The Two-Component Signaling System VraSR$_{SS}$ Is Critical for Multidrug Resistance and Full Virulence in *Streptococcus suis* Serotype 2

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ABSTRACT *Streptococcus suis* has received increasing attention for its involvement in severe human infections worldwide as well as in multidrug resistance. Two-component signaling systems (TCSSs) play important roles in bacterial adaptation to various environmental stimuli. In this study, we identified a novel TCSS located in *S. suis* serotype 2 (SS2), designated VraSR$_{SS}$, which is involved in bacterial pathogenicity and susceptibility to antimicrobials. Our data demonstrated that the $yvqF_{SS}$ gene, located upstream of $vraSR_{SS}$, shared the same promoter with the TCSS genes, which was directly regulated by VraSR$_{SS}$, as shown in electrophoretic mobility shift assays. Notably, YvqF$_{SS}$ and VraSR$_{SS}$ constitute a novel multidrug resistance module of SS2 that participates in resistance to certain groups of antimicrobials. Further analyses showed that VraSR$_{SS}$ inactivation significantly attenuated bacterial virulence in animal models, which, coupled with the significant activation of VraSR$_{SS}$ expression observed in host blood, strongly suggested that VraSR$_{SS}$ is an important regulator of SS2 pathogenicity. Indeed, RNA-sequencing analyses identified 106 genes that were differentially expressed between the wild-type and $\Delta vraSR_{SS}$ strains, including genes involved in capsular polysaccharide (CPS) biosynthesis. Subsequent studies confirmed that VraSR$_{SS}$ indirectly regulated the transcription of CPS gene clusters and, thus, controlled the CPS thickness shown by transmission electron microscopy. Decreased CPS biosynthesis caused by $vraSR_{SS}$ deletion subsequently increased bacterial adhesion to epithelial cells and attenuated antiphagocytosis against macrophages, which partially clarified the pathogenic mechanism mediated by VraSR$_{SS}$. Taken together, our data suggest that the novel TCSS, VraSR$_{SS}$, plays critical roles for multidrug resistance and full virulence in SS2.

KEYWORDS *Streptococcus suis*, capsular biosynthesis, multidrug resistance, two-component signaling system, virulence

Streptococcus suis is an important swine pathogen responsible for a wide range of diseases, including meningitis, septicemia, endocarditis, arthritis, and sudden death (1). Thirty-three reference serotypes have been identified based on capsular polysaccharide (CPS) (2), which is recognized as an important virulence factor in *S. suis* (3, 4). *S. suis* serotype 2 (SS2) is considered one of the most prevalent serotypes worldwide and an increasingly important zoonotic pathogen for humans (1). Two large-scale outbreaks of SS2 in humans with severe clinical symptoms emerged in 1998 and 2005 in China, during which many cases exhibited streptococcal toxic shock syndrome (5).

Two-component signaling systems (TCSSs), comprised of a membrane-bound sensor histidine kinase (HK) and a cytoplasmic response regulator (RR), are thought to play key roles in bacterial colonization and virulence (6, 7). The C-terminal catalytic and

ATP-binding (CA) domain in HK protein phosphorylates the histidine residue of the dimerization and histidine phosphotransfer (DHp) domain when encountering certain external signals. Subsequently, the phosphoryl group is transferred to the receiver (REC) domain in the RR protein, and the helix-turn-helix (HTH) domain subsequently binds the target DNA promoters to induce or repress the expression of downstream genes (8, 9). The genome of SS2 encodes at least 13 TCSSs (10), among which RevS, Salk/SalR, CovR, CiaRH, Ihk/Irr, VirR/VirS, NisK/NisR, and 1910HK/RR have been reported to regulate the expression of key virulence factors or alter the bacterial metabolism, thereby contributing to the virulence of SS2 (11–18). RevS was the first TCSS in SS2 that was found to be involved in the pathogenesis of infected piglets (11), and CovR was identified as a global virulence regulator by repressing several virulence factors, such as CPS, sortase A, DNase streptodornase, laminin-binding protein, and hemolysin (13). SalK/SalR is located in the 89K pathogenicity island and was previously found to be required for the overall virulence of Chinese isolates of highly pathogenic SS2 (12). Ihk/Irr and VirR/VirS were shown to contribute to the virulence of SS2 by altering the bacterial cell metabolism (15, 16). However, it remains unclear which underlying mechanisms are employed by CiaRH, NisK/NisR, and 1910HK/RR to contribute to SS2 virulence (14, 17, 18).

To date, TCSSs implicated in antimicrobial resistance have not been reported in SS2. VraSR, a well-known TCSS, was identified to interact with its upstream gene, *yvqF*, to confer resistance to diverse cell wall-targeting antibiotics in *Staphylococcus aureus* (19, 20) and shares high sequence identity with one of the predicted TCSSs (ZY05719_02080/ZY05719_02085) in the virulent SS2 strain ZY05719. Four genes (*orf1*, *yvqF*, *vraS*, and *vraR*) form an autoregulatory operon in *S. aureus*, among which *orf1* played no observable role, whereas the gene products YvqF and VraSR acted together to recognize members of cell wall-targeting antibiotics; thus, these proteins were proposed to function as a three-component system (20). ZY05719_02075, encoded by the upstream gene of *zy05719_02080/zy05719_02085*, was predicted to be a transmembrane protein but only shared 26% amino acid identity with the YvqF protein of *S. aureus*. It will be worthwhile to determine whether this VraSR homolog from *S. aureus* or other TCSSs regulate the multidrug resistance in SS2. With the massive use of multiple antimicrobial agents for *S. suis* prophylaxis and therapy, the emergence of resistance to tetracyclines, macrolides, β-lactams, and aminoglycosides has been frequently reported in *S. suis* worldwide (21–25). Indeed, antibiotic resistance determinants for tetracyclines, aminoglycosides, β-lactams, quinolones, and macrolides have been sequentially identified in *S. suis*, as have several integrative and conjugative elements (ICEs) carrying these resistance determinants (24, 26–33). Additionally, a multidrug gene, *cfr*, conferring resistance to diverse antimicrobials, including phenicols, lincosamides, oxazolidinones, pleuromutilins, streptogramin A, and macrolides, was detected on the pStrcfr plasmid in *S. suis* (34). Although these related genes can clarify the underlying mechanism for most antibiotic resistance in *S. suis*, the resistance mechanisms to the cell wall-targeting antimicrobials have remained unclear for this Gram-positive coccus.

