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Harnessing recombination to speed adaptive evolution in *Escherichia coli*

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ABSTRACT

Evolutionary engineering typically involves asexual propagation of a strain to improve a desired phenotype. However, asexual populations suffer from extensive clonal interference, a phenomenon where distinct lineages of beneficial clones compete and are often lost from the population given sufficient time. Improved adaptive mutants can likely be generated by genetic exchange between lineages, thereby reducing clonal interference. We present a system that allows continuous *in situ* recombination by using an *Escherichia coli* F-based conjugation system lacking surface exclusion. Evolution experiments revealed that Hfr-mediated recombination significantly speeds adaptation in certain circumstances. These results show that our system is stable, effective, and suitable for use in evolutionary engineering applications.

1. Introduction

Evolutionary engineering encompasses a range of powerful methodologies that have been harnessed to improve a range of microbial phenotypes of industrial interest (Smith and Liao, 2011; Basso et al., 2011; Cadiere et al., 2011; Wisselink et al., 2010). One of the principal challenges that reduces the effectiveness of adaptive evolution experiments in producing enhanced phenotypes of interest is competition between clonal populations of microbes with different but beneficial mutations (Gerrish and Lenski, 1998; Elena and Lenski, 2003; De Visser and Rozen, 2006; Sniegowski and Gerrish, 2010). This phenomenon, known as clonal interference, pits mutants with different beneficial mutations against one another in a contest for survival. Due to the lack of a mechanism for sexual recombination in most microbes, this competition generally results in the loss of adaptive genotypes from the population over time. Recombination between these lineages, in contrast, would reduce clonal interference by allowing multiple lineages to combine beneficial mutations into a single background, preventing the extinction of these alleles (Crow and Kimura, 1965). The loss of genotypic history due to clonal interference impedes understanding of industrially relevant complex phenotypes (Patnaik, 2008) by limiting our knowledge of the underlying molecular mechanisms governing the phenotype in question. Beneficial mutations must be acquired sequentially in an asexual population as well, theoretically limiting the overall pace of adaptation. Sexual populations avoid this problem by continuously exchanging beneficial mutations through mating, resulting in increased adaptation rates (Kim and Orr, 2005), reduced mutational load (Rice, 1998), along with many other benefits (De Visser and Elena, 2007).

Currently, the only broadly applicable method available for inducing recombination in asexual organisms is protoplast fusion (Stephanopoulos, 2002), where two cells physically merge together and produce recombinant progeny via interchromosomal recombination (Petri and Schmidt-Dannert, 2004). Even though this technique has been successfully applied to improve several desirable complex phenotypes (Patnaik et al., 2002; Zhang et al., 2002; Dai and Copley, 2004; Otte et al., 2009; Winkler et al., 2010; Reyes et al., 2012), protoplast fusion generates low yields of stable recombinants (0.05–0.7%) (Dai et al., 2005; Winkler et al., 2010) and many of these individuals revert to parental phenotypes over time (Hotchkiss and Gabor, 1980). Another, typically overlooked, disadvantage of genome shuffling is that the method requires the interruption of adaptive evolution, so that direct selection for improved recombinants is not usually possible. Allowing for continuous genetic exchange *in situ* sidesteps this issue entirely by allowing strains with competing beneficial mutations to exchange genetic information while remaining under the desired selective pressure.

DNA transfer between *Escherichia coli* cells is mediated by fertility plasmids such as F, originally discovered by Esther Lederberg and colleagues in the early 1950s as a "sexual factor" that could be physically transferred between different *E. coli* strains (Cavalli et al., 1953). F-conjugation is unique in that it supports mating in liquid media, rather than on filters or agar as required for other conjugative systems (Ippen-Ihler and Minkley, 1986), making it well-suited for use in evolutionary engineering. Decades of subsequent work by generations of scientists uncovered various aspects of F biology, including its occasional integration into the host chromosome to create high frequency

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recombination (Hfr) strains, gene function within the transfer (*tra*) operon (Frost et al., 1994), and eventually biotechnological applications of F such as exploration of genetic interactions or bacterial artificial chromosomes (Typas et al., 2008; Sambrook and Russell, 2001). Hfr strains are capable of transferring chromosomal DNA from the integrated F origin of transfer (Snyder and Champness, 2007), making these strains useful for strain construction. Overall, the F plasmid is well characterized and amenable to engineering for the creation of a sexual recombination system in *E. coli*. Hfr strains and yeast evolution incorporating mating have also been previously used without modification to experimentally demonstrate that recombination leads to improved evolutionary outcomes, in certain circumstances (Cooper, 2007; Gray and Goddard, 2012; Nakajima, 2012).

High frequency recombination strains are of particular interest as they are capable of transferring chromosomal DNA from the donor to an F^- recipient. The process of Hfr mating at its core involves the formation of a mating bridge between the Hfr donor and F^- recipient, followed by transfer of single stranded DNA derived from the host chromosome, as demonstrated in Fig. 1. The recipient has a high probability of integrating the transferred DNA via homologous recombination due to the genetic similarity of most *E. coli* strains (Babić et al., 2008). Hfr strains can theoretically transfer their entire chromosome, but in practice this rarely occurs due to DNA breakage or disruption of the mating pair. Mating specificity is controlled by two proteins within the *tra* operon (TraS, TraT) which efficiently act to reduce Hfr–Hfr DNA transfer by 100–300 fold via surface exclusion (Willetts, 1974; Achtman et al., 1977; Garcillán-Barcia and de la Cruz, 2008); genetic transfer in a mixed population of Hfr and F^- cells is therefore unidirectional. Efficient bidirectional (*i.e.* Hfr $\rightleftharpoons$ Hfr mating) conjugation therefore requires manipulation of *tra* expression and the gender proteins to ensure that Hfr–Hfr matings could occur under typical growth conditions. Several studies have shown that F^+–F^+ and Hfr–Hfr matings can occur if TraS or TraT are mutated (Willetts, 1974; Achtman et al., 1977). An Hfr strain with inactive TraST proteins is therefore capable of efficiently transmitting and receiving chromosomal DNA from other individuals in the population, representing a form of sexual exchange in an otherwise asexual organism.

