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METHODS

Construction of a Gene Knockdown System Based on Catalytically Inactive ("Dead") Cas9 (dCas9) in *Staphylococcus aureus*

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ABSTRACT There has been an absence of an efficient method of gene knockdown in the important human pathogen *Staphylococcus aureus* like RNA interference in eukaryotes. The previously developed antisense RNA technology is mainly applied for forward genetic screening but is rather limited in specific gene knockdown because of the lack of rational antisense RNA design strategies. Here we report an efficient and specific system for gene knockdown in *S. aureus* based on the type II clustered regularly interspaced short palindromic repeat (CRISPR) system from *Streptococcus pyogenes*. We can achieve gene silencing with the coexpression of dCas9, an RNA-guided DNA binding protein, and a small guide RNA complementary to the target gene. With this system, we have successfully silenced diverse sets of genes varying in size and expression level in different *S. aureus* strains. This system exhibited high-efficiency knockdown of both essential and nonessential genes, and its effect is inducible and reversible. In addition, the system can repress the expression of multiple genes simultaneously and silence an entire operon or part of it. This RNA-guided DNA targeting system thus provides a simple, rapid, and affordable method for selective gene knockdown in *S. aureus*.

IMPORTANCE *Staphylococcus aureus* is an important human and animal pathogen that can cause a diversity of infectious diseases. Molecular genetic study of *S. aureus* has provided an avenue for the understanding of its virulence, pathogenesis, and drug resistance, leading to the discovery of new therapies for the treatment of staphylococcal infections. However, methodologies developed for genetic manipulation of *S. aureus* usually involve either low efficiency or laborious procedures. Here we report an RNA-guided system for gene knockdown in *S. aureus* and show its high efficiency and simplicity for selective gene silencing in different strains of *S. aureus*. This simple, rapid, and affordable system may serve as a promising tool for functional gene study in *S. aureus*, especially for the study of essential genes, thus facilitating the understanding of this pathogen and its interaction with its hosts.

KEYWORDS CRISPRi, gene knockdown, *Staphylococcus aureus*, dCas9

Staphylococcus aureus is an opportunistic pathogen that can cause a diversity of infectious diseases in humans and animals. The global emergence and spread of antibiotic-resistant *S. aureus* pose serious challenges to the treatment of infections it causes (1). Genetic studies of *S. aureus* have provided an avenue for molecular understanding of the virulence, pathogenesis, and drug resistance of the organism, contributing to the development of new strategies for the treatment of infections it causes. Most of the studies have centered on virulence and antibiotic resistance genes, and the functions of these genes were evaluated mainly by targeted gene inactivation (2). So

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far, a plethora of methodologies have been developed for the study of the molecular genetics of *S. aureus* (3), among which there are two main kinds of techniques for targeted gene inactivation, i.e., gene knockout, which directly deletes or disrupts a gene in the genome, and gene knockdown, which represses gene expression but does not destroy the gene (2, 4). Allelic exchange is now the technique most commonly used to achieve gene knockout in *S. aureus* (5). Unfortunately, natural allelic replacements occur at a rather low rate in *S. aureus* and a counterselection system or an extensive screening process is required to screen for the desired mutants, making this method time-consuming and costly (5, 6). Moreover, gene knockout cannot be employed for the study of essential genes, which play important roles in the survival and pathogenesis of *S. aureus* (7).

Several efficient strategies have been developed for the knockdown or conditional knockout of essential genes in *S. aureus* in the past 20 years. One strategy involves controllable expression of an essential gene by inserting the inducible promoter into the region upstream of its chromosomal site (8), which also requires arduous cloning like allelic exchange. The second strategy uses targetrons derived from a *Lactococcus lactis* Ll.LtrB group II intron to create conditional mutations of both essential and nonessential genes in *S. aureus* (6). The temperature sensitivity of these targetrons requires that the mutant phenotype can only be manifested at high temperatures, which may not be proper for studying the physiological function of the gene. The third strategy, also the only one that does not alter the genome sequence, can repress gene expression by producing antisense RNA (asRNA) complementary to the target gene mRNA. Gene silencing by asRNA has been adopted for the study of essential genes in both eukaryotes and prokaryotes (9–11). It has been used to construct a random asRNA library and screen for essential genes in *S. aureus* (7). However, there remain some problems when it is employed to study specific genes because of the absence of rational asRNA design strategies, leading to a high failure rate and laborious construction and screening for efficient asRNAs (12, 13). Therefore, new techniques that are efficient, simple, and economical are still needed for the functional study of both essential and nonessential genes in *S. aureus*.

Recent work on the clustered regularly interspaced short palindromic repeat (CRISPR) system, an adaptive immune system of prokaryotes, has led to the development of novel strategies for gene knockout and knockdown in both eukaryotes and prokaryotes (14, 15). The CRISPR system has been found in most archaea and bacteria, providing the host with immunity against foreign nucleic acids like phage and plasmid nucleic acids (16). Of the various CRISPR systems characterized so far, the type II system has been best studied and exploited for its simplicity and potential as a genome editing tool (17). It requires only a single protein, called Cas9, for target DNA cleavage, while other systems need a multimeric complex of proteins. A naturally occurring CRISPR-Cas9 system needs two RNAs, a CRISPR RNA (crRNA) for target DNA binding and a *trans*-activating crRNA (tracrRNA) for Cas9 binding, to form hybrids and bind with Cas9 to mediate the cleavage of target DNA. The engineered CRISPR-Cas9 system has circumvented the problem of expression of two RNAs by designing a single guide RNA (sgRNA) that mimics the crRNA-tracrRNA complex (18). Other than sequence complementarity between the sgRNA and the target DNA, a short sequence (5′-NGG-3′) that immediately follows the targeting DNA sequence is also essential for determining the specificity of the system (19, 20). Since its emergence, this engineered CRISPR-Cas9 system has been applied to perform genome editing across a wide variety of cell types and whole organisms (17).

Recently, this CRISPR-Cas9 system has been modified for target gene regulation rather than disruption. The key component of this modified system is a catalytically dead Cas9 (dCas9) lacking endonuclease activity, which contains two silencing mutations of the Cas9 nuclease domains (D10A and H841A) (14). When the dCas9-sgRNA complex binds to the specific gene target, it does not cleave the DNA like wild-type Cas9 but blocks RNA polymerase from binding to the promoter or moving forward on the DNA to achieve transcriptional repression. This modified system, known as CRISPR

interference (CRISPRi), has shown high efficiency in gene silencing in both *Escherichia coli* (14) and mycobacteria (21). We consider this system a potentially powerful tool for functional studies of specific genes, especially essential genes in *S. aureus*, yet there have been no reports of its application in staphylococci.

Here we implemented dCas9 and sgRNA to construct an efficient system for gene knockdown in *S. aureus*. *E. coli-S. aureus* shuttle plasmid pSD1 was first constructed for constitutive expression of the sgRNA and conditional expression of dCas9 under the control of an anhydrotetracycline (ATc)-inducible promoter (22), making this system both inducible and reversible. To achieve specific gene knockdown, we only need to clone the gene-specific complementary sequences into the sgRNA expression cassette in the plasmid. Restriction enzyme SapI has been chosen to make this cloning quite simple. With this system, we have successfully silenced diverse sets of genes varying in size and expression level in different *S. aureus* strains with relatively high efficiency compared to that of the asRNA method. We have also demonstrated that this system can repress the expression of multiple genes simultaneously, silence an entire operon or part of it, and be used for functional studies of essential genes.

