CRISPR–Cas10 assisted editing of virulent staphylococcal phages

S.M. Nayeemul Bari, Asma Hatoum-Aslan*
Department of Biological Sciences, University of Alabama, Tuscaloosa, AL, United States
*Correspondence author: e-mail address: ahatoum@ua.edu

Contents

Abstract

Phages are the most abundant entities in the biosphere and profoundly impact the bacterial populations within and around us. They attach to a specific host, inject their DNA, hijack the host's cellular processes, and replicate exponentially while destroying the host. Historically, phages have been exploited as powerful antimicrobials, and phage-derived proteins have constituted the basis for numerous biotechnological applications. Only in recent years have metagenomic studies revealed that phage genomes harbor a rich reservoir of genetic diversity, which might afford further therapeutic and/or biotechnological value. Nevertheless, functions for the majority of phage genes remain unknown, and due to their swift and destructive replication cycle, many phages are intractable by current genetic engineering techniques. Whether to advance the basic understanding of phage biology or to tap into their potential applications, efficient methods for phage genetic engineering are needed. Recent reports have shown that

Methods in Enzymology
ISSN 0076-6879
https://doi.org/10.1016/bs.mie.2018.10.023

CRISPR–Cas systems, a class of prokaryotic immune systems that protect against phage infection, can be harnessed to engineer diverse phages. In this chapter, we describe methods to genetically manipulate virulent phages using CRISPR–Cas10, a Type III-A CRISPR–Cas system native to *Staphylococcus epidermidis*. A method for engineering phages that infect a CRISPR-less *Staphylococcus aureus* host is also described. Both approaches have proved successful in isolating desired phage mutants with 100% efficiency, demonstrating that CRISPR–Cas10 constitutes a powerful tool for phage genetic engineering. The relatively widespread presence of Type III CRISPR–Cas systems in bacteria and archaea imply that similar strategies may be used to manipulate the genomes of diverse prokaryotic viruses.

Abbreviations

Cas	CRISPR-associated
CRISPR	clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
dNTP	deoxyribonucleotide triphosphate
HIA	heart infusion agar
NEB	new England Biolabs
ORF	open reading frame
PCR	polymerase chain reaction
PFU	plaque-forming units
PNK	polynucleotide kinase
TSA	tryptic soy agar
TSB	tryptic soy broth
U	unit(s)

1. Introduction

Phages are ubiquitous in nature and have profound impacts on the ecosystems within and around us. They are major conduits of gene exchange and key drivers of bacterial turnover and evolution (Bergh, Børsheim, Bratbak, & Heldal, 1989; Breitbart & Rohwer, 2005; Brüssow, Canchaya, Hardt, & Bru, 2004; Suttle, 2007; Waldor & Mekalanos, 1996; Zinder & Lederberg, 1952). Phages attach to a specific bacterial host, inject their DNA, and execute a replication program that can lead to the destruction of the host within minutes of infection. Since their discovery over a century ago (D'Herelle, 1917; Twort, 1915), phages were among the first model organisms to be used in modern molecular biology and have been exploited for a myriad of applications that span across disciplines (Pires, Cleto, Sillankorva, Azeredo, & Lu, 2016). Whole phages can be used as precision

antimicrobials (Wittebole, De Roock, & Opal, 2013) and biosensors for pathogen detection in food and the environment (Singh, Poshtiban, & Evoy, 2013). Phage display of peptides or other conjugates on their capsids has enabled targeted drug delivery, vaccine development, and affinity screening of random peptides (Molek & Bratkovič, 2015). Phages have even been used as scaffolds to build nanoscale devices (Molek & Bratkovič, 2015). Furthermore, phage-derived enzymes, such as T4 DNA ligase and T7 RNA polymerase, have been used for decades as molecular tools and continue to benefit molecular genetics research. Recent metagenomic analyses have revealed that phage genomes harbor tremendous genetic diversity and that the majority of phage genes have yet unknown functions (Ofir & Sorek, 2018). Thus, there still remains significant potential to advance the basic understanding of phage biology and the development of novel phage-inspired technologies.

A robust method for phage genetic engineering is critical to advancing phage research. The engineering of temperate/lysogenic phages, which insert their genome into the host chromosome and remain dormant for generations, can be feasibly accomplished by applying the same genetic techniques available for the specific host organism. In contrast, virulent/lytic phages exhibit a swift replication cycle that destroys the host within minutes of infection, thus making these phages difficult, if not impossible to engineer by most genetic techniques. However, recent reports have shown that the class of prokaryotic immune systems known as CRISPR–Cas can be used as efficient tools to engineer virulent phages that infect diverse bacterial hosts (recently reviewed in Hatoum-Aslan, 2018).

CRISPR–Cas systems protect against phage infection and the stable incorporation of other foreign nucleic acids (Marraffini, 2015). In the general pathway, CRISPR loci maintain an archive of past nucleic acid invaders in the form of short (30–40 nucleotides) invader-derived sequences called spacers integrated in between DNA repeat sequences of similar length. Spacers are transcribed and processed into mature CRISPR RNAs (crRNAs), which form an effector complex with one or more CRISPR-associated (Cas) nucleases. The resulting ribonucleoprotein complex senses and destroys foreign nucleic acids harboring sequences complementary to the crRNA, called protospacers. CRISPR–Cas systems are relatively widespread in the prokaryotic world, with six distinct types (I–VI) and dozens of subtypes found across diverse bacterial and archaeal *phyla* (Koonin, Makarova, & Zhang, 2017).

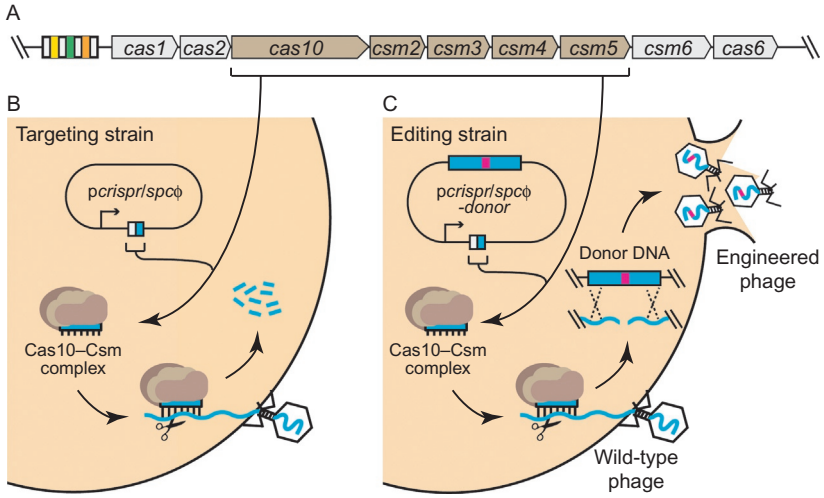

Fig. 1 The Type III-A CRISPR–Cas system in *S. epidermidis* RP62a, known as CRISPR–Cas10. (A) This system encodes three spacers (*colored rectangles*), four repeats (*light gray rectangles*), and nine *cas/csm* genes. The genes *colored brown* encode the proteins that form the Cas10–Csm effector complex. This system can be harnessed to engineer phage genomes using a two-step approach. In the first step (B) a targeting strain is constructed, which bears a plasmid-borne spacer matching the phage of interest. The spacer is confirmed to be functional by challenging the targeting strain with the phage of interest. In the second step (C) an editing strain is constructed, which bears a modified plasmid encoding both the targeting spacer and a donor DNA construct containing desired mutations. Phage editing is performed by amplifying the phage on the editing strain. Phages that recombine with the donor DNA construct and acquire the mutations escape further targeting by CRISPR–Cas10 and complete their replication cycle.

