Inactivation of a Mismatch-Repair System Diversifies Genotypic Landscape of *Escherichia coli* During Adaptive Laboratory Evolution

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Adaptive laboratory evolution (ALE) is used to find causal mutations that underlie improved strain performance under the applied selection pressure. ALE studies have revealed that mutator populations tend to outcompete their non-mutator counterparts following the evolutionary trajectory. Among them, mutS-inactivated mutator cells, characterized by a dysfunctional methyl-mismatch repair system, are frequently found in ALE experiments. Here, we examined mutS inactivation as an approach to facilitate ALE of *Escherichia coli*. The wild-type *E. coli* MG1655 and mutS knock-out derivative (ΔmutS) were evolved in parallel for 800 generations on lactate or glycerol minimal media in a serial-transfer experiment. Whole-genome re-sequencing of each lineage at 100-generation intervals revealed that (1) mutations emerge rapidly in the ΔmutS compared to in the wild-type strain; (2) mutations were more than fourfold higher in the ΔmutS strain at the end-point populations compared to the wild-type strain; and (3) a significant number of random mutations accumulated in the ΔmutS strains. We then measured the fitness of the end-point populations on an array of non-adaptive carbon sources. Interestingly, collateral fitness increases on non-adaptive carbon sources were more pronounced in the ΔmutS strains than the parental strain. Fitness measurement of single mutants revealed that the collateral fitness increase seen in the mutator lineages can be attributed to a pool of random mutations. Together, this study demonstrates that short-term mutator ALE extensively expands possible genotype space, resulting in versatile bacteria with elevated fitness levels across various carbon sources.

Keywords: adaptive laboratory evolution, mismatch repair, *Escherichia coli*, genotype space, phenotype microarray

INTRODUCTION

Bacterial cells are under constant selection pressure to adapt to ever changing environmental conditions. The adaptive response to changing conditions is based on finding fitness-enhancing genetic changes. Adaptive laboratory evolution (ALE) experiments using microbes have identified such mutations and horizontal gene transfer as the key determinants underpinning the emergence
of adaptive traits such as antibiotic resistance (Dragosits and Mattanovich, 2013). While adaptive evolution results in the rewiring of cellular regulatory and metabolic networks, mutation rate itself may also be subject to adaptation through a selection pressure on the genes associated with DNA repair (Rosche and Foster, 1999; Giraud et al., 2001a; Sprouffske et al., 2018).

It has been well documented that the emergence of mutator lineages during adaptive evolution results in a more rapid adaptation compared to wild-type strains (Sniegowski et al., 1997; Giraud et al., 2001a; Notley-McRobb et al., 2002; Barrick et al., 2009; LaCroix et al., 2015; Shibai et al., 2017). *In silico* prediction of mutation rate dynamics based on the canonical *Escherichia coli* mutation rates revealed that a 10–100-fold increase in mutation rates facilitates rapid adaptation in the bacterial population (Taddei et al., 1997). Likewise, it has been empirically shown that mutator strains with an intermediary range of mutability harnessed the fittest lineages following adaptation in minimal and rich media alike, and that too high a mutation rate adversely affects successful adaptation (Loh et al., 2010; Ishizawa et al., 2015; Sprouffske et al., 2018), and in some instances, survival (Shibai et al., 2017). Interestingly, mutators generate a plethora of random mutations many of which exert no imminent effect on host fitness. However, mounting evidence suggests the role of non-adaptive mutations in shaping novel adaptive phenotypes in the process of adaptation (Wagner, 2008; Szappanos et al., 2016).

In a prokaryotic population with basal mutation rate, most randomly arising mutations are subjected to loss due to genetic drift. However, in the presence of mutators, selection pressure further promotes the accumulation of intermediary mutations, expanding the pool of genetic reservoir that may serve as alternative, non-adaptive environments. Together, our results suggested that moderate mutator strains, such as *mutS*-inactivation, confer a higher-on-average fitness advantage on alternative, non-adaptive environments.

**MATERIALS AND METHODS**

**Strain Construction and Growth Conditions**

All strains used are *E. coli* K-12 MGI655 and its derivatives (Supplementary Table S1). The *mutS* mutator strain (*ΔmutS*) was constructed by a one-step inactivation method as described previously (Datsenko and Wanner, 2000). Homologous lambda recombination was used to obtain single nucleotide modification in the wild-type and *ΔmutS* mutator strain genomes. The mutant and the wild-type gene constructs were amplified from the genomic DNA of either wild-type or the end-point populations carrying the variant of interest with appropriate primers (Supplementary Table S2). A second PCR was performed using plasmid pKD13 as the template using proper primers (Supplementary Table S2). The two PCR amplicons were fused into gene-FRT-neo<sup>R</sup>-FRT by overlap extension PCR (Urban et al., 1997). Downstream procedures including λ-Red recombination expression, FLP-FRT-mediated marker elimination and single clone isolation were performed by the same protocol for a one-step inactivation method (Datsenko and Wanner, 2000). Lastly, the presence of the desired variants – *add* (947C>T), *xdhB* (860insG), and *ilvH* (124A>G) – and the corresponding wild-type counterparts were confirmed using Sanger sequencing.

