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A Processive Protein Chimera Introduces Mutations Across Defined DNA Regions In Vivo

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Supporting Information Placeholder

ABSTRACT: Laboratory-timescale evolution in vivo relies on the generation of large, mutationally diverse gene libraries to rapidly explore biomolecule sequence landscapes. Traditional global mutagenesis methods are problematic because they introduce many off-target mutations that are often lethal and can engender false positives. We report the development and application of the MutaT7 chimera, a potent and highly targeted in vivo mutagenesis agent. MutaT7 utilizes a DNA-damaging cytidine deaminase fused to a processive RNA polymerase to continuously direct mutations to specific, well-defined DNA regions of any relevant length. MutaT7 thus provides a mechanism for in vivo targeted mutagenesis across multi-kb DNA sequences. MutaT7 should prove useful in diverse organisms, opening the door to new types of in vivo evolution experiments.

Traditional in vivo mutagenesis strategies, which are critical for studying and using evolution in living systems, rely on exogenous mutagens (e.g., high energy light or chemicals) or expressing mutagenic enzymes (e.g., XL1-Red or the MP6 plasmid). These global mutagenesis strategies can yield high mutation rates and diverse genetic landscapes. However, extensive mutations throughout the genome are problematic in many contexts, especially in directed evolution experiments. Off-target mutations outside the intended DNA region are often toxic when they occur in essential portions of the genome, which is a problem that limits library size and engenders rapid silencing of mutagenic plasmids. Global mutagens also introduce “parasite” variants into DNA libraries, originating from mutations outside the gene of interest that allow an organism to circumvent selection schemes.

Targeted in vivo mutagenesis strategies have the potential to overcome these deficiencies. DNA-damaging enzymes fused to de-activated Cas9 nucleases can edit bases at specific genetic loci, but require many gRNAs to tile mutagenic enzymes throughout a target DNA that may be multi-kb in length. Moreover, the guide RNAs must be redesigned after each evolution round introduces new mutations in the target DNA. Another example is the use of an error-prone polI variant to selectively mutagenize genes on ColE1 plasmids, although this method is limited to E. coli and can target mutations within only a few kb of the ColE1 origin. Error-prone replication mediated by the Ty1 retrotransposon specifically in yeast can also selectively mutate < 5 kb genetic cargoes inserted into the retrotransposon. Other targeted mutation methods in yeast include oligo-mediated genome engineering, which can be labor-intensive, and an orthogonal replication system, which has a relatively low mutation rate.

We rationalized that a processive, DNA-traversing biomolecule tethered to a DNA-damaging enzyme could provide a generalizable solution to the problem of targeting mutations across large, yet still well-defined, DNA regions. Monomeric RNA polymerases possess inherently high promoter specificity and processivity. Cytidine deaminases are potent DNA-damaging enzymes that can act on single-stranded DNA substrates during transcription. We envisioned that a chimeric “MutaT7” protein consisting of a cytidine deaminase (rApo1) fused to T7 RNA polymerase (T7-pol) would, therefore, allow us to target mutations specifically to any DNA region lying downstream of a T7 promoter. Provided the T7 promoter is not present elsewhere in the genome.

To begin, we used a lacZ expression assay to show that T7-Pol tolerated an rApo1 N-terminal fusion and still efficiently transcribed tens of kilobases (Figure S1). Next, we integrated the MutaT7 gene under control of a weak promoter into the genome of E. coli lacking uracil N-glycosylase (Aung) (Figure S2 and Table S1). Deleting ung inhibits repair of deoxyuridine to deoxycytidine and increases mutagenesis rates, especially in the context of cytidine deaminases. We assayed for targeted mutagenesis using a codon reversion assay based on reporter plasmids either having

Figure 1. (a) Schematic illustrating global versus targeted mutagenesis. (b) The MutaT7 construct and the targeted mutagenesis cycle.
or lacking a T7 promoter sequence upstream of silent drug resistance genes with ACG triplets in place of ATG start codons (Figures 2a and S3–S5a). The kanamycin resistance gene (KanR) was placed immediately downstream of the T7 promoter. In this assay, successful C→T mutagenesis at the KanR start codon yields kanamycin-resistant colonies. Global mutagens such as the MP6 plasmid yielded high levels of kanamycin-resistant colonies regardless of the T7 promoter, consistent with a lack of promoter-based targeting (Figure 2b). In contrast, MutaT7 strains attained significant kanamycin resistance only when reporter plasmids possessed a T7 promoter upstream of the KanR gene (Figure 2b and Table S2). Expression of a catalytically dead version of MutaT7 (drApo1–T7) yielded kanamycin resistance frequencies similar to background levels, indicating that T7 activity alone was not responsible for the observed increase in kanamycin resistance (Figure 2b).

