Harnessing stepping-stone hosts to engineer, select, and reboot synthetic bacteriophages in one pot

Graphical abstract

Highlights

- A stepping-stone host-assisted phage engineering framework is established
- Genome assembly, editing, and rebooting are achieved in a single cell type
- The stepping-stone supports cross-genus and cross-order phage rebooting
- Rebooting outcome is associated with phage gene number and polymerase availability

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In brief

Cheng et al. develop a widely applicable framework, SHAPE, that utilizes user-friendly bacteria as stepping-stone hosts to complete multiplex manipulations of phage engineering in one pot. This framework makes tailored phages more approachable and has potential in phage therapy.
Report
Harnessing stepping-stone hosts to engineer, select, and reboot synthetic bacteriophages in one pot

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SUMMARY
Advances in synthetic genomics have led to a great demand for genetic manipulation. Trimming any process to simplify and accelerate streamlining of genetic code into life holds great promise for synthesizing and studying organisms. Here, we develop a simple but powerful stepping-stone strategy to promote genome refactoring of viruses in one pot, validated by successful cross-genus and cross-order rebooting of 90 phages infecting 4 orders of popular pathogens. Genomic sequencing suggests that rebooting outcome is associated with gene number and DNA polymerase availability within phage genomes. We integrate recombinering, screening, and rebooting processes in one pot and demonstrate genome assembly and genome editing of phages by stepping-stone hosts in an efficient and economic manner. Under this framework, in vitro assembly, yeast-based assembly, or genetic manipulation of native hosts are not required. As additional stepping-stone hosts are being developed, this framework will open doors for synthetic phages targeting more pathogens and commensals.

MOTIVATION
Synthetic genomics, a powerful approach to understand life and develop therapeutic agents, includes two crucial stages: genome synthesis and rebooting genome into life, both of which can be troublesome. For synthetic viruses, for example, engineering and rebooting are divided into separate steps and often involve multiple hosts or platforms, which is rather laborious and costly. Simplifying and integrating the two stages is a compelling approach to synthesize genomes easier and faster. We aimed to develop an alternative stepping-stone strategy to achieve genome refactoring of viruses in one pot in an efficient and economic manner, the stepping-stone host-assisted phage engineering (SHAPE) framework.
INTRODUCTION

Advances in synthetic biology are a boon for a deeper understanding of life and exploring novel therapeutic agents for diseases (Coradini et al., 2020; Elowitz and Lim, 2010). Efforts to synthesize genomes have spawned a wide range of synthetic species from viruses to bacteria and, more recently, complex eukaryotic chromosomes (Cello et al., 2002; Chan et al., 2005; Fredens et al., 2019; Gibson et al., 2010; Oldfield et al., 2017; Shen et al., 2017; Smith et al., 2003; van der Sloot and Tyers, 2017). Synthetic genomics usually require two crucial stages: genome synthesis and rebooting DNA into life (Coradini et al., 2020). De novo synthesis of genomes usually required a bottom-up approach involving cumbersome, expensive, and hierarchical assembly from smaller to larger fragments in vitro or in vivo (Baker, 2011; Chan et al., 2005; Fredens et al., 2019; Gibson et al., 2010; Oldfield et al., 2017; Shen et al., 2017; Smith et al., 2003; van der Sloot and Tyers, 2017). Previous studies have relied heavily on the yeast Saccharomyces cerevisiae to assemble chunks up to 10 kb or larger (Ando et al., 2015; Gibson et al., 2010; Oldfield et al., 2017; Shen et al., 2017; Thi Nhu Thao et al., 2020; van der Sloot and Tyers, 2017; Vashee et al., 2017). Bringing synthetic genomes into life is challenging. For synthetic viruses, for example, to reboot viral DNA into life, previous studies introduced the synthetic genome into natural hosts (Chan et al., 2005; Fredens et al., 2019; Gibson et al., 2010; Oldfield et al., 2017; Shen et al., 2017; Thi Nhu Thao et al., 2020; van der Sloot and Tyers, 2017; Vashee et al., 2017). However, this approach is hampered because many natural hosts are hard to manipulate. Simplifying the two stages is a compelling approach to synthesize genomes easier and faster.

