cas9 Enhances Bacterial Virulence by Repressing the *regR* Transcriptional Regulator in *Streptococcus agalactiae*

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ABSTRACT Clustered regularly interspaced palindromic repeats (CRISPR) and their associated *cas* genes have been demonstrated to regulate self-genes and virulence in many pathogens. In this study, we found that inactivation of *cas9* caused reduced adhesion and intracellular survival of the piscine *Streptococcus agalactiae* strain GD201008-001 and significantly decreased the virulence of this strain in zebrafish and mice. Further investigation indicated that the *regR* transcriptional regulator was upregulated in the $\Delta cas9$ mutant. As *regR* mediates the repression of hyaluronidase, a critical factor involved in opening the blood-brain barrier (BBB) in mice, *cas9*-mediated repression of *regR* transcription is important for *S. agalactiae* to open the BBB and thereby cause meningitis in animals. This study expands our understanding of endogenous gene regulation mediated by CRISPR-Cas systems in bacteria.

KEYWORDS *Streptococcus agalactiae*, *cas9*, bacterial virulence, *regR* transcriptional regulator, hyaluronidase

Streptococcus agalactiae, also known as group B streptococcus (GBS), is a Gram-positive zoonotic bacterium. It causes pneumonia, meningitis, and sepsis in human neonates and rare cases of necrotizing fasciitis in infants and adults (1, 2). *S. agalactiae* is the common pathogen causing both clinical and subclinical mastitis in dairy cattle in many countries, such as the United States (3), China (4), and Brazil (5), and there have also been reports of isolation of this bacterium from dogs and cats (6–8). In recent years, in addition to infecting mammals, *S. agalactiae* has become an emerging pathogen in aquatic environments and infects many species of cultivated fish, causing septicemia and meningoencephalitis, especially on tilapia farms (8–10). An increasing number of virulence genes have been identified over the last few years. However, the bacterial pathogenic mechanism remains largely unknown.

Recently, the clustered regularly interspaced palindromic repeat (CRISPR)-Cas system has been recognized as a factor essential for virulence control in many pathogens. This system is composed of CRISPR loci and flanking CRISPR-associated (*cas*) genes. The CRISPR-Cas system has been found in 40% of bacterial genomes and 90% of archaeal genomes and has been demonstrated to be an adaptive bacterial defense system against invading foreign nucleic acids derived from phages, plasmids, and viruses (11–13). The latest classification for CRISPR-Cas systems includes two classes based on the architecture of the effector modules, which are further subdivided into six types and various subtypes (14, 15).

Although the CRISPR-Cas system was demonstrated to be a resistance system against foreign nucleic acids, more and more research has indicated that CRISPR-Cas systems also play an important role in regulating self-genes and virulence in pathogens. Sampson et al. (16) demonstrated that the type II CRISPR-Cas system in *Francisella novicida* is essential for evading detection by a host conjugation receptor, and the *cas9* deletion mutant exhibited a significant decrease in the ability to adhere to, invade, and replicate in epithelial cells. Similar results were also observed in *Neisseria meningitidis*

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and *Campylobacter jejuni* (16–18). These findings suggest that *cas9* plays an important role in controlling virulence in several pathogens. In addition to *cas9*, *cas2* is essential to allow *Legionella pneumophila* to be harbored within amoebae (16, 19). It is interesting to note that even though both *cas9* and *cas2* belong to the same type II CRISPR-Cas system, *cas9* is not helpful for the intracellular survival of *L. pneumophila* and *cas2* is not essential for *F. novicida* virulence (16, 19). This indicates that even though type II CRISPR-Cas systems have similar functions in different pathogens, the functions of *cas* can be quite different.

By analysis of the *S. agalactiae* strains that have been sequenced to date, two different CRISPR-Cas systems were found in *S. agalactiae* strains: the type II-A system is ubiquitous and the type I-C system is present in only 20% of isolated strains (20, 21). Although the existence of these two systems has been clearly demonstrated, their function and impact on the virulence of *S. agalactiae* are still unknown. A study from Liu et al. (22) identified a type II-A CRISPR-Cas system in the chromosome of *S. agalactiae* strain GD201008-001, the Chinese epidemic piscine strain, using the CRISPR-Finder web tool (<http://crispr.i2bc.paris-saclay.fr/Server/>). This CRISPR-Cas system consists of four *cas* genes, *cas9*, *cas1*, *cas2*, and *csn2*, and the CRISPR array contains nine repeats interspaced by eight spacers. *Cas9* is known to be the main component and the largest protein in type II-A CRISPR-Cas systems, and it is essential for CRISPR RNA (crRNA) processing and spacer acquisition (23).

To investigate the role of the *cas9* gene in *S. agalactiae* virulence and its molecular mode of action, we knocked out this gene and identified the target genes regulated by *cas9* based on a transcriptomic analysis. We additionally investigated the regulation mechanism of *cas9*. This study will contribute to the understanding of the sophisticated virulence mechanisms that *S. agalactiae* has evolved and help to develop new approaches for the prevention and treatment of *S. agalactiae* infection.

RESULTS

Lack of the *cas9* gene does not influence bacterial growth *in vitro*. The result of the growth kinetics assay showed that the growth kinetics of the $\Delta cas9$ mutant did not exhibit any difference from those of the wild-type strain when the bacteria were cultured in Todd-Hewitt broth (THB) (see Fig. S1 in the supplemental material), indicating that in a nutrient-rich environment, the deletion of the *cas9* gene may not affect *S. agalactiae* growth.

***cas9* is involved in bacterial adherence to endothelial cells and survival in macrophages.** To elucidate the role of *cas9* in bacterial adhesion, we analyzed the relative abilities of *S. agalactiae* strains to adhere to brain microvascular endothelial cells. bEnd3 cells were lysed at 2 h after incubation with *S. agalactiae* strains at a multiplicity of infection (MOI) of 1:1. The ability of the $\Delta cas9$ mutant to adhere to bEnd3 cells was 2.5-fold lower than that of the wild-type strain (Fig. 1A), and this ability was partly restored in complemented strains, indicating that *cas9* is required for *S. agalactiae* adherence to endothelial cells.

Furthermore, we investigated the role of *cas9* in bacterial internalization by RAW 264.7 macrophages. We found that the $\Delta cas9$ mutant displayed resistance to being ingested by macrophages that was deficient compared to that of the wild-type or complemented $\Delta cas9$ ($C\Delta cas9$) strain, but this reduction was not statistically significant (Fig. 1B). However, the survival rate of the $\Delta cas9$ mutant in macrophages was significantly decreased compared to that of the wild-type or $C\Delta cas9$ strain (Fig. 1C). At 6 h after being taken up by the macrophages, the survival rate of the $\Delta cas9$ mutant was only approximately 28%; in contrast, it was 60% for the wild type and 55% for the $C\Delta cas9$ strain. These findings indicate that *cas9* is not essential for bacterial resistance to ingestion by macrophages but is required for survival within macrophages.