The powerful antiphage immunity provided by CRISPR–Cas systems can be exploited as a counter-selection mechanism to eliminate the wild-type version of a phage of interest and thus enrich for phages that have acquired desired mutations (Hatoum-Aslan, 2018). This chapter describes, in detail, methods to engineer lytic phages that infect *Staphylococcus epidermidis* RP62a using its native Type III-A CRISPR–Cas system, called CRISPR Cas10 (Fig. 1A). This system contains three spacers and nine CRISPR-associated *cas* and *csm* genes, seven of which (*cas10-cas6*) are required for the destruction of nucleic acid sequences contained within existing spacers (Hatoum-Aslan, Maniv, Samai, & Marraffini, 2014). The effector complex for this system, called Cas10–Csm, is composed of five protein subunits (Cas10, Csm2, Csm3, Csm4, and Csm5) and a crRNA (Hatoum-Aslan, Samai, Maniv, Jiang, & Marraffini, 2013). The accessory nucleases Cas6 and Csm6 have roles in crRNA processing

(Hatoum-Aslan, Maniv, & Marraffini, 2011; Hatoum-Aslan et al., 2014) and phage transcript degradation (Jiang, Samai, & Marraffini, 2016), respectively. This system can be harnessed to edit phage genomes using a two-step process (Bari, Walker, Cater, Aslan, & Hatoum-Aslan, 2017). In the first step, a "targeting strain" is created that bears the plasmid p*crispr/spcϕ*, which contains a single repeat and a spacer targeting the phage of interest at or near the genomic location to be mutated (Fig. 1B). This targeting strain is first challenged with phage to confirm that the newly introduced spacer promotes efficient immunity against the phage of interest. In the second step, an "editing strain" is created that bears the plasmid p*crispr/spcϕ-donor*, which contains the targeting spacer plus a "donor DNA" construct (Fig. 1C). The donor DNA construct contains phage-derived sequences with the desired mutations flanked on both sides by wild-type sequences of homology. Phage editing involves propagating phages on the editing strain—only those phages that successfully recombine with the donor DNA construct and acquire the mutations will escape CRISPR–Cas10 immunity and complete their replication cycle. These putative recombinants are then purified and screened for the presence of the desired mutation. Fig. 2 summarizes the steps involved in phage editing using this system. This chapter also describes a recombinant CRISPR–Cas10 system that can be used in a similar fashion to edit phages that infect a CRISPR-less *Staphylococcus aureus* host. Detailed protocols are found in the sections below, using as an example the editing of *S. epidermidis* RP62a phage Andhra (Cater et al., 2017).

2. Phage targeting in *S. epidermidis*

In order to program CRISPR–Cas10 to destroy the phage of interest, the protein and nucleic acid requirements for immunity must be considered. As mentioned earlier, crRNAs are processed by the endoribonuclease Cas6 (Hatoum-Aslan et al., 2011, 2014). Additional non-Cas nucleases also contribute to crRNA maturation (Walker, Chou-Zheng, Dunkle, & Hatoum-Aslan, 2016), and it remains unclear whether these are important for immunity. Mature crRNAs are bound by Cas10, Csm2, Csm3, Csm4, and Csm5 to form the Cas10–Csm complex (Fig. 3A) (Hatoum-Aslan et al., 2013). This effector complex detects and destroys DNA and RNA targets in a transcription-dependent manner (Goldberg, Jiang, Bikard, & Marraffini, 2014; Samai et al., 2015), thus necessitating that the crRNA be complementary to the coding (nontemplate) strand of the DNA as well as the corresponding mRNA. The accessory nuclease Csm6 is also required to

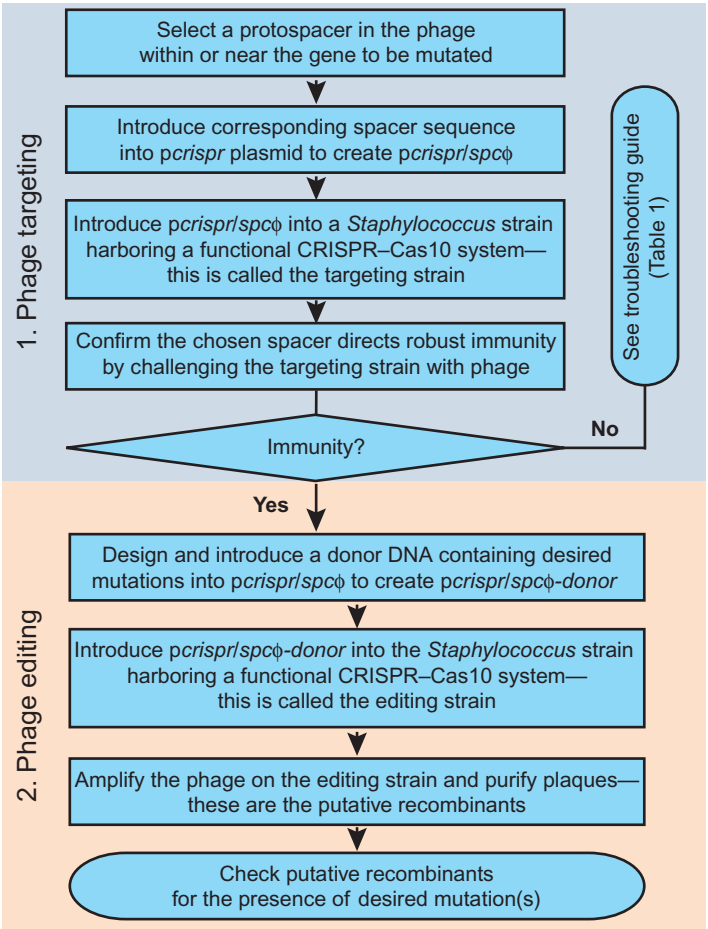

Fig. 2 A flow chart outlining the steps to phage engineering using CRISPR–Cas10.

degrade phage transcripts (Jiang et al., 2016). In addition, a sequence requirement exists in the eight nucleotides adjacent to the protospacer, a region called the "anti-tag"—the anti-tag should bear little or no complementarity to the opposing eight nucleotides on the 5′-end of the crRNA, termed the "5′-tag" (Fig. 3A). This unique feature of Type III systems safeguards against autoimmunity, or self-targeting of the CRISPR locus within the bacterial chromosome (Marraffini & Sontheimer, 2010). An example of a functional protospacer in phage Andhra with a permissive anti-tag sequence is shown in Fig. 3B. In this example, only a single position in the anti-tag harbors complementarity to the tag (nucleotides underlined in bold) and thus still allows for robust immunity (Bari et al., 2017).

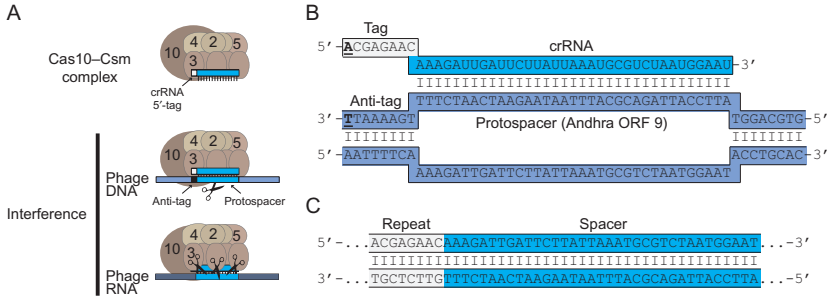

Fig. 3 Nucleic acid elements required for CRISPR–Cas10 immunity. (A) An illustration of the Cas10–Csm complex is shown on *top*, highlighting the 5′-end of the mature crRNA. The 5′-tag comprises eight nucleotides derived from the upstream repeat sequence and bears no resemblance to the targeted nucleic acid. The *bottom panel* shows the nucleic acid requirements for interference. During interference, phage DNA and RNA sequences complementary to the spacer are degraded by different subunits in the Cas10–Csm complex. An important requirement that licenses interference is the absence of complementarity between the crRNA 5′-tag and the opposing region adjacent to the protospacer, called the anti-tag. (B) An example of a permissive protospacer in phage Andhra where the anti-tag shares only one nucleotide of complementarity with the tag (nucleotides in *bold* and *underlined*). (C) The spacer that corresponds to the protospacer in (B) is shown, along with the portion of the repeat that encodes the crRNA 5′-tag.

