Using Retron Recombineering for Efficient and Targeted Bacteriophage Genome Modification

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by

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Certificate

This is to certify that this dissertation entitled **Using Retron Recombineering for Efficient and Targeted Bacteriophage Genome Modification** towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by **Darshini Mohan Poola** at The J. David Gladstone Institutes under the supervision of Seth Shipman, Ph.D. (Associate Investigator, Gladstone Institutes and Associate Professor, Department of Bioengineering and Therapeutic Sciences, UC San Francisco) during the academic year 2023-2024.

Seth Shipman, Ph.D.

Date: May 9, 2024

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Seth Shipman, Ph.D.

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This thesis is dedicated to Amma, Daddu, and Tanu

Declaration

I hereby declare that the matter embodied in the report entitled **Using Retron Recombineering for Efficient and Targeted Bacteriophage Genome Modification** are the results of the work carried out by me at the Department of Data Science and Biotechnology, The J. David Gladstone Institutes, under the supervision of Seth Shipman, Ph.D., and the same has not been submitted elsewhere for any other degree. Wherever others contribute, every effort is made to indicate this clearly, with due reference to the literature and acknowledgement of collaborative research and discussions.

Darshini Mohan Poola

Date: May 9, 2024

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List of Abbreviations

IPTG	Isopropyl β- d-1-thiogalactopyranoside
LB medium	Luria-Bertani medium
LPS	lipopolysaccharide
MMB	LB medium supplemented with 0.1 mM $MnCl_2$ and 5 mM $MgCl_2$
MOI	multiplicity of infection
msd	multicopy single-stranded DNA
msr	multicopy single-stranded RNA
OD600	optical density at 600 nm
PFU	plaque-forming unit
RBP	receptor-binding protein
RT	Reverse Transcriptase
SSAP	single-strand annealing protein
SSB	single-strand DNA-binding protein

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Contributions

Contributor name	Contributor role
Darshini Poola, Seth Shipman	Conceptualization Ideas
Darshini Poola, Santi Bhattarai-Kline, Chloe Fishman, Seth Shipman	Methodology
Darshini Poola, Seth Shipman	Software
Darshini Poola, Seth Shipman	Validation
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Seth Shipman	Funding acquisition

Abstract

Bacteriophages have emerged as promising biological tools not only for combating infectious bacteria but also for facilitating the exploration of intra- and inter-protein interactions. However, conventional methods for editing phage genomes have been inefficient, involving tedious screening, rigorous counterselection, or *in vitro* construction processes. These limitations hinder our ability to modify phages effectively, thus restricting innovation in this field. In this study, we introduce a scalable approach for engineering phage genomes using recombitrons — modified bacterial retrons capable of generating recombineering donor ssDNA. Coupled with single-stranded binding and annealing proteins, these retrons facilitate the integration of donor DNA into target sequences within phage genomes or phagemids. Notably, this method enables efficient genome modifications across multiple phages and sites simultaneously without requiring counterselection. Furthermore, the process is continuous, allowing edits to accumulate in the phage genome over time, and multiplexable, enabling the introduction of distinct mutations from different editing hosts in a mixed culture. We demonstrate the utility of this approach by applying it to analyze intra-protein epistasis in the T7 phage tail fiber. Specifically, we generate host-specific/tolerant mutants in the gp17 tail fiber protein and employ a combinatorial variant library to infect *E. coli* mutants with truncated lipopolysaccharide coats — a condition often observed in multi-drug resistant bacteria. Our findings underscore the efficacy and scalability of retron recombineering for not only modifying endogenous phage proteins but also exogenous ones cloned into phagemids. This method significantly expands molecular biologists' toolkit for genome editing and enhances our understanding of fundamental biological processes, including protein-protein interactions and epistasis.

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Outside the laboratory walls, California's peace-bestowing trails and enchanting waters have become a second home. Be it a disproven hypothesis in the lab or a gloomy weekend at the house, I could always rely on these to uplift my spirits. To my cherished friends from IISER-Pune, who, despite the miles that separated us, made sure to check on me, I owe a debt of gratitude beyond measure.

Last but certainly not least, to my beloved parents and brother, whose unwavering support and sacrifice have been the bedrock of my journey, I offer my deepest love and appreciation. Your enduring faith in me fuels my determination to continue striving for excellence.

Introduction

Background and rationale

In the ever-evolving landscape of biotechnology, the intricate interplay between genetic manipulation and therapeutic advancement is paramount. Amidst this backdrop, the challenge of orchestrating multisite edits within bacteriophage genomes stands as a formidable hurdle, yet one with profound implications for combatting multidrug-resistant bacteria. The escalating prevalence of antimicrobial resistance has spurred a growing interest in exploring bacteriophages as a potential alternative therapy. However, the use of naturally occurring phages may present certain limitations, including inefficiency in eradicating the target bacterial host and the potential for adverse side effects. Both natural and engineered phage cocktails have been used in the past to treat infections by multi-drug resistant *Acinetobacter baumannii* and *Mycobacterium abscessus* (Schooley *et al*., 2017, Dedrick *et al*., 2019). Two primary strategies have emerged for the engineering of phages: in vivo genome modification and ex vivo synthetic genomics.

Genome modification involves building and selecting/recovering engineered phages using recombination systems. (Ellis *et al*., 2001, Copeland *et al*., 2001) However, recovery is limited by the inability to use antibiotics as selectable markers, and the low recombination efficiency in homologous recombination-based engineering leads to time-consuming and labor-intensive screening for recombinant phages. CRISPR-Cas-based technologies offer a potential solution for the enrichment of mutant progeny, but constraints on their use include the requirement for bacteria to have a characterized native CRISPR-Cas system or be capable of expressing a heterologous system and the existence of multiple phage resistance mechanisms (Box *et al*., 2016, Bari *et al*., 2017, Hupfeld *et al*., 2018, Møller-Olsen *et al*., 2018, Ramirez-Chamorro, *et al*., 2021). Limitations in achieving high transformation efficiencies with large phage genomes in most hosts during bacteriophage recombineering of electroporated DNA (BRED) have led to the use of variations such as bacteriophage recombineering with infectious particles (BRIP) (Wetzel *et al*., 2021).

Synthetic genomics involves constructing phages from known rules of phage biology, but this approach is still in its infancy. The YAC-assisted assembly method is efficient for modifying genomes, but the process of restarting the phage genomes has been limited to transforming host bacteria, which only works for bacteria that are highly transformable (Ando *et al*., 2015, Bodner *et al*., 2021). Other possible approaches, such as the use of bacterial L-forms and cell-free methods, may provide a feasible way to restart assembled phages, but it is unclear if these methods can be universally applied to all bacterial species (Shin *et al*., 2012, Kilcher *et al*., 2018). Ultimately, the ability to trace a successfully engineered phage particle from an extensive library of mutants during rebooting is crucial for retaining the correlation between genotype and phenotype in *in vitro* phage tail engineering, which is currently a limiting factor

Retrons and their application in recombineering

Initially encountered in the 1980s within bacterial DNA preparations, retrons represent a unique class of genetic elements characterized by their RNA-DNA hybrid structure (Yee *et al*., 1984). These hybrids consist of a structured RNA strand (msr) and a DNA strand (msd) linked by a 2'-5' phosphodiester bond (Figure 1A). The msr-msd cassette is accompanied by a specialized reverse transcriptase (RT), making retrons distinctive among genetic elements. Retrons encode msr and msd within a single transcriptional cassette, with msr located 5' of msd (Inouye *et al*., 1989). The msr and msd form a highly structured transcript before reverse transcription, with stable hairpins in the msr and a hairpin with mismatched or wobble base pairs in the msd (Lampson *et al*., 2005, Xie *et al*. 2017). The 5' and 3' ends of the msr and msd transcript hybridize and serve as a primer for reverse transcription, which is catalyzed by the retron RT (Shimamoto *et al*. 1995a, Shimamoto *et al*. 1995b). Cellular RNAse H activity degrades the template of the msd during reverse transcription, and the resulting msDNA is further processed nucleolytically (Shimamoto *et al*. 1995b). The msr sequence and secondary structure are important for RT recognition, and expressing the RT of one retron and the msr of another does not produce msDNA, except for highly homologous retrons (Inouye *et al*. 1999).

Retrons have been repurposed in phage engineering as potent instruments for genome editing (Fishman *et al*., 2023). Retron recombineering uses RT-derived ssDNA as a donor, utilizing the

retron msd region, which undergoes transcription followed by targeted reverse transcription. (Figure 1B) The outcome is the generation of multicopy satellite DNA, employed as a recombineering donor for introducing sequence alterations into the replicating phage genome. The initial setup involves a modified retron-Eco1 ncRNA that produces a ~90-base RT-DNA within the editing bacterial strain, and the induced overexpression of ancillary proteins — an *E. coli* single-stranded binding protein (SSB), the CspRecT single strand annealing protein (SSAP), and the dominant-negative mutL E32K variant (Aronshtam *et al*., 1996) suppressing mismatch-repair of single-base mutations (Wannier *et al*., 2020) (Figure 1C). SSB binds and destabilizes internal helices, facilitating interaction with the SSAP, which further aids RT-DNA annealing to the lagging strand at a replication fork, incorporating the sequence into the newly replicated phage genome (Fishman *et al*., 2023) (Figure 1D). With a significant decrease in the technical challenges associated with phage library creation, applications such as examining epistasis within phage genomes and diversifying phage display libraries to increase the probability of identifying better mutants become viable by combining retron recombineering strains in various permutations for simultaneous multiplexed editing at more than one site in the target sequence.

The T7 tail fiber – a model to study phage host range

The interaction between phages and their bacterial hosts relies on shared receptors, crucial for infection and virulence (Bertozzi Silva *et al*., 2016; Rousset *et al*., 2018). Phage surface receptor-binding proteins (RBPs) play a central role, enabling attachment to various cell surface molecules such as proteins, polysaccharides, lipopolysaccharides (LPS), and carbohydrate-binding structures (Nobrega *et al*., 2018). Through genetic modifications and evolution, phages demonstrate notable adaptability in RBPs, expanding their host range across different ecological niches (Ando *et al*., 2015; Dedrick *et al*., 2019; Dunne *et al*., 2019; Yehl e*t al*., 2019). Phage RBPs remain the center of numerous mechanistic, structural, and evolutionary studies and are prime targets for engineering. (Holtzman *et al*., 2020). However, a more systematic and detailed grasp of how RBP mutations affect phage activity and host range is needed. Directed evolution, while insightful, tends to enrich only a small group of 'winners,' making it difficult to obtain a comprehensive mutational landscape of the RBP. Screening

involving random mutagenesis generate multi-mutant variants whose individual effects cannot be easily discerned. Other approaches, such as swapping homologous RBPs, lead to the gain of function; however, the underlying molecular determinants of function can be challenging to elucidate. In summary, in spite of the exceptional functional potential of phage RBPs, how systematic alterations to their sequence influence the overall functional landscape of a phage remains to be studied in detail.

