Effect of genetic background on diversification of *Pseudomonas aeruginosa*

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Table of Contents

Acknowledgments iii

Abstract v

Résumé vi

1.0 Introduction 1

2.0 Materials and Methods 10

- 2.1 Bacterial strains and growth media 10
- 2.2 Evolution experiment 10
- 2.3 Colony morphology 11
- 2.4 Competitive fitness 12
- 2.5 Population density 12
- 2.6 Phylogeny 13
- 2.7 Statistical analyses 13

3.0 Results 15

- 3.1 Differences in diversification in a simple versus complex environment 15
- 3.2 Effect of ancestor population size on diversification 15
- 3.3 Relationship between gains in fitness and extent of diversity 16
- 3.4 Effect of niche of origin on diversification 16

4.0 Discussion 17

5.0 Conclusion 25

Tables and Figures 26

References 35

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Abstract

Life on Earth is incredibly diverse. The process of diversification that gives rise to this diversity is not the same for all lineages. Diversification is often driven by ecological opportunity. *Pseudomonas aeruginosa* is an opportunistic pathogen present in a variety of environments that causes chronic lung infections in cystic fibrosis (CF) patients. It diversifies rapidly within the CF lung and CF lung-like environments. Here we aim to assess both ecological and genetic factors in diversification of several strains of *P. aeruginosa.* We evolved 12 replicate populations of 8 different strains of *P. aeruginosa* in a nutritionally complex (LB) and simple environment (MIN) for 750 generations. We then measured diversity over time by observing the number of colony morphologies in each population every 250 generations. We also measured competitive fitness relative to the ancestor for endpoint populations. To provide a more complete analysis, phylogeny was factored into our statistical models. First, we found no significant differences in diversification between populations evolved in LB versus MIN media. Ancestor population size had no significant effect on diversification. We found that in both selection environments, CF strains diversified less than environmental strains, but this difference was marginally significant and only present when comparing these two niches directly and excluding acute strains. Finally, we found no correlation between gains in fitness and endpoint diversity. Our results suggest that diversification is limited by niche specialization (domestication) of *P. aeruginosa* to the CF lung.

Résumé

La vie sur Terre est incroyablement diverse. Le processus de diversification à l'origine de cette diversité n’est pas le même pour toutes les lignées. La diversification est souvent motivée par l’opportunité écologique. *Pseudomonas aeruginosa* est une bactérie pathogène opportuniste qui forme des infections chronique chez les personnes souffrant de fibrose kystique (FK, mucoviscidose). Elle se diversifie rapidement dans le poumon FK est les environnements similaires. Nous avons évalué comment le contexte écologique et génétique influence la diversification de plusieurs souches de *P. aeruginosa*. Nous avons évolué 12 populations de 8 souches différentes de *P. aeruginosa* dans des environnements nutritionnels complexes (LB) et simples1 (MIN). Nous avons ensuite mesuré la diversité des populations en examinant la morphologie des colonies toutes les 250 générations. Nous avons aussi mesuré la fitness compétitive des populations finales par rapport à leurs ancêtres. Afin de fournir une analyse plus complète, la phylogénie a été prise en compte dans nos modèles statistiques. Nous n’avons pas trouvé de différence significative entre les populations évoluées dans les milieux LB et MIN. La taille de la population ancestrale n'avait pas d’effet significatif sur la diversification. Nous avons trouvé que les souches FK ce sont moins diversifiée que les souches environnementales dans les deux environnements de sélection (LB et MIN), mais cette différence était marginalement significative et seulement présente en excluant les souches aiguës. Enfin, nous n'avons trouvé aucune corrélation entre le gain de fitness et la diversité finale. Nos résultats suggèrent que la diversification est limitée par la spécialisation de niche (domestication) de *P. aeruginosa* dans le poumon FK.

1.0 Introduction

The diversity of life on Earth is astounding, with over 1 million eukaryotic species having been identified and described and 8.7(+/- 1.3) million predicted to exist (Mora et al., 2011). This diversity increases drastically when accounting for prokaryotic species. The most recent estimate of the number of bacteria and archaea is 2.2– 4.3 million species (Louca et al., 2019). With this much diversity on the planet, a common question that comes up in evolutionary biology is how did we end up with all these species? How did life get so diverse?

1.1 Theory

Diversification is the process of genetic divergence within a species or population giving rise to new groups with characteristics that are different from the ancestor. The process of diversification is often characterized by adaptive radiation. This is a process where a common ancestor diverges into many species with unique morphologies and phenotypes inhabiting a variety of environments (Schluter, 2000). Diversification is a complex process where many factors, both ecological and genetic, can influence how quickly or how much a lineage may diversify.

Not all lineages are created equal when it comes to the rate and extent of diversification. Some taxa are incredibly diverse whereas others are not. For example, in the plant kingdom, the family Asteraceae has an astounding 23 000 species versus the family Ginkgoaceae has only one species (Barreda et al., 2015). Different taxa diversify at different rates (Caron & Pie, 2020) and to different extents. Rate and extent of diversification are not always correlated. Some lineages diversify rapidly but are ultimately

limited in how much they can diversify (e.g. sticklebacks) and others become very diverse (e.g. cichlid fish).

Ecological opportunity, unused or underutilized resources or niche space, is one of the most important factors driving diversification (Kassen, 2014; Schluter, 2000). When an ecological niche becomes available (i.e. because of extinction or changes to the environment), this provides an opportunity for another species to diversify and fill available niches. Niche availability can come from either a population colonizing a new location or environmental changes in that population's current location. The most notable example of this, of course, is the case of the Galapagos finches (Grant & Grant, 2008; Lack, 1947). There are several species of finch with unique beak morphologies endemic to the islands. They are believed to have originated from the same ancestor and developed unique beak shapes over time as a result of exploiting difference resources. Another example is that of three-spine stickleback (*Gasteroseus aculeatus*). These fish inhabit either the benthic or limnetic zones in lakes. In the case of two lakes in British Columbia, the specialization of these fish to either the benthic or limnetic zone was reflected in their morphologies with two distinct morphotypes based on their habitat (Schluter, 2000).

Trade-offs are another key factor in the process of evolution and diversification. Trade-offs occur when an increase in one trait is accompanied by a decrease in another trait (Garland, 2014). In an evolutionary context, this translates as an increase in fitness due to the increase of one trait accompanied by a simultaneous decrease in fitness due to the decrease of another trait. This process is important for diversification as trade-offs may allow for higher fitness in one environment but lower fitness in another. This leads to niche specialization and thus, divergence (Kassen, 2014). Evolutionary trade-offs often promote

diversification. This has been shown in bacteria (Ferenci, 2016), sticklebacks (Schluter, 1995), ants (Blanchard & Moreau, 2017), and Galapagos finches (Herrel et al., 2009).

Another important factor in the process of diversification is population size, as this can influence the amount of genetic variation available to selection. A study with *Escherichia coli* showed that diversity was higher in populations with higher maximum population sizes (Stevens et al., 2007). In mutation-driven adaptation, population size (N) is important for determining the supply of mutation. Given a constant mutation rate (μ), mutation supply rate is greater in larger populations than in smaller populations. Thus, diversification rates are higher in larger populations when the source of genetic variation is mutation.

1.2 Experimental evolution

The process of evolutionary diversification generally cannot be observed in the span of a single human life for most large, long-lived organisms. Turning to species with much shorter generation times, however, allows us to track evolutionary diversification in real time using a technique called experimental evolution. While many species have generation times short enough to conduct evolutionary experiments in the lab or field, such as fruit flies in the genus *Drosophila*, or aphids (superfamily Aphidoidea), the most commonly used species for experimental evolution are microbes such as bacteria, single celled eukaryotes like yeast, and bacteriophages, viruses that infect bacteria. The generation times for microorganisms can be as short as a few hours, or less than an hour under optimal conditions. Another major advantage of using microorganisms is the ability to cryogenically preserve these organisms, giving us microbial version of a living fossil record. Microbial experimental evolution (MEE) has emerged as a compelling tool to study the evolutionary process in the laboratory (Kassen, 2014).

