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Functional Definition and Global Regulation of Zur, a Zinc Uptake Regulator in a *Streptococcus suis* Serotype 2 Strain Causing Streptococcal Toxic Shock Syndrome▽†

Youjun Feng,[1,3]‡ Ming Li,[2,4] Huimin Zhang,[1] Beiwen Zheng,[1,3] Huiming Han,[1,3] Changjun Wang,[2] Jinghua Yan,[1] Jiaqi Tang,[2] and George F. Gao[1]*

Center for Molecular Immunology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China[1]; Department of Epidemiology, Research Institute for Medicine of Nanjing Command, Nanjing 210002, China[2]; Graduate University, Chinese Academy of Sciences, Beijing 100049, China[3]; and Department of Microbiology, Third Military Medical University, Chongqing 630030, China[4]

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Zinc is an essential trace element for all living organisms and plays pivotal roles in various cellular processes. However, an excess of zinc is extremely deleterious to cells. Bacteria have evolved complex machineries (such as efflux/influx systems) to control the concentration at levels appropriate for the maintenance of zinc homeostasis in cells and adaptation to the environment. The Zur (zinc uptake regulator) protein is one of these functional members involved in the precise control of zinc homeostasis. Here we identified a *zur* homologue designated 310 from *Streptococcus suis* serotype 2, strain 05ZYH33, a highly invasive isolate causing streptococcal toxic shock syndrome. Biochemical analysis revealed that the protein product of gene 310 exists as a dimer form and carries zinc ions. An isogenic gene replacement mutant of gene 310, the Δ310 mutant, was obtained by homologous recombination. Physiological tests demonstrated that the Δ310 mutant is specifically sensitive to Zn^{2+}, while functional complementation of the Δ310 mutant can restore its duration capability, suggesting that 310 is a functional member of the Zur family. Two-dimensional electrophoresis indicated that nine proteins in the Δ310 mutant are overexpressed in comparison with those in the wild type. DNA microarray analyses suggested that 121 genes in the Δ310 mutant are affected, of which 72 genes are upregulated and 49 are downregulated. The transcriptome of *S. suis* serotype 2 with high Zn^{2+} concentrations also showed 117 differentially expressed genes, with 71 upregulated and 46 downregulated. Surprisingly, more than 70% of the genes differentially expressed in the Δ310 mutant were the same as those in *S. suis* serotype 2 that were differentially expressed in response to high Zn^{2+} concentration, consistent with the notion that 310 is involved in zinc homeostasis. We thus report for the first time a novel zinc-responsive regulator, Zur, from *Streptococcus suis* serotype 2.

Streptococcus suis 2 is a gram-positive pathogenic bacterium capable of infecting both piglets and humans and causing serious diseases such as arthritis, meningitis, and septicemia (58). It has spread to nearly 20 countries, resulting in more than 400 human cases of severe infection worldwide (38). In particular, two recent outbreaks (in 1998 and 2005) of severe human *S. suis* serotype 2 infections in China were characterized by streptococcal toxic shock syndrome, implying that highly invasive variants of *S. suis* serotype 2 might be emerging in Asia (63, 72). Virulence factors related to the pathogenesis of *S. suis* serotype 2 have been partially elucidated and include capsular polysaccharide (56), suilysin (37), muraminidase-released protein (42), and extracellular factor (59). Very recently, our group (8) reported the whole genome sequences of two virulent *S. suis* serotype 2 isolates (05ZYH33 and 98HAH12).

Similar to other transition metals such as iron, manganese, and nickel, zinc is recognized as an essential trace element for all living organisms, including a variety of bacterial pathogens (9). It plays critical roles in various cellular processes and physiological functions by either serving as a catalytic cofactor for numerous enzymes or maintaining the structure scaffold of metal-proteins (4, 10). However, an excess of zinc ion is toxic to normal physiological processes because it can trigger the formation of hydroxyl radicals, resulting in severe damage to DNA, proteins, and lipids (53). Consequently, the intracellular level of zinc must be precisely regulated to reach a dynamic balance (24, 53). Bacteria have evolved complex machineries (such as efflux/influx systems) to maintain zinc homeostasis (24). To survive and complete their infection cycles, pathogenic bacteria must compete with their hosts for the limited resource of Zn^{2+} in common niches (20, 24).

The zinc uptake regulator, Zur, is one of the key components contributing to the molecular systems by which zinc homeostasis is controlled (47). In fact, Zur has been classified as a subgroup of the Fur (ferric uptake regulator) family, which comprises at least five members, namely, Fur (for Fe^{2+}) (15, 25), Zur (for Zn^{2+}) (34, 47), Nur (for Ni^{2+}) (1), Mur (also called TroR) (51) (for Mn^{2+}) (14, 49), and PerR (for peroxide) (31, 66). Since the first discovery of the Zur protein in *Escherichia coli* (47), the functional members of the Zur family have been extended to a wide range of bacteria, such as *Bacillus*

* Corresponding author. Mailing address: Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China. Phone: 86-10-64807688. Fax: 86-10-64807882. E-mail: gaof@im.ac.cn.

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‡ Present address: Department of Microbiology, University of Illinois at Urbana-Champaign, Urbana, IL 61801.

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Characteristics[a]	Function	Reference(s) or source
Strains			
05ZYH33	Virulent Chinese *S. suis* serotype 2 isolate	Genome isolation, microarray analysis, animal infection	8, 63
05HAS68	Avirulent of Chinese *S. suis* serotype 2 isolate	Animal infection experiments	8, 63
Δ310 mutant	Gene 310 knockout mutant	Functional analysis	This work
CΔ310	Complemented Δ310 strain	Functional analysis	This work
DH5α and BL21(DE3)	Genetically modified *E. coli*	Cloning and expression host	In this lab
Plasmids			
pGEM-T vector	~3.0 kb; Amp^r	T vector	Promega
pMD-18T	~2.7 kb; Amp^r	T vector	Takara
pSET1	5.2 kb; Cm	*E. coli-S. suis* shuttle vector	61
pET28a (+)	~5.0 kb; Kan^r	His tag fusion expression vector	Novagen
pGEX-6P-1	4.9 kb; Amp^r	GST fusion expression vector	Amersham
pGEM::310R	~4.2 kb; Amp^r	With the 310R fragment	This work
pMD::Spc	~3.8 kb; Amp^r Spc^r	With Spc^r gene	This work
pMD::Spc-310R	~5.0 kb; Amp^r Spc^r	pMD-18:Spc with the 310R fragment	This work
pMD::Spc-310LR	~6.2 kb; Amp^r Spc^r	A mosaic plasmid designed to knock out gene 310	This work
pET28a::310	~5.8 kb; Kan^r	Recombinant expression plasmid to produce His_6-fused 310 protein	This work
pGEX-6P::310	5.4 kb, Amp^r	Recombinant expression plasmid to produce GST-fused 310 protein	This work
pSET1::C310	6.0 kb; Cm	Recombinant plasmid used for functional complementation of the *S. suis* serotype 2 mutant, Δ310	This work

[a] Amp^r, ampicillin resistance; Kan^r, kanamycin resistance; Spc^r, spectinomycin resistance; Cm, chloramphenicol resistance.

subtilis (17, 18), *Listeria monocytogenes* (11), *Staphylococcus aureus* (34), *Salmonella enterica* (7), *Mycobacterium tuberculosis* (40, 43), and *Xanthomonas campestris* (62). In *B. subtilis*, Zur regulates not only zinc uptake but also the mobilization of zinc through ribosomal proteins (44). Very recently, ribosome proteins were revealed to be regulated by Zur proteins in both *Streptomyces coelicolor* (46, 55) and *M. tuberculosis* (40). Tang et al. (62) showed that Zur is involved in extracellular polysaccharide production and is required for full virulence in *Xanthomonas campestris*. Similarly, Zur was found to be involved in the pathogenesis of *Salmonella enterica* (7) and *Xanthomonas campestris* (62). In contrast, *zur* fails to exhibit obvious roles in the pathogenicity of *S. aureus* (34). Following the solution of the crystal structure of Fur from *Pseudomonas aeruginosa* in 2003 (50), Lucrelli et al. (36) characterized the architecture of Zur that is central to zinc homeostasis in *M. tuberculosis*, providing structural insights into the interplay between Zur and its target DNA sequence (the Zur box). Intriguingly, Zur has also been suggested to act as an indirect activator by repressing two regulatory RNAs (*rhyA* and *rhyB*) (36), which is distinct from its general role as a repressor (25, 53).