RESULTS

Construction of dCas9 and sgRNA expression plasmid pSD1. To implement the CRISPRi system in *S. aureus*, ATc-inducible expression plasmid pRMC2 (22) was used to express the d*cas9* gene derived from *Streptococcus pyogenes cas9* and custom-designed sgRNA in *S. aureus*. The expression of d*cas9* is under the control of the ATc-inducible promoter P_{tetO}, while the expression of sgRNA is controlled by the constitutive promoter P_{pflB} from *S. aureus* (23) (Fig. 1). sgRNA consists of mainly two regions, the 5′ variable region for target DNA binding and the 3′ constant region for dCas9 binding (Fig. 1A). The constant region can also work as its own transcription terminator. Target-specific sgRNA expression can be achieved by cloning the coding sequence of the variable region into the sgRNA expression cassette (Fig. 1A). To avoid the introduction of extra nucleotides during the assembly of the sgRNA expression cassette, a special restriction enzyme, SapI, that produces editable overhangs was used (Fig. 1B). In the modular sgRNA expression cassette, there was a cloning box with two SapI sites between the promoter and coding sequence of the sgRNA constant region (Fig. 1C). The overhangs of the two SapI sites upon digestion have been designed to coincide with the sequence of the promoter (5′-TAG-3′) and the coding sequence of the sgRNA constant region (5′-GTT-3′) (Fig. 1C), so that the inserted fragment with two 5′ overhangs of 5′-CTA-3′ and 5′-AAC-3′ can ligate with the promoter and coding sequence of the constant region without leaving a scar (Fig. 1D). Since pRMC2 already contains a SapI site outside the multiple cloning sites, a single nucleotide mutation was first introduced into the SapI site to destroy it. The modular sgRNA expression cassette and the d*cas9* gene fragment with a ribosome binding site (RBS) and a transcription terminator were then cloned into the multiple cloning sites (Fig. 1E). The resulting plasmid was designated pSD1. For specific gene knockdown, target-specific complementary oligonucleotide sequences were synthesized, annealed, and cloned into SapI-digested pSD1 as described in Materials and Methods (Fig. 1D). Of note, the pSD1 plasmid without the insertion of the target-specific fragment can also express an sgRNA whose variable region is coded by the SapI cloning box. BLAST analysis showed that this sgRNA has no specific target in *S. aureus* (consecutive identity sequence of $<$10 bases), which makes it a proper negative control for analysis of target-specific sgRNA knockdown efficiency.

Knockdown efficiency of CRISPRi in different *S. aureus* strains. Next, we detected the efficiency of this CRISPRi-mediated gene knockdown system in different *S. aureus* strains. First, the alpha-toxin gene (*hla*) in *S. aureus* strain NCTC 8325 was chosen as the target and four sgRNAs that bind to different regions of the gene were designed (Fig. 2A). The alpha-toxin virulence of the cell would decrease upon *hla* expression reduction. We determined the Hla protein level and alpha-hemolytic activity by Western blot and alpha hemolysis assays, respectively. The results showed that in the absence of ATc, in which case dCas9 is not supposed to be expressed, strains expressing

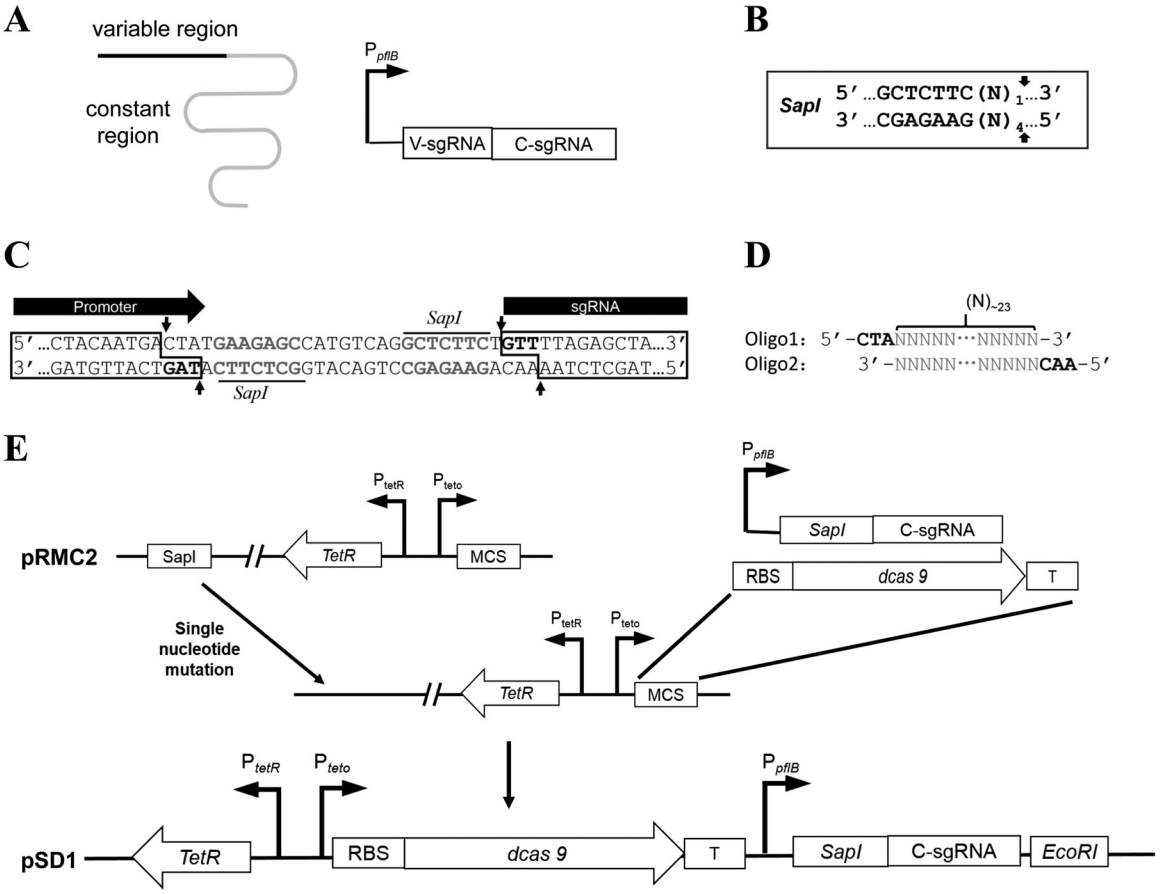

FIG 1 Schematic outline of the construction of dCas9 and sgRNA expression plasmid pSD1. (A) Schematic representation of sgRNA and organization of sgRNA expression cassette. The black line represents the sgRNA variable region for target DNA binding, and the gray line represents the constant region for dCas9 binding. (B) Recognition sequence and SapI cutting sites. The arrows indicate the cutting sites, and N represents any one of the four nucleotide bases. (C) Partial sequence of the modular sgRNA expression cassette. The gray bold nucleotides are the SapI recognition sequences, and arrows indicate the SapI cutting sites. Two 5′ overhang regions of 3 nt each (black bold) are produced upon SapI digestion. (D) Target-specific oligonucleotides coding for the sgRNA variable region. The gray sequences of the two oligonucleotides are complementary to each other, about 23 nt. A hybrid form of the two oligonucleotides has two 5′ overhangs (in black) for cloning into the SapI-digested sgRNA expression cassette. (E) Schematic diagram of plasmid pSD1 construction. Abbreviations: T, transcription terminator; MCS, multiple cloning site; V-sgRNA, coding sequence of the sgRNA variable region; C-sgRNA, coding sequence of the sgRNA constant region.

sgRNAs targeting *hla* showed no observable difference in the Hla protein level and alpha-hemolytic activity from the wild-type strain and the control strain expressing the invalid control sgRNA; but in the presence of 100 ng/ml ATc, strains expressing sgRNA binding to the nontemplate strand of the *hla* gene (sgRNA1, sgRNA2, and sgRNA3) showed a remarkable decrease in their Hla protein and alpha-hemolytic activity levels compared to those of the wild-type and control strains, whereas the strain expressing sgRNA binding to the template strand (sgRNA4) showed no obvious decrease (Fig. 2B). These data indicate that the CRISPRi system is inducible, and only sgRNA binding to the nontemplate DNA strand exhibited strong silencing efficiency, which is consistent with an earlier report on *E. coli* (14). Since the repression of gene expression by dCas9 occurs at the transcriptional level, we detected the *hla* mRNA levels in both the knockdown and control strains by quantitative reverse transcription (qRT)-PCR. Consistent with the Western blot and alpha hemolysis assay results, strains expressing sgRNA1, sgRNA2, and sgRNA3 exhibited a >100-fold decrease in the *hla* mRNA level after treatment with 100 ng/ml ATc, while the strain expressing sgRNA4 showed a much less extensive decrease (~2.5-fold) (Fig. 2C). Meanwhile, it was also observed that the three high-efficiency knockdown strains showed an ~3-fold decrease in *hla* mRNA even without ATc induction (Fig. 2C). We speculate that this was caused by leaky expression of dCas9

