A dual gene-specific mutator system installs all transition mutations at similar rates \textit{in vivo}.

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Running title: Expanding \textit{in vivo} mutagenesis spectrum
ABSTRACT

Targeted in vivo hypermutation accelerates directed evolution of proteins through concurrent DNA diversification and selection. Among recently developed methods, the systems employing a fusion protein of a nucleobase deaminase and T7 RNA polymerase present gene-specific targeting. However, their mutational spectra have been largely limited to exclusive or dominant C:G→T:A mutations. Here we describe eMutaT7transition, a new gene-specific mutator system, that installs all the transition mutations (C:G→T:A and A:T→G:C) at comparable rates. By using two mutator proteins in which two efficient deaminases, PmCDA1 and TadA-8e, are separately fused to T7 RNA polymerase, we obtained similar numbers of C:G→T:A and A:T→G:C mutations at a sufficiently high rate (~3.4 × 10⁻⁵ mutations per base per generation or ~1.3 mutations per 1 kb per day). Through eMutaT7transition-mediated TEM-1 evolution for antibiotic resistance, we generated many mutations also found in clinical isolates. Overall, with a fast mutation rate and wider mutational spectrum, eMutaT7transition is a potential first-line method for gene-specific in vivo hypermutation.

Keywords: Directed evolution / Deaminase / in vivo hypermutation / Synthetic biology / T7 RNA polymerase
INTRODUCTION

Directed evolution is a powerful approach that mimics natural evolution to improve biomolecular activity (Arnold, 1998; Packer & Liu, 2015). Traditional directed evolution relies on in vitro gene diversification such as error-prone PCR or randomized oligonucleotide pools (Packer & Liu, 2015). In contrast, continuous directed evolution (CDE) adopts in vivo hypermutation, allowing simultaneous gene diversification, selection, and replication in cells; this technique significantly enhances the depth and scale of biomolecular evolution (Molina et al., 2022; Morrison et al., 2020; Rix & Liu, 2021). As random mutagenesis in the genome is highly deleterious to cells, in vivo hypermutation methods should aim to introduce mutations in a relatively narrow region around the target gene (Molina et al., 2022).

Various methods for targeted in vivo hypermutation have been reported recently, based distinct molecular principles and thus presenting assorted target ranges (Molina et al., 2022; Morrison et al., 2020; Rix & Liu, 2021). Several CRISPR-Cas-based methods (e.g., EvolvR (Halperin et al., 2018), CRISPR-X (Hess et al., 2016), base editors (Komor et al., 2016; Ma et al., 2016; Nishida et al., 2016), and prime editors (Anzalone et al., 2019)) install mutations in smaller regions of a gene. OrthoRep (Ravikumar et al., 2018) uses an orthogonal error-prone DNA polymerase and generates mutations on a plasmid. Virus-based methods, such as PACE (Esvelt et al., 2011) and mammalian cell-based systems (Berman et al., 2018; English et al., 2019), mutate the entire viral genome. Considering that most directed evolution experiments focus on a single protein, the ideal target range is a single gene of interest. Gene-specific targeting was achieved through homologous platforms that use chimeric mutator proteins, generated by fusing a nucleobase deaminase to an orthogonal T7 RNA polymerase (T7RNAP) (Alvarez et al., 2020; Butt et al., 2021; Chen et al., 2020; Cravens et al., 2021; Moore et al., 2018; Park & Kim, 2021).
The deaminase-T7RNAP system was first reported in bacteria (MutaT7; (Moore et al., 2018)) and further extended to mammalian cells (TRACE; (Chen et al., 2020)), yeast (TRIDENT; (Cravens et al., 2021)), and plants (Butt et al., 2021). We previously demonstrated that the mutation rate of MutaT7 could be enhanced 7- to 20-fold with a more efficient cytidine deaminase, Petromyzon marinus cytidine deaminase (PmCDA1) (Park & Kim, 2021). This PmCDA1_T7RNAP mutator (previously termed eMutaT7, but here renamed eMutaT7PmCDA1) generated ~4 mutations per 1 kb per day (~9.4 × 10^{-6} mutations per base per generation) in Escherichia coli, representing the fastest gene-specific in vivo mutagenesis. The major limitation of eMutaT7PmCDA1 is a narrow mutational spectrum: it mainly generates C→T mutations on the coding strand and, with the Shoulders group’s dual promoter/terminator approach that allows transcription in both directions, introduces C→T and G→A mutations (C:G→T:A) (Moore et al., 2018; Park & Kim, 2021). Mutations could be expanded to A→G and T→C (A:T→G:C) with engineered tRNA adenosine deaminases, TadA-7.10 (Alvarez et al., 2020; Gaudelli et al., 2017) and yeTadA1.0 (Cravens et al., 2021), but they either had a ~200-fold slower mutation rate (~5 × 10^{-7} mutations per base per generation) (Alvarez et al., 2020) than eMutaT7PmCDA1, or presented C:G→T:A as dominant mutations (~95%) in nonselective conditions when combined with PmCDA1_T7RNAP (Cravens et al., 2021).

