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Rapid and Efficient Genome Editing in *Staphylococcus aureus* by Using an Engineered CRISPR/Cas9 System

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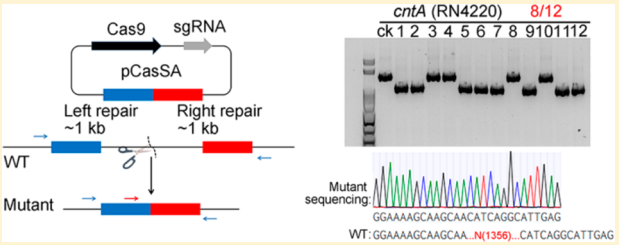
Supporting Information

ABSTRACT: *Staphylococcus aureus*, a major human pathogen, has been the cause of serious infectious diseases with a high mortality rate. Although genetics is a key means to study *S. aureus* physiology, such as drug resistance and pathogenesis, genetic manipulation in *S. aureus* is always time-consuming and labor-intensive. Here we report a CRISPR/Cas9 system (pCasSA) for rapid and efficient genome editing, including gene deletion, insertion, and single-base substitution mutation in *S. aureus*. The designed pCasSA system is amenable to the assembly of spacers and repair arms by Golden Gate assembly and Gibson assembly, respectively, enabling rapid construction of the plasmids for editing. We further engineered the pCasSA system to be an efficient transcription inhibition system for gene knockdown and possible genome-wide screening. The development of the CRISPR/Cas9-mediated genome editing and transcription inhibition tools will dramatically accelerate drug-target exploration and drug development.



INTRODUCTION

Staphylococcus aureus, one of the major human pathogens, causes a variety of infectious diseases, ranging from minor skin infections to life-threatening diseases, such as necrotizing pneumonia, endocarditis, and toxic shock syndrome.[1,2] The success of this pathogen in infection is largely attributed to its sophisticated regulatory pathways to coordinately express virulence factors.[3−5] Moreover, the emergence of antibiotic-resistant strains, such as methicillin-resistant and vancomycin-resistant *S. aureus*, has rendered the bacteria as the cause of the infectious diseases with the highest mortality rate in the United States since 2007,[6,7] emphasizing a dire need for novel drug-target exploration and drug development.

Access to unexplored drug targets would benefit greatly from the development of novel genome editing and screening tools, which would enable efficient discovery and validation of uncharacterized genes and pathways responsible for bacterial virulence and drug resistance. Nevertheless, although several allele-replacement plasmid systems (e.g., pMAD, pKOR1, and pIMAY) have been developed and widely utilized, genetic manipulation of *S. aureus* is always time-consuming and labor-intensive.[8−10] Typically, knocking out a gene in *S. aureus* requires two separated crossover steps (left in Figure 1). First, the editing plasmid is integrated into the target locus by homologous recombination, which is achieved by incubating the cells at a nonpermissive temperature (e.g., 37 or 42 °C). Second, the excision of the integrated plasmid is promoted by growing the cells at a permissive temperature (e.g., 30 °C), and the loss of the editing plasmid in the genome is facilitated by a counter selection method (e.g., expression of anti-*secY* RNA).

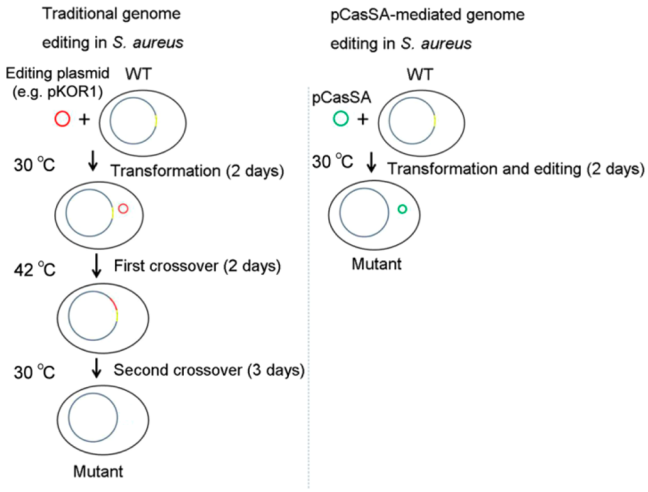


Figure 1. Comparison of the traditional genome editing method and the CRISPR/Cas9-mediated genome editing method in *S. aureus*.

