Published in final edited form as:

ACS Synth Biol. 2017 December 15; 6(12): 2316–2325. doi:10.1021/acssynbio.7b00240.

Strategies for Editing Virulent Staphylococcal Phages Using CRISPR-Cas10

S. M. Nayeemul Bari, Forrest C. Walker, Katie Cater, Barbaros Aslan, and Asma Hatoum-Aslan*

Department of Biological Sciences, University of Alabama, Tuscaloosa, Alabama 35487, United States

Abstract

Staphylococci are prevalent skin-dwelling bacteria that are also leading causes of antibiotic-resistant infections. Viruses that infect and lyse these organisms (virulent staphylococcal phages) can be used as alternatives to conventional antibiotics and represent promising tools to eliminate or manipulate specific species in the microbiome. However, since over half their genes have unknown functions, virulent staphylococcal phages carry inherent risk to cause unknown downstream side effects. Further, their swift and destructive reproductive cycle make them intractable by current genetic engineering techniques. CRISPR-Cas10 is an elaborate prokaryotic immune system that employs small RNAs and a multisubunit protein complex to detect and destroy phages and other foreign nucleic acids. Some staphylococci naturally possess CRISPR-Cas10 systems, thus providing an attractive tool already installed in the host chromosome to harness for phage genome engineering. However, the efficiency of CRISPR-Cas10 immunity against virulent staphylococcal phages and corresponding utility as a tool to facilitate their genome editing has not been explored. Here, we show that the CRISPR-Cas10 system native to *Staphylococcus epidermidis* exhibits robust immunity against diverse virulent staphylococcal phages. On the basis of this activity, a general two-step approach was developed to edit these phages that relies upon homologous recombination machinery encoded in the host. Variations of this approach to edit toxic phage genes and access phages that infect CRISPR-less staphylococci are also presented. This versatile set of genetic tools enables the systematic study of phage genes of unknown functions and the design of genetically defined phage-based antimicrobials that can eliminate or manipulate specific *Staphylococcus* species.

Graphical abstract

*Corresponding Author: Tel: 205-348-4097. Fax: 205-348-1786. ahatoum@ua.edu.

ORCID

Asma Hatoum-Aslan: 0000-0003-2395-8900

Author Contributions

Author contributions: S.M.N.B., F.C.W., K.C., and B.A. performed experiments, S.M.N.B., F.C.W., B.A., and A.H.-A. designed experiments, A.H.-A. conceived the study and wrote the manuscript, and all authors have read, edited, and approved the manuscript.

Notes

The authors declare the following competing financial interest(s): A.H.-A. has filed a provisional application with the US Patent and Trademark Office on this work.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.7b00240. Supplementary Figures S1–S3 and Tables S1–S5 (PDF)

Keywords

Type III-A CRISPR-Cas; CRISPR-Cas10; bacteriophage; genome editing; staphylococci

Staphylococci are dominant residents of human skin that play critical roles in health and disease. *S. epidermidis* is a ubiquitous skin commensal that promotes health by educating the immune system and preventing colonization by more aggressive skin pathogens;^{1–4} however, this organism is also responsible for the majority of infections associated with medical implants.⁵ *S. aureus* can cause a range of antibiotic-resistant infections, from moderate to fatal, in a variety of body sites,⁶ and asymptomatic nasal carriage in about one-third of the population constitutes a major risk factor for more serious, invasive infections.^{7–9} Since the declining discovery rate of new antibiotics cannot keep up with the rate at which these bacteria acquire resistance, the development of alternatives to conventional antibiotics has become imperative. Furthermore, the opposing impacts of related *Staphylococcus* species underscore the critical need for antimicrobials with exquisite specificity.

Bacterial viruses (phages) attack a single host or subset of related hosts within the same genus,¹⁰ making them ideal for use as precision antimicrobials. Staphylococcal phages are classified into three morphological families and harbor discrete genome lengths: *Podoviridae* (<20 kb), *Siphoviridae* (~40 kb), and *Myoviridae* (>125 kb).¹¹ While over 68 staphylococcal phages have been sequenced to date,^{11,12} the majority exhibit a temperate lifestyle that is unsuitable for antimicrobial applications. Temperate staphylococcal phages, which belong to the family *Siphoviridae*, can integrate into the host chromosome and promote pathogenicity by mobilizing virulence factors and pathogenicity islands.^{13,14} Fewer than 30% of sequenced staphylococcal phages are naturally virulent, belonging to the families *Myoviridae* and *Podoviridae*.¹¹ These phages exhibit a swift reproductive cycle that destroys the host within minutes of infection. While optimal for antimicrobial applications,^{15,16} virulent staphylococcal phages also carry an inherent risk of eliciting detrimental side-effects—over half their genes have unknown functions,¹¹ and their molecular interactions with the bacterial host remain poorly understood. As examples of such side-effects, virulent phages have the potential to facilitate horizontal gene transfer,^{17,18} promote biofilm formation,¹⁹ and/or elicit unanticipated immune responses.²⁰ These issues are compounded by the need to use cocktails of diverse phages for antimicrobial applications to curb the emergence of phage-resistant pathogens.^{15,16} Thus, gaining a better understanding of virulent phages and engineering phage-based antimicrobials with well-defined genetic components are expected to alleviate safety concerns, regulatory constraints, and

manufacturing challenges associated with the implementation of whole-phage therapeutics.
21

