SEXUAL RECOMBINATION AND THE DEVELOPMENT OF COMPLEX PHENOTYPES

A Dissertation

by

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ABSTRACT

Adaptive laboratory evolution facilitates the development and study of complex phenotypes. In an evolving population, individuals with mutations conveying a fitness benefit are selected for, and become enriched, in the environment. However, the rate of adaptation can be limited by the frequency of beneficial mutations; and competition amongst co-occurring beneficial mutations can lead to a loss of information. In this work, we describe the use of horizontal gene transfer (HGT) in conjunction with modulating mutation rate to more rapidly develop complex phenotypes in *E. coli*. We first characterize a previously developed "genderless" strain of *E. coli* proficient in continuous HGT during liquid culture. We next examine a few steps that can be taken to broaden and enhance the characteristics of this strain. We then introduced an inducible mutator system to the genderless strain in order allow modulation of mutation rate to enhance the supply of mutations during ALE. The strain was evolved in several well-characterized experimental environments to determine the influences of HGT and mutation rate on the rate of adaptation. The results indicate HGT and increasing mutation rate can act together to speed adaptive laboratory evolution, in many adaptive landscapes (environment). We then leveraged the HGT to more rapidly combine different complex phenotypes, to help expedite strain development of more industrially focused phenotypes. Finally, less developed works, which focus on applying different aspects of ALE toward strain development, are briefly discussed.

DEDICATION

To my Grandfather who never had the opportunity to finish his studies

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Except where cited, the work conducted for the dissertation was completed by George Peabody independently with the following exceptions. James Winkler performed several evolutions and preliminary work for the section on combining phenotypes and constructing the genderless strain. Angie Nathalia Lizarazo Román assisted with the work on evolving a strain with a genetic circuit (appendix 2). Javier Rivero and Hao Li assisted in the experimentation to test and evolve the genderless strain with inducible mutation rate (section 3). David A Castro, Weston Fountain, and Enzo Leiva Aravena were instrumental to completing the work on characterizing the advantages and frequency of the genderless strain (section 2).

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NOMENCLATURE

GFP	Green Fluorescent Protein
PCR	Polymerase Chain Reaction
DNA	Deoxy-Ribo Nucleaic Acid
RNA	Ribo Nucleic Acid
IPTG	Isopropyl β-D-1-thiogalactopyranoside
HGT	Horizontal Gene Transfer
TAL	Tri Acetic Lactone
PG	Phloroglucinol
TM	Trimethoprim
CM	Chloramphenicol
IL	Ionic Liquid, 1-ethyl-3-methylimidaolium chloride
OS	Osmotic Stress
ALE	Adaptive Laboratory Evolution
oriT	origin of Transfer
TET	Tetracycline
OD_{600}	Optical Density at 600 nano-meters

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INTRODUCTION

General Background

The generation of biofuels and other industrially desirable chemicals can often be achieved through the use of biocatalysts. Biocatalysts offer a versatile and environmentally friendly alternative to chemical synthesis. For more complex molecules, biocatalysts is often the only feasible assembly method (Białecka-Florjańczyk & Kapturowska, 2012; Bornscheuer et al., 2013; Chemler, Yan, & Koffas, 2006; Misawa, 2011; Ruiz et al., 2012; Sandmann, Albrecht, Schnurr, Knörzer, & Böger, 1999). However, industrial production of bio-based compounds is often faced with challenges such as low levels of product yield and titer, negative impacts due to toxicity of industrially desirable operating conditions, and process related issues such as contamination and scale up. Academic research has traditionally focused primarily on increasing titer and yield via metabolic engineering approaches. Although the optimization of biosynthesis through metabolic engineering, protein engineering and rational strain design is necessary to generate biocatalysts with increased yields and titers, less attention has been paid towards scale-up performance and biocatalyst robustness in industrial conditions, which can impact yield, titer, and productivity. Biocatalyst robustness is typically a highly complex phenotype, involving multiple genes and mechanisms. Several recent advances in high throughput technologies have enabled the effective study of tolerance phenotypes (Bantscheff, Lemeer, Savitski, & Kuster, 2012; Metzker, 2010; Putri, Yamamoto, Tsugawa, & Fukusaki, 2013).

Adaptive laboratory evolution (ALE) is a powerful technique for complex phenotype development, and is the focus of this work. ALE relies upon mutation and selection to evolve a population towards local or global optima along the fitness landscape of the chosen environment. ALE thereby applies the natural principles of evolution to the lab by controlling the growth conditions of the evolving population to increase cellular fitness through acquisition of beneficial mutations. Each mutation or combination of mutations are connected in a hypercube of genomic space, where the various mutational combinations lead to variable increases in fitness (S. Wright, 1932).

ALE has demonstrated substantial success in producing strains that are highly tolerant to various industrially relevant inhibitory conditions (Almario, Reyes, & Kao, 2013; Atsumi et al., 2010; Cooper, 2007; Demeke et al., 2013; McDonald, Rice, & Desai, 2016; Minty et al., 2011; Oide, Gunji, Moteki, & Yamamoto, 2015; Parreiras, Breuer, Narasimhan, & Higbee, 2014; Reyes, Gomez, & Kao, 2014; Royce et al., 2015; Shui et al., 2015; Wallace-Salinas & Gorwa-Grauslund, 2013; Y. Wang et al., 2014; Winkler, Garcia, Olson, Callaway, & Kao, 2014; J. Wright et al., 2011; X. Wu, Altman, Eiteman, & Altman, 2014). Prior to the genomics era, the underlying molecular mechanisms for tolerance in ALE developed strains were difficult to decipher. With recent advances in omics technologies, such as next generation genome sequencing and transcriptomics (*e.g.* RNA-seq, microarrays), proteomics, and metabolomics, more detailed molecular characterizations of these complex phenotypes have become possible (Bantscheff et al., 2012; Cooper, 2007; Metzker, 2010; Putri et al., 2013; Schifano, Edifor, & Sharp, 2013; Winkler & Kao, 2012). These successes have facilitated the

study of mechanisms behind tolerance phenotypes, which provide a platform for subsequent rational strain development, and can be potentially translated to production strains. Combined with high throughput genomics approaches, ALE has become a powerful inverse strain engineering technique for complex phenotypes, as it is a robust and simple to implement method. However, the stochastic and global nature of ALE makes deciphering results difficult; and like most other approaches, the inherent phenotypic landscape and mutational dynamics of the organism-of-interest limit the space this method can reasonably explore on a laboratory/human scale.

Previous work has attempted to enhance ALE by either addressing the aforementioned drawbacks or enhancing its stronger features to make ALE a more powerful and applicable technique for strain development. One example is the introduction of sexual recombination within an evolving population, which serves as a method to accelerate the adaptation in complex phenotypes (Colegrave, 2002; Colegrave, Kaltz, & Bell, 2002; Cooper, 2007; Toprak et al., 2011; Winkler & Kao, 2012). Sexual recombination can alleviate clonal interference, the phenomena of competition between co-arising beneficial mutations in separate asexual lineages (Huovinen, 1987; MacIntyre & Clegg, 2010). Introduction of sexual recombination to asexual populations can combine competing mutations and increase the space explored by the ALE is increased, expediting and enhancing the evolutionary trajectory.

Although the advantages of sex in alleviating clonal interference in evolving populations and ALE are well documented, surprisingly several aspects of the advantages of sexual recombination have yet to be elucidated or studied experimentally.

This work will focus on examining a few of the other benefits of sexual recombination with respect to ALE. It has been shown that the rate of adaptive evolution can be increased with increasing mutation rate (either via the use of a mutator strain or induced mutagenesis) (Desai, Fisher, & Murray, 2007; Giraud, 2001; Palmer et al., 2015; Taddei et al., 1997). However, high rate of mutation can lead to several undesirable consequences. One consequence is the development of unstable populations, where too many neutral or deleterious mutations arise in a beneficial genetic background leading to reduction or elimination of the desirable phenotype. Others examples include hitchhiking, where strongly beneficial mutations arise in a population with a deleterious mutation, essentially coupling the two and leading to the fixation of the deleterious mutation. Another example includes the possible influences of Muller's Ratchet wherein deleterious mutations are fixed in a population through random chance leading to a net loss in fitness. Sexual recombination has the potential to combat these drawbacks by repairing deleterious alleles to regenerate a least loaded class and by preserving the wild type alleles.

Industrial strains are often exposed to the presence of several inhibitory environmental conditions at once. However, no significant research has focused on incorporating multiple diverse tolerance phenotypes/mechanisms into one organism to efficiently generate poly-tolerant strains

Approaches to Tolerance Phenotype Development

A key topic of industrial and academic research has been the development of microbial strains as a means to improve the efficiency of biocatalyst-based production of chemicals and fuels. However, the genetic complexity of biological systems is such that there is a vast combinatorial space of genotypes that can influence a desirable phenotype. Knowledge of the interactions between genetic determinants associated with complex phenotypes is limited. Complex phenotypes are challenging to decipher since frequently, the measured attribute (*e.g.* tolerance) is controlled by alterations in multiple genomic or regulatory determinants resultant expression or function shifts steer strain behavior towards the measurable phenotypic changes. The impacts of each alteration are difficult to quantify and decipher from the resulting complex changes to cellular regulation and phenotypes. In complex phenotypes, elucidating and understanding the changes leading to the behavioral perturbations is idyllic, but difficult given our current tool set and incomplete cellular understanding.

Fortunately, recent technological advances, primarily in sequencing technologies, have facilitated method development aimed at phenotype interrogation. Advances in discovery and characterization of complex tolerance phenotypes and the associated relationships with genotype have allowed a more in depth understanding of cellular mechanisms and regulation than ever before. In brief, these new methods include advances in transcriptomics, plasmid or chromosomal genomic libraries, targeted study of genes of interest, and ALE.

One of the earliest high-throughput methods for identifying genes involved in enhanced tolerance phenotypes is transcriptomics, a method in which the entire (transcribed) RNA content of a sample population is probed to observe responses to chemical insults or other stress conditions. This approach is commonly used to determine transcriptional regulatory perturbations associated with a given stress response for further analysis, and provides insights into how a cell responds to environmental stressors. Recent applications of this method include studies of tolerance related to: butanol (Anfelt, Hallstrom, Nielsen, Uhlen, & Hudson, 2013; Rutherford et al., 2010; Winkler & Kao, 2011; 2012), ethanol (J. Wang et al., 2012; S. Yang et al., 2013), salt stress (S. Choi, Jung, Jeon, & Park, 2013; Villarino, Bombarely, Giovannoni, Scanlon, & Mattson, 2014), and hydrolysates (Applebee, Joyce, Conrad, Pettigrew, & Palsson, 2011; J. Yang et al., 2012). However transposon libraries do not represent the only method to perturb cellular function in a broad screen for genetic determinants, and are often subject to significant noise as a result of the large sample sizes and low accuracy of the method.

Genomic libraries also take a whole genome approach to phenotype development. Genomic libraries scan a chosen (meta)genome to identify genes involved in a tolerance phenotype, which can be used to launch mechanism assessment and to evaluate how gene dosage affects various phenotypes. Genomic libraries are generally split into two categories: plasmid or nonintegrated libraries, and integrated genomic libraries. Plasmid based genomic libraries are created by fragmenting the genomic DNA of the organism-of-interest to various sizes, followed by cloning of the fragments into

plasmids. By overexpressing random genomic DNA fragments followed by subsequent selection in the condition-of-interest to enrich for fragments that positively influence fitness, genomic library-based enrichment methods have been used to successfully identify genes related to various tolerance phenotypes. Recently characterized determinants using this method have been found in the following backgrounds: butanol (Applebee, Herrgard, & Palsson, 2008; Reyes, Abdelaal, & Kao, 2013), furfural (Applebee et al., 2011; Glebes et al., 2014b), acid stress (Cheng et al., 2014; Nicolaou, Gaida, & Papoutsakis, 2011) and ethanol (Hong et al., 2010; Nicolaou, Gaida, & Papoutsakis, 2012; Woodruff et al., 2013). However, other types of genomic libraries are often applied since plasmid based genomic libraries are limited to set levels of over expression extra-chromosomally. Other methods implement different ways to perturb gene expression in measurable ways.

The alternative to plasmid based genomic libraries, chromosomal genomic libraries, broadly fall into two categories: transposon insertion libraries and integration-based libraries. In contrast to the increased expression provided by plasmid-based libraries, transposon insertion libraries usually result in gene disruptions, as gene function is perturbed by the insertion of transposon elements in regulatory elements and coding sequences. Applications of this method include: ethanol (Goodarzi et al., 2010; Haldimann & Wanner, 2001; Kieboom, Dennis, de Bont, & Zylstra, 1998; H.-S. Kim, Kim, Yang, & Choi, 2011; Yoshikawa et al., 2009), furfural (H.-S. Kim, Kim, Kim, & Choi, 2012), methyl viologen (S. Biswas & Biswas, 2011), and ellagic acid (Freddolino, Goodarzi, & Tavazoie, 2014). A more rational method of generating an integration-

based library is the popular technique Trackable Multiplex Recombineering (TRMR). TRMR utilizes high-throughput DNA synthesis to directly modify each promoter sequence on the chromosome through homologous recombination to generate a library of both induced and repressed gene expression constructs for all genes in *E. coli* (Warner, Reeder, Karimpour-Fard, Woodruff, & Gill, 2010). TRMR has been applied to numerous inhibitory conditions such as: hydrolysates (Glebes, Sandoval, Gillis, & Gill, 2014a; Sandoval et al., 2012), furfural (Warner et al., 2010), and acetate and low pH (Herman & Modrich, 1981; Sandoval et al., 2012). Additionally, integrated libraries can be constructed using the Conditional-Replication, Integration, and Modular (CRIM) system developed by Wanner lab (Feinstein & Low, 1986; Haldimann & Wanner, 2001; Lanzov, Bakhlanova, & Clark, 2003). Fragmented genomic DNA fragments are ligated into CRIM plasmids, which can then be integrated directly to the chromosome at specific phage attachment sites for characterization. These methods all serve as ways to identify genetic determinants associated with tolerance phenotypes but still manage only to influence gene dosage and fail to examine other forms of developing tolerance.

Once determinants have been identified with one of the above methods, further strain tolerance can be formulated with more rational approaches focusing around an identified element. For example, if a gene or set of genes is known to impact a tolerance phenotype, those gene(s) can be used to study natural mechanisms by which microbes adapt to said stressors and improve targeted strain engineering. Some of these mechanisms for tolerance have been identified; for example, inhibitor degradation, cellular reconstruction (such as altered membrane composition to alleviate solvent or

osmotic stress), production of efflux pumps or similar mechanisms to maintain homeostasis, and modulation of existing cellular responses to counter effects of toxicity. The use of efflux pumps (Dunlop et al., 2011; Hamon & Ycart, 2012; Rosche & Foster, 2000) and chaperones (groESL) (Tomas, Welker, & Papoutsakis, 2003; Q. Wang, Venkataramanan, Huang, Papoutsakis, & Wu, 2013; Zingaro & Papoutsakis, 2013), have both been studied in depth for their tolerance enhancing effects in certain biofuels and butanol respectively. In addition, protein engineering can be applied to further improve target proteins identified as genetic determinants of tolerance. In protein engineering, mutant libraries of the gene(s) identified as targets are generated and screened for mutants that confer greater tolerance efficacy such as on furfural (Guzmán et al., 2015; H. Zheng et al., 2013) and pH temperature and oxidative stress (Applebee et al., 2011; Garcia Ruiz, Gonzalez Perez, Ruiz Dueñas, Martínez, & Alcalde, 2012). Another example of protein engineering involves global regulators that modulate the expression of tens to hundreds of genes increasing tolerance to biofuels (Alper, Moxley, Nevoigt, Fink, & Stephanopoulos, 2006; Applebee et al., 2011) and pH (Basak, Geng, & Jiang, 2014; Conrad et al., 2009). Their modification, through mutation or differential expression, allows large perturbations in cellular metabolism to be generated efficiently. Unfortunately, each of these methods requires rational targeting in the form of perquisite knowledge to be applied effectively.

Another way to generate highly tolerant or productive strains for use in production or industry without any significant prior strain knowledge is the use of adaptive evolution. The proposed research will focus heavily on the use of adaptive

evolution as it holds several advantages over more rational techniques outlined above, partly due to the low level of understanding of cellular phenotypes/mechanisms. Adaptive evolution harnesses natural selection in designed growth conditions to screen for mutations that generate phenotypes that are better suited to the experimental growth conditions. Through the clever use of selective pressures, ALE has been proven successful in generating highly tolerant strains and higher producers (Cooper, 2007; Fong et al., 2005; Guadalupe-Medina et al., 2013; Reyes et al., 2014; Winkler & Kao, 2012).

ALE experiments operate by introducing the strain of interest to the selective pressure (a toxic or growth limiting condition). As strain of interest grows and accumulates mutations, the mutations that increase fitness in the selected environment are enriched in the population. This leads to the development of a more fit final population composed of the cells whose mutations confer growth benefits in the environment relative to the ancestral strain. However, despite the robustness of ALE, some disadvantages exist. For example, in nearly all ALE experiments, asexual organisms or sexual organisms evolved asexually are used; and because sexual reproduction is not utilized, beneficial mutations occurring in different genetic backgrounds have no chance of coexisting. Evidence indicates that for asexual organisms such as bacteria, this leads to a loss of information due to competition. Cells with mutations conferring fitness benefits are out competed by simultaneously occurring mutations conferring stronger fitness benefits. Thus, as the fittest cell overtakes the whole population with its particular mutational background in what is known as a clonal

sweep, possible beneficial mutations are lost. This phenomenon is known as clonal interference is supported to some degree in ALE experiments (J. A. G. M. de Visser, 2005; McDonald et al., 2016). However, clonal sweeps are slow in large laboratory populations, which remain relatively heterogeneous in shorter laboratory time scales. So, the ability to rescue the beneficial mutations exists. ALE experiments also require careful experimental design so that the selection is for phenotypes that are tolerant to the desired inhibitor. ALE, like most all of the other mentioned strain design techniques, is also limited in the phenotypic landscape it can explore by the inherent characteristics of the studied organism. However, despite these few shortcomings, ALE represents one of the most attractive modes of strain engineering. It consistently generates highly tolerant strains through mechanisms often more complex than other methods are capable of detecting (*e.g.* subtle mutations leading to expression difference on the protein, RNA and DNA levels), and has been successfully applied to numerous conditions including biofuels (Haft et al., 2014; Minty et al., 2011; Portnoy, Bezdan, & Zengler, 2011; Reyes, Almario, Winkler, Orozco, & Kao, 2012; X. Shao et al., 2011), hydrolysates (Almario et al., 2013; Linville et al., 2013), osmotic stress (Winkler et al., 2014).

Within an ALE experiment, several choices play a key role in the success of the evolution. The choice and implementation of the selective pressure(s), or environmental conditions, are critical towards guiding the subject organisms toward developing tolerance to the intended condition. ALE also requires careful selection of the organism of study; some organisms are more suited for production or have natural tolerance that can be enhanced to identify the mechanisms more easily. The organism choice is also

important for shaping the dynamics of the evolution as the type of reproduction, (sexual or asexual, and ploidity, haploid or diploid), will influence the rate and complexity of the evolved phenotypes and the difficulty in finding the genetic determinants influencing the phenotype. In particular, sexual recombination represents a potentially significant factor in ALE due to the vague understanding of the influence of recombination on evolution both in the lab and nature.

Sexual Recombination

Historically, the benefits and origins of sexual recombination have been hotly debated (MacIntyre & Clegg, 2010). Outwardly, sexual recombination presents several obvious advantages and disadvantages (J. A. G. M. de Visser & Elena, 2007). For the purposes of this research we will only consider two types of sexuality: strictly asexual and asexual with the ability to sexual recombine. Therefore, characteristics associated with sexual obligates (organisms requiring sex to reproduce such as humans) will not be considered.

The first consideration toward an understanding of the influence of sexual recombination in evolution, and by proxy life, is to examine the drawbacks traditionally associated with sexual recombination. Typically, sexual recombination in general is cited as detrimental due to its fitness cost, as energetically, the requirement of finding and successfully mating incurs two obstacles: the 2 vs 1 argument and the physical act of mating over direct asexual division. Several of these issues only partially apply to our system, the evolution of obligate asexuals with HGT capabilities. In particular, the 2 vs.

1 argument (which states that 2 separate organisms work to reproduce and generate one offspring in sexual recombination, but asexual reproduction requires only a simple division) does not apply. The argument claims asexual reproduction leads to twice as many organisms in the same reproduction period from an asexual system than that of a sexual one. Because the bacteria we are studying reproduce solely asexually, this problem with sexual reproduction does not apply. Additionally, because the bacteria are not facultative sexually reproducing, no mating is required for growth indicating that the fitness cost of finding a mate is not applicable to this system. However, in order to confer an advantage in ALE, sexual recombination must occur. Therefore the meeting of two genotypes is necessary for recombination to be beneficial within an evolving population, and any fitness costs of finding a partner to recombine with are unique to sexual strain and not the asexual. Additionally, the fitness cost associated with maintaining active sexual recombination machinery also represents a disadvantage of sexual recombination and should be considered. Of the several drawbacks of sexual reproduction only the fitness costs of sex are directly applicable to the considered system of asexual organisms with the ability to recombine.

Although there are notable drawbacks to sexual production, many advantages also result from sex. The main advantages of sexual recombination within asexual evolving populations are postulated to stem from several phenomena. Sexual recombination theoretically offers advantages by: allowing a quicker exploration of the phenotypic landscape which allows phenotypes to be saved, removing deleterious mutations in strong genotypic backgrounds, and preventing and reversing Muller's

ratchet. Each advantage will be discussed with respect to asexual reproducing populations.

One of the advantages sexual recombination in asexual growth offers was proposed by Fisher/Muller (Fisher Muller) or even earlier as the Vicar of Bray hypothesis (MacIntyre & Clegg, 2010). The theory suggests that in asexual populations, over long periods of time, presumably only the genotype of the fittest mutant will fix in the population leading to the extinction of other genotypes present in the population. This implies that if there is any genetic diversity or other advantageous but less beneficial mutations that have arisen in a cell which is not of the fittest genotype, they will be lost over time. This leads to a loss of information over time if the sample population is not in a mutational regime where mutation is very rare and the population is largely homogenous. The advantage of sexual recombination can be visualized for this instance in the below Figure 1 reproduced from (Winkler & Kao, 2012).

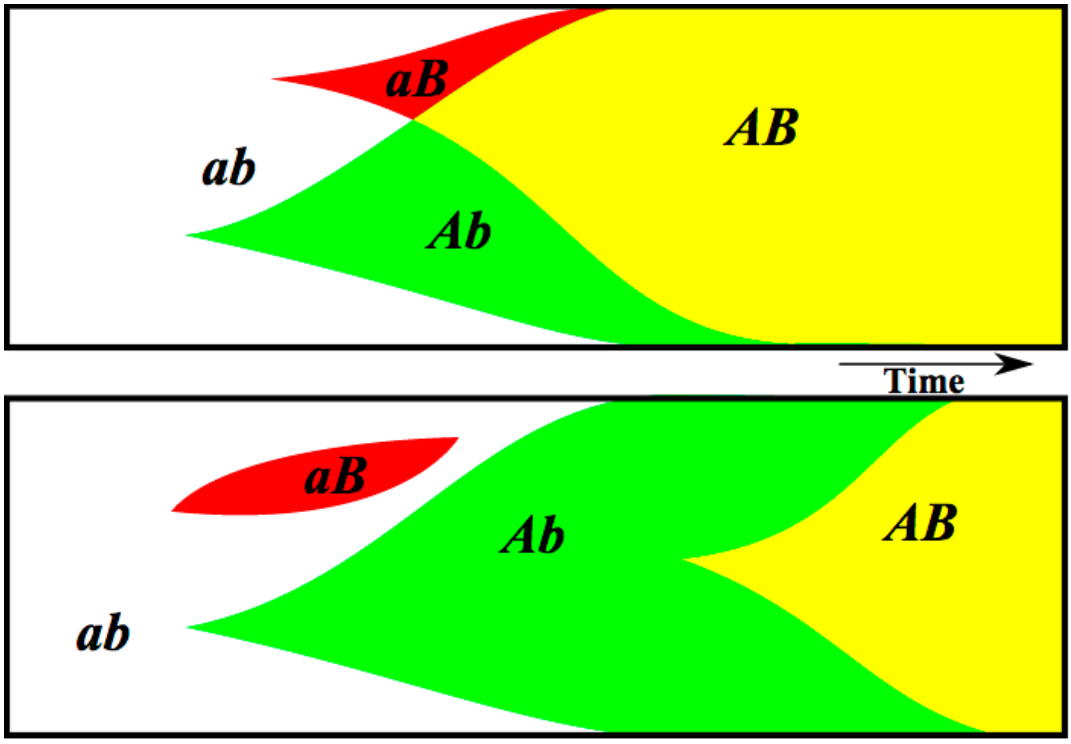

Figure 1: As time progress in the figure mutations accumulate changing the genotype from lower to upper case. The upper figure is the sexual population the lower is the asexual. Upper case letters indicate increased fitness and thus take over the population. In the sexual strain the A and B genotypes quickly recombine to a fitness maximum where as in the asexual bottom strain the A population fixes and must wait for the B mutation to again spontaneously arise.

Theoretically, sexual recombination allows a much quicker exploration of the available genotypic landscape by combining the available beneficial mutations and possibly generating a fitter mutant more rapidly. On the contrary, in a strictly asexual population each beneficial (fitter) mutant/genotype must wait for a spontaneous synergistic mutation to arise in order to further increase in fitness. This advantage of sexual recombination has been attributed to several experimental observations. For example, Birdsell and Wills observed that sexual recombination in *Saccharomyces cerevisiae* greatly enhanced evolution (Birdsell & Wills, 1996). In *S. cerevisiae* a MATa/MATα

diploid is sexual competent where as a homozygote (MATa/MATa) is not. The authors evolved both homo and heterozygote strains in competition. They found that the hetero, sexual, strains, when allowed one round of meiosis, consistently evolved to be more fit more quickly. In a heterogeneous genomic background, the difference was even more pronounced. Additional studies of sexual systems indicate that sexuality confers up to 5x greater rate of adaptation in certain populations sizes relative to asexual systems (Birdsell & Wills, 1996; Colegrave, 2002; Colegrave et al., 2002; Cooper, 2007; Maynard Smith, 1971).

Additional support comes from various models of the advantages of sexual recombination. For example, Crow and Kimura demonstrated, through theoretical simulation, that as either the population size, or the ratio of mutation rate to size of the selective advantage increases, the benefit of sexual recombination increases (Crow & Kimura, 1969).

Sexual recombination is claimed to exhibit another benefit, it can save or preserve phenotypes that may have future value. For example, if an organism has two phenotypes: one fitter in environment A, and the other in B, the A phenotype is not needed in B environment, but may be need in the future. Phenotype A can be preserved and not expressed in a diploid organism's second copy of a gene(s) (Scheuerl & Stelzer, 2017). However, this advantage is generally lost in haploid organisms, haploids lose the information storing capabilities of diploidity by carrying only one copy of each gene, though it is believed that recombination can maintain mutations through selective sweeps though the preservation of genes advantageous in other environments is likely

not a factor in the experimentation (Hamilton, Axelrod, & Tanese, 1990; Heisler & Curtsinger, 1990).

A very powerful advantage of sexual recombination is not only to combine beneficial mutations in different genotypes of asexual organisms that could normally not interact genetically, but also to remove deleterious mutations from fit genotypes. If a strongly beneficial mutation arises in a mutant with a mildly deleterious mutation, excepting the extremely rare back-mutation, the two mutations are coupled indefinitely without sexual recombination. Through repair with the wild type, sexual recombination can reduce the accumulations of mildly deleterious mutations and thereby reduce genetic load within a population. For example Mcdonald *et al.* demonstrated a reduction in hitchhiking deleterious mutations in sexually propagated yeast (McDonald et al., 2016). In a continuation of the previously described study with *S. cerevisiae* and the MATa/MATα homo/hetero alleles, the authors observed a decline in growth rate among the mating deficient strains over many generations but the mating strains yielded no such loss of fitness (Selk & Wills, 1998). Several simulations have demonstrated similar results, indicating that the genetic load of deleterious mutations is lower amongst sexual populations due to recombination (D. Charlesworth, Morgan, & Charlesworth, 1990), and this reduction effect is more pronounced amongst larger populations (Bataillon & Kirkpatrick, 2000).

The effect of population size is more effectively discussed with respect to a different phenomena known as Muller's ratchet (Muller, 1964). Muller's Ratchet is the accumulation of deleterious mutations in a population through factors such as genetic

drift or random chance. In an evolving population mutation leads to a distribution of mutation levels in the cells where the least loaded class has no deleterious mutations. When at any point the least mutated class accumulates an additional mutation or is lost to drift, the ratchet clicks forward, and the entire population is now irreversibly less fit. This process in theory continues until at some point the whole population completely loses fitness and is unable to reproduce, a situation named meltdown. Within large populations of asexual organisms the effects of the ratchet are very slow where as in small populations where there is generally a larger genetic load (Kimura, Maruyama, & Crow, 1963; Lynch, Conery, & Burger, 1995a) and stronger stochastic effects, the influence of the ratchet is much more pronounced (Bachtrog & Gordo, 2004). Muller's ratchet can be entirely avoided in a sexual population, provided any single mutation does not fix, because recombination can regenerate or create an organism containing none of the deleterious mutation accumulated over time. Thus, sex can effectively reverse and reduce the influence of Muller's ratchet under many circumstances. This has been demonstrated experimentally with the Φ6 RNA virus, (Chao, Tran, & Tran, 1997). When the virus was propagated through a series of low population bottlenecks, then subjected to recombination or no recombination, the populations with recombination exhibited substantial fitness gains over the control without mating. The effects of Muller's Ratchet have been extensively studied with computer simulations that lead to similar conclusions (Bell, 1988; Felsenstein, 1974; Gabriel, Lynch, & Burger, 1993; Lynch, Conery, & Burger, 1995a). The presence of Muller's Ratchet within a population relies heavily on the presence of genetic drift.

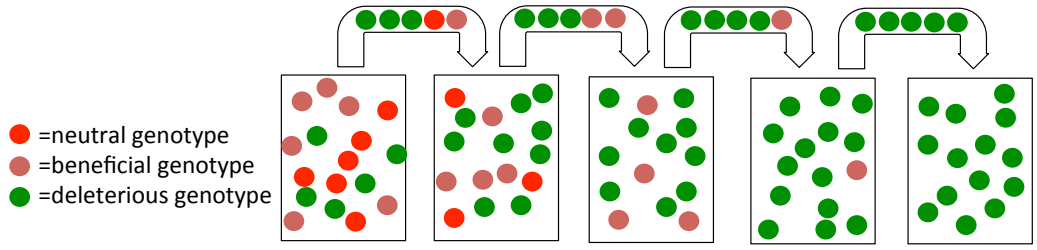

Figure 2: Illustration of Muller's ratchet caused by genetic drift. The deleterious genotype fixes as the population is propagated due to sample stochasticicty.

In conclusion, the influence of sexual recombination on evolution and adaptation has been studied extensively to decipher the reason behind its existence. The two theories most pertinent to this work that have arisen as a consequence of this search are, sexual recombination's ability to avoid Muller's Ratchet and ability to increase the rate of adaptation by alleviating clonal interference. The exploration of these two advantages will be the focus of much of the proposed work.

F-plasmid

In this work a sexually proficient strain of *E. coli* is utilized extensively. This strain harnesses the abilities of a chromosomally integrated F-plasmid. The F-plasmid is a well studied method of cellular conjugation in *E. coli.* The F-plasmid was originally characterized as having the ability to enable conjugation between F+ (F-plasmid carrying) and F- (F-plasmid deficient strains). The F+ strain would transfer a DNA copy of the F-plasmid to the F- strain, resulting in the F- strain converting to F+ (J. Lederberg, Cavalli, & Lederberg, 1952). Physically in the conjugation, the F-plasmid DNA is

secreted from the F+ strain through a type 4-secretion system and a pili connecting the F+ to the F- strain. The F-plasmid DNA is nicked at the origin of transfer (oriT) by *traI*, a relaxase, and the DNA protein complex is then pumped out of the cell with the help of *traD* which facilitated connection to the secretion system (la Cruz, Frost, Meyer, & Zechner, 2010). The single stranded copy of the F-plasmid would be duplicated and circularized so the previously F- strain would become a F+ strain. For a more in-depth description of the mating bridge, see (la Cruz et al., 2010), (Achtman, Morelli, & Schwuchow, 1978) and (Babić, Lindner, Vulić, Stewart, & Radman, 2008). The F-plasmid also harbors genes which inhibit the conjugation between F+ and other F+ cells. The *traST* genes generate surface exclusion and inhibit mating in two ways, the TraS protein blocks external DNA entry into the cell, and the TraT inhibits the formation of the previously described pili (Achtman, Kennedy, & Skurray, 1977; Garcillán-Barcia & la Cruz, 2008). In previous studies, Winkler et al. demonstrated that a knock out of these two genes allows F+, F+ bi-directional mating (Achtman et al., 1977; Jalajakumari et al., 1987; Willetts, 1974). The previously described method of conjugation only serves to spread the F-plasmid, however on rare occasions the F-plasmid will integrate into the genome, resulting in a HFR strain. Conjugation between HFR and non-HFR strains results in the chromosome of the HFR strain being transferred to the non-HFR strain. The actual transfer is frequently interrupted before the entire chromosome can be transferred as the mating efficiency decreases exponentially away from the oriT (Smith, 1991). The F-plasmid can integrate at numerous locations which result in different rates of transfer and strain phenotypes from the integration. This work focuses on the HFR

strain with the F-plasmid integrated in the *tra* (or tryptophan biosynthesis) operon (François, Conter, & Louarn, 1990). The HFR strains have been utilized to generate recombinant *E. coli* for double knock out construction (Typas et al., 2008), and adaptive evolution (Cooper, 2007; Winkler & Kao, 2012).

BENEFITS OF A RECOMBINATION-PROFICIENT *ESCHERICHIA COLI* SYSTEM FOR ADAPTIVE LABORATORY EVOLUTION[1]

Summary

Adaptive laboratory evolution typically involves the propagation of organisms asexually to select for mutants with desired phenotypes. However, asexual evolution is prone to competition amongst beneficial mutations (clonal interference) and the accumulation of hitchhiking mutations (Muller's ratchet). The benefits of horizontal gene transfer towards overcoming these known disadvantages of asexual evolution was characterized in a strain of *Escherichia coli* engineered for superior sexual recombination (genderless). Specifically, we experimentally validated the capacity of the genderless strain to reduce mutational load and recombine beneficial mutations. We also confirmed that inclusion of multiple origins of transfer influences both the frequency of genetic exchange throughout the chromosome and the linkage of donor DNA. We built a simple kinetic model to estimate recombination frequency as a function of transfer size and relative genotype enrichment in batch transfers; the model output correlated well with experimental data. Our results provide strong support for the advantages of utilizing the genderless strain over its asexual counterpart during adaptive laboratory evolution.

