *M. maripaludis* populations. All of the evolved *D. vulgaris* and *M. maripaludis* populations were able to enhance coculture growth, indicating that one or more new mutations became prevalent in each of these populations during evolution. In some cocultures, both species could cause improvements similar to those of the fully evolved cocultures so that the relative contributions of mutations in each species to the growth improvements of D_EM_E cocultures is unclear. We therefore tested whether there were interactions between the mutations causing improvements in *D. vulgaris* and *M. maripaludis* beyond what might be predicted from their independent effects using methods developed for detecting epistatic interactions (31). The growth improvements of D_EM_A and D_AM_E (relative to D_AM_A) cocultures were used to calculate multiplicative ($D_EM_A/D_AM_A \times D_AM_E/D_AM_A$) and additive ($D_EM_A/D_AM_A + D_AM_E/D_AM_A - 1$) null models for combinations of mutational effects.

The observed improvements in growth rate and yield of all H-evolved cocultures were lower than could be predicted from additive and multiplicative models (Supplementary material, Table S3). In cocultures H3, H5, and H6, growth rate improvements were significantly lower than both null models ($p<0.05$ in two-tailed, two-sample t-test, n=4) and in H1, H2, and H6 yield improvements were significantly lower. This result indicates that in the heterogeneous environment, there is a tendency toward antagonistic interactions between mutations affecting syntrophic growth efficiency in the coevolving populations. In U-evolved cocultures,

antagonistic interactions between mutational effects were not universal.. Growth rate was lower than predicted by both null models for eight U-evolved cocultures and yield was lower in nine, but these differences were not statistically significant, with the exception of coculture U12. This coculture had a significantly lower growth rate than predicted by both models. In 5 U-evolved cocultures, the observed improvements in growth were either the same or slightly higher than predicted.

Discussion

There are few empirical examples of the initial stages of adaptation to mutualism. By experimentally imposing a mutualism and then monitoring its evolution, we were able to demonstrate rapid improvement in productivity and stability in response to the challenges of a new interdependent relationship. The evolved mutualism grew up to 80% faster and produced up to 30% more biomass than the ancestral pairings. Although evolutionary changes in both species contributed to improvements, the contribution of each population varied with the environment in which the mutualism evolved. Most significantly, all *M. maripaludis* populations that evolved in a heterogeneous environment contributed to a faster growth rate, whereas the contribution of those evolved in a uniform environment was highly variable, with some not contributing to improvement. The study also suggested that there are substantial challenges associated with the early stages in the evolution of this mutualism. This characteristic was demonstrated by initially erratic growth that led to extinction in 2 out of 24 cocultures.

When populations first engage in a mutualistic relationship, they must adapt to new growth conditions and are therefore most likely using preexisting traits for new functions. One of the

first adaptations for mutualism may therefore be optimization of these traits for mutualistic performance. In support of this hypothesis both species in nearly every coculture appear to have substituted mutations that improved the overall productivity of syntrophy. Cocultures could grow faster and produce more cells even though the resources remained constant throughout the experiment. Each species contributed to one or both of these community-level changes, presumably because they were able to more efficiently use the available resources and hence, acquire more energy for growth.

In an obligate mutualism, growth may not occur if both interacting populations are not at a minimum density (4, 5), the positive feedback between populations can lead to unsustainable levels of growth (4), and evolution may cause substantial fluctuations in the population densities of commensals (32). Here, we showed that the growth dynamics of communities were erratic during the early evolution of an experimentally imposed obligate syntrophy. The cause of this erratic growth is unclear, but the extinction of two cocultures demonstrated significant ecological consequences. The surviving mutualisms eventually evolved stable, predictable responses to batch culture growth.

As populations evolve in mutualisms or other interactions, they acquire mutations that may affect not only their own fitness but also the environment for their coevolving partner. The coevolving partner may acquire mutations that mitigate or enhance these changes, depending on how they affect its fitness. This process underlies interactions between genotypes such as those described by Heath and Tiffin (33) between *Sinorhizobium medicae* and *Medicago truncatulata* genotypes. A surprising result from our study was the tendency towards antagonistic interactions between

coculture growth-enhancing mutations in some *D. vulgaris* and *M. maripaludis* populations. These interactions between mutational effects could indicate an ecological constraint on growth of the syntrophy that limits the combined effects of two efficient syntrophs, each of which is capable of improving growth of both species, bringing syntrophy to near maximal levels. The species could also be actively competing for a limiting resource. For example, evolved *D. vulgaris* may obtain more resources than the ancestor (e.g., incorporating more lactate into cellular carbon), thereby limiting growth opportunities for evolved *M. maripaludis* when they are together. In this scenario, *M. maripaludis* would have higher fitness without its evolved partner.

