The Orphan Response Regulator CovR: a Globally Negative Modulator of Virulence in *Streptococcus suis* Serotype 2[†]

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Streptococcus suis serotype 2 is an emerging zoonotic pathogen responsible for a wide range of life-threatening diseases in pigs and humans. However, the pathogenesis of *S. suis* serotype 2 infection is not well understood. In this study, we report that an orphan response regulator, CovR, globally regulates gene expression and negatively controls the virulence of *S. suis* 05ZYH33, a streptococcal toxic shock syndrome (STSS)-causing strain. A *covR*-defective ($\Delta covR$) mutant of 05ZYH33 displayed dramatic phenotypic changes, such as formation of longer chains, production of thicker capsules, and increased hemolytic activity. Adherence of the $\Delta covR$ mutant to epithelial cells was greatly increased, and its resistance to phagocytosis and killing by neutrophils and monocytes was also significantly enhanced. More importantly, inactivation of *covR* increased the lethality of *S. suis* serotype 2 in experimental infection of piglets, and this phenotype was restored by *covR* complementation. Colonization experiments also showed that the $\Delta covR$ mutant exhibited an increased ability to colonize susceptible tissues of piglets. The pleiotropic phenotype of the $\Delta covR$ mutant is in full agreement with the large number of genes controlled by CovR as revealed by transcription profile analysis: 2 genes are positively regulated, and 193 are repressed, including many that encode known or putative virulence factors. These findings suggested that CovR is a global repressor in virulence regulation of STSS-causing *S. suis* serotype 2.

Streptococcus suis serotype 2 is an important swine pathogen mainly associated with arthritis, endocarditis, meningitis, pneumonia, and septicemia (49, 66). It is also a major zoonotic agent for humans in contact with colonized, otherwise healthy pigs or their by-products, causing life-threatening infections. Recently, we reported an unprecedented epidemic in China in 2005, which resulted in 204 human cases (69). In particular, 18.6% of these human patients died from streptococcal toxic shock syndrome (STSS), implying that this important pathogenic species has evolved some new mechanisms to enhance its pathogenicity. To further pursue this issue, we have decoded the complete genomic sequences of two highly virulent *S. suis* serotype 2 isolates and identified a new pathogenicity island, referred to as 89K, which is prevalent in STSS-causing *S. suis* serotype 2 isolates (11). Very recently, we provided genetic evidence that 89K functions as a pathogenicity island and even claimed high virulence of Chinese isolates (46). Efforts are still being made in our joint research to achieve a comprehensive

understanding of the pathogenesis of *S. suis* serotype 2 infection.

Two-component signal transduction systems (TCSs) play important roles in bacterial gene expression (including that of many virulence-related genes) in response to a variety of environmental stimuli (19). In general, a TCS consists of a sensor kinase and an effector, or response regulator, which is generally a DNA-binding protein that modulates the expression of certain target genes. In *S. suis* serotype 2, at least 15 TCSs have been identified (11). Among them, only the orphan response regulator RevS (14) and the SalK-SalR (46) system have been reported and shown to positively control *S. suis* serotype 2 virulence. It is known that bacterial virulence is tightly regulated by both positive and negative feedback mechanisms. Therefore, environmental regulation of virulence gene expression in *S. suis* serotype 2 via TCSs deserves to be further addressed.

The newly sequenced genome of *S. suis* 05ZYH33, a highly pathogenic strain isolated from a Chinese STSS patient, enabled the identification of an orthologue of the CovR (for control of virulence) regulator (also known as CsrR, for capsule synthesis regulator) known to be involved in the virulence of *Streptococcus pyogenes* (group A streptococcus [GAS]) and *Streptococcus agalactiae* (group B streptococcus [GBS]) (22, 42). Unlike the other streptococcal CovS-CovR systems, *covR* is not located in the genome of *S. suis* 05ZYH33 next to a cognate histidine kinase; therefore, CovR was considered to be an orphan response regulator. In GAS, the global regulatory

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	Source/reference
Strains		
<i>S. suis</i> serotype 2		
05ZYH33	Virulent strain isolated from a dead patient with STSS	Laboratory collection
$\Delta covR$	Isogenic <i>covR</i> mutant of strain 05ZYH33; <i>Spc</i> ^r	This study
C $\Delta covR$	Complemented strain of $\Delta covR$; <i>Spc</i> ^r <i>Cm</i> ^r	This study
05HAS68	Avirulent strain isolated from a healthy pig	Laboratory collection
<i>E. coli</i>		
DH5 α	Host for cloning vector	Promega
Plasmids		
pMD18-T	TA cloning vector; <i>Amp</i> ^r	TaKaRa
pMD18- <i>covR</i>	pMD18-T with a PCR-derived fragment containing the <i>covR</i> gene and its flanking regions; <i>Amp</i> ^r ,	This study
pMD:: <i>covR</i>	pMD18- <i>covR</i> with a spectinomycin resistance gene inserted into the unique XhoI site of the <i>covR</i> gene; <i>Amp</i> ^r <i>Spc</i> ^r	This study
pSET1	<i>E. coli</i> - <i>S. suis</i> shuttle vector; <i>Cm</i> ^r	68
pSET2	<i>E. coli</i> - <i>S. suis</i> shuttle vector; <i>Spc</i> ^r	68
pSET1:: <i>covR</i>	pSET1 containing the intact <i>covR</i> gene and its upstream promoter; <i>Cm</i> ^r	This study

^a *Amp*^r, ampicillin resistant; *Cm*^r, chloromycetin resistant; *Spc*^r, spectinomycin resistant.

two-component system CovS-CovR controls the expression of nearly 15% of all chromosomal genes, including many that encode surface and secreted proteins mediating pathogen-host interactions, such as hyaluronic acid capsule, cysteine protease (pyrogenic exotoxin), streptokinase, streptolysin S, and streptodornase (22). Moreover, CovR was shown to play a central role in gene regulatory networks by influencing the expression of genes encoding transcriptional regulators, including other TCSs. Additionally, consistent with increased virulence gene transcription, a *covR*-defective mutant exhibited enhanced virulence in the mouse and increased resistance to human polymorphonuclear leukocyte (PMN)-mediated killing (17, 18, 27, 45). Similarly, in the case of GBS, a close relative of GAS, CovS-CovR also has a pleiotropic effect, influencing the transcription of about 7% of GBS genes (42). Interestingly, a $\Delta covSR$ mutant showed remarkable phenotypic changes, such as increased hemolytic activity and hyperadhesion to epithelial cells. In contrast to the pathogenic role of CovS-CovR in GAS, a mutant in the *covSR* locus in GBS was attenuated in virulence in an intraperitoneal-injection model (31, 42). As in *S. suis* 05ZYH33, CovR in *S. mutans* is also an orphan response regulator and has been shown to be essential for biofilm development and cariogenesis (28).

