ENVIRONMENTAL DRIVERS AND EVOLUTIONARY CONSEQUENCES OF HORIZONTAL GENE TRANSFER IN SOIL BACTERIA

By

Heather Kittredge

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Integrative Biology—Doctor of Philosophy
Ecology, Evolutionary Biology and Behavior—Dual Major

2021

ABSTRACT

ENVIRONMENTAL DRIVERS AND EVOLUTIONARY CONSEQUENCES OF HORIZONTAL GENE TRANSFER IN SOIL BACTERIA

By

Heather Kittredge

Horizontal gene transfer (HGT) is a driving force in bacterial evolution and could drive rapid adaptation in bacterial communities. Natural transformation is one mechanism of HGT that allows bacteria to pick up extracellular DNA (eDNA) from the environment and integrate it into their genome. But the rate of HGT in natural environments, and the role this process plays in facilitating rapid adaptation remains unknown. As climate change threatens the stability of environments worldwide, understanding how quickly bacteria can adapt to novel environments is essential. My dissertation research characterizes the environmental drivers and evolutionary consequences of natural transformation in a highly transformable model soil bacterium *Pseudomonas stutzeri*.

Despite decades of research on understanding HGT at the molecular level, less is known about the ecological drivers of HGT. To understand the soil conditions relevant for transformation, I first measured eDNA in the field over a short-term drying rewetting disturbance (Ch. 2). I found that eDNA increased in response to the rewetting disturbance but quickly disappeared from soil, suggesting a small portion of this eDNA could be transformed by bacterial cells recovering from the disturbance. To test the efficiency of transformation under the conditions in which eDNA disappeared, I created a novel microcosm system for quantifying transformation in soil (Ch. 3). Here, I inoculated soil with live antibiotic-susceptible, and dead antibiotic-resistant *P. stutzeri*. I

then tracked the evolution of antibiotic resistance over a range of soil conditions and eDNA concentrations. Transformation drove the evolution of antibiotic resistance across a wide range of soil moistures and increased in response to larger inputs of dead cells (eDNA source), with antibiotic resistance repeatedly appearing in antibiotic free soil.

Despite the prevalence of transformation across bacterial species, the evolutionary origins and consequences of transformation are still largely unknown. Transformation presumably provides a fitness benefit in stressful or continuously changing environments, but few studies have quantified changes in transformation in response to adaptive evolution. Here, I evolved *P. stutzeri* at different salinities and tested how the growth rate and transformation efficiency changed in response to salt adaptation (Ch. 4). Overall, the growth rate increased in response to adaptation, but the transformation efficiency declined, with only ~50% of the evolved populations transforming eDNA at the end of experiment – as opposed to 100% of ancestral populations transforming eDNA.

Overall, my dissertation research elucidates the factors driving transformation in soil, setting the stage for future experiments to scale up estimates of transformation to the whole community level. I find that transformation occurs under most soil conditions and allows genetic variants to arise at low frequencies in the absence of selection. I also report novel experimental evidence that transformation efficiency can change dramatically, and in a highly variable manner, over just ~330 generations. Taken together, this body of research highlights a role for transformation in many natural systems of ecological significance, and points to dead cells as an important but often overlooked source of genetic diversity.

This thesis is dedicated to Jake.
Thank you for always challenging the ‘ecological perspective’.

And to my family who always supported my dream to ‘play in the dirt’.

And to Sarah Evans whose unconditional support over the last 5 years made this dissertation possible!

ACKNOWLEDGEMENTS

First and foremost, I would like to thank Sarah Evans for her incredible support in research and in life. I will forever be grateful for the intellectual freedom you granted me in my research pursuits. I would also like to thank my committee for supporting my diverse research interests, your perspectives provided many unique and interdisciplinary insights. Finally, a thank you to the KBS community and especially to the members of the Evans lab, whose good energy and jovial attitudes made this experience that much better.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

ANOVA	Analysis of Variance
AR	Antibiotic resistance
ARG	Antibiotic resistance genes
eARG	Extracellular antibiotic resistance genes
eDNA	Extracellular DNA
$Gent_R$	Gentamicin resistance
GLBRC	Great Lakes Bioenergy Research Center
HGT	Horizontal gene transfer
Kan_R	Kanamycin resistance
LB	Lysogenic Broth
NaCl	Sodium Chloride
NMDS	Nonmetric multidimensional scaling
OTU	Operational Taxonomic Unit
PERMANOVA	Permutational multivariate analysis of variance
PMAxx	Propidium monoazide xx
qPCR	Quantitative PCR
R2A	Reasoner's 2A Agar
rRNA	Ribosomal ribonucleic acid

CHAPTER 1:

INTRODUCTION

Understanding how populations respond to rapid environmental change is a fundamental goal in ecology and evolutionary biology. Although bacteria are among the most ubiquitous organisms on Earth and there are many model systems for studying bacterial evolution in the laboratory, we still have a poor understanding of how quickly bacteria can adapt to changes in their natural environment [1], [2]. As climate change threatens to dramatically alter environments worldwide, understanding how bacterial populations will respond to these changes is essential in predicting effects on broader ecosystem processes [3], [4]. Bacteria are likely to respond faster than other organisms to environmental change due to their short generation times and their unique ability to move genetic elements among and within species through horizontal gene transfer (HGT) [5], [6]. The exchange of genes between and within bacterial species is of fundamental importance to the evolution of prokaryotic genomes, as evidenced by the rapid dissemination of antibiotic resistance genes in recent decades [7], [8]. Although this dissemination of foreign genes is extensively studied at the cellular level, little is known about the rate of HGT in natural environments, and the role this process plays in facilitating bacterial responses to environmental change [9], [10].

1.1 NATURAL TRANSFORMATION

Horizontal gene transfer (HGT) is the lateral exchange of genetic material between potentially unrelated living cells. It is a driving force in bacterial evolution, with estimates of HGT-driven gene gain and loss at least comparable to the rate of point mutations [6], [11]–[17]. HGT facilitates the acquisition of foreign DNA through three

main mechanisms: conjugation (pilus-mediated plasmid transfer requiring direct cell contact), transduction (DNA transfer by bacteriophages) and natural transformation (uptake of extracellular DNA).

Although HGT has been studied for decades, little is known about the relative contribution of each mechanism of HGT to bacterial evolution. My dissertation research focuses on natural transformation and its role in the soil environment. The acquisition of genes through natural transformation can accelerate genome evolution and increase a bacterium's probability of adapting and surviving to an existing or changing environment [10], [14], [18]. However, to date, transformation has predominately been studied in the lab and its relevance in accelerating genome evolution in natural environments remains largely unknown.

1.2 REGULATION OF COMPETENCE

The success of transformation depends on the development of competence – the physiological state of bacterial cells able to acquire free DNA from the environment [19]. Bacteria can be constitutively or conditionally competent in the laboratory. The induction of competence can be regulated by the cell cycle or induced by environmental cues [20]. However, these cues have been difficult to quantify experimentally since many bacteria seem unable to carry out transformation under laboratory conditions. In species that do undergo transformation in the lab, external factors that influence competence and transformation include temperature, pH, growth phase, nutrient concentration, and stress [21]–[23].

In soil, the induction of competence may be restricted by metabolic inactivity and lack of resources, as many soil bacteria are starved or living in a dormant state [24],

[25]. In particular, water and nutrient limitations could prevent transformation, as competence often requires high cell densities [23], [26]. Transformation has been shown to occur across many different environmental microorganisms [23], [27]–[29]. However, little is known about the environmental cues that regulate transformation for most bacterial species *in situ*. My dissertation research specifically addresses this knowledge gap by testing how transformation efficiency varies under different soil conditions, with a special focus on water availability which is one of the strongest controls on microbial processes in soil [30].

1.3 EXTRACELLULAR DNA IN THE ENVIRONMENT

In soil systems, transformation is often assumed to be infrequent compared to other modes of gene exchange such as conjugation and transduction. This is because extracellular DNA (eDNA) degrades rapidly in soil, reducing substrate availability for transformation [31]. However, eDNA can readily bind to clay and sand minerals, where it is protected from degradation but still available for transformation [22], [32]. This protective mechanism can generate large pools of eDNA, that in rare cases, exceed the intracellular DNA pool or the live fraction of the community [33]. This eDNA adsorbs to soil particles within 90-120 minutes of being released into soil and can persist for 6 months to years depending on the environmental conditions [22], [34], [35].

Laboratory studies show that eDNA concentration is a key driver of transformation, but it is unknown whether this translates to soil [36]. *In vitro* studies have shown that transformation increases in spatially structured environments (solid vs. liquid media), but even these poorly mimic the heterogenous soil environment, where cell-cell contact is likely restricted and eDNA availability could limit transformation. In this case,

more eDNA might be required to achieve the same transformation efficiencies observed in the lab. Indeed, conjugation rates are suppressed through genetic drift in spatially structured environments [37]. However, gene transfer is also more pervasive between bacteria that inhabit the same environment [38]. Because large pools of eDNA in soils could overcome spatial barriers, quantification of soil eDNA pools and their effect on transformation are critical for understanding bacterial evolution in nature.

1.4 EVOLUTIONARY ORIGINS OF TRANSFORMATION

Whether or not bacteria can survive in the absence of HGT has been debated for years [39]–[41]. Several theories exist to explain the evolutionary origins and potential benefits of transformation (reviewed in Seitz and Blokesch 2013). 1) Incoming eDNA can be used as a nutrient resource, 2) eDNA can serve as a template for genome repair or 3) eDNA can increase genetic diversity via recombination. A new amendment to this final theory suggests that transformation facilitates genetic recombination to rid bacterial genomes of selfish genetic elements [43], [44]. While transformation is predominately cited as a mechanism that evolved to diversify bacterial genomes, the evolutionary origins, and consequences of transformation are idiosyncratic.

The presence of cellular machinery dedicated to protecting transformed eDNA from degradation inside the cell, suggests that eDNA is not acquired purely for the nutrient benefit [42], [45]. In addition, bacteria use discriminatory mechanisms that allow them to preferentially kill and transform eDNA from close relatives – implying certain eDNA is preferable [46], [47]. This provides further support for the theory that transformation promotes genetic exchange within bacterial species and may in some scenarios act like meiotic sex in eukaryotes. However, the most convincing evidence

that transformation is a mechanism of genetic recombination, comes from population genetic models, [11], [48], [49] and experimental evolution studies [50], [51], which show that bacteria capable of transformation can have higher rates of population-level adaptation than non-transformers.

Despite this evidence, transformation also presents increased risks of acquiring deleterious DNA or unnecessarily expanding a bacterium's genome [52]. Seminal theoretical work concluded that, 'sex with dead cells was better than no sex at all' [53]. However, experimental research suggests a more complex relationship between fitness and transformation, in which the benefits of transformation are highly dependent on the fitness landscape and the environment [50], [54], [55]. Transformation can be beneficial in stressful or novel environments, but the fitness benefits are often small [54]. A major gap in this body of work is quantification of transformation efficiency after adaptive evolution, as previous work has disproportionately focused on differences in population-level fitness between competent and non-competent lineages without screening for differences in transformability across evolved lineages [50], [51]. Consequently, it has yet to be determined if transformation is subject to selection, and furthermore, what selective forces could act to increase or decrease transformation efficiency in natural systems.

1.5 *PSEUDOMONAS STUTZERI*: MODEL SYSTEM

Pseudomonas stutzeri is a highly transformable soil bacterium previously used to study transformation in soil [23], [28], [56], [57]. The highly transformable lineage was originally isolated from soil and acquires eDNA at a high efficiency under a variety of conditions. *P. stutzeri* is broadly relevant as it exchanges genes with several

opportunistic pathogens, including *Pseudomonas aeruginosa* and enterobacteria [58]. In addition, *P. stutzeri* represents an environmental vector for antibiotic resistance genes to spread to new pathogenic hosts in soil [59]. My dissertation research specifically explores the potential for *P. stutzeri* to act as a vector in the transformation of antibiotic resistance genes in soil.

1.6 RESEARCH QUESTIONS

More research is needed to understand the occurrence and importance of transformation in soil. Thus, the goal of my dissertation is to improve our understanding of the environmental conditions that promote transformation in natural systems. I address the following research questions:

1. How does the availability of soil eDNA change over a drying rewetting pulse disturbance?

2. What soil conditions promote transformation and do these conditions coincide with eDNA availability (determined in Q1).

3. How does the growth rate and transformation efficiency of *P. stutzeri* change in response to salt adaptation?

CHAPTER 2:

EXTRACELLULAR DNA MASKS MICROBIAL RESPONSES TO A PULSE DISTURBANCE

2.1 ABSTRACT

A major goal in microbial ecology is to predict how microbial communities will respond to global change. However, DNA-based sequencing that is intended to characterize live microbial communities includes extracellular DNA (eDNA) from dead cells. This could obscure relevant microbial responses, particularly to pulse disturbances, which kill bacteria and have disproportionate effects on ecosystems. Here, I characterized bacterial communities before and after a drying rewetting pulse disturbance, using an improved method for eDNA exclusion. I found that eDNA removal was important for detecting subtle yet significant changes in microbial abundance, diversity, and community composition across the disturbance. However, eDNA removal was less important for detecting differences between crop types and disturbance regimes. The size of the eDNA pool mainly affected estimates of bacterial abundance, while eDNA pools enriched in unique sequences (e.g. sequences not found in the live community), mainly affected estimates of community structure – irrespective of size. Consequently, eDNA inclusion made bacterial communities appear as though they had the same structure before and after the disturbance, when the live fraction was in fact different. Overall, pulse disturbance studies have a high risk of eDNA bias and should remove eDNA to improve predictions of ecosystem responses in future climates.

2.2 INTRODUCTION

Large pools of prokaryotic extracellular DNA (eDNA) can accumulate in the environment as bacteria die [23], [26]. This eDNA is included in molecular

characterizations of ‘live’ communities, potentially altering estimates of bacterial abundance, diversity, and community composition. Despite this danger, we know surprisingly little about the conditions that make microbial characterizations vulnerable to eDNA bias. Here, I consider a result to be ‘biased’ by eDNA if the biological interpretation of the result is altered by eDNA removal (i.e. if eDNA alters the ability to detect a change or the size of that change). Experiments testing bacterial responses to lethal disturbances could be especially susceptible to eDNA bias because bacterial death can increase necromass-derived eDNA. Here, I test whether eDNA alters our ability to characterize bacterial abundance, diversity, and community structure following soil drying rewetting, a stressful and likely lethal, pulse disturbance [60], [61]. To extend the generality of these findings, I also examine why eDNA bias emerges. Based on previous studies, I hypothesize that eDNA is most likely to bias results when eDNA pools are (i) large and (ii) enriched in sequences not present in the live community [33], [62].

To test this, I sampled soil bacteria that were subject to extreme drying rewetting in the field for 6 months. Rainout shelters allowed us to impose a moisture extreme outside of historical norms for the region (28 days of drought and 80 mm rain event). I collected soil samples at 3 timepoints across a drying-rewetting event: at the end of the drought (~6hrs before rewetting, t1), 1hr after rewetting (t2) and 18hrs after rewetting (t3). Rapid rewetting after long dry periods has been shown to cause bacterial death [63]–[65], so I expected eDNA pools to increase in the first hour after rewetting, but to decline after 18hrs, as increases in soil moisture stimulate eDNA decay [66]. I repeated this sampling in contrasting soils (conventionally-tilled corn monoculture and perennial

switchgrass monoculture), since differences in soil moisture and bacterial communities could affect eDNA pool dynamics and thus sensitivity to eDNA bias [67]–[69].

To isolate the effects of eDNA, I eliminated eDNA pools in one of two paired soil samples using the chemical propidium monoazide (PMAxx) which binds to eDNA and prevents amplification (modifying and improving the efficacy of methods in Carini et al. [33], Figure A2.1). I sequenced the 16S rRNA gene to characterize the live and extracellular fractions of the soil bacterial community.

2.3 MATERIAL AND METHODS

2.3.1 SITE AND SOIL COLLECTION

I exposed soil bacterial communities to ambient drying rewetting (6.6mm of rain every 3 days) and extreme drying rewetting (80mm of rain every 28 days) between April 3rd and September 17th in experimental Biofuel Cropping System plots located at the U.S. Department of Energy's Great Lakes Bioenergy Research Center (GLBRC) at the Kellogg Biological Station in Southwest, Michigan (42°23′47″N, 85°22′26″W, 288 m a.s.l.). On September 20th of 2017 I collected soil cores (10cm depth by 5cm diameter) from continuous corn (*Zea mays*) and continuous switchgrass (*Panicum virgatum,* variety "Cave-in-Rock") plots at three-time points; 6-hrs before, 1-hr after, and 18-hrs after a rewetting event, which also coincided with the end of a 28-day drought. The soils are well-drained mesic Typic Hapludalfs developed from glacial till and outwash consisting of co-mingled Kalamazoo (fine-loamy, mixed, semiactive) and Oshtemo (coarse-loamy, mixed, active) series (Crum and Collins 1995) with intermixed loess [70]. Bulk soil was brought back to the lab, sieved at <2mm and homogenized. Gravimetric soil moisture was determined on sieved soils dried at 60°C for 72 hrs. Soil moisture

increased in response to the extreme rewetting event (Figure 2.1A: Corn $p<0.0001$; Switchgrass $p=0.035$), while ambient rewetting had no effect on soil moisture (data not shown).

2.3.2 REMOVAL OF EXTRACELLUALR DNA

Methods for quantifying eDNA were modified from Carini et al. [33]. Two DNA extractions were completed for each soil sample using the DNeasy PowerSoil kit from Qiagen (previously PowerSoil DNA Isolation Kit by MO BIO). A paired sample design was used where one DNA extraction tube was treated with the chemical propidium monoazide (PMAxx Dye, 20mM in H_2O from Biotium) which prevents amplification of eDNA. The other DNA extraction was left untreated to quantify the total DNA pool. Both tubes were treated identically through the PMAxx activation steps. For each soil sample, 0.50 grams of soil was weighed out and divided evenly between the two DNA extraction tubes (0.25 g each). Prior to loading soil into the DNA extraction tubes, I removed the bead beating beads from sample tubes. The beads were removed for the steps involving light activation of PMAxx to prevent potential cell lysis. Then 500µl of the Powerbead solution (solution that comes in the tubes with the beads) was returned to the tube. In a dark room, 3µl of PMAxx was added to the 500µl of PowerBead Solution in the transparent bead beating tubes. Samples were homogenized for 1 minute and exposed to a 1000 W halogen light source for ten minutes while undergoing frequent homogenization (1000-Watt Halogen Telescoping Twin Head Tripod Work Light in a tinfoil lined cabinet). After light exposure, I returned the Powerbeads and the remaining PowerBead solution (~250µl) to the DNA extraction tube. The DNA was then extracted according to manufacturer's instructions, however, samples treated with PMAxx were

handled in low light conditions – minimal ambient light from windows – to minimize the binding of PMAxx to DNA released during the DNA extraction. The concentration of PMAxx was selected for this experiment because it optimized eDNA removal at higher concentrations of eDNA. However, this also led to increased variation in estimates of eDNA in smaller eDNA pools (Figure A2.1). In addition, previous work has shown that agricultural soils have low eDNA concentrations compared to deciduous or coniferous forest soils (Carini et al. 2016). Indicating future studies should optimize the PMAxx concentration to their specific study. In general, I recommend completing the DNA extraction (following PMAxx activation) in low light conditions or a dark room, as this reduces the probability that excess PMAxx (which is light activated) will bind to intracellular DNA that is released in the first step of the DNA extraction.

2.3.3 QUANTITATIVE PCR

I performed quantitative PCR in triplicate using 96-well plates on an Thermofischer thermocycler. I used 3 technical replicates for each PMA-treated and un-treated soil sample (Figure A2.1C). The total reaction volume was 20 µl with the following reaction mixture: 1 µl of each F and R primer (515f/806r at 10mM), 10 µl of iQ SYBR Green 2X Supermix (BIO-RAD), 4 µl sterile water, and 4 µl template DNA. The cycling conditions were: 95 °C for 15 min, followed by 40 cycles of 95°C 30 s; 50 °C 30 s; 72 °C 30 s. I generated melting curves for each run to verify product specificity by increasing the temperature from 60-95°C. Reactions were compared to standard curves developed using purified *Pseudomonas stutzeri 28a24* genomic DNA. For all qPCR reactions the linear relationship between the log of the copy number and the threshold cycle value was reported at $R^2 > 0.99$. Outlier analysis was performed on qPCR

replicates. I removed qPCR replicates that were 2 cycles above or below the other two replicates – this resulted in the removal of 1 qPCR replicate from 3 samples (samples 2_PMA, 6_Total and 42_PMA in Figure A2.1C (denoted by *)). Two soil samples with negative eDNA estimates were removed from the third time point analysis in both corn and switchgrass soil as they fell outside of the standard curve and were biologically inaccurate (sample 34 and 44, highlighted with gray bars in Figure A2.1C). Across the entire data set, soil samples treated with PMAxx had lower variation across qPCR replicates than untreated soil samples (Figure A2.1B).

2.3.4 AMPLICON SEQUENCING AND BIOINFORMATICS

I characterized bacterial communities using high throughput barcoded sequencing on the Illumina MiSeq platform at the Research Technology Support Facility (RTSF) Genomics Core at Michigan State University. Sequencing was done in a 2 × 250bp paired end format using a MiSeq v2 500 cycle reagent cartridge. The V4 hypervariable region of the 16S rRNA gene was amplified using dual-index, Illumina compatible primers 515F and 806R as described in Kozich et al. [71]. Completed libraries were normalized using Invitrogen SequalPrep DNA Normalization plates, then pooled and cleaned up using AmpureXP magnetic beads. The 16S reads were quality filtered and merged using the USEARCH pipeline (http://drive5.come/usearch/) and Cutadapt was used to remove primers and adapter bases before reads were filtered and truncated to 250bp. OTUs were clustered at the 97% sequence identity level and classified using UPARSE. Two samples with low read number were removed, making the lowest read number 7723. Singletons, chloroplasts, and mitochondria were removed (312 total) resulting in 15,062 bacterial OTUs and 2,259,918 reads across all samples.

All analyses were performed and visualized using bacterial counts that did not undergo rarefaction [72].

2.3.5 STATISTICAL ANALYSES

I tested for differences among univariate response variables (e.g. soil moisture, Shannon diversity, and bacterial abundance or number of 16S rRNA gene copies) using Type III sum of squares ANOVA with a post-hoc Tukey HSD correction test ($p < 0.05$) using the lme4 and agricolae packages in R. For all analyses, I report average values across the four field replicates, except for the qPCR data, where I first calculated the number of 16S rRNA gene copies for each technical replicate (n=3 qPCR replicates, Figure A2.1C). I calculated the percent eDNA by subtracting the number of 16S rRNA gene copies in the live fraction from the total number of copies, and then dividing by the total number of copies [(Total-Live)/(Total)*100].

I compared statistical differences in bacterial community composition using permutational analysis of variance (PerMANOVA) (9999 permutations) on weighted Unifrac distance matrices (Bray-Curtis distance) using the Adonis function in the vegan R package. I visualized community composition using Non-metric multidimensional scaling (NMDS) using phyloseq and ggplot2. I examined the proportion of taxa present in both the live community and eDNA pool using the Venn Diagram function in R. To assess shared membership in the eDNA pool I performed a Venn Diagram analysis on each field replicate and calculated the mean proportion of taxa specific to the eDNA pool at each time point. I report the proportion of taxa in this analysis instead of the raw number of taxa to account for changes in the total number of taxa across the disturbance. To estimate the effect of eDNA removal, I calculated standardized effect

sizes on soil samples with and without eDNA, according to biascorrected Hedges' $g*$ values [73] using the effsize package in R.

2.4 RESULTS AND DISCUSSION

Understanding when eDNA affects microbial community characterizations is important for accurately linking microbial dynamics to belowground processes [68], [74]. By comparing analyses in which eDNA was excluded to samples in which it was included (the approach of most studies), I was able to identify the microbial responses most sensitive to eDNA bias in two different soils – informing when future studies might invest in eDNA removal. Overall, I found that eDNA masked changes in bacterial community composition and abundance that occurred across drying-rewetting in corn soil (Table 2.1, Figure 2.1B,D,F). In contrast, eDNA inclusion did not mask responses to drying-rewetting in switchgrass soil, but there were fewer responses to mask, as soil moisture was more stable and may have buffered communities from drying-rewetting stress (Figure 2.1A).