Bacteriophages are fascinating organisms that play a key role in genetics and molecular biology and were crucial in establishing the central dogma of molecular biology because of their highly compact genomes and less complicated biological processes. More recently, engineered phages have emerged as versatile biological agents that efficiently detect and control multidrug-resistant (MDR) bacteria because of their advantages in tunable host range, killing efficiency, toxin expression, and so on (Ando et al., 2015; Ciceri et al., 2014; Dedrick et al., 2019; Kilcher et al., 2018; Lemire et al., 2018; Lu and Collins, 2007, 2009; Yehl et al., 2019; Yosef et al., 2015). A variety of strategies have been proposed for phage engineering (Table S1; Kilcher and Loessner, 2019). These strategies fall into four categories: (1) genome editing and rebooting in native hosts, (2) genome assembly in yeast and rebooting in native or non-native hosts, (3) genome assembly in vitro and rebooting in native or cross-genus hosts, and (4) genome assembly in vitro and rebooting in a cell-free system. Representatives of strategy (1) are Bacteriophage Recombinase of Electroporated DNA (BRED) and BRED combined with CRISPR-Cas9 (CRISPY-BRED), which transform target DNA into native hosts by electroporation, accelerate DNA recombination and/or promote counterselection of recombinant phage genomes via plasmid-carried exogenous systems, and reboot engineered genomes in native hosts (Marinelli et al., 2008; Wettzel et al., 2021). Representative of strategy (2) is the yeast platform, which achieved genome rebooting of T7-family phages in Escherichia coli 10G (Ando et al., 2015; Latka et al., 2021). Representative of strategy (3) is the L-form bacteria platform, which successfully rebooted 9 Listeria phages, 2 Bacillus phages, and 2 Staphylococcus aureus phages in Listeria L-form cells (Kilcher et al., 2018; Meile et al., 2020). Representative of strategy (4) is the cell-free transcription-translation (TX-TL) system, which has been applied successfully to genome rebooting of 4 coliphages (MS2, phiX174, T7, and T4) (Garamella et al., 2016; Noireaux and Liu, 2020; Rustad et al., 2018; Shin et al., 2012). Strategy 1 has been used widely in phage engineering and applied successfully to host strains with a well-developed genetic manipulation system, but it is challenging when the host strain is MDR or biofilm productive. The applicable scales of strategies (2)–(4) remain to be fully evaluated because of the limited number of phages tested.

Here we take this a step further to expand the application of non-native hosts as inspired by strategies (2) and (3). Motivated by the term “stepping-stone” in evolutionary biology and virology, which is sometimes used to describe the notion of a virus taking advantage of an intermediate host to reach the final host, we adapted the term and upgraded the stepping-stone host-assisted strategy to a simple but powerful framework to promote virus synthesis in one pot. As a proof of concept, we build the first versions of stepping-stone hosts and tested cross-genus and cross-order rebooting of 126 T7/ non-T7-family phages that originally infect common clinical MDR strains of Klebsiella pneumoniae, Salmonella enterica, Pseudomonas aeruginosa, and Acinetobacter baumannii. We also find underlying factors correlating with whether these phages are successfully rebooted by genomic sequencing of all 126 phages and analyzing “genotype-phenotype” association. Application of the stepping-stone host-assisted phage engineering (SHAPE) framework is highlighted by achieving phage engineering, including genome assembly and genome editing, and rebooting in one pot. SHAPE is a simple, efficient, and broadly applicable framework to build synthetic phages. This work provides insights into discovering more stepping-stone hosts to expand the application of SHAPE to the general field of synthetic genomics, where process improvement must be done to promote simple and fast streamlining of genetic code into life.

RESULTS

Construction and optimization of the stepping-stone host

First we tried an easy-to-manipulate strain, E. coli DH10B, as the first stepping-stone host. The laboratory E. coli K-12 strain DH10B is a MC1061 derivative, specifically designed for higher-efficiency cloning, and carries mutations that embrace large DNA uptake, enhance DNA stability, and protect foreign DNA from restriction systems (Durfee et al., 2008). To test the feasibility of the stepping-stone host DH10B, we first applied DH10B to reboot phage CPB0329, a K. pneumoniae phage isolated against clinical MDR strains. CPB0329 was chosen because it shares moderate homology with coliphage T7 (coverage, 65%; identity, 73.2%), and the T7 phage life
cycle is host independent (Qimron et al., 2010). As expected, the stepping-stone host DH10B successfully rebooted T7-family K. pneumoniae phage CPB0329 with high efficiency (Figure S1).

It has been reported that some viruses carry their own tRNAs to compensate for the deviation between host tRNA composition and virus codon use and to attain higher fitness (Bailly-Bechet et al., 2007; Delesalle et al., 2016). This led us to ask whether supplementary tRNAs could help phage rebooting in a non-native host. To test this, we optimized the stepping-stone host by introducing pRARE, a plasmid encoding six rare tRNAs and four common tRNAs in E. coli (Umlauf et al., 2015). The rebooting efficiency of K. pneumoniae phage CPB0329 was around one order of magnitude higher in DH10B/pRARE than in DH10B (Figure 1A). We observed that the rebooting efficiency decreased in DH10B and DH10B/pRARE after 24 h of recovery time immediately after transformation. It is likely that the optimal recovery time is affected by the balance between phage protein expression and degradation.