1.3 Important/relevant findings from MEE

Arguably, the most well-known example of microbial experimental evolution is the long-term evolution experiment (LTEE) conducted by the Lenski lab at Michigan State University since the late 1980s (Lenski et al., 1991). Many important findings on the process and dynamics of evolution, diversification, and adaptation have come from the LTEE using *Escherichia coli* (Lenski et al., 1991; Lenski & Travisano, 1994). Another important example of MEE comes from Rainey and Travisano (1998). Using *Pseudomonas fluorescens* they demonstrated the effect of ecological opportunity on adaptive radiation. In a spatially heterogenous environment, *P. fluorescens* diverged into unique niche-specialists with different morphologies (Rainey & Travisano, 1998). Studies on the process of diversification in microorganisms have often been focused on ecological opportunity as a driver. For example, ecological opportunity was found to be an important factor in the process of diversification as well as the maintenance of diversity in an experimental evolution study of *P. fluorescens* (Barrett & Bell, 2006).

However, most studies employing MEE, and indeed many in the field of microbiology more generally, are conducted with a single founding strain. These strains are chosen either because they are, or closely resemble, the “type strain” for a species (the strain on which the species description is based) or because they grow and reproduce well in lab conditions (e.g. *E. coli* K-12). Studies that use multiple strains are often derived mutants of one parent strain (i.e. isogenic to the wild-type strain save for the presence of a specific, defined mutation) rather than unique lineages isolated from different environments. For example, a study of antibiotic resistance in *Pseudomonas aeruginosa* used different antibiotic resistant mutants of PA14 to determine the impact of genetic background and initial fitness on compensatory evolution (Hernando-Amado et al., 2022). Focusing attention on one or a few

closely related strains can potentially bias our inferences about the factors contributing to diversification, as strains that are more distantly related or isolated from distinct environments are likely to have different traits and so may behave differently from the type or laboratory strain. To date, few studies have accounted directly for divergence among founding strains in MEE studies of adaptive evolution and diversification.

1.4 Pseudomonas aeruginosa

Pseudomonas aeruginosa is a ubiquitous, Gram-negative opportunistic pathogen found in a wide variety of environments including soil, water, as well as human, animal, and plant hosts (Folkesson et al., 2012). *Pseudomonas aeruginosa* can cause acute infections in humans, typically infecting burn wounds and the urinary tract, and is also known for causing chronic lung infections in humans with cystic fibrosis (CF). Chronic infections are, by definition, notoriously hard to treat in part due to *P. aeruginosa*'s strong resistance to antibiotics, although other putatively patho-adaptive traits likely play a role as well. The majority of CF airway infections are thought to originate by colonization from environmental sources (i.e., in the shower, sink, or hot tub) or from highly transmissible 'epidemic' strains. The transition from environmental strain to chronic infection is thought to involve a series of putatively patho-adaptive trait changes, as environmental strains are unlikely to be well-adapted to lung environment. The CF lung environment is a very complex environment, with gel-forming mucins covering the cilia on the respiratory epithelium and a variety of carbon sources (Folkesson et al., 2012; Yang et al., 2011). This environment is very stressful for *P. aeruginosa* with reactive oxygen species (ROS) causing oxidative stress, mucous causing osmotic stress, constant attacks by the immune system, and regular antibiotic use (Dettman et al., 2013; Dettman & Kassen, 2021; Yang et al., 2011) .To manage these stressors and adapt to the hostile environment of the CF lung, a suite of trait changes take place. Trait

changes undergone by *P. aeruginosa* include loss of motility, loss of virulence factors, increased antibiotic resistance, and increased biofilm production when adapting to the CF lung environment (Folkesson et al., 2012). These traits make strains of *P. aeruginosa* isolated from the CF lung very different from strains isolated from environmental sources, or acute infections.

Previous work has shown that *P. aeruginosa* undergoes rapid evolutionary diversification upon colonization of the CF lung (Folkesson et al., 2012; Jorth et al., 2015). The strong selection pressure of the CF lung environment drives rapid adaptation and diversification into the phenotype mentioned above (Sousa & Pereira, 2014). Additionally, *P. aeruginosa* isolated from the CF lung often lives in biofilms rather than a free-living, planktonic state. This biofilm lifestyle results in greater phenotypic diversification due to the heterogenous environment provided by the biofilms (Sousa & Pereira, 2014; Stewart & Franklin, 2008). Mutator phenotypes, those with up to ~1000 fold higher mutation rates than non-mutators, are much more common in biofilms, leading to greater genetic diversity (Oliver et al., 2000; Sousa & Pereira, 2014). Phenotypic diversity can itself be very high in the CF lung. One study found 75 unique phenotypic sub-types from 400 CF isolates, based on 9 phenotypic traits (Ashish et al., 2013). Another study found 15 unique morphotypes from 235 isolates taken from a single CF patient (Clark et al., 2015). In general populations of *P. aeruginosa* found in the CF lung environment are incredibly diverse.

Like *E. coli* and *P. fluorescens*, *P. aeruginosa* has also been used for microbial experimental evolution. Experimental evolution studies with *P. aeruginosa* often focus on evolution of antibiotic resistance given its tendency to develop antibiotic resistance and the threat posed by the rise of antibiotic resistant pathogens (Pendleton et al., 2013). The development of antibiotic resistance occurs in the presence of antibiotics (Jørgensen et al.,

2013; Wardell et al., 2019) and occasionally even in the absence of antibiotics in the environment (Schick & Kassen, 2018). Although antibiotic resistance and pathogenesis is often the focus of clinical research on *P. aeruginosa*, it has also been studied in the context of diversification, especially in regards to infection of the CF lung (Davies et al., 2017; Schick & Kassen, 2018; Sousa & Pereira, 2014; Winstanley et al., 2016).

As previously mentioned, experimental evolution studies are often limited to only one strain of a bacterial species and studies with *P. aeruginosa* are no exception. Many *P. aeruginosa* studies use one of two strains, PA14 or PAO1, which were originally isolated from acute infections of wounds. These strains have become the workhorses of *P. aeruginosa* research. While they are not genetically identical (Dettman & Kassen, 2021), many traits are similarly expressed in laboratory studies (Shewaramani & Kassen, 2022) with the exception of virulence, with PA14 being "hypervirulent" relative to PAO1 (Grace et al., 2022). Given that strains isolated from the CF airway can be highly genetically divergent and are not restricted to a particular subclade of the *P. aeruginosa* phylogeny (Dettman & Kassen, 2021), it remains an open question how much of what we know about patho-adaptation in the CF airway is biased by studying these two strains and not others. Answering this question is the main objective of this thesis.

1.5 Study outline, hypotheses, and predictions

The impact strain-to-strain variation may have on the process of diversification is unclear. With this in mind, we ask whether distinct strains of *P. aeruginosa* diversify to different extents in the presence or absence of ecological opportunity. We tracked evolutionary diversification in colony morphology using eight different strains of *P. aeruginosa* over ~750 generations in a complex nutrient environment and a simple nutrient environment. The strains were isolated from a range of sources including the environment

(soil, water), chronic infections of the CF airway, and the commonly used lab strains PAO1 and PA14, which were originally isolated from acute infections.

This experimental design allows us to evaluate the effects of ecology and genetics on diversification. On the ecological side, we test the hypothesis that ecological opportunity drives diversification by contrasting the number of distinct colony morphotypes in nutrient complex vs simple environments; we predict the former will support more colony morph variation than the latter. We evaluate three distinct hypotheses associated with genetic constraints on diversification. The first is that the rate and extent of diversification is governed by access to mutational variation mediated through absolute fitness, as measured by population size in the founding strain. In mutation-driven adaptation, as in this experiment, larger populations will supply more genetic variation to selection for a given mutation rate and so should more readily diversify than smaller populations. Given that this is population is in a closed, experimental environment, there are no external sources of mutation. The second hypothesis is that populations that are initially maladapted to their environment (i.e. lower on a fitness peak) will have access to alternative pathways to reach a peak, whereas populations that are initially better adapted to the environment will be limited in the number of pathways they can access. We predict that populations that are initially maladapted will diversify more and thus gains in competitive fitness relative to their ancestor will be positively correlated with extent of diversity. The third is that diversification is governed by the history of selection and niche specialization. Niche specialization is underlain by fitness trade-offs, which previous work on the evolutionary dimensions of chronic infections in the CF airway shows, are often the result of loss-of-function mutations and gene deletions, such as the loss of quorum sensing and motility-related genes (Folkesson et al., 2012; Schick & Kassen, 2018). This hypothesis suggests that CF isolates

will be constrained in their ability to diversify because they lack functional genes, or even key genes entirely, supporting further adaptive diversification in the presence of ecological opportunity relative to environmental strains.

