CRISPR-based engineering of phages for in situ bacterial base editing

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Investigation of microbial gene function is essential to the elucidation of ecological roles and complex genetic interactions that take place in microbial communities. While microbiome studies have increased in prevalence, the lack of viable in situ editing strategies impedes experimental progress, rendering genetic knowledge and manipulation of microbial communities largely inaccessible. Here, we demonstrate the utility of phage-delivered CRISPR-Cas payloads to perform targeted genetic manipulation within a community context, deploying a fabricated ecosystem (EcoFAB) as an analog for the soil microbiome. First, we detail the engineering of two classical phages for community editing using recombination to replace nonessential genes through Cas9-based selection. We show efficient engineering of T7, then demonstrate the expression of antibiotic resistance and fluorescent genes from an engineered λ prophage within an 	extit{Escherichia coli} host. Next, we modify λ to express an APOBEC-1-based cytosine base editor (CBE), which we leverage to perform C-to-T point mutations guided by a modified Cas9 containing only a single active nucleolytic domain (nCas9). We strategically introduce these base substitutions to create premature stop codons in-frame, inactivating both chromosomal (lacZ) and plasmid-encoded genes (mCherry and ampicillin resistance) without perturbation of the surrounding genomic regions. Furthermore, using a multigenera synthetic soil community, we employ phage-assisted base editing to induce host-specific phenotypic alterations in a community context both in vitro and within the EcoFAB, observing editing efficiencies from 10 to 28% across the bacterial population. The concurrent use of a synthetic microbial community, soil matrix, and EcoFAB device provides a controlled and reproducible model to more closely approximate in situ editing of the soil microbiome.

phage engineering | CRISPR | base editing | soil microbiome

The foundation of classical functional genomics is centered around the introduction of mutations and their associated phenotypic alterations; however, experiments are typically performed in isolation from native environments and corresponding indigenous microorganisms. Genetic investigation of microbiomes offers the potential to elucidate the complex biological roles and functional genomics of individual members of microbial networks therein (1–6). In general, a paucity of in situ editing approaches exist due to the inherent complexity of microbiomes and the environmental contexts in which they evolve (7). The soil rhizosphere represents an array of diverse and agriculturally relevant microbes, and bolstering our knowledge on the genomics, ecological roles, and metabolic networks of soil community members could have a powerful effect on future food sustainability and bioenergy efforts (8–13). The diversity and physical composition of the soil microbiome present significant challenges for direct genetic interrogation and manipulation of a single species in a community context. Fabricated ecosystems (EcoFABs) are specialized three-dimensional printed enclosures specifically created to foster reproducibility among microbiome studies (14, 15). EcoFABs can be loaded with precise amounts of medium, culture, and solid matrices to more closely replicate ecosystems of interest and are easily prepared for research studies of the soil microbiome. Targeted editing of community members is limited by the challenge of species-specific delivery, as well as the precision required to locate and edit a specific gene without unintended disruption of nearby loci. Recently, a generalizable platform was developed to address these problems by utilizing various delivery techniques to measure transposon insertion across a synthetic soil community (16). While this study was focused on the evaluation of conjugation, electroporation, and natural transformation for delivery to a broad range of organisms, transduction was not evaluated due to the host specificity of phage tropism. Like conjugation, phage-based delivery has the benefit of self-propagation, but with the added caveat of species- or strain-level specificity.

Phages exhibit a characteristically narrow host range, typically limited to a set of species or strains, and are capable of host detection even in highly complex ecosystems (17). Limited host range, in combination with self-replication, make phages ideal

Significance

Microbial consortia possess a wealth of genetic information; however, current approaches to deciphering genetics in a community context are largely constrained by low delivery efficiencies and a breadth of targeting specificities. Here, we describe a phage-based approach for precision editing of an individual gene within a target host organism in a community context. Engineering bacteriophage λ to contain a cytosine base editor, we inactivate host chromosomal and plasmid-based genetic targets through the strategic introduction of single-nucleotide mutations without Cas-based double-strand DNA cleavage. Next, we establish species- and site-specific editing within a synthetic soil community by using a fabricated ecosystem. Phage-assisted delivery, together with base editing, offers an important in situ editing approach for the genetic interrogation of microbial community members.

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prokaryotic delivery vectors while also controlling for unintended community off-targeting. Since the initial demonstration of precision targeting by CRISPR-Cas systems (18, 19), engineered phages have been enhanced with exogenous CRISPR-Cas payloads for delivery to a specific host and the subsequent targeting of a specific genetic sequence within. These early engineered phages employed Cas targeting for sequence-specific ablation of several important human pathogens, including Staphylococcus aureus, enterohemorrhagic Escherichia coli, and Clostridioides difficile (20–25). These approaches generally fall into two categories. The first category is the engineering of temperate phages to carry CRISPR-Cas systems, effectively compounding antibacterial efficiency through added nucleolytic Cas activity. The second is the use of phages purely as a vector for the delivery of a plasmid or other genetic elements, with capsids lacking the genetic instructions for phage replication. In the second category, the CRISPR-Cas elements encoded on the plasmid are solely responsible for any antibacterial activity.

Double-strand cleavage induced by Cas9 targeting leads to species- or strain-specific depletion of the target bacterial host population due to inefficiencies of bacterial DNA repair pathways, and cells escaping cleavage have been reported to contain a variety of inconsistent genomic deletions surrounding the target site (26). To move beyond targeted degradation of nucleic acid toward more sophisticated genome editing modalities, Cas effectors have been modified in novel and creative ways, enabling a variety of new genetic manipulations, including site-specific activation, repression, and single-nucleotide mutations (27–31). Recently, progress has been made in phage-based host editing through the delivery of a nuclease-deficient deactivated Cas9 (dCas9), which is catalytically dead but has preserved its ability to target and bind DNA at specified loci with programmable guides. In situ use of dCas9 was demonstrated for gene-specific repression in the gut microbiome (32). The aforementioned study uses lysogenic viral integration of engineered λ to express the modified nuclease to induce repression through transient binding to the target site without permanent modification of the target gene.

