THE EVOLUTION OF ECOLOGICAL INTERACTIONS DURING ADAPTIVE DIVERSIFICATION IN *PSEUDOMONAS AERUGINOSA*

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Abstract

Ecological opportunity—the availability of open niche space to an evolving lineage—has long been thought to modulate the extent of adaptive diversification. Many microbial evolution experiments have confirmed that ecological opportunity drives diversification of initially homogeneous populations into communities of ecologically distinct sub-lineages (ecotypes). Interactions among ecotypes are crucial for both community function and the maintenance of the ecological diversity produced during adaptive diversification, however the factors influencing the evolution of these interactions remain unexplored. We assessed the influence of ecological opportunity on this process by studying communities of the bacterium *Pseudomonas aeruginosa* that were evolved in either nutritionally complex (COM) or simple (SIM) environments. We measured the net ecological interactions in these communities by comparing the cellular productivity and competitive fitness of whole communities from each environment to that of their component isolates in both complex and simple media. On average, COM communities had both higher productivity and fitness than their component isolates in complex media, indicating that the components of these communities share net positive interactions. The same was not true of SIM communities, which did not differ in either measure from their component isolates. Follow-up experiments revealed that high fitness in two COM communities was driven by rare variants (frequency < 0.1%) that secrete compounds during growth which inhibit PA14, the strain used as a common competitor for fitness assays. Taken together, our results suggest that environments with high levels of ecological opportunity drive diversification into ecotypes that share net positive ecological interactions. The strong effect of diversity on productivity and fitness we found in newly diversified communities has a number of implications for evolutionary ecology as well as the treatment of *P. aeruginosa* infections.

On pense depuis longtemps que les opportunités écologiques, c’est-à-dire la disponibilité d`un espace de niche ouverte pour une lignée en évolution, modulent l’ampleur de la diversification adaptative. De nombreuses expériences d’évolution microbienne ont confirmé que l’opportunité écologique entraîne la diversification de populations initialement homogènes en communautés de sous-lignées écologiquement distinctes (écotypes). Les interactions entre écotypes sont cruciales à la fois pour la fonction de la communauté et pour le maintien de la diversité écologique produite au cours de la diversification adaptative, mais les facteurs influençant l'évolution de ces interactions restent inexplorés. Nous avons évalué l'influence de l'opportunité écologique sur ce processus en étudiant des communautés de la bactérie *Pseudomonas aeruginosa* qui ont évolué dans des environnements avec des nutriments complexes (COM) ou simples (SIM). Nous avons mesuré les interactions écologiques nettes dans ces communautés en comparent la productivité cellulaire et la compétitivité des communautés entières de chaque environnement à celles des isolats qui les composent dans des milieux simples ou complexes. En moyenne, les communautés COM avaient à la fois une productivité et une aptitude plus élevées que les isolats qui les composent dans des milieux complexes, ce qui indique que les composants de ces communautés partagent des interactions positives nettes. Il n'en va pas de même pour les communautés SIM, qui ne diffèrent pas dans des mesures des isolats qui les composent. Des expériences de suivi ont révélé que la valeur adaptative élevée dans deux communautés COM était due à des variantes rares (fréquence < 0,1 %) qui sécrètent des composés pendant la croissance qui inhibent PA14, la souche utilisée comme concurrent commun pour les essais d’aptitudes. Dans l'ensemble, nos résultats suggèrent que les environnements présentant des niveaux élevés d'opportunités écologiques favorisent la diversification vers des écotypes qui partagent des interactions écologiques positives nettes. Le fort effet de la diversité sur la

productivité et l'aptitude que nous avons trouvée dans les communautés nouvellement diversifient a un certain nombre d'implications pour l'écologie évolutive ainsi que pour le traitement des infections à *P. aeruginosa*.

Introduction

Life on Earth is incredibly diverse. Studies of eukaryotic diversity typically estimate that there are on the order of 2–100 million species on the planet (Mora et al., 2011; Costello, Wilson and Houlding, 2012; Larsen et al., 2017). A recent study including prokaryotes estimated the number of species to be between 1–6 billion, with the majority being single-celled (Larsen et al., 2017). Understanding how this vast diversity evolved has long obsessed evolutionary biologists, especially since the maintenance of diversity seems to conflict with natural selection. Natural selection is expected to purge diversity in evolving lineages, preserving only the fittest type in their environment. How then could populations have repeatedly diversified through time, producing the diversity of life we see today?

The ecological theory of adaptive diversification resolves the apparent conflict between natural selection and diversification (Schluter, 2000; Kassen and Rainey, 2004; Kassen, 2014). It states that phenotypic diversity emerges as a result of divergent natural selection of sub-lineages in different niches (Schluter, 2000; Kassen and Rainey, 2004; Kassen, 2014). Under this paradigm, diversity can emerge and be maintained because different genotypes have higher fitness under different environmental conditions. For example, morphologically distinct species pairs of freshwater sticklebacks (*Gasterosteus spp.*) occupy either littoral or benthic zones of recently formed coastal lakes in British Columbia, Canada, and each species is more efficient at foraging in their respective habitat relative to the other species (Schluter, 1993). Similarly, strains of the marine cyanobacteria, *Prochlorococcus spp.*, varying in their pigment content are favoured at different water depths where they encounter different light and temperature conditions (Moore, Rocap and Chisholm, 1998). This is often referred to as a genotype-by-environment (GxE) interaction in fitness because the most fit genotype depends on the environment in which fitness is measured (Kassen and Rainey, 2004). One of the central conditions of the ecological theory of diversification is that the extent of adaptive diversification of a lineage is governed by the number of open niches it encounters in its environment, which is referred to as ecological opportunity (Schluter, 2000; Losos and Mahler, 2010; Kassen, 2014).

Examples of rapid diversification in nature are broadly consistent with the ecological theory of adaptive diversification (Schluter, 2000). Unfortunately, natural diversification events often lack replication, controls, and complete evolutionary records of the groups involved, which makes robustly testing the ecological theory difficult. For example, the 14 extant species of Lack's finches diversified from a single progenitor that arrived on the Galapagos Islands 3 million years ago (Grant and Grant, 2008). Consistent with the ecological theory, these finches rapidly diversified in an environment replete with ecological opportunity resulting from a dearth of interspecific competitors in their new home (Grant and Grant, 2008). However, no clone of the ancestral finch landed on a separate, species-rich archipelago where interspecific competition would have been high, nor an archipelago devoid of the niches that today's finch species have evolved to fill. Was the diversification of finches in the Galapagos Islands an inevitable consequence of the environment they encountered, or a chance event that would not be replicated with more trials? It is impossible to say with confidence since it only happened once. Additionally, in the absence of a full fossil record, researchers are left to infer how diversification took place primarily based on the evidence that remains in the traits and genomes of extant species rather than being able to observe diversification as it happened. A true test of the ecological theory of adaptive diversification requires a system in which many replicate lineages evolved in environments differing in ecological opportunity and left behind a full evolutionary record, such that ancestral types can be understood in detail.

Microbial experimental evolution (MEE) has filled this gap. MEE is a method in which replicate populations of microbes are cultured in controlled environments for many generations, allowing researchers to study the process of evolution in these environments over time. The strains and culture conditions used in MEE often result in large population sizes and rapid generation times, which allows significant adaptation to occur within a short timeframe (Kassen, 2014; Lenski, 2017). Additionally, many microbial species can be stored indefinitely in suspended animation, allowing ancestral and evolved strains to be easily compared (Kassen, 2014; Lenski, 2017). Thus far, MEE studies have generally supported the ecological theory of adaptive diversification. For example, Rainey and Travisano (1998) found that populations of the bacterium *Pseudomonas fluorescens* rapidly diversified into three ecologically distinct sub-lineages (ecotypes) in unshaken microcosms. In this system, ecological opportunity was conferred by an oxygen gradient that developed between the air-surface interface and the bottom of the microcosm. Diversification was prevented when the oxygen gradient, and thus the ecological opportunity, was eliminated by shaking the microcosm (Rainey and Travisano, 1998). In another study, isolates from replicate populations of *P. fluorescens* that evolved for ~900 generations in media containing many carbon sources evolved a higher GxE interaction in fitness across carbon sources than isolates from populations evolved with fewer carbon sources (Barrett, MacLean and Bell, 2005). This suggested that the former populations diversified into ecotypes that were maintained as a result of specialization on different abiotic aspects of their growth medium (Barrett, MacLean and Bell, 2005). Several MEE studies have also demonstrated adaptive diversification resulting from organism-generated niches. For example, several studies have found that populations of *Escherichia coli* cultured in media containing glucose as the sole carbon source diversify into two ecotypes, one specialized on glucose and one specialized on the metabolic by-products excreted by its glucose-specialist neighbours (Helling, Vargas and Adams, 1987; Rosenzweig et al., 1994; Turner, Souza and Lenski, 1996; Treves, Manning and Adams, 1998; Rozen and Lenski, 2000). In this case, ecological opportunity is generated by closely related sub-types rather than abiotic variation, as in the previous examples. These studies demonstrate that ecological opportunity generated either by abiotic or biotic sources drives adaptive diversification in microbial populations, as predicted by the ecological theory.

Adaptive diversification produces communities composed of closely related ecotypes occupying different niches within the same environment. Biological communities are complex systems whose overall performance is defined by both the traits of community members individually and the ecological interactions they share (Loreau, 1998; Kirwan et al., 2009; Gorter, Manhart and Ackermann, 2020). Measures of community performance are diverse and include measures of biomass production, nutrient retention, and overall abundance (Bell, 1990, 1991; Tilman et al., 1997; Loreau et al., 2001). The interactions among community members may vary from mutually beneficial to mutually harmful, and the net sum of these interactions is responsible for the difference between the average performance of community members grown in monoculture and the performance of the community grown together (Kirwan et al., 2009; Gorter, Manhart and Ackermann, 2020). For example, resource partitioning—a positive interaction among community members resulting from divergent resource specialization—is thought to be mostly responsible for the positive relationship between species diversity and performance that has been found repeatedly in experimentally constructed communities (Tilman et al., 1997; Loreau and Hector, 2001; Cardinale et al., 2011; Thompson et al., 2018). Given the strong influence of ecological interactions on community performance, the evolution of ecological interactions during adaptive diversification should be of considerable interest to ecologists and agronomists.