The disturbance responses most vulenrable to eDNA bias differed from expectation. I expected eDNA bias to increase after bacterial death (timepoint t1 to t2), instead it was driven by the successional recovery of the disturbance-effected community (t1-t3 in corn soil, see Table 2.1, Figure 2.1A).This post-disturbance community (t3) was larger and compositionally distinct from the drought community (t1). However, these signatures of bacterial resilience were not detected in analyses that included eDNA, and consequently made it appear as though corn communities were largely unaffected by the disturbance (Table 2.1).

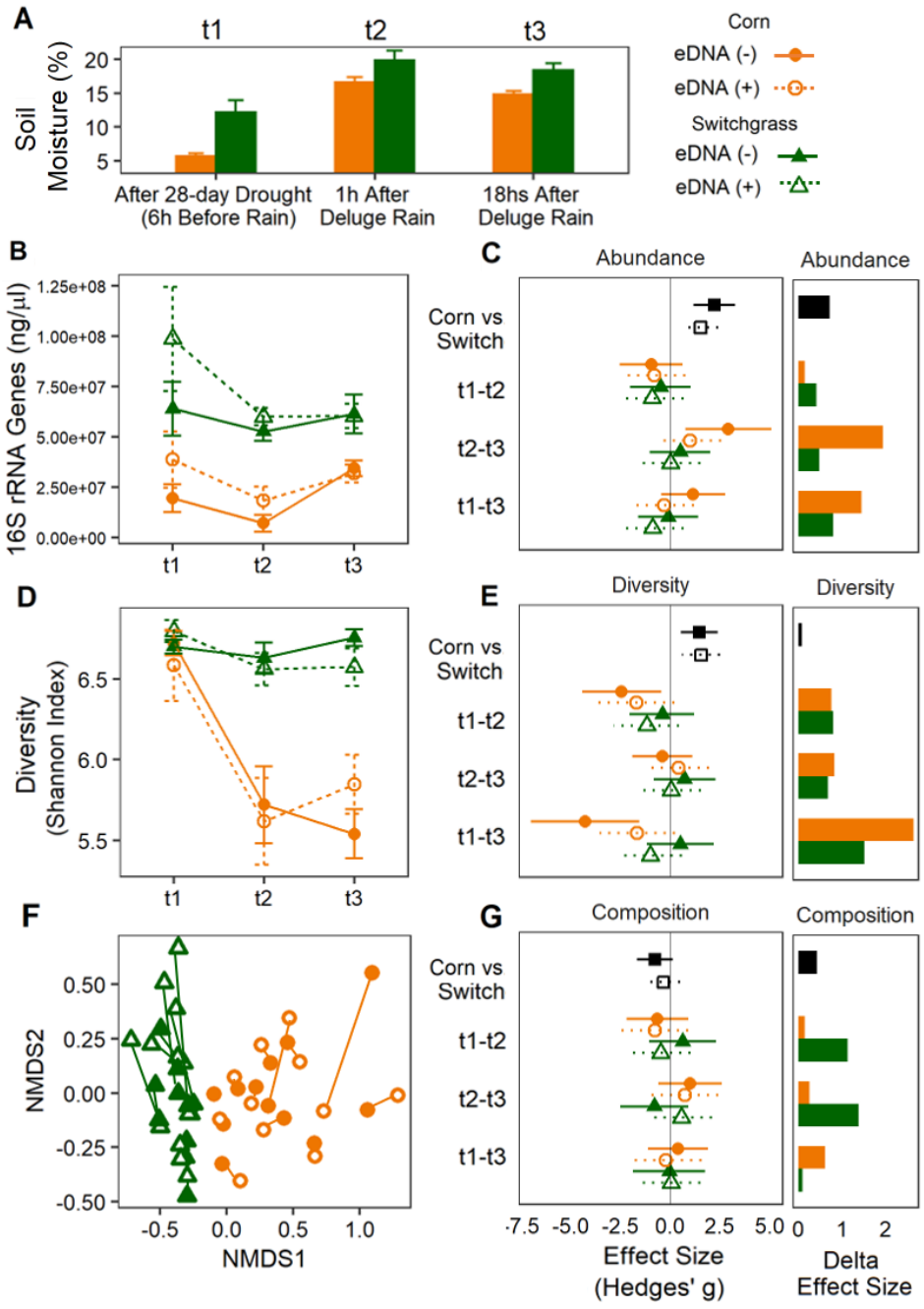

Figure 2.1: Effect of eDNA removal on bacterial responses to drying rewetting. (A) Soil moisture (B) average bacterial abundance (16S rRNA genes) and (D) average Shannon diversity including eDNA (dotted lines) or excluding eDNA (solid lines). (F) NMDS plot showing the composition of bacterial communities with eDNA (open points) or without eDNA (opaque points), with lines connecting paired soil samples. (C-G) Effect size for changes in (C) abundance, (E) diversity and (G) community composition including eDNA (open points, dotted lines) and excluding eDNA (opaque points, solid lines) with lines showing the 95% confidence interval. Positive effect sizes correspond to a larger value in switchgrass soil or an increase across the disturbance. The third panel shows the magnitude of change in effect size after excluding eDNA (A-G; n=4 field replicates).

Table 2.1: Results impacted by eDNA bias. Significant results that were masked by eDNA inclusion or already significant results that became more significant after eDNA removal. The rank refers to the effect of eDNA removal with 1 being the largest change in effect size after eDNA removal. (-) corresponds to p-values without eDNA and (+) corresponds to p-values with eDNA.

Treatment	Measure	Time	Δ Effect Size	Rank	(-)	(+)
Corn	Abundance	t2-t3	1.939	2	0.003	0.16
Corn	Diversity	t1-t2	0.76	10	0.007	0.047
Corn	Diversity	t1-t3	2.63	1	0.0004	0.05
Corn	Composition	t1-t2	0.14	19	0.05	0.076
Corn	Composition	t1-t3	0.61	13	0.033	0.085

Overall, I did not find support for hypothesis (i); large eDNA pools did not drive eDNA bias, but I did find support for hypothesis (ii). That is, eDNA bias was largely driven by a divergence between the live community and eDNA pool (except for changes in abundance which are inherently impacted by size). Here, eDNA bias increased when the eDNA pool was enriched in sequences not in the live community – even when the eDNA pool was small (compare Figure 2.2A, B). It may be that as the live community shifted in response to rewetting, the composition of the eDNA pool continued to reflect the drought community (Figure 2.2A, B**,** see expanded results). This caused a divergence between the live community and the eDNA pool that masked a significant shift in the live fraction of the corn community post-disturbance (Figure 2.2B) and altered conclusions about which taxa were sensitive to the disturbance (Table A2.2).

In addition to masking signficant results, eDNA also has the potential to create false positives – as was evident in switchgrass soil – where eDNA inclusion inflated changes in bacterial diversity and community composition across the disturbance (although not signficantly). In switchgrass communities eDNA inclusion generally inflated the effect of the disturbance (Figure 2.1E,G), making switchgrass communities

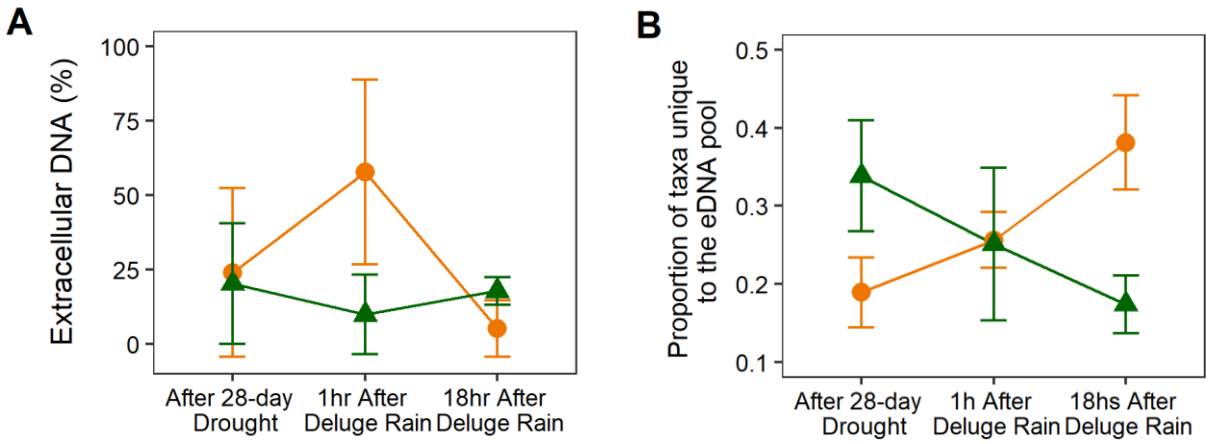

Figure 2.2: Changes to the eDNA pool in response to drying rewetting. (A)The eDNA (%) in response to soil rewetting for corn (orange circles) and switchgrass (green triangles) soil. (B) The number of eDNA-specific sequences (those found only in the eDNA pool at that sampling point) across the disturbance. (A-B) Bars and points show the average and error bars show the standard error (n=4 field replicates).

appear less resilient to drying-rewetting than was true in the live-fraction of the community – the opposite effect of eDNA in corn soil. Not only were trends inconsistent across crop type, but eDNA inclusion skewed trends across the disturbance. For instance, diversity decreased in corn soil in the 18hrs after rewetting, however, eDNA inclusion created the perception that diversity increased. To further complicate matters, the effect of eDNA removal (as measured by effect size) was often of similar magnitude in corn and switchgrass communities, despite eDNA only masking siginficant responses in corn soil. Ultimately, highlighting how difficult it is to predict the magnitude and direction of change associated with including eDNA in microbial community chracterizations.

Overall, our study suggests that eDNA removal is necessary for understanding microbial responses to short-term or pulse disturbances, particularly when changes in the live community may be subtle and occur over rapid timescales. However, eDNA removal may be less important for detecting large differences across land use types, as

even across the disturbance, corn and switchgrass communities were compositionally distinct – irrespective of eDNA removal. Since many global change studies are interested in the resistance and resilience of specific micorbial communities, including under more varied rainfall, pulse disutrbance studies should remove eDNA, as this will improve predictions of bacterial resilience and ecosystem function in future climates.

APPENDIX

APPENDIX

2.5 SUPPLEMENTAL RESULTS

In general, corn communities were more sensitive than switchgrass communities to the rewetting disturbance. This was evidenced by the significant drop in diversity from t1-t2 in corn soil. As well as a 56% decline in the number of 16S copies during this same window in corn soil and contrasted a 6% decline in switchgrass soil. Consequently, eDNA increased 34% in corn soil from t1-t2 (Figure 2.2A), even though the number of 16S copies in the eDNA pool actually dropped in both soils in response to rewetting (Figure 2.1B). This jump from 24-58% eDNA is the largest in the dataset and masks a marginally significant shift in community composition (Table 2.1). Then between t2-t3 in corn soil, the number of 16S copies in the live fraction of the community increased significantly (Figure 2.1B), far outweighing the decline that followed rewetting. This signature of disturbance recovery is undetectable in analyses that include eDNA though, as eDNA inflated the size of the corn community at t1 (Figure 2.2B) – making it appear as though the corn community was the same size before and after the disturbance.

At t3, only 5% eDNA remained in corn soil, yet the eDNA pool was more divergent from the live community than at the previous two sampling points (Figure 2.2A,B). This suggests that the risk of eDNA bias is not solely caused by large eDNA pools. I predict that following the disturbance, only certain bacterial taxa were able to recover and increase in abundance. Thus, creating a community that looked quite different than the t1 drought community. With only ~600 taxa remaining in the corn community at t3 – in contrast to ~1400 at t1 – it may be that rapid responders, likely with

a high 16S copy number, are in the first stages of a successional recovery event. However, this ecologically relevant and functionally important disturbance dynamic, is completely undetectable in analyses that include eDNA – making it appear as though bacterial abundance and community composition are the same before and after the disturbance.

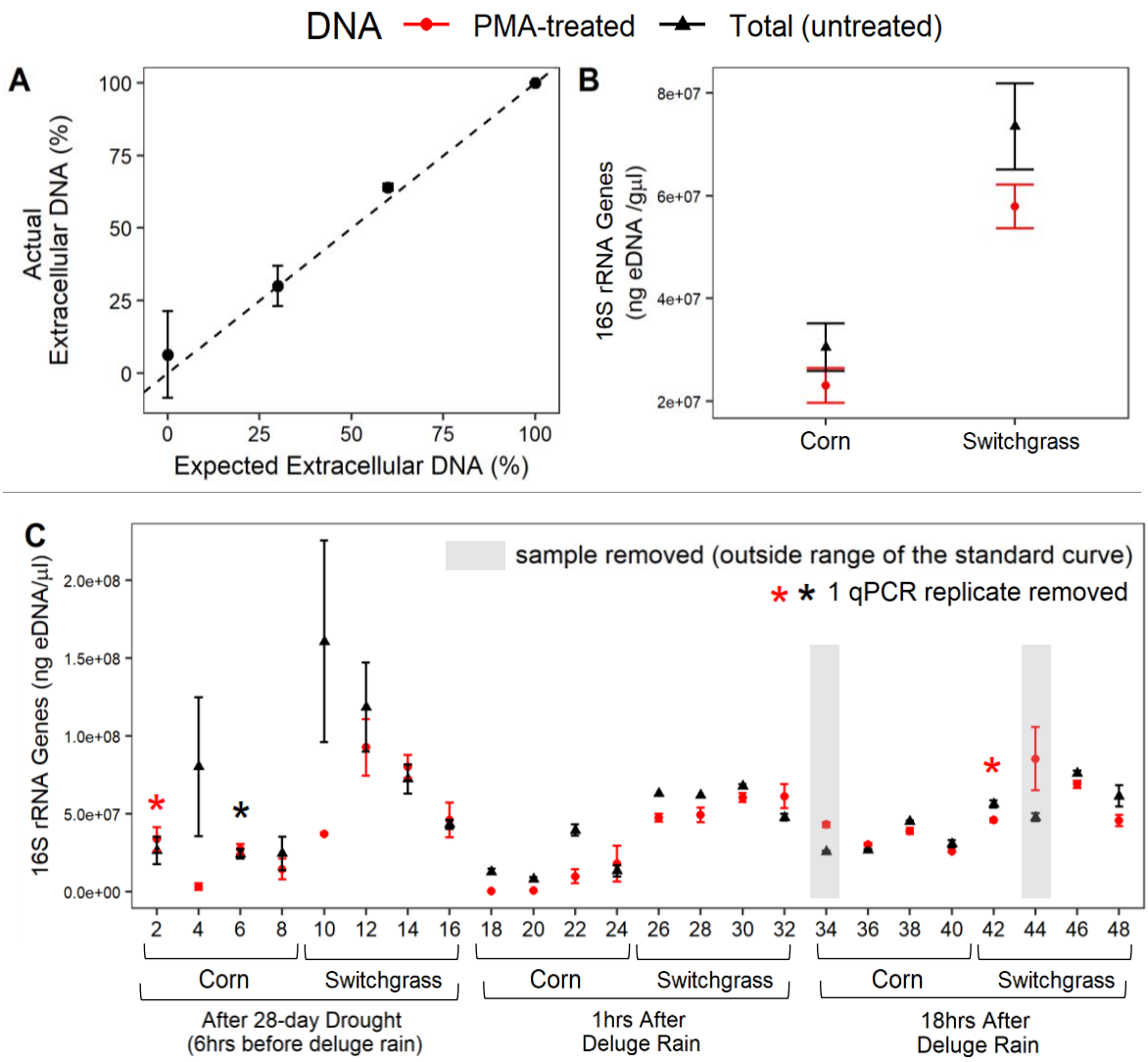

Figure A2.1: Technical aspects of eDNA removal. (A) Standard curve for the PMA-treatment in mock communities of live and dead *Pseudomonas stutzeri* inoculated into switchgrass soil (*Panicum virgatum,* variety "Cave-in-Rock"). Points show the average eDNA (%) in each mock community regressed against the expected eDNA (%), error bars show the standard error of the mean and the dashed line corresponds to x=y (n=3 soil replicates and n=3 qPCR replicates). (B) Average number of 16S rRNA gene copies in the PMA-treated (red circles) and untreated soil (black triangles) samples across the time series for corn and switchgrass soil with the error bars showing the standard error (Corn n=46 and Switchgrass n=47). (C) The average number of 16S rRNA gene copies across the 3 technical replicates (n=3 qPCR replicates) for each PMA-treated and untreated soil sample. The corresponding point in the time series and its crop type are shown below the plot. Two samples that fell outside the standard curve were removed and are highlighted in gray. Red asterisks indicate a PMA-treated technical replicate was removed and a black asterisk indicate an untreated technical replicate was removed.

Table A2.1: Expanded table of effect sizes and p-values for eDNA removal. Complete list of effect sizes and p-values for changes in abundance, diversity, and community composition across the disturbance. C corresponds to corn and S to switchgrass. The rank refers to the effect of eDNA removal with 1 being the largest change in effect size after eDNA removal. (-) corresponds to p-values without eDNA and (+) corresponds to p-values with eDNA. Results are listed in the same order as in Figure 2.1C, E, G. Positive effect sizes correspond to a larger value in switchgrass soil or an increase across the disturbance (t2 higher than t1).

			Effect Size (hedges' g)				p-value	
Treatment	Measure	Time	(-)	(+)	Δ	rank	(-)	(+)
C vs S	Abundance	C vs S	2.23	1.52	0.71	11		
C	Abundance	t1-t2	-0.957	-0.81	0.147	18		
S	Abundance	t1-t2	-0.488	-0.903	0.415	16		
C	Abundance	t2-t3	2.93	0.991	1.939	**2**	0.003*	0.16
S	Abundance	t2-t3	0.499	0.021	0.478	14		
C	Abundance	t1-t3	1.16	-0.29	1.45	**4**		
S	Abundance	t1-t3	-0.09	-0.88	0.79	9		
C vs S	Diversity	C vs S	1.47	1.55	0.08	21		
C	Diversity	t1-t2	-2.45	-1.69	0.76	10	0.007	0.047
S	Diversity	t1-t2	-0.385	-1.18	0.795	8		
C	Diversity	t2-t3	-0.39	0.436	0.826	7		
S	Diversity	t2-t3	0.72	0.046	0.674	12		
C	Diversity	t1-t3	-4.29	-1.66	2.63	**1**	0.0004	0.05
S	Diversity	t1-t3	0.51	-1	1.51	**3**		
C vs S	Composition	C vs S	-0.77	-0.35	0.42	15		
C	Composition	t1-t2	-0.64	-0.78	0.14	19	0.05	0.076
S	Composition	t1-t2	0.63	-0.49	1.12	6		
C	Composition	t2-t3	0.99	0.74	0.25	17		
S	Composition	t2-t3	-0.8	0.58	1.38	**5**		
C	Composition	t1-t3	0.38	-0.23	0.61	13	0.033*	0.085
S	Composition	t1-t3	-0.05	0.04	0.09	20		

Table A2.2: The top 15 taxa driving changes in response to soil rewetting. Dominant taxa driving shifts across the 3 sampling points – when eDNA is excluded and included in analyses. Taxa or OTUs that are incorrectly identified in the presence of eDNA are italicized.

Taxa Driving Responses to Soil Rewetting	
(-) eDNA excluded	**(+) eDNA included**
Corn Bulk Soil	
Mizugakiibacter	Mizugakiibacter
Sphingomonas	Thermosporotrichaceae
Thermosporotrichaceae	*Rhodanobacter*
Acidobacterium	Sphingomonas
Xanthomonadaceae	Acidobacterium
Bryobacter	Bryobacter
Bryobacter	*Chitinophagaceae*
Acidothermus	*Mizugakiibacter*
Sphingobium	*Chitinophagaceae*
Sphingomonas	*Castellaniella*
Deltaproteobacteria (GR_WP33-30)	Bryobacter
Xanthomonadaceae	*Acidobacteriaceae_[Subgroup_1]*
Acidobacteria (Subgroup_6)	*Acidobacteriaceae_[Subgroup_1]*
DA101_soil_group	*Acidobacteriaceae_[Subgroup_1]*
Sphingomonas	*Bradyrhizobium*
Switchgrass	
DA101_soil_group	*unclassified*
Sphingomonas	*Bradyrhizobium*
Holophagae	*Luteolibacter*
Nitrosomonadaceae	*Sphingomonas*
Acidobacteria (subgroup_4)	*Flavobacterium*
Acidobacteria (subgroup_4)	*Cellvibrio*
Acidobacteria (subgroup_4)	Acidobacteria (subgroup_12)
Acidobacteria (subgroup_12)	*Haliangium*
Sphingomonas	*Variibacter*
Haliangium	*Pseudomonas*
Acidobacteria (subgroup_6)	*Chloroflexi*
Reyranella	*Latescibacteria*
DA101_soil_group	*Acidobacteria (subgroup_6)*
Acidobacteria (subgroup_12)	DA101_soil_group
Acidobacteriaceae_[Subgroup_1]	DA101_soil_group

CHAPTER 3:

HORIZONTAL GENE TRANSFER FACILITATES THE SPREAD OF EXTRACELLULAR ANTIBIOTIC RESISTANCE GENES IN SOIL

3.1 ABSTRACT

It is now clear that the environment plays a major role in the dissemination of antibiotic resistance genes (ARGs), which are ubiquitous in the environment and pose a serious risk to human and veterinary health. While many studies focus on the spread of *live* antibiotic resistant bacteria to the environment, less is known about the contribution of extracellular ARGs to the evolution of antibiotic resistance in natural systems. In this study, I inoculate antibiotic-free soil with extracellular ARGs (eARGs) from dead *Pseudeononas stutzeri* cells and track the evolution of antibiotic resistance via natural transformation – a mechanism of HGT involving the genomic integration of eARGs. I find that transformation facilitates the rapid evolution of antibiotic resistance even when eARGs are rare (0.25 μg g^{-1} soil). However, when eARGs are abundant, transformation increases substantially. In general, transformation occurred under most soil conditions tested and was only inhibited at very high soil moistures (>30%). Finally, I show that transformed eARGs are just as successful as live antibiotic resistant invaders, when challenged with a low dose of antibiotic. Overall, this work demonstrates that dead bacteria are an overlooked path to antibiotic resistance, and that disinfection alone is insufficient to stop the spread of ARGs. More generally, the spread of ARGs in antibiotic-free soil, suggests that transformation allows genetic variants to establish at low frequencies in the absence of selection.

3.2 INTRODUCTION

Antibacterial resistance is a global threat to public health and could have a higher death toll than cancer by 2050 [75]. To reduce the impacts of antibiotic resistance on human health, we need to understand how antibiotic resistance genes (ARGs) move through the environment [76], [77]. However, the evolution of antibiotic resistance has traditionally been viewed as a clinical problem, and consequently less is known about when and how novel antibiotic resistant pathogens emerge from natural systems [78], [79]. ARGs in the environment are particularly concerning because they pose a significant threat to food and water resources [77] and can spread to new hosts through horizontal gene transfer (HGT) [79]–[82]. The spread of ARGs via HGT is a major mechanism in the rise of antibiotic resistance [7] but the environmental variables that promote the transfer of ARGs remain poorly understood [76], despite well-documented instances of ARGs moving from the environment to the clinic [83]–[85].