Here, we report on eMutaT7transition, a new dual mutator system that introduces all transition mutations (C:G→T:A and A:T→G:C) at comparable rates. The eMutaT7transition system uses two mutators, eMutaT7PmCDA1 and eMutaT7TadA-8e. The latter is the fusion of T7RNAP and a recently evolved E. coli adenosine deaminase, TadA-8e (Richter et al, 2020), which had much higher mutational activity than the previously evolved TadA-7.10 (Gaudelli et al., 2017) (Fig 1). We optimized the expression of the two mutators and a uracil glycosylase inhibitor, and demonstrated that the frequencies of the C:G→T:A and A:T→G:C mutations were not significantly different. Furthermore, overall mutation rate was not markedly reduced. eMutaT7transition also promoted rapid continuous directed evolution of antibiotic resistance.
with various transition mutations, suggesting that it is a viable alternative for gene-specific in vivo hypermutation with an improved mutational spectrum.

RESULTS AND DISCUSSION

**emUTaT7**T**adA-8e** promotes rapid gene-specific in vivo hypermutation

To date, TadA-8e is the most efficient TadA variant, presenting a rate constant ($k_{app}$) 590 times higher than that of the previous TadA-7.10, and has been successfully used for genome editing (Richter et al., 2020). To evaluate their efficiency in gene-specific in vivo hypermutation, we fused TadA-8e and TadA-7.10 to the N-terminus of T7RNAP, creating eMutaT7TadA-8e and eMutaT7TadA-7.10, respectively (Fig 2A). As in the previous characterization of eMutaT7PmCDA1 (Park & Kim, 2021), we expressed the mutator and induced hypermutation in the target gene, pheS A294G, which was inserted between T7 promoter and T7 terminator in a low-copy-number plasmid. We determined mutational suppression of the pheS A294G toxicity by counting viable cells in the presence of p-chlorophenylalanine (p-Cl-Phe), which is toxic to cells containing intact pheS A294G. We performed 20 rounds of in vivo hypermutation (4 h growth and 100-fold dilution to a new medium for a single round) without p-Cl-Phe and then sampled cells at different time points for the cell viability assay (Fig EV1A). We found that eMutaT7TadA-8e suppression frequencies were ~1000-fold higher than eMutaT7TadA-7.10 frequencies after 8 h, indicating that the former induces gene-specific hypermutation much faster than the latter (Fig 2B).

To examine whether eMutaT7TadA-8e generates mutations in the target gene, we randomly selected three clones from cells that had undergone 20 rounds of hypermutation and sequenced the target gene by Sanger method. We also included as negative controls cells that had an empty vector, expressed TadA-8e without T7RNAP, or contained the eMutaT7TadA-8e plasmid without induction (Fig 2A). Notably, we found ~6.7 mutations per
clone (~4.0 × 10⁻⁵ mutations per base per generation; ~1.6 mutations per 1 kb per day) in the
eMutaT7TadA-8e-expressing cells, while eMutaT7TadA-7.10-expressing cells and negative
controls did not exhibit mutations (Figs 2C and EV1B). This mutation rate is approximately
80-fold higher than that of eMutaT7TadA-7.10 (Alvarez et al., 2020) and only 2.4-fold lower than
that of eMutaT7PmCDA1 (Park & Kim, 2021). Interestingly, we identified nine A→G (45%) and
11 T→C (55%) mutations on the coding strand, indicating that eMutaT7TadA-8e causes
mutations on both DNA strands (Figs 2C and EV1B). We observed that eMutaT7TadA-8e
neither noticeably reduced cell viability (Fig EV1C) nor induced rifampicin resistance (Fig
EV1D). This result suggests that eMutaT7TadA-8e does not generate significant off-target
mutations in the genome.

Deletion of genes associated with hypoxanthine repair does not significantly increase
eMutaT7TadA-8e activity

In the eMutaT7PmCDA1 system, deletion of a gene encoding a uracil-DNA glycosylase (UNG)
enhanced the mutation rate (Park & Kim, 2021). UNG removes uracil (deaminated cytosine)
and initiates the base excision repair pathway (Lindahl, 1974). Likewise, we hypothesized
that the deletion of genes encoding hypoxanthine (deaminated adenine)-removal enzymes
would further increase eMutaT7TadA-8e mutation rate. We prepared a strain in which two
genes involved in hypoxanthine repair, nfi (Guo et al., 1997; Yao et al., 1994) and alkA
(Saparbaev & Laval, 1994), are deleted and analyzed eMutaT7TadA-8e-mediated
hypermutation (Fig 3A). Twenty rounds of targeted hypermutation revealed that mutations in
the Δnfi ΔalkA strain did not increase significantly from wild-type levels (average 9 and 6.3
mutations per clone on average, respectively) (Figs 3B and EV2). Because a DNA repair
enzyme often reduces mutation rates by more than an order of magnitude (Beletskii &
Bhagwat, 1996; Duncan & Weiss, 1982; Foster et al, 2015; Schaaper, 1993) and
construction of a gene deletion strain requires additional experimental steps, we concluded
that the Δnf ΔalkA strain has no obvious advantage over the wild-type strain for eMutaT7PmCDA1 activity.

Optimized expression of uracil glycosylase inhibitor increases eMutaT7PmCDA1 activity
Although we co-expressed a UNG inhibitor (UGI) with eMutaT7PmCDA1 from the plasmid pHyo094, we did not obtain an efficiency level that matched the Δung strain (Park & Kim, 2021). Proper UGI expression can greatly expand eMutaT7PmCDA1 utility by avoiding the ung deletion. To enhance UGI activity, we initially tested a new constitutive promoter for ugi or a triply fused protein, UGI_PmCDA1_T7RNAP. However, both were less efficient than the Δung strain (Fig EV3). Next, we optimized the ribosomal binding site (RBS) of ugi (Salis et al, 2009) (Fig 3C), and obtained a suppression frequency indistinguishable from that of the Δung strain (Fig 3D). Thus, we were able to avoid ung deletion for efficient eMutaT7PmCDA1-mediated mutagenesis.