**Cross-genus and cross-order rebooting of phages against MDR bacteria**

To examine the scope of applying DH10B as a stepping-stone host, we rebooted 126 bacteriophages from the T7 family and non-T7 family, which infect clinical MDR K. pneumoniae (Kp), S. enterica (Se), P. aeruginosa (Pa), and A. baumannii (Ab) (Figure S2). Kp and Se phages represent cross-genus rebooting (Escherichia » Klebsiella, Salmonella), whereas Pa and Ab phages represent cross-order rebooting (Enterobacterales » Pseudomonadales), and all phages were unable to infect DH10B. Our results showed that the stepping-stone host DH10B successfully rebooted 93.3% (28 of 30) Kp phages, 90% (28 of 31) Se phages, 52.3% (23 of 44) Pa phages, and 52.4% (11 of 21) Ab phages (Figure 1B). Overall, the success rate of cross-genus rebooting is higher than that of cross-order rebooting. Among the successfully rebooted phages, 77.8% do not belong to the T7 family, which is an improvement over prior research (Ando et al., 2015; Latka et al., 2021). All 44 Pa phages were isolated against the same type strain, PA01, but their rebooting outcome varied distinctly.

Only a handful of synthetic phages have been reported previously, and the generalization ability of the synthetic methods and the underlying factors were not clear. We explored the underlying factors affecting phage rebooting efficiency by genomic sequencing of all 126 tested phages and analyzing the association between genetic features and rebooting results (Figure 1C). To briefly summarize, the 126 tested phages are representative of K. pneumoniae, Siphoviridae, Podoviridae, a novel Ackermannviiridae family, as well as an unassigned family (Figure 1C). Their genomes range from 20,741–299,545 bp (Figure 1C), with a range of GC contents from 37.7%–64.6% (Figure S3B). Among all tested phages, 90 were successfully rebooted, including Myoviridae, Siphoviridae, Podoviridae, and an unassigned family (Figure S3A). Their genomes range from 20.7–156.8 kbp with 37.7%–64.6% GC content (Figures S3B and S3C). We analyzed the correlation between rebooting outcome and genes crucial for the phage life cycle, such as integrase, DNA polymerase, RNA polymerase, and tRNAs, via Fisher’s exact test (Table S2). We found that tRNA and integrase (indicating a lytic or temperate lifestyle) show insignificant correlation with rebooting outcome (tRNA, p = 0.09491; integrase, p = 0.1827). The availability of DNA polymerase instead of RNA polymerase showed significant correlation with rebooting outcome (DNA polymerase, p = 0.04362; RNA polymerase, p = 0.5257). We examined the correlation between rebooting outcome and genomic features, including genome size, gene number, average protein length, transcriptional orientation, and transcriptional strand switch (Table S2). We found that the gene number of a phage shows negative correlation with its rebooting outcome (p = 0.006151, r = –0.243), suggesting that phages with fewer genes rather than smaller genomes are more likely to be rebooted successfully within the stepping-stone host. These results demonstrated that the stepping-stone host represents a highly versatile strategy accommodating cross-genus and even cross-order rebooting of phages. We showed that larger-scale experimentation could help to elucidate the underlying phage biology and drive the generalizability of the framework.

**One-pot genome assembly and rebooting of synthetic phages**

With the success of phage rebooting in the stepping-stone host, we explored the potential of the stepping-stone host in phage genome engineering (Figure 2). In general, synthetic biology includes two sub-fields: de novo synthesis of an organism and re-engineering an existing organism (Wang et al., 2018). We first tested de novo genome synthesis in the stepping-stone host. Putting together multiple chunks using existing methods can be time consuming, troublesome, and expensive (Baker, 2011). However, the capability of assembling multiple large fragments in vivo, especially into the full-length genome of viruses, by bacteria has not been tested.

To perform in vivo assembly, we utilized the plasmid pKD46 carrying the λ-Red recombination system, which has been validated extensively in engineering the E. coli genome (Datsenko and Wanner, 2000). We first evaluated its function in DH10B by assembling a 3.2-kb plasmid from two DNA fragments with or without arabinose induction of the λ-Red recombination system. We found that the assembly efficiency of DH10B without induction was 1,000-fold lower than that with induction (Figure S4). Then, to demonstrate phage genome assembly, we dissected Kp phage CPB0260 into 4 fragments of ~9.5 kb, sharing 40- to 60-bp overlaps with each other to facilitate homologous recombination. The results showed that the stepping-stone host carrying a functional recombination system supports assembly and rebooting of phage CPB0260 from four synthetic segments (Figure 3A). Considering that the assembly efficiency is dramatically affected by the number of DNA fragments and the size of DNA (Huang et al., 2017), we tested synthesizing Kp phage CPB0174, 45,798 bp in length, from 5, 10, 11, and 12 fragments. Assembling and rebooting CPB0174 from 5 and 10 fragments succeeded, but synthesizing the phage from 11 fragments and more failed, suggesting that the current settings of SHAPE could support in vivo assembly of phages with up to 10 DNA fragments (Figures 3B and 3D). To demonstrate the ability of synthesizing larger genomes, we also
tried to assemble and reboot a Pa phage of over 92,000 bp, CPB0739, and succeeded (Figure 3C). Compared with previously reported in vitro genome assembly or vector-based genome assembly in yeast, followed by a separate step of rebooting, the SHAPE framework simplifies the phage engineering procedures and reduces labor and experiments costs. It also indicates the...
great potential of using bacteria to assemble large genome fragments for other synthetic organisms.