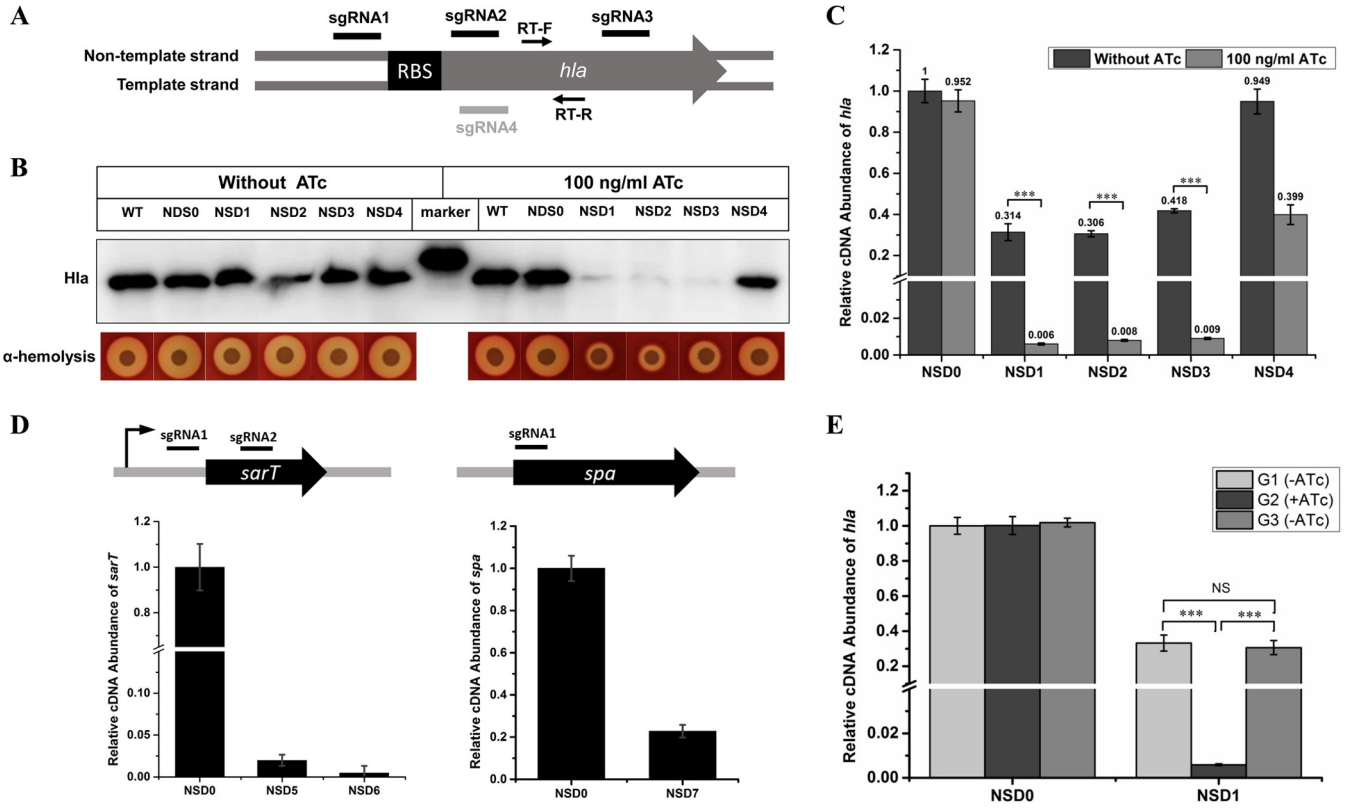

FIG 2 Knockdown efficiency of CRISPRi in *S. aureus* NCTC 8325. (A) Genetic organization of *hla* gene and binding sites of different sgRNAs. Arrows represent a pair of primers for qRT-PCR. (B) Hla protein levels and alpha-hemolytic activities of knockdown and control strains with or without ATc induction were measured by Western blot and alpha hemolysis assays, respectively. (C) Relative transcription level of *hla* in knockdown strains with or without ATc induction. (D) Knockdown of *sarT* and *spa* in NCTC 8325 by CRISPRi. (E) The knockdown effect of the CRISPRi system is reversible. The first generation of NSD0 and NSD1 was grown in the absence of ATc to an OD_{600} of 2 and then diluted 1:100 in ATc-containing TSB to obtain the second generation. The second generation was grown to an OD_{600} of 2 and then washed with TSB and diluted 1:100 in TSB lacking ATc to obtain the third generation. The third generation was also grown to an OD_{600} of 2. The *hla* expression levels in the different generations were measured by qRT-PCR. NSD0 was used as a control. The error bars indicate the standard errors of the means of three biological replicates. NS, not significant ($P > 0.05$); ***, $P < 0.001$. Abbreviations: WT, wild-type NCTC 8325; NSD0, NCTC 8325 carrying plasmid pSD1; NSD1, NCTC 8325 with the *hla* sgRNA1; NSD2, NCTC 8325 with the *hla* sgRNA2; NSD3, NCTC 8325 with the *hla* sgRNA3; NSD4, NCTC 8325 with the *hla* sgRNA4; NSD5, NCTC 8325 expressing the *sarT* sgRNA1; NSD6, NCTC 8325 expressing the *sarT* sgRNA2; NSD7, NCTC 8325 expressing the *spa* sgRNA1; G1 to G3, first, second, and third generations of bacteria.

since inducible promoters like P_{tetO} always have some basal expression (24). Another two genes, *sarT* and *spa*, with different sizes and expression levels in NCTC 8325 have also been successfully silenced by CRISPRi (Fig. 2D).

Given that the CRISPRi system is inducible, its knockdown effect should be induced in the presence of the inducer and reversed when the inducer is removed. To test whether our CRISPRi system is reversible, we cultured *hla* knockdown strain NSD1 in the absence of ATc to the mid-exponential phase (regarded as the first generation). The bacterial cells were then diluted 1:100 with ATc-containing medium and allowed to grow to the mid-exponential phase again (second generation). The second generation of NSD1 was washed with tryptic soy broth (TSB) to remove ATc and then diluted 1:100 with the medium lacking ATc and also allowed to grow to the mid-exponential phase (third generation). As expected, *hla* expression was significantly repressed in the second generation of NSD1 when ATc was present, but after ATc was removed, *hla* expression was restored to the normal level in the third generation of NSD1 (Fig. 2E). This result demonstrates that the knockdown effect of this CRISPRi system is inducible and reversible.

Then we detected the efficiency of the CRISPRi system in methicillin-resistant *S. aureus* (MRSA) strain N315. The drug resistance gene *mecA* was chosen as the target, and an sgRNA binding to the nontemplate strand of *mecA* near the start codon was designed (Fig. 3). Since *mecA* is mainly responsible for beta-lactam resistance in MRSA

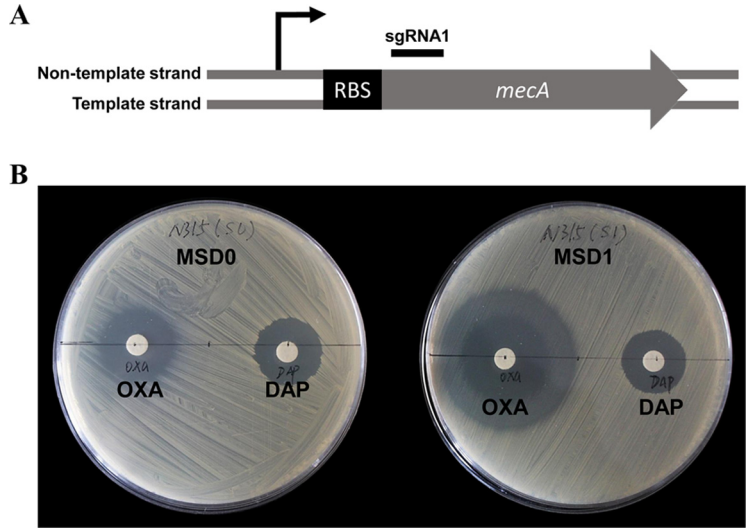

FIG 3 Knockdown of *mecA* in *S. aureus* N315 by CRISPRi. (A) Genetic organization of the *mecA* gene and the sgRNA binding site. (B) Oxacillin susceptibilities of knockdown (MSD1) and control (MSD0) strains detected by disk diffusion test. Daptomycin (a non-beta-lactam antibiotic) was used as a control. Abbreviations: OXA, oxacillin; DAP, daptomycin; MSD0, N315 carrying plasmid pSD1; MSD1, N315 expressing the *mecA* sgRNA1.

strains, knockdown of this gene should enhance the susceptibility of MRSA strains to beta-lactam antibiotics such as oxacillin. We used the disk diffusion method to measure the oxacillin susceptibility levels of the knockdown and control strains and used a non-beta-lactam antibiotic, daptomycin, as a control. The growth inhibition zone around oxacillin on the knockdown strain plate was significantly larger than that on the control strain plate, while the growth inhibition zones around daptomycin on both strain plates were similar in size (Fig. 3), indicating that the reduction of the beta-lactam resistance of N315 was caused by specific knockdown of *mecA*. Taken together, these data demonstrate that CRISPRi can be widely used for gene knockdown in different *S. aureus* strains.