The efficiency of a mutualism based on byproduct exchange is affected by how easily goods can be transferred between interacting populations. In our experiments, one species maintains thermodynamically permissible conditions as a byproduct of feeding while the other produces a metabolic byproduct, hydrogen. The transfer of this metabolite to *M. maripaludis* was probably most efficient in the uniform environment where it was vigorously dispersed by mixing. In contrast, if hydrogen is not efficiently transferred between species in the heterogeneous environment (as might require their close proximity), its accumulation in the headspace during growth would reduce availability in liquid (23). Inefficient hydrogen transfer is consistent with the observation that ancestral cocultures were slower and less productive in the heterogeneous environment.

Cocultures that evolved in the heterogeneous environment overcame this obstacle. They could grow as fast in the heterogeneous environment as all evolved cocultures could grow in the uniform environment. This evolved capacity required a special adaptation that was evidently not

acquired by the uniform-evolved cocultures, which could not grow at maximal rates in the heterogeneous environment. Evolutionary responses to this challenge were confined to *M. maripaludis*, the species that used hydrogen for growth. All of the *M. maripaludis* populations from the heterogeneous evolution environment improved coculture growth rate, but few of the uniform-evolved *M. maripaludis* had this capacity. In contrast, this variable had a more subtle effect, if any, on adaptation in *D. vulgaris*.

Other research with microbial systems has shown that the diversification of populations into new niches (8, 34), the evolution of exploitative relationships (7) and community diversity (6, 9) are affected by heterogeneous distributions of resources and populations that limit the diffusion of metabolites in communities. Our results confirm the importance of metabolite transfer rates on evolution of microorganisms. *M. maripaludis* relied on a diffusible metabolic byproduct for growth, and it had a different evolutionary response in the heterogeneous environment where this resource must be transferred through diffusion or in some way enhanced by interspecies contact. In contrast, *D. vulgaris* relied on lactate, a soluble growth substrate that would be evenly dispersed in either heterogeneity treatment and it showed a similar evolutionary response in both environments.

In conclusion, using experimental evolution of a model microbial mutualism, we were able to demonstrate several evolutionary responses of nascent mutualisms that may be predicted intuitively but have rarely been examined empirically. This model system for studying mutualistic interactions is now poised to address a variety of issues relating to evolution of interacting populations, including testing how quickly coevolving populations become

specialized to one another, the effects of adaptation to mutualism on solitary fitness, and also the genetic and physiological basis of adaptations to mutualism.

Methods

Strains and culture conditions. All cultures were grown at 37°C. *D. vulgaris* Hildenborough (ATCC 29579) was obtained from Dr. T.C. Hazen (Lawrence Berkeley National Labs). We isolated a clone of this strain, D1 and a spontaneous Nalidixic acid resistant derivative of that clone, D2 to use as ancestors in the evolution experiment. *D. vulgaris* was grown on plates with LS4D media (30) or in 10 ml CCMA (23) with 4.3g/L $NaSO_4$ in a Balch tube (18x 150 mm glass tube with a narrow opening to hold a 1-inch thick rubber septum) with a 80% N_2, 20% CO_2 headspace. See supplementary material for detailed recipes. All media were buffered with bicarbonate to maintain a pH of 7.2.

We isolated clone M1 of *M. maripaludis* S2 (29) and a neomycin resistant clone (M2) from this population to use as ancestors in the evolution experiments. Clone M2 lost its neomycin resistance during culturing. *M. maripaludis* was cultured in 5 ml CCMA without lactate and supplemented with 0.82g/L acetate, 1g/L casamino acids, and a higher sulfide concentration (0.5 mg NaS • $9H_2O$) in Balch tubes pressurized to 30psi with 20% CO_2, 80% H_2 and incubated in a horizontal position with shaking (300 rpm). Syntrophic cocultures were initiated by adding 0.1 ml (>1 x 10^7 cells) each of stationary-phase cultures of *D. vulgaris* and *M. maripaludis* to 20 ml CCMA (no sulfate) in a 30 ml Balch tube under 20% CO_2, 80% N_2 atmosphere.