The tip domain of the T7 tail fiber protein gp17 is its distal region that makes first contact with the host LPS (Gonzalez-García *et al*., 2015, Molineux, 2001, Qimron *et al*., 2006). It serves as a hotspot for gain-of-function variations due to its pivotal role in influencing the fitness of a phage during host adsorption. Novel gain-of-function variants targeting a resistant host can be discovered by exposing a collection of tail fiber variants to selection on a host resistant to infection (Huss *et al*., 2021). The *E. coli* genes rfaG and rfaD are essential in the biosynthesis of surface LPS, a recognized receptor for T7 in *E. coli*. The gene rfaG facilitates glucose transfer to the outer core of LPS, and deletion strains lacking this gene exhibit deficiencies in the outer core of LPS. Conversely, rfaD encodes a critical epimerase necessary for the assembly of the LPS inner core. Recent studies utilizing Deep Mutational Scanning, such as the work by Huss *et al*. using ORACLE, have comprehensively determined the molecular determinants of activity and host range (Huss *et al*., 2021). We aim to build upon this foundation and further investigate the effect of multi-site combinatorial mutations on the efficacy of RBP binding to and infecting strains with mutated LPS.

Employing multiplexed retron recombineering beyond phage proteins

Phage display has revolutionized the field of molecular biology by providing a powerful method for discovering affinity reagents (Smith and Petrenko, 1997). This technique involves the insertion of a gene encoding a protein of interest into a phage coat protein gene. Consequently, the phage displays the protein on its outer surface while harboring the gene for the protein internally, establishing a direct link between genotype and phenotype. By exploiting this feature,

researchers can screen the displayed proteins against various molecules such as proteins, peptides, or DNA sequences to identify interactions.

The screening process, termed *in vitro* selection, resembles natural selection and allows for the screening and amplification of large libraries of proteins. The size and diversity of these libraries significantly impact the likelihood of isolating clones with desired properties, such as strong binding affinity or recognition of diverse epitopes. However, constructing large and diverse libraries presents challenges. Most libraries typically contain 10^9–10^10 members, requiring numerous electroporations and substantial amounts of DNA, which are limited by bacterial transformation efficiency. Common methods for library construction, such as PCR-based techniques, entail drawbacks such as the need for large quantities of digested plasmid DNA and a potential decrease in transformation efficiency due to UV exposure during gel extraction. Despite advancements, methods like Kunkel mutagenesis and rolling circle amplification still necessitate multiple electroporations and are limited to single-site point mutants (Huang *et al*., 2023).

In addressing these challenges, multiplexed retron recombineering emerges as a promising approach for efficient combinatorial library preparation. Retron recombineering exploits the retron system to introduce mutations into phagemid DNA with high efficiency. When combined with multiplexed strategies, it simplifies library construction by reducing the number of steps required and streamlining the workflow.

Central to phage display technology are phagemid vectors like pComb3XSS (Andris-Widhopf *et al*., 2000). These vectors are specifically designed to express fusion proteins and incorporate elements that facilitate efficient display and secretion. Leveraging the pIII capsid protein, these vectors enable the display of larger inserts compared to traditional phage vectors, thus broadening the scope of applications, particularly in targeted imaging and therapy. The versatility of the system extends to phage fitness-independent recombineering, wherein the lac operator-repressor inducible expression system allows for controlled expression, while the inclusion of an OmpA leader sequence ensures the secretion of expressed proteins into the periplasm (Andris-Widhopf *et al*., 2000). These features streamline the production and recovery

of recombinant proteins, thereby enhancing the scalability and throughput of phage display experiments.

Scope of this project

This project endeavors to achieve three primary objectives aimed at advancing the efficacy and capabilities of retron recombineering technology. The first goal is to enhance the efficiency and versatility of the retron recombineering technique by elucidating a specific set of variables critical for its application in editing novel phages. Currently, our retron recombineering protocol is optimized primarily for editing efficiencies in phage λ (Fishman et. al, 2023). We aim to expand the applicability of retron recombineering across a broader spectrum of phage types — starting with T7 and M13. This expansion will take place through a comprehensive analysis of the physical, biochemical, and molecular factors underlying the retron recombineering technique.

By systematically dissecting these factors, it becomes possible to refine and adapt the protocol to accommodate the unique characteristics of diverse phages, thereby extending the reach of retron recombineering in genetic manipulation endeavors. Specifically, simultaneously generating multi-site combinatorial edits would serve as a valuable tool in several applications, such as understanding the fundamental biology behind intra-protein epistasis, identifying mutants that target multidrug-resistant bacteria, and expanding the repertoire of phage display libraries — all of them accelerating the possibility of uncovering novelties in their respective fields.

As an illustration of the utility of multiplexed retron recombineering in fundamental biology, the second objective focuses on investigating the epistatic interactions within T7 phage-host systems. This entails generating host-specific or tolerant mutations at four distinct sites within the gp17 gene tail fiber of the T7 phage. Subsequently, these combinatorial variant mutant libraries will be employed to infect *E. coli* LPS-truncated deletion strains BW25113ΔrfaG and BW25113ΔrfaD, with the wild-type BW25113 strain serving as a control. By systematically exploring intra-protein epistasis in a high-throughput manner, this research aims to significantly augment our theoretical and practical comprehension by serving as a basis for both inter- and intra-protein biochemical interactions. Moreover, the identification of such epistatic relationships

holds promise for facilitating the development of predictive models, thus potentially furnishing researchers with robust tools for anticipating and manipulating biological interactions.

In addition to the aforementioned objectives, our study endeavors to diversify the scope of targetable proteins, transcending the limitations imposed by multiplexed editing of endogenous phage proteins. This strategic expansion is driven by the imperative to mitigate our reliance on phage fitness, thereby disentangling it from the process of recombineering. To achieve this, we draw inspiration from the versatile technique of phage display, an established methodology renowned for its efficacy in probing protein-protein interactions. By relocating our focal point from the phage genome to a phagemid housing an inducible expression system, we effectively eliminate the variable of phage fitness from the experimental framework. This innovative approach not only circumvents potential constraints imposed by the natural biology of the phage but also empowers us to manipulate and study target proteins with enhanced precision and flexibility. By adopting such a methodological refinement, we aim to unlock new avenues for protein targeting and manipulation, thereby advancing the frontiers of molecular biology and biotechnology.

In essence, this project represents a concerted effort to advance the state-of-the-art genetic engineering technique of retron recombineering, particularly through its enhancement and diversification of retron recombineering technology. By addressing these objectives, we aim to not only expand the toolkit available to molecular biologists for genome editing but also to deepen our understanding of fundamental biological processes, such as protein-protein interactions and epistasis. Such endeavors are pivotal for pushing the boundaries of scientific knowledge and facilitating the development of innovative approaches to address biological challenges.

Materials and Methods

Propagation of Phage Variants

Phages utilized in this study were obtained from ATCC and NEB stocks, including phage λ WT #23724-B2, T7 #BAA-1025-B2, and M13KO7 NEB#N0315S. Culturing of the phages was conducted at 37°C in Luria-Bertani (LB) medium supplemented with 0.1 mM $MnCl_2$ and 5 mM $MgCl_2$ (MMB), along with appropriate antibiotic selection until reaching an optical density at 600 nm (OD600) of 0.25, employing established methodologies. Upon reaching the desired OD600, indicative of phage proliferation, the cultures were centrifuged at 4000 rpm for 10 minutes to separate the bacterial debris and phage particles. Subsequently, the supernatant containing the phage particles was subjected to filtration through a 0.2 μm filter to eliminate any residual bacterial cells, thereby ensuring the purity of the phage preparations.

In the case of phage λ, a modified strain, phage λΔcI, was utilized in this study. The modification involved the introduction of two early stop codons within the cI gene, which is pivotal in regulating lysogeny. This modification was achieved using recombinant DNA technology. Annotation of the genomic edits introduced in phage λΔcI was performed by comparison with the wild-type reference sequences available in the NCBI GenBank database for phage λ (accession number: J02459.1) and T7 (accession number: V01146.1).

Construction of Plasmids

The cloning procedures were performed in *E. coli* NEB®5-alpha (NEB#C2987U). The vector pORTMAGE-Ec1 (Addgene #138474) was modified to pCF.111 by integrating an additional SSB protein for T7 experiments. Plasmids designated for RT-DNA synthesis, housing the retron-Eco1 RT and ncRNA with elongated a1/a2 regions, were derived from pSLS.492 (Addgene #184957). To introduce specific donor sequences for single-site modifications, corresponding primers were utilized, and these sequences were integrated into the RT-DNA-encoding region of the ncRNA. This substitution process adhered to the methodology established by Jacobus *et al.* (Jacobus *et al.*, 2015).

Upon receipt of the plasmids, they were verified through restriction digest analysis and Sanger sequencing to confirm the successful integration of the desired modifications. *E. coli* NEB®5-alpha competent cells were transformed with the recombinant plasmids using standard heat shock methods. Putative cloned transformants were selected on LB agar plates supplemented with appropriate antibiotics. Following colony selection, plasmids were extracted using commercial kits according to the manufacturer's instructions. Subsequently, the extracted plasmids were further confirmed via restriction digest analysis and Sanger sequencing. This ensured the fidelity and accuracy of the constructed vectors before downstream applications such as retron expression and single-site modifications.

Plaque Assays

Plaque assays were conducted in accordance with the methodology outlined by Mazzocco *et al.* (Mazzocco *et al.*, 2009). and quantified utilizing the full plate plaque assay procedure, as delineated by Kropinski *et al.* (Kropinski *et al.*, 2009). Bacterial cultures were incubated with shaking overnight at 37°C and utilized as hosts for phage propagation. For small drop plaque assays, 200 µl of the bacterial culture was thoroughly mixed with 2 mL of melted MMB agar (composed of LB supplemented with 0.1 mM $MnCl_2$, 5 mM $MgCl_2$, and 0.75% agar) and plated onto MMB agar plates. Serial 10-fold dilutions of phage lysate in MMB were prepared, and 2 µl drops of each dilution were spotted onto the bacterial lawn. The plates were air-dried for 20 minutes at room temperature and then cultured overnight at 37°C to allow plaque formation.