Further, the evolution of ecological interactions during adaptive diversification is crucial to the maintenance of the ecological diversity produced during this process. For ecological diversity to be maintained, ecotypes must sufficiently reduce niche overlap; if niche overlap is not sufficiently reduced, any ecological diversity is expected to be only transient until a beneficial mutant occurs in the population and sweeps to fixation, eliminating diversity (Dykhuizen, 1990; Cohan, 2005). In the latter case, the function of whole communities is not expected to differ from that of their components grown alone because the components share the same niche and do not interact. Alternatively, if ecotypes evolve a sufficiently strong GxE interaction for fitness across different resources present in their environment (niche differentiation), a beneficial mutant that occurs in the genetic background of one sub-lineage cannot sweep to fixation and thus diversity can be maintained in the long run (Cohan, 2001; Kassen, 2002; Cohan, 2005; Good, Martis and Hallatschek, 2018). If ecotypes specialize on purely abiotic aspects of their environment, this should lead to resource partitioning among ecotypes, and thus communities that have higher performance than any of their components grown alone (Loreau and Hector, 2001; Cardinale, Palmer and Collins, 2002; Kirwan et al., 2009). Alternatively, if ecotypes evolve to occupy organism-constructed niches (as in the above examples of metabolic cross-feeding in *E. coli*), this should lead to facilitation among ecotypes, which is also expected to produce communities that have higher performance than their component types grown in monoculture (Cardinale, Palmer and Collins, 2002; Kirwan et al., 2009). Importantly, the positive effect of resource partitioning on community performance is only expected to persist in environments containing the abiotic resources on which ecotypes are specialized. Conversely, facilitation should be maintained in alternate environments since niches are provided by the community itself. Thus, comparing the performance of communities and their components across different environments can reveal a) whether the ecological diversity present in the community is truly adaptive (ie. whether ecotypes occupy different niches and thus share net positive interactions), and b) the relative contribution of resource partitioning and facilitation to the maintenance of ecological diversity within the community (ie. testing the extent to which higher performance of communities relative to their components depends on the environment in which it is measured). Despite the relevance of the evolution of ecological interactions to both community-level traits and the maintenance of ecological diversity, it remains poorly understood (Andrade-Domínguez et al., 2014).

The limited MEE work in this area has found that ecological diversification is generally accompanied by the evolution of net positive ecological interactions (Brockhurst et al., 2006; Poltak and Cooper, 2011; Ellis et al., 2015; Flynn et al., 2016). For example, Poltak and Cooper (2011) found that two replicate lines of the bacterium *Burkholderia cenocepacia* evolved in environments with high ecological opportunity conferred by spatial structure diversified into three colony morphotypes. Cultures of the three types grown together had higher biofilm productivity than any of the morphotypes grown in monoculture, demonstrating that the morphotypes shared predominantly positive interactions (Poltak and Cooper, 2011). Follow-up experiments showed that both spatial niche partitioning within biofilms (resource partitioning) and metabolic cross-feeding (facilitation) were responsible for the positive relationship between diversity and biofilm productivity in these communities (Poltak and Cooper, 2011; Ellis et al., 2015). Similarly, another study from the same group found that replicate populations of the bacterium *Pseudomonas aeruginosa* evolved in spatially structured microcosms diversified into several morphotypes that typically had higher biofilm productivity when grown in co-culture than in monoculture (Flynn et al., 2016). These studies are a useful first step, however they are insufficient to determine the influence of ecological opportunity on the evolution of interactions during adaptive diversification because interactions were only examined in lines evolved in environments with high levels of ecological opportunity. To accomplish this next step, a well-replicated comparison of the performance of communities and their components in microbial lines evolved in environments varying in ecological opportunity is required.

To fill this gap, we took advantage of lines of *P. aeruginosa* that were experimentally evolved in either nutritionally complex (COM) or nutritionally simple (SIM) environments for ~220 generations in a previous study (Schick and Kassen, 2018). COM environments consisted of a medium containing diverse carbon and nitrogen sources and thus conferred high abiotic ecological opportunity. Conversely, SIM environments consisted of minimal media supplemented with glucose as the sole carbon source and thus conferred little abiotic ecological opportunity. COM lines evolved higher within-community phenotypic variation than SIM lines, suggesting ecological opportunity drove diversification, although the adaptive significance of this variation was not examined in that study (Schick and Kassen, 2018). To examine the net effect of ecological interactions among the components of COM and SIM communities, we compared two complementary measures of performance in whole communities to the average performance of a sample of isolates taken from each. We first compared the cellular productivity of communities and their component isolates, as this measure closely resembles measures of performance used in previous studies of diversity–community performance relationships, both in microbes (Bell, 1990, 1991; Poltak and Cooper, 2011; Ellis et al., 2015; Flynn et al., 2016) and in plants (Tilman et al., 1997). Next, we compared the competitive fitness of communities and their component isolates. We chose to measure competitive fitness because it is directly relevant to the forces maintaining diversity in communities. One disadvantage of competitive fitness as a measure of community performance is that it may be peculiar to the strain used as a common competitor. This issue is unavoidable unless competitive fitness is averaged across many different common competitors. In keeping with standard practice in MEE studies, we used the ancestral strain of the evolution experiment as the common competitor in this study. This is the most sensible choice because, depending on the rate of adaptation in the evolving lines, the ancestral genotype should be either still present or only recently outcompeted in the evolved communities and thus highly relevant to the dynamics of competition therein.

We found that the cellular productivity and competitive fitness of COM communities, but not SIM communities, was higher than that of their component isolates, suggesting that the COM environment drove the evolution of net positive interactions. A follow-up experiment revealed that high competitive fitness in certain COM communities was driven by hidden diversity not picked up in our isolate samples. This led us to investigate how competitive fitness varied across a wide gradient of diversity in two COM communities, which showed that high fitness in these lines was driven by rare variants (RVs) present at frequencies lower than 0.1%. A subsequent spent media assay revealed that RVs cause high competitive fitness by secreting molecules into the media during growth that inhibit the growth of the ancestral strain, which was used as a common competitor in competitive fitness assays in this study. Overall, our results indicate that ecological opportunity drives adaptive diversification into ecotypes that share positive interactions for two measures of performance.

Methods

Experimental evolution and generation of isolate library and isolate mixes

The origin of the evolved communities is described in detail in Schick and Kassen (2018). Briefly, 60 replicate lines of *P. aeruginosa* strain PA14 were propagated at 37°C in either COM or SIM environments, with 30 lines in each environment. COM environments consisted of synthetic cystic fibrosis media (SCFM), which is a defined media composed of the essential chemical elements of sputum from cystic fibrosis patients (Palmer, Aye and Whiteley, 2007). The SIM environment consisted of M9 media (7.78 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl, 493 mg/L $MgSO_4$, 14.7 mg/L $CaCl_2$) supplemented with 4 g/L of glucose as the sole carbon source (MIN media). Half of the founding strains were marked with the *lacZ* gene such that they could be distinguished unmarked strains on agar plates supplemented with x-galactosidase (x-gal). Populations were propagated in 1.5 mL batch cultures with daily serial transfers of 1% of the previous day's culture into fresh media in unshaken 24-well cell culture plates (Costar® 24-well Clear TC-treated Multiple Well Plates). Microcosms were left unshaken to promote spatial heterogeneity in environmental conditions, which may in turn promote diversification (Rainey and Travisano, 1998; Habets et al., 2006; Martin et al., 2016). Samples from each population were stored every four days in 20% glycerol at –80°C over the course of the 32-day experiment (~220 generations). Unless otherwise stated, bacterial cultures in this study were grown in unshaken 1.5 mL microcosms at 37°C in 24-well culture plates.

To generate working stocks of 8 randomly selected lines from each environment, we inoculated Lysogeny Broth (LB) microcosms with a small volume of frozen stock from the stored endpoint lines from Schick and Kassen (2018). We allowed the microcosms to grow for 24 hrs before storing the cultures in 20% glycerol at –80°C. We generated a library of isolates from the working stocks of each line by plating a diluted sample from the frozen culture onto a MIN agar plate (MIN media + 1.3% agar (w/v); Oxoid Bacteriological Agar) at a density that gives ~100 colonies. From each plate, we randomly selected 22 colonies and inoculated them into LB microcosms which grew for 24 hrs before they were stored in 20% glycerol at –80°C. We also generated equal mixes of all 22 isolates (henceforth referred to as "isolate mixes") from each of the 8 examined COM lines by first growing all 22 isolates from each line in individual LB microcosms for 24 hrs. Then, for each line, we combined 45.5 μL from each of the 22 cultures into one 1.5 mL Eppendorf tube and stored these mixes in 20% glycerol at –80°C.

Cellular productivity assay

We revived evolved communities and isolates by transferring a small volume of frozen stock from each of the stored cultures into microcosms filled with either LB or MIN media. We used LB instead of SCFM to represent a complex environment for this assay and the *Competitive fitness assay* (below) for its ease of preparation. Like SCFM, LB is a complex media with many carbon and nitrogen sources (Sezonov, Joseleau-Petit and D'Ari, 2007). MIN media was used to represent a simple environment in both assays. After 24 hrs of growth, we transferred 1% of the mixture into a fresh microcosm containing the same media for another 24-hr growth period. We then diluted the cultures in MIN media by a factor of 10^6 and plated 100 μL from each on MIN agar plates to ensure they would produce a countable number of colonies. After 48 hrs of growth at 37°C, we counted the number of colonies on each plate, which we used as our measure of cellular productivity. We measured the productivity of all 22 isolates from each line in each environment without replication. Isolates were measured without replication because we chose to maximize the diversity of isolate measured in lieu of making repeated measurements on individual isolates. We measure the productivity of each community in each environment with 8-fold replication.

Competitive fitness assay

As above, we revived evolved communities and isolates as well as the ancestral strain from frozen stocks in fresh LB or MIN microcosms and allowed them to grow for 24 hrs. We then generated co-cultures by mixing an equal volume of each evolved strain with the oppositely marked ancestral strain, which acted as a common competitor in this experiment (*lacZ* marked ancestral strain for unmarked evolved strains and vice-versa), by adding 50 µL of each evolved and ancestral bacterial into 900 µL of the same media in 1 mL microcosms. Next, we diluted these mixtures into additional fresh 1 mL microcosms containing the same media at a 5-fold dilution factor and allowed these co-cultures to grow together for a 24-hr period, resulting in ~5.6 generations of growth in the mix. Before and after the co-culture period, we diluted and plated a sample of each mixed culture on MIN agar plates with x-gal (effective dilution factor 10^6). We recorded the number of marked and unmarked colonies at both time points (*lacZ*-marked colonies appear blue on agar containing x-gal), and used these counts to calculate fitness with the following formula:

$$s = \frac{\ln\left(\frac{n1_f}{n1_i}\right) - \ln\left(\frac{n2_f}{n2_i}\right)}{No.\ Generations} \qquad (1)$$

Where s represents the selection coefficient, $n1_f$ and $n1_i$ represent the number of colonies of the focal bacteria at the final and initial timepoint, and $n2_f$ and $n2_i$ represent the number of colonies of the competitor bacteria at the final and initial timepoint (Dykhuizen and Hartl, 1983; Melnyk, Wong and Kassen, 2015). The selection coefficient reflects the natural logarithm of the geometric growth rate of the ratio between focal bacteria and the competitor per generation. If the selection coefficient is 1, this reflects an e-fold increase in the ratio between focal and competitor bacteria each generation.