In this study, we redesignated the ZY05719_02075 and ZY05719_02080/ZY05719_02085 proteins YvqF$_{SS}$ and VraSR$_{SS}$, respectively. Our results demonstrated that VraSR$_{SS}$ contributes to multidrug resistance in SS2 by directly regulating the accessory protein YvqF$_{SS}$. In addition, we found that the inactivation of VraSR$_{SS}$ significantly attenuates the virulence of SS2. Further RNA-sequencing (RNA-Seq) analyses identified 52 genes in the ΔvraSR$_{SS}$ mutant that were markedly downregulated compared with that of the wild-type (WT) strain. Of note, more than 15 downregulated genes are known to be involved in the biosynthesis of CPS. Transmission electron microscopy and capsule staining confirmed that the ΔvraSR$_{SS}$ strain indeed reduced the CPS thickness significantly. These results suggest that VraSR$_{SS}$ is a novel TCSS contributing to antimicrobial drug susceptibility and the full virulence of SS2.
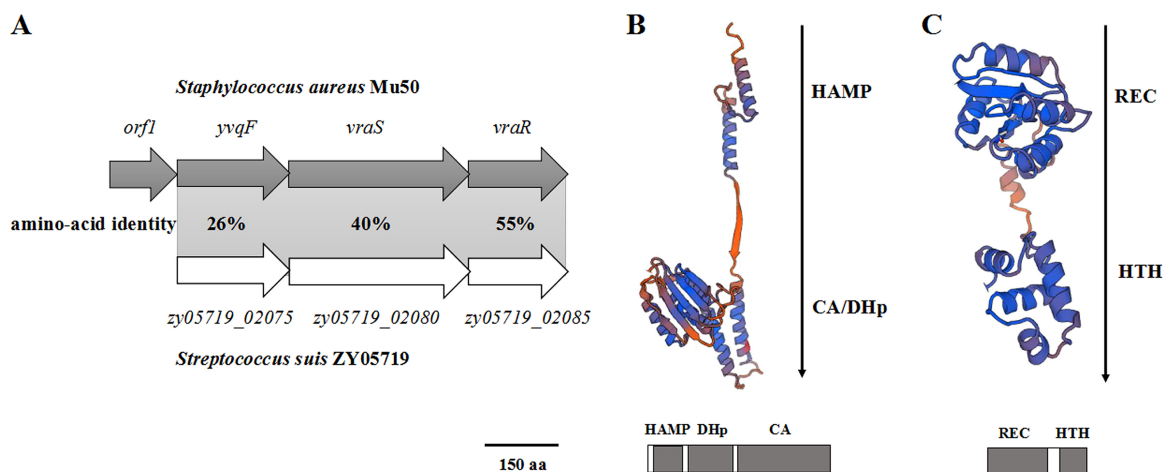
FIG 1 Identification of a novel TCSS in SS2 strain ZY05719. (A) Homology analyses of the ZY05719_02075, ZY05719_02080, and ZY05719_02085 proteins by the protein BLAST algorithm. The identities of the amino acid sequences encoded by each gene are shown between SS2 strain ZY05719 and *S. aureus* strain Mu50. (B) Predicted three-dimensional structure and conserved domains of the ZY05719_02080 protein, as determined using the SWISS-MODEL online server. The HAMP domain that potentially mediates transmembrane signal transduction, the DHp domain containing a conserved histidine residue, and the CA domain were identified by sequence alignment and are labeled accordingly. (C) Three-dimensional structure and conserved domains of the ZY05719_02085 protein, predicted using the SWISS-MODEL online server. A REC domain catalyzing phosphoryl and an HTH domain used for DNA binding were identified by sequence alignment and are labeled accordingly.

RESULTS

Identification of a potential TCSS in SS2. Homology analysis of the ZY05719_02080 and ZY05719_02085 proteins revealed that their amino acid sequences shared 40% and 55% identity with the VraS and VraR proteins of *S. aureus* (Fig. 1A) (19), respectively, suggesting that they also constitute a functional TCSS in *S. suis*. Domains and three-dimensional structures of ZY05719_02080 and ZY05719_02085 proteins were predicted using the SWISS-MODEL online server (https://www.swissmodel.expasy.org/). ZY05719_02080 was predicted to be a typical sensor kinase harboring a DHp domain, CA domain (Fig. 1B), and HAMP domain that may mediate transmembrane signal transduction. As shown in Fig. 1C, a REC domain catalyzing phosphoryl and an HTH domain used to conduct DNA binding were predicted to be present in ZY05719_02085, and a similar domain architecture is commonly found in most RRs. Such observations are consistent with the characteristics of a TCSS. Additionally, DNA sequence analyses were performed using the BLAST search service in NCBI, and *zy05719_02080* and *zy05719_02085* did not show any homology to any characterized HK or RR genes in *Streptococcus* species, suggesting that ZY05719_02080/ZY05719_02085 is an unknown TCSS in *S. suis*; thus, here it was redesignated VraS$_{SS}$/VraR$_{SS}$.

The TCSS *vraSR$_{SS}$* gene and the upstream *zy05719_02075* gene constituted a novel multidrug resistance module in SS2. It should be noted that the protein encoded by *zy05719_02075*, located upstream of the *vraS$_{SS}$* gene, shows 26% sequence identity to the YvqF protein in *S. aureus* (Fig. 1A), which interacted with VraSR to contribute to resistance against cell wall-targeting antimicrobials (20). To assess whether ZY05719_02075 also functions in the resistance to antimicrobials, we examined the susceptibility profile of the Δ*zy05719_02075* strain against selected antimicrobial agents *in vitro*. As shown in Table 1, the Δ*zy05719_02075* strain was more sensitive than the WT strain to a wide range of antimicrobials, including ampicillin, amikacin, ceftriaxone, gentamicin, kanamycin, penicillin, streptomycin, vancomycin, bacitracin, colistin, polymyxin B, and nisin, and this deficiency was completely restored by complementation. These results indicated that *zy05719_02075* functioned as a novel multidrug resistance gene in *S. suis* and showed a broader spectrum of antimicrobial resistance than *yvqF* of *S. aureus*; thus, here *zy05719_02075* was redesignated *yvqF$_{SS}$*.

We then asked whether the multidrug resistance associated with YvqF$_{SS}$ was dependent on the regulation by VraSR$_{SS}$ and constructed a *vraSR$_{SS}$* mutant strain.