An important, but often overlooked source of environmental ARGs is extracellular DNA (eDNA) [86], [87]. Extracellular ARGs (eARGs) enter the environment through active secretion or bacterial death, and once there can integrate into new bacterial genomes through a mechanism of HGT called natural transformation. Soil harbors one of the largest environmental reservoirs of eARGs [88], [89] and is home to many antibiotic producing bacteria that could select for the maintenance of eARGs in new hosts [79]. Since eARGs can persist in soil for more than 80 days – in comparison to less than 1 day in aquatic environments – the odds of a transfer event and subsequent spread, are high in soil [34]. Overall, understanding the occurrence of these transfer events will be important for the early detection of multi-drug resistance, especially since

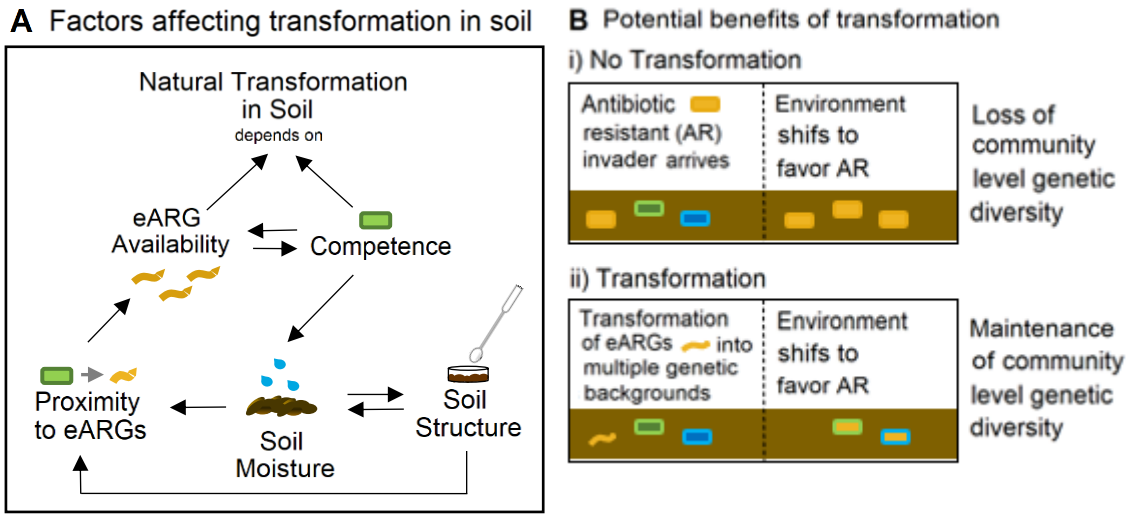

Figure 3.1: Soil characteristics likely to affect natural transformation. (A) Transformation requires cellular competence and the presence of eARGs. But is also likely to depend on the soil moisture, soil structure, and proximity to eARGs. Arrows show possible interactions between soil characteristics and point towards the effected variable. (B) In this conceptual framework, a novel ARG enters soil via a live invader (i) or via transformation (ii). In the transformation scenario, the blue and green cells susceptible to the antibiotic acquire the ARG via transformation which allows the ARG to move into multiple genetic backgrounds. This preserves community-level genetic diversity that would have been lost if a single antibiotic resistant invader rose to fixation in the presence of an antibiotic.

11 of the 12 priority antibiotic resistant pathogens are known or predicted to be naturally transformable [90].

In soil, transformation is likely to depend on the concentration of eARGs, as transformation generally increases with the availability of eDNA when measured in the lab (Figure A2.1A). However, transformation may proceed differently in complex environments like soil. For instance, soil is spatially heterogenous and spatial barriers have been shown to limit the transfer of plasmids via conjugation [37]. In addition, eDNA can persist in soil for longer than other environments, but its stability is still likely to depend on soil properties like water content [66]. These same soil properties may also impact the tendency for eDNA to undergo transformation via effects on recipient cells. In particular, the conditions which favor eDNA stability may differ from those that favor

competence – the physiology state of transforming cells. For instance, wetter soils may favor active growth which often induces competence, while in contrast eDNA degradation increases with water availability and could prevent transformation. Finally, the availability of moisture could directly impact processes like biofilm formation which is known to facilitate gene transfer [91], [92]. See Figure 3.1A for a conceptual diagram of soil characteristics likely to influence transformation.

The conditions that promote transformation in soil could be rare, but even infrequent transformation events could lead to the emergence of novel drug-resistance, especially under a selective pressure [93], [94]. For this reason, antibiotics in the environment have gained increasing attention over the last decade [8]. In fact, if an antibiotic provides a strong enough selective pressure, transformed eARGs could reach high abundances in a population. While live antibiotic resistant cells will generally establish more successfully than an equal abundance of eARGs (DNA), due to the rarity of transformation, a strong enough selective pressure could equal the playing field such that eARGs are just as successful as live invaders. Transformed alleles also have the added advantage of moving into multiple genetic backgrounds [39], [40], [95], this ultimately preserves community-level genetic diversity and changes population-level outcomes (see Figure 3.1B).

The transfer of eARGs into multiple genetic backgrounds is often facilitated by mobile genetic elements, which are a major driver in the emergence of multidrug resistance [96], [97]. These genetic parasites insert themselves into bacterial genomes and generally appear at high abundances in soils enriched in ARGs [98], [99]. While transformation can disseminate mobile genetic elements into unrelated bacteria at high

frequencies, the prevelance of this phenomenon is poorly characterized outside of laboratory conditions. Here, I address this knowledge gap, by incoulating agricultural soils with eARGs carried on a mobile genetic element (miniTn7 transposon), and track the evolution of antibiotic resistance into populations of *Pseudomonas stutzeri* – a model organism for studying transformation in soil (Sikorski et al. 1998, see Figure 3.2 for the experimental design).

Overall, I show that eARGs from dead bacteria are an important, but often overlooked source of antibiotic resistance in natural systems. Specifically, I find that the availability of eARGs drives the evolution of antibiotic resistance and that transformation is prevalent under a wide range of soil conditions – only decreasing at very high soil moistures and in homogenized soils. More broadly though, I show that antibiotic resistant transformants repeatedly establish in antibiotic-free soil. Together, this provides novel *in situ* evidence that HGT is an evolutionary force that expands the adaptive potential of bacterial communities by facilitating the spread of non-selected antibiotic resistance genes.

3.3 METHODS

3.3.1 SITE AND SOIL COLLECTION

Soil cores (10cm depth by 5cm diameter) were collected in October 2018 and April 2019 from the Great Lakes Bioenergy Research Center (GLBRC) scale-up fields located at Lux Arbor Reserve Farm in southwest Michigan (42°24’ N, 85°24’ W). Plots were established as perennial switchgrass monocultures (*Panicum virgatum L*) in 2013, and before that were in a corn–soybean rotation for more than 10 years. The soils developed on glacial outwash and are classified as well-drained Typic Hapludalf, fine-

loamy, mixed, mesic (Kalamazoo series) or coarse-loamy, mixed, mesic (Oshtemo series) or loamy sand, mixed, mesic (Boyer series) [100]. Soils were sieved at 2mm and autoclaved in two cycles (60 minutes at 121°C; gravity cycle) separated by a 24-hr window to target dormant and spore-forming cells resuscitated during the first autoclave cycle.

3.3.2 BACTERIAL CULTURES AND EXTRACELLULAR DNA

Soil microcosms were inoculated with *Pseudomonas stutzeri*, strain 28a24 [101]. Prior to inoculation, the bacterial cultures were grown at 30°C on an orbital shaker (120 rpm) for 24hrs in liquid luria broth (LB) media to a concentration of 10^6 CFU/mL. All LB media used throughout the experiment followed a recipe of 10% tryptone, 5% yeast extract, and 5% NaCl (solid media contained 1.5% agarose). Stocks of antibiotic resistant extracellular DNA (eARGs) were made from a mutant *P. stutzeri*, strain encoding a gentamicin resistance gene and a LacZ gene (Tn7 transposition of pUC18-mini-Tn7T-Gm-lacZ into strain 28a24, see Choi and Schweizer 2006). eDNA was also made from the wildtype *P. stutzeri* to act as a negative control. The gentamicin resistant *P. stutzeri* cells were genetically identical to the wildtype *P. stutzeri* cells, except for the presence of the antibiotic resistance gene. The batch cultures for eDNA stocks were prepared under the same conditions specified above but were grown for 48hrs and then resuspended in sterile nanopure water. The cells for eDNA stocks were killed via heat shock (90°C for 1hr) and confirmed dead by plating. The final concentrations of eDNA ranged from 25-50 ng and was appropriately diluted for each experiment (DNA concentrations were determined using Qubit fluorometric quantification and Invitrogen

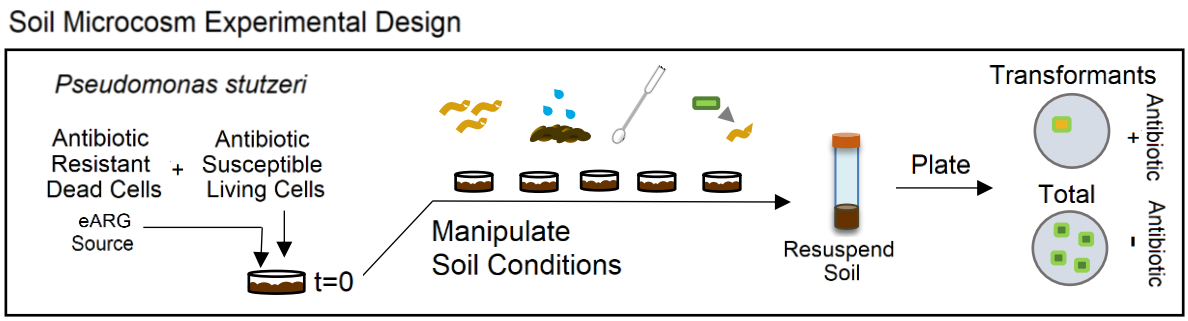

Figure 3.2: General experimental design for soil microcosms. At time zero soil was inoculated with live antibiotic susceptible *P. stutzeri* and dead antibiotic resistant *P. stutzeri* – providing a source of eARGs. Soils were then exposed to a variety of soil conditions and the number of transformants periodically counted by resuspending soil in a slurry and plating onto selective media.

Quant-iT PicoGreen dsDNA Assay Kit). *P. stutzeri's* transformation efficiency plateaus at ~5ng eDNA under standard laboratory conditions (Figure A3.1A).

3.3.3 SOIL MICROCOSMS

Soil microcosms were established in small 60 x 15mm petri dishes using 10 grams of dry, sterile, switchgrass soil. Except for the experiment in Figure 4, which used large 150 x 15mm petri dishes filled with 100 grams of soil. On day 0 of each experiment, the center of the microcosm was inoculated with 2mL of wildtype *P. stutzeri* cells suspended in liquid LB at a concentration of 10^6 CFU/mL. Immediately after adding cells, I inoculated the soil with eDNA encoding gentamicin resistance. To control for contamination or evolution of gentamycin resistance via mutation, two additional treatments were included in every experiment; 1) 5µg of eDNA g^{-1} soil made from gentamicin susceptible *P. stutzeri* cells, and 2) sterile water without eDNA. Since transformants never appeared in the control treatments the results are not shown.

All microcosms were maintained at ~23°C and the soil was never mixed unless directly specified (e.g. in Figure 3.4B). All microcosms were initially inoculated to ~40% soil moisture on day 0, and then dried to 20% soil moisture, where they were

maintained until the next eDNA addition (except in the experiments manipulating soil moisture where the soil was dried according to the treatment-level soil moisture). In each experiment, I counted the number of transformants, and the population size every 5 days, and then added more eDNA to simulate periodic inputs of eARGs. After eDNA additions, I gradually dried the soils over the next ~4hrs back to 20% soil moisture.

To establish a baseline for transformation in the soil system, I quantified transformation every 24hrs for 5 days after inoculation with eDNA. I ran parallel assays on petri dishes using LB and Reasoner's 2A agar (R2A) using a concentration of 5µg eDNA g^{-1} soil. Each treatment had 8 replicates (Figure 3.3A).

To understand the relationship between the availability of eDNA and transformation, I varied the concentration of eDNA in soil between 5, 2.5, 1.25, 0.25 µg of eDNA $gram^{-1}$ soil (Figure 3.3B, C, D). I used these concentrations as they conservatively represent ~10%, 5%, 2.5% and 0.5% of the total eDNA pool in soil per [103]. I kept the concentration of eDNA low, as only a small percentage of eDNA will generally carry antibiotic resistance genes. The experiment lasted 15 days and eARGs were added on day 0, 5, and 10, with each treatment consisting of 8 replicate soil microcosms.

To determine how soil moisture affected transformation, I maintained soil microcosms at 5, 10, 20, 30 or 40% gravimetric soil moisture over a period of 10 days (Figure 3.4A, i.e. soil moisture = [weight after water addition – dry weight] / dry weight]*100). In this experiment, all the microcosms were inoculated with an intermediate concentration of eDNA (2.5µg g^{-1} soil) and eARGs were added on day 0 and 5. I report the number of transformants present on day 10, using 8 replicate

microcosms per treatment. To understand if the physical structure of the soil was important for transformation, I manipulated the physical structure of the soil by mixing the soil every 2hrs, 8hrs or never, throughout a 48hr period (Figure 3.4B). Mixing was carried out using a sterile spatula. In each microcosm, I kept the eDNA concentration constant at 5 µg g^{-1} soil and the soil moisture constant at 10%, with each treatment consisting of 4 replicates.

To determine if transformation was dispersal limited under different soil moistures, I established 8 pools of eDNA in large microcosms maintained at 10, 20, 30 or 40% soil moisture (Figure 3.5). Half of the eDNA pools contained eARGs (*P. stutzeri* + gentamicin resistance), and the other half did not (*P. stutzeri* wildtype). The 8 pools were located 1.25, 3.80, 5 or 7 cm from the center of the plate. Each eDNA pool was inoculated with 2µg eDNA g^{-1} soil (400 µl total volume) and *P. stutzeri* was inoculated to the center of the microcosm (2mL total volume). To prevent dispersal during inoculation, I dripped the eDNA and bacterial cells into the soil in 200µl aliquots. The experiment ran for 5 days, using 4 replicate microcosms per soil moisture. At the end of the experiment, soil was collected from the center of each eDNA pool to count the number of transformants and total cells.

3.3.4 ANTIBIOTIC RESISTANCE GENES IN LIVE VS. DEAD CELLS

In a final laboratory experiment, I tested how an equal concentration of ‘eARGs’ and ‘live antibiotic resistant invaders’ establish in populations of *P. stutzeri* subjected to three different selective regimes (Figure 3.6). Initially, I established two equal populations of kanamycin resistant *P.stutzeri* cells (DAB837 in [104]). To one of the two Kan_R populations, I added 60,000 ‘live’ gentamicin resistant *P. stutzeri* cells ($Gent_R$). To

the other population, I added 60,000 'dead $Gent_R$' cells which provided a source of eARGs. Therefore, on day 0 of the experiment, the two treatments contained 4% $Gent_R$ cells and 0% $Gent_R$ cells, respectively. The kanamycin and gentamicin resistance genes were both carried on a miniTn7 transposon, which allowed me to track the frequency of gene replacement (just $Gent_R$) vs gene addition ($Gent_R$ + Kan_R) in transformed cells (Figure 3.6B).

I performed parallel experiments in liquid LB under 3 selective regimes: 0%, 10% or 25% of the lethal dose of gentamicin (equivalent to 0, 5 or 12.5 mg/ml gentamicin, respectively). Populations were founded in 1mL of media and the gentamicin added at t=0. I ran the experiment for 10 days, providing 1 mL of fresh LB media to each population every 24hrs. On day 5, I removed the selective pressure and transferred the populations at a 1:4 dilution to liquid LB with no gentamicin. Each day I counted the number of Kan_R and $Gent_R$ genotypes using serial dilution and selective plating of 10µl dots. I counted the total number of cells on solid LB media (no antibiotic), the number of $Gent_R$ cells on solid LB media with gentamycin (50 µg/ml) + Xgal (20 µg/ml), and the number of Kan_R + $Gent_R$ cells on solid LB media with kanamycin (50 µg/ml) + gentamycin (50 µg/ml) + Xgal (40 µg/ml). I report the frequency of $Gent_R$ genotypes ($Gent_R$ cells/total cells) every 24hrs over the course of 10 days. In addition, on day 7 of the experiment, I counted the number of cells that were both Kan_R and $Gent_R$ (Figure 3.6B).

3.3.5 COUNTING TRANSFORMANTS

To determine the number of transformants in the soil microcosm experiments, I weighed out 0.2g of soil from each microcosm and placed it into a 1.5mL centrifuge

tube. To each tube I added 180µl of liquid LB and vortexed for 10 seconds (~10^{-1} dilution). After allowing the soil to settle for 10 minutes, I transferred the supernatant to a 96-well plate and diluted out to 10^{-6} or 10^{-9} depending on the experiment and the expected number of cells. In the experiments that manipulated eDNA concentration and soil moisture, I plated 50µl cell suspensions. For the remaining experiments I plated 10µl dots. All plating was done on petri dishes with solid LB (to count the total population size) or solid LB + gentamycin (50 µg/ml) + Xgal (40 µg/ml) (to count transformants in soil). Plates were incubated at 30°C and the number of colonies counted after 48-72hrs. The number of cells is reported g^{-1} soil, except in Figure 4 where it is reported per eARG pool (0.2g soil) and calculated according to the following equation: Cells per unit = Cells μl^{-1} x [Soil slurry volume (200µl) / Soil Mass in slurry (g)].

I report the number of transformants g^{-1} soil, as it is an environmentally relevant metric, and transformation is not affected by population size in populations larger than 10,000 *P. stutzeri* cells (Figure A3.1B). The only exceptions are Figure 3.3A where the population sizes varied between soil and lab assays and in Figure 3.4B where the population size fell below 1000 cells. In these instances, I instead report the Transformation Frequency = log(transformants)/log(population size).

3.3.6 STATISTICAL ANALYSES

Prior to analyses all data were verified to meet assumptions of normality and homogeneity of variance. Data that did not conform to assumptions of homogeneity of variance were log transformed when appropriate. Results from soil microcosm studies were analyzed by either one-way or two-way ANOVA followed by Tukey's post hoc with test variable (i.e. soil manipulation and sampling day) as a fixed effect using the R *stats*

package (R core team 2018). Experiments with multiple sampling days were analyzed by two-way ANOVA, except in certian instances, when the test variables were analyzed individually by sampling day (e.g.Figure 3.4A). Results from the soil microcosm experiment in (Figure 3.4) were analyzed by two-way ANOVA with the 'distance to eARGs' and 'soil moisture' as fixed effects. When signficant, interactions between test variables were included in the model. Results from laboratory experiments (Figure 3.6) were anlayzed using two-way ANOVA with the treatment (Live vs Dead cells) and selection regime (0, 10, 25% lethal dose gentamicin) as fixed effects. Results were based on the frequency of gentamicin resistant genotypes present in each population at the end of the experiment. Differences between all test variable groups were considered significant at $\alpha \leq 0.05$.

3.4 RESULTS

Inoculating eARGs into soil facilitated gene transfer through natural transformation. Transformants appeared in soil within 24 hours after eARG addition and evolved in the presence of just 0.25 µg eDNA g^{-1} soil, which, conservatively-estimated, is only a fraction (1/100) of eDNA in field soil (reviewed by [103]; also see [105]). Increasing the amount of eARGs increased the number of transformants in soil, suggesting larger pools of eARGs pose a greater risk to the spread of antibiotic resistance. While transformation occurred under a wide range of soil conditions, rates were highest at intermediate soil moistures (5-20%) and increased with the availability of eARGs.

First, I tested how the soil environment impacted transformation by comparing the number of transformants that evolved in soil versus on low (R2A) and high-nutrient

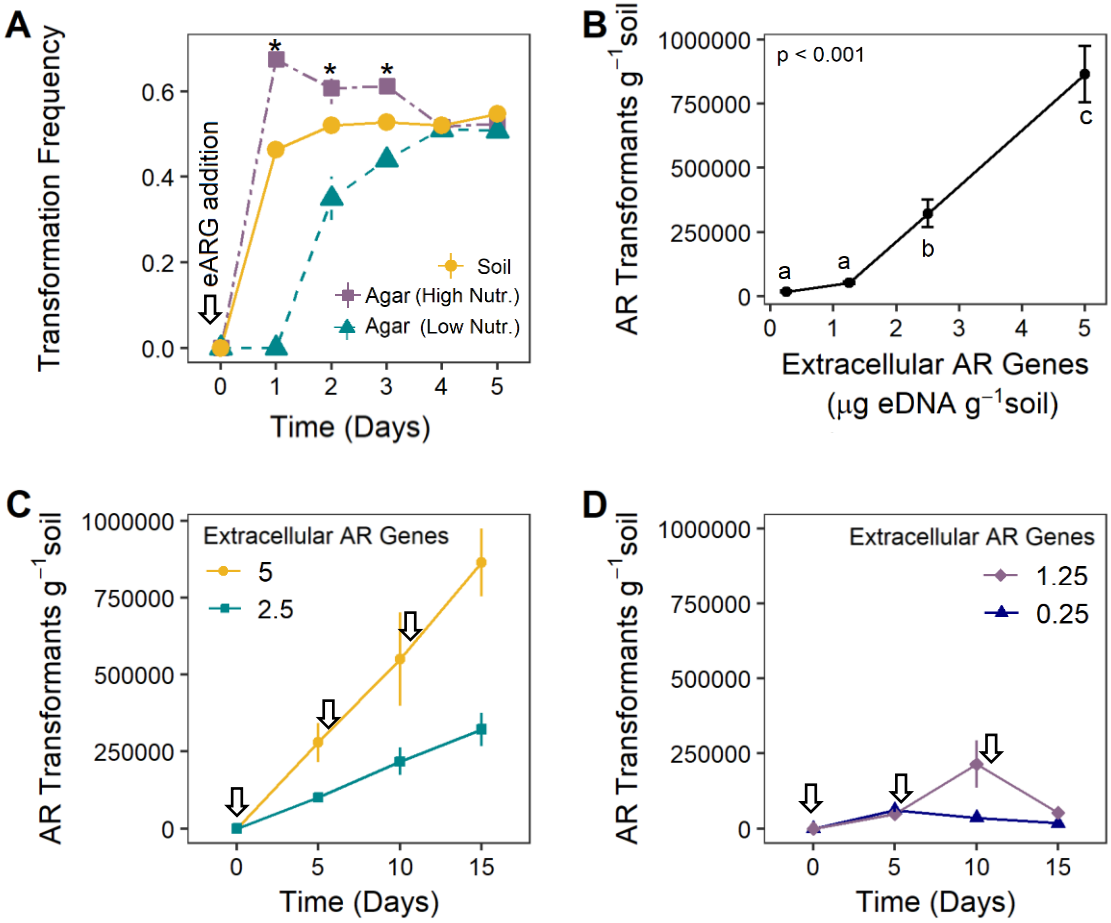

Figure 3.3: The relationship between eDNA availability and transformation. (A) Comparison of transformation in soil versus on petri dishes (High Nutrient = Luria Broth (LB); Low Nutrient = Reasoner's 2A Agar (R2A)). Transformation Frequency = [Log(Transformants)/ Log(Recipient Cells)]. Asterisks indicate that the transformation frequency varied across the 3 treatments on that day. (B) The relationship between eARG concentration and the appearance of transformants in soil (day 15 from 3.3C). The eDNA concentration ranged from <1% to 10% of a standard soil eDNA pool. (C-D) Time series tracking the appearance and maintenance of transformants when soil was supplemented with period inputs of eARGs at either, (C) 5, 2.5, (D) 0.25, or 1.25 μg eDNA g^{-1} soil. eARGs were added on day 0, 5, and 10 after counting transformants. The arrows indicate the timing of the eARG additions. (A-D) Points/bars represent the average number of transformants g^{-1} soil and error bars show the standard error of the mean (n=8 replicates).

(LB) agar petri dishes. I found that transformants appeared in soil and petri dishes at a similar frequency. However, it took 4 days for the frequency of transformants in soil to equal the number of transformants on high and low nutrient agar plates (Figure 3.3A). This shows that soil is not a significant barrier to transformation, but that transformation

may initially proceed slower in soil than under laboratory conditions (24-72hrs, $p<0.001$ across the 3 treatments).

One of the strongest controls on transformation was the availability of eARGs. I found that transformation scaled linearly with the concentration of eDNA but only in soils inoculated with at least 2.5 µg of eDNA g^{-1} soil (Figure 3.3B, $p<0.001$). Periodic inputs of large concentrations of eDNA (>2.5 µg), increased the number of transformants by an equal magnitude (Figure 3.3C). While periodic inputs of small concentrations of eDNA (<1.25 µg), did not uniformly increase the number of transformants (Figure 3.3D).

