BIODIVERSITY

High functional diversity stimulates diversification in experimental microbial communities

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There is a growing awareness that biodiversity not only drives ecosystem services but also affects evolutionary dynamics. However, different theories predict contrasting outcomes on when do evolutionary processes occur within a context of competition. We tested whether functional diversity can explain diversification patterns. We tracked the survival and diversification of a focal bacterial species (*Pseudomonas fluorescens*) growing in bacterial communities of variable diversity and composition. We found that high functional diversity reduced the fitness of the focal species and, at the same time, fostered its diversification. This pattern was linked to resource competition: High diversity increased competition on a portion of the resources while leaving most underexploited. The evolved phenotypes of the focal species showed a better use of underexploited resources, albeit at a cost of lower overall growth rates. As a result, diversification alleviated the impact of competition on the fitness of the focal species. We conclude that biodiversity can stimulate evolutionary diversification, provided that sufficient alternative niches are available.

INTRODUCTION

Biodiversity has always fluctuated during Earth's history, with phases of massive extinction followed by adaptive radiation (*1*). In the light of the current massive global species loss at the hands of recent human development, there is growing interest in predicting how species loss affects the structure and functioning of ecosystems on an evolutionary scale. Evolution of species and the emergence of new taxa can restore ecosystem processes that have been lost during extinction waves (*2*), and understanding how changes in diversity patterns feedback to evolutionary rates may provide new tools to predict the shape of tomorrow's ecosystems and design conservation strategies.

Biotic interactions such as competition and predation are major drivers of evolutionary processes (*3*), suggesting possible feed back loops between biodiversity and evolutionary diversification. However, the net effect of competition on diversification processes is highly variable and different studies have reported contrasting outcomes. On one hand, a range of studies suggests that evolution will slow down with increasing numbers of competing taxa because available niches will be exploited more completely, thereby preventing the establishment of a novel phenotype (*4*–*6*). On the other hand, competition may also stimulate adaptive radiation (*3*, *7*–*9*). In natural ecosystems, biodiversity hotspots function as cradles for new species (*10*), suggesting that speciation rates may increase with species richness (that is, that biodiversity favors further diversification) (*11*). We propose that contrasting observations on the role of competition in evolutionary dynamics can be unified by investigating biodiversity-evolution relationships over a gradient of species richness and niche differentiation.

Here, we explored the evolutionary dynamics of a focal bacterial species growing in communities of different biodiversity levels, from one species (focal species competing with itself) to eight species of the genus *Pseudomonas*. This model system allowed us to explore different ecological scenarios at high functional and taxonomical resolution while drawing on a large pool of life history strategies (*12*). We manipulated the functional diversity (FD) of the background community using an index integrating both the number of competitors and niche partitioning (*13*). FD is commonly used as predictor of community functioning, and we expected that it may also predict evolutionary processes (*14*). For instance, FD is highest in species-rich communities where each species uses different subsets of the available niche space, which likely alters evolutionary diversification processes.

We specifically addressed whether higher biodiversity promotes or restricts evolutionary diversification in the focal species. We focused on the first step of evolutionary processes, the generation of mutants harboring altered phenotypes. We expected that biodiversity affects evolutionary dynamics by altering the establishment and maintenance of evolved phenotypes. High biodiversity is often associated with increased resource competition (*15*), yet, at the same time, many species may compete for few shared niches, leaving alternative niches underexploited (*16*). We therefore expected that novel phenotypes are more likely to be established at high biodiversity because of the novel phenotypes escaping competition by using underexploited resources more efficiently (*17*).

We used the XerD site-specific recombinase as a model molecular mechanism generating mutations (*18*). Site-specific recombinases generate important mutation steps by reshuffling the genome. They rapidly generate new phenotypes and play an essential role for survival in new or competitive environments (*19*–*21*). Although this enzyme is not the only mechanism generating mutations, its deactivation slows down evolutionary rates at the tested time scale (*19*). We therefore used a functional mutant lacking XerD as a reference to estimate the fitness gain conferred by the evolution of new phenotypes under various conditions. First, we addressed whether high biodiversity accelerates diversification and characterized the phenotypic changes occurring during the diversification process. Second, we assessed whether and when the novel phenotypes take over the ancestral phenotype, considering the

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spread of novel phenotypes as an indicator for the pressure on the focal species to evolve new features. Finally, we characterized the ancestral strain and evolved phenotypes and tested whether evolved phenotypes occupy a different niche and consume different resources than the ancestral strain.

