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Benefits of a Recombination-Proficient *Escherichia coli* System for Adaptive Laboratory Evolution

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ABSTRACT

Adaptive laboratory evolution typically involves the propagation of organisms asexually to select for mutants with the desired phenotypes. However, asexual evolution is prone to competition among beneficial mutations (clonal interference) and the accumulation of hitchhiking and neutral mutations. The benefits of horizontal gene transfer toward overcoming these known disadvantages of asexual evolution were 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 the 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 the experimental data. Our results provide strong support for the advantages of utilizing the genderless strain over its asexual counterpart during adaptive laboratory evolution for generating beneficial mutants with reduced mutational load.

IMPORTANCE

Over 80 years ago Fisher and Muller began a debate on the origins of sexual recombination. Although many aspects of sexual recombination have been examined at length, experimental evidence behind the behaviors of recombination in many systems and the means to harness it remain elusive. In this study, we sought to experimentally validate some advantages of recombination in typically asexual *Escherichia coli* and determine if a sexual strain of *E. coli* can become an effective tool for strain development.

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 increasing genetic diversity via the use of a mutagen (UV, ethyl methanesulfate [EMS], etc.) or a mutator strain (1, 2). Due to the ease of experimentation, in the majority of cases, microbes are propagated asexually (even when with use of sexual organisms such as *Saccharomyces cerevisiae*). However, there are two known limitations associated with asexual evolution: clonal interference wherein competition among beneficial mutations leads to a loss of information and Muller's ratchet where fixation of deleterious mutations through genetic drift leads to an irreversible decline in strain fitness. One of the theorized benefits of sexual recombination is the reduction of interclonal competition by combining beneficial genotypes that coexist in the population, thereby mitigating the negative impacts of clonal interference and enhancing the rate of strain improvement (3). Similarly, a sexually competent strain can minimize Muller's ratchet by reducing the accumulation of neutral and deleterious mutations through recombination between different genotypes (4, 5).

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 in adaptation. In general, sexual recombination has been demonstrated to be helpful in speeding adaptation in large populations with higher mutation rates from both experimental and computational analyses (6–11). Depending on the model/system parameters, sexual recombination has also been shown to aid in increasing genetic diversity by generating new genotypes via recombination (12, 13); conversely, it has also been predicted to lead to a breakup of desirable characteristics (14–16). In addition, previous theoretical work suggests that at a particular mutation rate, there exists an

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optimal recombination rate that maximizes the rate of adaptation (7, 12). Muller's ratchet has been predicted to be a function of effective population size and rates of recombination and mutation (17–19), and at a given population size, recombination is believed to reduce the risk of accumulation of deleterious mutations (19, 20). Furthermore, there is strong evidence for increased mutation rates, leading to mutational meltdowns in certain simulated and experimental populations (21–23). Computational work on evolving populations has further shown that high mutation rates and small effective population sizes in asexual populations exacerbate the effects of Muller's ratchet, and in the presence of sexual recombination, this effect is reduced (17, 19, 20, 24). With the availability of affordable next-generation sequencing technologies, experimental evidence supporting Muller's ratchet is accumulating. For example, Lang et al. found that hitchhiking of neutral mutations is prevalent in asexually evolving populations of *S. cerevisiae* (25); more recent work in *S. cerevisiae* by McDonald et al. found that hitchhiking mutations were more prevalent in asexually evolving populations than in evolving populations subjected to periodic recombination (5).

While there have been extensive computational analyses, due to limited available experimental methodologies, detailed verifications of the effects of recombination during ALE are lacking (especially in bacteria). Although the 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 (5, 26–30). To address this limitation, we had previously developed a "genderless" strain of *Escherichia coli* that is capable of continuous bidirectional conjugation during adaptive laboratory evolution (10).

Conjugation is a set of natural processes 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^-) (31). 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 (32). At low frequencies, the F plasmid is spontaneously integrated into the chromosome to form high-frequency recombination (HFR) strains (33). In HFR strains, chromosomal DNA can be transferred from the donor to the recipient cell (33). 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 (9), 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 (10). 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 the frequency of HFR transfer throughout the chromosome, as little existing work has focused on characterizing and expanding the use of genetic transfer in the F plasmid conjugation system (34–38). Our results suggest that the additional oriTs can be harnessed by the genderless strain to increase the coverage of the 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 (10), which are derivatives of BW25113 K-12 *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*::Kan^r (kanamycin resistance) in the KEIO collection (39) and moving it to the genderless and asexual control strains (see Table 1 for genotypes). The mutation rates of the resultant Δ*mutS* strains HFR-2xorit-SFX- Δ*mutS*::Kan^r (genderless mutator) and BW25113 2xoriT Δ*mutS*::Kan^r (asexual 2xoriT mutator) were measured using the standard fluctuation test (40) and were estimated to be 200 to 300 times higher than that for the nonmutator wild-type (WT) strain, which are within the expected range for the Δ*mutS* genotype (41, 42).

The two extra oriTs in the chromosome of HFR-2xoriT-SFX- genderless strain were removed using the procedure outlined by Datsenko and Wanner (43) 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 and oriTv_mbhA_f and oriTv_hyfC_r and oriTv_hyfC_f in Table 2).