In testing these hypotheses, we must take care to distinguish shared inheritance from mechanism as explanations for the response of diversification. Strains that are more closely related, perhaps because they come from the same clade, are more likely to show similar phenotypic responses. In terms of diversification, similar patterns of diversification could result because the spectrum of mutational variation available to selection will be more similar between strains that are more closely related. Increasing genotypic divergence across a phylogeny can lead to differences in the spectrum and extent of diversification. To account for shared evolutionary history in our analyses we used phylogenetically informed analyses, testing the mechanisms discussed above with and without the effect of phylogeny. If the effect of any given mechanism remains significant after accounting for phylogeny, we can more confidently say that the mechanism in question is a driver of diversification in this system.

2.0 Materials and Methods

2.1 Bacterial strains and growth media

We conducted our experiments using strains of *Pseudomonas aeruginosa* isolated from various sources. A total of eight strains were used, three from chronically infected CF airways, three from environmental sources, and two commonly used lab strains PA14 and PAO1, originally isolated from acute infections (Table 1). The three strains isolated from chronically infected CF airways—referred to as CF strains—were JD313, JD322, and JD328 (Table 1). The three environmental strains used were Jp54, Jp1140, and Pae111 (Table 1). Two versions of each strain (*lacZ*-marked and unmarked) were diluted and plated on Lysogeny Broth (LB) agar and grown for 24hrs at 37°C. The gene *lacZ* encodes for beta-galactosidase. This enzyme catalyzes the hydrolysis reaction of beta-galactosides into monosaccharides. X-gal is a type of beta-galactoside, that when broken down produces a blue colour. This is called X-gal staining and is often used as a marker gene to detect successful plasmid transformation (Burn, 2012). In our case, we mark bacteria with *lacZ* so that we are able to differentiate between two different cultures when grown together on agar with X-gal. A single colony was chosen at random from each plate and grown in 1.5 mL of liquid LB media overnight at 37°C. Liquid cultures were then stored at -80°C in 20% glycerol for later use.

2.2 Evolution experiment

All eight strains were evolved in two different environments, a complex environment (LB) and a simple environment (M9 minimal media; hereafter designated MIN). LB is a complex environment consisting of yeast extract, tryptone, and sodium chloride providing many carbon sources to be exploited by bacteria whereas MIN has only one carbon source; glucose. Both unmarked and *lacZ*-marked versions of each strain were used in the

experiment. Plates were set up in a checkerboard pattern (Figure 1) by alternating *lacZ*-marked and unmarked to be able to identify cross-contamination between cultures later. Twelve replicate populations of each founding strain (six *lacZ* and six unmarked), derived according to the protocol described in the previous section, were propagated in each treatment. The evolution experiment consisted of daily serial transfer of each replicate population diluted 1:100 in fresh media and under static (unshaken) conditions for 24hrs at 37°C. Each day 15μL of culture was transferred into 1.5mL of fresh growth media. The dilution factor ensures each population experiences at least ~6.64 generations per day. The experiment was continued for 110 days corresponding to ~750 bacterial generations. Populations were archived at -80 C every 250 generations in 1.5mL Eppendorf tubes in 20% glycerol for future experiments.

2.3 Colony morphology

Each evolved population was taken from frozen cultures and grown overnight in their respective selection environments at 37°C under static conditions. Overnight cultures were then diluted 10^{-6} for both MIN- and LB-evolved populations respectively and plated on M9 minimal media agar, following Schick and Kassen (2018). Agar plates were incubated for 48 hours at 37°C. Colonies were then categorized based on shape, size, and surface texture (nomenclature based on Rainey and Travisano, 1998 classification). For example, a colony that was small and smooth would be considered a unique morphology and different from another colony that was small and wrinkly. Richness was calculated using the vegan package in R by treating each unique colony morphology as a "species" (Oksanen et al., 2022).

2.4 Competitive fitness

Competitive fitness assays were conducted using flow cytometry. Ancestor strains were all marked with yellow fluorescent protein (YFP) to be able to distinguish between cells from ancestor populations and cells from evolved populations with flow cytometry. Overnight cultures of evolved populations and YFP-marked ancestors were grown from frozen culture overnight at 37°C under static conditions. Evolved populations were then mixed with their corresponding YFP-marked ancestor in a 50:50 ratio for all strains except clinical which were mixed at a 30:70 (evolved:ancestor) ratio to create the t_0 mixes and then frozen in 20% glycerol. Flow cytometry was performed on the mixtures using the Gallios Flow Cytometer (Beckman Coulter) and Kaluza for Gallios acquisition software (Beckman Coulter). Filter-sterilized minimal salts were used as a buffer solution. These mixes were then co-cultured overnight at 37°C under static conditions to create the t_{24} and then frozen in 20% glycerol. Flow cytometry was performed on the t_{24} cultures in the same manner as described above. Cell counts were taken from the flow data using the Kaluza Analysis Software (Beckman Coulter). Selection coefficient (*s*) was calculated using the following formula.

$$s = \frac{\ln\left(\frac{evolved\ final}{evolved\ initial}\right) - \ln\left(\frac{ancestor\ final}{ancestor\ initial}\right)}{\#generations}$$

2.5 Population density

To get an estimate of ancestor population size, colony forming units (CFU) per mL was measured for all ancestor strains in both selection environments. Two replicates of all ancestor strains were grown overnight from frozen culture at 37°C under static conditions in 24-well plates, in both LB and MIN. Cultures were diluted with serial dilutions by a factor of 10^6 then 0.1mL was plated on to MIN agar. Like the protocol for colony morphology, agar

plates were incubated for 48 hours at 37°C. After 48 hours, colonies were counted for each plate. Finally, CFU per mL for each strain was calculated using the following formula;

$$CFU/mL = \frac{colony\ count * dilution\ factor}{volume\ plated\ (mL)}$$

2.6 Phylogeny

An alignment of all eight strains used in the experiment as well as an additional strain (PA7, another wound isolate) that was used as an outgroup (following Shewaramani and Kassen, 2022) was extracted from an alignment of 1000 *Pseudomonas aeruginosa* genomes from 381 core genes (Dettman & Kassen, 2021). The alignment was originally done by Dettman and Kassen (2021) using MUSCLE (version 3.8, Edgar, 2004). A tree was created from the alignment of the nine strains using Geneious Prime 2022.2.2 (Figure 2).

2.7 Statistical analyses

All statistical analyses and figures were done in R and R studio (R core team, 2021).

We used the vegan package in R to calculate richness from colony morphology data (Oksanen et al., 2022). To test for differences in diversity (richness/number of colony morphs) while accounting for phylogenetic relationships between strains brms (Bayesian Regression Model using Stan) was used (Bürkner, 2017, 2018, 2021). The phylogenetic variance-covariance matrix was used in the model to account for phylogenetic relatedness (Paradis & Schliep, 2019). Fixed effects are medium (MIN or LB), niche (environmental, acute, CF), and ancestor population size (CFU/mL), random effects are strain and population ID (each replicate population), and the response variable is richness (number of colony morphs). Each model was run for 100 000 iterations, with the first 50 000 discarded

as warmup iterations. We tested each factor of interest (medium, niche, ancestor population size) individually using brms by running each model with and without phylogeny attached to strain. We also tested the interaction between niche and medium with and without phylogeny.

To test for a correlation between diversity (richness) and fitness (selection coefficient) we used a multivariate brms with niche and medium as fixed effects, strain and population ID as random effects, and richness (number of colony morphs) and selection coefficient as response variables. Like the models described above, these models were run with and without phylogeny attached to strain. These models were run for 250 000 iterations to improve effective sample size, with the first 125 000 discarded as warmup iterations.

3.0 Results

3.1 Differences in diversification in a simple versus complex environment

To estimate how much of an effect ecological opportunity has on the extent of diversification of *P. aeruginosa* we compared number of colony morphology types (richness) over time in MIN vs LB. At the end of the experiment, after 750 generations of evolution, average richness for LB-evolved populations was 1.68 and for MIN-evolved populations was 1.60 (Figure 3).