Since *S. epidermidis* RP62a encodes a functional CRISPR–Cas10 system in its genome (Marraffini & Sontheimer, 2008), the protein requirements for interference are met in this strain. However, a spacer targeting the specific phage of interest must be introduced into this strain on the plasmid p*crispr*. Fig. 3C shows the spacer sequence corresponding to the protospacer in Fig. 3B that would need to be inserted into p*crispr* in this example. The sections below describe how to (i) select a functional protospacer region in the phage of interest; (ii) introduce the corresponding spacer sequence into p*crispr* using inverse polymerase chain reaction (PCR); and (iii) confirm that the constructed targeting strain harboring the new spacer exhibits robust immunity.

2.1 Selecting functional protospacers for CRISPR–Cas10 interference

As explained earlier, a CRISPR–Cas10 protospacer must reside on the coding (nontemplate) strand of the DNA, be actively transcribed, and harbor little or no complementarity between the adjacent anti-tag and the 5′-tag of the crRNA. Protospacers should be 35 nucleotides in length and ideally overlap with the region of the phage genome to be mutated. A protospacer

may also be selected within 500 nucleotides of the region to be edited, but this would necessitate screening of recombinants at the end of the editing process to identify phages that have acquired the desired mutation(s) (Bari et al., 2017). Protospacers exhibiting these characteristics may be selected manually or by using a Python script that was developed in order to simplify the process. The script identifies in a given gene all potential protospacers that harbor zero complementarity between the anti-tag and crRNA tag region. The following are the steps to using the script:

1. Go to github using the link: https://github.com/ahatoum/CRISPR-Cas10-Protospacer-Selector
2. Download and place the two Python files (MainScript.py, GNfunctions.py) in the same directory.
3. Open MainScript.py in an editor. Edit the tag and gene sequences in 5′–3′ directions to reflect the actual tag sequence (if using a different Type III system with a different 5′-tag) and the coding (nontemplate) strand of the phage gene sequence of interest, respectively. The chosen gene should be actively transcribed at some point during the replication cycle.
4. Run the code using any Python interpreter (version 3.0 or higher). Running the script will create a "Results.txt" file in the same directory that presents the tabulated results. The results appear in four columns as follows: protospacer coordinate (beginning in 5′ direction), protospacer sequence, Anti-tag sequence, spacer sequence to be cloned into p*crispr*.

2.2 Constructing a "targeting strain" that destroys the phage of interest

Once an appropriate protospacer is selected, a corresponding spacer (the reverse complement of the protospacer) needs to be introduced into p*crispr* (Fig. 4A). p*crispr* is a 3228 nucleotide plasmid containing the leader region of the *S. epidermidis* RP62a CRISPR locus and a single repeat. The plasmid also encodes the *cat* (chloramphenicol acetyl transferase) gene, which confers chloramphenicol resistance. Our previous work has shown that a *S. epidermidis* strain bearing p*crispr* with a single spacer encoded downstream of the repeat sequence enables efficient immunity against the corresponding phage (Bari et al., 2017). The spacer can be introduced into p*crispr* using inverse PCR (Fig. 4B). In this method, forward and reverse PCR primers

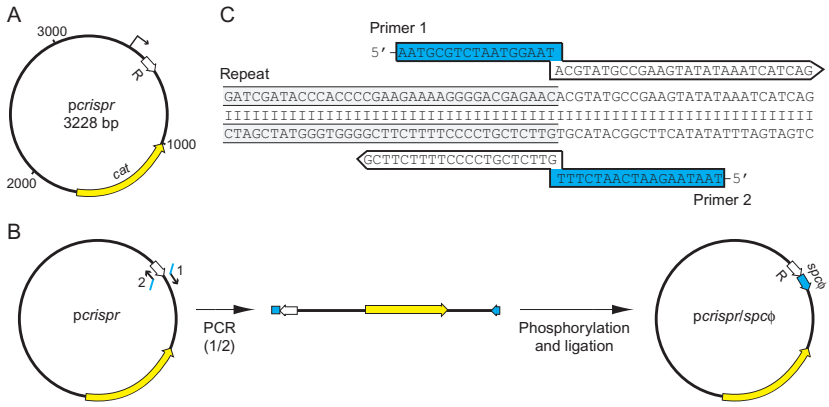

Fig. 4 Constructing p*crispr/spcϕ*. (A) A map of the base plasmid p*crispr* is shown highlighting the chloramphenicol acetyl transferase gene (*cat*), CRISPR–Cas10 repeat (R), and promoter (*arrow*). (B) The process of inserting a new spacer into p*crispr* using inverse PCR is shown. In this process, primers 1 and 2, which harbor portions of the new spacer as 5′-overhangs (*blue*), are used to amplify the entire plasmid. The PCR product is then phosphorylated on its 5′-ends and ligated to form a new plasmid containing a new spacer sequence (spcϕ). The resulting plasmid with newly introduced spacer is called p*crispr/spcϕ*. (C) The sequences of primers 1 and 2 are shown for inserting the exemplar Andhra-targeting spacer in Fig. 3C. The sequence in between the primers corresponds to the repeat sequence (*highlighted in light gray*) and adjacent plasmid sequence, in between which the new spacer should be inserted.

(nonoverlapping) are designed to anneal precisely at the repeat-plasmid junction in which the spacer is to be inserted. Each primer will also contain a portion of the spacer on their 5′-ends, such that between the two primers, the whole spacer sequence is present (see example primers in Fig. 4C). A PCR is next performed using these primers and p*crispr* as a template. The resulting PCR product should encompass the entire plasmid and contain spacer sequences on both ends. The PCR product is then phosphorylated and ligated to recircularize the plasmid and reform a complete spacer sequence. The resulting plasmid is called pcrispr/*spcϕ*. The following are detailed instructions for making pcrispr/*spcϕ* using inverse PCR and introducing it into *S. epidermidis* to generate the targeting strain:

1. Design and obtain a pair of PCR primers that anneal in the repeat region (reverse primer) and the sequence directly adjacent (forward primer) with overhangs on their 5′-ends corresponding to the entire spacer sequence (Fig. 4C).

2. Perform a PCR using these primers and the plasmid p*crispr* as a template. PCR should be carried out with high fidelity Phusion DNA polymerase (NEB), which generates PCR products with blunt ends. The PCR should contain 1–2 ng of the template DNA, 0.5 μ*M* of each primer, 200 μ*M* of each dNTP (A, C, G, T), 1 × Phusion buffer, and 0.25 U of Phusion DNA polymerase. Use a thermocycler to run the reaction with the following PCR program: 95°C for 1 min, 1 cycle; 95°C for 10 s, 55°C for 10 s, and 72°C for 3 min, 30 cycles; and 72°C for 6 min, 1 cycle.
3. Purify the PCR product using a PCR purification kit (Omega Biotek) and measure the concentration.
4. Phosphorylate the 5′-ends of the PCR product using T4 polynucleotide kinase (PNK, NEB): combine 0.5–1.0 pmol (1–2 μg if using p*crispr*) of the purified PCR product with 1 × T4 DNA ligase buffer (same as PNK buffer but contains adenosine triphosphate) and 10 U of T4 PNK. Incubate the mixture at 37°C for 30 min.
5. Circularize the PCR product by adding 400 U of T4 DNA ligase (NEB) and adjusting the concentration of buffer to 1 × with an appropriate volume of 10 × T4 DNA ligase buffer and nuclease-free water. Incubate the mixture at room temperature overnight.
6. Dialyze the ligation reaction and transform electrocompetent *S. aureus* RN4220 cells with the entire reaction mixture. Detailed protocols for sample dialysis, preparation of electrocompetent *Staphylococcus* cells, and nucleic acid transformation can be found in Section 5.