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[1] Reprinted with permission from: Peabody, G., Winkler, J., Fountain, W., Castro, D. A., Leiva-Aravena, E., & Kao, K. C. (2016). Benefits of a Recombination-Proficient Escherichia coli System for Adaptive Laboratory Evolution. *Applied and Environmental Microbiology*, *82*(22), 6736–6747,

Introduction

Adaptive laboratory evolution (ALE) has often been used to successfully develop strains for industrially relevant phenotypes in a variety of organisms, typically with microbes such as yeast and bacteria. ALE is generally robust and does not require significant existing knowledge of the organism of interest. This method involves short or long-term propagation of an organism under a selective pressure of interest to select for mutants with desired traits. One strategy often used to expedite ALE experiments is by increasing genetic diversity via the use of a mutagen (UV, EMS, etc) or a mutator strain (Parekh, Vinci, & Strobel, 2000; Winkler & Kao, 2014). Due to the ease of experimentation, in the majority of cases, microbes are propagated asexually (even when using sexual organisms such as *S. cerevisiae*). However, there are two known limitations associated with asexual evolution: clonal interference wherein competition amongst beneficial mutations leads to a loss of information, and Muller's ratchet where fixation of deleterious mutations through hitchhiking and population bottlenecks leads to irreversible decline in strain fitness. One of the theorized benefits of sexual recombination is the reduction of interclonal competition by combining beneficial genotypes that co-exist in the population, thereby mitigating the negative impacts of clonal interference and enhancing the rate of strain improvement (R. A. Fisher, 1930). Similarly, a sexually competent strain can minimize Muller's ratchet by reducing the accumulation of neutral and deleterious mutations through recombination between different genotypes (Kimura & Maruyama, 1966; McDonald et al., 2016).

The support for these two theories stems from theoretical analysis using simulations and modeling along with limited experimental evidence. Criteria such as, mutation rate, effective population size and structure, frequency of mating, and environmental condition (topography of adaptive landscape), have all been found to impact the effectiveness of sexual recombination on adaptation. In general, sexual recombination has been demonstrated to be helpful in speeding adaptation in large populations with higher mutation rates in both experimental and computational analyses (Colegrave, 2002; Cooper, 2007; Y. Kim, 2005; Lobkovsky, Wolf, & Koonin, 2016; Takahata, 1982; Winkler & Kao, 2012). Depending on the model/system parameters, sexual recombination has also been shown to aid in increasing genetic diversity by generating new genotypes via recombination (Keightley & Otto, 2006; Moradigaravand et al., 2014); conversely it has also been predicted to lead to a breakup of desirable characteristics (Barton & Otto, 2005; J. A. G. M. de Visser, Park, & Krug, 2009; Moradigaravand & Engelstädter, 2012). In addition, previous theoretical work suggests that at a particular mutation rate, there exists an optimal recombination rate that maximizes the rate of adaptation (Lobkovsky et al., 2016; Moradigaravand et al., 2014). Muller's ratchet has been predicted to be a function of effective population size and rates of recombination and mutation (Gabriel et al., 1993; Lynch, Conery, & Burger, 1995a; Muller, 1964), and at a given population size, recombination is believed to reduce the risk of accumulating deleterious mutations (Lynch, Conery, & Burger, 1995b; Muller, 1964). Furthermore, there is strong evidence for increased mutation rates leading to mutational meltdowns in certain simulated and experimental populations (Bachtrog &

Gordo, 2004; Bruggeman, Debets, Wijngaarden, deVisser, & Hoekstra, 2003; Zeyl, Mizesko, & De Visser, 2001). Computational work on evolving populations has further shown that high mutation rates and small effective population sizes in asexual populations exacerbates the effects of Muller's ratchet, and in the presence of sexual recombination this effect is reduced (Gabriel et al., 1993; Lynch, Conery, & Burger, 1995b; Muller, 1964; Takeuchi, Kaneko, & Koonin, 2014). With the availability of affordable next-gen sequencing technologies, experimental evidence supporting Muller's ratchet is accumulating. For example, Lang *et al* found hitchhiking of neutral mutations to be prevalent in asexually evolving populations of *S. cerevisiae* (Lang et al., 2013); more recent work in *S. cerevisiae* by McDonald *et al* found hitchhiking mutations to be more prevalent in asexually evolving populations than evolving populations subjected to periodic recombination (McDonald et al., 2016).

While there have been extensive computational analyses, due to limited available experimental methodologies, detailed verifications of the effects of recombination during ALE is lacking, especially in bacteria. Although existing methods for genetic exchange such as protoplast fusion (in bacteria and yeast) and sporulation (in *S. cerevisiae*) have been successfully used to generate beneficial recombinants in microbial systems, they require the interruption of the ALE experiment and may subsequently impose undesirable selective pressures (Hou, 2009; McDonald et al., 2016; Patnaik et al., 2002; Reyes et al., 2012; Shi, Wang, & Wang, 2009; Y.-X. Zhang et al., 2002). To address this limitation, we had previously developed a "genderless" strain of *E. coli* that

is capable of continuous bi-directional conjugation during adaptive laboratory evolution (Winkler & Kao, 2012).

Conjugation is a natural process by which bacterial cells can exchange DNA, among which the F conjugation system in *E. coli* is the best studied. Cells containing an F plasmid (F^+) are capable of transferring the plasmid to a neighboring cell that does not contain the F plasmid (F^-) (J. Lederberg et al., 1952). During conjugation, the F conjugation machinery forms a mating bridge, allowing single-stranded DNA, beginning at the origin of transfer (oriT), to be transferred to the recipient F^- cell (Firth, Ippen-Ihler, & Skurray, 1996). At low frequencies, the F plasmid is spontaneously integrated into the chromosome to form high frequency recombination (HFR) strains (Smith, 1991). In HFR strains, chromosomal DNA can be transferred from the donor to the recipient cell (Smith, 1991). When DNA is transferred from the donor to the recipient cell, homologous recombination can occur allowing chromosomal mutations to be transferred horizontally. Based on the Hfr strain and prior work by the Cooper lab (Cooper, 2007), we removed the surface exclusion *traS* and *traT* (SFX) genes to generate the "genderless" strain, and experimentally demonstrated that continuous *in situ* sexual recombination enhances the speed of ALE in complex fitness landscapes (Winkler & Kao, 2012). In this work, we investigate several aspects of the previously developed sexually proficient "genderless" strain to further elucidate the mechanisms by which sexual recombination enhances ALE. We examined the effect of introducing additional oriTs on genomic coverage of HFR transfer, as little existing work has focused on characterizing the genomic coverage and expanding the use of genetic transfer in the F

system (Clark, 1963; Fu, Tsai, Luo, & Deonier, 1991; Simon, 1984; Typas et al., 2008; Yakobson & Guiney, 1984). Our results suggest that the additional oriTs can be harnessed by the "genderless" strain to increase the coverage of genetic material transferred. To characterize the benefits of sexual recombination in the context of applications in ALE, we also examined how our strain impacts Muller's ratchet and demonstrated the capability of our sexual system to potentially reduce accumulation of mutations and to repair deleterious mutations at an appreciable level. Furthermore, we also examined the applicability of a kinetic model in simulating the mating capabilities of our "genderless" strain.

Materials and Methods

Strain construction

All strains were developed from the HFR-2xoriT-SFX- (sexual) "genderless" strain and the BW25113 2xoriT (asexual) strains previously constructed (Winkler & Kao, 2012), which are derivatives of BW25113 K12 *E. coli* (F-, Δ(araD-araB)567, lacZ4787(del)::rrnB-3, LAM-, rph-1, Δ(rhaD-rhaB)568, hsdR514). Mutator versions of the sexual and asexual strains were constructed by removing the *mutS* gene of the mismatch repair system via standard P1 phage transduction from strain BW25113 Δ*mutS*::KanR in the KEIO collection (Baba et al., 2006) to the genderless and asexual control strains. The mutation rates of the resultant Δ*mutS* strains HFR-2xSFX-Δ*mutS*::KanR (genderless mutator) and BW25113 2xoriT Δ*mutS*::KanR (asexual 2xoriT mutator) were measured using the standard fluctuation test (Hall, Ma, Liang, & Singh,

2009), and were estimated to be 200-300 times higher than the non-mutator wild-type (WT) strain, which are within the expected range for the Δ*mutS* genotype (Boe, Tolker-Nielsen, Eegholm, Spliid, & Vrang, 1994; Herman & Modrich, 1981).

The two extra oriTs in the chromosome of HFR-2xoriT-SFX- genderless strain were removed using the procedure outlined by Datsenko and Wanner (Datsenko & Wanner, 2000) using template plasmid pKD32 to generate strain HFR-Δ2xoriT-SFX-. Successful oriT removal was confirmed with antibiotic selection (chloramphenicol) and subsequent removal of antibiotic resistance marker with plasmid pCP20 was confirmed with PCR verification (primers oriTv_mbhA_r & oriTv_mbhA_f and oriTv_hyfC_r & oriTv_hyfC_f in Table 1).

Table 1: List of primers used in this study.

Usage	Target	Direction	Name	Sequence 5' to 3'
Construction	mbhA oriT	Foreword	mbhA_H1	TTGTCAACAGCGTCAGTAAATCCGAGCGTGAAAGCATTATCGCCGCGCTGTGTAGGCTGGAGCTGCTTC
Construction	mbhA oriT	Reverse	mbhA_H2rc	GCACCACTTTATCCCCATGCTGACCAAAGTATTGATACAGCGTATCGGAATGGGAATTAGCCATGGTCC
Construction	hyfC oriT	Foreword	hyfC_H1	ACCCCACTTTTTACGGGTATTTCCCGGCAGATACGCGCGCGTATGCACTGTGTAGGCTGGAGCTGCTTC
Construction	hyfC oriT	Reverse	hyfC_H2rc	CCAGGTCACATGGTGAATGAGTAAAAAACGCCCGCGTGCCAGCGTGTTTATGGGAATTAGCCATGGTCC
Construction	Cat in G6272	Foreword	G6272_H1	TGAGGGCCAG GGAACAAGTG GCGAAAATCG TATCAAAGAATGATCCAGATGTGTAGGCTGGAGCTGCTTC
Construction	Cat in G6272	Reverse	G6272_H2rc	CCGCTGGTTTTCCGCTAATGGTTTATTTCCTCTTTCTTTCCCGGCAAGAAATGGGAATTAGCCATGGTCC
Construction	Cat in ybhl	Foreword	ybhl_H1	ATGGACAGATTCCCACGTTCTGATTCAATCGTACAACCCCGGGCTGGCTTGTGTAGGCTGGAGCTGCTTC

Table 1: Continued

Usage	Target	Direction	Name	Sequence 5' to 3'
Construction	Cat in ybhl	Reverse	ybhl_H2rc	AACGGCGGTTGCCGAAGATCCGCAACAACATCAGGAACAGGTTGATGAAGATGGGAATTAGCCATGGTCC
Verification	ybhl	Foreword	ybhl f	ATGGACAGATTCCCACGTTC
Verification	ybhl	Reverse	ybhl r	CGAAGATCCGCAACAACATC
Verification	G6272	Foreword	G6272 f	GGAACAAGTGGCGAAAATCG
Verification	G6272	Reverse	G6272 r	CCGCTGGTTTTCCGCTAATG
Verification	mbhA oriT	Foreword	oriTv_mbhA_f	CAGAAACCTCGGAAATACGC
Verification	mbhA oriT	Reverse	oriTv_mbhA_r	GCATTGCTCACCTCTCAACA
Verification	hyfC oriT	Foreword	oriTv_hyfC_f	GGCTGGCCAAAGAAATACAG
Verification	hyfC oriT	Reverse	oriTv_hyfC_r	AGATCAGCGACAACATGCAC
Verification	ybhl alternate	Foreword	C1_veri_2	CCTTGTCGCCTTGCGTATAA
Verification	G6272 alternate	Reverse	C2_veri_2	CCTACCTGTGACGGAAGATC
Verification	tn10	Foreword	tetA_f_qp	CCACTCCCTATCAGTGATAGAG
Verification	tn10	Reverse	tetA_r_qp	CGGAATAACATCATTTGGTGACG
Verification	tn10 out	Foreword	TnA_5v	GTTTTTGTTGTGATGTAGGCAT
Verification	tn10 out	Reverse	TnC_5v	TTAAAGTGATGATAAAAGGC
Verification	glnA	Foreword	glnA_veri_f	GCCTCAGGCATTAGAAATAGCG
Verification	glnA	Reverse	glnA_veri_r	CGTAATGGATCGCCAGGTTG
Verification	hisB	Foreword	hisB_veri_f	CGCTGGCAAACGAAGAAGTC
Verification	hisB	Reverse	hisB_veri_r	GGCATTGCGTAGCTGTGAAC

Six strains of BW25113 each containing a single antibiotic marker were constructed by inserting antibiotic resistance genes at different locations within the chromosome: *hisB*::KanR and *glnA*::KanR (kanamycin resistance) markers were transduced from the KEIO collection, the *zje*::Tn10 and *zdi*::Tn10 (tetracycline resistance) makers were transduced from strains BW6156 and BW5659 (Wanner, 1986), respectively, and the *ybh1*::cat and *G6272*::cat (chloramphenicol resistance) markers

constructed using the method outlined in (Datsenko & Wanner, 2000) using primers listed inTable 1. Three of the antibiotic markers A: *ybhl*::cat, B: *zje-2005*::Tn10, and C: *hisB*::KanR are located at ~11 minutes away from the chromosomal locations of each of the 3 oriTs in HFR-2xoriT-SFX- and were individually introduced into strains HFR-2xoriT-SFX- and HFR-Δ2xoriT-SFX-. The resulting 6 strains each contained a single antibiotic resistance marker and were verified with colony PCR (see Table 1 for list of primers) and resistance to the appropriate antibiotic. The three HFR-2xoriT-SFX- strains with individual markers A, B and C were mated together to isolate a recombinant that contains all three markers. The isolated HFR-2xoriT-SFX- recombinant with all 3 markers (HFR-2xoriT-SFX- *ybhl*::cat, *zje-2005*::Tn10 and *hisB*::KanR) was named 3m11. Similarly, the three HFR-Δ2xoriT-SFX- strains containing individual markers A, B and C were mated together to isolate strain 3m11Δ2xoriT that contains all 3 markers. The other 3 antibiotic markers D: *G6272*::cmR, E: *glnA*::KanR, and F: *zdi-57*::Tn10, located at ~17 minutes away from each of the 3 oriTs in the chromosomal were also individually introduced into HFR-2xSFX- and HFR-Δ2xoriT-SFX- to generate 6 strains each containing a single antibiotic resistance marker, and verified with resistance to the appropriate antibiotic and colony PCR (see Table 1 for list of primers used). Each set of 3 single-marked strains of HFR-2xSFX- and HFR-Δ2xoriT-SFX- were mated as described above to generate the triple-marked strains 3m17 and 3m17Δ2xoriT, respectively.

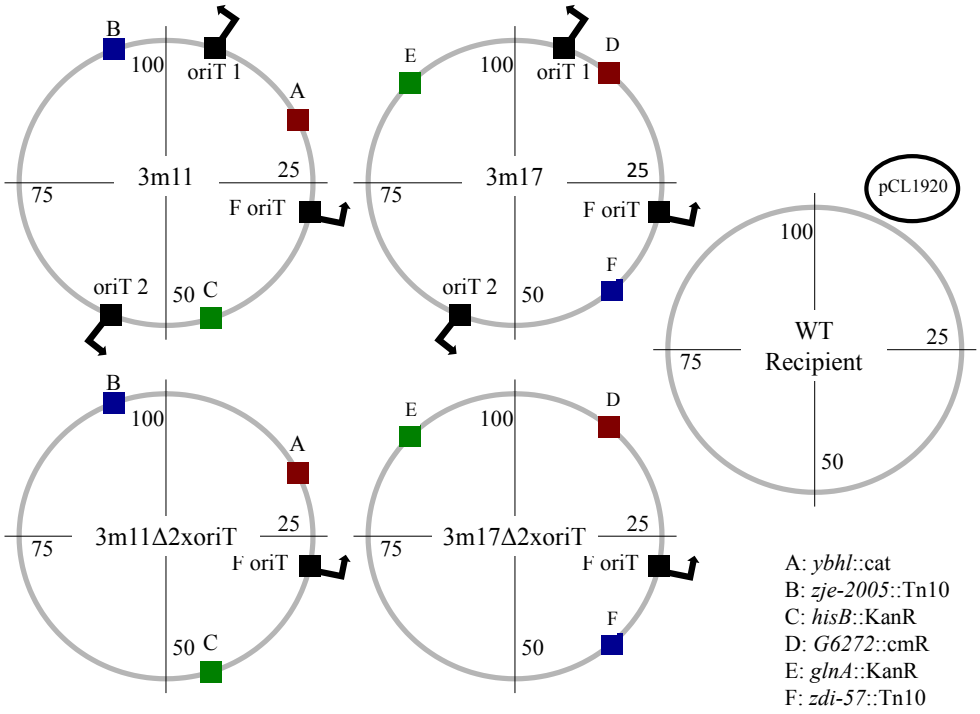


Figure 3: Chromosomal maps of the 3-marker strains. The positions labeled oriT are the origins of transfer, and the arrows indicate the directionality of DNA transfer. The markers are color-coded based on antibiotic resistance markers: chloramphenicol (red), tetracycline (blue), kanamycin (green).

Short mating assay protocol

Wild type [BW25113] cell carrying plasmid pCL1920 (Lerner & Inouye, 1990) encoding *aadA* for streptomycin/spectromycin resistance (BW25113/pCL1920) was used as a recipient for measuring conjugation and recombination efficiencies. The 3m17 and 3m17Δ2xoriT strains were used as donors. Overnight cultures of each strain were grown from single colonies in Luria Broth (LB) supplemented with the appropriate antibiotics. Each culture was normalized to OD_{600} ~1, diluted 100 fold into 10 mL of LB supplemented with the appropriate antibiotics and incubated at 37ºC at 225 rpm until

mid-exponential phase (OD_{600} ~ 0.5). Cells were pelleted by centrifugation at 5000xg for 3 minutes and re-suspended to normalized OD_{600} ~ 0.5 in fresh pre-warmed LB without antibiotics. One mL of donor (either 3m17 or 3m17Δ2xoriT) and 1 ml of recipient (BW25113/pCL1920) were mixed in approximately equal ratios and incubated for 105 minutes at 37ºC without agitation to allow mating. Then 1 µl of streptomycin stock solution (50 mg/ml) was added to 0.5 mL of the mating culture and vortexed vigorously for 1 minute to disrupt mating. 100 µl of the mating mixture was immediately plated onto three separate LB plates supplemented with 100µg/ml of streptomycin combined with one of the following, 1) chloramphenicol, 2) tetracycline, or 3) kanamycin, to select for recipient cells that have inherited at least one of the three antibiotic markers from the triple-marker donor strain (see Step 1 in Figure 4).

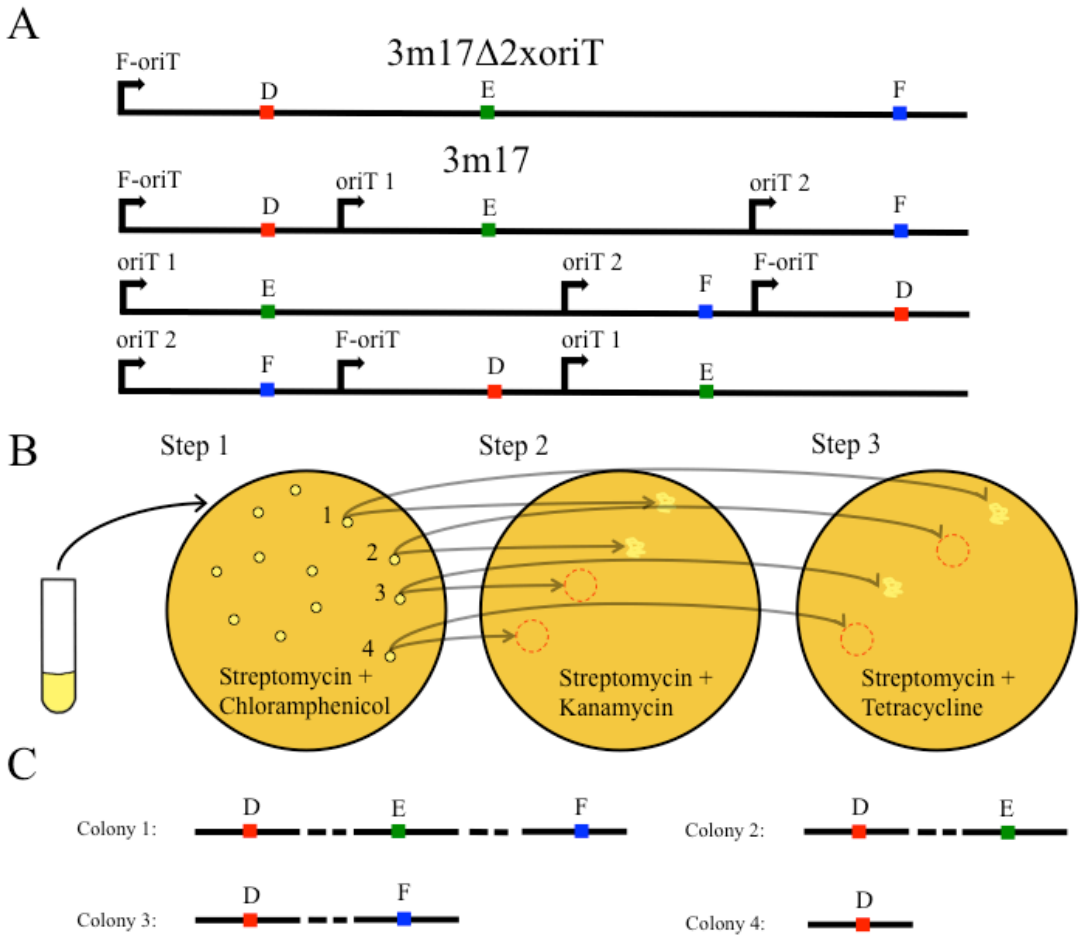


Figure 4: Short mating experiment. A) A schematic of the theoretical transfer orders used for the origin(s) of transfer in strains 3m17 and 3m17Δ2xoriT is depicted. B) Schematic of the short mating experiment. Step 1, Mixed cultures of the 3m17 or 3m17Δ2xoriT donor and recipient are plated on dual-selection plates, streptomycin resistance (to select for recipient) and one of the antibiotic resistances encoded by the triple-marked donor, for illustration purposes, chloramphenicol was chosen. Steps 2 and 3, the resultant colonies are streaked in replicate onto plates to select for resistance to one of the other three antibiotic resistances, in this case either kanamycin (2) or tetracycline (3). For example, colony 1 has all three markers, colony 2 has markers for chloramphenicol and kanamycin, colony 3 has markers for chloramphenicol and tetracycline, and colony 4 only has the chloramphenicol marker. C) The assumed marker composition for colonies 1-4 in the schematic.

The plates were incubated for 24 hours at 37ºC, and 50 colonies from each plate were randomly picked and plated onto 2 different selection plates, each containing 100 μg/ml of streptomycin and an antibiotic for one of the other two markers that were not initially assayed for, to allow selection for recombinants that have inherited more than one marker from the donor strain (see Steps 2 & 3 in Figure 4). The frequency of recombinants with co-transferred markers was calculated based on the number of colonies that displayed growth after 24 hour incubation at 37ºC.

Long mating assay protocol

A longer mating assay was devised to replicate mating in ALE conditions using 3m17, 3m17Δ2xoriT, 3m11, and 3m11Δ2xoriT as donors. BW25113/pCL1920 was used as the recipient. Single colonies of each strain were cultured overnight in LB. Overnight cultures were normalized to OD_{600} ~1.0, and 20 μl of one of the donors and the recipient cell type were co-inoculated into 2 mL of fresh LB. Each donor and recipient pair (at an initial OD_{600} ~ 0.01) was allowed to mate in liquid culture at 37ºC and 225 rpm. After exactly 24 hours, the final mixture was plated on 4 different types of plates as follows. Total cell concentration was estimated by plating 50 μL of a 10^{-6} diluted culture onto a non-selective LB plate. To estimate the number of recipient cells that received each marker from the donor strain, the overnight culture was diluted ~10^{-4} and plated on three types of dual-selective plates, each containing 100 μg/ml of streptomycin and either 1) chloramphenicol, 2) tetracycline, or 3) kanamycin. The final colony counts on the LB and selective plates were used to estimate the frequency of recombinants in the population.

Evolution experiment

Six independent populations of the mutator strains, HFR-SFX-2xoriT-ΔmutS:;KanR (genderless mutator) and BW25113-2xoriT ΔmutS::KanR (asexual 2xoriT mutator), were serially passaged for approximately 850 generations in LB media. The OD_{600} of each population was measured daily before passaging. In each passage, cultures were diluted ~10^{-6} fold in 6 serial dilutions of 10^{-1} each by pipetting 10 µl of the mixture into 90 µl of fresh media. 10 µl of the final diluted mixture (10^{-6}) was inoculated into 2 mL of fresh LB. Based on the dilution used, ~50-100 cells are estimated to be transferred during each serial passage; we choose this harsh bottleneck to increase drift. The strains were assayed weekly for significant fitness changes in reference to the parental strain by measuring the average population growth rate in LB at 37ºC using a 96-well plate reader with shaking and incubation capabilities (TECAN Infinite M200). Every 4 transfers, frozen stocks of each population were prepared in 15% (v/v) glycerol and stored at -80ºC, and the genderless mutator populations were assayed to confirm their ability to mate.

Screening of growth impaired mutants

Mutants with growth impairments on minimum medium were isolated from the final transfer of the evolved populations. Population samples were revived from frozen stocks by inoculating approximately 10 µl of the frozen stock into 2 mL of LB media for overnight growth at 37ºC at 225 rpm. The overnight cultures were diluted 10^{-6} fold and then plated on non-selective LB plates to isolate single colonies. 500 individual colonies from each population were randomly chosen and re-streaked onto both M9 dropout

media plates (see Table 2 for amino acid supplementation) and LB plates and incubated for 48 hours at 37ºC. Colonies that grew on LB plates but exhibited growth impairment (lack of observable growth after 48 hours) on the M9 dropout plates were further verified for growth impairment phenotype (see Figure 5: Visualization of growth impaired phenotype on dropout plates.).

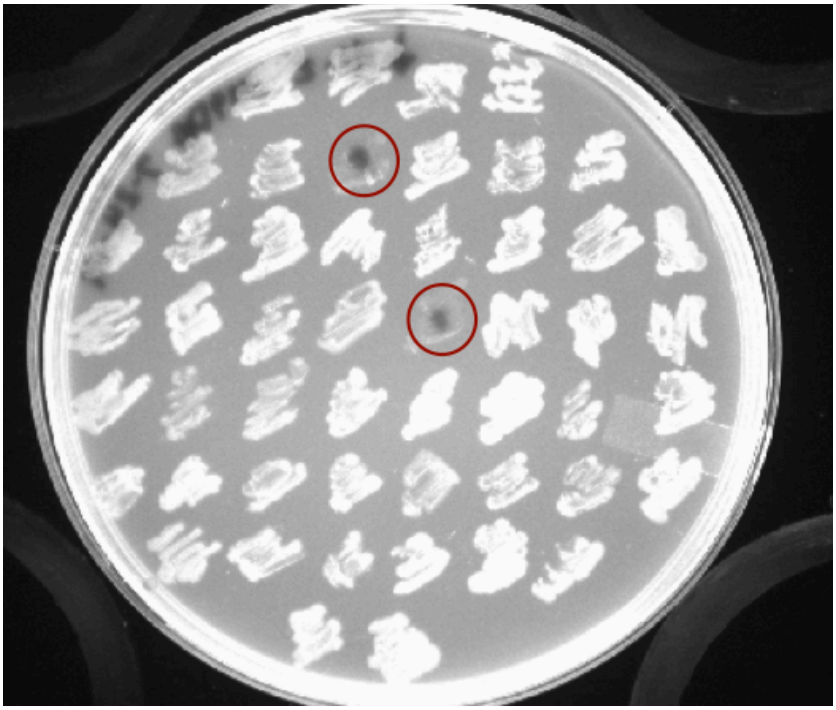


Figure 5: Visualization of growth impaired phenotype on dropout plates.

For verification of clones with impaired growth on M9 dropout media, a single colony from LB plate was inoculated into two separate cultures, in 2 mL of M9 dropout medium and 2 mL of LB. The cultures were incubated at 37ºC and 225 rpm; those that exhibited $OD_{600} < 0.1$ after 24 hours of growth were confirmed as growth deficient in the M9 dropout medium. Frozen stocks of growth-deficient strains were prepared from the replicate LB cultures.

Table 2: Drop out media amino acid composition.

Amino Acid	mg/L
Alanine	129
Cysteine	0
Aspartic acid	93
Glutamic acid	110
Phenylalanine	91
Glycine	131
Histidine	42
Isoleucine	111
Lysine	0
Leucine	171
Methionine	0
Asparagine	0
Proline	0
Glutamine	110
Arginine	148
Serine	63
Threonine	89
Valine	46
Tryptophan	245
Tyrosine	30

Complementary mating of growth deficient strains

Two growth-impaired and recombinant-proficient strains isolated from independently evolved populations were paired to determine if divergent populations

could repair the accumulated mutations causing growth deficiency. Cells were streaked onto LB plates from frozen stocks, and single colonies were picked and cultured overnight in LB media. The overnight cultures of each growth-impaired strain were normalized to OD_{600} of ~1.0 and mixed in different proportions in 2 mL of fresh LB media for a total inoculum size of ~10^7 cells. The cells were incubated at 37ºC for 24 hours. The resulting cultures were then normalized to OD_{600} ~1.0, spun down, and resuspended in fresh M9 dropout media, 10 µl of each was transferred into 2 mL of M9 dropout media to determine if the final culture contained any recombinants. $OD_{600} > 0.1$ after 24 hours of incubation at 37ºC and 225 rpm was used as the criteria to determine mixtures that contained successful recombinants.

Complementary mating of single marker strains

Overnight cultures of strains SMD and SMB (see Table 3) were cultured from single colonies in LB with the appropriate antibiotic at 37ºC and 225 rpm for 24 hours. The overnight cultures were normalized to an OD_{600} of ~1, and 200 fold dilutions of each strain was used to co-inoculated a single test tube of 10 mL of LB (for a final mixture with OD_{600} of ~0.01). Control cultures with single strains were diluted 100 fold into 10 mL LB. The subcultures were incubated at 37ºC and 225 rpm for 24 hours. Each culture was periodically assayed for the presence of recombinants by diluting an appropriate volume of sample into fresh liquid LB and immediately plating 50 µl onto LB plate supplemented with tetracycline and chloramphenicol; the sample was also plated on an LB only plate to estimate the total number of cells. The dilution factors used were tailored to the expected number of recombinants and cells. In the mating with

varying ratios of the two different single marker strains, the same protocol was followed except the initial mix of cells in the subculture was modified to maintain the same overall starting OD_{600} but changing the ratio of the two genotypes. A second pair of mating using strains SMA and SMF (see Table 3) were also utilized to confirm the frequency of recombinant formation as a function of initial ratio of donor/recipient strains.

Table 3: Strains used in this study.