Recent pioneering work in base editing has introduced a novel genome editing mechanism to the growing reserve of CRISPR-Cas tools (27, 28). Base editors facilitate targeted deamination of a single base without the adverse fitness effects of double-strand DNA (dsDNA) breakage associated with the use of other Cas effectors. Due to the direct deamination of the target base, a repair template is unnecessary, greatly simplifying payload construction. Base editors consist of a nCas9 or dCas9 fused to either a cytidine deaminase, capable of catalyzing a C-to-T point mutation through a uracil intermediary, or an adenosine deaminase, catalyzing an A-to-G conversion through an inosine intermediary (27, 28). Through nCas9 or dCas9 targeting, the deaminase is guided to a precise locus of interest to generate a substitution mutation within a 5-nucleotide (nt) editing window in the guide sequence. The RNA-guided nucleotide precision facilitated by base editing offers unprecedented levels of control over gene composition and functional inquisition, while limiting depletion of the bacterial host by avoiding dsDNA genomic cleavage, which is not efficiently repaired by bacterial DNA repair pathways (33).

Here, we deployed engineered phages to deliver CRISPR-Cas payloads for targeted community editing, both in vitro and within an EcoFAB apparatus. First, we investigated the feasibility of T7 and λ engineering for delivery of exogenous payloads to the E. coli host. Next, we show that λ lysogeny is an effective mechanism for the expression of exogenous genes, and exploit this to deliver a cytosine base editor (CBE) to the E. coli host to generate genetic knockouts through targeted point mutations. Finally, we demonstrate in situ editing within a community context through the use of an EcoFAB loaded with a synthetic soil medium.

Results

T7 Phage Engineering. To investigate the feasibility of exogenous payload insertion into a lytic phage genome, we performed Cas9-mediated modification of bacteriophage T7. Expanding on previously reported CRISPR-based T7 engineering approaches (34), we used a single-step recombination/selection approach, leveraging a single plasmid containing both the CRISPR-Cas machinery and repair template for homology-directed repair. First, we selected a putative insertion site within the T7 genome (position 6,458 on the chromosome, SI Appendix, Fig. S1A) and then added the corresponding 30-nt spacer to the CRISPR array of the pCas9 plasmid expressing Streptococcus pyogenes cas9. The expression of this spacer guides Cas9 for targeted selection against phages that do not contain the edit of interest at the specified insertion site (Fig. 1A) (35). The selected insertion site is just downstream of the T7-encoded RNA polymerase, within a cluster of nonessential genes that will minimize disruption to the phage life cycle when perturbed (Fig. 1B). Next, we constructed a repair template to serve as a substrate for homologous recombination. To maximize recombination efficiency, we selected 500-nt homologous arms flanking the T7 insertion site and added them to the repair template on either side of the red fluorescent gene, mCherry, under the control of the p70a promoter. For simplicity, we added the repair template into the pCas9 plasmid, resulting in a single engineering plasmid, pTRK1286. E. coli MC1061 was transformed with pTRK1286 and then infected with T7 by standard plaque assay. Plaques were checked for the presence of the insert via PCR and then propagated in plasmid-free MC1061 for 3 rounds of plaque purification to ensure purity. After PCR detection of purified phages, 100% of the resulting plaques tested were found to contain the 857-nt mCherry insertion (SI Appendix, Fig. S1 B and C). The purified stock of engineered T7 was subsequently used to infect MC1061 for the delivery of mCherry; however, no fluorescence was detected during infection. Speculatively, the lack of fluorescence could be due to the short life cycle of T7 (∼25 min) (36), which causes the host to lyse quickly, potentially preventing the sufficient accumulation of the fluorescent protein. Alternatively, the inserted gene could be poorly transcribed or translated at this locus in the genome. Next, we hypothesized that T7 could be coerced to function as a delivery vector if it expressed a CRISPR array encoding a self-targeting spacer that would degrade the T7 genome shortly after host infection to prevent cell lysis. We expanded the repair template to include a CRISPR array with repeat sequences matching the type I-E repeats found in MC1061 to repurpose its endogenous CRISPR-Cas machinery for T7 degradation. This addition brought the total length of the repair template to 2.2 kilobases (kb), which is greater than T7’s reported capsid packaging limit (37). Subsequent infection experiments were performed; however, no successful engineered phages could be detected. Due to the limited capsid capacity of T7, in combination with the additional engineering steps required to convert T7 to a suitable delivery vector, further work on T7 was abandoned in favor of phase λ. Although the utilization of T7 was ultimately not appropriate for the purposes of host editing here, its amenability to CRISPR-based engineering and ability to efficiently take up small payloads via homology-directed repair make it a valuable tool for molecular biology and future community editing studies.

λ Phage Engineering. Next, we sought to demonstrate Cas9-mediated engineering of phase λ through the insertion of an
Cas9 array targeting an intergenic region next to the subsequent Cas9 selection. Next, we inserted a spacer into the ing an ideal locus for the insertion of exogenous genes and for components necessary for Cas9-based targeting of nonedited phages, as well as a repair template, which is inserted into the EagI site. (mCherry insertion in the T7 genome. (Fig. 1

 successively edited and the positive transformants were infected with gentamicin medium, selecting for host cells harboring the

 Fig. 1. Cas9-mediated phage engineering of T7 and λ. (A) Vector map of the generic construct used to engineer both T7 and λ. This construct contains the components necessary for Cas9-based targeting of nonedited phages, as well as a repair template, which is inserted into the EagI site. (B) The site of mCherry insertion in the T7 genome. (C) The insertion site of the gfp and gmR genes into the λ genome. Three nonessential genes (ea47, ea31, and ea59) from the b region are replaced through homologous recombination. (D) Microscope images from time points from λ::gfp:gmR infection of E. coli c600. Light microscope: Upper row. Fluorescence: Lower row. (E) Fluorescent growth curve generated from the infection of c600 with λ::gfp:gmR and wild-type λ. Shaded regions adjacent to each line indicate SE.