The overall process often requires 1 week, or even longer if any failure occurs in the aforementioned steps.

Fortunately, the recent discovery of the CRISPR/Cas9 system provides a simple, sequence-specific platform for generating a double-strand DNA break in the target genome, and therefore, it is possible to select the double-crossover events in one step (right in Figure 1).[11−18] The CRISPR/Cas9

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system was originally discovered in the bacterial immune system and is one of the key means utilized by bacteria to fight against invading viruses. The system requires three components for successful cleavage of foreign DNAs: the CRISPR RNA (crRNA), the trans-activating RNA (tracrRNA), and the Cas9 protein. The crRNA is the spacer in the CRISPR array that directly targets the DNA locus to ensure specificity, while the tracrRNA is a short RNA that forms a complex with crRNA and recruits the Cas9 protein for editing.[19] To further simplify the system, the crRNA and tracrRNA are fused together to generate a single synthetic guide RNA (sgRNA), which has been demonstrated to exhibit editing efficiencies similar to those using the separated RNAs. The Cas9 protein is an RNA-guided endonuclease that cleaves the target DNA. It consists of a nuclease (NUC) lobe as well as an α-helical recognition (REC) lobe (Figure 2a).[20,21] The NUC lobe contains three domains: the HNH nuclease domain, which cleaves the target strand of DNA (complementary to the guide RNA); the RuvC-like nuclease domain, which cleaves the nontarget DNA strand; and a protospacer-adjacent motif (PAM)-interacting domain (PI domain), which contributes to the DNA target specificity of Cas9 through base-specific interactions with the PAM region of DNA. The helical REC lobe contains regions that play a role in the recognition of guide RNA−target DNA heteroduplexes and cognate sgRNA scaffolds. As shown in Figure 2b, the sgRNA forms a complex with the Cas9 protein and directs the Cas9 protein to the genomic DNA locus by base-pairing with the sequence that is adjacent to a PAM. The Cas9/sgRNA complex creates a double-strand DNA break within the base-pairing region, stimulating the DNA repair pathway. Sequence-specific deletions, point mutations, or insertions can be achieved through recombination of the target locus with exogenously supplied DNA "donor templates" during the process of homology-directed repair. Thus, simply by customizing an approximately 20 nucleotide (nt) region of the sgRNA, the CRISPR/Cas9 system is capable of targeting any sites of interest in the genome when a PAM is present in the DNA sequence (NGG in the case of *Streptococcus pyogenes* CRISPR/Cas9),[11] making it an easily programmable tool for specific genome editing.

As evidenced by the successes of using CRISPR/Cas9 for genome editing in a variety of organisms, such as mammalian cells,[13] *Escherichia coli*,[22] and *Saccharomyces cerevisiae*,[23] we envisioned that the CRISPR/Cas9 system would enable rapid and efficient genome editing in *S. aureus* via engineering of the system to be adaptable in this organism.

In the present work, we have developed a CRISPR/Cas9-mediated genome editing tool (pCasSA) in *S. aureus*. The technique enables rapid and efficient genome editing, including gene deletion, single-base substitution mutation, and gene insertion in *S. aureus*. By mutation of the active sites of Cas9 protein, Asp10 and His840, to Ala, the pCasSA system was further developed to be a highly efficient transcription-inhibition system (pCasiSA), which will be an effective tool for fast and accurate screening of genes and pathways of interest in *S. aureus*.

RESULTS AND DISCUSSION

To harness the CRISPR/Cas9 system for genome editing in *S. aureus*, we designed and constructed the CRISPR/Cas9 expression plasmid pCasSA (Figure 3a). A well-studied Cas9 protein, *Streptococcus pyogenes* Cas9 (SpCas9), was chosen to construct the pCasSA system.[20,21] Inspired by the successful