Here, we show that the orphan response regulator CovR plays a crucial role in negatively controlling the virulence of *S. suis* serotype 2. A genome scale overview of the CovR regulatory network indicates that CovR is a global virulence regulator in *S. suis* serotype 2.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *S. suis* serotype 2 strains were grown in Todd-Hewitt broth (THB) (Difco Laboratories, Detroit, MI) and plated on THB agar (Biotrading) containing 6% (vol/vol) sheep blood. *E. coli* strains were grown in Luria broth and plated on Luria broth containing 1.5% (wt/vol) agar. If required, antibiotics were added in the following concentrations: 50 μ g/ml of spectinomycin (Sigma, St. Louis, MO) for the *S. suis* serotype 2 mutant strain, 5 μ g/ml of chloramphenicol (Sigma, St. Louis, MO) and 50 μ g/ml

spectinomycin for the complemented strain, and 100 μ g/ml of spectinomycin, 10 μ g/ml of chloramphenicol, and 100 μ g/ml of ampicillin (Boehringer, Mannheim, Germany) for *Escherichia coli*.

Construction of a *covR* knockout mutant. To construct the mutant strain $\Delta covR$, the *covR* gene was insertionally inactivated in *S. suis* serotype 2 strain 05ZYH33 with a spectinomycin resistance cassette. A 3.4-kb DNA fragment containing the *covR* gene and its flanking regions was amplified from 05ZYH33 genomic DNA with primers LA and RA (Table 2 lists the sequences of the primers used) and then cloned into the pMD18-T cloning vector (TaKaRa, Japan) to create pMD-*covR*. Subsequently, a 1.1-kb spectinomycin resistance cassette (amplified from the plasmid pSET2) was inserted into a unique XhoI site within the *covR* coding sequence in pMD-*covR* to generate the *covR* knockout vector pMD::*covR*. Since the *spc* gene does not carry a terminator, insertion of *spc* is expected to be nonpolar. Plasmid pMD::*covR* was used to electrotransform *S. suis* 05ZYH33 as described previously (64). Spectinomycin-resistant transformants were selected on THB supplemented with 0.5% yeast extract containing the appropriate antibiotic, and PCR analysis with flanking primers lying outside the homologous regions and reverse transcription (RT)-PCR detection were carried out to confirm that *covR* inactivation had occurred by double-crossover recombination.

Complementation of the *covR* inactivation. For complementation assays, a DNA fragment containing the entire *covR* gene and its upstream promoter was amplified using primers C-F and C-R, which introduced EcoRI and PstI sites, respectively. After digestion with the appropriate restriction enzymes, the resulting fragment was cloned into the *E. coli*-*S. suis* shuttle vector pSET1 (68) at the EcoRI/PstI sites to generate the *covR*-complementing plasmid pSET1::*covR*. This vector was electrotransformed directly into the $\Delta covR$ mutant to obtain the complemented strain C $\Delta covR$ using our previously reported method (46).

Transmission electron microscopy (TEM). The samples from agar-grown bacteria (05ZYH33 and the $\Delta covR$ strain) were prepared by conventional methods. Briefly, bacterial cells were fixed in 5% glutaraldehyde for 2 h, postfixed with 1% osmium tetroxide for 1 h, dehydrated in ethanol, and embedded in Epon-812 epoxy resin. Thin sections were poststained with uranyl acetate and lead citrate and examined with a JEM-1010 transmission electron microscope (Jeol Ltd., Tokyo, Japan) at an accelerating voltage of 100 kV. Capsule thickness measurements were made on 20 to 25 randomly chosen cells of each strain.

Titration of hemolytic activity. Hemolysin units in the culture supernatant were determined as described by Jacobs et al. (30), with slight modifications. Briefly, 2% sheep red blood cells in phosphate-buffered saline (PBS) were prepared, and 150 μ l of the red blood cells was added to serial twofold dilutions of culture supernatant of 05ZYH33 and the $\Delta covR$ strain. The microplate was incubated at 37°C for 2 h and then centrifuged at 600 $\times$ g for 10 min; 150 μ l of supernatant was transferred to a new plate and read at 540 nm with a micro-ELISA reader (Bio-Tek, Synergy HT). Hemolysin units were determined as the