 exogenous payload containing sequences for both green fluorescent protein (gfp) as well as gentamicin resistance (gmR). λ is a temperate phage and will integrate into the genome of the host under certain conditions where it can remain dormant, replicating with the E. coli host genome (38). While in this lysogenic state, λ expresses only a single gene, cI, which represses all other λ-encoded genes. Despite cI-controlled repression of λ genes, exogenous genes under control of non-cI-regulated promoters can be efficiently expressed (31). λ maintains a well-characterized region near the center of the genome termed the “b region” (39). This region contains several previously described nonessential genes (lon, orf-401, orf-314, orf-194, ea47, ea31, and ea59), creating an ideal locus for the insertion of exogenous genes and for subsequent Cas9 selection. Next, we inserted a spacer into the pCas9 array targeting an intergenic region next to the ea47 gene (Fig. 1C). λ has a ~48-kb linear dsDNA genome, which circularizes upon entry into the host cell but is relinearized at the cos site prior to capsid packaging. Prior studies have shown that the λ-encoded terminase will efficiently package linear DNA fragments that range from 38 kb to 53 kb, resulting in a maximum effective payload insertion size of ~5 kb (40). Next, we constructed a repair template containing gfp and gmR sequences flanked by 40-nt homologous arms for recombination. This two-gene exogenous payload is 2,125 nt in length, which is well under the presumed maximum insertion limit. Previous λ engineering efforts have demonstrated that homologous arms as short as 40 nt are sufficient for recombination and can be easily added to the 5′ end of primers to facilitate plasmid construction (32). The resulting plasmid, pTrK1287, was transformed into MC1061, and the positive transformants were infected with λ, yielding successfully edited λ phages (λ::gfp:gmR). λ::gfp:gmR was used to infect E. coli c600 cells, a classical λ host strain, and then plated on gentamicin medium, selecting for host cells harboring the engineered phage within its genome. The resulting colonies were screened via PCR and were found to contain the engineered prophage and displayed the green fluorescent phenotype under a fluorescent microscope. Time series infection experiments show continued gfp expression and propagation throughout the culture (Fig. 1D and E). These results indicate successful insertion and expression of the gfp and gmR sequences while in the prophage state.

 Due to the capsid capacity and architecture of the λ genome, we were able to increase the genomic footprint of the engineering payload to include a much larger base editor construct. Coupled with the gmR sequence for antibiotic selection, the total length of the base editor payload is ~6 kb. Since this value is larger than the capsid capacity limit (~5 kb), we designed homologous arms so that recombination would replace ~4 kb of nonessential genes in the b region (Fig. 2A). The CBE used here consists of the rat cytidine deaminase APOBEC1 fused to the N terminus of Cas9 using the XTEN linker (31). The Cas9 nickase (SpCas9D10A) maintains a catalytically active HNH domain that increases base editing efficiency by nicking the nontarget DNA strand to aid in the resolution of any potential U:G heteroduplexes (27). To minimize the total size of the payload, we decided to avoid base editor constructs containing one or more copies of the uracil DNA glycosylase inhibitor tag, which functions to inhibit the base-excision repair activity of uracil DNA glycosylase.

 Initial attempts to engineer the CBE into the phage genome employed 40-nt homologous arms with Cas9 selection pressure at the same locus flanking the ea47 gene as previously described (Fig. 2A); however, no engineered phages were successfully generated. To improve editing efficiency, we modified our approach in two ways. First, an additional spacer targeting a separate locus in the b region (the ea31 gene) was added to the repeat-spacer array to provide additional selection pressure. Next, larger 500-nt
homologous arms were added to the repair template site to facilitate more efficient recombination, generating the final engineering plasmid, pTRK1288 (Fig. 2B). pTRK1288 was transformed into c600, and positive transformants were infected with λ, creating engineered phages harboring the full CBE sequence (λ::CBE). c600 was subsequently infected with λ::CBE, and prophage integration was confirmed by long-read nanopore sequencing (Fig. 2C).

### Base Editing the E. coli Chromosome

Following the successful integration of the CBE payload into the λ genome, we sought to perform phage-based targeted editing of MG1655, a strain of E. coli K-12. Again, leveraging lysogeny, the engineered λ genome will integrate into the host, expressing the base editor components. Guided by Cas9’s guide RNA, the base editor will bind the target gene and catalyze the C-to-T conversion (Fig. 3A). The topology of the deaminase linker, and Cas9 creates a small active window from positions 3 to 8 on the 5’ side of the 20-nt target site (Fig. 3B) (31). To facilitate lacZ targeting, a 20-nt spacer complementary to the MG1655 lacZ sequence was inserted as the CBE single-guide RNA (sgRNA) to create λ::CBElacZ. This spacer was strategically designed to bring the deaminase within range of an in-frame CAG codon, which upon cytosine editing will generate the desired LacZ protein. Additionally, a strain of λ was engineered to contain the CBE with a nontargeting spacer (λ::CBE*ST) as well as the gmR sequence as a negative control for downstream experiments.

The MG1655 host was coincubated with λ::CBElacZ in broth for 3 h at 37°C with a multiplicity of infection (MOI) of 1 and then plated on selective medium containing ChromoMax (IPTG/X-Gal solution) for blue/white screening. Resulting overnight colonies showed a mix of blue and white phenotypes (Fig. 3C). The presence of the λ::CBElacZ prophage was confirmed by PCR and Sanger sequencing. Colony genotypes were then determined via Sanger sequencing, and specific mutations were called if the edited peak in the resulting chromatogram was singular or dominant, as multiple genotypes within a single colony can be represented by the presence of multiple peaks. We observed the expected CAG-to-TAG mutation for all white colonies, indicating successful phage delivery and base editing of the host genome (Fig. 3D). Sanger results underwent additional analysis by EditR (41) to determine the percentage of edited reads per colony, yielding an 81 to 98% C-to-T editing rate for each colony presenting a white phenotype (Fig. 3E). Of 65 colonies tested across replicates (n = 4) including both blue and white phenotypes, 76.3% ± 11.6% (mean ± SD) contained the CAG-to-TAG mutation.

Next, we investigated the genotypes of blue colonies indicative of functional LacZ activity, and observed three distinct point mutations. The most frequent genotype observed was a CAG-to-AAG point mutation, leading to a glutamine-to-lysine substitution, found in 17.8% ± 10.6% of colonies. Next and less frequently, we saw escape from editing or the reversion back to the wild-type genotype (CAG) in 2.7% colonies. In a single colony, we observed a CAG-to-GAG mutation (1.3% ± 2.3%), leading to a glutamine-to-glutamic acid substitution. In all colonies tested, a single dominant peak was present in the Sanger chromatograms, representing a single editing outcome. Notably, many colonies appear to be in transition between the wild-type CAG genotype and the edited TAG genotype. These colonies appear blue on the plate despite their uniform TAG genotype, likely due to residual blue product (5,5'-dibromo-4,4'-dichloro-indigo) produced from X-Gal cleavage by functional β-galactosidase prior to lacZ being edited. When restreaked and propagated overnight, the majority of the these colonies appeared white.