Although there were small differences that were present across generations between MIN- and LB-evolved populations, this difference was not formally significant. A brms showed no significant difference in diversification between LB and MIN evolved populations without phylogeny (estimate=-0.04, 95%CI=-0.18 - 0.11) or with phylogeny (estimate=-0.03, 95%CI=-0.17 - 0.11). The interaction between niche and environment did not have a significant effect on diversification without phylogeny (MIN:CF estimate=0.10, 95%CI=-0.26 – 0.46; MIN:Environmental estimate =0.04, 95%CI=-0.29 – 0.37) or with phylogeny (MIN:CF estimate=0.11, 95%CI=-0.25 – 0.46; MIN:Environmental estimate =0.04, 95%CI=-0.28 – 0.37).

3.2 Effect of ancestor population size on diversification

Ancestor population size (CFU/ml) varied greatly between strains, especially in LB (Figure 4). In LB Pae111 had the highest CFU/ml but PAO1 had the highest CFU/ml in MIN, and in both environments JD322 had the lowest CFU/ml (Figure 4). However, there was no significant relationship between ancestor population size and diversification. Population size was not statistically significant in a brms without (estimate=-0.03, 95%CI=-0.04 - 0.11) and with phylogeny (estimate=0.03, 95%CI=-0.04 - 0.10).

3.3 Relationship between gains in fitness and extent of diversity

On average, all strains gained fitness (selection coefficient, s>0) after 750 generations of evolution in both LB and MIN (Figure 7). A multivariate brms showed no statistically significant correlation between the extent of diversity (richness) and gains in fitness (selection coefficient). The among strain correlation (estimate=-0.22, 95%CI=-0.96-0.81) and the residual correlation (estimate=0.29, 95%CI=0.25-0.80) were both not statistically significant before phylogeny and after accounting for phylogeny (among: estimate=-0.10, 95%CI=-0.94-0.85) and (residual: estimate=0.30, 95%CI=-0.24 - 0.77) (Figure 8). These results should be interpreted with caution as the models could not produce an acceptable effective sample size (ESS) and may not have converged properly.

3.4 Effect of niche of origin on diversification

We compared rate and extent of diversification between strains originating from different niches (environmental, CF, and acute). In LB, environmental strains diversified the most, followed by acute strains, and CF strains diversified the least (Figure 5). In MIN, again environmental strains diversified the most (Figure 5). Although there was a consistent trend of environmental strains diversifying more than CF or acute strains, this difference was not formally significant. Both brms models showed no significance either without phylogeny for CF strains (estimate=-0.06, 95%CI=-0.31 - 0.19) and environmental strains (estimate=0.15, 95%CI=-0.08 - 0.40), or with phylogeny for CF strains (estimate=-0.06, 95%CI=-0.29 - 0.19) and environmental strains (estimate=0.15, 95%CI=-0.06 - 0.37).

Chronic infections are thought to result from colonization by environmental strains, so the main contrast of interest for our study is between CF and environmental isolates. In this case, the difference in richness is near-significant without phylogeny (estimate=0.22, 95%CI=-0.02 - 0.45), and with phylogeny (estimate=0.21, 95%CI=-0.00 - 0.42).

4.0 Discussion

Diversification is a complex process that can be driven by multiple factors. Some of these factors are much better understood than others. Ecological opportunity has been well-studied as a driving factor of diversification (Kassen, 2014; Schluter, 2000) and MEE studies have largely confirmed that diversity tends to be higher in the presence of ecological opportunity than its absence (Kassen, 2014). However, the effect of genetic background on diversification has not been studied to the same extent as other factors driving diversification.

4.1 Summary of main results

Here we evolved eight different lineages of *Pseudomonas aeruginosa* in two different environments over 750 generations to examine the potential effect of genetic background on diversification (Figure 6). To get an estimate of diversity of evolved populations we examined colony morphology of evolved populations every 250 generations. We used number of colony morphology types (richness) as an estimate of population diversity. Here we investigated several hypotheses regarding the effects of ecology and genetic background (genetic “constraints”) on diversification. First, we examined the hypothesis that ecological opportunity drives diversification by comparing richness between the simple (MIN) and complex (LB) environment. Although there were small differences between the two environments that were present across generations, we found no significant differences in diversification between LB and MIN-evolved populations, after phylogeny was accounted for. As for genetic constraints, we first investigated the hypothesis that absolute fitness, measured by initial population size of the founding strains, determines the access to mutation and thus diversification. We found no significant relationship between population size and diversification once phylogeny was accounted for. Second, we investigated the

hypothesis that CF strains are domesticated to the CF lung through gene loss and loss-of-function mutations, thus inhibiting their ability to diversify. We compared diversification between strains from different niches; acute isolates, CF isolates, and environmental isolates. We found no significant differences between the three niches. However, when comparing environmental strains and CF strains on their own we found that environmental strains diversified more than CF strains and that this difference was marginally statistically significant. Third, we investigated the hypothesis that populations which are initially maladapted to environment have greater access to alternative paths to higher fitness and will therefore become more diverse by accessing these alternative pathways. We found no significant correlation between gains in fitness and extent of diversity.

4.2 No difference in diversification between complex and simple nutritional environments

Ecological opportunity is one of the major drivers of diversification. We hypothesized that LB media, being a complex, nutrient rich environment, would provide greater ecological opportunity than MIN media, which contains a single carbon source, and so allow for more diversification in LB. While diversity was slightly higher in LB-evolved populations than MIN-evolved populations in both environments at the end of our experiment, a result in line with previous findings in MEE and with a similar experiment that included one of our strains (PA14) (Schick & Kassen, 2018), the difference was not formally significant. It may be that our measure of diversity, which is based on conspicuous (by eye) differences in colony morphology, is too crude to capture the full range of trait changes occurring in nutrient rich environments like LB. Previous work with PA14, for example, revealed striking differences between a second nutrient rich environment (synthetic CF medium) and the same minimal medium in a range of traits associated with patho-adaptation to the CF airway like motility, virulence factor production and biofilm formation (Schick & Kassen, 2018). Measuring these

and other traits would be an important next step in evaluating this hypothesis more directly. Alternatively, LB may constitute a more modest spectrum of ecological opportunity than SCFM (synthetic cystic fibrosis medium), despite both being nutrient rich compared to the MIN environment. SCFM contains many free ions, amino acids, in addition to glucose, iron sulfate and lactate (Palmer et al., 2007). LB is composed of yeast extract, tryptone, and NaCl. Yeast extract, like SCFM, is very nutritionally rich, containing polysaccharides, peptides, vitamins, amino acids. It has been shown that *P. aeruginosa* diversifies rapidly in the CF lung and CF lung-like environments (Ashish et al., 2013; Clark et al., 2015; Schick & Kassen, 2018). In our experiment *P. aeruginosa* did diversify, however it may not have diversified to the same extent as it would have in SCFM. So, although both of these environments are nutritionally rich, with many carbon sources to be exploited, there may be something unique about SCFM and the CF lung environment that allows for high levels of diversification. A direct test of this hypothesis would involve contrasting the extent of diversification in LB and SCFM, ideally with a larger number of replicates as our study, with just 8-12 replicates per strain, is likely under-powered compared to the Schick and Kassen study that used 30 replicates per treatment. However, it may be that we should reject our hypothesis in this case and consider factors other than ecological opportunity that may be driving diversification in *P. aeruginosa*.

4.3 Population size does not drive diversification.

Population size (N) is an important factor to consider in the context of diversification. In mutation-driven adaptation, mutation supply rate is the main source of variation and is determined by population size (N) and mutation supply rate (μ). Depending on if $N\mu>1$ or $N\mu<1$ this will influence diversification. Strong selection weak mutation (SSWM) conditions are when a beneficial mutation arises and quickly sweeps and fixes in the population. This

occurs when the population size (N) multiplied by the mutation rate (μ) is smaller than 1. These conditions lead to successional mutations. Conversely, Nμ is greater than 1, we have clonal interference where a weak beneficial mutation begins to sweep but other, slightly more beneficial, weak mutations out-compete the initial mutation and prevent it from fixing in the population (Desai & Fisher, 2007). If a population was under SSWM conditions, a beneficial mutation would have quickly fixed in the population, reducing diversity. In the case of clonal interference, there would be more diversity as many slightly beneficial mutations would persist in the populations. So, if some populations are so large that there is clonal interference and some are small enough to be in SSWM conditions, we would see an effect of ancestor population size on diversification.