In attempts to explore the functional genomics of *S. suis* serotype 2 and the molecular pathogenesis of invasive streptococcal toxic shock syndrome infection, we identified a Fur/Zur-like homologue referred to as gene 310. Subsequently, we carried out systematic investigations to characterize gene 310. Our data demonstrate clearly that gene 310 is a functional member of the Zur family from *S. suis* 05ZYH33. Moreover, global regulation of Zur in *S. suis* serotype 2 is presented, for the first time, highlighting its relationship with zinc homeostasis at the genomic level.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids are detailed in Table 1. *Streptococcus suis* strains (e.g., 05ZYH33) were grown in Todd-Hewitt broth (THB) (Difco Laboratories, Detroit, MI) or plated on THB agar containing 5% (vol/vol) sheep blood (63); 100 μg/ml of spectinomycin (Spc) (Sigma) was used to select for *S. suis* transformants. *E. coli* strains DH5α and BL21 were cultured in Luria-Bertani (LB) liquid medium or plated on LB agar. If necessary, either 50 μg/ml of ampicillin (Sigma) or 50 μg/ml of kanamycin (Sigma) was utilized to screen *E. coli* transformants (16).

Bioinformatics search of *zur*/*fur*-like gene candidate. To identify *zur*/*fur*-like genes from the genome of *S. suis* 2, an in silico search was carried out using the *E. coli zur*/*fur* gene sequence as search models (62). One putative open reading frame (ORF) with the coding number 310 was recognized as a possible *zur*/*fur*-like gene and was subjected to further bioinformatics analysis, including Blast-P and ClustalW.

Overexpression and purification of protein 310. A pair of primers (310-F and 310-R) (Table 2) was designed to amplify gene 310, which was then cloned into pGEM-T vector (Takara) for direct DNA sequencing. Subsequently, it was subcloned into the two kinds of prokaryotic expression vectors [pET28(a) (Novagen) and pGEX-6P-1 (Amersham)], generating the recombinant plasmids pET28::310 and pGEX-6P::310, respectively (Table 1). pET28::310 and pGEX-6P::310 both were transformed into competent cells of *E. coli* BL21(DE3) for production of recombinant 310 protein.

A soluble protein encoded by gene 310 (named protein 310) was obtained as described previously (16) with some minor modifications. After sonication, the clarified bacterial supernatant was loaded onto affinity columns (for the His tag version, a nickel-ion [Ni^+] affinity column [Qiagen], for the glutathione *S*-transferase [GST]-fused version, a glutathione Sepharose 4B column [Amersham]). The recombinant His_6-tagged protein 310 was eluted in elution buffer with 50 mM imidazole after removing the contaminant proteins with washing buffer containing 20 mM imidazole. The GST-fused 310 protein was washed with 1× phosphate-buffered saline (PBS) and then eluted with 20 mM reduced glutathione. PreScission proteinase was used to cleave the N-terminal GST tag to obtain the 310 protein with GST removed.

Both versions of the acquired 310 protein (310 with GST removed and His-tagged 310) were concentrated by ultrafiltration (5-kDa cutoff) and exchanged from 1× PBS buffer into the exclusion buffer (20 mM Tris, 150 mM NaCl, pH

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

Category and primer	Sequence[a]	Restriction site or product size (bp)	Function or gene
General PCR amplification or detection			
310-F	5′-ATGGATCCATGGAACTCCATTCTCAC-3′	BamHI	Amplify gene 310
310-R	5′-CCGCTCGAGTTAGTTCTGGCAATCAGG-3′	XhoI	
310L-F	5′-CCAACATATGGATAAGCTCTTCATCGCC-3′	NdeI	Amplify left arm of gene 310
310L-R	5′-CCCAAGCTTAAGACGAATATCCAAGCG-3′	HindIII	(310L)
310R-F	5′-TCCCCCGGGAGTTTCCTCCTTTCGTTAT-3′	EcoRI	Amplify right arm of gene 310
310R-R	5′-CGGAATTCGAATCTCCTGGTGAGTC-3′	SmaI	(310R)
Spc-F	5′-GGATCCGTTCGTGAATACATGTTATA-3′	—[b]	Spc^r gene
Spc-R	5′-CTGCAGGTTTTCTAAAATCTGAT-3′	—	
P-F	5′-GTCGTGATGATGTGGCTGT-3′	—	310 with both 310L/R partial
P-R	5′-AGTCATGTCCGTCGTAGG-3′	—	fragments
C310-F	5′-AGGCTGCAGGAAAAGTCATGTCCGTCGTAGG-3′	PstI	Amplify gene 310 containing its
C310-R	5′-CCTGAATTCAGCCGCTTGGATATTCGTCTT-3′	EcoRI	promoter (690 bp)
310-F2	5′-GAACACGTCATCCAACATCTA-3′	—	Detect transcription of gene
310-R2	5′GTGCCCCATAAAGTCGTAAT-3′	—	310 via RT-PCR of its partial coding sequence (252 bp)
0112-F	5′-GTTACACAGCATACTGCATTTGC-3′	—	Detect transcription of gene
0112-R	5′-CAAGAGGATTAAGAACGTCCAAC-3′	—	0112
Real-time RT-PCR evaluation			
0155-F	5′-GTTCTTGACGGACCACACC-3′	227	GAPDH
0155-R	5′-GCAGCGTTTACTTCTTCAGC-3′		
0017-F	5′-CCAGGGTATCTAATCCTG-3′	216	0017
0017-R	5′-GGCGGTTTGATAAGTCTG-3′		
1355-F	5′-CACAGTCTCTTTCGCTTG-3′	220	1355
1355-R	5′-GGTTTATCAAATCCTGTGC-3′		
1968-F	5′-GTCAAGGTAGGAGATCTTG-3′	234	1968
1968-R	5′-CGCATCTTGTTCTGGATC-3′		
1998-F	5′-CAGGTGTAACCCTTAGTC-3′	228	1998
1998-R	5′-CTGGATAGAGGTCACTG-3′		
0019-F	5′-GAATGCATCGCTTCGTAG-3′	198	0019
0019-R	5′-CTCAATGAACTCACGATGC-3′		
0293-F	5′-CGCCCTGCCCTGTCACTTGT-3′	201	0293
0293-R	5′-GACCCGTCCGGCAGATACCA-3′		
0303-F	5′-GTGGCAACTTCCTACTGTC-3′	246	0303
0303-R	5′-GACCAAGTCTGACAGATTG-3′		
0319-F	5′-CTGACAGCTCTGACCATC-3′	247	0319
0319-R	5′-CGTAGTCAACATCGCTAAG-3′		
1912-F	5′-GATTCAATTGCCCCAGACCT-3′	220	1912
1912-R	5′-AGCAAGTACCCCACAGACGA-3′		
2187-F	5′-CAATCTAGCGAACTAGCAG-3′	230	2187
2187-R	5′-GTGAAGTAGCTTGCTTAGA-3′		

[a] The underlined sequences are the restriction sites.
[b] —, absence of restriction enzyme site.