Next, we infected MG1655 with λ::CBElacZ in Luria broth (LB; in vitro) and generated lacZ amplicons directly from

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**Fig. 2.** λ engineered to contain a CBE. (A) The insertion site of the CBE as well as the gmR sequence into the λ genome. Four genes from λ’s b region are replaced through homologous recombination: Orf-314, ea47, ea31, and ea59. (B) Vector map of pTRK1288, the engineering plasmid used to generate λ::CBE. (C) Long-read nanopore sequencing coverage generated from λ::CBE integration into the c600 genome.
culture and without antibiotic selection to check base editing efficiency throughout the entire population during various time points over 16 h (0 h, 4 h, 8 h, and 16 h). No editing was observed from 0 to 8 h, and the first detectable progress was at 16 h, where we observed an average percentage C-to-T editing of 11.3% ± 0.2% and an average percentage C-to-A editing of 12.1% ± 0.1% (n = 3). Expanding on this finding, we wanted to check editing efficiency across the E. coli population within the EcoFAB apparatus without antibiotic selection. We loaded it with sterile quartz sand and LB and then performed the same infection of MG1655 with λ::CBElacZ. Again, no significant editing was observed from 0 to 8 h; however, at 16 h, we saw an average percentage of C-to-T editing of 28% ± 0.1% across the bacterial population, with an average percentage of C-to-A editing of 10% ± 0.3% (n = 2). No CAG or GAG codons were detectable in either tube or EcoFAB experiments at 16 h.

To approximate the rate of λ lysogeny, we repeated the infection of MG1655 with λ::CBEmCherry, this time plating on both selective (20 μg/mL gentamicin) and nonselective medium (LB only). Experiments without antibiotic selection showed ~10-fold higher CFU/mL than experiments plated with antibiotics, indicating a ~10% rate of lysogeny under these experimental conditions (3-h phage/host coinoculation at 37 °C, nonshaking, no cation additives in medium) (SI Appendix, Fig. S2).

While we did observe successful base edits, the low level of penetrance seen here is unlikely to provide sufficient genetic perturbation for phenotypic studies that require a high degree of editing. Moving forward, this proof of concept could be significantly bolstered through strategies that optimize lysogeny and penetrance across the bacterial population.

**Base Editing Plasmid-Based Targets.** After demonstrating lacZ editing within the chromosome, we expanded our analysis to include plasmid-based targets and selected pTRK600, which encodes the fluorescent mCherry gene, as the first candidate for editing (SI Appendix, Fig. S3). Again, we employed the strategy of strategically editing a specific site in the mCherry gene to introduce a premature stop codon that would generate a nonfunctional protein. Using the protocol described above, we generated λ::CBEmCherry, then coincubated λ::CBEmCherry with MG1655 harboring pTRK600, and plated it on selective medium. After a 16-h incubation, resulting colonies, presumably lysogens, were viewed under the microscope and displayed the edited phenotype with a noticeably small percentage of cells actively fluorescing (Fig. 4A). Genotypes of resulting colonies were checked via Sanger sequencing. Interestingly, instead of finding high-efficiency edits, as observed with the lacZ chromosomal targeting represented by single peaks, we observed dual peaks within the chromatogram at the editing locus, indicating the presence of both the wild-type “C” genotype, as well as the edited “T” genotype in a single colony (Fig. 4B). Analysis with EditR revealed editing percentages much lower than previous chromosomal editing (Fig. 4C). To investigate the temporal component of base editing plasmid sequences, colonies with
dual-peak genotypes were restreaked and grown overnight, followed by another round of PCR and Sanger sequencing. After additional growth, the edited genotype was more represented in the population (Fig. 4D). Parent colonies displayed 20 to 30% editing. In general, restreaked colonies displayed much higher editing rates of 40 to 100%.

Next, we selected plasmid-encoded ampicillin resistance for knockout by using the classical λ E. coli host c600. This strain contains the glnV44 genotype (also called supE44), an amber stop codon suppressor mutation that causes any UAG stop codons to be read through and replaced with glutamine (CAG) (42). The p70a-deGFP plasmid (Daicel Arbor Biosciences) containing ampR and deGFP sequences was transformed into c600 (Fig. 5A). Engineered λ containing a complementary spacer to the ampicillin resistance sequence was generated (λ::CBEampR), coincubated in broth with c600 containing p70a, and then plated on an ampicillin/gentamicin medium. Due to the glnV44 mutation, c600 will continue to grow on ampicillin despite mutation of the ampicillin resistance sequence, enabling verification of the edited genotype. Colonies were Sanger sequenced and again yielded the dual wild-type and edited genotypes (Fig. 5B). We saw CAG-to-TAG editing in 71.5% ± 13.1% colonies (n = 2) and targeting escape or repair back to the wild-type codon in 28.5% ± 13.1% of colonies.

Next, we grew MG1655 that does not contain the glnV44 mutation, with p70a in broth containing ampicillin, and then introduced λ::CBElacZ at an MOI of 1. During the first ∼8 to 10 h of infection, lambda is primarily engaged in the lytic life cycle and is lysing host cells, as indicated by the lack of increased green fluorescence (Fig. 5C) and the significant drop in the optical density at 600 nm (OD600) during these first hours of infection. This lytic period is then followed by the growth of lysogens, which harbor engineered lambda. Ultimately, we observed the loss of resistance to ampicillin, evidenced by a decreased OD600nm and a drop in green fluorescence over time.

Demonstration of Community Editing. To provide an application proof of concept and demonstrate delivery and base editing in E. coli within a microbial community context, we assembled a minimal synthetic mixed soil community consisting of E. coli MG1655 and two additional soil-relevant strains, namely, Klebsiella oxytoca M5a1 and Paraburkholderia bryophila 376MFSHa3.1, that were previously isolated from a rice rhizosphere and an Arabidopsis thaliana rhizosphere, respectively (43). This community was assembled in vitro and coinfected with λ::CBElacZ in broth for 3 h, and then they were plated on selective medium for blue/white screening. E. coli MG1655 lysogenized with λ::CBElacZ will grow on our selective medium of choice, 20 μg/mL of gentamicin, while ensuring the inhibition of Klebsiella oxytoca M5a1 and Paraburkholderia bryophila 376MFSHa3.1, which are gentamicin susceptible (SI Appendix, Fig. S4).