Knockdown of multiple genes simultaneously by CRISPRi. The targeting specificity of CRISPRi is totally determined by sgRNA, and one knockdown plasmid can harbor multiple sgRNA expression cassettes. We explored whether this system can knock down multiple genes simultaneously in *S. aureus*. sgRNA1 targeting *spa* and sgRNA2 targeting *hla* were expressed separately or together in NCTC 8325 (Fig. 4A). When expressed alone, each of these two sgRNAs can specifically silence its target gene but not affect the expression of the other gene. When the two sgRNAs were expressed together, both genes were efficiently repressed, though at a lower rate than when they were expressed alone (Fig. 4A), which may have resulted from the competitive binding of two sgRNAs to dCas9. These results suggest that sgRNA targeting is specific and this system can be used to knock down multiple genes simultaneously in *S. aureus*. We also found that by expressing multiple sgRNAs targeting the same gene, enhanced gene knockdown can be achieved (Fig. 4B).

Knockdown of operon genes by CRISPRi. Since dCas9-sgRNA represses gene expression by blocking RNA polymerase from binding or moving forward on the DNA, in an operon where several genes are cotranscribed, sgRNA targeting the first gene or the promoter should silence the expression of the whole operon, while sgRNA targeting other positions should strongly repress the expression of genes downstream of the position but have a less pronounced effect on upstream genes because transcription stops at the targeting site. To determine the efficacy of the CRISPRi system on operon gene knockdown in *S. aureus*, we first chose the *ccrAB* operon in MRSA strain N315 as the target. The *ccrAB* operon contains only two genes, *ccrA* and downstream gene *ccrB*, which are cotranscribed from a promoter upstream of *ccrA* (25). We designed four

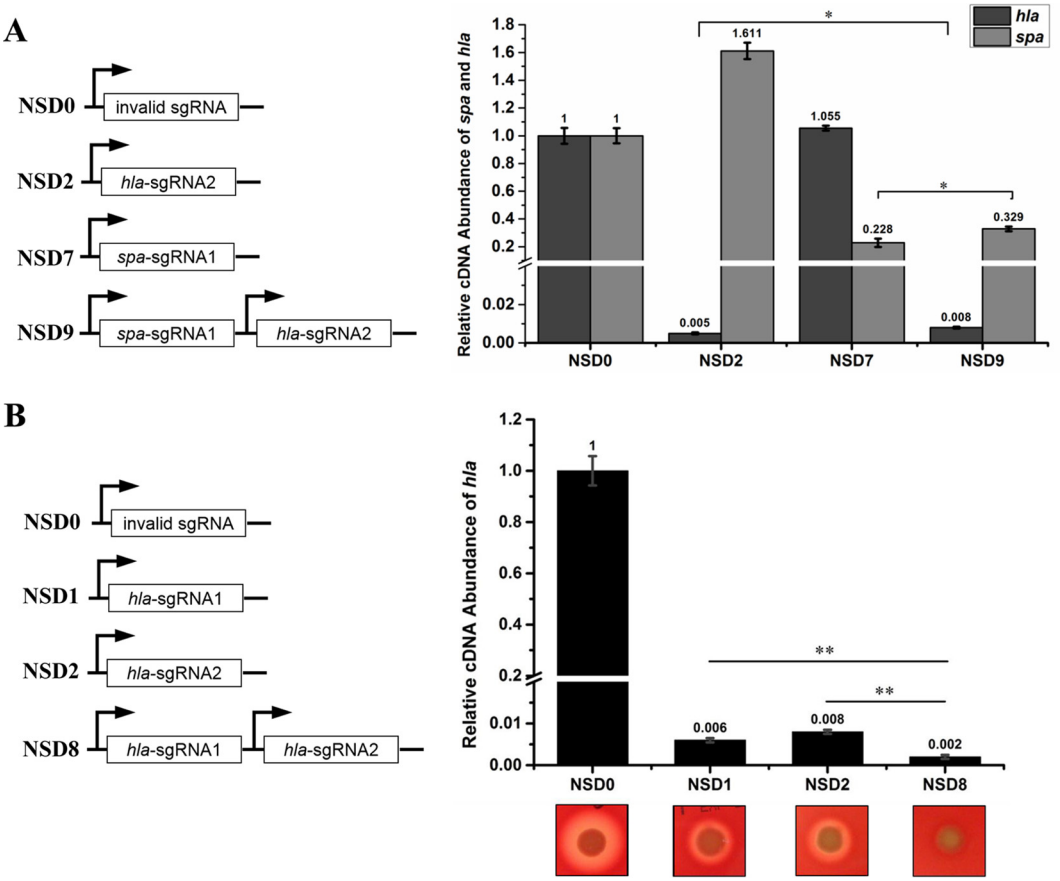

FIG 4 The CRISPRi system can simultaneously target multiple genes or sites. (A) Knockdown of *hla* and *spa* simultaneously by CRISPRi. Both genes are efficiently silenced in the double-knockdown strain. (B) Enhanced knockdown of *hla* by expression of both sgRNA1 and sgRNA2. The *hla* knockdown levels in the single- and double-knockdown strains were determined by alpha hemolysis and qRT-PCR. The alpha hemolysis data are representative of three independent experiments. The error bars indicate the standard errors of the means of three biological replicates. *, $P < 0.05$; **, $P < 0.01$. Abbreviations: NSD0, NCTC 8325 carrying plasmid pSD1; NSD1, NCTC 8325 expressing the *hla* sgRNA1; NSD2, NCTC 8325 expressing the *hla* sgRNA2; NSD7, NCTC 8325 expressing the *spa* sgRNA1; NSD8, NCTC 8325 expressing both the *hla* sgRNA1 and the *hla* sgRNA2; NSD9, NCTC 8325 expressing both the *hla* sgRNA2 and the *spa* sgRNA1.

sgRNAs, i.e., two pairs each of which targeted the same gene, and detected the two genes' expression levels in the knockdown and control strains (Fig. 5A). The qRT-PCR result showed that sgRNAs targeting *ccrA* (sgRNA1, sgRNA2) can remarkably repress the expression of both genes. Meanwhile, sgRNAs targeting *ccrB* (sgRNA3, sgRNA4) can significantly repress *ccrB* expression but exhibited rather slight repression of *ccrA* (Fig. 5A).

We further applied this system to knock down a bigger operon with more genes in *S. aureus* NCTC 8325. Homology analysis and RT-PCR identified a conserved operon with six genes in *S. aureus*. The operon contains a predicted essential gene, *era*, and five other genes whose functions in *S. aureus* have not been revealed (26, 27). Four sgRNAs targeting different genes in the operon were designed, and it was observed that they could strongly repress the expression of genes downstream of their target sites but exhibited limited inhibition of the expression of upstream genes, and the inhibition was even weaker with longer distances between the upstream gene and the target site (Fig. 5B). Taken together, these results indicate that the CRISPRi system can be used flexibly to knock down an entire operon or part of it by targeting different positions of the operon. Theoretically, an essential gene knockdown should lead to a growth defect of the bacterial cell, but in our experiment, no growth inhibition was observed in all four *era*-containing operon knockdown strains, which suggests that *era* may not be essential in *S. aureus* NCTC 8325.