When the eDNA concentration was held constant and soil microcosms were incubated at 5, 10, 20, 30 or 40% soil moisture, I found that transformation was highest at 10% soil moisture (though 10% did not significantly differ from 5% or 20%) (Figure 3.4A, $p < 0.001$). However, on day 5 before the second eDNA addition there were significantly fewer transformants at 5% soil moisture than 10% and 20% (data not

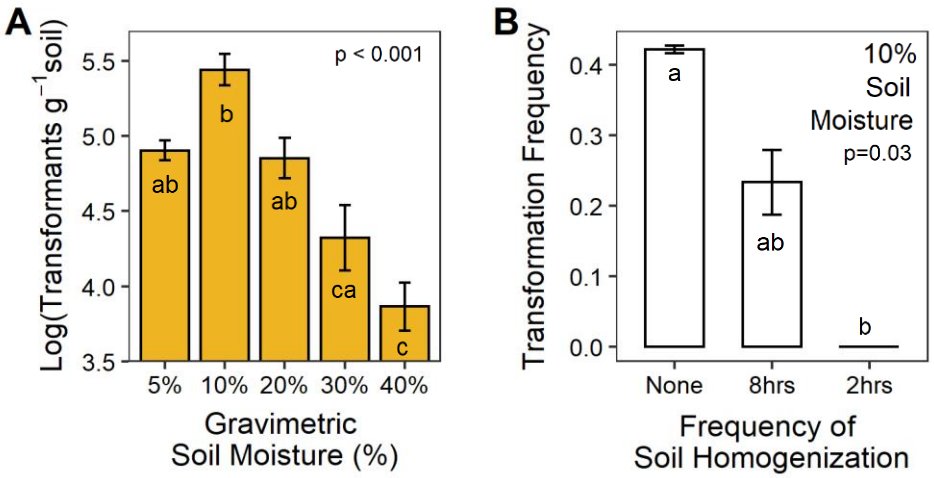

Figure 3.4: The relationship between soil moisture and transformation. (A) The number of transformants in soil incubated at 5%, 10%, 20%, 30% or 40% soil moisture over a 10-day experiment. (B) The relationship between the frequency of soil homogenization and the evolution of transformants at 10% soil moisture. Homogenization was conducted every 2hrs, 8hrs or never – over a 48hr window. Bars represent the average number of $\log_{10}$(transformants g^{-1} soil) and error bars show the standard error of the mean (A: n=8, B: n=4 replicates).

shown). To elucidate what factors might increase transformation at 10% soil moisture, I performed a 48-hr transformation assay where I disturbed the soil matrix every 2h, 8h, or left the soil undisturbed (Figure 3.4B). Homogenizing the soil every 2h completely prevented transformation from occurring, while homogenizing every 8h reduced the frequency of transformation events compared to a non-homogenized control (p=0.03).

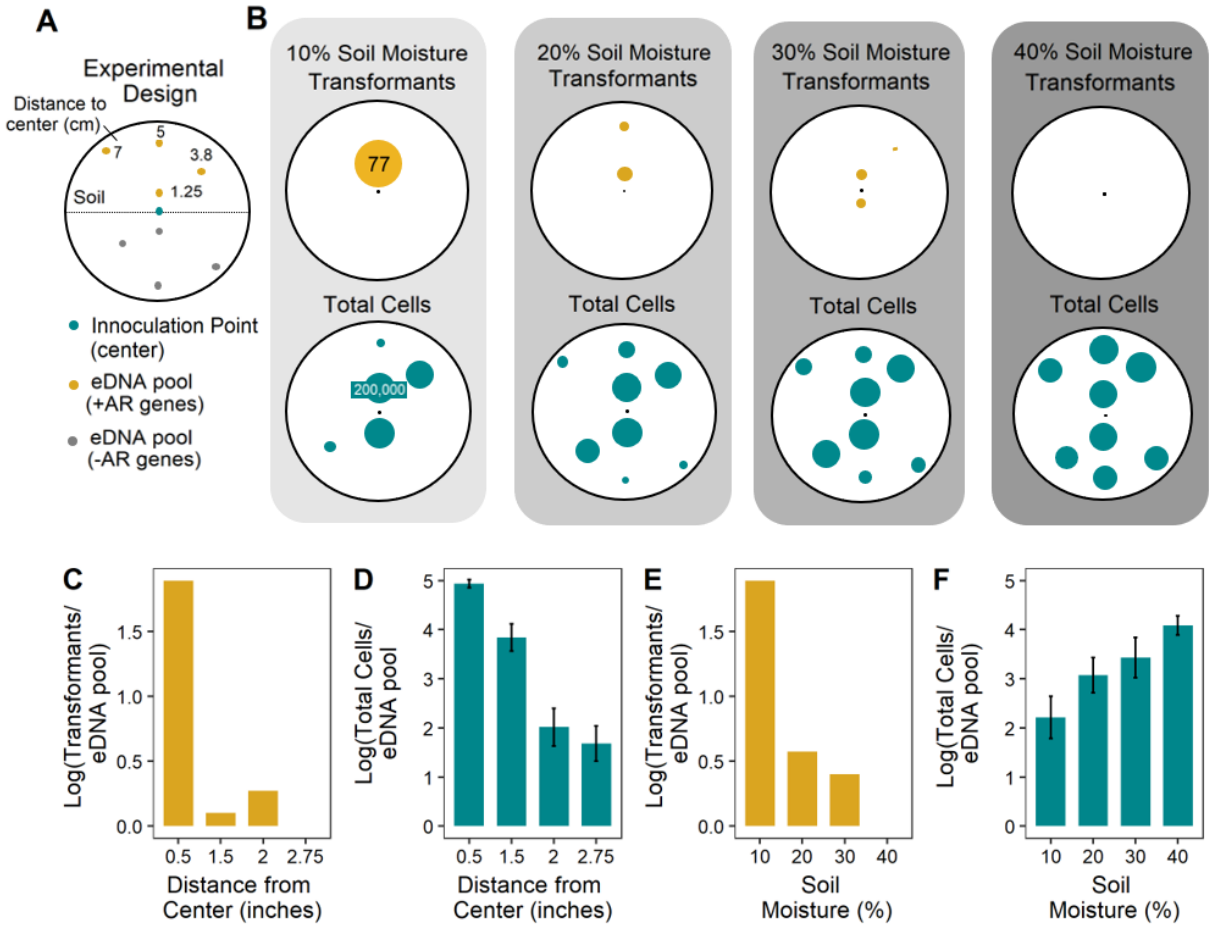

Figure 3.5: The relationship between dispersal and transformation. (A) Location of eDNA pools in soil microcosms set-up in 150 x 15mm petri dishes. Transformable cells were added to the center of the plate; the top 4 eDNA pools have eARGs (yellow) and the bottom 4 do not (gray). (B) The top panel shows the average number of transformants and the bottom panel shows the average cell count in each eDNA pool after 5 days of dispersal. The size of the dot increases as the number of cells increases. (C) The total transformants and (D) the average cells at each distance, pooled across the four soil moistures. (E) The total transformants and (F) the average cells at each soil moisture, pooled across the four distances. (C,E) represent the sum of transformants across the replicates. Error bars show the standard error of the mean (n=4 replicates).

Next, I tested the effect of separating *P. stutzeri* cells from local eARG sources and found that this spatial separation posed a substantial barrier to transformation in soils maintained at 10, 20, 30 or 40% soil moisture (Figure 3.5). Even in wet soils, where dispersal to eARG sources was high, there were no transformants in any of the eDNA pools (dispersal at 10% vs 40% soil moisture, $p=0.0289$, Figure 3.5B-F). Meanwhile at 10% soil moisture there were significantly more transformants than at any other soil moisture. However, these transformants only appeared in the closest eDNA pool, with *P. stutzeri* unable to disperse to the most distant eARGs located 7cm away (transformants *soil moisture, $p<0.001$, Figure 3.5B-F). Generally, dispersal and transformation happened at intermediate rates at 20 and 30% soil moisture.

In a final experiment, I used a lab-based assay to compare the establishment of an equal concentration of 'eARGs' or 'live antibiotic resistant invaders' in populations of *P. stutzeri* (Figure 3.6A). The recipient cells were all kanamycin resistant (Kan_R), while the eARGs and live invaders encoded gentamicin resistance ($Gent_R$). In almost all instances, transformed $Gent_R$ genes replaced native Kan_R genes, with gene addition ($Gent_R + Kan_R$) occurring at very low frequencies (<1% of transformants) and only under the highest dose of antibiotic (Figure 3.6B). In the absence of a selective pressure and at 10% of the lethal dose of antibiotic, live invaders reached higher frequencies than antibiotic resistant transformants (Figure 3.6C, $p<0.001$). However, when the selective pressure increased to 25% of the lethal dose, both the live invader and transformed eARGs reached a high frequency in the population (Figure 3.6D**,** $p<0.001$). Although antibiotic resistant transformants took 24 hours longer than live invaders to establish at high frequencies under the highest does of gentamicin (Figure 3.6D).

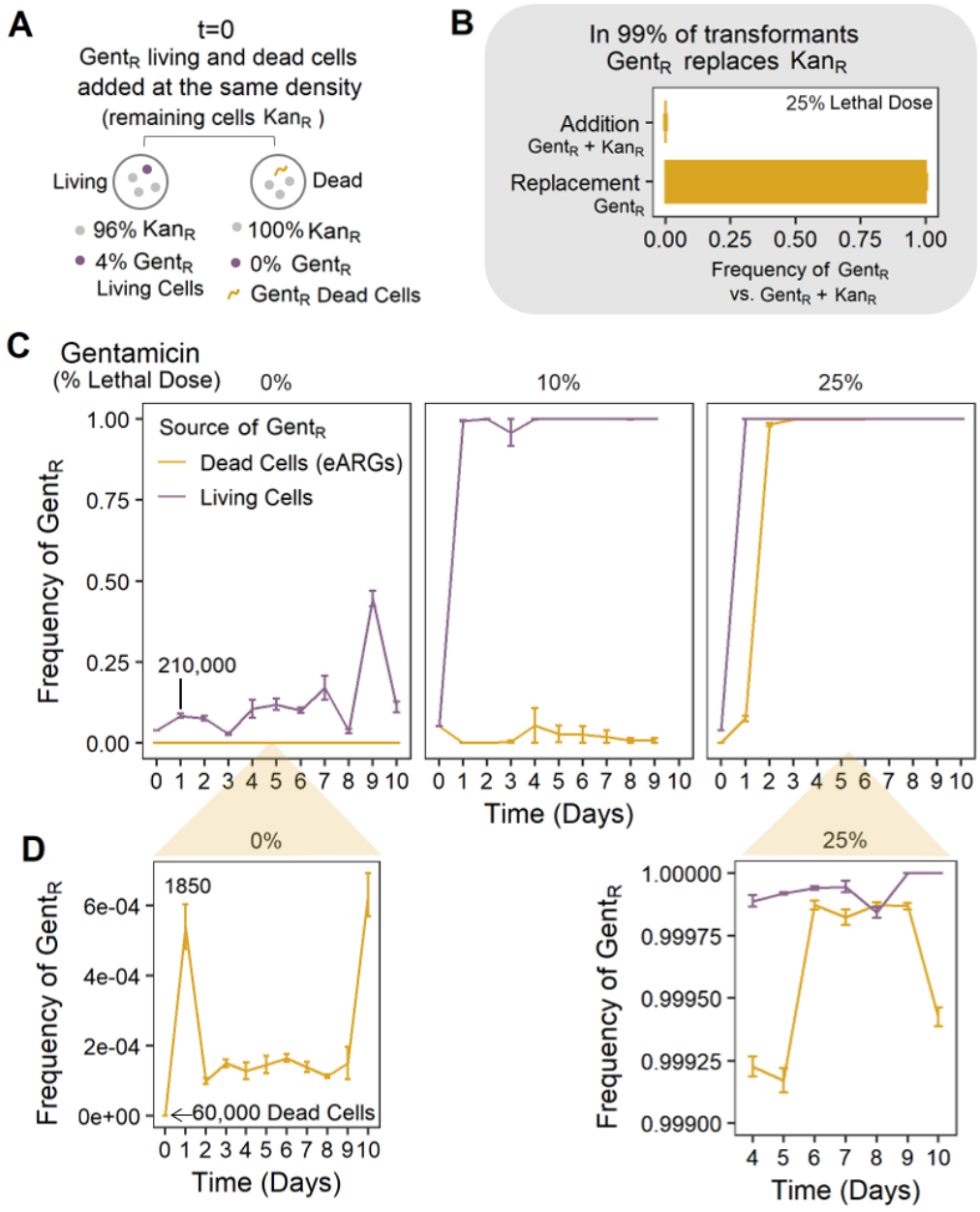

Figure 3.6: Transformation vs. invasion of antibiotic resistant genes. (A) Frequency of kanamycin (Kan_R) and gentamicin resistance ($Gent_R$) at t=0. 'Live' treatments started at 96% Kan_R and 4% $Gent_R$ (left). 'Dead' treatments started at 100% Kan_R and 0% $Gent_R$ + $Gent_R$ eDNA (right). (B) Gene addition (maintenance of Kan_R + $Gent_R$) was only detected at low frequencies in populations exposed to 25% of the lethal dose of gentamicin. (C) The average frequency of $Gent_R$ under three selection regimes: 0%, 10%, and 25% of the lethal dose of gentamicin. (D) Zoomed in perspective of panel 1 and panel 3 from C. (C-D) Error bars show the standard error of the mean (n=4 replicates).

3.5 DISCUSSION

In order to reduce the impacts of antibiotic resistance on human health, we need a better understanding of the ecological dimensions that promote the transfer of eARGs in natural systems [106]. In this study, I show that high concentrations of eARGs in soil increase the number of transformants, ultimately increasing the prevalence of antibiotic resistant bacteria in antibiotic-free soil. I find that transformants appear under most conditions typical for terrestrial soils (10-20% moisture), however, transformation efficiency decreases at high soil moistures and with soil mixing. In addition, I find that a low dose of antibiotic allows eARGs to establish with the same success as a live antibiotic resistant invader. Overall, the sustained biological activity of eARGs, even after bacteria death, suggests eARG removal should be incorporated into plans to combat antibiotic resistance.

Several studies have now posited that the spread of antibiotic genes into diverse bacterial lineages occurs via widespread HGT [8], [82]. Here, I show that eARGs supplied by dead bacteria are readily transferred into soil bacteria, with the potential for HGT scaling linearly with the abundance of eARGs. My findings provide novel evidence that large concentrations of environmental eARGs can drive the evolution of antibiotic resistance, and this information should be incorporated into our approach to combating antibiotic resistance. For instance, many disinfection methods focus on killing live bacteria, but may be more effective if they consider the persistence of eARG pools, which I find can be equally effective at spreading ARGs. This may explain why practices like composting manure prior to application on agricultural fields has been found to both increase and decrease the occurrence of ARGs, depending on the native bacterial

community [107], and other soil conditions [99]. Interestingly, manures composted at high temperatures – which promotes degradation of eDNA – can be most effective in reducing ARGs [108], supporting my findings that DNA degradation is a critical factor in reducing environmental concentrations of ARGs.

Despite the dangers of low-levels of eARGs persisting in soil for an extended time, the fate of most extracellular DNA is likely degradation [34]. While low levels of eDNA can persist in soil for 80+ days, 99% is degraded in the first ~7 days [66], [109]. Consequently, the most important role of soil conditions in regulating transformation may be the effect of moisture on the rate of eDNA decay, and likely explains why transformation declined at higher soil moistures (Figure 3.4, 3.5). Transformation was previously shown to decline at 35% soil moisture in *Acinetobacter calcoaceticum* [110], however, transformation was only measured above 18% soil moisture. In contrast, *P. stutzeri* had the highest transformation efficiency between 5 and 20% soil moisture (Figure 3.5A, 3.5). This finding provides novel evidence that transformation occurs at lower soil moistures than previously thought, but the relationship between soil moisture and transformation could vary widely across bacterial species, depending on how competence is regulated. It is also important to note that agricultural soils rarely exceed 30% soil moisture in the field, and in fact it has only happened twice since 1989 in the region where these soils were collected (Figure A3.2). However, wetland soils often exceed 30% soil moisture, suggesting that transformation rates may vary widely across soil habitats.

The increase in transformability at lower soil moistures could also be due to increased exposure to eDNA in drier conditions. Lower moisture conditions have

previously been shown to increase conjugation rates in *P. putida*, as unsaturated soils create fragmented habitats that lengthen the duration of cell contact between bacteria [111], [112]. While transformation does not require cell-cell contact, it does require cell-eDNA contact which increases the duration of time near eARGs and could promote transformation. However, drier soils could also promote transformation via biofilm formation, which is a common response to drought and increases microbial survivorship at low soil moistures [113]. Importantly, biofilms also enhance the efficiency of gene transfer [91] and could be a critical precursor to transformation in soil – as in the lab, *P.stutzeri* exhibits much higher transformation efficiencies in sessile or biofilm communities than planktonic communities (Figure A3.1C).

If biofilms are critical for transformation in soil it could explain why soil homogenization prevented transformation, as disturbing the biofilm structure every 2hrs would prevent mature biofilms from establishing and therefore could prevent transformation [114]. Future studies could use fluorescent proteins or confocal laser scanning microscopy to better quantify the relationship between biofilm establishment and transformation efficiency in soil [115].

Historically, the contribution of eARGs to antibiotic resistance was assumed to be low, primarily because transformed eARGs are deleterious, and theoretically transformants should not increase in abundance in the absence of selection. However, the presence of antibiotics in the environment could provide a selective pressure that enables transformed eARGs to reach high frequencies in a community. In fact, my research provides unique evidence that low doses of antibiotics allow transformed eARGs to establish in the population and produces a population trajectory analogous to

an invasion by live antibiotic resistant cells. This highlights that controlling the release of antibiotics into the environment is critical for preventing the emergence of novel antibiotic pathogens – even if there are *no live antibiotic resistant bacteria* in the community. An important future research direction will be determining the antibiotic concentrations at which eARGs represent a major source of antibiotic resistance in soil. Answering this question will require an increased understanding of antibiotic concentrations in soil and their effect on bacterial communities. This study lays the groundwork for future studies, which should examine the concentration of eARGs, and the sub-inhibitory concentrations of antibiotics which promote the rise of antibiotic resistant transformants in soil.

Taken together, my study reveals the most important variables for understanding the transmission of eARGs in soil and sets the stage for future experiments to scale up estimates of transformation to the whole community level. Here, I used a sterile soil system, inoculated with a single bacterium, as to prevent competitive interactions, and ensure the soil was antibiotic-free. Transformation may be lower in multi-species communities, where competitive interactions would limit access to eARGs and limit the success of transformants.

Regardless, this work provides novel evidence that eARGs from dead bacteria are an overlooked, but important route in the emergence of antibiotic resistance. I conclude that transformation occurs under most soil conditions and show that transformed eARGs are just as successful as live antibiotic resistant invaders, when challenged with a low dose of antibiotic. Overall, this demonstrates that disinfection alone is insufficient to prevent the spread of ARGs through environmental reservoirs.

Furthermore, special caution should be taken in releasing antibiotics into the environment, even if there are no *live* antibiotic resistant bacteria in the community, as transformation allows DNA to maintain its biological activity past microbial death.

APPENDIX

APPENDIX

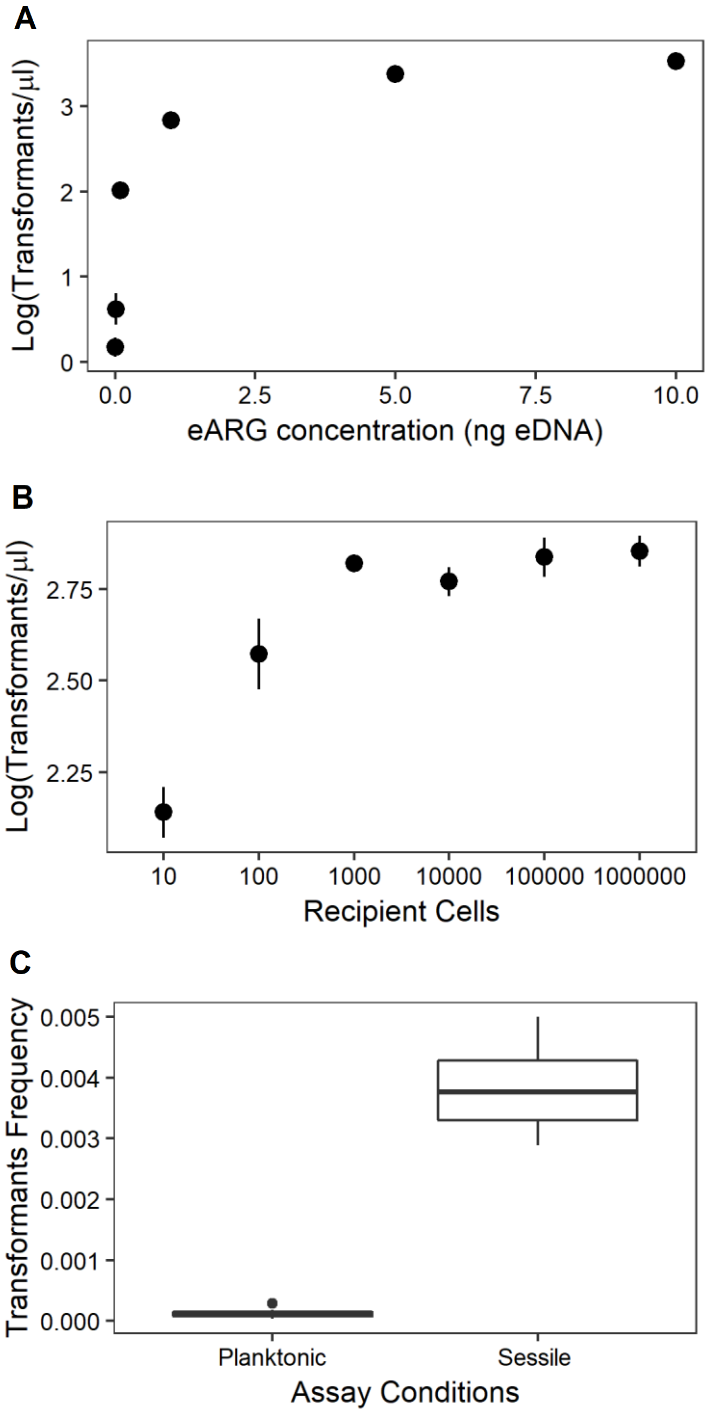

Figure A3.1: Transformation assays under laboratory conditions. (A) The relationship between transformation and the concentration of eDNA under laboratory conditions (0.001, 0.01, 0.1, 1, 5, 10 ng/µl eDNA). (B) The effect of total population size on the number of transformants. (C) Comparison of transformation in sessile (biofilm or surface attached) communities vs. planktonic communities.

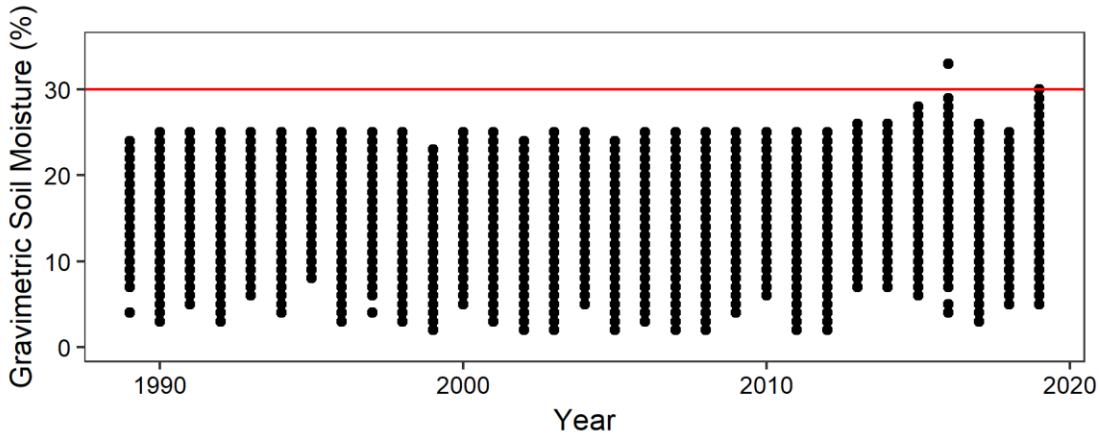

Figure A3.2: Gravimetric soil moisture from 1989 to 2019 Southwest, MI (regular measurements from April-November). Data from the Kellogg Biological Station Long-term ecological research center main cropping system. The red line shows 30% soil moisture and represents the soil moisture where transformation starts to decline.