8.0). Subsequently, they were subjected to gel filtration analysis using a Superdex 200 column (Pharmacia) fixed on an Äkta purifier system (Pharmacia) and monitored at a flow rate of 0.5 ml/min in running buffer (which is same as the exclusion buffer). The peak was collected, visualized by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then stained with Coomassie brilliant blue R250 (Sigma, St. Louis, MO). The apparent molecular weights were estimated by comparison with standard protein markers (Sangon, Shanghai, China) run on the same gel (16).

Chemical cross-linking assays. Chemical cross-linking experiments were performed to identify the natural state of protein 310. In brief, the purified recombinant protein (approximately 10 mg/ml) was subjected to chemical cross-linking with ethylene glycol bis-succinimidylsuccinate (EGS) (Pierce). The reaction mixtures were incubated for 1 h on ice at different concentration of EGS (0, 2, 5, and 10 mM) and quenched with 50 mM glycine. Finally, cross-linked samples were analyzed by 15% SDS-PAGE (39).

Deletion and functional complementation of gene 310. To identify the function of gene 310 in *S. suis* serotype 2, we inactivated gene 310 using the strategy of homologous suicide plasmid integration. We constructed a gene 310 knockout vector, pMD::Spc-310LR, carrying the Spc resistance gene (Spc^r) (Table 1) and electrotransformed it into competent cells of 05ZYH33 as described by Smith et al. (57) and Li et al. (32) with minor modifications. Colony PCR assay was used to examine Spc^r transformants with a series of specific primers (Table 2). To further confirm the mutant, total RNA was extracted from *S. suis* serotype 2 cultures at an optical density at 600 nm (OD_{600}) of 0.8 with Trizol reagent (Invitrogen) and purified using the RNeasy minikit (Qiagen). Reverse transcription-PCR (RT-PCR) was utilized as described by Chen et al. (8). A mutant of interest, the Δ310 mutant was eventually obtained, which was also verified by direct DNA sequencing.

To further perform the functional complementation of the Δ310 mutant, a DNA fragment of gene 310 together with its upstream promoter was amplified by PCR using a pair of primers with specific restriction enzyme sites (C310-F and C310-R) (Table 1) and then was cloned directionally into the *E. coli-S. suis* shuttle vector pSET1, generating the recombinant plasmid pSET::C310 (Table 1). After the verification of direct DNA sequencing, pSET1::C310 was electrotransformed into the Δ310 mutant. The complemented strains of the Δ310 mutant (CΔ310 mutant) were screened on THB agar plates with double selection pressure of Spc and chloramphenicol, following the method we reported recently (32).

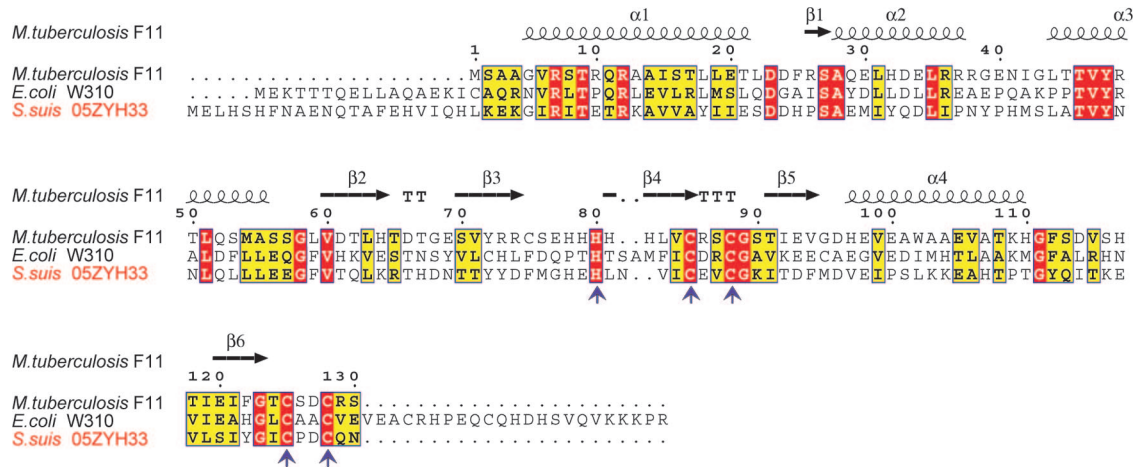

FIG. 1. Multiple alignment of protein 310 from *S. suis* 05ZYH33 with the related Zur regulators at the amino acid level. The multiple alignment was conducted using the ESPript software together with ClustalW online. The Zur proteins correspond to *S. suis* 05ZYH33 (YP_001197678), *M. tuberculosis* F11 (2O03A), and *E. coli* W3110 (BAE 78048). Three types of protein secondary structure are as follows: α, alpha-helix (with cartoon helixes); β, beta-sheet; T, turn. *S. suis* serotype 2 strain 05ZYH33 is highlighted in red. According to the structural architecture of the *M. tuberculosis* Zur protein, five conserved residues (H80, C85, C88, C125, and C128) that are critical for the specific binding to zinc ions are indicated with blue arrows.

Western blot analysis of protein 310 expression in *S. suis* 2. Total bacterial protein was prepared by sonication from three strains of *S. suis* 2, i.e., the wild type (WT) (05ZYH33), the Δ310 mutant, and the Δ310 mutant complemented strain (CΔ310 mutant). They were then separated by 15% SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane for the Western blot-based detection of protein 310 as we described previously (16). Here, the first antibody is polyclonal anti-310 protein rabbit serum, which was obtained according to the customary immunization protocol for New Zealand White rabbits (33).

Tests of the sensitivity of the Δ310 mutant to divalent cations. Since 310 is a member of the *zur/fur* family, we aimed to test its function. Salts as sources of six different divalent cations, i.e., $MnCl_2$, $FeSO_4$, $MgCl_2$, $CaCl_2$, $CuSO_4$, and $ZnSO_4$ were mixed with THB liquid medium in appropriate concentrations. The growth of the Δ310 mutant cultivated in this above mixed medium was compared to that of the WT by measuring the OD_{600} (62, 70). Similarly, the assays of toxicity of divalent cations to *S. suis* serotype 2 strains were performed using chemically defined medium (CDM).

Measurement of zinc ions. To examine whether protein 310 contains zinc, the purified protein 310 after the desalting treatment was tested by inductively coupled plasma atomic emission spectrometry (ICP-AES) (model Vista-Mpx; Varin, Japan). For the measurement of bacterial zinc content, both strains (the WT and the Δ310 mutant) were grown in 100 ml of THB (and CDM) liquid medium with 150 mM $ZnSO_4$. Bacterial cells harvested by centrifugation were washed thrice with PBS containing 0.5 mM EDTA to remove externally bound Zn^{2+} as described by Tang et al. (62). The cell density was adjusted with sterilized double-distilled water to an OD_{600} of 1.0. The bacterial zinc content was determined by ICP-AES. Finally, the zinc contents in double-distilled water, THB, and CDM were tested using the same instrument.

Proteomics analysis. Two bacterial strains (the WT and the Δ310 mutant) were cultivated in 200 ml of THB liquid medium and colleted by centrifugation at 4°C to an OD_{600} of 0.8. The total proteins were extracted from pellets as described by Wang et al. (68). To optimize two-dimensional electrophoresis (2-DE), a preliminary experiment was first performed on a 7-cm gel with a linear range of pH (3.0 to 10.0). Then, total proteins of *S. suis* serotype 2 were separated in triplicate via 2-DE on 17-cm gels in which the pH ranged from 3.0 to 7.0.

The 2-DE images were acquired with an Image Scanner (Amersham Biosciences) in transmission mode. The image analysis was carried out by a combination of manual visualization and software analysis with Image Master Elite version 4.1 (Amersham Biosciences). To gain comparable data for quantitative analysis, several key parameters in the image analysis were fixed as constant (67). The average spot intensity was normalized to the total spot volume with a multiplication factor of 100 (68). Spots on 2-DE with different intensity were carefully excised, dehydrated with acetonitrile, and digested in gel with trypsin solution. The digested products were subjected to further analysis by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry. Based on the acquired peptide sequences, database searches were conducted with MASCOT software 1.9 (Matrix Science) against the protein database of *S. suis* serotype 2 in the Beijing Genomics Institute.