   *Note that *S. epidermidis* RP62a will not directly take up PCR-derived plasmids. Therefore, *S. aureus* RN4220, which is receptive to transformation with plasmids derived from PCR products, is used as a passage strain. Once the plasmid is stably introduced into *S. aureus* RN4220, it can be prepared and transferred into *S. epidermidis*.
7. Pick several *S. aureus* transformants and perform colony PCR using primers that anneal upstream and downstream of the inserted spacer. Sequence the PCR products to confirm that the spacer has been inserted properly. Recommended primers for PCR and sequencing of the newly inserted spacer in p*crispr* are:

   forward: 5′-TTGTCAAAAAAAGTGACATATCATATAATCTT GTAC.

   reverse: 5′-ACTGTACTTTTTACAGTCGGTTTTCTAATG.

8. Isolate pcrispr/*spcϕ* from a *S. aureus* transformant in which the spacer sequence has been confirmed and introduce the plasmid into *S. epidermidis* RP62a or the desired CRISPR–Cas10 containing host that is susceptible to the phage to be edited. The *Staphylococcus* strain containing a CRISPR–Cas10 system and pcrispr/*spcϕ* is called the targeting strain.

2.3 Testing the targeting strain for antiphage immunity

Once constructed, the targeting strain is challenged with the phage of interest to confirm that CRISPR–Cas10 can now recognize and destroy the phage. To test this, the double-agar overlay method can be used (Cater et al., 2017):

1. Dilute an overnight culture of the targeting strain 1:20 in 4 mL Heart infusion agar (HIA) prepared at 50% the recommended concentration (here onward referred to as HIA top agar). Add $CaCl_2$ to a final concentration of 5 m*M*.
2. Overlay this mixture atop a 100 mm petri plate containing Tryptic soy agar (TSA) plus 5 m*M* $CaCl_2$. Prepare a separate overlay containing the strain harboring the p*crispr* plasmid as negative control.
3. Obtain a lysate of the phage of interest with a titer of approximately 1×10^9 PFU/mL and serially dilute it 10-fold, out to 10^{-7}.
4. Spot 10 μL of each dilution atop the overlays and allow spots to dry at room temperature for about 15 min.
5. Incubate plates at 37°C overnight and score for plaques on the following day.

Robust CRISPR–Cas10 immunity is indicated by the absence of phage plaques on the targeting strain, with the negative control plaque count reflecting the phage titer. Partial clearing may appear where higher concentrations of the phage were spotted on the targeting strain due to the action of lysins present in the phage lysate (a phenomenon known as lysis from without); however, if immunity is intact, few/no plaques should appear where more dilute lysate is spotted. In contrast, if it appears as though CRISPR–Cas10 immunity is entirely absent (i.e., there are similar plaque counts on the targeting and negative control strains) or defective (i.e., there are one or two orders of magnitude fewer plaques on the targeting strain vs the negative control), there may be several reasons for such observations. See Table 1 for a troubleshooting guide that lists possible reasons for failure at this stage and suggested courses of action.

Table 1 Troubleshooting guide.

Observation	Potential problem(s)	Suggested course of action
Inability to recover *Staphylococcus* transformants containing a p*crispr* based plasmid with a new spacer inserted	Spacer might be targeting one or more chromosomal loci in the bacterium, causing cell death	Choose a different protospacer region and try to introduce the corresponding spacer.
Inability to recover *Staphylococcus* transformants containing a p*crispr-spcϕ* based plasmid with the donor DNA inserted	Phage-derived sequence might encode a protein that is toxic to the cells	Shorten the homology arms (to a length no shorter than 100 nucleotides) or employ a distal editing approach to avoid including a whole toxic gene
Targeting strain does not exhibit immunity to phage of interest (i.e., there is no reduction in plaque count after challenging targeting strain with phage as compared to the negative control)	Protospacer/spacer designed incorrectly	Double-check to make sure that the spacer will bind to both the coding strand and the mRNA
	Gene/region of interest is not transcribed	Select a protospacer in an adjacent region that is known to be transcribed and employ distal editing approach
	If using a native CRISPR–Cas10 system other than the one described herein, it might not be functional	Use the heterologous p*crispr-cas/Δcas1Δcas2* system which is known to be functional
	Phage and/or mobile elements in the cell might contain anti-CRISPR protein(s) against Type III systems. Although such inhibitors have not been reported for Type III systems, it is reasonable to speculate that they do exist	Use a different CRISPR–Cas Type for phage editing, or a different approach altogether

Targeting strain exhibits weak immunity to phage of interest (i.e., there is modest (1–2 orders of magnitude) reduction in plaque count after challenging targeting strain with phage as compared to the negative control)	Phages that escape immunity might have acquired random mutations at or near the protospacer region. While we have not observed such mutants, it is feasible that spontaneous deletions may occur in the phage genome across nonessential loci, and such mutants would escape Type III CRISPR–Cas immunity	Purify escaping phages and check for mutations on or near the protospacer. Recall that a mutation that inactivates the promoter driving expression of the protospacer region may also lead to phage escape from CRISPR–Cas10 immunity. Even if such mutations occur, it is still possible to recover the desired mutant by following the editing protocols described
No plaques present after culturing phage on editing strain	Intended mutation might impact phage viability	Introduce into the editing strain a plasmid that harbors a wild-type copy of the gene of interest with silent mutations in the protospacer. Try again to get the desired edits into the phage

3. Phage editing in *S. epidermidis*

Once a targeting strain has been created and confirmed to elicit robust antiphage immunity, the p*crispr/spcϕ* plasmid is then further modified to harbor a donor DNA construct—the modified plasmid is called p*crispr/spcϕ-donor*. The donor DNA construct consists of phage-derived sequences that span (i) the protospacer region targeted by CRISPR–Cas10 and (ii) the region to be edited along with desired mutations. The protospacer should also be flanked on both sides by approximately 250 nucleotides of wild-type phage sequences (called homology arms). Note that homology arms as short as 100 nucleotides can still work, but with lower efficiency (Bari et al., 2017). Fig. 5A shows examples of two possible donor DNA construct configurations, in which (i) the protospacer overlaps with the desired mutations (ideal scenario) and (ii) the protospacer lies distal to the desired mutation (alternative).