Evolution experiment Four cocultures consisting of D1 and M1, D1 and M2, D2 and M1, and D2 and M2 were each used as inoculum (0.2 ml) for six independent cultures. Three were incubated without shaking (Heterogeneous environment) and three in a horizontal position with shaking at 300 rpm (Uniform environment). Thus, the evolution experiment was started with 12 independently evolving cocultures in each heterogeneity treatment. Every seven days, coculture density was measured in a spectrophotometer at $OD_{600\ nm}$, and transferred to fresh media (1% inoculum) if they achieved their 'maximum density' of 0.25-0.35 OD. Otherwise they were left to incubate until the next weekly transfer. If the coculture density declined from one week to the next it was transferred even though the maximum density was low (0.1 to 0.16 OD_{600nm}). At the 45^{th} 100-fold dilution (300 doublings of the syntrophic community, 6.6 per transfer) and several prior intervals, samples of each evolved coculture were stored in 10-20% glycerol at -80^{o}C. In the heterogeneous lines, a significant number of cells were concentrated at the bottom of the culture tubes at each transfer interval, so these tubes were shaken vigorously to redistribute cells prior to transfer. Purity of the cocultures was checked periodically by plating on R2A and by microscopic examination.

Assay of coculture growth rate and yield. Coculture growth rate and yield was assayed for all 12 cocultures from the uniform environment and only 8 cocultures from the heterogeneous environment, because two cocultures in the heterogeneous environment went extinct, and two others had not reached their 45^{th} transfer until after the assays were completed.

Freezer stocks of the evolved cocultures at transfer 45 and all four ancestral cocultures were each used to inoculate a balch tube containing media typically used to propagate *D. vulgaris* and

antibiotic against *M. maripaludis* (5µg/ml puromycin), and another tube containing media for *M. maripaludis* and antibiotic against *D. vulgaris* (1mg/ml spectinomycin). The resulting separated *D. vulgaris* and *M. maripaludis* populations were used to inoculate two each of the following combinations per evolved coculture: D_EM_E, D_EM_A, D_AM_E, and D_AM_A. All of these cocultures were incubated in their evolution environment. Two extra D_EM_E replicates were made for each evolved coculture and incubated in the alternate environment that they did not evolve in, resulting in a total of 200 cocultures per temporal block (400 total for the entire assay). After cocultures maintained their maximum density for three days, they were transferred (1%) into fresh CCMA and their density was recorded periodically until they had once again maintained a maximum density for three days. Coculture yield (maximum OD_{600} nm reached by the coculture) and growth rate were estimated independently for each replicate growth curve. The coculture growth rate was the slope (obtained from several data points) of the linear portion of the curve obtained by plotting ln(OD_{600} nm) = time.

Statistical analyses. Evolutionary changes in coculture growth rate and yield were obtained by dividing each measurement of a coculture containing one or more evolved population (D_EM_E, D_EM_A, or D_AM_E) by a randomly chosen measure from the same temporal block of that coculture's direct ancestor (D_AM_A). The natural log of this ratio was used to complete the mixed model ANOVAs using the Mixed procedure and the satterthwaite approximation of degrees of freedom in SAS version 9.1 (35). Formal ANOVA tables and a detailed description of the statistical models are presented in the supplementary material (Tables S1 and S2).

***D. vulgaris* and *M. maripaludis* density estimation**. Using the same methods in assays of coculture growth rate and yield, species populations were separated and used to make D_EM_E, and D_AM_A cocultures for all 20 evolved lines and 4 ancestral pairings. We counted density of *D. vulgaris* and *M. maripaludis* in cocultures that had been at stationary-phase densities for three days using a petroff-hauser counting chamber and a light microscope at 400x magnification. *D. vulgaris* (vibriod) and *M. maripaludis* (cocci) were identified by cellular morphology. This experiment was repeated three times.