Expression microarray-based analysis. On the basis of the 05ZYH33 genome sequence, oligonucleotide probes with an average length of 35 nucleotides were designed to match all the putative ORFs (2,194 in total) using Array Designer 2.0 and then synthesized onto complementary metal oxide semiconductor matrix utilizing an in situ electrochemical synthesis technique (Combimatrix). To gain insights into the response of *S. suis* serotype 2 to Zn^{2+}, we selected another isolate that behaved like the WT in the presence of 200 μM Zn^{2+} (referred to as WT/Zn^{2+}). Total RNAs from these three samples (WT, Δ310 mutant, and WT/Zn^{2+}) were utilized to perform RT-PCR, and the generated cDNAs were labeled with Cy3 dCTP during reverse transcription. After prehybridization, microarray slides were hybridized with the relevant samples at 65°C for 4 to 16 h (13). This was repeated four times for three samples.

All hybridization slides were scanned with a GenePix 4100A scanner after appropriate washing, and the average pixel intensity values were quantified using GenePix Pro 4.1. Statistical analysis (*t* test and *P* values) was carried out, and genes with more than twofold change ratios were regarded as candidate targets (13).

Real-time quantitative RT-PCR. To validate the results of the microarray data, 10 randomly selected genes (Table 2) were subjected in triplicate to quantitative RT-PCR utilizing Sybr green detection in a Rotor Gene 6000 (Corbett) (75). For each sample (WT, Δ310 mutant, and WT/Zn^{2+}), 2 μg of total RNA was used for first-strand cDNA synthesis with a commercial RT kit (Promega) as recommended by the manufacturer, and the primers (Table 2) were designed according to the published genomic sequence of 05ZYH33 (8). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control. Bacterial DNAs in a dilution series, as standard samples, were applied to generate the standard quantitative curve, and double standard curves were utilized to perform the quantitative analysis of the genes of interest. The average ratio of target genes' transcription levels was calculated with the software of Rotor Gene 6.0.

RESULTS

Bioinformatics-based identification of a *zur/fur*-like gene, 310, from *S. suis* serotype 2 strain 05ZYH33. The recently determined genome sequence of *S. suis* 05ZYH33, a virulent Chinese isolate of *S. suis* serotype 2 (8), has facilitated the further development of functional genomics. Bioinformatics analysis identified an ORF termed 310 located at bp 302393 to 301941 on the negative strand of the genome (not shown). The

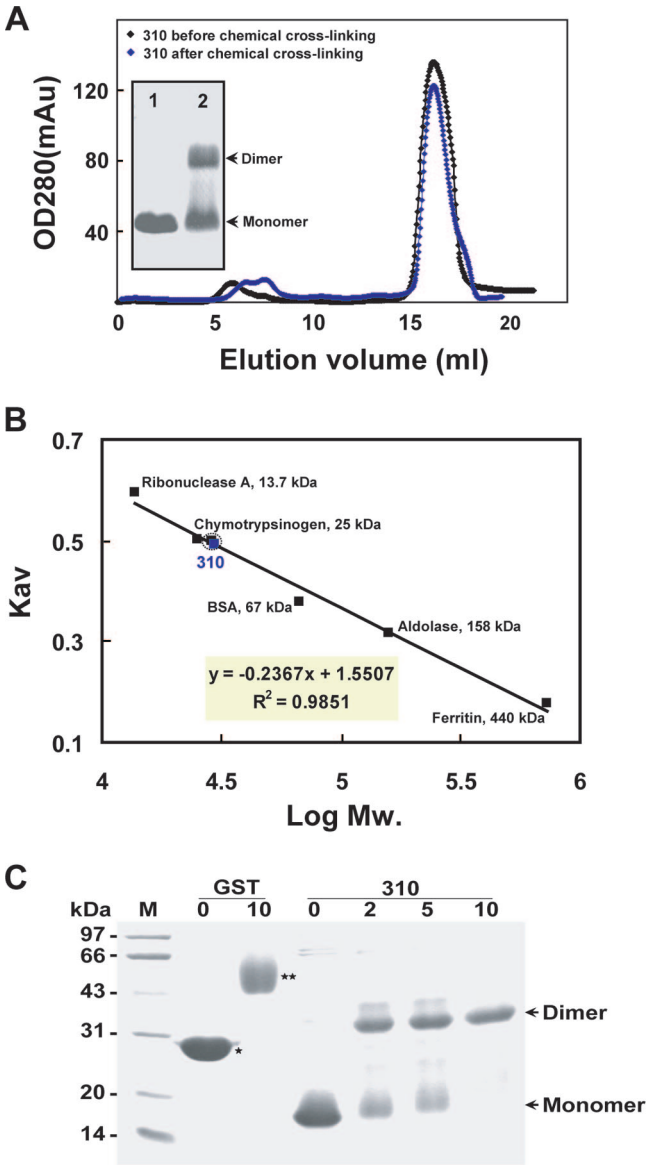

FIG. 2. Existence of protein 310 as a dimer. (A) FPLC profile of protein 310. The purified protein 310 (before and after chemical cross-linking) was subjected to gel filtration on a Superdex 200HR 10/30 column. The samples from the two different peaks were visualized on a 15% SDS-PAGE gel. Lane 1, protein 310 from before chemical cross-linking; lane 2, protein 310 from after chemical cross-linking. The protein of interest is indicated by the arrow. (B) Elution behavior of standard proteins. Standardized proteins (Pharmacia) were run on a Superdex 200HR 10/30 to relatively determine the molecular mass of protein 310. Protein 310 (same as in panel A) is highlighted circled. (C) Chemical cross-linking assay of protein 310. The samples were separated by 15% SDS-PAGE following chemical cross-linking. GST, a typical dimer protein, was used as a positive control in the chemical cross-linking experiment. The GST monomer is indicated by an asterisk, while the GST dimer is highlighted with double asterisks. 0, 2, 5, and 10, concentrations of EGS (mM). Protein 310 occurs only as a monomer in vitro on SDS-PAGE without addition of EGS. With the increase of EGS, the intensity of the monomer is decreased, while the dimer is enhanced. Protein 310 before and after the gel filtration behaves similarly in the chemical cross-linking experiments. Also, protein 310 was demonstrated to carry zinc ions by ICP-AES (Table 3).

TABLE 3. Determination of zinc contents in protein 310 and *S. suis* serotype 2 strains

Protein or strain	Zinc content[a]
310 protein	
GST removed	0.43 ± 0.03 A
His tagged	0.62 ± 0.02 A
WT strain	
THB	0.28 ± 0.05 A
CDM	0.31 ± 0.06 B
Δ310 mutant	
THB	1.45 ± 0.13 A
CDM	1.66 ± 0.10 B

[a] Data means ± standard deviations for triplicate samples and are in milligrams per liter for protein 310 and in micrograms per 10^{10} cells for strains. Different letters in each column (A and B) indicates significance differences (A, $P \leq 0.01$; B, $P \leq 0.005$). The concentration of both version of protein 310 was quantified to be 1 mg/ml. The zinc contents for double-distilled water, CDM, and THB were determined to be <2, 160, and 1,141 μg/liter, respectively.

deduced product of 310 (named protein 310 here) consisted of 151 amino acids (aa) exhibiting 28.4% and 35.1% amino acid similarity to the Zur proteins of *E. coli* and *M. tuberculosis*, respectively (Fig. 1). Further analysis suggested that protein 310 contains a potential DNA-binding motif (helix-turn-helix) at its N terminus (not shown) and possesses nearly 38% α-helices in its secondary structure (Fig. 1), implying that it is possibly a transcription factor.