3.1 Designing the donor DNA construct

As mentioned earlier, the ideal setup is that the protospacer overlaps with the region to be mutated; however, mutations may also be introduced in regions distal to the protospacer. Regardless of the position of the desired mutation(s), the donor DNA construct must encompass both the protospacer and the region of the phage genome to be mutated plus the desired mutations. In the example presented here for phage Andhra, the protospacer directly overlaps with the region to be mutated, with the desired mutations being eight silent mutations across the protospacer (Fig. 5B). To facilitate screening of putative recombinants, a restriction endonuclease site (BstZ171) has been added with the silent mutations. With this design, phages that take up the mutations would simultaneously lose the protospacer sequence and thus acquire the ability to escape CRISPR–Cas10 immunity. In an alternative design, where desired mutations are distal to the protospacer, the donor DNA construct should still harbor silent mutations across the protospacer to allow recombinant phages to escape CRISPR–Cas10 immunity (Fig. 5A). In the latter setup, recombinant phages may or may not have picked up the desired mutations, but all should have acquired the silent mutations across the protospacer. As an important note, a minimum of five mutations should be introduced across the protospacer, regardless of the placement of desired mutations, in order for the phage to escape CRISPR–Cas10 immunity

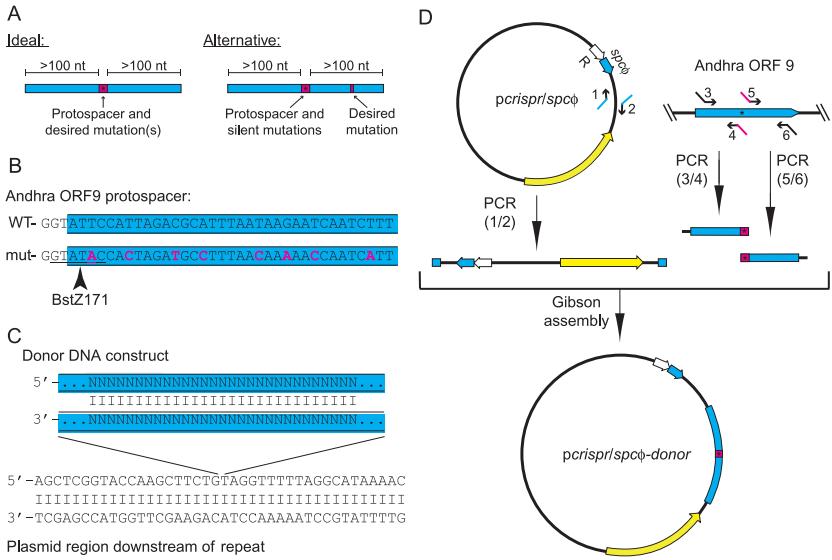

Fig. 5 Constructing p*crispr/spcϕ-donor*. (A) Two possible designs for the donor DNA construct are shown. In the ideal scenario (*left*), the desired mutations overlap with the protospacer. In an alternative configuration (*right*), the desired mutations lie distal to the protospacer. In the latter case, at least five silent mutations must also be introduced in the protospacer to allow phages to escape immunity. Mutations are shown in *magenta*, and protospacers are indicated by *asterisks*. In both cases, the protospacer must be flanked by sequences homologous to the phage on both sides. Homology arms should be no shorter than 100 nucleotides in length. (B) The exemplar Andhra ORF9 protospacer is shown in the 5′-3′ direction (*top*), with silent mutations (*magenta*) that can be introduced to subvert immunity (*bottom*). The mutations also introduce a BstZ171 restriction site (*underlined*) that can facilitate the screening of putative recombinants. (C) The region of the p*crispr* plasmid downstream of the repeat is shown in which it is safe to insert the donor DNA construct. (D) The process of constructing p*crispr/spcϕ-donor* using Gibson assembly. In this process, three PCRs are performed using indicated primers (1–6) on indicated templates (p*crispr/spcϕ* or phage DNA). Each of the primers contains 5′-overhangs corresponding to adjacent regions in the plasmid to be constructed, such that the resulting PCR products would contain, on both ends, 15–25 nucleotides of sequence identical to the adjacent region in the construct. The products are then combined in a 1:1:1 ratio and assembled using the Gibson assembly mix, which contains three enzymes: T5 exonuclease, Phusion polymerase, and Taq ligase. The assembled plasmid with spacer plus donor DNA is called p*crispr/spcϕ-donor*.

(Pyenson, Gayvert, Varble, Elemento, & Marraffini, 2017). Therefore, if a point mutation is desired in the protospacer region, at least four additional silent mutations should be introduced in the vicinity in order for recombinant phages to survive immunity.

In previous work, silent mutations have been successfully introduced into diverse phages at several genetic loci (Bari et al., 2017). Although gene deletions have not been attempted, it is anticipated that they can be easily created, provided the gene is expressed, yet not essential for phage replication. If a deletion is desired, the most straightforward approach would be to choose a protospacer within the gene to be knocked out, and use the flanking regions as the donor DNA construct. In this setup, silent mutations are not required, as the protospacer would be eliminated entirely should recombination occur between the donor DNA and the phage. In an alternative approach, an effective knockout can be created without changing genome size by introducing premature stop codon(s) near the beginning of the open reading frame.

3.2 Constructing an "editing strain" that enriches for phage recombinants

Once the most appropriate donor DNA construct is designed, it must be introduced into the plasmid p*crispr/spcϕ* to create p*crispr/spcϕ-donor*. Fig. 5C shows the region in pcrispr/*spcϕ* that lies directly downstream of the repeat and spacer where it is safe to introduce the donor DNA without disrupting important elements in the plasmid. This insertion can be accomplished by obtaining a synthetic construct, or by assembling the necessary DNA fragments using Gibson assembly (Gibson et al., 2009). In our relatively simple example, the plasmid can be easily created using a 3-piece Gibson assembly strategy (Fig. 5D). The protocol is as follows:

1. Design and obtain three sets of PCR primers that will amplify (i) the backbone plasmid (primers 1 and 2 in Fig. 5D), (ii) upstream of the protospacer region (primers 3 and 4 in Fig. 5D), and (iii) downstream of the protospacer region (primers 5 and 6 in Fig. 5D). Each primer should include 5′-end overhangs that overlap with 15–25 nucleotides of the adjacent region in the plasmid to be constructed. The reverse primer that amplifies the upstream region of the protospacer and the forward primer that amplifies the downstream region (primers 4 and 5 in Fig. 5D) should be designed in such a way that the 5′-overhangs of the primers carry the protospacer sequence with desired mutation plus several silent mutations in order to allow the recombinant phages to escape CRISPR–Cas10 immunity.
2. Perform three separate PCRs using (i) primers 1 and 2 with pcrispr/*spcϕ* as template, (ii) primers 3 and 4 with phage genomic DNA as template, and (iii) primers 5 and 6 with phage genomic DNA as template (Fig. 5D). Use high fidelity Phusion DNA polymerase (NEB) according to the

protocol described in Section 2.2. The elongation times for the PCRs may be adjusted to account for the different fragment sizes (1 min of elongation time per 1 kb of DNA)

3. Purify the PCR products and measure their concentrations.
4. Combine all three fragments with Gibson assembly master mix (NEB) according to the manufacturer's instructions. In order to assemble 2–3 fragments, 0.02–0.5 pmol of each PCR product is used. For transformation into *S. aureus* > 0.06 pmol of the purified PCR products is ideal. Higher assembling efficiency can be achieved by using a two- to three-fold excess of the smaller inserts over the vector; however, the total volume of purified PCR product should not exceed 20% of the total reaction volume.
5. Incubate the Gibson assembly mixture at 50°C for 30 min.
6. Dialyze the assembled product and transform into the *S. aureus* RN4220 passage strain as described in Section 5.
7. Pick several transformants and confirm that the donor construct has been correctly inserted using colony PCR followed by sequencing. The same primers listed in Section 2.2 can be used for PCR and sequencing of the donor DNA insert.
8. Isolate pcrispr/*spcϕ-donor* from a *S. aureus* transformant in which the donor DNA construct has been confirmed and introduce the plasmid into *S. epidermidis* RP62a or the desired CRISPR–Cas10 containing strain that is susceptible to the phage to be edited. The *S. epidermidis* strain harboring pcrispr/*spcϕ-donor* is termed the editing strain.