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Figure legends

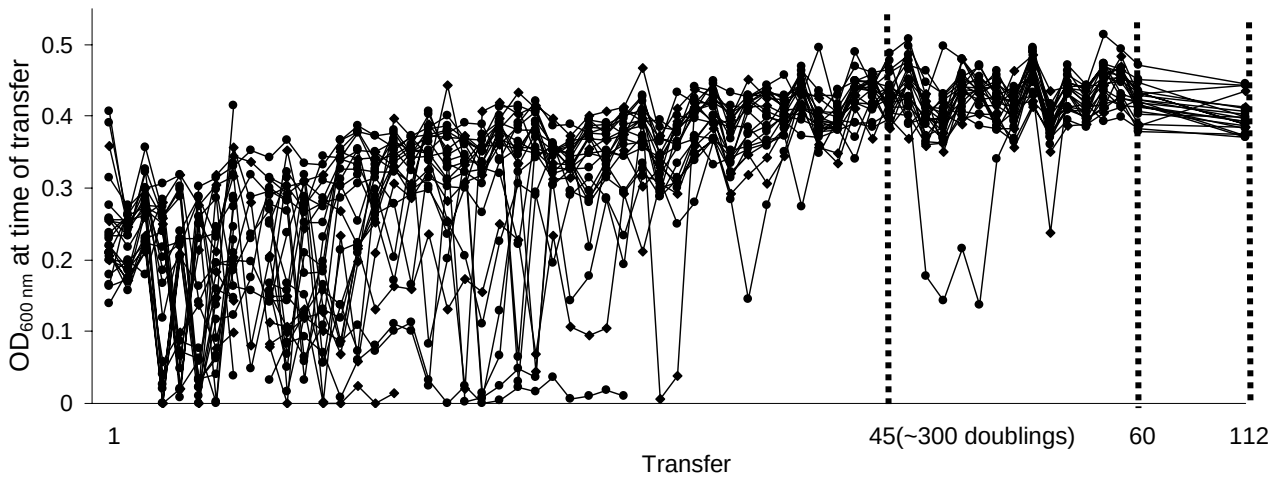
Figure 1. Coculture density at each transfer during the evolution experiment.

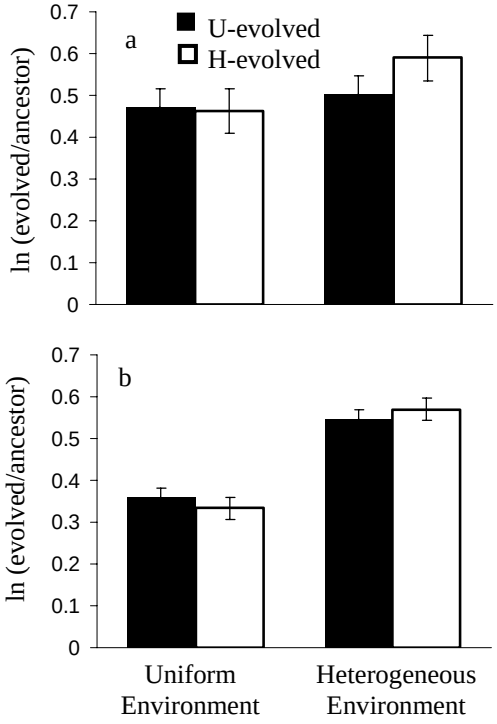
Figure 2. Improvements in growth rate and yield of cocultures after 300 generations of evolution. The growth rate (a) and yield (b) of uniform (solid bars) or heterogeneous (open bars) evolved cocultures and their ancestors was measured in both evolution environments. Bars

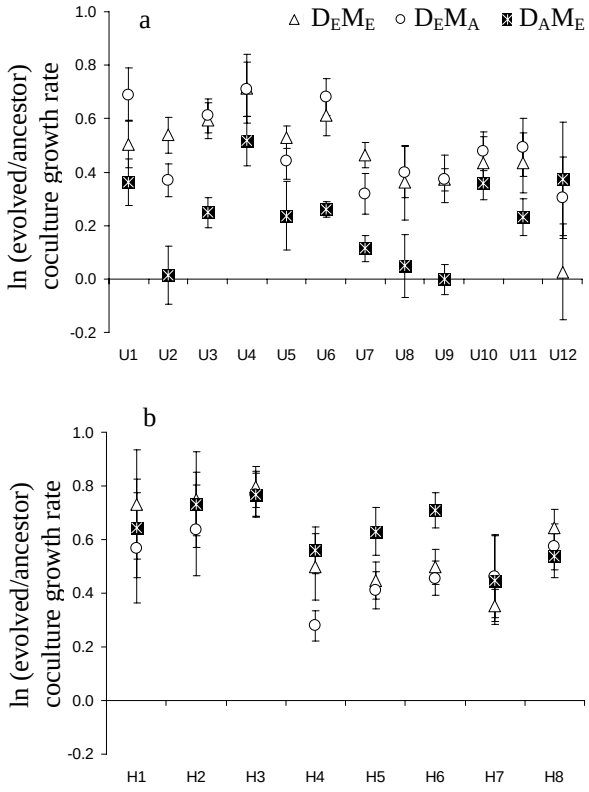
indicate least-squared means from the ANOVA results reported in Table S1 and error bars indicate standard error.