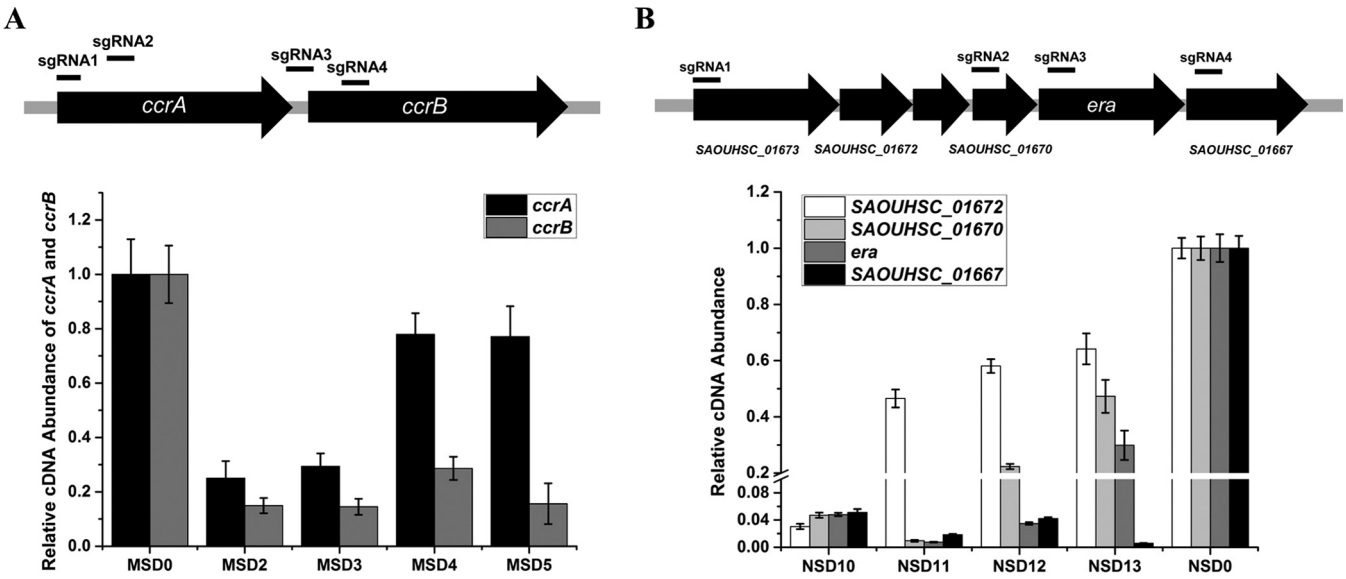

FIG 5 Whole or partial operon knockdown by CRISPRi. The knockdown effect of CRISPRi on the *ccrAB* operon in *S. aureus* N315 (A) and the *era*-containing operon in *S. aureus* NCTC 8325 (B) was determined by selectively targeting different operon genes. The relative transcription levels of different operon genes in different knockdown strains were measured by qRT-PCR. The error bars indicate the standard errors of the means of three biological replicates. Abbreviations: MSD0, N315 carrying plasmid pSD1; MSD2, N315 expressing the *ccrAB* sgRNA1; MSD3, N315 expressing the *ccrAB* sgRNA2; MSD4, N315 expressing the *ccrAB* sgRNA3; MSD5, N315 expressing the *ccrAB* sgRNA4; NSD0, NCTC 8325 carrying plasmid pSD1; NSD10, NCTC 8325 expressing the *era* sgRNA1; NSD11, NCTC 8325 expressing the *era* sgRNA2; NSD12, NCTC 8325 expressing the *era* sgRNA3; NSD13, NCTC 8325 expressing the *era* sgRNA4.

Study of essential genes by CRISPRi. One principal advantage of gene knockdown over gene knockout is that it can also be used to study essential genes. We thus used the CRISPRi system for knockdown of essential genes in *S. aureus* to determine its efficacy in the functional study of essential genes. Three experimentally confirmed essential genes, *murE*, *rpsC*, and *walR*, in *S. aureus* RN4220 have been targeted (7, 8, 28). Growth of these knockdown strains was detected at first. As expected, the knockdown strains showed appreciable growth inhibition in the presence of 100 ng/ml ATc (Fig. 6B), whereas in the absence of ATc, only marginal inhibition was observed compared with the control strain (Fig. 6A). This result proves that the CRISPRi system can also be used for efficient knockdown of essential genes and further demonstrates that the knockdown efficiency of the system is inducible. We then conducted a functional analysis of the *walR* knockdown strain. WalR and WalK compose a highly conserved two-component system that positively regulates autolysin synthesis and biofilm formation in *S. aureus* (29). To prevent the severe growth defect of the knockdown strain from hindering the functional study of essential genes, we used a lower concentration (20 ng/ml) of ATc to mitigate the growth inhibition of the *walR* knockdown strain (Fig. 6C). We examined the Triton X-100-induced autolytic activities of the *walR* knockdown strain and the control strain. Consistent with the function of the *walKR* two-component system, the *walR* knockdown strain exhibited strikingly high resistance to Triton X-100-induced autolysis compared with the control strain (Fig. 6D). Therefore, CRISPRi can serve as a powerful method for the functional study of essential genes.

DISCUSSION

The absence of an efficient gene knockdown method in prokaryotes like RNA interference in eukaryotes severely hindered the study of essential genes in bacteria, especially in the important human pathogen *S. aureus*. The functions of many essential genes in *S. aureus* are still unknown, which strongly hampers vaccine development and drug discovery. asRNA technology has been adapted for bacteria as a gene silencing method since the end of last century (30, 31). While antisense regulation by small noncoding RNAs has been found to be a ubiquitous natural phenomenon in bacteria, the mechanisms of action are largely unknown (32, 33). Even in the best-studied

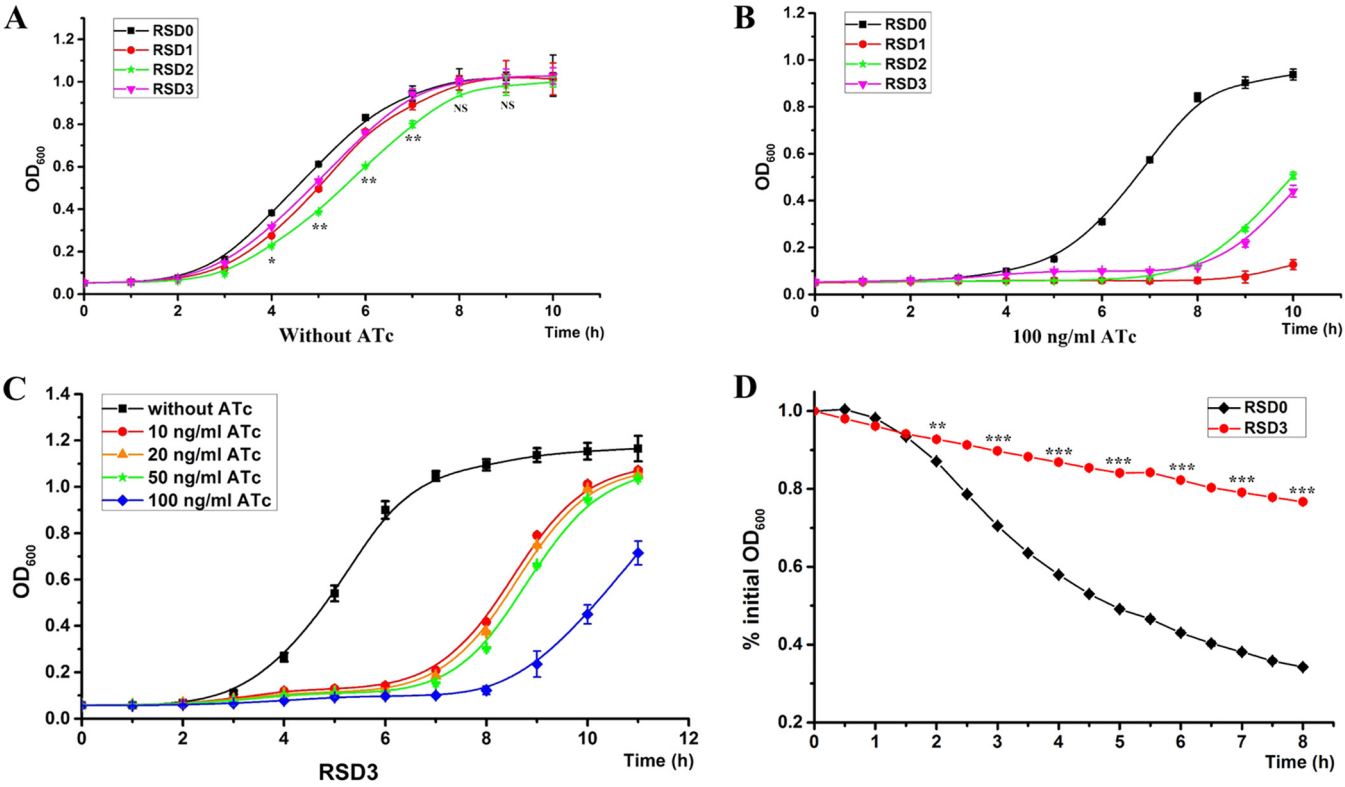

FIG 6 Functional study of essential genes in *S. aureus* by CRISPRi. (A) Growth curves of control and essential gene (*murE*, *rpsC*, and *walR*) knockdown strains without ATc induction. (B) Growth curves of control and essential gene knockdown strains with the induction by 100 ng/ml ATc. (C) Growth curves of a *walR* knockdown strain (RSD3) with the induction by different concentrations of ATc. A low concentration of ATc is enough to induce high-level gene knockdown. (D) Triton X-100-induced autolysis of a *walR* knockdown strain (RSD3) and a control strain (RSD0). The error bars indicate the standard errors of the means of four biological replicates. NS, not significant ($P > 0.05$); *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Abbreviations: RSD0, RN4220 carrying plasmid pSD1; RSD1, RN4220 expressing sgRNA targeting *murE*; RSD2, RN4220 expressing sgRNA targeting *rpsC*; RSD3, RN4220 expressing sgRNA targeting *walR*.

species, *E. coli*, the mechanisms are not fully understood, which makes rational asRNA design quite challenging (13). Moreover, the asRNA design principle developed from *E. coli* was not applicable for gene silencing in Gram-positive bacteria (12), so the only approach by which to get optimal asRNA for a target gene in a Gram-positive pathogen like *S. aureus* is screening of a library of randomly produced asRNAs (13), making this method both time-consuming and costly. Despite this, the asRNA technique was still broadly used in *S. aureus* since there are no better options for gene knockdown in that organism (34–36).