TABLE 1 Sensitivity of the strains to antimicrobials

Antimicrobial	MIC (μg/ml)					Classification
	ZY05719	$\Delta vraSR_{SS}$	C$\Delta vraSR_{SS}$	$\Delta yvqF_{SS}$	C$\Delta yvqF_{SS}$	
Amikacin	32	4	32	4	16	Aminoglycosides
Gentamicin	4	0.5	4	1	4	Aminoglycosides
Kanamycin	16	2	16	4	8	Aminoglycosides
Streptomycin	128	32	128	64	128	Aminoglycosides
Ampicillin	1/8	$\leq$1/32	1/8	1/16	1/8	β-Lactams
Penicillin	1/32	$\leq$1/64	1/32	$\leq$1/64	1/32	β-Lactams
Ceftriaxone	4	0.5	4	2	4	β-Lactams
Vancomycin	0.5	0.25	0.5	0.5	0.5	Peptides
Bacitracin	8	0.5	4	0.5	4	Peptides
Colistin	256	32	256	128	256	Peptides
Polymyxin B	128	16	64	64	128	Peptides
Nisin	256	64	256	128	256	Peptides

Subsequent study showed that the MICs of all antimicrobials for the $\Delta vraSR_{SS}$ strain were close to that of the $\Delta yvqF_{SS}$ strain and were significantly lower than that for the WT strain. Complementation restored resistance to these antimicrobials to levels similar to that of the WT strain, which further confirmed the susceptibility phenotype of $vraSR_{SS}$ deletion. In addition, we conducted quantitative real-time PCR (qRT-PCR) assays, which verified that the $yvqF_{SS}$ deletion did not affect expression of the downstream gene, $vraSR_{SS}$ (see Fig. S1 in the supplemental material). These results suggested that VraSR$_{SS}$ regulates the transcription of $yvqF_{SS}$ to control multidrug resistance in SS2.

VraSR$_{SS}$ directly regulated the expression of $yvqF_{SS}$. The closely encoding neighborhood between $yvqF_{SS}$ and $vraSR_{SS}$, coupled with the mechanism of VraR in autoregulation (20), prompted us to consider that these three genes are controlled by one promoter. Indeed, prediction by the BProm program SoftBerry followed by RT-PCR analysis indicated that $yvqF_{SS}$-$vraSR_{SS}$ formed a single operon (Fig. 2A) and thus shared one promoter. Furthermore, $vraSR_{SS}$ deletion significantly decreased the expression of $yvqF_{SS}$ (Fig. 2B), while reintroducing $vraSR_{SS}$ into the mutant recovered $yvqF_{SS}$ expression to WT levels (Fig. 2B), suggesting that VraSR$_{SS}$ indeed regulated $yvqF_{SS}$ expression in SS2. To determine the mechanisms of VraSR$_{SS}$-mediated transcriptional regulation of the $yvqF_{SS}$ gene, we analyzed the upstream region of $yvqF_{SS}$ to identify a potential promoter region for the regulator-binding assay. Electrophoretic mobility shift assay (EMSA) showed that the VraR$_{SS}$ protein could bind to the upstream region of this operon but not the control fragment from 16S rRNA (Fig. 2C), which indicated that VraR$_{SS}$ directly regulated the expression of $yvqF_{SS}$ and the downstream TCSS genes, thus controlling multidrug resistance in the SS2 strain ZY05719.

VraSR$_{SS}$ is required for the full virulence of SS2. TCSSs enable bacteria to sense, respond, and adapt to a wide range of environments, stressors, and growth conditions (6, 7). Many TCSSs have been reported to respond to host stimulation, thereby contributing to the pathogenic process. Our results showed that $vraSR_{SS}$ transcription significantly increased *in vivo* by more than 4.1-fold compared to its expression level *in vitro* (Fig. 3A), suggesting that VraR$_{SS}$ regulates pathogenicity in SS2. To verify this possibility, an animal challenge test was performed using BALB/c mice injected with 5×10^8 CFU of different bacterial strains. We found that the mice infected by the $\Delta vraSR_{SS}$ strain showed a significantly higher survival rate (100%), with or without slight clinical signs, compared with the survival of mice infected by the WT strain (10%), which showed acute clinical signs, such as shivering, rough hair coat, and depression. Although the BALB/c mouse model has been widely used for *S. suis* virulence studies, the model remains controversial (35–38). Thus, we then employed a zebrafish model to repeat the *S. suis* infection assays, as it has been identified as a convincing model for evaluating virulence with *Streptococcus* spp. (39, 40). Similarly, the 50% lethal dose (LD$_{50}$) value of the $\Delta vraSR_{SS}$ strain (6.84×10^6 CFU/fish) was significantly higher than that of the WT strain (3.09×10^5 CFU/fish) in the zebrafish infection model (Table S1). These observations, coupled with the complete restoration of virulence by comple-
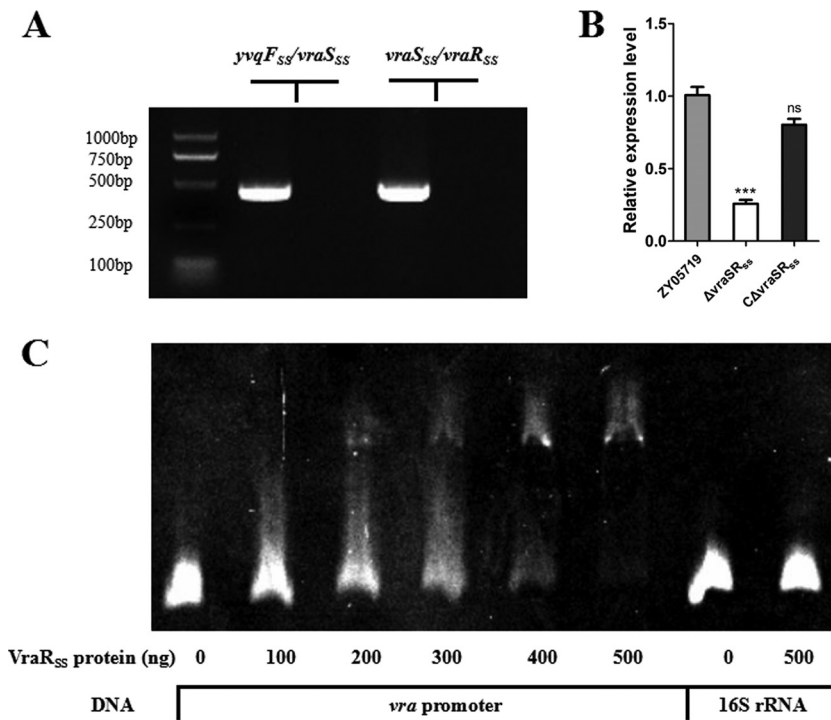
FIG 2 VraSR$_{SS}$ directly regulated the expression of YvqF$_{SS}$ and itself in SS2. (A) The $yvqF_{SS}$, $vraS_{SS}$, and $vraR_{SS}$ genes formed an operon, as determined by RT-PCR. An RNA sample that was not reverse transcribed served as a negative control. (B) Expression levels of $yvqF_{SS}$ in the WT, $\Delta vraSR_{SS}$, and complemented strains were measured by qRT-PCR. qRT-PCR expression values are shown as the means plus standard deviations (error bars) from at least three independent experiments. The unpaired two-tailed Student's t test was used for statistical analysis (ns, $P > 0.05$; ***, $P < 0.001$). (C) An EMSA showing VraR$_{SS}$ binding to the promoter region of the $yvqF_{SS}$ operon. The purified VraR$_{SS}$ fusion protein was added to each reaction mixture at different concentrations. DNA probes containing the $yvqF_{SS}$ operon promoter region were used at 80 ng per reaction mixture, and fragments amplified from 16S rRNA served as a negative control.

mentation to the WT strain levels in both mouse and zebrafish models, indicated that the virulence of SS2 was significantly attenuated by VraSR$_{SS}$ inactivation.