RESULTS

Fitness benefits of recombination-driven diversification

The percentage of the focal species at the end of the experiment decreased with FD ($F_{1,181}$ = 38.9, P < 0.001; Fig. 1A), suggesting that high niche preemption in diverse communities increased the competition for resources. However, this decrease varied with the genetic background of the focal species: The growth of the rec$^+$ strain exceeded that of the rec$^-$ mutant ($F_{1,181}$ = 25.6, P < 0.001 for the genetic background), suggesting that diversification conferred a fitness advantage under the studied experimental conditions. Further, the growth advantage of the wild type over the mutant depended on FD ($F_{1,92}$ = 54.6, P < 0.001 for the genetic background × FD interaction): At low FD, the two strains showed a similar fitness, whereas the growth advantage of the rec$^+$ strain increased with FD (up to 10-fold in the most functionally diverse communities) (Fig. 1B). The growth advantage of the rec$^+$ strain was correlated with the frequency of evolved phenotypes at the end of the experiment ($F_{1,92}$ = 8.4, P = 0.005), suggesting that adaptive radiation conferred a fitness gain under resource competition. Supporting this conclusion, the rec$^+$ strain reached higher density when grown in isolation than the rec$^-$ mutant at low resource availability, whereas the density of the two strains was similar at high nutrient availability, suggesting that diversification increased the exploitation of scarce resources (fig. S1).

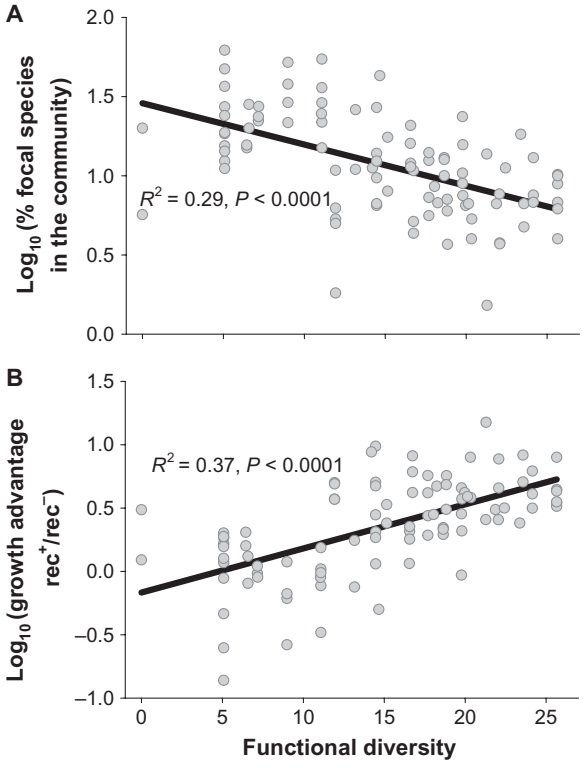

Fig. 1. Relationship between functional diversity (FD) and the growth of the focal species *P. fluorescens* F113. (**A**) Decline of the focal wild-type *P. fluorescens* F113 strain (rec$^+$) with increasing FD of the background bacterial community. (**B**) Effect of FD on the competitive advantage of the wild type (rec$^+$) focal species over its isogenic *xerD*$^-$ mutant (rec$^-$) impaired in site-specific recombination and showing slow evolutionary rates. Competitive advantage was defined as the ratio between the relative fitness of the rec$^+$ and rec$^-$ strains (compared to the background community). Each community was set up independently twice, with the rec$^+$ and rec$^-$ strain as focal species.