Six strains of BW25113, each containing a single antibiotic marker, were constructed by inserting antibiotic resistance genes at different locations within the chromosome: *hisB*::Kan^r and *glnA*::Kan^r (kanamycin resistance) markers were transduced from the KEIO collection; the *zje*::Tn*10* and *zdi*::Tn*10* (tetracycline resistance) markers were transduced from strains BW6156 and BW5659 (44), respectively; and the *ybhL*::*cat* and *G6272*::*cat* (chloramphenicol resistance) markers were constructed using the method outlined in reference 43 using the primers listed in Table 2. Three of the antibiotic markers, A (*ybhL*::*cat*), B (*zje-2005*::Tn*10*), and C (*hisB*::Kan^r), are located at ~11 min 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 2 for the 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*::Tn*10*, and *hisB*::Kan^r) was named 3m11 (see Fig. 1 for representative chromosomal maps). 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*::Cm^r [chloramphenicol resistance]), E (*glnA*::Kan^r), and F (*zdi-57*::Tn*10*), located at ~17 min away from each of the 3 oriTs in the chromosome were also individually introduced into HFR-2xoriT-SFX- 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 the list of primers used). Each set of 3 single-marker strains of HFR-2xoriT-SFX- and HFR-Δ2xoriT-SFX- was mated as described above to generate the triple-marked strains 3m17 and 3m17Δ2xoriT, respectively (see Fig. 1 for representative chromosomal maps).

TABLE 1 Strains used

Name or description	Strain	Genotype/features[a]	HFR	Reference or source
JW3841	BW25113	Δ*glnA*::*kan*	N	39
JW2004	BW25113	Δ*hisB*::*kan*	N	39
QP1	BW25113	Δ*ybhL*::*cat*	N	This work
QP2	BW25113	Δ*G6272*::*cat*	N	This work
BW6156	BW6156	*zje-2005*::Tn*10* (Tet^r)	Y	CGSC
BW5659	BW5659	*zdi-57*::Tn*10* (Tet^r)	Y	CGSC
Antibiotic cassette	BW25113	pKD32	N	43
Helper plasmid	BW25113	pKD46	N	43
Removal plasmid	BW25113	pCP20	N	43
WT recipient	BW25113	pCL1920	N	45
Genderless	BW25113	Δ*mbhA*::oriT Δ*hyfC*::oriT *trp*::F[Δ*traST*] (Gen^r)	Y	This work
WT 2xoriT	BW25113	Δ*mbhA*::oriT Δ*hyfC*::oriT	N	This work
Genderless mutator	BW25113	Δ*mbhA*::oriT Δ*hyfC*::oriT *trp*::F[Δ*traST*] (Gen^r) Δ*mutS*::*kan*	Y	This work
Asexual 2xoriT mutator	BW25113	Δ*mbhA*::oriT Δ*hyfC*::oriT Δ*mutS*::*kan*	N	This work
QP3	BW25113	Δ*mbhA*::oriT Δ*hyfC*::*cat* trp::F[Δ*traST*] (Gen^r)	Y	This work
QP4	BW25113	Δ*mbhA*::*cat* Δ*hyfC*::oriT trp::F[Δ*traST*] (Gen^r)	Y	This work
QP5	BW25113	Δ*mbhA* Δ*hyfC*::oriT *trp*::F[Δ*traST*] (Gen^r)	Y	This work
QP6	BW25113	Δ*mbhA* Δ*hyfC*::*cat trp*::F[Δ*traST*] (Gen^r)	Y	This work
Genderless Δ2xoriT	BW25113	Δ*mbhA* Δ*hyfC trp*::F[Δ*traST*] (Gen^r)	Y	This work
QP7	BW25113	Δ*mbhA* Δ*hyfC trp*::F[Δ*traST*] (Gen^r) Δ*ybhL*::*cat*	Y	This work
QP8	BW25113	Δ*mbhA* Δ*hyfC trp*::F[Δ*traST*] (Gen^r) Δ*G6272*::*cat*	Y	This work
QP9	BW25113	Δ*mbhA* Δ*hyfC trp*::F[Δ*traST*] (Gen^r) *zje-2005*::Tn*10* (Tet^r)	Y	This work
QP10	BW25113	Δ*mbhA* Δ*hyfC trp*::F[Δ*traST*] (Gen^r) *zdi-57*::Tn*10* (Tet^r)	Y	This work
QP11	BW25113	Δ*mbhA* Δ*hyfC trp*::F[Δ*traST*] (Gen^r) Δ*glnA*::*kan*	Y	This work
QP12	BW25113	Δ*mbhA* Δ*hyfC trp*::F[Δ*traST*] (Gen^r) Δ*hisB*::*kan*	Y	This work
3m11Δ2xoriT	BW25113	Δ*mbhA* Δ*hyfC trp*::F[Δ*traST*] (Gen^r) Δ*ybhL*::*cat zje-2005*::Tn*10* (Tet^r) Δ*hisB*::*kan*	Y	This work
3m17Δ2xoriT	BW25113	Δ*mbhA* Δ*hyfC trp*::F[Δ*traST*] (Gen^r) Δ*G6272*::*cat* Δ*glnA*::*kan zdi-57*::Tn*10* (Tet^r)	Y	This work
SME	BW25113	Δ*mbhA*::oriT Δ*hyfC*::oriT *trp*::F[Δ*traST*] (Gen^r) Δ*glnA*::kan	Y	This work
SMC	BW25113	Δ*mbhA*::oriT Δ*hyfC*::oriT *trp*::F[Δ*traST*] (Gen^r) Δ*hisB*::*kan*	Y	This work
SMA	BW25113	Δ*mbhA*::oriT Δ*hyfC*::oriT *trp*::F[Δ*traST*] (Gen^r) Δ*ybhL*::*cat*	Y	This work
SMD	BW25113	Δ*mbhA*::oriT Δ*hyfC*::oriT *trp*::F[Δ*traST*] (Gen^r) Δ*G6272*::*cat*	Y	This work
SMB	BW25113	Δ*mbhA*::oriT Δ*hyfC*::oriT *trp*::F[Δ*traST*] (Gen^r) *zje-2005*::Tn*10* (Tet^r)	Y	This work
SMF	BW25113	Δ*mbhA*::oriT Δ*hyfC*::oriT *trp*::F[Δ*traST*] (Gen^r) *zdi-57*::Tn*10* (Tet^r)	Y	This work
3m11	BW25113	Δ*mbhA*::oriT Δ*hyfC*::oriT *trp*::F[Δ*traST*] (Gen^r) Δ*ybhL*::*cat zje-2005*::Tn*10* (Tet^r) Δ*hisB*::*kan*	Y	This work
3m17	BW25113	Δ*mbhA*::oriT Δ*hyfC*::oriT *trp*::F[Δ*traST*] (Gen^r) Δ*G6272*::cmR Δ*glnA*::*kan zdi-57*::Tn*10* (Tet^r)	Y	This work