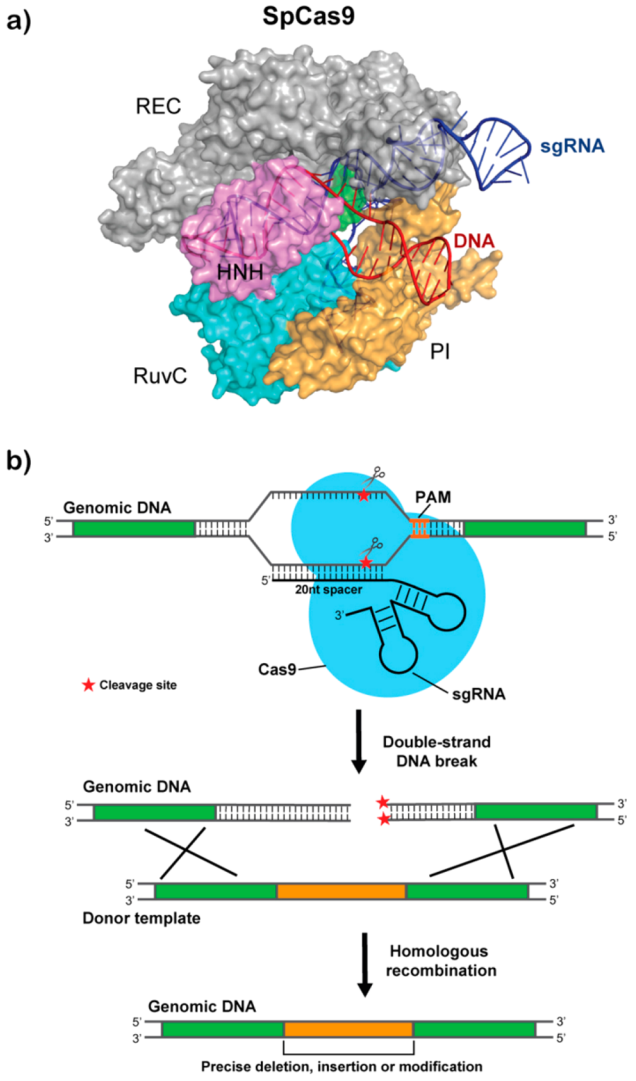

Figure 2. Crystal structure of the Cas9 protein and the scheme of Cas9-mediated sequence-specific genome editing. (a) Crystal structure of *Streptococcus pyogenes* Cas9 (SpCas9; PDB ID 4UN3). The domains of the SpCas9 protein are annotated, and the orientation of the target DNA strand and the sgRNA are also shown. (b) Detailed mechanism of CRISPR/Cas9-mediated genome editing in bacteria. The Cas9/sgRNA complex creates a double-strand DNA break in the genome. To survive, the bacterial cells have to undergo a homologous-recombination-based repair process with the utilization of donor templates. In theory, only the cells whose genomes are repaired can survive after editing.

development of the CRISPR/Cas9-mediated genome editing method in *Streptomyces* species, in which the expressions of Cas9 protein and sgRNA are driven by a strong *rpsL* promoter,[24] we utilized an *S. aureus rpsL* promoter to drive the expression of the Cas9 protein. Because the transcription start site of the *rpsL* promoter in *S. aureus* has not been determined and the presence of extra nucleotides at the 5′ end of sgRNA may reduce the targeting efficiency, we used the well-studied strong promoter *cap 1A* instead of the *rpsL* promoter to drive the expression of sgRNA.[25] One striking feature of this system is the presence of two seamless cloning sites (Figure 3b): the *Bsa*I sites are used for one-step assembly of spacers by Golden Gate assembly,[26] and the *Xba*I and *Xho*I sites are used for one-step Gibson-assembly-mediated cloning of repair arms