FD and the spread of novel phenotypes

The frequency of evolved morphotypes retrieved from the focal species at the end of the experiment increased with FD: When the focal species was competing with itself or in a community of low FD, the ancestral phenotype dominated. The extent to which the ancestral phenotype was replaced by the novel ones increased with increasing FD of the community (Fig. 2). No novel phenotypes were detected in the rec$^-$ strain (of 100 randomly picked colonies), confirming that site-specific recombination was a key mechanism for rapid evolution of new phenotypes at the tested experimental time scale (48 hours). The effect of FD on the frequency of novel phenotypes remained highly significant (P < 0.0001) even when fitted after the presence/absence of each single genotype, indicating that the observed patterns were not due to a sampling effect (that is, the inclusion of a particular genotype in the community) (*15*).

Resource use patterns

Focal species. Re-isolated phenotypes grouped well into discrete resource use clusters matching colony morphology (Fig. 3A), indicating that the morphotype reflected differences in resource use. Compared to the ancestral strain, the evolved phenotypes used the available resource spectrum more equitably (that is, were trophic generalists) ($F_{1,16}$ = 158.7, P < 0.0001 for the Shannon index of resource use; Fig. 3B). At the same time, their overall resource use was reduced (Fig. 3C), suggesting a trade-off between generalism and overall resource use. Further, the more a phenotype differed from the ancestral one (measured as multivariate Euclidian distance), the more generalistic was its resource use pattern but the lower its overall growth rate

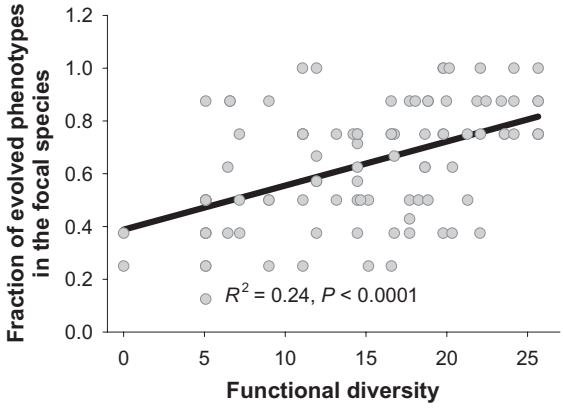

Fig. 2. Relationship between functional diversity (FD) and the diversification of the focal species *P. fluorescens* F113. Diversification was defined as the fraction of evolved phenotypes within the focal species after growth in communities of varying diversity for 48 hours. Evolved phenotypes were discriminated from the ancestral phenotype on the basis of colony morphology.