[a] Tet^r, tetracycline resistance; Gen^r, gentamicin resistance.

Short mating assay protocol. WT (BW25113) cells carrying plasmid pCL1920 (45)-encoding *aadA* for streptomycin/spectromycin resistance (BW25113/pCL1920) were used as recipients 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-Bertani (LB) medium supplemented with the appropriate antibiotics. Each culture was normalized to an optical density at 600 nm (OD_{600}) of ~1, diluted 100-fold into 10 ml of LB medium supplemented with the appropriate antibiotics and incubated at 37°C at 225 rpm until mid-exponential phase (OD_{600} of ~0.5). Cells were pelleted by centrifugation at 5,000 × *g* for 3 min and resuspended to a normalized OD_{600} of ~0.5 in fresh prewarmed LB medium without antibiotics. One milliliter of donor (either 3m17 or 3m17Δ2xoriT) and 1 ml of recipient (BW25113/pCL1920) were mixed in approximately equal ratios and incubated for 105 min at 37°C 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 min to disrupt mating. Next, 100 μl of the mating mixture was immediately plated onto three separate LB plates supplemented with 100 μg/ml of streptomycin combined with either (i) chloramphenicol, (ii) tetracycline, or (iii) 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 Fig. 2). The plates were incubated for 24 h at 37°C, 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 and 3 in Fig. 2). The frequency of recombinants with cotransferred markers was calculated based on the number of colonies that displayed growth after 24 h of incubation at 37°C.

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 medium. Overnight cultures were normalized to an OD_{600} of ~1.0, and 20 μl of one of the donors and the recipient cell type was coinoculated into 2 ml of fresh LB medium. Each donor and recipient pair (at an initial OD_{600} of ~0.01) was allowed to mate in liquid culture at 37°C and 225 rpm. After exactly 24 h, the final mixture was plated on 4 different types of plates as follows. The total cell concentration was estimated by plating 50 μl of a 10^{-6} diluted culture onto a nonselective 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 (i)

TABLE 2 List of primers used

Name	Target	Direction	Sequence (5′ to 3′)	Usage
mbhA_H1	*cat*	Forward	TTGTCAACAGCGTCAGTAAATCCGAGCGTGAAAGCATTATCGCCGCGCTGT GTAGGCTGGAGCTGCTTC	Construction
mbhA_H2rc	*cat*	Reverse	GCACCACTTTATCCCCATGCTGACCAAAGTATTGATACAGCGTATCGGAAT GGGAATTAGCCATGGTCC	Construction
hyfC_H1	*cat*	Forward	ACCCCACTTTTTACGGGTATTTCCCGGCAGATACGCGCGCGTATGCACTGTG TAGGCTGGAGCTGCTTC	Construction
hyfC_H2rc	*cat*	Reverse	CCAGGTCACATGGTGAATGAGTAAAAAACGCCCGCGTGCCAGCGTGTTTAT GGGAATTAGCCATGGTCC	Construction
G6272_H1	*cat*	Forward	TGAGGGCCAG GGAACAAGTG GCGAAAATCG TATCAAAGAATGATCCAGATGTG TAGGCTGGAGCTGCTTC	Construction
G6272_H2rc	*cat*	Reverse	CCGCTGGTTTTCCGCTAATGGTTTATTTCCTCTTTCTTTCCCGGCAAGAAA TGGGAATTAGCCATGGTCC	Construction
ybhl_H1	*cat*	Forward	ATGGACAGATTCCCACGTTCTGATTCAATCGTACAACCCCGGGCTGGCTTGTG TAGGCTGGAGCTGCTTC	Construction
ybhl_H2rc	*cat*	Reverse	AACGGCGGTTGCCGAAGATCCGCAACAACATCAGGAACAGGTTGATGAAGAT GGGAATTAGCCATGGTCC	Construction
ybhl f	*ybhL*	Forward	ATGGACAGATTCCCACGTTC	Verification
ybhl r	*ybhL*	Reverse	CGAAGATCCGCAACAACATC	Verification
G6272 f	*G6272*	Forward	GGAACAAGTGGCGAAAATCG	Verification
G6272 r	*G6272*	Reverse	CCGCTGGTTTTCCGCTAATG	Verification
oriTv_mbhA_f	mbhA oriT	Forward	CAGAAACCTCGGAAATACGC	Verification
oriTv_mbhA_r	mbhA oriT	Reverse	GCATTGCTCACCTCTCAACA	Verification
oriTv_hyfC_f	hyfC oriT	Forward	GGCTGGCCAAAGAAATACAG	Verification
oriTv_hyfC_r	hyfC oriT	Reverse	AGATCAGCGACAACATGCAC	Verification
C1_veri_2	*ybhL*	Forward	CCTTGTCGCCTTGCGTATAA	Verification
C2_veri_2	*G6272*	Reverse	CCTACCTGTGACGGAAGATC	Verification
tetA_f_qp	Tn*10*	Forward	CCACTCCCTATCAGTGATAGAG	Verification
tetA_r_qp	Tn*10*	Reverse	CGGAATAACATCATTTGGTGACG	Verification
TnA_5v	Tn*10* out	Forward	GTTTTTGTTGTGATGTAGGCAT	Verification
TnC_5v	Tn*10* out	Reverse	TTAAAGTGATGATAAAAGGC	Verification
glnA_veri_f	*glnA*	Forward	GCCTCAGGCATTAGAAATAGCG	Verification
glnA_veri_r	*glnA*	Reverse	CGTAATGGATCGCCAGGTTG	Verification
hisB_veri_f	*hisB*	Forward	CGCTGGCAAACGAAGAAGTC	Verification
hisB_veri_r	*hisB*	Reverse	GGCATTGCGTAGCTGTGAAC	Verification