To determine whether the *cas9* gene plays a role in macrophage injury caused by *S. agalactiae*, lactose dehydrogenase (LDH) release was measured. At 4 h postinfection at an MOI of 1:1, we observed that the relative number of lysed RAW 264.7 cells infected with the $\Delta cas9$ mutant was significantly lower, exhibiting 2.7-fold and 1.7-fold de-

FIG 1 Infection of brain microvascular endothelial cells and macrophages by *S. agalactiae* wild-type (WT), $\Delta cas9$, and C $\Delta cas9$ strains at an MOI of 1. (A) Infection of bEnd3 brain microvascular endothelial cells. At 2 h postinfection, cells were washed and lysed for measurement of the number of CFU. (B) Infection of RAW 264.7 macrophages. At 1 h postinfection, cells were extensively washed to remove extracellular bacteria after antibiotic treatment. Then the cells were lysed for measurement of the number of CFU. (C) Intracellular bacterial survival in RAW 264.7 macrophages infected with *S. agalactiae*. Phagocytosis was allowed to proceed for 1 h before the addition of antibiotics for 1 h. At each time point, cells were washed and lysed to measure the number of CFU of intracellular bacteria. (D) Effect of bacterial cell-mediated cytotoxicity, as measured by LDH cytotoxicity assays. RAW 264.7 macrophages were infected with the indicated strains for 4 h to measure the level of LDH release into the supernatant. Error bars represent SDs. Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

creases compared to the number of cells infected with the wild-type and C $\Delta cas9$ strains, respectively (Fig. 1D). Together, our intracellular infection results clearly demonstrate the importance of *cas9* in the colonization and survival of *S. agalactiae* in host cells.

***cas9* enhances bacterial virulence in vivo.** To investigate the role of *cas9* in virulence, zebrafish were injected intraperitoneally (i.p.) with the wild-type, $\Delta cas9$, or C $\Delta cas9$ strain. The mortality of the zebrafish was recorded twice a day for 7 days after the injection (Table 1). The 50% lethal dose (LD₅₀) value of the $\Delta cas9$ strain (1.92×10^4 CFU) was 63-fold higher than that of the wild-type strain (3.03×10^2 CFU). In the

TABLE 1 Calculation of LD₅₀ for zebrafish

Dose of challenge (no. of CFU/0.02 ml)	No. of dead animals/total no. tested after infection with:		
	Wild type	$\Delta cas9$ mutant	C $\Delta cas9$ mutant
2×10^6	14/14	14/14	14/14
2×10^5	14/14	12/14	14/14
2×10^4	14/14	11/14	14/14
2×10^3	10/14	8/14	12/14
2×10^2	6/14	4/14	5/14
2×10^1	3/14	0/14	1/14

FIG 2 Survival percentage and bacterial distribution in different organs of mice infected by the *S. agalactiae* wild-type (WT), $\Delta cas9$, and $C\Delta cas9$ strains. Mice were infected with 500 CFU of *S. agalactiae*. (A) The survival of the mice was monitored every 4 h. (B to D) At 16 h postinfection, spleens (B), blood (C), and brains (D) were harvested for counting of the number of CFU. The bacterial loads in blood are expressed as the number of CFU per milliliter, and those in the spleen and brain are expressed as the number of CFU per gram of tissue. Bars represent the means for six infected mice. Error bars represent SDs. Asterisks indicate significant differences (**, $P < 0.01$; ***, $P < 0.001$).

$C\Delta cas9$ strain, the virulence was mostly restored, with the LD_{50} value (3.72×10^2 CFU) being 51-fold lower than that of the $\Delta cas9$ strain.

Furthermore, we investigated the mortality of mice infected with the strains described above (Fig. 2A). Mice infected with the wild-type or $C\Delta cas9$ strain rapidly succumbed to disease and death, with 100% mortality occurring within 36 h. In contrast, in the mice infected with $\Delta cas9$ strains, the first case of death occurred at 24 h postinfection, and the mortality rate was approximately 60% at 40 h. Additionally, no new deaths were observed by day 7. To better understand the effects of *cas9* during systemic infection, the bacterial burdens in the blood, spleen, and brain were calculated. At 16 h postinfection, the level of colonization with the $\Delta cas9$ strain in the spleen and blood as well as in brain tissue was significantly reduced, with a 4-fold decrease in the spleen (Fig. 2B), a 4.8-fold decrease in the blood (Fig. 2C), and a 5-fold decrease in the brain (Fig. 2D) compared to the level of colonization in mice infected with the wild-type strain being seen. These results provide further evidence of the important role that *cas9* can play in *S. agalactiae* pathogenesis.

Transcriptomic analysis of the *cas9* knockout mutant. As the main component of a type II-A system, *cas9* encodes an RNA guide endonuclease. We hypothesized that there may exist some target genes that can be cleaved by *cas9* or regulated indirectly and thereby influence bacterial virulence. To better understand the mechanism of gene regulation, we explored the differences between the wild-type and $\Delta cas9$ strains by transcriptomic analysis. Whole-cell RNA was harvested from both the wild-type and $\Delta cas9$ strains cultured in THB for high-throughput RNA sequencing (RNA-Seq). The data obtained were screened for genes with a change in expression of at least 2-fold and a P value of less than 0.05. As shown in Table 2, a total of 29 genes showed a significant

TABLE 2 Differential transcription of genes by the $\Delta cas9$ mutant and the wild-type *S. agalactiae* strain

Function and gene	Gene and/or possible function	Regulation	NCBI reference sequence
Prophage lambdaSa04			
Gene 1	Minor structural protein	Down	WP_000796407.1
Gene 2	DNA methylase	Down	WP_006739362.1
Gene 3	Carbamoylsarcosine amidase	Down	WP_017650699.1
Gene 4	Phage tail protein; cl17486	Down	WP_015057816.1
Gene 5	TP901 family tail tape measure protein	Down	WP_000918338.1
Gene 6	HK97 family portal protein	Down	WP_017769756.1
Gene 7	phi13 family major tail protein	Down	WP_006738724.1
Gene 8	Terminase, large subunit	Down	WP_006268711.1
Gene 9	Site-specific recombinase resolvase	Down	WP_003083348.1
Gene 10	<i>clpP</i> , portal proteins	Down	WP_017769756.1
Gene 11	HK97 family major capsid protein	Down	WP_017646689.1
Gene 12	DNA primase, virulence-associated protein E	Down	WP_017768029.1
Gene 13	Site-specific recombinase resolvase	Down	WP_002906257.1
Gene 14	SNF2 family helicase, DEAD/DEAH box helicase	Down	WP_003083276.1
Gene 15	Holin	Down	WP_000661350.1
Gene 16	DNA polymerase I	Down	WP_017769760.1
Membrane			
Gene 17	YSIRK signal domain/LPXTH anchor domain surface protein	Down	WP_000944926.1
Gene 18	Uncharacterized membrane protein	Up	NP_688147.1
Metabolism/stress response			
Gene 19	<i>N</i> -Acetylmuramoyl-L-alanine amidase	Down	WP_000143477.1
Gene 20	<i>metK</i> , methionine adenosyltransferase	Down	WP_003058380.1
Gene 21	Thiamine biosynthesis protein ApbE	Up	NP_688090.1
Gene 22	Peptidoglycan-binding protein LysM	Up	NP_689133.1
Gene 23	Lipoyl-binding domain-containing protein	Up	NP_687909.1
Gene 24	<i>treC</i> , alpha-amylase	Up	NP_687228.1
Transport, gene 25	<i>treP</i> , PTS ^a trehalose-specific transporter subunit IIBCA	Up	NP_687227.1
DNA replication, gene 26	DNA replication and repair protein RecF	UP	NP_689141.1
Regulatory			
Gene 27	RegR family transcriptional regulator	Up	NP_688885.1
Gene 28	TetR family transcriptional regulator	Down	WP_000445921.1
Unknown function, gene 29	Domain of unknown function	Up	NP_688316.1