NAME	STRAIN	GENOTYPE/FEATURES	HFR	SOURCE
JW3841	BW25113	ΔglnA::kan	N	Keio
JW2004	BW25113	ΔhisB::kan	N	Keio
QP1	BW25113	ΔybhI::cat	N	This Work
QP2	BW25113	ΔG6272::cat	N	This Work
BW6156	BW6156	zje-2005::Tn10 (tetR)	Y	CGSC
BW5659	BW5659	zdi-57::Tn10 (tetR)	Y	CGSC
Antibiotic Cassette	BW25113	pKD32	N	Wanner
Helper Plasmid	BW25113	pKD46	N	Wanner
Removal Plasmid	BW25113	pCP20	N	Wanner
Wild type Recipient	BW25113	pCL1920	N	see citation
Genderless	BW25113	ΔmbhA::oriT ΔhyfC::oriT trp::F[ΔtraST] (genR)	Y	This Work
Wild type 2xoriT	BW25113	ΔmbhA::oriT ΔhyfC::oriT	N	This Work
Genderless mutator	BW25113	ΔmbhA::oriT ΔhyfC::oriT trp::F[ΔtraST] (genR) ΔmutS::kan	Y	This Work
Asexual 2xoriT mutator	BW25113	ΔmbhA::oriT ΔhyfC::oriT ΔmutS::kan	N	This Work
QP3	BW25113	ΔmbhA::oriT ΔhyfC::cat trp::F[ΔtraST] (genR)	Y	This Work
QP4	BW25113	ΔmbhA::cat ΔhyfC::oriT trp::F[ΔtraST] (genR)	Y	This Work
QP5	BW25113	ΔmbhA ΔhyfC::oriT trp::F[ΔtraST] (genR)	Y	This Work
QP6	BW25113	ΔmbhA ΔhyfC::cat trp::F[ΔtraST] (genR)	Y	This Work
Genderless Δ2xoriT	BW25113	ΔmbhA ΔhyfC trp::F[ΔtraST] (genR)	Y	This Work

Table 3: Continued

NAME	STRAIN	GENOTYPE/FEATURES	HFR	SOURCE
QP7	BW25113	ΔmbhA ΔhyfC trp::F[ΔtraST] (genR) ΔybhI::cat	Y	This Work
QP8	BW25113	ΔmbhA ΔhyfC trp::F[ΔtraST] (genR) ΔG6272::cat	Y	This Work
QP9	BW25113	ΔmbhA ΔhyfC trp::F[ΔtraST] (genR) zje-2005::Tn10 (tetR)	Y	This Work
QP10	BW25113	ΔmbhA ΔhyfC trp::F[ΔtraST] (genR) zdi-57::Tn10 (tetR)	Y	This Work
QP11	BW25113	ΔmbhA ΔhyfC trp::F[ΔtraST] (genR) ΔglnA::kan	Y	This Work
QP12	BW25113	ΔmbhA ΔhyfC trp::F[ΔtraST] (genR) ΔhisB::kan	Y	This Work
3m11Δ2xoriT	BW25113	ΔmbhA ΔhyfC trp::F[ΔtraST] (genR) ΔybhI::cat zje-2005::Tn10 (tetR) ΔhisB::kan	Y	This Work
3m17Δ2xoriT	BW25113	ΔmbhA ΔhyfC trp::F[ΔtraST] (genR) ΔG6272::cat ΔglnA::kan zdi-57::Tn10 (tetR)	Y	This Work
SME	BW25113	ΔmbhA::oriT ΔhyfC::oriT trp::F[ΔtraST] (genR) ΔglnA::kan	Y	This Work
SMC	BW25113	ΔmbhA::oriT ΔhyfC::oriT trp::F[ΔtraST] (genR) ΔhisB::kan	Y	This Work
SMA	BW25113	ΔmbhA::oriT ΔhyfC::oriT trp::F[ΔtraST] (genR) ΔybhI::cat	Y	This Work
SMD	BW25113	ΔmbhA::oriT ΔhyfC::oriT trp::F[ΔtraST] (genR) ΔG6272::cat	Y	This Work
SMB	BW25113	ΔmbhA::oriT ΔhyfC::oriT trp::F[ΔtraST] (genR) zje-2005::Tn10 (tetR)	Y	This Work
SMF	BW25113	ΔmbhA::oriT ΔhyfC::oriT trp::F[ΔtraST] (genR) zdi-57::Tn10 (tetR)	Y	This Work
3m11	BW25113	ΔmbhA::oriT ΔhyfC::oriT trp::F[ΔtraST] (genR) ΔybhI::cat zje-2005::Tn10 (tetR) ΔhisB::kan	Y	This Work
3m17	BW25113	ΔmbhA::oriT ΔhyfC::oriT trp::F[ΔtraST] (genR) ΔG6272::cmR ΔglnA::kanR zdi-57::Tn10 (tetR)	Y	This Work

Model of bi-directional mating

The Matlab package Simbiology was used to model cell growth and mating kinetics using the mass action kinetic law and the following reactions (where S is a carbon source, A and B are cells with a single maker, C is a cell with both markers and N is a cell with no markers):

Growth

$$1: \qquad A + S \xrightarrow{k_1} 2\,A$$

2: $B + S \overset{k_1}{\rightarrow} 2\,B$

3: $C + S \overset{k_1}{\rightarrow} 2\,C$

4: $N + S \overset{k_1}{\rightarrow} 2\,N$

Mating

A1:	$A + N \overset{k_2}{\rightarrow} A + A$	B1:	$B + N \overset{k_2}{\rightarrow} B + B$
A2:	$A + B \overset{k_2}{\rightarrow} A + A$	B2:	$B + A \overset{k_2}{\rightarrow} B + C$
A3:	$A + B \overset{k_2}{\rightarrow} A + C$	B3:	$B + A \overset{k_2}{\rightarrow} B + B$
A4:	$A + B \overset{k_2}{\rightarrow} A + N$	B4:	$B + A \overset{k_2}{\rightarrow} B + N$
A5:	$A + C \overset{k_2}{\rightarrow} A + C$	B5:	$B + C \overset{k_2}{\rightarrow} B + C$
C1:	$C + N \overset{k_2}{\rightarrow} C + C$	N1:	$N + A \overset{k_2}{\rightarrow} N + N$
C2:	$C + N \overset{k_2}{\rightarrow} C + A$	N2:	$N + B \overset{k_2}{\rightarrow} N + N$
C3:	$C + N \overset{k_2}{\rightarrow} C + B$	N3:	$N + C \overset{k_2}{\rightarrow} N + A$
C4:	$C + A \overset{k_2}{\rightarrow} C + C$	N4:	$N + C \overset{k_2}{\rightarrow} N + B$
C5:	$C + B \overset{k_2}{\rightarrow} C + C$	N5:	$N + C \overset{k_2}{\rightarrow} N + N$

and initial values:

$$S_o = 10^9 \;\; A_o = 5.0 * 10^6 \; B_o = 5.0 * 10^6 \; C_o = 0 \;\; N_o = 0 \;\; in\; molecule$$

$$k_1 = 2.0 * 10^{-9}\ k_2 = 10^{-10}\ in\ \ (molecule * sec)^{-1}$$

Several assumptions were made in the development of the model: 1) lag phase was incorporated by reducing the growth rate by 10^{-4} for the initial 30 minutes, 2) the initial rate constants for recombination (k_2) values were modified (reduced by 50 fold) to match the experimental data, when the substrate was 20% consumed (OD of ~ 0.4); the 50 fold reduction in k_2 value appears to be consistent with previous predictions (Collins & Broda, 1975; Cullum, Collins, & Broda, 1978), 3) the reaction rate constants for reactions 1-4 (cell growth) and A1-A5, B1-B5, C1-C5, N1-N5 (cell mating) are the same. The model was run for 24 simulated hours in approximately 250 time steps (determined by the solver).

Results

Genetic coverage with additional origins of transfer

We have previously engineered the HFR strain HFR-SFX-2xoriT (genderless) that lacks surface exclusion machinery, and demonstrated the mating system to be effective for leveraging *in situ* recombination to increase adaptation rates during ALE experiments for phenotypes with complex adaptive landscapes (Winkler & Kao, 2012). Since the transfer frequency of genetic material decays exponentially as distance increases from the oriT (Smith, 1991), one of the features added to the strain is two additional origin of transfers (for a total of 3 oriTs including the one from the F factor). The additional oriTs were expected to increase coverage of genomic regions further

away from the F factor. However, the benefit of the additional oriTs and their relative activities compared with the oriT from the F factor has yet to be assessed. In this experiment, we aimed to more closely determine if the two additional oriTs are active and if their presence can effectively increase the coverage of genome transfer on a laboratory time scale (more similar to time scales of an ALE experiment).

To determine the influence of additional oriTs on the frequency of genetic transfer of alleles at various positions in the chromosome, we generated triple marker strains with antibiotic resistance markers located approximately 11 minutes (strain 3m11 containing markers A, B, and C) or 17 minutes (strain 3m17 containing markers D, E, and F) from each of the 3 oriTs. We also deleted the two extra oriTs from the triple marker strains to generate strains 3m17Δ2xoriT and 3m11Δ2xoriT, which only contain the oriT native to the F (F-oriT). The absolute marker transfer rates between the 3m17, 3m11, 3m17Δ2xoriT and 3m11Δ2xoriT strains were compared after 24 hours of mating. Since there is an exponential decay in probability of transfer as marker distance increases from an oriT, we hypothesized that the strains with the additional 2xoriTs (3m11 and 3m17) would yield higher recombination frequency in markers E, B, C, and F (see Figure 3 for marker locations) compared with strains 3m17Δ2xoriT and 3m11Δ2xoriT. To assess the ability of the "genderless" strains to mate and transfer a marker through horizontal genetic exchange, we mixed the F^- BW25113/pCL1920 recipient carrying a selectable marker with each of the triple-marker strains, 3m11, 3m17, 3m11Δ2xoriT, and 3m17Δ2xoriT, and measured the frequency at which recipient cells have inherited each of the markers from the donors. The data showed that the

presence of the two additional oriTs resulted in increased transfer of markers furthest away from the F-oriT compared to the strains without the additional oriTs (see Figure 6) (p-values of 0.0267 and 0.001 for the furthest 17 (marker F) and 11 (marker C) minute markers, respectively, using the one-tailed Student t-test). This supports our hypothesis that the additional oriTs are active and helps to increase the absolute coverage of genetic transfer during normal culture conditions.

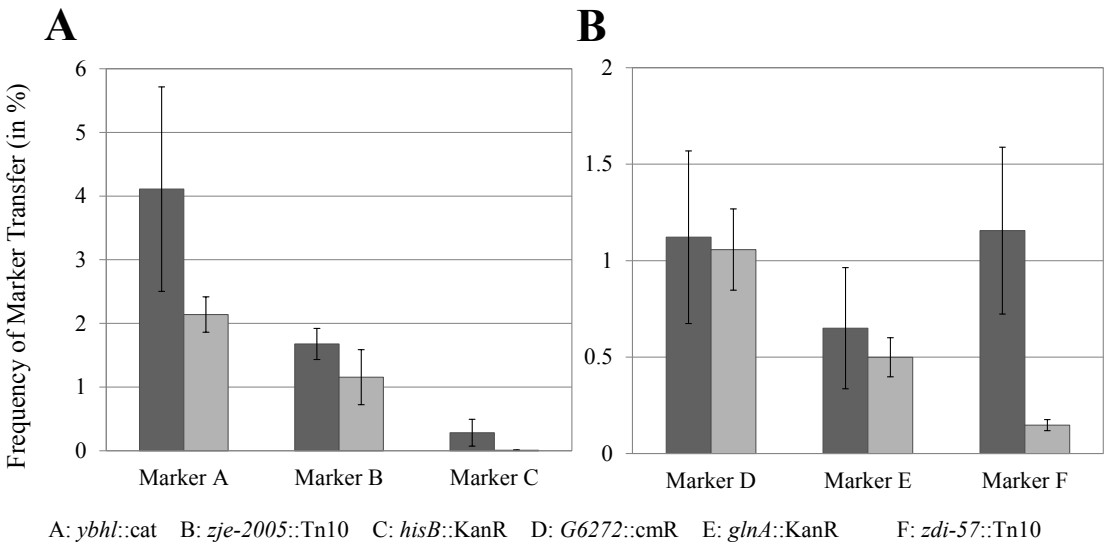


Figure 6: Percentage of recipient cells that received a marker from the triple-marker donor strain in the long mating assay. A) The frequency of isolating recombinants of either markers A, B or C in the final recipient populations using donor strains 3m11 (dark gray) and 3m11Δ2xoriT (light gray). B) The frequency of isolating recombinants containing either markers D, E or F in the final recipient populations using donor strains 3m17 (dark gray) and 3m17Δ2xoriT (light gray).

Identify possible bias in oriT usage

Having established the net influence of the oriTs on genetic transfer of the three antibiotic markers, we next investigated any bias in oriT usage between the 3 oriTs, and if the additional oriTs have an effect on frequency of co-transfer of alleles. There are

three primary factors influencing the frequency of observing co-transferred markers: distance from upstream oriT, the order of the markers, and distance between the markers. When the distance of a marker from the oriT increases, the frequency of the recipient cell inheriting the donor marker decreases as the probability of interrupted mating or degradation of DNA increases. Similarly, as the distance between two markers increases, the chance of both markers being co-transferred also decreases. Thus we expect that in the 3m17Δ2xoriT strain that contains just the F-oriT, successful recombinants that have inherited marker E would have a higher frequency of co-inheriting marker D (closest marker downstream of F-oriT), and recombinants containing marker F would also have a higher frequency of co-inheriting markers D and E.

To determine the correlation between the number of oriTs and marker co-transfer frequencies, we measured the frequency of marker co-transfer between the wild-type F^- recipient BW25113/pCL1920 and a triple-marker donor strain with (3m17) or without (3m17Δ2xoriT) the extra oriTs. To minimize the effect of multiple mating events, we performed a short-term mating experiment using a mating period of 105 minutes. Successful recombinants were first screened for the presence of at least one of the three donor markers, followed by further screens to determine the frequency of co-inheritance of additional markers. If the oriTs were active, we would expect to see a different pattern of marker co-transfer frequency between strains 3m17 and 3m17Δ2xoriT. In the 3m17Δ2xoriT strain without the additional oriTs, each downstream marker is inevitably linked to markers closer to the F-oriT. Thus we would expect linkage between the later

markers E and F with the first marker D. However as shown in Figure 7, there appears to be low linkage between these markers. The significant distance between marker F and both markers D and E likely explains the low observed linkage of marker F to the upstream markers. With the addition of oriT 1 and 2, increased marker co-transfer is expected see Figure 7, as DNA transfer events can initiate from any oriT as illustrated in Figure 3. In 3m17, DNA transfer events that initiate at oriT-F would yield a pattern similar to that in the 3m17Δ2xoriT strain. However, DNA transfer events that initiate at each of the 2 additional oriTs in strain 3m17 are expected to have their own unique pattern of marker linkage compared to strain 3m17Δ2xoriT. Genetic transfer that originates from oriT 1 is expected to result in some level of co-transfer of markers E and F. Due to the closer proximities of D, E, and F markers, DNA transfer events that initiate from oriT 2 are expected to result in higher frequency of marker co-transfer between these markers, especially marker pairs F-D and D-E.

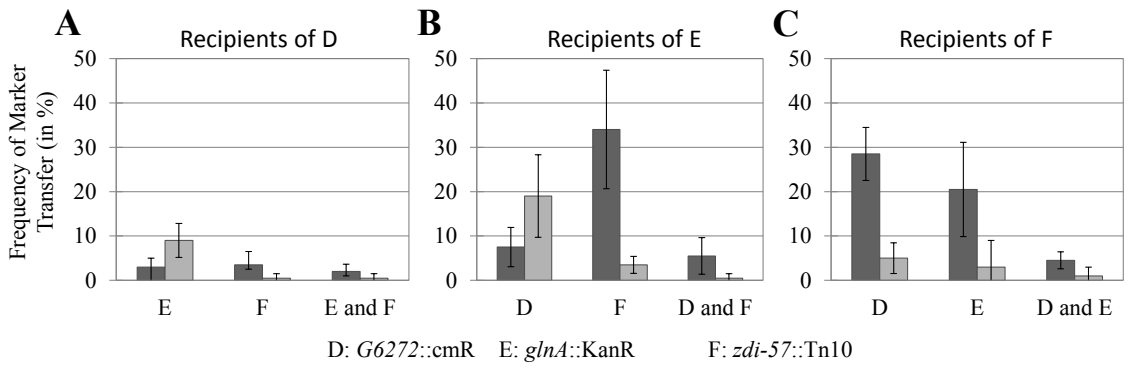


Figure 7: Frequency of marker co-transfer after 105 minutes of mating between donor strains 3m17 (dark gray) or 3m17Δ2xoriT (light gray), and recipient strain WT/pCL1920 (streptomycin resistance). A) Percentage of recipient cells that inherited marker D and also co-inherited either marker E or F, or both markers E and F. B) Percentage of recipient cells that inherited marker E and also co-inherited marker D or F, or both markers D and F. C) Percentage of recipient cells that inherited marker F and also co-inherited either marker D or E, or both markers or D and E.

The data showed that the addition of the two oriTs significantly altered the frequency of marker co-inheritance (see Figure 7). In some cases, the expected pattern of marker co-inheritance was observed, for example isolates of marker F are linked with both D and E. The noted differences in the patterns of co-transfer suggests that the additional oriTs were active in initiating transfer during conjugation, as the frequency of co-inheritance is higher in the strains with the extra oriTs in the majority of cases.

Recombination-proficient strain is capable of reducing the effects of Muller's ratchet

One of the key postulated benefits of sexual recombination is the reduction of Muller's ratchet. To determine whether the recombination-proficient strain can effectively achieve this during an ALE experiment, we generated a mutator version our

strain by deleting *mutS*, the sliding clamp component of the mismatch repair system (Groothuizen et al., 2015; Lamers et al., 2000), and carried out an ALE experiment in rich medium (LB) for ~850 generations. The mutation rate of the Δ*mutS* strain was increased by ~200-300 fold in both genderless mutator and asexual 2xoriT mutator strains over the non-mutator counter-parts HFR-2xoriT-SFX and BW25113-2xoriT, respectively. We implemented a severe bottleneck of 50-100 cells per transfer, which has been shown to accelerate Muller's ratchet by increasing the stochasticity of the experiment and consequently the frequency of transferring and fixing deleterious mutations. A severe bottleneck also reduces the frequency of selective sweeps of beneficial mutations and the associated hitchhiking mutations (Gabriel et al., 1993; Handel & Bennett, 2008; Lynch, Conery, & Burger, 1995a; Muller, 1964; Raynes, Halstead, & Sniegowski, 2013; Wahl & Gerrish, 2001; Wahl & Zhu, 2015). Periodic examination of mating abilities revealed no loss of conjugation capacity at the population-level over the course of evolution. No significant change in average population fitness compared with the unevolved parental strains was observed during the evolution (see Figure 8), indicating mutational meltdown was not observed in our experiments.

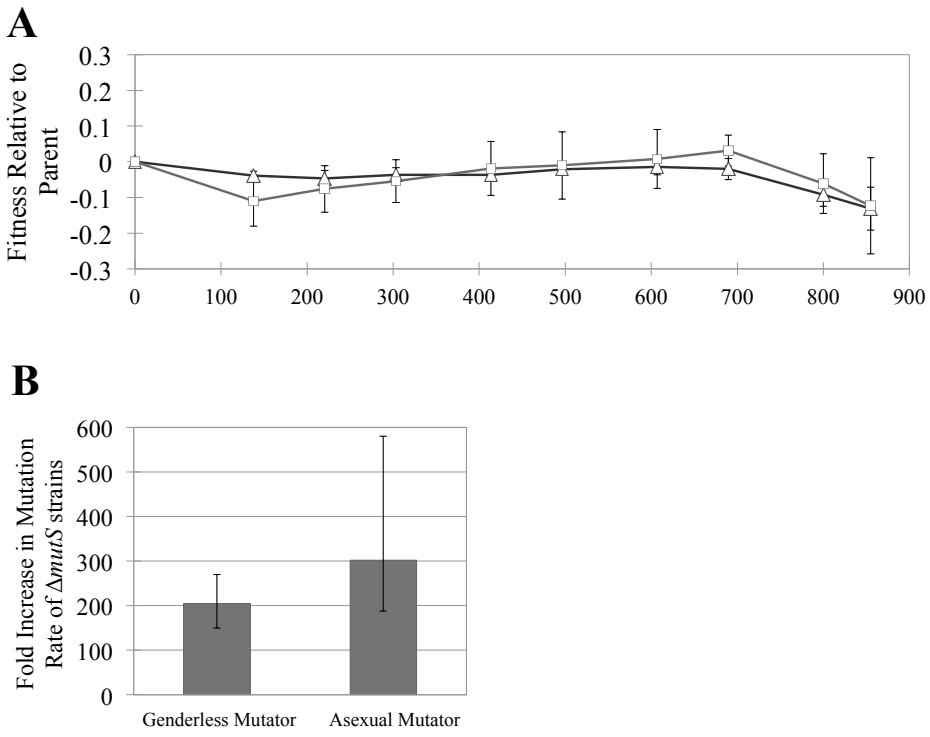


Figure 8: Mutation rates and fitness for the adaptive evolution of the asexual 2xoriT mutator strain and genderless mutator strain on LB. A) Mean population fitness changes of evolving populations (averaged across all 6 parallel populations per strain); and asexual 2xoriT mutator (open triangle) and genderless mutator (open square). B) Measured mutation rates relative to the ancestral strain for the genderless mutator and asexual 2xoriT mutator strains.

Since no gross fitness defects or advantages were observed at the population level in rich medium during the course of evolution, and most mutations in *E. coli* are observed to be slightly deleterious (Kibota & Lynch, 1996), we assumed that mutations that lead to biosynthetic deficiencies are present in the evolving population are not during serial passage in LB. Many auxotrophic strains with single deletions in biosynthetic genes were tested and found to be neutral or slightly disadvantageous in LB (data not shown). We postulated that the sexual populations would have a lower

mutational load, and thus a lower probability of accumulating mutations that result in biosynthetic deficiencies. To elucidate whether there exists a higher number of growth deficient mutants in the asexual 2xoriT mutator populations compared with the recombination-proficient genderless mutator populations, we randomly isolated 500 colonies from each of the 12 populations (6 parallel populations per strain) at the end of the ALE experiment and tested each for growth deficiency on minimum media plates supplemented with all amino acids except cysteine, lysine, methionine, asparagine, and proline (M9 dropout media). Genotypes unable to grow on this M9 dropout media are likely to have defects in the biosynthesis of at least one of the amino acids not supplemented. The data showed an increase in the frequency of asexual genotypes deficient in the biosynthesis of at least one amino acid compared to the sexual strains with a p-value of 0.048 using the one tailed Student t-test see Table 4.

Table 4: Number of growth deficient colonies per 500 colonies assayed for each of the 6 sexual and asexual evolved populations.

GENOTYPE	SEXUAL	ASEXUAL
POPULATION	GROWTH DEFICIENT COLONIES PER 500	
1	3	42
2	0	17
3	2	3
4	3	13
5	5	6
6	1	6
AVERAGE	**2.33**	**14.5**
STANDARD DEVIATION	1.75	14.4

We further examined whether recombination is the likely cause of the lower observed mutational load by pairing sexual isolates that exhibit growth deficiencies in the M9 dropout media to determine if recombinants can repair the growth deficiency. Each pair was allowed to mate for 24 hours in LB medium in replicate cultures, and 0.5% of the final culture volume was sampled to determine whether recombinants that were able to restore wild type growth in M9 dropout media were present. We found that 2 different pairs of mutants from separate populations were always able to successfully generate recombinants that are able to grow in the M9 dropout media.

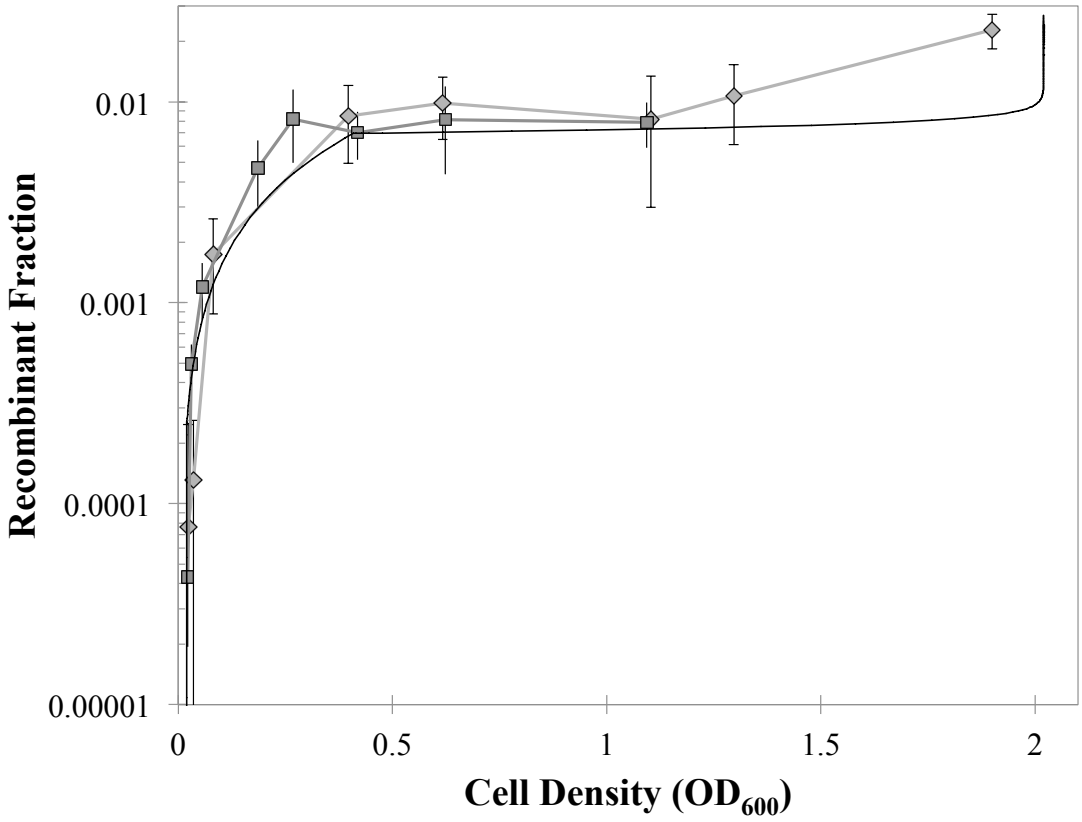


Figure 9: Changes in the fraction of the population that contains the desired recombinant as a function of cell density in batch culture. Experimental data for 2 pairs of single-marker strains are shown (diamond and square). Modeling data shown in solid black line.

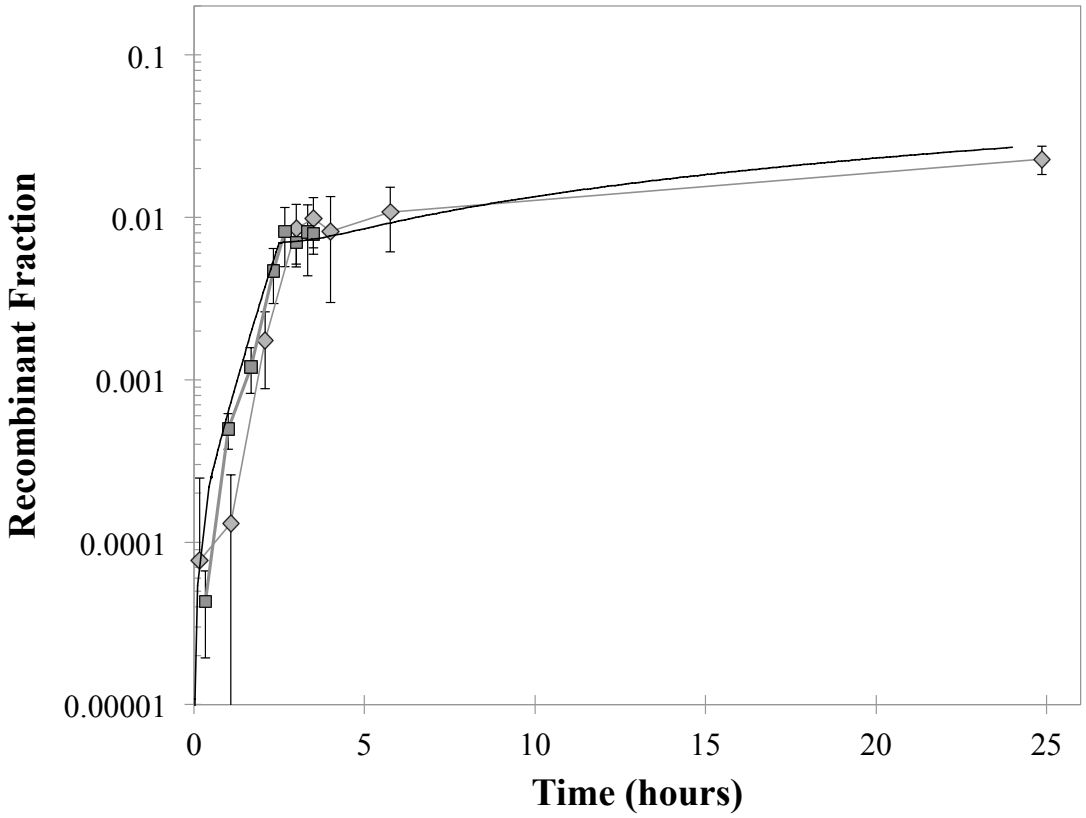


Figure 10: Changes in the fraction of the population that contains the desired recombinant as a function of time in batch culture. Experimental data for 2 pairs of single-marker strains are shown (diamond and square). Modeling data shown in solid black line.

Upon observing that divergent populations were able to recombine effectively, we sought to determine the kinetics of recombination *in situ*. To establish a basic understanding of the mating capabilities of the genderless strain, we co-cultured two genderless strains, each containing only a single marker, and experimentally quantified the frequency of dual marker recombinants in the population over time. We also modeled the kinetics of recombinant formation using mass action kinetics model for cell growth and mating. The model was able to closely reproduce the experimental data for

both growth rate and frequency of mating for two single marker strains as shown in Figure 9 and Figure 10. The results showed that the rate of change in the fraction of recombinants in the population increased more rapidly during early exponential phase (hours 0.5 to 4.5 see Figure 11), reaching ~1% of the population within the first 5 hours of batch culture. The rate of increase in the fraction of recombinants slowed down significantly as the population reaches stationary phase (at ~ 5 hours).

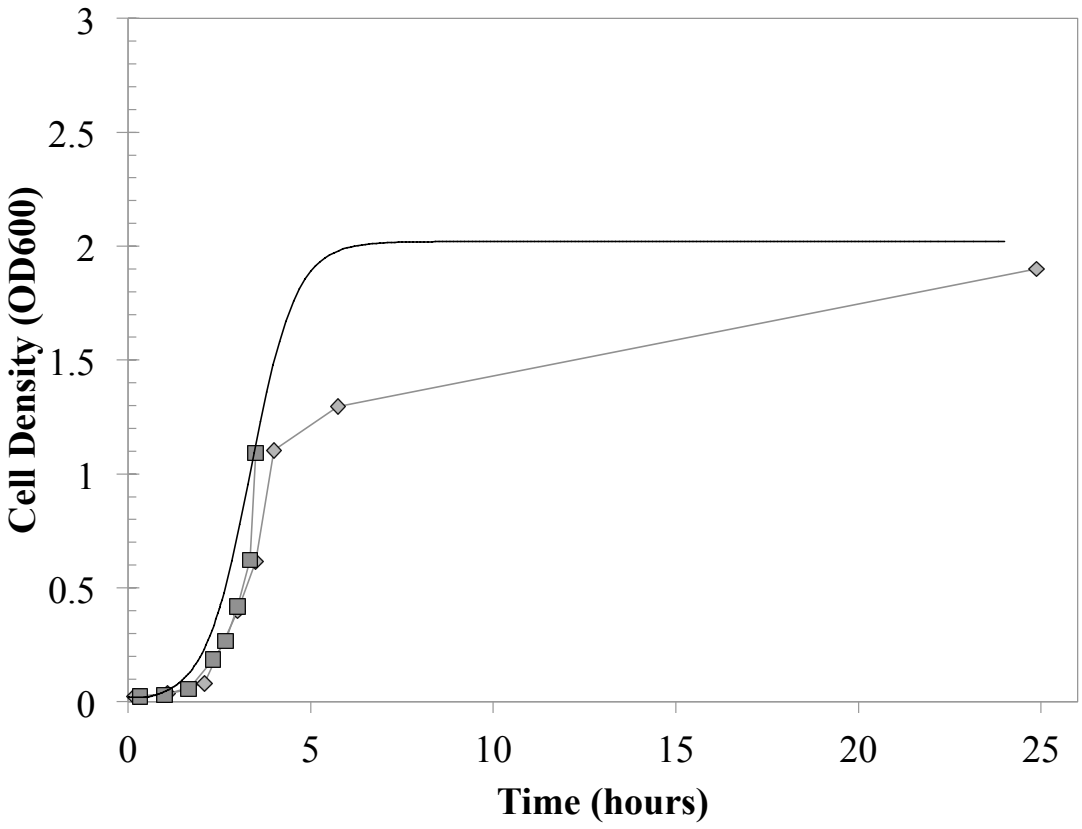


Figure 11: The measured OD_{600}s for trials 1 & 2 and the corresponding model's prediction. Trial 2 (Square), Trial 1 (diamond), black line (model).

Table 5: The model was run with the below input values for A, B and N. The output recombinant fraction is shown.

Trial	A	B	N	Starting number of cells	Fraction of recombinants
1	5000000	5000000	0	10^7	0.026256683
2	50000000	50000000	0	10^8	0.060411549
3	500000	500000	0	10^6	0.019174898
4	100000	100000	9800000	10^7	0.0000105
5	1000000	1000000	8000000	10^7	0.0010503

We next asked if the model could effectively predict whether changes in the relative proportion of the starting genotypes would significantly impact the frequency of desired recombinant formation. By keeping the total initial cell concentration the same, the relative frequencies of the two different single maker strains was varied from 1:1 to 1:10000. Our experimental results showed that a logarithmic decrease in the relative frequencies of the two mating strains led to a logarithmic decrease in the frequency of observing desired recombinants in the final culture. The model illustrates the frequency of observing desired recombinants is proportional to the product of the relative frequency of each genotype. Additionally, the data from the model matched the experimental results well; it successfully captured the trend of decreased formation of successful recombinants as the relative frequency of one strain was decreased (as seen in Figure 12).

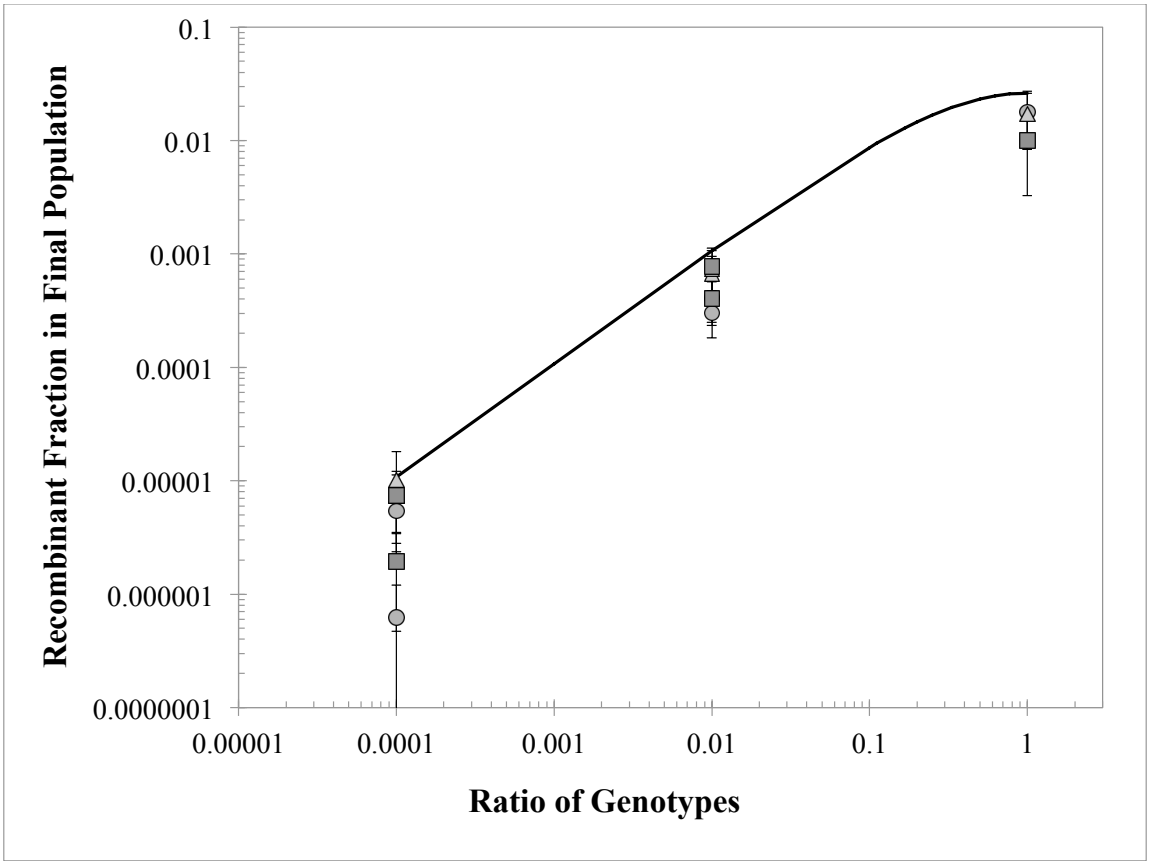


Figure 12: The fraction of the population that contains the desired recombinant versus the ratio of the two starting genotypes for two different pairs of starting genderless single-marker strains. Square and circle: two replicate experiments using starting strains SMB and SMD (markers B and D respectively). Triangle: experiment with starting strains SMA and SMF (markers A and F respectively). Black line: model data.