Virulent staphylococcal phages are intractable by most current genetic engineering techniques.²² Classical strategies that rely solely on homologous recombination between the phage genome and a donor DNA construct are inefficient owing to low recombination rates and massive screening efforts required to recover the desired mutant.²³ Other strategies that involve the transformation of bacterial hosts with whole phage genomes^{24,25} are unsuitable for use in natural *Staphylococcus* isolates, which exhibit low/no competence.²⁶ However, recent reports have shown that CRISPR-Cas (Clustered regularly-interspaced short palindromic repeats-CRISPR-associated) systems in distinct bacteria can facilitate phage editing.^{27–30} CRISPR-Cas systems are a diverse class of prokaryotic immune systems that use small CRISPR RNAs (crRNAs) and Cas nucleases to detect and destroy phages and other nucleic acid invaders.^{31–36} In these systems, CRISPR loci maintain an archive of short (30–40 nucleotide) invader-derived sequences called “spacers” integrated between similarly sized DNA repeats. The repeat-spacer array is transcribed and processed to generate crRNAs that each specify a single target for destruction. CrRNAs combine with one or more Cas nucleases to form an effector complex, which detects and degrades nucleic acid sequences (called “protospacers”) complementary to the crRNA. CRISPR-Cas systems are remarkably diverse, with two broad Classes and six Types (I–VI) currently described.^{37,38} Types I and II CRISPR-Cas systems have recently been used in conjunction with homologous recombination to facilitate phage editing,^{27–30} however, the general applicability of this approach in other organisms using distinct CRISPR-Cas systems remains unknown.

In this study, we explored the utility of the Type III-A CRISPR-Cas system native to *S. epidermidis* RP62a (here onward called CRISPR-Cas10) as an engineering platform for virulent staphylococcal phages. This system has three spacers (*spcI–3*) and nine CRISPR-associated (*cas* and *csm*) genes (Figure 1a) that block plasmid transfer³⁶ and phage infection^{39,40} with a ribonucleoprotein complex called Cas10-Csm.⁴¹ This system degrades both DNA and RNA protospacers in a transcription-dependent manner,^{39,42} thus providing an opportunity for temperate phages to escape immunity and integrate peacefully into the host chromosome, provided their lytic genes remain silenced.³⁹ The efficiency of CRISPR-Cas10 immunity against naturally virulent staphylococcal phages and corresponding utility as a tool to facilitate editing of these phages has not been explored. Here, we describe a general two-step approach to harness CRISPR-Cas10 and host-encoded recombination machinery to edit virulent staphylococcal phages (Figure 1b, c). Variations of this approach were also developed to edit phage genes that are toxic to the host (such as genes that encode lysins, cell wall hydrolytic enzymes), and to show that a heterologous CRISPR-Cas10 system encoded on a plasmid can be used to edit phages that attack a *S. aureus* strain devoid of a natural CRISPR-Cas system. Additionally, in order to facilitate the design of CRISPR-Cas10 targeting constructs, a Python script was developed to identify all optimal protospacers in a given phage gene. This versatile set of genetic tools enables (i) the systematic study of genes of unknown function in staphylococcal phages and (ii) the design of phage-based antimicrobials with well-defined genetic components.

RESULTS AND DISCUSSION

CRISPR-Cas10 Elicits Robust Targeting of Virulent Staphylococcal Phages

The effectiveness of CRISPR-Cas10 as a counter-selection tool to facilitate virulent phage editing relies upon the efficiency at which this system can eliminate virulent phages. Therefore, we first tested CRISPR-Cas10 immunity against representatives from both virulent staphylococcal phage families: *Podoviridae* phage Andhra⁴³ and *Myoviridae* phage ISP.¹² Since the *S. epidermidis* CRISPR-Cas10 system lacks natural spacers targeting these phages, the system had to be reprogrammed to target Andhra and ISP. In order to reprogram CRISPR-Cas10 to recognize these phages, we used the plasmid *pcrispr*, which contains a single repeat-spacer unit from CRISPR-Cas10,⁴⁴ as a backbone to create a suite of *pcrispr/spc* plasmids, which encode single spacers that target a variety of protospacer loci spanning the genomes of Andhra and ISP (Figure 2a, b). Nine protospacer regions were selected (Table S1) according to the two criteria that permit the recognition and destruction of foreign DNA by Type III-A CRISPR-Cas systems.^{39,45} First, 35-nucleotide protospacers were selected with little or no complementarity between the “antitag” region adjacent to the protospacer and the corresponding eight-nucleotide tag sequence on the 5′-end of the crRNA (5′-ACGAGAAC). Second, protospacers were selected in coding regions, with corresponding crRNAs designed to bind to the coding DNA strand (and the mRNA). To test for a potential targeting bias toward genes transcribed early or late in the phage replication cycle, protospacers were selected in putative early genes (encoding DNA polymerases) and late genes (encoding cell wall hydrolytic enzymes) in both phages (Table S1). The resulting targeting plasmids (*pcrispr/spcA1*–*/spcA6* and *pcrispr/spcI1*–*/spcI3*) were introduced into *S. epidermidis* LAM104, a variant of *S. epidermidis* RP62a that lacks *spc1*–*3* of the native CRISPR locus.³⁶ *S. epidermidis* LAM104 strains harboring a *pcrispr/spc* plasmid are called “targeting strains” (Figure 1b). A control targeting strain bearing *pcrispr/spcI* was also included, which contains *spcI* of the native CRISPR locus (Table S1), a plasmid-targeting spacer unrelated to any known phage.³⁶