^aPTS, phosphotransferase system.

difference in expression between these two strains, with 20 genes being downregulated and 9 genes being upregulated in the $\Delta cas9$ strain compared to their levels of expression in the wild-type strain. Then, 11 of these 29 genes (5 of the upregulated genes and 6 of the downregulated genes) were randomly selected for further verification by quantitative real-time reverse transcription-PCR (qRT-PCR), and the quantitative results were consistent with the transcriptomic data (Fig. 3). Interestingly, 16 of the downregulated genes were found to be located in a prophage, lambdaSa04, indicating a strong positive correlation between the *cas9* expression level and this prophage. The other four downregulated genes were two genes involved in metabolism and stress responses, one gene encoding membrane surface protein, and a regulator gene (*tetR*). On the other hand, the predicted functions of the nine upregulated genes were quite different; four of the genes were related to metabolism and stress responses, and the others encoded proteins belonging to different transporter, transcriptional regulator, and DNA replication families.

***regR* transcription is suppressed by *cas9*.** Because CRISPR-Cas systems can specifically mediate the degradation of their target nucleic acid sequences (24, 25), we analyzed the similarity between the sequences of these differentially transcribed genes and those on a CRISPR array to find the potential target genes. By comparing the sequences of the nine upregulated genes, we found that only the *regR* transcriptional regulator gene contained two regions that hybridized with the CRISPR array. One region, from nucleotides (nt) 934 to 956, partly hybridized with spacer 2 and repeats

FIG 3 qRT-PCR validation of the differentially regulated genes in the *S. agalactiae* wild-type (WT) and $\Delta cas9$ strains. Eleven genes, i.e., minor structural protein (MSP), DNA methylase, HK97, holin, SNF2, *clpP*, *regR*, *recF*, *apbE*, *treP*, and *treC*, were selected and amplified using real-time RT-PCR. The real-time RT-PCR data for each gene are relative to those obtained for the 16S rRNA control. Data points represent the means $\pm$ SDs from three independent experiments. Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

(Fig. 4A), while the other, from nt 10 to 28, hybridized with repeats (Fig. 4B). We hypothesized that this gene might be targeted by crRNA and be cleaved by *cas9*. To further evaluate our hypothesis, an RNA degradation assay was performed. When mRNA production was prevented by rifampin treatment, *regR* transcript levels were rapidly reduced in the wild-type strain. In contrast, in the $\Delta cas9$ strain, the *regR* transcript was slowly degraded (Fig. 4C), indicating that *cas9* is required for its degradation.

Hyaluronidase activity is suppressed by *regR* in vitro. The regulator *regR* has been reported to have the ability to repress hyaluronidase activity in *Streptococcus pneumoniae* (26). Interestingly, our previous study reported that hyaluronidase is

FIG 4 *cas9* is required for the degradation of *regR* mRNA. (A and B) Schematic of predicted hybridization fragments between *regR* and CRISPR: 23 bp of *regR* mRNA hybridized with the 11-bp spacer 2 and 9-bp repeat sequences (A), and 19 bp of *regR* mRNA hybridized with a 16-bp repeat sequence (B). (C) Time course of *regR* stability in the indicated strains after treatment with rifampin. Error bars represent SDs. Asterisks indicate significant differences (**, $P < 0.01$).

FIG 5 Hyaluronidase activity in wild-type (WT) *S. agalactiae*, its derivative *cas9* and *regR* mutants (the $\Delta cas9$, $\Delta regR$, and $\Delta cas9-regR$ mutants), the complemented strains (the $C\Delta cas9$, $C\Delta regR$, and $CregR-\Delta cas9$ mutants), and the *regR*-overexpressing strain (the OE *regR* strain). Supernatants of bacteria cultured overnight were harvested and diluted with assay buffer containing hyaluronic acid sodium. Thirty minutes later, stop buffer was added to stop the reaction and the absorbance values at 400 nm were determined. (B) Time course of *hylB* stability in the indicated strains after treatment with rifampin. (C) The transcription levels of *hylB* in the indicated strains were measured by quantitative RT-PCR. Error bars represent SDs. Asterisks indicate significant differences (**, $P < 0.01$; ***, $P < 0.001$).

encoded by the *hylB* gene and is an essential virulence factor that plays an important role in opening the blood-brain barrier (BBB) in the host animal (27). To determine the relationship between *regR* and hyaluronidase in *S. agalactiae*, we investigated the activity of hyaluronidase in different strains. Hyaluronidase is an endoglycosidase that belongs to the family of cell wall-anchored proteins, the activity of which can be determined in culture supernatants (28). *S. agalactiae* strains were cultured in THB, and culture supernatants were harvested for analysis. The deletion of the *cas9* gene caused a 4-fold decrease in hyaluronidase activity compared to that observed in the wild-type strain (Fig. 5A). However, the RNA degradation assay for the *hyl* gene (Fig. 5B) and the qRT-PCR data (Fig. 5C) showed that there was no significant difference between the $\Delta cas9$ and wild-type strains, indicating that *cas9* does not have a direct impact on transcription of the hyaluronidase gene. Then, we knocked out the *regR* gene in both the wild-type and $\Delta cas9$ strains. We found that in the $\Delta cas9$ mutant, inactivation of the *regR* gene restored hyaluronidase activity to the same level seen in the wild-type strain, but this recovery was abolished when *regR* was complemented in the $\Delta cas9-regR$ double deletion mutant. For a better understanding of the regulatory role of *regR* for hyaluronidase, we constructed an *S. agalactiae* strain overexpressing *regR* and analyzed its hyaluronidase activity. Compared to the wild-type strain, the strain overexpressing *regR* exhibited significantly repressed hyaluronidase activity (Fig. 5A). All the data

FIG 6 The virulence of $\Delta cas9$ was rescued in $cas9-regR$ double deletion mutants. (A) Mice were i.p. injected with 500 CFU of the indicated strains, and the mice were monitored for mortality every 4 h. (B to D) At 16 h postinfection, brains (B), blood (C), and spleens (D) were harvested for counting of the number of CFU. Bacterial loads in blood are expressed as the number of CFU per milliliter, and bacterial loads in the spleen and brain are expressed as the number of CFU per gram of tissue. Bars represent the means for six infected mice at 16 h postinfection. Error bars represent SDs. Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

indicated that the repression of hyaluronidase activity in the $\Delta cas9$ strain was caused by upregulation of the *regR* gene.