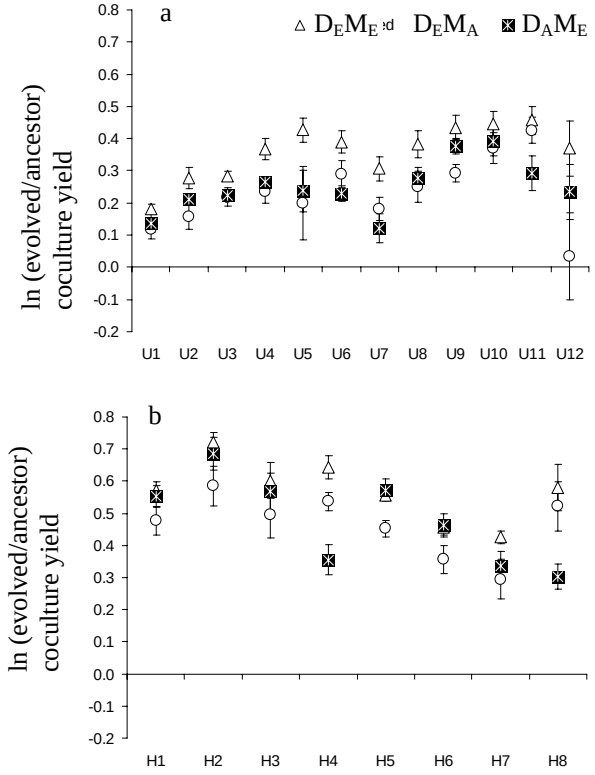
Figure 3. Improvements in growth rate of each coculture caused by both evolved species together (open triangles), evolved *D. vulgaris* only (open circles), or evolved *M. maripaludis* only (white stars on solid squares). The average of four replicate measures for cocultures from the uniform environment and the heterogeneous environment are plotted separately in panels a and b, respectively. Error bars indicate 95% confidence intervals.

Figure 4. Improvements in yield of each coculture caused by both evolved species together. Symbols and error bars have the same meaning as in Fig 3. The average of four replicate measures for cocultures from the uniform environment and the heterogeneous environment are plotted separately in panels a and b, respectively.


0.5
0.4
0.3
0.2
0.1
0
OD600 nm at time of transfer
1
45(~300 doublings)
60
112
Transfer


a
U-evolved
H-evolved
ln (evolved/ancestor)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
b
ln (evolved/ancestor)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Uniform Environment
Heterogeneous Environment


a
D_EM_E
D_EM_A
D_AM_E
ln (evolved/ancestor) coculture growth rate
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
U1
U2
U3
U4
U5
U6
U7
U8
U9
U10
U11
U12
b
ln (evolved/ancestor) coculture growth rate
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
H1
H2
H3
H4
H5
H6
H7
H8


a
D_EM_E
D_EM_A
D_AM_E
ln (evolved/ancestor) coculture yield
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
U1 U2 U3 U4 U5 U6 U7 U8 U9 U10 U11 U12
b
ln (evolved/ancestor) coculture yield
H1 H2 H3 H4 H5 H6 H7 H8

Media used to grow cocultures and *D. vulgaris*

LS4D media was used to grow *D. vulgaris* on plates to isolate ancestral clones.

per Liter
7.1 g $NaSO_4$
11.2 g of 60% Na lactate
1.6g $MgCl_2 \bullet 6H_2O$
1.0g NH_4Cl
0.4g K_2HPO_4
0.1g $CaCl_2$
1ml Thauer's vitamins
1 ml Trace minerals
9.1g PIPES
1mg Resazurin
5 ml Titanium Citrate Solution
15 g agar