3.3 Editing phages

Once the editing strain is created, the phage of interest can be forced to acquire the mutations in the donor DNA by simply amplifying the phage on the editing strain:

1. Combine the phage with 500 μL of a mid-log culture of the editing strain at a multiplicity of infection of 1 in Tryptic soy broth (TSB) plus 5 m*M* $CaCl_2$.
2. Allow the mixture to incubate for 1 h at 37°C without shaking. As a control, the corresponding targeting strain is also cultured with the phage under the same conditions.
3. Add the phage-host mixture to 4 mL HIA top agar plus 5 m*M* $CaCl_2$ and overlay the mixtures onto 100 mm petri plates containing TSA and 5 m*M* $CaCl_2$.
4. Incubate the plates at 37°C overnight and score for plaques on the following day.

3.4 Genotyping putative recombinants

A good indication of successful editing is the appearance of plaques on the plate with the editing strain, and no visible plaques on the targeting strain (for troubleshooting, see Table 1). If the control plate has no plaques, the plaques on the editing plate are likely all recombinants that have taken up the mutations in the protospacer which were provided on the donor DNA. Bear in mind that if a distal editing approach is being used, in which the protospacer and desired mutation(s) are separated, then not all phage recombinants would have necessarily acquired desired mutation(s). Regardless of the approach, putative recombinants should be purified and confirmed to have taken up the intended mutations. This can be accomplished by following the steps below:

1. Pick several well-isolated plaques from the editing plate with micropipette tips and place the agar in sterile microcentrifuge tubes containing 500 μL sterile TSB. Vortex at high speed for 1 min to allow phages to be released from the agar into the media.
2. Centrifuge the tubes at $8000 \times g$ for 2 min to pellet the agar and cells.
3. Prepare 10-fold serial dilutions of the supernatant out to 10^{-7} and spot on HIA top agar overlays of the targeting strain as described in Section 2.3. Allow the plates to incubate at 37°C overnight.
4. Pick one well-isolated plaque derived from each putative recombinant and repeat steps 1–2 above.
5. Combine into a sterile 15-mL conical tube the entire phage-containing supernatant with 2 mL of an overnight culture of the targeting strain diluted 1:100 in fresh TSB. Add $CaCl_2$ to a final concentration of 5 m*M*.
6. Incubate the culture at 37°C for 6 h with agitation.
7. Pellet the cells by centrifuging at $4000 \times g$ for 5 min and filter the supernatant through 0.45 μm filter. The filtered supernatant is the phage lysate.
8. Determine the titer of the phage lysate by spotting 10-fold dilutions onto a top agar overlay containing the targeting strain. The phage titer should be $>1 \times 10^8$ PFU/mL before proceeding to the next step. If the titer is $<1 \times 10^8$ PFU/mL, repeat the phage amplification procedure in steps 5–7 with the new lysate in order to boost the titer.
9. Extract phage DNA directly from the phage lysate:
   a. Combine 200 μL of each filtered lysate with 8 μL of 0.5 M EDTA (pH 8.0), 10 μL of 10% SDS, and 2 μL of proteinase K (20 mg/mL).
   b. Invert the tubes to mix and pulse spin to bring the contents of the tube to the bottom.

c. Incubate the mixtures at 55°C for 1 h.
d. Add an equal volume of a mixture of Phenol, Chloroform, and Isoamyl alcohol (25:24:1) to each digested lysate.
e. Vortex the mixtures at high speed for 1 min.
f. Centrifuge the mixtures at 17,000 × *g* for 5 min and transfer the aqueous (top) layers into fresh tubes.
g. Mix the aqueous layers with 2.5 volumes of 100% ethanol and 0.1 volumes of 3.0 M sodium acetate, pH 5.2.
h. Place the samples in ice for 10 min.
i. Centrifuge the samples at 17,000 × *g* for 5 min and discard the supernatants.
j. Wash pellets by adding 1 mL of 75% ethanol and gently inverting.
k. Centrifuge at 17,000 × *g* for 5 min.
l. Gently aspirate the supernatants without disturbing the pellets and allow the pellets to air-dry for 10–20 min.
m. Dissolve pellets in 30 μL of nuclease-free water.

10. Perform a PCR using a 1:10 dilution of the phage DNA as template and primers that will amplify the protospacer region of the phage genome.
11. Purify the PCR products and determine if the intended mutation(s) are present by digesting the products with the appropriate restriction enzyme and/or sequencing the entire PCR product.

4. Editing phages that infect CRISPR-less *Staphylococcus* hosts

The protocols outlined above assume that the desired *Staphylococcus* host already harbors an endogenous and functional CRISPR–Cas10 system. If the chosen host does not possess its own CRISPR–Cas system, or if its CRISPR–Cas system is nonfunctional, phages can still be edited with the same protocols described earlier, except using the plasmid p*crispr-cas/Δcas1Δcas2* (Fig. 6A). This plasmid encodes the seven CRISPR-associated genes required for immunity (*cas10-cas6*) in addition to a repeat sequence that is transcribed by the native promoter for this system. A targeting construct can be designed and created with this base plasmid by inserting the desired spacer downstream of the repeat (Fig. 6B) according to the protocols in Section 2. Recommended primers for PCR and sequencing of the newly inserted spacer in pcrispr-*cas/Δcas1Δcas2/spcϕ* are:

forward: 5′-AATAATGTATTTACGCTGGGGC.
reverse: 5′-CCCCTAGAAATTAATCAATGCGTATTTTATTCAA AATCTAC.