T7 promoter-dependent KanR mutagenesis by MutaT7 shows that mutagenesis can be targeted to a desired DNA region near a T7 promoter. Because T7-pol is highly processive, we anticipated mutations would also be introduced further downstream of the T7 promoter. We assayed for MutaT7 processivity by inserting a tetracycline-resistance (TetR) gene with an inactive, ACG start codon ~1.6 kb downstream of the KanR gene (Figure 2a). We observed high levels of MutaT7-dependent tetracycline resistance only in reporter strains having the T7 promoter, consistent with targeted and processive introduction of mutations across a lengthy, multi-kb DNA region (Figure 2b). Global mutagens again generated tetracycline-resistant colonies at high frequency in all cases, irrespective of the T7 promoter (Figure 2b).

Targeted mutagenesis using the processive MutaT7 chimera requires not just recruitment to a DNA locus, but also termination at the end of targeted DNA. To address termination, we used KanR/TetR reporter plasmids in which we separated the silent, start codon-defective resistance genes by one or more T7 terminators (Figure 2a). Upon assaying for drug resistance, we found that four copies of the T7 terminator fully constrained mutagenesis to the intended upstream KanR gene (Figure S6). Using this terminator array, we observed tetracycline resistance for MutaT7 strains similar to background levels, whereas kanamycin resistance remained high (Figure 2b). Global mutagens again induced high levels of kanamycin- and tetracycline-resistance, irrespective of the terminator array (Figure 2b).

To further assess whether MutaT7 induces mutagenesis specifically on the target DNA, we evaluated the evolution of resistance to rifampicin and fosfomycin. Resistance can derive from diverse genomic mutations such that the appearance of resistant colonies correlates with off-target mutation rates in the genome. Figure 2.

**Figure 2.** (a) Drug resistance start codon reversion reporter assay for measuring extent of mutagenesis at specific DNA loci. (b) Codon reversion reporter assay data for combinations of mutagen and reporter plasmids. Mutagens include deactivated drApo1 fused to T7 RNA polymerase (drApo1–T7; negative control), unfused rApo1 (rApo1), targeted mutagen (MutaT7), and global mutagen (MP6). (c) Extent of off-target mutagenesis assessed by rifampicin and fosfomycin. Resistance can derive from diverse genomic mutations such that the appearance of resistant colonies correlates with off-target mutation rates in the genome.
analogous to cheating parasites in directed evolution schemes. Selection on either rifampicin- or fosfomycin-treated plates revealed that MutT7-expressing samples displayed drug resistance frequencies comparable to background. In contrast, high frequencies of antibiotic resistance were observed in all global mutagenesis samples (Figures 2c and S5b–c).

We next attempted to use MutT7 to evolve ectopically expressed folA gene variants that confer trimethoprim resistance. The folA gene encodes dihydrofolate reductase, and folA mutations are just one of many potential routes to trimethoprim resistance.\(^{10}\) We used either global mutagenesis or MutT7 to mutagenize E. coli carrying a T7-targeted episomal copy of folA. We then Sanger-sequenced colonies that grew on trimethoprim plates. We observed that 29 of 44 trimethoprim-resistant colonies obtained using MutT7 had a mutation known to confer resistance\(^11\) in the episomal folA promoter (Tables S3 and S4, Figure S7). In contrast, none of the 43 trimethoprim-resistant colonies obtained using the global mutagen contained mutations in the episomal folA gene. Instead, they presumably gained trimethoprim resistance via undesired mutations in the E. coli genome. The ability of MutT7 to generate a high rate of true positives in the desired episomal gene target, whereas global mutagenesis exclusively generated cheaters (false positives), highlights a key advantage of MutT7.

In theory, another benefit of targeted mutagenesis is the capacity to attain much larger library sizes by avoiding toxic mutations in essential, off-target genes. Based on the apparently low off-target mutagenesis rate of MutT7, we hypothesized that E. coli carrying MutT7 would have significantly higher viability than bacteria treated with global mutagens. Indeed, consistent with prior work,\(^2\) we observed very low viability in all populations treated with global mutagens. In contrast, populations expressing MutT7 possessed viability similar to untreated cells (Figures 2d and S5d). We also found that the total number of kanamycin-resistant colonies was similar between MutT7 and globally mutagenized samples (Figure 2e) despite the somewhat lower mutagenesis rate of the MutT7 construct relative to MP6 (Figure 2b; the average kanamycin resistance frequency for MP6 was \(6.7 \times 10^{-6}\) versus \(5.7 \times 10^{-5}\) for MutT7). This observation highlights that the use of MutT7 to maximize on-target mutations while simultaneously minimizing off-target mutations results in larger productive library sizes.