TABLE 2. Primers used for PCR amplification and real-time PCR evaluation

Primer	Sequence (5'–3') ^a	Target gene or location
Conventional PCR		
LA	CTCAACAATCTGACTCATCAAGAATCG	<i>covR</i> gene and its flanking regions
RA	CTTCACTGACTGGAACCAAGGCG	
SPC-F	GCCTCGAGGTTTCGTAATACATGTTATAATAAC (XhoI)	Spectinomycin resistance gene
SPC-R	GCCTCGAGGTTTCTAAAAATCTGATTACC (XhoI)	
LU	GATAGGACCCAGCCAAAATACC	Outside the homologous regions of <i>covR</i>
RD	TACAACCGCTCTGCTGATAAAACA	
C-F	GGCTGCAGCCTCTCTACTCTAAATCA (PstI)	<i>covR</i> gene and its upstream promoter
C-R	CTGAATTCTGTACCAACATTCTCAGC (EcoRI)	
CovR-F	ATGGCTAAGAAAATTTTGATTGCT	Internal region of <i>covR</i>
CovR-R	TCAATCGCGCATGGCATATCCGAC	
Real-time PCR		
0330-1	AGCTAGTTTATATGACCCCCACAC	05SSU0330
0330-2	GCGATCCCCAACTGCTTCAACC	
1285-1	GGCATGGAAAGAAGGGCTGAAT	05SSU1285
1285-2	CACCTACCGATGACCAAAACCAA	
2011-1	AGAGGCTGGCACTTATGACGAG	05SSU2011
2011-2	GACCGACGCCGACTTCCTTGAT	
1668-1	AAGGCAGCAGACGGTGAGAGGA	05SSU1668
1668-2	TAGCCAGTGCAAGCCAGTAACCA	
0566-1	CCCAACCCTACTGCCCTTTTA	05SSU0566
0566-2	GCAACCATCGCATCACATTTT	
0581-1	CAGCGTCGTGAGCAACAAAAC	05SSU0581
0581-2	AGCTGGCGAATAGTATCCTCAAT	
1074-1	AGGGGTCTTCAATACTTCGCTCAT	05SSU1074
1074-2	GCCGCACCAGTTACACCAAAAAT	
1367-1	GGTGTCAGATGTGCGTCGTG	05SSU1367
1367-2	ATATCCAGGCGGTCGGCTAAT	
1660-1	GGGGCCATTTAAGGACCAT	05SSU1660
1660-2	TGAGTACCGAAAGGCATTATCTA	

^a Unique restriction sites (identified in parentheses) are in boldface.

reciprocal of the highest dilution of hemolysin that induced at least 50% lysis of erythrocytes.

Adherence assays. For cell adhesion assays, bacteria were centrifuged, washed three times with PBS, and resuspended at 10^6 CFU/ml in RPMI 1640 medium without antibiotics as previously described (74, 75). Human umbilical vein endothelial cells (HUVEC) were isolated by collagenase digestion of umbilical veins from undamaged sections of fresh cords. The human laryngeal epithelial cell line Hep-2 (CCTCC GDC004) and HUVEC were cultured at 37°C and 5% CO₂ in RPMI 1640 medium with 10% heat-inactivated fetal bovine serum (FBS) (Gibco-BRL). Cell monolayers (HUVEC and Hep-2) were infected at a multiplicity of infection of 10 bacteria per cell. Before the adhesion experiment, the broth cultures were vigorously vortexed with a vortex mixer to disperse any aggregates and to generate short bacterial chains containing a maximum of six cocci, as visualized by phase-contrast microscopy. The plates were centrifuged at $800 \times g$ for 10 min and incubated in RPMI 1640 medium without FBS for 1 h at 37°C with 5% CO₂. The monolayers were washed five times with PBS and incubated with 20 μ l of 0.05% trypsin/0.03% EDTA for 10 min at 37°C. After the addition of 80 μ l of ice-cold deionized water, the cells were detached and disrupted by scraping the bottoms of the wells. Serial dilutions of the cell lysates were plated onto THB agar to enumerate viable bacteria. The percent adherence was calculated as follows: (CFU on plate/CFU in original inoculum) $\times$ 100%. Assays were performed in duplicate and were repeated at least three times.

Cell-killing experiments. PMNs (also called neutrophils) and monocytes (MONOs) were isolated from heparinized venous blood of healthy human volunteers or healthy piglets using the method of dextran sedimentation as described by Baltimore et al. (1). Bacteria were opsonized in 10% normal human or pig serum and incubated with human or pig PMNs at a ratio of 10:1 (bacteria-cell) for 1 h. For each strain, controls included samples containing PMNs and heat-inactivated sera and samples lacking PMNs but containing human or pig sera. Cells were lysed with 0.1% saponin (20 min on ice), and serial dilutions of the lysates were plated on THB agar. Colonies were counted, and the percentage of *S. suis* serotype 2 bacteria that survived was determined as follows: (CFU_{PMN+}/CFU_{PMN-}) $\times$ 100% (36). The MONO-mediated killing assay was

performed in a manner similar to the PMN-killing assay. Each assay was performed in triplicate.

Experimental infections of piglets. For virulence studies, 3-week-old specific-pathogen-free (SPF) piglets (six pigs/group) were challenged intravenously with the Δ *covR* strain and the complemented strain C Δ *covR* at a dose of 10^8 CFU/piglet. The parental wild-type strain, 05ZYH33, and an avirulent strain, 05HAS68, served as positive and negative controls, respectively. The infected piglets were monitored for clinical signs, and their survival times were recorded. All animal experiments were performed in a biosafety level 3 facility and approved by the local ethical committee.

Competitive-infection assay. To precisely distinguish virulence in the wild-type strain, 05ZYH33, and the mutant Δ *covR* strain, 50% lethal doses of both strains would have to be determined, and a large number of piglets would be required. For ethical reasons, this is not acceptable. To circumvent this problem, a competitive-infection assay was used to compare the capabilities of the Δ *covR* mutant and its parental strain to colonize different tissues of piglets. A group of six piglets were inoculated intravenously with a mixture of wild-type and mutant bacteria at a ratio of 1:1 (5×10^7 CFU). During the experiment, the piglets that developed typical *S. suis* serotype 2 infection symptoms were sacrificed for bacterial-colonization analysis by using previously reported methods (15).

Pathological examination by light and electron microscopy. To examine the differences in pathological changes produced by the wild-type strain, 05ZYH33, and the Δ *covR* mutant, kidney and heart tissues from the autopsy specimens of piglets were selected to prepare sections for light microscopy and TEM observation by using methods described previously (69).

Microarray-based comparative analysis of the Δ *covR* strain. DNA microarray design and synthesis, as well as cDNA labeling and hybridization, were performed as previously described (46). Statistically significant differences were defined as those with a *t* test *P* value of less than 0.05 and a ratio of change threshold of at least 2 standard deviations compared to the median ratio for each strain.