For ALE experiment, cells were cultured in M9 minimal medium supplemented with 2 mM of MgSO<sub>4</sub>, 0.1 mM of CaCl<sub>2</sub>, and either 0.2% (w/v) of lactate or 0.2% (v/v) glycerol as the sole carbon source. Cells were incubated at 30°C unless stated otherwise. For growth rate measurement on non-adaptive carbon/nitrogen sources, M9 minimal medium containing 0.2% 2'-Deoxyadenosine (w/v) + 0.2% lactate (v/v), 0.2% inosine (w/v) + 0.2% glycerol, 0.2% L-Threonine + 0.2% lactate and 0.2% pyruvate (w/v) were used. For fitness assay, cells were pre-cultured in M9 minimal medium containing appropriate carbon or nitrogen sources then serially passaged for two times (Shachrai et al., 2010). To quantitatively determine cellular growth kinetics, cell density was measured on EPOCH2 plate readers (BioTek, Winnoski, VT, United States) and Synergy H1 (BioTek, Winnoski, VT, United States) at 30°C with shaking (807 rpm double orbital) for 24 h, using default 48-well
configuration. Absorbance at OD<sub>600</sub> nm was measured at 10-min intervals. The resulting data output was analyzed on the Gen5 software.

**Adaptive Laboratory Evolution**

A total of eight independent lineages comprising two biological replicates from each strain in either M9 lactate or M9 glycerol media. All lineages were adaptively evolved for ∼800 generations. An arbitrarily chosen single colony on LB agar plate was inoculated on 3 mL LB medium then incubated overnight; this batch served as the ancestral stock cells and was kept frozen in −80°C, 20% (v/v) glycerol. Simultaneously, 100 µL of the overnight culture was serially transferred to 3 mL of either 0.2% glycerol or lactate M9 minimal media up until 800 accumulative generations were reached. The passaging was accompanied with serially passaged to fresh M9 media up until 800 accumulative generations were reached. The passaging was accompanied with an intermittent decrease in initial OD<sub>600</sub> to compensate for the adaptive increase in growth rates. Cell pellets from every passaging was performed until one of the two strains was eliminated from the competition ALE as indicated by the absence of corresponding amplicon band(s) from the PCR reaction.

The ratio of the competing populations over time was quantified using real-time qPCR. Since the amount of genomic DNA is proportional to cell density, by determining the relative quantity of genomic DNA from the mix-culture, the ratio of heterogeneous populations present at specific time points was derived. As mutS deletion was the sole identifier of ΔmutS mutator strain, we designed two primer sets, one targeting mutS gene and the other one flanking the scar region on the mutS<sup>−</sup> genome (Supplementary Table S2), yielding amplicons of 108 and 106 bp, respectively. To demonstrate the feasibility of this experiment, wild-type and ΔmutS mutator strain were mixed in the ratios of 1:9, 3:7, 5:5, 7:3, and 9:1 based on the optical density at 600 nm. Each qPCR reaction was performed according to the ALE method described above. The sole genetic marker was mutS gene to differentiate between the two strains. Strain profiles in mix-culture were periodically confirmed using colony PCR with two primer sets – a universal primer set hybridized to both wild-type and mutS<sup>−</sup> genome, and a “mutS<sup>+</sup>” primer set specific to mutS gene that discriminates MG1655 wild-type from mutS<sup>−</sup> genome (Supplementary Table S2). Each PCR reaction was set up total 20 µL reaction volume, comprised of 10 µL of Accupower Master Mix (Bioneer, Daejeon, South Korea), 0.4 µL of forward and reverse primers, and 0.5 µL of ALE culture. Serial passaging was performed up until one of the two strains was eliminated from the competition ALE as indicated by the absence of corresponding amplicon band(s) from the PCR reaction.

**Whole Genome Resequencing**

A total of 66 samples were sequenced including (i) 64 population samples at 100 generation intervals from the eight independently evolved lineages and, (ii) two parental strains of E. coli MG1655 wild-type and ΔmutS mutator strain. Genomic DNA (gDNA) was isolated using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, United States) as per the manufacturer's
protocol. The isolated gDNA was checked using a NanoDrop™
2000 (ThermoFisher Scientific, Waltham, MA, United States) to
assure UV absorbance ratio between 1.8 ~ 2.0 and visualized
under 1% agarose gel. The DNA concentration was quantified
via a Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, CA,
United States) using a Qubit™ dsDNA HS Assay Kit (Invitrogen,
Carlsbad, CA, United States). For each sample, 2,200 ng input
dNA was suspended in 56 µL of TE Buffer (pH 8.0) and sheared
to target peak size of 400–500 bp using the Covaris S220 Focused-
ultrasonicator (Covaris, Woburn, MA, United States) according
to manufacturer’s recommendations, except that sonication
duration was subjected to variation. NGS library was prepared
using a TruSeq DNA PCR-Free kit (Illumina FC-121-3002)
following the manufacturer’s guide. Resulting libraries were
sequenced with rapid-run mode as 50 cycle single-ended reaction
in the Illumina Hiseq 2500 system.