Identification of VraSR$_{SS}$-regulated genes related to virulence via transcriptional analysis. RNA-Seq analysis was performed to further explore the underlying
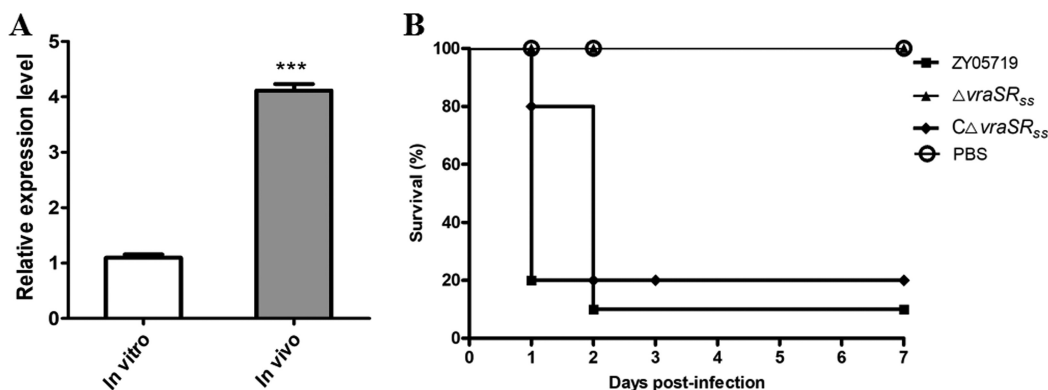

FIG 3 VraSR$_{SS}$ contributed to the full virulence of SS2. (A) Levels of $vraSR_{SS}$ gene expression during THB culture (*in vitro*) and animal infection (mouse blood, *in vivo*) were analyzed by qRT-PCR. The unpaired two-tailed Student's t test was used for statistical analysis (***, $P < 0.001$). (B) Deletion of $vraSR_{SS}$ resulted in significant attenuation of mortality in SS2 infection. Randomized groups of 10 SPF BALB/c mice were challenged with different bacterial strains at a dose of 5×10^8 CFU/mouse. Another 10 SPF BALB/c mice injected with PBS served as negative controls. The survival rates were monitored for 7 days after challenge. Significant differences in survival between different groups were analyzed by log-rank (Mantel-Cox) test ($P < 0.05$).
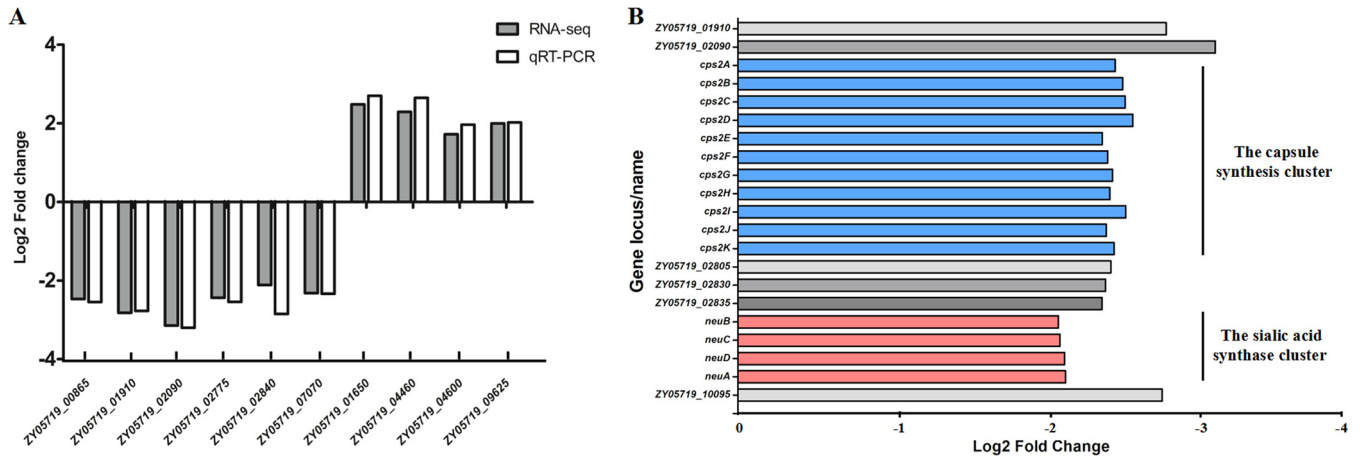
FIG 4 Identification of VraSR$_{SS}$-regulated genes via transcriptional analysis. (A) Comparison of gene regulation by RNA-Seq analysis or qRT-PCR. qRT-PCR was used to validate the expression level changes of 10 selected genes, including 4 upregulated genes and 6 downregulated genes, identified by RNA-Seq analysis. Each sample was run in triplicate, and the reference gene *parC* was detected as a control. Relative fold changes in expression were calculated using the $2^{-\Delta\Delta CT}$ method. (B) The VraSR$_{SS}$-regulated genes identified by transcriptional analysis were involved in CPS biosynthesis. The expression levels of CPS biosynthesis-related genes were significantly lower in the $\Delta vraSR_{SS}$ strain.

mechanism whereby VraSR$_{SS}$ regulates the pathogenicity of SS2. The results showed that 54 genes were significantly upregulated and 52 were significantly downregulated in the $\Delta vraSR_{SS}$ strain compared to the levels for the WT strain (Table S2). To confirm the changes in RNA transcription levels, qRT-PCR was performed to verify the expression profile of 10 selected genes in the WT and $\Delta vraSR_{SS}$ strains. The relative expression levels of six downregulated genes and four upregulated genes identified by RNA-Seq were consistent in the qRT-PCR assays, which demonstrated the reliability of the RNA-Seq transcriptional data (Fig. 4A). These regulated genes can be classified into different functional categories, including ABC transporters, protein folding, and capsular synthesis. Although the expression levels of several well-known virulence factors, like fibronectin and fibrinogen-binding protein (FBPS), muramidase-released protein (MRP), and extracellular protein factor (EF), were unchanged, the expression level of gene clusters driving CPS biosynthesis were significantly decreased in the $\Delta vraSR_{SS}$ strain (Fig. 4B), suggesting that VraSR$_{SS}$ regulates virulence by controlling CPS biosynthesis in SS2.