CCMA was used to grow cocultures, and modified by adding sulfate or acetate and yeast extract to grow *D. vulgaris* and *M. maripaludis*, respectively. These modifications are described in the methods section of the published manuscript.

pH 7.2, containing per Liter
2.3 g NaCl
5.5 g $MgCl_2 \bullet 6H_2O$
0.14g $CaCl_2 \bullet 2H_2O$
0.5 g NH_4Cl
0.1g KCl
4.32 ml 60% Sodium DL-Lactate
1 mg/Resazurine
0.192g K_2HPO_4
2.1g $NaHCO_3$
1 ml Trace Minerals
1 ml Thauer's Vitamins
0.18g L-Cysteine Hydrochloride
0.078 mg $NaS \bullet 9H_2O$)

The Trace minerals solution used in these experiments contained as follows:

per liter
1.0 g $FeCl_2 \cdot 4H_2O$
0.5 g $MnCl_2 \cdot 4H_2O$
0.3 g $CoCl_2 \cdot 4H_2O$
0.2 g $ZnCl_2$
0.05 g $Na_2MoO_4 \cdot 4H_2O$

0.02 g H_3BO_3
0.1 g $NiSO_4 \cdot 6H_2O$
0.002 g $CuCl_2 \cdot 2H_2O$
0.006 g $Na_2SeO_3 \cdot 5H_2O$
0.008 g $Na_2\ WO_4 \cdot 2H_2O$
12.8 g Nitriloacetic acid (pH 6.5).

The Thauer's vitamin solution used in these experiments contained:

per liter
0.02 g biotin
0.02 g folic acid
0.1 g pyridoxine HCl
0.05 g thiamine HCl
0.05 g riboflavin
0.05 g nicotinic acid
0.05 g DL pantothenic acid
0.05 g *p*-aminobenzoic acid
0.01 g vitamin B12.

Statistical models

The following statistical model was used to test whether the heterogeneity of the evolution environment affected the magnitude of change in evolved cocultures: ln (D_EM_E/D_AM_A growth rate or yield) = Evolution Environment + Block + Assay Environment + EvolEnv*AssayEnv + Coculture(EvolEnv). Coculture is a random factor nested within Evolution Environment referring to any effect of particular evolved cocultures.

To test whether whether the composition (D_EM_E, D_EM_A, D_AM_E) of the coculture affects growth improvments relative to D_AM_A, and if these differences are specific to uniform or heterogeneous-evolved cocultures, we used the following statistical model: ln (D_EM_E, D_AM_E, D_EM_A/D_AM_A growth rate or yield) = Evolution Environment + Block + Composition + EvolEnv*Comp + Coculture(EvolEnv) + Comp*Coculture(EvolEnv). Coculture(EvolEnv) and Comp*Coculture(EvolEnv) are both random factors. The effect Comp*Coculture(EvolEnv) served as the error term for tests of the fixed effects on the magnitude of change in coculture yield. However, when the same model was applied to coculture growth rate, the covariance for this parameter was '0'. Fixed effects were therefore tested against experimental error variance in the growth rate ANOVA.

Figure S1. Abundance of *D. vulgaris* and *M. maripaludis* during stationary-phase in evolved and ancestral cocultures. *D. vulgaris* (vibroid) and *M. maripaludis* cells(coccoid) in ancestral (A) and U-evolved cocultures (U1-12) grown in the uniform environment (a), and ancestral and H-evolved cocultures (H1-8) grown in the heterogeneous environment (b) were identified by morphology. The average densities of *D. vulgaris* and *M. maripaludis* in evolved and ancestral cocultures in the unform (c) and heterogeneous (d) environments are also shown. Error bars indicate standard deviation in a and b, and 95% confidence intervals in c and d.