In order to test the efficiency of CRISPR-Cas10 immunity in the presence of each spacer, corresponding targeting strains were challenged with phages by spotting phage dilutions atop lawns of each strain. As expected, the control targeting strain bearing *pcrispr/spcI* remained susceptible to both Andhra (Figure 2c, d) and ISP (Figure 2e, f), as evidenced by the appearance of phage plaques. Equally susceptible was the targeting strain that harbored *pcrispr/spcA4*, which targets the same protospacer region as *pcrispr/spcA2*, but encodes crRNAs complementary to the noncoding (*i.e.*, template) strand of the protospacer. This observation is consistent with previous studies that showed CRISPR-Cas10 immunity only occurs in the presence of base-pair complementarity between the crRNA and the coding DNA strand, along with its corresponding mRNA.^{39,42} One exception seems to be *pcrispr/spcA3*, which targets a noncoding strand, yet still provides immunity. This could be explained by the presence of bidirectional transcription at the targeted locus due to leakage from the adjacent gene, which is transcribed in the opposite direction. Nonetheless, when coding strands are targeted, CRISPR-Cas10 affords complete protection against Andhra and ISP at all tested loci (Figure 2d, f), as evidenced by the absence of phage plaques, even in the presence the most concentrated phage lysate (10⁹ pfu/mL). Notably, spacers targeting

putative early genes (*spcA2* and *spcII*) and late genes (*spcA3* and *spcI3*) were equally effective in directing complete protection against phage infection.

The altogether absence of phages that naturally escape CRISPR-Cas10 immunity (CRISPR escaper mutants, or CEMs) is striking. CEMs are phages that have acquired random mutations in the protospacer and/or adjacent regions that allow escape from CRISPR-Cas immunity. The evolution of CEMs has been well documented in organisms that harbor Types I and II CRISPR-Cas systems.^{34,46,47} This occurs because immunity in these systems relies upon perfect complementarity between the crRNA and protospacer in a short (6–8 nucleotide) seed sequence^{47–49} and a protospacer adjacent motif (PAM).⁵⁰ Therefore, even a single nucleotide substitution within the seed or PAM can allow phages to naturally escape interference without acquiring the desired mutations. The appearance of CEMs was observed at varying frequencies when Types I and II CRISPR-Cas systems were used to edit phages.^{27–30} In contrast, neither a PAM nor a seed sequence has been identified for CRISPR-Cas10.^{40,45} This system is also extremely tolerant to mismatches between the crRNA and protospacer during antiphage immunity.⁴⁰

CRISPR-Cas10 Immunity Facilitates the Recovery of Virulent Phage Recombinants

The efficient immune response that CRISPR-Cas10 mounts against Andhra and ISP, and consequent failure of these phages to naturally escape immunity, suggest this system could provide a robust counter-selection mechanism to facilitate recovery of phage recombinants that have acquired desired mutations from a donor DNA construct. To test this, we introduced donor DNA constructs (called “donor sequences”) into targeting plasmids *pcrispr/spcA2* and *pcrispr/spcII*, which encode crRNAs that specify immunity against the DNA polymerase genes of Andhra (ORF 9) and ISP (ORF 61), respectively. The donor sequences are composed of 500 nucleotide homology arms flanking the protospacer with several silent mutations introduced into the protospacer region (Figure 3a, b). The silent mutations are designed to allow phage escape from CRISPR-Cas10 immunity and also add a unique restriction enzyme cut site. The *pcrispr/spcA2* and *pcrispr/spcII* plasmids were used as backbones to create *pcrispr/spcA2-donor* and *pcrispr/spcII-donor*, respectively. These plasmids, which contain both a targeting spacer and a donor sequence, were introduced into *S. epidermidis* LAM104. *S. epidermidis* LAM104 strains harboring a *pcrispr/spcφ-donor* plasmid are called “editing strains” (Figure 1c).