VraR$_{SS}$ indirectly regulated the expression of gene clusters driving CPS biosynthesis in SS2. CPS has been proven to be a critical virulence factor in SS2, and the N-acetylneuraminic acid (sialic acid) linked to the end of the CPS chain also played a role in SS2 virulence (3, 4, 41–43). Our RNA-Seq data revealed that both the capsule synthesis cluster (*cps2A-K*) and the sialic acid synthase cluster (*neuBCDA*) were significantly downregulated in the $\Delta vraSR_{SS}$ compared to the WT strain (Fig. 4B and 5A). qPCR results confirmed similar decreases in the gene expression levels of *cps2C*, *cps2F*, and *neuB* in the $\Delta vraSR_{SS}$ strain, while complementation completely restored transcription-level deficiencies caused by the *vraSR$_{SS}$* deletion (Fig. 5B). To confirm that CPS biosynthesis was attenuated in $\Delta vraSR_{SS}$, transmission electron microscopy (TEM) was performed to visualize the colony morphology. As shown in Fig. 5C, inactivation of VraSR$_{SS}$ caused a significant decrease of capsule thickness compared to that in the WT strain, which generally matched the transcriptomics and qPCR assay results. This effect on capsule formation was confirmed at the population level by crystal violet staining (Fig. 5D). As expected, complementation nearly restored the capsule thickness to that of the WT strain. We also conducted EMSAs to examine whether VraR$_{SS}$ directly regulated expression of the *cps* and *neu* cluster. The promoter of the *vra* operon and the fragments amplified from the 16S rRNA gene served as controls. As shown in Fig. 5E, neither the promoter region of the *cps* cluster nor that of the *neu* cluster was shifted by the recombinant VraR$_{SS}$ protein, and only the upstream region of the *yvqF$_{SS}$* operon
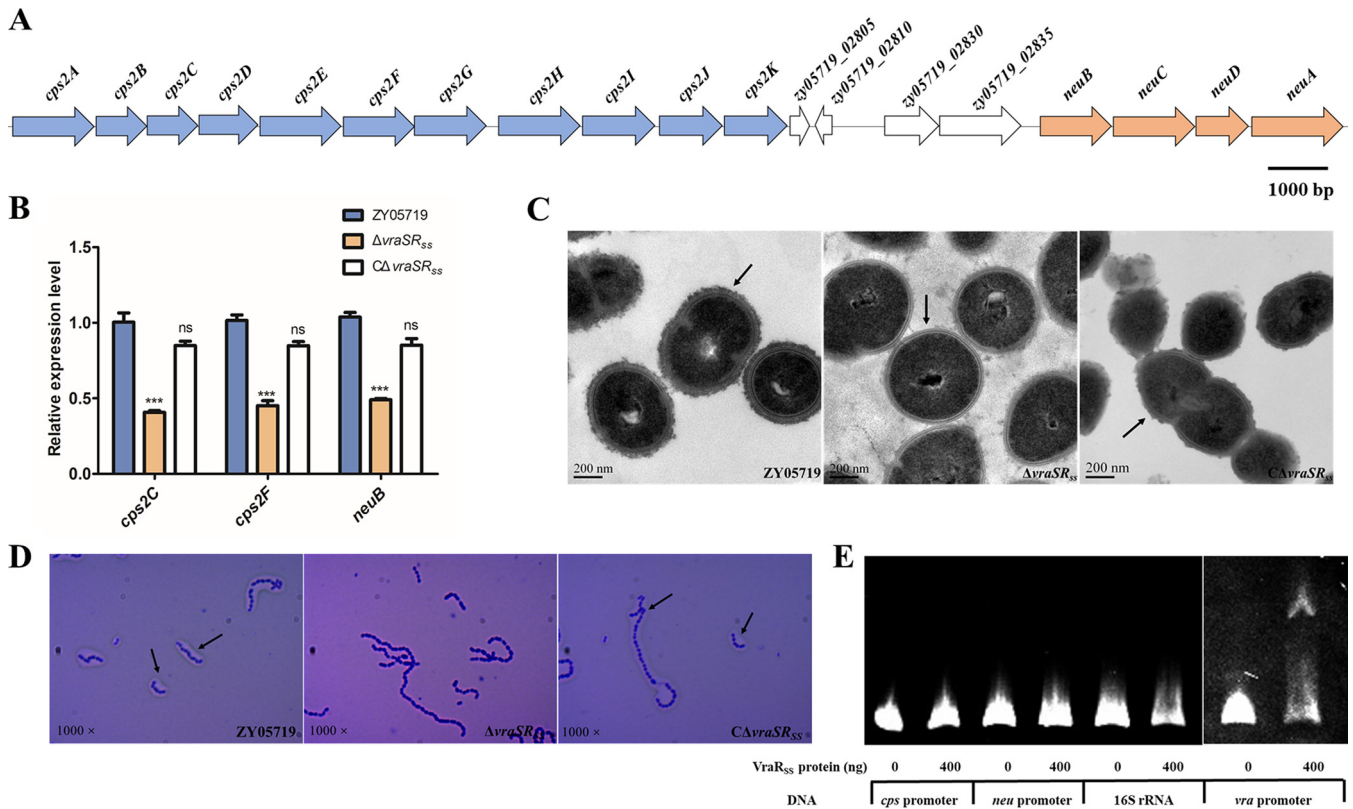
FIG 5 VraSR$_{SS}$ indirectly regulated expression of the CPS biosynthesis gene cluster in SS2. (A) Schematic diagram of the genetic organization of the CPS synthesis and the sialic acid synthase clusters. The arrows indicate the direction of transcription. (B) Expression levels of *cps2C*, *cps2F*, and *neuB* in ZY05719, Δ*vraSR$_{SS}$*, and CΔ*vraSR$_{SS}$* strains were measured by qRT-PCR. The unpaired two-tailed Student's *t* test was used for statistical analysis (ns, $P > 0.05$; ***, $P < 0.001$). (C) Transmission electron micrographs of the ZY05719, Δ*vraSR$_{SS}$*, and CΔ*vraSR$_{SS}$* strains. The scale bars indicate the magnification size. (D) Photomicrographs of the capsules of the ZY05719, Δ*vraSR$_{SS}$*, and CΔ*vraSR$_{SS}$* strains stained with crystal violet (1,000×). (E) VraR$_{SS}$ indirectly regulated the CPS biosynthesis gene cluster. Either the *cps* or *neu* promoter region could be shifted by the recombinant VraR$_{SS}$ protein in EMSAs. DNA probes containing the *yvqF$_{SS}$* operon promoter region were used as a positive control, and fragments amplified from 16S rRNA served as a negative control.

(positive control) was clearly bound. These results suggested that VraSR$_{SS}$ controlled capsular biosynthesis via indirect regulation to contribute to the full virulence of SS2. Future work will need to be performed to identify the downstream factor(s) of VraSR$_{SS}$ and its regulatory mechanism for the *cps* and *neu* clusters.

