Synthetic engineering and biological containment of bacteriophages

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The serious threats posed by drug-resistant bacterial infections and recent developments in synthetic biology have fueled a growing interest in genetically engineered phages with therapeutic potential. To date, many investigations on engineered phages have been limited to proof of concept or fundamental studies using phages with relatively small genomes or commercially available "phage display kits". Moreover, safeguards supporting efficient translation for practical use have not been implemented. Here, we developed a cell-free phage engineering and rebooting platform. We successfully assembled natural, designer, and chemically synthesized genomes and rebooted functional phages infecting gram-negative bacteria and acid-fast mycobacteria. Furthermore, we demonstrated the creation of biologically contained phages for the treatment of bacterial infections. These synthetic biocontained phages exhibited similar properties to those of a parent phage against lethal sepsis in vivo. This efficient, flexible, and rational approach will serve to accelerate phage biology studies and can be used for many practical applications, including phage therapy.

biological containment | bacteriophage | cell-free genome engineering | phage therapy | synthetic biology

Phages are natural enemies of bacteria. Virulent phages infect bacteria and produce progeny, eventually killing their hosts and releasing progeny phages into the extracellular environment. In recent years, with the global spread of drug-resistant bacterial infections, phage-based therapy (or "phage therapy") has gained attention. Clinical trials investigating wild-type (WT) phages screened from the environment are underway. Moreover, the applications of engineered phages such as those carrying a biofilm-degrading enzyme (1), synthetic gene networks boosting the effect of antibiotics (2), CRISPR–Cas9 nucleases to produce sequence-specific antimicrobials (3, 4), and engineered host determinants for targeted bacterial population editing (5–7) are being explored. Engineered phages carrying fluorescent or luminescent genes can also be used as highly sensitive bacterial detection systems (8).

Despite the potential utility of phage design and rebooting, a single, streamlined, development platform has not been reported. Two existing methodologies enable the design-oriented rebooting of phages. The first methodology is a yeast-based approach, in which the phage genome is assembled from DNA fragments in yeast cells. The resulting genome is extracted and introduced into an appropriate host bacterium to reboot a phage. The Endy group used this method to create a fully decompressed synthetic ΦX174 (9), and the Lu group engineered phages with a designed host range (5). Although promising, this method requires multiple time-consuming processes and high transformation efficiency of the host bacterium, and the phage genome must be linked to a vector for maintenance in yeast. This can complicate the interpretation of results, especially when rebooting fails. The second methodology is an in vitro approach first demonstrated by Smith, who assembled the ΦX174 WT genome, installed it in *Escherichia coli*, and rebooted a functional phage (10). Kilcher et al. developed synthetic phages that infect gram-positive bacteria, including *Listeria*, *Bacillus*, and *Staphylococcus* (11). The method also enables phage engineering and is less restrictive than the yeast-based approach. To enhance engineered phage use, studies on efficient single platforms and related design guidelines are needed.

Here, we present a robust and flexible phage rebooting platform combining in vitro genome assembly and installation into appropriate host bacteria or a cell-free transcription and translation (TXTL) system. In addition, we developed guidelines for preparation of two unique biologically contained phages in anticipation of practical application.

Significance

In this study, we used an efficient and simple synthetic phage engineering platform to generate tailor-made and biologically contained phages for the treatment of drug-resistant and difficult-to-treat bacterial infection. The biocontained phages were genetically virion gene deficient but phenotypically functional and rescued septic mice in a manner similar to that of its parent wild-type phage without producing progeny. Our platform enabled the rebooting of custom-made and biocontained phages from diverse phage families, accelerating a safe synthetic phage therapy for combating drug-resistant superbugs.

Results

Rebooting of Diverse Phage Families from Genomes Assembled In Vitro. We first aimed to reboot various WT phages infecting gram-negative bacteria and acid-fast mycobacteria from in vitro–assembled genomes. The entirety of the assembled phage genome was amplified using PCR from a template phage genome or chemically synthesized DNA. Primers were designed to generate each adjacent PCR fragment with 28–65 bp homologous regions. These fragments were assembled using the Gibson Assembly and then installed into host bacteria by electroporation, resulting in rebooted phages (Fig. 1*A*).

We tested our strategy using the model coliphage T7, which has a linear genome and exhibits linear concatemerization during replication (12). We constructed a linear genome (39,937 bp) from four PCR fragments and then installed it in *E. coli* via electroporation to reboot the functional phage T7 (Fig. 1*B*). We used this strategy to reboot other T7-like phages, namely coliphage T3 (38,208 bp) (12), *Salmonella* phage SP6 (43,769 bp) (12, 13), and *Pseudomonas* phage gh-1 (37,359 bp) (12, 14), which were expected to have genome structures and life cycles similar to those of T7. We succeeded in rebooting T3 from four DNA fragments in *E. coli* (Fig. 1*C*), SP6 from four fragments in *Salmonella* Typhimurium LT2 (Fig. 1*D*), and gh-1 from four fragments in *Pseudomonas putida* (Fig. 1*E*). These results showed that our design rule and approach were functional for rebooting phages from linear genomes.

In reality, most phage genomes that enter the host bacterium in a linear form are converted to a circular form prior to replication (11). Therefore, we designed fragments that allowed end-joining to reboot phages from assembled circular genomes (Fig. 1*A*). We successfully rebooted coliphage λ (48,502 bp) from five fragments in *E. coli* (Fig. 1*F*) and *Salmonella* phage P22 (41,724 bp) from four fragments in *S.* Typhimurium LT2 (Fig. 1*G*).