***cas9* is required for *regR* repression-mediated virulence enhancement.** Because we observed a marked defect in BBB penetration by the $\Delta cas9$ strain in infected mice, as assessed by bacterial burden analysis, we sought to address whether inactivation of *cas9* attenuated bacterial virulence by upregulating the transcript levels of the *regR* gene. We investigated the mortality of mice infected with the wild-type, $\Delta cas9$, $\Delta regR$, or $\Delta cas9-regR$ strain. The results showed that the virulence of the $\Delta cas9-regR$ strain was clearly increased compared with that of the $\Delta cas9$ strain, as infection with this strain led to a total mortality 12.5% higher than that caused by infection with the $\Delta cas9$ strain at 28 h postinfection (Fig. 6A). This indicated that the bacterial attenuation caused by the *cas9* deletion could be partly restored by inactivation of *regR*. However, virulence was not restored to the same level as that of the wild-type strain, which caused a mortality of 100%. Furthermore, the bacterial burden assay indicated that at 16 h postinfection, colonization by the $\Delta cas9-regR$ strain in the brain was significantly higher than that by the $\Delta cas9$ strain, showing an approximately 2.7-fold increase (Fig. 6B). The bacterial loads in blood from $\Delta cas9-regR$ strain-infected mice were also 2-fold higher than those in blood from mice infected with the $\Delta cas9$ mutant (Fig. 6C). Similar to the results from the mortality study, the bacterial loads in the brain and blood from mice infected with the $\Delta cas9-regR$ strain were not restored to the same levels as those observed in mice infected with the wild-type strain. In contrast to the results for the brain and blood, the bacterial load in the spleen was significantly lower (40-fold decrease) in $\Delta cas9-regR$

FIG 7 Cytokine transcription in the spleens of BALB/c mice i.p. injected with the indicated strains. At each time point, the spleens were harvested and tissue homogenates were used to quantify the IL-6 (A) and TNF- α (B) levels by qRT-PCR. Mice injected with PBS served as a control. The mRNA levels of the cytokines were normalized to the levels of β -actin and expressed as *n*-fold increases with respect to the values for the control. Error bars represent SDs. Asterisks indicate significant differences (**, $P < 0.01$; ***, $P < 0.001$).

strain-infected mice than in $\Delta cas9$ mutant-infected mice (Fig. 6D). We hypothesize that the reduced colonization in the spleen might be due to the drastic innate immune response to the infection by *regR* mutants.

When a comparison between the $\Delta cas9-regR$ and $\Delta regR$ mutants was made, we found that the $\Delta regR$ mutant caused 100% of mice to die by hour 28, while 40% of mice survived after $\Delta cas9-regR$ mutant infection (Fig. 6A). Regarding the bacterial loads in the three tissues analyzed, the group infected with the $\Delta regR$ mutant displayed higher loads than the group infected with the $\Delta cas9-regR$ mutant, although none of the differences were statistically significant (Fig. 6B to D). This suggests that the *regR* gene might be one of the factors that influences the bacterial virulence of the $\Delta cas9$ strain. Other differentially expressed genes found in the transcriptomic analysis may also play a role in the regulation of bacterial virulence.

cas9 plays an important role in triggering a proinflammatory response. To determine the effect of *cas9* inactivation on the innate immune response, the transcription levels of interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF- α) were assayed. Spleen tissues were harvested from mice infected with the wild-type, $\Delta cas9$, $\Delta regR$, or $\Delta cas9-regR$ strain to extract the RNA for qRT-PCR analysis. The results showed that infection with any one of the four *S. agalactiae* strains led to significantly increased levels of transcription of IL-6 and TNF- α compared to those in the uninfected control mice. At 5 h postinfection, mice infected with bacteria produced low levels of IL-6 and TNF- α , but from 10 h to 15 h postinfection, infected mice showed a significant increase in the transcription levels of these two cytokines (Fig. 7A). The inactivation of *cas9* upregulated IL-6 compared to the levels of IL-6 induced by that of the wild-type strain:

IL-6 expression was 8-fold higher at 10 h postinfection and 2-fold higher at 15 h postinfection. Furthermore, the deletion of *regR* from the *cas9* mutant resulted in the downregulation of IL-6 expression to levels near those in mice infected with the wild-type strain. Additionally, there was no significant difference in the IL-6 level between $\Delta cas9-regR$ strain- and $\Delta regR$ strain-infected mice. In contrast to the results for IL-6, there was no significant difference in TNF- α levels between mice infected with the various strains before 10 h postinfection. However, at 15 h, mice infected with the $\Delta cas9$ mutant showed a 2.3-fold decrease in TNF- α levels compared to those in mice infected with the wild type, and in mice infected with the *cas9* mutant lacking *regR* or the wild-type strain lacking *regR*, the TNF- α level was increased to levels significantly higher than those in mice infected with the wild type (Fig. 7B). These results indicate that inactivation of *regR* may stimulate the transcription of TNF- α .

DISCUSSION

CRISPR-Cas systems are important for bacteria to defend against phages, plasmids, and other foreign DNA. In addition, some recently published studies suggested that in pathogenic host-associated bacteria, there might be a role for the type II CRISPR-Cas system in virulence (16, 19). However, little is known about the definite mechanism of virulence regulation by the CRISPR-Cas system. The type II CRISPR-Cas system has three subtypes, which share the conserved *cas* genes: *cas9*, *cas1*, and *cas2*. Cas9 is the hallmark protein of type II systems and is essential for these systems' immune mechanisms and virulence regulation (29). Based on these facts, we knocked out the *cas9* gene from a highly virulent *S. agalactiae* strain, GD201008-001, and tried to determine the relationship between *cas9* and bacterial virulence.