Mutation Profiling
Sequencing data were processed on a CLC Genomics Workbench
(CLC bio, Aarhus, Denmark). Adapter sequences were trimmed
using a Trim Sequneq Tool in NGS Core Tools with a quality
limit of 0.05. Reads containing more than two ambiguous
bases were filtered and the resulting reads were mapped
to the E. coli MG1655 reference genome (NCBI accession
NC_000913.3) with following parameters: mismatch cost: 2, indel
cost: 3, length and similarity fractions: 0.9. Variant calling was
performed using a Quality-based Variant Detection Tool with
the following parameters: neighborhood radius: 5, maximum
gap and mismatch counts: 5, minimum neighborhood and
central qualities: 30, minimum coverage: 10, minimum variant
frequency: 10%, and maximum expected alleles: 4. Non-specific
matches were ignored, and bacterial and plant plastid genetic
codes were used. Variants in non-specific regions and repeat
regions (i.e., rRNA and transposases) were discarded. The variants
from the biological replicates were merged together to
generate an exhaustive view of the genotypic landscaped shaped
in the course of adaptive evolution. We considered gene, rather
than gene position, as a unit for mutation. As for the mutations
that overlap between the biological replicates, mutations with
higher frequency was chosen.

Phenotype Microarray
We comparatively analyzed the growth capacity of E. coli
MG1655 and the evolved strains on non-evolutionary carbon
sources in Biolog PM1 MicroPlate™ (Biolog Inc., Hayward, CA,
United States), which contains 95 different carbon sources as a
sole nutrient source. The frozen cell stocks were inoculated in
3 mL of 0.2% lactate or glycerol M9 minimal media, cultured
overnight at 37°C with agitation. Two technical replicates were
tested per sample. Cells were collected using a centrifuge, the cell
pellets re-suspended in 6 mL of Inoculating Fluid A (Biolog Inc.,
Hayward, CA, United States) with OD600 nm of approximately
0.4 (42% T cell). To make 85% T cell suspension, 4 mL of 42% T
cell suspension was transferred to 20 mL of Inoculating Fluid A.
100 µL of 85% T cell suspension was loaded onto each well on
the PM01 plate. To quantitatively determine cellular growth kinetics,
cell density was measured on EPOCH 2 plate readers (BioTek,
Winnskoi, VT) at 30°C with shaking (807 rpm double orbital)
for 24 h, using default 96-well configuration.

Analysis of PM01 Data
Doubling time measured from the negative-control sample (well
A01 of the Biolog PM01 plate) grown in nutrient-free Inoculating
Fluid A was used as the threshold for growth rate. Substrates
with doubling time equal or less than the threshold value were
designated as “no growth rate” and were excluded for further
analysis. Specific growth rate of each substrate was estimated
using the BioTek Gen5 software. Growth rate improvement or
 retardation of the end-point strains relative to the ancestral
strains was calculated as log2 (fold-change), and statistical
significance was computed between the technical replicates
using the student’s independent t-test (normal distribution
was assumed), and logarithmically expressed in –log10 (P-
value). The substrates with statistically non-significant fitness
changes (P > 0.05, Student’s independent t-test) were omitted
from subsequent analysis. Those with significant changes were
categorized into four groups: (i) Gain of Function – refers to
substrates on which the adapted strains acquired the ability to
grow; (ii) Loss of Function – instance where the cell growth
becomes diminished in the adapted strains; (iii) Fitness gain–
denoting substrates on which significant enhancement in the
growth capacity is seen following adaptive evolution; and (iv)
Fitness loss – substrates with significant retardation in growth
rate following adaptive evolution.

RESULTS
Adaptive Laboratory Evolution in Lactate
or Glycerol Minimal Media
Two biological replicates from each of E. coli K-12 MG1655
wild-type and mutS knock-out derivate (ΔmutS mutator) strains
were propagated in M9 minimal medium containing either
lactate or glycerol. ALE yielded two lactate-adapted wild-types,
two lactate-adapted mutators, two glycerol-adapted wild-types
and two glycerol-adapted mutators evolved for 800 generations
using serial transfer. To characterize the fitness trajectories
accompanying the ALE, doubling time per passage was estimated
as a function of accumulated generations (Wiser and Lenski,
2015). The fitness trajectories revealed that the ALE was the
most rapid in the first 200 generations (Figure 1A). By the
800th generation, fitness increase reached a plateau in all ALE
lineages. Although the fitness trajectories were altogether similar
between the two strains, the early trajectories showed that the
doubling time of ΔmutS mutator strain decreased faster than
that of the wild-type strain. Specific growth rates of the end-
point populations in each lineage were then calculated to assess
the outcome of mutator-driven evolution on adaptive fitness
(Figure 1B). Specific growth rate of the ΔmutS end-point
population was 2.45 ± 0.17 fold higher than that of its parental
strain, which was only marginally higher than that of the wild-
type lineage (2.29 ± 0.09 fold increase). Taken together, despite
of some differences in the rate of fitness gain in the course of ALE,
the final fitness levels attained by the end-point populations of the wild-type and the ΔmutS mutator strains were comparable.

Mutator Strain Outcompetes Its Wild-Type Counterpart in Competition Experiments

To recapitulate the observation that increased mutagenic potential confers a selective advantage on a short-term adaptation (Chao and Cox, 1983; Giraud et al., 2001a), we performed competitive growth experiments between the wild-type and the ΔmutS mutator strains and evaluated the relative fitness levels of the two strains. The two strains were mixed in 1:1 ratio on M9 lactate or glycerol minimal media respectively, at starting OD<sub>600</sub> nm of 0.05 in duplicate. In these growth conditions, the strains share limited resources and compete for survival until a dominant strain takes over the population during a short-term competition. In all four mixed cultures, the ΔmutS mutator strains eventually outcompeted their DNA repair-proficient counterparts (Figure 2A). In each of lactate or glycerol serial culture, ΔmutS mutator strains completely displaced the wild-type strains (<0.1%) approximately by the 200th generations, indicating that acquisition of beneficial mutations was faster in the ΔmutS mutator strains than in the wild-type strains.