Real-time PCR evaluation. Microarray data were subjected to further confirmation in nine chosen genes with real-time PCR assays as described previously

(60). Triplicate assays were performed in two-step multiplex RT-PCRs with SYBR premix Ex *Taq* (TaKaRa) on an Opticon 2 (MJ Research) using RNA from two independent cultures of each strain. Target threshold cycle values were normalized to average threshold cycle values for an internal reference transcript, glyceraldehyde-3-phosphate dehydrogenase, which is a housekeeping gene that is constitutively expressed in *S. suis* serotype 2. The mean ratio changes in target gene transcription were calculated as described by Livak and Schmittgen (47).

Statistical analysis. All assays were performed in triplicate and repeated at least three times on different days. Statistical analysis was performed using Student's *t* test. *P* values were considered significant if *P* was <0.05.

RESULTS

Bioinformatics-based identification of a *covR* gene in *S. suis* 05ZYH33. In the genome of *S. suis* 05ZYH33, the peptide encoded by 05SSU1732 exhibits 46% and 59% amino acid sequence identity with the CovR response regulators of *S. pyogenes* and *Streptococcus sanguinis* SK36, respectively, belonging to the widespread OmpR family, and hence, 05SSU1732 was renamed *covR*. However, in the vicinity of *covR*, no cognate histidine kinase was identified (see Table S1 in the supplemental material). Therefore CovR was considered to be an orphan response regulator in *S. suis* 05ZYH33. The nucleotide sequence of the *covR* gene of 05ZYH33 is almost identical to those of *S. suis* serotype 2 strains 89/1591 and P1/7. PCR analysis amplifying the *covR* locus revealed that *covR* is present in all 35 known serotypes of *S. suis* (types 1 to 34 and type 1/2), with the one exception of serotype 20 (data not shown).

Construction and characterization of a *covR*-defective mutant. To investigate the role of CovR in *S. suis* 05ZYH33, an isogenic *covR* knockout mutant was constructed. The *covR* coding sequence was cloned and disrupted at amino acid position 152 by inserting a spectinomycin resistance gene before its transfer into the 05ZYH33 chromosome by allelic exchange (Fig. 1A). The double-crossover event was confirmed by PCR (Fig. 1B) and DNA sequencing (data not shown), as well as by RT-PCR analysis (Fig. 1C).

Prior to evaluating the effect of *covR* inactivation on the virulence of *S. suis* 05ZYH33 in vivo, we first examined the growth characteristics of the $\Delta covR$ mutant in vitro. A slight difference in growth of the wild-type and mutant strains was reproducibly observed. The $\Delta covR$ mutant grew at a lower rate than the parent strain in THB both with and without 10% FBS (Fig. 2). More interestingly, the mean chain length of the $\Delta covR$ strain was found to be much longer than that of the wild-type strain under the same growth conditions (Fig. 3A). TEM revealed that the capsule of the $\Delta covR$ strain is thicker than that of its parental strain, 05ZYH33 (Fig. 3B). The thickness of the capsular material for 05ZYH33 and the $\Delta covR$ strain ranged between 37 and 74 nm and between 55 and 112 nm, respectively.

When inoculated on sheep blood agar plates, the $\Delta covR$ strain tended to induce bigger hemolytic zones than the wild-type strain (Fig. 4A). This observation was confirmed by measuring hemolytic activities in a photometric assay. The hemolytic titer of the $\Delta covR$ mutant (2^8 unit) was approximately twofold higher than that of 05ZYH33 (2^7 unit) (Fig. 4B), suggesting that *covR* inactivation would enhance the hemolytic activity of *S. suis* serotype 2.

Hyperadhesion of the $\Delta covR$ strain to epithelial and endothelial cells. Adherence of pathogenic bacteria to the mucosal

FIG. 1. Construction of an isogenic *covR* mutant of *S. suis* 05ZYH33. (A) Diagram of the *covR* locus from *S. suis* 05ZYH33 and strategy for insertional inactivation of *covR*. The spectinomycin resistance gene, indicated by a thick black arrow, was inserted in the unique *Xho*I site of *covR*. The thin arrows indicate the primer positions used for the construction and identification of the *covR* knockout mutant ($\Delta covR$). (B) Confirmatory PCRs of the $\Delta covR$ mutant. The primer pairs used in the PCR analysis are indicated above the lanes. Genomic DNAs from the mutant $\Delta covR$ strain (lanes 1, 3, 5, and 7) and the wild-type strain, 05ZYH33 (lanes 2, 4, 6, and 8), were used as templates. The DNA molecular size marker is a 1-kb DNA ladder (lane M). (C) RT-PCR analysis of *covR* gene transcripts. Total RNAs were extracted from independent *S. suis* serotype 2 cultures of the wild type (lane 1), the $\Delta covR$ mutant (lane 2), and the complemented strain, $\Delta covR$ (lane 3). cDNAs generated from these RNA samples were subjected to RT-PCR analysis with *covR*-specific primers (CovR-F and CovR-R). The RT-PCR products were analyzed by electrophoresis on a 1.0% agarose gel (lanes 1, 05ZYH33; lanes 2, $\Delta covR$ strain; lanes 3, $\Delta covR$). The 1-kbp DNA ladder marker is shown on the right (lane M).

surface is considered to be an essential step in the infectious process. To determine whether the lack of CovR affected the cellular adhesion of *S. suis* serotype 2, the adherence efficiencies of the wild-type strain and the $\Delta covR$ mutant to human epithelial (Hep-2) or endothelial (HUVEC) cells were compared. As shown in Fig. 5, the $\Delta covR$ mutant showed significantly more adhesion to both Hep-2 cells and HUVEC (*P* < 0.01) than the wild-type strain, indicating the role of CovR as an important mediator in the cellular-adhesion process.