Direct plating of phages atop lawns of the corresponding editing strains failed to allow plaque formation (not shown); however, when editing strains were infected with their respective phages in liquid culture for as few as 2 min, plaques were observed (Figure 3c, d). Plaque numbers on the editing strains increased with time, likely due to multiple phage replication cycles occurring over the longer time periods. Importantly, no plaques resulted when the corresponding targeting strains were cocultured with phages under identical conditions, suggesting all phages replicating on the editing strains have likely acquired the mutations. To confirm this, 20 putative recombinants were selected for each phage, and their genomes were PCR amplified in regions encompassing the protospacers. PCR products from ten putative recombinants were subjected to digestion with the appropriate restriction enzymes (Figure 3e, f, and not shown), and the remaining ten were sequenced. Strikingly,

100% of selected plaques contained the intended mutations in exclusion of any others within the sequenced region (flanking 400+ nucleotides). To show this technique can be applied to distinct genetic loci transcribed in the opposite direction, Andhra ORF10 (a putative late gene) was edited using the same approach, and similar results were obtained (Figure S1). Recombination efficiencies overall were low (10^{-5} at best, Table S2), perhaps due to a kinetic advantage for CRISPR cleavage over recombination events at the targeted locus. Nonetheless, the more than 99% efficiency of CRISPR-Cas10 immunity against wild-type phages effectively revealed the rare recombinants.

Alternative Strategies to Edit Toxic Phage Genes

Since phage genomes encode proteins that are toxic to the bacterial host, (such as lysins, which degrade cell walls), such genetic loci might be refractory to overexpression on *pcrispr/spc ϕ -donor* plasmids, thus hampering this approach. To overcome this issue, we first determined the minimal homology arm length required to facilitate recombination. The plasmid *pcrispr/spcA2-donor*, which contains 500 nucleotide homology arms, was used as a backbone to create similar plasmids with 250, 100, or 35 nucleotide homology arms (Figure 4a). Coculturing Andhra with editing strains harboring these constructs showed that 100 nucleotides on either side of the protospacer were sufficient to facilitate homologous recombination (Figure 4b). This shorter length thus minimizes the length of phage-derived sequences needed in the *pcrispr/spc ϕ -donor* plasmids. We also investigated the use of this system to introduce mutations distal to the targeted region, which would allow more flexibility in the selection of phage-derived sequences to include in the *pcrispr/spc ϕ -donor* plasmids. To test this, *pcrispr/spcA2-donor/distal* was created, which bears silent mutations at regular intervals distal to the mutant protospacer (Figure 4c and Figure S2). The editing strain harboring this plasmid was cocultured with Andhra, and phages from 31 random plaques were sequenced across the donor region. As expected, 100% of the recombinant phages selected acquired the mutations at the protospacer in order to escape CRISPR-Cas10 immunity (Figure 4d and Table S3). Importantly, a subset of these phages also acquired distal mutations, up to 470 nucleotides from the protospacer. Notably, the mutations incorporated at position -470 occur in ORF10, which encodes a lytic enzyme that is toxic to *S. epidermidis* and *S. aureus* strains.⁴³ By minimizing the length of the donor sequence and allowing flexibility in the placement of the desired mutation(s) relative to the protospacer, these alternative strategies facilitate editing of toxic phage genes.

Editing *S. aureus* Phages with a Heterologous System

Since many staphylococci lack native CRISPR-Cas systems,⁵¹ we wondered if a heterologous CRISPR-Cas10 system would enable access to phages that attack CRISPR-less hosts. To test this, we created the targeting and editing plasmids *pcrispr-cas/spc ϕ* and *pcrispr-cas/spc ϕ -donor*, respectively. Both plasmids encode the *S. epidermidis* CRISPR-Cas10 system with deletions in *cas1* and *cas2*, which are dispensable for immunity.⁵² These plasmids were introduced into *S. aureus* RN4220, which is naturally devoid of a CRISPR-Cas system. A similar two-step approach was used to test the efficiencies of targeting and editing of phage ISP, which also replicates on *S. aureus*.¹² We observed that similarly to antiphage immunity in *S. epidermidis*, CRISPR-Cas10 affords robust protection against ISP when overexpressed in the *S. aureus* background (Figure S3a, b). Coculturing ISP with the

editing strain thus enabled the recovery of numerous recombinant phages (Figure S3c, d). Interestingly, the editing efficiency in *S. aureus* is 2–3 orders of magnitude lower than that observed in the native *S. epidermidis* background (Table S2), which could likely be due to differences in the homology-directed repair mechanisms in these two organisms. Nonetheless, the robust immunity mounted by CRISPR-Cas10 in this heterologous system effectively revealed the rare recombinants.