Note that in order to more accurately capture the trend in the experimental results, the rate constant for recombination in the model was reduced during mid exponential phase. The decrease in mating frequency has been previously observed by Collins *et al* (Collins & Broda, 1975; Cullum et al., 1978) and Cullum *et al* (Collins & Broda, 1975; Cullum et al., 1978) as recipient populations sizes reach or exceed ~5.0 x 10^7 cells; the authors speculated that fertility becomes the limiting factor in horizontal gene transfer rather

than cell-to-cell contact (Collins & Broda, 1975; Cullum et al., 1978). Building off the success of the model in recapitulating the results of having one rare genotype, we also briefly tested the model with alternate starting cell concentrations (10 fold higher and 10 fold lower), and with two rare mutant genotypes with a wild-type background genotype (both A and B at 1% or 0.1% of the population). The results (see Figure 13: Cells were cultured and mated as in the Materials and Methods but with varying starting concentrations as detailed in Table 5. After 24 hours of the growth the culture was serial diluted 10 fold and 5 μl of each dilution was spotted onto dual selective plates (Chloramphenicol and tetracycline). and Table 5), while qualitative, suggests that the model retained a high degree of predictability even in these alternate conditions.

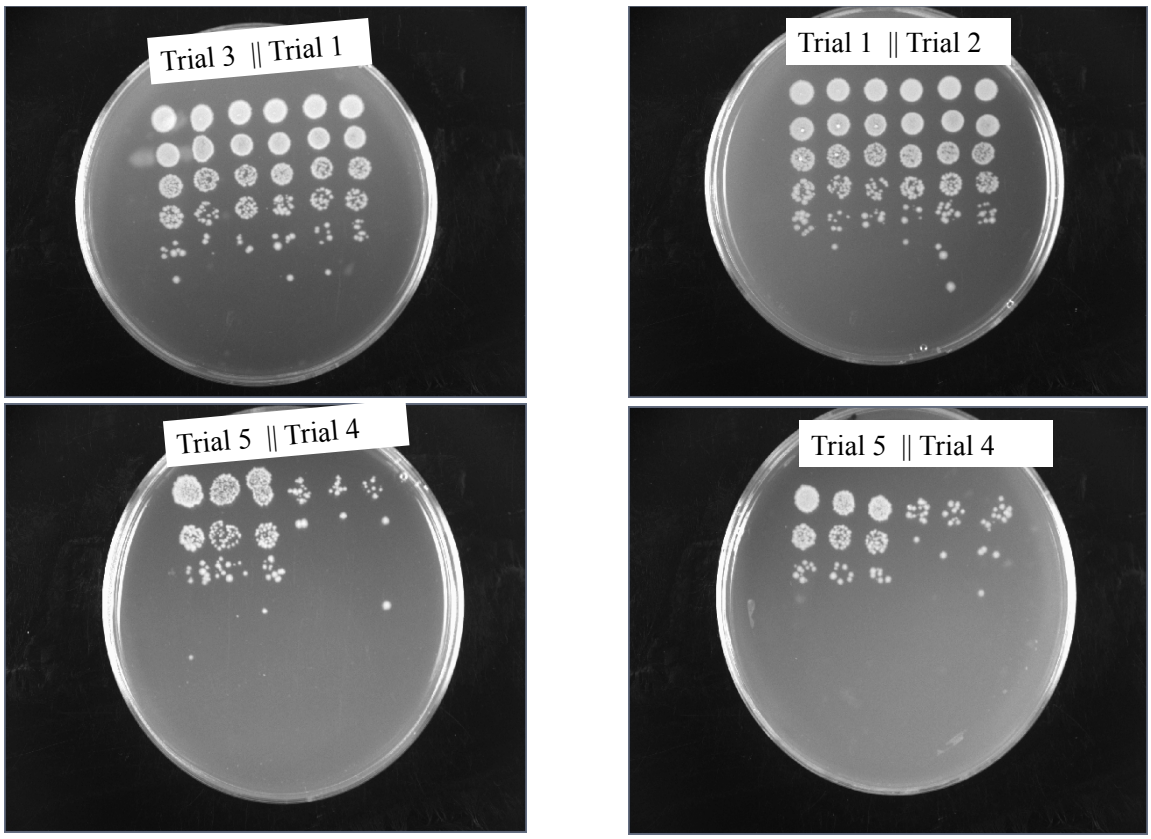


Figure 13: Cells were cultured and mated as in the Materials and Methods but with varying starting concentrations as detailed in Table 5. After 24 hours of the growth the culture was serial diluted 10 fold and 5 µl of each dilution was spotted onto dual selective plates (Chloramphenicol and tetracycline).

Discussion

The incorporation of recombination offers potential benefits for adaptive laboratory evolution, via reduction of Muller's ratchet and more rapid strain development per the Fisher Muller model. Prior work has successfully applied recombination to expedite adaptive laboratory evolution (Cooper, 2007; Patnaik et al., 2002; Reyes et al., 2012; Shi et al., 2009; Winkler & Kao, 2012; Y.-X. Zhang et al.,

2002). However due to limitations in available experimental systems, it has been difficult to examine these concepts outside of theoretical realms. Here we sought to characterize some unexplored properties of our previously developed genderless strain, capable of continuous bi-directional conjugation. We examined the use of additional integrated oriTs to reduce bias in genome transfer and the effects of recombination in an ALE setting (including the genomic transfer efficiency, coverage, and frequency in our "genderless" system), and also reductions in Muller's ratchet through the removal of deleterious mutations.

Our results showed that the additional oriTs indeed function in our recombinant-proficient strains, and their presence alters the frequency at which genetic material distal to the native F-oriT is transferred. We determined that the extra oriTs increase the frequency of transfer of specific genetic markers as the distance between those markers and the original F-oriT increases, by acting as alternate initiation site of DNA transfer. This data implies that by increasing the number of oriTs in the genome, the functional genetic coverage of a recombination-proficient strain in an ALE experiment may be expanded, justifying this approach as a way to enhance genome coverage during horizontal gene transfer. Interestingly, we observed an unexpected lower frequency of transfer for the hisB::KanR marker C (Figure 6), which may be related with the histidine auxotrophy it confers.

We further assessed the relative use of each oriT and found that a strain with the 2 additional oriTs co-transferred distant genetic markers much more frequently than the strain with only the F-oriT. The ability of the oriT to function in *trans* is supported by

previous studies characterizing the oriT region for plasmid transfer, where the oriT was placed on a plasmid separate from a chromosomally encoded F factor (Fu et al., 1991), and prior use of conjugation in library construction (Typas et al., 2008). However, we were unable to determine whether there was significant preference by the *trans* acting F factor mediated machinery toward individual oriTs. This may be due to effects of nearby genetic features on the relative frequencies of DNA initiation at each oriT site. Therefore, due to the complexity of the three possible initiation points and unknown transfer lengths and frequencies, deducing a more detailed model of the additional oriT functionality from our data is beyond the scope of this work. Additional study into the activity of the oriTs could be fruitful in determining optimum locations for enhancing the genetic coverage of our genderless strain.

To determine if our "genderless" strain can effectively recombine beneficial mutations and remove deleterious mutations over a prolonged ALE experiment, we evolved our HFR-SFX-2xoriT strain and its asexual counterpart in an environment more susceptible to the negative impacts of Muller's ratchet. A small transfer size was used to increase genetic drift, and increased mutation rate was used to increase the frequency of deleterious/neutral mutations (Wahl & Zhu, 2015). This allowed us to exaggerate the differences in mutational accumulation between sexual and asexual strains within an experimentally feasible time scale. Previous reports in the literature indicate that small effective population sizes and high mutation rates are much more sensitive to Muller's ratchet (Gordo & Charlesworth, 2000). Therefore we used a small effective population size of (calculated with the harmonic mean (Wahl & Gerrish, 2001)) ~120 and a mutator

strain. However, we failed to observe mutational meltdown in any of our populations over the course of ~850 generations; though a similar mutational meltdown experiment in yeast resulted in only one meltdown in a longer timeframe (Zeyl et al., 2001). However, we did find that *in situ* recombination during ALE likely reduced the genetic load in the evolving population, as the evolved sexual populations accumulated fewer detectable mutations in our auxotrophic assay, relative to the asexual populations. Presumably, recombination reduces Muller's ratchet by allowing deleterious or neutral mutations to be repaired, and reduces the frequency of neutral or deleterious hitchhiker mutations linked to beneficial genotypes by allowing genetic exchange with a wild-type allele. Our results lend veracity to the theoretical work that sexual recombination can reduce the effects of Muller's ratchet. We also determined that mating between auxotrophic isolates from independently evolved sexual populations were also able to effectively repair the auxotrophies. Notably, this implies our recombination-proficient strain is capable of recombining alleles between different genotypes and can potentially be used to reduce clonal interference, providing further evidence for the benefit of sexual recombination theorized by the Fisher Muller model.

To expand our understanding of how the frequency of two initial donor genotypes impacts the frequency of desired recombinant formation, we experimentally measured the fraction of desired recombinants in the population after 24 hours of batch growth using varying ratios of starting donor genotypes. We utilized single marker "genderless" strains to constrain the desired recombination being quantified. Additionally, we modeled the impact of initial donor ratio on successful recombinant

formation using a mass action kinetics model to simulate cell growth and mating during batch culture. The model was able to recapitulate the experimental data and more accurately depict the decline in recombinant fraction in the final population as a function of relative frequency of each marker, consistent with prior observations that the frequency of recombination was proportional to the donor and recipient frequencies in the population (Andrup, 1998). Moreover, our data indicates the probability of generating a desired recombinant to be relatively high when one genotype is present at a relatively low frequency in the population. For example, our results suggested that a mutation present in ~1% of the initial population of a typical serial batch culture will transfer the mutation to 1 in 1000 cells that do not initially contain the mutation within 24 hours of growth. This further implies that even when one particular genotype dominates the population, the likelihood of generating a hybrid with a mutation from a smaller sub-population is relatively high. The converse (that of mutational repair) is supported by the reduced mutational load observed in our evolved sexual populations, as hitchhiking or drift enriched deleterious and neutral mutations can potentially be repaired via recombination.

The kinetic model was also able to reproduce the rate of recombinant formation during batch culture. The modeling data showed a rapid increase in the frequency of desired recombinant during the initial stages of cell growth. This result supports the theory that the fraction of recombinants during cell growth is highly contingent on "founders" or recombinants that are formed towards the beginning of the exponential phase and is consistent with what is to be expected when the growth rate is much greater

than the rate of recombination. In other words, the initial rapid increase in recombinant frequency is dominated by replication of recombinants rather than by additional successful conjugation and recombination events. As the cells approach late exponential and early stationary phase, we experimentally observed a rapid drop in conjugation rate. This expected drop in conjugation frequency was also built into the model. However, despite the decrease in conjugation rate, the model predicts that the fraction of desired recombinants continues to increase, and that this increase is dominated by additional mating events rather than clonal expansion of the recombinant genotype. The model-predictions of recombinant fraction versus time were validated experimentally (Figure 10). When modulating additional parameters of our system, such as the starting cell concentration and the rarity of two mutant genotypes of interest, we found that the model was predictive. These results indicate that despite the lower conjugation and recombination rate per cell at higher cell densities, higher initial cell densities yield higher numbers of recombinants; similarly, lower initial cell densities exhibit the opposite effect. Furthermore, the model lends insight into the mating frequency of rare mutations. We found that without a selective advantage, two rare mutants may need to be present in ~10% of the population to generate recombinants at a ~0.1% frequency in the final population. We hope that despite the simplicity of the constructed kinetic model, it can be utilized to tune the evolutionary parameters such as relative frequency of different genotypes, transfer size, number of generations per transfer, and transfer time to be utilized with our genderless strain to achieve the desired amount of recombination.

Our study provided evidence that *in situ* recombination during ALE experiments can offer several advantages toward the use of this approach for strain development. We demonstrated that the use of the recombination-proficient strain can successfully repair or combine various genotypes to reduce Muller's ratchet and increase genetic diversity as postulated by the Fisher-Muller model. We also found sexual recombination to be an effective route for reducing some of the negative impacts of increasing mutation rate in an evolving population. There has been ample evidence that mutator strains can significantly expedite the rates of adaptation in ALE experiments by increasing the availability of beneficial mutations. However, increased mutation rate often leads to increased frequency of hitchhiking neutral and deleterious mutations that can reduce the overall fitness of the clone; and the large number of mutations present can make downstream molecular analysis laborious. By introducing recombination in a mutator background, some of these concerns may be partially alleviated. In this work we successfully deduced a more detailed understanding of the F conjugation system for sexual recombination in a genderless strain of *E. coli*, and experimentally demonstrated the potential benefits of recombination on ALE.

ELUCIDATING THE INFLUENCE OF HORIZONTAL GENE TRANSFER AND MUTATION RATE ON ADAPTIVE LABORATORY EVOLUTION IN *ESCHERICHIA COLI*

Summary

Dependent on the fitness landscape, sexual recombination and mutation rate are theorized to play different roles in adaptive evolution; however, direct experimental support is limited. Here we examined how these factors impact the rate of adaptation by taking advantage of a genderless strain of *E. coli* capable of continuous *in situ* sexual recombination. We chose antibiotic challenges as differential adaptive landscapes, one representative of a more rugged landscape with multiple possible determinants, which increase tolerance, the other with a limited fitness landscape. The results showed that the populations with increased mutation rate, and capable of sexual recombination, outperformed all the other populations. Further characterization of two sexual and two asexual populations with increased mutation rate revealed increased maintenance of beneficial mutations in the sexual versus asexual populations, demonstrating capability of the genderless strain to alleviated clonal interference. Furthermore, the molecular signature of a mating event was experimentally identified within the sexual population combining two beneficial mutations thereby alleviating clonal interference. Additional evidence is also presented suggesting effects of stochasticity underlying the results.

Introduction

The use of adaptive laboratory evolution for phenotype development relies on the principles of Darwinian evolution, wherein individuals with mutations conveying a fitness benefit can be selected for, and enriched. As an asexual bacterial population evolves and accumulates mutations in a given environment, mutations imparting beneficial phenotypes expand as a subpopulation; the introduction of sexual recombination (Colegrave, 2002; Cooper, 2007; Gray & Goddard, 2012; Winkler & Kao, 2012; Zeyl & Bell, 1997) and increased mutation rate (Chao & Cox, 1983; Gentile, Yu, Serrano, Gerrish, & Sniegowski, 2011; Notley-McRobb, Seeto, & Ferenci, 2002; Raynes, Gazzara, & Sniegowski, 2011; Tröbner & Piechocki, 1985) have both been shown to facilitate swifter adaptation in certain fitness landscapes. However, how mutation rate and sexual recombination impacts the evolutionary trajectory along the fitness landscape of a given environment, is largely unexplored experimentally. In addition, the impact of mutations and their interactions, epistasis, on a cell's fitness outside a few well-characterized examples in specific environments, is limited (Barrick & Lenski, 2013; Deatherage, Kepner, Bennett, Lenski, & Barrick, 2017; Toprak et al., 2011), review (Mackay, 2013). Existing studies on microbial evolution has yielded significant insights into the dynamics of evolving populations. Because the availability, frequency, and strength of beneficial and deleterious mutations varies depending on the environment, adaptive laboratory evolution in different environments can yield different intra-population dynamics. There is strong experimental evidence to support the hypothesis that clonal interference shapes population structure during microbial adaptive

evolution, wherein many beneficial mutations co-arise and compete as subpopulations (see Figure 14-2) (Kao & Sherlock, 2008; Miralles, Gerrish, Moya, & Elena, 1999). The presence of horizontal gene transfer (HGT) is theorized to have two advantages for an evolving population: reducing clonal interference (the competition between beneficial mutations in an evolving asexual population) thereby speeding evolution (see Figure 14-4), and to break apart hitchhiking (deleterious or neutral) mutations from strong beneficial mutations. . Additionally, an increased mutation rate is expected expedite the rate of fitness improvement by increasing the availability of beneficial mutations (see Figure 14-3). Recent work has approached the relationships between HGT and mutation rate (Cooper, 2007) and further elucidated the impact of HGT on the molecular signatures of evolution in other microbes (McDonald et al., 2016). However, given the rarity of HGT in simpler and faster growing organisms, the advantages of HGT, especially over a range of mutation rates, is significantly less clear (Cooper, 2007).

Here we aim to determine the influences of both sexual recombination (horizontal gene transfer) and mutation rate on evolving populations of *E. coli* in well studied fitness landscapes. Previously, we have harnessed a "genderless" strain of *E. coli*, capable of horizontal gene transfer by harnessing F-plasmid machinery, and demonstrated that *in situ* horizontal gene transfer can increase the rate of adaptation during adaptive laboratory evolution (ALE) experiments in complex adaptive landscapes (Winkler & Kao, 2012). In this work, we introduced an inducible mutator system into the genderless strain. The resulting strain, capable of HGT and with a modular mutation rate, was evolved, along with an asexual counterpart, in several different environments.

Each of these backgrounds has been selected to represent different expected fitness landscapes allowing us to determine when sexual recombination and mutation rate provide an advantage to evolving populations of cells.

Two antibiotics were chosen as challenges for evolution with glycerol as the carbon source. Previous work on *E. coli* adaptation to chloramphenicol (CM) provides qualitative evidence of a smooth adaptation rate indicative of many smaller beneficial mutations (Toprak et al., 2011), we have previously demonstrated sexual strains have an advantage in more rapidly accumulating resistance when grown in M9 medium supplemented with CM. Therefore, the adaptive landscape for CM resistance is likely complex, and adaptation to CM is expected to significantly benefit from HGT and slightly benefit from increased mutation rate. Trimethoprim (TM) targets the DHFR protein responsible for folic acid synthesis (Huovinen, 1987). Previous work has identified TM tolerance occurs primarily through large fitness sweeps from sequential mutations in *folA* (Palmer et al., 2015). Not surprisingly, in previous work the use of the genderless strain had no benefit to a population of *E. coli* subjected to TM challenge on M9 (Winkler & Kao, 2012). Thus, the TM adaptive landscape is less complex and only increased mutation rate is expected to enhance the rate of adaptation to TM. The third evolution was performed without antibiotic challenge, solely for improved growth rate on glycerol. The Palsson lab has identified consistent evolutionary trajectories for more rapid growth on glycerol, wherein a few larger leaps in relative fitness of ~30-80% were reproducibly observed from mutations in *glpK* and *rpoB/C* (Cheng et al., 2014). Mutations in *glpK*, which encodes for glycerol kinase, have been linked to altering three

primary functions leading to better growth: auto-regulation, catabolite repression and enzyme activity (Applebee et al., 2008; 2011; Cheng et al., 2014). Mutations observed in *rpoB/C*, likely lead to complex global regulatory changes (Cheng et al., 2014). Correspondingly, as *E. coli* evolve toward faster growth on glycerol minimal media, we expect a few large jumps in fitness, populations will most benefit from increased mutation rate; HGT should provide little benefit when there are few, very strong, beneficial determinants. Additionally, we expect all of the evolved populations will settle on a similar final fitness after both strong mutations fix.

From the evolution experiments in each fitness landscape, we found HGT synergizes with increasing mutation rate together to speed evolution in some environments tested. We also observed strong evidence of an *in situ* recombination event that alleviated clonal interference and generated a superior genotype in a sexual *E. coli*.

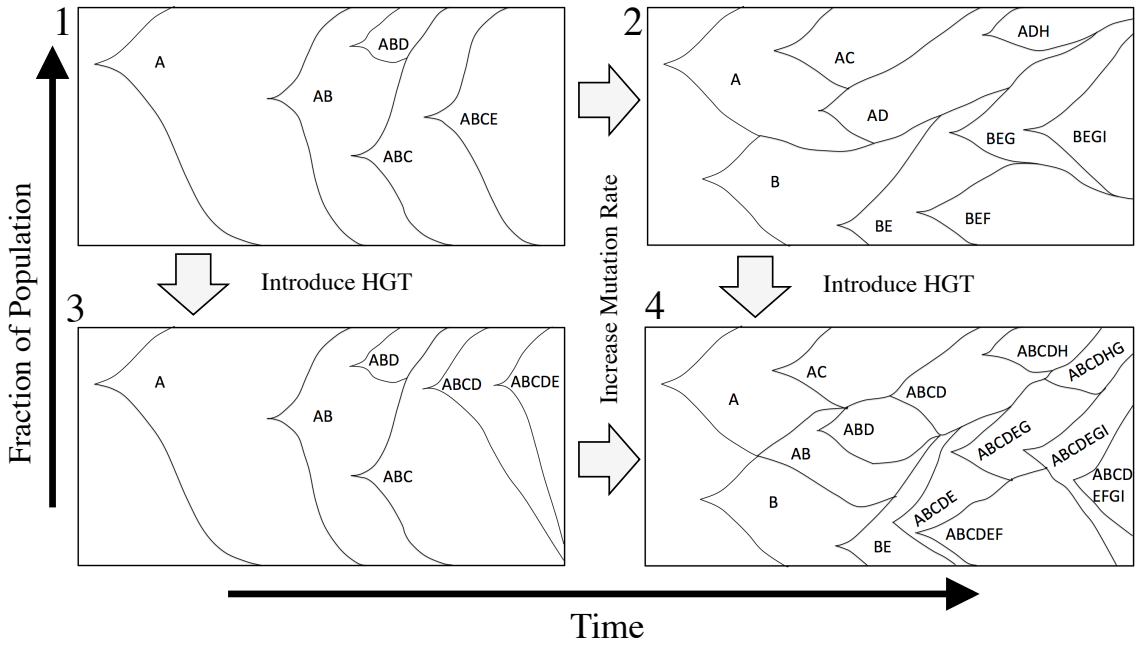


Figure 14: Sketch of evolutions in different environments. 1) Mutationally limited fitness landscape, most mutations fix in a sweep, very little competition. 2) Increased mutation rate over (1), more competition and faster evolution observed, but some mutations are lost. 3) Adding HGT to (1), no strong influence on evolution observed. 4) Both HGT and increase in mutation rate from (1), much more rapid evolution and less loss of information.

Materials and Methods

Strain Construction

The *dam* gene was inserted behind the arabinose promoter of PLA2 (Haldimann & Wanner, 2001) and integrated into the chromosome at the λ phage attachment site of the previously constructed HFR-2xoriT-SFX (genderless) and BW25113 2xoriT strains (Winkler & Kao, 2012) to generate the conditional mutators, HFR-2xoriT-SFX-P_{bad}*dam* (sexual) "genderless" and the BW25113 2xoriT-P_{bad}*dam* (asexual), strains. Both strains are ara-, Δ(araD-araB)567. The mutation rates of each strain were determined by standard fluctuation tests (Hamon & Ycart, 2012; Rosche & Foster, 2000) with

0.00005% (w/v) arabinose induction. Each test was performed on glycerol minimal media (G9) which is identical to M9 minimal media except glucose is replaced with 5% (v/v) glycerol as the carbon source and supplemented with 50 mg/L tryptophan.

Glycerol Evolution

Six single colonies for each genotype (genderless and asexual) were grown up in LB media overnight. An appropriate dilution from each culture was used to inoculate ~10^7 cells into two test tubes, one test tube containing 5 mL of glycerol minimal media (G9) the other with 5 mL of G9 media with 0.00005% (w/v) arabinose, grown at 37ºC and 275 rpm. The cultures were then propagated into fresh, pre-warmed media when the OD_{600} value approached approximately 0.5 (~7 generations) to maintain the cells in log phase growth. For each serial transfer, an initial OD_{600} of ~0.004 was used. Samples collected every other serial transfer were cryo-preserved at -80ºC in 15% glycerol.

Chloramphenicol (CM) and Trimethoprim (TM) Evolutions

The antibiotic challenged evolutions were performed similarly as in the glycerol evolution with the following exceptions. After the first 4 serial transfers, the cultures were propagated with antibiotic challenge (starting concentration 1.33 μg/mL CM or 0.32 μg/mL TM). If a culture exhibited growth rate greater than 0.583 doubles/hour (7 generations in 12 hours) the antibiotic concentration was increased by 25%, otherwise the culture was transferred to fresh media with the same antibiotic concentration. If a culture exhibited improvements in antibiotic resistance for 3 consecutive transfers, the concentration was ramped up by 50% instead of 25% for all subsequent transfers until a transfer with no improvement was observed.

Exponential Fitting

Exponential rate of improvement and lag were estimated for the two antibiotic evolutions, using the ft function in MATLAB with parameters for ramp rate, a, and lag time, b, in $C_{ab}=C_{abo}\, e^{(a(x+b))}$.

Sequencing

Population samples were grown up from 20 µl frozen stock on LB media; isolates were selected from colonies of the population streaked onto LB plates then grown up on LB. Genomic DNA was extraction from either single isolates or population samples using ZR Fungal/Bacterial gDNA miniPrep (Zymo Research). NGS library preparations and sequencing were performed by the Texas A&M Institute for Genome Sciences and Society core facility at Texas A&M University using 75 bp single end read on the Illumina NextSeq 500. 500 million reads passed quality control with an average coverage for each population sample of 500 reads/bp. The sequencing data was aligned to ecoli BW25113 reference (Grenier, Matteau, Baby, & Rodrigue, 2014) with breseq (Deatherage & Barrick, 2014).

Table 6: Primers utilized in this section.

Name	Sequence 5'->3'
P1	CCG AAG TAT ACG GTC AGA CTA ACA TTG CCG
P2	TTC CGA AGT ATA CGG TCA GAC TAA CAT TGC CA
P3	CTC GCC AGT GTT CAT CAG CAT AAA GCA G
P4	CCAG ACA ATG GCG TTA TAG ATA GGC
P5	GCC AGA CAA TGG CGT TAT AGA TAG GA
P6	CTC CAC GCT GGT AGA AGT GCT G
P7	TAC GCG TGC CGC CTG TGC
P8	GAA TAC GCG TGC CGC CTG TGT
P9	CCG ATT ACA CCA ACG CCT CTC GTA C
P10	CCC CAT ACA AAG CGG TAC TGA AAA AAC T
P11	CCC CAT ACA AAG CGG TAC TGA AAA AAC C
P12	CAC GGC TGA GCT TTT CGC AC
P13	TTT GCC AGC GAA GGG ATC TTC C
P14	TTT GCC AGC GAA GGG ATC TTC T
P15	GTT GAT GAA GCT TGA GCC GTA GG
P16	GGT AAA CAG GGT CGT TTC CGT CTG AA
P17	GGT AAA CAG GGT CGT TTC CGT CTG AG
P18	GCG GAT AAC TTC GTC CAG GAT ATC C
P19	CGTAAAAACCCGCTTCGGCG
P20	ACATCCCGCAACCTGCGATT
P21	CCTCGCTGGGTGTGGAAGTC
P22	AGCAGGCGGGTGAAACAGTC
P23	CAT CGC TTC CAG CAC GTC AC
P24	GAT TGG TCT ACT GAT TGC GGT CAT TG
P25	GCGAAGATTGTGGCTCCACCCAGCATATACTGCTGCTTGACGAATTTTATATC AAA GGG AAA ACT GTC CAT AT
P26	CGGATAAGCCTCGCTTTCCGGCACGTTCATCACGAAAAATATTGCTGTAATGT GAC GGA AGA TCA CTT CG
P27	CAACGCATAGTTCACTTCGCCAGTCGGGCCGCAGGCGATGGTGGTCAGCAATC AAA GGG AAA ACT GTC CAT AT
P28	TTCAGAACAAAAAGCTTCGCTGTAATATGACTACGGGACAATTAAACATGTGT GAC GGA AGA TCA CTT CG
P29	GGCAAGGATTCGATACTATTCCTGTGTAACTTTCTTAAGGAACGAGAATGATC AAA GGG AAA ACT GTC CAT AT
P30	TGGCGAAACCCACCTTAAGGTGGGTTTTGTTATTTTGAGGGCTGAGGAAGTGT GAC GGA AGA TCA CTT CG
P31	CAGTAGAATTCATG AAG AAA AAT CGC GCT TTT TTG
P32	CAC TTG AAT TCC ATC CGC TTC TCC TTG AGA ATT A
P33	TTGTCGGTGAACGCTCTCCT
P34	CACATTGATTATTTGCACGG
P35	CGTTCATCTTTCCCTGGT
P36	AGGATGCGTCATCGCCATTA
P37	GGCATCACGGCAATATAC
P38	TCTGGTCTGGTAGCAATG
P39	ACTTAACGGCTGACATGG
P40	ACGAGTATCGAGATGGCA

Allele Frequency Tracking

Genomic DNA was extracted as above. qRT-PCR was performed on the genomic DNA using the DyNAmo HS SYBR Green qPCR kit following the manufacturer's instructions, using both mutant and wild type primers for each mutation with three technical replicates each (See

Table 6) (Little, 2001; Mesrian Tanha, Mojtabavi Naeini, Rahgozar, Rasa, & Vallian, 2015). The allele specific primers were validated by constructing a standard curve with different percentages of mutant and wild type gDNA, see Figure 15.

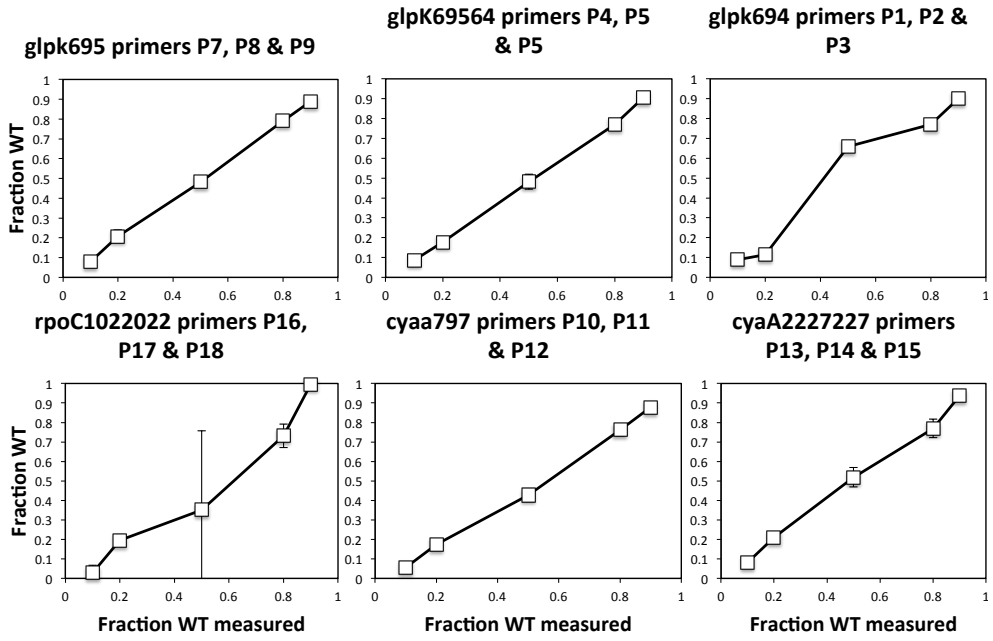


Figure 15: Calibration curve for each pair of allele specific primers used in this study.

Genotype Tracking

Frozen stock of GIG1Time 3 population was diluted and plated on LB. Allele specific PCR was performed on individual isolates to determine the presence of mutations *cyaA2227, cyaA797 glpK695*, and *glpK288*. Colonies were selected and resuspended in 50 µl of sterile water. The colony suspension was tested for each mutation using both wild type and mutant allele specific primers. Mutations were

determined from the resultant band distribution. One isolate of each identified genotype was further verified with Sanger sequencing.

Population Fitnesses

96 randomly chosen colonies of the population of interest were inoculated into G9 media for growth for 24 hours. 2 µl of the culture was transferred to a 96-well microtiter plate with 98 µl G9 media. Plate reader with shaking and incubation capabilities (TECAN Infinite M200) was used to track the growth in each well at 37°C with 270 rpm orbital shaking. To eliminate edge effects, we excluded the edges of the 96-well microtiter plates.

Strain Reconstruction

We reconstructed several mutations identified in GIG1 Time=3 evolution in wild-type strains using the procedure outlined by Datsenko and Wanner and the *cat-sacB* selection-counterselection cassette (Datsenko & Wanner, 2000; Thomason, Sawitzke, Li, Costantino, & Court, 2014). The selection was performed on LB plates with 12 µg/mL CM and the counter selection was performed on plates LB plates with no NaCl and 6% (w/v) sucrose. All DNA was amplified with primers from

Table 6. All mutations constructed in the BW25113 background were verified with Sanger sequencing.

Construct Fitnesses

Construct fitnesses were measured using the same method as the population isolates.

Selection Experiment with Genderless Mutant Constructs

Single mutants of the genderless strain was cultured overnight in LB from individual colonies. 5 µl of the overnight culture was normalized and used to inoculate fresh cultures in 5 ml of G9 in test tubes and cultured for 5 hours at 37ºC and 275 rpm. Each individual culture was normalized to OD600 ~1 and pooled. 19 µl of the pooled culture was inoculated into fresh G9 according to the protocol for the glycerol evolution outlined above for 6 serial transfers.

Results

Constructing an inducible mutator phenotype in the asexual and genderless strains

Conditional mutator versions of the previously developed genderless strain and asexual control were constructed to allow modulation of mutation rate. The genderless strain contains a chromosomally integrated F-plasmid, with the surface exclusion genes *traST* knocked out. This allows effective *in situ* HGT; for detailed characterization see Winkler *et al* and (Peabody et al., 2016). The DNA adenine methylase (*dam*) gene was

cloned behind the arabinose inducible promoter and integrated into the *E. coli* chromosome using the CRIM plasmid system (Haldimann & Wanner, 2001) for easy excision. Overexpression of *dam* has been shown to increase mutation rate by reducing the time a cell has to harness methyl-directed mismatch repair (Herman & Modrich, 1981). It should be noted this method of increasing the mutations rate is orthogonal to the frequency of recombination (Feinstein & Low, 1986; Lanzov et al., 2003). When induced with 0.0005% (w/v) arabinose, the mutation rate is increased in both the genderless and asexual control strains on glycerol minimal media by 22.69 (Upper CI and 32.64 Lower CI 13.98) and 17.73 (Upper CI and 26.77 Lower CI 9.94), respectively. Additionally, we confirmed the genderless strain maintained the ability to mate when grown on glycerol minimal media (data not shown).