CHAPTER 4:

CHANGES IN TRANSFORMATION AFTER SALT ADAPTATION

4.1 ABSTRACT

The exchange of genes between potentially unrelated bacteria is termed horizontal gene transfer (HGT) and is a driving force in bacterial evolution. Natural transformation is one mechanism of HGT where extracellular DNA (eDNA) from the environment is recombined into the host genome. The widespread conservation of transformation in bacterial lineages implies there is a fitness benefit. However, the nature of these benefits and the evolutionary origins of transformation are still unknown. Here, I examine how ~330 generations or 100 days of serial passage in either constant or increasing salinities impacts the growth rate and transformation efficiency of *Pseudomonas stutzeri*. While the growth rate generally improved in response to serial transfer, the transformation efficiency of the evolved lineages varied extensively, with only 39-64% of populations undergoing transformation at the end of adaptive evolution. In comparison, 100% of the ancestral populations were able to undergo natural transformation. I also found that evolving *P. stutzeri* with different cell lysates (or populations of dead cells) minimally affected the growth rate and transformation efficiency, especially in comparison to the pervasiveness with which transformation capacity was lost across the evolved populations. Taken together, I show that the efficiency of eDNA uptake changes over relatively rapid timescales, suggesting that transformation is an adaptive and selectable trait that could be lost in environments where it is not beneficial.

4.2 INTRODUCTION

Natural transformation is a mechanism of horizontal gene transfer whereby bacteria acquire extracellular DNA (eDNA) from the environment and recombine it into their genomes. Transformation plays a key role in bacterial evolution [11], [116]; however, the fitness benefits of transformation remain unknown, despite extensive study. While it is generally accepted that transformation can facilitate adaptation through genetic recombination, the consequences of this genetic exchange can be both beneficial and costly [52]. The theoretical benefits of transformation are similar to meiotic sex and include speeding up adaptation, combining beneficial genes into one genome, and separating beneficial mutations from deleterious loads [11], [48], [95], [117], [118]. However, extracellular DNA from dead bacteria can also carry an increased mutational load or promote the spread of selfish genes [53], [119]. Consequently, the fitness advantages of transformation and the environmental conditions in which they are conferred have been difficult to quantify experimentally (see Table 4.1).

There are several potential explanations for the evolutionary maintenance of transformation [reviewed in 42]. Several of them posit that transformation evolved as a byproduct of acquiring DNA for nutrients [120]–[123] or genome repair [45], [124], [125]. However, the presence of cellular machinery dedicated to protecting extracellular DNA (eDNA) from degradation inside the cell, suggests that eDNA is not acquired purely for the nutrient benefit [42], [45]. In addition, many bacterial taxa preferentially kill and transform eDNA from close relatives, a process somewhat analogous to the exchange of DNA in eukaryotic sex [46], [47].

Table 4.1: Review of transformation-mediated fitness effects. Experimental evolution studies that have quantified the fitness effects of transformation.

Citation	Bacterial Taxa	Adaptation Conditions	Transformation increased adaptation	Exogenous DNA provided	Changes in transformation
Bacher et al. 2006 [55]	*Acinetobacter baylyi*	High Salinity & Temperature	No	No	Decreased
Baltrus et al. 2007 [50]	*Helicobacter pylori*	Novel Laboratory Conditions	Yes	No	Not reported
Perron et al. 2012 [126]	*Acinetobacter baylyi*	Periodic Antibiotics (3-4x/wk)	Yes, when provided resistance genes	Yes	Not reported
Engelmoer et al. 2013 [51]	*Streptococcus pneumoniae*	Periodic Antibiotic (kanamycin 2x/wk)	Yes	No	Not reported
Utnes et al. 2015 [127]	*Acinetobacter baylyi*	Novel Laboratory Conditions	Yes, but only during early stationary phase	Yes	Not reported
Mcleman et al. 2016 [128]	*Acinetobacter baylyi*	Parasitic Phage	Yes, from phage-sensitive or resistant DNA	Yes	Not reported

Transformation is also similar to eukaryotic sex in that it is primarily beneficial in stressful or continuously changing environments. Population genetic models [11], [48], [49] and experimental evolution studies [50], [51] have shown that transformation is beneficial in rapidly fluctuating or stochastic environments where transformable cells can outcompete non-transformers [43], [129]. Theoretically, this is because transformation can increase genetic variation, thereby increasing the efficiency of natural selection [130]. Transformation does not always provide a fitness benefit in stressful environments though, as Bacher et al. [55] found that competent lineages of

Acinetobacter baylyi did not adapt to novel laboratory conditions faster than their non-competent competitors, and repeatedly lost the ability to transform eDNA.

While several other studies have shown that transformation is beneficial in stressful environments (see Table 4.1), it is still unclear how the availability of beneficial genes might alter transformation-mediated fitness effects. For instance, antibiotic resistance only evolved via transformation when antibiotic resistance genes were provided, while phage resistance evolved in the presence of phage-sensitive or -resistant DNA [126], [128]. Since sequence similarity improves the efficiency of homologous recombination, it is generally accepted that transformation is most prevalent between closely related organisms [131]. However, sharing genes with close relatives could limit the acquisition of novel gene combinations and ultimately limit adaptation.

Here, I aim to better understand the evolutionary benefits of transformation by evolving *Pseudomonas stutzeri* – a highly transformable soil bacterium – in either constant or increasing salt concentrations for 100 days, while supplying different sources of eDNA (cell lysates or dead cells). At the end of the experiment, I quantify the growth rate, population size, and transformation efficiency (transformants/µg eDNA) of the evolved populations and compare this to the same measurements in the starting isolate or ancestor. I specifically address the following questions: 1) Does the transformation efficiency increase in response to evolving in a variable – relative to a constant environment (increasing salinity vs. constant low salinity)? 2) Does evolving with dead halophiles or dead *Pseudomonads* better facilitate adaptation to high salt concentrations?

4.3 METHODS

4.3.1 SERIAL DILUTION EXPERIMENT

I serially transferred *Pseudomonas stutzeri*, strain 28a24 for ~330 generations (100 days) in 96-well microtiter plates [see 101 for whole genome sequence]. Cultures were serially transferred every 24hrs at a 1:10 dilution and maintained at 26°C. For the first 50 days (~170 generations) of the experiment, all populations were transferred as one treatment in a constant salt media (1.5% salinity; 10g/L tryptone, 5g/L yeast extract, and 15g/L NaCl). After 50 days (~170 generations), the experiment was shut down due to the global covid-19 pandemic, and populations preserved in 40% glycerol at 20°C. Four weeks later, populations were revived and serially passed at 1.5% salinity for 4 days before the experiment was 're-started' on day 51 (~170 generations). At this point, I split the experiment into two treatments. The original treatment was maintained at a low constant salinity for the remainder of the experiment (1.5% salt media from day 1 to 100). The new treatment, which I refer to as the increasing salinity treatment was

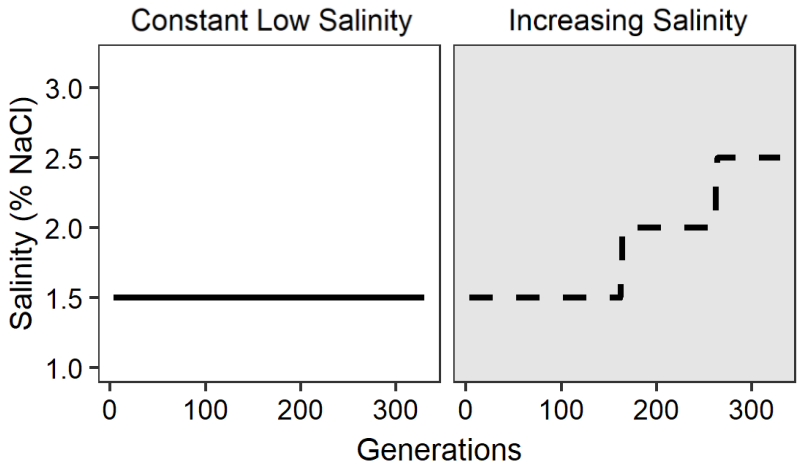

Figure 4.1: The serial transfer conditions for the two evolution treatments. The left panel shows the conditions for populations adapted to a constant low salinity environment (1.5% salinity) and the right panel shows the conditions for populations adapted to increasing salinities (1.5% to 2.5% salinity).

transferred to a 2% salt media (20g/L NaCl), where it was serially passed for 100 generations. Then on day 81, I increased the salt concentration to 2.5%, were it stayed for 67 generations until Day 100 (see Figure 4.1 for the serial transfer conditions). The constant low and increasing salinity treatments had 96 replicates each. In addition, during each transfer (every 24hrs), populations were supplemented with eDNA via whole populations of dead bacteria – which equated to 5ng of genomic eDNA each transfer. I refer to these as cell lysates as they contain DNA and other cellular components (see Table S4.1 for a detailed list of the cell lysate sources). The experimental design is detailed in Figure 4.2.

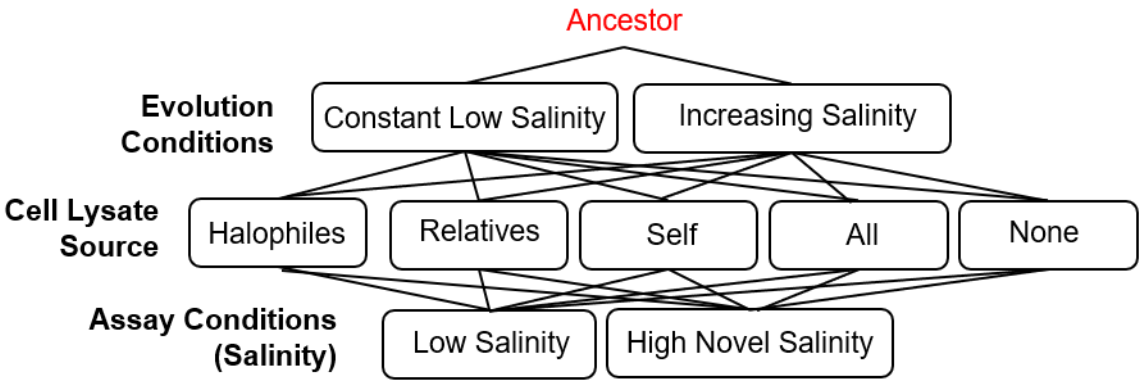

Figure 4.2: Graphical representation of the experimental design. The first tier represents the two serial transfer conditions (n=96). Within each of these treatments, I supplemented populations with different cell lysates – there were twelve total cell lysate treatments, grouped into five categories (n=8 x 12). The third tier represents the assay conditions in which I measured the growth rate, population size, and transformation capacity at the end of the experiment.

4.3.2 PREPARATION OF CELL LYSATES

Individual cell lysates were prepared in 100mL batch cultures in liquid LB on a shaker table at 120rpm and 30°C (10g/L tryptone, 5g/L yeast extract, and 5g/L NaCl). After 48hrs, each culture was plated to confirm there was no contamination (10 µl replicate dots plated 3x). Each culture was then heat shocked at 90°C for 1hr. After heat shock, each culture was plated to confirm all the cells were dead. If bacterial strains still

had viable colonies, these cultures went through another round of heat shock at 100-110°C, which was sufficient to kill the remaining cells. The heat shocked cultures were then spun down and resuspended in sterile nanopore water. The lysates were filtered through a 0.22µm filter and standardized to a concentration of 1ng DNA/µl using a Qubit 2.0 fluorometer (Life Technologies, USA). There were 12 cell lysate treatments with 8 replicates each. See Table S4.1 for expanded list of cell lysates.

4.3.3. GROWTH RATE DETERMINATION

All assays were conducted on the ancestral population, and populations that evolved for 100 days (~330 generations). Strains of *P. stutzeri* were revived from 40% glycerol storage (-80°C) and diluted 1:10 in liquid LB media (0.5% salinity: 10g/L tryptone, 5g/L yeast extract, and 5g/L NaCl) in 250-µl microwell plates. After revival, the populations were transferred every 24hrs at a 1:10 dilution in 0.5% salinity for 4 transfers. After the fourth transfer, I moved the populations to two separate salt

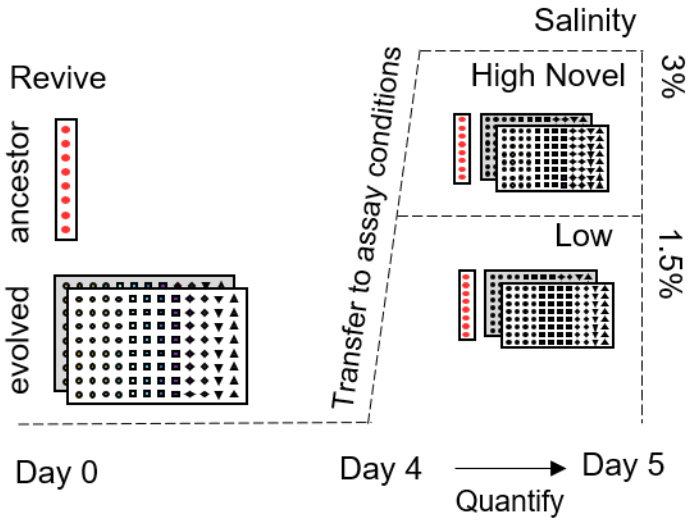

Figure 4.3: Revival and assay conditions. The ancestral and evolved lineages (day 100 populations) were revived and transferred in 0.5% salinity for 4 transfers. On the 4th transfer, the populations were moved to the low (1.5%) and high (3%) salinity environment. I then quantified the growth rate, transformation capacity, and population size over the next 24hrs (between day 4 & 5 after the revival).

environments in 250-µl microwell plates, to quantify the growth rate and transformation capacity (Figure 4.3). In the constant low salinity treatment, 10 of the 96 populations never revived. In the increasing salinity treatment, 1 population never revived and 1 was contaminated, these are not reported in the results (see Table S4.2 for a full list).

Each 24hr assay was conducted at low salinity (1.5% salinity: 10g/L tryptone, 5g/L yeast extract, and 15g/L NaCl) and high salinity – a novel and stressful environment for *P. stutzeri* (3% salinity: 10g/L tryptone, 5g/L yeast extract, and 30g/L NaCl) (Figure 4.3). For each population, I monitored absorbance at 600nm for 24hrs using a Biotek Synergy HGT (Winooski, VT) microplate reader. The growth curve data was fit to a standard form of the logistic equation using the Growthcurver package in R studio. I used the logistic equation to describe the population size N_t at time t:

$$N_t = \frac{K}{1 + (\frac{\mathrm{K} - N_0}{N_0})e^{-rt}}$$

4.3.4 TRANSFORMATION EFFICIENCY AND FREQUENCY

Cultures were revived following the same protocol used for growth rate determination (described in 4.3.3). I quantified the transformation efficiency by tracking the acquisition of gentamicin resistance into the evolved and ancestral *P. stutzeri* populations which were gentamicin susceptible. The eDNA encoding gentamicin resistance was prepared from a mutant strain of *P. stutzeri*, strain 28a24, which carries a gentamycin resistance gene and LacZ gene fused to a miniTn7 transposon (Tn7 transposition of pUC18-mini-Tn7T-Gm-lacZ). To begin the assay, I transferred 20µl from each evolved and ancestral population into 180µl of fresh LB media containing 1.5% and 3% salinity. I added genomic extracellular DNA (eDNA) resuspended in nanopore

water to each population and incubated at 30°C. After 24hrs I performed a serial dilution and titers were determined on selective media (LB + gentamycin [50 µg/ml] + Xgal [40 µg/ml]) and non-selective media (LB) using triplicate 10µl dots. Population level transformation efficiency was determined by dividing the average number of transformants in a population by the µg of eDNA (0.02µg). I also report population level transformation frequencies by dividing the average number of transformants by the total number of cells or the population size.

4.4.5 STATISTICAL ANALYSES

Prior to analysis, I checked that data met assumptions of normality and homogeneity of variance. I corrected for increased homogeneity of variance across population sizes using a log transformation. I analyzed bacterial growth rates and populations sizes using two-factor ANOVA, with Evolution Conditions, Assay Conditions, and their interaction as factors (see Figure 4.2 for factors). For each evolution treatment – constant salinity versus increasing salinity – I used a two-factor ANOVA with the Assay Conditions, Cell Lysate treatment, and their interaction as factors. I determined differences in transformation capacity using a general linearized model with a negative binomial distribution to account for positive skew. To determine statistical differences in the number of non-transforming populations (zeros) I used a two-part hurdle model from the hurdle package in R, as it specifies one process for zero counts and one process for positive counts, and is commonly used for positively skewed data with lots of zeros [132], [133].

4.4 RESULTS

4.4.1 GROWTH RATE

P. stutzeri adapted to changes in salinity after ~330 generations of serial transfer. Both evolution treatments (constant vs. increasing salinity) on average grew faster than the ancestor in the high salt environment. However, populations evolved in the increasing salinity environment grew faster in both the low and high salt environment (Figure 4.4A; Salinity $p < 0.001$; Treatment*Salinity $p = 0.052$). In addition, populations exposed to the gradual increase in salt, exhibited higher growth rates than those adapted to the constant salt concentration but only when tested at the lower salinity (Figure 4.4A**;** $p= 0.0468$). Interestingly, both of the evolved populations had larger population sizes in the high salt environment – relative to the low salt environment and to the ancestor (Figure 4.4B; $p <0.001$). This was surprising given the evolved populations grew significantly slower in that environment compared to the low salinity

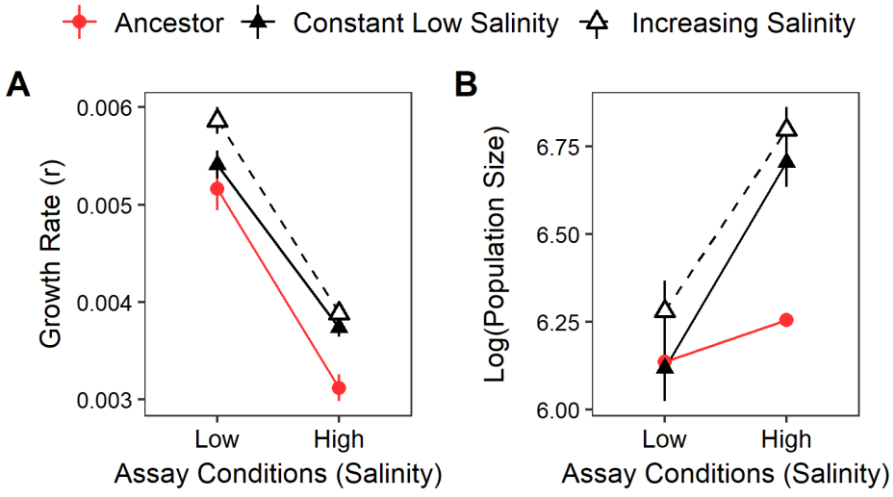

Figure 4.4: Effects of adaptive evolution. (A) Growth rate and (B) log transformed population size for the ancestor (red circles), constant low salinity (black closed triangles), and increasing salinity evolution treatments (black open circles) in low (1.5%) and high (3%) salinity. The points show the average across the ancestral (n=8) and evolved populations, and the error bars indicate the standard error (constant low, n = 86; increasing n = 94)**.**

4.4.2 LOSS OF TRANSFORMABILITY

Evolved populations exhibited a significant loss of transformation capacity relative to the ancestor (Figure 4.5; constant low salinity, p=0.005; increasing salinity, p=0.02). At the end of the experiment between 39% and 64% of evolved populations – depending on the treatment and test conditions still underwent transformation. In the remaining populations there were no transformants at a detectable level. In addition, there was a striking similarity between the two evolution treatments in terms of how many populations underwent transformation (Figure 4.5). In both evolution treatments, there were significantly more populations undergoing transformation when tested at the higher salinity, despite no known difference in genotype (Figure 4.5; p <0.001 for both treatments).

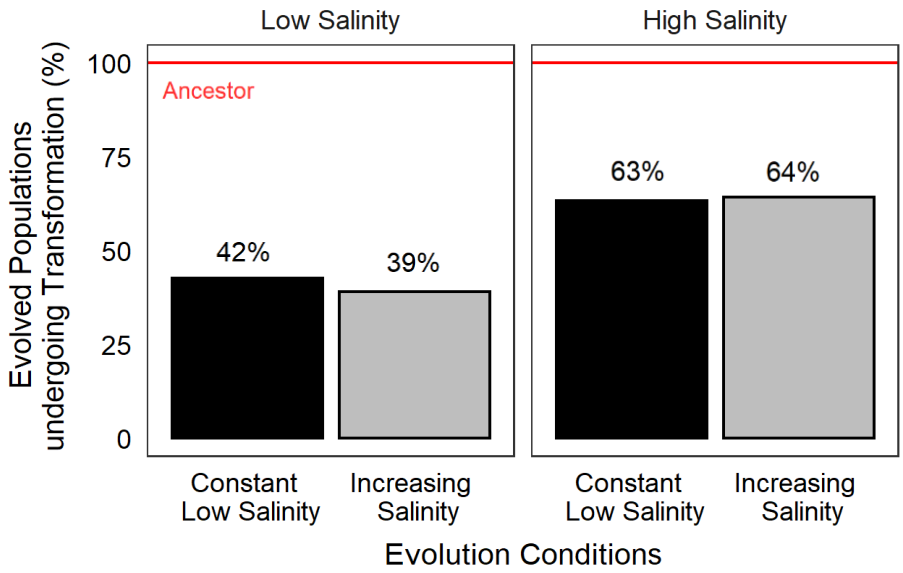

Figure 4.5: Loss of transformability. The number of evolved populations with a detectable number of transformants in the low and high salinity test environment for populations evolved in constant or increasing salinities (count data). The red line corresponds to the beginning of the experiment when transformants could be detected in 100% of the ancestral populations.

4.4.3 TRANSFORMANTS AND TOTAL CELLS

There was no relationship between the number of transformants and the number of recipient cells in independently evolving populations (Figure 4.6). This trend was true across the treatments, as well as agreeing with preliminary work showing that transformation is not limited by population size in larger populations – such as the ones in this experiment (Figure A4.1A**;** above 10,000 cells). Therefore, I report the number of

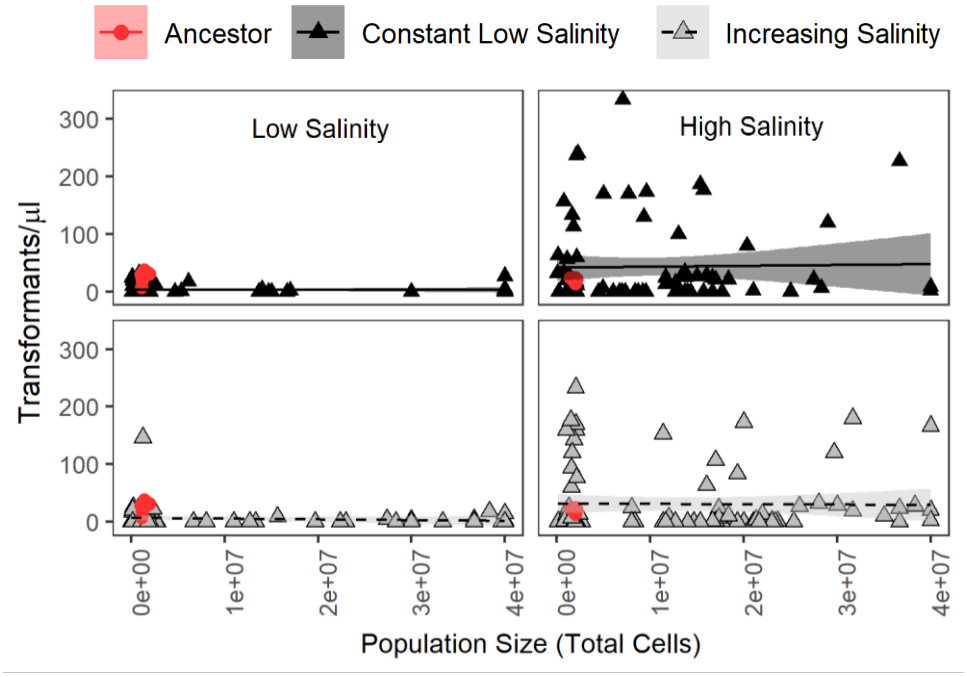

Figure 4.6: The relationship between transformants and total cells. In the ancestral and evolved populations, the number of transformants does not increase as the population size or total number of cells increases. Each point represents an individual population-level measurement. The linear relationship between transformants and total cells is indicated by the lines with the shaded areas showing the 95% confidence interval (n=8 ancestor, n=86 constant low and n=94 in the increasing salinity treatment).

transformants standardized by the amount of eDNA (transformation efficiency). However, I also report (in Figure 4.7B) the number of transformants standardized by the number of recipient cells (transformation frequency). This is done to account for the fact that, on average, there were significantly more transformants and recipient cells at higher salinities, suggesting the increase in the average number of transformants could

be correlated with the increase in the average number of recipient cells – despite there being no evidence of such a correlation within the individual populations.