We propose to harness this phenomenon by engineering an effectively "genderless" Hfr strain that is competent as a DNA donor and recipient in conjugation with other Hfr individuals for use in adaptive evolution. This approach exploits the ability of Hfr strains to efficiently transfer chromosomal DNA that has been leveraged on countless occasions since their identification. Mating will occur continuously under selective conditions without the need for any external intervention. Genetically heterogeneous populations of genderless cells will constantly exchange genetic material, potentially increasing the rate of adaptation within a population and improving evolutionary outcomes for improved tolerance or growth. Unlike other methods for recombining *E. coli* genomes, mating can occur under a constant, arbitrary selective pressure, reducing the need to screen potential recombinants generated by other procedures while directly selecting for positive epistasis among existing mutations automatically.

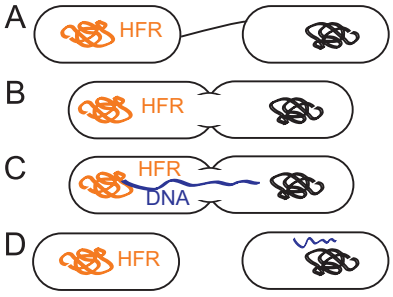

Fig. 1. A summary of the general F-mediated conjugation process in *E. coli*. **A.** The pilus of the male (Hfr) strain, which contains an F plasmid integrated into the genome, latches onto a F^- recipient. **B.** After cell-cell contact is made, a mating bridge is formed to allow for stable DNA transfer. Note that *traS* and *traT* are thought to inhibit this step by interfering with the mating bridge. **C.** Single-stranded DNA is transferred from the donor to recipient from the origin of transfer on the donor chromosome. **D.** Once transfer is complete or disrupted in some way (mechanically, chemically), the donor and recipient separate. Any transferred DNA may be integrated into the recipient chromosome via homologous recombination.

2. Methods and materials

2.1. Strain construction

All strains used for evolution experiments in this study are derivatives of BW25113 (Coli Genetic Stock Center, CGSC). A list of all strains used in this study is given in Table 1. The origin of transfer sequence from X892 (CGSC) was PCR amplified (Phusion, NEB) and cloned into the Pst1 site of the pKD13 (*kan*) and pKD32 (*cat*) disruption plasmids developed by Datsenko and Wanner (2000). Clones containing origin of transfer (oriT) fragments in the same orientation were used in all subsequent experiments. Two pseudogenes identified on EcoCyc (Keseler et al., 2011), *mbhA* (5.39 min) and *hyfC* (56.08 min), were chosen to be replaced by the oriT cassettes. To create strains BW25113-A and B containing oriT sites at these locations, the cassettes from pKD13 and pKD32 were amplified using previously described procedures (Baba et al., 2006) and were then subsequently transformed into BW25113/pKD46. Transformants were verified with PCR and sequenced (MCLAB, CA) to ensure no mutations occurred in the oriT region. Following this verification step, P1 transduction was used to transfer the *Δ*(hyfC)::[oriT cat] marker from strain B to A, creating BW25113 2xOriT. CAG3101 (Typas et al., 2008) and CAG31031 *Δ*(*traST*) were separately used to introduce F into the genome of BW25113 2xOriT via conjugation to construct Hfr-2xSFX+ (active surface exclusion) and Hfr-2xSFX− (*Δ*(*traST*), surface exclusion deficient). Both strains were screened to ensure that an additional *oriT* linked to *tetAR* was not transferred as well. The 2xOriT, Hfr-2xSFX+, and Hfr-2xSFX− strains were then transformed with pCP20 and incubated at 43 °C overnight to eliminate the antibiotic markers in the cassettes. The final strains (hereafter referred to as the evolution strains) have either no antibiotic resistance cassettes (2xOriT) or are gentamicin resistant (Hfr-2xSFX+,−). Presence and orientation of the cassettes and the F plasmid in these strains was verified by PCR.

2.2. Strain characterization

Microplate assays (TECAN) were used to assess the growth phenotypes of the evolution strains and to test their sensitivity to several inhibitors (chloramphenicol, trimethoprim, butanol, acetate). Growth assays were performed in M9 minimal media supplemented with 50 μg/ml tryptophan and 0.5% (w/v) glucose. Antibiotic tolerance was evaluated in M9 media supplemented with 0−8 μg/ml of drug (chloramphenicol (CM), streptomycin (STR), trimethoprim (TM)). Hfr-2xSFX− was slightly more sensitive to trimethoprim than the other evolution strains and did not initially tolerate TM concentrations greater than 0.8 μg/ml. Hfr-2xSFX+ was also found to be approximately 50% more resistant to streptomycin prior to the evolution experiment. The CM tolerances of the strains were similar. These differences could be the result of jackpot mutations or metabolic changes triggered by the presence of the F plasmid in the strains. The chloramphenicol concentration that reduced the final biomass yield (optical density)