CRISPR-Cas10 Protospacers Are Densely Packed Across Phage Genomes

The results obtained thus far show CRISPR-Cas10 can be used as a powerful tool for phage genome editing. However, protospacer selection for CRISPR-Cas10 interference is subjected to at least two constraints: targeted regions must be (i) actively transcribed,³⁹ and (ii) harbor little or no complementarity between the antitag and the opposing 8-nucleotide tag on the 5'-end of crRNAs.⁴⁵ Since staphylococcal phage genomes are densely packed with coding sequences,¹¹ the former constraint is unlikely to constitute a severe limitation. However, we wondered if the requisite absence of complementarity between the crRNA 5'-tag and protospacer-adjacent antitag would limit access to significant regions of phage genomes. To test this, a Python script was developed to identify in a given gene all permissible 35-nucleotide protospacers that harbored zero complementarity between the protospacer adjacent antitag region and crRNA 5'-tag, which constitutes the strictest condition for a permissible protospacer. All 20 genes from Andhra and 20 genes from ISP (selected at random) were analyzed to identify all such protospacers that are predicted to be permissible for CRISPR-Cas10 interference. Strikingly, an average of 12.1 ± 2.8 and 12.8 ± 2.6 permissible protospacers were identified per 100 nucleotides of coding sequence in Andhra and ISP, respectively (Table S4). Notably, this value represents the minimum number of protospacers since some complementarity between the tag and antitag is tolerated (Table S1 and ref 45).

To date, CRISPR-Cas10 has remained underexplored for genetic applications, likely owing to its remarkable complexity. The transcription dependence of this system, which provides a mechanism for temperate phages to evade immunity, calls into question the utility of CRISPR-Cas10 as an editing tool for other types of phages. This work presents the first systematic study of CRISPR-Cas10 immunity against virulent staphylococcal phages and demonstrates CRISPR-Cas10 effectively facilitates the recovery of rare phage recombinants containing desired mutations. The set of genetic tools described herein thus enable the systematic study of genes of unknown function in virulent staphylococcal phages through the introduction of point mutations and premature stop codons. Importantly, since many staphylococci naturally possess CRISPR-Cas10 systems, or can express a functional system on a plasmid, these tools can be applied to phages that infect diverse hosts. Given that phage genomes are replete with protospacers that are permissible for CRISPR-Cas10 targeting (Table S4), and editing can also be accomplished up to 470 nucleotides distal from the protospacer (Figure 4), these tools enable virtually unrestricted access to the genome sequence space in virulent phages. To facilitate the implementation of this technique, the Python script that identifies all permissible CRISPR-Cas10 protospacers in a given gene and corresponding spacers to be cloned into targeting constructs has been made available at <https://github.com/ahatoum/CRISPR-Cas10-Protospacer-Selector>. It is anticipated that these

tools will become useful for applications beyond phage editing as CRISPR-Cas10 systems become increasingly appreciated for their unique properties and exploited for broader applications.

MATERIALS AND METHODS

Strains and Growth Conditions

S. epidermidis RP62a⁵³ and LAM104³⁶ were grown in Brain Heart Infusion broth (BHI) (Difco). *S. aureus* RN4220 was grown in Tryptic Soy Broth (TSB) (Difco). Media were supplemented with the following antibiotics as needed: 10 µg/mL chloramphenicol (for selection of *pcrispr* and *pcrispr-cas* based plasmids) and 15 µg/mL neomycin (for selection of *S. epidermidis*). Phage Andhra was discovered in-house,⁴³ and phage ISP was a generous gift from Luciano Marraffini. For phage propagation, *S. epidermidis* was grown in BHI plus 5 mM CaCl₂ to an early logarithmic phase at 37 °C with shaking. Phages were added at a multiplicity of infection (MOI) of 0.1 and incubated for an additional 6 h at 37 °C. The culture was pelleted at 8000g for 5 min and the supernatant was filtered through a 0.45 µm filter. Phages were enumerated by spotting 10-fold dilutions on Heart Infusion Agar (HIA) (Hardee Diagnostics) containing overnight cultures of *S. epidermidis* (1:100 dilution) and 5 mM CaCl₂ overlaid atop Tryptic Soy Agar (TSA) (Difco) plates also containing 5 mM CaCl₂. High titer phage lysates were maintained at 4 °C.

Spacer Design

Spacers *A1*, *A2*, *A5*, *A6*, and *II–I3* (Table S1) were designed in accordance with the two criteria that are essential for the targeting of foreign DNA by the Type III-A CRISPR-Cas system.^{39,45} Briefly, spacers were designed to target protospacer regions that bore little or no complementarity between the eight nucleotide tag on the 5′-end of the crRNA (5′-ACGAGAAC) and the corresponding “antitag” region adjacent to the protospacer, especially in the −4, −3, and −2 positions (5′-GAA). In addition, spacers were designed to encode crRNAs with base-pair complementarity with the coding strand (as well as the corresponding mRNA.) As negative controls, spacers *A3* and *A4* were deliberately designed to defy the latter rule—these targeted the putative noncoding (template) strand. Nonetheless, *spcA3* permitted efficient immunity, likely due to bidirectional transcription at the targeted locus (see main text for details).