We found no significant relationship between ancestor population size (CFU/mL) and diversification. Population size varied greatly between strains with Pae111 and PAO1 having the highest CFU/mL and JD322 having the smallest (Figure 3). Population size (CFU/mL) were in the 100s of millions to billions in LB, in 10s of millions in MIN, so regardless of environment, the population sizes were rather large. The estimated genome-wide mutation rate (μ) of *P. aeruginosa* is 0.52×10^{-3} per genome per generation (Dettman et al., 2016). So, even in the case of the strain with the smallest population size (JD322 in MIN with an initial population size (N) of 10 000 000 CFU/mL) $N\mu >> 1$, therefore populations are not in SSWM conditions. This means we may be seeing clonal interference in all population simply due to how high the initial population sizes of the ancestor are. Considering that there were not added selection pressures to either the LB or MIN environment, selection would be weak.

4.4 Domestication to the CF lung limits further diversification.

The one mechanism influencing diversification for which we do have some, admittedly limited, evidence is genetic constraints associated with niche specialization. In particular, environmental strains tended to diversify more than CF isolates across all conditions, and independent of phylogeny. This contrast is of interest here because most chronic infections result from colonization by environmental strains. The lack of a phylogenetic signal in our data is not unexpected as previous work (Dettman & Kassen, 2021) shows that CF isolates can be derived from any clade across the phylogeny and estimates of selection based on comparative genomic data (dN/dS) suggests strains isolated from the CF airway experience stronger positive selection than those from environmental or acute sources. Our data suggests the strong selection imposed by the CF airway leads to specialization that evidently can compromise further diversification. The proximate mechanism behind this effect likely stems from specialization derived from loss-of-function mutations and gene loss that often accompany rapid adaptation to novel environments, including the CF airway. Consequently, the spectrum of genetic variation available for further adaptive diversification is likely to be limited, especially in key genes associated with patho-adaptation. Evaluating this hypothesis further could involve more direct estimates of the spectrum of variation that arises in putatively patho-adaptive traits like biofilm formation or virulence factor production. It may be useful to measure the ability of isolates to process different carbon sources that are available in LB to get a better idea of population diversity. As LB is composed of yeast extract, tryptone, and NaCl, the carbon sources available are primarily amino acids with very little sugar present (Sezonov et al., 2007). Following Sezonov's (2007) methodology with *E. coli*, we could conduct bioassays by growing *P. aeruginosa* on agar supplemented with various carbon sources to determine the ability of evolved isolates to metabolize each one. For example, high performance liquid chromatography (HPLC) showed that glutamine, glutamic acid, and proline were in

relatively high concentrations in LB, perhaps making these potential metabolites of interest (Sezonov et al., 2007). This may give us an idea of how different individuals in the population may be exploiting different sources of nutrition in this complex environment. Measuring other traits such as motility (twitch and/or swim), growth rate, and biofilm production, may also be useful for getting a more accurate picture of population diversity. These traits were measured by Schick and Kassen, and while they were selected based on their relevance to patho-adaptation, they may also be general indicators of what is going on in a population.

Although we have some evidence that adaptation to the CF lung limits further diversification, it is important to note that we have limited statistical ability to test the effect of niche specialization on diversification in general. As we only had CF strains as our specialized strains, n=1 for this variable. If we were to test the hypothesis that niche specialization limits diversification in greater depth, we would need to test strains that are specialized to a variety of environments, not just the CF lung environment.

4.5 Gains in fitness not correlated with extent of diversity.

We found no significant correlation between gains in fitness (measured by selection coefficient relative to the ancestor) and extent of diversity (richness after 750 generations) after accounting for phylogeny. This was unexpected as we anticipated that populations that were initially maladapted to their environment (i.e. those with greater gains in fitness over time) would become more diverse from exploiting alternative pathways to greater fitness. To our knowledge, there are few studies that explicitly test the relationship between fitness and diversity using experimental evolution. A 2003 study using *Pseudomonas fluorescens* found a negative relationship between adaptation and diversification after a week of evolution (Buckling et al., 2003). Similar to our study, they measured diversity by assessing colony

morphology on agar plates. They found that over time, relative fitness to the ancestor strain increased while diversity (number of genotypes) decreased (Buckling et al., 2003). They hypothesized that the reason for this limited ability to diversify with increasing relative fitness was due to niche specialization. Our results do not align with these results as we showed no relationship (positive or negative) between relative fitness to the ancestor and diversity. However, the 2003 study had a duration of only a week (6 transfers) and fitness and diversity were measured daily (Buckling et al., 2003), whereas our experiment lasted 750 generations (~110 transfers) and we only measured endpoints for fitness relative to the ancestor. So, this negative relationship between population diversity and fitness may be present in the short term but not in the long term. As we cryogenically preserved our populations every 7 days, it would be possible to examine this relationship on a shorter timescale for a future study. However, the results and hypothesized mechanism of adaptation limiting diversification via niche specialization is not entirely inconsistent with our results (Buckling et al., 2003). We also hypothesized that CF strains would be limited in their ability to diversify as they are already specialized to the CF lung, and we found some limited support for this hypothesis. Perhaps the reason why we are not seeing a positive or negative relationship between fitness and diversity in our evolved populations is we are only measuring one metric of fitness, change in fitness relative to the ancestor. We are not considering potential trade-offs in fitness between different traits. It has been shown that trade-offs do promote diversity in bacteria (Ferenci, 2016). It is possible that if we were to measure several different traits in our evolved populations (as mentioned above), we may see increases in fitness in some traits and reduction of fitness in others.

Another metric of diversity to consider is genetic diversity which is something we were not able to measure in our evolved populations. This may be important to consider as

it would give the most complete picture when it comes to population diversity, especially compared to our much simpler measurements based on colony morphology. Several studies in various organisms have shown that high genetic diversity in populations yields greater population fitness. This is a particularly important concept in conservation. This positive relationship has been shown in the field in butterflies (Vandewoestijne et al., 2008) and in the lab using crustaceans (Markert et al., 2010). Although, to our knowledge this has not been tested with experimental evolution, it may be worth exploring in the future. A future experiment could have the populations sequenced to determine population diversity which may give us a fuller picture of the overall diversity in these populations. Along with multiple measures of fitness, this may improve our ability to test our hypotheses.

Finally, it is also important to note that there is no exact consensus on the relationship between diversity (species richness) and fitness (productivity). This lack of consensus has been a topic of discussion for decades. All kinds of relationships have been shown in literature; positive, negative, no relationship, or unimodal (Mittelbach et al., 2001). Unimodal relationships, where intermediate levels of diversity yield the highest productivity, tend to be the most commonly observed (Mittelbach et al., 2001). A striking example of this unimodal relationship is the global relationship between diversity and productivity in phytoplankton which is mediated by predator-prey interactions (Vallina et al., 2014). Because of the variability of these relationships it is hard to accurately predict what the relationship will be for a given population.

5.0 Conclusion

Overall, our results are somewhat inconclusive. Most results were non-significant or marginally significant. An unexpected result was that there were no significant differences in diversification between a complex and simple nutritional environment. We also saw no significant relationship between ancestor population size and diversification nor between extent of diversity and population fitness. However, we did show a small, but consistent trend of environmental strains diversifying more than both acute and CF strains in simple and complex nutritional environment. Our results demonstrate the effect that niche specialization, specifically domestication to the CF lung, may limit further diversification.

With a bacterial fossil record of 750 generations of evolution from 8 different strains of *P. aeruginosa* in two selection environments, there is a lot of potential for future experiments with these evolved populations. Phenotypic diversity could be observed in greater depth by measuring multiple traits (growth rate, motility, etc.). It could also be measured on a finer temporal scale, with populations having been frozen every ~50 generations. Additionally, with sequencing, we could examine specific mutations and genetic diversity over time. There is already ongoing work looking into parallelism in the MIN-evolved populations. It may also be useful to evolve populations in SCFM and LB concurrently to examine the differences between these two rich nutritional environments when it comes to diversification.