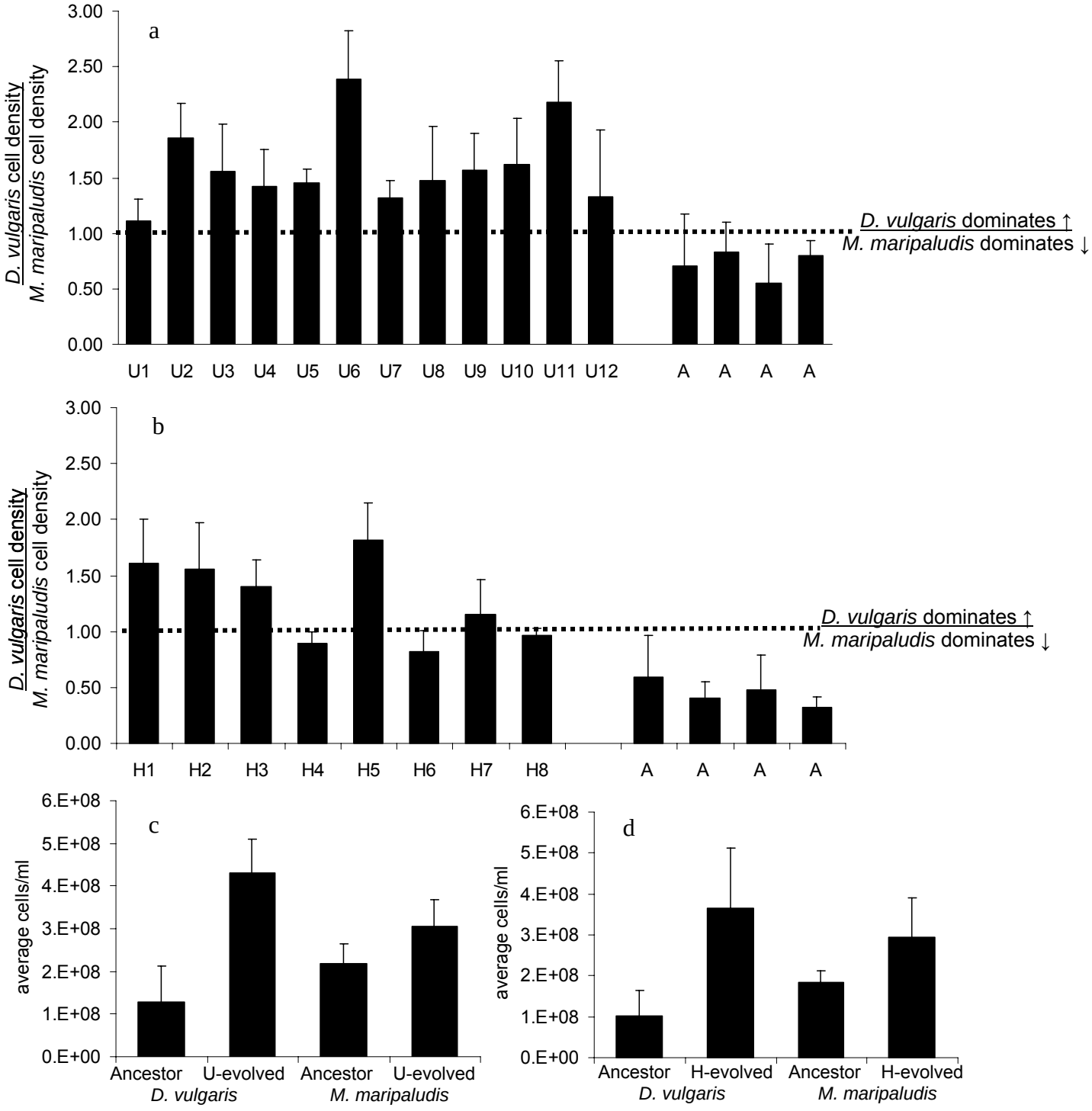

a
D. vulgaris cell density / M. maripaludis cell density
3.00
2.50
2.00
1.50
1.00
0.50
0.00
U1 U2 U3 U4 U5 U6 U7 U8 U9 U10 U11 U12
A A A A
D. vulgaris dominates ↑
M. maripaludis dominates ↓
b
D. vulgaris cell density / M. maripaludis cell density
3.00
2.50
2.00
1.50
1.00
0.50
0.00
H1 H2 H3 H4 H5 H6 H7 H8
A A A A
D. vulgaris dominates ↑
M. maripaludis dominates ↓
c
average cells/ml
6.E+08
5.E+08
4.E+08
3.E+08
2.E+08
1.E+08
0.E+00
Ancestor U-evolved Ancestor U-evolved
D. vulgaris
M. maripaludis
d
average cells/ml
6.E+08
5.E+08
4.E+08
3.E+08
2.E+08
1.E+08
0.E+00
Ancestor H-evolved Ancestor H-evolved
D. vulgaris
M. maripaludis