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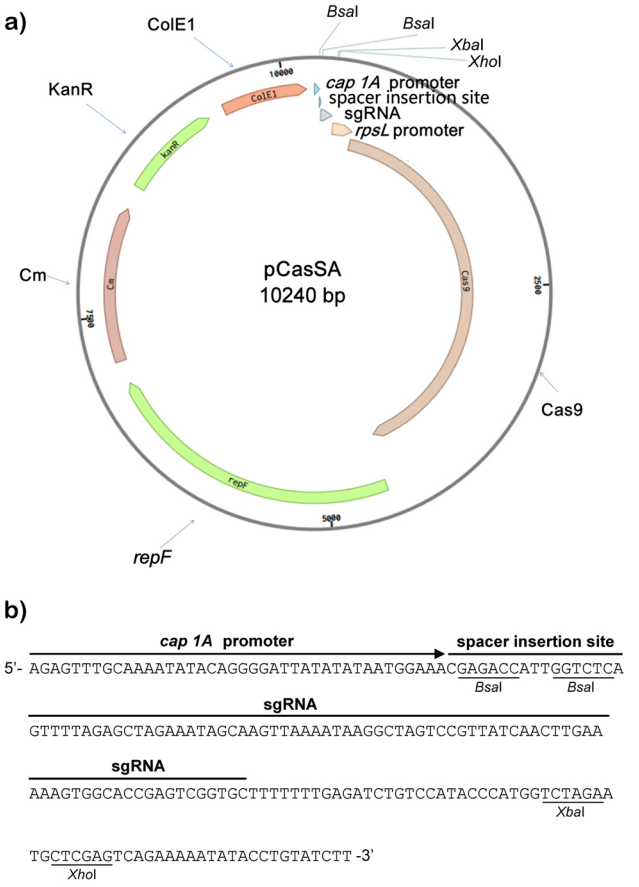

Figure 3. Map and the coloning sites of the pCasSA plasmid. (a) Map of the pCasSA plasmid. *Bsa*I sites, Golden Gate assembly of spacers; *Xba*I and *Xho*I sites, Gibson assembly of repair arms; *cap 1A* promoter, the sgRNA expression promoter; *rpsL* promoter, the Cas9 protein expression promoter; *repF*, an *S. aureus* temperature-sensitive origin for plasmid curing after editing; *Cm*, the chloramphenicol-resistance marker in *S. aureus*; KanR, the kanamycin-resistance marker in *E. coli*; ColE1, a replication origin for *E. coli*. (b) Sequence of the cloning sites of the pCasSA plasmid.

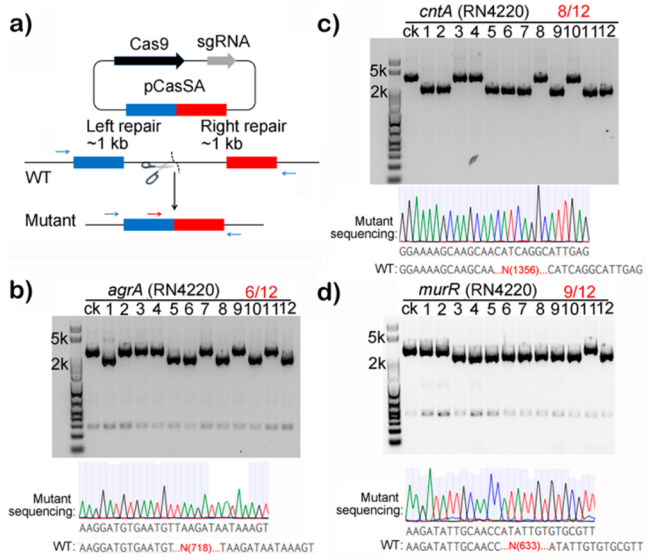

Figure 4. CRISPR/Cas9-mediated genome editing in the *S. aureus* RN4220 strain. (a) Schematic illustration of the editing procedures. The blue arrows are the primers utilized for PCR validation of the editing efficiency. The red arrow is the primer used for sequencing. (b) pCasSA-mediated disruption of the *agrA* gene in the RN4220 strain. The editing efficiency was 6/12. The lane labeled "ck" is the PCR product from the wild-type strain as a control. (c) pCasSA-mediated disruption of the *cntA* gene in the RN4220 strain. The editing efficiency was 8/12. (d) pCasSA-mediated disruption of the *murR* gene in the RN4220 strain. The editing efficiency was 9/12.

for homologous-recombination-mediated repair after double-strand DNA break.[27] We also introduced a temperature-sensitive origin, *repF*, in this system, which may enable fast curing of this plasmid after editing.[9]