In order to confirm the robustness and flexibility of our methodology, we also rebooted the model mycophage D29 (49,136 bp) (15, 16), a phage with a high-GC (63.5%) genome that infects acid-fast *Mycobacterium* spp. We prepared five DNA fragments to construct the circular genome and then successfully rebooted the functional mycophage D29 in *Mycobacterium smegmatis* mc^{2}155 (Fig. 1*H*). We also succeeded in rebooting the uncharacterized mycophage B1 (45,961 bp) (16, 17) from five DNA fragments (Fig. 1*I*) and GS4E (49,823 bp) (16, 17) from five fragments (Fig. 1*J*) in *M. smegmatis* mc^{2}155.

In this study, we also examined the possibility of cross-genus rebooting of non-coliphages from genomes assembled in vitro. It was reported that among the phages that cannot infect *E. coli* 10G (*SI Appendix*, Fig. S1*A*), SP6 and gh-1 can be rebooted in 10G (5). As expected, both SP6 and gh-1 were rebooted in *E. coli* from genomes assembled in vitro and subsequently infected their original host cells (*SI Appendix*, Fig. S1 *B* and *C*), while the others were not rebooted. Given that obtaining these results and cross-genus rebooting via 10G requires additional steps and time, we used
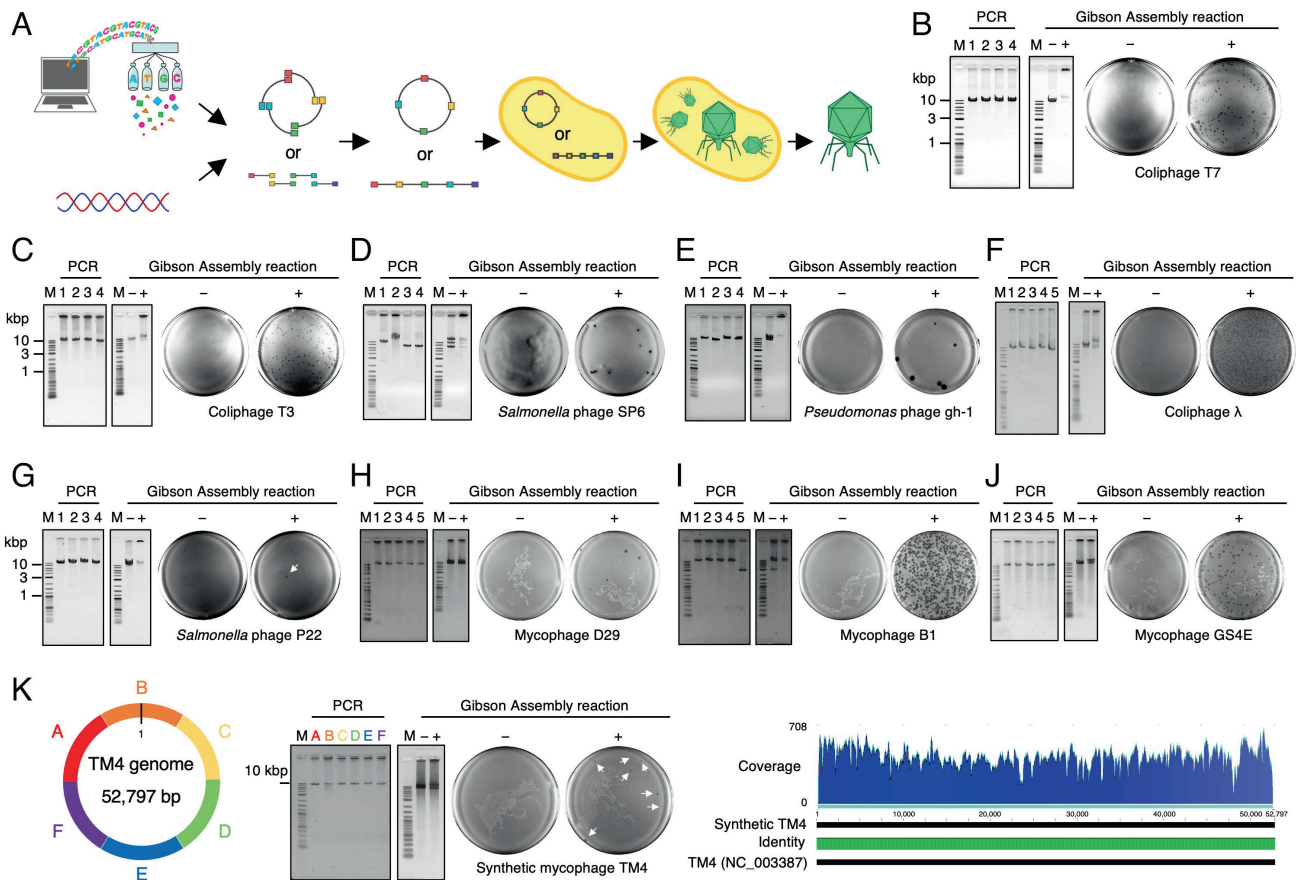

Fig. 1. Rebooting of WT phages from genomes assembled in vitro. (*A*) Schematic illustrating the workflow for rebooting phages from genomes assembled in vitro via electroporation. DNA fragments exhibiting homology with adjacent fragments were amplified from the phage genome or from chemically synthesized DNA. PCR fragments were assembled using the Gibson Assembly and then electroporated into host bacteria to reboot phages. (*B–J*) Rebooting of various phages from genomes assembled in vitro by electroporating into host bacteria. (*K*) Rebooting of phage TM4 from chemically synthesized DNA fragments by electroporating into *M. smegmatis* mc^{2}155. White arrows indicate plaques. M, Quick-Load 1 kb Plus DNA Ladder (NEB, Japan).

appropriate original host bacteria for all rebooting experiments described.

Rebooting of a Mycophage Controlled by a Chemically Synthesized Genome Assembled In Vitro.