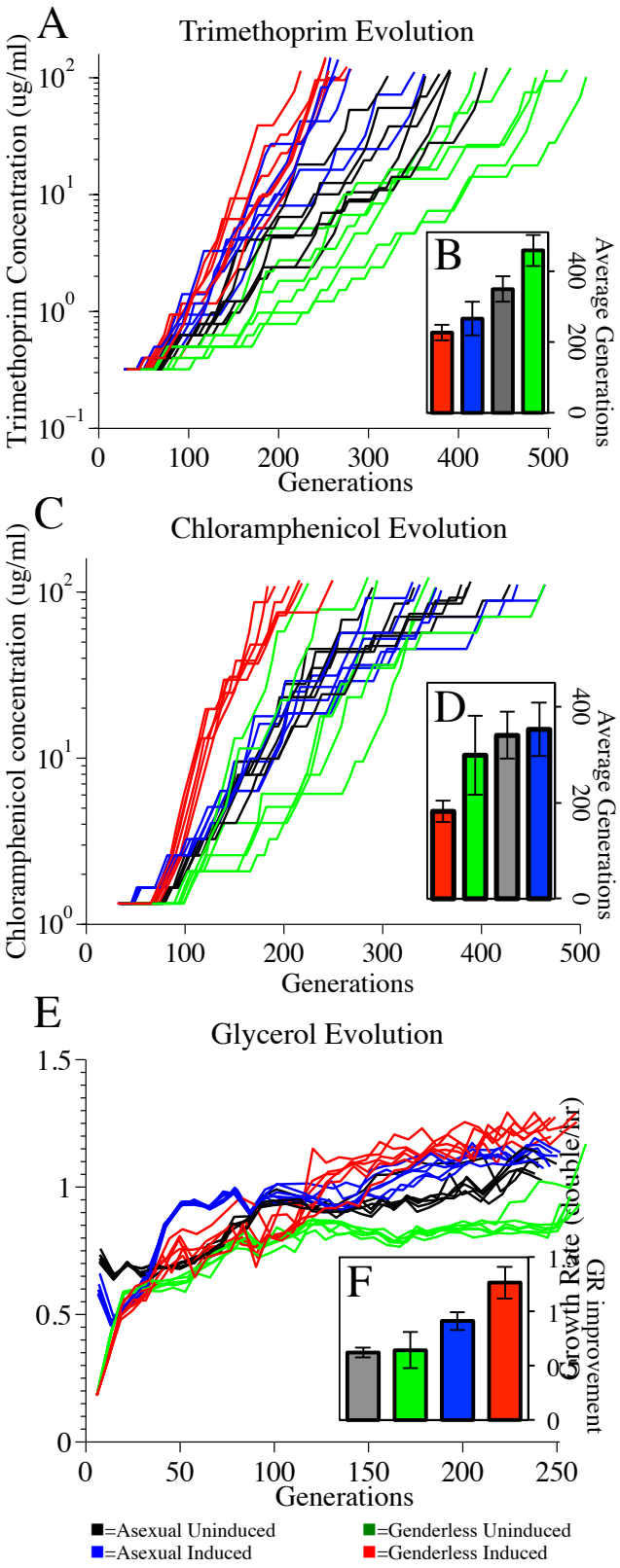

A
Trimethoprim Evolution
Trimethoprim Concentration (ug/ml)
10^2
10^1
10^0
10^{-1}
0
100
200
300
400
500
Generations
B
Average Generations
400
200
0
C
Chloramphenicol Evolution
Chloramphenicol concentration (ug/ml)
10^2
10^1
10^0
0
100
200
300
400
500
Generations
D
Average Generations
400
200
0
E
Glycerol Evolution
1.5
1
0.5
0
0
50
100
150
200
250
Generations
Growth Rate (doubles/hr)
F
GR improvement
1
0
■=Asexual Uninduced
■=Asexual Induced
■=Genderless Uninduced
■=Genderless Induced

Figure 16: A visualization of each evolution with glycerol minimal media. Black asexual populations, Blue asexual indicued populations, Green genderless ara- populations, Red genderless ara+ populations. A) Chloramphenicol tolerance versus generations for each population. B) Average number of transfers to reach CM tolerance 100 for each group of populations. C) Trimethoprim tolerance versus generations for each population. D) Average number of transfers to reach TM tolerance 100 for each group of populations. E) Growth rate versus generations for each evolved population. F) Relative improvement of growth rate over transfer 3 average for each group of populations. All errors are STD.

Evolutions with Chloramphenicol and Trimethoprim challenge produce expected characteristics

We compared the strains with or without HGT each with or without induction of the mutator phenotype using the following four strains/conditions: genderless with arabinose induction (genderless ara+), genderless without arabinose induction (genderless ara-), asexual with arabinose induction (asexual ara+), and asexual without induction (asexual ara-). In each evolution, all populations were maintained in exponential growth via serial batch transfer during mid-log phase; consequently, the population fitness is ascribed by growth rate during exponential phase with minimal influence of the length of growth lag and cell survival during stationary phase. An exponential ramping scheme for the antibiotic challenges was implemented to maintain the evolving populations in the presence of an antibiotic concentration consistent with the populations true antibiotic tolerance (Toprak et al., 2011; Winkler & Kao, 2012). For each strain/condition, 6 replicate populations were evolved in a glycerol minimal media supplemented with either chloramphenicol (CM) or trimethoprim (TM). Each population was first adapted to glycerol without antibiotic supplementation for 4 serial transfers.

Using a target CM tolerance of 100 μg/mL, in the evolutions a significantly more rapid improvement in tolerance was observed in the genderless ara+ populations relative to both the asexual ara+ or ara- populations (p-values <0.001) and the genderless ara- population (p-value<0.05), (see Figure 16). The genderless ara- populations displayed a similar rapid improvement in tolerance as to the genderless ara+ populations but was preceded by a prolonged periods of lag. The asexual ara+ and ara- populations performed similarly (see Figure 16).

We quantified the evolutionary data by fitting antibiotic tolerance versus generations to a two-term exponential, one fitted term for rate of exponential improvement "a" and the other for time lag before improvement "b", $C_{ab}=C_{abo}\, e^{(a(x+b))}$. The value of the fitted terms "a" and "b" for the genderless ara- populations are much higher than the asexual strains (p-value<0.05). The genderless ara+ populations have larger exponential terms (p-value<0.05), Figure 17. The results suggest that HGT facilitates a more swift increase in resistance, probably by combining compatible determinants of CM tolerance. The increased mutation rate in genderless ara+ populations likely provided an increased supply of mutations for generating beneficial recombinants, thereby reducing the lag before improvement in tolerance.

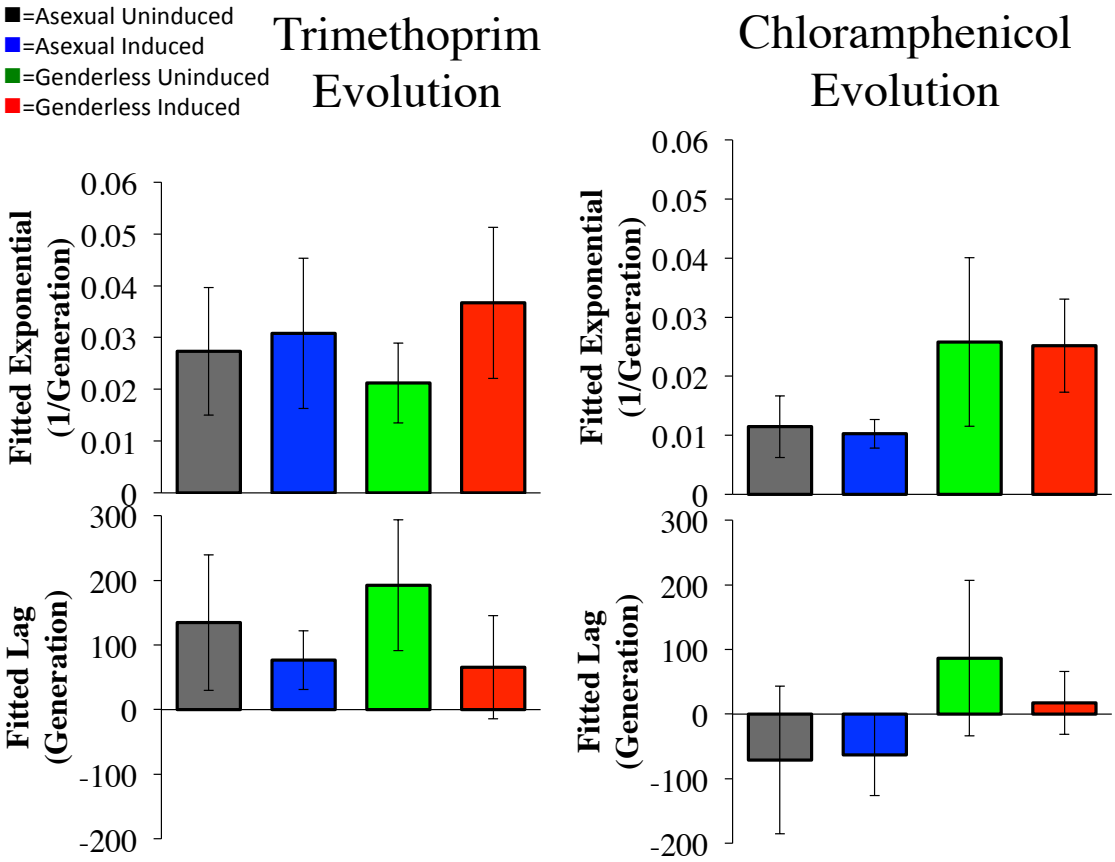


Figure 17: Comparison of terms fit from evolutionary data. Tolerance versus generations was fit to a two term exponential, one term for lag before improvement, the other for the exponential rate of improvement.

The four strains/conditions were evolved in TM with a target tolerance of 100 µg/mL (see Figure 16). The fitted "b" parameter of the ara+ populations exhibit more rapid tolerance development over their ara- counterparts (p-value <0.001). The sexual ara+ population has a slightly more rapid increase in fitness but it is not statistically significant. As expected, TM fitness landscape lends little advantage to HGT but an increased mutation rate is advantageous.

Sexual Evolution for faster Growth rate on Glycerol

E. coli is not well adapted to glycerol as a primary carbon source; therefore, during adaptive evolution to TM and CM, the populations may have also been adapting to growth on glycerol. Each strain/condition were also evolved for faster growth on glycerol to assess the impacts of HGT and mutation rate on rates of adaptation on this carbon source. Both the genderless ara- and genderless ara+ populations exhibited lower initial fitnesses on glycerol compared with their asexual counterparts, possibly due to a metabolic adaptation period caused by the metabolic burden of the conjugation machinery (Guzmán et al., 2015). It takes approximately 3 serial transfers for the genderless strain to physiologically adapt to the glycerol minimal media. This metabolic transition period is reproducibly observable if populations grown on glycerol media are transferred to rich media and then back to glycerol media (data not shown). Therefore, relative fitness was calculated using the specific growth rates after the third transfer as baseline. The ara+ populations reached fitness plateaus more rapidly than their ara- counterparts (see Figure 16). In the genderless populations, the genderless ara+ reached the first plateau in fitness increase at generation ~50, whereas the genderless ara- reached a similar fitness at generation 80. The genderless ara+ reached another plateau at generation ~130, but the genderless ara- never reached this fitness peak by the termination of the experiment. A similar trend was observed between the asexual populations, the asexual ara+ reached plateaus at generations 60 and 150, while the asexual ara- reached a similar plateau much later, at generations 90 and 225.

Prior studies have determined that mutations in *rpoB/C* and *glpK* are primarily responsible for *E. coli* adaptation on glycerol (Applebee et al., 2008; 2011; Cheng et al., 2014) Assuming the fitness landscape of *E. coli* on glycerol is simple, as in the case with trimethoprim, we would expect the benefit of HGT to be minimal. Thus, it was unexpected that the genderless ara+ populations had a higher final growth rate than the asexual ara+ (p-value $< 10^{-4}$). The genderless strain can potentially allow for the best combination of co-arising *rpoC* and *glpK* mutations to be generated, and could allow for weaker beneficial mutations to be preserved through mutational sweeps, avoiding clonal interference and leading to better adaptation. To assess these possibilities, we assayed the fitness diversity in the final populations; if the genderless populations preserved diversity we would expect the final populations to have a larger distribution of fitnesses relative to the asexual ara+ populations. The measured growth rate distributions correlate well with the average growth rates of the final evolved populations (see Figure 18).

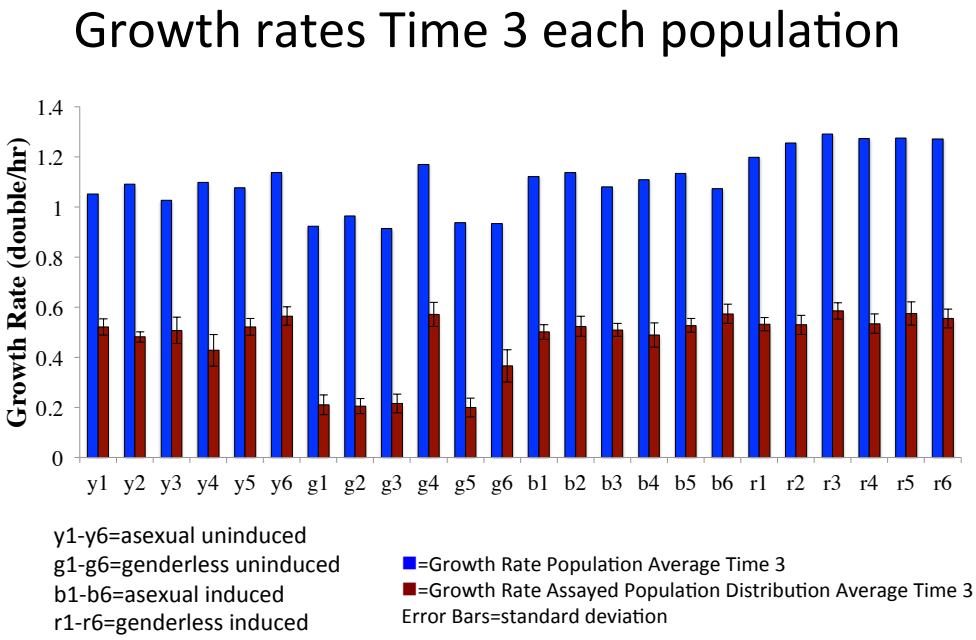


Figure 18: The measured growth rate distributions and measured population growth rates at Time 3.

However, there was no significant difference in the variances of the genderless and asexual ara+ populations (F-test >0.05). See Figure 19 for population distributions.

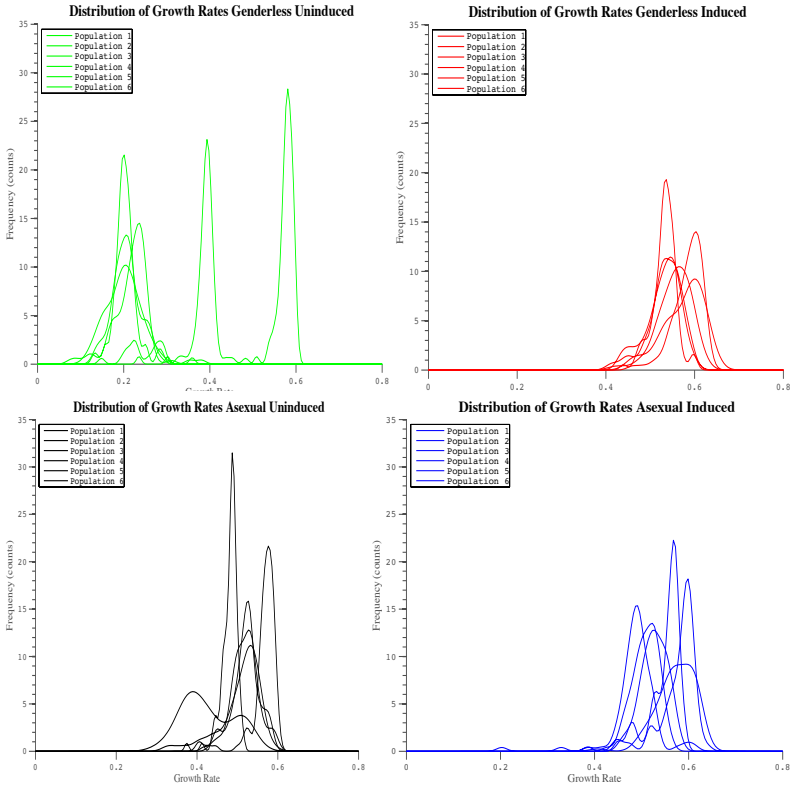


Figure 19: Growth rate distributions for assay of population distribution of growth rates.

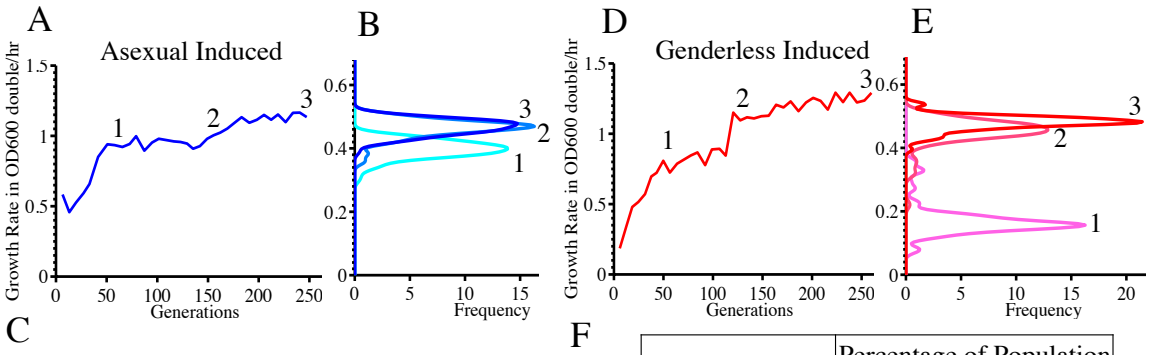
As fitness distributions do not definitively provide information regarding beneficial mutations in the population, we sequenced population samples at 3 time points from evolving populations genderless ara+, 1 and 2 (GIG1 and GIG2 respectively), and asexual ara+, 1 and 2 (AIG1 and AIG2 respectively). Each time point chosen corresponded to an increase in population fitness to a plateau mentioned previously and the final population. After removing extraneous mutations, each population contained 1-10 mutations that were detected at a statistically significant level, and in all sequenced populations (genderless and asexual), mutations in both *glpK*, and ropB/rpoC were observed (see Table 7).

Table 7: Curated list of mutation observed in this study.

Gene	Mutation	Location	Population
ygfA/traM	A→C	intergenic (-220/-194)	Genderless P2 TP1
traJ	.→T	coding (124/690 nt)	Genderless P2 TP1&2
mraZ	C→T	238	Genderless P1 TP2
lpxC/secM	G→T	intergenic (+91/-140)	Genderless P1 TP3
hrpB/mrcB	T→G	intergenic (+85/-111)	Genderless P2 TP3
cof/ybaO	T→.	intergenic (+34/-119)	Genderless P1 TP3
glsA	G→A	829	Genderless P1 TP2
miaB	G→A	1306	Genderless P2 TP2
ubiF/glnX	G→T	intergenic (+153/+1)	Genderless P1 TP3
treA	A→G	1372	Genderless P2 TP2
azoR1	-12 bp	coding (150-161/606 nt)	Genderless P1 TP2&3
curA	A→C	519	Genderless P1 TP1
rsxC	C→A	2012	Genderless P2 TP2
ccmB	G→A	149	Genderless P2 TP1
pdxK	G→A	315	Genderless P2 TP1
sseA/sseB	G→T	intergenic (+371/+447)	Asexual P2 TP2
gspB	C→T	202	Genderless P2 TP1&2
bcsQ	A→C	pseudogene (218/753 nt)	Asexual P1 TP1
yhjY	G→T	6	Asexual P1 TP2
cyaA	T→C	797	Genderless P1 TP2&3
cyaA	A→G	1378	Genderless P2 TP2
cyaA	C→T	2227	Genderless P1 TP3
glpK	A→G	1330	Asexual P2 TP1
glpK	C→T	695	Asexual P2 TP2, Genderless P1 TP2&3
glpK	C→T	694	Asexual P1 TP1, Genderless P1 TP2
glpK	C→T	692	Asexual P2 TP2
glpK	G→T	565	Asexual P2 TP1,2&3
glpK	C→A	288	Genderless P1 TP2&3
glpK	C→T	196	Genderless P2 TP2
glpK	G→A	164	Asexual P1 TP1, Genderless P2 TP2
rpoB	G→T	1330	Asexual P1 TP2
rpoB	C→T	1576	Asexual P2 TP1
rpoB	A→C	1903	Asexual P2 TP3
rpoB	+64 bp	coding (1927/4029 nt)	Asexual P2 TP2&3
rpoC	-18 bp	1401	Genderless P2 TP2
rpoC	A→G	1022	Genderless P1 TP1,2&3, Genderless P2 TP1&2
leuQ	.→G	noncoding (80/87 nt)	Genderless P1 TP1

This was not unexpected, as the Palsson group identified mutations in *glpK* in 47/50 independent ALE experiments (Applebee et al., 2011); the *glpk288* and *glpK692* mutations we identified in our evolved populations have been previously observed (Applebee et al., 2011). Interestingly, mutations in *cyaA* (adenylate cyclase) were observed only in the two genderless populations. Adenylate cyclase is responsible for production of cAMP and is crucial for alternative carbon source utilization. Mutations in

cyaA have not been previously reported in glycerol-evolved *E. coli*, but have been observed in lactate-evolved *E. coli* (Conrad et al., 2009). The remaining mutations identified had no obvious connection to growth rate on glycerol, and will not be analyzed in detail in this work. To connect the dynamics of the evolution to the sequencing data, at each sequenced time point we measured the fitness distributions of the populations, see Figure 20 for a representative asexual and genderless population.



C

	Percentage of Population		
Time	1	2	3
glpK 695 C→T	19.9	-	-
glpK 692 C→T	13.8	-	-
glpK 565 G→T	27.8	87.2	100.0
rpoC 1927 +64 bp	-	38.1	100.0

F

	Percentage of Population		
Time	1	2	3
rpoC 1022 A→G	12.0	83.0	100.0
cyaA 797 T→C	-	24.0	45.1
cyaA 2227 C→T	-	-	29.8
glpK 695 C→T	-	11.3	44.1
glpK 288 C→A	-	23.9	42.5
glpK 694 C→T	-	15.6	-

Figure 20: A vignette of 3 evolution metrics side by side. A) Population fitness of a single asexual induced population. Three time points are marked for reference as 1, 2, and 3 respectively B) Population fitness distribution at the three time points, 1, 2 and 3. C) Population level sequencing data for observed mutations at time points 1, 2, and 3. D) Population fitness of a single genderless induced population. Three time points are marked for reference as 1, 2, and 3 respectively E) Population fitness distribution at the three time points, 1, 2 and 3. F) Population level sequencing data for observed mutations at time points 1, 2, and 3.

In AIG1 the population average fitness exhibited a large increase between generation 60 (Time = 1) and generation 150 (Time = 2), with no significant further increase between Time = 2 and generation 250 (Time = 3), depicted in Figure 20. Population-level sequencing data from AIG1 revealed 3 different mutations in *glpK* at Time = 1. Prior to Time = 2, a mutant containing the *glpK565* mutation likely acquired a mutation in *rpoC*, which expanded as a subpopulation to reach 100% frequency. No additional mutations were detected in this population by the end of the evolution experiment. Therefore, it is not surprising that the population fitness was maintained

between Time = 2 and Time = 3, as the superior genotype (containing both *glpK565* and *rpoC1927*) outcompeted all other less fit mutations and swept the population. On the other hand, the genderless populations retained more heterogeneity, see data for population GIG1 as an example, Figure 20. Initially in population GIG1, an *rpoC1022* mutation started to expand, reaching ~12% frequency by generation 50 (Time = 1), and 100% (fixation) by generation 250 (Time = 3). At generation 130 (Time = 2), several additional mutations (one in *cyaA* and three in *glpK*) reached significant frequencies, drastically increasing the population fitness. Contrary to the asexual ara+ population, where a single pair of mutations fixed, in the genderless ara+ population, the population fitness increased and a diversity of mutations were maintained until the end the termination of the experiment.

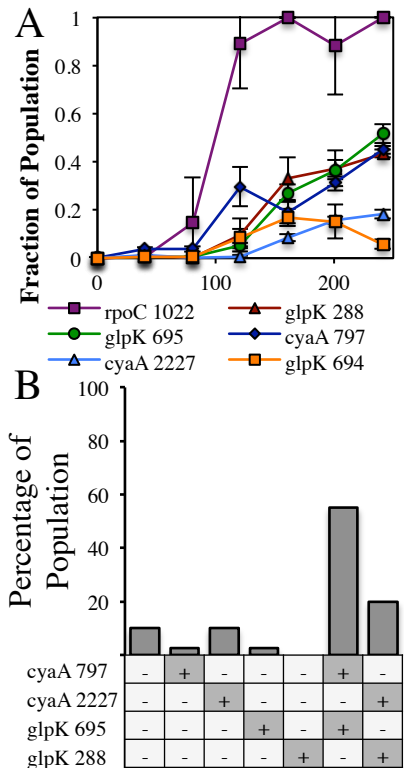


Figure 21: Evolution dynamics for the sample representative genderless induced population evolved for faster growth rate on glycerol. A) Allele specific PCR observed mutation frequencies in the evolution over time. B) Genotype frequencies for 4 assayed genes in the final population from 100 isolates tested with allele specific PCR.

Resolving the presence of an in situ HGT event

As the genderless population is expected to recombine mutations from independent clones in the population, we aimed to determine if recombination indeed occurred between different subpopulations. First, the 6 mutations identified in GIG1 were tracked by allele specific PCR (see Figure 21-A). The results confirmed the *rpoC1022* mutation fixes in the population by Time 3 and *glpK694* is largely lost by Time =3. The combined frequencies of the remaining 4 mutations, *glpK288* and *glpK695* and *cyaA2227* and *cyaA797* were confirmed to be well above 100%, indicating some of

these four mutations must coexist as pairs of mutations in a single genotype. To identify the different combinations that are present in the population, we randomly picked 96 isolates from Time = 3 population and used allele specific PCR to determine the genotypes for these 4 mutations in the colony isolates (see Figure 21-B). Most of the isolates contain either a single mutation (*glpK288*, *glpK694,* or *cyaA797*) or a pair of mutations (either *glpK695*+*cyaA797* or *glpK288*+*cyaA2227*). Notably, individual colonies with single mutations in either *glpK695* or *cyaA797* and isolates with both of these two mutations were identified; this data suggests that either a sexual recombination event occurred between the subpopulations with individual mutations in *glpK695* and *cyaA797*, to generate recombinant with both mutations, or the *glpK695* mutation spontaneously arose in a mutant with the *cyaA797* mutation or vice versa. Given the low probability of an identical mutation occurring spontaneously in more than one subpopulation, it is more probable that the *glpK695*+*cyaA797* mutant arose from a recombination event.

Ascertaining the fitness landscape

To determine the accessible fitness landscape of this particular population, we reconstructed each mutation and combination of mutated genes into the wild-type BW25113. The growth rates of each single, double, and triple mutant constructs were compared relative to the wild type on G9 media. As shown in Table 8, all of the single mutants exhibited a statistically significantly improved growth rate compared to the wild-type (p-value $<10^{-3}$; Student's t-test), showing that all mutations identified in *rpoC*,

glpK, and *cyaA* are beneficial, though the *glpK* mutations showed the highest fitness followed by *rpoC* then the *cyaA* mutations.

Table 8: P-values for all comparisons between single mutant constructs.

	Bw2513	BW rpoC1022	BW cyaA797	BW cyaA2227	BW glpK695	BW glpK288	BW glpK694
Bw2513	1	-	-	-	-	-	-
BW rpoC1022	2.15006E-07	1	-	-	-	-	-
BW cyaA797	0.010719811	0.000720132	1	-	-	-	-
BW cyaA2227	0.000199932	0.0445072	0.135234285	1	-	-	-
BW glpK695	9.25713E-19	4.75568E-08	4.45606E-13	1.85318E-10	1	-	-
BW glpK288	1.17319E-15	0.000234953	5.63269E-10	3.1E-07	2.13747E-06	1	-
BW glpK694	1.60895E-17	7.17077E-07	1.93653E-12	1.16125E-09	0.195440807	0.00210698	1

The double mutants exhibited greater growth rates than their single mutant counterparts (p-values $<10^{-3}$), suggesting reciprocal sign epistasis is not present; except for when *cyaA2227* was combined with *glpK694* and when *cyaA797* was combined with *glpK695*, where no additional increase in fitness were observed beyond that of the single *glpK* mutation Table 9.

Table 9: P-values for all comparisons between double/triple mutant constructs and their composite mutations.

Genotype	WT	rpoC	cyaA	glpK	rpoC+cyaA	rpoC+glpK	cyaA+glpK
BW rpoC1022 cyaA2227	4.49084E-10	7.23439E-05	1.11777E-06	-	-	-	-
BW rpoC1022 cyaA797	6.22704E-09	0.000178303	3.59762E-07	-	-	-	-
BW cyaA2227 glpK694	8.66131E-14	-	6.00979E-11	0.047253838	-	-	-
BW cyaA2227 glpK288	1.46903E-14	-	4.23446E-08	0.001215923	-	-	-
BW cyaA2227 glpK695	1.48636E-07	-	5.42056E-06	0.114946422	-	-	-
BW cyaA797 glpK694	1.70628E-10	-	7.70233E-06	0.452683223	-	-	-
BW cyaA797 glpK288	2.3915E-11	-	7.42402E-08	0.001735857	-	-	-
BW cyaA797 glpK695	1.94349E-16	-	1.11534E-09	0.026144699	-	-	-
BW rpoC1022 glpK694	8.45842E-19	6.61383E-14	-	2.42838E-09	-	-	-
BW rpoC1022 glpK288	2.65796E-21	9.23296E-17	-	7.63896E-18	-	-	-
BW rpoC1022 glpK695	2.04913E-18	3.89374E-14	-	3.83341E-09	-	-	-
BW rpoC1022 cyaA2227 glpK694	1.34146E-11	3.54112E-10	1.10332E-10	2.54147E-08	1.86205E-07	0.000383709	5.97253E-07
BW rpoC1022 cyaA2227 glpK288	9.00332E-11	2.05583E-09	8.03019E-10	2.34552E-08	9.9447E-07	0.015928754	1.16206E-06
BW rpoC1022 cyaA2227 glpK695	6.85661E-11	4.06061E-10	3.97674E-10	3.11442E-08	1.48151E-07	0.000566317	8.12214E-06
BW rpoC1022 cyaA797 glpK694	7.5229E-13	2.22787E-11	1.07335E-12	1.10678E-09	2.10036E-08	2.84496E-05	1.27758E-09
BW rpoC1022 cyaA797 glpK288	1.29348E-19	4.70226E-12	8.24372E-14	2.08273E-15	3.06857E-10	1.03902E-06	1.28928E-11
BW rpoC1022 cyaA797 glpK695	6.97941E-15	1.00223E-11	2.00332E-13	1.38691E-10	8.20924E-09	0.000190156	9.76296E-09

All triple mutants exhibited fitness values that are statistically significantly improved over their possible double mutant counterparts (p-values<0.02) see Figure 22.

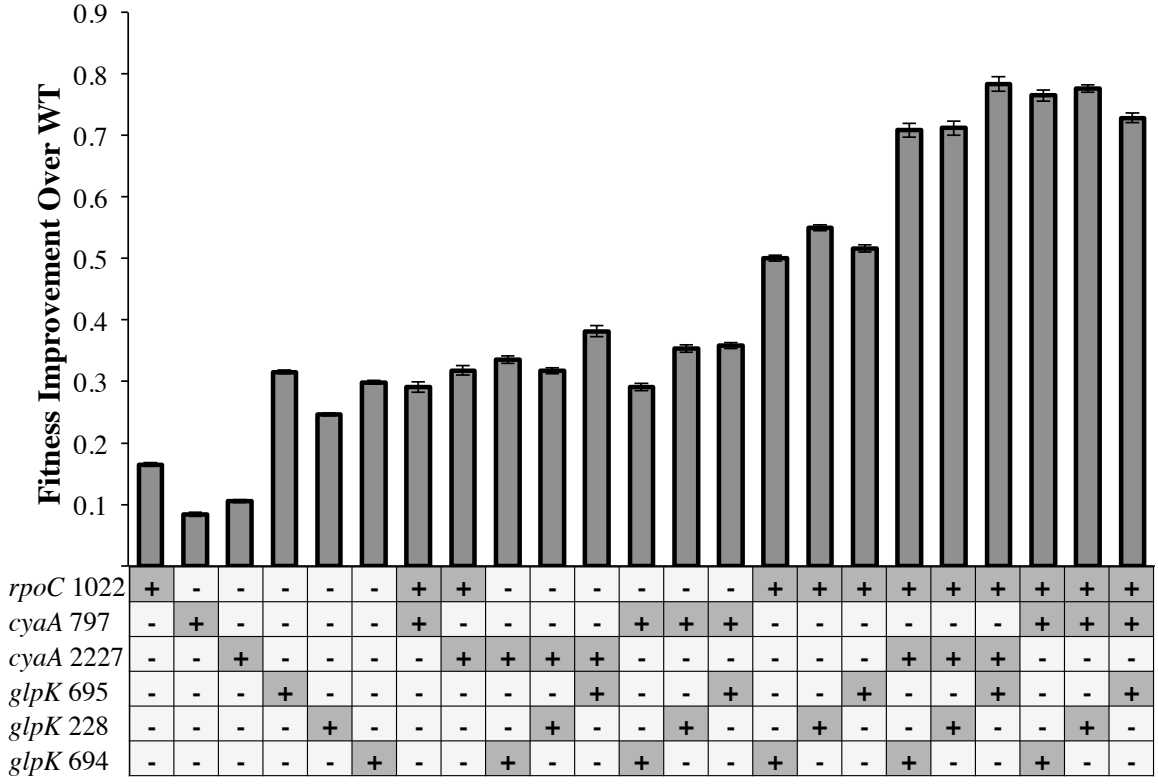


Figure 22: Relative fitness improvement of each constructed mutant over the wild type BW25113.

Epistatic interactions between different mutations were quantified by comparing the fitness of each double or triple mutants against the sum of its component individual mutations. Results showed that all combinations of mutations with an *rpoC1022* mutation yielded positive epistasis, whereas combinations without *rpoC1022* yielded variable epistasis (see Figure 23).

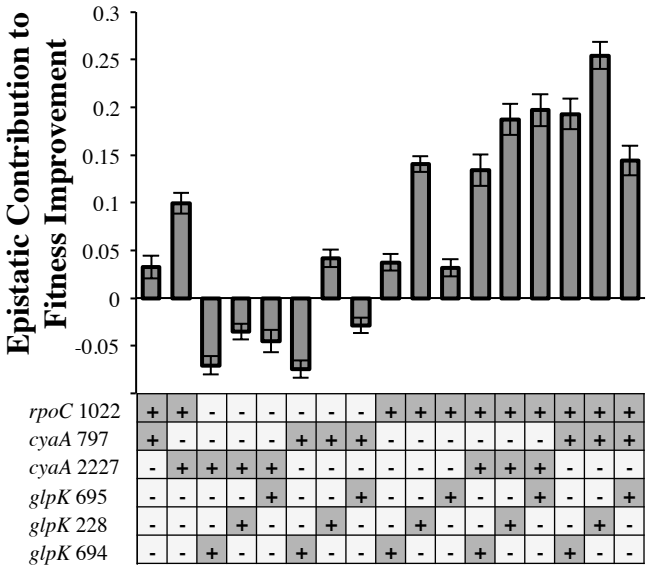


Figure 23: Epistatic contribution toward relative fitness improvement of each construct with multiple mutations relative to its composite single mutations.