Construction of *S. epidermidis* Targeting Strains

Spacers were introduced into targeting plasmids with inverse PCR using *pcrispr*⁴⁴ as template and the primers listed in Table S5. Following PCR, products were purified using the EZNA Cycle Pure Kit (Omega). Purified PCR products were 5′ phosphorylated by T4 polynucleotide kinase (NEB) and circularized by T4 DNA ligase (NEB). Ligated constructs were first transformed into *S. aureus* RN4220, a passage strain, *via* electroporation and selected on TSA supplemented with chloramphenicol. Several transformants were checked for the presence of the appropriate spacer by colony PCR and subsequent sequencing of PCR products using primers A200 and F016 (Table S5). Confirmed *pcrispr/spcφ* constructs were purified using the EZNA Plasmid Miniprep Kit (Omega) and transformed into *S. epidermidis* LAM104 for targeting experiments.

Construction of *S. epidermidis* Editing Strains

Donor plasmids *pcrispr/spcA2-donor* and *pcrispr/spcA3-donor* were created in two steps using Gibson assembly⁵⁴ and inverse PCR with primers indicated in Table S5. Briefly, Gibson assembly was first used to introduce wild-type phage-derived sequences into *pcrispr/spcφ* constructs to make *pcrispr/spcφ-Andhra* constructs. To do this, PCR products were generated using *pcrispr/spcφ* constructs as templates for the backbone and phage genomic DNA as template for the inserts using primers N057–N060 (for *pcrispr/spcA2-Andhra*) and N124–N127 (for *pcrispr/spcA3-Andhra*). PCR products were purified as above and Gibson assembled. Assembled constructs were transformed into *S. aureus* RN4220 by electroporation. Transformants were confirmed for the presence of the phage-derived sequences by colony PCR and sequencing of PCR products using primers A200 and F016. In the second step, inverse PCR (as described above) was used to introduce silent mutations into confirmed *pcrispr/spcφ-Andhra* constructs using primers N055 and N056 (for *pcrispr/spcA2-donor*) and N144 and N145 (for *pcrispr/spcA3-donor*). To create donor plasmid *pcrispr/spcII-donor*, a 3-part Gibson assembly was performed with *pcrispr/spcII* as template for the backbone, phage ISP DNA as template for the two inserts, and primers F316–F321 (Table S5). To create Andhra donor plasmids with varying homology arm lengths, plasmid *pcrispr/spcA2-donor* was used as a template to create plasmids *pcrispr/spcA2-donor/250* and *-donor/100* by Gibson assembly with primers N114–N117, and N118–N121, respectively (Table S5). Plasmid *pcrispr/spcA2-donor/35* was created by inverse PCR using *pcrispr/spcA2* as template and primers N061 and N062 (Table S5). Plasmid *pcrispr/spcA2-donor/distal* was created by a two-piece Gibson assembly using *pcrispr/spcA2-donor* as a template for the backbone, synthetic construct A454 (Invitrogen, Figure S2) as template for the donor sequence, and primers N057–N060 (Table S5). All ligated/Gibson assembled donor plasmids were transformed first into *S. aureus* RN4220. Several transformants were checked for the presence of desired constructs using colony PCR and sequencing, and confirmed plasmids were purified and introduced into *S. epidermidis* LAM104 for editing experiments.

Construction of *S. aureus* Targeting and Editing Strains

The *pcrispr-cas/spcI* plasmid was constructed with Gibson assembly using primers listed in Table S5, which were used to combine the *cas* genes from *pcrispr-cas/ cas1 cas2*⁶² (PCR amplified with primers F065 and F066) with the single repeat-spacer unit and plasmid backbone of pGG3³⁹ (PCR amplified with primers F064 and F067), thus generating a single repeat/spacer CRISPR array in a *cas1/2* background. The *pcrispr-cas/spcII* targeting plasmid was created by Gibson assembly, which was used to assemble *spcII* from *pcrispr/spcII* (amplified with PCR primers F354 and F355) with the backbone of *pcrispr-cas/spcI* (amplified with PCR primers F060 and F353). The *pcrispr/spcII-donor* editing plasmid was created by Gibson assembly using the *pcrispr-cas/spcII* plasmid as backbone (amplified with primers F367 and F370) and the recovery sequence from *pcrispr/spcII* (amplified with primers F368 and F369). All assembled constructs were transformed into *S. aureus* RN4220 and their sequences were confirmed *via* colony PCR and sequencing with primers A405 and F064. *S. aureus* RN4220 strains with confirmed constructs were used in targeting and editing experiments.