We generated mycophage TM4 (52,797 bp) (18) from six chemically synthesized DNA fragments (A–F) based on its public sequence data (TM4: NC_003387). The fragments were amplified to exhibit an overlap of 28–38 bp with adjacent parts, purified, and then circularized in vitro (Fig. 1K). The assembled genome was installed into *M. smegmatis* mc^{2}155. The functional synthetic TM4—controlled by a chemically synthesized genome—was rebooted (Fig. 1K). The whole synthetic TM4 genome was read by MiSeq and confirmed to be 100% identical to the referenced TM4 (Fig. 1K), demonstrating successful phage rebooting from digitized genome sequence information.

Creating Engineered Phages.

To evaluate the utility of our phage rebooting platform, we subsequently created engineered phages. P22 is a temperate *Salmonella* phage, and its lysogenic state is stably maintained by the C2 repressor (19, 20). We designed a lytic P22 phage (P22$_{\Delta c2}$) by deleting the *c2* repressor gene (Fig. 2A). The designed DNA fragments of phage P22$_{\Delta c2}$ were assembled in vitro, and the assembled circular P22$_{\Delta c2}$ genome was installed into *S.* Typhimurium LT2 for rebooting (Fig. 2B). The deletion of the *c2* gene in the synthetic P22$_{\Delta c2}$ was confirmed using PCR

(Fig. 2C). The genetically engineered P22$_{\Delta c2}$ was confirmed to be constitutively lytic, forming clear plaques (Fig. 2D), whereas the parental P22 exhibited turbid plaques. These results indicated that our in vitro engineering strategy efficiently converted P22 from a temperate to a virulent state.

We next aimed to modulate the host range of the phage T3 by swapping its host determinant using an in vitro assembly strategy. A previous study (5) reported that T3 cannot infect *E. coli* strain BW25113, whereas T7 can (in a T7 tail fiber–dependent manner). Therefore, we aimed to design a T3 genome replacing the T3 tail fiber gene with that of T7 (Fig. 2E). The resulting genome fragments were amplified and assembled in vitro and then installed into *E. coli* (Fig. 2F). As shown in Fig. 2G, the resulting synthetic T3 with a T7 tail fiber (T3$_{T7(gp17)}$) infected the nonpermissive *E. coli* strain BW25113 in a manner similar to that of T7, whereas the parental WT T3 could not infect this strain.

Furthermore, we engineered the mycophage D29 with a genetic payload. We attempted to add the NanoLuc (Nluc) gene (21). To consider the packaging capacity of D29, we designed the genome by replacing the nonessential genes *72* and *73* (D29 genome coordinates 45,387–45,859 also known as gene *82/71*) (22) with P$_{hsp60}$-Nluc (Fig. 2H). The designed genome was amplified from the D29 genome and pMVhsp-Nluc, assembled in vitro, and then installed into *M. smegmatis* mc^{2}155 to reboot the engineered mycophage D29$_{\Delta 72-73::Nluc}$ (Fig. 2I). The deletion of genes *72 and 73* and insertion of Nluc gene were confirmed using PCR (Fig. 2J).
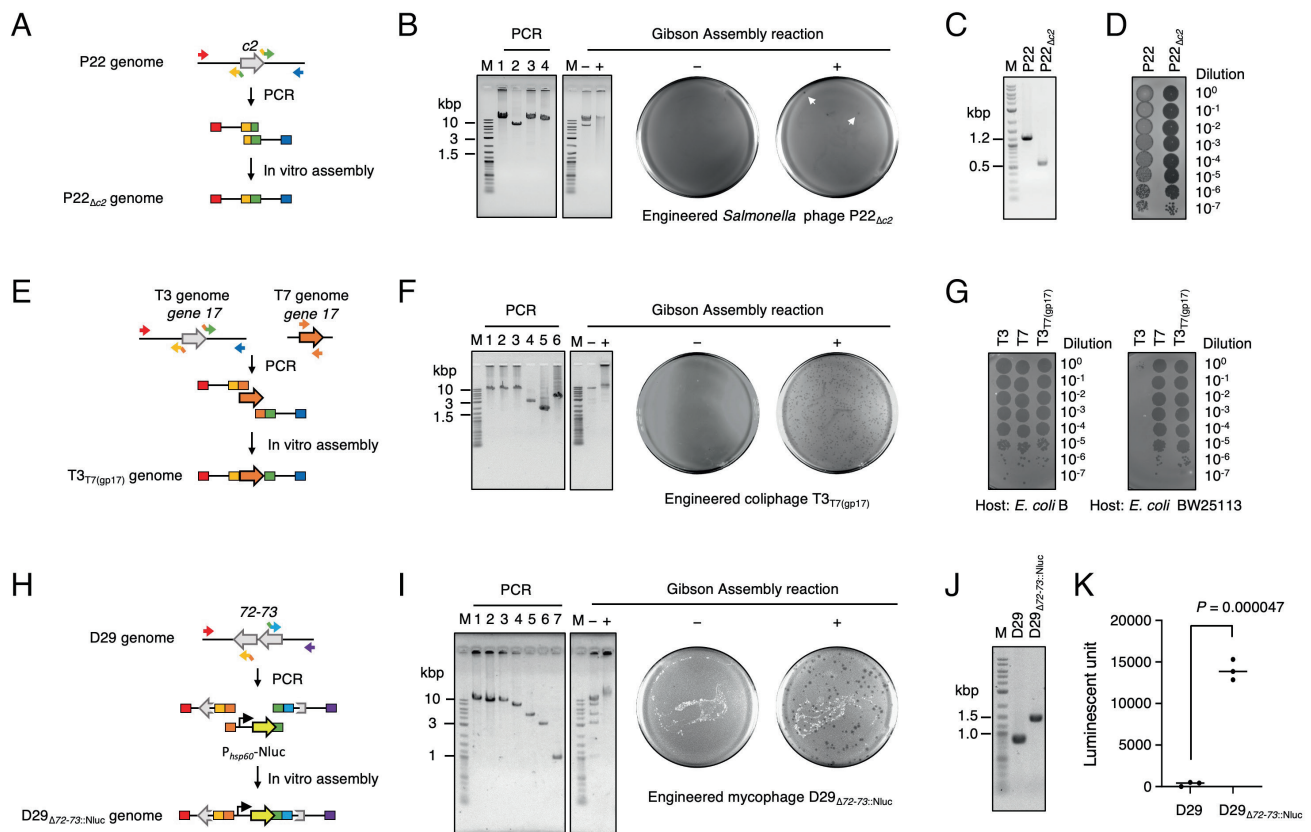