**Efficient phage engineering with genome payload and reduction**

To perform genome editing of a T7-family Kp phage, CPB0329, we first tried to insert an exogenous DspB module (Lu and Collins, 2007; Figure 4A) into the phage genome mediated by the λ-Red recombination system alone. However, only wild-type phages were detected by PCR verification of more than 20 single clones (Table S3). Inspired by previous studies, we then utilized the CRISPR-Cas9 system for counterselection (Schilling et al., 2018; Shen et al., 2018; Wetzel et al., 2021). pCas carrying the CRISPR-Cas9 system under control of a constitutive promoter was co-transformed into the stepping-stone host with pSgRNA carrying the single guide RNA (sgRNA) and DNA substrate for recombineering. pSgRNA supports flexible cloning of new DNA substrates and sgRNA sequences.

See also Figure S4 and Table S1.

Figure 2. Schematic the workflow of SHAPE
SHAPE has two functions: in vivo assembly and DNA editing. For in vivo assembly, plasmid pKD46 is transformed into the stepping-stone host. The synthetic DNA fragments are transformed into the stepping-stone host harboring plasmid pKD46, and de novo synthetic phages are produced by the stepping-stone host and amplified on a lawn of a natural host. For DNA editing, sgRNA and DNA substrates are cloned in the pN20 vector. The resulting pSgRNA is co-transformed with pCas into the stepping-stone host. The phage genome is transformed into the stepping-stone host harboring the two plasmids, and engineered phages are produced by the stepping-stone host and amplified on a lawn of a natural host.
The SHAPE platform could accomplish three molecular reactions in one pot: recombination of designed DNA substrates, negative selection of native phage genomes, and rebooting of viral particles from engineered phage genomes (Figure 2).

With λ-Red recombination and CRISPR-Cas9 systems, we tried to engineer the genome of CPB0329 with a payload (Figure 4A) and reduction by knocking out a non-essential ligase gene (Masamune et al., 1971) (Figure 4B). To ensure successful counterselection, we designed different sgRNAs for independent experiments. Distinct engineering efficiencies were observed among these sgRNAs (Table S3; Figure 4E), suggesting that the design of sgRNAs can be crucial for counterselection efficiency by SHAPE. Two non T7-family Kp phages, CPB0170 (46,784 bp, Siphoviridae) and CPB0171 (46,784 bp, Siphoviridae), were also engineered. In this case, a DspB module was inserted downstream of a major capsid gene within the CPB0170 genome or between two hypothetical genes with opposite transcriptional directions within the CPB0171 genome (Figures 4C and 4D). Again, we observed 90%–100% and 60%–100% engineering efficiency in the two cases, respectively (Figure 4E). In some cases, the engineering efficiency can be enhanced with an additional 15 h of recovery time after transformation (Table S3). These data illustrate the high efficiency of SHAPE in genome editing of T7- and non-T7-family phages.

**DISCUSSION**

We designed and implemented an easy and efficient framework: a stepping-stone strategy for synthetic phages. SHAPE incorporates a series of reactions in a single cell type and only requires the most widely used cloning techniques and laboratory conditions, which overcomes the limitation of native-host-based strategies (BRED or CRISPY-BRED) and simplifies the processes of
SHAPE powerfully complements current approaches. For example, employing the technique of in vitro genome assembly and rebooting in L-form bacteria seems to be a wise choice for phages targeting Gram-positive bacteria, particularly *Listeria*, *Bacillus*, and *Staphylococcus*, as demonstrated by Kilcher et al. (2018). As for phages targeting Gram-negative bacteria, three approaches can be chosen. If the native host bacteria are easy to manipulate and compatible recombineering systems are available, a native-host-based strategy would be a fair option; if the native host bacteria are easy to manipulate but no compatible recombineering systems are available, yeast/in vitro assembly and native-host-based rebooting are applicable. In other cases, the benefits of SHAPE could be overwhelming, especially for engineering phages targeting *Klebsiella*, *Salmonella*, *Pseudomonas*, and *Acinetobacter* phages, as verified in this study.

With development of more stepping-stone hosts, SHAPE could be applicable for phage engineering targeting more pathogens and commensals. An example is gut commensal bacteria, many of which play a key role in human chronic diseases such as inflammatory bowel disease (IBD), type II diabetes, and liver disease (Clemente et al., 2012). Most gut commensal bacteria are strictly anaerobic and require a special growth medium and environment. Massive expansion of gut bacteriophages identified *in silico* has created new capabilities to further investigate interactions among phages, gut bacteria, immunity, and disease (Benler et al., 2021; Camarillo-Guerrero et al., 2021; Devoto et al., 2021).
et al., 2019; Gregory et al., 2020; Nayfach et al., 2021; Yutin et al., 2018). But efforts to isolate gut bacteriophages have proven difficult, and only a limited number of gut phages have been isolated to date (Guerin et al., 2018, 2021; Hryckowian et al., 2020; Porter et al., 2020). In these cases, SHAPE might be an interesting option for building synthetic phages to treat chronic gut microbiome-related diseases (Dong et al., 2020; Duan et al., 2019; Kabwe et al., 2021; Yuan et al., 2019; Zheng et al., 2019).

