Managing and engineering microbial communities relies on the ability to predict their composition. While progress has been made on predicting compositions on short, ecological timescales, there is still little work aimed at predicting compositions on evolutionary timescales. Therefore, it is still unknown for how long communities typically remain stable after reaching ecological equilibrium, and how repeatable and predictable are changes when they occur. Here, we address this knowledge gap by tracking the composition of 87 two- and three-species bacterial communities, with 3–18 replicates each, for ~400 generations. We find that community composition typically changed during evolution, but that the composition of replicate communities remained similar. Furthermore, these changes were predictable in a bottom-up approach—changes in the composition of trios were consistent with those that occurred in pairs during coevolution. Our results demonstrate that simple assembly rules can hold even on evolutionary timescales, suggesting it may be possible to forecast the evolution of microbial communities.
Microbes are engines of biogeochemical cycles\(^1\), agents of health and illness\(^2\), and key players in food and biotechnological industries\(^3\). They are seldom found in a biotic void but rather form complex and diverse communities of multiple interacting species. The ongoing realization of the significant role microbial communities play in various environments\(^4\)-\(^6\), along with the potential that lies in the ability to manipulate and design them, has motivated research aiming to disentangle the rules that govern microbial ecology\(^7\).

The ability to rationally design or manipulate communities depends on our ability to predict their composition. Currently, predicting the structure and dynamics of microbial communities commonly relies on fitting specific ecological models, quantitative mechanistic models, or using genome-scale metabolic models\(^8\)-\(^11\). Challenges in fitting such models\(^11\) and concerns regarding their validity\(^12\) have recently led us to develop an alternative non-parametric predictive framework, which was validated using laboratory experiments in microbial microcosms\(^13\),\(^14\), and in the gut of C.\ e\ l\ a\ g\ a\ n\ s\(^15\). In this bottom-up framework, the composition of multispecies communities is inferred using the measured pairwise interactions between the members of the community. However, while this approach and other notable frameworks had made progress, none of these take into account changes in species abundances that could occur during evolution; thus, the timescales at which these frameworks remain predictive are still unclear.

Understanding the dynamics of community composition over evolutionary timescales is crucial for designing sustainable microbial communities; adaptations that occur while species coevolve as a community, could potentially result in loss of diversity or lead to loss of desired functions\(^16\). Experiments involving microbial communities have demonstrated that changes in interspecies interactions and metabolic activities are common when species evolve in a community, and are often different than those that occur when species evolve in isolation\(^17\)-\(^24\). Yet we still lack a clear understanding of how prevalent such changes are, and how they affect community composition.

Evolutionary changes in community composition may not be repeatable, making them challenging to predict. At ecological timescales, community composition is typically highly deterministic—repeated experiments converge to similar compositions from the identical initial conditions\(^13\). Many features of single-species evolution are also often found to be strikingly repeatable\(^25\)-\(^34\), however, it is not clear whether similar repeatability occurs when species coevolve within communities. A recent experimental study found that the composition of 96-replicate communities composed of six species of soil bacteria was significantly altered during coevolution, and that replicates clustered into four distinct types that varied in species abundances\(^35\). In another study that measured the dynamics of a three-species multitrophic microbial community for three months, variation between replicates remained strikingly low for the duration of the experiment\(^36\). How coevolution affects the predictability of community-level features, including community composition, is still an under-explored question.

Here, we use experimental evolution of 87 two- and three-species bacterial communities, with 3-18 replicates each, to test the prevalence, repeatability, and predictability of changes in community composition over ~400 generations of coculturing. We find that over these timescales community composition typically changes significantly, and that variability between replicate communities typically increases. However, replicate communities remain similar enough to allow predictions to be made. Specifically, we show that a simple assembly rule based on the outcomes of pairwise coculturing, originally developed for ecological timescales\(^13\), can be expanded for evolutionary timescales. In contrast, we find that predictions based on species’ growth parameters, which have reasonable accuracies at ecological timescales, have no predictive power at evolutionary timescales.