We measured the fitness of 22 isolates in LB and 11 isolates in MIN media from each of the 16 lines without replication. We measured the fitness of communities with 8-fold replication in LB and 4-fold replication in MIN. We used fewer replicates in MIN because this environment tended to yield less variable fitness values.

To investigate whether the diversity captured in our 22 isolate samples were representative of the diversity contained in six focal COM lines (6.1, 6.2, 6.8, 6.12, 6.15, 6.17), we compared the fitness of communities and isolate mixes from each line in LB, with the fitness being measured as above. Community and isolate mix fitness measurements were replicated four times.

Dilution-diversity experiment

We designed this method based on similar experiments with natural microbial communities (Wertz et al., 2006, 2007; van Elsas et al., 2012) in order to understand the relationship between diversity and fitness in two focal COM communities (6.1 and 6.2). We revived these communities by transferring a small volume of frozen stock into LB microcosms, as described above. Following 24 hrs of growth, we ran a 10-fold dilution series on these cultures using the wells of a 24-well cell culture plate, diluting with fresh LB and generating a dilution gradient that varied in density from ~3×10^9 to ~3×10^2 CFU/mL. We then took a 150 µL sample from each of the final six wells of the dilution series, which varied in density from ~3×10^7 to ~3×10^2 CFU/mL. As such, the densest sample contained ~$4.5\text{x}10^6$ cells and the least dense sample contained ~45 cells. These density estimates are based on the cellular density of COM communities following 24 hrs of growth in LB (see Supplementary Figure 1A). We then allowed the community samples to grow for 24 hrs before storing them in 20% glycerol at –80°C. Although the density of community samples varied across the dilution gradient, this growth period should have allowed them to reach similar densities before storage.

For each of the two focal lines, we performed three replicate dilution series from cultures grown independently from frozen stocks. Each replicate produced a series of six community samples varying over a wide range of diversity, since each was produced by a progressively smaller bottleneck of the original community. We measured the competitive fitness of each community sample as described above (see *Competitive fitness assay*) in duplicate, producing six total fitness measurements for each community at each dilution level. This allowed us to determine at what bottleneck size the competitive fitness of community samples changed from community-like (which is to say relatively high) to isolate mix-like (which is to say relatively low).

Sterile spent media assay

We performed a sterile spent media assay to investigate whether RVs conferred high fitness in two focal COM lines (6.1 and 6.2) by secreting molecules into the growth media that either facilitated the growth of other evolved genotypes and/or inhibited the growth of the ancestral strain. This assay was performed on community samples of both focal lines from the *Dilution-diversity experiment*. Specifically, we focussed on two community samples from each line that were taken from adjacent dilution levels in which the less dilute sample contained the RV (RV^+) and the more dilute sample was RV^-, as inferred by their fitness values in the *Dilution-diversity experiment*. We grew the RV^+ and RV^- samples from frozen stocks in LB microcosms for 6 hrs before centrifuging them in 1.5 mL Eppendorf tubes at 4000 rpm for 10 min. A 6-hr growth period was used to ensure the community samples would be in the exponential phase of growth when their spent media was collected. Following centrifugation, we transferred supernatant from each tube into fresh 1.5 mL Eppendorf tubes using sterile 3 mL syringes (BD) and 0.22 µm disposable syringe filters (Millex-GP), removing any remaining cells.

As we prepared the sterile spent media, we grew two evolved isolates from each focal line as well as PA14 for 24 hrs in LB microcosms. We then adjusted the optical density (OD_{600}) of these cultures to 0.1 by diluting them in LB at dilution factors proportional to their initial density readings. Next, we added the density-adjusted cultures into one of three growth treatments with 5-fold replication: RV^+ supernatant, RV^- supernatant, or no supernatant control (2 focal lines × 3 growth treatments × 3 focal lines × 5 replicates = 90 total growth curve replicates). The RV^+ and RV^- supernatant treatments consisted of 120 µL of 1.5× LB, 60 µL supernatant (either RV^+ or RV^-), and 20 µL of the density-adjusted bacterial cultures. The no supernatant control consisted of 180 µL of LB and 20 µL of the density-adjusted bacterial cultures. 1.5× LB was used in the former two treatments to ensure an equal density of nutrients was supplied from the growth media across all treatments. Once these ingredients were mixed in separate well of a 96-well plate (Corning 96-well Polypropylene Microplate), the plate was placed in a plate reader (BioTek PowerWave XS2 Microplate Spectrophotometer) which measured the optical density (OD_{600}) of each culture every 10 min over a 12-hr period. The plate reader incubated the cultures at 37°C and shook the plate prior to each reading in order to minimize the development of biofilms that may have interfered with density readings.

Four total growth curve replicates were lost due to contamination (one from line 6.2 isolate 1, two from 6.2 isolate 2, and one from PA14 grown in RV^+ spent media from line 6.2).

Statistics

We used the statistical software R to generate all figures and fit all statistical models (R Core Team, 2020).

To test whether the effect of diversity on cellular productivity and fitness in evolved lines was influenced by the selective environment, we constructed separate linear mixed-effects models (LMMs) for each trait using the R package lme4 (Bates et al., 2015). Trait values (either cellular productivity or fitness) were coded as continuous response variables and selective environment (COM vs. SIM), diversity category (isolate vs. community), and the interaction between the two were coded as categorical predictor variables. A unique ID was assigned to each evolved line (Line ID) and was added as a random effect in each model to control for the variation in the trait value associated with each of the 16 evolved lines. The interaction between selective environment and diversity category was the term of interest to test our statistical hypothesis (ie. does the influence of diversity on the trait in question differ among selective environments?). In the productivity model, the response variable was log-transformed to ensure homoscedasticity of the model's residuals.

We constructed similar LMMs to test whether the effect of diversity on cellular productivity and fitness depended on the environment in which the traits were measured. We ran these models separately for COM and SIM lines and had trait values coded as continuous response variables. The environment in which the trait was measured (LB vs. MIN), the diversity category, and the interaction between the two were coded as categorical predictor variables and Line ID was again added as a random effect. Similar to the models described above, the interaction term was the factor of interest for testing the statistical hypothesis (ie. does the influence of diversity on the trait in question differ among test environment?). As above, productivity values were log-transformed in their respective models.

We used Welch's Two Sample t-tests to test whether the fitness of six COM communities differed from that of mixes of 22 isolates taken from those communities. To control for the elevated chance of Type I error resulting from multiple comparisons, we lowered the critical α-value used to gauge statistical significance from 0.05 to 0.0083 (Bonferroni correction; six t-tests).

To test whether the growth of evolved isolates and the ancestral strain was affected by sterilized spent media from RV^+ and RV^- community samples, we first modeled the growth of each replicate culture using two-parameter logistic growth models fit via non-linear least squares using the *nmlrt* package (Nash, 2016). We then extracted the carrying capacity (K) estimates from each curve. One extremely high estimate of K was corrected to the highest reasonable OD_{600} value of 2. Next, we constructed six separate simple linear models with K as a continuous response variable and spent media condition (levels: RV^+ spent media, RV^- spent media, control) as a categorical predictor for each relevant combination of evolved line and focal bacteria (evolved isolate 1, evolved isolate 2, and PA14 for each of the two COM lines examined). We ran separate ANOVAs on the models to test in each case whether spent media condition explained a significant proportion of variation in K. In the cases in which the effect of spent media condition was significant, we proceeded to run post-hoc Tukey tests to determine which spent media treatments led to different average K-values. As above, we lowered the critical α-value used to gauge statistical significance from 0.05 to 0.0083 to account for multiple comparisons (Bonferroni correction; six possible post-hoc tests).

Results

Cellular productivity increases with diversity in COM lines but not SIM lines

To explore how the extent of abiotic ecological opportunity influenced the evolution of ecological interactions during diversification, we first compared the cellular productivity of COM- and SIM-evolved communities to their component isolates in both selective environments. As expected, the effect of diversity on cellular productivity was greater in COM lines than SIM lines when measured in their respective selective environments (Figure 1; LMM, selective environment:diversity category interaction, $t = 3.41$, $P < 0.001$). In COM lines, community cellular productivity was an average of 27.5% higher than isolate cellular productivity in complex media (Figure 1A; emmeans pairwise contrast, est. [95% CI]: 27.5%

[14.3–42.2%], $t_{460} = 4.36$, $P < 0.001$), but SIM community and isolate cellular productivity did not differ when measured in simple media (Figure 1B; emmeans pairwise contrast, est. [95% CI]: −2.5% [−12.6–8.7%], $t_{460} = -0.45$, $P = 0.97$). The positive effect of diversity on cellular productivity among COM lines was dependent on the test environment (LMM, test environment:diversity category interaction, $t = 3.87$, $P < 0.001$), as we did not detect a difference between community and isolate cellular productivity when COM lines were measured in simple media (Figure 2A; emmeans pairwise contrast, est. [95% CI]: −9.5% [−20.0–2.3%], $t_{467} = -1.60$, $P = 0.38$). Conversely, the relationship between diversity and cellular productivity did not differ among SIM lines across test environments (LMM, test environment:diversity category interaction, $t = 0.10$, $P = 0.92$), as community and isolate cellular productivity also did not differ in SIM lines when measured in complex media (Figure 2B; emmeans pairwise contrast, est. [95% CI]: −1.7% [−12.5–10.5%], $t_{468} = -0.29$, $P = 0.99$).

Notably, the productivity of COM communities, which evolved for 220 generation in complex media, was 62.8% lower than that of the ancestral strain in this environment (Supplementary Figure 1A; Tukey post-hoc contrast, est. [95% CI]: 62.8% [52.4–73.1%], $P < 0.001$). In simple media, the productivity of SIM communities did not differ from the ancestral strain (Supplementary Figure 1B; Tukey post-hoc contrast, $P = 0.23$).