Phage Targeting and Genome Editing

To test the efficiency of targeting by *pcrispr/spcφ* or *pcrispr-cas/spcφ* plasmids, overnight cultures of targeting strains were diluted 1:40 in HIA top agar plus 5 mM CaCl₂. The mixture was overlaid atop a TSA plate containing 5 mM CaCl₂. After allowing the top agar to set (~10 min at room temperature), 10-fold serial dilutions of targeted phages were spotted on the top agar, and phage lysate drops were allowed to dry at room temperature ~15 min. Plates were incubated overnight at 37 °C, and phage plaques were enumerated the following day. To test the efficiency of phage editing in the presence of various donor plasmids, editing strains were combined with their appropriate phages at MOI = 1 and cocultured for indicated times at 37 °C without shaking. As controls, corresponding targeting strains were also cocultured under the same conditions. Phage-host mixtures were diluted 1:20 in HIA top agar plus 5 mM CaCl₂, and then overlaid atop TSA plates containing 5 mM CaCl₂. Top agar was allowed to set and plates were incubated overnight at 37 °C. Plaques were enumerated the following day. All experiments were conducted in triplicate.

Genome Extraction and Confirmation of Recombinant Phages

To confirm the presence of desired mutations in putative recombinant phages, 20 plaques were selected from the phage-editing strain coculture plates. Individual plaques were picked from the top agar, placed into 500 μ L of TSB, and vortexed for 1 min to extract phages from plaques. Phages released into the supernatant were propagated by incubating with the corresponding targeting strains (1:100 dilution of overnight culture) for 6 h in BHI plus 5 mM CaCl₂. Cells were pelleted, and phage lysates were passed through 0.45 μ m filters. Filtered lysates were combined 1:1 with phenol, chloroform, isoamyl alcohol (25:24:1) and vortexed for 1 min. Mixtures were centrifuged at 17 000g for 5 min, and aqueous layers were recovered into a fresh tube. Aqueous layers were then mixed with 100% ethanol (2.5 vols) and 3.0 M Na-acetate pH 5.2 (1/10 vol). Samples were kept in ice for 10 min and centrifuged at 17 000g for 5 min. DNA pellets were washed with 1 mL 75% ethanol and air-dried for 10 min. Pellets were dissolved in 30 μ L of distilled H₂O and used as templates for PCR amplification with primers N146 and N147 for Andhra or F317 and F319 for ISP (Table S5). Ten PCR products were subjected to digestion with appropriate restriction enzymes (as indicated in figure legends) and the remaining ten were sequenced using indicated primers (Table S5).

Python Script for Protospacer Selection

A Python script (MainScript.py) was developed that takes a gene sequence (in 5′–3′ direction) and crRNA 5′-tag (in 5′–3′ direction) as user inputs, and as outputs, produces all possible 35-nucleotide protospacers that exhibit zero complementarity between the protospacer adjacent antitag region and the crRNA 5′-tag. The reverse complement of the tag is first obtained to generate an eight-nucleotide comparison template. Within a loop, a window of eight nucleotides that progressively moves rightward is copied from the gene sequence and compared to the template derived from the user's tag. In this comparison, when corresponding nucleotides are “not” equal to each other, a logic true is produced. Then the results of these Boolean comparisons are subjected to a logic AND operation among

themselves, which yields true only when there is no match at any position between the nucleotides in the moving window and the comparison template. As the loop proceeds, each time the logic AND operation yields true, the beginning 5'-end coordinate of the moving window is recorded in an array with respect to the original gene sequence input, and a "possibility" counter is incremented by one. Each of these coordinates are required to be greater than 35 nucleotides into the gene (measuring from the 5'-end of the gene). Once this loop is completed, another loop begins, in which 35 nucleotides to the left of each recorded coordinate is extracted from the gene sequence—this is called the protospacer. The reverse complement of the protospacer is also generated as an output to indicate the corresponding spacer sequence that would need to be cloned into targeting and editing constructs. The Python source code and instructions to run the code are available at <https://github.com/ahatoum/CRISPR-Cas10-Protospacer-Selector>.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by start-up funds from the University of Alabama (UA) College of Arts and Sciences; a grant from the UA College Academy of Research, Scholarship, and Creative Activity (CARSCA); and a grant from the National Institutes of Health [5K22AI113106-02] awarded to A.H.-A.

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Figure 1.