To test the functionality of the pCasSA system, we first transformed an empty pCasSA plasmid as well as a 20 base pair (bp) spacer-introduced pCasSA plasmid (pCasSA-*agrA*_spacer: the spacer can target the *agrA* gene) into a laboratory *S. aureus* strain RN4220. The introduction of the *agrA* spacer will produce an intact sgRNA that can direct the Cas9 endonuclease to the *agrA* gene locus. The CRISPR/Cas9 complex will then generate a double-strand DNA break at that locus of the genome, leading to the death of the bacterial cells. Consistent with our expectation, more than 10^3 colonies were obtained for a single transformation with the empty plasmid, whereas fewer than 10 colonies could be observed for the same transformation with the plasmid containing the spacer (Figure S1), strongly indicating the high efficiency of CRISPR/Cas9-mediated *S. aureus* genome cleavage. We then assembled the repair arms (~1 kb each) into the spacer-introduced plasmid to assess the editing efficiency (Figure 4a). As shown in Figure 4b, the efficiency of deleting the *agrA* gene by this system is ~50% (6/12), as confirmed by both polymerase chain reaction (PCR) screening and sequencing. We also tested the efficiencies of the pCasSA system for the deletions of three other genes, *cntA* (Figure 4c), *murR* (Figure 4d), and *cymR* (Figure S2), and obtained editing efficiencies of 8/12, 9/12, and 4/12, respectively, therefore demonstrating the high editing efficiency of this system in the *S. aureus* RN4220 strain.

To cure the pCasSA plasmid after editing, we picked one colony from the RN4220 strain that contained the desired *agrA* gene deletion. The colony was first cultured at 30 °C and then diluted into fresh medium and incubated at a nonpermissive plasmid replication temperature (42 °C) in the absence of antibiotics until the culture was evident. A fraction of the culture was streaked onto a tryptic soy broth (TSB) agar plate and incubated at 37 °C overnight. Four individual colonies from the plate were randomly picked and streaked onto two different TSB agar plates in the presence or absence of the selection marker chloramphenicol. All four colonies grew normally on the plate without the antibiotic, whereas no obvious growth was observed on the plate in the presence of the antibiotic (Figure S3), thus confirming that the plasmid can be easily cured after editing. Because high-temperature treatment and the presence of antibiotics in the traditional genome editing methods favor the selection of secondary *sae* mutants,[28] we sequenced the *sae* sites of five different RN4220 *agrA* mutants to check the secondary mutations at *sae* sites. We did not observe any additional mutations in any of the samples tested (Figure S4), possibly because the plasmid curing step (the only step that uses a high temperature in the pCasSA-mediated genome editing method) did not introduce any antibiotics for cell culturing.

Because of the presence of numerous mutations,[29] *S. aureus* RN4220 is not an optimal strain for physiology studies. In addition, the presence of the mutations may affect the efficiency

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of DNA homologous recombination, therefore affecting the overall editing efficiency. To expand the utility of the pCasSA system, we tested the editing efficiencies of this system in two clinically isolated *S. aureus* strains, Newman and USA300. The editing plasmids from the RN4220 strains that carried the desired mutations were transformed into the Newman and USA300 strains by electroporation[30] and phage transduction,[31] respectively. Then we used PCR, sequencing, and the hemolysis assay (loss of the hemolytic activity is a major phenotype for *agrA* disruption[3]) to validate the deletions. We observed similar gene-deletion efficiencies in the Newman strain (*agrA* [Figure 5a], *cntA* [Figure 5b], *spa* [Figure S5a],