chloramphenicol, (ii) tetracycline, or (iii) 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-2xoriT-SFX- Δ*mutS*::Kan^r (genderless mutator) and BW25113-2xoriT Δ*mutS*::Kan^r (asexual 2xoriT mutator), were serially passaged for approximately 850 generations in LB medium. 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 medium. Then 10 μl of the final diluted mixture (10^{-6}) was inoculated into 2 ml of fresh LB medium. Based on the dilution used, ~50 to 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 medium at 37°C 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% (vol/vol) glycerol and stored at −80°C, and the genderless mutator populations were assayed to confirm their ability to mate.

Screening of growth-impaired mutants. Mutants with growth impairments on minimal 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 medium for overnight growth at 37°C at 225 rpm. The overnight cultures were diluted 10^{-6}-fold and then plated on nonselective LB plates to isolate single colonies. A total of 500 individual colonies from each population were randomly chosen and restreaked onto both M9 dropout medium plates supplemented with the amino acids (mg/liter) 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), and tyrosine (30) and replicate LB plates and incubated for 48 h at 37°C. Colonies that grew on LB plates but exhibited growth impairment (lack of observable growth after 48 h) on the M9 dropout plates were further verified for the growth impairment phenotype (see Fig. S1 in the supplemental material). For verification of clones with impaired growth on M9 dropout medium, a single colony from an LB plate was inoculated into two separate cultures, in 2 ml of M9 dropout medium, supplemented identically to the dropout plates, and 2 ml of LB medium. The cultures were incubated at 37°C and 225 rpm; those that exhibited an OD_{600} of <0.1 after 24 h 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.

Complementary mating of growth-deficient strains. Two growth-impaired and recombinant-proficient isolates from independently evolved populations were paired to determine if genotypes from 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 medium. The overnight cultures of each growth-impaired strain were normalized to an OD_{600} of ~1.0. Then equal volumes of the cultures were mixed and trans-

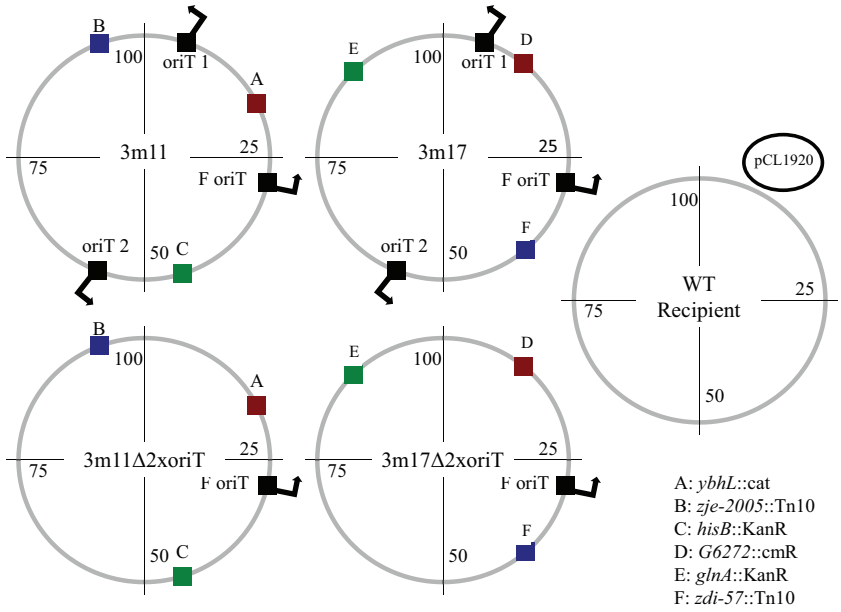

FIG 1 Chromosomal maps of the triple-marker strains. The positions labeled oriT are the origins of transfer, and the arrows indicate the directionality of DNA transfer. The markers are color-coded based on antibiotic resistance markers: chloramphenicol (red), tetracycline (blue), and kanamycin (green).

ferred to fresh LB medium with a starting OD_{600} of ~0.01; unmixed cultures were passaged individually as controls. The cells were incubated at 37°C for 24 h. The resulting cultures were then normalized to an OD_{600} of ~1.0, spun down, and resuspended in fresh M9 dropout medium, and 10 μl of each was transferred into 2 ml of M9 dropout medium to determine if the final culture contained any recombinants. An OD_{600} of >0.1 after 24 h of incubation at 37°C and 225 rpm was used as the criterion to determine mixtures that contained successful recombinants.