The resulting overnight colonies displayed the mixed blue and white phenotype, indicative of successful lysogeny and editing. Sanger sequencing verified point mutations within the lacZ sequence, qualitatively confirming phage-assisted base editing within a synthetic community context. Nine of 18 total colonies checked contained the TAG mutation, 5 of 18 contained the AAG mutation, and 1 contained the wild-type "C" genotype, while 3 colonies simultaneously contained both TAG and AAG mutations, albeit at low percentages.

![Fig. 4. Base editing plasmid-based mCherry. (A) Microscope images of MG1655pTRK600 colonies that have been lysogenized with λ::CBE<sup>mCherry</sup>, which targets and truncates the mCherry protein. Light microscope: Upper row. Fluorescence: Lower row. (B) Chromatogram displaying mCherry base edits. The dual peak consists of an edited peak (T in red), as well as the wild-type peak (C in blue). (C) C-to-T editing percentages by base position within the mCherry spacer. The expected base position of the edit is position 5. Editing percentages higher than the gray dashed line are statistically significant as determined by EditR (P < 0.05). (D) C-to-T percent editing efficiencies calculated by EditR from three parent colonies that were restreaked, grown overnight, and checked for editing progression.](https://doi.org/10.1073/pnas.2206744119)
Next, we sought to create a reproducible model ecosystem that more closely approximates a natural rhizosphere environment, to provide a qualitative proof-of-concept experiment for in situ implementation. We deployed EcoFAB devices filled with sterile white quartz sand to act as a synthetic soil analog (Fig. 6A). We created the three-member synthetic community by syringe injection of each member into the EcoFAB device, followed by the addition of λ::CBElacZ. The EcoFAB community was incubated for 4 h and then samples were extracted and plated on selective medium for blue/white screening (Fig. 6B). Overnight colonies displayed both blue and white phenotypes (Fig. 6C). Editing was verified by Sanger sequencing, yielding the desired TAG mutation in 79.6% ± 14.1% colonies (Fig. 6D). The λ::CBElacZ mutation was found in 18.6% ± 12.4% of colonies (n = 2), and only a single colony contained the wild-type CAG codon. These results highlight the ability of λ to seek out its host within a community context in situ, despite the presence of multiple organisms and varying natural environmental conditions presented by the constraints of other physical media.

Discussion

In the work presented here, we demonstrate programmable genetic manipulation of specific bacteria within a synthetic soil community by using phage delivery of base editors. Leveraging phage lysogeny, we deploy a CBE for precise perturbation of both chromosomal and plasmid gene targets in a host cell. Furthermore, we use an EcoFAB device to recapitulate characteristics of the soil rhizosphere with a silica soil analog and show that engineered phage can deliver a functional base editing payload to a specific host within a mixed microbial community context. The specific base editor employed here has also shown editing activity in Pseudomonas (31) and could putatively be applied to more diverse microbial systems.

Phage-based targeted editing is an effective method for making precision knockouts in a diverse mixed community and will improve efforts to interrogate gene and microbial functions in the complex rhizosphere environment. Beyond the introduction of premature stop codons, point mutations enable investigation into other genetic elements such as regulatory sequences specifically for organisms that are recalcitrant to genetic engineering or traditional DNA transformation protocols, while limiting the fitness consequences of dsDNA cleavage. Gain-of-function mutations are a particularly interesting topic of exploration due to their prevalence in the rise of antibiotic resistance, and one study has already used base editing to generate rifampicin resistance in E. coli through specific mutation of rpoB (44). Although we developed phage-based targeted editing to specifically address challenges associated with community editing, this technology could also improve the throughput of traditional CRISPR-based monoculture editing by first enabling stable integration of CRISPR-Cas cargo via lysogeny, followed by the introduction of plasmid-based guides.

Due to the reliance on lysogeny as a delivery modality, this community editing strategy is limited to temperate phage. Without significant engineering efforts, obligately lytic phages are not ideal delivery vectors since the primary outcome of infection is cell death. Recently, the filamentous phage M13 was engineered to deliver a CRISPR-Cas payload to E. coli for
disruption of target genes within the murine gut microbiome (26). Filamentous phage (inoviruses) do not strictly subscribe to lytic or lysogenic infection cycles but rather rely on extrusion through the bacterial membrane without lysis to establish chronic infections that allow continuous virion production without killing the host cell (45). Although cultivated inoviruses are known to infect hosts from only 5 bacterial phyla and 10 genera, machine learning approaches have recently highlighted a wealth of diverse and pervasive inoviruses across various ecosystems that could prove promising tools for future microbiome editing studies (46).

The primary constraint we encountered when deploying base editors was target availability. Finding a suitable target for the generation of a premature stop codon first requires satisfying the Cas effector guide requirement by finding an appropriate PAM in a gene of interest. Next, a potential stop codon precursor (CAG, CAA, CGA) must be identified within the active window of the base editor that is consistent with the reading frame of the target gene. While these requirements greatly restrict target availability, some flexibility can be reintroduced by replacing the Cas effector to alter the PAM requirement and by changing the length of the sgRNA and linker to modify the active window of the base editor (27). Due to recent groundbreaking research into base editors, a variety of base editing options exist, including cytosine editors (C to T), adenine editors (A to G), and some that can perform both cytosine and adenine conversions simultaneously (27, 28, 47). Ultimately, ongoing efforts to advance base editing technology will facilitate access to a wider range of genetic loci for more adaptable targeting, increased point mutation availability, and improved on-target nucleotide conversion efficiency.