Tables and Figures

Niche	Strain	Source	Isolation Location	Country	Isolation year
Environmental	Pae111	Soil	Maysville, KY	USA	2010
	Jp54	Sea water	Suruga Bay	Japan	2004
	Jp1140	Sea water	Pacific Ocean	Japan	2003
CF	JD313	CF patient	Hamilton, ON	Canada	2008
	JD322	CF patient	Toronto, ON	Canada	2006
	JD328	CF patient	London, ON	Canada	2006
Acute	PA14	Wound	Boston, MA	USA	1970s*
	PAO1	Wound	Melbourne, VIC	Australia	1955

Table 1. Ancestral strains of *P. aeruginosa* used for the selection experiment. JD322 is Ontario Epidemic Strain A, JD313 and JD328 are Ontario Epidemic Strain B (Dettman and Kassen, 2021). *Exact isolation date unknown, first publication documenting PA14 comes from 1977 (Mathee, 2018; Schroth et al., 2018).

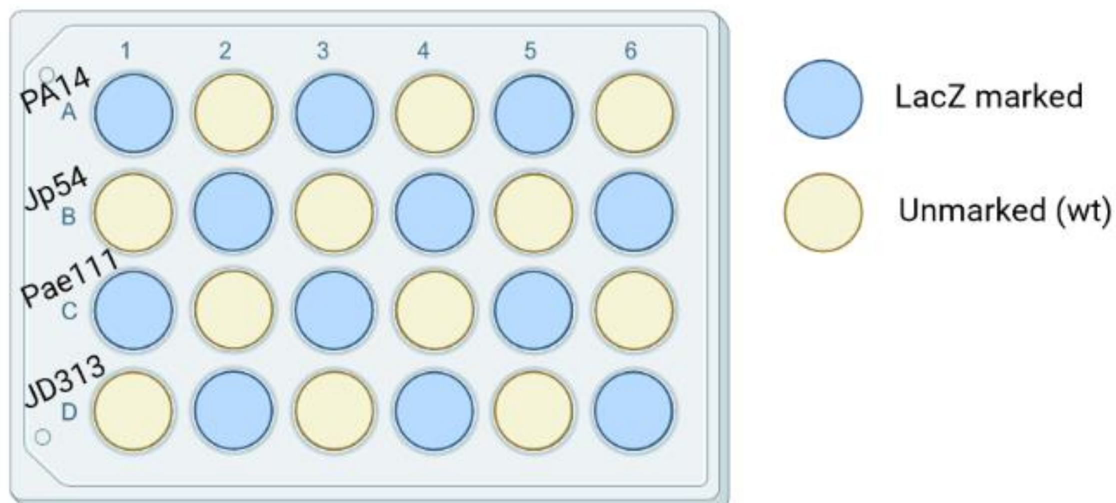

Figure 1. Example of plate layout used for selection experiment. Each strain occupied one row on a 24-well plate, alternating its *lacZ* marked and wt version. This was done for all strains in both LB and MIN. Created with BioRender.com.

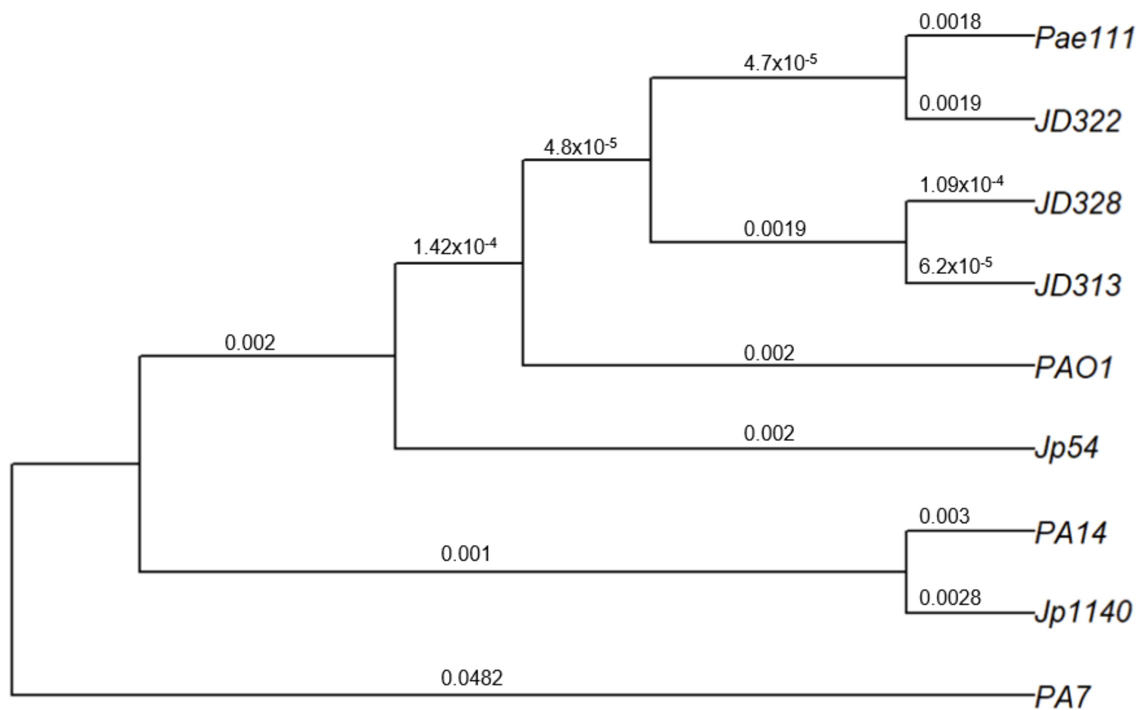

Figure 2. Phylogenetic tree of all strains of *Pseudomonas aeruginosa* used in the selection experiment as well as PA7 which was used as an outgroup to root the tree. Sequences of 381 core genes were aligned with MAFFT 7.471 (Katoh and Standley, 2013; Dettman and Kassen, 2021) and the tree was generated using Geneious Prime 2022.2.2. Branch lengths show estimated nucleotide substitutions per site.

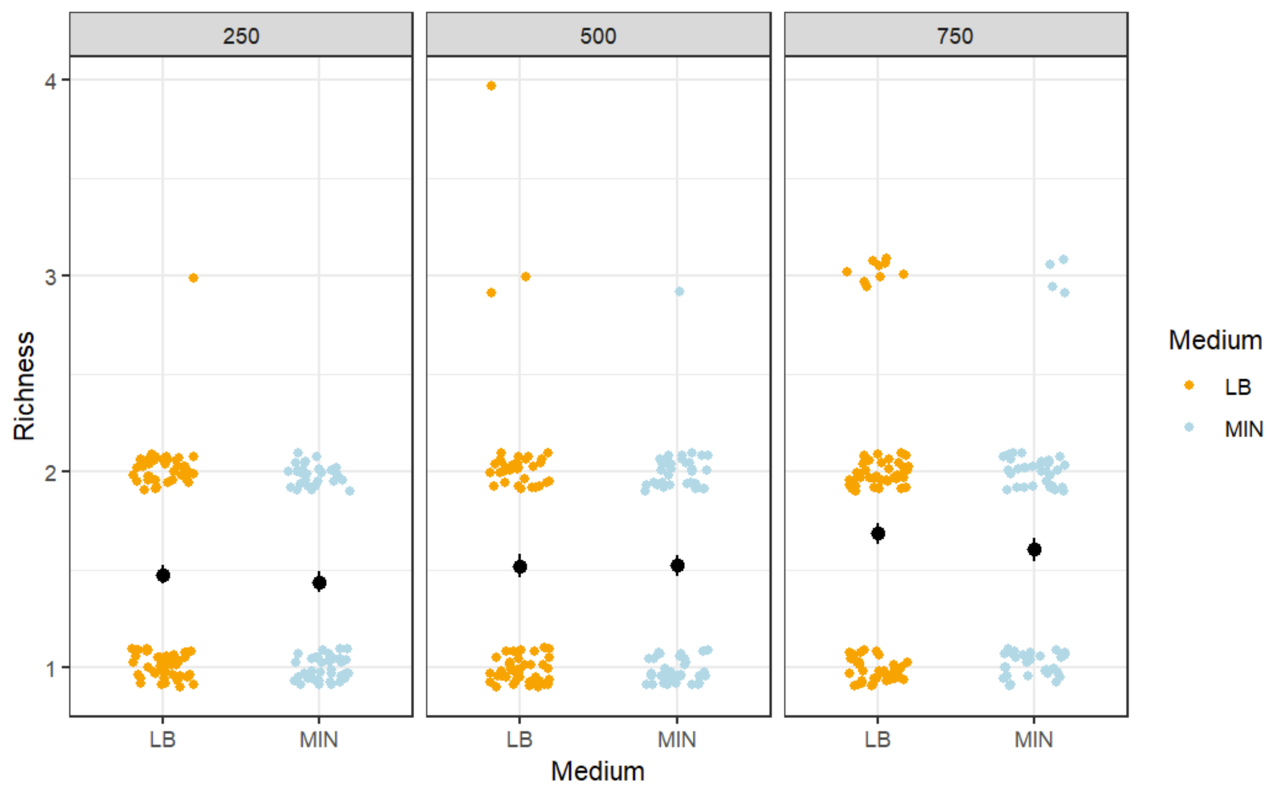

Figure 3. Strip charts of population diversity (number of colony morphologies, richness) in LB (orange) and MIN (blue) at each major time point. Error bars show means +/- SEM.