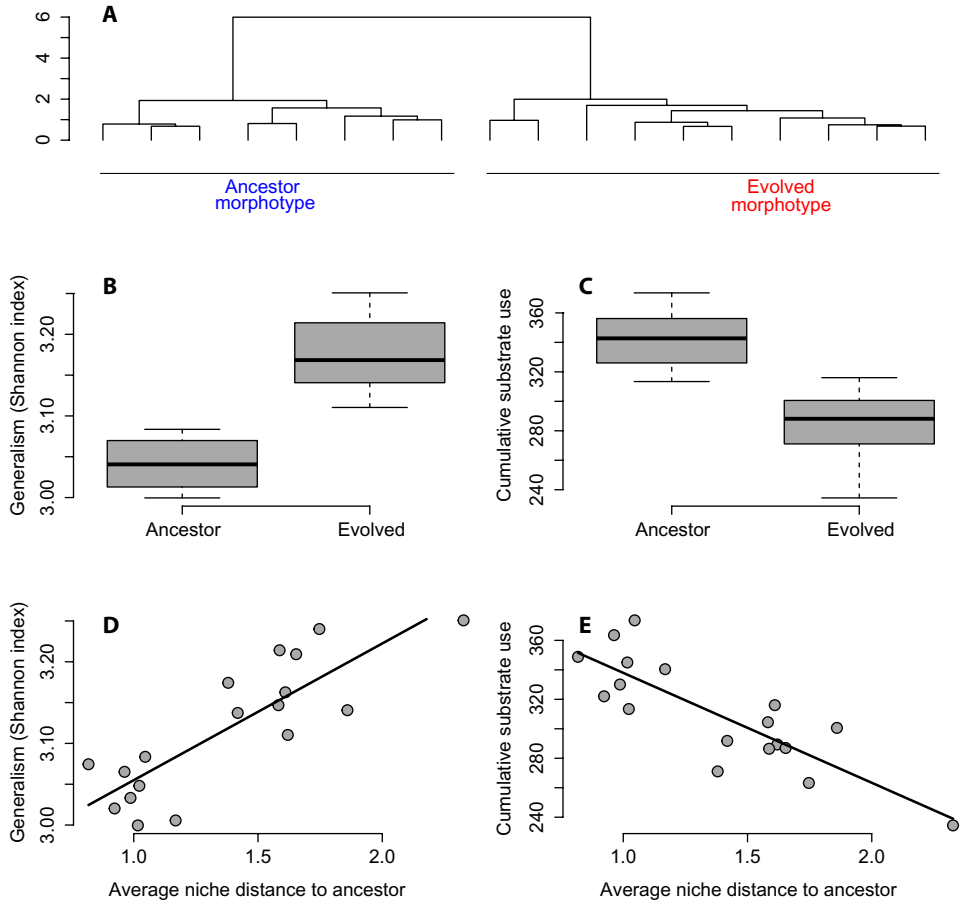

Fig. 3. Characterization of the ancestral and evolved morphotypes of the focal species at the end of the experiment. (**A**) Recovered colonies formed two metabolic profile clusters in line with colony morphology. (**B**) Evolved morphotypes showed a more generalist resource use pattern than the ancestral strain, suggesting niche expansion during the diversification process. (**C**) Cumulative resource use potential of the evolved morphotypes was reduced compared to the ancestral strain, suggesting that acquisition of a new niche was accompanied by negative effects on overall resource uptake and metabolism. (**D** and **E**) Morphotypes with resource use patterns more distant from the ancestral strain showed a more generalistic resource use pattern yet a reduced overall resource use.

(Fig. 3, D and E). This suggests that evolving to use a wider spectrum of resources comes at the cost of reduced fitness and competitive strength.

Background communities. Trophic links were skewed and followed a log-normal distribution (Fig. 4A). In addition, resource use patterns were not randomly distributed: All of the tested lineages competed for the same set of core resources while differentially using satellite resources. The associated differential resource preemption is illustrated by computing FD as a function of genotypic richness differentiating the two subsets of resources; increasing genotypic richness resulted in higher FD on satellite resources but not on core resources (Fig. 4B). This suggests that new phenotypes in a community increases competition for main resources, with their success in establishing themselves in the community being based on the exploitation of little used (satellite) resources.

DISCUSSION

The relationship between biodiversity and ecosystem functioning has been intensively investigated at ecological time scales (*22*), yet little is known on how biodiversity change affects ecosystems at evolutionary time scales. Biodiversity has been linked to both increases and decreases in diversification rates (*3*–*5*, *7*, *11*). The present study aimed to explore the mechanisms responsible for these variable outcomes by examining experimental communities of varying diversity in respect to adaptive radiation and changes in resource use. This reductionist approach allowed us to disentangle underlying mechanisms that are difficult to track in complex natural ecosystems. We manipulated resource competition by establishing a gradient in FD, including competition with clone mates as reference. We assessed the net impact of biodiversity on diversification as the fitness advantage conferred by the recombinase, allowing rapid manipulation of the genetic material and potential replacement of the ancestral focal species by novel phenotypes. Finally, we inspected changes in the resource use spectrum as a potential mechanism underlying biodiversity-diversification relationships.

High biodiversity stimulated the replacement of the ancestral phenotype of the focal species by novel ones, suggesting that novel phenotypes more efficiently exploited the given resource spectrum. Multispecies communities tend to exploit complex resources more intensively, leaving