While the use of base editing to generate targeted mutations is broadly applicable across prokaryotes, the use of phage delivery vectors limits generalizability due to their characteristic host specificity, requiring engineering of a specific phage for each community member of interest. While this can be prohibitive, narrow host range also facilitates delivery specificity within a mixed community. In this study, we were able to engineer T7 and λ due to decades of prior investigation into gene function and essentiality, highlighting the importance of continued work in phage isolation, characterization of the virome, and functional genomics. As cas technologies continue to mature, developing a more thorough understanding of phage packaging limits and locus editing amenability will enable the delivery of larger candidate payloads for more sophisticated editing outcomes, such as the mobilization of large donor sequences into programmable loci using CRISPR-associated transposon systems (48). Importantly, future studies should quantitatively determine the efficiency of editing in a microbial community context and identify the factors that limit and promote the penetrance and resilience of edits in complex microbial consortia.

The microbiome is an integral component to the function and health of ecosystems and elucidating the complex genetic interactions within these communities requires specialized targeted editing approaches. The combination of engineered phage with CRISPR-Cas technologies provides robust delivery and editing modalities to diverse microbiota. As interest in soil taxa has risen in recent years due to the agricultural implications of the changing climate, improved in situ techniques can enable and improve the functional interrogation of rhizosphere communities which could be increasingly significant to the evaluation of future plant fitness models, increasing crop production, and improving nitrogen fixation.

Materials and Methods

DNA Manipulations. Plasmid DNA from E. coli was extracted using the QiAprep Spin Miniprep Kit (Qiagen) following the standard manufacturer's recommendations. Primers for PCR, as well as single-stranded DNA oligos for duplex annealing, were synthesized by Integrated DNA Technologies (IDT). Colony screening was performed by generating PCR amplicons following standard PCR protocols with OneTaq DNA polymerase (New England Biolabs [NEB]).
generate high-quality amplicons for cloning, Q5 Hot Start High-Fidelity Polymerase (NEB) was used. All PCR products were visualized in 0.8% agarose gels. All Sanger DNA sequencing for amplicon, construct, and base editing confirmation was performed by Genewiz. Restriction digestions were carried out using restriction enzymes from NEB with 1 μg of purified plasmid DNA in a final volume of 50 μL. Digests were performed at 37 °C for 30 min, followed by gel extraction using Monarch DNA Gel Extraction Kit (NEB). DNA ligations were done at a 3:1 insert:vector ratio with 50 ng of vector in a final volume of 10 μL of Instant Sticky-end Ligase Master Mix (NEB) following the manufacturer’s recommendations. Annealed oligos with 4 bp overhangs for ligation compatibility were generated by resuspending single-stranded DNA oligonucleotides in Duplex Buffer (IDT) to a concentration of 100 μM. A total of 2 μg of each oligo was mixed and brought to a volume of 50 μL using Duplex Buffer. Oligos were annealed by incubating the mix at 95 °C for 2 min, then 25 °C for 45 min, and followed by a 4 °C incubation for 1 h. Annealed oligos were transformed immediately or stored at −20 °C.

Bacterial Strains and Growth Conditions. E. coli strains c600, MC1061, and MG1655 used in this study were propagated in LB (Difco) under aerobic conditions at 250 rpm or on LB agar (1.5% w/v) plates at 37 °C aerobically. The same growth conditions were used for the propagation of Klebsiella oxytoca M5a1 and Paraburkholderia braiphylla 376M5Sha3.1. All strains used in this study are listed in SI Appendix, Table S4.

Construction of Phage Engineering Plasmids. The plasmid pCas9 was obtained from Addgene (#42876) and used as a backbone for all subsequent plasmid construction. To add new spacers to pCas9, double-stranded DNA in the form of synthetic annealed oligos (SI Appendix, Table S1) were ligated into EagI-digested pCas9. The ligation was transformed into ribudium chloride-treated competent MC1061 cells with heat shock at 42 °C for 1 min, and followed by a 2-min incubation on ice. A total of 1 ml of SOC medium (NEB) was added to the competent cells, followed by recovery at 37 °C, and shaking aerobically at 250 rpm for 1.5 h. Recovered cells were plated on LB agar with 25 μg/mL chloramphenicol. The resulting colonies were screened for spacer insertion via colony PCR using pCas9_spacer_F and R primers (SI Appendix, Table S2) and confirmed using Sanger sequencing.

The mCherry repair template used for T7 engineering was synthesized by IDT (Si Appendix, Table S3), containing 500-nt homologous arms. The larger T7-encoded RNA polymerase (SI Appendix, Table S1). A1 % inoculum from overnight culture was added to 4 mL of LB and grown to log phase. A total of 300 μL of log-phase cells were added to 100 μL of T7 serial dilutions in saline magnesium (SM) buffer (100 mM sodium chloride, 8 mM magnesium sulfate heptahydrate, and 50 mM Tris-Cl [pH 7.5]) following the double agar layer plaque assay protocol (39), then mixed with 8 μL molten top agar (LB with 0.75% agar), and poured onto LB agar plates to solidify. Double agar layer plates were incubated overnight aerobically at 37 °C. Individual plaques were checked for engineering by PCR (SI Appendix, Table S2), and positive plaques were picked with a p10 tip and resuspended in 100 μL SM buffer. Isolated engineered phages in buffer were used in the next round of double agar layer plaque assays and repeated a total of 3 times to ensure phage purity. Individual plaques were again checked for the presence of the appropriate insertion by PCR and Sanger sequencing. A crude phage plate lysate was generated by soaking double agar layer plaque assay plates in 5 mL SM buffer and incubating at room temperature for 3 h shaking at 84 rpm. The SM buffer/physi mixtures was passed through a 0.22-μM filter to generate the T7::mCherry crude lysate stock.

To engineer λ to express gfp and gentamicin resistance, c600 containing pTRK1287 was grown aerobically overnight, shaking at 37 °C, in LB with 25 μg/mL chloramphenicol. pTRK1286 contains 500 nt homologous arms complementary to the λ genome and contains a Cas9 guide targeting a site downstream of the λ-encoded RNA polymerase (SI Appendix, Table S1). A 1% inoculum from overnight culture was added to 4 mL of LB and grown to log phase. A total of 300 μL of log-phase cells were added to 100 μL of T7 serial dilutions in saline magnesium (SM) buffer (100 mM sodium chloride, 8 mM magnesium sulfate heptahydrate, and 50 mM Tris-Cl [pH 7.5]) following the double agar layer plaque assay protocol (39), then mixed with 8 μL molten top agar (LB with 0.75% agar), and poured onto LB agar plates to solidify. Double agar layer plates were incubated overnight aerobically at 37 °C. Individual plaques were checked for engineering by PCR (SI Appendix, Table S2), and positive plaques were picked with a p10 tip and resuspended in 100 μL SM buffer. Isolated engineered phages in buffer were used in the next round of double agar layer plaque assays and repeated a total of 3 times to ensure phage purity. Individual plaques were again checked for the presence of the appropriate insertion by PCR and Sanger sequencing. A crude phage plate lysate was generated by soaking double agar layer plaque assay plates in 5 mL SM buffer and incubating at room temperature for 3 h shaking at 84 rpm. The SM buffer/physi mixtures was passed through a 0.22-μM filter to generate the T7::mCherry crude lysate stock.