Dual expression system introduces all transition mutations at comparable rates
We examined whether the two deaminases could simultaneously install both C:G→T:A and A:T→G:C mutations at similar rates. Initially, we tested two triple-fused proteins, PmCDA1_TadA-8e_T7RNAP and TadA-8e_PmCDA1_T7RNAP, in which two deaminases were attached to the N-terminus of T7RNAP in different orders (Fig 4A). Sequencing of clones after 20 rounds of in vivo hypermutation revealed that PmCDA1_TadA-8e_T7RNAP installed more A:T→G:C mutations (84%) than C:G→T:A (16%), whereas TadA-8e_PmCDA1_T7RNAP generated more C:G→T:A (96%) than A:T→G:C (4%) (Figs 4B and EV4A). This result indicates that the deaminase closer to T7RNAP is more active. Shorter or longer linker lengths between enzymes did not significantly reduce the gap (Appendix Fig S1).
Next, we tested the expression of two mutators, eMutaT7\textsuperscript{PmCDA1} and eMutaT7\textsuperscript{TadA-8e}, from a single plasmid (Fig 4C). The pDae079 plasmid, in which the eMutaT7\textsuperscript{TadA-8e} gene is located in front of the eMutaT7\textsuperscript{PmCDA1} gene, yielded the same amounts of A:T→G:C (50%) and C:G→T:A (50%) mutations (\(p = 1.0\)). In contrast, the pDae080 plasmid, which reversed the order of the two mutators, disproportionately generated C:G→T:A (85%) over A:T→G:C (15%) (\(p = 0.0058\); Figures 4D and S4B). As expected, weaker UGI expression without optimized RBS significantly reduced C:G→T:A mutations in the wild-type strain (\(p = 0.0041\)) but produced comparable numbers of mutations in the \(\Delta\)ung strain (A:T→G:C, 38%; C:G→T:A, 62%; \(p = 0.25\); Figs 4D and EV4B). We thus selected pDae079 for eMutaT7\textsuperscript{transition}, which appears to install transition mutations at a rate of \(\sim3.4 \times 10^{-5}\) mutations per base per generation (\(\sim1.3\) mutations per 1 kb per day), approximately 2.8-fold slower than that of eMutaT7\textsuperscript{PmCDA1} (Park & Kim, 2021).

**eMutaT7\textsuperscript{transition} evolves TEM-1 with various transition mutations**

We previously demonstrated that eMutaT7\textsuperscript{PmCDA1} promoted rapid continuous directed evolution of TEM-1 for resistance against third-generation cephalosporin antibiotics, cefotaxime (CTX) and ceftazidime (CAZ) (Park & Kim, 2021). Here, we tested eMutaT7\textsuperscript{transition} in the same way. We used the dual promoter/terminator approach to install both C→T and G→A mutations (Moore et al., 2018; Park & Kim, 2021). By sequentially increasing antibiotic concentrations during multiple rounds of \textit{in vivo} hypermutation, we elevated minimum inhibitory concentrations (MICs) from 0.05 to 400–1600 µg/mL in 80 h for CTX (Fig 5A) and from 0.4 to 4000 µg/mL in 48 h for CAZ (Fig 5B).

Sanger sequencing of several resistant clones revealed that their mutational spectra were more diverse than those obtained with eMutaT7\textsuperscript{PmCDA1} (Fig EV5). Adenine deamination
(A:T→G:C) generated I45V, H151R, I171M, I171T, and M180T, whereas cytosine deamination (C:G→T:A) generated E102K, R162H, G236S, and E237K (Figs 5C, D, and EV5). I45V, H151R, and M180T have been reported to increase enzyme stability (Bershtein et al., 2008; Brown et al., 2010; Kather et al., 2008; Palzkill, 2018). Additionally, E102K, R162H, I171M/T, G236S, and E237K are involved in resistance to CTX or CAZ (Barlow & Hall, 2003; Driffield et al., 2006; Palzkill, 2018; Salverda et al., 2010; Sowek et al., 1991; Zaccolo & Gherardi, 1999). Other mutations are not found in clinical or laboratorial isolates (H24R and V42A), are found in the wild-type allele (I82V), or have unknown functional effect (T267A) (Salverda et al., 2010). These results suggest that eMutaT7transition indeed covers a wider protein mutational space for evolution.

In conclusion, this study described a new mutator system that combines eMutaT7PmCDA1 and eMutaT7TadA-8e, called eMutaT7transition. This new system has advantages over previous deaminase-T7RNAP mutators. First, eMutaT7transition expands the mutational spectrum to all transition mutations (C:G→T:A and A:T→G:C). eMutaT7PmCDA1 can mediate 8.4% of all amino acid changes (32 out of total 380 changes), but eMutaT7transition expands them to 19% (74 changes). Accordingly, we observed in TEM-1 evolution experiments several A:T→G:C mutations that have been previously identified in clinical or laboratorial isolates. Second, all transition mutations are produced at similar rates. This outcome was made possible by the use of two efficient deaminases, PmCDA1 and TadA-8e, along with appropriate expression of the two mutators and a DNA glycosylase inhibitor. In contrast, TRIDENT generated considerably more C:G→T:A mutations (~95%) in yeast (Cravens et al., 2021).