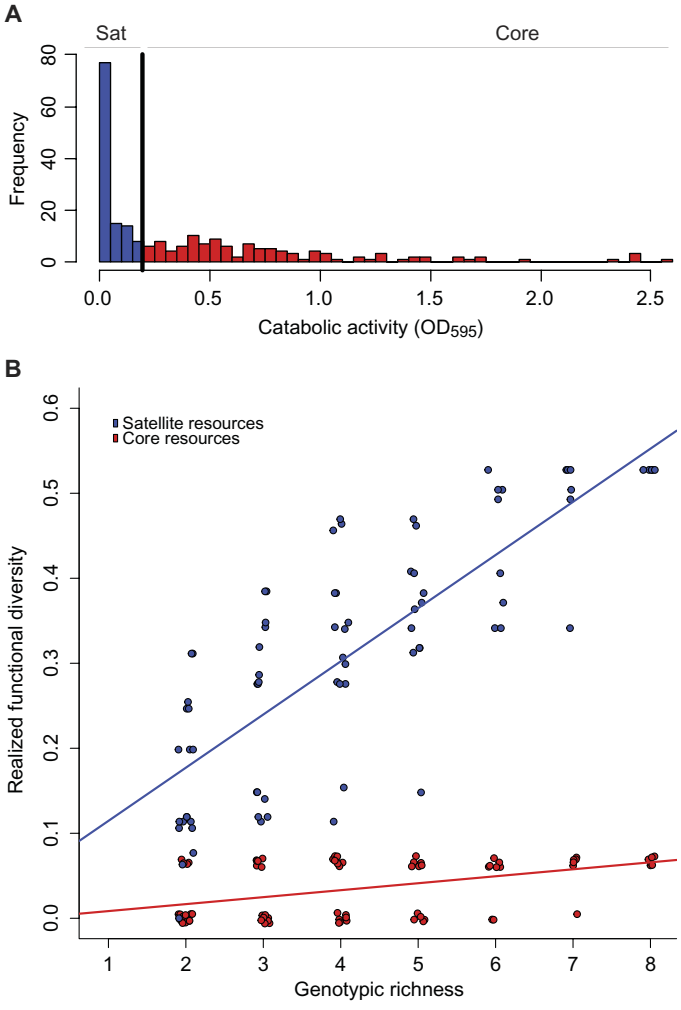

Fig. 4. Distribution of resource use patterns and consequences for the realized FD. (**A**) Most substrates were used at low rates by bacteria [satellite resources (Sat), catabolized less intensively than the median resource (left of vertical line), blue], and most catabolic activity was linked to a subset of resources [core resources, catabolized more intensively than the median (right of vertical line), red]. (**B**) Relationship between genotypic richness and FD on core resources (red) and satellite resources (blue).

few resources unused (*23*). This resource preemption can reduce the fitness of individual species as biodiversity increases (*15*, *24*). This matches well with our observations and suggests that the evolution of novel phenotypes allows for a better exploitation of additional resources, thereby escaping a tangled bank situation on the resources (*25*). However, high diversity may also result in a more complete use of resources, which may restrict diversification (*4*, *26*). Replacement of the ancestral phenotypes by newly evolved phenotypes in diverse communities suggests that the benefit of evolving to more efficiently use underexploited resources outweighs the disadvantages of increased completion in highly diverse communities. Notably, evolved phenotypes essentially gained the ability to better use resources consumed at a low rate by the ancestral phenotype rather than acquiring the ability to use novel resources. This ability may prove useful in escaping competition in many ecosystems, in which species compete for a common set of resources and niches (*16*). The decline of the focal species in species-rich communities was likely associated with increased resource competition. At the same time, high FD was associated with a fitness advantage of the wild type over the mutant impaired in XerD recombinase. This suggests that the fitness advantage is based on the ability to rapidly generate new phenotypes at high biodiversity because this allows the enhanced use of resources marginally used by the ancestral strain.

Diversification was limited by fitness trade-offs, providing an explanation for the low frequency of evolved phenotypes in the absence of competition. Fitness trade-offs are typical for radiation processes and contribute to species coexistence (*27*, *28*). In the present experiment, the more the evolved phenotypes differed from the ancestral phenotype, the lower their overall growth rate was. In the absence of competition, the evolved phenotypes could coexist with the ancestral phenotype; however, their lower growth rate kept them at low frequency. As diversity increases, competition reduces the growth of the ancestral strain, allowing the novel phenotypes to be established in the community using resources underexploited by the competing species.