4.4.5 HIGH VARIATION IN TRANSFORMATION EFFICIENCY

At the end of the experiment the transformation efficiency (transformants/µg DNA) was significantly lower in the low salt environment, regardless of the evolution conditions (Figure 4.7A; $p < 0.0001$). However, the populations that transformed eDNA and evolved at constant salinity, did so at a higher efficiency than the ancestor – but only when tested in the high salt environment (Figure A4.2; $p = 0.0216$). When the number of transformants was standardized by the number of recipient cells, there were no statistically significant differences in transformation frequency (Figure 4.7B). Although numerically the transformation frequency was highest for populations evolved in constant low salinities but moved to the high salinity environment for the transformation assay.

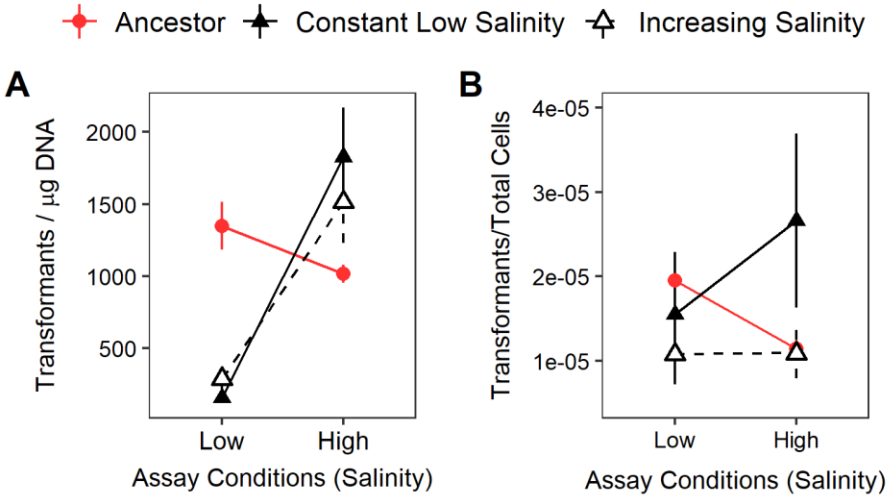

Figure 4.7: Changes in transformation in response to experimental evolution. (A) Transformation efficiency (transformants/µg DNA) and (B) transformation frequency (transformants/total Cells) for the ancestor (red circles) and evolved populations (constant low salinity = solid black lines; increasing salinity = dashed lines). The points show the average across the ancestral (n=8) and evolved (constant low = 86, increasing =94) populations, and the error bars indicate the standard error (averages include transforming and non-transforming populations).

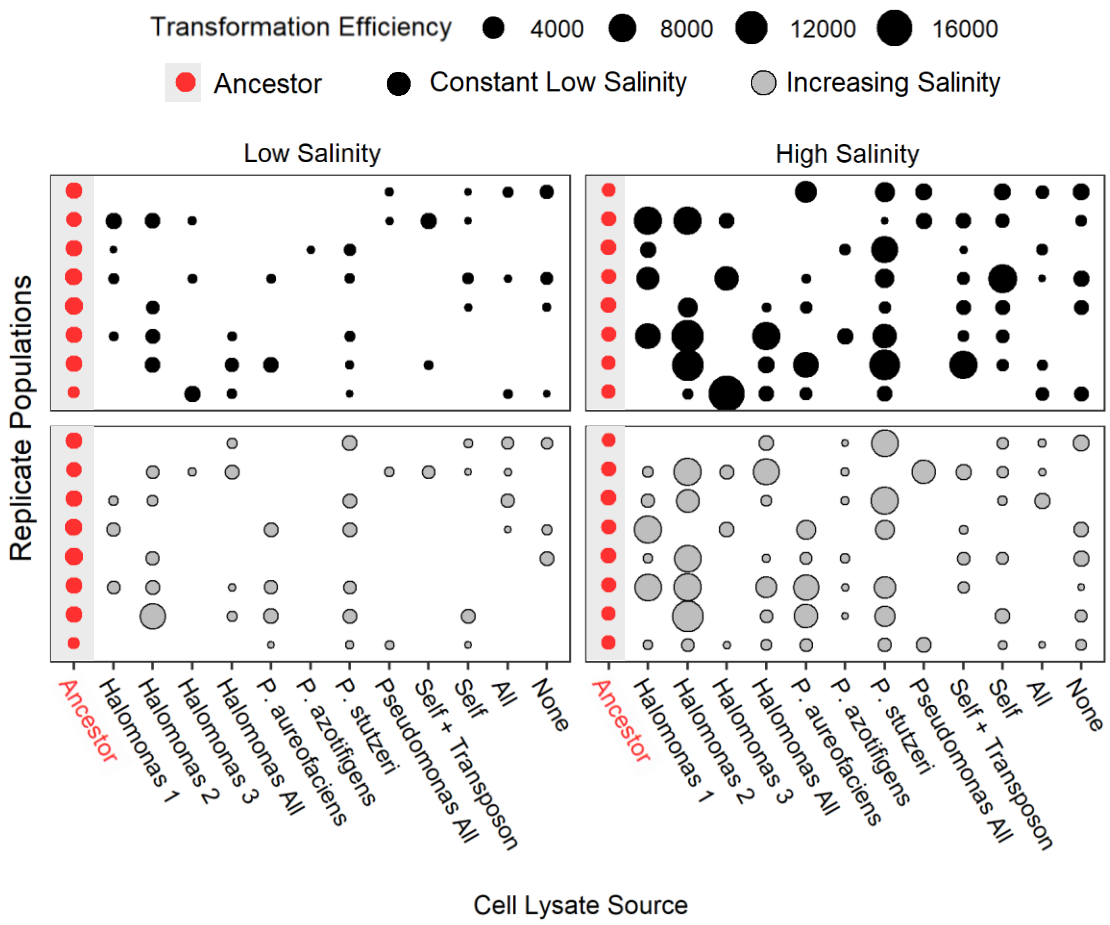

Figure 4.8: Variation in transformation efficiency. Transformation efficiency for the ancestral and evolved populations in low and high salinities. Higher transformation efficiencies are denoted by larger circles with the populations evolved at a constant low salinity in the top panel and those evolved in increasing salinities in the bottom panel. The cell lysates are shown on the x-axis and the replicate populations on the y-axis corresponding to a 96-well plate layout (n=8 replicates per cell lysate source).

Evolving with cells lysates or populations of dead cells did not affect transformation efficiency in a uniform manner (Figure 4.8; Figure A4.3; see Table A4.3 for expanded results). In general, the transformation efficiency was higher in the high salt environment. However, standardizing by the population size indicated there was no difference in transformation frequency between the low and high salt environment – as the number of transformants and the number of total cells was larger in the high salt environment (Figure A4.4). Moreover, there were no consistent changes in growth rate

or population size with the addition of different cell lysates (Figure A4.5; see Table A4.4 and A4.5 for expanded results). There was a high level of congruency between the two evolution treatments in terms of which populations underwent transformation (Figure 4.8; comparing the top and bottom panels). These effects may have appeared early in the experiment, since the two treatments diverged from a single set of evolving populations on day 50 of the experiment.

4.4.6 TRADE-OFF BETWEEN GROWTH RATE AND TRANSFORMATION

There was no trade-off between the transformation efficiency and the growth rate (Figure 4.9; the same being true for transformation frequency – data not shown). While there may have been tradeoff between growth rate and transformation within individual strains, I was unable to detect such a tradeoff in the population-level measurements conducted here.

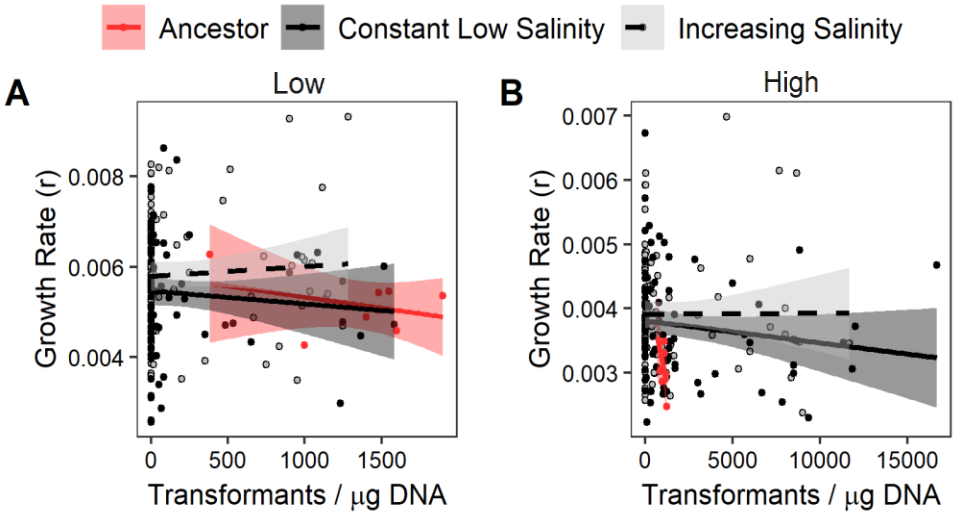

Figure 4.9: Trade-off between growth rate and transformation efficiency. Average population-level growth rate regressed against the transformation efficiency in the (A) low salinity and (B) high salinity environments. Each point represents an individual population-level measurement. The linear relationship between transformants and total cells is indicated by the line, with the shaded area showing the 95% confidence interval (n=8 ancestor, n=86 constant low and n=94 in the increasing salinity treatment).

4.5 DISCUSSION

Understanding the evolutionary origins and fitness consequences of transformation can shed light on the larger question of why organisms undergo genetic recombination, and to what extent the traits governing selection are themselves selected upon. Here, I conclude that evolving *P. stutzeri* with different sources of eDNA or evolving them in constant versus increasing salinities did not have a large effect on growth rate or transformation efficiency. However, I did find that the transformation capacity – the ability for evolved populations to transform eDNA – changed dramatically over just ~330 generations or 100 days of serial transfer.

By the end of the experiment, around 50% of the evolved populations did not transform any of the provided extracellular antibiotic resistance genes, although the exact percentage varied from 36-61% depending on the treatment. This was true, regardless of whether the number of transformants was reported as the transformation efficiency (transformants/µg eDNA) or the transformation frequency (transformants/total cells). I report both metrics here to account for differences in average population size in the low versus high salinity environment, even though the total population size only limits transformation in very small populations of *P. stutzeri* (smaller than those reported here; Figure A4.1B). Overall, I focus the discussion on the variation in transformation capacity across the evolved lineages, as this is true irrespective of how the data is analyzed (transformation efficiency vs. transformation frequency).

Several other bacterial species, in addition to *P. stutzeri*, undergo transformation irrespective of the population density. For instance, *Vibrio parahaemolyticus* and *V. campbellii* both undergo transformation in the absence of quorum sensing which is the

ability to regulate gene expression with population size. Meanwhile, their close relative *V. cholerae,* and *Streptococcus* species both require quorum sensing for successful transformation [29], [134]. Interestingly, the genetic features that underpin the differences in quorum sensing across *Vibrio* species have yet to be identified. Similarly, different isolates of the same bacterial species often exhibit large differences in their transformation capacity, with the genetic variation underpinning these differences often impossible to discern. For instance, isolates of *P. stutzeri* collected from different soil environments had highly variable transformation frequencies, with about one-third of isolates considered non-transformable [28]. Similar observations have been made in *Vibrio* species that inhabit different environments, and spurred the recent suggestion that transformation may be lost in environments where it is no longer beneficial [29], [135].

Hence, it is possible that transformation was not maintained in several of the evolved lineages because it was not providing a fitness benefit during experimental evolution. Weak selection for transformation could have been due to the application of only a mild stress or due to infrequent fluctuations in the environment. For instance, previous studies that found transformation was beneficial, tended to shift the environment every 2-3 transfers [51], [126], as opposed to every 20-30 transfers as was done in this study. Therefore, an interesting follow-up study would be to compare the distribution of transformation phenotypes after evolving *P. stutzeri* in a constant optimal environment versus a constant but very stressful environment – to better understand how stress, or fluctuations in stress, shape the evolution of transformation.

Another possibility is that transformation only provides a fitness benefit in response to very specific stressors. For instance, several studies that found transformation was beneficial, exposed evolving populations to periodic inputs of sub-inhibitory concentrations of antibiotics [51], [126]. Because transformation allows the reversible integration of resistance genes, and antibiotics are usually transiently present in the environment, transformation could be a mechanism well-suited to handling antibiotic stress. In contrast, transformation may be less beneficial in response to stressors like changes in osmotic pressure which are encoded by large and connected gene networks. In general, more work needs to be done on the specific stressors that transformation confers a benefit to, as prokaryotic genes appear to adapt to either vertical or horizontal transmission, meaning that not all processes may be well-adapted to evolve via horizontal gene transfer [136].

A final consideration is the role of osmotic pressure in altering the efficiency of eDNA uptake. Populations adapting to high osmolarity environments generally have a large fraction of mutations in genes associated with cell wall synthesis [137]. Therefore, it could be that changes in the cell wall altered the pilus structure which captures eDNA from the environment [138]. *P. stutzeri* has two pili that interact to regulate transformation. The type IV pilus acquires eDNA from the environment, while the second pilus is believed to translocate eDNA into the cytoplasm and when knocked out decreases transformation ~90% [139]. Therefore, changes in the cell wall in response to salt stress could have altered the interaction between these two pili, creating the gradient of transformation capacity evident in the evolved lineages. Follow-up

investigations which involve whole genome sequencing, will hopefully elucidate if mutations in osmoregulatory genes could have altered transformation capacity.

Despite evidence of high variation in transformation capacity in many bacterial lineages, very few studies have quantified changes in transformation during experimental evolution. To date, six experimental evolution studies have focused on the fitness effects of transformation but only one study has quantified transformation before and after experimental evolution. In that one study, transformation did not provide a fitness benefit and the evolved lineages repeatedly lost the capacity to undergo transformation [55]. Several other studies have identified transformation-mediated fitness benefits (primarily in stressful environments), but none of them quantified the prevalence of transformation at the end of the experiment (Table 4.1).

Yet, quantifying transformation after adaptation could help disentangle the benefits of transformation from the overall benefits of competence – which is the physiological state a bacterium must enter to undergo transformation and often initiates multiple stress responses alongside transformation. For instance, Pseudomonads also use the Type IV pilus for flagellum-independent movement or twitching motility [139]. While *Bacillus subtilis*, a well-studied soil-dweller, upregulates transformation as part of a general stress response prompted by DNA damage or antibiotics [121]. Therefore, future studies that quantify changes in transformation efficiency during and after experimental evolution, will be critical in disentangling the specific benefits of transformation within the larger regulatory network of competence.

Overall, this study provides novel experimental evidence that the ability to undergo transformation can change over relatively short timescales and may be more

plastic across space and time than is generally accepted. Most intersting is the substantial decrease in transformation efficiency in the low salt environment where *P. stutzeri* evolved, suggesting that transformation did not provide a fitness benefit during salt adaptation. Taken together, this work suggests that transformation is an adaptive, selectable trait, that may increase or decrease rapidly in response to selection.

APPENDIX

APPENDIX

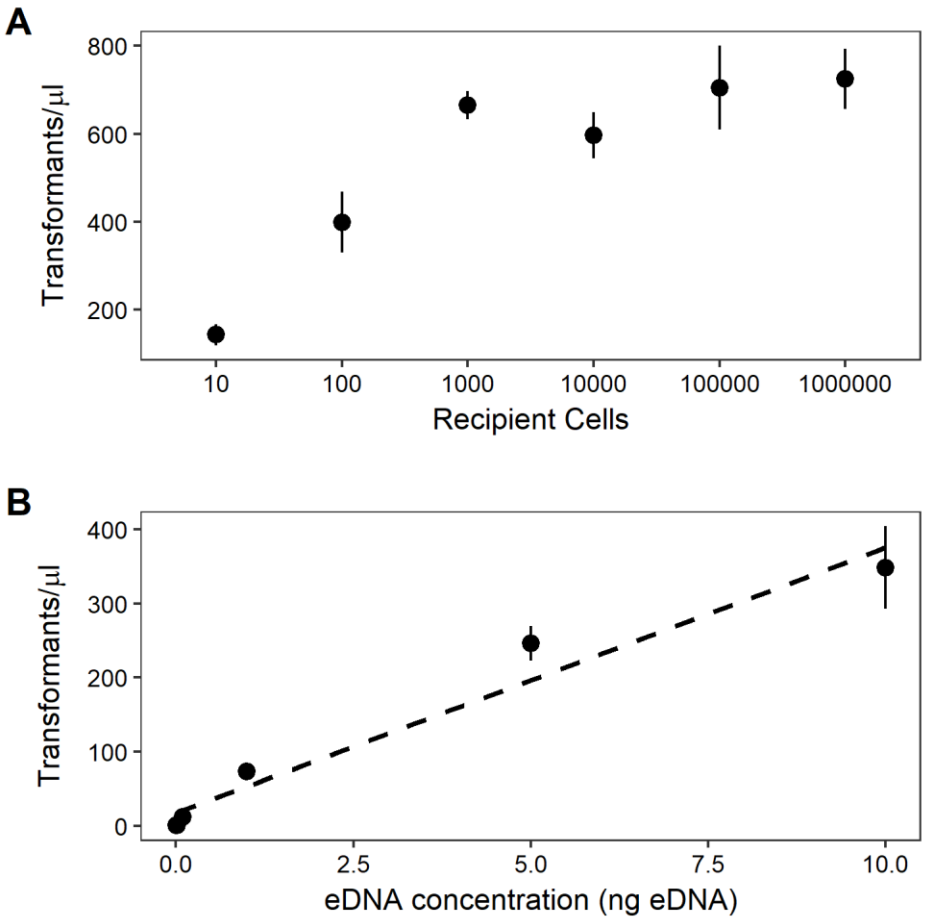

Figure A4.1: Preliminary transformation assays. (A) The effect of total population size on the number of transformants when the eDNA concentration is held constant. (B) The relationship between the concentration of eDNA and the number of transformants under laboratory conditions (0.001, 0.01, 0.1, 1, 5, 10 ng/µl eDNA) – in large populations (~1,000,000 recipient cells).

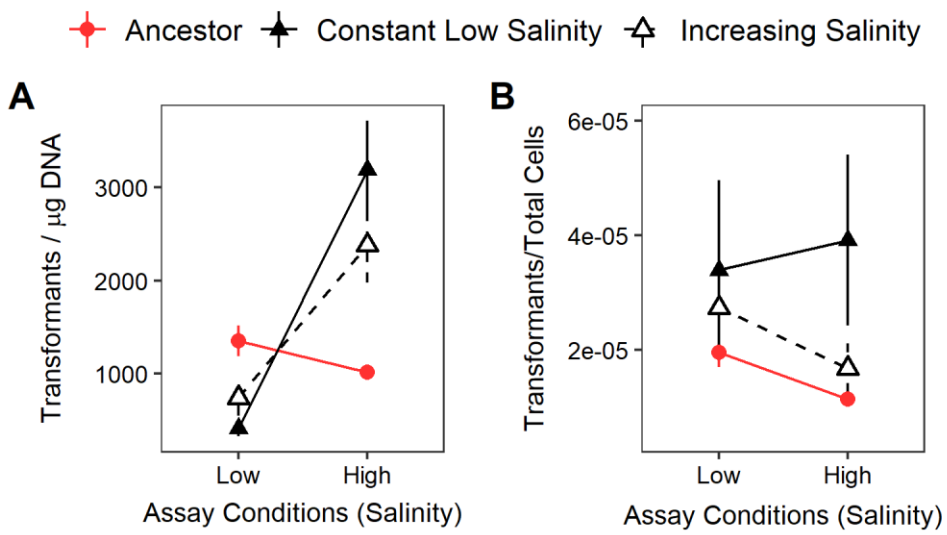

Figure A4.2: Transformability in the populations that transformed eDNA. (A) Transformation efficiency (transformants/µg DNA) and (B) transformation frequency (transformants/total Cells) for the ancestor (red circles) and evolved populations (constant low salinity = solid black lines; increasing salinity = dashed lines). The points show the average across the ancestral (n=8) and evolved (constant low = 86, increasing =94) populations, and the error bars indicate the standard error (averages include only populations that underwent transformation).

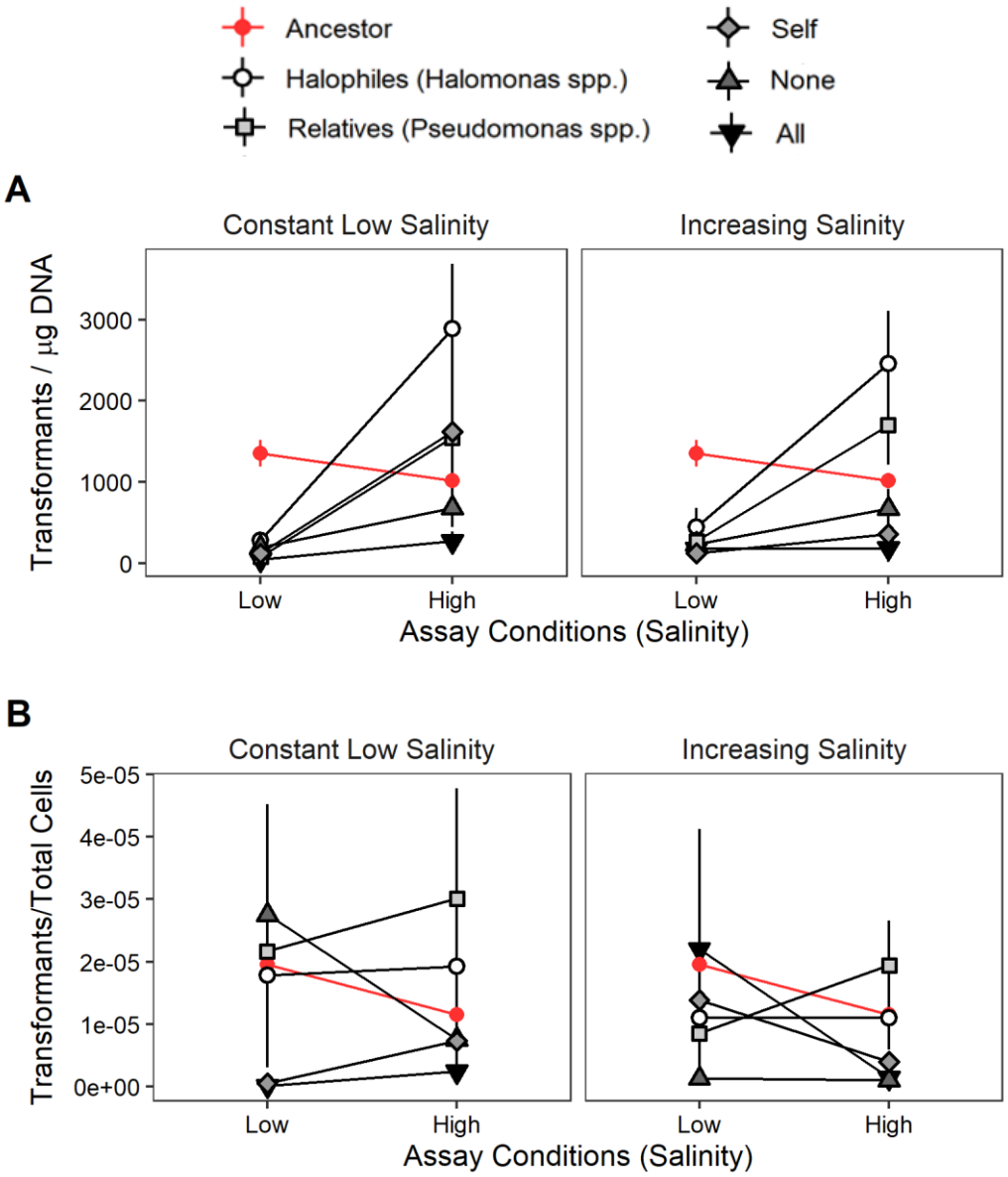

Figure A4.3: Effect of cell lysates on transformation. (A) Transformation efficiency, and (B) transformation frequency for each cell lysate treatment. The ancestor (red circles), the constant low salinity (left panel) and the increasing salinity treatments (right panel, dashed lines) are shown at low and high salinity (n=8 replicate populations across treatments, excluding treatments that went extinct, see table S4.2).