Competitive fitness increases with diversity in COM lines but not SIM lines

We next compared the competitive fitness relative to the ancestral strain of whole communities and isolates from COM- and SIM-evolved lines in both environments. In line with our expectations and the productivity results, we found that the effect of diversity on competitive fitness was greater among COM lines than SIM lines when measured in their selective environments (Figure 3; LMM, selective environment:diversity category interaction, $t = -5.36$, $P < 0.001$). Strikingly, the selection coefficient of COM communities was an average of 0.23 higher than that of COM isolates in complex media (Figure 3A; emmeans pairwise contrast, est. [95% CI]: 0.23 [0.18–0.28], $t_{313.2} = 9.17$, $P < 0.001$). Conversely, we did not detect a difference between the fitness of SIM communities and isolates when measured in simple media (Figure 3B; emmeans pairwise contrast, est. [95% CI]: 0.01 [−0.05–0.07], $t_{310} = 0.28$, $P = 0.99$).

Like the pattern for productivity, the relationship between diversity and competitive fitness differed across test environments for COM lines (LMM, test environment:diversity category interaction, $t = -2.32$, $P = 0.020$). In this case COM communities had an even greater competitive fitness advantage over their component isolates in simple media than in complex media (Figure 4A; emmeans pairwise contrast, est. [95% CI]: 0.34 [0.26–0.42], $t_{284} = 8.32$, $P < 0.001$), suggesting the interactions driving high fitness do not require the nutritional diversity present in COM media. The relationship between diversity and fitness also differed across environments in SIM lines (LMM, test environment:diversity category interaction, $t = 4.13$, $P < 0.001$), which was driven by higher fitness of SIM communities relative to their component isolates in complex media (Figure 4B; emmeans pairwise contrast, est. [95% CI]: 0.15 [0.11–0.19], $t_{336} = 7.44$, $P < 0.001$).

High fitness in four COM communities is driven by hidden diversity

The strong influence of diversity on the competitive fitness of COM lines indicated that components of certain COM communities shared strong positive interactions. To test whether these interactions were among the genotypes we captured within our isolate library, we compared the fitness of COM communities to equal mixes of the 22 isolates taken from each. If the positive effect of diversity on fitness was driven by isolates picked up in our isolate library, communities and isolate mixes should have similar average fitness. We limited this test to the six lines in which community fitness exceeded isolate fitness in complex media (Figure 3A). We found that communities had higher fitness than isolate mixes in 4 out of 6 cases (Figure 5; t-tests, $P < 0.0005$), suggesting that high fitness in these communities was driven by variants not contained within the isolate mixes.

High competitive fitness in two COM communities is driven by extremely rare variants

We chose to more closely examine the relationship between diversity and competitive fitness in two COM lines (6.1 and 6.2) in which community fitness exceeded isolate mix fitness, suggesting high community fitness was driven by hidden diversity. We measured the fitness of samples from these communities that were produced by bottlenecks that varied in size across six orders of magnitude. We reasoned that samples produced from smaller bottlenecks (ie. higher dilution factors) contained lower levels of diversity. In both lines, we found that competitive fitness decreased dramatically in a stepwise manner at a threshold dilution factor (Figure 6). In line 6.1, fitness shifted from high to low in all three replicate dilution series between dilution factors 10^4 and 10^5, representing samples consisting of approximately 45,000 and 4,500 cells following the bottleneck, respectively (Figure 6a). In line 6.2, fitness shifted from high to low between dilution factors 10^4 and 10^5 in one replicate dilution series and between dilution factors 10^5 and 10^6 in the two others (between ~4,500 and ~450 cells samples; Figure 6b). These results strongly suggested that high community fitness in these two COM lines was driven by rare variants (RVs) that were diluted to extinction at higher dilution factors in this assay. Based on the estimated bottleneck size at which RVs appeared to be lost and assuming that a single RV cell passing through the bottleneck is sufficient to cause high fitness, we estimated that their frequency in each line is <0.1%.

Rare variants from two COM lines inhibit the growth of PA14

We next explored the mechanism by which RVs influence community fitness in COM lines 6.1 and 6.2. Since many microbial interactions are mediated by small molecules secreted into the media during growth (Hibbing et al., 2010), we tested whether the cell free spent media from RV^+ community samples increased the carrying capacity of two isolates taken from the same line (facilitation) and/or decreased the carrying capacity of the ancestral strain, which was used as the common competitor in competitive fitness assays (antagonism). Both of these non-mutually exclusive mechanisms could explain the strong influence of RVs on community fitness in these lines.

In both lines, the ancestral strain had a higher carrying capacity in pure LB than in LB treated with RV^+ spent media (Figure 7; Tukey post-hoc contrast, $P < 0.005$), but not in LB treated with RV^- spent media (Figure 7; Tukey post-hoc contrast, $P > 0.14$). This is consistent with the hypothesis that RVs contribute to high fitness by inhibiting the growth of the common competitor via molecules secreted into the media during growth. Unexpectedly, the carrying capacity of one isolate from line 6.1 and both isolates from line 6.2 was lower in both spent media treatments relative to pure LB (Figure 7a, 7d, and 7e; Tukey post-hoc contrast, $P < 0.001$). This suggests that, rather than facilitating their neighbouring genotypes, variants captured in both

RV^+ and RV^- community samples secrete compounds into the media during growth that inhibit common variants in their community.

Discussion

There is mounting evidence that ecological opportunity drives diversification via divergent natural selection, generating communities of ecologically distinct sub-lineages (Rainey and Travisano, 1998; Schluter, 2000; Barrett, MacLean and Bell, 2005; Habets et al., 2006; Kassen, 2009; Losos and Mahler, 2010; Poltak and Cooper, 2011; Kassen, 2014). However, the influence of ecological opportunity on the evolution of interactions among nascent ecotypes is poorly understood despite their importance for community function and the maintenance of ecological diversity. Here, we examined the evolution of ecological interactions in lines of *Pseudomonas aeruginosa* evolved in nutritionally complex or simple environments in two complementary ways. First, we compared the cellular productivity of evolved communities to their component isolates. We found COM communities are more productive on average than their component isolates in complex media, but the same is not true in SIM lines measured in simple media. Second, we compared the competitive fitness of evolved communities to their component isolates, finding that the fitness of COM communities, but not SIM communities, was much greater than that of their component isolates in their respective selective media. Further exploration of the relationship between diversity and competitive fitness in two COM lines revealed that high fitness was driven by rare variants present at frequencies lower than 0.1%. A subsequent sterile spent media assay demonstrated that rare variants influence community fitness by secreting molecules into the media during growth that strongly inhibit PA14, the strain used as a common competitor in competitive fitness assays. All in all, our results demonstrate that ecological opportunity drives diversification into ecotypes that share net positive interactions.

Our results suggest that resource partitioning contributes to the positive interactions among the components of COM communities, but that antagonism is the dominant interaction shaping the competitive fitness of microbial communities evolved in this environment. Additionally, we provide compelling evidence that the traits of simple microbial communities composed of recently diverged ecotypes can be strongly influenced by the presence of extremely rare variants, underscoring the importance of understanding the traits of microbial populations and communities in their entirety rather than from a small sample of isolates. Finally, our results have important implications for the ecological significance of phenotypic diversification during chronic infection of the lungs of individuals with cystic fibrosis by *P. aeruginosa*.

We found that evolution in nutritionally complex media produced communities that were both more productive and had higher fitness than their component isolates. These results are broadly consistent with the hypothesis that evolution in environments with high levels of ecological opportunity leads to niche differentiation of sub-lineages, generating communities with net positive interactions. The results of the cellular productivity assay suggest that resource partitioning specifically drove positive interactions among COM community members, as we found that the difference in cellular productivity between COM communities and isolates was not maintained when measured in simple media. This may have occurred because MIN media does not contain the resources on which COM community members are specialized, which would reduce the benefits of resource partitioning, and thus the benefit of diversity, on productivity. Similar results were found in another MEE study in which ecological opportunity was conferred through the addition of a polystyrene bead to growth media, which provided a substrate on which complex biofilms could form (Poltak and Cooper, 2011). The authors examined two lines that evolved independently in this environment in detail, finding a positive effect of diversity on productivity in both, which resulted in part from spatial partitioning within biofilms. In one line the beneficial effect of diversity on productivity was reduced when no bead was added to the media, presumably because this removed the biofilm niches in which ecotypes were specialized, although this pattern was not repeated in the other line (Poltak and Cooper, 2011). Taken together, our results along with those from Poltak and Cooper (2011) support the notion that ecological opportunity drives diversification into ecotypes that are more productive when grown together than in monoculture as a result of resource partitioning.

While resource partitioning appeared to drive higher productivity in COM lines relative to their component isolates, the same cannot be said for the competitive fitness. Unexpectedly, we found that the competitive fitness advantage of COM communities relative to their components was higher in simple media than in complex media. This suggests that the ecological interactions that led to high fitness of COM communities in complex media were not dependent on the specific resource profile of this environment and thus were unlikely to be the result of resource partitioning, at least for resources specific to the complex media. However, the competitive fitness results are consistent with the components of COM communities occupying organism-generated niches, leading to facilitation and, thus, positive interactions among ecotypes regardless of the resource profile of their environment. Subsequent results discussed below suggest that high fitness of COM communities was not driven by either of these mechanisms, but rather as an indirect result of antagonism between RVs and the ancestral strain.

Another unexpected result was that the fitness of SIM communities was higher than their component isolates in complex media. While this result may have been driven by positive interactions among ecotypes composing these communities, this is unlikely given that SIM communities did not have higher fitness than their components in their selective environment where those interactions would have evolved. A more likely driver of the positive relationship between diversity and competitive fitness among SIM lines in complex media is selection among genotypes during the competition assay itself. In this context, selection refers to an increase in the relative frequency of genotypes in SIM communities that have higher fitness than the average community member during growth in complex media (Bell, 1990, 1991; Loreau and Hector, 2001). It would not be surprising for this to occur in the non-selective environment, as populations are expected to contain greater variation in fitness in environments to which they have not adapted (Remold and Lenski, 2001; Fry and Heinsohn, 2002; Martin and Lenormand, 2006). Also consistent with selection driving this pattern is that the fitness of SIM communities in complex media never exceeded the fitness of its most fit genotype, suggesting that higher community fitness could be explained by the over-representation of genotypes with higher fitness (Figure 4B; Bell, 1990, 1991).