De novo genome assembly is one of the bottlenecks of genome writing, although the progress of genome writing technologies have been witnessed (Chari and Church, 2017; Farzadfard et al., 2021). Compared with the dramatical rise in throughput and drop in cost of genome reading (sequencing), efforts to improve genome writing technologies are still required (Chari and Church, 2017; Wang et al., 2018). At present, it is still laborious and costly to perform large-scale genome synthesis. The SHAPE framework integrates multiple steps in one pot and provides insights into reducing the cost and labor of genome writing. With further standardization of the framework and advances in automation (Hilson et al., 2019; Hololwko et al., 2021), fewer modules and higher throughput can be realized in biofoundries to enable massively parallel construction of synthetic organisms.

Limitations of the study
The study presented version 1.0 of SHAPE, and there is room for updates of a few aspects. For instance, SHAPE’s efficiency is associated with transformation efficiency, which reflects the stepping-stone host’s ability to take up large phage genomes; therefore, in vitro circularization or spermidine treatment might be useful to improve the transformation efficiency of large DNA (Gosule and Schellman, 1976). Using a stepping-stone host with high transformation efficiency (e.g. Stellar-competent cells), could also be helpful. To some degree, the engineering efficiency of SHAPE is limited by the design of sgRNA. The recently developed near-Protospacer Adjacent Motif (PAM)-less CRISPR-Cas9 variants might be used in the next version of SHAE to avoid the NGG-PAM limitation of Cas9 targeting (Walton et al., 2020), which would provide higher flexibility in programming phages. Multiple DNA substrates and sgRNAs could be introduced in pSgRNA, enabling multi-site modifications in one step. Most importantly, diverse stepping-stone hosts must be validated to generalize application of the SHAPE framework in genome assembly, editing, and rebooting of random phages.

We did not demonstrate application of tailored phages for clinically relevant purposes in this study, but hopefully distinctive designs and applications of synthetic phages can be pursued among the readers of this paper. The study also did not investigate whether the genetic factors correlating with phage rebooting are actually the cause, and this can be done through rigorous experimental verification, including exogenous expression of genes in the stepping-stone host cells. To better understand the mechanisms of phage rebooting, more phage-host pairs can be tested, especially in non-native hosts. We believe that gathering “genotypic-phenotypic” mapping data via SHAPE application, by us and by the research community working with a wide variety of bacteria and phages, will provide a better understanding of phage biology. This, in turn, will drive the evolution of SHAPE and could eventually lead to universal application of this strategy by selecting stepping-stone hosts with rationale.

STAR METHODS
Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.crmeth.2022.100217.

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AUTHOR CONTRIBUTIONS
M.X. conceived the study. L.C. and M.X. designed the experiments. W.S., YayunW., X.H., S.Y., Y.M., Y.P., N.-K.W., and Y.L. isolated the bacteria and phages. L.C., Z.D., H.T., B.X., W.L., L.K., YunW., and Y.S. carried out all other experiments. L.C., W.S., B.X., and J.L. performed bioinformatics and computational analyses. L.C., Z.D., and M.X. interpreted the results and wrote the manuscript.