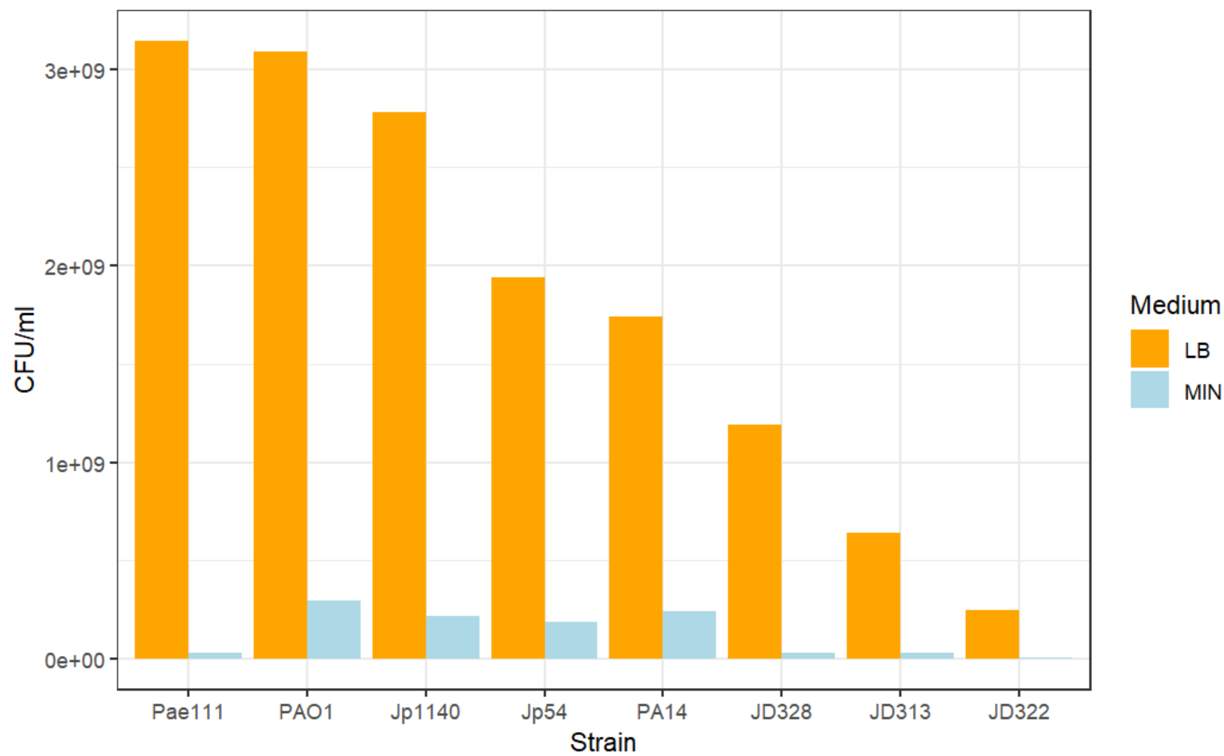

Figure 4. Population size of ancestor strains show as colony forming units (CFU) per mL. Orange bars show CFU/mL in LB and blue bars show CFU/mL in MIN.

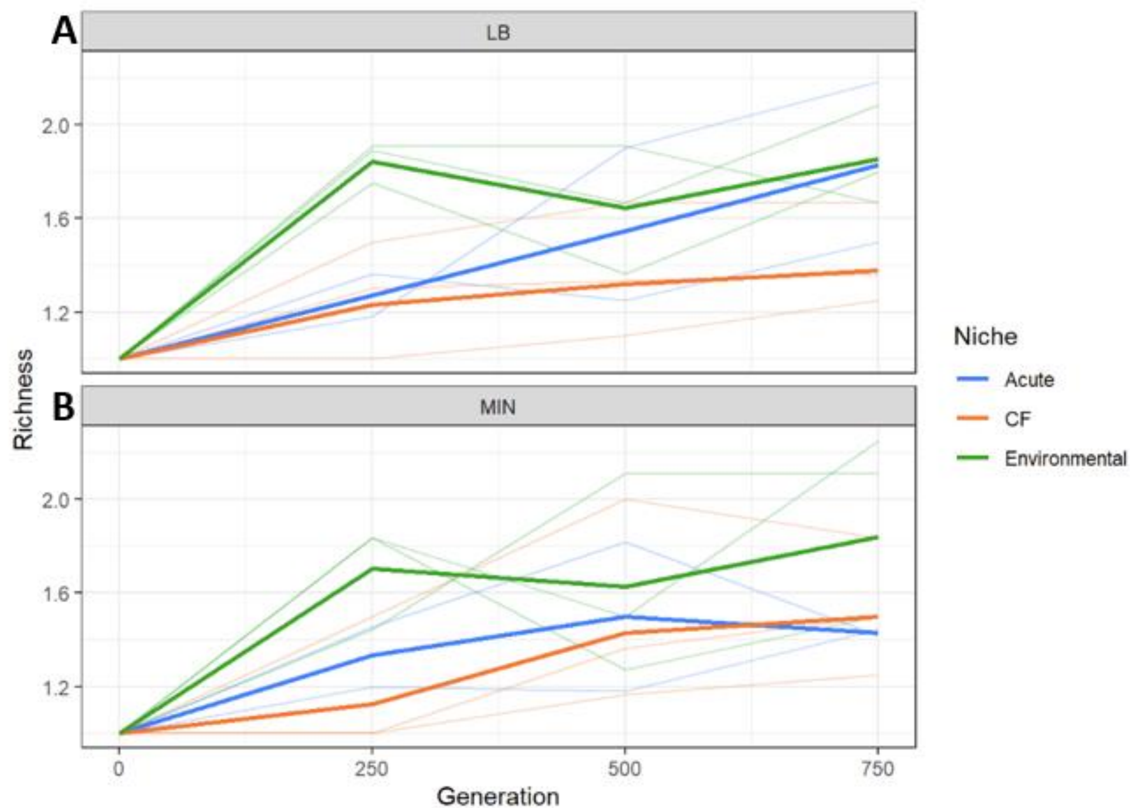

Figure 5. Plot of colony morphology types (richness) over time of *P. aeruginosa* populations evolved in LB (A) and minimal media (B). Thick, dark lines represent mean richness by niche. Thin, faint lines represent means of each strain.