In this study, we provide a much more efficient and simpler tool for selective gene silencing in *S. aureus* by using sgRNA-guided dCas9. With a well-characterized mechanism of action and a general blueprint for sgRNA design, the CRISPRi system can theoretically be easily and efficiently used to inactivate any target gene. We showed that this system can successfully silence various genes in different *S. aureus* strains, with a much higher success rate and better efficiency than the asRNA method. By using this system, we achieved >100-fold repression of *hla* expression in *S. aureus* NCTC 8325 (Fig. 2), while early studies using asRNAs targeting *hla* could only downregulate the expression of *hla* approximately 16-fold (30, 37). Moreover, the CRISPRi system is inducible and reversible (Fig. 2E), allowing efficient control of its knockdown effect. We also demonstrated that CRISPRi can serve as a powerful tool for the identification and functional study of essential genes in *S. aureus* (Fig. 6). We believe that it will greatly promote research on genes essential for bacterial growth and pathogenesis.

Another significant advantage of the CRISPRi system over other RNA-guided silencing systems is the low off-target effect, which has been confirmed by whole-transcriptome analyses of both bacteria and eukaryotes (14, 15). Since dCas9-sgRNA requires no host factors for function, this naturally low off-target effect is supposed to be constant in

different organisms, including *S. aureus*. It has been revealed that the specificity of the CRISPRi system is determined jointly by the 5′-NGG-3′ sequence and at least a 12-bp consecutive sgRNA-DNA stretch (14). For bacteria with a small genome like *S. aureus* (~2.8 Mb), this 14-nucleotide (nt)-long region is enough to determine a unique target site, but to avoid potential off-target effects, a BLAST analysis of sgRNA in the genome is always recommended to rule out additional potential binding sites (with 14-nt base pairing with sgRNA including 5′-NGG-3′).

The CRISPRi system can also knock down multiple genes simultaneously, which is vitally valuable for molecular study of *S. aureus*. Since most cellular processes associated with drug resistance and pathogenicity in *S. aureus* involve a series of genes, the effect of a particular gene on cellular activity might be limiting (2). While inactivation of multiple genes by allelic replacement or asRNA is laborious, it can be easily achieved by CRISPRi by expressing multiple sgRNAs together. Since a single sgRNA expression cassette is only ~300 bp, it is possible for one knockdown plasmid to carry several sgRNA expression cassettes. Although there is, to some extent, competition between different sgRNAs, each of them still retains high knockdown efficiency (Fig. 4A). For genes that are difficult to silence by a single sgRNA, enhanced knockdown can also be achieved by expressing multiple sgRNAs targeting different sites of the same gene (Fig. 4B).

Operons play important roles in the regulation of metabolism and virulence in pathogenic bacteria. In fact, it has been recognized that 62% of the genes in *S. aureus* are located within operons (38). Therefore, genetic study of operons is crucial to understand the molecular mechanisms of the virulence and pathogenesis of *S. aureus*. The CRISPRi system has been proved to be a powerful tool for the study of operon genes. It can be used flexibly to knock down an entire operon or part of it (Fig. 5). Moreover, it can also serve as a simple method for the confirmation of operons predicted by *in silico* or experimental approaches.

Despite all of these advantages of the CRISPRi system, it is also worth noting that a leaky effect may exist for sgRNAs with high knockdown efficiency. In our study, we found that sgRNAs with low repression rates did not display an obvious leaky effect, while sgRNAs with high repression rates usually showed a significant leaky effect (Fig. 2C and 6A; see Fig. S1 in the supplemental material). However, we also found that the leaky efficiency of an sgRNA is very limited compared to the induced knockdown efficiency (usually <10%), so it will not influence the inducibility of the knockdown effect and functional study of the target gene.

In conclusion, here we report an efficient gene knockdown method based on dCas9 in *S. aureus*. We believe that this simple, rapid, and affordable selective gene knockdown system will serve as a promising tool for the study of molecular genetics in *S. aureus*, thus facilitating understanding of the pathogen and the fight against the infections it causes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. The *E. coli* strain was grown in Luria-Bertani (LB) medium with shaking at 220 rpm or on LB agar plates at 37°C. *S. aureus* strains were grown in TSB (Difco) with shaking at 220 rpm or on TSB agar plates at 37°C. When needed, 150 μg/ml ampicillin sodium salt for the *E. coli* strain or 15 μg/ml chloramphenicol (Chloromycetin) for *S. aureus* strains was added to the culture medium.

Preparation of electrocompetent *S. aureus* cells. *S. aureus* cells from a 15% glycerol stock were streaked onto a TSB agar plate and incubated at 37°C. A single colony was picked and incubated in 5 ml of TSB at 37°C overnight. One milliliter of the overnight culture was added to 100 ml of TSB in a 500-ml flask and shaken at 37°C until an optical density at 600 nm (OD_{600}) of 0.4 was reached. The culture was put on ice for 5 min and then transferred to a sterile, round-bottom centrifuge tube. The cells were collected by centrifugation at 2,500 × *g* at 4°C for 10 min, the supernatant was discarded, the cells were resuspended gently in 10 ml of ice-cold 0.5 M sucrose, and the suspension was kept on ice for 5 min. The centrifugation and resuspension steps were repeated twice. The cells were then resuspended in 1 ml of ice-cold 0.5 M sucrose and kept on ice for 15 min. Finally, 100-μl aliquots were prepared in sterile microcentrifuge tubes and frozen in liquid nitrogen. The competent cells obtained were stored at −80°C.

Plasmid extraction and transformation in *S. aureus*. Plasmids were isolated from all *S. aureus* strains with a plasmid purification kit (Sangon Biotech) in accordance with the manufacturer's instructions, except that the cells were pretreated with digestion buffer containing 40 U/ml lysostaphin, 10