Full plate plaque assays were initiated by mixing 200 µl of the overnight bacterial culture with 20 µl of phage lysate. Serial dilutions of the phage lysate were carried out to attain plaque counts ranging from 200 to 10. Following a 5-minute incubation period at room temperature without agitation, the bacterial-phage mixture was combined with 2 mL of melted MMB agar and evenly spread onto MMB agar plates. After air-drying for 20 minutes at room temperature, the plates were kept overnight at 37°C to enable plaque development. Subsequently, plaque-forming units (PFUs) were enumerated to determine the phage titer.

Sanger sequencing of phage plaques was conducted by selecting individual plaques generated from the full plate assay as described above. Plates containing the plaques were dispatched to

Azenta/Genewiz for sequencing, utilizing one of the NextSeq-compatible primers employed for assessing the same genomic region. Sequencing data were analyzed using Geneious software through alignment to the genomic region encompassing the editing site on the phage genome. This facilitated the identification and characterization of any genomic alterations or mutations present within the sequenced phage isolates.

Retron Recombineering, Sequencing, and Analysis

The bMS.346 strain, which was developed from *E. coli* MG1655 through the introduction of premature stop codons to deactivate the exoI and recJ genes, served as the foundational strain for all phage λ and T7 retron recombineering experiments conducted in this study. Subsequently, the ER2738 strain (NEB#E4104), a derivative of *E. coli* NM522 carrying the fhuA2 mutation facilitating selection of the F' episome using tetracycline, was exclusively utilized for phagemid editing with M13KO7 helper phage. Phage retron recombineering cultures were maintained in an MMB and incubated with shaking at 37°C. The induction of genetic elements and maintenance of genetic constructs were facilitated by the addition of specific inducers and antibiotics. Specifically, inducers such as 2 mg/mL L-arabinose, 1 mM Isopropyl β-d-1-thiogalactopyranoside (IPTG), and 1 mM m-toluic acid, alongside antibiotics including 10 μg/mL doxycycline, 35 μg/mL kanamycin, and 100 μg/mL carbenicillin, were incorporated into the culture medium at the specified concentrations.

Experiments involving T7 phage multiplexing were conducted in 25 mL cultures, initiated at an OD600 of 0.25, with a phage multiplicity of infection (MOI) of 0.1, unless alternative specifications were explicitly stated. Phagemid editing utilizing M13KO7 was performed in 500 μL cultures under identical conditions. Initially, phages were propagated in the host strain designated for editing, namely bMS.346 or ER2738, as applicable. Post-infection, the cultures were permitted to grow for an incubation period of 16 hours. Subsequently, to eliminate bacterial cells and cellular debris, the culture was subjected to centrifugation at 4000 rpm for a duration of 10 minutes. For subsequent analysis via amplicon-based sequencing, the resulting lysate was mixed in equal proportions with nuclease-free water and subjected to heat treatment at 95°C for 5 minutes to ensure lysis of remaining bacterial cells and to release phage genomic DNA. The lysate was then utilized as a template in PCR amplification, employing primers flanking the

targeted editing site on the phage genome. Following PCR, the resulting amplicons were indexed and sequenced utilizing the Illumina NextSeq 1000/2000 platform. Quantitative determination of editing efficiency was achieved through computational analysis of the obtained sequencing data. The editing percentage was calculated by dividing the number of sequences displaying the desired editing outcome by the total count of reads encompassing both wild-type and edited sequences.

Enrichment of Phage in Non-Editing Hosts

To assess the impact of multiplex-edited phages on bacterial cultures, a total of 1-10 million multiplex-edited phages were introduced into exponentially growing bacterial cultures, initiated at an OD600 of 0.4 in 25 mL volumes. Phages were propagated using their respective enrichment hosts over a 16-hour duration under continuous shaking at 37°C to facilitate optimal phage replication. For subsequent PCR amplification and sequencing, an appropriate template amount was crucial. To ensure robust sequencing depth, the amount of enriched phages utilized as the PCR template exceeded the anticipated number of reads. Specifically, double the quantity of enriched phage relative to the expected number of reads was employed. For instance, if 1 million reads were anticipated, 2 million phages were utilized as the template for Illumina sequencing PCR. This methodology ensured adequate representation of phage genomes within the sequencing library, minimizing the risk of sequence dropout and ensuring reliable characterization of phage populations.

Editing Rate Quantification

In this study, we developed a customized Python workflow to accurately quantify edits identified from amplicon sequencing data. The workflow underwent several stages to ensure robustness and specificity in detecting edits while minimizing false positives. Initially, reads were filtered to include only those containing flanking nucleotide sequences located outside the RT-Donor region on the phage genome. This step was crucial to exclude reads originating from RT-DNA or plasmid, which could confound the analysis.

Following the initial filtering step, reads were further processed by trimming off the left and right sequences immediately flanking the edit site. This trimming process was essential for precisely delineating the region of interest and minimizing noise in subsequent analyses. Subsequently, reads containing the inside flanking sequences in the correct order, with an appropriate distance between them, were identified. The distance criterion varied depending on the type of edit under investigation, ensuring flexibility and adaptability of the workflow across different experimental conditions.

Based on the alignment of inside flanking sequences, reads were then categorized into three distinct groups: wild-type, edit, or other. This categorization allowed for the accurate quantification of edit events within the sequencing data while accounting for potential sequencing artifacts or background noise. Finally, the edit percentage was calculated as the ratio of edited reads to the total number of reads containing flanking sequences. This metric provided a quantitative measure of the prevalence of edits within the analyzed dataset, facilitating comparisons across samples and experimental conditions.

Results and Discussion

T7 requires an intact LPS core for successful, irreversible adsorption

Two LPS-truncated mutants were used to assess the importance of the complete LPS structure in T7 receptor-binding domain recognition. These mutants included strains lacking either the outer core (resulting from the deletion of the rfaG gene) or both the outer and inner cores (achieved through deletion of the rfaD gene). Upon infecting these mutants with T7 phage, a significant reduction in the number of T7 plaques was observed compared to the wild-type LPS strain (Figure 2A). Specifically, the decrease was approximately 30% in the case of LPS outer core mutants and approximately 60% when both outer and inner LPS cores were absent (Figure 2B). These findings underscore the indispensable role of the LPS core in facilitating successful T7 infection.

Building upon prior research by Huss *et al.*, who developed a library of gp17 point mutants and identified residues pivotal for phage-host interactions through enrichment assays in LPS-truncated host strains, we sought to expand our understanding of these interactions. Residues experiencing a significant reduction in substitutions were classified as intolerant, while those displaying enrichment in either one of the mutant hosts were categorized as host-specific. The remaining residues were deemed tolerant. However, this method was limited to analyzing point mutants, whereas phage fitness advantage often stems from the combined effect of mutations at multiple sites. For our study, we identified four top hits on the gp17 protein, along with their respective host-specific and tolerant substitutions (Figure 2C). Subsequently, these mutations were integrated into the retron recombineering system, enabling the generation of editing mixtures targeting all four sites for either host-specific or tolerant substitutions alongside synonymous controls. Following three rounds of multiplexed editing, the pool of edited phages was utilized to infect LPS-truncated strains, with the wild-type LPS strain serving as a control. The enrichment of combinatorial edits was quantified in each post-infection pool (Figure 2D). These findings aimed to set a base to study the nuanced role of specific gp17 residues in

phage-host interactions, highlighting the potential for targeted manipulation to study phage-host interaction and intra-protein epistasis.

Editing efficiencies differ across phages, number of sites, and mutation types

We investigated the recombineering efficiencies in phages λ and T7, aiming to elucidate factors influencing editing outcomes. Our results revealed notable disparities in editing efficiencies between the two phages, with phage T7 exhibiting nearly a third of the editing efficiency of phage λ for a synonymous single-base edit (Figure 3A). This inconsistency can be attributed to the significant difference in burst size between the two phages, with T7 yielding nearly four times more progeny per infected host compared to phage λ. The higher burst size of T7 likely contributes to a dilution effect on editing efficiencies. Additionally, the reliance of phage λ on host replication machinery, unlike T7, may confer advantages in the context of retron recombineering, especially concerning compatibility with overexpressed host proteins.

Further testing of multiplexed editing at four sites of the T7 tail fiber protein gp17 in mixed cultures unveiled intriguing dynamics. Synonymous mutations demonstrated slightly greater cumulative efficiencies compared to host-specific/tolerant edits, both lower than the efficiency of a synonymous edit made in an unmixed culture targeting a single site of the T7 genome (Figure 3B). Selection pressures acting on host-specific/tolerant edits within the editing strain or differences in the number of diverse strains for host-specific/tolerant edits and synonymous ones might account for this observation.

Motivated by the overarching objective of enhancing editing efficiencies in T7, we explored various conditions. Previous studies have shown that the addition of 3% ethanol for IPTG induction has improved induction by improving membrane permeability for the uptake of inducer (Chhetri *et al*., 2015). However, adding 3% ethanol during induction of editing bacterial mixtures did not significantly impact editing efficiency, suggesting that RT-DNA availability might not be a limiting factor. However, overnight editing at a lower temperature (30°C) augmented cumulative editing efficiencies substantially, possibly by mitigating protein

misfolding and reducing T7 burst size. This finding was reinforced by a positive correlation between editing efficiency and decreased T7 multiplicity of infection (MOI), which also had a positive effect on editing efficiencies (Figure 3C).

Subsequent studies into the growth dynamics of phage-infected overnight cultures underscored the impact of T7 replication time on editing efficiencies. Bacterial cultures infected with T7 exhibited earlier clearance compared to those infected with phage λ at equivalent MOIs (Figure 3D). This disparity suggests fewer replication cycles for T7-infected cultures, thereby maintaining the relatively constant low rate of edits per generation. Strategies to modulate T7 burst size, including temperature adjustments and genetic modifications, hold promise for further enhancing editing efficiencies (Nguyen *et al*., 2014). These findings shed light on the fine interplay between phage characteristics, host dynamics, and environmental factors in shaping retron-based recombineering outcomes.

Single-site edit efficiencies improve with a decrease in temperature and MOI in T7 phage

Improving the recombitron technology for multiplexed phage genome editing requires careful optimization of multiple variables, including temperature, duration, and MOI. Based on the previous result, an MOI of 10^-6 was used, except where specified otherwise. Editing efficiency was measured after reducing the temperature to 30°C and the duration of editing to 6 hours. The results showed that the rate of synonymous edit in gene 3.5 in individual cultures targeting a single site almost doubled under the optimized conditions (Figure 4A). Moreover, the presence of single-site edits after multiplexed editing in mixed cultures showed marginal improvement (Figure 4B), although this enhancement did not extend to multiple-site edits (Figure 4C). The observed hindrance in stacking edits in mixed cultures simultaneously targeting multiple sites is likely attributed to the reduced phage replication rate and burst size at lower temperatures, thus providing more time for the recombitron to integrate into the lagging strand of replicative DNA. This results in fewer phages being carried over in every subsequent round of editing, thereby bottlenecking the diversity of edits propagated and further reducing the total efficiency of multiple-site edits.