Complementary mating of single-marker strains. Overnight cultures of strains SMD and SMB (Table 1) were grown from single colonies in LB with the appropriate antibiotic at 37°C and 225 rpm for 24 h. The overnight cultures were normalized to an OD_{600} of ~1, and a 200-fold dilution of each strain was used to coinoculate a single test tube of 10 ml of LB medium (for a final mixture with an OD_{600} of ~0.01). Control cultures with single strains were diluted 100-fold into 10 ml of LB medium. The subcultures were incubated at 37°C and 225 rpm for 24 h. Each culture was periodically assayed for the presence of recombinants by diluting an appropriate volume of sample into fresh liquid LB medium and immediately plating 50 μl onto an LB plate supplemented with tetracycline and chloramphenicol; the sample was also plated on an LB plate with no supplements 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 various ratios of the two different single-marker strains, the same protocol was followed except that the initial mix of cells in the subculture was modified to maintain the same overall starting OD_{600} but with a change in the ratio of the two genotypes. A second pair for mating using strains SMA and SMF (Table 1) was also utilized to confirm the frequency of recombinant formation as a function of initial ratio of donor/recipient strains.

Model of bidirectional mating. The Matlab package SimBiology was used to model cell growth and mating kinetics using the mass action kinetic law (see Fig. 3 for model reactions). Several assumptions were made in the development of the model: (i) lag phase was incorporated by reducing the growth rate by 10^{-4} for the initial 30 min; (ii) 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 (an OD_{600} of ~0.4) and the 50-fold reduction in k_2 value appears to be consistent with previous predictions (46, 47); and (iii) the reaction rate constants for reactions 1 to 4 (cell growth) and A1 to A5, B1 to B5, C1 to C5, and N1 to 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-2xoriT-SFX- (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 (10). Since the transfer frequency of genetic material decays exponentially as distance increases from the oriT (33), 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 that of 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 min (strain 3m11 containing markers A, B, and C) or 17 min (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 h of mating. Since there is an ex-

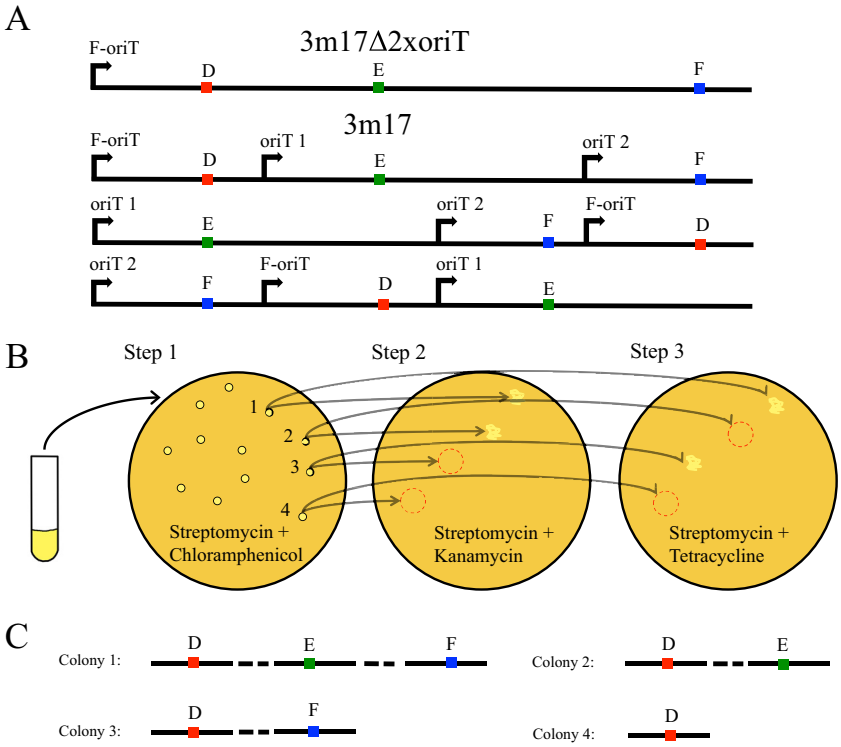

FIG 2 Short mating experiment. (A) Schematic diagram of the theoretical transfer orders used for the origin(s) of transfer in strains 3m17 and 3m17Δ2xoriT. (B) Schematic diagram of the short mating experiment. In step 1, mixed cultures of the 3m17 or 3m17Δ2xoriT donor and recipient are plated on dual-selection plates, streptomycin resistance (to select for recipient) and one of the antibiotics encoded by the triple-marked donor; for illustration purposes, chloramphenicol was chosen. In steps 2 and 3, the resultant colonies are streaked as replicates onto plates to select for resistance to one of the other three antibiotics, 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 has only the chloramphenicol marker. (C) Assumed marker composition for colonies 1 to 4 in the schematic diagram.

Growth

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

2: $B + S \xrightarrow{k_1} 2\,B$

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

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

Mating

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

Initial values

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

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

FIG 3 The set of reactions used in the Matlab model. S is a carbon source, A and B are cells with a single marker, C is a cell with both markers, and N is a cell with no markers.

ponential decay in the probability of transfer as the marker distance increases from an oriT, we hypothesized that the strains with the additional 2xoriTs (3m11 and 3m17) would yield higher recombination frequencies in markers E, B, C, and F (see Fig. 1 for marker locations) than 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 the markers furthest away from the F-oriT compared to those of the strains without the additional oriTs (Fig. 4) (P values of 0.0267 and 0.001 for the furthest 17-min [marker F] and 11-min [marker C] markers, respectively, using the one-tailed Student t test). This supports our hypothesis that the additional oriTs are active and help to increase the absolute coverage of genetic transfer during normal culture conditions.