After finding that the high fitness of COM communities was driven by hidden diversity, we employed a new method to measure the competitive fitness of samples taken from two COM lines across a gradient of bottleneck sizes. The *Dilution-diversity experiment* revealed that competitive fitness decreases in a stepwise manner at a threshold dilution level, strongly suggesting that high community fitness in these lines is driven by rare variants that were stochastically diluted out in smaller community samples. Based on the dilution level at which competitive fitness decreased in this experiment, we inferred that the frequency of the RVs was < 0.1% in both lines (Figure 6). These results demonstrate that the traits of communities composed of recently diverged ecotypes can be dramatically altered by the presence of extremely rare variants. The rarity of these variants makes them unlikely to be picked up in typical isolate libraries of experimentally evolved microbial populations or detected by typical deep sequencing techniques (Barrett, MacLean and Bell, 2005; Habets et al., 2006; Poltak and Cooper, 2011; Bailey and Kassen, 2012; Herron and Doebeli, 2013; Traverse et al., 2013; Flynn et al., 2016; Schick and Kassen, 2018), raising the possibility that ecologically important diversity may be missed in many MEE studies. This problem is paralleled by studies of natural microbial communities, which are replete with rare species, some of which have been shown to play important functional roles (Wertz et al., 2006, 2007; Pester et al., 2010; van Elsas et al., 2012; Jia, Dini-Andreote and Falcão Salles, 2018). The detection and characterization of ecologically and evolutionarily important rare variants remains a major challenge for microbial ecologists in both lab and field settings. Methods similar to the *Dilution-diversity experiment* (referred to as dilution-to-extinction in earlier studies of natural communities; Wertz et al., 2006, 2007; Van Elsas et al., 2012) show promise for studying the impact of rare variants on microbial community function.

Our sterile spent media assay revealed that RVs increased community fitness in two COM lines by inhibiting the growth of PA14, the ancestral strain and common competitor for the competitive fitness assays in this study (Figure 7). We did not investigate the molecular mechanism underpinning this antagonistic interaction, although this is of considerable interest given that *P. aeruginosa* is an opportunistic pathogen causing substantial morbidity and mortality among individuals with cystic fibrosis (CF) and is often recalcitrant to antibiotic treatment (Sousa and Pereira, 2014; Winstanley, O'Brien and Brockhurst, 2016; Pang et al., 2019). One clue into the mechanism is that it appears to be independent of nutritional complexity, given that COM communities had high fitness in both complex and simple media (Figure 3A; Figure 4A). Additionally, the mechanism must not be contact-dependent, since all cells were removed from RV$^+$ spent media before PA14 was inhibited in its presence. One possibility is that, in a mechanism similar to a recently reported antagonistic interaction between *P. aeruginosa* and *Staphylococcus aureus* (Jancheva and Böttcher, 2021), the RVs may be secreting a molecule causing the induction of prophages within the genome of PA14. Alternatively, RVs may kill PA14 through secretion of bacteriocins, which are highly specific proteinaceous toxins produced by many species of bacteria including *P. aeruginosa* (Riley and Wertz, 2002; Inglis et al., 2009). The specificity of killing by bacteriocins is consistent with our results as RV$^+$ spent media inhibited PA14 but not isolates descended from PA14 over a relatively short evolutionary period (~220 generations; Figure 7). Follow up work on our focal lines will examine the mechanism through which RV$^+$ sterile spent media inhibits the growth of PA14.

Regardless of the mechanism, our results suggest that antagonism was more likely to evolve in lines cultured in nutritionally complex media. The selective advantage of inhibiting PA14 seems clear; PA14 was the ancestral strain of the evolution experiment and thus mutants able to inhibit its growth may have had a relative fitness advantage early on during the evolution period. What remains puzzling is why similar selective pressures did not drive the evolution of antagonism in SIM communities, especially considering the benefits of antagonism appeared to be even greater in MIN media (Figure 4A). One possibility is that differences between the COM and SIM environments other than nutritional complexity drove the evolution of antagonism in COM communities. An environmental difference deserving further attention is the difference in density of PA14 supported in SCFM relative to MIN media. PA14 can grow to densities exceeding 5×10^9 CFU/mL in SCFM (Palmer, Aye and Whiteley, 2007), but our cellular productivity results suggest that PA14 grows to an average density of just 4.7×10^8 CFU/mL in MIN media (Supplementary Figure 1B). Evolving at higher densities may have contributed to the evolution of antagonism in two non-mutually exclusive ways. First, higher population sizes support a higher rare beneficial mutation supply rate (Burch and Chao, 1999; De Visser and Rozen, 2005), which may have made mutations causing antagonism more likely to occur in COM than SIM communities. Second, growth at higher densities in unshaken complex media seemed to support thicker biofilms at the air-media interface (Pers. Obs.). Microbial biofilms are highly structured environments in which bacteria are bound together by a carbohydrate-rich extracellular matrix (Davey and O’Toole, 2000). Previous work has demonstrated that the selective advantage of secreting antimicrobial compounds is enhanced by spatial structure because it allows locally high concentrations of inhibitory compounds to build up near producers (Chao and Levin, 1981; Nadell, Drescher and Foster, 2016). Thus, it is possible that the higher microbial density in SCFM supported greater biofilm formation which, in turn, promoted the evolution of antagonism by providing spatial structure. A future experiment should compare lines of PA14 evolved in SCFM or another complex media at varying nutrient densities to assess whether nutritional complexity or growth density drive the evolution of antagonism in this species.

P. aeruginosa isolates taken from the sputum of individuals with CF display remarkable phenotypic and genetic diversity (Mowat et al., 2011; Winstanley, O'Brien and Brockhurst, 2016; Feliziani et al., 2014). Within the lungs of a single patient, multiple *P. aeruginosa* sub-lineages can persist together for many years without any one type sweeping to fixation, hinting that sub-lineages may occupy different niches in the lung and thus be maintained by negative frequency dependent selection (Feliziani et al., 2014; Winstanley, O'Brien and Brockhurst, 2016). It has been suggested that the evolution of phenotypic diversity in *P. aeruginosa* communities during chronic infection in the lungs of CF patients may contribute to how difficult they are to eradicate by medical intervention (Sousa and Pereira, 2014). Consistent with within-lung diversification in *P. aeruginosa*, the COM lines used in our study evolved high levels of phenotypic diversity in media intended to mimic the nutritional composition of the CF lung (Schick and Kassen, 2018). We found that genotypes in these lines shared predominantly positive interactions, leading to higher population growth and fitness of evolved communities as a whole relative to their component types (Figure 1A, Figure 3A). If *P. aeruginosa* genotypes present in chronically infected CF lungs share similar positive interactions, this may explain why phenotypically diverse communities of this species are so difficult to treat. Future work examining the ecological interactions among clinical isolates of *P. aeruginosa* sampled simultaneously from single individuals would be extremely valuable in determining the ecological significance of phenotypic and genetic variation of this species during chronic infection in the CF lung.

Given the extensive phenotypic diversity of *P. aeruginosa* communities during chronic infection, it is now recognized that characterizing an individual's infection based on a single clinical isolate can be misleading (Winstanley, O’Brien and Brockhurst, 2016). This is because clinical isolates from a single individual routinely vary in many clinically relevant phenotypes (Workentine et al., 2013; Winstanley, O’Brien and Brockhurst, 2016). The present study suggests that even measuring phenotypes in a large sample of clinical isolates from a single individual may be misleading, since we show that the traits of communities composed of recently diverged *P. aeruginosa* ecotypes cannot necessarily be predicted from the traits of a handful of isolates measured in monoculture. While we only measured the effect of diversity on productivity and fitness, there is no reason to believe that the same pattern could not apply to clinically relevant phenotypes like biofilm production, toxin production, and even antimicrobial resistance. Clinically important phenotypes measured in mixtures of many isolates from a single infected individual may provide a more accurate picture of how *P. aeruginosa* communities are behaving within patients’ lungs, which may in turn make these measurements more predictive of disease prognosis than measurements of single isolates.

There has been some debate among ecologists on whether species within a community should be thought of as ecologically distinct or equivalent (Bell, 2001; Hubbell, 2001; Ricklefs, 2004). Proponents of the former model believe that patterns of species diversity are shaped by ecological processes like competition and that the maintenance of diversity in communities relies on niche differences among species (Bell, 2001). Others argue that patterns of species diversity can be entirely understood from purely neutral, stochastic processes (ecological drift) that do not require ecological differences between species (Bell, 2001; Hubbell, 2001; Ricklefs, 2004). While the latter model may sound counter-intuitive, neutral community models recapitulate many of the patterns of species diversity predicted by more familiar models that allow for ecological differences among species (Bell, 2001; Hubbell, 2001). Microbial evolution experiments including the present work that have examined the origin and maintenance of diversity starting from initially clonal populations can be thought of as models of community assembly by *in situ* mutation. Therefore, these MEE studies can be used to test the predictions of models that assume that ecological differences among species are important or unimportant to patterns of diversity. If ecological differences among species are important to the maintenance of diversity, phenotypic diversification and the stable maintenance of sub-lineages should be accompanied by ecological differentiation, which is expected to lead to net positive interactions among coexisting sub-lineages. Our work adds to long list of MEE studies which have refuted the neutral theory by finding that the maintenance of diversity in simple microbial communities is driven by ecological differentiation and the evolution of positive ecological interactions among sub-lineages (Helling, Vargas and Adams, 1987; Rosenzweig et al., 1994; Turner, Souza and Lenski, 1996; Rainey and Travisano, 1998; Rozen and Lenski, 2000; Barrett, MacLean and Bell, 2005; Poltak and Cooper, 2011; Flynn et al., 2016). These results underscore both the importance of ecological interactions among species in structuring ecological communities and the utility of MEE to test hypotheses related to community ecology.

One of the most consistent patterns in community ecology is that the performance of communities tends to increase with biodiversity, and this phenomenon is often attributed to niche differences among community members (Tilman et al., 1997; Loreau and Hector, 2001; Cardinale et al., 2011; Thompson et al., 2018). We show here for the first time that this relationship emerges naturally as initially clonal populations evolve in complex environments but not in simple environments. This suggests that lineages occupying simple environments do not diversify ecologically because there are few niches available to support diverse ecological strategies. Conversely, complex environments are able to support sub-lineages occupying different niches, leading to a positive relationship between diversity and community performance. The positive relationship between diversity and cellular productivity in COM communities seemed to be driven by resource specialization because it was only present when productivity was measured in complex media. Conversely, competitive fitness was much higher in COM communities than their component isolates across both complex and simple media, suggesting that facilitation among community members drove this pattern. However, high fitness in COM communities seemed to be driven primarily by antagonism between RVs and the common competitor, suggesting this pattern is peculiar to the competitor strain used to measure fitness and thus may not be generalizable to other systems. Nonetheless, our study provides compelling evidence that community assembly resulting from the diversification of initially clonal populations is highly dependent on the number niches available to the evolving lineage.