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To engineer λ to contain the APOBEC-1 CBE, c600 culture containing pTRK1288 was grown aerobically overnight, shaking at 37 °C in LB with 20 μg/mL gentamicin. pTRK1287 contains 40 nt homologous arms complementary to the λ system and contains a Cas9 guide targeting a site flanking the ea47 gene in the b region (SI Appendix, Table S2). The double agar layer plaque assay was performed as described above using 300 μL of log-phase cells, grown from 1% inoculum of overnight culture (c600) in LB, mixed with 100 μL of serially diluted lambda phage in buffer. Bacterial cells and phage were combined with 8 mL molten top agar (LB with 0.75% agar) and plated onto LB agar plates to solidify and were incubated overnight aerobically at 37 °C. Individual plaques were checked for the inserted gfp and gentamicin resistance cassettes by PCR (SI Appendix, Table S2), and individual positive plaques were picked with a p10 tip and resuspended in 100 μL SM buffer. The double agar layer plaque assay was repeated with the newly resuspended engineered phages a total of three times for phage purity. A crude plate lysate phage stock was generated from the third round of plaque assay plates with SM buffer, as described above.

To engineer λ to contain the APOBEC-1 CBE, c600 culture containing pTRK1288 was grown aerobically overnight, shaking at 37 °C in LB with 20 μg/mL gentamicin. pTRK1287 contains 500 nt homologous arms complementary to the Orf314 and ea59 genes, and expresses one spacer targeting a region flanking the ea47 sequence and a second spacer targeting the ea31 gene (SI Appendix, Table S1) for additional selection pressure. We grew c600 on LB plates to log phase from 1% overnight inoculum in LB and then
mixed 300 μL of these cells with 100 μL of serially diluted λ in SM buffer. This bacterium/phage mixture was combined with 8 mL molten top agar (LB with 0.75% agar), poured onto LB agar plates to solidify, and then incubated aerobically at 37 °C overnight. A crude plate lysate of engineered phage stock was generated from overnight plates using 5 mL SM buffer, incubated at room temperature, shaking, for 3 h, and then passed through a 0.22 μM filter.

**Experimental Lysogeny with Engineered λ (E. coli Only).**

*In vitro experiments.* MG1655 cells were grown to midlog phase in LB, and then 100 μL cells were added to 100 μL phage crude lysate (either λ::gfp::gmR or λ::CBE) and incubated at 37 °C for 3 to 6 h (MOI = 1). A total of 100 μL of serial dilutions was spread plated on LB agar plates containing 20 μg mL⁻¹ gentamicin and then incubated aerobically overnight at 37 °C. Resulting colonies were checked for the presence of engineered prophage by PCR. Base editing was checked by PCR and Sanger sequencing (SI Appendix, Table S2).

*EcoFAB experiments.* A total of 3.0 g white quartz sand (Sigma-Aldrich, 50 to 70 mesh particle size) was loaded into the EcoFAB, followed by autoclaving for sterilization. MG1655 cells were grown in LB overnight, and then 40 μL cells was added to 2.5 mL LB and injected into the EcoFAB by syringe through the lower loading port. The EcoFAB was incubated for 4 to 16 h at 37 °C aerobically, without shaking. A total of 100 μL of serial dilutions was extracted by syringe and spread plated on LB agar plates containing 20 μg mL⁻¹ gentamicin and then incubated aerobically overnight at 37 °C. Resulting colonies were checked for the presence of engineered prophage and base editing by PCR (SI Appendix, Table S2). To generate time series editing efficiencies for both in vitro and EcoFAB experiments, 100 μL samples were extracted at 0 h, 2 h, 4 h, 6 h, 8 h, and 16 h of growth. A total of 1 μL was taken from each sample (in duplicate or triplicate) to serve as a template for colony PCR to check λ::CBE editing across the population (SI Appendix, Table S2). Amplicons were sent for Sanger sequencing, and EdIt was used to analyze base editing penetrance.

**Experimental Lysogeny with Engineered λ (Synthetic Soil Community).**

*In vitro experiments.* E. coli MG1655, Klebsiella oxytoca MSa1, and Paraburkholderia bryophila 3766MSha3.1 were grown individually overnight in LB at 37 °C aerobically, shaking at 250 rpm. A total of 40 μL cells from each overnight culture was added to 4 mL LB, followed by the addition of 200 μL λ::CBEλ22 stock. This community was incubated at 37 °C aerobically for 3 h, shaking. A total of 100 μL of serial dilutions was plated on LB agar plates with 20 μg mL⁻¹ gentamicin and incubated overnight at 37 °C. Resulting colonies were screened for editing by PCR (SI Appendix, Table S2) and verified by Sanger sequencing.

*EcoFAB experiments.* A total of 3.0 g of white quartz sand was loaded into the EcoFAB, followed by autoclaving for sterilization. A total of 2.5 mL LB was injected into the EcoFAB’s lower loading port by syringe. E. coli MG1655, Klebsiella oxytoca MSa1, and Paraburkholderia bryophila 3766MSha3.1 were grown individually overnight in LB at 37 °C aerobically, shaking at 250 rpm. A total of 40 μL cells from each overnight culture was added to the EcoFAB by injection from a 1-mL syringe, followed by the injection of 200 μL of λ::CBEλ22 stock. EcoFABS were incubated at 37 °C aerobically without shaking, for 4 h. A total of 100 μL was extracted from the EcoFAB using a 1-mL syringe, then plated on LB agar plates with 20 μg mL⁻¹ gentamicin, and incubated overnight 37 °C aerobically, shaking. Resulting colonies were screened for editing by PCR (SI Appendix, Table S2).