Downregulation of CPS biosynthesis caused by *vraSR$_{SS}$* deletion is responsible for the increased adhesion to bEnd.3 cells and attenuated antiphagocytosis against RAW 264.7 cells. Adhesion to the host cell surface and antiphagocytosis against macrophages are considered essential steps in the pathogenesis of bacterial infections (44). Previous studies have confirmed that loss of capsule in SS2 could impair antiphagocytosis against macrophages and increase adhesion to epithelial cells (45, 46). Here, the WT, a Δ*vraSR$_{SS}$* complementary strain, and a capsular deficient mutant strain (Δ*cps*) were tested to assess whether VraSR$_{SS}$ inactivation would cause similar effects. As shown in Fig. 6, both the *vraSR$_{SS}$* and *cps* mutant strains displayed significantly increased adhesion to bEnd.3 cells ($P < 0.001$) but significantly attenuated antiphagocytosis against RAW264.7 cells versus the WT strain. Notably, the *vraSR$_{SS}$*-complemented strain showed complete restoration of the above-described changes to levels similar to that of the WT strain. These results further confirmed that VraSR$_{SS}$ could control capsular biosynthesis to be involved in antiphagocytosis and host cell adhesion, thereby playing key roles in the full virulence of SS2.

DISCUSSION

TCSSs are comprised of a membrane-bound HK protein and a cytoplasmic RR protein, which represent one of the major mechanisms used by bacterial cells to adapt
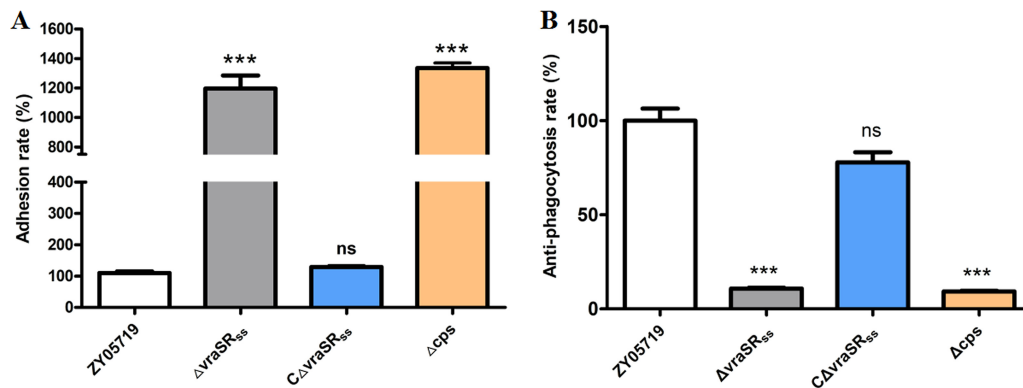
FIG 6 VraSR$_{SS}$ is involved in SS2 adhesion to bEnd.3 cells and resisting phagocytosis by RAW 264.7 cells. (A) Effect of the *vraSR$_{SS}$* mutation on the ability of SS2 to adhere to bEnd.3 cells. The adhesion rate of the ZY05719 strain was significantly lower than that of the Δ*vraSR$_{SS}$* and Δ*cps* strains. The *vraSR$_{SS}$*-complemented strain showed adhesion restored to the level of the WT strain. (B) Effect of the *vraSR$_{SS}$* deletion on the ability of SS2 to resist phagocytosis by RAW 264.7 cells. The antiphagocytosis rate of the ZY05719 strain was significantly higher than that of the Δ*vraSR$_{SS}$* and Δ*cps* strains. The *vraSR$_{SS}$*-complemented strain showed an ability restored to the level of the WT strain. The data are shown as the means and standard deviations of the results from three independent experiments performed in triplicate. Two-tailed unpaired Student's *t* tests were used for statistical analysis (***, $P < 0.001$).

to harsh environmental stimuli, such as pH, temperature, and antimicrobial drugs (7, 47). VraSR, a TCSS identified in *S. aureus*, was initially characterized as the master regulator of vancomycin resistance that functions by interacting with the membrane-anchored protein YvqF, and it was subsequently demonstrated to respond to various cell wall-targeting antimicrobials, including vancomycin, β-lactams, and certain cationic peptides (19, 20, 48). VraSR$_{SS}$ is a homolog carried by *S. suis* that shares ~50% sequence identity with the VraSR of *S. aureus*. The upstream gene *yvqF$_{SS}$* also encodes a membrane-anchored protein but shares very low homology with the YvqF protein of *S. aureus*.

Our results showed that VraSR$_{SS}$ inactivation caused a direct and significant down-regulation of the *yvqF$_{SS}$* gene and confirmed that either *yvqF$_{SS}$* or *vraSR$_{SS}$* deletion significantly increased the susceptibility to diverse antimicrobial agents. Notably, YvqF$_{SS}$ and VraSR$_{SS}$ showed a broader spectrum than their homologs in *S. aureus* in terms of multidrug resistance, which are classified based on differing chemical structures and compositions, including aminoglycosides (amikacin, gentamicin, kanamycin, and streptomycin), β-lactams (ampicillin, penicillin, and ceftriaxone), and peptides (vancomycin, bacitracin, colistin, polymyxin B, and nisin). As reported previously, VraSR in *S. aureus* responds to cell wall-targeting antibiotics but not aminoglycoside antimicrobials (20). The β-lactam- and peptide-based antimicrobials target the cell wall to kill bacteria, while the aminoglycosides employ a different antibacterial mechanism by inhibiting protein synthesis (49). Although a previous study demonstrated that *S. suis* resistance to β-lactams occurs due to the alteration of penicillin-binding proteins (32), data generated for VraSR$_{SS}$ and the accessory YvqF$_{SS}$ in this study reveal a novel mechanism of this antibiotic resistance. Perhaps the membrane-anchored protein YvqF$_{SS}$ was involved in the cell wall biosynthesis pathway in *S. suis*, and the decreased production of YvqF$_{SS}$ might damage the integrity of both cell membrane and cell wall, thus enhancing the permeability of bacterial cells to aminoglycosides and increasing susceptibility to this class of antimicrobials. Unlike the plasmid-located multidrug resistance gene *cfr*, the chromosomal *yvqF$_{SS}$* gene is not easily transferred during interbacterial communication and is directly regulated by the TCSS VraSR$_{SS}$ to more precisely respond to antibiotics. Recently, TCSSs have become increasingly recognized as potential targets of adjuvant antibiotic therapies, because their regulation of virulence and responses to cell envelope stress could affect the proliferation of bacterial pathogens in the host (50). For example, walkmycin B and waldiomycin showed antibiotic activity against *Bacillus subtilis* and *S. aureus* by inhibiting HK protein WalK

autophosphorylation (51, 52). Therefore, an inhibitor of VraSR$_{SS}$ could reduce the mortality associated with *S. suis* infection.