Until the 46th generation of the lactate competition experiment, the wild-type strains constituted up to 80% of ~4 × 10<sup>4</sup> cellular population largely owing to its faster initial growth rate (Figure 2A). By the 72nd generation, the ΔmutS mutator strains occupied more than half of the population, then gradually displaced the wild-type counterpart down to < 0.1% by the 202nd generation (Figure 2A). The fluctuation on previous strain-independent ALE experiment results (Figure 1) coincided with these results, where the wild-type strains showed superior growth rate than the ΔmutS mutator strain on M9 lactate at the starting point, it became reversed by the 50th generations. Meanwhile, until the 25th generation of the glycerol competition experiment, the ΔmutS mutator strains occupied about three quarter of the population. This was expected as the specific growth rate of the ancestral ΔmutS mutator strains was faster than the ancestral wild-type strains on M9 glycerol (Figure 1B). Yet, this was immediately followed by a marked increase in the wild-type population making up nearly 80% of the total population. Such rapid fitness increase in the wild-type strains was also evident in the individual ALE experiments, where the growth rate of the wild-type strains evened with that of the ΔmutS mutator strains by the 40th generation on M9 glycerol media condition (Figure 1A). In subsequent generations, the ΔmutS mutator strains serially displaced the wild-type counterparts down to 0.1% by the end of the short-term laboratory evolution experiments (Figure 2A). These results confirm that the mutation-prone strain is capable of obtaining a selective advantage on cell growth compared with the wild-type counterparts during a short-term adaptive evolution.

Inactivation of a DNA Repair System Expands the Genotype Space

To understand the genetic basis of enhanced adaptability in ΔmutS mutator populations, we determined the mutation profiles of the ancestral, evolving and end-point populations of the wild-type and the ΔmutS mutator lineages. Whole genome re-sequencing was performed for all eight lineages at 100-generation intervals, allowing us to analyze mutation trajectories (Figure 3). As expected, the ΔmutS mutator strains accumulated mutations more rapidly than the wild-type strains. The ΔmutS mutator strains produced in overall 246 mutations that mapped to 158 genes and 36 intergenic regions, while the wild-type strains harbored 62 mutations mapped to 27 genes and 11 intergenic regions, suggesting that inactivation of mutS brings about fourfold increase in mutation rates in bacteria. Such changes in the mutation rate were comparable to, or slightly higher than, the rates reported in other mutS-inactivated strains of Lactobacillus casei (Overbeck et al., 2017) and E. coli C321ΔA (Wannier et al., 2018). As it was known, frequency of transition mutations were far more pronounced over transversion mutations in all ΔmutS mutator lineages (Supplementary Table S3; Modrich, 1991; Tsuru et al., 2015).

We identified a group of genes or intergenic regions recurrently mutated across two or more independent ALE lineages, where selective pressures account for such genetic parallelism in the mutation profiles (Bull et al., 1997;
Cooper et al., 2001, 2003; Barrick et al., 2009). The mutated genes were then categorized in accordance to their functional niche – whether the effect of a mutation is confined in homogeneous condition (known as specialist), or potent across a broader range of heterogeneous environments (called generalist) (Kassen, 2002), as summarized in Supplementary Table S3. Examples of generalist mutations in E. coli include rpoB (RNAP subunit β) and rph (truncated RNase PH) which are implicated in translational machinery. In our current design, the sole carbon substrates – lactate or glycerol – supplemented in the minimal media impose selection pressures that favor specialist mutations to arise. So for instance, mutation in glycerol kinase encoding gene glpK is known as the specialist mutation in glycerol minimal medium (Nat Genet, 2006, 38, 1406-1412; Nat Commun, 2017, 8:2112), while mutations in ppsA encoding phosphoenolpyruvate synthetase is thought to rewire lactate metabolism in E. coli (Genome Res, 2005, 15, 1365-1372) thus serving as lactate-specialist mutation. Subsequent analysis revealed that the mutator and the wild-type lineages shared a large proportion of generalist, as well as specialist mutations in a carbon-dependent manner (Supplementary Table S3). Yet, most of the random mutations were found in the mutator lineages (sum of random mutations in all mutators n = 167; in all wild-type strains n = 16).

The whole-genome re-sequencing demonstrated that the mutations were most prolific in the end-point populations (Figure 3 and Supplementary Table S3). Using the functional analysis using the COG database, we compared the enriched mutations in the end-point populations against the E. coli K-12 MG1655 genes. With an assumption that mutation occurs at random throughout the genome, the enrichment levels imply over-representation of relevant functional gene categories or classes. In particular, genes involved in amino acid transport and metabolism (E), secondary metabolites biosynthesis, transport and catabolism (Q) and transcription (K) were more enriched in the mutated genes of end-point populations than the wild-type MG1655 genes, indicating mutation hot spots (Chattopadhyay et al., 2009; Lourenco et al., 2016).