In the present experiment, we focused on site-specific recombinases as a source of mutations. Their ability to excise, move, and invert large pieces of DNA within the genome (*29*) rapidly generates new phenotypes (*19*, *30*). Site-specific recombinases form the core of rapid adaptive processes in the rhizosphere (*19*, *21*) and in gut bacteria (*20*), as well as in pathogens (*31*). In the experimental system used, site-specific recombinases played an important role in short-term evolution; no novel phenotypes occurred in the mutant lacking XerD. However, this does not exclude the possibility that other mechanisms were involved in the observed pattern. Point mutations certainly also took place, but as they occur at a lower frequency, they are likely more important in long-term adaptive processes (*32*). Further, long-term experimental evolution experiments with varying biodiversity levels may help disentangle the relative contribution of different mutation mechanisms for bacterial evolution. Evolutionary and ecosystem processes have long been treated as distinct, but recent studies on grassland communities (*33*) and the present study suggest that it is time for unification.

The choice of simplified bacterial communities as a model allowed detailed insight into diversification processes and their underlying mechanisms. However, the simplified system and settings also have limitations. We focused on resource competition as the major driver of diversification processes, thereby ignoring other interactions that also drive evolutionary processes, such as predation and mutualism. We targeted trait variation in competing strains because this variation is essential to allow further evolutionary processes to take place, but the evolutionary trajectory will depend on the type of selective regime. Future studies that consider more complex interactions are needed to shed light on the relative importance of different types of interactions for diversification in microbial systems (*34*) and extrapolate findings to natural settings in the field. Further, we did not assess the ability of the evolved phenotypes to invade the communities when rare. This might be justified as we sampled evolved phenotypes after they had established themselves in the resident community, which necessarily involved invasion. However, more detailed studies are necessary to disentangle the frequency-dependent fitness of novel phenotypes in communities of varying diversity. Resource use of bacterial strains was based on the Biolog system, which is well established and provides a good proxy for predicting competitive interactions (*35*). However, this approach only represents a snapshot of the catabolic potential of bacteria ex situ (*28*), and its use in explaining growth kinetics in complex bacterial communities is limited (*36*).

Considering these limitations, we used the measurements to characterize bacterial phenotypes rather than their resource use in situ.

Our results suggest that competition for resources is a good predictor of diversification processes in bacterial communities. However, this does not preclude that other mechanisms were also involved in the observed patterns. For instance, changes in life history strategies may also have driven diversification processes (*31*). For instance, the lower growth rate of the evolved phenotypes may have been caused by a shift in the balance between growth and stress resistance (*37*). However, lower population growth of more generalist phenotypes indicated that the trade-off between population growth and the ability to use a wide spectrum of resources was involved in the observed diversification processes (*28*). Further, cross-feeding and niche construction may have contributed to the observed diversification processes (*38*, *39*). Extending the current study and including a wider range of traits involved in trophic and nontrophic interactions are promising perspectives in developing a more general eco-evolutionary theory (*40*).

Overall, our results suggest that biodiversity can stimulate the evolution of novel phenotypes, provided that (i) part of the resources get increasingly scarce at high diversity, reducing the fitness of the ancestral strain, and (ii) that evolved phenotypes gain access to underutilized resources. Here, we took single resources as different niche dimensions (*41*). We stressed that diversification processes increase at high species diversity; however, the relationship is most likely nonlinear. Presumably, at some point, the effect of diversity on diversification will saturate or even reverse as all resources are being consumed, resulting in a hump-shaped biodiversity-diversification function. The changes in colony morphology and resource use patterns were detected after growing the bacteria for at least 10 generations after the end of the experiment, indicating that the changes were inherited and simply not acclimation effects. Considering the exceptional diversity of natural bacterial communities, our experimental approach only explored the low diversity end of this relationship. However, because of the nested structure and compartmentalization of many food webs, where subsets of species are linked with subsets of resources only, we expect the positive biodiversity-diversification relationship to be important even at high species richness.