Conclusion

From Lack’s finches to Caribbean anoles to marine cyanobacteria, adaptive diversification has produced much of the diversity of life on Earth (Moore, Rocap and Chisholm, 1998; Schluter, 2000; Losos et al., 2006; Grant and Grant, 2008). This process is driven by divergent natural selection in environments with high levels of ecological opportunity (Schluter, 2000). Much remains poorly understood regarding how environmental conditions drive the evolution of interactions among nascent ecotypes during adaptive diversification, despite the importance of these interactions to the maintenance of ecological diversity and to globally significant ecosystem functions. For example, *Prochlorococcus spp.*, the most abundant photosynthetic organisms on Earth, are able to occupy a wide range of water depths as a result of adaptive diversification for different light and temperature levels (Moore, Rocap and Chisholm, 1998; Partensky, Hess and Vaulot, 1999; Biller et al., 2015). Here we provide the first study comparing net ecological interactions in communities generated by diversification in contrasting environmental conditions. We found that high levels of ecological opportunity conferred through nutritional complexity drove to the evolution of net positive ecological interactions during diversification in lines of *Pseudomonas aeruginosa*, leading to communities that had higher cellular productivity and competitive fitness than their component isolates. Our hope is that this study will spur on additional research on the factors influencing the evolution of ecological interactions among nascent ecotypes during adaptive diversification.

Figures

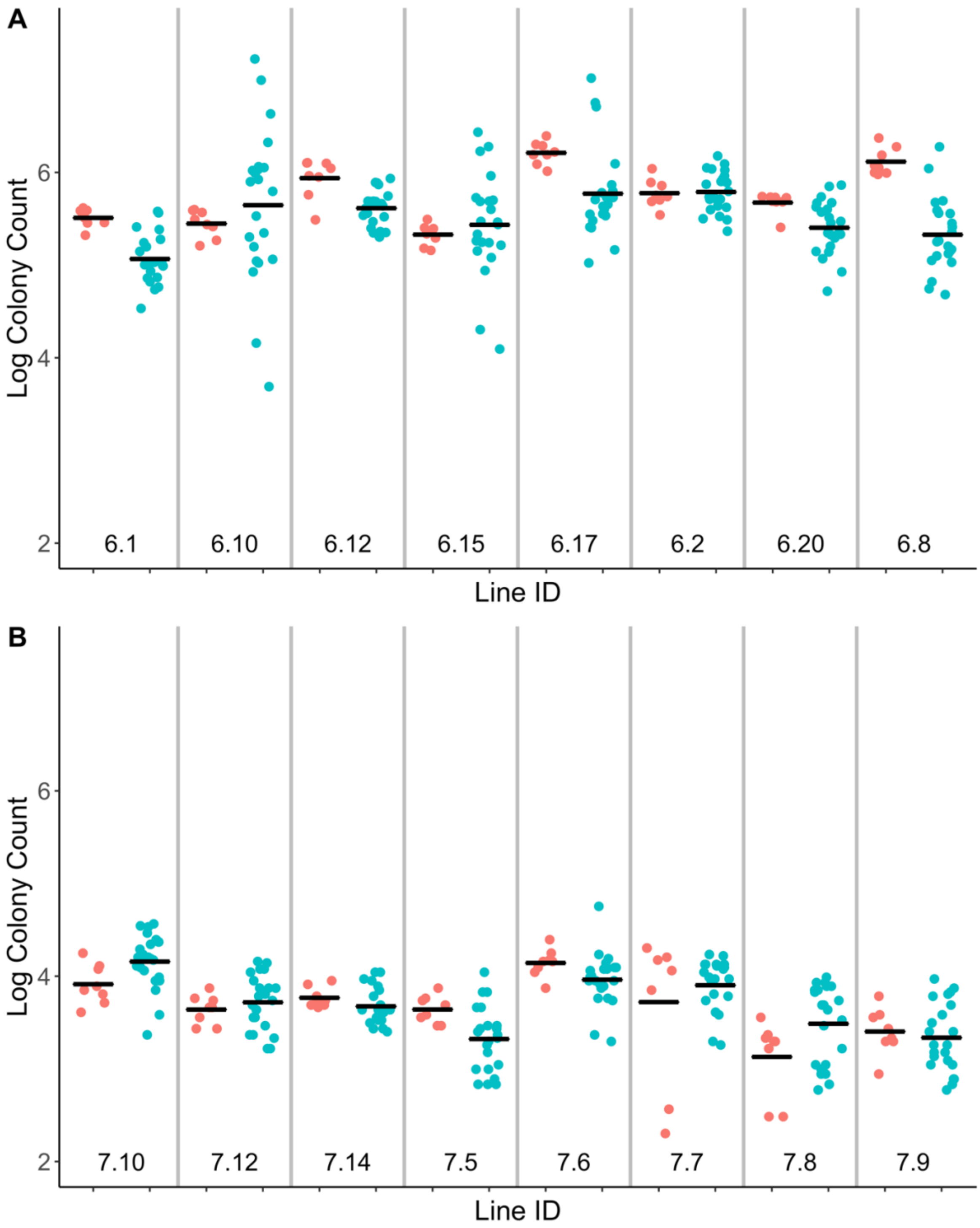


Figure 1: Cellular productivity of **A**. COM lines and **B**. SIM lines measured in their selective environment. Whole communities are shown in red and isolates taken from those communities are shown in blue. Black horizontal bars denote the mean cellular productivity for each group.

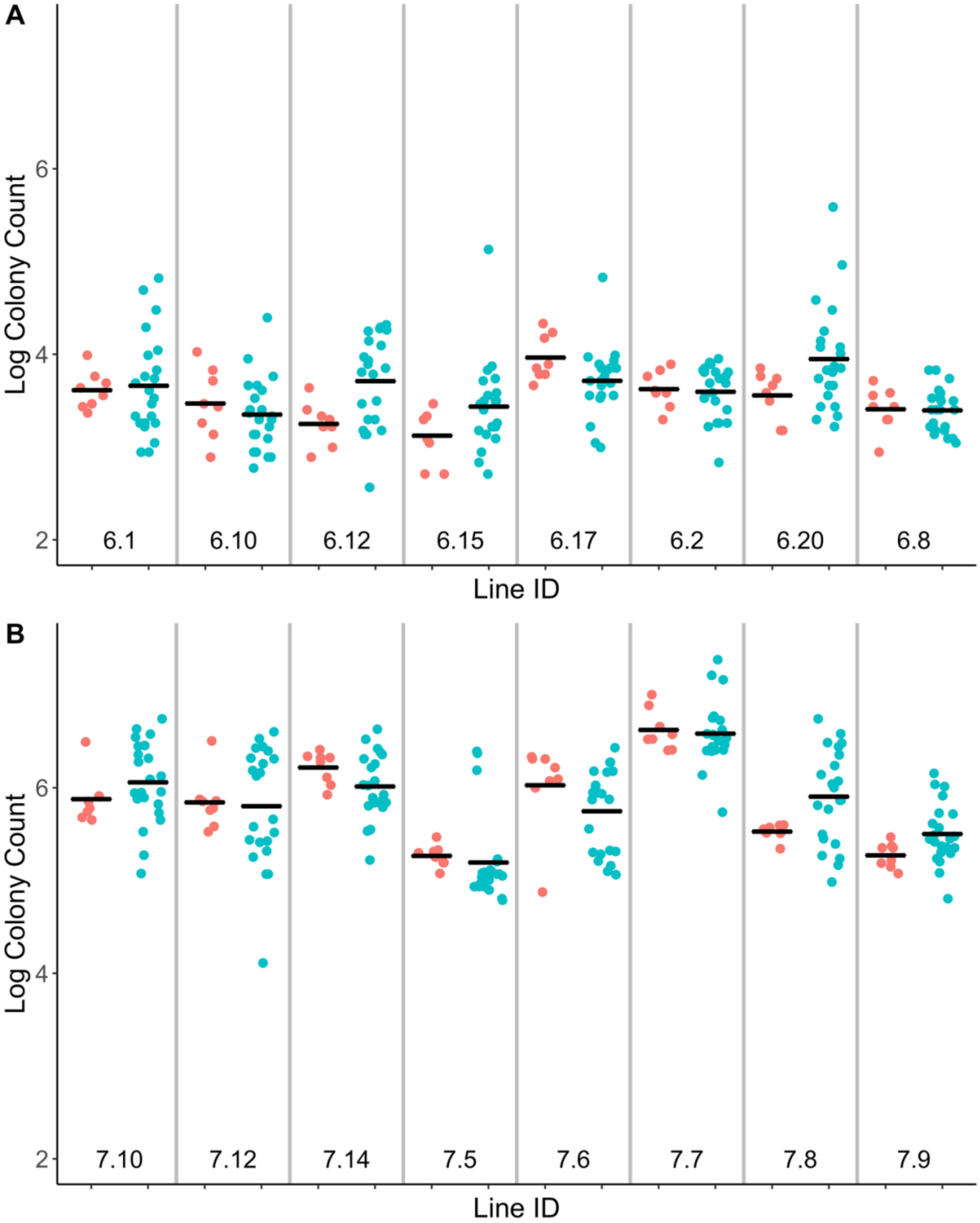


Figure 2: Cellular productivity of **A**. COM lines and **B**. SIM lines measured in their non-selective environment. Whole communities are shown in red and isolates taken from those communities are shown in blue. Black horizontal bars denote the mean cellular productivity for each group.