**Short-Term ALE Exerts No Observable Fitness Burden on Mutator Strains**

To demonstrate whether the ΔmutS mutator strain still sustains its competitive edge after undergoing 800 generations of adaptive evolution, we performed a new round of competition experiment involving the end-point lineages of ΔmutS and wild-type evolved under lactate or glycerol minimal medium. Two mix-cultures of i. lactate-evolved ΔmutS and wild-type and; ii. glycerol-evolved ΔmutS and wild-type strains were allowed to competitively evolve in their respective adaptive media. In all instances of competition experiments, the end-point ΔmutS strains outcompeted their wild-type competitors by the 201th and 250th generations in lactate- and glycerol-evolved populations respectively (Figures 4A,B). This corroborates similar competition experiment performed in nutritionally rich mouse gut showed that mutS-defective E. coli pre-adapted in the intestinal environment for 20 days also managed to replace most of its isogenic wild-type competitors in mouse gut (Giraud et al., 2001a).

**Fitness Gain on Alternative Carbon Sources Is More Pronounced in the Mutator Lineages Than the Wild Type**

In an effort to relate genotypic changes to phenotypes, we assayed the fitness of the end-point populations across a broad panel of single-carbon environments. As specific growth rate is a commonly proposed representative of bacterial fitness (Nur, 1987), we measured the specific growth rates of both ancestral and end-point populations on 95 different non-adaptive substrates (as described in Methods). It was previously described that the mutT hypermutator strain in the long-term evolution experiment and the mutS mutator strain that evolved in mouse gut adversely affected fitness landscape on alternative environments presumably due to an accumulation of deleterious mutations (Giraud et al., 2001a; Leiby and Marx, 2014). Yet, fitness assay of mutL mutator strain adaptively

**FIGURE 2** | Competition experiment between E. coli parental wild-type and ΔmutS strains. Ratio-wise representation of the proportion of the parental wild-type versus the parental ΔmutS strains in the course of competition experiment in (A) lactate or (B) glycerol minimal medium. Each bar represents the ΔmutS/wild-type ratios from the propagating cultures sampled intermittently.
FIGURE 3 | Continued
evolved over 3,000 generations against antibiotics and heavy metals, revealing a stochastic nature of the mutator strain in relation to fitness acquisition or loss in alternative environments (Sprouffske et al., 2018). Phenotype transitions between the ancestral and the evolved strains were summarized in Table 1, where digits in each category refer to the number of relevant substrates in the PM01 microplate. Growth rate data revealed that the number of substrates in Fitness Gain category was similar between the end-point populations of ΔmutS mutator strains (n = 42 for lactate, n = 32 for glycerol), and the wild-type (n = 32 for lactate, n = 33 for glycerol) (p > 0.5, Pearson’s chi-squared test). However, the number of substrates with fitness increase above log2 (fold-change) of 0.5 was significantly higher in ΔmutS mutator strains (n = 37 for lactate, n = 16 for glycerol) than in wild-type (n = 17 for lactate, n = 15 for glycerol) (p < 0.0015, Pearson’s chi-squared test). Notably, the number of substrates that fell above log2 (fold-change) > 1 was markedly higher in the end-point strains of ΔmutS mutator (n = 8 for lactate, n = 4 for glycerol) than in the wild-type (n = 2 for lactate, n = 0 for glycerol) (p < 0.02, Pearson’s chi-squared test) (Figure 4). Carbon substrates with log2 (fold-change) > 0.5, 1.0, p-value < 0.05 are listed in Supplementary Table S4.

Apart from the changes in growth kinetics, we observed cases where populations acquired or lost the ability to thrive on alternative carbon or nitrogen substrates. Emergence of such collateral, non-adaptive traits was first realized in the long-term evolution experiments where glucose-adapted strains acquired the ability to metabolize citrate which is a non-utilized carbon source in the wild-type E. coli B (Blount et al., 2008). As opposed to the mutators in the long-term evolution experiments, ΔmutS mutator strains in this study showed no visible differences in
terms of novel gain or loss of function phenotypes following adaptation between the end-point population of the wild-type and the ∆mutS mutator lineages (p > 0.5 and p > 0.1, Pearson’s chi-squared test, for gain and loss of function, respectively). The number of substrates in Gain of Function category was in overall greater than that in Loss of Function. Together, this line of evidence indicated that a short-term ALE does not inflict metabolic capacity of MMR-inactivated mutator strains as seen in the long-term evolution experiments (Leiby and Marx, 2014); rather, this seems to maximize the chance of survival on alternative environments.

Fitness Analysis of Single Mutations on Alternative Environments

To assess the contributions of individual mutations to fitness on non-adaptive carbon sources, we selected mutation candidates by inferring their roles in carbon and amino acid metabolism (Table 2). Growth rates of the engineered strains harboring a single mutation and their wild-type controls were measured on M9 minimal medium containing adaptive (AC), non-adaptive carbon sources (NC) or both carbon sources (AC + NC). For example, inosine and adenosine which are purine nucleosides are unable to be utilized as sole growth substrates by E. coli, instead they can be co-utilized in the presence of other growth substrates such as aspartate (Xi et al., 2000). Therefore, growth rates on inosine and adenosine were measured in M9 minimal medium supplemented with adaptive carbon substrate (AC + NC). Hypoxanthine hydroxylase encoded by xdhB constitutes a subdomain of xanthine dehydrogenase and is known to be associated with adenosine degradation pathway in E. coli (Xi et al., 2000). The mutation on xdhB (860ins861G) from the mutator end-point on glycerol ALE was characterized by a frameshift mutation inflicting the 3’ end of the protein coding region of xdhB. The mutation was also identified during the ALE of genome-recoded E. coli where selective pressures on amber stop codons may have implicated with read-through of termination codons including that of xdhB (Wannier et al., 2018). This evidence suggests the frameshift mutation on xdhB is a generalist mutation that impacts fitness via tuning cellular translation efficiency. In our study, xdhB mutant (860ins861G) exhibited higher growth rate in the non-adaptive environment (AC + NC), but lower growth rate in adaptive condition (AC), than its wild-type complement (Table 2). On the other hand, gene encoding deoxyadenosine deaminase add is involved in nucleotide biosynthesis and metabolism (Remy and Love, 1968). A point mutation in add was identified in the mutator end-point on lactate ALE. This mutation (947C>T) characterizes C is changed to T at nucleotide 947. *ins* represents insertion, and *fs* represents frameshift. **SD** denotes standard deviation.