Before beginning the cellular and animal infection assays, we analyzed the growth kinetics of the $\Delta cas9$ mutant in THB and determined that bacterial growth was not affected due to the inactivation of *cas9*. Then, we selected bEnd3 brain microvascular endothelial cells and RAW 264.7 macrophages to perform cellular infections. Before bacteria invade the central nervous system (CNS), they must first penetrate the BBB. Endothelial cells are primary elements of the BBB and form capillaries and the tight junctions between cells (30). Our study showed that a deficiency of the *cas9* gene reduced bacterial attachment to bEnd3 cells, suggesting that *cas9* might be an important factor in opening the BBB. Additionally, we found that the inactivation of *cas9* caused a decrease in intracellular bacterial titers in macrophages. However, this reduction in bacterial titer in RAW 264.7 macrophages was not due to deficiencies in initial phagocytosis of the bacteria but was caused by reduced resistance to digestion and repressed cytotoxic effects on macrophages. Intracellular survival in macrophages effectively reduces phagocyte clearance and therefore helps pathogens to evade detection by host immune systems and to be transported between tissues (31, 32). These results raised the question of whether the observed reduced adhesion and intracellular survival would cause reduced virulence in *S. agalactiae*.

To answer this question, we used zebrafish and mouse models to evaluate the virulence changes caused by deletion of *cas9*. Zebrafish are an ideal experimental model for various streptococcal species (33–35). We observed that the $\Delta cas9$ mutant exhibited a 63-fold enhancement of the LD₅₀ compared to that of the wild-type strain in zebrafish and took longer to cause death in mice. We harvested the spleens, blood, and brains from infected mice and calculated the bacterial loads in the different tissues and blood. The deficiency of *cas9* caused *S. agalactiae* to be more easily eliminated by the immune system, as evidenced by the significantly reduced bacterial density observed in the blood and spleen. Additionally, the reduced bacterial burden observed in the brain suggested an impaired ability of the $\Delta cas9$ mutant to enter the brain.

To address the virulence mechanism of *S. agalactiae* that is regulated by *cas9*, we performed a transcriptomic RNA-Seq analysis of the wild-type and $\Delta cas9$ mutant strains. Notably, our data showed that 16 of 20 significantly downregulated genes belong to a prophage, lambdaSa04. Bacteriophages have critical roles in genome diversification and the virulence evolution of pathogens (36). There is some experimental evidence

pointing to an interplay between CRISPR and prophage acquisition (13, 23, 37, 38). However, to our knowledge, associations of *cas9* with an acquired prophage have not yet been described in any published studies. Our future work will address this. The functions of the nine upregulated genes were quite different. For example, *lysM* is related to cell hydrophobicity and sensitivity to osmotic or oxidative stress (39), *recF* has an important role in homologous recombination and DNA damage repair (40), and *apbE* encodes a membrane-associated lipoprotein (41, 42). Together, the transcriptomic results suggested that *cas9* has a wide range of comprehensive regulatory functions in *S. agalactiae* that warrant further study.

Considering the endonuclease activity of *cas9* and its specificity, we focused on the sequences of the upregulated genes and predicted that a transcription regulator, *regR*, has two domains that may hybridize with a CRISPR array. Then, we performed an RNA degradation assay to prove our hypothesis. Compared to the degradation of *regR* mRNA observed for the wild-type strain, *regR* mRNA was rapidly degraded in the $\Delta cas9$ mutant, indicating that *cas9* is required for the degradation of *regR* mRNA. Interestingly, *regR* was reported to be a global regulator in *S. pneumoniae*, and importantly, it is known to repress hyaluronidase activities (26). Hyaluronidase has been demonstrated to play an important role in bacterial invasion and breaking through the BBB (27, 43, 44). Therefore, we sought to address whether there may be any associations among *cas9*, *regR*, and hyaluronidase in *S. agalactiae*. We directly analyzed hyaluronidase activity and found that the concentration of hyaluronidase contained in the supernatant from the *cas9* mutant was significantly lower than that in the supernatant from the wild-type strain. However, the concentration was restored to near wild-type levels when *regR* was also deleted, and this restoration was abolished after *regR* was complemented in the $\Delta cas9-regR$ double mutant. Then, we analyzed whether *cas9* could also degrade the mRNA of the *hylB* gene, which encodes hyaluronidase, and found that a deficiency in *cas9* did not impact the stability of *hylB* mRNA, suggesting that *cas9* has no direct endonuclease activity on *hylB*. Furthermore, qRT-PCR data showed that deletion of *cas9* had no effect on *hylB* transcript levels. These results indicate that Cas9 acts by directly degrading the mRNA of *regR* and thereby enhances hyaluronidase activity in *S. agalactiae*. Our previous study found that hyaluronidase is an important virulence factor for *S. agalactiae*, which helps this bacterium penetrate the BBB in mice (45). Based on these findings, we hypothesized that the reduced virulence in the $\Delta cas9$ mutant might be due to inactive hyaluronidase caused by enhanced *regR* transcription. One thing to be noted, however, is that other factors regulated by *regR* but not explored here might also contribute to bacterial virulence. *regR* has been reported to be a global regulator in *S. pneumoniae* (26), and its physiological effect concerns both competence and virulence expression. In *Bradyrhizobium japonicum*, *regR* can regulate almost 620 genes directly or indirectly under denitrifying conditions (46). However, the involvement of RegR in gene expression has not been investigated in *S. agalactiae*. Future studies will specifically address this.

Additionally, we found that the virulence of the $\Delta cas9-regR$ double mutant was higher than that of the $\Delta cas9$ mutant in mice. In addition, infection with the $\Delta cas9-regR$ mutant resulted in higher bacterial loads in the blood and brain than infection with the $\Delta cas9$ mutant did, indicating that the deletion of *regR* improved the transmission potential of $\Delta cas9$ and the ability of the strain to open the BBB and enter the CNS. However, the virulence of the $\Delta cas9-regR$ mutant was still lower than that of the wild-type strain and the $\Delta regR$ mutant, indicating that the interplay among *cas9*, *regR*, and hyaluronidase might be just one of the reasons for the virulence reduction in the $\Delta cas9$ mutant. Regarding our observations from the transcriptomic data, a total of 29 genes exhibited differential expression. Therefore, we hypothesized that genes other than *regR* may also play important roles in bacterial virulence. Notably, in contrast to the results in the brain and blood, further inactivation of *regR* in the $\Delta cas9$ mutant did not increase the bacterial load in the spleen, which was significantly lower than that observed in the spleens of mice infected with the $\Delta cas9$ mutant. The bacterial load was lower in mice infected with the $\Delta cas9$ mutant than in mice infected with the $\Delta regR$

mutant. This finding indicates that *regR* is more involved in *S. agalactiae* colonization of the spleen. However, the regulation mechanism of *regR* is unknown.