Of special note, the *rpoC1022+glpk228* combination yielded strong positive epistasis. Likewise, all of the triple mutants, which all have the *rpoC1022* mutation, display strong positive epistasis. To confirm that the adaptive landscape for glycerol identified in this work is not specific to the BW25113 background, we also reconstructed these mutations in MG1655 as well. The results showed similar fitness trends as those observed in BW25113 (see Figure 24).

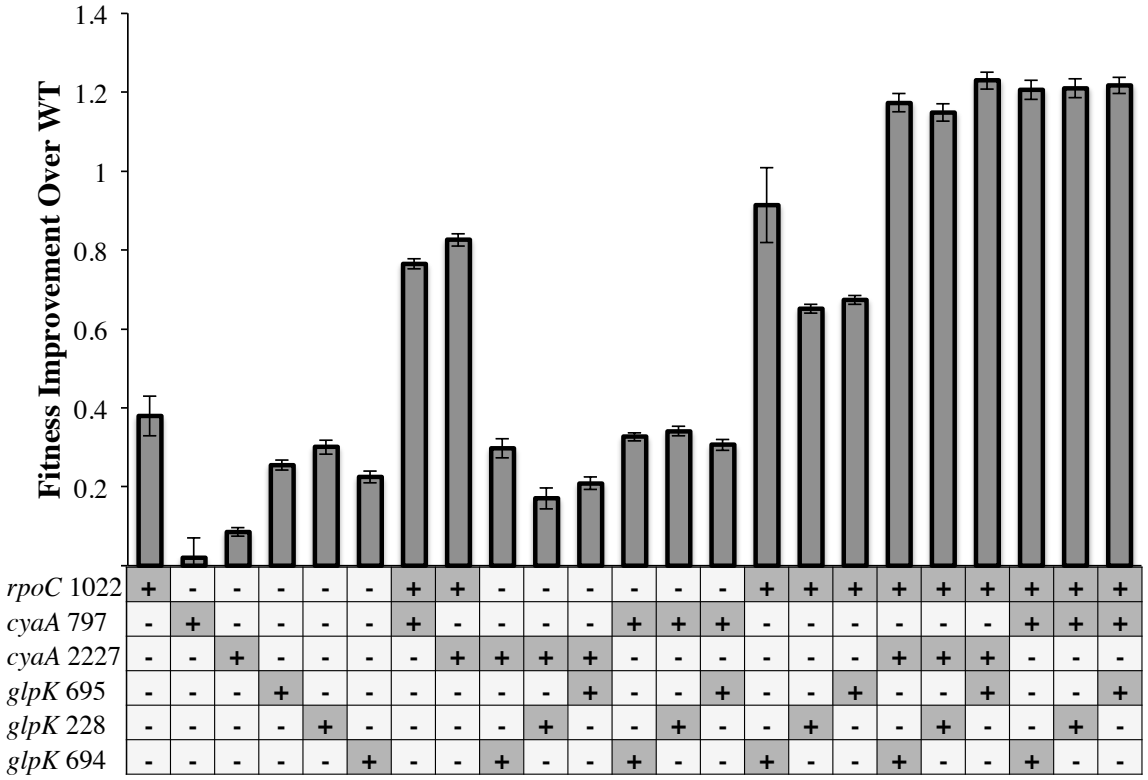


Figure 24: Relative fitness improvement of each constructed mutant in MG1655 over the wild type MG1655.

The combinations of three mutations we observed in the adaptive evolution, *rpoC1022* with either *glpK695+cyaA797* or *glpK228+cyaA2228*, represent just two of the peaks on the fitness landscape, but not necessarily the local maxima. To gain further insight into the potential benefit of HGT to simultaneously generate and select for beneficial combinations of mutations, we reconstructed the 6 individual mutations identified from the adaptive evolution in the genderless strain and used them to seed a short selection experiment on glycerol. After 8 transfers (55 generations of selection), each population maintained several mutations that reached moderate frequencies (though *rpoC* seems to be consistently maintained). The sum of the frequencies of all mutations

in each population was ~2, indicating the average number of mutations in each cell in our final populations was ~2, strongly suggesting that recombination occurred (see Table 10).

Table 10: Mutant frequency as fraction of population for 6 replicates initially composed of equal fractions of each mutant and evolved for ~55 generation in glycerol minimal media.

	Mutation Fraction of Population					
Replicate	1	2	3	4	5	6
rpoC1022	0.911±0.039	0.839±0.055	0.868±0.025	0.936±0.019	0.821±0.011	0.805±0.019
cyaA797	0.003±0001	0.002±0.001	0.001±0.001	0.001±0.001	0.007±0.002	0.003±0.001
cyaA2227	0.498±0.056	0.181±0.018	0.252±0.028	0.326±0.056	0.455±0.031	0.088±0.008
glpK695	0.007±0.001	0.019±0.006	0.015±0.004	0.01±0.005	0.155±0.056	0.018±0.006
glpK288	0.399±0.106	0.672±0.022	0.832±0.012	0.592±0.063	0.367±0.019	0.84±0.008
glpK694	0.426±0.055	0.26±0.073	0.109±0.034	0.495±0.097	0.504±0.06	0.151±0.025
Total	2.24±0.13	1.97±0.09	2.07±0.05	2.36±0.13	2.3±0.09	1.9±0.03

Discussion

The overall objective of this work was to determine the influence of HGT and increased mutation rate as a tool for ALE. The genderless strain, capable of continuous *in situ* HGT, was further modified with an inducible mutator phenotype. The combination of both HGT and an increased mutation rate was beneficial in expediting adaptive evolution in all fitness landscapes tested. The genderless ara+ populations reached the CM target tolerance 50.4 % more rapidly, the TM target tolerance 16.8 % faster, and achieved a 13.6 % higher growth rate on glycerol minimal media then the next best performing strain/condition. The results strongly suggest that the combination of HGT and increased mutation rate is capable of enhancing ALE in microbial systems independent of challenge.

In all antibiotic challenge evolutions, populations without an increased mutation rate were observed to have extended lag before phenotype improvement; this is indicative of mutationally limited regimes where the mutational supply is too low for HGT to be beneficial. This was especially evident with the CM challenge, where the genderless ara- population improved more quickly than either asexual populations but was subjected to an extended delay. Increased mutation rate was expected to be beneficial in a CM challenge, however the asexual ara+ populations did not perform better than the asexual ara-. We conjecture that increased mutation rate is only marginally beneficial in this case and with additional replicates, the asexual ara+ and ara- may become statistically separable.

Next generation sequencing of the glycerol evolved populations consistently identified the expected *rpoC* and *glpK* mutations in all sequenced populations and was able to provide strong insight into the different intra-population dynamics occurring upon the introduction of HGT; specifically, we uncovered the strongest evidence to our knowledge of HGT to alleviate clonal interference. To examine the observed mutations, each was reconstructed into the wild type BW25113. Many of our conclusions hinge on similarity of BW25113 and MG1655, because the growth advantages of each mutation/combination of mutations was consistent, we are confident the adaptive landscape of BW25113 is similar to MG1655 and the conclusions of Palsson work about the fitness landscape of glycerol are applicable.

The sequenced GIG1 population maintained multiple different combinations of mutations in various subpopulations including those of lesser fitness benefit

(*rpoC1022+cyaA797* versus *rpoC1022* with either *glpK288* or *glpK695* [fitness differences p-values<10^{-4}]), and eventually produced a pair of triple mutant subpopulations from these. On the other hand, in the asexual population, all the lesser-fit mutations besides the two that fixed were lost. While the ability to pass genetic material through HGT bi-directionally has been well established in *E. coli* (Cooper, 2007; Winkler & Kao, 2012), the molecular signature of HGT to alleviate clonal interference has not been conclusively documented. In recent work by Mcdonald *et al.* the ability of yeast to purge hitchhikers was demonstrated, but no direct evidence of combining beneficial alleles was observed (McDonald et al., 2016). Similarly, in a recent work a recombination event was observed, but the relative impact of the recombination remains indeterminate due to the complexity of determining fitness in their fitness landscape. In population GIG1, we observed the evidence of recombination, the co-existence of two different alleles in separate mutants and the combination of both alleles in one mutant; where subpopulations containing either *rpoC1022+cyaA797* or *rpoC1022+glpK695* and another subpopulation containing *rpoC1022+glpK695+cyaA797* were identified in the same population sample. While this observation may also be explained by very rare mutational events in which an identical mutation occurred twice in different sub-populations in the same evolution, the more probable explanation is the occurrence of recombination event. To mathematically weigh the two hypotheses, we compared the frequency of generating the desired mutant in a population composing of 5% genotype A and 5% genotype B, conditions similar to those of generation 125 (see Figure 21-A). We modified the parameters of our previously developed model to incorporate the

experimental conditions, population size, mutant fitness, etc. of this work, and compared the expected number of double mutants formed from recombination events to the expected number of spontaneous double mutants. Results showed that the chance of the double mutant arising from mating is ~5 orders of magnitude more frequent than from spontaneous mutation. However, if recombination events are sufficiently frequent, it is surprising we did not observe all other combinations of mutations at Time 3; upon termination of the experiment, the GIG1 population had settled primarily on two of the four possible combinations of *glpK* and *cyaA* mutations. We hypothesize that two factors inhibited the establishment of the other possible genotypes by Time 3. First, the fitness landscape available to recombination (only) is flat, there is large improvement from double to triple mutant; the improvement from the fittest double mutant to least fit triple mutant is 7.1% (the average improvement from double to triple mutant is 17%) however between triple mutants (the highest peaks on the fitness landscape) the largest difference in fitness is only 4.3%. Accordingly, any recombinants established initially would rapidly expand limiting the opportunity for alternate triple mutants to form. Second, we posit the distance between the two genes of interest *glpK* and *cyaA*, of about ~10-30kb, is small enough to dampen the frequency of generating recombinants. Our prediction is further supported by the results of the selection experiment where the results from 6 independent populations revealed the generation and enrichment of several random combinations of mutations, indicating underlying stochasticity. If recombination was more frequent, such that all possible combinations can be generated rapidly, we would have expected a more consistent mutational profile in each population.

Overall, our results showed that an increased mutation rate was able to provide an advantage in environments where limited beneficial determinants present themselves, and HGT was similarly able to improve ALE in environments where beneficial determinants are weaker and more frequent. When combined, the benefits of both led to the observed near universal improvement in ALE. Additionally, strong evidence for the mechanism of the advantage of HGT, a recombination event was observed between two subpopulations to found a more fit sub-population.

COMBINING PHENOTYPES WITH THE GENDERLESS CHARACTER

Summary

The ultimate goal in industrial strain design is to implement a robust production genotype for a commercialized process. Currently, most research focuses on two approaches, 1) improving production through optimized biomass, yield, and titer, or 2) by improving robustness to industrial conditions; however, forays between one goal into the other have been limited. Even within these categories often progress is very one-dimensional, ignoring non-ideal conditions and the potential applicability and robustness of a developed phenotype. To address this, we have generated a method to rapidly combine different desirable traits to avoid incompatible mechanisms and create production strains more rapidly. Our system harnesses the power of horizontal gene transfer in a "genderless" *E. coli* strain to combine groups of phenotypes composed of different mechanisms en-masse and find functional recombinants rapidly. Here we demonstrate a proof of concept with the combination of several different tolerance phenotypes.

Introduction

The production of bio-based commodities is a rapidly developing field, aided by the development of several powerful strain engineering tools (CRISPR, MAGE, TRMR, RAGE etc…), next generation sequencing and the necessary computational techniques to understand the massive amounts of data (Goodwin, McPherson, & McCombie, 2016;

Jakočiūnas, Jensen, & Keasling, 2016). However, progress in phenotype development has been somewhat limited by the difficulty in producing non-growth coupled compounds in the high yields and titers necessary to compete with existing chemical synthesis (S. K. Lee, Chou, Ham, Lee, & Keasling, 2008; Peralta-Yahya & Keasling, 2010). High yields have been reported for a variety of compounds in strict laboratory settings using expensive feedstocks and ideal growth conditions (see (Hara et al., 2014) for a recent review). To transition into industrial settings, strong producers need robustness to a variety of conditions such as: growth on alternative feedstocks, less ideal conditions like osmotic stress, pretreatment remnants, and high sugar concentrations (Lynd, 1996; Piotrowski et al., 2014). Additionally, often at high levels of production there can be significant product or intermediate toxicity (Keasling, 2010).

Rational engineering of tolerance phenotypes has been met with limited success due the complexity of the working space and limits of current cellular modeling. Other less rational approaches such as adaptive laboratory evolution have seen more success (see (Winkler & Kao, 2014)). Adaptive Laboratory Evolution couples the careful choice of a selective environment, in which the desirable phenotype will have a fitness advantageous, to selection on genotypic diversity within a population. Cells with beneficial mutations will expand in the population over time. These beneficial mutants can be utilized directly or studied for future improvement. Generally speaking, ALE is a highly advantage method because the toxicity of various inhibitors is poorly understood, as no *a priori* knowledge about the condition of interest is needed.

ALE has been applied toward the development of several mechanisms for tolerance to various toxic compounds for a variety of different stressors in many different microbes (Almario et al., 2013; Demeke et al., 2013; Koppram, Albers, & Olsson, 2012; Royce et al., 2015; Xu et al., 2015; L. Zhu, Cai, Zhang, & Li, 2013). Industrially relevant conditions are often composed of several different inhibitors. However, combining mechanisms represents a hurdle as considerations such as antagonist pleiotropy between conditions and mechanisms of tolerance have been observed, but insufficient knowledge is available to guide rational combinatorial strain engineering. For example, two different mechanisms of butanol tolerance have been shown to exhibit antagonistic pleiotropy (Reyes et al., 2013; Schrag, Perrot, & Levin, 1997) and similarly, recent work by Deatherage *et al* (Deatherage et al., 2017) has unveiled how even slight changes in operating conditions can modulate the advantage of different mutations, though the mutations may still be beneficial in both environments, the extent of the advantage can be drastically attenuated. Some broad screening-based approaches have been attempted to isolate multi-tolerant microbes (Alper & Stephanopoulos, 2007; Appukuttan et al., 2016; Çakar, Seker, Tamerler, Sonderegger, & Sauer, 2005; S. I. Jensen, Lennen, Herrgård, & Nielsen, 2015; Lennen & Herrgard, 2014; Oide et al., 2015; D.-Q. Zheng et al., 2011), and in a few instances cross-tolerance has been examined (Demeke et al., 2013; Dragosits & Mattanovich, 2013; Dragosits, Mozhayskiy, Quinones-Soto, Park, & Tagkopoulos, 2013; Reyes et al., 2013; Sandoval et al., 2012; X. Wu et al., 2014), but in general work linking the multiple steps of generating a production strain is lacking.

Here we propose to use two tools to rapidly generate multi-tolerant strains and sidestep the difficulties of rationally designing a ramping scheme for multiple inhibitors. First, we propose the use of ALE to generate multiple populations evolved for different inhibitors in parallel, using our previously developed genderless *E. coli* capable of HGT. It is assumed that each evolved population will be heterogeneous, with multiple mechanisms for the desired phenotype. Second, we will take our evolved genderless populations, and combine the different tolerance phenotypes through mass mating events and subsequently screen for recombinants with desired combined phenotypes. Our work aims to avoid several issues currently present in the development of muti-tolerant strains, such as antagonistic pleiotropy, and the rational design of multi-inhibitor challenges for adaptive evolution. We first tested the ability of our genderless strain to combine two phenotypes by mixing populations previously evolved for CM tolerance, a complex fitness landscape (Toprak et al., 2011; Winkler & Kao, 2012), and glycerol, which has two known driver mutations for increased fitness (Applebee et al., 2008; Cheng et al., 2014). The mixed (mass-mated) populations were then compared to their self-mated counterparts and previously developed populations of the genderless strain evolved for CM tolerance with a glycerol carbon source (positive control). Next, we transitioned our mating system to less well-characterized, but industrially relevant environments, the Ionic Liquid 1-ethyl-3-methylimidaolium chloride (IL) and osmotic stress of high NaCl concentrations. ILs have promising applications in pretreatment of lignocellulosic biomass (Elgharbawy, Alam, & Moniruzzaman, 2016), they are capable of rapid biomass pretreatment and recyclable. The IL we have chosen has not been studied

extensively as an inhibitor of cell growth, with only one study noting an increased tolerance to IL with the overexpression of an efflux pump (Frederix et al., 2014). Osmotic tolerance is another desirable characteristic in biocatalysis; high osmotic tolerance can facilitate cost saving through the use of waste streams high in inhibitors such as NaCl (Rumbold et al., 2009). Osmotic tolerance has been more heavily characterized, previous strain engineering efforts include increased production of native osmoprotectants (Purvis, Yomano, & Ingram, 2005) and expressing foreign determinants (Rozwadowski, Khachatourians, & Selvaraj, 1991). Additionally, osmotic tolerance has been studied in the context of other inhibitors (Appukuttan et al., 2016; Winkler et al., 2014), and exhibits some cross talk with other stressors; however, identified mechanisms of osmotic tolerance have varied responses to other stressors (Dragosits et al., 2013).

Methods

Evolution Experiments

We performed several evolution experiments to generate the seed populations for the cross-tolerance mating. Each evolution utilized the genderless strain, and when applicable the mutation rate of the genderless strain was increased by overexpressing the *dam* gene behind an arabinose promoter and adding 0.0005% (w/v) arabinose (see materials and methods, section 3).

Chloramphenicol with Glucose Carbon Source

The genderless strain was evolved with a chloramphenicol challenge when grown on glucose as sole carbon source. Six replicate populations of the genderless

strain were grown from single colonies. The cultures were inoculated into minimal media supplemented with CM. Each populations was transferred every 24 hours and CM concentration adjusted as previously described (Winkler & Kao, 2012). The populations used for the mating experiments in this study were taken from the populations evolved for 15 serial passages, see (Winkler & Kao, 2012) for additional information and evolution details.

Osmotic Inhibitor Evolved Populations

The genderless strain was evolved with an osmotic challenge and a glucose carbon source. 6 replicate populations of the genderless strain were grown up from single colonies and serially passaged every 24 hours as described (Winkler et al., 2014). The cultures were supplemented with NaCl as an osmotic challenge. The osmotic challenge was occasionally ramped, up to a maximum of 0.8 M NaCl. The populations used for the mating experiments in this study were taken from the populations evolved for 7 passages. See (Winkler et al., 2014) for complete evolution details.

Growth Rate on Glycerol Populations

The genderless strain was evolved for more rapid growth rate on glycerol, no antibiotic challenge. The evolution was briefly described before (see materials and methods, section 3). 6 replicate genderless inducible mutator isolates were grown up in LB and transferred into glycerol. The cultures were induced with 0.0005% w/v arabinose to increase the mutation rate. Each passage, cultures were grown until the OD_{600} reached approximately 0.5 at which point they were diluted to an OD_{600} of 0.004 to maintain the populations in exponential growth for approximately 250 generations.

Chloramphenicol Tolerance with Glycerol Carbon Source

The genderless strain was evolved for growth on glycerol with a CM challenge. The evolution was briefly described before (see materials and methods, section 3). 6 replicate isolates of the genderless inducible mutator strain were grown up on LB and inoculated into glycerol media. The cultures were induced with 0.0005% w/v arabinose to increase the mutation rate. The cultures were maintained in exponential phase with an increasing CM challenge based on the OD_{600} after 12 hours. The cells were passaged approximately 200 generations.

Ionic Liquid Evolved Populations

The genderless strain was evolved for ionic liquid tolerance on a glucose carbon source. 6 replicate isolates were grown on M9 media supplemented with IL. The populations were serially propagated every 24 hours into fresh media by diluting approximately 100 fold each transfer. The ionic liquid was supplemented at 100 mM and ramped up to 150 mM at transfer 6 when the populations reached a target OD_{600}. The populations used for the mating experiments in this study were taken from the populations evolved for 8 serial passages

Fitness Assays During Evolution

The fitness of the evolving populations were determined periodically throughout the osmotic and ionic liquid evolutions. 3 overnight cultures of the wild type were grown up from colonies in M9 for 24 hours. The wild type cultures and three technical replicates of each of the evolving populations were diluted to OD_{600} of 1.0 and 2 µl was added to 98 µl of M9 supplemented with the current concentration of challenge to three

replicate wells of a 96 well plate. The plate was measured every 5 minutes in the TECAN automated plate reader, with 270 rpm orbital shaking and incubation at 37^{o}C between measurements.

Mating protocol Chloramphenicol and Glycerol

3 different populations (evolution replicates) each of the glycerol evolved, chloramphenicol in glycerol evolved and the chloramphenicol in glucose-evolved evolutions were grown up from frozen stocks in LB. The glycerol and chloramphenicol glucose evolved populations were randomly paired and mixed onto a LB plate for mass mating. Each individual population was also patched onto LB plates and incubated for 24 hours for self-mating. The cells from the plates were scraped and resuspend in 0.5 mL PBS. 5 µl of each cell suspension was inoculated into glycerol minimal media supplemented with 1.33 µg/mL CM. This concentration was chosen to allow some growth for all of the sampled populations. After 24 hours of growth, 2 µl of the culture was inoculated into 3 separate wells of a 96 well plate harboring 98 µl of glycerol minimal media supplemented with 3 µg/mL CM. The plate was measured every 5 minutes in the TECAN automated plate reader, with 270 rpm orbital shaking and incubation at 37^{o}C between measurements.

Mating Protocol Ionic Liquid and Osmotic Stress

6 separate replicate populations of each of the Ionic Liquid Evolved and Osmotic Evolved evolutions were grown up on LB from frozen stock. Then each set of LB cultures from separate evolutions were normalized to OD_{600} of 1.0 and pooled equally into 1 single culture. The two pooled cultures (one of all 6 of the IL evolved populations,

the other of all 6 of the OS evolved populations) were mixed together, and individually plated onto LB plates. After 24 hours the cultures were scraped and resuspended into 0.5 mL PBS. The cell suspension was inoculated into M9 media containing 100 mM ionic liquid and 0.45 M NaCl. This concentration was chosen to allow growth for all of the sampled populations. After 48 hours of growth the resultant cultures were normalized to OD_{600} 1.0 and diluted 100 fold into M9 media containing 140mM ionic liquid and .495 M NaCl where final growth was tracked.

Results

Known Inhibitor Combining Phenotype Test

It has been previously established that the genderless strain can rapidly combine pairs of genetic markers *in situ*. However, very little work has focused on the combination of less rational targets such as tolerance mechanisms and growth enhancing mutations. To assess the capability of the genderless strain to integrate multiple phenotypes into a single clone efficiently, we choose to utilize the populations previously evolved for a higher growth rate on glycerol as a substrate and populations evolved for enhanced tolerance to chloramphenicol in glucose, for the initial test. Populations of the genderless strain evolved in the presence of both environmental factors (glycerol and chloramphenicol) have also been developed previously, and will be used as a positive control for comparison with the proposed mating scheme for developing dual complex phenotypes. Chloramphenicol challenge presents as a complex adaptive landscape with multiple smaller beneficial mutations providing tolerance.

Genetic changes enhancing growth rate on glycerol are limited instead to a handful of genes. In order to successfully grow rapidly in chloramphenicol challenge, we expect that cells within the population would need to acquire at least one mutation for improved growth rate on glycerol and several mutations conferring tolerance to the chloramphenicol challenge. To test the feasibility of inter-population genderless strain mating, we grew up frozen stock of the glycerol and chloramphenicol evolved strains and the glycerol and chloramphenicol evolved reference. All the populations used for mating were taken from the final population's frozen stock of the respective evolution. See Figure 25, Figure 26, and Figure 27 for the evolution dynamics.

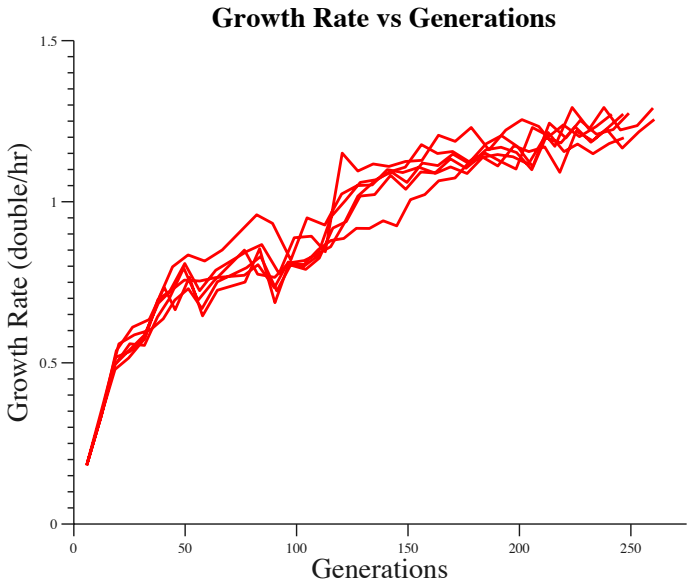


Figure 25: Evolution dynamics of genderless strain evolved for faster growth rate on glycerol.

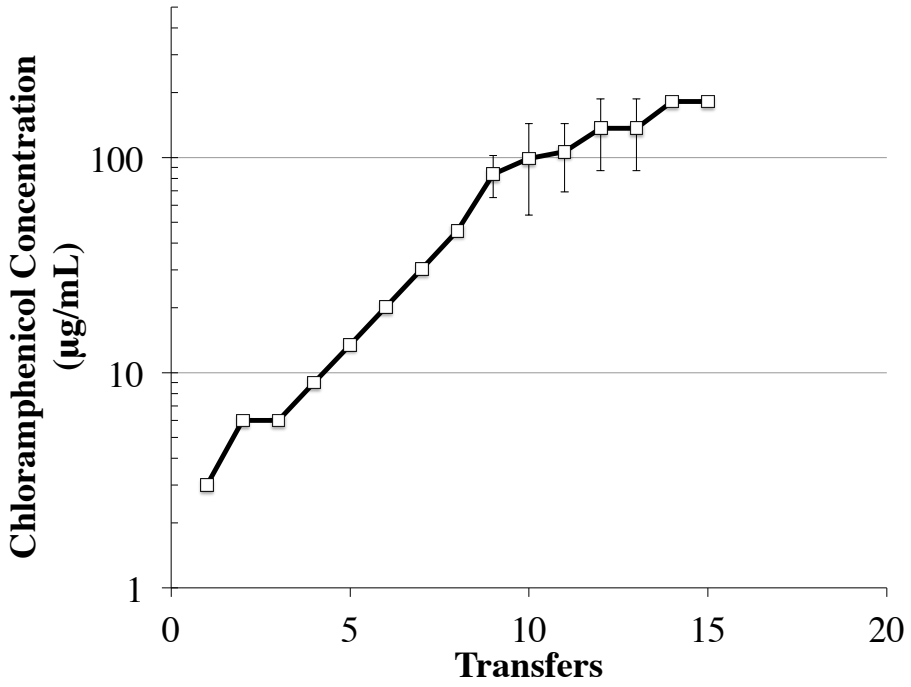


Figure 26: Evolution dynamics of the genderless strain grown on glucose with a chloramphenicol challenge, reproduced from (Winkler et al., 2014).

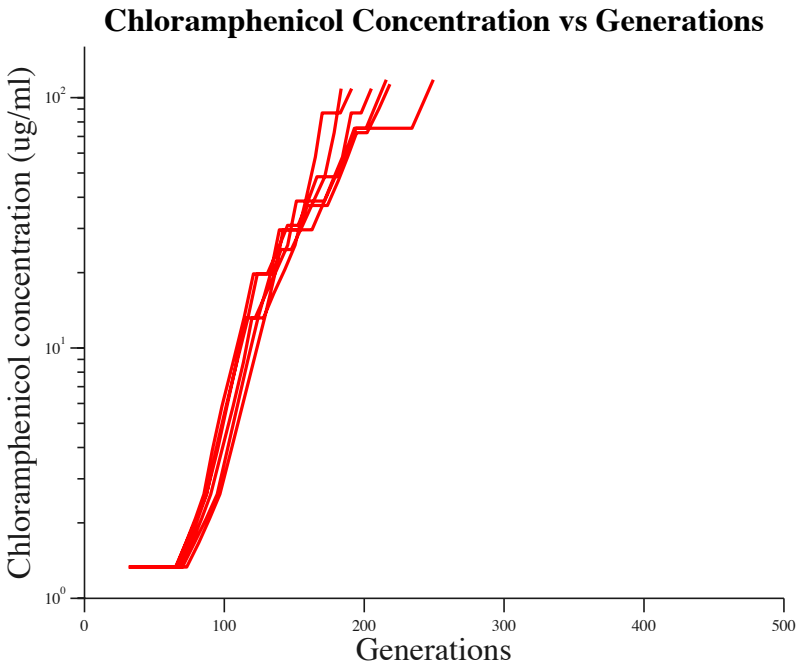


Figure 27: Evolution dynamics of the genderless strain evolved for higher chloramphenicol tolerance with glycerol as the carbon source.

Each resuspended population was mated and screened at a low level of chloramphenicol challenge in a glycerol minimal media. The resulting culture was propagated to a higher chloramphenicol challenge to assay the population for faster growth rate in the presence of the challenge. This was compared to the growth rate observed in the glycerol and CM evolved populations Figure 28.

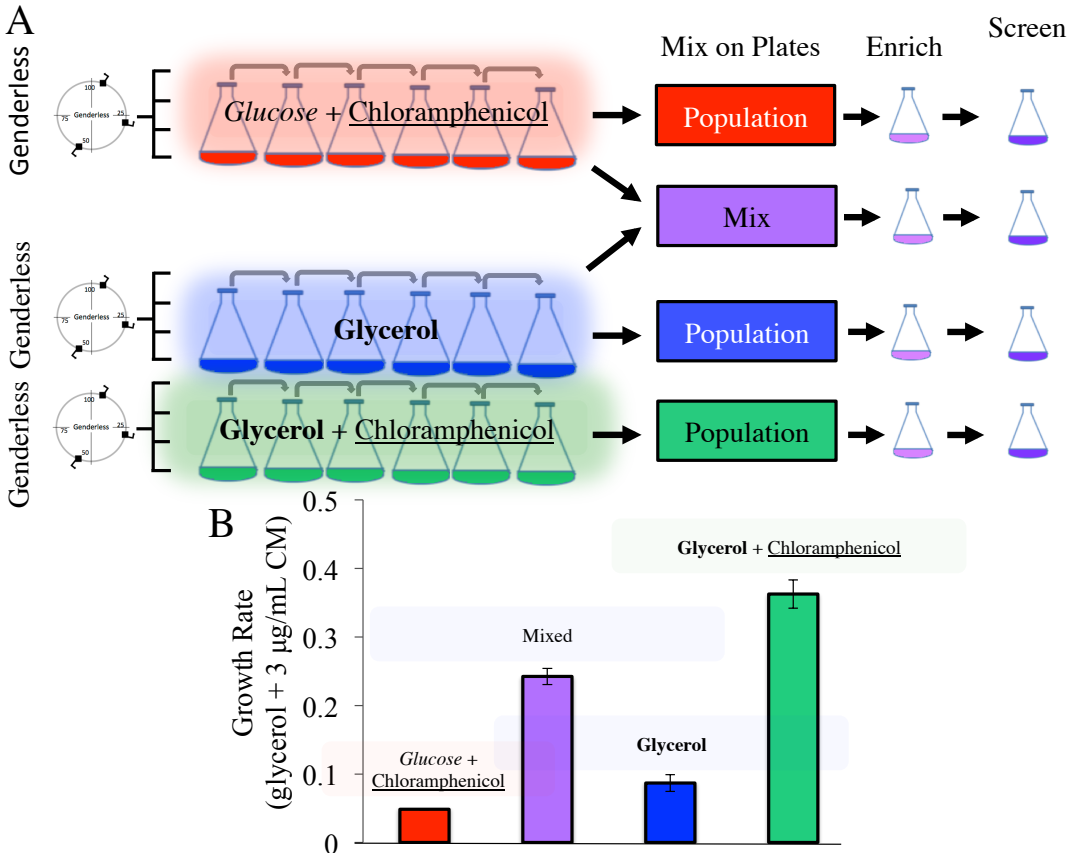


Figure 28: The inter-population mating schema for the genderless strain evolved under pre-characterized conditions. A) The genderless strain was evolved in 3 conditions, glucose with a ramping chloramphenicol challenge, for faster growth rate on glycerol, and for a ramping chloramphenicol challenge with glycerol as the carbons source. The products of the individual conditions were mated together on plates. The populations were then propagated in a low then high chloramphenicol and glycerol challenge. B) The average growth rates were measured for each sets of populations in the higher chloramphenicol and glycerol transfer. Error bars are standard deviations of 3 biological and 2 technical replicates.

The average growth rate of the populations from the screen at a high level of CM with glycerol as the carbon source indicates the mixed populations were able to significantly outperform the individual populations (p-value $<10^{-3}$).

Unknown Inhibitor Combining Phenotype Test

Though the mating of two separate populations from different evolutions was successful at combining the phenotypes of two previously characterized adaptive landscapes, often little is known about a tolerance phenotype or mechanism. To characterize the capability of the genderless strain to combine unknown mechanisms with unknown complexity we chose to examine several industrially relevant conditions, osmotic tolerance, and stress from an Ionic Liquid, a hydrolysate pretreatment chemical. Osmotic stress on the genderless strain has been previously characterized (Winkler et al., 2014) allowing us to simply assess the appropriate challenge level. However, the range of concentrations of Ionic Liquid the wild type genderless strain could grow on was unknown. A calibration curve for the growth rate of the genderless strain challenged with IL was constructed, see Figure 29.

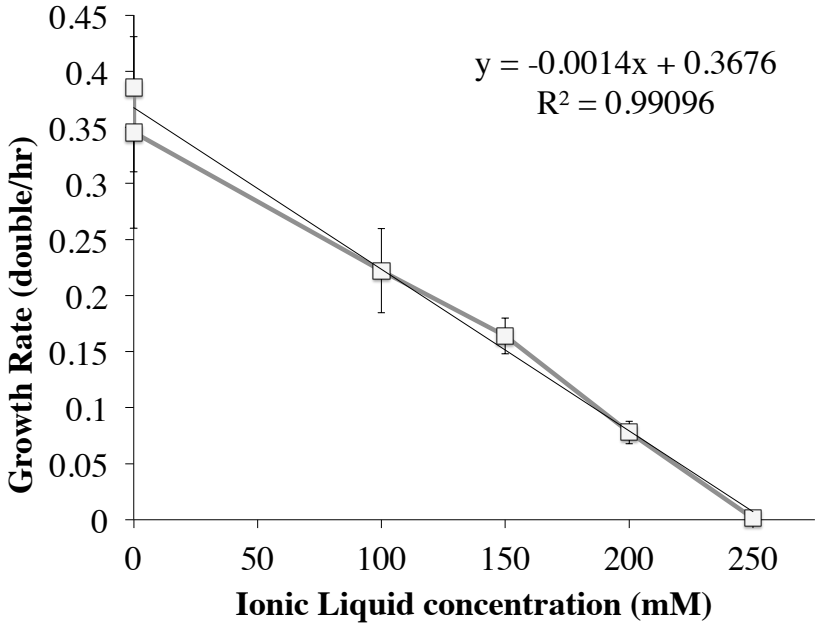


Figure 29: Growth rate of wild type genderless strain in M9 media challenged with Ionic Liquid. Error bars represent standard deviations (n=3).