Biochemical characterization of protein 310. To better understand protein 310, the complete coding sequence of gene 310 was cloned into pET28(a). The purified, recombinant, soluble 310 protein (before and after chemical cross-linking) was analyzed by gel filtration. The fast protein liquid chromatography (FPLC) profile of both 310 protein samples on Superdex 200 showed that they eluted at approximately same position at ~16.2 ml (~35 kDa) (Fig. 2A). The samples collected from these two peaks (with and without chemical cross-linking) were separated by 15% SDS-PAGE, which showed that (i) protein 310 has a molecular mass of ~17 kDa, which is consistent with the theoretical estimation of the amino acid content of a monomer (Fig. 2A and B), and (ii) protein 310 exists as a dimer after the chemical cross-linking assay (Fig. 2A). Protein 310 before and after gel filtration was also subjected to further analysis of chemical cross-linking. The position of protein 310 shifted from monomer to a mixture of monomer and dimer (an intermediate state) to dimer after addition of EGS (Fig. 2C), suggesting that the 310 protein is indeed a dimer, a typical biochemical characteristic of the Zur family (26). Subsequently, ICP-AES measurement confirmed that protein 310 contains zinc (Table 3), which is in consistent with structural findings for *M. tuberculosis* Zur protein (36). The above data provide indirect biochemical evidence that protein 310 is a member of the Zur family.

Physiological function of gene 310. In order to elucidate the role of 310, the Δ310 mutant was obtained from more than 200 *S. suis* transformants. The correct genotype of the Δ310 mutant was systemically confirmed by multiple approaches, such as PCR (Fig. 3A and B), RT-PCR (Fig. 3C), Western blotting (Fig. 3D), and direct DNA sequencing.

Several biological properties were addressed, including morphology, hemolytic activity, and sensitivity to H_2O_2. No obvious morphological difference was observed between the Δ310

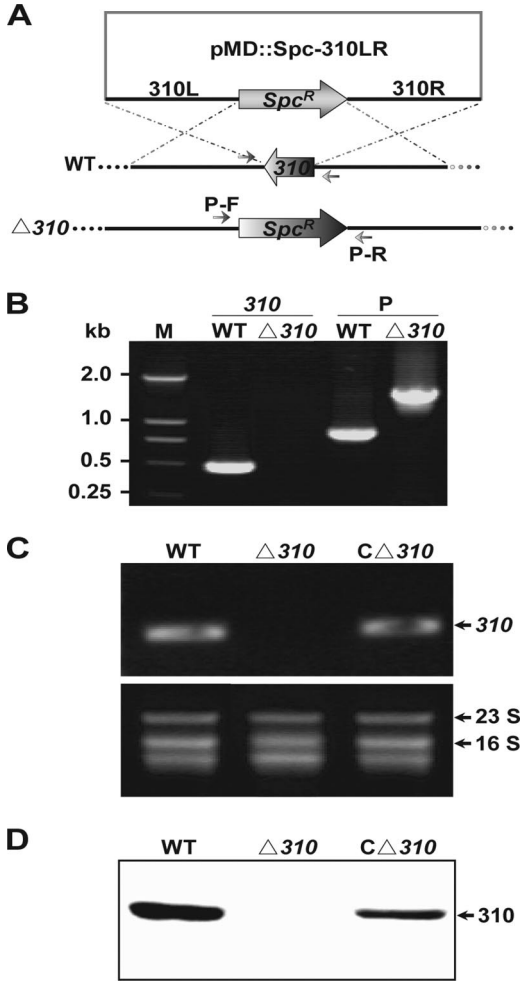

FIG. 3. Identification of the Δ310 mutant, the isogenic mutant of gene 310 in *S. suis* serotype 2. (A) Gene 310 knockout from the *S. suis* serotype 2 chromosome. pMD::Spc-310LR is the recombinant vector constructed to specifically inactivate gene 310. 310L and 310R indicate the left and right borders of gene 310. A pair of specific primers (P-F and P-R) located in both sides adjacent to gene 310 are indicated by arrows and were used for PCR detection of existence of gene 310 in the genome of *S. suis* serotype 2. (B) Multiple-PCR analysis of the Δ310 mutant. The PCR products were separated by electrophoresis on a 1.0% agarose gel stained with ethidium bromide. P, PCR product amplified with primers P-F and P-R. Gene 310 in the Δ310 mutant has been replaced by an Spc^r gene, without affecting either boundary sequence (not shown). (C) RT-PCR analysis of the Δ310 mutant. RT-PCR products of gene 310 were separated by electrophoresis on a 1.0% agarose gel (top). Total RNAs of *S. suis* serotype 2 strains (WT, the Δ310 mutant, and CΔ310) were determined by electrophoresis on a 1.0% agarose gel (bottom). 23 S, 23S ribosome subunit; 16 S, 16S ribosome subunit. (D) Western blot analysis. Protein 310 is shown to be expressed in the WT and the complemented strain, CΔ310, but not in the gene 310 isogenic mutant.

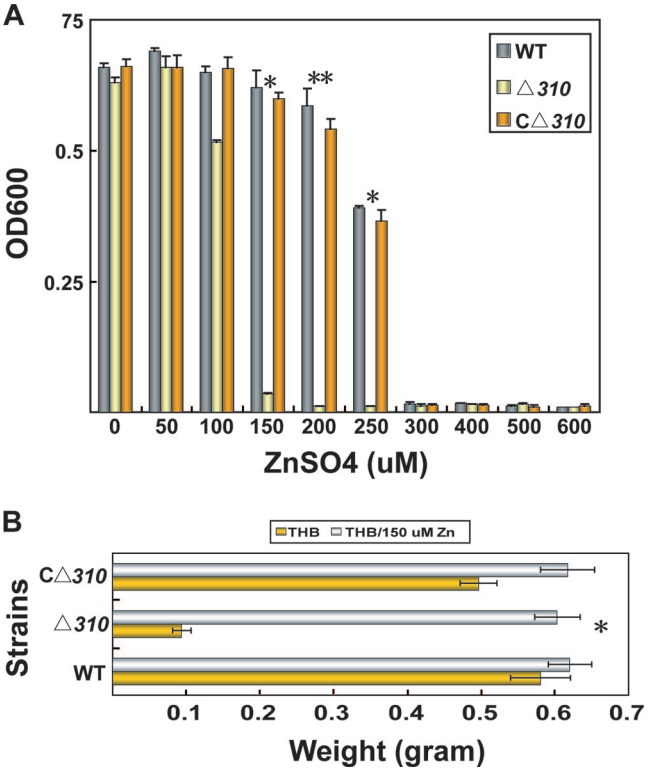

FIG. 4. Role of gene 310 in the sensitivity of the Δ310 mutant to Zn^{2+}. (A) Sensitivity of the Δ310 mutant to Zn^{2+}. An overculture of each *S. suis* serotype 2 strain (60 μl, cell density adjusted to an OD_{600} of 0.7) was inoculated into 3 ml of THB liquid medium supplemented with $ZnSO_4$ to a final concentration of 0, 50, 100, 150, 200, 250, 300, 400, 500, or 600 μM. After 12 h of stationary culture at 37°C bacterial cells were subjected to 1 min of shaking at 160 rpm to reach density uniformity before measurement of bacterial cell density. The cell density was measured spectrometrically at 600 nm. (B) Defect of Δ310 mutant growth with high concentrations of zinc ions. The dry weights of 200-ml bacterial cultures were measured. Prior to calculating the weight, the cells were harvested by centrifugation, washed with 1× PBS three times, and then air dried for 20 min. The values are expressed as the means ± standard deviation from five repeats. **, $P \leq 0.001$; *, $P \leq 0.01$.

mutant and the WT under the culture conditions used (not shown). To further investigate the physiological function of gene 310, six divalent metal ions (Mg^{2+}, Fe^{2+}, Zn^{2+}, Ca^{2+}, Mn^{2+}, and Cu^{2+}) were assessed for their toxicity to *S. suis* serotype 2. For each of the five ions Mg^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+}, and Fe^{2+}, both strains (WT and Δ310 mutant) were found to grow well even at an abnormally high concentration (800 μM), indicating that gene 310 was not involved in the tolerance to them. The Δ310 mutant was highly resistant to Fe^{2+} at the same level the WT, suggesting that 310 is not involved in iron metabolism.