In *F. novicida*, Cas9-mediated innate immunity inhibition has been reported to be involved in bacterial pathogenicity by repressing production of an immunogenic membrane protein, BLP, which dampens Toll-like receptor 2 (TLR2) activation and thereby reduces TLR2-dependent IL-6 secretion (16). In agreement with this, the present study also found that the strain in which *cas9* was inactivated triggered higher levels of expression of IL-6 in the spleen than the wild-type strain of *S. agalactiae*. This finding agrees well with that of our previous study, which found that the inactivation of *hylB* in *S. agalactiae* enhanced IL-6 levels in the spleen (27). While a similar result for IL-6 expression was obtained with the Δ *hylB* and Δ *cas9* mutants, the level of expression of TNF- α observed in mice infected with the Δ *cas9* mutant was opposite that observed in mice infected with the Δ *hylB* mutant. In our study, the inactivation of *cas9* caused a significant decrease in TNF- α expression. Therefore, we cannot determine whether the Cas9-mediated alteration of IL-6 and TNF- α expression is relevant to hyaluronidase. Proinflammatory cytokine production is a complex process, which might involve multiple factors. Additionally, the Δ *cas9* and Δ *regR* mutants showed opposite results regarding IL-6 and TNF- α expression, while the expression of the two cytokines was partially balanced in mice infected with the Δ *cas9-regR* strain. This led us to speculate that the Cas9-mediated alteration of proinflammatory cytokine levels might be mainly caused by the upregulation of *regR* expression. Nevertheless, this hypothesis remains to be investigated in future studies.

Notably, the inactivation of *regR* had no significant impact on the bacterial load in the brain but caused a significant reduction in the bacterial load in the spleen compared to that observed in mice infected with the wild-type strain. This phenomenon may be explained by the fact that *regR* deletion causes a high level of TNF- α , which contributes to breaching of the BBB but protects the spleen from bacterial colonization (47–49).

In conclusion, this is the first report indicating that Cas9 plays an important role in *S. agalactiae* pathogenesis by repressing the *regR* regulator, which further elevates hyaluronidase secretion. However, how *cas9* specifically degrades the mRNA of endogenous genes and which spacer plays the primary role in targeting genes still require further study. Unraveling of these mechanisms will certainly provide deeper insight into virulence regulation by the CRISPR-Cas system in *Streptococcus*.

MATERIALS AND METHODS

Ethics statement. Animal experiments were implemented according to animal welfare standards and were approved by the Ethical Committee for Animal Experiments of Nanjing Agricultural University, Nanjing, China. All animal experiments complied with the guidelines of the Animal Welfare Council of China.

Cell lines, strains, plasmids, and growth conditions. RAW 264.7 macrophages (ATCC) were cultured in Dulbecco's modified Eagle medium (DMEM) with high glucose (Gibco, Invitrogen Corp., Carlsbad, CA) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS; Gibco). bEnd3 cells (ATCC) were cultured in DMEM with high glucose (Gibco) supplemented with 15% (vol/vol) heat-inactivated FBS (Gibco).

The bacterial strains and plasmids used in this study are listed in Table 3. *S. agalactiae* strain GD201008-001, which is β -hemolysin/cytolysin positive and belongs to serotype Ia and multilocus sequence type (MLST) ST-7, was isolated in 2010 from tilapia with meningoencephalitis from a fish farm in Guangdong Province, China (22). GD201008-001 was grown in Todd-Hewitt broth (THB; Basingstoke, Hampshire, England), on THB medium with 1.5% (wt/vol) agar, or in THB medium containing 5% (vol/vol) sheep blood. *Escherichia coli* strain DH5 α was used as the host for plasmids and was cultured in Luria-Bertani (LB) broth or on LB agar medium. The antibiotic spectinomycin (Spc; Sigma Co., St. Louis, MO) was added to the medium or broth, when necessary, at 100 μ g/ml for *S. agalactiae* and 50 μ g/ml for *E. coli*.

Construction of *S. agalactiae* mutants. To delete the *cas9* gene from *S. agalactiae* GD201008-001, pSET4S, a thermosensitive suicide vector carrying fused upstream and downstream arm fragments, was constructed. By using the primers Δ *cas9*-A/ Δ *cas9*-B and Δ *cas9*-C/ Δ *cas9*-D, these two arms were amplified and further, utilizing the primers Δ *cas9*-A/ Δ *cas9*-D, allowed to fuse into one fragment without the *cas9* gene. All primers are listed in Table S1 in the supplemental material. Both the fusion fragment and pSET4S were digested by the same restriction enzymes, SmaI/SphI, and ligated by T4 DNA ligase to generate the Cas9 deletion vector, pSET4S-*cas9*. The pSET4S-*cas9* candidates were transformed into

TABLE 3 Characteristics of bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
<i>S. agalactiae</i>		
GD201008-001 serotype Ia strain	A virulent strain of <i>S. agalactiae</i> isolated from tilapia with meningoencephalitis in China	Collected in our laboratory
$\Delta cas9$ mutant	<i>cas9</i> gene deletion mutant of GD201008-001	This study
$\Delta cas9$ complemented strain ($\Delta cas9$)	GD201008-001 $\Delta cas9$ complemented with vector pSET2:: <i>cas9</i> ; Spc ^r	This study
$\Delta regR$ mutant	<i>regR</i> gene deletion mutant of GD201008-001	This study
$\Delta cas9-regR$ mutant	<i>cas9</i> and <i>regR</i> gene double-deletion mutant of GD201008-001	This study
$\Delta regR$ complemented strain ($\Delta regR$)	GD201008-001 $\Delta regR$ complemented with vector pSET2:: <i>regR</i> ; Spc ^r	This study
$\Delta regR$ complemented $\Delta cas9$ strain ($\Delta regR-\Delta cas9$)	GD201008-001 $\Delta cas9-regR$ complemented with vector pSET2:: <i>regR</i> ; Spc ^r	This study
<i>regR</i> -overexpressing strain (OE <i>regR</i>)	<i>regR</i> gene-overexpressing wild-type strain GD201008-001 containing the vector pSET2:: <i>regR</i> ; Spc ^r	This study
<i>E. coli</i> DH5 α	Used for production of recombinant plasmids	Invitrogen
Plasmids		
pSET4S	Thermosensitive suicide vector for gene replacement in <i>Streptococcus</i> replication of pG ⁺ host3 and pUC19; <i>lacZ'</i> Spc ^r	50
pSET2	<i>E. coli-Streptococcus</i> shuttle vector; Spc ^r	51
pSET4S:: <i>cas9</i>	Recombinant vector with pSET4S background, designed for knockout of <i>cas9</i> ; Spc ^r	This study
pSET2:: <i>cas9</i>	Complementation vector with pSET2 background containing the promoter followed by full-length <i>cas9</i> ORF	This study
pSET4S:: <i>regR</i>	Recombinant vector with pSET4S background, designed for knockout of <i>regR</i> ; Spc ^r	This study
pSET2:: <i>regR</i>	Complementation vector with pSET2 background containing the promoter followed by the full-length <i>regR</i> ORF	

E. coli DH5 α for propagation and were verified by PCR and nucleotide sequence analysis before being electrotransformed into *S. agalactiae* GD201008-001 competent cells, which were selected for on Todd-Hewitt (TH) agar medium with 100 μ g/ml Spc (50). Successful double-crossover mutagenesis of the $\Delta cas9$ mutant was verified by PCR and quantitative real-time reverse transcription-PCR (qRT-PCR) (the primers are listed in Table 4). *regR* gene deletion mutants of the wild-type and $\Delta cas9$ strains and their respective complemented strains were constructed by the same protocol using the primers in Table S1 in the supplemental material.