DECLARATION OF INTERESTS
The authors have filed a patent application.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Bacterial and virus strains** | | |
| *Escherichia coli* DH10B | Thermo Fisher Scientific | N/A |
| *Escherichia coli* DH10B/pSgRNA+pCas | This work | N/A |
| *Escherichia coli* DH10B/pRARE | This work | N/A |
| *Escherichia coli* DH10B/pRARE+pKD46 | This work | N/A |
| See Table S2 | This work | N/A |
| **Chemicals, peptides, and recombinant proteins** | | |
| Q5 High-Fidelity 2X Master Mix | NEB | M0494S |
| DNase I | SIGMA | Cat # DN25 |
| RNaseA | Invitrogen | Cat # 12091021 |
| Proteinase K | NEB | P8107S |
| BsaI-HFv2 | NEB | R3733S |
| BbsI-HF | NEB | R3539S |
| T4 DNA ligase | NEB | M0202S |
| L(+)-Arabinose | Sangon Biotech | Cat # A610071-0025 |
| Agarose | BIO ROAD | Cat # 1613101 |
| PEG8000 | SIGMA | P5413-2KG |
| 0.5M EDTA | INVITROGEN | Cat # AM9261 |
| SDS Solution | INVITROGEN | Cat # AM9820 |
| **Critical commercial assays** | | |
| AxyPrep DNA Gel Extraction Kit | AXYGEN | AP-GX-250 |
| Zymoclean Large Fragment DNA Recovery | ZYMO RESEARCH | Cat # 4045 |
| TIANprep Rapid Mini Plasmid Kit | TIANGEN | Cat # 4992192 |
| GENECLEAN Turbo Kit | MP Biomedicals | Cat # MP111102400 |
| Qubit™ dsDNA HS Assay Kit | INVITROGEN | Q32854 |
| Qubit™ dsDNA BR Assay Kit | INVITROGEN | Q32850 |
| **Oligonucleotides** | | |
| See Table S2 | This work | N/A |
| DspB module | *De novo* synthesized | WP_00546617.1 |
| **Recombinant DNA** | | |
| pSgRNA | This work | N/A |
| MX5001 | This work | N/A |
| MX5003 | This work | N/A |
| MX5004 | This work | N/A |
| MX5005 | This work | N/A |
| **Software and algorithms** | | |
| Snapgene v1.1.3 | Snapgene Software | https://www.snapgene.com/ |
| ImageJ | Schneider et al., 2012 | https://imagej.nih.gov/ij/ |
| CRISPick | Sanson et al., 2018 | https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design |
| sgRNAcas9 | Xie et al., 2014 | N/A |
| Fastp | Chen et al., 2018a | N/A |
| SOAPnuke | Chen et al., 2018b | https://github.com/BGI-flexlab/SOAPnuke |
| SPAdes v3.13.0 | Bankevich et al., 2012 | N/A |
| tRNAscan-SE v2.0.9 | Chan et al., 2021 | N/A |

(Continued on next page)
**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Minfeng Xiao (xiaominfeng@genomics.cn).

**Materials availability**
This study did not generate new unique reagents. Plasmids generated in this study are available from the lead contact with a completed material transfer agreement.

**Data and code availability**
- All data generated in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this work paper is available from the lead contact upon request.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

Kanamycin resistant and temperature sensitive plasmid pCas expresses Cas9 protein continuously and lambda Red recombinaseing proteins under the control of inducible arabinose promoter. Chloramphenicol resistant vector pSgRNA contains sgRNA and recombinaseing DNA substrates. Ampicillin resistant vector pKD46 only expressing lambda Red recombinaseing proteins under the control of inducible arabinose promoter has been described previously (Datsenko and Wanner, 2000). E. coli strain DH10B in this research was adopted as a stepping-stone host. DH10B/pRARE was constructed via transforming chloramphenicol resistant plasmid pRARE (Umlauf et al., 2015) that encodes six rare tRNAs into DH10B. DH10B harboring pCas was cultured in LB broth with 50 μg mL⁻¹ kanamycin at 30°C, and DH10B harboring pN20 or pSgRNA was cultured in LB broth with 25 μg mL⁻¹ chloramphenicol at 37°C. DH10B harboring pCas and pSgRNA was cultured in LB broth with 50 μg mL⁻¹ kanamycin and 25 μg mL⁻¹ chloramphenicol at 30°C. DH10B/pRARE harboring pKD46 was cultured in LB broth with 100 μg mL⁻¹ ampicillin and 25 μg mL⁻¹ chloramphenicol at 30°C. All other strains were cultured in LB broth at 37°C.

**METHOD DETAILS**

**Isolation and sequencing of phages**

*Isolation of natural phages.* Phages used in this study were isolated from sewage of 14 cities in China with a wide range of bacterial strains (Table S2). 50 mL of sewage water was centrifuged at 5,000 g for 20 min at 4°C to and the supernatant was passed through a 0.45 μm membrane filter (VWR). 40 mL of filtered sewage was co-incubated with 500 μL of overnight bacterial culture in a 250 mL flask containing 10 mL 5x LB broth, at 37°C, 220 rpm for overnight. 200 μL of the filter-sterilized culture was mixed with 100 μL of log-phase bacteria culture. Double-overlay agar assays were performed, and the plates were incubated at 37°C for overnight to obtain phage plaques. The phages were further purified, i.e. until all phage plaques are uniform, using double-overlay agar assays which usually takes 3-5 rounds of purification.