eMutaT7transition had a ~2.8-fold slower mutation rate (~3.4 × 10⁻⁵ mutations per base per generation; ~1.3 mutations per 1 kb per day) than eMutaT7PmCDA1 (Park & Kim, 2021). However, it was still sufficiently fast to support rapid continuous directed evolution of TEM-1. Moreover, as also observed with eMutaT7PmCDA1, evolved TEM-1 variants often contained 2-
to 3-fold more mutations than expected from the nominal rate; variants resistant to CTX and CAZ showed rates of ~2.6 and ~3.9 mutations per 1 kb per day, respectively (Fig EV5). Future research should aim to include transversion mutations in the mutational spectrum without significantly sacrificing mutation rate. With its good mutation rate and wider mutational spectrum, we believe that eMutaT7\(^{\text{transition}}\) can become the method of choice for gene-specific \textit{in vivo} hypermutation.
MATERIALS AND METHODS

Materials

All PCR experiments were conducted with KOD Plus neo DNA polymerase (Toyobo, Japan). T4 polynucleotide kinase and T4 DNA ligases were purchased from Enzynomics (South Korea). Plasmids and DNA fragments were purified with LaboPass™ plasmid DNA purification kit mini, LaboPass™ PCR purification kit, and LaboPass™ Gel extraction kit (Cosmogenetech, South Korea). Sequences of all DNA constructs in this study were confirmed by Sanger sequencing (Macrogen, South Korea and Bionics, South Korea). Antibiotics (carbenicillin, chloramphenicol, kanamycin, streptomycin, and tetracycline), arabinose, and Isopropyl β-D-1-thiogalactopyranoside (IPTG) were purchased from LPS solution (South Korea). Cefotaxime and ceftazidime were purchased from Tokyo chemical industry (Japan). H-p-Chloro-DL-Phe-OH (p-Cl-Phe) was purchased from Bachem (Switzerland).

Plasmid and E. coli strain construction

Plasmids, Escherichia coli strains, and primers used in this study are listed in Appendix Tables S1-S3, respectively. Genes for adenine deaminase TadA-7.10 (Gaudelli et al., 2017) and TadA-8e (Richter et al., 2020) were synthesized from Gene Universal (USA). Genes for the Petromyzon marinus cytidine deaminase (PmCDA1), XTEN linker, and T7 RNA polymerase (T7RNAP) were amplified from the plasmid expressing eMutaT7 (pHyo094) (Park & Kim, 2021). Genes for adenine deaminases (TadA-7.10 or TadA-8e), linker, and T7RNAP were linked by in vivo assembly (IVA) cloning (Garcia-Nafria et al, 2016). Plasmids expressing PmCDA1_XTEN_T7RNAP (eMutaT7PmCDA1), TadA-8e_XTEN_T7RNAP (eMutaT7TadA-8e), and UGI were cloned by IVA cloning. Also, genes encoding triply-fused proteins, UGI_PmCDA1_T7RNAP, PmCDA1_TadA-8e_T7RNAP, and TadA-8e_PmCDA1_T7RNAP, were also constructed by IVA cloning.
All plasmids expressing variants of mutators or targets (mutation, deletion, and insertion) were constructed using the site-directed mutagenesis PCR method. (Reikofski & Tao, 1992) Plasmids expressing eMutaT7 and UGI in different conditions (deletion of UGI, an optimized ribosomal binding site for UGI, or a constitutive promoter for UGI) were made on pHyo094. Plasmids harboring TadA-8e were made on pDae029. Plasmids expressing PmCDA1_TadA-8e_T7RNAP with different linkers were constructed on pDae036.

For evolution of antibiotic resistance, a target plasmid (pGE158) was constructed from pHyo245, which contains the \textit{pheS} \textsubscript{A294G} gene between dual promoter/terminator pairs in a low-copy-number plasmid (Park & Kim, 2021): Ampicillin resistance gene in pHyo245 was replaced with tetracycline resistance gene and \textit{pheS} \textsubscript{A294G} was replaced with the \textit{TEM-1} gene by IVA cloning. Tetracycline resistance gene was amplified from the plasmid pREMCM3 (Melancon & Schultz, 2009) and the \textit{TEM-1} gene was obtained from pHyo182 (Park & Kim, 2021).

W3110 \textit{Δ}alkA \textit{Δ}nfi strain (cDJ085) was constructed by homologous recombination method (Sharan \textit{et al}, 2009) The \textit{alkA} and \textit{nfi} genes in W3110 were replaced with the streptomycin resistance gene and the kanamycin resistance gene, respectively. 30 \textmu g/mL of streptomycin or kanamycin was used for selection. Proper gene deletion was confirmed by colony PCR using 2X TOP simple\textsuperscript{TM} DyeMIX-Tenuto (Enzynomics).