Fig. 2. Rebooting of engineered phages from designer genomes assembled in vitro. (*A–D*) Rebooting of engineered P22 from in vitro–assembled genomes. (*A*) Overview of P22 genome engineering. (*B*) DNA fragments amplified from the P22 genome were assembled and installed into *Salmonella* LT2. White arrows indicate plaques. (*C*) Confirmation of P22Δ*c2* genome structure using PCR. (*D*) Plaque formation assay of WT P22 and P22$_{\Delta c2}$. (*E–G*) Rebooting of synthetic T3 with T7 tail fiber, T3$_{T7(gp17)}$, from genomes assembled in vitro. (*E*) Overview of T3 genome engineering. (*F*) DNA fragments amplified from the T3 and T7 genomes were assembled and installed into *E. coli* 10G. (*G*) Plaque formation assays to confirm the host range expansion of T3$_{T7(gp17)}$. (*H–K*) Rebooting of engineered mycophage D29 from genomes assembled in vitro. (*H*) Overview of D29 genome engineering. (*I*) PCR fragments amplified from the D29 and P$_{hsp60}$-Nluc fragment were assembled and installed into *M. smegmatis* mc^{2}155. (*J*) Confirmation of D29$_{\Delta 72-73::Nluc}$ genome structure using PCR. (*K*) Evaluation of Nluc expression from D29$_{\Delta 72-73::Nluc}$ (*n* = 3). M, Quick-Load 1 kb Plus DNA Ladder (NEB, Japan).

D29$_{\Delta 72\text{-}73::\text{Nluc}}$ expressed the intended genetic payload and showed luminescent signal depending on the Nluc activity (Fig. 2K).

Cell-Free Engineering and Cell-Free Rebooting of Phage Genomes. As an additional study, we coupled in vitro genome assembly with a cell-free TXTL system (Fig. 3A). The T7 genome was assembled in vitro and employed in a cell-free TXTL system to reboot the phage particles. We found that $2.37 \pm 0.12 \times 10^5$ plaque-forming units (PFUs) (*n* = 4) of T7 phage per μg of in vitro–assembled genome were present on the *E. coli* 10G lawn (*SI Appendix*, Fig. S2), indicating that functional T7 phages were successfully designed, assembled, and rebooted without living bacterial cells. Furthermore, we generated a genetically modified synthetic phage, T7$_{lacZ}$, under cell-free conditions. Considering the findings that T7 can package its genome ranging from 85% (33,946 bp) to 103% (41,135 bp) into head particles (12), we inserted 3,092 bp of the fragment containing the *lacZ* gene and Shine–Dalgarno sequence into the WT T7 genome and deleted or truncated the nonessential genes (*0.4, 0.5, 0.6AB, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7,* and *1.8*) in a single step, such that the size of the designed genome did not exceed the packaging capacity (Fig. 3B) (12, 23). The T7$_{lacZ}$ genome was synthesized via in vitro amplification and assembly of seven DNA fragments, and then, its phage particle was rebooted using the cell-free TXTL system (Fig. 3C). We found that $2.90 \pm 0.07 \times 10^3$ PFUs/μg (*n* = 3) of T7$_{lacZ}$ phage were rebooted on the *E. coli* 10G lawn. 10-fold serially diluted T7$_{lacZ}$ lysate was spotted on *E. coli* 10G grown on lysogeny broth (LB) agar plates containing X-gal. T7$_{lacZ}$ formed clear blue plaques with a killing efficiency comparable to that of WT T7 (Fig. 3D).

Creation of Biologically Contained Bacteriophages. Although engineered phages are useful for proof of concept (POC) and fundamental studies, their potential to escape into the natural environment after application has created a need for containment strategies. Of particular interest to the field is the engineering of biocontained phages that replicate only under specific conditions, e.g., in the presence of a secondary drug. However, chemical strategies are ineffective for containment because the mutation rates of engineered phages, while low, are never zero, resulting in the escape of mutants. Regardless of the discussion as to whether viruses (including phages) are living organisms, phages are biological entities with genome and can proliferate in a host bacterium. Therefore, any embedded biocontainment system must be extremely robust to prevent the release of even a small fraction of engineered phages, which could grow to dominate the ecosystem. Here, we demonstrate two rational methods to generate biocontained phages.