The acquisition of vancomycin resistance in *S. aureus* is often accompanied by reduced virulence. A recent study found that VraSR directly regulated the function of the Agr quorum-sensing system and, thus, inhibited virulence in *S. aureus* (53). However, our data showed that *vraSR$_{SS}$* expression was significantly higher *in vivo* than *in vitro*, which prompted us to consider its potential roles in pathogenesis. Indeed, the deletion of the *vraSR$_{SS}$* gene significantly attenuated the virulence of SS2 in both the mouse and zebrafish models, suggesting that VraSR$_{SS}$ is an important TCSS in mediating responses to host stimulation and for the expression of many virulence factors *in vivo*. RNA-Seq analyses identified 106 genes in the ΔvraSR$_{SS}$ strain with significantly different transcription levels compared with those in the WT strain, which helped elucidate the underlying regulatory mechanism of VraSR$_{SS}$ in SS2 strain ZY05719. Of these 106 genes, more than 15 downregulated genes were from the same clusters and related to capsular biosynthesis. It is well known that CPS are key virulence factors in SS2 and play critical roles in antiphagocytic processes against host macrophage phagocytosis and killing (3, 4). Further study showed that *vraSR$_{SS}$* deletion caused the results consistent with CPS deficiency in increasing bacterial adhesion to epithelial cells and attenuating antiphagocytosis against macrophages, which partially explained the attenuated bacterial pathogenicity in animal models.

To further examine the process whereby VraSR$_{SS}$ regulates CPS biosynthesis, we visualized the bacterial morphology by TEM and capsule staining. The results confirmed that VraSR$_{SS}$ inactivation significantly decreased the capsule thickness in SS2 compared to that in the WT strain, which was consistent with the transcriptional data and the attenuated virulence observed in animal challenge assays. In addition, our EMSA showed that VraR$_{SS}$ did not bind the *cps* and *nue* promoter regions from the SS2 capsule biosynthesis cluster. This finding, coupled with the qRT-PCR results displaying a significant downregulation of clustered genes related to CPS expression in ΔvraSR$_{SS}$ compared to the WT and complementary strains, suggested that TCSS VraSR$_{SS}$ indirectly regulates CPS biosynthesis.

Environmental signals can also modulate the expression of virulence factors, and *S. suis* growth in Todd-Hewitt broth (THB) exhibits a weaker invasion ability than the growth in culture medium with fewer nutrients (46). Not only transcriptional regulation of the *cps* gene cluster but also the availability of glucose or other carbohydrates affects CPS biosynthesis (54). Our RNA-Seq results highlighted several sugar transporter genes (*zy05719_10085* to *zy05719_10100*) that were significantly downregulated in the ΔvraSR$_{SS}$ strain, suggesting that VraSR$_{SS}$ controlled CPS biosynthesis by regulating carbohydrate acquisition in SS2 cells. In addition, given that a previous report showed that the global virulence regulator CovR could repress CPS biosynthesis (13), we compared the transcriptome data between ΔvraSR$_{SS}$ and ΔcovR strains to clarify the potential correlation between both TCSSs. Unlike VraSR$_{SS}$, which upregulated transcription of the whole CPS biosynthesis cluster (*cps2A-K* and *neuBCDA*), CovR downregulated the transcription of only two genes (*cps2C* and *neuA*) in the cluster (13), suggesting that these TCSSs have regulated CPS biosynthesis through different mechanisms. Notably, VraSR$_{SS}$ are activated during systemic infection (Fig. 3), which may help relieve the repression of CovR on *cps2C* and *neuA* for CPS biosynthesis, thus increasing the bacterial fitness in host bloodstream for full virulence.

In summary, a novel TCSS (VraSR$_{SS}$) associated with multidrug resistance and virulence was identified in SS2. We demonstrated that VraSR$_{SS}$ directly regulated the expression of the membrane-anchored protein encoded by *yvqF$_{SS}$* to mediate multidrug resistance and contributed to the full virulence of SS2 by indirectly regulating CPS biosynthesis. However, the external signal that activates HK and the underlying mechanism of VraSR$_{SS}$ in regulating CPS biosynthesis remain unknown and require further exploration in our future work.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and plasmids. The SS2 strain ZY05719 was originally isolated from a diseased pig during an outbreak in the Sichuan Province of China. The strain was grown at 37°C in Todd-Hewitt broth (THB; Becton-Dickinson, USA) or Todd-Hewitt broth agar (THA) and harvested at the mid-exponential growth phase (optical density at 600 nm [OD_{600}] of 0.6). *Escherichia coli* strains DH5α and BL21(DE3) were grown on Luria-Bertani (LB) agar plates or in LB broth at 37°C. When antibiotics (Sigma-Aldrich) were required, the following concentrations were added to the medium: for *S. suis*, spectinomycin (Spc) at 100 μg/ml; for *E. coli*, Spc at 50 μg/ml and kanamycin (Kan) at 50 μg/ml. All strains and plasmids used in this study are listed in Table S3 in the supplemental material.

Recombinant DNA techniques. The pSET4S vector was used to construct the target gene deletion mutants as previously described (55). The upstream and downstream regions of target genes were amplified from genomic DNA of the ZY05719 strain and fused together by overlap extension PCR. After digestion with respective endonucleases, the fusion fragments were inserted into pSET4S vector with the same endonucleases sites. ZY05719 competent cells were prepared and electrotransformed with the recombinant vector, after which the resultant strains were selected at 28°C in Spc and then grown at 37°C without Spc. The deletion of target genes was verified by PCR and sequencing. To construct the complemented strains, the target genes (including their putative promoter sequences) were amplified from the ZY05719 strain and independently inserted into the pSET2 plasmid. The recombinant pSET2 plasmids then were electroporated into mutant competent cells.

To express the recombinant VraR$_{SS}$ protein, the coding region of *vraR$_{SS}$* was amplified from genomic DNA and inserted into the pET28a vector. After confirming the DNA sequence, the recombinant pET28a-*vraR$_{SS}$* vector was transformed into BL21(DE3) cells. The sequences of all primers used in this study are shown in Table S4.

Bioinformatics analysis. DNAStar Lasergene 7 software and the BLAST program (available from the NCBI website, http://blast.ncbi.nlm.nih.gov/) were used to analyze the DNA sequences. The three-dimensional structures of VraS$_{SS}$ and VraR$_{SS}$ were predicted using the SWISS-MODEL online server (https://www.swissmodel.expasy.org/) (56–59). The BProm program (SoftBerry) was used to predict the promoter region.

Antimicrobial susceptibility testing. The MIC of an antimicrobial substance was defined as the lowest concentration resulting in no detectable bacterial growth. The MICs of antimicrobials (Sigma-Aldrich) were determined by performing the standardized microdilution assay in a 96-well microtiter plate containing 100 μl THB medium in each well. The strains were grown to mid-exponential growth phase, diluted to an OD_{600} of 0.05, and used to inoculate wells containing THB with standard 2-fold (vol/vol) dilutions of the antimicrobial of interest. Plates were incubated at 37°C for 24 h. Each assay was repeated independently three times.