The pSET2 shuttle cloning vector was utilized to generate a *cas9* complementation vector (51); a 4,578-bp fragment containing an open reading frame (ORF) of *cas9* from the genome was amplified and ligated to the pSET2 vector. Prior to being electrotransformed into the $\Delta cas9$ mutant, the recombinant plasmid was transformed into *E. coli* DH5 α for propagation. Complementation vector-transformed $\Delta cas9$ mutants were cultured on Spc-containing TH agar medium, and positive clones were verified by PCR and qRT-PCR. The complemented strain of the $\Delta regR$ mutant was constructed using the same approach. The *regR* complementation vector was also introduced into the wild-type strain to generate the *regR*-overexpressing strain.

qRT-PCR, transcriptomic analysis, and sequence analysis. Quantitative real-time reverse transcription-PCR (qRT-PCR) was carried out as described previously (27). The RNA of bacterial cells in mid-log phase was extracted with an EZNA total RNA kit I (Omega, Beijing, China). The RNA was then reverse transcribed into cDNA using HiScript II QRT Supermix (Vazyme, China). Two-step relative qRT-PCR was used for measuring the level of mRNA expression. The 16S rRNA housekeeping gene was used as the internal control gene. The primers used for the qRT-PCR assays are listed in Table 4. SYBR green PCR was performed using a SYBR Fast quantitative PCR master mix (Kapa) following the protocol of the manufacturer and carried out in triplicate by using an ABI 7500 RT-PCR system. Changes in gene transcription were determined using the comparative cycle threshold ($2^{-\Delta\Delta CT}$) method (52).

A Vahtstm mRNA-Seq (v2) library preparation kit for the Illumina system (Vazyme, China) was used to generate the transcriptome library for RNA sequencing. Transcriptome reads were mapped against the reference sequence of *S. agalactiae* GD201008-001 using TopHat2 software. The Cuffdiff program was used to find differentially expressed genes. Differentially expressed genes were identified as those with a *P* value of <0.05 and a fold change in expression between two samples of >2.

To evaluate the sequences that were hybrids of regulated genes and the CRISPR array, sequences of upregulated genes were analyzed with the BLASTN program to compare them to sequences from the CRISPR array for *S. agalactiae* GD201008-001 at the NCBI website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), using the parameters that the application automatically sets for short queries.

In vitro growth kinetics assay. For the growth kinetics assay, overnight *S. agalactiae* cultures of the wild-type, $\Delta cas9$ mutant, and complemented strains were prepared and equalized by dilution adjustments. The cell densities (based on the optical density at 600 nm [OD₆₀₀]) in THB were measured every 2 h from 0 h to 12 h.

***S. agalactiae* intracellular survival and adherence assay.** For the intracellular survival assay, RAW 264.7 macrophages were cultured in 10% FBS-containing DMEM at 37°C in an atmosphere with 5% CO₂.

TABLE 4 Primers used for qRT-PCR

Primer	Sequence (5'-3')	Function
<i>cas9</i> -up-F	AATATGCCAGCTTTCCTTC	An upstream fragment of the <i>cas9</i> ORF
<i>cas9</i> -up-R	ACCCACAGAAAGGCACATA	
<i>cas9</i> -self-F	CAATAGGCCTTGACATCGG	A fragment of the <i>cas9</i> ORF
<i>cas9</i> -self-R	TTCAAGCGTCTATCTGCAGC	
<i>cas9</i> -down-F	GTTGGCGAACC GTTGTG	A downstream fragment of the <i>cas9</i> ORF
<i>cas9</i> -down-R	CCAAACGTTTAATCAGCATGG	
<i>regR</i> -F(RT)	TGATGTAGGCTGAATAATGAAA	A fragment of the <i>regR</i> ORF
<i>regR</i> -R(RT)	TATGATGCCAAAAGTGAAGA	
<i>hylB</i> -F	AACGCCAAAAACATCAAAGA	A fragment of the <i>hylB</i> ORF
<i>hylB</i> -R	TGAAGCCAAGCCAGACTCTC	
MSP-F	TGTCCCCATCAAATAAGCAG	A fragment of the minor structural protein ORF
MSP-R	TACCACCAAATCCTACACCAT	
DNA methylase-F	GATAATCTCGTCTCCGCCAC	A fragment of the DNA methylase ORF
DNA methylase-R	TTTTACATTTGTTGACCTCCTCCAG	
<i>treP</i> -F	ATTTCTTCGCTCATCCCTTC	A fragment of the <i>treP</i> ORF
<i>treP</i> -R	TGCATTCTTTACACTGCACCAG	
<i>treC</i> -F	GCAATGTCTGCTGCTGAAGTAC	A fragment of the <i>treC</i> ORF
<i>treC</i> -R	TGTGACTCGTAAGATGGGGACT	
HK97-F	CTGCCACTTTGTGAGCGTTTG	A fragment of the HK97 ORF
HK97-R	TGATTGGTTATTCGCCTATTGC	
holin-F	CTGCTGCCATGAGACCTGTGA	A fragment of the holin ORF
holin-R	AGGACTTATCGGATCAATCTTTGG	
SNF2-F	TTTTCTTTATCCGTCAGTCGC	A fragment of the SNF2 ORF
SNF2-R	GTAGGAATGGCAATGTCGTCT	
<i>clpP</i> -F	CGAGTAGTGGGAAGAAGAAA	A fragment of the <i>clpP</i> ORF
<i>clpP</i> -R	TCAGGTAAGAGCGGATAGAG	
<i>recF</i> -F	ATCACGATGAGGTCCGATGCT	A fragment of the <i>recF</i> ORF
<i>recF</i> -R	TATCCAAGCCCTAAATGATGAAGC	
<i>apbE</i> -F	GTGGTCCAGATTTACAGTCG	A fragment of the <i>apbE</i> ORF
<i>apbE</i> -R	CAGGTACTTATGAACGCCGTCT	
16S rRNA-F	CGACGATACATAGCCGACCT	A fragment of 16S rRNA
16S rRNA-R	CCGCTCACTTGGTAGATTTTCC	
IL-6-F	CCACTTCACAAGTCGGAGGCTTA	A fragment of IL-6 in mice
IL-6-R	GCAAGTGCATCATCGTTGTTTCATAC	
TNF- α -F	AAGCCTGTAGCCACGTCGTA	A fragment of TNF- α in mice
TNF- α -R	GGCACCCTAGTTGGTTGTCCTTG	
β -actin-F	TGACAGGATGCAGAAGGAGA	A fragment of β -actin in mice
β -actin-R	GCTGGAAGGTGACAGTGAG	

For the invasion assay, RAW 264.7 cells at a density of 10^5 cells/ml were seeded in 24-well plates a day before the experiment. *S. agalactiae* wild-type, $\Delta cas9$, and complemented strains were grown in THB until mid-log phase at 37°C. Bacterial cells were harvested by three rounds of centrifugation at $5,000 \times g$ for 5 min, in which they were resuspended in phosphate-buffered saline (PBS) for the first two rounds, followed by a final resuspension in serum-free DMEM. The cell monolayers were washed three times with PBS prior to being infected with bacteria at a multiplicity of infection (MOI) of 1:1 and were then incubated at 37°C for 1 h. Extracellular bacteria were removed by washing with PBS five times, refilling the wells with 5 μ g/ml penicillin G-containing 1% FBS–DMEM, and incubating the plates at 37°C in 5% CO₂ for 1 h, which represented the zero time point. At the subsequent 2-, 4-, 6-, 8-, and 12-h time points, infected cells were washed and lysed. Lysates were serially diluted in PBS and plated on TH agar medium, and the number of CFU was counted after incubation at 37°C overnight.