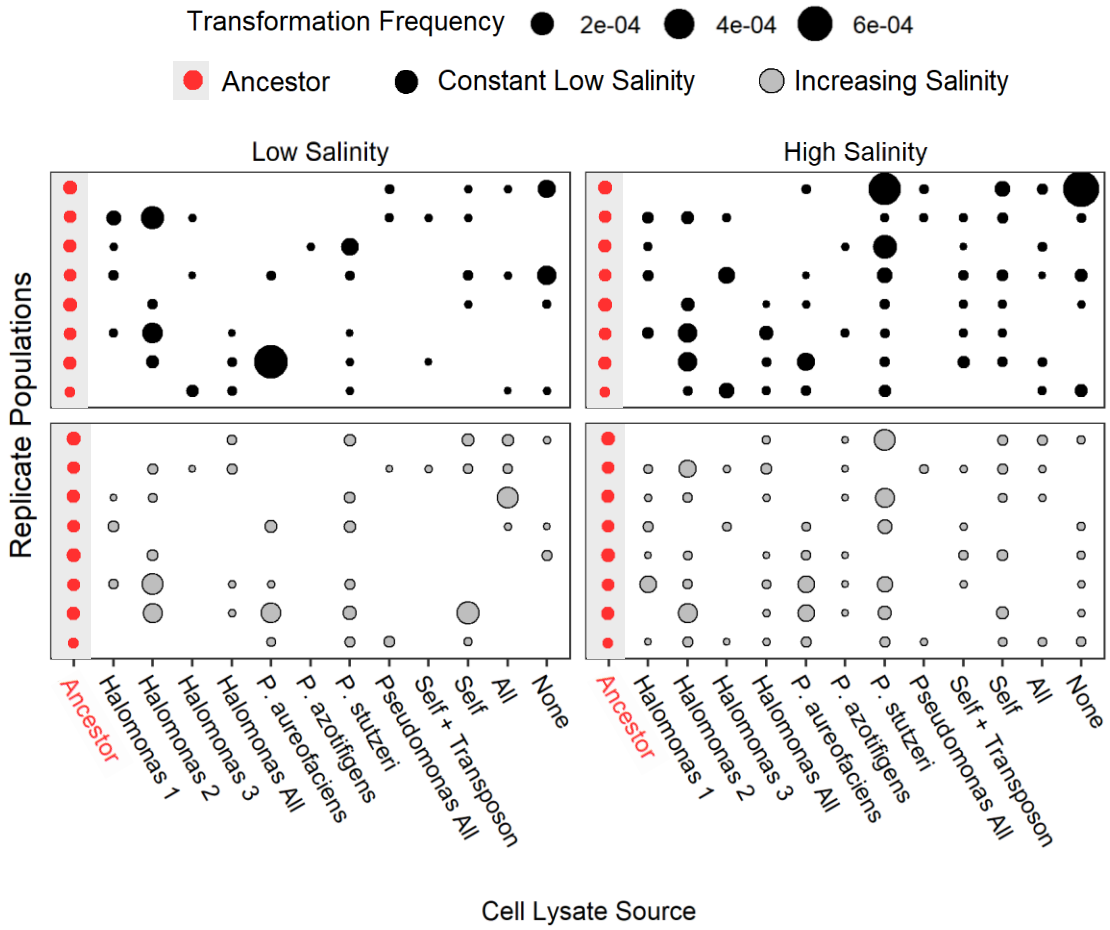

Figure A4.4: Variation in transformation frequency. Transformation frequency for the ancestral and evolved populations in low and high salinities. Higher transformation frequencies are denoted by larger circles with the populations evolved at a constant low salinity in the top panel and those evolved in increasing salinities in the bottom two panels. The cell lysates are shown on the x-axis and the replicate populations on the y-axis corresponding to a 96-well plate (n=8 replicates per cell lysate).

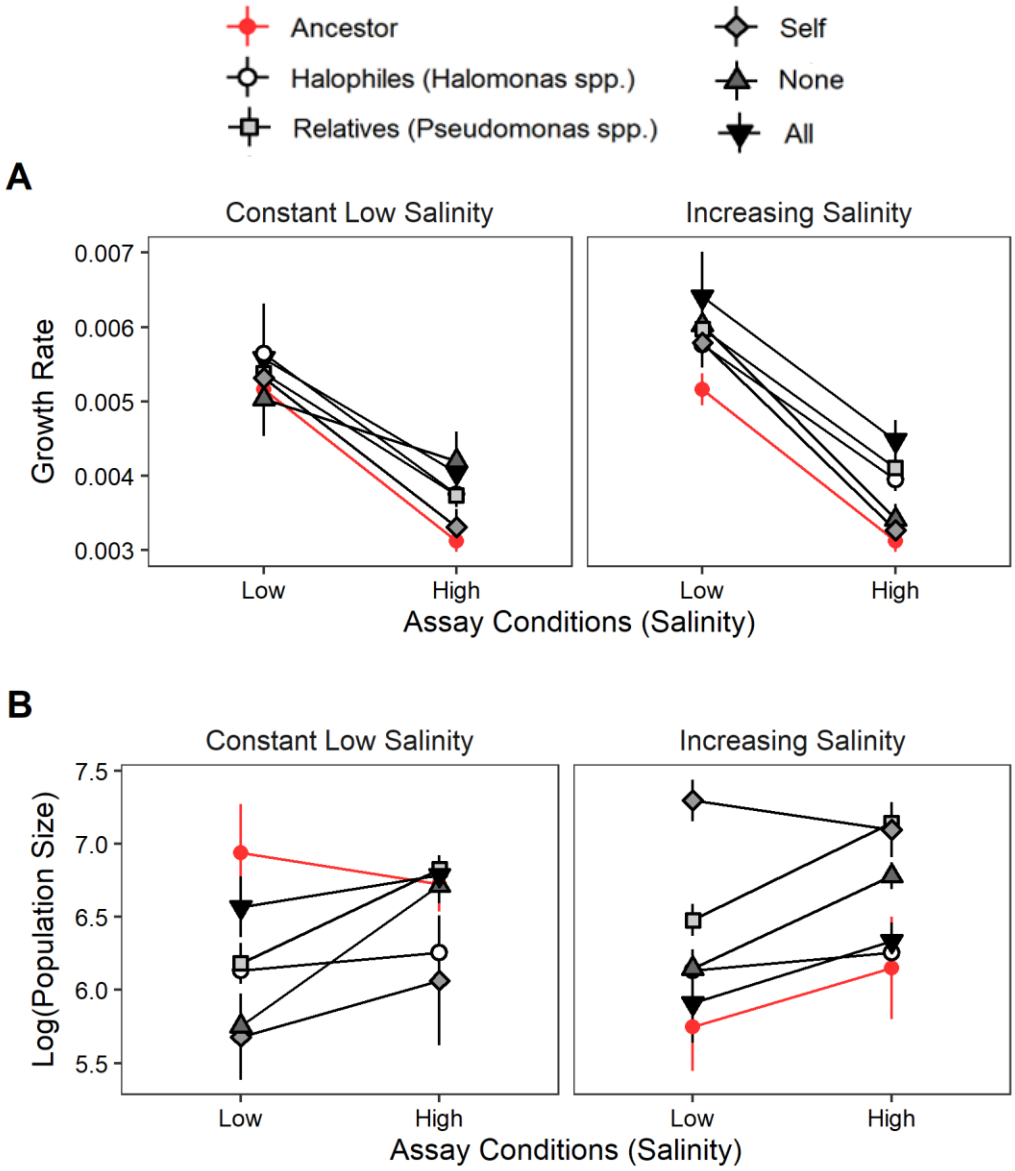

Figure A4.5: Effect of cell lysates on growth rate and population size. (A) Growth rate, and (B) log transformed population size for each cell lysate treatment. The ancestor (red circles), the constant low salinity (left panel) and the increasing salinity treatments (right panel, dashed lines) are shown at low and high salinity (n=8 replicate population across treatments).

Table A4.1: Cell lysate sources. The halophiles were collected from salt springs in the Namib dessert. The conductivity of the spring at the time of collection is listed for *Halomonas spp*. The *Pseudomonas* relatives were purchased from the German culture collection (accessions number listed).

Strain	Cell Lysate Category	Strain Source	Conductivity (mS/cm)
Halomonas spp. 1 (*Halomonas taeanensis*)	Halophile	Namib Springs Aub Canyon	7.3
Halomonas spp. 2 (*Halomonas*)	Halophile	Namib Springs Kai-As	1
Halomonas spp. 3 (*Halomonas*)	Halophile	Namib Springs Swartmodder	190
Halomonas spp. All (1+2+3)	Halophile		
Pseudomonas chlororaphis subsp. *aureofaciens*	Relative	DSM6698	
Pseudomonas azotifigens	Relative	DSM17556	
Pseudomonas stutzeri JM300	Relative	DSM10701	
Pseudomonas spp. All	Relative		
Self-Lysate + Transposon (*P. stutzeri* + miniTn7)	Self	Baltrus lab	
Self-Lysate (*P. stutzeri*)	Self	Baltrus lab	
None	None		
All	All		

Table A4.2: Populations that did not revive. List of populations that did not revive at the end of the experiment. One population was contaminated (3c).

96-Microplate Well	Evolution Treatment	Cell Lysate Treatment
1a	Constant Low Salinity	Halomonas spp. 1 (*Halomonas taeanensis*)
2a	Constant Low Salinity	Halomonas spp. 2 (*Halomonas*)
3a	Constant Low Salinity	Halomonas spp. 3 (*Halomonas*)
4a	Constant Low Salinity	Halomonas spp. All (1+2+3)
4b	Constant Low Salinity	Halomonas spp. All (1+2+3)
5c	Constant Low Salinity	*Pseudomonas chlororaphis* subsp. *aureofaciens*
6g	Constant Low Salinity	Pseudomonas azotifigens
6h	Constant Low Salinity	Pseudomonas azotifigens
10c	Constant Low Salinity	Self Lysate (*P. stutzeri*)
11b	Constant Low Salinity	None
1a	Increasing Salinity	Halomonas spp. 1 (*Halomonas taeanensis*)
3c	Increasing Salinity	Halomonas spp. 3 (*Halomonas*)

Table A4.3: Expanded transformation efficiency results. Letters for pairwise comparisons across the cell lysate treatments with p-values listed at the bottom of the column.

	Transformation Efficiency			
	Constant Salinity		Increasing Salinity	
	1.50%	3.00%	1.50%	3.00%
Ancestor	a	a	b	a
Self Lysate	b	a	a	a
No Lysate	b	a	ab	a
Halophile Lysate	b	a	ab	a
Relatives Lysate	b	a	a	a
All spp.	b	a	ab	a
	p<0.0001	p=0.055	p=0.0207	p=0.0506

Table A4.4: Expanded growth rate results. Letters for pairwise comparisons across the cell lysate treatments with p-values listed at the bottom of the column.

	Growth Rate (r)			
	Constant Salinity		Increasing Salinity	
	1.50%	3.00%	1.50%	3.00%
Ancestor	a	a	a	a
Self Lysate	a	ab	ab	ab
No Lysate	a	b	a	abc
Halophile Lysate	a	b	ab	bc
Relatives Lysate	a	b	b	c
All spp.	a	b	ab	c
	p=0.697	p<0.001	p=0.08	p<0.001

Table A4.5: Expanded population size results. Letters for pairwise comparisons across the cell lysate treatments with p-values listed at the bottom of the column.

	Log_{10} (Populations size)			
	Constant Salinity		Increasing Salinity	
	1.50%	3.00%	1.50%	3.00%
Ancestor	abc	ab	a	ab
Self Lysate	bc	ab	a	ab
No Lysate	ab	a	b	c
Halophile Lysate	abc	b	ab	c
Relatives Lysate	a	ab	a	bc
All spp	c	ab	a	a
	p=0.008	p=0.02	p<0.001	p<0.001

CHAPTER 5:

CONCLUSIONS

The goal of my dissertation was to understand if natural transformation promotes rapid adaptation in soil bacteria. I first studied eDNA dynamics after a drying-rewetting disturbance as increases in eDNA from bacterial death could promote transformation. Overall, I found that eDNA cycled rapidly through soil in response to drying rewetting, ultimately disappearing from soil as bacterial communities recovered from the disturbance (Ch. 2). Interestingly, most sequences present in the eDNA pool were also present in the live community, suggesting that eDNA – even if slightly deleterious – could promote genetic admixing within bacterial species recovering from a disturbance. I also used this dataset to test if including eDNA in microbial community characterizations led to false conclusions about live bacterial communities. I showed that including eDNA in analyses minimally affected most conclusions about bacterial sensitivity but did mask subtle signatures of bacterial resilience and recovery post-disturbance that could ultimately skew predictions of ecosystem stability.

In a second study, I quantified transformation in soil microcosms to determine the environmental drivers of eDNA acquisition (Ch. 3). I found that *P. stutzeri* could transform eDNA under most soil conditions – including those seen across the drying rewetting disturbance. I show that eDNA encoding antibiotic resistance genes can be transformed at high rates in soil. These findings suggest that widespread efforts to reduce the spread of antibiotic resistance genes in the environment should incorporate methods that eliminate both live and extracellular sources of drug resistance.

In a final laboratory experiment, I tested the fitness effects of transformation by adapting *P. stutzeri* to high salt concentrations. After ~330 generations of adaptation, I quantified the growth rate and transformation efficiency of the evolved populations. I found that *P. stutzeri* grew faster than the ancestor at high salt concentrations, but that the transformation efficiency was greatly diminished (Ch. 4). Overall, ~50% of the evolved populations did not undergo transformation, despite the original or ancestral populations *all* undergoing transformation. This suggests that transformation was not responsible for salt adaptation and may have been selected against during experimental evolution. In general, this work contributes to the larger question of why bacteria undergo transformation, and sets the stage for future experiments to investigate the question: under what conditions do transformable cells outcompete their non-transformable counterparts?

Taken together, I find that eDNA cycles through soil rapidly after a pulse disturbance and is readily transformed under these soil conditions. I also find that the transformation efficiency can change dramatically over just ~330 generations, suggesting that transformation capacity may be an adaptive trait that diminishes in the absence of selection. Overall, this body of research supports the hypothesis that transformation is periodically adaptive but often maladaptive and is likely important in spatially structured environments where new niches regularly open for invasion [11]. It also suggests that we need to expand our view of the community metagenome to include extracellular DNA which is biologically active after bacterial death through the process of natural transformation.

BIBLIOGRAPHY

BIBLIOGRAPHY

[1] E. Low-Décarie *et al.*, “Community rescue in experimental metacommunities,” *Proc. Natl. Acad. Sci.*, no. 10, p. 201513125, 2015.

[2] N. D. Youngblut, A. Shade, J. S. Read, K. D. Mcmahon, and R. J. Whitaker, “Lineage-specific responses of microbial communities to environmental change,” *Appl. Environ. Microbiol.*, vol. 79, no. 1, pp. 39–47, 2013.

[3] S. D. Allison and J. B. H. Martiny, “Resistance, resilience, and redundancy in microbial communities,” *Proc. Natl. Acad. Sci.*, vol. 105, no. Supplement 1, pp. 11512–11519, 2008.

[4] D. Nemergut, A. Shade, and C. Violle, “When, where and how does microbial community composition matter?,” *Front. Microbiol.*, vol. 5, no. SEP, pp. 2012–2014, 2014.

[5] R. Jain, M. C. Rivera, J. E. Moore, and J. A. Lake, “Horizontal gene transfer accelerates genome innovation and evolution,” *Mol. Biol. Evol.*, vol. 20, no. 10, pp. 1598–1602, 2003.

[6] H. Ochman, J. G. Lawrence, and E. a Groisman, “Lateral gene transfer and the nature of bacterial innovation.,” *Nature*, vol. 405, no. 6784, pp. 299–304, 2000.

[7] C. J. H. Von Wintersdorff *et al.*, “Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer,” *Front. Microbiol.*, vol. 7, no. FEB, pp. 1–10, 2016.

[8] J. Martinez, “Antibiotics and Antibiotic Resistance Genes in Natural Environments,” *Science (80-.).*, vol. 321, 2008.

[9] F. M. Cohan, “Bacterial speciation: Genetic sweeps in bacterial species,” *Curr. Biol.*, vol. 26, no. 3, pp. R112–R115, 2016.

[10] S. D. Quistad, G. Doulcier, and P. B. Rainey, “Experimental manipulation of selfish genetic elements links genes to microbial community function,” *Philos. Trans. B*, 2019.

[11] E. V. Koonin, “Horizontal gene transfer: essentiality and evolvability in prokaryotes, and roles in evolutionary transitions,” *F1000Research*, vol. 5, no. 0, p. 1805, 2016.

[12] W. Hao and G. Brian Golding, “The fate of laterally transferred genes: Life in the fast lane to adaptation or death,” *Genome Res.*, vol. 16, no. 5, pp. 636–643, 2006.

[13] P. R. Marri, W. Hao, and G. B. Golding, “Gene gain and gene loss in

Streptococcus: Is it driven by habitat?," *Mol. Biol. Evol.*, vol. 23, no. 12, pp. 2379–2391, 2006.

[14] P. R. Marri, W. Hao, and G. B. Golding, "The role of laterally transferred genes in adaptive evolution," *BMC Evol. Biol.*, vol. 7, no. SUPPL. 1, pp. 27–29, 2007.

[15] R. W. Nowell, S. Green, B. E. Laue, and P. M. Sharp, "The extent of genome flux and its role in the differentiation of bacterial lineages," *Genome Biol. Evol.*, vol. 6, no. 6, pp. 1514–1529, 2014.

[16] M. Vos and X. Didelot, "A comparison of homologous recombination rates in bacteria and archaea," *ISME J.*, vol. 393, pp. 199–208, 2009.

[17] R. Jain, M. C. Rivera, J. E. Moore, and J. a Lake, "Horizontal gene transfer in microbial genome evolution.," *Theor. Popul. Biol.*, vol. 61, no. 4, pp. 489–495, 2002.

[18] N. Frazão, A. Sousa, M. Lässig, and I. Gordo, "Horizontal gene transfer overrides mutation in Escherichia coli colonizing the mammalian gut," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 116, no. 36, pp. 17906–17915, 2019.

[19] O. Johnsborg, V. Eldholm, and L. S. Ha, "Natural genetic transformation : prevalence , mechanisms and function," vol. 158, 2007.

[20] M. Blokesch, "Natural competence for transformation," *Curr. Biol.*, vol. 26, no. 21, pp. R1126–R1130, 2016.

[21] K. L. Meibom, M. Blokesch, N. A. Dolganov, C.-Y. Wu, and G. K. Schoolnik, "Chitin Induces Natural Competence in Vibrio cholerae," 2005.

[22] M. Khanna and G. Stotzky, "Transformation of Bacillus subtilis by DNA bound on montmorillonite and effect of DNase on the transforming ability of bound DNA," *Appl. Environ. Microbiol.*, vol. 58, no. 6, pp. 1930–1939, 1992.

[23] M. G. Lorenz and W. Wackernagel, "Bacterial gene transfer by natural genetic transformation in the environment.," *Microbiol. Rev.*, vol. 58, no. 3, pp. 563–602, 1994.

[24] S. E. Jones and J. T. Lennon, "Dormancy contributes to the maintenance of microbial diversity," *Proc. Natl. Acad. Sci.*, vol. 107, no. 13, pp. 5881–5886, 2010.

[25] J. W. Sorensen and A. Shade, "Dormancy dynamics and dispersal contribute to soil microbiome resilience," *Philos. Trans. R. Soc. B Biol. Sci.*, vol. 375, no. 1798, 2020.

[26] D. J. Levy-Booth *et al.*, "Cycling of extracellular DNA in the soil environment," *Soil Biol. Biochem.*, vol. 39, no. 12, pp. 2977–2991, 2007.

[27] J. De Vries and W. Wackernagel, “Integration of foreign DNA during natural transformation of Acinetobacter sp. by homology-facilitated illegitimate recombination,” *PNAS*, vol. 99, no. 4, pp. 2094–2099, 2001.

[28] J. Sikorski, N. Teschner, and W. Wackernagel, “Highly different levels of natural transformation are associated with genomic subgroups within a local population of Pseudomonas stutzeri from soil,” *Appl. Environ. Microbiol.*, vol. 68, no. 2, pp. 865–873, 2002.

[29] C. A. Simpson, R. Podicheti, D. B. Rusch, A. B. Dalia, and C. Van Kessel, “Diversity in Natural Transformation Frequencies and Regulation across Vibrio Species,” vol. 10, no. 6, pp. 1–16, 2019.

[30] W. J. Parton, D. W. Anderson, C. V Cole, and J. W. B. Stewart, “Simulation of soil organic matter formations and mineralization in semiarid agroecosystems,” *Spec. Publ. Georg. Agric. Exp. Station.*, 1983.

[31] K. M. Nielsen, P. J. Johnsen, D. Bensasson, and D. Daffonchio, “Release and persistence of extracellular DNA in the environment,” *Environ. Biosafety Res.*, vol. 6, no. 1–2, pp. 37–53, 2007.

[32] E. Gallori *et al.*, “Transformation of Bacillus subtilis by DNA bound on clay in non-sterile soil,” *FEMS Microbiol. Ecol.*, vol. 15, no. 1–2, pp. 119–126, 1994.

[33] P. Carini, P. J. Marsden, J. W. Leff, E. E. Morgan, M. S. Strickland, and N. Fierer, “Relic DNA is abundant in soil and obscures estimates of soil microbial diversity,” *Nat. Mic*, vol. 53, no. 9, p. 680840, 2016.

[34] M. G. Lorenz and W. Wackernagel, “Adsorption of DNA to sand and variable degradation rates of adsorbed DNA.,” *Appl. Environ. Microbiol.*, vol. 53, no. 12, pp. 2948–2952, 1987.

[35] L. S. England, L. Hung, and J. T. Trevors, “Persistence of Pseudomonas aureofaciens strains and DNA in soil,” *Soil Biol. Biochem.*, vol. 29, no. 9–10, pp. 1521–1527, 1997.

[36] J. Sikorski, S. Graupner, M. G. Lorenz, and W. Wackernagel, “Natural genetic transformation of Pseudomonas stutzeri in a non-sterile soil,” *Microbiology*, vol. 144, no. 2, pp. 569–576, 1998.

[37] P. Freese, K. Korolev, J. Jimenez, and I. Chen, “Genetic Drift Suppresses Bacterial Conjugation in Spatially Structured Populations,” *Biophys. J.*, vol. 106, no. February, pp. 944–954, 2014.

[38] C. S. Smillie, M. B. Smith, J. Friedman, O. X. Cordero, L. A. David, and E. J. Alm, “Ecology drives a global network of gene exchange connecting the human microbiome,” *Nature*, vol. 480, no. 7376, pp. 241–244, 2011.