Despite these challenges, it is clear that the reduction in temperature prevented the edit dilution effect observed at 37°C for single-site-targeting homogeneous bacterial cultures infected with T7. In the case of individual cultures targeting a single site in the phage genome, the reduced dilution effect ensured that a greater fraction of edited phages were carried over in every subsequent round of editing. However, this effect did not occur in the case of mixed cultures targeting multiple sites, explaining the drop in the percentage of each double-site combination of edits. Overall, this highlights the importance of carefully optimizing the phage growth dynamics, the number of sites targeted, and the duration of editing to achieve maximum efficiency in multiplexed phage genome editing using the recombitron technology. These findings represent a significant development in the field of phage genome editing and provide a basis for further optimization to improve the efficiency of multiplexed phage genome editing.

Phage retron recombineering — a dependable and effective way of studying combinative epistatic interactions

In this study, we employed phage retron recombineering as a pioneering approach to delve into the intricate dynamics of phage-host interactions at an amino acid-level resolution, focusing mainly on epistasis within the T7 receptor-binding protein gp17. Leveraging previous deep mutational scanning efforts on gp17, which identified key amino acids dictating phage-host interactions using LPS mutants, residues were classified based on their response to substitution (Huss *et al*., 2021). Those exhibiting a significant decrease upon substitution were categorized as intolerant, while those showing depletion in one host but enrichment in another were termed functional/host-switching. The remaining residues were labled host-tolerant. Earlier methods were confined to single-point mutants, and we recognized that the advantageous effects for a phage often stem from combinations of mutations at multiple sites. We pinpointed four pivotal residues located on the exterior loops of gp17 and their respective substitutions associated with host-switching or host-tolerance. Incorporating these findings into our retron recombineering platform, we engineered a bacterial mixture comprising eight strains targeting all four sites for substitution. The combinatorially edited pool of phages was subsequently utilized to infect LPS-truncated bacterial strains (Figure 5A).

Upon infecting bacterial strains expressing truncated LPS cores (ΔrfaG and ΔrfaD) with the phage mixture edited at the four gp17 sites, we observed that double-site host-switching/tolerant combinations exhibited an enrichment nearly 10-100 times higher than their corresponding single-site mutants. We quantified epistasis by subtracting the log-transformed enrichment/depletion of individual single-site mutants from that of the double-site mutants (Figure 5C, Figure 5D). We found some instances of epistasis among the double mutants after selection through the wild-type strain.

For instance, we observed that glycines within the gp17 exterior loops were relatively intolerant to substitution, likely due to their hydrophobic nature and critical role in mitigating steric hindrance (Figure 5B). However, when G521 was mutated concurrently with another residue like V544, the combined effect led to a significantly higher enrichment in the hydrophilic residue-favoring ΔrfaG mutant — effects that would have remained obscure with solely point mutant libraries (Figure 5C). Our approach not only validates the additive extrapolation of log-transformed fitness from single-site mutations to simultaneous multiple-site mutations but also unveils distinctive positive and negative epistatic interactions between host-switching and host-tolerant edits, all without resorting to barcoding or mutant isolation. This underscores the potential of our approach to unravel the biochemical underpinnings of enriched multi-site mutants, which can be further explored through established techniques such as in silico structural analysis, ELISA, NMR, and x-ray studies. Taken together, these findings underscore phage retron recombineering as a dependable and effective means of probing combinative epistatic interactions, where eight simple plasmids could be used to quickly generate 24 distinct, precise phage mutants for direct phenotyping without a step of counterselection or isolation, offering promising prospects for the development of targeted therapies against diseases caused by multidrug-resistant bacteria.

Using constitutively expressing recombitron machinery for phage genome editing

In our ongoing efforts to enhance the efficiency and applicability of retron recombineering technology, we explored the potential of two novel plasmid architectures, which were repurposed from previously unpublished data, for introducing synonymous edits in gene 3.5 and gene 18.5 of T7 bacteriophage. The first plasmid, designed with a modified Eco1 retron under the control of a J23115 promoter, in conjunction with the CspRecT single-strand annealing protein (SSAP), demonstrated promising results. The choice of the J23115 promoter, known for its optimal balance between promoter strength and bacterial fitness, proved advantageous (Figure 6A). The second plasmid utilized a modified Kva1 retron driven by a FAB45 promoter, along with the Beta SSAP, leveraging previous findings by Liu *et al*., which showcased high editing efficiencies with retrons in bacterial genomes (Figure 6B). Notably, the work of Khan *et al*. also provided insights into a potent Kva retron with efficient editing capabilities in both *E. coli* and phage λ genomes (Figure 6C). Despite initial attempts to integrate the CspRecT SSAP with the Kva recombitron, faced with challenges suggesting potential toxicity, we adopted the Beta SSAP instead (unpublished work). Schematic representations of both constitutive plasmids are presented in Figure 6D.

Upon applying these plasmids to induce a three-residue edit at targeted sites within T7 gene 3.5 and gene 18.5, the Eco1 recombitron exhibited nearly a tenfold increase in efficiency compared to the Kva recombitron after a single round of editing. It's worth noting that the necessity for three-residue edits arose due to the absence of the dominant-negative mutL E32K, which typically corrects single-base mismatches and could potentially diminish single-base edited fractions. However, both approaches fell short of the efficiency demonstrated by the two-plasmid inducible recombitron machinery, which targets single residues at corresponding sites (Figure 6E, Figure 6F). Plausible explanations for this disparity include the inherently lower efficiency of triple-residue edits relative to single-residue edits and the absence of *E. coli* single-strand DNA-binding protein (SSB) in the new system, as suggested by previous investigations conducted by Fishman *et al*. (Figure 6G). These findings highlight the promise of constitutive

plasmids in further optimizing retron recombineering technology, thereby expanding its utility for novel experimental applications.

Editing phagemids — disentangling multiplexed editing and phage fitness

In our investigation within the T7 system, we have made significant strides in elucidating critical considerations pivotal for a multiplexed editing strategy aimed at dissecting inter- and intra-protein interactions with residue-level precision. One notable revelation from our endeavors is the discernible limitation of reduced phage fitness on editing the T7 tail fiber. The reliance of our method on multiple infection cycles skewed the editing efficiencies. Nevertheless, the integrity of our results remains intact owing to the meticulous sequencing, with the quantified pre-enrichment pool systematically normalized against the post-enrichment one. Notably, challenges emerged from the absence of certain mutation combinations within the pre-enrichment pool, thereby preventing the accurate quantification of their infection efficacy post-enrichment. Attempts to address this issue by expressing wild-type T7 tail fiber in trans failed to yield the anticipated enhancements in edit combination representation. This anomaly could be attributed to the codon-optimized gp17 protein being positioned downstream of two other proteins—CspRecT and *E. coli* SSB—under the same promoter, potentially hindering the adequate production of gp17 relative to the rate of T7 phage replication despite the presence of a ribosome-binding site upstream of the gene. It is plausible that this approach may prove more efficacious with phages characterized by lower replication rates and burst sizes. Alternatively, the adoption of multitrons presents a promising avenue for circumventing these challenges, thereby obviating the necessity for multiple infections in facilitating edit stacking.

In pursuit of optimizing the phage as an adept vehicle for multiplexing purposes, we drew inspiration from the principles of the phage display system. This system utilizes a phagemid housing a fusion M13 coat protein pIII, enabling the presentation of peptides on the M13 phage surface and facilitating subsequent binding and enrichment analyses (Figure 7A). However, traditional phage display libraries are limited to point mutants rather than combinatorial ones. To address this limitation, we directed our attention to the pComb3XSS plasmid, which harbors the

pIII fusion protein. Modifications to phagemids would exert minimal to no impact on phage fitness, considering that the fusion pIII protein is IPTG-inducible and remains unexpressed during the editing process. This strategic approach effectively decouples the editing and selection stages. Furthermore, this methodological advancement is not confined to endogenous phage proteins; it enables the cloning of any peptide into the pIII fusion, serving as a platform for the construction of combinatorial mutant libraries. The integration of these techniques promises to significantly enhance the versatility and efficiency of phage-based multiplexing strategies.

Prior to commencing this study, several optimizations were undertaken. The M13KO7 helper phage in conjunction with the F^+ ER2738 bacterial strain were chosen for the experiment based on their compatibility and the ability of the bacterium to withstand the tempest of the retron recombineering machinery. Three specific sites spanning approximately 350 bases within the pIII fusion coding region of pComb3XSS were targeted for modification. Recombitron plasmids were meticulously designed from previously validated constitutive plasmids. Compatibility between the bacterial strain and recombitron plasmids was ensured before proceeding. Subsequent to bacterial transformation with both the phagemid and recombitron plasmid constructs, a two-hour incubation period facilitated culture growth (follow the schematic in Figure 7C). The introduction of the M13KO7 helper phage, bearing a phagemid with a weak p15A replication origin, was then executed to enable multiplexing. Notably, the pComb3XSS phagemid features a robust f1 origin of replication, rendering it highly favored by the M13KO7 phage. Importantly, M13KO7 exhibits infectivity both with and without phagemid uptake, meeting a fundamental criterion for our multiplexed experiment. Following overnight culturing of phage-infected bacteria, the supernatant enriched with phage particles was harvested and utilized to infect a fresh culture of bacteria harboring the recombitron plasmid but lacking the phagemid.

The optimization of key parameters in our system under time margins of the study yielded promising results, showcasing the potential efficacy of our approach. Initial experiments involved testing two constitutive plasmids to independently target two sites on the phagemid (Figure 7B). Notably, the Eco1 recombitron under the J23115 promoter outperformed the alternative plasmid, mirroring the efficiency observed with T7. Further optimization focused on

achieving strand specificity is crucial for enhancing editing efficiency, particularly in single-stranded DNA targeting the lagging strand of replicative DNA. Earlier studies showed that T7 exhibited superior editing on a single strand due to its unidirectional replication, unlike phage λ, which lacks strand bias owing to bidirectional replication. These were reinforced when recombitrons targeting both strands of the phagemid demonstrated equivalent efficiency, consistent with the bidirectional replication origin pBR322. Notably, the proximity to replication origins significantly influenced editing efficiencies, with sites closer to bacterial origins displaying higher efficiency before infection, while those closer to the phage f1 origin exhibited increased efficacy after infection, potentially attributed to rolling circle replication. This underscores the functioning of recombitrons even during M13 infection, suggesting the multiplexing potential of this system. Concerns regarding phage-mediated interference with editing were addressed through sequencing pre- and post-infection phagemid pools, revealing consistent edit frequencies across targeted sites before and after infection, as depicted in Figure 7G. These findings affirm the robustness of our method, paving the way for further exploration and application in genome editing.