EMSAs. EMSAs were performed to analyze the binding of VraR$_{SS}$ to the DNA probe. The recombinant VraR$_{SS}$ protein was expressed in BL21 cells from the pET28a-*vraR$_{SS}$* vector and purified using nickel-nitrilotriacetic acid spin columns (GE Healthcare). The DNA probes were obtained by PCR amplification and purified using a gel extraction kit (TaKaRa). The negative-control probe was amplified from 16S rRNA. Increasing amounts of native purified VraR$_{SS}$ protein (0 to 500 ng) were added to the DNA probe (80 ng) in binding buffer (10 mM Tris base, 50 mM KCl, 5 mM MgCl$_2$, 1 mM dithiothreitol, 0.05% Nonidet P-40, 2.5% glycerol, pH 7.5). The reaction mixtures were incubated at 37°C for 30 min and then loaded onto a 6% polyacrylamide gel in 0.5× TBE buffer (44.5 mM Tris base, 44.5 mM boric acid, 1 mM EDTA; pH 7.5) at 200 V for 45 min. After electrophoresis, the gel was stained in 0.5× TBE buffer with ethidium bromide for 20 min.

Animal tests. To investigate the survival curves of mice, 10 specific-pathogen-free (SPF) BALB/c mice (female, 6 weeks old) were challenged by intraperitoneal injection with the strains of interest at a dose of 5×10^8 CFU/mouse. Another 10 SPF BALB/c mice were injected with an equal volume of sterile phosphate-buffered saline (PBS) as a negative control. Clinical signs and survival rates were monitored for 7 days after challenge.

In addition, to calculate the LD$_{50}$ values, 15 zebrafish per group were challenged by intraperitoneal injection with 20 μl of 10-fold-diluted bacteria, ranging from 5×10^4 to 5×10^7 total CFU/fish. Another group was injected with an equal volume of sterile PBS as a negative control. Mortality was monitored for 7 days postchallenge. This experiment was repeated three times. LD$_{50}$ values were calculated using the method of Reed and Muench (60).

All animal experiments were conducted in the Laboratory Animal Center of the Nanjing Agricultural University with the approval of the Laboratory Animal Monitoring Committee of Jiangsu Province.

RNA isolation, transcriptome sequencing, RT-PCR, and qRT-PCR. RNAs from the ZY05719 and Δ*vraSR$_{SS}$* strains were extracted using TRIzol (TaKaRa) according to the manufacturer's instructions. After removing contaminating DNA, the extracted RNAs were sent to Novogene (Tianjin, China) for RNA-Seq analysis. Each strain was analyzed using three biological replicates. The sequencing library was constructed and sequenced using the Illumina HiSeq 2000 platform, as previously described (61). To control the false discovery rate for the transcriptome data, comparisons with estimated fold changes of at least 3 (log$_2$ fold changes of at least 1.6) and *q* values of ≤0.05 were declared significant.

RT-PCR and qRT-PCR were performed to validate the RNA transcription levels determined by RNA-Seq. Total RNAs were isolated from strains cultured in THB and from the infected blood of mice. After extraction as described above, total RNAs were reverse transcribed by following the recommended protocol of the HiScript II first-strand cDNA synthesis kit (Vazyme). The QuantStudio 6 Flex real-time PCR system (Thermo Fisher Scientific) and SYBR Premix *Ex Taq* (TaKaRa) were used per the manufacturers' instructions. The sequences of primers used for qRT-PCR are shown in Table S4, and the housekeeping

gene (*parC*) was used as a control (62). Each sample procedure was repeated three times. Relative fold changes in expression were calculated using the $2^{-\Delta\Delta CT}$ method (63).

For the cotranscription test, total RNAs were extracted and reverse transcribed as described above. Primers were designed to span the open reading frames (ORFs) $yvqF_{SS}$ and $vraS_{SS}$, as well as $vraS_{SS}$ and $vraR_{SS}$. RNA was purified and reverse transcribed to prepare cDNA. One microgram of total RNA samples (without reverse transcription) served as a control to confirm that the samples were free of contaminating DNA. The RNA and matching cDNA samples then were used as PCR templates.

TEM and capsule staining. The colony morphology was analyzed by TEM. Briefly, bacteria growing in mid-exponential phase were harvested by centrifugation at $5,000 \times g$ for 10 min and fixed in 2.5% glutaraldehyde for more than 2 h. The samples were dehydrated in propylene oxide for 10 min, embedded in epoxy resin, and examined using a Hitachi H-7650 system (Hitachi) according to the manufacturer's instructions. This experiment was performed independently three times.

To visualize the bacterial capsules at the population level, capsule staining was performed as previously described (64). A drop of bacterial culture was placed on a microscope slide and spread in a thin film using another slide. After gentle flame fixation, the smear was stained with 0.1% crystal violet and gently heated. Subsequently, the dye was washed with 20% copper sulfate. The slide was air dried and examined immediately under oil immersion.

Cell experiments. Mouse brain microvascular endothelial cells (bEnd.3) were used to perform adhesion assays, and murine macrophage-like RAW264.7 cells were used to study antiphagocytosis of SS2. These cells were cultured in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS), and maintained at 37°C with 5% CO_2. The strains were grown to mid-exponential growth phase (OD$_{600}$ of 0.6) and washed twice in PBS.

Adhesion assays were performed as previously reported (65). The bEnd.3 cells were cultured in 24-well cell plates and washed three times with PBS. The bacteria were suspended in DMEM without antibiotics to a density of 1×10^7 CFU/ml. After infecting the cells in each well with 1 ml of the bacterial suspension, the plates were centrifuged at $800 \times g$ for 15 min and incubated at 37°C for 90 min. Subsequently, the infected cells were washed five times and trypsinized at 37°C for 10 min. Serial dilutions of the cell lysate were plated onto THA, and the plates were incubated overnight at 37°C. Each assay was repeated independently three times.

For the antiphagocytosis assay, the strains were incubated with RAW264.7 macrophages at a bacterium-to-cell ratio of 100:1. After coculturing for 1.5 h, the infected cells were washed three times with PBS and incubated in DMEM containing gentamicin (100 μg/ml) and penicillin (5 μg/ml). After coculturing for another 1.5 h, the macrophages were washed three times and lysed with water. Serial dilutions of the cell lysate were plated onto THA. Each assay was repeated independently three times.

Data analysis. All experiments were performed at least three times, and the data were analyzed using an unpaired two-tailed Student's *t* test, or the log-rank (Mantel-Cox) test, with the GraphPad software package. For all tests, a *P* value of <0.05 was considered statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI .00096-18.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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