The adherence assay was performed as described previously (53). bEnd3 brain microvascular endothelial cells were cultured in DMEM supplemented with 15% FBS at 37°C in 5% CO₂. For the adherence assay, bEnd3 cells at a density of 10^5 cells/ml were seeded in 24-well plates a day before the experiment. Bacterial and cell monolayers were processed in the same way described above for the intracellular survival assay. Infected cells were incubated at 37°C in 5% CO₂ for 2 h and were then washed 5 times with PBS before being lysed. The lysates were serially diluted in PBS and plated on TH agar medium, and the number of CFU was counted after overnight incubation at 37°C.

Cytotoxicity assay. A lactate dehydrogenase (LDH) cytotoxicity assay was performed as previously described but with some modifications. The CytoTox 96 nonradioactive cytotoxicity assay (Promega) was utilized to measure LDH levels. Bacteria were cultured and diluted as described above. RAW 264.7 macrophages cultured in 96-well plates were infected with 100 μ l of bacterial suspension at an MOI of 1:1. After centrifugation at $800 \times g$ for 10 min, the plates were incubated for 4 h at 37°C in 5% CO₂. Cells were lysed by Triton X-100 at a final concentration of 1% (vol/vol) to serve as the positive control for maximum release. The amount of LDH released by untreated cells and bacteria was measured as the control for spontaneous

release. The LDH release value (absorbance at 492 nm) was read by a microplate reader. The percentage of cell cytotoxicity was calculated using the formula $100 \times [(sample\ LDH\ release - spontaneous\ LDH\ release) / (maximum\ LDH\ release - spontaneous\ LDH\ release)]$, as shown in the manufacturer's protocol.

LD₅₀ determination in zebrafish. The zebrafish used in this study were raised for over a week before being challenged, and their care and feeding followed established protocols (54). Before being injected into the zebrafish, bacterial cells in late log phase in THB were harvested by two rounds of centrifugation and resuspension in phosphate-buffered saline (PBS). Zebrafish were anesthetized with 90 mg/liter tricaine methanesulfonate (MS-222) and were then intraperitoneally (i.p.) injected with 20 μ l of 10-fold serially diluted suspensions of bacteria (10^3 to 10^8 CFU/ml). Each treatment group included 14 zebrafish. Fish in the control group were injected with an equal volume of PBS. Mortality was recorded twice per day for the next 7 days. The 50% lethal dose (LD₅₀) values were calculated by the Reed-Muench method (55).

Murine infections. For the bacterial burden assay, female BALB/c mice (5 to 7 weeks of age) were bought from the Experimental Animal Center of Yangzhou University. Mice were infected with 5×10^2 CFU of the indicated strains (the wild-type, $\Delta cas9$, $\Delta regR$, or $\Delta cas9-regR$ strain) in sterile PBS. Each treatment group had six mice. At 16 h postinfection, brains, spleens, and blood were harvested, weighed, and homogenized in PBS. The homogenized samples were serially diluted and plated to enumerate the CFU. For survival experiments, groups of six mice were infected i.p. with 5×10^2 CFU of the above-indicated strains and monitored for death at each 4-h interval postinfection until 7 days passed. For the innate immunity assay, four groups of mice were infected with 5×10^2 CFU of one of the strains indicated above. At 5 h, 10 h, and 15 h postinfection, the spleens of six mice in each group were harvested and homogenized in lysis buffer for RNA extraction. RNA was extracted with the EZNA total RNA kit I (Omega, Beijing, China) and was then reverse transcribed to cDNA using the HiScript II QRT supermix (Vazyme, China). The primers used for the qRT-PCR assay are listed in Table 4. SYBR green PCR was performed using the SYBR Fast quantitative PCR (qPCR) master mix (Kapa) following the protocol described by the manufacturer. The levels of IL-6 and TNF- α mRNA were normalized to the levels of β -actin mRNA.

RNA degradation assay. RNA degradation assays were performed and modified as previously described (56). Overnight cultures of bacteria were subcultured into 10 ml of THB and grown to an OD₆₀₀ of 1.0. Rifampin was supplemented into the medium to a final concentration of 500 μ g/ml, and the cultures were incubated at 37°C. At the time points indicated above, aliquots were taken for RNA extraction.

Hyaluronidase activity measurement. Hyaluronidase activity was measured as described previously (57). Overnight cultures of bacteria were diluted to an OD₆₀₀ of 1.0, and after centrifugation, the hyaluronidase content in the supernatant was assayed as follows. The substrate for the assay was hyaluronic acid sodium dissolved in the assay buffer (150 mM NaCl, 200 mM sodium acetate, pH 6) at a concentration of 1 mg/ml. Assay buffer was used to dilute 500 μ l of the supernatant from each strain to 1 ml, and the diluted sample was incubated with 100 μ l of the substrate at 37°C. Thirty minutes later, 1 ml of stop buffer (2% NaOH, 2.5% cetrimide) was added to stop the reaction. Assay results were calculated by comparing the absorbance at 400 nm to that of a blank comprised of acetate buffer (1 ml) and stop buffer (1 ml). Various concentrations of commercial hyaluronidase from bovine testes were used as positive controls, and the turbidity of the reaction was negative relative to the enzymatic activity of hyaluronidase. The data from the positive controls were used to calculate the standard curve.

Statistical analysis. Data were analyzed with SPSS Statistics (version 20.0) software. Multiple comparisons were performed by analysis of variance (ANOVA) for the analysis of qRT-PCR results. The nonparametric Mann-Whitney U test was used to analyze the results of the animal experiments and intracellular assays. A *P* value of <0.05 indicated a significant difference, and all error bars in the figures represent the standard deviations (SDs) from independent experiments.

Data availability. The RNA-Seq data generated from this study were submitted to the NCBI Sequence Read Archive (SRA) under accession numbers SRR6269131 to SRR6269134.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/IAI.00552-17>.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.2 MB.

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