**OD and Fluorescent Measurements.** All OD600nm and fluorescent measurements were carried out using a flat-bottom 96-well plate (Corning 96) and plate reader (OmeGa). All experiments were performed in triplicate. λ::gfp::gmR experiments were carried out by adding 100 μL of midlog c600 cells (grown in LB) to plate wells, followed by 100 μL of λ::gfp::gmR, wild-type λ, or SM buffer. Fluorescent measurements were taken at 485 nm and were measured once an hour for a total of 24 h, and 37 °C was maintained throughout the duration of the experiment. λ::CBEλ22 experiments were performed by adding 2 μL of midlog c600λ22 cells to each well; followed by 150 μL LB, and then 50 μL of λ::CBEλ22::CBEλ22, or SM buffer. Fluorescent measurements were taken at 485 nm, and OD measurements were taken at 600 nm once an hour for 48 h in total; 37 °C was maintained at all times during the experiment.

**Other Plasmids.** pCas9 was a gift from Luciano Marraffini (Addgene plasmid # 42876; addgene.org/42876; RRID:Addgene_42876); pCasPA-BEC was a gift from Quanjiang Ji (Addgene plasmid # 113349; addgene.org/113349; RRID: Addgene_113349).

**Data, Materials, and Software Availability.** All annealing oligo sequences, primer sequences, and repair template sequences, as well as targets used in the analysis are contained within the supplemental information. To check for the presence of a suitable codon that can be modified by Cris Tol base editing to generate an in-frame stop codon, gene sequences were analyzed and selected using custom code available at https://github.com/CRISPRlab/CRISPRbaseedit (49).

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1. I. Cho, M. J. Blaser, The human microbiome: At the interface of health and disease. *Nat. Rev. Genet.* 13, 326-270 (2012).
2. Y. Yan, L. H. Nguyen, E. A. Franzosa, C. Huttenhower, Strain-level epidemiology of microbial communities and the human microbiome. *Genome Med.* 12, 71 (2020).
3. S. E. Hannula, H. K. Ma, I. E. Perez-Jaramillo, A. Pineda, T. M. Bezemer, Structure and ecological function of the soil microbiome affecting plant-soil feedbacks in the presence of a soil-borne pathogen. *Environ. Microbiol.* 22, 660-676 (2020).
4. M. Käser et al., Unraveling the complexity of soil microbiomes in a large-scale study subjected to different agricultural management in styria. *Front. Microbiol.* 11, 1052 (2020).
5. S. Nayfach et al., iMGS Data Consortium, A genomic catalog of Earth’s microbiomes. *Nat. Biotechnol.* 39, 499-509 (2021).
6. A. Pascual-García, G. Bonhoeffer, T. Bell, Metabolically cohesive microbial consortia and ecosystem functioning. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 375, 20190245 (2020).
7. R. U. Sheth, Y. Cabil, S. P. Chen, H. M. Wang, Manipulating bacterial communities by in situ directed microbiome engineering. *Trends Genet.* 32, 189-200 (2016).
8. Y. Ding, T. R. Northen, A. Khalil, A. Halkett, E. A. Schmelz, Getting back to the grass roots: Harnessing specialized metabolites for improved crop resilience. *Curr. Opin. Biotechnol.* 70, 1174-185 (2021).
9. S. Selle et al., ‘Cis-engineering’ a natural drug hit. *Nature* 591, 429-433 (2021).
10. D. B. Brown et al., Defining the core-Arabidopsis thaliana root microbiome. *Nature* 488, 86-90 (2012).
11. A. Sawyer et al., Cultivar and phosporus effects on switchgrass yield and rhizosphere microbial diversity. *Appl. Microbiol. Biotechnol.* 103, 1973-1987 (2019).
25. I. Yosel, M. Mamor, R. Kiro, U. Oimron, Temperate and lytic bacteriophages programmed to sensitize and kill antibiotic-resistant bacteria. *Proc. Natl. Acad. Sci. U.S.A.* **112**, 7267–7272 (2015).

26. K. N. Lam et al., Phage-delivered CRISPR-Cas9 for strain-specific depletion and genomic deletions in the gut microbiome. *G3* Rep. **37**, 109930 (2021).

27. A. C. Komor, Y. B. Kim, M. S. Packer, J. A. Zuris, D. R. Liu, Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature* **533**, 420–424 (2016).

28. N. M. Gaudelli et al., Programmable base editing of AT to GC in genomic DNA without DNA cleavage. *Nature* **551**, 464–471 (2017).

29. Y. Liu, X. Wan, B. Wang, Engineered CRISPRa enables programmable eukaryote-like gene activation in bacteria. *Nat. Commun.* **10**, 3693 (2019).

30. J. S. Hawkins, S. Wong, J. M. Peters, R. Almeida, L. S. Qi, Targeted transcriptional repression in bacteria using CRISPR interference (CRISPRi). *Methods Mol. Biol.* **1311**, 349–362 (2015).

31. W. Chen et al., CRISPR/Cas9-based genome editing in *Pseudomonas aeruginosa* and cytidine deaminase-mediated base editing in *Pseudomonas* species. *iScience* **6**, 222–231 (2018).

32. B. B. Hsu et al., In situ reprogramming of gut bacteria by oral delivery. *Nat. Commun.* **11**, 5030 (2020).

33. M. S. Dillingham, S. C. Kowalczykowski, RecBCD enzyme and the repair of double-stranded DNA breaks. *Microbiol. Mol. Biol. Rev.* **72**, 642–671 (2008).

34. A. M. Grigoryte et al., Comparison of CRISPR and marker-based methods for the engineering of Phage T7. *Viruses* **12**, 193 (2020).

35. M. L. Lemay, D. M. Tremblay, S. Moineau, Genome engineering of virulent lactococcal phages using CRISPR-Cas9. *ACS Synth. Biol.* **6**, 1351–1358 (2017).

36. F. W. Studier, Bacteriophage T7. *Science* **176**, 367–376 (1972).

37. I. J. Molineux, "The T7 group" in *The Bacteriophages*, R. Calendar, Ed. (Oxford University Press, 2006), pp. 277–301.