### Results

We conducted a high-throughput evolutionary experiment, propagating 87 two- and three-species communities for ~400 generations in well-mixed microcosms containing M9 minimal-media supplemented with three carbon sources—galacturonic acid, acetate, and serine (Fig. 1A–C, “Methods”). All communities consist of subsets of a library of 16 heterotrophic soil bacterial species (Supplementary Table 1). Multiple replicates (3–18, Supplementary Data 1) of each community, as well as of the 16 monocultures, were cultured in 200 µl minimal media and passaged through 38 cycles of 48 h of growth and 1500-fold dilution into fresh media (Fig. 1, Methods). The relative abundances of each species within communities were measured at 9 timepoints throughout the experiment by plating and counting colonies, which are morphologically distinct for each species (Fig. 2A, Supplementary Figs. 1, 2). To increase the chance that the communities included in the evolution experiment consist of species that coexist on evolutionary timescales, we only included pairs that were found to coexist for ~60 generations in a preliminary experiment, and trios composed of such pairs (Methods). In most communities all species survived throughout the evolution experiment (>74% of all communities; >83% of pairs and >65% of trios).

Community composition typically changes over evolutionary timescales. The first few dozen generations are characterized by significant shifts in community composition (Fig. 2A,B, Supplementary Fig. 4A). These rapid changes are consistent with previous studies\(^13\),\(^37\), and likely reflect a transition from the arbitrary initial abundances that species were inoculated at to an ecological equilibrium. This is supported by the fact that over these timescales, pairs inoculated at varying initial fractions converge to similar compositions (Supplementary Fig. 3), and that changes decelerate considerably after ~50-~70 generations (Fig. 2B, Supplementary Fig. 4A), suggesting that these timescales were sufficient for most communities to reach an ecological equilibrium.

Compositions reached at ecological timescales typically change during coevolution. Although the rate of change in community composition drops, communities continue to change slowly (Fig. 2B, Supplementary Figs. 4A, 5). These alterations accumulate such that as time goes by most communities diverge significantly from their ecological states (Fig. 2C, Supplementary Fig. 4B). While most communities change significantly, some communities remain strikingly stable throughout the entire experiment (Fig. 2A,C, Supplementary Figs. 1, 2, 4B), but it is still not clear what features contribute to their stability. These results underscore our limited ability to extrapolate community composition to evolutionary timescales from ecological data, and stress the need to adjust prediction rules for these timescales.

Community composition changes over evolutionary timescales due to heritable alterations in species’ phenotypes. In order to evaluate whether compositional changes that occur on longer timescales are caused by evolutionary changes or by long-term ecological dynamics, we re-isolated strains from 30 pairs that evolved for ~400 generations. Pairs of evolved strains, as well as ancestral strains, were then cocultured again for ~53 generations from the same initial conditions used to initiate the evolution experiment. We observed that while ancestral strains reproduced the compositions reached at ecological timescales during the evolutionary experiment, evolved strains reached significantly
Pair compositions predict trio compositions over evolutionary determinism and are thus potentially predictable. Although coevolution has a diverging effect on community composition, post-ecological alterations display some level of variability between replicate communities (Fig. 3A, Supplementary Fig. 8A). Most communities (74%), had more similar compositions during the first ~70 generations than at generation ~400 (Fig. 3B, Supplementary Fig. 8B). Nonetheless, in replicate-to-replicate variability at generation ~400 than at generation ~70 (Fig. 3B, Supplementary Fig. 8B). Nonetheless, in subsequent changes were less repeatable and often resulted in increased variability between replicate communities (Fig. 3A, Supplementary Fig. 8A). Most communities (74%), had more replicate-to-replicate variability at generation ~400 than at generation ~70 (Fig. 3B, Supplementary Fig. 8B). Nonetheless, in most communities replicates remained more similar to each other than expected by chance throughout the experiment (Mann–Whitney p value ~8 \times 10^{-19} at generation ~400, 87 communities, Supplementary Fig. 8A, “Methods”). Moreover, the species whose abundance increased by the largest factor during coevolution tended to be conserved across replicate communities (permutation test p value <5 \times 10^{-4}, 87 communities; Fig. 3C, Supplementary Fig. 8C, “Methods”). These results reveal that although coevolution has a diverging effect on community composition, post-ecological alterations display some level of determinism and are thus potentially predictable.