[39] J. Felsenstein and S. Yokoyama, “The evolutionary advantage of recombination. II. Individual selection for recombination,” *Genetics*, vol. 83, no. 4, pp. 845–859, 1976.

[40] H. J. Muller, “The relation of recombination to mutational advance,” *Mutat. Res. - Fundam. Mol. Mech. Mutagen.*, vol. 1, no. 1, pp. 2–9, 1964.

[41] D. Misevic, R. E. Lenski, and C. Ofria, “Sexual reproduction and Muller’s ratchet in digital organisms,” *Ninth Int. Conf. Artif. Life*, pp. 340–345, 2004.

[42] P. Seitz and M. Blokesch, “Cues and regulatory pathways involved in natural competence and transformation in pathogenic and environmental Gram-negative bacteria,” *FEMS Microbiol. Rev.*, vol. 37, no. 3, pp. 336–363, 2013.

[43] G. Carvalho *et al.*, “Bacterial transformation buffers environmental fluctuations through the reversible integration of mobile genetic elements,” pp. 1–12, 2019.

[44] M. Blokesch, “In and out — contribution of natural transformation to the shuffling of large genomic regions,” *Curr. Opin. Microbiol.*, vol. 38, pp. 22–29, 2017.

[45] C. Johnston, B. Martin, G. Fichant, P. Polard, and J. P. Claverys, “Bacterial transformation: Distribution, shared mechanisms and divergent control,” *Nature Reviews Microbiology*, vol. 12, no. 3. 2014.

[46] S. Guiral, T. J. Mitchell, B. Martin, and J. Claverys, “Competence-programmed predation of noncompetent cells in the human pathogen Streptococcus pneumoniae : Genetic requirements,” *PNAS*, 2005.

[47] J. W. Veening and M. Blokesch, “Interbacterial predation as a strategy for DNA acquisition in naturally competent bacteria,” *Nat. Rev. Microbiol.*, vol. 15, no. 10, pp. 621–629, 2017.

[48] N. Takeuchi, K. Kaneko, and E. V. Koonin, “Horizontal gene transfer can rescue prokaryotes from Muller’s ratchet: Benefit of DNA from dead cells and population subdivision,” *G3 Genes, Genomes, Genet.*, vol. 4, no. 2, pp. 325–339, 2014.

[49] M. Lynch, R. rger, D. Buthcer, and W. Gabriel, “The mutation meltdown in asexual populations,” *J.Hered.*, vol. 84, no. February, pp. 339–344, 1993.

[50] D. A. Baltrus, K. Guillemin, and P. C. Phillips, “Natural Transformation increases the rate of adaptation in the human pathogen Helicobacter pylori,” *Evolution (N. Y).*, vol. 62, no. 1, pp. 39–49, 2007.

[51] D. J. P. Engelmoer, I. Donaldson, and D. E. Rozen, “Conservative Sex and the Benefits of Transformation in Streptococcus pneumoniae,” *PLoS Pathog.*, vol. 9, no. 11, pp. 1–7, 2013.

[52] D. A. Baltrus, “Exploring the costs of horizontal gene transfer,” *Trends Ecol. Evol.*,

vol. 28, no. 8, pp. 489–495, 2013.

[53] R. J. Redfield, “Evolution of Bacterial Transformation: Is Sex With Dead Cells Ever Better Than No Sex at All?,” 1988.

[54] D. J. P. Engelmoer and D. E. Rozen, “Competence increases survival during stress in streptococcus pneumoniae,” *Evolution (N. Y).*, no. 1, 2011.

[55] J. M. Bacher, D. Metzgar, and de C.-L. Valerie, “Rapid Evolution of Diminished Transformability in Acinetobacter baylyi □,” *J. Bacteriol.*, vol. 188, no. 24, pp. 8534–8542, 2006.

[56] S. Graupner, V. Frey, R. Hashemi, and M. G. Lorenz, “Type IV Pilus Genes pilA and pilC of Pseudomonas stutzeri Are Required for Natural Genetic Transformation , and pilA Can Be Replaced by Corresponding Genes from Nontransformable Species,” vol. 182, no. 8, pp. 2184–2190, 2000.

[57] M. G. Lorenz and W. Wackernagel, “High frequency of natural genetic transformation of Pseudomonas stutzeri in soil extract supplemented with a carbon/energy and phosphorus source,” *Appl. Environ. Microbiol.*, vol. 57, no. 4, pp. 1246–1251, 1991.

[58] S. Domingues, K. Harms, W. F. Fricke, P. J. Johnsen, G. J. da Silva, and K. M. Nielsen, “Natural Transformation Facilitates Transfer of Transposons, Integrons and Gene Cassettes between Bacterial Species,” *PLoS Pathog.*, vol. 8, no. 8, 2012.

[59] M. Camiade *et al.*, “Antibiotic resistance patterns of Pseudomonas spp. isolated from faecal wastes in the environment and contaminated surface water,” *FEMS Microbiol. Ecol.*, vol. 96, no. 2, pp. 1–15, 2020.

[60] R. L. Barnard, C. A. Osborne, and M. K. Firestone, “Responses of soil bacterial and fungal communities to extreme desiccation and rewetting,” *ISME J.*, vol. 7, no. 11, pp. 2229–2241, 2013.

[61] S. J. Blazewicz, E. Schwartz, M. K. Firestone, S. J. Blazewicz, E. Schwartz, and M. K. Firestone, “Growth and death of bacteria and fungi underlie rainfall-induced carbon dioxide pulses from seasonally dried soil Published by : Wiley on behalf of the Ecological Society of America Stable URL : http://www.jstor.org/stable/43494786 Growth and death of bac,” vol. 95, no. 5, pp. 1162–1172, 2017.

[62] J. T. Lennon, M. E. Muscarella, S. A. Placella, and B. K. Lehmkuhl, “How , When , and Where Relic DNA Affects Microbial Diversity,” vol. 9, no. 3, pp. 1–14, 2018.

[63] E. K. Bünemann, B. Keller, D. Hoop, K. Jud, P. Boivin, and E. Frossard, “Increased availability of phosphorus after drying and rewetting of a grassland soil: Processes and plant use,” *Plant Soil*, vol. 370, no. 1–2, pp. 511–526, 2013.

[64] M. Van Dinh, A. Guhr, A. R. Weig, and E. Matzner, “Drying and rewetting of forest floors: dynamics of soluble phosphorus, microbial biomass-phosphorus, and the composition of microbial communities,” *Biol. Fertil. Soils*, vol. 54, no. 6, pp. 761–768, 2018.

[65] S. U. Khan, P. S. Hooda, M. S. A. Blackwell, and R. Busquets, “Microbial Biomass Responses to Soil Drying-Rewetting and Phosphorus Leaching,” *Front. Environ. Sci.*, vol. 7, no. September, pp. 1–9, 2019.

[66] S. H. Sirois and D. H. Buckley, “Factors governing extracellular DNA degradation dynamics in soil,” *Environ. Microbiol. Rep.*, vol. 11, no. 2, pp. 173–184, 2019.

[67] S. Karlowsky, A. Augusti, J. Ingrisch, M. K. U. Akanda, M. Bahn, and G. Gleixner, “Drought-induced accumulation of root exudates supports post-drought recovery of microbes in mountain grassland,” *Front. Plant Sci.*, vol. 871, no. November, pp. 1–16, 2018.

[68] D. Naylor and D. Coleman-Derr, “Drought stress and root-associated bacterial communities,” *Front. Plant Sci.*, vol. 8, no. January, pp. 1–16, 2018.

[69] E. A. de Nijs, L. C. Hicks, A. Leizeaga, A. Tietema, and J. Rousk, “Soil microbial moisture dependences and responses to drying–rewetting: The legacy of 18 years drought,” *Glob. Chang. Biol.*, vol. 25, no. 3, pp. 1005–1015, 2019.

[70] M. D. Luehmann *et al.*, “Loamy, two-storied soils on the outwash plains of southwestern lower Michigan: Pedoturbation of loess with the underlying sand,” *Ann. Am. Assoc. Geogr.*, vol. 106, no. 3, pp. 551–571, 2016.

[71] J. J. Kozich, S. L. Westcott, N. T. Baxter, S. K. Highlander, and P. D. Schloss, “Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the miseq illumina sequencing platform,” *Appl. Environ. Microbiol.*, vol. 79, no. 17, pp. 5112–5120, 2013.

[72] P. J. McMurdie and S. Holmes, “Waste Not, Want Not: Why Rarefying Microbiome Data Is Inadmissible,” *PLoS Comput. Biol.*, vol. 10, no. 4, 2014.

[73] L. V. Hedges and I. Olkin, “Nonparametric estimators of effect size in meta-analysis,” *Psychol. Bull.*, vol. 96, no. 3, pp. 573–580, 1984.

[74] R. N. Upton, E. M. Bach, and K. S. Hofmockel, “Belowground response of prairie restoration and resiliency to drought,” *Agric. Ecosyst. Environ.*, vol. 266, no. April, pp. 122–132, 2018.

[75] J. O’Neill, “Review on Anitmicrobial Resistance. Tackling drug-resistant infections globally,” vol. 7, no. 3, p. 110, 2016.

[76] A. C. Singer, H. Shaw, V. Rhodes, and A. Hart, “Review of antimicrobial resistance in the environment and its relevance to environmental regulators: Final

Report and Recommendations," *Front. Microbiol.*, vol. 7, no. NOV, pp. 1–22, 2016.

[77] R. L. Finley *et al.*, "The scourge of antibiotic resistance: The important role of the environment," *Clin. Infect. Dis.*, vol. 57, no. 5, pp. 704–710, 2013.

[78] B. Berglund, "Environmental dissemination of antibiotic resistance genes and correlation to anthropogenic contamination with antibiotics," *Infect. Ecol. Epidemiol.*, vol. 5, no. 1, p. 28564, 2015.

[79] E. Peterson and P. Kaur, "Antibiotic resistance mechanisms in bacteria: Relationships between resistance determinants of antibiotic producers, environmental bacteria, and clinical pathogens," *Front. Microbiol.*, vol. 9, no. NOV, pp. 1–21, 2018.

[80] X. Jiang *et al.*, "Dissemination of antibiotic resistance genes from antibiotic producers to pathogens," *Nat. Commun.*, vol. 8, pp. 1–7, 2017.

[81] J. Bengtsson-Palme, E. Kristiansson, and D. G. J. Larsson, "Environmental factors influencing the development and spread of antibiotic resistance," *FEMS Microbiol. Rev.*, vol. 42, no. 1, pp. 68–80, 2018.

[82] R. S. McInnes, G. E. McCallum, L. E. Lamberte, and W. van Schaik, "Horizontal transfer of antibiotic resistance genes in the human gut microbiome," *Curr. Opin. Microbiol.*, vol. 53, pp. 35–43, 2020.

[83] L. Poirel, J. M. Rodriguez-Martinez, H. Mammeri, A. Liard, and P. Nordmann, "Origin of plasmid-mediated quinolone resistance determinant QnrA," *Antimicrob. Agents Chemother.*, vol. 49, no. 8, pp. 3523–3525, 2005.

[84] L. Poirel, P. Kämpfer, and P. Nordmann, "Chromosome-encoded ambler class a β-lactamase of Kluyvera georgiana, a probable progenitor of a subgroup of CTX-M extended-spectrum β-lactamases," *Antimicrob. Agents Chemother.*, vol. 46, no. 12, pp. 4038–4040, 2002.

[85] G. D. Wright, "Antibiotic resistance in the environment: A link to the clinic?," *Curr. Opin. Microbiol.*, vol. 13, no. 5, pp. 589–594, 2010.

[86] P. Dong, H. Wang, T. Fang, Y. Wang, and Q. Ye, "Assessment of extracellular antibiotic resistance genes (eARGs) in typical environmental samples and the transforming ability of eARG," *Environ. Int.*, vol. 125, no. October 2018, pp. 90–96, 2019.

[87] D. Mao *et al.*, "Persistence of extracellular DNA in river sediment facilitates antibiotic resistance gene propagation," *Environ. Sci. Technol.*, vol. 48, no. 1, pp. 71–78, 2014.

[88] H. Heuer and K. Smalla, "Manure and sulfadiazine synergistically increased

bacterial antibiotic resistance in soil over at least two months," *Environ. Microbiol.*, vol. 9, no. 3, pp. 657–666, 2007.

[89] C. T. T. Binh, H. Heuer, M. Kaupenjohann, and K. Smalla, "Piggery manure used for soil fertilization is a reservoir for transferable antibiotic resistance plasmids," *FEMS Microbiol. Ecol.*, vol. 66, no. 1, pp. 25–37, 2008.

[90] N. A. Lerminiaux and A. D. S. Cameron, "Horizontal transfer of antibiotic resistance genes in clinical environments," *Can. J. Microbiol.*, vol. 65, no. 1, pp. 34–44, 2019.

[91] S. Molin and T. Tolker-Nielsen, "Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilisation of the biofilm structure," *Curr. Opin. Biotechnol.*, vol. 14, no. 3, pp. 255–261, 2003.

[92] J. S. Madsen, M. Burmølle, L. H. Hansen, and S. J. Sørensen, "The interconnection between biofilm formation and horizontal gene transfer," *FEMS Immunol. Med. Microbiol.*, vol. 65, no. 2, pp. 183–195, 2012.

[93] C. Ding and J. He, "Effect of antibiotics in the environment on microbial populations," *Appl. Microbiol. Biotechnol.*, vol. 87, no. 3, pp. 925–941, 2010.

[94] Q. Chang, W. Wang, G. Regev-Yochay, M. Lipsitch, and W. P. Hanage, "Antibiotics in agriculture and the risk to human health: How worried should we be?," *Evol. Appl.*, vol. 8, no. 3, pp. 240–247, 2015.

[95] T. F. Cooper, "Recombination speeds adaptation by reducing competition between beneficial mutations in populations of Escherichia coli," *PLoS Biol.*, vol. 5, no. 9, pp. 1899–1905, 2007.

[96] K. Palmer and M. Gilmore, "Multidrug-Resistant Enterococci Lack CRISPR- cas," *MBio*, vol. 1, no. 4, pp. 1–10, 2010.

[97] S. Babakhani and M. Oloomi, "Transposons: the agents of antibiotic resistance in bacteria," *J. Basic Microbiol.*, vol. 58, no. 11, pp. 905–917, 2018.

[98] Y. G. Zhu *et al.*, "Diverse and abundant antibiotic resistance genes in Chinese swine farms," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 110, no. 9, pp. 3435–3440, 2013.

[99] X. Qian, J. Gu, W. Sun, X. J. Wang, J. Q. Su, and R. Stedfeld, "Diversity, abundance, and persistence of antibiotic resistance genes in various types of animal manure following industrial composting," *J. Hazard. Mater.*, vol. 344, pp. 716–722, 2018.

[100] A. K. Bhardwaj, P. Jasrotia, S. K. Hamilton, and G. P. Robertson, "Ecological management of intensively cropped agro-ecosystems improves soil quality with sustained productivity," *Agric. Ecosyst. Environ.*, vol. 140, no. 3–4, pp. 419–429,

2011.

[101] B. A. Smith, K. M. Dougherty, and D. A. Baltrus, “Complete Genome Sequence of the Highly Transformable Pseudomonas stutzeri Strain 28a24.,” *Genome Announc.*, vol. 2, no. 3, p. 2014, 2014.

[102] K.-H. Choi and H. P. Schweizer, “mini-Tn7 insertion in bacteria with single attTn7 sites: example Pseudomonas aeruginosa.,” *Nat. Protoc.*, vol. 1, no. 1, pp. 153–161, 2006.

[103] E. M. Morrissey *et al.*, “Dynamics of extracellular DNA decomposition and bacterial community composition in soil,” *Soil Biol. Biochem.*, vol. 86, pp. 42–49, 2015.

[104] A. Romanchuk *et al.*, “Bigger is not always better: Transmission and fitness burden of ~1MB Pseudomonas syringae megaplasmid pMPPla107,” *Plasmid*, vol. 73, pp. 16–25, 2014.

[105] G. Pietramellara, J. Ascher, F. Borgogni, M. Ceccherini, G. Guerri, and P. Nannipieri, “Extracellular DNA in soil and sediment: fate and ecological relevance.” pp. 219–235, 2009.

[106] T. Zhang *et al.*, “Editorial: The Environmental Dimension of Antibiotic Resistance,” *FEMS Microbiol. Ecol.*, vol. 96, no. 8, pp. 1–2, 2020.

[107] A. Ezzariai *et al.*, “Human and veterinary antibiotics during composting of sludge or manure: Global perspectives on persistence, degradation, and resistance genes,” *J. Hazard. Mater.*, vol. 359, no. July, pp. 465–481, 2018.

[108] H. Li *et al.*, “Effects of bamboo charcoal on antibiotic resistance genes during chicken manure composting,” *Ecotoxicol. Environ. Saf.*, vol. 140, no. February, pp. 1–6, 2017.

[109] C. W. McKinney and R. S. Dungan, “Influence of environmental conditions on extracellular and intracellular antibiotic resistance genes in manure-amended soil: A microcosm study,” *Soil Sci. Soc. Am. J.*, vol. 84, no. 3, pp. 747–759, 2020.

[110] K. M. Nielsen, M. D. M. van Weerelt, T. N. Berg, A. M. Bones, A. N. Hagler, and J. D. van Elsas, “Natural transformation and availability of transforming DNA to *Acinetobacter calcoaceticus* in soil microcosms,” *Appl. Environ. Microbiol.*, vol. 63, no. 5, pp. 1945–1952, 1997.

[111] R. Tecon, A. Ebrahimi, H. Kleyer, S. E. Levi, and D. Or, “Cell-to-cell bacterial interactions promoted by drier conditions on soil surfaces,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 115, no. 39, pp. 9791–9796, 2018.

[112] A. Dechesne, G. Wang, G. Gülez, D. Or, and B. F. Smets, “Hydration-controlled bacterial motility and dispersal on surfaces,” *Proc. Natl. Acad. Sci. U. S. A.*, vol.

107, no. 32, pp. 14369–14372, 2010.

[113] J. T. Lennon and B. K. Lehmkuhl, “A trait-based approach to bacterial biofilms in soil,” *Environ. Microbiol.*, vol. 18, no. 8, pp. 2732–2742, 2016.

[114] X. S. Ding, B. Zhao, Q. An, M. Tian, and J. S. Guo, “Role of extracellular polymeric substances in biofilm formation by Pseudomonas stutzeri strain XL-2,” *Appl. Microbiol. Biotechnol.*, vol. 103, no. 21–22, pp. 9169–9180, 2019.

[115] M. Wilson, R. Mcnab, and B. Henderson, *Chapter 14 - Bacterial Cell Biology and Development*. 2017.

[116] O. H. Ambur, J. Engelstädter, P. J. Johnsen, E. L. Miller, and D. E. Rozen, “Steady at the wheel: Conservative sex and the benefits of bacterial transformation,” *Philos. Trans. R. Soc. B Biol. Sci.*, vol. 371, no. 1706, 2016.

[117] Y. Kim and H. A. Orr, “Adaptation in sexuals vs. asexuals: Clonal interference and the Fisher-Muller model,” *Genetics*, vol. 171, no. 3, pp. 1377–1386, 2005.

[118] J. C. Mell and R. J. Redfield, “Natural Competence and the Evolution of DNA Uptake Specificity COMPETENCE,” vol. 196, no. 8, pp. 1471–1483, 2014.

[119] R. J. Redfield, M. R. Schragt, and A. M. Dean, “The Evolution of Bacterial Transformation: Sex With Poor Relations,” vol. 38, 1997.

[120] D. Moradigaravand and J. Engelsta, “The Evolution of Natural Competence : Disentangling Costs and Benefits of Sex in Bacteria,” vol. 182, no. 4, 2013.

[121] J. Claverys, M. Prudhomme, and B. Martin, “Induction of Competence Regulons as a General Response to Stress in Gram-Positive Bacteria,” 2006.

[122] R. J. Redfield, “Genes for breakfast: The have-your-cake and-eat-lt-too of bacterial transformation,” *J. Hered.*, vol. 84, no. 5, pp. 400–404, 1993.

[123] S. Sinha, J. Mell, and R. Redfield, “The availability of purine nucleotides regulates natural competence by controlling translation of the competence activator Sxy,” vol. 88, no. May, pp. 1106–1119, 2013.

[124] R. E. Michod, M. F. Wojciechowski, and M. A. Hoezler, “DNA repair and the evolution of transformation in the bacterium Bacillus subtilis,” *Genetics*, vol. 118, no. 1, pp. 31–39, 1988.

[125] X. Charpentier, P. Polard, and J. Claverys, “Induction of competence for genetic transformation by antibiotics : convergent evolution of stress responses in distant bacterial species lacking SOS ?,” *Curr. Opin. Microbiol.*, vol. 15, no. 5, pp. 570–576, 2012.

[126] G. G. Perron, A. E. G. Lee, Y. Wang, W. E. Huang, and T. G. Barraclough,

"Bacterial recombination promotes the evolution of multi-drug-resistance in functionally diverse populations," *Proc. R. Soc. B Biol. Sci.*, vol. 279, no. 1733, pp. 1477–1484, 2012.

[127] A. L. G. Utnes *et al.*, "Growth phase-specific evolutionary benefits of natural transformation in Acinetobacter baylyi," pp. 2221–2231, 2015.

[128] A. Mcleman, P. Sierocinski, E. Hesse, A. Buckling, and G. Perron, "No effect of natural transformation on the evolution of resistance to bacteriophages in the Acinetobacter baylyi model system," *Nat. Sci. Reports*, no. November, pp. 6–11, 2016.

[129] N. D. Palmer and R. A. Cartwright, "Strong Episodic Selection for Natural Competence for Transformation Due to Host-Pathogen Dynamics," pp. 1–22, 2018.

[130] N. H. Barton and B. Charlesworth, "Why Sex and Recombination ?," vol. 281, no. September, pp. 1986–1991, 1998.

[131] S. M. Soucy, J. Huang, and J. P. Gogarten, "Horizontal gene transfer: Building the web of life," *Nat. Rev. Genet.*, vol. 16, no. 8, pp. 472–482, 2015.

[132] U. Gonzales-Barron, M. Kerr, J. J. Sheridan, and F. Butler, "Count data distributions and their zero-modified equivalents as a framework for modelling microbial data with a relatively high occurrence of zero counts," *Int. J. Food Microbiol.*, vol. 136, no. 3, pp. 268–277, 2010.

[133] H. Hofstetter, E. Dusseldorp, A. Zeileis, and A. A. Schuller, "Modeling Caries Experience: Advantages of the use of the hurdle model," *Caries Res.*, vol. 50, no. 6, pp. 517–526, 2016.

[134] E. Shanker and M. J. Federle, "Quorum sensing regulation of competence and bacteriocins in Streptococcus pneumoniae and mutans," *Genes (Basel).*, vol. 8, no. 1, 2017.

[135] M. Jaskolska, S. Stutzmann, C. Stoudmann, and M. Blokesch, "QstR-dependent regulation of natural competence and type VI secretion in Vibrio cholerae," *Nucleic Acids Res.*, vol. 46, no. 20, pp. 10619–10634, 2018.

[136] A. Novick and W. F. Doolittle, "Horizontal persistence and the complexity hypothesis," *Biol. Philos.*, vol. 35, no. 1, pp. 1–22, 2020.

[137] S. Cesar *et al.*, "Bacterial Evolution in High-Osmilarity Environments," vol. 11, no. 4, pp. 1–17, 2020.

[138] S. Graupner, V. Frey, R. Hashemi, M. G. Lorenz, G. Brandes, and W. Wackernagel, "Type IV pilus genes pilA and pilC of Pseudomonas stutzeri are required for natural genetic transformation, and pilA can be replaced by

corresponding genes from nontransformable species," *J. Bacteriol.*, vol. 182, no. 8, pp. 2184–2190, 2000.

[139] S. Graupner, N. Weger, M. Sohni, and W. Wackernagel, "Requirement of Novel Competence Genes pilT and pilU of Pseudomonas stutzeri for Natural Transformation and Suppression of pilT Deficiency by a Hexahistidine Tag on the Type IV Pilus Protein PilAI," vol. 183, no. 16, pp. 4694–4701, 2001.