This study provides the first mechanistic explanation of biodiversity-diversification relationships in simplified communities, allowing a resolution that is very difficult to achieve in natural systems. Although caution should be exercised when transferring results from microcosm studies to real-world ecosystems, our results provide evidence that reduced biodiversity may compromise the ability of communities to respond in an evolutionary way to environmental changes. Thus, species loss not only may impair ecosystem functioning through short-term losses of functions but also can result in an "evolutionary debt" by slowing down the evolution of trait variation in the remaining organisms. Thus, species loss may prevent adaptations to changing environmental conditions that are needed to maintain ecosystem functioning in the long term.

MATERIALS AND METHODS

Focal species

We used *Pseudomonas fluorescens* F113 as the focal species. This strain, like other proteobacteria, rapidly diversifies into a discrete number of phenotypes that can be discriminated by their colony morphology, which is later referred to as morphotypes (*19*).

Manipulation of mutation rates

We used an isogenic mutant lacking the site-specific recombinase XerD, a DNA-manipulating enzyme that speeds up mutation rates. In contrast to the wild type, which rapidly diversifies into morphologically distinct phenotypes (*19*), the *xerD*-deficient mutant shows strongly reduced appearance of novel phenotypes. In pilot experiments, we did not find evolved phenotypes in this mutant at the time scale of the experiments used in this study. Therefore, we refer to it as nonrecombining ("rec^-"), in contrast to the recombining wild type ("rec^+"). We use the rec^- as a baseline to evaluate bacterial fitness when diversification is slowed down and the fitness difference between rec^- and rec^+ strains as proxy for the advantage of recombination-mediated diversification. Both strains carry a resistance gene to kanamycin, allowing separation from the background community.

Background communities

Total initial bacterial density was the same in each community. For each of the experiments, bacteria from a single colony were picked and grown in LB medium at 20°C for 12 hours and harvested in the early exponential phase to circumvent accumulation of mutations, which mainly occurs in the plateau phase of bacterial growth (*19*). Bacteria were washed three times with 0.85% NaCl and adjusted to an optical density (OD_{600}) of 0.5. The focal species did not contain newly evolved morphotypes at the beginning of the experiment (100 colonies screened).

Competition experiments

The rec^+ or rec^- strains of *P. fluorescens* F113 were added separately to each of the established communities at a constant frequency of 20% focal species and 80% of the mixture of other genotypes. Focal species abundance was chosen to reflect the average initial abundance of each species across the setup diversity gradient. Each community was set up twice, using the rec^+ or rec^- strain, respectively, resulting in a total of 190 communities (table S1). Note that the design includes one treatment, with the focal species competing with clone mates (F113 only) as reference.

Bacteria were grown in microtiter plates containing 150 µl of 1:5 diluted PGY broth (peptone, 2 g/liter; glucose, 2 g/liter; and yeast extract, 1 g/liter), a medium we previously used for experiments on biodiversity-ecosystem functioning relationships with the same bacteria (*42*). Plates were incubated with agitation (500 rpm) at 25°C. After 48 hours (late plateau phase), total bacteria and focal species were enumerated by serial dilution plating on LB plates with or without kanamycin (25 µg/ml).

Resource depletion experiments

To investigate resource limitation as a potential mechanism explaining the competitive advantage of the recombining strain, we simulated nutrient depletion by growing rec^+ and rec^- strains individually in a dilution series of PGY (total resources ranging from 5×10^{-3} to 5 g/liter). After 48-hour incubation at 25°C, the density of each strain was measured by dilution plating, and the advantage of recombination was expressed as density(rec^+)/density(rec^-).

Characterization of resource use patterns

For functional characterization of morphotypes, we used Biolog EcoPlates (Biolog Inc.). They allow characterization of the resource use spectrum of bacteria and have been shown to allow insight into competitive interactions at the experimental conditions used (*15*, *35*, *42*). However, as ex situ measurement on the basis of bacterial growth, Biolog profiles may not reliably reflect the in situ resource use of bacterial

strains and, therefore, have to be interpreted with caution (*36*). We measured substrate coloration (OD_{595}) at regular intervals for 60 hours and computed the area under the curve (AUC) for each isolate and substrate using the audpc() function in the R package "agricolae." We used AUC values as the index of resource use and took it to compute the FD of each community (weighted by the relative abundances of all genotypes present including the focal one) using the treedive function from the R package "vegan." FD encompasses both the number of species present and their ability to use different resources, allowing the estimation of (potential) resource competition patterns in each community (*13*).