\textbf{In vivo hypermutation}

Three biological replicates of W3110 or the \textit{Δ}ung strain (cHYO057) harboring a mutator plasmid and a target plasmid (pHyo182 for a single promoter) were grown overnight in LB medium with 35 \textmu g/mL chloramphenicol and 50 \textmu g/mL carbenicillin (cycle #0). On the following day, the overnight cultures were diluted 100-fold in a fresh LB medium supplemented with 35 \textmu g/mL chloramphenicol, 50 \textmu g/mL carbenicillin, 0.2% arabinose, and
0.1 mM IPTG in a 96-deep well plate (Bioneer, South Korea) and incubated at 37°C with shaking (cycle #1). Bacterial cells were diluted every 4 hours and this growth cycle was repeated up to 20 times for accumulation of mutations. At the end of cycle, a fraction of cells were stored at -80°C with 15% glycerol. To identify mutations in the target gene, cells at cycle #20 were streaked on LB-Agar plates with 35 μg/mL chloramphenicol and 50 μg/mL carbenicillin. Three or six colonies were randomly chosen for isolation of target plasmids. The target genes in the purified target plasmids were sequenced by Sanger sequencing. Mutations were counted in the region between 147-bp upstream and 138bp-downstream of the pheS_A924G gene (total 1269 bp). Primer 314 and 315 were used for amplification and sequencing of the target gene that has a single promoter system.

**PheS_A294G suppression assay**

Suppression frequency of the pheS_A294G toxicity was determined as previously described (Park & Kim, 2021). Cells obtained at the endpoint of each cycle (overnight culture for cycle #0) were diluted to OD$_{600}$ ~ 0.2. Serial 10-fold dilutions of cells (5μl) using LB broth were placed on YEG-agar plates with or without additives (1.6 mM p-Cl-Phe, 0.2% arabinose, and 0.1 mM IPTG) and grown overnight at 37°C. On the following day, the number of colonies on each condition was counted to calculate the suppression frequency. The suppression frequency was calculated as $N_1/N_0$ ($N_1$: colony forming unit (CFU) in the p-Cl-Phe plates and $N_0$: CFU in plates without p-Cl-Phe).

**Assays for cell viability and off-target mutagenesis**

Cell viability and off-target mutagenesis were assayed as previously described (Park & Kim, 2021). Overnight cultures of the cells harboring the plasmid expressing eMutaT7$_{TadA-8e}$, no mutator, or MP6 were diluted 100-fold in LB supplemented with 35 μg/mL chloramphenicol and grown to a log phase (OD$_{600}$ = 0.2-0.5) at 37°C. Cells were diluted to OD$_{600}$ ~ 0.2 and serial 10-fold dilutions of cells (5μl) using LB broth were placed on LB-agar supplemented
with 35 μg/mL chloramphenicol and 0.2% arabinose. After overnight growth at 37°C, the number of colonies on the plates were counted to calculate CFU/mL. To evaluate the off-target mutagenesis via rifampicin resistance, cells taken at cycle #0 and cycle #20 were grown to log phase in LB supplemented with 35 μg/mL chloramphenicol and 50 μg/mL carbenicillin, and subjected to viability assay on plates with or without rifampicin (50 μg/mL).

**Mutation rate calculation**

Mutation rates were calculated and presented as mutations per base per generation and mutations per 1 kb per day. The sizes of the gene region are 1269 bases for pheS_A294G and 1121 bases for TEM-1. One growth cycle, which is 100-fold growth in four hours, indicates 6.64 generations (log₂100). The mutation rate of TadA-7.10_T7RNAP from Fernandez group’s report (Alvarez et al., 2020) was calculated as \((3.0 \times 10^{-4} \text{ variant calling frequency from Figure 4f}) / (200 \text{ base}) / (3 \text{ generation}) = 5 \times 10^{-7} \text{ mutations per base per generation.}

**TEM-1 evolution and identification of the evolved mutants**

TEM-1 evolution experiments were performed as previously described (Park & Kim, 2021). Strains were grown in LB medium supplemented with 6 μg/mL tetracycline, 35 μg/mL chloramphenicol, 0.2% arabinose, and 0.1 mM IPTG. Cells were grown without selection pressure at the initial cycle. Then, multiple cultures were grown with different concentrations of an antibiotic (cefotaxime and ceftazidime) at the same time and the culture grown at the highest antibiotic concentration (OD₆₀₀ > 1) were used for the next round of evolution. After the final cycle, the target plasmids were purified and re-inserted into fresh W3110 cells harboring the T7RNAP-expressing plasmid (pHyo183) for validation of antibiotic resistance. Twelve colonies were randomly selected for MIC measurement and those with high MIC values (five colonies with 400-1600 μg/mL MIC for CTX, three colonies with 4000 μg/mL MIC for CAZ) were subjected to the target gene sequencing by Sanger method.
MIC determination

MIC values were measured as previously described (Park & Kim, 2021). Cells were grown overnight in LB medium supplemented with 6 μg/mL tetracycline, 35 μg/mL chloramphenicol. They were diluted 10,000-fold into fresh LB broth with increasing concentrations of antibiotics (2-fold) in 96-deep well plates, and grown at 37°C with shaking (290 rpm) overnight. Final cell density (OD$_{600}$) was measured by M200 microplate reader (TECAN, Switzerland).

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AUTHOR CONTRIBUTIONS

DS and SK developed the initial concept; DS, GE, and HWK performed experiments and analyzed the results; SK supervised the work throughout; DS and SK wrote the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.
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FIGURE LEGENDS

Figure 1. Schematic of the eMutaT7\textsuperscript{transition} system. eMutaT7\textsuperscript{transition} uses two chimeric mutators, eMutaT7\textsuperscript{PmCDA1} and eMutaT7\textsuperscript{TadA-8e}, and co-expresses a uracil-glycosylase inhibitor (UGI). The two mutators are expressed with 0.2% arabinose, and target gene transcription is induced with 0.1 mM IPTG. When T7 RNA polymerases transcribe the target gene between T7 promoter and T7 terminator, deaminase enzymes introduce C:G→T:A (cytidine deaminase) or A:T→G:C (adenine deaminase) mutations. UGI promotes C:G→T:A mutations via preventing uracil removal in DNA (Wang et al., 1991).