Table S1. Mixed model ANOVA testing the effects of heterogeneity in the evolution or assay environment on growth improvements of evolved cocultures.

	num df	den df	F	p
Growth rate				
Evolution Environment	1	18	0.41	0.531
Block	1	136	0.08	0.772
Assay Environment	1	136	5.35	0.022
EvolEnv*AssayEnv	1	136	2.03	0.157
Yield				
Evolution Environment	1	18	0.00	0.969
Block	1	136	3.28	0.073
Assay Environment	1	136	228.9	<0.0001
EvolEnv*AssayEnv	1	136	3.29	0.072

Table S2. Mixed model ANOVA testing for differences in effect of evolved *D. vulgaris*, *M. maripaludis*, or the combination of both species on coculture growth improvements.

	num df	den df	F	p
Growth rate				
Evolution Environment	1	18	10.1	0.005
Block	1	214	1.44	0.232
Composition	2	214	4.51	0.012
EvolEnv*Comp	2	214	15.2	<0.0001
Yield				
Evolution Environment	1	18	33.2	<0.0001
Block	1	178	0.04	0.84
Composition	2	35.7	19.8	<0.0001
EvolEnv*Comp	2	35.7	0.2	0.819

The term 'composition' tests whether D_EM_A, D_AM_E, or D_EM_E cocultures have different levels of improvement relative to the ancestor, and the interaction between evolution environment and composition (EvolEnv*Comp) tests whether these relationships are different in the uniform and heterogeneous-evolved cocultures.

Table S3. Tests for synergistic or antagonistic interactions between mutations in *D. vulgaris* and *M. maripaludis*.

Coculture Uniform	Observed GR	Add GR	Obs − Add GR	Mult GR	Obs − Mult GR	Observed Yield	Add Yield	Obs − Add Yield	Mult Yield	Obs − Mult Yield
U1	1.7	2.4	−	2.9	−*	1.2	1.3	−	1.3	−
U2	1.7	1.5	+	1.5	+	1.3	1.4	−	1.4	−
U3	1.8	2.1	−	2.4	−	1.3	1.5	−	1.6	−
U4	2.0	2.7	−	3.4	−*	1.4	1.6	−	1.7	−
U5	1.7	1.8	−	2.0	−	1.5	1.5		1.5	
U6	1.8	2.3	−	2.6	−	1.5	1.6	−	1.7	−
U7	1.6	1.5	+	1.5	+	1.4	1.3	+	1.4	
U8	1.4	1.5	−	1.6	−	1.5	1.6	−	1.7	−
U9	1.5	1.4	+	1.4	+	1.5	1.8	−	2.0	−
U10	1.5	2.0	−	2.3	−	1.6	1.9	−	2.1	−*
U11	1.5	1.9	−	2.1	−	1.6	1.9	−	2.1	−
U12	1.0	1.8	−*	2.0	−*	1.4	1.3	+	1.3	+
Heterogeneous										
H1	2.1	2.7	−	3.4	−*	1.8	2.3	−*	2.8	−*
H2	2.1	3.0	−	3.9	−*	2.1	2.8	−*	3.6	−*
H3	2.2	3.3	−*	4.6	−*	1.8	2.4	−	2.9	−*
H4	1.6	2.1	−	2.3	−	1.9	2.1	−	2.4	−
H5	1.6	2.4	−*	2.8	−*	1.7	2.3	−	2.8	−
H6	1.6	2.6	−*	3.2	−*	1.6	2.0	−*	2.3	−*
H7	1.4	2.2	−	2.5	−*	1.5	1.7	−	1.9	−
H8	1.9	2.5	−	3.0	−*	1.8	2.0	−	2.3	−

The observed improvements in growth rate (GR) and yield of evolved cocultures was the mean ratio of $D_E M_E / D_A M_A$ for the indicated coculture. This value was tested against predicted improvements based on either an additive (add) or multiplicative (mult) null model for the combination of $D_E M_A / D_A M_A$ and $D_A M_E / D_A M_A$. The difference between observed and predicted improvements is indicated by +/- symbols and marked by * if it was significant ($p<0.05$) in a two-tailed, two-sample t-test, n=4.