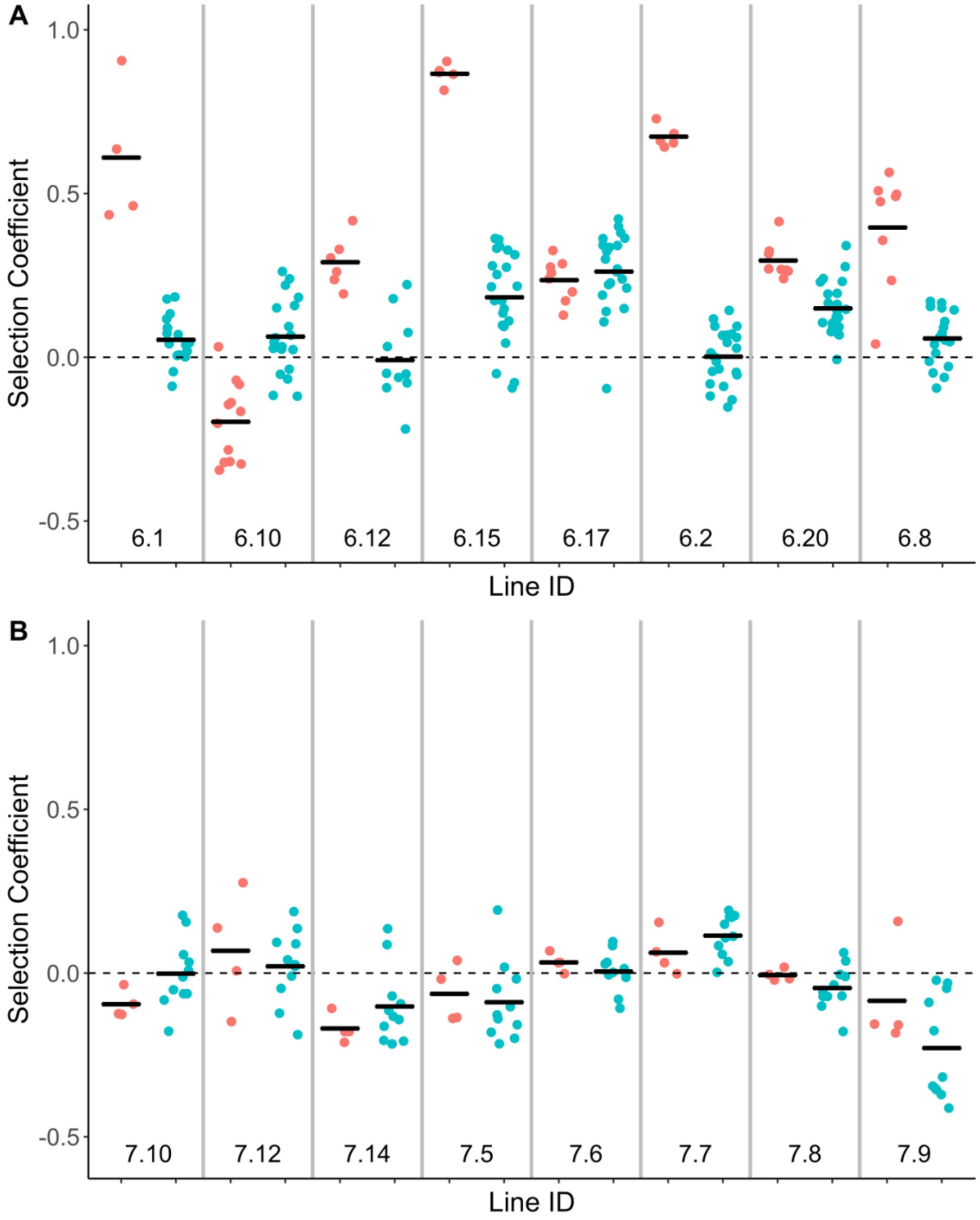


Figure 3: Competitive fitness of **A**. COM lines and **B**. SIM lines measured in their selective environment. Whole communities are shown in red and isolates taken from those communities are shown in blue. Black horizontal bars denote the mean competitive fitness for each group. The black dashed line denotes a competitive fitness value equal to the competitor strain.

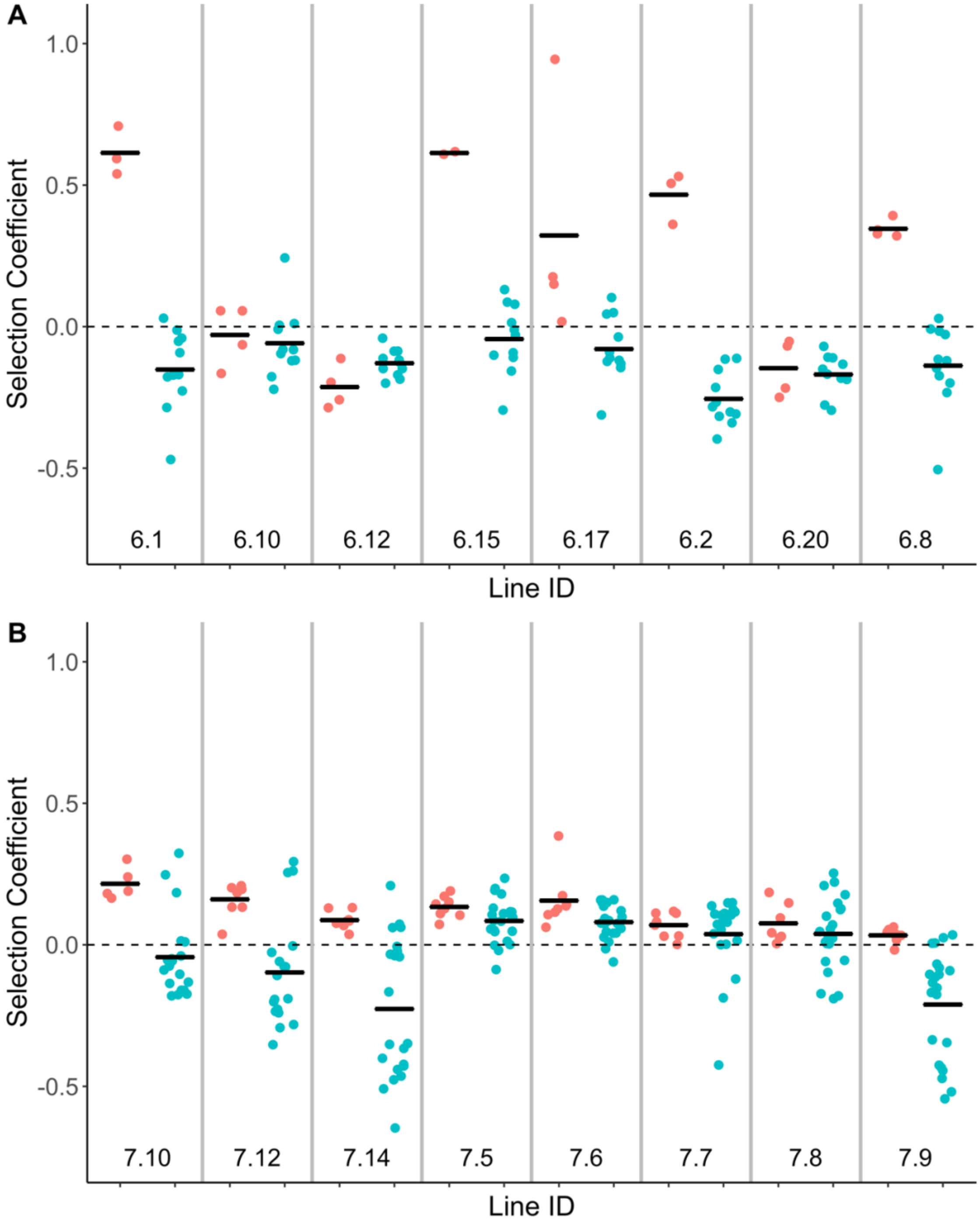


Figure 4: Competitive fitness of **A**. COM lines and **B**. SIM lines measured in their non-selective environment. Whole communities are shown in red and isolates taken from those communities are shown in blue. Black horizontal bars denote the mean competitive fitness for each group. The black dashed line denotes a competitive fitness value equal to the competitor strain.

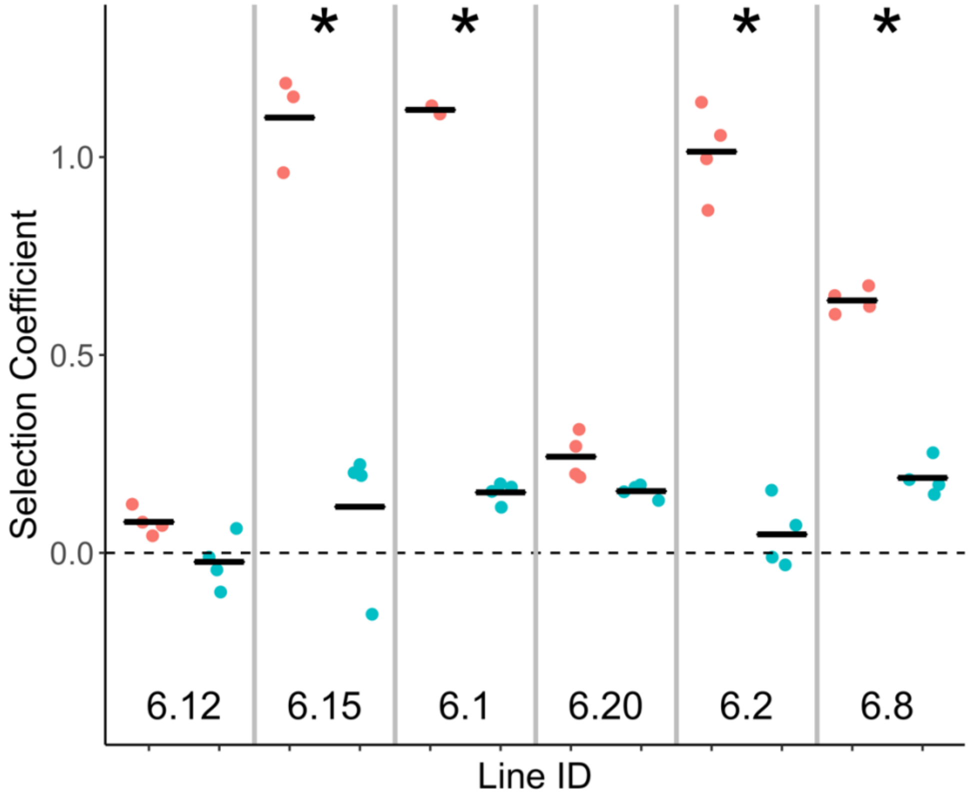


Figure 5: Competitive fitness of COM communities (red) and isolate mixes (blue) from six focal lines measured in complex media. Black horizontal bars denote the mean competitive fitness for each group. The black dashed line denotes a competitive fitness value equal to the competitor strain. * denotes a statistically significant difference in competitive fitness between communities and isolate mixes from a Welch's Two-Sample t-test (α = 0.0083).