Figure 2. eMutaT7\textsuperscript{TadA-8e} rapidly introduces A→G and T→C mutations in the target gene. A Scheme of the tested mutators and conditions.

B Frequency of the \textit{pheS} A294G toxicity suppression at each mutagenesis cycle for cells expressing eMutaT7\textsuperscript{TadA-7.10} or eMutaT7\textsuperscript{TadA-8e}.

C Number of A→G (red) or T→C (orange) mutations found in three clones obtained after 20 mutagenesis cycles. Data are presented as dot plots with mean ± standard deviation (SD) (n = 3). **P < 0.01, ***P < 0.001; Student’s \textit{t}-test.

Figure 3. Optimization of eMutaT7\textsuperscript{TadA-8e} or eMutaT7\textsuperscript{PmCDA1}-mediated \textit{in vivo} hypermutation.

A eMutaT7\textsuperscript{TadA-8e} activity was tested in wild-type or Δ\textit{nfi} ΔalkA strains.

B Number of mutations found in three clones from the two samples in (A) after 20 mutagenesis cycles.

C eMutaT7\textsuperscript{PmCDA1} activity was tested without or with an optimized ribosomal binding site for \textit{ugi} in wild-type or Δ\textit{ung} strains.

D Suppression frequency of the \textit{pheS} A294G toxicity at each mutagenesis cycle for cells evolved under the four different conditions shown in (C). Data are presented as dot plots with mean ± standard deviation (SD) (n = 3). ***P < 0.001, Student’s \textit{t}-test.
**Figure 4. eMutaT7\textsuperscript{transition} rapidly introduces all transition mutations at similar rates.**

A Two triply-fused mutators were tested for equivalent incorporation of A:T→G:C and C:G→T:A mutations.

B Mutation counts in six clones from samples shown in (A) after 20 mutagenesis cycles.

C Four dual mutator systems were tested for equivalent incorporation of A:T→G:C and C:G→T:A mutations.

D Mutation counts in six clones from samples in (C) after 20 mutagenesis cycles. Data are presented as dot plots with mean ± standard deviation (SD) (n = 6). **P < 0.01, ***P < 0.001; Student's t-test.

**Figure 5. Continuous directed evolution of TEM-1 for antibiotic resistance using eMutaT7\textsuperscript{transition}.**

A, B Evolutionary pathways of TEM-1 for resistance against CTX (A) or CAZ (B). Each number indicates an antibiotic concentration in a culture. Strikethrough indicates no growth.

C, D Structure of TEM-1 (PDB, 1axb) showing a covalent inhibitor (yellow), the active site (green), A:T→G:C mutations in evolved TEM-1 (red), and C:G→T:A mutations in evolved TEM-1 (blue). Mutations in orange and purple boxes indicate those that increased enzyme activity and stability, respectively.
EXPANDED VIEW FIGURE LEGENDS

Figure EV1. Characterization of eMutaT7\textsuperscript{TadA-8e}.

A A representative result of the \textit{pheS}\textsubscript{A294G} toxicity suppression experiment without or with \textit{p}-\textit{Cl}-Phe. Strains harboring the \textit{pheS}\textsubscript{A294G} target gene were subjected to \textit{in vivo} hypermutation in five different conditions: expressing eMutaT7\textsuperscript{TadA-7.10}, eMutaT7\textsuperscript{TadA-8e}, no protein, TadA-8e, or no eMutaT7\textsuperscript{TadA-8e} from the cognate plasmid. Cells taken at particular growth cycle were grown in LB media (without arabinose and IPTG) until OD\textsubscript{600} reached \sim 0.2. Serial 10-fold dilutions of cells were spotted and grown on YEG-agar plates supplemented with or without 1.6 mM \textit{p}-\textit{Cl}-Phe. Growth of cells expressing \textit{pheS}\textsubscript{A294G} is inhibited in the presence of \textit{p}-\textit{Cl}-Phe. Only the eMutaT7\textsuperscript{TadA-7.10}- and eMutaT7\textsuperscript{TadA-8e}-expressing cells had T7 RNA polymerase and thus showed growth inhibition in the presence of \textit{p}-\textit{Cl}-Phe.

B A list of mutations found in samples shown in Figure 2C.

C Viability of cells expressing no protein, eMutaT7\textsuperscript{TadA-8e}, or MP6.

D Off-target mutation level of cells expressing eMutaT7\textsuperscript{TadA-8e} or no protein was estimated by rifampicin resistance frequency. The dotted line represents spontaneous rifampicin resistance level.

Data are presented as dot plots with mean ± standard deviation (SD) (n = 3). ***P < 0.001; Student's t-test.

Figure EV2. The deletion of inosine glycosylases did not elevate the mutation rate of eMutaT7\textsuperscript{TadA-8e}.

A Number of A>C (red) and T>C (orange) mutations in samples shown in Figure 3B.

B A list of mutations in (A).

Data are presented as dot plots with mean ± standard deviation (SD) (n = 3).

Figure EV3. eMutaT7\textsuperscript{PmCDA\textsubscript{1}} optimization.
A Five tested conditions for eMutaT7PmCDA1-mediated hypermutation: no UGI in wild-type strain (1), UGI expression under the original promoter in wild-type (2) or Δung (3) strains, UGI expression under a new constitutive promoter (J23100) in wild-type strain (4), and a triple fusion of UGI, PmCDA1, and T7RNAP in the wild-type strain (5).