However, the Δ310 mutant grew slowly in THB medium supplemented with 150 and 200 μM Zn^{2+}, whereas the WT and complemented (CΔ310) strains grew well under the same conditions (Fig. 4A). When the concentration of Zn^{2+} was increased to 250 μM, the growth rates of both the WT and CΔ310 strains were also hampered, and they all were killed when the concentration of Zn^{2+} was higher than 300 μM (Fig. 4A). On the other hand, the biomass of the Δ310 mutant was decreased greatly (Fig. 4B). Similar sensitivity of the Δ310 mutant to Zn^{2+} was found in CDM (not shown).

Quantitative analysis of bacterial zinc revealed that the zinc content in the Δ310 mutant is obviously higher than that in the WT (growing in either THB or CDM with suppression of Zn^{2+}). This implies that deletion of 310 is responsible in part for the dysfunction of zinc metabolism in *Streptococcus suis*

TABLE 4. Mass spectrometry identification of the protein spots identified by 2-DE analysis

Protein spot	Expression ratio, Δ310 mutant/ WT (fold)	No. of peptide fragments (sizes, aa)	Functional category	Code in *S. suis* serotype 2 genome	Length (aa)
24	3.12	2 (16, 17)	Ribosome-binding factor A	05SSU1845	136
30	3.35	4 (41, 8, 13, 47)	AtpB	05SSU1177	168
327	5.72	3 (22, 16, 11)	5,10-Methylene-tetrahydrofolate dehydrogenase/methenyl tetrahydrofolate cyclohydrolase	05SSU0498	282
352[a]		2 (10, 22)	ACC beta subunit	05SSU1797	288
362	3.04	5 (19, 23, 13, 19, 9)	3′-Exo-DNase	05SSU0672	276
373[b]	4.33	6 (11, 31, 14, 15, 8, 11)	GTP-binding protein Era	05SSU1397	299
427	3.41	2 (20, 17)	GTP-binding translation factor	05SSU0006	371
437	3.06	2 (17, 10)	GTP cyclohydrolase I[c]		187
657	3.37	2 (26, 20)	Dak phosphatase[c]		191

[a] Protein spot 352 is found only in 2-DE of the Δ310 mutant.
[b] The expression level of protein spot 373 (Era, a GTP-binding protein) was found to be correlated to its transcription level.
[c] Information for this peptide fragment was from protein database search results with poor confidence.

cells (Table 3). Indeed, a similar effect has been noted in the *zur* isogenic mutant of *Xanthomonas campestris* pv. campestris (62). These data strongly demonstrate that gene 310 is a functional member of the *zur* family that controls zinc homeostasis.

Global regulation of gene 310 in *S. suis* serotype 2. Because 310 was characterized as Zur, a zinc-responsive transcription factor, it was of interest to investigate Zur-mediated, genome-wide regulation in *S. suis* serotype 2. 2-DE was first applied to compare the differential expression profiles of the Δ310 mutant and the WT. Protein spot 352 was found only in the Δ310 mutant and was identified as an acetyl coenzyme A carboxylase (ACC) beta subunit (Table 4). Eight other protein spots whose densities in the Δ310 mutant were three times greater than those in the WT were also observed (Table 4).

The limited information acquired by 2-DE prompted us to employ DNA microarray analysis to further investigate this issue. An expression microarray of *S. suis* serotype 2 that covers 2,194 genes of 05ZYH33 was designed. In this study, a twofold difference in relative transcription level was selected as a threshold for analysis of microarray data, as described by Ichikawa et al. (27). The effectiveness of the microarray data was confirmed by real-time quantitative RT-PCR (Table 5). Globally, 5.6% of the genes represented on the microarray ($n = 121$) were differentially transcribed in the Δ310 mutant compared with the WT. Among them, 72 genes were upregulated, and 49 genes were downregulated (Fig. 5A). These genes were involved in metabolism, information storage/process, and cellular signaling/defense. For positively regulated genes in the Δ310 mutant, there were 23 genes categorized as proteinase genes, 8 genes related to DNA metabolism/repair, and 5 surface-associated protein genes, as well as many genes for unknown/hypothetical proteins (Table 6). Likewise, the negatively regulated genes in the Δ310 mutant included the above members. Of note, 10 ABC transporters, which are usually associated with influx and efflux of metabolic molecules (71) and metal ions (6), were positively regulated in the Δ310 mutant, and 2 ABC-type transporters were negatively regulated in the Δ310 mutant (Table 6). A Zn-dependent NADPH:quinone reductase was revealed to be upregulated in the Δ310 mutant, posing the possibility that zinc ions may be directly competed by protein 310 in bacterial cells (Table 5; see Table S1 in the supplemental material). Additionally, metal-responsive transcription factors were involved in Zur regulation (Table 6; see Tables S1 and S2 in the supplemental material). Combined with genome-wide search for zinc-related genes (Table 7), semiquantitative RT-PCR analysis of the Δ310 mutant prompted us to link gene 310 directly to a zinc ABC transporter (05SSU0112), implying that protein 310 probably functions as a zinc-responsive negative regulator in this case (Fig. 6), which would be in close agreement with *E. coli* Zur regulator (47).

TABLE 5. Real-time quantitative RT-PCR assays of microarray data

Gene code	Functional annotation	Avg ratio[a]			
		(WT/Zn^{2+})/WT		Δ310 mutant/WT	
		Microarray	RT-PCR	Microarray	RT-PCR
05SSU0017	Unknown	0.16	0.29	0.19	0.10
05SSU1355	ATP-binding protein of ABC transporter	0.35	0.39	0.34	0.87
05SSU1968	DNA nuclease	0.41	0.77	0.39	0.22
05SSU1998	Phosphoglycerol transferase	0.17	0.19	0.17	0.53
05SSU0019	Putative cell shape-determining protein	2.6	1.56	2.73	1.22
05SSU0293	Permease of ABC transporter	2.66	1.74	2.69	1.65
05SSU0303	Lipoate-protein ligase A	2.46	2.09	2.58	3.23
05SSU0319	Zn-dependent NADPH:quinone reductase	2.16	1.89	2.05	3.95
05SSU1912	Predicted membrane protein	3.41	2.54	3.2	4.39
05SSU2187	ATPase of ABC transporters	3.46	3.22	3.81	1.80

[a] The GAPDH gene (a housekeeping gene) served as the reference gene for real-time quantitative RT-PCR experiments. For the 10 randomly selected genes, the quantitative RT-PCR results are relatively in accordance with the microarray data. These data represents the means of triplicate values.