The genderless strain was sensitive to the IL in mM range. An evolution in Ionic Liquid was then performed to generate the seed populations of the genderless strain tolerant to IL, see Figure 30.

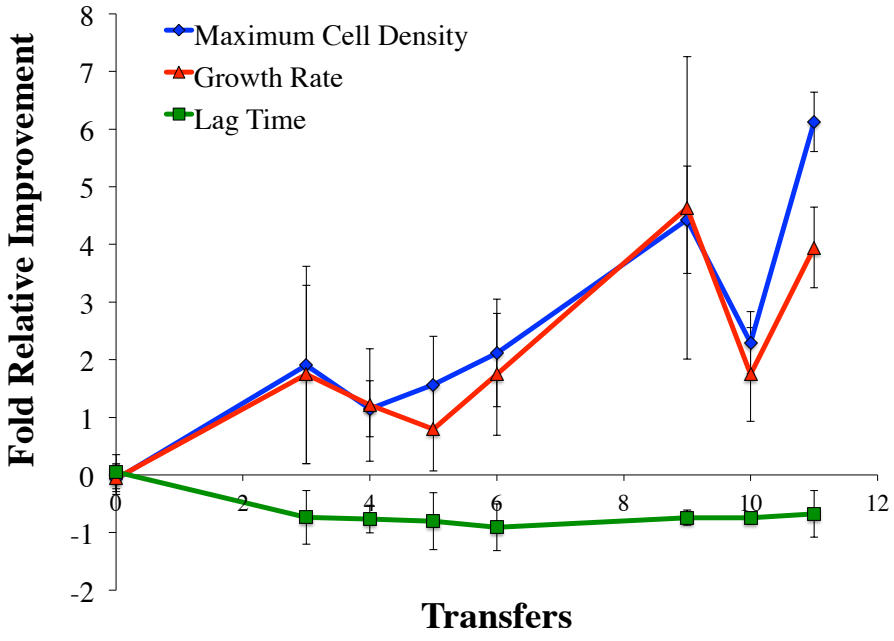


Figure 30: Evolution dynamics of the genderless strain evolved on IL relative to the WT. Relative final cell density is in blue, relative growth rate in red, and relative lag time in green. Error bars represent standard deviations.

Similarly, we had previously evolved the genderless strain for tolerance to osmotic stress (Winkler et al., 2014), for the evolutionary dynamics see Figure 31.

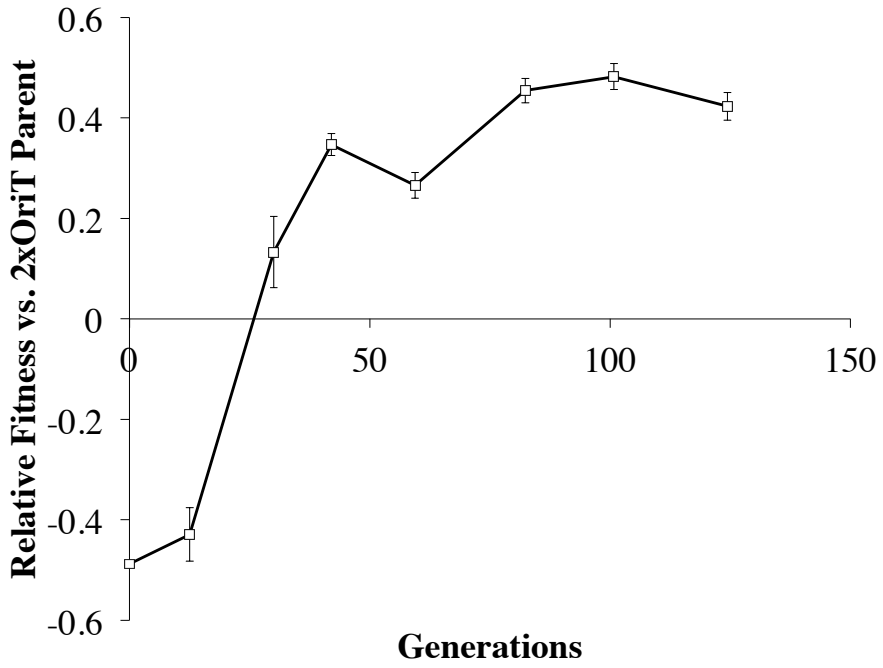


Figure 31: Evolution dynamics of genderless strain evolved for osmotic tolerance, fitness relative to wild type with 2xoriT plotted versus generations, reproduced from (Winkler et al., 2014).

To combine the osmotic tolerance phenotype and the ionic liquid tolerance phenotype, we choose to use population samples from when each of the groups of populations exhibited an apparent increase in tolerance. This corresponded with the 7th transfer from each evolution and approximately 50 generations. A similar protocol as was previously described was followed to combine the osmotic and ionic liquid tolerant phenotypes. However, in this case only final optical density was tracked see Figure 32 for details.

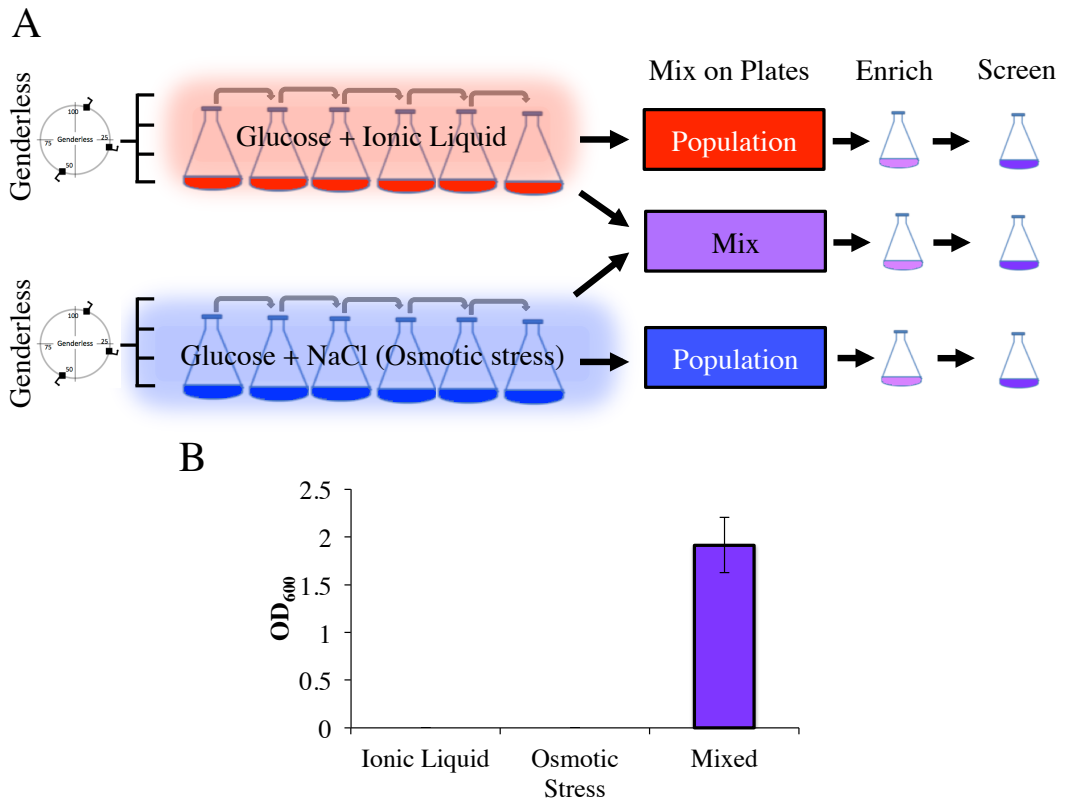


Figure 32: Schema of combining unknown inhibitors. A) Evolutions of genderless strain for osmotic and ionic liquid tolerance, followed by mating, enrichment and screening. B) Measured cell density from screen at higher concentration of inhibitors, error bars are standard deviations.

The mixed populations were able to grow in the increased inhibitor concentrations; the un-mixed populations were not able to grow, indicating multi-tolerant phenotypes were accrued in the mixed populations.

Discussion

Strains developed for various phenotypes have been demonstrated to rapidly combine using the HGT proficient genderless strain. Although spontaneous *de novo* mutations or mutations with cross-tolerance effects in the pre-mixed seed populations may be the source of the cross tolerant phenotypes in the mixed populations, because the individual populations are taken as controls, we believe that the multi-tolerance stems from recombination events. The multi-tolerant phenotypes are very rapidly generated, therefore we further speculate that the frequency of the successful recombinant events for improved tolerance is sufficiently high, and the final populations are composed of multiple different recombinants, possibly harboring different combinations of tolerance mechanisms from the various seed populations. We have previously identified a couple of genes that confer a growth rate advantage in glycerol, and CM has been identified as a complex background with multiple different beneficial mutations. Thus any combination of these two genotypes will likely incorporate several different mutations from of each background into a single genotype. However, the rarity of these events must also be considered, and we postulate that the advantage of this method is 1) much larger than the natural mutation rate to get any single genotype, and 2) recombination frequency is much higher than the frequency of multiple mutations occurring in a single strain in a similar time frame. An alternative approach is to evolve strains in the presence of multiple inhibitors. However, given the difficulty of gauging the exact limiting inhibitor during an evolution, the proposed method provides an alternative that avoids uncertainty in challenge level. The implementation of this method also allows stock strains

displaying desirable phenotypes to be further developed to generate phenotypes on-demand in a plug and play manner. To be more applicable to real world systems, additional research is needed to study the possibility of incorporating additional phenotype (more than 2 total), and in preserving desirable characteristics such as high production phenotypes throughout the mating process.

IMPROVING THE GENDERLESS STRAIN

Summary

The use of horizontal gene transfer has been demonstrated to effectively increase the rate of phenotype improvement in ALE. Here we examine how to improve an *E. coli* strain capable of HGT, the genderless strain. We take several approaches toward enhancing the genderless strain for improved phenotype development; we aim to improve the frequency of successful mating events by modulating physical and genetic variables and also to improve strain robustness by removing auxotrophies in the wild type genderless strain.

Introduction

Adaptive laboratory evolution is frequently leveraged for phenotype development in microbes, wherein the natural process of mutation, and selection upon mutation by the environment can produce and enrich desirable phenotypes. ALE can be enhanced by increasing the mutation rate which acts to increase the speed of the evolution by expanding the available diversity to select upon (M. de Visser, Zeyl, Gerrish, Blanchard, & Lenski, 1999; Sniegowski, Gerrish, & Lenski, 1997). Alternatively, diversity can be manufactured via horizontal gene transfer where the existing diversity is harnessed and mixed combinatorially to increase the mutational space explored in an evolving population (Y. Kim, 2005; McDonald et al., 2016; Raynes et al., 2011). We have previously developed a “genderless” strain of *E. coli* capable of

HGT (Winkler & Kao, 2012). The genderless strain utilizes an integrated F-plasmid to transfer single stranded chromosomal DNA to neighboring cells. Once received, the DNA can be spontaneously integrated into the chromosome via homologous recombination (Lloyd & Buckman, 1995). The genderless strain has been successfully used to speed adaptive laboratory evolution in complex adaptive landscapes, and with an increased mutation rate (Winkler & Kao, 2012). However, the frequency of recombination is still limited. In our previous work, we identified that during cell growth, cell to cell interactions are only limiting at low cell concentrations. This indicates that above a certain cell concentration the limiting factor in recombination is related to uptake of DNA or integration breaks (Cullum et al., 1978). In order to reduce these limitations we investigated two parameters that could enhance recombination frequency in the genderless strain. First we investigated the use of non-native recombination machinery such as the lambda red genes that have been used extensively for efficient chromosomal editing and represents a possible mechanism of enhancing recombination frequency even for ssDNA (Datsenko & Wanner, 2000; Mosberg, Lajoie, & Church, 2010). Second, we attempted to improve the native machinery. Other groups have previously identified a mutant of a native recombination gene, *recA*, capable of increased recombination, recA441. Mutations in *recA* have been reported to increase or decrease homologous recombination (Lavery & Kowalczykowski, 1990; 1992; Lloyd, 1978). However, the increased homologous recombination is connected with a more easily induced SOS response (Kowalczykowski, Dixon, Eggleston, Lauder, & Rehrauer, 1994; Lavery & Kowalczykowski, 1992; Witkin, McCall, Volkert, & Wermundsen,

1982). The mutant we chose to study, *recA441*, has been determined to slightly increase the SOS response. In order to construct the *recA441* mutation, we found the following substitutions glutamic acid to lysine at 38 and isoleucine to valine at residue 298 (Knight, Aoki, Ujita, & McEntee, 1984) reported in the literature, we surmise are the following mutations *recA* 115 G->A and *recA* 502 A->G.

In another approach to improving the genderless strain we aimed to reduce its growth defect relative to the wild type F- counterpart. The genderless character is integrated in the tryptophan biosynthesis pathway, potentially contributing to the genderless strains reduce growth compared to the wild type. By reconstructing the wild type growth on tryptophan, the growth of the genderless strain can be improved.

Here we aim to improve the genderless strain by examining another system of homologous DNA integration, alternative growth schemes, altered native machinery of homologous recombination and by repairing the trp- phenotype.

Materials Methods

Strain construction Alternative repair machinery

Two genderless strains with single antibiotic markers at 11 minutes from either an F-plasmid integration site or an origin of transfer were chosen for bi-directional mating, strains SMD Δ*mbhA*::oriT Δ*hyfC*::oriT *trp*::F[Δ*traST*] (genR) Δ*ybh*I::*cat* and SMB Δ*mbhA*::oriT Δ*hyfC*::oriT *trp*::F[Δ*traST*] (genR) *zje-2005*::Tn10 (tetR). To introduce the lambda red genes exo, beta, and gam to the mating strains SMD and SMB we transformed them with plasmid pKD46 which has ampicillin resistance, a

temperature sensitive oriV and the three lambda red genes behind an arabinose inducible promoter.

Strain construction Mutation of native repair pathways

We constructed the two mutations identified for the *recA441* genotype into the wild type BW25113 strain using the procedure outlined by Datsenko and Wanner and the *cat-sacB* selection-counterselection cassette (Thomason et al., 2014) as previously described (Datsenko & Wanner, 2000). To generate the counter selection cassette we used primers Pp10 and Pp11, the repair DNA was constructed with primers Pp12 and Pp13 and verified with Pp14 and Pp15. The mutations were verified with primers Pp6-9.

Strain Construction Repair of Auxotrophy

To repair the auxotrophy of the genderless strain we used the method of datsenko and wanner (Datsenko & Wanner, 2000). The plasmid pkd46 was transformed into the genderless strain, followed by the repair DNA following standard protocols. To construct the repair DNA we used primers Pp1 and Pp2. The insertion was verified with primers Pp3, Pp4 and Pp5, see Table 11.

Table 11: Primers used for constructing *recA441*, and to repair genderless trp-auxotrophy

Primer	Sequence 5'->3'
Pp1	TCCGGGTTTTCGCGCATCATTTTCAACGCATGGGCCAGGGCGTGGGAGGAGGATTTTTCCGGCTTCATTAA AGAAAG
Pp2	GGCTTCAAGGGCTTCATCATC
Pp3	GGATGATCCCTTCGTGCAG
Pp4	CCGTAAGAGGCTATCTGGC
Pp5	CCTTCTCTTTCCAGAATCTGCG
Pp6	CATGCGCCTGGGTGAAGACCGTTCCATGGATGTGG
Pp7	CATGCGCCTGGGTGAAGACCGTTCCATGGATGTGA
Pp8	ATCGGTCAGGGTAAAGCGAATGCGACTGCCTGGCTG
Pp9	GTCGGTCAGGGTAAAGCGAATGCGACTGCCTGGCTG
Pp10	TTGGTAAAGGCTCCATCATGCGCCTGGGTGAAGACCGTTCCATGGATGTGTCAGAAGAACTCGTCAAGAA
Pp11	TTC CGG GTT ATC TTT CAG CCA GGC AGT CGC ATT CGC TTT ACC CTG ACC GAtttatattccccagaacatca
Pp12	TTGGTAAAGGCTCCATCATGCGCCTGGGTGAAGACCGTTCCATGGATGTGAAAA CCA TCT CTA CCG GTT CGC TTT
Pp13	TTC CGG GTT ATC TTT CAG CCA GGC AGT CGC ATT CGC TTT ACC CTG ACC GACCT TCT CAC CTT TGT AGC TGT ACC AC
Pp14	GCAGTGAAGAGAAGCCTGTCG
Pp15	TCT GCC GTA GCA TCA ATC TCT TC

Mating Protocols

The mating protocol used in this work has been previously been described with the following exceptions. Where plasmids were used, antibiotics were supplied as needed. For mating in alternate growth conditions, the cultures were either grown as usual, grown as usual for 6 hours then transferred to an incubator with no shaking, or only grown in an incubator with no shaking. For the plate based mating, the same

inoculum size as in liquid mating was spotted onto an LB plate with no selection. After 24 hours of growth the spot was scraped and resuspended into 0.5 mL PBS.

Results

Alternate Recombination Machinery

To test if the *E. coli*'s native homologous recombination machinery could be enhanced to provide greater frequency of recombination for the genderless strain, we expressed the lambda red phage proteins during a test mating experiment. Two strains were grown up and mixed 50-50, each harboring a single chromosomally integrated antibiotic marker and the lambda red expression system on the pkd46 plasmid. They were then mixed together and split into two cultures, one with induction of the lambda red system, the other without. The frequency of recombinants after 24 hours was determined via serial dilutions. In the cultures with arabinose induction, and therefore lambda red expression, the frequency of recombination decreased 1000 fold, signifying the two mechanisms of recombination interact negatively, see Figure 33.

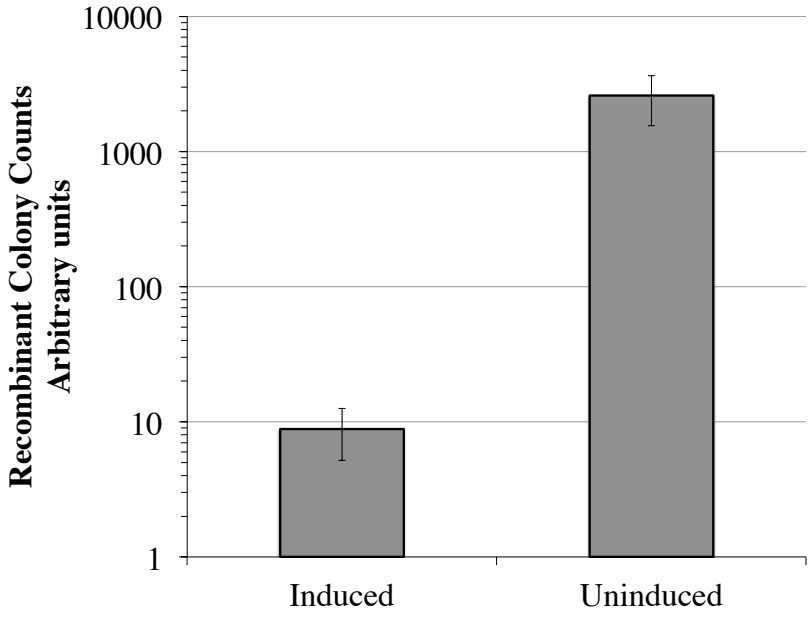


Figure 33: Frequency of recombinants with the lambda red genes induced on plasmid pkd46 versus uninduced, arbitary colony counts normalized to final cell density.

Unconventional Growth for Increased Recombination

As previously observed, the frequency of recombination is dependent on cell density and is likely dependent on the duration of cell-cell interactions (Collins & Broda, 1975; Cullum et al., 1978). We altered the growth dynamics of the cultures to attempt to reduce the break-up of cell-cell interactions by reducing the shaking experienced by the culture, and even by allowing the cells to grow on plates where cell-cell interactions should be prolonged even further. We mated the populations in 4 conditions: the standard culturing for mating, the standard conditions for mating until the end of exponential phase, without shaking, and on a plate. We found that the plate based mating had the highest efficiency ($p\text{-value}<0.05$) where as the stagnant culture without shaking

had the lowest (p-value<0.01), see Figure 34. The cultures in the shaker until the end of exponential phase and the standard mating protocol were indistinguishable.

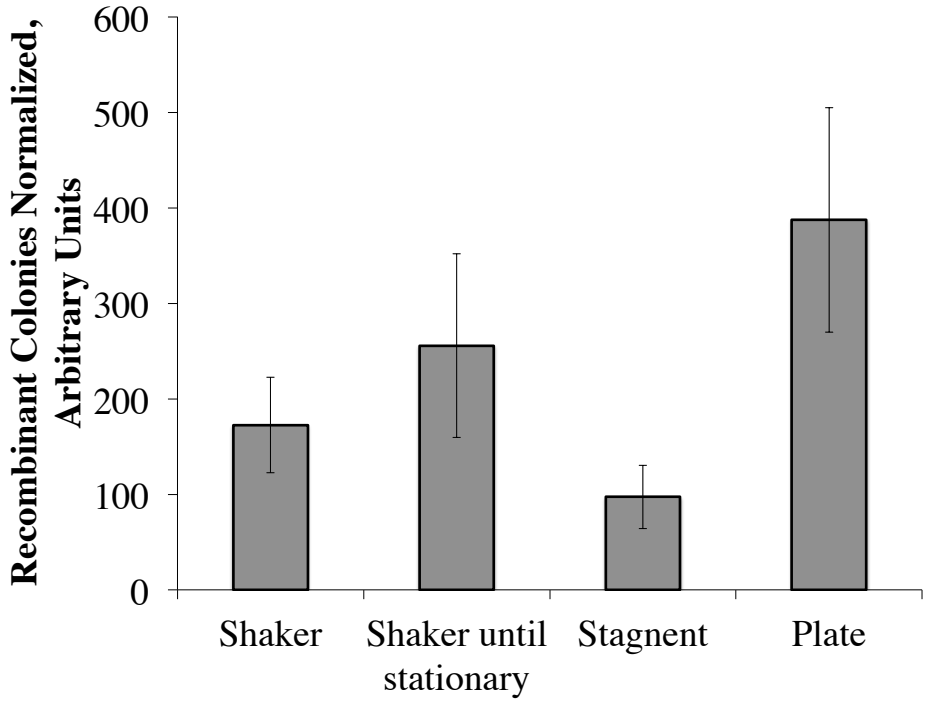


Figure 34: Frequency of recombinants normalized by final OD_{600} for four mating conditions at 37°C: in shaker for 24 hours, 8 hours shaker 16 hours stationary, stagnent culture, and on an LB plate.

Mutation of native repair pathways

Some work has indicated a higher recombination frequency can be achieved with a mutant *recA*. To test if the genderless strain could more frequently incorporate chromosomal DNA if the recipient genotype was the *recA* mutant we first constructed the *recA441* mutation into wild type BW25113. We then measured the frequency of

recombination between a genderless strain donor carrying an antibiotic marker, and the recipient, either the wild type control or *recA441*, see Figure 35.

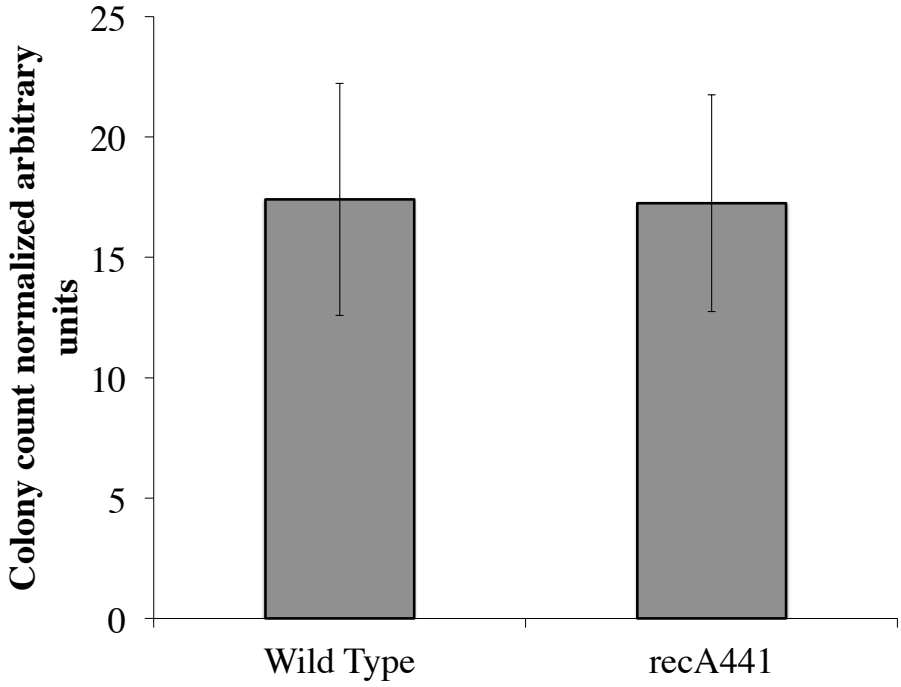


Figure 35: Frequency of recombinants between genderless donor and recipient (either wild type or recA441).

We found that the frequency of recombination was essentially identical. This indicated the *recA441* genotype was either constructed incorrectly or did not increase the frequency of incorporating transferred DNA.

Repair of Auxotrophy

We aimed to remove the auxotrophy in the genderless strain in order to eliminate the need for amino acid supplementation to minimal media for growth. Next generation sequencing data from previous work (Winkler et al., 2014) was harnessed to locate the exact location of the HFR integration event. It was determined base pairs

1311564:1311572 of *trpB*, sequence TGCTCTTTA, were duplicated and present at either side of the F-plasmid insertion. To restore the wild type trp operon, we introduced the repair cassette which included the remainder of the truncated *trpB*, *trpA* and the terminator loop followed by homologies to the region directly before the *trpB* duplication, see Figure 36.

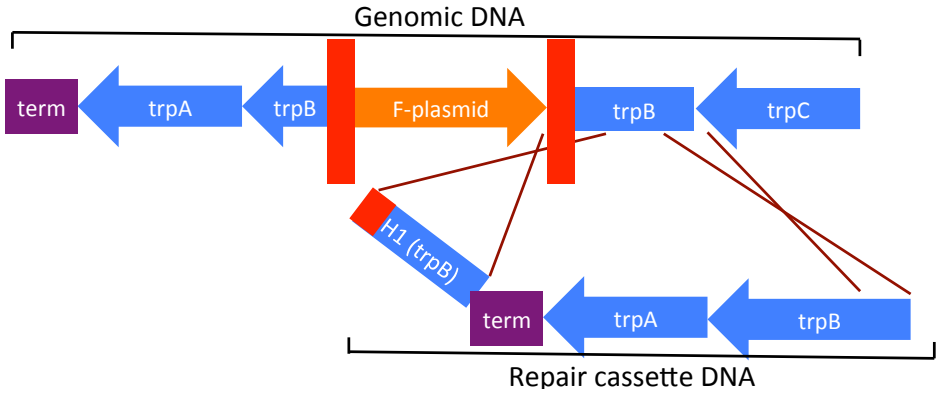


Figure 36: Diagram of the trp- phenotype repair strategy.

Once repaired using standard chromosomal integration strategies, the strain was tested for prototrophy and improved growth. Although the wild type ability to grow on un-supplemented minimal media was restored, no improvement of the genderless trp+ strain's ability to grow was observed, Figure 37.

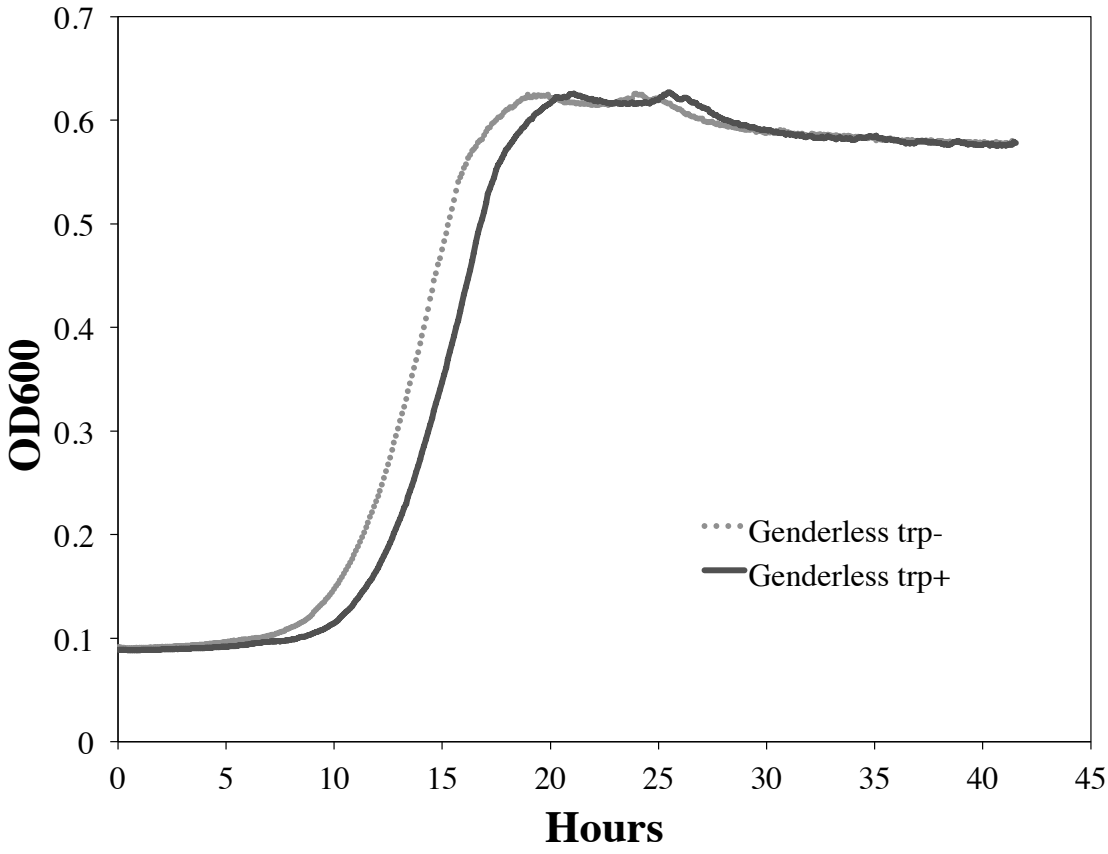


Figure 37: Comparison of genderless trp+ to the genderless trp- strain, grown on M9 minimal media. The genderless trp- was supplemented with 50 µg/mL of tryptophan. No significant difference in growth is detected between them.

Discussion

The genderless strain has a strong advantage when evolving in some fitness landscapes because it can combine beneficial mutations between genomes. However, it suffers several drawbacks such as the trp- phenotype and reduced growth relative the F- wild type. We took several approaches to enhance the genderless strains advantage and increase the frequency of recombination. Previously it had been observed that during initial growth, cell-cell contact is the rate-limiting step to recombinant formation, after

which homologous recombination becomes the limiting factor. To enhance the frequency of collisions, we altered the culturing conditions by increasing the amount of time with reduced shaking and even eliminating the liquid culturing. More recombinants were generated when we reduced the shaking speed after exponential phase was complete. However it should be noted the improvement was marginal and the final cell density observed when the cultures are removed from shaking after 8 hours is drastically reduces (about 2 fold, data not shown). Although this method appears to enhance recombination it appears to come at the cost of adversely impacting the robustness of cell growth. The plate based mating saw the greatest relative improvement in recombinant production. This was largely expected as the cells are moving much slower, further the relative distance between any two cells is much lower vastly increasing the chances of recombination occurring.

The alternative to cell-cell interactions and DNA uptake being the limiting factors in recombinant formation would be the incorporation of foreign genetic material being limiting. In order to remove this potential limitation, we attempted to improve the native machinery for increased recombination and to enhance the native machinery with the lambda red recombination genes. When we overexpressed the lambda red genes, the frequency of recombination was vastly reduced. The lambda red system had a very high efficiency of recombination with short dsDNA introduced from electroporation and so this result is unexpected. We believe this is due to several factors. The gam gene is a repressor of some DNA repair functions including some aspects of the native homologous recombination system in *E. coli* (Karu, Sakaki, Echols, & Linn,

1975). Thus the expression of gam likely eliminated the native recombination, and only recombinants utilizing the lambda red system were observed. The lambda red system is expected to have a very high recombination frequency for ssDNA (Nevoigt, Fassbender, & Stahl, 2000). However, it has been noted that the efficiency of the lambda red system decreases drastically with DNA size (Maresca et al., 2010). Given the F-plasmid machinery is transferring large fragments of DNA and the native degradation pathways are reduced by gam, the ultimate recombination rate would be much lower than expected. Alternative to another recombination mechanism, we attempted to improve the frequency of recombination by using a mutated *recA* shown to improve recombination frequency previously (Lavery & Kowalczykowski, 1990; 1992; Lloyd, 1978). Unfortunately, we were unable to reproduce the expected results and improve the recombination frequency.

Additionally, we attempted to relieve some of the growth deficit of the genderless strain relative to the wild type. The genderless strain is trp- due to an interruption of the trp operon. We successfully repaired the trp operon, however this failed to improve the growth potential of the genderless strain relative to the wild type. Therefore, the growth defect of the genderless strain in certain conditions is likely due to the metabolic burden of expression the tra genes and any effects associated with DNA transfer and conjugation.

Conclusions

Though these works have had limited influence on improving the genderless strain, we have received some insights that can guide future steps. We observed that plate based mating is highly efficient indicating an intermittent plate based mating step within future evolutions could be advantageous, especially when improvement is stagnant, the increased recombination could act to regenerate diversity within a population. To attempt to implement the lambda red recombination machinery again, we plan to remove gam hopefully maintaining the native recombination frequency to combine the two mechanisms additively. Additionally, the directionality of ssDNA appears to impact its recombination frequency, to capitalize on this we plan on adding oriTs in the clockwise direction to try to maximize recombination frequency (Ellis, Yu, DiTizio, & Court, 2001).