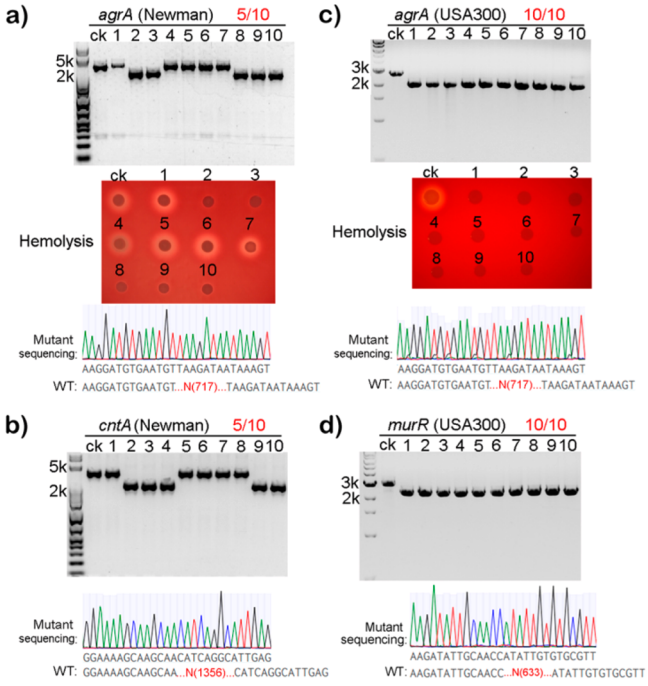

Figure 5. CRISPR/Cas9-mediated genome editing in the clinically isolated *S. aureus* Newman and USA300 strains. (a) pCasSA-mediated disruption of *agrA* gene in the Newman strain. The editing efficiency was 5/10. The lane labeled "ck" is the PCR product from the wild-type strain. Loss of hemolysis activity indicates the deletion of the *agrA* gene. (b) pCasSA-mediated disruption of the *cntA* gene in the Newman strain. The editing efficiency was 5/10. (c) pCasSA-mediated disruption of the *agrA* gene in the USA300 strain. The editing efficiency was 10/10. Loss of hemolysis activity indicates the deletion of the *agrA* gene. (d) pCasSA-mediated disruption of the *murR* gene in the USA300 strain. The editing efficiency was 10/10.

and *murR* [Figure S5b]) and much higher editing efficiencies in the USA300 strains (*agrA* [Figure 5c], *murR* [Figure 5d], and *cymR* [Figure S5c]) compared with those in the laboratory RN4220 strain, thus confirming the broad applications of this method for genome editing in a variety of *S. aureus* strains.

Mechanistic studies of gene and pathway functions may require the constructions of single-base substitution mutants as well as insertions, such as a reporter system to monitor the activity of a promoter of interest. To test the feasibility of this method in creating single-base substitution mutants, we first created a premature stop codon in the *agrA* gene of the editing plasmid by Gibson assembly (Figure 6a). Next, we transformed the plasmid into the RN4220 strain and then to the Newman strain (the plasmid was extracted from the RN4220 strain that carried the desired mutation). We observed very high editing

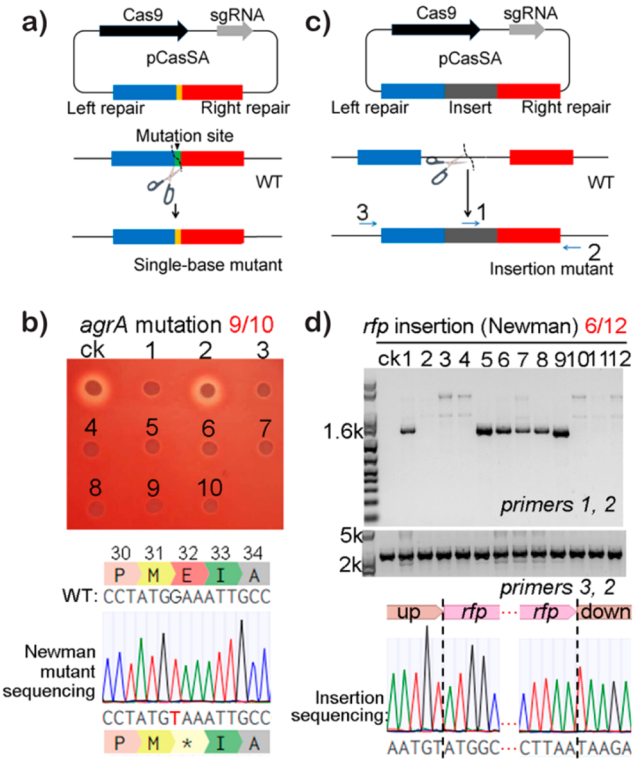

Figure 6. CRISPR/Cas9-mediated single-base substitution mutation and gene insertion in the *S. aureus* Newman strain. (a) Scheme showing the procedures for single-base substitution mutation in *S. aureus* by pCasSA. (b) pCasSA enables highly efficient single-base substitution mutation in the Newman strain. A premature stop codon is introduced the *agrA* gene. The mutation site is colored red. The mutation efficiency was 9/10, as confirmed by hemolysis assay and sequencing. (c) Scheme showing the procedures for gene insertion into *S. aureus* by pCasSA. The blue arrows are the primers used for PCR validation. (d) pCasSA enables efficient gene insertion in the Newman strain. The *agrA* gene was replaced by the *rfp* gene. The efficiency for *rfp* gene insertion was 6/12 in the Newman strain.