A two-step approach for CRISPR-Cas10 assisted editing of virulent staphylococcal phages. The native *S. epidermidis* RP62a CRISPR-Cas locus (a) is composed of four repeats (white rectangles) three spacers (numbered rectangles) and nine CRISPR-associated *cas* and *csm* genes. Genes that encode members of the Cas10-Csm effector complex are indicated with a bracket. This system can be harnessed to facilitate phage genome editing in a two-step approach that involves the creation of a targeting strain (b) and an editing strain (c). In the first step, a plasmid called *pcrispr/spcφ* is constructed, which bears a single repeat and a spacer complementary to the phage of interest. This targeting construct is introduced into *S. epidermidis*, and the resulting *S. epidermidis*-*pcrispr/spcφ* strain is termed the targeting strain (b). The targeting strain is challenged with the phage by spotting phage lysate on top agar overlays to confirm that the selected spacer indeed protects against phage infection *via* CRISPR-Cas10 immunity. In the second step, *pcrispr/spcφ* plasmids that elicit efficient immunity are used as a backbone to construct *pcrispr/spcφ-donor* plasmids (c). Donor plasmids retain the targeting spacer, and have an additional phage-derived “donor” sequence (green rectangle), which bears desired mutations in the protospacer region (magenta stripes) flanked by sequences (>100 nucleotides) homologous to the phage genome on both sides. This donor construct is introduced into *S. epidermidis*, and the resulting *S. epidermidis*-*pcrispr/spcφ-donor* strain is termed the editing strain (c). This strain is combined with phages in liquid culture for various amounts of time, during which Cas10-Csm cleavage of the phage genome stimulates homology-directed repair (dashed lines) using the donor region in *pcrispr/spcφ-donor* as a repair template. Having incorporated the desired mutations, recombinant phage genomes can thus escape further cleavage and complete the infection cycle. The CRISPR-Cas10 system native to *S. epidermidis* LAM104, a derivative of RP62a with a deletion in *spc1–3* of the CRISPR locus,³⁶ was used as the background to create both the targeting and editing strains shown in the main figures of the paper. Phage editing was

also conducted in a *S. aureus* RN4220 background by cloning the *S. epidermidis* CRISPR-Cas10 system on a plasmid and using a two-step approach similar to the one described above (data shown in Figure S3).

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Figure 2. CRISPR-Cas10 elicits robust immunity against virulent phages at multiple genetic loci. Schematic representations of genomes of phages Andhra (a) and ISP (b). Genome coordinates are indicated on top, and open reading frames (ORFs) transcribed in the rightward and leftward directions are indicated with colored arrows (magenta and green, respectively). Spacers that were tested in this study (*spcA1-A6* and *spcI1-I3*) are indicated with black arrows in the shaded track below each targeted ORF. Targeting *S. epidermidis* strains harboring indicated *pcrispr/spc* plasmids were challenged with phages Andhra (c, d) and ISP (e, f) by spotting 10-fold dilutions of each phage atop lawns of corresponding targeting strains. Panels (c) and (e) show a representative plate, while panels (d) and (f) show an average number of plaque forming units (pfu) per milliliter in three independent trials ($\pm$ S.D.). Where bars are absent, pfu/mL was below the limit of detection.

Figure 3.

CRISPR-Cas10 enables the recovery of recombinant phages. The protospacer regions of Andhra ORF9 (a) and ISP ORF 61 (b) are shown in the 5′–3′ direction. The wild-type sequence appears on top, and mutant variants included in donor plasmids appears below. Mutated nucleotides are shown in magenta and restriction enzyme recognition and cut sites added with the mutations are highlighted in green and indicated with an arrow, respectively. Editing *S. epidermidis* strains harboring indicated *pcrispr/spcφ-donor* plasmids were cocultured with phages Andhra (c) and ISP (d) for varying amounts of time as shown. As controls, targeting *S. epidermidis* strains harboring indicated *pcrispr/spcφ* plasmids were cocultured with appropriate phages in parallel for the same amounts of time. Following the coculturing period, phage-host mixtures were plated and plaques were enumerated on the following day. Experiments were carried out in triplicate and average pfu/mL (±S.D.) are shown for targeting strains (black bars) and editing strains (gray bars). Where bars are absent, pfu/mL was below the limit of detection. (e, f) Ten plaques were selected from each 60 min coculture plate (with the editing strains), and phage genomes were purified, PCR amplified across the edited region, and PCR products were subjected to digestion with indicated restriction enzymes. Digests were resolved on a 1% agarose gel and visualized with ethidium bromide. Restriction digests from eight out of ten selected plaques for phages Andhra (e) and ISP (f) are shown. Wild-type phages were included as a negative control, and uncut and cut DNA fragments are indicated with arrows/brackets.

Figure 4.

Alternative approaches to facilitate editing of phage-derived toxic genes. (a) Variants of *pcrispr/spcA2-donor* plasmids with homology arm lengths of 500, 250, 100, or 35 nucleotides are shown. (b) Editing strains harboring *pcrispr/spcA2-donor* plasmids with indicated homology arm lengths were cocultured with phage Andhra for 60 min, and resulting plaques were enumerated (gray bars). The experiment was carried out in triplicate and average pfu/mL ($\pm$ S.D.) are shown. (c) A variant of the *pcrispr/spcA2-donor* plasmid called *pcrispr/spcA2-donor/distal* is shown, which contains silent mutations at regular intervals from the protospacer region. Positions of mutations are shown with magenta bars (refer to Figure S2 for the sequence). (d) An editing *S. epidermidis* strain bearing this plasmid was cocultured with phage Andhra for 60 min and the mixture was plated. On the following day, 31 plaques were selected, phage genomes were extracted and PCR amplified across the donor sequence region, and scored for the presence or absence of silent mutations at each position (refer to Table S3 for breakdown of mutations per phage). Shown are the fraction of phages that acquired mutations at each position.