The first is a knowledge-based approach for creating synthetic transducing particles. The phage T7 was chosen as a well-characterized model for the POC study. The T7 machinery recognizes a packaging signal sequence (Pac) to store or "package" its genome into a T7 head particle. The Pac is composed of PacB and PacC. Terminase small subunit gp18 recognizes PacB in concatemeric genome and interacts with the large subunit gp19–prohead complex to translocate DNA into phage head. After headful packaging, gp19 cleaves at PacC in concatemeric T7 phage genome, resulting in the maturation of T7 head (24–26). The workflow for creating synthetic transducing particles is shown in *SI Appendix*, Fig. S3A. First, we designed and assembled a synthetic T7 phage genome without Pac using in vitro genome assembly. Next, a plasmid carrying the Pac and "your favorite gene" (*yfg*) was constructed and introduced into *E. coli*. T7-based synthetic transducing particles (T7$_{\Delta Pac}$) were created by electroporating the genome into *E. coli* cells harboring the plasmid. In the cell, the genome without Pac produced progeny virions and packaged the plasmid with Pac into head particles, resulting in T7$_{\Delta Pac\text{-}yfg}$. Using this approach, we
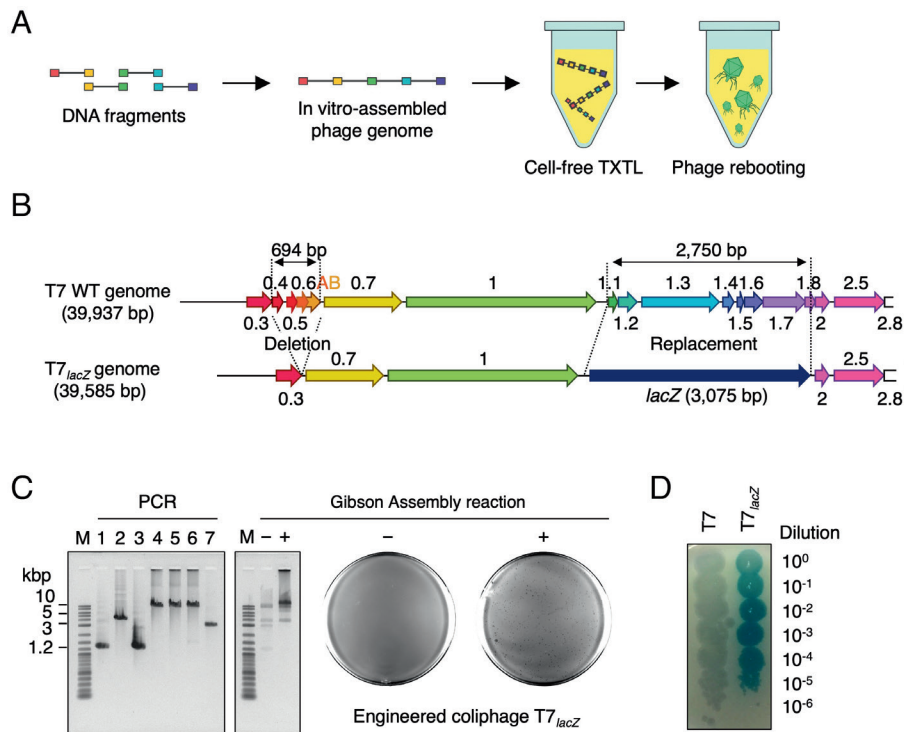

Fig. 3. Cell-free engineering and cell-free rebooting of phages. (*A*) Workflow for the cell-free rebooting of phages from genomes assembled in vitro. Phage genome fragments were amplified and then assembled via the Gibson Assembly. Phages were produced in a cell-free TXTL system from a genome assembled in vitro. (*B*) Design of synthetic T7$_{lacZ}$ genome. (*C*) Rebooting of synthetic T7$_{lacZ}$ from a genome assembled in vitro in a cell-free TXTL system. (*D*) Plaque formation assay on an X-gal plate to confirm *lacZ* expression from synthetic T7$_{lacZ}$ genome. M, Quick-Load 1 kb Plus DNA Ladder (NEB, Japan).

generated T7$_{\Delta Pac\text{-}lacZ}$, which packaged a plasmid carrying *lacZ*. Lysate was mixed with *lacZ*-deficient *E. coli* and plated on LB plates containing X-gal. As a result, 1.6×10^4 ($\pm 3.1 \times 10^3$) CFUs/mL T7$_{\Delta Pac\text{-}lacZ}$ were produced, and all emerged colonies appeared blue (*SI Appendix*, Fig. S3B). T7$_{\Delta Pac\text{-}lacZ}$ did not produce plaques on the bacterial lawn (*SI Appendix*, Fig. S3C), indicating biological containment. Additionally, we investigated whether our platform was able to create a T7$_{\Delta Pac}$-loaded CRISPR–Cas system. We designed the system to sense and cut a chloramphenicol resistance gene, *cat*, for sequence-specific killing of target bacteria. Using the approach described for T7$_{\Delta Pac\text{-}lacZ}$, we prepared a biocontained T7$_{\Delta Pac\text{-}CRISPR\text{-}cat}$. As shown in *SI Appendix*, Fig. S3D, T7$_{\Delta Pac\text{-}CRISPR\text{-}cat}$ killed *E. coli* B $\Delta lacZ$::*cat*, in which *lacZ* was replaced by *cat* but not the parent *E. coli*. Although this approach requires a deep understanding of phage packaging mechanisms, it allows phages to be easily converted into programmable transducing particles carrying desired payloads.