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description[a]	Source or reference
Strains		
*E. coli Trans*1-T1	Clone host strain, F^- ϕ80(*lacZ*)ΔM15 Δ*lacX74 hsdR* (r_K^- m_K^+) Δ*recA1398 endA1 tonA*	TransGen
S. aureus		
RN4220	8325-4, restriction-negative strain	Laboratory stock
NCTC 8325	Wild type	Laboratory stock
N315	HA-MRSA	Laboratory stock
RSD0	RN4220 carrying plasmid pSD1	This study
RSD1	RN4220 carrying plasmid pSD1-*murE*	This study
RSD2	RN4220 carrying plasmid pSD1-*rpsC*	This study
RSD3	RN4220 carrying plasmid pSD1-*walR*	This study
NSD0	NCTC 8325 carrying plasmid pSD1	This study
NSD1	NCTC 8325 carrying plasmid pSD1-*hla1*	This study
NSD2	NCTC 8325 carrying plasmid pSD1-*hla2*	This study
NSD3	NCTC 8325 carrying plasmid pSD1-*hla3*	This study
NSD4	NCTC 8325 carrying plasmid pSD1-*hla4*	This study
NSD5	NCTC 8325 carrying plasmid pSD1-*sarT1*	This study
NSD6	NCTC 8325 carrying plasmid pSD1-*sarT2*	This study
NSD7	NCTC 8325 carrying plasmid pSD1-*spa1*	This study
NSD8	NCTC 8325 carrying plasmid pSD1-*hla1&2*	This study
NSD9	NCTC 8325 carrying plasmid pSD1-*spa1hla2*	This study
NSD10	NCTC 8325 carrying plasmid pSD1-*era1*	This study
NSD11	NCTC 8325 carrying plasmid pSD1-*era2*	This study
NSD12	NCTC 8325 carrying plasmid pSD1-*era3*	This study
NSD13	NCTC 8325 carrying plasmid pSD1-*era4*	This study
MSD0	N315 carrying plasmid pSD1	This study
MSD1	N315 carrying plasmid pSD1-*mecA1*	This study
MSD2	N315 carrying plasmid pSD1-*ccrAB1*	This study
MSD3	N315 carrying plasmid pSD1-*ccrAB2*	This study
MSD4	N315 carrying plasmid pSD1-*ccrAB3*	This study
MSD5	N315 carrying plasmid pSD1-*ccrAB4*	This study
Plasmids		
pRMC2	Shuttle vector, ATc inducible, Ap^r Cm^r	22
pSD1	pRMC2 derivative with dCas9 and sgRNA expression cassette	This study
pSD1-*hla1*	pSD1 with sgRNA1 targeting *hla*	This study
pSD1-*hla2*	pSD1 with sgRNA2 targeting *hla*	This study
pSD1-*hla3*	pSD1 with sgRNA3 targeting *hla*	This study
pSD1-*hla4*	pSD1 with sgRNA4 targeting *hla*	This study
pSD1-*hla1&2*	pSD1 with sgRNA1 and sgRNA2 targeting *hla*	This study
pSD1-*sarT1*	pSD1 with sgRNA1 targeting *sarT*	This study
pSD1-*sarT2*	pSD1 with sgRNA2 targeting *sarT*	This study
pSD1-*spa1*	pSD1 with sgRNA1 targeting *spa*	This study
pSD1-*spa1hla2*	pSD1 with sgRNA1 targeting *spa* and sgRNA2 targeting *hla*	This study
pSD1-*mecA1*	pSD1 with sgRNA1 targeting *mecA*	This study
pSD1-*ccrAB1*	pSD1 with sgRNA1 targeting *ccrAB*	This study
pSD1-*ccrAB2*	pSD1 with sgRNA2 targeting *ccrAB*	This study
pSD1-*ccrAB3*	pSD1 with sgRNA3 targeting *ccrAB*	This study
pSD1-*ccrAB4*	pSD1 with sgRNA4 targeting *ccrAB*	This study
pSD1-*era1*	pSD1 with sgRNA1 targeting operon containing *era*	This study
pSD1-*era2*	pSD1 with sgRNA2 targeting operon containing *era*	This study
pSD1-*era3*	pSD1 with sgRNA3 targeting operon containing *era*	This study
pSD1-*era4*	pSD1 with sgRNA4 targeting operon containing *era*	This study
pSD1-*murE*	pSD1 with sgRNA targeting *murE*	This study
pSD1-*rpsC*	pSD1 with sgRNA targeting *rpsC*	This study
pSD1-*walR*	pSD1 with sgRNA targeting *walR*	This study

[a]Abbreviations: Ap^r, ampicillin-resistant; Cm^r, chloramphenicol-resistant.

mg/ml lysozyme, and 10% (vol/vol) glycerol for 30 to 60 min. Plasmids were transformed into all *S. aureus* strains by electroporation. Plasmid DNA (100 to 500 ng) and electrocompetent *S. aureus* cells (100 μl) were mixed and placed in a Gene Pulser cuvette with a 0.2-cm electrode gap. The settings for electroporation were as follows: voltage, 2.5 kV; capacitance, 50 μF; resistance, 200 Ω. After electroporation, 400 μl of TSB was added to the cuvette immediately and it was put on ice for about 15 min. The cells were then transferred into a 1.5-ml Eppendorf tube and incubated with shaking (220 rpm, 37°C) for 1 h before being spread onto a TSB plate.

Design and construction of the modular sgRNA expression cassette. To construct a modular sgRNA expression cassette that can be easily used for cloning and expression of target-specific sgRNA

without any extra nucleotides, first the transcriptional start site-defined promoter P_{pflB} was amplified by PCR from the *S. aureus* RN4220 genome with primers PpflB-SmaI-F and PpflB-R. A SapI cloning box with two SapI sites was amplified with primers sgRNA-SapI-F and sgRNA-SapI-R, and the coding sequence of the sgRNA constant region was amplified with primers sgRNA-F and sgRNA-R. The three DNA fragments were then assembled by SLiCE in one step (39). The overhangs of the two SapI sites upon digestion have been designed to coincide with the sequence of the promoter (5′-TAG-3′) and the coding sequence of the sgRNA constant region (5′-GTT-3′), so the two 5′ overhangs of the coding sequence of the sgRNA variable region, 5′-CTA-3′ and 5′-AAC-3′, can respectively ligate with the promoter and coding sequence of the constant region without leaving a scar. The sequences of the primers used in sgRNA expression cassette construction are shown in Table 2.

Design and construction of dCas9 and sgRNA expression plasmid pSD1. To construct plasmid pSD1 for constitutive expression of the sgRNA and conditional expression of dCas9, first the original SapI site in plasmid pRMC2 (22) was mutated by overlap PCR with primers PRMC2-NcoI-F, PRMC2-SapI-R, PRMC2-SapI-F, and PRMC2-EcoRI-R. The sgRNA expression cassette was then amplified by PCR from the SLiCE product with primers sgRNA-slice-BglII-F and sgRNA-slice-EcoRI-R and cloned into BglII-EcoRI double-digested plasmid pRMC2. This cloning also introduced an SmaI site upstream of the sgRNA expression cassette for the subsequent insertion of a d*cas9* gene fragment. Finally, the d*cas9* gene fragment with an RBS from *S. aureus* and a strong transcription terminator (BioBrick part BBa_B0015) was amplified from a previously constructed plasmid with primers dcas9-BglII-F and dcas9-SmaI-R and inserted into BglII-SmaI double-digested plasmid pRMC2-sgRNA. The final plasmid, pSD1, is about 11 kbp. The sequences of the primers used for plasmid construction are shown in Table 2.

Construction of the specific gene knockdown plasmid. To achieve knockdown of an individual gene, the 5′-NGG-3′ sequence was first identified in the template strand of the target gene. The 23 nt immediately upstream of the 5′-NGG-3′ were then taken and added to the 3′ end of 5′-CTA-3′ to create sgRNA oligonucleotide 1, and the reverse complementary sequence of the 23 nt was taken and added to the 3′ end of 5′-AAC-3′ to create sgRNA oligonucleotide 2. A BLAST search was performed to detect the specificity of the sgRNA. Subsequently, the two oligonucleotides (20 μM) were annealed and then cloned into SapI-digested plasmid pSD1.

To construct a double-knockdown plasmid, two single-knockdown plasmids were first constructed. One sgRNA expression cassette was then amplified by PCR with promoter-specific forward primer sgRNA-cassette-F and terminator-specific reverse primer sgRNA-cassette-R. The PCR product was digested with EcoRI and then cloned into the other plasmid downstream of the existing sgRNA expression cassette.

The plasmids were first introduced into restriction-negative *S. aureus* strain RN4220 for modification and subsequently transformed into experimental strains. The sequences of the primers and sgRNA oligonucleotides used for plasmid construction are shown in Table 2.

Total RNA isolation and real-time qRT-PCR. Total RNA was extracted by RNAiso Plus in accordance with the manufacturer's instructions (TaKaRa). Residual DNA was digested with RNase-free DNase I (TaKaRa). RT was carried out with the PrimeScript First Strand cDNA synthesis kit (TaKaRa), and real-time PCR was performed with SYBR Premix *Ex Taq* (TaKaRa) by using a StepOne real-time system (Applied Biosystems). The quantity of cDNA was normalized to the abundance of *pta* cDNA (40). All of the qRT-PCR assays were repeated at least three times.

Western blot assay of alpha-toxin. Stationary-phase culture supernatant was collected and heated for 10 min at 95°C. The samples were then separated by 12% SDS-PAGE and electrotransferred onto a polyvinylidene difluoride membrane (GE, Piscataway, NJ). The protein was detected with a rabbit anti-alpha-toxin antibody (diluted 1:1,000; Sigma), followed by a horseradish peroxidase-conjugated sheep anti-rabbit antibody (diluted 1:10,000; Pierce). The images were obtained with ImageQuant LAS 4000 (GE).

Assay for alpha hemolysis. The alpha-hemolytic activities of the *S. aureus* NCTC 8325 control strain and the *hla* knockdown strains were assayed on sheep blood agar. The OD_{600}s of overnight culture were measured and adjusted to the same level. One-microliter culture volumes were then spotted onto sheep blood agar plates with or without ATc. The plates were left to dry for about 2 min and incubated at 37°C for 24 h.

Oxacillin susceptibility analysis. The oxacillin susceptibilities of the *S. aureus* N315 control strain and the *mecA* knockdown strain were analyzed by disk diffusion test as previously described (41). Cultures of the knockdown and control strains were both diluted to an OD_{600} of 2.0. Sterile swabs were then used to streak the cultures onto Mueller-Hinton agar plates (Difco) with 100 ng/ml ATc to form bacterial lawns. The plates were left to dry for about 5 min. Disks containing oxacillin (128 mg/ml, 2.5 μl) and daptomycin (16 mg/ml, 2.5 μl) were then attached to the plates with sterile tweezers. The plates were incubated overnight at 37°C.