*DNA extraction.* Phage lysates were prepared by lysing 40 mL of logarithmically growing cells with the appropriate phage at a MOI of 0.1–0.01 and incubating the cultures until clearance. Lysates were centrifuged at 10,000 g for 10 min, sterilized with 0.45 μm membrane filters (VWR), incubated with 10 μg mL⁻¹ DNase and RNase at 37°C for 1 h. Inactivate DNase and RNase at 65°C for 15 min. Lysates were treated with precipitate solution (10% PEG-8000, 1 M NaCl final) at 4°C overnight. Phage particles were collected by spinning down the lysate at 10,000 g, 4°C for 20 min, suspended in 200 μL of TE buffer (0.5 M EDTA pH8, 0.1 M Tris·HCl pH7.4 final), and incubated with 0.5% (w/v) SDS and 10 μL of 20 mg mL⁻¹ Proteinase K in 56°C for 2 h. Genomic DNA was extracted from the supernatants using GENECLEAN Turbo Kit according to the manufacturer’s instructions (MP Biomedicals), and the concentration was determined by Qubit™ dsDNA BR Assay (Invitrogen).
**Genome sequencing and assembly.** Paired-end libraries with an insert size of 200–400 bp were constructed and sequenced on the MGISEQ-2000 (MGI, BGI-Shenzhen) platform to obtain about 1000x clean data (phase genome). Reads were filtered with SOAPnuka (Chen et al., 2018b) (https://github.com/BGI-flexlab/SOAPnuka) and fastp (Chen et al., 2018a), and clean reads were assembled with SPAdes v3.13.0 (Bankevich et al., 2012). Annotation of phage genomes was conducted using prodigal, BLASTp searches against NCBI nr database (snapshot of 2019-07-17), and HMM searches against UniProt/Swiss-Prot database (snapshot of 2019-07-17).

**Preparation of competent cells.** Genome rebooting of phages in stepping-stone hosts. Phage genomic DNA were gently mixed with 200 μL of competent cells, ice-bathed for 30 min, heat-shocked at 42 °C for 2 min, and then ice-bathed for another 3 min. The transformants were recovered in 1 mL of pre-warmed LB broth at 37 °C, 220 rpm for 4 h or over a period of 48 h to assess rebooting kinetics in DH10B and DH10B/pRARE. 5 mM Ca^{2+} was supplemented in the culture of *K. pneumoniae* phages, 5 mM Ca^{2+} and Mg^{2+} were supplemented in the culture of *S. enterica* phages, 5 mM Mg^{2+} was supplemented in the culture of *A. baumannii* phages or *P. aeruginosa* phages. 5% (v/v) chloroform was added to the culture and vortexed rigorously to lyse the cells and release phage particles. After centrifugation at 12,000 g for 5 min, 300 μL of supernatants were mixed with 200 μL of log-phase host bacteria and incubated at 37 °C for 1–5 h. Specifically, *K. pneumoniae* or *S. enterica* phages were incubated for 1–3 h, while *A. baumannii* phages or *P. aeruginosa* phages were incubated for 4–5 h. The incubation step was skipped in order to measure the rebooting efficiency in Figure 1A. Double-overlap agar assays were performed, and the plates were incubated at 37 °C or room temperature (22 °C) to obtain plaques. 5 mM Ca^{2+} was supplemented in the top-layer agar for *K. pneumoniae* phages, 5 mM Ca^{2+} and Mg^{2+} were supplemented in the top-layer agar for *S. enterica* phages, 5 mM Mg^{2+} was supplemented in the top-layer agar for *A. baumannii* phages or *P. aeruginosa* phages. Phage rebooting experiments were carried out at least three times. Negative control was included to avoid false reboot caused by contamination.

**In vivo assembly of phages.** Bacteriophage genomes were split into four to twelve DNA fragments with 40 bp to 300 bp overlaps. DH10B/pRARE plus pKD46 was used for *in vivo* assembling. DH10B/pRARE+pKD46 was inoculated in 3 mL of LB broth containing 100 μg mL^{-1} ampicillin and 25 μg mL^{-1} chloramphenicol at 30 °C overnight. The overnight culture was diluted 1:100 with fresh LB broth containing 10 mM arabinose, 100 μg mL^{-1} ampicillin and 25 μg mL^{-1} chloramphenicol and grown upon reaching absorbance OD_{600} = 0.5–0.6 at 30 °C or 37 °C. Competent cells were then prepared, 1.5–3 g of Kp phage genomic DNA was transformed into competent cells, ice-bathed for 2 min, then ice-bathed for another 3 min. The transformants were recovered in 1 mL of pre-warmed LB broth at 37 °C, washed with 12.5 mL of sterilized ice-cold CaCl\(_2\) solution, and suspended in 500 μL of 0.1 M sterilized ice-cold CaCl\(_2\) solution.

**Rebooting of phages in stepping-stone hosts.** Phage genomic DNA were gently mixed with 200 μL of competent cells, ice-bathed for 30 min, heat-shocked at 42 °C for 2 min, and then ice-bathed for another 3 min. The transformants were recovered in 1 mL of pre-warmed LB broth at 37 °C, 220 rpm for 6 h. After 5% chloroform treatment and centrifugation, 200 μL of supernatants were incubated with 200 μL of log-phase host bacteria and incubated at 37 °C for 1–5 h. Specifically, *K. pneumoniae* or *S. enterica* phages were incubated for 1–3 h, while *A. baumannii* phages or *P. aeruginosa* phages were incubated for 4–5 h. The incubation step was skipped in order to measure the rebooting efficiency in Figure 1A. Double-overlap agar assays were performed, and the plates were incubated at 37 °C or room temperature (22 °C) to obtain plaques. 5 mM Ca^{2+} was supplemented in the top-layer agar for *K. pneumoniae* phages, 5 mM Ca^{2+} and Mg^{2+} were supplemented in the top-layer agar for *S. enterica* phages, 5 mM Mg^{2+} was supplemented in the top-layer agar for *A. baumannii* phages or *P. aeruginosa* phages. Phage rebooting experiments were carried out at least three times. Negative control was included to avoid false reboot caused by contamination.