The second method involves deleting an essential structural gene from the phage genome. To create synthetic biocontained phages that can infect and kill target bacteria while remaining nonproliferable, a phage genome lacking virion gene(s) was designed and introduced into *E. coli* carrying a plasmid expressing the corresponding gene(s) (Fig. 4A). Specifically, we constructed a synthetic T7 phage genome without the genes *10AB*, which encodes major and minor capsids comprising the head, and installed them in *E. coli* expressing the genes (Fig. 4 *B* and *C*). In the cells, T7 virions were produced by the genome, and head particles were expressed from the plasmid, thus enabling the creation of the phenotypically functional but genetically head-deficient T7$_{\Delta head}$ (Fig. 4D). When 10-fold serially diluted T7$_{\Delta head}$ lysates were spotted on a lawn of *E. coli* expressing *10AB*, they formed plaques at an efficiency comparable to that of WT T7. In contrast, T7$_{\Delta head}$ did not form plaques on *E. coli* that did not express *10AB* (Fig. 4E). We considered that the cell lysis observed at a high-density spot represented a typical lysis from without (LO) phenomenon because individual plaques were not formed. Next, we aimed to confirm whether biocontained phages expressing exogenous genetic payloads could be created by deleting other virions. We constructed a synthetic T7 genome without tail genes through the deletion of the adaptor gene *11* and the nozzle gene *12*, as well as the replacement of the tail fiber gene *17* with *lacZ* (*SI Appendix*, Fig. S4A). Using the same approach used for T7$_{\Delta head}$, we created T7$_{\Delta tail\text{::}lacZ}$ (*SI Appendix*, Fig. S4B) and confirmed its genome structure (*SI Appendix*, Fig. S4C) and functionality. As shown in *SI Appendix*, Fig. S4D, T7$_{\Delta tail\text{::}lacZ}$ formed clear blue plaques on *E. coli* expressing tail genes at an efficiency comparable to that of WT T7. In contrast, T7$_{\Delta tail\text{::}lacZ}$ did not form plaques on *E. coli* that did not express the genes but formed blue LO. These results suggest that deleting a virion gene induced the loss of phage proliferation, forming biocontained phages.

Biocontained Phages Rescue Mice from Lethal Sepsis. To further investigate cell-free engineering and rebooting, and to confirm
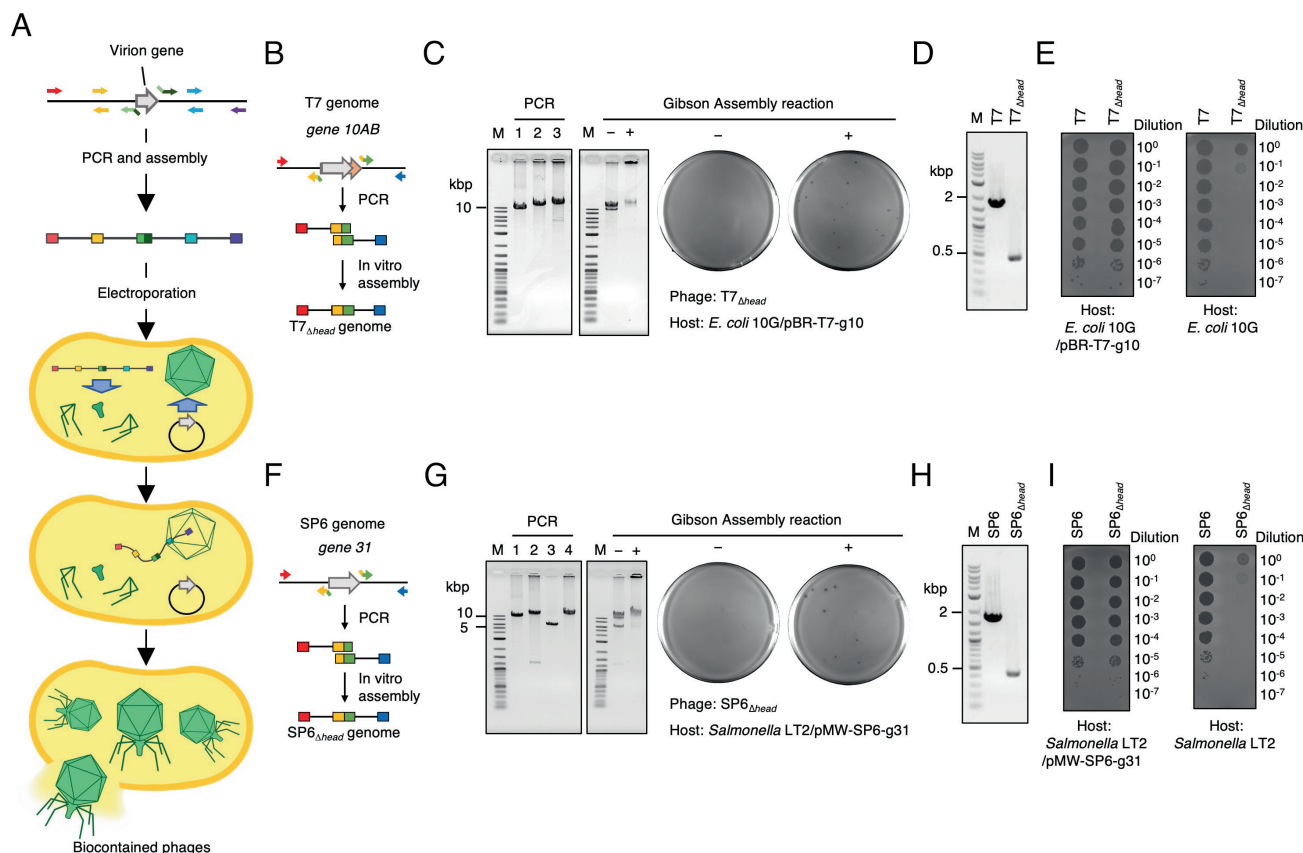

Fig. 4. Creation of biocontained phages. (*A*) Schematic illustrating the workflow for creating biocontained phages. A phage genome without a virion gene was assembled and introduced into host bacteria harboring a plasmid carrying the corresponding gene. A synthetic phage genome lacking the virion gene produced progeny phages because the virion protein was supplied from the plasmid. Biocontained progeny phages were released from lysed cells. (*B–I*) Creation of T7$_{\Delta head}$ and SP6$_{\Delta head}$. (*B, F*) Overview of phage genome engineering. (*C, G*) PCR-amplified DNA fragments were assembled and installed into host bacteria expressing the capsid gene. (*D, H*) Confirmation of the deletion of head gene(s) in biocontained phages using PCR. (*E, I*) Plaque formation assays. M, Quick-Load 1 kb Plus DNA Ladder (NEB, Japan).