We next turned to DNA sequencing to better understand the processivity and targeting of MutT7 mutagenesis. We allowed an E. coli population expressing MutT7 and the episomal Kan\(^\beta\)Ter\(^\delta\) reporter plasmid to drift in the absence of selection pressure for 15 days prior to isolation of episomal DNA from clones (Figure S3a). Sanger sequencing of the target episomal region revealed mutations at multiple loci throughout the Kan\(^\beta\) target gene, independent of selection pressure (Figure S8 and Table S5). In a separate experiment where the target DNA consisted of an episomal rpsL allele (initially sensitive to streptomycin) downstream of a T7 promoter (Figure S9a), we further evaluated the processivity of MutT7.

Sanger sequencing of streptomycin-resistant DNA isolated from a MutT7-expressing strain of E. coli again revealed that multiple mutations appeared throughout the targeted rpsL gene, with ~90% C→T mutations and ~10% G→A mutations (Figure S9b and Table S6).

Next, we employed Illumina sequencing to identify mutations anywhere in the episomal reporter DNA sequence obtained from clones of the E. coli populations in Figure 3a. This experiment assesses on- versus off-target mutagenesis across a ~10 kb stretch of DNA containing only ~1 kb of intended target DNA. MutT7 samples displayed many mutations throughout the episome when the terminator array was removed but the T7 promoter was maintained (Figure 3b; see also Table S7). Treatment with the MP6 global mutagen also led to mutations throughout the entire episomal DNA. In contrast, mutations in MutT7 strains appeared almost exclusively within the Kan\(^\beta\) target gene when both a promoter and terminator array were present, even after 15 days of continuous culturing (Figure 3b; see also Table S7). Upon normalizing on- and off-target mutation rates, we observed that the few off-target mutations found on plasmids with a terminator from MutT7 strains were present only to the same extent as in the control sample not treated with any mutagen (Figure 3c, red striped bars).

A disadvantage of MutT7 is its limited mutational spectrum and an apparent strand bias observed in our sequencing results showing that we obtained predominantly C→T transitions in the sense strand using a single T7 promoter (Figure 3e and Tables S5–S7). We hypothesized that the mutational spectrum could be doubled by introducing a second T7 promoter that would recruit MutT7 to the 3′ end of the target DNA and enable processive activity in the opposing direction. Indeed, we found that installing an additional antisense T7 promoter led to the accumulation of both G→A and C→T mutations throughout the target gene during continuous culturing (Figure S10a–b and Table S8). Furthermore, the average number and range of mutations per clone increased over time (Figure S10c). The latter observation indicates that, in contrast to global mutagenesis methods where the organism often rapidly silences mutagen expression, the high on-target to off-target mutation ratio of MutT7 enabled long-term maintenance of mutagen expression in cells.

We note that repair of deoxyuridine must be prevented to observe significant mutagenesis with MutT7 (also observed with other cytidine deaminase-based systems\(^1\)–\(^3\)). Although we used ung cells to address this issue in the aforementioned experiments, a more flexible alternative is to co-express MutT7 with the uracil glycosylase inhibitor (UGI; a protein that can inhibit UNG activity in many prokaryotes and eukaryotes\(^4\)–\(^8\), \(^12\)). Such co-expression resulted in a high rate of mutagenesis similar to that achieved using ung cells (see Figure S11). UGI thus eliminates the need to delete ung to achieve efficient mutagenesis with MutT7, significantly increasing the flexibility of our system.

In summary, the processively acting MutT7 chimera can selectively direct mutations to large, yet well-defined, regions of DNA.
in vivo. We anticipate that utilizing other base editing enzymes in concert with cytidine deaminase will significantly widen the mutational spectrum of MutaT7 and further enable the creation of rich and diverse DNA libraries in vivo. Moreover, DNA-modifiers fused to T7 could facilitate targeted epigenetic studies. The ubiquitous applicability of T7-poll in diverse organisms suggests that MutaT7 will prove useful in a broad range of evolutionary and synthetic biology settings.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website.

Complete experimental methods and supplementary figures and tables (PDF)
- Table S2 - Mutation assay data (XLSX)
- Table S4 - folA Sanger data (XLSX)
- Table S5 - Sanger Kan 96 (XLSX)
- Table S6 - Sanger rpsL (XLSX)
- Table S7 - Mutations by NGS (XLSX)
- Table S8 - Sanger Dual promoter (XLSX)

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Graphical Abstract