**TABLE 1** | Tabulation of the Biolog PM01 substrates in accordance to the growth profiles in the eight end-point ALE populations and the corresponding ancestral populations.

|                | Glycerol | Lactate |
|----------------|----------|---------|
|                | WT       | ∆mutS   |
| Gain of function | 8        | 7       | 4       | 4       |
| Loss of function | 0        | 1       | 1       | 4       |
| No growth       | 14       | 15      | 19      | 19      |
| Non-significant | 40       | 39      | 39      | 26      |
| Fitness gain (total) | 33       | 32      | 32      | 42      |
| Fitness gain (0.5 < log2(Fold-Change) < 1.0) | 15       | 16      | 17      | 37      |
| Fitness gain (log2(Fold-Change) > 1.0)     | 0        | 4       | 2       | 8       |
| Fitness loss     | 0        | 1       | 0       | 0       |

*Gain of function*: substrates that became accessible (utilizable) by the end-point strains; “Loss of function”: substrates that became inaccessible (non-utilizable) by the end-point strains; “Non-significant”: substrates where changes in growth rates between the ancestral and corresponding end-point strains were non-significant; “Fitness gain”: substrates with significant increase in growth rates in the end-point populations relative to the ancestral strains. They are further classified in accordance to fold-change values in the growth rates; “Fitness loss”: substrates with significant decrease in growth rates in the end-point populations relative to the ancestral strains.

**TABLE 2** | The candidate mutations and functionally associated metabolic substrates.

| Gene | Mutation* | Nutrient supplements | Growth rate (±SD)** |
|------|-----------|----------------------|---------------------|
| add  | A316V     | Lactate              | 0.022 ± 0.002       |
|      | (947C > T)| Lactate + 2’-Deoxy   | 0.096 ± 0.003       |
|      |           | Adenosine            | 0.087 ± 0.004       |
| xdhB | A287fs    | Glycerol             | 0.069 ± 0.003       |
|      | (860ins861G) | Inosine + Glycerol   | 0.058 ± 0.004       |
| ilvH | T42A      | Lactate              | 0.180 ± 0.006       |
|      | (124A > G)| l-trehalose + Lactate| 0.143 ± 0.002       |
|      |           | Pyruvate             | 0.207 ± 0.010       |
|      |           |                      | 0.182 ± 0.007       |
|      |           |                      | 0.141 ± 0.009       |

*Single nucleotides substitutions are designated by “*”. For example, 947C>T denotes that C is changed to T at nucleotide 947. *ins* represents insertion, and *fs* represents frameshift. **SD** denotes standard deviation.
**DISCUSSION**

Several studies have shown that in microbial cells, elevated mutation rates can be exploited to facilitate ALE toward better fitness compared with non-mutator cells (Sniegowski et al., 1997; Giraud et al., 2001a; Notley-McRobb et al., 2002; Barrick et al., 2009; LaCroix et al., 2015). Often, MMR-impaired cells have been used as the mutator strain in numbers of ALE applications (Luan et al., 2013; Overbeck et al., 2017; Wannier et al., 2018) because MMR-inactivation were known to generate moderate mutators in *E. coli* (Taddei et al., 1997; Rosche and Foster, 1999; Tenaillon et al., 1999; Loh et al., 2010; Sprouffske et al., 2018). In laboratory settings, the desired ALE results can be achieved when strains become best-adapted for survival in an unfavorable condition. In nature’s perspective, however, evolutionary success also entails the capacity to effectively occupy a range of potential niche. While it is not plausible for a cell to harbor maximum fitness capacity in all circumstances (Law, 1979), microbial cells nonetheless retain the potential to adapt in a new set of conditions. They do this by reserving a small subset of mutator populations that provide a constant influx of random allelic variants (LeClerc et al., 1996; Matic et al., 1997; Rosche and Foster, 1999). This parallels with the idea of stepwise accumulation of intermediary mutations that lead to the emergence of novel phenotypes (Wagner, 2008; Szappanos et al., 2016). In this work, we explored the evolutionary contingencies of mutator ALE by analyzing collateral fitness capacities in our end-point populations across a panel of alternative environments.