B Frequency of the pheS_A294G toxicity suppression at each mutagenesis cycle for cells evolved under the five different conditions shown in (A).

Data are presented as dot plots with mean ± standard deviation (SD) (n = 3). **P < 0.01, ***P < 0.001; Student’s t-test.

Figure EV4. A full list of mutations in samples shown in Figure 4B (A) and 4D (B).

Figure EV5. A full list of mutations found in samples shown in Figure 5A (A) and 5B (B).
**Figure 1**

- **mutator**
  - + 0.2% arabinose

- **target**
  - target gene
  - T7 promoter
  - T7 terminator

- **mRNA**
  - Uracil glycosylase
  - + 0.1mM IPTG

- **TadA-8e**: Adenine Deaminase
- **PmCDA1**: Cytidine Deaminase
- **T7RNAP**: T7 RNA Polymerase
- **UGI**: Uracil-glycosylase inhibitor

Legend:
- Red: A:T -> G:C
- Blue: C:G -> T:A
Figure 2

A.

1. (pDae028): 
   - TadA-7.10
   - eMutaT7
   - Log10(Suppression frequency)
   - Time(hr)

2. (pDae029): 
   - TadA-8e
   - eMutaT7
   - Log10(Suppression frequency)
   - Time(hr)

3. (pBAD33): 
   - TadA-8e
   - Empty vector
   - Log10(Suppression frequency)
   - Time(hr)

4. (pDae032): 
   - TadA-8e
   - No induction
   - Log10(Suppression frequency)
   - Time(hr)

B.

- Log10(Suppression frequency) vs. Time(hr)
- n.s*** ** *** *** ***
- Mutator
- 1 2

C.

- Number of mutations
- A>G
- T>C
- Mutator
- 1 2 3 4 5
Wild type

Δung

5 (pHyo094): 3 (pDae014): 4 (pHyo094): 6 (pDae069):

log10(Suppression frequency)

Number of mutations

Strain Mutator

Figure 3
Figure 5

(A) Cefotaxime (CTX) (μg/mL) starting MIC = 0.05

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(B) Ceftazidime (CAZ) (μg/mL) starting MIC = 0.4

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(C) Evolved TEM-1 against CTX

(D) Evolved TEM-1 against CAZ

**Legend:**
- **Red:** A:T>G:C; **Blue:** C:G>T:A; **Orange box:** increase the activity; **Purple box:** increase the stability
Figure EV1

A

YEG

[p-Cl-phe]

3 (Empty vector)

1 (eMutaT7TadA-7.10)

4 (TadA-8e)

2 (eMutaT7TadA-8e)

5 (No induction of eMutaT7TadA-8e)

Ten fold dilutions

B

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Amino acids were numbered according to the protein sequence of pheS_A294G
A>G mutation on the coding strand, red; T>C mutation on the coding strand, orange
* mutations not made on pheS_A294G gene orf but made between a T7 promoter and a T7 terminator

C

D

log$_{10}$(Rifampicin resistance frequency)

0h
(cycle#0)
80h
(cycle#20)

Empty vector (3)
+eMutaT7TadA-8e (2)
Spontaneous resistance

n.s

***

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this version posted June 12, 2022. ; https://doi.org/10.1101/2022.06.09.495438
doi: bioRxiv preprint
Cell strain-clone# | A>G mut# | T>C mut# | total# | average | mutation rate
--- | --- | --- | --- | --- | ---
1-1 | -144*, D20D, Y31H, R111R | 4 | 4 | 6.3 | 1.5 mut•day⁻¹•kb⁻¹
1-3 | -64*, -17*, G33G, R167R | 5 | -64*, -17* | 9 | 6.3 | 1.5 mut•day⁻¹•kb⁻¹
2-1 | -133*, -131*, -65*, -63*, N196D | 5 | D25D, Y137H, F326L | 3 | 8 | 9 | 2.1 mut•day⁻¹•kb⁻¹
2-2 | -131*, -109*, -98*, -92*, -6*, V173V, T216A, X328X | 8 | -87*, -17*, L24S, A81A | 4 | 12 | 9 | 2.1 mut•day⁻¹•kb⁻¹
2-3 | -133*, -131*, -98*, M1V, N26D, E102E | 6 | -17* | 1 | 7 | 9 | 2.1 mut•day⁻¹•kb⁻¹

Amino acids were numbered according to the protein sequence of pheS_A294G
A>G mutation on the coding strand, red; T>C mutation on the coding strand, orange
* mutations not made on pheS_A294G gene orf but made between a T7 promoter and a T7 terminator
**Figure EV3**

A

![Diagram showing mutator and strain relationships](image)

1 (pDae014): PmCDA1 → Wild type
2 (pHy094): T7RNAP → Wild type
3 (pHy094): J23100 → Δung
4 (pDae043): T7RNAP → Wild type
5 (pDae044): PmCDA1 → Wild type

B

![Graph showing suppression frequency](image)

- **log_{10}(Suppression frequency)**
- Mutators: 1, 2, 3, 4, 5
- Time points: 0h, 4h, 8h, 12h, 16h, 20h

Statistical significance:
- ****: p < 0.01
- ***: p < 0.001
- n.s.: not significant

Legend:
- PmCDA1
- T7RNAP
- Δung
- UGI

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Amino acids were numbered according to the protein sequence of phesS_A294G