Following optimization of the pertinent factors, the present study endeavored to expand the method to accommodate multiplexed edits on the phagemid. Despite limitations encountered at the current depth of amplicon sequencing, which precluded the isolation of multi-site simultaneous edits, the data generated reveals promising prospects for the advancement of this technology along such avenues. The edited percentages are relatively low, warranting cautious interpretation. Disparities in editing frequencies across sites are evident, attributed to inherent biases in the recombitron machinery favoring sites proximal to the origin of replication, akin to observations in phage T7 and λ. Furthermore, variations in the efficacy of recombitrons, particularly when modified by the integration of foreign sequences like the edit and its flanking homology, contribute to the observed heterogeneity. Nevertheless, these discrepancies find plausible explanations rooted in fundamental biological processes. Firstly, the generation of single-site edits in the absence of mutL E32K likely attenuates edited percentages owing to active mismatch repair mechanisms. Secondly, comparisons with previous multiplexed post-editing pools, achieved after three rounds of editing, underscore the non-linear accumulation of edits. Thus, minimal editing in the initial round does not necessarily predict

analogous outcomes after subsequent rounds. In sum, while acknowledging these intricacies, the technology poised herein exhibits remarkable potential, signifying a promising avenue for impactful future endeavors.

Perspective

So far, we have successfully showcased multiplexed retron recombineering to elucidate the underlying biology of phage genome-encoded proteins. The subsequent endeavor involves extending this methodology by employing phagemids to analyze proteins not native to the phage. This expansion aims to furnish detailed mechanistic insights at the residue level concerning transcription factors, phage-host interactions, and clinically significant peptides. Ongoing investigations in our laboratory are yielding promising outcomes in this endeavor.

An unexplored aspect of the retron recombineering system pertains to its portability. Presently, the system exhibits optimal functionality within the *E. coli* genome and its associated phages. Further inquiry is warranted to explore the feasibility of employing recombitrons across diverse bacterial species and phages. Such investigations would also unveil factors conducive to enhancing editing efficiencies, a prerequisite for effective multiplexing.

Recent efforts in our laboratory have endeavored to compile a comprehensive inventory of identified retrons (Khan *et al.* 2024). This undertaking represents a significant stride toward identifying superior tools for recombineering. Moreover, the conceptualization of multitrons offers a potential avenue for reducing reliance on successive infections for stacking multi-site edits (González-Delgado *et al.* 2024).

Retrons, both as a biological entity and a tool in bioengineering, collectively harbor considerable potential for continued exploration and therapeutic applications.

Figures

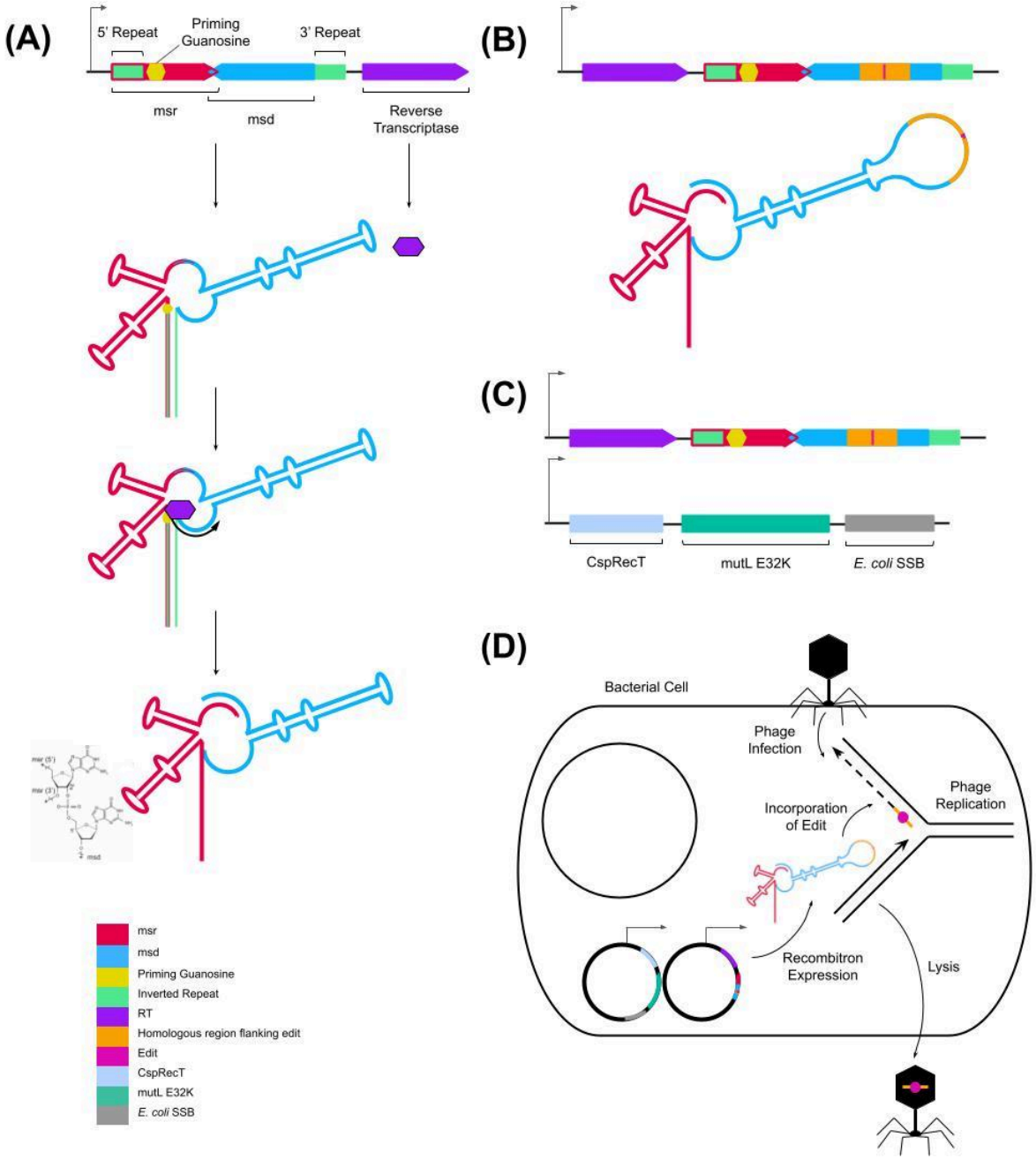

(A)
5' Repeat
Priming Guanosine
3' Repeat
msr
msd
Reverse Transcriptase
(B)
(C)
CspRecT
mutL E32K
E. coli SSB
(D)
Bacterial Cell
Phage Infection
Incorporation of Edit
Phage Replication
Recombitron Expression
Lysis
msr
msd
Priming Guanosine
Inverted Repeat
RT
Homologous region flanking edit
Edit
CspRecT
mutL E32K
E. coli SSB

Figure 1: (A) The general transcribed sequence of a retron along with the encoded products, where the reverse transcription of the msd region primed by a specific guanosine base in a conserved sequence, resulting in a branched msr-RT-DNA structure bound by a 2'-5' phosphodiester linkage, (B) the repurposed retron machinery to incorporate edits in the replicating phage genome, (C) a schematic representation of the reverse-transcribed repurposed RT-DNA, (D) the mechanism of integrating an edit of interest into the lagging strand of a replicating phage genome