Increased resistance of the $\Delta covR$ strain to phagocytosis and killing by neutrophils and MONOs. The ability of PMNs and MONOs to kill a diverse array of bacterial pathogens is essential for innate host defense (3). Therefore, we investigated the

FIG. 2. Comparison of the in vitro growth rates of the wild-type strain, 05ZYH33, and the $\Delta covR$ mutant. (A) Bacteria were cultured in THB at 37°C. (B) Bacteria were cultured in THB containing 10% FBS at 37°C. The results shown are representative of three independent experiments. OD_{600} , optical density at 600 nm.

ingestion and killing of the wild-type and mutant strains by human and porcine PMNs and MONOs. In PMN-mediated killing assays, a higher percent survival of $\Delta covR$ bacteria ($64\% \pm 10\%$) than of 05ZYH33 ($42\% \pm 11\%$) ($P < 0.05$) was observed after a 1-hour incubation (Fig. 6), and significantly more mutant strain bacteria ($72\% \pm 12\%$) than wild-type bacteria ($45\% \pm 10\%$) ($P < 0.05$) were associated with human MONOs. Similar phenomena were observed in porcine PMN and MONO-mediated killing assays (data not shown). These results indicate that *covR* disruption leads to increased resistance of the pathogen to phagocytosis and killing by PMNs and MONOs or production of bacterial factors that alter normal PMN and MONO function during bacterium-cell interactions. For example, in GAS, the *covRS* mutant had enhanced activity of the protease SpyCEP (*S. pyogenes* cell envelope protease) to cleave granulocyte chemotactic protein 2 (GCP-2) and growth-related oncogene α (GRO α), two potent chemokines involved in the

activation of neutrophils, thus impairing the host innate immune response (67).

Enhanced virulence of $\Delta covR$ in a piglet infection model. An experimental infection model in SPF piglets was designed to assess the role of *CovR* in virulence. As an initial comparison of virulence, groups of six piglets were challenged intravenously with either the wild-type strain, 05ZYH33; the $\Delta covR$ mutant; the complemented strain, $\Delta covR$; or an avirulent strain, 05HAS68 (Fig. 7). We noticed that all six piglets infected with 05ZYH33 developed most of the typical disease symptoms, including high fever, limping, swollen joints, shiv-

FIG. 3. Cell morphology of the *S. suis* wild-type strain, 05ZYH33, and the mutant strain, $\Delta covR$. (A) Light microscope morphology of *S. suis* strains using Gram staining (magnification, $\times 1,000$). (B) Transmission electron micrographs of bacteria cultured in THB containing 10% FBS. The capsule is indicated by the arrows.

FIG. 4. Hemolytic-activity analysis of the $\Delta covR$ mutant and its parental strain, 05ZYH33. (A) Hemolytic activities of *S. suis* strains streaked on THB agar plates containing 6% sheep blood and incubated for 48 h at 37°C. (B) Titration of hemolytic activities of *S. suis* serotype 2 culture supernatants. The horizontal line indicates the highest dilution of hemolysin that induced at least 50% lysis of erythrocytes. OD_{540} , optical density at 540 nm.

FIG. 5. Hyperadhesion of the $\Delta covR$ mutant to human epithelial (Hep-2) cells and endothelial cells (HUVEC). The results were determined after a 1-h coinoculation of various *S. suis* strains with Hep-2 cells and HUVEC at a multiplicity of infection of 10, followed by extensive washing of nonadherent bacteria and cell lysis to retrieve 100- μ l aliquots of total cell-associated bacteria for viable plate counts. The results shown are the means $\pm$ standard deviations of three independent experiments. *, $P < 0.01$.

ering, central nervous system failure, and respiratory failure within 24 h. Five of them died on day 2 postinfection and the last one on day 3. The bacteria could be reisolated from tissues of the piglets, including the heart, lung, and liver. In contrast, all piglets inoculated with the avirulent strain 05HAS68 as a negative control did not show any clinical symptoms, and no deaths occurred over a 14-day period of observation. However, piglets challenged with the $\Delta covR$ mutant died within 9 to 14 h without showing any obvious signs of disease, such as high fever and central nervous system failure. It seemed that the $\Delta covR$ mutant could cause sudden death. All of the piglets infected with the complemented strain $C\Delta covR$ died, with a

FIG. 6. Enhanced resistance of the $\Delta covR$ strain to phagocytosis and killing by neutrophils and MONOs. The wild-type and mutant strains were incubated with PMNs or MONOs at a bacteria-to-cell ratio of 10:1, respectively. The cells were lysed after 1 h of incubation, and the survival percentage of each strain was calculated as follows: (CFU_{PMN+}/CFU_{PMN-}) or $(CFU_{MONO+}/CFU_{MONO-}) \times 100\%$. The data are expressed as the means $\pm$ standard deviations of three independent experiments. *, $P < 0.05$.

FIG. 7. Pig infection experiments. Groups of six SPF piglets were challenged intravenously with approximately 10^8 CFU of the indicated strains. The survival time for each piglet is indicated. Each datum point represents one piglet.

median survival time of 2 days, similar to that observed in the wild-type group.

In response to the sudden death caused by the $\Delta covR$ strain, pathological examination was carried out by light and electron microscopy. For the wild-type-infected group, the glomeruli in the cortical area were enlarged and the cells, especially capillary endothelial cells, proliferated obviously and became swollen, with resulting narrowed capillary lumens and vascular collapse (Fig. 8A). Degeneration and exfoliation of the renal tubular epithelium were observed, and the microvilli of the renal tubular epithelium were decreased or lost (Fig. 8A and E). Chondriosomes were swollen with the disruption of mitochondrial cristae (Fig. 8E). In heart tissue, the cardiac muscle fibers were arranged regularly, although accompanied by sarcoplasmic lysis, decreased muscle bundles, and scarce transverse striation (Fig. 8C). Swollen mitochondria with disrupted cristae and dilated endoplasm were also observed (Fig. 8G). Apoptotic cells were occasionally seen. For the mutant-infected group, pathological alterations similar to those in the wild-type-infected group were observed, but to a more serious degree. More importantly, disseminated intravascular coagulation in kidney tissue and focal hemorrhages in the stratum of the heart could be exclusively identified in the mutant-infected group (Fig. 8B and D).