Biolog resource use patterns were log-normal distributed, meaning that a few resources were used intensively, although most were only used at low rates. To account for this skewed distribution (see Results), we included an additional analysis: We divided resources into two subsets—"core" (upper 50% based on the median consumption across the eight genotypes) and "satellite" resources (lower 50%). This classification illustrates that few resources were used by all tested bacteria, whereas other resources were used by only certain bacterial strains at much lower rates. To assess niche complementarity in respect to these two subsets of resources, we recalculated FD indices separately for core and satellite resources.

Characterization of evolved and ancestral phenotypes in the focal species

We assessed the diversification of the focal species on the basis of morphotype abundance following an established colony morphology classification method developed by Sánchez-Contreras *et al.* (*43*). On the basis of the typology they proposed, we assigned colonies of type C (compact and opaque colonies with reduced swarming behavior) to the ancestral morphotype, as reported by the reference studies. In a pilot experiment, we showed that all colonies of the focal species were of type C at the moment of assembling the communities. Colonies of types F and S (diffuse colonies with enhanced swarming) were assigned to evolved morphotypes appearing when bacteria are grown for several days on the roots of plants and, to a lesser extent, in a liquid medium (*43*). We picked 10 colonies from the focal species from each treatment (1900 in total) and transferred them to soft TSA [tryptic soy broth (3 g/liter) and 0.5% agar], the low agar and nutrient concentration exacerbating differences in colony morphology. After 48-hour incubation at 25°C, morphotypes were categorized according to colony morphology into ancestral and evolved morphotypes following the classification described by Sánchez-Contreras *et al.* (*43*). To validate the value of morphotype classification as proxy for other traits, we randomly selected 18 colonies, recorded their morphotype, and measured their substrate utilization profiles with Biolog plates as described above. The whole characterization process lasted for at least 10 bacterial generations, suggesting that the observed changes were inherited and not merely transient metabolic shifts in response to competitors. On the basis of these measurements, we assessed whether evolved morphotypes differed in their resource use patterns from the ancestral phenotype. The sum of AUC values of all tested substrates was used to reflect the overall capability of bacterial strains to take up and metabolize resources.

Statistical analyses

Relative fitness (ω) of the focal species (rec^+ and rec^-) compared to the total community was determined on the basis of Malthusian growth parameters as $\omega = \ln(X_{final}/X_{initial})/\ln(Y_{final}/Y_{initial})$, where X and Y are the abundances (colony-forming units per milliliter) of the focal species and the remaining community, respectively. Values <1 reflect lower growth than the background community. The ratio of the relative fitness ($\omega_{rec+}/\omega_{rec-}$) was used as proxy to quantify the net benefit associated with rapid evolution in each community. Diversification was defined as the percentage of evolved morphotypes in the focal species as recovered at the end of the experiment.

The effect of FD on the focal species was analyzed with a linear model assuming log-normal (relative fitness) or negative binomial data distribution (percentage evolved phenotypes). To verify whether the observed effects were not due to sampling effect (that is, the inclusion of a particular strain in a given community), we fitted FD after each of the genotypes (presence/absence) using sequential GLM with type I sum of squares. Sampling effects were assumed not to be critical if the FD effect remained significant when fitted after single genotypes (*15*). All analyses were performed in R version 3.0.2 (R Core Development Team, Vienna, Austria).

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/2/6/e1600124/DC1

table S1. Assembly of communities of *P. fluorescens* of different phylogenetic diversity and FD for studying the evolution of *P. fluorescens* F113 and its $XerD^-$ isogenic mutant (focal species).

table S2. Summary of the communities setup.

fig. S1. Effect of resource concentration (dilution series of PGY medium) on the relative growth of *P. fluorescens* F113 (rec^+) over its *xerD* isogenic mutant (rec^-).

fig. S2. Maximum likelihood tree based on *phlD* sequences depicting the phylogenetic relationships between the eight studied *Pseudomonas* lineages.

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