Pair compositions predict trio compositions over evolutionary timescales. It has been previously established that two-species competition experiments could often predict the composition of multispecies communities\(^\text{13,14}\). While these experiments suggest that most ecological outcomes could be explained by pairwise interactions, it is still unknown whether similar predictability holds at evolutionary timescales. In order to assess predictability at evolutionary timescales, we employed an approach that was previously used to predict the composition of three-species communities from pairwise data at ecological timescales. In this approach, the fraction of each species in a trio is predicted to be the weighted geometric mean of its pairwise fractions from the same time point as the trio were significantly better than an uninformed guess (0.73 \pm 0.03). In contrast, predictions based on the pair fractions at generation ~70 significantly during these timescales (Fig. 2C), the accuracy of these predictions deteriorated with time; predictions of three-species community compositions after ~400 generations, based on pair fractions at ~70 generations were not significantly better than an uninformed guess (0.76 accuracy, Fig. 4B, Supplementary Fig. 9, Mann–Whitney p value = 0.1). In contrast, predictions based on the pair fractions from the same time point as the trio were significantly more accurate than the uninformed guess throughout the whole experiment, and only slightly deteriorated with time.
Furthermore, in 82% of trios the species whose abundance increased by the largest factor since ecological timescales could be accurately predicted based on the species’ abundance increase in pairs (binomial test with \( p = 1/3 \), \( p \) value = 0.004). These results demonstrate that the previously established assembly rule could be adjusted for predicting the composition of communities on evolutionary timescales, and suggests that high-order interactions are not major determinants of community composition during coevolution in our system.

**Growth of individually-evolved species does not predict community composition.** While pairwise competitions predict the composition of multispecies communities, measuring the pairwise compositions of multiple pairs over evolutionary timescales is laborious and in many cases infeasible. Monoculture data, such as species’ growth rates and carrying capacities, can be acquired more readily and was previously shown to correlate with the species’ competitive ability\(^{13-15}\). Therefore, we next tested whether such monoculture data may provide a more accessible alternative for predicting the structure of multispecies communities.

We found that carrying capacities and growth rates could not accurately predict species’ fractions or the identity of the dominant species in evolved coculture (Figs. 4, 5, Supplementary Figs. 11, 12, 13, 14). The accuracy of predicting species fractions based on the carrying capacities of individually-evolved species
was comparable to that of an uninformed guess throughout the evolution experiment (Fig. 4B). The identity of the dominant species in two-species cocultures could be predicted with some accuracy at ecological timescales by the ancestral strains’ carrying capacities and growth rates (Fig. 5A, Supplementary Figs. 11A, D, 14A, B, “Methods”). However, after evolving for ~400 generations, the identity of the dominant species could not be accurately predicted by the ancestral strains’ growth ability, nor by the growth ability of strains that were evolved in monoculture (Fig. 5B, Supplementary Figs. 11B, C, E, F, 5, “Methods”). The deterioration in the predictive ability of the ancestral strain’s growth ability after coevolution is consistent with the fact that almost all species increased in growth during ~400 generations of evolution (Supplementary Figs. 15, 5). The fact that the growth abilities of both individually-evolved strains and coevolved strains re-isolated from 21 pairs (Supplementary Fig. 13) also failed to predict pair compositions suggest that the evolved compositions are less determined by differences in growth capabilities than the ancestral compositions.