*Escherichia coli* MG1655 wild-type and ΔmutS mutator strains were adaptively evolved in M9 medium with lactate or glycerol as the sole carbon sources. Fitness levels of the end-point strains increased between 2.2 and 2.7-fold (Figure 1B), which is comparable to fitness increase observed in ALE performed for similar timescales under lactate, glycerol and glucose minimal media (Fong et al., 2005a,b). Generally, it has been known that mutators evolved in short-term give rise to more robust populations compared with their isogenic non-mutator counterparts (LaCroix et al., 2015; Overbeck et al., 2017; Wannier et al., 2018). As opposed to what was expected, there was no appreciable difference in adaptive capacity in terms of fitness gain between the end-point wild-type and ΔmutS mutator strain (Figure 1B). This was intriguing given the significant divergence in their mutation profiles (Figure 3 and Supplementary Table S3). The wild-type lineages generated in overall 62 mutations, which is a quarter of what was identified in all four ΔmutS mutator strains (246 mutations). In addition, unlike what is considered as usual fitness trajectory of *E. coli* mutators (characterized by a sharp leap in adaptive fitness distinct from that of the non-mutators) (Taddei et al., 1997; LaCroix et al., 2015), the fitness trajectories of the ΔmutS mutator strains closely resembled that of the wild-type (Figure 1A). Yet again, the ΔmutS mutator strains outcompeted its wild-type counterpart in the competition (Figure 2) despite the growth defect intrinsic in mutators (Ishizawa et al., 2015), demonstrating the selective advantage of mutator populations in adaptation. This observation corroborated the result of similar competition experiments performed in mouse gut (Giraud et al., 2001a) and in glucose chemostat with lag-to-displacement of 60 ± 3.6 generation (Chao and Cox, 1983). The temporal fluctuations in the population (Figure 2A) is likely attributed to “adaptation window” characterized by a rapid increase in genetic heterogeneity in a population with varying degree of fitness levels (Barrick et al., 2009; Van Cleve and Weissman, 2015). In such circumstances, mutator populations retain selective advantage for survival in that they can leverage their increased mutation rates, which comes with higher chance acquisition of beneficial mutations, to accelerate adaptation (Sniegowski et al., 1997; Giraud et al., 2001a; Notley-McRobb et al., 2002; Barrick et al., 2009; LaCroix et al., 2015). Together, the results illustrate a strong competitive advantage of mutator strains over wild-type strains in short-term competition.

Variant detection at 100 generation intervals enabled analysis of mutation profile at high resolution. Mutagenic potential of mutS inactivation was as high as four-fold than that of basal mutation rate (Supplementary Table S3). Mutation rates of ΔmutS mutator strain are roughly comparable with that of mutT mutator phenotype in the long-term evolution experiments and ΔmutS derivative of recoded *E. coli* strain evolved in short-term, each of which harnessed 62 mutations per 1,000 generations and ∼55 mutations after 1,100 generation respectively (Barrick et al., 2009; Wannier et al., 2018). The mutation rates in the wild-type lineages lied well within the range of those in similar ALE experiments (1 to 40 mutations in ~1000 generations and 2 to 3 mutations in ~660 generations) (Herring et al., 2006; Barrick et al., 2009; Conrad et al., 2009; Wannier et al., 2018). A group of causative genes reproducibly mutated across independent lineages were classified accordingly (Supplementary Table S3). We first categorized the genes or intergenic regions recurrently mutated in the wild-type and ΔmutS mutator lineages, then classified the mutations with regards to their ecological niche width (Supplementary Table S3; Kassen, 2002).

First, the mutations that persist in multiple heterogeneous conditions characterized by a broader niche width is termed “generalist mutations” (Kassen, 2002). The generalist mutations identified in this study were rpoB (RNA polymerase subunit β), hfq (RNA-binding protein), rph (truncated RNASH), ptsP (mannose-specific PTS enzyme IIC component), crp (DNA-binding transcriptional dual regulator), and the intergenic region between pyrE (orotate phosphoribosyltransferase) and rph. The variants of rpoB and hfq are highly frequent in the *E. coli* populations adaptively evolved in minimal media (Herring et al., 2006; Conrad et al., 2009; LaCroix et al., 2015), largely owing to their essential role in the transcription (rpoB) (Kobayashi et al., 1990) and growth rate dependent effect on bacterial fitness (hfq) (Maharjan et al., 2013). Numbers of disabling mutations (frame-shift and non-sense mutations) on ptsP, a component of a PTS chain, identified in three independent lineages is consistent with the finding that adaptive evolution of *E. coli* that lacks the PTS system (including deletion of ptsP) led to an extensive re-wiring of metabolic flux that contributed to a significant increase in growth rate (Aguilar et al., 2012). Deletion in the intergenic region of pyrE-rph is particularly frequent in the adaptive evolution of *E. coli* MG1655 in that...
the mutation compensates for an intrinsic defect of K-12 strains in pyrimidine biosynthesis (Jensen, 1993; Conrad et al., 2009; LaCroix et al., 2015). These generalist mutations, except for crp, were mapped to both the wild-type and the ΔmutS mutator lineages (Supplementary Table S3).