the usefulness of biocontained phages for practical applications, we created the nonreplicative *Salmonella* phage SP6 for phage therapy experiments. We constructed the SP6 phage genome without gene *31*, which encodes a capsid protein. The genome was installed in the *S*. Typhimurium strain LT2 expressing gene *31* (Fig. 4 *F* and *G*). The deletion of the gene *31* in the synthetic SP6$_{\Delta head}$ was confirmed using PCR (Fig. 4*H*). The resulting high-titer SP6$_{\Delta head}$ lysate was serially diluted 10-fold and spotted on a lawn of parental or gene *31*–expressing LT2. SP6$_{\Delta head}$ formed plaques on a lawn of LT2 expressing gene *31* but not on the parental LT2 (Fig. 4*I*).

Next, we analyzed the bactericidal activity of SP6$_{\Delta head}$ against LT2. SP6$_{\Delta head}$ weakly but significantly inhibited the growth of LT2 at multiplicity of infection (MOI) 1 (Fig. 5*A*). The killing efficiency was higher at MOI 10; however, the efficiency at MOI 100 was almost the same as that at MOI 10. This tendency was similar to that observed for WT SP6. We also examined the titer or PFU every 30 min in the killing assays (Fig. 5*B*). SP6$_{\Delta head}$

showed no increase in PFUs during these assays, indicating biological containment.

To confirm whether nonreplicative biocontained phages can be used for phage therapy, SP6$_{\Delta head}$ was tested against a murine *S*. Typhimurium LT2-induced sepsis model. 6-wk-old C57BL/6 female mice were intraperitoneally infected with 10^5 CFUs of LT2. After 30 min, 5×10^7 PFUs of WT SP6, SP6$_{\Delta head}$, or LB was intravenously injected into the mice (Fig. 5*C*). None of the control mice survived for more than 3 d. In contrast, the survival rate of mice treated with SP6$_{\Delta head}$ was 62.5% after 6 d. There was no statistical significance between WT SP6 and SP6$_{\Delta head}$ ($P = 0.3593$). These results indicated that nonreplicative biocontained phages efficiently killed target bacteria and significantly improved the survival rate of mice with lethal sepsis as well as WT SP6. Importantly, SP6$_{\Delta head}$ collected from treated mice did not form plaques on lawns of *S*. Typhimurium LT2 but formed plaques on lawns of LT2 expressing gene *31*. These results indicated that the phages were biocontained and active when used in vivo.
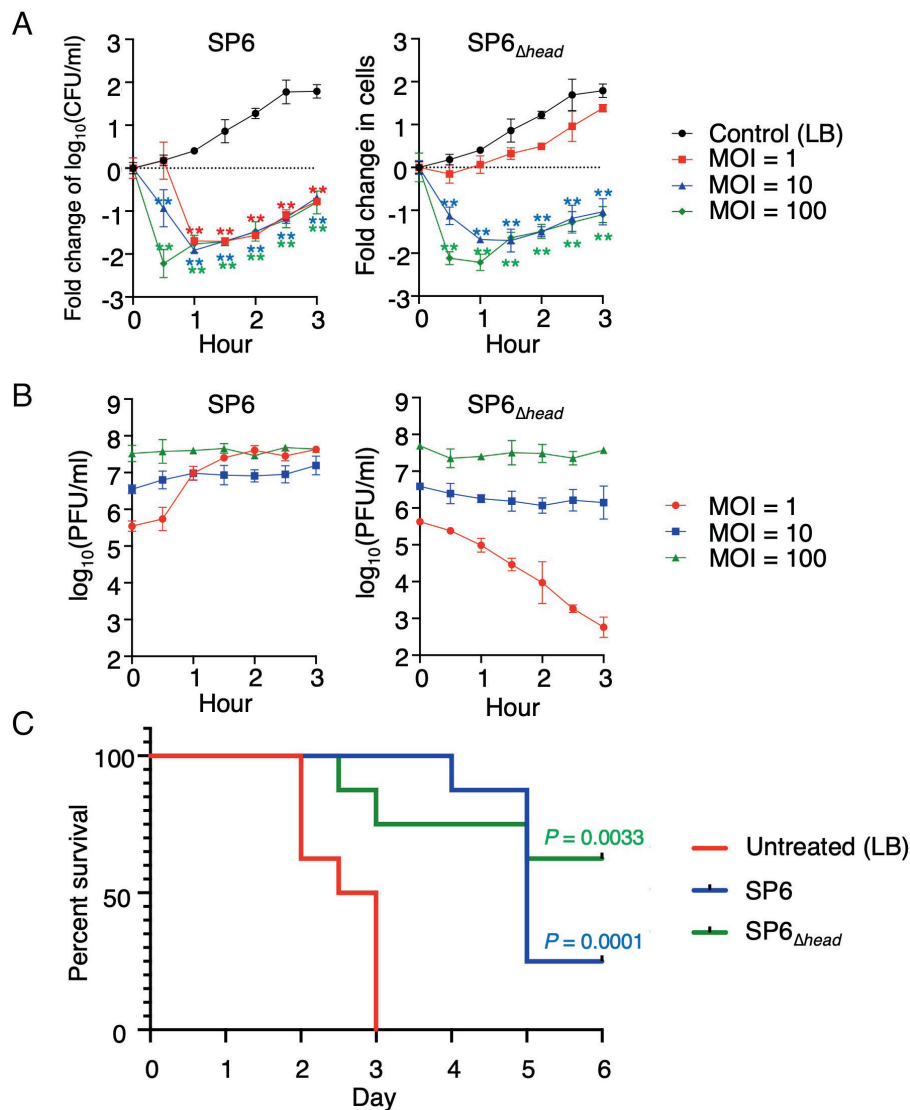