In the second series of experiments, a competitive-infection assay was adopted to further compare the virulence of the wild-type strain, 05ZYH33, and that of the $\Delta covR$ mutant by challenging a group of six piglets with a 1:1 mixture of wild-type and mutant bacteria. From various tissue samples of infected piglets, the percentage of each strain within the population was calculated. As shown in Fig. 9, the percentages varied greatly in different tissue samples. In most of the organs

FIG. 8. Pathological examination of infected piglets. Kidney and heart tissues from the autopsy specimens of piglets infected by the wild-type strain, 05ZYH33, and the $\Delta covR$ mutant were prepared for light microscopy and TEM observation in order to examine the differences in the pathological changes produced by the two strains.

examined, including heart, liver, kidney, and brain, the numbers of mutant bacteria reisolated were considerably higher than those of the wild-type strain ($P < 0.05$). In contrast, the $\Delta covR$ mutant colonized the lung tissue to a lesser degree than the wild-type strain ($P < 0.05$). Additionally, similar numbers of wild-type and mutant bacteria were reisolated from lymph nodes ($P > 0.05$), suggesting that both strains colonized this organ with the same efficiency.

Taken together, these results indicated that inactivation of *covR* enhances the capability of *S. suis* 05ZYH33 to disseminate and cause a systemic infection.

DNA microarray identification of CovR-regulated genes. To identify CovR-regulated genes and also putative virulence-associated transcriptional alterations in the $\Delta covR$ mutant, a DNA microarray-based comparative-transcriptomics approach was applied. As expected, the expression levels of flanking genes of *covR* were found to be unaltered in the $\Delta covR$ mutant, thus confirming that insertion of the spectinomycin cassette into *covR* does not have a polar effect on downstream genes. In total, the inactivation of *covR* led to 195 differentially expressed genes spread throughout the genome (8.9%) (see Ta-

FIG. 9. Differential colonization abilities of the wild-type and $\Delta covR$ mutant strains in susceptible tissues of piglets. The $\Delta covR$ mutant was mixed with the wild type at a ratio of 1:1 and inoculated intravenously into six SPF piglets. When typical *S. suis* serotype 2 infection symptoms developed, the piglets were sacrificed, the bacteria recovered from various tissues were enumerated, and the percentage of each strain within the population was calculated. The data are expressed as the means and standard deviations of three independent experiments. *, $P < 0.05$.

ble S2 in the supplemental material), with a twofold cutoff, indicating a global regulatory role of this orphan response regulator. Of these, 193 genes were CovR repressed and 2 genes were positively regulated by CovR. These regulated

TABLE 3. Summary of gene categories with differential transcription in the $\Delta covR$ mutant compared with the wild-type strain, 05ZYH33, as assessed by DNA microarray analysis

Functional classification	No. downregulated	No. upregulated
1. Enzymes		
1.1 Cell processes and signaling		13
1.2 Metabolism		39
1.3 Information storage and processing		19
1.4 Poorly characterized		13
1.5 Unknown function		7
2. ABC-type transport systems		
2.1 Cell processes and signaling		4
2.2 Metabolism		12
2.3 Poorly characterized		2
3. Transcriptional regulators	1	8
4. Virulence-related factors		
4.1 Capsular polysaccharide-related protein		3
4.2 Hemolysins and related proteins		1
4.3 Sortase or sortase-like protein		2
4.4 CMP- <i>N</i> -acetylneuraminic acid synthetase		1
5. Other proteins		
5.1 Cell processes and signaling		4
5.2 Metabolism		4
5.3 Information storage and processing		14
5.4 Poorly characterized		13
5.5 Unknown or hypothetical protein	1	34
Total	2	193

FIG. 10. Correlation of DNA microarray and real-time PCR results. The changes in the relative gene transcription ($\Delta covR$ to 05ZYH33) of nine selected genes obtained by DNA microarray and real-time PCR analyses were $\log_2$ transformed, and the values were plotted against each other to evaluate their correlation.

genes can be classified into several broad categories (Table 3) and include many that encode membrane-associated transport proteins, enzymes involved in intermediate metabolism, regulatory proteins, and secreted or surface components, including several proven or putative virulence factors.

Validation of the microarray results by real-time PCR analysis. In order to independently confirm the microarray data, the relative transcript levels of nine selected genes were measured by quantitative real-time PCR analysis. These nine genes were chosen because the majority of them were identified as encoding factors influencing bacterium-host interactions. Of these, seven genes (05SSU0330 [*lbp*], 05SSU1668 [*hly*], 05SSU1074 [*srtA*], 05SSU0566 [*cps2C*], 05SSU0581, 05SSU1285, and 05SSU2011) were upregulated and the other two (05SSU1367 and 05SSU1660) were downregulated in the $\Delta covR$ mutant. These genes identified as differentially expressed by microarray analysis were consequently confirmed to exhibit altered transcript levels by real-time PCR assays. As shown in Fig. 10, there was a strong positive correlation ($R^2 = 0.9$) between the data obtained by these two different techniques.

DISCUSSION

Although it has been known for a number of pathogens, including GAS, GBS, and *S. mutans*, that CovS/CovR (or CovR) play a pivotal role in the regulation of virulence, the function of this system in *S. suis* has so far received little attention. In the present study, we carried out the first detailed functional investigation of CovR in the pathogenesis of *S. suis* 05ZYH33. Unlike most TCS members of histidine kinase response regulator pairs, CovR was identified as an orphan response regulator in 05ZYH33, similar to that of *S. mutans* (28). This suggests that other phosphorelay systems might be involved in the phosphorylation of CovR or that, in the absence of CovS, an alternative sensor kinase or some low-molecular-

weight compounds, such as acetyl phosphate, might serve as phosphodonors (51, 81).

To determine the role of CovR in *S. suis*, we constructed a $\Delta covR$ mutant, and remarkable phenotypic changes were readily observed, such as increased hemolytic activity, longer chains, and thicker capsule walls. In light of the interactions between *S. suis* and host epithelial or endothelial cells, we tested the ability of the $\Delta covR$ mutant to adhere to these cells and found that adherence of the $\Delta covR$ mutant to both epithelial cells (Hep-2) and endothelial cells (HUVEC) was greatly increased. Charland et al. demonstrated that *S. suis* could adhere to brain microvascular endothelial cells and HUVEC, especially brain microvascular endothelial cells (10). A previous study had shown that *S. suis* was able to adhere to different epithelial cells and that the levels of adherence were, in general, similar in human and animal-derived cells (41). As a confirmation of this, we observed that levels of adherence of *S. suis* 05ZYH33 to the above-mentioned cells from piglets and humans were comparable in our preexperiments.