Growth curve measurement. Overnight cultures of the RN4220 control strain and the essential gene knockdown strains in TSB without ATc were diluted to an OD_{600} of 0.05 into new TSB with or without ATc and allowed to grow in 96-well plates with shaking (220 rpm) at 37°C. Culture growth was monitored at 1-h intervals with a microplate reader (Elx800; Bio-Tek). For each sample, four biological replicates were analyzed.

Triton X-100-induced autolysis assay. Bacterial cells grown overnight were diluted to an OD_{600} of 0.05 in TSB with or without 20 ng/ml ATc and allowed to grow with shaking (220 rpm) at 37°C to the early exponential phase (OD_{600} of 0.8). Cells were harvested by centrifugation at 5,000 $\times$ *g* for 10 min, washed three times with 50 mM Tris-HCl (pH 7.5), and resuspended in 50 mM Tris-HCl (pH 7.5). The resuspended cells (100 μl) were added to 96-well plates, and the same volume of 50 mM Tris-HCl (pH 7.5) containing

TABLE 2 Sequences of primers used in this study

Primer	Sequence (5′–3′)
PRMC2-NcoI-F	CATTCTCTGGTATTTGGACTCCTG
PRMC2-SapI-R	AGTCAGTGAGCGAGGAAGCGTAAGAGCGCCCAATAC
PRMC2-SapI-F	GTATTGGGCGCTCTTACGCTTCCTCGCTCACTGACT
PRMC2-EcoRI-R	AGCTTGATGGTACCGTTAACAGA
PpflB-SmaI-F	TAGCCCCGGGATATACTCCTAAATTAACTT
PpflB-R	TAGTCATTGTAGCATGTTTGTTGTG
sgRNA-SapI-F	CACAACAAACATGCTACAATGACTATGAAGAGC CATGTCAGGCTCTTCTGT
sgRNA-SapI-R	ACTTGCTATTTCTAGCTCTAAAACAGAAGAGCCT GACATGGCTCTTCATAG
sgRNA-F	GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAA
sgRNA-R	AAAAAAGCACCGACTCGGTGCCACTTTTTCAAG TTGATAACGGACTAGCCTTATTT
sgRNA-slice-BglII-F	GAAGATCTTAGCCCCGGGATATACTCC
sgRNA-slice-EcoRI-R	CGGAATTCAAAAAAGCACCGACTCGG
sgRNA-cassette-F	CGGAATTCATATACTCCTAAATTAACTT
sgRNA-cassette-R	CGGAATTCAAAAAAGCACCGACTCGG
dcas9-BglII-F	GAAGATCTGGGAGGCCGTTTCATGGATAA
dcas9-SmaI-R	TCCCCCGGGTATAAACGCAGAAAGGCCCAC
hla-sgRNA1-oligo1	CTAGTTGAATAAATTCTTTATGAAACA
hla-sgRNA1-oligo2	AACTGTTTCATAAAGAATTTATTCAAC
hla-sgRNA2-oligo1	CTACATTAGCGACAGGATTCATTAATA
hla-sgRNA2-oligo2	AACTATTAATGAATCCTGTCGCTAATG
hla-sgRNA3-oligo1	CTACGGGTTCCAAGAATCTCTATCATA
hla-sgRNA3-oligo2	AACTATGATAGAGATTCTTGGAACCCG
hla-sgRNA4-oligo1	CTACTCAGTAACAACAACACTATTGCT
hla-sgRNA4-oligo2	AACAGCAATAGTGTTGTTGTTACTGAG
mecA-sgRNA1-oligo1	CTAACAACTACAACTATTAAAATAAG
mecA-sgRNA1-oligo2	AACCTTATTTTAATAGTTGTAGTTGT
spa-sgRNA1-oligo1	CTATCGTGTTGCGCAGCATTTGCAGC
spa-sgRNA1-oligo2	AACGCTGCAAATGCTGCGCAACACGA
sarT-sgRNA1-oligo1	CTATAACTCCAAATATATTTAGTTTG
sarT-sgRNA1-oligo2	AACCAAACTAAATATATTTGGAGTTA
sarT-sgRNA2-oligo1	CTATTACGCTTTTAACAATTCTAGAT
sarT-sgRNA2-oligo2	AACATCTAGAATTGTTAAAAGCGTAA
ccrAB-sgRNA1-oligo1	CTAATAGCCTATGACTTGTTTCATAT
ccrAB-sgRNA1-oligo2	AACATATGAAACAAGTCATAGGCTAT
ccrAB-sgRNA2-oligo1	CTAGATATGTTGAATATGATGTTTTT
ccrAB-sgRNA2-oligo2	AACAAAAACATCATATTCAACATATC
ccrAB-sgRNA3-oligo1	CTACGTTTATGCAATCGATGATTGCA
ccrAB-sgRNA3-oligo2	AACTGCAATCATCGATTGCATAAACG
ccrAB-sgRNA4-oligo1	CTAATATATCTTTAACTTCAAAATGA
ccrAB-sgRNA4-oligo2	AACTCATTTTGAAGTTAAAGATATAT
era-sgRNA1-oligo1	CTAATATCGTCTATTTGTATAATTCC
era-sgRNA1-oligo2	AACGGAATTATACAAATAGACGATAT
era-sgRNA2-oligo1	CTACCCTAAAATAACGCTATAAAATG
era-sgRNA2-oligo2	AACCATTTTATAGCGTTATTTTAGGG
era-sgRNA3-oligo1	CTAACAAATGTTGACTTTCCTACATT
era-sgRNA3-oligo2	AACAATGTAGGAAAGTCAACATTTGT
era-sgRNA4-oligo1	CTACTGCTTTGATGATAATCCCTTTT
era-sgRNA4-oligo2	AACAAAAGGGATTATCATCAAAGCAG
murE-sgRNA-oligo1	CTAATACTAGCTACTCTTAATGTGTC
murE-sgRNA-oligo2	AACGACACATTAAGAGTAGCTAGTAT
rpsC-sgRNA1oligo1	CTAATAATACCAACACGAAGTCCGAT
rpsC-sgRNA-oligo2	AACATCGGACTTCGTGTTGGTATTAT
walR-sgRNA-oligo1	CTATTAAATTCTAAAATATCAGCAAT
walR-sgRNA-oligo2	AACATTGCTGATATTTTAGAATTTAA
RT-pta-F	AAAGCGCCAGGTGCTAAATTAC
RT-pta-R	CTGGACCAACTGCATCATATCC
RT-hla-F	AAAGTAGGCTGGAAAGTGAT
RT-hla-R	TAGCGAAGTCTGGTGAAAA
RT-spa-F	AAGATGGTAACGGAGTACATGTCG
RT-spa-R	CAAGTTCTTGACCAGGTTTGATC
RT-ccrA-F	GCACAGTTATTAGAAGAAGATA
RT-ccrA-R	GCCATATTGATTGTTGACA
RT-ccrB-F	CAATACCACGAATACACTT
RT-ccrB-R	CATCACATAATCTTCAATCAC
RT-era2-F	TGCTTTAGAAGAAGATGAGCCAGAG
RT-era2-R	GTCCGTAATTGTTTGCTTGTTCT

(Continued on next page)

TABLE 2 (Continued)

Primer	Sequence (5′–3′)
RT-era4-F	GTTAGAAAAGCACAACAAGAATC
RT-era4-R	TAGCTCGTTCAGCACATATCG
RT-era5-F	AAGGTGTTATGACAAGAGATGACG
RT-era5-R	AGCGACTTTCATCATATAGTCACC
RT-era6-F	GGTGCAAAAGTACCACTTATGGC
RT-era6-R	TTCCCATACCTCGCCACTGATT
pRMC2-seq-F	CACAGATGCGTAAGGAGA

0.1% (vol/vol) Triton X-100 was added to each sample well quickly. The plates were incubated at 37°C with shaking (220 rpm). Cell autolysis was determined by measuring the progressive decrease in OD_{600} at half-hour intervals with a microplate reader (Elx800; Bio-Tek). For each sample, four biological replicates were analyzed.

Statistical analysis. F tests of two samples for variance were performed. Unpaired two-tailed *t* tests for equal or unequal variance were then performed to calculate the significant differences (*P* values). All tests were performed by the data analysis tool in Microsoft Excel.

Accession number(s). The annotated pSD1 sequence has been submitted to GenBank, and the accession number is KX685167.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.00291-17.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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