Discussion
We observed that during ~400 generations of coevolution communities diverged from their ancestral compositions. However, it is not clear whether community compositions would continue to change at longer timescales of thousands of generations. Long-term evolutionary experiments in E. coli showed that following an initial period of rapid adaptation, evolutionary changes can continue for tens-of-thousands of generations at a lower rate32. In our experiment, changes in community composition slowed down slightly after ~200 generations (Figs. 2B, C, 3A), and it remains to be seen whether and on what timescales communities will reach evolutionary stable compositions.

While we found that community composition changed significantly in a repeatable manner in our experiment, there was considerable variability in stability and repeatability across the different communities (Figs. 2, 3, Supplementary Figs. 1, 2). It has been suggested that the strength and sign of interactions in a community could have a major influence on the evolutionary stability of the community20,38. Other factors, including the shape
of the fitness landscape, species’ evolvability, and their metabolic similarity could also affect the evolutionary trajectory of the community. Further work is needed in order to understand what makes certain communities evolutionary stable, and what makes some communities more evolutionary repeatable than others.

We have focused on the evolutionary dynamics and predictability of community composition, rather than community-level properties or traits of individual species within communities. While species abundances tend to change in a repeatable and predictable manner during coevolution, it is not clear whether the same is true for the underlying phenotypic and genotypic changes. For example, different mutations or changes in resource utilization may lead to similar changes in community composition. In contrast, the evolution of community-level properties, such as productivity, may be even more repeatable and predictable, as they have been shown to be less variable than species composition in natural communities. Furthermore, the fact that similar changes in community composition occurred in pairs and in trios may indicate that adaptation was not very sensitive to the specific biotic context and may have been driven by the abiotic conditions. However, the growth ability of individual species was only predictive of community composition on ecological timescales (Fig. 5, Supplementary Fig. 13). This suggests that when species coevolve, more complex interactions than competition for the supplied resources play a larger role in determining community composition. For example, species may adapt to consume or to better tolerate their partners’ secretions. Elucidating the selective forces that act on species within communities remains an outstanding challenge.

Evolution in natural communities may differ significantly from our experiments, which involved highly simplified communities in stable laboratory conditions. In particular, our experiments were conducted in a well-mixed environment, thus reducing the possibility for spatial structure, which has been suggested to play an important role in the evolution of species interactions. In addition, higher-order interactions may play a more significant role in shaping community composition in more species-rich communities, making predictions based on pairwise coevolution more challenging.
more challenging. Furthermore, our assembly rule may be less predictive of communities involving more than one trophic-level, as was recently observed in an evolutionary experiment involving bacteria, a protist, and a phage.

In summary, microbial communities have the potential to be harnessed for numerous applications as plant growth promoters, bioremediation agents, biomanufacturing agents, or probiotic therapeutics. However, even in the absence of external perturbations, communities could change significantly over time and lose their desired functions. Our data highlights the need for knowledge of the evolutionary dynamics for a sustainable design of microbial communities, as it underlies the prevalence and timescales at which compositional changes occur. However, our findings suggest that compositional changes could still be predicted in a bottom-up approach from pairwise interactions even after hundreds of generations of coevolution. It still remains to be determined how well these predictions scale-up to more diverse communities, how would different environmental conditions such as fluctuations and spatial structure influence these results, and the timescales at which these predictions still hold.

**Methods**

**Strains and media.** The set of 16 strains used in this experiment contains environmental isolates along with strains from the ATCC collection (Supplementary Table 1). The strains were chosen based on two criteria: a distinct colony morphology when plated on an NB agar plate; and ability to coexist for ~60 generations with at least two other strains in our collection.

All cultures were grown in M9 minimal salts media containing 1X M9 salts, 2 mM MgSO₄, 0.1 mM CaCl₂, 1X trace metal solution (Teknova), supplemented with 0.003, 0.001, 0.08, 0.32, 0.67. One species’ growth (H77) was not measured and therefore the two communities that included it were removed from this analysis, and accuracies were evaluated from the data of 42 pairs. Source data are provided as a Source Data file.