To induce diseases, *S. suis* must be able to survive in the bloodstream after its transmission via the respiratory tract. An early theory suggested that *S. suis* is taken up by MONOs, allowing the bacteria to survive and travel intracellularly in the circulation (80). Neutrophils may play an important role in the pathogenesis of infection, given that infiltration by neutrophils and mononuclear cells is frequently observed in lesions caused by *S. suis* (8). Our work shows that the $\Delta covR$ mutant survived more than the wild type in human PMNs and MONOs. It is reasonable to explain this phenomenon by the thicker capsule of the $\Delta covR$ strain, since it has been reported that the capsular polysaccharide (CPS) of *S. suis* protects against phagocytosis and against the bactericidal activity of leukocytes (6, 9, 48, 59, 62, 63, 79). The work of Segura et al. demonstrated that *S. suis* serotype 2 is able to interact with MONOs of human origin, inducing the release of large amounts of the proinflammatory cytokines (61).

In a piglet intravenous-challenge model, the $\Delta covR$ mutant has been shown to exhibit significantly enhanced virulence compared to the wild-type strain, as judged from several aspects, including the survival times of infected piglets, colonization abilities, and pathological observations. To examine the pathological changes in piglets that died from the $\Delta covR$ mutant, optical microscopy and TEM were used. Many organs, including alveolar epithelium, hepatic cell, kidney, and cardiac muscle, exhibited cristate disruption of the chondriosomes (Fig. 9 and data not shown). Pathological changes were particularly manifested in kidney and cardiac muscle, although pathological observations were similar in the two groups. More strikingly, the $\Delta covR$ mutant-infected group died 9 h after inoculation while the wild-type-infected group died at 2 days. The autopsy specimen from the $\Delta covR$ mutant-infected group showed clearly visible disseminated intravascular coagulation in kidney tissue, abdomens, and backs of piglets (data not shown), whereas the wild-type-infected group did not. All of these results indicate that the $\Delta covR$ mutant is more lethal than the wild-type strain, and the $\Delta covR$ strain upregulated many virulence genes related to physical and pathological changes.

The role of CovR in the pathogenesis of *S. suis* was studied in an experimental infection model in piglets. Since we were

unable to determine a 50% lethal dose for *S. suis*, it was decided to compare the virulence of the $\Delta covR$ mutant with that of the wild-type strain in a competitive-infection assay in piglets. This kind of cocolonization experiment has been successfully applied to determine the virulence of mutants of *Actinobacillus pleuropneumoniae* and *S. suis* in piglets (14, 15, 21). The data showed that the $\Delta covR$ strain was capable of colonizing the most-examined organs, including heart, liver, kidney, and brain, with higher efficiency than the wild-type strain, which indicates that some components regulated by *CovR* may play roles in the colonization process. The reason for the mutant strain colonizing the lung less efficiently than the wild-type strain is not known but may be related to tissue-specific effects.

Considering the global regulatory role of *CovR* in both GAS and GBS, an expression-profiling analysis of *S. suis* 05ZYH33 and the $\Delta covR$ mutant was carried out to determine which *S. suis* serotype 2 virulence genes belong to the *CovR* regulon. This analysis revealed that transcription of as many as 8.9% of all chromosomal genes ($n = 195$) was affected by *covR* disruption. Of note, several proven or putative virulence-associated genes were included, such as *cps2C* (one open reading frame of the CPS biosynthesis operon, 05SSU0566), the sialic acid synthase-coding gene (05SSU0581), *srtA* (sortase A; 05SSU1074), *sda* (DNase streptodornase; 05SSU2011), *lbp* (laminin-binding protein; 05SSU0330), and *hly* (hemolysin; 05SSU1668).

The CPS of *S. suis* serotype 2 is composed of five kinds of sugars—the monosaccharides galactose, glucose, *N*-acetyl-D-glucosamine, rhamnose, and *N*-acetylneuraminic acid (sialic acid) (34)—and is so far the only proven critical virulence factor, based on the observation that unencapsulated isogenic mutants showed increased hydrophobicity and phagocytosis using murine and porcine phagocytes. More importantly, unencapsulated mutants were found to be completely avirulent and rapidly cleared from circulation in pig and mouse models of infection (9, 63). As an important component of CPS, sialic acid has been proven to be related to virulence for other bacterial agents of meningitis (50, 78). The CPS biosynthesis (*cps*) locus of *S. suis* serotype 2 consists of 14 open reading frames, among which *cps2C* could be involved not only in the chain length determination of the serotype 2 capsule, but also in export of the polysaccharide (63). Consistent with the phenotypic changes of the $\Delta covR$ mutant, with formation of longer chains and production of thicker capsules, transcripts of the *cps2c* gene were more abundant in the absence of *CovR*. Additionally, a gene (05SSU0581) encoding a putative sialic acid synthase that may be involved in capsule development was also overexpressed in the $\Delta covR$ mutant. Since it has been generally accepted that there is a close correlation between encapsulation and resistance to phagocytosis or bactericidal effects, it is reasonable that the $\Delta covR$ mutant exhibits enhanced capabilities for resistance to phagocytosis and killing by human PMNs and MONOs compared to *S. suis* 05ZYH33.

Bacterial adherence to host cell surfaces is the first step in colonization and the establishment of an effective infection. At this initial stage of infection, proteins on the surfaces of bacterial cells may function as adhesins for specific adherence (5, 20, 53, 57). In this process, sortase A (SrtA, also referred to as sortase) plays a key role in the cleavage and anchoring of surface proteins with an LPXTG motif to the cell wall (4, 72).