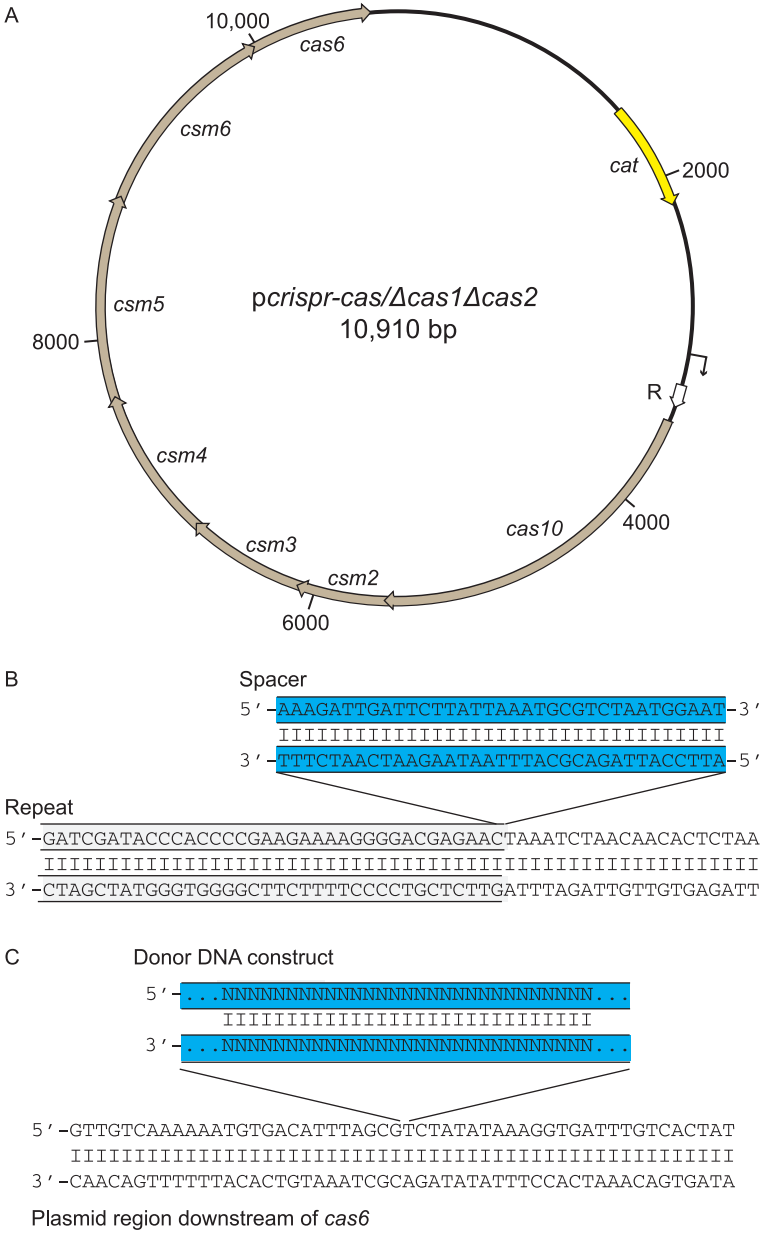

Fig. 6 An alternative system for engineering CRISPR-less hosts. (A) A map of the base plasmid p*crispr-cas/Δcas1Δcas2* is shown highlighting the chloramphenicol acetyl transferase gene (*cat*), promoter (*arrow*), repeat (R), and seven *cas/csm* genes required for interference (*colored brown*). (B) The repeat (*highlighted gray*) and adjacent plasmid sequences are shown, in between which new spacers should be inserted which target the phage of interest. The Andhra ORF9 spacer sequence is shown as an example. (C) The region in the plasmid downstream of *cas6* is shown, in which it is safe to introduce the donor DNA construct.

Once the functionality of the targeting construct is confirmed, it can be modified to incorporate the donor DNA downstream of *cas6* (Fig. 6C) according to the protocols in Sections 3.1 and 3.2. Recommended primers for the PCR and sequencing of the donor DNA construct in p*crispr-cas/Δcas1Δcas2/spcϕ-donor* are:

forward: 5′-TTTAGTTGTCAAAAAATGTGACATTTAGCG.

reverse: 5′-TTATCTTTGATGATGCAATATATTAAGCAGCA AGAG.

Once the editing strain is created, phage editing and genotyping can be carried out according to the protocols described in Sections 3.3 and 3.4. This heterologous system has been successfully used to edit multiple genetic loci in phage ISP using *S. aureus* RN4220 as host (Bari et al., 2017).

5. Transforming *Staphylococcus* bacteria

Staphylococcus species can be very challenging to transform due to their thick cell wall and multiple internal barriers to gene transfer such as restriction modification and CRISPR–Cas systems. *S. aureus* RN4220 is a restriction-defective mutant that does not have its own CRISPR–Cas system, thus making it useful as a passage strain for PCR-constructed plasmids. Supercoiled plasmids purified from this strain can be easily transferred into wild-type *S. epidermidis* isolates. The protocols below describe how to prepare the nucleic acid samples and *Staphylococcus* cells for successful transformation using electroporation.

5.1 Preparing electrocompetent cells

The following protocol can be used to prepare electrocompetent *S. epidermidis* and *S. aureus* cells. Once prepared, they may be kept frozen at −80°C and used up to 3 months later.

1. Grow bacteria overnight in 10 mL of TSB with appropriate antibiotics.
2. In a sterile Erlenmeyer flask, dilute the entire overnight culture with fresh TSB until the optical density at 600 nm reaches 0.5.
3. Incubate the diluted culture at 37°C for 30 min.
4. Arrest the cell growth by incubating the culture on ice for 20 min. From this step onward, everything should be kept on ice and centrifugation should be performed at 4°C.
5. Transfer equal volumes of the chilled culture into two different 50-mL conical tubes and centrifuge at $4000 \times g$ for 10 min.

6. Discard the supernatant, resuspend the pellets in an equal volume of ice-cold distilled water, and centrifuge at $4000 \times g$ for 10 min.
7. Repeat step 6.
8. Discard the supernatant, resuspend pellets in 0.05 volume of ice-cold 10% glycerol, and combine the resuspended cells into a single tube.
9. Centrifuge at $4000 \times g$ for 10 min.
10. Discard the supernatant, resuspend the pellet in 0.04 volumes of ice-cold 10% glycerol, and centrifuge at $4000 \times g$ for 10 min.
11. Pour off the supernatant and resuspend the pellet in 0.005 volume of ice-cold 10% glycerol.
12. Distribute 50 μL aliquots into sterile microcentrifuge tubes and use immediately or freeze at −80°C.

5.2 Preparing the nucleic acid sample

Many steps in the process of phage editing described earlier require the transfer of PCR-derived plasmids into *Staphylococcus* cells. Some steps also require the transformation of plasmid "minipreps" into cells. Regardless of the source of nucleic acid being transferred, the samples must first be dialyzed before using them in an electroporation protocol. The following are the steps to dialyze plasmids prior to transformation:

1. If the sample is a PCR-derived plasmid (ligated inverse PCR product or Gibson assembly product), dilute the sample 1:1 with nuclease-free water. If the sample is a plasmid miniprep, use 2–3 μg of miniprep and dilute to a final volume of 40 μL with nuclease-free water.
2. Float a 0.02-μm filter disc (13 mm diameter) in a petri dish filled with distilled water, and carefully pipette the entire sample onto the center of the filter disc.
3. Allow the sample to dialyze (i.e., float on the disc) for 20 min and aspirate the sample. It may be placed into a sterile tube and saved for later or placed directly into the tube of competent cells for electroporation.

5.3 Transforming bacteria using electroporation

The following protocol describes how to transform *S. epidermidis* and *S. aureus* cells with dialyzed nucleic acid samples using electroporation:

1. Thaw the competent cells on ice for 5 min, and then at room temperature for an additional 5 min (this step can be skipped if freshly prepared competent cells are being used).

2. Centrifuge the competent cells at 4000 × *g* at 4°C for 1 min, discard the supernatant, and resuspend the pellet in 50 μL of 10% glycerol containing 500 m*M* sucrose.
3. Mix the entire amount of the dialyzed sample with the competent cells and transfer the mixture into a 2 mm electroporation cuvette (VWR). Prepare a negative control without DNA sample added.
4. Electroporate cells using the following program: voltage, 2100 V; capacitance, 25 μF; and resistance, 100 Ω.
5. Immediately following the electric pulse, add 1 mL of sterile TSB containing 500 mM sucrose into the cuvette.
6. Transfer the media containing electroporated bacterial cells into a 15-mL conical tube and allow the cells to recover by incubating at 37°C for 1 h with agitation.
7. Plate 200 μL of the recovered culture on selective media and incubate plates at 37°C overnight.
8. On the next day, score for colonies. The negative control plate should have none, while a typical *S. epidermidis* or *S. aureus* transformation should have tens or hundreds of colonies, depending on the quality and concentration of the plasmid added to the cells.

6. Conclusions

The protocols described herein are expected to advance the basic understanding of *Staphylococcus* phages while enabling the development of more powerful phage-based antimicrobials. Staphylococci are leading causes of drug-resistant infections (Furuya & Lowy, 2006) and phages that infect and destroy these organisms have tremendous therapeutic potential (Borysowski, Łobocka, Międzybrodzki, Weber-Dqbrowska, & Górski, 2011). Virulent phages from both lytic *Staphylococcus* phage families (*Myoviridae* and *Podoviridae*) have been successfully edited using these protocols with 100% recovery of desired mutations in putative recombinants, thus demonstrating that CRISPR–Cas10 constitutes a powerful tool to engineer *Staphylococcus* phages (Bari et al., 2017). Since Type III CRISPR–Cas systems are relatively widespread across bacterial and archaeal *phyla* (Koonin et al., 2017), this general approach is expected to be applicable to phages that infect diverse hosts.