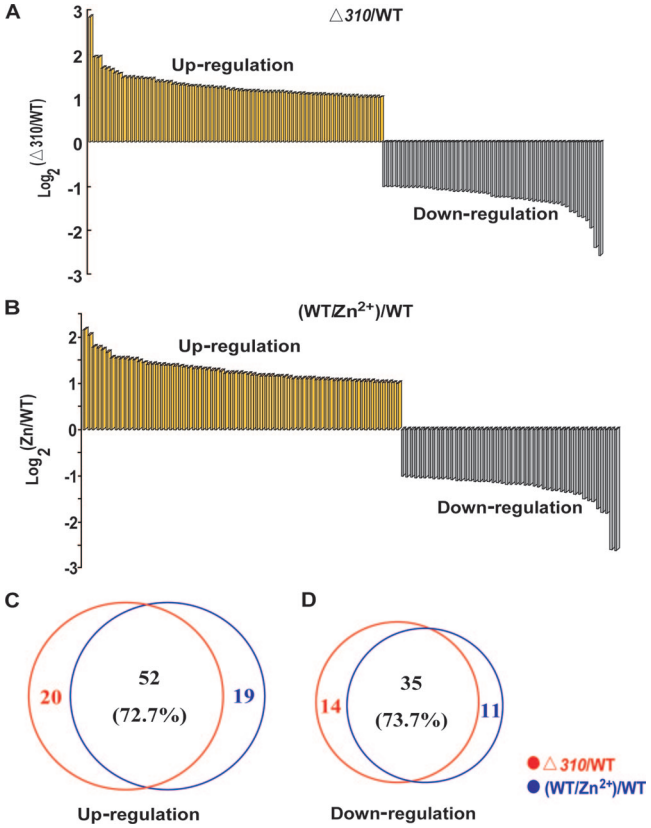

FIG. 5. Global regulation of gene 310 and evaluation of genome-wide response of *S. suis* serotype 2 to Zn^{2+} suppression. (A) Differentially expressed profile of the Δ310 mutant compared with the WT. (B) Genome-wide response of *S. suis* serotype 2 to the presence of Zn^{2+}. The upregulated genes are in yellow, and the downregulated genes are in gray. The criteria for selecting differentially expressed genes were a fold change of ≥ 2 and a *P* value of ≤0.05. For every bacterial sample, the hybridization was repeated in four unique DNA microarrays, and the signal intensity was quantified with GenePix Pro 4.1. Values are expressed as the means from four repeats. (C) Comparative analysis of the upregulated genes from the Δ310 mutant and WT/Zn^{2+}. The red circle indicates the total number of genes upregulated in the Δ310 mutant compared with the WT. The blue circle represents the total number of genes upregulated in WT/Zn^{2+} compared with the WT. A total of 52 upregulated genes are shared by the Δ310 mutant and *S. suis* serotype 2 WT/Zn^{2+}. A total of 20 upregulated genes are specific to the Δ310 mutant, and 19 upregulated genes are found only in WT/Zn^{2+} (for details, see Tables S1 and S3 in the supplemental material). (D) Comparative analysis of the downregulated genes from the Δ310 mutant and WT/Zn^{2+}. A total of 35 downregulated genes were shared by the Δ310 mutant and *S. suis* serotype 2 WT/Zn^{2+}. A total of 14 downregulated genes are specific to the Δ310 mutant, and 11 negatively regulated genes are present only in WT/Zn^{2+} (for details, see Tables S2 and S4 in the supplemental material).

Correlation of gene 310 with Zn^{2+} suppression of *S. suis* serotype 2. The bacterial culture of *S. suis* serotype 2 treated with Zn^{2+} (200 μM) (abbreviated WT/Zn^{2+} here) was also analyzed by DNA microarray analysis. In total, there were 117 genes whose transcription profiles were affected during the process in response to high levels of Zn^{2+}. Dual regulation was confirmed to relate to stimulus of zinc ion, which included 71 positively regulated genes and 46 negatively regulated genes (Table 6 and Fig. 5B). To our surprise, more than 70% of genes affected by zinc in the specific transcriptome of WT/Zn^{2+} were also affected by deletion of 310, which in turn provides evidence that gene 310 is a novel zinc-dependent regulator. In detail, 52 of the upregulated genes in WT/Zn^{2+} overlapped with those in the Δ310 mutant (Fig. 5C), and the downregulated genes in WT/Zn^{2+} covered 73.7% of those in the Δ310 mutant (Fig. 5D).

DISCUSSION

Streptococcus suis infections are one of the primary and secondary bacterial diseases causing serious economic losses in pig industries worldwide each year (58, 64). These pathogens are generally categorized into 35 serotypes on the basis of the difference in their capsular antigens, of which serotype 2 is a prevalent serotype most frequently isolated from diseased piglets and human patients (58). There is an increasing amount of data suggesting that *S. suis* serotype 2 has evolved into a highly invasive entity, raising great concerns over global public health (38). Unfortunately, the control of *S. suis* serotype 2 infections by vaccines and antimicrobials has been hampered partly due to the increased tolerance of *S. suis* serotype 2 to antimicrobials together with current limited knowledge on its pathogenesis (38). Therefore, a better understanding of the different aspects of *S. suis* serotype 2, especially pathogenesis, will facilitate the development of effective therapeutics which can prevent and/or treat *S. suis* serotype 2 infections.

We identified gene 310 from *Streptococcus suis*, encoding a functional member of the Zur family. Bioinformatics analysis revealed that this protein comprises two conserved domains in the family of Fur/Zur proteins: a hypothetical DNA-binding motif at its N terminus and a putative C-terminal dimerizing module (2). Sensitivity assays with divalent transition metal ions demonstrated that gene 310 is a zinc-responsive regulator

TABLE 6. Genome-wide display of the genes differentially expressed in the Δ310 mutant and *S. suis* serotype 2 WT/Zn^{2+}

Functional category	Δ310 mutant/WT		(WT/Zn^{2+})/WT	
	No. down-regulated	No. up-regulated	No. down-regulated	No. up-regulated
Unknown proteins	10	13	9	12
Surface/membrane-related proteins	6	5	6	6
Proteinases	12	23	11	20
Replication/transcription/translation-related factors	4	3	5	3
Ribosome-related proteins	1	0	1	0
Components of type IV secretion system	1	0	0	0
Transposases/integrases	2	2	0	3
Cps2-related proteins	1	0	0	0
ABC-like transporters	2	10	1	13
DNA metabolism/repairs	4	8	6	5
Electron transfer-related protein	0	1	1	1
Chaperonin	0	1	0	1
Cell shape-related proteins	0	2	0	1
No annotation	6	4	6	6

TABLE 7. Genome-wide search for genes with involvement of zinc ions

Code	Length (aa)	GC (%)	Strand[b]	Functional annotation	Expression (fold)[c] in Δ310 mutant
05SSU0112[a]	503	39.36	+	Zinc ABC transporter, zinc-binding lipoprotein	2.2 ↓
05SSU0330	306	41.39	+	ABC-type metal ion transporter, zinc-binding lipoprotein	1.8 ↓
05SSU1083	355	42.07	−	Uncharacterized ABC-type transport system, surface lipoprotein	1.6 ↑
05SSU1302	277	43.2	−	Zinc transporter, ZIP	1.1 ↑
05SSU1771	189	40.74	−	ABC-type metal ion transport system, surface antigen	1.3 ↑
05SSU2086	317	40.69	−	ABC-type metal ion transport system, manganese/zinc-binding lipoprotein	1.1 ↓
05SSU0153	609	42.09	+	Putative membrane-bound neutral zinc metallo-endopeptidase	1.1 ↓
05SSU0279	341	45.65	−	Zinc-dependent alcohol dehydrogenase	1.4 ↓
05SSU0319[a]	333	46.45	+	NADPH:quinone reductase and related Zn-dependent oxidoreductase	2.1 ↑
05SSU0728	596	42.51	−	Peptidase M, neutral zinc metallopeptidase	1.2 ↓
05SSU1022	1926	41.76	−	Surface-bound neutral zinc metallopeptidase	1.6 ↓
05SSU1084	134	37.81	−	Cytidine/deoxycytidylate zinc-binding deaminase	1.5 ↑
05SSU1388	372	47.67	+	Zinc-containing alcohol dehydrogenase	1.2 ↑
05SSU1478	219	40.79	−	Neutral zinc metallopeptidase	1.3 ↓
05SSU1607	313	45.79	+	Zinc binding mannose-6-phosphate isomerase	1.2 ↓
05SSU1622	161	43.79	+	Cytidine/deoxycytidylate deaminase, zinc-binding region	1.8 ↑
05SSU1657	311	43.84	+	Neutral zinc metallopeptidase	1.4 ↓
05SSU1962	419	42.96	−	Hypothetical zinc metalloprotease	1.7 ↑
05SSU1976	152	47.15	−	Putative neutral zinc metallopeptidase	1.1 ↑
05SSU2032	184	48.98	−	Zinc-binding carbonic anhydrase	1.1 ↑
05SSU2053	173	44.32	−	Zinc-binding cytidine/deoxycytidylate deaminase	1.3 ↓
05SSU2082	630	47.88	−	Hypothetical zinc metalloproteinase	1.2 ↓
05SSU2179	404	35.56	−	Hypothetical zinc protease	1.4 ↑
05SSU2180	417	35.57	−	Predicted Zn-dependent peptidase	1.6 ↓