In several important gram-positive pathogens, it has been demonstrated that a mutant deficient in the *srtA* gene caused defects in the cell wall sorting of such surface proteins, including some surface-associated adherence factors, and consequently led to a significant decrease in either adhesion or virulence (35, 40, 82). Recently, Vanier et al. demonstrated that an *S. suis* mutant strain lacking the *srtA* gene had reduced capacity to adhere to porcine brain microvascular endothelial cells, as well as plasma fibronectin, cellular fibronectin, and collagen type I (76). However, they found that disruption of *srtA* had little effect on the virulence of *S. suis* in a mouse intraperitoneal model of infection. In contrast to this, our recent experiments with the $\Delta srtA$ mutant clearly showed that SrtA is essential for *S. suis* serotype 2 virulence in a piglet model (77). The reason for these conflicting results is unclear but may be related to experimental differences, such as the use of different bacterial strains and animal models. Using DNA microarray analysis and quantitative RT-PCR, we found that the transcription level of the *srtA* gene in the $\Delta covR$ mutant was much higher than that in the wild-type strain. Moreover, transcription of the gene 05SSU0330 encoding laminin-binding protein (LBP), an important surface protein adhesin that has been proven to promote adhesion of GAS to Hep-2 cells and invasion of GBS into the bloodstream (65, 70), was also repressed by *CovR*. As confirmation of these findings, the adherence and colonization abilities of the $\Delta covR$ mutant were found to be significantly increased compared to those of the wild-type strain, 05ZYH33. Furthermore, in agreement with the increased hemolytic activity of the $\Delta covR$ mutant and extensive hemorrhage in multiple organs at autopsy in piglets infected with the $\Delta covR$ strain, the *hly* gene encoding hemolysin is upregulated in the absence of *CovR*.

Bacteria have selected mechanisms to express only the appropriate subset of genes conferring a growth or survival advantage in a given situation (12). The expression of many bacterial genes is regulated at the initiation of transcription by regulators that, in response to specific environmental and/or cellular signals, bind at the promoters of target genes to activate or repress them (23). The genome of *S. suis* 05ZYH33 contains approximately 51 genes that encode homologues of transcriptional regulators, including 15 two-component regulatory systems (11). Many genes encoding proven or putative transcriptional regulators were differentially expressed in the $\Delta covR$ mutant (Table 3; see Table S2 in the supplemental material). 05SSU0447 encodes a protein called fatty acyl-responsive regulator (P30 protein), which has been identified as a fatty acid and fatty acyl-coenzyme A-responsive DNA-binding protein, autoregulates its own expression, and may modulate citric acid cycle expression in response to long-chain fatty acids (55). It belongs to transcriptional regulators of the GntR family, which are involved in virulence gene control in *Brucella melitensis*. Attenuated mutants of the GntR family were identified and confirmed to replicate at lower rates in mice and to be attenuated in cellular models (25, 44). 05SSU0706 encodes a protein belonging to the DeoR family. Some regulators of the DeoR family are involved in sugar metabolism, but many proteins of this family have no experimentally defined function (52, 56). While Haine et al. proposed that GntR4 (in the GntR family) and DeoR1 (in the DeoR family) are direct or indirect activators of *virB* transcription in *B. melitensis* (25), a *B. suis*

deoR1 mutant was shown to be strongly attenuated in human macrophages (37), which is consistent with a possible role of *deoR1* in *virB* activation in this cellular model. The upregulation of 05SSU0447 and 05SSU0706 indicates that both of them might be involved in virulence gene control in *S. suis*.

The phosphoenolpyruvate-carbohydrate phosphotransferase system (PTS) was originally described as a sugar phosphorylation system (39) and plays a major role in sugar transport and in the regulation of essential physiological processes in many bacteria (16, 54, 58, 71). In addition to their roles in sugar transport, PTS proteins participate in many other physiological processes, such as signal transduction, chemotaxis, regulation of carbon metabolism, and coordination of carbon and nitrogen metabolism (2, 16, 54). Recent studies have also shown that PTS components are associated with bacterial virulence: signature-tagged mutagenesis screenings have identified PTS genes as virulence factors in some pathogenic streptococci and salmonellae (26, 32, 43, 73). There are 27 genes encoding PTS components (11) in the genome of *S. suis* 05ZYH33, and expression levels of 05SSU0398, 05SSU0710, 05SSU1038, 05SSU1401, and 05SSU1780 are upregulated in the Δ *covR* mutant. 05SSU0706, a gene upstream of 05SSU0710 that encodes a transcriptional regulator of sugar metabolism, is also upregulated. Thus, it seems plausible that these PTS proteins not only take part in metabolism, but are also involved in virulence gene control in *S. suis*.

Transcripts of 05SSU0625 and 05SSU0627, which encode histone acetyltransferase HPA2 and related acetyltransferases and carbamate kinase, respectively, together with the transcripts of 05SSU0631 and 05SSU01652, which encode arginine repressors belonging to the arginine deiminase system (ADS), are all upregulated in the mutant strain compared to the wild type. The ADS can be considered a system that protects oral streptococci and *S. pyogenes* against acidic stress (7, 13). Thus, the ADS might facilitate *S. suis* survival within the different niches of the host and thereby probably contributes to *S. suis* pathogenesis (24). 05SSU1636 encodes lactoylglutathione lyase (LGL), which is an enzyme that catalyzes the conversion of toxic methylglyoxal, derived from glycolysis, to *S*-D-lactoylglutathione (29, 33). Methylglyoxal inhibits the growth of cells in all types of organisms. In *S. mutans*, LGL functions in the detoxification of methylglyoxal, resulting in increased acidity (38). In this study, upregulation of 05SSU1636 might contribute to the enhanced virulence of the Δ *covR* strain.

In conclusion, this investigation has provided initial insight into the global regulatory role of CovR in *S. suis* serotype 2, especially its negative control of *S. suis* serotype 2 virulence. However, further studies are necessary to define a specific CovR recognition and binding sequence and are currently being pursued in our laboratory. Determining the nature of the interaction between CovR and its target promoters is the first step toward understanding the CovR signaling pathway and identifying genes directly regulated by CovR.

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