Fig. 5. Evaluation of containment and bactericidal activity of biocontained phages in vitro and in vivo. (*A*) Killing curves of *Salmonella* LT2 treated with WT SP6 or SP6$_{\Delta head}$, and (*B*) changes in the amount of WT SP6 or SP6$_{\Delta head}$. PFU was calculated from the number of plaques on a lawn of LT2 harboring pMW-SP6-g31. The data are presented as the mean of three independent experiments; error bars represent SD. * and ** indicate *P* values of < 0.05 and < 0.01, respectively, as calculated using the Student *t* test between the control and MOI = 1, 10, or 100. (*C*) Phage therapy experiment. Treatment of murine sepsis model using WT SP6 or SP6$_{\Delta head}$. *P* values were calculated by performing a log-rank test between control and WT SP6 or SP6$_{\Delta head}$.

Discussion

In this study, we demonstrated the development of natural and designer phages infecting gram-negative bacteria and acid-fast mycobacteria through in vitro genome assembly. To date, only phages infecting gram-positive bacteria have been created from in vitro genome assembly using Kilcher's synthetic platform (11). Such applications in phages infecting gram-negative bacteria have been limited to coliphages ΦX174 (10) and T7 (27). In the present study, we showed successful rebooting of *Salmonella*, *Pseudomonas*, and mycophages, in addition to the coliphages, from in vitro genome assembly. We also created the functional mycophage TM4 from chemically synthesized DNA based on sequence information in a cell-free environment. Our results reveal a platform that is broadly applicable for phage engineering, opening a possibility to create fully tailor-made phages for various applications.

We performed electroporation to generate most of the phages used in this study. However, it is extremely difficult to reboot functional phages using host bacteria with low transformation efficiency. In this case, the cell-free TXTL system was a powerful tool. The cell-free rebooting of coliphage MS2, ΦX174, T7, and T4 using a phage genome has been reported (28–30). Methods for preparing a TXTL system from bacteria such as *E. coli*, *Streptomyces* spp., *P. putida*, and *Vibrio natriegens* have also been reported (28, 31–35) but are inadequate to accommodate cell-free creation of other phages. Although *E. coli* shows relatively high transformation efficiency, as a POC, we demonstrated the cell-free rebooting of a designer phage by using a TXTL system. Rebooting of phages through a TXTL system may allow for the preparation of phage lysates on demand in hospitals, and/or for industrial mass production of phages, as it enables functional phages to proliferate without host bacteria. Ultimately, a universal TXTL is needed to facilitate synthetic phage development in the future.

We developed two methods to create biocontained phages and limit the escape of synthetic phages into the environment. The first, based on synthetic transducing particles, involves a mutant phage genome deprived of its packaging signal and a plasmid DNA to complement it. While this technology is functional and can be utilized for bacteria detection, sterilization, and gene manipulation, the number of transducing particles produced from a rebooting reaction is completely dependent on the efficiency of electroporation. This is a major limitation of this strategy, especially for the phage therapy, which needs high-titer phage lysates. The second, based on biologically contained phages, was easier to convert than that of the synthetic transducing particles. The biocontained phages can be rebooted by installing a modified phage genome without a virion gene into bacteria expressing the corresponding gene, and high-titer phage lysates can be prepared on the strain. In addition, by replacing a virion gene with *yfg*, biocontained phages can be customized for functions such as bacteria detection, increased killing efficiency, and toxin neutralization. Regulatory approval of engineered phages is expected to be more complex than that of WT phages, but biological containment methods will facilitate the transition to clinical applications.

In this study, in vitro genome assembly was performed to improve understanding of phage biology and expand the possible clinical applications. The methods for phage engineering presented here will contribute significantly to the development of specific, targeted phage for clinical use.

Materials and Methods

E. coli, *S.* Typhimurium LT2, and *P. putida* were grown in LB (1% Bacto Tryptone [BD], 0.5% Bacto Yeast Extract [BD], and 1% NaCl [Nacalai Tesque]) at 30°C or 37°C. *M. smegmatis* mc²155 was cultured in Middlebrook 7H9 liquid medium (BD) supplemented with Middlebrook albumin dextrose catalase (ADC) (BD) and 0.05% Tween 80 (Nacalai Tesque) at 37°C. Detailed methods for construction of transcomplementation plasmids (*SI Appendix*, Fig. S5), generation of synthetic TM4 genome from chemically synthesized DNA fragments, construction of the *E. coli* B Δ*lacZ* mutant, preparation of phage lysates, extraction of phage genomes, in vitro genome assembly, preparation of electrocompetent cells, rebooting of phages in the host bacterium, rebooting of phages in a cell-free TXTL system, determination of PFUs, assay for bacterial killing, phage therapy for murine sepsis, genome sequencing, and computational analysis and transduction are described in the *SI Appendix, Materials and Methods*. The phage and bacterial strains used in this study are shown in *SI Appendix*, Table S1. The plasmids and primers used in this study are listed in *SI Appendix*, Tables S2 and S3. PCR conditions and adjacent homologous sequences for in vitro assembly are shown in SI Appendix, Tables S4 and S5. All WT and synthetic phages used in this study were sequenced (*SI Appendix*, Table S6).

Data, Materials, and Software Availability. All study data are included in the article and/or *SI Appendix*.

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