A>G mutation on the coding strand, red; T>C mutation on the coding strand, orange; C>T mutation on the coding strand, blue; G>A mutation on the coding strand, cyan

*mutations not made on phesS_A294G gene orf but made between a T7 promoter and a T7 terminator

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<td>6-3</td>
<td></td>
<td>8</td>
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<td>8</td>
<td>4.5 mut•day⁻¹•kb⁻¹</td>
</tr>
<tr>
<td>6-4</td>
<td>-98*, -92*, -87*, -73*, -64*, -2*, N26D, Y31C, S2S, S2S</td>
<td>4</td>
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<td>4</td>
<td>4.5 mut•day⁻¹•kb⁻¹</td>
</tr>
<tr>
<td>6-5</td>
<td>-109*, -92*, -76*, -17*, N26D, N72N, Y198C</td>
<td>7</td>
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<td>7</td>
<td>4.5 mut•day⁻¹•kb⁻¹</td>
</tr>
<tr>
<td>6-6</td>
<td></td>
<td>4</td>
<td></td>
<td>4</td>
<td>4</td>
<td>4.5 mut•day⁻¹•kb⁻¹</td>
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<tr>
<td>7-1</td>
<td>V109V</td>
<td>1</td>
<td></td>
<td>1</td>
<td>1</td>
<td>7.5 mut•day⁻¹•kb⁻¹</td>
</tr>
<tr>
<td>7-2</td>
<td>-17*, D25G, T112A</td>
<td>3</td>
<td></td>
<td>3</td>
<td>3</td>
<td>7.5 mut•day⁻¹•kb⁻¹</td>
</tr>
<tr>
<td>7-3</td>
<td>M1T</td>
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<td>1</td>
<td>1</td>
<td>7.5 mut•day⁻¹•kb⁻¹</td>
</tr>
<tr>
<td>7-4</td>
<td>-92*, -32*, -17*, N26D, +20*</td>
<td>5</td>
<td></td>
<td>5</td>
<td>5</td>
<td>7.5 mut•day⁻¹•kb⁻¹</td>
</tr>
<tr>
<td>7-5</td>
<td>-144*, -17*, S2S, N61D, R99R, R232R</td>
<td>6</td>
<td></td>
<td>6</td>
<td>6</td>
<td>7.5 mut•day⁻¹•kb⁻¹</td>
</tr>
<tr>
<td>7-6</td>
<td>-98*</td>
<td>1</td>
<td></td>
<td>1</td>
<td>1</td>
<td>7.5 mut•day⁻¹•kb⁻¹</td>
</tr>
</tbody>
</table>

Amino acids were numbered according to the protein sequence of phesS_A294G

A>G mutation on the coding strand, red; T>C mutation on the coding strand, orange; C>T mutation on the coding strand, blue; G>A mutation on the coding strand, cyan

*mutations not made on phesS_A294G gene orf but made between a T7 promoter and a T7 terminator
### Mutation list of evolved TEM-1 against cefotaxime (CTX)

<table>
<thead>
<tr>
<th>Clone #</th>
<th>A&gt;G</th>
<th>mut#</th>
<th>T&gt;C</th>
<th>mut#</th>
<th>C&gt;T</th>
<th>mut#</th>
<th>G&gt;A</th>
<th>mut#</th>
<th>total#</th>
<th>MIC (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>H151R</td>
<td>1</td>
<td>V42A, M180T</td>
<td>2</td>
<td>-25*, M180T</td>
<td>2</td>
<td></td>
<td>2</td>
<td>5</td>
<td>400</td>
</tr>
<tr>
<td>5</td>
<td>H151R, T267A</td>
<td>2</td>
<td></td>
<td>2</td>
<td></td>
<td>0</td>
<td>-81*, G236S</td>
<td>2</td>
<td>6</td>
<td>400</td>
</tr>
</tbody>
</table>

Average mutation rate: 9.6 mut day⁻¹ kb⁻¹

### Mutation list of evolved TEM-1 against ceftazidime (CAZ)

<table>
<thead>
<tr>
<th>Clone #</th>
<th>A&gt;G</th>
<th>mut#</th>
<th>T&gt;C</th>
<th>mut#</th>
<th>C&gt;T</th>
<th>mut#</th>
<th>G&gt;A</th>
<th>mut#</th>
<th>total#</th>
<th>MIC (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 2</td>
<td>-32*, A16A**, I82V, H151R</td>
<td>4</td>
<td>-33*, -25*, S128S, N272N</td>
<td>4</td>
<td></td>
<td>0</td>
<td>R162H, E237K</td>
<td>2</td>
<td>10</td>
<td>4000</td>
</tr>
<tr>
<td>2, 3</td>
<td>-32*, H24R, I45V, H151R</td>
<td>4</td>
<td>I171T, N272N</td>
<td>2</td>
<td></td>
<td>0</td>
<td>R162H, E237K</td>
<td>2</td>
<td>8</td>
<td>4000</td>
</tr>
</tbody>
</table>

Average mutation rate: 8.7 mut day⁻¹ kb⁻¹

Amino acids were numbered according to the protein sequence of TEM-1 including signal sequence. Silent mutations, grey; The mutations which increase the MIC of CTX and CAZ, orange; The mutations that increase the enzyme stability, purple

* mutations not made on TEM-1 gene orf but made between a T7 promoter and a T7 terminator
** mutations made on signal sequence (23AA)
*** mutation on start codon from ATG to GTG