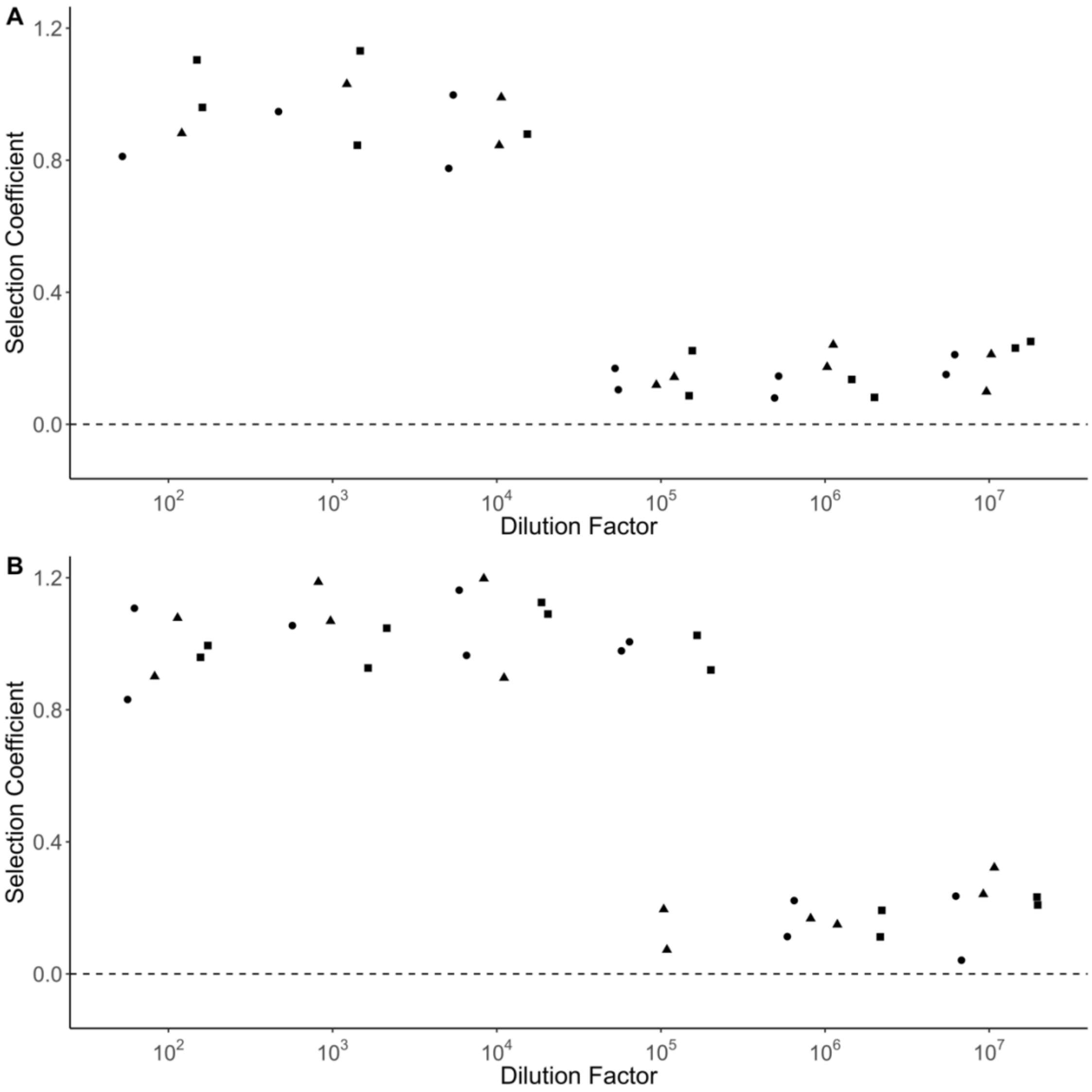


Figure 6: Competitive fitness of samples from **A**. COM line 6.1 and **B**. COM line 6.2 taken across a gradient of bottleneck size spanning six orders of magnitude. Shapes denote the replicate dilution series from which samples were taken. Each sample was measured in duplicate (both values shown). The black dashed line denotes a competitive fitness value equal to the competitor strain.

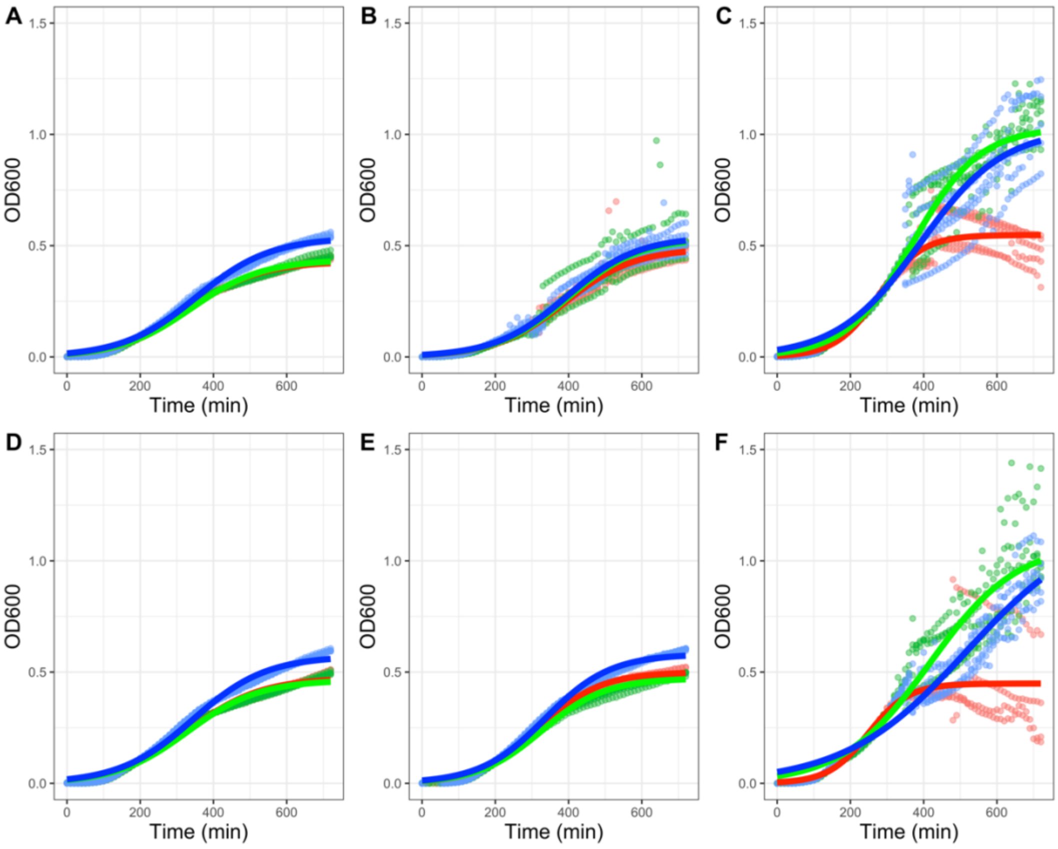
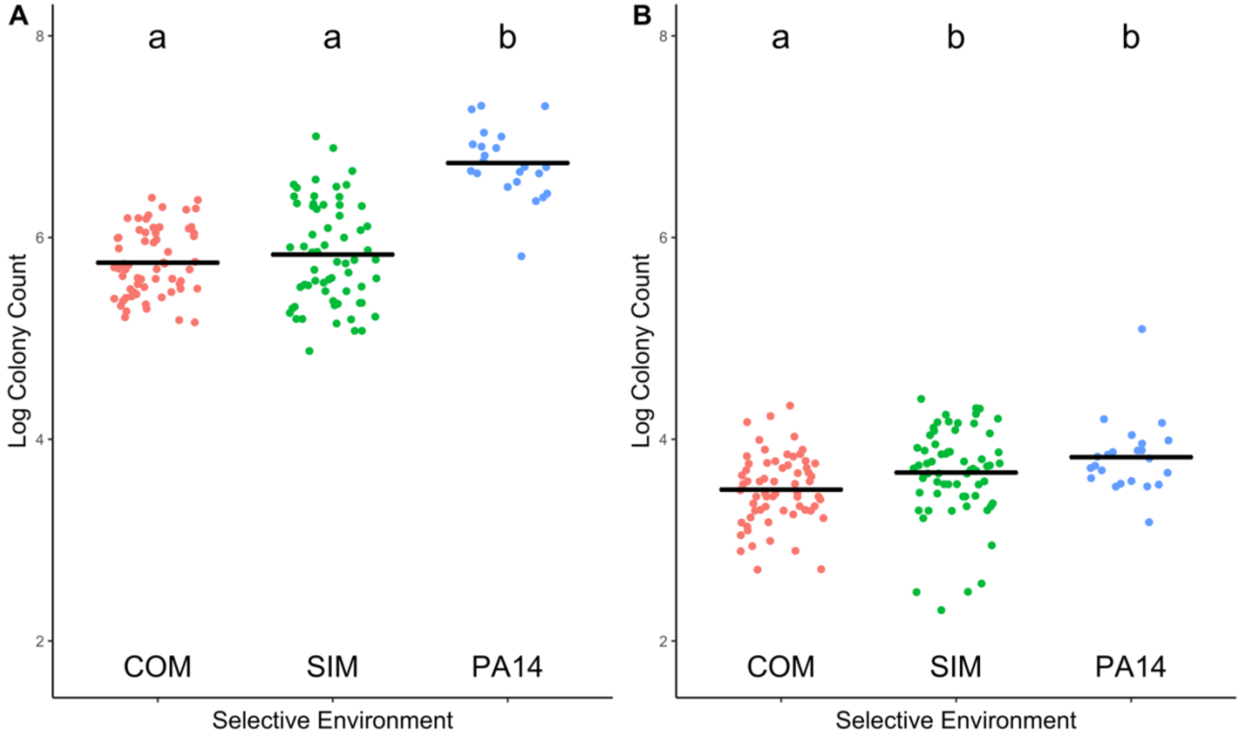


Figure 7: Growth curves of evolved isolates and PA14 grown in LB supplemented with RV^+ spent media (red), RV^- spent media (green), or no spent media (control; blue). **A-B**. Two evolved isolates from COM line 6.1 and **C**. PA14 grown in spent media from COM line 6.1. **D-E**. Two evolved isolates from COM line 6.2 and **F**. PA14 grown in spent media from COM line 6.2. Curves were generated using the R package *growthcurver* and represent an average of all replicate curves for each treatment (Sprouffsk, 2020).

Supplementary Figures



Supplementary Figure 1: Cellular productivity of COM communities (red), SIM communities (green), and PA14 (blue) in **A**. LB and **B**. MIN media. Black horizontal bars denote the mean cellular productivity for each group. Letters denote significant differences from post-hoc Tukey tests (α = 0.0083).

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