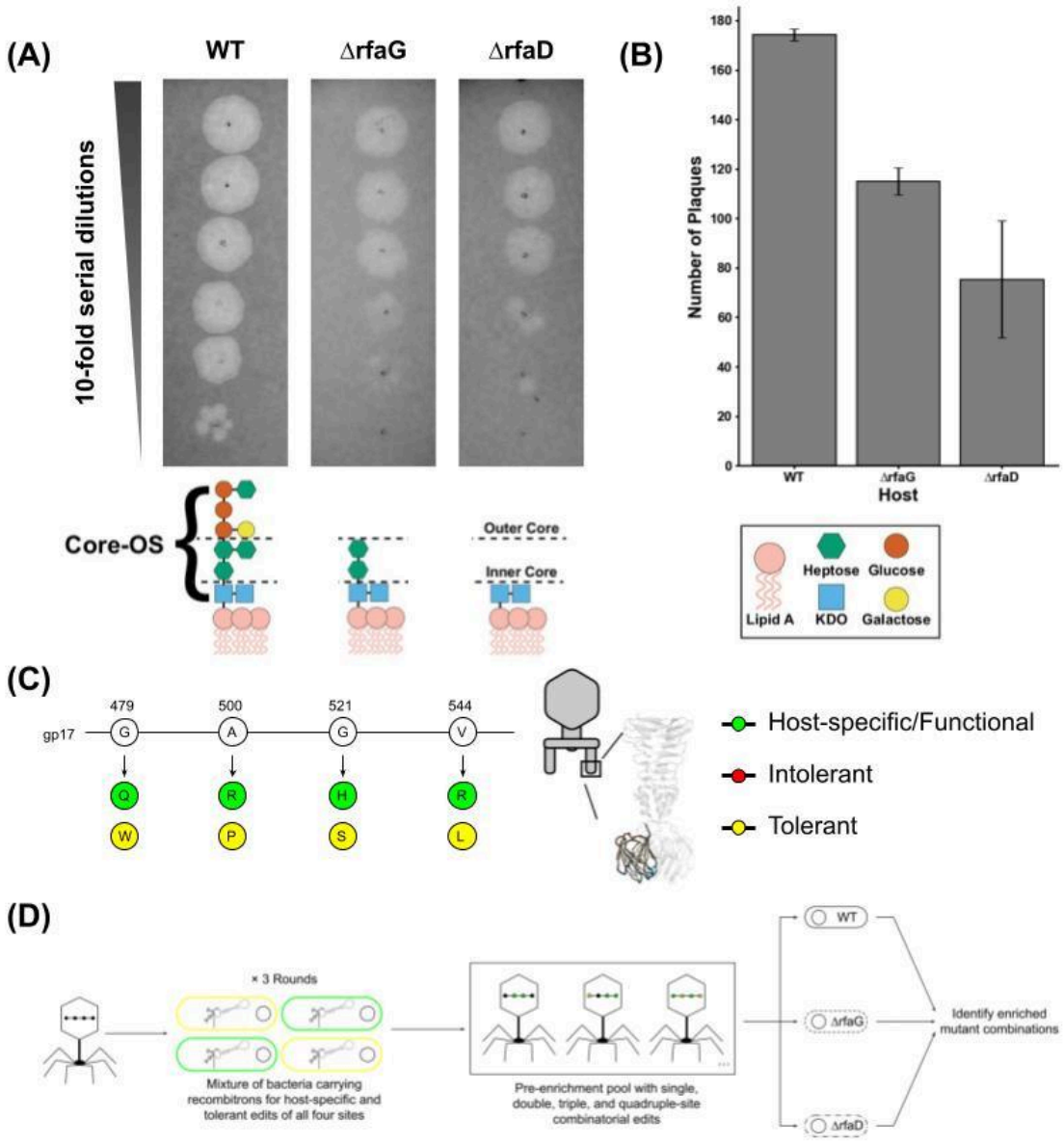
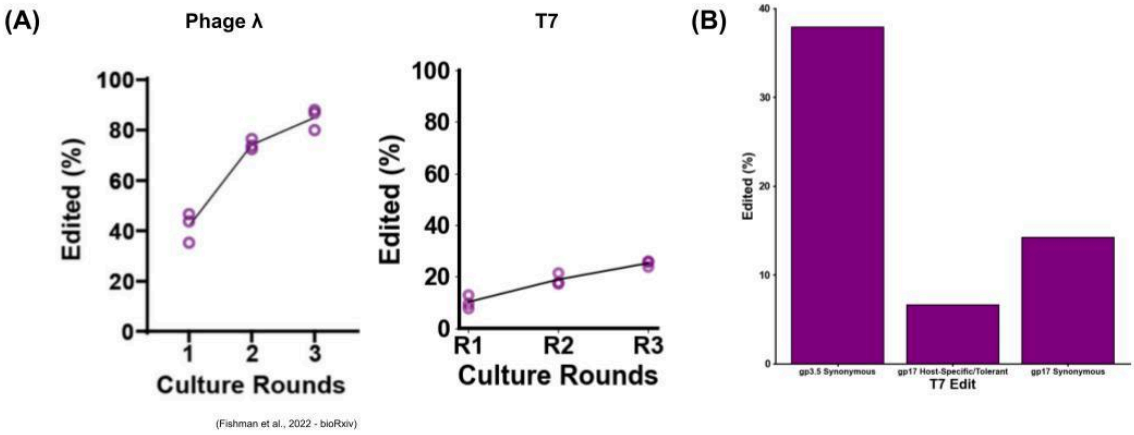
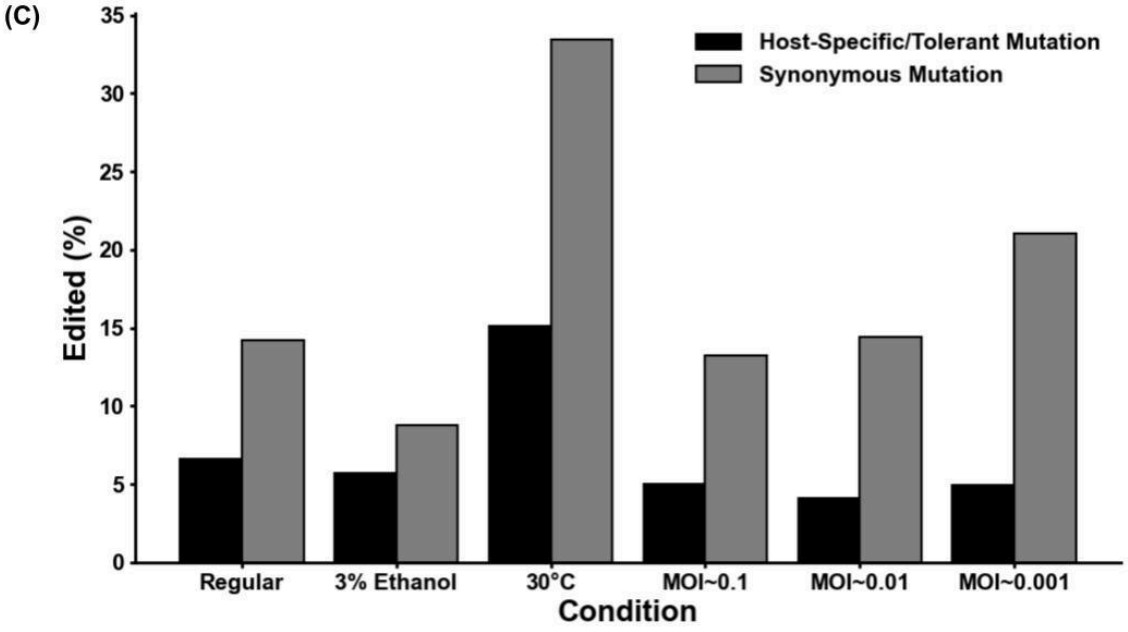

(A)
WT
ΔrfaG
ΔrfaD
10-fold serial dilutions
Core-OS
Outer Core
Inner Core
(B)
Number of Plaques
180
160
140
120
100
80
60
40
20
0
WT
ΔrfaG
ΔrfaD
Host
Heptose
Glucose
Lipid A
KDO
Galactose
(C)
gp17
479
500
521
544
G
A
G
V
Q
R
H
R
W
P
S
L
Host-specific/Functional
Intolerant
Tolerant
(D)
× 3 Rounds
Mixture of bacteria carrying recombitrons for host-specific and tolerant edits of all four sites
Pre-enrichment pool with single, double, triple, and quadruple-site combinatorial edits
WT
ΔrfaG
ΔrfaD
Identify enriched mutant combinations

Figure 2: (A) T7 spotted on the two LPS-truncated mutant strains used in this study with the wild-type LPS strain as a control, (B) a quantification of the difference in the efficiency of T7 plaquing on wild-type and LPS-mutant strains, (C) four sites selected on the gp17 protein and their corresponding host-specific and tolerant edits, (C) a schematic of the general workflow to generate multiplexed simultaneous host-specific and tolerant edits at the four sites of the gp17 protein and the subsequent enrichment of favorable combinations after the infection of LPS-truncated mutants by the resulting edited mixture of T7 phages


(A)
Phage λ
T7
Edited (%)
Culture Rounds
1
2
3
R1
R2
R3
(Fishman et al., 2022 - bioRxiv)
(B)
Edited (%)
gp3.5 Synonymous
gp17 Host-Specific/Tolerant
gp17 Synonymous
T7 Edit


(C)
Host-Specific/Tolerant Mutation
Synonymous Mutation
Edited (%)
Regular
3% Ethanol
30°C
MOI~0.1
MOI~0.01
MOI~0.001
Condition

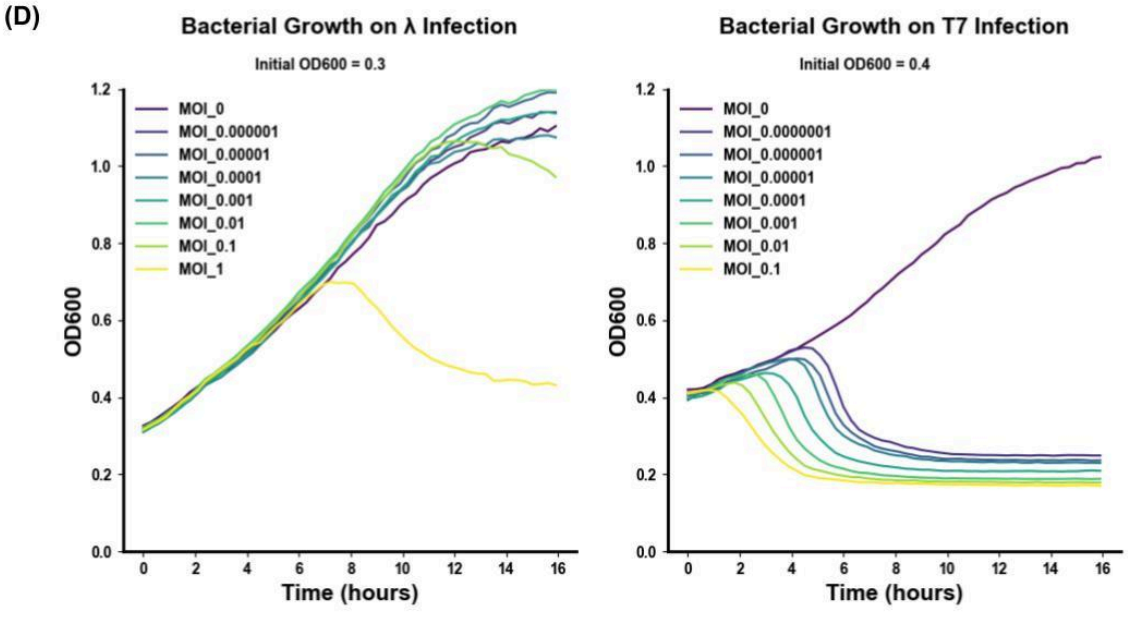

(D)
Bacterial Growth on λ Infection
Initial OD600 = 0.3
MOI_0
MOI_0.000001
MOI_0.00001
MOI_0.0001
MOI_0.001
MOI_0.01
MOI_0.1
MOI_1
OD600
Time (hours)
Bacterial Growth on T7 Infection
Initial OD600 = 0.4
MOI_0
MOI_0.0000001
MOI_0.000001
MOI_0.00001
MOI_0.0001
MOI_0.001
MOI_0.01
MOI_0.1
OD600
Time (hours)

Figure 3: (A) A comparison of editing efficiencies between phage λ and T7 for a single-site synonymous substitution after three rounds of editing, (B) a comparison of the editing efficiency of the single-site synonymous edit and the cumulative efficiencies of all four host-specific/tolerant and synonymous edits, (C) A comparison of the T7 editing efficiencies across the various conditions studied, (D) a comparison of the overnight growth curves of bacterial cultures infected with phage λ and T7 at different MOIs