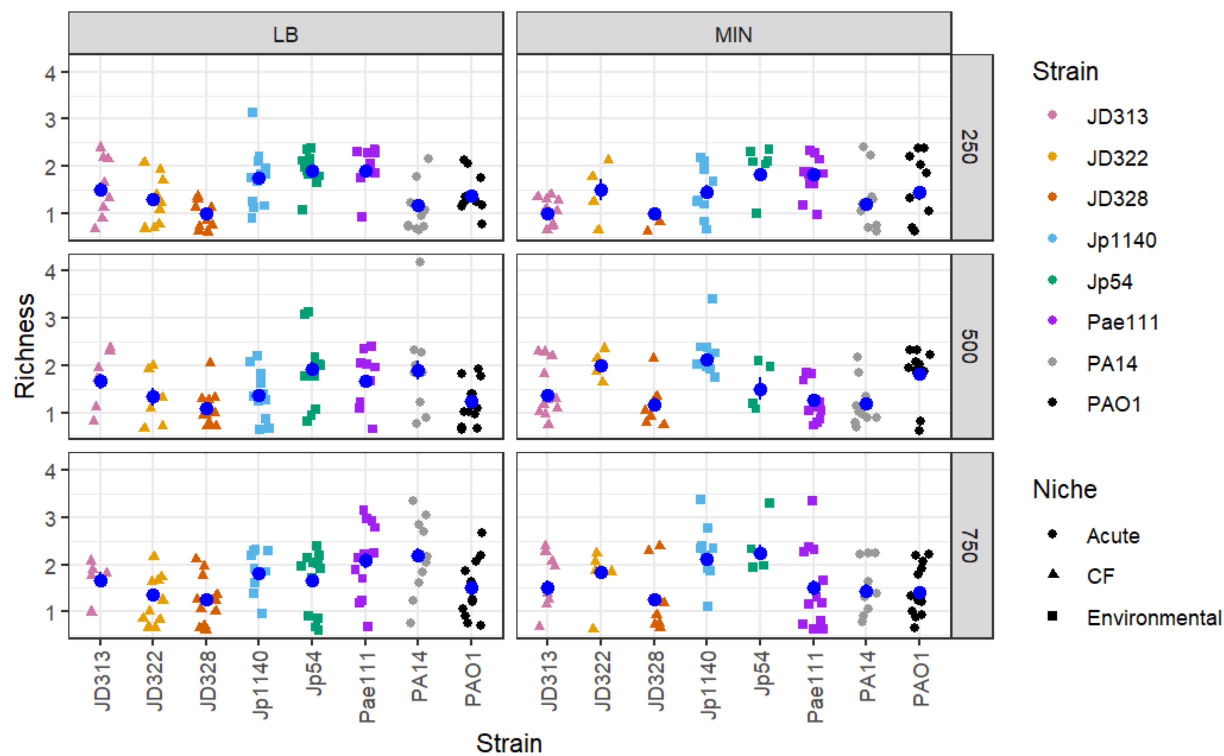

Figure 6. Strip charts of population diversity of evolved populations by strain, generation, and medium. Colour represents ancestor strain and shape represents niche. Left side shows LB-evolved populations and right side shows MIN-evolved populations. Error bars represent means +/-SEM.

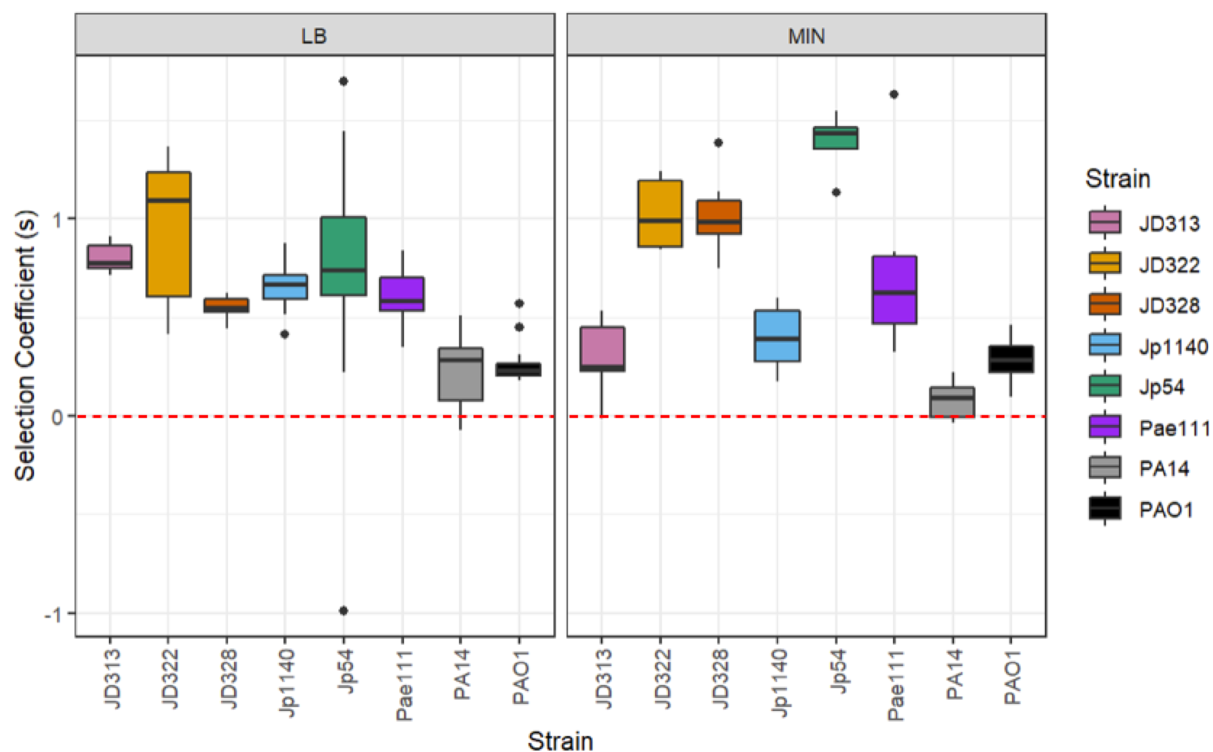

Figure 7. Box plots of population fitness (selection coefficient) of evolved populations by strain and medium. Colour represents ancestor strain and shape represents niche. Left side shows LB-evolved populations and right side shows MIN-evolved populations.

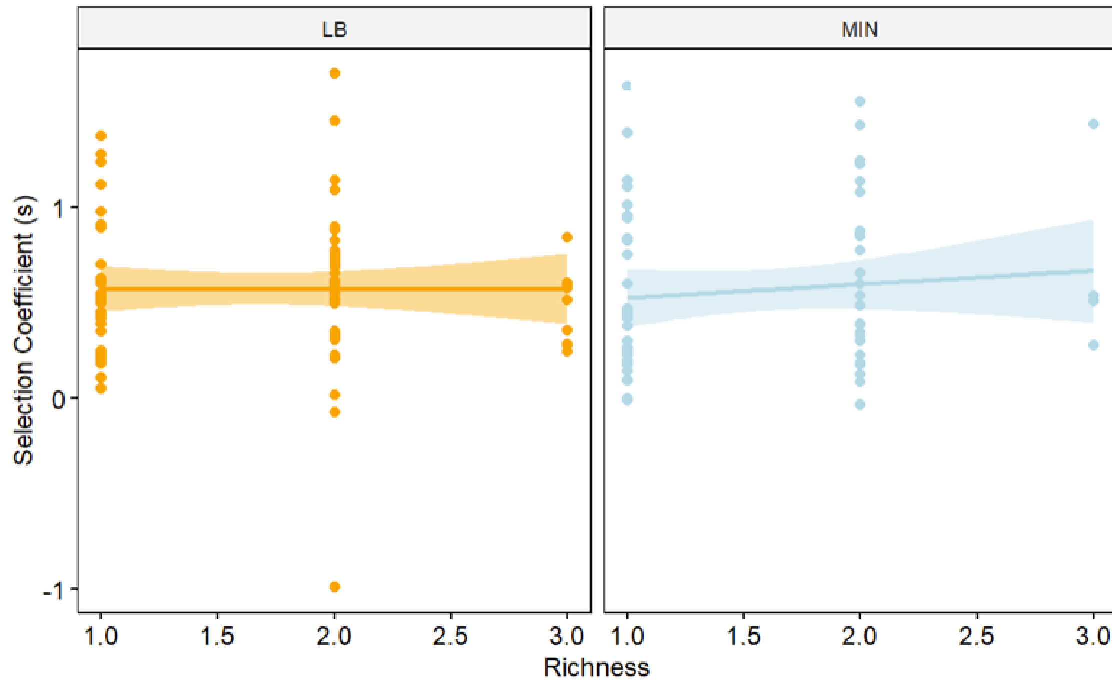

Figure 8. Relationship between population fitness (selection coefficient) and diversity (richness) after 750 generations of evolution. Left side (orange) shows LB-evolved populations and right side (blue) shows MIN-evolved populations.

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