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 the frequency of cotransfer of alleles. There are three primary factors influencing the frequency of observing cotransferred markers: distance from the upstream oriT, the order of the markers, and distance be-

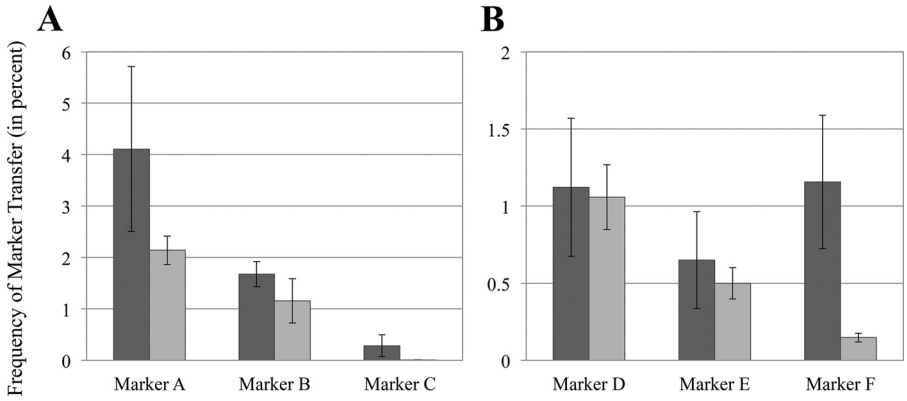

FIG 4 Percentage of recipient cells that received a marker from the triple-marker donor strain in the long mating assay. (A) Frequency of isolating recombinants of marker A, B, or C in the final recipient populations using donor strains 3m11 (dark gray) and 3m11Δ2xoriT (light gray). (B) Frequency of isolating recombinants containing marker D, E, or F in the final recipient populations using donor strains 3m17 (dark gray) and 3m17Δ2xoriT (light gray).

tween 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 the two markers increases, the chance of both markers being cotransferred 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 coinheriting marker D (closest marker downstream of F-oriT), and recombinants containing marker F would also have a higher frequency of coinheriting markers D and E.

To determine the correlation between the number of oriTs and marker cotransfer frequencies, we measured the frequency of marker cotransfer 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 min. 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 coinheritance of additional markers. If the oriTs were active, we would expect to see a different pattern of marker cotransfer 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 and the first marker D. However, as shown in Fig. 5, 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 cotransfer is expected (Fig. 5), as DNA transfer events can initiate from any oriT as illustrated in Fig. 1. In 3m17, DNA transfer events that initiate at F-oriT 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 cotransfer 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 cotransfer between these markers, especially marker pairs F-D and D-E.

The data showed that the addition of the two oriTs significantly altered the frequency of marker coinheritance (Fig. 5). In some cases, the expected pattern of marker coinheritance was observed; for example, isolates of marker F are linked with those of both D and E. The noted differences in the patterns of cotransfer suggest that the additional oriTs were active in initiating transfer during conjugation, as the frequency of coinheritance is higher in the strains with the extra oriTs in the majority of cases.

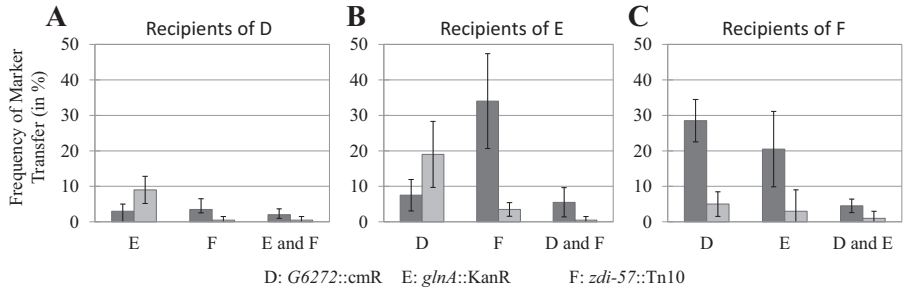

FIG 5 Frequency of marker cotransfer after 105 min of mating between donor strain 3m17 (dark gray) or 3m17Δ2xoriT (light gray) and recipient strain WT/pCL1920 (streptomycin resistance). (A) Percentage of recipient cells that inherited marker D and also coinherited either marker E or F or both markers E and F. (B) Percentage of recipient cells that inherited marker E and also coinherited marker D or F or both markers D and F. (C) Percentage of recipient cells that inherited marker F and also coinherited either marker D or E or both markers D and E.