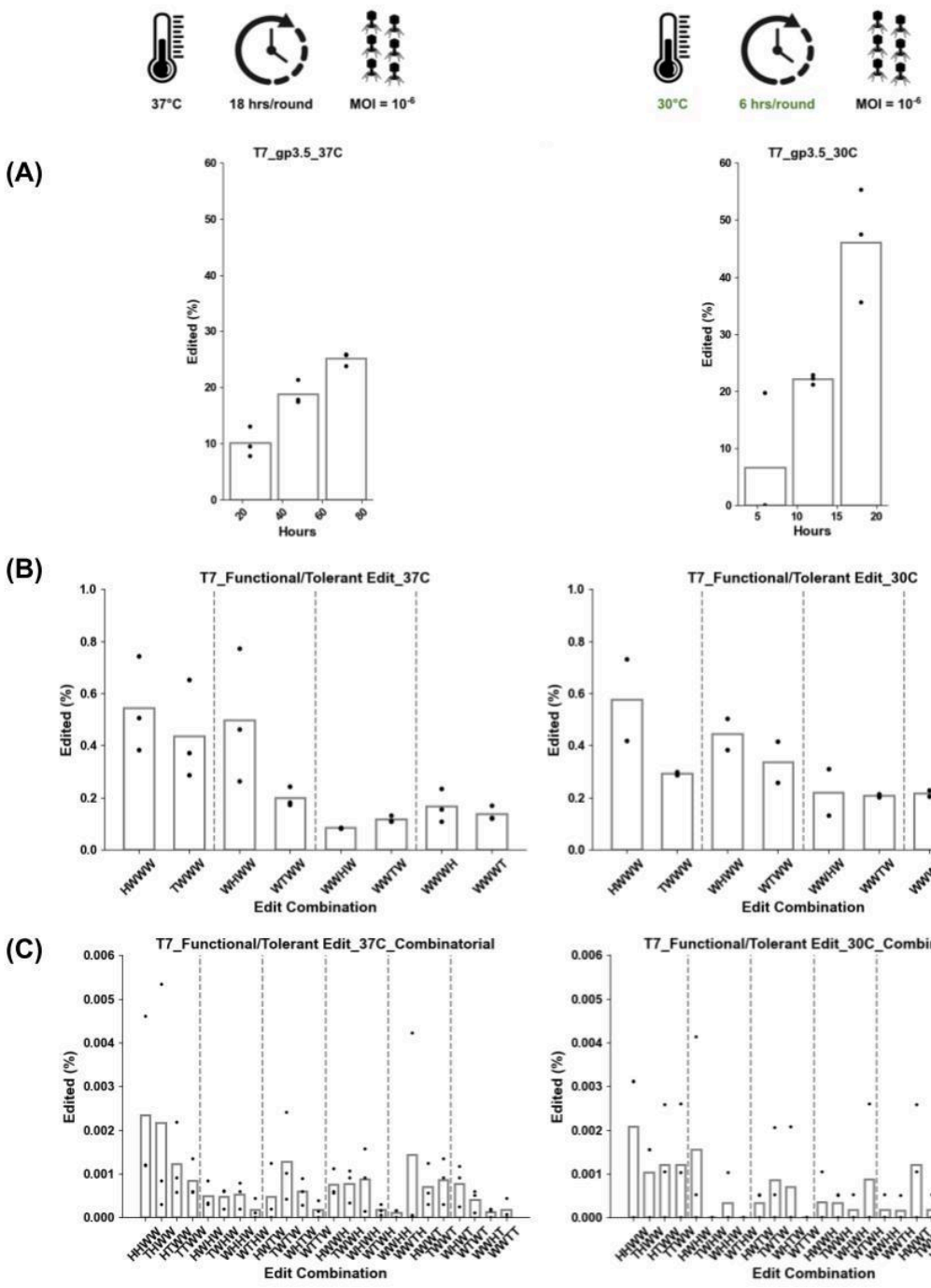

37°C
18 hrs/round
MOI = 10-5
30°C
6 hrs/round
MOI = 10-5
(A)
T7_gp3.5_37C
Edited (%)
Hours
T7_gp3.5_30C
Edited (%)
Hours
(B)
T7_Functional/Tolerant Edit_37C
Edited (%)
HWWW
TWWW
WHWW
WTWW
WWHW
WWTW
WWWH
WWWT
Edit Combination
T7_Functional/Tolerant Edit_30C
Edited (%)
HWWW
TWWW
WHWW
WTWW
WWHW
WWTW
WWWH
WWWT
Edit Combination
(C)
T7_Functional/Tolerant Edit_37C_Combinatorial
Edited (%)
Edit Combination
T7_Functional/Tolerant Edit_30C_Combinatorial
Edited (%)
Edit Combination

Figure 4: A comparison of editing efficiencies between cultures incubated at 37C for 18 hrs/round and those at 30C for 6 hrs/round for (A) a single-base synonymous edit in gene 3.5, (B) the single-site edits and (C) the double-site combinatorial edits from the multiplexed editing experiments with eight bacterial editors (four carrying functional edit retron donors and four carrying tolerant edit retron donors) targeting the four sites

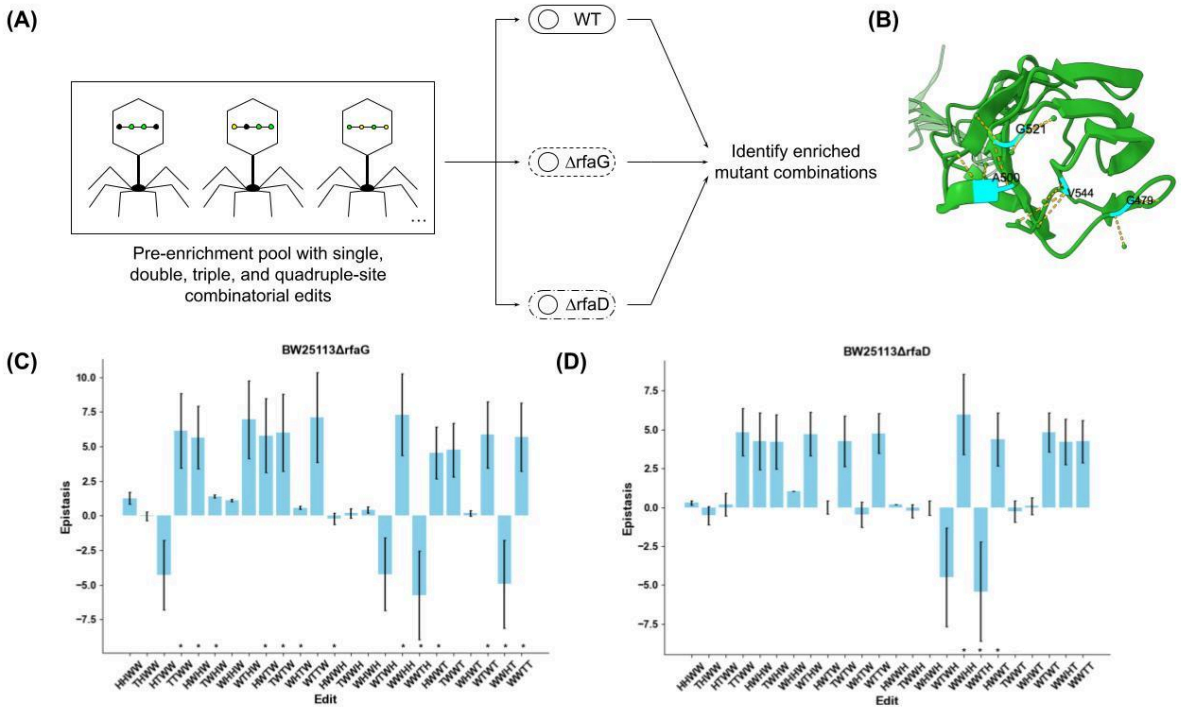

(A)
WT
ΔrfaG
ΔrfaD
Identify enriched mutant combinations
Pre-enrichment pool with single, double, triple, and quadruple-site combinatorial edits
(B)
G521
A509
V544
G479
(C)
BW25113ΔrfaG
Epistasis
Edit
(D)
BW25113ΔrfaD
Epistasis
Edit

Figure 5: (A) A schematic of the workflow for enrichment of combinatorial mutants, (B) the three dimensional protein folded structure of a section gp17 with the targeted residues highlighted, and quantification of epistasis by subtracting the log-transformed enrichment/depletion of individual single-site mutants from that of their corresponding double-site combinations in (C) ΔrfaG and (D) ΔrfaD