**In vivo assembly of synthetic genomes.** Bacteriophage genomes were split into four to twelve DNA fragments with 40 bp to 300 bp overlaps. DH10B/pRARE plus pKD46 was used for *in vivo* assembling. DH10B/pRARE+pKD46 was inoculated in 3 mL of LB broth containing 100 μg mL^{-1} ampicillin and 25 μg mL^{-1} chloramphenicol at 30 °C overnight. The overnight culture was diluted 1:100 with fresh LB broth containing 10 mM arabinose, 100 μg mL^{-1} ampicillin and 25 μg mL^{-1} chloramphenicol and grown upon reaching absorbance OD_{600} = 0.5–0.6 at 30 °C or 37 °C. Competent cells were then prepared, 1.5–3 g of Kp phage genomic DNA was transformed into competent cells, ice-bathed for 2 min, then ice-bathed for another 3 min. The transformants were recovered in 1 mL of pre-warmed LB broth at 37 °C, washed with 12.5 mL of sterilized ice-cold CaCl\(_2\) solution, and suspended in 500 μL of 0.1 M sterilized ice-cold CaCl\(_2\) solution.

**Genome editing of phages.** Construction of pSgRNA plasmid. sgRNAs targeting the engineering site were designed using CRISPRko (Sanson et al., 2018) and sgRNAcas9 (Xie et al., 2014). sgRNA oligos were annealed to form double-strands with 4 nt sticky ends. DNA substrate for recombinering was constructed depending on specific engineering demand. 100 bp of homologous fragments upstream and downstream of the engineering site were amplified with primers carrying BbsI restriction site. For deletions, a sgRNA and two 100-bp homologous fragments were cloned into vector pN20 using golden gate assembly to generate the final homologous arms. To insert DspB module, a sgRNA, two 100-bp homologous fragments and synthetic DspB module were cloned into vector pN20 using golden gate assembly to generate DspB module flanking 100-bp homologous arms. The synthetic DspB module contained a copy of CPB0329 major capsid promoter and RBS, upstream of a gene encoding biofilm-dispersing enzyme - dspb from *Aggregatibacter actinomycetemcomitans* HK1651 (ResSeq: NZ_CP007502.1). Plasmids were prepared with TIANprep Rapid Mini Plasmid Kit (TIANGEN).

**Engineering of phages in stepping-stone hosts.** pSgRNA and pCas were co-transformed into 200 μL of competent DH10B cells and recovered in 1 mL of LB broth at 30 °C for 1.5 h. Transformants were selected on LB agar containing 50 μg mL^{-1} kanamycin and 25 μg mL^{-1} chloramphenicol. A positive transformant was inoculated in 3 mL of LB broth containing 50 μg mL^{-1} kanamycin and 25 μg mL^{-1} chloramphenicol at 30 °C overnight. The overnight culture was diluted 1:100 with fresh LB broth containing 10 mM arabinose, 50 μg mL^{-1} kanamycin and 25 μg mL^{-1} chloramphenicol and grown upon reaching absorbance OD_{600} = 0.5–0.6, i.e., for 6 h at 30 °C. Competent cells were then prepared, 1.5–3 μg of Kp phage genomic DNA was transformed into 200 μL of competent cells. The transformants were recovered in 1 mL of pre-warmed LB broth at 37 °C, 220 rpm for 3 h or 37 °C.
220 rpm for 3 h then place on the bench at room temperature (22°C) for 15 h. Spot test assays were performed, and the plates were incubated at 37°C for 4–16 h to obtain plaques. One to ten plaques were picked for screening using PCR, and positive plaques were then purified for three rounds for further PCR verification.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

As shown in Figure 1A, rebooting efficiency was defined as phage plaque forming units per fmol of genomic DNA. All rebooting efficiencies are log10 transformed, presented as the mean, and the error bars represent the standard deviation from triplicate measures. As shown in Results “Cross-genus and cross-order rebooting of phages against MDR bacteria”, correlation analysis of rebooting outcome (success or failure) and genome feature of 126 phages was performed by point-biserial in R. 5 parameters representing genome features were used in our analysis: (i) Transcription orientation, the ratio of the number of phage genes in the longest stretch of consecutive genes in the same direction to the total number of genes in phage genome, (ii) Average protein length, (iii) Gene numbers, (iv) Genome size, (v) Transcriptional strand switch, the ratio of the number of transcriptional strand switches to the total gene number in phage genomes. Fisher’s exact test was performed to determine associations between rebooting outcome and DNA polymerase or RNA polymerase or Integrase or tRNA.