Funding

A.H.-A. would like to acknowledge funding from the National Institutes of Health [K22AI113106], an NSF CAREER award [No. 1749886], and start-up funds from the University of Alabama.

References

Bari, S. M. N., Walker, F. C., Cater, K., Aslan, B., & Hatoum-Aslan, A. (2017). Strategies for editing virulent staphylococcal phages using CRISPR-Cas10. *ACS Synthetic Biology*, *6*(12), 2316–2325. https://doi.org/10.1021/acssynbio.7b00240.

Bergh, O., Børsheim, K. Y., Bratbak, G., & Heldal, M. (1989). High abundance of viruses found in aquatic environments. *Nature*, *340*(6233), 467–468.

Borysowski, J., Łobocka, M., Międzybrodzki, R., Weber-Dqbrowska, B., & Górski, A. (2011). Potential of bacteriophages and their Lysins in the treatment of MRSA. *BioDrugs*, *25*(6), 347–355.

Breitbart, M., & Rohwer, F. (2005). Here a virus, there a virus, everywhere the same virus? *Trends in Microbiology*, *13*(6), 278–284. https://doi.org/10.1016/j.tim.2005.04.003.

Brüssow, H., Canchaya, C., Hardt, W., & Bru, H. (2004). Phages and the evolution of bacterial pathogens: From genomic rearrangements to lysogenic conversion. *Microbiology and Molecular Biology Reviews*, *68*(3), 560–602. https://doi.org/10.1128/MMBR.68.3.560.

Cater, K., Dandu, V. S., Bari, S. M. N., Lackey, K., Everett, G. F. K., & Hatoum-Aslan, A. (2017). A novel staphylococcus podophage encodes a unique lysin with unusual modular design. *mSphere*, *2*(2), 1–9. https://doi.org/10.1128/mSphere.00040-17.

D'Herelle, F. (1917). Sur un microbe invisible antagoniste des bacilles dysentériques. *Comptes Rendus Académie des Sciences*, *165*, 373–375.

Furuya, E. Y., & Lowy, F. D. (2006). Antimicrobial-resistant bacteria in the community setting. *Nature Reviews Microbiology*, *4*, 36–45. https://doi.org/10.1038/nrmicro1325.

Gibson, D. G., Young, L., Chuang, R.-Y., Venter, J. C., Hutchison, C. A., & Smith, H. O. (2009). Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nature Methods*, *6*(5), 343–345.

Goldberg, G. W., Jiang, W., Bikard, D., & Marraffini, L. A. (2014). Conditional tolerance of temperate phages via transcription-dependent CRISPR-Cas targeting. *Nature*, *514*(7524), 633–637. https://doi.org/10.1038/nature13637.

Hatoum-Aslan, A. (2018). Phage genetic engineering using CRISPR–Cas systems. *Viruses*, *10*, 335. https://doi.org/10.3390/v10060335.

Hatoum-Aslan, A., Maniv, I., & Marraffini, L. A. (2011). Mature clustered, regularly interspaced, short palindromic repeats RNA (crRNA) length is measured by a ruler mechanism anchored at the precursor processing site. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(52), 21218–21222.

Hatoum-Aslan, A., Maniv, I., Samai, P., & Marraffini, L. A. (2014). Genetic characterization of antiplasmid immunity through a type III-A CRISPR-cas system. *Journal of Bacteriology*, *196*(2), 310–317.

Hatoum-Aslan, A., Samai, P., Maniv, I., Jiang, W., & Marraffini, L. A. (2013). A ruler protein in a complex for antiviral defense determines the length of small interfering CRISPR RNAs. *Journal of Biological Chemistry*, *288*(39), 27888–27897.

Jiang, W., Samai, P., & Marraffini, L. A. (2016). Degradation of phage transcripts by CRISPR-associated RNases enables type III CRISPR-Cas immunity. *Cell*, *164*(4), 710–721. https://doi.org/10.1016/j.cell.2015.12.053.

Koonin, E. V., Makarova, K. S., & Zhang, F. (2017). Diversity, classification and evolution of CRISPR-Cas systems. *Current Opinion in Microbiology*, *37*, 67–78.

Marraffini, L. A. (2015). CRISPR-Cas immunity in prokaryotes. *Nature*, *526*(7571), 55–61. https://doi.org/10.1038/nature15386.

Marraffini, L. A., & Sontheimer, E. J. (2008). CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA. *Science*, *322*(5909), 1843–1845. https://doi.org/10.1126/science.1165771.

Marraffini, L. A., & Sontheimer, E. J. (2010). Self vs. non-self discrimination during CRISPR RNA-directed immunity. *Nature*, *463*(7280), 568–571. https://doi.org/10.1038/nature08703.Self.

Molek, P., & Bratkovič, T. (2015). Bacteriophages as scaffolds for bipartite display: Designing Swiss army knives on a nanoscale. *Bioconjugate Chemistry*, *26*(3), 367–378. https://doi.org/10.1021/acs.bioconjchem.5b00034.

Ofir, G., & Sorek, R. (2018). Contemporary phage biology: From classic models to new insights. *Cell*, *172*(6), 1260–1270. https://doi.org/10.1016/j.cell.2017.10.045.

Pires, D. P., Cleto, S., Sillankorva, S., Azeredo, J., & Lu, T. K. (2016). Genetically engineered phages: A review of advances over the last decade. *Microbiology and Molecular Biology Reviews*, *80*(3), 523–543. https://doi.org/10.1128/MMBR.00069-15.

Pyenson, N. C., Gayvert, K., Varble, A., Elemento, O., & Marraffini, L. A. (2017). Broad targeting specificity during bacterial type III CRISPR-Cas immunity constrains viral escape. *Cell Host & Microbe*, *22*, 343–353.

Samai, P., Pyenson, N., Jiang, W., Goldberg, G. W., Hatoum-Aslan, A., & Marraffini, L. A. (2015). Co-transcriptional DNA and RNA cleavage during type III CRISPR-cas immunity. *Cell*, *161*(5), 1164–1174. https://doi.org/10.1016/j.cell.2015.04.027.

Singh, A., Poshtiban, S., & Evoy, S. (2013). Recent advances in bacteriophage based biosensors for food-borne pathogen detection. *Sensors (Switzerland)*, *13*(2), 1763–1786. https://doi.org/10.3390/s130201763.

Suttle, C. A. (2007). Marine viruses–major players in the global ecosystem. *Nature Reviews. Microbiology*, *5*(10), 801–812. https://doi.org/10.1038/nrmicro1750.

Twort, F. W. (1915). An investigation on the nature of ultra-microscopic viruses. *The Lancet*, *186*(4814), 1241–1243. https://doi.org/10.1016/S0140-6736(01)20383-3.

Waldor, M. K., & Mekalanos, J. J. (1996). Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science (New York, N.Y.)*, *272*(5270), 1910–1914. https://doi.org/10.1126/science.272.5270.1910.

Walker, F. C., Chou-Zheng, L., Dunkle, J. A., & Hatoum-Aslan, A. (2017). Molecular determinants for CRISPR RNA maturation in the Cas10–Csm complex and roles for non-Cas nucleases. *Nucleic Acids Research*, *45*, 2112–2123. https://doi.org/10.1093/nar/gkw891. pii: gkw89.

Wittebole, X., De Roock, S., & Opal, S. M. (2013). A historical overview of bacteriophage therapy as an alternative to antibiotics for the treatment of bacterial pathogens. *Virulence*, *5*(1), 226–235. https://doi.org/10.4161/viru.25991.

Zinder, N. D., & Lederberg, J. (1952). Genetic exchange in Salmonella. *Journal of Bacteriology*, *64*, 679–699.