**Evolution experiment.** Frozen stocks of individual species were streaked out on nutrient agar Petri plates and grown at 28 °C. After 48 h single colonies were picked and incubated into 15 ml falcon tubes containing 3 ml nutrient media (3 g/L peptone BD dico, BD Bioscience; 3 g/L yeast extract BD dico, BD Bioscience; 15 g/L agar Bacto, BD Bioscience) and counting colonies. For that, the cultures were diluted to an OD of 2.4 × 10⁻⁵. 10⁵ and 10⁰ µl of the diluted culture was plated on NB plates and spread using glass beads. Plates were incubated at 28 °C for 48 h and colonies were counted manually. The distribution of the number of colonies counted at each plate to infer community composition is found in Supplementary Fig. 11.

We chose the communities based on a preliminary experiment that was conducted by the same protocol for six transfers. In this experiment, 114 of 171 possible pairs of a set of 19 strains (3 strains were not included in the evolution experiment) were cocultured. Pairs that had coexisted for the duration of this experiment, and were confidently distinguishable by colony morphology, and trios that are composed of these pairs, were used for the coevolutionary experiment. We started the evolutionary experiment with 51 pairs and 51 trios, and removed communities that did not coexist for the first ~70 from the final analysis. If a replicate was suspected to be contaminated it was also excluded from further analysis.

**Ecological experiments.** We supplemented the data of the evolutionary experiment with two ecological competition experiments with the same experimental condition. In order to assess whether communities typically reach an ecological equilibrium within ~50–70 generations (Supplementary Fig. 3), we cultured eight of the pairs that were used in the evolutionary experiment. This experiment was initiated in the same way as the evolutionary experiment, only that after the species starters were normalized they were inoculated at the varying initial fractions - 9:1, 5:5, 1:9. Because the normalization depended on optical density, there is a variation in the actual initial fractions between different pairs. Community composition was then measured on six transfers during this experiment: 0, 1, 2, 4, 5, and 6.

In order to assess whether changes in composition are due to heritable changes in species’ phenotypes, we used strains that were re-isolated from 31 evolved pairs, and 13 pairs of ancestral strains (Supplementary Figs. 6, 7). Strains were replicated from glycerol stocks into the experimental media and grown for 24 h. The starters were normalized to initiate the competition assay at OD = 10⁻⁴ in fresh M9 media. Species were mixed at equal volume and were propagated for five cycles. Community composition was measured at initial conditions, and at the end of the final cycle (5).

**Quantification of repeatability.** In order to quantify the qualitative repeatability of different replicate communities we first identified which species was the maximally increasing member at each replicate, that is, which species had increased its abundance by the largest factor between generation 70 and 400. Then, we quantified the frequency of the replicates that had the same maximally increasing member for each community. This measure always produces a value between 1 and 1/n where n is the number of species in the community. We checked the distribution of the repeatability scores against the null hypothesis that the factor by which species’ abundances increased during evolution is independent of the species or the community. For this, we shuffled the factor of change in relative abundance across all samples, for pairs and trios separately, and quantified the new repeatability scores of the shuffled data. Data of the null hypothesis were generated over 2000 times, and the p value was given by the probability to get a mean equal or above the real data mean.

We used the average Euclidean distance of replicates from the median replicate in order to quantify the variability between replicate communities. In order to check whether the distribution of variabilities is similar to what can be expected of random communities, in which each species in the community is just as likely to have a reduced abundance as to have a replaced abundance, we drew a uniform Dirichlet distribution with q = 1. We then checked the statistical difference between the two distributions using one-sided Mann–Whitney U test.
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**Author contributions**

N.M. and J.F. designed the study and wrote the manuscript, N.M., N.T., and Y.S. performed the experiments, and N.M performed the analysis.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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