efficiencies in both strains (12/12 for the RN4220 strain [Figure S6a] and 9/10 for the Newman strain [Figure 6b]). To test the functionality of this system in creating insertion mutants, we assembled the *rfp* (red fluorescence protein) gene along with the repair arms (*agrA* upstream and *agrA* downstream) into the editing plasmid by Gibson assembly (Figure 6c). We transformed the plasmid into the RN4220 strain and then to the Newman strain. The insertion efficiencies were 5/9 and 6/12 in the RN4220 strain (Figure S6b) and the Newman strain (Figure 6d), respectively. Together, these experiments demonstrated that the pCasSA system is capable of creating single-base substitution mutants and insertion mutants in *S. aureus* with high efficiencies.

One of the major advantages of using the CRISPR/Cas9 system for genome editing is the relative ease of assembly of the genome-targeting module (the spacer in the case of CRISPR/Cas9) compared with other genome editing tools, such as transcription activator-like effector (TALEN)[32,33] and zinc-finger nuclease (ZFN).[34] By means of the high-throughput DNA synthesis technique, a pool of spacers can be synthesized easily. Through the use of Golden Gate assembly, the pCasSA system is amenable to the assembly of a pool of spacers simultaneously, thus enabling fast and accurate construction of a CRISPR/Cas9 library for genome-wide screening with the use

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of the catalytically dead Cas9 protein (dCas9). As evidenced by the success of using dCas9 for transcription inhibition in other organisms,[35,36] we created a transcription inhibition system (pCasiSA) in *S. aureus* by mutating the active sites of Cas9 protein, Asp10 and His840, to Ala (Figure 7a; these mutations

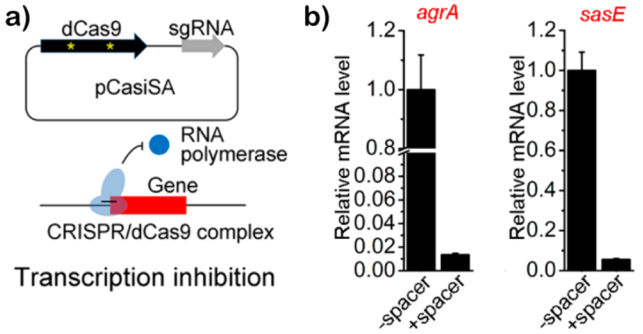

Figure 7. CRISPR/Cas9-mediated transcription inhibition in the *S. aureus* Newman strain. (a) Scheme showing the procedures for transcription inhibition in *S. aureus* by pCasiSA. (b) pCasiSA enables efficient transcription inhibition in the Newman strain. The experiments were performed on the *agrA* and *sasE* genes. Quantitative real-time PCR was used to quantify the mRNA levels.

abolish cleavage but do not impair DNA binding). Next, we assessed the efficiency of this system for transcription inhibition by comparing the transcription levels of the target genes in the cells carrying the empty pCasiSA plasmid or the pCasiSA plasmid with the spacers of the target genes. As shown in Figure 7b, the transcription levels of *agrA* and *sasE* genes were dramatically reduced after the introduction of the spacers, thus demonstrating the great capacity of the pCasiSA system for transcription inhibition. The development of the pCasiSA system would allow accurate genome-wide as well as defined gene library screening, which cannot be achieved by traditional screening tools such as transposon-mediated screening in *S. aureus*.

CONCLUSIONS

We have engineered the CRISPR/Cas9 system of *S. pyogenes* to be adaptable for genome editing in *S. aureus* and demonstrated the high editing efficiencies and easy use of this method for gene deletion, single-base substitution mutation, and gene insertion. We also developed a highly efficient transcription inhibition system, which may enable fast and accurate screening of genes and pathways that are responsible for the phenotypes of interest. The introduction of modern DNA assembly techniques into the system would greatly reduce the time and effort required for precise DNA manipulations. Further utilizations and optimizations of the pCasSA and pCasiSA systems should dramatically accelerate a wide variety of investigations in *S. aureus* and related organisms, such as drug development, enzymology, natural product mining, gene characterization, and other basic science research in microbiology as well as interdisciplinary research in chemical biology and synthetic biology.