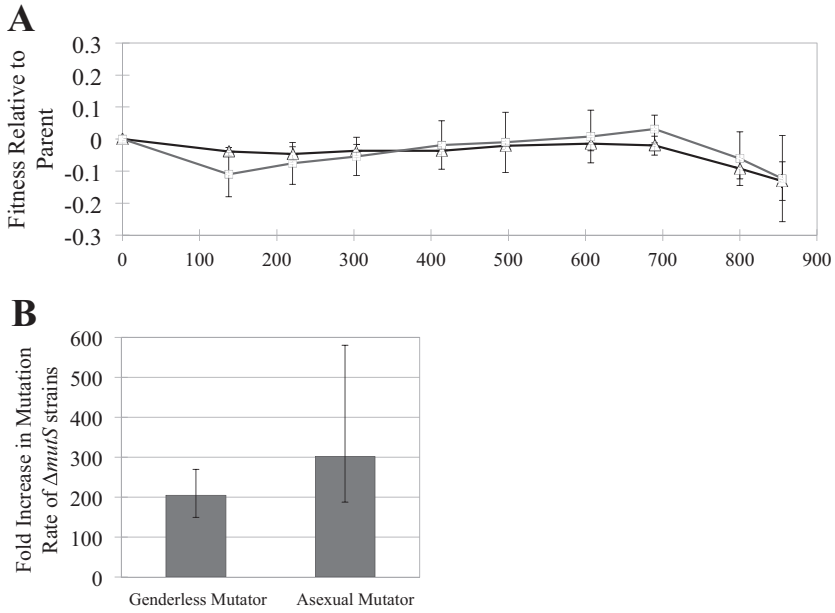

FIG 6 Mutation rates and fitness for the adaptive evolution of the asexual 2xoriT mutator strain and genderless mutator strain on LB medium. (A) Mean population fitness changes of evolving populations (averaged across all 6 parallel populations per strain) and asexual 2xoriT mutator (triangle) and genderless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

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 of our strain by deleting *mutS*, the mismatch recognition component of the mismatch repair system (48, 49), 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- to 300-fold in both the genderless mutator and the asexual 2xoriT mutator strains over that of the nonmutator counterparts HFR-2xoriT-SFX and BW25113-2xoriT, respectively. We implemented a severe bottleneck of 50 to 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 (17–19, 50–53). 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 that of the unevolved parental strains was observed during the evolution (Fig. 6), indicating mutational meltdown (wherein accumulation of deleterious mutations within a population leads to fitness decline and eventual population extinction) was not observed in our experiments.

Since no significant fitness changes 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 (54), we assumed that mutations that lead to biosynthetic deficiencies present in the evolving population were likely not beneficial during serial passage in LB medium. Many auxotrophic strains with single deletions in biosynthetic genes were tested and found to be neutral or slightly disadvantageous in LB medium (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 than in 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 minimal medium plates supplemented with all amino acids except cysteine, lysine, methionine, asparagine, and proline (M9 dropout medium). Genotypes unable to grow on this M9 dropout medium 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 that of the sexual strains with a *P* value of 0.048 using the one-tailed Student *t* test (Table 3).

We further examined whether recombination is the likely

TABLE 3 Number of growth-deficient colonies per 500 colonies assayed for each of the 6 asexual and sexual populations

	No. of growth-deficient colonies per 500 colonies assayed	
Genotype	Sexual	Asexual
1	3	42
2	0	17
3	2	3
4	3	13
5	5	6
6	1	6
Average	2.33	14.5
SD	1.75	14.4

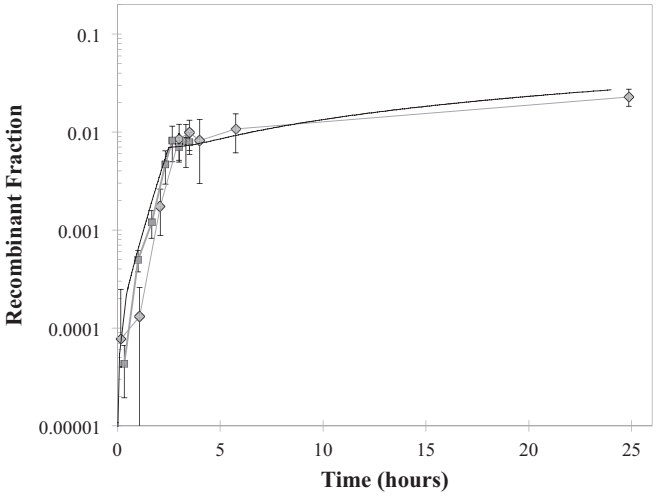

FIG 7 Changes in the fraction of the population that contains the desired recombinant as a function of time in batch culture. Experimental data for 2 pairs of single-marker strains are shown (diamond and square). Modeling data are shown by the solid black line.

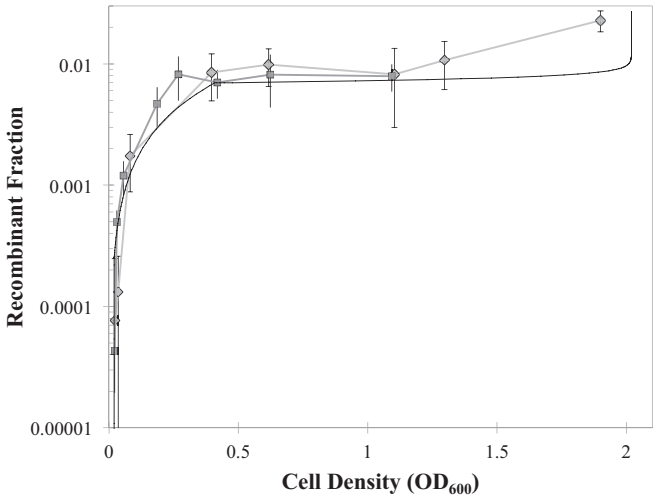

FIG 8 Changes in the fraction of the population that contains the desired recombinant as a function of cell density in batch culture. Experimental data for 2 pairs of single-marker strains are shown (diamond and square). Modeling data are shown by the solid black line.

cause of the lower observed mutational load by pairing sexual isolates that exhibit growth deficiencies in the M9 dropout medium to determine if recombinants can repair the growth deficiency. Each pair was allowed to mate for 24 h 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 WT growth in M9 dropout medium 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 medium.

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 cocultured 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 a 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 Fig. 7 and 8. 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 Fig. S3 in the supplemental material), reaching ~1% of the population within the first 5 h of batch culture. The rate of increase in the fraction of recombinants diminished significantly as the population reached stationary phase (at ~5 h).