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

In this work, the use of sexual recombination to enhance phenotype development in microbial factories has been heavily characterized. Initially, we determined that there are several key factors playing a role in the frequency of genetic exchange both influenced by internal and external forces. The role of HGT has been determined to reduce genetic load in evolving populations. The positive impact of this cannot be understated as often the downstream characterization of successful evolutions is the most time consuming and expensive step. Not only can a reduced genetic load positively impact characterization, but also open the door to an increased mutation rate without many of the typical drawbacks associated with being a mutator. We examined this in several different adaptive landscapes and found that a strain proficient in HGT and with an increased mutation rate outperformed all other combinations of those factors. Further still, there is little direct evidence of recombination events occurring to reduce clonal interference, to address this, we were able to track down independent lineages all but confirming successful recombination occurred. These factors strongly supported the concept that recombination can combine various determinants. A natural extension of various factors for a single phenotype would be the combination of factors from various different phenotypes. The genderless strain was shown to be able to successfully combine phenotypes from different populations into a population of multi-tolerants.

Recommendations

In order to progress the work combining phenotypes, a study developing a stronger workflow improving modularity and the number of phenotypes that can be incorporated into a single organism is needed. A study of this kind would be a breakthrough and advance the ability to generate multi-tolerant strains and provide the requisite populations for a genomics study characterizing the compatibility of mechanisms of tolerance, a problem that has not been tackled with any depth. Additionally, the use of evolution can be limited to growth-coupled phenotypes. In the appendix, the use of biosensors to couple growth to production of a molecule of interest is discussed. The future of this technology, coupled with microfluidics and an emulsion-based evolution is a very promising direction for the field of strain development as we are still many years from developing strong whole cell models.

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APPENDIX A: STUDY OF THE COMPLEX PHENOTYPE OF PHLOROGLUCINOL STRESS ON *E. COLI*

Introduction

Phloroglucinol (PG) is an attractive compound for commercial scale biosynthesis. PG is a potential precursor for numerous different medicinal applications and easily used as a building block for nitrogen based impact resistant explosives. The complexity and the expense of current chemical synthesis methods provide strong motives for a bio-based approach. Current attempts to utilize biocatalysts for production have met with relative success. Zha *et al.* Achkar *et al.* and Cao *et al.* have reported yields of 1.28, 0.78 and 3.8 g/L titer respectively by inserting the *phlD* gene, responsible for PG biosynthesis from malonyl-CoA, into *E. coli* and optimizing metabolic pathways so as to shunt carbon flux towards PG synthesis (Achkar, Xian, Zhao, & Frost, 2005; Cao, Jiang, Zhang, & Xian, 2011; Zha, Rubin-Pitel, Shao, & Zhao, 2009). However, several groups have reported that high levels of production may be inhibited by the nature of PG. Specifically, (Cao et al., 2011) and (Zha et al., 2009) indicated that the toxicity of PG inhibited their yields. PG is not naturally found at high levels and its use beyond possible niche cell wall synthesis is unknown. Therefore it is imperative to determine its influence on cellular growth and the way in which cellular functions are impaired or perturbed to modify industrial strains appropriately for production. Here we used transcriptomics to study the influence of phloroglucinol on the growth of *E. coli* in an attempt to determine genetic determinants for further study.

Materials and Methods

Inhibition calculation

6 individual colonies of BW25113 were inoculated into LB for overnight growth. 5 µl of overnight culture was inoculated into 5 mL of LB with varying amounts of phloroglucinol (0-4 g/L). Each culture was then spread between 2 wells of a 96 well plate. The 96 well plate was put into a TECAN plate reader, with 270 rpm orbital shaking and 37^{o}C incubation.

Microarray

The transcriptomic study was performed as follows. BW25113 *E. coli* were grown at 37^{o}C in a 30 mL chemostat with a dilution rate of 0.2, a 5 hour generation time. The cells were grown in a modified M9, with no metal mix or 10% $CaCl_2$. 4 biological replicates were grown in either a control, no inhibitor condition, or supplemented with 1 g/L PG. Culture total RNA was harvested after 8 generations via vacuum filtration of the biomass and resuspension into 5 ml RNA later. The RNA was processed as in (Winkler & Kao, 2011). Genes were determined as up or down regulated by comparing the experimental, PG supplemented cultures, to the control cultures.

Knockout and Up-regulation

To construct the gene knockouts, we directly used the KEIO collection stock which are the appropriate wild type, BW2513 (Baba et al., 2006). The strains with

plasmids overexpressing different genes were constructed by extracting plasmid DNA from the appropriate ASKA collection clone (Kitagawa et al., 2006). The harvested plasmids were transformed into the BW25113 wild type used for the microarray data, using chemically competent cells, see (Green & Sambrook, 2012) for more details.

Results

An initial examination of the level of inhibition of growth exhibited in *E. coli* in the presence of PG was assayed by growing the wild type BW25113 in the presence of varying levels of PG, see Figure 38. Corresponding with the cited growth inhibition, PG reduces growth at concentrations of 1-3 g/L in M9 media.

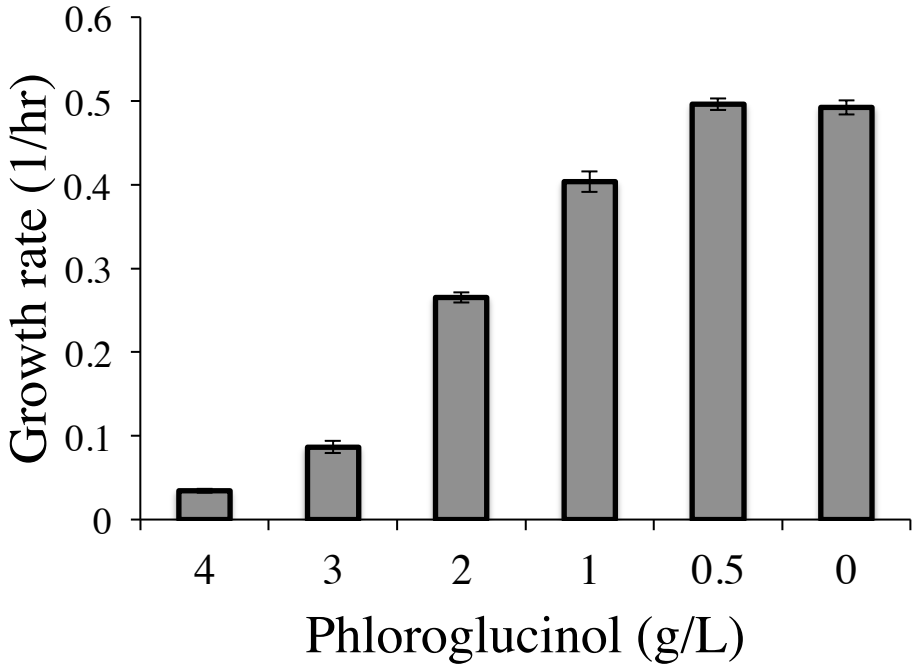

Figure 38: Growth rate of BW25113 on phloroglucinol in rich media. Error bars are standard deviation.

After determining the appropriate inhibitory concentration of PG on *E. coli*., a transcriptomic study was performed. We harvested total RNA from inhibited and uninhibited cultures grown in a chemostat for several generations. From the microarray data, a total of 23 genes were identified for further study based on their expression levels, Table 12.

Table 12: Table of genes chosen for additional study based on the microarray data. On the left is the genes that were statistically significantly up or down regulated (p-value<0.05), a one word description of each gene is given in the right column.

hdeA	acid
hdeB	acid
hdeD	acid
gadA	acid
gadB	acid
gadC	acid
gadW	acid
bhsA	copper
dps	iron
ompF	transport
ybfA	unknown
ymgE	unknown
rspB	stationary
rspA	stationary

Table 12: Continued

gatZ	galactitol
marB	drug
marR	drug
marA	drug
wrbA	quinone
yqjH	iron
yqjI	iron
patA	Putrescine
ydcS	Putrescine

Numerous genes were identified with increased expression that relate to acid tolerance, *gadA-C,E,W,X* and *hdeABD*. hdeABD proteins all play roles in acid resistance, hdeB is a chaperone in acid stress, *hdeA* produces a protein that plays an unknown role in resistance to low pH (Kern, Malki, Abdallah, Tagourti, & Richarme, 2006). hdeD is protein required for the ydeO induced acid resistance (Peng, Oliver, & Wood, 2005). gadAB form a glutamate decarboxylase that is coupled to gadC, an antiporter, to export 4-aminobutanoate and uptake L-glutamate to increase internal pH and survive acid conditions (J. Reizer, Ramseier, Reizer, Charbit, & Saier, 1996). These genes and their directly regulator *gadW* were chosen for study, the more global transcriptional regulators *gadE,X* were ignored for study. Two genes related to

putrescine uptake and catabolism, *patA* and *ydcS*, were also up-regulated. Putrescine is a polyamine with ill-defined regulatory roles within all cells. Curiously, one of the intermediates in the break down of putrescine is 4-aminobutryate. Putrescine is broken into 4-aminobutanal via patA and coupled to the conversion of 2-oxoglutarate to L-glutamate (Schneider & Reitzer, 2012). Via patD the 4-aminobutanal is broken into 4-aminobutanoate, the anti-porter substrate of gadC. ydcS is a putrescine ABC transporter (Schneider, Hernandez, & Reitzer, 2013).

Drug exporters *marARB* are reported to be associated with drug efflux oxidative stress and organic solvents (Alekshun & Levy, 1999; Cohen, Hächler, & Levy, 1993; White, Goldman, Demple, & Levy, 1997). Each of these genes exhibited negative expression relative to the control, no PG samples. It is possible that the reduced efflux increased PG tolerance if the exporters were leaky. However, this response is unexpected. Previous research has suggested that the *marARB* genes are induced when exposed to phenolic compounds and PG has a phenol-ring as the backbone for its structure. The counterintuitive response of the cell, and reduced expression for all three genes warranted further study so they were selected for their influence on growth in the presence of PG. Even more interesting is the opposite effect over expression of *marA* had on production levels of PG as reported by (Cao et al., 2011).

Several genes related to iron uptake and processing showed significantly reduced expression levels in the experimental PG group. *yqjH* and *yqjI* its regulator both exhibited reduced expression *yqjI* is the transcriptional regulator of *yqjH* which is a ferric reductase reducing iron for cellular use (S. Wang, Wu, & Outten, 2010). The

overexpressed *dps* is another gene related to iron, however it was up-regulated. It sequesters iron to serve as an antioxidant and is highly expressed in stationary phase (Rychlewski, Zhang, & Godzik, 1999).

The gene *wrbA* had high levels of overexpression, wrbA is reported to have quinone oxidoreductase activity. A quinone has a phenol ring and two ketone groups. This resembles the physical structure of PG, wrbA could have some substrate promiscuity and dehydrate PG (Patridge & Ferry, 2006).

All of the genes *gatA-D,YZ* displayed strong overexpression in the presence of PG. These genes are responsible for galactitol catabolism. *gatZ*, as opposed to all of the genes, was selected for study due to time constraints(J. Reizer et al., 1996).

The genes *rspAB* related to stationary phase regulation in *E. coli* were highly overexpressed (Huisman & Kolter, 1994). They are overexpressed to reduce the starvation sigma factor production in stationary phase. This response is contrary to what is expected as σ^S expression is associated with several stress responses.

The *bhsa* gene exhibited the lowest level of expression among all surveyed genes. bhsA is related to stress response and membrane permeability to copper (X. S. Zhang, Garcia-Contreras, & Wood, 2007). *ompF* was highly overexpressed. ompF facilitates the uptake of small molecules such as sugars, ions, and amino acids (Cowan et al., 1992) Two highly overexpressed genes, *ymgE* and *ybfA*, have no literature background describing their cellular function and were chosen based on their high level of consistent expression in each biological replicate.

To study the selected individual genes influence on survival of PG stress, the genes' corresponding overexpression or knockouts were isolated from the KEIO collection (Baba et al., 2006) and ASKA plasmid collection (Kitagawa et al., 2006). The ASKA collection isolates were harvested for plasmid after overnight growth in LB with chloramphenicol. Each of the genes was examined for growth benefits relative to the appropriate control, wild type genotype for KEIO and an empty plasmid control for the ASKA clones. We measured the growth rates of each in the presence of PG see Figure 39 and Figure 40.

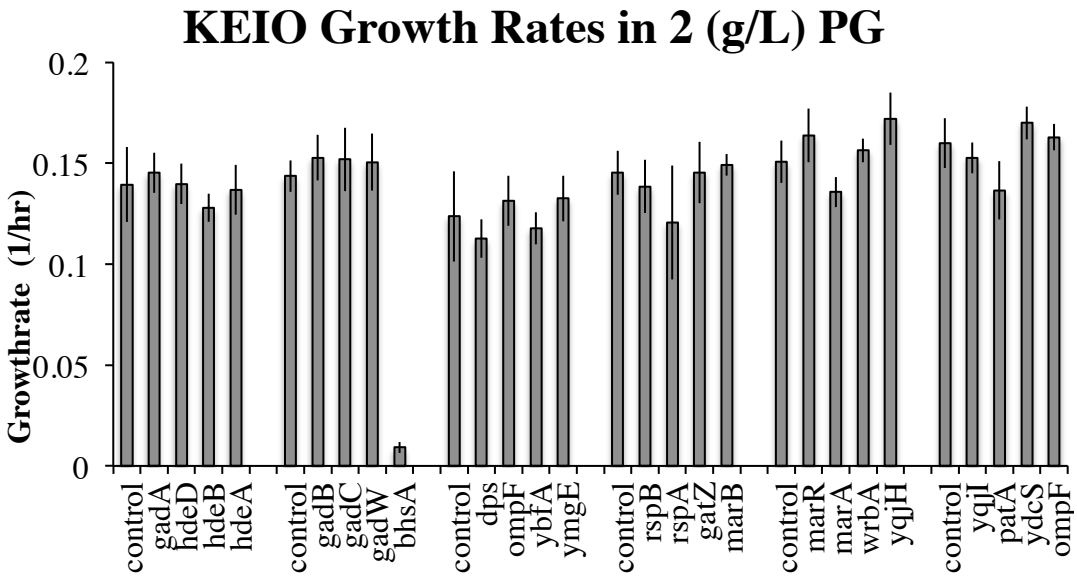

Figure 39: Growth rates of gene knockouts on 2 (g/L) phloroglucinol in minimal media.

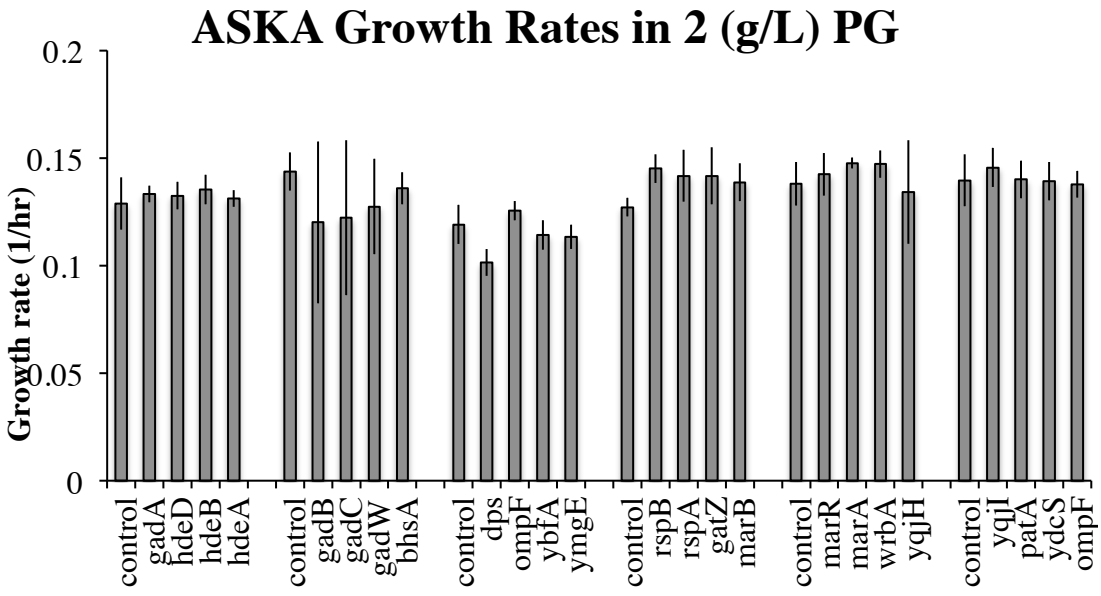

Figure 40: Growth rates of BW25113 carrying the ASKA plasmid for the corresponding gene with IPTG inducible expression on 2 (g/L) phloroglucinol in minimal media; induction was at 0.1mM IPTG.

There were no significant differences in growth rate between the control strains and the knockouts or overexpression of the 23 genes excepting *bhsA*, which displayed poor growth in the KO with and without the PG selective pressure.

Discussion

The results indicate the adaptive landscape of PG is highly complex where likely few single determinants, which strongly influence the development of a tolerant phenotype. To approach the development of an *E. coli* phenotype tolerant to PG, it may be necessary to introduce foreign genetic determinants. One approach would involve

screening for environmental samples that exhibit high levels of PG tolerance and to utilize their genomic DNA in a meta genomic library screen for PG tolerance.

APPENDIX B: TRI-ACETIC ACID LACTONE PRODUCTION VIA GENETIC CIRCUIT

Introduction

4-hydroxy-6-methyl-2-pyrone, triacetic acid lactone (TAL), is a target for biosynthesis that can be used to produce multiple intermediate and end products(Chia, Schwartz, Shanks, & Dumesic, 2012). TAL is naturally synthesized by a type III polyketide synthase (Tang et al., 2013). TAL can be utilized in a variety of reactions to generate attractive end products like sorbic acid, 1,3-pentadiene (Cardenas & Da Silva, 2014) and 1,3,5-trihydroxybenzene (phloroglucinol).

The Cirino group has previously developed a methodology for altering the binding site of a protein to select for biosensor mutants which are sensitive to alternative agonists. Tang *et al.* took *araC*, the arabinose regulatory protein, and found a mutant sensitive to TAL, *araC** (Tang, Fazelinia, & Cirino, 2008). If a biosensor for a target compound can be synthesized, it can effectively be coupled to growth with a genetic circuit to responding to the production of the compound. We utilized the *araC** mutant to couple the production of TAL to antibiotic resistance and thus survival. This allows an evolving population of cells to be selected for higher production of TAL.

In this work we also investigate the applicability of this type of genetic circuit to microfluidic-based evolution. Microfluidic evolutions and screens have been successfully used for more easily observable phenotypes (Agresti et al., 2010). However, little work has focused on transitioning the vast array of biosensors into growth-coupled

circuits and using them to evolve for improved production with high throughput microfluidic based screening.

Materials and Methods

Strains and plasmids

All strains used in this work were derived from HF19 (Tang et al., 2008). To this strain an arabinose inducible promoter driving the expression of the *bla* gene, for ampicillin resistance, was integrated into the chromosome to create strain SQ12. Three different plasmids containing the remaining pieces of the genetic circuit were constructed, ppcp1103, ppcc1155, and ppcc1101. Each plasmid has apramycin resistance gene for maintenance, lacIq repressed gene 2-PS for triacetic lactone synthesis, and a constitutively expressed *araC** that binds with TAL instead of arabinose to sense production. The plasmids have the following differences, ppcc1155 has no *araC** gene, and ppcc1103 has a mutation in 2-PS to increase its efficiency. Each plasmid was transformed into the SQ12 background. Two additional strains were constructed, SQ12-ppcc1155 and SQ12-ppcc1103, which were transfected with a constitutively expressed GFP (Reyes et al., 2012).

Evolutions

The strains SQ12-ppcc1155 and SQ12-ppcc1101 were evolved in rich media for approximately 100 generations with ampicillin challenge. The media was supplemented with 40 mM IPTG and 50 μg/mL apramycin. Every 24 hours the populations were

passaged and the final OD_{600} was measured. If a culture's final OD_{600} reached above a certain OD_{600} (transfer 1*0.75) the ampicillin concentration was ramped up 200 μg/mL. Strains SQ12 and HF19 were evolved similarly.

rt-PCR

rt-PCR was used to quantify the *bla* gene expression of the initial and final populations. Cells were grown up and harvested as in (Winkler & Kao, 2011) in rich media at mid exponential phase with 40 mM IPTG and 50 μg/mL apramycin. RNA was extracted and purified. The relative change in expression of *bla* was compared to the housekeeping gene *rrsA* and then each normalized ΔCq value was compared between the initial and final population (Livak & Schmittgen, 2001).

Droplet Growth Characterization

Single colonies of the SQ12::GFP-ppcc1155 and SQ12::GFP-ppcc1103 strains were inoculated into rich media and grown up overnight with 50 μg/mL streptomycin and 50 μg/mL apramycin. Each culture was diluted 600 fold into fresh rich media with 40 mM IPTG, 400 μg/mL ampicillin, and 50 μg/mL apramycin. The cultures were grown at 275 rpm with orbital shaking in a 96 well plate at 37^{o}C. Similarly the same cultures were emulsified to hold 8-10 cells per droplet and grown in a microfluidic chamber. The growth was tracked by area averaged fluorescence measurements.

TAL measurements

Isolates of interest were grown up in rich media with 40 mM IPTG and 50 μg/mL apramycin overnight. The cultures were diluted to OD_{600} 0.01 in fresh media and grown for 24 hours. The cells were filtered out and the remaining culture run on the HPLC for 40 minutes. A reference curve was constructed with standards of TAL.

Results

The initial genetic circuit to couple growth to triacetic lactone production was constructed such that strains that produced more TAL, would be able to turn on the arabinose induced *bla* gene with the mutant *araC**, providing them resistance to exogenous ampicillin challenge. We hypothesized, as the populations evolved, sub-populations with a higher production of TAL would be able to induce a larger production of *bla*, have higher resistance to ampicillin, and out compete lower producing cells in the population. To test this hypothesis, we evolved two genotypes, those with the *araC* mutant required for TAL sensing, and those without, strains SQ12-ppcc1103 and SQ12-ppCC1101 respectively. The cells were evolved with a ramping ampicillin challenge dependent on final cell density after 24 hours of growth. We expected that the strain with the *araC** gene would be able to more rapidly accumulate ampicillin resistance while simultaneously producing more TAL. The evolution metrics are plotted below in Figure 41.

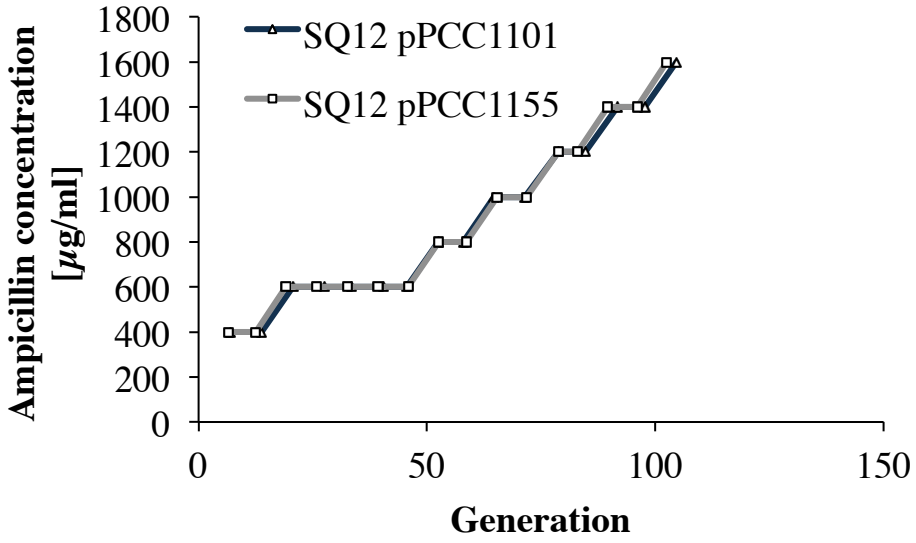

Figure 41: Evolution metrics for the SQ12 strain with two different plasmids, ppcc1155 and ppcc1103. Each was grown on rich media with ramping ampicillin concentration. Ampicillin concentration versus generations is plotted

Unfortunately, the control populations accumulated ampicillin resistance just as rapidly the experimental populations. The strains could be either mutating to express the ampicillin resistance gene constitutively, bypassing the genetic circuit, or accumulating mutations to give it spontaneously higher ampicillin tolerance independent of the circuit. To test if the mutations were related to *bla* expression, decoupling ampicillin resistance gene expression from TAL production, we performed rt-PCR on the evolved isolates to determine if they had higher transcript copy number. We graphed the relative expression of *bla* at the first and last time point relative to a reference gene in Figure 42.

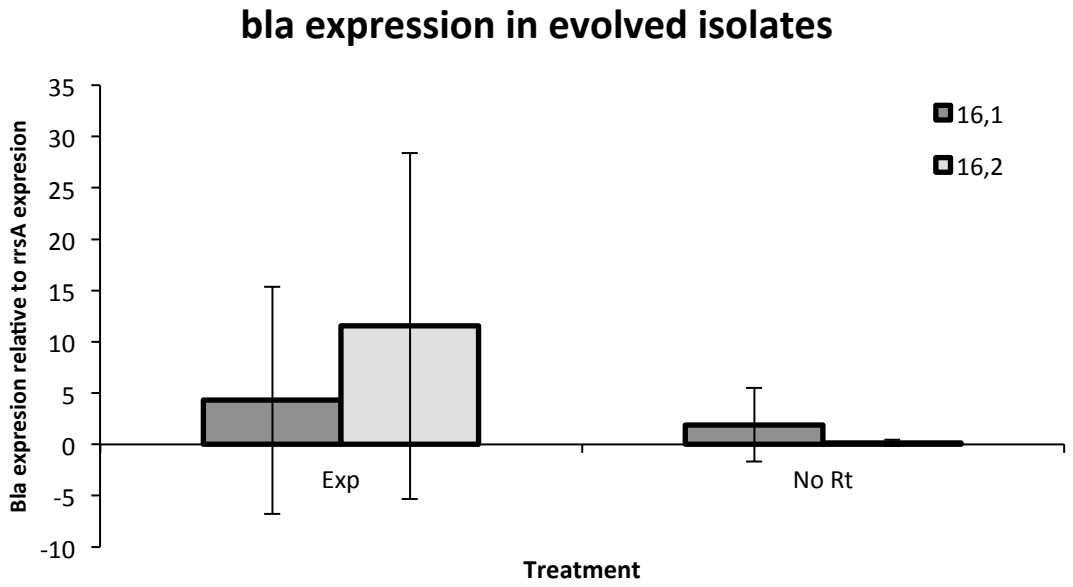

Figure 42: *bla* relative expression from qrt-PCR for two isolates from 2 different final populations between the first and last transfers.

We found that the *bla* gene expression was not significantly perturbed between the two time points indicating that the accumulated ampicillin resistance was likely not connected to the genetic circuit see Figure 42. To probe the alternative hypothesis, that the strains were accumulating spontaneous resistance through alternative mechanisms of tolerance we evolved 2 control strains with no genetic circuit, wild type SQ12 and the parental strain HF19. Due to leaky expression of the *bla* gene, SQ12 had a stronger initial resistance to ampicillin.

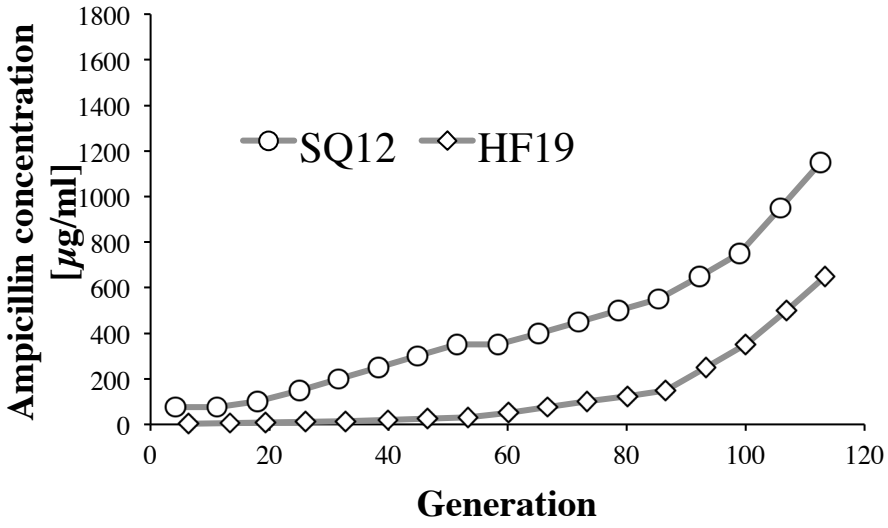

Figure 43: Evolution metrics for the strains SQ12 and HF19. Each was grown on rich media with ramping ampicillin concentration. Ampicillin concentration versus generations is plotted.

From Figure 43, it is clear that resistance accumulates quite rapidly for the native strains without the genetic circuit or even the *bla* gene, indicating the evolution with the genetic circuit was not successful due to the speed at which natural tolerance develops.

The strains were developing tolerance very rapidly to ampicillin when grown together, allowing cheaters to potentially thrive. We wished to study if a microfluidic approach, separating the cheaters from the producers could be a viable alternative. We first aimed to determine if difference could be detected between the complete and partial genetic circuits in a micro-droplet growth. We initially determined that the largest difference between the strains with the complete genetic circuit and those without is the duration of the lag phase before strain growth Figure 44.

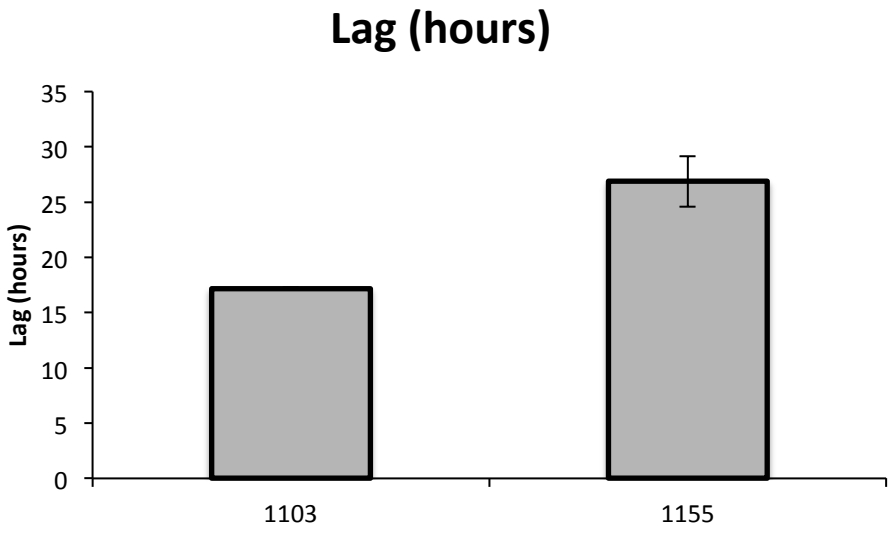

Figure 44: Lag time for 2 strains in 400 µg/mL ampicillin with 50 µg/mL apramycin and 40 mM IPTG. 1103 is SQ12::GFP-ppcc1103, 1155 is SQ12::GFP-ppcc1155.

To test if this difference could be observed in another laboratory and inside a micro-droplet, we grew the fluorescent variants of strains SQ12-ppcc1103 and SQ12-ppcc1155 in a plate reader and inside micro-droplet emulsion with each droplet contacting approximately 10 cells.

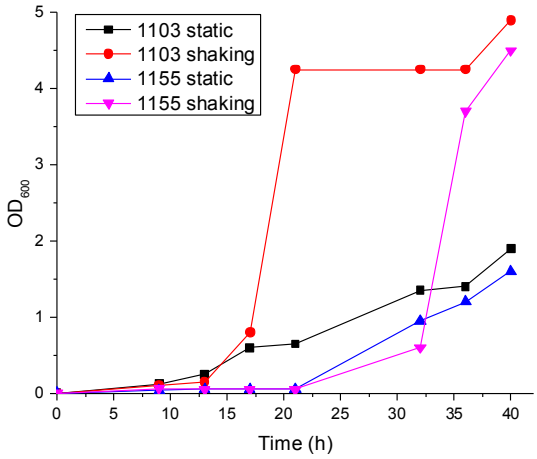

Figure 45: Growth curves for static and shaking growth of 2 different strains in in 400 µg/mL ampicillin with 50 µg/mL apramycin and 40 mM IPTG in the Han lab. 1103 is SQ12::GFP-ppcc1103 and 1155 is SQ12::GFP-ppcc1155.

We found the lag time between the micro-plate reader and the plate readers was highly consistent Figure 45.

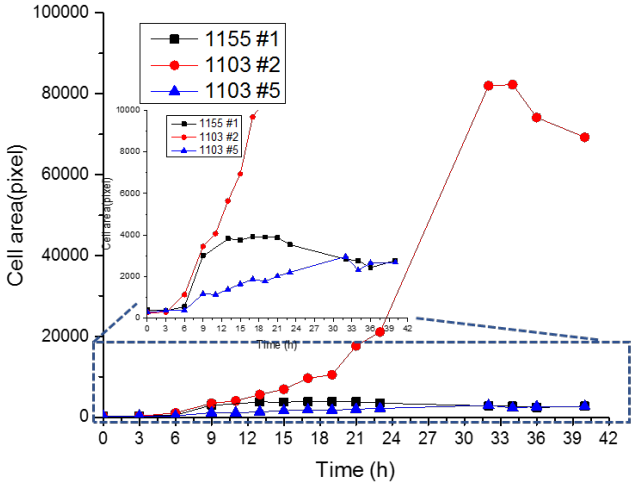

Figure 46: Growth curves based on average droplet fluorescence for several different droplets containing 2 different strains in 400 µg/mL ampicillin with 50 µg/mL apramycin and 40 mM IPTG. 1155 in black is SQ12::GFP-ppcc1155, 1103 in blue and red is SQ12::GFP-ppcc1103.

The cells were then grown in micro-droplets of the same media and fluorescence area over time tracked. It was determined that some fraction of the population of cells behaved as expected, however another fraction of the population grew more similar to the control incomplete genetic circuit, see Figure 46.

Discussion

The use of biosensors as parts of genetic circuits to couple growth to production of any sensible molecule could provide a powerful tool for strain engineering for screening with both evolutionary and rational approaches. Here we have demonstrated the construction of a genetic circuit for coupling TAL production to strain growth. However, the model circuit was unable to successful evolve higher production levels.

We observed a high tolerance increase in the control strains, equivalent to that of the TAL producer. This is likely due to the high rate of spontaneous mutations increasing ampicillin tolerance relative to the frequency of mutations that increase TAL production.

Fortunately, the evolved populations did not evolve tolerance by mutating the genetic circuit directly, as we observed no change in the level of expression of the *bla* gene in the evolved isolates. We believe this indicates the circuit could be a functional candidate for ALE if the growth couple phenotype, in this case *bla*, is less prone to strong mutations increasing tolerance. To determine if we could transition this type of genetic circuit to a more functional platform that will eliminate cheaters, we tested if the genetic circuit would function in a micro emulsion and droplet based growth. The test system functioned adequately, it may be capable of high throughput screening and evolutions using growth coupled genetic circuits.