EXPERIMENTAL SECTION

Bacterial Strains, Primers, Plasmids, and Growth Conditions. All of the bacterial strains and plasmids used in this study are listed in Table S1. The primers used in this study were purchased from Sangon Biotech (Shanghai, China) and are listed in Table S2. *E. coli* strains were grown in Luria–Bertani (LB) broth, and *S. aureus* strains were cultured in TSB. Antibiotics were added at the following concentrations: kanamycin, 50 μg/mL for the *E. coli* strains; chloramphenicol, 5 μg/mL for the *S. aureus* RN4220 strain and 10 μg/mL for the *S. aureus* Newman and USA300 strains.

Construction of pCasSA and pCasiSA Plasmids. The pCasSA plasmid was constructed with the following procedures: The *rpsL* promoter was PCR-amplified from the genomic DNA of the *S. aureus* Newman strain. The gene encoding the Cas9 nuclease was amplified from the pCas9 plasmid.[22] A temperature-sensitive origin, *repF*, and the chloramphenicol-resistance marker were amplified from the pKOR1 plasmid.[9] The origin ColE1 and the kanamycin-resistance marker were amplified together from the pCRISPR plasmid.[22] The five fragments were assembled into a plasmid via Golden Gate assembly. The promoter *cap 1A* along with the sgRNA fragment was synthesized as a gBlock and inserted into the *Sal*I/*Xho*I sites of the aforementioned plasmid, resulting in the final pCasSA plasmid. The success of the construction of the pCasSA plasmid was verified by PCR, enzyme digestion, and sequencing.

The transcription inhibition system (pCasiSA) was constructed by mutating the active sites of the Cas9 protein, Asp10 and His840, to Ala. The plasmid pCasSA was separated into three parts. Each part was PCR-amplified using the primers containing the desired mutations. Then the three DNA fragments were assembled by Gibson assembly to form the pCasiSA plasmid. The constructed pCasiSA plasmid was further verified by sequencing.

Electroporation. Overnight culture from a single colony of *S. aureus* (1 mL) was diluted into 100 mL of TSB and incubated at 30 °C. When the optical density at 600 nm (OD_{600}) of the culture reached 0.3 to 0.4, the cells were chilled on ice for 10 min and then harvested by centrifugation at 5000 rpm for 5 min. The supernatant was discarded, and the cells were resuspended by pipetting gently with 20 mL of sterile ice-cold 0.5 M sucrose. The centrifugation and resuspension steps were repeated twice. Finally, the cells were resuspended in 1 mL of 0.5 M sucrose, and 50 μL aliquots were frozen in liquid nitrogen and stored at −80 °C.

For electroporation, 50 μL of competent cells were thawed on ice for 5 min (more colonies could be obtained when fresh competent cells were used). Then the cells were mixed with 1−2 μg of plasmid DNA and transferred into a 1 mm electroporation cuvette (Bio-Rad) at room temperature. After being pulsed at 21 kV/cm, 100 Ω, and 25 μF, the cells were incubated in 1 mL of TSB at 30 °C for ∼1.5 h before being plated on a TSB agar plate containing chloramphenicol. The plate was incubated at 30 °C overnight.

Hemolysis Assay. Different *S. aureus* strains were grown in TSB at 30 °C overnight. The next day, 1 μL of culture of each strain was loaded onto a sheep blood plate. The plate was incubated at 30 °C for 24 h before being photographed.

Real-Time PCR. A 5 mL aliquot of culture of the *S. aureus* strain was incubated at 30 °C. When the OD_{600} reached 1.0, the cells were harvested by centrifugation and lysed by Fastprep (6.0 m/s for 40 s) at room temperature. The total RNA was isolated using RNAiso Plus (Takara) following the manufacturer's protocol. RNA (1 μg) was reverse-transcripted to cDNA using a PrimeScript RT reagent kit (Takara). Quantification of the cDNA was carried out on a LightCycler 96 system (Roche) using the SYBR Premix Ex *Taq*II kit (Takara). The expression levels of *agrA* and *sasE* genes were normalized to that of the reference gene 16S rRNA.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b13317.

Detailed experimental procedures and supplementary figures and tables (PDF)

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Notes

The authors declare no competing financial interest.

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