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-marker strains were varied from 1:1 to 1:10,000. 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 that 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 Fig. 9). 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. A decrease in mating frequency has been previously observed in the Broda laboratory (46, 47), as recipient population sizes reach or exceed ~5.0×10^7 cells; the authors speculated that fertility becomes the limiting factor in horizontal gene transfer rather than cell-to-cell contact (46, 47). Building off the success of the model in recapitulating the results of having one

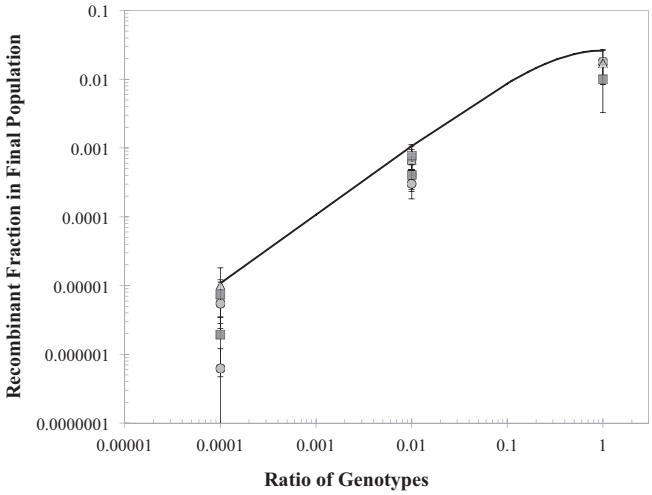

FIG 9 The fraction of the population that contains the desired recombinant versus the ratio of the two starting genotypes for two different pairs of starting genderless single-marker strains. Square and circle, two replicate experiments using starting strains SMB and SMD (markers B and D, respectively); Triangle, experiment with starting strains SMA and SMF (markers A and F, respectively); black line, modeling data.

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 combined with a wild-type background genotype (both A and B at 1% or 0.1% of the population). The results (see Fig. S2 and Table S1 in the supplemental material), while qualitative, suggest that the model retained a high degree of predictability even in these alternate conditions.

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 (9, 10, 26–29). However, due to limitations in available experimental systems, it has been difficult to examine these concepts outside theoretical realms. Here we sought to characterize some unexplored properties of our previously developed genderless strain, capable of continuous bidirectional 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 an alternate initiation site of DNA transfer. These data imply 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*::Kan^r marker C (Fig. 4), which may be related to the histidine auxotrophy it confers. Thus, in addition to the three factors we listed as primary determinants for transfer frequency (distance from the upstream oriT, order of the markers, and distance between the markers), a fourth factor exists, for which any significant fitness advantages or disadvantages imparted by a marker may also impact the observed transfer frequency.

We further assessed the relative use of each oriT and found that a strain with the 2 additional oriTs cotransferred 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 (35) and prior use of conjugation in library construction (34). However, we were unable to determine whether there was a significant preference by the *trans*-acting F factor-mediated machinery toward individual oriTs. This may be due to the 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-2xoriT-SFX- 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 an increased mutation rate was used to increase the frequency of deleterious/neutral mutations (51). This allowed us to exaggerate the differences in mutational accumulation between the 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 (55). Therefore, we used a small effective population size of (calculated with the harmonic mean [50]) ~120 and a mutator strain. However, we failed to observe mutational meltdown in any of our populations over the course of ~850 generations; a similar mutational meltdown experiment in yeast resulted in only one meltdown in a longer time frame (22). 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 those in the asexual populations. Theoretically, recombination reduces Muller's ratchet by allowing deleterious or neutral mutations to be repaired and also reduces the frequency of deleterious hitchhiker mutations linked to beneficial genotypes by allowing genetic exchange with a wild-type allele. Our results point toward a lower mutational accumulation in the sexual strain; however, given the nonsignificant fitness changes between the asexual and sexual populations, additional work is needed to conclude with 100% certainty that recombination reduces the effects of Muller's ratchet. We determined that mating between auxotrophic isolates from independently evolved sexual populations was also able to effectively repair the auxotrophies. Notably, this implies that our recombination-proficient strain is capable of recombining alleles between different genotypes and could potentially 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 the two initial donor genotypes impacts the frequency of the desired recombinant formation, we experimentally measured the fraction of desired recombinants in the population after 24 h of batch growth using various ratios of starting donor genotypes. We utilized single-marker genderless strains to constrain the desired recombination being quantified. Additionally, we modeled the impact of the 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 the recombinant fraction in the final population as a function of the 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 (56). Moreover, our data indicate that the probability of generating a desired recombinant is 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 1,000 cells that do not initially contain the mutation within

24 h 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 subpopulation 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 toward 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 phases, we experimentally observed a rapid drop in the conjugation rate. This expected drop in conjugation frequency was also built into the model. However, despite the decrease in the 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 the recombinant fraction versus time and cell density were validated experimentally (Fig. 7 and 8). When modulating additional parameters of our system, such as the starting cell concentration and the rarity of the 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 provides 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 an ~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 a recombination-proficient strain can repair or combine various genotypes, which may lead to reduced accumulation of deleterious or neutral mutations, although recombination can also act to increase genetic diversity as postulated by the Fisher-Muller model. We therefore postulate that sexual recombination is an effective route for reducing some of the negative impacts of increasing mutation rate in an evolving population. There is ample evidence that mutator strains can significantly expedite the rates of adaptation in ALE experiments by increasing the availability of beneficial mutations. However, an 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.

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