[a] Gene whose expression profile is altered at least twofold in the Δ310 mutant.
[b] +, positive strand of *S. suis* serotype 2 chromosome; −, negative strand of *S. suis* serotype 2 chromosome.
[c] ↓, decreased expression; ↑, elevated expression.

gene, referred to as *zur* (Fig. 4). Our biochemical data (Fig. 2) showed that the full-length product of gene 310 exists as a dimer, which is consistent with the crystal structures (48) and previous biochemical analysis (2, 12) of other members of this superfamily. Although the deletion of *fur*/*zur* was found to impair the growth of *Actinobacillus pleuropneumoniae* (28), the Δ310 mutant exhibited growth similar to that of its parental strain (not shown). Subsequent microarray results provided

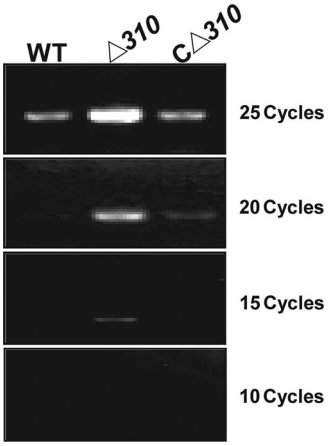

FIG. 6. Transcriptional analysis of gene 0112, a zinc-binding lipoprotein in ABC-type transporters. On the basis of the combined information, gene 0112, a component of the ABC zinc transporter (also called ZnuA), is proposed to be a possible element which can be directly regulated by gene 310 in *Streptococcus suis*. Semiquantitative RT-PCR was used for transcriptional analysis of gene 0112. The DNA fragment of interest is 235 bp in length.

one possible explanation for this, in that the overexpression of Era, which is required for bacterial growth (45), may remedy the defect caused by the deletion of gene 310 in the Δ310 mutant to some extent. The inactivation of gene 310 seemed to not affect the bacterial phenotype, such as hemolytic activity. In contrast to those regulators sensing oxidative stress in this family, e.g., PerR (31, 69), our data did not support the involvement of gene 310 in H_2O_2 sensitivity (not shown). Retrospectively, Dpr (*D*ps-like *p*eroxide *r*esistance protein) was suggested to confer on *S. suis* serotype 2 resistance to H_2O_2 (52). However, the role of *zur* in pathogenesis is controversial (7, 34, 62) In our piglet infections, *zur* had no apparent effect on *S. suis* serotype 2 virulence, which is consistent with results seen for *S. aureus* (34).

Given that Zur is a global regulator in bacteria, we tried to dissect its regulatory networks at the levels of the proteome and transcriptome. At the proteomics level, we identified only nine protein spots (Table 4). One protein dot (no. 352) that is present only in the Δ310 mutant was confirmed to be the beta subunit of ACC. ACC is a central metabolic enzyme that catalyzes the committed step in fatty acid biosynthesis. Recently, the ACC structures of both *S. aureus* and *E. coli* revealed a zinc-binding motif (5). Therefore, it is reasonable that Zur-mediated downregulation of ACC can be attributed to its interaction with zinc. On the other hand, there are five other proteins whose expression level in the Δ310 mutant was three times higher than that in the WT. First, it is reasonable that the two proteins (ribosome-binding factor A [spot 24] and GTP-binding translation factor [spot 427]) were derepressed in the Δ310 mutant. Several studies have indicated that zinc is required for the structural stability of translation initiation fac-

tors that generally contain ribosome-binding domains (22, 35). It is possible that ribosome-binding factors compete with a translation initiation factor for the resources of ribosomes (19, 21). Second, Era (a GTP-binding protein) was revealed by 2-DE to be negatively regulated by Zur. Era has been demonstrated to interact with 16S rRNA (23) and mediate the assembly of the 30S ribosome unit (54), and ribosomal proteins have been suggested to be related to Zur protein and zinc ions (46, 55). Therefore, it is logical that Era can be linked to Zur in *S. suis* serotype 2. Certainly, the total number of affected proteins elucidated by 2-DE seemed to be much less than that one might expect.

Further microarray analysis showed that the gene 310-mediated dual-regulation network is globally involved in 121 genes, of which 72 were upregulated and 49 were downregulated in the Δ310 mutant (Fig. 5A). Most genes affected by *zur* encode putative proteinases, of which 23 were upregulated and 12 were downregulated (Table 6). They included some Zn^{2+}-dependent members (e.g., NADPH:quinone reductase and lipase) involved in the basic metabolism of carbohydrates, nucleic acids, and fatty acids (10). There were more than 20 unknown genes which are influenced by gene 310. Membrane/surface proteins have been suggested to contribute greatly to the invasiveness and immunogenicity of pathogens (16, 41). We observed that they are also subject to dual regulation by Zur (some are stimulated, and others are repressed). In particular, a hyaluronidase with an LPXTG motif, which plays roles in pathogenicity of group A streptococci (60), was also found to be negatively regulated by Zur. However, *cps2C*, which is involved in the production of capsular polysaccharide, a virulence determinant of *S. suis* serotype 2 (56), is positively regulated by Zur. It seemed that virulence manifestation is linked to the regulation of Zur in *S. suis*. Transcription factors and activators (e.g., *alsR* and *plcR*) were also affected by Zur, implying the existence of indirect regulation mediated by Zur in *S. suis* serotype 2 (40, 74).

The transcriptome of *S. suis* serotype 2 under the control of Zn^{2+} exhibited 117 differentially expressed genes compared with that under normal culture condition (Fig. 5B). Intriguingly, we noticed that more than 70% of the genes regulated in response to Zn^{2+} overlapped with those regulated by Zur protein in *S. suis* serotype 2 (Fig. 5C). Two points of interest are as follows. First, it is reasonable that the expression of a 60-kDa chaperonin (see Table S3 in the supplemental material) is elevated greatly in response to the emerging stress of zinc ions at a high level (73), which is similar to what is observed in the Δ310 mutant. Second, not only were most ABC-type transporters regulated by Zn^{2+}, but membrane proteins, proteinases, and unknown proteins were also affected, which in turn validates the Zur-mediated regulation network. It is well known that MscS (the mechanosensitive channel of small conductance) plays a critical role in osmoregulation in prokaryotic microorganisms (3, 65). Similarly, microarray results revealed that treatment with Zn^{2+} can repress the transcription of *mscS* in *S. suis* serotype 2. Unexpectedly, MreC, a cell shape determinant (29, 30), was found to be positively regulated but not negatively regulated by Zur, suggesting that *S. suis* serotype 2 has evolved morphological machineries to adapt to the dramatic change of environmental zinc ions. In addition, transcription regulators were affected by zinc, suggesting that their target genes may be indirectly controlled by Zur (40).

In summary, we defined a functional *zur* gene from *S. suis* serotype 2. We attempted to define the genome-wide regulation network of Zur by 2-DE and DNA microarray analysis. Based on microarray analysis of the Δ310 mutant compared with *S. suis* serotype 2 treated with zinc ions, we gained, for the first time, a glimpse of the cross talk between gene 310 and Zn^{2+}, which in turn provides genome-wide evidence that gene 310 is a functional member of the *zur* family.

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