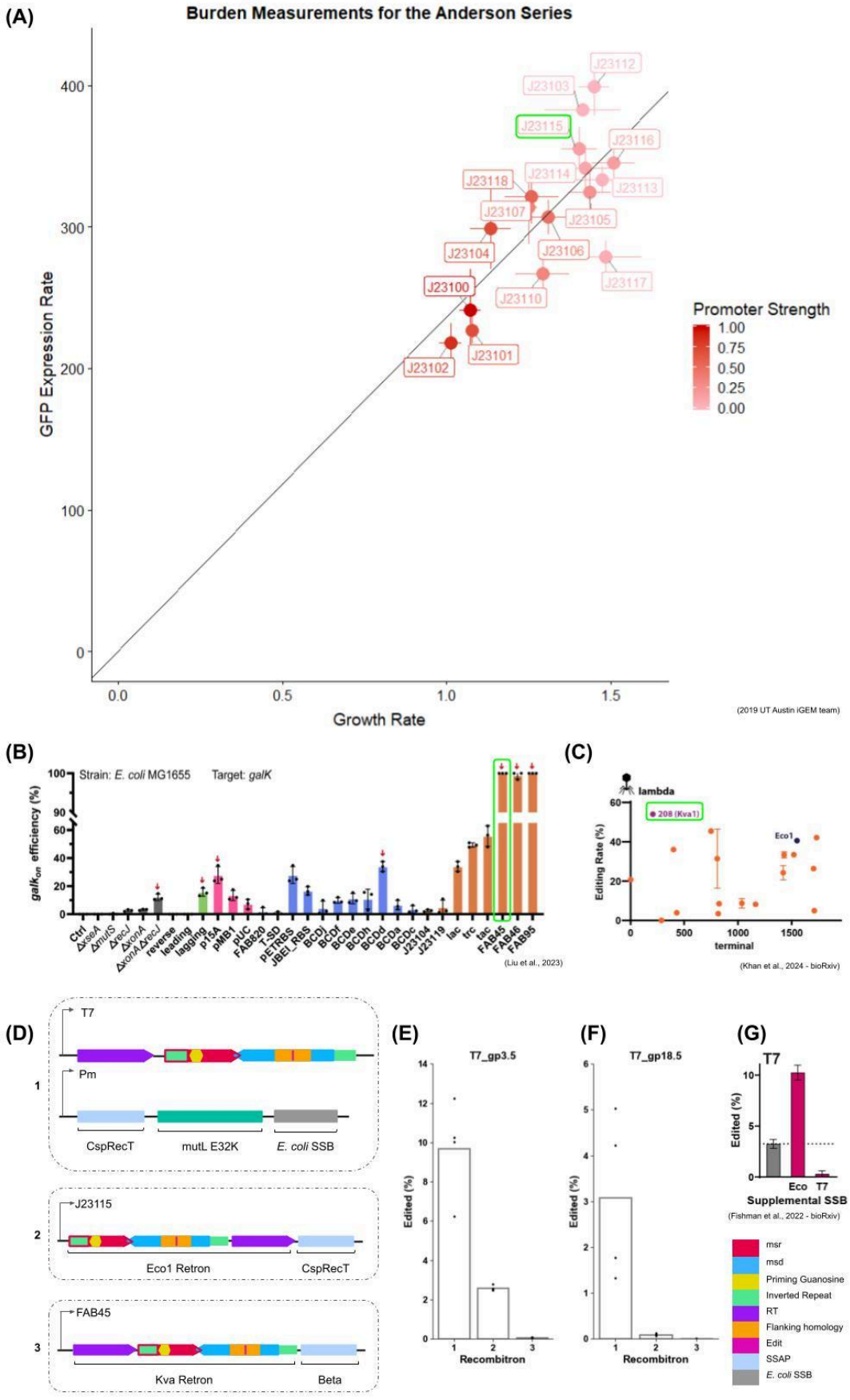

(A)
Burden Measurements for the Anderson Series
GFP Expression Rate
Growth Rate
Promoter Strength
1.00
0.75
0.50
0.25
0.00
J23112
J23103
J23115
J23116
J23114
J23113
J23118
J23107
J23105
J23104
J23106
J23117
J23100
J23110
J23101
J23102
(2019 UT Austin iGEM team)
(B)
Strain: E. coli MG1655
Target: galK
galKon efficiency (%)
Ctrl
ΔrecA
ΔmutS
ΔrecJ
ΔxonA
ΔxonA ΔrecJ
reverse
leading
lagging
p15A
pMB1
pUC
FAB620
T-SD
PETRBS
JBEI_RBS
BCDi
BCDf
BCDe
BCDh
BCDd
BCDa
BCDc
J23104
J23119
lac
trc
tac
FAB45
FAB46
FAB95
(Liu et al., 2023)
(C)
lambda
208 (Kva1)
Eco1
Editing Rate (%)
terminal
(Khan et al., 2024 - bioRxiv)
(D)
T7
Pm
CspRecT
mutL E32K
E. coli SSB
J23115
Eco1 Retron
CspRecT
FAB45
Kva Retron
Beta
(E)
T7_gp3.5
Edited (%)
Recombitron
(F)
T7_gp18.5
Edited (%)
Recombitron
(G)
T7
Edited (%)
Eco
T7
Supplemental SSB
(Fishman et al., 2022 - bioRxiv)
msr
msd
Priming Guanosine
Inverted Repeat
RT
Flanking homology
Edit
SSAP
E. coli SSB

Figure 6: (A) A study plotting promoter strength of various promoters against their effect on bacterial growth rate – a rationale to use J23115 for the constitutively expressed recombitron, (B) A study testing various plasmid components to identify ones that offer the best editing efficiencies in bacterial genomes – a rationale to use FAB45 for the constitutively expressed recombitron, (C) a previous study from our lab comparing the editing efficiencies of diverse retrons in the genome of – a rationale to test Kva1 for the new system, (D) the final cassettes of [1] the inducible two-plasmid recombitron used in former experiments, [2] the constitutively expressed recombitron with the J23115 promoter, Eco1 retron, and CspRecT SSAP, and [3] the constitutively expressed recombitron with the FAB45 promoter, Kva retron, and Beta SSAP, A comparison of editing efficiencies using the three plasmids in T7 at (E) gene 3.5 and (F) gene 18.5, (G) a comparison of the editing efficiencies in T7 with and without the *E. coli* SSB and in the presences of T7 SSB supplemented

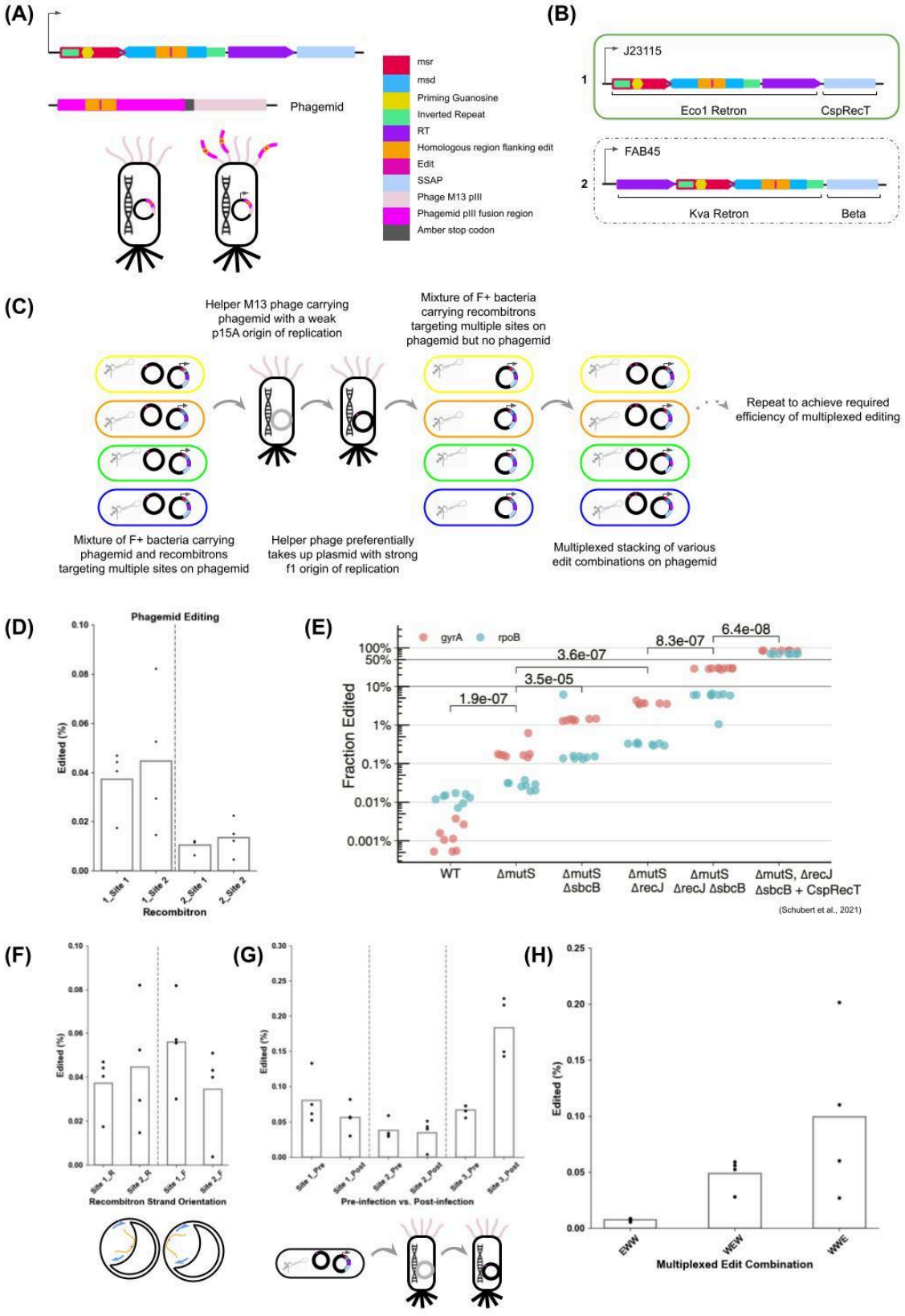

(A)
Phagemid
msr
msd
Priming Guanosine
Inverted Repeat
RT
Homologous region flanking edit
Edit
SSAP
Phage M13 pIII
Phagemid pIII fusion region
Amber stop codon
(B)
1
J23115
Eco1 Retron
CspRecT
2
FAB45
Kva Retron
Beta
(C)
Helper M13 phage carrying phagemid with a weak p15A origin of replication
Mixture of F+ bacteria carrying recombitrons targeting multiple sites on phagemid but no phagemid
Repeat to achieve required efficiency of multiplexed editing
Mixture of F+ bacteria carrying phagemid and recombitrons targeting multiple sites on phagemid
Helper phage preferentially takes up plasmid with strong f1 origin of replication
Multiplexed stacking of various edit combinations on phagemid
(D)
Phagemid Editing
Edited (%)
1_Site 1
1_Site 2
2_Site 1
2_Site 2
Recombitron
(E)
gyrA
rpoB
Fraction Edited
1.9e-07
3.5e-05
3.6e-07
8.3e-07
6.4e-08
WT
ΔmutS
ΔmutS ΔsbcB
ΔmutS ΔrecJ
ΔmutS ΔrecJ ΔsbcB
ΔmutS, ΔrecJ ΔsbcB + CspRecT
(Schubert et al., 2021)
(F)
Edited (%)
Site 1_R
Site 2_R
Site 1_F
Site 2_F
Recombitron Strand Orientation
(G)
Edited (%)
Site 1_Pre
Site 1_Post
Site 2_Pre
Site 2_Post
Site 3_Pre
Site 3_Post
Pre-infection vs. Post-infection
(H)
Edited (%)
EWW
WEW
WWE
Multiplexed Edit Combination

Figure 7: (A) A schematic of the recombitron and phagemid cassettes along with the helper M13KO7 phage in its induced and uninduced state for the phagemid-encoded pIII fusion coat protein, (B) a schematic of the two constitutive plasmids tested for phagemid editing, (C) a representative workflow for the phagemid multiplexed editing, (D) a comparison of the editing efficiencies of both the recombitron plasmids at two editing two different sites on the pIII fusion region; both forward and reverse strand targeting donors have been used in either case, (E) a previous study underscoring the importance of the genomic deletions in the modified *E. coli* strain used of editing when the presence of an F plasmid was not a necessity, (F) a comparison of the forward and reverse forward and reverse strand targeting donors at two different sites on the pIII fusion, (G) a comparison of the editing efficiencies before and after infection by the M13KO7 helper phage at three different sites on the phagemid, (H) the editing efficiencies of each combinatorial edit (only single-site edits could be quantified at the current depth of sequencing) in a single round of multiplexed editing with a mixture of bacterial editors targeting the same three sites as in (G)

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