Second, the mutations that convey beneficial effect in a confined homogenous environment is called “specialist mutations” (Kassen, 2002). Well-established specialist mutations in lactate ALE include mutations on ppsA, cyaA and ydcI (Conrad et al., 2009). Among them, ppsA encodes phosphoenolpyruvate synthetase which is associated with conversion of pyruvate into phosphoenolpyruvate or vice versa. Adenylyl cyclase encoding gene cyaA is associated with CAMP production and is implicated in the utilization of non-PTS carbon sources. For this reason, cyaA mutants are frequently seen in ALE experiments in non-PTS carbons such as lactate and glycerol (Herrington et al., 2006; Conrad et al., 2009; Peabody et al., 2017). Moreover, ydcI is a gene that encode putative DNA-binding transcriptional repressor that is thought to be implicated in cellular stress response (Solomon et al., 2014). The recurrence of ydcI mutants in other ALEs on minimal and rich media (Conrad et al., 2009; Liu et al., 2017) also suggests their role in globally mediated regulation in fitness improvement through some unknown mechanisms. The variants of all three genes were identified across lactate-evolved wild-type and the ΔmutS mutator lineages (Supplementary Table S3). As for the glycerol specialist mutations, previous studies demonstrated causal link of glpK (glycerol kinase) and rpoC (β’ subunit of RNA polymerase) mutation in growth rate improvements in glycerol ALE (Conrad et al., 2011; Sandberg et al., 2017). As an essential component of RNA polymerase, rpoC is thought to be selected in virtually any sub-optimally fit bacterial population (Murphy and Cashel, 2003). The variants of glpK and rpoC were also mapped across glycerol-evolved wild-type and the ΔmutS mutator lineages (Supplementary Table S3).

Third, there were only few causative variants identified under wild-type specific (crp) and ΔmutS-specific (trkH) categories. The variant of crp was reported to contribute to fitness increase in E. coli strains adaptively evolved in non-preferential carbon sources including lactose (Quan et al., 2012). K⁺ transporter protein encoded from trkH is involved in ion efflux regulation. It was reported that a non-synonymous mutation on trkH arose from an antibiotic challenge resulted in mild resistance against non-target antibiotics (Lazar et al., 2013). Emergence of an off-target, collateral effect against antibiotics in ALE experiments was also documented in mutator populations adaptively evolved under nutrient limitations for 3000 generations (Sproufske et al., 2018).

Lastly, in addition to the generalist and specialist mutations, the remaining 183 mutations were found to be mutually exclusive and mapped uniquely to one of the eight ALE lineages (Supplementary Table S3). These mutations were designated as random mutations. Notably, nearly all of the random mutations were found in the mutS-inactivated lineages (n = 167), where lactate- and glycerol-adapted ΔmutS mutator lineages harbored 83 and 84 of random mutations, while the corresponding wild-type lineages retained only 4 and 12 random variants respectively. Interestingly, genes that have not been registered in the ALE mutation metadata database (ALEdb) were newly identified in the mutator lineages (Phaneuf et al., 2019). It is also worth noting that, whereas the profiles of the generalist and the specialist mutations were largely consistent across all eight ALE lineages, distribution of the random mutations was highly biased toward the mutator lineages.

Genetic load incurred by a gradual accumulation of deleterious mutations is often presented as an evolutionary tradeoff in mutator populations in exchange for their accelerated pace of evolution. Mutators inflicted with genetic burden are either outcompeted by fitter sub-lineages (Giraud et al., 2001a), or rescued with the emergence of anti-mutator genotypes that ameliorate the genetic burden (Wielgoss et al., 2013). The fact that the evolved mutator strains managed to outcompete the isogenic wild-type controls indicates that short-term evolution in minimal medium imposed no observable genetic burden to the mutator strains (Figures 4A,B). This seems to indicate that the cost of mutations accumulated in the mutator lineages through a short-term evolution did not outweigh the benefit (Giraud et al., 2001a).

With evidence suggesting that a successive accumulation of random mutations may, in turn, leads to secondary adaptive mutation (Wagner, 2008; Lynch and Abegg, 2010; Szappanos et al., 2016), growth rate measurement on a number of non-adaptive carbons was performed to prove whether these lines of genotypic changes translates to phenotypic changes. Previous studies on the populations in the long-term evolution experiments observed an extensive antagonistic pleiotropy of the 20,000 and 50,000 generation populations on a range of non-specialized environments, where the performance of mutator strains on non-adaptive environments was worse off than wild-type strains (Leiby and Marx, 2014). It is speculated that global metabolic erosion observed in the mutator lineage results from the gradual accumulation of mild-to-moderately deleterious non-synonymous mutations imposing a severe fitness burden (DePristo et al., 2005; Wielgoss et al., 2013). In this study, however, it was observed that the mutators adaptively evolved in short-term performed better across a spectrum of alternative nutrient substrates than the wild-type strains. It is possible that the sheer abundance of beneficial generalist mutations that affect global regulatory network drew the difference in the fitness profile. Nonetheless, single mutation analysis of add (947C > T), xdhB (860ins861G) and ilvH (124A > G) identified in the end-point populations of ΔmutS mutator lineages demonstrated fitness advantage on respective non-adaptive nutrient substrates (Figure 4). Together, this evidence seems to suggest that random expansion of genotype space driven by short-term laboratory evolution of mutators facilitates adaptation across a broader spectrum of fitness landscapes.

Overall, a moderate increase in mutation rates confers evolutionary edge in survival across a span of heterogeneous environments. In addition, short-term mutator ALE may serve as a tool of choice in generating versatile microbes with a strong survival advantage in fluctuating environments without observable genetic load. Use of high-throughput genotype and phenotype screening techniques enabled identification of random mutations likely causative in alternative environments.
DATA AVAILABILITY

Whole genome resequencing data generated during the current study are available in the EMBL Nucleotide Sequence Database (ENA) with primary accession number PRJEB32586.

AUTHOR CONTRIBUTIONS

B-KC conceived and supervised the study. B-KC, MK, and KK designed the study. MK and KK performed the experiments, analyzed the data, and wrote the manuscript with contributions from B-KC, DC, SC, SK, and BP.

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SUPPLEMENTARY MATERIAL

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